THE GENETIC DISSECTION OF NEU-MEDIATED TRANSFORMATION

By

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A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfillment of the Requirements

for the Degree

Doctorate of Philosophy

McMaster University

GENETIC DISSECTION OF NEU-MEDIATED TRANSFORMATION

Doctorate of Philosophy (1999) McMaster University

(Biology) Hamilton, Ontario

TITLE: The Genetic Dissection Of Neu-Mediated Transformation

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NUMBER OF PAGES: xix, 265

ABSTRACT

The ErbB-2/Neu receptor tyrosine kinase (RTK) plays a causal role in mammary tumourigenesis in a significant proportion of women, yet mechanistically does so in ill defined manner. The carboxy-terminus of the receptor, containing several phosphotyrosine residues, is thought to mediate transformation through interactions with cytoplasmic SH2/PTB-containing signaling molecules. To assess the role of each tyrosine phosphorylation site in cellular transformation, I created and analysed several series of phosphorylation mutants. While mutation of individual sites (Y1028, Y1144, Y1201, Y1226/7, Y1253) had little effect on *neu*-mediated transformation, the simultaneous mutation of each known Neu autophosphorylation site rendered the receptor transformation impaired. I assessed the role of each tyrosine phosphorylation site in cellular transformation, by restoring individual tyrosine residues to this transformation debilitated neu mutant. Reversion of any one of four mutated sites (Y1144, Y1201, Y1226/7, Y1253) restored wild-type transforming activity. These transforming "add-back" mutants displayed Ras-dependent signaling, which was further correlated with the ability of two of these "add-back" mutants to bind either the GRB2 (to Y1144) or SHC (to Y1227) adaptor molecules known to couple RTKs to Ras. Microinjection experiments suggest that Y1144 and Y1227 mediate Grb2 dependent and independent signals respectively. Additionally, several proteins were found to interact with the terminal phosphorylation site (Y1253) and using finer mutagenesis, transformation from Y1253 was correlated with the binding of an unknown 34kDa protein.

By contrast, restoration of tyrosine 1028 to transforming add-back mutants suppressed the transformation. Mechanistically transformation repression correlated with a reduction in the ability to bind Shc and Grb2. As decreased Grb2 association was reversible by protein tyrosine phosphatase (PTP) inhibition, these data suggest that tyrosine 1028 acts to decrease transformation, in part, through the activation of PTPs. I used a RT-PCR approach to isolate PTPs expressed in the mammary gland to identify

PTPs relevant in Neu signaling. One candidate, Shp-1, is overexpressed in mammary tumours and epithelial cells derived thereof and inhibits Neu-mediated transformation genetically upstream of Ras.

These data argue not only that the transformation by activated *neu* is mediated primarily through Ras by multiple signaling proteins and is regulated in a negative manner by the Shp-1 PTP, but it also provides much insight in rational drug targets for ErbB-2-mediated human diseases .

Acknowledgments

This is the page where I'm supposed to give thanks those who helped

Acknowledgments - continued

While at McMaster, I have met a great number of people who have enriched my experience and provided guidance and humor thus making my studies, on the whole, most enjoyable. My supervisor, Bill Muller, has endured more from me than most anyone should and over the years he has become a mentor, at times an audience for my mania and a great friend. Bill has given me the freedom to pursue my ideas under the umbrella of his research focus thus providing nurturing environment for exploration. As a result, I can be considered a Bill Muller product perhaps akin to Dr. Frankenstein's infamous creation.

I am grateful to the various members of my ever evolving committee Drs. Silvia Bacchhetti, Jim Smiley, John Hassell and Michael Rudnicki who have provided input that has help shaped my approach to science and this work. Pat Hayward must be thanked for the long chats and keeping me abreast of the impending deadlines: I am sorry for wrecking the department average.

I will always have fond memories of Uzma Ashraf and Harold Kim, two kind souls I have had the pleasure knowing and of Monica Graham, who has weathered many a storm of my personality yet remains a friend. I will not forget (without extensive therapy) many of the fun moments with Bill, Monica, Harold, Marc Webster, John Hutchinson, Mike Raugh and Don White and hope that I in return have made your time more enjoyable. I obligated to Monica Graham for keeping the ship running and I must "thank" the army of undergrads who worked with me as well as all members of our lab both past and present.

I am grateful to my parents who have provided the impetus and means for success. To Rick for love and support, "a heartfelt gratitude" hardly seems worthy. It seems all the childhood "shots" to the head had little effect! Thanks to the Beth and Mel LeCouter and the LeCouter family for their unwavering support and love. Most importantly, to the loves of my life, Jennifer and Ainsley, I cannot begin to express the joy you bring. I will be ever indebted to you both as you have given me perspective on what is important in life, that is love and family. You all mean the world to me.

Dedication

To Ainsley,

With your birth came love

As you grow so does my heart

CONTRIBUTIONS BY OTHERS

Some of the work described directly or indirectly in my thesis was generated with the aid of some very adept colleagues both within our lab and in different institutions. As I have benefited greatly from their assistance, I wish to acknowledge their generous contributions.

The microinjection experiments in Table 3.4 and in Figure 3.12 were performed in the laboratory of Michael F. Moran (Banting and Best Research Institute, Toronto, Canada) by Zhixaing Wang, a post-doctoral fellow Neil Warner, as a fourth year student in our lab, carried out the Rap1A transformation reversion experiment presented in Table 3.4. In the laboratory of Philip E. Branton (McGill, Montreal, Canada), R. Bruce Rowely carried out pNPPase experiments (Figure 5.6B). Jennifer LeCouter, in the laboratory of Micheal A. Rudnicki (McMaster University) generated the two deletion mutants used in Table 3.2. Philip Leder (Harvard), Peter M. Siegal and Chantale T. Guy provided murine tumor tissues and Robert Cardiff (UC Davis) supplied human breast samples used in Figure 5.4.

As fourth year students in our laboratory, Neera Jeyabala, Peter M. Siegal and JoAnn Attard generated YE-APEY and YE-DPEY, sequenced *ptp*M2, and subcloned the YAX_3B mutants respectively.

Oligonucleotides synthesis and DNA sequencing of Neu-autophosphorylation mutants were respectively performed by Dinsdale Gooden and Brian Allore (MOBIX, McMaster University, Hamilton).

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List of Used Abbreviations

aa	amino acid(s)
BrdU	5-bromo-2'-deoxyuridine
BSA	bovine serum albumin
CA	carbonic anhydrase
CDK	cyclin dependent protein kinases
CHAPS	3-[(3-cholamidopropyl)-dimethyl-ammnio]-1-propanesulfonate
CSF-1	colony stimulating factor 1
DAG	diacylglycerol
DCIS	ductal carcinoma *in situ*
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ER	estrogen receptor
Erk	extracellular regulated kinases
FGF	fibroblast growth factor
GAP	GTPase Activating protein
GDS	guanine nucleotide dissociation factors
GEFs	guanine nucleotide exchange factors
Grb	growth factor receptor bound protein
GST	glutathione S-transferase
HRP	horse raddish peroxidase
IB	immunoblots
IP	immunoprecipitations
IP_3	inositol 1,4,5-tris-phosphate
IPTG	isopropyl-β-D-thiogalactopyranoside
LTR	long terminal repeat
mAbs	monoclonal antibodies
MAPK	mitogen activated kinase
MBP	myelin basic protein
MEK	mitogen activated kinase kinases
MMTV	murine mammary tumour virus
MT	middle tumour antigen
NDF	neu differentiation factor also known as neuregulin
NPP	nitrophenol phosphate
NRG	neuregulin
nt	nucleotide(s)
PBS	phosphate buffered saline
PDGFR	platelet derived growth factor receptor
PGK	phosphoglycerate kinase
PGK	phosphoglycerol kinase
$PI(3,4,5)P_3$	phosphatidylinositol (3,4,5) tris-phosphate
$PI(4,5)P_2$	phosphatidylinositol (4,5)-bis-phosphate
PI3'K	phosphatidylinositiol 3'kinase
PIP_3	phosphatidylinositol (3,4,5) tris-phosphate
PKC	protein kinase C
PLC	phospholipase C
PMA	phorbol 12-myristate 13-acetate
PMSF	phenylmethylsulfonyl fluoride
pNPP	p-nitrophenol phosphate
PR	progesterone receptor
PTB	protein tyrosine binding
PTK	protein tyrosine kinase
PTP	protein tyrosine phosphatase

pTyr	phosphotyrosine
PVDF	polyvinylidene diflouride
PyV	Polyomavirus
Rb	retinoblastoma gene product
RTK	receptor protein tyrosine kinase
SH2	Src homology 2
Shc	SH2 and collagen homology proteins
sos	son of sevenless
STAT	signal transducer and activator of transcription
TBS	tris buffered saline
TCA	trichloroacetic acid
TCR	T-cell receptor
TM-PTP	Transmembrane protein tyrosine phosphatase
TPA	12-*O*-tetradecanophorbol-13-acetate

Chapter 1

Introduction

Women with proliferative disorders of increasing severity (atypical hyperplasias to *in situ* carcinomas to invasive carcinomas) compared to women with proliferative disorders lacking atypia or those with normal epithelium are at increased risk of subsequent breast cancer development (Dupont and Page 1985). Thus it appears that mammary tumorigenesis, like colorectal tumour formation (Fearon and Vogelstein 1990; Vogelstein and Kinzler 1993), may arise in a step-wise fashion involving the acquisition of several distinct genetic perturbations, each contributing to full malignancy. One gene, *erbB*-2, appears to be responsible for the genesis of a significant number of mammary carcinomas.

1.2 The ErbB Family Of Receptor Tyrosine Kinases

1.2.1 The Neu/ErbB-2 proto-oncogene

DNA transfer experiments from chemically-induced (ethylnitrosourea, ENU) rat neuroglioblastomas (Schubert *et al.* 1974) into NIH-3T3 fibroblasts results in the morphological transformation of the latter cells (Shih *et al.* 1979) and correlates with the emergence of a 185kDa phosphoprotein of recipient cell lines (Padhy *et al.* 1982). Isolation of the cDNA encoding the oncogene revealed that *neu* (for neuroblastoma, Schehter *et al.* 1984) is structurally related to the epidermal growth factor receptor (EGFR) tyrosine kinase (Bargmann *et al.* 1986b). Upon sequence comparison with the subsequently cloned normal cellular gene, the oncogene was found to differ by a single nucleotide (Bargmann *et al.* 1986a). This substitution results in a non-conservative amino acid change (Val to Glu) within the transmembrane domain and confers upon the receptor a constitutive ligand-independent kinase activity (see below and Bargmann and Weinberg 1988). The oncogenic form is referred to as "activated *neu*" or neu^{NT} whereas the wild-type rat gene is termed *neu*.

Concurrently, a human gene was isolated on the basis of homology with the viral v-*erbB* oncogene by low stringency screens of cDNA libraries (Coussens *et al.* 1985) and as an amplified locus in a human salivary adenocarcinoma cell line (Yamamoto *et al.* 1986; Semba *et al.* 1985). This gene, named *erbB-2* or *Her2*, displays high sequence homology with *neu* and represents the human ortholog. The ErbB family of receptor tyrosine kinases (RTKs) now contains four members: ErbB-1/EGFR, ErbB-2/Neu, ErbB-3 and ErbB4.

1.2.2 ErbB-2 in human cancers

ErbB-2 displayed 100% amino acid sequence identity to a partial genomic clone of a locus that had two interesting properties. The locus contained sequence similarity but not identity to the EGFR and it appeared to be amplified in a human mammary carcinoma line (King *et al.* 1985). These data prompted Slamon and colleagues to determine if *erbB*-2 gene alterations exist in primary breast cancers (Slamon *et al.* 1987). Their data suggest that the *erbB*-2 locus is amplified in 20-30% of human tumors. Interestingly however, the activating point mutation (V664E) within the transmembrane encoding region has not been found (Lemoine *et al.* 1990; Slamon *et al.* 1989) likely due to a requirement of two nucleotide alterations to produce the same amino acid change in the human gene. Instead, increased expression of wild-type *erbB*-2 is thought to result in human malignancy.

In addition to breast malignancies, gene amplification and elevated expression has been observed in a variety of carcinoma predominantly those of secretory epithelial origin. While overexpression in ovarian and gastric tumours is consistently observed, it has also been detected in a number of non-small cell lung, salivary, bladder-derived, colon and pancreatic tumours, suggesting deregulated *erbB*-2 expression may be responsible for a variety of human pathologies (reviewed in Hynes and Stern 1994).

1.2.3 ErbB-2 as a prognostic marker of recurrent breast cancer

While *erbB*-2 hyperexpression is observed in a variety of adenocarcinoma, it may serve as a useful prognostic marker of aggressive breast and ovarian cancers. Larger epidemiology studies demonstrate an

association of *erbB*-2 amplification and elevated expression of with a poor clinical prognosis in breast cancer patients (reviewed in Gullick 1990; Dhingra and Hortobagy 1996). Indeed, overexpression is detected in 50% of *in situ* and 14% of invasive human ductal carcinomas (reviewed in Mansour *et al.* 1994) while elevated expression is rarely observed in benign breast disorders (hyperplasias and dysplasias) (Allred *et al.* 1992), suggesting that expression of *erbB*-2 leads to a more aggressive tumour phenotype. This hypothesis is consistent with the observation of elevated S-fractions in cases of ductal carcinoma *in situ* (DCIS) (Barnes *et al.* 1990) and in S-phase labeling in invasive comedo DCIS (Borg *et al.* 1989) which overexpress the ErbB-2 protein. Additionally, *erbB*-2 overexpression in ovarian and lung adenocarcinomas correlates with reduced survival (reviewed in Hynes and Stern 1994; Tzahar and Yarden 1998).

In large studies, ErbB-2 shows prognostic abilities in predicting overall survival for node positive but not node negative patients (Ravdin 1995). The correlation of increased ErbB-2 expression and poor prognosis may reflect the current treatment modalities in that ErbB-2 prognosticates mean disease free survival amoungst node positive individuals. As these patients are routinely given chemotherapy/tamoxifen (hormonal and adjuvant therapies), the correlation may reflect treatment resistance and ErbB-2 hyperexpression. Interestingly, node positive patients with ErbB-2 overexpression derive greater benefits from high dose chemotherapies (cyclophosphamide, doxorubicin, fluorouracil) (Muss *et al.* 1994) and increased expression is also indicative of a poor adjuvant hormone treatment response in a subset of individuals (Tripathy and Benz 1994; Mansour *et al.* 1994; Slamon *et al.* 1989, reviewed in Hynes and Stern 1994). Conversely, ErbB-2 negativity correlates with tamoxifen responsiveness. This clinical observation is mirrored in cultured mammary epithelial cell experiments and likely reflects ErbB-2-induced transcriptional repression of the estrogen receptor gene (Benz *et al.* 1992). While evidence suggests ErbB-2 positive tumours or cells derived thereof, display increased resistance to radiation and specific chemotherapeutic agents, some agents (taxol) appear more effective in killing ErbB-2 positive cells than non-expressors in culture (reviewed in Baselga *et al.* 1997).

There has been much controversy of the prognostic utility of ErbB-2 in node negative individuals, the group that would benefit most from an effective prognostic marker (see Dhingra and Hortobagy 1996; Ravdin 1995; Hynes and Stern 1994). Recently, ErbB-2 overexpression has been demonstrated to be prognostic in predicting disease recurrence in node negative patients (Andrulis *et al.* 1998). Thus, in conjunction with other diagnostic markers (tumour size and grade and lymph node, menopausal and ER/PR status), ErbB-2 levels may indicate which treatment modalities will be most effective for the individual patient. Additionally, the prevalence of ErbB-2 overexpression suggests that ErbB-2 itself would be an effective target for future therapeutics.

1.2.4 ErbB family members in proliferation and oncogenic transformation

A potential oncogenic role of the wild-type receptor was inferred by the demonstration that overexpression of *erbB*-2 was capable of transforming NIH-3T3 fibroblasts in the absence of ligand (Hudziak *et al.* 1987; Di Fiore *et al.* 1987b). Additionally, elevated expression of the EGFR transforms NIH-3T3 cells but does so in a ligand-dependent fashion (Velu *et al.* 1987; Di Fiore *et al.* 1987a). In fibroblasts lacking detectable expression of the ErbB family members, expression of ErbB-2 or EGFR in response to ligand was sufficient in mediating transformation. Moreover, coexpression of ErbB-1 and ErbB-2 lead to a synergistic EGF-induced transforming response. In fact, NDF, a ligand for ErbB-3 and -4 (see section 1.2.7), could effect transformation in cells coexpressing either ErbB-1 or -2 with either ErbB-3 or -4 but not in cells expressing each of the receptors alone (Zhang *et al.* 1996; Cohen *et al.* 1996). Although the lack of transformation by ErbB-3 could be attributed to a decreased kinase activity, ErbB-4 was readily tyrosine phosphorylated in response to NDF-treatment (Zhang *et al.* 1996; Cohen *et al.* 1996). These data suggest that ErbB-1 and ErbB-2 are mitogenic whereas ErbB3 and ErbB-4 are not.

In addition to these effects on transformation, ErbB-2 increases proliferative potential of coexpressed family members. Derivatives of an IL3-dependent cell line (Ba/F3) were made to express each ErbB family member individually or in pairwise combinations. Expression of individual ErbB family

RTKs was insufficient to induce cellular proliferation in the presence of NDF/NRG1, yet proliferative responses were obtained when ErbB-2 was coexpressed with either ErbB3 or ErbB-4 (Riese II *et al.* 1995). Similar results were obtained in 32D myeloid cells (Pinkas-Kramarski *et al.* 1996). Treatment of the Ba/F3-derived cells with ligands capable of binding EGFR resulted in maximal proliferation when Neu was coexpressed (Riese II *et al.* 1996). Moreover, treatment of particular mammary epithelial lines with NDF induces differentiation and not proliferation (Peles *et al.* 1992a; Beerli *et al.* 1995). Taken together these data suggest that ErbB family members harbour distinct capabilities to induce cellular proliferation and differentiation and that ErbB-2 appears to be particularly mitogenic.

1.2.5 Transformation of the murine mammary gland through expression of Neu

Taken together, the previous data strongly suggest deregulated *erbB-2/neu* expression plays a critical role in the genesis of a variety of carcinomas. Because amplification of three genes topoisomerase 2A (*TOP2A*) (Smith *et al.* 1993), Grb7(Stein *et al.* 1994) and *THRA1* (van de Vijver *et al.* 1987) in close proximity to *erbB*-2at 17q21 (Coussens *et al.* 1985) has been documented, it is possible that *erbB*-2 itself may not be responsible for tumour formation. For example, given that the topoisomerase 2A (TOP2A) gene is often coamplified with ErbB-2 and that doxorubicin, the most efficacious chemotherapeutic for breast cancers, targets TOP2A (Smith *et al.* 1993), it is possible that ErbB-2 treatment resistance reflects *TOP2A* amplification and not ErbB-2 activity. To address the causality of elevated *erbB*-2 expression in mammary carcinogenesis, researchers have induced expression of Neu in murine mammary glands via transgenic and retroviral technologies. Expression of constitutively active Neu in the mammary epithelium of transgenic mice (Muller *et al.* 1988; Bouchard *et al.* 1989) or retrovirally infected Rats (Wang *et al.* 1991) results in the rapid induction of mammary tumours that histologically resemble human comedo-carcinomas (Cardiff and Muller 1993). Interestingly, human comedo-carcinomas express elevated ErbB-2 levels (Morrison 1994), have a higher proliferative rate and are clinically more severe than other DCIS (Patchefsky *et al.* 1989; Bieche and Lidereau 1995). Transgenic mice expressing elevated levels of wild-

type Neu develop focal breast tumours of similar comedo-type morphology with a delayed relative onset (Guy *et al.* 1992). 70% of these animals analysed possess metastatic lesions of mammary origin within their lungs after a prolonged period of time. It appears that the majority of tumours arise due to somatic mutation of the transgene (Siegel *et al.* 1994 and Chan, Siegel and Muller unpublished results), which oddly have not been detected in over 150 human breast tumours tested to date. These altered forms, but not the parental wild-type Neu, are tyrosine phosphorylated indicating that they have become catalytically activated (Siegel *et al.* 1994). Indeed, tyrosine phosphorylated forms of ErbB-2 have been detected in primary breast tumour extracts (Wildenhain *et al.* 1990; DiGiovanna and Stern 1995). The results of tissue culture and transgenic experiments, coupled with the epidemiological evidence, define a causal role for *neu/erbB*-2 in the genesis of mammary carcinoma. Moreover, these murine systems should provide useful models to test the efficacy of therapeutic agents in ErbB-2-induced tumours.

1.2.6 The structure of Neu/ErbB-2 and ErbB family members

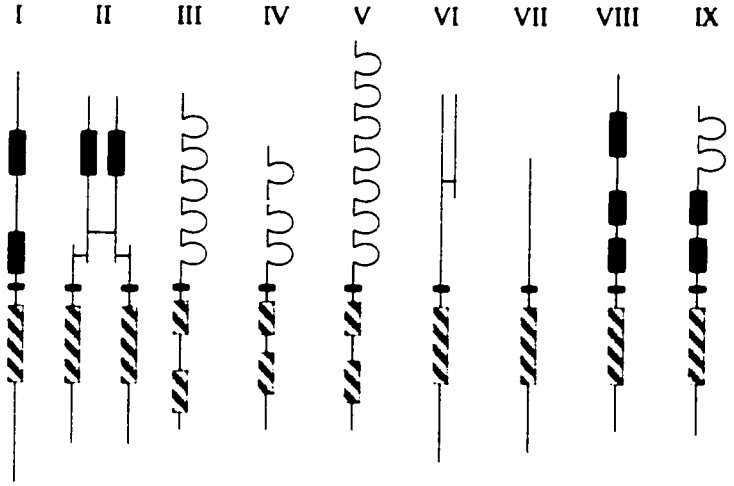
As previously mentioned, ErbB-2 has significant homology with epidermal growth factor receptor tyrosine kinase and along with ErbB-3 (Kraus *et al.* 1989; Plowman *et al.* 1990) and ErbB-4 (Plowman *et al.* 1993), form the class I/ErbB tyrosine kinase family (Carraway III and Cantley 1994) (Figure 1.1A). Structurally, these glycoproteins consist of an extracellular ligand binding domain containing two cysteine rich regions, a single transmembrane hydrophobic sequence and a non-interrupted protein tyrosine kinase (PTK) catalytic domain which is followed by a carboxy-terminal region harbouring several tyrosine residues (Figure 1.1B). The overall structure of ErbB family members is conserved throughout evolution and is reflected in the structures of the Caenorhabditis *elegans (C. elegans) let-23* and *Drosophila DER* gene products.

The ligand binding domain can be subdivided into four subdomains: two cysteine rich regions (subdomains II and IV) are not responsible for directly interacting with ligand but appear to coordinate the flanking ligand binding regions (subdomains I and III). It is these latter regions that confer ligand

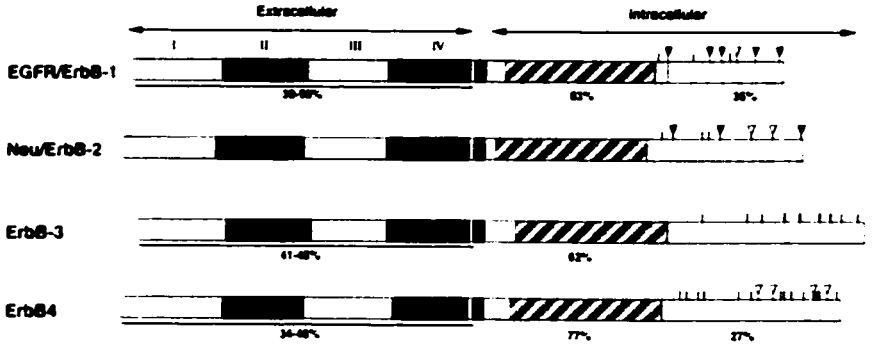
Figure 1.1 Vertebrate receptor tyrosine kinases: ErbB family member structure.

(**A**) Depicted are the overall structures of the distinct RTK subfamilies modified from Fantl et al (Fantl *et al.* 1993). The following structural features are highlighted: tyrosine kinase domains (diagonal striped boxes), cysteine rich domains (red boxes), transmembrane domains (black boxes), immunoglobulin-like domains (semi-circles), fibronectin type III domains (horizontal striped green boxes) and acid rich regions (open box). Horizontal lines represent disulphide bonds. (**B**) Schematic representation of the ErbB RTK family depicting the functional domains and degree of amino acid identity to ErbB-2. The extracellular portion is depicted as containing four subdomains (I-IV) of which two are cysteine rich regions (red). A single transmembrane domain (black) and the tyrosine kinase domain (striped) are indicated. Known or suspected tyrosine phosphorylation sites within the carboxy-terminal region are depicted by closed and open triangles respectively, while additional tyrosine residues are indicated by vertical lines.

A


I
II
III
IV
V
VI
VII
VIII
IX

B


Extracellular
Intracellular
I
II
III
IV
EGFR/ErbB-1
Neu/ErbB-2
ErbB-3
ErbB4

specificity to each receptor (Lax *et al.* 1988 reviewed in Tzahar and Yarden 1998). The extracellular subdomains of ErbB-2/Neu share the highest homology to EGFR (39-50%).

The hydrophobic transmembrane domain forms an alpha-helix *in vitro* (Gullick *et al.* 1992), bisects the protein and is followed by a 38 amino acid juxtamembrane region. Within the juxtamembrane domain are conserved phosphorylation sites for protein kinase C (PKC) (with sites in EGFR, ErbB-2) and mitogen activated kinases (MAPK) Erk1 and Erk2 (EGFR, ErbB-2, ErbB-4). While it is unclear if phosphorylation by MAPK has physiological ramifications, TPA induced phosphorylation of EGFR T654, presumably through PKC, results in a reduction of kinase activity through decreased high affinity EGF binding sites without internalization of the receptor (Lin *et al.* 1986; Livneh *et al.* 1988). TPA also induces a decrease in Neu kinase activity (Dobashi *et al.* 1989; Cao *et al.* 1991), perhaps reflecting a negative feedback independent of receptor internalization.

As with all RTKs the catalytic domain exhibits the highest region of similarity (63-83%) between ErbB family members. It is however of interest that two residues which are either highly conserved (E759H) or invariant (D834N) amoungst protein kinases are not conserved in ErbB-3 (Kraus *et al.* 1989; Plowman *et al.* 1990). Of these, the aspartic acid, invariant amoungst all protein kinases (Hunter 1991), is located in the active site of protein kinase A and plays a critical role in the phosphotransferase reaction (Knighton *et al.* 1991): ErbB-3 contains an asparagine residue in the analogous position. Significantly, alteration of this aspartic acid to asparagine abolishes catalytic activity of the v-fps and c-kit PTK (Moran *et al.* 1988) and is partially responsible for the severely impaired kinase activity of ErbB-3 (Guy *et al.* 1994b; Prigent and Gullick 1994). Restoration of these two residues to glutamic and aspartic acid does not, however, reconstitute catalytic activity (Prigent and Gullick 1994).

The C-terminal region is quite heterogeneous amoungst family members (227-362 residues) with highest homology existing between EGFR and ErbB-2. This region is characterized as being proline rich and contains several conserved stretches of 3-7 amino acids centered around tyrosine residues. The C-terminal tail has the ability to impart receptor-specific signaling to chimeric PTKs (Di Fiore *et al.* 1990).

1.2.7 Receptor activation

1.2.7.1 *Ligand binding and heterodimerization*

Numerous ligands exist for the ErbB family members (reviewed in Tzahar and Yarden 1998). EGFR binds several distinct ligands each containing an EGF homology motif: these include EGF, transforming growth factor α (TGFα), amphiregulin, betacellulin, heparin binding EGF-like growth factor (reviewed in Hynes and Stern 1994; Tzahar and Yarden 1998). Neuregulins, the ligands for ErbB-3 and ErbB-4, represent multiple isoforms of the same gene and are also known as gp30 (Lupo *et al.* 1990), heregulins (Holmes *et al.* 1992), Neu differentiating factor (NDF) (Peles *et al.* 1992a; Wen *et al.* 1992), glial growth factor (Marchionni *et al.* 1993) and acetylcholine receptor inducing activity (ARIA) (Corfas *et al.* 1993; Falls *et al.* 1993). By contrast, no ligand has been cloned for ErbB-2/Neu although several activities have been purified for some time (Dobashi *et al.* 1991; Lupo *et al.* 1992; Samanta *et al.* 1994; Wu *et al.* 1994). A factor, termed Cripto, has been cloned and which bears significant homology to EGF. It does not activate the known ErbB family members as assessed by increased phosphotyrosine content yet it does activate components of the Ras-Raf-MEK-MAPK cascade. Thus it has been suggested that an additional ErbB RTK may exist (Kannan *et al.* 1997). The use of multiple ligands for a single ErbB receptor is evolutionarily conserved. This is exemplified in *Drosophila* DER where at least three activating ligands (*spitz, vein, gurken*) exist as well as a DER antagonist, *argos*, of which a mammalian counterpart has yet to be described (Schweitzer and Shilo 1997).

Ligand-binding induces homo- or hetero- dimerization of receptor family members (Yarden and Schlessinger 1987; Heldin 1995). It is unclear whether monomeric ligand induces a conformational change (Lemmon *et al.* 1997) exposing a putative dimerization domain or whether the ligand contains two binding regions and simultaneously interacts with both monomers and thus being functionally bivalent (Tzahar *et al.* 1997). If the bivalency model holds true, it is quite unlike that observed for the growth hormone receptor where a single ligand has two receptor binding sites and displays nM affinities for both sites (Wells 1996). For the ErbB family ligands tested there exists a high affinity binding site (K_D~nM) and a

low affinity (~uM) binding site (Tzahar *et al.* 1997). Regardless of the mechanism involved, ErbB-2 coexpression with EGFR demonstrates increased EGF-ligand affinity (Sliwkowski *et al.* 1994; Karunagaran *et al.* 1996).

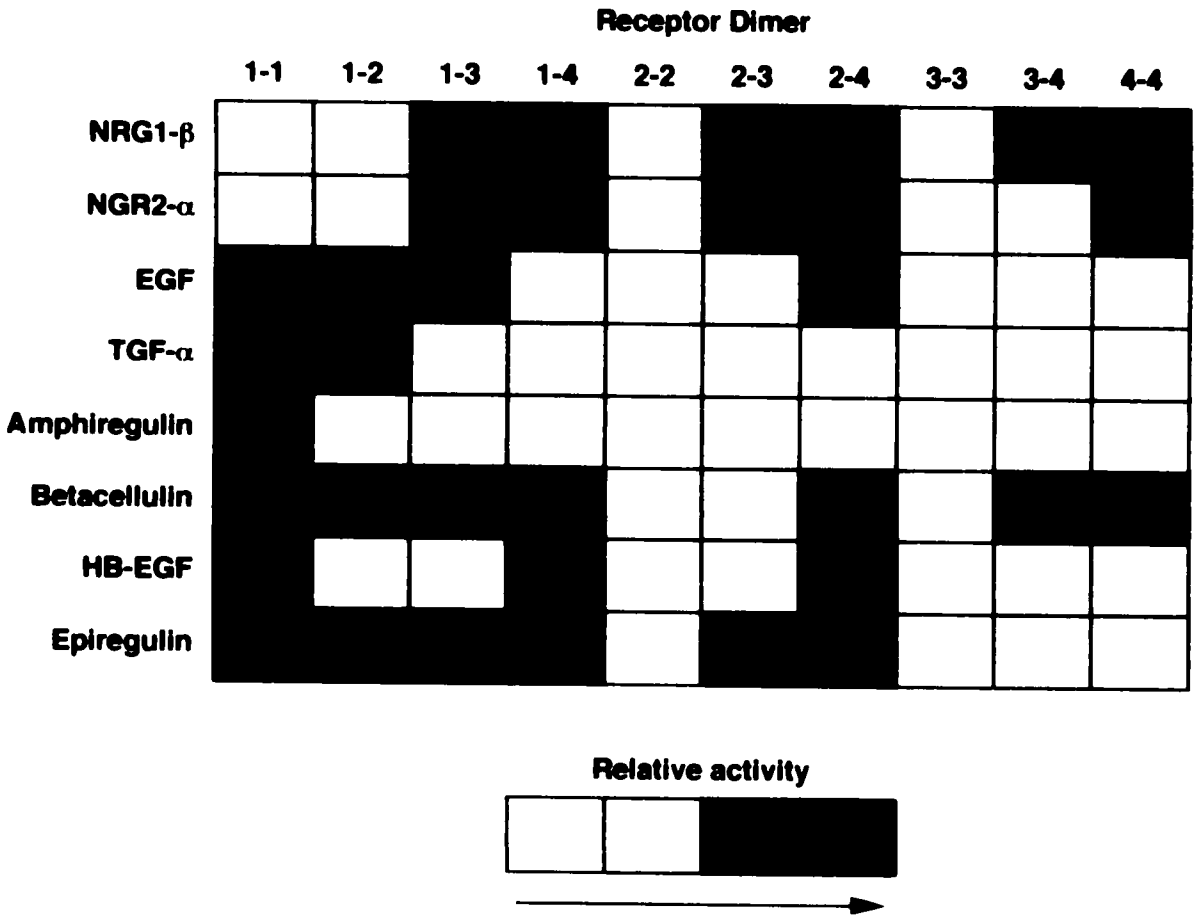
Different ligands can induce formation of specific heterodimerization partners. For example, although ErbB-2 does not directly bind EGF or NRG1, EGF induces the formation of EGFR/ErbB-2 heterodimers (Wada *et al.* 1990) whereas NRG1 isoforms induce ErbB-2/ErbB-3 heterodimers (Tzahar *et al.* 1997). The data thus far suggest that ErbB-2 is preferentially recruited to form heterodimers with each of the ErbB family members in response to treatment with the known ErbB ligands (Tzahar and Yarden 1998). While ErbB3 is a ligand for NRG1, it displays a low affinity (~2nM) which is dramatically increased to 0.02nM by ErbB-2 expression (Sliwkowski *et al.* 1994; Pinkas-Kramarski *et al.* 1996). Moreover, coexpression of ErbB-2, but not EGFR, with ErbB3 leads to a concomitant deceleration in the off rate of the ligand (Pinkas-Kramarski *et al.* 1996). Of these dimer pairs, it appears that EGF- or NRG1-induced heterodimers with ErbB-2 are more stable and possess increased signaling capacities over homodimers. Additionally, NRG1-induced ErbB-3/ErbB-4 heterodimers are the least stable and effective complexes. Other heterodimer combinations have intermediate proliferative signaling abilities (Tzahar and Yarden 1998). The specificities of ligand-induced activation of the ErbB family members is summarized in Figure 1.2. Thus the increased proliferative potential of ErbB-2 heterodimers is, in part, reflective of increased ligand binding abilities.

1.2.7.2 *Auto- and trans-phosphorylation of ErbB family members*

Ligand-induced dimerization increases catalytic activity which is first manifested by trans- and auto- phosphorylation of distinct tyrosine residues primarily located within the carboxy-tail region. This activation presumably is a result of a particular intracellular conformational change as dimerization alone is not sufficient for Neu PTK activation (Burke *et al.* 1997). Several lines of evidence support transphosphorylation of dimerization partners and the functional consequences. Coexpression of wild type EGFR and a kinase deficient Neu mutant or a kinase deficient EGFR and wild-type ErbB-2 resulted in

Figure 1.2 ErbB specificity of EGF-like ligands.

Each column represents one of ten possible dimeric complexes of ErbB proteins. The extent of response to various ligands (horizontal rows) is shown by coloured boxes; red color indicates a potent response, wand white stands for no response. Response that are either detectable at high ligand concentration, or require extreme overexpression, are shown as yellow or orange boxes. Note that ErbB-3 homodimers bind all NRGs, but their response is defective due to an inactive kinase domain. ErbB-2 fails to interact with any of these ligands and thus is unresponsive as a homodimer. Figure and legend modified from (Tzahar and Yarden 1998).


Receptor Dimer
1-1
1-2
1-3
1-4
2-2
2-3
2-4
3-3
3-4
4-4
NRG1-β
NGR2-α
EGF
TGF-α
Amphiregulin
Betacellulin
HB-EGF
Epiregulin
Relative activity

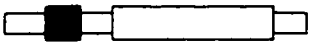
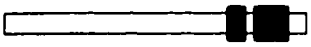
tyrosine phosphorylation of both heterodimer partners in an EGF-dependent manner (Kashles *et al.* 1991; Qian *et al.* 1994; Spivak-Kroizman *et al.* 1992). Additionally, overexpression of kinase deficient EGFR can prevent transformation by EGFR in fibroblasts (Kashles *et al.* 1991). Transphosphorylation is inferred as the heterodimers that form are not tyrosine phosphorylated. ErbB-3 homodimers have little catalytic activity when expressed in the absence of other family members yet are readily phosphorylated when coexpressed with a family member in a ligand dependent manner (Riese II *et al.* 1995; Tzahar *et al.* 1997). Finally, retention of ErbB-2 in the endoplasmic reticulum through the use of ER-retained single chain mAbs suggests that surface expression of ErbB-2 is required for high efficiency EGFR, ErbB-3 and ErbB-4 tyrosine phosphorylation in response to their respective ligands in several cell lines (Graus-Porta *et al.* 1995; Beerli *et al.* 1995).

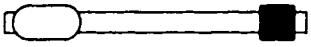
1.3 Specificity of Receptor Tyrosine Kinase Signal Transduction

In response to growth factor stimulation, the predominant tyrosine phosphorylated proteins are often the RTKs themselves, presumably through autophosphorylation. The primary function of RTK tyrosine phosphorylation is to generate binding sites for cytoplasmic or plasma membrane associated proteins involved in transducing proliferative or differentiating signals to the nucleus. Each "signaling protein" contains modular domains enabling direct interaction with specific receptors in a phosphotyrosine-dependent and sequence-specific manner (Pawson 1995). Each of these has an approximately 100 amino acid Src homology 2 (SH2), originally defined in Src family PTKs (Sadowski *et al.* 1986), or the more recently described ~160 residue protein tyrosine binding/interacting (PTB/PID) domain (van der Geer *et al.* 1995), originally discovered in the SH2 and collagen homology (Shc) protein both of which direct receptor recognition. SH2 containing proteins can be loosely grouped into two classes: enzymes and non-enzymatic proteins (Figure 1.3). In addition, SH2 domains are also found in a group of transcription factors. Class I SH2 proteins harbour diverse enzymatic activities which include protein tyrosine kinase (Src PTKs) and phosphatase (Shp) activities and enzymes involved in phospholipid metabolism (phospholipases (PLCγ)

Figure 1.3 SH2 domain containing proteins

Representative SH2 containing proteins of a variety of families. Each protein is depicted as containing one or more of the following functional/structural domains: Protein tyrosine interaction motifs (SH2 ■ and PTB ▭ domain) SH3 (■), PH (▯), guanine nucleotide exchange factor (▥) and GTPase and (▤) domains, protein kinases (▨) and phosphatases (◪) and phospholipid lipases and phosphatases (▧) and ▨) catalytic domains. SH2 containing proteins are grouped into two classes those harbouring enzymatic activities (class I) and those which do not (class II) with the enzymatic activity of the protein or associated protein(s) indicated. The consensus SH2 and PTB binding sites (Songyang *et al.* 1993; Songyang *et al.* 1994; Songyang *et al.* 1995b; Holland *et al.* 1997) are depicted where X indicates no specific amino acid was selected for. Where two phosphotyrosine interaction motifs are present both consensus sequences are given with the top sequence referring to the amino most motif. Not depicted are SH2-proteins whose functions are not well understood and are thus not easily grouped into either class. These class III proteins likely do not associate with enzymes and serve structural roles.

Class I - Enzymes		Enzymatic activity	Consensus Binding Site
Src		protein tyrosine kinase	Y E E I
Bek		protein tyrosine kinase	
Syk		protein tyrosine kinase	Y x x x Y QEI eqt L
Shp		protein tyrosine phosphatase	Y F x F Y IV x VI
PLCγ		phospholipase	Y IVL Ed LIV Y VI IL PIL
SHIP		lipid phosphatase	Y ? ? ?
Ras-GAP		Ras GTPase activating protein	Y x x P Y x x x
Vav		GTP exchange factor	Y x x x
Stat		transcription factor	Y x x x

Class II - Adaptor Proteins		Associated Enzyme	
Shc		Grb2-sos exchange complex	N x x Y Y EI x ILM
Grb2		sos, Ras GTP exchange factor	Y qy E y
p85		p110, lipid kinase	Y MIV x M Y mli x M
Crk		C3G, Rap GTP exchange factor	Y D H P
Nck		sos, Ras GTP exchange factor	Y D E P

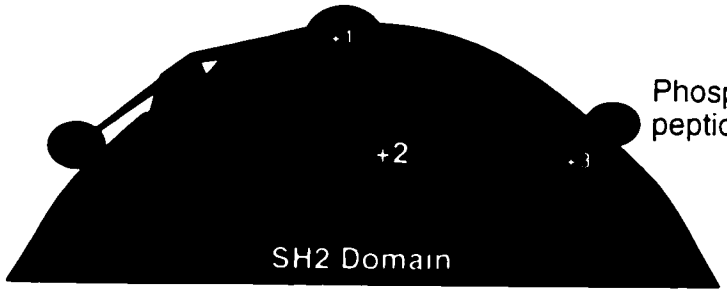
and phospholipid specific phosphatases (SHIP)). Class II SH2 proteins have no known enzymatic activity or homology with the catalytic domains of enzymes. Invariably these proteins contain distinct conserved domains enabling intermolecular protein interactions. For example, SH3 domains which recognize specific proline rich linear sequences or themselves contain sequences targeting them for interaction with other proteins (e.g. conserved phosphorylation sites and proline rich SH3 binding motifs) (Pawson 1995; Pawson and Scott 1997). Often class II proteins are complexed with enzymatic "subunits" and are thus called adaptor proteins. This is exemplified by the p85 regulatory subunit of phosphatidylinositol 3'OH kinase (PI3'K) and the growth factor bound 2 (Grb2) protein which bind to receptors through SH2 domains and to enzymes through other distinct motifs: p85 to the 110kDa catalytic PI3'K through a conserved sequence between the SH2 domains and Grb2 to the Sos exchange factor through one of two SH3 domains (see section 1.4).

The crystal structures for several SH2 and PTB domains demonstrate these modules recognize phosphotyrosine motifs in a distinct manner (Figure 1.4). The SH2 domain contains a bipartite binding site: a basic cleft recognizes the phosphotyrosine residue and a variable binding surface which recognizes the +1 to +5 amino acids carboxyl to the phosphotyrosine forming specific interactions with the SH2 surface and in some cases an additional cleft (Overduin *et al.* 1992; Waksman *et al.* 1992; Waksman *et al.* 1993). For recognition the tyrosine must be phosphorylated and increased affinity (to $K_d \sim 10^{-8}$M) requires that a specific carboxyl-terminal sequence be present. Use of degenerate peptide mixtures reveals different consensus binding sequences for a number of SH2 domains: those in p85 select methionine (Met) at +3 whereas Grb2 has a distinct preference for asparagine (Asn) at the +2 position (Songyang *et al.* 1993; Songyang *et al.* 1994). PTB domains on the other hand appear to recognize peptide sequences which form β-turns; in class I PTB domains tyrosine phosphorylation is a prerequisite for high affinity binding while class II domains do not require phosphorylation (Li *et al.* 1997b; Sudol 1998). Again the phosphotyrosine is embedded in a basic phosphotyrosine binding pocket but the -3 to -1 residues preceding phosphotyrosine form a β-turn, wrapping around the PTB surface (Zhou *et al.* 1995; Eck *et al.* 1996). Depending on the

Figure 1.4 SH2 and PTB domain structure and binding specificity

Schematized are a canonical SH2 and PTB domain (green) bound to their respective peptide substrates (red with a blue tyrosine residue). By convention, each residue is number relative to the phosphorylated tyrosine (...-2, -1, pY, +1, +2...). The SH2 domain is based on that crystallized with the Src SH2 consensus phosphorylation site from Polyomavirus middle T antigen Y315 (Waksman *et al.* 1993). In this case the phosphorylated tyrosine residue and the +3 amino acid relative to it are found buried within individual pockets. Specificity of interaction is increased via the phosphotyrosine and the residues to the +1 to +3 (up to +5) positions. Type I PTB domains such as that in Shc and IRS-1 recognize phosphorylated peptides immediately preceded by a β-turn. The residues from -8 to +1 appear to interact with the purified PTB domain. The PTB schematic is based on IRS-1 and Shc crystal structures (Zhou *et al.* 1995; Eck *et al.* 1996).

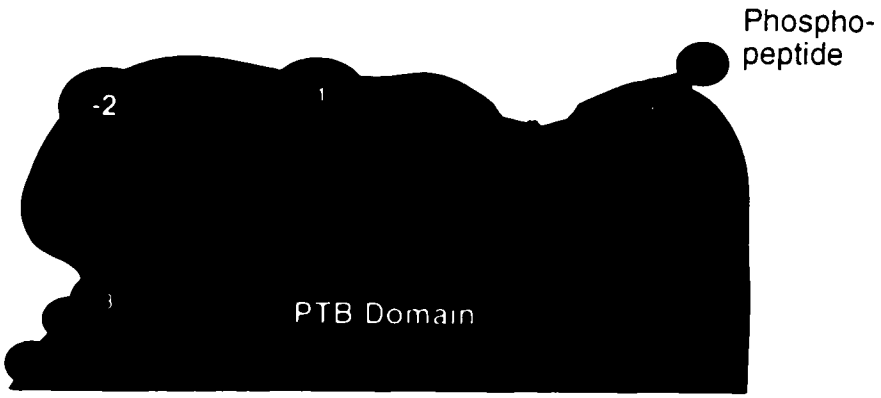
SH2 Domain Specificity
(based on Src SH2 crystal structure)



P
-3 -2 -1 Y +1 +2 +3

COOH-Terminal Specificity

PTBDomain Specificity
(based on SHC and IRS-1 PTB crystal structure)



P
-5 -4 -3 -2 -1 Y +1 +2 +3

NH_2-Terminal Specificity

PTB domain, residues from -8 to -5 form specific generally hydrophobic interactions with the PTB domains. These domains also differ in binding kinetics in that SH2 domains bind with a high affinity but possess a high off rate relative that of tested PTB domains (Laminet *et al.* 1996). Thus each SH2 and PTB containing protein is expected to bind particular target sequences with specific affinities.

Binding to tyrosine phosphorylated RTKs can affect SH2/PTB proteins in a variety of ways, altering subcellular localization (e.g. Sos), stimulating enzymatic activity (e.g. PI3'K) or inducing tyrosine phosphorylation of the signaling protein (e.g. PLCγ) (Pawson 1995). Taken together with the unique SH2/PTB binding specificities, sequence variation about a receptor's tyrosine phosphorylation sites provides a mechanism whereby a RTK can recruit specific SH2/PTB signaling molecules thereby activating distinct intracellular signal pathways. It is thus neither surprising that phosphotyrosine sites are often conserved amoungst RTKs of the same family nor that each RTK often contains distinct sites of phosphorylation. For example, EGFR contains three consensus Grb2 binding sites, ErbB-3 contains six potential p85 PI3'K binding sites, while both contain binding sites for Shc.

Interestingly, several SH2/PTB containing proteins possess oncogenic potential or were isolated by virtue of homology to retroviral transforming or human oncogenes and are thus thought to play positive regulatory roles in transformation and proliferation. Indeed, Shc, Crk and Nck are oncogenic when overexpressed (Chou, Pelicci *et al.* 1992; Matsuda *et al.* 1992). Moreover, v-*crk* v-*fps*, v-*abl* and v-*src* are transforming oncogenes in avian retrovirus while blasts cells of 95% of chronic myelogenous leukemic patients (stem cell neoplasia) contain a chromosomal translocation in the abl PTK gene (Groffen *et al.* 1984; Ben-Neriah 1986). The Polyoma DNA tumour virus middle T antigen, which while possessing potent transforming activities, does not itself possess detectable enzymatic activities but rather exerts its oncogenic effects through its association with c-Src family of tyrosine kinases (Courtneidge and Smith 1983; Kornbluth *et al.* 1986; Kypta *et al.* 1988; Cheng *et al.* 1988), PI3'K (Whitman *et al.* 1985; Courtneidge and Hebner 1987) and Shc (Campbell *et al.* 1994; Dilworth *et al.* 1994).

1.4 SH2/PTB containing proteins involved in ErbB-2 signaling

Although there are many reports itemizing the signaling proteins which interact with different receptors, the role each molecule plays in transducing a proliferative, transforming or differentiating signal is less clear. To dissect the pathways involved in RTK signal transduction one can remove the sites of phosphorylation on the receptor or attempt to antagonize the function of specific signaling molecules through the use of competitive inhibitors, microinjection of neutralizing antibodies or chemical inhibitors of enzymatic activities. Alternatively, assays can be carried out in cells in which the signaling molecule has been genetically ablated, but this requires the generation of multiple targeted lines of cells for each signaling protein of interest. Each method has its advantages and disadvantages, thus it is prudent to employ multiple approaches to assess the role of any given protein in a transduction pathway. Perhaps the best characterized RTK is the platelet derived growth factor receptor-β (PDGFR). PDGFR associates with ten different SH2/PTB-containing proteins at eleven phosphorylation sites (reviewed in Heldin 1997; Heldin *et al.* 1998). Point mutation of individual tyrosine phosphorylation sites reveals that the binding sites for Src are absolutely required for catalytic activity and as a result for PDGF induced proliferation (Mori *et al.* 1993). No other site appears to be required for PDGFR signaling by this approach, suggesting multiple sites of phosphorylation are required for signaling (Ronnstrand *et al.* 1992; Fantl *et al.* 1992; Kazlauskas *et al.* 1992; Valius *et al.* 1993). A PDGFR mutant lacking five phosphorylation sites (termed F5) retains Src interactions, lacks interaction with p85 PI3'K, phospholipase C-g1 (PLCγ1), the SH2-containing protein tyrosine phosphatase Shp-2 and the Ras-GTPase activating protein (Ras-GAP), harbours full catalytic activity but possesses debilitated proliferative potential. Reversion of the p85 PI3'K or the PLCγ1 binding sites in F5 derived mutants termed "add-back" receptors, reconstitutes PDGF-induced DNA synthesis and cell proliferation demonstrating that either site and by inference, either pathway, can independently mediate a proliferative signal (Valius and Kazlauskas 1993).

While such an approach has not been attempted for ErbB family members, a number of SH2/PTB proteins appear to play specific and important roles in ErbB signaling. These intracellular signaling

molecules include: PLCγ1 (Fazioli *et al.* 1991; Jallal *et al.* 1992; Segatto *et al.* 1992), c-Src (Luttrell *et al.* 1994; Muthuswamy *et al.* 1994), Crk (Hempstead *et al.* 1994) and Grb7 (Stein *et al.* 1994) which interact with Neu through specific phosphotyrosine residues. An additional set of SH2-containing proteins that associate with Neu include Ras-GAP (Fazioli *et al.* 1991; Jallal *et al.* 1992), Shc (Segatto *et al.* 1993), Grb2 (Janes *et al.* 1994) and Nck (Dougall *et al.* 1996). These latter proteins modulate the activity of Ras by either promoting the formation of an active Ras-GTP complex or by accelerating the hydrolysis of Ras-GTP to its inactive Ras-GDP state (see section 1.5).

1.4.1 Phospholipase C- γ1

Phospholipase C (PLC) isoenzymes hydrolyse phosphatidylinositol 4,5-bis-phosphate (PIP_2) to produce inositol 1,4,5-tris-phosphate (IP_3) and diacylglycerol (DAG), second messengers responsible for increasing intracellular calcium levels and activation of protein kinase C isoforms (PKC) respectively. The SH2-containing PLCγ1/2 are tyrosine phosphorylated by RTKs (Margolis *et al.* 1989) and this phosphorylation is essential for increased activity *in vitro* and *in vivo* in response to growth factor stimulation (Nishbe *et al.* 1990; Kim *et al.* 1991). While PLCγ activity is required for Ras and serum induced DNA synthesis (Smith *et al.* 1990), the physiologic significance of activation is not entirely understood. PLCγ associates with both EGFR and ErbB-2 and is tyrosine phosphorylated in response to receptor phosphorylation (Fazioli *et al.* 1991; Peles *et al.* 1991) although this phosphorylation does not correlate with mitogenicity (Fazioli *et al.* 1991). Although PLCγ1 appears to mediate a proliferative signal from PDGFR as assessed by add-back mutant expression in some cells (Valius and Kazlauskas 1993) and microinjection experiments of SH2 domains or neutralizing antibodies in fibroblasts (Roche *et al.* 1996), there is growing evidence that PLCγ is not a positive mediator of RTK signaling. The inclusion of the PLCγ1 binding site to a PDGFR add-back mutant suppresses Ras and PI3'K activation (Klinghoffer *et al.* 1996). Mutation of the PLCγ binding site on the fibroblast growth factor (FGF) receptor prevents PLCγ association and activation but not mitogenesis (Mohammadi *et al.* 1992; Peters *et al.* 1992). Moreover,

increased PLCγ expression in fibroblasts results in an increase in PDGF- and FGF-induced PIP_2 hydrolysis but does not increase growth factor-induced mitogenesis (Margolis *et al.* 1990; Cuadrado and Molloy 1990). Notwithstanding, PLCγ1 is detectably overexpressed and tyrosine phosphorylated in a high percentage of primary mammary carcinoma but not in nonmalignant breast tissues. Moreover, all tested tumours containing tyrosine phosphorylated PLCγ1 also possessed increased EGFR or ErbB-2 levels (Arteaga *et al.* 1991). Thus, while PLCγ appears to be a substrate of ErbB-2, it's role in signaling and tumour physiology is, as yet, ill-defined.

1.4.2 c-Src

c-Src is the homolog to the v-Src avian retroviral oncogene. It appears to not only play a role in mammary tumorigenesis but potentially a role exists in ErbB family signaling as well. Src activity is elevated in human and murine primary breast carcinoma and cells derived thereof (Jacobs and Rubsamen 1983; Ottenhoff-Kalff *et al.* 1992; Rosen *et al.* 1996; Muthuswamy *et al.* 1994). Additionally, cortactin, a v-Src substrate (Wu *et al.* 1991), is the product of the EMS-1 locus (Schuuring *et al.* 1993) on human chromosome 11q13, a region frequently amplified in malignant mammary carcinoma (Schuuring *et al.* 1992). Transgenic mice expressing an activated c-*src* allele develop heritable mammary tumors (Webster *et al.* 1995), while a functional c-*src* allele is required to elicit mammary tumour formation in transgenic mice expressing PyV-MT in the mammary epithelium (Guy *et al.* 1994a). Thus regulation of Src activity is necessary and sufficient for deregulated mammary growth although other genetic events may be needed for full oncogenic transformation (see Webster *et al.* 1995).

Src appears to play a critical role in the signal transduction from specific RTKs. Microinjection of Src-neutralizing antibodies or catalytically inactive forms of Src prevents DNA synthesis in response to PDGF and EGF but not to colony stimulating factor 1 (CSF-1) activation of their cognate receptors (Twamley-Stein *et al.* 1993; Roche *et al.* 1995). Mutagenesis of the Src binding sites of the PDGFR results in a loss of detectable receptor kinase activity and thus PDGF-induced proliferation (Mori *et al.* 1993). The

role of Src family members in ErbB signaling is however unclear. Src associates indirectly with EGFR and directly to Neu through its SH2 domain (Luttrell *et al.* 1994; Muthuswamy *et al.* 1994; Muthuswamy and Muller 1995) and is somewhat activated in response to activated Neu expression in fibroblasts (Muthuswamy and Muller 1995). Moreover, EGF-induced DNA synthesis, transformation and tumorgenicity are potentiated in cells expressing elevated c-Src levels (Luttrell *et al.* 1988; Maa *et al.* 1995). Taken together, it appears that Src may play a role in ErbB-2/Neu signaling, but the nature of this relationship awaits further experimentation and will be greatly aided by mapping of the Src binding site.

1.4.3 Phosphatidylinositol 3'OH Kinase

The p85 subunit of PI3'K binds to the phosphotyrosine containing-consensus sequence YxxM (Songyang *et al.* 1993). While elevated levels of PI3'K activity have been demonstrated in some cell lines overexpressing Neu/ErbB-2 (Scott *et al.* 1991; Peles *et al.* 1992b), it appears to be indirect and dependent on ErbB-3 levels (see Almandi *et al.* 1995; Carraway III *et al.* 1995). Transient cotransfection into 293 cells with *erbB*-2 and *erbB*-3, but not *erbB*-2 or *erbB*-3 alone, resulted in an increased immunoprecipitable PI3'K activity (Wallasch *et al.* 1995). Although a consensus YMxM motif is found in Neu/ErbB-2, this YxxM sequence resides in the kinase domain and is present in each ErbB family member as well as a number of Src, Abl, PDGFR and insulin receptor family members (Hanks *et al.* 1988) suggesting it plays a structural role. Moreover it has not been identified as a site of tyrosine phosphorylation in ErbB-2 (Hazan *et al.* 1989; Segatto *et al.* 1990) or the EGFR (Downward *et al.* 1984; Rotin *et al.* 1992). ErbB-3 contains six YxxM motifs in the carboxy-tail and although the sites of ErbB-3 tyrosine phosphorylation have yet to be examined, elevated PI3'K activity is immunoprecipitated with antibodies to ErbB-3 but not EGFR or ErbB-2 in response to receptor activation (Soltoff *et al.* 1994; Wallasch *et al.* 1995). Additionally, p85 associates with ErbB-3 in response to NDF/EGF and tyrosine phosphorylated peptides derived from these six potential p85 binding sites inhibit this association (Prigent and Gullick 1994). As a result, it is thought

that PI3'K activity may play a role in Neu/ErbB-2 signaling but does so through recruitment and phosphorylation of ErbB-3 (Sepp-Lorenzino *et al.* 1996).

1.4.4 Grb2, Shc, Ras

An additional set of SH2-containing proteins which associate with Neu include Ras-GAP (Fazioli *et al.* 1991; Jallal *et al.* 1992), Shc (Segatto *et al.* 1993) and Grb2 (Janes *et al.* 1994) and Nck (Dougall *et al.* 1996). These latter proteins modulate the activity of Ras by either promoting the formation of an active Ras-GTP complex or by accelerating the hydrolysis of Ras-GTP to its inactive Ras-GDP state (Lowy and Willumsen 1993). For example, Ras-GAP, which directly interacts with Neu (Fazioli *et al.* 1991; Jallal *et al.* 1992; Muthuswamy and Muller 1995), stimulates the intrinsic Ras GTPase activity and results in the downregulation of Ras activity (section 1.5).

By contrast, the 23kDa Grb2 protein lacks catalytic activity, containing a single SH2 domain flanked by two SH3 domains (Lowenstein *et al.* 1992). These latter domains allow constitutive association with Sos GDP/GTP exchange proteins which promote the conversion of Ras-GDP to the active Ras-GTP state (Gale *et al.* 1993; Egan *et al.* 1993; Li *et al.* 1993; Rozakis-Adcock *et al.* 1993; Buday and Downward 1993; Chardin *et al.* 1993). Three mammalian Shc isoforms exist: p66 generated from alternate use of an upstream exon and p52 and p46 produced by differential translation initiation (Migliaccio, 1997). Each protein contains an amino terminal PTB, a carboxyl SH2 domain and a central collagen homology domain (CH1). Shc proteins are thought to promote Ras activation by indirectly recruiting Grb2 to receptor tyrosine kinases (Pelicci *et al.* 1992; McGlade *et al.* 1992b; Rozakis-Adcock *et al.* 1992) through a CH1 domain located phosphorylated tyrosine residue producing a consensus binding site for the Grb2 SH2 domain (Songyang *et al.* 1994; Salcini *et al.* 1994; Gotoh *et al.* 1997).

It is of interest that elevation of Ras-dependent signaling has been demonstrated in a variety of mammary tumour-derived cell lines. In many of these lines, Grb2 levels are correspondingly elevated and often associated with tyrosine phosphorylated Shc proteins (Janes *et al.* 1994; Daly *et al.* 1994). Moreover,

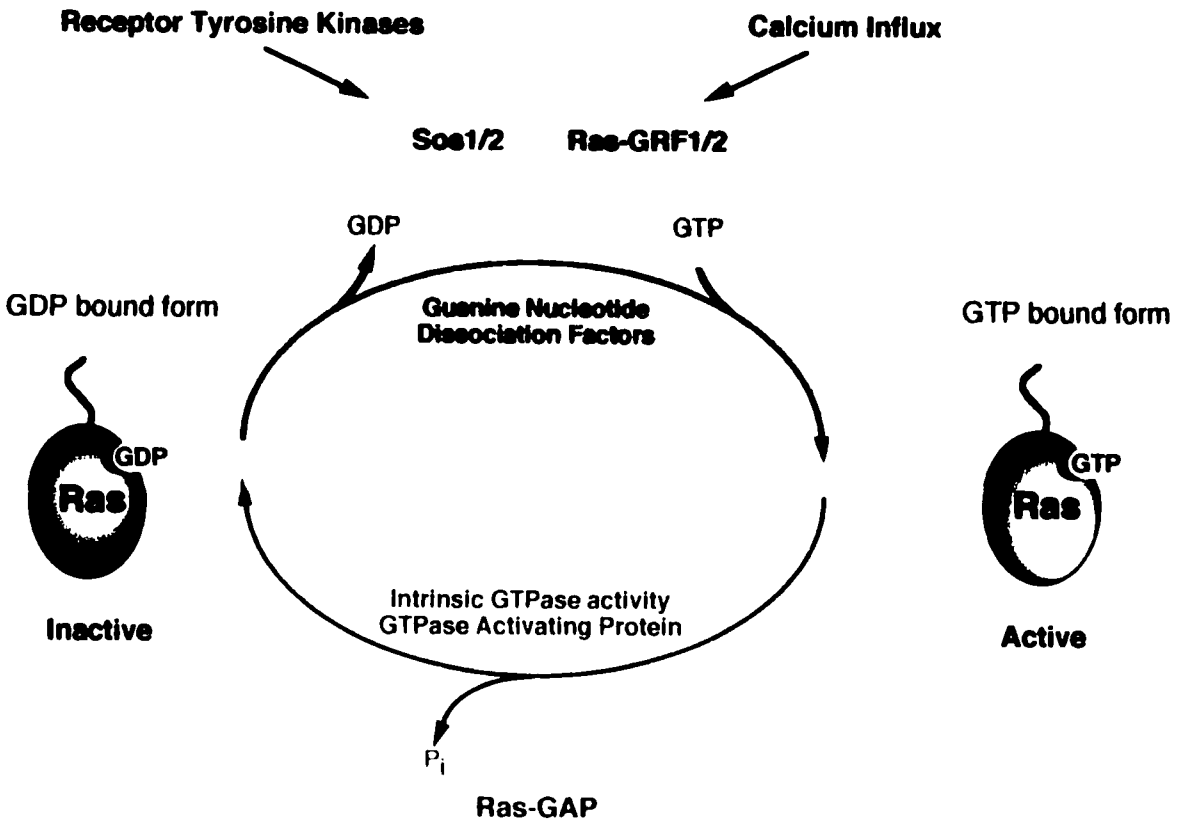
Shc proteins are found tyrosine phosphorylated in all tested breast tumours analyzed (Pelicci et al. 1995). Expression of Grb2 or Shc in the mammary epithelium of transgenic mice does not however lead to the rapid development of mammary carcinoma perhaps due to a lack of "upstream activation" (M. Rauh, C. Tortorice, V. Blakmore, W.J. Muller, unpublished). Mammary specific expression of a PyV-MT mutant incapable of Shc association dramatically impairs the ability of MT to induce multifocal metastatic tumours. Interestingly, in a proportion of the solitary tumours that do arise, Shc binding is reconstituted via transgene mutation (Webster *et al.* 1998). Both Grb2 and Shc reside at chromosomal regions amplified in a human cancers (Daly *et al.* 1994; Huebner *et al.* 1994). Taken together, Grb2 and Shc likely play a role in mammary tumourigenesis, although their precise roles in ErbB-2 mediated events is as yet undetermined.

1.5 Regulation of Ras Function

Ras proteins are evolutionarily conserved GTPases which play a pivotal role in a wide variety of cellular processes ranging from proliferation to differentiation, transformation, apoptosis and yeast mating (Dobrowolski *et al.* 1984; Feig and Cooper 1988; Simon *et al.* 1991; Kayne and Sternberg 1995; Hagag *et al.* 1986; Szeberenyi *et al.* 1990; Trent *et al.* 1996). Their importance in such phenomena has placed the regulation and mechanism of action of these proteins under intense research. Ras GTPases act as molecular switches alternating between "active" GTP-bound and "inactive" GDP-bound states (Figure 1.5). The balance between these states is maintained by the cumulative activities of two classes of enzymes: guanine nucleotide exchange factors (GEFs), which promote the exchange of GDP for GTP, thereby activating Ras, and GTPase activating proteins (GAPs), which accelerate Ras' intrinsic GTPase activity, leading to the hydrolysis of GTP shuttling Ras to its inactive form (Lowy and Willumsen 1993; McCormick 1995; Katz and McCormick 1997; Bourne *et al.* 1991). Oncogenic mutants often lack GTPase activity and due to the difference in cellular GTP/GDP levels, are found in the GTP bound state. Association with the plasma membrane occurs through a post-translationally bound lipid (farnesyl or gerynylyl) covalently bound to the C-terminus through a CAAX motif (A=aliphatic and X=met or ser) (Willumsen *et al.* 1984; Gutierrez *et al.*

Figure 1.5 Ras regulation

Ras proteins are GTPases found predominantly as either GDP or GTP bound. The GDP bound form is considered inactive. Guanine nucleotide exchange factors (GEFs) such as the Sos and Ras-GRF proteins dramatically increase the dissociation rate of GDP from the Ras. This allows the rapid passive loading of GTP due to higher intracellular concentrations. Ras' intrinsic GTPase activity slowly hydrolyses GTP to GDP, an activity that is increased 5 logs by Ras GTPase activating proteins such as Ras-GAP (or NF1 not shown). This returns Ras to its inactive GDP bound state.


Receptor Tyrosine Kinases
Calcium Influx
Sos1/2
Ras-GRF1/2
GDP
GTP
GDP bound form
Guanine Nucleotide
Dissociation Factors
GTP bound form
GDP
Ras
GTP
Ras
Intrinsic GTPase activity
GTPase Activating Protein
Inactive
Active
P_i
Ras-GAP

1989; Hancock *et al.* 1989). This modification is required for Ras function as viral mutants lacking this modification are transformation incompetent (Willumsen *et al.* 1984). The preponderance of data now suggests that GTP-bound Ras proteins are active because they assume a conformation which allows for interaction with several so-called effector proteins (see below and Bourne *et al.* 1991).

While mammalian Ras proteins are found mutated in many primary cancers, activating mutations are not considered to play a dramatic role in the etiology of breast carcinoma and at best play a minor role in disease progression (Rochlitz *et al.* 1989; Bos 1989).

1.5.1 Positive regulation of Ras by nucleotide exchange factors

The genetic determinants of Ras regulation have been extensively studied in both *C. elegans* and *Drosophila* by exploiting genetic screens. The sevenless RTK (*sev*) is required for normal ommitidia development in the developing *Drosophila* eye. Genetic and biochemical evidence have revealed two proteins, Grb2 and Sos, coordinately act to stimulate Ras activation. The *son of sevenless* (*sos*) gene functions within *sevenless*-expressing cells in a cell autonomous fashion genetically downstream of the *sev* (Simon *et al.* 1991) and upstream of the *Drosophila Ras*1 genes (Rogge *et al.* 1991; Bonfini *et al.* 1992) allowing normal ommitidial development. *sos* sequence analysis reveals homology to the catalytic subunit of *Saccharomyces cerevisae* cdc25, a RAS1 exchange factor. Moreover, biochemical analysis of the mammalian Sos protein (mSos1) reveals that it indeed is a Ras specific GDP/GTP exchange factor (Gale *et al.* 1993; Chardin *et al.* 1993).

The mammalian growth factor receptor bound (Grb) protein, Grb2, displays extensive homology with two genes: the *C. elegans sem5* gene which genetically lies between the *C. elegans* EGFR (*let*-23) and Ras (*let*-60) in vulval development (Clark *et al.* 1992) and to the *Drosophila DRK* gene which is essential for *sev* signaling to *Ras*1 (Olivier *et al.* 1993). Consisting of a single SH2 domain flanked by a pair of SH3 domains, Grb2 has no known biochemical activity (Lowenstein *et al.* 1992), but forms SH2-RTK interactions through specific tyrosine phosphorylated residues containing an asparagine (N) in the +2

position (YxNx) (Songyang *et al.* 1993; Songyang *et al.* 1994). In addition to being placed on the same genetic pathway emanating from RTKs to Ras, Grb2 and Sos physically interact forming a stable cytosolic complex (Egan *et al.* 1993; Gale *et al.* 1993; Rozakis-Adcock *et al.* 1993; Buday and Downward 1993; Li *et al.* 1993; Chardin *et al.* 1993). This interaction is mediated by Grb2 SH3 binding to specific proline-rich motifs within Sos proteins (Rozakis-Adcock *et al.* 1993). It is felt that the role Grb2 plays in Ras activation is to recruit Sos to the plasma membrane whereby it can act on Ras. Of particular interest is the observation that there is no increase in the total cellular Sos activity in response to growth factor stimulation but Sos does move from the soluble to the particulate fraction (Buday and Downward 1993), suggesting subcellular relocalization is regulated. Plasma membrane localization of Sos does appear to be sufficient for Ras activation in culture (Aronheim *et al.* 1994; Quilliam *et al.* 1994). A similar strategy targeting Grb2 to the plasma membrane has not been described but would prove the existence of a similar role and should result in the RTK-independent activation of Ras.

A number of other mammalian Ras exchange factors have been identified: these include Ras-GRF1/2, Smg-GDS, and C3G (Shou *et al.* 1992; Fam *et al.* 1997; Tanaka *et al.* 1994). The latter proteins, Smg-GDS and C3G (Gotoh *et al.* 1995) do display some Ras-GEF activity *in vitro*, yet are much more active on other members of the Ras superfamily. Ras-GRF1/2 are primarily expressed in the brain and while displaying specificity towards Ras, these GEFs are regulated by intracellular calcium levels through a calmodulin-binding IQ domain (Farnsworth *et al.* 1995; Fam *et al.* 1997). As a result, Ras-GRF1/2 primarily function downstream of seven membrane proteins. Of the known exchange factors, only Sos proteins are Ras-specific, contain proline rich motifs enabling indirect RTK interaction and genetically are positioned between RTKs and Ras. Consequently, Sos proteins thought to play a pivotal role in the Ras-exchange activity directly regulated by RTK signaling.

1.5.2 Negative regulation of Ras by GAP

p120 Ras-GAP was first identified as a cytosolic activity capable of increasing Ras' intrinsic GTPase activity and was subsequently cloned and named Ras-GTPase activating protein (Ras-GAP). Three proteins classes with GAP activity have been identified: the SH2-containing Ras-GAP, the gene product of the neurofibromatosis type 1 disease locus (NF1) and those with either PH or IQ domains (reviewed in McCormick 1995). Ras-GAP dramatically accelerates Ras-GTPase activity *in vitro* (10^5 fold, reviewed in Bourne *et al.* 1991) and when overexpressed or constitutively membrane bound inhibits Ras-dependent transformation (Clark *et al.* 1993; Huang *et al.* 1993a). Ras-GAP expression also inhibits the transforming abilities of v-*src* and v-*fms* suggesting these proteins require Ras activity in the transformation process (Nori *et al.* 1991; DeClue *et al.* 1991) as has been indicated by Ras-dependent proliferation studies (Smith *et al.* 1986).

Ras-GAP has properties of a Ras effector protein (section 1.5.3) in that it interacts directly with Ras in a GTP-dependent manner (Scheffzek *et al.* 1997 and references therein) and in response to specific PTK activation associates with two tyrosine phosphoproteins (Ellis *et al.* 1990): p62-DOK (Carpino *et al.* 1997; Yamanashi and Baltimore 1997) and p190-RhoGAP (Settleman *et al.* 1992). The formation of p62/p190/Ras-GAP complexes does not however require Ras activation (Pronk *et al.* 1993). Moreover, there are no reports which demonstrate a requirement for Ras-GAP to elicit Ras-dependent signaling. On the contrary, fibroblasts derived from Ras-GAP nullizygous mice show hyper-responsive Ras activation following PDGF treatment (van der Geer *et al.* 1997). Additionally, Ras effector domain mutants exist which lack Ras-GAP association yet are transformation competent (reviewed in Marshall 1993). Thus, while Ras-GAP clearly plays a role in Ras signaling, the nature of such a role is likely antagonistic.

1.5.3 Ras Effector Proteins

Ras proteins appear to elicit mitogenic signaling through the direct interaction of several proteins which may include Raf, PI3'K, Ral-GDS, Ras-GAP and MEK proteins as well as others (reviewed in Marshall 1995; Katz and McCormick 1997) (Figure 1.6). Biochemically, it appears that there are at least

three independent pathways emanating from Ras (White *et al.* 1995; Khosravi-Far *et al.* 1996; Rodriguez-Viciana *et al.* 1997). The most intensely studied of these pathways is the Ras-dependent activation of the MAPK cascade.

1.5.3.1 *Raf*

Ras interacts with c-Raf in a Ras-GTP-dependent fashion through its effector domain recruiting the Ser/Thr kinase to the plasma membrane (Zhang *et al.* 1993; Moodie *et al.* 1993; Van Aelest *et al.* 1993; Vojtek *et al.* 1993) where Raf becomes activated (Stokoe *et al.* 1994; Leevers *et al.* 1994) by an additional ill-defined signal(s) (Marias *et al.* 1995; Williams *et al.* 1992 reviewed in Morrison and Cutler Jr. 1997). Raf in turn binds, phosphorylates and activates dual specificity MAPK kinases, MEK 1/2 (Kyriakis *et al.* 1992; Dent *et al.* 1992; Huang *et al.* 1993b; Moodie *et al.* 1993; Van Aelest *et al.* 1993). These proteins catalytically stimulate MAPKs, termed extracellular regulated kinases (ERK1/2), through the phosphorylation of two evolutionarily conserved residues within the kinase domain (Cobb and Goldsmith 1995). ERKs then translocate to the nucleus where they phosphorylate transcription factors leading to an increase in transcriptional activity (Hill and Treisman 1995). *Drosophila* homologues to each kinase in the cascade appear to function in a linear genetic pathway paralleling biochemical activation.

Dominant inhibitory mutants of Raf are sufficient in blocking Ras-transformation in culture (Qui *et al.* 1995) and a Ras-effector mutants defective in binding Raf are transformation defective (White *et al.* 1995; Khosravi-Far *et al.* 1996; Rodriguez-Viciana *et al.* 1997). Thus Raf displays properties expected of a *bona fide* Ras effector protein.

1.5.3.2 *Ras activation of Phosphatidylinositol 3-OH kinase*

Phosphatidylinositol 3-OH kinase (PI3'K) is composed of an 85kDa regulatory (p85) and 110kDa catalytic (p110) domain, the latter of which interacts with Ras in a GTP-dependent manner (Rodriguez-Viciana *et al.* 1994). Coexpression of oncogenic Ras and PI3'K leads to an increase in PI3'K products (phosphatidylinositol (3,4,5) tris-phosphate [PI(3,4,5)P_2] and phosphatidylinositol (3,4) bis-phosphate) [PI(3,4)P_2]) in a Ras-dependent fashion (Rodriguez-Viciana *et al.* 1996). Significantly, a single amino acid

Figure 1.6 Ras Effector Proteins

In response to receptor tyrosine kinase activation, GDP-bound Ras is activated by the membrane localized Son of sevenless protein through the cdc25 guanine nucleotide exchange factor (GEF) domain. Schematized are the most well characterized Ras effector proteins: PI3'Kinase, the serine/threonine kinase Raf and the Ral-GDS exchange proteins. Ral-GDS proteins activate PLC-D. Raf activation, in part through membrane localization is responsible for the sequential activation of the MAP kinase, MEK1 and the Erk1/2 MAP kinases. PI3'kinase products activate the serine/threonine AKT/PKB kinase in turn activating S6kinase. These lipid can bind to the plekstrin homology domain within Sos proteins activating the adjacent Dbl domain which functions as a Rac-specific GEF. GTP-bound Rac associates with and activates the PAK protein kinase which results in the activation of a protein kinase cascade leading to the activation of Jun or stress-activated kinases (JNK/SAPK).

mutation in p110 abolishes Ras-induced but not unstimulated enzymatic activity also prevents Ras-p110 interaction (Rodriguez-Viciana *et al.* 1996). In addition, PI3'K has been placed downstream of Ras through the use of PDGFR autophosphorylation mutants (Klinghoffer *et al.* 1996) and V12-Ras "effector" domain mutants. The latter of which lead to the concomitant loss of Ras-p110 association, PI3'K activation and cellular transformation (Rodriguez-Viciana *et al.* 1997). Moreover, use of dominant interfering p85 mutants demonstrates a requirement for PI3'K activity in Ras-induced transformation. While the mechanism of PI3'K activation is unclear it may involve recruitment to the membrane as plasma membrane targeting of p110 increases cellular PI3'K activity (Hu *et al.* 1995a). While these data suggest that PI3'K is an effector of Ras function, the known proteins responsive to the products of PI3'K (e.g. AKT, S6 kinase, Rac) do not appear to act mitogenically (Burgering and Coffer 1995; Franke *et al.* 1997; reviewed in Toker and Cantley 1997). PI3'K itself and Akt do however appear to be involved in cellular survival and under specific circumstances have anti-apoptotic properties (Yao and Cooper 1995; Dudek *et al.* 1997; Kennedy *et al.* 1997; Khwaja *et al.* 1997; Kuaffmann-Zeh *et al.* 1997; Kulik *et al.* 1997).

Activation of PI3'K may have distinct consequences. While PI(3,4)P_2 binds and activates AKT (Franke *et al.* 1997; Toker and Cantley 1997), PI(3,4,5)P_3 binds SH2 and PTB domains in a manner which is directly competitive with phosphopeptides (Zhou *et al.* 1995; Rameh *et al.* 1995) and thus may function to inhibit intermolecular interactions of signaling molecules and their binding partners. It appears that PI3'K products may initiate the activation of an additional MAP kinase pathway through the GTPase Rac, a member of the Rho family of Ras GTPases (Rodriguez-Viciana *et al.* 1997; Nimual *et al.* 1998). Rac interacts with and stimulates the protein kinase PAK (Manser *et al.* 1994) and through an unidentified protein activates a Ser/Thr kinase cascade of MEKK, SEK, JNK (Minden *et al.* 1995; Coso *et al.* 1995; Lamarche *et al.* 1996; Joneson *et al.* 1996a; Westwick *et al.* 1997). Ras-induced activation of Rac-signaling is wortmannin sensitive (Nimual *et al.* 1998) implicating PI3'K in this process. Indeed, the Ras effector mutant C40, which interacts with p110 but not Raf or Ral-GDS, activates JNK activity and membrane ruffling (Joneson *et al.* 1996b; Rodriguez-Viciana *et al.* 1997; Nimual *et al.* 1998).

Interestingly, this activation of JNK appears to occur through the Ras Sos exchange factor. In addition to a cdc25 Ras GEF domain, Sos harbours a Dbl homology (DH) and plekstrin homology (PH) domain which function as Rho family-specific GEFs and in part to bind phospholipids like those produced by PI3'K, respectively (Whitehead *et al.* 1997; Pawson and Scott 1997). In the Dbl family members the PH domain acts as a negative regulator of exchange factor activity (Whitehead *et al.* 1997). Interestingly, expression of the Sos DH domain alone leads to Rac activation which is inhibited by the inclusion of the PH domain. This PH-mediated inhibition is relieved by the expression of Ras-C40 (Nimual *et al.* 1998). Thus taken together these data suggest that Ras induction of Rac activation occurs through derepression of the Sos DH domain via the products of PI3'K. This provides a cogent explanation of a Rac requirement in the Ras-dependent transformation (Qui *et al.* 1995).

1.5.3.3 *Ral-GDS*

Ral-GDS proteins along with the distantly related Rlf exchange factor mediate a third distinct genetic pathway from oncogenic Ras (reviewed in Campbell *et al.* 1998). These proteins are GEFs for Ral GTPases and are activated in response to activated Ras expression (Urano *et al.* 1996; Wolthuis *et al.* 1997). Rlf synergizes with Ras to activate a Ras-dependent promoter and activation can be carried out by a constitutively activate Rlf allele (Wolthuis *et al.* 1997). Ras effector mutants deficient in Ral-GDS association possess impaired transformation potential (Rodriguez-Viciana *et al.* 1997) and expression of active Rlf alleles complement such Ras effector mutants in transformation assays (Urano *et al.* 1996). Thus Ral-GDS members have the hallmarks of *bona fide* Ras effector proteins.

1.6 Protein Tyrosine Phosphatases-Structure

Protein tyrosine phosphorylation is regulated by the coordinate actions of protein tyrosine kinases (PTKs) and phosphatases (PTPs). This protein modification is critical to the regulation of growth and cell cycle control, differentiation, cytoskeletal architecture as well as aspects of metabolism (e.g. insulin responses). Since the discovery of the Src PTK in the early eighties much of the attention in phosphotyrosyl regulation has focused on kinases and only recently have the roles and identity of particular PTPs been realized. This supergene family is predicted to contain approximately 500 members (Tonks and Neel 1997) which can be broadly grouped into two categories: atypical PTPs and "classical" PTPs. The latter of which can be loosely defined as transmembrane and non-transmembrane PTPs (TM- and nonTM-PTPs). While some non-classical PTPs are thought to play pivotal roles in the regulation of MAP kinase activation and cell cycle progression or as the virulence factor of the pbuebontic plague, the role of the majority of classical PTPs is as yet unrealized.

Amino acid sequence alignments demonstrate several elements within the 200-300 residue classical PTP domain are highly conserved particularly the PTP signature motif [I/V]HCxAGxxR[S/T]G (Denu *et al.* 1997). This motif resides at the base of a cleft, the depth of which allows only phosphotyrosine but not phospho-serine or -threonine access to the catalytic cysteine which acts as a nucleophile attacking the phosphorous atom in the reaction (Barford *et al.* 1994). Interestingly, this cysteine is required for catalytic activity and while mutation to serine results in ablation of catalytic activity, cysteine to serine mutants often retain the ability to bind their phosphotyrosine substrates (Bliska *et al.* 1991; Bliska *et al.* 1992; Garton *et al.* 1996; Flint *et al.* 1997; Sun *et al.* 1993). These properties confer to these C→S mutants "substrate-trapping" abilities. Alteration of an additional conserved residue results in mutants with similar properties (Flint *et al.* 1997; Tiganis *et al.* 1998). Mutation of the conserved arginine within the signature motif results in loss of catalytic and substrate binding abilities, as the large positively charged residue directly interacts with the negatively charged oxygen atoms (Flint *et al.* 1997). These latter mutants are hypothesized to function as true null alleles whereas substrate-trapping mutants

theoretically can function in both a positive and negative manner due to the ability to protect target phosphotyrosine residues from dephosphorylation and presumably preclude phosphotyrosine mediated interactions (Sun *et al.* 1993).

PTP domains are found in structurally diverse proteins which may also include SH2 domains, fibronectin- and immunoglobulin- like repeat elements. Interestingly, other than SH2 domains, PTPs bear little homology to the non-catalytic regions of PTKs.

1.6.1 Transmembrane PTPs

Transmembrane PTPs (TM-PTPs) contain an extracellular region, followed by a single hydrophobic transmembrane region and intracellularly one or two PTPs domains with the membrane proximal domain harbouring the majority of catalytic activity and the distal domain perhaps involved in substrate targeting (Mourey and Dixon 1994). The extracellular domains and number of PTPs domains are used to group TM-PTPs into at least five to eight TM-PTPs types (Mourey and Dixon 1994). The ectodomains of many TM-PTPs contain structural motifs reminiscent of proteins involved in cellular adhesion. Indeed, four of five *Drosophila* TM-PTPs contain "adhesion-like" motifs and are expressed exclusively in the nervous system (Tian *et al.* 1991; Yang *et al.* 1991; Hariharan *et al.* 1991b). Of these, two genes clearly are involved in neuronal targeting, a process requiring complex cell-cell interactions (Desai *et al.* 1996). In addition, a loss-of-function mutation in the *Drosophila* LAR gene results in improper neuron migration and innervation of target muscles (Krueger *et al.* 1996). Moreover, the human PTPζ/β proteins contain in their extracellular regions a carbonic anhydrase-like (CA) domain which lack critical catalytic residues. This CA domain functions to bind contactin, a plasma membrane glycosylated phosphatidylinositol molecule found on the surface of particular cells involved in neuronal cell recognition (Peles *et al.* 1995). The extracellular domains of PTPμ and PTPκ contain immunoglobulin- and fibronectin III- like repeats which are involved in homo- but not hetero- typic binding of these PTPs expressed on different cells (Brady-Kalnay *et al.* 1993; Gebbink *et al.* 1993; Sap *et al.* 1994; Zondag *et al.* 1995).

Additionally, these proteins are thought to play an as yet ill-defined role in regulating cytoskeletal architecture by virtue of their presence with cadherins which are involved in adherin junction and catinins which link cadherins to the actin cytoskeleton (Fuchs *et al.* 1996; Kypta *et al.* 1996). Taken together, these data suggest that TM-PTPs may play roles in cell-cell mediated events although a considerable amount of work is required to elucidate these roles.

Perhaps the most well-characterized PTP is the transmembrane PTP, CD45 which is expressed on the surface of all cells of hematopoietic origin. CD45 expression is required for cultured T-, B-and natural killer- cell activation (Pingel and Thomas 1989; Weaver *et al.* 1991; Justement *et al.* 1991; Bell *et al.* 1993; reviewed in Thomas 1995; Neel 1997). Mice made deficient for CD45 lack proper thymocyte maturation and harbour B-cell abnormalities as well (Kishihara *et al.* 1993; reviewed in Neel 1997). Mechanistically, CD45 appears to play a critical role in the T-cell receptor (TCR) activation of Src family kinases (Lck/Fyn) through dephosphorylation of the C-terminal inhibitory phosphotyrosine (McFarland *et al.* 1993; Ostergaard *et al.* 1989; reviewed in Thomas 1995). More recently, substrate-trapping mutants have been implicated CD45 in negative TCR signaling via its association with ζ-chain of TCR CD3 (Furukawa *et al.* 1994).

Although a potential ligand for CD45 is as yet forthcoming, early experiments crosslinking CD45 molecules with antibodies suggested tha: CD45 capping lead to an inhibition of TCR signaling (Ledbetter *et al.* 1991). Given its positive role in TCR activation this suggested that PTP activity is inhibited by this capping. More recently using EGFR-CD45 chimeras, dimerization of CD45 was demonstrated to decrease PTP activity and TCR function (Desai *et al.* 1993). Structurally, the monomers within such a dimer are thought to mutually inhibit each other via an "inhibitory wedge" inserted into the catalytic cleft of the dimer partner (Majeti *et al.* 1998). Unlike RTKs, dimerization induced inhibition may be a general mechanism for TM-PTP activity control as this has been described for some (Bilwes *et al.* 1996) but not all TM-PTPs (Hoffmann *et al.* 1997) Thus CD45 is thought to play both positive and negative roles in TCR signaling and serves as a paradigm for TM-PTP signaling.

1.6.2 Non-transmembrane PTPs

The first PTP to be biochemically purified and microsequenced was PTP1B. Since this time cDNAs encoding a number of classical non-transmembrane PTPs have been identified in *Drosophila*, *C. elegans*, dictosylium and mammalian organisms. In addition to the PTP domain, these proteins contain a number of other recognizable domains. These include SH2 domains, PEST sequences, proline rich motifs which are SH3/WW domain targets, ezrin repeats and sequences homologous to retinaldehyde binding proteins. While the physiological role these PTPs play in signal transductive events is as yet unknown, biochemical studies proteins of PTP1B and SH2-containing phosphatase (Shp) families have revealed specific roles.

PTP1B and TCPTP are both targeted to the cytoplasmic face of the endoplasmic reticulum (Frangoli *et al.* 1992) by virtue of a short hydrophobic C-terminal sequence, yet both of these PTPs can reside elsewhere in the cell either by virtue of alternative splicing to produced a truncated protein (TCPTP; Tillmann *et al.* 1994) as well as proteolytic cleavage (PTP1B; Frangoli *et al.* 1993). *In vitro* phosphatase assays demonstrate PTP domains appear promiscuous in their substrate specificity although crystal structures (Jia *et al.* 1995; Hof *et al.* 1998) and use of substrate-trap mutants (Flint *et al.* 1997) suggest that the *in vivo* activities may be tightly regulated in part through direct protein-protein interactions with the substrate. This is exemplified by the requirement of an intact SH3-binding site on both the PTP1B and PEST phosphatases in order to recognize the SH3-containing p130Cas protein as a substrate *in vivo* (Garton *et al.* 1996; Liu *et al.* 1998). PTP1B-p130Cas interactions are likely physiologically relevant as association is a prerequisite for PTP1B-dependent transformation suppression (Liu *et al.* 1998). Both PTP1B and TCPTP substrate-trap mutants have identified the EGF receptor as an *in vivo* substrate, yet only the TCPTP PTP domain interacts with tyrosine phosphorylated Shc proteins (Flint *et al.* 1997; Tiganis *et al.* 1998). TCPTP selectively interacts with one but not both of the Shc tyrosine phosphorylation sites (Tiganis *et al.* 1998) demonstrating specificity. Thus, while *in vitro* PTP assays suggest little substrate specificity exists, the use of substrate-trapping mutants reveals that this may not be the case *in vivo*.

Shp-1 and -2 represent the two known mammalian SH2 containing protein tyrosine phosphatases. Mutation of Shp-1 and Shp-2 genes both are functionally recessive yet each gene has quite distinct biological functions in mammalian development (Shultz and Sidman 1987; Shultz *et al.* 1993; Saxton *et al.* 1997). *Motheaten* and *motheaten viable* represent two loss of function alleles of the murine *shp-1* gene. Homozygous *motheaten* mice die shortly after birth (2-3 weeks) due to multiple hematopoietic deficits which is coincident with its major site of expression (Shultz and Sidman 1987; Shultz *et al.* 1993; Tsui *et al.* 1993). *Motheaten viable* mice survive to approximately 64 days, have defects in melanocyte production, and hyperplasia or expansion of myeloid and some lymphoid lineages. This likely reflects the negative role *shp-1* plays in hematopoietic cell signaling. *Motheaten* and *motheaten viable* mice die of hematopoietic cell induced lung pnemonitis not caused by T or B cell (Yu *et al.* 1996) but is due to macrophage infiltration of the lung (Koo *et al.* 1993). Mechanistically, Shp-1, through its SH2 domains, physically associates with the Kit RTK (Yi and Ihle 1993), a molecule required for proper macrophage development. Genetically, *motheaten* mutants function to ameliorate macrophage loss from *white spotting locus* mutants in the Kit RTK (Lorenz *et al.* 1996; Paulson *et al.* 1996). While macrophage specific Shp-1 substrates are not known, it is clear that Shp-1 is a negative regulator of this and other hematopoietic cell type signaling (reviewed in Thomas 1995; Unkeless and Jin 1997; Neel and Tonks 1997; Neel 1997).

Targeted ablation of Shp-2 is incompatible with embryonic development beyond day E8.5 to E9.5. These embryos perish due to an apparent failure to gastrulate properly as evidenced by a perturbation of axial mesoderm derived structures (Saxton *et al.* 1997). In *Drosophila* (Perkins *et al.* 1992), nematodes (Gutch *et al.* 1998) and Xenopus (Tang *et al.* 1995) Shp homologs exist and are involved proper development. In C. elegans, *PTP-2*, the Shp homolog, is required for oogenesis and functions as a positive mediator of *let*-23 signaling in vulval development (Gutch *et al.* 1998). Loss of function mutations in the *Drosophila corkscrew* gene attenuate *sev* RTK signaling and proper ommatidia formation (Perkins *et al.* 1992; Allard *et al.* 1996). Expression of a catalytic mutants of SH-PTP2, a Xenopus Shp homolog, dramatically induces anteriorization of injected embryos (Tang *et al.* 1995). Thus taken together these data

argue for evolutionary conservation of SH2-containing phosphatase function and furthermore suggest that Shp phosphatases may play positive and negative regulatory roles in a variety of developmental programs.

1.7 The role of PTPs in oncogenesis

The etiology of tumours has been molecularly defined for but a number of cancers. Aberrant regulation of protein tyrosine phosphorylation appears to be play a critical role in the development of a number of human cancers (e.g. Philadelphia chromosome in CML). Thus it is not surprising that PTPs play pivotal roles in malignancy in humans as well as murine systems. Recently three PTPs have been implicated in the development of cancers.

Forced expression of rPTPα in Rat embryo fibroblasts leads to the acquisition of a transformed phenotype with expressing lines forming tumours in immunocompromised mice (Zheng *et al.* 1992). Interestingly, cell lines expressing rPTPα possess elevated Src kinase activity which correlated with the dephosphorylation of the c-Src negative regulatory tyrosine 527 *in vivo* and directly *in vitro* with the purified rPTPα PTP domain (Zheng *et al.* 1992; den Hertog *et al.* 1993). Taken together these data suggest that rPTPα may function as a proto-oncogene although to date there is no evidence suggesting that it is mutated or aberrantly expressed in human tumours.

Cyclin dependent protein kinases (CDKs) are negatively regulated by tyrosine and threonine phosphorylation within the ATP binding pocket of their kinase domains. In yeast and mammalian cells CDC25 dephosphorylates these residues functioning as a positive activator of CDKs and thus the cell cycle (reviewed in Draetta and Eckstein 1997). Mammalian cells contain three highly homologous but distinct CDC25 genes A, B and C. Microinjection of anti-CDC25A and C antibodies leads to cell cycle arrest at different points in the cell cycle suggesting distinct roles for these two phosphatases in mammalian cells (Draetta and Eckstein 1997). While CDC25B RNA expression is undetectable in normal breast tissue, 32% of neoplastic tissue overexpress CDC25B RNA displaying a strong correlate with microvessel density and histological tumour grade (Galaktionov *et al.* 1995). Moreover, forced expression of CDC25A and B, but

not C, transforms primary fibroblasts in cooperation with either V12Ras expression or Rb deficiency (the retinoblastoma gene product), thus demonstrating oncogenic potential (Galaktionov *et al.* 1995). Interestingly, c-*myc* also cooperates with Ras to transform primary fibroblasts (Land *et al.* 1983) and c-*myc* activates both CDC25A and CDC25B transcription (Galaktionov *et al.* 1996). Given that full activation of CDKs requires both dephosphorylation by CDC25 and the binding of cyclins (Morgan 1995), it is intriguing that elevated levels of cyclin D1 are also observed in primary breast tumours (Buckley *et al.* 1993; Bartkova *et al.* 1994) and that mammary specific expression of cyclin D1 in transgenic mice leads to widespread mammary epithelial hyperplasias later giving rise to focal mammary tumors (Wang *et al.* 1994). Thus, the CDC25 genes are proto-oncogenes *in vitro* and *in vivo*.

Cowden disease (CD) is an autosomal dominant disorder predisposing individuals to developing tumours of the breast, thyroid and skin. Lhermitte-Duclos disease (LDD), is a constellation of nervous tissues hyperproliferative disorders which include hamartomas, benign differentiated tumours. Interestingly, multiple tissue hamartomas are the hallmark of CD (also known as multiple hamartoma syndrome) suggesting the two syndromes may be related. Indeed, linkage analysis for both CD and LDD localized a single locus to 10q23 indicating the same gene may be responsible. A candidate tumour suppressor (PTEN, MMAC1, TEP1) maps to this same locus and is mutated in a variety of tumour-derived cell lines and sporadic tumours of the breast, prostate and brain (Li *et al.* 1997a; Liaw *et al.* 1997). Sequence analysis suggests and subsequent biochemical analyses demonstrate that PTEN is a dual specificity PTPs and where tested disease specific mutations result in a loss of PTP activity (Myers *et al.* 1997). Coupled with the observed loss of heterozygosity observed for some CD patients, these data suggest that PTEN functions as a tumour suppressor gene requiring its catalytic activity to do so. Analysis of PTEN-deficient mouse embryonic fibroblasts suggest that PTEN likely is not an inhibitor of proliferation but is a positive regulator of apoptosis initiated be specific agents (Stambolic *et al.* 1998). At the molecular level, PTEN dephosphorylates $PI(3,4,5)P_3$ (Maehama and Dixon 1998; Stambolic *et al.* 1998), a product of PI3'K required for AKT/PKB activation (Franke *et al.* 1997; Toker and Cantley 1997). As such, PTEN is

thought to prevent AKT-mediated apoptotic protection leading to transformation. Although, the phenotype of mice made deficient in the PTEN gene have not been published, these data suggest that PTEN functions as a negative regulator of tumour formation.

Given the number of PTKs implicated in the development or progression of human tumours, the role of PTPs in oncogenesis has not been fully realized and thus warrants further investigation.

1.8 Summary of intent

The role of Neu autophosphorylation sites in transformation has been the source of some contention. A number of SH2/PTB containing proteins such as PLCγ1, Ras-GAP, Grb2, Grb7, Shc, and Src- and Shp- family members, through physical association with Neu, have been implicated in Neu-mediated signaling. In chapter 3, I demonstrate that Neu mediates independent transforming signals from four of five tyrosine phosphorylation sites. The molecular mechanism appears to involve the independent use of Grb2, Shc and additional molecules from distinct phosphorylation sites to activate Ras. I have demonstrated that a single phosphorylation site in Neu binds to several proteins, including a novel PTB containing protein termed DOKR. Additionally, evidence is presented that Src binding does not correlate with transformation potential from Neu. Chapter 4 details the discovery of a negative regulatory tyrosine phosphorylation site that acts to decrease the ability of the receptor to couple with positive signaling molecules. This effect is vanadate sensitive, suggesting that a protein tyrosine phosphatase is activated by this residue. In chapter 5, I describe the cloning and characterization of an SH2 containing protein tyrosine phosphatase (PTPM1), now called Shp-1. Elevated Shp-1 expression is observed in human and murine mammary tumours and plays a negative role in Neu-mediated transformation. Moreover, Shp-1 associates with distinct proteins in different cell types. Taken together this thesis contains many novel observations which clearly define for the first time the multiple mechanisms by which Neu mediates transformation. These observations may have profound pharmacological implications as to rational drug design to target ErbB-2 hyperexpressing tumours with efficacy.

Chapter 2

Materials and Methods

2.1 RNA Analysis

2.1.1 Reverse Transcription Polymerase Chain Reaction (Rt-PCR).

Total cellular RNA was isolated from whole tissue by a modified guanidinium thiocyanate method (Sambrook *et al.* 1989; Muller *et al.* 1988). 10μg of total RNA was heat denatured (65°C, 10min) and immediately incubated on ice prior to use as a template for random-primed reverse transcription. Single stranded cDNA was produced with 200 units of mouse Moloney Leukemia Virus (MLV) reverse transcriptase in a 20μl volume containing 1μg pdN6, 1mM dNTP, 2.5mM MgCl2, 13.5uM DTT, 1xPCR buffer (Promega), and 5 units RNAsin. Incubation was carried out at 37°C for 60min and the reaction terminated by a 10min 95°C heat treatment. PCRs were carried out by adjusting the above reaction to a total volume of 100μl containing 0.6mM dNTP, 1mM $MgCl_2$, 0.2uM of each PCR primer (primers F1 and R1), 1xPCR buffer, and 2.5 units of Thermus aquaticus (Taq) polymerase, overlayed with 40μl of mineral oil. Thirty cycles of amplification were performed (95°C for 60 sec, 42°C for 120 sec, and 65°C for 180 sec) in a Perken-Elmer Cetus thermocycler followed by an 8min 72°C incubation to ensure complete extension of the final products of amplification. DNA of the expected size (~500bp) was isolated and digested with *Bam*HI and *Eco*RI prior to ligation into Bluescript SK+ (Stratagene). The ligation mixes were transformed into *E.coli* (XL1-Blue or DH5α) by standard protocols (Sambrook *et al.* 1989) and clones harbouring inserts of the expected size were grouped on the basis of restriction analysis or single nucleotide sequence. A representative of each group was then subjected to sequence analysis by the

dideoxynucleotide chain termination method following the protocol of the manufacturer (United States Biochemicals).

2.1.2 Northern analysis

20μg of total cellular RNA was heat denatured at 65°c for 10min in Northern sample buffer (48% formamide (v/v), 6.5% formaldehyde (v/v), 1xMOPS), fractionated through a 1% agarose gel containing 0.7 formaldehyde and 1xMOPS, and transferred to a GeneScreen membrane (Dupont) by capillary action as described in Sambrook et al (Sambrook *et al.* 1989). The GeneScreen membranes were hybridized at 42°C overnight in 10ml deionized formamide, 4ml (50%) dextran sulphate, 3ml 10% SDS, 100mg/ml salmon sperm DNA containing a ^{32}P-labeled probe made from a randomly primed of the clone PTPM1 PCR product and were washed as described (Sambrook *et al.* 1989).

2.1.3 RNAse protection analyses

RNAse protection analyses were carried out according to the procedure of Melton et al (Melton *et al.* 1984) using 20μg of total RNA per assay. T3 RNA polymerase driven anti-sense riboprobes were generated from linearized pBluescript plasmids. A PTPM1 specific riboprobe was generated from a NaeI-linearized plasmid harbouring a portion of the original PCR product (corresponding to nts 1096-1296 of PTPM1) to produce a 571 nt probe containing 200 nts specific to the PTPM1 message.

2.2 PCR Mutagenesis, DNA Constructs and cDNA Library Screening

2.2.1 Generation of Neu autophosphorylation mutants

2.2.1A *Single site mutants*

Neu cDNAs harboring the wild-type or V664E transmembrane mutation were a generous gift of Robert Weinberg (Bargmann *et al.* 1986b). Mutations in carboxyl-terminal autophosphorylation sites were generated by standard polymerase chain reaction protocols for oligonucleotide-directed mutagenesis. Following the introduction of an *Eco*RI site immediately 3' to the stop codon (nucleotide 3800), the *Nco*I-

*Eco*RI fragment (nucleotides 3030 to 3800) was cloned into pSL301 (InVitrogen) to facilitate subsequent sequence analyses. Mutagenic oligonucleotides pairs for each tyrosine were as follows: site A (Y1024) (forward AB2937 and reverse AB2938); site B (Y1144) (forward AB2939 and reverse AB2940); site C (Y1201) (forward AB2941 and reverse AB2942); site D (Y1225/6) (forward AB2943 and reverse AB2944); site E (Y1253) (forward AB2945 and reverse AB 2946) (Table 2.1). The conditions for amplification were as described (Siegel *et al.* 1994) with the following exceptions. Amplification was performed for 20 cycles of 45 sec at 95°C, 1min 30 sec at 37°C and 2min 30 sec at 73°C. The first amplification products were excised from agarose gels, boiled for 5min in TE (10mM Tris pH7.6, 1mM EDTA) and an aliquot was used for a subsequent round of amplification. Amplifications were carried out to create plasmids with single amino acid alterations or multiple simultaneous alterations (e.g. tyrosines at sites C and E were simultaneously mutated by initially carrying out three reactions [T7 primer and AB 2942; AB2941 and AB2946; AB2945 and T3 primer] an aliquot of each of the products were mixed and PCR amplified externally using T3 and T7 oligonucleotides). Standard sub-cloning procedures were utilized to bring mutations together within the same plasmid. All cloned PCR amplified regions were sequenced in their entirety by Brian Allore at the MOBIX Central Facility of McMaster University.

2.2.1B *Generation of Double add-back mutants*

pSL301 was digested with XhoI and SalI, gel purified and self-ligated to produce pSΔSX. The *Nco*I-*Eco*RI fragment from pSL-YA was subcloned into pSΔSX to create pSΔSX-YA. Double add-back mutants were produced by cloning *Xba*I-*Eco*RI fragments from pSL- YB, YC, YD and YE into pSΔSX-YA producing pSΔSX- YAB, YAC, YAD and YAE.

2.2.1C *Generation of YB, YC, YD, YE second site mutants*

A similar mutagenic PCR strategy as described above was used on pSΔSX-NYPD to introduce specific mutations into codon N1146 whilst simultaneously reverting F1144 to Y to create four pSΔSX-derived plasmids substituting Asp (N) for Leu, Val, Pro and His. In a similar fashion, P1199 and P1256 were converted Asp (D) and Ala (A) while reconstituting F1201Y and F1258Y respectively. To accomplish

Table 2.1 Oligonucleotides used

	PTPM1/Shp-1 oligonucleotides			Restriction sites shared (+/-)
F1		ATGGATCCGGA(C/T)TA(C/T)ATNAA(C/T)GC	sense	BamHI
R1		CCAAGCTTGGGNGC(G/A)CT(G/A)CA(G/A)TG(G/C)A	antisense	HindIII
F4		ACCGGATCCAA(A/G)TG(T/C)G(A/C)I(C/G)A(A/G)TA(T/C)TGG	sense	
AB1944	C453S forward	CATTGTCCACTCGAGCGCCGGCATC	sense	XhoI
Bd1	C453S reverse	GATGCCGGCGCTCGAGTGGACAATG	antisense	XhoI
	Glu-Gln	GGAATTCCATGGAATACATGCCCATGGAGGTGAGGTGGT-TTCACCGG		
	Autophosphorylation mutants			
AB 2937	site A	GAAGAGTTTCTAGTGCCCCAGCAGGG	sense	BfaI
AB 2938	site A	GGGCACTAGAAACTCTTCAGCGTC	antisense	BfaI
AB 2939	site B	CGAGTTTGTTAACCAATCAGAGG	sense	HpaI
AB 2940	site B	TTGGTTAACAAACTCGGGCTGGGGC	antisense	HpaI
AB 2941	site C	CTGAATTTTTTGGTACCGAGAGAAGGC	sense	KpnI
AB 2942	site C	CGGTACCAAAAAATTCAGGGTTCTCCAC	antisense	KpnI
AB 2943	site D	CCTCTTCTTCTGGGACCAGAACTCATCG	sense	EarI
AB 2944	site D	GTCCCAGAAGAAGAGGTTGTCAAAGG	antisense	EarI
AB 2945	site E	TGAGTTTCTAGGCCTGGATGTACC	sense	-AvrII/BlnI
AB 2946	site E	CAGGCCTAGAAACTCAGGGTTCTC	antisense	-AvrII/BlnI
	Site A-derived Mutants			
AB6679	L1025 - YXVP	GAA GAG TAT NNN GTG CCC CAG CAG GGA TTC	sense	
AB6682	L1025 - YXVP	CTG GGG CAC NNN ATA CTC TTC AGC GTC	antisense	
AB6680	V1026 - YLXP	GAG TAT CTG NNN CCC CAG CAG GGA TTC TTC	sense	
AB6683	V1026 - YLXP	CTG CTG GGG NNN CAG ATA CTC TTC AGC	antisense	
AB6681	P1027 - YLVX	TAT CTG GTG NNN CAG CAG GGA TTC TTC TCC CCG	sense	
AB6684	P1027 - YLVX	TCC CTG CTG NNN CAC CAG ATA CTC TTC AGC	antisense	
	Site B-derived Mutants			
AB6000	N1146 ADVH	GAGTATGTG (C/G)(T/A)C CAATCAGAGGTTCAGCCT	sense	
AB6001	N1146 ADVH	TGATTGG(T/A)(C/G)CACATACTCGGGCTGGGGCT	antisense	
	Site C-derived Mutants			
AB4838	P1199(DAPH)	GCTGTGGAGAAC(G/C)(A/C)TGAATACTTAGTAC	sense	
AB4849	P1199(DAPH)	CTAAGTATTCA(T/G)(C/G)GTTCTCCACAGCA	antisense	
	Site D-derived Mutants			
AB6002	N1224(A/D)	GCCTTTGACG(C/A)CCTGTATTACTGGGACCAGAAC	sense	
AB6003	N1224(A/D)	GTAATAGACG(G/T)GTCAAAGGCTGGGCTGAAG	antisense	
AB7347	Y1226F	GAC AAC CTC TTT TAC TGG GAC CAG	sense	
AB7346	Y1226F	CTGGTCCCAGTAAAAGAGGTTGTC	antisense	
AB7349	Y1227F	GAC AAC CTC TAT TTC TGG GAC CAG	sense	
AB7348	Y1227F	CTGGTCCCAGAAATAGAGGTTGTC	antisense	
	Site E-derived Mutants			
ABxxxx	N1255(DA)		sense	
AByyyy	N1255 (DA)		antisense	
AB4827	P1256(DAPH)	GCAGAGAAC(G/C)(A/C)T GAGTACCTAGGCCT	sense	
AB4828	P1256(DAPH)	TAGGTACTCA(T/G)(C/G)GTTCTCTGCAGT	antisense	
	Oligos for sequencing			
AB6685	forward before NcoI	TTGGTGTCAGAATTTTCACGTA	sense	
AB6676	forward on NcoI	CATCCAGCCCCATGGACAGTACCTTC	sense	
AB6677	reverse after BglII	GTCACCATCAAACACATCGGAG	antisense	
AB6233	forward before BglII	CTGACACTGGGCCTGGAG	sense	
AB6232	reverse after XbaI	GAAGGCAAAAACGTCTTTGAC	antisense	
AB7421	forward before XbaI	CTG CCT CCT GTC CGG CCT GCT	sense	
AB2916/7420	reverse with stop codon and EcoRI site	CAC GAA TTC TCA TAC AGG TAC ATC CAG GCC	antisense	
AB9434	reverse oligo in pJ4W	TCACACCACAGAAGTAAGGTTCCTTCACAA	antisense	
	oligos for fusion proteins			
AB1945	PTP1C forward N-terminal	GAAGAATTC C ATGGTGAGGTGGTTTCACC M V R W F H	sense	EcoRI
AB1947	PTP1C reverse N-terminal	CACGAATTCCTGGTGGGGTCCGAGCAGTTC	antisense	EcoRI
AB1948	PTP1C C-terminal	CCCGAATTC C ATGGAGAGGTGGTACCACGGC M E R W Y H G	sense	EcoRI
AB1946	PTP1C reverse C-terminal	CACGAATTCCTGGTGGGGTCCGAGCAGTTC	antisense	EcoRI
AB5676	SHC forward	CGTGGAATTCAGGACATGAACAAGCTG	sense	EcoRI
AB5674	SHC reverse at PTB	TGGGAATTCTCAGTAGTACTGATGGTC	antisense	EcoRI
AB5676	SHC reverse at end	CAGCTGAATTCACACTTTCCGATCCAC	antisense	EcoRI
AB1950	GAP forward	CGGGAATTCCATGGTGAGGTGGTATCACGGAAAACTTG	sense	EcoRI
AB1951	GAP reverse	CACGAATTCATTGAGTACTTGTTCTTGATCCTG	antisense	EcoRI

this, PCR was carried out over nucleotides (*Xba*I-3800) with mutagenic oligonucleotides AB6000/AB6001, AB4838/4839 and AB4827/4828. Neera Jeybala. created N1255A/D mutants in a similar fashion using oligonucleotides ABxxxx and AByyyy. To create Y1226, Y1227 and N1224A/D the same procedure was used with mutagenic oligonucleotides AB7349/AB7348, AB7347/AB7346 and AB6002/AB6003 respectively. The *Bgl*II-*Xba*I or *Xba*I-*Eco*RI regions were sequenced to confirm fidelity of the cloned PCR-amplified sequences.

2.2.1D *Generation of YAxB second site mutants*

Three sets of reactions was used to introduce complete degeneracy into codons L1029, V1030 and P1031 within the plasmid pSΔSX-YAB using mutagenic oligonucleotides AB6679/AB6682, AB6680/AB6683 and AB6681/AB6684 and external oligonucleotides AB6676/AB6677 (see Table 2.1). PCRs were cloned as *Nco*I-*Bgl*II fragments into pSΔSX-YAB to create pSΔSX-derived plasmids $pYAX_1B$, $pYAX_2B$ $pYAX_3B$. The latter two sets of plasmids were screened for inserts by JoAnn Attard (a fourth year student). Plasmids were sequenced over the *Nco*I-*Bgl*II region to confirm fidelity of the amplified sequences and to determine the mutations obtained.

2.2.2 Shp-1/PTPM1 cDNA Isolation

The BamHI/EcoRI fragment of PCR-MI was used to generate a random-primed 32P-cDNA probe that was used to screen a Lambda-Zap thymus cDNA library (Stratagene). $1.2x10^6$ bacteriophage were screened and following the primary screen, 50 secondary positives displaying enrichment. Of these, cDNAs from 17 secondary plaques were rescued into Bluescript plasmid by M13 helper virus infection according to the manufacturers protocol. Positive isolates was confirmed by Southern blot hybridization to the PTPM1 PCR-derived probe for twelve independently derived plasmids (data not shown). Two clones were chosen for nucleotide sequence analyses as they contained the largest inserts, similar in size to the mRNA transcript (~2400 nucleotides), they hybridized to the *ptpM1* PCR product, produced a product

when used as a template for PCR amplification with different degenerate PTP-specific oligonucleotides and produced an approximately 70kDa protein in reticulolysate in vitro translations reactions (data not shown).

2.2.3 Generation of Shp-1/PTPM1 C453S

To create a cDNA containing the C453S mutation PCR were performed as above with mutagenic oligonucleotides F4/Bd-1 and AB1944/T3 both using pB-B20 as a template. The 300 and 700bp fragments were gel-purified and used in a subsequent PCR with F4 and T3. The 1000bp product was gel purified, digested with *Nco*I and *Sac*I and ligated with a *Nco*I-*Sac*I digested pB-B20 to create pC453S.

2.2.4 Generation of Mammalian Expression Plasmids

To facilitate cloning of autophosphorylation mutants an *Nco*I site within the 5'-noncoding sequence of the *neuNT*, harbouring the activating point mutation in the transmembrane encoding sequences Bargmann *et al.* 1986b was destroyed. The 5'-portion of *neuNT* was excised from pGem7-*NeuNT* (provided by P. Seigel) as a HinDIII/EagI fragment and subcloned into the *Hin*DIII/*Eag*I sites of pBluescript-SK$^-$ to create pB-NT-1.6$_{H3/Eg}$. This plasmid was linearized with *Nco*I, the ends made blunt with S1 nuclease/Klenow treatment (Sambrook *et al.* 1989) and was then self-ligated to form pB-NTΔN-1.6$_{H3/Eg}$. The *Hin*DIII/*Eag*I fragment from pB-NTΔN-1.6$_{H3/Eg}$ was reintroduced into pGem7-NeuNT to create pG-NTΔN. pGNeuΔN encoding the wild-type cDNA was created in a similar fashion. The 4.3KB *Hin*DIII/*Eco*RI fragment encompassing the entire *neu* coding region from pGNeuΔN was placed under the transcriptional control of the murine Moloney Leukemia Virus (MMoLV) long terminal repeat (LTR) into the corresponding sites of pJ4Ω (Morgenstern and Land 1990). This plasmid was termed pJNT whereas pJNeu refers to the cDNA containing V664. Autophosphorylation mutants were subsequently cloned into pJNT from pSL301- and pSΔSX- derived plasmids as *Nco*I-*Eco*RI fragments to produce expression plasmids with an activated form of *neu* under MMoLV-LTR control. pJNT-YAX$_3$B plasmids were subcloned by J.-A. Attard. Two deletion mutants were generated by Jennifer E. LeCouter from pJNT by digestion with *Nco*I followed by a "blunt end" reaction with Klenow. *Eco*RI-*Hin*DIII fragments of pJNT-

derived plasmids were cloned into the corresponding sites of pCNDA3 (InVitrogen) to create a series of mammalian expression vectors with Neu and its derivatives under the transcriptional control of the Simian Virus 40 (SV40) promoter/enhancer and each containing a Neomycin resistance marker. *Eco*RI fragments containing either the wild-type or C453S Shp-1/PTPM1 cDNAs were cloned into the corresponding site in pJ4Ω to create pJ-Shp1 and pJ-C453S respectively.

2.2.5 Other Mammalian Expression Plasmids Used

A plasmid (PGK-puro) containing the puromycin resistance gene under the transcriptional control of the phosphoglycerate kinase (PGK) promoter was the gift of M.A. Rundicki while pEJ-Ras and SV2-Neo were generously obtained from Dr. J.A. Hassell. pKRev-1 was described previously (Kitayama *et al.* 1989).

2.3 In vitro association assays

2.3.1 Generation of GST plasmids.

Several GST-fusion proteins were generated by PCR for Shp-1; these include GST-Shp1, gN-SH2, gC-SH2 and gNC-SH2. The oligonucleotides used were Bd1/Bd-2, AB1945/AB1947, AB1948/AB1946, AB1945/AB1946. Each PCR product was cloned through oligonucleotides incorporated *Eco*RI sites into pGEX.3X (Pharmacia) and transformed into XL-I Blue bacteria. Similarly, PCR products encoding the open reading frame of p55-Shc (aa 1-469), the Shc PTB domain (aa 1-160) and the Ras-GAP SH2-SH3-SH2 domains were produced with oligonucleotides AB5675/AB5676, AB5675/Ab5674 and AB1950/AB1951 respectively and were cloned into the *Eco*RI sites of GSTag (Ron and Dressler 1992) and/or pGEX-3X through PCR-engineered restrictions sites. Positive clones were screened on the basis of restriction analysis and production of the expected sized proteins. The murine Shc cDNA was a gift of Dr. T. Pawson (University of Toronto) while GEX-2T, pGEX-3X, GSTag and the human Ras-GAP cDNA were generously given given by Dr. J.A. Hassell (McMaster University). GSTag-Grb2 encodes the entire Grb2 protein and GSTag-Shc SH2 (Stein *et al.* 1994) were generously provided by B. Margolis (New York

State University). GSTag-Src contains the SH2 domain of Src and was derived (Muthuswamy and Muller 1995) from pGEX2T-Src-SH2, a gift from Dr. R.B. Rowely (Bristoll Myers Squibb, New Jersey).

2.3.2 Purification of bacterially produce recombinant proteins.

Overnight bacterial cultures harboring the various GST-fusion plasmids were inoculated to fresh cultures of LB-amp at a 1:20 dilution and were grown at 37°C to an O.D. of 1.0. IPTG was added to a final concentration of 0.5-1mM and incubation was continued a further 2-3 hours. Pelleted bacteria were resuspended in 1/50 volume of PBS containing 5mM DTT and 10mg/ml aprotinin, 10mg/ml leupeptin, and 1mM PMSF and were lysed by sonication. Lysates were cleared by two rounds of centrifugation at 13000rpm 4°C and the supernatant was incubated with 80µl of a 1:1 slurry of glutathione sepharose (CLB4, Pharmacia) for 5-15min at room temperature in an end-over-end rotator. The beads were washed 5 times in 1xPBS in the presence of protease inhibitors and GST-fusion proteins were eluted with 2 volumes of PBS containing 300mg/ml free glutathione on ice for 5min. Eluates were collected and tested for the presence of protein using the Bradford assay following manufacturers instructions (BioRad).

2.3.3 In vitro binding assays

1mg of total cellular extracts from starved and serum-induced cells was incubated with ~2-5µg immobilized GST-fusion proteins for 3-6 hours at 4°C in an end-over-end rotator. The beads were washed three times in HNTG buffer containing 1mM Na_3VO_4 and following the addition of and equal volume of 2x sample loading buffer, affinity purified proteins were subjected to SDS-PAGE.

2.3.4 Direct Blot Assay

Following SDS-8.5% PAGE, Neu immunoprecipitates were electrophoretically transferred to polyvinylidene diflouride (PVDF) membranes (Immobilon-P; Millipore) and blocked overnight at 4°C or at room temperature for 1hr in block buffer (20mM HEPES [pH 7.5], 5mM $MgCl_2$, 1mM KCl, 5mM DTT 10% sodium azide) containing 5% skim milk. Fusion proteins were purified and immobilized on

glutathione sepharose beads (Pharmacia) essentially as described (Smith and Johnson 1991). Immobilized GSTag-fusion proteins were washed three times in DK buffer (50mM KH_2PO_4, 10mM $MgCl_2$, 5mM NaF, 4.5mM DTT). These were incubated in reaction buffer (50mM KH_2PO_4, 10mM $MgCl_2$, 5mM NaF, 4.7mM DTT) containing 500μCi $^{32}P\gamma$-ATP (6000Ci/mM) and 20μg of protein kinase A (Sigma, P-2645) at 30°C for 30min after which time an additional 20μg of protein kinase A was added. Unincorporated nucleotides were removed by washing six times in ice cold 1XPBS containing 5mM NaF and 5mM EDTA. Proteins were eluted with glutathionine as above. Blots were probed for 3hr at room temperature with radiolabeled fusion proteins at $1.5x10^6$cpm in block buffer. The membranes were washed three times in TBS-T and analyzed by autoradiography.

2.4 In vitro translation.

The entire PTPM1 cDNA, cloned into the EcoRI site of pGEM7ZF+ (Promega), was linearized at various sites within the cDNA and the multicloning region. These templates were transcribed using 100 units of SP6 RNA polymerase in a reaction containing 1x transcription buffer (40mM Tris HCl pH8.3, 6mM MgCl2, 2mM spermadine, and 10mM NaCl), 10mM DTT, 0.1mg/μl BSA, 10 units RNAsin, 0.5mM (ATP,CTP,UTP, and GpppG), and 0.5mM GTP. Incubation was carried out at 40°C for 60min. RNA was precipitated and one third of the product was denatured for 10min at 65°C, and added to a rabbit reticulolysate in vitro translation reaction (Promega) in the presence of 90μCi [^{35}S]-methionine (Amersham) following the manufacturers protocols. One fifth of this reaction was electrophoresed through a 10% SDS/polyacrylamide gel and processed by standard techniques.

2.5 Cells and Cell Culture

2.5.1 Generation and culture of Cell lines

Rat1 and NIH-3T3 fibroblasts were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, penicillin, streptomycin and fungizone. Expression plasmids were introduced into Rat1 cells along with PGK-puro by the method of Graham and van der Eb (Graham

and Van der Eb 1973) as modified by Wigler (Wigler *et al.* 1977) at a 40:1 ratio of expression plasmid to puromycin resistance plasmid (PGK-puro). Alternatively electroporation was used to introduce DNA into cells (see electroporations). Transfected cells were selected in supplemented DMEM containing either 3.0μg/ml puromycin or 400-800μg/ml geneticin. In each case multiple clones were isolated and used for further characterization.

2.5.2 Focus Forming Assays

2.5.2A *Electroporations*

Rat1 fibroblasts were harvested by trypsinization, washed 4 times with 1xPBS at room temperature and normalized to 10^7 cells per electroporation cuvette (BioRad GenePulsar Cuvette with 0.4cm electrode gap, cat#165-2088) containing 50μg of each mutant plasmid DNA along with 2μg PGK-puro in a total volume of 800μl. Cells were electroporated in a BioRad electroporator with voltage 1000V and a capacitance of 25uF. Typically, a time constant of approximately 0.4-0.5msec was obtained. Electroporated cells were immediately transferred to 100ml supplemented DMEM, were seeded at 10^6 cells per plate and were maintained in supplemented DMEM for 14 days with the media being changed every third day. The plates were stained with Giemsa following manufacturer's instructions. Relative transformation potential and standard error were calculated by the respective equations:

$$\%NT = 100\% \times \frac{\frac{1}{n_1}\sum_{n1=1} foci(test_{n1})}{\frac{1}{n_{NT}}\sum_{nNT=1} foci(n_{NT})} \quad \text{and} \quad SE_{test} = 100\% \times \frac{\sqrt{\frac{1}{n_1}\sum_{n1=1}([\overline{foci}(test)] - [foci(test_{n1})])^2}}{\frac{1}{n_{NT}}\sum_{nNT=1} foci(n_{NT})}$$

Where n_1 and n_{NT} are the number of plates obtained with the test and NT plasmids respectively and $\overline{foci}(test)$ and $foci(test_{n1})$ are the mean number of foci and the number of foci on a given plate respectively.

2.5.2B *Lipofectamine Transfections*

Focus assays were performed by seeding Rat1 fibroblasts at $3x10^5$ cells per well in six well Nunc tissue culture dishes. This plating regime produces 80% confluency the following day. Plasmid DNA was included to 1.1μg per 6μl lipofectamine reagent mixing as per manufacturers instruction. Liposomes containing DNA were added to cells in 1ml DMEM lacking serum and incubated at 37°C for 2-2.5hrs. The reagent mixture was removed and the cells washed once in DMEM and once in DMEM containing 10%FBS which was replace with 3-4mls of DMEM supplemented with antimicrobial agents and 5%FBS. When the cells reached confluency they were split into either two 60mm plates or a single 100mm dishes which were subsequently treated as detailed above.

2.5.3 Soft Agar Assays

2.5.3A *Rat1-derived cell lines*

Growth in soft agar was assessed by plating 1000 viable cells as assessed by trypan blue exclusion in 0.25% agarose, supplemented 1xDMEM containing 5% FBS on top of 0.5% agarose in 1X supplemented DMEM containing 5% FBS on 60mm plates. Plates were maintained for 21 days, stained overnight with 10mg/ml of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide Sigma catalog # M2128) in 0.25% agarose, 1xDMEM and counted macroscopically.

2.5.3B *Pooled stable NIH-3T3 or Rat1 Fibroblasts Clones*

pCDNA3 or pCDNA3-derived plasmids harboring the different Neu mutants were electroporated into NIH-3T3 or Rat1 fibroblasts as described above. Following 21 days selection in 800μg/ml Geneticin (GibcoBRL), 10^5 cells were plated in DMEM containing 5% FBS and 0.25% agarose and were incubated at 37°C for 28 days. The colonies were stained and counted as above.

2.6 DNA synthesis assay - BrdU Incorporation

DNA synthesis was assayed after metabolic incorporation of 5-bromo-2'-deoxyuridine (BrdU; Cell Proliferation Kit, Amersham) essentially as described previously (Wang and Moran 1996). Cells growing on glass cover slips were deprived of serum overnight (~18 h) and then non-injected or microinjected antibodies to Ras (Y13-259, 2 mg/ml) (Dobrowolski *et al.* 1984; Mulcahy *et al.* 1985) together with biotinylated GST (1.3 mg/ml). After incubation at 37° for 2 h, fresh medium containing 10% FBS and BrdU was added, and the coverslips were incubated at 37° for 18 h. Following fixation, BrdU incorporated into DNA was visualized with a BrdU antibody and a rhodamine-labeled secondary antibody to mouse immunoglobulin. Microinjected cells were identified by staining with FITC labeled avidin (Jackson Immunology Laboratory). Not less than 100 cells were injected on each coverslip and DNA synthesis was determined as the percentage of microinjected cells staining positively for BrdU incorporation. Equivalent results were obtained from two independent experiments, and results from one such experiment are shown. In non-injected cells the percentage of BrdU-labeled cells was determined for not less than 200 cells counted from randomly selected fields of view. Cells were visualized for simultaneous red and green fluorescence by using the appropriate filter sets (Carl Zeiss Ltd.).

2.7 Protein Analyses

2.7.1 Preparation of cell lysates.

For starvation experiments, cells were washed twice in PBS and incubated in DMEM containing 0.5% calf serum for 24 to 72 hours. Serum induction was effected with DMEM containing 20% calf serum, 1mM Na_3VO_4 where indicated for 60 minutes unless stated otherwise. Cells were washed twice in ice cold 1xPBS and lysis was carried out on ice for 20min following the addition of lysis buffer (see immunoprecipitations). Lysates were cleared by centrifugation at 13000 RPM and 4°C for 10min and protein concentrations of the supernatant were determined using the Bradford assay (BioRad) following the manufacturers instructions using a standard protein concentration curve (2-20µg of BSA (Pierce)) generated for each experiment.

2.7.2 Antibodies

Polyclonal antisera to PTPM1, termed αDD1 and αDD2, were generated by repeated injections of New Zealand White rabbits with a cysteine-linked C-terminal keyhole limpet (KLH) conjugated peptide containing the sequence (EEKVKKQRSADKEK-C-KLH). Initial immunization of 500μg antigen in complete Freud's adjuvant were given intramuscularly and twenty one days following the primary immunization, a 500μg boost in incomplete Freud's adjuvant was administered subcutaneously. Subsequent boosts were given at 3 week intervals and 10 days following each boost, 20-40ml bleeds were obtained by the central animal facility staff at McMaster University. Bleeds positive for PTPM1 immunoreactivity were aliquoted and frozen at -80°C. Antisera was used at 10μl/500μg for immunoprecipitations (IP) and from 1:1000-1:500 for immunoblots (IB). DOKR-specific antisera (used at 20μl/1mg in IPs) were a generous gift of Dr. D.J. Dumont and N. Jones (AMGEN, Toronto), while an anti-PTPM1/Shp-1 polyclonal (AV2) was provided by A. Veillette (McGill) and was used at 1:1000 in IBs and 5μl/500μg for IPs. Polyclonal antisera to SHPS-1 was provided by Fujioka *et al.* 1996 and was used at a 1:250 dilution for IBs.

Neu IPs were performed with a mouse monoclonal antibody (mAb) (7.16.4; 1μl ascites fluid/500μg) (Drebin *et al.* 1985) while being detected in immunoblots using mAbs from Oncogene Science (AB3; 1:1000 for IB), or Transduction Labs (E19420; 1:300) or anti-sera made to the AB3 epitope (pAB3; unpublished, generated by P. Siegel; 1:500; cPTAENPEYGLDVPV). Shc immunoprecipitations were carried out with a mixture of polyclonal antisera (0.1μg of S14630, Transduction Labs and 0.1μg of 51-636, Upstate Biotechnology Inc per 100μg lysate). Shc was detected using a rabbit polyclonal or mouse monoclonal from Transduction Labs (S14630; 1:1000). Grb2 was immunoprecipitated with rabbit polyclonal antisera (C23, Santa Cruz) and was detected in immunoblots with either a rabbit polyclonal sera from Santa Cruz (C23; 1:400) or a mixture of mAbs at 1:1000 each from Upstate Biotechnology Inc (05-226) and Transduction Labs (g16720). Antiphosphotyrosine antibodies were from Upstate Biotechnology Inc (1:1000; 4G10) or Transduction Labs (Py20; 1:1000 for IB).

In each case horse radish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit secondary antibodies were obtained from Jackson Laboratories and were used at a 1:1000 dilution. Where indicated

125I-conjugated anti-rabbit or anti-mouse antibodies were purchased from ICN and were used at 100μCi/ml. The specific antibodies used in each immunoprecipitation and immunoblot is indicated in the figure legends and were used at the concentrations recommended by suppliers unless otherwise indicated above.

2.7.3 Immunoprecipitations

Confluent plates were washed twice in ice cold 1x PBS and the cells were then lysed for 20min either in modified TNE lysis buffer (mTNE: 50mM Tris HCl [pH 8.0], 150mM NaCl, 1%NP40, 10mM NaF, 10mM sodium orthophosphate, 2mM EDTA) mCHAPS lysis buffer (50mM Tris HCl [pH 8.0], 50mM NaCl, 0.7% 3-[(3-cholamidopropyl)-dimethyl-ammnio]-1-propanesulfonate (CHAPS), 10mM NaF, 10mM sodium orthophosphate, 2mM EDTA) or PLC lysis buffer (McGlade *et al.* 1992a) each containing 1mM sodium orthovanadate, 10μg/ml leupeptin, 10μg/ml aprotinin. Lysates were clarified through centrifugation at 4°C for 15min and protein concentrations were normalized following quantitation by the Bradford assay (BioRad). Equivalent amounts of protein (as indicated in the figure legends) were incubated with the appropriate antibodies and protein A or G sepharose beads (Pharmacia) for 3 hr at 4°C. Shc immunoprecipitations were carried out with a mixture of polyclonal antisera (0.1μg and 0.1μg / 100μg lysate; cat# S14630, Transduction Labs and 51-636, Upstate Biotechnology Inc) pre-absorbed to protein A sepharose from lysates prepared using mCHAPS lysis buffer. Neu immunoprecipitates were performed with a mouse monoclonal antibody (mAb 7.16.4; 1μl/500μg) (Drebin *et al.* 1985) in mTNE. The immunoprecipitates were subsequently washed 5 to 7 times with lysis buffer and were resuspended in 1x sodium dodecyl sulfate (SDS) gel loading buffer. For Grb2-Neu coimmunoprecipitations, cells were lysed in PLC lysis buffer and proteins were immunoprecipitated with 7.16.4 mAbs conjugated to protein G sepharose (Harlow and Lane 1988) as Grb2 comigrates with the antibody light chain. Immunoprecipitates were washed 7 times in PLC lysis buffer, boiled in 1xSDS gel loading buffer lacking reducing agents and the beads were pelleted by centrifugation. The supernatant was subsequently boiled following the addition of β-mercaptoethanol to 0.8M.

2.7.4 Immunoblot procedures

For immunoblot analyses, immunoprecipitates or 20-40 μg (as indicated) of total cell lysates were electrophoresed on SDS-polyacrylamide gels and the proteins were transferred onto PVDF membranes. Phosphotyrosine immunoblots were blocked in Tris buffered saline , 0.05% Tween-20 (TBS-T; 20mM Tris-HCl [pH 7.5], 150mM NaCl), 3% BSA (fraction V; Sigma) overnight at 4°C or at room temperature for 1hr. Membranes were probed with antiphosphotyrosine antibodies (4G10, Upstate Biotechnology Inc. or Py20, Transduction Labs) in blocking buffer for 1-3 hr at room temp, washed four times for 15min in TBS-T and once for 15min in TBS-T containing 3% skim milk. Membranes were then incubated with HRP-conjugated anti-mouse antibodies followed by four 15min washes in TBS-T and were visualized by enhanced chemiluminescence (ECL, Amersham) following the manufacturers instruction. Immunoblots for Neu were performed using mAbs AB3 (1:1000; Oncogene Science) or for Shc-coimmunoprecipitations (1:300; E19420, Transduction Labs). Anti-sera (unpublished) made to the epitope of the AB3 mAb was used in Figure 3.3A to detect Neu. In each case HRP-conjugated anti-mouse or anti-rabbit secondary antibodies (1:1000; Jackson Laboratories) were used.

2.7.5 Kinase Assays

2.7.5A *Neu Auto-kinase activity*

Neu was immunoprecipitated as above from cells lysed in mTNE lysis buffer. Following four washes in ice cold mTNE, the beads were washed in twice in ice cold 1x kinase buffer (100mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] [pH 7.0], 5mM $MnCl_2$). All traces of kinase buffer were removed and 1X kinase buffer containing 10μCi of $^{32}P\gamma$-ATP was added. The reactions were carried out at 30°C for 20min and were terminated with the addition an equal volume of boiling 2x SDS-PAGE loading buffer containing 5mM EDTA. The products were analyzed by SDS-9%PAGE under reducing Laemmli conditions and the gels were processed as previously described (Muthuswamy *et al.* 1994).

2.7.5B *Neu Kinase activity towards an exogenous substrate*

Neu was immunoprecipitated from 2.5mg of the indicated lysates in lysis buffer using 4µl of 7.16.4 mAb. This ensures that the antibody is limiting and thus each immunoprecipitate contained equivalent amounts of Neu. Immunoprecipitates were carried out in duplicate from independently lysed cells. Following four washes in ice cold lysis buffer, the beads were washed in twice in ice cold 1x kinase buffer (100mM HEPES [pH 7.0], 5mM $MnCl_2$). Each immunoprecipitate was divided into five equal aliquots. All traces of kinase buffer were removed and ice cold 1X kinase buffer containing 10µCi of $^{32}P\gamma$-ATP and 2µg myelin basic protein (MBP) (UBI) was added. Reactions were carried out at 4°C and were terminated at the indicated times with the addition an equal volume of boiling 2x SDS-PAGE loading buffer containing 5mM EDTA. The products were analyzed by SDS-(4-15%) gradient PAGE and were processed as above. $^{32}P\gamma$-incorporation into MBP was quantified on a PhosphoImager. The mean counts from duplicate immunoprecipitates from duplicate cell lines are represented.

2.7.6 PTPase Assays.

2.7.6A *^{32}P-labeled substrates*

Raytide peptide (Oncogene Science) and myelin basic protein (Sigma) were labelled *in vitro* with purified c-Src (Oncogene Science) following the manufacturer's protocol. PTPase assays were carried out in PTPase buffer (20mM Tris pH7.2, 2mM EDTA, 2mM EGTA, 7.5% glycerol, 2.5mM β-mercaptoethanol). 20µl of labelled MBP or Raytide peptide were added to 10µl of total bacterial lysates and the mixtures were made to 50µl with PTPase buffer. Incubation was carried out for 10min at 37°C and the trichloroacetic acid (TCA)-soluble counts were measured. The data is presented as a percentage released relative to a blank [cpm(released)-cpm(blank)]/[cpm(total)-cpm(blank)]x100%.

2.7.6B p-Nitrophenol phosphate (pNPP) assay

Aliquots of bacterial extracts harbouring either pGEX.3X or pGEX.PTPM1 were diluted to 450µl with buffer A (20mM Immidizol pH7.2, containing benzamide HCl, 2mM EGTA, 2mmEDTA, 10mM β-mercaptoethanol) and incubated at 37°C following the addition of 50µl 200mM pNPP. The reaction was terminated via the addition of sodium bicarbonate to 1M. Both the amount of total bacterial extract and time of reaction were varied independently. 150µg of protein was used in assays in which the incubation time was varied and an incubation time of 60min was used in assays in which the protein concentration was varied. Note the pNPPase assays were carried out by R.B. Rowely in P.E. Branton's lab (McGill University). PTPM1 immunoprecipitates were incubated with 200µl 200mM pNPP at 37°C for 1hr.

Chapter 3

Distinct Neu Tyrosine Phosphorylation Sites Mediate Ras-Dependent Transformation Through Multiple Adapter Proteins

3.1 INTRODUCTION

Elevated expression of the EGFR family members has been implicated in the etiology of human ovarian and breast cancers (see section 1.2; reviewed in Hynes and Stern 1994; Mansour *et al.* 1994). In particular, amplification and consequent overexpression of the *neu/erbB-2* RTK are observed in a large proportion (20-30%) of primary human breast cancers (Slamon *et al.* 1987; Slamon *et al.* 1989; Sainsbury *et al.* 1988). Consistent with these observations, expression of either wild-type *neu* or a constitutively active mutant (V664E) in the mammary epithelia of transgenic mice induces metastatic mammary tumors (Muller *et al.* 1988; Bouchard *et al.* 1989; Guy *et al.* 1992). The induction of mammary tumors in twild-type *neu* transgenic mice correlates with elevated levels of tyrosine phosphorylated Neu.

These studies clearly demonstrate that Neu activation can induce mammary carcinomas yet the molecular mechanism for the potent transforming activity of *neu* is poorly understood. Following receptor dimerization, autophosphorylation takes place at several tyrosine residues in the cytoplasmic terminus of Neu (see below) (Hazan *et al.* 1989; Segatto *et al.* 1990), providing potential binding sites for cytoplasmic SH2 or PTB containing signaling molecules (see section 1.3). Specifically, Src binds to Neu and its activity is increased moderately in response to activated Neu expression (Muthuswamy and Muller 1995), Ras-GAP, PLCγ and Shc proteins associate with Neu and are tyrosine phosphorylated in Neu expressing cells (Fazioli *et al.* 1991; Peles *et al.* 1991; Jallal *et al.* 1992; Segatto *et al.* 1992; Segatto *et al.* 1993; Xie *et al.* 1995) and Grb2 and Grb7 directly interact with the receptor (Janes *et al.* 1994; Stein *et al.* 1994).

This work however fails to provide functional evidence of the role these interactions has in the transformation process.

While the binding sites on Neu for these SH2 and PTB containing proteins are not established, deletion or mutation of Neu tyrosine autophosphorylation sites can dramatically affect the transforming activity of *neu* (Segatto *et al.* 1990; Di Fiore *et al.* 1990; Akiyama *et al.* 1991; Mikami *et al.* 1992; Ben-Levy *et al.* 1994). However, these studies differ in their conclusions concerning the relative importance of these tyrosine autophosphorylation sites in *neu*-mediated transformation. In one set of experiments, alteration of three or five tyrosine phosphorylation sites in Neu resulted in a progressive debilitation of transforming activity (Segatto *et al.* 1992). In other studies, mutation of tyrosine 1248 in an activated version of human ErbB-2 resulted in a transformation defective molecule suggesting that this site was essential for the transforming activity of neu (Akiyama *et al.* 1991). It has also been reported that addition of this single tyrosine phosphorylation site to a transformation defective, carboxyl truncation mutant restored the transforming potential to activated *neu*. However, mutation of this site alone did not ablate transforming activity (Ben-Levy *et al.* 1994). These observations suggest that phosphorylation of this residue in Neu was both sufficient and necessary for its transforming potential. By contrast, alteration of the analogous tyrosine residue in the wild-type ErbB-2 protein did not effect its transforming potential (Segatto *et al.* 1990). These discrepancies may, in part, be explained by the use of different versions of the gene (human versus rat, wild-type versus activated alleles), the cell types used (Rat or NIH-3T3 fibroblasts) or in the levels of expression of the genes as expression levels have profound effects on the properties of other transforming oncogenes (such as Ras; Hua *et al.* 1997).

To systematically address the role of tyrosine autophosphorylation sites in *neu*-mediated transformation, I employed a strategy initially described for the platelet derived growth factor receptor (PDGFR). In these studies, individual tyrosine residues were restored to a mitogenically inactive mutant PDGF receptor containing tyrosine to phenylalanine substitutions at the known tyrosine phosphorylation sites (add-back mutants, Valius and Kazlauskas 1993). Using a similar strategy, I generated a catalytically

activated Neu molecule (V664E) in which tyrosine phosphorylation sites (Hazan *et al.* 1989; Di Fiore *et al.* 1990) were similarly altered. This mutant, termed Neu tyrosine phosphorylation deficient (NT-NYPD), was severely impaired in its capacity to transform Rat1 and NIH-3T3 fibroblasts. To elucidate the function of individual tyrosine autophosphorylation sites, a series of add-back mutants were generated in which individual tyrosine phosphorylation sites were restored to the NT-NYPD mutant. Restoration of any one of four individual tyrosine residues to the NT-NYPD mutant completely reestablished the transforming activity of this mutant. Interestingly, these transforming mutants require Ras activity to induce proliferative signals. To elucidate the molecular basis for the apparent functional redundancy of the four tyrosine phosphorylation sites, I analyzed the ability of these mutants to associate with signaling molecules that have been implicated in Neu-mediated transformation. The results which follow demonstrate that Neu uses multiple proteins to initiate transforming signals.

RESULTS

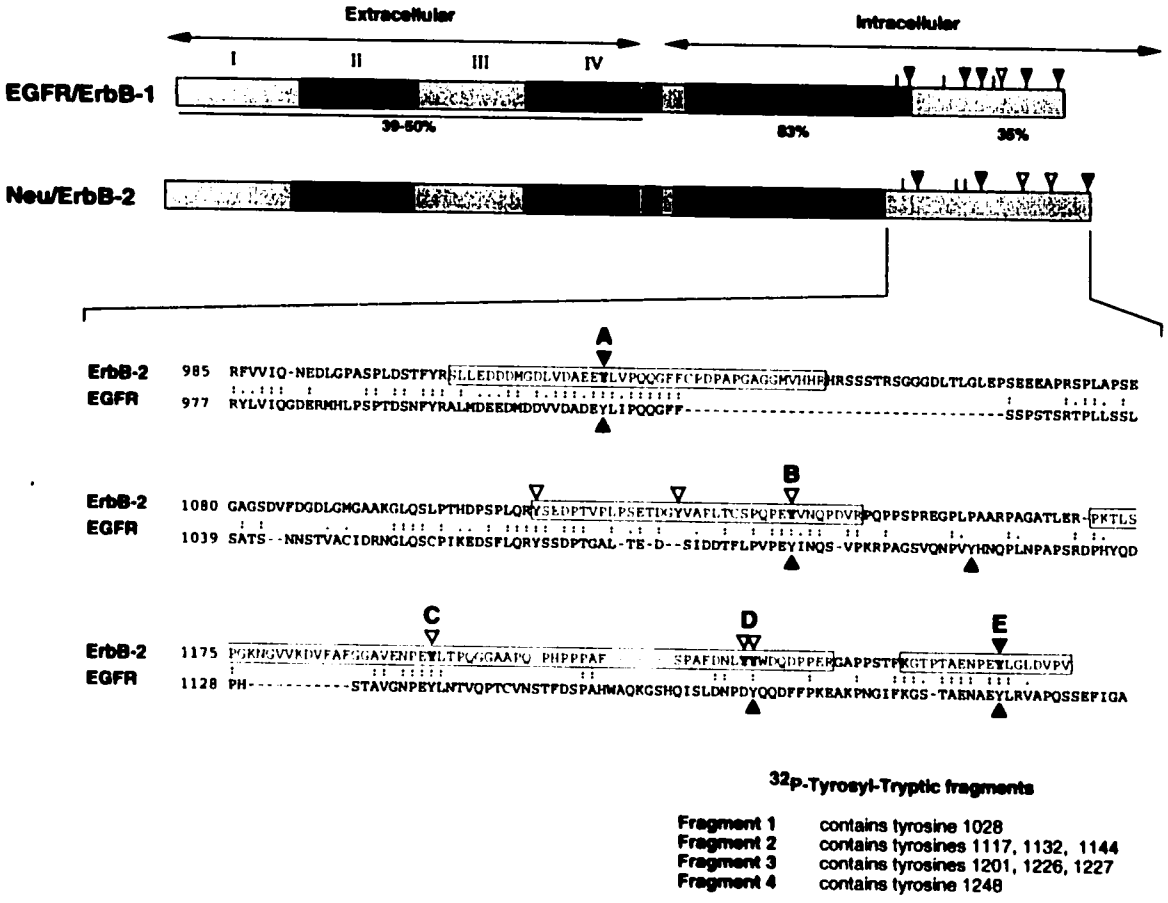
3.2 Neu mediates transformation through carboxy terminal phosphorylation sites

3.2.1 Alteration of individual Neu tyrosine autophosphorylation sites has a modest effect on cellular transformation

Given the importance of the tyrosyl-phosphorylation sites in RTK-mediated proliferative signal transduction and the role Neu/ErbB-2 plays in human disease, I chose to genetically dissect the mechanisms by which Neu initiates a transforming signal by assessing the transformation potential of Neu autophosphorylation site mutants. Previous data demonstrate that ErbB-2 is phosphorylated on at least four tyrosine residues. Phosphoamino analysis of tryptic fragments derived from ErbB-2 reveal that the four tyrosine-phosphorylated fragments each map to the carboxyl-tail of ErbB-2 (Hazan *et al.* 1989). Tryptic fragments 1 and 4 contain single tyrosines allowing unequivocal identification of the phosphorylated residue (Y1028 and Y1248) (see Figure 3.1). Fragments 2 and 3, however, each contain three tyrosines making the determination of the phosphorylation status of these residues ambiguous. Of the three tyrosines in fragment 3 (Y1201, Y1226, Y1227), one (Y1227) is collinear with a *bona fide* tyrosyl-phosphorylation site within the EGFR (Figure 3.1). Similarly, tyrosine 1144 in fragment 2 is collinear with Y1068, a phosphorylation site within the EGFR. Thus I have chosen to target Y1028, Y1144, Y1248 for mutagenesis. As it is difficult to determine whether Y1226 or Y1227 or both are phosphorylated, I chose to simultaneously alter both residues. The remaining tyrosine within this tryptic fragment (Y1201) follows a similar amino acid motif (ENPE-Y) to that found upstream of Y1248. As acidic amino acids upstream of tyrosine residues often target tyrosines for phosphorylation (Songyang *et al.* 1995a), Y1201 was also chosen for mutagenesis. The remaining tyrosines of tryptic fragment 2 were not chosen for the following reasons. Tyrosine 1117 harbours no nearby acidic residues to the amino side and gas phase sequence analyses of fragment 2 reveals a tyrosine at position 1132 (Hazan *et al.* 1989). As this method is incapable

Figure 3.1 ErbB-2/Neu contains autophosphorylation sites clustered in the carboxy tail.

Schematic representation of the EGFR and Neu/ErbB-2 depicting the functional domains and degree of amino acid identity to ErbB-2. The extracellular portion is depicted as containing four subdomains (I-IV) of which two are cysteine rich regions (red). A single transmembrane domain (black) and the tyrosine kinase domain (striped) are indicated. Known or suspected tyrosine phosphorylation sites are depicted by closed and open triangles respectively, while additional tyrosine residues are indicated by vertical lines. **(Bottom)** Alignment of the carboxy-terminal regions of human EGFR and ErbB-2. Boxed are the phosphotyrosine-containing tryptic fragments in ErbB-2. *Bona fide* tyrosyl-phosphorylation sites are emphasized with black triangles while open triangles highlight residues on the same tyrosine-phosphorylated proteolytic fragment containing multiple tyrosines.



^{32}P-Tyrosyl-Tryptic fragments

Fragment 1	contains tyrosine 1028
Fragment 2	contains tyrosines 1117, 1132, 1144
Fragment 3	contains tyrosines 1201, 1226, 1227
Fragment 4	contains tyrosine 1248

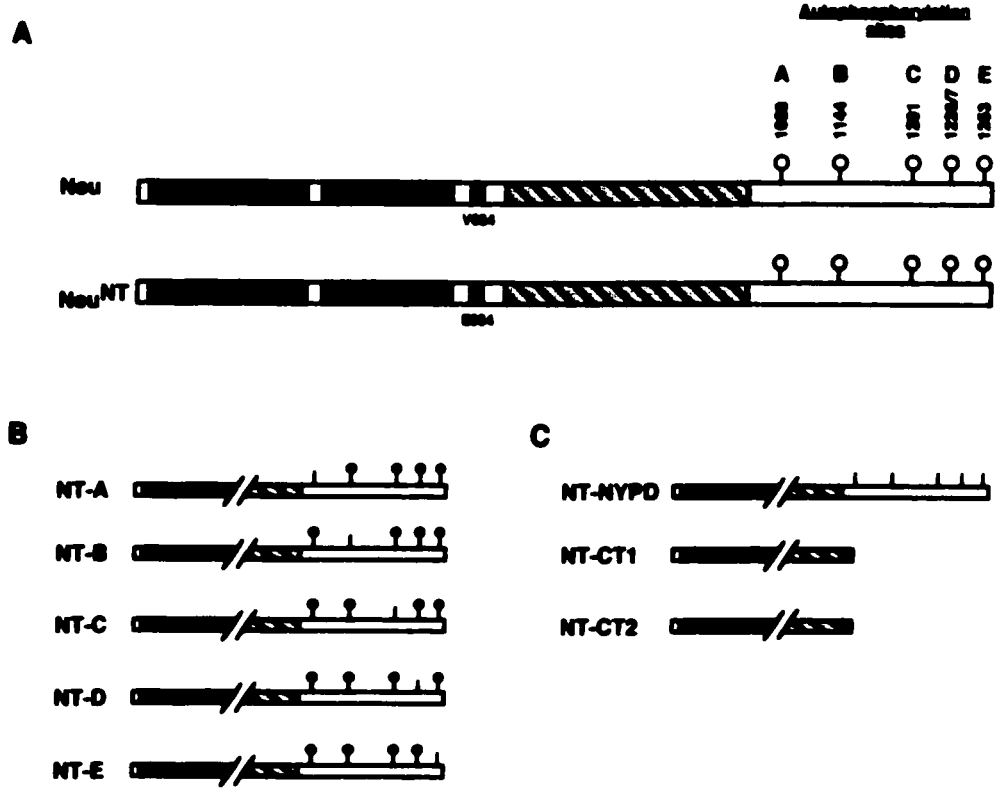
of amino acid determination when tyrosine residues are phosphorylated, tyrosine 1132 is either not phosphorylated or phosphorylated at low stoichiometry. Thus I have chosen five sites to target: Y1028, Y1144, Y1201, Y1226/7 and Y1248 (Figure 3.1).

I have generated a series of Neu molecules in which the carboxyl-terminal tyrosine phosphorylation sites have been converted to phenylalanine residues (Figure 3.2A). This residue was chosen as its side chain contains a benzene ring which most closely resemble the phenolic ring of tyrosine and is not phosphorylated owing to the lack of a suitable electron donor (-OH) found in tyrosine. Because a ligand for Neu has yet to be described, these "autophosphorylation" mutations were introduced into a *neu* cDNA harbouring the activating point mutation in the transmembrane domain that renders Neu constitutively kinase active (V664E, Bargmann and Weinberg 1988; Bargmann *et al.* 1986a) through increased homodimerization of mutant receptors (Weiner *et al.* 1989). For the purpose of clarity, activated Neu mutants lacking individual tyrosine phosphorylation sites are termed NT-A (tyrosine residue 1028 to phenylalanine), NT-B (Y1144F), NT-C (Y1201F), NT-D (Y1226/7F) and NT-E (Y1253F) (Figure 3.2B).

To assess the transforming capacities of the various mutant *neu* constructs, these mutants were placed under the transcriptional control of murine Moloney leukemia virus (MMoLV) long terminal repeat (LTR) and tested for their ability to transform Rat1 fibroblasts in culture. Mutation of any single site (NT-A through NT-E) has minimal effects on transformation potential compared to the unaltered activated cDNA (NT). For example, single site mutations in sites B to E result in modest impairment of focus forming activity (75-85% of NT, Table 3.1). By contrast, removal of site A (NT-A) resulted in a modest but reproducible 1.6 fold elevation of focus forming activity compared to the activated *neu* construct. Similar results were obtained when Rat1-derived cell lines were tested for the ability to grow in an anchorage independent manner (data not shown). These results suggest that alteration of any single tyrosine phosphorylation site does not drastically interfere with the ability to transform Rat1 cells.

Figure 3.2 Schematic representation of Neu RTK autophosphorylation point and deletion mutants.

(A) The structure of Neu and activated Neu (Neu ^{NT}), containing the transmembrane mutation V664E, are depicted. Tyrosine phosphorylation sites at residues 1028 (site A), 1144 (site B), 1201 (site C), 1226/7 (site D) and 1253 (site E) are indicated (Y). (B) Tyrosine residues were converted to phenylalanine residues (F) to create a series of phosphorylation point mutants. (C) NT-NYPD contains mutations at each of the indicated tyrosine phosphorylation sites. NT-CT1 carries a stop codon immediately following methionine 1005 and NT-CT2 is a frame shift deletion mutant terminating with the sequences ^{1005}MHGQYLLPFTAGR. where the underlined amino acids differ from that of Neu. All mutants described herein were derived from Neu^{NT}.


A
A
B
C
D
E
Neu
Neu^NT
B
NT-A
NT-B
NT-C
NT-D
NT-E
C
NT-NYPD
NT-CT1
NT-CT2

3.2.2 The carboxy terminal region contains tyrosine phosphorylation sites required for transformation

To determine whether the carboxyl region containing the known autophosphorylation sites was required for transformation, *neu* mutants were constructed in which all of the tyrosine phosphorylation sites were either converted to phenylalanine residues (NT-NYPD) or were deleted (NT-CT1 and NT-CT2) (Figure 3.2C) and the resulting mutants were tested for their capacity to transform Rat1 fibroblasts. Simultaneous conversion of these tyrosines to phenylalanine residues dramatically impaired the ability to transform Rat1 fibroblasts (8% the level of activated *neu*), a measure of loss of contact inhibition, or to form colonies in soft agar which reflects an ability to grow in an anchorage independent fashion (Table 3.2). Moreover, NT-NYPD expressing lines grew to stable saturation densities, unlike the NT counterparts (data not shown). While these changes indicate that NT-NYPD has diminished transforming abilities, DNA synthesisby this mutant revealed no difference from the Rat1 fibroblast controls (Table 3.2, Rat1=78% and NT-NYPD=76% versus NT=97%), suggesting that these phosphorylation sites contribute to a proliferative signal as well. Interestingly, deletion of the entire carboxyl terminus completely abolished the ability of *neu* to induce focus formation (NT-CT1 and NT-CT2, Table 3.2). Relief of contact inhibition and anchorage independent growth were assessed in NIH-3T3 fibroblasts yielding similar results (Table 3.2). These observations suggest that while individual autophosphorylation sites are dispensable for transformation, multiple sites of tyrosine phosphorylation appear to be required for efficient *neu*-mediated transformation.

3.2.3 NT-NYPD has reduced levels of tyrosine phosphorylation *in vivo* yet harbors kinase activities similar to activated Neu

It is possible that the reduction in the transforming activity of NT-NYPD relative to NT is due to an impairment of the catalytic activity or stability of NT-NYPD. Neu was immunoprecipitated from stable Rat1 fibroblasts expressing NT or NT-NYPD. One third of each immunoprecipitate was subjected to

Table 3.1 Transformation of Rat-1 cells with *neu* autophosphorylation point mutants

Construct	Focus assay 1		Focus assay 2		Focus assay 3		Relative transforming ability[e]
	Average no. of foci[a]	% Transformation of NT[b]	Average no. of foci[a]	% Transformation of NT[b]	Average no. of foci[c]	% Transformation of NT[d]	
Neu	0	0	0	0	0	0	0
NT	167 ± 13	100	287 ± 23	100	198 ± 20	100	100
NT-A	367 ± 33	220	400 ± 19	140	287 ± 29 265 ± 21	145 134	160 ± 35
NT-B	95 ± 13	57	293 ± 21	102	150 ± 15	76	78 ± 19
NT-C	221 ± 21	133	143 ± 10	50	138 ± 11 172 ± 23	70 87	85 ± 30
NT-D	111 ± 22	67	213 ± 10	74	166 ± 14	84	75 ± 7
NT-E	134 ± 15	81	248 ± 13	87	161 ± 16	81	83 ± 3

Three independent focus assays were performed using Rat-1 fibroblasts. The first and second experiments were performed using independent plasmid preparations, while the third experiment was conducted with DNA from an focus assay 2. The nomenclature describing the mutants is defined in Figure 1. All cDNAs were placed under the transcriptional control of the Moloney murine leukemia virus long terminal repeat with the parental vector being pJ4Ω. Use of pJ4Ω did not result in focus formation. Data not available is denoted by n.a.

[a]Values represent the mean number of foci/plate counted on six plates ± standard deviation.

[b]Values are the ratios of the mean numbers of foci obtained for each construct with respect to that obtained with NeuNT (NT) multiplied by 100%.

[c]Values are the mean number of foci/plate counted on six plates ± standard deviation. Where two numbers are given, independent DNAs were used in the same focus assay and the values represent the mean number of foci/plate counted on each of six plates ± standard deviation.

[d]Values are the ratios of the mean numbers of foci obtained for each construct with respect to that obtained with NeuNT (NT) multiplied by 100%. Where independent DNAs were used within the same focus assay the mean of the two ratios is given.

[e]Values are the weighted mean transforming abilities from the three experiments ± standard deviations.

Table 3.2 Transformation and Proliferative Potential of *Neu* Deletion and NT-NYPD Autophosphorylation Mutants

Construct[f]	Rat1			NIH-3T3	
	Relative Focus Formation Potential (in % NT)[a]	Relative Soft Agar Colony Formation[b]	DNA proliferation (% BrDU+ cells)[c]	Relative Focus Formation Potential (in % NT)[d]	Soft Agar colony formation[e]
control	0	0	78	0	-
NT	100	100	97	100	+
NT-NTPD	8 ± 1	11	76	0 ± 0	-
NT-CT1[g]	0 ± 0	n.a.	n.a.	n.a.	n.a.
NT-CT8	0 ± 0	n.a.	n.a.	n.a.	n.a.

[a] Independent focus assays were performed using Rat-1 fibroblasts. All cDNAs were placed under the transcriptional control of the Mcloney murine leukemia virus long terminal repeat with the parental vector being pJ4Ω. Use of pJ4Ω did not result in focus formation. Focus formation assays were performed in Rat1 cells using at least five (control, NT, NT-NYPD) or two (NT-CT1, NT-CT2) independent plasmid preparations in seven (control, NT, NT-NYPD) or two (NT-CT1, NT-CT2) independent experiments. Values are the mean transforming abilities ± standard error calculated as described in Chapter 2.

[b] Colony formation in 0.25% agarose was assessed as in methods. The mean number of colonies formed per 10^3 cells (± standard error) obtained from two independent experiments each with five plates of duplicate cell lines were used to calculate the relative soft agar colony formation. This is the ratio of the mean no. of colonies obtained for each mutant relative to NT multiplied by 100%. The cell lines used were Rat1, NT(3,6) and NT-NYPD (6,22).

[c] Percent DNA synthesis is the percentage of BrdU+ staining cells in Rat1, NT6 or NT-NYPD 22 cell lines.

[d] Independent focus assays were performed using NIH-3T3 fibroblasts as in (*a*). Values are the mean transforming abilities ± standard error.

[e] NIH-3T3 fibroblasts were electroporated with pCDNA3 (control) or pCDNA3-derived plasmids containing the indicated Neu mutants. Following three weeks of G418 selection, 10^5 cells were plated in 0.25% agarose and colony formation was assessed four weeks later. Colony formation is indicated by (+) and a lack of cell growth is designated (-).

[f] Control plasmids pJ4Ω were used for focus forming assays in NIH-3T3 and Rat1 fibroblasts while Rat1-cells were used in soft agar colony formation and DNA synthesis assays. For soft agar colony formation in NIH-3T3 cells, G418 resistant cells from pCDNA3 containing NIH-3T3 cells were used.

[g] Data not available is denoted by n.a.

SDS-PAGE followed by immunoblot analysis with anti-phosphotyrosine antibodies (Figure 3.3B). Comparison of the tyrosine phosphorylation (pTyr) content to the Neu levels (Figure 3.3A) of NT-NYPD to that of NT expressed in Rat1 cells suggests that the pTyr levels of NT-NYPD are reduced relative to that of NT. This reduction is also observed in NIH-3T3 fibroblasts (data not shown). The remaining portion of each immunoprecipitate was subjected to an *in vitro* autokinase assay. Multiple cell lines expressing NT-NYPD mutant possessed similar levels of tyrosine kinase activity by comparison to activated Neu expressing fibroblasts, while the parental Rat1 cells harboured no detectable Neu kinase activity (Figure 3.3C).

While these data suggest that NT-and NT-NYPD harbour similar kinase activities using itself as a substrate, there is the possibility that differences in kinase activity may be manifested towards an exogenous substrate. To this end, I performed a series of *in vitro* kinase assays using an exogenous substrate, myelin basic protein (MBP). These assays were carried out quickly at 4°C in order to observe the initial reaction rates. The initial rates of incorporation of ^{32}P into MBP were similar for NT and NT-NYPD, both of which are 3 fold higher than that found in Neu immunoprecipitates from Rat1 lysates ($4.0x10^5$ and $3.7x10^5$ versus $1.3x10^5$ units/min) (Figure 3.4). Kinetic differences between endogenous and exogenous Neu in these lines is similar to that reported in the literature (Mikami *et al.* 1992), the background presumably reflecting activation of the kinase through immunoprecipitation-mediated dimerization. These kinetic analyses failed to reveal significant differences in the catalytic activities of NT and NT-NYPD towards an exogenous substrate under conditions of linear incorporation, suggesting the differences in the transforming activities of NT-NYPD and NT cannot be attributed to this property of the kinase. One cannot, however, discount the possibility that subtle alterations in the catalytic activities or specificities, not revealed by standard analyses, may exist.

Figure 3.3 NT-NYPD and NT molecules harbour similar kinase activities.

Neu was immunoprecipitated from lysates (containing 1.5mg of protein) obtained from Rat1 or the Rat1-derived stable cell lines expressing NT or NT-NYPD. One third of each immunoprecipitate was electrophoresed on an SDS-8.5% gel, transferred to a PVDF membrane and probed with anti-phosphotyrosine mAbs (**B**). The secondary antibodies were inactivated and the membrane was subsequently probed with anti-Neu antisera (**A**). The remaining two thirds of each immunoprecipitate was incubated with ^{32}P-γ-ATP in an *in vitro* kinase assay and the products were electrophoresed as in (B) (kinase assay) (**C**).

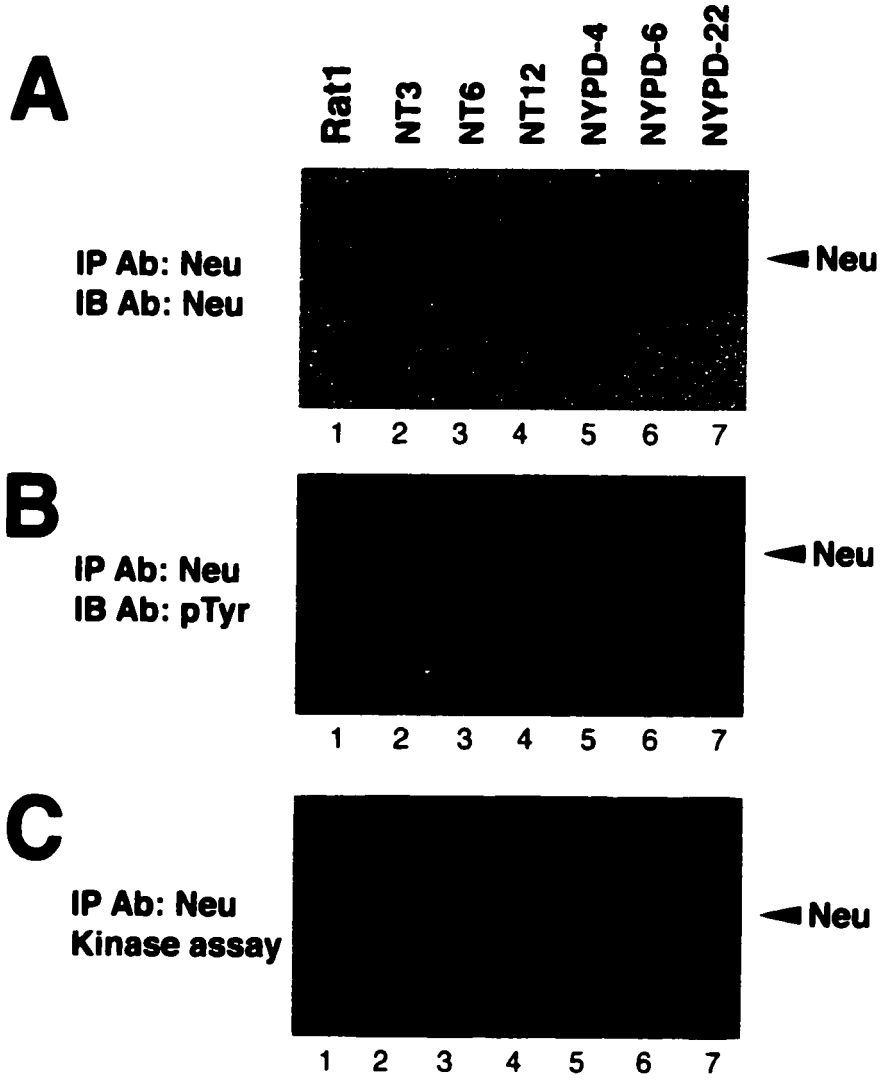

A
Rat1
NT3
NT6
NT12
NYPD-4
NYPD-6
NYPD-22
IP Ab: Neu
IB Ab: Neu
Neu
1 2 3 4 5 6 7
B
IP Ab: Neu
IB Ab: pTyr
Neu
1 2 3 4 5 6 7
C
IP Ab: Neu
Kinase assay
Neu
1 2 3 4 5 6 7

Figure 3.4 NT and NT-NYPD display similar kinetic activities towards an exogenous substrate.

In vitro kinase activities of Neu immunoprecipitates from lysates obtained from Rat1 or the Rat1-derived NT (lines 3 and 6) and NT-NYPD (lines 4 and 22) stable cell lines. Immunoprecipitates were subjected to an *in vitro* kinase assay using myelin basic protein (MBP) as an exogenous substrate (see materials and methods). The amount of radioactivity incorporated into MBP was quantitated by PhosphoImager analyses from duplicate immunoprecipitates isolated from two independent cell lines. The mean counts are represented with standard deviation indicated by error bars within a single experiment. The rates of incorporation are Rat1 $1.3x10^5$ units/min, NT $4.0x10^5$ units/min and NYPD $3.7x10^5$ units/min. Inset is a representative of MBP phosphorylation (arrowhead) for the time points and cell lines indicated.

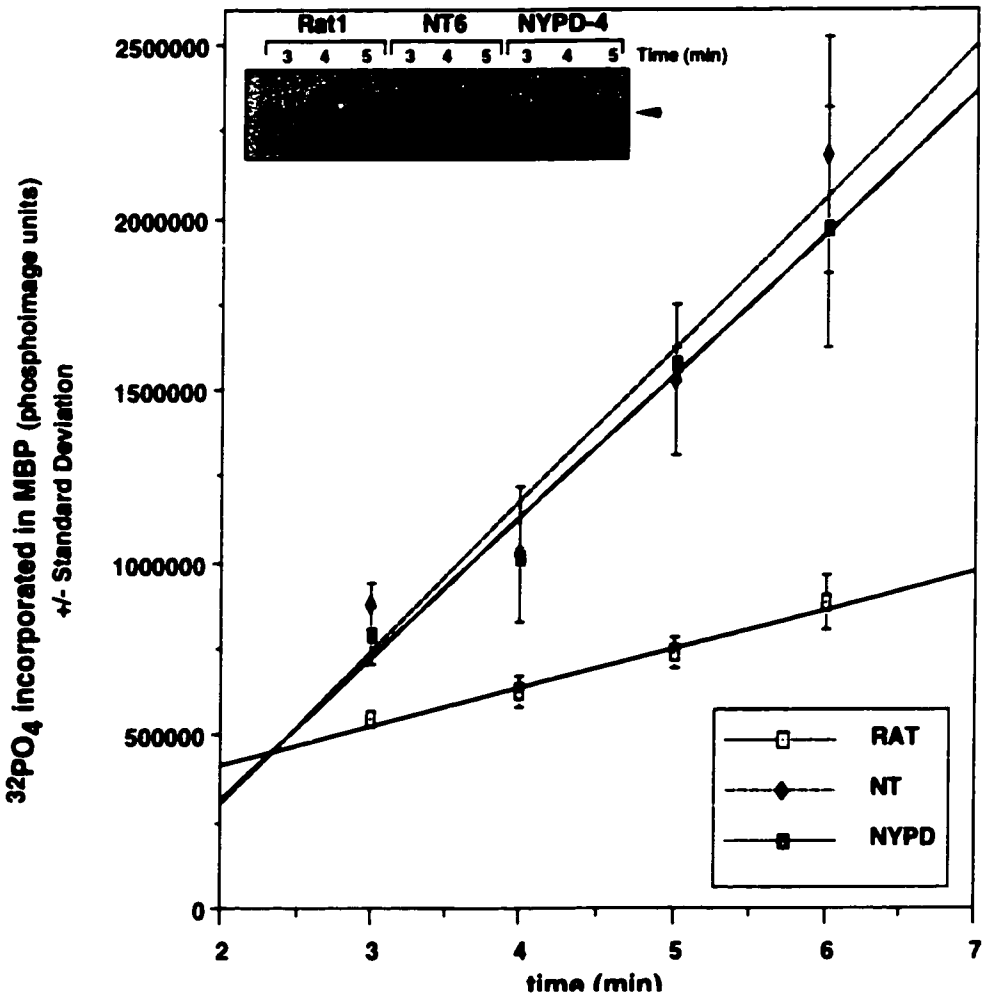

Rat1
NT6
NYPD-4
3 4 5 3 4 5 3 4 5
Time (min)
2500000
2000000
1500000
1000000
500000
0
2
3
4
5
6
7
time (min)
32PO4 incorporated in MBP (phosphoimage units)
+/- Standard Deviation
RAT
NT
NYPD

Table 3.3 Transformation properties of add-back mutants

	Focus Formation[b]		Soft Agar Colony Formation[c]		
			Cell Lines	Pooled Transfectants	
Construct	Rat1	NIH-3T3	Rat1	Rat1	NIH-3T3[d, e]
Control[f]	0	0	0	0	-
NT	100	100 ± 5	100 ± 6	100	+
NT-NYPD	8 ± 1	2 ± 0	11 ± 5	12 ± 2	-
NT-YA	0 ± 0	1 ± 1	0 ± 0	0 ± 0	-
NT-YB	117 ± 12	n.a	116 ± 22	111 ± 2	+
NT-YC	108 ± 9	147 ± 9	99 ± 7	115 ± 10	+
NT-YD	122 ± 9	248 ± 10	76 ± 22	130 ± 9	+
NT-YE	114 ± 8	187 ± 11	95 ± 19	108 ± 10	+

[a] Focus formation assays were performed in Rat1 cells as in Table 3.1 using at least four independent plasmid preparations. Relative transforming activity was calculated as in Table 3.1 (± standard error) using the mean values of the % NT transformation of each construct. Seven (control, NT, NT-NYPD, NT-YC, NT-YD, NT-YE) or five (NT-YA, NT-YB) independent experiments were conducted for each mutant.

[b] Focus formation assays were performed in NIH-3T3 cells as above. Data represents relative transforming activity. These data represent a single experiment using six plates and are normalized to represent the percentage of NT transformation for each construct ± standard deviation.

[c] Colony formation in 0.25% agarose was assessed as in methods. The mean number of colonies formed per 10^3 cells (± standard error) was obtained from two independent experiments each with five plates using duplicate cell lines for each form of Neu. The cell lines used were Rat1, NT(3,6), NT-NYPD (6,22), NT-YA (23, x4c), NT-YB (6,7), NT-YC (2,12), NT-YD (6,22) NT-YE (2,4). The relative soft agar colony formation is the ratio of the mean no. of colonies obtained for each mutant relative to NT multiplied by 100%.

[d] Rat1 fibroblasts were electroporated with pCDNA3 (control) or pCDNA3-derived plasmids containing the indicated Neu mutants. Following three weeks of G418 selection, 10^5 cells were plated in 0.25% agarose-DMEM and colony formation was assessed four weeks later (material and methods). The mean number of colonies formed per 10^5 cells (± standard error) was obtained from two independent experiments each with five plates of duplicate electroporations.

[e] NIH-3T3 fibroblasts were electroporated as in (d) with pCDNA3 (control) or pCDNA3-derived plasmids containing the indicated Neu mutants. Colony formation (+) following 4 weeks or a lack of cell growth (-) are indicated.

[f] Control plasmids pJ4Ω or pJNeu were used for focus forming assays and Rat1 cells or NIH-3T3 cells were used in soft agar colony formation assays.

3.2.4 Multiple autophosphorylation sites independently mediate a transforming signal

While no individual phosphorylation site is absolutely required for *neu*-mediated transformation, it is possible that multiple phosphorylation sites independently contribute to the transformation process. To identify whether particular tyrosine residues were sufficient in mediating a transforming signal, individual phenylalanine substitutions in the NT-NYPD mutant were reverted to tyrosine residues to create a series of add-back mutants possessing only one of these sites. For simplicity, I have termed these add-back mutants NT-YA (tyrosine at 1028), NT-YB (tyrosine 1144), NT-YC (tyrosine 1201), NT-YD (tyrosines 1226 and 1227) and NT-YE (tyrosine 1253) (Figure 3.5A).

Restoration of any one of four tyrosine autophosphorylation sites (NT-YB, NT-YC, NT-YD, or NT-YE) results in focus forming activities comparable to those observed in the parental activated *neu* cDNA in Rat1 fibroblasts (Figure 3.5B and Table 3.3). To ensure the validity of these results these electroporations were performed five to seven times using at least four independent plasmid preparations each yielding similar results. As an independent measure of transformation, anchorage independent growth assays were performed. Multiple Rat1 fibroblasts-derived cell lines expressing the various add-back mutants (see section 3.4 for cell line characterization) were placed in agarose and colony forming ability was assessed (Figure 3.6). While these results recapitulate those found with focus forming assay, I consistently observed smaller colonies formed in lines expressing NT-YB compared to those from the other transforming mutants. It should also be noted that NT-NYPD lines form very small colonies containing generally less than twenty cells which were not scored macroscopically. The ability to grow in an anchorage independent manner was not acquired through the cloning and passaging of these lines, as pooled colonies from electroporated Rat1 cells behave in a similar manner (Table 3.3, see legend and materials and methods). Notably, pooled Rat1 cells expressing NT-YB appeared to form colonies of various sizes suggesting the small colonies formed from stable lines reflect the particular clones used in these assays.

Figure 3.5 Add-back mutants: focus formation in Rat1 fibroblasts

(A) Schematic representation of Neu add-back mutants. Each add-back mutant is derived from NT-NYPD and contains a single tyrosine phosphorylation site and phenylalanine residues at the four remaining sites. For example NT-YB contains a tyrosine at residue 1144 and phenylalanines at residues 1028, 1201, 1226/7 and 1253. All mutants contain the activating V664E mutation from Neu^{NT}. **(B)** Effect of Neu add-back mutant expression in Rat1 fibroblasts in focus formation assays. Rat1 fibroblasts were electroporated with pJ4Ω or pJ4Ω-derived plasmids, plated at a density of 10^6 cells per 100mm tissue culture dish and were maintained in a monolayer for 14 days prior to being stained for DNA (materials and methods). A representative plates is shown to illustrate the relative transforming abilities and morphologies of the add-back mutants compared to activated Neu (NT).

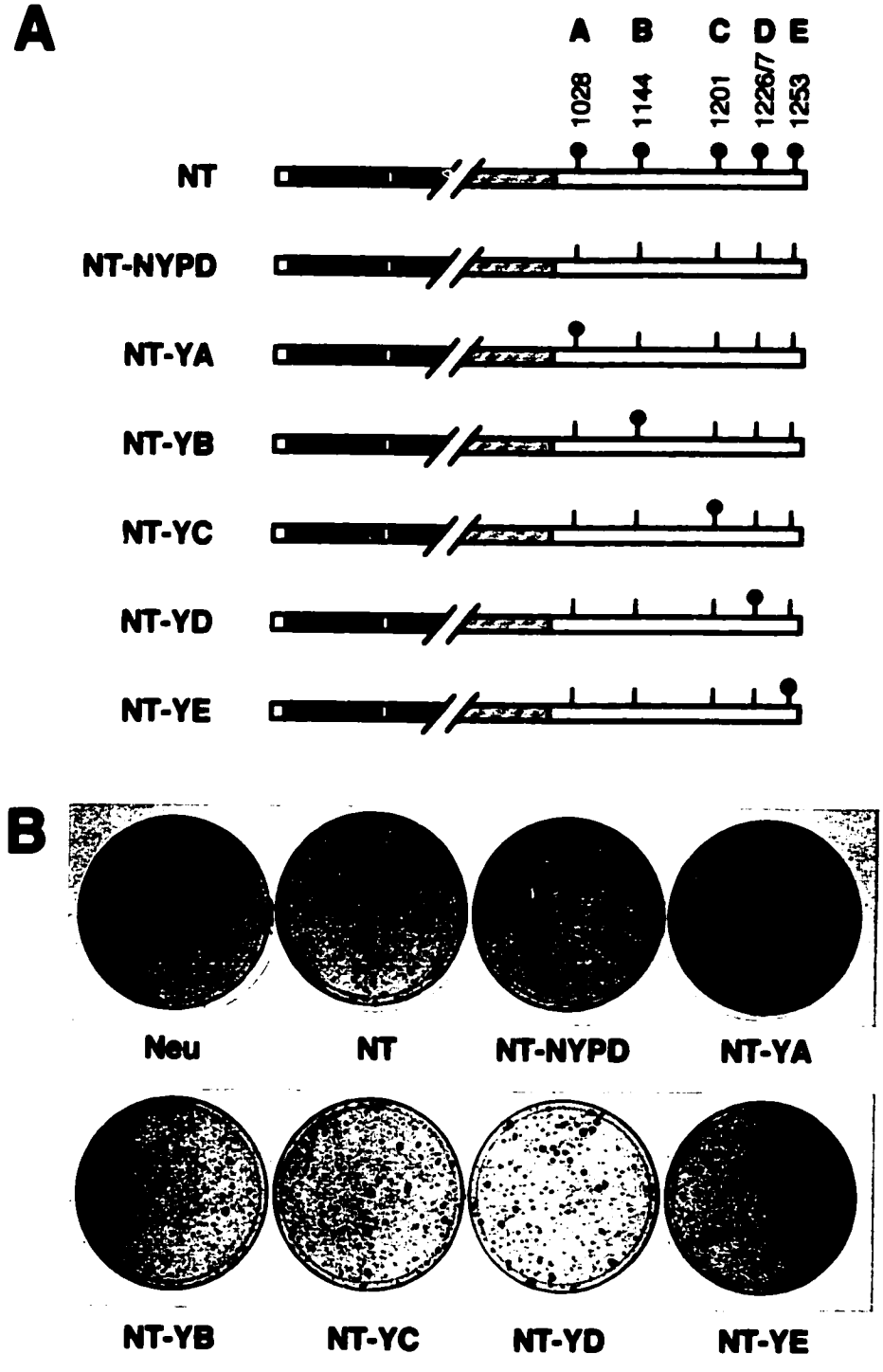
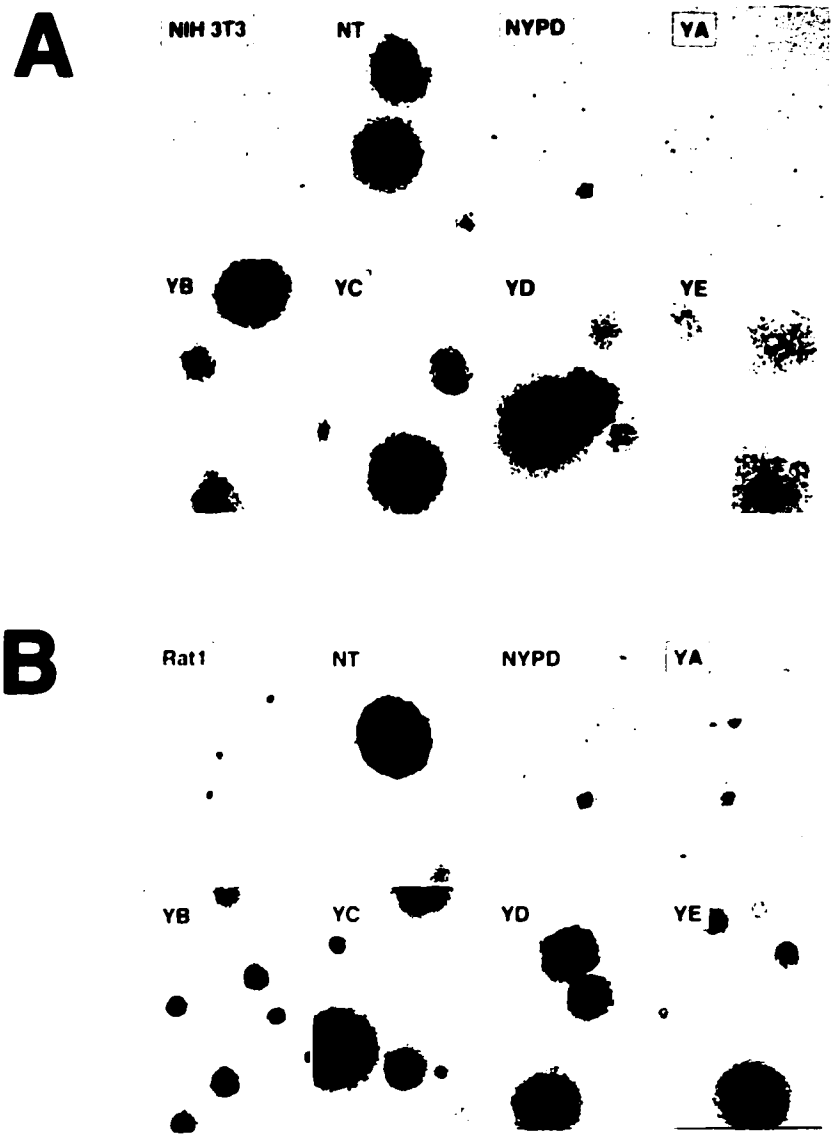

A
A
B
C
D
E
1028
1144
1201
1226/7
1253
NT
NT-NYPD
NT-YA
NT-YB
NT-YC
NT-YD
NT-YE
B
Neu
NT
NT-NYPD
NT-YA
NT-YB
NT-YC
NT-YD
NT-YE

Figure 3.6 Soft agar colony formation of NIH-3T3 and Rat1 fibroblasts expressing different add-back Neu mutants.

(Top panels) Clonal Rat1-derived were cells seeded in 0.25% agarose, maintained in a humidified incubator at 37°C for 21 days and photographed. Shown are representative colonies for Rat1 or lines expressing NT, NT-NYPD or the indicated NT-derived add-back mutants. Each is at the same magnification. The lines photographed are NT-6, NT-NYPD22, NT-YA23, NT-YB7, NT-YC2, NT-YD22 and NT-YE4. (Lower panels) NIH-3T3 fibroblasts were electroporated with pCDNA3 or pCDNA3-derived plasmids containing the indicated Neu mutants. Following 3 weeks of G418 selection, 10^5 drug-resistant cells were seeded in 0.25% agarose, maintained in a humidified incubator at 37°C for 28 days and photographed. Representative colonies for NIH-3T3 and derived pooled colonies expressing NT, NT-NYPD or the indicated NT-derived add-back mutants are displayed. Each is at the same magnification. Note that the morphology seen in NT-YE cells is seen in different fields in the NT colonies but not in any of the other transforming mutants.


A
NIH 3T3
NT
NYPD
YA
YB
YC
YD
YE
B
Rat1
NT
NYPD
YA
YB
YC
YD
YE

To ensure that these transformation data do not reflect cell-type specific phenomena, I performed the same assays using NIH-3T3 cells. The transforming activity of these mutants in NIH 3T3 fibroblasts in focus and soft agar colony formation are comparable (Table 3.3 and Figure 3.6). Pooled colonies expressing activated Neu form colonies of two different morphology; dense spherical colonies and amorphous extended colonies (not shown in Figure 3.6). Interestingly, the latter morphology was exclusively observed in colonies formed by NT-YE expressing cells in NIH-3T3 pooled colonies. The results of these experiments suggest that activated *neu* can induce transformation through multiple functionally redundant tyrosine phosphorylation sites by several criteria (sites B, C, D and E, Table 3.3). By contrast to these transforming add-back mutants, restoration of tyrosine residue 1028 (site A) to NT-NYPD completely suppressed the basal transforming activity of the NT-NYPD mutant (NT-YA) and mutation of this single tyrosine residue alone in activated *neu*, creating NT-A, results in a reproducible elevation of transformation activity (Table 3.1). Thus, unlike tyrosine residues B through E, site A interferes with the transforming activity of *neu*.

3.3 Interaction of c-Src with add-back mutants.

Studies of the Polyoma virus (PyV) demonstrate c-Src (Src) is a positive mediator of middle T antigen (MT) mediated transformation in tissue culture and plays a critical role in MT-induced mammary tumor formation (Guy *et al.* 1994a). Moreover, our lab has demonstrated that Src specifically interacts with Neu and is catalytically activated by expression of activated Neu in Rat-2 fibroblasts (Muthuswamy *et al.* 1994; Muthuswamy and Muller 1995). These data suggest that Src may bind specifically to one or more of the four transforming add-back mutants and mediate a transforming signal. To address this possibility, Rat1-derived cell lines expressing the various add-back mutants were generated. Each mutant was stabley expressed, tyrosine phosphorylated and kinase active (see Dankort *et al.* 1997). Given that Src interacts directly with Neu, I tested whether the add-back mutants interacted directly with Src via a direct blot assay.

The procedure for this direct blot approach is summarized in Figure 3.7. Briefly, the cDNA encoding a protein or portion thereof is cloned into a glutathione S-transferase expression vector which contains an in frame consensus protein kinase A (PKA) site (Ron and Dressler 1992). The fusion protein is expressed in bacteria and purified on glutathione beads. The immobilized protein is then radiolabeled to high specific activities by incubating the beads with $^{32}P\gamma ATP$ and PKA which are later removed by extensive washing. The radiolabeled fusion protein is eluted from the beads and used to probe a membrane containing electrophoretically separated proteins which is then washed as an immunoblot. Following autoradiography, proteins detected on the blot must directly interact with the radiolabeled fusion protein and is thus termed a "direct blotting" or "Far-western" assay.

To test whether the site of Src interaction with Neu mapped to phosphorylation sites A-E, Neu immunoprecipitates made from the indicated cell lines were subjected to direct blot analysis using radiolabeled GST-Src-SH2. As expected, the fusion protein bound to Neu derived from activated Neu expressing cells but not to that derived from the parental Rat1 fibroblasts but also interacted with the NT-NYPD protein as well as each add-back mutant (Figure 3.8A). Moreover, immunoprecipitation of c-Src under the mild conditions from Rat1 parental and derived lines expressing either NT or NT-NYPD demonstrated that Src associates *in vivo* with both activated Neu and NT-NYPD (Figure 3.8c). Thus while Src appears to directly interact with Neu, it does so at a site(s) other than the known phosphorylation sites (A-E).

3.4 Ras plays a pivotal role in Neu-mediated signaling

3.4.1 All sites require Ras function to initiate DNA synthesis

Because the Ras signaling pathway is thought to play an important role in Neu-mediated transformation (Segatto *et al.* 1993; Ben-Levy *et al.* 1994; Janes *et al.* 1994), we tested the requirement of Ras activity in Neu-mediated proliferation. One means of establishing whether Ras signaling is involved in Neu-mediated cell proliferation is through microinjection of the anti-Ras neutralizing antibody, Y13-259,

Figure 3.7 Far-western / affinity blot analysis

(A) cDNAs are cloned in frame with glutathione S-transferase in pGSTag (Ron and Dressler 1992) which contains a protein kinase A (PKA) consensus phosphorylation site. The presence of an upstream Lac operator sequence allows for the IPTG induction of transcripts encoding the GSTag fusion proteins in bacteria. **(B)** Fusion proteins are purified and immobilized on glutathione sepharose. **(C)** Purified PKA and $^{32}P\gamma$-ATP are used to phosphorylate the PKA site and following the reaction these are remove by extensive washing of the immobilized fusion protein. **(D)** Excess reduced glutathione is used to elute the GST-fusions from the beads. Blots containing electrophoretically separated proteins are then probed with the ^{32}P-radiolabelled fusion protein (D) or phosphotyrosine as a control **(F)**.

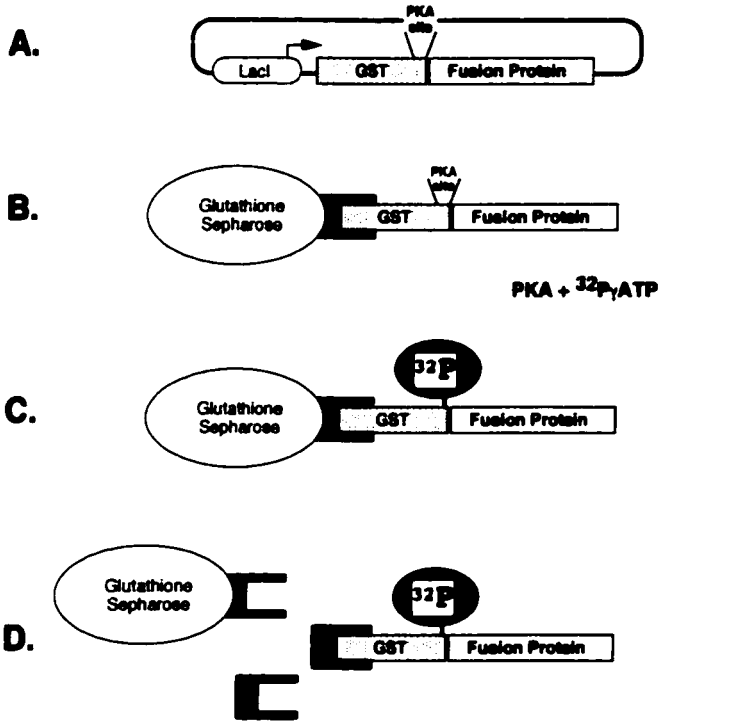
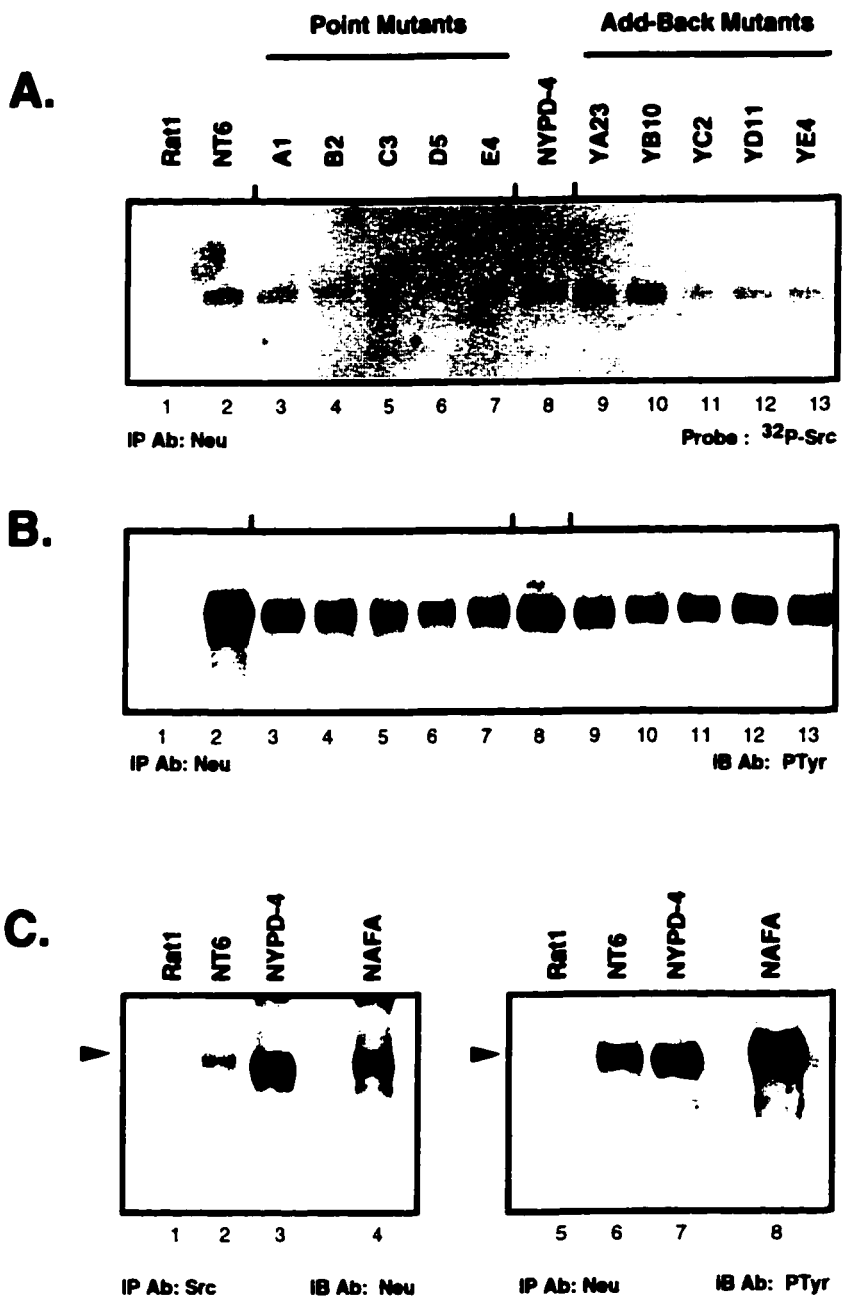

A.
PKA site
LacI
GST
Fusion Protein
B.
Glutathione Sepharose
PKA site
GST
Fusion Protein
PKA + 32PγATP
C.
32P
Glutathione Sepharose
GST
Fusion Protein
D.
Glutathione Sepharose
32P
GST
Fusion Protein
E.
SHC
Neu
: IP Ab
Lysate
NT
Rat1
NT
1 2 3 4
Probe: 32P-labeled gst-fusion
F.
SHC
Neu
: IP Ab
Lysate
NT
Rat1
NT
1 2 3 4
IB Ab : PTyr

Figure 3.8 c-Src interacts with both NT and NT-NYPD in vitro and in vivo.

Neu was immunoprecipitated from 1.5 mg of protein lysate derived from the indicated cell lines. **(A)** Two thirds of each precipitate was electrophoresed on an SDS-8.5% gel, transferred to a PVDF membrane and probed with a ^{32}P-radiolabeled GST-Src SH2 fusion protein. **(B)** The remaining portion of the immunoprecipitate was subjected to anti-phosphotyrosine immunoblot analyses. **(C)** Src was immunoprecipitated from 2.0mg of protein lysates of Rat1, Rat1-derived NT or NYPD or NAFA mammary epithelial cells and were immunoblots were developed using Neu specific antibodies. **(D)** Neu immunoprecipitates of the same protein lysates (from 500ug) were subjected to antiphosphotyrosine immunoblot analyses. The migration of Neu is indicated by the arrowhead.


A.
Point Mutants
Add-Back Mutants
Rat1
NT6
A1
B2
C3
D5
E4
NYPD-4
YA23
YB10
YC2
YD11
YE4
1 2 3 4 5 6 7 8 9 10 11 12 13
IP Ab: Neu
Probe : 32p-Src
B.
1 2 3 4 5 6 7 8 9 10 11 12 13
IP Ab: Neu
IB Ab: PTyr
C.
Rat1
NT6
NYPD-4
NAFA
1 2 3 4
IP Ab: Src
IB Ab: Neu
Rat1
NT6
NYPD-4
NAFA
5 6 7 8
IP Ab: Neu
IB Ab: PTyr

into Rat1-derived cell lines expressing each of the add-back mutants. Microinjection of this antibody has been shown to specifically inhibit Ras-mediated signaling events leading to DNA synthesis (Dobrowolski *et al.* 1984; Mulcahy *et al.* 1985). Y13-259 injected and non-injected cells were then monitored for induction of DNA synthesis by BrdU incorporation. DNA synthesis was observed in over 90% of the control cells expressing transforming mutants of Neu compared to 78% observed in parental Rat1 cells while NT-NYPD and NT-YA expressing cell lines induced intermediate levels of DNA synthesis (Table 3.4). Interestingly, microinjection of Ras neutralizing antibodies inhibited BrdU incorporation in all tested cell lines, whereas microinjection of a non-neutralizing anti-Ras antibody (Y13-238) under these conditions does not inhibit DNA synthesis in fibroblasts (Wang and Moran 1996). Inhibition of DNA synthesis in Rat1 cells by Y13-259 likely is a result of an impairment of signaling stimulated by serum factors such as lysophosphatidic acid which require Ras activity to induce DNA synthesis (Jalink *et al.* 1994). These data suggest that Neu-induced proliferation occurs through multiple tyrosine residues each impinging on Ras.

3.4.2 Effect of expression of Ras-interfering gene Rap1A/KRev-1 on Neu mediated signaling

While microinjection of Ras-inhibitory antibodies into cells expressing the various Neu autophosphorylation mutants indicates a role for Ras in the induction of DNA synthesis, it was unclear whether or not Neu utilizes Ras to mediate transformation. We chose to test this through the coexpression of one of two Ras genetic inhibitors: Rap1A and N17-Ras. Rap1A/K*rev*-1 (Pizon *et al.* 1988) encodes a Ras-related protein with 100% identity to the Ras "effector" domain and has the ability to revert v-Ki-*ras* transformed cells when overexpressed (Kitayama *et al.* 1989). Overexpression is thought to titrate critical Ras substrates to a Rap1A-substrate complex where it presumably does not activate these proteins thereby functioning as a dominant competitive inhibitor of normal Ras function. Indeed, expression of an activated form of Rap1A inhibits EGF-mediated Erk activation yet has little effect on Ras-GTP levels (Cook *et al.* 1993). Moreover, Rap1A expression inhibits Ras-induced but not Raf-induced activation of the c-*fos*

Table 3.4 Ras plays a central role in Neu-mediated signaling

	Prolifeation (%BrdU positive Nuclei)[d]		Relative Tranformation Potential[a,b]		
	Control	Anti-Ras Y13-259 injected	SV2-Neo[c]	Rap1A	%SV2-Neo Transformation
Vector	78	5.9	0	0	n.a.
NT	97	8.2	100	33 ± 4	33 ± 4
NT-NYPD	76	5.0	42 ± 3	25 ± 4	61 ± 5
NT-YA	83	5.6	n.t.	n.t.	n.a.
NT-YB	98	6.6	83 ± 8	24 ± 1	30 ± 4
NT-YC	95	9.9	146 ± 4	112 ± 9	76 ± 4
NT-YD	97	7.9	126 ± 13	37 ± 1	30 ± 3
NT-YE	92	6.9	124 ± 20	44 ± 4	37 ± 10
SV40 LT	n.a.	n.a.	100	106 ± xx	106

Focus formation assays were performed in Rat1 cells using lipofectamine reagent as described in materials and methods using two independent plasmid preparations.

[a] Transforming activity was calculated relative to that obtained from activated Neu for each of three independent experiments each containing duplicates of three independently transfected plates for each condition (i.e. six plates per experiment in each of three experiments). The relative transforming potential is the mean transformation potential of the three experiments (± standard error).

[b] Neu expressing plasmids (100ng) driven from the Murine Moloney Leukemia Virus LTR were cotransfected with 1.0ug of SV2Neo or kRev1. Empty vector (pJ4Ω) was used as a negative control.

[c] The percentage of control was determined as the ratio of transforming activities observed when cotransfected with pKRev1 relative to that found with SV2-Neo multiplied by 100%

[d] Rat1-derived cells were serum deprived and were left uninjected or were microinjected with Ras inhibitory antibodies (Y13-259). Cells were later stimulated with serum in the presence of BrdU and the percentage of cells staining positive for BrdU is presented and determined as in materials and methods.

promoter suggesting Rap1A functions genetically downstream of Ras and upstream of Raf (Sakoda *et al.* 1992). These properties make Rap1A a genetic inhibitor of Ras signaling when coexpressed.

Rat1 cells were cotransfected by lipid-mediated transfection with either an empty expression vector (pSV2-Neo) or one encoding Rap1A (pKRev-1) along with the transforming add-back mutants (NT YB, YC, YD, YE). It should be noted that we have determined that the conditions used are well within the linear range of transformation (Neil Warner, Dankort, Muller). Activated Neu-mediated transformation was decreased to 33% that of control empty vector (i.e. a 3-fold reduction in transformation) when Rap1A was coexpressed (Table 3.4), suggesting a role for Ras in this process. Jelinek and Hassell have previously demonstrated that Rap1A expression in Rat1 fibroblasts is not lethal and does not decrease the number of cells which stabley integrate a second plasmid encoding a selectable marker (Jelinek and Hassell 1992), suggesting the reduction in foci observed is a result of Ras signal impairment and not due to lethality. Interestingly, cotransfection of pKRev-1 had a differential effect on transformation by the add-back mutants, inhibiting NT-YB, NT-YD and NT-YE much more dramatically than NT-YC (Table 3.4). Rap1A mediated transformation repression appears to be relatively specific as Rap1A had little effect on simian virus 40 large T antigen mediated transformation. We were however, unable to cause complete Rap1A-induced reversion; presumably in part due to an inability to increase the amount of pKRev-1 DNA in the transfection to more than 10-fold that of Neu. Indeed, to effectively inhibit PyV MT-mediated transformation in Rat1 cells a 500 fold excess pKRev-1 plasmid is required to observe a 50% decrease in transformation efficiency (Jelinek and Hassell 1992). Typically the number of foci obtained in this study was much lower. These results indicate that Neu utilizes Ras to mediate transformation and that, unlike the observation that Ras function was required to induce DNA synthesis from all the add-back mutants, it appears phosphorylation sites B, D and E appear to mediate transformation in a Ras-dependent manner. Similar transformation assays using N17-Ras yielded uninterpretable results due to lethality, as assessed by inhibition of SV40 -large T antigen transformation by N17-Ras (Table 3.4).

Thus two different Ras antagonist inhibit signaling from activated Neu, NT-YB, NT-YD and NT-YE and while transformation from NT-YC was not inhibited by Rap1A, signaling was impaired by Ras-neutralizing antibody injection. Taken together these data demonstrate that Neu can mediate Ras activation through the use of multiple independent tyrosine phosphorylation sites.

3.5 Grb2 binds directly to site B (tyrosine 1144) and mediates a transforming signal

3.5.1 Grb2 coimmunoprecipitates with add-back mutants YB and YD

Three guanine nucleotide exchange factors for Ras proteins exist; Ras-GEF, Ras-GNRF and mSos proteins. Of these, Sos proteins are genetically and biochemically involved in mediating Ras activation in response to several RTKs (see Section 1.5). Three adaptor proteins, Grb2, Crk (Matsuda *et al.* 1994) and Nck (Hu *et al.* 1995b), function to link RTKs to Sos through SH3-Sos and SH2-RTK interaction. As unique amino acid sequences surround each Neu autophosphorylation site, it is likely that different add-back mutations couple to Ras through multiple adaptor proteins. To explore this hypothesis, I examined the capacity of the different add-back mutants to associate with Grb2. Lysates containing activated Neu, add-back mutants (NT-YA through NT-YE) and NT-NYPD were immunoprecipitated with Neu-specific antibodies and were subjected to immunoblot analyses with Grb2-specific antisera. Grb2 is complexed with Neu in immunoprecipitates from cell lines expressing activated Neu, NT-YB and NT-YD, yet not in lines expressing NYPD, YA, YC, and YE mutants (Figure 3.9A), despite similar tyrosine phosphorylated Neu (Figure 3.9B) and Grb2 (Figure 3.9C) levels in these immunoprecipitates and lysates, respectively. These observations demonstrate that the Grb2 adaptor protein physically associates with activated Neu through tyrosine residues 1144 (B site) and 1226/27 (D site).

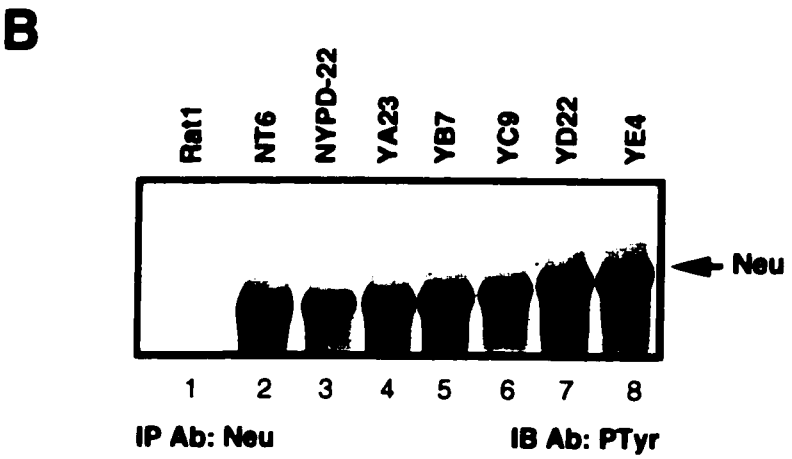
3.5.2 Site B is both necessary and sufficient for direct Grb2 binding.

While Grb2 binds site B and D *in vivo*, only the sequences surrounding tyrosine 1144 (site B) (YVNQ) conform to a Grb2 binding site (Songyang *et al.* 1993; Songyang *et al.* 1994), implying that binding to site D is indirect. Because Grb2 can bind tyrosine kinases either directly (Stein *et al.* 1994;

Figure 3.9 Grb2 associates with Neu *in vivo* at distinct autophosphorylation sites.

Neu was immunoprecipitated from 2.5mg of protein lysates of Rat1 (lane 1) or Rat1-derived cell lines (lanes 2-8). The immunoprecipitates were electrophoresed on a SDS-(6-15%) gradient gel, and transferred to a PVDF membrane. The membrane was cut and the bottom half, containing proteins of 18 to 80kDa, was probed with Grb2-specific rabbit polyclonal sera (**A**). The arrow indicates the position of Grb2. The upper portion of the membrane was probed with anti-phosphotyrosine antibodies (**B**) and the arrow indicates the migration of Neu. (**C**) Equivalent amounts (20ug) of the same protein lysates were subjected to immunoblot analyses with Grb2-specific antisera.

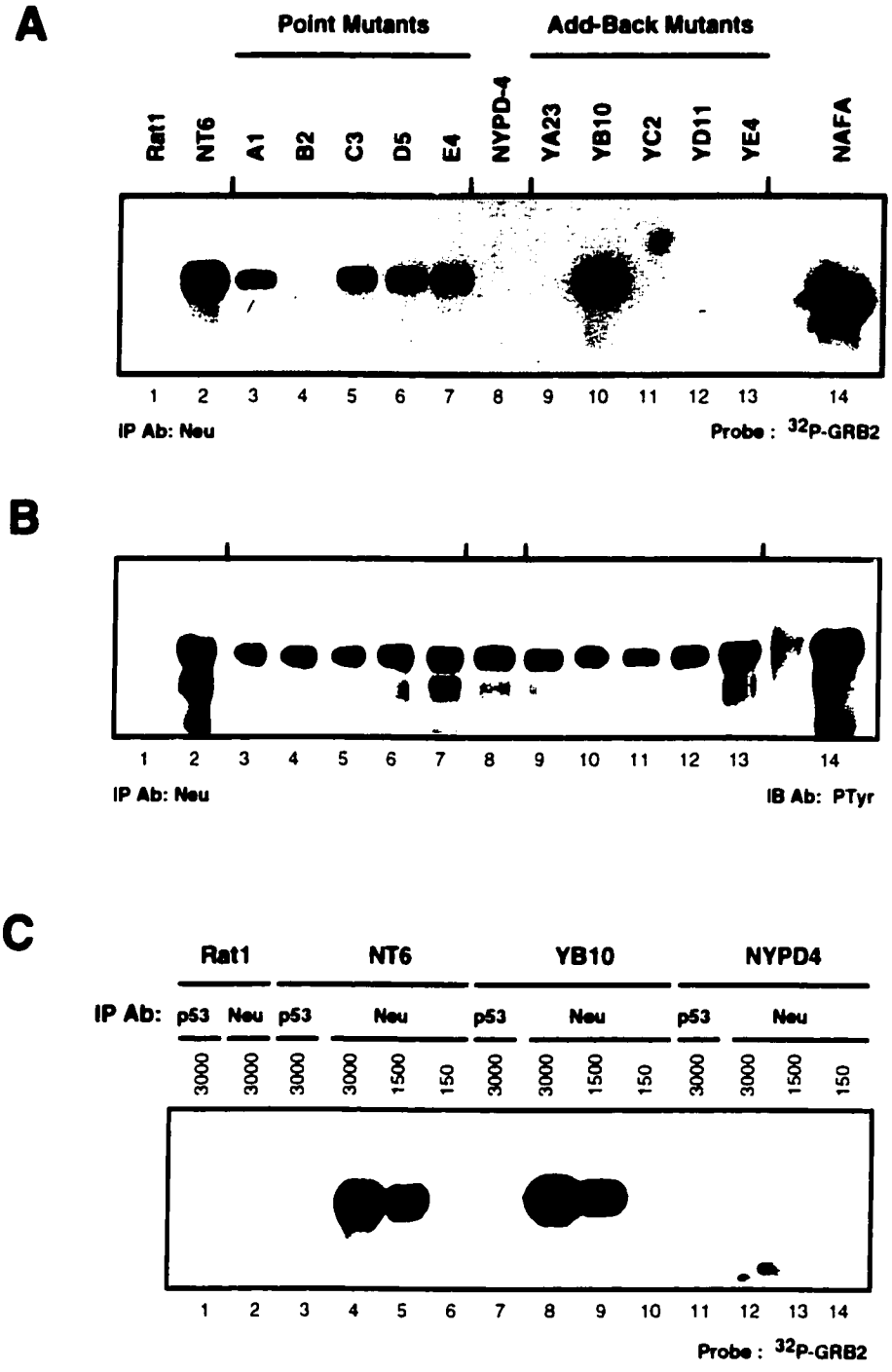

A
Rat1
NT6
NYPD-22
YA23
YB7
YC9
YD22
YE4
GRB2
1 2 3 4 5 6 7 8
IP Ab: Neu
IB Ab: GRB2


B
Rat1
NT6
NYPD-22
YA23
YB7
YC9
YD22
YE4
Neu
1 2 3 4 5 6 7 8
IP Ab: Neu
IB Ab: PTyr


C
Rat1
NT6
NYPD-22
YA23
YB7
YC9
YD22
YE4
GRB2
1 2 3 4 5 6 7 8
IB Ab: GRB2

Figure 3.10 Grb2 associates with Neu directly and specifically through tyrosine 1144 (site B).

Neu was immunoprecipitated from 1.5 mg of protein lysate derived from the indicated cell lines. (A) Two thirds of each precipitate was electrophoresed on an SDS-8.5% gel, transferred to a PVDF membrane and probed with a ^{32}P-radiolabeled GST-Grb2 fusion protein. (B) The remaining portion of each immunoprecipitate was subjected to anti-phosphotyrosine immunoblot analyses. A marker was loaded between lanes 13 and 14. (C) 3mg, 1.5mg or 150ug of protein were immunoprecipitated with either p53 (lanes 1, 7, 11) or Neu (lanes 2,3, 4-6, 8-10, 12-14)-specific antibodies from the lysates of the indicated cell lines. The immunoprecipitates were subjected to direct blot analyses with ^{32}P-GST-Grb2 as in (A).


A
Point Mutants
Add-Back Mutants
Rat1
NT6
A1
B2
C3
D5
E4
NYPD-4
YA23
YB10
YC2
YD11
YE4
NAFA
1 2 3 4 5 6 7 8 9 10 11 12 13 14
IP Ab: Neu
Probe : 32P-GRB2
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14
IP Ab: Neu
IB Ab: PTyr
C
Rat1
NT6
YB10
NYPD4
IP Ab: p53 Neu p53 Neu p53 Neu p53 Neu
3000 3000 3000 3000 1500 150 3000 3000 1500 150 3000 3000 1500 150
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Probe : 32P-GRB2

Arvidsson *et al.* 1994; Pendergast *et al.* 1993) or indirectly (Li *et al.* 1994) through its interaction with tyrosine phosphorylated proteins, I determined which site(s) Grb2 directly interacts. Using the direct blot assay, I examined whether the different point and add-back mutants could directly interact with the Grb2 adaptor. Protein blots containing Neu immunoprecipitates derived from Rat1 fibroblast expressing activated Neu, point mutants or add-back mutants were probed with a ^{32}-P-radiolabeled GST-Grb2 protein. Comparison of direct Grb2 binding to activated Neu and derived point mutants revealed that each bound radiolabeled GST-Grb2 similarly, save for NT-B (Figure 3.10A, lanes 2-7), despite having similar levels of tyrosine phosphorylated Neu in each immunoprecipitate (Figure 3.10B). Conversely, GST-Grb2 did not bind to NT-NYPD nor to any of the add-back mutants with the exception of NT-YB, which bound strongly (Figure 3.10A, lanes 8-13). The inability to detect direct Grb2-Neu interaction from the NT-NYPD mutant or the NT-YA, NT-YC, NT-YD, and NT-YE lysates (Figure 3.10A, lanes 8,9 and lanes11-13) was not due to a lack of tyrosine phosphorylated Neu, as each immunoprecipitate contained comparable levels of the phosphoprotein (Figure 3.10B, lanes 8-14).

To ensure GST-Grb2 binding specificity, increasing amounts of Neu derived from cell lines expressing wild-type activated Neu, NT-YB and NT-NYPD proteins were tested for their capacity to bind the radiolabeled fusion protein. Neither the NT-NYPD nor the parental Rat1 Neu immunoprecipitates bound the Grb2 probe at the highest protein concentrations tested while strong GST-Grb2 binding to NT and NT-YB was observed (Figure 3.10C). As expected, nonspecific control containing p53 immunoprecipitates failed to interact with the radiolabelled fusion protein. Taken together, these observations suggest that phosphorylation of tyrosine 1144 (site B), but not Y1226/7 (site D), is both necessary and sufficient for the direct binding of Grb2 to activated Neu.

3.5.3 Grb2 binding correlates with transformation potential from site B. (Y1144)

While the binding of Grb2 to NT-YB correlates with transformation (i.e. compared to NT-NYPD), it is conceivable that an additional protein interacts with this phosphorylation site to mediate

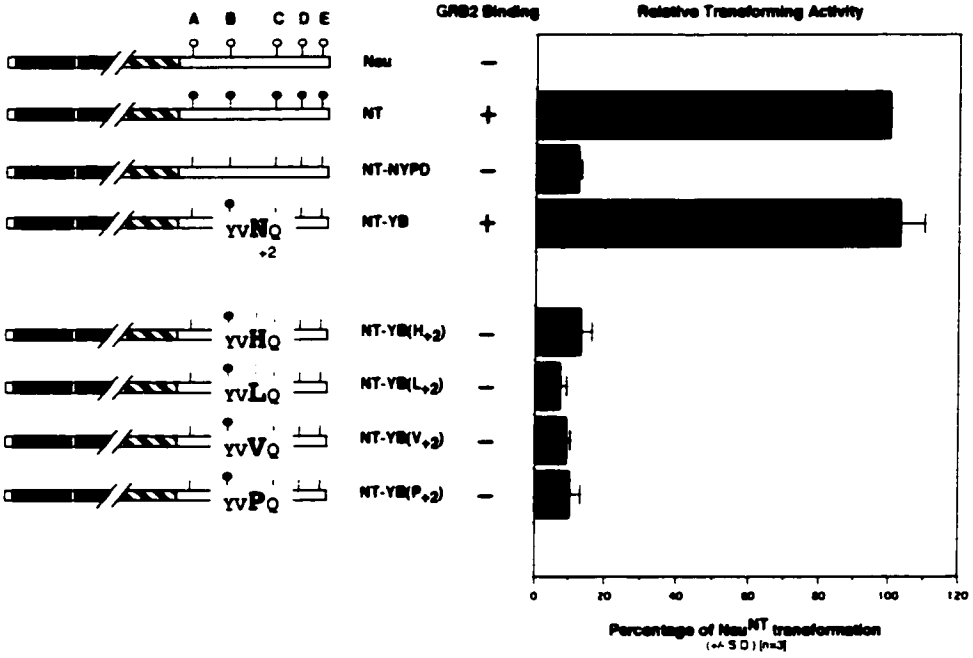
transformation. Binding of the Grb2 SH2 domain to a degenerate peptide pool reveals a requirement for asparagine (N) in the +2 position to form a consensus binding sequence of YxNx where x indicates that no residue was strongly selected for or against (Songyang *et al.* 1994). To strengthen the correlation between transformation and Grb2 binding, I introduced mutations at the codon encoding N1146 (the asparagine in the +2 position) into NT-YB. Four mutants termed NT-YB(L_{+2}), NT-YB(V_{+2}), NT-YB(P_{+2}) and NT-YB(H_{+2}) were created (Figure 3.11 upper panels) to ensure that any phenotype seen observed was due to the loss of the asparagine (and presumably Grb2 association) and not due to the acquisition of a new binding site for a different SH2 containing molecule. Interestingly, alteration of N1146 to either leucine, valine, proline or histidine resulted a dramatic reduction in the focus forming activities relative to the parental NT-YB molecule (Figure 3.11). This decrease in transformation potential was correlated with a decrease in Grb2 binding to the "NT-YB second site" mutants (Figure 3.11 lower panels). The differences in Grb2-Neu binding observed are not due to differences in the amounts of tyrosine phosphorylated Neu nor Grb2 found in each immunoprecipitate and lysate respectively. In fact, there was consistently more of each NT-YB_{+2} mutant expressed relative to the parental NT-YB lines (compare lanes 2 and 4 to lanes 5 through 8). Taken together with the transformation data, it appears that Grb2 binding is required for efficient NT-YB mediated transformation.

3.5.4. Microinjection of Grb2 SH2 domain inhibits DNA proliferation from site B. (Y1144)

I have demonstrated that activated Neu molecules which directly interact with Grb2 have the ability to transmit a transforming signal. While use of finer mutagenesis of the Grb2 binding site reveals that the binding of Grb2 strongly correlates with the transforming potential, it is possible that Grb2 is not required to mediate signals from NT-YB *in vivo*. We chose to test whether this was the case by microinjecting Rat1 lines expressing the various add-back mutants with a GST-Grb2-SH2 domain fusion protein. This fusion protein has been demonstrated to abrogate Grb2 function under these conditions and

Figure 3.11 Binding of Grb2 to proline 1146 is required for NT-YB mediated transformation

(Upper left panel) Depicted are mutants derived from NT-YB. Mutants were made to alter the asparagine (N1146) in the Grb2 binding site (YVNQ) of NT-YB to a histidine (H), leucine (L), valine (V) and proline (P) creating NT -YB(H^{+2}), -YB(L^{+2}), -YB(V^{+2}) and -YB(P^{+2}) respectively. Grb2 binding was assessed by coimmunoprecipitation. Strong binding (+) and a lack of association (-) are indicated to summarize data from lower panels. **(Upper right panel)** Relative transforming activity is indicated graphically and calculated as describe in materials. Values represent the mean of the relative transforming abilities from the three independent experiments ± standard error. **(Lower panels)** Neu was immunoprecipitated from 2.0mg of protein lysates of Rat1 (lane 1) or Rat1-derived cell lines (lanes 2-8). The immunoprecipitates were electrophoresed on a SDS-(6-15%) gradient gel, and transferred to a PVDF membrane. The membrane was cut and the bottom half, containing proteins of 18 to 80kDa, was probed with Grb2-specific rabbit polyclonal sera as indicated. The arrow highlights the migration of Grb2. The upper portion of the membrane was probed with anti-phosphotyrosine antibodies and the arrow indicates the migration of Neu. Equivalent amounts (20ug) of the same protein lysates were subjected to immunoblot analyses with Grb2-specific antisera.


A B C D E
GRB2 Binding
Relative Transforming Activity
Neu
NT
NT-NYPD
NT-YB
NT-YB(H+2)
NT-YB(L+2)
NT-YB(V+2)
NT-YB(P+2)
0 20 40 60 80 100 120
Percentage of NeuNT transformation

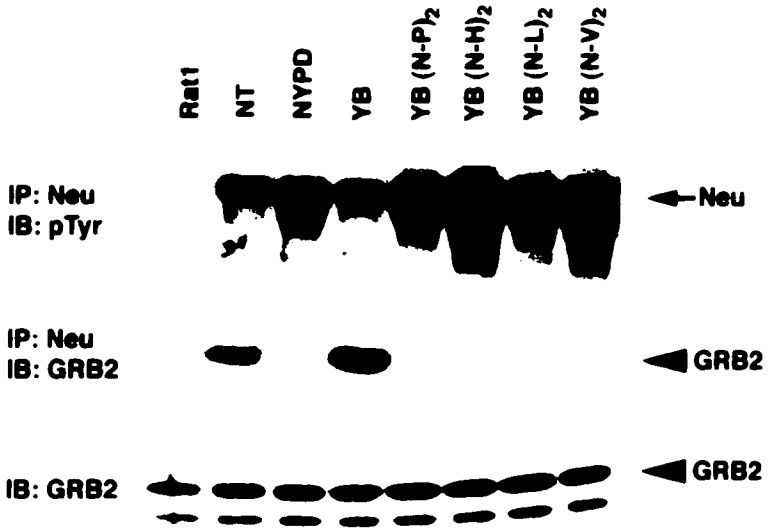
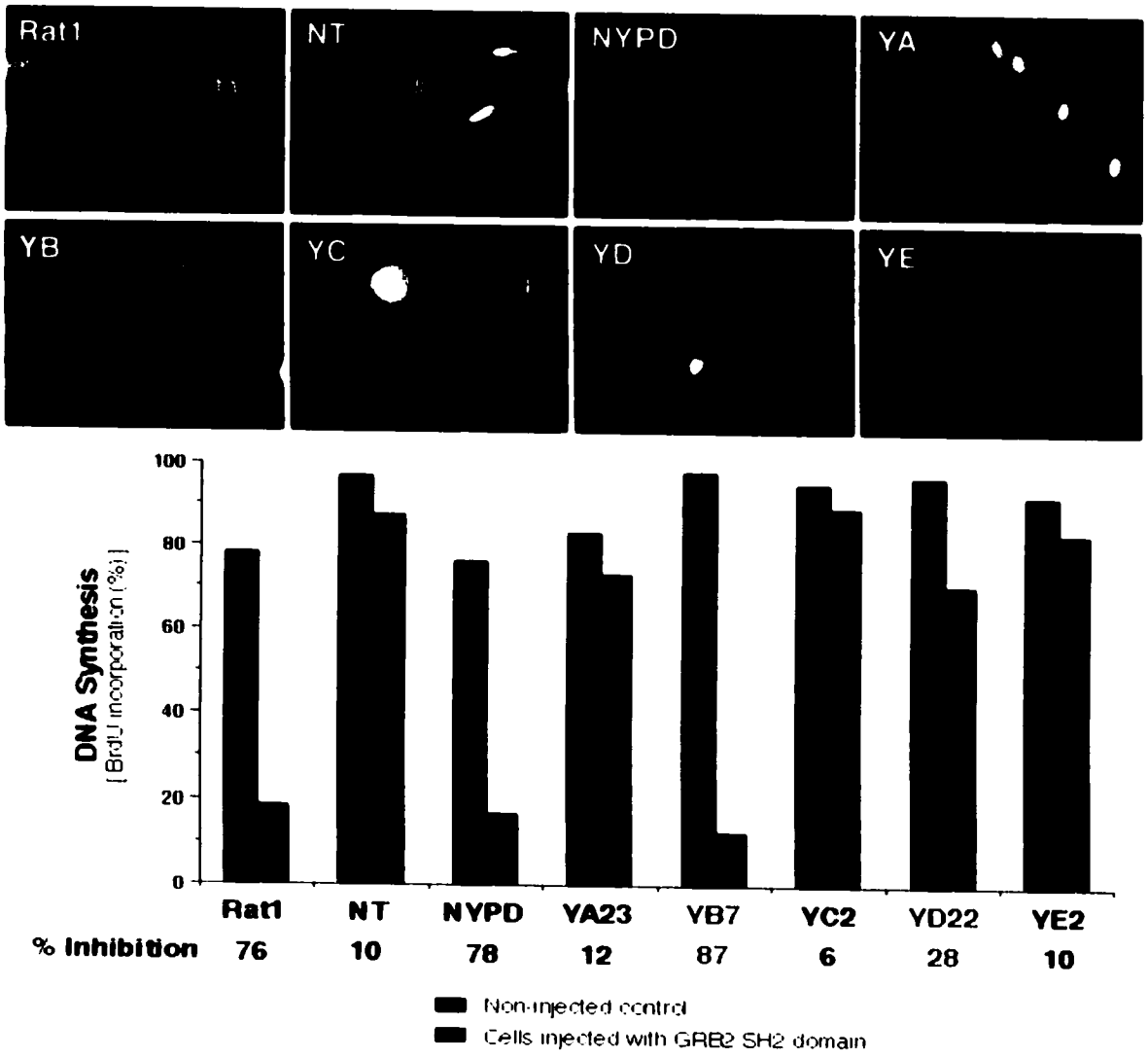

Rat1
NT
NYPD
YB
YB (N-P)2
YB (N-H)2
YB (N-L)2
YB (N-V)2
IP: Neu
IB: pTyr
Neu
IP: Neu
IB: GRB2
GRB2
IB: GRB2
GRB2

Figure 3.12 Effect of Grb2 SH2 domain microinjection on DNA synthesis from add-back cells lines.

(upper panel) Cells rendered quiescent through serum deprivation were either uninjected or microinjected with 1ug biotinylated Grb2 SH2 domain and released from G_0 via addition of serum at which time BrdU added. 18 hours later cells which had entered S-phase were scored as BrdU+ nuclei (rhodamine red labeled) while microinjected cells were visualized with FITC (green) labeled avidin. A representative field of view containing injected cells for each indicated cells line is shown. **(lower panel)** The percentage of BrdU positive injected or non-injected cells are graphically depicted. DNA synthesis was determined as the percentage of microinjected cells (not less than 100 cells for each line) staining positively for BrdU incorporation (as described in materials and methods). Equivalent results were obtained from two independent experiments, and results from one such experiment are shown.


Rat1
NT
NYPD
YA
YB
YC
YD
YE
DNA Synthesis
(BrdU incorporation (%))
100
80
60
40
20
0
Rat1
NT
NYPD
YA23
YB7
YC2
YD22
YE2
% Inhibition
76
10
78
12
87
6
28
10
Non-injected control
Cells injected with GRB2 SH2 domain

functions by competing for Grb2 binding sites creating non-productive complexes as it lacks SH3 domains which interact with Grb2 effector proteins, mSos and dynamin (Wang and Moran 1996).

Microinjection of the Grb2-SH2 domain into activated Neu expressing cells had little effect on the ability to mediate DNA synthesis (Figure 3.12) suggesting that there exists at least one Grb2-independent signal emanating from Neu. In fact microinjection of GST-Grb2-SH2 into either NT -YA, -YC, or -YE had negligible effects on proliferation. Interestingly, GST-Grb2-SH2 microinjection strongly inhibited DNA synthesis in NT-YB cells while having a somewhat intermediate effect in NT-YD expressing cells. Specifically, DNA synthesis was reduced to 90% for NT, 72% for YD and 13% for YB relative to the uninjected controls (Figure 3.12, lower panel). These data argue that Grb2 mediates a positive proliferative signal from Neu and while not required in the context of the activated "wild-type" molecule it clearly plays a critical role in mediating the effects of the site B add-back mutant. Additionally, it would appear that Grb2 activity is only partially required for site D mediated events.

3.6 Shc binds to site D (tyrosines 1226/1227) and mediates a transforming signal

3.6.1 Shc binds to site D directly

3.6.1.1 *Shc interacts with tyrosines 1226/1227 in vivo*

Grb2-direct blot experiments suggest that Grb2 directly interacts with tyrosine 1144 (site B, Figure 3.10), while coimmunoprecipitation studies demonstrate that tyrosines 1226 and 1227 (site D) were also capable of indirectly interacting with Grb2 (Figure 3.9). One potential mechanism by which this might occur is by indirect Grb2 binding through an adaptor protein. For example, Grb2 could couple with activated Neu through its interaction with the Shc adaptor protein (Rozakis-Adcock *et al.* 1992). Because Shc proteins become tyrosine phosphorylated upon activation of many tyrosine kinases and that this phosphorylation leads to the generation of a Grb2 binding site (Salcini *et al.* 1994; Rozakis-Adcock *et al.* 1992), I first set out to examine the phosphorylation status of the Shc proteins (p46, p52 and p66) isolated from the add-back mutant lines. Shc was poorly tyrosine phosphorylated in control Rat fibroblasts yet

highly phosphorylated in NT expressing cells (Figure 3.13A). When Shc from NT-NYPD and the add-back lines were analyzed in a similar manner, invariably Shc was found phosphorylated in all cases. It should be noted that the phosphorylation of the p66 isoform was not cell line specific as additional assays revealed its phosphorylation from every cell line expressing an activated Neu allele analyzed (data not shown). Additionally, Shc immunoprecipitates from NT and NT-YD cell lysates contain a higher molecular weight protein with a mobility similar that of activated Neu (~200kDa) (lane 6 and 8). This ~200kDa protein was not observed in Shc immunoprecipitates derived from NT-NYPD cells, nor from any of the add-back mutants (lanes 3-7) save for NT-YD (lane 6). Thus the tyrosine specific association of the 200kDa phosphoprotein with YD coupled with the observation that Grb2 bound indirectly to this site, strongly implicate YD as a Shc binding site and suggests that pp200 is in fact the Neu receptor kinase.

To explore this possibility that pp200 is Neu, Shc immunoprecipitates from add-back mutant expressing Rat1 cells were subjected to immunoblot analyses with Neu-specific antibodies. Complexes containing Neu and Shc were detected in the lysates of NT-YD and activated Neu (NT) lines (Figure 3.13D lanes 2 and 7). By contrast, Neu was not detected in Shc immunoprecipitates derived from NT-NYPD, NT-YA, NT-YB, NT-YC, or NT-YE expressing cell lines although equivalent levels of Shc proteins and tyrosine phosphorylated Neu were detected in all Neu expressing cell lines examined (Figures 3.13 B and C). Consistent with the notion that phosphorylation of Shc leads to the generation of a Grb2 binding site, Grb2 was found in association with Shc in these same immunoprecipitates assessed by anti-Grb2 immunoblot analyses of the same immunoprecipitates (Figure 3.13E). Interestingly, Shc-Grb2 complexes are formed in cell lines expressing Neu add-back mutants unable to bind Shc (NT-YA, NT-YB, NT-YC, and NT-YE, Figure 3.16 D and E). Therefore, tyrosine phosphorylation of Shc and Grb2-Shc complex formation does not appear to require stable Shc association with Neu.

To assess whether site D was required for Shc binding, Neu derived from cell lines expressing the different point mutants was tested for Shc association. Shc interacted with each of the point mutants but failed to interact with NT-D (Figure 3.14A) despite possessing comparable levels of Shc proteins and

Figure 3.13 Shc proteins associate with Neu *in vivo* at a single autophosphorylation site.

(A) Shc proteins were immunoprecipitated from 1.0mg of protein lysates derived from the indicated cell lines and were analysed by antiphosphotyrosine immunoblot analyses. The arrows indicate the migration of the 66, 52 and 46 kDa Shc proteins and the arrowhead highlights an associated ~200kDa phosphoprotein. **(B)** Equivalent amounts (20ug) of protein lysates were subjected to immunoblot analyses with Shc-specific polyclonal antibodies. (C) Neu was immunoprecipitated from 500ug of the indicated lysates and the immunoprecipitates were subjected to anti-phosphotyrosine immunoblot analyses. **(D)** Shc proteins were immunoprecipitated as in (A) and the immunoprecipitates were electrophoresed on a SDS-(6-15%) gradient gel, transferred to a PVDF membrane which was later cut in half. The top half, containing proteins 80kDa and above, was probed with Neu-specific mAbs. The migration of Neu is indicated. **(E)** The lower portion of the membrane in (D) containing proteins 18 to 80kDa was probed with Grb2-specific mAbs. Arrows indicated the 66, 52 and 46 kDa forms of Shc and arrowheads highlight the migration of Neu. Note the same lysates and loading pattern were used for the blots B through E. Equivalent Shc levels were obtained in control experiments using the same lysates used in (A); data not shown.

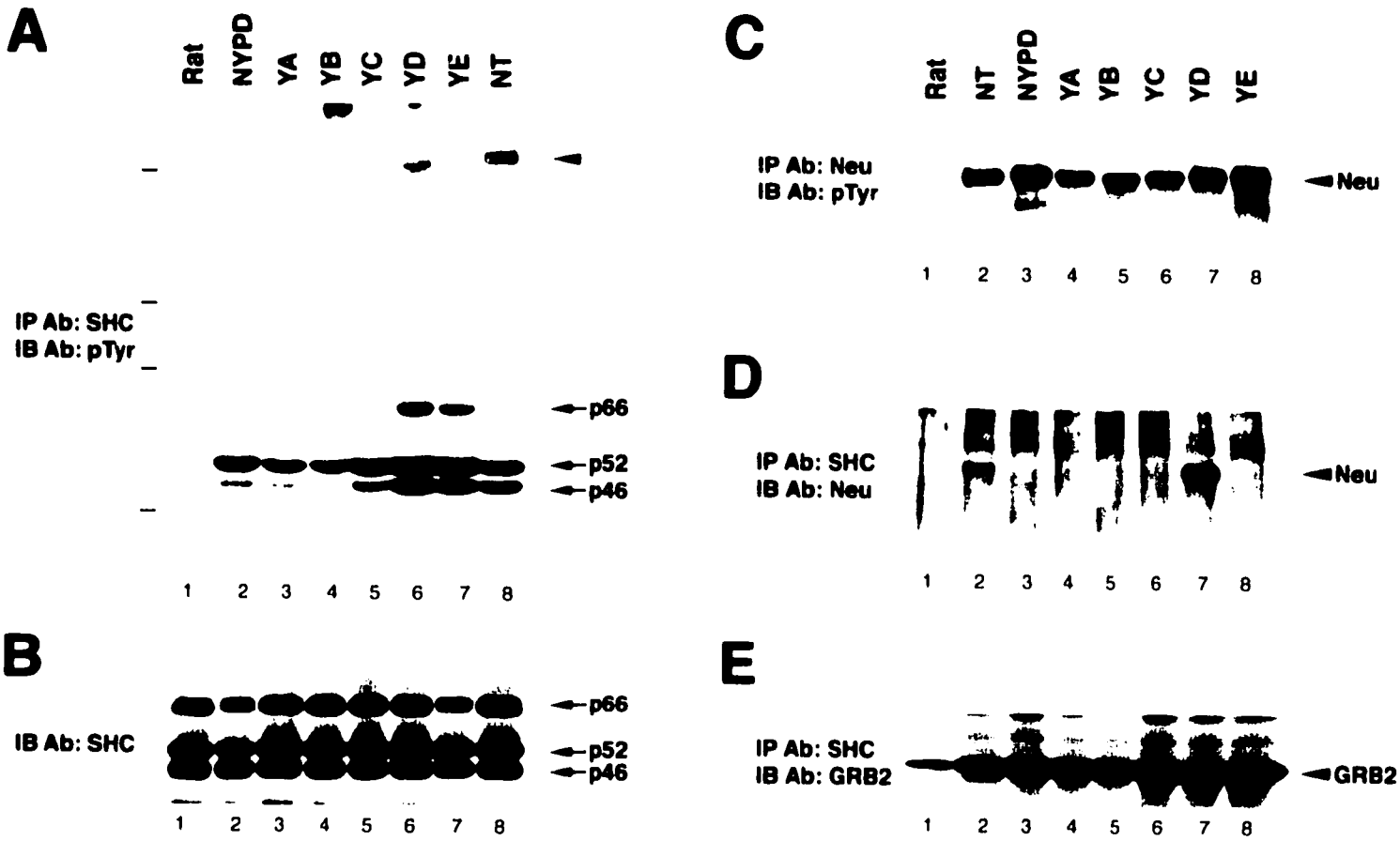
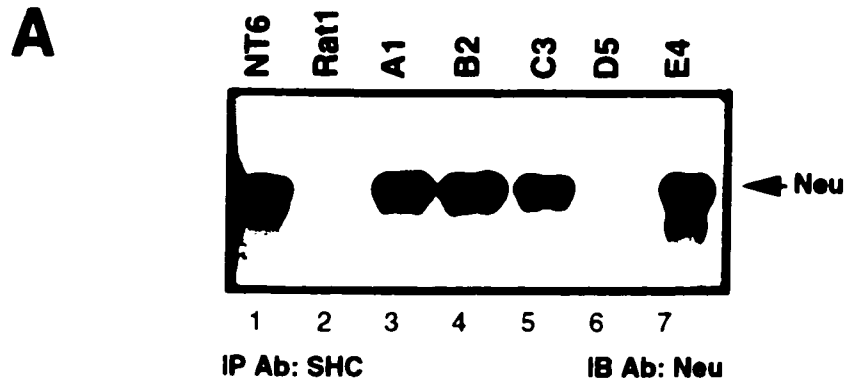
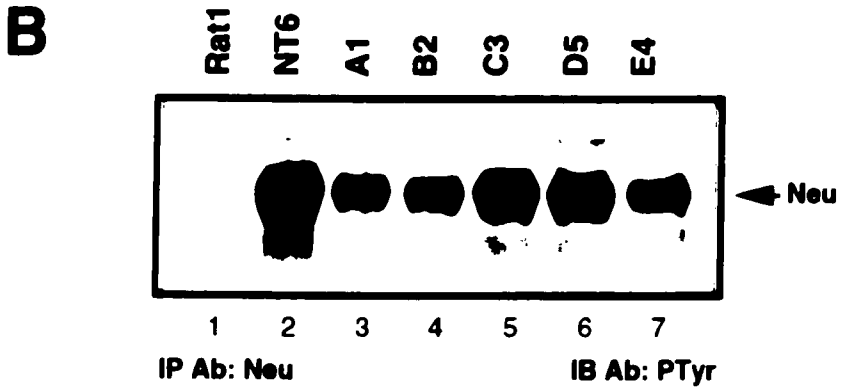
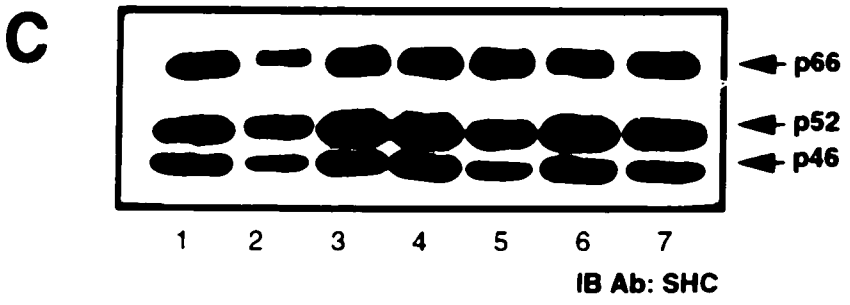

A
Rat
NYPD
YA
YB
YC
YD
YE
NT
IP Ab: SHC
IB Ab: pTyr
p66
p52
p46
1 2 3 4 5 6 7 8
B
IB Ab: SHC
p66
p52
p46
1 2 3 4 5 6 7 8
C
Rat
NT
NYPD
YA
YB
YC
YD
YE
IP Ab: Neu
IB Ab: pTyr
Neu
1 2 3 4 5 6 7 8
D
IP Ab: SHC
IB Ab: Neu
Neu
1 2 3 4 5 6 7 8
E
IP Ab: SHC
IB Ab: GRB2
GRB2
1 2 3 4 5 6 7 8

Figure 3.14 Shc association with Neu requires a single autophosphorylation site (site D)

(A) Shc proteins were immunoprecipitated and immunoblotted for Neu as in figure 3.13. **(B)** Neu was immunoprecipitated from 500ug of the same lysates and the immunoprecipitates were subjected to anti-phosphotyrosine immunoblot analyses. Indicated is the migration of Neu. **(C)** Equivalent amounts (20ug) of the same protein lysates were subjected to immunoblot analyses with Shc-specific polyclonal antibodies. Indicated are the 66, 52 and 46 kDa forms of Shc.


A
NT6
Rat1
A1
B2
C3
D5
E4
Neu
1 2 3 4 5 6 7
IP Ab: SHC
IB Ab: Neu


B
Rat1
NT6
A1
B2
C3
D5
E4
Neu
1 2 3 4 5 6 7
IP Ab: Neu
IB Ab: PTyr


C
p66
p52
p46
1 2 3 4 5 6 7
IB Ab: SHC

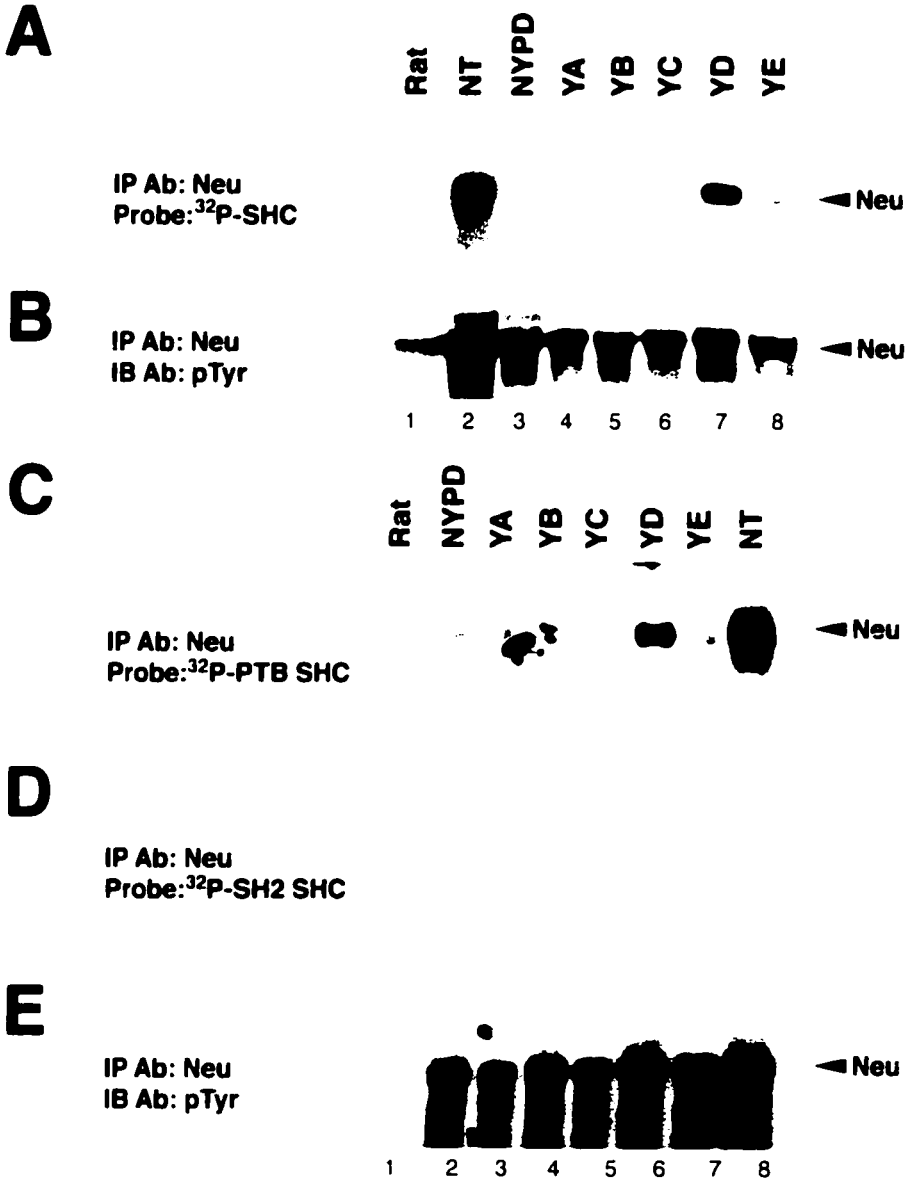
tyrosine phosphorylated Neu (Figures 3.14B and C). These observations demonstrate that not only is site D sufficient for Neu/Shc, interaction it is also required. Taken together, these data suggest that the Grb2 found in Neu immunoprecipitates derived from the NT-YD cell line (Figure 3.9) is due to its interaction with Shc and further demonstrate that Shc associates with Neu through the receptor phosphorylation site D (tyrosines 1226,1227).

3.6.1.2 *Shc binds through its PTB domain to site D directly*

The data presented thus far demonstrate that Shc proteins associate *in vivo* with site D. To determine if Shc directly interacts with site D, immunoblots containing Neu add-back immunoprecipitates were probed with a ^{32}P-radiolabeled GST-Shc fusion protein. Consistent with the coimmunoprecipitation studies, NT and NT-YD cell lines strongly bound the radiolabeled GST-Shc protein (Figure 3.15A, lanes 2 and 7). Longer exposures of the autoradiograph revealed weak binding of ^{32}P-radiolabeled GST-Shc to NT-YE. Given that Shc proteins contain two protein domains (PTB and SH2) capable of mediating phosphotyrosine-Shc interactions, I sought to determine which domain was responsible for this direct interaction. To this end, ^{32}P-radiolabeled GST-fusion proteins containing either the Shc PTB domain or the SH2 domain were used to probe blots containing Neu add-back immunoprecipitates. Although equivalent amounts of tyrosine phosphorylated Neu was detected in each immunoprecipitate (Figure 3.15E), the ^{32}P-Shc-PTB domain (Figure 3.15C) but not the Shc-SH2 domain probe (Figure 3.15D) bound to Neu. Moreover, the PTB domain bound to NT and NT-YD (lanes 6 and 8) but not the other add-back mutants in this assay. The same SH2 fusion protein has been shown to interact with the EGFR (Blaikie *et al.* 1994). Taken together, these data demonstrate that *in vitro* and *in vivo* the primary site of interaction on Neu for Shc is site D (tyrosine residues 12226/27) and Shc-Neu interactions are mediated not through the SH2 but through the PTB domain.

Figure 3.15 Shc associates with Neu directly at site D through its PTB domain

(A) Neu was immunoprecipitated from 2.0 mg of protein lysate derived from the indicated cell lines. Three fourths of the immunoprecipitate was electrophoresed on an SDS-8.5% gel, transferred to a PVDF membrane and probed with a ^{32}P-radiolabeled GST-Shc fusion protein. **(B)** The remaining portion of the immunoprecipitate was subjected to immunoblot analyses with anti-phosphotyrosine mAbs. **(C)** Neu was immunoprecipitated from 3.5 mg of protein lysate derived from the indicated cell lines. Two equal portions (equivalent to 1500ug each) of the immunoprecipitate were electrophoresed on an SDS-8.5% gel, transferred to a PVDF membrane and probed with ^{32}P-radiolabeled GST-fusion proteins of either the PTB- **(C)** or SH2- **(D)** domains. **(E)** The remaining portion of the immunoprecipitate (equivalent to 500ug of original lysate) was subjected to immunoblot analyses with anti-phosphotyrosine mAbs. The arrows indicate the migration of Neu.

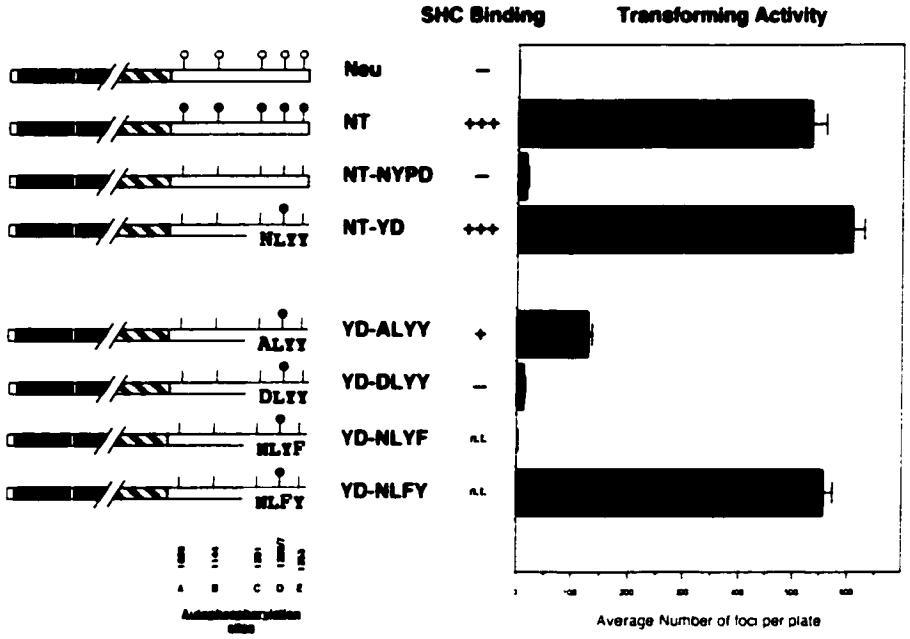

A
Rat
NT
NYPD
YA
YB
YC
YD
YE
IP Ab: Neu
Probe:32P-SHC
Neu
B
IP Ab: Neu
IB Ab: pTyr
Neu
1 2 3 4 5 6 7 8
C
Rat
NYPD
YA
YB
YC
YD
YE
NT
IP Ab: Neu
Probe:32P-PTB SHC
Neu
D
IP Ab: Neu
Probe:32P-SH2 SHC
E
IP Ab: Neu
IB Ab: pTyr
Neu
1 2 3 4 5 6 7 8

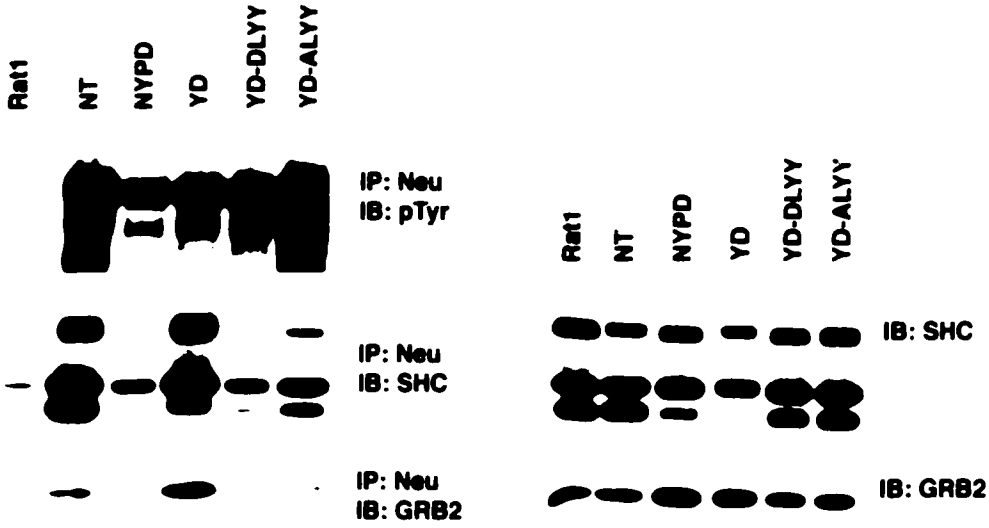
3.6.2 Shc binding correlates with transformation potential from site D (Y1227)

The observation that Shc-Neu interactions are mediate in a PTB-dependent manner implies that the residues of Neu required for Shc binding to be different from those which SH2 domains bind. Specifically, SH2 domains recognize sequences to the carboxy-side of a phosphorylated tyrosine residue whereas PTB domains recognize sequences predominantly to the amino side of the phosphotyrosine (see Section 1.3 and Figure 1.4). The consensus binding sequence determined for the Shc PTB domain is xΦxNPxY where Φ is a large hydrophobic residue (reviewed in Harrison 1996; Pawson and Scott 1997; Sudol 1998). Indeed, Shc-PyV MT interactions require both the tyrosine (Y315) and the asparagine at the -3 position (N312). Comparing the sequences surrounding site D (^{1224}NLYY^{1227}) to those found in the Shc binding site of MT (^{312}NPSY^{315}), both N12244 and Y1227 appear to create a partial PTB binding site for Shc (NxxY). Given that it is difficult to determine whether Y1226 or Y1227 or both are phosphorylated by tryptic peptide analyses, I simultaneously altered both residues in making the add-back mutants. To determine which tyrosine residue (or both) is (are) required for site D mediated transformation, these tyrosines were individually mutated in the context of NT-NYPD to create YD-NLFY and YD-NLYF. Additionally, N1224 was changed to alanine or aspartic acid in the context of NT-YD creating YD-ALYY and YD-DLYY respectively (Figure 3.16 upper panels). Consistent with the notion that Shc interacts through its PTB domain with Neu, alteration of N1224 to alanine abolished transformation from site D (Figure 3.16 upper panels) whereas aspartic acid change resulted in an impairment of transformation intermediate that of NT-NYPD and NT-YD. Moreover, the presence of a tyrosine at residue 1227 is sufficient to mediate transformation from site D whereas Y1226 is unable to do so. To determine whether mutations of N1224 affected Neu-Shc interactions, I assessed the levels of Shc found in Neu immunoprecipitates of cell lines expressing these various "NT-YD-second site" mutants. Although low Shc levels are found in Neu immunoprecipitates derived from NT-NYPD lines, elevated Shc levels were detected in Neu immunoprecipitates from NT and NT-YD expressing cells (Figure 3.16 lower panels). Mutation of N1144 to aspartic acid reduced Shc binding to levels observed for NT-NYPD while

Figure 3.16 Binding of Shc to site D is required for NT-YD mediated transformation.

(Upper left panel) Neu molecules harbouring mutations of the Shc binding site (NLYY) are indicated in the same fashion as depicted in figure 3.2. The asparagine (N, residue 1224) in the NLYY motif has been changed to an alanine (YD-ALYY) or an aspartic acid residue (YD-DLYY). The tyrosine residues at 1226 or 1227 were converted to phenylalanine residues to create YD-NLFY and YD-NLYF respectively. All mutants contain the activating transmembrane mutation V664E with sites A, B C and E mutated to phenylalanine. Shc binding was assessed by coimmunoprecipitation and strong (+++), intermediate (+) or a lack of association (-) are indicated. Mutants not tested for binding are indicated by n.t. **(Upper right panel)** The mean number of foci ± standard error is indicated graphically and is representative of three independent experiments. **(Lower left panels)** Neu was immunoprecipitated from 2.0 mg of protein lysates of Rat1 (lane 1) or Rat1-derived cell lines (lanes 2-7). The immunoprecipitates were electrophoresed on a SDS-(6-15%) gradient gel, and transferred to a PVDF membrane. The membrane was cut and the top third, containing proteins larger than 80kDa, was probed with anti-phosphotyrosine antibodies, the middle portion of the blot containing proteins 32 to 80kDa was probed with Shc-specific antisera and the remaining third containing proteins under 32kDa was probed with Grb2-specific rabbit polyclonal sera as indicated. **(Lower right panels)** Equivalent amounts (20ug) of the same protein lysates were subjected to immunoblot analyses with Shc-specific or Grb2-specific antisera.


SHC Binding
Transforming Activity
Neu
NT
NT-NYPD
NT-YD
YD-ALYY
YD-DLYY
YD-NLYF
YD-NLFY
NLYY
ALYY
DLYY
NLYF
NLFY
Average Number of foci per plate


Rat1
NT
NYPD
YD
YD-DLYY
YD-ALYY
IP: Neu
IB: pTyr
IP: Neu
IB: SHC
IP: Neu
IB: GRB2
IB: SHC
IB: GRB2

an alanine in this position reduced to levels intermediate that of NT and NT-NYPD (lanes 5-7). The reduced Shc binding was not a consequence of reduced receptor or Shc protein expression (Figure 3.16 lower panels). This reduced Shc association correlated with a reduction in the amount of Grb2 coupled with receptor in the same immunoprecipitates which cannot be ascribed to Grb2 levels within these cells (Figure 3.16 lower panels). Taken with the transformation data for these mutants, these data demonstrate a good correlation between Shc binding and transformation from site D.

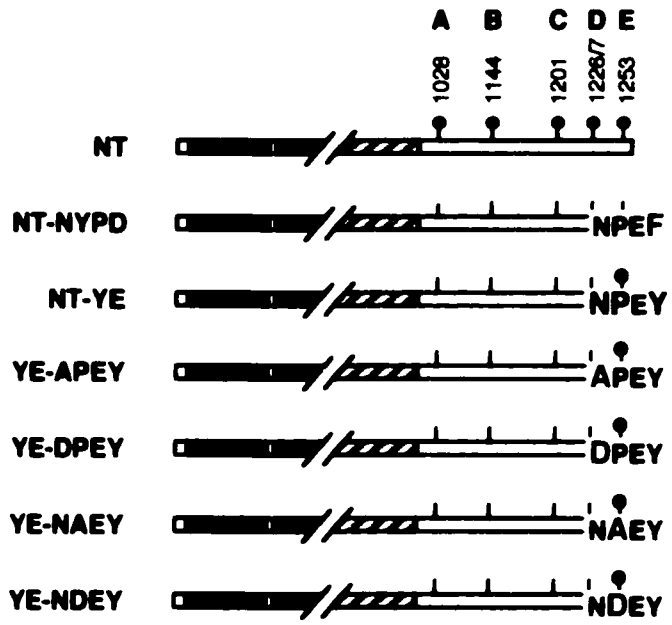
3.7 DOKR, a novel adaptor protein, binds directly to site E (tyrosine 1253) yet does not mediate a transforming signal

A short stretch of amino acid identity around tyrosines 1201 and 1253 exist (sites C [ENPEYLTP] and E [ENPEYLDL] respectively). The presence of a β-turn-forming NPxY motif (Trub *et al.* 1995) in the core Shc (Songyang *et al.* 1995b) and IRS-1 PTB domain binding sites (Wolf *et al.* 1995; Eck *et al.* 1996) suggests that one or more PTB containing proteins mediates transformation from sites C and E. Mutations were made to codons encoding the conserved asparagine (N1250) and/or the conserved proline (P1201, P1251) in the context of NT-YC or NT-YE to those encoding either alanine or aspartic acid in the context of NT-YC or NT-YE creating YC-NAEY, YC-NDEY, YE-NAEY, YE-NDEY, YE-APEY and YE-DPEY (Figure 3.17 and not shown). These mutants were transfected into Rat1 fibroblasts along with NT-YC and NT-YE and their transformation potential was determined by focus formation activity. Both NT-YC "second site" mutant possessed debilitated transformation potential (approximately 40% of the parental; data not shown), suggesting a requirement for a proline at this position. Asparagine 1250 appears to be required for transformation from site E whereas alteration of proline 1251 to alanine, but not aspartic acid, reduced the transforming activity of NT-YE (Figure 3.17). The requirement of an intact NPxY motif argues that a PTB containing protein(s) may in fact mediate the signal(s) emanating from these sites.

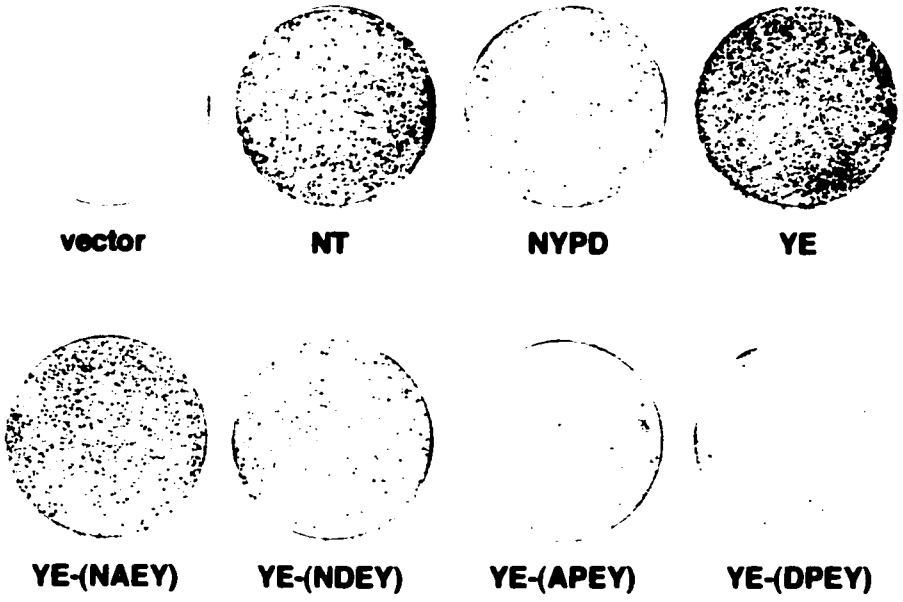
Figure 3.17 An intact NPxY motif is required for transformation from NT-YE.

Neu molecules harbouring mutations of proline are indicated in the same fashion as depicted in figure 3.18. The proline (P, residue 1199) in the NPEY motif at site E has been changed to an alanine YE-NAEY or an aspartic acid residue YE-NDEY in the context of NT-YE. Similarly, asparagine 1250 has been changed to an alanine or aspartic acid creating YE-APEY and YE-DPEY respectively. Focus formation assays were obtained in Rat1 fibroblasts and representative plates are shown in the lower panel. Similar results were obtained in three independent experiments. In three experiments, the following transforming frequencies ± standard error were obtained pJ4 (0±0), NT (100±0), NYPD (17±4), YE (118±9), YE-NAEY (98±11), YE-NDEY (30±4) and in a single experiment YE-APEY (6±1) and YE-DPEY (4±1).

A


A
B
C
D
E
1028
1144
1201
1226/7
1253
NT
NT-NYPD
NPEF
NT-YE
NPEY
YE-APEY
APEY
YE-DPEY
DPEY
YE-NAEY
NAEY
YE-NDEY
NDEY

B

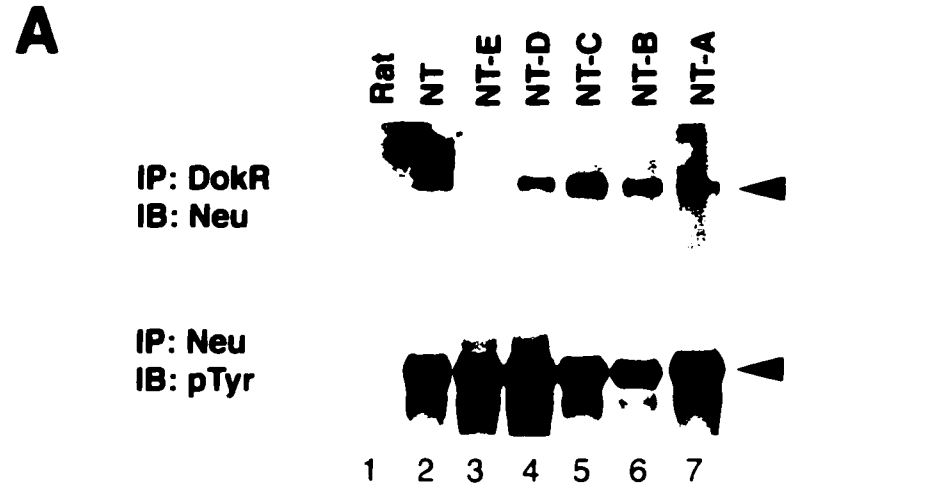

vector
NT
NYPD
YE
YE-(NAEY)
YE-(NDEY)
YE-(APEY)
YE-(DPEY)

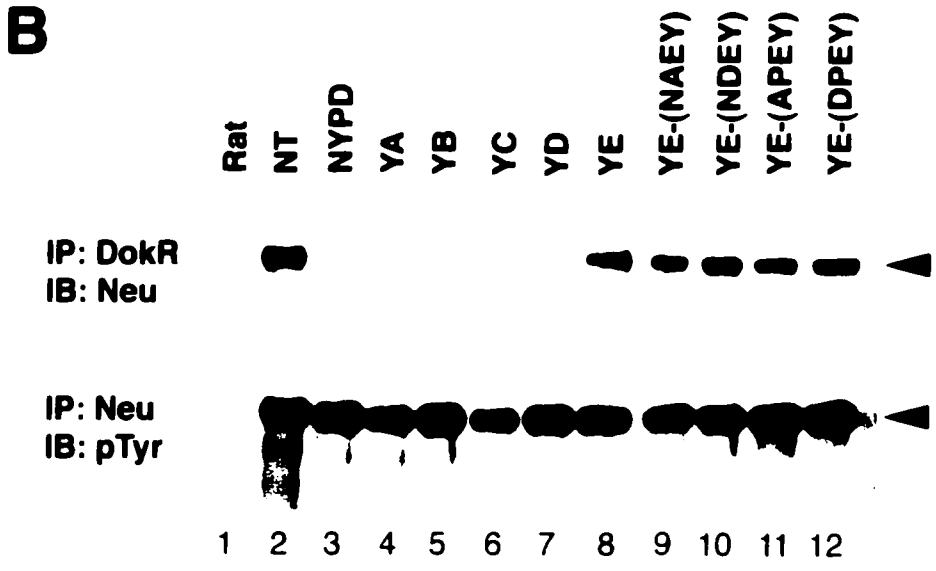
Several proteins implicated in proliferation/differentiation have been demonstrated to contain PTB-like domains including IRS-1, Numb, c-Cbl and mDab1 (O'Neil *et al.* 1994; Gustafson *et al.* 1995; Li *et al.* 1997b; Bonita *et al.* 1997; Howell *et al.* 1997). The sequence similarities between the tyrosine interaction domains of these proteins is quite low making a PTB designation difficult. A novel cDNA encoding a putative PTB-containing protein was cloned via its association with the Tie-2/Tek RTK in a yeast two-hybrid screen (Jones and Dumont 1998). Sequence analysis revealed homology to p62DOK and is termed DOKR for DOK related protein. DOK is a 62kDa Ras-GAP associated protein which is tyrosine phosphorylated in response to transforming and mitogenic PTK activation (Ellis *et al.* 1990) as well as in blast cells from CML patients (Carpino *et al.* 1997). Further analysis suggest that DOKR interacts with Tie-2/Tek through an amino terminal domain (Jones and Dumont 1998) which bears homology to the IRS-1 PTB-like domain. Moreover DOKR is expressed in murine fibroblasts lines. Thus since DOKR contains a PTB domain and is expressed in fibroblast lines suggested it may be a candidate for interaction with sites C or E.

To explore the possibility that DOKR physically interacts with Neu *in vivo*, the former was immunoprecipitated from Rat1-derived lines expressing Neu point mutants. DOKR was associated with activated Neu derived from wild-type as well as all point mutant expressing cell lines with the exception of NT-E which bound poorly (Figure 3.18A). Conversely, similar DOKR coimmunoprecipitations with Neu add-back mutants demonstrates interaction with NT-YE yet not to NT-NYPD nor the other add-back mutants (Figure 3.18B compare lane 8 to lanes 4-7). The inability to detect DOKR-Neu complexes is not due to differences in the levels of tyrosine phosphorylated Neu (Figure 3.18A and B). Given that the available DOKR antisera does not function in immunoblots, I have been unable to ascertain whether there are differences in the amount of DOKR expressed in these lines. These data demonstrate that tyrosine 1253 (site E) is both necessary and sufficient for Neu-DOKR complex formation. The role of DOKR in site E mediated transformation however is unclear as DOKR-Neu complexes are readily detected in all the NT-YE-derived mutants (YE-NAEY, YE-NDEY, YE-APEY and YE-DPEY) (Figure 3.18B lanes 9-12).

Figure 3.18 DOKR associates with Neu *in vivo* with tyrosine 1253 (site E)

(A) DOKR was immunoprecipitated from 1.0mg of protein lysates derived from the indicated Neu point mutant expressing cell lines. (upper panel) The immunoprecipitates were analyzed by anti-Neu immunoblot analysis (lower panel) Neu was immunoprecipitated from 500ug of the same lysates and the immunoprecipitates were subjected to anti-phosphotyrosine immunoblot analyses. **(B)** (upper panel) DOKR immunoprecipitates from the indicated cell lines were subjected to anti-Neu immunoblot analysis as in (A). Pooled lines were used for YE -NAEY, -NDEY, -APEY and -DPEY mutants. (lower panel) The phosphotyrosine content of Neu was assessed by anti-phosphotyrosine immunoblot analyses as in (A). Indicated is the migration of Neu.


A
Rat
NT
NT-E
NT-D
NT-C
NT-B
NT-A
IP: DokR
IB: Neu
IP: Neu
IB: pTyr
1 2 3 4 5 6 7

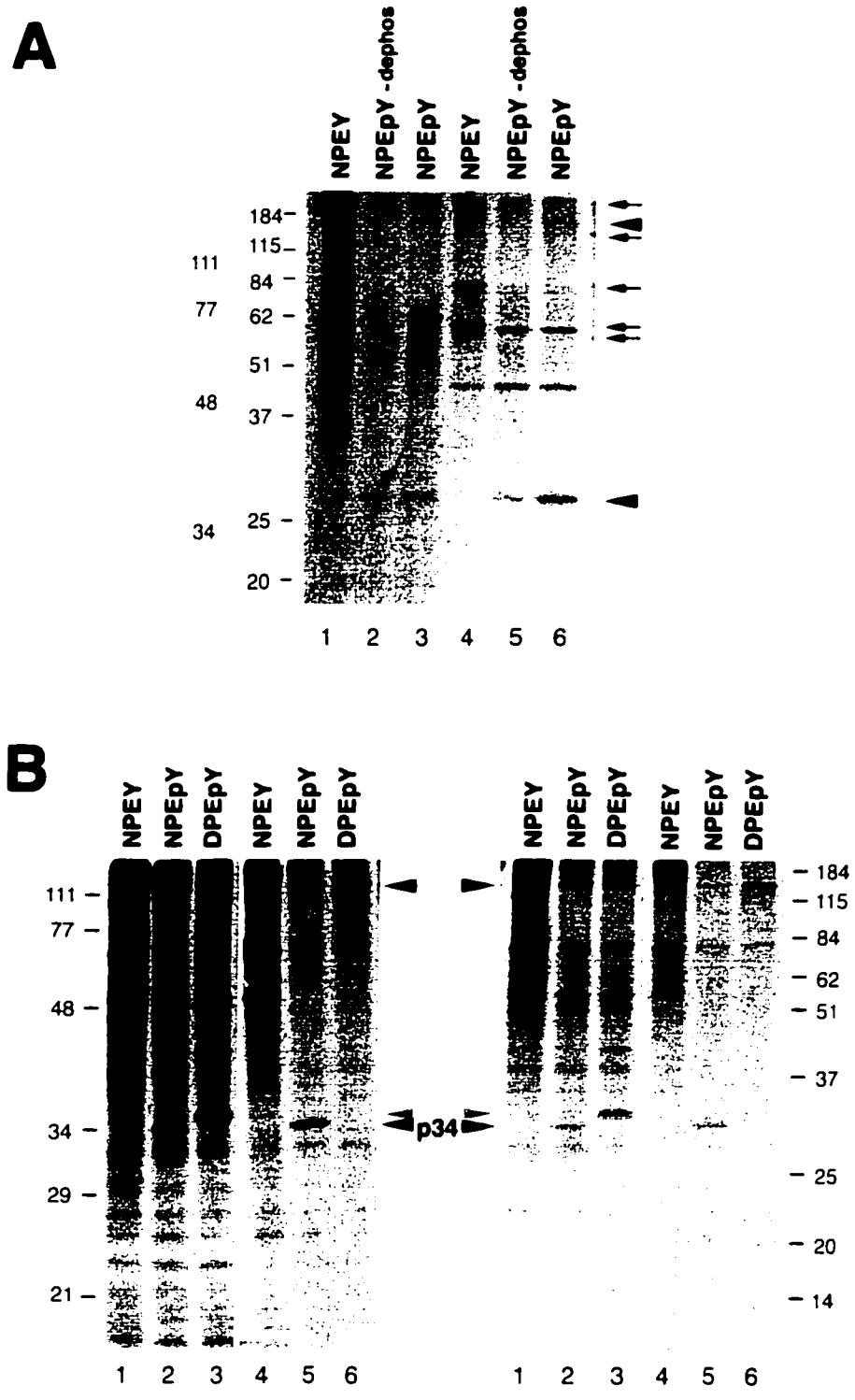

B
Rat
NT
NYPD
YA
YB
YC
YD
YE
YE-(NAEY)
YE-(NDEY)
YE-(APEY)
YE-(DPEY)
IP: DokR
IB: Neu
IP: Neu
IB: pTyr
1 2 3 4 5 6 7 8 9 10 11 12

Moreover, protein derived from *in vitro* translated DOKR cDNAs does not interact with a tyrosine phosphorylated peptide spanning site E (data not shown). Taken together, these data suggest that while DOKR physically associates with Neu through site E, it does so indirectly and that DOKR interaction does not correlate with transformation through site E.

Given that DOKR-Neu interaction did not correlate with transformation, I have undertaken a search for site E interacting proteins. To this end, I have used chemically synthesized phosphorylated and nonphosphorylated peptides which span site E from the -11 to the +7 position relative to the phosphotyrosine residue (wild-type NPEY peptide: FEGTPTAE**NPEY**LGLDVPV) in *in vitro* association assays. The amino terminus of each peptide is covalently linked to biotin providing a means of immobilizing the peptides via streptavidin-conjugated agarose beads. To these immobilized peptides, equivalent amounts of Rat1 or NIH-3T3 ^{35}S-methionine labeled extracts were incubated, the beads were extensively washed and specifically associated proteins were subjected to SDS-PAGE followed by autoradiography. Consistently, two proteins of approximately 150kDa and 34kDa appear to associate with the phosphorylated but not the nonphosphorylated peptide (Figure 3.19A lanes 3 and 6). Neither protein is similar to the 52 and 80kDa DOKR proteins. It is unclear whether these proteins are tyrosine phosphorylated as phosphotyrosine immunoblots have been uninformative. The identity of these proteins is as yet unknown, but immunoblot analysis has ruled out Nck or CrkII as the 34kDa protein (data not shown). The 34kDa protein was detected in each experiment from labeled extracts of quiescent monolayers, growing cells and serum deprived and stimulated extracts (data not shown). Given that the YE-DPEY mutation was nontransforming, a tyrosine phosphorylated peptide was synthesized to contain the N1250D mutation (DPEpY peptide: FEGTPTAE**DPEpY**LGLDVPV). The 34kDa, but not the 150kDa protein, failed to interact with this DPEpY-peptide when isolated from Rat1 fibroblasts. Moreover, in some experiments a 36kDa protein associated with the phosphorylated DPEY-peptide but not the phosphorylated or nonphosphorylated wild-type peptides (Figure 3.19B lanes 2 and 3). Interestingly, similar experiments using Jurkat T-cells revealed association of a comigrating 34kDa protein to the tyrosine phosphorylated

Figure 3.19 Phosphopeptides containing tyrosine 1253 (site E) affinity purify several cellular proteins

(A) Immobilized peptides (1ug) were incubated with ^{35}S-methionine labeled extracts (500ug) of NIH-3T3 (lanes 1-3) and Rat1 (lanes 4-6) fibroblasts. Specifically associated proteins were electrophoresed on a SDS-(12%) polyacrylamide gel and were visualized by autoradiography. Approximate molecular weights are indicate to the left. The peptides (FEGTPTAENPEYLGLDVPV) used were either synthesized non-phosphorylated (NPEY; lanes 1 and 4), synthesized phosphorylated (NPEpY; lanes 3 and 6) or were synthesized phosphorylated but were dephosphorylated prior to binding assay (NPEpY-dephos; lanes 2 and 5). Arrowheads highlight the migration of two phosphotyrosine-dependent associated proteins whereas arrows represent non-specific proteins. (B) Proteins from ^{35}S-methionine labeled Rat1 (lanes 1-3) and Jurkat T-cell (lanes 4-6) extracts were affinity purified on non-phosphorylated (NPEY) and phosphorylated (NPEpY) wild-type peptides as well as to a phosphorylated peptide containing a N1250D mutation (DPEpY) as in (A). Black arrowheads indicate the migration of p34 and the mobility of an approximately 150kDa phosphotyrosine dependent protein. Grey arrows indicate the migration of a fibroblast-specific 36kDa protein which associates with the DPEpY peptide.


A
NPEY
NPEpY -dephos
NPEpY
NPEY
NPEpY -dephos
NPEpY
184
115
111
84
77
62
51
48
37
34
25
20
1 2 3 4 5 6
B
NPEY
NPEpY
DPEpY
NPEY
NPEpY
DPEpY
NPEY
NPEpY
DPEpY
NPEY
NPEpY
DPEpY
111
77
48
34
29
21
p34
184
115
84
62
51
37
25
20
14
1 2 3 4 5 6
1 2 3 4 5 6

wild-type but not DPEpY peptide (Figure 3.19B lanes 5 and 6). Phosphotyrosine immunoblot analysis of peptide purified proteins has failed to demonstrate tyrosine phosphorylation of these (or any) specifically associated proteins (data not shown). These data suggest that a number of proteins associate with site E through the phosphorylation of tyrosine 1253 but the *in vitro* binding of a single 34kDa protein correlates with transforming potential from this site.

3.8 A novel in vitro association assay reveals CrkII interacts with site C.

While coimmunoprecipitation analyses generally reflect *in vivo* interactions, it is often difficult to determine the appropriate conditions under which two proteins bind from cell lysates. Moreover, often different lysis conditions are required for each set of proteins. To more rapidly identify candidate signaling molecules and the Neu tyrosine phosphorylation sites to which they interact, I developed an *in vitro* association assay based on the work of Pawson and colleagues (Larose and Pawson 1993) using tyrosine phosphorylated GST-fusion proteins. *E. coli* do not contain tyrosine kinases, thus I made use of recombinant bacterial strain, TKB1, harboring an inducible Elk (Lhotak and Pawson 1991) RTK domain. Plasmids encoding GST-fusion proteins were transformed into TKB1 or the parental PTK-deficient BL21 bacteria and the fusion proteins were induced with IPTG, which is also induces expression of the Elk PTK domain in TKB1 but not BL21 bacteria (Figure 3.20A). Fusion proteins are purified and immobilized on glutathione sepharose beads and specifically interacting cellular proteins are then enriched from cell lysates by standard affinity purification procedures. Proteins which bind are visualized by immunoblot analyses with antisera generated to suspected interacting proteins. The use of unphosphorylated and phosphorylated fusions along with a comparison of results obtained with specific tyrosine to phenylalanine mutations ensures specificity of observed protein interactions.

Three GST fusion proteins were constructed to individually contain phosphorylation sites Y1201, Y1226/7 and Y1253 (gT-YC, YD and YE respectively) as well as a fusion mutated at each of the three sites (gT-F3) (Figure 3.20B). While Coomassie staining revealed roughly equivalent levels of proteins from

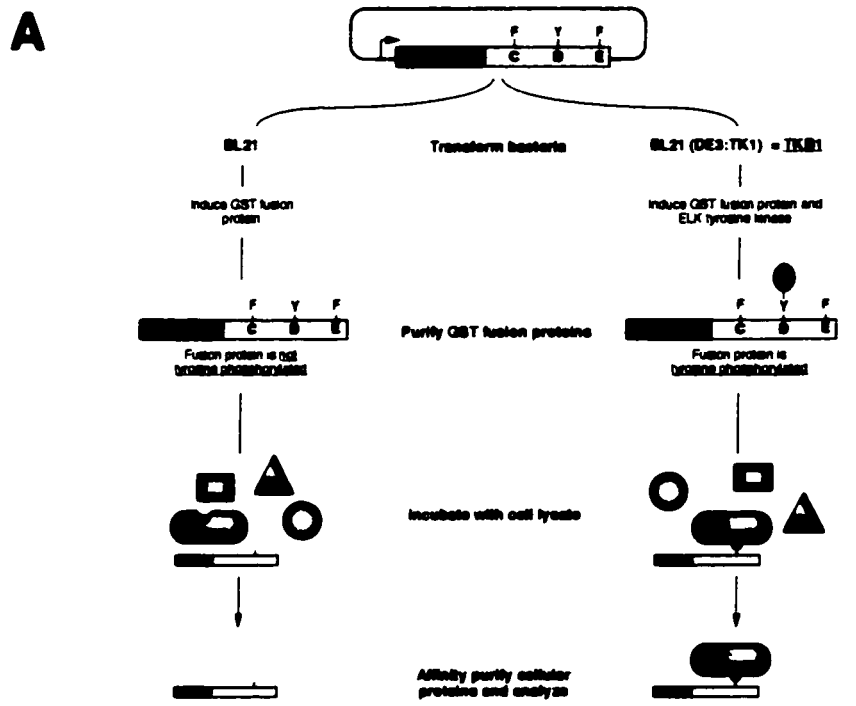
BL21 and TKB1 bacteria, immunoblot analyses revealed that gT-YC, gT-YD gT-YE were tyrosine phosphorylated when purified from TKB1 but not from the parental BL21 strain (Figure 3.21A and B). The phosphoproteins found in the TKB1s expressing gT-F3 likely represent contaminating bacterial proteins as these have not been repeatedly observed in other experiments. Thus TKB1 can efficiently induce the tyrosine phosphorylation of GST fusion proteins but does not phosphorylate GST itself.

The feasibility of this approach was tested by determining whether Shc proteins specifically interact with GST-fusions containing tyrosine phosphorylated site D. Lysates from exponentially growing NIH-3T3 fibroblasts were incubated with gT-YC, gT-YD, gT-YE and gT-F3 purified from BL21 or TKB1 and specifically associated proteins analyzed by immunoblot analyses with Shc specific antisera (Figure 3.21C). Shc did not interact with fusion proteins produced in the BL21 strain yet efficiently bound gT-YD when expressed in TKB1. The lack of binding cannot be attributed to the amounts nor the level of tyrosine phosphorylation of the fusion proteins.

Having demonstrated the specificity of this *in vitro* approach, I screened affinity purified proteins which have been implicated in Ras activation, namely Crk and Nck, both of which can interact with Sos proteins (Matsuda *et al.* 1994; Hu *et al.* 1995b). An aliquot of the same purified proteins were subjected to immunoblot analysis with Crk-specific antibodies. CrkII was not detectably bound to unphosphorylated proteins yet repeatedly was retained on gT-YC beads (Figure 3.21D), suggesting a specific interaction. Neither Nck nor Ras-GAP, however, were purified by this approach (data not shown). Crk was variably detected in association with gT-YD at much lower relative amounts (compare lanes 4 and 6). Thus, CrkII but not Nck, can interact with tyrosine 1201 *in vitro* when phosphorylated.

Figure 3.20 ***In vitro*** **association assay with bacterially produced tyrosine phosphorylated Neu fusion proteins.**

(A) Plasmids encoding GST fusion proteins were transformed into BL21 or BL21 bacteria containing the Elk tyrosine kinase domain (TKB1) under the transcriptional control of the *lac* operon. In both strains IPTG induces expression of the GST fusion proteins and in TKB1 also the expression of Elk tyrosine kinase domain. Fusion proteins were immobilized and used to affinity purify specific mammalian proteins from cell lysates. The blue cellular protein interacts specifically with the tyrosine phosphorylated GST-fusion whereas the other proteins do not. **(B)** GST fusions were constructed to contain residues 1171-1260 of Neu and each contains a single phosphorylation site at residues 1201, 1226/7 and 1253 to create gT-YC, gT-YD and gT-YE respectively. gT-F3 contains tyrosine to phenylalanine mutations at each of the three phosphorylation sites.


A
F Y F
C D E
BL21
Transform bacteria
BL21 (DE3:TK1) + TKB1
Induce GST fusion protein
Induce GST fusion protein and ELK tyrosine kinase
Purify GST fusion proteins
Fusion protein is not tyrosine phosphorylated
Fusion protein is tyrosine phosphorylated
Incubate with cell lysate
Affinity purify cellular proteins and analyze

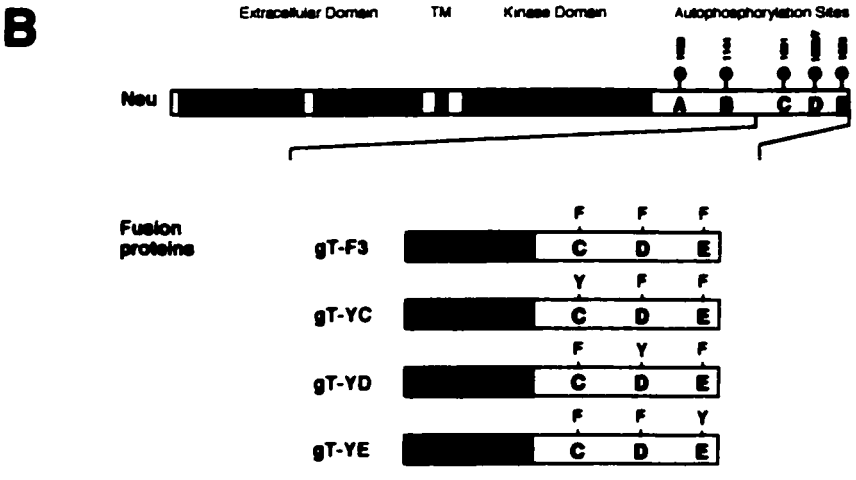
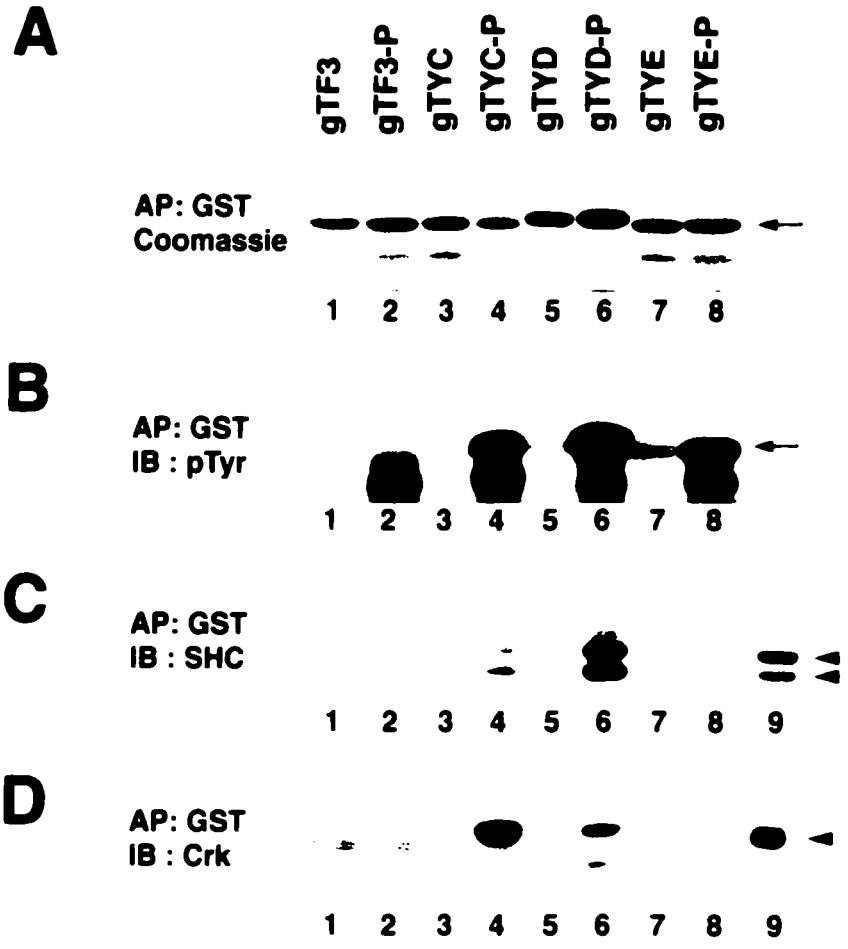

B
Extracellular Domain
TM
Kinase Domain
Autophosphorylation Sites
Neu
A B C D E
Fusion proteins
gT-F3
gT-YC
gT-YD
gT-YE
F F F
Y F F
F Y F
F F Y
C D E

Figure 3.21 Phosphorylation site C interacts with CrkII when expressed as a bacterial tyrosine phosphorylated fusion protein

(A) One twentieth of the purified fusion proteins isolated from BL21 (odd lanes) or TKB1 (even lanes) bacteria were used to assess protein integrity by Coomassie stain of the indicated fusion proteins. **(B)** One third of each purified fusion protein was subjected to anti-phosphotyrosine immunoblot analyses. The arrow indicates the migration of each fusion protein. Note faint bands observed in the odd numbered lanes represents non-specific binding of the secondary antibody. **(C and D)** Equivalent amounts of each remaining immobilized fusion protein were incubated with cellular extract from quiescent NIH-3T3 fibroblasts. The beads were washed 4-6 times in PLC lysis buffer and the resulting affinity purified proteins were analyzed by immunoblot analyses with Shc- (C) or Crk- (D) specific antibodies. 25ug of the same protein lysates was loaded in lane 9 and arrowheads denote the migration of the p46 and p52 Shc isoforms and CrkII.


A
gTF3
gTF3-P
gTYC
gTYC-P
gTYD
gTYD-P
gTYE
gTYE-P
AP: GST
Coomassie
1 2 3 4 5 6 7 8
B
AP: GST
IB : pTyr
1 2 3 4 5 6 7 8
C
AP: GST
IB : SHC
1 2 3 4 5 6 7 8 9
D
AP: GST
IB : Crk
1 2 3 4 5 6 7 8 9

3.9 DISCUSSION

3.9.1 Several phosphorylation sites appear functionally redundant

The work in this chapter represents the first demonstration that an ErbB family member utilizes multiple signaling molecules to initiate a mitogenic or transforming signal. To identify the signaling pathways involved in *neu*-mediated transformation, I derived a number of mutants in the carboxyl terminus of Neu focusing on the known tyrosine phosphorylation sites. Using site directed mutagenesis, I generated two series of phosphorylation mutants: point mutants, in which individual tyrosine residues were replaced with phenylalanines (Figure 3.2A) and add-back mutants in which individual tyrosine residues were reconstituted to a mutant *neu* molecule possessing mutations in all the known tyrosine phosphorylation sites (NT-NYPD, Figure 3.5). Loss of any single phosphorylation site had little effect on Neu transforming potential, resulting in transformation efficiencies between 75 to 85% (Table 3.1) or in the case of site A point mutant, a reproducible elevation of transformation. Simultaneous mutation or deletion of all of these sites results in a dramatic reduction in the transforming potential of activated *neu* as assessed by several criteria (NT-NYPD and NT-CT, Table 3.2). The fact that restoration of tyrosine residues at positions B, C, D or E to NT-NYPD completely restores the transforming potential of activated *neu* demonstrates that Neu induces cellular transformation through multiple ostensibly redundant tyrosine phosphorylation sites (Table 3.3).

These observations are consistent with reports by other groups that the deletion of carboxyl terminus of Neu ablates transformation activity (Di Fiore *et al.* 1990; Segatto *et al.* 1990; Segatto *et al.* 1992; Mikami *et al.* 1992). More recently, Ben-Levy and colleagues attempted to identify the important tyrosine residues involved in transformation by grafting oligonucleotides encoding individual tyrosine autophosphorylation sites to a cDNA encoding a transformation defective *neu* molecule possessing a carboxyl-terminal truncation (Ben-Levy *et al.* 1994). Using this approach, only the sequence encoding tyrosine phosphorylation site E (tyrosine 1253) was able to restore the transforming activity of activated

neu. By contrast, I have found that three other tyrosine phosphorylation sites (tyrosine 1144 [site B], tyrosine 1201 [site C] and tyrosines 1226/7 [site D]) can efficiently mediate a transforming signal. There are several possible explanations for these discordant observations. For example, it is conceivable that the inability of the other sites to functionally substitute in this former study reflects an inadequacy to be efficiently phosphorylated due to either their sequence or spatial context. Alternatively, if these sites are tyrosine phosphorylated, their lack of transformation potential may reflect an inability to couple properly with their cognate substrates. Furthermore, it is also possible that sequences located outside these phosphorylation sites not included in these grafted mutants contribute to high efficiency coupling of substrates with activated Neu. Indeed, each of these grafted phosphorylation sites contain four residues to the amino side of the tyrosine, and five or six carboxyl residues which are immediately followed by a stop codon. While this should be sufficient to allow efficient SH2 interactions, some PTB binding determinants, like those of IRS-1, extend to the -8 position relative to the phosphotyrosine (Wolf *et al.* 1995; Eck *et al.* 1996). Lastly, the binding of PDZ-domain containing proteins to the carboxy-terminus of transmembrane proteins has been recently shown to play a role in clustering of transmembrane proteins including RTKs (Hock *et al.* 1998; reviewed in Sudol 1998). There appear to be several classes of PDZ proteins which require the presence of a hydrophobic carboxy-terminal residues, generally ending in valine, in each of these transmembrane proteins with which they interact. Interestingly, Neu would be predicted to bind to class III PDZ proteins, as it terminates in two hydrophobic residues followed by a valine (-VPV). Thus, it is possible that this motif is required for proper RTK clustering and thus activation. It should be noted that the only the "grafted" site E phosphorylation site harbours such a carboxy-terminal sequence (Ben-Levy *et al.* 1994) thus it is possible that the other grafted mutant do not assume proper clustered conformation. Whatever the explanation, my data clearly demonstrate that Neu can induce cellular transformation through multiple seemingly redundant tyrosine phosphorylation sites.

3.9.2 Requirement for Ras activity by transforming add-back mutants

The observed functional redundancy between the various autophosphorylation sites in *neu*-mediated transformation can, in part, be accounted for by their capacity to bind different adaptor molecules coupling the Neu RTK to the Ras pathway. Microinjection of Ras neutralizing antibodies (Y13-259) and expression of Ras antagonist, Rap1A, demonstrate a requirement for Ras activity downstream of activated Neu and the transforming add-back mutants NT-YB, NT-YD and NT-YE (Table 3.4). Y13-259 microinjection, but not Rap1A expression, was sufficient to inhibit signaling from NT-YC. As Y13-259 microinjection is routinely used to assess Ras-requirements in mammalian cells, we believe that NT-YC signaling does require Ras.

Rap1A is identical to Ras in its effector domain and although Rap1A is used as a Ras antagonist, it is mechanistically unclear how it does so (reviewed Bos 1997). Rap1A-induced Ras inhibition requires an intact effector domain (Kitayama and Noda 1990) and while in its GTP-bound state, Rap1A interacts with two Ras effector molecules, Raf (Nassar *et al.* 1995; Hu *et al.* 1997) and Ral-GDS (Urano *et al.* 1996), yet this does not result in their activation (Urano *et al.* 1996; Hu *et al.* 1997). There are no published reports demonstrating Rap1A interactions with PI3'K although this has been cited as an unpublished observation (Nassar *et al.* 1995). Oddly, while Rap1A is not a Ras-GAP substrate, *in vitro* binding assays indicate Rap1A-GTP binds Ras-GAP with a apparent 100-fold higher affinity resulting in an increase in Ras-GTP levels *in vitro* (Frech *et al.* 1990) and *in vivo* (Cook *et al.* 1993). Genetically, gain of function mutations in *Drosophila* Rap1 (*Dras3, roughened* allele) are downstream *sevenless* and result in loss of R7 ommatidial development; a phenotype reminiscent of inactivating *ras* alleles (Hariharan *et al.* 1991a). Thus by all accounts Rap1 functions as a natural Ras inhibitor. Additionally, Rap1, unlike Ras, is geranylgeranylated (Buss *et al.* 1991) and in fibroblasts is not found at the plasma membrane but in other particulate fractions of the cell (Beranger *et al.* 1991) providing an excellent mechanism of titrating cytosolic effector molecules away from Ras to inactive complexes. While the preponderance of data suggests that Rap1 inhibits Ras activity by titrating critical Ras effector molecules, there are several possibilities to explain NT-YC's

Rap1A transformation independence. These add-back mutants may differentially activate downstream Ras effector molecules. Alternatively, NT-YC may activate a Rap1-GTPase activating protein. Finally, hyper-stimulation of Rap1A may lead to proliferative responses.

(i) It is possible that NT-YC, but not the other transforming add-back mutants, preferentially activates one of the Ras effector molecules, one which is not efficiently targeted by Rap1A. This hypothesis proposes that in addition to GTP loading of Ras, each Ras effector molecule can be activated by "upstream" signals. For example, while Ras-recruitment of Raf to the plasma membrane is necessary for full Raf activation, it is clear that an additional signal is required for maximal Raf activity (Williams *et al.* 1992). It appears likely this signal, which can be provided by v-Src expression, is a plasma membrane bound protein kinase (Williams *et al.* 1992; Stokoe *et al.* 1994; Stokoe and McCormick 1997). Additionally, although PI3'K is activated by oncogenic Ras (Rodriguez-Viciana *et al.* 1994; Rodriguez-Viciana *et al.* 1996), two PDGFR add-back mutants capable of activating Ras do not possess elevated PIP_3 production in response to PDGF treatment (Valius and Kazlauskas 1993; Valius *et al.* 1993). This suggests that endogenous Ras activation is insufficient for PI3'K activation. Moreover, PDGFR add-back mutants which bind PI3'K specifically, do not induce elevated PI3'K products unless Ras is activate as well (Klinghoffer *et al.* 1996). The use of a specific PI3'K inhibitor (wortmannin) in these experiments positions PI3'K activation below that of Ras. Taken together, these data demonstrate that efficient activation of Ras effector molecules under physiological conditions requires signals generated "upstream" of Ras whereas viral/oncogenic forms of Ras may more efficiently couple to Ras effector molecules.

At least two Ras effector molecules are simultaneously required to elicit efficient v-Ras transformation (Rodriguez-Viciana *et al.* 1997). Thus, it is possible that Rap1A differentially inhibits activation of these effector molecules and furthermore that NT-YC utilizes an effector molecule inefficiently sequestered by Rap1A. Which of the three known Ras effector molecules are critical Rap1 targets is unknown but could easily be tested through coexpression of Rap1 (or Rap1-V12) and the RasV12-effector mutants generated by Wigler and colleagues (White *et al.* 1995; Khosravi-Far *et al.* 1996;

Rodriguez-Viciana *et al.* 1997). Alternatively, effector domain mutations could be made in a GTPase deficient Rap1A (Rap1A-V12) and used to determine which Ras effector is required to inhibit transformation from each add-back mutant and Ras-V12. The requirement of each Ras-effector pathway in NT-YC-mediated transformation can be tested through the use of inhibitory Raf, Ral and PI3'K/Rac mutants. In fact, kinase deficient Raf inhibits transformation from activated Neu and appears to specifically inhibit NT-YE-mediated transformation (N. Warner and Muller, unpublished observations) implying that these add-back mutants differentially activate Ras effector molecules.

(ii) It is conceivable that NT-YC, but not the other transforming add-back mutants, recruits or activates a Rap1A-specific GAP. Given that Rap1A must be in the GTP-bound form to bind to the tested Ras-effector molecules, activation of a Rap1A GTPase would allow Ras-specific antagonists (Y13-259 mAbs) to inhibit NT-YC signaling while being resistant to Rap1A overexpression. Indeed several specific Rap1-GAPs exist yet RTK association or activation has not been tested (reviewed in Bos 1997). This hypothesis can be directly tested by assessing the Rap-GAP activity from cell lysates expressing the various add-back mutants.

(iii) CrkII associates *in vitro* with site C (Figure 3.22), CrkII binds to C3G, a Rap1A specific exchange factor (Tanaka *et al.* 1994; Gotoh *et al.* 1995), and thus NT-YC would be expected to have increased GTP-Rap1A levels. This form of Rap1A binds to and activates RafB (Gotoh *et al.* 1995). Moreover, Rap1A, but not Ras, interacts with a novel protein which has structural motifs suggesting a role in membrane/cytoskeletal regulation (Serebriiskii *et al.* 1997). It is conceivable that the hyperactivation of one or both of these proteins is sufficient to overcome the Ras inhibitory effects in response to Rap1A expression.

Thus, while Ras activity is clearly required for NT- YB, YD and YE signaling, a careful analysis of Ras effector protein/pathways activation is required to distinguish between the above possibilities. The relative levels of GTP and GDP bound to Ras should be ascertained to unequivocally demonstrate that the transforming add-back mutants do activate Ras-GTP loading. The use of PC12 neurite outgrowth can be

explored as this is an established Ras-dependent process (Hagag *et al.* 1986) and PC12 cells harbouring an inducible N17-Ras allele have been routinely used to study this process (Szeberenyi *et al.* 1990). These experiments are currently being pursued.

3.9.3 Grb2 mediates signaling from site B (Y1144)

I have presented evidence that Grb2 can bind directly to activated Neu through tyrosine 1144 (site B) and indirectly through its interaction with the Shc adaptor protein through tyrosines 1226/1227 (site D) although these may have different consequences. These observations extend earlier data showing that Neu complexes with both the Grb2 and Shc adaptor proteins (Segatto *et al.* 1993; Janes *et al.* 1994; Kavanaugh and Williams 1994). Consistent with these analyses, it has been demonstrated that point mutation of the comparable site to B in ErbB-2 (tyrosine 1139) interferes with the capacity of this receptor to couple with Grb2 *in vitro* (Ricci *et al.* 1995). My data further demonstrate that this site is not only required for binding of Grb2, but is also in fact sufficient for its direct association with Grb2 (Figures 3.9 and 3.10). These observations are also consistent with studies conducted with the closely related EGFR. In these studies, indirect peptide competition analyses suggested that the principle binding site for Grb2 on the EGFR is tyrosine 1068 (Batzer *et al.* 1994). Interestingly, the sequence following this phosphotyrosine residue closely resembles those found surrounding tyrosine 1144 in Neu (PVPEpYINQ and PQPEpYVNQ respectively). Moreover, the Grb2 SH2 domain displays a strong preference for peptides containing an asparagine in the +2 position, relative to the phosphorylated tyrosine (Songyang *et al.* 1994), as is found at site B. Four different substitutions of this asparagine (N1146) render NT-YB transformation impaired while simultaneously preventing the association of Grb2 to the receptor (Figure 3.11). Finally, microinjection of the Grb2 SH2 domain, which acts as a competitive inhibitor (Wang and Moran 1996), into NT-YB expressing cells prevented DNA synthesis induction (Figure 3.12). Taken together with the data linking Grb2 to Sos (see section 1.5), these data strongly indicate that transforming signal originating at tyrosine 1144 is conveyed to Ras through the Grb2-Sos complex.

Due to the nature of the experiments employed, the possibility exists that this signal is mediated by an additional molecule(s) with binding specificities similar that of Grb2. The SH2-containing protein Grb7 (Margolis *et al.* 1992) is often co-overexpressed with ErbB-2 in human breast tumours and can directly interact with tyrosine phosphorylated Shc and Neu/ErbB-2 where it competes *in vitro* for Grb2 binding sites (Stein *et al.* 1994). Grb7 binding has been mapped to tyrosine 1139 (site B) of ErbB-2 by *in vitro* peptide competition (Janes *et al.* 1997). Using two different commercially obtained antisera, I have been unable to detect Grb7 in Rat1 fibroblasts. To date there is no direct evidence linking Grb7 to Ras activation and as a result I believe Grb2 functions to mediate both a proliferative and transforming signal from site B. To formally define Grb2's role in NT-YB mediated transformation, assays should be carried out in $grb2^{+/+}$ and $grb2^{-/-}$ derived cells or through antisense RNA approaches. While the latter is technically feasible, $grb2^{-/-}$ embryos fail to implant in the uterine wall (Chen *et al.* 1998) preventing the isolation of $grb2^{-/-}$ fibroblasts until tissue specific or temporally induced $grb2^{-/-}$ knock-out mice are produced. Alternatively, it may be possible to isolate $grb2^{-/-}$ fibroblasts from chimeric embryos made from homozygous null $grb2^{-/-}$ ES cells as these cells will be resistant to G418 treatment whereas the wild-type cells will be drug sensitive.

An alternative approach to test whether Grb2, and not a molecule with similar binding affinities, mediates transformation from site B involves the use of complementary mutation of the Grb2 SH2 domain. Alteration of specific residues within the SH2 domain of Src alters the binding specificityp to that of the Grb2 SH2 domain and Grb2 can similarly be altered to Src SH2 sites (Marengere *et al.* 1994). Given that mutation of the asparagine in the Grb2 binding site (YvNq) ablated transformation from site B, one could screen for Grb2 SH2 domain mutants which bind to this altered sequence (e.g. YvHq). If Grb2 is responsible for site B-mediated transformation, coexpression of these Grb2 mutants would be expected to restore transformation to the NT-YB(+2) mutants which it binds. These data would provide an unambiguous demonstration of Grb2's role in signaling from this site.

3.9.4 Shc mediates signals from site D (Y1227)

I have also presented evidence that activated Neu can indirectly recruit Grb2 through its interaction with Shc at site D (tyrosines 1226 and 1227) (Figure 3.13). Consistent with these analyses, Kavanaugh and colleagues have provided indirect peptide competition evidence that the site analogous to D (Y1222) in c-ErbB-2 is the primary site of interaction of ErbB-2 with the Shc PTB domain. Lower affinity interaction with sites analogous to C and E (Y1196 and Y1248 respectively) were also observed (Kavanaugh *et al.* 1995). Similar *in vitro* studies with point mutants of ErbB-2 have suggested that sites C, D and E are the major determinants required for Shc binding (Ricci *et al.* 1995). I have further demonstrated that phosphorylation site D is both sufficient and necessary to form complexes of activated Neu and Shc *in vivo* (Figures 3.13-14) and that interaction is direct, mediated through the Shc PTB domain (Figure 3.15). It should be noted that although early indications indicated an NPxY motif was required for Shc PTB binding, a proline to leucine alteration at the -2 position allows for Shc PTB interaction (Kavanaugh *et al.* 1995; Laminet *et al.* 1996) and neither proline nor leucine at this position was selected for or against Shc-PTB binding from a degenerate peptide library (Songyang *et al.* 1995b). Although mutation of site D involves the conversion of two sequential tyrosine residues located at positions 1226 and 1227, it is likely tyrosine residue 1227 is the tyrosine residue responsible for binding Shc since this latter residue conforms to the reported Shc PTB consensus binding site NxxY (Kavanaugh *et al.* 1995; Batzer *et al.* 1995; Songyang *et al.* 1995b). Mutation of the conserved tyrosine or asparagine residue in this motif results in impairment of site D mediated transformation and for the asparagine mutants, drastically reduces Shc binding to Neu (Figure 3.16). Thus there appears to be a tight correlation between Shc-Neu interaction and transformation.

Although my data demonstrate that site D is the primary site of interaction with Shc, mutants incapable of binding Shc are still capable of inducing Shc tyrosine phosphorylation (Figure 3.13) and inducing the formation of Shc/Grb2 complexes (Figure 3.20C). One possible explanation for these observations is that since these mutant receptors remain capable of binding Src (Figure 3.8) and that

activated Src alleles induce Shc phosphorylation (McGlade *et al.* 1992b), Neu-induced activation of Src (Muthuswamy and Muller 1995) may be responsible for Shc phosphorylation. The requirement of Src in the phosphorylation of Shc mediated by NT-NYPD expression can be tested in *src-/-* fibroblasts. Alternatively, it is theoretically possible that the observed tyrosine phosphorylation of Shc in the absence of direct interaction is due to heterodimerization of these mutant Neu molecules with other ErbB family members. No reports exist which examine the ability of molecules with the activating transmembrane mutation to heterodimerize with normal ErbB receptors although a single report indicates that oncogenic neuNT may require wild-type family members to efficiently transform fibroblasts (Cohen *et al.* 1996). Regardless of the mechanism involved, tyrosine phosphorylation of Shc and consequent Grb2 association are insufficient to induce wild-type levels of transforming activity since the NT-NYPD and NT-YA mutants are severely impaired in their transforming activity yet induce both Shc phosphorylation and Shc-Grb2 complex formation.

The mechanism whereby Shc mediates proliferative signals has been attributed to Grb2 recruitment and the presumed activation of Ras (Rozakis-Adcock *et al.* 1992; Rozakis-Adcock *et al.* 1993). We have demonstrated a site D-mediated Ras requirement for transformation and DNA synthesis induction (Table 3.4). Additionally, the preceding data demonstrate Shc simultaneously interacts with tyrosine 1227 and Grb2 and while Shc binding and transformation are correlated, Grb2 SH2 domain microinjection had little effect on YD-mediated proliferation (Figure 3.12). The principal Grb2 binding site on Shc (Y317; Salcini *et al.* 1994) is not found within *Drosophila* Shc (dShc; Lai *et al.* 1995) indicating that dShc may signal independently of Grb2. Additional Shc phosphorylation sites have been localized to residues 239 and 240 (Gotoh *et al.* 1996; Gotoh *et al.* 1997; van der Geer *et al.* 1996) which are found in dShc (Lai *et al.* 1995). In some cells types and certainly *in vitro*, tyrosine 239 binds Grb2 (van der Geer *et al.* 1996; Harmer and DeFranco 1997; Tiganis *et al.* 1998): in others Y317 is responsible for Grb2 binding (Salcini *et al.* 1994; Gotoh *et al.* 1997; Ishihara *et al.* 1997). Functionally, tyrosine 317 and tyrosines 239/240 have distinct roles. While all three sites are required for enhanced EGF-induced DNA proliferation, when

overexpressed phosphorylation mutants elicit distinct activities: Y317 mediates Erk activation while Y239/240 are indispensable for EGF-induced c-myc mRNA accumulation and protection against apoptosis in an IL3 dependent cell line (Gotoh *et al.* 1996; Gotoh *et al.* 1997). These latter effects may be mediated by one or more proteins which specifically interact with Y239 (van der Geer *et al.* 1996). I have attempted to address the role of Shc phosphorylation in Neu-mediated signaling but have been unable to inhibit transformation from NT, NT-YD or PyV-MT with either Shc -Y315F or YY239/240FF mutants. Thus additional experiments with a triple Shc phosphorylation mutant should be carried out to determine if Shc phosphorylation does play a role in Neu signaling. Alternatively, given that there is no direct evidence that Shc activates Ras activation, a molecule with similar Shc binding specificity may bind site D and mediate Ras activation. Shc deficient mice exist but die in utero at approximately E10.5 (Lai and Pawson 1998). It would be useful to examine the transforming properties of these mutants in embryonic fibroblasts from $shc^{+/-}$ and $shc^{-/-}$ to test such a hypothesis.

3.9.5 Site E associates with several potential signal regulators

NIH-3T3 fibroblasts expressing the YE add-back mutant display an interesting phenotype when grown in an anchorage independent fashion (Figure 3.6). Unlike the other add-back mutants which form spherical densely packed colonies in soft agar, all NT-YE expressing colonies are irregularly shaped and loosely packed with the appearance of cells migrating outwards. This phenotype was observed in some colonies formed by NT-expressing NIH-3T3 cells but was not observed in the other add-back transfectants. This is intriguing as this NIH-3T3 phenotype is characteristic of metastatic lines. Coupled with the Ras dependency (Table 3.4) and indications of PTB-mediated transformation (Figure 3.17), these data warranted a search for proteins which may mediate transformation from this site.

While the orthalagous site in EGFR (Y1173) has been shown to interact with PLCγ (Rotin *et al.* 1992) and that PLCγ recruitment to RTKs can mediate Ras-dependent signaling (Valius and Kazlauskas 1993) through a yet identified mechanism, it is unlikely that this is the major mediator of YE signaling.

Firstly, EGFR Y1173 is one of three PLCγ binding sites conserved between Neu and EGFR yet it represents a low affinity PLCγ binding site (Rotin *et al.* 1992). Secondly, there is growing evidence that PLCγ functions as a negative regulator of mitogenesis and transformation (Klinghoffer *et al.* 1996; Obermeier *et al.* 1996; Partanen *et al.* 1998; see section 1.4.1 and Chapter 4). Moreover, microinjection experiments suggest that PLCγ is required downstream of Ras (Smith *et al.* 1990). Lastly, mutational analysis suggests that an NPxY motif is required for transformation from this site (Figure 3.17). These mutations would not be expected to affect PLCγ binding (Songyang *et al.* 1993). Thus PLCγ is not likely to play a positive role in Neu signaling from site E: it may however play a negative role.

A number of PTB containing proteins exist which may mediate signaling from both this site and tyrosine 1201 (site C). I excluded those PTB-containing proteins known not to be expressed in the fibroblasts used herein (mDab1, F65.1) as well as those which are genetic inhibitors of RTK signaling (cbl; Sli) Meisner *et al.* 1997; Howell *et al.* 1997). Additionally, mNumb fusion proteins fail to interact with activated Neu *in vitro* (in collaboration with J. McGlade, AMGEN Inst. Toronto, Canada). IRS-1/2 are ubiquitously expressed, contain a PTB domain and function downstream of the insulin receptor (IR). Upon IR activation, IRS-1 binds to an NPxY motif in the IR through its PTB domain and becomes tyrosine phosphorylated at several sites whereby it functions as an adapter molecule recruiting several cytoplasmic signaling molecules (p85, Syp: reviewed in Myers and White 1996). IRS-1 binding is involved in Ras activation by the insulin receptor, making this a candidate site C/E effector molecule. IRS-1 PTB domains require a large hydrophobic residue in the -8 position relative to phosphotyrosine (Wolf *et al.* 1995; Eck *et al.* 1996). Given that site C and E respectively contain a glycine and threonine at these positions suggests that IRS-1 is incapable of interacting with high affinity to either site. I have however not assessed the role of IRS proteins in Neu mediated signaling although this warrants further investigation.

Ras-GAP associates with two tyrosine phosphorylated proteins, p190, a Rho-GAP (Settleman *et al.* 1992), and p62, in v-src expressing Rat2 or in EGF treated EGFR-expressing Rat1 fibroblasts (Moran *et al.* 1991). Myristolation Y527F-Src mutants (G2A) possess elevated PTK activities, are non-transforming

owing to inappropriate subcellular localization and fail to induce p62 tyrosine phosphorylation (Bouton *et al.* 1991) suggesting p62 phosphorylation is involved in the transformation process. p62 (p62DOK the Ras-GAP associated protein) was recently cloned as a prominently phosphotyrosine containing protein in leukemic cells derived from CML patients (Yamanashi and Baltimore 1997). The authors noted that in addition to an amino terminal plekstrin homology domain, thought to play a role in transient lipid-mediated membrane association (Pawson 1995), a small region (30 residues) is distantly similar to the IRS-1 PTB domain. Closer examination of the sequence suggests that DOK contains an IRS-like PTB domain which are distinct from that of Shc (Dankort unpublished). The lack of suitable reagents has prevented analysis of DOK in Neu signaling.

A DOK related protein (DOKR), cloned via a yeast two hybrid screen for Tie2/Tek-RTK-associated proteins, harbours an amino terminal receptor binding domain (Jones and Dumont 1998). DOKR is expressed in NIH3T3 fibroblasts and preliminary data demonstrates it is overexpressed in MMTV-Neu mammary tumours relative to the adjacent epithelium (data not shown). Somewhat surprisingly, DOKR appears to specifically interact with site E but not C even though the surrounding sequences are quite similar (site C: $_{-8}$GGAVENPEYLVP$_{+3}$ and site E: $_{-8}$TPTAENPEYLGL$_{+3}$). While DOKR-Neu complexes demonstrated a specificity for site E, this interaction did not correlate with transformation from site E. Specifically, NPxY motif mutants severely impaired in their transformation potential remain capable of DOKR binding (Figures 3.17, 3.18). Taken together these data represent the first demonstration that DOK-family members interact with ErbB-RTKs and furthermore suggest that they are not positive mediators of signal transduction from the Neu RTK.

p62DOK phosphorylation is not inhibited by Ras-N17 expression consistent with the notion that p62DOK phosphorylation is upstream of Ras (Pronk *et al.* 1993). In addition to interactions with Ras-GAP, p62DOK associates with PLCγ (Maa *et al.* 1994) and the C-terminal Src kinase (Csk) which phosphorylates and inactivates Src kinases (Neet and Hunter 1995; Nada *et al.* 1991). Oddly, each of these proteins is a negative regulator of signaling and coupled with my data it is possible that DOKR may recruit

inhibitory signaling molecules to Neu. Circumstantial evidence suggests this may be the case in Neu signaling. Specifically, NT-YE-derived mutants capable of DOKR association (APEY and DPEY) transform at frequencies below that of NT-NYPD (Figure 3.17). Experiments demonstrating a negative role for DOKR in Neu mediated transformation through site E can be carried out by coexpression of DOKR and the add-back mutants. Additionally, mutation of residues surrounding site E which prevent DOKR association would be predicted to harbour increased transforming abilities.

I have demonstrated that at least two additional proteins (p150 and p34) are capable of interacting with site E *in vitro* in a phosphotyrosine-dependent manner. p150 appears to bind to tyrosine phosphorylated peptides containing the DPEY mutation that ablates transformation from site E. Coupled with the observation that *in vitro* translated DOKR fails to directly interact with site E suggests that DOKR may bind to p150. It is intriguing to note that this protein is similar in size to PLCγ and that a DOK related protein interacts with PLCγ (Maa *et al.* 1994). This possibility is being investigated. Interestingly p34 associates with only wild-type tyrosine phosphopeptides to site E yet not to the DPEpY peptide suggesting that p34 is an excellent candidate molecule mediating signaling from site E. I am currently purifying this protein for the purposes of identifying its molecular sequence.

3.9.6 A potential role for CrkII in Neu signaling

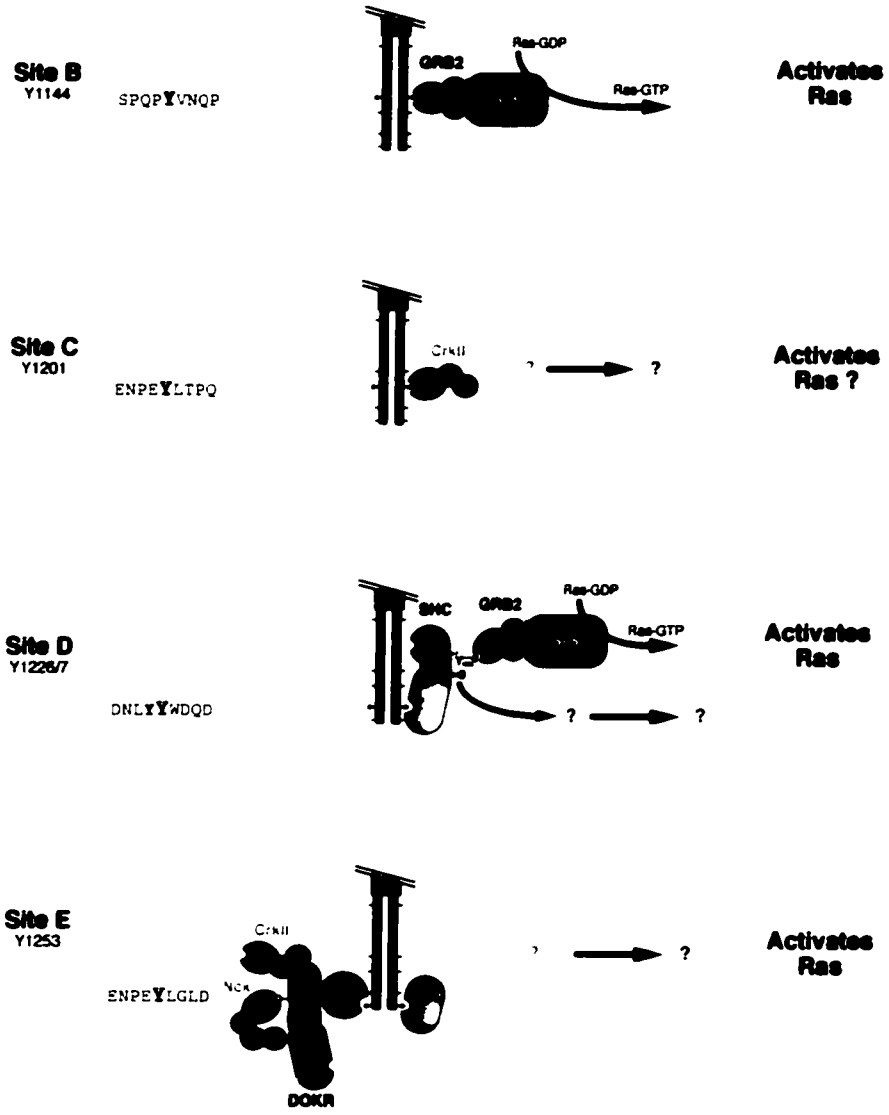
I developed a novel *in vitro* association assay based on the experiments of Pawson and colleagues who demonstrated that infection of bacteria with a lambda bacteriophage encoding a PTK lead to the efficient tyrosine phosphorylation of fusion proteins (Larose and Pawson 1993). GST itself is not phosphorylated under these conditions (data not shown). Thus by removing all but one tyrosine residue in the fusion portion allows for assessment of tyrosine phosphorylation. This approach allows for the rapid screening of candidate interacting proteins and simultaneously provides a means of defining the *in vitro* binding sites. Using fusion proteins containing either site C, D or E, Shc associates preferentially with site D and CrkII with site C. The predicted Crk SH2 binding site demonstrates a strong selection for a proline

residue in the +3 position (Songyang *et al.* 1993) which is found at site C (YLTP). The role Crk proteins play in EGFR signaling is unclear as use of dominant-inhibitory Grb2 but not Crk mutants inhibit EGF mediated Erk activation (Tanaka *et al.* 1995). These data can be reconciled by the observation that the corresponding tyrosine in EGFR is not a predicted Crk SH2 binding site (YLNT) and furthermore that Crk proteins may not activate Erks. Mammalian Crk proteins were isolated as cellular counterparts to the *v-crk* retroviral oncogene (Mayer *et al.* 1988; Tsuchie *et al.* 1989) and exist as two differentially spliced isoforms: CrkI contains an SH2 and SH3 domain and CrkII has an additional C-terminal SH3 domain (Matsuda *et al.* 1992). Overexpression of CrkI but not CrkII induces fibroblast transformation (Matsuda *et al.* 1992). A role of Ras in Crk signaling has been realized. First, microinjection of PC12 cells with CrkI/II induces neurite outgrowth in a Ras-dependent manner (Tanaka *et al.* 1993). Secondly, use of farnesylation inhibitors and expression of Ras-N17 demonstrate a Ras requirement for v-*crk*-induced cellular transformation. Oddly, there was little activation of Erk activity in response to efficient v-Crk expression perhaps suggesting other Ras effector molecules are used by v-Crk (Greulich and Hanafusa 1996). Lastly, a chemically induced CrkII mutant was isolated on its ability to inhibit EGF-mediated transformation of NRK fibroblasts. Cell lines expressing this CrkII mutant (Crk-23) have reduced inducible Ras-GTP levels and are also resistant v-*erbB* and *erbB-2* transformation but not to transformation via oncogenic *raf* or *ras* expression (Kizaka-Kondoh *et al.* 1996). EGF-mediated transformation is restored by wild-type CrkII but not CrkI, Grb2 or Nck expression. Thus taken together, these data position CrkII between Neu and Ras and further implicate Crk as a mediator of site C transformation. Whether CrkII-Neu complexes form *in vivo* and if so, are direct (or through a PTB containing protein) are the subject of future studies.

In summary, the key results of this chapter demonstrate multiple SH2/PTB containing proteins interact with individual Neu tyrosine phosphorylation sites. When present singly these sites and by inference the pathways they activate, are sufficient transform recipient cells in many cases in a Ras-dependent manner (Figure 3.22).

Figure 3.22 Schematic representation of positive signals which emanate from Neu autophosphorylation sites.

Indicated are the amino acid sequences surrounding each autophosphorylation site, the proteins identified to bind these site *in vitro* (black and white) and *in vivo* (colour) and the possible mechanism by which the individual sites function.


Site B
Y1144
SPQPYVNQP
GRB2
Ras-GDP
Ras-GTP
Activates Ras
Site C
Y1201
ENPEYLTPQ
Crkll
?
?
Activates Ras ?
Site D
Y1226/7
DNLYYWDQD
SHC
GRB2
Ras-GDP
Ras-GTP
?
?
Activates Ras
Site E
Y1253
ENPEYLGLD
Crkll
NCK
DOKR
?
?
Activates Ras

Chapter 4

Tyrosine 1028 (Site A) Represses Transformation in a Sequence Specific Fashion

4.1 INTRODUCTION

Although the presence of negative regulatory tyrosine phosphorylation sites is widely accepted for Src-family kinases, there is limited evidence indicating the existence of negative regulatory tyrosine phosphorylation sites in growth factor receptors. Alteration of a specific tyrosine residue in the carboxyl terminus of the c-Fms RTK results in its biological activation. It is, however, unclear whether this residue it is a *bona fide* autophosphorylation site (Roussel *et al.* 1987).

While four of the Neu tyrosine autophosphorylation sites are functionally redundant for *neu*-mediated transformation, addition of tyrosine phosphorylation site A (tyrosine 1028) to the NYPD mutant abolished the basal transforming properties of this mutant. Moreover, mutation of this residue in activated Neu results in an increase of transforming potential (Chapter 3). These observations suggest that tyrosine 1028 negatively regulates Neu transformation.

RESULTS

4.2 Restoration of tyrosine phosphorylation site A interferes with *neu*-mediated transformation in a specific and cis-acting fashion.

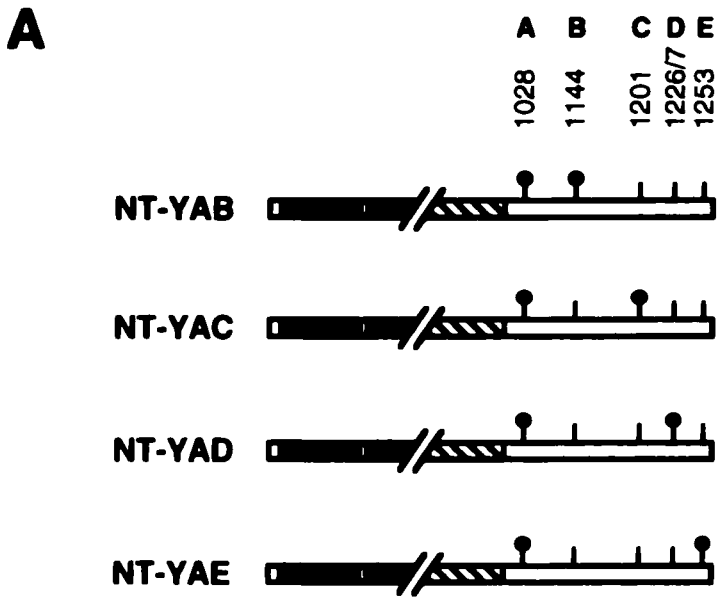
To further investigate the specificity by which site A interferes with *neu*-mediated transformation, tyrosine 1028 (site A) was restored in *cis* to the transforming add-back mutants, NT-YB, NT-YC, NT-YD and NT-YE, creating a series of double add-back mutants which each contain phosphorylation site A and one of the remaining four sites (Figure 4.1). The specific transforming activities of these double add-back mutations was then assessed in Rat1 fibroblasts. Restoration of site A to the NT-YB, NT-YC, and NT-YE mutants severely impaired the transforming activities of these mutants (Figure 4.1B). In particular, addition of site A to the NT-YB mutant (NT-YAB) virtually abolished its transforming activity whereas the NT-YAC and NT-YAE mutants were less severely effected (Table 4.1). In addition, the morphology of foci that were scored in these double add-back mutants were pinpoint by comparison to the larger foci observed in the parental add-back mutations (Figure 4.1B). By contrast to these observations, addition of site A to the NT-YD mutant resulted in a modest reduction (less than two fold) in its specific transforming activity. Unlike the pinpoint foci detected in the other double-add-back mutations, the foci induced by NT-YAD were morphologically comparable to those induced by activated *neu* (Figure 4.1B). Thus unlike the other add-back mutants, the inclusion of site A has a minimal effect on the ability of the NT-YD mutant to transform Rat1 cells. Taken together, these data suggest that site A interferes with Neu-mediated transformation in a cis-acting and specific fashion.

4.3 Double add-back mutant NT-YAB and NT-YB have similar kinase activities

Transformation inhibition induced by the inclusion of tyrosine 1028 (site A) to the transforming add-back mutants (YB) may be a result of a reduced catalytic activity of these double add-back mutants. To address this possibility, I determined the initial rates of kinase activity of the NT-YB and

Figure 4.1 Transformation of mutant Neu molecules is suppressed by site A.

(A) Illustrated are a series of mutant Neu molecules (double add-back mutants) each containing a tyrosine at residue 1028 and a single tyrosine at one of the remaining autophosphorylation sites. A mutant containing tyrosines at 1028 and 1144 and phenylalanines at residues 1201, 1226/7, and 1253 is termed NT-YAB. Note that all mutants contain the activating transmembrane mutation E664V. (B) Representative plates from focus assay 2 (Table 4.1) are shown to illustrate the relative transforming abilities and morphologies of the add-back mutants compared to activated Neu (NT) (upper and middle panels). The lower panel illustrates the transformation repression mediated by site A in a series of double add-back mutants.


A
A
B
C
D
E
1028
1144
1201
1226/7
1253
NT-YAB
NT-YAC
NT-YAD
NT-YAE

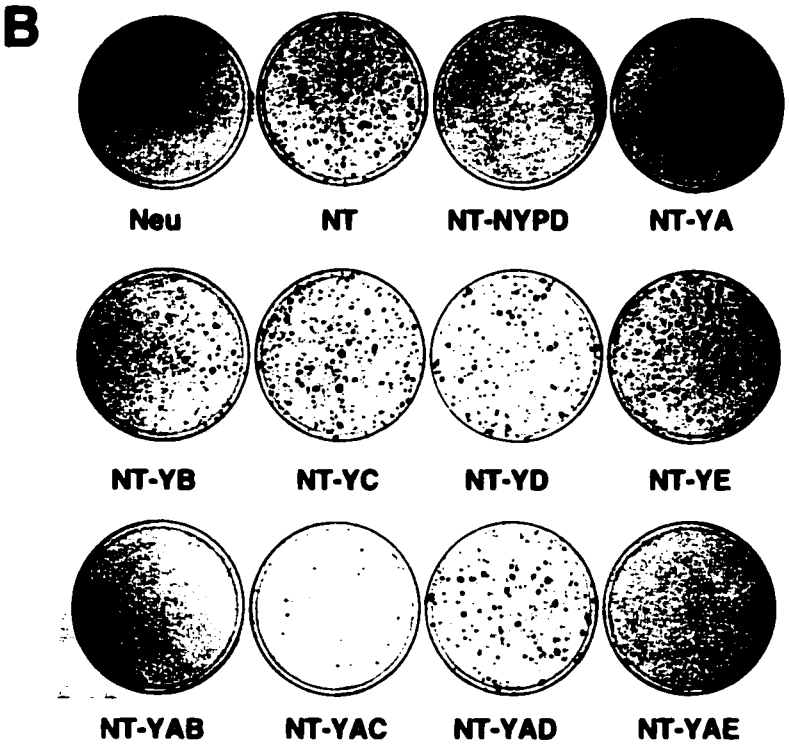

B
Neu
NT
NT-NYPD
NT-YA
NT-YB
NT-YC
NT-YD
NT-YE
NT-YAB
NT-YAC
NT-YAD
NT-YAE

Table 4.1 Site A inhibits Neu mediated transformation in Rat-1 cells

Construct	Focus assay 1		Focus assay 2		Relative transforming ability[c]
	Average no. of foci[a]	% Transformation of NT[b]	Average no. of foci[a]	% Transformation of NT[b]	
Neu	0	0	0	0	0
NT	258 ± 25	100	232 ± 51	100	100
NT-NYPD	20 ± 5	8	11 ± 1	5	7 ± 2
NT-YA	0 ± 0	0	n.a.	n.a.	0
NT-YB	185 ± 19	72	264 ± 20	114	93 ± 29
NT-YC	297 ± 11	115	238 ± 11	103	109 ± 8
NT-YD	268 ± 29	104	198 ± 29	85	95 ± 13
NT-YE	323 ± 31	125	206 ± 31	89	107 ± 25
NT-YAB	1 ± 1	0	4 ± 3	2	1 ± 1
NT-YAC	36 ± 1	14	33 ± 18	14	14 ± 0
NT-YAD	159 ± 18	62	114 ± 24	49	56 ± 9
NT-YAE	35 ± 10	14	60 ± 9	26	20 ± 8

Two independent focus assays were performed using Rat-1 fibroblasts. Independent plasmid preparations were used for each experiment. Data not available is denoted by n.a..

[a]Values represent the mean number of foci per plate counted on six plates ± standard deviation.

[b]Values are the ratios of the mean numbers of foci obtained for each construct with respect to that obtained with NeuNT (NT) multiplied by 100%.

c Values are the wieghted mean transforming abilities from the two experiments ± standard deviations.

NT-YAB mutants in immunoprecipitation kinase assays using MBP as an exogenous substrate. The results of these analyses revealed that the NT-YAB mutant displayed activities comparable to NT-YB (Figure 4.2). The rate of incorporation was two to three fold above that obtained in parental Rat1 fibroblasts. Thus the differences in transforming activity between NT-YB and NT-YAB cannot be accounted by major differences in catalytic activity.

4.4 Residues adjacent to tyrosine 1028 are required for transformation repression.

The presence of similar kinase activity in the double add-back YAB mutant argues that repression cannot be attributed to this property of the kinase. Interestingly, sequence alignment of the residues surrounding Y1028 in Neu with the homologous sites found within ErbB-2, EGFR, ErbB-3, ErbB-4 v-ErbB and the platyfish (*Xiphophorus maculatus*) XMrk, the *C. elegans* Let-23 and the *Drosophila* DER gene products reveal remarkable similarities (Figure 4.3). The Torso RTK negative regulatory site is included for comparison and it too has similar conserved residues. In each case a leucine and proline are respectively found in the +1 and +3 positions relative to the tyrosine. This is consistent with the presence of a SH2 binding site at site A. To test this hypothesis, I generated three series of NT-YAB derived mutants possessing complete degeneracy in the +1 +2 or +3 codon relative to Y1028 by PCR-mediated mutagenesis. These mutants were cloned into an expression vector and tested for the relative transforming efficiency as above (Figure 4.4). Interestingly, mutation of leucine to some, but not all residues, resulted in an increase in transforming activities to that similar of NT-YB, suggesting derepression occurred. There were, however, no distinct requirements of a specific amino acid size, charge or shape. A similar result was found for alterations in V1030. Of particular interest was that alterating proline 1031 to either glycine, serine or alanine had little effect on transforming activity, yet all other tested mutants appeared to be derepressed. In each case residues larger than the original proline possessed elevated transforming activities whereas transformation repression required residues smaller than the proline. Taken together these data suggest that repression mediated from site A occurs in a sequence specific fashion, requiring a small residue at the +3 position.

Figure 4.2 NT-YAB and NT-YB display similar kinetic activities towards an exogenous substrate.

In vitro kinase activities of Neu immunoprecipitates from lysates (containing 10.0mg of protein) obtained from Rat1 or the Rat1-derived stable cell lines. Kinase activity was determined as described in Figure 3.5. The amount of radioactivity incorporated into MBP was quantitated by PhosphoImager analyses from duplicates. The resulting averages are displayed along with standard deviation. The slopes of the best fit line are Rat ($9.58x10^4$ units/min), YB ($29.27x10^4$ units/min), YAB1 ($32.37x10^4$ units/min) and YAB4 ($18.00x10^4$ units/min: not shown).

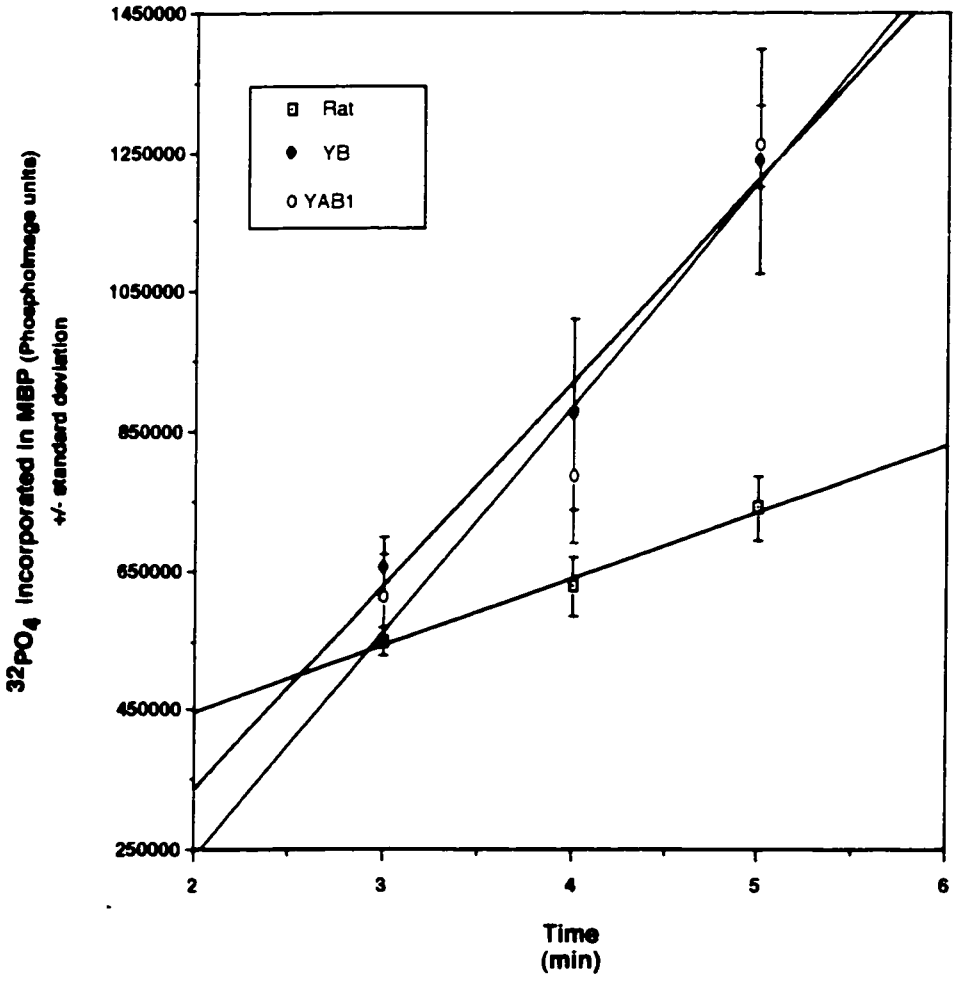

1450000
1250000
1050000
850000
650000
450000
250000
2
3
4
5
6
Rat
YB
YAB1
32PO4 incorporated in MBP (Phosphoimage units)
+/- standard deviation
Time
(min)

Figure 4.3 Alignment of sequences surrounding site A (Y1028) orthalagous sites in RTKs.

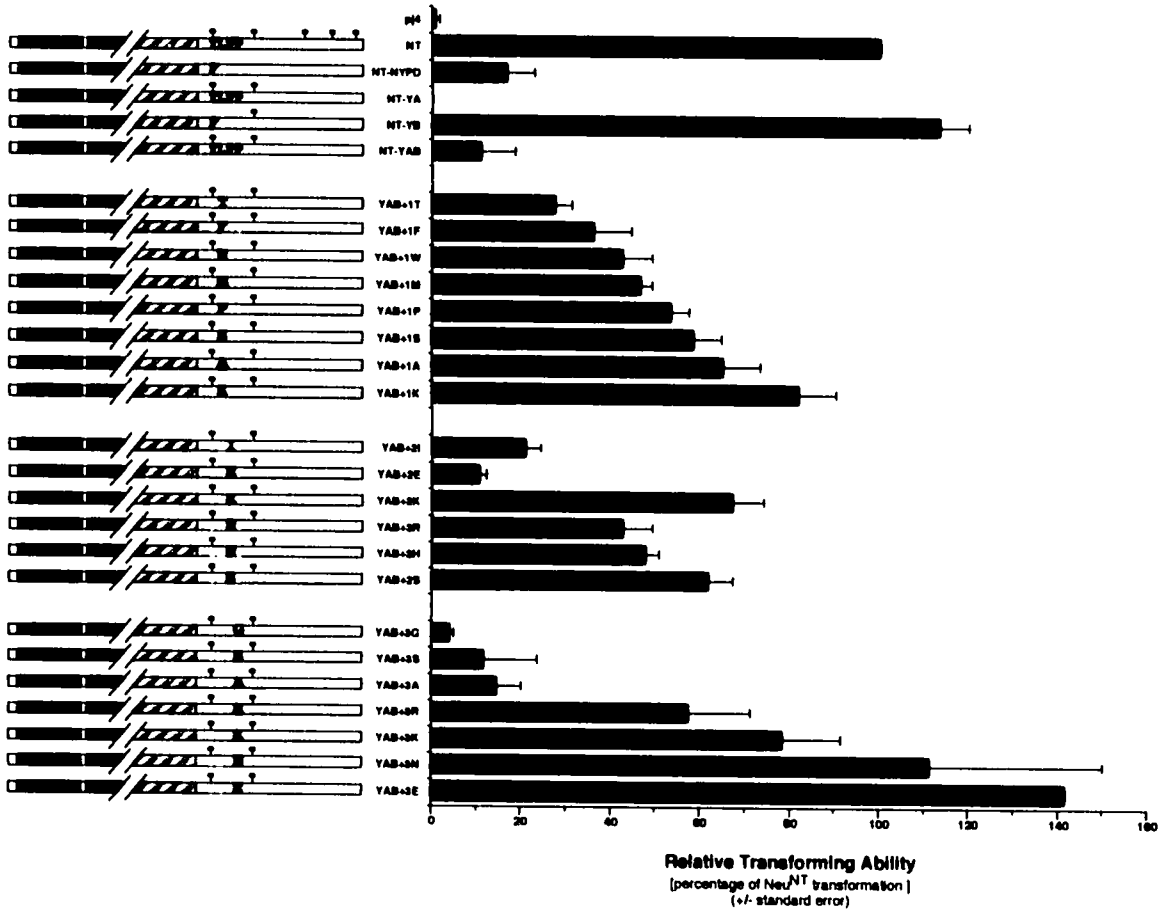
Alignment of the residues -4 to +5 relative to the tyrosine 1028 in Neu. The receptors are human ErbB-2, human and chicken EGFR, *Xiphophorus* XMrk and *Drosophila* DER and Torso. While Torso is not an ErbB family member this tyrosine phosphorylation site is demonstrated to mediate a negative signal from the receptor (Cleghon *et al.* 1996). Residues absolutely conserved are in bold conserved hydrophobic and acidic residues are indicated by ϕ and ψ respectively.

NEU	Y1028	DLVDAEE**YL**V**P**QQGFF
hErbB2	Y1023	DLVDAEE**YL**V**P**QQGFF
EGFR	Y992	DVVDADE**YL**I**P**QQGFF
verbB	Y436	DIVDADE**YL**V**P**HQGFF
XMRK	Y1007	DVVDADE**YL**L**P**YKRIN
Der	Y1261	LPVDEDD**YL**M**P**TCQPG
Let23	Y1242	TAQEDNS**YL**I**P**KTKEV
ErbB3	Y1149	EEEDVNG**Y**VM**P**DTHLK
ErbB4	Y1140	GELDEEG**Y**MT**P**MRDKP
TORSO	Y918	VPCEEEL**YL**E**P**LN*
consensus		**...ψψψ.YLϕP..**

Figure 4.4 Mutations in the +1, +2 and +3 residues adjacent to tyrosine 1028 (site A) effects site A mediated repression.

Depicted are mutants made to individually alter the leucine (L1029), valine (V1030) and proline (P1031) residues immediately following tyrosine 1028 at site A. Each is derived in a NT-YAB background containing tyrosines 1028 and 1144 and are given the designations NT-YA(x1)B. Relative transforming activity is indicated graphically and calculated as describe in materials. Values represent the mean relative transforming abilities from the three independent experiments ± standard error.

Site A Represses Transformation In A Sequence Specific Manner



4.5 Ras-GAP Does Not Mediate Transformation Repression From Site A

That each transforming add-back mutant requires Ras to mediate proliferative signals coupled with the observation that site A inhibits transformation from sites B through E, suggests that an inhibitor of Ras activity may be activated in response to tyrosine 1028 phosphorylation. Of the identified Ras inhibitors, Ras-GAP is the best candidate for mediating such a repressive signal as it binds directly to RTKs through its SH2 domains and in the case of the PDGFR, decreases receptor-mediated signal transduction to Ras (Valius and Kazlauskas 1995; Klinghoffer *et al.* 1996). Overexpression of Ras-GAP inhibits signaling from the PDGFR and membrane targeted Ras-GAP suppresses transformation by Ras (Huang *et al.* 1993a). Ras-GAP associates with Neu/ErbB-2 (Fazioli *et al.* 1991; Jallal *et al.* 1992) through its SH2 domains when tested *in vitro* (Muthuswamy and Muller 1995). Additionally, the Ras-GAP SH2 domains have a consensus binding sequence of YxxP (Holland *et al.* 1997) which is similar to YLVP found at site A.

To explore the possibility that Ras-GAP mediates the aforementioned repression, I tested the ability of a GST fusion protein containing the SH2 and SH3 domains of Ras-GAP to directly interact with activated Neu and NT-NYPD by direct blot analyses. These immunoprecipitates contained similar levels of tyrosine phosphorylated Neu (Figure 4.5C) and as demonstrated previously, Grb2 interacted with Neu-NT but not NT-NYPD (Figure 4.5B). Conversely, the radiolabeled GST-GAP probe interacted with both forms of Neu (Figure 4.5A). These data demonstrate that Ras-GAP binds directly to Neu at an unidentified phosphorylation site and that Ras-GAP is likely not the site A coupled substrate.

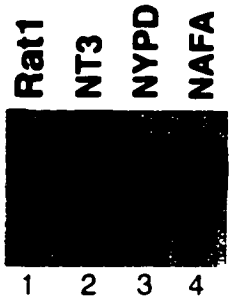
4.6 Site A Inhibits Signaling Molecules From Binding

Another possible mechanism by which site A may interfere with Neu-mediated transformation is by interfering with the ability of Neu to couple with its downstream effectors. To explore this possibility, I tested whether inclusion of site A to NT-YB (NT-YAB) or NT-YD (NT-YAD) interfered with the capacity of these molecules to bind either Grb2 or Shc through coimmunoprecipitation analyses. As previously

Figure 4.5 Ras-GAP directly interacts with activated Neu and NT-NYPD

Neu was immunoprecipitated from 3.0 mg of protein lysates of Rat1 (lane 1) or Rat1-fibroblasts expressing NT (lanes 2-3) or NT-NYPD (lanes 4-5). Equal aliquots of each immunoprecipitate was electrophoresed on an SDS-8.5% gel, transferred to a PVDF membrane and probed with ^{32}P-radiolabeled GST-fusion proteins containing the Ras-GAP-SH2 domains (A) or Grb2 (B). (C) The remaining portion of each immunoprecipitate was subjected to immunoblot analyses with anti-phosphotyrosine mAbs. The arrows indicate the migration of Neu.

A



IP : Neu
IB : pTyr

B

IP: Neu
blot: ^{32}P-GST-GAP

C

IP: Neu
blot: ^{32}P-GST-GRB2

determined, Grb2 was absent in Neu immunoprecipitates derived from Rat1 or NT-NYPD expressing cells, yet abundant in those derived from NT, NT-YB and NT-YD expressors (Figure 4.6C). Significantly, Grb2 binding to NT-YAB or NT-YAD appeared to be consistently reduced relative to NT-YB and NT-YD (Figure 4.6C, compare lanes 4 and 6 to lanes 5 and 7 respectively). These differences are not reflective of Grb2 levels in these lysates (Figure 4.6D) nor in the amount of tyrosine phosphorylated Neu found in each immunoprecipitate (Figure 4.6A). The reduced amount of Grb2 bound to NT-YAD appears to be a result of a decreased Shc binding to the receptor (Figure 4.6B) and not due to differences in the amount of Grb2 bound to Shc as equivalent amounts were detected from each of these cell lines (data not shown). Additionally, the differences in Shc binding between the NT-YD and NT-YAD mutants cannot be attributed to either the levels of tyrosine phosphorylated Neu or Shc (Figures 4.6A and D). Taken together, these data demonstrate that site A appears to modulate the ability of both Shc and Grb2 to couple to Neu .

4.7 Inhibition Of Grb2 Binding In NT-YAB Is Vanadate Sensitive

Although similar Neu-phosphotyrosine levels are detected between add-back and double add-back mutants, it is possible that the reduced Grb2 binding to NT-YAB results from a site A activated protein tyrosine phosphatase (PTP) activity. To investigate the role of such a presumptive PTP in this process, cells were pretreated with the PTP inhibitor (Gordon 1992) sodium orthovanadate (herein termed vanadate) prior lysis. In each case, vanadate pretreatment lead to an increase in Neu tyrosine phosphorylation. The level of increased receptor tyrosine phosphorylation however greatly differed amoungst the different mutants. Moreover, vanadate pretreatment lead to a specific increase in Grb2 binding to NT-YAB while only modestly effecting binding to NT-YB (Figure 4.7). These data suggest that the decrease in Grb2 binding and perhaps transformation from site B in the double add-back mutants NT-YAB is a result of the increased activity of a vanadate sensitive PTP.

Figure 4.6 Site A decreases binding of Grb2 to sites B and D *in vivo*.

Neu was immunoprecipitated from 2.0 mg of protein lysates of Rat1 (lane 1) or Rat1-derived cell lines (lanes 2-7). The immunoprecipitates were electrophoresed on a SDS-(6-15%) gradient gel, and transferred to a PVDF membrane. The membrane was cut and the top third, containing proteins larger than 80kDa, was probed with anti-phosphotyrosine antibodies (**A**) The middle portion of the blot containing proteins 32 to 80kDa was probed with Shc-specific antisera (**B**) and the remaining third containing proteins under 32kDa was probed with Grb2-specific rabbit polyclonal sera (**C**). Equivalent amounts (20ug) of the same protein lysates were subjected to immunoblot analyses with Shc-specific (**D**) or Grb2-specific (**E**) antisera.

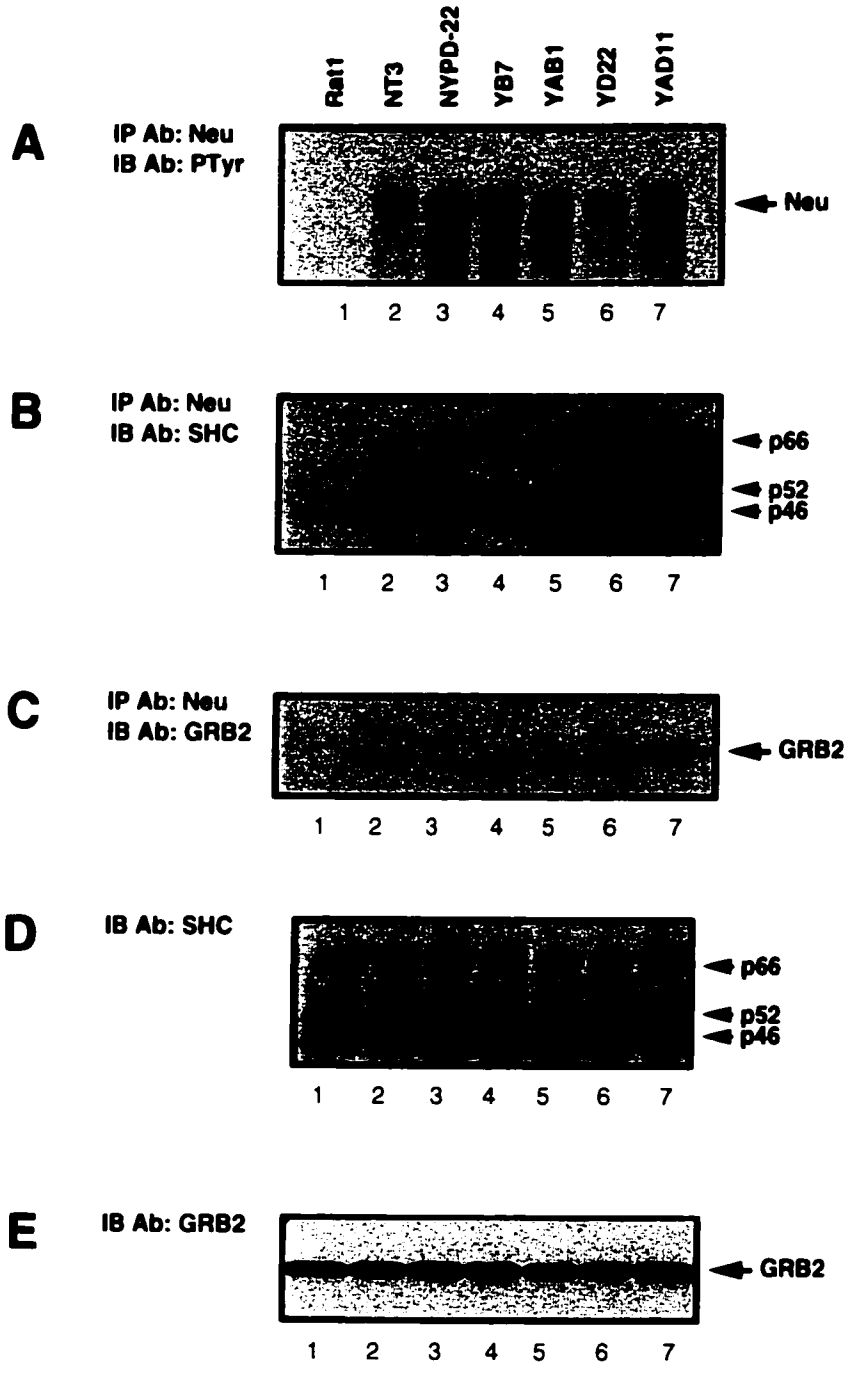
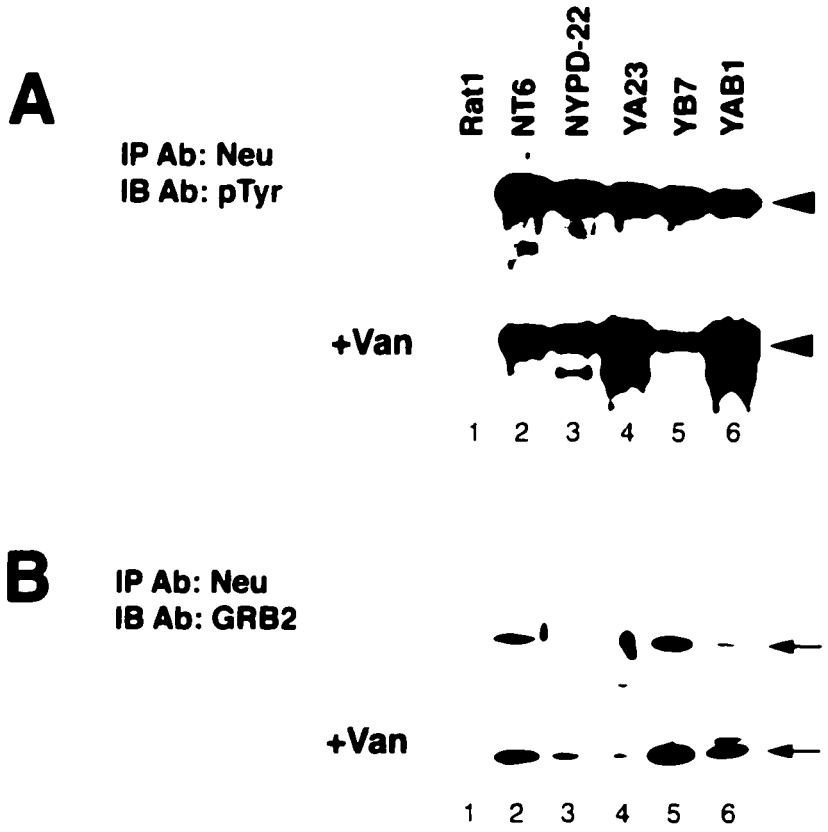

Rat1
NT3
NYPD-22
YB7
YAB1
YD22
YAD11
A
IP Ab: Neu
IB Ab: PTyr
Neu
1 2 3 4 5 6 7
B
IP Ab: Neu
IB Ab: SHC
p66
p52
p46
1 2 3 4 5 6 7
C
IP Ab: Neu
IB Ab: GRB2
GRB2
1 2 3 4 5 6 7
D
IB Ab: SHC
p66
p52
p46
1 2 3 4 5 6 7
E
IB Ab: GRB2
GRB2
1 2 3 4 5 6 7

Figure 4.7 Inhibition of protein tyrosine phosphatase activity restores Grb2 binding to NT-YAB.

Rat1 (lanes 1, x) or the indicated Rat1-derived fibroblasts were maintained in 5%FBS-DMEM and either left untreated or incubated with 1mM sodium orthovanadate for 20min. Neu was immunoprecipitated from 2.0 mg of protein lysates, the immunoprecipitates were electrophoresed on a SDS-(6-15%) gradient gel, and transferred to a PVDF membrane. The membrane was cut and the top half, containing proteins larger than 80kDa, was probed with anti-phosphotyrosine antibodies (**A**) while the bottom portion of the blot containing proteins under 80kDa was probed Grb2-specific rabbit polyclonal sera (**B**).


A
IP Ab: Neu
IB Ab: pTyr
Rat1
NT6
NYPD-22
YA23
YB7
YAB1
+Van
1 2 3 4 5 6
B
IP Ab: Neu
IB Ab: GRB2
+Van
1 2 3 4 5 6

4.8 DISCUSSION

In addition to identifying the tyrosine phosphorylation sites that are positively involved in transformation, I have identified a tyrosine phosphorylation site (site A, tyrosine 1028) that suppresses the transforming potential of activated *neu*. Evidence supporting this contention stems from the observation that mutation of site A results in a moderate but consistent elevation of the transforming potential of activated *neu* (Table 3.1). Conversely, restoration of site A to the NT-NYPD mutant or NT-YB, NT-YC and NT-YE severely suppressed the transforming activities of these molecules (Tables 3.3 and 4.1). Although the transforming activities of all add-back mutants are affected in *cis* by addition of site A, restoration of site A to the NT-YD mutant diminished morphological transformation to a lesser extent than the other mutants (Table 4.1, Figure 4.1). Biochemical analyses suggest that the transformation defect observed in these double add-back mutants is not a reflection of their kinase activities (Figure 4.2) but can be, in part, explained by interfering with their capacity to couple with signaling molecules, Grb2 and Shc (Figure 4.6). The majority of Grb2 binding can be restored to NT-YAB by the inhibition of protein tyrosine phosphatase activity (Figure 4.7). Perhaps the differential resistance of the add-back mutants to the negative regulatory effects of the site A reflects the binding of PTB domain rather than the SH2-phosphotyrosine interactions that may occur at the other phosphorylation sites. Specifically, site B and D bind the SH2 domain of Grb2 and the Shc PTB domain respectively, while the sites C and E likely represent PTB-like binding sites (Chapter 3). In this regard it has been reported, that the Shc PTB domain has similar affinities but a considerably slower kinetics for tyrosine phosphorylated peptides than a typical SH2 domain (Laminet *et al.* 1996) suggesting that PTB domains remain bound, unlike SH2 domains, which are thought to rapidly exchange. Thus in the face of a constitutive protein tyrosine phosphatase activity, PTB domains would be expected to protect their binding sites better than those of SH2 domains. Alternatively, sites C, D and E may activate signaling pathways that are refractory to the inhibitory influence of site A.

The molecular mechanism by which this site mediates repression is unclear. Transformation repression from site A requires a specific residues in the +2 and +3 residues relative to Y1028 (Figure 4.4). There appeared to be a strong preference for proline and other small residues at the +3 position and for particular amino acids at the +2 position. While these data do not prove that repression is mediated by a SH2 containing molecule, they are consistent with this assertion. In the EGFR, the analogous tyrosine residue juxtaposes a domain involved in calcium influx and receptor internalization (CaIn domain) (Chen *et al.* 1989). Kinase deficient forms of EGFR or microinjection of anti-phosphotyrosine antibodies demonstrate the requirement of receptor activation and tyrosine phosphorylation of a cellular protein (perhaps the receptor itself) in these processes. The involvement of tyrosine phosphorylation of this site itself has not been clearly shown to affect internalization however (Chen *et al.* 1989). It is thus possible that the presence of a tyrosine at site A initiates receptor internalization. While this may be the case for EGFR, it is unlikely to fully explain the decreased transformation potential of these Neu mutants as all other ErbB-RTKs appear to be refractory to ligand-mediated internalization when expressed as EGFR-ErbB chimeras (Baulida *et al.* 1996).

The evidence indicating an existence of negative regulatory autophosphorylation sites in RTKs is scant. Infection of newly hatched chicks with replication competent retroviruses expressing wild-type c-ErbB (the chicken EGFR ortholog) induces leukemia but does not produce solid tumours. Interestingly, retroviruses containing a c-ErbB Y447F point mutant (analogous to a NT-A point mutant) rapidly induce sarcoma formation, while four other phosphorylation mutants failed to do so despite retaining their leukemia inducing abilities (Chang *et al.* 1995). These data are consistent with the hypothesis of a negative regulatory phosphorylation site at a position analogous to site A.

In an elegant study, Lesa and Sternberg genetically determined that of the eight potential tyrosine phosphorylation sites within the Let-23 carboxy terminal region, five sites play positive signaling roles in different tissues while an additional site mediates negative regulation of the receptor (Lesa and Sternberg 1997). Interestingly, this site negatively regulates both viability and vulva differentiation but has

little effect on fertility indicating tissue specificity. The Drosophila RTK *torso* has two major autophosphorylation sites: one which genetically functions as a positive mediator of *torso* function and one site which when mutated functions as a gain of function mutant (Cleghon *et al.* 1996). While the molecular mechanism responsible has not been determined, the binding of either mammalian Ras-GAP or PLCγ1 to the negative regulatory autophosphorylation site (Y319) *in vitro* correlates with the induction of a negative developmental signal emanating from this site. Activated Neu is known to associate with Ras-GAP (Fazioli *et al.* 1991; Jallal *et al.* 1992) and while site A does conform to a consensus Ras-GAP SH2 binding site (YxxP) (Holland *et al.* 1997), the fact that Ras-GAP binds to NT-NYPD *in vitro* (Figure 4.5) and *in vivo* (H. Kim and W.J. Muller, unpublished) suggests that Ras-GAP is an unlikely candidate.

Within the EGFR family, mutation of the comparable tyrosine residue to site A in the EGFR (tyrosine 992) appears to moderately increase EGF-induced mitogenesis from the EGFR (Gotoh *et al.* 1994). Of the five sites of tyrosine phosphorylation in the EGFR (Y992, Y1068, Y1086, Y1148 and Y1173) PLCγ interacts with at least three: Y992, Y1068 and Y1173 (Rotin *et al.* 1992). Significantly, while Y992 represents a minor EGFR phosphorylation site (Honegger *et al.* 1988), it represents the highest affinity PLCγ binding site (Rotin *et al.* 1992). The sequences surrounding Y1028 of Neu, Y992 in EGFR and Y319 in Torso are strikingly similar (Figure 4.3) and are consistent with the predicted consensus PLCγ1 binding these sites [Y-V/I-I/L-P/I/V] (Songyang *et al.* 1993). Circumstantial evidence indicates a potential mechanism which proposes that PLCγ1 binding to this residue may act in a negative fashion. The inclusion of a higher affinity PLCγ1 binding site to EGFR Y992 decreass its ability to induce proliferation (Obermeier *et al.* 1996). Interestingly, this correlates with an increased MAP kinase activity (presumably through PKC mediated Raf activation (Kolch *et al.* 1993)) and EGFR dephosphorylation (Obermeier *et al.* 1996). A negative role for PLCγ in fibroblast growth factor receptor 1 (FGFR1) signaling has been inferred by analysing mice with this gene mutated. *Fgfr1* mutants lacking the PLCγ binding site function as gain of function or hypermorphic mutations; manifesting a phenotype in the heterozygous, hemizygous and homozygous state (Partanen *et al.* 1998). In culture, mutation of the PLCγ binding site in FGFR does not

inhibit mitogenesis (Mohammadi *et al.* 1992; Peters *et al.* 1992) nor does increased expression of PLCγ increase the proliferative responses of FGFR in response to ligand (Cuadrado and Molloy 1990). Together with the observation that increased PLCγ expression does not increase PDGF-mediated proliferation, these data suggest that the correlation of proliferation and PLCγ-PDGFR interactions reflects the activity of another molecule binding to this site. Moreover these data indicate that, at the very least, PLCγ is a negative regulator of RTK signaling from receptors other that PDGFR family members.

Treatment of cells with phorbol esters, such as phorbol 12-myristate 13-acetate (PMA) or 12-*O*-tetradecanophorbol-13-acetate (TPA), rapidly activates calcium-dependent PKC through direct interaction with the regulatory region of PKC in much the same way DAG does. Direct PKC activation results in the phosphorylation of both the EGFR (T654, Lin *et al.* 1986) and Neu (Cao *et al.* 1991). In both cases, this correlates reduced receptor signaling through a reduction in kinase activity (Cao *et al.* 1991) and, in the case of the EGFR receptor, internalization and a loss of high affinity EGF binding (Lin *et al.* 1986; Livneh *et al.* 1988). Interestingly, TPA treatment prevents EGF-induced DNA synthesis of wild-type EGFR but not of EGFR carrying a threonine 654 to alanine substitution (Livneh *et al.* 1988). Moreover, inhibition of PLCγ activity either through expression of a enzymatically inert competitive inhibitor or by treating cells with the PLCγ-specific pharmacological inhibitor U73122 increases EGFR-mediated responses (Chen *et al.* 1996b). PKC was implicated in this process through the use of a PKC inhibitor (calphostin C) and through the use of a EGFR T654A mutant. Additionally, PKC activation reduces the phosphotyrosine content of EGFR-ErbB-2 chimeras, an effect nullified by the presence of a PTP inhibitor (Seedorf *et al.* 1995). Thus, the receptor is subject to PKC-mediated dephosphorylation events and not due to decreased RTK activity. Taken together, these data strongly argue that PLCγ activation of PKC leads to ErbB receptor downregulation through dephosphorylation. Moreover they predict the existence of a PKC activated PTP. Recently the EGFR has been demonstrated to be a potential substrate of the ubiquitously expressed PTP1B and TC-PTP (Flint *et al.* 1997; Tiganis *et al.* 1998). It is, however, unclear whether the activities of these phosphatases are regulated by PKC or EGF activation. Additionally, an EGF-inducible

tyrosine phosphatase activity exists which specifically dephosphorylates EGFR and Neu but does not recognize either tyrosine phosphorylated PLCγ or Ras-GAP as substrates (den Hertog *et al.* 1993). Unfortunately, the identity of this PTP acitivity is as yet unknown.

To test whether such site A → PLCγ → PKC → PTP or site A → PLCγ → PTP repressive pathways exist, several experiments can be conducted. First, this hypothesis requires that site A is indeed a PLCγ binding site. If PLCγ is recruited to site A, then one would predict that YAB(+1,2,3) mutants which are derepressed will have lost the ability to couple PLCγ. This can be tested biochemically with standard techniques. Secondly, treatment of add-back and double add-back mutants with PLCγ and/or PKC specific pharmacological inhibitors would address the catalytic requirements of these molecules in mediating repression. These assays would likely measure DNA synthesis rather than transformation to minimize drug toxicity effects. Biochemically, PKC activation (through TPA) or PLCγ/PKC inactivation would be predicted to respectively decrease or increase Grb2 and Shc binding to NT-YAB and NT-YAD. Fourth, expression of dominant inhibitory PLCγ molecules would be predicted to alleviate site A mediated repression. Finally, PKC agonists should decrease Neu interactions with Grb2 and Shc and that this would be reversible with PTP inhibition.

Alternatively, it is conceivable that site A directly recruits and activates a tyrosine phosphatase. In the erythropoietin and IgG-Fc binding (FcgRIIB) receptors, mutation of specific tyrosine phosphorylation sites results in hypersensitivity to ligand stimulation and prolonged receptor-associated tyrosine kinase activity. It is intriguing that in both these receptors, these phosphorylation sites are responsible for recruitment of the SH2 domain containing Shp-1 tyrosine phosphatase (also known as PTP1C, SH-PTP1 and HCP) (D'Ambrosio *et al.* 1995; Klingmuller *et al.* 1995). Shp-1 appears to negatively regulate signaling from several transmembrane proteins such as the c-Kit RTK and interleukin-3 receptor (Yi and Ihle 1993; Lorenz *et al.* 1996; Paulson *et al.* 1996; Yi *et al.* 1993) and genetic studies indicate Shp-1 attenuates signaling from the T cell (Plas *et al.* 1996) and the colony-stimulating factor-1 (CSF-1) (Chen *et al.* 1996a) receptors. Although there appears to be little sequence similarity between

these receptors and Neu, in certain cell types, specific complexes of Shp-1 and Neu have been detected (Vogel *et al.* 1993). Moreover, evidence indicates that binding of Shp-2 (also known as SH-PTP2, PTP1D and Syp) to the PDGF receptor results in its increased phosphatase activity and that this increase could be recapitulated *in vitro* by incubating purified Shp-2 with tyrosine phosphorylated peptides containing the Shp-2 binding site (Vogel *et al.* 1993; Valius *et al.* 1993; Lechleider *et al.* 1993). In this regard, treatment of NT-YAB cells with sodium orthovanadate results in increased Grb2 binding to levels comparable to that found in treated NT-YB cells (Figure 4.7). I have however been unable to demonstrate Shp-1/Neu interactions exist *in vitro* or *in vivo* using many approaches, suggesting that if Shp-1/Neu complexes exist (Vogel *et al.* 1993), they may be relatively unstable. Thus, the precise molecular mechanism by which site A functions to down regulate Neu-mediated transformation will require further experimentation.

Chapter 5

Isolation, Cloning And Characterization Of A Protein Tyrosine Phosphatase, PTPM1/Shp-1.

5.1 INTRODUCTION

Regulation of protein tyrosine phosphorylation plays a critical role in controlling both cell growth and differentiation. The balance of tyrosine phosphorylation is controlled by the cumulative activities of protein tyrosine phosphatase (PTPs) and kinases (PTKs). Disregulation of this balance can result in the malignant transformation of cells. Indeed, the mammary gland appears to be exquisitely sensitive to the activities of PTKs. For example, mammary epithelial-specific expression of either Polyomavirus (PyV) middle T antigen (MT) or activated Neu results in the synchronous rapid acquisition of multifocal tumours, whereas those expressing either c-*myc* or oncogenic v-Ha-*ras* do not (reviewed in Dankort and Muller 1996). In MT-expressing transgenic mice, a functional *src* PTK allele is required to elicit tumour formation (Guy *et al.* 1994a). Moreover, elevated activities of several tyrosine kinases have been observed in breast tumours including members of the Src (Jacobs and Rubsamen 1983; Ottenhoff-Kalff *et al.* 1992; Rosen *et al.* 1996), insulin-like growth factor receptor (reviewed in Kolibaba and Druker 1997; Peyrat and Bonneterre 1992) and epidermal growth factor receptor (EGFR)/ErbB kinase families. ErbB family members are thought to play a pivotal role in the human disease (Sections 1.2.2 and 1.2.3 and Slamon *et al.* 1987; Slamon *et al.* 1989; Andrulis *et al.* 1998; reviewed in Hynes and Stern 1994; Mansour *et al.* 1994; Ravdin 1995; Dhingra and Hortobagy 1996).

While the biochemical reactions involved suggest that PTPs simply oppose PTK activities, PTPs can act both as positive and negative regulators of cellular responses (proliferation, protection against apoptosis). Cyclin dependent kinase (CDK) activity is required for cell cycle progression through the G1-S border and is subject to multiple levels of both positive and negative regulation (reviewed in Morgan 1995). Negative regulation is effected through association with members of the p21 or p16 CDK inhibitor proteins and by phosphorylation of conserved threonine and tyrosine residues found within the ATP binding fold of CDKs. Phosphorylation of these sites precludes ATP binding. Activation of CDKs, as the name implies, occurs through cyclin binding as well as dephosphorylation of these inhibitory phosphorylation sites by CDC25-related protein phosphatases A, B and C (Draetta and Eckstein 1997). In primary cells, CDC25 A and B cooperate with oncogenic Ras (V12) to induce transformation. Moreover, elevated CDC25B expression is detected in approximately one third of the primary breast tumours tested (Galaktionov *et al.* 1995). In contrast, PTEN, also known as MMAC1, was isolated as a candidate tumour suppressor gene mapping to human chromosome 10q22-23, a region identified by linkage analysis to be responsible for predisposition to a constellation of autosomally dominant hyperproliferative disorders (Nelen *et al.* 1996). The constellation of tumours include breast cancer and female carriers are at increased risk of subsequent breast cancer development. PTEN, like CDC25, is a dual specificity phosphatase (Myers *et al.* 1997). It is found deleted and mutated in derived tumour samples and where tested mutations impair or ablate catalytic activity (Li *et al.* 1997a; Liaw *et al.* 1997; Myers *et al.* 1997; Steck *et al.* 1997; Rhei *et al.* 1997). Thus, protein phosphatases appear to play both positive and negative roles in the etiology of breast cancers.

Given the potential importance of protein tyrosine phosphatases in regulating mammary epithelial proliferation, I attempted to clone and identify PTPs expressed in the mammary epithelium using a degenerate PCR approach. Here I describe the cloning and characterization of PTPM1, now termed Shp-1, as a mammary expressed PTP. Shp-1 (Matthews *et al.* 1992) (formally called PTP1C (Shen *et al.* 1991), SH-PTP1 (Plutzky *et al.* 1992), HCP (Yi *et al.* 1992)) is the founding member of the Src homology 2 (SH2) domain containing protein tyrosine phosphatase family which, in addition, contains the mammalian Shp-2

(formally termed PTP1D, Syp, SH-PTP2, SH-PTP3 and PTP2C), the *Xenopus XShp-2*, the *Drosophila corkscrew* and the *C. elegans PTP-2* genes (Freeman *et al.* 1992; Tang *et al.* 1995; Perkins *et al.* 1992; Gutch *et al.* 1998).

The available data thus far suggest that Shp-1 inhibits cellular signaling, as demonstrated for the erythropoietin (EPO) receptor Klingmuller *et al.* 1995), interferon α (INFα) receptor (David *et al.* 1995), T cell receptor (TCR) (Plas *et al.* 1996), interleukin-3 (IL-3) receptor (Yi *et al.* 1993) as well as responses from the c-Kit RTK (white spotting locus) (Lorenz *et al.* 1996; Paulson *et al.* 1996). Each of these responses is limited to hematopoietic cells, although Shp-1 expression is detected sporadically in a limited number of a variety of cell lines tested.

In this chapter, I demonstrate that Shp-1 is expressed in mammary epithelial cells where it becomes tyrosine phosphorylated and interacts with two tyrosine phosphorylated proteins. Elevated expression is observed in both murine and human primary breast tumours and expression of wild-type but not catalytically inactive Shp-1 inhibits transformation by constitutively active Neu/ErbB-2.

RESULTS

5.2 Isolation of several PTP partial cDNAs, PCR amplification of PTP partial cDNAs.

To clone PTPs expressed in the mouse mammary gland, partial PTP cDNAs were isolated by degenerate PCR cloning (Wilks *et al.* 1989) using degenerate oligonucleotides made to conserved regions in PTPs (Figure 5.1). RNAs derived from virgin though lactating mammary glands were reverse transcribed, subjected to PCR with these degenerate oligonucleotides and the corresponding PCR products were cloned into plasmids. Sequencing of 43 clones yielded six distinct PTP-encoding sequences, of which three clones (*ptpM1*, *M2* and *M3*) were novel at the time of isolation (Table 5.1). Specifically, *ptpM5*, and *ptpBr1* are identical to the amino-terminal PTP domains of transmembrane PTPs; leukocyte common antigen related proteins LAR (Streuli *et al.* 1988) and LRP (Matthews *et al.* 1990) respectively, while *ptpM4* displayed 94% identity with the human PTPζ (Krueger and Saito 1992), presumably representing the murine homolog of this PTP.

The three remaining clones, while being PTP-like, bore higher homology to existing PTPs than to one another and dendogram analyses suggested PTPM1 and M2 represented cytoplasmic PTPs while PTPM3 displayed more homology to the membrane spanning variety (Figure 5.1). Similar types of catalytic domain sequence analyses are predictive in protein kinase catalytic specificities and subfamily designations (Hanks *et al.* 1988; Hunter 1991). Subsequently, *ptpM2* has been cloned and it appears to have characteristics of a cytoplasmic PTP (MEG2) where its expression is found in a number of hematopoietic and epithelial cell lines (Gu *et al.* 1992). *ptpM3* displays high homology to a partial human cDNA of a predicted transmembrane PTP and is encoded by an approximately 15kB transcript (PTPty35 Yi *et al.* 1991) not yet been cloned in its entirety.

Figure 5.1 Oligonucleotide used for PCR amplification / Alignment of known PTP domains

Alignment of known PTP domains when experiments were initiated. Indicated are the oligonucleotides used for PCR amplification. In places of degeneracy nucleotides are listed vertically and N=G, A, T, and C incorporated at equal molecular ratios. D1 and D2 refer to the amino and carboxyl PTP domains of putative transmembrane PTPs. Mouse proteins are leukocyte common antigen (MLCA; Charbonneau *et al.* 1988), LCA related proteins, LAR (MLAR; Streuli *et al.* 1988) and LRP (MLRP; Matthews *et al.* 1990), the rat STEP gene (RSTEP; Lombroso *et al.* 1991), and the human PTP1B (HPTPB1; Charbonneau *et al.* 1988) and T cell PTPs (HTCPTP; Cool *et al.* 1989). DD PTPM2 is MEG2 (Gu *et al.* 1992) and DD PTPM3 and DD PTPM4 represent the mouse homologs of the human PTPty35 (Yi *et al.* 1991) and PTPζ (Krueger and Saito 1992) genes respectively. Human PTP1C (HPTP1C; Shen *et al.* 1991) is shown for comparison. Alignment was modified from (Streuli *et al.* 1989).

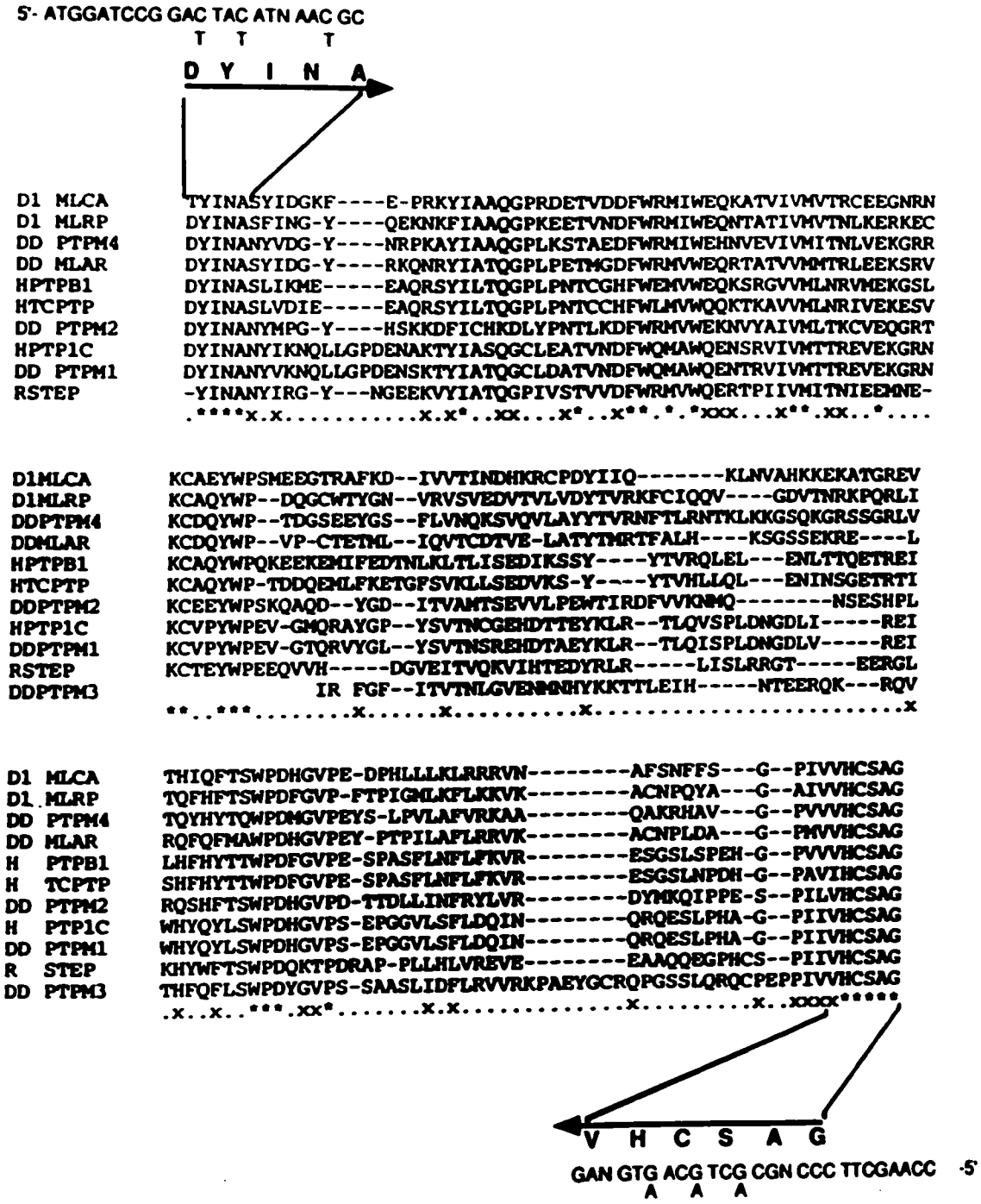

5'- ATGGATCCG GAC TAC ATN AAC GC
T T T
D Y I N A
D1 MLCA TYINASYIDGKF----E-PRKYIAAQGPRDETVDDFWRMIWEQKATVIVMVTRCEEGNRN
D1 MLRP DYINASFING-Y----QEKNKFIAAQGPKEETVNDFWRMIWEQNTATIVMVTNLKERKEC
DD PTPM4 DYINANYVDG-Y----NRPKAYIAAQGPLKSTAEDFWRMIWEHNVEVIVMITNLVEKGRR
DD MLAR DYINASYIDG-Y----RKQNRYIATQGPLPETMGDFWRMVWEQRTATVVMMTRLEEKSRV
HPTPB1 DYINASLIKME-----EAQRSYILTQGPLPNTCGHFWEMVWEQKSRGVVMLNRVMEKGSL
HTCPTP DYINASLVDIE-----EAQRSYILTQGPLPNTCCHFWLMVWQQKTKAVVMLNRIVEKESV
DD PTPM2 DYINANYMPG-Y----HSKKDFICHKDLYPNTLKDFWRMVWEKNVYAIVMLTKCVEQGRT
HPTP1C DYINANYIKNQLLGPDENAKTYIASQGCLEATVNDFWQMAWQENSRVIVMTTREVEKGRN
DD PTPM1 DYINANYVKNQLLGPDENSKTYIATQGCLDATVNDFWQMAWQENTRVIVMTTREVEKGRN
RSTEP -YINANYIRG-Y---NGEEKVYIATQGPIVSTVVDFWRMVWQERTPIIVMITNIEEMNE-
.****x.x.....x.x*..xx...x*..x**.*.*xxx...x**.xx..*....
D1MLCA KCAEYWPSMEEGTRAFKD--IVVTINDHKRCPDYIIQ-------KLNVAHKKEKATGREV
D1MLRP KCAQYWP--DQGCWTYGN--VRVSVEDVTVLVDYTVRKFCIQQV----GDVTNRKPQRLI
DDPTPM4 KCDQYWP--TDGSEEYGS--FLVNQKSVQVLAYYTVRNFTLRNTKLKKGSQKGRSSGRLV
DDMLAR KCDQYWP--VP-CTETML--IQVTCDTVE-LATYTMRTFALH----KSGSSEKRE----L
HPTPB1 KCAQYWPQKEEKEMIFEDTNLKLTLISEDIKSSY----YTVRQLEL---ENLTTQETREI
HTCPTP KCAQYWP-TDDQEMLFKETGFSVKLLSEDVKS-Y----YTVHLLQL---ENINSGETRTI
DDPTPM2 KCEEYWPSKQAQD--YGD--ITVAMTSEVVLPEWTIRDFVVKNMQ--------NSESHPL
HPTP1C KCVPYWPEV-GMQRAYGP--YSVTNCGEHDTTEYKLR--TLQVSPLDNGDLI-----REI
DDPTPM1 KCVPYWPEV-GTQRVYGL--YSVTNSREHDTAEYKLR--TLQISPLDNGDLV-----REI
RSTEP KCTEYWPEEQVVH-----DGVEITVQKVIHTEDYRLR-----LISLRRGT-----EERGL
DDPTPM3 IR FGF--ITVTNLGVENMNHYKKTTLEIH-----NTEERQK---RQV
..*.....x.....x.....x.....x
D1 MLCA THIQFTSWPDHGVPE-DPHLLLKLRRRVN--------AFSNFFS---G--PIVVHCSAG
D1 MLRP TQFHFTSWPDFGVP-FTPIGMLKFLKKVK--------ACNPQYA---G--AIVVHCSAG
DD PTPM4 TQYHYTQWPDMGVPEYS-LPVLAFVRKAA--------QAKRHAV---G--PVVVHCSAG
DD MLAR RQFQFMAWPDHGVPEY-PTPILAFLRRVK--------ACNPLDA---G--PMVVHCSAG
H PTPB1 LHFHYTTWPDFGVPE-SPASPLNFLFKVR--------ESGSLSPEH-G--PVVVHCSAG
H TCPTP SHFHYTTWPDFGVPE-SPASFLNFLFKVR--------ESGSLNPDH-G--PAVIHCSAG
DD PTPM2 RQSHFTSWPDHGVPD-TTDLLINFRYLVR--------DYMKQIPPE-S--PILVHCSAG
H PTP1C WHYQYLSWPDHGVPS-EPGGVLSFLDQIN--------QRQESLPHA-G--PIIVHCSAG
DD PTPM1 WHYQYLSWPDHGVPS-EPGGVLSFLDQIN--------QRQESLPHA-G--PIIVHCSAG
R STEP KHYWFTSWPDQKTPDRAP-PLLHLVREVE--------EAAQQEGPHCS--PIIVHCSAG
DD PTPM3 THFQFLSWPDYGVPS-SAASLIDFLRVVRKPAEYGCRQPGSSLQRQCPEPPIVVHCSAG
.x..x..***.xx*.....x.x.....x.....x..xxxx*****
V H C S A G
GAN GTG ACG TCG CGN CCC TTCGAACC 5'
A A A

Table 5.1 Protein tyrosine phosphatases isolated by RT-PCR

Clone	Gene Name	Number isolated	RNA Source[b]
ptpM1	Shp-1	35	Mammary: Virg., 4M, 16M
ptpM2	MEG2	1	Mammary: 4M
ptpM3	PTP-ty35	3	Mammary: 12M
			MMTV-Neu (202) BT
			Embryo: 16E
ptpM4	PTPζ[a]	1	MMTV-Neu (202) BT
ptpM5	LAR[a]	1	Mammary: 12M
ptpBr1	LRP[a]	2	MMTV-Neu (202) BT

[a] ptpM4, ptpM5 and ptpBr1 represent the amino terminal PTP domains of the indicated transmembrane phosphatases.

[b] Total RNA isolated from the 2nd 3rd and 4th mammary glands of virgin (virg.), day 4, 12 and 16 FVB mice are designated virg., 4M, 12M and 16M respectively and as was day 16 embryonic RNA (16E). Breast tumour (BT) RNA was isolated from a MMTV-neu (202) mouse.

5.3 RNA Expression pattern of cloned cDNAs

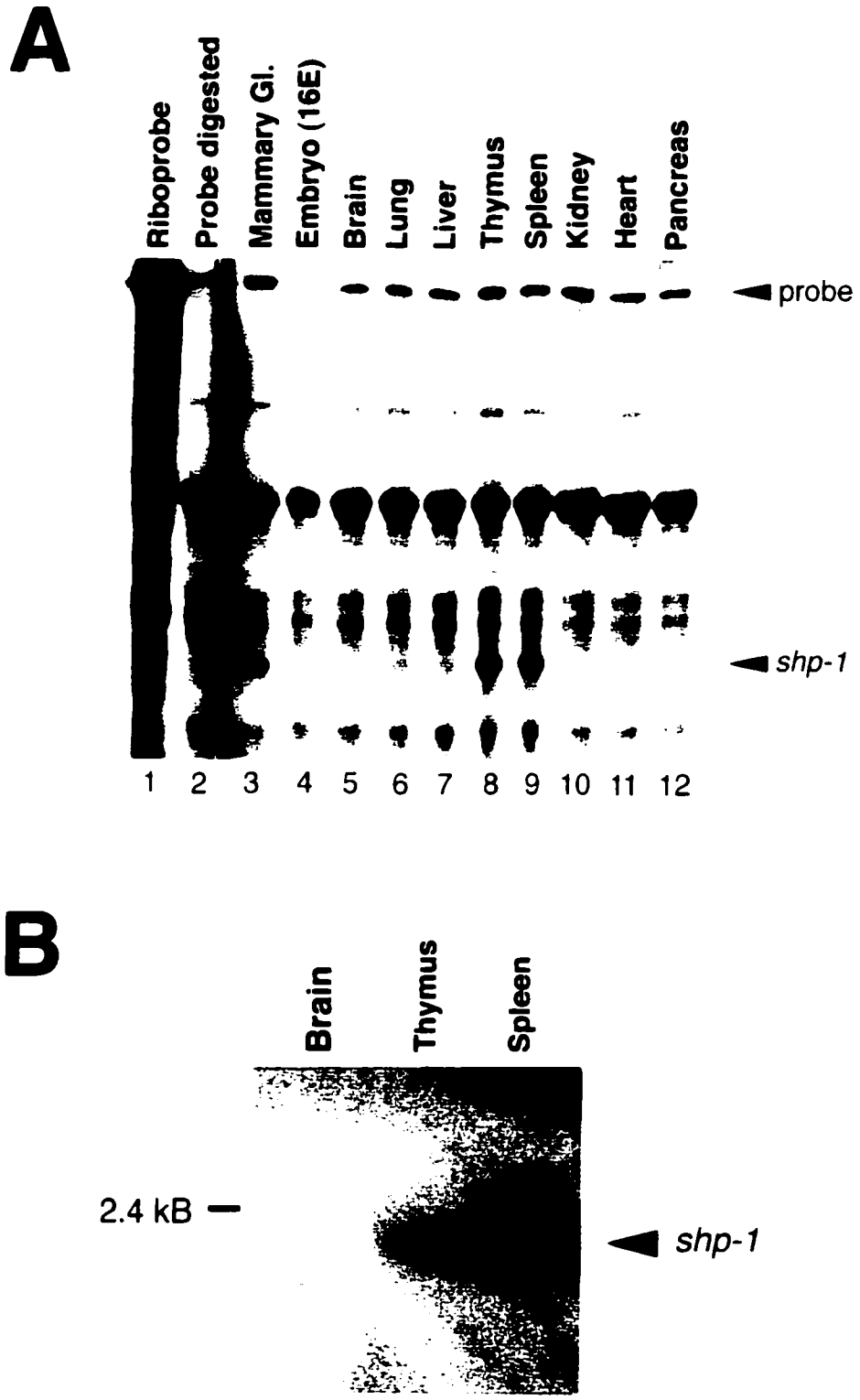
ptpM1 was repeatedly isolated from a variety of mammary derived RNA sources with degenerate oligonucleotides directed to different conserved regions of the PTP domain (data not shown), thus I examined the tissue specific expression of *ptpM1*, by performing RNAse protection analyses with *ptpM1* specific riboprobes. Consistent with the RT-PCR analyses, *ptpM1*, transcripts were readily detected in mammary gland derived RNAs (Figure 5.2A). In addition, high levels of *ptpM1* expression could be detected in spleen and thymus. Lower levels of *ptpM1*, could be detected in RNA isolated from the lung, kidney, and in day 16 embryos upon longer exposure of the autoradiographs (data not shown). Two lymphoid cell lines were tested for expression and *ptpM1* RNA was found in EL4, a T-cell line (data not shown). Northern blot analysis of thymus and spleen derived RNA suggested that the *ptpM1* transcript was 2.3Kb (Figure 5.2B). *ptpM2* and *ptpM3* had a less rigid tissue specificity than *ptpM1* displaying expression throughout many of the adult mouse tissues and in total embryos (Siegel, Dankort, and Muller; and data not shown).

5.4 Cloning of the cDNA: Isolation and sequence analysis of cDNA.

Because of its relatively narrow tissue distribution and novel sequence, I sought to isolate the full-length cDNA for *ptpM1*. Screening of a thymus cDNA library with the 400bp *ptpM1*-PCR product yielded two clones, A5 and B20, which were chosen for further sequence analysis. These two cDNAs harbour a 1785nt open reading frame encoding a 595 amino acid protein of predicted molecular weight 68 kDa (Figure 5.3A). There exists a near perfect mammalian translation initiation consensus sequence (AGGATGG: Kozak 1986) at nucleotide 196 preceded by three in frame stop codons. The other two reading frames are closed by multiple stop codons. This methionine is preferentially use by the translation machinery *in vitro* over the next encoded in frame methionine at nucleotide 707 (GTTATGT) as demonstrated with translation of truncated cDNAs (Figure 5.3B). The open reading frame is followed by three in frame stop codons, a polyadenylation signal sequence (AAUAAA) at nt2184, as well as a 27

Figure 5.2 RNA expression profile of *ptpm1*

(A) 20ug of total cellular RNA from the indicated tissues or cells was subjected to RNAse protection analysis using a riboprobe directed to nucleotides 1096-1236 of the *ptpm1* PTP domain. The intact riboprobe migrates at 506 nucleotides (probe) while the 140 nucleotide protected fragment is indicated by the arrowhead (*shp-1*). No RNA (probe) or yeast tRNA were included as controls for probe integrity and specificity respectively. **(B)** 2ug of polyA selected RNA from the indicated murine tissues was electrophoresed through a formaldehyde/agarose gel, transferred to a GeneScreen membrane and probed with a ^{32}P-labeled *ptpm1*-specific probe. The message size was interpolated from RNA molecular weight standards.


A
Riboprobe
Probe digested
Mammary Gl.
Embryo (16E)
Brain
Lung
Liver
Thymus
Spleen
Kidney
Heart
Pancreas
probe
shp-1
1 2 3 4 5 6 7 8 9 10 11 12
B
Brain
Thymus
Spleen
2.4 kB
shp-1

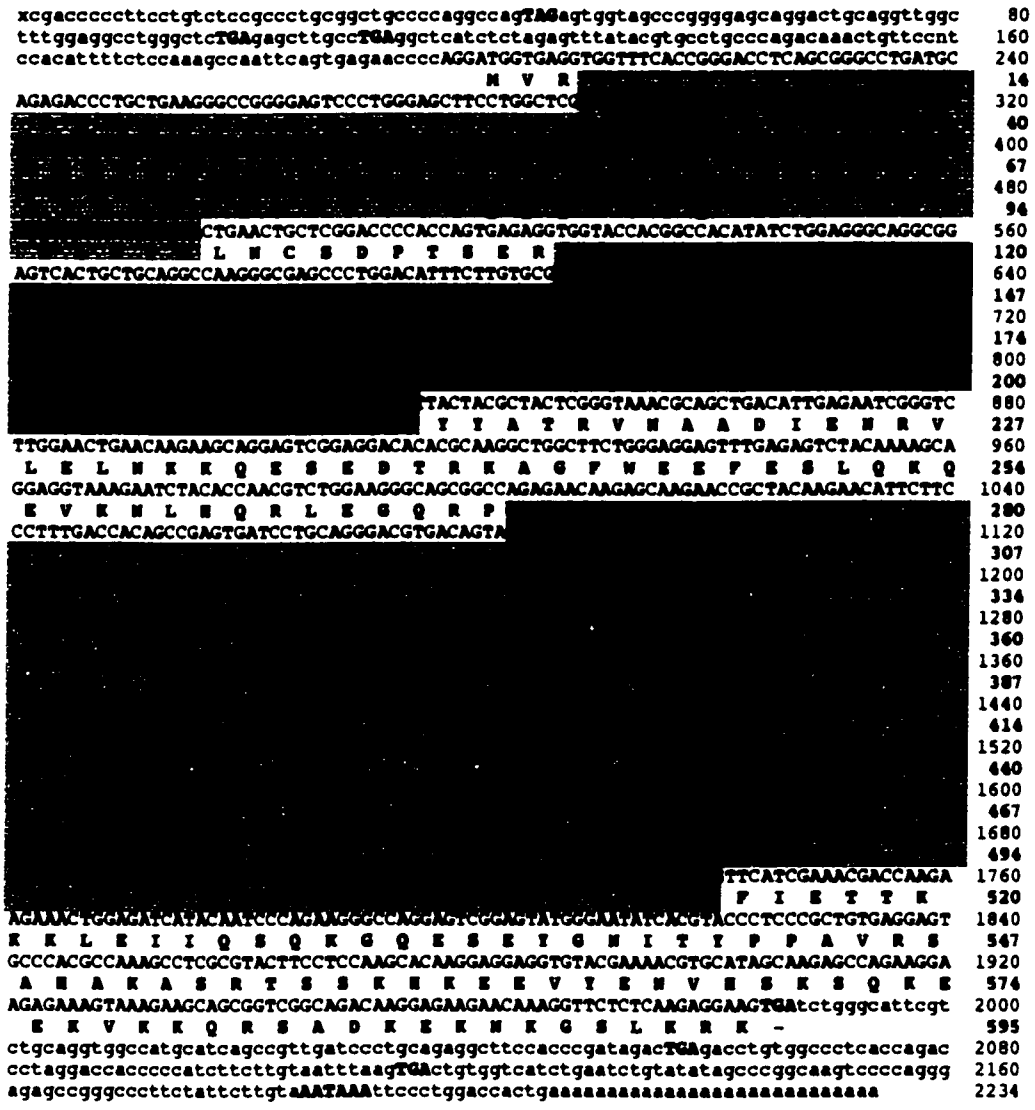
nucleotide poly-adenosine tract. The fact that this open reading frame is flanked by in frame stop codons, followed by a poly-adenosine tract, and is roughly the same size as the *ptpM1* transcript detected by northern blot hybridization analysis suggests that the entire *ptpM1* cDNA was isolated.

Sequence analysis demonstrates that the catalytic domain of *ptpM1* is identical to that of the PCR product. Examination of the amino acid sequence of PTPM1 revealed that, in addition to a single PTP domain, there exist two tandem regions of internal homology similar to the SH2 domain (Sadowski *et al.* 1986). Further inspection reveals no hydrophobic regions suggesting that PTPM1 is not a membrane spanning PTP but is, as the PTP domain alignment suggests, a cytoplasmic protein. *ptpM1* encodes a protein consisting largely of two amino-terminal SH2 domains separated by 10 amino acids, followed by a 57 amino acid "linker" region possessing little homology to previous described proteins, a 246 amino acid PTP domain, and an 81 amino acid highly charged carboxy-terminus (Figure 5.3A). Comparison to known SH2 domains demonstrates that SH2 domains possess similar (55%) homology to each another as well as to the COOH-terminal SH2 domain of the Ras-GAP protein and the SH2 domain of Nck (52% and 51%) (Figure 5.3C). The presence of a small hydrophobic residue at the SH2 βD5 position (defined by Waksman *et al.* 1993) of each SH2 domain suggest they are class III SH2 domains. Hydrophobic or small residues at βD5 allow class III, but not class I SH2 domains to form an elongated hydrophobic phosphopeptide binding pockets capable of interacting with an extended recognition motif for hydrophobic residues while class I SH2 domains recognize polar residues in the +1, +2 or +3 positions (Waksman *et al.* 1993; Songyang *et al.* 1993). At the time of isolation, these structural motifs suggested that PTPM1 represented the first of a new class of SH2-containing proteins. While novel at the time of isolation of the cDNA, this gene has been cloned by several groups (PTP1C Shen *et al.* 1991; Shp Matthews *et al.* 1992; SH-PTP1 Plutzky *et al.* 1992; HCP Yi *et al.* 1992) and has more recently been termed Shp-1. Henceforth PTPM1 will be referred to as Shp-1.

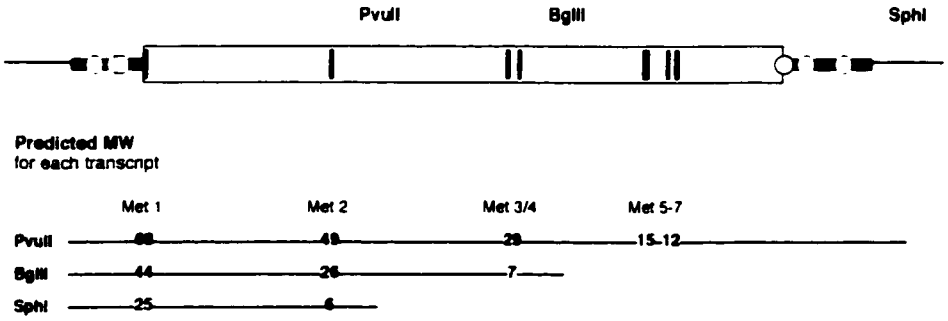
Figure 5.3 Sequence, SH2 alignment and *in vitro translation* of *ptpm1* cDNA

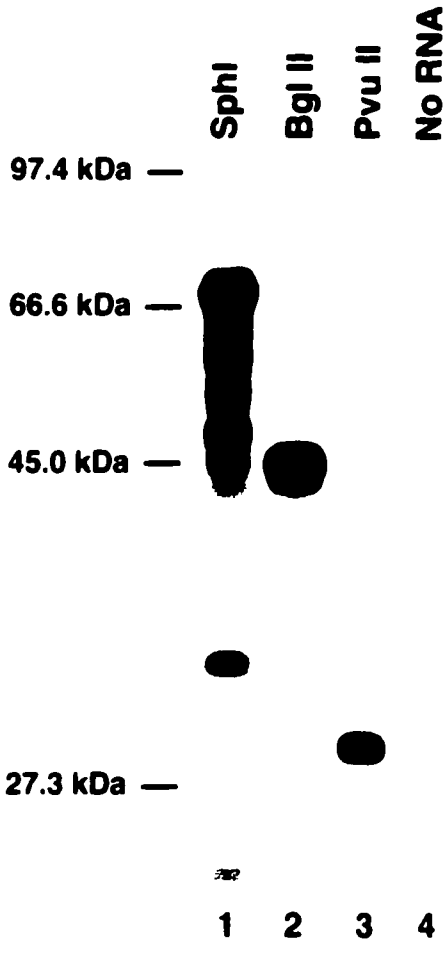
(A) Two independent overlapping clones (A5, B20) were sequenced in their entirety and correspond to nucleotides 1-2225 and 121-2234 respectively. An open reading frame of 595 amino acids (single residue designation positioned below corresponding codon) beginning at a translation initiation consensus sequence. This open reading frame is preceded and followed by in frame stop codons and a poly-adenylation sequence (bold and capitalized): the other reading frames are closed. The SH2 and PTP domains are boxed and the protein is schematized to scale below. **(B)** The cDNA represented by the horizontal bold line and open reading frame is schematized as a box flanked by in frame stop codons (circles). Each methionine within the ORF is indicated by a bold vertical line. Three SP6 RNA polymerase-driven RNA transcripts were made terminating at either an *Sph*I site outside the cDNA or internal *Bgl*II and *Pvu*II sites as indicated. The predicated molecular weights of the polypeptides produced from each methionine are positioned on the each transcript below the corresponding methionine (numbered 1 through 7). The diagrammed RNAs were subjected to *in vitro* translation in the presence of ^{35}S-methionine. Equivalent volumes of the translated products were electrophoresed through a SDS-10%-PAG and visualized by autoradiography. Molecular weight standards indicate size. **(C)** Alignment of SH2 domains using CLUSTAL alignment program and subsequently refined manually. Residues which make contact with phosphotyrosine from the Src /Abl SH2 crystal structures are indicated with an asterisk below the alignment. Parameters were K-Tuple=1, gap penalty=10, window size=10, filtering level=10, open gap and unit gap cost=10. The suffix N or C refers to amino and carboxyl SH2 domains in proteins containing two SH2 domains. The proteins are positioned by relatedness in that Abl and Csk SH2 domains are more closely related than Abl and Shc SH2 domain.

A

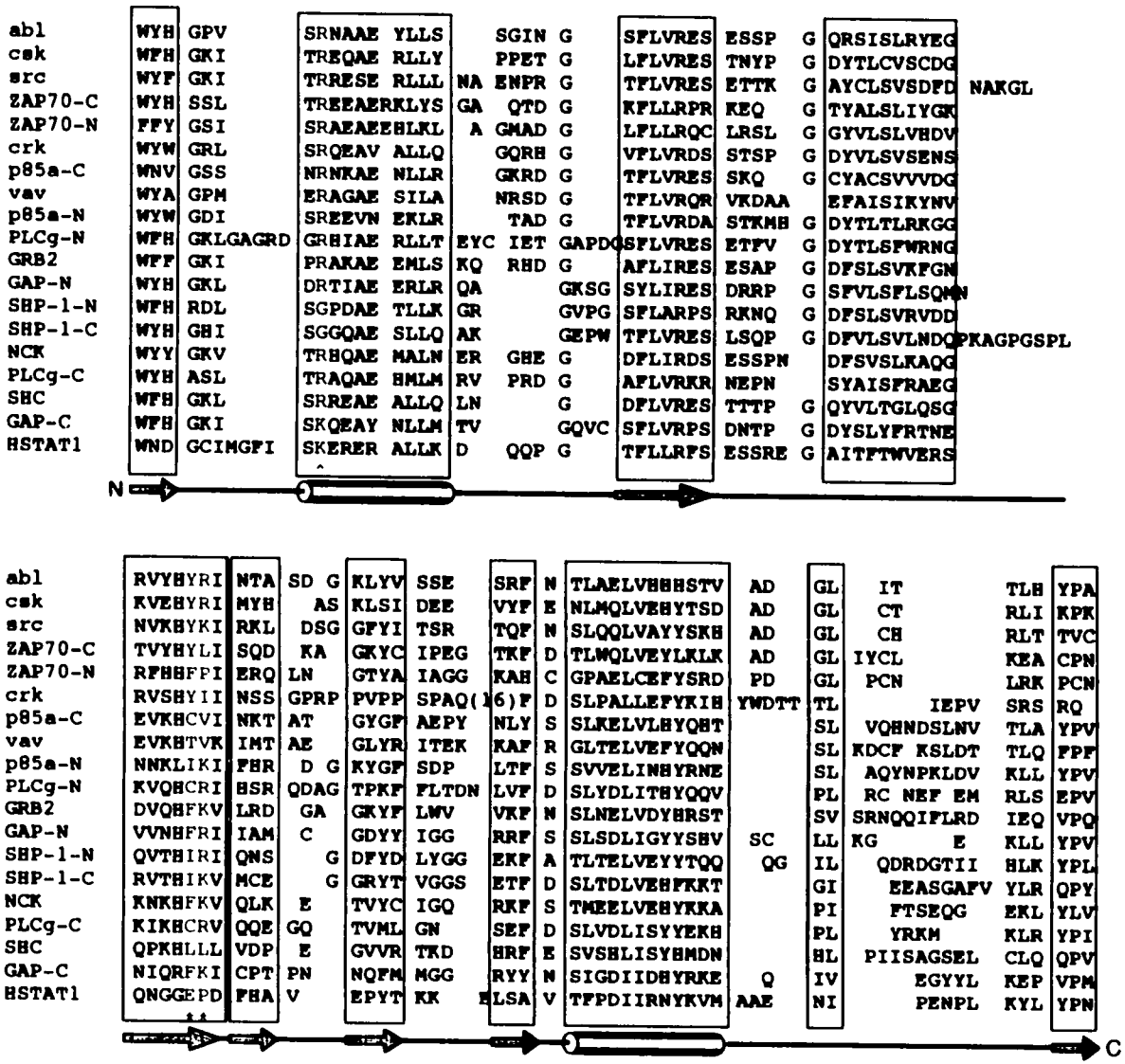


B


PvuII
BglII
SphI
Predicted MW
for each transcript
Met 1
Met 2
Met 3/4
Met 5-7
PvuII
BglII
SphI
44
25
15-12
7
6


SphI
Bgl II
Pvu II
No RNA
97.4 kDa
66.6 kDa
45.0 kDa
27.3 kDa
1
2
3
4

C


abl WYH GPV SRNAAE YLLS SGIN G SFLVRES ESSP G QRSISLRYEG
csk WFH GKI TREQAE RLLY PPET G LFLVRES TNYP G DYTLCVSCDG
src WYF GKI TRRESE RLLL NA ENPR G TFLVRES ETTK G AYCLSVSDFD NAKGL
ZAP70-C WYH SSL TREEAERKLYS GA QTD G KFLLRPR KEQ G TYALSLIYGK
ZAP70-N FFY GSI SRAEAEEHLKL A GMAD G LFLLRQC LRSL G GYVLSLVHDV
crk WYW GRL SRQEAV ALLQ GQRH G VFLVRDS STSP G DYVLSVSENS
p85a-C WNV GSS NRNKAE NLLR GKRD G TFLVRES SKQ G CYACSVVVDG
vav WYA GPM ERAGAE SILA NRSD G TFLVRQR VKDAA EFAISIKYNV
p85a-N WYW GDI SREEVN EKLR TAD G TFLVRDA STKMH G DYTLTLRKGG
PLCg-N WFH GKLGAGRD GRHIAE RLLT EYC IET GAPDQ SFLVRES ETFV G DYTLSFWRNG
GRB2 WFF GKI PRAKAE EMLS KQ RHD G AFLIRES ESAP G DFSLSVKFGN
GAP-N WYH GKL DRTIAE ERLR QA GKSG SYLIRES DRRP G SFVLSFLSQHN
SHP-1-N WFH RDL SGPDAE TLLK GR GVPG SFLARPS RKNQ G DFSLSVRVDD
SHP-1-C WYH GHI SGGQAE SLLQ AK GEPW TFLVRES LSQP G DFVLSVLNDQPKAGPGSPL
NCK WYY GKV TRHQAE MALN ER GHE G DFLIRDS ESSPN DFSVSLKAQG
PLCg-C WYH ASL TRAQAE HMLM RV PRD G AFLVRKR NEPN SYAISFRAEG
SHC WFH GKL SRREAE ALLQ LN G DFLVRES TTTP G QYVLTGLQSG
GAP-C WFH GKI SKQEAY NLLM TV GQVC SFLVRPS DNTP G DYSLYFRTNE
HSTAT1 WND GCIMGFI SKERER ALLK D QQP G TFLLRFS ESSRE G AITFTWVERS
N
abl RVYHYRI NTA SD G KLYV SSE SRF N TLAELVHHHSTV AD GL IT TLH YPA
csk KVEHYRI MYH AS KLSI DEE VYF E NLMQLVEHYTSD AD GL CT RLI KPK
src NVKHYKI RKL DSG GFYI TSR TQF N SLQQLVAYYSKH AD GL CH RLT TVC
ZAP70-C TVYHYLI SQD KA GKYC IPEG TKF D TLWQLVEYLKLK AD GL IYCL KEA CPN
ZAP70-N RFHHFPI ERQ LN GTYA IAGG KAH C GPAELCEFYSRD PD GL PCN LRK PCN
crk RVSHYII NSS GPRP PVPP SPAQ(16)F D SLPALLEFYKIH YWDTT TL IEPV SRS RQ
p85a-C EVKHCVI NKT AT GYGF AEPY NLY S SLKELVLHYQHT SL VQHNDSLNV TLA YPV
vav EVKHTVK IMT AE GLYR ITEK KAF R GLTELVEFYQQN SL KDCF KSLDT TLQ FPF
p85a-N NNKLIKI FHR D G KYGF SDP LTF S SVVELINHYRNE SL AQYNPKLDV KLL YPV
PLCg-N KVQHCRI HSR QDAG TPKF FLTDN LVF D SLYDLITHYQQV PL RC NEF EM RLS EPV
GRB2 DVQHFKV LRD GA GKYF LWV VKF N SLNELVDYHRST SV SRNQQIFLRD IEQ VPQ
GAP-N VVNHFRI IAM C GDYY IGG RRF S SLSDLIGYYSHV SC LL KG E KLL YPV
SHP-1-N QVTHIRI QNS G DFYD LYGG EKF A TLTELVEYYTQQ QG IL QDRDGTII HLK YPL
SHP-1-C RVTHIKV MCE G GRYT VGGS ETF D SLTDLVEHFKRT GI EEASGAFV YLR QPY
NCK KNKHFKV QLK E TVYC IGQ RKF S TMEELVEHYKKA PI FTSEQG EKL YLV
PLCg-C KIKHCRV QQE GQ TVML GN SEF D SLVDLISYYEKH PL YRKM KLR YPI
SHC QPKHLLL VDP E GVVR TKD HRF E SVSHLISYHMDN HL PIISAGSEL CLQ QPV
GAP-C NIQRFKI CPT PN NQFM MGG RYY N SIGDIIDHYRKE Q IV EGYYL KEP VPM
HSTAT1 QNGGEPD FHA V EPYT KK ELSA V TFPDIIRNYKVM AAE NI PENPL KYL YPN
C

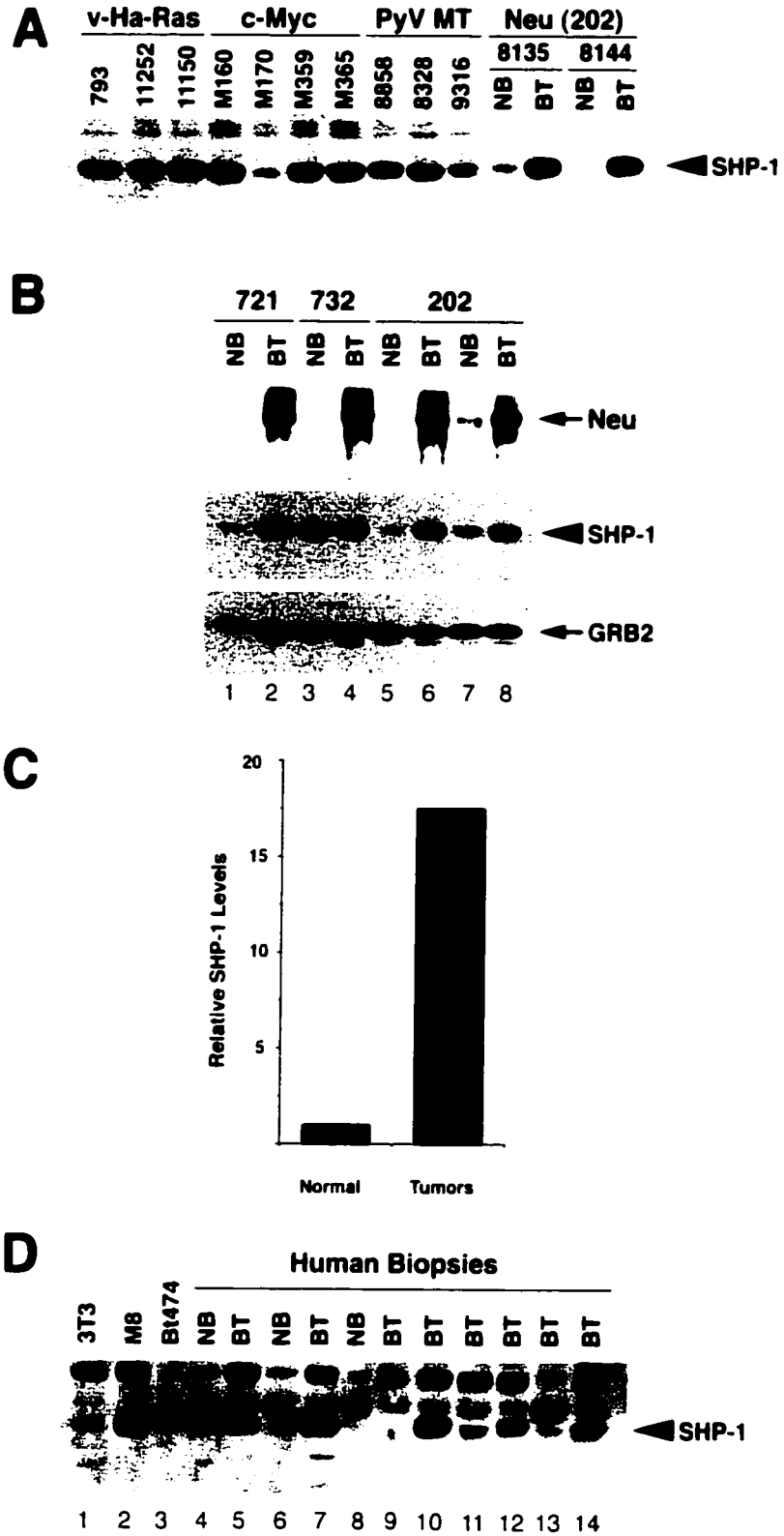
5.5 Mammary gland specific expression of Shp-1

5.5.1 Shp-1 expression is elevated in tumours of mammary origin

The presence of *shp-1* transcripts in the mammary gland was intriguing given our long standing interest in understanding the molecular events leading to mammary carcinoma formation. To further explore the significance of mammary epithelial specific expression of Shp-1 in mammary tumorigenesis, I assessed whether Shp-1 levels were altered during mammary tumor progression. To this end, I performed immunoblot analyses with Shp-1 specific antibodies on mammary tumors derived from several transgenic strains of mice which develop heritable mammary tumours due to the directed expression of c-Myc, v-Ha-Ras, Neu or the Polyomavirus middle T antigen oncogene products (reviewed in Dankort and Muller 1996). Elevated levels of Shp-1 were detected in the majority of tumours isolated from these mice (Figure 5.4A). Interestingly analyses of MMTV-Neu transgenic mice further demonstrated that in contrast to the Neu-induced tumors, which expressed elevated levels of Shp-1, the adjacent morphologically normal tissue did not (Figure 5.4A). To confirm that elevated Shp-1 levels were not confined to single MMTV-neu transgenic strain, additional matched normal and tumor mammary samples from three independent strains of MMTV-neu mice were analyzed for Shp-1 expression. Shp-1 levels were elevated in the tumours of 31 of 35 mice, compared to the adjacent epithelium (Figure 5.4B and not shown) and as quantitated by ^{125}I-conjugated secondary antibodies tumours possessed significantly higher levels of Shp-1 (17.5 fold) releative to the adjacent morphological normal epithelium (n=19)(Figure 5.4C). This difference in Shp-1 levels was not due to differences in protein loading since all the samples possessed comparable levels of Grb2 protein (Figure 5.4B). Elevated Shp-1 expression was also detected in 9 of 15 samples human breast tumour biopsies (Figure 5.4D and data not shown). In this small sample size, there was no correlation between the Shp-1 levels and the expression of ErbB-2, ErbB-1, progesterone or estrogen receptors, nor was there a correlation with disease pathology (data not shown). Taken together these observations suggest that Shp-1 levels are elevated in a large percentage of human and murine breast tumours.

Figure 5.4 Shp-1 expression is elevated mammary tumours

(**A**) 25ug of mammary tumour cell lysates derived from the indicated MMTV-transgenic mice was subjected to anti-Shp-1 immunoblot analysis. BT and NB refer to breast tumour and the macroscopically normal surrounding breast tissue derived from the same animal respectively. (**B**) Protein lysates were analyzed from three independent strains of MMTV mice (Guy *et al.* 1992) for Neu, Grb2 and Shp-1 expression as indicated. (C) Lysates of 19 matched samples from 16 MMTV-Neu mice (strain 202) were electrophoresed through 6-12% gradient SDS-polyacrylamide gels and transferred to a PVDF membrane. The membrane was cut in half and the top half containing proteins larger than 45kDa were analyzed for Shp-1 expression while the bottom portion of the blot was probed with Grb2-specific rabbit polyclonal antisera. The amount of each protein was quantified using ^{125}I-conjugated anti-rabbit secondary antibodies by PhosphoImager analysis. The fold increase of Shp-1 expression in tumours relative to adjacent normal tissue was calculated using Grb2 levels as a protein control as Grb2 levels accurately reflect protein levels in this system (M. Rauh and W.J. Muller unpublished results). Depicted is mean fold increase in Shp-1 expression. The mean was 17.5 with a standard error of 5.3 SEM (not shown). This difference is statistically significant to a p value <0.007 by a Student's T test. (**D**) Shp-1 expression was evaluated as in (A) from 11 human breast biopsies. Morphologically normal tissue (NB) samples were obtained for three of the eight depicted breast tumour samples and are loaded to the left of the matched BT. BT474 is a human mammary epithelial cell line.


A
v-Ha-Ras
c-Myc
PyV MT
Neu (202)
8135
8144
793
11252
11150
M160
M170
M359
M365
8858
8328
9316
NB
BT
NB
BT
SHP-1
B
721
732
202
NB
BT
NB
BT
NB
BT
NB
BT
Neu
SHP-1
GRB2
1 2 3 4 5 6 7 8
C
Relative SHP-1 Levels
20
15
10
5
Normal
Tumors
D
Human Biopsies
3T3
M8
Bt474
NB
BT
NB
BT
NB
BT
BT
BT
BT
BT
BT
SHP-1
1 2 3 4 5 6 7 8 9 10 11 12 13 14

5.5.2 Expression in cell lines derived from murine and human mammary tumors

shp-1 is expressed in most hematopoietic cell lineages including myeloid and lymphoid lineages, but is not detected in a variety of other cell types leading to the assertion that *shp-1* is a hematopoietically-restricted gene (Yi *et al.* 1991; Matthews *et al.* 1992). Because Shp-1 is abundantly expressed in the most cells of hematopoietic origin, it is possible that the mammary specific expression I observed was due to contaminating lymph nodes resident in the isolated mammary glands. Additionally, infiltrating macrophages and lymphocytes could explain the tumour specific elevation of Shp-1 observed. To eliminate these possibilities, I screened several mammary tumour derived epithelial cell lines by immunoblot analysis with Shp-1 specific antisera. A protein with an approximate 68kDa was detected in each murine-epithelial cell line that comigrated with Shp-1 expressed from the mouse cDNA in NIH-3T3 cells (Figure 5.5). As previously reported, no Shp-1 immunoreactivity was observed in the parental NIH 3T3 cells. Human Shp-1 is consistently retarded in its migration but was detected in the tested human mammary tumour cell lines (Bt474, MCF7, SkBR3, T47D) requiring longer exposures to reveal expression in T47D as is the case in murine C127 cells (Figure 5.5 lower panel). The identity of Shp-1 in these mammary epithelial cell lines has been confirmed by competition with the immunizing peptide and the use of three independent antisera directed to different portions of Shp-1 (data not shown). These data argue that observed expression of Shp-1 is epithelial cell specific.

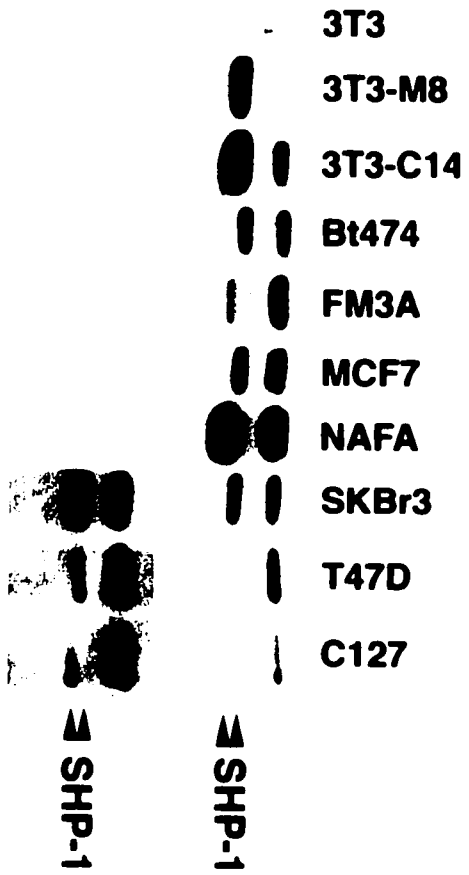
5.6 Shp-1 has protein tyrosine phosphatase activity

5.6.1 Bacterial-produced Shp-1 possesses PTP activity *in vitro*.

To demonstrate that *shp-1* encodes a protein with PTP activity, the *shp-1* open reading frame was fused in frame to glutathione S-transferase (GST). Bacteria have no PTK or PTP activities, thus PTP assays were carried out using total protein extracts derived from independent bacterial isolates containing recombinant GST-Shp1 as well as those harbouring GST alone. Raytide peptide and myelin basic protein

Figure 5.5 Shp-1 is expressed in cells of mammary epithelial origin

35ug of total cellular protein was electrophoresed on a SDS-7.5% polyacrylamide gel, and transferred to a PVDF membrane. The membrane was probed with Shp-1-specific rabbit polyclonal sera. Shp-1 expression in human (Bt474, MCF7, SkBR3, T47D) and rodent (FM3A, NAF, C127) mammary derived lines is indicated. NIH-3T3 fibroblasts expressing the mouse Shp-1 cDNA are indicated as 3T3-M8 and 3T3-C14. Lower panel represents a longer exposure of a portion of the same immunoblot.


3T3
3T3-M8
3T3-C14
Bt474
FM3A
MCF7
NAFA
SKBr3
T47D
C127
SHP-1
SHP-1

(MBP) were used as exogenous substrates following phosphorylation on tyrosine *in vitro* with purified $pp60^{c\text{-}src}$ and $^{32}P\gamma$-ATP. The use of purified Src ensures that phosphorylation takes place specifically on tyrosine residues. Incubation of the ^{32}P-Tyr containing substrates with the GST-Shp1 but not GST extracts caused the release of acid soluble $^{32}PO_4$, demonstrating that this protein harbours phosphatase activity *in vitro* (Figure 5.6A). A time- and dose- dependent linear response was observed in reactions containing GST-Shp1 but not GST alone using the phosphotyrosine-analog, p-nitrophenol phosphate (pNPP), as a substrate in PTP assays (Figure 5.6B). While pNPP is not a physiologic substrate of PTPs, its conversion to nitrophenol phosphate correlates well with PTP activity at the pH used (Tonks *et al.* 1988).

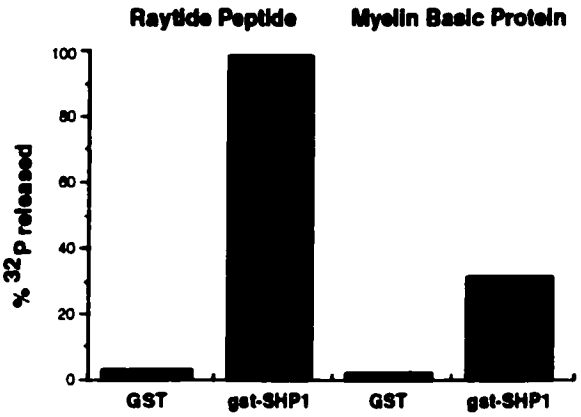
5.6.2 Shp-1 requires cysteine 453 for catalytic activity *in vitro*.

An invariant cysteine residue found in all PTP domains is required for catalytic activity because it functions as a nucleophile attacking the phosphorous atom in the dephosphorylation reaction (Barford *et al.* 1994 reviewed in Denu *et al.* 1997). It also appears to directly participate in the formation of an enzyme-substrate complex forming a phospho-cysteine catalytic intermediate (Pot and Dixon 1992). Mutation of this residue results in loss of catalytic activity of PTPs (Streuli *et al.* 1989). PCR was used mutate the conserved cysteine (amino acid 453) to a serine producing Shp-1 C453S (Shp-1^{C453S}). The Shp-1^{C453S} cDNA was subcloned, along with the wild-type cDNA, into the expression vector pJ4Ω and were transfected along with pSV2neo into NIH-3T3 fibroblasts. Stable lines expressing either Shp-1 or Shp-1^{C453S} were isolated (data not shown). Shp-1 immunoprecipitates derived from these cell lines were subjected to PTP assays using pNPP as a substrate. NIH-3T3 cells contain little, if any, immunoprecipitable Shp-1 PTP activity. However in all cell lines expressing wild-type Shp-1, pNPPase activity roughly correlates with the level of expression (Figure 5.7A and B). Pre-incubation of Shp-1 peptide antisera with the non-conjugated immunizing peptide not only abolishes immunoprecipitation of Shp-1, but also PTP activity, demonstrating that the PTP activity observed is in fact

Figure 5.6 Bacterially produced Shp-1 possesses protein tyrosine phosphatase and pNPPase activity *in vitro* .

The Shp-1 open reading frame was cloned in frame into pGEX-3X and expressed as a 92kDa GST-Shp1 fusion protein in XL1-Blue bacteria upon IPTG induction. PTP activity in total bacterial extracts were determined. (**A**) Raytide peptide and myelin basic protein were phosphorylated *in vitro* with $^{32}P\gamma$-ATP and $pp60^{c\text{-}src}$ prior to adding equivalent amounts of duplicate bacterial extracts containing GST or GST-Shp1. Following a 5 minutes incubation, acid soluble ^{32}P was quantitated and represented as the percentage of ^{32}P released relative to a blank. The percentage of ^{32}P-release from the Raytide peptide and myelin basic protein was 3.1 and 2.1 for GST and 98.6 and 31.7 for GST-Shp1 respectively. (**B**) Independent bacterial protein extracts were incubated with p-nitrophenol phosphate (pNPP) for the indicated time and the reaction was terminated at the indicated time. The amount of extract and incubation time were 150ug and 60 minutes respectively, unless otherwise indicated. The amount of dephosphorylation was measured by the absorbance at 410nm. The assay is representative of two independent experiments.

A


Raytide Peptide
Myelin Basic Protein
% 32P released
100
80
60
40
20
0
GST
gst-SHP1
GST
gst-SHP1

B

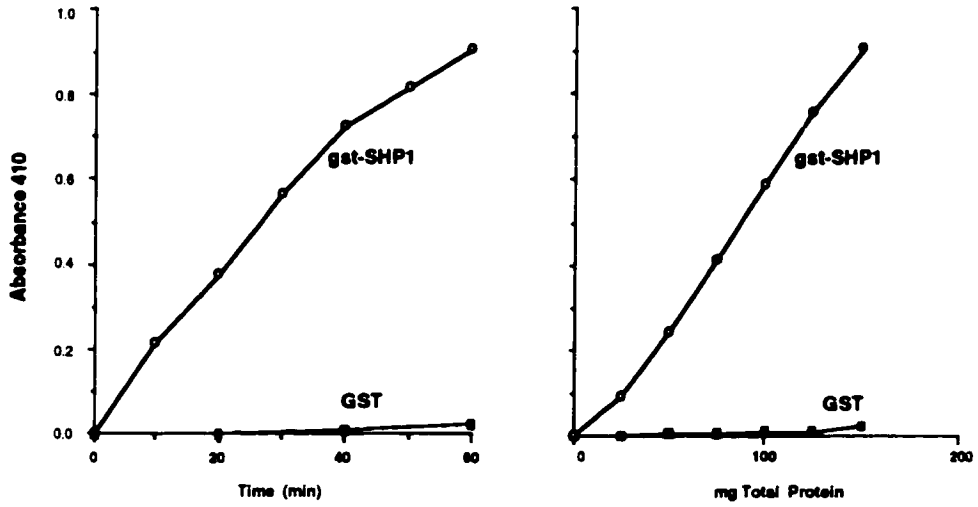
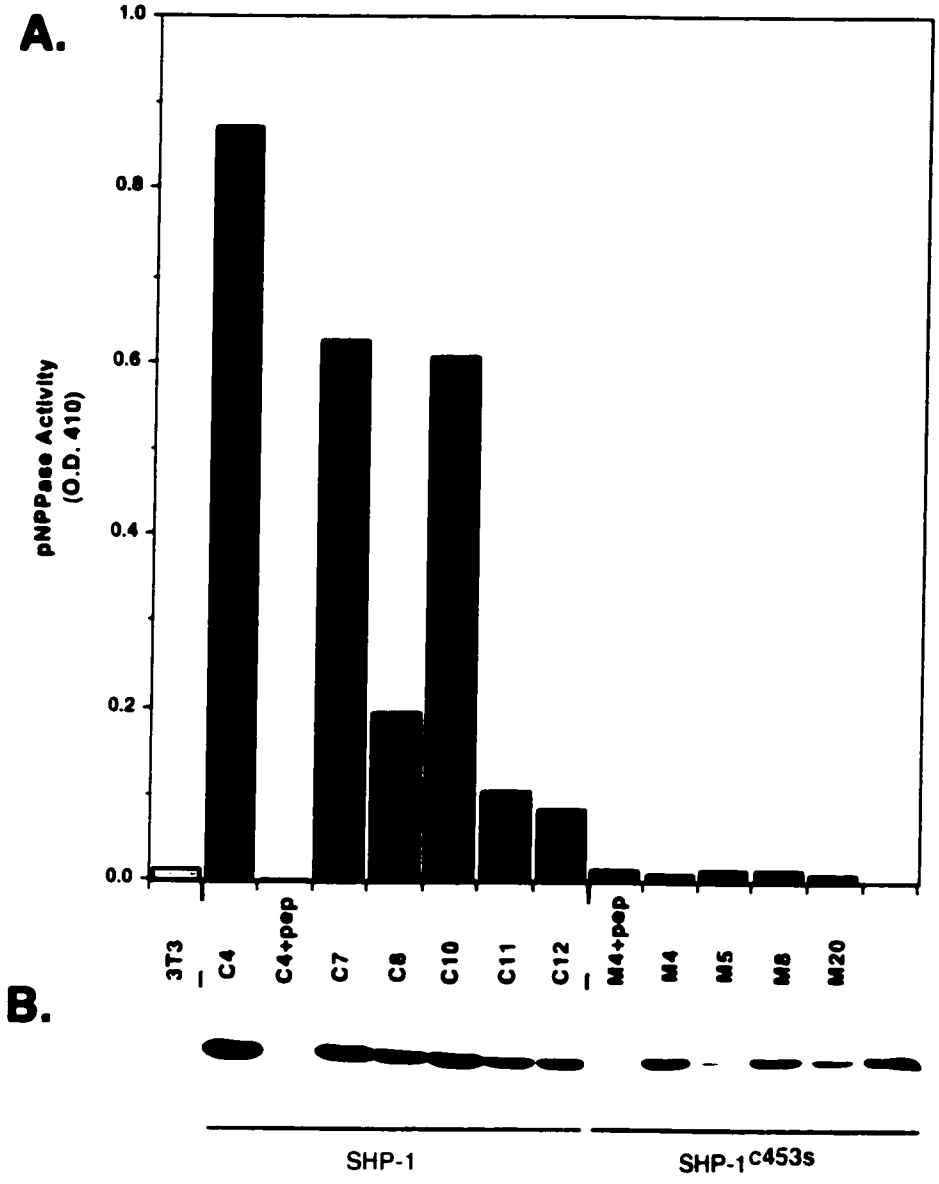

Absorbance 410
1.0
0.8
0.6
0.4
0.2
0.0
gst-SHP1
GST
0
20
40
60
Time (min)
gst-SHP1
GST
0
100
200
mg Total Protein

Figure 5.7 Cysteine 453 is required for Shp-1 catalytic activity *in vivo*

Shp-1 was immunoprecipitated with Shp-1-specific peptide antisera from the indicated NIH-3T3-derived fibroblast lines expressing either wild-type or C453S Shp-1. Peptide (1ug) was included in the indicated immunoprecipitates (+pep). **(A)** Half of each immunoprecipitate was incubated with pNNP for 45min. The reaction was stopped and conversion of pNNP to NPP was quantified by absorbance at 410nm. These data are representative of three independent experiments. **(B)** Shp-1 levels in the remaining portion of each immunoprecipitate was assessed by immunoblots with Shp-1-specific antisera (AV-2).


A.
1.0
0.8
0.6
0.4
0.2
0.0
pNPPase Activity
(O.D. 410)
3T3
C4
C4+pep
C7
C8
C10
C11
C12
M4+pep
M4
M5
M8
M20
B.
SHP-1
SHP-1C453S

Shp-1 specific. Moreover, although similar levels of the Shp-1^{C453S} mutant were detected in several lines (compare Shp-1 levels of C8, C11 and C12 to those of M4, M8 and M21), these immunoprecipitates had no tyrosine phosphatase activity (Figure 5.7A). Taken together, these data confirm that the cloned *shp-1* gene does encode a protein phosphatase and that C453S mutation indeed ablates catalytic activity of Shp-1.

5.7 Shp-1 is tyrosine phosphorylated and interacts with several tyrosyl phosphoproteins

5.7.1 Shp-1 is tyrosine phosphorylated in response to growth factor activation

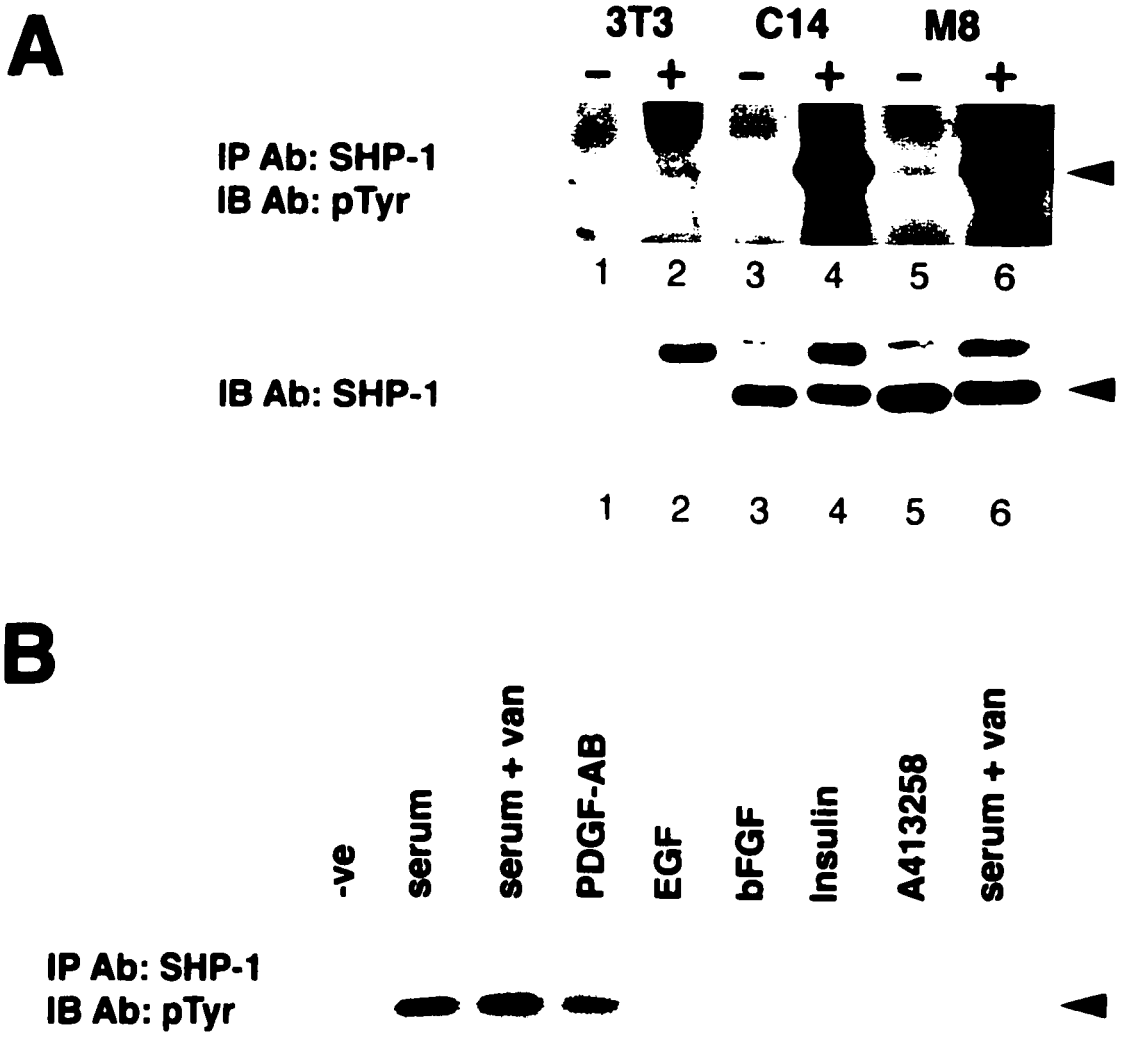
SH2 containing proteins are often RTK substrates becoming rapidly tyrosine phosphorylated following growth factor stimulation (Sections 1.3 and 1.4 and Chapter 3). Indeed, Shc, PLCγ1, Ras-GAP, and the PI3'K p85 become tyrosine phosphorylated after serum-deprived fibroblasts are stimulated with serum. To determine if Shp-1 was tyrosine phosphorylated, serum deprived NIH-3T3-derived fibroblasts were stimulated with serum and treated with vanadate to inhibit PTP activity and Shp-1 immunoprecipitates were subjected to phosphotyrosine immunoblot analysis. In these quiescent cells, Shp-1 and the Shp-1^{C453S} mutant were not appreciably tyrosine phosphorylated, yet upon serum stimulation, Shp-1 became tyrosine phosphorylated (Figure 5.8A and B). In several experiments, Shp-1^{C453S}, but not wild-type Shp-1, was tyrosine phosphorylated in serum-deprived cells, suggesting Shp-1 can auto-dephosphorylate (data not shown). Additional experiments demonstrated that Shp-1 phosphorylation can be detected at the earliest time points analyzed (5min). Growth factor treatment reveals that Shp-1 is phosphorylated in response to PDGF stimulation but not to EGF treatment in 3T3-derived lines (Figure 5.10C). Thus Shp-1 is tyrosine phosphorylated in response to growth factor addition.

5.7.2 Shp-1 associates with several tyrosine phosphorylated proteins in response to serum stimulation.

Shp-1 physically and functionally interacts with several hematopoietic expressed transmembrane proteins such as the c-KIT RTK (Yi and Ihle 1993; Lorenz *et al.* 1996; Paulson *et al.* 1996), interleukin-3

Figure 5.8 Shp-1 is tyrosine phosphorylated in response to growth factor stimulation.

Shp-1 was immunoprecipitated with Shp-1-specific antisera (AV-2) from serum-deprived (-) or serum- and vanadate- stimulated (+) NIH-3T3 fibroblast lines indicated. Immunoprecipitates were subjected to SDS-PAGE and analyzed by **(A) (lower panel)** Shp-1 peptide antisera or **(upper panel)** anti-phosphotyrosine immunoblot analysis. **(B)** Serum starved NIH-3T3 parental or derived (M5 clone which express Shp-1^{C453S}) fibroblasts were stimulated with serum, serum and vanadate, growth factors or the calcium ionophore A413258 for 8 min. Shp-1 immunoprecipitates were subjected to anti-phosphotyrosine immunoblot analysis. In each case, the arrowhead highlights Shp-1 migration.

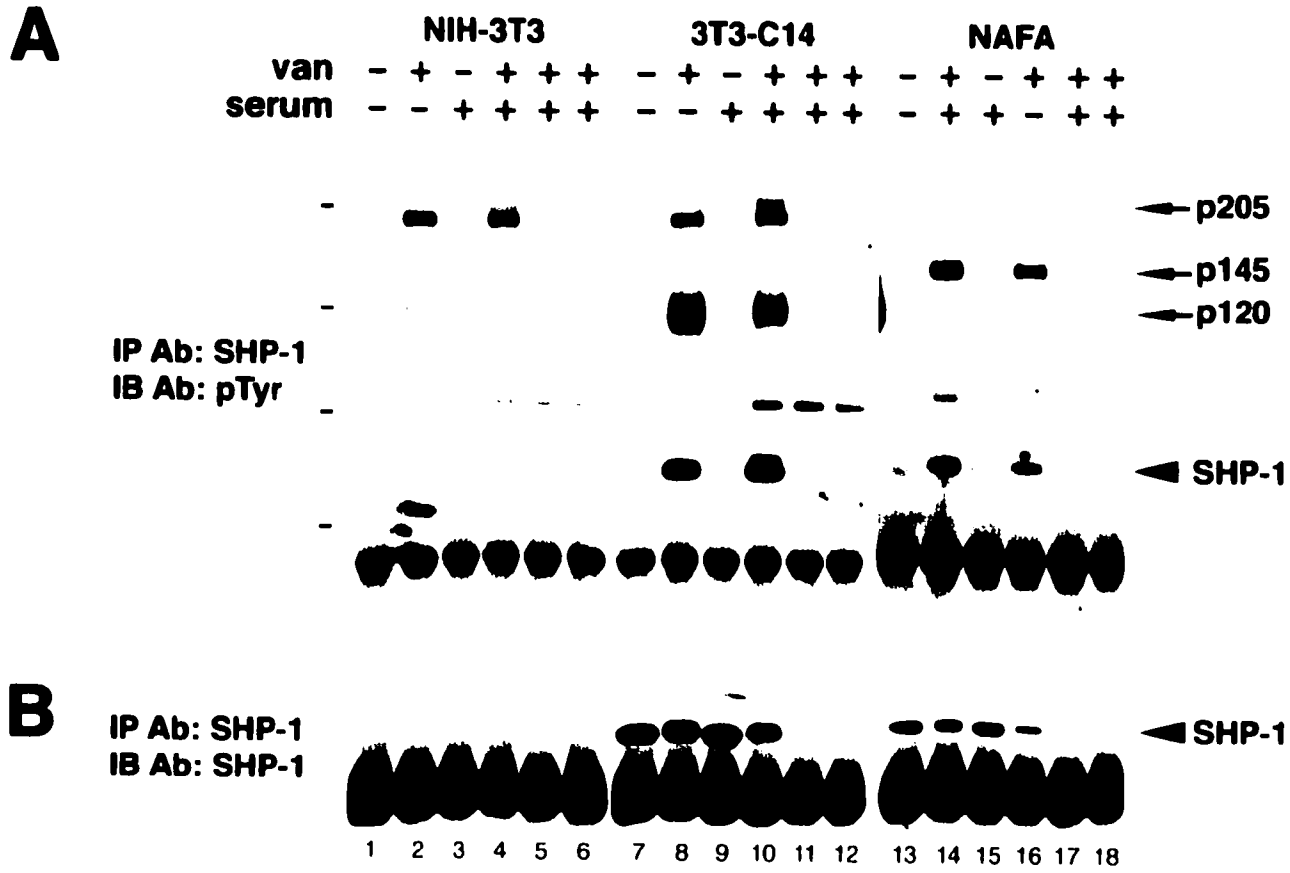

A
3T3
C14
M8
- + - + - +
IP Ab: SHP-1
IB Ab: pTyr
1 2 3 4 5 6
IB Ab: SHP-1
1 2 3 4 5 6
B
-ve
serum
serum + van
PDGF-AB
EGF
bFGF
Insulin
A413258
serum + van
IP Ab: SHP-1
IB Ab: pTyr

receptor (IL-3R; Yi *et al.* 1993) and erythropoietin receptor (EPOR; Klingmuller *et al.* 1995). Moreover, genetic studies indicate Shp-1 attenuates signaling from the T cell (Plas *et al.* 1996) and the colony-stimulating factor-1 (CSF-1) (Chen *et al.* 1996a) receptors indirectly, presumably through association with specific tyrosine phosphorylated proteins (Timms *et al.* 1998). These experiments however, have been limited to cells of lymphoid and myeloid lineages.

To determine whether comparable Shp-1 interactions occur in mammary epithelial cells, I performed immunoblot analyses with antiphosphotyrosine specific antibodies on Shp-1 immunoprecipitates from either Shp-1 expressing NIH fibroblasts or Shp-1 expressing NAFA mammary tumour cells. The NAFA epithelial cell line was derived from an activated Neu induced mammary tumor and expresses high levels of activated Neu protein (Muller *et al.* 1988). To accomplish this, Shp-1 immunoprecipitates were prepared from serum deprived cells that were left untreated or were treated with serum and/or sodium vanadate. Vanadate treatment enhanced tyrosine phosphorylation of Shp-1 as well as the specific association of Shp-1 with a phosphotyrosyl protein of approximately 145kDa (p145) (Figure 5.9A lane 16). Although the addition of serum had little effect on the profile of phosphotyrosine-containing proteins in Shp-1 immunoprecipitates, serum and vanadate treatment revealed a higher molecular weight protein of approximately 205kDa (p205) which associated with Shp-1 (lane 14). Neither protein was observed when the Shp-1-specific antisera was neutralized with the immunizing peptide nor in immunoprecipitates using preimmune sera from the same rabbit (lanes 17 and 18). The 205kDa tyrosine phosphorylated protein is not Neu since immunoblot analyses of these Shp-1 immunoprecipitates have failed to reveal specific Neu/Shp-1 complexes (data not shown). Interestingly, NIH-3T3 fibroblasts engineered to express Shp-1 interact with a different subset of tyrosine phosphoproteins, suggesting that Shp-1 forms cell-type specific association with phosphoproteins. In fibroblasts that express Shp-1, but not parental 3T3 cells, Shp-1 appears to interact with a 120kDa phosphoprotein (p120) (lanes 8, 10) and in response to serum and vanadate treatment with a larger 205kDa protein (lane 10). Whether the NAFA-derived and fibroblast-derived 205kDa protein are the same remains to be determined. These observations suggest that Shp-1

Figure 5.9 Shp-1 associates with distinct tyrosyl-phosphoproteins in a cell type dependent manner.

Shp-1 was immunoprecipitated from serum deprived cells (-) or those stimulated with serum and/or vanadate (+) from NIH-3T3 (lanes 1-6), NIH-3T3 fibroblasts expressing wild-type Shp-1 (line C14) (lanes 7-12) or NAF epithelial cells (lanes 13-18). Immunoprecipitation was carried out using preimmune sera (lanes 6, 12, 18) or Shp-1-specific peptide antisera (lanes 1-4, 7-10, 13-16) or Shp-1-specific peptide antisera neutralized with immunizing peptide (lanes 5, 11, 17) using 1.5mg of protein lysate. Immunoprecipitates were subjected to SDS-8%-PAGE and analyzed by anti-phosphotyrosine (**A**) or anti-Shp-1 (AV-2 antisera) (**B**) immunoblot analysis. Arrowheads denote the migration of Shp-1 and arrows highlight the positions of Shp-1-associated proteins.


A
NIH-3T3
3T3-C14
NAFA
van
serum
p205
p145
p120
IP Ab: SHP-1
IB Ab: pTyr
SHP-1
B
IP Ab: SHP-1
IB Ab: SHP-1
SHP-1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

becomes tyrosine phosphorylated in response to several mitogenic signals and interacts with different tyrosyl-phosphoproteins in a cell type specific manner.

5.7.3 *In vitro* association of cellular phosphotyrosyl-proteins with Shp-1.

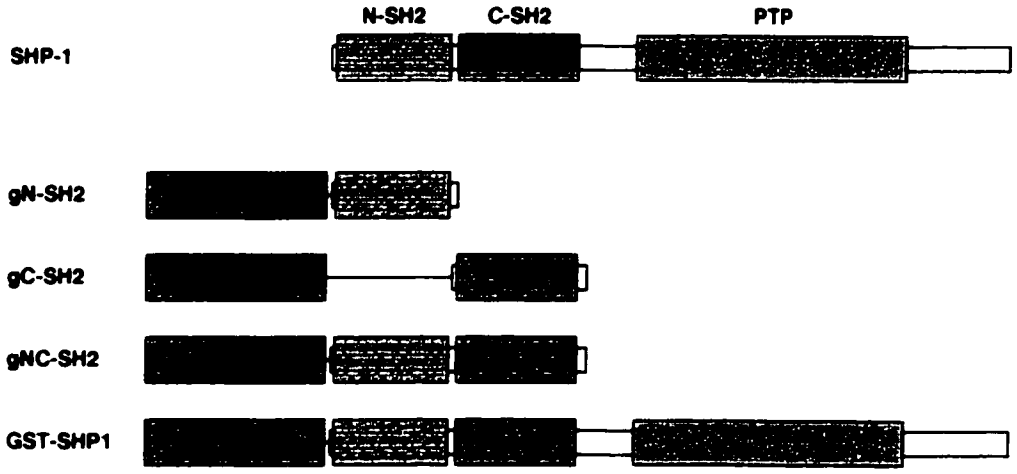
The association with pp120, pp145 and pp205 in a serum/vanadate dependent manner is consistent with the hypothesis that these interactions are mediated by phosphotyrosine. This proposes that Shp-1 either associates with these phosphoproteins through its SH2 domains or the associated protein contains a phosphotyrosine interacting motif which interacts with one or more Shp-1 phosphorylation sites. To explore the former hypothesis, GST fusions containing the entire Shp-1 open reading frame (GST-Shp1), the amino (gN-SH2), carboxy (gC-SH2) or both SH2 domains (gNC-SH2) (Figure 5.10A) were constructed to use *in vitro* affinity purification assays. These assays, while not performed with endogenous proteins nor with physiologic protein concentrations, do reveal similar protein-protein interactions as those obtained by immunoprecipitation of endogenous proteins (Cantley 1991).

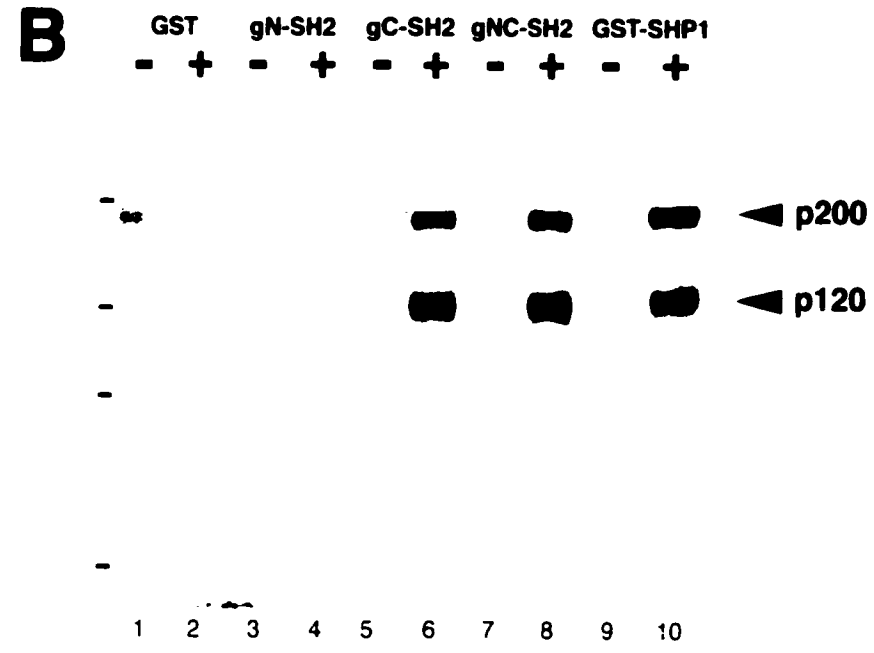
Extracts from serum deprived or stimulated 3T3 fibroblasts were incubated with a number of GST fusion proteins (Figure 5.10A). These fibroblasts were chosen over of mammary epithelial cell lines as they contain low Shp-1 levels reducing the possibility of competition with endogenous Shp-1 for target protein interactions as has been observed for PLCγ1 (Anderson *et al.* 1990). Two high molecular weight tyrosyl-phosphoproteins of approximately 120kDa and 190- specifically associate with GST-Shp1, but not the GST control, in a serum stimulated manner (Figure 5.10B). These proteins will be referred to as Shp-1/PTP1C-Associated Phosphoproteins, CAP120 and CAP200. Association of GST-Shp1 with CAP120 and CAP200 is detectable within 1 min of serum stimulation in the presence of vanadate (data not shown). Both CAP120 and CAP200 interact with the carboxy terminal SH2 domain (Figure 5.10B) and additional experiments demonstrate that can CAP120 and CAP200 independently associate with the carboxy SH2 domain *in vitro* .

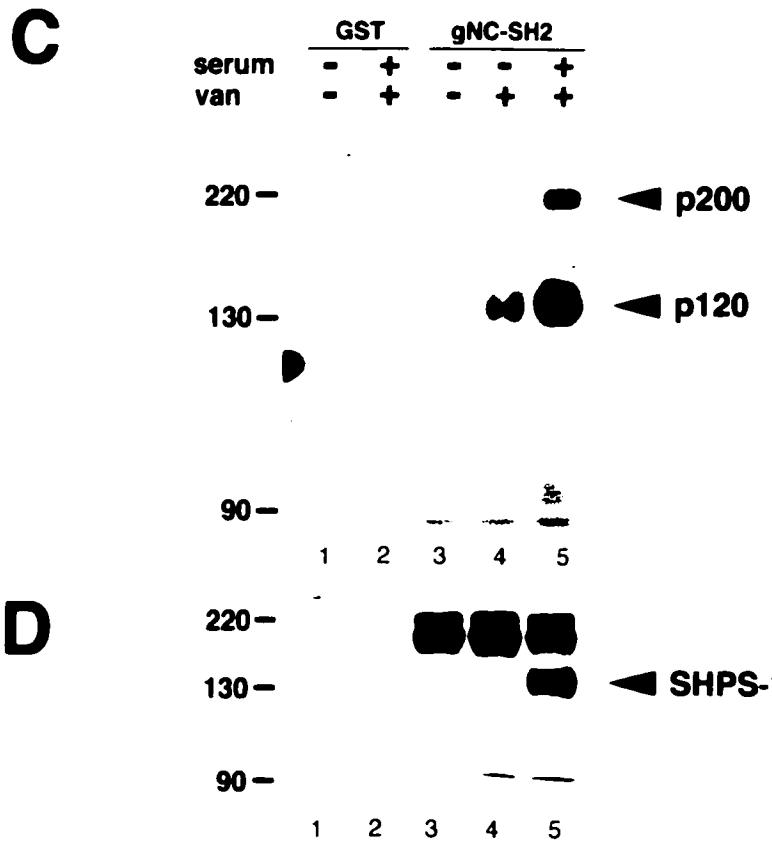
Figure 5.10 Shp-1 associates with SHPS-1 in fibroblasts through its carboxy SH2 domain *in vitro*.

(A) Schematic representation of the GST-fusion proteins constructed. SH2 domains are depicted as shaded boxes and the PTP domain is black. Horizontal lines do not represent amino acids but symbolize that the boxes (coding regions) are fused. **(B)** NIH-3T3-derived lysates were incubated with immobilized bacterial fusion proteins (A), purified cellular proteins were subject to SDS-PAGE and analyzed by antiphosphotyrosine immunoblot analysis. **(C)** Lysates of quiescent NIH-3T3 fibroblasts that were mock, serum or serum and vanadate treated as indicated were incubated with GST (lanes 1,2) or gNC-SH2(lanes 3-5). Half of the affinity purified cellular proteins were subject to SDS-PAGE and analyzed by antiphosphotyrosine immunoblot analysis. **(D)** The remaining portion of each affinity purification was subjected to anti-SHPS-1 immunoblot analysis with affinity purified antisera.

A




B
GST
gN-SH2
gC-SH2
gNC-SH2
GST-SHP1
- + - + - + - + - +
p200
p120
1 2 3 4 5 6 7 8 9 10


C
GST
gNC-SH2
serum - + - - +
van - + - + +
220
130
p200
p120
D
90
1 2 3 4 5
220
130
90
SHPS-1
1 2 3 4 5

5.7.4 The pp120 Shp-1 associated protein is SHPS-1

I was interested in identifying CAP120 and CAP200 in hopes that this would shed light on Shp-1's physiologic role. It is clear that CAP120 is not p120-Ras-GAP or the v-Src substrates p120 or p125-FAK nor is CAP200 p210-tensin, p180PDGFR or p185Neu. Recently, a cDNA, termed SHPS-1, encoding a Shp-2 interacting protein has been cloned (Fujioka *et al.* 1996; Kharitonenkov *et al.* 1997). SHPS-1 is tyrosine phosphorylated in v-*src*-transformed cells and serum or LPA stimulation of fibroblasts (Fujioka *et al.* 1996). SHPS-1 tyrosine phosphorylation is detected following EGF, PDGF and insulin treatment of a variety of cell lines (Fujioka *et al.* 1996; Kharitonenkov *et al.* 1997). Molecular cloning of SHPS-1 cDNAs, reveals that there are at least four family members each containing a conserved putative signal sequence followed by three immunoglobulin-like structures and a hydrophobic potential transmembrane domain. There appear to be two classes of SHPS-1 proteins, also known as signal-regulating proteins (SIRPs; Kharitonenkov *et al.* 1997), those that contain a presumptive intracellular region harbouring four conserved tyrosine residues (SIRPα of which SHPS-1 is a member) and those which terminate following the putative transmembrane stop sequence (SIPRβ Kharitonenkov *et al.* 1997). The sequences surrounding these tyrosines bear significant homology to one another, to known Shp-1/2 binding sites on IRS-1 and conform to consensus immunoreceptor tyrosine-based inhibition motif sequences (ITIMs; Unkeless and Jin 1997). Interestingly, forced expression of SIRPα-1 (also called SHPS-1) is sufficient to prevent transformation from the v-*fps* PTK in recipient fibroblasts (Kharitonenkov *et al.* 1997).

Lysates from control, serum and serum and vanadate treated NIH-3T3 fibroblasts were incubated with either immobilized GST or gNC-SH2 which contains both Shp-1 SH2 domains. Specifically bound proteins were analyzed by immunoblot analysis with antiphosphotyrosine or SHPS-1-specific antibodies. CAP120 from cells treated with serum with or without vanadate bound gNC-SH2 although there was considerably more CAP120 detected in the former (Figure 5.10C). Interestingly, the binding of CAP120 to gNC-SH2 correlated with the detection of SHPS-1 in these affinity purifications (Figure 5.10D). SHPS-1 did not bind GST alone (lanes 1-2) but was detected in lysates of serum quiesced cells (data not shown).

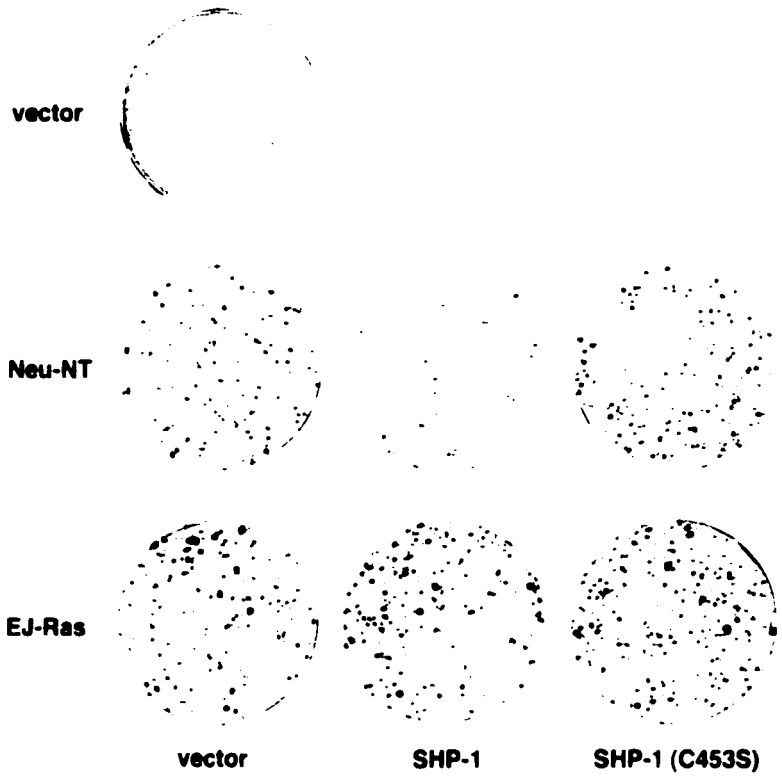
5.8 Shp-1 requires catalytic activity to act as a dominant inhibitor of activated Neu-mediated transformation

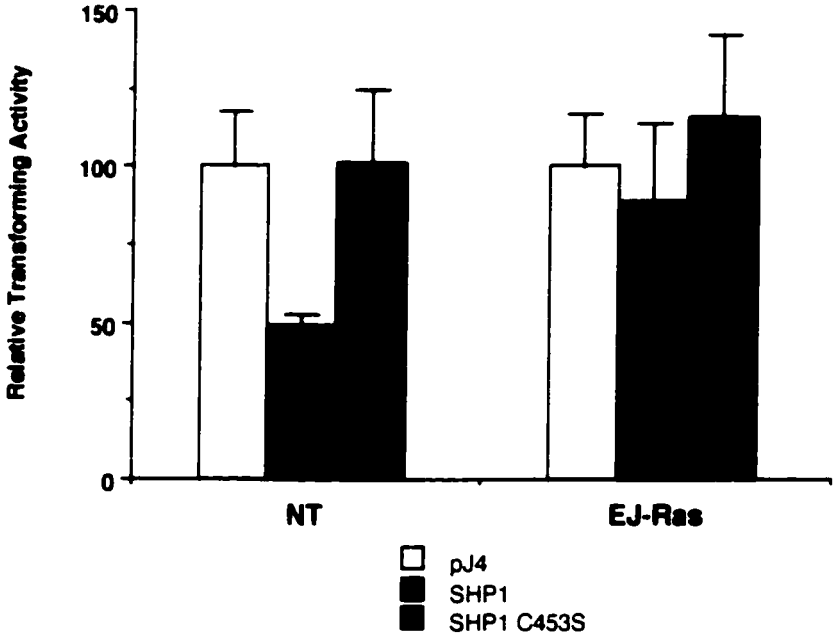
Shp-1 levels are elevated in Neu induced tumours and Shp-1 was phosphorylated when isolated from vanadate treated epithelial cells expressing oncogenic Neu (Figures 5.4 and 5.9). Moreover, Shp-1 associates with several proteins one of which, SHPS-1, has been shown to inhibit transformation by a viral PTK. Taken together with the previous described inhibitory role in RTK signaling (Lorenz *et al.* 1996; Paulson *et al.* 1996), I examined whether *shp-1* expression could interfere Neu-mediated transformation.

To explore this possibility, fibroblasts were transfected with expression vectors encoding Neu-NT, add-back mutants NT-YB through NT-YE or oncogenic Ras along with either an empty vector or one encoding wild-type Shp-1. Shp-1 expression inhibited NT-mediated transformation (Figure 5.11) but did not alter the transforming activities mediated from sites B through E (data not shown). In contrast to inhibition of Neu transformation, no specific inhibition of Ras-mediated transformation was observed. Transformation inhibition required Shp-1 catalytic activity, as the C453S mutant failed to inhibit Neu-mediated transformation in these experiments. Given that we and others have shown that Neu signals primarily through Ras in these fibroblasts (Dankort *et al.* 1997; Ben-Levy *et al.* 1994), these observations suggest that Shp-1 specifically antagonizes Neu signaling upstream of Ras.

Figure 5.11 Shp-1 catalytic activity is required to inhibit Neu NT-mediated transformation.

100ng of either pJNT or pEJ-Ras were cotransfected in liposomes into Rat1 fibroblasts along with 1ug of pJ4Ω, pJ4Ω-encoding wild-type or catalytically inactive Shp1c453s. Transforming potential of the indicated combinations of plasmids were normalized (± standard error) to that observed in the presence of 1ug of the empty pJ4Ω expression vector. These data are the result of a single experiments with 3 plates receiving each plasmid combination.


vector
Neu-NT
EJ-Ras
vector
SHP-1
SHP-1 (C453S)


Relative Transforming Activity
150
100
50
0
NT
EJ-Ras
pJ4
SHP1
SHP1 C453S

5.9 DISCUSSION

The data presented in this chapter demonstrate the isolation and characterization of a mammary expressed SH2-containing protein tyrosine phosphatase. My objective was to isolate mammary PTPs. To this end, I used a degenerate PCR approach to clone cDNAs encoding a then novel PTP now called *Shp*-1 (Figure 5.3). In addition to Shp-1, the transmembrane PTP LAR was also isolated by this RT-PCR approach. Consistent with its mammary specific expression, mice made homozygous for a targeted LAR mutation display abnormal mammary gland development (Schaapveld *et al.* 1997). Although it is widely held that Shp-1 is a hematopoietic specific phosphatase (reviewed in Neel 1997; Neel and Tonks 1997; Byon *et al.* 1997), its expression has been detected in a variety of cell lines including those from embryonic kidneys (293 Su *et al.* 1996) embryonic carcinoma (F9: Tsuneizumi *et al.* 1994), cervical carcinoma (A431: Tomic *et al.* 1995, HeLa: Plutzky *et al.* 1992), hepatoma (Uchida *et al.* 1994; Plutzky *et al.* 1992) and in 2 of 4 lung carcinoma derived lines (Yi *et al.* 1992; Plutzky *et al.* 1992). I have further detected Shp-1 transcripts in the mammary glands of virgin mice (Figure 5.2), elevated Shp-1 protein levels expressed in mammary derived tumour tissue (Figure 5.4) and in 8/8 mammary epithelial cell lines (Figure 5.5). Consistent with these observations, Shp-1 was originally cloned from a mammary cell line library (Shen *et al.* 1991). Moreover, low levels of Shp-1 transcripts were also detected in a single mammary cell line (MCF7 Yi *et al.* 1991). Taken together, these data strongly argue that Shp-1 is also a mammary expressed PTP.

In addition to the conserved PTP domain, Shp-1 contains two amino terminal SH2 domains (Figure 5.3C). As these domains mediate protein-phosphotyrosine interaction, I set out to identify the proteins which specifically interacted with Shp-1 in response to growth factor activation. In the course of these experiments, I found that Shp-1 was tyrosine phosphorylated in response to serum stimulation of both NAFA epithelial cells and NIH-3T3 derived fibroblasts. Shp-1 tyrosine phosphorylation can be induced with PDGF in these fibroblasts (Figure 5.10). I have been unable to detect tyrosine phosphorylated Shp-1 in transgenic mouse breast tumours, perhaps reflecting autodephosphorylation of the PTP. This is based on

the observation that tyrosine phosphorylation of wild-type Shp-1 is detectable only in the presence of PTP inhibitors and that catalytically inactive Shp-1 (C453S) was often found tyrosine phosphorylated in in the absence of vanadate treatment (Figure 5.8 and data not shown). These data are consistent with the hypothesis that Shp-1 can auto-dephosphorylate. This can be tested using a GST-C453S substrate trap mutant. This fusion would be predicted to interact with tyrosine phosphorylated Shp-1 isolated from serum stimulated cells.

In NIH-3T3 fibroblasts engineered to express Shp-1, Shp-1 inducibly associated with two tyrosine phosphorylated proteins of approximately 120 and 200 kDa, although the latter protein nearly comigrates with a nonspecific band (Figure 5.9 lane 4 versus 10). *In vitro* two proteins of similar size associated with the C-terminal SH2 domain (Figure 5.10B). Notably, the CAP120 protein binds *in vitro* to Shp-1 SH2 domains and is immunoreactive with anti-SHPS-1 antisera (Figure 5.10D) suggesting the 120kDa phosphoprotein which coimmunoprecipitates with Shp-1 is in fact SHPS-1. SHPS-1 (also known as SIRP1α) was originally isolated as a potential Shp-1 substrate (Fujioka *et al.* 1996; Kharitonenkov *et al.* 1997). In macrophages, CSF-1 treatment induces the tyrosine phosphorylation of Shp-1 and association with several phosphoproteins. Although CSF-1R/Shp-1 interactions were not observed, BIT1, a SHPS-1 family member, associates with both Shp-1 and CSF-1R and it is hyperphosphorylated in response to CSF-1R stimulation in macrophages isolated from *shp-1* deficient mice compared to wild-type controls (Chen *et al.* 1996a; Timms *et al.* 1998). Moreover, SHPS-1 (SIRP1α) expression inhibits transformation mediated by v-*fms*, a retrovirally transduced CSF-1R homolog (Kharitonenkov *et al.* 1997). Taken together these data suggests that Shp-1 may negatively regulate receptor tyrosine kinases in part through its association with SHPS/SIRP family members. It will be interesting to determine whether SHPS-1 interacts with Neu or is tyrosine phosphorylated in Neu transformed cells as would be predicted by these data.

In contrast to the observations made with fibroblasts, Shp-1 associates with an approximately 145kDa tyrosine phosphorylated protein in the NAFA mammary tumour cell line following vanadate treatment. This association is not restricted to NAFA cells since C127 mammary epithelial cells also

possess a similar Shp-1/145kDa complex following serum/vanadate stimulation (data not shown). These observations argue that Shp-1 associates with distinct cell type specific factors following growth factor stimulation. Although I have not identified p145, one possible candidate is the SH2 domain containing inositol 5'-phosphatase (SHIP) (Damen *et al.* 1996; Lioubin *et al.* 1996). Like, Shp-1, SHIP is thought to be a hematopoietic specific gene. Enzymatically it displays phosphatase activity towards the 5'-phosphate of 1,3,4,5-tetraphosphate and phosphatidylinositol 3,4,5-trisphosphate (Lioubin *et al.* 1996; Damen *et al.* 1996). Indeed, it has recently reported that the complexes involving the closely related Shp-2 phosphatase and SHIP can be detected in hematopoietic cells following IL-3 or EPO receptor activation or BCR-Abl expression (Liu *et al.* 1997; Sattler *et al.* 1997). IL-3 induced complexes appear to occur through the SHIP-SH2 domain and a phosphotyrosine residue in the carboxyterminus of Shp-2 (Liu *et al.* 1997). The functional consequences of Shp-2/SHIP interactions were not determined and awaits coexpression analyses. Future studies with SHIP specific antisera should allow us to address the possibility that the 145kDa phosphoprotein is SHIP.

Previous studies have suggested that one of the primary functions of Shp-1 is to attenuate tyrosine kinase signals emanating from activated receptors. Indeed, Shp-1 is a negative regulator of signaling from the erythropoietin, interferon α, T cell and IL-3 receptors (Klingmuller *et al.* 1995; David *et al.* 1995; Plas *et al.* 1996; Yi *et al.* 1993) as well as the c-Kit RTK (Lorenz *et al.* 1996; Paulson *et al.* 1996). More recently, there has been a report suggesting that Shp-1 binds to the ErbB family member, EGFR and attenuates receptor signaling through its association with phosphotyrosine residue 1173 (Keilhack *et al.* 1998). Although I have not been able to detect specific complexes of activated Neu and Shp-1, I have demonstrated that elevated expression of wild-type but not catalytically inactive Shp-1 can significantly interfere with Neu-induced transformation (Figure 5.11). One possible explanation for these results is that Shp-1 may be indirectly be recruited to activated Neu through the formation of Neu/EGFR heterodimer. Indeed previous studies have demonstrated that Neu can form specific heterodimers with the EGFR (Wada *et al.* 1990, reviewed in Carraway III and Cantley 1994; Alroy and Yarden 1997; Tzahar and Yarden 1998).

Interestingly elevated expression of Shp-1 had little effect on transformation mediated by activated Ras. These data suggest that Shp-1 functions to attenuate Neu signals either upstream of Neu or in a parallel pathway with Ras. Interestingly, the *Drosophila corkscrew* and the *C. elegans PTP-2* genes also appear to function genetically downstream of RTKs. That active *ras* alleles can rescue *corkscrew* or *PTP-2* loss of function mutations, position a these PTPs genetically upstream of *ras* (Perkins *et al.* 1992; Gutch *et al.* 1998) although a downsteam effect has been reported (Allard *et al.* 1996) . Thus these observations suggest that the observed elevated expression of Shp-1 in mammary tumors is involved in the negative regulation of Neu-mediated signaling.

The observation that Shp-1 levels are elevated in breast tumours coupled with its ability to inhibit Neu mediated transformation seems counter intuitive. In culture, expression of activated Neu alleles leads to a decrease in the number of drug resistant colonies formed in cotransfection experiments and this correlates inversely with the degree of transformation (data not shown), suggesting that elevated levels of Neu signaling may have a toxic effect on the cell. Perhaps elevation of Shp-1 attenuates Neu signaling, preventing cell death. Alternatively, it is possible that Shp-1 may play other roles in the developing tumour.

Whereas Shp-1 expression can be detected in normal and tumor derived mammary epithelium, the precise role of Shp-1 in controlling normal mammary epithelial proliferation and differentiation remains unclear. In this regard, the Shp-1 gene is disrupted in two naturally occurring mutants, *motheaten* (*me*) and *motheaten viable* (*mev*), which both are the result of point mutation induced aberrant splicing (Shultz *et al.* 1993; Tsui *et al.* 1993). While *me/me* mice do not contain detectable Shp-1 protein, functioning as a genetically null allele, two forms of Shp-1 are found in *mev/mev* mice, both of which have severely retarded PTPase activities (Shultz *et al.* 1993; Kozlowski *et al.* 1993). Although virtually all hematopoietic lineages are affected in these mice (reviewed in Shultz and Sidman 1987; Neel and Tonks 1997), there have been no reports of abnormal mammary gland development or breast tumour formation in homozygous *motheaten viable* (*mev/mev*) mice. However, due to the limited lifespan of these naturally occurring mouse mutations

(60 days on average), these strains would not likely survive long enough for tumor progression to occur. The human *shp-1* gene resides at 12p12-p13 and is thought to play a role in human lymphoma formation as this locus sustains translocations and deletions in association with acute lymphocytic leukemia (Yi *et al.* 1992). This region however has not previously been identified as an LOH in human breast cancer genesis or progression (Morrison 1994; Bieche and Lidereau 1995) but this does not preclude the possibility of mutation of *shp-1* gene in these processes. Future studies with transplanted epithelium from *mev/mev* or *me/me* strains or tissue specific ablation of the *shp-1* gene will provide insight into the role of Shp-1 in tumor progression or mammary gland development.

Chapter 6

Summary and Conclusions

6.1 ErbB-family members in development

Biochemical analyses suggest that Neuregulins actions are mediated in part through ErbB-2/ErbB-4 heterodimers. More recently, gene targeting reveals that these ErbB-family members function together in cardiac development. Prior to septum formation, the mammalian heart consists of a single ventricle (reviewed in Fishman and Chien 1997). Within this structure develop finger-like projections called trabeculae that function to increase oxygenation of the ventricle at around mouse gestation day 10.5. Trabeculae later coalesce to form a septum separating two ventricles. The formation of ventricular trabeculae is aborted in mice lacking either ErbB-2, ErbB-4 or neuregulin 1 (Gassmann *et al.* 1995; Lee *et al.* 1995; Meyer and Bichmeier 1995) and presumably is the cause of embryonic lethality in homozygous null animals at approximately day 11 *in utero*. Neuregulin 1, but not its receptors (ErbB-3, ErbB-4) is expressed in the endocardial endothelium and the endocardial cushions, the latter of which develop into valves (Meyer and Bichmeier 1995). Interestingly, ErbB-2 (Lee *et al.* 1995) and ErbB-4 (Gassmann *et al.* 1995; Meyer and Bichmeier 1995) are expressed in the underlying myocardium and the myocardial cells destined to form the ventricular trabeculae. Proper valve formation is delayed in all three deficient mice. Thus, it would appear that in the heart, proper development requires neuregulin expression in the endothelium to inducetrabeculation via ErbB-2/ErbB-4 heterodimers. ErbB-3 is not expressed in either the myocardium or the endocardial endothelium (Meyer and Bichmeier 1995) and while most ErbB-3 deficient embryos die between E11.5 and E13.5, they perish not due to heart malformation, but due to a lack of proper Schwann cell development/survival (Riethmacher *et al.* 1997). This is intriguing in that Neu was originally isolated as a Schwannoma-derived oncogene (Shih *et al.* 1981; Bargmann *et al.* 1986b).

Additionally, the development of several cranial neural crest-derived sensory ganglia is lacking in ErbB-2 deficient mice at the time of death (Lee *et al.* 1995). These same neural crest-derived ganglia are reduced in size in NRG1 deficient animals suggesting the two genes function together here.

ErbB family members appear to play a role in the functional innervation of motor neurons. Soluble Neuregulin (NRG1) is concentrated at synapses through both motor neuron endplates synthesis and surface release from muscle fibers at synapses (Jo *et al.* 1995; Goodearl *et al.* 1995). Moreover, muscle cells express and concentrate ErbB-2, ErbB-3 and ErbB-4 at neuromuscular junctions (Zhu *et al.* 1995; Altiok *et al.* 1995; Moscoso *et al.* 1995). NRG1, which was independently isolated as an acetylcholine receptor (AChR) inducing activity (ARIA), induces AChR transcripts in muscle fibers which when translated is localized to synaptic sites (Chu *et al.* 1995). AChR synthesis is thought to involve ErbB-4 and possibly ErbB-2, but not ErbB-3 activity, since while surviving ErbB-3 deficient mice lack Schwann cells, neuromuscular junctions remain functional to at least E15.5. NRG1 induced AChR transcription requires Erk1 but not PI3' nor S6 kinase activities (Altiok *et al.* 1997). ErbB-2 and ErbB-4 deficient mice die prior to the functional innervation of muscles which begins at E15, thus the genetic role of these receptors in AChR expression, muscular synapse formation and neuronal survival awaits further experimentation. ErbB-2 expression in neuromuscular junctions and in many secretory epithelial tissues suggests that it may play crucial roles in adult tissues which is not discernible through the study of non-conditional nullizygous animals. Thus it would be quite interesting to assess the requirement of Neu/ErbB-2 in the adult animal through temporally or spatially regulated "knock-out" strategies.

6.2 ErbB-2 signals through multiple effector molecules to activate Ras

The work presented in Chapter 3 demonstrates Neu recruits multiple proteins to effect Ras activation. The reasons for this are not intuitively obvious, but there are several possibilities which are consistent with these observations. First, it is possible that the adaptor proteins used by Neu/ErbB-2 may be spatially or temporally restricted in their pattern of expression. Second, Neu/ErbB-2 may be

differentially phosphorylated in a temporal and/or spatially specific manner. Lastly, recruitment of distinct adaptor proteins may result in the differential activation of Ras effector proteins.

Hypothesis I: Differential effector protein expression

Given Neu/ErbB-2's expression pattern in the adult and developing embryo it is quite possible that Neu/ErbB-2 recruits several functionally redundant adaptor proteins not expressed in all the tissues which Neu/ErbB-2 functions. For example, perhaps Neu/ErbB-2 uses Shc to mediate signals in the heart for trabeculation and valve formation but requires Grb2 recruitment for AChR expression in skeletal muscle. This hypothesis can be evaluated in two ways.

First, the different add-back or point mutants can be tested for their ability to complement lethality observed in a nullizygous background through a "knock-in" strategy (Hanks *et al.* 1995). This will demonstrate which sites are sufficient or required for normal embryonic development and perhaps reveal a tissue specific requirement for individual phosphorylation sites. Preliminary evidence indicates that mice homozygous for the Neu-D point mutant "knocked-in" allele die at birth unlike the nullizygous animals which perish *in utero* (Lee *et al.* 1995). This lethality appears specific in that similar homozygous "knock-in" point mutations for site B and site A develop to term and appear to be healthy (W.R. Hardy and W.J. Muller unpublished observation).

A second approach makes use of the observation that heterozygous *erbB-2*$^{+/-}$ animals appear normal (Lee *et al.* 1995). Cre recombinase, placed under the transcriptional control of the *erbB-2* gene via a "knock-in" approach, can be used to excise critical exons within target adaptor encoding genes as has been demonstrated for other genes (Orban *et al.* 1992; Gu *et al.* 1994; Shibata *et al.* 1997). This will enable one to genetically ablate an adaptor molecule's expression only within tissues expressing ErbB-2. For example, *grb2*$^{-/-}$ embryos fail to implant in the uterine wall (Chen *et al.* 1998), thus the requirement of *grb2* in trabeculation or neuronal development will require tissue specific or temporally regulated *grb2* ablation. To test the requirement of *grb2* in *erbB-2* mediated signaling, the *grb2* locus can be flanked with unidirectional loxP sites and *grb2*$^{flx/flx}$; *erbB-2*$^{wt/cre}$ mice can be analysed for developmental deficiencies.

Alternatively, dominant inhibitory mutants of SH2/PTB signaling molecules can be expressed in *erbB-2* expressing tissues with a similar knock-in approach although embryonic lethality may require the analysis of many chimeric animals. This chimeric embryonic lethality can theoretically be circumvented by placing the dominant inhibitory molecules under the transcriptional control of the tetracycline repressor (Gossen and Bujard 1992) and the tetracycline repressor "knocked-in" to the *erbB-2* locus. In this manner, the dominant inhibitory molecule expression is temporally and spatially restricted.

Hypothesis II: Neu may be differentially phosphorylated

Another potential explanation for the recruitment of seemingly redundant pathways is that these sites may be differentially phosphorylated spatially throughout the developing animal or temporally in response to activation. Given the ability of Neu/ErbB-2 to form homo- and hetero- dimers, it is conceivable that different partners or ligands induce phosphorylation of distinct tyrosine residues. Evidence to support this notion comes from chimeric receptor studies. By far western blot analyses, Grb7 binds efficiently to ErbB-2 isolated from human mammary lines or NeuNT expressing fibroblasts but only poorly to the EGFR-ErbB-2 chimeric following EGF-stimulation (Stein *et al.* 1994). Additionally, Src induces the phosphorylation of a EGFR creating a p85 binding site (Stover *et al.* 1995). One can compare Neu/ErbB-2 phosphotryptic peptide maps obtained from cells expressing Neu/ErbB-2 to those containing ErbB-1/2, ErbB-2/3 and ErbB-2/4 (with the other receptors in excess of ErbB-2 by at least four fold) in response to different ligands (EGF, TGFα, NRG, *etc.*). If the hypothesis is correct, Neu should be differentially phosphorylated and thus will signal through different adaptor proteins. Recent evidence has demonstrated that ErbB-2 is differentially phosphorylated in response to EGF or NRG treatment (Olayioye *et al.* 1998). This hypothesis can be tested in the developing mouse through the use of specific antisera raised to the individual phosphorylation sites (DiGiovanna and Stern 1995).

Hypothesis III: Differential activation of Ras effector molecules

It is also possible that Neu/ErbB-2 activates Ras using different adaptor proteins and the result is differential signaling through Ras. This requires that these adaptors somehow influence the ability of Ras to equally signal to Ras effectors. Perhaps Ras effector molecules themselves are directly affected by the activation or recruitment of Neu associated proteins (see sections 1.5.3 and 3.9.2). An example of this is the observation that Raf not only requires Ras association but also activation through an unknown kinase (Stokoe and McCormick 1997). While Ras effector mutant studies suggest that PI3'K p110 association is sufficient for oncogenic Ras-induced PI3'K activation, it appears that simultaneous recruitment of p85 to RTKs is required for elevated PI3'K activity (Klinghoffer *et al.* 1996).

Alternatively, it is also possible that an adaptor protein or the exchange factor it uses (if there are yet discovered GEFs) prevents or augments the binding and/or activity of one Ras effector molecule over another. This is not to say that Ras itself takes on different conformations. While this hypothesis is quite intriguing it is also quite difficult to prove experimentally and will likely require advances in yeast protein-protein interaction technologies beyond the current three hybrid systems which now exist (Xu *et al.* 1997).

A systematic comparison of Ras effector pathways activities should confirm or deny the possibility that these add-back mutants differentially activate Ras effector molecules. Moreover, this hypothesis predicts that distinct add-back mutants will be differentially sensitive to Ras effector molecule inhibitors. Data to support this contention stems from observations made in our lab demonstrating that NT-YE transformation was inhibited by the expression of kinase deficient Raf whereas the other transforming add-back mutants were not (Neil Warner and W.J. Muller unpublished observations).

Clearly these three scenarios are not mutually exclusive. For example, it is conceivable that ErbB-2 is differentially phosphorylated in different tissues each expressing a distinct subset of SH2/PTB proteins allowing ErbB-2 to activate a subset of Ras effector proteins. It is interesting to note that mammary-specific expression of YB and YD has distinct phenotypic consequences. Preliminary evidence suggests that MMTV-YD-induced mammary tumours are indistinguishable from MMTV-Neu-induced

comedocarcinomas but are relatively non-metastatic whereas MMTV-YB-induced mammary tumours have a higher metastatic potential and possess a papillary histology (Dankort, R. Cardiff, Muller, unpublished observations). Thus as the Grb2 microinjection experiments suggest (Figure 3.12), it appears that signaling through tyrosines 1144 and 1227 is quite distinct. Much more work is required to clarify these issues.

6.3 Inhibition of Neu mediated transformation

In Chapter 4, I demonstrated that an autophosphorylation site (Y1028, site A) acts to repress transformation by four other known autophosphorylation sites. Genetically, mice made homozygous for the Neu-A point mutation (*erbB2*$^{neuA/neuA}$) appear to function as hypermorphic alleles (W.R. Hardy and W.J. Muller, unpublished observation). Specifically 30% of mice hemizygous for a null allele and for a point mutation at site A (erbB2$^{-/neuA}$) develop to term and are phenotypically normal. In contrast, many of the mice hemizygous for a null allele and wild type (erbB2$^{-/neu}$) die *in utero* and the 5% of mice that develop to term are paralyzed. These data support the notion that site A is a negative regulator of Neu/ErbB-2.

The presence of this phosphorylation site impairs the ability of Neu to physically couple to the tested Neu effectors Grb2 and Shc (Figure 4.1). Mechanistically, this can be explained by a site A activated PTP activity, as vanadate treatment dramatically increased phosphorylation of receptors containing site A and restored binding of Grb2 to NT-YAB (Figures 4.6 and 4.7). The identity of the site A "effector molecule" is as yet unknown. The most likely candidate would be PLCγ as it binds the collinear phosphorylation site on the EGFR and in the EGFR PLCγ appears to function as a negative regulator of signaling (Rotin *et al.* 1992; Obermeier *et al.* 1996; Chen *et al.* 1996b). PLCγ may signal receptor dephosphorylation in at least two ways. First PKC mediated phosphorylation of Neu in the juxtamembrane region leads to a decreased phosphorylation of the receptor (Dobashi *et al.* 1989; Cao *et al.* 1991; Marte *et al.* 1995; Altiok *et al.* 1995). Alternatively, inositol tris-phosphate (IP_3) itself may activate a tyrosine phosphatase. In this regard, it is interesting that *shp-1* expression inhibits activated Neu-mediated

transformation (Figure 5.11) and that anionic phospholipids, such as phosphatidylinositol, activate Shp-1 phosphatase activity *in vitro* several thousand fold (Zhao *et al.* 1993). *shp-1* inhibition of Neu-mediated transformation required catalytic activity and appeared to be genetically "upstream" of Ras. Moreover, *shp-1* coexpression had little effect on transformation by the add-back mutants YB through YE. Each of these mutants lack tyrosine 1028 (site A) and as such it is possible that *shp-1*-mediated repression requires activation from site A. This hypothesis predicts that the point mutant NT-A should be refractory to *shp-1*-mediate transformation repression and that the double add-back mutants such as NT-YAD would be sensitive to *shp-1* expression. Although I have been repeatedly unsuccessful at demonstrating Shp-1/Neu interactions, this complex has been found *in vivo* (Vogel *et al.* 1993) and *in vitro* Shp-1 binds to the EGFR at a site collinear with E (Keilhack *et al.* 1998). It is possible that while Shp-1 may bind to site E, its elevated catalytic activation requires signals from site A. Clearly, phosphatase assays can be used to assess this notion.

6.4 ErbB-2 as a rational target for therapeutic intervention

That Neu can mediate several independent transforming signals which seem to culminate in Ras activation suggests attempts to block individual SH2/PTB-Neu interactions would prove futile. As such there are two rational drug targets for ErbB-2 overexpressing tumours, those being ErbB-2 and Ras. A number of methods have been recently employed to target ErbB-2 in mammary tumour derived cells. Antibodies which induce receptor downregulation have a marked effect on proliferation of ErbB-2 expressing mammary carcinoma lines in culture (Drebin *et al.* 1985; Hudziak *et al.* 1989; Xu *et al.* 1993; Pietras *et al.* 1994; Hurwitz *et al.* 1995; Klapper *et al.* 1997) as well as inducing increased cisplatin sensitivity (Hancock *et al.* 1991; Arteaga *et al.* 1994; Pietras *et al.* 1994). Recent phase II trials with humanized versions of these antibodies have demonstrated efficacy in node positive patients and in 12% of those treated reduced tumour load to less than 50% (Baselga *et al.* 1996). A second generation of ErbB-2 therapeutics have been based on the observation that ErbB-2 bound extracellular antibodies are often

internalized along with the receptor. This second generation of potential drugs have covalently linked bacterial endotoxins to downregulating antibodies. Thus when ErbB-2 expressing cells are treated with such agents there is a rapid internalization followed by cell death. These approaches have been proven effective in mouse tumour models using genetically engineered cells lines (Wels *et al.* 1995; King *et al.* 1996).

As several ErbB family members may be overexpressed in breast carcinoma, a means to simultaneously target more than one ErbB protein has been affected via the production of endotoxin-linked bivalent antibodies (Schmidt *et al.* 1996) or endotoxin-linked ErbB-family ligands (Fiddes *et al.* 1995). Unfortunately, the use of linked endotoxins would be predicted to kill non-transformed ErbB-2^{+} cells elsewhere in the body. Perhaps a more specific drug would be a chemotherapeutic molecule covalently linked (see Arap *et al.* 1998) to an internalizing antibody. This would be expected to specifically deliver a chemotherapeutic to cells expressing elevated ErbB-2 and killing only the mitotically active cells. Other methods include the use of antisense technologies to reduce the amount of receptor expressed (Vaughn *et al.* 1995) and ErbB specific kinase inhibitors (Osherov *et al.* 1993; Zhang and Hung 1996).

These data also suggest that Ras may serve as a logical target for therapeutic intervention in ErbB-2 overexpressing tumours. Although Ras mutations are infrequent in breast, ovarian and cervical carcinoma development and progression (Rochlitz *et al.* 1989; reviewed in Bos 1989), it is a logical target in ErbB-family-mediated carcinoma. Ras induced transformation requires that Ras be membrane associated through a covalently bound farnesyl lipid (section 1.5). Inhibition of this process through farnesyltransferase inhibitors effectively prevents Ras transformation (reviewed in Cox and Der 1997). Surprisingly, these inhibitors are not toxic in cell culture assays. This approach however would be cytostatic in that treatment would not be predicted to kill tumour cell only halt their growth as long as the therapeutic agent is present. Alternatively, a multipronged approach is in order. The use of anti-ErbB and anti-Ras therapeutics in combination may have synergistic effects when coupled with conventional treatments where appropriate.

While this study has yielded several intriguing results, revealing both positive and negative regulation of an oncogene, the molecular intricacy of Neu/ErbB-2 signaling will likely be realized only within the context of the other three family members and their ligands. Furthermore, dissection of signaling from Neu/ErbB-2 in the developing or adult organism will require the sophisticated manipulation of the murine genome but may provide insight into a variety of roles this gene serves in mammals. This information will prove useful in designing potent and specific drug targets of ErbB-2-induced carcinoma.

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