CHARACTERIZATION AND PHOSPHORYLATION SITE MAPPING OF HUMAN PLECKSTRIN

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Karen Leigh Craig, B.Sc.

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AUTHOR: Karen Leigh Craig, B.Sc. (Univeristy of Waterloo)

SUPERVISOR: Dr. Calvin B. Harley

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ABSTRACT

Pleckstrin is the major substrate of PKC in activated platelets, therefore a role for pleckstrin in mediating platelet responses such as secretion has been widely hypothesized. Previous cloning of the pleckstrin gene indicated that it encoded a unique protein sequence containing no obvious structural, catalytic or otherwise functional motifs, apart from several candidate PKC phosphorylation sites and homology between the N and C terminal domains.

The role of pleckstrin in mediating cytoskeletal changes that occur during platelet activation and secretion was investigated based on published data implicating pleckstrin in directly inhibiting actin polymerization in its unphosphorylated form. There were no significant effects on the kinetics of actin polymerization in vitro using recombinant pleckstrin or pleckstrin purified from platelets. In accordance with this observation, overexpression of pleckstrin in COS cells did not appear to affect the subcellular distribution or filament length of actin compared to uninfected cells. Also, pleckstrin did not significantly co-localize with actin prior to or after exposure to phorbol 12-myristate 13-acetate (PMA) concentrations known to induce maximal phosphorylation of pleckstrin. It was therefore concluded that pleckstrin does not play a direct role in regulating actin polymerization.

Based on limited sequence homology to dynamin (which is involved in endocytosis), I postulated that pleckstrin might regulate vesicle motility, a key requirement for platelet degranulation. Pleckstrin immunofluorescently labelled in transfected COS cells did not appreciably co-localize with either ER or Golgi structures, the precursors of secretory vesicles, or with microtubules, however some punctate fluorescence was

observed that co-localized with the ER. Immunfluorescent detection of pleckstrin in peripheral blood cells indicated that pleckstrin is most highly abundant in platelets, moderately expressed in polymorphonuclear cells and absent or lowly expressed in lymphocytes.

Pleckstrin's prevailing characteristic, its phosphorylation, was examined in greater detail. Wild-type recombinant pleckstrin overexpressed in COS cells displayed a 5 fold stimulation of ^{32}P incorporation in response to PMA treatment. To exclude the possiblity of phosphorylation by kinases other than PKC, the effect of staurosporine on inhibiting pleckstrin phosphorylation was examined. Staurosporine inhibited pleckstrin phosphorylation in the heterologous COS cell system with an IC_{50} = 10 nM, similar to that reported to inhibit pleckstrin phosphorylation in the platelet. Additionally, identical phosphopeptide maps were generated of wild-type pleckstrin isolated from HL-60, HEL or transfected COS cells indicating that pleckstrin expressed recombinantly in COS cells was by all known measures functionally competent. However, a pleckstrin mutant deleted for 42 residues including the most probable phosphorylation sites was de-regulated for phosphorylation, displaying a high basal level of phosphorylation, no response to PMA, and increased association with cytoskeletal or membraneous components.

To precisely map the phosphorylation sites of pleckstrin, 8 serine or threonine residues were converted to alanine using site-directed mutagenesis. Two of these mutants, S113A and S117A, consistently displayed a lower level of PMA-induced phosphorylation compared to the wild-type protein. Tryptic phosphopeptide maps of three pleckstrin mutants, S113A, T114A and S117A indicated that phosphorylation of pleckstrin occurs almost exclusively on S113 and S117; these sites accounted for 46 and 52% respectively of the incorporated phosphate. A minor, variable amount of phosphorylation on T114 was observed. These phosphorylation sites were confirmed by phosphopeptide mapping synthetic peptides corresponding to residues 108-120 of pleckstrin, and by performing

phosphoamino acid analysis on the tryptic phosphopeptides generated in these maps.

Lastly, overexpression of each of the phosphorylation site mutants of pleckstrin in COS cells indicated that one of them, S113A, might possess a dominant activity that perturbs membrane/cytoskeletal structures. This mutant induced large, semi-circular ruffles or "lobes" in transfected COS cells in two independent experiments.

In conclusion, the function of pleckstrin is still entirely unknown. It is quite clear however that pleckstrin is an excellent substrate for PKC. Phosphorylation of pleckstrin likely affects its function, a hypothesis supported in this work by the unusual phenotype of COS cells overexpressing the S113A mutant.

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LIST OF ABBREVIATIONS

2-D	2-dimensional
ADP	adenosine diphosphate
AMP	adenosine monophosphate
AP	alkaline phosphatase
ATP	adenosine triphosphate
β-ARK	beta adrenergic receptor kinase
BCIP	5-bromo-4-chloro-3-indoyl phosphate p-toluidine salt
BSA	bovine serum albumin
Btk	bruton's tyrosine kinase
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
C-terminal	carboxy terminal
CAT	chloramphenicol transacetylase
CM	carboxy methyl
CNBr	cyanogen bromide
$cPLA_2$	cytosolic phospholipase A_2
Da	dalton
DAG	diacylglycerol
DEAE	diethylaminoethyl
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleoside triposhosphates
ds	double stranded
ECL	enhanced chemiluminescence
EDTA	ethylenediamine tetraacetic acid
ER	endoplasmic reticulum
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
fMLP	N-formyl met leu pro
G-6-P	glucose 6-phosphatase
GAP	GTPase activating protein
GDP	guanosine diphosphate
GTP	guanosine triphosphate
GST	glutathione *S*-transferase
H-7	1-(5-isoquinolinesulfonyl)-2-methylpiperazine
HEL	human erythroleukemia
HRP	horseradish peroxidase
HSV	herpes simplex virus
IC_{50}	inhibitory concentration (50% of maximal)
IEF	isoelectric focusing
Ig	immunoglobulin
IP_3	inositol trisphosphate
K_M	Michaelis-Menton constant
kDa	kilodalton
MAP kinase	mitogen activated protein kinase
MAP2	microtubule associated protein 2

MARCKS	myristoylated alanine-rich C kinase substrate
M_r	relative molecular mass
mRNA	messenger ribonucleic acid
N-terminal	amino terminal
NADPH	nicotinamide adenine dinucleotide phosphate
NBT	p-nitro blue tetrazolium
NK	natural killer
NMR	nuclear magnetic resonance
NP-40	nonidet P-40
PAGE	polyacrylamide gel electrophoresis
PBL	periferal blood leukocytes
PDGF	platelet derived growth factor
PH	pleckstrin homology
pI	isoelectric pH
PI	phosphatidylinositol
PI3K	phosphatidylinositol 3' kinase
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKC	protein kinase C
PLC	phospholipase C
PLD	phospholipase D
PMA	phorbol 12-myristate 13-acetate
PMSF	phenylmethylsulphonyl fluoride
pp	phosphoprotein
RA	retinoic acid
RBL	rat basophilic leukemia
RNA	ribonucleic acid
RPTK	receptor protein tyrosine kinase
SDS	sodium dodecyl sulphate
SH2	src homology 2
SH3	src homology 3
SV40	simian virus 40
TAE	tris-acetate-edta
TBE	tris-borate-edta
TBS	tris buffered saline
tk	thymidine kinase
TLC	thin layer chromatography
TPA	12-O-tetradecanoyl phorbol 13-acetate
TRITC	tetramethylrhodamine isothiocyanate
Tw20	tween-20
Tx-100	triton X-100
WT	wild-type
YT	yeast tryptone

INTRODUCTION

1.1 Mechanisms of Signal Transduction

The term "signal transduction" in biology refers to the transmission of chemically encoded information directionally from one medium (or macromolecule) to another. It is comparable to electronic circuitry, with many positive and negative signals converging to, or diverging from interconnecting pathways. The sum of these signals results in an appropriately generated response unique to that cell type, species and environmental demand, and can range from short-term effects such as membrane depolarization and hormone secretion to permanent alterations in gene transcription and cellular proliferation (Egan & Weinberg, 1993).

Biochemically, signal transmission involves either quantitatively increasing the absolute amount of the messenger or increasing the proportion of messenger that is in an active form. For example, new molecules may be generated extremely quickly in energetically favourable reactions from pre-existing substrates (e.g. inositol trisphosphate, cyclic AMP). Alternatively, the activity of existing molecules (e.g. proteins) may be altered by phosphorylation, subcellular re-localization, ligand-induced conformational changes, or by degradation. These latter examples also represent fast and energetically inexpensive ways to alter signalling processes, in contrast to invoking new protein synthesis.

1.1.1 Phosphoinositide Based Signalling

In 1953, ^{32}P was discovered to be rapidly incorporated into pancreas tissue phospholipids in response to acetylcholine (Hokin & Hokin, 1953). It is now well known that this phospholipid turnover results in part from the hydrolysis of a specific

phospholipid, phosphatidylinositol 4,5-bisphosphate (PI4,5P), to generate the two second messengers diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3), constituting the basis of a highly utilized signal transduction pathway. The IP_3 second messenger acts to raise intracellular calcium concentrations by allosterically activating an ion channel protein (Ferris et al., 1989) located in the endoplasmic reticulum or sarcoplasmic reticulum membranes of smooth muscle cells. Upon IP_3 binding, calcium is released from these intracellular stores into the cytoplasm (reviewed in Berridge, 1993; Miyazaki, 1995). This transient increase in cytosolic calcium affects a great number of structural proteins and enzymes, many through interaction with the small calcium-binding protein calmodulin.

The other component of the phosphoinositide (PI)-generated signal, DAG, serves to activate protein kinase C (PKC). In the presence of elevated levels of calcium, DAG and membrane components such as phosphatidylserine, PKC becomes maximally activated and phosphorylates several target proteins. In many instances, the bifurcating signal generated from PI hydrolysis acts co-ordinately to impliment cellular changes. For example, cytokine gene expression activated by the T cell receptor is dependent on a transcription complex called the NF-AT (nuclear factor of activated T cells). Whereas one subunit of this complex is dephosphorylated by the calcium/calmodulin-dependent phosphatase calcineurin, the other subunit is newly synthesized in a strictly PKC-dependent manner (Szamel & Resch, 1995).

Phospholipid-derived second messengers arise from the activity of several types of receptor coupled phospholipases (reviewed in Lee & Rhee, 1995). In platelets, thrombin receptor activation is coupled to phospholipase C (PLC) stimulation through a heterotrimeric G protein. The activated receptor stimulates GDP to GTP exchange on the alpha subunit of the G protein (G_α), which when bound to GTP has reduced affinity for the $\beta\gamma$ subunit. Phospholipase C_β can be activated by binding either the free $\beta\gamma$ subunit or the GTP-bound α subunit, resulting in hydrolysis of PIP_2 to generate DAG and IP_3. Other

phospholipases can also play a role in PKC activation. For example, DAG can be generated from hydrolysis of phosphatidylcholine by PLD, and longer, sustained increases in DAG levels are believed to be produced through this enzyme (Nishizuka, 1995). PKC is also stimulated by arachidonic acid, generated by the action of yet another phospholipase, cytosolic phospholipase A_2 ($cPLA_2$) (see Fig. 1.1).

Full platelet activation by thrombin involves dramatic morphological changes in the platelet including shape change, aggregation, adhesion and secretion of two types of secretory vesicles (Huang & Detwiler, 1986). The above events depend in large part on activation of PI-based signalling pathways that lead to PKC activation, however more recent reports suggest PKC can be activated cryptically by tyrosine kinase based signalling pathways (see section 1.1.3). Dramatic phosphorylation of a protein of apparant molecular mass 40-47 kDa, pleckstrin, the subject of this thesis, was noted to correlate temporally with platelet activation (see section 1.3), and is therefore implicated in mediating some of the above physiological responses. One such proposed role for pleckstrin is the regulation of actin polymerization (Hashimoto et al., 1987).

Intriguingly, the newly recognized PH domains of pleckstrin (Haslam et al., 1993; Mayer et al., 1993) have been postulated to bind PIP_2 (see section 1.4.3), and this particular PI has been extensively implicated in the regulation of actin-binding proteins (see section 1.1.4). Therefore PIP_2 may also influence pleckstrin function in addition to whatever effects phosphorylation (mediated by PIP_2 hydrolysis and activation of PKC) has on pleckstrin function. Additionally, many PH domain-containing proteins also contain dbl homology domains that stimulate small G proteins, and are therefore implicated in growth control and regulation of cytoskeletal dynamics (Cerione & Zheng, 1996). Therefore a link between phosphorylation of pleckstrin and changes in the platelet cytoskeleton is tenable.

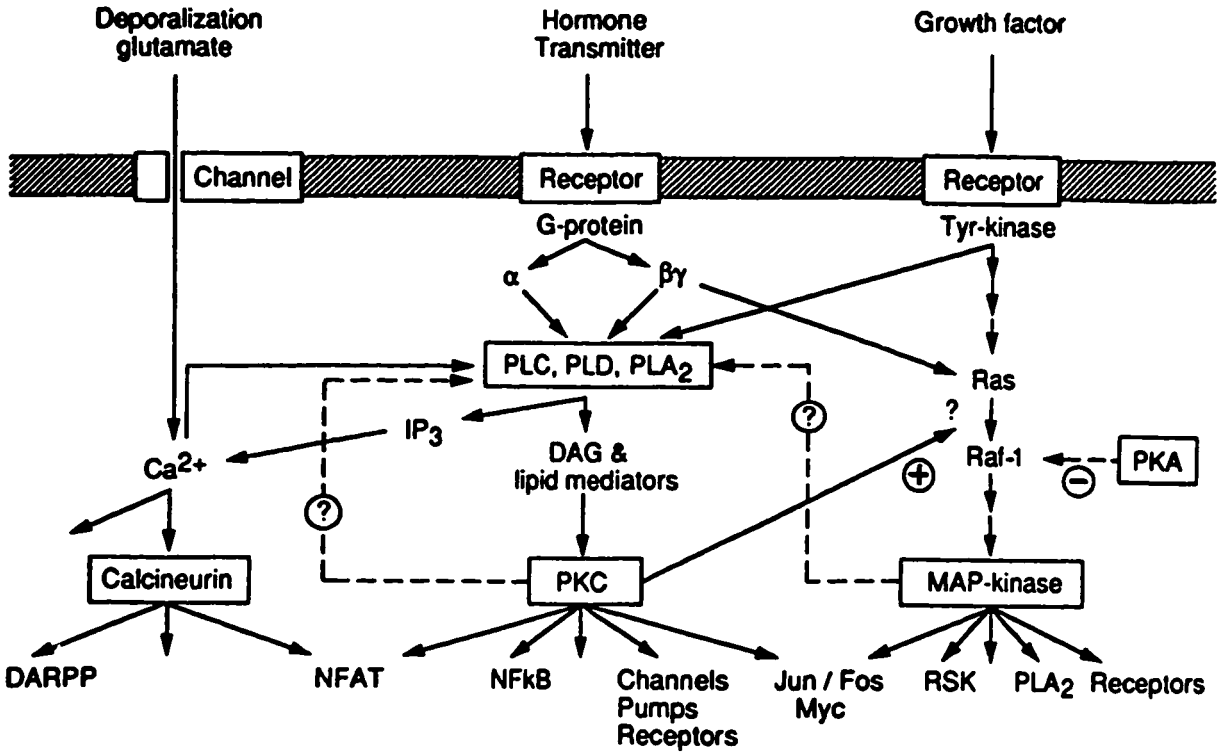

Figure 1.1 - Signalling networks involving PKC. Reproduced from Nishizuka, 1995.

1.1.2 Tyrosine Kinases in Signalling

Another major signalling mechanism involves the activation of cytosolic and transmembrane receptor protein tyrosine kinases (RPTKs). Mitogenic responses are often associated with activation of RPTKs and many oncogenes represent mutated versions of normal cellular proteins that may act at almost any point along RPTK-based signalling pathways (reviewed in Cantley et al., 1991). The generally accepted model of RPTK activation involves initial ligand binding, then receptor dimerization and autophosphorylation occur. Phosphorylated receptors are activated and can phosphorylate substrates and/or bind many SH2-containing proteins via their own autophosphorylated regions. SH2 domain/phosphotyrosine interactions provide a mechanism for the receptor to localize specific substrates and targets to the plasma membrane (reviewed in van der Geer et al., 1994). RPTKs activate ras by recruiting sos to the plasma membrane through the grb2 adaptor. Once ras is activated it can physically associate with and activate raf-1. Raf then phosphorylates mitogen activated protein kinase kinase (MAPKK or MEK), a dual specificity kinase that in turn activates MAP kinase by phosphorylation at both a tyrosine and threonine residue. Identification of this central kinase cascade resulted from an analysis of pathways controlling developmental programs in *Drosophila* and *C. elegans* and nutrient sensing in yeast (reviewed in Egan & Weinberg, 1993).

1.1.3 Cross-Talk between Tyrosine Kinase and PI-Based Signalling

A major target and substrate of RPTKs is phosphatidylinositol 3' kinase (PI3K) (van der Geer et al., 1994). PI3K activity is found associated with many cytosolic transforming oncogenes (e.g. middle T antigen, src) and is a necessary element of their oncogenic potential. This enzyme converts PI4-P and PI4,5-P_2 to PI3,4-P_2 (PIP_2) and PI3,4,5-P_3 (PIP_3) respectively. The 3'phosphatidylinositides are poor substrates for known phospholipases, therefore it is not known whether these products or their derived

inositol phosphates have a second messenger function. The only data supporting a second messenger function for these compounds is the observation that PIP_3 can activate the PKC isozymes ε, η and ζ in vitro (Nakanishi et al., 1993; and see below). Intriguingly, PKC appears to be activated in thrombin-stimulated platelets by a mode partly inhibitable by wortmannin, a specific PI3K inhibitor. Furthermore, addition of synthetic $PI3,4,5P_3$ was shown to induce pleckstrin phosphorylation, presumably through direct activation of PKC (Zhang et al., 1995).

Cross-talk occurs between phospholipid based and RPTK based signalling pathways at several levels. For example, PLCγ binds to and is phosphorylated by several RPTKs, which leads to its activation and generation of phospholipid-derived second messengers that activate PKC (reviewed in Nishizuka, 1995). Also, $cPLA_2$ can be activated by certain RTPKs through activation of MAP kinase or directly by PKC, which phosphorylates $cPLA_2$ on a particular serine residue (Lin et al., 1993). PKCα also feeds into the central ras growth regulatory pathway by directly phosphorylating and activating raf, a kinase downstream of ras (Kolch et al., 1993; and see Fig. 1.1). In other systems PKC appears to function upstream of ras through modulation of rasGAP activity. Although PKC does not appear to phosphorylate GAP directly, GAP from 12-O-tetradecanoyl phorbol 13-acetate (TPA) treated cells has a reduced ability to stimulate ras GTPase activity (Downward et al., 1990).

1.1.4 Role of Phosphoinositides and G Proteins in Cytoskeletal Organization

In platelets, activation involves shape change, aggregation and ultimately secretion. These dramatic physiological changes require re-organization of the actin cytoskeleton and are induced by agonists such as thrombin or collagen acting through the phosphatidylinositol pathway. Although PKC can phosphorylate many actin regulatory

proteins (Wilkinson, 1991), phosphatidylinositols may have a role in regulation of the actin cytoskeleton separate from the effects of calcium and PKC since many types of radiolabelled lipids were discovered to bind platelet cytoskeletal proteins (Burn, 1988). PIP_2 in particular can bind to several cytoskeletal associated proteins, such as profilin, gelsolin, villin and talin (reviewed in Janmey, 1994). Actin under physiological conditions should be fully polymerized but it is instead maintained in a 50% unpolymerized state by the existence of many actin binding and filament capping proteins. Much of the free actin in cells is complexed 1:1 with profilin and this complex can be dissociated by PIP_2, allowing a net polymerization to occur. In phosphoinositide based signalling, the initial phase of reorganization of the cytoskeleton involves release of actin from calcium-sensitive actin-plasma membrane cross-linking proteins (Janmey, 1994). Then to produce shape change, locomotion and pseudopod protrusion a second phase involving the net polymerization of actin is necessary, which may be triggered by synthesis of phosphoinositides.

The small ras related GTPases rac and rho have been implicated in cytoskeletal reorganization (reviewed in Hall, 1994; Ridley, 1995). Microinjected rac regulates growth factor induced membrane ruffling (Ridley et al., 1992) whereas microinjected rho causes formation of actin stress fibers (Ridley & Hall, 1992). rac is also a component of the NADPH oxidase system (Ridley, 1995). PKC action may lie upstream of rac and rho since many rac and rho-dependent processes (eg. lymphocyte aggregation, superoxide production, membrane ruffling) are stimulated by PKC activators. Phosphatidyloinositol 3-kinase (PI3K) is also involved in cytoskeletal dynamics since membrane ruffling and chemotaxis induced by the PDGF receptor requires an intact binding site for PI3K on the receptor. Furthermore, PI3K has been shown to function upstream of rac since wortmannin, blocks PDGF-induced membrane ruffling, but not ruffling induced by microinjected rac (Ridley et al., 1992). How rac and rho mediate cytoskeletal and

submembrane structural changes is unknown but not surprizingly many other ras-related G proteins have been implicated in controlling the intracellular vesicle traffic associated with both constitutive and regulated exocytosis (reviewed in Ferro-Novick & Novick, 1993).

1.2 Protein Kinase C

1.2.1 Structural Aspects

PKC was first identified by Nishizuka and colleagues as a cyclic nucleotide-independent serine/threonine protein kinase (Takai et al., 1977; Inoue et al., 1977). It has since its discovery been postulated to play a significant role in tumour promotion, gene transcription, exocytosis, ion channel function (reviewed in Kaczmarek, 1987) and even long term potential (memory) (Abeliovich et al., 1993a) and spatial and learning facets in brain (Abeliovich et al., 1993b). Twelve PKC isoforms have been discovered making PKC the largest known serine/threonine kinase family (Parker, 1992). PKC isoforms fall into two main categories: the conventional calcium-dependent α, β, γ group and the nonconvential PKC's, δ, ε and η. PKC ζ and yeast PKC comprise yet another distinct subfamily. All PKC isoforms have an N-terminal regulatory domain and a highly conserved C-terminal catalytic domain. The C1 region of the regulatory domain contains a pseudosubstrate site (House & Kemp, 1990) that folds over the catalytic site presumably preventing substrate from binding. A cysteine-rich diacylglycerol or phorbol ester binding motif also resides within the C1 region.

To activate PKC, DAG, phospholipid and calcium are required. Evidence exists that ζ and possibly λ are not activated by DAG or phorbol ester, and ζ, as stated earlier, may be activated by the product of PI3K action, PI-3,4,5P_3 (Nakanishi et al., 1993). After DAG binds to the C1 region, the enzyme's affinity for calcium is lowered to physiological concentrations and a conformational change occurs moving the pseudosubstrate region away from the catalytic site, facilitating substrate binding and phosphorylation. The δ, ε,

ζ, η isoforms differ by lacking the C2 region that is believed to contain a calcium-dependent phospholipid binding activity, and correspondingly the interaction of these isoforms with lipid membranes is calcium-independent (reviewed in Huang, 1989).

1.2.2 Activators and Inhibitors of PKC

Certain phorbol ester derivatives such as TPA mimic DAG and compete for binding to the C1 region of PKC. Since TPA is membrane permeable and relatively non-hydrolyzable, this compound can constitutively activate PKC. Activation of PKC results in its translocation to a particulate fraction, whereas any remaining soluble PKC does not appear to be active (Blackshear, 1988). Prolonged phorbol ester treatment is known to result in downregulation of PKC through a proteolytic susceptibility of the activated enzyme. For example, exposure of murine fibroblasts to 16 nM TPA for 16 hours leads to a 95% reduction in both PKC protein and activity (Blackshear, 1988). Similar phorbol ester binding sites have been found in other proteins (eg. N-chimerin), indicating the possibility that processes independent of PKC activation may occur under phorbol ester treatment.

Beside phorbol esters, other tools employed to study PKC function are natural product and synthetic PKC inhibitors. A serious caveat with these inhibitors is that they are relatively non-specific, capable of inhibiting several different kinases at similar concentrations. For example, the H-7 inhibitor [1-(5-isoquinolinesulfonyl)-2-methylpiperazine], was used for many years to implicate PKC in regulating various cellular processes (Schachtele et al., 1988; Nath & Powledge, 1988). However, other effects of H-7 were seen apart from its effect on PKC (Love et al., 1989; Conquer & Mahadevappa, 1990; Goodman et al., 1990). In one study, H-7 was only 50% effective at inhibiting PKC activation in *E. coli*-exposed macrophages at a concentration of 100 nM (Gbarah et al., 1989). Staurosporine has replaced H-7 as a more effective PKC inhibitor (Tamaoki et

al., 1986), yet even this compound lacks specificity, having identical IC_{50} values for the tyrosine kinase v-src and PKC (6 nM; O'Brian & Ward, 1990). Staurosporine is now superceeded by more selective PKC inhbitors such as the fungally-derived calphostin C and cercosporin (Tamaoki & Nakano, 1990).

1.2.3 Localization and Function of Specific PKC Isoforms

Since there is stronger homology between individual PKC isoforms of different species than between isoforms within a species, it has been postulated that unique isoforms have arisen to fill tailored functions within the cell (reviewed in Dekker & Parker, 1994; Hug & Sarre, 1993). In fact the localization, cofactor requirements and possibly substrate specificity are different for the different isoforms. For example, the tissue distribution of α, β and ζ is widespread, whereas γ, η and θ expression is limited to only a few tissues. Fibroblast and neuroblastoma cell lines lack PKC βI and βII expression (Hug & Sarre, 1993) whereas PKCα is absent from some myeloid cell types (Grabarek et al., 1992). Lack of expression of ε is also characteristic of myeloid lineages (neutrophils, macrophages, megakaryctes & platelets), but not erythroid lineages (Hug & Sarre, 1993).

Particular isoforms also behave distinctly in their subcellular localization depending upon the state of the cell. For example, PKCα and β relocalize to the particulate (membrane and cytoskeleton) fraction in platelets after thrombin treatment whereas δ does not. PKCα can alternatively display a nuclear relocalization in certain situations such as when fibroblasts are treated with thrombin or TPA (Hug & Sarre, 1993; Thomas et al., 1988). βII also has the ability to translocate to the nucleus in HL-60 cells where it is believed to phosphorylate lamin B (Hocevar & Fields, 1991). In support of these observations, a potential nuclear targeting motif has been discovered in the regulatory domain of PKC (Malviya & Block, 1992).

Because phorbol esters constitutively activate and yet also deplete PKC, it has been

disputed whether the long-term effects of phorbol esters are consequences of PKC activation or of PKC disappearance (Blackshear, 1988). Equally confusing, in some cases only the α, but not the β isoforms are depleted by prolonged phorbol ester treatment, therefore caution should be used in assessing studies claiming to deplete cellular PKC by this method (Hug & Sarre, 1993; Farese et al., 1992). With the cloning of various PKC isoforms it has however become possible to address the issue of PKC's role in mediating the sometimes tumorigenic effects of prolonged phorbol ester treatment. Overexpressed βI can induce transformation of rat fibroblasts whereas similarily expressed α has yet to produce a tumorigenic phenotype in the cell lines tested (Borner & Fabbro, 1992). In the continued presence of TPA, the βI-expressing cells were even more strongly tumorigenic, as evidenced by a higher number of foci formed in soft agar. In other cell types however, βI induced growth inhibition and differentiation (Borner & Fabbro, 1992). Therefore, the effects of long-term TPA treatment appear to reflect a combination of cell type and isoform expression within that cell type.

In cell free systems, PKC isoforms can phosphorylate many substrates, yet no one substrate phosphorylation event can clearly be attributed to activation of one particular isoform (Dekker & Parker, 1994). However in vitro studies indicate that some isoforms display different activities towards histone IIIS, an observation that appears to result from differences in effectiveness of particular psuedosubstrate regions to compete against substrate for binding (Dekker & Parker, 1994).

1.2.4 Targets of PKC

There has been tremendous difficulty in trying to identify physiologically relevant substrates of PKC (Berridge, 1987). PKC can phosphorylate many cytoskeletal proteins (eg. vinculin, talin), but how this may mediate structural changes is not always obvious (Borner & Fabbro, 1992). High levels of PKC are found in brain tissues and some PKC

substrates specific to brain have been identified. The most interesting of these is neuromodulin, which is localized to the growth cones of developing neurites, and has been implicated in axonal regeneration and synaptic plasticity (Benowitz & Routtenberg, 1987). PKC regulates neuromodulin function by phosphorylating a site in neuromodulin which destroys the ability of this protein to bind calmodulin (Apel et al., 1990). Another PKC substrate, the myristolyated alanine-rich C kinase substrate (MARCKS) also binds Ca^{2+}/calmodulin in a phosphorylation dependent manner (Graff et al., 1989). MARCKS is localized to focal contact regions of the plasma membrane where it may serve to cross-link actin to focal contacts (reviewed in Aderem, 1992). A PKC-dependent phosphorylation event releases MARCKS from the membrane into the cytosol where it remains associated with actin but can no longer cross-link it. In this way, PKC might control the local cytoskeletal dynamics at the plasma membrane that are required for filopodia formation.

PKC can regulate gene transcription by directly phosphorylating several transcription factors (reviewed in Meek & Street, 1992). It can also regulate the nuclear localization and therefore activity of the NF-κB transcription factor by phosphorylating its cytosolic anchor, IkB, in a variety of cells (Baeuerle & Baltimore, 1988). PKC activation also indirectly induces dephosphorylation of a threonine residue in the c-jun protein that allows c-jun to associate with the promoter elements of TPA-responsive genes (Boyle et al., 1991). Undoubtedly the effects of PKC activation are pleiotrophic, resulting in either transient responses, or permanent changes in cellular function through alterations in gene transcription.

1.3 Original Characterization of Pleckstrin

1.3.1 Evidence of a Role for Pleckstrin (p47) Phosphorylation in Platelet Secretion

Protein phosphorylation is a major mechanism of regulating components of signalling pathways. The platelet undergoes regulated secretion in response to several physiological activators, and thus phosphorylation of key platelet proteins was postulated in part to mediate this dramatic cellular response (Greengard, 1978). Phosphorylation state differences in platelet proteins were first characterized by comparing the intensities of ^{32}P-labelled proteins separated by SDS-PAGE from pre and post stimulated platelets (Lyons et al., 1975). Out of numerous labelled bands, only 2 migrating at 20 and 40 kDa became appreciably phosphorylated during activation by thrombin. This increase was 2-6 fold for both bands and occurred half maximally 10-14 seconds after thrombin addition, correlating well with the timing of secretion as monitored by tritiated 5-hydroxytryptamine (serotonin) release (Lyons et al., 1975). The effect was dependent on an intact plasma membrane since thrombin treatment of sonicated platelets could not induce this phosphorylation, providing evidence for a transmembrane signalling component(s). Agents that elevate intracellular cAMP (e.g. phosphodiesterase inhibitor) were known to antagonize platelet activation and these also blocked phosphorylation of the 20 and 40 kDa proteins, again correlating phosphorylation with secretion and ruling out phosphorylation by a cAMP-dependent kinase.

Other groups reported similar findings in platelets (Haslam & Lynham, 1977), and mast cells (Sieghart et al., 1978). A 200% increase in phosphorylation of the 40,000 to 47,000 Da region of the gel was reported to occur under collagen, thrombin and calcium ionophore treatment (Haslam & Lynham, 1977). This phosphorylation was shown to be intimately associated with the secretory response since aggregation induced without secretion by ADP or fibrinogen did not increase p47 (pleckstrin) phosphorylation. Others

confirmed that both aggregation and secretion correlated temporally with pleckstrin phosphorylation, whereas initial platelet shape change did not (Verhoeven et al., 1985).

The absolute requirement of pleckstrin phosphorylation for secretion was questioned as some activators of platelet shape change (e.g. concanavalin A) induced phosphorylation of pleckstrin without inducing the release reaction (Bennett et al., 1979). Therefore, although pleckstrin phosphorylation may be necessary for secretion, it is not sufficient for this process. Further investigation showed that partial secretion could actually occur in the absence of pleckstrin phosphorylation, under conditions of high calcium and aspirin (Rittenhouse & Horne, 1984), questioning even the necessity of pleckstrin phosphorylation for secretion. In contrast, the requirement for calcium in triggering release is clear (Haslam & Lynham, 1977; Bennett et al., 1979; Walker & Watson, 1993). However, concentrations of calcium sufficient to produce maximal pleckstrin phosphorylation did not produce maximal secretion (Haslam & Davidson, 1984), again implying that pleckstrin phosphorylation does not strictly correlate with secretion. Finally, in a clinical study, a patient's platelets that were defective for the aggregation response to weak agonists displayed a slower rate of pleckstrin phosphorylation under conditions which still elicited normal levels of secretion (Speiser-Ellerton & Weiss, 1990). In summary, pleckstrin's role in secretion is presently undefined. Evidence exists that pathways separate from ones that activate PKC may have a role in eliciting a full secretion response in combination with PKC-dependent events (Coorssen et al., 1990).

1.3.2 Evidence for Pleckstrin Phosphorylation by PKC

Once it was discovered that thrombin stimulated platelets rapidly produce DAG, a PKC stimulator, pleckstrin was realized as a likely substrate for the newly activated PKC. Kawahara and colleagues showed that pleckstrin phosphorylation could be stimulated by

exogenous phospholipase C and blocked by the PKC inhibitors chlorpromazine and dibucaine (Kawahara et al., 1980). Additionally, it was determined that pleckstrin was the major substrate of platelets phosphorylated in vitro by purified PKC (Kawahara et al., 1980). Fingerprint analysis of pleckstrin phosphorylated in vivo in response to thrombin stimulation, or by purified PKC in vitro was identical, indicating that PKC is indeed likely responsible for pleckstrin phosphorylation in vivo (Sano et al., 1983).

1.3.3 Pleckstrin Phosphorylation as an Index of PKC Activation

Although the function of pleckstrin is unknown, numerous groups have used the degree of pleckstrin phosphorylation as an indirect measure of PKC activity in the platelet (Saitoh et al., 1989; Nguyen et al., 1991; Kroll et al., 1993). Phosphorylation of pleckstrin concomitant with other physiological platelet responses was used as evidence for instigating PKC in regulating platelet activating factor-induced platelet responses (Murphy & Westwick, 1992), synthesis of intracellular histamine (Saxena et al., 1991) and questioned PKC's role in metabolism of inositol phosphate derivatives (Watson et al., 1988). More recently it has been used to assess PKC's role in cathepsin G-induced platelet activation (Si-Tahar et al., 1996) and megakaryocytic differentiation of HEL cells (Hong et al., 1996).

Pleckstrin has also been used in in vitro assays of PKC activity, with the argument that it is a more physiological and selective PKC substrate than the commonly used histone III-S (Sutherland & Walsh, 1989). Interestingly, pleckstrin was not phosphorylated by purified aortic PKC stimulated by cis-unsaturated fatty acids, whereas histone was (Dell & Severson, 1989). Phosphorylation of pleckstrin by this PKC preparation was more sensitive to phosphatidylserine and calcium concentrations than was histone phosphorylation, intimating perhaps that specific PKC isoforms do have physiologically relevant substrate preferences and that pleckstrin might be a better substrate for the calcium-

dependent kinases (α,β,γ) than histone. In support of this, the potential phosphorylation site of pleckstrin at residues 108-110 most closely resembles the PKCα, βI and βII pseudosubstrate regions. However, pleckstrin phosphorylation by a calcium-independent kinase has been detected (Tsukuda et al., 1988), which may be attributable to the δ and ζ PKC isoforms that are expressed in platelets (Hug & Sarre, 1993).

1.3.4 Original Biochemical Characterization of Pleckstrin

Pleckstrin was originally purified from platelets by preparative SDS-PAGE and estimated to comprise 0.65% of total platelet protein (Lyons & Atherton, 1979). The stoichiometry of phosphorylation was 0.3-0.5% mole of phosphate/mole of protein (another group reported up to 4.3 mole phosphate could be incorporated per mole pleckstrin in vitro (Connolly et al., 1986)). The amino acid composition of pleckstrin was sufficiently different from actin, which migrates near pleckstrin by SDS-PAGE, and tubulin to rule out identity with these major cytoskeletal proteins. It also did not co-migrate with partially purified cAMP binding proteins, indicating it was not the regulatory subunit of cAMP-dependent kinase (Lyons & Atherton, 1979).

Pleckstrin was determined to be cytosolically localized by its presence in the 90,000 g supernatant of disrupted platelets (Fox et al., 1979). Phosphorylated pleckstrin from thrombin treated platelets isoelectrically focused on 2-D gels as 7-9 individual spots between pH 6.5 and 6.1 (Imaoka et al., 1983), suggesting that pleckstrin forms multiple phosphorylated isomers. Hypophosphorylated pleckstrin focused between pH 6.6 and 6.8 (Imaoka et al., 1983) in agreement with a previous report of 6.82 (Lyons & Atherton, 1979). Phosphoamino acid analysis indicated that pleckstrin contained 0% phosphotyrosine, 83% phosphoserine and 17% phosphothreonine, consistent with phosphorylation by a serine/threonine specific kinase such as PKC. All incorporated

phosphate was localized to a 14,800 Da V8 protease resistant fragment (Imaoka et al., 1983).

1.3.5 Erroneous Identities of Pleckstrin

A 5'-phosphomonoesterase activity specific for inositol 1,4,5-trisphosphate was purified from platelets (Connolly et al., 1985) and subsequently proposed to be pleckstrin (Connolly et al., 1986), based upon the following evidence: i) the esterase could be phosphorylated by PKC in vitro, which increased its activity, ii) this protein co-migrated by SDS-PAGE with the 40 kDa protein (pleckstrin) phosphorylated in response to thrombin when mixed with ^{32}P labelled platelet proteins, iii) one dimensional ^{32}P labelled peptide maps of these two proteins were the same.

Since increases in IP_3 concentration generated from receptor stimulated PI turnover mobilizes intracellular calcium, phosphorylation of this esterase by Ca^{2+}-activated PKC would provide a negative feedback mechanism by increasing hydrolytic activity towards IP_3. However, it is not likely a protein comprising 0.65% of total cellular protein would function solely as a highly specific esterase, its activity being only 3 fold increased by phosphorylation. Phosphorylation of this esterase in vivo under physiological conditions (eg. thrombin stimulation) was not examined. A likely explanation for the conclusions in Connolly et al., is the co-purification of pleckstrin and a minor esterase component of the same apparent M_r, which was originally characterized as a 38 kDa, not a 40 to 47 kDa protein (Connolly et al., 1985). Subsequent to this report, a 5'-phosphomonoesterase activity was partially chromatographically separated from pleckstrin (Dr. R.J. Haslam, personal communication).

Pleckstrin was proposed to be a lipocortin possessing anti-phospholipase A_2 (PLA_2) activity, based on the observation that a 40 kDa protein possessing anti-PLA_2 activity in neutrophils was phosphorylated in a PKC-dependent manner (Touqui et al.,

1986). Phosphorylation suppressed the anti-PLA_2 activity of this lipocortin, supporting a model allowing mobilization of arachidonic acid to occur after platelet and PKC activation. The hypothesis that pleckstrin and this lipocortin were indistinguishable was based on a monoclonal antibody to lipocortin V that partially reduced the amount of the phosphorylated 40 kDa band (extent not quantitated). As it was subsequently shown that pleckstrin is not a lipocortin (Tyers et al., 1988) these results suggest that pleckstrin may not represent the major 40 kDa phosphorylated band in neutrophils.

Yet another group reported that pleckstrin was indistinguishable from the α subunit of pyruvate dehydrogenase (Chiang et al., 1987). This protein is released from the mitochondrial inner membrane during platelet disruption by freezing or sonication. Using affinity chromatography, a 42 kDa peptide component of pyruvate dehydrogenase was purified and phosphorylation of this peptide during platelet activation correlated with diminished pyruvate dehydrogenase activity. Evidence against this protein being pleckstrin included its less than 1.5 fold stimulation of phosphorylation during platelet activation, and its association with a pyruvate dehydrogenase kinase in the cell that clearly was not PKC. Therefore the only evidence linking the α subunit of pyruvate dehydrogenase to pleckstrin was similarity in apparent molecular weight and ability to be phosphorylated.

Pleckstrin purified from resting platelets was shown to inhibit actin polymerization in vitro (Hashimoto et al., 1987). However, phosphorylated pleckstrin purified from thrombin treated platelets was not inhibitory. Therefore pleckstrin phosphorylation concomitant with platelet activation was proposed to orchestrate the massive reorganization of actin filaments that is a necessary antecedent to secretion. This putative function of pleckstrin could not be ruled out based on pleckstrin's primary sequence, since pleckstrin could represent a novel actin binding protein (Tyers et al., 1988). This hypothesis was additionally conceivable since an actin binding protein would be expected to be relatively

abundant, as is pleckstrin in platelets. Although it was subsequently shown that pleckstrin does not bind actin (Sasaki et al., 1988), an indirect role for pleckstrin in modulation of actin dynamics has not been ruled out.

1.3.6 Molecular Characterization of Pleckstrin

With the cloning of the pleckstrin cDNA from retinoic acid differentiated HL-60 cells (Tyers et al., 1988), two of the preceding identities for pleckstrin were clearly wrong: the deduced pleckstrin protein sequence showed no similarity to the human lipocortin V or pyruvate dehydrogenase sequences. The pleckstrin sequence was unique and contained several highly probable PKC phosphorylation sites as would be expected of a major PKC substrate. A potential Ca^{2+} binding EF hand motif at residues 292-320 was identified by computer comparison of the pleckstrin sequence with known EF hands (Tyers et al., 1989). Attempts to demonstrate calcium binding for pleckstrin were not successful (Tyers, Ph.D. thesis), and it is now known that this region resides within a pleckstrin homology domain (see below), which contains no EF hand loop in its tertiary structure.

Pleckstrin appears to be expressed exclusively in the heamatopoietic cells of higher vertebrates. Pleckstrin expression is highest in the platelet, with variable levels seen in other heamatopoietic cell lines possibly partly reflecting their degree of differentiation (Tyers et al., 1988). For example, pleckstrin abundance is low in human promyleocytic HL-60 cells but increases 4 fold (to 0.07% of total cellular protein) in response to 1μM retinoic acid (RA) treatment, which induces a granulocytic phenotype (Tyers et al., 1987). TPA-induced monocytic differentiation did not however reproducibly lead to increased pleckstrin expression. Others have confirmed the presence of pleckstrin in mature granulocytes, T and B cells, and its absence from erythrocytes (Gailani et al., 1990).

1.4 Modular Domains in Signal Transduction: Pleckstrin Homology Domains

Signal transduction depends critically on specific components of signalling pathways making regulated interactions with effector proteins (reviewed in Pawson, 1995; Cohen et al., 1995). In addition to simply activating other components, these interactions may serve to relocalize proteins to the plasma membrane to facilitate their interaction with other membrane bound components, or to change their activity by post-translational modifications such as phosphorylation. Two semi-conserved protein-protein interaction motifs common to certain subsets of signalling molecules have been discovered. The src-homology 2 (SH2) domain binds phosphotyrosine-containing sequences, and SH3 domains bind proline rich motifs. The pleckstrin homology domain (PH) domain has been identified as a third major prevalent motif in signalling proteins (Haslam et al., 1993; Mayer et al., 1993). Recently another distinct phosphotyrosine interaction domain (the PID motif), has been discovered (Bork & Margolis, 1995). Also referred to as the PTB (phosphotyrosine binding) domain, this motif is present in the insulin receptor substrate 1 (IRS-1) protein and the adaptor protein shc (Lemmon et al., 1996). Crystallographic analysis has revealed that the 3-dimensional structure of the PTB domain is superimposible on the PH domain, therefore questioning whether or not PTB domains are truly distinct from PH domains (Eck et al., 1996).

1.4.1 Identification of Pleckstrin Homology Domains

Sequence alignment of pleckstrin with itself highlighted a region of homology between the N and C termini (Tyers et al., 1988). The C terminal region also scored weakly but significantly against a region in rasGAP just C-terminal to its SH2-SH3-SH2 motif (Tyers, Ph.D. thesis). Subsequently the human sec7 homolog (Liu & Pohjadak, 1992) and dynamin were noted to have similarity to pleckstrin within this same region.

These regions are now collectively known as pleckstrin homology (PH) domains, prevailingly found in proteins that are either regulators of small G proteins (eg. rasGAP, sos, Tiam-1), or are kinases (rac, tec, btk) or are involved in cytoskeletal aspects of the cell (dynamin and β-spectrins)(Musacchio et al., 1993; Gibson et al., 1994), suggesting PH domains mediate specific functions required for cellular signalling. Fig. 1.2 shows the primary sequence of some representative PH domains, and the secondary structure of the dynamin PH domain. Relatively conserved residues shaded in Fig. 1.2 have been shown from structural studies to be important for maintainance of the secondary and tertiary folding of the domain. The low sequence conservation of the PH domain (any one PH domain is at most 21% identical any other PH domain), delayed the identification of many other PH domains already present within protein sequence databases (Gibson et al., 1994). The number of PH domains, now comparable to SH2 domains, will likely exceed the latter since many SH2 domains have been selectively identified by homology screening (Shaw, 1996).

1.4.2 PH Domain Structure

Using heteronuclear NMR, the three dimensional structure of the N-terminal PH domain of pleckstrin has been solved (Yoon et al., 1994). Two other PH domain structures, that of β-spectrin (Macias et al., 1994) and dynamin (Ferguson et al., 1994) have also been solved by homonuclear NMR and X-ray crystallography respectively. All three structures consist of two orthogonal beta sheets and a long C-terminal alpha helix that closes off the wide end of the wedge structure produced by the beta sheets (reviewed in Wagner, 1994; see Fig. 1.3). Like SH2 and SH3 domains (Cohen et al., 1995), the PH domain folds into a compact globular form with the N and C terminal residues closely positioned. This self-contained folding supports the notion that the PH domain functions as a module able to insert into proteins without perturbing overall protein structure or

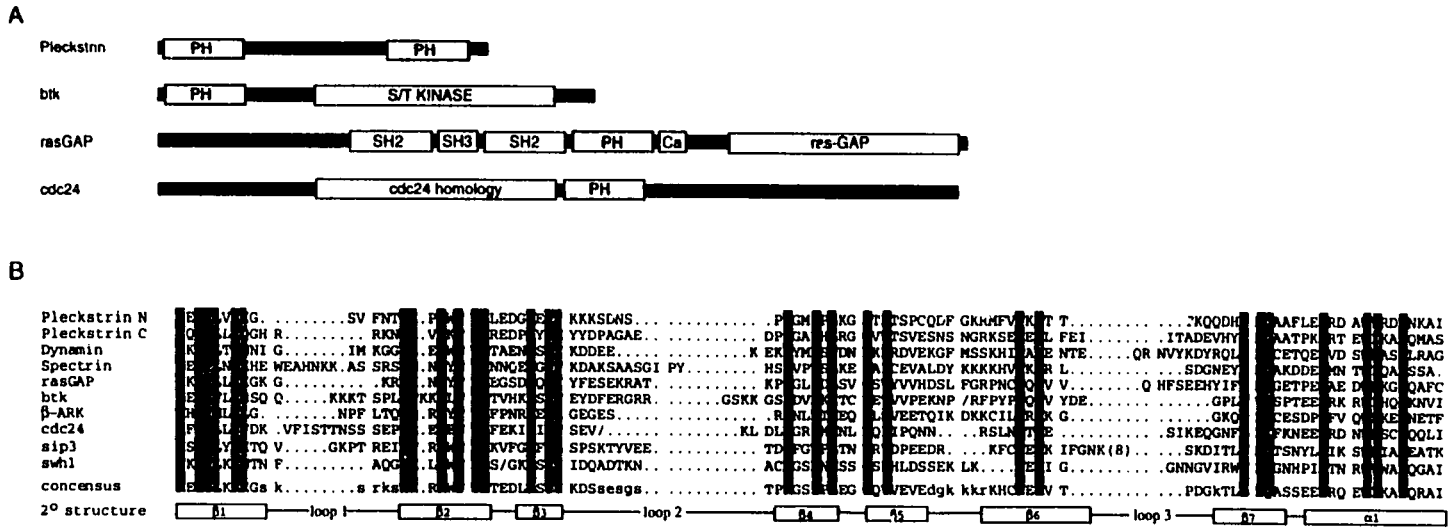

Figure 1.2 - The PH domain module.

A) Line diagrams of some proteins that contain PH domains. B) Protein sequence alignment of some representative PH domains with conserved residues shaded grey and the invariant tryptophan dark grey. The secondary structure of the dynamin PH domain is shown below. Adapted from Ferguson et al., 1994.

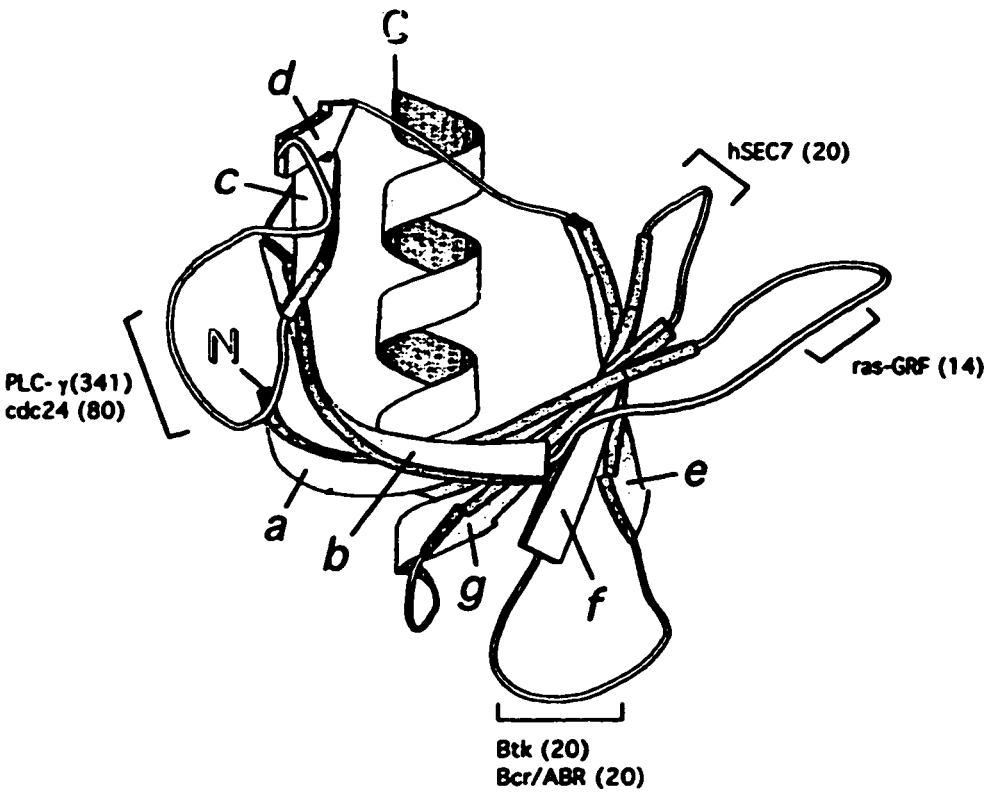

Figure 1.3 - Three-dimensional representation of the PH domain.

This model is based upon the solution structures of both the PH domain of β-spectrin and the N-terminal pleckstrin PH domain. The PH domains of some proteins have variously sized insertions (as indicated) in the loop regions between particular β-strands. The beta strands labelled *a* through *g* above correspond to the beta structures designated β1 - β7 in Fig. 1.2. Reproduced from Macias et al., 1994.

function (Gibson et al., 1994). Three variable loops exist which can tolerate large insertions, such as a SH2-SH2-SH3 motif in phospholipase Cγ (Fig. 1.3). Only one residue, a tryptophan, is invariant with two exceptions: Tiam1/2 has a phenylalanine at this position, although this sequence alignment is uncertain (Gibson et al., 1994); and quite intriguingly, 2 out of 5 individual pleckstrin cDNA's transcribed from HL-60 mRNA coded for an arginine at this site (Tyers et al., 1988). It may be possible that this mutation (identifiable as a Sma I restriction fragment length polymorphism (Tyers, Ph.D. thesis)), is either partially causitive of, or results from the transformed phenotype of HL-60 cells.

Like pleckstrin itself, there is no known function for the PH domain. However, genetic evidence suggests that at least one PH domain is essential to signalling processes controlling proper haematopietic development. The genetic lesions causative of the immunodeficient phenotype of XID mice and a similar condition in humans, agammaglobinaemia, have been mapped to the PH domain of the mouse bruton's tyrosine kinase (btk) gene and its human homolog atk. Some of these lesions are insertions or deletions predicted to cause gross structural perturbations of the PH domain. More informative is a point mutation at a predicted semi-conserved surface exposed residue (arg_{20} to cys in mice; arg to his in humans) that results in a similar phenotype, suggesting this residue is critical to PH domain function.

1.4.3 Theories of PH Domain Function

Despite the fact that the structure of several PH domains have been solved (Yoon et al., 1994; Macias et al., 1994; Ferguson et al., 1994), no highly convincing data exist to support any particular function for the PH domain. PH domains have been postulated to function as membrane anchors via a capacity to bind PIP_2 (Harlan et al., 1994). By monitoring NMR chemical shifts of PH domains in the presence of increasing lipid concentrations, Fesik and co-workers showed that the N-terminal PH domain of pleckstrin

bound specifically to PIP_2 whereas other inositol derivates were not bound (Harlan et al., 1994). The interaction appeared primarily electrostatic, and is functionally speculative in the absence of a hydrophobic lipid binding region of the PH domain (Ferguson et al., 1994). These experiments required the presence of high detergent concentrations to prevent precipitation of pleckstrin's N-terminal PH domain (Harlan et al., 1994), which may not reflect physiological conditions.

Other considerations cast doubt on the significance of PIP_2 binding as regards PH domain function. Since nearly 2/3rds of pleckstrin consists of PH domains, it is difficult to imagine such a large portion of a protein being solely dedicated to membrane anchoring. Also, pleckstrin has been characterized as cytosolic (Fox et al., 1979; Imaoka et al., 1983), which is inconsistent with a membraneous localization. The low sequence conservation among PH domains is also intuitively inconsistent with a hypothesis dictating that all PH domains bind the same small ligand. Thus, it is probable that PH domains have alternate functions that are modulated by, or entirely separable from, PIP_2 binding. Since pleckstrin is predominantly composed of PH domains, an elucidation of pleckstrin function may in the future reveal general characteristics endogenous to all PH domains.

PH domains have been reported to bind $\beta\gamma$ subunits of heterotrimeric G proteins. Based on deletion analysis, the interaction of β-adrenergic receptor kinase (βARK) with $G_{\beta\gamma}$ was delineated to a region comprising the alpha helix of the βARK PH domain and sequences C-terminal to the PH domain (Koch et al., 1993). The necessity of PH domains in mediating this interaction is doubtful since a peptide spanning only the last 9 amino acids of the PH domain but containing 17 residues C-terminal to the PH domain effectively blocked this interaction (Koch et al., 1993). In a different report, nine unique PH domain-GST fusion proteins were shown to bind $G_{\beta\gamma}$ to varying extents in vitro, however, high concentrations of purified components were used in this assay (500 nM GST fusion; 183

nM $G_{\beta\gamma}$) (Touhara et al., 1994). However, the discovery that β isomers of phospholipase C contain PH domains strengthens the βγ binding hypothesis since this class of enzymes is known to be directly activated by βγ subunits (Parker et al., 1994). Recently, overexpression of several individual PH domains in COS-7 cells was shown to inhibit the $G_{\beta\gamma}$ dependent activation of PLC (Luttrel et al., 1995). The lack of PH domain specificity in this report may more realistically reflect the sequestration of PLC's substrate, PIP_2 by these overexpressed PH domains, rather than their direct binding to βγ subunits. Overexpression of pleckstrin was also shown to inhibit phosphoinositide hydrolysis (Abrams et al., 1995a), an effect apparently dependent on both an intact N-terminal PH domain and an unaltered phosphorylation region encompassing serines 113, 117 and threonine 114 of pleckstrin (Abrams et al., 1995b).

Two reports suggest that PH domains have affinity for particular PKC isoforms (Yao et al., 1994; Konishi et al., 1994). The PH domain of Btk appears to bind PKC since both anti-Btk serum and a Btk PH domain-GST fusion precipitate several PKC isoforms from mast cell lysates (Yao et al., 1994). The PH domain of rac kinase may mediate association of rac with PKC ζ, possibly via an interaction between the regulatory domain of PKC and the β1 and β2 strands of the PH domain (Konishi et al., 1994). It is not clear whether these PH domain-PKC interactions are functional in the sense of a distinct signalling purpose or are manifestations of enzyme-substrate interactions, since PKC may phosphorylate these and many other PH domain containing proteins, most noteably pleckstrin. Another theory to explain these PKC - PH domain interactions involves the PH domain of btk indirectly interacting with PKC by binding the WD40 repeats of a PKC associated protein, RACK (receptor for activated C kinase). Precedence for such a theory is largely derived from PH domain interactions with the WD40-containing β subunits of heterotrimeric G proteins (Shaw, 1996).

In direct analogy to the specific binding of SH2 domains to phosphotyrosine containing peptide sequences, PH domains have been postulated to recognize phosphorylated serine or threonine residues in the context of variable peptide sequences (Gibson et al., 1994). This theory was drawn from some superficial similarities between SH2 and PH domains, such as their similar size and their prevalence in an overlapping set of signalling proteins, that are often themselves targets for tyrosine (SH2 domain proteins) or serine/threonine (PH domain containing proteins) phosphorylation. The noted low sequence conservation among PH domains, in conjunction with the 3 variable loop regions of PH domains proposed to encapsulate a positively charged ligand binding cleft, is consistent with the binding of negatively charged phosphoserine/threonine residues within the context of a variable peptide sequence. Although this hypothesis is extremely intriguing (by analogy to the impact SH2-phophotyrosine recognition has on our understanding of signal transduction), in the absense of any supporting biochemical evidence it remains purely speculative.

1.5 Rationale and Scope of This Work

Phosphorylation events are crucial to protein signalling pathways, yet the effect phosphorylation has on protein structure and function is often not known. In fact, it is estimated that 1/3rd of all proteins are phosphorylated, and approximately 2000 protein kinase genes appear to exist in the human genome (Hunter, 1995). Since pleckstrin is the major PKC substrate of platelets, both its existence and phosphorylation are assumed to be critical to aspect(s) of platelet function that occur after thrombin stimulation.

The first half of this thesis describes the purification of recombinant pleckstrin. This was undertaken in part to facilitate in vitro experimentation on putative pleckstrin functions, and partly to provide a source of antigen to produce anti-pleckstrin antibodies essential for further functional characterization of pleckstrin. The second part of this thesis

concerns expression of pleckstrin in a heterologous mammalian system. Transient transfection of monkey kidney epithelial (COS) cells allowed for high level expression of human recombinant pleckstrin. Wild-type pleckstrin and various pleckstrin site specific and deletion mutants were then examined in this heterologous system with respect to subcellular localization and potential phenotypic effects. Finally, the in vitro and in vivo phosphorylation sites of pleckstrin were mapped.

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals and Reagents

The following is a list of certain chemicals and reagents used in this work and their source.

Chemical or Reagent	Supplier
Acrylamide	BioRad
Agarose - Low Melting Temperature	FMC
Ammonium Persulfate	BioRad
Ampicillin	Sigma
ATP	BRL/Gibco
BCIP	BioRad
Bis-acrylamide	BioRad
DEAE-dextran	Pharmacia
1,4-Diazabicyclo[2.2.2]-octane	Sigma
sn-1,2-dioleoylglycerol	Sigma
dNTP's	BRL/Gibco
Dry skim milk powder	Carnation
Glutathione	Sigma
Glycine	Biorad
H-7	Sigma
NBT	BioRad
NP-40	Sigma
Phenol	USB
Phosphatidyl serine	Sigma
polyvinylpyrrolidone, M_r 360,000	Sigma
Retinoic Acid	Sigma
Rhodamine conjugated phalloidin	Sigma
SDS	Biorad
Staurosporine	Sigma
Tetracyclin	Sigma
TPA	LC Services
Tris	Biorad
Triton X-100	Sigma
Urea	Biorad

2.1.2 Radiochemicals

[α^{35}S]-dATP (1000 Ci/mmol), [^{32}P]-orthophosphate (9000 Ci/mmol) and [γ^{32}P]-ATP (3000 Ci/mmol) and [α^{32}P]-dATP (3000 Ci/mmol) were purchased from ICN.

[^{35}S]-methionine (1000 Ci/mmol), was purchased from Amersham.

2.1.3 Enzymes, Proteins and Immunologicals

The following lists essential proteinaeous reagents used in this work and their source

Enzyme/Protein or Kit	Source
Bovine serum albumin	Sigma
Calf intestinal phosphatase	Pharmacia
CNBr-activated Sepharose	Pharmacia
DNA sequencing kit	USB
DNAse I	Sigma
Enhanced Chemiluminescence Kit	Amersham
fMLP peptide	Sigma
S-hexylgluathione coupled agarose	Sigma
Immunologicals:	
goat anti-rabbit HRP	Amersham
goat anti-rabbit AP	BioRad
goat anti-chicken AP	Zymed
rabbit anti-CAT	Dr. J.P. Capone Dept. of Biochemistry McMaster Univ.
rabbit anti-G-6-P	Sigma
rabbit anti-GST	Dr. J.A. Hassel Dept. of Biology McMaster Univ.
rabbit anti-MAP2	Dr. C. Langdon, Dept. of Immunology, McMaster Univ.
mouse anti-tubulin	Dr. C. Langdon
FITC-goat anti-rabbit IgG	Zymed
FITC-anti-chicken IgY	Zymed
FITC-anti-mouse IgG	Sigma
TRITC-goat anti-rabbit IgG	Zymed
TRITC-anti mouse IgG	Sigma
Klenow fragment of DNA polymerase	Pharmacia
Mung bean nuclease	Pharmacia
Protein A-Sepharose	Pharmacia
Protein Kinase C (mixture of α, β, γ isoforms)	UBI
Recombinant a-PKC	UBI
Restriction endonucleases	BRL, NEB or Pharmacia
T4 DNA ligase	BRL
T4 polynucleotide kinase	BRL
Trypsin (Cat. # T8642)	Sigma
Vent DNA polymerase	New England Biolabs

2.1.4 Eukaryotic Cell Lines and Bacterial Strains

HL-60 (human promyleocytic) cells were subcultured from either passage 18 or 20 liquid nitrogen stocks originally obtained from the ATCC. HEL (human erythroleukemia) cells were obtained from the laboratory of Dr. R.J. Haslam, Dept of Pathology, McMaster Univ. K562 promegakaryocytic cells were from liquid nitrogen stocks originally obtained from Dr. Clarke, Dept. of Pathology, McMaster Univ. COS-1 African green monkey kidney epithelial cells were obtained from the laboratory of Dr. H.P. Ghosh, Dept. of Biochemistry, McMaster Univ. RBL (rat basophilic leukemia) cells were obtained from Dr. Blennerhasset, Pathology Dept., McMaster Univ.

Bacterial strains used for transformation were either HB101 or DH5α competent cells purchased from Gibco/BRL. JM109 and BMH 71-18 mutS were supplied as part of a mutagenesis kit (Promega "Altered Sites" kit).

2.1.5 Oligonucleotides and Peptides

The following oligonucleotides (FPLC purified) and peptides were synthesized by the Central Facility of the Institute for Molecular Biology at McMaster University. These oligonucleotides correspond to the sense strand of the pleckstrin gene. Underlined nucleotides mismatch the pleckstrin sequence and create the amino acid mutations listed.

Oligodeoxyribonucleotide	Mutation Introduced
5' CTA TAA GAA GAA AGC TGA CAA CAG CC	S40A
5' AAA AGT GAC AAC GCC CCC AAA GGA A	S43A
5' TAA GAT CAC TGC GAC CAA A	T73A
5' CAA GGA AAG CTA CCA G	S113A
5' AGG AAA TCT GCC AGG AG	T114A
5' CCA GGA GGG CCA TTC G	S117A
5' TTA TAT TTG GCC ATG AAA	S132A
5' ATG AAA GAC GCT GAA AAA	T136A

Polypeptide	Corresponding Residues of Pleckstrin
1. ARKSTRRSIRL-NH_2	110-120
2. KFARKSTRRSIRL-NH_2	108-120
3. KFARKSTRRAIRL-NH_2	108-120 (S117A)

2.2 RECOMBINANT DNA TECHNOLOGY

2.2.1 Growth, Maintainance and Transformation of Bacteria

Bacteria were grown either on YT 1.5% agar plates or in YT liquid culture at 37°C (YT; 8 g/L bactotryptone, 5 g/L bacto yeast extract, 2.5 g/L NaCl, pH 7.5) and placed at 4°C for short term storage. Long term storage of bacteria was performed by mixing 850 µl of freshly saturated bacterial culture with 150 µl of glycerol and placing at -70°C .

Competent bacteria (50 µl) were transformed with approximately 50 ng plasmid DNA by incubating the cells and DNA together on ice for 30 min, then heat shocking for 20 s at 37°C. The bacteria were allowed to recover in 1 ml of YT plus 9 mM $MgCl_2$ at 37°C for 45 min before spreading onto plates containing the appropriate antibiotic (e.g. ampicillin, chloramphenicol or tetracyclin).

2.2.2 Purification, Quantitation and Analysis of DNA

To screen several individual bacterial colonies for the presence of the correct recombinant plasmid, standard small scale preparations of plasmid DNA (mini-preps) were performed using an alkaline lysis method (Sambrook et al., 1989). Up to 1 µg of purified plasmid DNA was subsequently digested with the appropriate restriction endonucleases by standard methods (Sambrook et al., 1989) in a final volume of 20 µl. The reactions were stopped with 2 µl of 10 X FDM (1 mg/ml each bromophenol blue & xylene cyanol, 50 mM EDTA, 90% formamide) and 5 µl of the digest analysed by electrophoresis on 1 X TBE

(45 mM Tris-borate, 1 mM EDTA), 1% agarose gels. After staining the gels in 50 μg/L ethidium bromide in 1 X TBE for 30 min, the DNA was visualized by fluorescent UV light and photographed using Polaroid film. DNA sizes and quantity were estimated by comparison to 1 kilobase ladder DNA (approximately 16 ng/band) (Gibco/BRL) which was electrophoresed beside restriction digests.

Large preparations of DNA (300 to 600 μg) were made by an extension of the alkaline lysis method that includes extra purification steps (Sambrook et al., 1989). Fast purifications of 100 μg of plasmid DNA were performed using Qiaex columns (Qiagen, Inc.).

A fluorometer (Sequoia-Turner model 450) was used to determine the DNA concentration of moderate and large scale plasmid preparations. Occasionally, absorbance of light at 260 nm in a Beckman spectrophotometer (model DU-64) was used to estimate ds (double stranded) DNA concentration using the relationship A = 1 @ 50 μg/ml ds DNA.

2.2.3 Subcloning and Mutagenesis

2.2.3.1 General Methods

ds DNA fragments used in ligation reactions were purified by one of the two following methods: Fragments separated on 1% agarose gels were purified by electrophoresing the DNA out of the ethidium bromide stained gel slice into a dialysis bag submersed in 1 X TBE in a darkened gel box. The DNA was then precipitated by addition of 0.1 volume sodium acetate (3 M, pH 5.2) and 2.5 volumes of ethanol. Alternatively, the DNA was first electrophoresed on 1% low melting point agarose (FMC) gels run at low voltage in 1 X TAE (40 mM Tris-acetate, 1 mM EDTA) at 4°C. Ethidium bromide stained gel slices containing the desired DNA fragments were melted at 65°C and the agarose phenol extracted several times prior to sodium acetate/ethanol precipitation of the DNA.

To improve intermolecular ligation efficiencies, DNA fragments derived from

vector sequences were treated with calf intestinal phosphatase prior to purification and ligation to "insert" DNA fragments. Ligations reactions were carried out in 10 µl volumes containing 1 µl of 10 X ligation buffer supplied by the manufacturer, 1 µl of 10 mM ATP and at least 100 ng of DNA composed of a 2:1 molar ratio or greater of insert to vector fragments (for a theoretical discussion see Sambrook et al., 1989, p. 1.63 to 1.67). T4 DNA ligase (1 µl) was then added to the ligation reactions which were incubated at 10 - 14 °C for 1 h followed by 15 h at 4 - 6 °C.

2.2.3.2 Construction of pEVp47(+), pEVp47(-), pEVp47ΔNsi, pEVp47ΔXba and pGEXp47

A 1093 bp Nco I fragment containing the entire pleckstrin coding region plus 44 bp of 3' untranslated sequence, derived from a previous pleckstrin prokaryotic expression construct (pKK233-p47) (Tyers et al., 1988) was treated with Klenow and dNTPs to make the ends flush, then ligated into the Sma I site of the eukaryotic expression vector pEVRF2 (Matthias et al., 1989), creating pEVp47(+) (Fig. 2.1). This cloning procedure fuses the first 9 codons of the HSV thymidine kinase gene to the first translated ATG of the pleckstrin cDNA, therefore translation initiation could potentially occur at either ATG. The sequence of the 9 potentially translated amino acids is MASWGSGYP. These residues would yield an extended polypeptide having a predicted M_r = 41,023. pEVΔNsi was generated by digesting pEVp47(+) with Nsi I, removing the 3' protruding nucleotides with mung bean nuclease, then digesting with Xba I, extending the Xba I site recessed nucleotides with Klenow and recircularizing the plasmid using T4 ligase. A radiolabelled oligonucleotide probe specific to a sequence located within the deleted region was used to negatively select for the correct recombinant by colony hybridization (Sambrook et al., 1989). pEVp47ΔXba was created by digestion of pEVp47(+) with XbaI followed by religation.

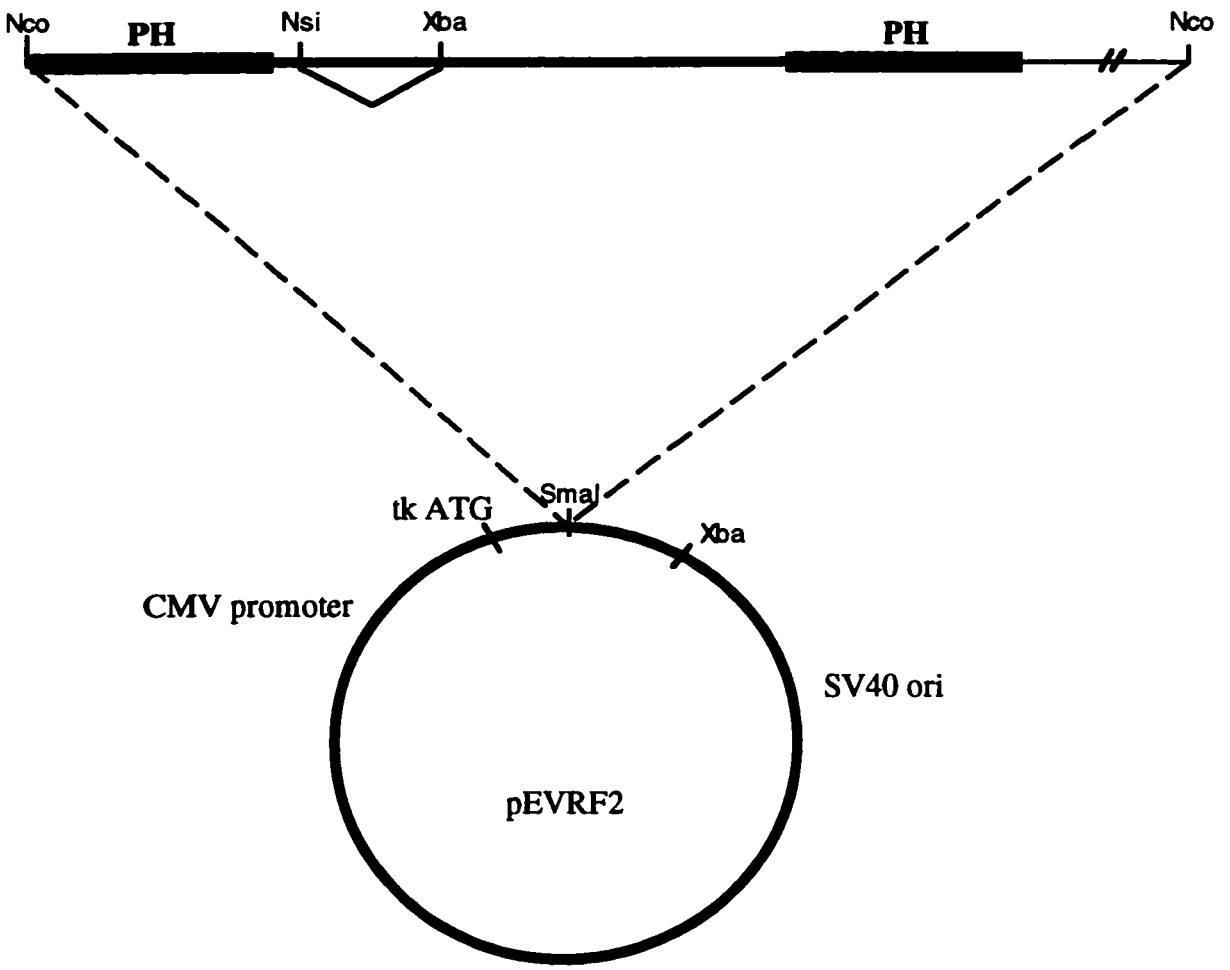

Figure 2.1 - Construction of pEVp47(+), pEVp47(-) and pEVp47ΔNsi.

A 1100 bp Nco I fragment from pKK233-p47 containing the entire pleckstrin coding region plus some 3' untranslated sequence (thin line) was gel purified, the ends filled in with DNA polymerase, then ligated into the Sma I site of pEVRF2 to create pEVp47(+). A clone was selected that contained the pleckstrin DNA inserted in the opposite orientation (pEVp47(-)). A deletion construct (pEVp47ΔNsi) was made by digesting pEVp47(+) with Nsi I, treating the DNA with mung bean nuclease, then partially digesting with Xba I and recircularizing the plasmid with T4 DNA ligase. Translation initiation can potentially occur from the pleckstrin start codon or the thymidine kinase (tk) ATG.

The prokaryotic expression vector pGEXp47 was created by ligating the Bam HI - Bgl II fragment from pEVp47(+) into the Bam HI site of the prokaryotic expression vector pGEX-2T (Pharmacia).

2.2.3.3 Site-Directed Mutagenesis

Mutagenesis was carried out by subcloning the Bam HI - Xba I fragment of pEVp47(+) containing the entire pleckstrin cDNA into the multiple cloning site of the pSelect-1 phagemid supplied with the Promega "Altered Sites" Mutagenesis Kit. After transformation into JM 109 *E. coli* cells, single-stranded template DNA was prepared after infection of the bacteria with helper phage R408. Six oligonucleotides were designed to change the codons for the following amino acids to alanine: ser 40 (AGT to GCT), ser 43 (AGC to GCC), thr 73 (ACG to GCG), ser 113 (TCT to GCT), thr 114 (ACC to GCC) and ser 117 (TCC to GCC, see section 2.1.5). The double stranded DNA synthesized in the mutagenesis reaction was transformed into *E. coli* strain BMH 71-18 mutS, which is repair deficient. Plasmid DNA was screened for the correct mutation by dideoxynucleotide sequencing. A 445 bp Bam HI - Xba I fragment corresponding to the N-terminal half of pleckstrin was digested out of the correctly mutated phagmids and subcloned back into the wild-type vector pEVp47(+), which had this 445 bp region removed. The subcloned regions were sequenced in their entirety by automated fluorescence sequencing (service provided by MOBIX) to ensure that no other mutations had been inadvertently created. All other sequencing was performed using standard dideoxyribonucleotide sequencing (Sambrook et al., 1989) and either [α^{32}P]-dATP or [α^{35}S]-dATP.

2.3 PROTEIN ANALYSIS

2.3.1 Quantification

Different methods for protein determination were used depending on the purpose for quantification. For quick analyses, the Bradford method was used as supplied in kit-format by Biorad and compared against BSA and lysozyme standards. For more accurate protein determination, Lowry assays were performed (Lowry et al., 1951). For determination of protein concentration when detergents (e.g. SDS) or other potentially interferring agents were present in the protein solution, a Pierce BCA kit was used. In instances of pure protein preparations, absorbance at 280 nm was used to calculate protein concentration.

2.3.2 SDS-PAGE and 2-Dimensional Isoelectric Focusing

Either 10 or 12.5% polyacrylamide separating gels were poured for a Biorad Mini-Protean system using standard buffer solutions and acrylamide mixes (Garfin, 1990). A 4% polyacrylamide stack layer was always poured above the separating gel to form a discontinuous system. Large Biorad gels (20 cm x 20 cm) were used for preparative SDS-PAGE.

A Biorad Mini-Protean/IEF system was used to isoelectrically focus proteins followed by separation by SDS-PAGE. The protein sample was dissolved in IEF loading buffer (O'Farrell, 1975), containing 8 M urea and ampholytes (Biorad). Samples were loaded onto 1.0 mm diameter tube gels composed of an equal ratio of pH 4.5-6 and pH 6-8 ampholytes and electrofocused at 750 V for 4 h. The pH gradient along the tube gels was determined by soaking a series of 0.5 cm pieces of a control tube gel in 1 ml of 2M urea for 1 h, then measuring the pH of this solution. 2-D gels containing [^{35}S]-methionine labelled proteins were soaked in En^{3}Hance (Dupont) prior to drying and exposure to film.

2.3.3 Purification of Anti-Pleckstrin Antibodies

Recombinant glutathione *S*-transferase (GST)-pleckstrin fusion protein expressed from pGEXp47 in *E. coli* was purified to apparent homogeneity by affinity chromatography on S-hexylglutathione coupled 4% beaded agarose (Sigma), essentially as described (Smith & Johnson, 1988). The protein was then emulsified in Freund's complete adjuvant and subcutaneously injected into New Zealand White rabbits (300 µg/rabbit). After 3 boosts the serum was collected and affinity purified by the following procedure. Purified GST-pleckstrin was coupled to cyanogen bromide activated Sepharose (Pharmacia), as per manufacturer's instructions to make an affinity column. After the resin was pre-equilibriated with 10 mM sodium phosphate, pH 6.8, 10 ml of serum was directly applied to the column and washed through with several bed volumes of phosphate buffer. Pleckstrin-specific antibodies were eluted with 100 mM glycine, pH 1.8, and neutralized in 1/20 volume 1 M phosphate buffer, pH 8.0 (Harlow & Lane, 1988). Each fraction was tested for its ability to immunoprecipitate pleckstrin from transiently transfected COS cells (see Fig. 3.3.3). Only the highly specific fractions were pooled for use in further experiments.

In addition to three rabbits, one egg-laying hen was immunized intramuscularily with GST-pleckstrin. The eggs were continuously collected and IgY partially purified from the yolks of a series of eggs laid roughly 1 week after each immunization (Patterson et al., 1962; Polson et al., 1980). Goat anti-chicken alkaline phosphatase conjugated antibody was used as a secondary antibody to detect the chicken anti-pleckstrin antibodies on western blots.

2.3.4 Western Blotting

Proteins run on SDS-polyacrylamide gels were transferred onto nitrocellulose, and incubated with a 1/200 dilution of the affinity purified anti-pleckstrin antibody in Tween-20

(Tw-20) buffer (0.1% Tw-20, 140 mM NaCl, 10 mM Tris-HCl, pH 7.5). After 2 h, the filters were washed in Tw-20 buffer for 30 min followed by 4 washes for 10 min each. The blots were then either developed using an ECL detection method (Amersham), or by a colorimetric method. For chemiluminescence, a 1/20,000 dilution of a goat anti-rabbit horseradish peroxidase secondary antibody (Amersham) was then incubated with the blot for 1 h, followed by several washes in Tw-20 buffer. The washed blot was developed with the reagents provided in the ECL kit, and exposed to Kodak XAR film for 1 min. For colorimetric development, a 1/7,500 dilution of a goat anti-rabbit alkaline phosphatase secondary antibody (Zymed) was incubated with the blot for 1 h, followed by several Tw-20 washes. The reactive bands were then detected by incubation in 5-bromo-4-chloro-3-indoyl phosphate p-toluidine salt (BCIP) and p-nitro blue tetrazolium chloride (NBT). For some blots, primary antibody was detected using ^{125}I-labelled Protein A (Amersham).

2.3.5 Immunoprecipitations

500 µl of cold lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5) was added to each PBS washed, cell pellet (see labelling conditions below). After a 30 min incubation on ice, the lysates were spun at 10,000 g for 10 min. Affinity purified anti-pleckstrin antibody (5 µl) was added to each lysate supernatant and gently rocked 16 h at 4°C. Swollen Protein A-Sepharose beads (20 µl, Pharmacia) were added to the lysate for the last hour of this procedure, then pelleted by low speed centrifugation and the supernatant removed. After 5 washes with 400 µl of lysis buffer, protein loading buffer was added to the beads, and the solubilized protein was loaded onto SDS-polyacylamide gels or IEF tube gels.

2.3.6 Actin Purification and Assay of Polymerization

Rabbit muscle actin as a stable acetone powder (11g) was received as a gift from C. Satori and Dr. R. Haslam, McMaster Univ. It had been brought to this stage of purification by the method of Pardee and Spudich (1982), and therefore further purification was performed based on this method. The final monomeric G actin concentration was determined to be 2.1 mg/ml by Bradford protein determination and by absorbance at 280 nm (extinction coefficient$_{280nm}$ = 47,800 $cm^{-1}M^{-1}$). A portion of this G actin solution was removed for assaying and the remainder polymerized to its more stable F (filamentous) form and stored at 4°C until required.

The extent of actin polymerization was measured using conventional capillary viscometry (Cooper & Pollard, 1982). An Ostwald semimicro viscometer of size 150 from Cannon Instrument Co. (State College, Pennsylvania) was used at ambient temperature. 600 µl samples were sucked up the capillary and the time for the sample to pass through the end point measured (t_s). Polymerization was then initiated by the addition of KCl to a final concentration of 30 mM, and flow time measurements taken over the course several minutes. Flow times were compared to the time for buffer only to pass through (t_b), and converted to specific viscosity values (sp. vis.) by the relationship sp. vis. = t_s/t_b - 1. In a typical experiment, the final concentration of actin used was 12 mM and that of pleckstrin 1/40th this amount as based on the experimental parameters of Hashimoto et al., 1987.

2.4 CELL CULTURE, TRANSFECTION and IMMUNOFLUORESCENCE

2.4.1 Maintenance of Cell Lines

HL-60 and HEL cell lines were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine. COS cells were grown in Dulbecco's medium containing 10% FBS. All cells were incubated in a 37°C, 5% CO_2 atmosphere. Suspension cells were maintained at 5 x 10^5 to 5 x 10^6 cells/ml, whereas

adherent COS cells were split by trypsinization every 4 or 5 days at a 1 to 20 dilution.

2.4.2 Transient Transfection of COS Cells

COS cells were transiently transfected by a DEAE-dextran technique (Selden, 1993). Briefly, 5 ml of serum-free medium containing 250 μg/ml DEAE-dextran (Pharmacia), 50 mM Tris-HCl, pH 7.5 and 25 μg plasmid DNA was added to each 75% confluent dish of COS cells and incubated at 37°C for 3 h. The medium was removed and 2 mls of 10% DMSO in phosphate buffered saline (PBS) was added to the dishes for 1 min to shock the cells. The cells were then washed in PBS and incubated in complete medium for two days to allow for recombinant protein expression.

2.4.3 Immunofluorescence

Non-adherent cells (HL-60) were allowed to attach to coverslips treated with poly-L-lysine whereas COS cells were directly grown on coverslips resting on the bottom of tissue culture dishes. HL-60, HEL or COS cells (2 days after transfection) were fixed by a 30 min incubation in 4% paraformaldehyde in PBS. After washing in PBS, cells were permeabilized with 1% Triton X-100 in PBS for 10 min, rinsed with PBS containing 0.2% Tween-20 (PBS/Tw20), then washed in the same buffer plus 1% BSA (PBS/Tw20/BSA). Affinity purified antibody was applied to the coverslips at a 1/200 dilution in PBS plus 3% BSA and incubated for 45 min in a humidified chamber. Excess primary antibody was then washed off with PBS/Tw20/BSA and a 1/20 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit secondary antibody (Zymed) was applied for 45 min. The coverslips were then washed in PBS/Tw20, followed by a rinse in PBS alone, then dried and mounted in 50% glycerol containing 2.5% w/v of 1,4-diazabicyclo[2.2.2]-octane (Sigma) to prevent photobleaching. Slides were examined by standard fluorescence microscopy (Jenamed), or by a BioRad MRC-600 laser scanning confocal microscope.

Rhodamine conjugated wheat germ agglutinin and concanavilin A were used to label the Golgi apparatus and rough ER respectively (Tartakoff & Vassalli, 1983).

2.5 PHOSPHOPEPTIDE MAPPING

2.5.1 In vivo Labelling of Phosphoproteins

Approximately 10^7 HL-60, HEL or COS cells per sample were washed twice in either phosphate or methionine-free media, then incubated at 37°C with 200 μCi of [^{32}P]-orthophosphate (ICN) (2h), or 100 μCi of [^{35}S]-methionine (Dupont/NEN) (6h). For staurosporine dose-response curves, PMA and or staurosporine in DMSO carrier solvent (or solvent alone for control cells) was added to each sample at 30 min or 1 h respectively, prior to harvesting. Cells were then lysed on ice in lysis buffer and pleckstrin immunoprecipitated as described above and loaded on a 12% SDS-polyacrylamide gel. PMA-induced phosphorylation was measured by subtracting the uninduced band from the PMA-induced band, after first subtracting the non-specific background phosphorylation in each lane, using Molecular Dynamics PhosphorImager software. The percent of PMA-induced phosphorylation was calculated by dividing the staurosporine treated band by the PMA-induced value, times 100.

2.5.2 In vitro Kinase Assays and Kinetics

Recombinant GST-pleckstrin fusion protein purified as described in section 2.3.3 was phosphorylated in a mixed miscele assay (Hannun et al., 1985) using a mixture of purified α,β and γ PKC isozymes from rat brain (Upstate Biotechnology, Inc. (UBI)) in a final volume of 25 μl. The reaction was stopped by addition of an equal volume of Lamelli loading buffer and run on an SDS-polyacrylamide gel. The proteins were transferred to nitrocellulose and the phosphorylated recombinant GST-pleckstrin band processed for phosphopeptide mapping as described in section 2.5.3.

The peptides ARKSTRRSIRL-NH_2, KFARKSTRRSIRL-NH_2 and KFARKSTRRAIRL-NH_2 were phosphorylated in vitro as described above. Unincorporated [γ^{32}P]-ATP was removed by ion-exchange chromatography on a DEAE-Sepharose column prior to trypsinization and phosphopeptide mapping.

Kinetic analysis of peptide phosphorylation by recombinant PKCα (UBI) was performed by incubating various concentrations of peptide with PKC at room temperature, then removing and spotting 5 µl aliquouts at specific time intervals onto phosphocellulose discs (p81 paper - Whatman, Clifton, NJ). The discs were immediately submersed in 75 mM phosphoric acid to stop the reaction (Casnellie, 1991). Unincorporated [γ^{32}P]-ATP was removed from the discs by several washes in 75 mM phosphoric acid, then the discs were air dried, exposed to PhosphorImaging cassettes and the amount of radioactivity remaining on each disc quantitated using a Molecular Dynamics PhosphorImager. Data analysis (graphing and calculation of K_M) was performed using FIG P software.

2.5.3 Phosphopeptide Mapping

Pleckstrin immunoprecipitated from in vivo labelled cells or from in vitro phosphorylation reactions was run on an SDS-polyacrylamide gel and electrophoretically transferred onto nitrocellulose. After exposure to film, the radioactive band was cut out and processed as described (Luo et al., 1991), by incubation in 0.5 % polyvinylpyrrolidone, M_r 360,000 (Sigma) in 100 mM acetic acid for 30 minutes at 37°C. The nitrocellulose was then washed 5 times with 1ml of water and incubated at 37°C in bicarbonate buffer containing 100 ng L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (Sigma) per µl, the concentration of which was doubled after 2 h and incubated for at least 8 more hours to ensure complete digestion. The soluble tryptic peptides were lyophilized then oxidized in performic acid. After the peptides were

dissolved in water and lyophilized twice more, they were dissolved in 3 µl pH 1.9 buffer comprised of H_2O/acetic acid/formic acid (897:78:25; v/v/v) and spotted onto 1 mm thick cellulose plates (EM Science) as described (Boyle et al., 1991). The plates were run on a Hunter Thin Layer Electrophoresis System at 1500 V for 35 min, air dried, then transferred to a chromatography tank containing phosphochromatography buffer comprised of n-butanol/H_2O/pyridine/acetic acid (15:12:10:3; v/v/v/v). The plates were developed twice in this buffer to increase the resolution in the chromatographic direction, then exposed to a PhosphorImager screen or directly to film. Phosphoamino acid analysis was performed on individual spots scraped from the plates as described (Boyle et al., 1991).

RESULTS AND DISCUSSION

PART A - In vitro Characterization of Human Recombinant Pleckstrin

The long-term goal of this project was to determine the function(s) of pleckstrin. One way this might be achieved is through testing several putative functions in vitro using purified pleckstrin. In anticipation that clue(s) to pleckstrin's function would eventually arise, recombinant pleckstrin was purified from *E. coli* for future biochemical analyses. Additionally, this recombinant source of pleckstrin was used to generate anti-pleckstrin antibodies required for a continuation of in vitro and in vivo studies on pleckstrin.

3.1 Purification of Recombinant Pleckstrin Expressed from the pKK233 Prokaryotic Expression Vector

The pleckstrin cDNA had been inserted into the pKK233 prokaryotic expression vector prior to the start of this project (Tyers et al., 1988). The apparent size of the polypeptide produced by this vector (pKKp47) was determined by running transformed *E. coli* lysate adjacent to platelet pleckstrin and protein standards on an SDS-polyacrylamide gel. Recombinant pleckstrin appeared to co-migrate with platelet pleckstrin at an apparent molecular mass of 42,000 Da, and accounted for 5-10% of total bacterial protein (Tyers et al., 1988).

To have a readily obtainable source of pure human pleckstrin (other than from platelets or cultured lymphoid cells), development of a purification scheme for recombinant pleckstrin was necessary. Initially a scheme based on that published for platelet pleckstrin

(Imaoka et al., 1983) was adopted for pleckstrin overexpressed in bacteria. When this scheme was attemped, a major difference between the platelet and bacterial systems immediately arose. Recombinant pleckstrin released during bacterial cell wall lysis by sonication was invariably partly or entirely degraded to lower molecular weight forms (Fig. 3.1.1). Any remaining full-length recombinant protein was found predominantly in the 10,000 g x 5 min pellet, indicating insolubility. The major degradation product was partially soluble since approximately half of it partitioned into the supernatant fraction. The routine strategy of including several protease inhibitors in the lysis buffer to prevent degradation was unsuccessful (see Table 3.1.1). The most frustrating observation was that proteolysis appeared to occur concomitant with cell lysis (or within a few seconds), with little degradation occurring afterwards.

Since recombinant pleckstrin was insoluble and susceptible to proteases, it became reasonable to hypothesize that it was present within the cells in the form of inclusion bodies. These are optically opaque densities often seen in bacteria overexpressing recombinant proteins (Williams et al., 1982). Usually the proteins in these aggregates are improperly folded, with exposed hydrophobic regions facilitating aggregation with other similarily exposed regions. Hence, upon cell lysis, these improperly folded polypeptides are succeptable to proteases to varying degrees. If well packed together, they can be pelleted out of the supernatant, an observation that has been exploited as an initial purification step for other recombinant proteins (for review, see Marston, 1986).

The methods put forth in the literature to solubilize proteins within inclusion bodies ubiquitously involve the use of strong denaturants such as urea or guanidinium hydrochloride (Marston et al., 1984; Weir & Sparks, 1987). Since not all of pleckstrin was pelletable (in other blots, up to 35% appears in the supernatant), and extensive degradation occurred, the sonication and centrifugation steps had to be circumvented. It was discovered that by adding 8 M urea directly to the washed bacteria, it was easy to

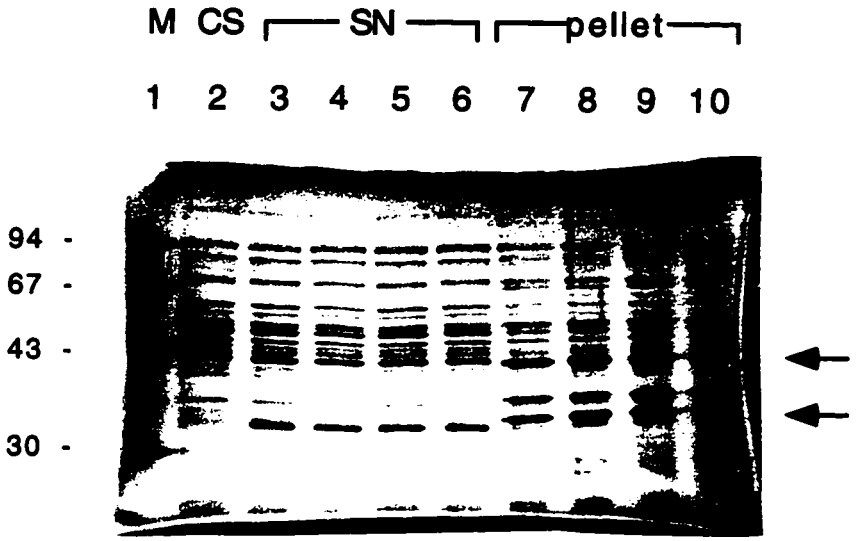

Figure 3.1.1 - Recombinant pleckstrin expressed from the pKK233-3 vector in *E. coli.*

Shown is an SDS-PAGE analysis of bacterial lysates expressing recombinant pleckstrin. The cells were lysed by sonication and spun for 5 min, 10,000 g. Supernant fractions (SN) from the spin were loaded in lanes 3 to 6, the pellet fractions loaded in lanes 7 to 10. Cells corresponding to lanes 3 & 7 were lysed in the presence of 0 mM PMSF; lanes 4 & 8, 0.1 mM PMSF; lanes 5 & 9, 1.0 mM PMSF; lane 6 & 10, 1.0 mM PMSF, and left on ice 2 h prior to analysis. In lane 2 control bacterial supernatant (CS) was loaded and in lane 1, molecular weight markers (M). Top arrow indicates full-length recombinant pleckstrin, which was only present in the pellet fractions. A major degradation product of pleckstrin (lower arrow) was identified in both supernatant (dark 35 kDa band) and pellet fractions (lighter 35 kDa underneath 2 darker bands) by western blotting (not shown).

PROCEDURE	OUTCOME	FIGURE
A) Lysis by sonication	degraded	Fig. 3.1.1
Protease Inhibitors included during sonication:		
PMSF 10mM	degraded	Fig. 3.1.1
Leupeptin	degraded	
Aprotinin	degraded	
EDTA	degraded	
B) Lysis in 8 M urea	intact	
After purified on a CM-Sepharose column equilibriated with 6 M urea	intact	Fig. 3.1.2
Then dialysis to remove urea in:		
Tris-HCl, pH 8	precip/deg	Fig. 3.1.3
deoxycholate / Tris-HCl, pH 8	precip/deg	
glutathione / Tris, pH 8	precip/deg	
0.5 M Na-acetate, pH 4.3	soluble/intact	Fig. 3.1.3
0.1% acetic acid, pH 2.3	precip/deg	Fig. 3.1.3
C) Further purify by preparative SDS-PAGE		
Then: Electroelute into Tris, pH 8	precip/deg	
Electroelute into 1% SDS	intact	
Lyophilize	intact	Fig. 3.1.3
SDS affinity column	precip/deg	

Table 3.1.1 - Procedures Used to purify recombinant pleckstrin overexpressed from the pKK233 vector in *E. coli*.

The various procedures are grouped into 3 main categories. In **A**), the bacteria were lysed by sonication in the presence of various protease inhibitors, none of which noticeably prevented the degradation of pleckstrin compared to untreated samples. In **B**), urea-denatured pleckstrin was 80 % purified by ion-exchange chromatography on a CM-Sepharose column. Subsequent removal of the denaturant without precipitation (precip) or degradation (deg) was difficult. In **C**), after further purification by preparative SDS-PAGE, pleckstrin was successfully electroeluted from the gel in a soluble form, but only in the continued presence of SDS. Any attempt to remove the SDS resulted in the precipitation or loss of the protein.

achieve complete bacterial lysis and simultaneouly solubilize all bacterial proteins without degradation of pleckstrin.

The clarified, urea-denatured lysate was then loaded on a CM-Sepharose column equilibriated in 6M urea, 50mM Tris-HCl, pH 8.3 and 1mM EDTA, as has been used successfully for others (Nagai et al., 1988). This column binds only relatively basic proteins whereas RNA, DNA and acidic proteins, which include the majority of bacterial proteins (Marston, 1986), should pass through. This behaviour was confirmed by the large 595 nm absorbance peak of the flow through fraction in Fig. 3.1.2A. Pleckstrin and a few other proteins were retained on the column at this pH, but with increasing salt pleckstrin was the major protein retained. In a similar manner polyarginine fusion proteins have been selectively purified by cation exchange chromatography (Sassenfeld & Brewer, 1984). The affinity of pleckstrin for the CM-Sepharose column allowed for the purification (to approximately 80%) of large quantities of denatured pleckstrin, in essentially one step.

Attempts to remove the urea using stepwise dialysis against lower urea concentrations were unsuccessful. Pleckstrin was often not recoverable after such an attempt, either from precipitation or degradation. Similarily, Giantini & Shatkin, 1987 documentated that bacterial $\sigma 3$ protein would precipitate upon decreasing the urea concentration from 4 M to 2 M during dialysis. Other unsuccessful modifications to the dialysis step that were tried are documented in Table 3.1.1. Partial success was achieved with 0.5 M sodium acetate buffer, pH 4.3 (Fig. 3.1.3B). It is possible that bacterially-expressed pleckstrin requires this lower pH and high ionic strength to renature, as does the zinc finger domain of transcription factor IIIA (Nagai et al., 1988). Mismatching of disuphide bonds have often been implicated in the improper folding and aggregation of proteins into inclusion bodies (Giantini & Shatkin, 1987). Contrarily, inclusion bodies can form from proteins not containing cysteine residues (Lee et al., 1988), and often renaturation can occur without the addition of reducing reagents (Marston et al., 1984).

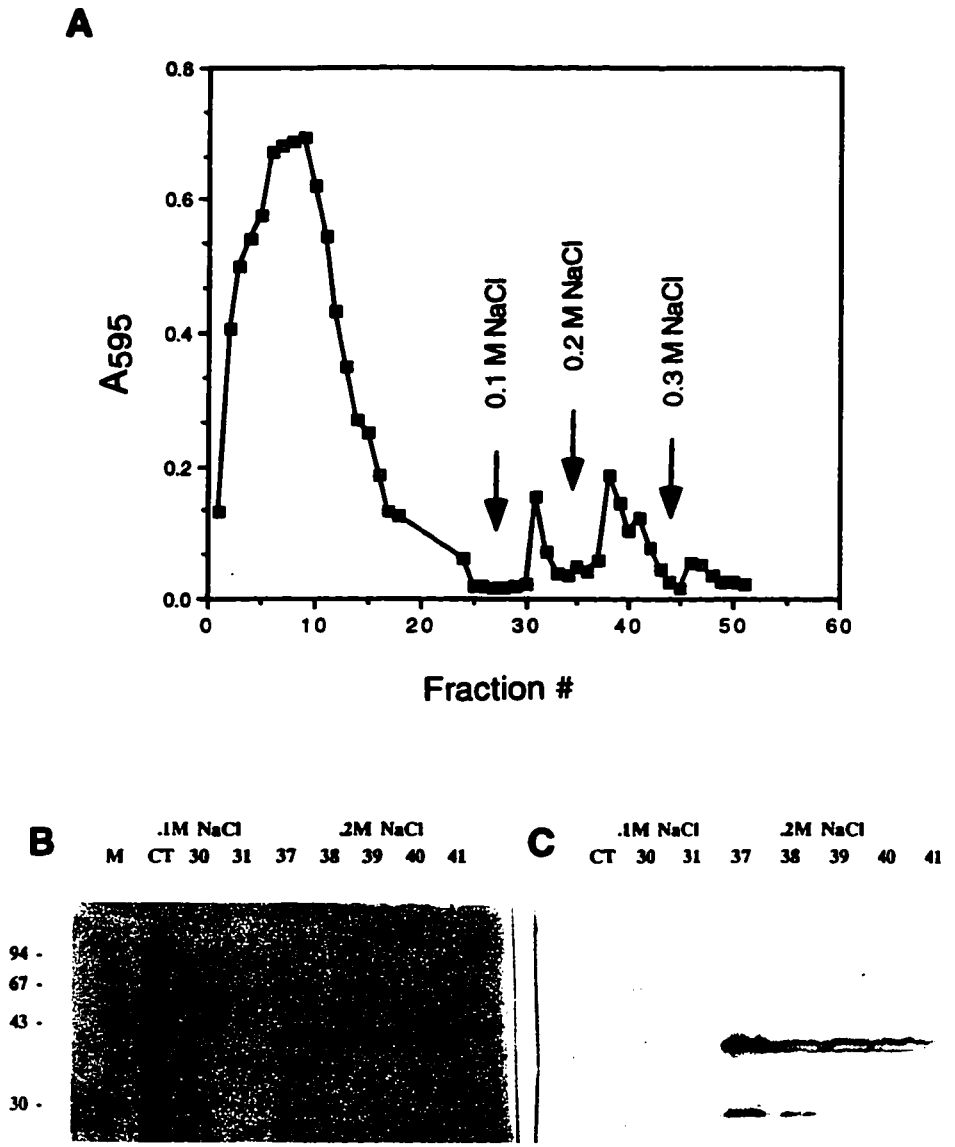

Figure 3.1.2 - Purification of recombinant pleckstrin by ion-exchange chromatography under denaturing conditions.

A total bacterial lysate containing pleckstrin was denatured in 8 M urea, spun 30 min, 10,000 g and loaded on a CM-Sepharose column equilibriated with 6 M urea and 40 mM Tris-HCl, pH 8.3. **A**, plot of protein absorbance at 595 nm (samples reacted with the Bradford reagent) versus fraction number. The NaCl concentration was increased stepwise as indicated. SDS-PAGE (**B**), and immunoblot analysis (**C**) of some of the fractions in **A**. Note that recombinant pleckstrin eluted in fractions 37-41, after the salt concentration was increased to 0.2 M.

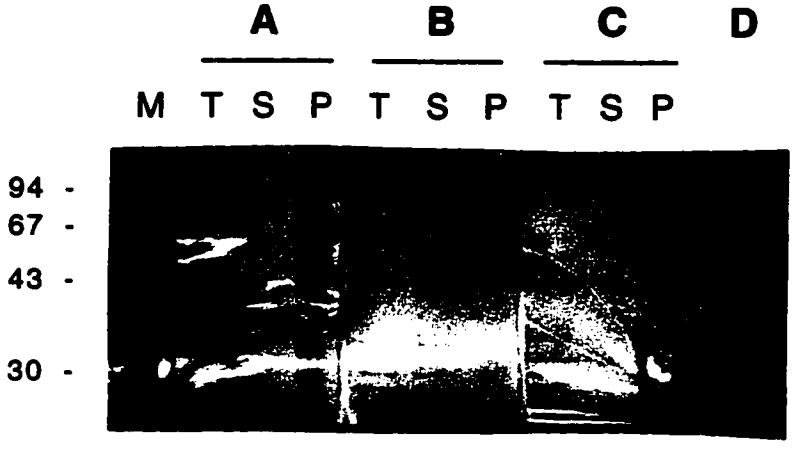

Figure 3.1.3 - Attempts to renaturate pleckstrin by dialysis.

After purification by ion exchange chromatography, the urea present in fractions containing pleckstrin was removed by dialysis against several different buffer types (**A** to **C**). The dialyzed solutions were spun for 5 min, 10,000 g, and T, S and P referring to total, supernatant and pellet fractions respectively were analysed by SDS-PAGE. **A**, Tris-HCl, pH 8. **B**, 0.5 M sodium acetate buffer, pH 4.3. **C**, 0.1% acetic acid buffer, pH 2.5. **D**, purified recombinant pleckstrin electroeluted from a preparative SDS-polyacrylamide gel, after initial purification by column chromatography.

In conclusion it appears that pleckstrin once denatured is not easily renatured. In fact, Surgue et al., 1990, have shown that unfolding of prochymosin in concentrated urea is not fully reversible, due to the slow formation of an irreversibly denatured form. This effect might be due to cis-trans proline residue isomerization, or to side chain carbamylation from the high urea concentration. It has also been documented that protein renaturation occurs most efficiently in dilute protein solution, favouring intra over inter molecular interactions, limiting the formation of improper intermolecular disulphide bonds (Marston, 1986; Rudolph & Lilie, 1996). This possibility was not fully explored in the work presented here.

To take denatured pleckstrin from 80% pure to apparent homogeneity, preparative SDS-PAGE was used (Fig. 3.1.3D). Pleckstrin was electroeluted into buffer containing 0.1% SDS, then used successfully for immunization, although antibody titres from this protocol were not very high (see section 3.3). The electroeluted source of pleckstrin was also assayed for inhibition of actin polymerization as described in section 3.4. Attempts to remove the SDS and renature pleckstrin by dialysis, by KCl precipitation or by "extrati D" SDS-affinity columns (Pierce) were unsuccessful, again indicative that the appropriate conditions for renaturation of bacterially-expressed recombinant pleckstrin had yet to be realized.

3.2 Purification of Recombinant Pleckstrin Expressed as a Glutathione *S*-Transferase Fusion Protein

Due to the difficulty in purifying pleckstrin expressed from the pKK233 vector, a more sophisticated prokaryotic expression vector was sought. The pGEX-2T system involves expressing recombinant proteins as fusions to the binding domain of glutathione *S*-transferase (GST) from the parasitic worm *Schistosoma japonicum* (Smith & Johnson,

1988). Twenty-two out of 26 eukaryotic proteins were soluble when expressed as GST fusions by this system (Smith & Johnson, 1988). This was remarkable since the majority of recombinant proteins expressed in *E. coli* either alone or as fusions up until 1986 were insoluble (Marston, 1986). Since the GST polypeptide appeared to have an excellent empirical record for directing the correct folding, solubility and stability of attached domains it was hypothesized that pleckstrin might also be soluble in the form of a GST fusion protein.

Cloning the pleckstrin cDNA into the pGEX-2T expression vector was accomplished by ligating the Bam HI - Bgl II fragment derived from pEVp47 in frame into the Bam HI site of pGEX-2T, to create a fusion protein of predicted M_r of 67,500. When expressed as a GST-fusion protein, pleckstrin was found in the supernatant fraction after sonication and centrifugation. It was also resonably resistant to proteolytic attack, with only EDTA required in the buffers to prevent proteolysis. The supernatant fraction was loaded onto an S-hexylglutathione agarose column and GST-pleckstrin was selectively bound as all other proteins passed through. GST-pleckstrin was then eluted by addition of 5 mM free glutathione (Fig. 3.2.1). The fusion protein obtained by this protocol was nearly 100% pure, with any lower molecular weight bands representing minor degradation products as determined by western analysis with anti-GST antibody (data not shown). Yields were typically 5 mg fusion protein per litre of bacterial culture.

A feature of some fusion proteins apart from ease of affinity purification is an engineered method to remove the carrier polypeptide. For pGEX-2T, the DNA sequence upstream of the multiple cloning site codes for six amino acids that comprise a thrombin cleavage site. This enables facile cleavage of the GST polypeptide after purification of the fusion protein. This feature is especially important if functional assays need to be performed on the native protein. When thrombin was added to purified GST-pleckstrin, extensive degradation occurred (data not shown) even though pleckstrin does not have any

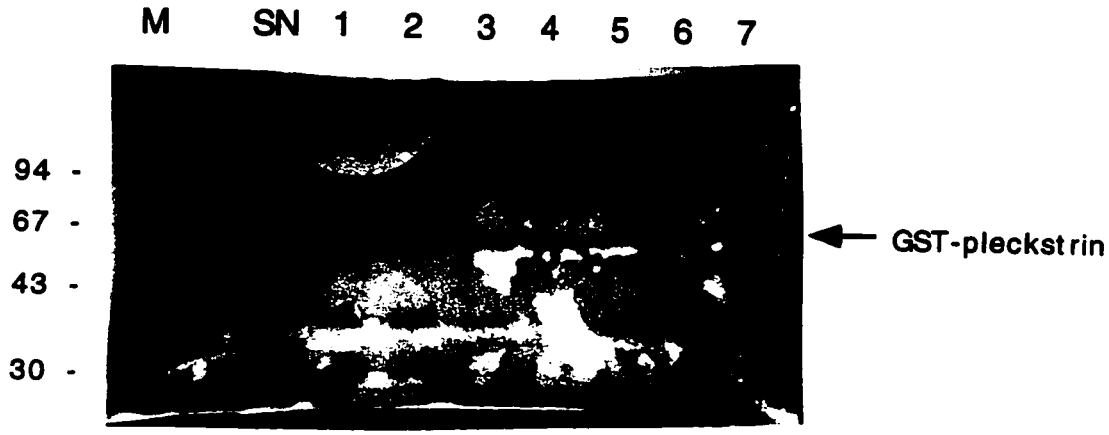

Figure 3.2.1 - Purification of GST-Pleckstrin by Affinity Chromatography on Glutathione Resin.

The supernatant (SN) of a bacterial lysate containing overexpressed GST-pleckstrin was loaded onto S-hexylglutathione resin in PBS plus 1 mM EDTA. Unbound proteins were eluted in PBS/EDTA, then GST-pleckstrin (67 kDa) was eluted by competition with 5 mM free glutathione in Tris-HCl, pH 8.0, in fractions 1 to 7. M, molecular weight markers.

recognizable thrombin cleavage sites (Chang, 1985). Possibly cleavage of the GST portion led to the instability or unfolding of pleckstrin, such that it was subsequently open to attack by trace amounts contaminating proteases, reminiscent of the scenerio outlined in the previous section. Perhaps a co- or post-translational phosphorylation event is necessary for maximal stability of pleckstrin. Such "silent" phosphorylations, so named because they do not turnover rapidly and are therefore difficult to detect, are believed to provide stability, as in the case of cAMP-dependent kinase (Taylor et al., 1993). Since *E. coli* does not have protein serine or threonine kinases, such a phosphorylation would be absent in recombinant pleckstrin.

Although recombinantly expressed pleckstrin on its own appeared unstable, it was not necessary for immunization purposes to remove the GST domain from the fusion protein, since the worm GST sequence does not necessarily produce cross-reacting antibodies to mammalian GST's (Smith and Johnson, 1988). Also, from a functional standpoint, the GST portion contained no consensus sites for phosphorylation by any known kinases (PC GENE signature search). Therefore, it was not necessary to remove the GST portion in order to perform the in vitro phosphorylation site mapping as detailed in section 3.7.

3.3 Generation of Affinity Purified Anti-Pleckstrin Antibodies

As mentioned earlier, antibodies to pleckstrin were needed in order to carry out further independent research on the biochemistry and function of pleckstrin. Therefore three New Zealand White rabbits were immunized with recombinant pleckstrin initially purified using the prokaryotic pKK233 expression system (section 3.1).

Since the titres of the immunized rabbits were low even after several injections (data not shown), a hen was obtained for immunization due to its generally superior immune

response to the more phylogenetically distant mammalian antigens (Gassmann et al., 1990). Even with a low quantity of antigen, titres in chickens can be seen as early as 16 days post initial injection and they tend to remain high without continued boosting (Polson et al., 1980). Additionally, the easily collected eggs contain as high or higher levels of IgG-like (IgY) antibodies as is present in hen serum. A 90% pure preparation of the IgG-like antibodies can then be obtained by a series of simple polyethylene glycol precipitations (Patterson et al., 1962), as shown in Fig. 3.3.1A. Anti-pleckstrin specific antibodies were seen by western blotting seven days after the second immunization, and although the titre was low, the response was very specific (Fig. 3.3.1B). This IgY preparation was as competent as rabbit anti-pleckstrin antibodies in the immunofluorescence detection of pleckstrin (data not shown), however since IgY does not bind to Protein A or G, its use in immunoprecipitations was precluded.

Once expression of recombinant GST-pleckstrin was undertaken (section 3.2), this plentiful source of antigen greatly increased the response in the rabbits (Fig. 3.3.2). The rabbit serum was further affinity purified so it could be used with confidence in electron microscopy work and immunoprecipitations. To achieve this, crude serum was passed over a column comprised of recombinant GST-pleckstrin covalently coupled to an agarose resin. Anti-pleckstrin specific antibodies were eluted from the column using a low pH buffer as described in section 2.3.3 and shown in Fig. 3.3.3. This affinity purification step removed essentially all non-specific antibodies as shown by the specificity of immunoprecipitations #7 to 17 as compared to pre-column immune serum immunoprecipitations (Fig. 3.3.3).

3.4 Role of Pleckstrin in Actin Polymerization

At the time this project began, there was one report implicating pleckstrin in directly

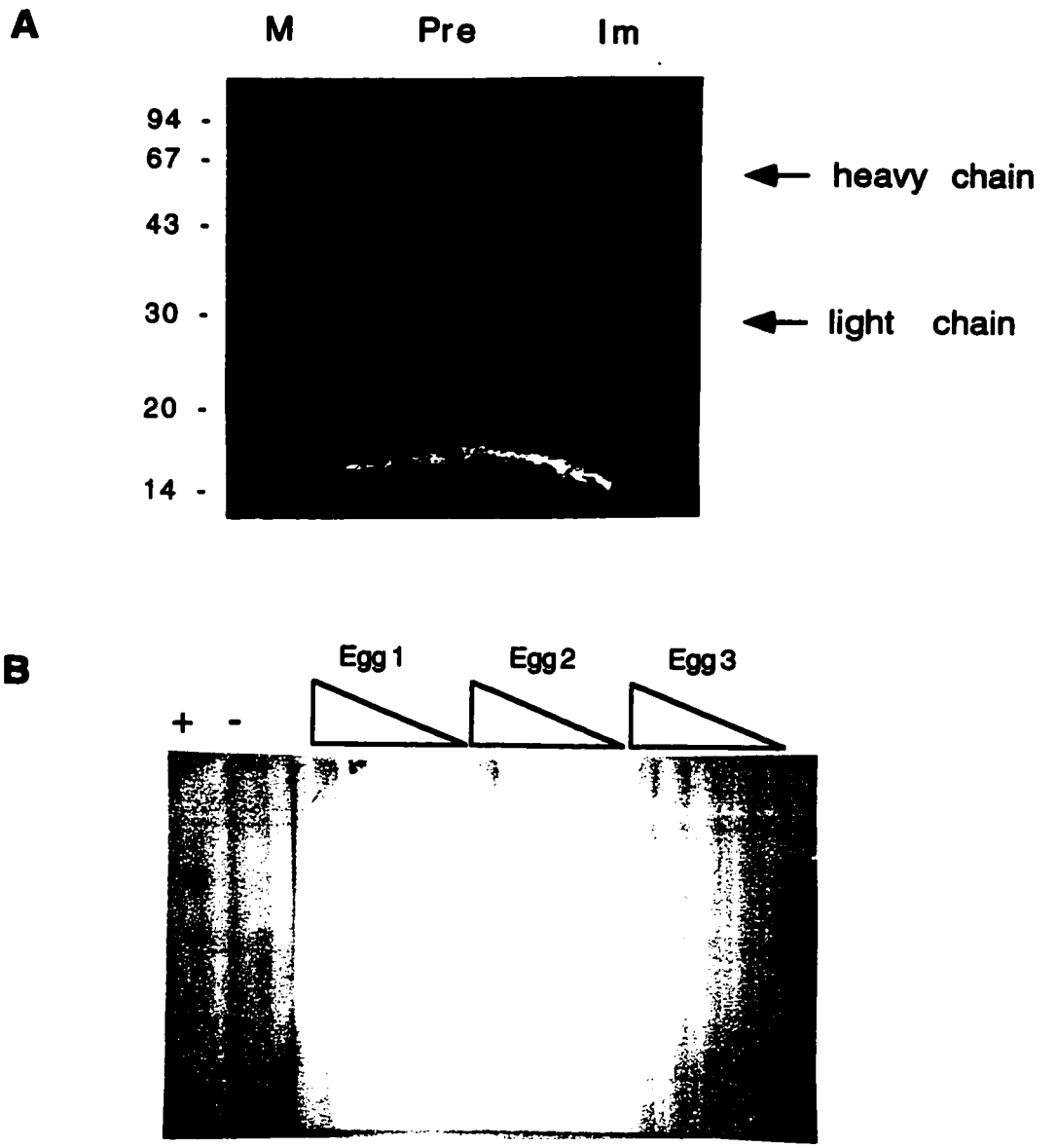

Figure 3.3.1 - Chicken Anti-Pleckstrin Antibodies.

A, Coomasie stained SDS-polyacrylamide gel of the IgY fraction purified from a hen's egg prior to immunization (Pre) and after (Im). The heavy and light chains of the IgY fractions are clearly apparent. B, Western blot analysis of the reactivity of IgY solutions purified from three individual eggs towards recombinant pleckstrin present in a total bacterial lysate. (+) represents crude rabbit anti-pleckstrin sera (1/400 dilution), (-) represents preimmune IgY (1/200), and dilutions of IgY solution from each egg are 1/20 to 1/12500 in 5 fold increments from left to right. Individual lanes were created using a BioRad multiwell immunoblot holder.

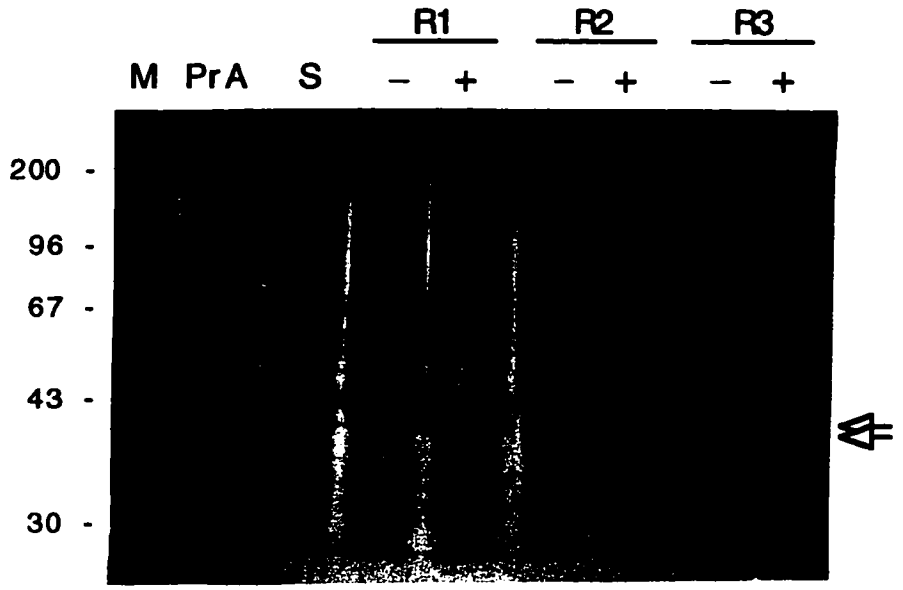

Figure 3.3.2 - Immune Response from Rabbits as Detected by Immunoprecipitation of Pleckstrin from Transfected COS Cells.

COS cells transfected with pEVp47 were labelled with ^{35}S-methionine and pleckstrin immunoprecipitated using either preimmune sera (-), or immune sera (+) from three rabbits (R1, R2, R3). Controls included an immunoprecipitation using Protein A - Sepharose beads only (PrA), or rabbit anti-pleckstrin sera obtained from C. Satori (S). The two pleckstrin bands (doublet) are indicated at the right.

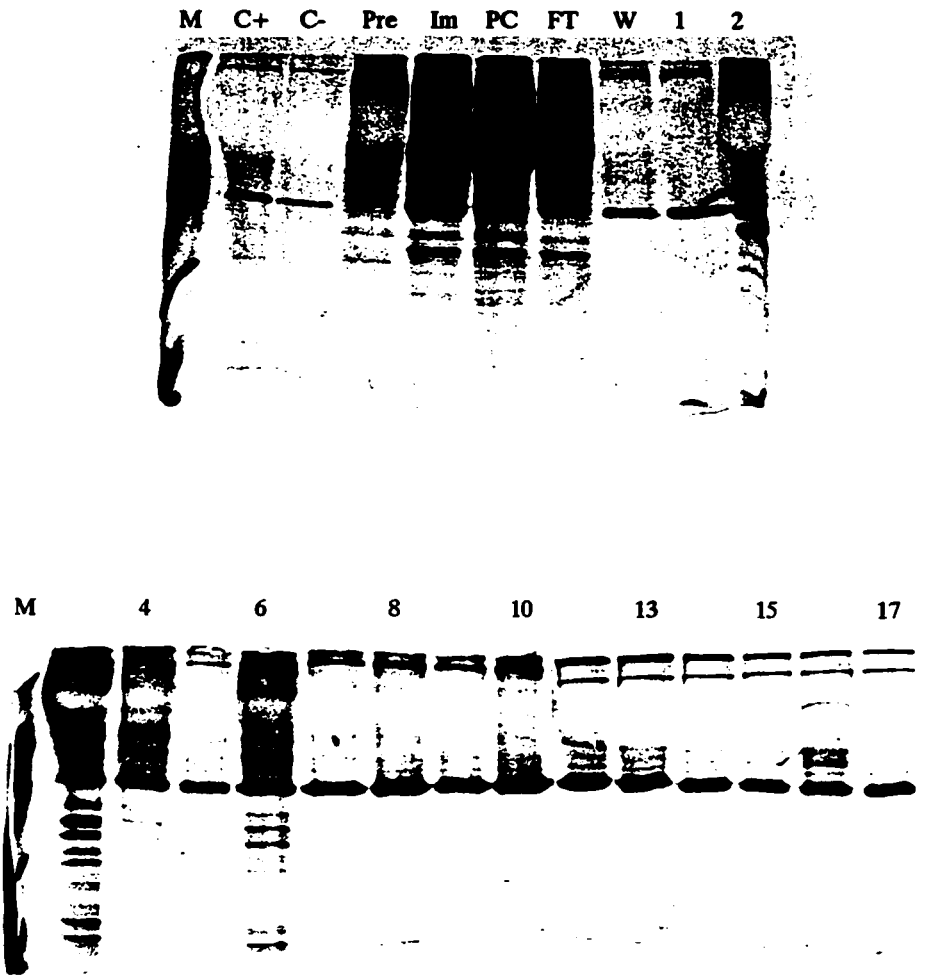

Figure 3.3.3 - Affinity Purification of Rabbit Anti-Pleckstrin Antibodies.

Ten ml of serum from rabbit #2 was passed through a GST-pleckstrin column (see Section 2.3.3) and each eluted fraction was tested for its ability to immunoprecipitate pleckstrin from transfected, [^{35}S]-methionine labelled COS cells. In C+ and C-, Protein A beads only were added to transfected and untransfected COS cell lysate respectively. Preimmune (Pre) and immune sera (Im) from rabbit #2 was tested, as was immune sera added to lysate precleared with preimmune sera (PC). FT and W refer to column flow through and wash fractions respectively. Peak activity was seen in fractions 7 to 17, as shown by the intensity and clarity of the doublet in these immunoprecipitations.

regulating the polymerization of actin (Hashimoto et al., 1987). These authors reported that pleckstrin purified from resting platelets inhibited actin polymerization in vitro. Conversely, pleckstrin isolated from thrombin treated platelets was not inhibitory. They hypothesized that unphosphorylated pleckstrin binds to actin monomers or filaments in the platelet preventing polymerization. Furthermore, the pleckstrin phosphorylated during platelet activation would hypothetically fail to maintain binding activity, resulting in the net polymerization of actin required to achieve platelet shape change and secretion.

These findings were re-examined by using recombinantly expressed pleckstrin, purified rabbit muscle actin (Pardee & Spudich, 1982) and a capillary viscometric assay (Cooper & Pollard, 1982). This system was ideal for testing Hashimoto's hypothesis because recombinantly expressed pleckstrin should be completely unphosphorylated due to a lack of appropriate phosphorylation reactions in bacteria. Rabbit muscle actin was purified to homogeneity as described in section 2.3.6, however, a minor amount of a previously characterized lower band called the protease resistent core was detected (Pardee & Spudich, 1982). Although Hashimoto's work was performed using a falling ball method (Pollard & Cooper, 1982), capillary viscometry is expected to yield similar results with the additional advantages of facility and economy. One caveat to the capillary method is that increased frequency of assaying increases the rate of polymerization. This is due to unavoidable shearing when the sample is sucked up the capillary, which paradoxically increases the rate of polymerization by creating more nucleation sites (Cooper & Pollard, 1982). Care was therefore taken to assay all samples with the same frequency.

Hashimoto et al., described three characteristics of their inhibitory activity. First, the extent of inhibition of polymerization observed was 80% or greater. Second, maximal inhibition could only be observed if the sample was pre-incubated with pleckstrin for 15 minutes or longer prior to initiating polymerization with salt. Third, inhibition plateaued at a molar ratio of pleckstrin to actin of 1:40. Therefore, when performing these assays, an

adequate preincubation period was included and a minimum of a 1:40 ratio of pleckstrin to actin was used.

Figure 3.4.1 shows the data obtained when actin was incubated with a total bacterial supernatant fraction comprised of 2 to 5% recombinantly expressed pleckstrin. This was compared against a control lysate not containing recombinant pleckstrin, and clearly there is no inhibition of polymerization. Since this source of pleckstrin was impure and partially insoluble, another attempt used purified recombinant pleckstrin eluted from a preparative SDS-polyacrylamide gel (as shown in Fig. 3.1.3D), but no inhibition of polymerization was seen (data not shown). A third attempt was made using pleckstrin purified from platelets (a gift of C. Satori), to control for the possibility that electroeluted, recombinant pleckstrin is not in a native, functional state (Fig. 3.4.2). Again, no effect on inhibition of actin polymerization was detected, so no further experimentation of this nature was performed.

There are several reasons why Hashimoto's hypothesis was attractive even prior to the execution of their experiments. Both a notable increase in phosphorylation of pleckstrin and a net increase in cellular filamentous actin content occur concomitant with platelet activation, therefore these events may be causally related. PMA treatment can elicit both of these events by activating PKC (Sano et al., 1983), which must somehow exert its effects via the proteins it phosphorylates. Therefore it is resonable to speculate that pleckstrin, the major target of PMA-inducible phosphorylation in the platelet, could be involved in mediating actin filament elongation. For precedence, the 20 kDa light chain of myosin is also phosphorylated during platelet activation, an event that causes it to interact with actin and mediate contraction (Wilkinson, 1991). In fact, several of the known actin binding or crosslinking proteins are phosphorylated. These include caldesmon and vinculin, the latter being a substrate for PKC. For the actin binding protein filamin, dephosphorylation of one of its two phosphorylated residues abolishes its ability to crosslink F-actin (Wilkinson,

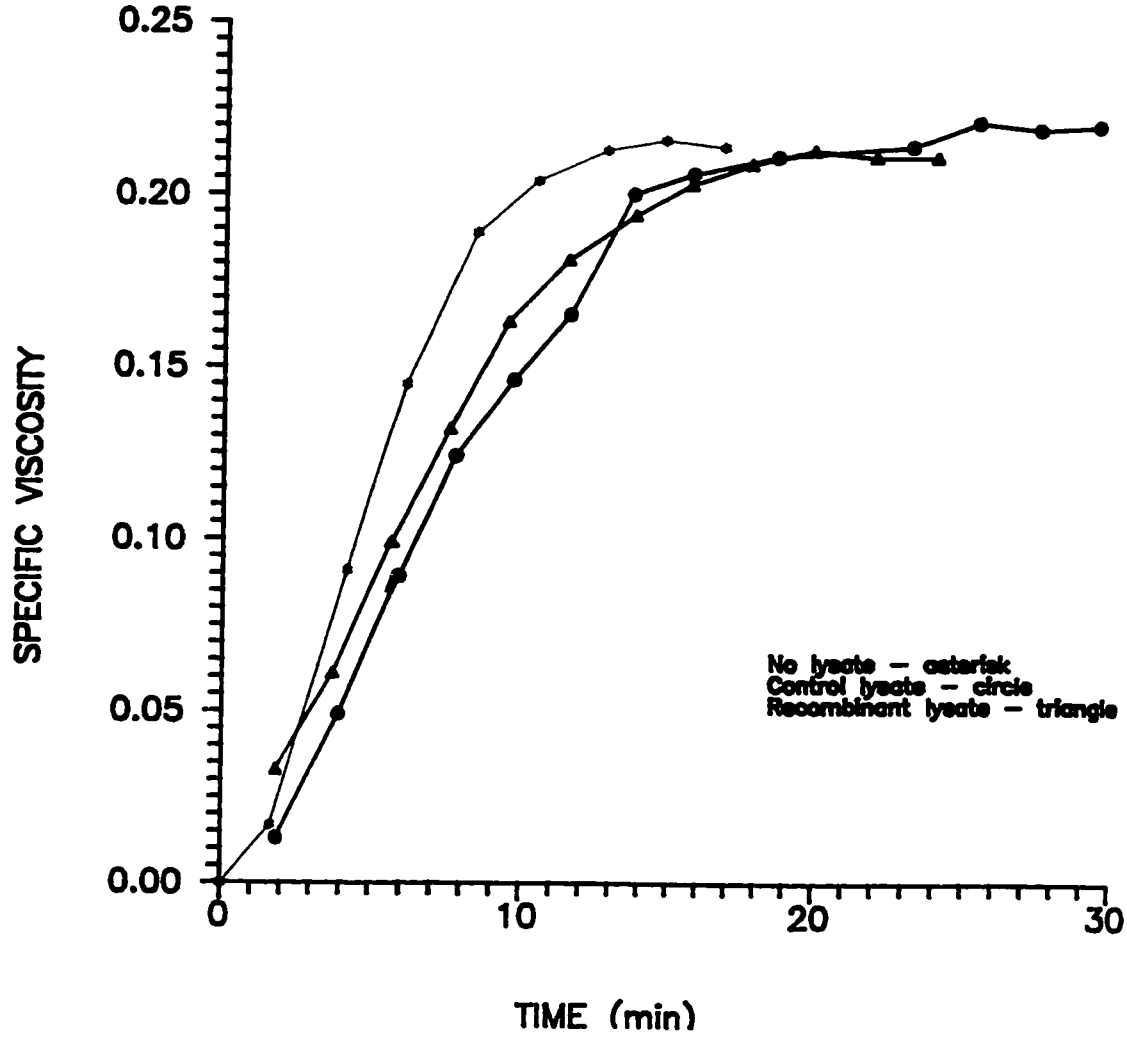

Figure 3.4.1 - Actin polymerization in the presence of recombinant pleckstrin in a bacterial lysate.

A crude bacterial lysate containing overexpressed recombinant pleckstrin (triangle) was compared to a lysate not containing pleckstrin (circle), or buffer alone (asterisk) for the ability to inhibit actin polymerization in an in vitro viscometric assay. The length of time for the actin solution to pass through an Ostwald capillary viscometer (after addition of KCl to 12 μM) was measured over time (x axis) and converted to specific viscosity (y axis, see section 2.3.6).

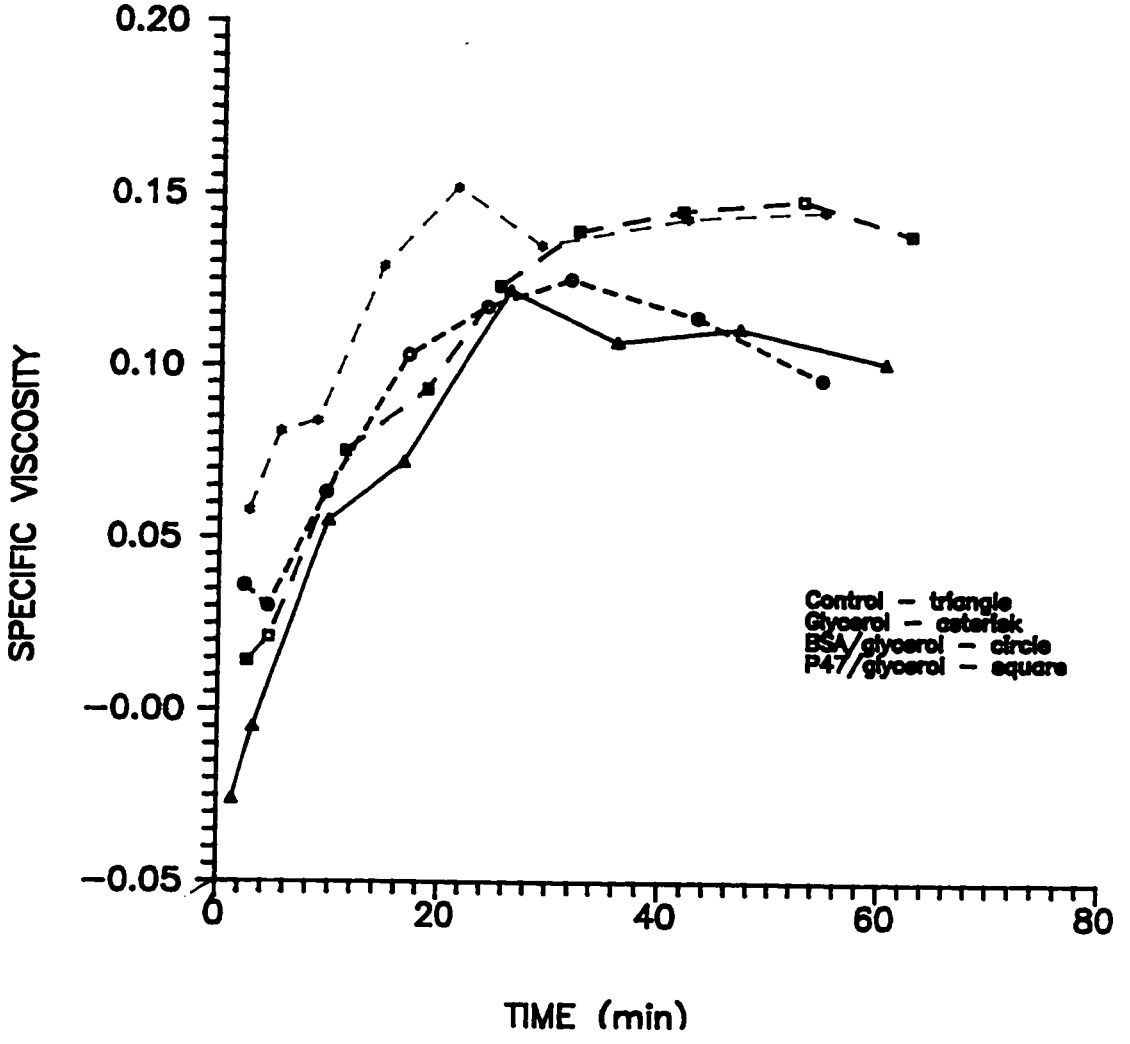

Figure 3.4.2 - Actin polymerization in the presence of pleckstrin purified from platelets.

Pleckstrin purified from platelets was added to an actin solution and the rate of polymerization measured as per Fig. 3.4.1. Controls indicated in the inset included a non-specific protein control (BSA) and/or glycerol control to normalize for any buffer effects attributable to the glycerol (3.1% final concentration) present in the pleckstrin preparation.

1991). Therefore it was plausible to believe that pleckstrin phosphorylation might also regulate actin polymerization.

The following are some empirical criticisms of Hashimoto's work. For pleckstrin to inhibit actin polymerization at a molar ratio of 40:1 (notably the exact ratio found in the platelet (20% actin (Weiss, 1982) to 0.5% pleckstrin (Lyons & Atherton, 1979; Imaoka et al., 1983))), it must be assumed that inhibition occurs not from merely binding soluble actin monomers (one would expect a 1:1 ratio), but by binding the ends of filaments to prevent elongation. Therefore, if bound to actin filaments in its unphosphorylated state, pleckstrin might be expected to be isolated in the TX-100 insoluble fraction during cell lysis (Philips et al., 1980). After activation of platelets and phosphorylation of pleckstrin, it would then be expected to partition into the soluble fraction. But this work and that of others (Fox et al., 1979) has shown that pleckstrin partitions into the soluble fraction irrespective of phosphorylation state (Fig. 3.8.1).

Hashimoto's polymerization inhibitor is likely a contaminant that co-fractionates with pleckstrin under their purification conditions, which they report produces only 80% pure pleckstrin. They claim that unphosphorylated pleckstrin elutes at much lower salt concentrations (85 mM vs 155 mM) than phosphorylated pleckstrin during their final purification step (Hashimoto et al., 1987), therefore, this could be the source of their contamination. It seems unlikely that these pleckstrin isoforms would elute so uniquely given the only slight net charge difference between the hypo and hyperphosphorylated forms (see section 3.5). Additionally, their discrimination of "unphosphorylated" pleckstrin is suspect given that hypophosphorylated pleckstrin would not be traced well using ^{32}P radiolabelling. It should be noted that the purification scheme of Imaoka et al. (1983), is more elaborate and yields purer final product (98%). It was this latter source of pleckstrin that was used in the actin polymerization assay shown in Fig. 3.4.2.

Shortly after this experimental re-analysis of Hashimoto's work, another report

from their group was published that showed an inability of unphosphorylated pleckstrin to be retained on actin-affinity columns (Sasaki et al., 1989). A 48 kDa protein that bound to the actin column in a calcium-dependent manner was determined not to be pleckstrin since it did not subsequently become phosphorylated by PKC in vitro. What they believe to be pleckstrin eluted with the unbound flow through fraction from the column. Therefore, there is no evidence supporting a direct interaction of pleckstrin with actin.

PART B - In Vivo Characterization of Human Pleckstrin: Expression, Phosphorylation and Subcellular Distribution

It is difficult task to elucidate the function of a completely novel protein. One avenue of investigation would be to examine the consequences of manipulating the natural expression level of the protein in vivo. Several ways to disrupt the natural expression of pleckstrin can be conceived. Using appropriate transient or stable transfections, particular cell lines could be created that overexpress pleckstrin either constitutively or inducibly in cell types that do or do not normally express pleckstrin. Conversely, transfections with antisense plasmids might sufficiently suppress pleckstrin expression in cells that natively express pleckstrin. Alteration in pleckstrin expression levels could then be correlated to changes in cell growth, morphology, differentiation capabilities and functional characteristics unique to that cell type. Even if successful, it would still be quite difficult to identify the particular molecular event(s) that pleckstrin over- or underexpression induces that led to the altered phenotype.

In mice, homologous recombination has been used successfully to ablate unique genes with consequences on organ development often being quite visible (Galli-Taliadoros et al., 1995). Since isolation of a genomic clone corresponding to the 5' end of the pleckstrin cDNA had been elusive (M. Tyers, Ph.D. thesis), gene knockout and promoter studies were not seen as feasible during the course of this work.

3.5 Expression of Pleckstrin in Transiently Transfected COS Cells

At the onset of this project, numerous attempts were made to subclone a 2 kbp Pvu

II cDNA fragment containing the entire coding region of pleckstrin into the pXM eukaryotic expression vector (Yang et al., 1986). During this time, four different subcloning strategies were employed, but none were successful. Clones that hybridized to the 2 kbp radiolabelled probe always yielded unusual restriction enzyme patterns containing 2.5 and 6 kbp fragments (data not shown).

A vector was obtained (pEVRF2) that is reported to yield high copy numbers in cells expressing the SV40 large T antigen (Matthias et al., 1989). COS-1 cells, derived from a African Green Monkey kidney epithelial cell line, overexpress the SV40 large T antigen and therefore can maintain a high copy number of this plasmid. These cells also display robust growth characteristics. The pleckstrin cDNA was readily subcloned into this vector by the methodology outlined in Fig. 2.1. The plasmid generated (pEVp47(+)), was used extensively for transiently transfecting COS cells in the work described in the following sections. The pleckstrin cDNA was also subcloned into the eukaryotic expression vectors pSVneo and pMEP4. The latter vector employs a metallothionein promoter to drive inducible gene expression. Unfortunatlely the levels of zinc reportedly required to achieve expression were found to be toxic to the COS cells (data not shown). Since there exist two unique restriction sites in the pleckstrin sequence on either side of the highly probable PKC phosphorylation site (amino acids 113-117; Tyers et al., 1988), a deletion mutant was constructed lacking this region (Nsi I - Xba I, Fig 2.1, deletion mutant termed pEVΔNsi). Expression of pleckstrin from pEVp47(+) and the deletion construct pEVΔNsi transfected into COS cells was compared to endogenous levels found in the human erythroleukemia (HEL), human promyelocytic HL-60 cell lines and in platelets (Fig. 3.5.1). The transiently transfected COS cells appeared to express approximately the same amount of pleckstrin as HL-60 cells. For some transfections the levels approached that of HEL cells, which express approximately 5 times more pleckstrin per mg protein than HL-60 cells. The high level of pleckstrin expression seen in HEL cells is not

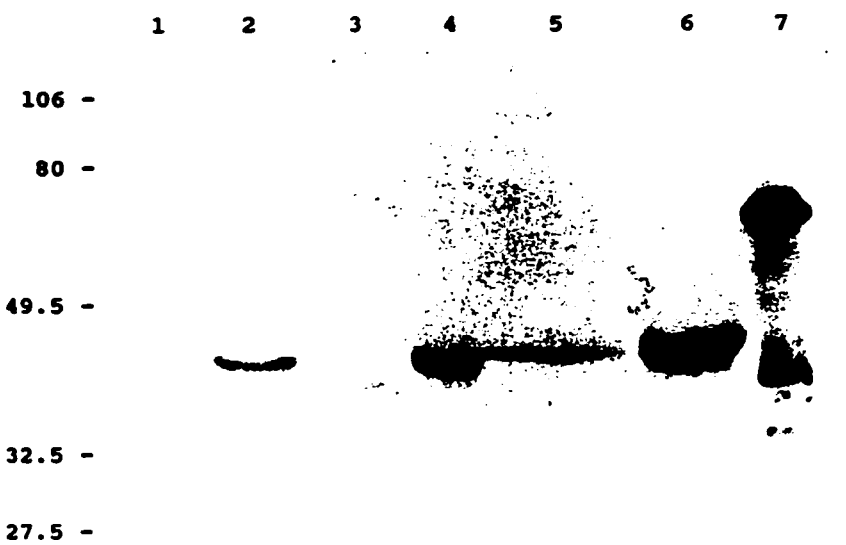

Figure 3.5.1 - Western Analysis of Pleckstrin Expressed Heterologously in COS Cells.

Soluble protein (25 μg) from the following sources was separated by SDS-PAGE, transferred to nitrocellulose and probed with rabbit anti-pleckstrin antibodies and ^{125}I-Protein A: lane 1, untransfected COS cells; lane 2, pEVp47(+) transfected COS cells; lane 3, pEVΔNsi transfected COS cells; lane 4, HEL cells; lane 5, HL-60 cells; lane 6, platelets; lane 7, purified recombinant GST-pleckstrin, which runs at an apparent molecular mass of 67 kDa.

surprising since they express many megakaryocyte-specific markers (Long et al., 1990). By running 25 ng of recombinant GST-pleckstrin on this blot the mass amount of pleckstrin expressed in the COS cells was estimated to be 0.02% of total cell protein. This value is comparable to the amount previously determined to be present in HL-60 cells (4.4 ng/25 μg total protein (Tyers et al., 1987)). It is important to note that the level of pleckstrin in each sucessfully transfected COS cell is actually approximately 20 times higher than this value (therefore 0.4% of total cell protein), since typically only about 5% of the cells become transfected (data not shown).

On some well resolved westerns, as in Fig. 3.5.2, two specific immunoreactive bands (a doublet) were observed for the wild-type clone. The higher band most likely represents translation initiation occurring at the ATG of the herpes simplex virus thymidine kinase (HSV tk) gene, which is 9 codons upstream from the pleckstrin start codon. This N-terminally extended polypeptide is predicted to be 937 Da larger than full-length pleckstrin, having the additional sequence MASWGSGYP. Indeed when measured, the difference in apparent molecular mass of the two bands is approximately 1 kDa. Additionally, when pleckstrin from an endogenous source is run alongside pleckstrin expressed from pEVp47 in COS cells, native pleckstrin lines up with the lower band (data not shown). Only one band for the deletion mutant was ever observed (Fig. 3.5.2, lane 8), and comparison against molecular mass standards indicated that this polypeptide most likely arose from translation initiation at the upstream ATG. A deletion mutant lacking the the C-terminal half of the pleckstrin coding region was also constructed, but expression from this clone (pEVp47ΔXba), could not be detected (Fig. 3.5.2, lane 7).

Another clone was selected that had the pleckstrin cDNA inserted in the opposite orientation (Fig. 2.1). When this "antisense" clone was co-transfected with the sense plasmid, it efficiently supressed pleckstrin expression derived from the sense plasmid at a

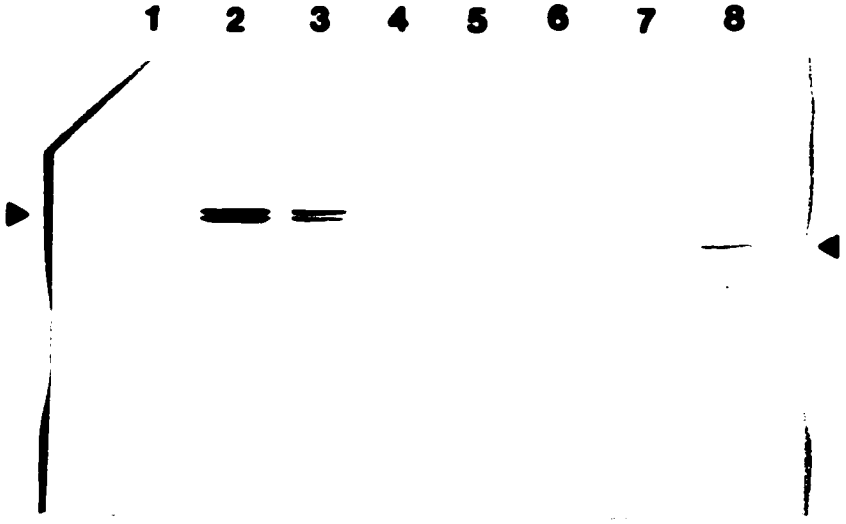

Figure 3.5.2 - Western Analysis Indicating Antisense Suppression of Pleckstrin Expression.

Protein samples from COS cells transfected with the following amounts of plasmid DNA were analyzed by western blotting (see section 2.4.2): lane 1, no DNA; lanes 2 & 3, 15 and 1.5 μg pEVp47(+) respectively; then increasing molar ratios of antisense plasmid (pEVp47(-)) to sense plasmid (pEVp47(+)) were used as follows; in lane 4, 10:1; lane 5, 50:1; lane 6, 100:1; using 15 μg sense plasmid in lane 4 and 1.5 μg in lanes 5 & 6. Lane 7, 25 μg of a C-terminally truncated pleckstrin construct (pEVp47ΔXba) for which protein expression was never detectable. Lane 8, 25 μg of the deletion construct pEVΔNsi.

molar ratio of 10:1 (Fig. 3.5.2, lane 4). This was promising since similar studies have reported a requirement for a 50 to 100 fold greater amount of antisense plasmid to completely abolish protein expression (Dougherty & Parks, 1995). One caveat however is that the antisense plasmid might be squelching transcription from the sense plasmid by soaking up limited transcription factors and not by RNA hybrid formation. If true one would not expect protein expression to be completely abolished but reduced 10 fold, since transcription factors should distribute in proportion to available target DNA's. A 10 fold excess of antisense RNA should however be able to bind all available sense RNA's, completely inhibiting protein expression. Since residual pleckstrin expession was not detected in co-transfections with antisense plasmid, inhibition was probably via RNA hybrid formation. The antisense plasmid would therefore be a useful tool for suppressing endogenous pleckstrin expression in white blood cell lines either transiently or stabily transfected with the antisense clone. However, transfection of heamatopoietic cell lines proved difficult (data not shown), and further efforts to generate these cell lines were abondoned.

To validate the heterologous COS expression system, it was necessary to prove that recombinant pleckstrin expressed in COS cells was functionally identical to that of native pleckstrin. In the absence of a functional assay for pleckstrin, phosphorylation in response to PKC activators in vivo was chosen as the best functional parameter to study. Phosphorylation of pleckstrin had been examined previously by 2-dimensional (2-D) isoelectric focusing (Imaoka et al., 1983). Isoelectric focusing would show two things. First, recombinantly expressed plecsktrin should have a pI identical to native pleckstrin. Second, its position in the 2-D gel should undergo a visible acidic shift after PKC activation, correlating with an increase in phosphorylation state. The 2-D analysis in Fig. 3.5.3 shows that pleckstrin immunoprecipitated from [^{35}S]-methionine-labelled, transfected COS cells displays the same pattern of multiple isomers in the pI range of 6.2 -

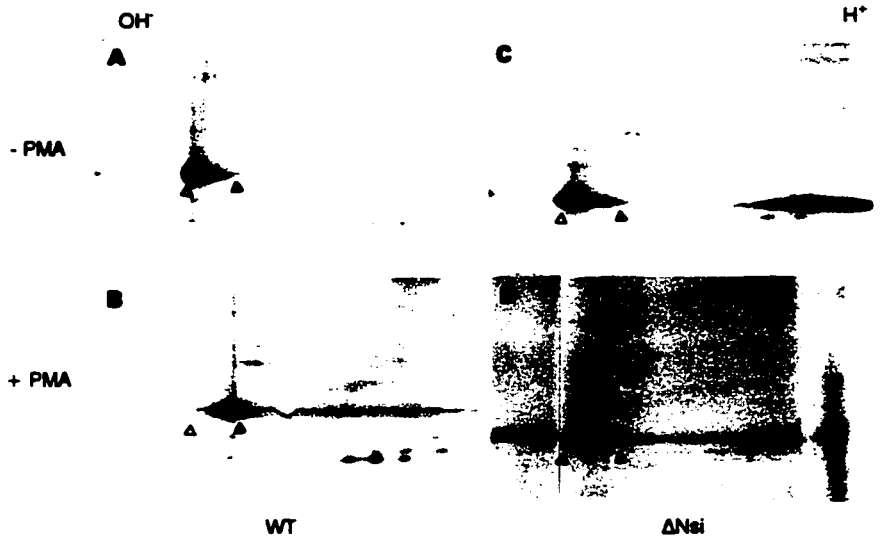

Figure 3.5.3 - 2-Dimensional Isoelectric Focusing of Wild-Type and Deletion Mutant Forms of Pleckstrin Expressed in COS Cells.

Wild-type pleckstrin (WT) or the deletion mutant (ΔNsi) were immunoprecipitated from ^{35}S-methionine labelled transfected COS cells and isoelectrically focused, then separated by SDS-PAGE. When PMA was added (100 nM, 30 min), the series of spots noticeably shifts in the acidic direction for the wild-type protein (compare **B** to **A**), but not as substantially for the deletion mutant (compare **D** to **C**). Open and closed arrowheads bracket the higher pI and lower pI isomers respectively.

6.7 as does native platelet pleckstrin (Imaoka et al., 1983). Additionally, pretreating the COS cells with PMA caused a distinct acidic shift in the abundance of the isomers (meaning the lower pI forms that are presumably more highly phosphorylated, increased in relative abundance at the expense of higher pI forms). It was promising to note that the putative 5' extended translation product produced a series of spots identical to the lower ones. This implies that the 9 neutrally-charged amino acids of the N-terminally extended polypeptide do not interfere with the phosphorylation characteristics of pleckstrin. The pleckstrin ΔNsi deletion mutant had a qualitatively different pattern of spots prior to PMA addition in comparsion to full-length pleckstrin. Furthermore these spots did not undergo the same dramatic acidic shift after PMA stimulation (Fig. 3.5.3).

A 2-D isoelectric focusing gel analysis was made directly comparing pleckstrin immunoprecipitated from COS cells with that from HL-60 cells. In both cases the pI of pleckstrin and its shift in response to PMA were identical (Fig. 3.5.4). In these particular gels fewer isomers of pleckstrin are seen than in the gels of Fig. 3.5.3. The multiple isoforms seen in previous gels (Fig. 3.5.3 and Imaoka et al., 1983) might be a result of carbamylation of pleckstrin in the high urea concentrations used during isoelectric focusing (Cooper, 1991). In fact, the distance measured in pI units between adjacent isomers (0.1 pH unit) corresponds more to that expected from a carbamylation modification than a phosphorylation (0.5 pH units, Cooper, 1991). Therefore, the stoichiometry of phosphorylation cannot be accurately assessed from these 2-D [^{35}S]-methionine-labelled protein gels. However, from the completeness of the acidic shift, it can be inferred that the vast majority of pleckstrin molecules become at least singly phosphorylated after PMA stimulation.

Phosphorylation of recombinant pleckstrin was also studied by ^{32}P-labelling transfected COS cells (see section 2.5.1). When total cell protein from transfected COS cells was compared to that from untransfected cells, a distinct peak of radioacitivty at

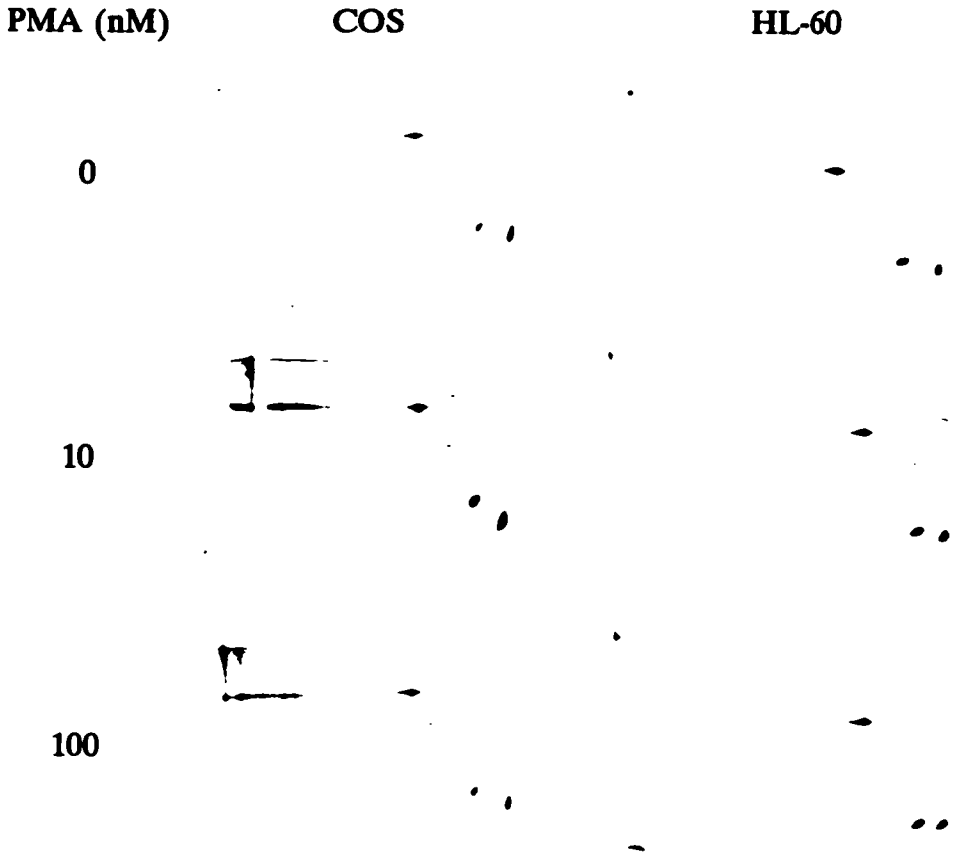

Figure 3.5.4 - 2-D Isoelectric Focusing Comparing Pleckstrin Isolated from HL-60 Cells to that from Transfected COS Cells.

Transfected COS cells or HL-60 cells were exposed to various concentrations of PMA (0, 10 or 100 nM) 30 min prior to harvesting. Pleckstrin was then immunoprecipitated from the cell lysates, isoelectrically focused and analysed by SDS-PAGE. The pleckstrin spot(s) (upper middle) shift to the right after 10 or 100 nM PMA treatment in both systems. The two spots in the lower right hand corner of the gels may represent immunoreactive degradation products of pleckstrin or cross-reacting proteins.

approximately 40 kDa appeared in the transfected lane (Fig. 3.5.5A). Integration of this peak by densitometry indicated that it accounted for 1% of the total protein-bound ^{32}P. Immunoprecipitation confirmed the presence of ^{32}P-labelled pleckstrin, which had the same migration in SDS polyacrylamide gels as the peak radioactivity in cell lysates (Fig. 3.5.5B). When the estimated fraction of cells transfected with the plasmid is considered, labelling of pleckstrin constitutes roughly 10% of the phosphate incorporated into soluble proteins these cells.

To confirm that phosphorylation of pleckstrin is PKC-mediated, staurosporine was added to the cells prior to PMA treatment. At a concentration of 10 nM, staurosporine inhibited the PMA-inducible phosphorylation of pleckstrin in COS cells by 50% (Fig. 3.5.6b,e). Examination of pleckstrin phosphorylation in HL-60 cells revealed a comparable staurosporine IC_{50} of 6 nM (Fig 3.5.6a,d), identical to the concentration quoted for PKC inhibition in vitro (O'Brien & Ward, 1990). Both the HL-60 and transfected COS cell values are within a standard deviation of the 28 nM IC_{50} reported for staurosporine inhibition of pleckstrin phosphorylation in the platelet (Saxena et al., 1991). Although staurosporine is not a specific PKC inhibitor, it has a lower IC_{50} for PKC than for PKA or most tyrosine kinases (O'Brien & Ward, 1990) and therefore should be a somewhat discriminatory agent when used close to its PKC IC_{50} value.

Unexpectedly, the deletion mutant was constitutively phosphorylated in the absense of PMA treatment (Fig. 3.5.6c,f). Addition of PMA did not noticeably increase the already high level of phosphorylation of the deletion mutant. It is not known which kinases are responsible for the constitutive phosphorylation of the deletion mutant or which sites were being phosphorylated.

Collectively, the above results indicate that recombinant pleckstrin expressed heterologously in COS cells is indeed functionally identical to natively expressed pleckstrin.

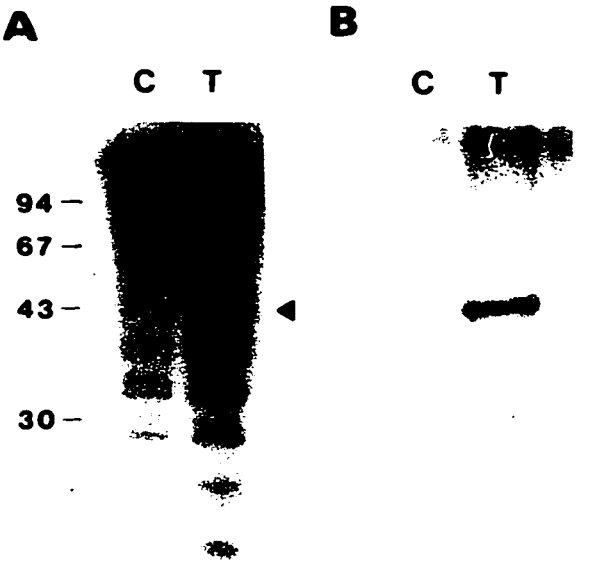

Figure 3.5.5 - Phosphorylation of Pleckstrin Expressed Heterologously in COS Cells.

A, ^{32}P-labelled total cellular protein, or **B**, immunoprecipitates using anti-pleckstrin antibodies from untransfected COS cells (C), or from pEVp47(+) transfected cells (T), were analysed by SDS-PAGE and exposed to film. The arrowhead indicates the location of ^{32}P-labelled pleckstrin, which is absent in untransfected COS cells.

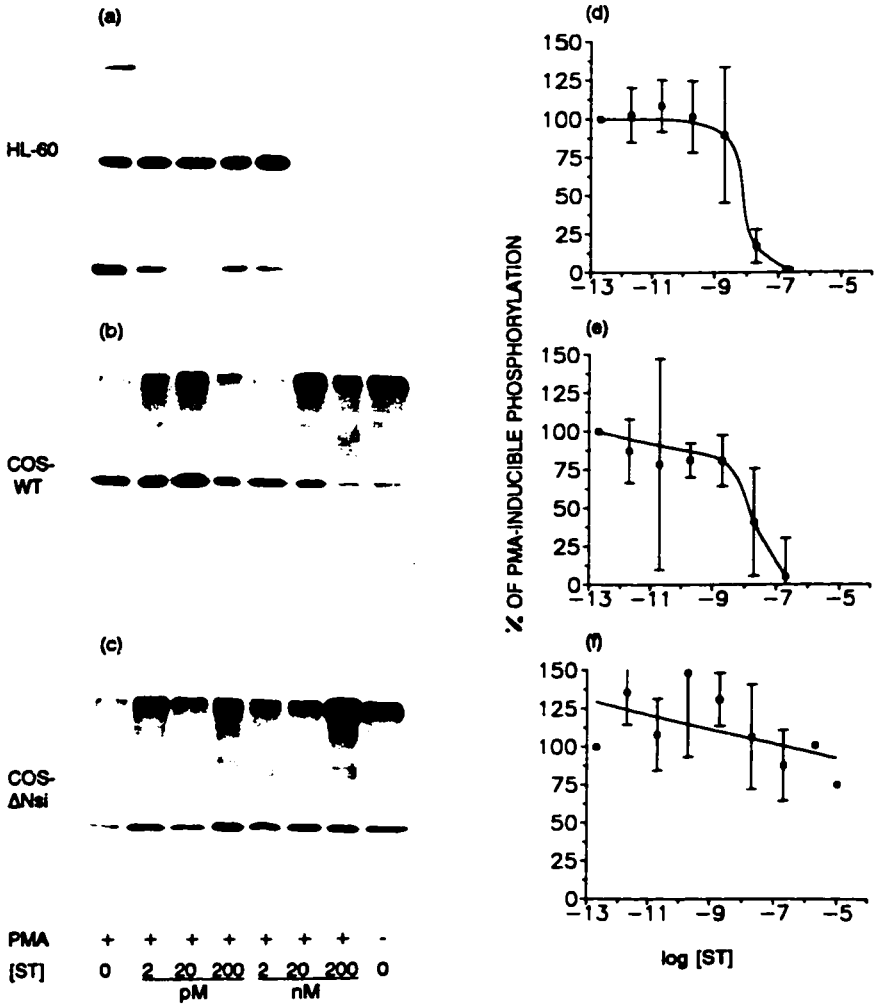

Figure 3.5.6 - Effect of Staurosporine on PMA-Induced Pleckstrin Phosphorylation.

Pleckstrin from ^{32}P-labelled HL-60 (a), pEVp47(+) (b), or pEVΔNsi (c) transfected COS cells treated with PMA (100 nM, 30 min) and staurosporine (ST, indicated concentrations, 1 h), was immunoprecipitated and analysed by SDS-PAGE. These experiments were repeated three times and quantitated using Molecular Dynamics PhosphorImager software, and the percent of PMA-inducible phosphorylation remaining at each concentration of ST then represented graphically in (d), (e) and (f). Since the deletion mutant displayed no PMA-inducible increase in phosphorylation (compare first and last lanes in (c)), 100 % phosphorylation in (f) refers to the level of phosphorylation in the presence of 100 nM PMA over background (instead of over 0 nM PMA).

3.6 - Construction, Expression, and Phosphorylation of 8 Phosphorylation Site Mutants of Pleckstrin

The pleckstrin protein sequence was analysed by PC GENE software and found to contain 11 potential PKC phosphorylation sites as shown in Table 3.6.1. Two of these sites (40 & 114) also scored significantly as potential cAMP or cGMP-dependent kinase phosphorylation sites as did sites 132 & 331 for caesin kinase II. Also included in Table 3.6.1 is a ranking of putative PKC phosphorylation sites generated previously using the ALIGN program (Tyers et al., 1989). This multiple alignment program was based upon data from only 4 known PKC phosphorylation sites. Re-analysis of potential phosphorylation sites using the criteria of Kennelly & Krebs, 1991, led to a slightly different ranking (Table 3.6.1, last column). Their conclusion after an analysis of 68 physiological PKC phosphorylation sites was that $(R/K_{1-3}, X_{2-0})$-S^*/T^*-$(X_{2-0}, R/K_{1-3})$ > S^*/T^*-$(X_{2-0}, R/K_{1-3})$ > or = $(R/K_{1-3}, X_{2-0})$-S^*/T^*, where X represents any amino acid and the subscript represents the number (not placement) of particular residues. It therefore appears that having at least one basic residue on each side of the phosphoacceptor, preferrably at the -2 and +2 position, is a stronger determinant of phosphorylation than having one or more basic residues on one side only.

Using the above analysis as a guideline, 8 residues within the N-terminal half of pleckstrin were selected for mutagenesis (S40, S43, T73, S113, T114, S117, S132 & T136). Since it was likely that one of S113, T114 or S117 would be an actual phosphorylation site (Table 3.6.1), and since it had been shown previously that all the ^{32}P was localized to a 14 kDa proteolytic fragment of pleckstrin (Imaoka et al., 1983), sites distal to these three, although fair candidates, were not selected for mutagensis.

During the creation of these point mutations, two different double stranded (ds) site-directed mutagenesis schemes were attempted. The first involved priming a single

Residue	Context	Score[a]	Rank[a]	Rank[b]
20	kgsvfn T wkpmwv			5
40*	efykkk S dnspkg	427[c]	5	6
43*	kkksdn S pkgmip	433	4	5
73*	fvfkit T tkqqdh	416	6	4
113*	qkfark S trrsir	455	2	1
114*	kfarks T rrsirl	470	1	1
117*	rkstrr S irlpep	450	3	1
132*	lgalyl S mkdtek			5
136*	ylsmkd T ekgike			2
170	wlvsnq S vrnrqe			5
331	yflqaa T ekgike			5
348	iqmasr T gk			3

a - based upon the program ALIGN
b - based upon the criteria of Kennelly & Krebs, 1991
c - this site was not selected by PC GENE
* - indicates the 8 sites choosen for subsequent mutation

Table 3.6.1 - Putative PKC phosphorylation sites in pleckstrin.

Shown are 12 putative PKC phosphorylation sites in the pleckstrin protein identified using the PROSITE subroutine of PC GENE. The scores of the 6 most likely PKC phosphorylation sites in pleckstrin identified previously using the program ALIGN (Tyers *et al.*, 1989) are also shown. It should be noted that site 40, identified by ALIGN, was not identified by PC GENE as a PKC site, probably because it lacks a C-terminal basic residue (Kennelly & Krebs, 1991). An alternate rank was assigned to each putative phosphorylation site based on the criteria of Kennelly and Krebs, 1991 (last column).

round of DNA polymerization to incorporate the mutagenic oligo, but insufficient amounts of the newly synthesized product were produced to make subcloning feasible (data not shown). Clearly PCR based mutagenesis is a natural extension and improvement upon the above method (Vallette et al., 1989). The second ds mutagenesis method involved the formation of DNA hybrids after denaturation/renaturation of overlapping linear DNA fragments, from which priming to incorporate mutagenic nucleotides can occur (Morinaga et al., 1984). However, subsequent transformation and colony hybridization using the mutant oligonucleotide led to the selection of several false positives (data not shown), likely due to difficulties in achieving hybridization conditions that can discriminate a single base change.

The Promega altered sites mutagenesis kit was subsequently purchased and the 445 bp Bam HI - Xba I fragment from pEVp47(+) subcloned into the p-Select 1 vector. Mutagenesis was followed as outlined in section 2.2.3.3, using the mutagenic oligonucleotides listed in section 2.1.5. Sequencing confirmed that the correct base change had been made for all 8 mutants (data not shown). Overall, > 75 % of the transformants had the mutation incorporated. To subclone the mutant 445 bp fragments back into pEVp47(+), an Xba I partial digest of the parental Bam HI linearized vector had to be performed since 2 Xba I sites are present in pEVp47(+), then the parental vector missing the 445 bp Bam HI - Xba I piece had to be carefully excised from a LMP gel. The entire 445 bp region of each mutant was sequenced after subcloning back into the pEVp47(+) expression vector to insure that no other mutations had inadvertently been created (data not shown).

The 8 mutant plasmids were transfected into COS cells and expression levels of all 8 mutant proteins were similar to wild-type, as shown in Fig. 3.6.1. These expression levels were consistent from transfection to transfection, and as per the wild-type protein, a doublet was seen for each mutant (section 3.5). To determine if these mutant proteins

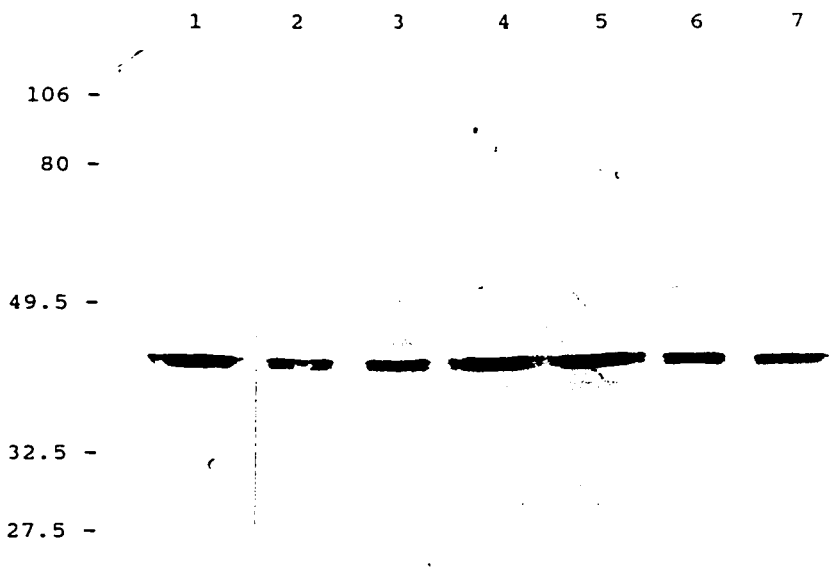

Figure 3.6.1 - Western Analysis of Site-Directed Pleckstrin Mutants Expressed in COS Cells.

COS cell lysate (25 μg) from transfections of the wild-type pleckstrin construct or 6 of the 8 site-directed mutant constructs was separated by SDS-PAGE and transferred to nitrocellulose. Lane 1, wild-type pleckstrin; lane 2, S40A; lane 3, S43A; lane 4, T73A; lane 5, S113A; lane 6, T114A; lane 7, S117A. The blot was developed by a colorimetric reaction using an alkaline phosphatase secondary antibody after incubation with the primary anti-pleckstrin antibody.

could be phosphorylated, each mutant-transfected dish of COS cells was ^{32}P-labelled, the mutant proteins immunoprecipitated and analyzed by SDS-PAGE. The level of phosphorylation was measured before and after PMA stimulation, and after inhibition with staurosporine. When compared to wild-type, only 2 mutants reproducibly displayed a subnormal level of phosphorylation after PMA treatment; S113A and S117A (Fig. 3.6.2). The percent reduction in PMA-induced phosphorylation of these two mutants ranged from 40 to 60% depending on the experiment.

3.7 Phosphorylation Site Mapping of Pleckstrin

There are several reasons to map the phosphorylation sites of pleckstrin. First, conclusive identification phosphorylated residues in pleckstrin will greatly facilitate interpretation of the role of phosphorylation in any future structure/function studies of pleckstrin. Second, identification of the kinases responsible for phosphorylating these sites in vivo leads to a better understanding of pleckstrin's role in various signalling pathways. Third, an expansion to the empirical data base of physiological phosphorylation sites increases our understanding of kinase specificity.

Phosphorylation site mapping is commonly performed by tryptic cleavage of the purified, ^{32}P-labelled protein of interest, followed by electrophoresis on a thin layer chromatographic (TLC) plate. The peptides are then further resolved by chromatography in the second dimension. Other peptide cleavage methods and alternate isotopes can often help in identifying the phosphopeptides (Boyle et al., 1991), and if a temperature controlled flat bed apparatus is used, very reproducible maps can be made.

A priori to mapping, the deduced pleckstrin amino acid sequence was analysed for potential tryptic cleavage sites using PC GENE software. A list was then compiled of all

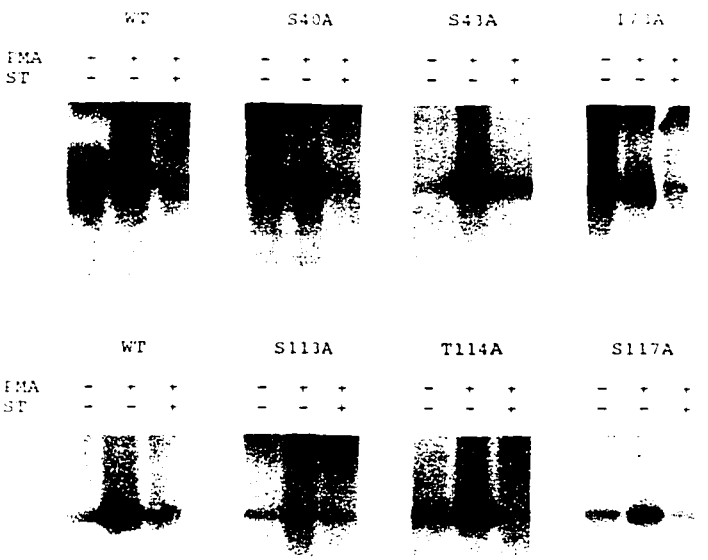

Figure 3.6.2 - In Vivo Phosphorylation of Site-Directed Mutants.

Shown are immunoprecipitations of the wild-type and 6 mutant forms of pleckstrin from [^{32}P]-orthophosphate labelled transfected COS cells that had been pretreated with PMA (100 nM, 30 min) (middle lane) and staurosporine (ST; 200 nM, 1 h) (last lane), or neither (first lane).

the potential tryptic fragments containing a serine or threonine (Table 3.7.1), since it had been previously shown that pleckstrin is phosphorylated on serine and threonine but not tyrosine (Imaoka et al., 1983). Assuming a phosphorylation state of one, the electrophoretic mobility of the tryptic peptides in pH 1.9 buffer was calculated based upon the relationship mobility = charge/mass$^{2/3}$ derived by Offord, 1966 (see Table 3.7.1). The relative ascent of these peptides in chromatography buffer was calculated by averaging the sum of the individual amino acids empirical migration in this buffer (Boyle et al., 1991). (This latter measure proved less accurate than electrophoretic mobility predictions, but still provided valuable relative positioning information). Trypsin theoretically cleaves pleckstrin into 40 fragments larger than one amino acid, of which 15 contain at least one serine or threonine (Table 3.7.1). The relative positions of these 15 peptides after theoretical 2-dimensional mapping are plotted graphically in Fig. 3.7.1.

To perform phosphorylation site mapping, pleckstrin was first immunoprecipitated and separated by SDS-PAGE, then transferred to nitrocellulose and incubated with trypsin (Luo et al., 1991). For a comparative analysis, maps were generated for wild-type pleckstrin isolated from three very different sources. As shown in Fig. 3.7.2, identical maps were produced by pleckstrin natively expressed in HEL cells, heterologously expressed in pEVp47(+) transfected COS cells and bacterially expressed pleckstrin phosphorylated in vitro. The major spots on these maps were labelled 1, 2 and 3. Since the map of recombinant GST-pleckstrin phosphorylated in vitro was identical to that for natively expressed pleckstrin, it can be assumed phosphorylation of pleckstrin by PKC in vitro adequately mimics phosphorylation of native pleckstrin in vivo. Additionally, the presence of the GST domain in the recombinant fusion protein did not interfere with phosphorylation of pleckstrin.

Occasionally, a fourth and fifth spot were seen on maps of both endogenously derived and recombinant pleckstrin (Fig. 3.7.3). These additional spots migrated to the

#	Residues	Sequence	Mobility	Hydro
1	15-37	G<u>S</u>VFN<u>T</u>WKPMWVVLLEDGIEFYK	1.00	.501
2	40-45	<u>S</u>DN<u>S</u>PK	1.24	.282
3	52-64	G<u>ST</u>L<u>TS</u>PCQDFGK	<.79	.380
4	71-75	I<u>TTT</u>K	1.34	.416
5	113-115	<u>ST</u>R	1.72	.307
6	117-119	<u>S</u>IR	1.69	.427
7	120-134	LPEPIDLGALYL<u>S</u>MK	0.69	.517
8	135-138	D<u>T</u>EK	1.45	.255
9	151-172	QEGLMIA<u>SS</u>LLNEGYLQPAGDM<u>S</u>K	0.52	.438
10	199-238	<u>S</u>AVDG<u>T</u>/<u>S</u>GFFCEEN<u>SS</u>DDDVILK	<.36	.431
11	294-307	GCVV<u>T</u><u>S</u>VE<u>S</u>N<u>S</u>NGR	<.76	.354
12	309-333	<u>S</u>EEENLFEII<u>T</u>ADEVH/LQAA<u>T</u>PK	0.97	.462
13	336-340	<u>T</u>EWIK	1.20	.452
14	341-347	AIQMA<u>S</u>R	1.11	.406
15	348-350	<u>T</u>GK	1.89	.263

Table 3.7.1 - Relative mobilities of predicted tryptic phosphopeptides of pleckstrin.

Of the 40 peptides predicted to be generated from complete tryptic hydrolysis of pleckstrin, 15 contain one or more serine or threonine residues (underlined), and are therefore listed above. The relative electrophoretic mobilities of these peptides in pH 1.9 buffer (and assuming a phosphorylation state of one), were calculated as: mobility = 100 x (charge)/(mass)$^{2/3}$ (Offord, 1966). Their predicted hydrophobicities (Hydro) and therefore their relative ascent in phosphochromatography buffer (Boyle *et al.*, 1991), are also listed. This information was used to plot the graph shown in Fig. 3.7.1.

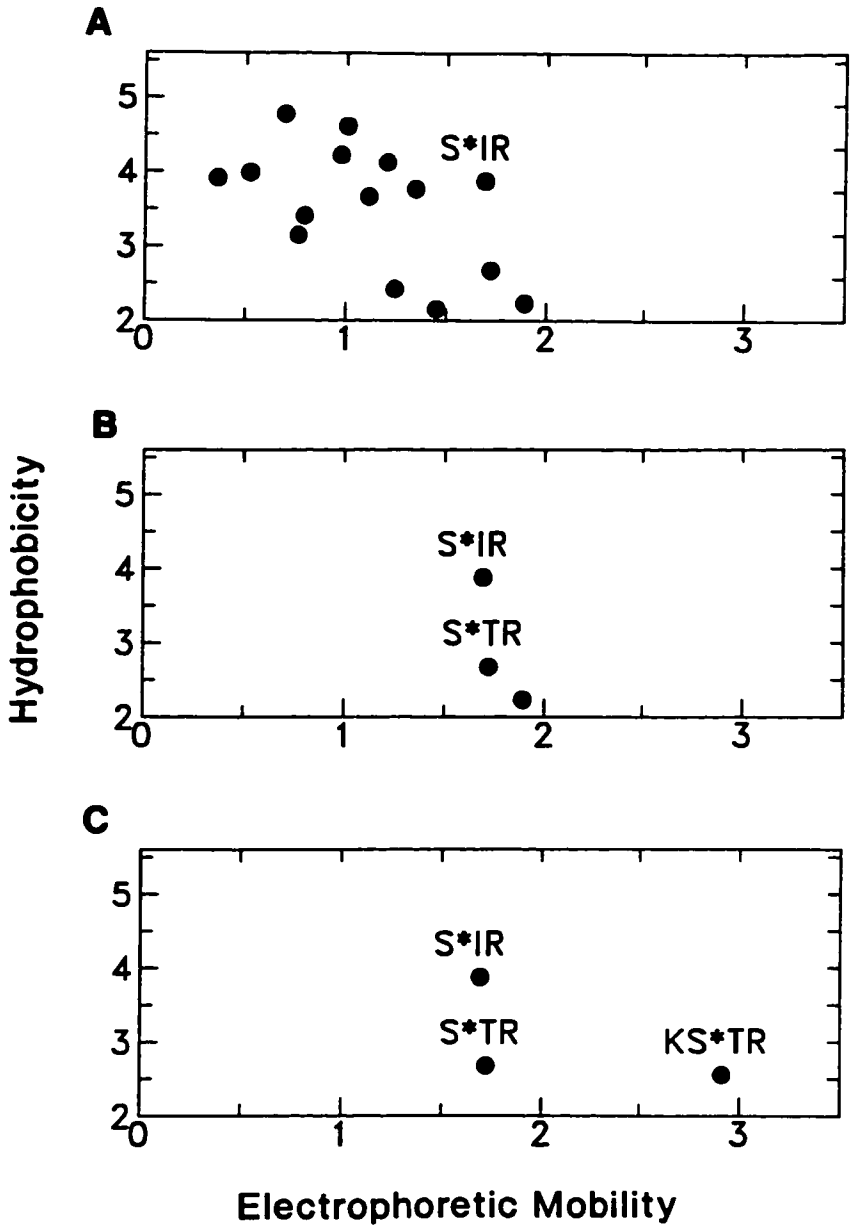

Figure 3.7.1 - Theoretical Phosphopeptide Mapping.

Using the calculated electrophoretic mobility and hydrophobicity values for each of the 15 tryptic peptides of pleckstrin listed in Table 3.7.1, the plot shown in **A** was generated. Identification of spot 1 from the map of S117A in Fig. 3.7.6 as being a tryptic peptide containing serine 117 (see results), leads to the identification of spot 1 as S*IR (amino acids 117-119). In **B**, the theoretical peptides having less mobility than S*IR in **A** are excluded since none appear on the empirical maps. Also, the spot corresponding to the peptide S*TR is now labelled in **B**. Included in **C** is the partial tryptic hydrolysis product KS*TR predicted to migrate to the position of spot 3 in the empirical mapping.

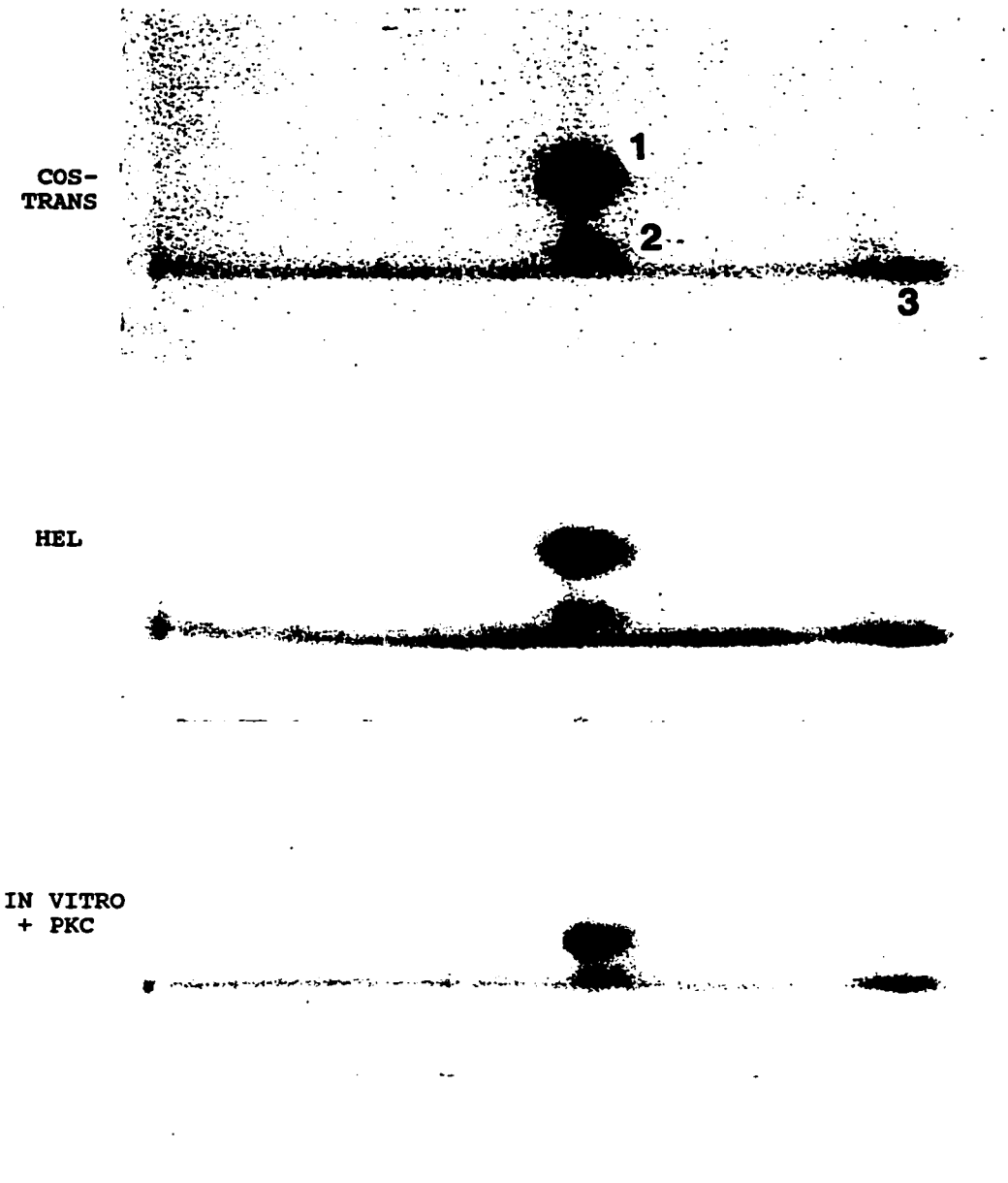

Figure 3.7.2 - Tryptic Phosphopeptide Mapping of Wild-Type Pleckstrin.

^{32}P-labelled pleckstrin, immunoprecipitated and transferred to nitrocellulose, was digested with trypsin and the resulting peptides separated first by electrophoresis in pH 1.9 buffer (origin at the left), then by chromatography in the vertical direction. Wild-type pleckstrin from the following sources was used: upper panel, COS cells transfected with the wild-type construct; middle panel, HEL cells; lower panel, GST-pleckstrin fusion protein purified from *E. coli* and phosphorylated in vitro with purified PKC. Three major spots labelled 1, 2 and 3 were repeatedly observed.

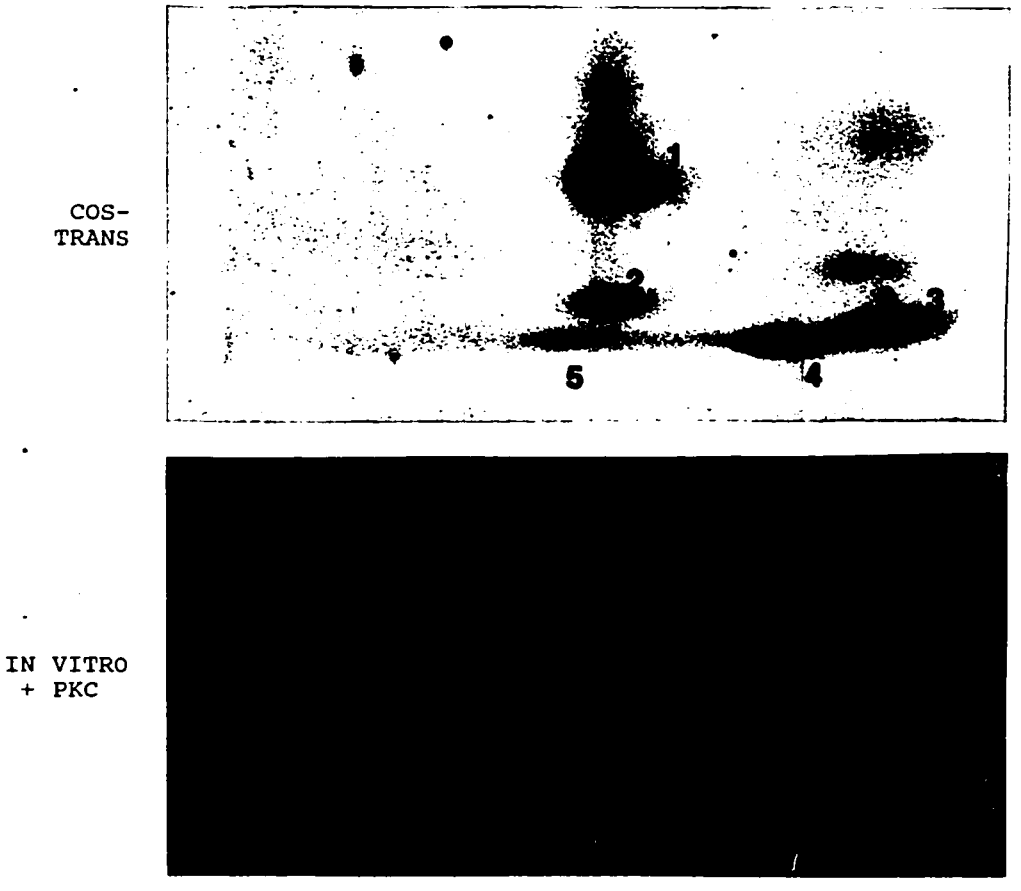

Figure 3.7.3 - Tryptic Phosphopeptide Maps with Additional Spots.

Tryptic phosphopeptide maps of wild-type pleckstrin from transfected COS cells (top panel) and in vitro phosphorylated GST-pleckstrin (lower panel) showing 2 additional spots located to the lower left of spots 2 and 3 (as marked in Fig. 3.7.2).

lower left of spots 2 and 3 respectively, consistent with the possibility that they are the doubly phosphorylated versions of spots 2 and 3 (Boyle et al., 1991). Since they were not routinely observed, further investigation into their identity was not practical. Phosphoamino acid analysis was performed on spots 1, 2 and 3 scraped from a representative wild-type pleckstrin TLC plate. As shown in Fig. 3.7.4, phosphoserine was detected in spots one and two. Analysis of spot three was unsucessful due to low recovery of ^{32}P from this sample.

The deletion mutant produced a smear on the TLC plate (Fig. 3.7.5). This mutant protein partially associates with the insoluble fraction after cell lysis in 1% NP-40 (see section 3.8), possibly reflecting a lipid association that may impede subsequent trypsinization and TLC analysis. Since the data of Fig. 3.6.2 shows a lower absolute phosphorylation level for mutants S113A and S117A, these two mutants were chosen for mapping first. Mutant T114A was also mapped since it scored high in theory as a potential site. As shown in Fig. 3.7.2, the major spot, spot 1 was completely absent on the map for mutant S117A (Fig. 3.7.6, bottom). It was therefore assumed that spot 1 corresponded to a tryptic peptide containing serine 117, likely the peptide S*IR (amino acids 117-119, asterik following the phosphorylated residue). Conversely for the mutant S113A, the reverse was observed with spot 1 being present and both spots 2 and 3 completely absent (Fig. 3.7.6, top). This indicated that both of these spots represent tryptic fragments containing a phosphorylated serine 113 in the wild-type protein. This data corroborated the phosphoamino acid analysis that indicated that spots 1 and 2 contain phosphoserine.

Since spot 1 likely contained serine 117 (see above), the peptide predicted to contain this residue, S*IR, was highlighted as a reference point on the theoretical tryptic phosphopeptide map (Fig. 3.7.1A). Since spots 2 & 3 empirically migrate further than spot 1 in the electrophoretic direction, all peptides migrating to the left of S*IR on the theoretical map could be disregarded as potential phosphorylation sites (Fig. 3.7.1B).

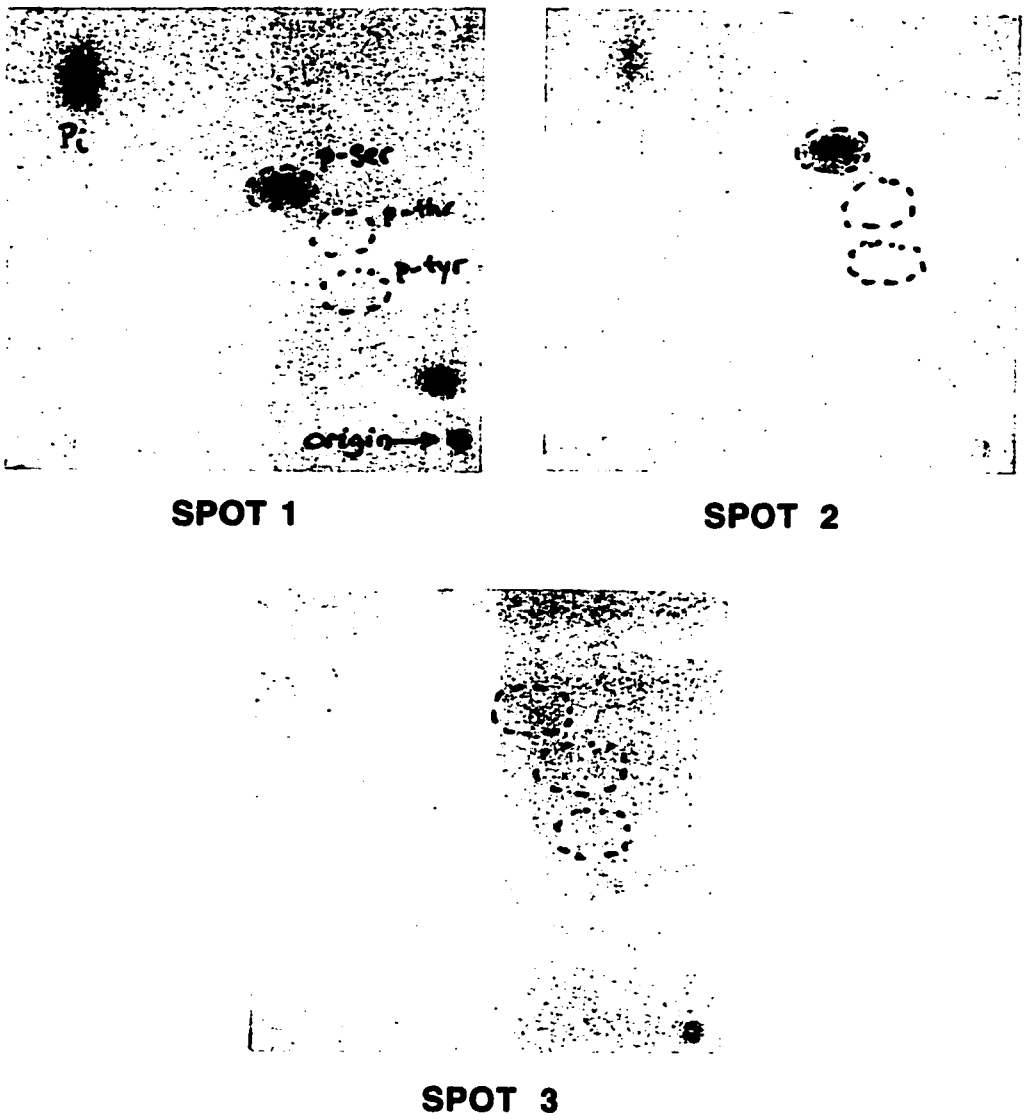

Figure 3.7.4 - Phosphoamino Acid Analysis of Spots 1, 2 & 3.

Spots 1, 2 & 3 were individually scraped from a phosphopeptide map of wild-type pleckstrin derived from transfected COS cells. The material was then hydrolyzed in acid and the products separated by electrophoresis in pH 1.9 buffer followed by electrophoresis in pH 3.5 buffer. Cold phosphoamino acid markers were co-run with each sample and visualized by ninhydrin spray. Spots 1 and 2 were both determined to contain phosphoserine.

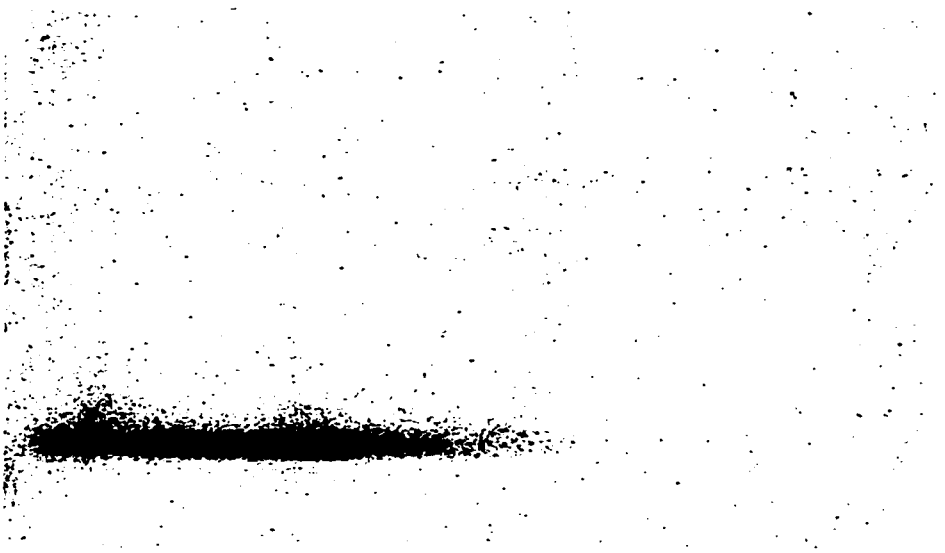

Figure 3.7.5 - Tryptic Phosphopeptide Map of the Pleckstrin Deletion Mutant.

Phosphopeptide mapping was performed as per Fig. 3.7.2 on the pleckstrin deletion mutant (ΔNsi). A repeat of this experiment produced the same results.

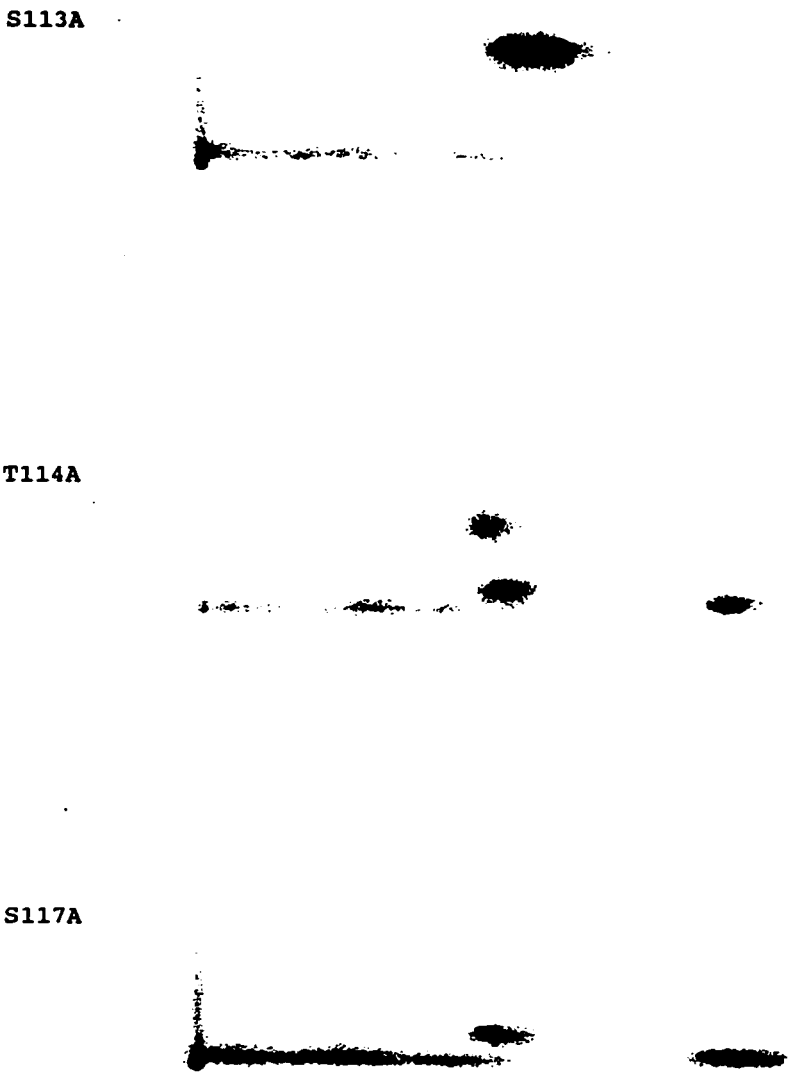

Figure 3.7.6 - Tryptic Phosphopeptide Maps of S113A, T114A & S117A Mutants.

COS cells were transfected with the pleckstrin constructs encoding the S113A, T114A or S117A mutations. After PMA addition (100 nM, 30 min), the mutant proteins were isolated and processed for mapping exactly as described for wild-type pleckstrin (see Materials & Methods), producing the maps shown above.

Empirically spot 2 migrates almost directly beneath spot 1, therefore when a corresponding theoretical peptide is sought, it is noted that directly beneath S*IR on the theoretical map is S*TR, the peptide predicted to be generated by tryptic cleavage containing serine 113. Therefore, due to its migration relative to spot 1 (S*IR), spot 2 was believed to correspond to the tryptic fragment S*TR (amino acids 113-115), remarkably corroborating the observed absense of spot 2 for mutant S113A.

Spot 3, assumed to contain serine 113 from mutational analysis, had no correlated theoretical spot until partial tryptic hydrolysis was included in the model. An analysis of how trypsin might hydrolyze the residues surrounding serine 113 in the sequence 109-FAR'K'STR'R'SIR'L -120 (' indicating where trypsin might cleave), suggests that S*TR and KS*TR have about equal probability of being formed initially. But since trypsin is inefficient at cleaving N-terminal lysine or arginine residues (Needleman, 1970), KS*TR once formed will be resistant to further cleavage. Using the same calculations, this partial fragment is predicted to migrate incredibly close to the position of spot 3 (Fig. 3.7.1C). (Theoretically KS*TR should migrate 1.69 times further than S*TR; empirically spot 3 migrated 1.72 times further than spot 2).

Analysis of mutant T114A showed that all 3 spots remained present (Fig. 3.7.6, middle). In fact, this mutant confirmed by different criteria that spots 2 and 3 contain a phosphorylated serine 113. Mutation of threonine 114 to alanine is predicted to change the migration pattern of the phosphopeptides containing this residue. Since threonine 114 and serine 113 are necessarily on the same tryptic peptides, S*TR (spot 2) and KS*TR (spot 3) would both be predicted to migrate differently for mutant T114A, where they are in fact S*AR and KS*AR. Indeed spots 2 and 3 were seen to migrate slightly further in the electrophoresis direction for this mutant and exactly to the extent predicted (Fig. 3.5.6, note spot 2 is no longer directly beneath spot 1 for the T114A mutant). Interestingly, mutation of threonine 114 to alanine reversed the site preference for serine 117 over serine 113 in the

wild-type protein from 58:42 (range = +/- 2% over 3 experiments) to 35:65 (Table 3.7.2). This preference reversal might be attributable to the creation of a more favourable consensus sequence around serine 113 and/or the generation of a less favourable sequence at serine 117. This particular mutant illustrates the potential consequences of cis-acting effects that might be created during site-directed mutagenesis.

Although mutagenesis has been supported as providing definitive proof of the identification of a phosphorylation site (Boyle et al., 1991), and although the predicted sequence of the tryptic fragments containing these target residues was supported by a comparison of the empirical map with the theoretical one, this does not provide direct proof of the amino acid sequence of the peptides in these spots. To resolve this problem, manual or automated Edman degradation is usually employed to determine which cycle releases peak radioactivity (Ikebe & Reardon, 1990; Adachi et al., 1992). In this particular case however, a release of radioactivity in the first round can not discriminate between S*IR, S*TR and S*TRR. Since phosphorylation of larger amounts of recombinant pleckstrin in vitro proved difficult due to enzyme instability, microsequencing was not attempted. Instead, confirmation of phosphorylation sites was provided by tryptic phosphopeptide mapping synthetic peptides corresponding to the region in pleckstrin in which the candidate phosphorylation sites are clustered. A peptide corresponding to amino acids 110-120 of the pleckstrin sequence (ARKSTRRSIRL) was phosphorylated in vitro, trypsinized and analyzed as per the full-length protein (Fig. 3.7.7). This peptide was presumably >99% phosphorylated on serine 117, as indicated by the prominence of spot 1. Since this peptide appeared so weakly phosphorylated on serine 113, an N-terminally extended version was synthesized with the belief that the extra residues would enhance phosphorylation at serine 113. Indeed the N-terminally extended peptide 108-KFARKSTRRSIRL-120 produced a map containing all the same spots seen in maps of full-length pleckstrin (Fig. 3.7.8A). Additional proof that spot 1 corresponds to S*IR was generated by mapping a third

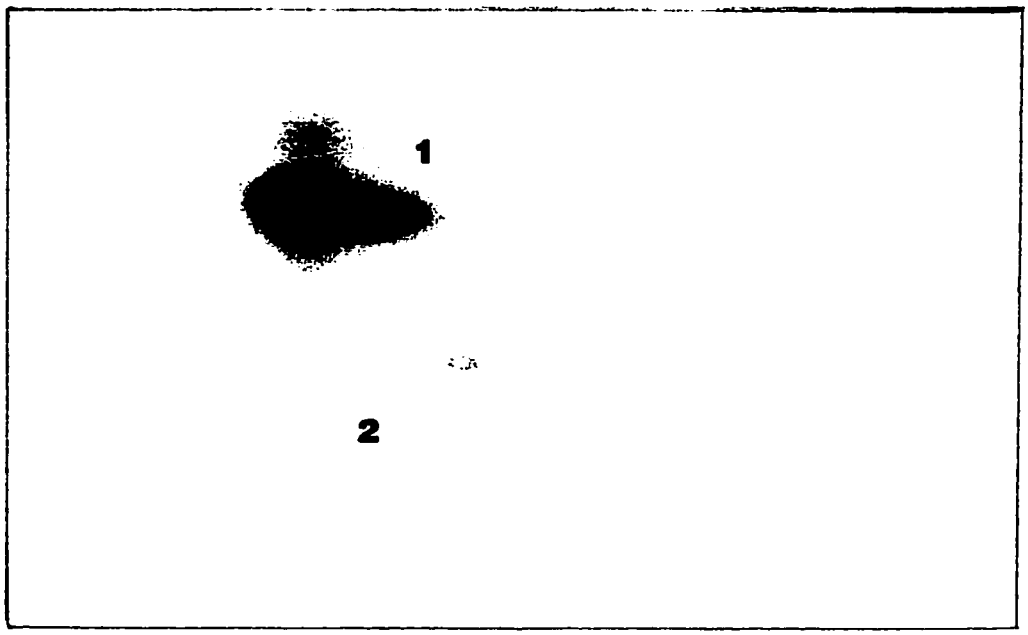

Figure 3.7.7 - Tryptic Phosphopeptide Mapping of Peptide 1.

The synthetic peptide ARKSTRRSIRL-NH_2 was phosphorylated in vitro with purified PKC, trypsinized, lyophilized, then spotted onto a TLC plate. Greater than 99% of the total radioactivity was confined to spot 1.

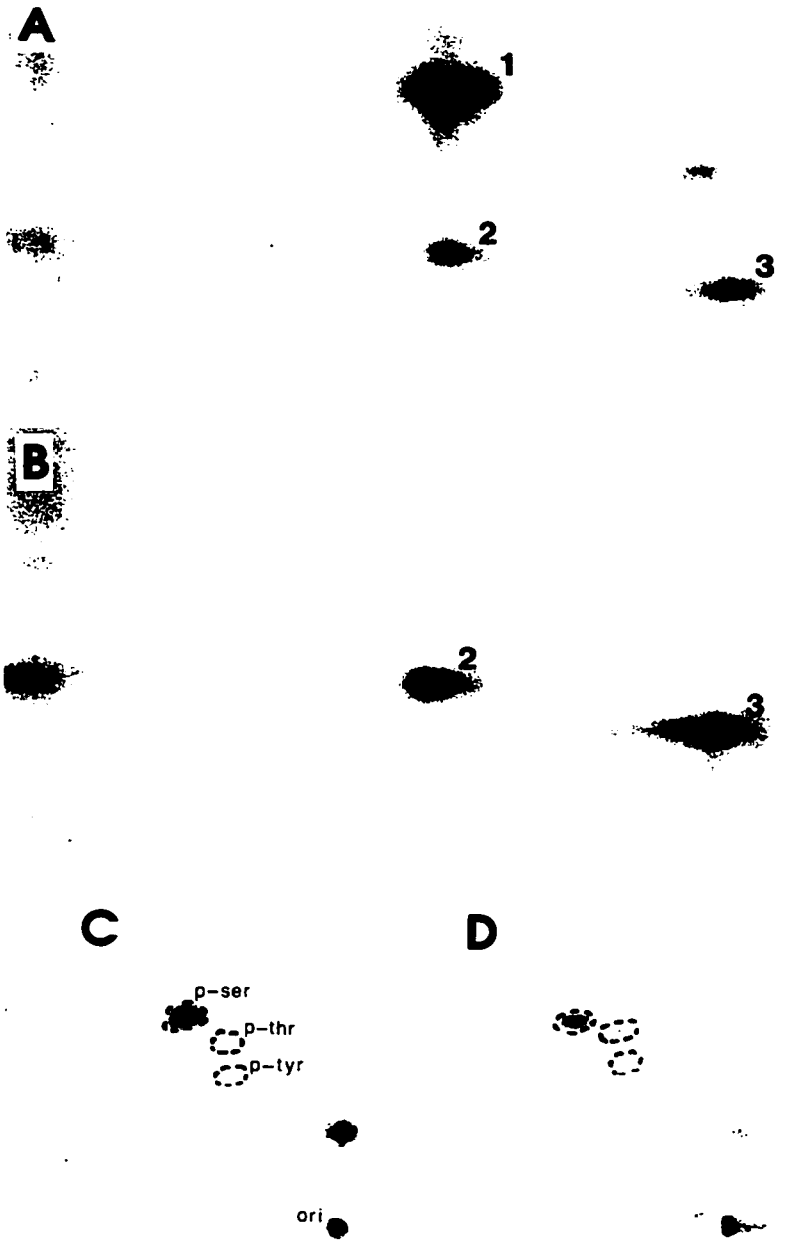

Figure 3.7.8 - Tryptic Phosphopeptide Mapping of Peptides 2 & 3.

A, phosphopeptide map of the peptide KFARKSTRRSIRL-NH_2 and **B**, the peptide KFARKSTRRAIRL-NH_2, both phosphorylated in vitro by PKC. **C** and **D**, phosphoamino acid analysis of spots 2 and 3 respectively, scraped from the plate shown in **B**.

synthetic peptide that had serine 117 replaced with alanine, analogous to the full-length mutant S117A. The map of this peptide lacked spot 1 (Fig. 3.7.8B). Therefore, definitively, spot 1 corresponds to a serine 117-containing peptide.

To determine whether spots 2 and 3 correspond to S*TR and KS*TR respectively or ST*R and KST*R respectively, these serine and threonine phosphoisomers not being resolvable (identical masses; nearly identical hydrophobicities), phosphoamino acid analysis on spots 2 and 3 from the S117A synthetic peptide was performed. As seen in Fig. 3.7.8C, spot 2 contained 100% phosphoserine. Spot 3, which contained predominantly phosphoserine, interestingly had a minor amount of phosphothreonine (6% of total phosphate incorporated into this peptide, Fig. 3.7.8D). This indicates that T114 can be phosphorylated to a minor extent, perhaps accounting for the previous detection of phosphothreonine in platelet pleckstrin (Imaoka et al., 1983).

Since both S113 and S117 score very high as candidate PKC phosphorylation sites, the binding constants of the pleckstrin-derived synthetic peptides for recombinant PKCα were probed. A hyperbolic substrate saturation curve was generated for each peptide and the Michaelis-Menton binding constant K_M interpolated from each curve-fitted graph using FIG P software (Fig. 3.7.9 & Table 3.7.2). The low K_M of 0.8 μM for peptide 2 is one of the lowest K_M's reported yet for a synthetic peptide substrate of PKC (Kemp & Pearson, 1991). Interestingly, the in vivo preference for phosphorylation on serine 117 over serine 113 was expressed even more strongly in vitro with the synthetic peptides. Replacing serine 117 with alanine in peptide 3 increased the Michaelis-Menton constant, again implying that PKC more strongly recognizes the residues surrounding serine 117 as opposed to serine 113. As a final comment on the site preference of S117 over S113 (although computer generated sequence analysis predicted T114 or S113 to be preferentially phosphorylated), 23 of 37 empirically determined PKC phosphorylation sites contain a hydrophobic residue immediately C-terminal to the phosphorylated residue

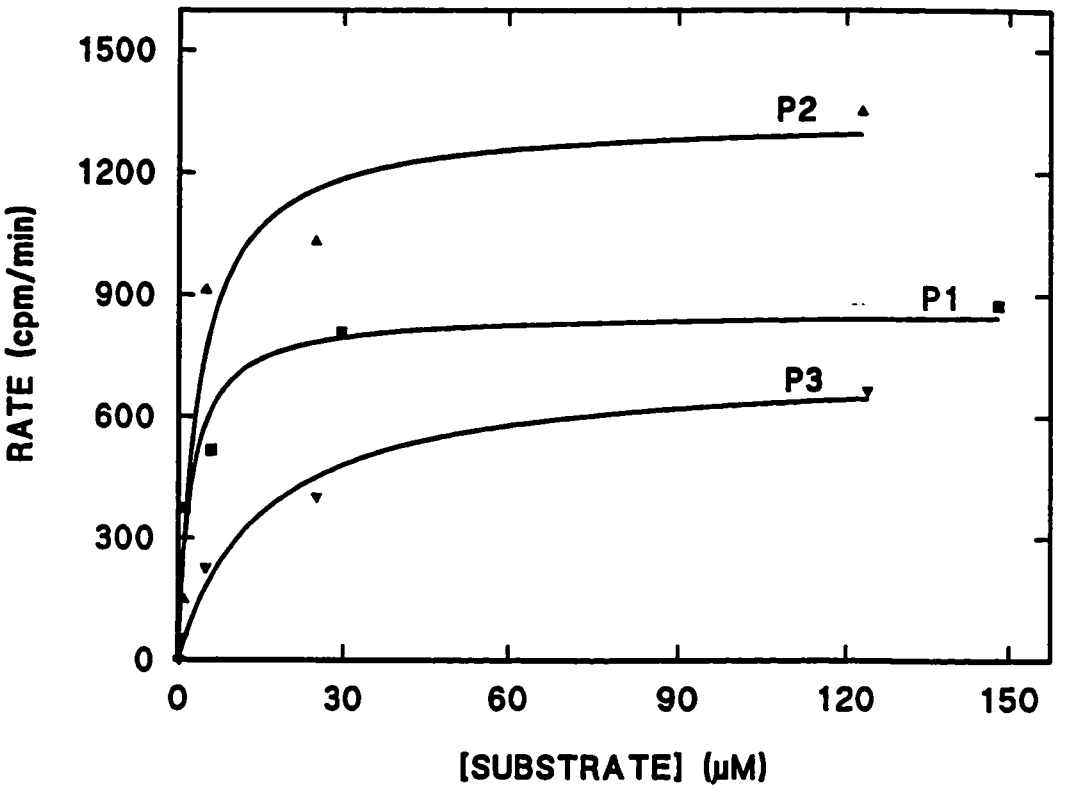

Figure 3.7.9 - Kinetic Analysis of Synthetic Peptide Phosphorylation.

Substrate saturation curves for the following peptides incubated with recombinant PKCα: P1, ARKSTRRSIRL-NH_2; P2, KFARKSTRRSIRL-NH_2; P3, KFARKSTRRAIRL-NH_2. FIG P software was used for curve fitting and calculation of K_M for each peptide.

Substrate	K_M (μM)	Radioactivity
ARKSTRRSIRL-NH_2	1.71 +/- .06	>99% spot 1
KFARKSTRRSIRL-NH_2	0.8 +/- .6	85% spot 1, 15% 2 & 3
KFARKSTRRAIRL-NH_2	15 +/- 4	100% spots 2 & 3
Wild-type pleckstrin	----------	58% spot 1, 42% 2 & 3
Mutant S113A	----------	100% spot 1
Mutant T114A	----------	35% spot 1, 65% 2 & 3
Mutant S117A	----------	100% spots 2 & 3

Table 3.7.2 - Binding constants and phosphorylation characteristics of pleckstrin-derived peptides and proteins.

The K_M values listed above for each peptide were derived from the substrate saturation curves shown in Fig. 3.7.9. Percent of radioactivity incorporated into each spot resolved by phosphopeptide mapping is also listed. A preference for serine 117 (spot 1) was observed in all cases except mutant T114A, which was preferentially phosphorylated on serine 113.

(Pearson & Kemp, 1991), which correlates well with a preference for S_{117}IR over S_{113}TR, and for enhanced S113 phosphorylation when T114 was mutated to alanine.

Interestingly, during kinetic analysis of these peptides, the stoichiometry of phosphorylation approached, but never surpassed a molar ratio of 1 phosphate: 1 peptide under conditions of increasing enzyme and decreasing peptide concentration. This implies that phosphorylation on S117 precludes to a certain extent phosphorylation on S113 and vice versa. Although the data with the synthetic peptides suggests phosphorylation on these residues is mutually exclusive, this observation cannot be confidently applied to in vivo phosphorylation of the full-length protein. The fifth and sixth spots seen on 2-D maps potentially representing KS*T*R (or S*T*RR) and KS*T*RR in addition to the approximately 50 % reduction in total phosphorylation of mutants S113A and S117A (and the multiple isoforms seen on IEF gels) argues against a single phosphorylation hypothesis, although many considerations make this a difficult point to address.

In summary, pleckstrin becomes phosphorylated by PKC almost exclusively on S113 and S117, but occasionally on threonine 114. A likely scenerio is that phosphorylation of both S113 and S117 occurs in vivo during maximal physiological stimulation. It will be interesting in the future to test the functional significance and/or redundancy of these individual phosphorylations using these site-directed mutants.

3.8 Subcellular Localization of Pleckstrin as Determined by Indirect Immunofluorescence

Subcellular immunofluorescence detection of pleckstrin was undertaken with the belief that a concise understanding of pleckstrin's localization within the cell would promote discovery of its function. Many proteins that influence cell structure are clearly associated with intermediate filaments, actin stress fibres or localized at cell-substratum

contact regions (Hansen et al., 1994; Widmer & Caroni, 1993). In addition to pleckstrin's localization within the cell, immunofluorescent examination of transfected COS cells could possibly reveal differences between transfected and untransfected cells. These differences would be attributed to overexpression of pleckstrin and could yield clues as to its activity. Additionally, cytoskeletal components can be visualized by the appropriate antibodies in double labelling experiments, revealing any structural perturbations induced by overexpression of the protein of interest. For example, immunofluorescent labelling of actin showed that rho stimulated actin stress fibre and focal adhesion formation rapidly after microinjection into fibroblasts (Ridley & Hall, 1992). In similar experiments microinjected rac stimulated pinocytosis and induced membrane ruffling (Ridley et al., 1992).

Pleckstrin's paramount feature is its phosphorylation by PKC. This event presumably alters its activity, possibly visible as 1) an alteration in its subcellular distribution or 2) an affect on cell structure or function or 3) both. MARCKS, like pleckstrin, is a PKC substrate of unknown function. It has been shown to display one of two distinct localizations dependent on its phosphorylation state. Unphosphorylated MARCKS is associated with punctate structures in macrophage filopodia, where it co-localizes with vinculin and talin, whereas phosphorylated MARCKS is seen as diffusely cytoplasmic (Rosen et al., 1990). These observations led to the hypothesis of a role for MARCKS in membrane cytoskeleton regulation. The P47ox protein of neutrophils is predominantly cytosolic, associating with membranes after phosphorylation (Clark et al., 1990), an event correlating with activation of the superoxide burst. Lastly, reversible phopsophorylation is responsible for the cell cycle dependent translocation of rab4 from endosomes to the cytosol (van der Sluijs et al., 1992).

3.8.1 Subcellular Localization of Pleckstrin in Transfected COS Cells

Subcellular localization of recombinant wild-type and deletion mutant forms of

pleckstrin was performed first by biochemical fractionation. Although platelet pleckstrin is completely soluble (Imaoka et al., 1983), much of the recombinant pleckstrin expressed in *E. coli* was insoluble, raising concerns about recombinant pleckstrin expressed in COS cells. Wild-type pleckstrin appeared completely soluble, as shown by its detection in the supernatant fraction of COS cells lysed in 0.5% NP-40 (Fig. 3.8.1). The deletion mutant by contrast was localized predominantly in the pellet fraction. This suggests the deletion mutant associates with either cytoskeletal components, nuclei, membranes or forms large self-aggregates. The biochemical fractionation was also performed before and after PMA addition to test whether or not pleckstrin's localization is phosphorylation-dependent. The solubility characteristics of both the wild-type and deletion mutant were unaltered after exposure to a PMA concentration known to induce maximal phosphorylation (Fig. 3.8.1).

Indirect immunofluoresence detection of pleckstrin in the transfected COS cells yielded information firstly about the success of the transfection technique. It was possible to estimate the percentage of cells successfully transfected by tabulating both fluorescing and non-fluorescing cells in several fields. Depending on the condition of the cells and the transfection technique used, up to 10% of the cells could be transfected (data not shown), and the expression levels within these cells were quite high as discussed in section 3.5.

Pleckstrin immunofluorescently detected in COS cells appeared predominantly cytosolic, although some punctate character closer to the nucleus was identified (Fig. 3.8.2). Oddly, the deletion mutant displayed the same apparent localization as wild-type pleckstrin, starkly contrasting the biochemical fractionation data (Fig. 3.8.2C,D). Wild-type pleckstrin appeared innocuous to COS cells since cells still expressing it were detected up to 3 weeks post transfection, whereas this was not true for deletion mutant transfected cells. At two days post transfection, 50% or more or the cells displayed cytoplasmic shedding, which progressed until small rounded balls of what appeared to be nuclear material was left (Fig. 3.8.3). It is not known to what extent this phenomenon was a non-

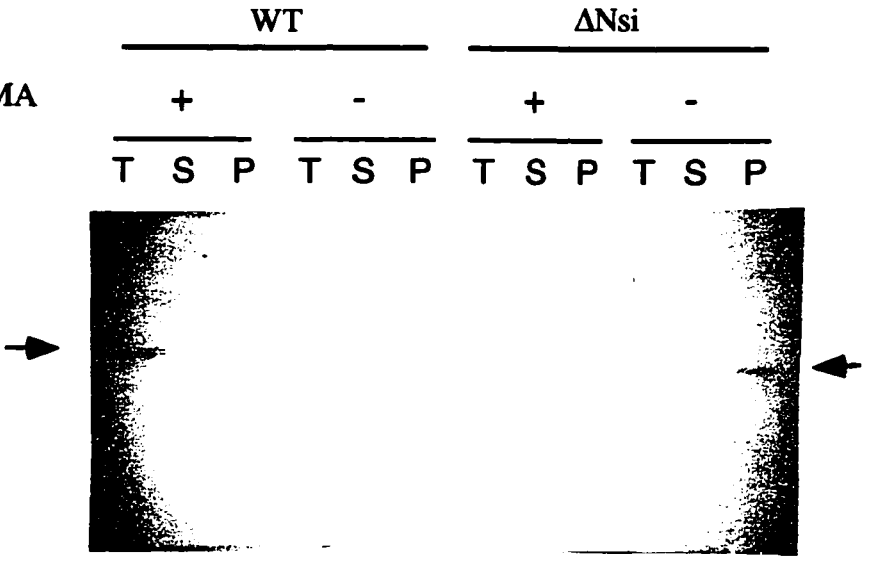

Figure 3.8.1 - Biochemical fractionation of pleckstrin.

Cos cells transfected with either wild-type pleckstrin (WT), or the deletion mutant (ΔNsi) were lysed in 1% NP-40 buffer (see section 2.3.5), and a total sample was taken (T). The lysates were then spun for 10 min, 10,000 g, and supernatant (S), or pellet (P) fractions analyzed by SDS-PAGE. (+) indicates that the COS cells had been treated with 100 nM PMA for 30 min prior to harvesting.

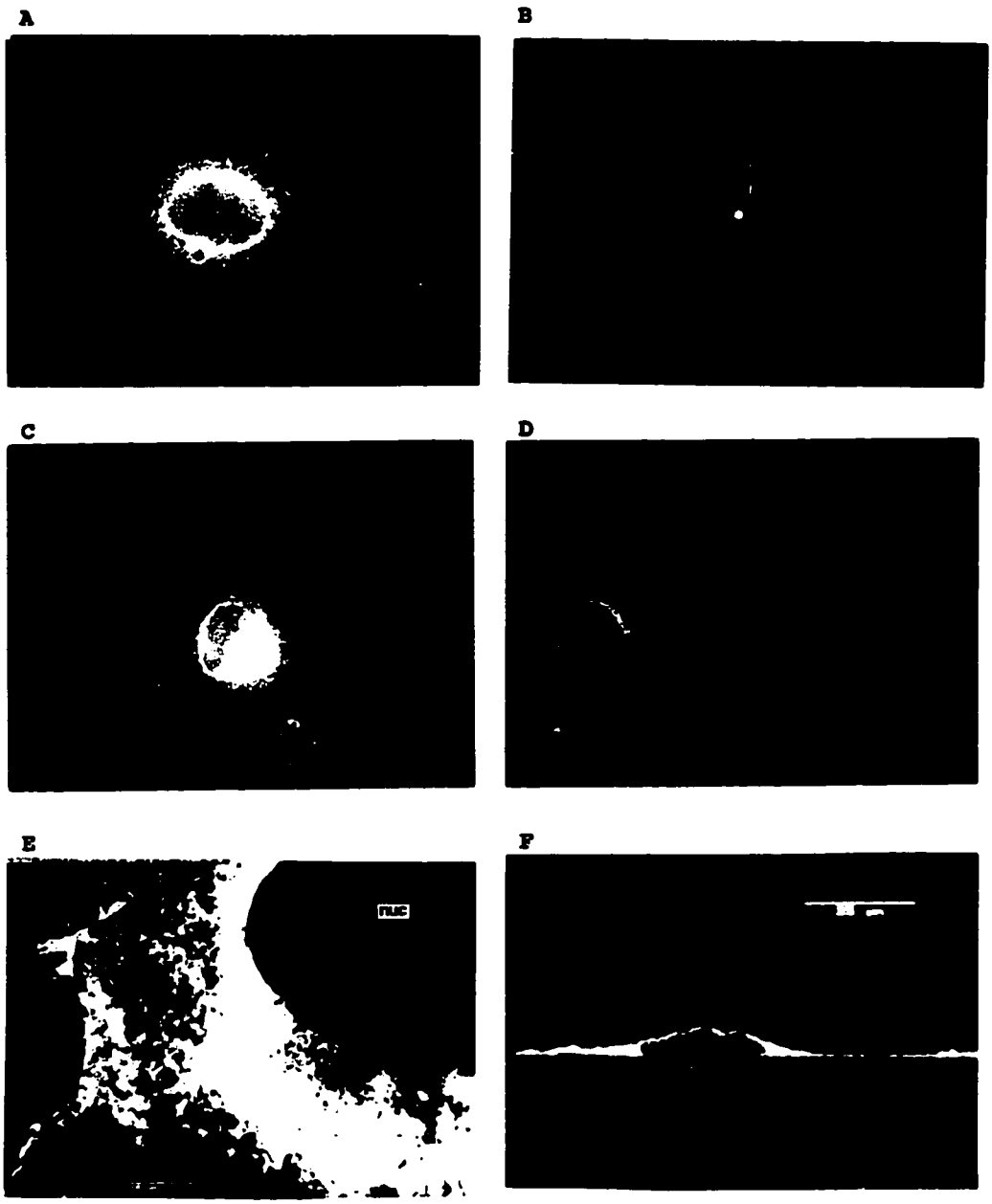

Figure 3.8.2 - Effect of PMA on Pleckstrin Localization in Transfected COS Cells.

Wild-type pleckstrin (A,B,E,F) or the deletion mutant (C,D) were expressed transiently in COS cells and detected by indirect immunofluoresence using rabbit anti-pleckstrin antibody and FITC-conjugated anti-rabbit IgG antibody. The cells were treated with either no PMA (A,C) or 100 nM PMA (B,D,E,F) 30 min prior to fixation and staining. E, confocal imaging of a PMA treated, wild-type pleckstrin transfected COS cell. F, Confocal imaging 2-D reconstruction of a vertical slice through the same COS cell as in E.

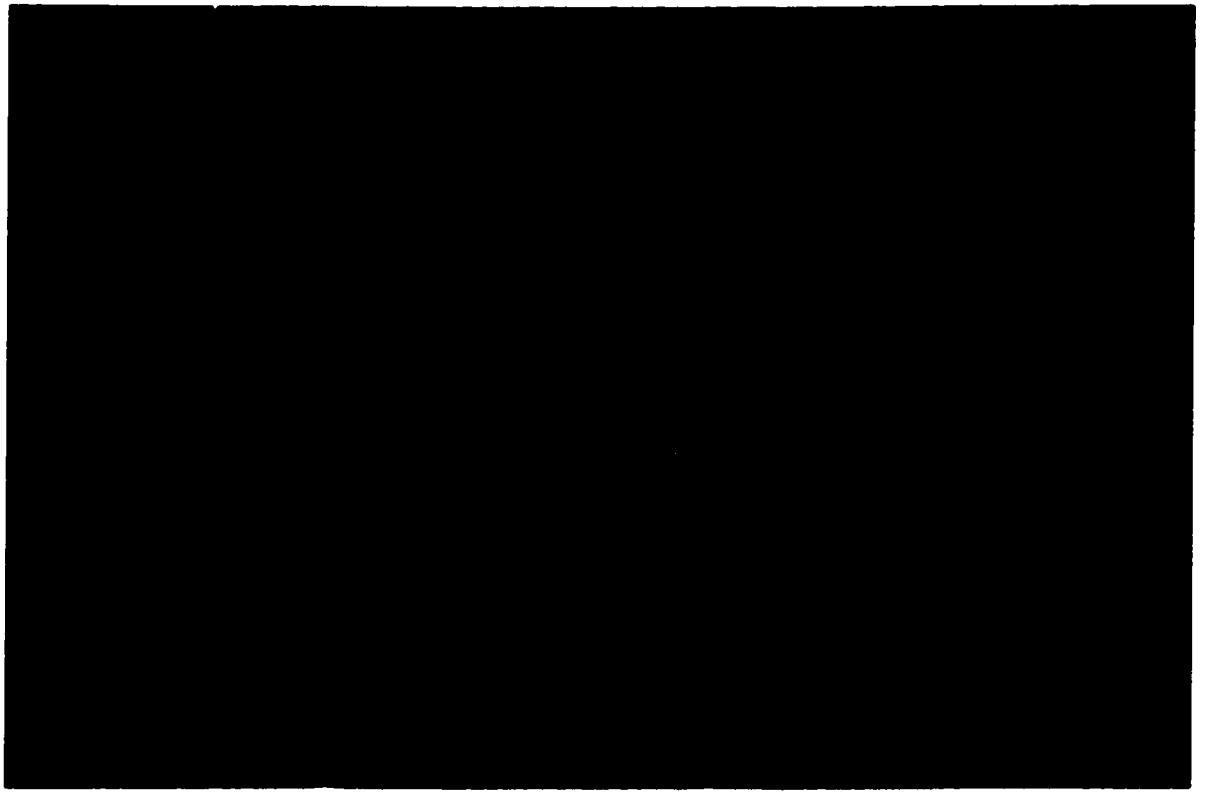

Figure 3.8.3 - Cytoplasmic Loss Induced by the Pleckstrin Deletion Mutant.

COS cells were transfected with the pleckstrin phosphorylation-region deletion construct pEVp47ΔNsi and processed for immunofluorescence staining 48 hr later. Above is a typical deletion construct transfected cell that appears to be shedding cytoplasm.

specific toxic response of the cells to overexpression of a foreign protein, or whether it was caused by a dominant pleckstrin activity specific to the deletion mutant. Interestingly, the cytoplasmic blebbing seen for the deletion mutant is reminiscent of megakaryocytic platelet shedding (Wilkinson, 1991). Often seen are long pseudopods that pinch off disk-like platelets along their length, similar to the deletion mutant transfected cells. Perhaps not so coincidentally, neutrophils can shed pseudoplatelets during acute inflammatory responses (Hanker & Giammara, 1983), and they also express relatively high levels of pleckstrin.

After PMA addition, there was no significant change in the fluorescence pattern, indicating no apparent alteration in pleckstrin localization (Fig. 3.8.2, B,D vs. A,C). There was however a slight increase in the punctate character of the immunofluorescence, and perhaps a slight increase in membrane ruffling (Fig. 3.8.2 B vs A). These two changes could realistically result from the pleiotropic effects of PMA treatment, not specifically from phosphorylation of pleckstrin. To determine if this effect was selective for transfected cells, the cytoplasm and demarcation of it in untransfected cells (or cells transfected with a negative control) needed to be visualized. Several controls were attempted including transfection of an expression vector for chloramphenicol transacetylase (CAT) followed by detection with an anti-CAT antibody, or alternatively labelling for endogenous glucose 6-phosphatase. In brief, the CAT staining was intensely punctate whereas glucose 6-phosphatase displayed a filamentous, tubulin-like distribution, therefore neither of these supposedly cytoplasmic proteins appeared as diffusely cytoplasmic and soluble as pleckstrin itself.

Since previous data implicated pleckstrin in regulating actin polymerization (see section 3.4), it was conceivable that pleckstrin might be found localized with free or filamentous actin. Additionally, overexpression of pleckstrin might visibly alter the monomeric to filamentous actin ratio in the cell. Therefore labelling for actin as well as pleckstrin (double labelling) using rhodamine conjugated phalloidin was performed. The

actin staining of the COS cells appeared quite similar to that seen by others (Ridley & Hall, 1992). Pleckstrin partially co-localized with actin near the nucleus, where shorter actin filaments were found, but did not co-localize with the prominent actin stress fibres in the rest of the cell or in cytoplasmic projections (Fig. 3.8.4). A comparison of the actin staining in transfected and untransfected cells indicated that the state of actin polymerization in each cell type was similar, both before and after PMA treatment. Double labelling for pleckstrin and tubulin also showed no correlation between the distribution of these proteins (data not shown).

To examine pleckstrin's subcellular distribution with greater precision, confocal microscopy was employed. Confocal imaging resulted in a more granular fluorescence pattern than that seen previously, and uncovered a region of greater fluorescence near the nucleus, perhaps localizing with the endoplasmic reticulum (ER) (Fig. 3.8.2E). The intense perinuclear fluorescence seen by regular immunofluorescence microscopy could now be attributed to the great cytoplasmic depth around the nucleus as revealed by a transverse confocal view of the cell (Fig. 3.8.2F), which shows how dramatically the nucleus rises above the flattened cytoplasm of the cell. The pleckstrin fluorescence in HL-60 and HEL cells also looked more granular by confocal microscopy in comparison to regular fluorescence (data not shown).

Homology identified between pleckstrin and rasGAP (Tyers, Ph.D. thesis) suggested that pleckstrin might similarly regulate a small GTPase. Since many members of the ras superfamily appear to have a role in membrane trafficking (Novick & Brennwald, 1993), and since a platelet's primary function involves regulated secretion, it was tenable that pleckstrin might have a role in vesicle movement via a G protein. Pleckstrin also displays homology within its PH domain to dynamin (Liu & Pohajdak, 1992; Haslam et al., 1993), a known microtubule motor believed to cross-link endocytotic vesicles to microtubules (Scaife & Margolis, 1990). Perturbations in regulated or constitutive

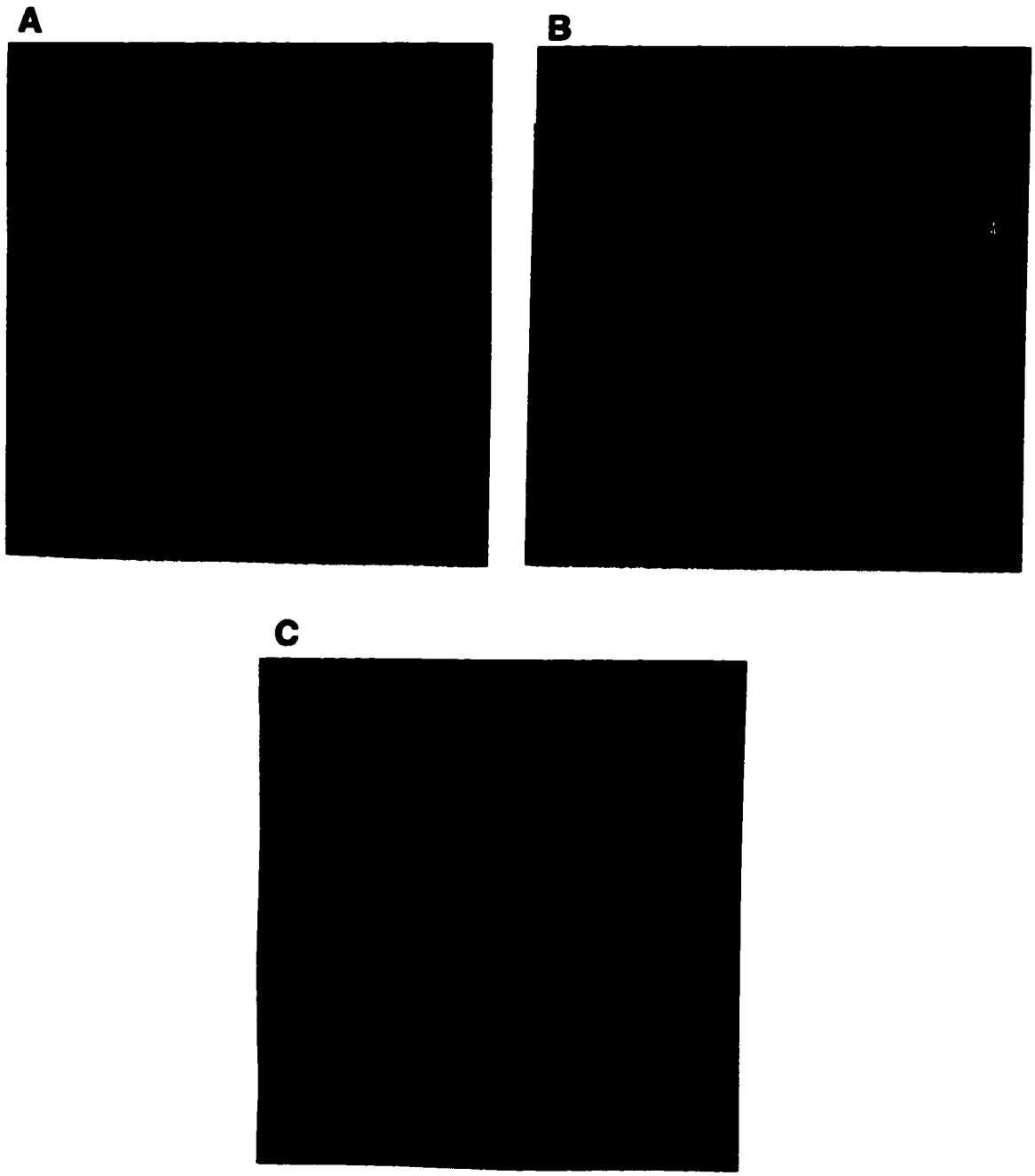

Figure 3.8.4 - Double Labelling for Actin and Pleckstrin in Transfected COS Cells.

A, FITC staining for pleckstrin and **B**, TRITC-phalloidin staining for actin in COS cells transfected with the wild-type pleckstrin construct. **C** is an overlay of both the fluorescein and rhodamine channels, with co-localizations appearing yellow.

secretion can be quite visible. For example, the yeast secretion defective mutant sec 7 causes a build up of Golgi structures, clearly visible by immunofluorescence (Franzusoff et al., 1991). Therefore pleckstrin was co-stained with rhodamine conjugated wheat germ agglutinin and concanavalin A to examine its localization with or perturbation of Golgi and ER structures respectively in transfected COS cells. Pleckstrin did not co-localize with Golgi structures, nor did it disrupt their appearance in transfected cells (Fig. 3.8.5). Labelled rough ER showed some overlap with pleckstrin close to the nucleus where more intense, partially punctate staining had previously been seen (Fig. 3.8.6). Although highly speculative, perhaps the last observation underlies a functional relationship between pleckstrin and vesicular structures that eventually fuse with the plasma membrane in secretory processes.

Seven out of the 8 phosphorylation site mutants displayed wild-type immunofluorescent staining before and after PMA addition (data not shown). One of them, S113A, occasionally induced a large semicircular rippling of the cell that appeared to spread out from the nucleus (Fig. 3.8.7). This phenomenon was observed in 2 out of 6 transfections, and when present, various degrees of this rippling or ruffling was seen in about 70% of the transfected cells. Reasons for its absence in some transfections might involve the health and confluence of the cells, or subtle culture conditions. It is known that growth factors and stimulators of ras induce ruffling (Ridley et al., 1992), yet the ruffling seen in conjunction with overexpression of S113A was much larger and smoother. Also, 19 out of 24 S113A-transfected cells displayed nuclear staining (ex. Fig. 3.8.7B), which was never observed for wild-type pleckstrin or the other mutants. Interestingly, serine 113 is one of the two clearly identified phosphorylation sites of pleckstrin, strengthening a functional significance for the above observation.

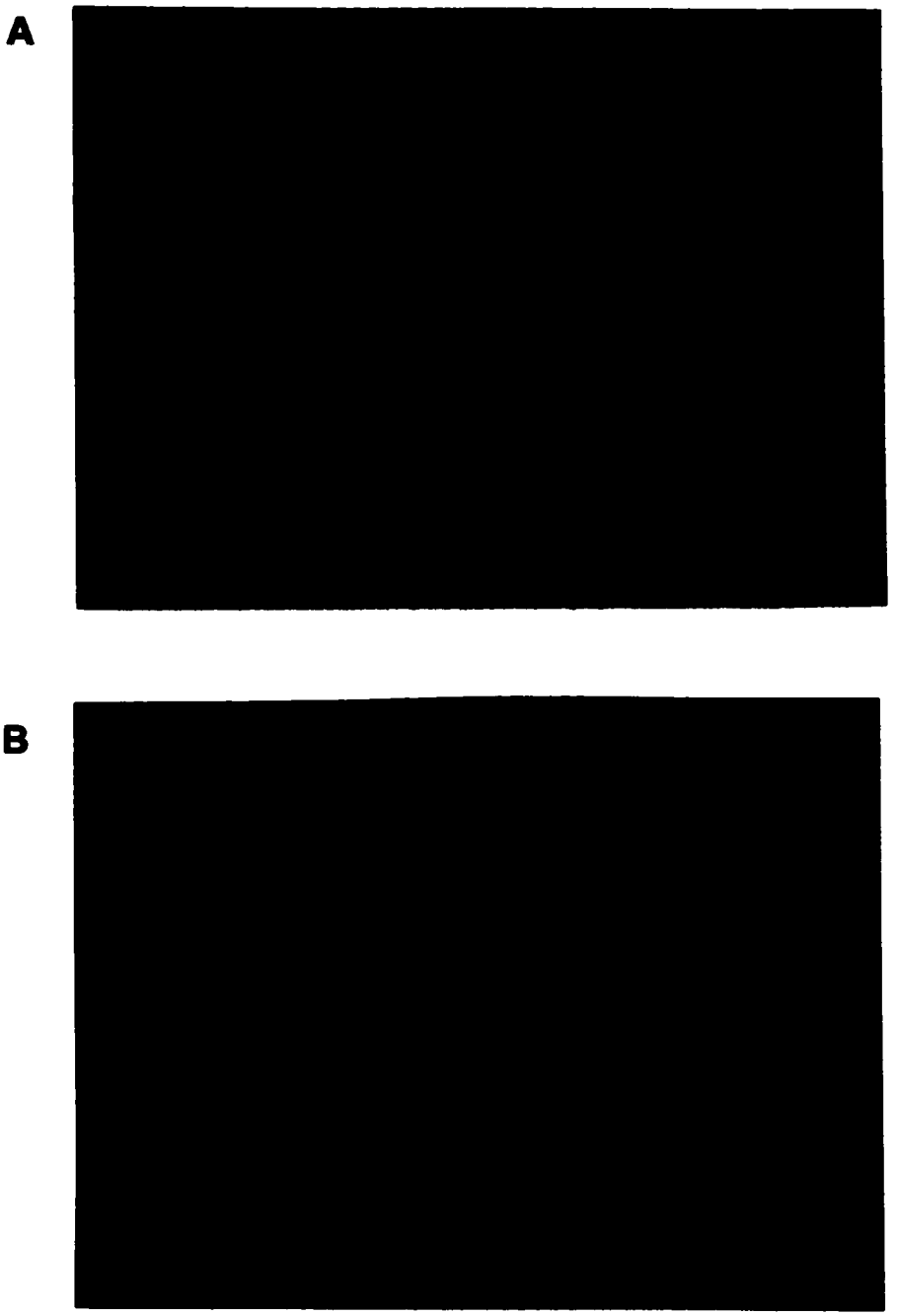

Figure 3.8.5 - Double Labelling for Pleckstrin and Golgi Structures.

A, immunofluorescence detection of pleckstrin in transfected COS cells and B, detection of Golgi structures in the same field of COS cells using rhodamine conjugated weight germ agglutinin.

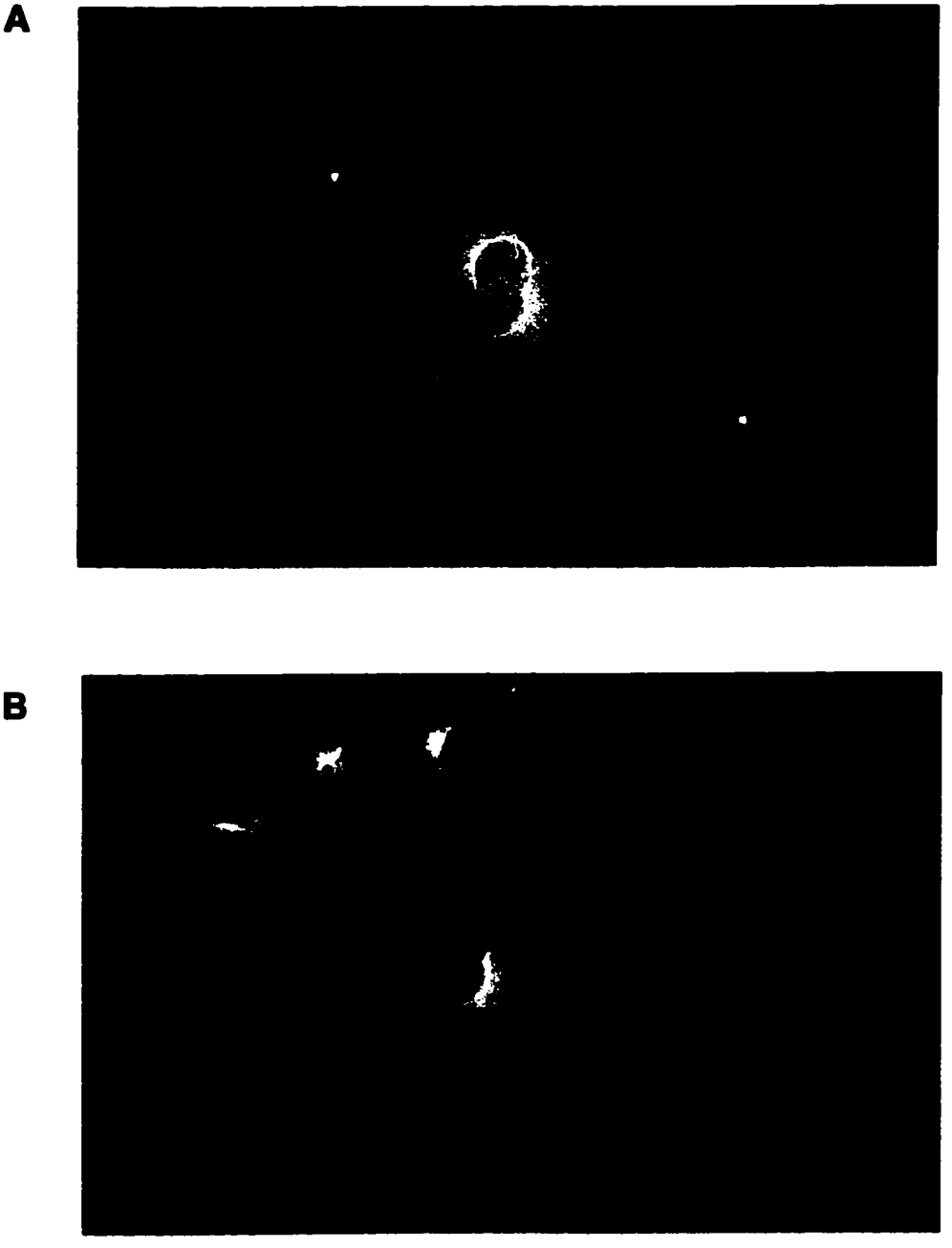

Figure 3.8.6 - Double Labelling for Pleckstrin and ER Structures.

A, immunofluorescence detection of pleckstrin in transfected COS cells and B, detection of ER structures in the same field of COS cells using rhodamine conjugated concanavalin A.

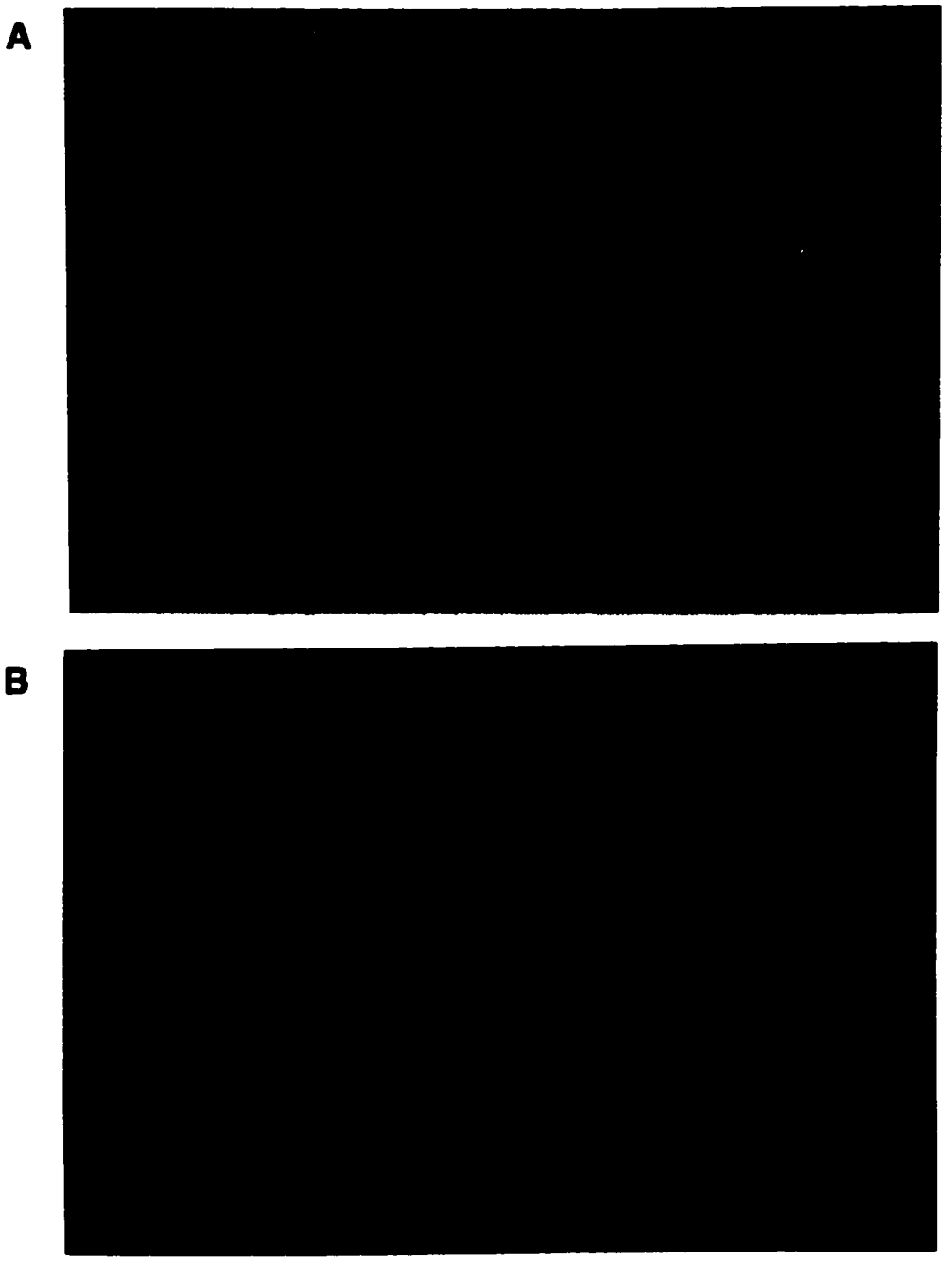

Figure 3.8.7 - Immunofluorescence Detection of Pleckstrin Phosphorylation Site Mutant S113A.

A and B are photographs of COS cells transfected with the pEVp47(S113A) construct and processed for immunofluorescence microscopy identically as per wild-type pleckstrin. Nuclear staining is prominent in B.

3.8.2 Immunofluorescence Detection of Pleckstrin in Haematopoietic Cells

To determine which haematopoietic lineages pleckstrin expression is restricted to, indirect immunofluorescent staining of pleckstrin in human peripheral blood leukocytes (PBLs) was performed in collaboration with Dr. C. Langdon. Contaminating platelets fluoresced very intensely, as expected (Fig. 3.8.8). Polymorphonuclear cells, which are comprised mostly of neutrophils, can be differentiated from lymphocytes by their larger size and lobated nucleus. These cell types expressed high levels of pleckstrin (Fig. 3.8.8). The roughly 50% of PBLs that did not express pleckstrin were morphologically indistinct from lymphocytes (smaller size, large round nucleus). Purification of a B cell population indicated that less than 10% of these cells fluoresced in the presence of the anti-pleckstrin antibody. The fluorescing cells were larger, possibly representing plasma B cells (data not shown). These cells are highly differentiated and actively secrete antibodies, consistent with a role for pleckstrin in facilitating secretion. Neutrophils and monocytes in particular have been shown to express the highest levels of pleckstrin of all the nucleated blood cells (Gailani et al., 1990), corroborating our observations. Differentiation of HL-60 cells with retinoic acid towards neutrophilic granulocytes is associated with increased pleckstrin expression (Tyers et al., 1987; Gailani et al., 1990). Therefore differentiation of proerythroblastic K562 cells (which do not express pleckstrin, Tyers et al., 1988) towards a megakaryocytic phenotype using PMA (Long et al., 1990) was attempted. Pleckstrin expression was not induced however (data not shown), possibly indicating that pleckstrin is specific to terminally differentiated lineages.

In summary, pleckstrin expression is predominant in granulocytic cells (neutrophils, basophils, eosinophils) and monocytes but absent from erythrocytes and some lymphocytes (B and T cells). These results partly conflict with the observation that several B (Raji) and T (HUT 78) derived cell lines have been shown to express pleckstrin (Tyers et al., 1988), but since abberant gene expression is characteristic of transformed

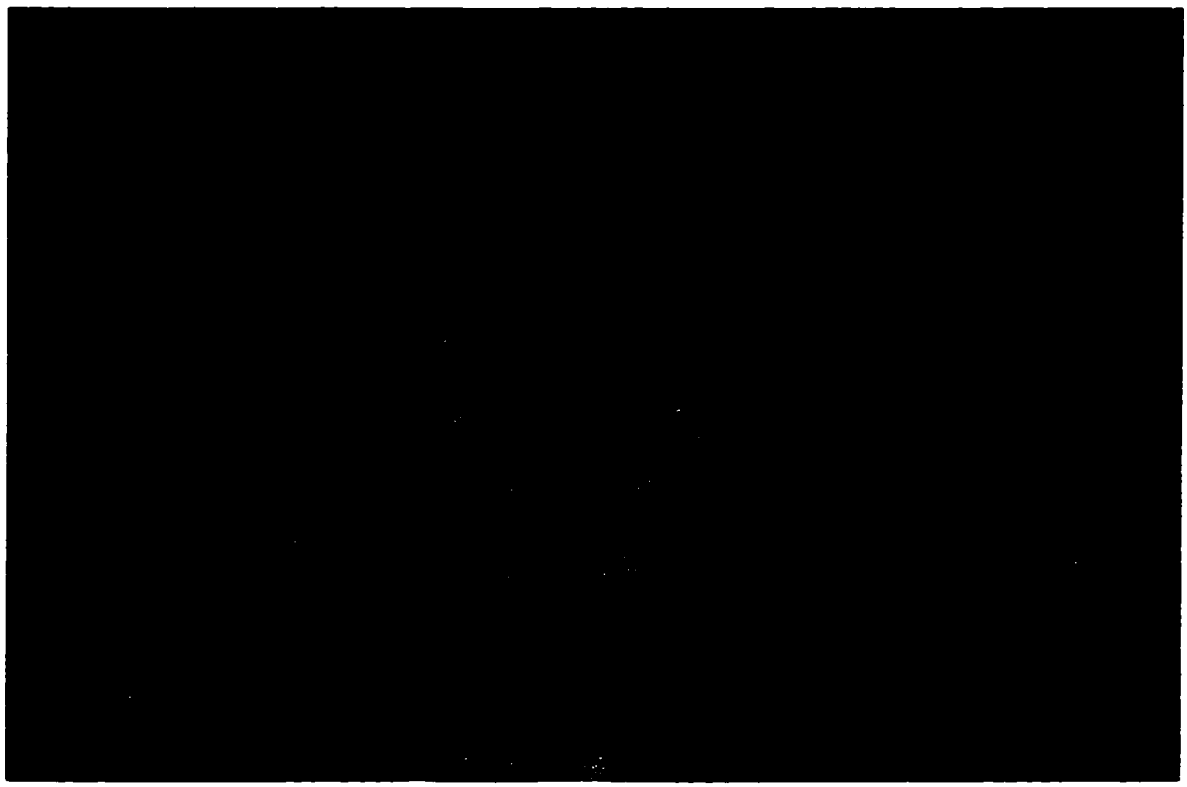

Figure 3.8.8 - Immunofluorescence Detection of Pleckstrin in Human Leukocytes and Platelets.

Human peripheral blood leukocytes were stained for pleckstrin by indirect immunofluorescence. The fluorescence beam was opened simultaneously with regular light so that both fluorescing and non-fluorescing cells could been seen. The small, intensely fluorescent objects are platelets.

cells this observation may not be acutely informative. Further exploration using selective marker antibodies to differentiate among the array of highly specialized T cells that express pleckstrin would provide more definitive information. Due to its apparently tightly controlled expression in blood cells, pleckstrin may itself become a haematopoietic lineage marker in the future.

Some of the fluorescence patterns observed (for example CAT, glucose 6-phosphatase and deletion mutant staining) were unexpected and therefore challenge the confidence immunofluorescence can provide in determining subcellular localization. A case in point is dynamin. Although it originally co-purified with microtubules (Obar et al., 1991), its immunofluorescence staining pattern in PC12 cells is diffusely cytoplasmic, displaying no co-localization with tubulin (Scaife & Margolis, 1990). Even taxol-induced microtubule re-arrangements did not alter the dynamin staining pattern (Scaife & Margolis, 1990).

It has been extoled that the majority of PKC substrates are associated with the plasma membrane where PKC is believed to be activated. Yet wild-type pleckstrin appears both biochemically and histologically cytosolic prior to and after phosphorylation. Perhaps transient membrane association or disassociation of either PKC or pleckstrin is sufficient to achieve fruitful interaction. In support of this, creation of a non-myristoylated form of MARCKS was found to be as readily phosphorylated as the native protein (Graff et al., 1989). Therefore, it would be erroneous to suggest that a specific localization pattern excludes or indicates any function for a particular protein.

SUMMARY AND PERSPECTIVES

Most proteins are purified, cloned and studied because they are known to possess an activity of interest to biochemists. Using this project as an example, it can be emphasized that there is an inherent difficulty in trying to discern the function of a completely novel protein. More challenges of this type will arise however as sequencing of the human genome gains momentum and identifies novel open reading frames.

Although during the course of studying pleckstrin its function was never revealed, a strong foundation was laid facilitating further research on this protein. Pleckstrin specific tools generated during this work include: an easily purified source of recombinant pleckstrin in the form of a GST-fusion protein, crude and affinity-purified polyclonal antibodies to recombinant pleckstrin, several plasmid constructs enabling the over or underexpression (antisense construct) of pleckstrin in eukaryotic cell lines and a phagemid vector from which site-directed mutants can be quickly generated.

Using these tools, preliminary investigation into the function of pleckstrin was carried out. Transfected COS cells were examined in detail regarding the subcellular localization of pleckstrin and potential structural effects on the cell from overexpression. Wild-type pleckstrin overexpression appeared innocuous to the COS cells whereas the deletion mutant displayed deleterious effects, either intrinsically or extrinsically. It was also shown that both the recombinant protein in vitro or pleckstrin expressed heterologously in vivo was phosphorylated identically to endogenously expressed pleckstrin from HEL or HL-60 cells. The phosphorylation sites of pleckstrin were concisely mapped using two different approaches; site-directed mutagenesis and marker peptides phosphorylated in vitro. Phosphorylation of pleckstrin did not alter its subcellular

localization, again failing to yield functional information. The only potential functionally correlatable observation was that mutant S113A, which lacks one of the major phosphorylation sites, induced an unusual membrane or cytoplasmic lobbing in transfected COS cells.

Future investigations can be categorized based upon the following three attributes of pleckstrin:

1) Pleckstrin has a very restricted tissue distribution.

It will be important to discern at what stages of hematopoiesis and in which cell types pleckstrin expression is up-regulated. There are clearly some cell types (for example B and T lineages) that do not express pleckstrin (section 3.8) and others that express high levels (platelets, neutrophils). Examination of immature and mature megakaryocytes from rat or mouse bone marrow might yield information regarding the timing of pleckstrin up-regulation prior to platelet formation, as has been obtained for other megakaryocyte-specific genes (Courtney *et al.*, 1991). Additionally, electron microscopy using the affinity purified antibody should be extended to other secretory hematopoietic cells such as neutrophils, to visualize pleckstrin localization prior to, during and after secretion (this antibody non-specifically labelled alpha granule matrices in platelets processed for electron microscopy, C. Langdon, unpublished results). However, a cytoplasmic association with secretory granules may implicate pleckstrin in signalling pathways that control secretion. This hypothesis could be tested by microinjecting anti-pleckstrin antibodies into model secretory cells such as rat basophilic mast cells. This latter avenue requires development of a good test system, which is not trivial.

The restricted expression pattern of pleckstrin suggests that the pleckstrin promoter contains haematopoietic-specific transcriptional elements. Therefore, once cloned, the pleckstrin promoter might make an excellent investigative tool for identifying factors responsible for transcriptional regulation of haematopoietically controlled genes. Finally,

replacement of the pleckstrin gene in mouse with the *lac Z* gene would provide comprehensive information on the pleckstrin expression pattern throughout development. More importantly, pleckstrin (-) mice might present obvious deficiencies in normal haematopoietic function (e.g. impaired thrombosis) likely without gross perturbation of developmental pathways required for mouse viability.

2) Pleckstrin is phosphorylated by PKC on serines 113, 117, and occasionally on threonine 114.

PKC phosphorylates both recombinant pleckstrin and the short pleckstrin sequence specific peptides efficiently in vitro (section 3.7). A pleckstrin-derived peptide would make an excellent tool for measuring PKC activity in lysates or immunoprecipitations. It then follows that substitution of the phosphorylatable serines with alanine might create an excellent inhibitor peptide. The specificities of the different PKC isoforms might be revealed using various peptide analogues to pleckstrin's phosphorylation domain. Also, determinants that control preference for serine 117 over serine 113 could be examined in detail by similarly constructed peptides.

Since serine 113 is a major phosphorylation site, serious examination of the strange cytoplasmic lobations occurring in the COS cells transfected with the S113A mutant should be undertaken. These cells could be double labelled for a variety of subcellular structures to help define the type and extent of morphological perturbations that occur in association with this mutant. The role of external growth factors in triggering the S113A mutant-associated lobbing should be examined since similar effects by growth factors on membrane ruffling have been observed (Ridley *et al.*, 1992). Pleckstrin is not native to COS cells, therefore the S113A construct should be expressed in other cell lines to test the universality of the lobation response. It is possible the S113A mutant represents a constitutively active form of pleckstrin that would act dominantly in the presence of endogenous wild-type pleckstrin in HL-60 cells. Once reliable methods for transfecting

suspension cells are developed, expression of these mutants in haematopoietically derived cell lines should be undertaken.

3) Pleckstrin contains a repeated domain (the PH domain) found in a variety of other signalling molecules.

By far the most significant discovery about pleckstrin to date has been the identification of a distinct domain found in many interesting signalling proteins that displays weak homology to pleckstrin's N and C termini (Haslam *et al.*, 1993; Mayer *et al.*, 1993). Within one year of the original identification, the number of proteins containing a PH domain increased from 15 to 71 (Gibson *et al.*, 1994). This was largely due to availability of protein sequence alignment programs capable of identifying weakly conserved sequences (Shaw, 1993).

The PH domain containing proteins generally fall into three functional categories (Gibson *et al.*, 1994). The largest group represents proteins involved in directly regulating small GTP binding proteins. It includes rasGAP, bcr and son-of-sevenless homologs, all of which regulate ras. The second group includes proteins from several distinct serine/threonine kinase families. It is interesting to note that the tec tyrosine kinase has a PH domain, indicating potential PH domain-mediated cross-talk between proteins signalling through serine kinases and those signalling through tyrosine kinases. The third group include cytoskeletal regulatory proteins such as dynamin, kinesin and ß-spectrin.

Superficially, PH domains appear restricted to proteins involved in signal transduction. For example, a few PH-domain containing proteins bind to receptors (insulin receptor substrate-1 (IRS-1), epidermal growth factor receptor-binding protein (Grb7)), a few are catalytically active (the kinases, phospholipase C-b,d,g), a few regulate the monomeric G proteins found at the plasma membrane or exclusively at intracellular membrane sites (bcr and rasGAP), and the remainder regulate cytoskeletal dynamics often implicated in growth control or secretion (dynamin). Therefore there could be several

signalling pathways that repeatedly invoke PH domain function from events initiated at the plasma membrane to responses that ultimately require cytoskeletal rearrangements such as secretion.

For pleckstrin, the phosphoserine or threonine binding hypothesis of PH domains (see Introduction) allows for some interesting speculation. Phosphorylation at S113 or S117 of pleckstrin might cause intramolecular recognition of either the N or C-terminal PH domain. This type of interaction has been shown for SH2 domains in src and is an important regulatory feature of this kinase (Malarkey et al., 1995). This theory could be tested by incubation of the phosphorylated peptide corresponding to residues 108-120 of pleckstrin with the intact unphosphorylated protein, or with the N or C terminal PH domains alone. Alternately, phosphorylation of pleckstrin might alter the conformation and therefore affinity of one or both PH domains for putative target proteins. Finally, PH domains may bind phosphatidylinositols in a manner competitive with serine or threonine phosphorylated proteinaeous targets as has been reported for the SH2 domain of PI3K and src (Rameh et al., 1995). Finally, the yeast dihybrid system could be used to screen for proteins that might bind to pleckstrin's PH domains.

Most proteins having a PH domain contain several other distinct motifs such SH2, SH3, or catalytic domains in addition to a PH domain. Pleckstrin is unique in that it has two PH domains separated by approximately 150 amino acids containing no recognizable functional motif. This is reminiscent of the crk proto-oncogene, which has only SH2 and SH3 domains yet can induce transformation when overexpressed (Sabe et al., 1995). Pleckstrin may similarily act to tether signalling proteins to one another or to the plasma membrane, however, no significant membrane localization for pleckstrin has been observed. Alternatively, pleckstrin might function as a regulatory domain, binding to and directly modulating the catalytic activities of other proteins.

One of the PH domain containing proteins, the proposed human homolog of the yeast sec7 protein (hsec7; Liu & Pohajdak, 1992) has homology to pleckstrin extending past the PH domain (underlined, see below), into the phosphorylation region of pleckstrin.

```
Pleckstrin 92 WVRDINKAIKCIEGGQKF-ARKSTRRS*IRLP 121
hSEC7     386 WIKCIKAAISRPDFYEMLAARKKKVSS*TKRH 398
```

Thus hsec7 has a putative PKC phosphorylation site that corresponds to serine 117 of pleckstrin (asterik and bold). This suggests that these two proteins might share a common function in this extended region, which might bear upon their PH domain function. hsec7, like pleckstrin, has a restricted tissue distribution, being most highly expressed in natural killer (NK) T cells (Liu & Pohajdak, 1992). It has been theorized that hsec7 is involved in re-orientating secretory granules towards targets to be lysed during NK/T cell conjugation (Liu & Pohajdak, 1992). Pleckstrin might therefore have an analogous role in the platelet.

Future work from many laboratories will undoubtedly focus on the role of the PH domain, and specific information regarding PH domain function in other proteins may shed light on pleckstrin function in general. If either the N or C terminal PH domain of pleckstrin is discovered to tightly bind lipid bilayers in vivo, then these protein modules may become useful as portable targetting motifs. In this scenerio, the activity of many signalling molecules that rely upon membrane localization for their activity could be manipulated, leading to an enhanced understanding of a wide range of signalling processes.

APPENDIX

5.1 Are There Proteins That Co-Immunoprecipitate With Pleckstrin?

Recent publications have emphasized the importance of protein-protein interactions in modulating signalling processes (reviewed in Pawson, 1995). Especially for proto-oncogenes or tumour suppressor proteins, identification of interacting proteins often leads to an understanding of how these proteins critically affect signalling pathways that control cell division.

Previous work revealed that immunoprecipitation of pleckstrin from [^{35}S]-methionine-labelled HL-60 cells co-precipitated one major protein with an apparent molecular mass of 190 kDa, and several minor proteins of approximately >100 (2), 66, 32 and 15 kDa (M. Tyers, Ph.D. thesis, 1988). Western analysis of total protein from retinoic acid differentiated HL-60 cells detected only pleckstrin and the 15 kDa protein. Therefore, the 15 kDa protein might be a proteolytic fragment of pleckstrin, an alternate transcription/translation product of the pleckstrin gene or simply an antigenetically cross-reacting, unrelated gene product. ^{32}P-labelling the cells prior to immunoprecipitation indicated that only pleckstrin and the 190 kDa protein are phosphoproteins, both apparently incorporating more label after PMA treatment. Since identification of these associating proteins had obvious potential in providing clues to pleckstrin's function, these observations were investigated in more detail.

The original immunoprecipitations were repeated using the same cell line, differentiation protocol (1μM RA; 7 d) and antiserum, and an identical pattern of bands was observed (Fig. A.1.1) as had been seen previously (M. Tyers, Ph.D. thesis, 1988). The

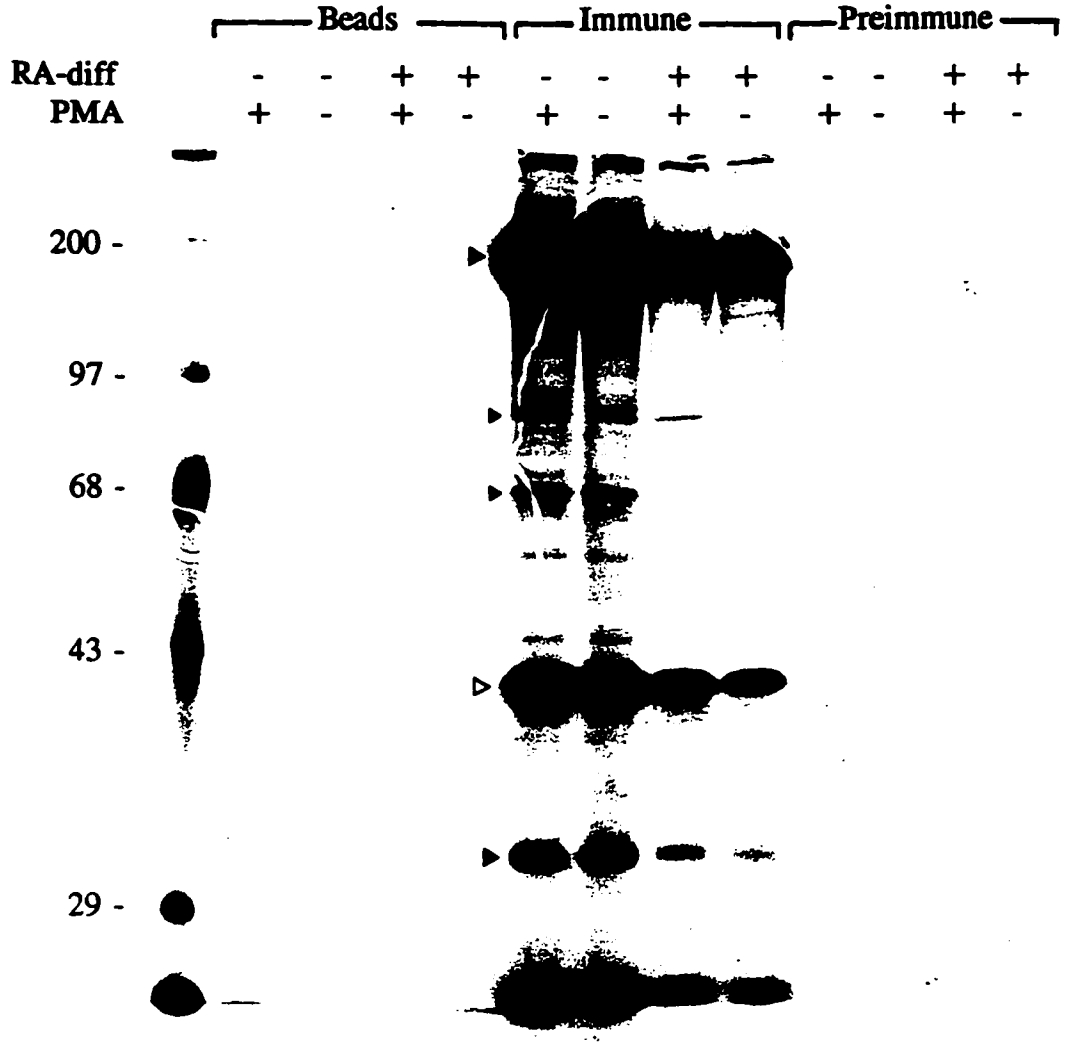

Figure A.1.1 - Immunoprecipitation of Pleckstrin from HL-60 Cells.

Pleckstrin from [^{35}S]-methionine-labelled retinoic acid-differentiated or undifferentiated HL-60 cells was immunoprecipitated using a rabbit anti-pleckstrin serum. Pleckstrin is identified by the open arrowhead; the 4 closed arrowheads indicate antigenetically unrelated proteins that co-immunoprecipitate with pleckstrin, of apparent molecular mass 190, 84, 68 and 32 kDa. Note the absense of these bands in control immunoprecipitations using preimmune serum or just Protein A-Sepharose beads. Brief treatment of the cells with PMA prior to lysis increased the amount of the 84 kDa co-precipitating protein. Induction of the cells towards a granulocytic phenotype with retionic acid did not appear to alter the pattern or relative amounts of co-precipitating proteins.

190 kDa band was slightly more intense than pleckstrin, but without Coomasie staining it was not possible to calculate the molar ratio of the 190 kDa protein to pleckstrin from this experiment. There did not appear to be any changes in the relative abundance of these proteins in retinoic acid treated cells, indicating no difference in associations after differentiation of the HL-60 cells towards a granulocytic phenotype. When PMA was added to the cells prior to harvesting, a minor 84 kDa co-immunoprecipitating band increased in intensity by a factor of 2 or greater. This may indicate that the 84 kDa protein has greater affinity for phosphorylated pleckstrin.

Controls for potential binding to preimmune sera or to Protein A-Sepharose beads were included in these experiments. Protein A-Sepharose beads pulled down a faint band having the same apparent molecular weight as pleckstrin (Fig. A.1.1, lane beside molecular weight standards). Preimmune sera precipitated a faint band that migrates just above pleckstrin that is also seen in immune samples, and a very faint band level with the 190 kDa co-immunoprecipitating phosphoprotein (pp190). These controls sufficiently indicate that pleckstrin and the major and minor ^{35}S-methonine-labelled proteins immunoprecipitate only in the presence of immune serum.

Since pp190 was apparently ^{32}P-labelled, this protein might be the tyrosine phosphorylated pp190 that associates with rasGAP (rhoGAP, Lancaster et al., 1994). Phosphotyrosine could not be detected at 190 kDa after the immunoprecipitated proteins were western blotted with an anti-phosphotyrosine antibody, suggesting pp190 may instead be serine or threonine phosphorylated. This band also did not react with an anti-microtubule associated protein 2 (MAP2; Tucker, 1990) antibody (data not shown).

With the long-term goal of cloning pp190, several attempts were made to scale up immunoprecipitation of pleckstrin from HL-60 cells so that enough pp190 could be isolated for sequencing. Unfortunately the amount of pleckstrin and pp190 immunoprecipitated never appeared to proportionately increase with increasing amounts of antibody and cell

lysate. Other attempts to isolate pp190 involved using affinity columns of purified recombinant GST-pleckstrin or alternatively the affinity purified anti-pleckstrin antibody. After passing HL-60 lysate over the anti-pleckstrin column, pleckstrin was bound strongly as expected, but pp190 was not detected (not shown).

It should be noted that affinity purified anti-pleckstrin antibody, was used for these latter experiments, not the original polyclonal serum used in the experiment shown in Fig. A.1.1. To test the amount of this highly specific antibody required to bind all the pleckstrin in a particular aliquot of cell lysate, serial dilution of the antibody in immunoprecipitations was performed. The results were startling in that a 40 kDa (same apparent molecular mass as pleckstrin) and a 200 kDa band immunoprecipitated even at extremely dilute antibody concentrations (Fig. A.1.2). Knowing that the nearly infinitely dilute antibody could not have precipitated these proteins, these bands must represent proteins with affinity for Protein A-Sepharose beads. Without antibody present, Protein A-Sepharose beads did immunoprecipitate a 40 and 200 kDa protein (Fig. A.1.2, first lane). In the presence of affinity purified antibody, the 40 kDa band intensifies, indicating immunoprecipitation of pleckstrin in addition to the unfortuitously co-migrating non-specific protein. Additional experiments determined that this non-specific 40 kDa protein precipitated in the absence of Sepharose beads, perhaps by sticking to the eppendorf tube (data not shown). Western analysis of control immunoprecipitations confirmed that the non-specific 40 kDa protein did not react with the anti-pleckstrin antibody (not shown). Due to its apparent size and characteristic of pelleting in immoprecipitations, this protein might be actin.

After the original experiment shown in Fig. A.1.1, there had been no reason to repeat the beads only control. Since the affinity purified antibody contains less total IgG than the sera, the Sepharose beads used in immunoprecipitations with the affinity purified antibody would likely have more available binding sites for non-specific protein(s). This might explain the dramatic increase in intensity of the non-specific proteins when the

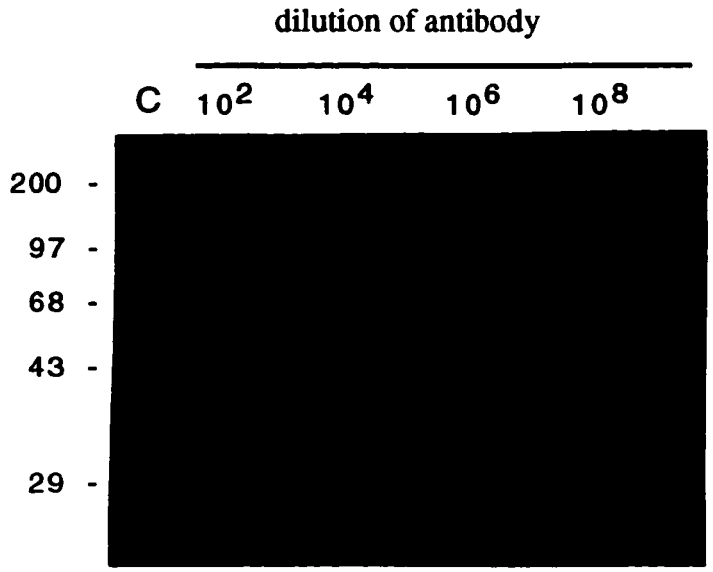

Figure A.1.2 - Immunoprecipitation of Pleckstrin using Serial Dilutions of the Affinity Purified Antibody.

Constant volumes of serially diluted affinity purified anti-pleckstrin antibody (to give a final dilution of 10^2 to 10^9 in 10 fold increments) were added to lysates of ^{35}S-methionine labelled HL-60 cells. Protein A-Sepharose beads were then added to the lysates and after 1 hr washed extensively in 1% NP-40 buffer (see section 2.3.5). The control lane (C), shows a prominent band at the same migration distance as pleckstrin (40 kDa), and a band at 200 kDa. At the 10^2 dilution, the 40 kDa band is more intense presumably due to the presence of pleckstrin. Unusual is the presence of the >200 kDa band in all dilution lanes but not in the control lane.

affinity purified antibody was used as compared to crude sera. Therefore, there is still reason to believe that the proteins orignally co-immunoprecipitated with pleckstrin really do specifically bind pleckstrin. Unfortunately, investigation of these proteins was confounded by non-specific precipitating proteins co-migrating with pleckstrin and pp190.

5.2 Location of an Intron in the Pleckstrin Gene

A 4.5 kbp Eco RI - Sal I fragment from genomic clone #53 (Tyers, Ph.D. thesis, 1988) had been shown to hybridize to the 581 bp Eco RI fragment spanning nucleotides 102 to 683 in the pleckstrin cDNA. Therefore this 4.5 kbp fragment was subcloned into pUC 118 and sequenced using the reverse universal primer. The sequence obtained matched the pleckstrin sequence from just downstream of the first Eco RI site to nucleotide 198 (amino acid 66). At this point the sequence diverged for 53 nucleotides, after which the sequence was not readable. The sequence at the divergence site matches that of an exon-intron splice site excellently (Mount, 1982), as shown below.

```
amino acid     r  m   f  v

cDNA        ...AGGATG/TTTGTG...
genomic     ...AGGATG/GTAAGT...

consensus   ...---AAG/GTAAGT...
                  C
                exon/intron
```

The location of this intron is just in front of the first residue of fifth subdomain of pleckstrin's N-terminal PH domain (Musacchio *et al.*, 1993). Therefore functional diversity may have arisen by swapping the first 4 or last 2 subdomains of the PH domain in pleckstrin and other PH domain containing proteins.

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LIST OF ABSTRACTS AND PUBLICATIONS

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Brumell, J.H., Ferguson, D., **Craig**, K., Tyers, M. & Grinstein, S. "Regulation of Pleckstrin Phosphorylation in Human Neutrophils" 1996 ASBMB/ASIP/AAI Joint Meeting

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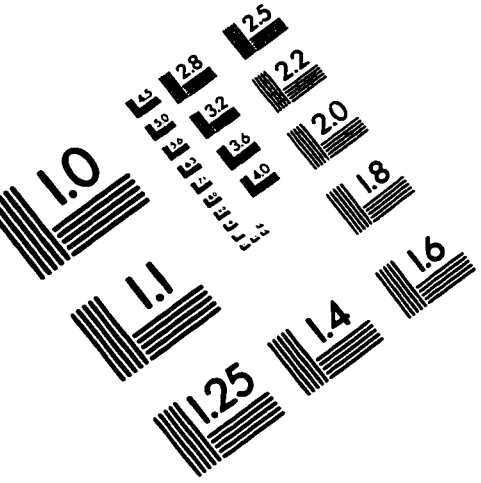
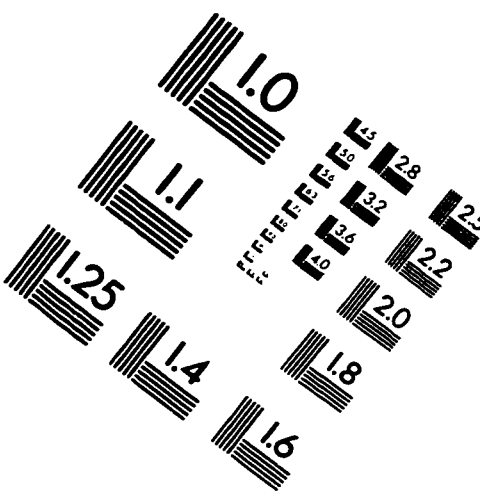
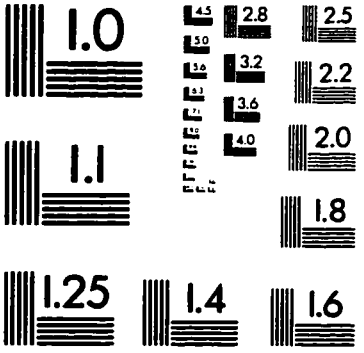
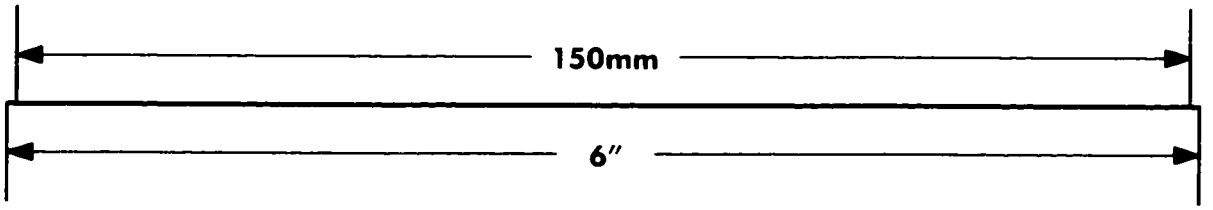
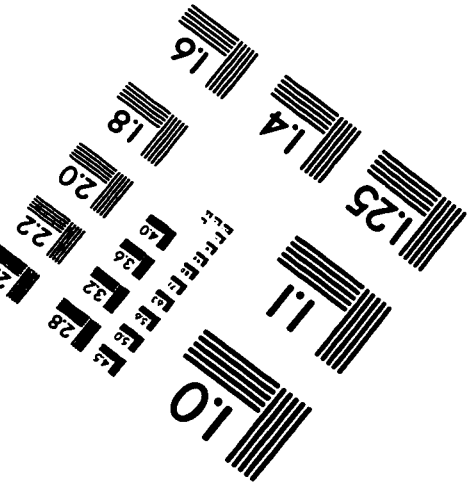
IMAGE EVALUATION
TEST TARGET (QA-3)

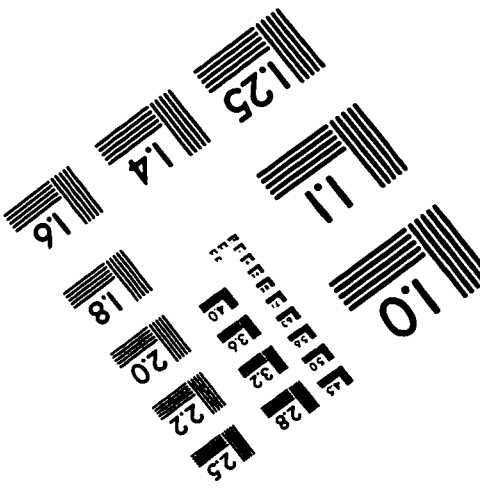












APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989