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Mammary Tumors Expressing the *neu* Proto-oncogene Possess Elevated c-Src Tyrosine Kinase Activity

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Amplification and overexpression of the *neu* (c-*erbB2*) proto-oncogene has been implicated in the pathogenesis of 20 to 30% of human breast cancers. Although the activation of Neu receptor tyrosine kinase appears to be a pivotal step during mammary tumorigenesis, the mechanism by which Neu signals cell proliferation is unclear. Molecules bearing a domain shared by the c-Src proto-oncogene (Src homology 2) are thought to be involved in signal transduction from activated receptor tyrosine kinases such as Neu. To test whether c-Src was implicated in Neu-mediated signal transduction, we measured the activity of the c-Src tyrosine kinase in tissue extracts from either mammary tumors or adjacent mammary epithelium derived from transgenic mice expressing a mouse mammary tumor virus promoter/enhancer/unactivated *neu* fusion gene. The Neu-induced mammary tumors possessed six- to eightfold-higher c-Src kinase activity than the adjacent epithelium. The increase in c-Src tyrosine kinase activity was not due to an increase in the levels of c-Src but rather was a result of the elevation of its specific activity. Moreover, activation of c-Src was correlated with its ability to complex tyrosine-phosphorylated Neu both in vitro and in vivo. Together, these observations suggest that activation of the c-Src tyrosine kinase during mammary tumorigenesis may occur through a direct interaction with activated Neu.

The *neu* (c-*erbB2*) proto-oncogene encodes a growth factor receptor tyrosine kinase that is highly homologous to the epidermal growth factor receptor (2, 8, 18, 26, 28, 40). Oncogenic activation of Neu can occur through multiple molecular mechanisms, including point mutations in the transmembrane domain (3), deletion of the extracellular domain (4), and overexpression (9, 10, 16). Activation of the Neu kinase results in its association with and tyrosine phosphorylation of a number of downstream signalling proteins. Moreover, the activities of phospholipase C-γ1, the GTPase-activating protein (GAP), and protein tyrosine phosphatase 1D are elevated following tyrosine phosphorylation by activated Neu (1, 11, 27, 39).

Elevated expression of Neu has been observed in 20 to 30% of primary breast cancers and has been inversely correlated with the survival of the patient (12, 23, 29, 30). Consistent with these observations, expression of the *neu* proto-oncogene in the mammary glands of transgenic mice results in the development of focal mammary tumors that metastasize with high frequency (15). Tumorigenesis in transgenic mice carrying the mouse mammary tumor virus (MMTV)/unactivated *neu* fusion gene closely correlates with activation of the intrinsic tyrosine kinase activity of Neu (15).

Another potent tyrosine kinase activity implicated in mammary tumorigenesis is that of c-Src. Analyses of primary human breast cancers have revealed that a large proportion of human breast tumors possess elevated c-Src activity (17, 22, 24). In one of these reports, elevation of c-Src activity was attributed to an increase in the specific activity of c-Src (24). Consistent with these observations, activation of c-Src kinase activity in the mammary epithelium of transgenic mice by expression of the polyomavirus (PyV) middle-T antigen results in rapid induction of metastatic mammary tumors (13). Given the potential importance of c-Src in mammary tumorigenesis, we assessed whether c-Src would play a role in Neu-mediated mammary tumorigenesis. To this end, protein extracts from normal or tumor tissues derived from MMTV/unactivated *neu* transgenic mice 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 six- to eightfold higher levels of c-Src kinase activity than did the adjacent mammary epithelium. To test the possibility that activation of c-Src occurs through direct physical association with Neu, a fusion protein containing the c-Src SH2 domain was incubated with cell lysates containing tyrosine-phosphorylated Neu in vitro. The results demonstrated that the c-Src SH2-containing fusion protein could specifically bind to tyrosine-phosphorylated Neu. Moreover, physical complexes between activated Neu and c-Src could be detected in vivo by immunoprecipitation and immunoblot analyses. These observations support the hypothesis that activation of the c-Src kinase may play an important role in Neu-mediated tumorigenesis and metastases.

MATERIALS AND METHODS

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, Princeton, N.J.). The GAP SH2 fusion DNA contains both SH2 domains flanking the SH3 domain (amino acids 181 to 451) and was inserted as an

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*Eco*RI fragment in pGEX 3X (Pharmacia). Construction of the MMTV promoter/enhancer-activated *neu* fusion gene (pMMTV/*neu* NT) has been described previously (21).

Immunoprecipitation and in vitro kinase assays. Tissue samples were ground to a powder under liquid nitrogen and lysed in TNE lysis buffer (50 mM Tris HCl [pH 7.6], 150 mM NaCl, 1% Nonidet P-40, 2 mM EDTA, 1 mM sodium orthovanadate, 2 mM dithiothreitol, 10 μg of leupeptin per ml, 10 μg of aprotinin per ml, 5 μg of *Nα-p*-tosyl-L-lysine chloromethyl ketone [TLCK] per ml, 10 μg of tosylsulfonyl phenylalanyl chloromethyl ketone [TPCK] per ml). The lysates were cleared by centrifugation at 12,000 × *g* for 10 min at 4°C. Immunoprecipitations were performed by incubating 750 μg of the protein lysate with either 300 ng of anti-Src antibody (Ab.1; Oncogene Science) or 300 to 400 ng of anti-Neu antibody (monoclonal antibody [MAb] 7.16.4) for 30 min at 4°C. Following incubation with protein G-Sepharose beads (Pharmacia) on a rotating platform at 4°C for 30 min, the precipitates were washed four times with TNE and used for further analysis. The c-Src immunoprecipitates were washed once with 2× kinase buffer (200 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.0], 10 mM $MnCl_2$) and were resuspended in 9 μl of 2× kinase buffer, 5 to 10 μCi of [γ-^{32}P]ATP (>45,000 Ci/mmol; Amersham), and 10 μg of acid-denatured enolase. After a 5-min incubation at room temperature, the reaction was terminated by adding an equal volume of sodium dodecyl sulfate (SDS)-gel loading buffer (62.5 mM Tris HCl [pH 6.8], 2% SDS, 5% glycerol, 0.7 M 2-mercaptoethanol, 0.25% bromophenol blue). We have previously determined that under these conditions, the rate of transfer of [γ-^{32}P]ATP onto acid-denatured enolase is within the linear range for at least 10 min (21a). The samples were electrophoresed on SDS–10% polyacrylamide gels, and the gels were incubated in 1 M KOH for 30 to 45 min at 45°C, dried, and subjected to autoradiography. The dried gels were also exposed to phosphor screens and quantitated by PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.) analysis.

Stable mammary epithelial cell lines expressing high levels of activated *neu* were derived from mammary tumors of MMTV/activated *neu* transgenic mice (21). The cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed in low-salt 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS) lysis buffer (50 mM Tris [pH 8.0], 0.7% CHAPS, 50 mM NaCl, 1 mM sodium orthovanadate, 10 μg of leupeptin per ml, 10 μg of aprotinin per ml, 5 μg of TLCK per ml, 10 μg of TPCK per ml). The lysates were cleared by centrifugation at 12,000 × *g* for 10 min at 4°C. Immunoprecipitations were done with 4.0 mg of total lysate and 1.2 μg of anti-Src antibody (Ab.1; Oncogene Science) at 4°C for 1.0 h. This was followed by incubation with protein G-Sepharose beads on a rotating platform at 4°C for 1.0 h. The immunoprecipitates were washed five times with lysis buffer containing either 50 or 150 mM NaCl, as specified. The control precipitates (normal rabbit serum [NRS] and p53) were washed with lysis buffer containing 50 mM NaCl. The immunoprecipitates were resuspended in SDS-gel loading buffer, resolved on an SDS–9% polyacrylamide gel, and immunoblotted for Neu as described below.

Immunoblotting. The c-Src immunoprecipitates were resuspended in SDS-gel loading buffer, and the proteins were resolved on an SDS–9% polyacrylamide gel. The proteins were transferred onto a polyvinylidene difluoride membrane (Millipore), using an immunoblot transfer apparatus (Bio-Rad). Following an overnight incubation in 3% skim milk at 4°C, the membrane was incubated with either an anti-Src (1:1,000; Oncogene Science) or anti-Neu (Ab.3; 1:1,000; Oncogene Science) antibody for 3 h. After being washed five times with PBS (140 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4), the membranes were incubated for 1 h with a 1:5,000 dilution of goat anti-mouse immunoglobulin G conjugated with horseradish peroxidase (Bio-Can Scientific). The membranes were washed five to seven times with PBS, and the proteins were visualized by an enhanced chemiluminescence system (Amersham).

Antiphosphotyrosine immunoblotting was performed in a similar fashion with the exception that the membrane was blocked overnight in 3% bovine serum albumin (BSA; Sigma) in Tris-buffered saline (TBS; 20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM KCl) and probed for 2 h with antiphosphotyrosine antibodies (1:500; Upstate Biotechnology, Inc. [UBI]) in 3% BSA in TBS. After being washed in TBS–0.05% Tween 20, the blots were incubated in 3% milk in TBS for 1 h. The membrane was incubated with goat anti-mouse immunoglobulin G, and proteins were visualized by the enhanced chemiluminescence detection system (Amersham).

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 dilutjon buffer (200 mM HEPES [pH 7.0], 10% glycerol, 0.1% Nonidet P-40) and resuspended in 60 μl of dilution buffer. The reaction was initiated by mixing 15 μl of the immunoprecipitate, 5 μl of substrate peptide (1.5 mM stock), and 5 μl of assay buffer (250 mM Tris-Cl [pH 7.0], 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°C for 20 min. The assay was terminated by the addition of 10 μl of glacial acetic acid. The contents were centrifuged at 3,000 rpm for 5 min, and 15 μl of the supernatant was spotted onto 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 NRS immunoprecipitate.

Affinity complex formation with fusion proteins. Rat-2 fibroblast cell lines expressing activated neu under MMTV transcriptional control were established by transfecting pMMTV/*neu* NT (21) into Rat-2 cells. Two representative clones (NT-11 and NT-12) were used for these analyses. To establish whether these cell lines expressed tyrosine-phosphorylated Neu, the cells were plated in either the presence or absence of dexamethasone (final concentration, 10^{-6} M), and the cell lysates were collected in 1.0 ml of phospholipase C lysis buffer (19) 3 days after plating. The lysates were cleared by centrifugation at 12,000 × *g* for 20 min, the samples were immunoprecipitated with Neu-specific MAb 7.16.4 and washed, and the immunoprecipitates were electrophoresed on SDS–9% polyacrylamide gels and immunoblotted with antiphosphotyrosine 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 (described below). After incubation of the samples for 90 min at 4°C on a rotating platform, the affinity-bound complexes were washed four times with HNTG buffer (19) and resuspended in 1× SDS-gel loading buffer. The samples were resolved on an SDS–9% polyacrylamide gel and blotted with anti-Neu antibody Ab.3 (Oncogene Science) as described above.

Competition assay using fusion proteins. *Escherichia coli* cells harboring GST-SH2 fusion proteins were grown, in-

duced with isopropythiogalacto-pyranoside (IPTG), and lysed as described by Smith and Johnson (31). The Sepharose-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 with MTPBS (150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4 [pH 7.3]) and resuspended in phospholipase C lysis buffer. 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 the column was washed four times with 4 to 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 (pH 8.0) containing 5 mM reduced glutathione (BDH). This soluble form of fusion protein will not be able to rebind glutathione-Sepharose beads (31).

Competition assays were performed by preincubating 500 μg of NT-11 or NT-12 cell lysates with 100 μg of soluble GST-SH2 fusion proteins for 90 min at 4°C. After the preincubation step, the cell lysates were incubated with 10 μg of GST-SH2 fusion proteins bound to glutathione-Sepharose beads for 90 min at 4°C as indicated. The beads were washed four times with HNTG buffer and resuspended in 1× SDS-gel loading buffer. The proteins were resolved on an SDS–8% polyacrylamide gel and immunoblotted with Neu-specific antibody Ab.3 (Oncogene Science).

RESULTS

Elevated c-Src tyrosine kinase activity in Neu-expressing mammary tumors. To test the possibility that activation of c-Src is involved in Neu-induced mammary tumorigenesis, we 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 (15). 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 (5). Although attempts were made to exclude overt tumors in the adjacent mammary epithelium, the surrounding epithelium occasionally exhibited histological features of hyperplasia and dysplasia (15). Protein extracts derived from both normal and tumor tissues were immunoprecipitated with Src-specific MAbs and subjected to in vitro kinase assays using acid-denatured enolase as a substrate. As shown in Fig. 1A, all tumors examined in this fashion exhibited elevated levels of c-Src tyrosine kinase activity by comparison with the matched adjacent mammary epithelium. 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 1).

The c-Src tyrosine kinase activity within the Neu-induced mammary tumors was also measured by the capacity of c-Src immunoprecipitates from these tissues to phosphorylate a modified cdc2 peptide (Lys-19–cdc-2[6–20]–NH_2) in vitro. 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 epidermal growth factor receptor (6). 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 1). Incubation of these c-Src immunoprecipitates with a control substrate peptide resulted in reduction of peptide phosphorylation to background levels (data not shown). To exclude the possibility that the elevated tumor-specific c-Src kinase activity was restricted to one MMTV/unactivated *neu* transgenic line, four matched samples from two other lines (N#721 and N#732) (15) were examined by in vitro kinase analysis. These mammary tumors also exhibited a comparable increase c-Src kinase activity (data not shown).

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 (Fig. 1B). Therefore, the elevation of c-Src tyrosine kinase activity observed in the mammary tumors

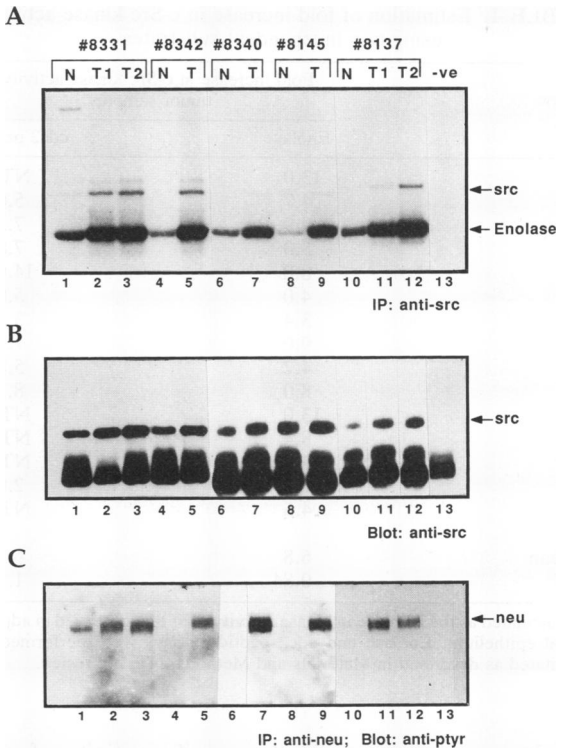

FIG. 1. Elevated c-Src kinase activity in Neu-induced tumorigenesis. (A) Tissue lysates derived from mammary tumors (T) or adjacent epithelium (N) were immunoprecipitated (IP) with c-Src-specific antibody Ab.1 (Oncogene Science), and 50% of the immunoprecipitate was subjected to in vitro kinase assays using acid-denatured enolase as an external substrate. The SDS-polyacrylamide gels were alkali treated and dried before exposure to X-ray film. NRS was used as a negative control (-ve). Arrows indicate positions 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 an anti-Src antibody. The position of the c-Src protein is indicated by an arrow. The lower broad band is cross-reactive immunoglobulin species. (C) Immunoprecipitation and immunoblot analyses of tissue extracts derived from tumor and adjacent epithelium with anti-Neu (MAb 7.16.4) and antiphosphotyrosine (anti-ptyr; UBI) antibodies. The position of the tyrosine-phosphorylated Neu protein is indicated by an arrow.

TABLE 1. Estimation of fold increase in c-Src kinase activity, using two independent substrates

Mouse no.	Fold increase in c-Src kinase activity in tumor samples[a]	
	Enolase	cdc2 peptide
288	13.0	NT
8137	4.7	5.0
8137	9.9	7.5
8142	5.0	7.8
8145	8.0	14.0
8331	4.0	5.0
8331	3.4	3.5
8334	9.0	7.4
8340	4.2	5.8
8342	8.0	8.5
8364	13.0	NT
8364	8.4	NT
8564	13.0	NT
8565	2.1	2.0
8567	4.5	NT
Mean	6.8	6.7
SE	0.84	1.1

[a] 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 in Materials and Methods. NT, not tested.

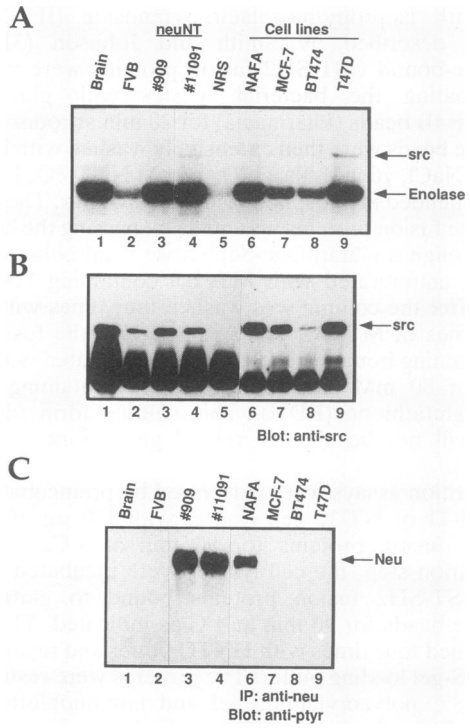

FIG. 2. Mammary tumors and derived tumor cell lines expressing activated Neu possess elevated c-Src kinase activity. (A) Protein extracts from mammary tumors derived from the MMTV/activated *neu* (21) mice or several mammary tumor cells lines were immunoprecipitated (IP) with Src-specific antibody Ab.1 (Oncogene Science) and subjected to in vitro kinase assays with exogenous enolase substrate. Also included are tissue extract derived from brain (positive control; lane 1) and tissue extract derived from normal Friend leukemia virus strain B (FVB) mammary tissue (negative control; lane 2). The tumor tissue was also incubated with nonspecific NRS (lane 5). The locations of c-Src and enolase substrate are indicated by arrows. (B) Immunoblot analyses of c-Src immunoprecipitates with Src-specific antibody Ab.1 (Oncogene Science). The location of c-Src is indicated by the arrow. (C) The same protein extracts were incubated with Neu-specific MAb 7.16.4, and the immune complexes were resolved through an SDS–9% polyacrylamide gel and blotted with phosphotyrosine-specific antibodies (UBI). The position of tyrosine-phosphorylated Neu is indicated by the arrow.

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 (15). 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 antiphosphotyrosine 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 Neu kinase activity correlated well with the activation of c-Src (Fig. 1C). The one sample of adjacent mammary tissue in which tyrosine-phosphorylated Neu could be detected (Fig. 1C, lane 1) also possessed elevated c-Src kinase activity (Fig. 1A, lane 1). Conceivably, the presence of kinase-active Neu in this tissue reflects 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 (Fig. 2A, lanes 3 and 4) (21) and in several mammary tumor-derived cell lines (lanes 6 to 9). By comparison with nontransgenic mammary epithelium (lane 2), the activated Neu-induced tumors (lanes 3 and 4) and two mammary tumor cell lines (lanes 6 and 9) possessed elevated c-Src activity. With the exception of the BT474 cell line, which expressed low levels of c-Src, immunoblot analyses revealed comparable levels of c-Src among the tissues and cell lines (Fig. 2B). Thus, as with 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 whether the increase of c-Src activity correlated with activation of Neu kinase, these extracts were immunoprecipitated with Neu-specific MAb 7.16.4 and then subjected to immunoblot analyses with antiphosphotyrosine antibodies (Fig. 2C). In both the activated Neu-induced tumors and a derived tumor cell line (NAFA), high levels of tyrosine-phosphorylated Neu were detected (lanes 3 to 6). 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 indicate that activation of Neu kinase activity is correlated with induction of c-Src activity. However, one mammary tumor cell line (T47D) 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.

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

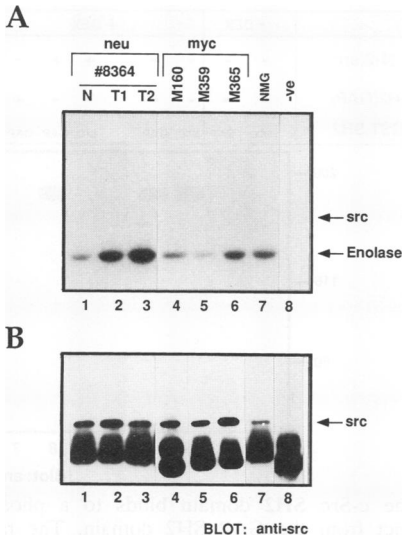

FIG. 3. Differential activation of c-Src in Neu- and c-Myc-expressing mammary tumors. (A) The tumor lysates were incubated with anti-Src antibody Ab.1 (Oncogene Science), and the immune complexes were purified and subjected to an in vitro kinase assay using acid-denatured enolase. The SDS-polyacrylamide gel was treated with KOH before autoradiography. N and T represents normal and tumor epithelium, respectively, from a *neu* transgenic mouse (lanes 1 to 3). M160, M359, and M365 were tumors from c-*myc* transgenic mice (lanes 4 to 6). Lane NMG, normal mammary epithelium from a nontransgenic mouse; lane -ve, NRS control. The position of c-Src as observed on longer exposure of the autoradiogram is indicated. (B) Immunoblot analyses of the same immunoprecipitated tissue samples with Src-specific antibodies. The broad lower band is due to cross-reaction with the c-Src antibody in the samples.

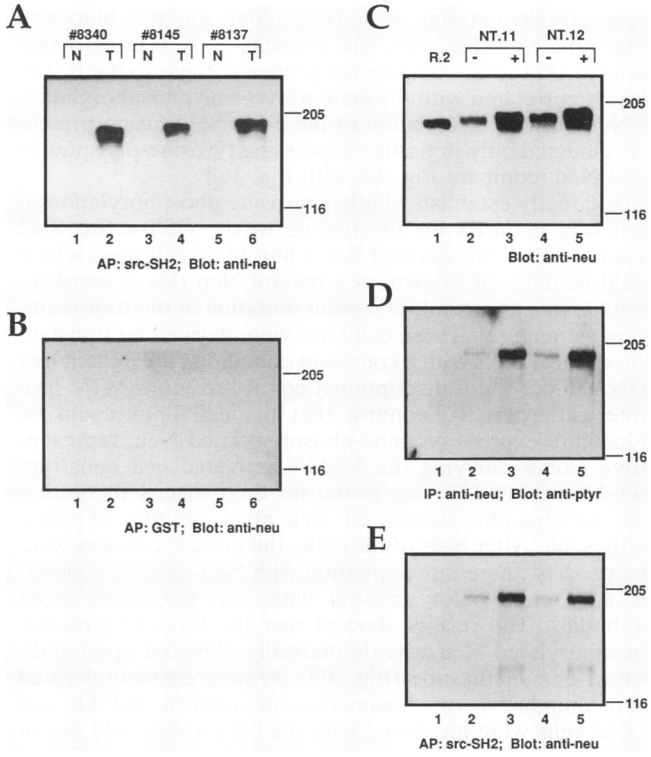

FIG. 4. The c-Src SH2 domain binds to tyrosine-phosphorylated Neu. (A) Tissue extracts from tumor (T) or adjacent epithelium (N) were incubated with Sepharose-bound GST–c-Src SH2 fusion protein. The bound material was eluted and immunoblotted with anti-Neu Ab.3 (Oncogene Science). The position of Neu is indicated with an arrow. AP, affinity purification. (B) The same protein extracts were passed through a GST column and treated as described above. (C) Stably transformed Rat-2 cell lines (NT-11 and NT-12) expressing activated *neu* under the control of the MMTV promoter/enhancer were derived. Two representative clones were plated in either the presence (+) or absence (−) of dexamethasone. Total protein lysates were resolved and blotted with anti-Neu antibody Ab.3 (Oncogene Science). (D) Identical lysates were incubated with anti-Neu MAb 7.16.4, and the immune complexes were resolved through an SDS–9% polyacrylamide gel and blotted for antiphosphotyrosine (anti-ptyr; UBI). IP, immunoprecipitation. (E) Similar protein extracts were incubated with a Sepharose-bound GST–c-Src SH2 fusion protein and were affinity purified as described in Materials and Methods. The purified complexes were separated through an SDS–9% polyacrylamide gel and blotted for anti-Neu (Ab.3; Oncogene Science).

If this hypothesis is correct, then mammary tumors induced by other oncogenes which function downstream of c-Src should not possess elevated c-Src activity. To test this possibility, we extended the in vitro kinase analyses to mammary tumors induced by the c-*myc* oncogene (34). Transgenic mice carrying the MMTV/c-*myc* fusion gene (TG.M) develop focal mammary tumors that arise next to normal transgene-expressing mammary epithelium (34). 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 significantly lower than those in the Neu-induced mammary tumors (Fig. 3A; compare lanes 2 and 3 and lanes 4 to 6). Quantitative measurement revealed that the Neu-induced mammary tumors possessed 4- to 7.5-fold-higher levels of c-Src kinase activity compared with c-Myc-induced mammary tumors (data not shown). 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 (Fig. 3A, lane 7). 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 in Neu- and c-Myc-expressing tumors (Fig. 3B; compare lanes 2 and 3 with lanes 4 to 6). Thus, activation of c-Src kinase may be required for transformation by the *neu* oncogene but is dispensable for tumorigenesis mediated by the c-*myc* proto-oncogene.

The SH2 domains of c-Src specifically associate with tyrosine-phosphorylated Neu in vitro and in vivo. Because the increased tyrosine kinase activity of c-Src in Neu-induced mammary tumors correlated with elevated Neu tyrosine kinase activity, we were interested in establishing whether c-Src could directly associate with the tyrosine-phosphorylated Neu receptor. To test this possibility, Neu-containing protein extracts derived from tumors and adjacent epithelium were examined for the capacity to bind Sepharose beads bearing a GST–c-Src SH2 fusion protein. The bound material was eluted, and the presence of Neu was detected by immunoblot analysis. As shown in Fig. 4A, 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 protein extracts with GST alone did not bind any detectable Neu (Fig. 4B). The capacity of Neu to associate with the 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 detected only in tissues expressing tyrosine-phosphorylated Neu (compare Fig. 4A with Fig. 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 as a result of a point mutation in the transmembrane domain (3). 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 and NT-12) were tested for the capacity to synthesize tyrosine-phosphorylated Neu after addition of dexamethasone. After lysis of the cells, the protein extracts were analyzed by immunoprecipitation with Neu-specific antibodies and immunoblot analysis with anti phosphotyrosine antibodies. The results showed that the level of tyrosine-phosphorylated Neu was dramatically elevated upon addition of dexamethasone (Fig. 4D). Protein extracts derived from uninduced or dexamethasone-induced NT-11 and NT-12 cells were incubated with the GST–c-Src SH2 fusion protein immobilized on Sepharose beads, and 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 (Fig. 4E). 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 (Fig. 4C).

We were interested in learning whether the phosphorylated tyrosine residue on Neu to which the Src SH2 domain bound was also used by other SH2-containing molecules involved proliferative signal transduction. Because GAP has been implicated as an important component of the Neu signal transduction pathway (11), we tested the capacity of a GST fusion protein carrying both GAP SH2 domains to bind to cell extracts from uninduced (Fig. 5, lane 2) and induced (lane 4) NT-11 Rat-2 cells. Consistent with the observations made with the c-Src SH2-containing GST fusion protein (Fig. 5, lanes 1 and 3), the GAP SH2-containing fusion protein associated with Neu from only the induced extracts. To examine whether the binding sites for the c-Src and GAP SH2 domains were distinct, we tested whether preincubation of a soluble c-Src SH2-containing protein (see Materials and Methods) with the protein extracts from the induced cells could prevent binding of Neu to a column containing either a Sepharose-bound GST-GAP SH2 fusion protein or a Sepharose-bound GST-Src SH2 protein. 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 fusion protein (lane 5) but did not interfere with the binding of Neu to a Sepharose-bound GST-GAP SH2 protein (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 Sepharose-bound GST-GAP SH2 fusion protein (lane 7) but had little effect on the capacity of

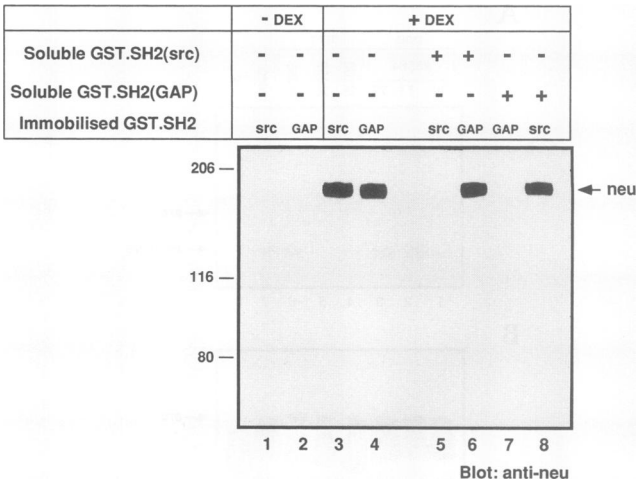

FIG. 5. The c-Src SH2 domain binds to a phosphotyrosine residue distinct from the GAP SH2 domain. The rat cell lines carrying the MMTV/activated *neu* fusion gene were grown in either the presence (+) or absence (−) (DEX) of dexamethasone. Cell lysates were preincubated in the presence (+) or absence (−) of either soluble GST-SH2 (Src) (lanes 5 and 6) or soluble GST-SH2 (GAP) (lanes 7 and 8). Following affinity purification using GST-SH2 (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 an SDS–8% polyacrylamide gel and immunoblotted for Neu (Ab.3; Oncogene Science). The position of Neu is indicated by the arrow.

Neu to bind the immobilized GST–c-Src SH2 fusion protein (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.

To confirm that the physical association of c-Src occurs in vivo, cell lysates derived from a mammary tumor cell line established from the MMTV/activated *neu* mice (NAFA [21]) were immunoprecipitated with Src-specific antibodies and immunoblotted with Neu-specific antibodies (Fig. 6A, lanes 3 and 4). This cell line has previously been shown to express high levels of activated Neu and possess elevated c-Src activity (Fig. 2). As shown in Fig. 6A, Neu could be detected in the c-Src immunoprecipitates (lane 3). This association could be disrupted by washing the immunoprecipitates with high-salt buffer (150 mM NaCl; lane 4). By contrast, immunoprecipitations with control antibodies (lane 1), NRS, or a nonspecific mouse MAb (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 and 60 kDa that comigrated with Neu and c-Src (Fig. 6B, lane 2). Together with the in vitro data, the observations provide compelling evidence that activation of c-Src in these cells occurs through its direct physical association with activated Neu.

DISCUSSION

Our results show that activation of c-Src is a frequent event in Neu-induced mammary tumors in transgenic mice. We also present evidence that activation of c-Src may occur through direct interaction of its SH2 domain with tyrosine-phosphorylated Neu. Together, these observations support

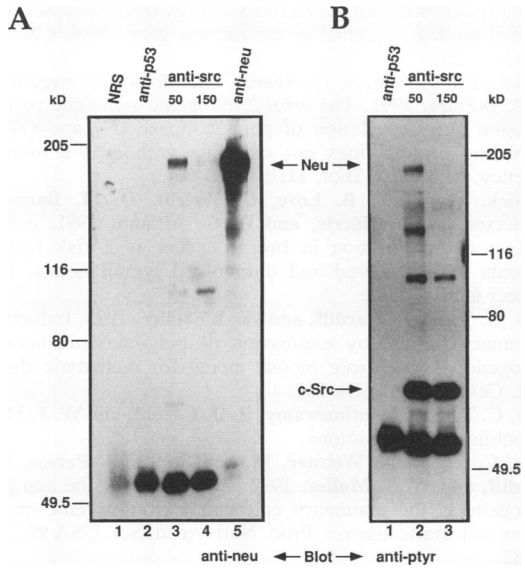

FIG. 6. c-Src physically associates with activated Neu in vivo. (A) Protein extracts derived from the NAFA mammary tumor cell line (21) (Fig. 2) were immunoprecipitated with Src-specific antibodies, and the immunoprecipitates were washed five times in lysis buffer containing either 50 (lane 3) or 150 (lane 4) mM NaCl. The immune complexes were resolved through an SDS–9% polyacrylamide gel and immunoblotted with Neu-specific antibody Ab.3 (Oncogene Science). The extracts were also immunoprecipitated with either NRS (lane 1) or a nonspecific mouse MAb (anti-p53; lane 2) as a negative control. An anti-Neu (MAb 7.16.4) immunoprecipitate served as a positive control (lane 5). (B) The identical immunoprecipitates were blotted with antiphosphotyrosine (anti-ptyr; UBI) antibodies. The positions of c-Src and Neu are indicated by arrows.

the contention that Neu may signal cell proliferation through activation of c-Src.

The dramatic elevation of the c-Src kinase activity in mammary tumors of MMTV/unactivated *neu* transgenic mice is likely due to the increase in the specific activity of c-Src because the amounts of c-Src protein present in the tumor and the adjacent epithelium were approximately the same. 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 (17, 22, 24). However, it is unclear in these studies whether elevated c-Src activity was accompanied by increased Neu tyrosine kinase activity. In this regard, we have recently examined four Neu-expressing human breast tumors 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 with adjacent mammary tissue (21b). 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.

The enhanced activity of c-Src observed in both Neu-induced human and Neu-induced murine mammary tumors is closely associated with tumor progression. For example, amplification and overexpression of Neu in human breast cancer appears to be inversely correlated with the survival of the patient (12, 23, 29). Moreover, mammary gland-specific expression of Neu in transgenic mice results in a high incidence of metastatic disease (15). Further support for the notion that activation of c-Src in the mammary epithelium is an important step in tumorigenesis derives from observations made with MMTV/PyV middle-T transgenic mice (13). Mammary gland-specific expression of PyV middle-T antigen results in the activation of the c-Src tyrosine kinase pathway in this tissue and leads to the appearance of multifocal mammary tumors which metastasize to the lung with high frequency. We have recently crossed the MMTV/PyV middle-T mice with mice containing a germ line disruption of c-*src* gene (33) to assess whether activation of c-Src is required for PyV middle-T-antigen-induced tumorigenesis. By contrast to the rapid tumor progression observed in parental MMTV/PyV middle-T strains, mice expressing the middle-T oncogene in the absence of functional c-Src rarely developed mammary tumors (14). These observations suggest that the function of c-Src is required for PyV middle-T-antigen-induced mammary tumorigenesis and further suggest that the mammary epithelium is particularly sensitive to the activation of signal transduction pathways involving c-Src.

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 (37, 38). 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 Neu-expressing 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 a specific phosphotyrosine residue(s) within the activated Neu receptor, suggesting that direct physical interaction between activated Neu and c-Src may alter the latter's intrinsic tyrosine kinase activity. Consistent with this hypothesis, both the platelet-derived growth factor (PDGF) and the colony stimulating factor 1 (CSF-1) receptor tyrosine kinases are known to associate with (20) and to activate members of the Src family kinases (7). Interestingly, cells that express a mutant CSF-1 receptor that is incapable of activating the c-Src pathway are nonresponsive to CSF-1 mitogenic stimulation (7). However, elevated expression of c-*myc* can restore CSF-1 responsiveness (25). It is conceivable that overexpression of c-*myc* in the mammary tumors derived from the MMTV/c-*myc* mice obviates the requirement for elevated c-Src activity in a similar fashion.

The association of SH2 domain of c-Src with tyrosine-phosphorylated Neu requires specific phosphotyrosine residues within Neu because preincubation with a soluble GAP SH2-containing fusion protein did not interfere with Neu's capacity to bind to a Sepharose-bound GST–c-Src SH2 protein (Fig. 5). These observations are consistent with the view that SH2 domains recognize specific tyrosine phos-

phopeptide sequences within autophosphorylated receptors (32). Conceivably, the elevation of c-Src activity observed in the Neu-induced mammary tumors is due to specific association of c-Src with Neu. Consistent with this view is the observation that tyrosine-phosphorylated Neu can be detected in c-Src immunoprecipitates derived from Neu-transformed mammary tumor cells (Fig. 6).

While it is unclear whether activation of c-Src is required for Neu-induced tumorigenesis, the importance of this interaction in PDGF receptor (PDGFR) signalling has recently been demonstrated by the observation that microinjection of either c-Src-specific antibodies or dominant negative mutants of c-Src can ablate PDGFR-mediated mitogenesis (36). Indeed, association of Src family members with the PDGFR results in phosphorylation of Src family members and correlates with their enzymatic activation (35). Although the activation of c-Src in Neu-induced mammary tumorigenesis might involve a similar process, the precise mechanism of action and its site of interaction on Neu remain to be defined.

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