GRB2 AND GRB7 IN MAMMARY TUMORIGENESIS

THE ROLE OF GRB2 AND GRB7 IN POLYOMAVIRUS MIDDLE T ANTIGEN- AND NEU-MEDIATED MAMMARY TUMORIGENESIS

By
CHRISTOPHER G. TORTORICE, B. Sc.

A Thesis
Submitted to the School of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree
Master of Science

McMaster University

MASTER OF SCIENCE (1997) McMaster University

(Biology) Hamilton, Ontario

TITLE: The role of Grb2 and Grb7 in polyomavirus middle T antigen- and Neu-mediated mammary tumorigenesis

AUTHOR: Christopher G. Tortorice, B. Sc. (Honours) (University of Windsor)

SUPERVISOR: Professor W.J. Muller

NUMBER OF PAGES: x, 82

Abstract

Activated protein tyrosine kinases, which have been implicated in the genesis of a number of human cancers, rely on a variety of protein-protein interactions to transmit their proliferative signals within the cell. These interactions are often mediated by Src homology 2 and 3 (SH2 and SH3) domains. A class of proteins which are mainly composed of such domains, termed adaptor proteins, has been identified.

The Growth factor receptor bound proteins Grb2 and Grb7 are SH2 domain adaptor proteins which have been shown to associate directly or in complex with many tyrosine kinases, including the c-ErbB-2/Neu receptor tyrosine kinase. While overexpression of either protein alone in rat fibroblasts is not transforming, human breast cancer cell lines exhibit Grb2 and Grb7 gene amplification, and mRNA and protein overexpression.

The role of Grb2 in polyomavirus middle T antigen-mediated mammary tumorigenesis has been examined utilizing gene targeting and transgenic approaches. Initial characterization of the progeny of matings involving $Grb2^{+/-}$ mice and MMTV/middle T transgenic mice indicated that delayed tumor kinetics may be the result of Grb2 dosage differences between $mT^{+};Grb2^{+/-}$ and mT^{+};Grb2+/+ animals. Transgenic animals expressing a dominant negative version of Grb2 in the mammary epithelium have been generated to explore an alternate method for disrupting signaling from middle T antigen.

The role of Grb2 and Grb7 in Neu-mediated mammary tumorigenesis is also being examined. Both MMTV/Grb2 and MMTV/Grb7 transgenic mice that express the transgene in the mammary epithelium have been identified by

ribonuclease protection analysis. Matings involving these strains and MMTV/*neu* mice should aid in determining the effects of overexpressing Grb2 or Grb7 on Neu-mediated mammary tumorigenesis.

Acknowledgements

Despite the often heard assertions of individual effort - "I did it my way" or "I really worked hard for this" - I have come to realize that no task can be accomplished in isolation. In fact, even a project as straightforward as this thesis could not have been completed without the guidance and encouragement of others. I would like to thank Dr. William Muller, my supervisor, for providing advice, encouragement, and an occasional dose of reality when I really needed one. I must also thank the members of my supervisory committee - Dr. John Hassell, Dr. Herb Schellhorn and Dr. Ana Campos - for their thoughtful comments with regard to my project.

I would like to specially acknowledge the members of the laboratory who are largely responsible for relating to me all the things which I have learned about lab work, including Peter Siegel, David Dankort and Marc Webster. Furthermore, I must thank Valerie Blackmore, Harold Kim, John Hutchinson and Mike Rauh, who made graduate studies much more fun than they otherwise would have been. I also must thank Monica Graham for her great patience during those early morning mouse tagging sessions.

I would like to take this opportunity to thank my parents and grandparents for the many years that they have lent me their support and love, even when I went off on some crazy mission to study biology at university. They have helped me through all of the frustrations, panics and annoyances of university life. Finally to my close friends Bill T., Brett, James, Francoise, Patrick, Alysan, all of my patient roommates, and the McMaster golf team players and coaches - thanks for all your kindness and loyalty. I couldn't have made it without you.

Contributions by others

The photomicrographs displayed in Figures 3-2 were prepared in the laboratory of Dr. Robert Cardiff in the Department of Medical Pathology, University of California, Davis.

MMTV/Grb2 mice were generated by Peter Siegel and Linda Wei, while M\MTV/P49L and MMTV/Grb7 mice were generated by P. Siegel; the DNA construct used to generate the MMTV/Grb7 mice was produced by David Dankort.

Monica Graham performed a large proportion of the Southern blot analyses and PCR reactions used to identify transgenic and knockout mice (described in section 2.2).

Table of Contents

List of Figures

List of Tables

Chapter 1: Introduction

Identification of the intracellular signaling molecules responsible for transmitting mitogenic stimuli remains a key issue in the study of oncogenesis. In particular, activated protein tyrosine kinases play critical roles in a number of human cancers (Mulligan et al., 1993; Hofstra et al., 1994; Nishikawa et al., 1994). These tyrosine kinases associate with a variety of other enzymatic and non-catalytic cytoplasmic proteins. Many such proteins are substrate molecules which become phosphorylated on tyrosine residues, while others bind to form protein complexes necessary for the propagation of intracellular signals.

Signal transduction is often mediated by the assembly of specific protein-protein complexes. The assembly of such complexes can be accomplished by small modular phosphotyrosine-dependent and -independent protein binding domains. The Src homology 2 (SH2) and 3 (SH3) domains are the most well characterized of these protein interaction domains (Koch et al., 1991; Pawson and Gish, 1992; Margolis, 1992; Schlessinger, 1994). SH2 domains are phosphotyrosine-binding modules (Koch et al., 1991; Pawson and Gish, 1992), while SH3 domains recognize proline-rich sequences (Ren et al., 1993); both are conserved among a wide variety of cytoplasmic proteins involved in the transmission of mitogenic signals. Oncogenic signaling by tyrosine kinases is thought to involve the participation of a number of SH2/SH3 domain proteins, including the Growth factor receptor-bound protein 2 (Grb2) (Daly et al, 1994; Ghishizhky et al, 1995; Tanaka et al, 1995) and Grb7 (Stein et al., 1994).

The identification of a novel SH3-SH2-SH3 protein, Sem-5, from the nematode worm *Caenorhabditis elegans* (Clark et al., 1992), along with the

identification of mammalian Grb2 (Lowenstein et al., 1992) and *Drosophila* Drk (Simon et al., 1993; Olivier et al., 1993) provided evidence that SH2 and SH3 containing proteins are highly conserved among both vertebrates and invertebrates. Functional analysis of these proteins has demonstrated that they perform analogous roles in signal transduction from activated tyrosine kinases. Mutations in *sem-5* have been shown to inhibit vulval development in C. *elegans* (Clark et al., 1992), and *drk* mutants impair signaling from the Sevenless receptor tyrosine kinase (Olivier et al., 1993). Taken together, these observations from *Drosophila* and C. *elegans* indicate that similar loss of function mutations in *grb2* might impair signaling from receptor tyrosine kinases in mammalian systems, and suggest possible therapies for the treatment of human cancers involving activated tyrosine kinases.

1.1 Activation of tyrosine kinases in transformation and tumorigenesis

In multicellular organisms, the control of cellular growth and differentiation is accomplished via a variety of extracellular stimuli. Many extracellular signals are transmitted by polypeptide growth factors such as the epidermal growth factor (EGF) and platelet-derived growth factor (PDGF). These growth factors elicit cellular responses by interacting with specific transmembrane cell surface receptors, several families of which transmit signals within the cell by functioning as protein tyrosine kinases (Panayotou and Waterfield, 1993). The identification of some viral oncogenes as altered growth factor receptor tyrosine kinases (RTKs), including v-ErbB as a truncated version of the EGF receptor (Downward et

al., 1984), led to a closer examination of the role of RTKs in growth control and oncogenesis (Ullrich and Schlessinger, 1990).

While RTKs normally function in the control of cell growth and differentiation, mutation of several mammalian RTKs has been shown to cause their unregulated biological activation (Bargmann et al., 1986; Roussel et al., 1988; Coulier et al., 1990). Chemical mutagenesis using pre- or neonatal BDIX rats produced a variety of neuro- and glioblastomas, and resulted in the isolation of the *neu* oncogene, a RTK closely related to the EGF receptor (Schechter et al., 1984; Schechter et al., 1985). The oncogenic activity of Neu was determined to be the result of a somatic point mutation in the transmembrane domain of the receptor (Bargmann et al., 1986).

Mutant versions of human RTKs that are oncogenically activated have also been engineered. Specifically, a point mutation introduced into the extracellular domain of the human colony stimulating factor (CSF-1) receptor by site-directed mutagenesis produced a CSF-1 receptor capable of transforming NIH 3T3 cells in a growth factor-independent manner (Roussel et al., 1988). Furthermore, human *trk* oncogenes can be generated by in-frame deletion, kinase domain duplication and point mutation (Coulier et al., 1990). Collectively, these studies indicated that multiple mechanisms for activating the transforming ability of RTKs existed.

To determine whether activated tyrosine kinases are capable of inducing tumors *in vivo*, transgenic mice have been used extensively. Expression of the activated c-*neu* oncogene under control of the mouse mammary tumor virus (MMTV) promoter led to the synchronous development of mammary adenocarcinomas that involved the entire mammary epithelium (Muller et al., 1988). In contrast, expression of an activated version of the c-Src cytoplasmic

tyrosine kinase under control of the MMTV promoter produced mammary epithelial hyperplasias which only rarely progressed to neoplasias (Webster et al., 1995).

Transgenic mice expressing an H2-L promoter driven, constitutively activated mutant c-*kit* construct developed acute leukemias or malignant lymphomas that ultimately led to their death (Kitayama et al., 1996). Recently, rat calcitonin gene-related promoter / calcitonin promoter-directed expression of a mutant *RET* receptor tyrosine kinase was shown to induce multifocal medullary thyroid carcinoma (Michiels et al., 1997). These tumors were morphologically and biologically similar to the human tumors observed in multiple endocrine neoplasia type 2. It is clear that many of these transgenic mouse strains provide valuable models for studying human malignancies, including transgenic mice expressing various forms of Neu (Muller et al., 1988; Guy et al., 1992b; P.M. Siegel and W.J. Muller, unpublished results) and mutant *RET* receptor (Michiels et al., 1997).

Activating mutations of RTKs have been identified in a number of human cancers. Deletions in the extracellular domain of the EGF receptor have been implicated in human gliomas (Nishikawa et al., 1994), and germ-line mutations of the *RET* receptor were reported in association with multiple endocrine neoplasia type 2A (Mulligan et al., 1993). In fact, two other dominantly inherited cancer syndromes, multiple endocrine neoplasia type 2B and sporadic medullary thyroid carcinoma, were also shown to be associated with mutation of the *RET* receptor (Hofstra et al., 1994). These mutations were demonstrated to activate the *RET* receptor via stabilization of *RET* dimers or alteration of *RET* catalytic properties (Santoro et al., 1995; Asai et al., 1995), confirming that the constitutive activation of RTKs can play a causal role in hereditary and sporadic human cancers.

1.2 SH2 and SH3 domains mediate protein interactions downstream of activated tyrosine kinases

Receptor tyrosine kinases, activated transiently by ligand binding or constitutively by mutation, phosphorylate specific tyrosine residues on target proteins; these targets include a variety of enzymatic and non-catalytic proteins, as well as the RTK itself (reviewed in Pawson, 1992; Kazlauskas, 1994). Receptor autophosphorylation provides a number of binding sites for the SH2 domains of cytoplasmic signaling proteins, allowing activated RTKs to couple with intracellular signaling pathways (Anderson et al., 1990).

SH2 domains were first identified as non-catalytic domains of cytoplasmic tyrosine kinases like Src and Fps (Sadowski et al., 1986; Pawson, 1988). These highly conserved domains of approximately 100 amino acids bind directly to phosphotyrosine (Matsuda et al., 1990), allowing the assembly of heteromeric protein complexes that control the activation of signal transduction pathways (Moran et al., 1990; Koch et al., 1991). The interaction between RTKs and SH2 domain-containing proteins was localized to the tyrosine phosphorylated carboxy-terminus of the receptors (Margolis et al., 1990). Furthermore, the amino acid sequence C-terminal to the phosphotyrosine on the receptor was discovered to be essential for the interaction between phosphatidylinositol 3'-kinase (PI3'K) and the PDGF receptor (Fantl et al., 1992). Thus, different SH2 domains bind to distinct phosphotyrosines on activated RTKs, enabling different receptors to activate a variety of signaling pathways based upon the sequences surrounding their autophosphorylation sites (Pawson, 1992).

Many of the SH2 domain proteins that couple to activated growth factor receptors exhibit enzymatic activity (Figure 1-1). The isolated SH2 domains of phospholipase Cγ1 (PLCγ1), which specifically phosphorylates phosphoinositides, Ras GTPase activating protein (GAP), which stimulates the intrinsic GTPase activity of Ras to return Ras from the active GTP-bound state to the inactive GDP-bound state, and members of the Src family of cytoplasmic tyrosine kinases, all interact with activated receptors (Anderson et al., 1990).

Several other proteins with enzymatic activity couple directly or via SH2-containing subunits to tyrosine phosphorylated RTKs. The cloning of an 85 kilodalton protein that contains two SH2 domains and one SH3 domain, and characterization of its interaction with the PDGF receptor, the tyrosine phosphorylated carboxy-terminus of the EGF receptor, and the 110 kilodalton subunit of PI3'K, led to the identification of p85 as a subunit of PI3'K (Escobedo et al., 1991; Otsu et al., 1991; Skolnik et al., 1991). p85 provides a direct link between PI3'K and activated RTKs via its two SH2 domains (McGlade et al., 1992).

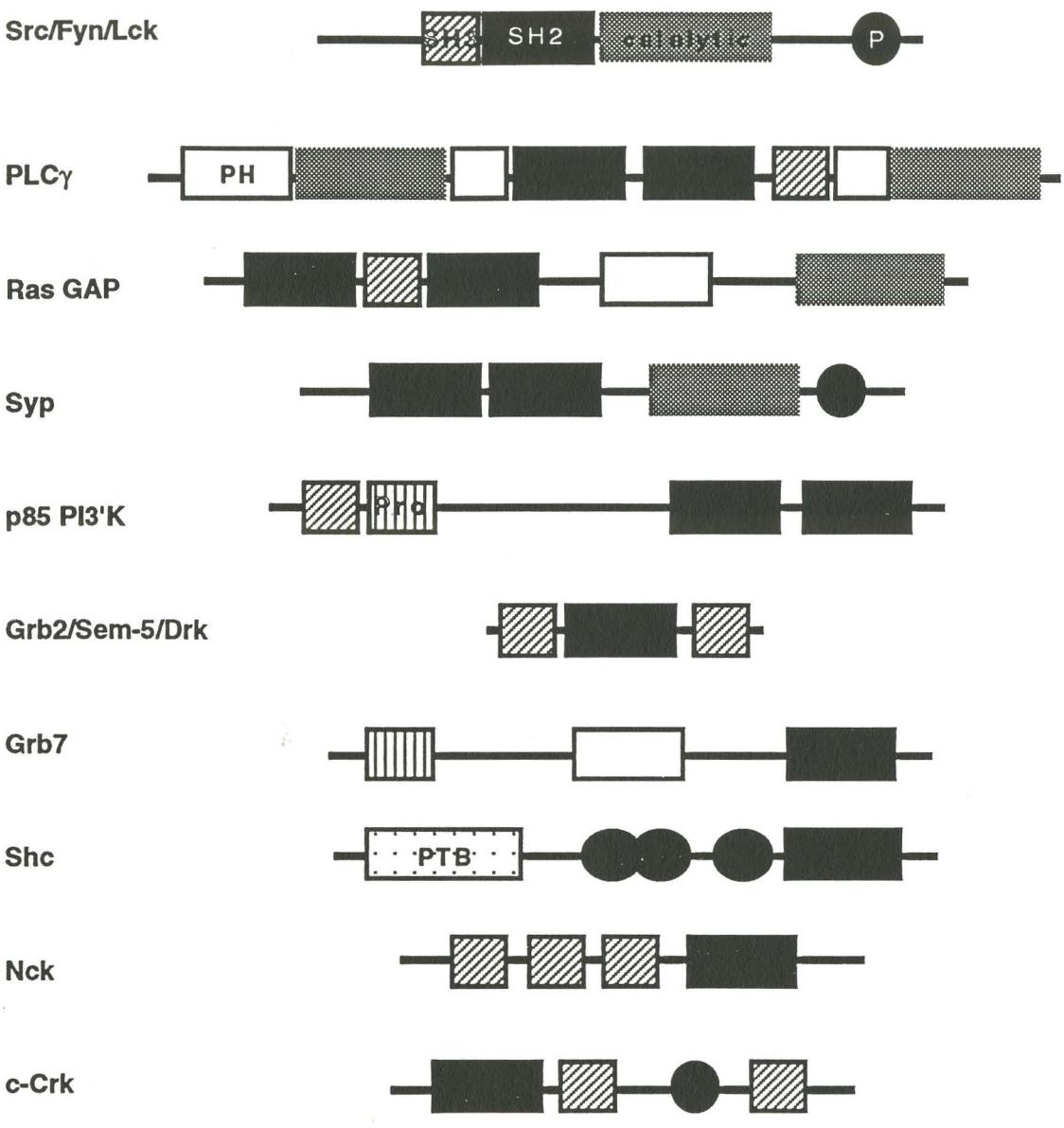
In addition, protein tyrosine phosphatases interact with growth factor receptors. Syp phosphotyrosine phosphatase, which possesses two SH2 domains, is a RTK substrate which is phosphorylated on tyrosine residues in response to growth factor stimulation. Syp directly binds to the PDGF and EGF receptors (Kazlauskas et al., 1993; Vogel et al., 1993), and indirectly interacts with the insulin receptor via insulin receptor substrate 1 (IRS-1) (Tanaka et al, 1996).

Many SH2 domain-containing proteins also possess a distinct sequence of approximately 50 amino acids, the SH3 domain (Figure 1-1). SH3 domains are involved in protein-protein interactions as well, and act by binding to proline-rich sequences (Ren et al., 1993). Much like the SH2 domain, SH3 domains were first

Figure 1-1: Proteins containing SH2 and SH3 domains

Many proteins involved in intracellular signaling possess modular protein-protein interaction domains, including the SH2 domain (black rectangles), SH3 domain (hatched squares), PH domain including the split PH domain of PLCγ (white rectangles) and PTB domain (stippled box). Some of these proteins have catalytic activity domains (gray rectangles) while others harbour proline-rich regions (vertically striped rectangles) or phosphotyrosine-containing SH2 binding sites (black circles).

Proteins containing SH2 and SH3 domains



observed in the Src family of tyrosine kinases (Stahl et al., 1988; Mayer et al., 1988), and are present in many RTK substrates, including PLCγ1, Ras GAP and the PI3'K subunit p85 (Pawson, 1988; Koch et al., 1991).

A number of SH2- and SH3-containing proteins, including the p85 subunit of PI3'K, lack catalytic domains. p85 contains two SH2 domains and one SH3 domain, and associates with activated RTKs, but does not exhibit detectable PI3'K or other enzymatic activity (Escobedo et al., 1991; Otsu et al., 1991; Skolnik et al., 1991). Instead, its primary role seems to involve functioning as an adaptor protein, linking the p110 catalytic subunit of PI3'K to activated RTKs. Other SH2/SH3 adaptor proteins that have been identified include Nck, Vav, Shc, c-Crk, Grb7 and Grb2 (Koch et al., 1991; Pawson, 1992; Margolis et al., 1992; Lowenstein et al., 1992).

Nck and Crk are two members of the family of SH2/SH3 adaptor proteins. Nck consists almost entirely of three SH3 domains followed by a single SH2 domain (Lehmann et al., 1990). Nck is ubiquitously expressed, binds to activated PDGF and EGF receptors in an SH2-dependent manner, and becomes phosphorylated on tyrosine, serine and threonine residues (Li et al., 1992, Meisenhelder and Hunter, 1992). Overexpression of *nck* transforms mammalian fibroblasts (Li et al., 1992; Chou et al., 1992), but the function of Nck in normal cellular processes remains to be fully determined. Interestingly, Nck is a nuclear as well as cellular protein, although its subcellular localization remains unaltered in response to growth factor or serum stimulation (Lawe et al., 1997).

Characterization of a viral transforming protein lacking catalytic activity, v-Crk (Matsuda et al., 1990; Mayer and Hanafusa, 1990), resulted in the identification of another cellular adaptor protein, c-Crk. Human Crk exists in two alternatively spliced forms, Crk-1 and Crk-2 (Matsuda et al., 1992). Crk-1

possesses one SH2 and one SH3 domain, while Crk-2 exhibits an SH2-SH3-SH3 organization. These proteins are thought to differ in their biological activity, since Crk-1-transformed fibroblasts grow as colonies in soft agar and form tumors in nude mice, while Crk-2 was only weakly or non-transforming when transfected into fibroblasts (Matsuda et al., 1992). Crk may transduce intracellular signals from Abl nonreceptor tyrosine kinase, as dominant inhibiting Crk mutants blocked activation of the Erk mitogen-activated protein kinase downstream of oncogenic Abl (Tanaka et al., 1995).

In probing for SH2-containing sequences, the *SHC* gene was isolated (Pelicci et al., 1992). *SHC* codes for three overlapping proteins of 46, 52 and 66 kilodaltons. The three Shc proteins share a number of conserved domains, including a carboxy-terminal SH2 domain and a 145 amino acid collagen homology region (Pelicci et al., 1992), as well as a unique amino-terminal phosphotyrosine binding (PTB) domain (Blaikie et al., 1994; Kavanaugh et al., 1994; van der Geer et al., 1995).

Shc proteins appear to be critical to intracellular signaling from RTKs. Upon activation of several RTKs, including EGF receptor (Pelicci et al., 1992), ErbB-2/Neu (Segatto et al., 1993), ErbB-3 (Prigent and Gullick, 1994), PDGF receptor (Yokote et al, 1994), Trk (Stephens et al., 1994) and Ret (Arighi et al., 1997), the widely expressed 46 and 52 kilodalton Shc proteins associate via their SH2 or PTB domains and are phosphorylated on tyrosine 317 (Salcini et al., 1994). This phosphotyrosine residue serves as a high affinity docking site for the SH2 domain of Grb2, and a lower affinity binding site for the SH2 domain of Grb7 (Stein et al., 1994). Shc appears to play a role in growth regulation, as its constitutive expression transformed fibroblasts in culture and formed tumors in nude mice (Pelicci et al., 1992). Overexpression of MMTV/Shc in transgenic mice

produced mammary epithelial hyperplasias, as well as tumors in a small number of multiparous females (V. Blackmore and W.J. Muller, unpublished observations). Further, Shc proteins were shown to be constitutively phosphorylated in a wide range of human carcinoma, melanoma, leukemia and lymphoma cell lines (Pelicci et al., 1995).

1.3 The Grb7 family of SH2 domain proteins

Growth factor receptor-bound protein 7, or Grb7, is a SH2 domain protein of 535 amino acids which was cloned from a T7 polymerase-based expression library using the tyrosine phosphorylated EGF receptor as a probe (Margolis et al., 1992). Grb7 has been demonstrated to belong to a family of proteins that includes Grb10 (Ooi et al, 1995) and Grb14 (Daly et al., 1996). These three SH2 domain proteins contain a central region with similarity to the C. *elegans* gene *mig-10*, a gene that is necessary for the embryonic migration of a subset of C. *elegans* neuronal cells (Ooi et al., 1995). This central region also possesses a conserved pleckstrin homology domain, which is thought to be involved in mediating protein-protein interactions (Touhara et al., 1994; Yao et al., 1994), or alternatively, in binding phosphoinositides (Harlan et al., 1994).

Members of the *grb7* gene family exhibit distinct patterns of expression. The tissue distribution of murine *grb7* mRNA is limited to liver, kidney, ovary and testis (Margolis et al., 1992), while *grb10* expression was found to be abundant in heart and kidney, with some transcript detected in brain and lung (Ooi et al., 1995). *grb14* is more widely expressed: mRNA was strongly expressed in testis, ovary, heart, liver, skeletal muscle, kidney and pancreas (Daly et al., 1996). In

contrast, other adaptor proteins like Nck (Li et al., 1992) and Grb2 (Lowenstein et al., 1992) are ubiquitously expressed. It seems likely that the Grb7 family proteins play more specialized roles in intracellular signaling, with specific family members carrying out distinct, tissue-specific functions (Daly et al., 1996).

A role for Grb7 in signal transduction has been investigated. Grb7 binds weakly to tyrosine phosphorylated Shc (Stein et al., 1994), and binding between Grb7 and the Syp protein tyrosine phosphatase has been demonstrated in a yeast two hybrid screen (Keegan and Cooper, 1996). Grb7 maps on mouse chromosome 11 to a region which also contains the Neu RTK. The homologous human locus, 17q, is often amplified in human breast cancer, leading to ErbB-2 overexpression. ErbB-2 overexpression is common in human cancers, and is correlated with poor patient prognosis (reviewed in Hynes and Stern, 1994). Grb7 binds tightly to Neu/ErbB-2 and is both amplified and overexpressed in human breast cancer cell lines and breast tumors (Stein et al., 1994). While the association between Grb7 and Neu/ErbB-2 in breast tumors suggests a basic signaling pathway involving both proteins, no Grb7 function has been elucidated.

1.4 The Grb2 adaptor protein

Grb2 is the mammalian homologue of the *Caenorhabditis elegans* Sem-5 and *Drosophila melanogaster* Drk proteins (Lowenstein et al., 1992; Clark et al., 1992; Simon et al., 1993; Olivier et al., 1993). These three proteins from distantly related organisms have been extremely well conserved, as evidenced by their degree of protein sequence similarity. 108 residues are identical among all three proteins (47%), and human Grb2 and C. *elegans* Sem-5 share 55% identity at the

amino acid level, while Grb2 and *Drosophila* Drk share 64% identity (Simon et al., 1993). Human and murine Grb2 differ at only one position, where methionine is substituted for valine at residue 154 (Downward, 1994).

Grb2, or growth factor receptor-bound protein 2, was initially isolated based upon its ability to bind to the tyrosine phosphorylated carboxy-terminus of the EGF receptor via its SH2 domain (Lowenstein et al., 1992). The *grb2* gene encodes a ubiquitously expressed polypeptide of approximately 25 kilodaltons that consists of a central SH2 domain flanked by two SH3 domains, and possesses no intrinsic enzymatic activity. Rather, Grb2/Sem-5/Drk seems to function as an adaptor, coupling activated tyrosine kinases to downstream effectors. For example, activation of Grb2 by oncogenic HER2 (Neu/ErbB-2) can occur through either direct binding between the HER2 RTK and Grb2, or indirect binding through the Shc adaptor protein (Hynes and Stern, 1994; Rozakis-Adcock et al., 1992).

Several mechanisms that enable Grb2 to interact with RTKs have been described. Grb2 binds directly via its SH2 domain to specific phosphotyrosine residues within the carboxy-terminus of a number of RTKs, including the EGF receptor (Okutani et al., 1994), Neu/ErbB-2 (Ricci et al., 1995; Dankort et al., 1997), and Met receptor (Maina et al., 1996). Grb2 also interacts indirectly with many RTKs by binding tyrosine phosphorylated Shc (Rozakis-Adcock et al., 1992; Okutani et al., 1994; Prigent and Gullick, 1994; Ricci et al., 1995; Dankort et al., 1997; Lorenzo et al., 1997). Further, the Syp cytoplasmic phosphotyrosine phosphatase mediates indirect binding between the PDGF receptor and Grb2 (Li et al., 1994).

Indirect binding between activated RTKs and Grb2 also involves a family of multifunctional docking proteins. In response to insulin stimulation, insulin

receptor substrate 1 (IRS-1) is phosphorylated on tyrosines and recruits a number of cytoplasmic proteins, including Grb2, in order to link the insulin receptor to intracellular signaling pathways (Skolnik et al., 1993). Similarly, a docking protein termed Grb2-associated binder-1 (Gab1) has been identified. Gab1 is a direct substrate of the insulin and EGF receptors, and interacts with a number of SH2 domain proteins (Holgado-Madruga et al., 1996).

By means of its amino-terminal SH3 domain, Grb2 recruits the guanine nucleotide exchange factor mSos (Sastry et al., 1995), producing heteromeric RTK-Grb2-Sos complexes. mSos stimulates downstream signaling by catalyzing GDP-GTP exchange on Ras (Egan et al., 1993); GTP-bound Ras activates a cascade of protein serine/threonine kinases, leading to the phosphorylation of transcription factors by mitogen-activated protein kinases (Marshall, 1994).

Demonstration of the ability of Grb2 to link activated RTKs to the Ras signaling pathway and stimulate DNA synthesis in cooperation with H-ras (Lowenstein et al., 1992) has led investigators to examine a role for Grb2 in oncogenesis. The *grb2* gene is located at the distal end of chromosome 17q (Huebner et al., 1994), a region duplicated in accelerated chronic myelogenous leukemia (CML) (Mitelman et al., 1991). Grb2 binds directly to phosphotyrosine 177 of the Bcr protein *in vivo* (Ma et al., 1997). Bcr-Abl fusion proteins, which induce oncogenesis in CML and acute lymphoblastic leukemia, bind Grb2 and activate the Ras signaling pathway for transformation of primary bone marrow cultures (Pendergast et al., 1993).

While overexpression of Grb2 in rat fibroblasts is not transforming, a number of human breast cancer cell lines exhibit *grb2* gene amplification, and mRNA and protein overexpression (Daly et al., 1994). Grb2 has been shown to couple the activated ErbB-2 RTK to the Ras signaling pathway in a number of

these breast cancer cell lines (Janes et al., 1994). Furthermore, Grb2 is required for neoplastic transformation induced by IRS-1 overexpression (Tanaka et al., 1996). Taken together, these observations indicate a possible requirement for Grb2 in transmitting oncogenic signals from several sources.

1.5 Grb2 homologues Drk and Sem-5 perform critical intracellular signaling roles in invertebrate development

Grb2 occupies a central position in the chain of protein-protein interactions that leads to the activation of Ras. A large number of cytoplasmic and receptor tyrosine kinases utilize Grb2-Sos to activate Ras, including Abl tyrosine kinase, the EGF receptor and the insulin receptor (Tanaka et al., 1995; Skolnik et al., 1993). It is not surprising that the Grb2 homologues Sem-5 and Drk share a conserved mechanism for activating Ras, and that disruption of this signaling cascade affects cellular processes. *Sem-5* and *drk* play critical roles in C. *elegans* and *Drosophila* development, and it has been shown that alterations in these genes lead to severe developmental defects (Clark et al., 1992; Simon et al., 1993; Olivier et al., 1993).

Vulval cell fate determination in C. *elegans* relies on the action of a number of gene products, many of which are homologues of components of mammalian RTK signaling pathways. Vulval precursor cells express the Let-23 RTK, a member of the EGF receptor family. The cognate ligand for Let-23 is Lin-3, a transforming growth factor-α-like protein expressed on the surface of the adjacent anchor cell. Interaction of Lin-3 with Let-23 initiates a differentiation program via which the precursor cell adopts a vulval cell fate. Downstream of Let-

23 lie Sem-5 and Let-60, which is a C. *elegans* Ras homologue (reviewed in Stern and DeVore, 1994). Mutations in Sem-5 have been shown to inhibit vulval development (Clark et al., 1992). These mutations altered amino acid residues that are highly conserved among SH2 and SH3 domains, suggesting that disruption of SH2 or SH3 domain function might serve to block tyrosine kinase-mediated signaling.

The *Drosophila* compound eye, which consists of approximately 800 identical units termed ommatidia, is another model system that has been used extensively in the study of developmental programs. Each ommatidium contains eight central photoreceptor neurons, designated R1 through R8, as well as four lens-secreting cone cells and eight accessory cells. Within each ommatidium, the R7 photoreceptor cell is the last to commit to the neuronal developmental pathway, and its recruitment has been widely investigated (reviewed in Simon, 1994; Zipursky and Rubin, 1994). During R7 cell development in the fruit fly retina, the Sevenless (Sev) RTK interacts with its ligand, Bride of sevenless (Boss), which is expressed on the surface of the R8 cell. The signal generated results in the activation of Ras1, and development of the precursor cell into an R7 photoreceptor cell. Drk and Son of sevenless (Sos) have been localized between Sev and Ras1 in genetic studies, and are thus implicated in the activation of Ras1 (Downward, 1994). Specifically, constitutive activation of Ras1 can bypass the requirement for Drk in photoreceptor cells (Simon et al., 1993). Conversely, mutations in *drk* reduce or block signaling from the Sev RTK. Although loss of function mutations of *drk* are homozygous lethal, heterozygotes have been utilized to demonstrate that *drk* function is required for signaling from activated Sev (Olivier et al., 1993; Raabe et al., 1995).

These observations from *Drosophila* and C. *elegans* provided insight into the roles of SH2- and SH3-containing adaptor proteins in developmental processes involving tyrosine kinase signaling. It seems likely that similar loss of function mutations in *grb2* might impair signaling from RTKs in mammalian systems. Further, possible therapies for the treatment of human cancers involving activated tyrosine kinases are suggested.

1.6 Transgenic mouse models of breast cancer

Two model systems for exploring the mechanism of mammary tumorigenesis are in use in our laboratory. Transgenic mice expressing the polyomavirus middle T antigen (mT) or the Neu RTK under transcriptional control of the MMTV promoter/enhancer provide animal models for the study of tyrosine kinase-mediated mammary tumorigenesis, as well as the development of secondary metastatic tumors in the lung (Guy et al., 1992a; Guy et al., 1992b). A number of lines of transgenic mice expressing a MMTV/unactivated *neu* construct were shown to develop focal mammary tumors, but only following relatively long periods of latency (Guy et al., 1992b). The elevated Neu tyrosine kinase activity observed in the mammary tumors of these mice was shown to occur by means of somatic mutation within the transgene (Siegel et al., 1994). These mutant *neu* transgenes possessing in-frame amino acid deletions in the extracellular domain of Neu were cloned. Several lines of transgenic mice expressing Neu deletion constructs (NDL) were generated, and found to heritably develop mammary tumors (P.M. Siegel and W.J. Muller, unpublished results).

Upon activation, the Neu RTK becomes phosphorylated on tyrosine residues, providing docking sites for downstream proteins bearing SH2 or PTB domains. Grb2 may interact directly with Neu/ErbB-2, or indirectly via Shc at specific phosphotyrosine residues located within the cytoplasmic carboxy-terminal tail of the receptor (Ricci et al., 1995; Dankort et al., 1997). Mutation of these binding sites negatively affects Neu-mediated transformation (Dankort et al., 1997). Neu/ErbB-2 also directly interacts with a number of other cellular proteins in order to activate a network of intracellular signaling pathways, including other EGF receptor family members, c-Src, phospholipase C-γ, PI3' kinase, phosphatidylinositol 4'-kinase, Grb7, Ras GAP, GAP-associated p190, Shc, Syp, protein tyrosine phosphatase 1C and Eps8 (reviewed in Hynes and Stern, 1994; Dougall et al., 1994).

Transgenic mice carrying the MMTV/mT transgene develop multifocal mammary tumors that involve the entire mammary epithelium (Guy et al., 1992a). Although the mT protein possesses no intrinsic tyrosine kinase activity, members of the c-Src family of cytoplasmic tyrosine kinases (c-Src, c-Yes, Fyn) are recruited and activated (Guy et al., 1994). The subsequent phosphorylation of mT on specific tyrosine residues allows the recruitment of a number of cellular proteins. Shc interacts with mT via a PTB domain-phosphotyrosine interaction that involves tyrosine 250 of mT (Campbell et al., 1994; Dilworth et al., 1994). Shc itself becomes phosphorylated on tyrosine, probably by mT-associated c-Src, enabling Grb2 association and stimulation of Ras via mSos (Rozakis-Adcock et al., 1992; Egan et al., 1993). Therefore, mT and its associated cytoplasmic tyrosine kinase transform cells in a manner functionally analogous to an activated RTK (Dilworth et al., 1994). mT is particularly useful for the study of oncogenic signaling since it activates a limited number of nonredundant pathways. The association of mT with

Src, Shc, PI3' kinase and potentially PLCγ provides a limited number of output pathways for study. Grb2 association with mT is likely limited to indirect binding via Shc, while RTKs may be able to activate Grb2 in several ways, including direct binding and indirect binding via Shc, Gab1, IRS-1 and Syp.

1.7 The role of Grb2 and Grb7 in polyomavirus middle T antigen- and Neu-mediated mammary tumorigenesis

The identification of a class of non-catalytic intracellular signaling molecules, or adaptor proteins, has resulted in many discoveries regarding signaling from activated tyrosine kinases. Since tyrosine kinase signaling is thought to be involved in several human cancers, both tyrosine kinases and components of downstream signaling cascades represent possible therapeutic targets. It is important for investigators to determine whether or not modulating the levels of signaling molecules can have positive or negative effects on tumor initiation, development and metastasis. To that end, I have studied the effects of modulating the mammary epithelial levels of Grb2 and Grb7 on tumorigenesis in transgenic mice.

To explore whether the dosage of Grb2 is important in middle T- or Neu-mediated mammary tumorigenesis, separate strains of MMTV/mT and MMTV/NDL mice have been mated with knockout mice lacking one copy of *grb2*. Mice completely lacking Grb2 are inviable, but Grb2+/- animals are viable and exhibit apparently normal growth and development (A. Cheng and T. Pawson, unpublished results). The female progeny of mT transgenic X Grb2+/- matings

were monitored for the onset of palpable mammary tumors. The results showed that mT^{+};$Grb2^{+/-}$ mice developed tumors at a slower rate than did mT^{+};$Grb2^{+/+}$ animals. A clear difference in tumor load between age-matched animals can also be observed at different time intervals. These results suggest that the Shc-Grb2-Sos-Ras pathway may be important for mT-mediated tumorigenesis.

Another means to study the role of Grb2 in mammary tumorigenesis involves introducing a dominant negative *grb2* gene into the mammary gland. Since *grb2* knockout mice are inviable, the previously described breeding experiments have used $Grb2^{+/-}$ animals, which all express the endogenous *grb2* gene. The use of interfering mutant Grb2 proteins may provide a more efficient method for blocking Grb2 function *in vivo*. To meet this objective, transgenic mice carrying a dominant acting mutant *grb2* gene under control of the MMTV promoter/enhancer have been generated (P. Siegel and W.J. Muller, unpublished results). A point mutation resulting in a proline to leucine mutation in the amino terminal SH3 domain of *grb2* renders the gene product Grb2-P49L incapable of interacting with mSos (Lowenstein et al., 1992); this mutation mimics a *sem-5* alteration that inhibits vulval development described by Clark et al. (1992). I have characterized one line of mice expressing this transgene, and mated these Grb2-P49L-1 mice with mT and NDL transgenic mice to assess the ability of Grb2-P49L to act as a dominant inhibiting mutant. Bigenic progeny coexpressing Grb2-P49L with mT or Neu in the mammary epithelium did not appear to differ from MMTV/mT or MMTV/NDL siblings in terms of mammary tumor development.

In addition, transgenic mice carrying the wild-type *grb2* cDNA under MMTV control have been generated. (L. Wei, P. Siegel and W.J. Muller, unpublished results). The expression pattern of the transgene has been determined for nine lines of mice, and lines expressing the transgene have been

monitored for mammary gland abnormalities. Overexpression of Grb2 does not appear to lead to mammary gland hyperplasia or neoplasia. Mating MMTV/Grb2 mice with MMTV/mT or MMTV/NDL transgenic mice should further reveal the role of Grb2 in tyrosine kinase-mediated tumorigenesis.

The observed interaction between Grb7 and Neu/ErbB-2 in human breast cancer cell lines and primary tumor samples (Stein et al., 1994) is also being investigated. Transgenic mice carrying the MMTV/Grb7 transgene have been generated (P. Siegel and W.J. Muller, unpublished results). Seven lines of Grb7 mice have been screened for transgene expression, and monitored for the development of mammary gland abnormalities. While overexpression of Grb7 in the mammary epithelium does not lead to any obvious abnormalities, mating MMTV/Grb7 and MMTV/NDL transgenic mice should help to determine the effects of overexpressing Grb7 on Neu-mediated mammary tumor development.

Chapter 2: Materials and Methods

2.1 DNA constructs

The pMMTV/Grb2 and pMMTV/Grb2-P49L constructs were kindly provided by R. Daly. Briefly, the plasmids were derived by inserting an *Eco* RI fragment possessing the wild type or mutant Grb2 cDNA into an MMTV LTR expression vector, p206. The MMTV component of p206 was derived from plasmid pA9 (Huang et al., 1981), while the SV40 transcriptional processing signals at the 3' end of the cDNA were obtained from plasmid CDM8 (Seed et al., 1987). The plasmids as received required modification, and were recloned by P. Siegel. The cDNA was sequenced to verify the mutation.

The Grb7 cDNA, provided by B. Margolis, was cloned into the p206 MMTV LTR expression vector by D. Dankort.

pSP65mT(HA), the polyomavirus middle T antigen riboprotection probe obtained from J. Hassell, contains a 203 bp *Hind* III/*Acc* I fragment of the polyomavirus early region (polyomavirus nucleotides 165 to 368) inserted into the *Hind* III/*Acc* I sites of pSP65 (Promega Biotech, Madison, Wisconsin).

The PGK-1 internal control plasmid was obtained from M. Rudnicki, and was generated by inserting an *Acc* I/*Pst* I fragment (nucleotides 939 to 1633) (Mori et al., 1986) into the *Pst* I site of pSP64 (Promega).

The plasmid pASV, which was assembled in order to produce a riboprobe to detect the SV40 polyadenylation signal present in the transgenes, was generated by W. Muller as described previously (Muller et al., 1988).

Plasmid pBS-Si, utilized for detecting Grb2 heterozygotes, was obtained from T. Pawson.

2.2 Generation and identification of transgenic and knockout mice

The MMTV/Grb2-P49L transgenic mice were generated by P. Siegel, while the MMTV/Grb2 mice were generated by L. Wei. DNA was first prepared for microinjection by digestion with *Sal* I and *Spe* I; the fragment was purified as described previously (Sinn et al., 1987), and transgenic animals were derived by microinjection as described by Guy et al. (1992a).

The MMTV/Grb7 transgenics were derived by P. Siegel in a similar manner, with the injection fragment being released via a *Sal* I/*Apa* LI digestion. The polyomavirus middle T antigen transgenic line, mT634, was generated as previously described (Guy et al., 1992a).

MMTV/NDL1-2 transgenic mice express an altered Neu cDNA which possesses a deletion in the extracellular domain proximal to the transmembrane domain. These transgenic animals were also generated by P. Siegel, utilizing the microinjection method described by Guy et al. (1992a) (P. Siegel and W.J. Muller, unpublished results).

Mice heterozygous for the *grb2* gene ($Grb2^{+/-}$) were generated by homologous recombination in embryonic stem cells, and kindly provided by T. Pawson. To identify $Grb2^{+/-}$ progeny, genomic DNA was extracted from a 0.5 cm tail clipping by overnight digestion in PK buffer (10 mM Tris-Cl [pH 8.0]-100 mM NaCl-10 mM EDTA [pH 8.0]-0.5% SDS) containing 0.2 mg/ml proteinase K (Canadian Life Technologies, Burlington, Ontario). Nucleic acids were isolated by

successive buffer saturated phenol and chloroform extractions, and precipitated in two volumes of absolute ethanol. The nucleic acid pellet was resuspended in 50 υl of 10 mM Tris [pH 7.5]-1 mM EDTA [pH 8.0], and 15 υl of the DNA solution was digested with 30 U of *Bam* HI for 1.5 hours at 37°C. Digested DNA was electrophoresed through a 1.0% agarose gel, and the gel was denatured under constant shaking for 40 minutes in 600 mM NaCl-200 mM NaOH and neutralized under constant shaking for 40 minutes in 600 mM NaCl- 1 M Tris (pH 7.6). Following Southern blot transfer (Southern, 1975), DNA was cross-linked to GeneScreen filters (Mandel Scientific, Guelph, Ontario) using a UV Stratalinker (Stratagene, La Jolla, California), and the filters were hybridized overnight at 60°C with the Grb2 genomic probe. In order to generate a probe to identify $Grb2^{+/-}$ animals, plasmid pBS-Si (Grb2) was digested with *Xba* I and *Eco* RI. The resulting 400 bp fragment was gel purified and radiolabelled with [α^{32}P]-dCTP (Mandel Scientific) by random priming. The next day, filters were washed once for 20 minutes at room temperature in 150 mM NaP-1%SDS, and once for 20 minutes at 60°C in the same buffer. Filters were air dried, and labelled probe/restriction fragment hybrids were detected by autoradiography using Kodak X-OMAT AR film (Eastman Kodak Company, Rochester, New York). The 12 kb recombinant band could be clearly distinguished from the 16 kb wild-type band.

Transgenic animals were also identied by Southern blot analysis. Genomic DNA was isolated from tail clippings as described previously, and 15 υl of the DNA solution was digested with 30 U of *Bam* HI for 1.5 hours at 37°C. Digested DNA was electrophoresed through a 1.0% agarose gel and denatured and neutralized as described above. Following Southern blot transfer (Southern, 1975) and cross-linking, filters were hybridized with a [α-^{32}P]-CTP labelled SV40 polyadenylation probe - the 750 bp probe fragment was released from plasmid

pSPA (SV40 polyadenylation sequence in pBluescript KS) by a combined *Bam* HI/*Eco* RI digest. Labelled fragments were detected by autoradiography using Kodak X-OMAT AR film (Eastman Kodak).

Alternatively, $Grb2^{+/-}$ and transgenic animals were identified by polymerase chain reaction (PCR) analysis, using oligonucleotide primers that amplified the inserted neo gene sequence ($Grb2^{+/-}$) or cDNA sequences (transgenic animals). For PCR analysis, genomic DNA was extracted from tail clippings as described above. 1 υl of DNA solution was amplified in a reaction solution that included 1 U Taq DNA polymerase (Canadian Life Technologies), 500 υM deoxynucleoside triphosphate mixture (dATP, dCTP, dGTP, dTTP), 3 mM $MgCl_2$ and oligonucleotide primers at a concentration of 1 υM.

2.3 RNA extraction and ribonuclease protection assays

Total RNA was isolated from tissues by guanidinium isothiocyanate extraction followed by cesium chloride gradient fractionation (Chirgwin et al., 1979). Pieces of tissue or whole small organs were homogenized in 3 ml of 4 M guanidinium isothiocyanate-25 mM sodium citrate-100 mM 2-mercaptoethanol, and either stored at -80°C or loaded immediately onto a 4.0 ml cesium chloride cushion (5.7 M CsCl-25 mM sodium acetate (pH 5.0)). Samples were centrifuged at 32 000 rpm for 18 hours at 20°C using a Beckman SW41.Ti swinging bucket type rotor (VWR Scientific, Toronto, Ontario). Following centrifugation, the supernatant was removed by aspiration and the RNA pellet was resuspended in 500 υl of diethyl pyrocarbonate (Sigma-Aldrich Canada, Oakville, Ontario) treated sterile water, and RNA was precipitated at -20°C by adding 50 υl of 3 M sodium

acetate and 1 ml of absolute ethanol. After resuspension in 100 υl of diethyl pyrocarbonate treated sterile water, RNA yield was determined by UV absorption at 260 nm.

To generate the antisense riboprobes, the template plasmids were digested using restriction endonucleases and the appropriate fragments utilized for *in vitro* transcription reactions (Melton et al., 1984). The antisense mT riboprobe was generated by linearizing the pSP65mT template plasmid (described above) with *Hind* III and transcribing with SP6 RNA polymerase. The PGK-1 internal control probe was produced by digesting the template plasmid (described above) with *Eco* NI, isolating the 640 bp fragment, and transcribing with SP6 RNA polymerase. Similarly, the SV40 polyadenylation (SPA) riboprobe was synthesized by linearizing the pASV template plasmid (described above) with *Hind* III and transcribing with SP6 RNA polymerase.

To carry out *in vitro* transcription reactions, 1 υg of template DNA was incubated with 30 U of SP6 RNA polymerase (Canadian Life Technologies) for 1.5 hours at 37°C in the presence of 1X SP6 transcription buffer-400 υM rATP/rCTP/rGTP-40 υM rUTP-8 mM dithiothreitol-1 υl RNAguard RNAase inhibitor (Pharmacia Biotech Canada, Baie D'Urfe, Quebec)-100 υCi [α-^{32}P] rUTP (Mandel Scientific). Samples were chilled on ice and subjected to digestion with 20 U of RNase-free DNase I (Boehringer Mannheim Canada, Laval, Quebec) in the presence of 10 mM $MgCl_2$ for 10 minutes at 37°C. Following phenol:chloroform extraction and ethanol precipitation, antisense probes were resuspended in 100 υl of diethyl pyrocarbonate treated sterile water.

Ribonuclease protection assays were performed by overnight hybridization of the various probes to 20 υg of total RNA in the presence of 1X PIPES buffer (40 mM piperazine-N,N'-bis[2-ethanesulfonic acid] [pH 6.4]-1 mM

EDTA [pH 8.0]-400 mM NaCl) and 80% deionized formamide. The next day, samples were subjected to ribonuclease digestion at room temperature for 30 minutes with 30 υg/ml RNase A (Sigma-Aldrich) and 2 υg/ml RNase T1 (Sigma-Aldrich) in digestion buffer (300 mM NaCl-10 mM Tris [pH 7.4]-5 mM EDTA [pH 7.5]). The enzymes were denatured using 100 υg proteinase K and 0.5% SDS, and the samples subjected to a phenol:chloroform extraction. Following ethanol precipitation, pellets were resuspended in ribonuclease protection sample loading buffer (80% formamide-10 mM EDTA [pH 8.0]-1 mg/ml xylene cyanol FF-1 mg/ml bromophenol blue), and denatured at 90°C for 5 minutes. Protected fragments were separated on urea-6% polyacrylamide gels. Gels were dried 1-2 hours at 80°C, and protected fragments were detected by autoradiography using Kodak X-OMAT AR film (Eastman Kodak) or PhosphorImager analysis (Molecular Dynamics, Sunnyvale, California).

2.4 Immunoprecipitation and *in vitro* kinase analyses

Tissue samples were flash frozen in liquid nitrogen and stored at -80°C until use. Frozen tissues were ground to a powder under liquid nitrogen, and lysed for 30 minutes on ice in TNE lysis buffer (50 mM Tris HCl [pH 8.0]-150 mM NaCl-1% Nonidet P-40-2 mM EDTA-1 mM sodium orthovanadate-2 mM dithiothreitol-10 υg/ml leupeptin-10 υg/ml aprotinin). The lysates were cleared by centrifugation at 13000 rpm for 15 minutes at 4°C. The protein concentrations of the whole tissue extracts were measured using the Bio-Rad protein assay (Bio-Rad Laboratories, Mississauga, Ontario). Immunoprecipitations were performed by incubating equal amounts of protein (500 υg) with an excess of anti-mT

antibodies (MAb 762) (Dilworth et al., 1994) and approximately 25 υg of protein G-Sepharose fast flow (Pharmacia) for 8 hours at 4°C on a rotating platform. mT immunoprecipates were washed three times with TNE lysis buffer and twice with 2X kinase buffer (20 mM HEPES [pH 7.0]-10 mM $MnCl_2$), and utilized in an enolase assay. The beads were resuspended in 9 υl of 2X kinase buffer, and 10 υCi of [γ^{32}P]- ATP and 10 υl of acid denatured enolase were added. Following a 5 minute incubation at room temperature, 20 υl of SDS gel loading buffer (625 mM Tris-HCl [pH 6.8]-2% SDS-5% glycerol-0.7 M 2-mercaptoethanol-0.25% bromphenol blue) were added, and proteins were electrophoresed on SDS-10% polyacrylamide gels. Gels were fixed in 30% methanol/7% acetic acid for 30 minutes, incubated 35 minutes at 45°C in 1M KOH, and incubated for 45 minutes in 30% methanol/7% acetic acid (several changes). Gels were dried 40 minutes at 80°C, and radiolabelled enolase was detected by autoradiography using Kodak X-OMAT AR film (Eastman Kodak).

2.5 Histological evaluation

Complete autopsies were performed as described by Muller et al. (1988). Tissues were fixed in 4% paraformaldehyde, and paraffin blocking, sectioning and hematoxylin and eosin staining were performed by Pathology Research Services, Chedoke-McMaster Hospital. To produce whole mounts, mammary glands were removed from the inner surface of the skin, retaining some peripheral connective tissue. The glands were spread on glass slides and allowed to air dry overnight. Glands were fixed and defatted overnight in acetone, then squashed between two slides and returned to acetone for two additional hours before staining, in order to

enhance the defatting process. Slides were stained in Harris modified hematoxylin (Fisher Scientific Canada, Nepean, Ontario), and destained with three or four changes of 2% hydrochloric acid/70% ethanol. Following a brief (30 to 40 second) wash in ammonium hydroxide, slides were transferred to 70% ethanol for several hours, followed by a transfer to 100% ethanol for several hours. Tissues were cleared overnight in xylenes (Caledon Laboratories, Georgetown, Ontario), mounted in Permount (Fisher Scientific) and covered with a coverslip. The images for Figures 3-2 and 3-3 were generated in the laboratory of Dr. Robert Cardiff at the University of California, Davis, using a Kontronics camera model 8012 on an Olympus BH2 microscope, and digitized using Photoshop 4.0. Whole mount images were captured at a magnification of 4x, while histological images were captured at a magnification of 10x.

2.6 Isolation of primary mammary epithelial cells

Mammary tumors were dissected from twelve to fourteen week old $mT^{+};Grb2^{+/+}$ and $mT^{+};Grb2^{+/-}$ female mice, and transferred to petri dishes containing 25 ml of sterile phosphate-buffered saline (PBS) and allowed to stand for a few minutes. The tissue was then transferred to a second dish containing sterile PBS, and cells were released by mincing and scraping with a scalpel. This cell suspension was added to 25 ml of 2X collagenase/dispase (0.5 mg/ml collagenase [Boehringer Mannheim], 5 mg/ml dispase [Boehringer Mannheim] in PBS) and stirred vigorously at 37°C for 30 minutes. Cells were washed twice in 50 ml of PBS, and plated in Dulbecco's modified Eagle medium (Gibco BRL) with 10% fetal bovine serum, 50 υg/ml gentamycin and fungizone.

Chapter 3: Results

Grb2 is involved in oncogenic signaling

Grb2 is one of a number of SH2 domain containing proteins that interacts with tyrosine phosphorylated receptor tyrosine kinases within cells. Grb2 couples RTKs to the Ras signaling pathway, resulting in the stimulation of DNA synthesis (Lowenstein et al., 1992). Since Grb2 occupies a central position in mitogenic signaling, several investigators have examined a role for Grb2 in oncogenesis. Localization of the *grb2* gene to the distal end of chromosome 17q - a region duplicated in chronic myelogenous leukemia - and positioning of Grb2 between the Bcr-Abl oncoprotein and Ras indicated a possible role for Grb2 in leukemia initiation or progression (Huebner et al., 1994; Mitelman et al., 1991; Pendergast et al., 1993).

Although overexpression of Grb2 in rat fibroblasts is not transforming, several human breast cancer cell lines exhibit *grb2* gene amplification, and mRNA and protein overexpression (Daly et al., 1994). Furthermore, Grb2 has been demonstrated to link the activated ErbB-2/Neu RTK to the Ras signaling pathway in a number of these breast cancer cell lines (Janes et al., 1994). Based upon these results, it seemed likely that Grb2 was involved in transmitting oncogenic signals in tyrosine kinase mediated mammary tumorigenesis. In previous studies the polyomavirus middle T antigen, which recruits and activates members of the c-Src family of cytoplasmic tyrosine kinases, has been used to model tyrosine kinase induced mammary tumorigenesis. A requirement for Grb2

has been examined in transgenic mice by modulating the levels of Grb2 available in mammary epithelial cells transformed by middle T antigen.

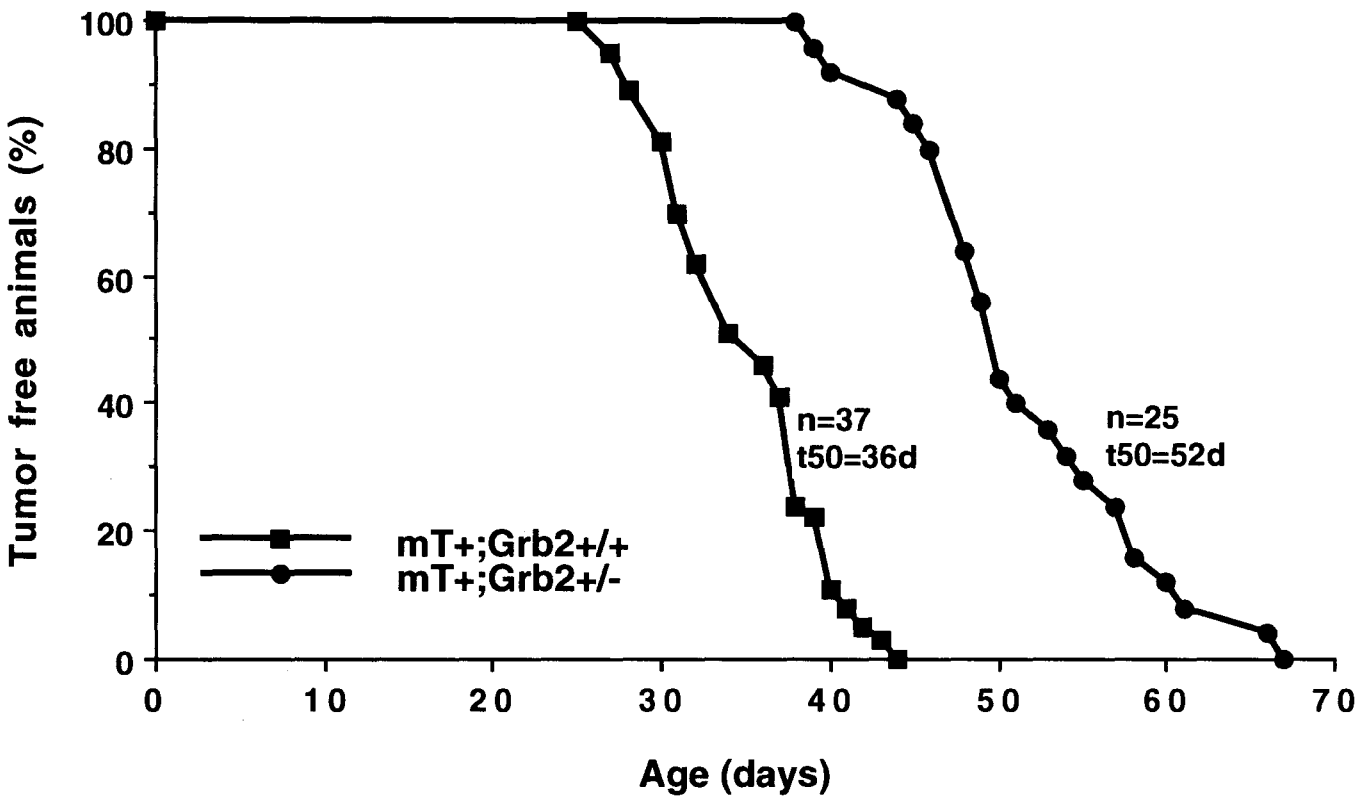
3.1 Delayed kinetics of tumor formation in mT transgenic animals heterozygous for *grb2*

To examine the role of Grb2 in polyomavirus mT antigen-mediated transformation, mice heterozygous for *grb2* ($Grb2^{+/-}$) were mated with MMTV/mT634 transgenic mice. The mT634 line has been well characterized with respect to transgene expression, the onset of tumor formation, and induction of metastatic disease (Guy et al., 1992a). Specifically, female mT634 transgenic animals heritably develop multifocal mammary adenocarcinomas, which are palpable in carriers by approximately five weeks of age. Approximately 94% of these tumor-bearing females developed detectable lung metastases by three months of age. The female progeny of mT634 X $Grb2^{+/-}$ matings were monitored for the development of palpable mammary tumors (Figure 3-1). The age at which fifty percent of virgin female $mT^{+};Grb2^{+/+}$ animals displayed palpable mammary tumors (t_{50}) was 36 days (n=37). Surprisingly, $mT^{+};Grb2^{+/-}$ females developed palpable mammary tumors with a t_{50} of 52 days. Despite the ability of mT antigen to activate a number of intracellular signaling pathways, it appears that the mT-Shc-Grb2-Sos-Ras pathway may be important for mT-mediated tumorigenesis.

The analysis of mammary gland whole mounts also revealed differences in tumor development. By comparison with wild-type mammary tissue, it was observed that mammary development in age-matched six week old $Grb2^{+/-}$

Figure 3-1: Kinetics of tumor occurrence of MMTV/mT634;Grb2$^{+/+}$ and MMTV/mT634;Grb2$^{+/-}$ mice.

Comparison of the kinetics of tumor formation between virgin female MMTV/mT634 transgenic carriers that were homozygous or heterozygous for the Grb2 gene. The age at which 50% of mice were found to have tumors (t_{50}) and the number of mice examined (n) are indicated.

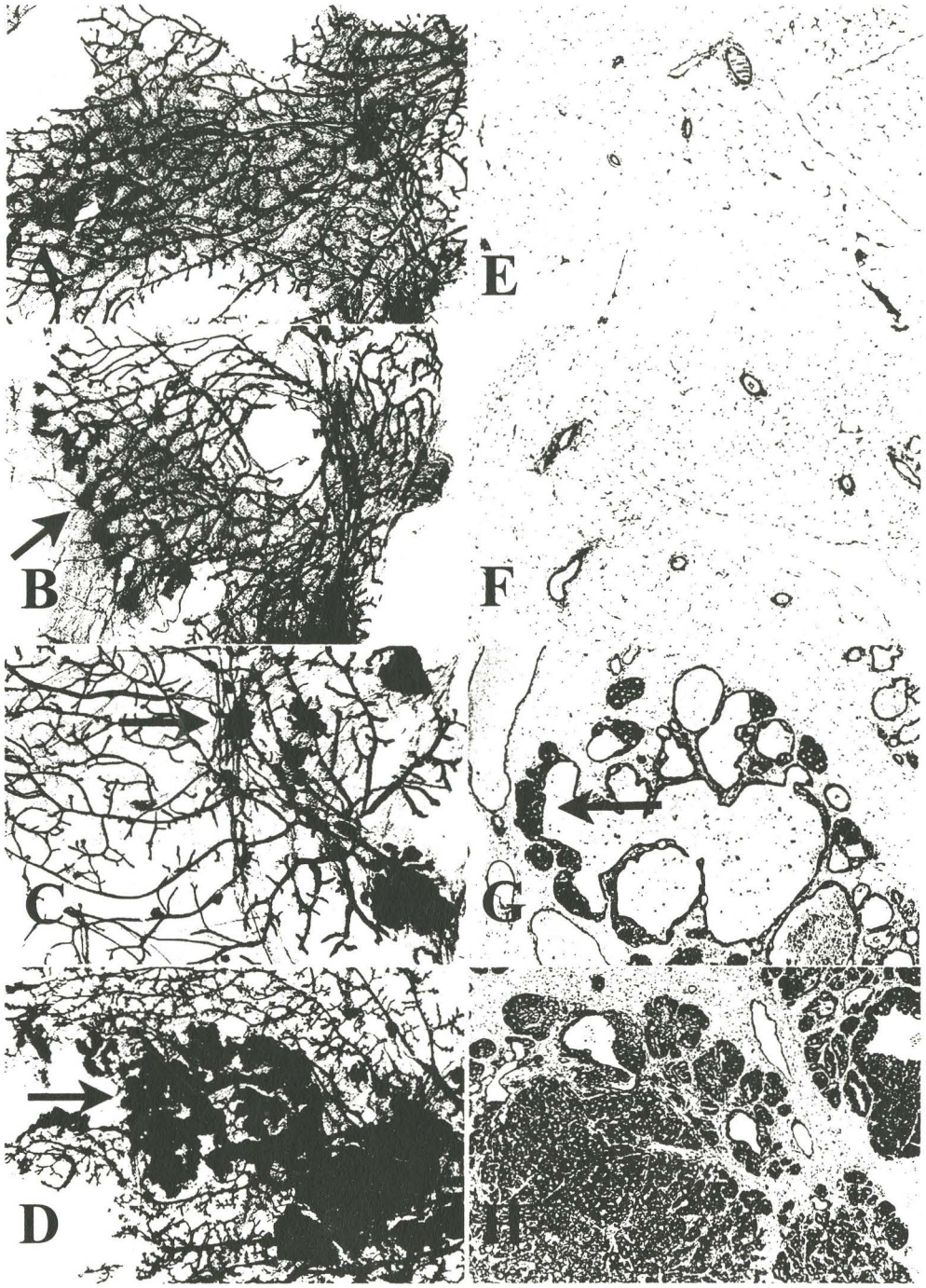

Tumor free animals (%)
100
80
60
40
20
0
0
10
20
30
40
50
60
70
Age (days)
n=37
t50=36d
n=25
t50=52d
mT+;Grb2+/+
mT+;Grb2+/-

females appears relatively normal (Figure 3-2A,B). While growth in the mammary tree from the $Grb2^{+/-}$ mouse has not filled the entire fat pad, the development of branching ducts, but small number of secondary and tertiary branches, is typical of the virgin mammary gland. This provides evidence that the observed tumor kinetics differences between $mT^{+};Grb2^{+/+}$ and $mT^{+};Grb2^{+/-}$ animals were likely not due to developmental defects in the $Grb2^{+/-}$ animals. The $mT^{+};Grb2^{+/+}$ glands exhibited fully developed mammary trees. Multiple focal lesions and abnormal branching of the mammary ducts can be observed (Figure 3-2D). These animals also frequently displayed pulmonary metastases (not shown). While whole mounts from $mT^{+};Grb2^{+/-}$ females also possessed dilated ducts and scattered small focal lesions (Figure 3-2C), the duct architecture with relatively few side branches more closely resembled the wild-type pattern. It seems likely that mammary tumors are initiated in the same manner in both MMTV/ $mT634^{+};Grb2^{+/+}$ and MMTV/$mT634^{+};Grb2^{+/-}$ animals, however abnormal growth is impaired in $Grb2^{+/-}$ mice.

Histological examination of mammary gland thin sections further revealed the differences in mT-mediated mammary tumor development. The endothelium that lines mammary ducts of both wild-type and $Grb2^{+/-}$ females normally consists of a single layer of mammary epithelial cells (Figure 3-2E,F). Age-matched $mT^{+};Grb2^{+/-}$ mice did not exhibit palpable mammary tumors, but did display some proliferation of the mammary epithelium, as evidenced by the multiple layers of dysplastic epithelium lining some of the cystic ducts and lobules (Figure 3-2G). Strikingly, $mT^{+};Grb2^{+/+}$ mice exhibited clinically visible mammary tumors, and normal mammary gland architecture was obscured by layers of dysplastic epithelium (Figure 3-2H).

Figure 3-2: Mammary gland whole mount and histological analysis of MMTV/mT634 X Grb2$^{+/-}$ mice.

Prints showing whole mount (A-D) (Magnification 5X) and histological (E-H) (Magnification 40X) images of mammary gland six weeks after birth. Mammary glands displayed were obtained from wild type (A and E), knockout Grb2+/- (B and F), transgenic Grb2+/- X mT634 (C and G) and transgenic mT634 (D and H) mice. Note that the growth in the mammary tree from the Grb2+/- mouse (B and F) has not filled the entire fat pad, has actively growing terminal buds (arrow) and is retarded in relation to the wild type (A) which has filled the fat pad and is quiescent. However, the mammary gland is histologically normal (E and F). The Grb2 animals crossed mT transgenics have fully developed mammary trees with dilated ducts with relatively few side branches and scattered small focal lesions (arrow) (C). Some of the cystic ducts and lobules are lined by multiple layers of dysplastic epithelium (arrow) (G). In contrast, the gland from the 6 week old mT transgenic mouse is fully developed with multiple focal lesions (arrow) (D) that are filled with dysplastic epithelium (H) and pulmonary metastases (not shown).


A
B
C
D
E
F
G
H

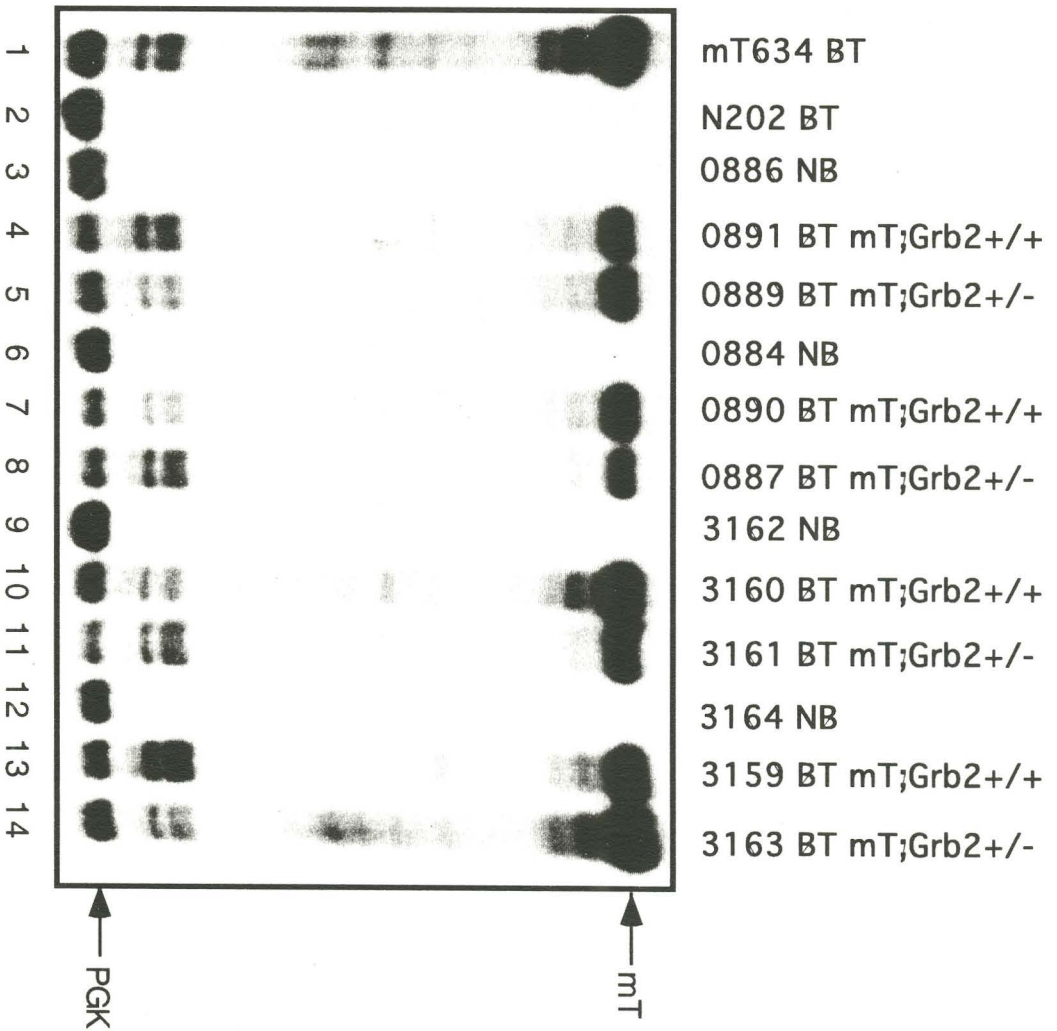
3.2 Polyomavirus middle T antigen expression and function in $Grb2^{+/+}$ and $Grb2^{+/-}$ mice

To ensure that differences in tumor kinetics were not due to altered MMTV/mT634 transgene expression, ribonuclease protection assays were carried out (Figure 3-3). 20 υg of total RNA from female animals of the various genotypes was hybridized with a transgene-specific probe that yields a 203-base protected fragment corresponding to the 5' portion of the mT cDNA. To ensure that approximately equal amounts of RNA from all tissue samples were analyzed, an antisense probe generated from the mouse phosphoglycerate kinase cDNA (PGK-1) was included in each reaction as an internal control. Extracts from wild-type mammary glands (NB) did not exhibit transgene expression (Figure 3-3, lanes 3,6,9,12). Approximately equal amounts of transgene transcript were detected in age-matched breast tumors (BT) from $mT^{+};Grb2^{+/+}$ and $mT^{+};Grb2^{+/-}$ mice (Figure 3-3, compare lanes 4 and 5, 7 and 8, 10 and 11, 13 and 14). After normalizing to PGK-1 levels, quantitation by PhosphorImager analysis confirmed that no significant differences in transgene levels could be detected between $mT^{+};Grb2^{+/+}$ and $mT^{+};Grb2^{+/-}$ breast tumors. RNA samples derived from mT634 and Neu breast tumors were included as positive and negative controls, respectively, and are shown in lanes 1 and 2 of Figure 3-3.

The polyomavirus middle T antigen interacts with c-Src family cytoplasmic tyrosine kinases, and the rapid induction of metastatic tumors in mT634 transgenic mice requires a functional c-Src (Guy et al., 1994). In order to confirm that the mT protein was functioning normally in $Grb2^{+/-}$ animals, the mT/Src family tyrosine kinase interaction was examined. Protein extracts from tumor tissue derived from age-matched $mT^{+};Grb2^{+/+}$ and $mT^{+};Grb2^{+/-}$ mice were immunoprecipitated with

Figure 3-3: RNase protection analysis of expression of MMTV/mT634 transgene RNA in the mouse mammary epithelium.

Tissues were derived from virgin female mice 10 to 12 weeks of age. Total RNA was isolated from both normal breast tissue (NB) and breast tumor tissue (BT) and analyzed. The antisense probe used in this RNase protection analysis protects a 203-nucleotide fragment indicated by mT and an arrow. An antisense probe, directed against mouse phosphoglycerate kinase (PGK-1) RNA, was utilized to control for equal RNA loading. The 124-nucleotide protected fragment is indicated by PGK and an arrow. RNA samples from MMTV/mT634 and MMTV/*neu* (N202) breast tumors were included as positive and negative controls, respectively.


1 2 3 4 5 6 7 8 9 10 11 12 13 14
mT634 BT
N202 BT
0886 NB
0891 BT mT;Grb2+/+
0889 BT mT;Grb2+/-
0884 NB
0890 BT mT;Grb2+/+
0887 BT mT;Grb2+/-
3162 NB
3160 BT mT;Grb2+/+
3161 BT mT;Grb2+/-
3164 NB
3159 BT mT;Grb2+/+
3163 BT mT;Grb2+/-
PGK
mT

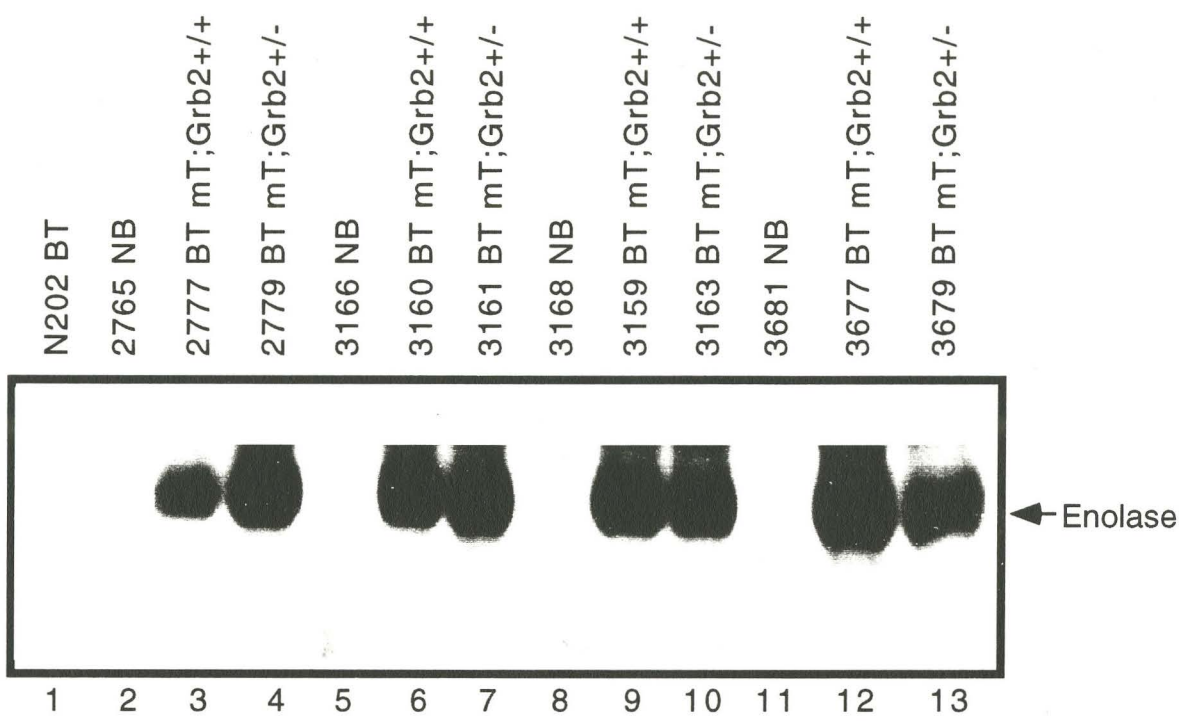
antibodies directed against mT, and *in vitro* kinase assays using acid denatured enolase as a substrate were performed (Figure 3-4). A prominent phosphorylated band corresponding to enolase was observed in mT634 tumor extracts, while incubation of a Neu tumor extract with the mT-specific antibody (Ab762) did not result in phosphorylation on enolase (Figure 3-4, lane 1). No clear difference between $Grb2^{+/+}$ and $Grb2^{+/-}$ mT tumors with respect to phosphorylation on enolase can be discriminated (Figure 3-4, compare lanes 3 and 4, 6 and 7, 9 and 10, 12 and 13). Quantitation by PhosphorImager analysis confirmed that no significant differences between the tumor extracts in terms of phosphorylation on enolase can be detected, although sample to sample variation can be observed. These results indicate that the mT antigen is likely functioning normally in $Grb2^{+/+}$ and $Grb2^{+/-}$ mice, by recruiting Src family tyrosine kinases for the subsequent activation of downstream signaling pathways.

3.3 Proliferative and metastatic abilities of mammary tumor cells

The mechanism by which decreased Grb2 dosage affects tumor growth and development remains unclear. It is possible that a decrease in Grb2 protein levels might simply slow the rate of proliferation of tumor cells by decreasing the proportion of GTP-bound Ras in these actively growing cells. To assess their proliferative ability, mammary epithelial cells from both $mT^{+};Grb2^{+/+}$ and $mT^{+};Grb2^{+/-}$ tumors have been established in culture. Attempts to derive primary cell lines have proved difficult, as $Grb2^{+/-}$ mammary epithelial cells proliferate slowly after isolation, and usually fail to proliferate after trypsinization. To circumvent the problems associated with manipulation of the $Grb2^{+/-}$ tumor cells,

Figure 3-4: Polyomavirus middle T antigen associated kinase activity in tumor and normal mammary epithelium.

Protein extracts from mammary tumors (BT) and control tissues (NB) derived from the MMTV/mT634 X Grb2$^{+/-}$ mice were immunoprecipitated with a mT-specific monoclonal antibody (MAb762) and subjected to *in vitro* kinase assays with exogenous enolase substrate. Included as a negative control is a tissue extract from a MMTV/*neu* tumor (lane 1). The location of the enolase substrate is indicated by an arrow.


N202 BT
2765 NB
2777 BT mT;Grb2+/+
2779 BT mT;Grb2+/-
3166 NB
3160 BT mT;Grb2+/+
3161 BT mT;Grb2+/-
3168 NB
3159 BT mT;Grb2+/+
3163 BT mT;Grb2+/-
3681 NB
3677 BT mT;Grb2+/+
3679 BT mT;Grb2+/-
Enolase
1 2 3 4 5 6 7 8 9 10 11 12 13

mixed cellular populations from $mT^{+};Grb2^{+/+}$ and $mT^{+};Grb2^{+/-}$ tumors were seeded directly into 24 well tissue culture dishes for the purpose of performing ^{3}H-thymidine incorporation assays. The levels of incorporation varied widely between tumors of the same genotype, as well as between wells representing cells from the same tumor. These variations were likely due to cell death versus proliferation between the various wells.

The metastatic potential of cells from mT634-mediated $Grb2^{+/+}$ and $Grb2^{+/-}$ tumors was also assessed. The lungs of animals from both sets of mice were examined for the appearance of pulmonary metastases. Preliminary results indicate that mT RNA can be detected in the lungs of 12 to 13 week old $mT^{+};Grb2^{+/+}$ female mice, but not in 10 week old mice of the same genotype (Figure 3-5). In contrast, mT RNA was only detected in a single lung sample from $mT^{+};Grb2^{+/-}$ mice. These observations were confirmed following histological examination of lung sections taken from the same mice (Table 3-1). Tumor-bearing $mT^{+};Grb2^{+/+}$ female mice frequently possessed histologically visible lung metastases at 12 weeks of age, while their $mT^{+};Grb2^{+/-}$ counterparts did not.

3.4 Expression of the MMTV/Grb2-P49L transgene and its role in MMTV/mT634 tumor development

In attempting to uncover a more efficient method for blocking Grb2 function *in vivo*, transgenic mice carrying a dominant acting mutant *grb2* gene under the control of the MMTV promoter/enhancer have been generated (P. Siegel and W.J. Muller, unpublished results). A total of two transgenic founders carrying the MMTV/Grb2-P49L transgene have been generated to date. Of these two strains,

Figure 3-5: Detection of pulmonary metastases in lung tissue from MMTV/mT634 x Grb2+/- mice.

Pulmonary metastases were detected by ribonuclease protection in lung samples derived from 10 to 13 week old mT634 X Grb2+/- mice. The antisense probe used in this analysis protects a 203-nucleotide fragment, and is indicated by mT and an arrow. An antisense riboprobe that protects a 124-nucleotide fragment of the mouse phosphoglycerate kinase 1 RNA was included as a RNA loading control, and is indicated by PGK and an arrow. Genotypes of the individuals tested are indicated above each lane. The ear tag numbers of the mice examined were 0886, 0891, 0889, 0888, 0890, 0887, 3162, 3160, 3161, 3164, 3159, 3163, 2778, 2777, and 2779 for lanes 1 through 15, respectively. No mT expression was detected in any wild-type samples (lanes 1, 4, 7, 10 and 13), nor was any mT expression detected in 10 week old mice (lanes 1-6). Expression was largely restricted to 12 week old or older, mammary tumor-bearing mT+;Grb2+/+ animals (lanes 8, 11, 14), although a faint signal was detected from a single mT+;Grb2+/- sample (lane 12).

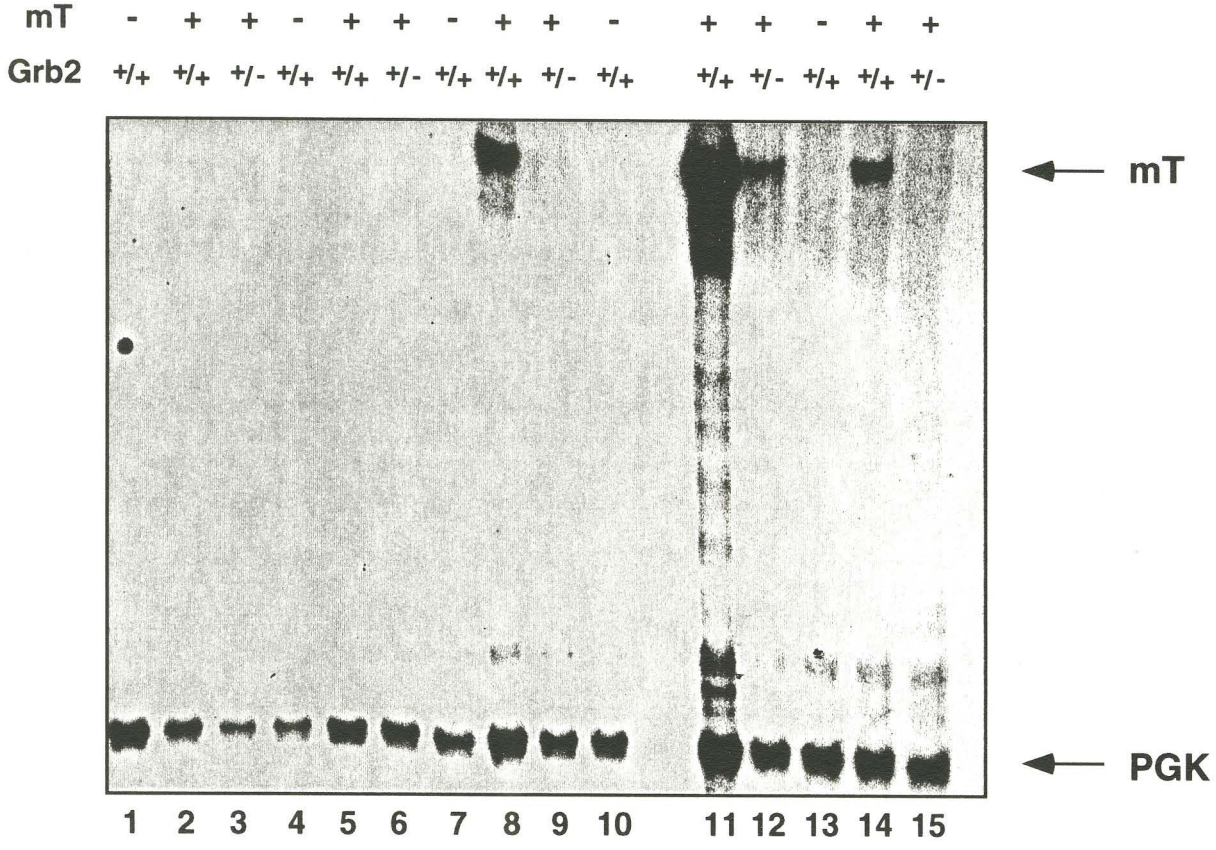

mT - + + - + + - + + - + + + - + +
Grb2 +/+ +/+ +/- +/+ +/+ +/- +/+ +/+ +/- +/+ +/+ +/- +/+ +/+ +/-
mT
PGK
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Table 3-1: Pulmonary metastases in mT634 X Grb2+/- mice

Lung tissue was extracted from ten to thirteen week old mT+;Grb2+/+ and mT+;Grb2+/- mice and examined by different methods for the presence of pulmonary metastases. RNase protection analyses were performed using 10 υg of total RNA isolated from lung tissue. The antisense riboprobe utilized in these studies is directed against middle T, and protects a 203-nucleotide fragment. The relative levels of transgene expression detected are indicated by +++ (high levels), ++ (moderate levels), + (low levels), +/- (very low levels) and - (not detected). Thin sections of lung tissue were also examined by histological methods for metastases. The presence of focal metastases is indicated by Y (present) or N (not present).

TABLE 3-1: Pulmonary metastases in mT634 X Grb2+/- mice

	mT+;Grb2+/+			mT+;Grb2+/-	
Ear Tag #	Transgene*	Histology	Ear Tag #	Transgene*	Histology
0890	-	ND	0887	-	ND
0891	-	ND	0889	-	ND
2777	+	Y	2779	-	N
3159	+++	Y	3161	-	N
3160	++	N	3163	+/-	N
Totals	3/5	2/3	Totals	1/5	0/3

*Transgene expression was determined by ribonuclease protection analysis using 10 ug of total RNA isolated from lung tissue (described in Chapter 2). The relative levels of mT transgene expression detected are indicated by +++ (high levels), ++ (moderate levels), + (low levels), +/- (very low levels) and - (not detected). ND represents Not Determined.

one (P49L-1) passed the transgene to its progeny in a Mendelian fashion. Transgene expression was assessed using 30 υg of total RNA derived from tissues of male and female carriers and hybridizing with a transgene-specific riboprobe that detects the SV40 polyadenylation and splicing signals. Transgene expression was noted in the mammary gland of female carriers, and salivary gland of male carriers. Examination of mammary gland whole mounts from the female carriers did not reveal any obvious abnormalities in development (C. Tortorice and W.J. Muller, unpublished observations).

Offspring from MMTV/mT634 X MMTV/Grb2-P49L matings have been monitored for the development of palpable mammary tumors (Figure 3-6). Virgin female mT634$^+$ and mT$^+$;P49L-1$^+$ mice developed palpable mammary tumors with t_{50} values of 35 and 41 days, respectively. It appears that expression of the MMTV/Grb2-P49L transgene has little or no effect on mT634-mediated tumorigenesis, as palpable tumors can be detected at early onset in MMTV/Grb2-P49L carriers.

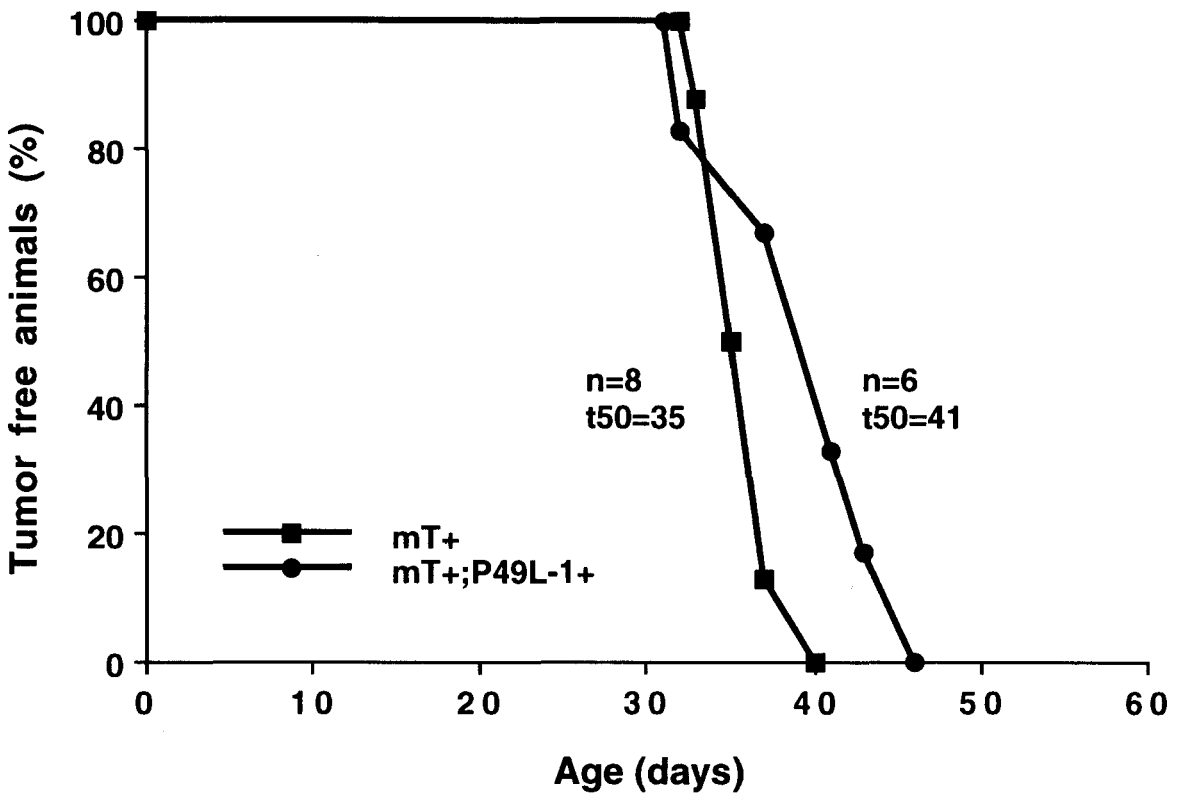
3.5 MMTV-directed overexpression of *grb2*

To further explore the effects of modulating Grb2 levels on mammary tumorigeneis, transgenic mice carrying the wild-type *grb2* cDNA under MMTV control have been generated (L. Wei, P. Siegel and W.J. Muller, unpublished results), A total of ten transgenic founders carrying the transgene were generated, nine of which passed the transgene to their progeny in a Mendelian fashion.

The tissue expression pattern of the *grb2* transgene was determined by carrying out ribonuclease protection assays. 20 υg of total RNA isolated from

Figure 3-6: Kinetics of tumor occurrence of MMTV/mT634 and MMTV/mT634;Grb2-P49L-1 mice.

Comparison of the kinetics of tumor formation between virgin female MMTV/mT634 transgenic carriers and virgin female MMTV/mT634 transgenic carriers also carrying MMTV/Grb2-P49L-1. The age at which 50% of the mice were found to have tumors (t_{50}) and the number of mice examined (n) are indicated.


Tumor free animals (%)
100
80
60
40
20
0
0
10
20
30
40
50
60
Age (days)
n=8
t50=35
n=6
t50=41
mT+
mT+;P49L-1+

male and female carriers was hybridized with a transgene-specific probe that yields a 784-base protected fragment corresponding to the 3' polyadenylation and splicing signals of the transgene. To ensure that roughly equal amounts of RNA from all tissue samples were analyzed, an antisense riboprobe generated from the mouse phosphoglycerate kinase cDNA (PGK-1) was included in each reaction as an internal control. Representative results for the Grb2-1 line are shown in Figure 3-7. Grb2-1 female #9115 exhibited a relatively high level of mammary gland expression (Figure 3-7, lane 6), and a moderate amount of salivary gland expression (Figure 3-7, lane 8). Transgene expression was not detected in any other tissues (Figure 3-7, lanes 1-5, 7, 9, 10). High level expression was detected in Grb2-1 male salivary gland and seminal vesicles (Figure 3-7, lanes 17, 18), while lower levels were observed only in male mammary gland, small intestine, spleen and testes (Figure 3-7, lanes 16, 19-21).

The results of a number of these RNase protection analyses are summarized in Table 3-2. Three of the nine lines generated, including Grb2-1, Grb2-3 and Grb2-6, expressed the transgene at relatively high levels in the female mammary gland. High levels of expression were also detected in both male and female salivary gland and male seminal vesicles from these lines. Expression was not detected in ovary (Table 3-2).

Overexpression of Grb2 in the mammary epithelium does not result in rapid transformation of the mammary gland, since mice up to six months of age do not display any evidence of tumors. However, whole mounts from the Grb2-6 line exhibit signs of epithelial proliferation reminiscent of mild hyperplasia. It remains possible that older mice may develop overt hyperplasias or tumors dependent upon MMTV-directed *grb2* overexpression.

Figure 3-7: RNase protection analysis of expression of the MMTV/ *grb2* transgene in the mammary epithelium

Tissues were derived from male and virgin female mice, and total RNA was isolated and analyzed. Representative results for the Grb2-1 line are displayed. The antisense probe used in this RNase protection analysis protects a 784-nucleotide fragment indicated by SPA and an arrow. An antisense probe, directed against mouse phosphoglycerate kinase (PGK-1) RNA, was utilized to control for equal RNA loading. The 124-nucleotide protected fragment is indicated by PGK and an arrow. Mamm. gl., saliv. gl. and sem. vesic. represent mammary gland, salivary gland and seminal vesicles, respectively.

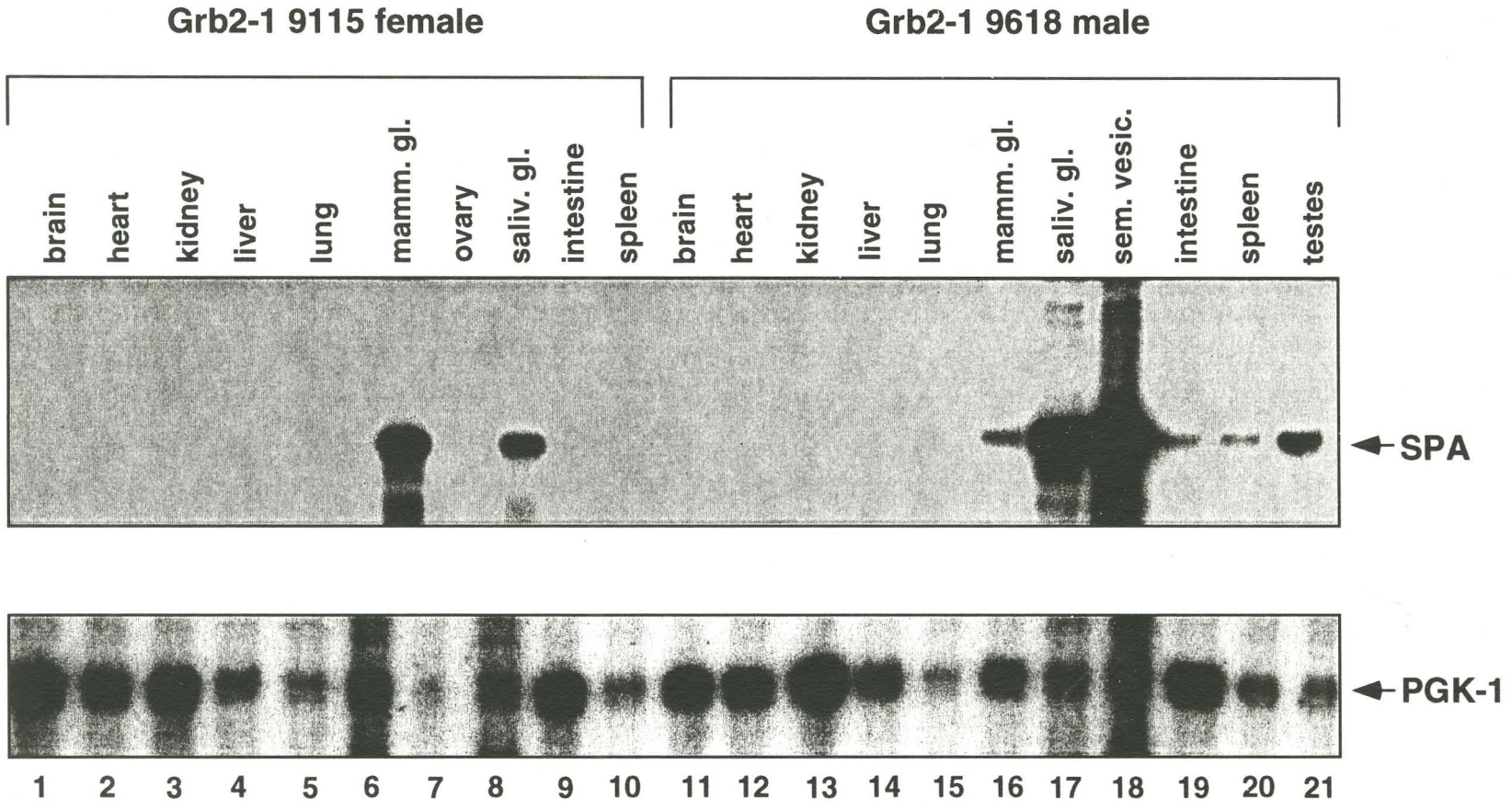

Grb2-1 9115 female
Grb2-1 9618 male
brain
heart
kidney
liver
lung
mamm. gl.
ovary
saliv. gl.
intestine
spleen
brain
heart
kidney
liver
lung
mamm. gl.
saliv. gl.
sem. vesic.
intestine
spleen
testes
SPA
PGK-1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21

Table 3-2: Transgene expression in MMTV/Grb2 mice

Ribonuclease protection analyses were performed on 20 υg of total RNA isolated from a variety of organs from both male and female MMTV/Grb2 transgene carriers. The antisense riboprobe used in these studies is directed against the SV40 component of the transgene and yields a 784-nucleotide protected fragment. An antisense probe directed against mouse phosphoglycerate kinase (PGK-1) RNA was utilized as an internal control.

TABLE 3-2: Transgene expression in MMTV/Grb2 mice*

Line	Expression of transgene				
	Mammary gland	Ovary	Salivary gland	Sem.vesicles	Testis
Grb2-1	+++	-	+++	+++	+
Grb2-2	-	-	-	ND	ND
Grb2-3	++	-	++	ND	ND
Grb2-4	+	ND	+	++	ND
Grb2-5	-	-	-	ND	ND
Grb2-6	+++	-	+++	+++	-
Grb2-8	-	-	-	ND	ND
Grb2-9	+	-	+	ND	ND
Grb2-10	+	-	+	ND	ND

*Transgene expression was determined by ribonuclease protection analysis using 20 υg of total RNA isolated from various organs (described in Chapter 2). The relative levels of transgene expression detected are indicated by +++ (high levels), ++ (moderate levels), + (low levels) and - (not detected). Sem. vesicles and ND represent Seminal vesicles and Not Determined, respectively.

A role for Grb7 in human breast cancer

Grb7 interacts with intracellular signaling proteins including tyrosine phosphorylated Shc and the protein tyrosine phosphatase Syp (Stein et al., 1994; Keegan and Cooper, 1996). Furthermore, Grb7 has been localized to a region on mouse chromosome 11 near the Neu RTK. The homologous human locus, 17q, is found amplified in human breast cancer. The ErbB-2/Neu overexpression that results from this amplification is common in human cancers, and has been correlated with poor patient prognosis (reviewed in Hynes and Stern, 1994). In addition, Grb7 and Neu/ErbB-2 bind tightly together, and both are overexpressed in human breast cancer cell lines and breast tumors (Stein et al., 1994). Transgenic mice overexpressing Grb7 in the mammary epithelium have been generated in order to assess Grb7 function in mammary tumorigenesis. The association between Grb7 and Neu/ErbB-2 in human breast cancer cell lines and breast tumors will be studied further using transgenic mice expressing both Grb7 and Neu.

3.6 Expression of *grb7* in the mouse mammary gland

In order to examine the role of Grb7 in mammary tumorigenesis, a Grb7 cDNA under transcriptional control of the MMTV promoter/enhancer was used to produce transgenic mice. A total of seven transgenic founders were generated carrying the MMTV/*grb7* transgene (P. Siegel and W.J. Muller, unpublished results). Of the seven lines generated, four passed the transgene to their progeny in a Mendelian fashion.

The tissue specificity of transgene expression was assessed by subjecting 20 υg of total RNA derived from 12 different tissues isolated from male and female animals, to ribonuclease protection analysis with a transgene-specific probe. A representative RNase protection is shown in Figure 3-8. Expression was detected only in total RNA samples derived from mammary gland and salivary gland of a Grb7-1 female (Figure 3-8, lanes 12 and 14). The transgene was also detected at high levels in some male tissues, including seminal vesicle and salivary gland (Figure 3-8, lanes 7,8). The results from a number of these RNase protection analyses are summarized in Table 3-3. Four of the seven lines - Grb7-1, Grb7-2, Grb7-3 and Grb7-7 - exhibited a strong level of expression in the mammary glands of female transgenic mice. Lower amounts of expression were detected in other tissues such as the ovary and salivary gland in lines Grb7-1, -2, -3 and -7, while very low amounts of expression could be detected in a number of organs in Grb7-3 mice after longer exposure of the autoradiograms (Table 3-3). The wide-spread, very low level expression observed across a range of tissues for Grb7-3 mice might represent expression in hematopoietic cells, where blood contamination of samples could result in the detection of transgene expression.

The expression of Grb7 in the mammary epithelium does not appear to result in transformation of the mammary gland or tumor formation, even after multiple pregnancies in mice up to 13 months of age. In several cases, however, litters of pups nursed by female MMTV/Grb7 transgenic mice have appeared to grow and develop more slowly than usual. The reduced size of several litters weaned from MMTV/Grb7 female mice may be the result of a lactation defect. Despite these observations, no clear morphological abnormalities were evident upon examination of mammary glands from FVB and Grb7 transgenic females at different stages of development.

Figure 3-8: RNase protection analysis of expression of the MMTV/ *grb7* transgene in the mammary epithelium

Tissues were derived from male and virgin female mice, and total RNA was isolated and analyzed. Representative results from the Grb7-7 and Grb7-1 lines are displayed. The antisense probe used in this RNase protection analysis protects a 784-nucleotide fragment indicated by SPA and an arrow. An antisense probe that protects a 124-nucleotide fragment of mouse phosphoglycerate kinase (PGK-1) RNA was utilized to control for equal RNA loading, and is indicated by PGK and an arrow. Mamm. gl., saliv. gl. and sem. vesic. represent mammary gland, salivary gland and seminal vesicles, respectively.

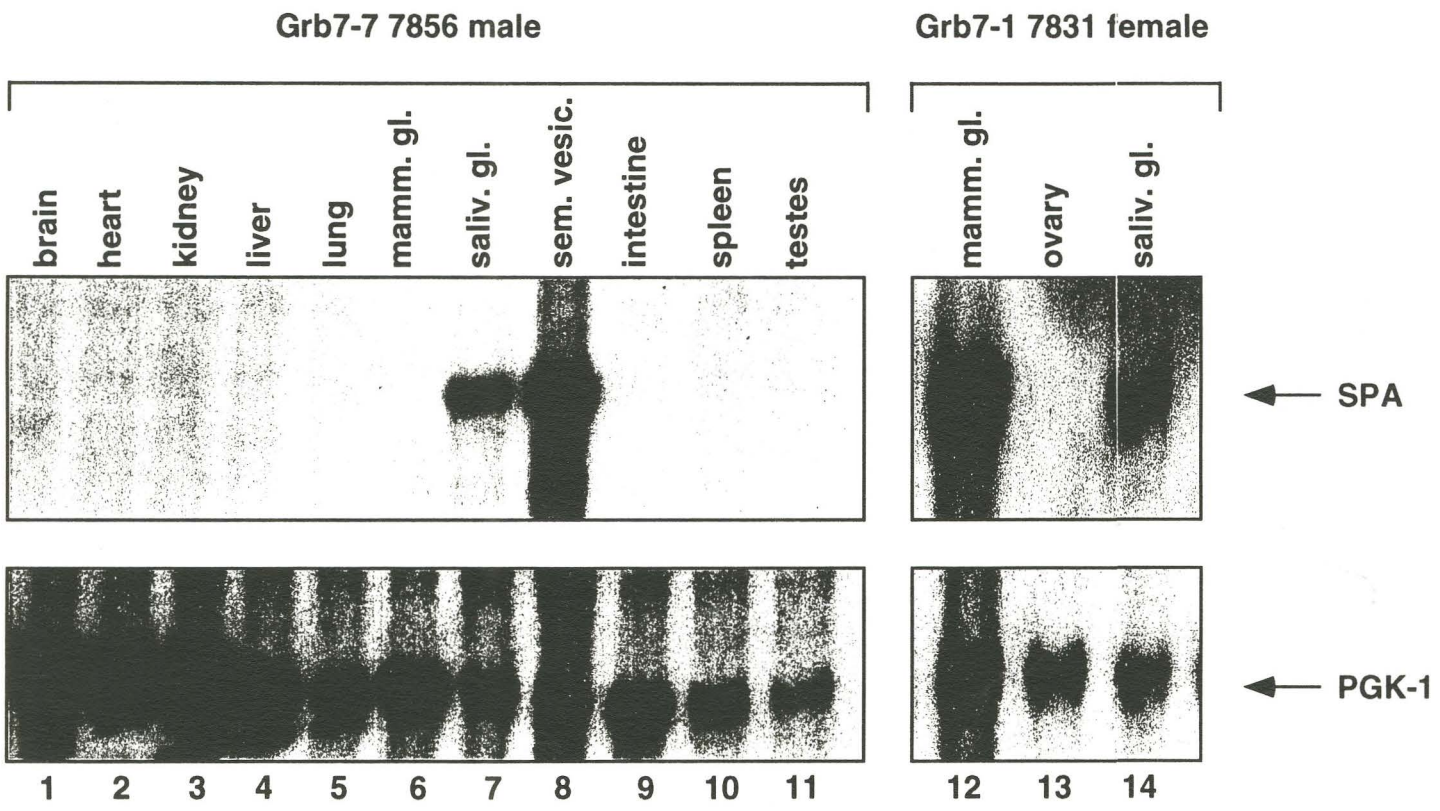

Grb7-7 7856 male
Grb7-1 7831 female
brain
heart
kidney
liver
lung
mamm. gl.
saliv. gl.
sem. vesic.
intestine
spleen
testes
mamm. gl.
ovary
saliv. gl.
SPA
PGK-1
1
2
3
4
5
6
7
8
9
10
11
12
13
14

Table 3-3: Transgene expression in MMTV/Grb7 mice.

Ribonuclease protection analyses were performed on 20 υg of total RNA isolated from a variety of organs from both male and female MMTV/Grb7 transgene carriers. The antisense riboprobe used in these studies is directed against the SV40 component of the transgene and yields a 784-nucleotide protected fragment. An antisense probe directed against mouse phosphoglycerate kinase (PGK-1) RNA was utilized as an internal control.

TABLE 3-3: Transgene expression in MMTV/Grb7 mice*

	Expression of transgene											
Line	Brain	Heart	Kidney	Liver	Lung	M.gland	Ovary	Saliv. gl.	Sem.v.	Intestine	Spleen	Testis
Grb7-1	-	-	-	-	-	+++	+	-	+	-	-	-
Grb7-2	-	-	-	-	-	+++	-	+	+	-	-	-
Grb7-3	-	+	+	+	+	++	+	+++	+	+	+	-
Grb7-4	ND	ND	ND	ND	ND	-	ND	-	ND	ND	ND	ND
Grb7-5	ND	ND	ND	ND	ND	-	-	-	ND	ND	ND	ND
Grb7-6	ND	ND	ND	ND	ND	+	-	+	ND	ND	ND	ND
Grb7-7	-	-	-	-	-	+++	+	+	+++	-	-	-

*Transgene expression was determined by ribonuclease protection analysis using 20 υg of total RNA isolated from various organs (described in Chapter 2). The relative levels of transgene expression detected are indicated by +++ (high levels), ++ (moderate levels), + (low levels) and - (not detected). M. gland, Saliv. gl., Sem. v. and ND represent Mammary gland, Salivary gland, Seminal vesicles and Not Determined, respectively.

3.7 The role of Grb2 in Neu-mediated tumorigenesis

Grb2 function in Neu-mediated mammary tumorigenesis was also investigated. Initially, MMTV/NDL1-2 male mice were mated with $Grb2^{+/-}$ female mice, and the progeny were monitored for the development of mammary tumors. The NDL1-2 mice were generated in an FVB/N genetic background, while the Grb2 heterozygotes were produced in a 129/CD1 genetic background. While NDL1-2 transgenic mice typically develop mammary tumors by four to six months of age (P. Siegel and W.J. Muller, unpublished observations), all NDL1-2 transgenic mice from the mixed mating failed to develop tumors. RNase protection analysis using a riboprobe specific to the SV40 polyadenylation/ splicing signals revealed that the NDL1-2 transgene was not being expressed in these animals. It appears likely that a rare genetic event has resulted in the silencing of transgene expression in the mixed genetic background. It seemed likely that the lack of transgene expression was due to an insertion site phenomenon, such that the expression problem should be MMTV/NDL1-2 line-specific, and the use of another MMTV/NDL transgenic strain should not produce a similar result. Recent results from our laboratory using another NDL strain, the NDL2-5 strain, suggest that the transgene expression and tumor development problems experienced with the NDL1-2 strain were a line-specific phenomenon. In the interim, male $Grb2^{+/-}$ mice were mated with female FVB animals for several generations, and the Neo cassette-disrupted *grb2* allele was selected at each generation. Isolation of the disrupted *grb2* allele into an FVB background may facilitate matings with MMTV/NDL FVB mice, while use of the NDL2-5 transgenic line has also produced encouraging results. Both approaches should allow future enquiry into the role of Grb2 in Neu-induced mammary tumorigenesis.

Chapter 4: Discussion

The transmission of many mitogenic stimuli requires tyrosine kinase activity, and the subsequent recruitment of intracellular signaling molecules. A class of signaling molecules termed adaptor proteins has emerged: these proteins lack catalytic activity but possess conserved protein-protein interaction domains. To establish that adaptor proteins represent targets for cancer therapy, it is important to determine whether or not modulating the levels of these intracellular signaling molecules has an effect on downstream signaling and oncogenesis.

Several lines of evidence indicate that Grb2 might serve as a useful target for therapeutic intervention. *Grb2* gene amplification and overexpression has been correlated with breast cancer and leukemia incidence in some studies (Daly et al., 1994; Huebner et al., 1994). Furthermore, Grb2 transmits intracellular signals from a number of tyrosine kinases thought to be involved in these human cancers, including Neu/ErbB-2 and the Bcr-Abl fusion oncoprotein (Janes et al., 1994; Pendergast et al., 1993). The expression of mutant Grb2 proteins in cell lines expressing oncogenic Bcr-Abl suppressed Ras activation and reversed the cell lines' transformed phenotype (Gishizky et al., 1995). As well, overexpressing mutant versions of Grb2 blocked activation of the Erk-1 MAP kinase by the EGF receptor and oncogenic Abl (Tanaka et al., 1995). It is also well established that the Grb2 homologues are required for signaling to Ras in invertebrate systems (Clark et al., 1992; Simon et al., 1993; Olivier et al., 1993).

Based upon these lines of evidence, I have investigated the effects of modulating the levels of Grb2 and Grb7 in the mammary epithelium on tumorigenesis in transgenic mice. Knockout mice heterozygous for *grb2* , transgenic mice overexpressing wild-type and dominant negative versions of Grb2, and transgenic mice overexpressing wild-type *grb7* , have been utilized. These *in vivo* models have provided insight into the intracellular signaling mechanisms involved in mammary tumorigenesis.

Knockout mice heterozygous for the *grb2* gene were mated with transgenic polyomavirus mT antigen-expressing mice, in an effort to limit the amount of Grb2 available to the activated cells. It is important to note that Grb2 null embryos do not survive to birth, but rather die early in development (A. Cheng and T. Pawson, personal communication). It is not surprising that Grb2 null mice are inviable, given the critical roles for Grb2 in a wide range of processes from diverse cell types. Grb2 is involved in insulin receptor signaling (Skolnik et al., 1993), Ras stimulation downstream of activated EGF receptor family members (Lowenstein et al., 1992; Rozakis-Adcock et al., 1992), regulation of synaptic proteins (McPherson et al., 1994), and Met receptor signaling in muscle development (Maina et al., 1996). What is unexpected, however, is that embryos heterozygous for *grb2* ($Grb2^{+/-}$) survive to birth and develop into adult mice that appear completely normal. After observing many generations of litters, no detectable differences between wild-type and $Grb2^{+/-}$ new-borns, weanlings or adults have been observed. More complete characterization of these mice has been completed by A. Cheng (A. Cheng and T. Pawson, personal communication).

Progeny of the MMTV/mT634 and $Grb2^{+/-}$ mating were weaned approximately 19 days after birth, and monitored for the development of palpable mammary tumors. Previously, female transgenic carriers of the MMTV/mT634 line were shown to develop detectable mammary tumors with an average onset of 34 ± 6 days (Guy et al., 1992a). Mammary gland neoplasias could be detected in $mT^{+};Grb2^{+/+}$ mice from this current mating as early as 27 days; collectively, these mice developed palpable mammary tumors with a t_{50} of 36 days in a single blind study of 37 individuals. In contrast, palpable mammary tumors were detected in $mT^{+};Grb2^{+/-}$ mice with a t_{50} of 52 days in a study of 25 individuals (see Figure 3-1). This difference in tumor onset was quite surprising given the roughly equivalent overall growth rates of the $Grb2^{+/+}$ and $Grb2^{+/-}$ mice. Instead, a more subtle effect on tumor growth or metastasis might have been expected.

In order to examine these mammary gland abnormalities, mammary gland wholemounts and thin sections were produced. Careful comparison of 6 week old wild-type and $Grb2^{+/-}$ mammary trees revealed few differences in development. The $Grb2^{+/-}$ mammary tree had not completely filled the fat pad, but was otherwise histologically normal (Figures 3-2A, 3-3A) (Dr. R. Cardiff, personal communication). This evidence helps to confirm that the observed tumor kinetics differences were likely not due to developmental defects in the $Grb2^{+/-}$ animals. Examination of $mT^{+};Grb2^{+/-}$ and $mT^{+};Grb2^{+/+}$ wholemounts and thin sections revealed dramatic differences. The $mT^{+};Grb2^{+/+}$ fat pads were filled with dysplastic epithelium, and mammary trees were obscured by numerous widely distributed focal lesions. These neoplasias were much less advanced in age-matched $mT^{+};Grb2^{+/-}$ glands (see Figures 3-2, 3-3).

Many of these transgenic animals were observed for longer periods of time. Mice ten to fourteen weeks old from the two groups were also clearly distinguishable. The $mT^{+};Grb2^{+/+}$ mice carried large tumor loads that were clearly visible in all ten fat pads, and which were often found to be fluid-filled and necrotic upon necropsy. Conversely, the $mT^{+};Grb2^{+/-}$ mice carried considerably smaller tumor loads which were only visible in a small percentage of the fat pads. Further, these $Grb2^{+/-}$ tumors were not determined to be necrotic upon necropsy.

Significantly, there appeared to be a difference in pulmonary metastases from these primary sites. Histological examination and ribonuclease protection analysis using total RNA samples derived from lung tissue were used to establish differences in metastatic activity. Preliminary results indicated that pulmonary metastases only rarely appeared in tumor-bearing $Grb2^{+/-}$ mice, while a majority of $mT^{+};Grb2^{+/+}$ mice displayed metastases by 7 weeks post-detection of palpable mammary tumors. About 94% of tumor-bearing female transgenic carriers of the parental mT634 strain were previously shown to develop pulmonary metastases by 3 months of age (Guy et al., 1992a). The mechanism responsible for the observed differences in metastatic activity between $mT^{+};Grb2^{+/+}$ and $mT^{+};Grb2^{+/-}$ animals remains unclear. It is possible that metastasis is dependent upon the development of a threshhold primary tumor mass which is only achieved much later in $Grb2^{+/-}$ individuals. Alternatively, the observed differences in metastatic activity might reflect a diminished capacity of individual cells to escape the primary site, enter the circulatory system and establish themselves in the lungs. A carefully documented study of metastasis in these animals should be carried out. Primary tumor size and the length of the tumor-bearing time period are critical factors in metastasis. Injecting primary

tumor cells into the tail veins of immunodeficient nude mice represents one alternative method for measuring the metastatic abilities of these cells.

To confirm that the observed differences in tumor kinetics and metastatic potential were not due to problems with the mT transgene or protein, control studies examining mT expression and function were carried out. Ribonuclease protection assays showed that approximately equal amounts of transcript were present in age-matched $mT^{+};Grb2^{+/+}$ and $mT^{+};Grb2^{+/-}$ breast tumors (Figure 3-4), and PhosphorImager analysis confirmed the assessment that no significant differences in transgene levels could be detected. The fact that the MMTV/mT transgene was expressed normally in progeny from the mating is not insignificant, given the transgene expression problems that arose when matings between $Grb2^{+/-}$ and MMTV/NDL1-2 mice were attempted (see Section 3.7). In addition, mT function was assessed. PyV middle T antigen-mediated tumorigenesis depends upon an interaction with c-Src family tyrosine kinases (Guy et al., 1994). *In vitro* kinase assays on enolase using mT immunoprecipitates were performed. No significant difference between $mT^{+};Grb2^{+/+}$ and $mT^{+};Grb2^{+/-}$ samples were discovered, based upon PhosphorImager analysis of the autoradiograms (Figure 3-5). It seems clear that the mT transgene is being properly expressed in the $Grb2^{+/-}$ mice, and that the mT protein is appropriately recruiting and activating cytoplasmic tyrosine kinases in these same mice.

These findings collectively allow one of the major questions of this present study to be addressed. Modulating the levels of intracellular signaling molecules can have marked effects on tumor initiation, development and metastasis. Specifically, decreasing the Grb2 dosage in cells negatively affects mammary tumor production and metastasis in transgenic mice. The underlying mechanism

for the observed differential tumor production remains unclear. It is possible that the rate of proliferation of $Grb2^{+/-}$ tumor cells might be directly affected. The decrease in Grb2 protein levels could result in a corresponding decrease in the proportion of Ras in the active or GTP-bound state. Attempts to assess the proliferative abilities of mammary epithelial cells derived from these mice have been largely unsuccessful. An *in vivo* method for identifying proliferating cells that relies on bromodeoxyuridine incorporation might be effectively utilized to avoid the technical difficulties associated with culturing primary cells.

The experiments discussed thus far have all used mice heterozygous for Grb2 in an effort to interfere with mT-mediated tumorigenesis. The use of heterozygotes is not ideal, since the mammary epithelial cells of these mice all express the endogenous *grb2* gene to some degree, and no interfering proteins or drugs have been used to block Grb2 function. Introducing a dominant acting, mutant *grb2* gene into the mouse mammary gland might provide a more efficient method for blocking Grb2 function. A number of mutant versions of *grb2* have been discovered in invertebrate systems. Following chemical mutagenesis, four different mutations in the SH2 or SH3 domains of the C. *elegans grb2* homologue *sem-5* were isolated that negatively affected sex myoblast migration or vulval induction (Clark et al., 1992). Two such mutations affected highly conserved SH3 residues, including one substitution of leucine for proline at codon 49 in the amino-terminal SH3 domain. This P49L mutation was engineered in *grb2* (R. Daly, personal communication) and has been utilized as part of a MMTV fusion gene to produce transgenic mice (P. Siegel and W.J. Muller, unpublished results).

Transgenic mice expressing this MMTV/Grb2-P49L construct were mated with MMTV/mT634 transgenic mice, and monitored for the development of palpable mammary tumors (Figure 3-7). It seems clear that expression of the MMTV/Grb2-P49L transgene had no effect on mT-mediated tumorigenesis. It is possible that MMTV/Grb2-P49L transgene expression is delayed in these animals, as occurs with other transgenic lines, including some of the MMTV/NDL lines and the MMTV/mT121 line (P. Siegel, C. Guy and W.J. Muller, unpublished observations). In such a situation, rapid-onset mT634-mediated tumorigenesis would remain unaffected. To investigate this possibility, a Grb2-P49L-specific riboprobe has been generated, in order to establish that the transgene is being expressed in these bigenic animals. Alternatively, use of the MMTV/mT121 transgenic line, with female carriers that exhibit delayed tumor kinetics by comparison with the mT634 line, may also be considered.

To demonstrate that the Grb2-P49L product can act as a dominant negative protein, preliminary cotransfection experiments using activated Neu and Grb2-P49L were performed. Neu-mediated focus formation was reduced when both constructs were transfected into Rat1 fibroblasts when compared with Neu transfectants alone. However, in mT transgenic mice, levels of P49L transgene expression may not have been sufficient to compete with endogenous Grb2 for Shc binding. To increase levels of expression, MMTV/Grb2-P49L-1 mice could be inbred to increase the transgene copy numbers; alternatively, the lines would have to be rederived. Grb2-P49L does not act as an effective dominant inhibiting protein in MMTV/mT634 mammary tumorigenesis.

Wild-type Grb2 and Grb7 cDNAs have been expressed in the mouse mammary epithelium to determine whether or not expression of these proteins

alone can lead to transformation and tumor formation. Grb2 overexpression in cultured cells does not lead to cellular growth or transformation, except when other components of the signaling pathway like Ras or the EGF receptor are also overexpressed (Downward, 1994). Not unexpectedly, MMTV-directed overexpression of *grb2* does not appear to induce transformation of the mammary gland. Despite the detection of relatively high levels of transgene expression (see Figure 3-8, Table 3-2), female mice up to six months of age did not display any mammary gland abnormalities. The marked Grb2 overexpression observed in a number of breast cancer cell lines by Daly et al. (1994) might, nonetheless, be important to the transformation process. While Grb2 expression alone may not cause transformation, Grb2 overexpression in cells expressing activated tyrosine kinases may play a role in upregulating signaling from these activated receptors. Mating MMTV/Grb2 transgenics with less potent mammary tumor models may provide insight into roles for Grb2 in transformation. For example, mating MMTV/Grb2 mice with MMTV/mT250 and MMTV/mTDb transgenic models, which express mutant versions of middle T impaired in transformation ability (Webster et al., submitted for publication), may provide a context for studying Grb2 function.

Grb7 is another protein, which due to its observed interaction with HER2/Neu in breast cancer cell lines (Stein et al., 1994), is thought to play a role in mammary tumorigenesis. Overexpressing Grb7 in Rat1 fibroblasts did not induce transformation. Furthermore, expression of the MMTV/Grb7 transgene (see Figure 3-9; Table 3-3) did not result in transformation of the mammary gland, even in female mice 13 months of age following multiple pregnancies. Possible lactation defects in MMTV/Grb7 females could not be confirmed in parallel studies

with nontransgenic mice. Grb7 could be involved in upregulating signaling in the presence of activated Neu/ErbB-2. Breeding experiments using MMTV/NDL and MMTV/Grb7 mice were inconclusive, as many of the mice of the $NDL^{+};Grb7^{-/-}$ and $NDL^{+};Grb7^{+/-}$ genotypes did not exhibit tumors. Since so little is known about Grb7 and its cellular function, production of Grb7 knockout mice could provide evidence as to the developmental or cellular role of Grb7. The Neu/Grb7 interaction may yet prove important in Neu-mediated mammary tumorigenesis.

SH2 and SH3 domain proteins may serve as targets for anticancer therapies. Overexpression of Grb2 does not seem to be directly involved in tumorigenesis, however, Grb2 is a critical component of signaling pathways downstream of RTKs. Therefore, blocking Grb2 function may prove useful in anticancer therapy. Due to the central position occupied by Grb2 in so many processes, it is hard to believe that interrupting Grb2 function could be accomplished without disrupting normal cellular processes. However, evidence from Grb2 knockout mice indicates that normal cellular processes can carry on seemingly unaffected in heterozygotes, while tumor development and growth is retarded. Therefore, disrupting Grb2 function may be an acceptable component of a multi-approach therapeutic strategy. Introducing small peptides that contain specific SH2 or SH3 domains into cells to compete for binding may be efficacious in slowing tyrosine kinase mediated tumorigenesis. The best method for expressing larger interfering proteins in cells may involve viral vector gene therapy. Adenovirus vectors have already been successfully utilized to insert large expression cassettes into cells, and may also be useful for expressing SH2- and SH3-containing dominant interfering proteins in tumor cells.

Summary

The discovery of Grb2 and Grb7 has led to an increasingly intense examination of the roles of non-catalytic proteins in intracellular signaling downstream of receptor tyrosine kinases. In particular, Grb2 seems to play a critical role in the transmission of certain mitogenic signals. Modulating the levels of Grb2 available in cells had a profound effect on the progression of polyomavirus middle T antigen induced mammary tumors and appearance of pulmonary metastases in transgenic mice. These results suggest that Grb2 might serve as a target for anticancer therapies. Neither Grb2 nor Grb7 appeared to transform the mammary gland of transgenic mice expressing the respective constructs. Analysis of the roles of each protein in ErbB-2/Neu-mediated mammary tumorigenesis should serve to further reveal the roles of Grb2 and Grb7 in oncogenesis.

Chapter 5: References

Anderson, D., C.A. Koch, L. Grey, C. Ellis, M.F. Moran and T. Pawson. 1990. Binding of SH2 domains of phospholipase Cγ1, GAP, and Src to activated growth factor receptors. *Science* **250**: 979-982.

Arighi, E., L. Albert, F. Torriti, S. Ghizzoni, M.G. Rizotti, G. Pelicci, B. Pasini, I. Bongarzone, C. Piutti, M.A. Pierotti and M.G. Borello. 1997. Identification of Shc docking site on Ret tyrosine kinase. *Oncogene* **14**: 773-782.

Asai, N., T. Iwashita, M. Matsuyama and M. Takahashi. 1995. Mechanism of activation of the ret proto-oncogene by multiple endocrine neoplasia 2A mutations. *Molec.Cell. Biol.* **15**: 1613-1619.

Bargmann, C.I., M-C. Hung and R.A. Weinberg. 1986. Muliple independent activations of the *neu* oncogene by a point mutation altering the transmembrane domain of p185. *Cell* **45**: 649-657.

Blaikie, P., J. Immanuel, N.L. Wu, V. Yajnik and B. Margolis. 1994. A region in Shc distinct from the SH2 domain can bind tyrosine phosphorylated growth factor receptors. *J. Biol. Chem.* **269**: 32031-32034.

Campbell, K.S., E. Ogris, B. Burke, W. Su, K.R. Auger, B.J. Druker, B.S. Schauffhausen, T.M. Roberts and D.C. Pallas. 1994. Polyoma middle tumor antigen interacts with SHC protein via the NPTY (Asn-Pro-Thr-Tyr) motif in middle tumor antigen. *Proc. Natl. Acad. Sci. USA* **91**: 6344-6348.

Chirgwin, J.M., E. Przybala, R.J. MacDonald and W.J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**: 5294-5299.

Chou, M.M., J.E. Fajardo and H. Hanafusa. 1992. The SH2- and SH3-containing Nck protein transforms mammalian fibroblasts in the absence of elevated phosphotyrosine levels. *Molec. Cell. Biol.* **12**: 5834-5842.

Clark S.G., M.J. Stern and H.R. Horvitz. 1992. C. *elegans* cell-signaling gene *sem-5* encodes a protein with SH2 and SH3 domains. *Nature* **356**: 340-344.

Coulier, F., R. Kumar, M. Ernst, R. Klein, D. Martin-Zanca and M. Barbacid. 1990. Human *trk* oncogenes activated by point mutation, in-frame deletion and duplication of the tyrosine kinase domain. *Molec. Cell. Biol.* **10**: 4202-4210.

Daly, R.J., M.D. Binder and R.L. Sutherland. Overexpression of the Grb2 gene in human breast cancer cell lines. *Oncogene* **9**: 2723-2727.

Daly, R.J., G.M. Sanderson, P.W. Janes and R.L. Sutherland. 1996. Cloning and characterization of *GRB14*, a novel member of the *GRB7* gene family. *J. Biol. Chem.* **271**: 12502-12510.

Dankort, D.L., Z. Wang, V. Blackmore, M.F. Moran and W.J. Muller. 1997. Distinct tyrosine autophosphorylation sites negatively and positively modulate Neu-mediated transformation. *Molec. Cell. Biol.* in press.

Dilworth, S.M., C.E.P. Brewster, M.D. Jones, L. Lanfrancone, G. Pelicci and P.G. Pelicci. 1994. Transformation by polyomavirus middle T antigen involves the binding and tyrosine phosphorylation of Shc. *Nature* **367**: 87-90.

Dougall, W.C., X. Qian, N.C. Peterson, M.J. Miller, A. Samanta and M.I. Greene. 1994. The *neu* oncogene: signal transduction pathways, transformation mechanisms and evolving therapies. *Oncogene* **9**: 2109-2123.

Downward, J., Y. Yarden, E. Mayes, G. Scrace, N. Totty, P. Stockwell, A. Ullrich, J. Schlessinger and M.D. Waterfield. 1984. Close similarity of epidermal growth factor receptor and v-*erb*-B oncogene protein sequences. *Nature* **307**: 521-527.

Downward, J. !994. The Grb2/Sem-5 adaptor protein. *FEBS Letters* **338**: 113-117.

Escobedo, J.A., S. Navankasattusas, W.M. Kavanaugh, D. Milfay, V.A. Fried and L.T. Williams. 1991. cDNA cloning of a novel 85 kD protein that has SH2 domains and regulates binding of PI3-kinase to the PDGF β-receptor. *Cell* **65**: 75-82.

Fantl, W.J., J.A. Escobedo, G.A. Martin, C.W. Tuck, M. delRosario, F. McCormick and L.T. Williams. 1992. Distinct phosphotyrosines on a growth factor receptor bind to specific molecules that mediate different signaling pathways. *Cell* **69**: 413-423.

Ghishizhky, M.I., D. Cortez and A. M. Pendergast. 1995. Mutant forms of growth factor-binding protein-2 reverse BCR-ABL-induced transformation. *Proc. Natl. Acad. Sci. USA* **92**: 10889-10893.

Guy, C.T., R.D. Cardiff and W.J. Muller. 1992a. Induction of mammary tumors by expression of polyomavirus middle T oncogene: a transgenic mouse model for metastatic disease. *Molec. Cell Bio.* **12**: 954-961.

Guy, C.T., M.A. Webster, M. Schaller, T.J. Parsons, R.D. Cardiff and W.J. Muller. 1992b. Expression of the *neu* proto-oncogene in the mammary epithelium of transgenic mice induces metastatic disease. *Proc. Natl. Acad. Sci. USA* **89**: 10578-10582.

Guy, C.T.,S.K. Muthuswamy, R.D. Cardiff, P. Soriano and W.J. Muller. 1994. Activation of the c-Src tyrosine kinase is required for the induction of mammary tumors in transgenic mice. *Genes and Devel.* **8**: 23-32.

Harlan, J.E., P.J. Hajduk, H.S. Yoon and S.W. Fesik. 1994. Pleckstrin homology domains bind to phosphatidylinositol-4,5-bisphosphate. *Nature* **371**: 168-171.

Hofstra, R.M.W., R.M. Landsvater, I. Ceccherini, R.P. Stulp, T. Stelwagen, Y. Luo, B. Pasini, J.W.M. Hoppener, H.K.P. van Amstel, G. Romeo, C.J.M. Lips and C.H.C.M. Buys. 1994. A mutation in the *RET* proto-oncogene associated with multiple endocrine neoplasia type 2B and sporadic medullary thyroid carcinoma. *Nature* **367**: 375-376.

Holgado-Madruga, M., D.R. Emlet, D.K. Moscatello, A.K. Godwin and A.J. Wong. 1996. A Grb2-associated docking protein in EGF- and insulin-receptor signaling. *Nature* **379**: 560-564.

Huang, A.L., M.C. Ogtrowski, D. Berard and G.L. Hager. 1981. Glucocorticoid regulation of Ha-MuSV p21 conferred by sequences from mouse mammary tumor virus. *Cell* **27**: 245-255.

Huebner, K., K. Kastry, T. Druck, A.E. Salcini, L. Lanfrancone, G. Pelicci, E. Lowenstein, W. Li, S-H. Park, L. Cannizzaro, P.G. Pelicci and J. Schlessinger. 1994. Chromosome locations of genes encoding human signal transduction adapter proteins Nck (*NCK*), Shc (*SHC1*) and Grb2 (*GRB2*). *Genomics* **22**: 281-287.

Hynes, N.E. and D.F. Stern. 1994. The biology of *erbB-2/neu/HER-2* and its role in cancer. *Biochim. Biophys. Acta* **1198**: 165-184.

Janes, P.W., R.J. Daly, A. deFazio and R.L. Sutherland. 1994. Activation of the Ras signalling pathway in human breast cancer cells overexpressing *erbB-2*. *Oncogene* **9**: 3601-3608.

Kavanaugh, W.M. and L.T. Williams. 1994. An alternative to SH2 domains for binding tyrosine-phosphorylated proteins. *Science* **266**: 1862-1865.

Kazlauskas, A., G-S. Feng, T. Pawson and M. Valius. 1993. The 64 kDa protein that associates with the platelet-derived growth factor receptor β subunit via Tyr-1009 is the SH2-containing phosphotyrosine phosphatase Syp. *Proc. Natl. Acad. Sci. USA* **90**: 6939-6942.

Kazlauslas, A. 1994. Receptor tyrosine kinases and their targets. *Curr. Opin. Gen. Devel.* **4**: 5-14.

Keegan, K. and J.A. Cooper. 1996. Use of the two hybrid system to detect the association of the protein-tyrosine-phosphatase, SHPTP2, with another SH2-containing protein, Grb7. *Oncogene* **12**: 1537-1544.

Kitayama, H., T. Tsujimura, I. Matsumura, K. Oritani, H. Ikeda, J. Ishikawa, M. Okabe, M. Suzuki, K. Yamamura, Y. Matsuzawa, Y. Kitamura and Y. Kanakura. 1996. Neoplastic transformation of normal hematopoietic cells by constitutively activating mutations of c-*kit* receptor tyrosine kinase. *Blood* **88**: 995-1004.

Koch, C.A., D. Anderson, M.F. Moran, C. Ellis and T. Pawson. 1991. SH2 and SH3 domains: Elements that control interactions of cytoplasmic signaling proteins. *Science* **252**: 668-674.

Lawe, D.C., C. Hahn and H.J. Wong. 1997. The Nck SH2/SH3 adaptor protein is present in the nucleus and associates with the nuclear protein SAM68. *Oncogene* **14**: 223-231.

Lehmann, J.M., G. Riethmuller and J.P. Johnson. 1990. Nck, a melanoma cDNA encoding a cytoplasmic protein consisting of the Src homology units SH2 and SH3. *Nucl. Acids Res.* **18**: 1048.

Li, W., P. Hu, E.Y. Skolnik, A. Ullrich and J. Schlessinger. 1992. The SH2 and SH3 domain-containing Nck protein is oncogenic and a common target for phosphorylation by different surface receptors. *Molec. Cell. Biol.* **12**: 5824-5833.

Li, W., R. Nishimura, A. Kashishiam, A.G. Batzer, W.J.H. Kim, J.A. Cooper and J. Schlessinger. 1994. A new function for a phosphotyrosine phosphatase - linking GRB2-Sos to a receptor tyrosine kinase. *Molec. Cell. Biol.* **14**: 509-517.

Lorenzo, M.J., G.D. Gish, C. Houghton, T.J. Stonehouse, T. Pawson, B.A.J. Ponder and D.P. Smith. 1997. RET alternate splicing influences the interaction of activated RET with the SH2 and PTB domains of Shc, and the SH2 domain of Grb2. *Oncogene* **14**: 763-771.

Lowenstein, E.J., R.J. Daly, A.G. Batzer, W. Li, B. Margolis, R. Lammers, A. Ullrich, E.Y. Skolnik, D. Bar-Sagi and J. Schlessinger. 1992. The SH2 and SH3 domain-containing protein GRB2 links receptor tyrosine kinases to ras signaling. *Cell* **70**: 431-442.

Ma, G., D. Lu, Y. Wu, J. Liu and R.B. Arlinghaus. 1997. Bcr phosphorylated on tyrosine 177 binds Grb2. *Oncogene* **14**: 2367-2372.

Maina, F, F. Casagranda, E. Audero, A. Simeone, P.M. Comoglio, R. Klein and C. Ponzetto. 1996. Uncoupling of Grb2 from the Met receptor *in vivo* reveals complex roles in muscle development. *Cell* **87**: 531-542.

Margolis, B., N. Li, A. Koch, M. Mohammadi, D.R. Hurwitz, A. Zilberstein, A. Ullrich, T. Pawson and J. Schlessinger. 1990. The tyrosine phosphorylated

carboxyterminus of the EGF receptor is a binding site for GAP and PLCγ. *EMBO J.* **9**: 4375-4380.

Margolis, B. 1992. Proteins with SH2 domains: transducers in the tyrosine kinase signaling pathway. *Cell Growth Differen.* **3**: 73-80.

Margolis, B., O. Silvennainen, F. Comoglio, C. Roonprapunt, E. Skolnik, A. Ullrich and J. Schlessinger. 1992. High-efficiency expression/cloning of epidermal growth factor receptor-binding proteins with Src homology 2 domains. *Proc. Natl. Acad. Sci. USA* **89**: 8894-8898.

Marshall, C.J. 1994. MAP kinase kinase kinase, MAP kinase kinase and MAP kinase. *Curr. Opin. Gen. Devel.* **4**: 82-89.

Matsuda, M., B.J. Mayer, Y. Fukui and H. Hanafusa. 1990. Binding of transforming protein, P47*gag-crk*, to a broad range of phosphotyrosine-containing proteins. *Science* **248**: 1537-1539.

Matsuda, M., S. Tanaka, S. Nagata, A. Kojima, T. Kumata and M. Shibuya. 1992. Two species of human *CRK* cDNA encode proteins with distinct biological activities. *Molec. Cell. Biol.* **12**: 3482-3489.

Mayer, B.J., M. Hamaguchi and H. Hanafusa. 1988. A novel viral oncogene with structural similarity to phospholipase C. *Nature* **332**: 272-275.

Mayer, B.J. and H. Hanafusa. 1990. Mutagenic analysis of the v-*crk* oncogene: requirement for SH2 and SH3 domains and correlation between increased cellular phosphotyrosine and transformation. *J. Virol.* **64**: 3581-3589.

McGlade, C.J., C. Ellis, M. Reedijk, D. Anderson, G. Mbamalu, A.D. Reith, G. Panayotou, P. End, A. Bernstein, A. Kazlauskas, M.D. Waterfield and T. Pawson. 1992. SH2 domains of the p85α subunit of phosphatidylinositol 3-kinase regulate binding to growth factor receptors. *Molec. Cell. Biol.* **12**: 991-997.

McPherson, P.S., H.J. Czernik, T.J. Chilcote, F. Onofri, F. Benefati, P. Greengard, J. Schlessinger and P. De Camilli. 1994. Interaction of Grb2 via its Src homology 3 domains with synaptic proteins including synapsin I. *Proc. Natl. Acad. Sci. USA* **91**: 6486-6490.

Meisenhelder, J. and T. Hunter. 1992. The SH2/SH3 domain-containing protein Nck is recognized by certain anti-PLCγ1 monoclonal antibodies, and its phosphorylation on tyrosine is stimulated by platelet-derived growth factor and epidermal growth factor treatment. *Molec. Cell. Biol.* **12**: 5843-5856.

Melton, D.A., P.A. Krieg, M.R. Rebagliati, T. Maniatis, K. Zinn and M.R. Green. 1984. Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Res.* **12**: 7035-7056.

Michiels, F-M., S. Chappuis, B. Caillou, A. Pasini, M. Talbot, R. Monier, G.M. Lenoir, J. Feunteun and M. Billaud. 1997. Development of medullary thyroid carcinoma in transgenic mice expressing the *RET* protooncogene altered by a multiple endocrine neoplasia type 2A mutation. *Proc. Natl. Acad. Sci. USA* **94**: 3330-3335.

Mitelman, F., Y. Kaneko and J. Trent. 1991. Report of the committee on chromosomal changes in neoplasia. *Cytogenet. Cell Genet.* **58**: 1053-1079.

Moran, M.F., C.A. Koch, D. Anderson, C. Ellis, L. England, G.S. Martin and T. Pawson. 1990. Src homology 2 domains direct protein-protein interactions in signal transduction. *Proc. Natl. Acad. Sci. USA* **87**: 8622-8626.

Mori, N., J. Singer-Sam, C.-Y. Lee and A.D. Riggs. 1986. The nucleotide sequence of a cDNA clone containing the entire coding region for the mouse X-chromosome-linked phosphoglycerate kinase. *Gene* **45**: 275-280.

Muller, W.J., E. Sinn, R. Wallace, P.K. Pattengale and P. Leder. 1988. Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-*neu* oncogene. *Cell* **54**: 105-115.

Mulligan, L.M., J.B.K. Kwok, C.S. Healey, M.J. Elsdon, C. Eng, E. Gardner, D.R. Love, S.E. Mole, J.K. Moore, L. Papi, M.A. Ponder, H. Telenius, A. Tunnacliffe and B.A.J. Ponder. 1993. Germ-line mutations of the *RET* proto-oncogene in multiple endocrine neoplasia type 2A. *Nature* **363**: 458-460.

Nishikawa, R., X.-D. Ji, R.C. Harmon, C.S. Lazar, G.N. Gill, W.K. Cavenee and H.-J.S. Huang. 1994. A mutant epidermal growth factor receptor common in human glioma confers enhanced tumoigenicity. *Proc. Natl. Acad. Sci. USA* **91**: 7727-7731.

Okutani, T., Y. Okabayashi, Y. Sugimato, K. Sakaguchi, K. Matuoka, T. Takenawa and M. Kasuga. 1994. Grb2/Ash binds directly to tyrosines 1068 and 1086 and indirectly to tyrosine 1148 of activated human epidermal growth factor receptors in intact cells. *J. Biol. Chem.* **269**: 31310-31314.

Olivier, J.P., T. Raabe, M. Henkemeyer, B. Dickson, G. Mbamalu, B. Margolis, J. Schlessinger, E. Hafen and T. Pawson. 1993. A Drosophila SH2-SH3 adaptor protein implicated in coupling the sevenless tyrosine kinase to an activator of Ras guanine nucleotide exchange, Sos. *Cell* **73**: 179-191.

Ooi, J., V. Yajnik, D. Immanuel, M. Gordon, J.J. Markov, A.M. Buchberg and B. Margolis. 1995. The cloning of Grb 10 reveals a new family of SH2 domain proteins. *Oncogene* **10**: 1621-1630.

Otsu, M., I. Hiles, I. Gout, M.F. Fry, F. Ruiz-Larrea, G. Panayotou, A. Thompson, R. Dhand, J. Hsuan, N. Totty, A.D. Smith, S.J. Morgan, S.A. Courtneidge, P.J. Parker

and M.D. Waterfield. 1991. Characterization of two 85 kD proteins that associate with receptor tyrosine kinases, middle-T/pp60$^{c\text{-}src}$ complexes, and PI3-kinase. *Cell* **65**: 91-104.

Panayotou, G. and W.D. Waterfield. 1993. The assembly of signaling complexes by receptor tyrosine kinases. *BioEssays* **15**: 171-177.

Pawson, T. 1988. Non-catalytic domains of the cytoplasmic protein-tyrosine kinases: regulatory elements in signal transduction. *Oncogene* **3**: 491-495.

Pawson, T. 1992. Tyrosine kinases and their interactions with signaling proteins. *Curr. Opin. Gen. Devel.* **2**: 4-12.

Pawson, T. and G.D. Gish. 1992. SH2 and SH3 domains: from structure to function. *Cell* **71**: 359-362.

Pelicci, G., L. Lanfrancone, F. Grignani, J. McGlade, F. Cavallo, G. Forni, I. Nicoletti, F. Grignani, T. Pawson and P.G. Pelicci. 1992. A novel transforming protein (SHC) with an SH2 domain is implicated in mitogenic signal transduction. *Cell* **70**: 93-104.

Pelicci, G., L. Lanfrancone, A.E. Salcini, A. Romano, S. Mell, M.G. Borrello, O. Segatto, P.P. DiFiore and P.G. Pelicci. 1995. Constitutive phosphorylation of Shc proteins in human tumors. *Oncogene* **11**: 899-907.

Pendergast, A.M., L.A. Quilliam, L.D. Cripe, C.H. Bassing, Z. Dai, N. Li, A. Batzer, K.M. Raban, C.J. Der, J. Schlessinger and M.L. Gishizky. 1993. *BCR-ABL*-induced oncogenesis is mediated by direct interaction with the SH2 domain of the *GRB-2* adapter protein. *Cell* **75**: 175-185.

Prigent, S.A. and W.J. Gullick. 1994. Identification of c-ErbB-3 binding sites for phosphatidylinositol 3'-kinase and SHC using an EGF receptor/c-erbB-3 chimera. *EMBO J.* **13**: 2831-2841.

Raabe, T., J.P. Olivier, B. Dickson, X. Liu, G.D. Gish, T. Pawson and E. Hafen. 1995. Biochemical and genetic analysis of the Drk SH2/SH3 adaptor protein of *Drosophila*. *EMBO J.* **14**: 2509-2518.

Ren, R., B.J. Mayer, P. Cicchetti and D. Baltimore. 1993. Identification of a ten amino acid proline-rich SH3 binding site. *Science* **259**: 1157-1161.

Ricci, A., L. Lanfrancone, R. Chiari, G. Berlado, C. Pertica, P.G. Natali, P.G. Pelicci and O. Segatto. 1995. Analysis of protein-protein interactions involved in the activation of the Shc/Grb2 pathway by the ErbB-2 kinase. *Oncogene* **11**: 1519-1529.

Roussel, M.F., J.R. Downing, C.W. Rettenmier and C.J. Sherr. 1988. A point mutation in the extracellular domain of the human CSF-1 receptor (*c-fms* proto-oncogene product) activates its transforming potential. *Cell* **55**: 979-988.

Rozakis-Adcock, M., J. McGlade, G. Mbamalu, G. Pelicci, R. Daly, W. Li, A. Batzer, S. Thomas, J. Brugge, P.G. Pelicci, J. Schlessinger and T. Pawson. 1992. Association of the Shc and Grb2/Sem5 SH2-containing proteins is implicated in activation of the Ras pathway by tyrosine kinases. *Nature* **360**: 689-692.

Sadowski, I., J.C. Stone and T. Pawson. 1986. A non-catalytic domain conserved among cytoplasmic tyrosine kinases modifies the kinase function and transforming activity of Fujinami sarcoma virus P130$^{gag\text{-}fps}$. *Molec. Cell. Biol.* **6**: 4396-4408.

Salcini, A.E., J. McGlade, G. Pelicci, I. Nicoletti, T. Pawson and P.G. Pelicci. 1994. Formation of Shc-Grb2 complexes is necessary to induce neoplastic transformation by overexpression of Shc proteins. *Oncogene* **9**: 2827-2836.

Santoro, M., F. Carlomagno, A. Romano, D.P. Bottaro, N.A. Dathan, M. Grieco, A. Fusco, G. Vecchio, B. Matoskova, M.H. Kraus and P.P. DiFiore. 1995. Activation of *RET* as a dominant transforming gene by germline mutations of MEN2A and MEN2B. *Science* **267**: 381-383.

Sastry, L., W. Lin, W.T. Wong, P.P. DiFiore, C.A. Scoppa and C.R. King. 1995. Quantitative analysis of Grb2-Sos1 interaction: the N-terminal SH3 domain of Grb2 mediates affinity. *Oncogene* **11**: 1107-1112.

Schechter, A.L., D.F. Stern, L. Vaidyanathan, S.J. Decker, J.A. Drebin, M.I. Greene and R.A. Weinberg. 1984. The *neu* oncogene: an *erbB* -related gene encoding a 185,000-M_r tumor antigen. *Nature* **312**: 513-516.

Schechter, A.L., M-C.Hung, L. Vaidyanathan, R.A. Weinberg, T.L. Vang-Feng, V. Francke, A. Ullrich and L. Coussens. 1985. The *c-neu* gene: an *erbB* -homologous gene distinct from and unlinked to genes encoding the EGF receptor. *Science* **229**: 976-978.

Schlessinger, J. 1994. SH2/SH3 signaling proteins. *Curr. Opin.. Gen. Devel.* **4**: 25-30.

Seed, B. and A. Aruffo. 1987. Molecular cloning of the CD2 antigen, the T cell erythrocyte receptor, by rapid immunoselection procedure. *Proc. Natl. Acad. Sci. USA* **84**: 3365-3369.

Segatto, O., G. Pelicci, S. Giuli, G. Digiesi, P.P. DiFiore, J. McGlade, T. Pawson and P.G. Pelicci. 1993. Shc proteins are substrates of ErbB-2 kinase. *Oncogene* **8**: 2105-2112.

Siegel, P.M., D.L. Dankort, W.R. Hardy and W.J. Muller. 1994. Novel activating mutations in the *neu* proto-oncogene involved in the induction of mammary tumors. *Molec. Cell. Biol.* **14**: 7068-7077.

Simon, M.A., G.S. Dodson and G.M. Rubin. 1993. An SH3-SH2-SH3 protein is required for p21^{Ras1} activation and binds to Sevenless and Sos proteins *in vitro*. *Cell* **73**: 167-177.

Simon, M.A. 1994. Signal transduction during the development of the *Drosophila* R7 photoreceptor. *Devel. Biol.* **166**: 431-442.

Sinn, E., W. J. Muller, P.K. Pattengale, I. Tepler, R. Wallace and P. Leder. 1987. Coexpression of MMTV/v-HA-ras and MMTV/c-myc genes in transgenic mice: synergistic action of oncogenes *in vivo*. *Cell* **49**: 469-475.

Skolnik, E.Y., B. Margolis, M. Mohammadi, E. Lowenstein, R. Fischer, A. Drepps, A. Ullrich and J. Schlessinger. 1991. Cloning of PI3 kinase-associated p85 utilizing a novel method for expression/cloning of target proteins for receptor tyrosine kinases. *Cell* **65**: 83-90.

Skolnik, E.Y., A. Batzer, N. Li, C-H. Lee, E. Lowenstein, M. Mohammadi, B. Margolis and J. Schlessinger. 1993. The function of GRB2 in linking the insulin receptor to Ras signaling pathways. *Science* **260**: 1953-1955.

Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98**: 503-517.

Stahl, M.L., C.R. Ferenz, K.L. Kelleher, R.W. Kriz and J.L. Knopf. 1988. Sequence similarity of phospholipase C with the non-catalytic region of src. *Nature* **332**: 269-272.

Stein, D., J. Wu, S.A.W. Fuqua, C. Roonprapunt, V. Yajnik, P. D'Eustachio, J.J. Moskow, A.M. Buchberg, C.K. Osborne and B. Margolis. 1994. The SH2 domain protein GRB-7 is co-amplified, overexpressed and in a tight complex with HER2 in breast cancer. *EMBO J.* **13**: 1331-1340.

Stephens, R.M., D.M. Loeb, T.D. Copeland, T. Pawson, L.A. Greene and D.R. Kaplan. 1994. Trk receptors use redundant signal transduction pathways involving Shc and PLCγ1 to mediate NGF responses. *Neuron* **12**: 1-20.

Stern, M.J. and D.L. DeVore. 1994. Extending and connecting signaling pathways in *C. elegans*. *Devel. Biol.* **166**: 443-459.

Tanaka , M., R. Gupta and B.J. Mayer. 1995. Differential inhibition of signaling pathways by dominant-negative SH2/SH3 adapter proteins. *Molec. Cell. Bio.* **15**: 6829-6837.

Tanaka, S., T. Ito and J.R. Wands. 1996. Neoplastic transformation induced by insulin receptor substrate-1 overexpression requires an interaction with both Grb2 and Syp signaling molecules. *J. Biol. Chem.* **271**: 14610-14616.

Touhara, K., J. Inglese, J.A. Pitcher, G. Shaw and R.J. Lefkowitz. 1994. Binding of G protein βγ subunits to pleckstrin homology domains. *J. Biol. Chem.* **269**: 10217-10220.

Ullrich, A. and J. Schlessinger. 1990. Signal transduction by receptors with tyrosine kinase activity. *Cell* **61**: 203-212.

van der Geer, P., S. Wiley, V. K-M. Lai, J.P. Olivier, G.D. Gish, R. Stephens, D. Kaplan, S. Shoelson and T. Pawson. 1995. A conserved amino-terminal Shc domain binds to phosphotyrosine motif in activated receptors and phophopeptides. *Curr. Biol.* **5**: 404-412.

Vogel, W., R. Lammers, J. Huang and A. Ullrich. 1993. Activation of a phosphotyrosine phosphatase by tyrosine phosphorylation. *Science* **259**: 1611-1614.

Webster, M.A., R.D. Cardiff and W.J. Muller. 1995. Induction of mammary epithelial hyperplasias and mammary tumors in transgenic mice expressing a murine mammary tumor virus / activated c-*src* fusion gene. *Proc. Natl. Acad. Sci. USA* **92**: 7849-7853.

Yao, L., Y. Kawakami and T. Kawakami. 1994. The pleckstrin homology domain of Bruton tyrosine kinase interacts with protein kinase C. *Proc. Natl. Acad. Sci. USA* **91**: 9175-9179.

Yokote, K., S. Mori, K. Hansen, J. McGlade, T.Pawson, C.H. Heldin and L. Claesson-Welsh. 1994. Direct interaction between Shc and platelet-derived growth factor β-receptor. *J. Biol. Chem.* **269**: 15337-15343.

Zipursky, S.L. and G.M. Rubin. 1994. Determination of neuronal cell fate: lessons from the R7 neuron of *Drosophila. Ann. Rev. Neurosci.* **17**: 373-397.