ROLE OF THE SRC FAMILY TYROSINE KINASES IN MAMMARY TUMORIGENESIS

By

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ABSTRACT

Although previous studies have shown that a large proportion of human breast cancers possess elevated c-Src kinase activity its role in mammary tumorigenesis is not well defined. Polyoma virus middle T antigen is known to activate members of Src family kinases (c-Src and c-Yes). Indeed, mammary gland specific expression of middle T antigen in transgenic mice is known to result in induction of mammary tumors. To test the role played by c-Src and c-Yes activation in mammary tumorigenesis we interbred transgenic mice expressing the middle T antigen with mice lacking either functional c-Src or c-Yes. Mice expressing middle T antigen in the absence of functional c-Src failed to develop mammary tumors while absence of c-Yes did not have any effect. This observation suggests that activation of c-Src kinase plays an important role in mammary tumorigenesis while c-Yes is dispensable for this process.

Activation of the RTK Neu is known to play an important role in breast cancer. Since c-Src tyrosine kinases appear to play a pivotal role in mammary tumorigenesis, I investigated whether the Src family members are involved in Neu mediated signaling and tumorigenesis. *In vitro* kinase assays revealed that c-Src and c-Yes kinase activities were elevated in Neu induced mammary tumors while Fyn kinase activity was not. The increase in kinase activity correlated with the ability of c-Src and c-Yes to associate with tyrosine phosphorylated Neu *in vivo* . This *in vivo* interaction is likely due to the ability of c-Src and c-Yes SH2 domains to directly associate with phosphorylated tyrosine(s) on Neu. Although other members of erbB

family also play a role in breast cancer, my results suggest that among the RTKs that are known to activate c-Src (EGFR and Neu), c-Src and c-Yes interact only with Neu and not EGFR. These observations suggest that Src family kinases are involved in Neu mediated signaling and tumorigenesis and also suggest that Neu plays a role in EGF mediated activation of c-Src.

Although both c-Src and c-Yes are activated by Neu and middle T antigen, only c-Src is required for middle T mediated tumorigenesis. Indeed c-Src associates with a novel protein, p89, in transformed epithelial cells while c-Yes does not. Future identification of p89 will enable us to understand the mechanism by which c-Src transforms mammary epithelium.

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CONTRIBUTIONS BY OTHERS

The experiments described in the following figures were performed by Chantale Guy, a fellow Ph.D. student in our lab.

the RNase protection analysis in Figure 3.3

the hematoxylin eosin stained tissue and tissue sections in Figure 3.4

the observations in Table 3.1 describing the tumor kinetics.

TABLE OF CONTENTS

CHAPTER 3: ACTIVATION OF THE C-SRC TYROSINE KINASE IS REQUIRED FOR THE INDUCTION OF MAMMARY TUMORS IN TRANSGENIC MICE

CHAPTER 4: MAMMARY TUMORS EXPRESSING THE *NEU* PROTO-ONCOGENE POSSESS ELEVATED C-SRC TYROSINE KINASE ACTIVITY

CHAPTER 5 DIRECT AND SPECIFIC INTERACTION OF C-SRC WITH NEU IS INVOLVED IN SIGNALING BY THE EPIDERMAL GROWTH FACTOR RECEPTOR

LIST OF FIGURES

CHAPTER 6

LIST OF TABLES

CHAPTER 1

INTRODUCTION

1.1 Growth Factors and their Receptors in mammary epithelial development

Mammary gland development in mice begins early during embryonic life. In the latter half of the 21 day gestation period, the epithelial cells of the mammary bud primordia proliferate to form the mammary cord or primitive duct which opens at the future nipple. During sexual maturation, the epithelial cells in ductal end structures called "end buds" undergo extensive proliferation. The ducts branch out and occupy the entire fat pad, leaving a zone of unoccupied fat between the ducts. Under proper hormonal stimulation during late pregnancy, this interductal space is occupied by "lobuloalveolar" structures which are formed by the proliferation of epithelial cells in the lateral buds that are spaced regularly along the side of the ducts. Following this morphological development, the epithelial cells undergo overt differentiation that results in the development of secretory epithelial cells which secrete milk proteins (Topper and Freeman, 1980).

Polypeptide growth factors are thought to play a role in the normal and neoplastic development of the mammary gland. For example surgical removal of the submandibular gland (sialoadenectomy), the major source of Epidermal Growth Factor (EGF) synthesis, results in reduced mammary gland growth and a 50% decrease in milk production. Conversely administration of EGF to these sialoadenectomised mice was able to rescue the mammary gland development (Okamoto and Oka, 1984). Consistent with these observations surgical implantation of slow-release cholesterol-based pellets containing either EGF or Transforming growth factor alpha (TGFα) in the mammary fat pad can stimulate lobuloalveolar development in a 4-5 week old virgin mammary gland (Vonderharr, 1987). Indeed, implantation of EGF containing Elvax pellets (ethylene/vinyl acetate polymer) in the mammary glands of ovariectomized mice results in formation of new end buds (Coleman et al., 1988). These observations suggest that EGF and TGFα have the ability to induce ductal as well as lobuloalveolar growth in the mammary gland. Further evidence supporting this view derives from transgenic mice ectopically expressing TGFα. Transgenic mice expressing TGFα from the tissue specific Mouse Mammary Tumor Virus (MMTV) promoter/enhancer, demonstrate a hyperplasia of the alveoli and terminal buds in virgin and pregnant transgenic mice (Matsui et al., 1990). Taken together these observations suggest that overexpression of these growth factors can induce neoplastic growth.

The EGFR family of cell surface receptors are also thought to play important role in normal morphogenesis and neoplastic growth of the mammary gland. The EGFR family of receptors include EGFR (Ullrich et al., 1984) erbB-2 (Neu) (Schechter et al., 1984, Coussens et al., 1985, King et al., 1985, Bargmann et al., 1986, Yamamoto et al., 1986), erbB-3 (Kraus et al., 1989, Plowman et al., 1990) and erbB-4 (Plowman et al., 1993a). These receptors possess a common structural organization, including an extracellular ligand binding domain consisting of two cysteine rich regions, a single transmembrane domain and a cytoplasmic domain harboring the highly conserved tyrosine kinase domain and cytoplasmic tail.

Recently it has been shown that mice mutant (*waved-2*) expressing a kinase defective form of EGFR possess lactation defect (Fowler et al., 1995). This observation suggests that a functional EGFR plays an important role in normal mammary gland development. However the role played by the other erbB family of receptors in normal mammary gland development is not yet known. Although the DNA sequence of the ligand for Neu (erbB-2) has not yet been determined, a protein which specifically induces receptor dimerization and kinase activity of Neu has been identified (Dobashi et al., 1991, Samanta et al., 1994). The Neu differentiation factor (NDF) or HRG (Holmes et al., 1992, Peles et al., 1992, Wen et al., 1992) is the ligand for both erbB-3 and erbB-4 (Plowman et al., 1993, CarrawayIII et al., 1994, Sliwkowski et al., 1994). The role played by any of these growth factors in mammary gland development remains to be addressed.

The hypothesis that EGF/EGFR signaling may be involved in supporting mammary tumor growth is further supported by the observati n that the observation that sialoadenectomised mice have reduced incidence of spontaneous mammary tumors (Kurachi et al., 1985, Tsutsumi et al., 1987). Consistent with this indirect evidence, EGFR expression has been detected in 45% breast cancer samples but is rarely due to gene amplification (Klijn et al., 1992) and EGFR expression does not seem to correlate with any particular histological type of cancer (Klijn et al., 1992).

Whether EGFR overexpression alone can induce mammary tumorigenesis is not known. However, overexpression and amplification of erbB-2 (Neu) has been observed in more than 20% of breast cancer patients and is correlated with poor clinical prognosis in node positive patients. ErbB-2 overexpression is known to be associated with camedo, large cell, ductal carcinoma (King et al., 1985, Slamon et al., 1987, Slamon et al., 1989, Paik et al., 1990, Gullick et al., 1991, Hynes and Stern, 1994).

Further evidence for the involvement of *neu* in the induction of mammary tumors derives from observations made with transgenic mice expressing a constitutively active form of Neu possessing a point mutation in the transmembrane domain (Bargmann et al., 1986), under the control of MMTV promoter/enhancer (Muller et al., 1988, Bouchard et al., 1989). In several of these transgenic strains, high level expression of activated *neu* resulted in the development of multifocal mammary tumors that affected every female carrier (Muller et al., 1988). Consistent with these studies, mice expressing the wild type *neu* transgene under the control of MMTV promoter/enhancer also develop mammary tumors (Guy et al., 1992b). However, in mice expressing wild type *neu*, the induction of mammary tumors correlates with the activation of the Neu tyrosine kinase that frequently occurs as a result of activating mutations in the transgene (Siegel et al., 1994). These observations suggest that the rate limiting step in the induction of mammary tumors expressing *neu* is the activation of its tyrosine kinase activity.

1.2 Src Homology 2 domain containing proteins in mammary tumorigenesis.

Activation of the receptor tyrosine kinase results in the phosphorylation of specific tyrosine residues in its cytoplasmic tail. These phosphorylated tyrosines serve as docking sites for intracellular signaling molecules with Src Homology 2 (SH2) domain. The SH2 domain is a module of approximately 100 amino acids in length and plays an important role in mediating protein-protein interactions (Pawson, 1995). The SH2 domains bind directly to phosphorylated tyrosine residues embedded in a protein motif (Matsuda, et al., 1990; Mayer and Hanafusa, 1990, Anderson et al., 1990, Koch et al., 1991). Although the SH2 domains bind directly to phosphotyrosine residue(s), it is believed that the amino acids immediately C-terminal to the phosphotyrosine +1 and +3 convey specificity and selectivity to the SH2 mediated binding to phosphorylated tyrosine (Songyang et al., 1993). Recent crystallographic analysis showed that the SH2 domain possesses a bipartite structure in which an invariant arginine forms a hydrogen bond with the phosphate atoms on the phosphorylated tyrosine residue. The second binding site is variable and makes contact with the amino acids C-terminal to the pTyr (Eck et al., 1993, Waksman et al., 1993, Pascal et al., 1994).

The SH2 domain is present in a variety of signaling proteins. One group of SH2 domain containing proteins are those that contain intrinsic enzymatic activity such as Src family of tyrosine kinases, Zap70, Tec kinase, phospholipase C gamma 1 (PLCγ1), protein tyrosine phosphatases (PTP1C, PTP1D, syp) and, GTPase activating protein (GAP) (Pawson, 1994, 1995).

Another group of proteins referred to as "adapter molecules" are those that do not possess any intrinsic biochemical activity but serve as adapters to transduce the signal from activated RTK to a non-SH2 containing protein. For example, p85 subunit of PI3'-kinase has no catalytic activity but upon binding to phosphorylated tyrosine on a receptor induces a conformational change that activates the kinase activity of the catalytic subunit p110 (Backer et al., 1992, Carpenter et al., 1993). Growth factor receptor binding protein (Grb2), Shc, Crk and, Nck are adapter molecules that link RTK activation to ras signaling pathway (Matsuda et al., 1994, Pawson, 1994, Hu et al., 1995). Some of these adapter molecules such as Grb2 and Nck exist in a constitutive complex with a guanine nucleotide exchange factor (mSos) even in the cytoplasm of unstimulated fibroblasts (Gale et al., 1993, Li et al., 1993, Rozakis-Adcock et al., 1993, Hu et al., 1995). Upon ligand activation this complex is recruited proximal to the plasma membrane due to the ability of the SH2 domains in the adaptor molecules to interact with phosphorylated tyrosines on the receptor. This adaptor/Sos complex catalyses the exchange of GTP for GDP in ras molecule and this conversion of ras-GDP to ras-GTP activates the Ras function. Recent observations indicate that the adaptor molecule Shc contains a novel pTyr binding domain (PTB) of approximately 200 amino acids (Kavanaugh and Williams, 1994). PTB domains appear to recognize a N-P-X-Y motif and seem to play an important role in the interaction between Shc and activated growth factor receptor erbB-2 (Kavanaugh et al., 1995). The physiological significance of this domain is yet to be determined. The SH2 domains are also present in transcription factors (signal transducers and activators of transcription, STAT) that are thought to be activated by cytokines and growth factors through their cognate receptors (Darnell et al., 1994).

A number of SH2 domain containing proteins are thought to play role in human breast cancer. For example increased expression of the adapter molecules Grb2 (Daly et al., 1994), Grb7 (Stein et al., 1994b) and PLCγ1 (Arteaga et al., 1991) have been observed in primary breast cancers. There has also been several reports suggesting that a large proportion of human breast cancers possess elevated c-Src tyrosine kinase activity (Jacobs et al., 1983, Rosen et al., 1986; Ottenhoff-Klaff et al., 1992).

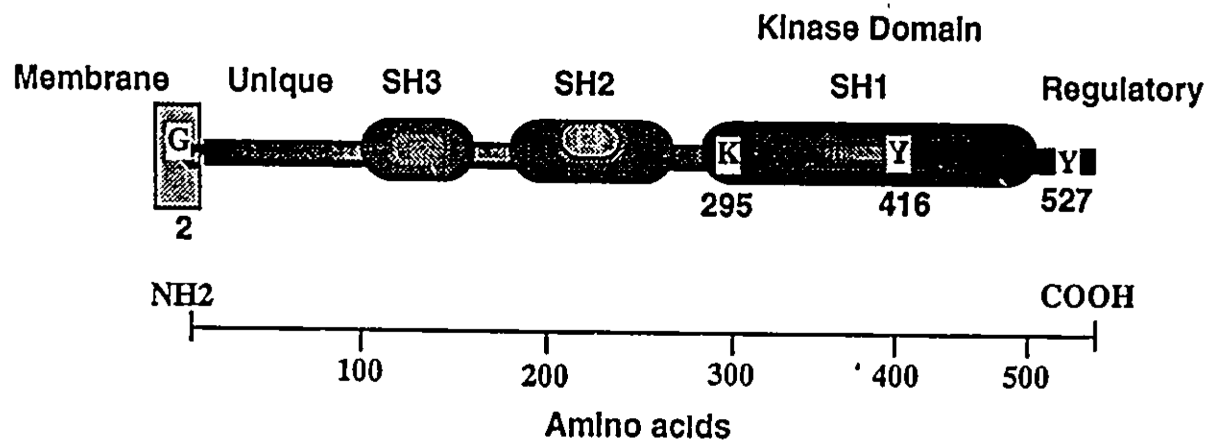
1.3 The Src family of protein tyrosine kinases

Members of the Src family belong to the non-receptor protein tyrosine kinase (NRPTKs) class of signaling molecules. The prototype of the this class, c-*src*, was identified as a cellular homologue of the transforming protein v-Src encoded by Rous Sarcoma virus (Stehelin et al., 1976, Brugge and Erickson, 1977). The subsequent identification that v-Src possesses intrinsic tyrosine kinase activity provided the first evidence that tyrosine kinases are directly involved in cellular transformation (Hunter and Sefton, 1980). Within two decades of the discovery of *c-src*, eight additional Src related genes have been identified, with molecular weights ranging from 55 to 62 kDa [*c-yes, fyn, c-fgr, lyn, lck, hck, blk* and *yrk*] (Superti-Furga and Courtneidge, 1995).

The members of the Src family share a common structural organization (Figure 1.1). All members of the Src family are associated with

Figure 1.1

Structural organization of the Src family members. A schematic diagram of c-Src protein tyrosine kinase showing major functional domains of the molecule. The numbers 2, 295, 416 and 527 correspond to the amino acid number of Glycine (G), Lysine (K), Tyrosine (Y) and Tyrosine respectively, see text for details. Tyr (Y) 416 is not discussed. SH represents Src Homology.



Kinase Domain
Membrane
Unique
SH3
SH2
SH1
Regulatory
G
K
Y
Y
2
295
416
527
NH2
COOH
100
200
300
400
500
Amino acids

the inner face of the cytoplasmic membrane via myristoylation of a glycine residue at position two (Schultz et al., 1985). Because mutations that interfere with myristoylation of v-Src are transformation defective, the membrane localization of v-Src is critical for its transforming properties (Cross et al., 1984; Kamps et al., 1985; Pellman et al., 1985). Recent observations have demonstrated that in addition to the glycine residue at position two, amino-terminal lysines at positions five, seven and nine forming a Gly-X-X-Lys-X-Lys-X-Lys motif also plays an important role in membrane binding of the Src family members (Silverman and Resh, 1992, Silverman et al., 1993)

Following this amino terminal sequence is a region of 50 to 80 amino acids that is unique to each member of the Src family (Parsons and Weber, 1989). This domain is thought to play a role in mediating protein-protein interactions. For example, the interaction between Lck and T cell surface molecules CD4 and CD8, and the association between Fyn and multiple units of CD3/TCR complex has been shown to occur through the N-terminal unique region of the Src family members (Shaw et al., 1990; Turner et al., 1990; Gauen et al., 1992).

The unique region is followed by three distinct domains of homology termed the Src homology 1 (SH1), Src homology 2 (SH2) and Src homology 3 (SH 3) regions (Sadowski et al., 1986). The SH1 is the catalytic tyrosine kinase domain that is conserved amongst all the members of protein tyrosine kinases including Abl, Fps/Fes, Tyk2, Syk/Zap, FAK, and Csk (Bolen, 1993). For a detailed description of the SH2 domain refer to section 1.2.

The SH3 domain is about 50 amino acids long and is also involved in mediating protein-protein interactions through a proline rich motif (Ren et al., 1993). All high affinity SH3 ligands identified so far contain a Pro-X-X-Pro motif (Ren et al., 1993, Yu et al., 1994). The functional significance of the SH2 and the C-terminal regulatory domain (Figure 1.1) in regulating the protein tyrosine kinase activity of the Src family members will be discussed below.

1.3.1 Regulation of Src protein tyrosine kinase activity

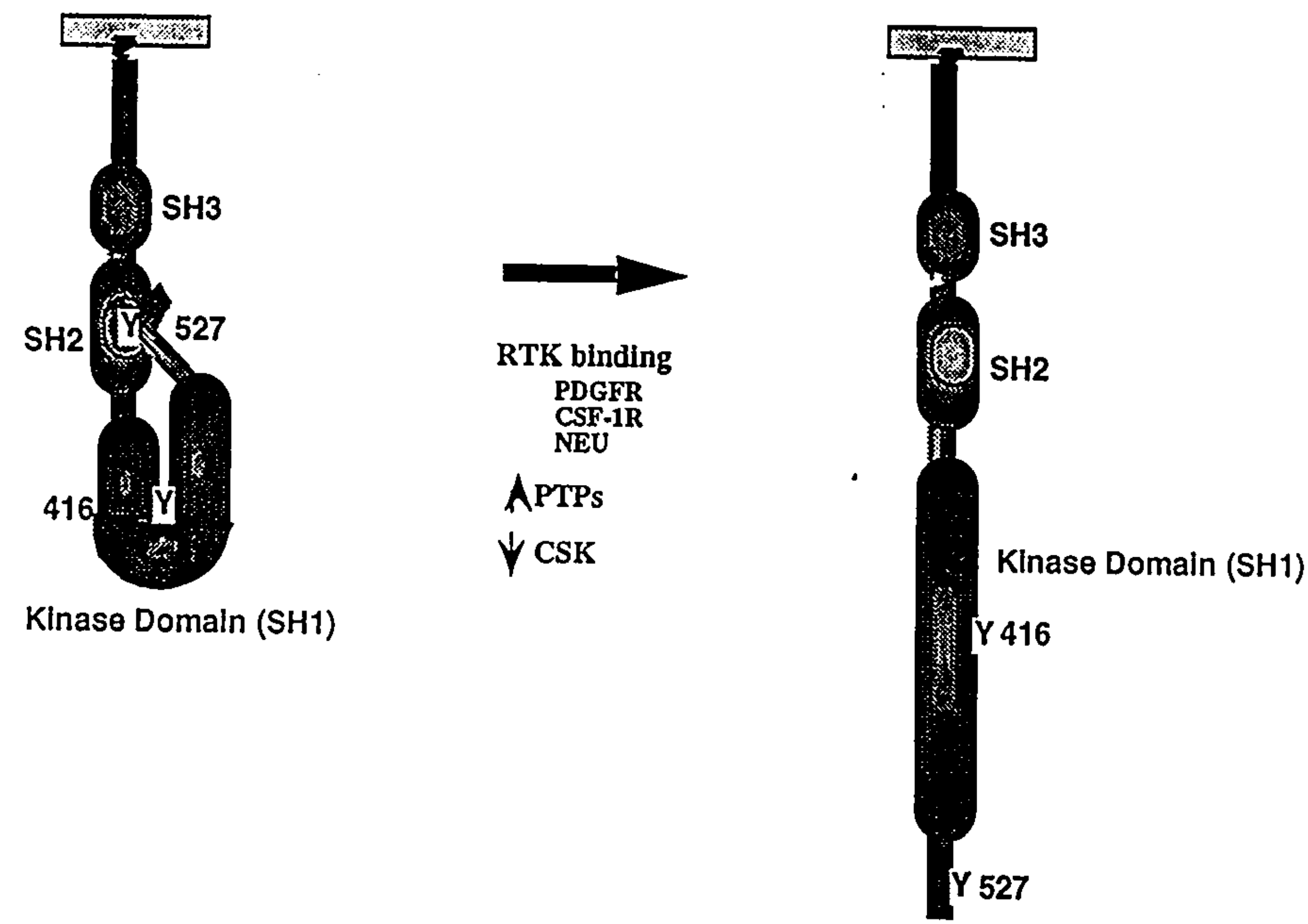
Although *v-src* was initially identified as a transforming gene, overexpression (app. 15 fold above the transforming levels of *v-src*) of its normal cellular homologue c-Src does not transform cells in culture (Parker et al., 1984, Iba et al., 1984, Shalloway et al., 1984, Tanaka and Fujita, 1986). However, (> 15 fold) overexpression of the wild type chicken c-Src can induce a partial transformation (focus formation without anchorage-independent growth or tumorigenesis) (Johnson et al., 1985). Interestingly, v-Src possesses at least ten fold higher *in vitro* protein kinase activity than c-Src (Iba et al., 1985, Coussens et al., 1985). The differences in the enzymatic activity of the virally encoded protein and the chicken cell encoded c-Src correlated with a number of mutations in the former (Takeya and Hanafusa, 1983, Hunter, 1987). In particular, a 19 amino acid sequence located in the carboxyl terminus of c-Src was replaced by an unique 12 amino acid sequence in v-Src (Takeya and Hanafusa, 1983). Replacing the unique C-terminal 12 amino acid of v-Src with the C-terminal 19 amino acid sequence of c-Src resulted in a reduction in the transforming ability of v-Src (Iba et al., 1984; Wilkerson et al., 1985). The importance of this region was further highlighted by the observation that two independent avian sarcoma virus isolates S1 and S2 have deletions involving the C-terminal amino acids (Ikawa et al., 1986).

The functional significance of the C-terminus was revealed by the observation that dephosphorylation of a tyrosine residue in the C-terminal half of c-Src leads to an increase in its tyrosine kinase activity (Courtneidge, 1985). This regulatory tyrosine was later identified as Tyr 527 in chicken c-Src (Cooper et al., 1986). Mutation of the Tyr 527 to Phe has been shown to increase both the transforming activity as well as the intrinsic kinase activity of c-Src (Piwnica-Worms et al., 1987, Cartwright et al., 1987, Kmiecik and Shalloway, 1987).

Phosphorylation of Tyr 527 in c-Src is thought to result in an intramolecular association between the SH2 domain and Tyr 527. As a consequence of this association, the catalytic activity of c-Src is repressed (Figure 1.2). Indirect support for this model derives from the observation that mutation or deletion of the SH2 domain of c-Src activates its kinase activity and results in cellular transformation (Hirai and Varmus, 1990, O'Brien et al., 1990, Seidel-Dugan et al., 1992). Further evidence supporting this model of c-Src regulation derives from the demonstration that phosphopeptides corresponding to the C-terminus of c-Src bind to activated but not to unactivated c-Src molecule (Roussel et al., 1991, Cobb and Parsons, 1993). More recently it was demonstrated that the c-Src SH3 domain may also play a role in the inhibition of c-Src kinase activity. It is

Figure 1.2

A potential model for the regulation of the tyrosine kinase activity of Src family members. The inactive molecule is shown on the left and the active molecule is shown on the right. See text for details. RTK: Receptor Tyrosine Kinase; PDGFR: Platelet Derived Growth Factor Receptor; CSF-1R: Colony Stimulating Factor-1 Receptor; PTPs: Protein Tyrosine Phosphatases; CSK: C-terminal Src Kinase.


SH3
SH2
Y
527
416
Y
Kinase Domain (SH1)
RTK binding
PDGFR
CSF-1R
NEU
PTPs
CSK
SH3
SH2
Kinase Domain (SH1)
Y 416
Y 527

thought that the phosphorylated Tyr 527 makes contact with the Src SH2 domain and this interaction is further stabilized by the Src SH3 domain (Murphy et al., 1993, Okada et al., 1993, Superti-Furga et al., 1993).

Interestingly, the amino acids around the Tyr 527 constitute a low affinity binding site for the Src SH2 domain (Songyang et al., 1993). Given the low affinity of the Src SH2 domain for the Tyr 527 site it is conceivable that phosphotyrosine residues located on other signaling molecules can compete with Tyr 527 for its interaction with the Src SH2 domain and thus provide a means for regulation of c-Src tyrosine activity by protein-protein interaction.

1.3.3 Proteins that negatively regulate c-Src kinase

The observation that a kinase defective c-Src mutant (Lys295Arg), when expressed in cells that lack functional c-Src, is also phosphorylated at Tyr 527 (Thomas et al., 1991) suggests that phosphorylation of Tyr 527 is not an autophosphorylation event but rather is carried out by a distinct tyrosine kinase. Consistent with this expectation, a carboxyl-terminal Src kinase (Csk) which specifically phosphorylates the Tyr 527 has been identified (Okada and Nakawada, 1989, Nada et al., 1991, Sabe et al., 1992). Germline inactivation of *csk* results in an 11 fold increase in the activity of the c-Src family of kinases and an embryonic lethal phenotype (Imamato and Soriano, 1993, Nada et al., 1993). However, phosphorylation of the C-terminal tyrosine (Tyr 527) of c-Src was still observed in cells lacking a functional Csk, implying that a family of Csk related kinases may exist

(Imamato and Soriano, 1993, Nada et al., 1993). Consistent with this notion novel Csk related kinases, leukocyte carboxyl-terminal *src* kinase(*lsk*) and, Ntk were recently isolated (McVicar et al., 1994, Chow et al., 1994). Csk has also been shown to be involved in regulating the activity of other members of the Src family (Okada et al., 1991, Bergman et al., 1992). Whether the Csk family of kinases has any role to play in regulating the kinase activity of Src family members during tumorigenesis remains to be assessed.

1.4.1 Activation of Src family kinases by RTKs

The activity of c-Src has been shown to be elevated in a major proportion of colon carcinomas (Bolen et al., 1987, Cartwright et al., 1990; Talamonti et al., 1993). Since activating mutations have not been detected in these tumors, activation of c-Src occurs by a mechanism that does not involve somatic mutations (Wang et al., 1991). There is considerable evidence to suggest that activation of c-Src kinase activity can occur through its interaction with either cellular or viral proteins.

Cellular proteins that belong to the receptor protein tyrosine kinases family, such as PDGF receptor (PDGFR) (Kypta et al., 1990), and the Colony stimulating growth factor-1 receptor (CSF-1R) (Courtneidge et al., 1993) are known to associate with Src family members. This association is thought to be mediated by the interaction between the SH2 domains of Src family members and one or more autophosphorylated tyrosines in the cytoplasmic domain of the receptor molecule (Twamley et al., 1992, Courtneidge et al., 1993). Recent evidence suggests that the tyrosine residues at position 579 and 581 in the PDGFR are responsible for interaction between the RTK and Src family members (Mori et al., 1993). The importance of the association of c-Src with receptor tyrosine kinases has recently been demonstrated by the observation that micro injection of fibroblasts with either dominant negative mutants of c-Src or antibodies that bind to and inactivate the Src family members effectively ablates PDGF mediated mitogenesis (Twamley-Stein et al., 1993). In addition to PDGFR c-Src is thought to be involved in Fibroblast growth factor-1 (FGF-1) mediated signaling. Like PDGFR c-Src associates with FGF-1 receptor upon FGF-1 stimulation of NIH3T3 cells through the c-Src SH2 domain (Zhan et al., 1994).

It is believed that PDGF or CSF-1 mediated activation of c-Src, c-Yes and Fyn tyrosine kinase activity is likely due to the ability of Src family members to associate with the tyrosine phosphorylated receptor molecule via their SH2 domain (Mori et al., 1993). Such an interaction would release the C-termini-mediated repression and result in catalytic activation of the kinase.

1.4.2 Viral proteins that activate c-Src

Viral proteins are also known to activate Src family of kinases. Polyoma virus (PyV) middle T antigen specifically associates with and activates the tyrosine kinase activity of c-Src family members (c-Src, c-Yes) (Courtneidge and Smith, 1983; Kornbluth et al., 1987). Moreover formation of these complexes appears to be critical for PyV middle T antigen to transform cells (Courtneidge and Smith, 1984; Cheng et al., 1986; Cook et al., 1990). PyV middle T antigen also associates with another ubiqutiously expressed Src family member, Fyn, but this association does not lead to an elevation in tyrosine kinase activity of Fyn (Kypta et al., 1988, Cheng et al., 1988a). Fine structure mapping of c-Src and PyV middle T interaction indicated that a region bounded by residues Asp-518 and Pro-525 in c-Src is required for complex formation (Cheng et al., 1988b). This association of c-Src to middle T antigen may facilitate the dephosphorylation of Tyr 527 by preventing the intramolecular interaction between the SH2 domain of c-Src and the Tyr 527. Interestingly, the Hamster Polyoma virus (HaPyV) middle T antigen does not associate with either c-Src or c-Yes while it binds especially to Fyn. The Fyn tyrosine kinase that is associated with HaPyV middle T antigen has elevated intrinsic kinase activity compared to that of the unbound Fyn (Courtneidge et al., 1991). The difference in ability of these middle T antigens to bind and activate different members of the Src family of kinases correlates with their distinct tumor profiles. HaPyV induces leukemia and lymphoma with short latency while the mouse PyV induces a wide range of tumors but never causes leukemia or lymphoma (for a review see Kiefer et al., 1994).

1.4.3 Activation of c-Src family by cell adhesion molecules

There is considerable evidence to suggest that c-Src is involved in signaling mediated by cell adhesion. Binding of cell surface integrins to their ligands in the extracellular matrix induces tyrosine phosphorylation of a focal adhesion kinase (FAK) and creates a binding site for c-Src (Cobb et al., 1994, Schaller et al., 1994, Xing et al., 1994) and Fyn (Cobb et al., 1994). This association is likely mediated by the SH2 domains of c-Src and Fyn (Cobb et al., 1994, Schaller et al., 1994, Xing et al., 1994). Recently Tyr 397 in FAK was shown to be the major autophosphorylation site and the binding site for c-Src SH2 domain (Schaller et al., 1994, Eide et al., 1995). Once activated c-Src in turn phosphorylates FAK (Calalb et al., 1995) at tyrosine residues. These tyrosine residues may in turn be involved in SH2 mediated binding of other signaling proteins.

1.4.4 Activation of c-Src by protein tyrosine phosphatases

Activation of c-Src can also occur through activation of specific protein tyrosine phosphatases (PTPases). Overexpression of a receptor-like protein tyrosine phosphatase (PTPα) in rat embryo fibroblasts results in dephosphorylation of Tyr 527 and activation of c-Src tyrosine kinase activity (Zeng et al., 1992). These data suggest that PTPα may be the tyrosine phosphatase responsible for dephosphorylating tyrosine 527.

However, it is likely that in any given cell type the kinase activity of Src family members is regulated by more than one mechanism.

1.5 Elevation of c-Src kinase activity in primary mammary tumors and tumor derived cell lines

Although expression of *c-src* at high levels does not induce transformation, the activation of c-Src is thought to play an important role in mediating cell proliferation (see section 1.4). In fact a number of primary tumors and tumor derived cell lines including breast, colon, melanoma and sarcoma have all been shown to possess elevated c-Src tyrosine kinase activity (Jacobs, and Rubsamen, 1983, Rosen et al., 1986, Barnekow et al., 1987, O'Connor et al., 1992, Ottenhoff-Klaff et al., 1992, Talamonti et al., 1993). Of these tumor types, activation of c-Src tyrosine kinase activity is thought to be a particularly common event in the genesis of breast carcinomas. Almost all the primary breast tumors tested exhibit elevated c-Src kinase activity when compared to normal breast tissues (Jacobs, and Rubsamen, 1983, Rosen et al., 1986, Ottenhoff-Klaff et al., 1992). In one of these studies the increase in c-Src kinase activity results from an increase in the specific activity of c-Src (Rosen et al., 1986).

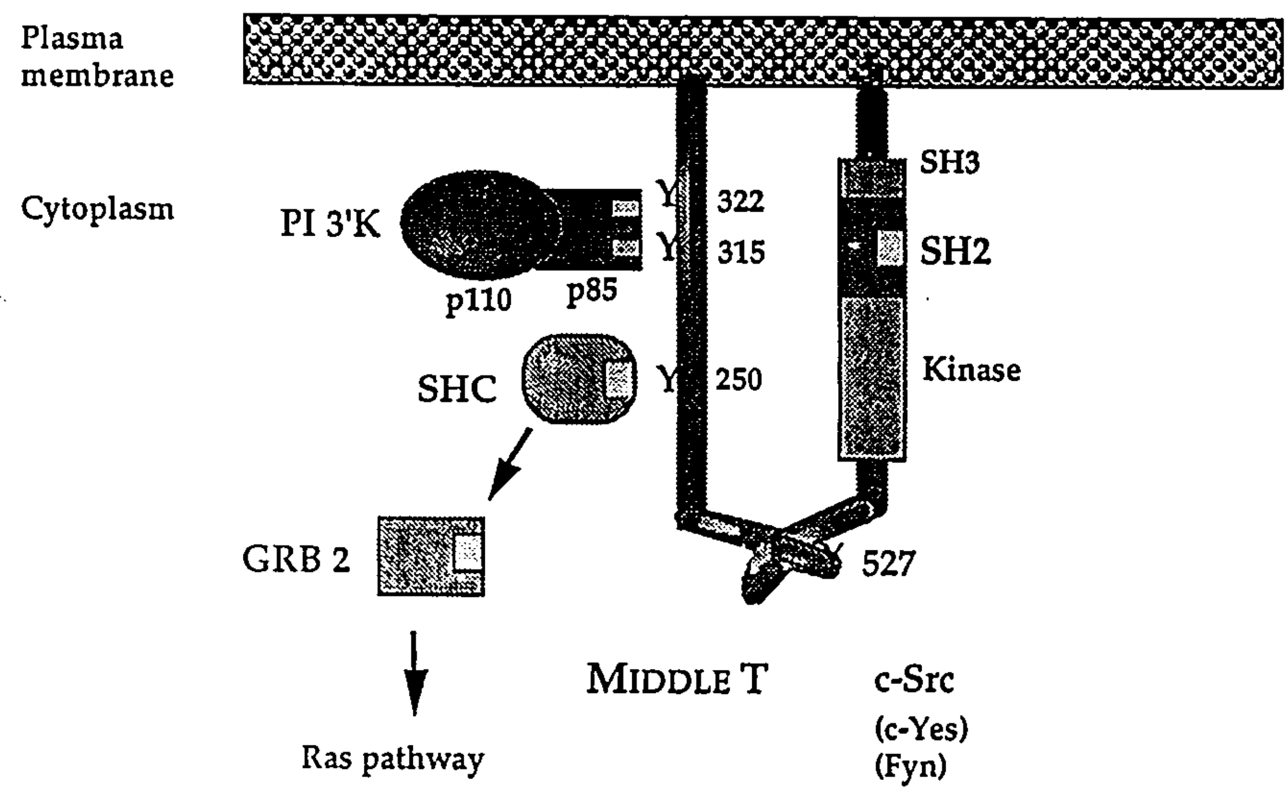
Overexpression of v-Src in mammary epithelial cells (IM2) results in loss of epithelial characteristics and loss of lactogenic hormone-induced terminal differentiation. IM2 cells overexpressing v-Src also lose their ability to repress the AP1 activity upon lactogen-mediated signal to differentiate (Jehn et al., 1992), suggesting that high levels of Src tyrosine kinase activity would repress the differentiation process by promoting proliferative signals.

1.6 Transgenic mouse models for testing the role of Src family in mammary tumorigenesis

Further evidence supporting the involvement of c-Src in mammary tumorigenesis derives from observations made with transgenic mice expressing the PyV middle T oncogene. Expression of PyV middle T under the control of the MMTV promoter/enhancer in transgenic mice leads to the development of multifocal mammary tumors that metastasize to the lung with high frequency (Guy et al., 1992a). The observation that mammary gland specific expression of PyV middle T antigen induces metastatic disease argues that the signaling pathways activated by PyV middle T are involved in promoting tumorigenesis. In fact, these mammary tumors possessed elevated c-Src and c-Yes kinase activities due to their association with the PyV middle T antigen (see chapter 3). In addition to the ability of PyV middle T to associate with and activate members of the Src family, middle T antigen is also known to associate with other cytoplasmic signaling molecules such as the P85 subunit of PI3' kinase (Courtneidge and Hebner, 1987; Whitman et al., 1985), protein phosphatase PP2A (Pallas et al., 1990; Walter et al., 1990), to an adapter molecule SHC which links tyrosine kinases to the ras signaling pathway (Dilworth et al., 1994) and to 14-3-3 proteins that are thought to play a role in activation of the protein kinase Raf (Pallas et al., 1994, Aitken, 1995) (Figure 1.3).

Figure 1.3

Cellular proteins that associate with polyoma virus middle T antigen. The middle T antigen associated tyrosine kinase activity is due to its ability to associate with the Src family members. Middle T antigen is phosphorylated on specific tyrosine residues by the Src/middle T complex. The Tyr 315 has been shown to mediate binding of the p85 subunit of the PI3'K, while the Tyr 250 mediates interaction between middle T and the shc/Grb2/mSos complex which in turn activate ras.



Plasma
membrane
Cytoplasm
PI 3'K
p110
p85
SHC
GRB 2
Ras pathway
Y
322
Y
315
Y
250
527
MIDDLE T
SH3
SH2
Kinase
c-Src
(c-Yes)
(Fyn)

1.6.1 c-Src is required for PyV middle T-mediated mammary tumorigenesis.

Although it is clear that activation of multiple signal transduction pathways by PyV middle T antigen plays a role in middle T induced mammary tumorigenesis, the relative contribution of these pathways to the transformed phenotype is unclear. Direct evidence supporting the involvement of c-Src in PyV middle T antigen mediated mammary tumorigenesis and metastases derives from the results of a study involving interbreeding of the MMTV/PyV middle T strains with mice bearing a germline mutation in c-Src or c-Yes. In chapter 3 of this thesis I will discuss results obtained from this study.

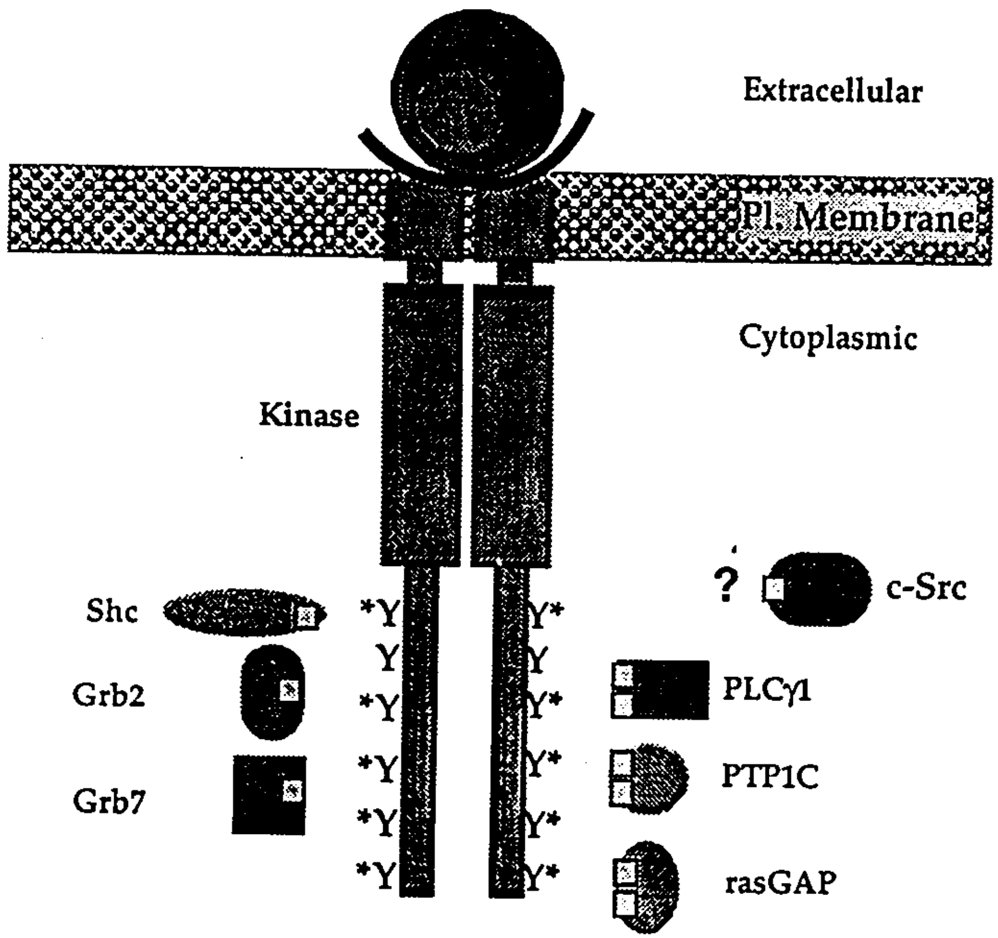
1.6.2 Involvement of c-Src and c-Yes in Neu-mediated mammary tumors

Receptor tyrosine kinases such as PDGFR and CSF-1R have been shown to activate the Src family of kinases (Kypta et al., 1989, Courtneidge et al., 1993). The erbB family of receptor tyrosine kinases are known to play an important role in human breast cancer (see section 1.1). Although evidence from transgenic mice and human breast tumor samples clearly suggests that mammary epithelium is sensitive to the activation of Neu (erbB-2) RTK, the actual mechanism by which Neu induces mammary tumorigenesis is not yet understood.

A number of intracellular signaling molecules such as Phospholipase C-γ1 (Fazioli et al., 1991, Peles et al., 1991, Segatto et al., 1992, Jallal et al., 1992), GTPase activating protein (GAP) (Fazioli et al., 1991, Jallal et al., 1992), Shc (Segatto et al., 1993, Kavanaugh et al., 1995), Grb2 (Janes et al., 1994), and Grb7 (Stein et al., 1994b) are involved in Neu signaling pathways (Figure 1.4). In chapters 4,5 and, 6 of this thesis I present evidence to demonstrate that c-Src and c-Yes, but not Fyn, kinase activity is elevated in Neu-induced mouse mammary tumors and this activation is likely due to the ability of SH2 domains of c-Src and c-Yes to interact directly with tyrosine phosphorylated Neu. These observations suggest that activation of c-Src and c-Yes tyrosine kinase may play a role in Neu-mediated mammary tumorigenesis.

Figure 1.4

Interaction between Neu and SH2 containing signaling proteins. Activation of the receptor either by mutation or by ligand leads to dimerization and activation of the tyrosine kinase activity. The receptor molecules in the dimer are thought to cross phosphorylate each other at specific tyrosine residues at the carboxyl tail. The adaptor molecules such as Grb2, shc, and Grb7 are known to bind directly to the receptor through their SH2 domain. Protein with enzymatic activity such as PLCγ 1, Protein tyrosine phosphatase 1C and ras GTPase activating protein were also shown to bind to Neu RTK. In this thesis I investigate whether c-Src is involved in the Neu signal transduction pathway.


Extracellular
Pl. Membrane
Cytoplasmic
Kinase
?
c-Src
Shc
Grb2
Grb7
PLCγ1
PTP1C
rasGAP
*Y
Y
Y*

CHAPTER 2

MATERIALS AND METHODS

2.1 DNA constructs.

Bacterial fusion protein bearing the SH2 domain of c-Src (amino acids 141 to 266) fused to glutathione-S-transferase (GST) was a generous gift of B. Rowley and J. Bolen (Bristol-Myers Squibb, New Jersey). The GAP SH2 fusion DNA contains both SH2 domains flanking the SH3 domain (amino acids 181 to 451) and was inserted as an EcoR1 fragment in pGEX 3X (Pharmacia). The GSTag SH2 fusion for c-Src was constructed by sub cloning a BamH1/EcoR1 fragment containing the SH2 domain from GST-c-Src SH2 (Muthuswamy et al., 1994) (amino acids 141 to 266) into a BamH1/EcoR1 site in pGSTag (Ron and Dressler 1992). pGSTag-GRB7 was a generous gift of Ben Margolis. The construction of MMTV promoter/enhancer activated Neu fusion gene (pMMTV/Neu NT) has been described previously (Muller et al., 1988).

2.2 Cell lines

Rat 2 fibroblast cell lines expressing activated Neu under MMTV transcriptional control were established by transfecting pMMTV/*neu* NT plasmid, that contains neomycin as a selectable marker, into Rat-2 cells. G418 resistant clones were isolated and two representative clones were used for further studies (NT.11 and NT.12). To induce the expression of activated *neu*, the NT.12 cells were grown in the presence of dexamethasone (final concentration 10^{-6} M) for a period of 24 to 36 hours. Fibroblast cells expressing PyV middle T antigen was derived by transfecting Rat 2 cell with pSV2-middleT plasmid. G418 resistant clones were selected and a clone mT.3 was used for this study. Mouse mammary epithelial cell line NAFA was derived from an activated *neu*-induced mouse mammary tumor (Muller et al., 1988) and was grown in modified DMEM containing 10% fetal bovine serum. Mammary epithelial cell line 1A2 was derived from a PyV middle T antigen induced mouse mammary tumor (provided by Christina Addison and Frank Graham), and was grown in RPMI containing 10% fetal bovine serum and 1x GMS-X (GIBCO BRL). Cell lines overexpressing human EGFR, R1/hER (Wasilenko et al 1991, kindly provided by Michael Weber) and A431 cells (ATCC) were induced with EGF (GIBCO BRL) (100ng per ml) for one minute, prior to lysis. Human breast cancer derived cell lines were obtained from ATCC and were cultured as recommended. All cell lines were grown in 100mm plates to confluency (5-6 x 10^6 cells) prior to lysis.

2.3 Antisera

A variety of antisera were used during these studies. Anti-Src (MAb327, epitope: SH3 domain, Oncogene Sci,), Anti-Src (MAb 7D10, epitope: aa 2-17 in the unique region, (Quality Biotech), Anti-Src (MAb GD11, epitope: aa 88-123, Upstate Biotech Inc.), Anti-Yes (polyclonal, yab-2, epitope aa 4-20 in the unique region, see below), Anti-Yes (polyclonal, epitope aa 12-78, generous gift of J. Bolen) Anti-Fyn (polyclonal, epitope aa 28-48, Santa Cruz Biotechnology Inc.), anti-Neu (MAb3, Oncogene Science), anti-Yes antibodies that cross react with both Src and Yes (generous gift from Marius Sudol), anti-phosphotyrosine (MAb 4G10, Upstate Biotechnology, Inc.), anti-EGFR (MAb, epitope aa 996-1022, Transduction Labs), anti-erbB-3 (MAb, epitope aa 1307-1323, C-17, Santa Cruz Biotechnology Inc.) or anti-erbB-4 (MAb, epitope aa 1291-1308, C-18, Santa Cruz Biotechnology Inc.)

Anti-Yes antiserum, yab-2, was generated by using a synthetic 16 amino acids long peptide (aa 4-20 murine c-Yes, IKSKEKSPAIKYTPEN) coupled to KLH (synthesized at Mobix central facility, McMaster University). Five month old male rabbits were given intramuscular injection with 500μg of antigen in 1:1 complete Freud's adjuvant. First boost was given four weeks after the initial injection with 500μg of antigen in 1:1 incomplete Freud's adjuvant. Subsequent boosts were given at four week intervals. Bleeds were collected two weeks after every boost.

2.4 Immunoprecipitation

2.4.1 Immunoprecipitation using mammary tissue samples

Tissue samples were ground to a powder under liquid nitrogen and lysed in TNE lysis buffer (50 mM Tris HCl pH7.6; 150 mM NaCl; 1% NP40; 2 mM EDTA; 1 mM sodium orthovanadate; 10 μg/ml leupeptin; 10 μg/ml aprotinin; 5 μg/ml TLCK; 10 μg/ml TPCK). The lysates were cleared by centrifugation at 12,000 xg for 10 min. at 4°C.

Immunoprecipitations were performed by incubating 1.0 to 1.5 mg of the protein lysate with 0.5 to 1.0 μg of respective antisera and 30 to 40 μl of Protein G Sepharose fast flow (Pharmacia) in a final volume of 500-600 μl for 2.0 hours at 4°C on a rotating platform. Following incubation, the Protein G Sepharose beads were washed four to five times with respective lysis buffer and used for further analysis. In some instances the NaCl concentration of the lysis buffer used for this wash was modified.

2.4.2 Immunoprecipitation using cell lysates

Cells in culture plates were rinsed three times with ice-cold 1x PBS (140 mM NaCl; 2.7 mM KCl; 4.3 mM Na_2HPO_4; 1.4 mM KH_2PO_4; 1mM sodium orthovanadate) and lysed in 750 μl of lysis buffer. Cells were lysed in TNE when the lysates were to be used for *in vitro* kinase assays, whereas for all coimmunoprecipitation analyses cells were lysed in low salt CHAPS lysis buffer [50 mM Tris (pH 8.0); 0.7% CHAPS; 50 mM NaCl; 1 mM sodium orthovanadate, 10 μg/ml leupeptin; 10 μg/ml aprotinin]. The cells were lysed for 20 to 25 min. on ice and the lysates were cleared by centrifugation at 12,000 xg at 4°C for 20 minutes. All the coimmunoprecipitation studies were done using fresh lysates. Immunoprecipitations were done as described above.

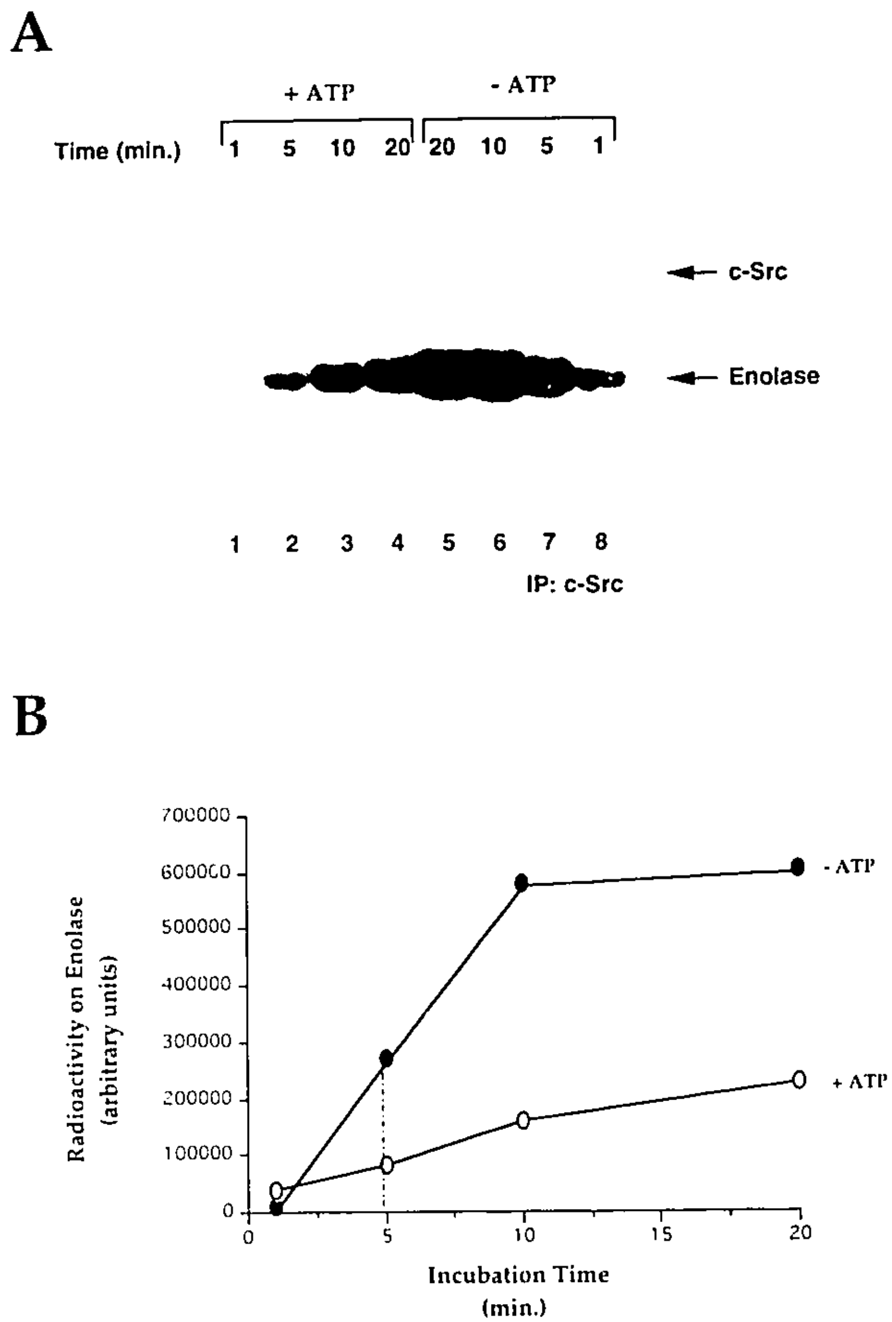
2.5 *in vitro* kinase assays

In order to identify the time of incubation required to obtain linear incorporation of γ-^{32}P ATP into c-Src, brain tissue lysates were prepared as described above and 600μg of protein was used. c-Src was immunoprecipitated and the immunoprecipitates were washed four times with TNE lysis buffer and once with 2 x kinase buffer (200 mM HEPES pH7.0; 10 mM $MnCl_2$). The immunoprecipitates were resuspended in 20μl of mix containing 10μl of 2 x kinase buffer; 1μl of (γ-^{32}P) ATP (>45000 Ci/mmol, 1.0 mCi/ml, Dupont, NEN) and 10 μg of acid denatured enolase in 10μl (Figure 2.1A, lanes 5-8). A parallel set of reactions were carried out with 2x kinase buffer supplemented with 20μM cold ATP (Figure 2.1A, lanes 1-4). Reaction were carried out for 1,5,10, or 20 minutes at room temperature. The reaction was terminated by adding an equal volume of 2x SDS gel loading buffer (1x: 62.5 mM Tris HCl pH6.8; 2% SDS; 5% glycerol; 0.7M 2-mercaptoethanol; 0.25% bromophenol blue) and was separated on a 10% SDS-PAGE. As shown on Figure 2.1A there was direct correlation between the amount of (γ-^{32}P) ATP transferred onto enolase and the reaction time. The amount of radioactivity transferred was quantitated using a PhosphorImager analysis and plotted on a graph (Figure 2.1B). The results indicate that a five minute reaction time in the absence of cold ATP is well within the linear range of incorporation (Figure 2.1B, see dotted line from X axis).

In vitro kinase assays using a part (1/3 rd) of the c-Src (Ab 327), c-Yes (gift from J. Bolen) or Fyn immunoprecipitates were carried out for five minutes at room temperature as described above. The samples were

Figure 2.1

(A) c-Src immunoprecipitates were incubated with acid-denatured enolase and γ–^{32}P ATP either in the presence (lanes 1-4) or absence of (lanes 5-8) of cold ATP. The position of enolase and c-Src are indicated by arrows. **(B)** The radioactivity transferred onto enolase in panel A was quantitated by PhosphorImager analysis and plotted . The vertical line (dotted) from X axis corresponds to the time point chosen for this study.


A
+ ATP
- ATP
Time (min.)
1 5 10 20 20 10 5 1
c-Src
Enolase
1 2 3 4 5 6 7 8
IP: c-Src
B
Radioactivity on Enolase (arbitrary units)
700000
600000
500000
400000
300000
200000
100000
0
0 5 10 15 20
Incubation Time (min.)
- ATP
+ ATP

electrophoresed on a 10% SDS polyacrylamide gel, the gel was incubated in 1M KOH for 30 to 45 min. at 45^{o}C, dried and subjected to autoradiography. The dried gels were also exposed to PhosphorImager screens and quantitated by PhosphorImager (Molecular Dynamics, Sunny vale, CA) analysis. The remaining 2/3 rd of the immunoprecipitates were used for immunoblots (see below), to assess the levels of immunoprecipitated protein from different samples.

2.5 .1 cdc2 peptide assay

The assays were performed as outlined by the manufacturer (UBI) with minor modifications. The c-Src immunoprecipitates were washed five times with TNE and once with dilution buffer (200 mM HEPES pH7.0; 10% Glycerol; 0.1% NP40) and resuspended in 60 µl of dilution buffer. The reaction was initiated by mixing 15 µl of the immunoprecipitate; 5 µl substrate peptide (1.5 mM stock) and 5 µl of assay buffer (250 mM Tris.HCl pH7.0; 125 mM $MgCl_2$; 0.25 mM Na_3VO_4) containing 5 µCi of (γ–^{32}P) ATP. The reaction mix was incubated at 30^{o}C for 20 min. The assay was terminated by the addition of 10 µl of glacial acetic acid. The contents were centrifuged at 3000 rpm for 5 min. and 15 µl of the supernatant was spotted on a P81 phosphocellulose filter paper (Whatman). The filter papers were washed extensively in 0.5% phosphoric acid and acetone prior to scintillation counting. Background levels were determined by running a parallel reaction with normal rabbit sera immunoprecipitate.

2.6 Immunoblotting

The immunoprecipitates were resuspended in SDS gel loading buffer and the proteins were resolved on a 9% SDS polyacrylamide gel. The proteins were transferred onto a PVDF membrane (Millipore) using an immunoblot transfer apparatus (BioRad). Following an overnight incubation in 3% skim milk at $4^{0}C$, the membranes were incubated with either anti-Src antibody (Ab 327, 1:1000, Oncogene Sci.) or anti-Fyn (1:750, Santa Cruz), or anti-Neu (Ab-3, 1:1000, Oncogene Sci.) or anti-EGFR (1:500, Transduction labs) or anti-Yes (1:1000) for 3 h. The membranes were washed five times with PBS for 5-10 minutes each. Subsequently the membranes were incubated for one hour with 1:5000 dilution of anti-mouse IgG or anti-rabbit IgG, conjugated with HRP (Bio-Can Scientific). The membranes were washed five times with PBS for 5-10 minutes each. The proteins were visualized by enhanced chemiluminescence (ECL) system (Amersham).

Anti-phosphotyrosine immunoblots were performed in a similar fashion with the exception that the membrane were blocked overnight in 3% BSA (Sigma) in TBS (20 mM Tris.HCl pH7.5; 150 mM NaCl; 5 mM KCl) and probed for three hours with anti-phosphotyrosine antibodies (4G10, 1:750, UBI) in 3% BSA in TBS. After washing the membranes in TBS; 0.05% Tween 20 (TBS-T), they were incubated in 3% milk in TBS for one hour. The membranes were incubated with anti-mouse IgG (1:5000) for 45-60 minutes and they were washed twice with TBS-T for 10 minutes each followed by three five minute washes with TBS alone. The proteins were visualized by the ECL detection system (Amersham). The suspension containing the primary antibody was stored at -20^{0}C for future use.

2.7 Preparation of GST fusion proteins

E. coli cells harboring GST.SH2 fusion proteins were grown, induced with IPTG and lysed as described (Smith and Johnson, 1991). The Sepharose beads bound GST.SH2 fusion proteins were prepared by incubating the bacterial lysates with glutathione Sepharose 4B beads (Pharmacia) for 20 min. at room temperature. The beads were then extensively washed (5-7 times) with MTPBS (150 mM NaCl; 16 mM Na_2HPO_4; 4 mM NaH_2PO_4 pH7.3) and were resuspended in MTPBS containing 10 μg/ml leupeptin; 10 μg/ml aprotinin.

The soluble form of the fusion proteins was made by passing the bacterial lysate through a glutathione Sepharose bead column which had been equilibrated with MTPBS containing 1% Triton X-100. After washing the column 4 times with 4-5 bed volumes of MTPBS 1% Triton X-100, the fusion proteins remaining bound to the column were eluted with 1 bed volume of 50 mM Tris.HCl pH8.0 containing 5 mM reduced glutathione (BDH). This soluble form of fusion protein is not able to rebind glutathione Sepharose beads (Smith and Johnson, 1991).

2.8 Affinity complex formation with fusion proteins

To establish whether these cell lines expressed tyrosine phosphorylated Neu, the cells were induced with dexamethasone (final conc. 10^{-6} M) for 36 hours and the cell lysates were collected in 1.0 ml of TNE lysis buffer. The lysates were cleared by centrifugation at 12,000 xg for 20 min. the samples were immunoprecipitated with Neu specific antibodies (7.16.4), washed and the immunoprecipitates were electrophoresed on 9% SDS PAGE and immunoblotted with anti-phosphotyrosine antibodies as described above.

In vitro association experiments were conducted by incubating 500 µg of cell lysates with 5 µg of GST SH2 fusion proteins bound to Sepharose beads. After incubation of the samples for 90 min. at 4^{o}C on a rotating platform, the affinity bound complexes were washed 4 times with HNTG buffer (Koch et al., 1992) and resuspended in 1 x SDS gel loading buffer. The samples were resolved on a 9% SDS polyacrylamide gel, blotted with anti-Neu antibody (Ab-3, Oncogene Sci.) as described above.

2.9 Competition assay using fusion proteins

Competition assays were performed by pre incubating 500 µg of NT cell lysates with 100 µg of soluble GST.SH2 fusion proteins for 90 min. at 4^{o}C. After the pre incubation step, the cell lysates were incubated with 10 µg of GST.SH2 fusion proteins bound to glutathione Sepharose beads for 90 min. at 4^{o}C as indicated. The beads were washed 4 times with HNTG buffer and resuspended in 1 x SDS gel loading buffer. The proteins were resolved on a 9% SDS polyacrylamide gel and immunoblotted with Neu-specific antibodies (Ab.3, Oncogene Sci.)

2.10 Direct binding assay

These assays were performed as described previously (Ron and Dressler 1992, Stein et al., 1994a) with minor modifications. Briefly, the GSTag fusions were labeled in vitro as follows: 10µl of beads harboring 15-20µg of fusion protein were washed once with Phosphorylation buffer (DK) (50mM Potassium phosphate, pH 7.15; 10mM $MgCl_2$; 5mM NaF; 4.5mM DTT) and resuspended in 60µl of reaction mixture containing 500µCi of γ-^{32}P ATP (6000Ci/mM, Dupont NEN) in DK buffer. The labeling reaction was started by adding 0.2 U/µl of Protein Kinase A (Sigma) and incubated at 30^{o}C for 30 min. The unincorporated nucleotides were removed by washing 4-5 times with 1X PBS supplemented with 5mM NaF. Immunoprecipitates and total cell lysates were resolved on a SDS-PAGE and transferred onto PVDF membranes. The membranes were incubated at room temperature for 3.0 h in blocking buffer (20mM Hepes, pH 7.5, 5mM KCl, 0.02% sodium azide, 5mM DTT, 5% Skim milk). The membranes were subsequently probed for 2hrs at room temperature in blocking buffer containing 1x 10^6 cpm/ml of probe and washed 4-5 times in TBS, 0.05% Tween-20.

CHAPTER 3

Activation of the c-Src tyrosine kinase is required for the induction of mammary tumors in transgenic mice

3.1 INTRODUCTION

The molecular basis of the events responsible for conversion of a normal cell to a tumor cell remains a major challenge in understanding oncogenesis. For example, deregulated expression of a number of oncogenes have been implicated in human breast cancer. Many of these oncoproteins appear t affect different mediators of mitogenic signal transduction pathways including growth factors (TGFα) (Matsui et al., 1990), receptors (erbB family) (King et al., 1985; Yokota et al., 1986; Slamon et al., 1987; Slamon et al., 1989) and transcription factors (c-Myc) (Escot et al., 1986). Expression of viral T antigens have been shown to transform cells in culture, and these viral antigens are being used as model systems to understand the process of cell transformation. For example expression of PyV middle T antigen under the control of MMTV promoter/enhancer results in rapid induction of multifocal mammary adenocarcinomas involving the entire mammary gland in every transgene carrier examined. In addition to the high penetrance and rapid development of mammary tumors, expression of the PyV middle T antigen leads to a high incidence of metastatic disease (Guy et al., 1992a).

The potent transforming properties of the PyV middle T antigen results from its capacity to associate with and activate a number of cellular enzymes. In particular, PyV middle T antigen can activate c-Src and, c-Yes tyrosine kinases by forming physical complexes with these proteins thus rendering them kinase active (Courtneidge and Smith, 1983; Kornbluth et al., 1987). In addition to its ability to associate with and activate different members of the Src family, the middle T oncogene is also known to interact with the 85-kDa subunit of the phosphatidylinositol-3'-kinase (Courtneidge and Hebner, 1987; Whitman et al., 1985), Shc (Dilworth et al., 1994) and protein phosphatase 2A (Pallas et al., 1990; Walter et al. 1990).

While it is clear that the interaction of PyV middle T antigen with these cellular proteins plays an important role in tumorigenesis, the relative contribution of each of these protein complexes to transformation remains to be defined. To directly assess the role of c-Src in PyV middle T antigen-induced mammary tumorigenesis, we have crossed transgenic mice carrying the MMTV/PyV middle T oncogene with mice carrying either disrupted c-*src* or *c-yes* alleles (Soriano et al., 1991; Stein et al., 1994a). This chapter deals with the results obtained from the study done in collaboration with Chantale Guy.

3.2 RESULTS

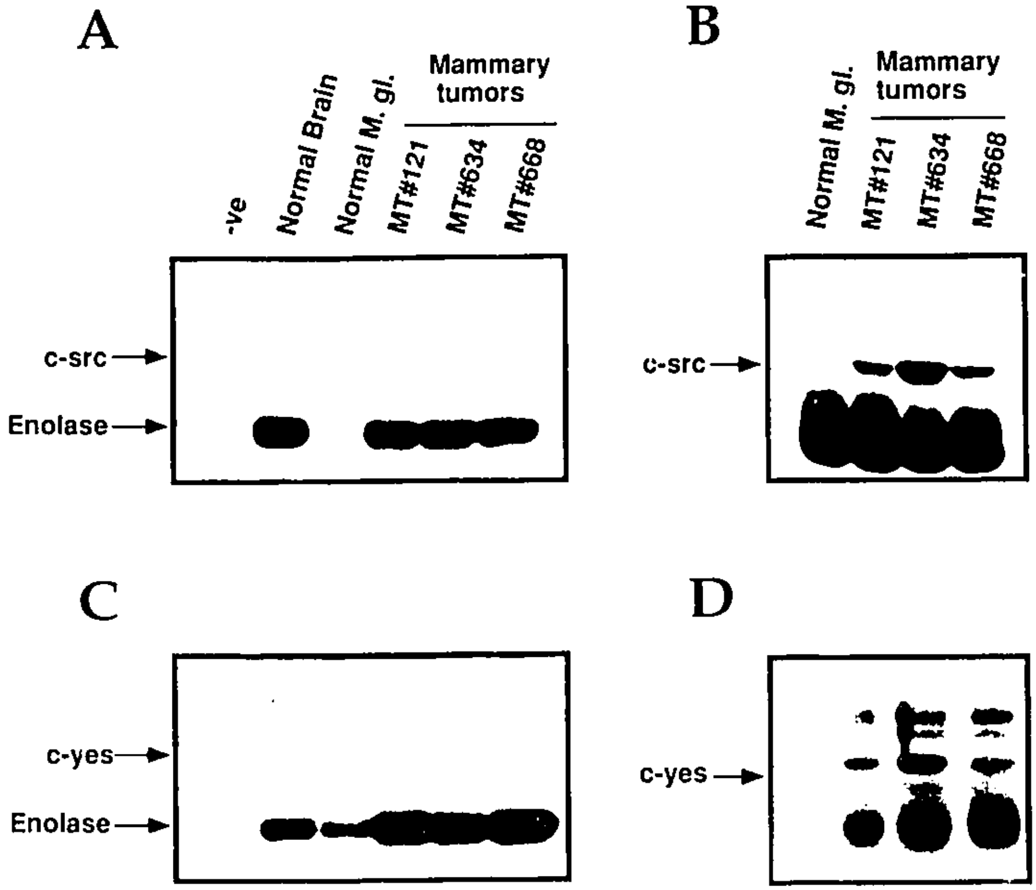
3.2.1 Expression of the PyV middle T oncogene in the mammary epithelium results in activation of c-Src and c-Yes tyrosine kinases.

PyV middle T antigen is known to associate with and activate both c-Src and c-Yes tyrosine kinases. To confirm that PyV middle T antigen-induced mammary tumors possess elevated c-Src and c-Yes kinase activity tumor tissue extracts from several MMTV/PyV middle T antigen transgenic strains (MT#121, MT#634, MT#668) were immunoprecipitated with either c-Src or c-Yes specific antibodies and subjected to *in vitro* kinase assays using acid denatured enolase as an external substrate (Figure 3.1A). These experiments were conducted under conditions where incorporation of γ–^{32}P ATP onto the enolase substrate occurs in a linear fashion (see Materials and Methods). As shown in Figure 3.1 all tumor samples had elevated c-Src (panel A) and c-Yes (panel C) kinase activity when compared to the normal mammary gland from a nontransgenic mice (Normal M.gl.). A non-specific control immunoprecipitate (rabbit serum) was used as a negative control (-ve). Quantitation by PhosphorImager analysis revealed that the tumor samples from the MMTV/PyV middle T antigen animals had on average a five fold greater c-Src and c-Yes kinase activities than the nontransgenic mammary epithelium. Although the increase in c-Src and c-Yes activities was modest, these values were consistently observed with multiple independent tumor extracts (n=9). To confirm that this increase in c-Src and c-Yes kinase activity was due to increase in the specific activity of the kinase and not due to increased protein expression, part of the

Figure 3.1 Activation of the c-Src family tyrosine kinases in the PyV middle T antigen-induced mammary tumors.

(A) *In vitro* kinase activities of mammary tumor extracts derived from the different MMTV/PyV middle T antigen transgenic strains including MT#121 (MT#742, 83 days of age), MT#634 (MT#616, 112 days of age), and MT#668 (MT#9313, 110 days of age). All tumors were isolated from multiparous female carriers. Each protein extract was immunoprecipitated with antibodies specific to c-Src, and c-Yes (lanes marked +). Normal rabbit sera was used as a nonspecific control antibody (lanes marked -). In addition, these analyses were conducted on positive control brain (normal brain) and nontransgenic mammary tissues (Normal M.gl.). The positions of c-Src and c-Yes kinases and exogenous enolase substrate are illustrated by the arrows.

(B) Immunoprecipitation of identical tumor and control tissue protein extracts with antisera directed against PyV middle T antigen followed by immunoblot analyses with c-Src or c-Yes specific antibodies. Also included are negative control protein extracts derived from nontransgenic mammary glands (M.gl.). The broad lower band is due to cross-reactive immunoglobulin band present in the immunoprecipitates.

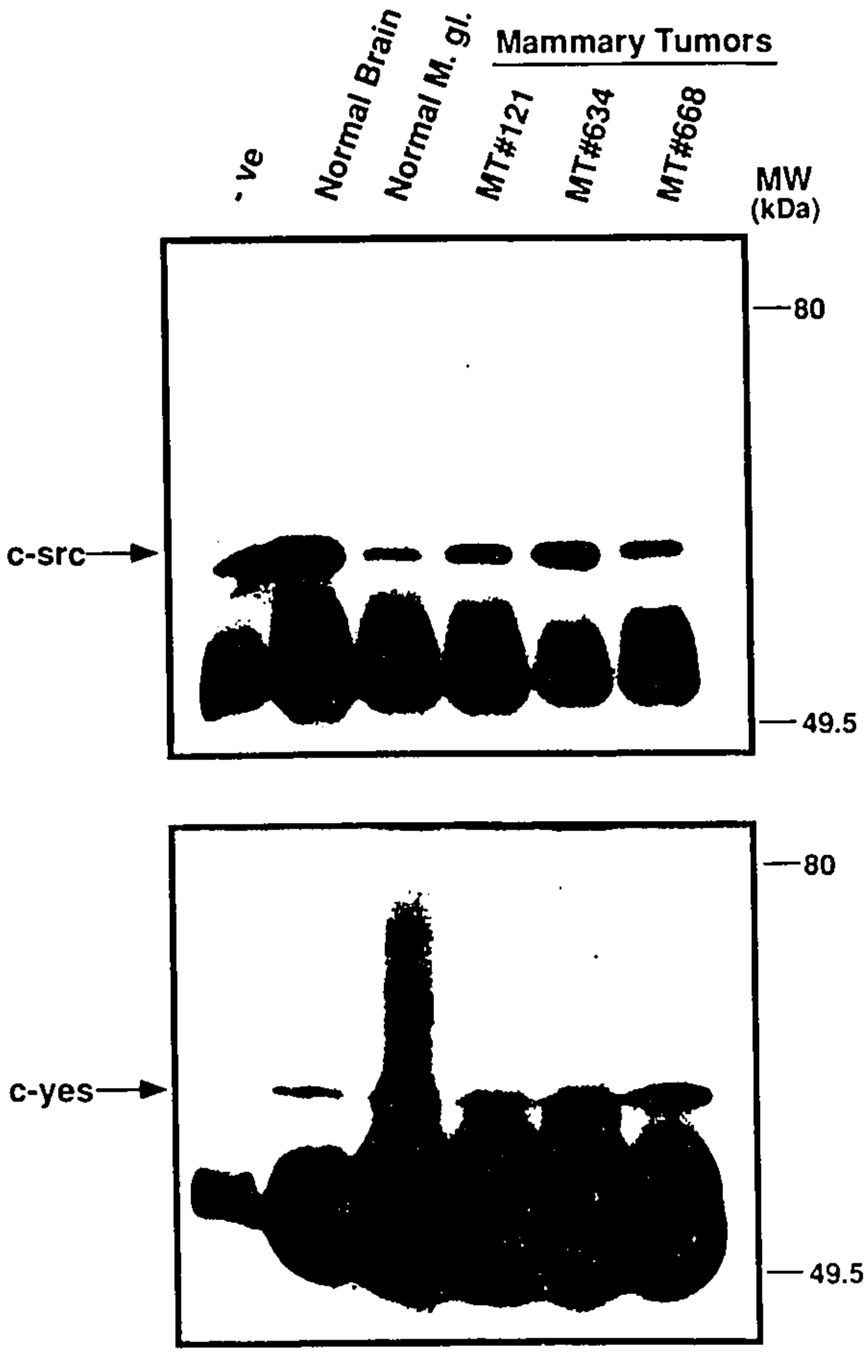

A
-ve
Normal Brain
Normal M. gl.
Mammary tumors
MT#121
MT#634
MT#668
c-src
Enolase
B
Normal M. gl.
Mammary tumors
MT#121
MT#634
MT#668
c-src
C
c-yes
Enolase
D
c-yes

immunoprecipitate used in Figure 3.1A and 3.1C were resolved on a SDS-PAGE and immunoblotted with either c-Src or c-Yes specific antisera. As shown in Figure 3.2 the Normal mammary gland (Normal M.gl.) and mammary tumors had comparable levels of c-Src and c-Yes protein suggesting that the observed five fold increase in kinase activity is a result of change in specific activity of the kinases. By contrast to c-Src and c-Yes, incubation of tumor and normal mammary gland extracts with a Fyn specific antibody failed to show evidence of enhanced Fyn kinase activity in the mammary tumors (Guy et al., 1994).

To determine whether the activation of c-Src and c-Yes correlated with their ability to associate with PyV middle T antigen, tumor extracts were immunoprecipitated with a PyV middle T antigen specific antisera and the immunoprecipitates were subjected to immunoblot analyses with antisera directed against either c-Src or c-Yes. The results of these analyses revealed that the presence of both the c-Src (Figure 3.1B) and c-Yes (Figure 3.1D) in the PyV middle T antigen immunoprecipitates. As expected, protein extracts derived from normal nontransgenic mammary epithelium failed to demonstrate the presence of either the c-Yes or c-Src due to the absence of the middle T antigen. Together, these results indicate that the PyV middle T antigen-induced mammary tumors possess elevated c-Src and c-Yes kinase activities and this is likely due to its ability to associate PyV middle T antigen.

Figure 3.2 Change in kinase activity is not a result of change in protein levels of c-Src and c-Yes.

A part of the immunoprecipitates used in the kinase assay (Figure 3.1) was resolved on SDS-PAGE and immunoblotted with either anti-Src (Top panel) or with anti-Yes (Bottom panel) specific antisera. Normal rabbit serum immunoprecipitate (-ve) was used as a negative control. All the tissue lysates are as those described in the legend for figure 3.1.



- ve
Normal Brain
Normal M. gl.
Mammary Tumors
MT#121
MT#634
MT#668
MW (kDa)
80
49.5
c-src
80
c-yes
49.5

3.2.2 A functional c-Src is required for the rapid induction of metastatic mammary tumors.

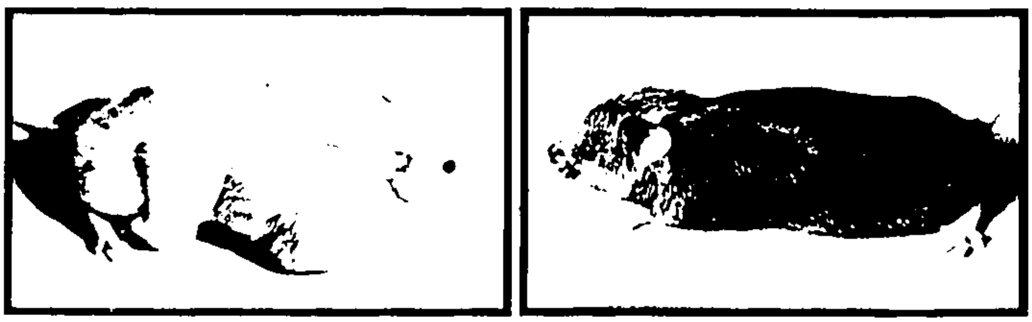
Although the PyV middle T antigen-induced mammary tumors possess elevated c-Src and c-Yes tyrosine kinase activities, it is unclear to what extent activation of each of these individual tyrosine kinases contributes to the overall transformed phenotype. To determine whether c-Src is required for PyV middle T antigen mediated mammary tumorigenesis, mice carrying a disrupted c-*src* gene (Soriano et al., 1991) were interbred with the MMTV/PyV middle T antigen transgenic mice (MT#634, Figure 3.1, Guy et al., 1992a). Using this approach, a variety of different genotypes of MMTV/PyV middle T antigen mice were generated including heterozygous transgene carriers in wild type c-*src* (MT/+, c-*src*+/+), heterozygous c-*src* (MT/+, c-*src* +/-) and null c-*src* backgrounds (MT/+, c-*src* -/-). The genotypes of each of these progeny were confirmed by Southern blot hybridization with appropriate transgene and c-*src* specific probes (Guy, 1994). All female transgenic progeny possessing at least one functional c-*src* allele developed multifocal mammary tumors that eventually enveloped the entire mammary epithelium by 120 days (Figure 3.3A). The onset of mammary tumor formation in transgenic mice carrying either both wild type c-*src* alleles (n=30) or heterozygous for the c-*src* mutation (n=33) were not significantly different (Table 3.1). By contrast, none of the MMTV/PyV middle T antigen transgenic mice homozygous for the c-*src* mutation (n=24) developed mammary tumors within this time frame (Table 3.1).

Figure 3.3 c-Src is required for the induction of mammary tumors in the MMTV/PyV middle T antigen transgenic mice.

(A) Transgenic mice carrying the PyV middle T oncogene in a wild type c-*src* (left panel, #8314 70 days of age) or null c-*src* (#7832,140 days of age) genetic backgrounds. Note the extensive mammary tumors in all mammary glands of the MT#8314 mouse and the lack of palpable tumors in the MT#7832 mouse.

(B) RNase protection analyses using 10 mg of total mammary tissue RNA isolated from multiparous females carrying the middle T antigen transgene in wild type, heterozygous and homozygous c-Src backgrounds. Mammary tissue extract from a c-*src*-/- nontransgenic animal was included as a negative control. The antisense probe used in this RNase protection analyses protects a 203-nucleotide fragment corresponding to the 5' end of the PyV middle T antigen cDNA. To ensure that equal amounts of RNA were loaded on the gels, a L32-4 antisense probe directed against the mouse ribosomal protein L32-..A was also included in the hybridization reaction. The L32 probe protects a 278 nucleotide fragment as indicated by the arrow.

A.



MT (+/-); c- *src* (+/+)   MT (+/-); c- *src* (-/-)

B.

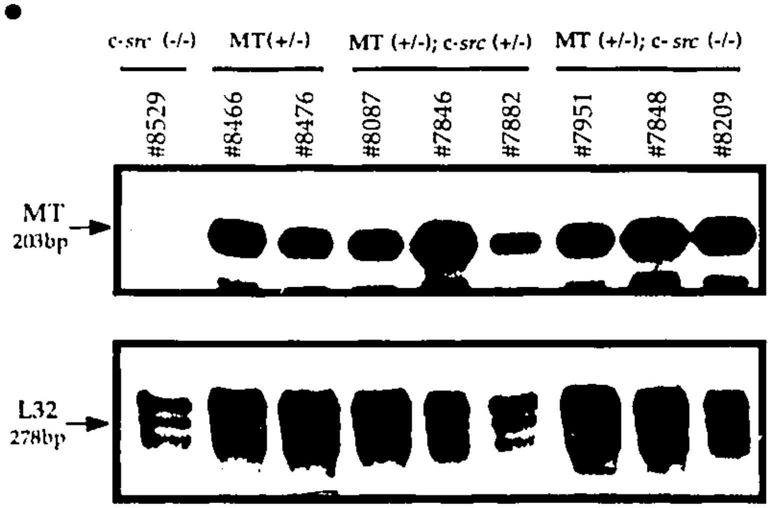


Table 3.1. Onset of tumors in the MMTV/PyV middle T mice in c-Src and c-Yes deficient background.

The number of animals tested for each genotype and the percentage of females which developed mammary tumors, after three months of age, are indicated. T_{50} value was calculated for each genotype and it corresponds to the time at which at least 50% of animals developed a palpable mammary tumor.

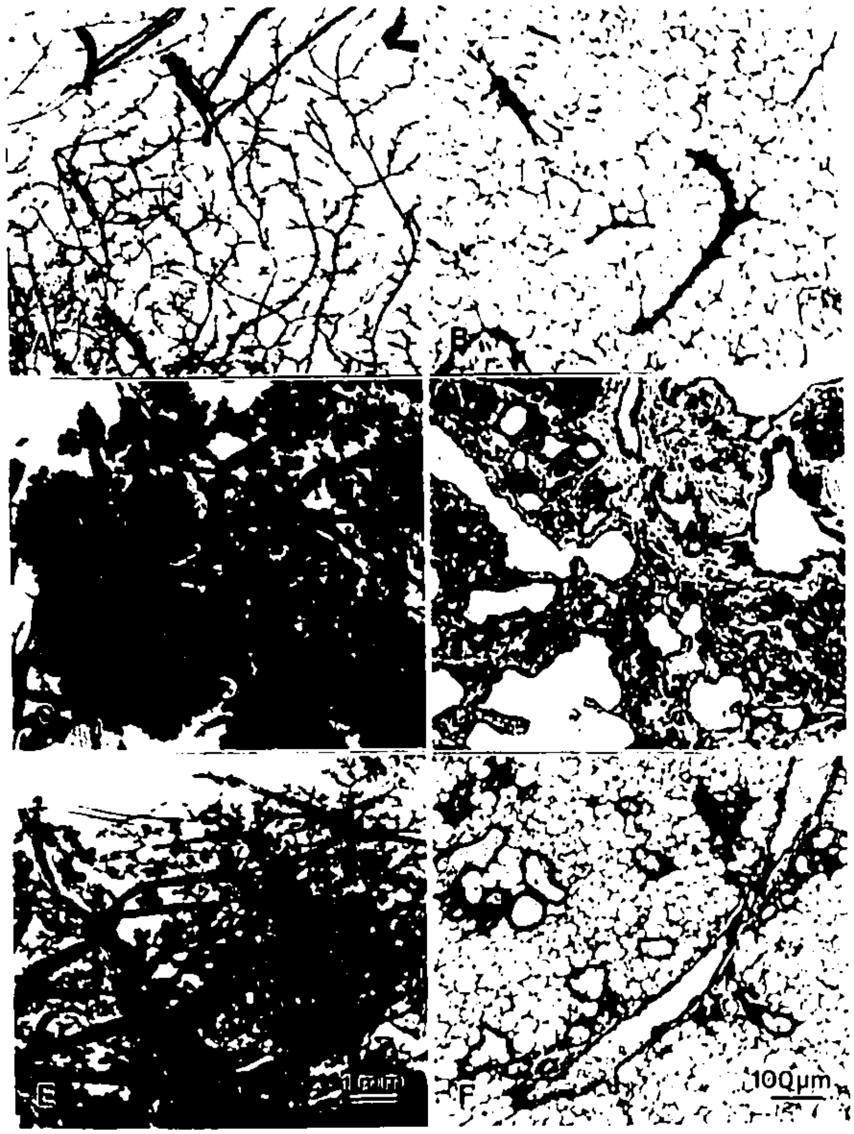
GENOTYPES	Number of animals	Percentage of animals with tumors	T_{50} for tumor formation (days)
MT +/-; src +/+	33	100	82
MT +/-; src +/-	30	100	86
MT +/-; src -/-	24	8	>200
MT +/-; yes +/+	30	100	58
MT +/-; yes +/-	26	100	58
MT +/-; yes -/-	31	100	80

The lack of tumor formation in middle T/+,*c-src* -/- mice was not due to difference in levels of transgene expression because RNase protection analysis (Guy et al., 1994) using 10μg of total RNA from mammary glands of multiparous mice using a transgene-specific probe yielded a 203-nucleotide protected fragment in all different *c-src* genotypes (Figure 3.3B). A mouse ribosomal protein probe (L32-4A) was used as an internal control. Consistent with the RNase protection analyses, immunoprecipitation/immunoblot analyses using PyV middle T antigen specific antisera revealed equivalent levels of PyV middle T antigen protein within the mammary epithelium of these mice (data not shown). Therefore, the inability of PyV middle T antigen mice to develop tumors in a c-*src* null genetic background was not due to differences in transgene expression.

The histological appearance of the mammary tissue derived from MMTV/PyV middle T antigen transgenic mice possessing at least one functional c-*src* allele exhibited dramatic differences in comparison to mammary tissue from transgenic mice homozygous for the disrupted c-*src* gene (Figure 3.4). By contrast to wild type FVB mammary glands (Figure 3.4A), wholemount examination of virgin mammary tissue from female MMTV/PyV middle T antigen mice heterozygous for the c-*src* mutation revealed the presence of multiple mammary adenocarcinomas as early as 60 days of age (Figure 3.4C). In older multiparous or virgin female transgenic mice homozygous for the disrupted c-*src* gene, mammary epithelial hyperplasias have been detected (Figure 3.4E). Although these mammary epithelial hyperplasias can eventually envelope the entire mammary fat

Figure 3.4 Histopathology of the MMTV/PyV middle T antigen mice carrying possessing disrupted c-*src* alleles.

A panel of photomicrographs showing the appearance of hematoxylin stained wholemount (left hand side: A,C,E, at 9X) and hematoxylin and eosin stained microscopic section (right hand side: B,D, and F at 90X) of virgin female FVB (A and B), MT (+/-); c-*src* (+/-) (MT#7698 at 75 days of age) (C and D) and MT(+/-); c-*src* (-/-) transgenic animal (MT# 7832 at 140 days of age) (E and F). Note the slender, non branching ducts of the wild type animal, the proliferative multilayered structures in the MT#7698 animal and the dilated complex ducts lined by a single epithelial layer in the MT#7832 animal.


A
B
C
D
E
1 mm
F
100µm

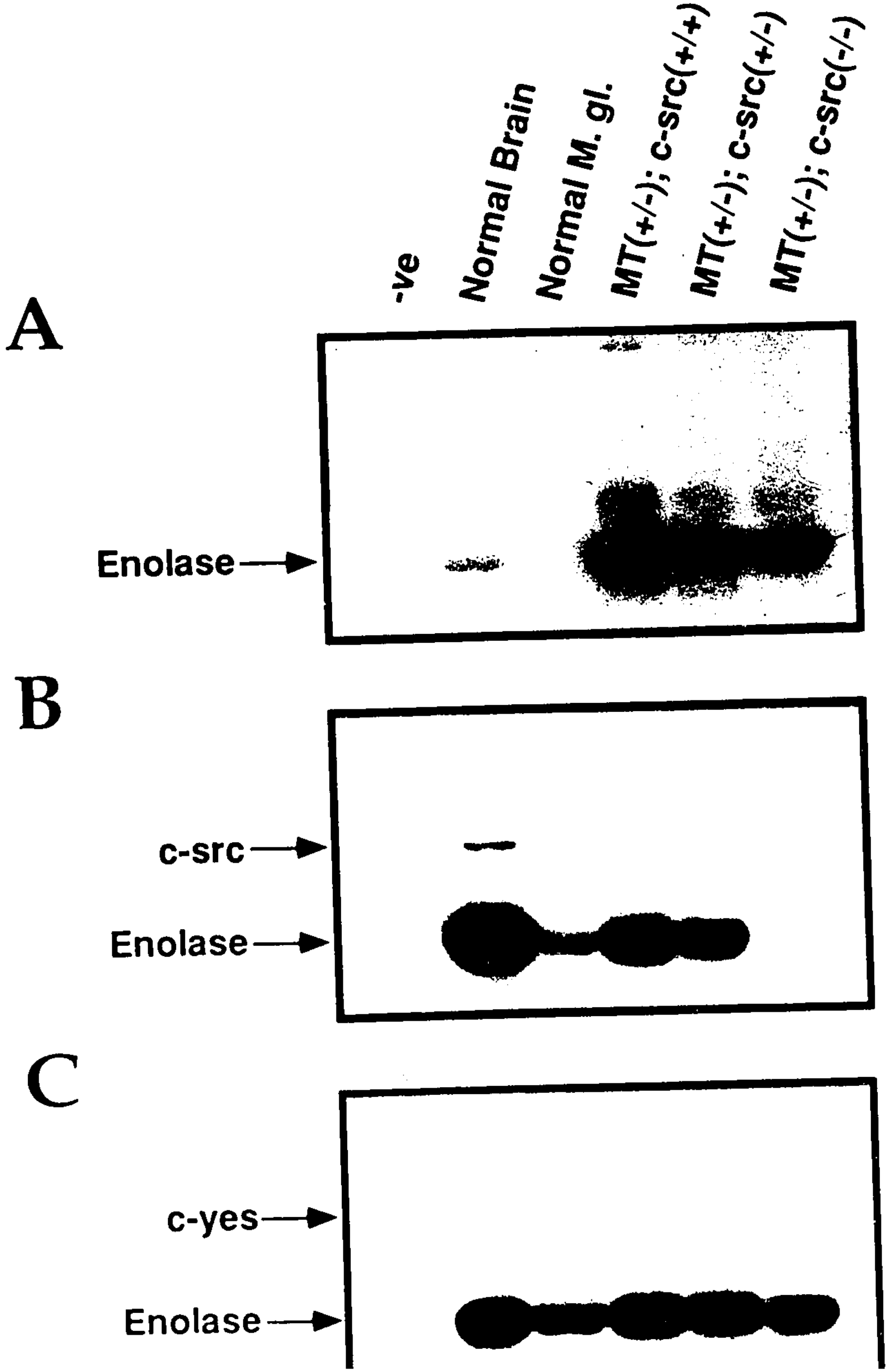
pad, they rarely progress to full malignancy. In fact, of the female transgenic mice lacking c-Src function that have lived to an age of 3 months or older (n=24), only two animals have developed focal mammary adenocarcinomas, and this occurred only after long latency (7 months of age) (Table 3.1). Consistent with these findings, the mammary epithelial hyperplasias observed in the c-*src* deficient background were histologically distinct from the middle T antigen-induced mammary tumors. By contrast to the proliferative multilayered epithelium observed in the PyV middle T antigen-induced mammary tumors, the hyperplasias observed in the c-Src null background were comprised of complex ducts lined by a single epithelial layer (Figures 3.4D and 3.4F).

3.2.3 Detection of PyV middle T antigen associated tyrosine kinase activity in the mammary tissue of c-Src deficient mice.

The epithelial hyperplasias observed in the in the c-Src deficient mice expressing the PyV middle T oncogene could conceivably result from activation of the PyV middle T antigen associated c-Yes tyrosine kinase. To test this possibility, *in vitro* kinase assays were performed on the mammary tissue derived from MMTV/PyV middle T antigen transgenic mice carrying either wild type or mutant c-*src* alleles with antisera directed against PyV middle T antigen, c-Src and c-Yes (Figure 3.5). Immunoprecipitation using PyV middle T antigen specific antisera and incubation of the immunoprecipitates with acid denatured enolase as an external substrate indicated that PyV middle T antigen associated activity could be detected in

Figure 3.5 Detection of PyV middle T antigen associated c-Yes kinase activity in mammary tissues defective in c-Src function.

Shown are, *in vitro* kinase activities of PyV middle T antigen. **(A).** c-Src **(B).** and c-Yes **(C).** derived from mammary tissue extracts from multiparous female MT (+/-); c-*src* (+/+) (MT#1907 at 90 days of age), MT (+/-); c-*src* (+/-) (MT#7849 at 150 days of age) and MT (+/-); c-*src* (-/-) (MT#9184 at 125 days of age). Protein extracts were incubated with Glu-Glu raised against PyV middle T antigen, a monoclonal antibody that recognizes c-Src (MAb 327, Oncogene Sci.) or a monoclonal antibody specific for c-Yes (3H9) (Sukegawa et al., 1990). In addition, these analyses were conducted on brain tissue (Normal Brain) which served as a positive control for c-Src and c-Yes activity. Nontransgenic mammary tissue was also included (Normal M. gl) as a negative control tissue. A negative control antibody used in these analyses is brain tissue incubated with normal rabbit sera (lane labeled "-ve"). The position of c-Src, c-Yes and the exogenous enolase substrate are illustrated by the arrows.


A
B
C
-ve
Normal Brain
Normal M. gl.
MT(+/-); c-src(+/+)
MT(+/-); c-src(+/-)
MT(+/-); c-src(-/-)
Enolase
c-src
Enolase
c-yes
Enolase

mice heterozygous or homozygous for the c-*src* mutation (Figure 3.5A). As expected, incubation of tissue extracts derived from either nontransgenic brain or nontransgenic mammary gland with the PyV middle T antigen specific antisera failed to exhibit significant PyV middle T antigen associated kinase activity. Quantitative analyses of the middle T antigen associated kinase activity (Figure 3.5A) revealed that the tissue extracts derived from either transgenic mice heterozygous or homozygous for the disrupted c-src allele possessed 60% and 40% of the levels observed in the mammary glands of transgenic mice possessing both wild type c-src alleles. To assess whether phosphorylation of PyV middle T antigen protein in the c-*src* null genetic background was due to the activation of the c-Yes tyrosine kinase, *in vitro* kinase assays were also conducted with antisera directed against c-Yes and c-Src. As expected *in vitro* kinase analyses with c-Src specific antibodies showed no evidence of c-Src kinase activity in mammary tissues obtained from transgenic mice lacking c-Src function (Figure 3.5B). However, comparable levels of c-Yes associated kinase activity could be detected in tissues from transgenic mice, carrying either wild type or disrupted c-*src* genes (Figure 3.5C). These observations suggest that the mammary epithelial hyperplasias observed in mice expressing MMTV/PyV middle T antigen in the absence of a functional c-Src is likely due to the activation of c-Yes tyrosine kinase by PyV middle T antigen.

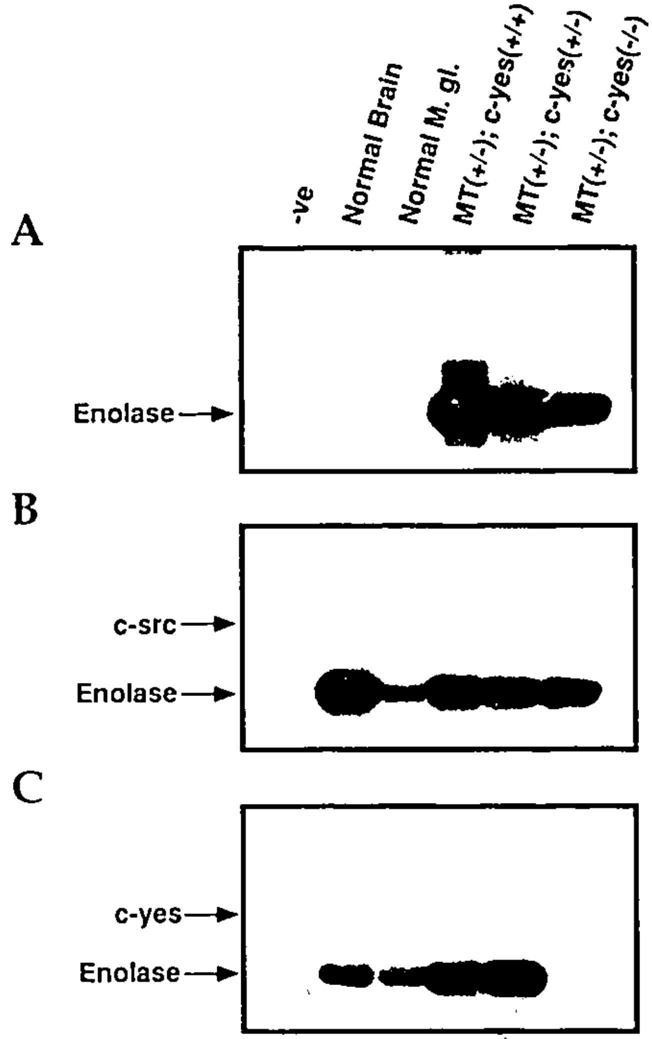
3.2.4 c-Yes is dispensable for PyV middle T antigen mediated mammary tumorigenesis

One possible interpretation of the cross between the MMTV/PyV middle T antigen and c-Src deficient strains is that transformation of the mammary epithelial cell by the middle T oncogene requires the activity of both the middle T antigen/c-Src and middle T antigen/c-Yes complexes to transform the mammary epithelial cell. To explore this possibility further, the MMTV/middle T antigen strains were crossed with c-Yes deficient mice. Unlike the c-Src deficient mice which suffer from osteopetrosis (Soriano et al., 1991), the c-Yes deficient mice display no obvious abnormalities (Stein et al., 1994a). As shown in Table 3.1, the onset of mammary tumor formation in mice carrying one or both wild type c-Yes alleles did not significantly differ. Interestingly, all female transgenic mice expressing the middle T antigen transgene in the c-Yes deficient background developed multifocal mammary tumors (Table 3.1). The mammary tumors that arose in the c-Yes deficient mice were histologically indistinguishable from tumors observed in the original MMTV/PyV middle T antigen strains (data not shown).

To test whether the tumors arising in the c-Yes deficient strains resulted from the activation of PyV middle T antigen associated c-Src kinase activity, *in vitro* kinase assays were conducted on mammary tissue derived from various genotypes with antisera specific for PyV middle T antigen, c-Src and c-Yes (Figure 3.6). Mammary tumors derived from the transgenic mice heterozygous or homozygous for the disrupted c-Yes alleles possessed 68% and 33% of the middle T antigen associated kinase activity observed in

Figure 3.6 Polyoma middle T antigen associated c-Src kinase activity in mammary tumors of mice lacking functional c-Yes.

Tissue extracts from mammary tumors of multiparous females MT(+/-); c-Yes (+/+) (MT#1907 at 90 days of age), MTV (+/-); c-Yes (+/-) (MT#39 at 138 days of age), MT (+/-); c-Yes (-/-) (MT#119 at 150 days of age) were used for *in vitro* kinase assays of middle T antigen **(A)**. c-Src **(B)**. c-Yes **(C)**. Protein extract were incubated with either Glu-Glu antibody which recognizes PyV middle T antigen, with monoclonal antibody recognizing Src (Ab.1 Oncogene Sci.), or with a monoclonal antibody recognizing Yes (3H9). Protein lysates from Brain and non-transgenic mammary gland (Normal M.gl.) were used as +ve and -ve controls respectively. Normal rabbit serum was incubated with lysates from Brain tissue to serve as non-specific control (-ve). The positions of Enolase, c-Src and c-Yes are indicated by arrows.


A
B
C
-ve
Normal Brain
Normal M. gl.
MT(+/-); c-yes(+/+)
MT(+/-); c-yes(+/-)
MT(+/-); c-yes(-/-)
Enolase
c-src
Enolase
c-yes
Enolase

the wild type c-Yes background respectively (Figure 3.6A). *In vitro* kinase analyses with c-Src and c-Yes specific antisera revealed comparable levels of c-Src kinase activity between the wild type and c-Yes deficient strains (Figure 3.6B). However, no detectable c-Yes associated kinase activity could be observed in the middle T antigen-induced mammary tumors derived from the c-Yes deficient mice (Figure 3.6C). Taken together, these findings argue that activation of the c-Yes kinase is not required for the induction of mammary tumors by the PyV middle T oncogene.

3.3 DISCUSSION

The observations presented here clearly show that, by contrast to the rapid development of mammary tumors observed in the MMTV/PyV middle T antigen mice heterozygous for a disrupted c-*src* gene (Soriano et al., 1991), mice expressing PyV middle T antigen in the absence of a functional c-Src rarely develop mammary tumors. However, these mice eventually develop benign mammary epithelial hyperplasias which correlated with the activation of the PyV middle T antigen associated c-Yes kinase. Conversely, mice expressing the middle T antigen transgene in a c-Yes deficient background (Stein et al., 1994a) develop multifocal mammary tumors with 100% penetrance. These observations strongly suggest that activation of a signal transduction pathway involving c-Src is required for PyV middle T antigen-mediated induction of mammary tumors in transgenic mice.

Consistent with observations made with middle T oncogene-expressing fibroblast cell lines (Courtneidge and Smith 1983, Kornbluth et al., 1987), association of PyV middle T antigen with c-Src and c-Yes resulted in activation of their intrinsic tyrosine kinase activities in middle T-induced mouse mammary tumors. While the Fyn tyrosine kinase is capable of associating with PyV middle T antigen, this protein-protein interaction does not result in a significant increase in its tyrosine kinase activity in fibroblasts (Kypta et al., 1988) or in PyV middle T antigen-induced mammary tumors (Guy and Muller, unpublished observations). By contrast, the closely related hamster PyV (HaPyV) encodes a middle T antigen product that is capable of associating and activating the Fyn tyrosine kinase but is not capable of associating with the c-Src and c-Yes tyrosine kinase (Courtneidge et al., 1991). Interestingly, unlike the mouse PyV which induce a variety of epithelial tumors including mammary tumors (Berebbi et al., 1990), expression of hamster PyV middle T antigen is associated with the induction of lymphoid tumors (Courtneidge et al., 1991). It is conceivable that the tumor type induced by these viral oncogenes may be dependent on the nature of the Src family member which is activated. Future experiments directed towards expressing the HaPyV middle T antigen in the mammary epithelium should allow this question to be addressed.

Although we could detect expression of middle T antigen encoded RNA and protein from the mammary glands of these c-Src deficient transgenic mice (Figures 3.3 and 3.5), these mice rarely developed mammary tumors. By contrast, all those transgenic mice which were heterozygous for the disrupted c-*src* allele or carrying both wild type c-*src* alleles developed multifocal mammary tumors with similar kinetics. Although the MMTV/PyV middle T antigen mice lacking c-Src function rarely develop mammary tumors, mammary epithelial hyperplasias were often observed in these animals (Figures 3.4E and 3.4F). Since elevated levels of c-Yes kinase activity can be detected in these tissues it is conceivable that these hyperplasias are the result of activation of c-Yes by PyV middle T antigen. However, given the infrequent occurrence of mammary tumors in these mice, the additional activation of c-Src appears to be required for the mammary cell to acquire the full malignant phenotype.

Unlike the MMTV/middle T antigen/c-Src deficient mice, transgenic mice expressing the middle T oncogene in a c-Yes deficient background develop multifocal mammary tumors with 100% penetrance (Table 1). The inability of the PyV middle T antigen /c-Yes complex to efficiently transform the mammary epithelium in the absence of c-Src does not appear to be the result of an overall lower level of PyV middle T antigen associated kinase activity since the levels of PyV middle associated kinase in either middle T/+, *c-src* -/- or middle T/+, *c-yes* -/- are atleast 50% of the levels present in the wild type (Figures 3.4A, 3.5A). One possible explanation for these observations is that the middle T/c-Src complex may have substrates that are distinct from the middle T/c-Yes complex and these are required for cellular transformation. In this regard, analyses of mammary gland extracts from the c-Src deficient, c-Yes deficient and wild type mice carrying the transgene with antiphosphotyrosine specific antibodies has revealed no obvious differences in the pattern of phosphorylated proteins between these tissues (data not shown). However, these analyses may not be sensitive enough to detect subtle differences in substrate specificity between c-Src and c-Yes.

Another cell type that is sensitive to transformation by middle T antigen is the endothelial cell (Bautch et al., 1987; Williams et al., 1988). By contrast to the mammary epithelial cell, transformation of the endothelial cell or established fibroblasts by PyV middle T antigen does not require functional c-Src (Thomas et al., 1993). However, endothelial expression of middle T antigen in a c-Yes deficient background resulted in reduction of the number of endothelial tumors which arose after a longer latency period (Kiefer et al., 1994). Conversely, in certain PyV transformed rat cell lines, inducible expression of antisense *c-src* construct results in the reduction of the tumorigenic properties of these lines (Amini et al., 1986). Hence, activation of closely related Src-family tyrosine kinases may have dramatically different outcomes in different cell types.

The results presented in this chapter clearly demonstrate that among the Src family members (c-Src and c-Yes) activation of c-Src tyrosine kinase is required for PyV middle T antigen mediated transformation of the mammary epithelium. Recently it was shown that expression of a constitutively active form of *c-src* (Y527F) under the control of MMTV promoter/enhancer can induce mammary gland hyperplasia that eventually form tumors after long latency (Webster et al., 1995). Taken together these observations suggests that activation of c-Src is necessary but not sufficient for rapid transformation of the mammary epithelial cells in transgenic mice. Thus the ability of middle T antigen to induce rapid transformation of mammary epithelial cells is likely due to its ability to activate a variety of signal transduction pathways in addition to activation of c-Src tyrosine kinase (see chapter 1.6).

Receptor tyrosine kinases are known to activate a number of signal transduction pathways. Consistent with this notion expression of kinase active form of Neu under the control MMTV promoter results in induction of mammary tumors in a single step kinetics (Muller et al., 1988). In the following chapters I present evidence that c-Src and c-Yes tyrosine kinases are also involved in signaling by activated Neu receptor tyrosine kinase.

CHAPTER 4

Mammary tumors expressing the *neu* proto-oncogene possess elevated c-Src tyrosine kinase activity

4.1 INTRODUCTION

Neu (c-erbB-2) is a member of the epidermal growth factor receptor (EGFR/c-erbB-1) family referred to as class I receptors (see chapter 1). In addition to *neu* and EGFR, the EGFR family include the recently identified erbB-3 (Kraus et al., 1989, Plowman et al., 1990) and erbB-4 (Plowman et al., 1993a) genes (for a review see CarrawayIII and Cantley, 1994). Overexpression of the EGFR family members has been implicated in a number of human cancers. For example, amplification and consequent overexpression of *neu* has been observed in a significant proportion of breast cancers (Slamon et al., 1987, 1989) and appears to be inversely correlated with the survival of the patient (Gullick et al., 1991, Paterson et al., 1991). Studies using transgenic mouse models suggest that the mammary epithelium is sensitive to the activation of Neu tyrosine kinase activity (see chapter 1) while the mechanism by which Neu transforms the mammary epithelium is unclear.

Transgenic mice expressing MMTV/PyV middle T antigen develop multifocal mammary tumors and this tumor formation requires the presence of a functional c-Src gene (Guy et al., 1992a, chapter 3). These observations suggest that activation of c-Src tyrosine kinase may play an important role in transformation of the mammary epithelium. Furthermore analyses of primary human breast cancers have revealed that a large proportion of human breast tumors possess elevated c-Src activity (Jacobs and Rubsamen 1983, Rosen et al., 1986, Ottenhoff-Kalff, et al., 1992). The mechanism by which c-Src tyrosine kinase activity is elevated in these primary tumors is not known. c-Src has been shown to be involved in signaling by RTKs such the PDGFR and CSF-1R and activation of these receptors results in elevation of Src kinase activity (see chapter 1). Since activation of the RTK Neu plays an important role in mammary tumorigenesis, I assessed whether c-Src would play a role in Neu-mediated signaling and mammary tumorigenesis. To this end, protein extracts from normal or tumor tissues derived from MMTV/unactivated *neu* transgenic mice (Guy et al., 1992b) were immunoprecipitated with Src specific antibodies and subjected to *in vitro* kinase assays. The results of these analyses revealed that the mammary tumor extracts contained 6 to 8 fold higher levels of c-Src kinase activity than the adjacent mammary epithelium. Moreover, physical complexes between activated Neu and c-Src could be detected *in vivo* by immunoprecipitation/immunoblot analyses. These observations support the hypothesis that activation of the c-Src kinase might play a role in Neu mediated mammary tumorigenesis.

4.2 RESULTS

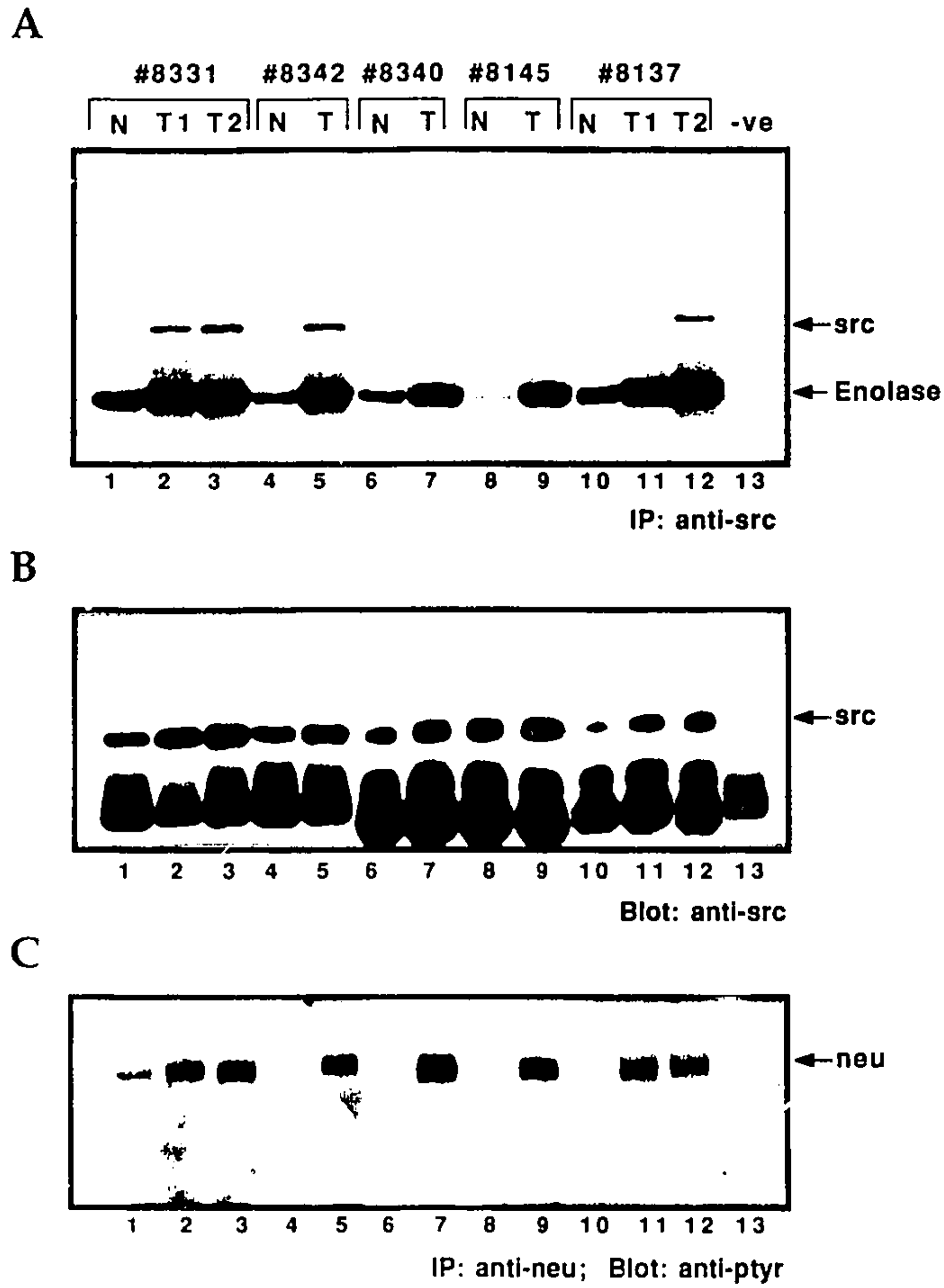
4.2.1 Elevated c-Src tyrosine kinase activity in Neu expressing mammary tumors.

To test the possibility that activation of c-Src may be involved in Neu-induced mammary tumorigenesis, I measured the tyrosine kinase activity of c-Src in mammary tumors and adjacent mammary tissues derived from the transgenic mouse strain (N#202) carrying an MMTV/unactivated *neu* fusion gene (Guy et al., 1992b). The mammary tumors that arise in these mice histologically resemble the human comedocarcinomas that are known to express high levels of the human Neu protein (Cardiff and Muller, 1993). Although attempts were made to exclude overt tumors in the adjacent mammary epithelium, the surrounding epithelium occasionally exhibited histological features of hyperplasia and dysplasia. Protein extracts derived from both normal and tumor tissues were immunoprecipitated with Src specific monoclonal antibodies and subjected to *in vitro* kinase assays using acid denatured enolase as a substrate. As shown in Figure 4.1A, all tumors (T) examined in this fashion exhibited elevated levels of c-Src tyrosine kinase activity by comparison to the matched adjacent mammary epithelium (N). Quantitation by PhosphorImager analysis revealed that the tumor samples had on average 6.8 fold higher c-Src kinase activity than the adjacent epithelium (Table 4.1).

The c-Src tyrosine kinase activity within the Neu-induced mammary tumors was also measured by the capacity of c-Src immunoprecipitates to phosphorylate a modified cdc2 peptide (Lys^{19}-cdc-2(6-20)-NH$_2$) *in vitro*.

Figure 4.1. Elevated c-Src kinase activity in Neu-induced tumorigenesis.

(A). Tissue lysates derived from mammary tumors (T) or adjacent epithelium (N) were immunoprecipitated with c-Src specific antibodies (Ab.1, Oncogene Sci.) and 50% of the immunoprecipitate was subjected to *in vitro* kinase assays using acid denatured enolase as an external substrate. The SDS PAGE gels were alkali treated and dried before exposure to X-ray film. Normal rabbit serum was used as a negative control (-ve). The arrows indicate the position of the phosphorylated c-Src and enolase proteins. **(B).** The remaining portion (50%) of the c-Src immunoprecipitate from mammary tumor or adjacent epithelium was immunoblotted with anti-Src antibody. The position of the c-Src protein is illustrated by an arrow. The lower broad band is cross reactive immunoglobulin species. **(C).** Immunoprecipitation/immunoblot analyses of tissue extracts derived from tumor and adjacent epithelium with anti-Neu (MAb 7.16.4) and anti-phosphotyrosine (UBI) antibodies. The position of the tyrosine phosphorylated Neu protein is illustrated by an arrow.


A
#8331
#8342
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N T1 T2 N T N T N T N T1 T2 -ve
src
Enolase
1 2 3 4 5 6 7 8 9 10 11 12 13
IP: anti-src
B
src
1 2 3 4 5 6 7 8 9 10 11 12 13
Blot: anti-src
C
neu
1 2 3 4 5 6 7 8 9 10 11 12 13
IP: anti-neu; Blot: anti-ptyr

Table 4.1 Activation of c-Src tyrosine kinase in Neu induced mammary tumors.

The fold increase of c-Src kinase activity in tumor samples was calculated as the increase in kinase activity over that observed in adjacent normal epithelium. Enolase and cdc2 peptide assays were performed and quantitated as described under Materials and Methods.

	Mouse #	Enolase	cdc2 Peptide		Mouse #	Enolase	cdc2 Peptide		Mouse #	Enolase	cdc2 Peptide
1)	288	13.0	NT	7)	8331	3.4	3.5	13)	8564	13.0	NT
2)	8137	4.7	5.0	8)	8334	9.0	7.4	14)	8565	2.1	2.0
3)	8137	9.9	7.5	9)	8340	4.2	5.8	15)	8567	4.5	NT
4)	8142	5.0	7.8	10)	8342	8.0	8.5				
5)	8145	8.0	14.0	11)	8364	13.0	NT		Mean	6.8	6.7
6)	8331	4.0	5.0	12)	8364	8.4	NT		S.E	0.84	1.1

S.E: Standard Error of the mean. NT: Not Tested.

This peptide has been previously shown to be a specific substrate for the Src family of tyrosine kinases but is a poor substrate for the receptor tyrosine kinases such as the EGFR (Cheng et al., 1992). Consistent with the results of the enolase analyses, c-Src immunoprecipitates from mammary tumors incubated with cdc-2 peptide demonstrated a 6.7 fold higher kinase activity than those observed with immunoprecipitates from adjacent mammary epithelium (Table 4.1). Incubation of these c-Src immunoprecipitates with a control substrate peptide resulted in reduction of peptide phosphorylation to background levels.

To determine whether the increase in c-Src kinase activity observed in the mammary tumors was due to elevated amounts of c-Src or due to changes in its specific activity, parallel immunoblot analyses with Src specific antibodies were performed on the immunoprecipitates from both tumors and adjacent epithelium. Comparable levels of c-Src protein were detected in tumor and adjacent epithelium by this approach. The observed variation in the c-Src protein levels does not seem to account for the increase in c-Src tyrosine kinase activity (Figure 4.1B). Therefore the elevation of c-Src tyrosine kinase activity observed in the mammary tumors was due to an increase in the specific activity of c-Src tyrosine kinase and not to an increase in the amount of the protein.

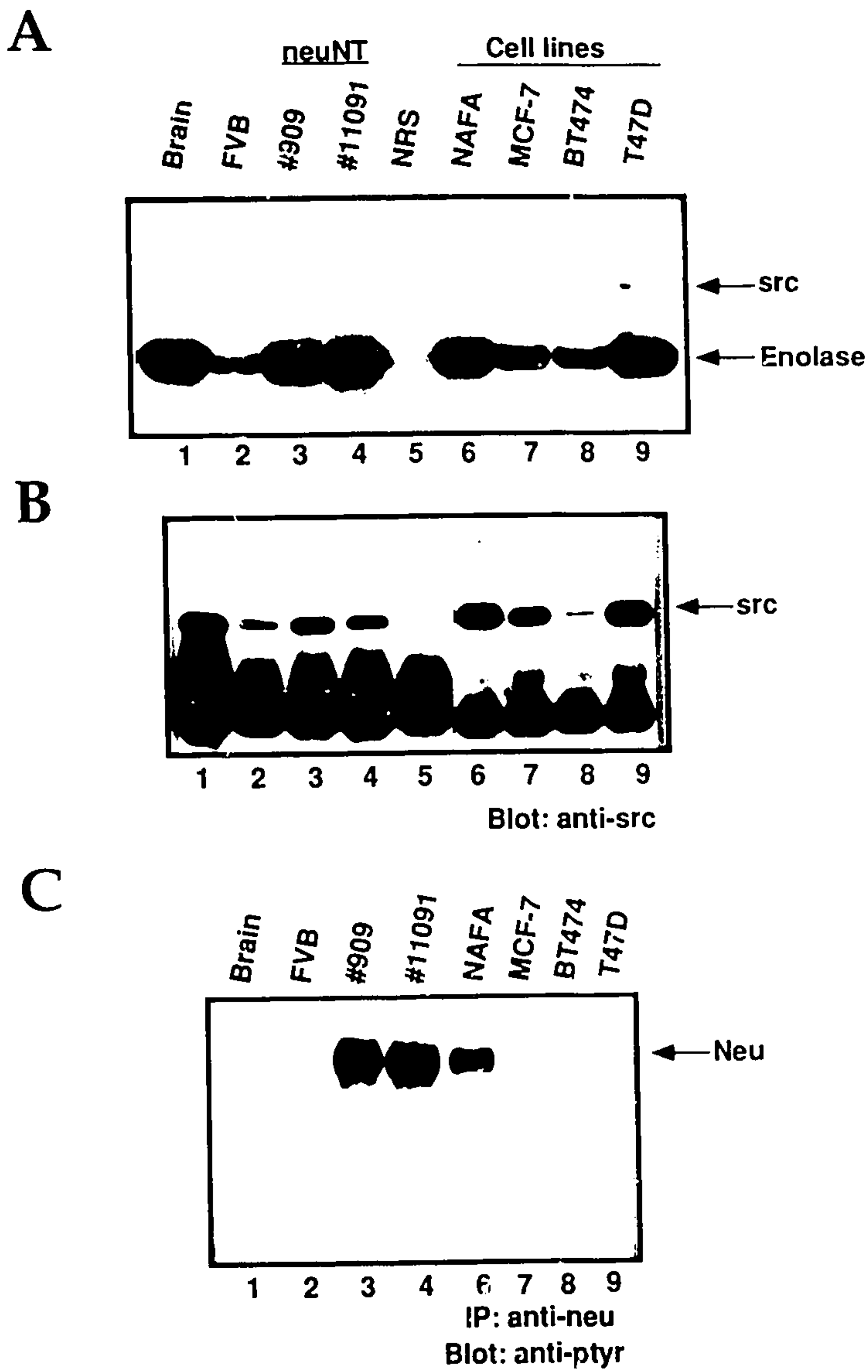
Mammary tumors that arise in N#202 female mice possess higher Neu intrinsic tyrosine kinase activity than the adjacent mammary epithelium (Guy et al., 1992b). To determine whether the activation of c-Src kinase was correlated with the activity of the Neu tyrosine kinase, protein extracts derived from tumor or tissues adjacent to the tumor were immunoprecipitated with Neu specific antibodies and then subjected to immunoblot analyses with anti-phosphotyrosine antibodies, as a measure of the state of activation of Neu. Although the level of tyrosine phosphorylated Neu observed in these tissues varied from one tissue sample to another, the presence of tyrosine phosphorylated Neu correlated well with the activation of c-Src (Figure 4.1C). The one sample of adjacent mammary tissue where tyrosine phosphorylated Neu could be detected (Figure 4.1C, Lane 1) also possessed elevated c-Src kinase activity (Figure 4.1A, lane 1). Conceivably the presence of kinase active Neu in this tissue may reflect the presence of microscopic tumors within this sample.

To further test the hypothesis that activation of c-Src is involved in Neu mediated tumorigenesis, we measured c-Src activity in primary tumors derived from the MMTV/activated *neu* mice (Figure 4.2A, lanes 3,4) (Muller et al., 1988) and in several mammary tumor derived cell lines (Figure 4.2A, lanes 6-9). By comparison to nontransgenic mammary epithelium (lane 2), the activated Neu-induced tumors (lanes 3,4) and two mammary tumor cell lines (lanes 6,9) possessed elevated c-Src activity. With the exception of the BT474 cell line which expresses low levels of c-Src, immunoblot analyses revealed comparable levels of c-Src among the tissues and cell lines. Thus like the tumors induced by unactivated Neu, the elevation in c-Src kinase activity in these activated Neu tumors reflects an increase in the specific activity of c-Src.

To determine if the increase of c-Src activity correlates with presence of tyrosine phosphorylated Neu, these extracts were immunoprecipitated with Neu specific antibodies (MAb7.16.4) followed by immunoblot analyses

Figure 4.2. Mammary tumors and derived tumor cell lines expressing activated Neu possess elevated c-Src kinase activity.

(A). Protein extracts from mammary tumors derived from the MMTV/activated *neu* mice or several mammary tumor cell lines were immunoprecipitated with Src specific antibodies (Ab.1, Oncogene Sci.) and subjected to *in vitro* kinase assays with exogenous enolase substrate. Also included as a positive control is tissue extracts derived from brain (Lane 1) and as negative controls tissue extract derived from normal FVB mammary tissue (Lane 2). The tumor tissue was also incubated with nonspecific normal rabbit sera (Lane 5). The location of c-Src and enolase substrate are indicated by the arrows. **(B).** Immunoblot analyses of c-Src immunoprecipitates with Src specific antibodies (Ab.1, Oncogene Sci.). The location of c-Src is indicated by the arrow. **(C).** The same protein extracts were incubated with Neu specific antibodies (7.16.4) and the immune complexes were resolved through a 9% SDS polyacrylamide gel and blotted with phosphotyrosine specific antibodies (UBI). The position of tyrosine phosphorylated Neu is indicated by the arrow.


A
neuNT
Cell lines
Brain
FVB
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NRS
NAFA
MCF-7
BT474
T47D
src
Enolase
1 2 3 4 5 6 7 8 9
B
src
1 2 3 4 5 6 7 8 9
Blot: anti-src
C
Brain
FVB
#909
#11091
NAFA
MCF-7
BT474
T47D
Neu
1 2 3 4 6 7 8 9
IP: anti-neu
Blot: anti-ptyr

with antiphosphotyrosine antibodies (Figure 4.2B). In both the activated Neu-induced tumors and a derived tumor cell line (NAFA) high levels of tyrosine phosphorylated Neu were detected (Lanes 3-5). By contrast, the MCF-7 and BT474 cell mammary tumor cell lines which possessed low c-Src activity failed to display evidence of activated Neu. These findings argue that activation of Neu kinase activity is correlated with induction of c-Src activity. However, one mammary tumor (T47D) cell line possessed elevated c-Src activity in the absence of activated Neu. Conceivably, the elevated c-Src activity observed in this cell line occurs through a mechanism independent of Neu activation.

4.2.2 Differential activation c-Src tyrosine kinase in Neu and c-Myc induced mammary tumorigenesis.

It is conceivable that the elevated c-Src kinase activity observed in the Neu- induced mammary tumors reflected the downstream involvement of c-Src in a Neu signal transduction pathway. If this hypothesis is correct, mammary tumors induced by oncogenes that function downstream of c-Src should not possess elevated c-Src activity. To test this possibility, I extended the *in vitro* kinase analyses to mammary tumors induced by the c-*myc* oncogene (Stewart et al., 1984). Transgenic mice carrying the MMTV/c-*myc* fusion gene (TG.M) develop focal mammary tumors that arise next to normal transgene-expressing mammary epithelium (Stewart et al., 1984). Examination of c-Src kinase activity in tumor specimens from the c-Myc-induced mammary tumors revealed that the levels of c-Src tyrosine kinase activity were not significantly elevated by comparison to the Neu-induced mammary tumors (Figure 4.3A, compare Lanes 2-3 and Lanes 4-6). Quantitative measurement revealed that the Neu-induced mammary tumors possessed 4 to 7.5 fold higher levels of c-Src kinase activity compared to c-Myc-induced mammary tumors. Moreover, the c-Src kinase activity observed in c-Myc-induced mammary tumors were comparable to the levels observed in the mammary epithelium of nontransgenic mice (Figure 4.3A, Lane 7, NMG). The inability to detect activated c-Src in the mammary tumors expressing c-Myc was not due to the absence of c-Src since immunoblot analyses of the immunoprecipitates from these tumor samples with Src specific antibodies revealed comparable levels of c-Src between Neu and c-Myc expressing tumors (Figure 4.3B, compare Lanes 2-3 with Lanes 4-6). Thus, it is likely that activation of c-Src kinase may be involved in transformation by the *neu* oncogene but is dispensable for tumorigenesis mediated by the *c-myc* proto-oncogene.

4.2.3 c-Src associates with Neu in vivo

To understand the mechanism by which c-Src is activated in Neu-induced mammary tumors I was interested to test whether c-Src could associate with Neu *in vivo*. Cell lysates derived from a mammary tumor cell line established from the MMTV/activated Neu mice (NAFA, Muller et al., 1988) were immunoprecipitated with Src specific antibodies (Ab 327) and immunoblotted with Neu specific antibodies (Figure 4.4A, Lanes 2,3). This cell line has previously been shown to express high levels of activated Neu

Figure 4.3. Differential activation of c-Src in Neu and c-Myc expressing mammary tumors.

(A). The tumor lysates were incubated with anti-Src antibody (Ab.1, Oncogene Sci.) and the immune complexes were purified and subjected to an *in vitro* kinase assay using acid denatured enolase. The SDS PAGE gel was treated with KOH before autoradiography. N and T represents normal and tumor epithelium respectively, from a *neu* transgenic mouse (Lanes 1,2,3). M160, M359, and M365 were tumors from c-*myc* transgenic mice (Lanes 4,5,6). The lane, "NMG" corresponds to normal mammary epithelium from a non-transgenic mouse and "-ve", corresponds to the normal rabbit serum (NRS) control. **(B).** Immunoblot analyses of the same immunoprecipitated tissue samples with Src specific antibodies. The position of c-Src as observed on longer exposure of the autoradiogram is illustrated by the arrow. The broad lower band is due to cross reaction with the c-Src antibody in the samples.

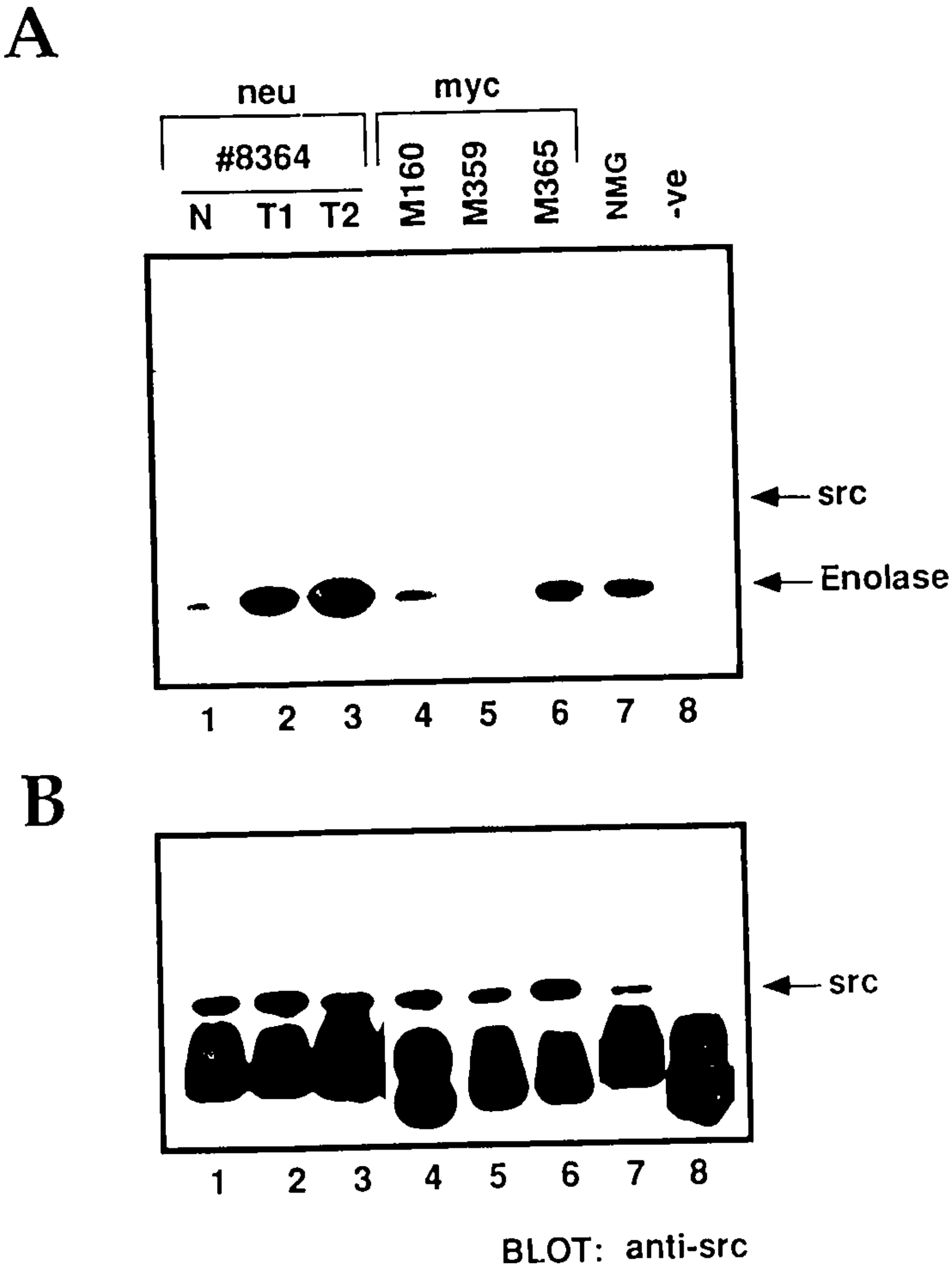
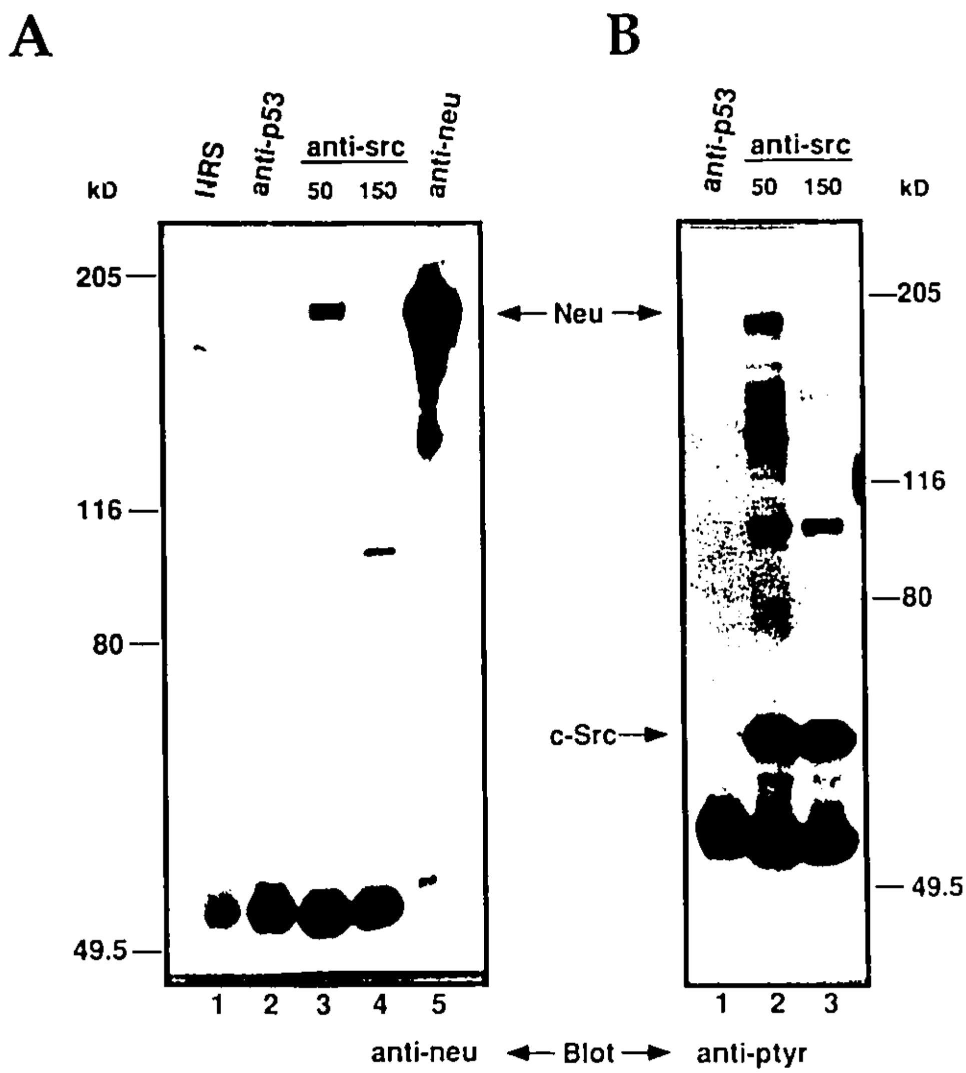

A
neu
myc
#8364
N
T1
T2
M160
M359
M365
NMG
-ve
src
Enolase
1
2
3
4
5
6
7
8
B
src
1
2
3
4
5
6
7
8
BLOT: anti-src

Figure 4.4 c-Src physical associates with activated Neu *in vivo*.

(A). Protein extracts derived from NAFA mammary tumor cell line (21: Figure 2) were immunoprecipitated with Src specific antibodies and the immunoprecipitates were washed 5 times in lysis buffer containing either 50 mM NaCl (Lane 3) or 150 mM NaCl (Lane 4). The immune complexes were resolved through a 9% SDS polyacrylamide gel and immunoblotted with Neu specific antibodies (Ab.3, Oncogene Sci.). The extracts were also immunoprecipitated with either normal rabbit sera (Lane 1) or nonspecific mouse monoclonal (anti-p53, Lane 2) as negative controls. An anti-Neu (7.16.4) immune precipitate serves as a positive control (Lane 5). **(B).** Identical immunoprecipitates were blotted with anti-phosphotyrosine (UBI) containing antibodies. The position of c-Src and Neu are indicated by the arrows.


A
B
HRS
anti-p53
anti-src
50
150
anti-neu
kD
205
116
80
49.5
Neu
c-Src
1
2
3
4
5
anti-neu
Blot
anti-ptyr

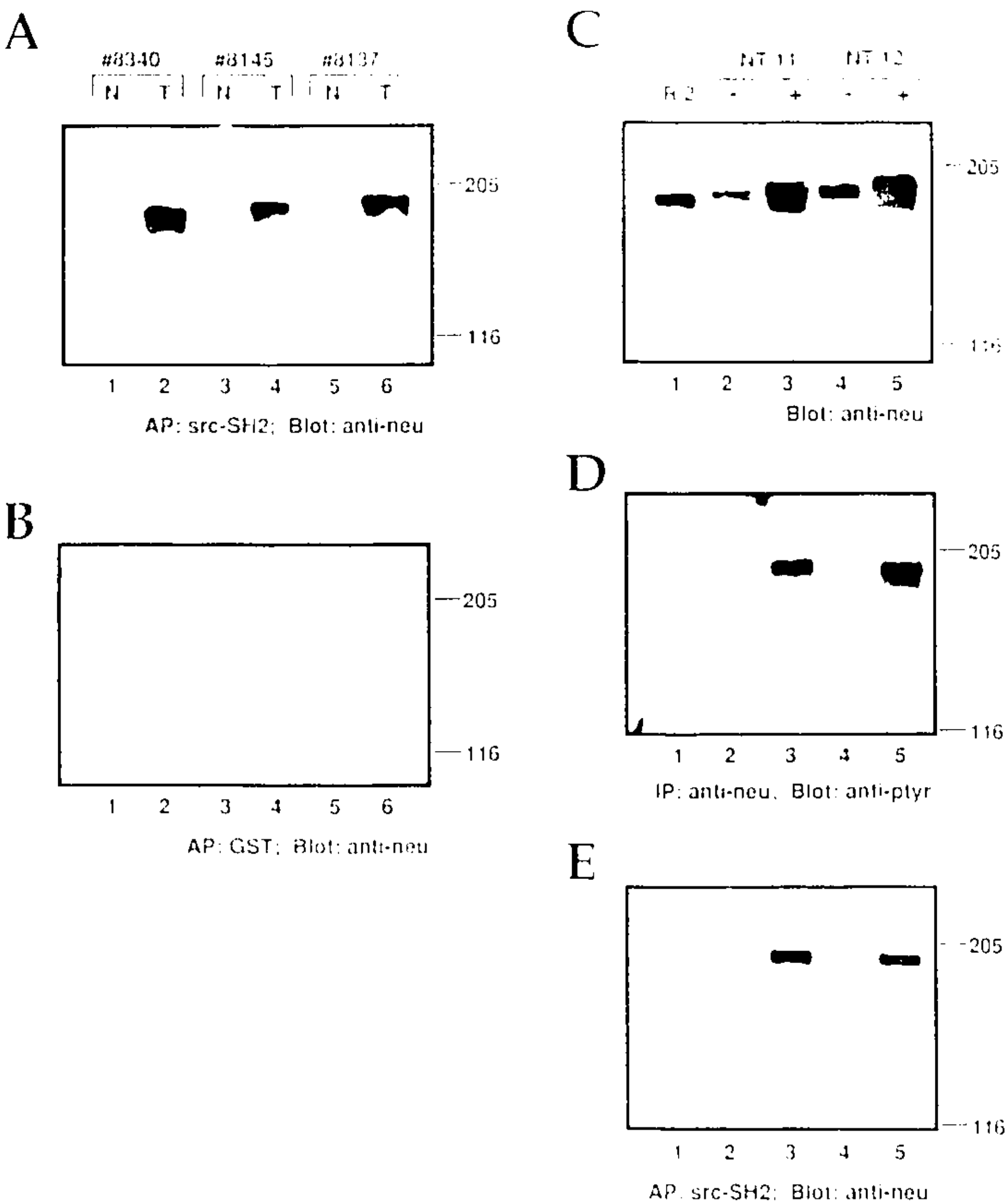
and to possess elevated c-Src activity (Figure 4.2). As shown in Figure 4.4A Neu could be detected in the c-Src immunoprecipitates (lane 3). By contrast, immunoprecipitations with control antibodies (lane 1) normal rabbit serum or nonspecific mouse monoclonal (lane 2) did not precipitate any detectable Neu. Consistent with these observations, immunoblot analyses of the c-Src immunoprecipitates with antiphosphotyrosine antibodies revealed tyrosine phosphorylated bands of 185 kDa and 60 kDa that comigrated with Neu and c-Src respectively (Figure 4.4B, lane 2) suggesting that c-Src associates with tyrosine phosphorylated Neu. But it is unclear whether c-Src requires the presence of tyrosine phosphorylated Neu for its ability to associate with Neu.

4.2.4 The SH2 domains of c-Src specifically associate with tyrosine phosphorylated Neu *in vitro*

Since c-Src associates with tyrosine phosphorylated Neu, it is possible that this association is mediated by the c-Src SH2 domain. To test this possibility, Neu containing protein extracts derived from tumors and adjacent epithelium were examined for their capacity to bind Sepharose beads bearing a GST-c-Src SH2 fusion protein. The proteins bound to the SH2 domain were eluted and the presence of Neu was detected by immunoblot analysis. As shown in Figure 4.5A, protein extracts derived from Neu-induced tumors bound to the GST-c-Src SH2 fusion protein (Lanes 2, 4, and 6). By contrast, this fusion protein failed to bind Neu derived from the adjacent epithelium (Lanes 1, 3 and 5). Incubation of these

Figure 4.5 The c-Src SH2 domain binds to tyrosine phosphorylated Neu.

(A). Tissue extracts from tumor (T) or adjacent epithelium (N) were incubated with Sepharose bound GST-c-Src-SH2 fusion protein. The bound material was eluted and immunoblotted with anti-Neu monoclonal antibody (Ab.3, Oncogene Sci.). **(B).** The same protein extracts were passed through a GST column and treated as described above. **(C).** Stably transformed Rat.2 cell lines (NT) expressing activated *neu* under the control of the MMTV promoter/enhancer were derived. Two representative clones were plated either in the presence (+) or absence (-) of dexamethasone (dex). Total protein lysates were resolved and blotted with anti-neu antibody (Ab.3, Oncogene Sci.). **(D).** Identical lysates were incubated with anti-Neu antibody (7.16.4) and the immune complexes were resolved through a 9% SDS polyacrylamide gel and blotted for anti-phosphotyrosine (UBI). **(E).** Similar protein extracts were incubated with a Sepharose bound GST-c-Src SH2 fusion protein and were affinity purified as described under Materials and Methods. The purified complexes were separated through a 9% SDS polyacrylamide gel and blotted for anti-Neu (Ab-3, Oncogene Sci.).


A
#8340
#8145
#8137
N T N T N T
205
116
1 2 3 4 5 6
AP: src-SH2; Blot: anti-neu
B
205
116
1 2 3 4 5 6
AP: GST; Blot: anti-neu
C
NT 11
NT 12
- + - +
205
1 2 3 4 5
Blot: anti-neu
D
205
116
1 2 3 4 5
IP: anti-neu, Blot: anti-ptyr
E
205
116
1 2 3 4 5
AP. src-SH2; Blot: anti-neu

protein extracts with GST alone did not bind any detectable Neu (Figure 4.5B). The capacity of Neu to associate with GST-c-Src SH2 fusion protein in these extracts was closely correlated with the state of tyrosine phosphorylation of Neu because association of the c-Src SH2 fusion protein was only detected in tissues expressing tyrosine phosphorylated Neu (compare Figure 4.5A with Figure 4.1C).

To directly establish whether tyrosine phosphorylation of Neu was required for its binding to the GST-c-Src SH2 fusion protein, we derived Rat-2 fibroblast cell lines which could be induced to express a mutant Neu that is constitutively active due to a point mutation in the transmembrane domain (Bargmann et al., 1986). These cell lines were derived by transfection of Rat-2 cells with a construct containing the mutant *neu* cDNA under the transcriptional control of the MMTV promoter/enhancer. To confirm that the cell lines could be induced to express tyrosine phosphorylated Neu, representative clones carrying the MMTV/activated *neu* construct (NT.11, NT.12) were tested for their capacity to synthesize tyrosine phosphorylated Neu upon dexamethasone administration. The cells were lysed and the protein extracts were analyzed by immunoprecipitation with Neu specific antibodies followed by immunoblot analysis with anti-phosphotyrosine antibodies. The results showed that the level of tyrosine phosphorylated Neu was dramatically elevated upon addition of dexamethasone (Figure 4.5D). The elevated levels of tyrosine phosphorylated Neu is due to the fact that the activated Neu protein expressed under the control of MMTV promoter/enhancer is 20 fold more active than the endogenous Neu (Bargmann and Weinberg, 1988). As expected, tyrosine phosphorylated Neu could not be detected in lysates derived from the Rat-2 parental cell line grown in the presence or in the absence of dexamethasone. This suggests that the increase in the levels of tyrosine phosphorylated Neu in NT.12 cells was due the ability of dexamethasone to induce expression of the activated *neu* gene under the control of the MMTV promoter/enhancer.

Protein extracts derived from uninduced or induced NT.11 and NT.12 cells were incubated with the GST-c-Src SH2 fusion protein immobilized on Sepharose beads, the affinity purified protein complexes were eluted, and immunoblotted with a Neu specific antibodies. The immobilized GST-c-Src SH2 fusion protein bound to tyrosine phosphorylated Neu but failed to bind Neu in its unphosphorylated state (Figure 4.5E). The inability to detect Neu in the uninduced cell extracts was not due to the absence of Neu in these cells because these cells express high levels of endogenous Neu as measured by immunoblot analysis (Figure 4.5C).

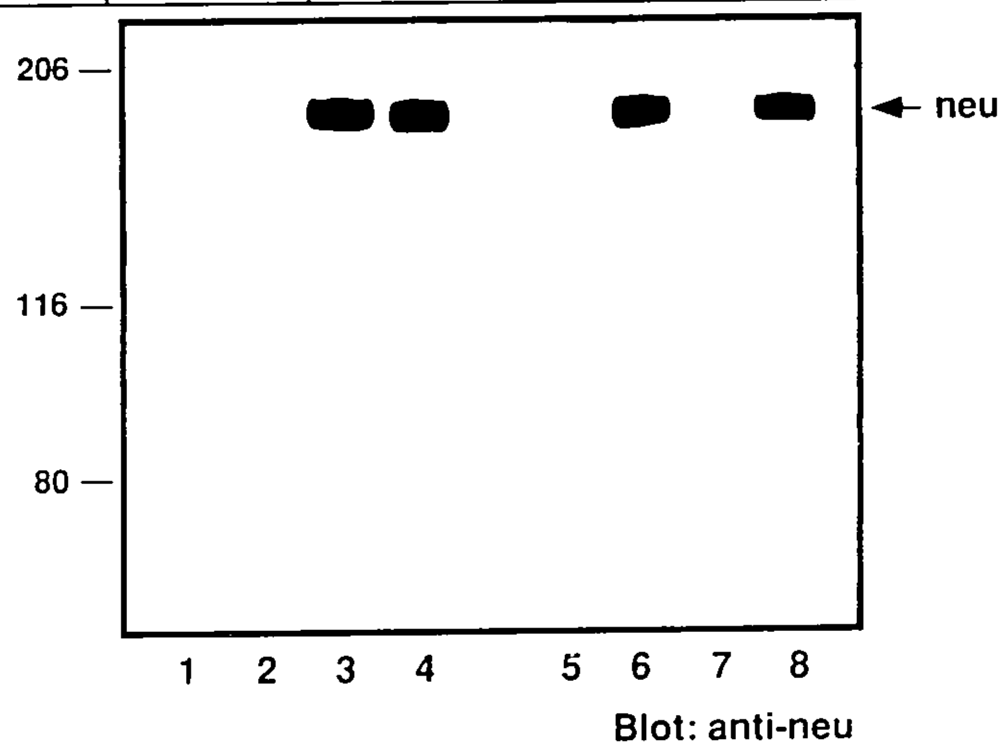
4.2.5 The c-Src SH2 binding site on Neu is distinct from the GAP SH2 binding site

I was interested to learn whether the phosphorylated tyrosine residue on Neu to which the Src SH2 domain bound, was also used by other SH2 containing molecules involved signal transduction. Because the GTPase activating protein (GAP) has been implicated as a component of the Neu signal transduction pathway (Fazioli et al., 1991), I tested the capacity of GST fusion protein carrying both the GAP SH2 domains to bind to cell extracts from uninduced (Figure 4.6, Lane 2) and induced NT.11 cells (Figure 4.6, Lane 4). Consistent with the observations made with the c-Src (Figure 4.6, Lanes 1,3), the GAP-SH2 containing fusion protein associated with tyrosine phosphorylated Neu. To examine whether the binding sites for the c-Src and GAP SH2 domains are distinct, I performed an *in vitro* competition assay. Cell extracts containing tyrosine phosphorylated Neu were preincubated with the soluble form of c-Src SH2 fusion protein (see Materials and Methods). The preincubated cell lysates were subsequently incubated with immobilized form of either GST-GAP SH2 or GST-c-Src SH2 fusion protein. The proteins bound to the immobilized form of fusion proteins were isolated and resolved on SDS-PAGE and immunoblotted with anti-Neu antibodies. The results showed that preincubation of tyrosine phosphorylated Neu with the soluble GST-c-Src SH2 fusion protein prevented binding of Neu to the immobilized GST-c-Src SH2 containing fusion protein (Figure 4.6, Lane 6) but did not interfere with the binding of Neu to immobilized GST-GAP SH2 containing protein (Figure 4.6, Lane 6). Conversely preincubation of the protein extracts from induced NT-11 cells with a soluble GST-GAP SH2 fusion protein efficiently interfered with the ability of tyrosine phosphorylated Neu to bind to the immobilized GST-GAP SH2 containing fusion protein (Figure 4.6, Lane 7) but had little effect on the capacity of Neu to bind the immobilized GST-c-Src SH2 fusion protein (Figure 4.6, Lane 8). These findings suggest that the c-Src SH2 domain binding site on Neu is distinct from that utilized by the GAP SH2 domain.

Figure 4.6 The c-Src SH2 domain binds to a phosphotyrosine residue distinct from the GAP-SH2 domain.

The rat cell lines carrying the MMTV/activated *neu* fusion gene were grown either in the presence (+ DEX) or absence of dexamethasone (- DEX). Cell lysates were preincubated either in the presence (+) or absence (-) either soluble GST-SH2(src) (lanes 5 and 6) or soluble GST-SH2 (GAP) (Lanes 7 and 8). Following affinity purification using GST.SH2-c-Src (Lanes 5 and 8) or GST-SH2 GAP fusion proteins (Lanes 6 and 7) immobilized on Sepharose beads, the affinity purified complexes were resolved by electrophoresis in 8% SDS polyacrylamide gel and immunoblotted for Neu (Ab-3, Oncogene Sci.). The position of the Neu is illustrated by an arrow.

	- DEX		+ DEX					
Soluble GST.SH2(src)	-	-	-	-	+	+	-	-
Soluble GST.SH2(GAP)	-	-	-	-	-	-	+	+
Immobilised GST.SH2	src	GAP	src	GAP	src	GAP	GAP	src



4.3 DISCUSSION

The results presented here show that activation of c-Src is a frequent event in Neu-induced mammary tumors in transgenic mice. I also present evidence to suggest that activation of c-Src might occur through interaction of its SH2 domain with tyrosine phosphorylated Neu. Together these observations support the contention that activation of c-Src is involved in Neu mediated signaling.

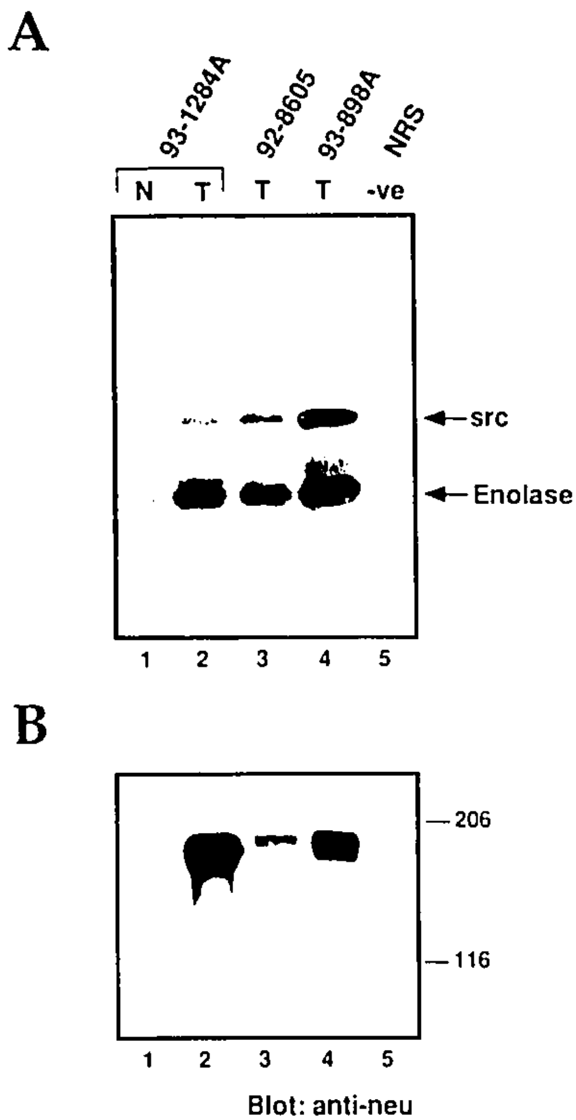
The dramatic elevation of c-Src kinase activity in mammary tumors of MMTV/unactivated *neu* transgenic mice is likely due to the increase in specific activity of c-Src because the variation in the amount of c-Src protein present in the tumor and the adjacent mammary epithelium does not account for the increase in c-Src kinase activity observed in the tumor samples. The increase in c-Src enzymatic activity closely correlated with tyrosine phosphorylation of Neu. The importance of activation of c-Src in Neu-induced mammary tumorigenesis is further supported by the observation that a large proportion of human breast tumors possess elevated c-Src kinase activity (Jacobs and Rubsamen 1983, Rosen et al., 1986, Ottenhoff-Kalff, et al., 1992). However, it is unclear in these studies whether elevated c-Src activity was accompanied by increased Neu tyrosine kinase activity. In this regard, I have examined few Neu-expressing human breast tumors (n=3) for evidence of elevated c-Src activity. Consistent with observations made with the Neu-induced transgenic tumors, every human breast tumor examined had elevated c-Src kinase activity compared to adjacent mammary tissue (Figure 4.7). However, careful examination of a larger number of human breast cancers will be required to establish whether the activation of c-Src correlates with elevated expression of Neu.

Although a large proportion of the mammary tumors possessed elevated c-Src activity, other murine mammary tumor samples did not. In particular, mammary tumors expressing the c-*myc* proto-oncogene possessed low levels of c-Src activity. Conceivably, the events involved in transformation of the c-*myc* expressing epithelial cell operates downstream of the c-Src kinase. In tissues such as the lymphoid compartment, c-*myc* induced tumorigenesis required the coexpression of either the pim-1 serine kinase or the product of the bmi locus (Van Louhuizen et al., 1989, Van Louhuizen et al., 1991). Whatever the mechanism by which c-*myc* induces mammary tumors, these observations suggest that elevated c-Src activity exhibited by mammary tumors is not simply due to acquisition of the transformed phenotype.

There is considerable evidence to suggest that the elevated c-Src activity in mammary tumors may result from activation of the Neu receptor kinase. In both Neu-expressing murine and human mammary tumors as well as derived cell lines we have consistently observed elevated c-Src activity. In addition, we were able to demonstrate that the c-Src SH2 domain binds to specific phosphotyrosine residue(s) within the activated Neu receptor suggesting that physical interaction between activated Neu and c-Src may alter the latter's intrinsic tyrosine kinase activity. Recently it was shown that *in vivo* physical complexes between c-Src and EGFR can be

Figure 4.7 c-Src kinase activity in human breast tumor samples.

Protein lysates were prepared from Normal (N) (lane 1) or tumor (T) (lanes 2-4) samples of human breast tissues. **(A).** Five hundred micrograms of protein was incubated with c-Src specific antisera (Ab 327). The immune complexes were washed and subject to *in vitro* kinase assay using acid denatured enolase as a substrate. Normal rabbit serum (NRS, lane 5) was used as a negative (-ve) control. **(B).** Fifty micrograms of total protein from the same samples were resolved on a SDS-PAGE gel and subjected to immunoblot analyses using anti-Neu antisera (Ab.3, Oncogene Sci.) Lane 5 corresponds to lysate from 3T3 cells that are known to express very low levels of Neu.


A
93-1284A
92-8605
93-898A
NRS
N
T
T
T
-ve
src
Enolase
1
2
3
4
5
B
206
116
1
2
3
4
5
Blot: anti-neu

detected in a breast cancer derived cell line (Luttrell et al., 1994). In the following chapter I present evidence that suggests, among the two erbB family members (EGFR and Neu) known to associate with and activate c-Src, Neu plays a central role in c-Src activation and EGF-mediated activation of c-Src tyrosine kinase may involve Neu RTK.

CHAPTER 5

Direct and specific interaction of c-Src with Neu is involved in signaling by the Epidermal Growth Factor Receptor

5.1 INTRODUCTION

EGF stimulation of cells expressing high levels of EGFR results in elevation of tyrosine kinase activity of the Src family members (c-Src, c-Yes and Fyn) (Osherov and Levitzki 1994). Moreover fibroblasts overexpressing c-Src are hyperresponsive to EGF mediated mitogenesis (Luttrell et al., 1988; Wilson et al., 1989). In the previous chapter I have presented evidence that suggests that physical complexes between c-Src and Neu can occur and these complexes correlate with elevated c-Src activity. Results from other labs have shown that c-Src associates with EGFR (Luttrell et al., 1994). Taken together these observations suggest that the c-Src tyrosine kinase may also be involved in signaling by these EGFR family members.

While c-Src can complex with both EGFR and Neu, it is unclear whether c-Src interacts directly with both of these tyrosine phosphorylated receptors. Because both Neu and EGFR can heterodimerize (King et al., 1988, Stern and Kamps, 1988, Kokai et al., 1989, Wada et al., 1990, Qian et al., 1992), it is conceivable that the observed complexes of c-Src with Neu and EGFR are due its interaction with only one of these receptors. It is also possible that c-Src/EGFR and c-Src/Neu association may involve intermediate proteins. To further elucidate the mechanism by which c-Src and EGFR family members interact, I examined the ability of a radiolabeled Glutathione-S-transferase (GST)-c-Src SH2 fusion protein to directly bind denatured tyrosine phosphorylated Neu. The results revealed that the c-Src SH2 fusion protein could directly bind Neu in tyrosine phosphorylation dependent manner. Interestingly, the c-Src SH2 fusion protein was unable to interact directly with tyrosine phosphorylated EGFR. To test the hypothesis that EGFR stimulated c-Src activity was through transphosphorylation of Neu, protein extracts derived from cells stimulated with EGF were immunoprecipitated with Neu, EGFR and Src specific antibodies and subjected to immunoblot analyses with anti-phosphotyrosine and anti-Neu antibodies. The results showed that EGF stimulation resulted in the formation of complexes between c-Src and tyrosine phosphorylated Neu. By contrast, EGF stimulation did not result in the formation of comparable complexes between EGFR and c-Src. These results suggest that activation of c-Src tyrosine kinase by these two closely related EGFR family members occurs through a direct and specific interaction of c-Src with tyrosine phosphorylated Neu.

5.2 RESULTS

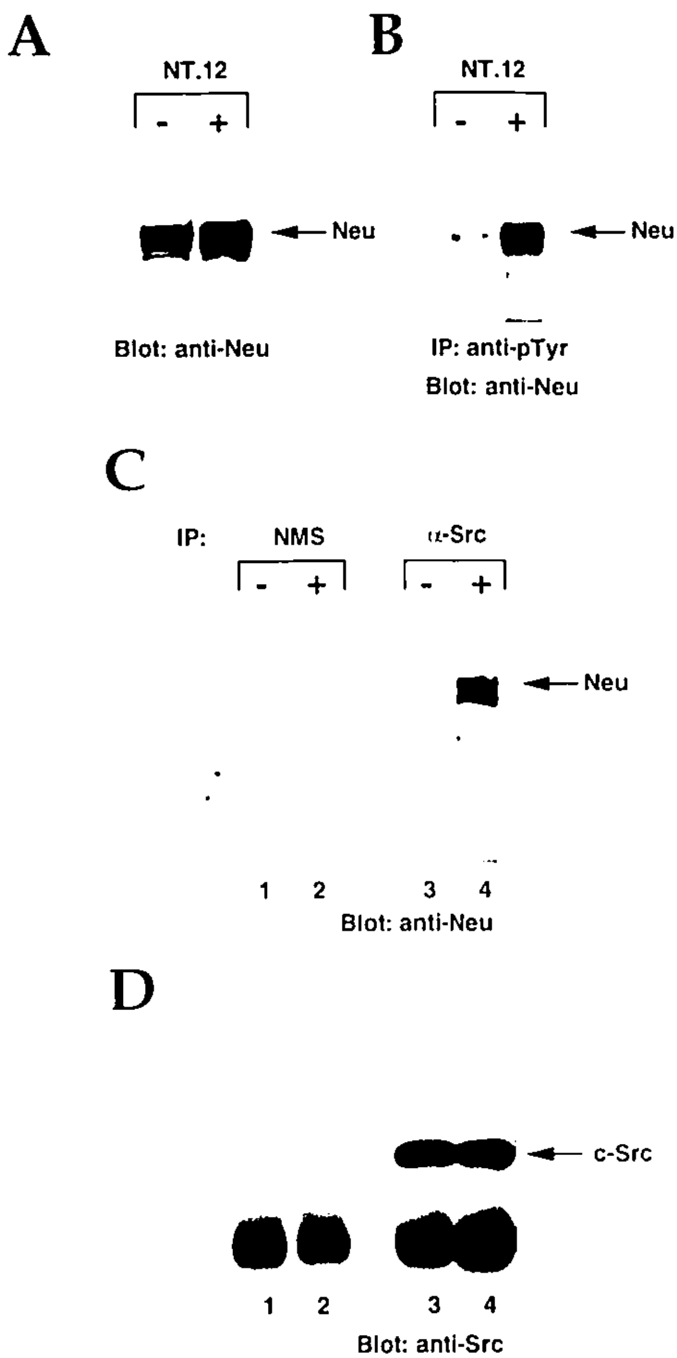
5.2.1 Association of c-Src with Neu is tyrosine phosphorylation dependent.

Although results presented in the previous chapter have shown that c-Src and Neu associate with each other it is unclear whether this *in vivo* association requires tyrosine phosphorylated Neu. To address this question, protein extracts derived from Rat-2 cell lines expressing activated *neu* (Val 664 to Glu, Bargmann et al., 1986) under the inducible MMTV promoter/enhancer (NT.12, see section 4.2) were subjected to immunoprecipitation and immunoblot analysis with c-Src and Neu specific antisera. To confirm that this cell line could be induced to express tyrosine phosphorylated Neu, lysates were prepared from cells grown either in the presence or absence of dexamethasone. Although dexamethasone induction of this cell line resulted in a marginal increase in the levels of total Neu (Figure 5.1A), the levels of tyrosine phosphorylated Neu were dramatically elevated upon stimulation with dexamethasone (Figure 5.1B).

To assess whether the *in vivo* association of Neu and c-Src required tyrosine phosphorylated Neu, c-Src was immunoprecipitated from both dexamethasone induced and uninduced cell lysates and subjected to immunoblot analyses with anti-Neu antibodies (Figure 5.1C), or with an antisera that recognizes c-Src (Figure 5.1D). The results revealed that c-Src complexed only with tyrosine phosphorylated Neu (Figure 5.1C, lane 4). The inability to detect Neu in the uninduced lysates was not due to

Figure 5.1 In vivo association between c-Src and Neu is dependent on the presence of tyrosine phosphorylated Neu.

NT.12 cells expressing activated Neu under the control of MMTV promoter/enhancer were grown both in the presence (+) and absence (-) of dexamethasone. **(A)** Anti-Neu immunoblot of total cell lysates. (**B**) The same batch of cell lysates were immunoprecipitated with anti-phosphotyrosine antibody and probed with anti-Neu antibodies. **(C)** c-Src was immunoprecipitated (α-Src, lanes 3 and 4) from the same batch of lysates and probed with anti-Neu antibodies. Normal mouse serum (NMS) was used as a non-specific control (lanes 1 and 2). **(D)** The blot in panel C was immunoblotted with an antibody that recognizes c-Src.


A
NT.12
- +
Neu
Blot: anti-Neu
B
NT.12
- +
Neu
IP: anti-pTyr
Blot: anti-Neu
C
IP: NMS α-Src
- + - +
Neu
1 2 3 4
Blot: anti-Neu
D
c-Src
1 2 3 4
Blot: anti-Src

difference in the levels of c-Src because comparable levels of c-Src were detected in both induced and uninduced extracts (Figure 5.1D compare lanes 3 and 4). These observations indicate that the physical association of c-Src with Neu is dependent on tyrosine phosphorylation of Neu.

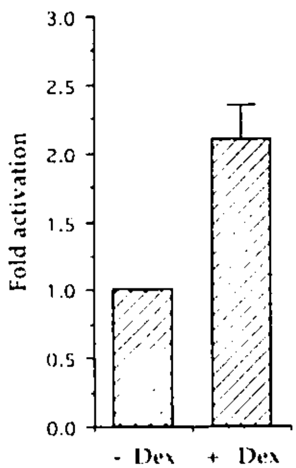
5.2.2 Expression of kinase active Neu results in elevation of c-Src tyrosine kinase activity

To assess whether the physical association of c-Src with Neu correlated with an increase in the specific activity of c-Src, I measured the capacity of c-Src immunoprecipitates to phosphorylate acid denatured enolase *in vitro.* The results revealed that the tyrosine kinase activity of c-Src increased by 2.3 fold in dexamethasone treated NT.12 cells when compared to that observed in untreated cells (Figure 5.2A). The increase in the c-Src kinase activity was due to a change in the intrinsic kinase activity of c-Src since control c-Src immunoprecipitates probed with anti-Src antibodies showed identical levels of c-Src protein both in uninduced and induced conditions (Figure 5.2B). No difference in c-Src kinase activity was observed in the Rat-2 parental cell line grown in the presence or absence of dexamethasone (data not shown). These observations suggest that activation of c-Src occurs as a direct consequence of its ability to complex with tyrosine phosphorylated Neu and genetically, c-Src functions downstream of Neu RTK.

Figure 5.2 Increase in the specific activity of c-Src upon expression of kinase active Neu.

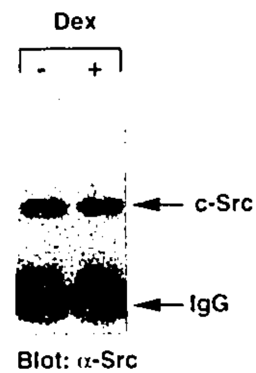
Lysates from NT.12 cells grown both in the presence (+Dex) and in the absence (-Dex) of dexamethasone (Dex) were immunoprecipitated with Src specific antibodies and the immunoprecipitates were incubated with γ-^{32}P ATP and acid denatured enolase as an external substrate. The radioactivity transferred onto the enolase was quantitated by PhosphorImager analyses. (A) The increase in c-Src kinase activity following Dex induction is shown as fold increase over the kinase activity observed in the absence of dexamethasone induction. The graph represents the average fold activation observed in four independent experiments. (B) A part of the immunoprecipitates used in the kinase assay (panel A) was immunoblotted with Src specific antisera (α-Src) and ^{125}I anti-mouse secondary antibody.

A



Fold activation
3.0
2.5
2.0
1.5
1.0
0.5
0.0
- Dex
+ Dex

B



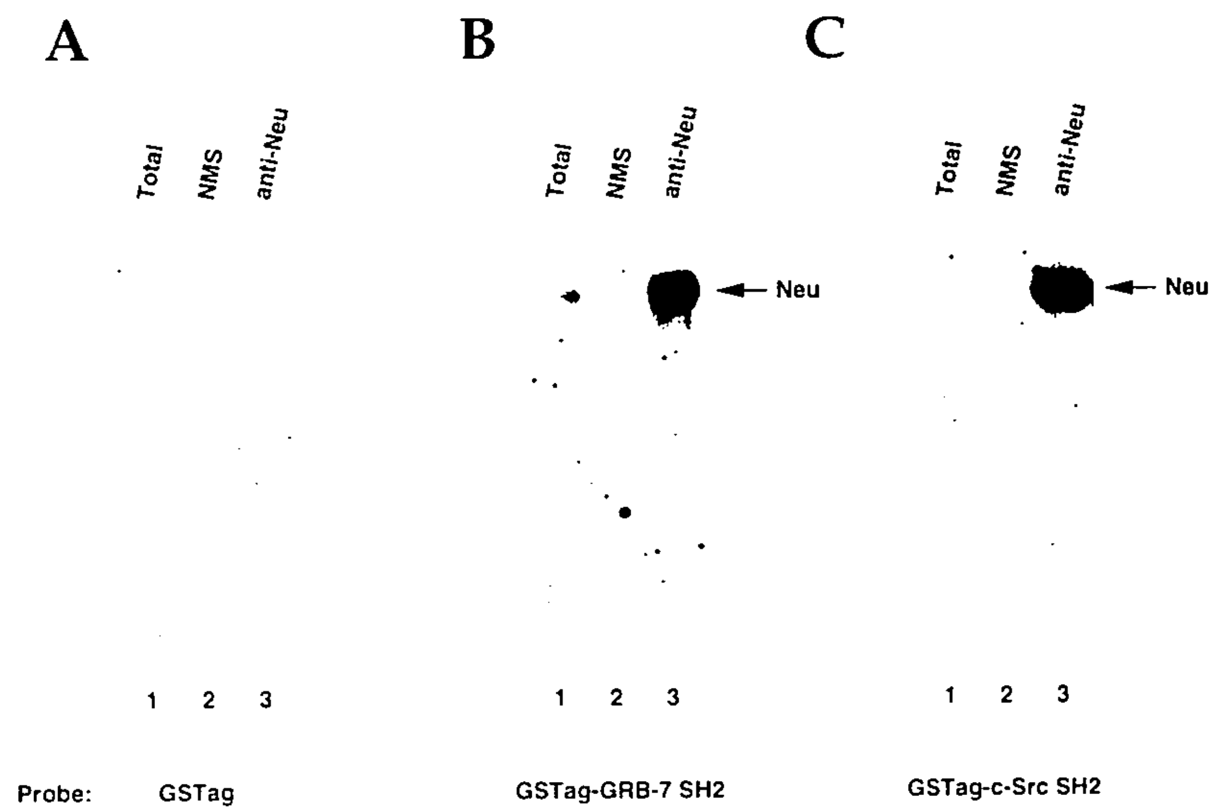
Dex
-
+
c-Src
IgG
Blot: α-Src

5.2.3 The SH2 domain of c-Src interacts directly with Neu.

Although these results suggest that c-Src associates with tyrosine phosphorylated Neu *in vivo*, it is unclear whether this association is a direct interaction of c-Src with Neu or occurs through the mediation of other protein(s). To determine whether the c-Src SH2 domain could directly bind to denatured Neu, total cell lysates, control immunoprecipitates and anti-Neu immunoprecipitates from a Neu expressing mammary epithelial cell line, NAFA, were resolved on SDS-PAGE, transferred onto PVDF membranes and probed with radiolabeled GST fusion protein containing the SH2 domain of c-Src (GSTag-c-Src SH2) as a probe (Figure 5.3). As shown in Figure 5.3A, radiolabeled GSTag alone did not bind to any protein in total cell lysates or to the Neu immunoprecipitate (lanes 1-3). As a positive control, I probed an identical membrane with a radiolabeled GSTag-Grb-7 SH2 fusion protein that has been previously demonstrated to directly bind Neu (Stein et al., 1994a). As expected, the Grb-7 SH2 domain bound to Neu both in total extracts and in Neu immunoprecipitates (Figure 5.3B, lanes 1 and 3). Similar analyses using radiolabeled c-Src SH2 (GSTag-c-Src SH2) fusion protein revealed that, like Grb-7, the c-Src SH2 containing fusion protein could directly interact with denatured Neu (Figure 5.3C, lanes 1 and 3). These observations indicate that the complexes observed between c-Src and Neu may occur through direct interaction of c-Src SH2 domain with Neu.

Figure 5.3 c-Src SH2 domain interacts directly with denatured Neu.

Anti-Neu immunoprecipitates (anti-Neu) and total lysates (Total) from a mammary epithelial cell line (NAFA) were resolved in a SDS-PAGE and blotted onto a PVDF membrane. Duplicate membrane strips were probed with radiolabeled GSTag alone (**A**, negative control) or GSTag-GRB-7 SH2 (**B**, positive control) or GSTag-c-Src SH2 (**C**). Normal mouse serum (NMS) immunoprecipitate was used a non-specific control.

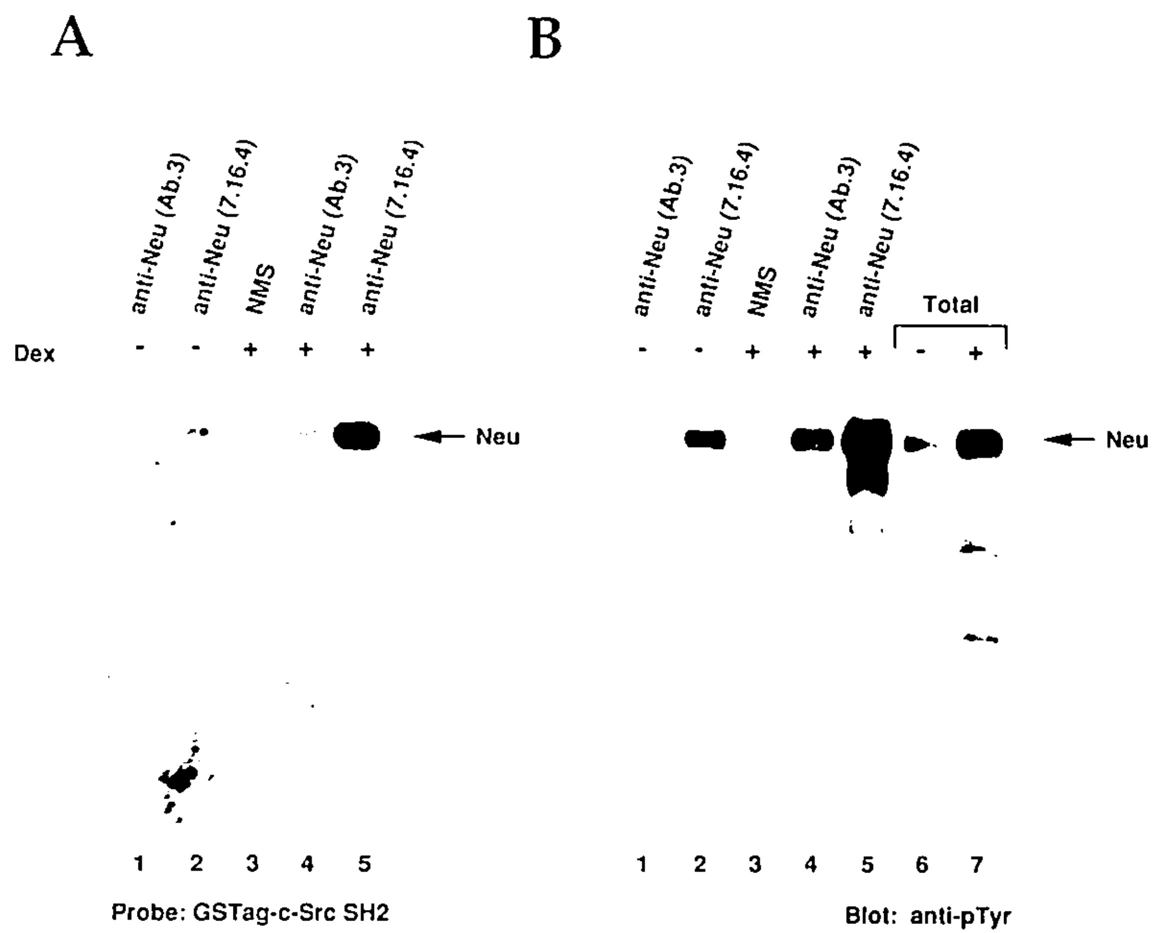

A
B
C
Total
NMS
anti-Neu
Total
NMS
anti-Neu
Total
NMS
anti-Neu
Neu
Neu
1 2 3
1 2 3
1 2 3
Probe:
GSTag
GSTag-GRB-7 SH2
GSTag-c-Src SH2

5.2.4 The direct binding of c-Src SH2 domain to Neu is dependent on tyrosine phosphorylation

To determine whether this interaction required tyrosine phosphorylated Neu, Neu was immunoprecipitated from dexamethasone induced or uninduced NT.12 cells (Figure 5.1A, B) using either antibodies raised against a C-terminal peptide (Ab-3) or the extracellular domain (7.16.4) of Neu (Drebin et al., 1984). The immunoprecipitates were resolved on a SDS-PAGE, transferred onto PVDF membrane and probed with a radiolabeled GSTag-c-Src SH2 fusion protein. As shown in Figure 5.4A, the GSTag-c-Src SH2 fusion protein bound weakly to Neu immunoprecipitated from the uninduced extracts (Lanes 1, 2). By contrast, Neu immunoprecipitates derived from the induced extracts bound the GSTag-c-Src SH2 domain stronger than the immunoprecipitates from uninduced lysates (Figure 5.4A, compare lanes 1 and 4; lanes 2 and 5). To assess whether the strength of interaction correlated with the extent of Neu tyrosine phosphorylation the same set of immunoprecipitates were subject to immunoblot analyses with anti-phosphotyrosine antibodies (Figure 5.4B). The results showed that the strength of the interaction between the radiolabeled c-Src SH2 fusion protein and Neu directly correlated with the state of Neu tyrosine phosphorylation. The difference in the amount of Neu protein immunoprecipitated by Ab.3 (lane 4) and 7.16.4 (lane 5) is due to the difference in the ability of the antibodies (Ab.3 and 7.16.4) to immunoprecipitate Neu. Taken together, these observations imply that the c-Src SH2 domain directly binds to Neu in a tyrosine phosphorylation dependent manner.

Figure 5.4 Direct binding of c-Src SH2 domain with denatured Neu is dependent on tyrosine phosphorylation of Neu.

Equal amount of lysates from NT.12 cells grown both in the presence (+) and absence (-) of dexamethasone (Dex) were immunoprecipitated with antibodies raised against the cytoplasmic tail (Ab.3) or the extracellular domain of Neu (7.16.4). (A) One-half of the immunoprecipitates was resolved on a SDS-PAGE, blotted onto PVDF membrane and probed with radiolabeled GST fusion protein containing c-Src SH2 domain (GSTag-c-Src SH2). (B) The remaining immunoprecipitates were immunoblotted with anti-phosphotyrosine antibodies (anti-pTyr). Total cell lysates (lanes 6 and 7) were used to demonstrate the extent of dexamethasone induction and also to show the presence of low levels of tyrosine phosphorylated Neu under uninduced conditions (lane 6). Normal mouse serum (NMS) was used as non-specific control.


A
B
Dex
anti-Neu (Ab.3)
anti-Neu (7.16.4)
NMS
anti-Neu (Ab.3)
anti-Neu (7.16.4)
- - + + +
Neu
1 2 3 4 5
Probe: GSTag-c-Src SH2
anti-Neu (Ab.3)
anti-Neu (7.16.4)
NMS
anti-Neu (Ab.3)
anti-Neu (7.16.4)
Total
- - + + + - +
Neu
1 2 3 4 5 6 7
Blot: anti-pTyr

5.2.5 Activation of c-Src in Neu-induced mammary tumors occurs through its interaction with Neu

The results described above suggest that activation of c-Src in Neu-induced mammary tumors correlate with its capacity to bind directly with Neu. However, it is conceivable that the other closely related EGFR family members could contribute to the elevated c-Src activity observed in these tumors. To test this possibility, I first determined whether other EGFR family members were expressed in NAFA cells and in primary Neu-induced mouse mammary tumors (Guy et al., 1992b). Immunoblot analyses on total cell lysates using specific antibodies to different EGFR family members (see Materials and Methods) revealed that NAFA cells expressed detectable levels of erbB-3 (Figure 5.5B, lane 3), while the primary Neu-induced tumors expressed detectable levels of EGFR, erbB-3 and, erbB-4 (Figure 5.5 A,B,C, lanes 1,2).

To assess whether these other family members might participate in binding to c-Src, protein extracts derived from either NAFA cells (Figure 5.6A) or primary Neu tumors (Figure 5.6C) were immunoprecipitated with antibodies directed against each of EGFR family members (EGFR, Neu, erbB-3, erbB-4) and subjected to direct binding analyses with the radiolabeled GSTag-c-Src SH2 protein. The results showed that the c-Src SH2 probe bound only to the Neu immunoprecipitates (Figure 5.6A, lane 3; Figure 5.6C lane 3). However, immunoblot analyses of the same immunoprecipitates with anti-phosphotyrosine antibodies indicated that Neu is the principal tyrosine phosphorylated EGFR family member (Figures 5,6B and 5.6D). After longer exposure of the blots, lower levels of

Figure 5.5 Expression of erbB family members in Neu-induced tumors and tumor derived cell line.

Total cell lysates derived from Neu-induced tumors (lanes 1,2 in panels A, B, C) or tumor derived cell line NAFA (lane 3, panels A, B, C) were immunoblotted with EGFR specific (A), erbB-3 specific (B) and erbB-4 specific (C) antisera. The positions of EGFR, erbB-3 and erbB-4 are indicated. For panel (D) refer to figure 5.6.

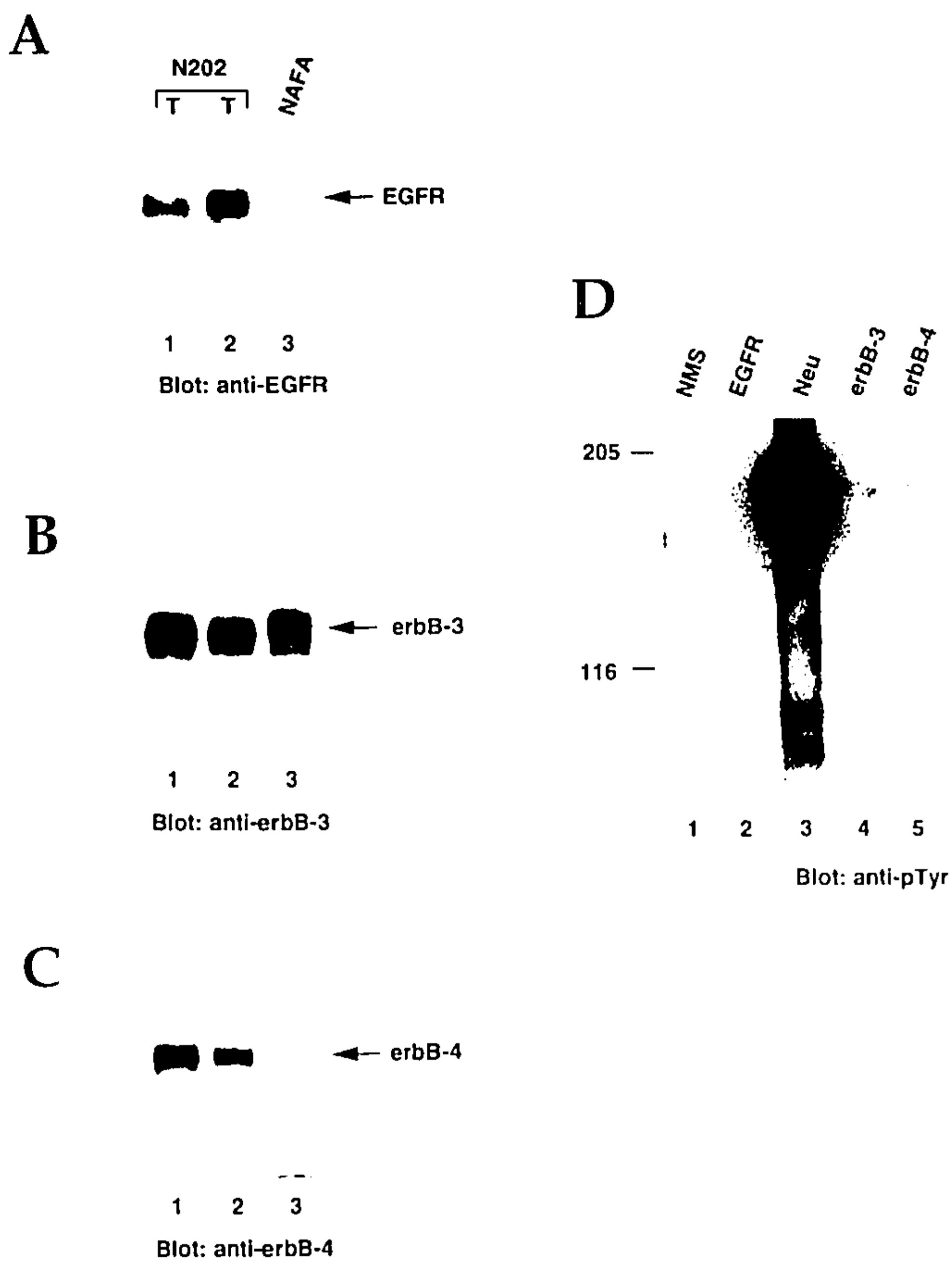
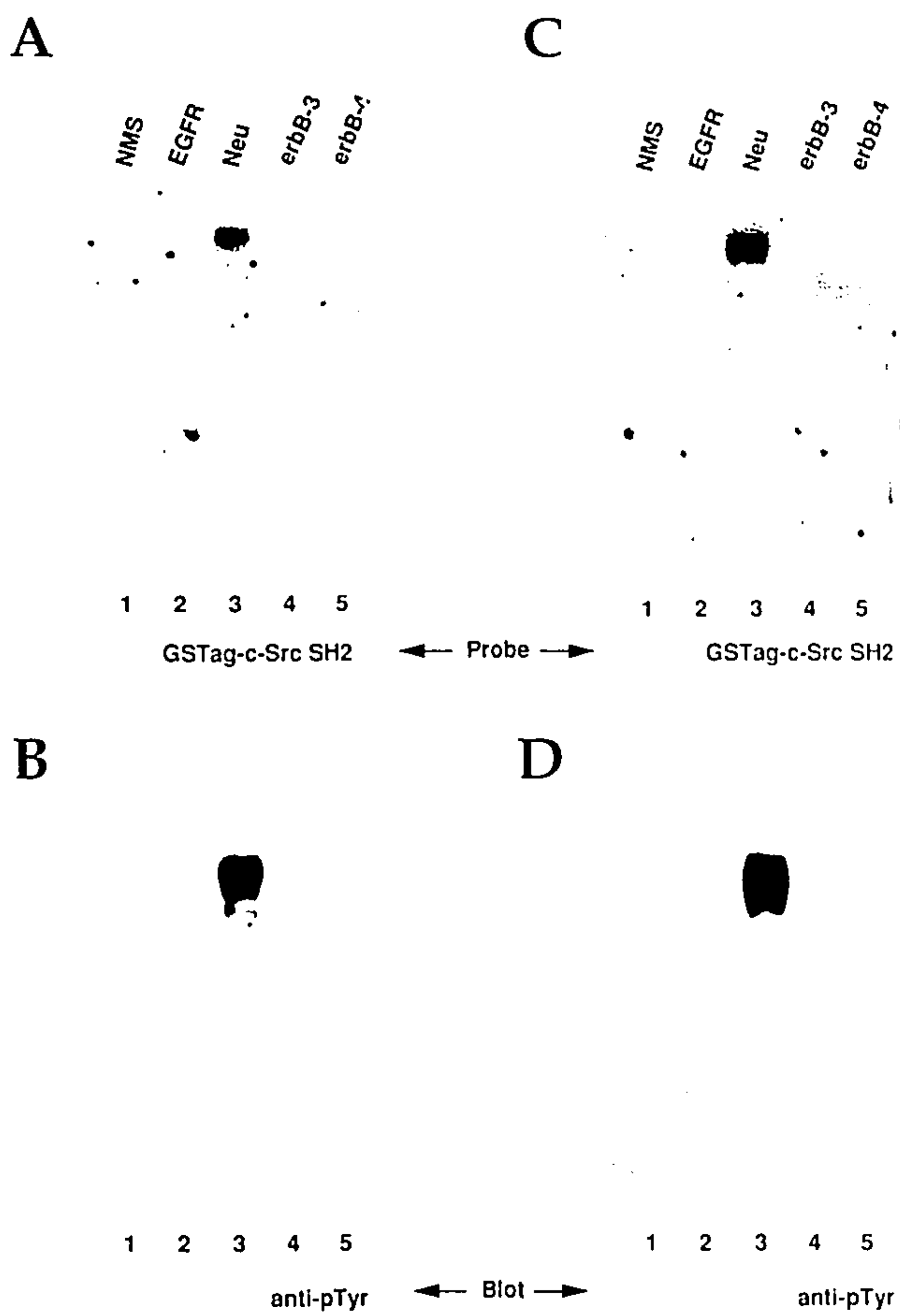

A
N202
T T
NAFA
EGFR
1 2 3
Blot: anti-EGFR
B
erbB-3
1 2 3
Blot: anti-erbB-3
C
erbB-4
1 2 3
Blot: anti-erbB-4
D
NMS
EGFR
Neu
erbB-3
erbB-4
205
116
1 2 3 4 5
Blot: anti-pTyr

Figure 5.6 The c-Src SH2 domain interacts with Neu in Neu-induced mammary tumors and derived cell line.

Each member of the EGFR family was immunoprecipitated using extracts from both Neu-induced mammary tumor and tumor derived epithelial cell line (NAFA). Half of the immunoprecipitates from the NAFA cells (A) and one half from the mammary tumor lysates (C) were probed with radiolabeled c-Src SH2 domain (GSTag-c-Src SH2). The remaining immunoprecipitates from NAFA cell lysates (B) and mammary tumor lysates (D) were probed with anti-phosphotyrosine antibodies (anti-pTyr). Normal mouse serum (NMS) was used as non-specific control. A longer exposure of the blot in panel D is shown in Figure 5.5.


A
C
NMS
EGFR
Neu
erbB-3
NMS
EGFR
Neu
erbB-3
erbB-4
1 2 3 4 5
GSTag-c-Src SH2
Probe
1 2 3 4 5
GSTag-c-Src SH2
B
D
1 2 3 4 5
anti-pTyr
Blot
1 2 3 4 5
anti-pTyr

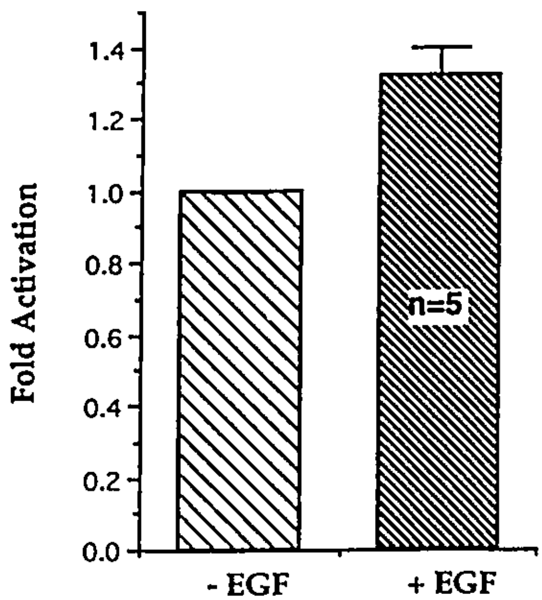
tyrosine phosphorylated EGFR and erbB-3 were detected in the primary tumor samples (Figure 5.5D). Nonetheless, no detectable binding of c-Src to these immunoprecipitates was observed. These analyses suggest that among the EGFR family members Neu is the principal tyrosine phosphorylated receptor that c-Src SH2 domain interacts with.

5.2.6 The c-Src-SH2 domain does not associate directly with activated EGFR *in vitro.*

The experiments described above indicate that in mammary tumor cells the association between c-Src and Neu is likely responsible for the elevated Src activity observed in these tumors (chapter 4). However, it is unclear whether the c-Src SH2 domain can associate directly with the other EGFR family members such as EGFR that is known to induce kinase activity of the Src family members (Osherov and Levitzki, 1994). Stimulation of fibroblasts overexpressing EGFR with EGF for 1.0 minute results in a three fold activation of the Src family of kinases using an antibody that recognizes c-Src, c-Yes and Fyn (Osherov and Levitzki, 1994). Similarly, I have observed a weak but consistent 1.4 fold activation of c-Src using c-Src specific antibodies (Ab327) following acute stimulation of Rat-1 fibroblasts overexpressing human EGFR (R1/hER, Wasilenko et al., 1991) (Figure 5.7). To assess whether the increase in c-Src activity correlated with the capacity of c-Src to interact directly with the activated EGFR, I tested the ability of c-Src SH2 fusion protein to interact with EGFR immunoprecipitates derived from either R1/hER or A431 cells after EGF

Figure 5.7 Stimulation of c-Src kinase activity by EGF.

R1/hER cell were stimulated with EGF for 1.0 minute, cell lysates were collected and c-Src was immunoprecipitated using c-Src specific antisera (Ab327). The immune complexes were subject to *in vitro* kinase assays using acid denatured enolase as an external substrate. The radioactivity transferred onto the enolase was quantitated by PhosphorImager analysis. Average fold increase of c-Src kinase activity observed in EGF stimulated cells is shown. The graph represents the average fold activation observed in five independent experiments.



Fold Activation
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
n=5
- EGF
+ EGF

stimulation. As shown in Figure 5.8A, I was unable to detect direct binding of c-Src SH2 domain to EGFR immunoprecipitates following EGF stimulation (lanes 2 and 4). By contrast, comparable analyses with Neu immunoprecipitates from the NAFA cell line demonstrated direct binding of the Src SH2 fusion protein to Neu (Figure 5.8A, lane 3). The inability to detect binding of c-Src SH2 to the EGFR was not due to lack of tyrosine phosphorylation of EGFR because immunoblot analyses of the immunoprecipitates with anti-phosphotyrosine antibodies clearly showed the presence of significant levels of tyrosine phosphorylated EGFR (Figure 5.8B, lanes 2 and 4). To confirm that the lack of association between EGFR and c-Src SH2 is not due to a difference in the levels of tyrosine phosphorylated Neu and EGFR I performed the analysis using comparable levels of tyrosine phosphorylated Neu and EGFR. The results showed that under conditions where equivalent levels of tyrosine phosphorylated receptors were present I was unable to detect binding of c-Src SH2 domain to EGFR while the c-Src SH2 domain bound to Neu (Figure 5.9, compare lanes 1,2 with 3,4).

5.2.7 Tyrosine phosphorylated EGFR can bind directly to Grb2 SH2 domain but not to c-Src SH2 domain.

To confirm that the tyrosine phosphorylated EGFR in the immunoprecipitates retains its ability to associate with a SH2 domain that is known to associate with EGFR, I assessed the ability of the adapter protein Grb2, which is known to associate with both phosphorylated EGFR

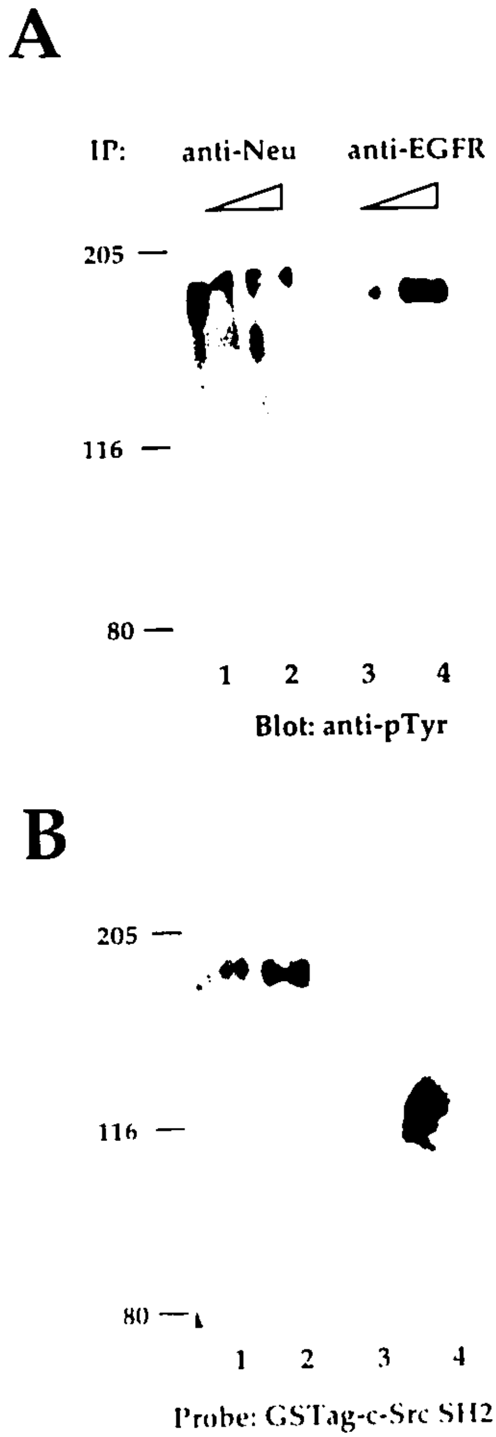
Figure 5.8 The c-Src SH2 domain does not interact directly with tyrosine phosphorylated EGFR.

Lysates from EGF treated A431 epithelial cells (lane 2) or R1/hER fibroblasts (lane 4) were immunoprecipitated with anti-EGFR antibodies (anti-EGFR) and Neu was immunoprecipitated from NAFA cell lysates (anti-Neu). (**A**) One half of the immunoprecipitates was resolved on a SDS-PAGE and probed with radiolabeled c-Src SH2 domain (GSTag-c-Src SH2). (**B**) The remainder of the immunoprecipitate was immunoblotted with anti-phosphotyrosine antibody (anti-pTyr). Normal mouse serum (NMS, lane 1) was used as a non-specific control. (IP: Immunoprecipitation). The molecular weight markers are in kDa.


A
IP :
NMS
anti-EGFR
anti-Neu
anti-EGFR
205 —
118 —
85 —
1 2 3 4
Probe: GSTag-c-Src SH2
B
205 —
118 —
85 —
1 2 3 4
Blot: anti-pTyr

Figure 5.9 The ability of c-Src SH2 to bind directly to Neu but not to EGFR is not due to differences in receptor protein levels.

Neu (lanes 1 and 2) and EGFR (lanes 3 and 4) were immunoprecipitated from increasing amounts of total lysates. **(A)** One part of the immunoprecipitate was used to determine the levels of tyrosine phosphorylated Neu and EGFR. **(B)** The remainder of the immunoprecipitate was resolved on SDS-PAGE and probed with radiolabeled GSTag-c-Src SH2. IP: Immunoprecipitation. Molecular weight markers are in kDa.


A
IP: anti-Neu anti-EGFR
205
116
80
1 2 3 4
Blot: anti-pTyr
B
205
116
80
1 2 3 4
Probe: GSTag-c-Src SH2

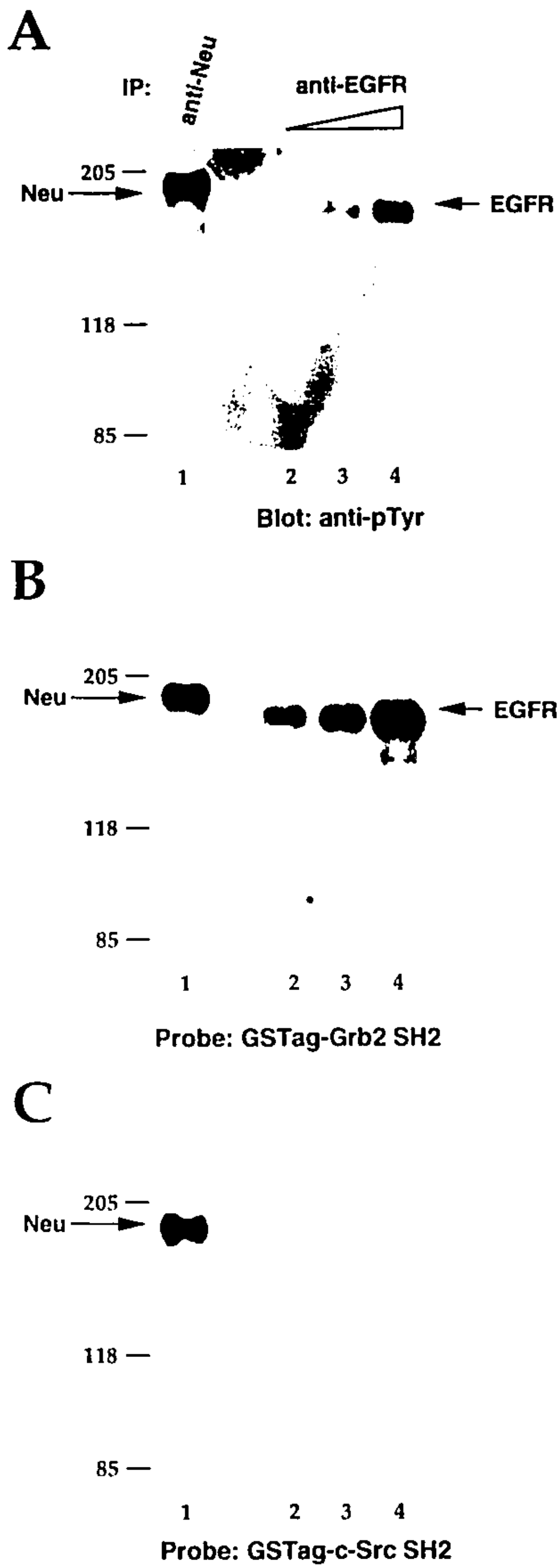
and Neu (Egan et al., 1993, Rozakis-Adcock, et al., 1993, Batzer et al., 1994, Janes et al., 1994), to associate with EGFR. As shown in Figure 5.10A (lanes 2-4), stimulation of R1/hER cells with EGF resulted in a marked tyrosine phosphorylation of EGFR. The same immunoprecipitates were electrophoresed through a SDS-PAGE gel, blotted onto PVDF membranes and, probed with either radiolabeled GSTag-Grb2 SH2 (Figure 5.10B) or GSTag-c-Src SH2 (Figure 5.10C) fusion proteins. The results showed that Grb2 SH2 domain binds directly to EGFR (Figure 5.10B, lanes 2-4) suggesting that the EGFR molecules in the immunoprecipitates retain their ability to associate with SH2 domains. However, as observed previously I was unable to detect association between the Src SH2 domain and the tyrosine phosphorylated EGFR (Figure 5.10C). In contrast control Neu immunoprecipitates bound both to Grb2 SH2 and c-Src SH2 domains (Figure 5.10B and C, lane 1). These observations strongly suggest that activation of c-Src by EGF may occur through a mechanism that does not require direct binding to the activated EGFR.

5.2.8 EGF treatment of EGFR overexpressing cells results in association of c-Src with tyrosine phosphorylated Neu.

Given that c-Src can bind directly to Neu and that Neu can heterodimerize with EGFR, one possible explanation for these observations is that EGF mediated activation of c-Src is occurring through a EGFR/Neu heterodimer. To explore this hypothesis, I examined whether EGF treatment could induce the formation of Neu/Src complexes

Figure 5.10 Tyrosine phosphorylated EGFR can bind directly to Grb2 SH2 domain but not to c-Src SH2 domain.

Increasing amounts of lysates from EGF treated R1/hER cells were immunoprecipitated with EGFR-specific (anti-EGFR) antibodies (lanes 2-4). The immunoprecipitates were equally divided, resolved on a SDS-PAGE and were immunoblotted with anti-pTyr antibodies (**A**), or probed with radiolabeled Grb2 SH2 domain (GSTag-Grb2 SH2) (**B**), or probed with radiolabeled c-Src SH2 domain (GSTag-c-Src SH2) (**C**). Lane 1 in panels **A**, **B**, and **C** correspond to Neu immunoprecipitate from NAFA cell lysate (anti-Neu). The molecular weight markers are in kDa.

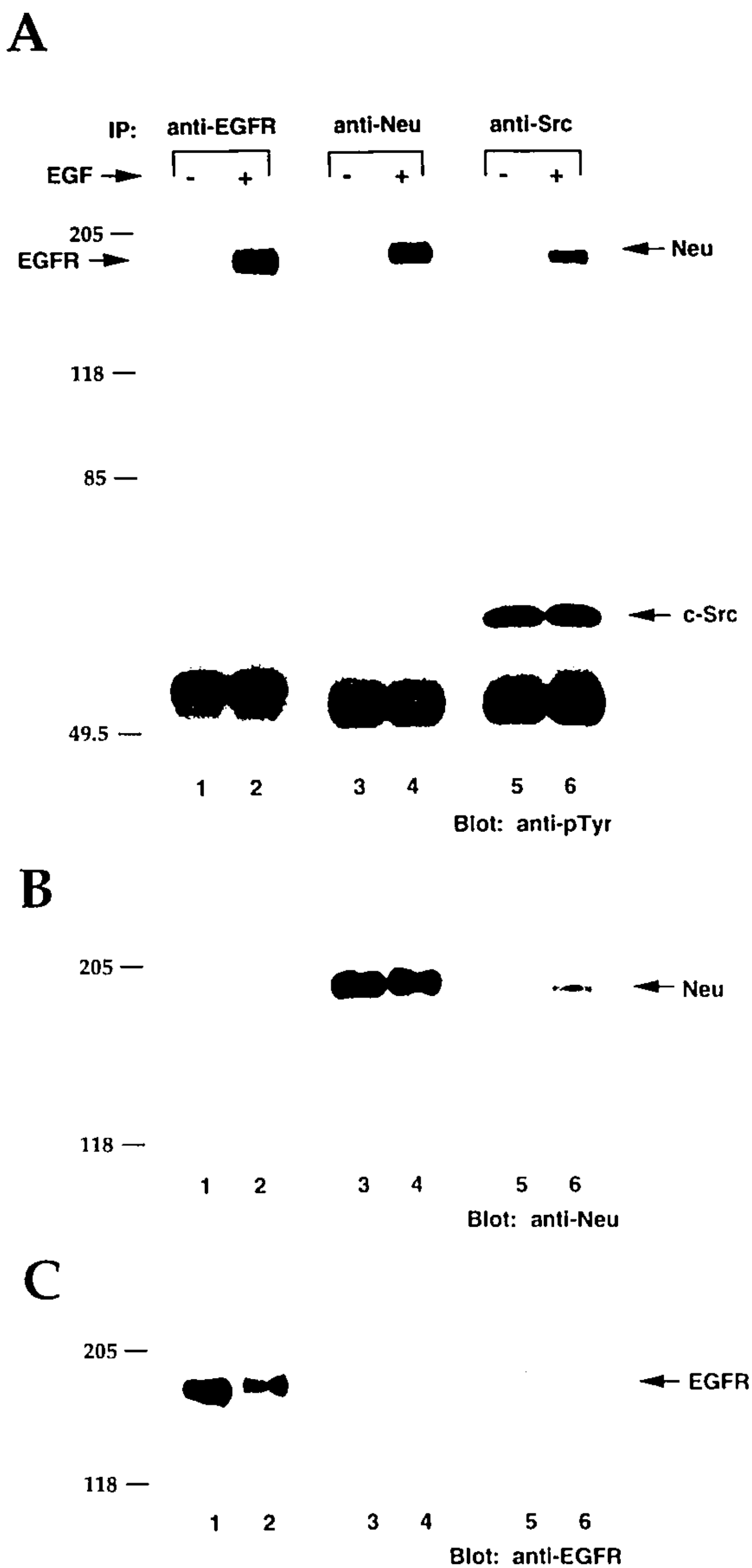

A
IP:
anti-Neu
anti-EGFR
205
Neu
EGFR
118
85
1
2
3
4
Blot: anti-pTyr
B
205
Neu
EGFR
118
85
1
2
3
4
Probe: GSTag-Grb2 SH2
C
205
Neu
118
85
1
2
3
4
Probe: GSTag-c-Src SH2

through tyrosine phosphorylation of the endogenous Neu present in R1/hER cells. To accomplish this, cell lysates derived from either unstimulated or EGF stimulated R1/hER cells were immunoprecipitated with EGFR, Neu or Src specific antisera and subjected to immunoblot analyses with anti-phosphotyrosine antibodies (Figure 5.11A). The results showed that EGF administration resulted in the rapid tyrosine phosphorylation of both the EGFR (Figure 5.11A, lanes 1 and 2) and Neu (lanes 3 and 4). Because EGF cannot directly bind Neu (King et al., 1988, Stern and Kamps 1988), the observed tyrosine phosphorylation of Neu is likely the result of transphosphorylation of Neu by the activated EGFR. Significantly, EGF stimulation resulted in the formation of a complex between c-Src and a 185 kDa tyrosine phosphorylated protein that comigrated with Neu (Figure 5.11A, compare lanes 4 and 6). By contrast, I was unable to detect any tyrosine phosphorylated protein that comigrated with the EGFR in these c-Src immunoprecipitates.

To confirm that the tyrosine phosphorylated protein was Neu and not EGFR, identical sets of immunoprecipitates were immunoblotted with either a Neu (Figure 5.11B) or EGFR specific antibody (Figure 5.11C). The results confirmed that EGF stimulation of these cells resulted in the formation of specific complex between c-Src and Neu (Figure 5.11B lane 6). However, no comparable EGFR/c-Src complex was detected in these EGF stimulated cells (Figure 5.11C, lane 6). Taken together, these observations strongly suggest that the activated EGFR stimulates c-Src through the Neu RTK.

Figure 5.11 EGF treatment results in specific association of c-Src with Neu.

Lysates were derived from R1/hER both before (-) and after (+) one minute induction with EGF. EGFR (anti-EGFR) or Neu (anti-Neu) or c-Src (anti-Src) were immunoprecipitated from the lysates and resolved on a SDS-PAGE gel. The immunoprecipitates were probed with anti-phosphotyrosine (A) or anti-Neu (B) or with anti-EGFR (C) antibodies. The autoradiograph in panel C was exposed almost 10 times longer than that in panel B. (IP: Immunoprecipitation). The molecular weight markers are in kDa.


A
IP: anti-EGFR anti-Neu anti-Src
EGF
- + - + - +
205
EGFR
Neu
118
85
c-Src
49.5
1 2 3 4 5 6
Blot: anti-pTyr
B
205
Neu
118
1 2 3 4 5 6
Blot: anti-Neu
C
205
EGFR
118
1 2 3 4 5 6
Blot: anti-EGFR

5.3 DISCUSSION

The results show that activation of c-Src by two EGFR family members, EGFR and Neu occurs through specific and direct interaction of c-Src with tyrosine phosphorylated Neu. The data also suggest that EGF stimulation results in association of c-Src with tyrosine phosphorylated Neu. Taken together, these observations suggest that Neu plays a central role in the activation of c-Src by these two closely related EGFR family members.

The expression of kinase active Neu in rat fibroblasts results in a reproducible 2.3 fold increase in the specific activity of c-Src (Figure 5.2). Although this is a relatively modest increase as compared to the 6.7 fold activation observed in *neu*-induced mammary tumors (Chapter 4), this increase in c-Src kinase activity is consistent with that observed in PDGFR and CSF-1R activation in fibroblast cell lines (Ralston and Bishop 1985, Kypta et al., 1990, Courtneidge et al., 1993). The increase in specific activity of c-Src correlated with the ability of c-Src to associate with Neu in a tyrosine phosphorylation dependent manner (Figure 5.1). These observations demonstrate a functional link between activation of the Neu RTK and the increase in c-Src tyrosine kinase activity and suggest that c-Src functions downstream of Neu in Neu-mediated signal transduction pathway.

The activation of the c-Src by Neu in Neu-induced mammary tumors and the mammary epithelial cell line appears to occur through a direct interaction of c-Src SH2 domain with Neu in a tyrosine phosphorylation dependent manner (Figures 5.3, and 5.4). It is possible that the direct interaction between c-Src and tyrosine phosphorylated Neu would render c-Src into a catalytically active conformation. However, formal proof for this hypothesis awaits future experimentation. Although immunoblot analyses indicated that other members of the EGFR family were expressed in these mammary tumors and derived cell line (Figure 5.5), further biochemical analyses revealed that these members are only weakly tyrosine phosphorylated (Figure 5.5 and 5.6). Therefore, it is likely the elevated c-Src activity observed in the Neu-induced mammary tumors and tumor derived cell line does not involve the participation of other members of the EGFR family.

While stimulation of EGFR does not appear to be directly involved in the activation of c-Src in these mammary epithelial cells, there is considerable evidence to suggest that activation of c-Src is involved in EGF mediated mitogenesis in other cell types. For example, EGF treatment of PC12 cells or mouse fibroblasts overexpressing EGFR activates Src family of kinases (Osherov and Levitzki 1994). Cells overexpressing c-Src show hyperresponsiveness to EGF mediated growth stimulation (Luttrell et al., 1988, Wilson et al., 1989) and micro-injection of antibodies that recognize c-Src, c-Yes and Fyn results in a strong inhibition of EGF-induced S phase entry of fibroblasts (Roche et al., 1995). Although these studies suggest that activation of c-Src tyrosine kinase plays an important role in EGF-induced mitogenesis, our results strongly suggest that c-Src does not directly interact with the activated EGFR (Figures 5.8, 5.9, 5.10 and 5.11). Rather, our observations suggest that activation of c-Src by the activated EGFR occurs through transphosphorylation of Neu (Figure 5.7, and 5.11). Consistent with our observations, under conditions where EGF activates Src family kinases, physical association between EGFR and Src family was not detected (Osherov and Levitzki 1994). By contrast to these observations, the formation of an activated EGFR and c-Src complex *in vivo* has been reported in MDA-MB-468 mammary tumor cells (Luttrell et al., 1994). However, whether this interaction involves Neu is unclear.

The observation that activation of c-Src in both EGFR and *neu* expressing cells occurs through tyrosine phosphorylated Neu has important implications in understanding the biological properties of these two closely related EGFR family members. For example, several studies have shown that under comparable conditions of expression and enzymatic activity, Neu is 100 fold more potent than EGFR in stimulating mitogenesis in NIH 3T3 fibroblasts (Di Fiore et al., 1987, Di Fiore et al., 1990). More recently, it has been shown that the c-Src substrate paxillin is tyrosine phosphorylated in cells expressing the cytoplasmic domain of Neu in a EGFR-erbB-2 chimera. Interestingly in cells expressing EGFR, tyrosine phosphorylated paxillin is not detected (Romano et al., 1994). These observations suggest that the differences in the mitogenic properties of Neu and EGFR could in part be explained by the inability of the latter receptor to couple with the Src pathway.

The notion that each of these EGFR family members has distinct signaling specificity is further supported by several recent studies. For example, it was shown that activation of the phosphatidylinositol-3' (PI-3') kinase by EGF, is mediated through the interaction of PI-3' kinase with erbB-3 in a EGFR/erbB-3 heterodimer (Soltoff et al., 1994). Consistent with these observations the 85 KDa subunit of PI-3' kinase associates with an EGFR-erbB-3 chimera upon EGF stimulation (Fedi et al., 1994, Prigent and Gullick 1994). These observations suggest that activation of these closely related RTKs results in the recruitment of distinct but complementary signaling pathways. Indeed coexpression of the EGFR and Neu can act synergistically to transform rodent fibroblasts (Kokai et al., 1989). Interestingly, a modified breast cancer cell line that does not expresses erbB-2 at cell surface, but expresses other erbB family receptors, is defective in EGF and Neu differentiation mediated factor (NDF) mediated signaling (Graus-Porta et al., 1995). This cell line show significant reduction in its ability to facilitate activation of mitogen-activated protein kinase and to promote induction of *c-fos* gene expression in response to EGF or NDF stimulation, when compared to the parental cell line that expresses all erbB family receptors including erbB-2 on the cell surface (Graus-Porta et al., 1995). Taken together these observations suggest that c-erbB-2 plays a central role in signaling by the erbB family of receptor tyrosine kinases.

The results presented in this and the previous chapter demonstrate that c-Src tyrosine kinase is involved in the Neu mediated signal transduction pathway. In addition to c-Src there are two other Src family members (c-Yes and Fyn) that are known to be expressed in a wide variety of tissues. In the following chapter I examine whether c-Yes and Fyn tyrosine kinases play a role in Neu mediated signaling and mammary tumorigenesis.

CHAPTER 6

Activation of the Src family of tyrosine kinases in Neu induced mammary tumors and their association with a distinct set of tyrosine phosphorylated proteins

6.1 INTRODUCTION

Among the Src family members, c-Src, c-Yes and Fyn are expressed in a wide range of tissues and are thought to be involved in growth factor mediated signal transduction pathways (Erpel and Courtneidge, 1995). The c-Src, c-Yes and Fyn tyrosine kinases have been shown to associate with RTK such as the Platelet derived growth factor receptor (PDGFR) (Kypta et al 1990), and the Colony stimulating growth factor-1 receptor (CSF-1R) (Courtneidge et al 1993). It is believed that PDGF or CSF-1 mediated activation of c-Src, c-Yes and Fyn tyrosine kinase activity is mediated by the ability of Src family members to associate with the tyrosine phosphorylated receptor molecule (Mori et al., 1993).

To better understand the role played by the Src family of tyrosine kinases in Neu-mediated mammary tumorigenesis I measured the kinase activity of c-Yes and Fyn in Neu-induced mouse mammary tumors and compared it with the kinase activity observed in adjacent morphologically normal mammary epithelium of the same animal. In this chapter I present evidence suggesting that c-Yes kinase activity was elevated by four fold in Neu-induced mammary tumors when compared to those in the adjacent nontumorous epithelium. By contrast, Fyn kinase activity was not elevated in Neu-induced mammary tumors. The increase in c-Yes tyrosine kinase activity correlates well with its ability to associate with Neu in a tyrosine phosphorylation dependent manner *in vivo*. This association is likely due to the ability of c-Yes SH2 domain to interact directly with denatured tyrosine phosphorylated Neu. I also present evidence suggesting that c-Yes and c-Src tyrosine kinases associate with distinct and overlapping sets of tyrosine phosphorylated cellular proteins in transformed mammary epithelial cells.

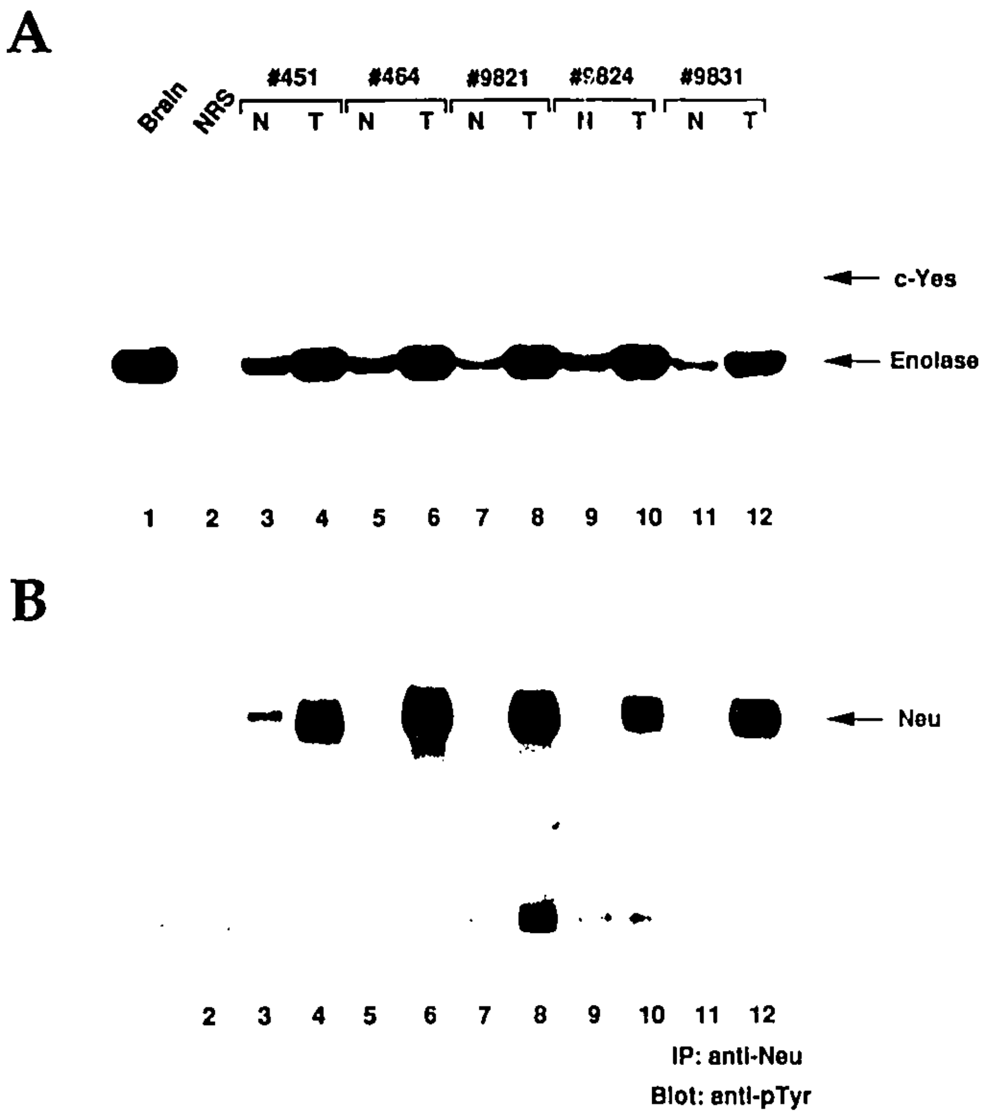
6.2 RESULTS

6.2.1 Neu-induced mouse mammary tumors possess elevated c-Yes kinase activity.

Mammary tumors and adjacent normal epithelium were isolated from transgenic mice expressing wild type Neu under the control of MMTV promoter/enhancer (Guy et al., 1992b). Tissue lysates were prepared and c-Yes immunoprecipitates were incubated with acid denatured enolase as an external substrate. As shown in Figure 6.1A all the lysates derived from the mammary tumors (lanes 4, 6, 8, 10, 12) possessed elevated c-Yes tyrosine kinase activity when compared to those derived from adjacent nontumorous epithelium (lanes 3, 5, 7, 9, 11). Analysis of additional eight matched sets of tumor and nontumorous

Figure 6.1 Neu-induced mouse mammary tumors possess elevated c-Yes kinase activity.

(A) c-Yes was immunoprecipitated from adjacent normal epithelium (N) or tumor (T) and the immunoprecipitates were incubated with γ-32P ATP and acid denatured Enolase. Tissue lysate from and Brain (lane 1) and a normal rabbit serum (NRS, lane 2) immunoprecipitate were used as positive and negative controls respectively. The position of Enolase and c-Yes are marked by arrows. **(B)** Neu was immunoprecipitated from the same lysates used in panel A and the immunoprecipitates were immunoblotted with antiphosphotyrosine antibodies. Numbers #451, #464, #9821, #9824, #9831 correspond to mice ear tag numbers.


A
Brain
NRS
#451
#464
#9821
#9824
#9831
N T N T N T N T N T
c-Yes
Enolase
1 2 3 4 5 6 7 8 9 10 11 12
B
Neu
2 3 4 5 6 7 8 9 10 11 12
IP: anti-Neu
Blot: anti-pTyr

epithelium also showed a consistent increase in c-Yes tyrosine kinase activity (Table 6.1). The radioactivity transferred onto the enolase was quantitated by PhosphorImager analysis. The results revealed that the mammary tumors possess on an average a 4.75 fold increase (n=13) in c-Yes tyrosine kinase activity when compared to nontumourous epithelium (Table 6.1). The increase in kinase activity showed tight correlation with the presence of tyrosine phosphorylated Neu (Figure 6.1B). These observations indicate that c-Yes may play a role in Neu-induced mammary tumorigenesis.

6.2.2 Fyn tyrosine kinase activity is not elevated in Neu-induced mammary tumors.

Another Src family member that is known to be widely expressed in various tissue types is the Fyn tyrosine kinase. As performed for c-Src (see chapter 4) and c-Yes I was interested to test whether Fyn tyrosine kinase activity was elevated in Neu-induced mammary tumors. To this end, Fyn was immunoprecipitated from both tumor and adjacent epithelial tissues and incubated with acid denatured enolase and γ-^{32}P ATP. Interestingly, the tumor tissues did not display elevated levels of Fyn tyrosine kinase activity compared to that observed in the adjacent normal epithelium (Figure 6.2A). This is not due to difference in the levels of Fyn protein or due to lack of tyrosine phosphorylated Neu since, control immunoblot analysis using part of the Fyn immunoprecipitates revealed that both tumors and adjacent epithelium possess significant levels of Fyn protein

Table 6.1 Fold increase in c-Yes kinase activity in Neu induced tumors.

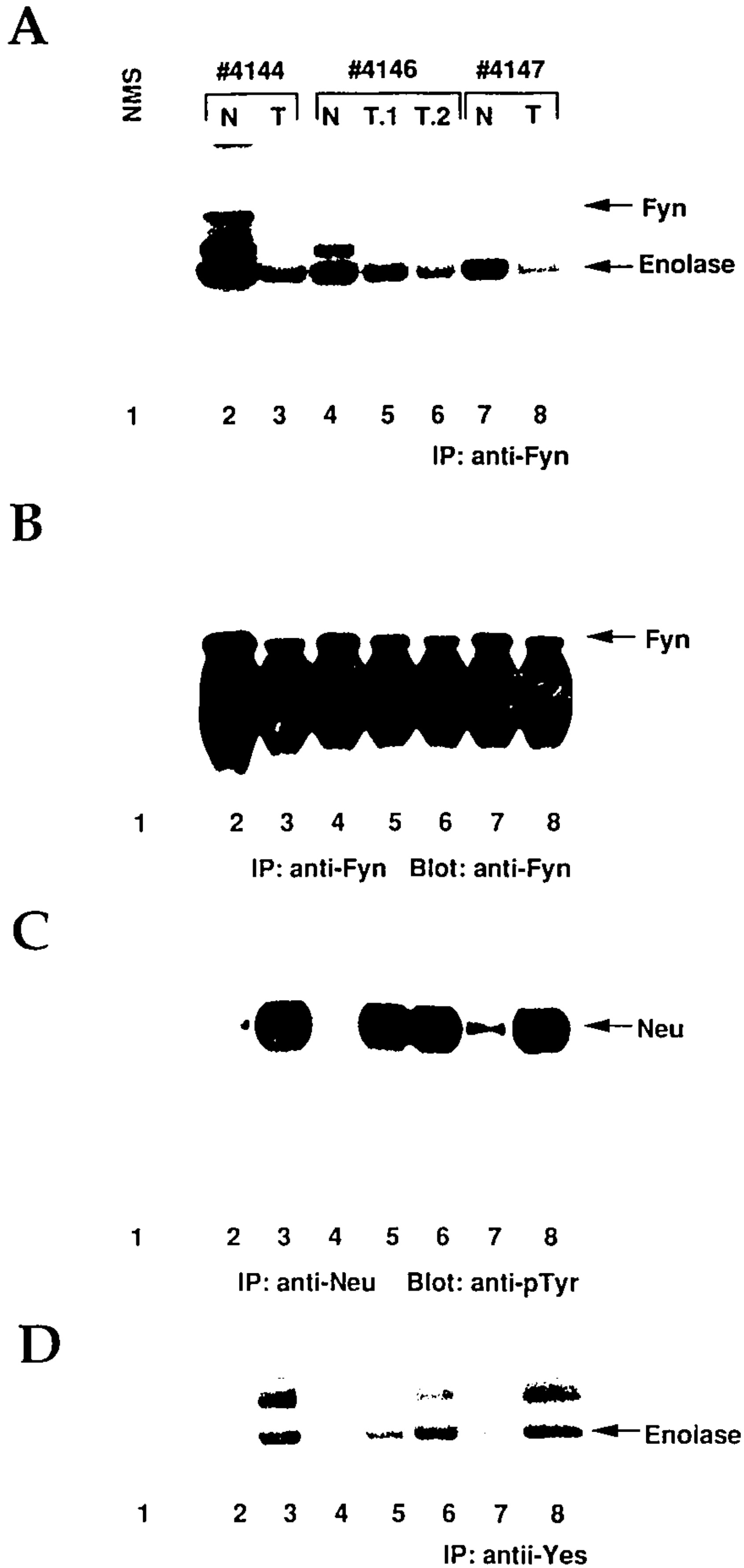
c-Yes was immunoprecipitated from tumor as well as adjacent morphologically normal mammary epithelium and subject to *in vitro* kinase assays. The radioactivity transferred on to enolase was quantitated by PhosphorImager analysis. The fold increase in kinase activity observed in the tumor samples over that observed in the adjacent normal mammary epithelium is shown. The numbers in column 1 correspond to mouse ear tag numbers.

Mouse number	Fold Activation
451	4.0
464	7.5
4144	2.7
4146	2.4
4147	2.6
7364	4.9
8340	6.0
8367	3.8
8367	5.6
8565	5.0
9821	6.7
9824	5.2
9831	5.5
N =	13
Mean	4.75
SD	1.6

S.D: Standard Deviation

Figure 6.2 Fyn tyrosine kinase activity is not elevated in Neu-induced mammary tumors.

Tissue samples were prepared from both mammary tumors (T) and adjacent nontumorous epithelium (N). **(A)** Fyn was immunoprecipitated from the tissue lysates and a part of the immunoprecipitate was incubated with acid denatured enolase and γ-^{32}P ATP. The position of Enolase and Fyn are indicated by arrows. **(B)** The remaining portion of the Fyn immunoprecipitate was resolved on SDS-PAGE and immunoblotted with anti-Fyn antibodies. **(C)** Neu was immunoprecipitated from the same batch of lysates and immunoblotted with anti-phosphotyrosine antibodies (anti-pTyr). **(D)** c-Yes was immunoprecipitated from the same batch of lysates used in A and subject to *in vitro* kinase assay using enolase as an external substrate. A normal mouse serum (NMS) immunoprecipitate was used as negative control (lane 1). The numbers #4144, #4146 and, #4147 correspond to mouse ear tag numbers.


A
NMS
#4144
#4146
#4147
N T
N T.1 T.2
N T
Fyn
Enolase
1 2 3 4 5 6 7 8
IP: anti-Fyn
B
Fyn
1 2 3 4 5 6 7 8
IP: anti-Fyn Blot: anti-Fyn
C
Neu
1 2 3 4 5 6 7 8
IP: anti-Neu Blot: anti-pTyr
D
Enolase
1 2 3 4 5 6 7 8
IP: antii-Yes

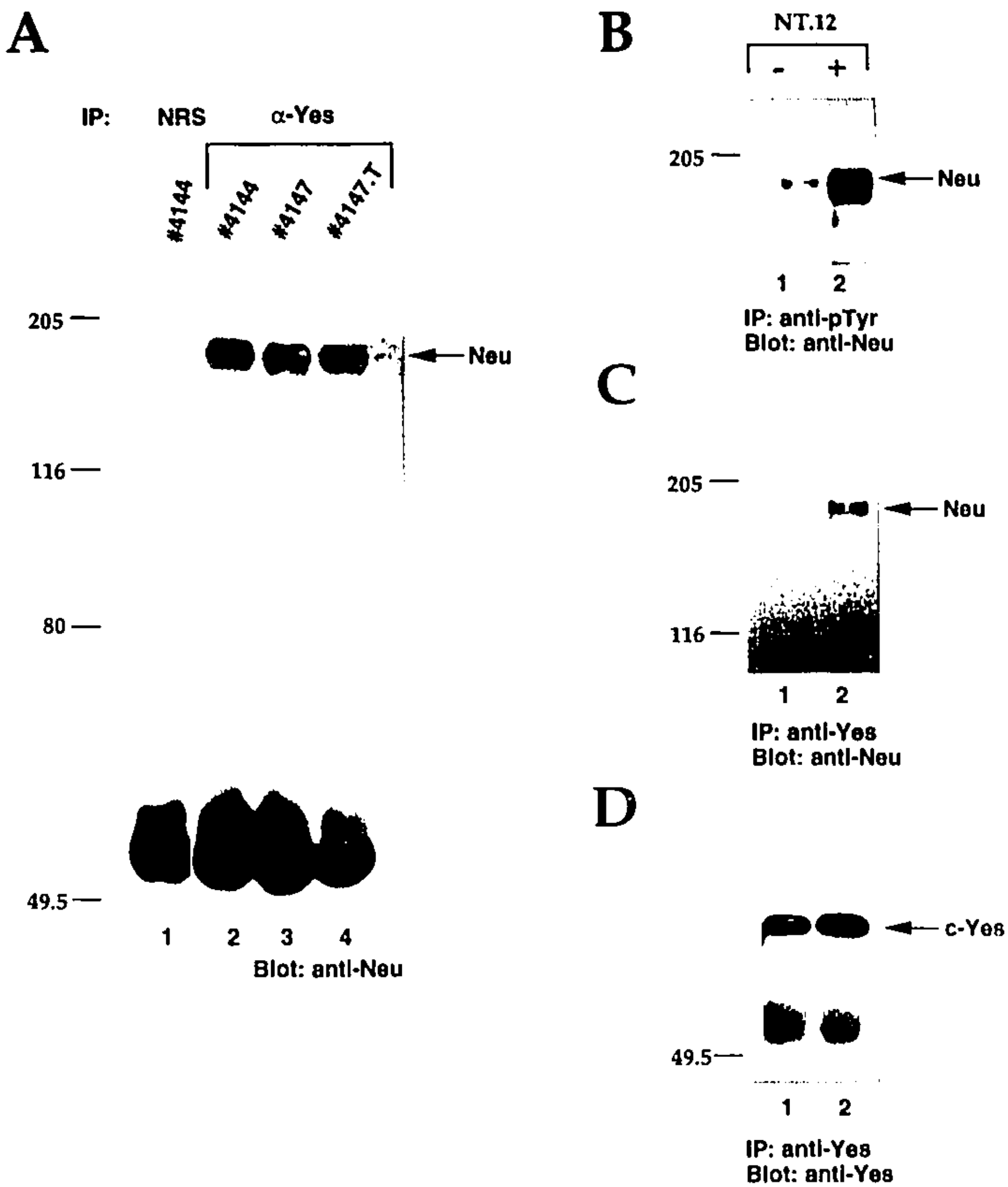
(Figure 6.2B) and anti-phosphotyrosine immunoblot analysis of Neu immunoprecipitates revealed that all the tumor samples possess high levels of tyrosine phosphorylated Neu (Figure 6.2C). To confirm that it is not a sampling difference, the same tumor lysates were assayed for c-Yes kinase activity. c-Yes immunoprecipitates from all the tumors possessed elevated kinase activity when compared to the adjacent nontumorous epithelium (#4144 3.0 fold; #4146 T1 3.0 fold, T2 3.4 fold) (Figure 6.2D). By contrast to the observations made with c-Src and c-Yes, Fyn tyrosine kinase activity was higher in the adjacent nontumorous epithelium than that observed in tumor samples (Figure 6.2A). This difference correlated with the difference in the amount of Fyn protein expressed in the nontumorous epithelium (Figure 6.2B). These observations suggest that Fyn may not play an important role in Neu-mediated mammary tumorigenesis.

6.2.3 c-Yes associates with Neu in a tyrosine phosphorylation dependent manner.

To understand the mechanism by which c-Yes kinase activity is elevated in Neu induced mammary tumors, I tested whether c-Yes can associate with Neu in tumor lysates. c-Yes was immunoprecipitated from tumor lysates and the immunoprecipitates were immunoblotted with anti-Neu antibodies. As shown in Figure 6.3A, c-Yes formed complexes with Neu in mammary tumors *in vivo*. Since increase in c-Yes kinase activity showed a tight correlation with the presence of tyrosine

Figure 6.3 c-Yes associates with Neu in tyrosine phosphorylation dependent manner.

(A) Tissue lysates were prepared from tumor sample using CHAPS lysis buffer. #4147 tumor was lysed both in CHAPS (lane 3) and TNE (#4147.T, lane 4). c-Yes was immunoprecipitated from 1.5 mg of total lysate and the immunoprecipitate was immunoblotted with anti-Neu antibodies. Normal rabbit serum (NRS) was used as a nonspecific control (lane 1). **(B)** Lysates were prepared from NT.12 cells grown in the absence (-) (lane 1) or presence (+) (lane 2) of dexamethasone. Phosphotyrosine containing proteins were immunoprecipitated and blotted with Neu specific antisera. **(C)** c-Yes was immunoprecipitated from 1.5 mg of the same batch of lysates and the immunoblotted with Neu specific antisera. **(D)** The same membrane from panel C was reprobed with and antisera that recognizes c-Yes. The molecular weight markers are indicated in kDa.


A
IP: NRS α-Yes
#4144 #4144 #4147 #4147.T
205
116
80
49.5
Neu
1 2 3 4
Blot: anti-Neu
B
NT.12
- +
205
Neu
1 2
IP: anti-pTyr
Blot: anti-Neu
C
205
116
Neu
1 2
IP: anti-Yes
Blot: anti-Neu
D
c-Yes
49.5
1 2
IP: anti-Yes
Blot: anti-Yes

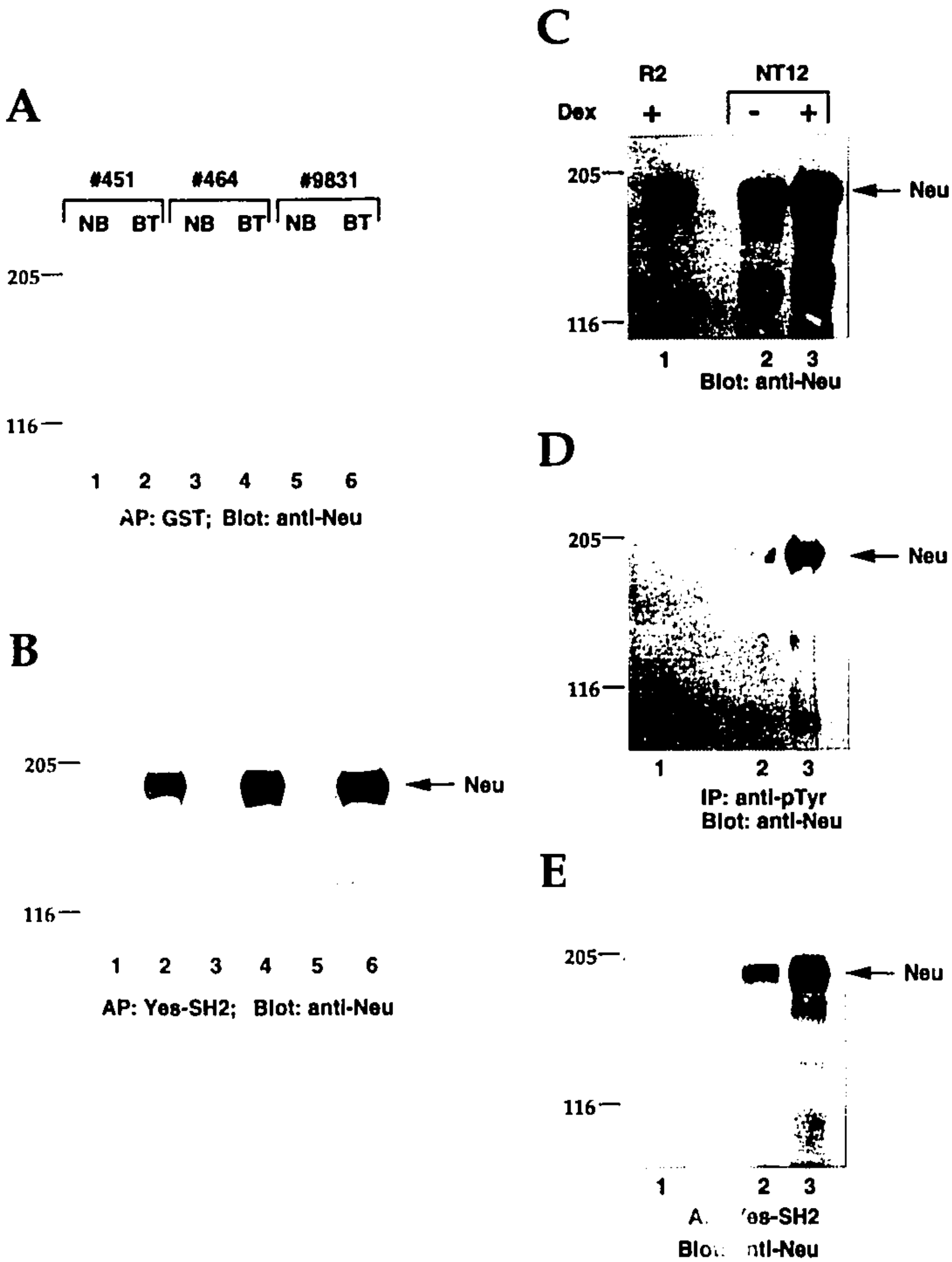
phosphorylated Neu it is possible that c-Yes requires tyrosine phosphorylated Neu for its ability to associate with Neu *in vivo*. To test this possibility, Rat.2 derived cells lines expressing the activated form of Neu (val 664 to glu, Bargmann et al., 1986) under the control of MMTV promoter (see Chapter 4) were utilized. The c-Yes was immunoprecipitated from both dexamethasone induced and uninduced NT.12 cell lysates and blotted with anti-Neu antibodies (Figure 6.3C). These results indicate that c-Yes was able to coimmunoprecipitate Neu only under conditions where there was tyrosine phosphorylated Neu (lane 2). The lack of association between c-Yes and Neu in the uninduced lysates (lane 1) was not due to difference in the amount of immunoprecipitable c-Yes protein because control immunoblot of the membrane in panel C with anti-Yes antibodies revealed that there was comparable levels of c-Yes protein under both the conditions (Figure 6.3D, compare lanes 1 and 2). These observations strongly suggest that c-Yes associates with Neu in a tyrosine phosphorylation dependent manner.

6.2.4 c-Yes SH2 domain alone associates with Neu in a tyrosine phosphorylation dependent manner.

Since it is clear that c-Yes associates with Neu *in vivo* and, this association requires tyrosine phosphorylated Neu it is likely that this interaction is mediated by the c-Yes SH2 domain. To test this possibility I incubated lysates from tumor and nontumorous tissues with a GST fusion protein containing the c-Yes SH2 domain. The proteins bound to the beads

Figure 6.4 c-Yes SH2 domain alone can associate with Neu in a tyrosine phosphorylation dependent manner.

The tissue lysates used in Figure 6.1 was incubated with either bacterially produced GST alone **(A)** or with a GST-c-Yes SH2 fusion (Yes SH2) **(B)**. The proteins bound to the fusion proteins were resolved in a SDS-PAGE and immunoblotted with anti-Neu antibodies. **(C)** Lysates were prepared from dexamethasone induced (+) Rat.2 cells (R2, lane 1) or from uninduced (-) and induced (+) NT.12 cells. Twenty five micrograms of total cell lysate was resolved on a SDS-PAGE and immunoblotted with anti-Neu antibodies. **(D)** The same batch of lysates used in panel C were either used to immunoprecipitate phosphotyrosine containing proteins **(D)**, or was incubated with GST fusion proteins containing the c-Yes SH2 domain **(E)** and were immunoblotted with Neu specific antisera. The molecular weight markers are indicated in kDa.


A
#451
#464
#9831
NB BT NB BT NB BT
205
116
1 2 3 4 5 6
AP: GST; Blot: anti-Neu
B
205
116
Neu
1 2 3 4 5 6
AP: Yes-SH2; Blot: anti-Neu
C
R2
NT12
Dex + - +
205
116
Neu
1 2 3
Blot: anti-Neu
D
205
116
Neu
1 2 3
IP: anti-pTyr
Blot: anti-Neu
E
205
116
Neu
1 2 3

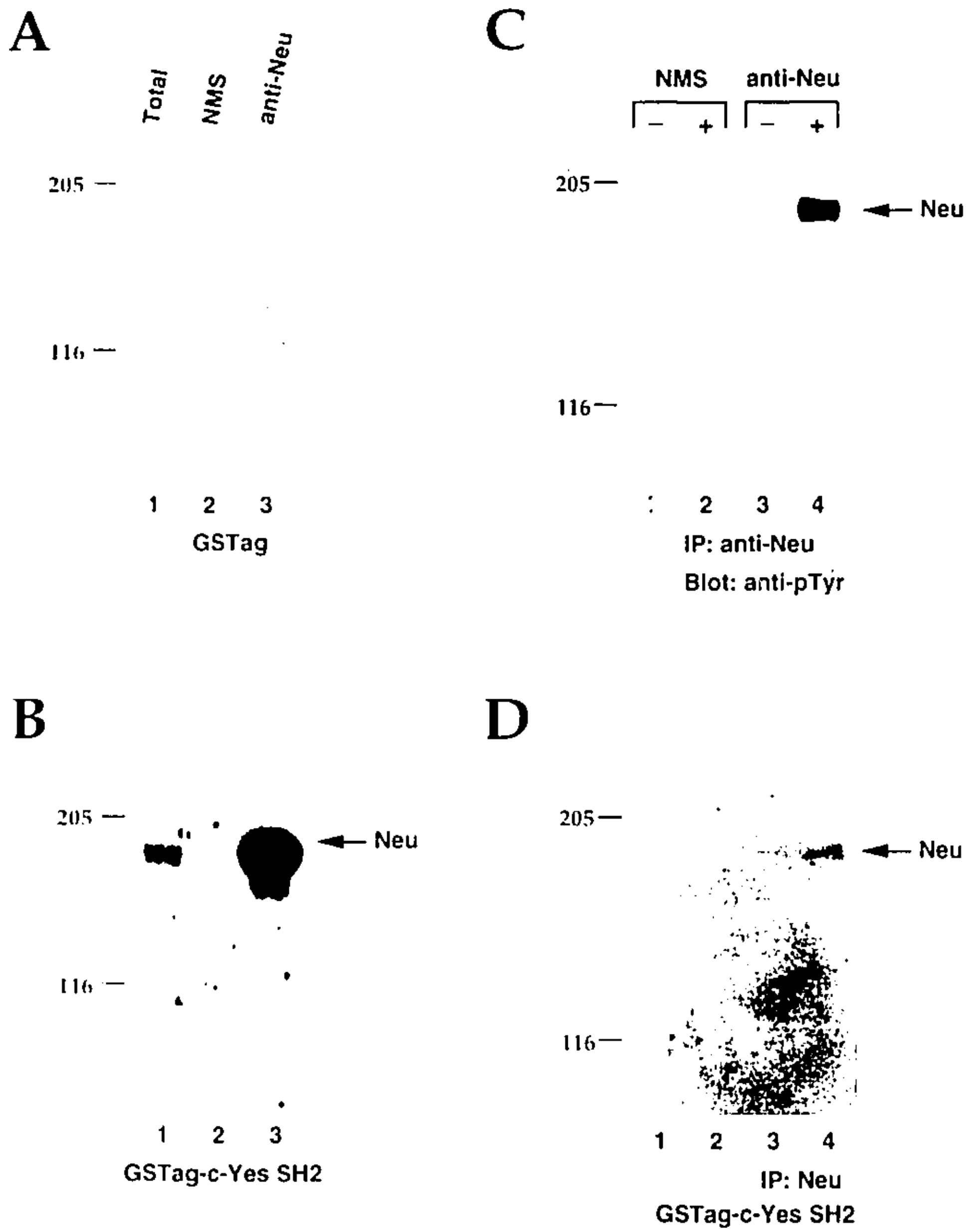
were resolved on a SDS-PAGE gel and immunoblotted with anti-Neu antibodies. As shown in Figure 6.4A GST alone did not associate with Neu in either normal or tumor epithelium. However, GST-c-Yes SH2 fusion protein associated with Neu in tumor tissue samples (Figure 6.4B). This *in vitro* association shows a strong correlation with the presence of tyrosine phosphorylated Neu (Figure 6.1B). To test whether tyrosine phosphorylated Neu is required for this *in vitro* association, we used lysates from dexamethasone induced NT.12 cells, uninduced NT.12 cells or Rat.2 parental cells. Immunoblot analysis using total lysates using anti-Neu antisera suggest that the parental cells line and uninduced NT.12 expresses significant levels of Neu protein (Figure 6.4C, lanes 1, 2) that is not tyrosine phosphorylated (Figure 6.4D, lanes 1, 2). Whereas, NT.12 cells induced with dexamethasone possess significant levels of tyrosine phosphorylated Neu (Figure 6.4D, lane 3) as a result of dexamethasone induced expression of activated *neu* from the MMTV promoter/enhancer. Fusion protein containing c-Yes SH2 domain was incubated with dexamethasone treated, untreated and Rat.1 cell lysates and the proteins bound were separated on a SDS-PAGE and probed with anti-Neu antibodies. As shown in Figure 6.4E, c-Yes SH2 domain associated only with tyrosine phophorylated Neu (lane 3) and it did not show any detectable association with unphosphorylated Neu present in the parental Rat.2 cell line (lane 1). The weak association observed in Figure 6.4E lane 2 is due to the presence of a detectable level of tyrosine phosphorylated Neu in uninduced NT.12 cells (Figure 6.4D lane 2). These observations suggest that the SH2 domain of c-Yes mediates the interaction between c-Yes and tyrosine phosphorylated Neu.

6.2.5 c-Yes SH2 domain interacts directly with denatured tyrosine phosphorylated Neu.

Although experiments in the previous sections suggest that c-Yes associates with Neu both *in vivo* and *in vitro* and that this association is likely mediated by the c-Yes SH2 domain, it is not clear if this association is a direct interaction of the SH2 domain with Neu or mediated by other cellular proteins. To test this possibility I probed PVDF membranes containing Neu immunoprecipitates with radiolabeled GSTag-c-Yes SH2 fusion protein. As expected, radiolabeled GSTag alone did not bind to any proteins in the total lysate or to the Neu immunoprecipitate (Figure 6.5A, lanes 1, 3) however, GSTag-c-Yes SH2 fusion protein bound to Neu both in the total lysate and in the Neu immunoprecipitate (Figure 6.5B, lanes 1,3). To confirm that this association also requires tyrosine phosphorylated Neu, Neu was immunoprecipitated from dexamethasone induced or uninduced NT.12 cell lysates. Figure 6.5C shows that treatment of NT.12 cells with dexamethasone induces the expression of activated Neu (lane 4). When an identical PVDF membrane was probed with radiolabeled GSTag-c-Yes SH2 fusion protein, the ability of c-Yes SH2 to bind directly to Neu showed strong correlation with the presence of tyrosine phosphorylated Neu. These observations indicate that c-Yes SH2

Figure 6.5 c-Yes SH2 domain interacts directly with denatured tyrosine phosphorylated Neu.

(A, B) Total NAFA cell lysate (lane 1) or normal mouse serum (NMS, lane 2) or a anti-Neu immunoprecipitate (lane 3) were resolved on a SDS-PAGE and transferred onto a membrane. Duplicate membrane strips were probed with either radiolabeled GSTag alone **(A)** or with radiolabeled GSTag-c-Yes SH2 domain **(B)**. NT.12 cells were grown both in the presence (+) and absence (-) of dexamethasone. Cell lysates were prepared and Neu was immunoprecipitated. One part of the immunoprecipitate was immunoblotted with anti-pTyr antibodies **(C)** and the other part was probed with radiolabeled GSTag-c-Yes SH2 fusion protein as a probe **(D)**. Normal mouse serum (NMS) was used as nonspecific control (lanes 1,2). The molecular weight markers are indicated in kDa.


A
Total
NMS
anti-Neu
205
116
1
2
3
GSTag
B
205
116
Neu
1
2
3
GSTag-c-Yes SH2
C
NMS
anti-Neu
−
+
−
+
205
116
Neu
2
3
4
IP: anti-Neu
Blot: anti-pTyr
D
205
116
Neu
1
2
3
4
IP: Neu
GSTag-c-Yes SH2

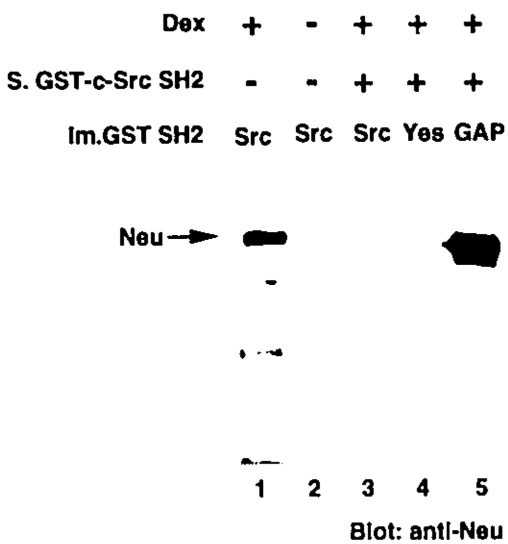
domain binds directly to denatured tyrosine phosphorylated Neu in a tyrosine phosphorylation dependent manner.

6.2.6 The SH2 domains of c-Yes and c-Src associate with the same site on tyrosine phosphorylated Neu.

Previous observations have shown that c-Src SH2 domain alone can bind directly to tyrosine phosphorylated Neu (see chapter 5). To determine if c-Yes and c-Src associate with the same site on the Neu molecule I performed an *in vitro* competition assay. GST SH2 fusion proteins containing either c-Yes or c-Src SH2 domains were made in bacteria and purified on a glutathione Sepharose column. The c-Src SH2 fusion protein was eluted from the Sepharose beads by incubating the purified fusion proteins in a buffer containing 5 mM glutathione. The eluted protein is referred to as Sol. GST-c-Src-SH2 while the uneluted/Sepharose bead bound fusion protein is referred to as Immobilized (Im.) GST-SH2. Cell lysates were derived from either dexamethasone induced or uninduced NT.12 cells. As expected, incubation of induced cell lysate containing tyrosine phosphorylated Neu with Im.GST-c-Src SH2 fusion resulted in an association between c-Src SH2 domain and Neu (Figure 6.6 , lane 1) and incubation of uninduced cell lysates did not result in association between c-Src SH2 domain and Neu (lane 2). The competition assay was conducted by preincubation of cell lysates derived from dexamethasone induced NT.12 cells with Sol.GST-c-Src SH2 fusion protein. These preincubated extracts were subsequently incubated with Im.GST SH2 fusions (Figure 6.6)

Figure 6.6 The SH2 domains of c-Yes and c-Src associate with the same site on tyrosine phosphorylated Neu.

NT.12 cells were grown in the presence (+) (lanes 1,3,4,5) or absence (-) lane 2) of dexamethasone (Dex). Two hundred micrograms (lanes 1,2) and 500μg of lysates were either incubated with the soluble form of GST-c-Src SH2 (Sol.GST-c-Src SH2) [(+) (lanes 3,4,5)]. Subsequent to the preincubation the cell lysates were incubated with immobilized (Im. GST-SH2) form of Src (lane 3) or Yes (lane 4) or GAP (lane 5) SH2 fusion proteins. Lanes 1,2 were incubated with Im.GST-SH2 alone. The proteins bound to the immobilized form of the fusion proteins were resolved on a SDS-PAGE and immunoblotted with anti-Neu antibodies.


Dex + - + + +
S. GST-c-Src SH2 - - + + +
Im.GST SH2 Src Src Src Yes GAP
Neu
1 2 3 4 5
Blot: anti-Neu

of either c-Src (lane 3) or c-Yes (lane 4) or GAP (lane 5) and the proteins bound to the immobilized SH2 fusions were purified and resolved on SDS-PAGE and immunoblotted with anti-Neu antibodies. As expected, preincubation of cell lysates with Sol. GST-c-Src SH2 fusion protein competes for the ability of Im. GST-c-Src SH2 to associate with Neu (Figure 6.6 lane 3). Interestingly, preincubation of Sol. GST-c-Src SH2 fusion protein also competed with the ability of Im. GST-c-Yes SH2 domain to associate with Neu (Figure 6.6 lane 4) suggesting that both c-Src and c-Yes compete for the same binding site *in vitro*. By contrast, preincubation with Sol. GST-c-Src SH2 had no effect on the ability of Im. GST-GAP SH2 fusion protein to bind to Neu (Figure 6.6 lane 5, chapter 4). These observations suggest that both Src family members (c-Src and c-Yes) compete for the same binding site on Neu to associate with the receptor.

6.2.7 The c-Src and c-Yes SH2-Neu interaction is SH2-pTyr mediated

To confirm that the association between c-Src and c-Yes and tyrosine phosphorylated Neu is a result of a SH2-pTyr interaction we tested whether the SH2 domain-Neu interaction could be competed using commercially available phosphorylated tyrosine as a competing agent. GST SH2 fusion of c-Src and c-Yes were incubated with cell lysates containing tyrosine phosphorylated Neu in the presence of increasing amounts of phosphotyrosine. Cellular proteins bound to the SH2 domains incubated under varying concentrations of phosphotyrosine were resolved and probed with anti-Neu antibodies. As shown in Figure 6.7 the presence of 16mM phosphotyrosine for c-Src SH2 domain (lane 5) and 50mM phosphotyrosine for c-Yes SH2 domain (lane 14) was able to compete with the ability of SH2 domains to interact with Neu while a non-specific competitor such as phosphoserine (50 mM) did not have any effect (Figure 6.7 lane 1 and 8). This observation was reproducible over three independent experiments. The amount of Neu bound to the SH2 domains under different conditions were quantitated on a PhosphorImager. Under conditions where there was no competitor (Figure 6.7, lane 2 and lane 9) c-Src SH2 domain bound four to five times more Neu than the c-Yes SH2 domain (Figure 6.8). Six to 8.0 mM of phosphotyrosine was sufficient to impart a 50% decrease in the amount of Neu associated with c-Src SH2 domain. However, c-Yes SH2 domain required about 38 to 42 mM of phosphotyrosine (Figure 6.8). These observations suggest two properties of the c-Src and c-Yes SH2 domains, one that the interaction between the SH2 domains and Neu is mediated by a phosphorylated tyrosine on the receptor and second that under these *in vitro* conditions c-Src SH2 domain binds four to five times more Neu molecules albeit with less affinity. The *in vivo* significance of this difference in affinity remains to be tested.

Figure 6.7 The c-Src and c-Yes SH2-Neu interaction is SH2-pTyr mediated

Purified GST fusion proteins containing c-Src SH2 domain (lanes 1-7) or c-Yes SH2 domain (lanes 8-14) were incubated with lysates containing tyrosine phosphorylated Neu in the presence of no competitor (lanes 2 and 9) or in the presence of increasing amounts of phosphotyrosine (lanes 3-7 and 10-14). The fusion proteins were also incubated with phosphoserine (50mM) as a non-specific control (lanes 1 and 8). The proteins bound to the SH2 domains were resolved on a SDS-PAGE and immunoblotted using anti-Neu antibodies.

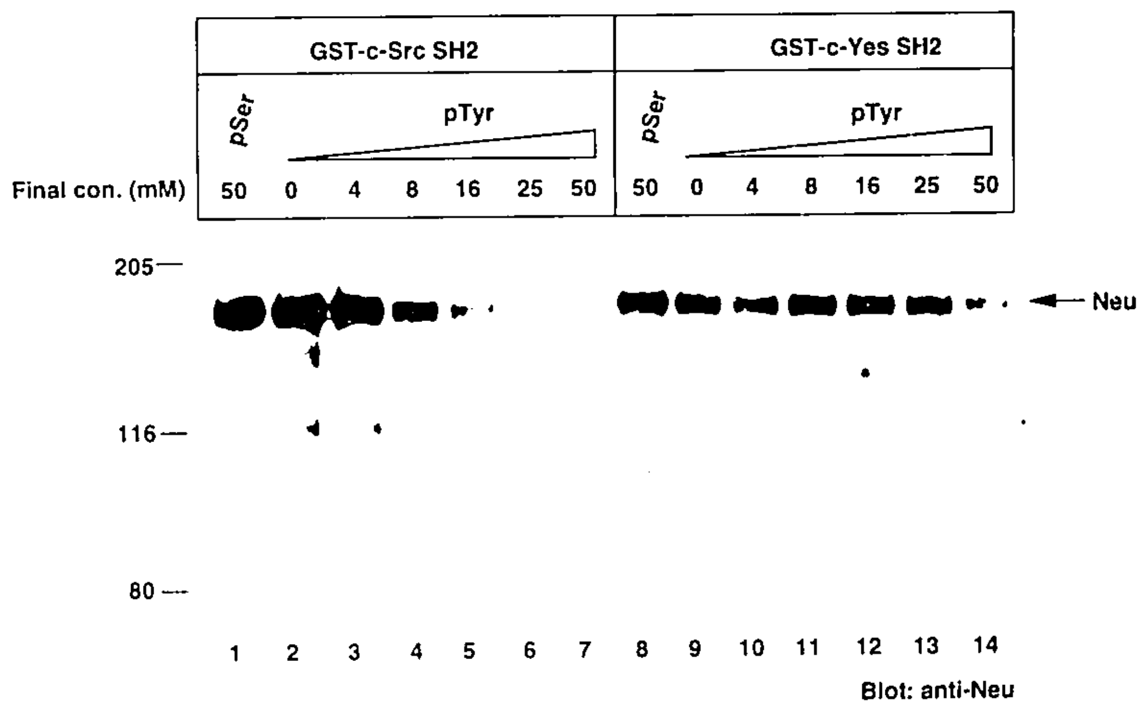
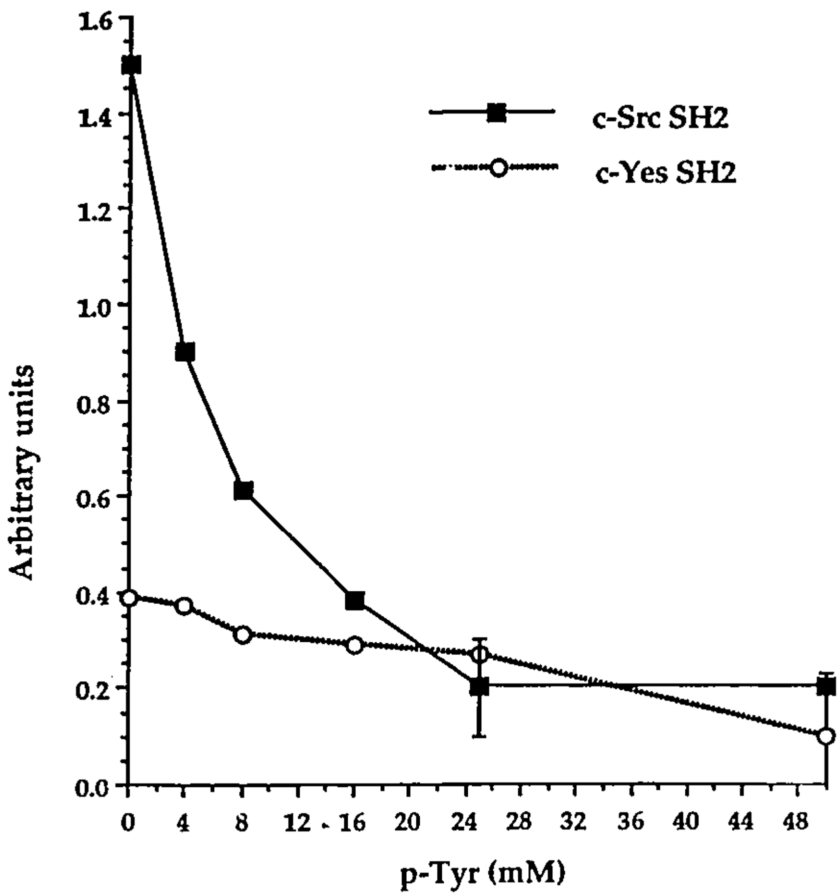

GST-c-Src SH2
GST-c-Yes SH2
pSer
pTyr
pSer
pTyr
Final con. (mM)
50 0 4 8 16 25 50 50 0 4 8 16 25 50
205
116
80
Neu
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Blot: anti-Neu

Figure 6.8 The SH2 domains of c-Src and c-Yes differ in their affinity of interaction with Neu.

The experiment in Figure 6.7 was repeated twice using ^{125}I anti-mouse as a secondary antibody and the amount of Neu in each lane was quantitated using a PhosphorImager and plotted in arbitrary units.



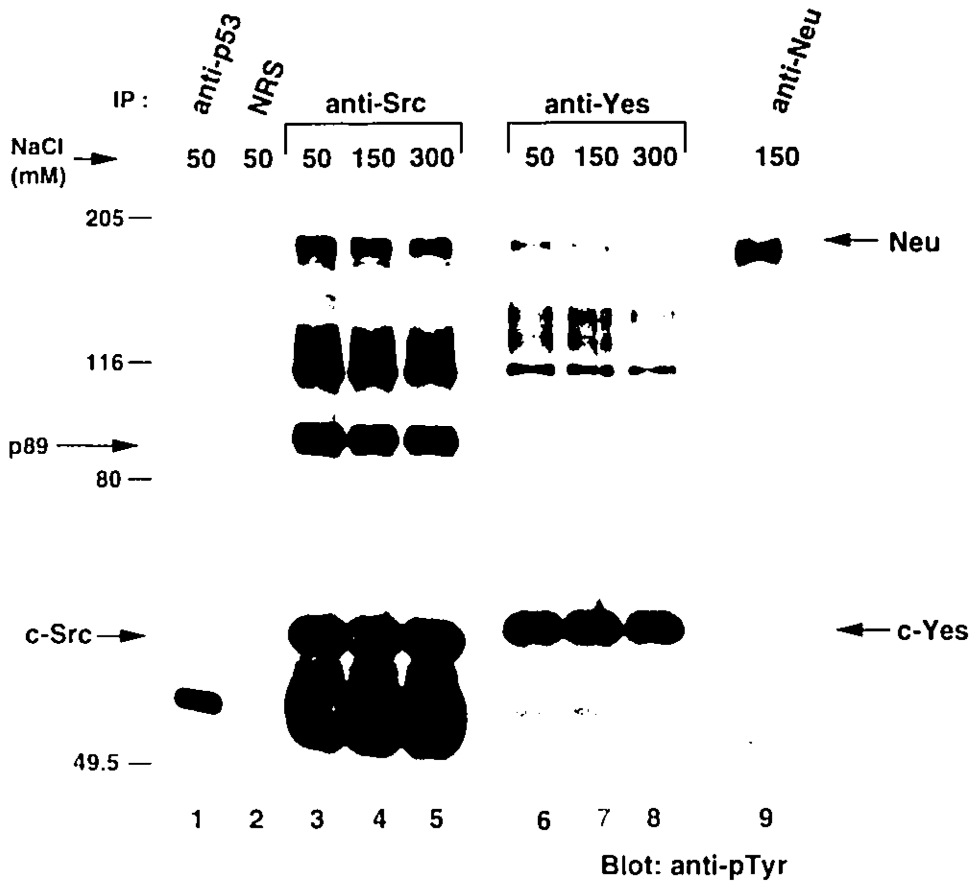
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Arbitrary units
c-Src SH2
c-Yes SH2
0
4
8
12
16
20
24
28
32
36
40
44
48
p-Tyr (mM)

6.2.8 c-Src and c-Yes tyrosine kinases associate with a distinct set of tyrosine phosphorylated proteins *in vivo* .

The results of the above analyses and those previously reported (see chapter 4) suggest that both c-Src and c-Yes kinases are involved in Neu-mediated mammary tumorigenesis. Although tyrosine phosphorylated Neu could be detected in physical complexes with both c-Src and c-Yes, it was unclear whether c-Src and c-Yes associate with similar or distinct proteins *in vivo*. To answer this question, protein extracts from Neu transformed mammary epithelial cells line, NAFA (Muller et al., 1988, chapter 4), were immunoprecipitated with either c-Src (MAb 7D10) (Figure 6.9 lanes 3-5) or c-Yes specific antibodies (Figure 6.9 lanes 6-8) and subjected to immunoblot analysis with anti-phosphotyrosine antibodies. As expected immunoprecipitation with nonspecific control antibodies did not immunoprecipitate any phosphotyrosine containing proteins (lanes 1,2). By contrast, immunoprecipitation with either c-Src or c-Yes specific antibodies, raised against their amino-terminal unique region, revealed a wide spectrum of phosphotyrosine containing proteins associated with these Src family members. The 185 kDa phosphoprotein found in both c-Src and c-Yes immunoprecipitates is likely Neu since it comigrates with Neu in anti-Neu immunoprecipitates (lane 9). Although both c-Src and c-Yes are associated with Neu, both Src family kinases appear to be associated with a distinct as well as overlapping sets of phosphotyrosine containing proteins. For example, a phosphotyrosine containing protein of approximately 89kDa (p89) was consistently found associated with c-Src but

Figure 6.9 c-Src and c-Yes associate with a distinct set of tyrosine phosphorylated proteins *in vitro*.

Equal amounts (2.0 mg) of NAFA cell lysates were incubated with anti-Src (lanes 3,4,5) or with anti-Yes (lanes 6,7,8) antibodies. The immunoprecipitates were washed in lysis buffer containing varying concentrations (mM) of NaCl as indicated. Anti-p53 (lane 1) and normal rabbit serum (NRS, lane 2) immunoprecipitates were used as negative controls. An anti-Neu immunoprecipitate (lane 9) using 500μg of lysate was also used. The immunoprecipitates were immunoblotted with anti-phosphotyrosine (anti-pTyr) antibodies. The positions of c-Src, c-Yes, p89 and, Neu are indicated. IP: Immunoprecipitate. The molecular weight markers are indicated in kDa.



IP :
anti-p53
NRS
anti-Src
anti-Yes
anti-Neu
NaCl (mM)
50
50
50 150 300
50 150 300
150
205
116
p89
80
c-Src
49.5
Neu
c-Yes
1 2 3 4 5 6 7 8 9
Blot: anti-pTyr

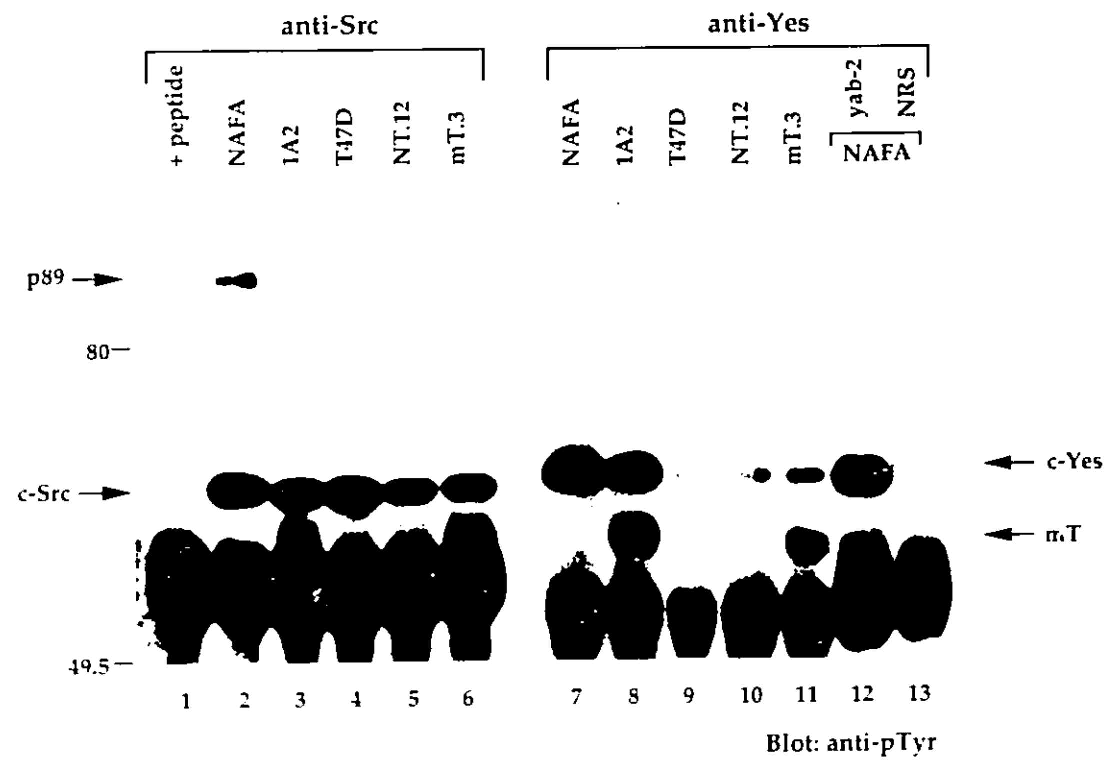
was absent in the c-Yes immunoprecipitates. When the immunoprecipitation was carried out in the presence of competing peptide (aa 2-17) neither c-Src nor p89 was observed in the immunoprecipitate (Figure 6.10 lane 1). In addition a low but detectable amounts of p89 was found associated with c-Src in an anti-Src immunoprecipitate with an independent MAb directed against the SH3 domain (Ab 327). In addition to this p89 several tyrosine phosphorylated proteins between 100-120 kDa were also found associated with c-Src and c-Yes. While the identity of these proteins are unknown, I determined that p89 was not p80/85 Src substrate, STAT3 (p91) or eps8 (90kDa) an EGFR associated phosphoprotein (Figure 6.11). These observations suggests that activation of c-Src and c-Yes by Neu results in their association with distinct and novel sets of tyrosine phosphorylated proteins.

6.2.9 c-Src associates with p89 only in transformed epithelial cells but not in transformed fibroblasts

In order to confirm that p89, c-Src associated protein, was not restricted to NAFA cells, I extended the analyses using two independently derived mammary epithelial cell lines that are known to possess elevated c-Src and c-Yes tyrosine kinase activity. c-Src and c-Yes immunoprecipitates from a mouse mammary epithelial cell line (1A2) derived from a PyV middle T antigen-induced mammary tumor (Guy et al 1992a, Addison and Graham, unpublished observations) and a human breast cancer cell line (T47D) that is known possesses

Figure 6.10 c-Src associates with p89 only in transformed epithelial cells but not in transformed fibroblasts.

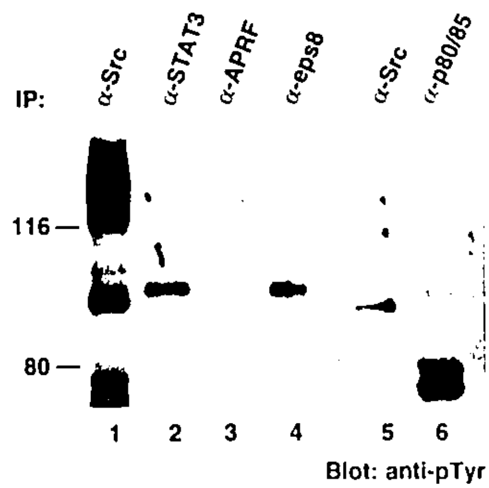
Cell lysates were prepared from transformed mammary epithelial cells NAFA (lanes 1,2,7,12,13), 1A2 (lanes 3,8) and T47D (lanes 4,9) or from transformed fibroblasts, NT.12 (lanes 5,10) and mT.3 (lanes 6,11) (see text for detailed descriptions of these cell lines). Equal amounts (750μg) of cell lysate was incubated with c-Src specific (lanes 1-6) or c-Yes specific (lanes 7-12) antisera. The immunoprecipitates were immunoblotted with antiphosphotyrosine (anti-pTyr) antibodies. The anti-Src antibody was preincubated on ice for 20 min. with 5-10 μM of aa 2-17 Src peptide prior to the addition of cell lysate (+peptide, lane 1). yab-2 is a Yes specific antisera directed against aa 4-20 of mouse c-Yes protein (lane 12). NRS: normal rabbit serum. The positions of c-Src, c-Yes, PyV middle T antigen (mT) and p89 are indicated. Molecular weight is in kDa.



anti-Src
anti-Yes
+ peptide
NAFA
1A2
T47D
NT.12
mT.3
NAFA
1A2
T47D
NT.12
mT.3
yab-2
NRS
NAFA
p89
80
c-Src
c-Yes
mT
49.5
1
2
3
4
5
6
7
8
9
10
11
12
13
Blot: anti-pTyr

Figure 6.11 P89 differs from proteins that have molecular mass of approximately 90kDa.

c-Src immunoprecipitate (lane 1, 5) was compared with STAT3 (p91) (lane 2), eps8 (lane 4) and p80/85 (lane 6) Src substrate. APRF is same as STAT3, the α-APRF antisera did not immunoprecipitate the mouse protein. All the immunoprecipitates (IP) were immunoblotted with anti-phosphotyrosine antibodies. Markers are in kDa.


IP:
α-Src
α-STAT3
α-APRF
α-eps8
α-Src
α-p80/85
116 —
80 —
1
2
3
4
5
6
Blot: anti-pTyr

elevated c-Src tyrosine kinase activity (see chapter 4) were analyzed. As shown in Figure 6.10, c-Src immunoprecipitates from either NAFA cells (lane 2), 1A2 (lane 3) or T47D (lane 4) coimmunoprecipitate a protein that migrates close to p89 in NAFA cell lysates. At present I do not know the reason for the difference in migration observed in c-Src immunoprecipitates from mouse (lanes 2,3) and human cell lysates (lane 4). However, c-Yes immunoprecipitates from NAFA, 1A2 or T47D (lanes 8,9,10) did not coimmunoprecipitate a protein that comigrates with p89. To rule out the possibility that our inability to detect association between c-Yes and p89 is due to the anti-Yes antisera used in these assays, I raised Yes-specific antisera (yab-2) against the mouse c-Yes protein using a peptide containing aa 4-20 in the amino terminal unique region of the molecule. Immunoprecipitation of c-Yes from NAFA cells using yab-2 followed by an anti-phosphotyrosine immunoblot showed that p89 was not associated with c-Yes (lane 13). This analysis also proved that yab-2 is specific for c-Yes since, the immunoprecipitates did not bring down any protein that comigrates with c-Src (compare lanes 2-6 with 13). To determine if c-Src and c-Yes associated with similar sets of substrates in all cell types transformed by Neu or PyV middle T, c-Src and c-Yes was immunoprecipitated from Neu (NT.12, lanes 5, 10) or PyV middle T (mT.3, lanes 6,11) antigen transformed Rat fibroblasts. Interestingly, under conditions where c-Src (lane 6) and c-Yes (lane 11) can associate with middle T antigen we were unable to detect any association with p89 in either NT.12 or mT.3 cell lines. This observation suggests that the c-Src and p89 interaction is specific to transformed mammary epithelial cells and not transformed fibroblasts.

6.3 DISCUSSION

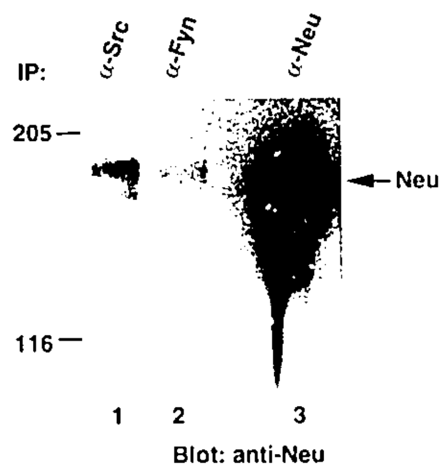
I have presented evidence that strongly suggests that among the ubiquitously expressed Src family members (c-Src, c-Yes and Fyn) only c-Src and c-Yes kinase activity is elevated in Neu-induced murine mammary tumors. The increase in c-Yes kinase activity correlates with its ability to associate with tyrosine phosphorylated Neu *in vivo*. This *in vivo* association is likely due to the ability of c-Yes SH2 domain to interact directly with Neu in a tyrosine phosphorylation dependent manner as shown *in vitro*. Interestingly, although the tyrosine kinase activities of both c-Src (chapter 4) and c-Yes are elevated in Neu-induced mammary tumors, they associate with distinct sets of tyrosine phosphorylated cellular proteins only in transformed mammary epithelial cells and not in transformed fibroblasts.

The observation that c-Yes kinase activity is elevated four to five fold in Neu-induced mammary tumors is consistent with the observation that Neu-induced mammary tumors posseses elevated c-Src kinase activity (chapter 4). Interestingly, unlike these two Src family members Fyn tyrosine kinase activity was not elevated in Neu-induced mammary tumors suggesting that Fyn tyrosine kinase may not play an important role in Neu-induced mammary tumorigenesis. This hypothesis is supported by the observation that PyV middle T antigen-induced mammary tumors also do not possess increased Fyn tyrosine kinase activity (Guy and Muller, unpublished observations). Interestingly, despite the lack of elevated Fyn kinase activity, complexes between Fyn and either PyV middle T (Kypta et al., 1988, Cheng et al., 1988) or Neu (Figure 6.12) can be detected. Consistent with these observations, only c-Src and c-Yes, but not any other Src family of kinases including Fyn activity was elevated in colon carcinoma derived cell lines and primary colon cancers when compared to untransformed cells (Park et al., 1993). Taken together these observations suggests that unlike c-Src and c-Yes, Fyn tyrosine activity may not play an important role in these epithelial tumors.

The increase in c-Yes tyrosine kinase activity in Neu-induced mammary tumors is likely due to the ability of c-Yes to directly associate with Neu in a tyrosine phosphorylation dependent manner (Figures 6.3, 6.4 and 6.5). These observations are consistent with those observed for PDGFR and CSF-1R which are known to activate c-Yes (Twamley et al., 1992, Courtneidge et al., 1993). Results reported in chapter 5 suggest that the increased c-Src kinase activity observed in Neu-induced tumors also results from the ability of c-Src SH2 domain to directly interact with Neu in a tyrosine phosphorylation dependent manner. *In vitro* competition analysis suggests that both the c-Src SH2 domain and the c-Yes SH2 domain bind to the same site on Neu (Figure 6.6). This is consistent with that observed for binding of Src family members to PDGFR where tyrosines 579 and 581 of the PDGFR are involved in mediating the association and activation of the Src family of kinases (Mori et al., 1993).

Figure 6.12 Fyn associates with Neu *in vivo*.

NAFA cell lysates (1.0 mg) were immunoprecipitated with either c-Src (lane 1) or Fyn (lane 2) antisera and immunoblotted with anti-Neu antibodies. An anti-Neu immunoprecipitate was used as control. The position of Neu is indicated and the markers are in kDa.


IP:
α-Src
α-Fyn
α-Neu
205
116
Neu
1
2
3
Blot: anti-Neu

Although the site of interaction between the Src family members and Neu is not known, evidence in section 6.2.7 suggests that this interaction is mediated by a phosphotyrosine and it is likely that Neu activates both c-Src and c-Yes by a similar mechanism.

I have also presented evidence to suggest that in transformed mammary epithelial cells c-Src and c-Yes associate with distinct and overlapping sets of tyrosine phosphorylated cellular proteins. It is likely that the higher molecular weight Src and Yes associated proteins may include Src substrates such as pp125 (FAK) (Cobb et al., 1994), p110, p120 or p130 (Kanner et al., 1991, Reynolds et al., 1992). Interestingly, a prominent protein with an approximate molecular weight of 89 kDa was observed (Figure 6.9 and 6.10) only in c-Src immunoprecipitates from transformed epithelial cell lysates. However, c-Src did not show any association with p89 in Neu or PyV middle T antigen transformed fibroblast cells suggesting that p89 is likely a mammary epithelial-specific c-Src substrate. These associated cellular proteins may play an important role in c-Src and c-Yes function and, the difference in the associated protein profile support the contention that c-Src and c-Yes differ in their ability to promote mammary tumorigenesis. The future identification of p89 may provide novel insights into the mechanisms involved in c-Src-mediated transformation of the mammary epithelium.

CHAPTER 7

CONCLUSION

The objective of this study is to understand the role played by activation of Src family tyrosine kinases in mouse mammary tumorigenesis and to define a role for the Src family of kinases in Neu mediated tumorigenesis and signal transduction.

Previous studies have shown that viral T antigens such as the PyV middle T activate a number of intracellular signaling pathways when expressed within a cell. This aberrant activation of signaling pathways is thought to play an important role in middle T antigen mediated transformation of cells (for a review see Keifer et al., 1994). PyV middle T antigen is known to associate with and activate the c-Src and c-Yes tyrosine kinase and this associated tyrosine kinase activity is required for its ability to transform cells. In addition to activating the Src family of tyrosine kinases middle T antigen also associates with and activates the PI3'kinase and Shc proteins (Kiefer et al., 1994). The SH2 domain of the p85 subunit of PI3' kinase has been shown to associate with Tyr 315 and that of Shc with Tyr 250 (N-P-T-Y motif) in the middle T molecule (Talmage et al., 1989, Cohen et al., 1990, Campbell et al., 1994, Dilworth et al., 1994). It is thought that Shc in turn binds to the Grb2/mSos complex and recruits this complex closer to the membrane. The membrane proximal recruitment of Grb2/mSos complex facilitates the mSos to activate Ras by catalyzing a GDP to GTP exchange. In addition to these signaling molecules PyV middle T antigen is also known to associate with a protein phosphatase, PP2A (Pallas et al., 1990, Walter et al., 1990), and 14-3-3 protein (Pallas et al., 1994) the significance of these associations is not yet understood.

Expression of PyV middle T antigen under the control of the MMTV promoter/enhancer in transgenic mice results in the rapid development of multifocal mammary tumors (Guy et al., 1992a). The potent transforming activity of PyV middle T in the mammary epithelium is likely due to its capacity to activate a number of signal transduction pathways (Figure 1.3). To determine the role played by individual members of the Src family (c-Src and c-Yes) in middle T mediated transformation we interbred MMTV/middle T transgenics with mice that lack either functional c-Src or functional c-Yes. By contrast to the rapid induction of mammary tumors observed in the parental MMTV/PyV middle T antigen transgenic strains, mammary gland-specific expression of the PyV middle T antigen in mice defective in c-Src function leads to the development of cystic hyperplasia of the mammary gland which rarely progress to full malignancy. Significantly, transgenic mice expressing PyV middle T antigen in the mammary epithelium of the c-Yes deficient or wild type mice develop multifocal metastatic mammary tumors at rates comparable to the parental MMTV/PyV middle T antigen strains (Guy et al., 1994). These observations indicate that a functional c-Src is required for PyV middle T antigen induced mammary tumorigenesis, and that the mammary epithelium is particularly sensitive to activation of the c-Src signal transduction pathway.

Analogous to the middle T antigen, receptor tyrosine kinases such as the well studied PDGFR and Neu are also known to activate a variety of signal transduction pathways upon activation. For example, activated Neu is known to activate a number of signaling pathways including Phospholipase C-γ1, GTPase activating protein (GAP), Shc, Grb2, Grb7 and PI3'kinase (Dougal et al., 1994). Interestingly several of these pathways are also activated by PyV middle T antigen. Given the ability of both Neu and middle T to transform the mammary epithelium, it is conceivable that activation of these pathways are causally linked to the phenotype. Because the c-Src pathway appeared to be pivotal for the transforming property of middle T, I investigated whether this pathway is involved in Neu mediated mammary tumorigenesis.

To accomplish this I made use of a mouse model system expressing the wild type Neu under the control of MMTV promoter/enhancer (Guy et al., 1992b). These transgenic mice develop focal mammary tumors adjacent to morphologically normal epithelium. The tumor tissues and adjacent epithelium were isolated fro. the transgenic mice and tissue lysates were used to analyze the relative levels of kinase activity of the Src family of kinases. The results clearly show that among the widely expressed Src family members (c-Src, c-Yes and Fyn) only c-Src and c-Yes kinase activities were elevated in Neu induced tumor samples compared to the adjacent morphologically normal epithelium (6.7 fold and 4.7 fold respectively). Interestingly, the Fyn tyrosine kinase activity was not elevated in Neu induced mammary tumors. These observations suggest that c-Src and c-Yes may play an important role in Neu mediated mammary tumorigenesis while Fyn may not play an important role.

In order to understand the mechanism by which Neu activates the tyrosine kinase activity of both c-Src and c-Yes, experiments were performed using cell culture systems involving rat fibroblasts and mammary tumor derived cell lines. Results presented in chapters 4, 5 and, 6 (Muthuswamy et al., 1994, Muthuswamy and Muller, 1995) suggest that both c-Src and c-Yes kinases associate with tyrosine phosphorylated Neu *in vivo* in tumor samples, tumor derived cell lines and, Neu transformed rat fibroblast cells. *In vitro* analyses suggest that the SH2 domains of c-Src and c-Yes associate directly with tyrosine phophosphorylated Neu. Although the exact c-Src SH2 binding site on Neu is not known preliminary results suggest that Tyrosines 1028, 1144, 1201, 1226/7 and 1253 are not involved in mediating the interaction (Dankort and Muller, unpublished observations). It is possible that the direct interaction between Src family members and tyrosine phosphorylated Neu would render Src kinases into a catalytically active conformation (see Figure 1.2) which may result in the observed increase in tyrosine kinase activity. A formal proof for this hypothesis awaits future experimentation. Consistent with this hypothesis, induced expression of kinase active form of Neu in rat fibroblasts results in a 2.3 fold increase in the intrinsic kinase activity of c-Src. This elevated kinase activity shows strong correlation with the ability of c-Src to associate with Neu *in vivo* and with the ability of c-Src SH2 domain to bind directly to Neu *in vitro*. Interestingly, PDGFR mutants (Y579F/Y581F) that do not bind the Src family members were found to lack their intrinsic kinase activity (Mori et al., 1993). Moreover, EGFR was constitutively phosphorylated on tyrosine in cells coexpressing v-Src and EGFR (Wasilenko et al., 1991). Although results presented here suggests that activation of Neu RTK is required for the activation of c-Src tyrosine kinase, it is unclear whether the association of Src with RTKs may play a role in enhanced activation of RTKs such as Neu and PDGFR.

The notion that the Src family of tyrosine kinases plays an important role in mammary tumorigenesis is further strengthen by a number of observations. Several studies have shown that almost all the human breast cancer specimens tested possess more than four fold increase in c-Src tyrosine kinase activity when compared to normal breast tissues (Figure 4.7, Jacobs and Rubsamen, 1983, Rosen et al., 1986, Ottenhoff-klaff et al., 1992, Muthuswamy and Muller, 1994). Furthermore, expression of the constitutively active form of c-Src (Y527F) under the control of MMTV promoter/enhancer in transgenic mice results in mammary epithelial hyperplasis that eventually form mammary tumors (Webster et al., 1995). Taken together these observations strongly argue that the Src family of tyrosine kinases, c-Src and c-Yes, plays an important role in mammary tumorigenesis.

Recent observations have shown that among the erbB family of receptors EGFR can associate with c-Src and EGF stimulation of cells expressing high levels of EGFR results in activation of the kinase activity of Src family members (Luttrell et al., 1994, Osherov and Levitzki, 1994). However, under conditions where c-Src kinase activity is elevated by EGF a physical complex between c-Src and EGFR was not observed suggesting that EGF mediated activation of c-Src may not involve association between EGFR and c-Src (Osherov and Levitzki, 1994). Although EGF does not bind directly to Neu (c-erbB-2) RTK, in cells expressing both EGFR and Neu addition of EGF has been shown to activate Neu (c-erbB-2) (King et al., 1988, Stern and Kamps, 1988). Given the ability of EGFR and Neu to heterodimerize, I was interested to test whether the Neu RTK plays a role in EGF mediated activation of c-Src tyrosine kinase. Interestingly the c-Src SH2 domain does not associate directly with tyrosine phosphorylated EGFR under conditions where it shows strong interaction with Neu suggesting that activation of c-Src by EGF is mediated by a mechanism that does not involve direct binding. Moreover, in established rat fibroblast cell lines expressing elevated levels of EGFR, EGF stimulation results in transphosphorylation of Neu and formation of complexes between c-Src and tyrosine phosphorylated Neu (Muthuswamy and Muller 1995). As observed for the c-Src SH2 domain the c-Yes SH2 domain also failed to associate with the tyrosine phosphorylated EGFR under conditions where it showed strong binding to tyrosine phosphorylated Neu (Figure 7.1). Taken together, these observations suggest that activation of Src tyrosine kinases by these two closely related EGFR family members results from a direct and specific interaction of c-Src and c-Yes with tyrosine phosphorylated Neu (Figure 7.2).

It is possible that the inability of EGFR to activate c-Src directly may be related to fact that EGFR is 100 fold less potent in its transforming ability compared to Neu (c-erbB-2). In this regard, chimeric receptors containing the ligand binding domain of EGFR and different portions of the c-erbB-2

Figure 7.1 c-Yes SH2 domain does not bind directly with activated EGFR.

Increasing amounts of Neu (lanes 1-3) and EGFR (lanes 4-6) were immunoprecipitated from NAFA cell lysates and EGF stimulated R1/hER cell lysates. The immunoprecipitates were resolved on SDS-PAGE and transferred onto Immobilon-P. Identical sets of membrane strips were probed with either radiolabeled GSTag-Grb2 (**A**) or GSTag-c-Yes SH2 (**B**). The results suggest that under conditions where Grb2 bound to EGFR I was unable to detect binding of c-Yes SH2 to EGFR while both Grb2 and c-Yes directly bound to Neu.

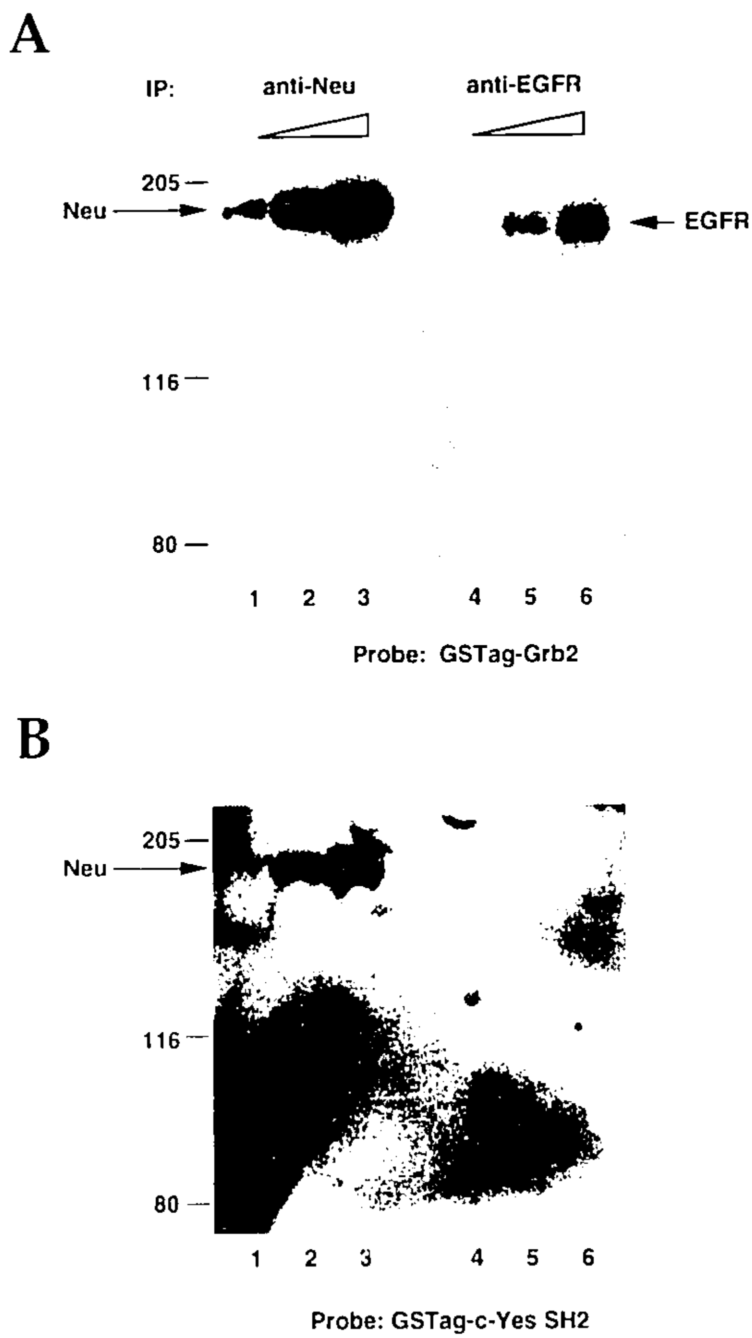
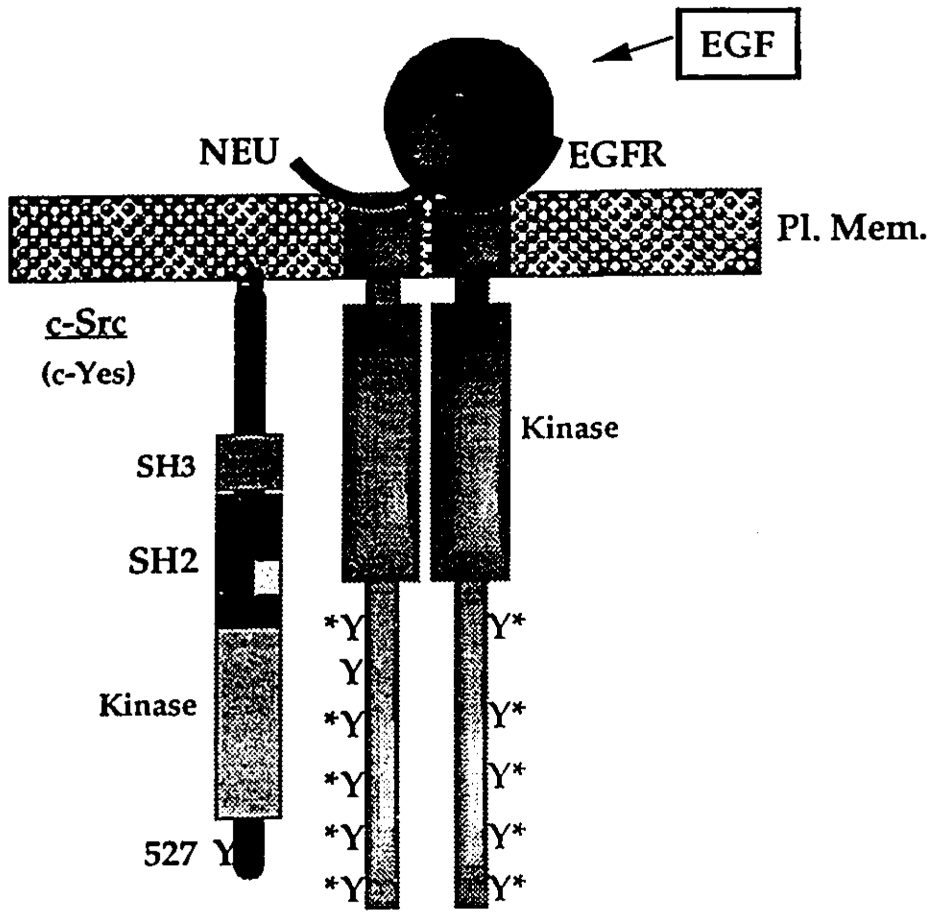

A
IP:
anti-Neu
anti-EGFR
205
Neu
EGFR
116
80
1
2
3
4
5
6
Probe: GSTag-Grb2
B
205
Neu
116
80
1
2
3
4
5
6
Probe: GSTag-c-Yes SH2

Figure 7.2 A model for EGF mediated activation of Src family kinases.


EGF
NEU
EGFR
Pl. Mem.
c-Src
(c-Yes)
Kinase
SH3
SH2
Kinase
527 Y
*Y
Y
*Y
*Y
*Y
*Y
Y*
Y*
Y*
Y*
Y*

cytoplasmic domain have been shown to mimic Neu in its transforming ability (Di Fiore et al., 1987). It will be interesting to determine whether such chimeric fusions facilitate direct binding of c-Src and promote EGF dependent activation of c-Src tyrosine kinase. Future identification of the c-Src binding site on Neu and reconstitution the c-Src binding site into an otherwise normal EGFR molecule will enable us to understand the biological importance of this biochemical observation.

Recent studies have shown that micro-injection of antibodies that recognize Src, Yes and Fyn kinases abolishes EGF and PDGF-induced S phase entry of fibroblasts (Twamley-Stein et al., 1993, Roche et al., 1995). These observations indicate that Src family members are required for PDGF and EGF-induced entry into cell cycle. The importance of elevated c-Src and c-Yes kinase activity in Neu-induced mammary tumors is yet to be determined, however, it is possible that Src family members (c-Src and c-Yes) play an important role in Neu-mediated mammary tumorigenesis. Future interbreeding between MMTV/*neu* transgenics and c-Src or c-Yes deficient mice would allow this question to be addressed.

Although Neu RTK activates tyrosine kinase activity of both c-Src and c-Yes, results presented in chapter 6 demonstrate that c-Src and c-Yes associate with distinct sets of cellular proteins. In particular, the phosphoprotein p89 was found associated with c-Src in mammary epithelial cells transformed by Neu or PyV middle T but was not observed in c-Yes immunoprecipitates. Moreover the association between c-Src and p89 was detected in transformed mammary epithelial cells but not in transformed fibroblasts suggesting that p89 may be a mammary epithelial specific c-Src substrate.

The notion that c-Src and c-Yes may function differently is supported by a number of observations. Expression of MMTV/PyV middle T antigen in the absence of c-Src results in dramatic reduction in induction of mammary tumorigenesis in transgenic mice whereas, expression of MMTV/middle T in the absence of c-Yes has no effect on the ability of middle T to induce mammary tumorigenesis (chapter 1, Guy et al., 1994). It is possible that the c-Src-specific association of p89 is required for the transformation of mammary epithelial cells. However, PyV middle T antigen can transform fibroblast cell lines lacking c-Src (Thomas et al., 1993). Since we did not observe any association between c-Src and p89 in PyV middle T antigen transformed fibroblast cells (Figure 6.10), it is likely that p89 is not required for transformation of fibroblasts and thus activation of either c-Src or c-Yes is sufficient for middle T-mediated transformation in fibroblasts. Interestingly, PyV middle T antigen-mediated transformation of endothelial cells is severely impaired in c-Yes deficient mice while lack of c-Src did not have any effect (Kiefer et al., 1994), again suggesting that c-Src and c-Yes have different roles in mediating cell transformation. Furthermore, mice lacking c-Src develop osteopeterosis due to defective osteoclast resorption while mice lacking functional c-Yes have no overt phenotype (Soriano et al., 1991, Lowe et al., 1993, Stein et al., 1994a) Taken together these observations suggest that these related kinases are not fully redundant and have kinase specific functions.

One possible hypothesis is that PyV middle T antigen, Neu or EGFR/Neu heterodimer activates c-Src tyrosine kinase which in turn, mediates mammary tumorigenesis by involving p89 (Figure 7.3). Although the biochemical nature of p89 is unknown, the association between p89 and c-Src may activate/repress p89 function that plays an important role in mammary tumorigenesis (Figure 7.3). It is possible that the lack of mammary tumor formation in the absence of c-Src is due to the inability of c-Yes to associate and activate/repress p89. However, it is unclear whether p89 is required for c-Src to mediate transformation. Identification and biochemical characterization of p89 will enable us to answer this question.

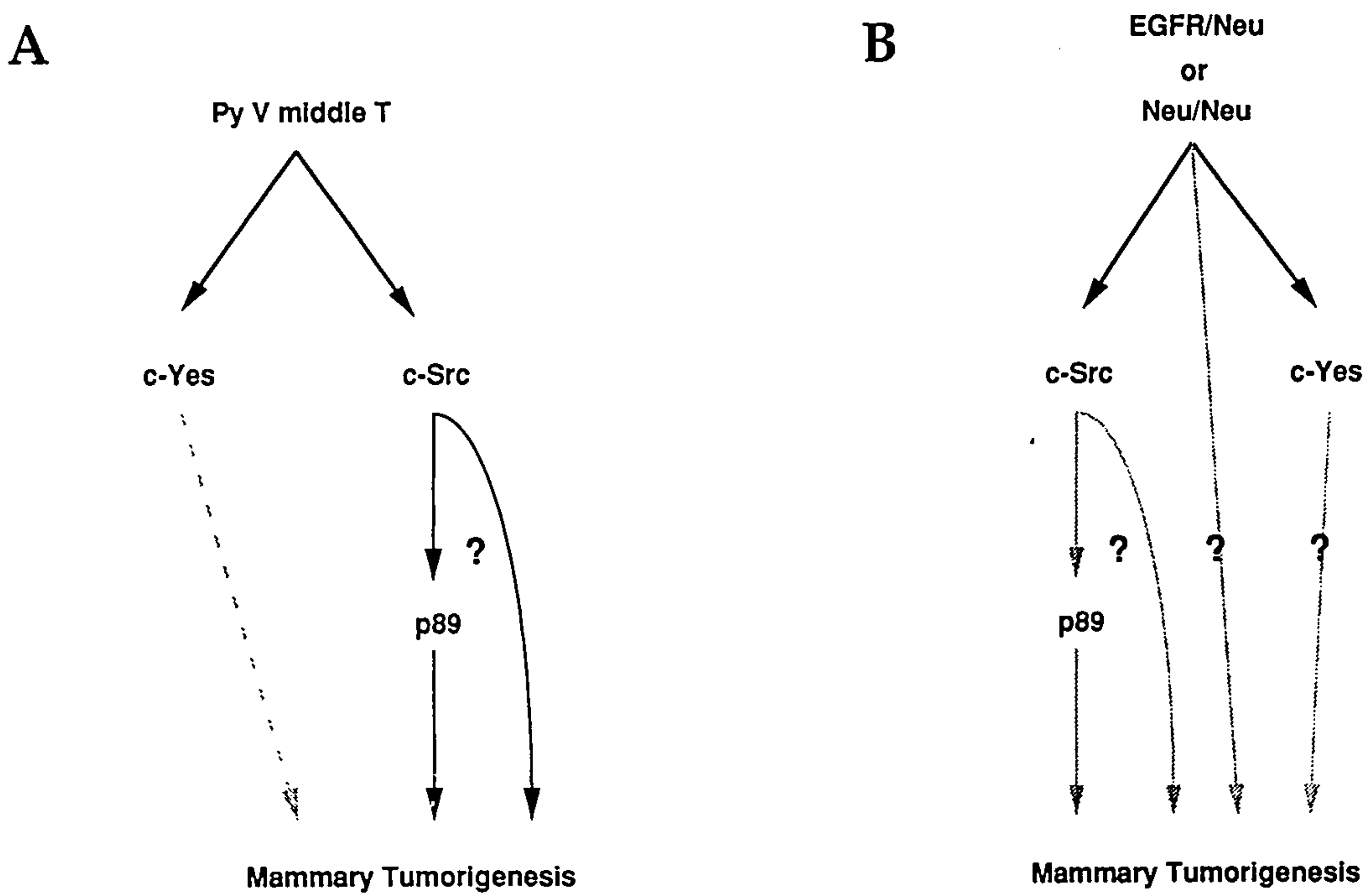
Figure 7.3 A model for c-Src tyrosine kinase in mammary tumorigenesis.

Both PyV middle T antigen **(A)** and Neu **(B)** activate (solid arrow) Src family members. c-Src associates with p89 in both Neu and middle T transformed mammary epithelial cells. It is unclear (indicated by '?') whether p89 is required for c-Src-mediated mammary tumorigenesis.

(A) Activation of c-Yes in the absence of c-Src is not sufficient for complete transformation of the mammary epithelium (indicated by dotted arrow)

(B) The importance of Src family kinases in Neu mediated mammary tumorigenesis is not known (indicated by light grey arrow)

Src family in Neu and middle T-mediated mammary tumorigenesis: A MODEL



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