

**MULTIMERIN,
A PLATELET AND ENDOTHELIAL CELL PROTEIN**

By

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**MULTIMERIN,
A PLATELET AND ENDOTHELIAL CELL PROTEIN**

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ABSTRACT

p-155 is a soluble platelet protein, with a reduced subunit mobility of 155 kDa, that was first identified using a monoclonal antibody raised against platelets. This thesis describes the identification and characterization of p-155, including its structure, biosynthesis, cells of origin, and cDNA sequence. Studies of the native p-155 protein indicated that it is comprised of variably sized, disulfide linked multimers of p-155 subunits, ranging in size from a 400 kDa trimer to large multimers, many millions of daltons in size. Based on its massive, multimeric structure, the native p-155 protein was designated as multimerin. Comparisons with other multimeric proteins found in platelets indicated that multimerin is a novel protein and also one of the largest proteins stored in platelets. In addition to platelets, multimerin was also found in endothelial cells. Biosynthetic metabolic labeling studies indicated that multimerin is synthesized by Dami cells (a megakaryocytic cell line), and by endothelial cells. Multimerin is a highly glycosylated protein with complex, N-linked carbohydrate accounting for 1/3 of its molecular mass. Cleveland mapping studies were used to investigate the relationship between the different sized multimerin subunits found in platelets and Dami cells. These studies demonstrated peptide homology, indicating that p-155 and p-170 (a larger but less abundant multimerin subunit found in platelets) originate from p-196, the multimerin precursor protein identified in metabolic labeling studies.

Multimerin antibodies were used to screen expression human endothelial cell libraries for multimerin cDNA clones and the most 5' cDNA clone was used to rescreen the library for complete 5' sequence. The complete cDNA sequence for multimerin was then determined. The multimerin cDNA sequence encodes a hydrophilic protein of 1228 amino acids with RGDS, EGF-like, partial EGF-like, and putative coiled-coil domains. In addition, the C-terminal region of multimerin resembles the globular head domain of complement C1q and collagens type VIII and X. These studies establish multimerin as a unique, multimeric platelet and endothelial cell protein. The massive size, RGDS motif, and repeating structure of multimerin suggest a possible role for this protein in adhesion.

This thesis is dedicated to David, Christopher and Geoffrey

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Hayward, C. P. M., Smith, J. W., Horsewood, P., Warkentin, T. E., and Kelton, J. G. (1991) p-155, A multimeric platelet protein that is expressed on activated platelets, *J. Biol. Chem.* 266, 7114-7120.

APPENDIX 2

Hayward, C. P. M., and Kelton, J. G. (1995) Multimerin: A multimerin protein stored in platelet alpha-granules. *Platelets* 6, 1-10.

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LIST OF SYMBOLS AND ABBREVIATIONS

The symbols and abbreviations used within the manuscripts are defined within these papers. Additional symbols and abbreviations contained within this thesis include:

α	alpha
β -TG	beta-thromboglobulin
cDNA	complementary deoxyribonucleic acid
EGF	epidermal growth factor
GPI-linked	glycosyl phosphatidyl inositol linked
IEF	isoelectric focusing
IgG	immunoglobulin G
kb	kilobases
kbp	kilobase pairs
kDa	kilodalton(s)
mRNA	messenger ribonucleic acid
NR/R	nonreduced/reduced
PAI-1	plasminogen activator inhibitor 1
PCR	polymerase chain reaction
PF4	platelet factor 4
PMA	phorbol 12-myristate 13-acetate
RGDS	arginine-glycine-aspartic acid-serine
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TSP	thrombospondin
2D	two dimensional
vWf	von Willebrand factor

1. INTRODUCTION

1.1 Platelet Proteins and Platelet Function

In normal blood vessels, platelets are nonadherent cells. At sites of vessel injury, platelets are activated by agonists and are transformed into adhesive cells that support coagulation. This change facilitates the binding of platelets to each other and to the adhesive proteins found in the injured blood vessel wall. Alterations in glycoproteins on the platelet membrane, including the activation of receptors and the binding of procoagulant and adhesive proteins to the platelet membrane, are an important part of this transformation (Nurden, 1993; Packham, 1994).

Platelet glycoproteins can be divided into three main categories: membrane glycoproteins, cytosolic proteins and soluble glycoproteins that are stored within platelet granules. A large number of transmembrane glycoproteins have now been identified (Tables 1 and 2). These proteins support a diverse range of platelet functions and include the receptors for adhesive ligands (Table 1) and the receptors for platelet-activating agonists (Table 2). The cytosol of platelets contains cytoskeletal proteins and proteins involved in metabolic pathways and intracellular signaling (Kroll *et al*, 1989; Brass *et al*, 1993; Furman *et al*, 1993). While the function of some of these proteins is well characterized, the role of many is still unknown.

Table 1. Platelet Membrane Glycoproteins

The platelet receptors for agonists are listed separately in Table 2

<i>Glycoprotein</i>	<i>Type</i>	<i>Function</i>	<i>Reference</i>
α IIb β 3	integrin	aggregation, adhesion	reviewed in Ginsberg <i>et al</i> 1993
α 2 β 1	integrin	adhesion collagen receptor	
α v β 3	integrin	adhesion vitronectin receptor	
α 6 β 1	integrin	adhesion laminin receptor	
α 5 β 1	integrin	adhesion fibronectin receptor	
glycoprotein IbIX complex	leucine rich glycoprotein	adhesion to von Willebrand factor	Lopez <i>et al</i> 1988 Hickey <i>et al</i> 1989 Sakariassen <i>et al</i> 1987
PECAM-1	immunoglobulin domain molecules	?	Newman <i>et al</i> 1990
P-selectin	selectin	platelet-leukocyte adhesion	Johnston <i>et al</i> 1989 Hamburger <i>et al</i> 1990
glycoprotein IV (CD 36)	-	adhesion to collagen, thrombospondin	Tandon <i>et al</i> 1989
glycoprotein V	leucine rich glycoprotein	complexed with GP IbIX ?thrombin receptor	Shimomura <i>et al</i> 1990
glycoprotein VI	-	collagen receptor	Moir <i>et al</i> 1992 Arai <i>et al</i> 1994
CD 9 (p24)	quadraspanin	?signal transduction	Lanza <i>et al</i> 1991
CD63 (granulophysin)	-	? (lysosomal membrane protein)	Niewenhuis <i>et al</i> 1987
CDw109	GPI-linked	?	Brasheem-Stein <i>et al</i> Haregewoin <i>et al</i> 1994
MIRL (CD59)	GPI-linked	complement defense	Morgan 1992
DAF	GPI-linked	complement defense	Nicholson-Weeler <i>et al</i> 1985 Kinoshita <i>et al</i> 1985
C8bp	GPI-linked	complement defense	Blaas <i>et al</i> 1988
HLA Class I	immunoglobulin domain molecule	immune response	Blumberg <i>et al</i> 1984
cC1qR	-	receptor for complement C1q, collagen-like domain	Peerschke <i>et al</i> 1988 Ghebrehwet <i>et al</i> 1992
gC1qR	-	receptor for complement C1q, globular domain	Peerschke <i>et al</i> 1994 Ghebrehwet <i>et al</i> 1994

Table 2. Platelet Membrane Receptors for Agonists

<i>Agonist</i>	<i>Platelet Receptor</i>	<i>Receptor Type</i>	<i>Reference</i>
thrombin	thrombin receptor	receptors with seven transmembrane domains	Vu <i>et al</i> 1991 Coughlin 1994
thromboxane A ₂	thromboxane A ₂ receptor	receptors with seven transmembrane domains	Hirata <i>et al</i> 1991
ADP	? aggregin	transmembrane protein	Coleman <i>et al</i> 1994
collagen	glycoprotein $\alpha_2\beta_1$ glycoprotein IV glycoprotein VI	integrin transmembrane protein transmembrane protein	Nieuwenhuis <i>et al</i> 1985 Tandon <i>et al</i> 1989 Moiri <i>et al</i> 1992 Arai <i>et al</i> 1994
epinephrine	α_2 adrenergic receptor	receptors with seven transmembrane domains	Regan <i>et al</i> 1986 Kobilka <i>et al</i> 1987
platelet activating factor (PAF)	PAF receptor	receptors with seven transmembrane domains	Honda <i>et al</i> 1991
immune complexes	Fc γ RII	immunoglobulin domain molecules	Rosenfeld <i>et al</i> 1985 Kelton <i>et al</i> 1987 Stuart <i>et al</i> 1987

Table 3. Soluble Glycoproteins Stored in Platelet Alpha-Granules

<i>Glycoprotein</i>	<i>Source</i>	<i>Function</i>	<i>Reference</i>
Fibrinogen	plasma	aggregation, adhesion	Handagama <i>et al</i> 1989, 1990 Harrison <i>et al</i> 1989
thrombospondin	megakaryocytes	aggregation, adhesion	Aiken <i>et al</i> 1987 Tuszynski <i>et al</i> 1988 Leung <i>et al</i> 1984
β TG	megakaryocytes	?heparin-binding protein	Kaplan <i>et al</i> 1979 Rucinski <i>et al</i> 1979
PF4	megakaryocytes	heparin-binding protein	Kaplan <i>et al</i> 1979 Rucinski <i>et al</i> 1979
von Willebrand factor	megakaryocytes	aggregation, adhesion	Koutts <i>et al</i> 1978
albumin	plasma	carrier protein	Handagama <i>et al</i> 1990
IgG	plasma	immune defense	Handagama <i>et al</i> 1990 George 1990
Factor V	megakaryocytes	coagulation	Chesney <i>et al</i> 1981
fibronectin	?plasma	adhesion	Zucker <i>et al</i> 1979
vitronectin	?plasma	?adhesion	Parker <i>et al</i> 1989 Preissner <i>et al</i> 1989
PAI-1	? megakaryocytes	inhibition of fibrinolysis	Booth <i>et al</i> 1988
anti-plasmin	plasma	inhibition of fibrinolysis	Gogstad <i>et al</i> 1983
α 2-macroglobulin	plasma	protease inhibitor	Nachman <i>et al</i> 1976
α 1-antitrypsin	plasma	protease inhibitor	Nachman <i>et al</i> 1976
high molecular weight kininogen	?megakaryocyte	protease inhibitor, coagulation cofactor	Schmaier <i>et al</i> 1986
histidine rich glycoprotein	?megakaryocyte	?	Leung <i>et al</i> 1983
C1 inhibitor	?megakaryocyte	inhibition of complement activation	Schmaier <i>et al</i> 1984
protein S	?	coagulation inhibitor	Schwarz <i>et al</i> 1985
osteonectin	?megakaryocyte	?	Stenner <i>et al</i> 1986 Breton-Gorius <i>et al</i> 1992
platelet derived growth factor	?megakaryocyte	?wound repair	Heldin <i>et al</i> 1979
transforming growth factor beta	?megakaryocyte	?wound repair	Assoain <i>et al</i> 1983
factor D	?	complement pathway	Kenney <i>et al</i> 1981
factor H	?	complement pathway	Kenney <i>et al</i> 1981
protease nexin-2	?megakaryocyte	protease inhibitor	Van Nostrand <i>et al</i> 1991

In addition to membrane and cytosolic glycoproteins, platelets contain soluble glycoproteins that are stored within alpha-granules and enzymes that are contained within lysosomes (George, 1990, Harrison and Cramer 1993b). Soluble proteins stored within platelets include adhesive proteins, such as von Willebrand factor, fibrinogen, and thrombospondin, coagulation factors, protease inhibitors, among others (Table 3). While some of the soluble proteins stored in platelets originate from the plasma, others are synthesized by megakaryocytes (Handagama *et al*, 1989; Handagama *et al*, 1990; Harrison *et al*, 1989; George, 1990; Harrison and Cramer 1993b).

Most platelet glycoproteins were identified by their electrophoretic mobility relative to the other proteins found in platelets. Initially, two dimensional nonreduced/reduced gel electrophoresis and isoelectric focusing techniques were used (Phillips and Poh Agin, 1977; Clemetson *et al*, 1979). In recent years, additional glycoproteins have been identified with the development of monoclonal antibodies, specific for platelet proteins, as investigative tools (Horsewood *et al*, 1993). Many of the newly characterized proteins are present in insufficient quantities to be detected by standard electrophoretic techniques or their migration properties failed to distinguish them as a separate protein.

Our knowledge of platelet glycoprotein function has come from several sources. In some inherited disorders, such as Glanzmann's thrombasthenia and the Bernard Soulier Syndrome, there is loss of a major glycoprotein that is evident on electrophoretic separations of platelet proteins (Nurden *et al*, 1974; Nurden *et al*, 1975; Phillips *et al*, 1975). Correlation of

these findings with an identified, abnormal platelet function led the integrin $\alpha_{IIb}\beta_3$ (glycoprotein IIb/IIIa) complex to be identified as the platelet receptor for fibrinogen, and the glycoprotein IbIX complex to be recognized as a platelet receptor for von Willebrand factor. For other glycoproteins, knowledge of their function has come from studies using antibodies and peptides to inhibit platelet functions. The cloning and sequencing of the cDNA for novel platelet proteins has also provided information about function. Indeed, the role of P-selectin as a receptor mediating platelet-leukocyte adhesion was first deduced from its cDNA sequence (Johnston *et al*, 1989).

The study of activation-induced changes in platelet glycoproteins has provided insights into platelet function. Changes have been identified in the conformation of integral membrane proteins that alter their ability to bind ligand (Shattil *et al*, 1985; Collier, 1985; Sims *et al*, 1991). In addition, platelet activation also changes the distribution of some platelet glycoproteins. For example, certain internal granule membrane proteins become expressed on the platelet surface, following fusion of internal granule membranes with the external membrane (McEver and Martin, 1984; Hsu-Lin *et al*, 1984, Nishibori *et al*, 1993; Harrison and Cramer, 1993b). Soluble proteins, stored within platelet granules, are released into the local environment, and some bind to the activated platelet membrane (Gralnick *et al*, 1984; Phillips *et al*, 1980; Parker and Gralnick, 1986; Harrison and Cramer, 1993b). These events in glycoprotein trafficking are important for supporting platelet function (Nurden and Nurden, 1993).

1.2 Mechanisms of Platelet Activation

Platelet activation can be defined as stimulation-induced changes that lead to enhanced adhesive and/or procoagulant properties. Platelets contain a variety of specific receptors that mediate their response to the external stimuli. A growing number of agonists have been identified that trigger platelet activation through specific receptors for these agonists (Packham, 1994). Some of these receptors have been cloned and sequenced. Receptors have been identified for thrombin, ADP, epinephrine, thromboxane A₂, collagen, immune complexes and platelet activating factor (Table 3). Platelets can also be activated by exposure to shear forces and by the binding of adhesive proteins to their receptors (Du *et al*, 1991; Ruggeri, 1993; Shattil, 1993).

Several events that occur with platelet activation have been used to detect or quantitate this process (Hsu-Lin *et al*, 1984; McEver and Martin, 1984; Shattil *et al*, 1985; Collier, 1985; George *et al*, 1986; Nieuwenhuis *et al*, 1987; Freilinger *et al*, 1988; Sims *et al*, 1991; Abrahms *et al*, 1990; Peerschke, 1992; Packham, 1994). Markers of platelet activation include:

I Anatomical Changes in Platelets:

1. Platelet aggregation.
2. Alterations in platelet morphology (pseudopod formation, release of granule contents, etc.)
3. Platelet microparticle formation.

II Markers of Granule Secretion

1. The secretion of alpha-granular proteins.

2. The secretion of dense-granule contents.
3. The expression, on the platelet surface membrane, of granular membrane proteins.
4. The binding of soluble glycoproteins, e.g. fibrinogen, to activated platelets.

III Biochemical Markers of Altered Platelet Function

1. Platelet procoagulant activity.
2. Activation-induced conformational changes in integral membrane glycoproteins.
3. Measurement of activation metabolites, protein phosphorylation, etc.

1.3 Glycoproteins as Markers of Platelet Activation

During platelet activation, changes occur in the glycoproteins expressed on platelet membrane. Measurements of these changes have been used as markers of the activation process. Activation-dependent platelet glycoproteins can be grouped into three major categories:

1. Integral membrane proteins that undergo conformational change with activation or ligand binding (Shattil *et al*, 1985; Collier, 1985; Freilinger *et al*, 1988; Sims *et al*, 1991; Du *et al*, 1991)
2. Integral granule membrane proteins that are expressed on the external platelet membrane following granule release

and membrane fusion (McEver *et al*, 1984, Hsu-Lin *et al*, 1984; Nieuwenhuis *et al*, 1987)

3. Soluble glycoproteins, stored within platelet granules, that are bound to the activated platelet membrane following their release from internal stores (Peerschke *et al*, 1980; Galnick *et al*, 1984; Phillips *et al*, 1980; Fernandez *et al*, 1982; Parker *et al*, 1986; Peerschke, 1992).

Monoclonal antibodies have proved useful for detecting activation-dependent alterations in platelet glycoproteins (Figure 1). Targets of these monoclonal antibodies can be integral granular membrane proteins or soluble proteins that bind to the platelet surface following activation. Other monoclonal antibodies detect conformational changes in integral membrane proteins that may be induced following platelet activation or ligand binding. These activation-dependent antibodies have been used as tools for detecting platelet activation *in vitro* (McEver *et al*, 1984; Hsu-Lin *et al*, 1984; Shattil *et al*, 1985; Coller, 1985; Nieuwenhuis *et al*, 1987; Freilinger *et al*, 1988; Sims *et al*, 1991; Du *et al*, 1991; Peerschke, 1992). In addition, activation-dependent antibodies have been used to detect *in vivo* platelet activation in patients (George *et al*, 1986; Abrams *et al*, 1990).

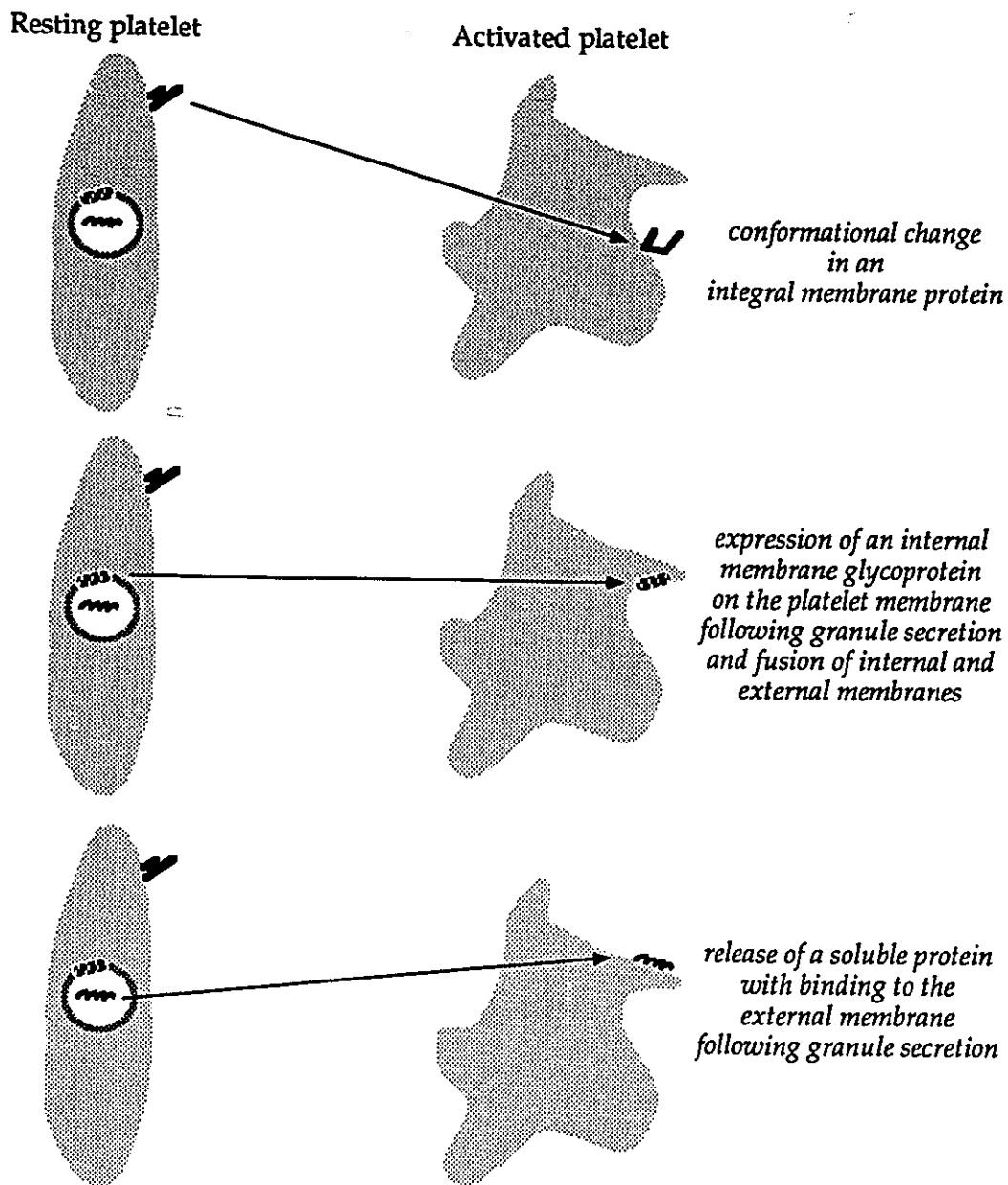


Figure 1. Activation-Induced Changes in Platelet Glycoproteins

1.4 The Importance of Soluble Glycoproteins Stored in Platelet Granules

Platelets contain, within their alpha-granules, a large number of proteins that are released during platelet activation (Table 2). Several lines of evidence indicate that many of these alpha-granular proteins are important for platelet function. First, patients with alpha-storage pool disease, have a deficiency of alpha-granular proteins that is associated with bleeding and a prolongation of the bleeding time (Gerrard *et al*, 1980; Levy-Toledano *et al*, 1982). Second, patients who have a selective alpha-granular protein deficiency, for example the deficiency of platelet von Willebrand factor, have a defect in platelet adhesion, despite normal plasma concentrations of von Willebrand factor (Mannucci *et al*, 1985; Gralnick *et al*, 1986; Fressinaud *et al*, 1994). A correlation has been observed between the bleeding time and intraplatelet stores of von Willebrand factor in patients with von Willebrand's disease, suggesting that the intracellular stores are important for hemostasis (Gralnick *et al*, 1986).

Isolated deficiencies of other granular proteins have not been described and our understanding of their importance is based primarily on experimental knowledge. These proteins appear to support a diverse range of functions. For example, thrombospondin, a trimeric disulfide-linked protein stored in platelets, stabilizes fibrinogen binding to the integrin $\alpha_{IIb}\beta_3$ (Tuszynski *et al*, 1988; Leung 1984). Platelets also contain, within their alpha-granules, approximately 1/4 of the circulating coagulation factor V which functions as a cofactor for the prothrombinase complex and accelerates thrombin formation (Tracy *et al*, 1985). Other alpha-granular proteins, such

as the fibrinolytic inhibitor PAI-1 (Booth *et al*, 1988), may be important for modulating the balance between thrombosis and fibrinolysis locally. Proteins, such as PF-4 and β -TG, may neutralize heparin-like molecules on the endothelial cell surface (Rucinski *et al*, 1979). Platelets also contain a number of growth-factors, such as PDGF, and TGF β_1 (Heldin *et al*, 1979; Assoain *et al*, 1983) that may be involved in wound repair.

Proteins contained within platelet alpha-granules can originate from one of two sources: endogenous synthesis by megakaryocytes or by endocytosis of plasma proteins (George, 1990; Harrison *et al*, 1993b). The proteins found in platelet alpha-granules that are endocytosed from plasma are present at a much higher concentration in plasma than in platelets (George, 1990). Fibrinogen, which is present in approximately a 2-3 fold higher concentration in platelets, is the only known exception to this rule (George, 1990). As individuals lacking the fibrinogen receptor $\alpha_{IIb}\beta_3$, are deficient in platelet fibrinogen, receptor-mediated endocytosis is the likely mechanism of fibrinogen uptake (Nurden *et al*, 1974; Phillips *et al*, 1975). For other proteins, such as albumin and IgG, the mechanism of uptake from plasma is less certain (Handagamma *et al*, 1989; Harrison *et al*, 1989).

Alpha-granular proteins which are synthesized by megakaryocytes are present in much higher concentration in platelets compared to plasma (George 1990). This may be an important mechanism to restrict or augment their actions at the sites of vessel injury. Additionally, the megakaryocyte-synthesized proteins may differ from the plasma forms of the same protein. For example, compared to plasma von Willebrand factor, the platelet von

Willebrand factor contains more of the high molecular weight multimers (Fernandez *et al*, 1982, Meyer *et al*, 1993) and binds with higher affinity to the platelet membrane (Williams *et al*, 1994).

1.5 The Identification of Multimerin (p-155):

A Novel, Activation-Dependent, Soluble Platelet Protein

The platelet glycoprotein multimerin (p-155) was first identified using a mouse monoclonal antibody, JS-1, that was raised against whole human platelets (Hayward *et al*, 1991a). The target of this monoclonal antibody was a platelet glycoprotein with a reduced mobility of 155 kDa (Hayward *et al*, 1991a). On the basis of this protein's mobility under reducing conditions, it was first designated as p-155 (Hayward *et al*, 1991a). Subsequent studies, contained within this thesis, identified the remarkable native multimeric structure of p-155 and this information led to its designation as multimerin (Hayward *et al*, 1991b).

Before my thesis project began, I had identified several features of p-155 that indicated p-155 was a unique platelet protein. The p-155 protein recognized by JS-1 was found to comigrate with glycoprotein Ia on reduced SDS-PAGE (Hayward *et al*, 1991a). However, glycoprotein Ia and p-155 were found to migrate differently using isoelectric focusing gels, and unlike glycoprotein Ia, p-155 was not complexed with glycoprotein IIa. Most importantly, immunodepletion studies indicated that p-155 was not glycoprotein Ia (Hayward *et al*, 1991a).

Investigations of the mobility of p-155, using nonreduced SDS-PAGE, indicated that p-155 was a disulfide-linked multimeric protein (Hayward *et al*, 1991a). Variability was observed in the size of the nonreduced, p-155 protein, indicating that it was composed of variably sized multimers (Hayward *et al*, 1991a). Using very low percentage acrylamide gels, the molecular weight of the nonreduced p-155 protein could not be determined, as it contained high molecular weight multimers that were too large to enter the resolving gel (Hayward *et al*, 1991a).

The initial investigations of this protein indicated that the expression of p-155 on the platelet surface was a marker of platelet activation (Hayward *et al*, 1991a). Quantitative studies of resting and thrombin stimulated platelets, using the radiolabeled monoclonal antibody, determined that the binding sites for JS-1 increased from 600 copies (resting platelets) to 4100 copies per platelet following activation (Hayward *et al*, 1991a). Investigation of the activation-associated changes in p-155 expression indicated that p-155 was a soluble protein (Hayward *et al*, 1991a). First, using Triton X-114 detergent partitioning to separate hydrophobic and hydrophilic platelet proteins (Clemetson *et al*, 1984), p-155 partitioned with the soluble proteins into the hydrophilic phase (Hayward *et al*, 1991a). Second, the p-155 bound to the surface of activated platelets could be eluted from the membrane by high concentration salt solutions, indicating that it was not an integral membrane protein (Hayward *et al*, 1991a). Third, p-155 was identified in the microparticle-free fraction of platelet releasate (Hayward *et al*, 1991a). On the basis of these findings, I postulated that p-155 was a soluble protein, stored

within platelet granules, that was released from intracellular stores during activation and then bound to the activated platelet surface.

The soluble nature of the p-155 protein suggested that it could be a plasma protein or a megakaryocyte synthesized protein. Using immunoblot analyses, no p-155 was detectable in the plasma or in other blood cells, suggesting that it might be synthesized by megakaryocytes (Hayward *et al*, 1991a).

Although these first studies of p-155 indicated that it was a unique platelet protein, other key questions had not yet been answered. The relationship between p-155 and other large, soluble, disulfide-linked multimeric platelet proteins (i.e., thrombospondin and von Willebrand factor) had not been investigated. In addition, important features of this protein, including its nonreduced structure, cell(s) of origin, biosynthesis, primary structure and function, were not yet known.

1.6 Objectives of the Current Study

The aim of this thesis project was to characterize p-155, which was later designated as multimerin (Hayward *et al*, 1991b). I postulated that knowledge of a new, and potentially unique, platelet activation marker might provide additional insights into the molecular mechanisms of hemostasis.

The first goal was to determine if p-155 (multimerin) was a unique, soluble, multimeric platelet protein. The next goal was to characterize the protein in detail, including investigations of its biosynthesis and amino acid sequence. The specific intentions of these studies were to:

- 1) Determine if p-155 is a novel platelet protein by comparing p-155 (multimerin) with the other known soluble, multimeric proteins that are stored within platelets and bind to activated platelets.
- 2) Evaluate the nonreduced, multimeric structure of p-155 (multimerin).
- 3) Determine if the multimerin stored within platelets originates from endogenous biosynthesis by megakaryocytes, and, if other cell types synthesize this protein. This objective included studies of multimerin biosynthesis using a megakaryocytic cell line and cultured human umbilical vein endothelial cells.
- 4) Obtain the deduced amino acid sequence of multimerin by isolating, cloning and sequencing the multimerin cDNA.

1.7 Thesis Outline

This thesis contains five manuscripts that represent my work to characterize multimerin. These include both published (Hayward *et al*, 1991a; Hayward *et al*, 1991b; Hayward *et al*, 1993, Hayward *et al* 1995c) and submitted manuscripts (Hayward *et al*, submitted for publication 1995b).

Chapter 2 contains an excerpt from the manuscript "p-155, a Multimeric Platelet Protein That Is Expressed on Activated Platelets" (Hayward *et al*, 1991a). This section describes the investigations that established p-155 as a unique, multimeric protein found in platelets. As part of this work, a procedure for preparing affinity purified p-155 was developed and the purified material was used to raise polyclonal antibodies against

p-155. Electrophoretic mobility and immunochemical techniques were used to distinguish p-155 from thrombospondin and von Willebrand factor. These investigations identified p-155 as a novel, multimeric platelet protein.

Chapter 3 includes the manuscript entitled "Multimerin: A Series of Large Disulfide-Linked Multimeric Proteins Within Platelets" (Hayward *et al*, 1991b). This chapter deals with the nonreduced structure of p-155, leading to the resolution of its multimeric structure. The discovery of its variably sized nonreduced structure led me to redesignate p-155 as multimerin - to reflect the multimeric structure of the native protein. This chapter describes comparisons of multimerin's size and multimeric structure with von Willebrand factor, thrombospondin and other platelet proteins. These studies identified multimerin as one of the largest proteins found in platelets and one of the largest proteins in the body.

Chapter 4 describes studies of multimerin biosynthesis. This chapter includes the manuscripts, "Multimerin is Found in the α -Granules of Resting Platelets and Is Synthesized by a Megakaryocytic Cell Line" (Hayward *et al*, 1993) and "Endothelial Cells Store Multimerin in Weibel-Palade Bodies and Possess Both Constitutive and Regulated Pathways for Multimerin Release" (Hayward *et al*, submitted for publication, 1995b). The investigations contained within this chapter identified the cells that synthesize multimerin. In addition, these studies investigated the glycosylation of multimerin and the relationships between the reduced major (155 kDa) and minor (170 kDa) subunits in the platelet multimerin multimers. A larger precursor protein was identified during the biosynthesis studies. Biosynthesis of multimerin

was shown to involve glycosylation of the precursor protein; proteolysis to a smaller polypeptide; and the formation of interchain disulfide bonds. Two cell types were shown to synthesize multimerin: a human megakaryocytic cell line and human endothelial cells. The identification of human cells that synthesized multimerin laid the groundwork for subsequent investigations of the multimerin cDNA. The manuscripts in chapter 4 contain immunohistochemistry and immunocytochemistry studies, performed by my co-authors and collaborators, that are not part of my thesis project. The insight and knowledge gained from these studies is gratefully acknowledged.

Chapter 5 describes the isolation, cloning and sequencing of the cDNA for human endothelial cell multimerin. This section of the thesis contains the manuscript "The cDNA Sequence of Human Endothelial Cell Multimerin: A Unique Protein with RGDS, Coiled-Coil, and EGF-like Domains and a Carboxyl-Terminus Similar to the Globular Domain of Complement C1q and Collagens Type VIII and X" (Hayward *et al*, 1995c). These studies were used to obtain the deduced amino acid sequence of multimerin. This work ultimately established multimerin as a unique protein, distinct from all previously sequenced proteins. Included in this chapter are Northern analyses that confirmed multimerin expression by endothelial cells and megakaryocytes. The identification of multimerin mRNA within platelets confirmed the hypothesis that platelet multimerin originates from endogenous biosynthesis by megakaryocytes. Included in this work are analyses of the deduced amino acid sequence, and a description of the identified multimerin subunit protein domains.

Chapter 6 discusses the thesis project, the implications of the scientific findings and their relevance to our knowledge of platelet proteins. Included in the Appendix are the manuscripts "p-155, a Multimeric Platelet Protein That Is Expressed on Activated Platelets" (Hayward *et al*, 1991a), and a review article: "Multimerin: A Multimeric Protein Stored in Platelet Alpha-granules" (Hayward *et al*, 1995a)

CHAPTER 2
p-155 (MULTIMERIN), A NOVEL, SOLUBLE, DISULFIDE-LINKED
PROTEIN FOUND IN PLATELETS

2.1 Introduction

The scientific studies contained within the manuscript "p-155, a Multimeric Platelet Protein That is Expressed on Activated Platelets" (Hayward *et al*, 1991a) are the first published descriptions of p-155 as a novel platelet protein. These studies represent my original research. I am grateful to John G. Kelton and the members of his laboratory group, James W. Smith, Peter Horsewood and Theodore E. Warkentin, for providing me with the monoclonal antibody for investigation, and for their assistance and scientific guidance during these investigations.

The article "p-155, a Multimeric Platelet Protein That is Expressed on Activated Platelets" (Hayward *et al*, 1991a) contains work done as part of my doctoral thesis along with investigations that I performed before commencing my doctoral studies (summarized in section 1.3). The work accomplished as part of my thesis project is presented as an excerpt of the original paper in section 2.2 of this chapter. In addition, section 2.2 contains an unpublished figure illustrating the purification of multimerin. The original article, in its entirety, is presented in Appendix 1, and has been

reprinted with the permission of the American Society for Biochemistry and Molecular Biology.

My earlier investigations of p-155 indicated that it was a novel platelet protein, distinct from the 155 kDa platelet glycoprotein Ia (Hayward *et al*, 1991a). Studies comparing the expression of p-155 on the membrane of resting and activated platelets indicated that p-155 was a marker of platelet activation (Hayward *et al*, 1991a). Triton X-114 partitioning, membrane elution studies and immunoblot analyses of platelet releasate indicated that p-155 was a soluble protein (Hayward *et al*, 1991a). Despite its soluble nature, no p-155 was detectable in the plasma or in other blood cells (Hayward *et al*, 1991a). Studies of the p-155 protein using nonreduced SDS-PAGE indicated that it was a disulfide-linked multimeric protein with variability in multimer size (Hayward *et al*, 1991a). The nonreduced protein contained multimers that were too large to be resolved by SDS-PAGE (Hayward *et al*, 1991a).

The focus of the thesis studies described in this chapter was to determine if p-155 was a novel, multimeric protein by comparing this protein to the known multimeric proteins that are found in platelets. As part of these investigations, a method for preparing purified p-155 was developed. This material was used to generate polyclonal anti-p-155 antibodies. The specificity of this antisera was compared with JS-1, the monoclonal anti-p-155. The purified p-155 was also tested to determine if p-155 was recognized by antisera specific for other multimeric platelet proteins. These studies identified multimerin as a unique protein, distinct from thrombospondin and von

Willebrand factor, two other disulfide-linked multimeric proteins that are expressed on activated platelets.

2.2 p-155, a Multimeric Protein That Is Expressed on Activated Platelets

Catherine P. M. Hayward, James W. Smith, Peter Horsewood, Theodore E. Warkentin, and John G. Kelton

This article contains studies, performed as part of my thesis, that were published in the manuscript:

p-155, A Multimeric Platelet Protein That Is Expressed on Activated Platelets, **J. Biol. Chem.** (1991) 266: 7114-7120 (Appendix 1)

Platelets respond to a large number of stimuli by undergoing complex biochemical and morphological changes. These changes are involved in physiological processes including adhesion, aggregation, and coagulation. Recent investigations identified an activation-dependent platelet protein, recognized by the monoclonal antibody JS-1, which was distinct from the 155 kDa platelet glycoprotein Ia. Because of its soluble, disulfide-linked multimeric structure, investigations were performed to determine if the monoclonal antibody JS-1 recognized a unique protein, or an activation marker expressed by a known, soluble, multimeric platelet protein. JS-1 affinity chromatography and preparative SDS-PAGE were used to prepare purified p-155 and polyclonal antibodies were raised against the purified protein. Both JS-1 and the polyclonal antisera recognized a 155 kDa protein in

immunoblot analyses of platelet lysates. Affinity purified p-155 was not recognized by antisera to von Willebrand factor or to thrombospondin, and p-155 differed from von Willebrand factor and thrombospondin in its reduced, subunit size. These studies identify p-155 as a unique, soluble, multimeric protein. The functional role of p-155 in platelets is not yet known.

INTRODUCTION

A variety of physiological and nonphysiological stimuli can initiate platelet activation. Platelet activation is associated with membrane alterations that allow platelets to bind to each other and to the vessel wall. The activation-induced alterations in platelet proteins can be categorized into three major groups: 1) conformational alterations in glycoproteins present on both resting and activated platelets (1), 2) exposure of activation-associated integral membrane glycoproteins by the fusion of granule membranes with the surface membrane (2) and 3) the surface binding of glycoproteins released from granule stores with activation (3-7).

Recently, we described a novel, activation-dependent multimeric platelet glycoprotein (M_r 155,000, reduced) that is present in platelet releasate and also becomes associated with the surface of platelets upon activation (8). The p-155 protein was found to exist, in the native state, as a series of varying sized, large multimers held together by disulfide bonds (8). Although p-155 and platelet glycoprotein Ia were found to migrate similarly on reduced sodium dodecyl sulfate-polyacrylamide gel electrophoresis,

immunodepletion and isoelectric focusing distinguished p-155 from glycoprotein Ia (8). Evidence for a soluble, nonintegral membrane-associated protein was obtained by Triton X-114 phase separation studies, membrane elution studies, and by demonstrating p-155 in the aqueous phase of platelet releasate (8). The p-155 protein was not present in detectable quantities in other blood cells or in plasma (8).

In this report, we describe affinity purification of p-155 and the preparation of polyclonal antisera specific for this protein. Investigations comparing the p-155 protein to known soluble, multimeric proteins that are stored in platelets, identified p-155 as a novel platelet protein.

METHODS AND MATERIALS

Monoclonal and Polyclonal Antibodies - The antibodies used for investigation included the murine monoclonal antibodies JS-1 (anti-p-155), and CH-1 (anti-thrombospondin) (8) and polyclonal antibodies against human von Willebrand factor (Dako Corp., Carpinteria, CA).

Preparation of Platelet Lysate - Blood from aspirin-free healthy volunteers was collected into acid citrate dextrose (ACD) (1:6, v:v). Platelets were gel-filtered in calcium-free Tyrode's buffer, pH 7.4, containing 0.35 g/100 ml bovine serum albumin, PGE₁ (0.3 μ M), and theophylline (1 mM). For immunoblotting, the gel-filtered platelets were pelleted and resuspended in lysing buffer containing 1% Triton X-100, 20 mM Tris HCl, 100 mM NaCl, pH 7.4, with 6.7 mM EDTA, 0.1 μ M leupeptin, 0.2 mM phenylmethylsulfonyl fluoride, 0.02 mg/liter soybean trypsin inhibitor, and 5 mM N-ethyl-

maleimide (final platelet concentration: 1×10^9 /ml). For some studies, Triton X-114 lysates (1%; final platelet concentration: 1×10^9 /ml) were prepared, to allow separation of hydrophilic platelet proteins from integral membrane proteins (9). Lysates (1% Triton X-114, 4 °C) were centrifuged ($30,000 \times g$ for 30 min, 4 °C) to remove cytoskeleton, and the lysate was incubated at 37 °C for 15 min to allow phase partitioning. The lysate was centrifuged ($110 \times g$ for 10 min, 24 °C) and the aqueous phase, containing p-155, was collected.

Affinity Purification of p-155 - Affinity purification of p-155 was performed using 5 mg of JS-1 linked to CNBr-activated Sepharose 4B (Pharmacia LKB, Uppsala, Sweden) according to the manufacturer's instructions. The aqueous phase of Triton X-114 lysate from 20 units of recently outdated Red Cross platelets (7-10 days old) was used to affinity purify p-155. Prior to eluting the platelet proteins bound to JS-1, the column was washed with 20 column volumes of PBS, pH 7.4 containing 5 mM N-ethylmaleimide. The bound proteins were eluted with 3.0 M magnesium chloride (elution volume 3-6 mls) and then dialyzed overnight against PBS containing 5 mM N-ethylmaleimide. The eluate was analyzed by reduced SDS-PAGE (50 μ l of purified protein/lane), silver staining and immunoblotting, using JS-1.

Preparation of Polyclonal Antisera to p-155 - Preparative SDS-PAGE was used in the final purification step before to immunization. The affinity purified p-155, prepared from 20 units of outdated platelet concentrates, was subjected to reduced SDS-PAGE (7%) in a single lane gel and transferred to nitrocellulose. A strip of nitrocellulose was analyzed by immunoblotting

with JS-1 to localize p-155 on the membrane. The region of the nitrocellulose containing p-155 was cut out of the membrane, dissolved in dimethyl sulfoxide, and used for immunization of rabbits as described (10).

Immunoblotting - Platelet lysates (50 μ l, 10^9 platelets/ml) were mixed with 2X reducing sample buffer (50 μ l of 0.125 M Tris, 0.1% glycerol, 2% SDS, 10% 2-ME, pH 6.8) and boiled for 2 minutes before separation on SDS-PAGE as described (11). Immunoblot analyses were performed as described (12). Briefly, the proteins were transferred to nitrocellulose, and the membrane was blocked with 3% bovine serum albumin in PBS, pH 7.4 before incubation with antibodies, diluted in 1% bovine serum albumin/PBS with 0.1% Tween 20. The antibody bound to the transblotted platelet protein was detected using a 1:750 dilution of alkaline-phosphatase-conjugated goat anti-mouse antibody or goat anti-rabbit (Jackson ImmunoResearch, BioCan Scientific Inc., Mississauga, Ontario, Canada).

RESULTS

Affinity Purification of p-155 - Silver stain analysis of the eluate from the JS-1 (monoclonal anti-p-155) affinity column identified a major band at 155 kDa, which was also recognized by JS-1 (Fig. 1). Compared to the starting material, only small quantities of other proteins were present in the affinity eluate. Because contaminating proteins were identified in the affinity purified p-155, preparative SDS-PAGE was used as the final purification step for generation of polyclonal antisera.

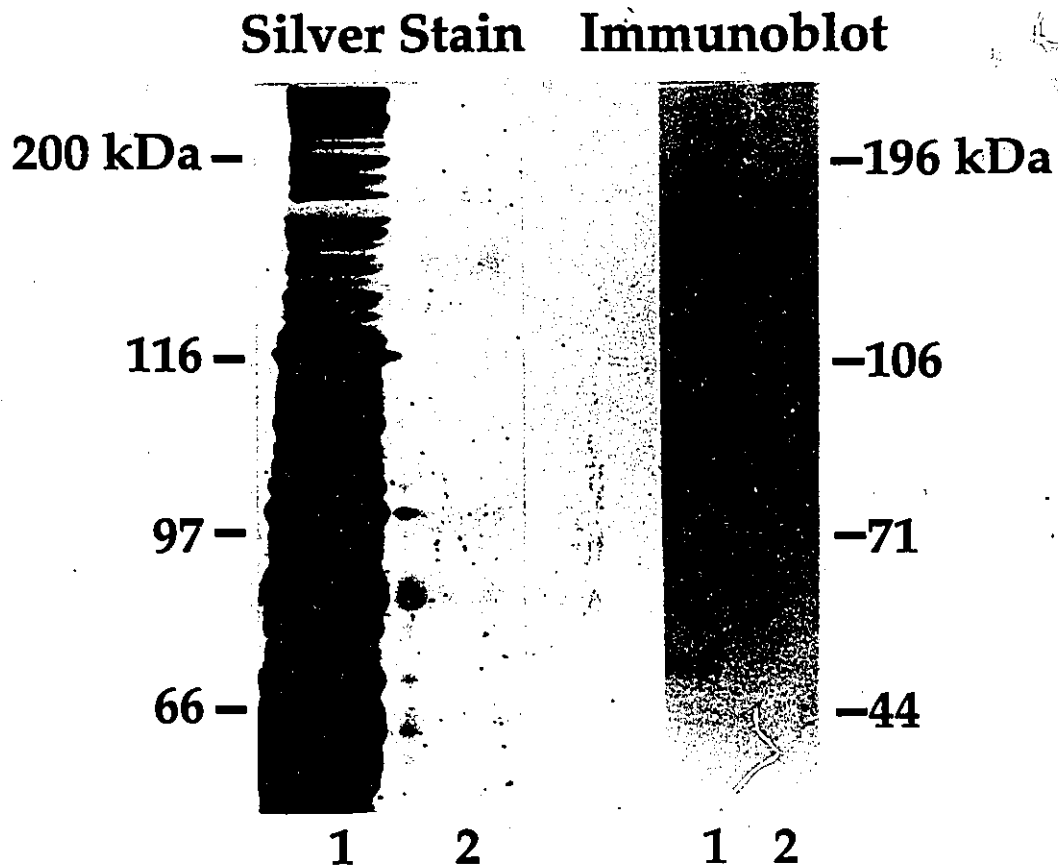


Figure 1 Silver Stain and Immunoblot Analyses of Affinity Purified p-155

The Triton X-114 extract of platelet lysate (lane 1) and JS-1 (monoclonal anti-p-155) affinity column eluate (lane 2) are compared. Samples were separated using reduced SDS-PAGE followed by silver stain analysis (left panel) or transferred to nitrocellulose for immunoblot analysis (right panel) using JS-1. The major protein identified in the affinity column eluate has a reduced mobility of 155 kDa. Immunoblot analysis indicates that the major protein in the affinity eluate is p-155.

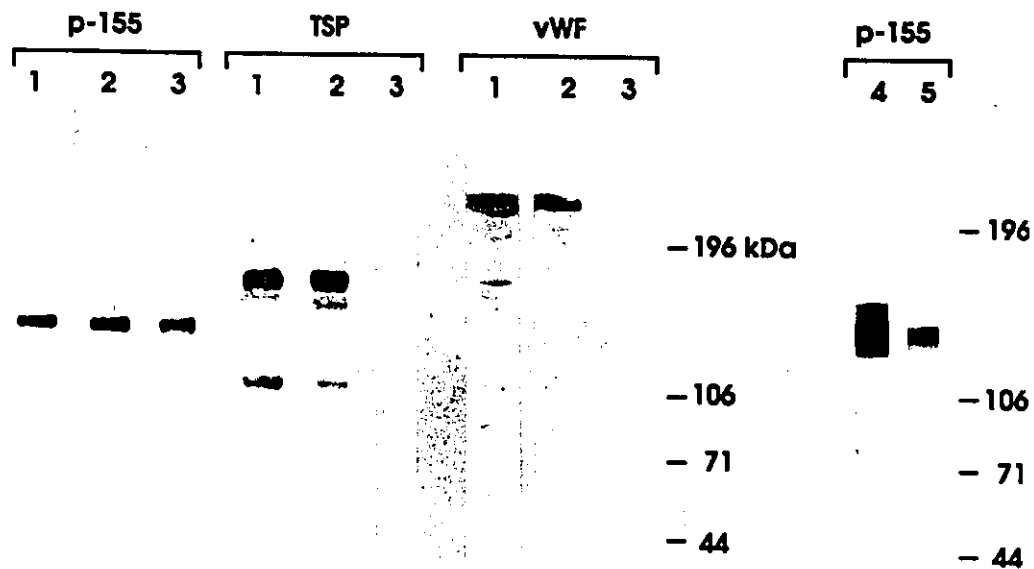


Figure 2 Comparison of the Reduced Subunits of p-155, Thrombospondin (TSP) and von Willebrand factor (vWf). This immunoblot demonstrates the different mobilities of p-155, thrombospondin, and von Willebrand factor. Samples were subjected to SDS-PAGE (reduced) followed by transfer to nitrocellulose and immunoblotting with JS-1 (anti-p-155), CH-1 (anti-thrombospondin), and anti-von Willebrand factor. Samples included resting platelet lysate (1% Triton X-100) (lane 1), Triton X-114 platelet lysate, aqueous phase (lane 2), and affinity-purified p-155 (lane 3). In lanes 4 and 5, the results of immunoblotting platelet lysate with JS-1 and polyclonal anti-p-155 (lane 5) are shown. This figure demonstrates that p-155 is not thrombospondin or von Willebrand factor.

Identification of the Platelet Protein Recognized by JS-1 - Immunoblot analyses of reduced platelet lysate using JS-1, identified a major band at 155 kDa. In addition, a minor band at 170 kDa was also identified by the monoclonal antibody (Fig. 2). The p-155 protein recognized by JS-1 was compared to thrombospondin and von Willebrand factor, two other large, multimeric disulfide-linked platelet proteins. Thrombospondin and von Willebrand factor differed from p-155 in their reduced subunit mobility. Furthermore, affinity purified p-155 was not recognized by antibodies to von Willebrand factor or to thrombospondin, further confirming the unique identity of this platelet protein.

Characterization of the Polyclonal Antibodies Raised Against Affinity Purified p-155 - Immunoblotting, using polyclonal antibodies raised against affinity purified p-155, identified a platelet protein with the same mobility as the p-155 recognized by JS-1 (Fig. 2). The polyclonal antisera was reactive with p-155 in immunoblot analyses at dilutions of 1:1000 (Fig. 2) to 1:10,000. These findings indicate that JS-1 recognizes a unique, p-155 platelet glycoprotein, and not a degradation product of either thrombospondin or von Willebrand factor.

DISCUSSION

Platelet activation can result in alterations to the surface structure of platelets and the binding of platelet and plasma proteins to the surface of these altered platelets. The study of these changes on the platelet surface can provide information not only about platelet physiology, but some changes

can act as markers of platelet activation in disease states. In this report, we describe a unique soluble, multimeric protein, p-155, that is expressed on the surface of activated platelets.

The p-155 protein was first identified using a monoclonal antibody (JS-1) that we had raised against whole platelets (8). The p-155 protein recognized by this antibody was found to exist in the native state as varying sized, large multimers composed of repeating subunits held together by disulfide bonds (8). We were unable to assign a size to the native protein, but comparison with thrombospondin suggested that the smallest multimer was a triplet (8). Triton X-114 partitioning and membrane elution studies indicated that p-155 was a soluble protein and immunoblot analyses identified p-155 in the microparticle-free fraction of platelet releasate (8). Studies of resting and activated platelets indicated an increased expression of p-155 with platelet activation, with the number of binding sites for JS-1 increasing from 600 to 4100 molecules/platelet (8). Although p-155 comigrated with the integral membrane glycoprotein Ia on reduced SDS-PAGE, immunodepletion and isoelectric focusing experiments indicated that p-155 was distinct from glycoprotein Ia (8).

Certain features of the p-155 protein recognized by JS-1 resemble a protein described by Bienz and Clemetson (13) and designated as Ia*. Similarities include migration characteristics on two-dimensional (nonreduced/reduced) SDS-PAGE, activation-induced surface expression, and the presence of both proteins in releasate. However, other aspects are quite different; p-155 is easily detected in the Triton-soluble phases of lysate

prepared from either resting or activated platelets (8). In contrast, Ia* was associated with the Triton-insoluble fraction of resting platelets (13). In addition, p-155 has an isoelectric point of 5.4-6.75 (8) whereas Ia* has an isoelectric point of 4.5-5.5 (13).

The intent of our studies was to prepare purified p-155, to generate polyclonal antisera specific for this protein, and to compare p-155 with the known soluble, disulfide-linked, multimeric platelet proteins. Using the aqueous phase of a Triton X-114 extract of platelets as the starting material, JS-1 affinity purification, and preparative SDS-PAGE, p-155 was isolated from the other platelet glycoproteins. Immunization of rabbits with this purified material resulted in the generation of polyclonal antisera, specific for p-155. Comparison of the immunoblot reactivity of JS-1 and the polyclonal anti-p-155 indicated that both antisera identify the same platelet protein.

Thrombospondin and von Willebrand factor are two soluble, disulfide-linked multimeric proteins that are stored within platelets (5, 6). With platelet activation, thrombospondin and von Willebrand factor are released and become bound to the platelet membrane (5, 6). Because of the similar disulfide-linked multimeric structure of p-155, we compared p-155 to thrombospondin and von Willebrand factor to determine if JS-1 recognized a novel platelet protein. Differences were seen in the reduced mobility of p-155, thrombospondin and von Willebrand factor. Furthermore, affinity purified p-155 was not recognized by antisera to either thrombospondin or von Willebrand factor, indicating that it was a unique protein. Finally, both monoclonal (JS-1) and polyclonal antibodies against p-155 identified the same

protein, indicating that the monoclonal antibody recognizes a different platelet glycoprotein and not a degradation product of thrombospondin or von Willebrand factor.

These studies establish p-155 as a novel, multimeric platelet protein, distinct from the known multimeric proteins in platelets. The activation-dependent nature of the membrane association of p-155 suggests that this multimeric protein may have a physiologic role in platelet activation. The functions of p-155 are currently under investigation.

REFERENCES

1. Collier, B. S. (1985) *J Clin Invest* 76, 101-108.
2. McEver, R. P., and Martin, M. N. (1984). *J Biol Chem* 259, 9799-9804.
3. Bennett, J. S, and Villaire, G. (1979). *J Clin Invest* 64, 1393-1401.
4. Fujimoto, T, Ohara,, S., and Hawiger, J. (1982). *J Clin Invest* 69, 1212-1222.
5. Parker, R. I., and Galnick, H. R. (1986). *Blood* 68, 732-736.
6. Aiken, M. L., Ginsberg, M. H., and Plow, E. F. (1987). *Blood* 69, 58-64.
7. Asch, M. L., and Podack, E. (1990). *J Clin Invest* 85, 1372-1378.
8. Hayward, C. P. M., Smith J. W., Horsewood, P., Warkentin, T. E., and Kelton, J. G. (1991). *J Biol Chem* 266, 7114-7120.
9. Bordier, C. (1981). *J Biol Chem* 256, 1604-1607.
10. Chiles, T. C., O'Brien, T. W., and Kilberg, M. S. (1987). *Anal Biochem* 163, 136-142.
11. Kelton, J. G., Smith, J. W., Horsewood, P., Humbert, J. R., Hayward, C. P. M., and Warkentin, T. E. (1990). *Blood* 75, 2172-2176.

12. Kelton, J. G., Smith, J. W., Santos, A. V., Murphy, W. G., and Horsewood, P. (1987). *Am J Hematol* 25, 299-310.
13. Bienz, D., and Clemetson, K. J. (1989). *J Biol Chem* 264, 507-514.

CHAPTER 3
INVESTIGATION OF THE MULTIMERIC STRUCTURE OF
MULTIMERIN

3.1 Introduction

My initial investigations of p-155 focused on determining if p-155 was a novel platelet protein. These studies demonstrated that p-155 was a soluble protein, stored within platelets, that is released following activation (Hayward *et al*, 1991a). Comparisons with von Willebrand factor and thrombospondin indicated that p-155 was an undescribed, disulfide-linked multimeric platelet protein (Hayward *et al*, 1991a). My early studies of the native p-155 indicated that it was a multimeric protein with variable molecular weight, however, its multimeric structure was difficult to resolve (Hayward *et al*, 1991a). Immunoprecipitates of p-155 contained material that was too large to enter even the lowest concentration, nonreduced polyacrylamide gels (Hayward *et al*, 1991a; Appendix 1) and heterogeneity was observed in its nonreduced mobility.

Consequently, the next studies focused on determining the multimeric composition of the p-155 protein. These investigations are described in the manuscript "Multimerin: A Series of Large Disulfide-Linked Multimeric Proteins Within Platelets" (Hayward *et al*, 1991b). The original article was published in the journal *Blood* and has been reprinted with the

permission of W. B. Saunders Co. I am grateful to Drs. Theodore E. Warkentin, and Peter Horsewood for their intellectual contributions.

Electrophoretic techniques, capable of sieving proteins greater than a million daltons in size, were used to determine the multimeric structure of p-155. Using a combination of agarose/acrylamide gel electrophoresis, radioimmunoprecipitation and immunoblot analyses, p-155 was resolved into a series of variably sized, disulfide-linked multimers. All the different sized multimers were found to be comprised of a predominant, p-155 subunit with smaller amounts of a p-170 subunit detected. We noted differences in the nonreduced mobilities of multimerin and von Willebrand factor, which were consistent with their assembly from different-sized subunit proteins. The smallest multimer of p-155 was a trimer. These studies also showed preferential binding of the largest p-155 multimers to the surface of platelets after activation.

Based upon the unusual migration characteristics of the p-155 protein, the native molecule was designated as multimerin, to reflect its massive, multimeric structure.

Multimerin: A Series of Large Disulfide-Linked Multimeric Proteins Within Platelets

Catherine P.M. Hayward, Theodore E. Warkentin, Peter Horsewood, and John G. Kelton

Platelets contain proteins with biochemical properties that are well adapted to promoting hemostasis. One important adhesive protein is von Willebrand factor (vWF), which is a very large protein comprised of a series of multimers, ranging from 860,000 to over 10 million daltons. In this report we describe a second platelet protein, p-155, which has a similar unique multimeric composition. Using agarose-acrylamide gel electrophoresis, platelet p-155 was shown to be composed of multimers ranging from less than 450 Kd to many million daltons. Based on this unique structure, we propose that the native molecule be designated as multimerin. Com-

A NUMBER OF PROTEINS are composed of identical subunits linked by disulfide bonds. The number of subunits may vary, some proteins are dimers (IgG), others are trimers (thrombospondin), and some are pentamers (IgM); but all of these proteins are made of a predictable number of subunits. There is an exception: von Willebrand factor (vWF) is unique in that it has a variable multimeric size. This protein exists as a series of multimers with molecular weights extending up to 20 million daltons¹ making this the largest platelet protein. The structure of vWF facilitates its role as an adhesive protein linking platelets to the vessel wall.²

To date, no other platelet protein existing as a series of multimers has been identified. In this report, we describe a second, extremely large platelet protein, p-155, which exists in the native state as a series of disulfide-linked multimers ranging in size from less than 450 Kd to many million daltons. We propose that the native protein be designated as multimerin to reflect this unusual structure. Platelets contain two soluble proteins with a complex multimeric composition, multimerin and vWF.

MATERIALS AND METHODS

Platelet preparation. Platelet lysate (1% sodium dodecyl sulfate [SDS], 2.5×10^9 platelets/mL) and releasate were prepared according to methods previously described.³ Following thrombin activa-

tion, 0.1 mmol/L leupeptin, 0.2 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 5 mmol/L N-ethyl maleimide were added to platelet releasate to prevent proteolysis. Surface radiolabeled activated platelets were prepared as described.³ Briefly, washed platelets (1×10^9 /mL) were activated for 10 minutes with 1 U/mL of bovine thrombin (Sigma, St Louis, MO), without agitation, then recombinant hirudin (Sigma), 4 U/mL, was added. Platelets were washed twice, surface radiolabeled with ¹²⁵I sodium iodide using lactoperoxidase,⁴ lysed, and subjected to immunoprecipitation.^{3,4}

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Subunit analysis. Immunoprecipitates of p-155 were prepared using monoclonal antibody (MoAb) JS-1 and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).³ JS-1 was raised by immunizing Balb/c mice with human platelets and identifies the p-155 protein by both immunoprecipitation and immunoblotting.³

Multimer analysis. Agarose-acrylamide gels were prepared using a modification of the method described by Ruggeri and Zimmerman,⁵ except that the stacking gel was omitted. Gels containing 1% acrylamide and 1.25% agarose (SeaPlaque agarose; FMC Bio-Products, Rockland, ME) or 1.5% acrylamide and 1.25% agarose were prepared using the described running gel buffers. Gels were polymerized with ammonium persulfate (0.025%) and N,N,N',N'-tetramethylethylenediamine (TEMED) (0.05%).

Samples were diluted in the agarose/acrylamide sample buffer (final concentrations: 10 mmol/L Tris, 2% SDS, 8 mol/L urea, 0.005% bromophenol blue; pH 8.0). Thirty-microliter aliquots were applied to each well. For immunoblotting experiments, the lanes were loaded with releasate (from 2×10^9 platelets), platelet lysate (1% SDS, 5×10^7 platelets), or 6 µg of human IgM (Sigma). Radioimmunoprecipitates were prepared with JS-1, washed, and eluted with agarose/acrylamide sample buffer (60 µL sample buffer/50 µL of packed Protein A beads). Gels were run at 25 V, constant voltage for 16 hours or until the buffer front was within 1 to 2 cm of the anodal end of the gel. After electrophoresis, gels were either transferred to nitrocellulose for immunoblotting or fixed with 15% trichloroacetic acid (TCA), dried (2 hours at 72°C) with cellophane membrane backing (Bio-Rad, Richmond, CA), and analyzed by autoradiography.

For two-dimensional gel electrophoresis (nonreduced/reduced), a lane from the agarose/acrylamide gels was cut from the gel, reduced for 10 minutes in SDS-PAGE sample buffer (0.0625 mol/L Tris, 0.05% glycerol, 1% SDS, pH 6.8), containing 5% 2-mercaptoethanol (2-ME) and 20% methanol, and transferred to the top of a 4% to 8% SDS-polyacrylamide gel. The agarose/acrylamide gel slice was abutted on the stacking gel, covered with reducing sample buffer, and electrophoresed.

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PLATELET MULTIMERIN

Immunoblotting was performed as described¹ using primary antibodies JS-1 (monoclonal anti-p-155),² rabbit anti-p-155, rabbit anti-vWF (Dako Corporation, Carpinteria, CA), CH-1 (monoclonal anti-thrombospondin),³ and alkaline phosphatase-conjugated antihuman IgM (Jackson ImmunoResearch, Biocan Scientific Inc, Mississauga, Ontario, Canada). The binding of the primary antibody to the transblotted protein was detected using alkaline phosphatase-conjugated goat antimouse IgG or goat antirabbit IgG (Jackson ImmunoResearch).

Silver staining was used to detect platelet proteins separated by nonreduced agarose/acrylamide gel electrophoresis followed by reduced SDS-PAGE.⁴

RESULTS

Investigation of the native structure of multimerin by agarose/acrylamide gel electrophoresis demonstrated the complex multimeric structure of this protein. Analysis of the nonreduced multimerin in platelet lysate by agarose/acrylamide gel electrophoresis followed by immunoblotting demonstrated discrete multimers of varying size (Figs 1 and 2). When analyzed by a 1.5% acrylamide/1.25% agarose gel, the smallest multimer of multimerin was demonstrated to migrate faster than thrombospondin (Mr 450 Kd, nonreduced⁷) (Fig 2). Under the same conditions, the second multimer migrated at a similar position to IgM. The majority of the multimers had a slower migration than IgM. Comparison with vWF in the same platelet lysate demonstrated differences both in multimer spacing and in the range of size distribution. The multimers were spaced closer than those of vWF and the multimers of multimerin tended to be smaller than the multimers of vWF. We were unable to assign specific molecular weights to the individual multimers because the R_f plots were not linear. The different migration positions of vWF and multimerin multimers confirm the unique identity of these two proteins.

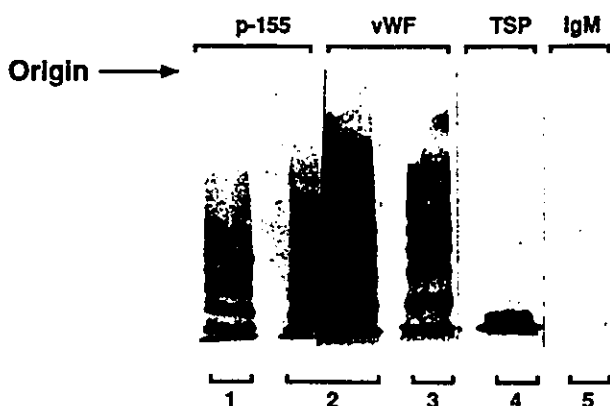


Fig 1. Comparison of the multimeric composition of multimerin and vWF. Platelet lysate (lanes 1 through 4) and purified IgM (lane 5) were separated on gels containing 1% acrylamide and 1.25% agarose. Following transfer to nitrocellulose, strips were subjected to immunoblotting using JS-1 (anti-p-155, lanes 1 and 2), anti-vWF (lanes 2 and 3), CH-1 (anti-thrombospondin, lane 4), and anti-IgM (lane 5). Lane 2 was a single, wide lane, bisected following transfer to nitrocellulose. This study demonstrates that multimerin resembles vWF in its complex multimeric composition but differs in multimer spacing and size distribution.

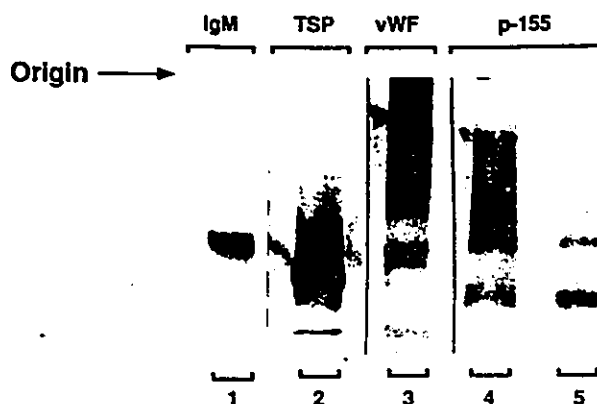
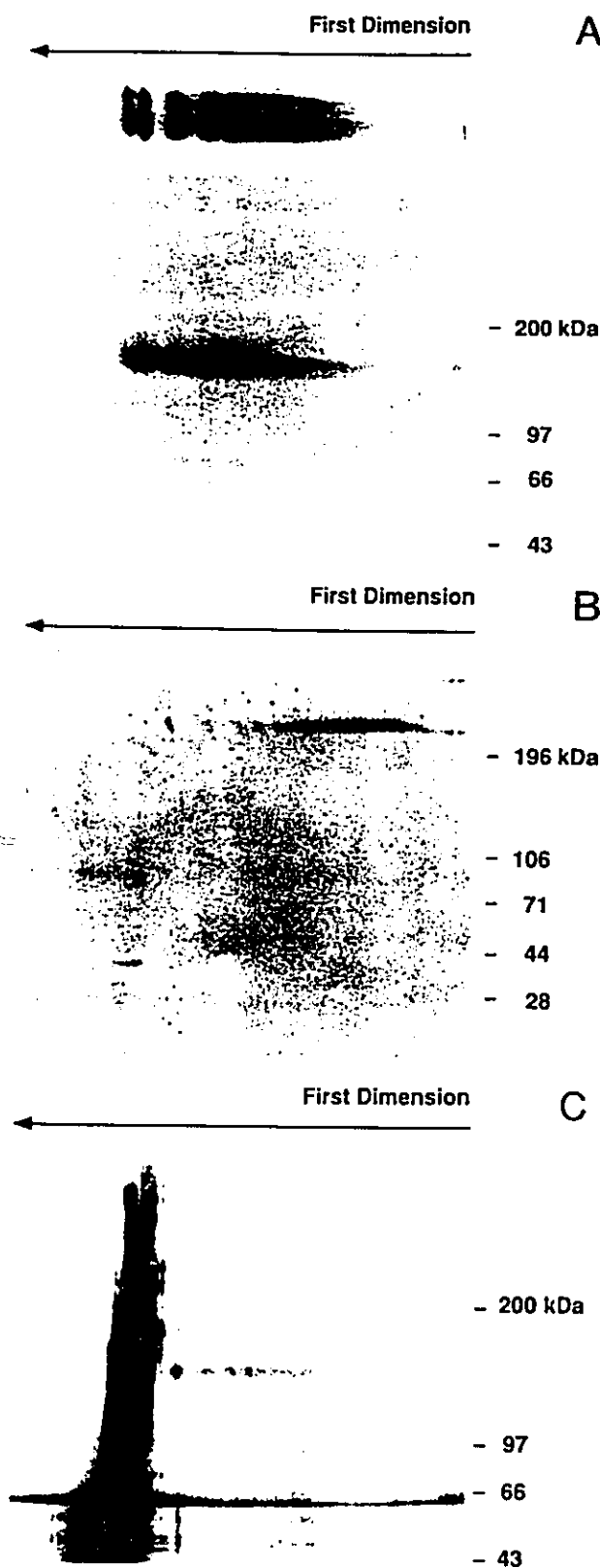


Fig 2. Resolution of the small multimers of multimerin by agarose/acrylamide gel electrophoresis. Platelet lysate (lanes 2 through 4), platelet releasate (lane 5), and purified IgM (lane 1) were separated on 1.5% acrylamide gels containing 1.25% agarose, transferred to nitrocellulose, then subjected to immunoblotting with anti-IgM (lane 1), CH-1 (anti-thrombospondin, lane 2), anti-vWF (lane 3), and JS-1 (anti-p-155, lanes 4 and 5). This study demonstrates that the smallest multimer of multimerin is smaller than thrombospondin and that platelet releasate contains mainly the small multimers compared with the native, internal protein.

The size of the intracellular multimerin was compared with the released protein. Platelets were lysed in the presence of proteolytic inhibitors. Other platelets were activated with thrombin and the releasate treated with the same proteolytic inhibitors. We have previously demonstrated that this does not alter the size of the p-155 subunit.³ The releasate contained more of the smaller multimers than platelet lysate (Fig 2). Multimers on the platelet surface were assessed by immunoprecipitation of surface ¹²⁵I-labeled, thrombin-activated platelets followed by agarose/acrylamide gel electrophoresis and autoradiography (Fig 3A, top). Multimerin expressed on the platelet surface showed a similar multimer pattern compared with the whole platelet lysate (Fig 1).

We have previously used differential reduction and SDS-PAGE to demonstrate variability in the native molecular weight of p-155.³ Most of the reduced protein is the 155-Kd subunit, but there is a small amount of a 170-Kd subunit (Fig 4). This suggests that partial reduction should generate three different sizes of dimers arising from the three combinations of these two subunits. Using conditions of increasing reduction, three intermediary bands were noted that were larger than the fully reduced p-155 protein but were smaller than the smallest nonreduced multimer. These intermediary bands were not evident under nonreducing conditions (Fig 4, upper panel). Full reduction of the three bands generated 170-Kd bands, 155- and 170-Kd bands, and 155-Kd bands, respectively (Fig 4, lower panel). The band at 200 Kd (Fig 4, upper panel, lanes A and B) was not seen consistently in p-155 immunoprecipitates and was not identified as a component of the individual multimers (Fig 3A). These results indicate that the smallest multimer of multimerin is a trimer. This conclusion is also supported by the demonstration that the smallest multimerin multi-



mer migrates faster than thrombospondin in a nonreduced gel (Fig 2).

Multimerin immunoprecipitates were subjected to nonreduced agarose/acrylamide gel electrophoresis followed by reduced SDS-PAGE. These studies demonstrated that the individual multimers were composed of the 155-Kd subunits (Fig 3A). Immunoblotting using either monoclonal or polyclonal anti-p-155 yielded identical findings. The MoAb JS-1 also identified a smaller amount of a 170-Kd protein in either immunoprecipitates or immunoblots³ (Fig 4). We were unable to demonstrate a selective association of the 170-Kd subunit with any one size of multimer. Similar analysis of vWF (Fig 3B) demonstrated a subunit composition of 220 Kd as previously described.⁸ Comparison of multimerin with vWF (Fig 1, 2, 3A and B) demonstrated that they differed in subunit size, multimer spacing, and multimer size distribution.

We investigated whether platelets contained any other large proteins using silver staining and nonreduced/reduced separation. Two extremely large, complex multimeric platelet proteins were identified (Fig 3C) corresponding to multimerin and vWF. No other platelet proteins with these migration characteristics were demonstrated. Additionally, no other proteins larger than multimerin or vWF could be identified.

DISCUSSION

The largest platelet protein is vWF, which is composed of disulfide-linked homodimers of 200-Kd subunits.⁸ Unlike almost all other multimeric proteins, which are composed of a fixed number of subunits linked by disulfide bonds, vWF is a series of multimers that range in size from 860 Kd to as large as 20 million daltons.¹ In this report, we describe another extremely large, variably sized, multimeric platelet protein, p-155, that resembles vWF in its complex multimeric composition.

Recently, we reported that p-155 is a soluble platelet protein that is expressed in low numbers on the surface of

Fig 3. (A) The subunit composition of the individual multimers of multimerin. Multimerin was immunoprecipitated from thrombin-activated surface ¹²⁵I-radiolabeled platelet lysate using JS-1. The multimerin immunoprecipitates were subjected to electrophoresis on 1% acrylamide/1.25% agarose gels under nonreducing conditions (upper panel) followed by reduced SDS-PAGE (lower panel) and autoradiography. This experiment demonstrates that the individual multimers of multimerin are composed of identical subunits. (B) The subunit composition of vWF. Platelet lysate was subjected to electrophoresis on 1% acrylamide/1.25% agarose gels under nonreducing conditions followed by reduced SDS-PAGE and transfer to nitrocellulose. vWF was identified following immunoblotting with rabbit anti-vWF. This study demonstrates that vWF, unlike multimerin, is composed of 220-Kd subunits. (C) Demonstration of complex multimeric proteins found in platelets. Platelet lysate was subjected to electrophoresis on 1% acrylamide/1.25% agarose gels under nonreducing conditions followed by reduced SDS-PAGE and silver staining. Horizontal bands below 66 Kd represent nonspecific staining. Two large, disulfide-linked, complex multimeric proteins are demonstrated with subunits of 220 Kd and 155 Kd. These subunits correspond to multimerin and vWF.

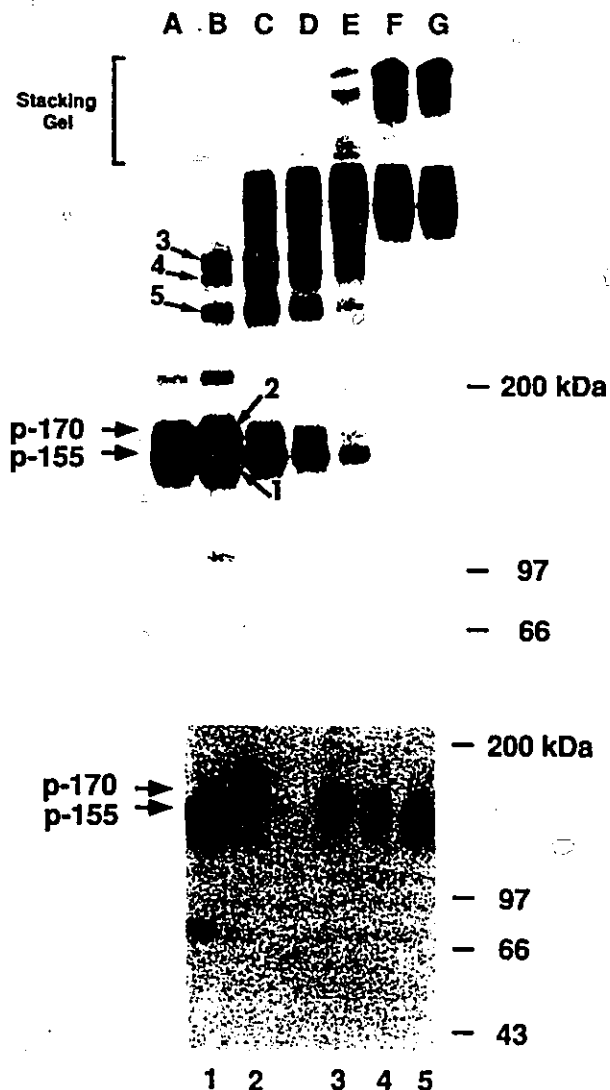


Fig. 4 Subunit composition of multimerin. Multimerin was immunoprecipitated from thrombin-activated surface 125 I-radiolabeled platelet lysate using JS-1. The multimerin immunoprecipitates were subjected to varying amounts of reduction and analyzed using 3% to 6% SDS-PAGE. The dithiothreitol (DTT) concentrations (mmol/L final) were: A-25, B-5, C-1, D-0.75, E-0.5, F-0.1, G-0. Bands labeled 1 through 5 (upper panel) were localized by autoradiography, cut from the gel, and electrophoresed under reducing conditions (lower panel). This experiment demonstrates that multimerin is composed of 155- and 170-Kd subunits and that partial reduction generates 170-170, 155-170, and 155-155 Kd dimers.

resting platelets (approximately 600 copies of JS-1 bound per platelet). However, following platelet activation, this protein is released and about 4,000 binding sites for JS-1 are expressed on the platelet surface.³ Further studies of the p-155 protein produced unexpected results and are the subject of this report. We found that p-155, like vWF, exists as a series of large multimers of increasing size. We suggest that the native protein, which is made of p-155 subunits, should be designated as multimerin, reflecting its multimeric composition.

Using nonreduced/reduced gel electrophoresis we demonstrated that all of the multimers are composed primarily of identical subunits (Fig 3A). Comparison with thrombospondin and IgM indicate that the multimers of p-155 range in size from a trimer of less than 450 Kd to many million daltons. The trimer migrates with an apparent molecular weight that is less than the predicted 465 Kd, based on three 155-Kd subunits. Other multimeric proteins, including thrombospondin,⁷ exhibit similar anomalous migration.

Multimerin is released by thrombin and its subunit size is unchanged by platelet activation³; however, platelet releasate contains much less of the high molecular weight multimers. The results suggest that the largest multimers of multimerin preferentially bind to the platelet surface. Similar findings have been reported for vWF.⁹

The unexpectedly massive size and unusual multimeric nature of multimerin led us to investigate whether platelets contained other large multimeric proteins besides multimerin and vWF. Platelet lysate was subjected to nonreduced/reduced electrophoresis followed by protein staining. Two large, disulfide-linked multimeric proteins were observed with subunit sizes of 220 Kd (vWF) and 155 Kd (multimerin), respectively. No other large proteins could be identified.

The body contains other extremely large proteins. For example, muscle contains several very large proteins, titan and nebulin.¹⁰ These proteins differ from the largest platelet proteins in that they are not multimeric. The multimeric nature of vWF and multimerin provides multiple functional sites for binding to the platelet surface and for binding to other ligands. Platelet proteins are well adapted to supporting platelet adhesion and aggregation. The contribution of multimerin to these events is under investigation.

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REFERENCES

1. Ruggeri ZM, Zimmerman TS: Variant von Willebrand's disease: Characterization of two subtypes by analysis of multimeric composition of factor VIII/von Willebrand factor in plasma and platelets. *J Clin Invest* 65:1318, 1980
2. Sakariassen KS, Bolhuis PA, Sixma JJ: Human blood platelet adhesion to artery subendothelium is mediated by factor VIII-von Willebrand factor bound to the subendothelium. *Nature* 279:636, 1979
3. Hayward CPM, Smith JW, Horsewood P, Warkentin TE, Kelton JG: p-155: A multimeric platelet protein that is expressed on activated platelets. *J Biol Chem* 266:7114, 1991
4. Kelton JG, Smith JW, Horsewood P, Humbert JR, Hayward CPM, Warkentin TE: Gov^b alloantigen system on human platelets. *Blood* 75:2172, 1990
5. Ruggeri ZM, Zimmerman TS: The complex multimeric composition of factor VIII/von Willebrand factor. *Blood* 57:1140, 1981
6. Merrill CR, Goldman D, Sedman SA, Ebert MH: Ultrasensi-

live stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. *Science* 211:1437, 1981

7. Lawler JW, Slayter HS, Coligan JE: Isolation and characterization of a high molecular weight glycoprotein from human blood platelets. *J Biol Chem* 253:8609, 1978

8. Wagner DD, Marder V: Biosynthesis of von Willebrand protein by human endothelial cells: Processing steps and their intracellular localization. *J Cell Biol* 99:2123, 1984

9. Fernandes MFL, Ginsberg MH, Ruggeri ZM, Battle FJ, Zimmerman TS: Multimeric structure of platelet factor VIII/von Willebrand factor. The presence of larger multimers and their reassociation with thrombin-stimulated platelets. *Blood* 60:1132, 1982

10. Horowitz R, Kempner ES, Bisher ME, Podolsky RJ: A physiological role for titin and nebulin in skeletal muscle. *Nature* 323:160, 1986

CHAPTER 4

STUDIES OF MULTIMERIN BIOSYNTHESIS

4.1 Introduction

Soluble proteins stored within platelet granules can originate from two sources: some platelet proteins are synthesized by megakaryocytes and are then stored within the platelet granules (Nachman *et al*, 1977; Ryo *et al*, 1983; George, 1990; Harrison *et al*, 1993b). Other proteins found in platelets are endocytosed from plasma and are then stored within platelet alpha-granules (Handagama *et al*, 1989; Harrison *et al*, 1989; Handagama *et al*, 1990; George, 1990; Harrison *et al*, 1993b). Most proteins that are synthesized by megakaryocytes are present at a much higher concentration in platelets than in plasma (George, 1990). Fibrinogen is the only known exception (George, 1990). Because multimerin could not be detected in plasma (Hayward *et al*, 1991a), this led me to postulate that multimerin was synthesized by megakaryocytes.

The identity of cell(s) that synthesize multimerin was next investigated. The human megakaryocytic cell line Dami cells (Greenberg *et al*, 1988) was chosen for the investigations of multimerin biosynthesis. In tandem with the studies of multimerin biosynthesis, investigations were undertaken to determine the relationship between p-155 and p-170, the two, different-sized multimerin subunits found in platelets. Protease digestions

and analyses of the carbohydrate components were used to explore the differences between these subunits, and to evaluate the processing of the multimerin subunits during biosynthesis. These studies are contained within the manuscript "Multimerin is Found in the α -Granules of Resting Platelets and Is Synthesized by a Megakaryocytic Cell Line" (Hayward *et al*, 1993). This paper was published in the Journal of Clinical Investigation and has been reprinted with the permission of the Society for Clinical Investigation. The manuscript contains immunohistochemical and immunoelectron microscopy investigations, performed by my collaborators, Dr. Ron H. Stead and Dr. Dorothy Ford Bainton, that are not part of my formal thesis.

These studies identified multimerin as a highly glycosylated platelet protein, containing primarily complex forms of N-linked carbohydrate. Using Cleveland mapping, peptide homology observed between p-155 and p-170, the multimerin subunits that were found in platelets. Deglycosylation studies of p-155 and p-170 indicated more complete proteolytic processing of p-155.

The studies of multimerin biosynthesis indicated the megakaryocytic cell line Dami cells synthesize multimerin following activation by PMA. Pulse-chase metabolic labeling studies and deglycosylation studies determined that multimerin originates from a larger polypeptide that undergoes N-glycosylation and proteolysis during biosynthesis. Cleveland mapping studies were used to study the differences between platelet and Dami cell multimerin. Peptide homology was observed

between the p-155 obtained from platelets and the larger, p-196, multimerin subunit that was secreted by Dami cells. Deglycosylation studies indicated more complete proteolysis of the multimerin found in platelets, although traces of the large precursor (p-196) could also be detected in platelets.

An issue requiring investigation was the intracellular storage site of platelet multimerin. Most soluble proteins found in platelets are stored within the alpha-granules. Platelet alpha-granules are known to contain three distinct zones that differ in their protein composition (Cramer *et al*, 1985; Harrison *et al*, 1993b). These include a central zone that contains the majority of the proteins stored within platelet alpha-granules, including IgG, albumin and fibrinogen, a nucleoid zone that contains proteoglycans and the proteins PF4 and beta-thromboglobulin, and an eccentric, electron-lucent zone that contains tubular structures and von Willebrand factor (Cramer *et al*, 1985; Wilbourne *et al*, 1993; Harrison *et al*, 1993b). The ultrastructure of the electron-lucent zone resembles that of Weibel-Palade bodies, an endothelial cell cytoplasmic organelle that stores von Willebrand factor (Weibel *et al*, 1964; Wagner *et al*, 1982; Cramer *et al*, 1985; Wilbourne *et al*, 1993; Harrison *et al*, 1993b). The electron microscopy studies demonstrated multimerin in an eccentric position within platelet alpha-granules, colocalizing with von Willebrand factor in the electron-lucent zone (Hayward *et al*, 1993).

An important goal of my thesis project was to identify cells that synthesize multimerin so that I could study the multimerin cDNA. An alternative source of multimerin cDNA was pursued because Dami cells (a malignant cell line with abnormal chromosomes) might contain mutant

genes (Greenberg *et al*, 1988); however, normal human megakaryocytes are difficult to harvest and culture, and human megakaryocyte cDNA libraries are not available. Based on the localization of multimerin to the region of platelet alpha-granules resembling Weibel-Palade bodies (Hayward *et al*, 1993), I postulated that multimerin might be stored in Weibel-Palade bodies and I investigated if human endothelial cells synthesize multimerin. My studies of multimerin biosynthesis by endothelial cells are described in detail in the manuscript "Endothelial Cells Store Multimerin in Weibel-Palade Bodies and Possess both Constitutive and Regulated Pathways for Multimerin Release". This manuscript has been submitted for publication (Hayward *et al*, 1995b). The immunohistochemistry and confocal investigations contained in the manuscript are not formally part of my thesis. I grateful to Drs. Thomas J. Podor, Ron H. Stead and Dr. Theodore E. Warkentin for their valued contributions and advice.

Multimerin was identified in the endothelium of both large and small, arterial and venous blood vessels. Investigations of multimerin biosynthesis by endothelial cells indicated similarities to the pattern of multimerin biosynthesis that had been observed with Dami cells. Similar to the multimerin stored in platelets, endothelial cells contained high molecular weight multimers of multimerin. Activation of endothelial cells, using agonists known to induce the release of Weibel-Palade body contents, caused a change in the distribution of multimerin, with binding of the released protein to the matrix. In contrast to the multimerin that was secreted constitutively into the culture medium, the multimerin released by

agonists was not found in the culture medium and was associated with insoluble matrix. This work indicated that human endothelial cells synthesize multimerin and possess both constitutive and regulated pathways for multimerin secretion.

Multimerin Is Found in the α -Granules of Resting Platelets and Is Synthesized by a Megakaryocytic Cell Line

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Abstract

In this report, we describe the intracellular localization of multimerin in platelets and its biosynthesis by Dami cells, a megakaryocytic cell line. Immunoelectron microscopy was used to examine frozen thin sections of resting and activated platelets. Multimerin was localized within the platelet α -granule in an eccentric position. Within activated platelets, multimerin was found in the surface-connected open cannalicular system and on the external plasma membrane. Light microscopic immunocytochemistry demonstrated multimerin in normal megakaryocytes and in Dami cells after stimulation with PMA. Confirmation of multimerin biosynthesis by Dami cells was obtained by metabolic labeling studies. Both platelet and Dami cell multimerin demonstrated several subunit sizes on reduced SDS-PAGE. However, peptide mapping confirmed structural homology between the different multimerin subunits. Glycosidase digestion demonstrated that multimerin is heavily glycosylated with mainly complex, N-linked carbohydrate. In contrast to the multimerin isolated from platelets, cultured Dami cells secreted mainly smaller multimers of the protein. Biosynthesis of multimerin by a megakaryocytic cell line supports endogenous biosynthesis by megakaryocytes as the origin of this platelet α -granule protein. (*J. Clin. Invest.* 1993. 91:2630-2639.) **Key words:** Dami cell • multimer • von Willebrand factor • bone marrow • immunocytochemistry

Introduction

Multimerin is an extremely large, soluble platelet glycoprotein that is expressed on the surface of activated platelets (1, 2). This protein exists as variably sized multimers that range from < 450 kD to many million daltons (1, 2). Multimerin and von Willebrand factor share a similar complex multimeric composition and our previous studies demonstrated that multimerin and von Willebrand factor are the two largest proteins in platelets (2). The individual multimers of platelet multimerin are comprised of p-155 and p-170 subunits, linked by disulfide bonds (2). During platelet activation, multimerin moves from the platelet interior and becomes expressed on the platelet sur-

face (1). The largest multimers of this protein remain bound to the platelet surface after activation and the smaller multimers are released (2).

The intent of the current study was to investigate the location and origin of platelet multimerin. Soluble proteins contained within platelets can originate from two sources: endogenous biosynthesis by megakaryocytes or endocytosis from plasma (3). In general, proteins synthesized by the megakaryocyte are present at a higher concentration in platelets than in plasma, whereas plasma-derived platelet proteins are present at a greater concentration in plasma than in platelets. Fibrinogen is the only known exception (3). Because we failed to detect multimerin in plasma, we postulated that this protein might be synthesized by megakaryocytes and stored within platelet granules for release upon activation. In this report, we describe the intracellular location of multimerin in platelets. Additionally, we demonstrate the presence of multimerin in normal megakaryocytes and its biosynthesis by Dami cells (4), a malignant megakaryocytic cell line.

Methods

Antibodies. Monoclonal antibody JS-1 and polyclonal rabbit antibodies against multimerin were used for immunoprecipitation and immunohistochemistry (1). Immunocytochemistry studies were performed using polyclonal antimultimerin and both monoclonal and polyclonal antibodies to von Willebrand factor (Dako Corp., Carpinteria, CA). These polyclonal antisera to multimerin and von Willebrand factor are non-cross-reactive and recognize reduced platelet proteins of 155 and 220 kD, respectively (1, 2). Additionally, using immunoblot studies, the polyclonal anti-von Willebrand factor does not recognize purified multimerin (1) and polyclonal antibodies to multimerin do not react with purified von Willebrand factor.

Platelet preparation. Washed platelets (1×10^8 /ml) were activated, without agitation, using bovine thrombin (1 U/ml; Sigma Immunochemicals, St. Louis, MO). After 10 min, recombinant hirudin (Sigma), 4 U/ml, was added. Activated platelets (1×10^9 /ml) were surface radiolabeled using 125 I and lactoperoxidase, washed, lysed, and used for immunoprecipitation (1, 5).

Dami cell preparation. Dami cells (4) were a generous gift of Dr. Sheryl M. Greenberg, Hematology Division, Brigham and Women's Hospital, Boston, MA. The cells were obtained from the American Type Culture Collection (CRL 9792), Rockville, MD. Cells were grown in RPMI 1640 medium supplemented with 5% fetal calf serum, 10 mM sodium bicarbonate, 10 mM HEPES, and 2 mM glutamine. For some experiments, Dami cells were activated by incubating the cells with 5 nM PMA. Cytochemistry studies were performed on Dami cells that were cultured on glass coverslips.

Dami cells (5×10^5 /ml) were surface labeled with 125 I (1, 5), washed, and solubilized in lysing buffer containing proteolytic inhibitors (1% Triton X-100, 0.1% SDS, 20 mM Tris, 100 mM NaCl, pH 7.4, with 10 mM EDTA, 0.1 μ M leupeptin, 0.2 mM PMSF, 0.02 mg/liter soybean trypsin inhibitor, and 5 mM N-ethyl maleimide; 1×10^5 cells/ml).

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Metabolic labeling was performed using [35 S]methionine (NEN/Dupont Canada Inc., Mississauga, Canada) in methionine-free medium. For 18-h labeling studies, 5 ml vol of medium containing 0.1 mCi/ml of [35 S]methionine were used. For pulse-chase experiments, 1 ml of culture medium containing 0.5 mCi of 35 S methionine was used.

Cleland mapping of the Dami cell multimerin was performed using supernatant from an 18-h labeling with Trans 35 S-label (0.5 mCi/5 ml; ICN Biomedicals Canada, Ltd., Montreal, Canada) and methionine-free, cysteine-free media. For some experiments, tunicamycin (1 μ g/ml; Boehringer Mannheim Canada, Laval, Quebec) was added to culture medium 3 h before labeling. The labeled Dami cells were solubilized in lysing buffer to the same final volume as the culture supernatant ($\sim 1 \times 10^5$ cells/ml).

Immunocytochemistry. Resting platelets and thrombin stimulated ($2 \text{ U}/10^9$ platelets in Tyrode's buffer containing 1 mM calcium, 5 min, 37°C.) platelets were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 for 3 h at 4°C. Cells were embedded in 2.3 M sucrose and frozen thin sections were cut from the cell block. Immunocytochemistry was performed as described (6, 7) using polyclonal rabbit antimultimerin (1:50) and goat anti-rabbit gold-10 (Amersham Corp., Arlington Heights, IL). A double-labeling experiment to localize multimerin and von Willebrand factor was performed using protein A, as described by Slot and Geuze (8). Rabbit antibody against multimerin was applied (dilution 1:10) and labeled with protein A-10. Before the addition of the second antibody, free protein A (0.05 mg/ml) was applied. Subsequently, rabbit antibodies to von Willebrand factor were added at a 1:10 dilution followed by protein A-5. A second experiment was also performed using the polyclonal multimerin antibody and goat anti-rabbit gold-5 and a monoclonal antibody against von Willebrand's factor (dilution 1:10) and goat anti-mouse gold. Controls consisted of a primary incubation with normal rabbit serum.

Immunocytochemistry was performed on normal bone marrow smears and on resting and PMA-activated Dami cells. Air-dried smears were fixed in acetone at 4°C for 15 min before blocking endogenous peroxidase activity with 0.5% hydrogen peroxide in methanol for 20 min. After further air drying, the slides were rinsed in Tris-buffered saline, pH 7.6 (TBS)¹ and incubated for 1 h in TBS with JS-1 (monoclonal antimultimerin; 1:100–1:1,000 dilution) or TBS alone (negative control). The slides were rinsed in TBS and incubated in biotinylated rabbit anti-mouse immunoglobulins (10 min) and streptavidin peroxidase conjugate (5 min; Histostain SP kit; Zymed Laboratories Inc., South San Francisco, CA). Peroxidase was demonstrated using aminoethylcarbazole in acetate buffer, pH 5.0. Nuclei were counterstained with hematoxylin.

Radioimmunoprecipitation. Immunoprecipitations of 1-ml vol of radiolabeled cell lysates or culture supernatant were performed using protein A sepharose beads (50 μ l) and either monoclonal (10 μ l JS-1) or polyclonal antibodies (50 μ l) against multimerin (1). To reduce nonspecific binding, samples from 18-h metabolic labeling experiments were subjected to two consecutive immunoprecipitations (9), using polyclonal antimultimerin. Beads were washed five times with lysing buffer (containing 0.2% Triton X-100), eluted with 100 μ l of lysing buffer containing 2% SDS, 2% Triton X-100, and 2% sodium deoxycholate, and boiled. Eluates were collected, diluted 10-fold with lysing buffer (containing 0.2% Triton X-100) and a second immunoprecipitation was performed. Immunoprecipitates were used for subunit and multimer analyses (1, 2). For pulse-chase experiments, single immunoprecipitations were performed using polyclonal antimultimerin.

Subunit analysis. Immunoprecipitates were analyzed by reduced SDS-PAGE and autoradiography or fluorography (1).

Multimer analysis. Immunoprecipitates were eluted with agarose/acrylamide sample buffer (final concentrations: 10 mM Tris, 2% SDS, 8 M urea, 0.005% bromophenol blue; pH 8.0). Samples were analyzed by nonreduced, agarose/acrylamide gel electrophoresis using 1.25%

agarose gels (SeaPlaque agarose; FMC Corp., FMC BioProducts, Rockland, ME) containing 1.5% acrylamide (2). Thrombospondin was used as a reference for multimer size (2). Individual multimer subunit composition was assessed by two-dimensional, nonreduced/reduced electrophoresis as described (2).

Protease mapping. Cleland mapping was used to compare the different multimerin subunits (10). Multimerin immunoprecipitates were prepared, reduced, and alkylated (1), followed by subunit separation using SDS-PAGE. The radiolabeled proteins were isolated from dried gels and protease digestion was performed in the stacking gel using V8 protease (0.05, 0.1, 0.5, and 5.0 μ g/lane), chymotrypsin (1, 10, and 50 μ g/lane) and trypsin (1, 10, and 50 μ g/lane). Peptides were resolved using 7–15% SDS-PAGE.

Carbohydrate analysis. The carbohydrate content of multimerin was assessed by exo and endoglycosidase digestions (9, 11). Multimerin immunoprecipitates were eluted with 0.1% SDS, 0.2% 2-mercaptoethanol containing protease inhibitors (final concentrations: 0.1 mg/ml benzamidine, 1 mM PMSF, 1 μ g/ml aprotinin, 10 μ g/ml leupeptin). The test samples were incubated with the different glycosidases, then analyzed by reduced SDS-PAGE. To exclude proteolytic degradation during the glycosidase incubations, control samples were incubated in buffer without glycosidase. Glycosidases used included: endoglycosidase H (Boehringer Mannheim; final concentrations: 50 mU/ml in 100 mM sodium phosphate buffer, pH 6.0, 18 h, 37°C), endoglycosidase F/N-glycosidase F-free and endoglycosidase F/N-glycosidase F (Boehringer Mannheim, for both glycosidases, final concentrations: 15 U/ml in 20 mM sodium phosphate buffer, pH 7.0, 18 h, 37°C), neuraminidase (Boehringer Mannheim, final concentrations: 2 U/ml in 20 mM sodium acetate buffer, pH 5.5, 1 h, 37°C) and O-glycanase (Genzyme Corp., Cambridge, MA; final concentrations: 30 mU/ml added to the neuraminidase treated protein, 5 h, 37°C). After incubation with the glycosidase, 100 μ l of 2 \times reducing sample buffer was added to all tubes, followed by reduced SDS-PAGE. For some studies, both N- and O-linked carbohydrates were removed by treating the immunoprecipitates with N-glycosidase F followed by neuraminidase and O-glycanase.

Results

Intracellular localization of multimerin in platelets. Immunoelectron microscopy performed on frozen thin sections of platelets demonstrated the presence of multimerin in an eccentric position within the α -granule of resting platelets (Fig. 1, *a* and *b*). Only small amounts of multimerin were detected on the plasma membrane of resting platelets. Using double labeling experiments with antibodies (either monoclonal or polyclonal) to von Willebrand factor and multimerin and two different sizes of gold, multimerin and von Willebrand factor colocalized to the same region of the α -granule (Fig. 1 *a*, *inset panel*, and *b*). No labeling of α -granules was observed in the negative controls.

Studies performed on frozen thin sections of thrombin-activated platelets demonstrated that the multimerin was located on the external plasma membrane and within the surface-connected cannalicular system after platelet activation (Fig. 1 *c*). There was no significant labeling of the surface-connected cannalicular system or plasma membrane in the negative controls.

Demonstration of multimerin in megakaryocytes and in Dami cells. Normal bone marrow megakaryocytes and resting and PMA-activated Dami cells were examined using immunocytochemical techniques and the monoclonal antibody to multimerin (Fig. 2). Normal megakaryocytes and platelets stained intensely for multimerin in a granular pattern. Other bone marrow cells were not multimerin immunoreactive. While we did not detect the presence of multimerin in resting Dami cells,

1. Abbreviation used in this paper: TBS, Tris-buffered saline.



Figure 1. Immunogold labeling of multimerin in frozen thin sections of resting and activated platelets. In resting platelets, labeled using polyclonal antibodies to multimerin and goat anti-rabbit gold-10 (panel *a*, $\times 50,000$), the gold particles were found within the alpha granule matrix, usually in an eccentric position (*large arrows*). Rare label was detected on the plasma membrane (*pm*). Double labeling studies of resting platelets (inset of an alpha granule, panel *a*, $\times 98,000$) were performed using polyclonal antibodies to multimerin (detection with protein A gold-10) and to von Willebrand factor (detection with protein A gold-5; *small arrows*). Multimerin (the large gold) and von Willebrand factor (the small gold) colocalized to the same region of the alpha granules. In panel *b* ($\times 60,000$), von Willebrand factor was detected with a monoclonal antibody and goat anti-mouse gold-10, and multimerin was detected with a polyclonal antibody and goat anti-rabbit gold-5. The results are similar to panel *a*, and short arrows point to the granules with eccentric double-labeling.

Thrombin-stimulated platelets (panel *c*, $\times 42,000$) exhibited shape change and aggregation. Immunocytochemistry using polyclonal antibodies to multimerin and goat anti-rabbit gold-10 demonstrated presence of gold in large vacuoles (*v*) many of which are probably sections of the surface-connected cannicular system. Smaller amounts of the label were localized to the external plasma membrane. No label was detected in the tight "contact zones" (*CZ*).



Figure 1 (Continued)

PMA-activated Dam1 cells showed a granular distribution of the protein. Background staining suggestive of an extracellular matrix distribution was observed for the PMA-activated Dam1 cells. Compared to the normal megakaryocytes, the PMA-activated Dam1 cells stained less intensely for multimerin. No staining was observed in the control slides processed without the monoclonal antibody.

To determine if endogenous biosynthesis was the source of multimerin in Dam1 cells, metabolic labeling studies were performed. Untreated and PMA-stimulated cells were metabolically labeled (18 h, [35 S]methionine) during the 1st, 2nd, and 3rd day of PMA stimulation. Multimerin immunoprecipitates were prepared from culture supernatants and cell lysates. Multimerin was not detected in the cell lysate or culture supernatant of resting Dam1 cells (Fig. 3). After stimulation with PMA, Dam1 cells synthesized and secreted multimerin. After 1 d of PMA stimulation, multimerin was detected both in the Dam1 cell lysate and culture supernatant and continued to be synthesized and secreted over the next several days of PMA stimulation. Greater quantities of multimerin were present in the culture supernatant compared to the cell lysate, indicating that the majority of the protein was secreted. Multimerin was

not detected on the surface of resuspended, washed, PMA-stimulated Dam1 cells (Fig. 3).

When the intracellular and secreted multimerin from an 18-h metabolic labeling were compared, the proteins differed in their reduced subunit size. The 170-kD band was the predominant protein in the cell lysate (Fig. 3). The secreted protein was comprised of 196- and 165-kD subunits, with a greater proportion of the larger subunit (Fig. 3). Both monoclonal and polyclonal antibodies to multimerin immunoprecipitated the two protein subunits from the culture supernatant. Comparison of the Dam1 cell multimerin with the platelet multimerin subunit revealed that the protein synthesized and secreted by the Dam1 cells had a larger subunit size (reduced) than the mature platelet protein.

Cleveland mapping of multimerin subunits. Cleveland mapping was used to investigate if the different subunits present in the reduced multimerin immunoprecipitates originated from a common precursor protein. Protease digestion of platelet p-155 and p-170 generated identical peptides (Fig. 4). Trypsin, chymotrypsin, and V8 protease digests showed extensive, but not complete, peptide homology between the p-155 platelet multimerin subunit and the p-196 Dam1 cell multimerin sub-

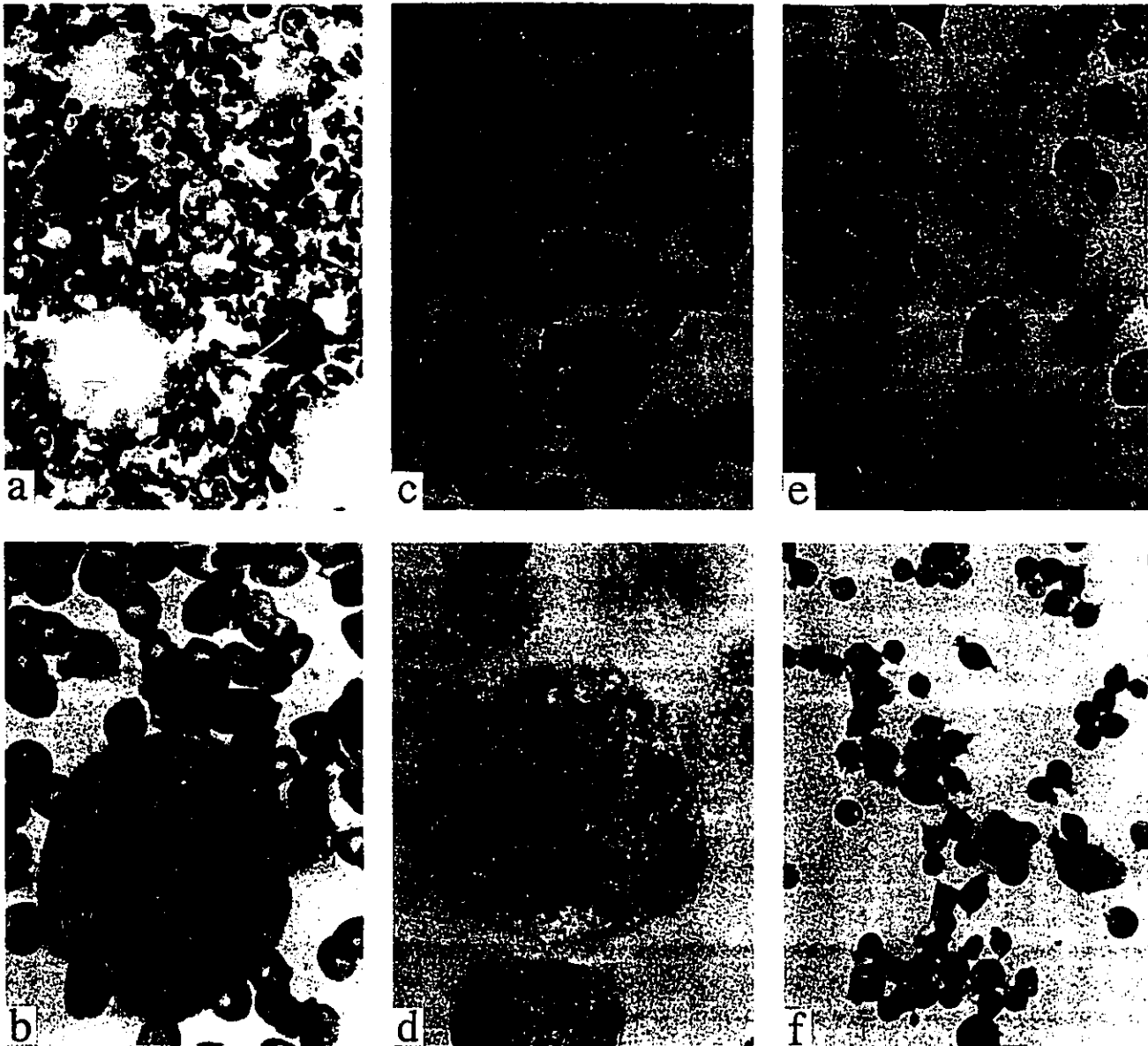


Figure 2. Immunocytochemistry demonstrating multimerin in megakaryocytes and in Dami cells. Immunocytochemistry was performed on normal bone marrow and on resting and PMA-stimulated Dami cells using JS-1 (monoclonal antimultimerin). Normal megakaryocytes exhibited intense staining for multimerin with a granular distribution (panel *a*, $\times 320$; panel *b*, $\times 800$). Only megakaryocytes and platelets were labeled. Resting Dami cells did not exhibit any staining with antibodies to multimerin (panel *f*, $\times 320$); however, after PMA stimulation, multimerin was evident in Dami cells (panel *c*, $\times 320$; panel *d*, $\times 800$). In most cells, a granular pattern of staining was observed with some evidence of extracellular staining, suggestive of a matrix distribution. Control slides, processed without JS-1, did not show any staining (panel *e*, $\times 320$).

unit (Fig. 4). The observed differences may be due to a longer peptide sequence in p-196, differences in glycosylation between the protein synthesized *in vivo* and *in vitro*, or the distribution of the different radiolabels on the individual peptides.

Carbohydrate composition of multimerin. Glycosidase digestions were performed to determine the carbohydrate composition of multimerin and to further investigate the differences in subunit sizes (Fig. 5). The platelet p-155 and p-170 multimerin subunits contained similar quantities of N-linked carbohydrate (Fig. 5, lane 4) and sialic acid (Fig. 5, lane 2). Removal of all N-linked carbohydrate by *N*-glycosidase F from p-170 and p-155 resulted in proteins with mobilities of 120 and

105 kD, respectively (Fig. 5, lane 4). Resistance of p-155 and p-170 to endoglycosidase F and endoglycosidase H (Fig. 5, lanes 6 and 11), indicated that p-155 and p-170 contain only the complex forms of N-linked carbohydrate. In contrast to the results obtained using *N*-glycosidase F, *O*-glycanase treatment resulted in only a minor shift in mobility of the p-155 subunit (Fig. 5, lane 21). Together, these results demonstrate that p-155 and p-170 multimerin subunits are highly glycosylated proteins that contain mainly complex N-linked carbohydrate. The failure of the deglycosylated subunits to comigrate (Fig. 5, lane 4) indicates that p-170 contains a larger polypeptide component than p-155.

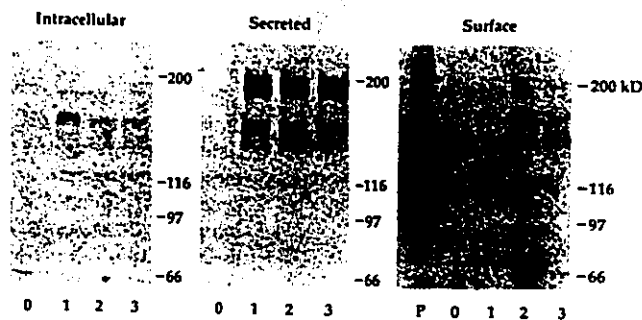


Figure 3. Damii cells synthesize multimerin after stimulation with PMA. Multimerin immunoprecipitates were prepared from ¹²⁵I surface radiolabeled Damii cells and [³⁵S]methionine metabolically labeled Damii cells. Cells were labeled during 0, 1, 2, or 3 d (lanes designated 0, 1, 2, 3) of PMA stimulation. Samples were analyzed by SDS-PAGE. For comparison, the multimerin from surface radiolabeled, activated platelets is shown in lane P. This study demonstrates that Damii cells synthesize and secrete multimerin after stimulation with PMA. Compared to activated platelets, PMA-stimulated, resuspended Damii cells do not express detectable amounts of multimerin on their surface. Bands below 155 kD in the platelet sample may represent coprecipitated proteins or degradation products.

Similar to platelet multimerin (Fig. 5, lane 15), the secreted Damii cell multimerin contained a large component of N-linked carbohydrate, evidenced by large mobility shifts of the p-196 and p-165 subunits after treatment with endoglycosidase F/N-glycosidase F (Fig. 5, lane 16). Additionally, the secreted Damii cell multimerin was resistant to endoglycosidase F (Fig. 5, lane 12) and to endoglycosidase H (Fig. 5, lane 10), indicating that, like platelet multimerin, all of the N-linked carbohydrate had been processed to complex forms. The Damii cell p-196 subunit migrated with an apparent reduced molecular mass of 145 kD (5% polyacrylamide gels; Fig. 5, lane 16) after removal of N-linked carbohydrate. As observed with the deglycosylated subunits of platelet multimerin, the smaller p-165 subunit of Damii cell multimerin contained a smaller polypeptide component than the larger p-196 subunit (Fig. 5, lane 16). Because the deglycosylated p-155 platelet multimerin subunit was smaller (Fig. 5, lane 15) than the deglycosylated subunits of multimerin from Damii cells (Fig. 5, lane 16), we postulate that there is more complete proteolytic processing of the protein derived from platelets.

The 196-kD Damii cell multimerin subunit contained sialic acid that was not completely removed by treatment with N-glycanase F (Fig. 5, lanes 16 and 18), suggesting the presence of O-linked carbohydrate. O-glycanase digestion resulted in a 9-kD mobility shift in the neuraminidase-treated 196-kD subunit (Fig. 5, lanes 20 and 22), confirming the presence of O-linked sugars on the p-196 subunit of multimerin. However, complex, N-linked carbohydrate is the predominant type.

Processing of multimerin during biosynthesis. Pulse-chase studies were undertaken to follow the processing of multimerin during its biosynthesis. Damii cells were metabolically labeled (20 min) and biosynthesis was followed in the culture supernatants and cell lysates at varying chase intervals after pulse labeling (Fig. 6). This study demonstrated that the first multimerin subunit synthesized was 170 kD. Subsequently the 196-kD protein became detectable in the cell lysate with the amount progressively increasing over the next hour. Secretion of the protein was first detected at 1 h and almost all of the synthe-

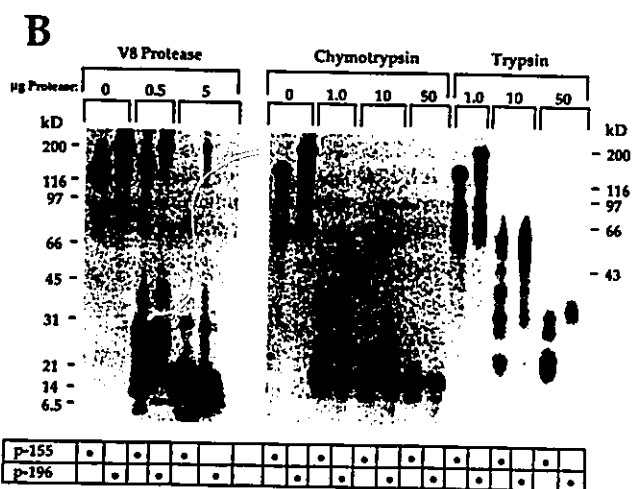
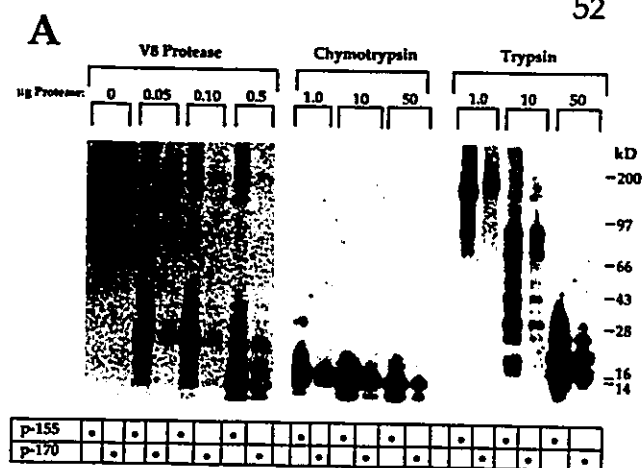


Figure 4. Peptide mapping of multimerin subunits. Multimerin immunoprecipitates prepared from ¹²⁵I surface labeled, activated platelets and from the culture supernatant of 18-h ³⁵S metabolically labeled, day 2 PMA-stimulated Damii cells were used to isolate the subunit proteins. Peptide mapping was performed by the method of Cleveland using V8 protease (0.05, 0.1, 0.5, and 5.0 μg/lane), chymotrypsin (1, 10, and 50 μg/lane) and trypsin (1, 10, and 50 μg/lane). (A) Comparison of platelet p-155 and p-170 multimerin subunits. This study demonstrates that the platelet multimerin subunits are highly related proteins with complete peptide homology. (B) Comparison of platelet p-155 and Damii cell p-196 multimerin subunits. This study demonstrates extensive peptide homology between the platelet p-155 and the larger Damii cell p-196.

sized multimerin was secreted within 3 h of biosynthesis. The secreted multimerin was primarily the 196-kD protein with smaller amounts of the 165-kD subunit.

We postulated that the increase in the molecular weight of multimerin subunit during biosynthesis was due to the addition of carbohydrate. Additionally, because the pulse-chase studies indicated rapid secretion, we postulated that the intracellular multimerin from 18-h labeling experiments was mainly the recently synthesized, precursor form of the protein. To address these issues, the multimerin prepared from 18-h metabolic labeled Damii cell lysate was compared to the precursor protein synthesized during a 0.5-h metabolic labeling (Fig. 7a). Both lysates contained a 170-kD multimerin subunit that was susceptible to endoglycosidase H digestion, indicating the

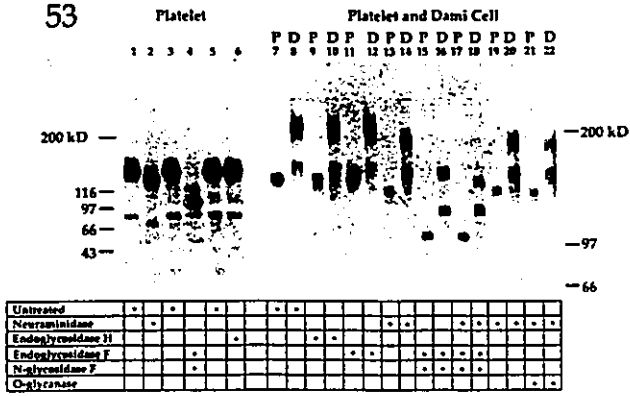


Figure 5. The carbohydrate composition of multimerin. The carbohydrate composition of platelet and Dami cell multimerin was assessed by digestion with endo and exoglycosidases. Platelet multimerin subunits were analyzed by 4–8% SDS-PAGE (lanes 1–6) and 5% SDS-PAGE was used to compare the Dami cell (D) and platelet (P) multimerin subunits (lanes 7–22). Both platelet (lanes 4 and 15) and Dami cell (lane 16) multimerin subunits are highly glycosylated with mainly complex, N-linked carbohydrate. The Dami cell p-196 multimerin subunit (lane 22) contains O-linked carbohydrate, evidenced by a mobility shift after treatment with O-glycanase. Comparison of the deglycosylated individual subunits demonstrates that there are differences in their polypeptide components (lanes 4 and 15–22).

presence of high mannose N-linked carbohydrate that had not been converted to complex forms. These results indicate that most of the multimerin found within Dami cells is the newly synthesized precursor that undergoes further processing of N-linked carbohydrate before secretion of the mature subunits.

Prevention of N-glycosylation in Dami cells with tunicamycin resulted in the biosynthesis of a smaller precursor pro-

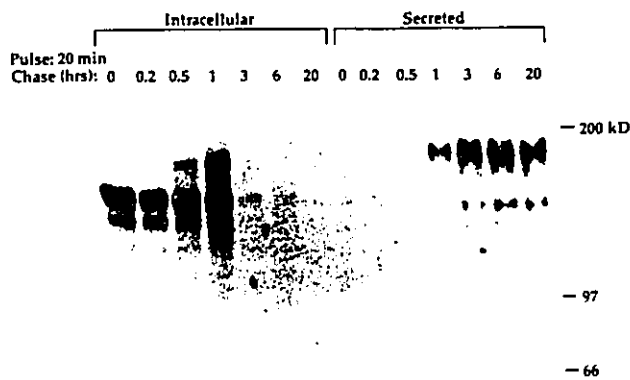


Figure 6. Pulse-chase study of multimerin biosynthesis by Dami cells. Day 2, PMA-stimulated Dami cells were pulse labeled for 20 min with [³⁵S]methionine. Multimerin immunoprecipitates were prepared from cell lysates and supernatants collected at 0, 0.2, 0.5, 1, 3, 6, and 20 h after pulse and analyzed by SDS-PAGE. This study demonstrates that multimerin is synthesized as a 170-kD subunit, followed by further processing to generate the secreted 196- and 165-kD subunits. The larger 196-kD subunit first appears in the cell lysate at 0.5 h and its appearance is rapidly followed by secretion of 196- and 165-kD subunits. Almost all of the synthesized protein was secreted by 3 h. The band at 140 kD in the cell lysate was not consistently seen in other pulse chase experiments and may represent a coprecipitated protein.

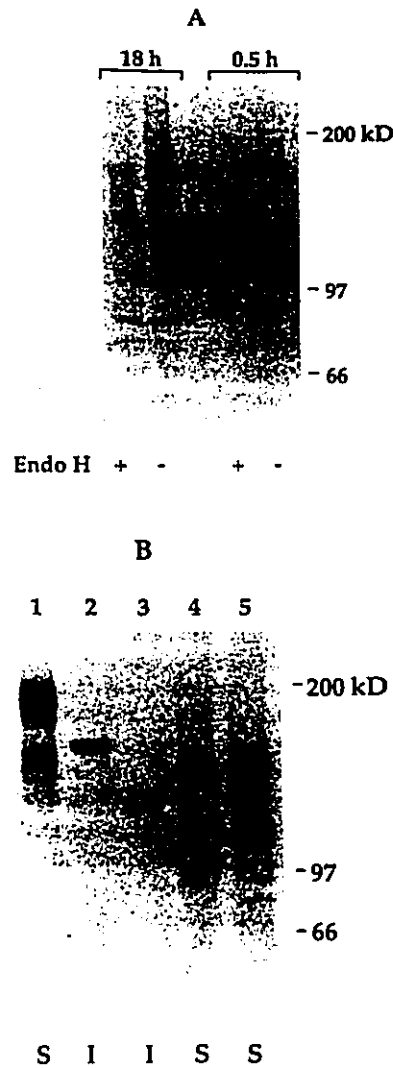


Figure 7. Comparison of intracellular and secreted Dami cell multimerin. The carbohydrate processing of multimerin was investigated using cell lysates and supernatants from 0.5 h (with and without tunicamycin) and 18-h [³⁵S]-methionine metabolically labeled, day 2 PMA-stimulated Dami cells. Immunoprecipitates were incubated with glycosidases or buffer, followed by reduced 5% SDS-PAGE. (A) The 170-kD intracellular multimerin subunit from an 18-h labeling study comigrated with the multimerin precursor synthesized during a 0.5-h labeling. Both proteins contained endoglycosidase H sensitive, high mannose N-linked carbohydrate. These findings indicate that the multimerin contained in the 18-h cell lysate is the recently synthesized, precursor form of multimerin. The multimerin immunoprecipitate from the 18-h lysate contained an additional endoglycosidase H resistant 130-kD protein,

not seen in previous 18-h lysates (Fig. 3). This may represent a coprecipitated protein. (B) The carbohydrate composition of the intracellular (I) and secreted (S) multimerin subunits were compared. The mature secreted protein consists of 196 and 165 kD subunits (lane 1, 18-h labeling) in contrast to the intracellular 170-kD subunit (lane 2, 0.5-h labeling). Prevention of N-glycosylation with tunicamycin resulted in the synthesis of a 132-kD protein (lane 3, 0.5-h labeling) that did not comigrate with either of the N-glycosidase F-treated, secreted subunits of multimerin (lane 5, 18-h labeling). Neuraminidase treatment further reduced the M_r of the N-deglycosylated, secreted multimerin (lane 4, 18-h labeling), indicating the presence of non-N-linked carbohydrate on the secreted 196-kD subunit.

This study demonstrates that formation of the mature, secreted protein involves conversion of high mannose N-linked carbohydrate to complex forms, the addition of O-linked carbohydrate and also indicates that proteolysis is required to generate the 165-kD subunit.

tein that migrated at 132 kD (Fig. 7 b, lane 3) in contrast to the 170-kD protein synthesized in the absence of tunicamycin (Fig. 7 b, lane 2). The endoglycosidase H treatment of the early precursor protein resulted in an equivalent reduction in apparent M_r (Fig. 7 a), suggesting that the 170-kD multimerin precursor contained only high mannose forms of N-linked carbohydrate.

Further studies were performed to investigate O-glycosylation during the biosynthesis of multimerin. The presence of O-linked carbohydrate was suggested by the higher M_r of the N-deglycosylated, 196-kD secreted multimerin subunit (Fig. 7 b, lane 5), compared with the 132-kD protein synthesized in the presence of tunicamycin (Fig. 7 b, lane 3). The presence of O-linked carbohydrate was confirmed by demonstrating that neuraminidase reduced the M_r of the N-deglycosylated p-196 (Fig. 7 b, lane 4; Fig. 5, lane 18) and O-glycanase further reduced the M_r of p-196 (Fig. 5, lane 22). The N-deglycosylated, secreted p-165 was smaller than the precursor protein synthesized in the presence of tunicamycin (Fig. 7 b, lanes 5 and 3), indicating that production of the smaller subunit of the secreted protein requires proteolysis. The observed differences in migration of the Dami cell p-165 and platelet p-170 multimerin subunits may reflect differences in either glycosylation or proteolysis.

Because the megakaryocytic cell line synthesized a larger multimerin subunit than found in platelets, we looked for evidence that the multimerin contained within platelets had originated from a larger precursor protein. A 200-kD band was occasionally observed in reduced multimerin immunoprecipitates from surface-labeled thrombin-activated platelets. To determine if this protein was a component of multimerin, two-dimensional nonreduced/reduced electrophoresis was performed. The 200-kD protein was present within the multimerin multimers, covalently linked to the p-155 and p-170 subunits by interchain disulfide bonds (Fig. 8).

Comparison of the multimeric composition of platelet multimerin and the multimerin secreted by Dami cells. Agarose/acrylamide gels were used to investigate the nonreduced structure of multimerin synthesized by Dami cells. Multimerin im-

munoprecipitates were prepared from Dami cell culture supernatants (18-h metabolic labelings) and from surface radiolabeled, thrombin-activated platelets. In comparison to the multimers from platelets, the constitutively secreted multimers from Dami cells were comprised mainly of the smallest multimers of the protein (Fig. 9). Two-dimensional, nonreduced/reduced electrophoresis demonstrated that the different sized subunits of Dami cell multimerin were linked by interchain disulfide bonds.

Discussion

Multimerin is an unusually large, disulfide-linked, multimeric protein that exhibits variability in its native multimeric size, ranging from less than 450 kD to many million Daltons (2). In our previous studies, we demonstrated that it was a unique, soluble platelet protein and demonstrated the activation-dependent nature of multimerin expression on the platelet surface (1). Further investigation demonstrated that multimerin resembles von Willebrand factor in its complex multimeric composition and that multimerin and von Willebrand factor are the two largest proteins in platelets (2). The purpose of the studies described in this report was threefold. The first studies were designed to determine the intracellular location of multimerin in platelets. The second series of investigations focused on identifying if the protein was present in megakaryocytes and whether it could be synthesized by Dami cells, a malignant megakaryocytic cell line. The third series of studies investigated the similarities and differences between the platelet and Dami cell forms of multimerin.

Previously, we have shown that multimerin is a soluble protein contained within platelets (1). Platelet activation results in a dramatic increase in the expression of multimerin on the platelet surface (1). In the current studies, we used immunoelectron microscopy to demonstrate that multimerin was contained within the α granules of resting platelets. Because the eccentric location of multimerin within the α granule resembled that of von Willebrand factor (12), dual labeling experi-

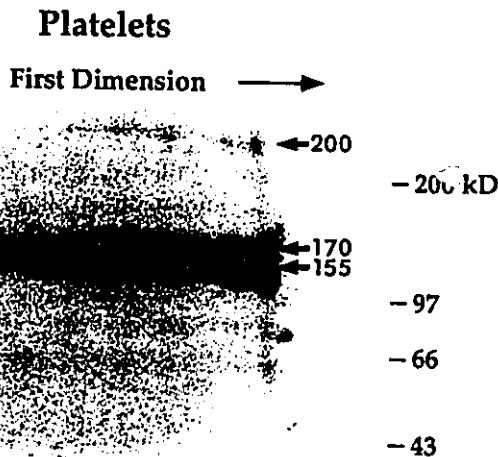


Figure 8. The composition of platelet multimerin multimers. The nature of the association of a 200-kD protein with platelet multimerin was investigated by two-dimensional nonreduced/reduced electrophoresis. Multimerin immunoprecipitates containing the 200-kD protein were prepared using ^{125}I surface radiolabeled activated platelets. Immunoprecipitates were separated by nonreduced agarose/acrylamide gel electrophoresis followed by reduced SDS-PAGE. The individual multimers of platelet multimerin contain mainly p-155 and p-170 subunits. However, the 200-kD protein also was identified as a minor component of the disulfide-linked multimers. The M_r of the 200-kD subunit was determined using 5% SDS-PAGE; anomalous migration was observed on 5–20% gradient gel shown in this figure.



Figure 9. Comparison of the multimeric composition of platelet and Dami cell multimerin. Multimerin immunoprecipitates were prepared using ^{125}I surface radiolabeled activated platelets (P), and culture supernatant from 18-h [^{35}S] methionine metabolically labeled day 2 PMA-stimulated Dami cells (D). Immunoprecipitates were analyzed by agarose/acrylamide gel electrophoresis. The arrow indicates the direction of migration. The position of thrombospondin (TSP, M_r 450 kD) is shown for reference. Compared to platelet multimerin, PMA-stimulated Dami cells secrete mainly the smaller multimers of multimerin.

ments with different sized protein A-gold were used to compare the distribution of these proteins. These studies demonstrated colocalization of multimerin and von Willebrand factor to the same region of the α granule (Fig. 1). In agreement with the measurable increase in multimerin on the platelet surface after platelet activation (1), multimerin was shown to redistribute to the open cannalicular system and to the external plasma membrane after activation, as well as being secreted to the extracellular medium.

The soluble nature of multimerin suggested one of two different pathways of synthesis: either endogenous biosynthesis by megakaryocytes or endocytosis of an exogenously synthesized protein. Usually, proteins that are endocytosed by megakaryocytes are present in a greater concentration in plasma than in platelets (3). In contrast, endogenously synthesized platelet proteins typically are present in greater concentration within platelets compared to plasma (3). Our previous studies failed to identify multimerin as a constituent of normal plasma (1). As a result, we postulated that multimerin was synthesized by megakaryocytes, then stored within granules.

Confirmation of endogenous biosynthesis by megakaryocytes was obtained using Dami cells, a megakaryocytic cell line. These cells synthesize a variety of platelet glycoproteins including von Willebrand factor (4). Immunohistochemistry demonstrated the presence of multimerin in normal megakaryocytes and in Dami cells (Fig. 2). Furthermore, using metabolic labeling, we demonstrated that PMA-activated Dami cells were able to synthesize multimerin (Fig. 3). The protein synthesized by the Dami cells was confirmed to be multimerin in two ways. First, two different antibodies against platelet multimerin (polyclonal and monoclonal) each immunoprecipitated the same protein. Second, Cleveland mapping demonstrated extensive peptide homology between platelet multimerin and the protein synthesized by Dami cells (Fig. 4). The Dami cell protein further resembled platelet multimerin in its soluble nature and disulfide-linked multimeric structure.

We have shown that platelet multimerin is primarily comprised of a 155-kD subunit with a smaller amount of a 170-kD subunit. Using protease digestion, peptide homology was demonstrated between these two proteins (Fig. 4). Glycosidase digestion of the p-155 and p-170 proteins demonstrated that both proteins were heavily glycosylated, containing mainly complex, N-linked carbohydrate (Fig. 5). Deglycosylation of p-155 and p-170 yielded different sized proteins, demonstrating that p-170 contains a larger polypeptide component. Together, these observations suggest that the p-155 protein is derived from the p-170 protein by proteolytic cleavage.

Next, pulse-chase metabolic labeling studies were used to follow the biosynthesis of multimerin. Multimerin was synthesized as a 170-kD precursor containing high mannose, N-linked carbohydrate, as evidenced by susceptibility to endoglycosidase H digestion (Figs. 6 and 7). Subsequent modification included the processing of N-linked carbohydrate from high mannose to complex forms and the addition of O-linked carbohydrate to generate a 196-kD subunit. Removal of all N-linked carbohydrate from the mature, 196-kD subunit produced a 51-kD shift in apparent molecular mass, which would indicate approximately 17 N-glycosylation sites on the multimerin precursor protein (11). Proteolysis resulted in the production of a smaller, 165-kD, secreted multimerin subunit.

In contrast to platelet multimerin, the protein synthesized

and secreted by Dami cells differed in its subunit sizes. The rapid secretion of multimerin synthesized *in vitro* by the PMA-activated Dami cells may account for less complete proteolytic processing compared to platelet multimerin synthesized *in vivo*. Similar incomplete proteolysis of von Willebrand factor secreted by cultured endothelial cells has been observed (13). Additionally, the malignant Dami cells may differ from normal megakaryocytes in their expression of the glycosyltransferases involved in N-glycosylation. This may account for the small differences in M_r observed between the Dami cell p-165 and the platelet p-170 multimerin subunits.

In contrast to platelet multimerin, which ranges in size up to many million Daltons (2), the multimerin synthesized and secreted by Dami cells contained less of the high molecular mass multimers (Fig. 9). The von Willebrand factor constitutively secreted by cultured endothelial cells also has been demonstrated to contain a predominance of the smaller multimers (13).

Our studies suggest that the platelet multimerin subunits originate from a larger precursor protein. We have occasionally observed that reduced platelet multimerin immunoprecipitates contain small quantities of a 200-kD protein that resembles the Dami cell p-196 (2). Using two-dimensional, nonreduced/reduced electrophoresis, we demonstrated that the 200-kD protein was a component of the disulfide-linked multimers of platelet multimerin (Fig. 8). Collectively, the evidence suggests that multimerin is synthesized both *in vivo* and *in vitro* as a larger precursor protein and that the mature, platelet multimerin undergoes proteolysis during biosynthesis and storage.

These studies indicate that multimerin is located within the α -granules of resting platelets and redistributes to the plasma membrane and surface-connected cannalicular system following activation. Multimerin is a heavily glycosylated protein that exists as variably sized, disulfide-linked multimers of related subunits. The synthesis of multimerin by the megakaryocytic cell line supports endogenous biosynthesis by megakaryocytes. Further confirmation of a megakaryocytic origin *in vivo* will require the demonstration of multimerin mRNA in normal megakaryocytes. Biosynthesis occurs through a process that involves the conversion of N-linked carbohydrate to complex forms, the addition of O-linked carbohydrate, proteolysis, and the formation of disulfide-linked multimers. The unusual repeating multimeric structure of multimerin, its localization to platelets, and its activation-induced expression on the platelet surface suggest a physiologic role in the cellular events associated with vascular injury.

Acknowledgments

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References

- Hayward, C. P. M., J. W. Smith, P. Horsewood, T. E. Warkentin, and J. G. Kelton. 1991. p-155, a multimeric platelet protein that is expressed on activated platelets. *J. Biol. Chem.* 266:7114-7120.
- Hayward, C. P. M., T. E. Warkentin, P. Horsewood, and J. G. Kelton.

1991. Multimerin: a series of large, disulfide-linked multimeric proteins within platelets. *Blood*. 77:2556-2560.
3. George, J. N. 1990. Platelet immunoglobulin G: its significance for the evaluation of thrombocytopenia and for understanding the origin of α -granule proteins. *Blood*. 76:859-870.
4. Greenberg, S. M., D. S. Rosenthal, T. A. Greeley, R. Tantravahi, and R. I. Handin. 1988. Characterization of a new megakaryocytic cell line: the Dami cell. *Blood*. 72:1968-1977.
5. Kelton, J. G., J. W. Smith, P. Horsewood, J. R. Humbert, C. P. M. Hayward, and T. E. Warkentin. 1990. Gov^{a/b} alloantigen system on human platelets. *Blood*. 75:2172-2176.
6. Bainton, D. F., L. J. Miller, T. K. Kishimoto, and T. A. Springer. 1987. Leukocyte adhesion receptors are stored in peroxidase-negative granules of human neutrophils. *J. Exp. Med.* 166:1641-1653.
7. McEver, R. P., J. H. Beckstead, K. L. Moore, L. Marshall-Carlson, and D. F. Bainton. 1989. GMP-140, a platelet α -granule membrane protein is also synthesized by vascular endothelial cells and is localized in Weibel-Palade bodies. *J. Clin. Invest.* 84:92-99.
8. Slot, J. W., and H. J. Geuze. 1984. Gold markers for single and double immunolabelling of ultrathin cryosections. In: *Immunolabelling for Electron Microscopy*. J. M. Polak and I. M. Vardell, editors. Elsevier Science Publishers, Oxford, p. 129.
9. Johnston, G. I., A. Kurosky, and R. P. McEver. 1989. Structural and biosynthetic studies of the granule membrane protein, GMP-140, from human platelets and endothelial cells. *J. Biol. Chem.* 264:1816-1823.
10. Cleveland, D. W., S. G. Fischer, M. W. Kirschner, and U. K. Laemmli. 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulphate and analysis by gel electrophoresis. *J. Biol. Chem.* 252:1102-1106.
11. Kornfeld, R., and S. Kornfeld. 1985. Assembly of asparagine-linked oligosaccharides. *Annu. Rev. Biochem.* 54:631-664.
12. Cramer, E. M., D. Meyer, R. le Menn, and J. Breton-Gorius. 1985. Eccentric localization of von Willebrand Factor in an internal structure of platelet α -granule resembling that of Weibel-Palade bodies. *Blood*. 66:710-713.
13. Wagner, D. D., and V. J. Marder. 1983. Biosynthesis of von Willebrand protein by human endothelial cells. Identification of a large precursor polypeptide chain. *J. Biol. Chem.* 258:2065-2067.

4.3 Endothelial Cells Store Multimerin in Weibel-Palade Bodies and Possess Both Constitutive and Regulated Pathways for Multimerin Release

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Multimerin is a large complex, multimeric glycoprotein that colocalizes with von Willebrand factor within platelet alpha-granules. In this report, we describe the biosynthesis, storage and secretion of multimerin by human endothelial cells. Immunohistochemistry identified multimerin in situ in small and large blood vessels. Confocal scanning laser microscopic analysis of endothelial cells localized multimerin to Weibel-Palade bodies. Treatment of endothelial cells with secretagogues (thrombin, PMA, ionophore) resulted in significant increases in the multimerin associated with the cell surface and in the pericellular matrix. The multimerin stored in Weibel-Palade bodies was enriched in high molecular weight multimers. In contrast to the small multimers of constitutively secreted multimerin, which were mainly found in the culture supernatant, the high molecular weight multimers of multimerin released from Weibel-Palade bodies bound to the Triton-insoluble matrix and were not detectable in the culture supernatant of

activated endothelial cells. The presence of multimerin in Weibel-Palade bodies suggests a role for this protein in the endothelial cell response to hemostatic and inflammatory stimuli. The absence of multimerin in plasma and its release by agonists may serve to restrict the function(s) of multimerin to sites of vessel injury.

INTRODUCTION

Multimerin is a large, soluble, complex multimeric protein that differs from vWf in its subunit and multimer sizes.^{1, 2, 3} The smallest multimerin multimer is a 400 kDa trimer but most of the multimers found in platelets are millions of daltons in size, forming one of the largest proteins that is found in platelets.² Following platelet activation and secretion, multimerin binds to the platelet surface but, unlike vWf, multimerin is not detectable in the plasma.^{1, 2, 3} The function of multimerin has been uncertain, however, our recent investigations indicate that multimerin is a factor V/Va binding protein and suggest a possible role as a carrier protein for the factor V that is stored within platelets.⁴ Recently, we completed the sequencing and cloning of the multimerin cDNA. These studies identified multimerin was a unique protein, with RGDS, EGF-like, and putative coiled-coil domains. In addition, the carboxyl-terminal region of multimerin was found to resemble the globular head domains of complement C1q and collagens type VIII and X.⁵

Multimerin is assembled from subunits that are linked by interchain disulfide bonds, to form multimers of variable size.^{1, 2, 3, 6} The

subunits are derived from a common precursor that undergoes proteolytic processing to form the mature protein.^{3, 6} Multimerin is a highly glycosylated protein with complex, N-linked carbohydrate accounting for approximately 1/3 of its molecular mass.⁶ In Dami cells, a megakaryocytic cell line, multimerin is synthesized as a 170 kDa precursor protein containing high-mannose forms of N-linked carbohydrate.⁶ This precursor undergoes further glycosylation to a p-196 protein.⁶ Subsequently, interchain disulfide bonds form between subunits, forming large homomultimers. Proteolytic processing of the subunits occurs^{3, 6} and the multimerin stored within platelets is composed mainly of mature, p-155 and p-170 subunits.^{1, 2, 3, 6} Only small amounts of p-196 are detectable in platelet multimerin.⁶

Using electron microscopy, we noted that multimerin and von Willebrand factor were located in the same region of platelet alpha-granules.⁶ Alpha-granules contain three distinct regions: a nucleoid zone, an intermediary zone and an eccentric, electron-lucent zone which contains tubular structures and vWf.^{7, 8, 9} The ultrastructure of this region closely resembles Weibel-Palade bodies, the storage site of vWf in endothelial cells.^{7, 10, 11} These observations suggested possible parallel cellular distributions for multimerin and vWf and led us to postulate that multimerin might be found within endothelial cells.

In this report, we demonstrate that multimerin is contained in endothelial cells both *in vitro* and *in vivo*. Additionally, we report colocalization of multimerin with vWf within Weibel-Palade bodies using confocal laser microscopy and provide evidence for a regulated pathway of

multimerin synthesis and release, which modulates expression of the protein on the cell surface and in the extracellular matrix.

MATERIALS AND METHODS

Reagents

MEM, methionine-free Dulbecco media, penicillin-streptomycin, goat anti-rabbit-FITC, goat anti-mouse-FITC were obtained from Gibco BRL, Burlington, Ont., Canada. Endothelial cell growth factor, N-glycanase F and neuraminidase were obtained from Boehringer Mannheim, Montreal, Que., Canada. O-glycanase was purchased from Genzyme, Cambridge, MA. Protein A Sepharose beads were obtained from Pharmacia Biotech Inc, Montreal. Porcine heparin was obtained from Leo Laboratories Inc, Pickering, Ont. Goat anti-rabbit-TR and goat anti-mouse-TR were purchased from Cappel, West Chester, PA. Dami cells¹², a human megakaryocytic cell line, were a generous gift of Dr. Sheryl M. Greenberg, Brigham and Women's Hospital, Boston, MA. Monoclonal and polyclonal vWf antibodies were purchased from Dako, Carpinteria, CA. Bovine skin gelatin, collagenase, PMA, ionophore A23187, leupeptin, soybean trypsin inhibitor, phenylmethylsulfonyl fluoride, N-ethyl maleimide, benzamidine, aprotinin were purchased from Sigma, St. Louis, Mo. Human thrombin was supplied by Enzyme Research Laboratories, South Bend, IND. Permafluor was obtained from Biomed, Foster, CA. ³⁵S-methionine was supplied by NEN DuPont, Mississauga, Ont. Metamorph/Northern Image software was obtained from EMPIX Imaging, Mississauga, Ont.

Cell Preparation

Human umbilical vein endothelial cells were isolated from collagenase digested umbilical cord segments as described.^{13, 14} Cells were grown in minimal essential media containing 20% human AB serum, 20 µg/ml endothelial cell growth factor, 90 µg/ml porcine heparin, 50 U/ml penicillin and 50 µg/ml streptomycin.

Dami cells, a human megakaryocytic cell line, were cultured using PMA as described.⁶ Platelet lysates and ¹²⁵I surface-radiolabeled, thrombin-activated platelets were prepared as previously described.^{1, 2, 15}

Antibodies

Monoclonal (JS-1) and polyclonal antibodies against multimerin^{1, 2}, and vWf and affinity purified polyclonal antibodies against PAI-1^{13, 16} were used for immunocytochemistry and immunoprecipitation. The multimerin and vWf antisera were non-crossreactive and identified distinct proteins by both reduced and nonreduced immunoblotting.^{1, 2, 6}

Immunocytochemistry

Immunoperoxidase histochemistry was performed as described using monoclonal (JS-1) and polyclonal anti-multimerin on frozen sections of normal tissue and cultured endothelial cells.⁶

For immunofluorescence studies, second passage endothelial cells were plated for 1-2 days in MEM media (containing 20% human AB serum without heparin and endothelial cell growth factor) on sterile glass coverslips

pre-coated with gelatin. For some experiments, the cells were stimulated (20 min at 37°C) with media containing either 1 U/ml human thrombin, 10 ng/ml PMA, or 10 μ M ionophore A23187. Control cells were incubated in medium without added agonist.

After treatment, the cells were rapidly fixed (5 min) with 3 % paraformaldehyde in PBS, pH 7.4 and incubated with alternating washes of PBS and 0.1 M glycine in PBS (10 min, RT) prior to antibody incubations. For some studies, cells were permeabilized with 0.025 % Triton X-100 (in PBS, 5 min, RT) after fixation. Cells were then incubated in blocking buffer (0.5 % BSA-PBS containing 50 mg/ml normal goat immunoglobulins, 30 min, RT) before incubation with primary antibodies (1/50 dilution in blocking buffer, 1 hr, 37 °C). Primary antibodies were detected using fluorochrome-conjugated secondary antibodies (1/20 dilution in blocking buffer, 1 hr, 37 °C) which included goat anti-rabbit-FITC, goat anti-rabbit-TR, goat anti-mouse-FITC and goat anti-mouse-TR . Slides were washed and mounted using Permafluor. Control slides were processed using normal mouse IgG, normal rabbit IgG, and no primary antisera. For some studies, cells were viewed and photographed on a standard epifluorescence microscope (Leica Laborlux S) fitted with a 63X planapo oil immersion lens (n.a. = 1.4), a 100 W mercury lamp, transmission filters for FITC (525 nm) and TR (600 nm long pass filter), and an automatic 35 mm camera.

Confocal Scanning Laser Microscopy (CSLM) and Image Analysis

Dual-labeling CSLM analysis of cultured endothelial cells, using Z-plane optical sectioning (200 nm/section), was performed to study the relative spatial distributions of intracellular and extracellular multimerin and vWf. Optical sectioning of cells was performed using a Zeiss LSM 10 equipped with a 63X planapo oil immersion lens (n.a. = 1.4). Dual-wavelength images were acquired using an argon ion laser (488 nm excitation), a helium/neon ion laser (543 nm excitation) and two matched long pass barrier filters for FITC (515-525 nm emission) and TR (575-640 nm emission) images. Image processing and analysis were performed on Metamorph/Northern Image software. Before analysis, all images were Z-plane line averaged and corrected for dark current and instrument background. Cells stained with non-immune primary antibodies were used to establish background intensity profiles.

Dual-labeling immunofluorescence studies (using monoclonal anti-multimerin and polyclonal anti-vWf as primary antibodies and FITC- and TR- conjugated secondary antibodies) were performed on nonpermeabilized and permeabilized monolayers of control and agonist-treated endothelial cells. Identical fields were analyzed for multimerin and vWf, with the final images containing data acquired from all of the Z-plane optical sections. For some studies, images were color encoded to demonstrate the relative staining intensity profiles or to demonstrate the location of the staining in the Z-plane.

Metabolic Radiolabeling

Second or third passage endothelial cells (confluent, 60 mm dishes) were used for 18 hr metabolic labeling with 3 ml of methionine-free Dulbecco media containing 0.1 mCi/ml ^{35}S -methionine and 2% fetal calf serum. For pulse-chase studies of multimerin biosynthesis, 1 ml of methionine-free culture medium containing 0.5 mCi of ^{35}S -methionine was used.⁶ Cells were solubilized in lysing buffer (30 min., 4 °C using 1% Triton X-100, 0.1% SDS, 20 mM Tris, 100 mM NaCl, pH 7.4 with 10 mM EDTA, 0.1 μM leupeptin, 0.2 mM PMSF, 0.02 mg/L soybean trypsin inhibitor and 5 mM N-ethyl maleimide; final volume 3 ml/dish). Conditioned media samples were collected into tubes containing the same concentration of leupeptin, PMSF and N-ethyl maleimide.

Studies, comparing the multimerin released by constitutive and regulated pathways, were performed using endothelial cells metabolically labeled for three days in medium containing 0.3 mCi of ^{35}S -methionine. Duplicate flasks were used for each condition and fractions were pooled prior to analysis. Following a 6 hour chase in medium without label, cells were washed twice in Hank's solution (without calcium or magnesium), then incubated (20 min, 37 °C) in serum-free medium in the absence or presence of 10 mM ionophore A23187 (0.1 % DMSO final, all flasks) or 1 U/ml of human alpha-thrombin. Culture supernatants and cell lysates were harvested at 20 minutes. Matrix fractions (Triton X-100 insoluble material) were solubilized (0.75 mls of lysing buffer, 1 % SDS/ T25 flask, 10 min). Equal volumes of resting and activated fractions and 0.3 ml (multimer) and 0.5 ml (subunit gels)

volumes of the 3 day and 6 hour fractions were used for immunoprecipitation.

Radioimmunoprecipitation

For radioimmunoprecipitation studies, one ml volumes of culture supernatants, and equivalent volumes of cell lysates and matrix fractions were used. Matrix fractions were renatured using lysing buffer containing Triton X-100 and deoxycholate (final: 1% Triton X-100, SDS 0.1%, 1% deoxycholate) and any insoluble material removed by centrifugation (30,000 g, 30 minutes) before antibody incubations. Proteins were immunoprecipitated as described using protein A beads (50 μ l) preincubated with either JS-1 (20 μ g), polyclonal anti-multimerin (50 μ l), polyclonal anti-vWf (50 μ l) or polyclonal anti-PAI-1 (50 μ l).^{1, 2, 6} To reduce non-specific binding in metabolically labeled preparations, double immunoprecipitations were performed as described.⁶ Immunoprecipitates were used for both subunit (5 % SDS-PAGE, reduced) and multimer (1.25 % agarose/ 1.5 % acrylamide gels) analyses.^{1, 2} Thrombospondin was used as a molecular mass reference for multimer gels.² Solubilized, ¹²⁵I surface radiolabeled, thrombin-activated platelets (1 ml volumes, 1×10^8 platelets/ml) were used for comparison studies.^{1, 2}

Carbohydrate Analysis

The carbohydrate content of multimerin was assessed as described.⁶ Briefly, multimerin immunoprecipitates, immobilized on protein A beads, were eluted with 0.1 % SDS, 0.2 % 2-mercaptoethanol with 0.1 mg/ml

benzamidine, 1 mM PMSF, 1 μ g/ml aprotinin and 10 μ g/ml leupeptin followed by treatment with buffer alone, N-glycosidase F (final concentrations: 15 U/ml in 20 mM sodium phosphate, pH 7.0, 1 hr, 37 °C), or N glycosidase F followed by neuraminidase (final concentrations: 2 U/ml in 20 mM sodium phosphate, pH 5.5, 1 hr, 37 °C) and O-glycanase (final concentrations: 30 U/ml added to the neuraminidase treated protein, 5 hr, 37 °C). Samples were analyzed using 5 % reduced SDS-PAGE.

RESULTS

Multimerin is Found in Endothelium in Situ

Immunohistochemistry, using frozen sections of normal human tissue, was used to investigate whether multimerin is found in endothelium. Multimerin was identified within the endothelium of a variety of different-sized venous and arterial blood vessels, including capillaries, venules, veins, arterioles, arteries and vaso vasorum (Fig. 1). Most vessels examined demonstrated staining for multimerin, but occasional vessels were observed that did not stain for multimerin. In some of the larger vessels, multimerin immunoreactivity was intense in the intima with progressively weaker staining in the media, indicating that multimerin was also in the subendothelial matrix. No staining of endothelium or subendothelium was seen with the negative controls, processed without the monoclonal antibody or with preimmune rabbit IgG. Apart from endothelium, subendothelium and platelets, no other cells or structures exhibited staining for multimerin.

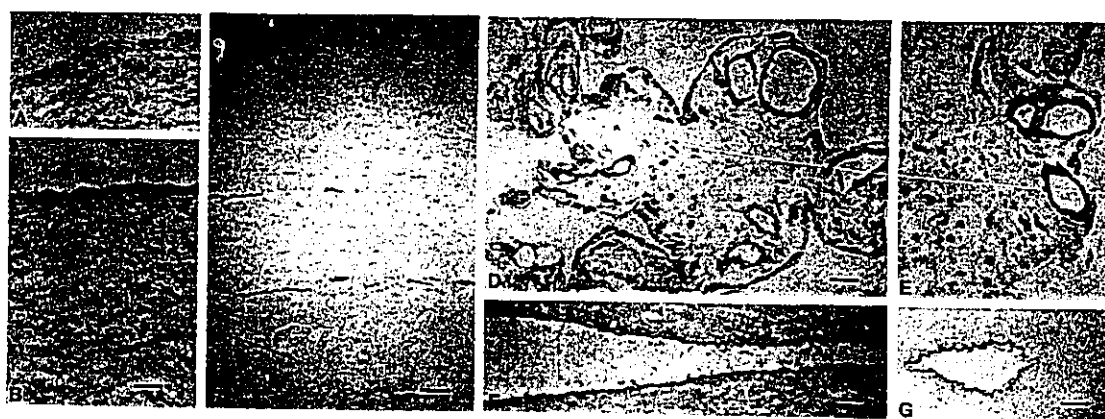


Figure 1

Distribution of Multimerin in Normal Blood Vessels and Endothelial Cells

Frozen sections of normal tissue including aorta (A, B), small bowel (C), placenta (D, E), carotid artery (F), and umbilical cord (G; vein is shown) were studied using monoclonal (B, C, D, F, G) and polyclonal anti-multimerin (E). No staining was observed with the negative controls, processed with preimmune serum (A). These photomicrographs demonstrate multimerin in the endothelium and subendothelium of blood vessels. Scale bars indicate 10 μ M (C, G) and 30 μ M (A, B, D, E, F).

Localization of Multimerin in Cultured Endothelial Cells

Following the identification of multimerin in umbilical veins in situ (Fig. 1G), immunofluorescent staining was used to study multimerin in cultured human umbilical vein endothelial cells. Nonpermeabilized and permeabilized preparations were compared to evaluate the extracellular and intracellular distribution of multimerin. In non-permeabilized preparations, the immunofluorescence staining for extracellular multimerin was associated with endothelial cell surfaces and with the extracellular matrix (Fig. 2 A). In contrast, the pericellular vWf staining was more punctate and fibrillar (Fig. 2 B). In Triton-permeabilized cells, multimerin was located in the perinuclear region and within rod-shaped cytoplasmic organelles (Fig. 2 C, E) which also stained for vWf (Fig. 2 D, G). Identical results were obtained using monoclonal and polyclonal antibodies to multimerin and using monoclonal and polyclonal antibodies to vWf. Similar staining patterns for multimerin were observed using single- and dual-labeled preparations and when multimerin was identified using either the FITC- or TR-conjugated secondary antibodies. No staining of Weibel-Palade body structures was observed with the negative controls (Fig. 2 F), or with antibodies to thrombospondin or type 1 plasminogen activator inhibitor (PAI-1), two proteins constitutively secreted by endothelial cells that are not stored in Weibel-Palade bodies.^{14, 17} These studies indicated that multimerin and vWf shared common intracellular storage granules, with differences in their extracellular distributions.

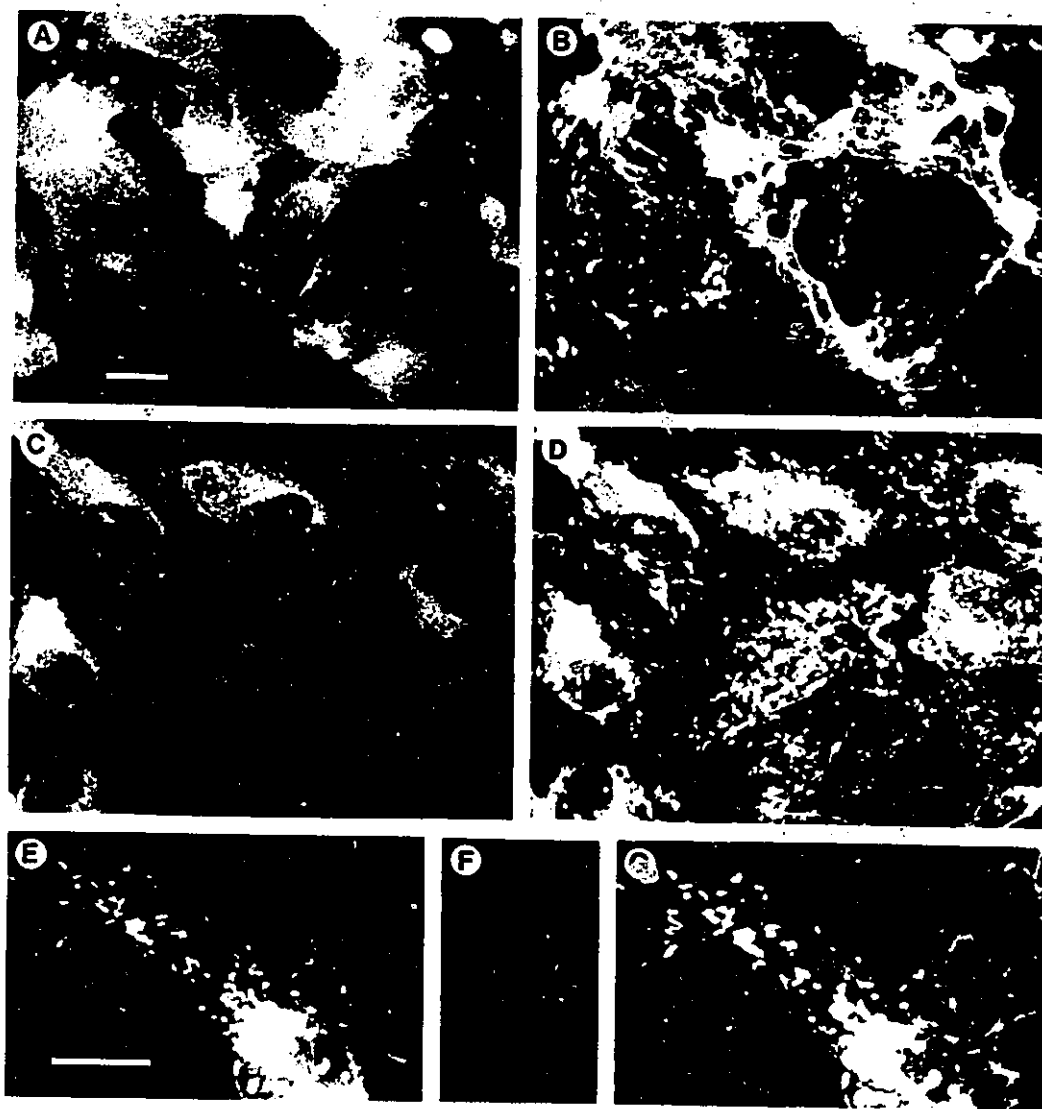


Figure 2

Immunocytochemistry Studies of Multimerin and vWf in Endothelial Cells

Permeabilized (c, d, e, f, g) and nonpermeabilized (a, b) quiescent endothelial cells were double-labeled using antibodies to multimerin (a, c, e) and vWf (b, d, g). This study demonstrates the presence of multimerin in intracellular organelles, colocalizing with vWf in Weibel-Palade bodies. No staining of Weibel-Palade bodies was observed in the negative controls (f). Scale bars indicate 10 μ M.

CSLM analysis, using optically sectioned microscopy images, was performed to evaluate the distributions of multimerin and von Willebrand factor in all three dimensions. Color was used to encode the Z-plane depth, with dark blue (3.5 mm) corresponding to staining at the level of the culture substratum-extracellular matrix and red (0 mm) corresponding to stained structures located more luminally. In permeabilized, quiescent endothelial cells (Fig. 3 A, B, C), both multimerin (Fig. 3A) and vWf (Fig. 3B) were noted in rod-shaped intracellular organelles located peripherally, within the cytoplasmic plane of the endothelial cells. Some of these organelles were located luminally, overlying the cell nucleus (yellow-green organelles in center field, Fig. 3 A, B). Additional vesicular staining, separate from the rod-shaped organelles, was noted for multimerin (Fig. 3 A and D).

Further comparisons were performed by overlaying the composite stacks of images for multimerin and vWf, looking for regions of overlap in their 3-dimensional distribution (Fig. 3 C). For these studies, regions of colocalization were color encoded in blue, regions staining only for multimerin were encoded in red, and regions staining only for vWf were encoded in yellow (Fig. 3 C). The overlap in the distribution of multimerin and vWf was within rod-shaped (2-4 mm long; <2 mm wide) cytoplasmic organelles, indicating storage of multimerin within Weibel-Palade bodies (Fig. 3 C). However, additional diffuse endothelial cell staining for multimerin was also observed, indicating some differences in the distribution of these proteins.

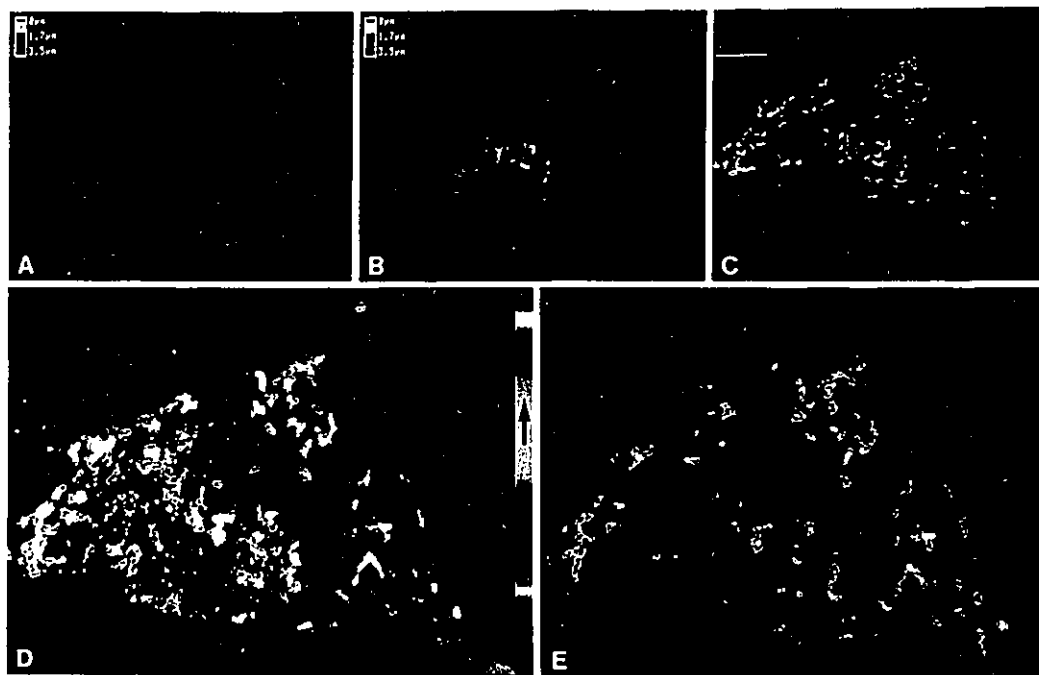


Figure 3
Analysis of the Distribution of Multimerin Within Quiescent Endothelial Cells

Three-dimensional CSLM image analysis of the distribution of multimerin (A) and vWf (B) in a double-labeled, permeabilized, quiescent endothelial cell. The Z-plane distribution of staining is color encoded with blue=basal, red=luminal. Both multimerin and vWf were located in intracellular organelles. Image analysis for regions of overlapping distribution is shown in C: codistribution is shown in blue, red indicates regions that contain only multimerin, and yellow indicates regions that contain only vWf. Multimerin and vWf colocalized inside Weibel-Palade bodies, however, additional regions staining only for multimerin were observed. Composite image stacks were also analyzed for the relative intensity profile (D, E) of each protein: the color-encoded scale bar (panels D, E) indicates the relative staining intensity. The highest intensity staining (white) for multimerin (D) and vWf (E) occurs within Weibel-Palade bodies. Scale bars (A, B) indicate 10 μ M.

Staining intensity profiles of double-labeled, permeabilized, quiescent cells were used to compare the pericellular distributions of multimerin and vWf, using data collected from the stack of Z-plane optical sections. In quiescent, permeabilized endothelial cells, the most intense staining was associated with Weibel-Palade bodies, indicating that the highest concentrations of multimerin and vWf were within these storage organelles (Fig. 3 D, E; shown as composite, color-coded images of pixel intensities).

Studies of Multimerin in Quiescent and Activated Endothelial Cell Matrices

Although multimerin and vWf colocalized within endothelial cells, differences were consistently observed in their pericellular distribution in both quiescent and activated endothelial cell monolayers. Quantitative confocal microscopy was used to characterize the changes in multimerin distribution with activation (Fig. 4). Double-labeled slides from quiescent and activated preparations (nonpermeabilized) were analyzed for multimerin and vWf expression, using a color-coded scale to indicate staining intensity (linear scale). All images were acquired at the identical contrast and brightness settings to permit comparisons for each fluorochrome.

Following treatment of endothelial cells with thrombin, ionophore A23187, or PMA (separate experiments) to induced Weibel-Palade release, a marked increase was observed in the pericellular staining for multimerin and vWf. This increased matrix staining was observed using both monoclonal and polyclonal antibodies against these proteins. Figure 4 shows representative images, incorporating all of the Z-plane optical sections of

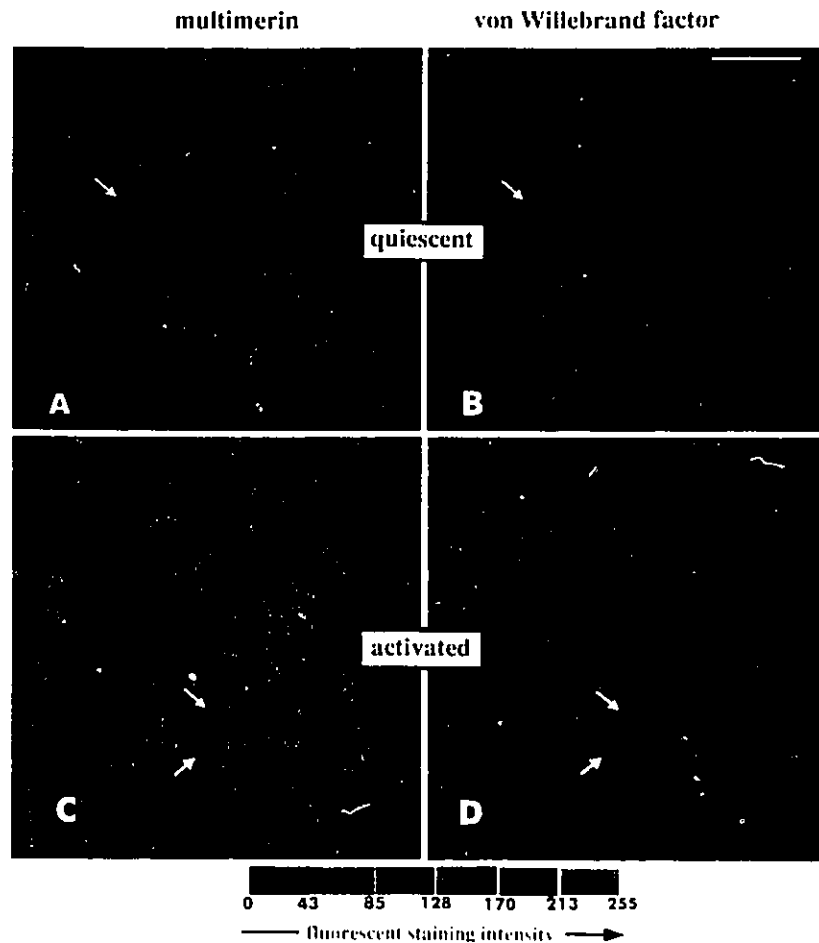


Figure 4
Studies of the Extracellular Distribution of Multimerin and vWf in Quiescent and Thrombin-Stimulated Endothelial Cells

Dual-labeled, quiescent (A, B) and thrombin-stimulated (C, D) endothelial cells were evaluated using CSLM to determine the pericellular distribution of multimerin (A, C) and vWf (B, D). All images were acquired at the same contrast and brightness settings to allow comparisons. Segmented histogram analysis was used to generate color-encoded images of relative fluorescent staining intensity, incorporated data from all of the Z-plane optical sections. The segmented, colored scale bar indicates the relative fluorescent intensities. Increases in both multimerin (C) and vWf (D) staining were apparent after activation with more intense multimerin staining associated with endothelial cells. The scale bar (panel B) corresponds to 50 μm .

nonpermeabilized, quiescent (Fig. 4 A, B) and thrombin-activated (Fig. 4 C, D) endothelial cell monolayers. The identical fields are shown for comparison of multimerin (Fig. 4 A, C) with vWf (Fig. 4 B, D). Quiescent, nonpermeabilized endothelial cells exhibited low level staining for multimerin on the cell surface (arrow, Fig. 4A) and in the matrix. In contrast to the distribution of multimerin, little pericellular vWf staining (arrow, Fig. 4B) was observed. Much more intense pericellular and matrix staining for both proteins was observed following activation and Weibel-Palade body release (Fig. 4 C, D). The vWf associated with the activated cells (arrows, Fig. 4 D) was located mainly in release patches as previously described.¹⁸ After activation, multimerin was also observed in release patches on the cell surface (arrows, Fig. 4 C), with more diffuse pericellular staining than vWf. Comparison of multimerin and vWf in the extracellular matrix of activated cells indicated similarities in their distribution. No increases in cell or matrix staining after activation were observed with the negative controls, processed with nonimmune mouse and rabbit IgG. These results indicate a regulated pathway for multimerin secretion which allows for a marked increase in the expression of multimerin on the cell surface and in the extracellular matrix. Comparison of the multimerin staining intensity profiles of control and Triton-extracted (1 % Triton X-100, 30 min., 4 °C, Fig. 5) ionophore-activated monolayers indicated that most of the pericellular multimerin was Triton-insoluble. Together, these results indicate binding of the multimerin released from Weibel-Palade bodies to the endothelial cell surface and extracellular matrix.

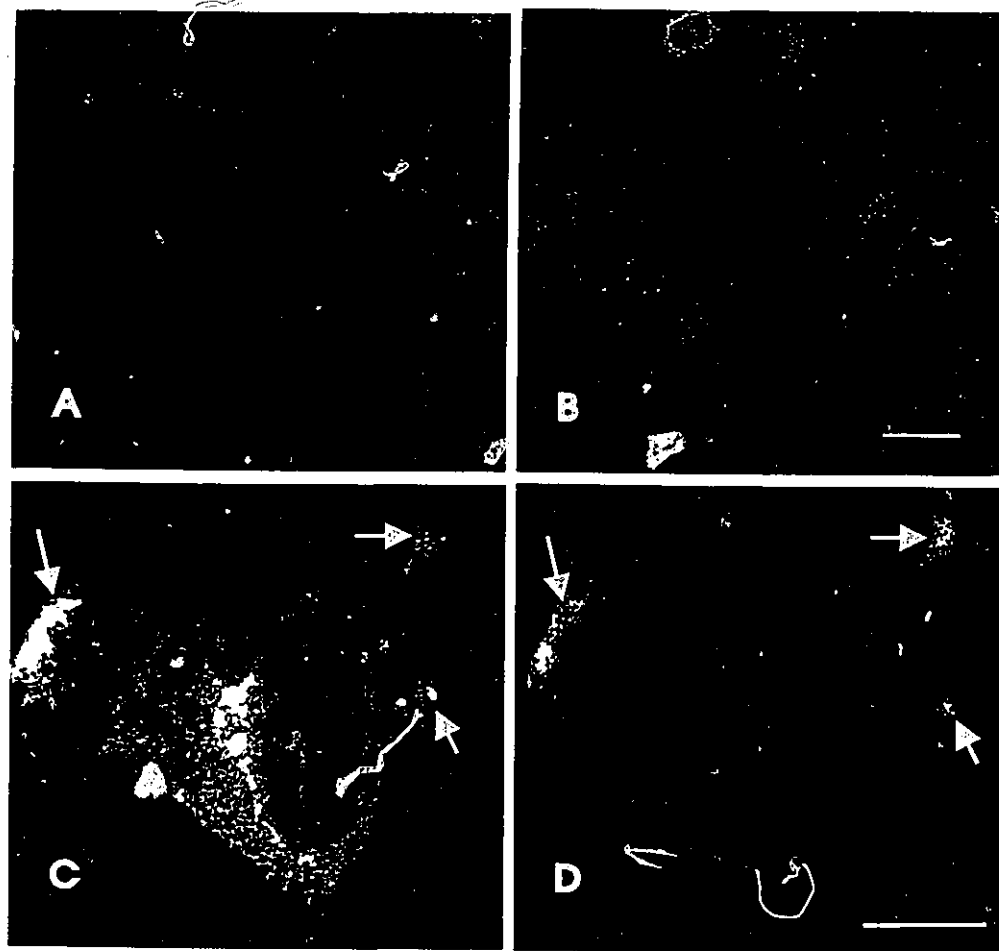


Figure 5
The Association of Multimerin with Endothelial Cells and Their Matrix
Following Weibel-Palade Body Release

Immunocytochemistry was used to evaluate the multimerin bound to the pericellular matrix after activation. Agonist-treated monolayers (ionophore A23187 treated cells, stained using monoclonal anti-multimerin are shown) demonstrated multimerin staining of the pericellular matrix that was resistant to extraction with 1% Triton X-100 (shown as staining intensity profiles, panel A-nonextracted, panel B - Triton extracted). After treatment with agonists, there was redistribution of Weibel-Palade bodies with coalescence of staining for multimerin (C) and the appearance of release patches (arrows) containing multimerin (C) and vWf (D) (panels C and D are staining intensity profiles of double-labeled, permeabilized, thrombin-treated cells). Images are color encoded for staining intensity, as per Fig. 3, panels D, E. Scale bars represent 50 μM (panels A, B) and 10 μM (panels C, D).

Investigations of Multimerin Biosynthesis by Endothelial Cells

The storage of multimerin within Weibel-Palade bodies led us to postulate that multimerin was synthesized by endothelial cells. Metabolic labeling studies were performed to test this postulate. Following metabolic labeling of cultured endothelial cells with ^{35}S methionine (18 hr), cell lysates and culture supernatants were examined for multimerin, using radioimmunoprecipitation.

Multimerin was identified in both the cell lysates and culture supernatants with greater quantities detected in the culture supernatant, indicating that most of the multimerin was constitutively secreted during an 18 hr metabolic labeling (Fig. 6). The subunit sizes of the labeled multimerin secreted by the cultured endothelial cells were 186 (5% SDS-PAGE) and 165 kDa (7% SDS-PAGE). Two-dimensional nonreduced-reduced electrophoresis indicated that the different sized subunits were linked by interchain disulfide bonds.

Pulse chase studies of multimerin biosynthesis by endothelial cells showed that multimerin was first synthesized as a 170 kDa precursor multimerin subunit, followed by the delayed appearance of a 186 kDa multimerin subunit in the cell lysate and supernatant (Fig. 7). Secretion of the protein began at 1 hour and was complete by 6 hours following biosynthesis. The secreted multimerin was composed of p-186 and p-165 kDa subunits with greater amounts of p-186 detected. The majority of the multimerin synthesized was constitutively secreted into the culture

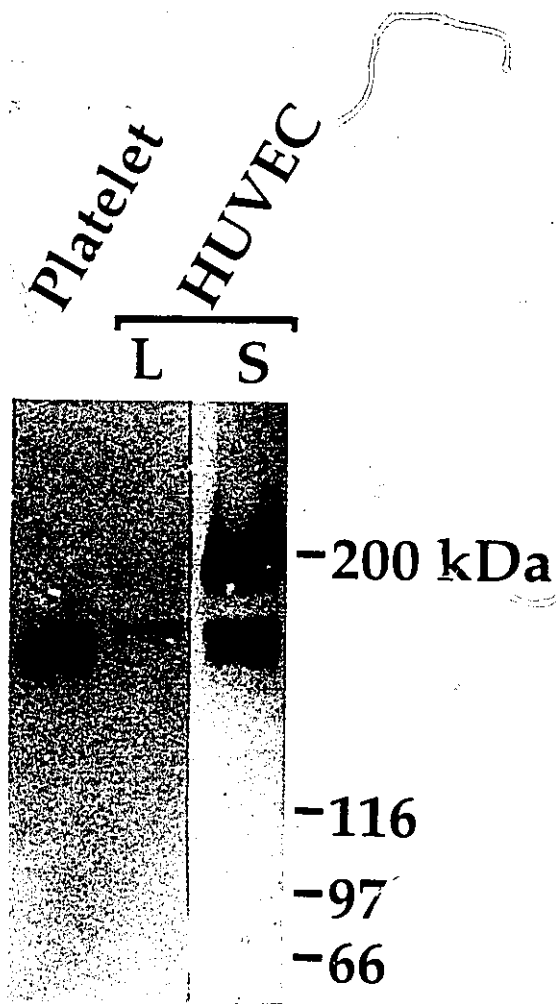


Figure 6

Metabolic Labeling Studies of Endothelial Cells

Endothelial cells were metabolically labeled for 18 hrs and then the intracellular (L) and secreted (S) multimerin were studied by immunoprecipitation. Radiolabeled platelet multimerin (P) is shown for comparison. These studies indicate that endothelial cells synthesize and constitutively secrete multimerin. The multimerin from platelets and endothelial cells differed in their reduced subunit mobilities with endothelial cells secreting large amounts of the nonproteolyzed (p-186) multimerin subunit.

supernatant (Fig. 6 and 7).

Biosynthesis of multimerin by endothelial cells (Fig. 7) was similar to the synthesis of multimerin by Dami cells.⁶ Endothelial cell multimerin contained primarily complex N-linked carbohydrate with smaller amounts of O-linked carbohydrate on the p-186 subunit. N-deglycosylation of the p-186 endothelial multimerin subunit resulted in a 45 kDa shift in its apparent molecular mass. Small but consistent differences were detected in the size of the mature multimerin subunits produced by Dami cells and endothelial cells. Carbohydrate analysis indicated that these differences were related to the extent of N-glycosylation.

Studies of the Multimerin Released by Regulated and Constitutive Pathways

We postulated that the multimerin destined for release by agonists might differ from the constitutively secreted multimerin in its subunit and multimer processing. Immunoblot studies of endothelial cell lysates demonstrated a similar multimer composition to the multimerin stored in platelets (Fig. 8). Both cells contained high molecular weight multimers of multimerin and differences were observed in the mobilities of endothelial cell multimerin and vWf.

Metabolic labeling studies were performed to compare the multimerin released by regulated and constitutive pathways. The pulse-chase studies indicated that constitutive multimerin secretion was complete by 6 hours. Cells were prepared by a 3 day metabolic labeling followed by a 6 hour cold-chase to remove any labeled multimerin and vWf destined for

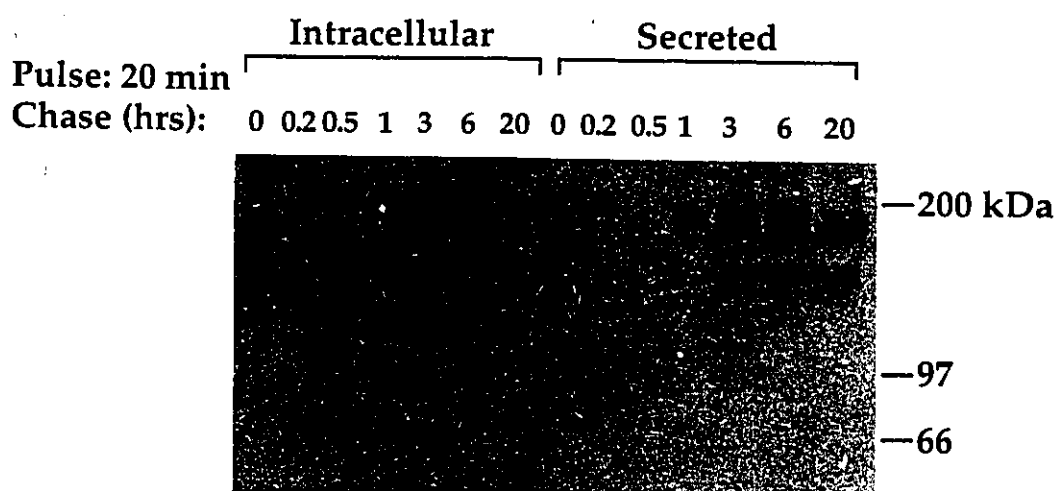


Figure 7

Pulse-Chase Studies of the Biosynthesis of Multimerin

Endothelial cells were metabolically labeled with ^{35}S methionine for 20 minutes and cell lysates (intracellular) and culture supernatants (secreted) were collected at different times following the pulse labeling. The samples were immunoprecipitated and the multimerin analyzed by reduced SDS-PAGE. This study demonstrates that multimerin originates from a 170 kDa precursor. This protein is processed to form the secreted 186 and 165 kDa subunits. Secretion of the protein began at 1 hr and was complete by 6 hrs.

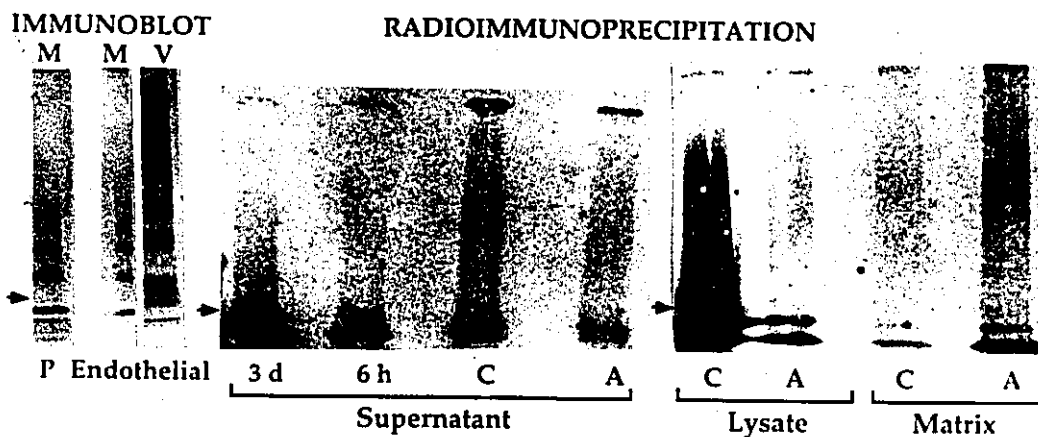


Figure 8

The Multimeric Composition of Endothelial Cell Multimerin.

Immunoblotting of the multimerin contained in platelet (P) and endothelial cell lysates showed a similar multimeric composition. Immunoblot comparison of endothelial cell vWf (V) and multimerin (M), performed on halves of the same lane, demonstrates the differences in their migration.

Radioimmunoprecipitations were used to compare the multimerin secreted by regulated and constitutive pathways. A 3 day metabolic labeling (constitutive) was followed by a 6 hr cold chase to remove labeled protein released by constitutive secretion and then ionophore-activated (A; regulated pathway) and quiescent (C) cell supernatants, lysates and renatured matrix fractions were harvested. Radioimmunoprecipitation findings indicate constitutive secretion of mainly the small multimers of multimerin (3 day supernatant and 6 hr chase), while resting cell lysates (C lysate; regulated pathway) contained high molecular weight multimers of multimerin. Arrows indicate the position of thrombospondin (M_r 450 kDa).

constitutive release. After washing, cultures were incubated with or without ionophore A23187. Control and activated culture supernatants, cell lysates and matrix fractions were harvested. Equivalent volumes of each fraction were used to prepare immunoprecipitates. Immunoprecipitates were analyzed on multimer (Fig. 8) and subunit gels (Fig. 9). Immunoprecipitation controls for these studies included vWf (known regulated pathway for secretion) and PAI-1 (exclusively constitutive pathway for secretion).

Multimer analyses indicated that the labeled multimerin stored in endothelial cells (Fig. 8, control lysate) was rich in high molecular weight multimers, in contrast to the small multimers released constitutively (Fig. 8, lanes 3d and 6h). Activation was associated with the loss of labeled multimerin from the lysate, but it was not recovered from the activated supernatant or the renatured matrix fractions.

Subunit analyses, using SDS-PAGE, indicated loss of the labeled multimerin subunits from the cell lysate (Fig. 9, activated (A) lysate) following treatment with ionophore. Equivalent findings were observed when thrombin was used as the agonist. While activation was associated with increased secretion of labeled vWf (v2,) and its propolypeptide (v3) into the culture supernatant (Fig. 9, A vs C supernatants), (Wagner *et al.*, 1987), little multimerin could be detected in the same supernatants. Even with prolonged exposures (3 weeks), labeled multimerin subunits could not be identified in the activated cell lysate or supernatant.

Similar to multimerin, metabolically labeled PAI-1 was constitutively secreted during the labeling and 6 hr chase period (Fig. 9, right

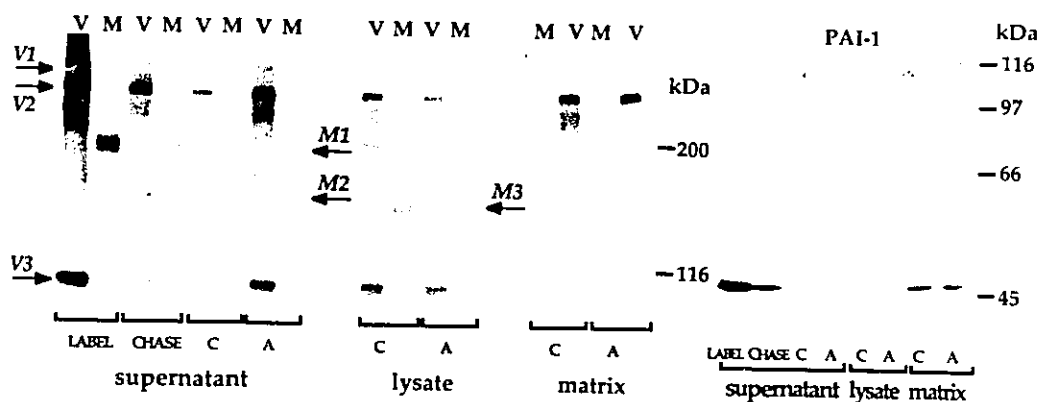


Figure 9
The Subunit Composition of Multimerin Released by Constitutive and Regulated Pathways

HUVECs were metabolically labeled and fractions harvested as in Fig. 8 (Fractions: LABEL-3 day supernatant; CHASE-6 hr cold chase; ionophore-activated (A) and quiescent (C) cell supernatants, lysates and renatured matrix fractions). Radioimmunoprecipitations, using antibodies to multimerin (M), vWf (V; known regulated pathway for secretion) (left panel), and PAI-1 (right panel; exclusively constitutive secretion), were analyzed by reduced SDS-PAGE. Arrows indicate the position of the multimerin (M) and vWf subunits (V). Similar to vWf, the multimerin destined for regular release (M3, p-155; control lysate) showed more extensive subunit proteolytic processing than the constitutively secreted protein (M1, p-186 and M2, p-165; LABEL and CHASE).

panel). However, no differences were observed between control and agonist-treated cell fractions, consistent with the lack of a pathway for stimulated PAI-1 secretion.

The loss of multimerin from the cell lysate without its release into the culture supernatant (Fig. 8 and 9) suggests that the majority of the multimerin released by agonist treatment was bound to Triton-insoluble components, either in the matrix or on endothelial cell surfaces. This postulate was consistent with the increases observed in pericellular multimerin staining after activation (Fig. 4C). Furthermore, no labeled multimerin was recovered in radioimmunoprecipitates prepared from ¹²⁵I surface labeled endothelial cells (quiescent and activated). These results indicate binding of the released multimerin to the Triton-insoluble pericellular matrix (Fig. 5C).

DISCUSSION

Multimerin is as an extremely large, multimeric protein, stored within platelets, and expressed on their surface following platelet activation.¹ Studies of the cDNA sequence have identified multimerin as a unique protein, unrelated to von Willebrand factor.⁵ The function of multimerin has been uncertain, but our recent investigations have identified multimerin as a factor V/Va binding protein that may function as a carrier protein for the factor V stored within platelets.⁴ Studies of resting platelet lysates indicated that all of the factor V coagulant activity was associated with multimerin and electron microscopy studies colocalized the factor V with multimerin within

alpha-granules.⁴ Although multimerin and factor V were found to be complexed in resting platelets, studies of thrombin stimulated platelets indicated that the majority of factor Va on the platelet membrane was not associated with multimerin.⁴

In our previous studies, we observed multimerin was stored in an eccentric location within platelet alpha-granules, colocalizing with vWf.⁶ The presence of multimerin and vWf in the region of the alpha-granule that resembles the ultrastructure of endothelial cell Weibel-Palade bodies led us to postulate that multimerin might also be found in Weibel-Palade bodies. The purpose of the investigations contained in this report was to determine if multimerin was present in endothelium *in vivo* and to characterize the biosynthesis and storage of multimerin by endothelial cells.

Immunohistochemistry studies indicated that multimerin was present in the endothelium and subendothelium of both large and small, venous and arterial vessels (Fig. 1). Apart from platelets located within vessels, no other structures stained for multimerin. In studies of cultured human umbilical vein endothelial cells, multimerin was identified in rod-shaped cytoplasmic organelles which had the morphological features of Weibel-Palade bodies.¹⁰ Double labeling studies, comparing multimerin with vWf, colocalized these proteins within Weibel-Palade bodies. Staining intensity profiles indicated that the highest concentrations of multimerin and vWf in quiescent endothelial cells were within the Weibel-Palade bodies (Fig. 3).

Previously, vWf and its propolypeptide were thought to be the only proteins found in Weibel-Palade bodies.^{11, 18, 19} Recently, the membrane of Weibel-Palade bodies has been demonstrated to contain P-selectin^{20, 21} and CD63.²² Histamine has also been localized to Weibel-Palade bodies.²³ The studies contained in this report demonstrate that Weibel-Palade bodies are the storage site of a second, soluble multimeric protein - multimerin. Failure of previous investigations to demonstrate the presence of multimerin, P-selectin, and CD63 in Weibel-Palade bodies may have been due to the insensitivity of the detection methods or loss of the proteins during the purification procedures.

Treatment of endothelial cells with agonists such as thrombin, PMA, A23187, fibrin, histamine, and C5b-9 are known to stimulate the release of vWf and redistribution of P-selectin to the cell surface.^{24, 25} Our studies indicate that Weibel-Palade body release is also associated with redistribution of multimerin. Double-labeling studies indicated that the release patches that appear on the cell surface following Weibel-Palade body release contained multimerin in addition to vWf. Weibel-Palade body release was associated with a marked increase in the multimerin staining of the endothelial pericellular matrix and this increase was confirmed by quantitative confocal analyses (Fig. 4). The multimerin bound to the pericellular matrix following Weibel-Palade body release was Triton-insoluble, indicating that multimerin is associated with the Triton-insoluble matrix following its release (Fig. 6). We consistently observed differences in the pericellular distribution of

multimerin and vWf, suggesting that these proteins interact with different ligands.

Investigations of multimerin biosynthesis by endothelial cells indicated that endothelial cells synthesize multimerin (Fig. 7). Pulse chase biosynthesis studies indicated similarities in multimerin biosynthesis by endothelial cells (Fig. 8) and Dami cells, a megakaryocytic cell line.⁶ Similar to Dami cell multimerin, endothelial cell multimerin is derived from a 170 kDa precursor protein (promultimerin) containing high mannose, N-linked carbohydrates which are further glycosylated to complex forms. However, differences were observed in the extent of multimerin N-glycosylation by endothelial cells and Dami cells, resulting in differences in the mobility of the multimerin subunits produced by these cells.

Because the subunit and multimer processing of vWf released by regulated and constitutive pathways are different, we postulated that there might be similar processing of multimerin and several parallels were observed. Similar to vWf,²⁶ multimerin released by the constitutive pathway was mainly found in the culture supernatant (Fig. 6 and 7) and was composed mainly of the small multimerin multimers (Fig. 8). In contrast, the multimerin destined for regulated release was enriched in high molecular weight multimers.

Comparison of the multimerin released by regulated and constitutive pathways revealed important differences in their fates. In contrast to the constitutive secretion of multimerin into the culture supernatant, the multimerin released from Weibel-Palade bodies was

primarily bound to the Triton-insoluble matrix and could not be detected in the culture supernatant (Fig. 8 and 9). The increased proportion of high molecular weight multimers (containing many ligand binding sites) in the multimerin destined for regulated release may be the reason it binds more avidly to activated endothelial cells and their matrices. However, other factors, such as activation of the endothelial multimerin receptor or alterations in the matrix ligands, may regulate multimerin binding.

The fate of the multimerin stored in platelets^{1, 2, 3} and endothelial cells is similar; most of the released multimerin binds to the cell and its matrix. This avid association to both platelets and endothelium is the likely explanation for the absence of multimerin in plasma. Because the multimerin bound to activated endothelial cells is Triton-insoluble, we speculate that multimerin binds to an endothelial receptor with links to the cytoskeleton.

We had anticipated that the sequence of multimerin might resemble that of vWf and explain their similar protein trafficking and complex multimeric structures. The propolypeptide sequence of vWf is thought to play a role in the targeting and storage of this protein.^{28, 29} Multimer assembly is also thought to be an important factor in the biogenesis of Weibel-Palade bodies.^{30, 31, 32} However, comparison of the cDNA sequence of multimerin to that of vWf reveals that they are distinct, unrelated proteins.⁵ Furthermore, the amino terminal region of multimerin bears no resemblance to that of vWf. The lack of homology in their

sequences indicates that other factors, perhaps their multimeric structures, might influence their similar protein trafficking.

The relationship of multimerin to the ultrastructure of the Weibel-Palade body is uncertain. The tubular structures contained within Weibel-Palade bodies are highly dependent on vWf. Transfection of vWf cDNA into other cells results in the formation of tubule-containing storage organelles resembling Weibel-Palade bodies.^{28, 32} Our recent investigations of several individuals with severe (type 3) von Willebrand's disease have shown that they are not deficient in platelet multimerin (unpublished observations[¶]). As platelets from patients with severe vWf deficiency have been shown to lack tubules,⁹ these data suggest that multimerin is associated with the matrix of Weibel-Palade bodies and the electron-lucent zone of platelet alpha-granules. These possibilities are under investigation.

The presence of another multimeric protein within Weibel-Palade bodies indicates an expanding role for this storage organelle. Within the vasculature, multimerin is restricted to platelets, endothelial cells and the subendothelium. Both platelets and endothelial cells sequester multimerin internally. Release of Weibel-Palade bodies alters the multimerin expression on the endothelial cell surface and in the pericellular matrix. The association of multimerin with activated platelets, endothelial cells and their pericellular matrix suggests a role in the events associated with vascular injury. We postulate that the multimerin bound to endothelial cell surfaces and their matrix may provide binding sites for factor V/Va. Furthermore, multimerin may have other functions, possibly adhesive, that are important for

hemostasis. The storage of multimerin within platelets and endothelial cells and its absence from plasma may allow restriction of multimerin function(s) to sites where there is vessel injury.

FOOTNOTES

¶ Hayward, C. P. M., Moore, J. C., and J. G. Kelton. Unpublished observations

***Abbreviations used in this paper:* von Willebrand factor, vWf; minimal essential media, MEM; fluorescein isothiocyanate, FITC; Texas Red, TR; phorbol 12-myristate 13-acetate, PMA; phosphate buffer saline, PBS; phenylmethylsulfonyl fluoride, PMSF; Sodium dodecyl sulfate polyacrylamide gel electrophoresis, SDS-PAGE; confocal scanning laser microscopy, CSLM.

REFERENCES

1. Hayward, C. P. M., Smith, J. W., Horsewood, P., Warkentin, T. E., and J. G. Kelton. 1991. p-155, a multimeric platelet protein that is expressed on activated platelets. *J. Biol. Chem.* 266: 7114-7120.
2. Hayward, C. P. M., Warkentin, T. E., Horsewood, P., and J. G. Kelton. 1991. Multimerin: a series of large, disulfide-linked multimeric proteins within platelets. *Blood* 77: 2556-2560.
3. Hayward, C. P. M. and J. G. Kelton. 1995. Multimerin: a multimeric protein stored in platelet alpha-granules. *Platelets*: 6: 1-6..

4. Hayward C. P. M., Furmaniak-Kazmierczak, E., Cieutat, A.-M., Moore, J. C., Bainton, D. F., Nesheim, M. E., Kelton, J. G. and, Côté, G. (1995) Factor V is complexed with multimerin in resting platelet lysates and colocalizes with multimerin within platelet alpha granules. *J. Biol. Chem.* 270: 19217-19224.
5. Hayward, C. P. M., Hassell, J. A., Denomme, G. A., Rachubinski, R. A., Brown, C., and Kelton, J. G. (1995) The cDNA sequence of human endothelial cell multimerin: A unique protein with RGDS, coiled-coil, and EGF-like domains and a carboxyl-terminus similar to the globular domain of complement C1q and collagens type VIII and X. *J. Biol. Chem.* 270: 18246-18251.
6. Hayward, C. P. M., Bainton, D. F., Smith, J. W., Horsewood, P., Stead, R. H., Podor, T. J., Warkentin, T. E., and J. G. Kelton. 1993. Multimerin is found in the α -granules of resting platelets and is synthesized by a megakaryocytic cell line. *J. Clin. Invest.* 91: 2630-2639.
7. Cramer E. M., Meyer D., le Menn R., and J. Breton-Gorius. 1985. Eccentric localization of von Willebrand factor in an internal structure of platelet α -granule resembling that of Weibel-Palade bodies. *Blood* 66: 710-713.
8. Harrison, P., and E. M. Cramer. 1993. Platelet α -granules. *Blood Reviews* 7: 52-62.
9. Wilbourne, B. R., Harrison, P., Lawrie, A. S., Savariau, E., Savidge, G. F., and E. M. Cramer. 1993. Porcine platelets contain an increased quantity of ultra-high molecular weight von Willebrand factor. *Br. J. Haemat.* 83: 608-615.

10. Weibel, E. R., and G. E. Palade. 1964. New cytoplasmic components in arterial endothelia. *J. Cell Biol.* 23: 101-112.
11. Wagner, D. D., Olmstead, J. B., and V. J. Marder 1982. Immunolocalization of von Willebrand protein in Weibel-Palade bodies of human endothelial cells. *J. Cell Biol.* 95: 355-360.
12. Greenberg, S. M., Rosenthal, D. S., Greeley, T. A., Tantravahi, R., and R. I. Handin. 1988. Characterization of a new megakaryocytic cell line: the Dami cell. *Blood* 72: 1968-1977.
13. Kassis, J., Hirsh, J., and T. J. Podor. 1992. Evidence that postoperative fibrinolytic shutdown is mediated by plasma factors that stimulate endothelial cell type I plasminogen activator inhibitor biosynthesis. *Blood* 80: 1758-1764.
14. Schleaf, R. R., Loskutoff, D. J. and T. J. Podor. 1991. Immunoelectron microscopic localization of type 1 plasminogen activator and inhibitor on the surface of activated endothelial cells. *J. Cell Biol.* 113: 1413-1423.
15. Kelton, J. G., Smith, J. W., Horsewood, P., Humbert, J. R., Hayward, C. P. M., and T. E. Warkentin. 1990. Gova^{a/b} alloantigen system on human platelets. *Blood* 75: 2172-2176.
16. Podor, T. J., Weitz, J. I., Hirsh, J., Shaughnessy, S. G., Foulon, D. M., Butcher, M. K., Seiffert, D., and D. J. Loskutoff. 1994. Vitronectin-mediated binding of type 1 plasminogen activator inhibitor (PAI-1) to vimentin exposed in damaged endothelial cells. *J. Clin. Invest: In press.*
17. Reinders, J. H., de Groot, P. G., Dawes, J., Hunter, N. R., van Heugten, H. A. A., Zanbergen, J., Gonsalves, M. D., and J. A. van Mourik. 1985.

- Comparison of secretion and subcellular localization of von Willebrand protein with that of thrombospondin and fibronectin in cultured human vascular endothelial cells. *Biochem. Biophys. Acta* 844: 306-313.
18. Wagner, D. D., Fay P. J., Sporn, L. A., Sinha, S., Lawrence, S. O., and V. J. Marder. 1987. Divergent fates of von Willebrand factor and its propolypeptide (von Willebrand antigen II) after secretion from endothelial cells. *Proc. Natl. Acad. Sci.* 84: 1955-1959.
 19. Ewenstein, B. M., Warhol, M. J. , Randin, R. I., and J. S. Pober. 1987. Composition of the von Willebrand factor storage organelle (Weibel-Palade body) isolated from cultured human umbilical vein endothelial cells. *J. Cell Biol.* 104: 1423-1433.
 20. Bonfanti, R., Furie B. C., Furie, B., and D. D. Wagner. 1989. PADGEM (GMP 140) is a component of Weibel-Palade bodies of human endothelial cells. *Blood* 73: 1109-1112.
 21. McEver, R. P., Beckstead, J. H., Moore, K. L., Marshall-Carlson, L., and D. F. Bainton. 1989. GMP-140, a platelet alpha-granule membrane protein is also synthesized by vascular endothelial cells and is localized in Weibel-Palade bodies. *J Clin Invest* 84: 92-99.
 22. Vischer, U. M., and D. D. Wagner. 1993. CD63 is a component of Weibel-Palade bodies of human endothelial cells. *Blood* 82: 1184-1191.
 23. Ueda, H., Doi, Y., Sakamoto, Y., Hamasaki, K., and S. Fujimoto. 1992. Simultaneous localization of histamine and Factor VIII-related antigen in the endothelium of the human umbilical vein. *Anat. Record* 232: 257-261.

24. Wagner, D. D. 1993. The Weibel-Palade body: the storage granule for von Willebrand factor and P-selectin. *Thromb. Haemost.* 70: 105-110.
25. Hattori, R. , Hamilton, K. K., Fugate, R. D., McEver, R. P., and P. J Sims. 1989. Stimulated secretion of endothelial von Willebrand factor is accompanied by rapid redistribution to the cell surface of the intracellular granule membrane protein GMP-140. *J. Biol. Chem.* 264: 7768-7771.
26. Sporn, L. A., Marder, V. J., and D. D. Wagner. 1986. Inducible Secretion of large, biologically potent von Willebrand factor multimers. *Cell* 46: 185-190.
27. Wagner, D. D., and V. J. Marder. 1984. Biosynthesis of von Willebrand protein by human endothelial cells: processing steps and their intracellular localization. *J. Cell Biol.* 99: 2123-2130.
28. Wagner, D. D., Saffaripour, S., Bonfanti, R., Sadler, J. E., Cramer, E. M., Chapman, B., and T. N. Mayadas. 1991. Induction of specific storage organelles by von Willebrand factor propolypeptide. *Cell* 64: 403-413.
29. Hournet, A. M., Saffaripour, S., Cramer, C. M., Tenza, D., and D. D. Wagner. 1993. Von Willebrand factor storage requires intact prosequence cleavage site. *Eur. J. Cell Biol.* 60: 31-41.
30. Verweij, C. L., Hart, M., and H. Pannekoek. 1987 Expression of variant von Willebrand factor (vWf) cDNA in heterologous cells: requirement of the propolypeptide on vWf multimer formation. *EMBO J.* 6: 2885-2890.

31. Wise, R. J., Pittman, D. D., Handin, R. I., Kaufman, R. J., and S. H. Orkin. 1988. The propeptide of von Willebrand factor independently mediates the assembly of von Willebrand multimers. *Cell* 52: 229-236.
32. Voorberg, J., Fontijn, R., Calafat, J., Janssen H., van Mourik, J. A. and H. Pannekoek. 1993. Biogenesis of von Willebrand factor-containing organelles in heterologous transfected CV-1 cells. *EMBO J.* 12: 749-758.

CHAPTER 5
CLONING AND SEQUENCING OF THE MULTIMERIN CDNA:
DETERMINATION OF THE DEDUCED
AMINO ACID SEQUENCE OF MULTIMERIN

5.1 Introduction

The preceding studies had established multimerin as a unique, soluble, complex multimeric protein. I had also identified two cell types that synthesized multimerin: megakaryocytes and endothelial cells (Hayward *et al*, 1993; Hayward *et al*, 1995b). The investigations of Dami cells and endothelial cells indicated a similar pattern of multimerin biosynthesis (Hayward *et al*, 1993; Hayward *et al*, 1995b). In studies of both cells, a large precursor subunit protein was identified that underwent glycosylation and proteolytic cleavage to produce the mature multimerin subunits found in platelets (Hayward *et al*, 1993; Hayward *et al*, 1995b). Traces of this precursor protein were also found in platelets (Hayward *et al*, 1993).

Before commencing the cloning and sequencing of the multimerin cDNA, the precursor protein was studied to determine its polypeptide size. Studies, using tunicamycin to inhibit N-glycosylation, indicated that the earliest multimerin precursor contained a 132 kDa polypeptide component (Hayward *et al*, 1993; Hayward *et al*, 1995b). Based upon this information, the cDNA for multimerin was predicted to contain a 3.6 kbp open reading frame.

The next step in studying multimerin was to clone and sequence its cDNA. These studies are described in the manuscript "The cDNA Sequence of Human Endothelial Cell Multimerin: A Unique Protein with RGDS, Coiled-coil, and EGF-like Domains and a Carboxyl Terminus Similar to the Globular Domain of Complement C1q and Collagens type VIII and X" (Hayward *et al*, 1995c). This paper was published in the Journal of Biological Chemistry and has been reprinted with the permission of the American Society for Biochemistry and Molecular Biology. The intellectual contributions of Drs. John A. Hassell, Gregory A. Denomme, and Richard A. Rachubinski and the technical assistance of Claudia Brown and Zhili Song in Dr. Kelton's laboratory are gratefully acknowledged. I am also grateful to Dr. J. Evan Sadler for the gift of endothelial cell libraries and Dr. Seth Darst (Rockefeller Institute) for the analyzing the multimerin sequence for coiled-coil structures.

Knowledge of the primary structure of many proteins has provided valuable insights into their structure and function. I postulated that the determination of the deduced amino acid sequence of multimerin would provide important clues about its function. The *in vitro* and *in vivo* demonstration that endothelial cells express multimerin (Hayward *et al*, 1995b) indicated that human endothelial cell libraries could be used to study the multimerin cDNA. The strategy I used was to screen cDNA libraries in λ gt11, using the monoclonal and polyclonal multimerin antibodies (described in Chapter 2). Following isolation of several cDNA clones, the most 5' clone was used to screen the library for more 5' sequences. The complete

multimerin cDNA sequence was derived from these overlapping clones. An in frame stop codon in the 5' untranslated region and the presence of a polyadenylation signal site and polyA tail in the 3' untranslated region indicated that a full length cDNA had been obtained. Data obtained by sequencing an internal peptide of purified multimerin confirmed that the cDNA sequenced encodes multimerin.

The multimerin cDNA encoded a 1228 amino acid protein. Sequence analysis predicted a hydrophilic protein with 23 potential N-glycosylation sites. RGDS, EGF-like and partial EGF-like domains were identified. Comparison with known proteins indicated that multimerin was a unique protein, unrelated to von Willebrand factor. Homology analyses identified similarities between multimerin and EGF-rich proteins in its EGF-like and partial EGF-like domains. The central portion of the sequence was found to resemble coiled-coil proteins including the rod-like tail of myosin heavy chain. Sequence analysis identified highly probable coiled-coil regions within the central portion of the multimerin sequence. The carboxyl terminal region of multimerin resembled the globular domain of the complement C1q and collagens type VIII and X. These studies confirm that multimerin is a unique protein. The domains identified may be important for multimerin structure and function.

The cDNA Sequence of Human Endothelial Cell Multimerin

A UNIQUE PROTEIN WITH RGDS, COILED-COIL, AND EPIDERMAL GROWTH FACTOR-LIKE DOMAINS AND A CARBOXYL TERMINUS SIMILAR TO THE GLOBULAR DOMAIN OF COMPLEMENT C1q AND COLLAGENS TYPE VIII AND X*

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Multimerin is a massive, soluble protein found in platelets and in the endothelium of blood vessels. Multimerin is composed of varying sized, disulfide-linked multimers, the smallest of which is a homotrimer. Multimerin is a factor V/Va-binding protein and may function as a carrier protein for platelet factor V. The cDNA for human multimerin was isolated from λ gt11 endothelial cell libraries using antibodies, and the isolated cDNA clones were used to obtain the full sequence. The full-length multimerin cDNA was 4.2 kilobase pairs. Northern analyses identified a 4.7-kilobase transcript in cultured endothelial cells, a megakaryocytic cell line, platelets, and highly vascular tissues. The multimerin cDNA can encode a protein of 1228 amino acids with the probable signal peptide cleavage site between amino acids 19 and 20. The protein is predicted to be hydrophilic and to contain 23 *N*-glycosylation sites. The adhesive motif RGDS (Arg-Gly-Asp-Ser) and an epidermal growth factor-like domain were identified. Sequence searches indicated that multimerin is a unique protein. Analyses identified probable coiled-coil structures in the central portion of the multimerin sequence. Additionally, the carboxyl-terminal region of multimerin resembles the globular, non-collagen-like, carboxyl-terminal domains of several other trimeric proteins, including complement C1q and collagens type VIII and X.

let lysates, multimerin is complexed with factor V, and immunoelectron microscopy studies indicate that factor V and multimerin are stored together within platelet α -granules.² However, following platelet activation and the release of multimerin and factor V, these two proteins dissociate.² These findings suggest that multimerin may play a role in the storage and stabilization of platelet (but not plasma) factor V and also indicate separate functions for these proteins on activated platelets. The avid association of multimerin with activated platelets (1, 4) and endothelial cells¹ suggests that there may be other functions, possibly adhesive, for multimerin once it is released from intracellular stores.

Multimerin is one of the largest proteins found in platelets and endothelial cells, with most of its multimers exceeding a million daltons in size (1-4).¹ The variable molecular weight of multimerin is due to differences in the number of multimerin subunits comprising the protein (1, 2, 4). Multimerin is highly glycosylated, with complex *N*-linked carbohydrate accounting for about one-third of its molecular mass (3). It is synthesized as a p-170 protein (132-kDa polypeptide component) containing high mannose, *N*-linked carbohydrates, which are then converted to complex forms (3). During biosynthesis, interchain disulfide bonds form to generate homotrimers and larger homomultimers (2-4). Proteolysis of the subunits occurs (without disrupting the multimeric structure), leading to the stable p-155 subunit that is stored in platelets (1-4).

A number of parallels exist in the protein trafficking and storage of multimerin and von Willebrand factor. Multimerin resembles von Willebrand factor in its complex, disulfide-linked multimeric structure (2, 4). However, unlike von Willebrand factor, which is assembled from dimers, the smallest multimer of multimerin is a 400-kDa homotrimer (2). Both proteins are stored within the electron-lucent zone of platelet α -granules and within the Weibel-Palade bodies of endothelial cells (3).¹ They are constitutively secreted as small multimers, and their intracellular stores are enriched in high molecular weight multimers (5).¹ However, unlike von Willebrand factor, multimerin is not detectable in plasma and differs in its subunits size and glycosylation (1, 3, 4).

In this report, we describe the isolation, sequencing, and deduced amino acid sequence of human endothelial cell multimerin cDNA. These studies identify multimerin as a unique protein, unrelated to von Willebrand factor, with RGDS, EGF-like, and coiled-coil domains, and a carboxyl-terminal region that resembles the trimeric, carboxyl-terminal globular do-

Multimerin is a large, soluble protein (1, 2) stored within platelet α -granules (3) and endothelial cell Weibel-Palade bodies.¹ Following activation of these cells, multimerin is released and binds to the cell surfaces of platelets (1-4), and endothelial cells,¹ and the extracellular matrix. *In vivo*, multimerin is restricted to megakaryocytes, platelets, and the endothelium and subendothelium of blood vessels, and it is not found in the plasma (3, 4).¹ Recent studies have identified multimerin as a factor V/Va-binding protein.² In resting plate-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) U27109.

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¹ C. P. M. Hayward, J. G. Kelton, R. H. Stead, T. E. Warkentin, and T. J. Podor, submitted for publication.

² Hayward, C. P. M., Furmaniak-Kazmierczak, E., Cieutat, A.-M.,

Moore, J. C., Bainton, D. F., Nesheim, M. E., Kelton, J. G., and Côté, G. (1995) *J. Biol. Chem.* 270, in press.

main components of complement C1q and collagens type VIII and X.

MATERIALS AND METHODS

cDNA Libraries—Human endothelial cell cDNA libraries in λ gt11 were obtained from Clontech (5' stretch human endothelial cDNA library; Palo Alto, CA) and additional human umbilical vein endothelial cell libraries (VII-91-4 and VII-91-5) were a generous gift from J. Evan Sadler (6) (St. Louis, MO).

Screening of λ gt11 cDNA Libraries—Libraries were screened for clones (7) expressing the multimerin protein using both monoclonal and polyclonal anti-multimerin antibodies (1) (1:1000 dilution), alkaline phosphatase-conjugated secondary antibodies (1:10,000 dilution, Promega, Madison, WI), and nitro blue tetrazolium-5-bromo-4-chloro-3-indolyl phosphate (Sigma) for detection. All of the clones identified by antibody screening were immunoreactive with both monoclonal and polyclonal multimerin antisera. Following cloning and sequencing of the initial isolates, the most 5' clone was labeled and used to rescreen the λ gt11 library for full-length cDNA clones (8, 9).

Lambda DNA was purified from plate lysates (10), digested with *Eco*RI, and the multimerin cDNA clones were subcloned into the *Eco*RI site of pGEM 7Zf+ (Promega).

PCR Amplification of λ Inserts—The multimerin cDNA inserts in λ gt11 were amplified using PCR and primers specific for λ gt11, as described previously (11). The inserts were subcloned and their flanking λ gt11 sequences were used to determine the orientation of the multimerin cDNAs (12). These clones were also used for sequencing across an internal *Eco*RI site, using multimerin-specific sequencing primers.

DNA Labeling—Multimerin cDNA inserts were labeled with [α - 32 P]ATP, using a random primer labeling kit (U. S. Biochemical Corp.), for use in library screening and Northern blotting. The 5' *Eco*RI fragments of the full-length multimerin cDNA (mm λ 17) and of mm λ 4 were used for Northern analyses.

DNA Sequencing—Double-stranded sequencing of overlapping clones was performed using manual (dideoxy sequencing with Sequenase, U. S. Biochemical Corp.) and automated sequencing of Qiagen miniprep DNA (Qiagen, Chatsworth, CA). Automated DNA sequencing was performed by the Central Facility of the Institute for Molecular Biology and Biotechnology, McMaster University, using an Applied Biosystems (model 373A) automatic DNA sequencer. Sequencing was done using dideoxy terminator technology with cycle sequence *Taq* according to the manufacturer's instructions. Primers used for sequencing included M13 universal forward and reverse primers. Multimerin-specific primers were used to fill in gaps in the aligned sequence of exonuclease III-deleted clones. PCR-derived clones were used only for studies of orientation and for sequencing across the internal *Eco*RI site.

Data Analyses—Sequence analyses and alignments were performed using the MacVector and Assembly programs (Eastman Kodak Co.) and PC Gene (IntelliGenetics, Inc., Mountain View, CA). Data bank searches (NCBI at National Library of Medicine, Bethesda, MD: nonredundant PDB+SwissProt+SPupdate+PIR+GenPept+GPupdate) were performed using the BLASTP algorithm. Further alignments of homologous sequences were performed using the Clustal program (DNA Star Ltd., London) and a PAM 250 matrix. Analyses for coiled-coil structures were performed by Dr. Seth Darst, Rockefeller Institute, using the program PEPCOIL (Genetics Computer Group, Madison, WI).

Protein Purification and Sequencing—Multimerin was purified from outdated platelet concentrates by affinity chromatography, as described (1). 15 μ g of the purified protein was used for preparative SDS-polyacrylamide gel electrophoresis (reduced) and transblotted onto a polyvinylidene difluoride membrane (Bio-Rad), following the manufacturer's instructions. The 155-kDa multimerin subunit was localized using Ponceau Red, excised from the membrane, and used to obtain internal amino acid sequence data. Protein digestion (lysylendopeptidase C), peptide separation, and amino acid sequencing were performed by the Harvard Microchemistry Facility (Cambridge, MA) using an ABI 477A protein sequencer with a 120A PTH-AA analyzer.

Northern Analyses—Northern analysis was performed as described (9, 13). RNA was isolated from first passage endothelial cells,¹ platelets (washed platelet pellet (1) from 30 ml of whole blood), and from resting and PMA-treated Dami cells (3) using TRIzol (Life Technologies, Inc.). 20 μ g of total RNA was loaded per lane (1% agarose gels), and RNA markers (Promega) were used to determine the transcript sizes. Hy-

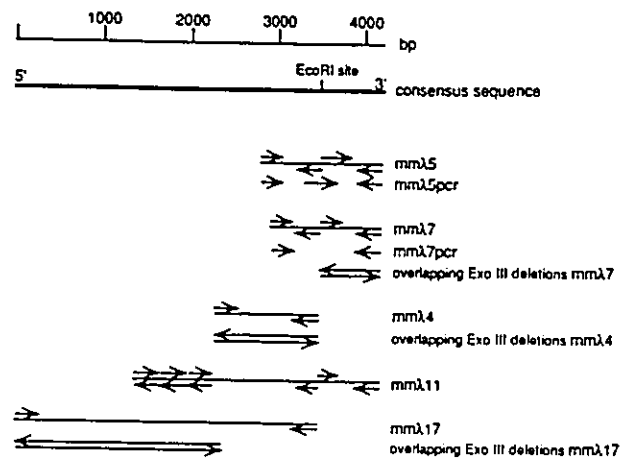


FIG. 1. Multimerin sequencing strategy. Arrows indicate the direction and distance of sequencing. Clones subcloned as PCR fragments are indicated with "pcr" in their name. The consensus clone length was 4212 bp.

bridization was performed using 32 P-labeled multimerin cDNA (mm λ 4 and mm λ 17) to analyze the expression of multimerin in cultured cells and in multiple tissues (multiple tissue Northern; Clontech), using high stringency washes, as recommended by the manufacturer. Multimerin expression was compared with actin (cDNA probe supplied by Promega) as the control.

RESULTS AND DISCUSSION

Molecular Cloning and Sequencing of Full-length Multimerin cDNA—The deduced protein sequence of multimerin was investigated by cloning and sequencing of the multimerin cDNA. As previous studies indicated synthesis of multimerin by endothelial cells,¹ human endothelial cell cDNA libraries in λ gt11 were chosen for these studies. Because the NH₂ terminus sequence of multimerin was found to be blocked, antibodies were used for screening. The full-length multimerin cDNA was predicted to contain a 3.6-kbp open reading frame, based upon the 132-kDa polypeptide component of the multimerin precursor (3).¹

Screening of the 300,000 plaques-forming units from the Clontech endothelial cell cDNA expression library yielded seven multimerin immunoreactive clones (mm λ 1–7). Sequencing of the 5' and 3' ends of mm λ 4, mm λ 5, and mm λ 7 identified overlap in their sequences, and all three isolates were recognized by both monoclonal (JS-1) and polyclonal multimerin antisera. All of the λ gt11 libraries screened were constructed using *Eco*RI adapters. Digestion of mm λ 2 and mm λ 5 liberated two *Eco*RI fragments, indicating the presence of at least one internal *Eco*RI site in the multimerin cDNA. To identify the orientation of the expressed clones in λ gt11 and the number of internal *Eco*RI sites, the complete mm λ 5 and mm λ 7 inserts were amplified using primer sites in the λ arms (11, 12) and subcloned into pGEM. Sequencing of the PCR-amplified inserts identified the orientation of the fragments and indicated that only one internal *Eco*RI site was present in mm λ 5. A 3' 729-bp fragment containing a polyadenylation signal site and terminating in a poly(A) tail (Fig. 2) was identified. The most 5' clone identified by antibody screening (mm λ 4, 1.4 kbp) terminated at the internal *Eco*RI site. Exonuclease III deletions of the overlapping clones (mm λ 4 and mm λ 7) were created to fully sequence the cDNA fragments in both directions (Fig. 1).

The radiolabeled mm λ 4 cDNA was used to screen the libraries VII-91-4 and VII-91-5 for a full-length cDNA clone. Two clones were isolated that contained additional 5' sequence: mm λ 11 (isolated from VII-91-4; 2.1- and 0.75-kbp *Eco*RI fragments) and mm λ 17 (isolated from VII-91-5; 3.7-kbp *Eco*RI fragment). The first isolate, mm λ 11, was used to obtain additional

¹ The abbreviations used are: PCR, polymerase chain reaction; PMA, phorbol 12-myristate 13-acetate; kbp, kilobase pair(s); bp, base pair(s); EGF, epidermal growth factor.

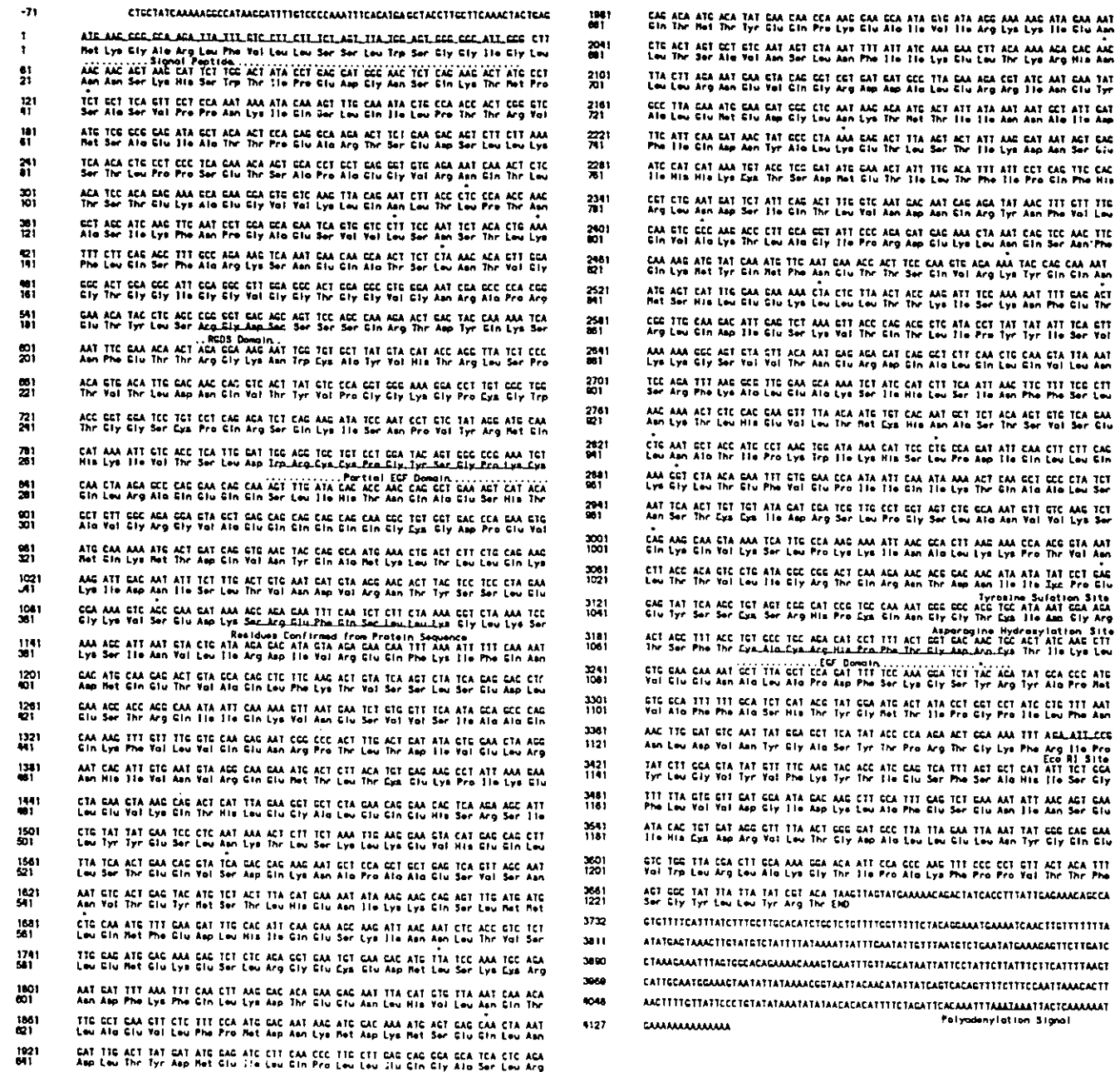


FIG. 2. Nucleotide sequence and deduced amino acid sequence of human endothelial cell multimerin. An in-frame stop codon in the 5'-untranslated region is underlined. An internal EcoRI site is indicated. The 3'-untranslated region contains a polyadenylation signal and terminates in a poly(A) tail. Amino acids 368-376 were confirmed by sequencing an internal peptide fragment of multimerin. The location of the most probable signal peptide is indicated. Potential N-linked glycosylation sites (dot) are noted, and cysteine residues are underlined. RGDS and EGF-like domains are indicated. A partial EGF-like domain, lacking the first cysteine of the EGF consensus sequence CXCXXXXXGXXC, is also indicated.

sequence 5' of mmA4, using multimerin-specific primers. Subsequently, exonuclease III deletions were created from mmA17, and the region 5' (and overlapping) of mmA4 and mmA11 was fully sequenced in both directions. Sequencing of the 5' end of mmA17 identified an in-frame stop codon prior to an initiation codon. This reading frame was consistent with the multimerin fusion proteins that had been identified by antibody screening of the λ gt11 library (12). These findings indicated that a complete cDNA sequence had been obtained. The sequence derived from the overlapping clones was 4212 bp in length.

The sequence of the human endothelial cell multimerin cDNA is shown in Fig. 2. The open reading frame is preceded by a 71-bp noncoding region containing an in-frame stop codon. The initiation ATG conforms to Kozak's consensus sequence for

initiation (14) and is followed by an open reading frame of 1228 codons and a 3'-noncoding region of 454 bp containing a polyadenylation signal and a poly(A) tail.

Deduced Protein Sequence of Multimerin and Homology with Other Proteins—The putative protein encoded by the multimerin cDNA was compared with amino acid sequence data obtained from purified platelet multimerin (Fig. 2). High confidence sequence data were obtained from an internal peptide fragment of platelet multimerin. These sequence data were in complete agreement with the predicted protein sequence (amino acids 368-376), indicating that the cDNA sequenced encodes multimerin.

The multimerin cDNA encodes a protein of 1228 amino acids with a calculated molecular mass of 138 kDa (Fig. 2). Analysis

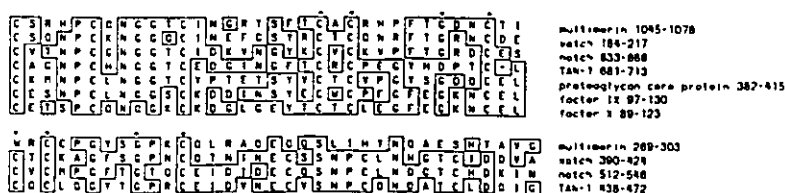


FIG. 3. Comparison alignments of multimerin with proteins containing EGF-like domains. The upper panel shows the comparison of the EGF-like domain in multimerin with the EGF-like domains of the highest scoring, homologous protein sequences. The lower panel is a comparison of a domain of multimerin with similarity to EGF-like domains in Xotch, Notch, and the human homologue TAN-1. This region of multimerin lacks the first cysteine residue of an EGF-like consensus sequence. Boxed residues indicate consensus residues, and an asterisk indicates the conserved residues in an EGF-like motif.

for the signal peptide cleavage site, using Prosite (PC Gene), indicated that the most probable cleavage site was between amino acids 19 and 20. The protein, minus the signal peptide, has a predicted molecular mass of 136 kDa, which is in close agreement with the 132-kDa nonglycosylated precursor identified by metabolic protein labeling studies of Dami cell (3) and endothelial cell multimerin.¹ Kyte-Doolittle plots indicated that the protein was hydrophilic, consistent with the partitioning of multimerin into the aqueous phase of Triton X-114 platelet extracts (1). Alignment of the multimerin polypeptide sequence to itself using MacVector and a PAM250 matrix failed to identify significant internal repeats within the multimerin protein sequence.

Analysis of the multimerin protein for functional domains using MacVector and PC Gene identified the adhesive motif RGDS (amino acids 186–189) (15, 16) and an EGF-like (17, 18) domain (amino acids 1065–1076). Consensus sequences for a tyrosine sulfation site (amino acid 1038) (19, 20) and an asparagine hydroxylation site (21) (amino acid 1058) were identified adjacent to the EGF-like domain. The protein contained 23 potential *N*-glycosylation sites (22–25), which is in close agreement with the 17–21 sites predicted by *N*-deglycosylation of endothelial cell and Dami cell multimerin (3).¹ The RGDS site was located in an unglycosylated region of the molecule with a high local flexibility score (MacVector, Karpus-Schulz analysis).

Search of the NCBI data banks using the BLASTP algorithm and BLOSUM 62 matrix indicated that multimerin was a novel protein. Assessment of the high scoring homologous sequences identified similarities between multimerin and a large number of proteins that contain EGF-like domains. The highest scoring elements were Xotch (26) and its homologues in other species. These homologues are transmembrane proteins, containing multiple EGF-like domains, and are important for neurogenic development. The highest scoring human proteins with homology to the EGF-like domain of multimerin included TAN-1 (27), a homologue of Xotch and Notch (28), fibroblast proteoglycan core protein (29), and coagulation factors IX (30–32) and X (33–35). Fig. 3 (upper panel) shows the comparison alignments for the EGF-like domain of multimerin. An additional region of homology between multimerin and Xotch, Notch, and TAN-1 proteins was identified spanning amino acids 265 and 303 of multimerin (Fig. 3, lower panel). This region of multimerin contains three cysteine residues but lacks the first cysteine in the EGF-like consensus sequence CXCXXXXGXXC. This domain is homologous with EGF-like domains in Xotch, Notch, and TAN-1 and in proteoglycan core proteins.

A number of proteins, including the rod-like tail of many myosin heavy chains (from a variety of species and tissue types) (36–38), macrogolgin (39), and the *tpv* oncogene (40), showed homology with regions in the central portion of multimerin between amino acids 317–1024. These homologous proteins are known to contain coiled-coil structures. Multiple sequence comparisons using the Clustal program (DNA Star) and

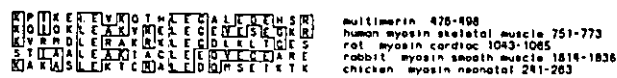


FIG. 4. Comparison alignments of multimerin with the rod-like tail of myosin heavy chains. Boxed residues indicate consensus residues.

a PAM 250 matrix revealed one region where residues were conserved between multimerin (amino acids 476–498) and many myosin heavy chain sequences (Fig. 4). The similarities between multimerin and a variety of coiled-coil proteins suggested that there could be coiled-coil structures within multimerin. This possibility was investigated using sequence analysis the program PEPCOIL. The multimerin polypeptide sequence contained regions of high probability for coiled-coil structures in the region of the protein that was similar to other coiled-coil proteins. The probable coiled-coil structures were located between amino acids: 317–375, 400–445, 668–738, and 818–873 (Fig. 5).

An additional region of significant homology was observed in the carboxyl terminus of the multimerin sequence. This region of multimerin was found to resemble the trimeric, carboxyl-terminal, non-collagen-like, globular domain of several other proteins. These included the A, B, and C chains of human and mouse complement C1q protein (41–46) and human α -1 collagen type VIII (47) and X (47–51). The protein sequence comparisons for this domain indicate conservation of hydrophobic and uncharged residues (Fig. 6). Electron microscopy studies of these homologous proteins have shown that their carboxyl-terminal domains form a globular-shaped head (53–55). In C1q, the globular domain is assembled from the carboxyl-terminal regions of an A, a B, and a C chain (56). The globular domains of C1q and collagens type VIII and X are implicated in protein interactions. This domain in the complement C1q protein is known to interact with the Fc portion of IgG and other complement activator molecules, leading to complement activation (56). In collagen type VIII, which is a heterotrimer of α -1 and α -2 chains, the COOH-terminal globular domain is implicated in the assembly of a mesh-like structure of collagen type VIII molecules (54). Collagen type X, a homotrimeric protein, contains a similar COOH-terminal globular domain (47–51). In collagen type X, the carboxyl-terminal globular domains associate to form a hexagonal mesh of collagen molecules (55).

A schematic summary of the various multimerin domains is shown in Fig. 7. Based upon the multimerin constructs that contained the epitope recognized by the monoclonal antibody JS-1, the JS-1 epitope was localized to the region containing amino acids 961–1139, which includes the EGF-like domain and a portion of the COOH-terminal globular domain.

Because of the many similarities observed in the multimeric structures and protein trafficking of multimerin and von Willebrand factor (2, 4),¹ we had anticipated that there could be similarities in their amino acid sequences. However, von Willebrand factor was not identified in the data bank searches

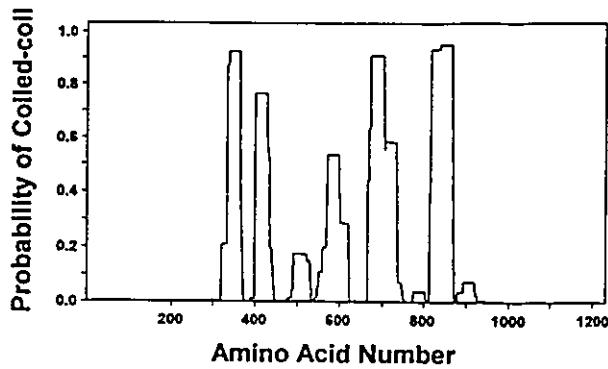


FIG. 5. The probability of coiled-coil structures in multimerin. A number of highly probable coiled-coil regions are identified between amino acids 317–873.

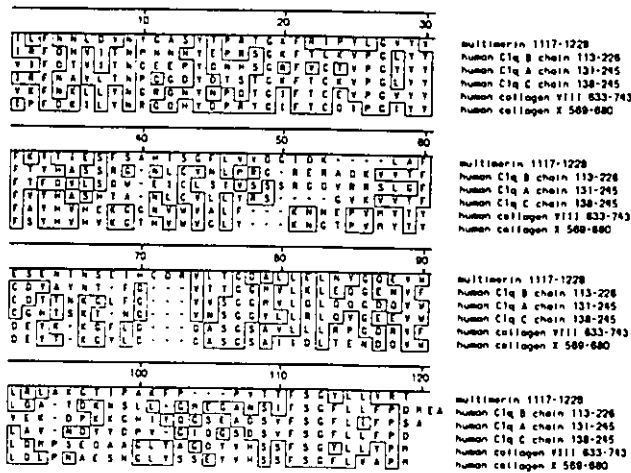


FIG. 6. Comparison alignments of multimerin with the COOH-terminal globular domains of complement C1q A, B, and C chains and human α -1 collagens type VIII and X. Boxed residues indicate consensus residues.

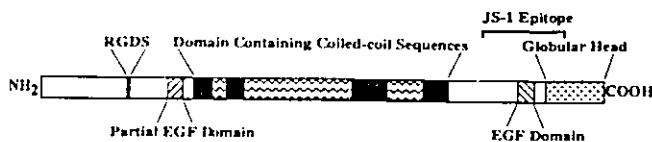


FIG. 7. Multimerin protein domains. Domains identified by protein analysis and the location of the monoclonal antibody JS-1 epitope are shown.

for homologous proteins, and direct comparison of the von Willebrand factor (57) and multimerin protein sequences failed to identify significant homologies. While the propeptide region has been postulated to be involved in the targeting of von Willebrand factor to Weibel Palade bodies (52, 58), the lack of similarity in the sequences of multimerin and von Willebrand factor suggest that other factors must account for their similar trafficking in platelets and endothelial cells.

Our earlier studies of multimerin biosynthesis identified a p-170 precursor protein, containing high mannose-linked carbohydrate (3). The 132-kDa polypeptide component of this p-170 multimerin precursor is in close agreement with the size of the deduced amino acid sequence minus its signal peptide (136 kDa). Based on these data, we have designated the multimerin precursor protein (minus the signal sequence) as promultimerin. During biosynthesis, the *N*-linked carbohydrates on promultimerin are converted to complex forms to produce a p-186 protein in endothelial cells and a p-196 protein in Dami cells (3).¹ The site(s) of proteolytic cleavage that produce the

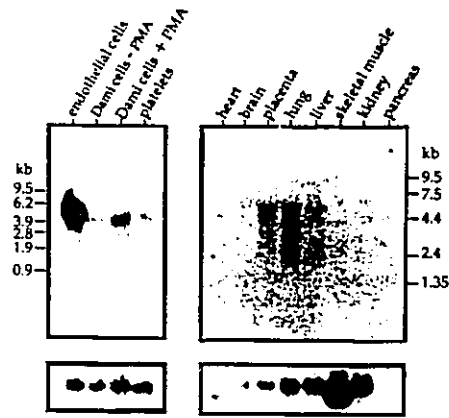


FIG. 8. Northern analysis of multimerin expression in cultured cells and in human tissues. The expression of multimerin (upper panels) and actin (lower panels) mRNA in isolated human cells and in human tissues. The left panels show cultured cells and platelets (20 μ g of total RNA/lane), and the right panels are a multiple tissue Northern (Clontech; 2 μ g of poly(A)⁺ RNA/lane). Resting (-PMA) and PMA-treated (+PMA) Dami cells are shown for comparison. A 4.7-kb transcript is identified in endothelial cells, PMA-treated Dami cells, platelets, and in lung, placenta, and liver (highly vascular tissues).

p-155 multimerin subunit (105-kDa polypeptide component) (3) that is stored within platelets (1) and endothelial cells¹ await identification. Because the EGF-like domain, globular C1q-like domain, and JS-1 epitope are located at the carboxyl end of the protein sequence, we postulate that the mature protein is produced by cleavage in the NH₂-terminal region.

Analyses of Multimerin RNA—Northern analysis of cultured cells and human tissues identified a 4.7-kb transcript using the multimerin cDNA probe (Fig. 8). Previous metabolic protein labeling studies indicated that multimerin is synthesized by endothelial cells¹ and by Dami cells (5) (a megakaryocytic cell line) after stimulation with PMA. The multimerin transcript was identified in cultured endothelial cells, Dami cells, and platelets. Comparison of resting and PMA-stimulated Dami cells indicated increased expression of the multimerin mRNA following stimulation of these cells with PMA. The identification of the multimerin transcript in platelets indicates that endogenous biosynthesis by megakaryocytes is the source of platelet multimerin. Comparison of multiple tissues identified the highest expression of multimerin in placenta, lung, and liver, three highly vascular tissues (Fig. 8). The detection of multimerin mRNA in vascular tissues is in agreement with the localization of multimerin in vascular endothelium *in situ* using immunohistochemistry.¹

The data presented in this report confirm that multimerin is a unique protein. Further studies are required to define the precise roles of multimerin's RDGS, coiled-coil, EGF-like, partial EGF-like, and C1q-like domains and to determine how the multimerin subunits are assembled into the large, disulfide-linked multimers. The coiled-coil and globular domains are likely sites for interchain associations. We postulate that the RGDS, EGF-like, and C1q-like domains will prove to be important sites for the interaction of multimerin with other proteins. Knowledge of the functions of these domains and of multimerin's tertiary structure may provide insights into the molecular mechanisms that control hemostasis. Additional clues may be provided by investigations of individuals who are deficient in multimerin.

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REFERENCES

1. Hayward, C. P. M., Smith, J. W., Horsewood, P., Warkentin, T. E., and Kelton, J. G. (1991) *J. Biol. Chem.* **266**, 7114-7120
2. Hayward, C. P. M., Warkentin, T. E., Horsewood, P., and Kelton, J. G. (1991) *Blood* **77**, 2556-2560
3. Hayward, C. P. M., Bainton, D. F., Podor, T. J., Smith, J. W., Stead, R., Horsewood, P., Warkentin, T. E., and Kelton, J. G. (1993) *J. Clin. Invest.* **91**, 2630-2639
4. Hayward, C. P. M., and Kelton, J. G. (1995) *Platelets* **6**, 1-10
5. Sporn, L. A., Marder, V. J., and Wagner, D. D. (1986) *Cell* **46**, 185-190
6. Ye, R. D., Wun, T.-C., and Sadler, J. E. (1987) *J. Biol. Chem.* **262**, 3718-3725
7. Young, R. A., and Davis, R. W. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80**, 1194-1198
8. Benton, W. D., and Davis, R. W. (1977) *Science* **196**, 180-182
9. Hunn, T. V., Young, R. A., and Davis, R. W. (1985) in *DNA Cloning: A Practical Approach* (Glover, D. M., ed) Vol. 1, pp. 49-78, IRL Press, Oxford
10. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) in *Molecular Cloning: A Laboratory Manual*, 2nd Ed., pp. 2.118-2.119, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
11. Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., and Erlich, H. A. (1988) *Science* **239**, 487-491
12. Galas, D. J., Calos, M. P., and Miller, J. H. (1980) *J. Mol. Biol.* **144**, 19-41
13. Fournay, R., Miyakoski, J., Day, R. S., III, and Paterson, M. C. (1988) *Focus* **10**, 5-6
14. Kozak, M. (1989) *J. Cell Biol.* **108**, 229-241
15. Ruoslahti, E., and Pierschbacher, M. D. (1986) *Cell* **44**, 517-518
16. D'Souza, S. E., Ginsberg, M. H., and Plow, E. F. (1991) *Trends Biochem. Sci.* **16**, 246-250
17. Davis, C. G. (1990) *New Biol.* **2**, 410-419
18. Kimura, H., Fischer, W. H., and Schubert, D. (1990) *Nature* **348**, 257-260
19. Huttner, W. B. (1987) *Trends Biochem. Sci.* **12**, 361-363
20. Rosenquist, G. L., and Nicholas, H. B., Jr. (1993) *Protein Sci.* **2**, 215-222
21. Stenflo, J., Ohlin, A.-K., Owen, W. G., and Schneider, W. J. (1988) *J. Biol. Chem.* **263**, 21-24
22. Marshall, R. D. (1972) *Annu. Rev. Biochem.* **41**, 673-702
23. Pless, D. D., and Lennarz, W. J. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74**, 134-138
24. Bause, E. (1983) *Biochem. J.* **209**, 331-336
25. Gavel, Y., and von Heijne, G. (1990) *Protein Eng.* **3**, 433-442
26. Coffman, C., Harris, W., and Kintner, C. (1990) *Science* **249**, 1438-1441
27. Ellisen, L. W., Bird, J., West, D. C., Soreng, A. L., Reynolds, T. C., Smith, S. D., and Sklar, J. (1991) *Cell* **66**, 649-661
28. Wharton, K. A., Johansen, K. M., Xu, T., and Artavanis-Tsakonas, S. (1985) *Cell* **43**, 567-581
29. Krusius, T., Gehlsen, K. R., and Ruoslahti, E. (1987) *J. Biol. Chem.* **262**, 13120-13125
30. Choo, K. H., Gould, K. G., Rees, D. J. G., and Brownlee, G. (1982) *Nature* **299**, 178-180
31. Jaye, M., De La Salle, H., Schamber, F., Ballard, A., Kohli, V., Findeli, A., Tolsloshev, P., and Lecocq, J. P. (1983) *Nucleic Acids Res.* **11**, 2325-2335
32. Jagadeeswaran, P., Lavelle, D. E., Kaul, R., Mohandas, T., and Warren, S. T. (1984) *Somatic Cell Mol. Genet.* **10**, 465-473
33. McMullen, B. A., Fujikawa, K., Kisiel, W., Sasagawa, T., Howald, W. N., Kwa, F. Y., and Weinstein, B. (1983) *Biochemistry* **22**, 2875-2884
34. Leytus, S. P., Chung, D. W., Kisiel, W., Kurachi, K., and Davie, E. W. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81**, 3699-3702
35. Fung, M. R., Hay, C. W., and MacGillivray, R. T. A. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82**, 3591-3595
36. Szez, C. G., Myers, J. C., Shows, T. B., and Leinwand, L. A. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 1164-1168
37. Feghaku, R., and Leinwand, L. A. (1989) *J. Cell Biol.* **108**, 1791-1797
38. Szez, L., and Leinwand, L. A. (1986) *Nucleic Acids Res.* **14**, 2951-2969
39. Seclig, H. P., Schranz, P., Schroeter, H., Wiemann, C., and Renz, M. (1994) *J. Autoimm.* **7**, 67-91
40. Mitchell, P. J., and Cooper, C. S. (1992) *Oncogene* **7**, 2329-2333
41. Reid, K. B. M. (1979) *Biochem. J.* **179**, 367-371
42. Reid, K. B. M., Gagnon, J., and Frampton, J. (1982) *Biochem. J.* **203**, 559-569
43. Reid, K. B. M. (1985) *Biochem. J.* **231**, 729-735
44. Sellar, G. C., Blake, D. J., and Reid, K. B. M. (1991) *Biochem. J.* **274**, 481-490
45. Petry, F., Reid, K. B. M., and Loos, M. (1991) *J. Immunol.* **147**, 3988-3993
46. Petry, F., Reid, K. B. M., and Loos, M. (1992) *Eur. J. Biochem.* **209**, 129-134
47. Muragaki, Y., Mattei, M. G., Yamaguchi, N., Olsen, B. R., and Ninomiya, Y. (1991) *Eur. J. Biochem.* **197**, 615-622
48. Apte, S., Mattei, M. G., and Olsen, B. R. (1991) *FEBS Lett.* **282**, 393-396
49. Reichenberger, E., Beier, F., LuValle, P., Olsen, B. R., von der Mark, K., Bertling, W. M. (1992) *FEBS Lett.* **311**, 305-310
50. Apte, S. S., Seldin, M. F., Hayashi, M., and Olsen, B. R. (1992) *Eur. J. Biochem.* **206**, 217-224
51. Thomas, J. T., Cresswell, C. J., Rash, B., Nicolai, H., Jones, T., Solomon, E., Grant, M. E., and Boot-Handford, R. P. (1991) *Biochem. J.* **280**, 617-623
52. Hournet, A. M., Saffaripour, S., Cramer, E. M., Tenza, D., and Wagner, D. D. (1993) *Eur. J. Cell Biol.* **60**, 31-41
53. Knobel, H. R., Villiger, W., and Isliker, H. (1975) *Eur. J. Immunol.* **5**, 78-82
54. Sawada, H., Konomi, H., and Hirokawa, K. (1990) *J. Cell Biol.* **110**, 219-227
55. Kwan, A. P. L., Cummings, C. E., Chapman, J. A., and Grant, M. E. (1991) *J. Cell Biol.* **114**, 597-604
56. Reid, K. B. M. (1986) *Essays Biochem.* **22**, 27-67
57. Titani, K., Kumar, S., Takio, K., Ericsson, L. H., Wade, R. D., Ashida, K., Walsh, K. A., Chopek, M. W., Sadler, J. E., and Fujikawa, K. (1986) *Biochemistry* **25**, 3171-3184
58. Wagner, D. D., Saffaripour, S., Bonfanti, R., Sadler, J. E., Cramer, E. M., Chapman, B., and Mayadas, T. N. (1991) *Cell* **64**, 403-413

CHAPTER 6

CONCLUSIONS

6.1 Thesis Objectives and Background Knowledge

This thesis describes the characterization of the human platelet protein p-155. P-155 is a new protein, with a massive, repeating, multimeric structure, that is one of the largest proteins found in platelets (Hayward *et al*, 1991a; Hayward *et al*, 1991b). Following the identification of its remarkable multimeric structure, this protein was designated as multimerin (Hayward *et al*, 1991b). Studies of multimerin biosynthesis and its cDNA sequence indicated that multimerin was a novel protein, synthesized by megakaryocytes and endothelial cells, with a unique protein sequence (Hayward *et al*, 1993; Hayward *et al*, 1995b; Hayward *et al*, 1995c).

Previously, multimerin was not recognized as a normal component of platelets and endothelial cells. I first identified multimerin using a monoclonal antibody, JS-1, raised against whole human platelets (Hayward *et al*, 1991a). Before beginning my doctoral studies, I had investigated the reactivity of this monoclonal antibody with human platelets. These studies indicated that the antibody JS-1 bound specifically to a platelet glycoprotein with a mobility of 155 kDa on reduced SDS-PAGE (Hayward *et al*, 1991a). Smaller quantities of a p-170 protein were also detected by the antibody. Both p-155 and p-170 were recognized by JS-1 in immunoblot analyses of platelet

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proteins, indicating that they were immunologically related proteins, perhaps derived from a common precursor protein (Hayward *et al*, 1991a).

The native protein recognized by JS-1 was extremely large and could not be resolved by nonreduced SDS-PAGE due to its massive size (Hayward *et al*, 1991a). Several large but different sized proteins could be identified in JS-1 immunoprecipitates of platelet proteins, using low percentage polyacrylamide gels (Hayward *et al*, 1991a). When these individual proteins were isolated from the nonreduced gels and rerun after full reduction, only p-155 and p-170 subunits were generated from each of the different sized proteins (Hayward *et al*, 1991a). These data indicated that the p-155 protein was multimeric, with variability in its nonreduced molecular weight (Hayward *et al*, 1991a).

When p-155 was compared with the known platelet glycoproteins, it comigrated with glycoprotein Ia on reduced SDS-PAGE (Hayward *et al*, 1991a). However, it differed from the transmembrane glycoprotein Ia in that p-155 was not complexed with glycoprotein IIa (Hayward *et al*, 1991a). Additionally, the p-155 protein identified by JS-1 was not recognized by antibodies specific for glycoprotein Ia, indicating that p-155 was a novel platelet protein (Hayward *et al*, 1991a).

Both radioimmunoprecipitation studies and direct binding studies indicated that the monoclonal antibody JS-1 recognizes a platelet activation-marker (Hayward *et al*, 1991a). The number of binding sites for JS-1 increased from 600 to 4100 following platelet activation by thrombin (Hayward *et al*, 1991a). Studies of the nature of the activation-induced increase in p-155 expression indicated that p-155 was a soluble platelet glycoprotein, released

from internal stores by platelet activation. Following release, multimerin bound to the surface of activated platelets (Hayward *et al*, 1991a).

Comparison of p-155 to the platelet glycoproteins that had been reported in the literature indicate some similarities of p-155 with a glycoprotein designated as Ia* (Hayward *et al*, 1991a; Bienz *et al*, 1989). Both p-155 and glycoprotein Ia* are disulfide-linked multimeric proteins with a reduced mobility of 155 kDa (Hayward *et al*, 1991a; Bienz *et al*, 1989). Both proteins are found in releasate and are expressed on the surface of activated platelets (Hayward *et al*, 1991a; Bienz *et al*, 1989). However, p-155 differed from glycoprotein Ia* by its migration on IEF gels and its association with the cytoskeleton (Hayward *et al*, 1991a; Bienz *et al*, 1989).

Many important features of p-155 had not been resolved before beginning my thesis studies. Perhaps the most important was the nonreduced structure of this protein. Other questions were the relationship of p-155 to other soluble, multimeric proteins in platelets, such as thrombospondin and von Willebrand factor. This was an important issue because these soluble multimeric proteins also are stored in platelets and bind to the platelet membrane following activation (Phillips *et al*, 1980; Fernandez *et al*, 1982; Ruggeri *et al*, 1983; Gralnick *et al*, 1984; Parker *et al*, 1986; Aiken *et al*, 1987).

The first goal of the thesis was to investigate if p-155 was a unique multimeric platelet protein. Following these investigations, the multimeric structure of the protein was investigated. The next stage of investigations focused on identifying the cells that synthesize multimerin and

characterizing its biosynthesis. These studies laid the groundwork for investigating the multimerin cDNA, which was the final step needed to determine if multimerin was a unique protein.

6.2 The Relationship of Multimerin to Known Soluble, Multimeric Platelet Glycoproteins

The initial studies of p-155 indicated that it was comprised of p-155 and p-170 subunits, linked by interchain disulfide bonds to form large, variably sized multimers (Hayward *et al*, 1991a). With platelet activation, the quantity of p-155 detected on the platelet membrane increased, indicating that multimerin bound to the platelet membrane following its release from intracellular stores (Hayward *et al*, 1991a). P-155 was compared to thrombospondin and von Willebrand factor, two known soluble, multimeric proteins stored in platelets that also bind to the platelet membrane (Hayward *et al*, 1991a).

Major differences were noted: thrombospondin and von Willebrand factor differed from multimerin in their reduced subunit size (Hayward *et al*, 1991a). Thrombospondin and von Willebrand factor were larger (reduced), with subunits of 185 and 220 kDa respectively (Hayward *et al*, 1991a). These proteins were also not recognized by JS-1 nor was affinity purified p-155 recognized by antisera to von Willebrand factor and thrombospondin (Hayward *et al*, 1991a). These data indicated that p-155 was distinct from the known soluble multimeric proteins found in platelets.

6.3 The Multimeric Composition of Multimerin

A number of proteins are known to be comprised of identical subunits, linked by interchain disulfide bonds. Most multimeric proteins contain a predictable number of subunits and are relatively small dimers (e.g. IgG), trimers (e.g. thrombospondin), or pentamers (e.g. IgM). Von Willebrand factor is an exception to this rule, being comprised of variably-size disulfide linked multimers up to 20 million daltons in size, making it one of the largest proteins in the body (Meyer *et al*, 1993). No other platelet proteins exhibit this property.

An early goal of my thesis was to determine the nonreduced structure of the p-155 protein. The variable size of the p-155 on nonreduced, low concentration acrylamide gels suggested that p-155 might resemble von Willebrand factor in its complex multimeric composition (Hayward *et al*, 1991a). Because the size of the nonreduced p-155 could not be resolved using even low percentage polyacrylamide gels, other techniques were needed to investigate the multimeric composition of p-155 (Hayward *et al*, 1991a).

The studies of the nonreduced p-155 protein produced an unexpected result: the native p-155 protein was found to be massive in size (Hayward *et al*, 1991b). Using denaturing-agarose/acrylamide gel electrophoresis, p-155 was resolved into a series of different sized proteins, most of which exceeded a million daltons in size (Hayward *et al*, 1991b). Two dimensional nonreduced/reduced electrophoresis indicated that each of the different sized multimers was comprised of the same p-155 and p-170 subunits (Hayward *et al*, 1991b). This information indicated that the multimers were assembled

from the same protein, with the larger multimers containing many more subunits.

P-155 had a distinct nonreduced migration pattern compared with thrombospondin (450 kDa), IgM (960 kDa) and von Willebrand factor (Hayward *et al*, 1991b). It resembled most closely von Willebrand factor, because it was a massive and complex multimeric protein (Hayward *et al*, 1991b). The smallest p-155 multimer was smaller than thrombospondin (450 kDa) (Hayward *et al*, 1991b). The "ladders" of platelet von Willebrand factor and p-155 multimers differed in their multimer spacing, consistent with their assembly from different-sized subunits (Hayward *et al*, 1991b). Based on the novel, remarkable multimeric structure of the native p-155 protein, the nonreduced p-155 protein was designated multimerin (Hayward *et al*, 1991b).

Von Willebrand factor is known to be assembled from homodimers, with the smallest multimer being a dimer of 220 kDa subunits (Meyer *et al*, 1993). To determine the multimeric composition of multimerin, a combination of agarose/acrylamide gel electrophoresis and graded reduction studies, using SDS-PAGE, were used. On agarose/acrylamide gels, the smallest multimer of multimerin migrated faster than thrombospondin, which is a trimer of 185 kDa subunits (Lawler, *et al*, 1978; Hayward *et al*, 1991b). Graded reduction demonstrated three intermediate products (subunit dimers) that were not present in the nonreduced or fully reduced preparations (Hayward *et al*, 1991b). When these intermediate products were isolated and examined under fully reduced conditions, they were found to be dimers of: p-170 and p-170; p-170 and p-155; and p-155 and p-155 (Hayward *et*

al, 1991b). No intermediates, other than dimers, were identified (Hayward *et al*, 1991b). These studies indicated that the smallest multimerin multimer is a trimer. Although it is not certain if multimerin is assembled as multimers of trimers, comparisons von Willebrand factor and multimerin indicate that multimerin is assembled from smaller units than the dimers (440 kDa) of von Willebrand factor (Hayward *et al*, 1991b). These findings suggest that the multimerin multimers might be made of multiples of trimers.

The truly massive size and multimeric composition of multimerin has important implications for function. When I compared the multimerin in resting platelet lysate to platelet releasate, major differences were found. The smallest multimers of multimerin were present in releasate, indicating preferential binding of the largest multimers to the platelet membrane during activation (Hayward *et al*, 1991b). Consistent with these findings, the radioimmunoprecipitates of multimerin prepared from surface-radiolabeled, thrombin activated platelets contained high molecular weight multimers of this protein (Hayward *et al*, 1991b). A similar preferential binding of large multimers to the platelet membrane has been observed for von Willebrand factor (Fernandez *et al*, 1982). The large, multimeric composition of these proteins is probably an important mechanism that provides multiple functional sites for binding to platelet proteins and other ligands.

6.4 Multimerin Biosynthesis

6.4.1 Multimerin Biosynthesis by a Megakaryocytic Cell Line

Important goals of my thesis were to determine the cell type(s) that synthesize multimerin and to characterize the biosynthesis of multimerin. Because immunoblot analyses of plasma failed to detect multimerin (Hayward *et al*, 1991a), I postulated that the multimerin found in platelets was produced by megakaryocytes. To prove the megakaryocytic origin of any platelet protein, one must either:

- 1) demonstrate the biosynthesis of the protein using megakaryocytes (or cell lines) (Ryo *et al*, 1983; Nachman *et al*, 1977)

or

- 2) demonstrate the message for the protein in platelet RNA (Handagama *et al*, 1990).

Because of difficulties in isolating sufficient human megakaryocytes for analyses, human megakaryocytic cell lines have been used to study the biosynthesis of human platelet proteins. This was the approach that I chose to investigate multimerin biosynthesis by megakaryocytes.

Dami cells are a human megakaryocytic cell line that expresses platelet glycoproteins. When these cells are treated with PMA, their morphology more closely resembles normal megakaryocytes and they express a number of platelet glycoproteins, including von Willebrand factor (Greenberg *et al*, 1988). These cells were used in studies of multimerin biosynthesis by megakaryocytes.

Metabolic labeling studies, using [³⁵S] methionine, were used to investigate multimerin biosynthesis by unstimulated and PMA-activated Dami cells. Radioimmunoprecipitation analyses, using multimerin antibodies, were used to analyze the cell lysates and culture media for the presence of multimerin. Multimerin was only synthesized if the cells were activated by PMA (Hayward *et al*, 1993).

Larger quantities of multimerin were detected in the Dami cell culture media than the cell lysate, indicating that most of the synthesized multimerin was secreted (Hayward *et al*, 1993). The multimerin produced by the Dami cells differed from the platelet multimerin in that it was composed mainly of small multimers with subunit sizes of 196 and 165 kDa (Hayward *et al*, 1993). Two dimensional gel electrophoresis indicated that the 196 and 165 kDa subunits were linked by interchain disulfide bonds (Hayward *et al*, 1993).

The biosynthesis of a larger, p-196, multimerin subunit by the Dami cells suggested that platelet multimerin subunits might originate from a larger precursor protein that undergoes proteolytic cleavage to produce p-155 and p-170 (the mature multimerin subunits found in platelets). Both monoclonal and polyclonal multimerin antibodies immunoprecipitated the p-196 and p-165 subunits from Dami cell culture supernatants (Hayward *et al*, 1993). Cleveland mapping studies (Cleveland *et al*, 1977) confirmed that the different multimerin subunits were related proteins. Peptide homology was found between p-155 and p-170, and between p-155 and p-196, indicating that they are related proteins (Hayward *et al*, 1993).

These data suggested that p-155 and p-170 came from a common precursor protein. The pattern of multimerin biosynthesis by Dami cells, suggested that platelet multimerin subunits might originate, *in vivo*, from a larger precursor protein. Multimerin immunoprecipitates from some donors had been observed to contain small quantities of a 200 kDa protein. I postulated that this could be the multimerin precursor protein synthesized by normal megakaryocytes. To investigate this possibility, two-dimensional nonreduced/reduced electrophoresis was used to determine if the 200 kDa protein was a coprecipitated protein or an integral component of the multimerin multimers. The 200 kDa protein migrated with the multimerin multimers in the first dimension, indicating that it was covalently linked to the other multimerin subunits (Hayward *et al*, 1993).

Pulse-chase biosynthesis studies indicated that multimerin is first synthesized by Dami cells as a 170 kDa subunit protein (Hayward *et al*, 1993). This precursor protein is subsequently modified to produce the 196 and 165 kDa multimerin subunits that were secreted into the culture supernatant (Hayward *et al*, 1993). Metabolic labeling studies of multimerin using Dami cells indicated that most of the multimerin was secreted following its synthesis (Hayward *et al*, 1993). In the pulse-chase metabolic labeling studies (20 minute labeling), secretion of the protein was observed by 1 hr and was complete by 3 hours of the chase (Hayward *et al*, 1993). Similarly, in an 18 hr metabolic labeling study, most of the synthesized protein was secreted into the culture supernatant (Hayward *et al*, 1993).

Carbohydrate analyses were performed to determine the carbohydrate composition of multimerin and to investigate the changes in the mobility of the multimerin subunits during biosynthesis. The multimerin in platelets and from Dami cells was highly glycosylated with complex N-linked carbohydrate accounting for approximately 1/3 of the molecular mass (Hayward *et al*, 1993). Multimerin was found to be synthesized as a 170 kDa precursor containing high-mannose forms of N-linked carbohydrate (Hayward *et al*, 1993). Processing of the 170 kDa precursor to a 196 kDa protein (p-196) was found to involve conversion of the high-mannose N-linked carbohydrates to complex forms (Hayward *et al*, 1993). Because removal of N-linked carbohydrate from p-196, using a glycosidase, produced a 51 kDa decrease in apparent molecular mass (Hayward *et al*, 1993), I concluded that there were 17 N-linked carbohydrates (assuming 3 kDa per N-linked carbohydrate) (Kornfeld *et al*, 1985) on the multimerin precursor protein. Only minor mobility shifts in the mobility of the multimerin subunits were observed following treatment with O-glycanase, indicating that the major forms of carbohydrate on multimerin were N-linked (Hayward *et al*, 1993). The precursor, synthesized in the presence of the N-glycosylation inhibitor tunicamycin, was 132 kDa. Based on an estimate of 110 daltons/amino acid, the protein was predicted to contain approximately 1200 amino acids.

Multimerin biosynthesis by Dami cells was determined to involve proteolytic processing, as p-165 contained a smaller polypeptide component than p-196 (Hayward *et al*, 1993). Similar studies of platelet multimerin

demonstrated that p-170 contained a larger polypeptide component than p-155. Differences were observed in the proteolytic processing of Dami cell and platelet multimerin (Hayward *et al*, 1993). The rapid secretion (within 3 hours) of multimerin by Dami cells may be the reason for its less complete proteolytic processing. Similar incomplete proteolytic processing has been observed with the von Willebrand factor constitutively secreted by cultured endothelial cells (Wagner *et al*, 1983)

These studies indicated that multimerin is first synthesized as a 170 kDa subunit (132 polypeptide component), containing high mannose forms of N-linked carbohydrate (Hayward *et al*, 1993). During biosynthesis, the N-linked carbohydrate is converted to complex forms (p-196) (Hayward *et al*, 1993). Proteolysis results in the formation of a 165 kDa subunit in Dami cells. Platelet multimerin is more extensively proteolytically processed, to form p-155 and p-170 subunits. The steps involved in multimerin biosynthesis are summarized in Figure 1.

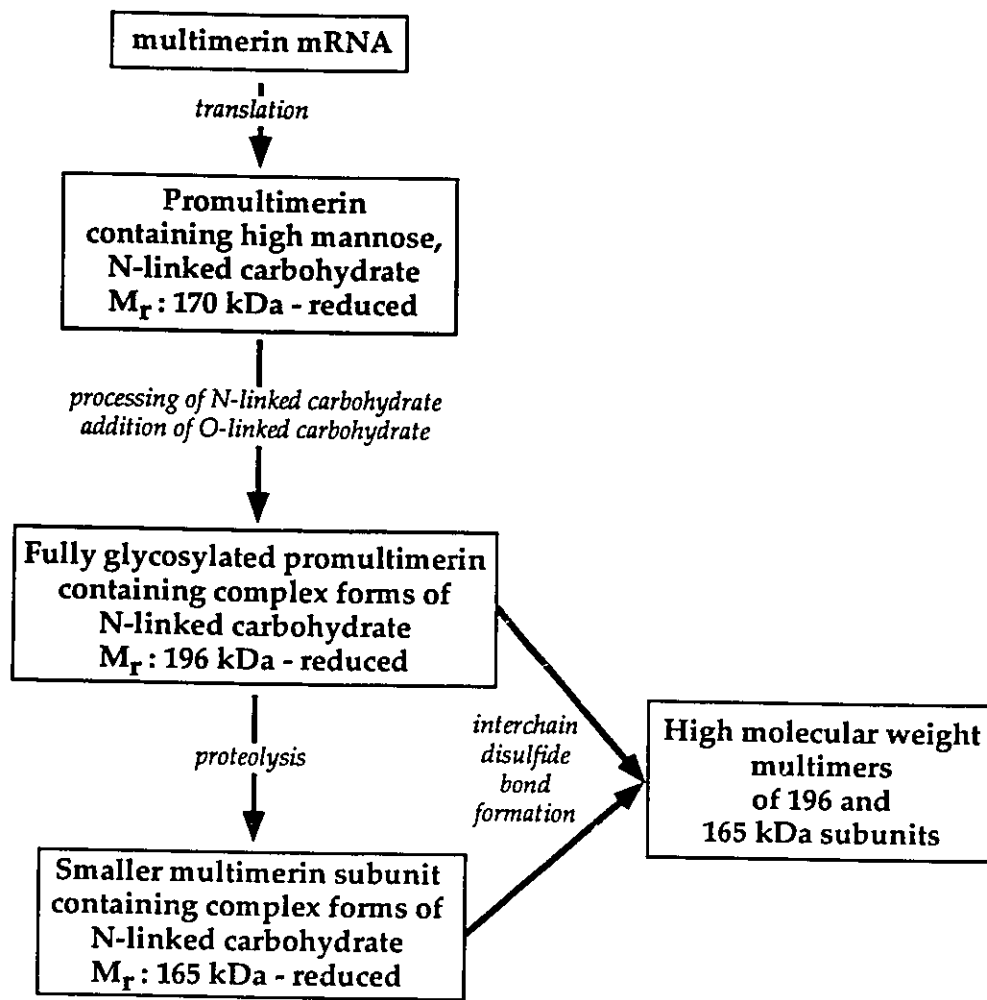


Figure 1. The Processing of Multimerin Subunits During Biosynthesis in Dami Cells

6.4.2 Multimerin Biosynthesis by Endothelial Cells

The next studies focused on studying the biosynthesis of multimerin by endothelial cells. Immunoelectron microscopy studies had indicated that multimerin was stored eccentrically within platelet alpha-granules (Hayward *et al*, 1993). Double labeling studies indicated colocalization of multimerin with von Willebrand factor in the electron-lucent region of platelet alpha-granules (Hayward *et al*, 1993). This region of alpha-granules resembles the Weibel-Palade bodies (Weibel *et al*, 1964) found in endothelial cells in both ultrastructure and protein content (Cramer *et al*, 1985; Wilbourne *et al*, 1993; Harrison *et al*, 1993b). Previously, von Willebrand factor was the only soluble protein known to be stored in the electron-lucent zone of platelet alpha-granules and in Weibel-Palade bodies (Cramer *et al*, 1985; Wilbourne *et al*, 1993; Harrison *et al*, 1993b). The presence of multimerin within the electron-lucent zone of platelet alpha-granules suggested the possibility that multimerin might also be found in endothelial cell Weibel-Palade bodies.

Immunohistochemistry studies indicated specific staining of large and small, arterial and venous blood vessels for multimerin (Hayward *et al*, 1995b). In addition, confocal microscopy studies of cultured human umbilical vein endothelial cells demonstrated that multimerin was found in the same cytoplasmic organelles as von Willebrand factor, identifying Weibel-Palade bodies as a storage organelle for multimerin (Hayward *et al*, 1995b; Wagner *et al*, 1982; Wagner *et al*, 1984; Wagner, 1993).

We next studied the distribution of multimerin following endothelial cell activation. We found that multimerin was secreted and

bound to the pericellular matrix after cell activation (Hayward *et al*, 1995b). These studies indicated that multimerin is stored in both platelets and endothelial cells and is secreted following cell activation. The secreted multimerin binds to platelet membranes and the periendothelial matrix.

Weibel-Palade bodies are now known to contain von Willebrand factor, multimerin, the membrane proteins P-selectin and CD63, and histamine (Wagner *et al*, 1982; Wagner *et al*, 1987, Wagner, 1993; Hayward *et al*, 1995b; McEver *et al*, 1989; Hattori *et al*, 1989; Ueda *et al*, 1992; Vischer *et al*, 1993). Together, these data suggest an expanding role for this organelle. Weibel-Palade body release is associated with alterations in endothelial cell function, including the secretion of large multimers of von Willebrand factor that support platelet adhesion (Wagner *et al*, 1986; Wagner, 1993). Weibel-Palade body secretion also leads to the appearance of P-selectin on the endothelial surface and enhanced leukocyte adhesion (Hamburger *et al*, 1990). The roles of Weibel-Palade body CD63 and histamine are uncertain (Wagner, 1993). The storage of multimerin in Weibel-Palade bodies could be an additional mechanism to alter endothelial cell function at sites of vessel injury.

As part of my thesis studies, I used metabolic labeling techniques to characterize multimerin biosynthesis by endothelial cells. Pulse-chase metabolic labeling studies indicated similarities in multimerin biosynthesis by Dami cells and endothelial cells. The secreted multimerin subunits from endothelial cells were 186 and 165 kDa in size (Hayward *et al*, 1995b) and

comparison with Dami cell multimerin indicated differences in the extent of N-glycosylation of multimerin by these cells (Hayward *et al*, 1995b).

Immunoblot studies were used to compare the multimerin in platelet and endothelial cell lysates. Both cell lysates containing the high molecular weight multimers (Hayward *et al*, 1995b). In contrast, the multimerin constitutively secreted by endothelial cells and Dami cells was comprised of mainly small multimers (Hayward *et al*, 1993; Hayward *et al*, 1995b). A similar constitutive secretion of small von Willebrand factor multimers by endothelial cells has been reported (Sporn *et al*, 1986).

Because differences were observed in the multimerin found in endothelial cell lysates and in culture supernatants, further studies were done to compare the regulated and constitutive pathways of multimerin secretion in these cells. Pulse-chase studies indicated that the constitutive secretion of multimerin was complete by 6 hours after biosynthesis. To evaluate the multimerin destined for regulated release, a three day metabolic labeling was performed, followed by a 6 hr chase to allow secretion of multimerin by the constitutive pathway. Cells were then treated with agonists to induce Weibel-Palade body release and compared to control, untreated endothelial cells. Cell lysates and culture supernatants were evaluated to follow changes in distribution of multimerin.

Compared to the constitutively secreted multimerin, the multimerin contained in the cell lysate after the three day metabolic labeling contained more high molecular weight multimers (Hayward *et al*, 1995b). Cell activation was associated with the loss of multimerin from the cell lysate

fraction, but it was not recovered from the culture supernatant (Hayward *et al*, 1995b). In contrast, analyses of the same fractions for von Willebrand factor indicated secretion of the stored von Willebrand factor into the culture supernatant of activated endothelial cells (Hayward *et al*, 1995b). No differences were observed between control and activated fractions analyzed for PAI-1, a protein synthesized by endothelial cells that is not stored in Weibel-Palade bodies (Hayward *et al*, 1995b; Podor *et al*, 1994).

Studies indicated that the multimerin released from Weibel-Palade bodies was mainly bound to the pericellular matrix, in contrast to the small multimers that were constitutively secreted into the culture supernatant (Hayward *et al*, 1995b). Although the specific cellular and matrix components that bind multimerin have not been identified, it is likely that the receptors or ligands are linked to the cytoskeleton and the extracellular matrix. The prevalence of high molecular weight multimers of multimerin within cells has implications for function. These large multimers, contain many ligand binding sites, and may bind more avidly to cell receptors and matrix proteins. However, other factors could regulate the binding of multimerin to cells and the matrix following activation. Activation of multimerin receptors or alterations in the matrix could influence multimerin binding. The identity of the multimerin cellular receptors and matrix binding proteins are not known.

These investigations indicate similarities in the protein trafficking of von Willebrand factor and multimerin. Both proteins are synthesized by megakaryocytes and endothelial cells and are stored within the electron-

lucent zone of platelet alpha-granules and endothelial cell Weibel-Palade bodies (Wagner *et al*, 1982; Wagner *et al*, 1983; Cramer *et al*, 1985; Harrison *et al*, 1993, Hayward *et al*, 1995b). In addition, they are both constitutively secreted as small multimers (Sporn *et al*, 1986; Hayward *et al*, 1995b). The intracellular stores of these proteins, that are destined for regulated release, are enriched in high molecular weight multimers (Sporn *et al*, 1986; Hayward *et al*, 1995b). The factors responsible for the differences between the constitutively secreted and stored multimers are not known, however, prolonged intracellular storage may enhance multimer formation.

Given the soluble nature of multimerin and its release from platelets and endothelial cells following cell activation, one might expect to find multimerin in plasma. However, the avid association of this protein to the cells and the pericellular matrix may explain why multimerin is not found in plasma. Although both multimerin and von Willebrand factor are constitutively secreted *in vitro*, it is not known if this pathway for secretion is important *in vivo*. Plasma von Willebrand factor contains more of the larger multimers in comparison to the von Willebrand factor constitutively secreted by endothelial cells which is mainly comprised of dimers (Wagner *et al*, 1983). It is possible that the conditions used to culture endothelial cells *in vitro* result in altered multimerin and von Willebrand factor protein trafficking.

Weibel-Palade bodies contain tubular structures (Weibel *et al*, 1964), which are highly dependent on the presence of von Willebrand factor in these organelles (Wagner *et al*, 1991; Wilbourne *et al*, 1993). Transfection of

von Willebrand factor cDNA into cells containing storage organelles results in the formation of granules resembling Weibel-Palade bodies: this evidence indicates a direct relationship between the formation of tubules and von Willebrand factor (Wagner *et al*, 1991; Voorberg *et al*, 1993). Furthermore, platelets from patients with severe von Willebrand's disease do not have tubular structures within the electron-lucent zone of their platelet alpha-granules (Wilbourne *et al*, 1993; Harrison *et al*, 1993). Whether multimerin contributes to the ultrastructure of this organelle is not yet known.

6.5 The Multimerin cDNA and Deduced Primary Structure

The primary sequence of many proteins has provided critical information about structure and function. I investigated the cDNA sequence for multimerin to obtain the deduced amino acid sequence and to confirm if multimerin was a unique glycoprotein. Potentially, this knowledge could also provide insights into function.

The demonstration of multimerin biosynthesis by endothelial cells was an important step towards cloning and sequencing the multimerin cDNA. Protein characterization indicated that multimerin was hydrophilic and highly glycosylated with complex, N-linked carbohydrates (Hayward *et al*, 1993, Hayward *et al*, 1995b). The polypeptide component of the multimerin precursor was 132 kDa (Hayward *et al*, 1993). This predicted a protein of approximately 1200 amino acids and a cDNA with an open reading frame of at least 3.6 kbp.

Human endothelial cell expression libraries in λ gt11 were chosen to investigate the multimerin cDNA. The amino-terminal sequence of multimerin could not be obtained from the purified protein because the protein was blocked (Hayward *et al*, 1995c). As a result, antibodies were used to screen the libraries for multimerin sequences. All clones identified by antibody screening reacted with both monoclonal and polyclonal multimerin antibodies (Hayward *et al*, 1995c). An internal Eco RI site was identified in several of the clones isolated by antibody screening (Hayward *et al*, 1995c). Because all of the libraries screened were constructed using Eco RI adapters, PCR was used to subclone the inserts from these clones without disrupting the Eco RI site. The PCR-derived clones were used only to establish the orientation of the fragments and to sequence across the internal Eco RI site. These studies indicated the presence of only one internal Eco RI site (Hayward *et al*, 1995c).

Using the most 5' clone identified as a probe, additional 5' multimerin cDNA sequences were obtained from the cDNA libraries (Hayward *et al*, 1995c). The full multimerin cDNA sequence was obtained by performing double-stranded sequencing of these overlapping clones. Techniques using nested Exo III deletions and internal primers for sequencing were used to obtain the full sequence (Hayward *et al*, 1995c). An in-frame stop codon was identified upstream of the initiation ATG, indicating that the full length cDNA had been obtained (Hayward *et al*, 1995c). The initiation ATG conformed to Kozak's consensus sequences for initiation (Kozak, 1989). The cDNA contained an open reading frame of 1228 codons, with the most

probable signal peptide cleavage site between amino acids 19 and 20 (Hayward *et al*, 1995c).

Several features of the multimerin cDNA were consistent with the properties of platelet and endothelial cell multimerin (Hayward *et al*, 1993; Hayward *et al*, 1995b; Hayward *et al*, 1995c). First, analysis of the sequence predicted a hydrophilic protein (Hayward *et al*, 1995c). Second, the sequence, minus the signal peptide, encoded a 136 kDa polypeptide (Hayward *et al*, 1995c) which was in close agreement with the 132 kDa precursor identified in studies of multimerin biosynthesis (Hayward *et al*, 1993). Third, the sequence encodes a highly glycosylated protein containing 23 potential N-glycosylation sites (Hayward *et al*, 1995c), which is consistent with the 17 N-linked carbohydrates identified in studies of the protein (Hayward *et al*, 1993; Hayward *et al*, 1995b). Based on the similarities between the 132 kDa precursor identified *in vitro* and the predicted amino acid sequence, the multimerin precursor (minus the signal sequence) was designated promultimerin (Hayward *et al*, 1995c).

Alignments of the polypeptide to itself did not identify any internal repeats within the sequence (Hayward *et al*, 1995c). Consequently, I focused on identifying functional and homologous domains. The motif RGDS, which is present in a variety of adhesive proteins (Ruoslahti *et al*, 1986; D'Souza *et al*, 1991), was identified in the multimerin sequence (Hayward *et al*, 1995c).

In addition to the RGDS site, several other domains were identified by sequence analysis. An EGF-like domain with adjacent consensus

sequences for asparagine hydroxylation and tyrosine sulfation was found. An EGF-like domain is a defined structural motif, that contains a number of cysteine residues that are linked by intrachain disulfide bonds within the EGF-like domain (Davis, 1990, Kimura et al, 1990). EGF-like domains have been identified in many proteins with diverse functions (Davis, 1990, Kimura et al, 1990). In some proteins, the EGF-like domain is involved in binding to other proteins, and in other proteins this domain have been implicated as a binding site for divalent cations (Davis, 1990, Kimura et al, 1990). Homology analysis identified similarities between other proteins containing EGF-like domains and two regions in the multimerin sequence: an EGF-like domain and a second region lacking the first cysteine in the EGF-like consensus sequence CXCXXXXXGXXC (Hayward *et al*, 1995c). This region of multimerin was designated as a partial EGF-like domain (Hayward *et al*, 1995c).

Homology analyses were used to determine if multimerin was a unique protein and if it is related to other sequenced proteins. Searches of the NCBI databanks indicated that the amino acid sequence of multimerin corresponded to a unique protein (Hayward *et al*, 1995c). Similarities were noted with other proteins in defined regions of the multimerin sequence (Hayward *et al*, 1995c). The highest scoring proteins, Xotch, Notch and the human homologue TAN-1, showed similarities to multimerin in the EGF-like and partial EGF-like domains (Wharton *et al*, 1985; Coffman *et al*, 1990; Ellisen *et al*, 1991). These other proteins are EGF-like domain rich, transmembrane proteins that are involved in neurogenic development (Wharton *et al*, 1985; Coffman *et al*, 1990; Ellisen *et al*, 1991).

Lower homology scores were noted for proteins containing coiled-coil regions; these proteins showed similarities to the central portion of the multimerin sequence (Saez *et al*, 1986; Feghaku *et al*, 1989; Saez *et al*, 1990; Mitchell *et al*, 1992; Seeling *et al*, 1994; Hayward *et al*, 1995c). Based on this information, I postulated that multimerin contains, within its central region, coiled-coil structures. Analyses of the multimerin sequence, performed by Dr. Seth Darst with the aid of the program PEPCOIL, identified several regions that were highly predictive of coiled-coil structures (Hayward *et al*, 1995c). Potentially, these sites might be involved in interchain interactions between multimerin subunits to form multimers or to stabilize the protein.

Significant homology was found between the carboxyl terminal region of multimerin and other proteins that contain a trimeric, carboxyl terminal globular domain (Knobel *et al*, 1975; Sawada *et al*, 1990; Kwan *et al*, 1991; Hayward *et al*, 1995c). These proteins include the A, B, and C chains of human and mouse complement C1q, and human alpha-1 collagens type VIII and X (Reid, 1979; Reid *et al*, 1982; Reid, 1985; Sellar *et al*, 1991; Petry *et al*, 1991; Muragaki *et al*, 1991; Apte *et al*, 1991; Reichenberger *et al*, 1991; Thomas *et al*, 1991; Apte *et al*, 1992; Petry *et al*, 1992). Alignments comparing the carboxyl terminus regions of these proteins indicated that there was conservation of hydrophobic and uncharged residues (Hayward *et al*, 1995c). These data suggest that multimerin contains a similar globular head structure. The globular domain of complement C1q and collagens type VIII and X is involved in protein-protein interactions (Sawada *et al*, 1990; Kwan *et al*, 1991; Reid, 1990). The globular domain of complement C1q is the binding site for

IgG and other complement activating molecules (Reid, 1990). The globular domains of collagens type VIII and X are involved in intermolecular interactions, between collagen molecules, to form mesh-like matrix structures (Sawada *et al*, 1990; Kwan *et al*, 1991). Potentially, the multimerin globular domain could be a site for interacting with other multimerin molecules, as is the case for collagen, or it could be involved in the binding of multimerin to other proteins, as occurs with complement C1q. Confirmation of a globular domain structure in multimerin awaits electron microscopic analyses of the protein's structure.

Complement C1q and collagens type VIII and X are trimeric proteins (Reid, 1986; Apte *et al*, 1991; Kapoor *et al*, 1988). Studies of the composition of multimerin indicated that the smallest multimer was a trimer (Hayward *et al*, 1991a, Hayward *et al*, 1991b). Sequences contained within the multimerin globular domain and perhaps within other regions of this protein might be important for tertiary structure. Although the smallest multimer was identified as a trimer (Hayward *et al*, 1991b), it is not known if trimers are the building blocks of the larger multimers. However, the size of the multimerin multimers, relative to those of von Willebrand factor, are consistent with a model of multimerin assembly from trimers.

Based on the protein sequence analyses, I defined several domains in promultimerin (Chapter 5, Figure 7) (Hayward *et al*, 1995c). An important issue remains: at what site(s) is promultimerin cleaved to produce the mature protein? Using data obtained from screening the expression library, the JS-1 epitope was localized near the carboxyl terminal region of the protein

(Hayward *et al*, 1995c). In addition, clones containing the 3' region of multimerin were reactive with the polyclonal multimerin antibody that was raised against the p-155 subunit (Hayward *et al*, 1995c). Based on this information, I postulate that the site of proteolysis that produces the mature protein is located in the amino-terminal region of promultimerin. Studies using deglycosidases indicate that p-155 and p-170 contain 105 and 120 kDa polypeptide components (Hayward *et al*, 1993); based on this information, the possible amino-terminal cleavage site, producing p-155, would be predicted to be near the RGDS site. It is not known if the RGDS site is found in the mature protein.

Studies of multimerin's distribution in human cells and tissues indicated that both endothelial cells and megakaryocytes synthesize and store multimerin (Hayward *et al*, 1993; Hayward *et al*, 199b). Northern analyses of multimerin expression indicated that the multimerin message is found in highly vascular tissues, consistent with the protein's distribution in the endothelium of blood vessels (Hayward *et al*, 1995c). In addition, the message for multimerin was detected in the RNA isolated from platelets (Hayward *et al*, 1995c). This indicates that the multimerin found in platelets is derived from endogenous biosynthesis by megakaryocytes.

No similarities were observed in the cDNA sequences of multimerin and von Willebrand factor (Hayward *et al*, 1995c). The amino-terminal region of von Willebrand factor is thought to be important for the targeting of von Willebrand factor to storage organelles and for multimer formation (Verweij *et al*, 1987; Wise *et al*, 1988; Wagner *et al*, 1991; Hournet *et al*, 1993;

Voorberg *et al*, 1993). Studies of von Willebrand factor indicate that multimerization of this protein occurs in the Golgi apparatus, before it appears in Weibel-Palade bodies (Vischer *et al*, 1994). The absence of a similar peptide sequence in multimerin indicates that other factors, perhaps their large multimeric structures, account for their similar targeting and storage. At present, these factors are unknown.

There are other important questions about the structure of multimerin that await resolution. How the subunit molecules assemble into the large disulfide-linked multimers, which domains are important for function, and which cysteines are involved in interchain disulfide bonds are not yet known. Nonetheless, the primary sequence information has provided an important tool for further structure-function analyses.

6.6 Implications for Future Research

Knowledge of platelet proteins and their role in supporting platelet function provides insight into the molecular mechanisms of hemostasis. The focus of my thesis was to characterize multimerin, including its cells of origin, biosynthesis and protein sequence. These studies led to the identification of a novel, massive protein that is found in platelets and endothelial cells and released from these cells by activation or injury. Although the function(s) of multimerin are at present uncertain, its size, multimeric structure, sequence, and binding to activated platelets and endothelial cells suggest that it may be important for adhesion.

The storage of multimerin within platelets and endothelial cells may be an important mechanism to restrict its action(s) to sites of vessel injury. It is also possible that multimerin may have functions, during its storage within platelet alpha-granules, that are separate from its roles on activated platelets and endothelial cells. Determining the function of multimerin is the subject of future studies. In considering function, an important question about multimerin must be asked: why is such a large protein stored inside platelets and endothelial cells? I believe that its multimeric structure and size are clues to its function. The presence of repetitive domains has the potential to concentrate and localize binding sites for cell receptors and ligands. Certainly, in the case of von Willebrand factor large multimers are the most potent for platelet adhesion and shear-induced aggregation. The RGDS and putative globular domains of multimerin suggest that this protein may also function in adhesion. Possible roles for multimerin include supporting platelet adhesion to subendothelium, endothelial cell adhesion to the subendothelial matrix, or the organization of the extracellular matrix.

Many proteins are recognized to have more than one function and can interact with several ligands and receptors. Examples include fibrinogen, which is important for fibrin formation and for platelet-platelet interaction and cell adhesion (Hantgan *et al*, 1984), and von Willebrand factor, which functions not only as an adhesive protein that binds to platelet and collagen but also as a carrier protein for factor VIII (Meyer *et al*, 1993). Multimerin, by analogy, may have more than one biologic function.

The factors that determine the targeting of multimerin to alpha-granules and Weibel-Palade bodies require further investigation. At present, no consensus sequence controlling targeting has been found in the megakaryocyte derived proteins that are stored in platelet alpha-granules or in the multimeric proteins stored in Weibel-Palade bodies. These data suggest that there may be separate mechanisms controlling multimerization and targeting.

Platelet alpha-granule and Weibel-Palade body release are mechanisms that facilitate local alterations in glycoprotein expression. Deficiencies in these intracellular glycoprotein pools results in defects in hemostasis; examples include alpha-storage pool disease and several forms of von Willebrand's disease. At present, it is not known what specific defects arise when there are deficiencies in the alpha-granular and Weibel-Palade body stores of multimerin. Recently, in collaboration with Drs. Graham Côté and Michael Nesheim at Queen's University, I identified multimerin as a factor V binding protein that is stored complexed with factor V in platelet alpha-granules (Hayward et al, 1995d). My subsequent investigations of a family, with an inherited deficiency in platelet factor V, determined that these individuals are deficient in platelet multimerin (manuscript in preparation). Serious bleeding in this family with multimerin deficiency suggests a role for multimerin in hemostasis. Further investigations are required to determine if this disorder is due to a defect in the multimerin gene. The contribution of the multimerin deficiency to the bleeding in this disorder is not yet known.

However, these data suggest new directions to pursue in determining multimerin function.

Knowledge, derived from studies characterizing multimerin as a novel platelet and endothelial protein, has provided the framework for future investigations of this protein. Understanding of the role of multimerin in hemostasis may provide additional insights into the molecular mechanisms by which platelets and vessel walls support hemostasis.

BIBLIOGRAPHY

- Abrams, C. S., Ellison, N., Budzinski, A. Z., and Shattil, S. F. (1990). Direct detection of activated platelets and platelet-derived microparticles in humans. *Blood* **75**, 128-128.
- Aiken, M. L., Ginsberg, M. H., and Plow, E. F. (1987) Mechanisms for expression of thrombospondin on the platelet cell surface. *Semin. Thromb. Hemost.* **13**, 307-316.
- Apte, S., Mattei, M. G., and Olsen, B. R. (1991). Cloning of human $\alpha 1(X)$ collagen DNA and localization of the COL10A1 gene to the q21-q22 region of human chromosome 6. *FEBS Lett.* **282**, 393-396
- Apte, S. S., Seldin M. F., Hayashi, M, and Olson, B. R. (1992) Cloning of the human and mouse type X collagen genes and mapping of the mouse type X collagen gene to chromosome 10. *Eur. J. Biochem.* **206**, 217-224
- Arai, M., Yamamoto, N., Moroi, M., Akamatsu, N., Fukutake, K., and Tanoue, K. (1994). *Br. J. Haematol.* **89**, 124-130.
- Asch, M. L., and Podack, E. (1990) Vitronectin binds to activated human platelets and plays a role in platelet aggregation. *J. Clin. Invest.* **85**, 1372-1378.
- Assoian, R. K., Komorija, A., Meyers, C. A., Miller, D. M., Sporn, M. B. (1983) Transforming growth factor-beta in human platelets. Identification of a major storage site, purification, and characterization. *J. Biol. Chem.* **258**, 7155-7160.
- Benton, W. D., and Davis, R. W. (1977) Screening λ gt recombinant clones by hybridization to single plaques in situ. *Science* **196**, 180-182.
- Bienz, D., and Clemetson, K. J. (1989) Human platelet glycoprotein Ia. One component is only expressed on the surface of activated platelets and may be a granule constituent. *J. Biol. Chem.* **264**, 507-514.

- Blaas, P., Berger, B, Weber, S, Peter, H. H., and Hansch, G. M. (1988) Enhanced stimulation of platelets by the terminal complement components is related to the lack of C8bp in the membrane. *J. Immunol.* **140**, 3045-3051.
- Blumberg, N., Masel, D., Mayer, T, Horan, P., and Heal, J. (1984) Removal of HLA-A, B antigens from platelets. *Blood* **63**, 448-450.
- Bonfanti, R., Furie B. C., Furie, B., and Wagner, D. D. (1989) PADGEM (GMP 140) is a component of Weibel-Palade bodies of human endothelial cells. *Blood* **73**, 1109-1112.
- Booth, N. A., Simpson, A. J., Croll, A., Bennett, B., and MacGregor, I R. (1988) Plasminogen activator inhibitor (PAI-1) in plasma and in platelets. *Br. J. Haematol.* **70**, 327-333.
- Brasheem-Stein, C., Nugent, D., and Bernstein, I. D. Characterization of an antigen expressed on activated human T-cells and platelets. *J. Immunol.* **140**, 2330-2333.
- Brass, L. F., Hoxie, J. A., and Manning, D. R. Signaling through G proteins and G protein-coupled receptors during platelet activation. *Thromb. Haemost.* **70**, 217-223.
- Breton-Gorius, J., Clezardin, P., Guichard, J., Debili, N., Malaval, L, Vainchenker W., Cramer, E. M., and Delmas, P. D. (1992) Localization of platelet osteonectin at the interface of the α -granule membranes in platelets and megakaryocytes. *Blood* **79**, 936-941.
- Chesney, C. M., Pifer, D., and Colman, R. W. (1981) Subcellular localization and secretion of factor V from human platelets. *Proc. Natl. Acad. Sci.* **78**, 5180-5184.
- Clemetson, K. J., Capitanio, A., and Luscher, E. F. (1979) High resolution two-dimensional gel electrophoresis of the proteins and glycoproteins of human blood platelets and platelet membranes. *Biochem. Biophys. Acta* **553**, 11-24.

- Clemetson, K. J., Bienz, D., Zahno, M. L., and Luscher, E. F. (1984) Distribution of platelet glycoproteins and phosphoproteins into the hydrophobic and hydrophilic phases in Triton X-114 phase partition. *Biochem. Biophys. Acta* **778**, 463-469.
- Cleveland, D. W., Fischer, S. G., Kirschner, M. W., and Laemmli, U. K. (1977) Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. *J. Biol. Chem.* **252**, 1102-1106.
- Coffman, C., Harris, W., and Kintner, C. (1990) Xotch, the *Xenopus* homologue of *Drosophila* notch. *Science* **249**, 1438-1441
- Coller, B. S. (1985) A new murine monoclonal antibody reports an activation-dependent change in the conformation and/or microenvironment of the platelet glycoprotein IIb/IIIa complex. *J. Clin. Invest.* **76**, 101-108.
- Coleman, R. W., Cook, J. J., and Niewiarowski, S. (1994) Mechanisms of platelet aggregation in *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 3rd Edition. Editors: Coleman, R. W., Hirsch, J., Marder, V. J., and Salzman, E. W. J. B. Lippincott, Philadelphia, PA.
- Coughlin S. R. (1994) Molecular mechanisms of thrombin signaling. *Semin. Hematol.* **31**, 270-277.
- Counts, R. B., Paskell, S. I., and Elgee, S. K. (1978) Disulfide bonds and the quaternary structure of factor VIII/von Willebrand factor. *J. Clin. Invest.* **62**, 702-709.
- Cramer, E. M., Meyer, D., le Menn, R., and Breton-Gorius, J. (1985) Eccentric localization of von Willebrand Factor in an internal structure of platelet α -granule resembling that of Weibel-Palade bodies. *Blood* **66**, 710-713.
- Davis C. G. (1990) The many faces of epidermal growth factor repeats. *New Biol.* **2**, 410-419
- D'Souza, S. E., Ginsberg, M. H., and Plow, E. F. (1991) Arginyl-glycyl-aspartic acid (RGD): A cell adhesion motif. *Trends Biochem. Sci.* **16**, 246-250

- Du, X., Plow, E. G., Freilinger III, A. L., O'Toole, T. E., Loftus, J. C., and Ginsberg, M. H. (1991). Ligands activate integrin $\alpha_{IIb}\beta_3$ (platelet GP IIb-IIIa). *Cell* **65**, 409-416.
- Ellisen, L. W., Bird, J., West, D. C., Soreng, A. L., Reynolds, T. C., Smith, S. D., and Sklar, J. (1991) TAN-1, the human homolog of *Drosophila* Notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. *Cell* **66**, 649-661
- Feghaku, R., and Leinwand, L. A. (1989). Molecular characterization of a developmentally regulated human perinatal myosin heavy chain. *J. Cell. Biol.* **108**, 1791-1797
- Fernandez, M. F. L., Ginsberg, M. H., Ruggeri, Z. M., Battle, F. J., and Zimmerman, T. S. (1982) Multimeric structure of platelet factor VIII/von Willebrand factor: The presence of larger multimers and their reassociation with thrombin-stimulated platelets. *Blood* **60**, 1132-1138.
- Freilinger III, A. L., Lam, S. C. T., Plow, E. F., Smith, M. A., Loftus, J. C., and Ginsberg, M. H. (1988) Occupancy of an adhesive glycoprotein receptor modulates expression of an antigenic site involved in cell adhesion. *J. Biol. Chem.* **263**, 12397-12402.
- Fressinaud, E., Federici, A. B., Castaman, G., Rothschild, C., Rodeghiero, F., Baumgartner, H. R., Mannucci, P. M., and Meyer, D. (1994) The role of platelet von Willebrand factor in platelet adherence and thrombus formation: A study of 34 patients with various subtypes of type I von Willebrand's disease. *Br. J. Haematol.* **86**, 327-332.
- Furman, M. I., Gardner, T. M., Goldschmidt-Clermont, P. J. (1993) Mechanisms of cytoskeletal reorganization during platelet activation. *Thromb. Haemost.* **70**, 229-232.
- George, J. N., Pickett, E. B., Saucerman, S., McEver, R. P., Kunicki, T. J., Kieffer, N., and Newman, P. J. (1986) Platelet surface glycoproteins. Studies on resting and activated platelets and platelet membrane microparticles in normal subjects and observations in patients during adult respiratory distress syndrome and cardiac surgery. *J. Clin. Invest.* **78**, 340-348.

- George, J. N. (1990) Platelet immunoglobulin G: Its significance for the evaluation of thrombocytopenia and for understanding the origin of α -granule proteins. *Blood* 76, 859-870.
- Gerrard, J. M., Phillips, D. R., Rao, G. H. R., Plow, E. F., Walz, D. A., Ross, R. Harker, L. A., White, J. G. (1980) Biochemical studies of two patients with the gray platelet syndrome. Selective deficiency of platelet alpha granules. *J. Clin. Invest.* 66, 102-109.
- Ghebrehiwet, B., Peerschke, E. I. B., Hong, Y., Munoz, P., and Gorevic, P. D. (1992) Short amino acid sequences derived from C1q receptor (C1q-R) show homology with alpha chains of fibronectin and vitronectin receptors and collagen type IV. *J. Leukocyte Biol.* 51, 546-556.
- Ghebrehiwet, B., Lim, B.-L., Peerschke, E. I. B., Willis, A. C., and Reid, K. B. M. (1994) Isolation, cDNA cloning, and overexpression of a 33-kDa cell surface glycoprotein that binds to the globular "heads" of C1q. *J. Exp. Med.* 179, 1809-1821.
- Gogstad, G. O., Stormorken, H., Solum, N. O. Platelet alpha 2-antiplasmin is located in the platelet alpha-granules. *Thromb. Res.* 31, 387-390.
- Gralnick, H. R., Williams, S. B., and Collier, B. S. (1984) Fibrinogen competes with von Willebrand factor for binding to the glycoprotein IIb/IIIa complex when platelets are stimulated with thrombin. *Blood* 64, 797-800.
- Gralnick, H. R., Rich, M. E., McKneown, L. P., Williams, S. B., Parker, R. I., Maisonneuve, P., Jeanneau, C., and Sultan, Y. (1986) Platelet von Willebrand factor: An important determinant of the bleeding time in type I von Willebrand's disease. *Blood* 68, 58-61.
- Greenberg, S. M., Rosenthal, D. S., Greeley, T. A., Tantravahi, R., and Handin, R. I. (1988) Characterization of a new megakaryocytic cell line: the Dami cell. *Blood* 72, 1968-1977.
- Hamburger, S. A., and McEver, R. P. (1990) GMP-140 mediates adhesion of stimulated platelets to neutrophils. *Blood* 75, 550-554.

- Handagama, P. J., Shuman, M. A., and Bainton, D. F. (1989) Incorporation of intravenously injected albumin, immunoglobulin G, and fibrinogen into guinea pig megakaryocyte granules. *J. Clin. Invest.* **84**, 73-82.
- Handagama, P., Rappolee, D. A., Werb, Z, Levin, J., and Bainton, D. F. (1990) Platelet α -granule fibrinogen, albumin, and immunoglobulin G are not synthesized by rat and mouse megakaryocytes. *J. Clin. Invest.* **86**, 1364-1368.
- Hantgan, R. R., Francis, C. W., and Marder, V. J. (1994) Fibrinogen structure and physiology. In: *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*, 3rd Edition. Editors: Coleman, R. W., Hirsch, J., Marder, V. J., and Salzman, E. W. J. B. Lippincott, Philadelphia, PA.
- Haregewoin, A., Solomon, H. R. C., Soman, G., Bergelson, J. M., Bhan, A. K., and Finberg, R. W. (1994) Cellular expression of a GPI-linked T cell activation protein. *Cell. Immunol.* **156**, 357-370.
- Harrison, P., Wilbourne, B., Debili, N., Vainchenker, W., Breton-Gorius, J., Lawrie, A. S., Masse, J.-M., Savidge, G. F. and Cramer, E. M. (1989) Uptake of plasma fibrinogen into the alpha granules of human megakaryocytes and platelets. *J. Clin. Invest.* **84**, 1320-1324.
- Harrison, P., and Martin Cramer, E. (1993b) Platelet α -granules. *Blood Reviews* **7**, 52-62.
- Hattori, R., Hamilton, K. K., Fugate, R. D., McEver, R. P., and Sims, P. J. (1989) Stimulated secretion of endothelial von Willebrand factor is accompanied by rapid redistribution to the cell surface of the intracellular granule membrane protein GMP-140. *J. Biol. Chem.* **264**, 7768-7771.
- Hayward, C. P. M., Smith, J. W., Horsewood, P., Warkentin, T. E., and Kelton, J. G. (1991a) p-155, a multimeric platelet protein that is expressed on activated platelets. *J. Biol. Chem.* **266**, 7114-7120.
- Hayward, C. P. M., Warkentin, T. E., Horsewood, P., and Kelton, J. G. (1991b) Multimerin: a series of large, disulfide-linked multimeric proteins within platelets. *Blood* **77**, 2556-2560.

- Hayward, C. P. M., Bainton, D. F., Smith, J. W., Horsewood, P., Stead, R. H., Podor, T. J., Warkentin, T. E., and Kelton, J. G. (1993) Multimerin is found in the α -granules of resting platelets and is synthesized by a megakaryocytic cell line. *J. Clin. Invest.* **91**, 2630-2639.
- Hayward, C. P. M., and Kelton, J. G. (1995a) Multimerin: A multimerin protein stored in platelet alpha-granules. *Platelets* **6**, 1-10.
- Hayward, C. P. M., Kelton, J. G., Stead, R. H., Warkentin, T. E., and Podor, T. J. (1995b) Endothelial cells store multimerin in Weibel-Palade bodies and possess both constitutive and regulated pathways for multimerin release. *Submitted for publication.*
- Hayward, C. P. M., Hassell, J. A., Denomme, G. A., Rachubinski, R. A., Brown, C., and Kelton, J. G. (1995c) The cDNA sequence of human endothelial cell multimerin: A unique protein with RGDS, coiled-coil, and EGF-like domains and a carboxyl-terminus similar to the globular domain of complement C1q and collagens type VIII and X. *J. Biol. Chem.* **270**, 18246-18251.
- Hayward C. P. M., Furmaniak-Kazmierczak, E., Cieutat, A.-M., Moore, J. C., Bainton, D. F., Nesheim, M. E., Kelton, J. G, and Côté, G. (1995d) Factor V is complexed with multimerin in resting platelet lysates and colocalizes with multimerin within platelet alpha granules. *J. Biol. Chem.* **270**, 19217-19224.
- Heldin, C. H., Westermark, B., and Wasteson, C. (1979) Platelet derived growth factor: Purification and partial characterization. *Proc. Natl. Acad. Sci.* **76**, 3722-3726.
- Hickey, M. J., Williams, S. A., and Roth, G. J. (1989) Human platelet glycoprotein IX: An adhesive prototype of leucine-rich glycoproteins with flank-center-flank sequences. *Proc. Natl. Acad. Sci.* **86**, 6773-6777.
- Hirata, M., Hayashi, Y., Ushikubi, F., Yokota, Y., Kageyama, R., Nakanishi, S., Narumiya, S. (1991) Cloning and expression of cDNA for a human thromboxane receptor. *Nature* **349**, 617-620.
- Honda, Z., Nakamura, I., Miki, I., Minami, M., Watanabe, T, Seyama, Y, Okado, H., Toh, H, Ito, K., Miyamoto, T., and Shimizu, T. (1991)

- Cloning by functional expression of platelet activating receptor from guinea pig lung. *Nature* 349, 342-346.
- Horsewood, P., Smith, J. W., and Kelton, J. G. (1993) Monoclonal antibodies against platelet glycoproteins. *Trans. Med. Rev.* 7, 156-172.
- Hournet, A. M., Saffaripour, S., Cramer, C. M., Tenza, D., and Wagner, D. D. (1993) Von Willebrand factor storage requires intact prosequence cleavage site. *Eur. J. Cell Biol.* 60, 31-41.
- Hoyer, L. W., and Shainhoff, J. R. (1980) Factor VIII-related protein circulates in normal human plasma as high molecular weight multimers. *Blood* 55, 1056-1059.
- Hsu-Lin, S., Berman, C. L., Furie, B. C., August, D., and Furie, B. (1984) A platelet membrane protein expressed during platelet activation and secretion. *J. Biol. Chem.* 259, 9121-9126.
- Jaffe, E. A., Hoyer, L. W., and Nachman, R. L. (1973) Synthesis of antihemophilic factor antigen by cultured human endothelial cells. *J. Clin. Invest.* 52, 2757-2764.
- Jaffe, E. A., Hoyer, L. W., and Nachman, R. L. (1974) Synthesis of von Willebrand factor by cultured human endothelial cells. *Proc. Natl. Acad. Sci.* 71, 1906-1909.
- Johnston, G. I., Cook, R. G., and McEver, R. P. (1989). Cloning of GMP-140, a granule membrane protein of platelets and endothelium: Sequence similarity to proteins involved in cell adhesion and inflammation. *Cell* 56, 1033-1044.
- Kaplan, K. L., Broekman, M. J., Chernoff, A., Leznick, G. R., and Drillings, M. (1979) Platelet alpha granule proteins: Studies on release and subcellular localization. *Blood* 53, 604-618.
- Kapoor, R., Sakai, L. Y., Funk, S., Roux, E, Bornstein, P., and Sage E. H. (1988) Type VIII collagen has a restricted distribution in specialized extracellular matrices. *J. Cell Biol.* 107, 721-730.
- Kelton, J. G., Smith, J. W., Santos, A. V., Murphy, W. G., and Horsewood, P. (1987) Platelet IgG Fc Receptor. *Am. J. Hematol.* 25, 299-310.

- Kelton, J. G., Smith, J. W., Horsewood, P., Humbert, J. R., Hayward, C. P. M., and Warkentin, T. E. (1990) G^v_{a/b} alloantigen system on human platelets. *Blood* 75, 2172-2176.
- Kenney, P. M., and Davis, A. E. (1981) Association of alternative complement pathway components with human blood platelets: Secretion and localization of Factor D and 1H globulin. *Clin. Immunol. Immunopath.* 21, 351-363.
- Kinoshita, T., Medof, M. E., Silber, R., and Nussenzweig, V. (1985) Distribution of decay accelerating factor in the peripheral blood of normal individuals and patients with paroxysmal nocturnal hemoglobinuria. *J. Exp. Med.* 162, 75-92.
- Knobel, H. R., Villiger, W., and Isliker, H. (1975) Chemical and electron microscopic studies of human C1q prepared by different methods. *Eur. J. Immunol.* 5, 78-82.
- Kobilka, B. K., Matsui, H., Kobilka, T. S., Yang-Feng, T. L., Francke, U., Caron, M. G., Lefkowitz, R. J., and Regan, R. W. (1987) Cloning, sequencing and expression of the gene coding for the human platelet α_2 -adrenergic receptor. *Science* 28, 650-656.
- Kornfeld, R., and Kornfeld, S. (1985) Assembly of asparagine-linked oligosaccharides. *Ann. Rev. Biochem.* 54, 631-664.
- Koutts, J., Walsh, P. N., Plow, E. F., Fenton II, J. W., Bouma, B. N., and Zimmerman, T. S. (1978) Active release of human factor VIII-related antigen by adenosine diphosphate, collagen and thrombin. *J. Clin. Invest.* 62, 1255-1263.
- Kozak M. (1989) The scanning model for translation: An update. *J. Cell. Biol.* 108, 229-241
- Kroll, M. H., and Schafer, A. I. (1989) Biochemical Mechanisms of Platelet Activation. *Blood* 74, 1181-1195.

- Kwan, A. P. L., Cummings, C. E., Chapman, J. A., and Grant, M. E. (1991) Macromolecular organization of chicken type X collagen in vitro. *J. Cell Biol.* **114**, 597-604
- Lanza, F., Wolf, D., Fox, C. F., Kieffer, N., Seyer, J. M., Fried, V. A., Soughlin, S. R., Phillips, D. R., and Jennings, L. K. (1991) cDNA cloning and expression of platelet p24/CD9: Evidence for a new family of multiple membrane spanning proteins. *J. Biol. Chem.* **266**, 10638-10645.
- Lawler, J. W., Slayter, H. S., and Coligan, J. E. (1978) Isolation and characterization of a high molecular weight glycoprotein from human blood platelets. *J. Biol. Chem.* **253**, 8609-8616.
- Legaz, M. E., Schmer, G., Counts, R. B., and Davie, E. W. (1973) Isolation and characterization of human factor VIII (antihemophilic factor). *J. Biol. Chem.* **248**, 3946-3955.
- Leung, L. L. K., Harpel, P. C., Nachman, R. L., and Rabellino, E. M. (1983) Histidine-rich glycoprotein is present in human platelets and it is cleared following thrombin stimulation. *Blood* **62**, 1016-1021.
- Leung, L. L. K. (1984) Role of thrombospondin in platelet aggregation. *J. Clin. Invest.* **74**, 1764-1772.
- Levy-Toledano, S., Caen, J. P., Breton-Gorius, J., Rendu, F., Cwyiner-Colenzer, C., Dupuy, E., Legrand, Y., Maclouf, J. (1982) Gray platelet syndrome: α -granule deficiency, its influence on platelet function. *J. Lab. Clin. Med.* **98**, 831-849.
- Lopez, J. A., Chung, D. W., Fujikawa, K., Hagen, F. S., Davie, E. W., and Roth, G. J. (1988) The α and β chains of human platelet glycoprotein Ib are both transmembrane proteins containing a leucine-rich amino acid sequence. *Proc. Natl. Acad. Sci.* **85**, 2135-2139.
- Lynch, D. C., Williams, R., Zimmerman, T. S., Kirby, E. P., and Livingston, D. M. (1983) Biosynthesis of the subunits of factor VIII R by bovine aortic endothelial cells. *Proc. Natl. Acad. Sci.* **80**, 2738-2742.
- Mannucci, P. M., Lombardi, R., Bader, R., Vianello, L., Ferecici, A. B., Solinas, S., Mazzucconi, M. G., and Mariani, G. (1985) Heterogeneity of type I

- von Willebrand's disease: Evidence for a subgroup with an abnormal von Willebrand factor. *Blood* **66**, 796-802.
- McEver, R. P., and Martin, M. N. (1984) A monoclonal antibody to a membrane glycoprotein binds only to activated platelets. *J. Biol. Chem.* **259**, 9799-9804.
- McEver, R. P., Beckstead, J. H., Moore, K. L., Marshall-Carlson, L., and D. F. Bainton. 1989. GMP-140, a platelet alpha-granule membrane protein is also synthesized by vascular endothelial cells and is localized in Weibel-Palade bodies. *J. Clin. Invest.* **84**, 92-99.
- Meyer, D., and Girma, J-P. (1993) von Willebrand factor: Structure and function. *Thromb. Haemost.* **70**, 99-104.
- Mitchell, P. J., and Cooper, C. S. (1992) Nucleotide sequence analysis of human tpr cDNA clones. *Oncogene* **7**, 2329-2333
- Moiri, M., Okuma, M., and Jung, S. M. (1992) Platelet adhesion to collagen-coated wells: analysis of this complex process and a comparison with the adhesion to metrigel-coated wells. *Biochem. Biophys. Acta*, **1137**, 1-9.
- Morgan, B. P. (1992) Isolation and characterization of the complement inhibiting protein CD59 antigen from platelet membranes. *Biochem. J.* **282**, 409-413.
- Muragaki, Y., Mattei, M. G., Yamaguchi, N., Olsen, B. R., and Ninomiya, Y. (1991) The complete primary structure of the human $\alpha 1$ (VIII) chain and assignment of its gene (COL8A1) to chromosome 3. *Eur. J. Biochem.* **197**, 615-622
- Nachman, R. L., and Jaffe, E. A. (1975) Subcellular platelet factor VIII antigen and von Willebrand factor. *J. Exp. Med.* **141**, 1101-1112.
- Nachman, R. L., and Harpel, P. C. (1976) Platelet α_2 -macroglobulin and α_1 -antitrypsin. *J. Biol. Chem.* **251**, 4512-4521.

- Nachman, R., Levine, R., and Jaffe, E. A. (1977) Synthesis of factor VIII antigen by cultured guinea pig megakaryocytes. *J. Clin. Invest.* **60**, 914-921.
- Newman, P. J., Berndt, M. C., Gorski, J., White, G. C., Lyman, S., Paddock, C. and Muller, W. A. (1990) PECAM-1 (CD31) cloning and relation to adhesion molecules of the immunoglobulin gene superfamily. *Science* **247**, 1219-1222.
- Nicholson-Weeler, A., March, J. P., Rosen, C. E., Spicer, D. B., and Austen, K. F. (1985) Surface membrane expression by human blood leukocytes and platelets of decay-accelerating factor, a regulatory protein of the complement system. *Blood* **65**, 1237-1234.
- Nieuwenhuis, H. K., Akkerman, J. W. N., Houdijk, W. P. M., and Sixma, J. J. (1985) Human blood platelets showing no response to collagen fail to express surface glycoprotein Ia. *Nature* **318**, 470-472.
- Nieuwenhuis, H. K., van Oosterhout, J. J. G., Rozemuller, E., van Iwaarden, F., and Sixma, J. J. (1987) Studies with a monoclonal antibody against activated platelets: Evidence that a secreted 53,000-molecular weight lysosome-like granule protein is exposed on the surface of activated platelets in the circulation. *Blood* **70**, 838-845.
- Nishibori, M., Cham, B., McNicol, A., Shalev, A., Jain, N., and Gerrard, J. The protein CD63 is in platelet dense granules, is deficient in a patient with Hermansky-Pudlak Syndrome, and appears identical to granulophysin. *J. Clin. Invest.* **1993**; **91**: 1775-1782.
- Nurden, A. T., and Caen, J. P. (1974) An abnormal platelet glycoprotein pattern in three cases of Glanzmann's thrombasthenia. *Br. J. Haematol.* **28**, 253-260.
- Nurden, A. T., and Caen, J. P. (1975) Specific roles for platelet surface glycoproteins in platelet function. *Nature* **255**, 720-722.
- Nurden, A. T., and Nurden, P. (1993) A review of the role of platelet membrane glycoproteins in the platelet-vessel wall interaction. *Balliere's Clinical Hematology* **6**, 653-690.

- Olson, J. D., Brockway, W. J., Fass, D. N., Bowie, E. J. W., and Mann, K. G. (1977) Purification of porcine and human ristocetin-Willebrand factor. *J. Lab. Clin. Med.* **89**, 1278-1294.
- Packham, M. A. (1994) The role of platelets in thrombosis and hemostasis. *Can. J. Physiol. Pharmacol.* **72**, 278-284.
- Parker, R. I., and Gralnick, H. R. (1986) Identification of platelet glycoprotein IIb/IIIa as the major binding site for released platelet-von Willebrand factor. *Blood* **68**, 732-736.
- Parker, C. J., Stone, O. L., White, V. F., and Bernshaw, N. J. (1989) Vitronectin (S protein) is associated with platelets. *Br. J. Haematol.* **71**, 245-252.
- Peerschke, E. I. B., Zucker, M. B., Grant, R. A., Egan J. J., and Johnson, M. M. (1980) Correlation between fibrinogen binding to platelets and platelet aggregability. *Blood* **55**, 841-847.
- Peerschke, E. I. B., and Ghrebrehwet, B. (1988) Identification and partial characterization of human C1q-binding sites. *J. Immunol.* **141**, 3505-3511.
- Peerschke, E. I. B. (1992) Recognition of platelet-associated fibrinogen by polyclonal antibodies: correlation with platelet aggregation. *Blood* **79**, 2028-2033.
- Peerschke, E. I. B., Reid, K. B. M., Ghebrehwet, B. (1994) Identification of a novel 33-kDa C1q-binding site on human blood platelets. *J. Immunol.* **152**, 5896-5901.
- Perret, B. A., Furlan, M., and Beck, E. A. (1979) Studies on factor VIII-related protein. Estimation of molecular size differences between factor VIII oligomers. *Biochem. Biophys. Acta* **578**, 164-174.
- Petry, F., Reid, K. B. M., and Loos, M. (1991) Gene expression of the A- and B-chain of mouse C1q in different tissues and the characterization of the recombinant A-chain. *J. Immunol.* **147**, 3988-3993

- Petry, F., Reid, K. B. M., and Loos, M. (1992) Isolation, sequence analysis and characterization of the cDNA clones coding for the C chain of mouse C1q. *Eur. J. Biochem.* **209**, 129-134
- Phillips, D. R., Jenkins, C. S. P., Luscher, E. F., and Larrieu, M. J. (1975). Molecular differences of exposed surface proteins on thrombasthenic platelet plasma membranes. *Nature* **257**, 599-600.
- Phillips, D. R., and Poh Agin, P. (1977) Platelet plasma membrane glycoproteins. Evidence for the presence of nonequivalent disulfide bonds using nonreduced-reduced two-dimensional gel electrophoresis. *J. Biol. Chem.* **252**, 2121-2126.
- Phillips, D. R., Jennings, L. K., and Prasanna, H. R. (1980) Ca⁺⁺ mediated association of glycoprotein G (thrombin-sensitive protein, thrombospondin) with human platelets. *J. Biol. Chem.* **255**, 11629-11632.
- Phillips, D. R., Charo, I. F., Parise, L. V., and Fitzgerald, L. A. (1988) The platelet membrane glycoprotein IIb-IIIa complex. *Blood* **71**, 831-843.
- Podor, T. J., Weitz, J. I., Hirsh, J., Shaughnessy, S. G., Foulon, D. M., Butcher, M. K., Seiffert, D., and Loskutoff, D. J. (1995) Vitronectin-mediated binding of type 1 plasminogen activator inhibitor (PAI-1) to vimentin exposed in damaged endothelial cells. *Submitted for publication.*
- Preissner, K. T., Holzhuter, S., Justus, C., and Muller-Berghaus, G. (1989) Identification and partial characterization of platelet vitronectin: Evidence for complex formation with platelet-derived plasminogen activator inhibitor-1. *Blood* **74**, 1989-1996.
- Regan, J. W., Nakata, H., DeMarinis, R. M., Caron, M. G., and Lefkowitz, R. J. (1986) Purification and characterization of the human platelet α_2 -adrenergic receptor. *J. Biol. Chem.* **261**, 3894-3899.
- Reichenberger, E., Beier, F., LuValle, P., Olsen, B. R., von der Mark, K., Bertling, W. M. (1992). Genomic organization and full-length cDNA sequence of human collagen X. *FEBS Lett.* **311**, 305-310

- Reid, K. B. M. (1979) Complete amino acid sequences of the three collagen-like regions present in subcomponent C1q of the first component of human complement. *Biochem. J.* **179**, 367-371
- Reid K. B. M., Gagnon, J., and Frampton, J. (1982) Completion of the amino acid sequences of the A and B chains of subcomponent C1q of the first component of human complement. *Biochem. J.* **203**, 559-569
- Reid, K. B. M. (1985) Molecular cloning and characterization of the complementary DNA and gene coding for the B-chain of subcomponent C1q of the human complement system. *Biochem. J.* **231**, 729-735
- Reid, K. B. M. (1990) Ig-binding domains of C1q. *Immunol. Today.* **11**, 387-388.
- Reinders, J. H., de Groot, P. G., Dawes, J., Hunter, N. R., van Heugten, H. A. A., Zanbergen, J., Gonsalves, M. D., and van Mourik, J. A. (1985) Comparison of secretion and subcellular localization of von Willebrand protein with that of thrombospondin and fibronectin in cultured human vascular endothelial cells. *Biochem. Biophys. Acta* **844**, 306-313.
- Rosenfeld, I. I. Looney R. J., Leddy, J. P., Phipps, D. C. Abraham, G. N., and Anderson, C. L. (1985) Human platelet Fc receptor for immunoglobulin G. *J. Clin. Invest.* **76**, 2317-2324.
- Rucinski, B., Niewiarowski, S., James, P., Walz, D. A., and Budzynski, A. Z. (1979) Anti-heparin proteins secreted by human platelets: Purification and characterization and radioimmunoassay. *Blood* **53**, 47-62.
- Ruggeri, Z. M., and Zimmerman, T. S. (1980) Variant von Willebrand's disease. Characterization of two subtypes by analysis of multimeric composition of factor VIII/von Willebrand factor in plasma and platelets. *J. Clin. Invest.* **65**, 1318-1325.
- Ruggeri, Z. M., Bader, R., and DeMarco, L. (1982) Glanzmann thrombasthenia: Deficient binding of von Willebrand factor to thrombin stimulated platelets. *Proc. Natl. Acad. Sci.* **79**, 6038-6041.

- Ruggeri, Z. M., DeMarco, L., Gatti, L., Bader, R., and Montgomery, R. R. (1983) Platelets have more than one binding site for von Willebrand factor. *J. Clin. Invest.* **72**, 1-12.
- Ruggeri, Z. M. (1993) Mechanisms of shear-induced platelet aggregation and adhesion. *Thromb. Haemost.* **70**, 119-123.
- Ruoslahti, E., and Pierschbacher, M. D. (1986) Arg-Gly-Asp: A versatile cell recognition signal. *Cell* **44**, 517-518
- Ryo, R., Nakeff, A., Shian Huang, S., Ginsberg, M., and Deuel, T. F. (1983) New synthesis of a platelet-specific protein: platelet factor 4 synthesis in a megakaryocyte-enriched rabbit bone marrow culture system. *J. Cell Biol.* **96**, 515-520.
- Saez, L., and Leinwand, L. A. (1986) Characterization of diverse forms of myosin heavy chain expressed in adult human skeletal muscle. *Nucleic Acids Res.* **14**, 2951-2969
- Saez, C. G., Myers, J. C., Shows, T. B., and Leinwand, L. A. (1990) Human nonmuscle myosin heavy chain mRNA: Generation of diversity through alternative polyadenylation. *Proc. Natl. Acad. Sci.* **87**, 1164-1168
- Sakariassen, K. S., Fressinaud, E., Girma, J. P., Meyer, D., Baumgartner, H. R. (1987) The role of platelet membrane glycoproteins and von Willebrand factor in adhesion of platelets to subendothelium and collagen. *Ann. N. Y. Acad. Sci.* **516**, 52-65.
- Sawada, H., Konomi, H., and Hirose, K. (1990) Characterization of the collagen in the hexagonal lattice of Descemet's membrane: Its relation to type VIII collagen. *J. Cell Biol.* **110**, 219-227
- Schleef, R. R., Loskutoff, D. J. and Podor, T. J. (1991) Immunoelectron microscopic localization of type 1 plasminogen activator and inhibitor on the surface of activated endothelial cells. *J. Cell Biol.* **113**, 1413-1423.
- Schmaier, A. H., Smith, P. M., and Colman, R. W. (1984) Platelet C1 inhibitor. A secreted granule protein. *J. Clin. Invest.* **75**, 242-250.

- Schmaier, A. H., Smith, P. M., Purdon, D., White, J. G., and Colman, R. W. (1986) High molecular weight kininogen: Localization in the unstimulated and activated platelet and activation by a platelet calpain(s). *Blood* **67**, 119-130.
- Schwarz, H. P., Heeb, M. J., Wencel Drake, J. D. and Griffin, J. H. (1985) Identification and quantitation of protein S in human platelets. *Blood* **66**, 1452-1455.
- Seelig, H. P., Schranz, P., Schroeter, H., Wiemann, C., and Renz, M. (1994) Macrogolgin - a new 376 kD Golgi complex outer membrane protein as a target of antibodies in patients with rheumatic disease and HIV infection. *J. Autoimm.* **7**, 67-91
- Sellar, G. C., Blake, D. J., and Reid, K. B. M., (1991). Characterization and organization of the genes encoding the A-, B- and C-chains of human complement subcomponent C1q. *Biochem. J.* **274**, 481-490
- Shattil, S. J., Hoxie, J. A., Cunningham, M., and Brass, L. F. (1985) Changes in the platelet membrane glycoprotein IIb/IIIa complex during platelet activation. *J. Biol. Chem.* **260**, 11107-11114.
- Shattil, S. J. (1993) Regulation of platelet anchorage and signaling by integrin $\alpha_{IIb}\beta_3$. *Thomb. Haemost.* **70**, 224-228.
- Shimomura, T., Fujimura, K, Maehama, S., Takemoto, M., Oda, K., Fujimoto, T., Oyama, R., Suzuki, M., Ichihara-Tanaka, K., Titani, K., Kuramoto, A. (1990) Rapid purification and characterization of human platelet glycoprotein V: The amino acid sequence contains leucine-rich repetitive modules as in glycoprotein Ib. *Blood* **75**, 2349-2356.
- Sims, P. J., Ginsberg, M. H., Plow, E. H., and Shattil, S. J. (1991) Effect of platelet activation on the conformation of the plasma membrane glycoprotein IIb-IIIa complex. *J. Biol. Chem.* **266**, 7345-7352.
- Sporn, L. A., Marder, V. J., and Wagner, D. D. (1986) Inducible secretion of large, biologically potent von Willebrand factor multimers. *Cell* **46**, 185-190.

- Stenner, D. D., Tracy, R. P., Riggs, B. L., and Mann, K. G. (1986) Human platelets contain and secrete osteonectin, a major protein of mineralized bone. *Proc. Natl. Acad. Sci.* **83**, 6892-6896.
- Stuart, S. G., Trounstein, M. L., Vaux, D. J. T., Koch, T., Martens, C. L., Mellman, I., and Moore, K. W. (1987) Isolation and expression of cDNA clones encoding a human receptor for IgG (Fc γ RII). *J. Exp. Med.* **166**, 1668-1684.
- Tandon, N. N., Kralisz, U., and Jamieson, G. A. (1989) Identification of glycoprotein IV (CD 36) as a primary receptor for platelet-collagen adhesion. *J. Biol. Chem.* **264**, 7576-7583.
- Thomas, J. T., Cresswell, C. J., Rash, B., Nicolai, H, Jones, T., Solomon, E., Grant M. E., and Boot-Handford, R. P. (1991) The human collagen X gene. Complete primary translated sequence and chromosomal localization. *Biochem. J.* **280**, 617-623
- Titani, K., Kumar, S., Takio, K., Ericsson, L. H., Wade, R. D., Ashida, K., Walsh, K. A., Chopek, M. W., Sadler, J. E., and Fujikawa, K. (1986) Amino acid sequence of human von Willebrand factor. *Biochem.* **25**, 3171-3184.
- Tracy, P. B., Eide, L. L., and Mann, K. G. (1985) Human prothrombinase complex assembly and function on isolated perispherical blood cell populations. *J. Biol. Chem.* **260**, 2119-2124.
- Tuszynski, G. P., Rothman, V. L., Murphy, A., Siegler, K., and Knudsen, K. A. (1988). Thrombospondin promotes platelet aggregation. *Blood* **72**, 109-115.
- Ueda, H., Doi, Y., Sakamoto, Y., Hamasaki, K., and Fujimoto, S. (1992) Simultaneous localization of histamine and Factor VIII-related antigen in the endothelium of the human umbilical vein. *Anat. Record* **232**, 257-261.
- Van Nostrand, W. E., Schmaier, A. H., Farrow, J. S., Cines, D. B., and Cunningham D. (1991) Protease nexin-2/amyloid beta-protein precursor in blood is a platelet-specific protein. *Biochem. Biophys. Res. Commun.* **175**, 15-21.

- Verweij, C. L., Hart, M., and Pannekoek, H. (1987) Expression of variant von Willebrand factor (vWf) cDNA in heterologous cells: requirement of the propolypeptide on vWf multimer formation. *EMBO J.* **6**, 2885-2890.
- Voorberg, J., Fontijn, R., Calafat, J., Janssen H., van Mourik, J. A. and Pannekoek, H. (1993) Biogenesis of von Willebrand factor-containing organelles in heterologous transfected CV-1 cells. *EMBO J.* **12**, 749-758.
- Vischer, U. M., and Wagner, D. D. (1993) CD63 is a component of Weibel-Palade bodies of human endothelial cells. *Blood* **82**, 1184-1191.
- Vischer, U. M., and Wagner, D. D. (1994) von Willebrand factor proteolytic processing and multimerization precede the formation of Weibel-Palade bodies. *Blood* **83**, 3536-3544.
- Vu, T.-K. H., Hung, D. T. Wheaton, V. I., and Coughlin, S. R. (1991). Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell* **64**, 1057-1068.
- Wagner, D. D., Olmsted, J. B., and Marder, V. J. (1982) Immunolocalization of von Willebrand protein in Weibel-Palade bodies of human endothelial cells. *J. Cell Biol.* **95**, 355-60.
- Wagner, D. D. and Marder, V. J. (1983) Biosynthesis of von Willebrand factor protein by human endothelial cells. Identification of a larger precursor polypeptide chain. *J. Biol. Chem.* **258**, 2065-2067.
- Wagner, D. D., and Marder, V. (1984) Biosynthesis of von Willebrand protein by human endothelial cells: processing steps and their intracellular localization. *J. Cell. Biol.* **99**, 2123-2130.
- Wagner, D. D., Fay P. J., Sporn, L. A., Sinha, S., Lawrence, S. O., and Marder, V. J. (1987) Divergent fates of von Willebrand factor and its propolypeptide (von Willebrand antigen II) after secretion from endothelial cells. *Proc. Natl. Acad. Sci.* **84**, 1955-1959.
- Wagner, D. D., Saffaripour, S., Bonfanti, R., Sadler, J. E., Cramer, E. M., Chapman, B., and Mayadas, T. N. (1991) Induction of specific storage organelles by von Willebrand factor propolypeptide. *Cell* **64**, 403-413.

- Wagner, D. D. (1993) The Weibel-Palade body: The storage organelle for von Willebrand factor and P-selectin. *Thromb. Haemost.* **70**, 105-110.
- Weibel, E. R., and Palade, G. E. (1964) New cytoplasmic components in arterial endothelia. *J. Cell. Biol.* **23**, 101-112.
- Wharton, K. A., Johansen, K. M., Xu, T., and Artavanis-Tsakonas. (1985) Nucleotide sequence from the neurogenic locus Notch implies a gene product that shares homology with proteins containing EGF-like repeats. *Cell* **43**, 567-581
- Wilbourne, B. R., Harrison, P., Lawrie, A. S., Savariau, E., Savidge, G. F., and Cramer, E. M. (1993) Porcine platelets contain an increased quantity of ultra-high molecular weight von Willebrand factor. *Br. J. Haemat.* **83**, 608-615.
- Williams, S. B., McKeown, L. P., Krutzch, H, Hansmann, K., Gralnick, H. R. (1994) Purification and characterization of human platelet von Willebrand factor. *Br. J. Haematol.* **88**, 582-591.
- Wise, R. J., Pittman, D. D., Handin, R. I., Kaufman, R. J., and Orkin, S. H. (1988) The propeptide of von Willebrand factor independently mediates the assembly of von Willebrand multimers. *Cell* **52**, 229-236.
- Zucker, M. B., Mosesson, M. W., Broekman, M. J., and Kaplan, K. L. Release of platelet fibronectin (cold-insoluble globulin) from alpha granules induced by thrombin or collagen; lack of requirement for plasma fibronectin in ADP-induced platelet aggregation. *Blood* **54**, 8-12.

p-155, a Multimeric Platelet Protein That Is Expressed on Activated Platelets*

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Platelets respond to a large number of stimuli by undergoing complex biochemical and morphological changes. These changes are involved in physiological processes including adhesion, aggregation, and coagulation. Platelet activation produces membrane alterations that can be recognized by monoclonal antibodies. In this report we describe a novel activation-dependent protein recognized by a monoclonal antibody, JS-1. The platelet glycoprotein was designated p-155 according to its apparent reduced molecular weight. p-155 exists in the native state as varying sized, large multimers held together by disulfide bonds. p-155 is released upon platelet activation and binds to the activated platelet surface. Although p-155 and platelet glycoprotein Ia migrate similarly on reduced sodium dodecyl sulfate-polyacrylamide gel electrophoresis, immunodepletion and isoelectric focusing distinguished p-155 from glycoprotein Ia. p-155 differed from von Willebrand factor and from thrombospondin in its reduced molecular weight. Additionally, immunoblotting of immunoprecipitated p-155 with antisera to von Willebrand factor and to thrombospondin confirmed the unique identity of p-155. Evidence for a soluble, nonintegral membrane-associated protein was obtained by Triton X-114 phase separation studies, membrane elution studies, and by the demonstration of the protein in the aqueous phase of platelet releasate. Both radioimmunoprecipitation and direct binding techniques demonstrated the activation-dependent nature of p-155. The protein could not be detected in other blood cells, endothelial cells, HEL cells, liver, or in plasma. The functional role of p-155 in platelets is not yet known.

A variety of physiological and nonphysiological stimuli can initiate platelet activation. Platelet activation is associated with membrane alterations that allow platelets to bind to each other and to the vessel wall. The activation-induced alterations in platelet proteins can be categorized into three major groups: 1) conformational alterations in glycoproteins present on both resting and activated platelets (1), 2) exposure of activation-associated integral membrane glycoproteins by the fusion of granule membranes with the surface membrane (2),

and 3) the surface binding of glycoproteins released from granule stores with activation (3-7).

In this report, we describe a novel activation-dependent, multimeric platelet glycoprotein (M_r 155,000, reduced) that is present in platelet releasate and also becomes associated with the surface of platelets upon activation.

MATERIALS AND METHODS

Preparation of Monoclonal Antibodies—The murine monoclonal antibody JS-1 was raised by immunizing mice with human platelets. BALB/c mice were given three intraperitoneal injections of 5×10^8 platelets 3 weeks apart. Spleen cells were harvested 3 days after the last injection. Fusions of spleen cells with SP2/0 myeloma cells were performed using the method of Galfré *et al.* (8). Culture supernatants were screened for platelet reactivity using an enzyme-linked immunoassay. Positive cultures were cloned twice by limiting dilution. Hybridomas were grown as ascites using pristane-primed BALB/c mice.

Murine monoclonal antibody Raj-1 (anti-GP I_b/IIIa) was raised by the same method. Specificity for GP I_b/IIIa was confirmed by radioimmunoprecipitation. No reactivity was seen by immunoprecipitation using platelets from a patient with Glanzmann's thrombasthenia lacking GP I_b/IIIa.

Monoclonal antibody CH-1 (anti-thrombospondin) was raised by immunizing mice with purified thrombospondin. Thrombospondin was purified as described (9). BALB/c mice were given three intraperitoneal injections of 25 mg 3 weeks apart. Culture supernatants were screened for reactivity against thrombospondin using an enzyme-linked immunoassay.

Monoclonal antibodies were purified from ascites fluid by protein A-Sepharose chromatography (Pharmacia LKB, Uppsala, Sweden). Unbound proteins were eluted with phosphate-buffered saline (PBS), pH 7.8, and bound IgG was eluted with 0.1 M glycine, pH 3.0. Purity exceeded 95% by both sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and HPLC analyses.

F(ab')₂ fragments were prepared by pepsin digestion at 37 °C in 0.1 M acetate buffer, pH 4.0, using a 2% weight equivalent of pepsin. Digestion was monitored by HPLC using a TSK 250 gel filtration column (Bio-Rad). F(ab')₂ fragments were separated from intact IgG and Fc fragments using Protein A chromatography. The final preparation of F(ab')₂ was demonstrated to be free of intact IgG and greater than 95% purity by SDS-PAGE and HPLC analyses.

Preparation of Resting Platelets—Blood from aspirin-free healthy volunteers was collected into acid citrate dextrose (ACD) (1:6, v/v). For experiments measuring p-155 on resting platelets, blood was collected into ACD containing PGE₁ (3.0 μM) and theophylline (1 mM). Platelet-rich plasma was harvested following centrifugation (100 × g for 15 min, 22 °C). Platelets were gel-filtered in calcium-free Tyrode's buffer, pH 7.4, containing 0.35 g/100 ml bovine serum albumin, PGE₁ (0.3 μM), and theophylline (1 mM). The platelets were used for radiolabeling or direct binding. For immunoblotting, gel-filtered platelets were pelleted and resuspended in lysing buffer con-

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The abbreviations used are: GP, glycoprotein; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography; ACD, acid citrate dextrose; PGE₁, prostaglandin E₁; 2-ME, 2-mercaptoethanol; DTT, dithiothreitol; HEL, human erythroid leukemia.

taining 1% Triton X-100, 20 mM Tris, 100 mM NaCl, pH 7.4, with 6.7 mM EDTA, 0.1 μ M leupeptin, 0.2 mM phenylmethylsulfonyl fluoride, 0.02 mg/liter soybean trypsin inhibitor, and 5 mM *N*-ethylmaleimide.

Preparation of Activated Platelets—For direct comparison with resting platelets, activated platelets were prepared as follows. Platelets collected without inhibitors were gel-filtered and 1×10^9 /ml were stimulated with 1 unit/ml bovine thrombin (Sigma). Ten minutes after the addition of thrombin, recombinant hirudin (Sigma), 4 units/ml, was added.

The platelet suspension was used for direct binding studies or washed twice in ACD/PBS, pH 6.2, before radiolabeling. For other experiments, platelets were obtained by differential centrifugation (1800 $\times g$ for 10 min, 22 °C), washed 3 times in ACD/PBS, then activated with thrombin.

Preparation of Platelet Release—Test platelets were washed 3 times in calcium-free Tyrode's solution, pH 6.2, and then resuspended in calcium-free Tyrode's, pH 7.4, at a count of 1×10^9 /ml. One aliquot was treated with thrombin, 6 units/ml for 10 min, and then hirudin, 24 units/ml, was added. The release fraction was collected after centrifugation (2000 $\times g$ for 10 min). Release fraction was ultracentrifuged (100,000 $\times g$ for 60 min, 4 °C) to remove platelet microparticles.

The cell pellets from the untreated and thrombin-stimulated platelets were solubilized in 1% SDS at a concentration of 10^9 platelets/ml. Samples were then analyzed using SDS-PAGE and immunoblotting.

Radioimmunoprecipitation—Platelets were radiolabeled with [¹²⁵I] sodium iodide using lactoperoxidase as described (10). Labeled platelets (10^9 /ml) were solubilized using 1% Triton X-100 in lysing buffer. Following lysis for 1 h at 4 °C, the samples were centrifuged (30,000 $\times g$ for 30 min, 4 °C) to remove the Triton-insoluble material. Immunoprecipitations of 1-ml volumes of radiolabeled lysates were performed using 50 μ l of Protein A-Sepharose CL-4B beads (1 Pharmacia) and 10 μ l of JS-1 ascites fluid.

Other antibodies used for radioimmunoprecipitation studies included Raj-1 (anti-GP IIb-IIIa), CH-1 (anti-thrombospondin), 12F1 (anti-GP Ia-IIa, a gift from Dr. V. Woods, San Diego, CA), and S-12 (anti-GMP-140, a gift from Dr. R. McEver, Oklahoma City, OK). After incubation at 22 °C for 1 h, beads were washed 5 times with lysing buffer containing 0.2% Triton X-100 and eluted with 100 μ l of 2 \times sample buffer (0.125 M Tris, 0.1% glycerol, 2% SDS, pH 6.8) and analyzed using SDS-PAGE. Sample buffer containing 2-mercaptoethanol (2-ME, 5% final) was used for analysis under reducing conditions. For some experiments, radiolabeled lysate from a patient with Glanzmann's thrombasthenia was used.

Immunoblotting—Platelet lysates were prepared similar to the method used for radioimmunoprecipitation. For investigation of the native structure of the protein, platelets were collected and gel-filtered with metabolic inhibitors and pelleted by centrifugation (1800 $\times g$ for 10 min, 22 °C) before solubilization in lysing buffer containing 1% Triton X-100.

Immunoblot studies were performed as described (11) except that 50- μ l aliquots of platelet lysate (10^9 /ml) were used. The nitrocellulose was blocked with 3% bovine serum albumin in PBS, pH 7.4, before incubation with antibodies, diluted in 1% bovine serum albumin/PBS with 0.1% Tween 20. The monoclonal antibody bound to the transblotted platelet proteins was detected using a 1:750 dilution of alkaline phosphatase-conjugated goat anti-mouse antibody (Jackson ImmunoResearch, BioCan Scientific Inc., Mississauga, Ontario, Canada). For studies comparing p-155 with von Willebrand factor, rabbit anti-von Willebrand factor (Dako Corp., Carpinteria, CA) was used.

For experiments using 3-6% nonreduced SDS-PAGE, gels were incubated in transfer buffer containing 5% 2-ME for 5 min to facilitate transfer of high molecular weight proteins.

Isoelectric Focusing—Isoelectric focusing (reduced) followed by SDS-PAGE was performed using the method of O'Farrell (12). Radioimmunoprecipitated samples were prepared using monoclonal antibodies 12F1 and JS-1 with thrombin-activated platelet lysate. The beads were washed once with 0.015 M sodium chloride containing 0.2% Triton X-100 before elution using 70 μ l of isoelectric focusing sample lysis buffer (9.5 M urea, 2% Triton X-100, 2% Ampholines, 5% 2-ME). LKB Ampholines (Pharmacia LKB) used were comprised of 1.6% pH range 5-7 and 0.4% pH range 3.5-10. A 20- μ l aliquot of the eluted sample was used for isoelectric focusing.

Investigation of the Nonreduced Structure of p-155—Immunoprecipitated samples (20 μ l), solubilized in nonreducing sample buffer, were separated using SDS-PAGE tube gels with a 3% stacking gel and 7.5% resolving gel. The tube gels were incubated for 30 min in

sample buffer containing 5% 2-ME and subjected to a second electrophoresis on 4-8% gradient SDS-PAGE. For some experiments, unlabeled platelet lysate was used, and nitrocellulose transfer and immunoblotting were performed following the second dimension separation. For other studies, radiolabeled platelet lysate from thrombin-activated platelets (15 μ l) was mixed with unlabeled platelet lysate (35 μ l), and the nitrocellulose was autoradiographed after immunoblotting with JS-1.

Effect of Reduction on p-155—3-6% and 3-12% gradient SDS-PAGE were used to assess the effect of increasing reduction on the mobility of p-155. Radioimmunoprecipitates were eluted with sample buffer containing increasing concentrations of dithiothreitol (DTT, 0, 0.1, 0.5, 1, 2, 5, 10, 25, 50, and 100 mM final). Following separation on SDS-PAGE, the bands were localized by autoradiography, cut from the dried gel, redissolved in reducing sample buffer, and subjected to a second separation on 3-6% SDS-PAGE. In separate experiments, aliquots of unlabeled Triton X-100 platelet lysate or cytoskeleton (Triton X-100-insoluble material dissolved in lysing buffer containing 1% SDS) were treated by identical DTT reduction followed by SDS-PAGE, nitrocellulose transfer, and immunoblotting.

The effect of alkylation on the migration of reduced p-155 was investigated. Immunoprecipitated p-155 was reduced with 10 mM DTT, treated with iodoacetamide (20 mM, final) followed by SDS-PAGE.

Phase Partitioning Using Triton X-114—Triton X-114 phase separation was used to separate hydrophilic platelet proteins from integral membrane proteins (13). Lysates (1% Triton X-114, 4 °C) were prepared for radioimmunoprecipitation and immunoblotting. Samples were centrifuged (30,000 $\times g$ for 30 min, 4 °C) to remove cytoskeleton, and the lysate was incubated at 37 °C for 15 min to allow phase partitioning. The lysate was centrifuged (110 $\times g$ for 10 min, 24 °C) and the aqueous and detergent phases collected. The detergent phase was diluted to a volume equal to the aqueous phase with lysing buffer containing 0.1% Triton X-100. Detergent and aqueous phases were analyzed by radioimmunoprecipitation or immunoblotting using JS-1.

Studies Investigating Whether p-155 Is an Integral or Peripheral Protein—To determine whether p-155 was a soluble, membrane-associated protein or an integral membrane protein expressed with activation (14), thrombin-activated platelets were prepared, radiolabeled, and then washed twice in PBS with inhibitors (6.7 mM EDTA, 1 mM theophylline, 3 μ M PGE₁, 5 mM *N*-ethylmaleimide, 0.2 mM phenylmethylsulfonyl fluoride, and 5 mM NaI). Aliquots of labeled platelets (1×10^9 /ml) were incubated in different eluting buffers (37 °C for 45 min). Eluting solutions tested included PBS (with inhibitors) and PBS to which either 1% Triton X-100, 3 M sodium chloride, or 1 M urea had been added. The samples were then centrifuged (2000 $\times g$ for 10 min), and the eluates were collected and dialyzed overnight against PBS containing 6.7 mM EDTA, 5 mM *N*-ethylmaleimide, 0.2 mM phenylmethylsulfonyl fluoride, and 5 mM NaI. Dialyzed eluates were subjected to immunoprecipitation using JS-1 or Raj-1 and analyzed by 3-6% gradient SDS-PAGE (reduced) and autoradiography.

Investigation of the Association of p-155 with the Cytoskeleton—Preliminary studies demonstrated that greater than 95% of the Triton-insoluble material in lysates was removed by centrifugation at 30,000 $\times g$ for 30 min at 4 °C. Investigations of the Triton X-114- or Triton X-100-insoluble fraction of platelet lysate were performed using immunoblotting. The pellet of Triton-insoluble material obtained by high speed centrifugation was resuspended in lysing buffer containing 2% SDS, sonicated for 3 min at maximum power using a Braun-Sonic 2000, and then analyzed by immunoblotting.

Binding Studies and Affinity Purification of p-155—The binding properties of the p-155 were studied using wheat germ lectin-Sepharose (Pharmacia) and heparin-Sepharose (Pharmacia). A 40% ammonium sulfate precipitate of Triton X-100 platelet lysate from 5×10^9 platelets was resuspended in 2 ml of lysing buffer, and 0.4-ml aliquots were incubated with 0.25-ml volumes of packed beads. Following a 1-h incubation at 22 °C, supernatants were collected, beads were washed 5 times with lysing buffer, and bound proteins were eluted using reducing sample buffer. Supernatants and eluates were analyzed by SDS-PAGE and immunoblotting.

Affinity purification of p-155 was performed using JS-1 linked to CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer's instructions. The aqueous phase of Triton X-114 lysate from 20 units of freshly outdated Red Cross platelets (7-10 days old) was used to affinity purify p-155. The bound protein was eluted with 3 M magnesium chloride and then dialyzed overnight against PBS

containing 5 mM *N*-ethylmaleimide, 10 mM iodoacetamide, and 0.2 mM phenylmethylsulfonyl fluoride.

Preparation of Polyclonal Antisera to p-155—Affinity-purified p-155 was subjected to reduced SDS-PAGE and transferred to nitrocellulose. A strip of nitrocellulose was used to localize p-155 by immunoblotting using JS-1. The corresponding region was cut from the nitrocellulose, dissolved in dimethyl sulfoxide, and used for immunization of rabbits as described (15).

The rabbit antisera was used for immunoblotting using an alkaline phosphatase-conjugated goat anti-rabbit IgG (Jackson Immuno-Research) for detection.

Direct Binding Studies—Binding studies were performed according to methods previously described (16) using ^{125}I -JS-1 F(ab')₂ and resting and activated platelets. Nonspecific binding was determined using a 50-fold excess of unlabeled JS-1 F(ab')₂. Dilutions of F(ab')₂ were prepared using calcium-free Tyrode's solution containing 0.35% bovine serum albumin. A 75- μl aliquot of platelet suspension was incubated with 25 μl of radiolabeled antibody and either 25 μl of unlabeled antibody or buffer. Following a 60-min incubation at 22 °C, 80 μl of the mixture (triplicate samples) was layered onto silicon oil, specific gravity 1.026 (Dow Corning), in an Eppendorf tube and centrifuged (12,000 $\times g$ for 3 min). The radioactivity of the pellet was determined.

Detection of p-155 in Other Cells—Human erythroid leukemia (HEL) cells, endothelial cells (and culture supernatant), and peripheral blood lymphocytes, granulocytes, monocytes, red cells, and plasma were tested by immunoblotting. HEL cells and endothelial cells were harvested from tissue culture flasks, washed twice in PBS, and lysed. Samples (10⁷ cells/ml) were analyzed by immunoblotting following separation using 7% SDS-PAGE (reduced).

Lymphocytes were harvested from heparinized venous blood (diluted 1:1 with PBS), layered onto Ficoll-Paque (Pharmacia LKB), and centrifuged (550 $\times g$ for 20 min, 22 °C). The cell layer from the interface was washed with PBS. The cell pellet was resuspended in 2 ml of RPMI-1640 (Gibco, Burlington, Ontario, Canada), treated with thrombin (100 units/ml), and centrifuged (1000 $\times g$ for 3 s) to remove platelet clumps. The lymphocytes were resuspended in fresh RPMI for counting, pelleted, and lysed.

Monocytes were prepared from the cell interface layer from the Ficoll separation. The cell suspension was washed once with PBS, resuspended in PBS, layered on 1.5 volumes of isotonic Percoll solution (Pharmacia Canada Ltd., Dorval, Canada), and centrifuged (1500 $\times g$ for 20 min, 22 °C). The monocytes were washed once with PBS and resuspended in 1 ml of RPMI. The cell suspension was then treated with 80 μM ADP, centrifuged (1000 $\times g$ for 3 s) to remove platelet clumps, washed once in RPMI, counted, and lysed.

Granulocytes were prepared by layering 2 ml of 6% Dextran 500 (Pharmacia) in Isoton (Coulter Electronic of Canada Ltd., Burlington, Ontario, Canada) onto 10 ml of heparinized blood. The cells were allowed to sediment for 1 h at 22 °C. The top layer was diluted 1:1 with PBS and layered over 5 ml of Ficoll-Paque. Following centrifugation (550 $\times g$ for 20 min, 22 °C), the mononuclear layer at the interface was discarded, and the pellet containing granulocytes and red cells was removed. The red cells were lysed, and the remaining cell pellet was washed twice, resuspended in RPMI, counted, and lysed.

Red cells were obtained by centrifuging blood collected in ACD, following removal of the platelet-rich plasma, at 1800 $\times g$ for 10 min. The red cells were washed twice in PBS, counted, and lysed.

Liver lysate was prepared by homogenizing 25 g of liver in 100 ml of lysing buffer at high speed for 10 min using a Brinkmann Polytron homogenizer. Triton X-100 was added to a final concentration of 1%. The sample was then processed following the method for platelet lysate preparation.

Additionally, HEL cells, endothelial cells, and phytohemagglutinin-activated T-cells were radiolabeled as described for platelets. Activated T-cells were prepared using the methods described by Brashem-Stein *et al.* (17). Lysates were immunoprecipitated with JS-1 and analyzed by SDS-PAGE.

RESULTS

Identification of the Platelet Protein Recognized by JS-1—Radioimmunoprecipitation studies using JS-1 and radiolabeled thrombin-activated platelets revealed two distinct bands on reduced gels with a major band at 155 kDa and a minor band at 170 kDa (Fig. 1). Alkylation using 20 mM iodoaceta-

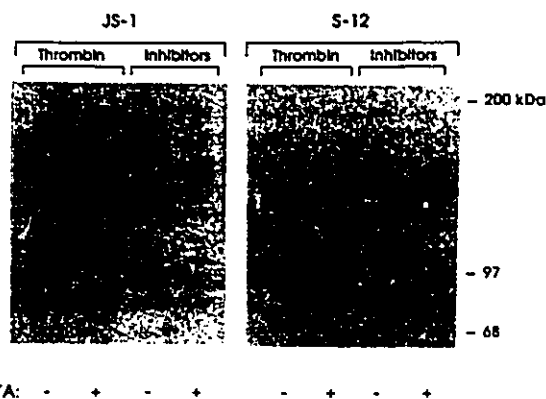


FIG. 1. Effect of platelet activation on p-155 and GMP-140 expression. This figure shows radioimmunoprecipitation studies using monoclonal antibodies JS-1 (anti-p-155) and S-12 (anti-GMP-140) and radiolabeled platelet lysates from thrombin-activated platelets and resting platelets (designated as inhibitors). EDTA had no effect on the expression of GMP-140 or p-155.

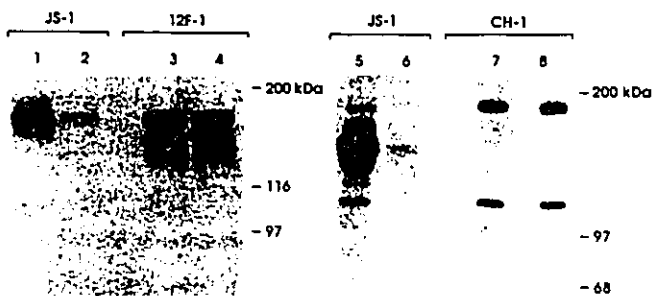


FIG. 2. Effect of p-155 immunodepletion on GP Ia-IIa and thrombospondin. The autoradiogram shows the effect of serial immunodepletions using lysate from radiolabeled thrombin-activated platelets. The immunodepletion was performed using JS-1 (lanes 2, 4, 6, and 8) or sham Protein A-Sepharose beads (lanes 1, 3, 5, and 7). Following immunodepletion, the supernatants were immunoprecipitated with either JS-1 (lanes 1, 2, 5, and 6), 12F1 (lanes 3 and 4) or CH-1 (lanes 7 and 8). 12F1 immunoprecipitates glycoproteins Ia (155 kDa) and IIa (135 kDa). CH-1 immunoprecipitates thrombospondin. This study indicates that p-155 is not Ia or thrombospondin.

mid following reduction did not alter the quantities of the 155- and 170-kDa proteins. The band at 82 kDa in Fig. 1 was not seen consistently with p-155 immunoprecipitates. Immunoblotting of reduced platelet lysate (collected and processed with metabolic and proteolytic inhibitors) gave the same findings as radioimmunoprecipitation with recognition of both the 170- and 155-kDa proteins by monoclonal antibody JS-1 (Fig. 4). Identical bands were observed in samples collected with and without metabolic inhibitors and with and without EDTA.

Because the 155-kDa subunit of p-155 migrated similarly to glycoprotein Ia on reduced SDS-PAGE, further studies were done to confirm the unique identity of p-155. Immunodepletion of radiolabeled lysate with JS-1 did not affect the amount of glycoprotein Ia detected by monoclonal antibody 12F1, which reacts with the glycoprotein Ia-IIa complex (18) (Fig. 2). Monoclonal antibody 12F1 but not JS-1 co-precipitated glycoprotein IIa. Additionally, JS-1 immunodepletion did not affect the amount of thrombospondin detected by radioimmunoprecipitation with CH-1 (a monoclonal antibody against thrombospondin) (Fig. 2).

Isoelectric focusing, performed under reducing conditions, confirmed that p-155 was distinct from Ia. The isoelectric focusing using 12F1 gave a pI of 5.8 for glycoprotein Ia with a pI of 5.25 for glycoprotein IIa (Fig. 3B). In contrast, p-155

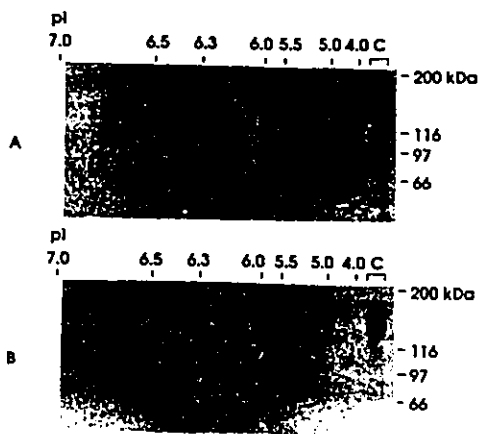


FIG. 3. Two-dimensional isoelectric focusing/SDS-PAGE of p-155 and glycoprotein Ia. Immunoprecipitates of platelet lysate from radiolabeled thrombin-activated platelets were prepared. In panel A, monoclonal antibody JS-1 and in panel B, 12F1 was used for the immunoprecipitation. The precipitated proteins were analyzed by isoelectric focusing followed by SDS-PAGE. Lane C is a control immunoprecipitated sample subjected to SDS-PAGE only.

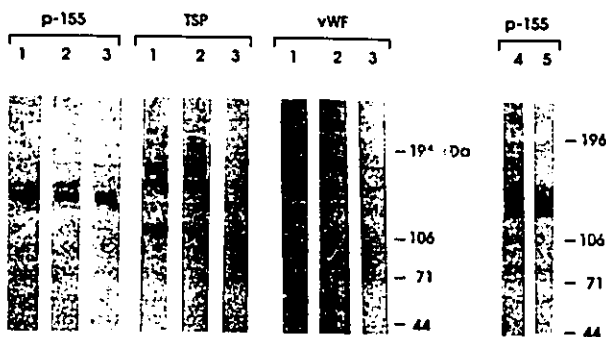


FIG. 4. Comparison of the reduced subunits of p-155, thrombospondin (TSP), and von Willebrand factor (vWF) by immunoblotting. The immunoblot demonstrates the different mobilities of p-155, thrombospondin, and von Willebrand factor. Samples were subjected to SDS-PAGE (reduced) followed by transfer to nitrocellulose and immunoblotting with JS-1 (anti-p-155), CH-1 (anti-thrombospondin), and anti-von Willebrand factor. Samples included resting platelet lysate (1% Triton X-100) (lane 1), Triton X-114 platelet lysate, aqueous phase (lane 2), and affinity-purified p-155 (lane 3). In lanes 4 and 5, the results of immunoblotting platelet lysate with JS-1 (lane 4) and polyclonal anti-p-155 (lane 5) are shown. This figure demonstrates that p-155 is not thrombospondin or von Willebrand factor.

exhibited a broad band extending from a pI of 5.4 to 6.75 with the major portion of the radiolabeled material between 6.5 and 6.7 (Fig. 3A). A slightly more acidic broad based band was seen at 170 kDa with an additional band at 150 kDa that had a pI of 6.6–6.75.

Immunoblotting distinguished p-155 from thrombospondin and von Willebrand factor, two other large, multimeric disulfide-linked platelet proteins. Thrombospondin and von Willebrand factor differed from p-155 in their reduced subunit mobility. Additionally, immunoprecipitated p-155 was not recognized by antibodies to von Willebrand factor or to thrombospondin (Fig. 4), further confirming the unique identity of this platelet protein. Immunoblotting using polyclonal antibodies to p-155 identified the same bands as JS-1 (Fig. 4). These findings demonstrate that JS-1 recognizes a unique platelet glycoprotein and not a degradation product of either thrombospondin or von Willebrand factor.

Under nonreducing conditions, p-155 exhibited markedly different migration characteristics from the reduced protein.

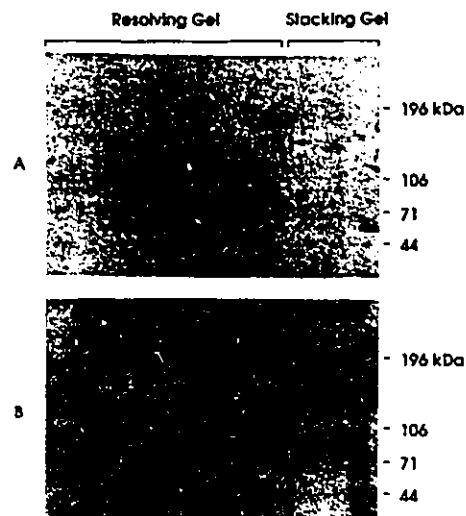


FIG. 5. Two-dimensional SDS-PAGE (nonreduced/reduced) of surface labeled, thrombin-activated platelets analyzed by immunoblotting and autoradiography. Radiolabeled thrombin-activated platelet lysate (1% Triton X-100) was mixed with unlabeled platelet lysate and then subjected to nonreducing SDS-PAGE. The tube gel was then reduced with 5% 2-mercaptoethanol and a second SDS-PAGE performed. After transfer to nitrocellulose, the proteins were analyzed by immunoblotting using JS-1 (panel A) and autoradiography (panel B). The positions of the first dimension stacking and resolving gels are shown across the top of the figure. The arrows indicate the position of p-155. p-155 analyzed by immunoprecipitation followed by reduced SDS-PAGE is shown to the right of panel B. This figure illustrates that p-155 is a minor component of the radiolabeled surface proteins.

Two-dimensional gel electrophoresis (nonreduced/reduced) demonstrated that in the nonreduced state, p-155 was too large to enter a 7.5% resolving gel and was retained in the stacking 3% gel (Fig. 5A). Following reduction, the protein migrated at 155 kDa. Using localization by immunoblotting with autoradiography, p-155 was demonstrated to be a minor component of the ^{125}I -labeled surface proteins on activated platelets (Fig. 5B). Two-dimensional SDS-PAGE using radioimmunoprecipitated p-155 gave similar findings with an additional minor band evident at 170 kDa following reduction.

Several bands in the high molecular weight region were detected using 3–6% and 3–12% gradient SDS-PAGE, when the immunoprecipitated p-155 was separated in the nonreduced state. Fig. 6A demonstrates the effect of increasing reduction on p-155. In a nonreduced state, radioactivity was present at the application site, within the stacking gel and in three separate bands in the upper region of the resolving gel. At low concentrations of DTT (0.1–0.5 mM), the lower band was more apparent. A smaller fourth band of M_r 255,000 was seen at DTT concentrations of 0.5–2.0 mM. With increasing reduction, more intense bands at 155 and 170 kDa were seen. The different molecular mass bands were cut from the gel and electrophoresed in the fully reduced state (Fig. 6B). All bands now migrated at 155 kDa. Faint bands were also evident at 170 kDa in lanes 4–8. The 155- and the 170-kDa bands migrated identically to their first separation when subjected to the second electrophoresis. Studies of platelet lysate reduced with increasing concentrations of DTT yielded identical intermediary bands (data not shown). Together, these data indicate that p-155 is an integral part of the high molecular weight components. The stepwise reduction pattern is consistent with disruption of interchain disulfide bonds.

Small amounts of radiolabeled material were evident below 155 kDa (Fig. 6A). Subsequent investigations using lysate precleared with Protein A beads did not demonstrate these

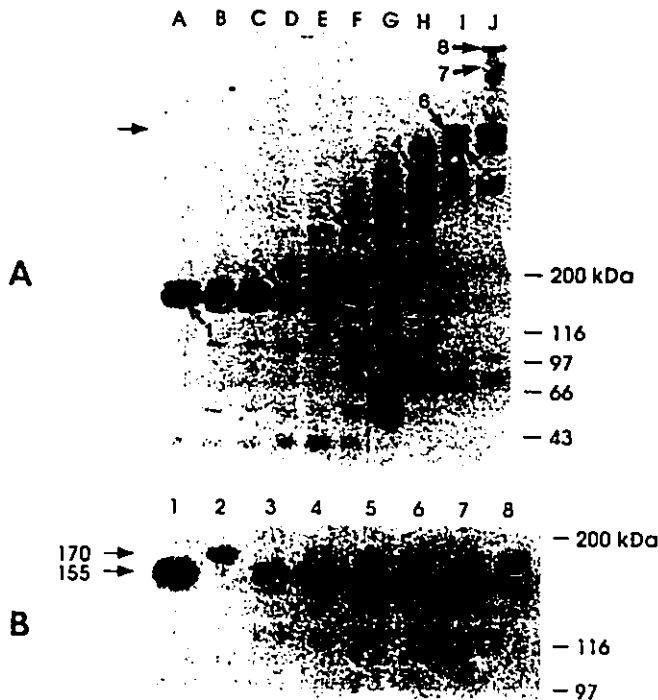


FIG. 6. The progressive reduction of p-155. This figure shows an autoradiograph produced by treating immunoprecipitated p-155 with increasing concentrations of DTT followed by 3–12% SDS-PAGE (panel A). The DTT concentrations (mM) used were: lane A, 100; lane B, 50; lane C, 25; lane D, 10; lane E, 5; lane F, 2; lane G, 1; lane H, 0.5; lane I, 0.1; lane J, 0. The arrow at the left side of the figure indicates the interface between the stacking and resolving gels. The bands labeled 1–8 were localized by autoradiography, cut from the gel, and electrophoresed under reducing conditions (panel B). Faint bands at 170 kDa were seen in lanes 4–8 but did not reproduce well in the photograph. This experiment illustrates that the native molecules are disulfide-linked multimers of varying size comprised of p-155 and p-170.

bands, suggesting nonspecific binding. As no other radiolabeled proteins were identified by analysis using either 3–6% SDS-PAGE or 3–12% SDS-PAGE, p-155 was demonstrated to contain only the 155- and 170-kDa subunits. Low molecular weight subunits less than 20 kDa or nonradiolabeled subunits cannot be excluded as components of the larger complexes. Without reduction, three different sized complexes were resolved by 3–12% SDS-PAGE, and additional material containing p-155 failed to enter the resolving gel. Using nonreduced 3–6% SDS-PAGE, thrombospondin migrated slightly slower than the smallest band in JS-1 immunoprecipitates. These data suggest that the native form of p-155 is a multimeric protein linked by disulfide bonds with variation in multimer size.

The glycoprotein nature of p-155 was confirmed by binding to wheat germ agglutinin. No binding to heparin-Sepharose was detected.

Effect of Platelet Activation on p-155 Expression—The influence of platelet activation on p-155 expression was investigated by radioimmunoprecipitation and direct binding studies. Only a faint band was detected using radioimmunoprecipitation when platelets were collected into metabolic inhibitors and gel-filtered (Fig. 1). In contrast, thrombin-activated platelets produced a marked increase in the amount of radioimmunoprecipitated p-155. Radioimmunoprecipitations of the same samples with S-12, a monoclonal antibody against the activation protein GM1-140, yielded similar findings.

Because preliminary work indicated that the intact monoclonal antibody JS-1 caused platelet activation in an Fc

receptor-dependent fashion, ^{125}I -F(ab')₂ fragments were used for direct binding experiments. Only 600 molecules of JS-1 F(ab')₂ bound to nonactivated platelets, whereas thrombin-stimulated platelets expressed approximately 4100 binding sites for JS-1 (Fig. 7). Scatchard analysis of thrombin-activated platelets (Fig. 8) indicated a high affinity binding ($K_d = 2.2$ nM) to a single class of binding sites.

Cellular Compartment of p-155—p-155 was detected in the aqueous phase using Triton X-114 partitioning and immunoblotting. No protein was detected in the detergent phase. Phase partitioning using Triton X-114 and surface-labeled platelets yielded identical findings. Using either Triton X-114 or Triton X-100 lysates, p-155 was also detected in the Triton-insoluble fraction by immunoblotting. When increasing dilu-

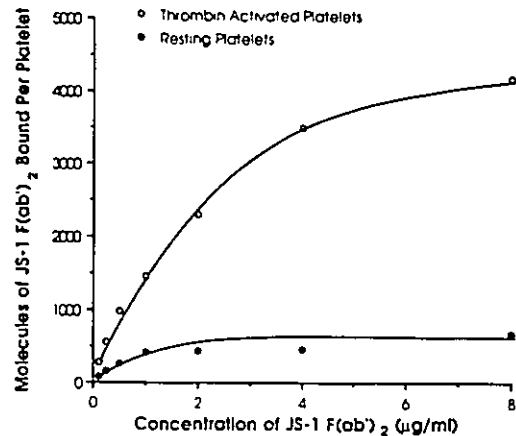


FIG. 7. Direct binding of radiolabeled JS-1 F(ab')₂ to resting and thrombin-activated platelets. Thrombin-activated platelets and resting platelets (collected and gel-filtered in the presence of PGE₁ and theophylline) were used for direct binding experiments. The specific binding of JS-1 F(ab')₂ per platelet is shown along the ordinate in relation to the concentration of JS-1 F(ab')₂ (abscissa). Nonspecific binding was determined using a 50-fold excess of unlabeled JS-1 F(ab')₂. All experiments were done in triplicate.

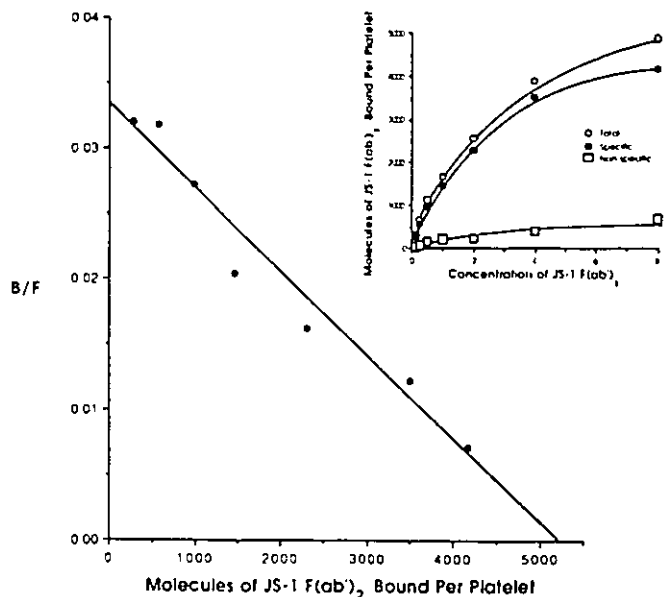


FIG. 8. Scatchard analysis of the binding of radiolabeled JS-1 F(ab')₂ to thrombin-activated platelets. Binding experiments were done in triplicate using a 50-fold excess of unlabeled JS-1 F(ab')₂ to determine specific binding. The inset shows the total, specific, and nonspecific binding data used for Scatchard analysis. This study indicates there are 5200 binding sites for JS-1 F(ab')₂ per activated platelet with K_d of 2.2 nM. B/F, bound/free.

tions of cytoskeleton and Triton X-100-soluble phases of platelet lysate were immunoblotted, reactivity was lost at similar dilutions suggesting that approximately equal amounts of p-155 were present in the two phases.

A small amount of labeled p-155 could be eluted from the platelet surface by incubation in isotonic buffer containing metabolic and proteolytic inhibitors and by 3 M salt (Fig. 9). Triton X-100 and urea solubilized large quantities of the protein. In contrast, glycoprotein IIb-IIIa, an integral membrane protein, could only be solubilized by Triton X-100. The solubilization of the protein in small quantities by isotonic buffer and high molar salt and in greater quantities by urea is consistent with a nonintegral, membrane-associated protein (14).

Immunoblotting using platelet releasate demonstrated the presence of p-155 (Fig. 10). Lysates made of the cell pellets

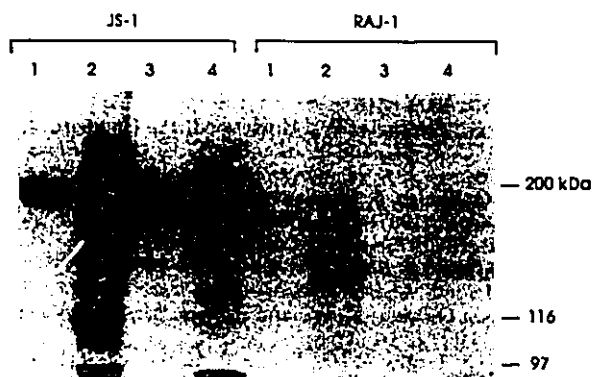


FIG. 9. The elution of p-155 from the platelet membrane. In this experiment, aliquots of radiolabeled, thrombin-activated platelets were incubated in the following eluting solutions: PBS (lane 1), or PBS to which Triton X-100 (lane 2), 3 M sodium chloride (lane 3), or 1 M urea (lane 4) were added. Following centrifugation, the supernatants were dialyzed and then immunoprecipitated with JS-1 or Raj-1 (anti-IIb-IIIa) before reduced SDS-PAGE and autoradiography. This experiment shows that p-155, unlike glycoproteins IIb-IIIa, can be eluted by 3 M sodium chloride or 1 M urea.



FIG. 10. Detection of p-155 in platelet releasate. Immunoblotting, using JS-1, demonstrating p-155 in platelet releasate (lane 1) and in the platelet lysate (10^8 platelets/ml, 1% SDS) from untreated (lane 3) and thrombin-treated platelets (lane 2) is shown. Samples were analyzed using SDS-PAGE and immunoblotting with JS-1. Platelet releasate from thrombin-stimulated platelets (10^{10} /ml) was prepared and ultracentrifuged to remove microparticles. This study demonstrates the presence of p-155 in platelet releasate. Additionally, following thrombin activation, most of the p-155 remains platelet-associated.

following thrombin-induced release revealed that large amounts of p-155 remained cell-associated after platelet activation (Fig. 10).

Comparison of the nonreduced/reduced structure of p-155 in releasate, lysate, and on the surface of thrombin-activated platelets indicated that the molecular weight bands in all samples were similar. Studies performed using the Triton-insoluble phase of platelet lysate yielded an identical DTT reduction pattern as the detergent phase (data not shown). These data suggest that the presence of p-155 with the Triton-insoluble material is not via a covalent attachment to cytoskeletal proteins.

Platelets from a patient with Glanzmann's thrombasthenia lacking glycoproteins IIb-IIIa demonstrated normal amounts of p-155.

Detection of p-155 in Other Cells—Using radioimmunoprecipitation and immunoblotting, p-155 could not be demonstrated in peripheral blood lymphocytes, activated and non-activated T-cells, granulocytes, monocytes, red cells, or plasma. Additionally, HEL cells, which express a number of platelet glycoproteins (19), did not express p-155. No reactivity with JS-1 was identified using endothelial cell lysates or culture supernatants. Immunoblotting using liver lysate with JS-1 was also negative.

DISCUSSION

Platelet activation can result in alterations to the surface structure of platelets and the binding of platelet and plasma proteins to the surface of these altered platelets. The study of these changes on the platelets surface can provide information not only about platelet physiology, but some changes can act as markers of platelet activation in disease states. In this report we describe a unique activation-dependent platelet glycoprotein, which we have designated as p-155 according to its apparent molecular weight in the reduced state.

The p-155 protein was first identified by a monoclonal antibody (JS-1) that we had raised against whole platelets. The purpose of the studies described in this report was the characterization of p-155. First, p-155 exists in the native state as varying sized, large multimers comprised of repeating subunits held together by disulfide bonds. We were unable to assign a size to the native protein, but comparison with thrombospondin suggests that the minimal multimer size is a triplet. Platelets contain other multimeric, disulfide-linked proteins that are released following platelet activation. Two examples are thrombospondin and von Willebrand factor. Thrombospondin differs from p-155 in its nonreduced-reduced mobilities. In addition, a monoclonal antibody against thrombospondin confirmed nonidentity with p-155. von Willebrand factor is similar to p-155 in its variability of multimeric size but has a different subunit size. Immunoblotting confirmed that p-155 and von Willebrand factor are distinct platelet glycoproteins. Furthermore, both von Willebrand factor and thrombospondin, unlike p-155, require divalent cations for membrane association.

A second characteristic of p-155 is that it is a soluble protein carried within platelets as shown by Triton X-114 phase-partitioning studies and the presence of p-155 in platelet releasate. p-155 can be released from platelets following stimulation by thrombin. Furthermore, membrane elution findings using activated platelets are also consistent with a nonintegral membrane-associated protein.

A third characteristic of p-155 is that its surface expression is activation-dependent. This was demonstrated in two ways. Resting platelets bound 600 molecules of JS-1, the monoclonal antibody against p-155. Following activation, approximately

4100 molecules of JS-1 bound to the platelet surface. Radioimmunoprecipitation studies confirmed the activation-dependent expression of p-155 on the platelet surface.

In spite of collection and processing of samples in metabolic and proteolytic inhibitors, we invariably observe that our monoclonal antibody JS-1 precipitated a minor band at 170 kDa as well as the dominant 155-kDa band. Because both proteins were recognized by immunoprecipitation and by immunoblotting using JS-1, we assume there is a common structural component. Common structural elements are also suggested by similar migration patterns using isoelectric focusing. The presence of both 155- and 170-kDa bands in immunoblots (where platelets were not activated by thrombin and were processed with proteolytic inhibitors) argues against *in vitro* thrombin-generated proteolysis as the origin of the 155-kDa protein. Further experiments will be required to determine if the differences between the two proteins are secondary to differences in protein or carbohydrate.

We were unable to detect p-155 in plasma, red cells, monocytes, neutrophils, endothelial cells, hepatocytes, and activated as well as nonactivated T-cells. Megakaryocytes can incorporate exogenously synthesized proteins into their granules (20), and some of the platelet granular proteins such as PF-4 are synthesized by megakaryocytes. While HEL cells do express some platelet glycoproteins (19), including some granular proteins such as von Willebrand factor, failure to detect p-155 in these cells could reflect a low rate of synthesis or lack of differentiation of the cell line.

Certain features of p-155 resemble a protein described by Bienz and Clemetson (21) and designated as Ia*. Similarities include migration characteristics on two-dimensional (non-reduced/reduced) SDS-PAGE, activation-induced surface expression, and the presence of both proteins in releasate. However, other aspects are quite different; p-155 is easily detected in the Triton-soluble phases of lysate prepared from either resting or activated platelets. In contrast, Ia* was associated with the Triton-insoluble fraction of resting platelets. In addition, p-155 has an isoelectric point of 5.4–6.75 whereas Ia* has an isoelectric point of 4.5–5.5.

The activation-dependent nature of the membrane associ-

ation of p-155 suggests a physiological role in platelet activation. The functional role of p-155 is currently under investigation.

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REFERENCES

1. Collier, B. S. (1985) *J. Clin. Invest.* **76**, 101–108
2. McEver, R. P., and Martin, M. N. (1984) *J. Biol. Chem.* **259**, 9799–9804
3. Bennett, J. S., and Villaire, G. (1979) *J. Clin. Invest.* **64**, 1393–1401
4. Fujimoto, T., Ohara, S., and Hawiger, J. (1982) *J. Clin. Invest.* **69**, 1212–1222
5. Parker, R. I., and Gralnick, H. R. (1986) *Blood* **68**, 732–736
6. Aiken, M. L., Ginsberg, M. H., and Plow, E. F. (1987) *Blood* **69**, 58–64
7. Asch, E., and Podack, E. (1990) *J. Clin. Invest.* **85**, 1372–1378
8. Galfre, G., Howe, S. C., Milstein, C., Butcher, G. W., and Howard, J. C. (1977) *Nature* **266**, 550–552
9. Lawler, J. W., Slayter, H. S., and Coligan, J. E. (1978) *J. Biol. Chem.* **253**, 8609–8616
10. Kelton, J. G., Smith, J. W., Horswood, P., Humbert, J. R., Hayward, C. P. M., and Warkentin, T. E. (1990) *Blood* **75**, 2172–2176
11. Kelton, J. G., Smith, J. W., Santos, A. V., Murphy, W. G., and Horswood, P. (1987) *Am. J. Hematol.* **25**, 299–310
12. O'Farrell, P. H. (1975) *J. Biol. Chem.* **250**, 4007–4021
13. Bordier, C. (1981) *J. Biol. Chem.* **256**, 1604–1607
14. Singer, S. J., and Nicolson, G. L. (1972) *Science* **175**, 720–731
15. Chiles, T. C., O'Brien, T. W., and Kilberg, M. S. (1987) *Anal. Biochem.* **163**, 136–142
16. Kelton, J. G., Murphy, W. G., Lucarelli, A., Garvey-Williams, J., Santos, A., Meyer, R., and Powers, P. (1989) *Br. J. Haematol.* **71**, 97–105
17. Brashem-Stein, C., Nugent, D., and Berstein, I. D. (1988) *J. Immunol.* **140**, 2330–2333
18. Pischel, K. D., Bluestein, H. G., and Woods, V. L. (1988) *J. Clin. Invest.* **81**, 505–513
19. Tabilio, A., Rosa, J.-P., Testa, U., Kieffer, N., Nurden, A. T., del Canizo, M. C., Breton-Gorius, J., and Vainchenker, W. (1984) *EMBO J.* **3**, 453–459
20. Handagama, P. J., Shuman, M. A., and Bainton, D. F. (1989) *J. Clin. Invest.* **84**, 73–82
21. Bienz, D., and Clemetson, K. J. (1989) *J. Biol. Chem.* **264**, 507–514

Multimerin: A Multimeric Protein Stored in Platelet Alpha-granules

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C. P. M. Hayward, J. G. Kelton

Glycoproteins play an important role in the platelet response to vessel injury. During platelet activation, there are alterations in the glycoproteins on the platelet membrane and these changes facilitate the transformation of platelets into adhesive, procoagulant cells. A key component of this process involves the release of proteins that are sequestered inside platelet granules. Some of proteins stored within platelets are derived from plasma, however, megakaryocytes synthesize and store other soluble proteins.^{1,2} Compared to plasma, platelet granules contain high concentrations of a number of proteins that are important for hemostasis, including von Willebrand factor, fibrinogen, and factor V.^{1,2} This intracellular store of proteins is vital for normal hemostasis and deficiencies of alpha-granular proteins can lead to bleeding.

Multimerin is a large adhesive protein synthesized by megakaryocytes and stored within platelet α -granules.³⁻⁵ This novel protein was first discovered in platelets, using a monoclonal antibody raised against human platelets.³ Multimerin is extremely large and is comprised of variably-sized, disulfide linked multimers.^{3,4} The building blocks of the multimers are the p-155 and p-170 subunits.^{3,4} These subunits are derived by proteolysis of a common precursor protein.⁵

Multimerin shares many similarities with von Willebrand factor, but, unlike von Willebrand

factor,^{6,7} it is not found in plasma.³ Within α -granules, multimerin is found in an eccentric location, colocalizing with von Willebrand factor.⁵ Platelet activation leads to multimerin release with expression of this protein on the activated platelet surface.³⁻⁵ Increased platelet surface expression of multimerin is a marker of platelet activation, both in vitro and in vivo.

While investigations have revealed the biochemical composition, biosynthesis, and intracellular location of multimerin, other aspects including its function remain uncertain. In this review, we will summarize our current knowledge of this novel protein.

The Discovery of Multimerin

Multimerin was first identified using the monoclonal antibody, JS-1, raised against whole human platelets.³ This antibody identifies a novel platelet glycoprotein by radioimmunoprecipitation and immunoblotting (Fig. 1).³ Using reduced SDS-PAGE, the protein is comprised of a 155 kDa subunit and smaller amounts of a 170 kDa subunit.³ The p-155 subunit comigrates with glycoprotein Ia but there are several important distinctions when these proteins are compared (Fig. 1).³ First, the p-155 protein recognized by JS-1 is not complexed with GP IIa. Second, nonreduced gel electrophoresis demonstrated that p-155 kDa and GP Ia have different migration characteristics. Additionally, when platelets are immunodepleted of GP Ia, p-155 is not altered in amounts, indicating that they are different proteins.³ These studies led to the initial designation of the protein recognized by JS-1 as p-155.³

Historically, many platelet proteins have been

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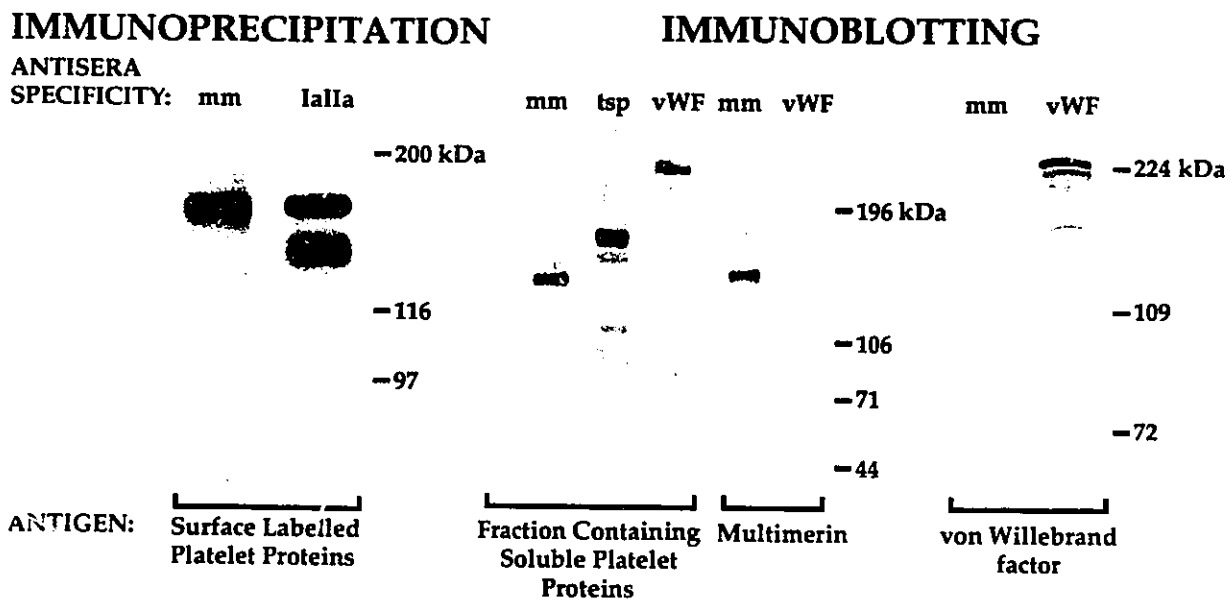


Fig. 1 Identification of multimerin using reduced SDS-PAGE. Radioimmunoprecipitation and immunoblot studies using the monoclonal antibody JS-1 and human platelets. This antibody identifies a 155 kDa protein expressed on the surface of activated platelets (left panel, lane mm). Unlike glycoprotein Ia (lane IaIa), multimerin is not associated with glycoprotein Ia. Comparison of multimerin (mm) to known soluble platelet glycoproteins (thrombospondin-tsp; von Willebrand factor-vWf) and to purified vWf indicates the unique nature of multimerin. Purified multimerin is not recognized by polyclonal antibodies to vWf.

identified by their mobility on nonreduced/reduced electrophoresis.⁸ More recently, monoclonal antibody technology has proved invaluable for identifying and characterizing less abundant proteins.⁹ Unlike many other recently identified proteins, p-155 is present in sufficiently high quantities in platelets to be detected by silver staining (Fig. 2, upper panel).⁴ However, the protein went undetected for many years possibly because its exceedingly large nonreduced size prevented it from entering most resolving gels (Fig. 2, lower panel).^{3,4} As a result, multimerin was only recently discovered when a monoclonal antibody raised against platelets identified its reduced subunit.

As shown in Figure 2 (upper panel), the two largest proteins in platelets are multimerin and von Willebrand factor.⁴ In their native form, both proteins are exceptionally large with multimer sizes of many million Daltons.^{4,10-13} The multimeric structure of p-155 (described subsequently) led us to designate it as multimerin.⁴

Multimerin—a Protein Expressed on Activated Platelets

A large number of soluble proteins have been identified which play a role in the adhesion and aggregation of platelets to the vessel wall, to other cells and to each other. These proteins include fibrinogen, von Willebrand factor and thrombospondin, among others. They are all stored in high concentrations within platelet α -granules and bind to specific receptors following platelet activation and granule release.^{1,2,14-20} This movement from internal stores to the activated platelet surface is important

for the transformation of platelets into adhesive cells at local sites of vessel injury and platelet activation.

Our early studies of this protein demonstrated that the expression of multimerin on the platelet surface requires platelet activation.³ Studies comparing proteins expressed on the surface of resting and activated platelets demonstrate a marked increase in the amount of the multimerin expressed on the platelet membrane following activation with thrombin, which parallels the increased expression of P-selectin.^{3,21,22} Quantitation studies with the radiolabelled monoclonal antibody JS-1 demonstrate that the number of binding sites for JS-1 on the platelet membrane increases from approximately 600 to 4500 copies with thrombin activation (Fig. 3).³ Investigations of the mechanism of this activation change pointed to release of a soluble, adhesive protein, from sequestered intracellular stores, with its subsequent binding to the activated platelet surface.³

Activation-dependent platelet proteins can be classified into three general groups (Fig. 4). The first group includes proteins that undergo conformational changes which can be detected by activation-specific, monoclonal antibodies. Examples include PAC-1 binding to GPIIb/IIIa ($\alpha_{IIb}\beta_3$).²³ A second group includes integral membrane proteins, located in platelet granules, that are expressed on the platelet surface following granule release and fusion of the granule and surface membranes (e.g. P-selectin, CD63).^{21,22,24} A third group of activation proteins are soluble adhesive proteins that bind to the surface of activated platelets. Examples of this group include fibrinogen, von Willebrand factor and thrombospondin.¹⁴⁻²⁰

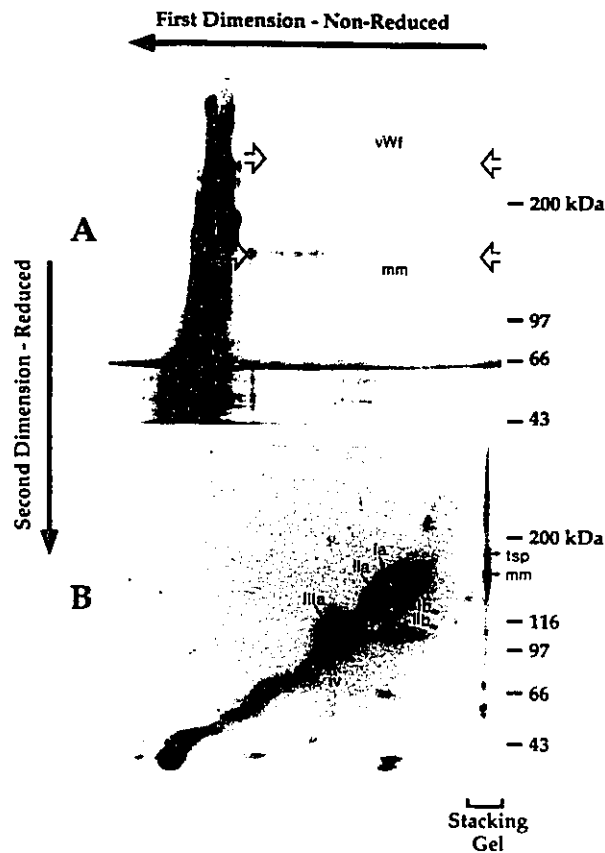


Fig. 2 Migration of multimerin using two-dimensional nonreduced/reduced electrophoresis. This figure shows the two-dimensional electrophoretic separation of platelet proteins using the traditional SDS-PAGE (nonreduced/reduced; lower panel) technique and using agarose acrylamide gels (nonreduced) in the first dimension followed by SDS-PAGE (upper panel). The upper panel is a silver stained gel and the lower panel is an autoradiogram of ^{125}I surface-radiolabelled platelet proteins. The major glycoproteins (Ia, IIa, IIb, IIIa, IV, mm—multimerin, tsp—thrombospondin, vWf—von Willebrand factor) identified by these separations are indicated. Gels which can sieve massive proteins (upper panel) are required to resolve the nonreduced multimerin into its individual multimers.

Three lines of evidence indicate that multimerin falls into the third category of platelet activation markers—i.e. it is a soluble, adhesive protein expressed on activated platelets.³ First, detergent partitioning studies demonstrate that multimerin is a soluble protein. Second, multimerin is found in the microparticle-free fraction of platelet releasate, indicating that it is released when platelets are activated. Third, unlike transmembrane proteins, multimerin is eluted off the activated platelet membrane by high molar salt solutions.

Because multimerin is not present in plasma, expression of this soluble protein on the activated platelet surface requires its release from sequestered internal stores. Multimerin has unique characteristics compared to other platelet glycoproteins that are released from platelets and rebind to the activated platelet membrane. For example, unlike thrombospondin, fibrinogen and von Willebrand factor, the

MULTIMERIN EXPRESSION

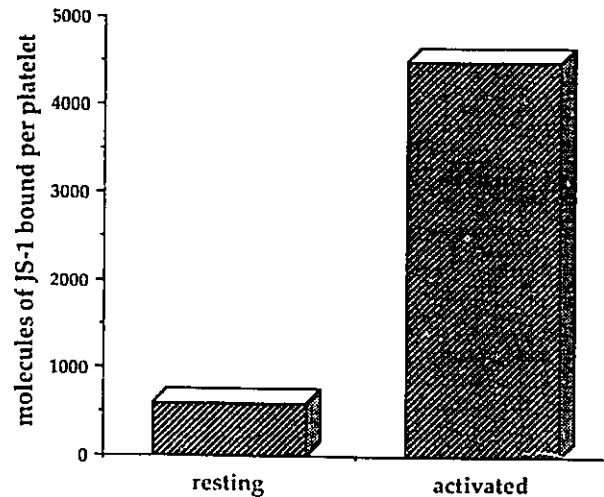


Fig. 3 Multimerin expression of resting and activated platelets. This figure shows the expression of multimerin on resting and activated platelets using the radiolabelled monoclonal antibody (JS-1) to multimerin. The values indicate specific binding. Following platelet activation, multimerin expression increases approximately 7-fold.

binding of multimerin to activated platelets is not inhibited by EDTA.^{3,14,18-20}

Other questions about the mechanism of multimerin expression on activated platelets remain unanswered. The identity of the platelet receptor for multimerin is unknown. The receptor is probably not an integrin, as EDTA does not inhibit the binding of multimerin to activated platelets. Additionally, platelets from a patient with Glanzmann's thrombasthenia, lacking glycoprotein $\alpha_{\text{IIb}}\beta_{\text{IIIa}}$, show normal multimerin expression.³

The Multimeric Nature of Multimerin

Under nonreducing conditions, multimerin is not resolved by SDS-PAGE due to its exceedingly large size.³ Using agarose-acrylamide gels (which can sieve very large proteins), p-155 separates into a series of varying-sized multimeric proteins that range in size from 400 kDa to many million Daltons (Fig. 5).⁴ The large size and variable multimeric nature of the protein led us to designate the native protein as multimerin.⁴ The multimerin multimers have a different mobility than those of von Willebrand factor (Fig. 5), indicating that they are not the same protein.⁴

Reduction of the individual multimers of multimerin liberates p-155 and p-170 subunits from all of the different sized multimers, indicating that the multimers are comprised of the same basic subunit proteins, linked by interchain disulfide bonds. The variation in multimerin multimer size reflects differences in the number of subunits that the individual multimers contain. In platelets, the smallest multimer is a 400 kDa trimer, but most multimers are millions of Daltons in size (Fig. 5).

MARKERS OF PLATELET ACTIVATION

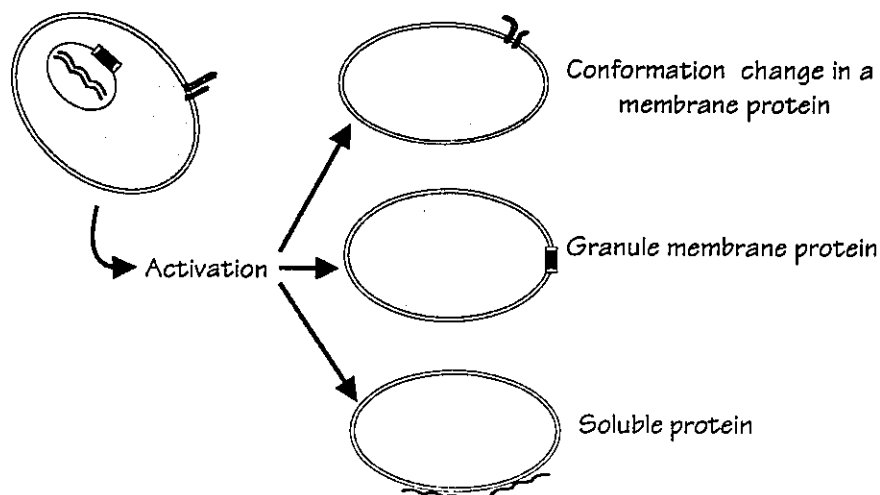


Fig. 4 Activation changes in platelet glycoproteins.

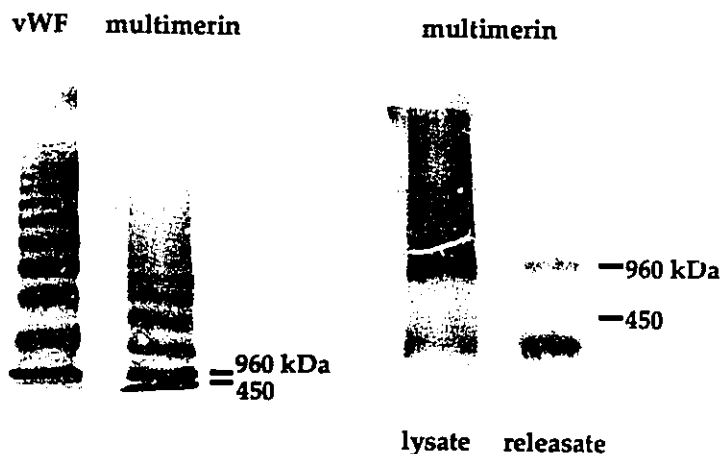


Fig. 5 The multimeric composition of platelet multimerin. An immunoblot analysis of the multimeric composition of multimerin in platelet lysate and releasate separated on 1.25% agarose gels containing 1% (left) and 1.5% acrylamide (right). The smallest multimerin multimer migrates at 400 kDa with most multimers exceeding 1 million Daltons. Compared to vWf, the multimerin multimers migrate with a different mobility. With platelet activation and multimerin release, there is selective binding of the largest multimers and only the smallest multimers are found in releasate.

Before our identification of multimerin, von Willebrand factor was thought to be unique in its complex multimeric structure¹⁰⁻¹³ but our investigations revealed that multimerin has similar multimeric properties to von Willebrand factor. Consequently, we searched for other large, multimeric platelet proteins. When platelet proteins are analyzed by nonreduced agarose/acrylamide gels followed by reduced separation using SDS-PAGE and silver staining, the only complex multimeric proteins evident are multimerin and von Willebrand factor (Fig. 2, upper panel).⁴ Multimerin and von Willebrand factor are the two largest proteins found in platelets and two of the largest proteins found in the body.

The multimeric structure of multimerin has important implications for function. Its repeating structure provides many binding sites for adhesion. Studies of

multimerin released from platelets indicate that the largest multimers are the most adhesive.⁴ Following platelet granule release, there is preferential binding of the largest multimers (containing many ligand binding domains) to the activated platelet surface and only the small multimers are found in releasate (Figs 5, 6).⁴

The many similarities between multimerin and von Willebrand factor led us to examine if multimerin is found in plasma.³ Immunoblotting and immunoassay studies have failed to detect multimerin in either plasma or serum. The explanation for these findings may very well relate to the avid association of this protein with activated platelets. In platelet releasate, multimerin is present in the soluble fraction and also in association with the platelet microparticles.³ Only small quantities of multimerin can be detected in the releasate.^{3,4} Most of the multimerin contained within

COMPARISON OF INTRACELLULAR AND RELEASED MULTIMERIN

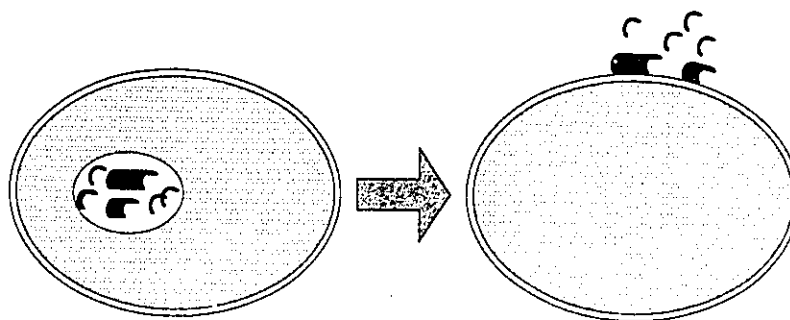


Fig. 6 The redistribution of multimerin with platelet activation. This diagram shows the changes in multimerin that occur following platelet activation and release of granule proteins. There is preferential attachment of the largest multimers (containing many more binding sites) to the activated platelet surface.

platelets remains associated with platelets after activation.^{3,4}

Multimerin and von Willebrand factor show many structural similarities but there are important differences that distinguish these proteins (summarized in the Table). Polyclonal antisera directed against multimerin and von Willebrand factor recognize proteins that have different nonreduced and reduced mobilities (Fig. 1).³⁻⁵ Other important differences between multimerin and von Willebrand factor include their subunit sizes and multimer sizes,^{3,4,6,7,10-13} glycosylation,^{5,25} the presence in plasma of von Willebrand factor but not multimerin,³ and the nature of their association with activated platelets.^{3,17,19,20} Finally, cloning and sequencing of the cDNA for multimerin (manuscript in preparation) indicates that multimerin is a novel protein, distinct from von Willebrand factor and all other proteins that have been sequenced.

Platelet proteins show a number of adaptations that favor their adhesive, hemostatic role. The similar repeating multimeric structure of multimerin and von Willebrand factor is an example of this adaptation.

Sequestration of these high molecular weight multimers inside platelets until activation may aid in restricting ligand interactions to sites of vessel injury.

The Relationship Between the Different Multimerin Subunits

Early investigations of multimerin revealed that it was multimeric and established the existence of two different, but immunologically related, subunits (p-155 and p-170).³ Both subunits are identified by the monoclonal antibody JS-1 and polyclonal antibodies raised against p-155 react with p-170.³ Both subunits are also dispersed throughout the different sized multimers of multimerin.^{3,4}

By protease digestions, p-155 and p-170 generate identical patterns of peptide digests.⁵ However, the peptide homology between p-155 and p-170 does not account for the differences in their molecular weights. Removal of all carbohydrate from the p-155 and p-170 proteins by glycosidases generates two different-sized, deglycosylated proteins, consistent with differences in their polypeptide-chain lengths.⁵

Table 1 Comparison of multimerin and von Willebrand factor

	Multimerin	von Willebrand factor
Molecular Weight		
Reduced	155 and 170 kDa	220 kDa
Nonreduced	Multimers ranging in size from 400 to many million Daltons	Multimers ranging in size from 440 kDa to 20 million Daltons
Smallest multimer size	trimer	dimer
Found in plasma	No	Yes
Stored in platelet alpha-granules	Yes, eccentric location	Yes, eccentric location
Located in normal blood vessels and in Weibel Palade bodies	Yes	Yes
Site of biosynthesis	Megakaryocytes Endothelial cells	Megakaryocytes Endothelial cells
Glycosylation	Highly glycosylated with: 17 complex N-linked sugars, accounting for approximately 1/3 of its apparent molecular weight	Glycosylated with: 10 O-linked sugars and 12 complex, N-linked sugars

Evidence indicates that p-155 is probably derived from p-170 by proteolytic cleavage. Because the subunit composition of multimerin in resting and activated platelets is similar, this processing must occur during biosynthesis or storage.

Occasionally, reduced platelet multimerin immunoprecipitates contain small amounts of a 200 kDa protein.^{4,5} Two-dimensional nonreduced-reduced migration studies indicate that the p-200 protein is linked to the other multimerin subunits by interchain disulfide bonds.⁵ Using metabolic labelling to follow the megakaryocyte biosynthesis of multimerin, we have now confirmed that the 200 kDa multimerin subunit is the precursor of p-155 and p-170.⁵ Within platelets, most of the protein is proteolyzed to the 155 kDa subunit. This proteolysis occurs without disrupting the multimeric structure of multimerin.

The Cellular Distribution of Multimerin

Multimerin is found within the platelets and megakaryocytes in the bone marrow. No other bone marrow cells stain for multimerin.⁵ Dami cells, a megakaryocytic cell line, contain multimerin in their cytoplasm, and stain with granular pattern for multimerin which resembles normal megakaryocytes. However, multimerin is found in Dami cells only after they are stimulated with PMA.⁵ This treatment alters the morphology of these cells to more closely resemble normal megakaryocytes and induces these cells to synthesize a number of proteins that are found in platelets.²⁶ The PMA-dependence of multimerin expression by Dami cells suggests that multimerin synthesis and storage occurs during the later stages of megakaryopoiesis.

Multimerin is Stored Within Platelet α -Granules

The soluble nature of multimerin and its expression on the platelet surface after activation indicate that multimerin is stored within platelet granules before its release. In collaboration with Dr Dorothy Bainton, immunogold labelling studies were used to determine the site of multimerin storage within platelets. Frozen thin-sections of resting platelets indicate that multimerin is located within the matrix of alpha-granules. A striking feature of multimerin's distribution within α -granules is its unusual, eccentric position within the granules (Fig. 7).⁵

Electron microscopy has identified a number of distinct regions within α -granules; these include the proteoglycan-rich nucleoid zone, the intermediary zone (containing most of the known alpha-granular proteins) and the eccentric, electronlucent zone, which contains tubular structures and von Willebrand factor.^{2,27,28}

The eccentric location of multimerin within α -granules resembles the distribution of von Willebrand factor.²⁷ In double labelling studies comparing these proteins, multimerin and von

Willebrand factor colocalize to the same region of the α -granule (Fig. 7).⁵ Ultrastructural studies of α -granules have localized von Willebrand factor to tubules within this organelle.^{2,27,28} The colocalization of multimerin with von Willebrand factor suggests an association of multimerin with the tubular structures or with the matrix of the electronlucent zone. To date, our investigations of multimerin have not suggested a direct association of multimerin with von Willebrand factor. Instead, we speculate that their colocalization within α -granules is due to similar mechanisms of protein trafficking and storage.

The Origin of Platelet Multimerin

Platelet α -granule proteins originate from two sources: synthesis by the megakaryocytes or uptake from the plasma.^{1,2} Proteins synthesized by megakaryocytes are present in higher concentration within platelets than in plasma.¹ Because multimerin is not found in plasma, we have postulated that the megakaryocytes must synthesize multimerin. To investigate this possibility, we have used metabolic labelling studies of PMA-activated Dami cells.⁵

We have found that Dami cells synthesize multimerin following activation with PMA.⁵ PMA is known to induce the synthesis of a variety of platelet proteins in Dami cells, including soluble proteins such as von Willebrand factor.²⁶ These data strongly support endogenous biosynthesis by normal megakaryocytes as the origin of platelet multimerin. Our postulate of megakaryocyte biosynthesis will be confirmed when the mRNA for multimerin is shown to be expressed in normal megakaryocytes or platelets.

Biochemical Characteristics of Multimerin

Platelet multimerin contains large amounts of complex N-linked carbohydrate.⁵ Deglycosylation of multimerin subunits reduces their molecular weight by approximately one third, indicating that the protein is highly glycosylated.⁵ Digestion with a variety of endo and exoglycosidases demonstrates that the main form of carbohydrate is N-linked and that the mature protein contains only complex forms of N-linked carbohydrate. Studies of megakaryocyte biosynthesis indicate only small amounts of O-linked carbohydrate on multimerin.⁵ Because the deglycosylated p-155 and p-170 subunits fail to comigrate,⁵ we conclude that p-155 is the result of more extensive proteolytic processing of the common precursor protein.

Multimerin Biosynthesis in Cultured Cells

We have used PMA-activated Dami cells to investigate the biosynthesis of multimerin in megakaryocytes. Using metabolic labelling followed by immunoprecipitation to isolate multimerin, the pro-



Fig. 7 Multimerin is stored within alpha-granules. In frozen thin-sections of resting platelets, multimerin (labelled with polyclonal anti-multimerin and goat-antirabbit gold-10; panel 1a, $\times 50\,000$) is found in the alpha-granule matrix, mainly in an eccentric position (large arrows). Double labelling studies (inset of 1a) comparing multimerin (polyclonal anti-multimerin; detection with protein A gold 10; the large gold) and vWf (polyclonal anti-vWf; detection with protein A gold 5; the small gold; small arrows) indicate colocalization of these proteins to the same region of platelet alpha-granules. Similar findings were observed when vWf was detected using a monoclonal antibody and goat anti-mouse gold-10 and multimerin was detected using polyclonal anti-multimerin and goat anti-rabbit gold-5 (panel 1b). (Reprinted with permission of The Society For Clinical Investigation from Hayward et al. *J Clin Invest* 1993; 91: 2630-2639.)

cess of multimerin biosynthesis was characterized (Fig. 8).⁵

Unstimulated Dami cells do not synthesize multimerin nor do they express the protein at levels detectable by immunochemistry.⁵ However, after activation with PMA, Dami cells stain strongly for multimerin and metabolically labelled multimerin is synthesized and secreted into the megakaryocyte culture medium.⁵ The mature multimerin secreted by Dami cells in culture is comprised of p-196 and p-165 kDa subunits. The biosynthesis of multimerin by the megakaryocytic cell line is summarized in Figure 8.

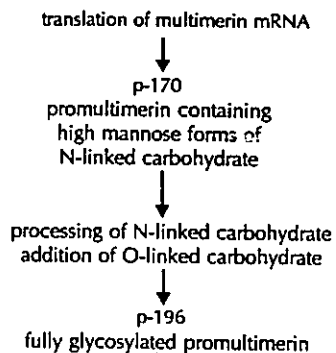
Pulse-chase metabolic labelling studies and carbohydrate analyses indicate that several events occur during the biosynthesis of multimerin.⁵ Multimerin is first synthesized as a 170 kDa early precursor which contains high-mannose forms of N-linked carbohydrate. Experiments using tunicamycin, which

inhibits the initial step in N-glycosylation, indicate that the polypeptide component of the multimerin precursor protein is 132 kDa.

Subsequently, the N-linked carbohydrate chains undergo further processing, converting the high-mannose sugars into the complex forms of N-linked carbohydrate, which is the type of carbohydrate found in the mature multimerin.⁵ This alteration in carbohydrate is associated with an increase in the size of the multimerin subunit from 170 kDa to 196 kDa. Only small amounts of O-linked carbohydrate are added during biosynthesis of p-196. Proteolysis of this multimerin subunit (p-196 or promultimerin) is required to generate the smaller p-165 multimerin subunit. The multimerin secreted by Dami cells is comprised of p-196 and p-165 subunits.⁵

During multimerin biosynthesis, interchain disulfide bonds form, covalently linking the multimerin

Subunit Processing



Multimer Formation

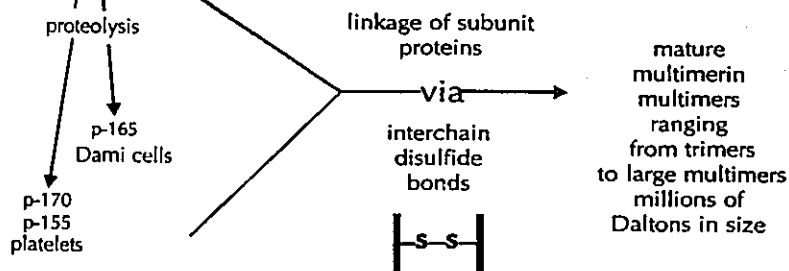


Fig. 8 Multimerin biosynthesis. The protein synthesis, glycosylation, proteolytic processing and multimerization steps involved in the biosynthesis of multimerin are outlined.

subunits. High molecular weight multimers form as a result of this process.⁵ Pulse-chase biosynthesis studies, analyzed on multimer gels, indicate that multimerization occurs just prior to secretion, suggesting that this event probably occurs in the Golgi apparatus (unpublished observations). The proteolytic processing of the multimerin subunits occurs without disrupting the multimeric structure of the protein.

The multimerin synthesized by Dami cells shares peptide homology with platelet multimerin.⁵ However, the multimerin synthesized by Dami cells differs from the multimerin stored in platelets in its subunit and multimeric composition.⁵ Deglycosylation and protease digestion experiments indicate more extensive proteolysis of the platelet form of multimerin which is comprised mainly of p-155 and a smaller amount of p-170.⁵ In contrast, the large, p-196 precursor (promultimerin) is the predominant multimerin subunit secreted by Dami cells.⁵ Occasionally, we have observed platelet multimerin immunoprecipitates containing small amounts of the large, multimerin subunit precursor.⁵ In both Dami cell and normal megakaryocytes, multimerin originates as a larger precursor protein (promultimerin). More complete proteolytic processing of multimerin occurs in platelets.³⁻⁵

Dami cells constitutively secrete only the smaller multimers of multimerin, in contrast to the extremely large multimers of the protein stored in platelet α -granules.^{4,5} Almost all of the multimerin contained within Dami cells is newly synthesized precursor protein; the majority of the synthesized multimerin

is secreted.⁵ This is in contrast to platelet multimerin, which is stored intracellularly (for days) until granule release occurs. The rapid secretion of the multimerin by Dami cells probably contributes to the less extensive proteolytic and multimeric processing. Protein trafficking in normal megakaryocytes and α -granules somehow facilitates the formation and storage of the higher molecular weight multimers of multimerin that are found in circulating platelets.

Potential Roles of Multimerin in Vessel Injury

The association of multimerin with the region within platelet α -granules resembling Weibel-Palade bodies²⁷ has led us to look for multimerin in endothelial cells. Our recent studies (manuscript submitted for publication) have identified multimerin within endothelial cell Weibel-Palade bodies and metabolic labelling studies confirm endothelial cell biosynthesis of multimerin. Both constitutive and agonist-induced pathways exist for multimerin release by endothelial cells. Following Weibel-Palade body release, there is a marked increase in the multimerin associated with the matrix and with the endothelial cell surface. The multimerin stored for release by agonists resembles platelet multimerin in that it is comprised of p-155 subunits and is enriched in the high molecular weight multimers of multimerin.

Parallels are seen in the biosynthesis of multimerin (ref. 5, and manuscript submitted for publication) and von Willebrand factor.²⁹⁻³⁴ Both proteins are made by endothelial cells and megakaryocytes and

stored in Weibel Palade bodies and platelet α -granules. The intracellular stores of multimerin and von Willebrand factor are enriched in high molecular weight multimers.

In both platelets³⁻⁵ and endothelial cells, multimerin is found within storage granules. Both cells secrete multimerin. After injury or activation, multimerin adheres to activated platelets³⁻⁵ and endothelial cells as well as their extracellular matrix. This sequestration of multimerin within the resting cells allows the amount of this protein found on cells surfaces and in the matrix to be upregulated at sites of cell injury or activation. Investigations are in progress to determine what function(s) multimerin has in the vascular response to injury.

Our knowledge of multimerin has expanded from the initial recognition of this protein to the elucidation of its cells of origin and biosynthesis. Multimerin and von Willebrand factor are stored in similar locations in platelet alpha-granules and in endothelial cell Weibel-Palade bodies. In both platelets and endothelial cells, the granular location of these proteins facilitates their rapid release following injury or activation. Multimerin expression on the cell surface serves as a marker of platelet and endothelial cell activation. The precise contribution of multimerin to the altered adhesive and procoagulant properties of activated platelets and endothelial cells is still unknown. Further insights are likely to be gained from structure-function studies and from identification of individuals who are deficient in multimerin.

Acknowledgements

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References

- George J N. Platelet immunoglobulin G: Its significance for the evaluation of thrombocytopenia and for understanding the origin of α -granule proteins. *Blood* 1990; 76: 859-870.
- Harrison P, Martin Cramer E. Platelet α -granules. *Blood Rev* 1993; 7: 52-62.
- Hayward C P M, Smith J W, Horsewood P, Warkentin T E, Kelton J G. p-155, a multimeric platelet protein that is expressed on activated platelets. *J Biol Chem* 1991; 266: 7114-7120.
- Hayward C P M, Warkentin T E, Horsewood P, Kelton J G. Multimerin: a series of large, disulfide-linked multimeric proteins within platelets. *Blood* 1991; 77: 2556-2560.
- Hayward C P M, Bainton D F, Smith J W, et al. Multimerin is found in the α -granules of resting platelets and is synthesized by a megakaryocytic cell line. *J Clin Invest* 1993; 91: 2630-2639.
- Legaz M E, Schmer G, Counts R B, Davie E W. Isolation and characterization of human factor VIII (antihemophilic factor). *J Biol Chem* 1973; 248: 3946-3955.
- Olson J D, Brockway W J, Fass D N, Bowie E J W, Mann K G. Purification of porcine and human ristocetin-Willebrand factor. *J Lab Clin Med* 1977; 89: 1278-1294.
- Phillips D R, Poh Agin P. Platelet plasma membrane glycoproteins. Evidence for the presence of nonequivalent disulfide bonds using nonreduced-reduced two-dimensional gel electrophoresis. *J Biol Chem* 1977; 252: 2121-2126.
- Horsewood P, Smith J W, Kelton J G. Monoclonal antibodies against platelet glycoproteins. *Trans Med Rev* 1993; 7: 156-172.
- Counts R B, Paskell S L, Elgee S K. Disulfide bonds and the quaternary structure of factor VIII/von Willebrand factor. *J Clin Invest* 1978; 62: 702-709.
- Perret B A, Furlan M, Beck E A. Studies on factor VIII-related protein. Estimation of molecular size differences between factor VIII oligomers. *Biochem Biophys Acta* 1979; 578: 164-174.
- Ruggeri Z M, Zimmerman T S. Variant von Willebrand's disease. Characterization of two subtypes by analysis of multimeric composition of factor VIII/von Willebrand factor in plasma and platelets. *J Clin Invest* 1980; 65: 1318-1325.
- Hoyer L W, Shainhoff J R. Factor VIII-related protein circulates in normal human plasma as high molecular weight multimers. *Blood* 1980; 55: 1056-1059.
- Phillips D R, Charo I F, Parise L V, Fitzgerald L A. The platelet membrane glycoprotein IIb-IIIa complex. *Blood* 1988; 71: 831-843.
- Ruggeri Z M, Bader R, DeMarco L, Glanzmann thrombasthenia: deficient binding of von Willebrand factor to thrombin stimulated platelets. *Proc Natl Acad Sci* 1982; 79: 6038-6041.
- Ruggeri Z M, DeMarco L, Gatti L, Bader R, Montgomery R R. Platelets have more than one binding site for von Willebrand factor. *J Clin Invest* 1983; 72: 1-12.
- Gralnick H R, Williams S B, Coller B S. Fibrinogen competes with von Willebrand factor for binding to the glycoprotein IIb/IIIa complex when platelets are stimulated with thrombin. *Blood* 1984; 64: 797-800.
- Phillips D R, Jennings L K, Prasanna H R. Ca⁺⁺ mediated association of glycoprotein G (thrombin-sensitive protein, thrombospondin) with human platelets. *J Biol Chem* 1980; 255: 11629-11632.
- Fernandez M F L, Ginsberg M H, Ruggeri Z M, Battle F J, Zimmerman T S. Multimeric structure of platelet factor VIII/von Willebrand factor: The presence of larger multimers and their reassociation with thrombin-stimulated platelets. *Blood* 1982; 60: 1132-1138.
- Parker R I, Gralnick H R. Identification of platelet glycoprotein IIb/IIIa as the major binding site for released platelet-von Willebrand factor. *Blood* 1986; 68: 732-736.
- McEver R P, Martin M N. A monoclonal antibody to a membrane glycoprotein binds only to activated platelets. *J Biol Chem* 1984; 259: 9799-9804.
- Hsu-Lin S, Berman C L, Furie B C, August D, Furie B. A platelet membrane protein expressed during platelet activation and secretion. *J Biol Chem* 1984; 259: 9121-9126.
- Shattil S J, Hoxie J A, Cunningham M, Brass L F. Changes in the platelet membrane glycoprotein IIb/IIIa complex during platelet activation. *J Biol Chem* 1985; 260: 11107-11114.
- Nishibori M, Cham B, McNicol A, Shalev A, Jain N, Gerrard J. The protein CD63 is in platelet dense granules, is deficient in a patient with Hermansky-Pudlak Syndrome, and appears identical to granulophysin. *J Clin Invest* 1993; 91: 1775-1782.
- Titani K, Kumar S, Takio K et al. Amino acid sequence of human von Willebrand factor. *Biochem* 1986; 25: 3171-3184.
- Greenberg S M, Rosenthal D S, Greeley T A, Tantravahi R, Handin R I. Characterization of a new megakaryocytic cell line: the Dami cell. *Blood* 1988; 72: 1968-1977.
- Cramer E M, Meyer D, le Menn R, Breton-Gorius J. Eccentric localization of von Willebrand Factor in an internal structure of platelet α -granule resembling that of Weibel-Palade bodies. *Blood* 1985; 66: 710-713.
- Wilbourne B R, Harrison P, Lawrie A S, Savariau E, Savidge G F, Cramer E M. Porcine platelets contain an increased quantity of ultra-high molecular weight von Willebrand factor. *Br J Haematol* 1993; 83: 608-615.
- Nachman R, Levine R, Jaffe E A. Synthesis of factor VIII antigen by cultured guinea pig megakaryocytes. *J Clin Invest* 1977; 60: 914-921.
- Jaffe E A, Hoyer L W, Nachman R L. Synthesis of antihemophilic factor antigen by cultured human endothelial cells. *J Clin Invest* 1973; 52: 2757-2764.
- Jaffe E A, Hoyer L W, Nachman R L. Synthesis of von

- Willebrand factor by cultured human endothelial cells. Proc Natl Acad Sci 1974; 71: 1906-1909.
32. Lynch D C, Williams R, Zimmerman T S, Kirby E P, Livingston D M. Biosynthesis of the subunits of factor VIII R by bovine aortic endothelial cells. Proc Natl Acad Sci 1983; 80: 2738-2742.
33. Wagner D D, Marder V. Biosynthesis of von Willebrand protein by human endothelial cells: processing steps and their intracellular localization. J Cell Biol 1984; 99: 2123-2130.
34. Sporn L A, Marder V J, Wagner D D. Inducible secretion of large, biologically potent von Willebrand factor multimers. Cell 1986; 46: 185-190.
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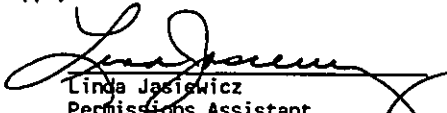
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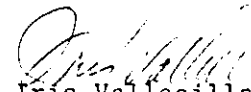
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Snerwood Park Alberta T8B 1C1

Fax 403 492-0450

I am completing a PhD thesis at McMaster University, entitled, "Characterization of Multimerin, a platelet and endothelial cell protein." I would like your permission to reprint in my thesis, the article listed below. Additionally, I am requesting your permission, as co-author, to allow the National Library of Canada to reprint this material in my thesis. A permission request has also been submitted to the Journal in which this article was published. Proper acknowledgement will be given to the portion which represents my thesis work. Please sign where indicated below and return this letter to me. Thank you for your attention.

Sincerely,

Catherine P. M. Hayward

*The cDNA sequence of human endothelial cell multimerin: A unique protein with RGDS, coiled-coil, and EGF domains, and a carboxyl terminus similar to the globular domain of complement C1Q and collagens type VIII and X. Submitted to J Biol Chem.

** submitted, not yet published*

Signed:

R. Rachubinski

Dated:

MARCH 13/95



McMASTER UNIVERSITY
DEPARTMENT OF PATHOLOGY
1200 Main Street West,
Hamilton, Ontario, Canada L8N 3Z5
Telephone: (416) 525-9140
Fax: (416) 577-0198

- SERVICE
- RESEARCH
- EDUCATION

March 13, 1995

Dr. C. Brown
3H42

I am completing a PhD thesis at McMaster University, entitled, "Characterization of Multimerin, a platelet and endothelial cell protein." I would like your permission to reprint in my thesis, the article listed below. Additionally, I am requesting your permission, as co-author, to allow the National Library of Canada to reprint this material in my thesis. A permission request has also been submitted to the Journal in which this article was published. Proper acknowledgement will be given to the portion which represents my thesis work. Please sign where indicated below and return this letter to me. Thank you for your attention.

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Signed: Claudia Brown
C. Brown

Dated: March 22 1995



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1200 Main Street West,
Hamilton, Ontario, Canada L8N 3Z5
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Fax: (416) 577-0198

- SERVICE
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March 9, 1995

Dr. T. J. Podor
Hamilton Civics Research Centre
HDGH

Dear Dr. Podor:

I am completing a PhD thesis at McMaster University, entitled, "Characterization of Multimerin, a platelet and endothelial cell protein." I would like your permission to reprint in my thesis, the articles listed below. Additionally, I am requesting your permission, as co-author, to allow the National Library of Canada to reprint this material in my thesis. A permission request has also been submitted to the Journal in which this article was published. Proper acknowledgement will be given to the portion which represents my thesis work. Please sign where indicated below and return this letter to me in the enclosed envelope. Thank you for your attention.

Sincerely,

Catherine P. M. Hayward

PERMISSION TO REPRINT AS STATED ABOVE FOR THE FOLLOWING ARTICLES:

Signed:

T. J. Podor

Dated:

March 13/95

Multimerin is found in the α -granules of resting platelets and is synthesized by a megakaryocytic cell line. J. Clin Inves 1993; 91:2630-2639.

*Endothelial cells store multimerin in Weibel-Palade bodies and possess both constitutive and regulated pathways for multimerin release.

*Submitted for publication.



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Hamilton, Ontario, Canada L8N 3Z5
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- SERVICE
- RESEARCH
- EDUCATION

Dr. Peter Horsewood
3N10E

March 9, 1995

Dear Dr. Horsewood:

I am completing a PhD thesis at McMaster University, entitled, "Characterization of Multimerin, a platelet and endothelial cell protein." I would like your permission to reprint in my thesis, the articles listed below. Additionally, I am requesting your permission, as co-author, to allow the National Library of Canada to reprint this material in my thesis. A permission request has also been submitted to the Journal in which this article was published. Proper acknowledgement will be given to the portion which represents my thesis work. Please sign where indicated below and return this letter to me in the enclosed envelope. Thank you for your attention.

Sincerely,

Catherine P.M. Hayward

PERMISSION TO REPRINT AS STATED ABOVE FOR THE FOLLOWING ARTICLES:

p-155, a multimeric protein that is expressed on activated platelets. J. Biol Chem 1991; 266:7114-7120

Multimerin: A series of large disulfide-linked multimeric proteins within platelets. Blood 1991; 77:2556-2560

Multimerin is found in the α -granules of resting platelets and is synthesized by a megakaryocytic cell line. J. Clin Invest 1993; 91:2630-2639.

Signed:

P. Horsewood

Dated:

16.3.95



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1200 Main Street West,
Hamilton, Ontario, Canada L8N 3Z5
Telephone: (416) 525-9140
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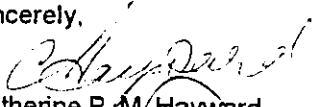
Dr. R. Stead
3N26

March 9, 1995

Dear Dr. Stead:

I am completing a PhD thesis at McMaster University, entitled, "Characterization of Multimerin, a platelet and endothelial cell protein." I would like your permission to reprint in my thesis, the articles listed below. Additionally, I am requesting your permission, as co-author, to allow the National Library of Canada to reprint this material in my thesis. A permission request has also been submitted to the Journal in which this article was published. Proper acknowledgement will be given to the portion which represents my thesis work. Please sign where indicated below and return this letter to me in the enclosed envelope. Thank you for your attention.

Sincerely,


Catherine P.M. Hayward

PERMISSION TO REPRINT AS STATED ABOVE FOR THE FOLLOWING ARTICLES:

Signed:  R. Stead

Dated: 20/3/95

Multimerin is found in the α -granules of resting platelets and is synthesized by a megakaryocytic cell line. J. Clin Inves 1993; 91:2630-2639.

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Mr. J. W. Smith
3H42

March 9, 1995

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Signed:

J.W. Smith

Dated:

16 March 95
