Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 83–87, January 1994
Cell Biology

Involvement of pp60$^{c\text{-}src}$ with two major signaling pathways in human breast cancer

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Communicated by Raymond L. Erikson, September 23, 1993 (received for review June 28, 1993)

ABSTRACT **The phosphotyrosine residues of receptor tyrosine kinases serve as unique binding sites for proteins involved in intracellular signaling, which contain SRC homology 2 (SH2) domains. Since overexpression or activation of the pp60$^{c\text{-}src}$ kinase has been reported in a number of human tumors, including primary human breast carcinomas, we examined the interactions of the SH2 and SH3 domains of human SRC with target proteins in human carcinoma cell lines. Glutathione *S*-transferase fusion proteins containing either the SH2, SH3, or the entire SH3/SH2 region of human SRC were used to affinity purify tyrosine-phosphorylated proteins from human breast carcinoma cell lines. We show here that in human breast carcinoma cell lines, the SRC SH2 domain binds to activated epidermal growth factor receptor (EGFR) and p185$^{HER2/neu}$. SRC SH2 binding to EGFR was also observed in a nontumorigenic cell line after hormone stimulation. Endogenous pp60$^{c\text{-}src}$ was found to tightly associate with tyrosine-phosphorylated EGFR. Association of the SRC SH2 with the EGFR was blocked by tyrosyl phosphopeptides containing the sequences surrounding tyrosine-530, the regulatory site in the SRC C terminus, or sequences surrounding the major sites of autophosphorylation in the EGFR. These results raise the possibility that association of pp60$^{c\text{-}src}$ with these receptor tyrosine kinases is an integral part of the signaling events mediated by these receptors and may contribute to malignant transformation.**

Signaling mediated by receptor tyrosine kinases, such as the epidermal growth factor receptor (EGFR), requires receptor autophosphorylation on tyrosine (1). These phosphotyrosine residues serve as unique binding sites for proteins that contain SRC homology 2 (SH2) domains. This protein motif recognizes phosphotyrosine in a sequence-specific manner, and the *in vivo* specificity of proteins for their cognate phosphotyrosine residue in the receptor is maintained in *in vitro* binding assays using the isolated SH2 domains of these proteins. Such domains are found in a number of proteins involved in intracellular signaling including p85, the noncatalytic subunit of phosphatidylinositol 3-kinase; GAP, the GTPase-activating protein of p21^{ras}; and phospholipase Cγ (2). Constitutive activation of these signaling pathways is apparent in many malignancies. Human breast cancers often overexpress two closely related receptor tyrosine kinases, EGFR or p185$^{HER2/neu}$, and amplification of these genes is correlated with poor clinical prognosis (3–5). It has recently been reported that many primary human breast tumors also show elevated activity of pp60$^{c\text{-}src}$ (6), suggesting that this protein may play a role in carcinoma of the breast. These results indicate that activation of downstream events mediated by both receptor and nonreceptor tyrosine kinases may be critical in some types of human neoplasia. Evidence for similar interactions between receptor and nonreceptor tyrosine kinases occurs in the response of normal cells to EGF. Cells that overexpress pp60$^{c\text{-}src}$ are phenotypically normal but hyperrespond to EGF as a mitogen (7). This enhanced mitogenic response is observed only if the SRC protein is membrane associated, enzymatically active, and contains an intact SH2 domain (8). We show here that the SH2 domain of the SRC protein binds both activated EGFR and p185$^{HER2/neu}$ from human breast carcinoma cell lines. In addition, endogenous pp60$^{c\text{-}src}$ tightly associates with tyrosine-phosphorylated EGFR. These results raise the possibility that pp60$^{c\text{-}src}$ may contribute to malignant transformation of the breast.

MATERIALS AND METHODS

Cell Culture and Hormone Stimulation. MDA-MB-468 and MDA-MB-231 (American Type Culture Collection) cell lines were grown in Leibovitz's L-15 medium (GIBCO) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified air atmosphere. SKBR3 (American Type Culture Collection) cells were grown in McCoy's 5A medium supplemented with 15% FBS at 37°C in 10% CO_2/90% air. The AGO3204 cell strain (kindly provided by T. Tlsty, University of North Carolina) was maintained in α-MEM (GIBCO) with Earle's salts supplemented with 10% FBS at 37°C in 5% CO_2/95% air. Cell lysates were prepared from subconfluent, logarithmic-phase cultures in ice-cold RIPA buffer [150 mM NaCl/50 mM Tris·HCl, pH 7.5/0.25% deoxycholate/0.1% Nonidet P-40/1 mM Na_3VO_4/1 mM phenylmethylsulfonyl fluoride (PMSF)/1 mM NaF/10 μg of aprotinin per ml/10 μg of pepstatin A per ml/50 μg of leupeptin per ml] and stored at −70°C. EGF stimulations were done on confluent monolayers that had been serum starved in medium supplemented with 0.1% bovine serum albumin (BSA) (Boehringer Mannheim) instead of FBS. Cultures were stimulated for the indicated time with 30 ng of EGF per ml (Collaborative Research) in serum-free medium and then lysed in ice-cold RIPA buffer.

Cloning and Expression of Recombinant Proteins. We used the pGEX bacterial expression vector to create fusion proteins that contained either the human SRC SH2 domain (amino acids 144–249), SRC homology 3 (SH3) domain (amino acids 87–143) or SH3/SH2 domains (amino acids 87–249), and the pGEX glutathione *S*-transferase (GST) protein. PCR amplimers were used to introduce restriction sites on either side of the human SRC coding sequence (kindly provided by D. Fujita, University of Calgary). The subcloned domains were gel purified and cloned into the pGEX-3X vector (Pharmacia) and transfected into XL1-Blue competent cells (Stratagene). The sequence of positive

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Abbreviations: EGFR, epidermal growth factor receptor; FBS, fetal bovine serum; BSA, bovine serum albumin; GST, glutathione *S*-transferase; EGFR tk, EGFR tyrosine kinase domain; mAb, monoclonal antibody; PDGFR, platelet-derived growth factor receptor.

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clones was verified by sequencing both strands with a ΔTaquence sequencing kit (United States Biochemical).

The recombinant EGFR tyrosine kinase domain (EGFR tk) purified from Sf9 cells as described (9) was a gift from Glaxo Group Research.

Purification of GST Fusion Proteins. Cultures of *Escherichia coli* expressing the pGex fusion proteins were induced with isopropyl β-D-thiogalactopyranoside for 3 hr as described in the manufacturer's protocol. Aliquots containing 50 ml of induced bacteria were centrifuged at 4000 × *g* at 4°C and the resulting pellets were stored at −70°C. The pellets were thawed and resuspended in TBS (150 mM NaCl/10 mM Tris·HCl, pH 7.5) containing detergent and protease inhibitors (1% Nonidet P-40/1 mM PMSF/2 mM EGTA/1 mM NaF/10 μg of aprotinin per ml/50 μg of leupeptin per ml). The samples were sonicated with three 10-sec bursts and clarified by centrifugation at 19,000 × *g* for 15 min at 4°C. Clarified lysates were incubated for 3 hr at 4°C with 4 ml of a 1:1 slurry of glutathione-Sepharose (Pharmacia) that had been washed three times and resuspended in TBS. The fusion proteins bound to glutathione-Sepharose were washed three times in TBS and resuspended to a 1:1 slurry in TBS containing 0.5% BSA.

Affinity Purification of Phosphotyrosyl Proteins from Cell Lysates. Cell lysates were thawed on ice, sonicated briefly, and clarified by centrifugation at 12,000 × *g* at 4°C for 10 min. The clarified lysate was adjusted to 5 mM dithiothreitol (DTT) and incubated with 50 μl of GST-Sepharose for 30 min at 4°C with gentle agitation. The precleared lysates were then incubated with 100 μl of immobilized bacterial fusion protein for 3 hr with gentle agitation at 4°C. The complexes were washed once with RIPA, once with RIPA adjusted to 0.1% SDS, and once with phosphate-buffered saline and analyzed by immunoblotting with anti-phosphotyrosine antibody as described below.

Immunoprecipitation and Western Immunoblots. Immunoblots with anti-receptor antisera were performed as described (10), using 10 ml of block buffer (10 mM Tris·HCl, pH 7.2/150 mM NaCl/0.05% Nonidet P-40/0.05% Tween-20/4% BSA) containing either 5 μl of polyclonal anti-EGFR (Upstate Biotechnology, Lake Placid, NY) and 4 μl of rabbit anti-sheep antiserum (Sigma) or 10 μl of anti-NEU2 antiserum (Oncogene Science; cAb-3). Bound EGFR was detected with block buffer containing 1 μCi of ^{125}I-labeled protein A per ml (1 Ci = 37 GBq) (Amersham), while bound anti-NEU2 antibody was detected with 1 μCi of ^{125}I-labeled sheep anti-mouse antiserum per ml (Amersham). Immunoprecipitations using anti-receptor antibodies were done with precleared cell lysates as described above. Lysates were incubated with either 10 μl of anti-EGFR antibody and 50 μl of protein G-Sepharose (Oncogene Science), 10 μl of anti-NEU1 (Oncogene Science; cAb-1) and 50 μl of protein A-Sepharose (Pharmacia), or 10 μl of anti-NEU2 and 50 μl of goat anti-mouse IgG coupled to Sepharose (Sigma). Immune complexes were incubated and washed as described above. Immunoblots were stripped by incubation in 62.5 mM Tris·HCl, pH 6.8/2% (wt/vol) SDS/100 mM 2-mercaptoethanol for 30 min at 70°C with constant agitation. The stripped blots were washed three times with rinse buffer, reblocked, and immunoblotted as described above. Enzyme-linked chemiluminescence detection was performed per the manufacturer's protocol (ECL; Amersham). The 13-amino acid peptides used in blocking experiments correspond to the C-terminal sequence of human SRC. Solid-phase synthesis and purification of the peptides were done according to the method described by Andrews *et al.* (10).

ELISA. EGFR tk was phosphorylated in buffer containing 10 mM Pipes (pH 7.2), 5 mM $MnCl_2$, 1 mM ATP, and 1 mM Na_3VO_4 for 20 min at 37°C. The reaction was stopped by the addition of EDTA to 10 mM. Phosphorylated EGFR tk diluted to a concentration of 40 μg/ml in TBS containing 1 mM Na_3VO_4 was used to coat 96-well plates (Nunc Maxisorp) at a vol of 50 μl per well. The plates were incubated for 1 hr at room temperature, washed three times with TBS, and blocked overnight at 4°C in blocking buffer [3% (wt/vol) BSA in TBS] containing 1 mM Na_3VO_4. Plates were washed an additional three times in TBS before use. Peptides corresponding to tyrosine residues in the EGFR C terminus were obtained from Bachem. Solid-phase synthesis and purification of Ac-Y*EEIE (where Y* is phosphotyrosine) were done according to the method described by Andrews *et al.* (10). Peptides (10 nM to 1 mM) were diluted in blocking buffer containing 1 mM Na_3VO_4 and 5 mM DTT and incubated with GST–SH3/SH2 fusion protein (6 μg/ml) for 10 min at room temperature. After incubation, 50 μl of the fusion protein solution was added to each well of EGFR tk-coated plates and incubated at room temperature for 1 hr with agitation. The plates were washed three times with TBS and incubated with a 1:2000 dilution of monoclonal antibody (mAb) 327 in blocking buffer at room temperature for 1 hr with agitation. The plates were washed three times with TBS and incubated with a 1:4000 dilution of alkaline phosphatase-linked anti-mouse IgG (Fc specific; Jackson ImmunoResearch) for 1 hr at room temperature with agitation, washed three times in TBS and once in AP buffer (100 mM Tris·HCl, pH 9.5/100 mM NaCl/5 mM $MnCl_2$), and incubated with 1 mg of *p*-nitrophenyl phosphate per ml in AP buffer for 20 min at room temperature. The extent of colorimetric reaction was measured on a Molecular Devices UVmax plate reader at 405 nm.

RESULTS

Detection of EGFR in Human Cell Lysates. To determine whether the SRC SH2 domain recognized human EGFR, we used two human breast carcinoma lines that overexpress EGFR, MDA-MB-468 and MDA-MB-231, and AGO3204, a nontumorigenic, simian virus 40-infected human lung fibroblast cell strain (11, 12). As shown in Fig. 1*A*, GST–SH2, but not GST alone, was able to precipitate phosphotyrosine-containing proteins from EGF-stimulated cells, including a prominent band of 170 kDa that comigrated with immunoprecipitated human EGFR (Fig. 1*A*). To verify that this 170-kDa protein was the EGFR, proteins precipitated from EGF-stimulated MDA-MB-468 cells by either GST or the GST fusion proteins were immunoblotted with either phosphotyrosine- or EGFR-specific antiserum (Fig. 1*B*). Only those constructs containing the SH2 domain were able to precipitate the tyrosine-phosphorylated 170-kDa band. Immunoblotting duplicate samples with antiserum specific for human EGFR confirmed that the 170-kDa protein was the EGFR (Fig. 1*B*). To estimate the amount of EGFR associated with the SH2 domain, the receptor was precipitated from lysates using either the GST–SH2 fusion protein or a polyclonal EGFR antibody and then immunoblotted with EGFR-specific antiserum. We compared the relative band intensity of the immunoblotted EGFR precipitated by a polyclonal anti-EGFR antibody with that precipitated by the GST–SH2 fusion protein. As shown in Fig. 1*B*, the GST–SH2 construct precipitated 10–30% of the amount of receptor brought down by the polyclonal anti-EGFR antibody. However, the EGFR precipitated by the GST–SH2 construct was highly tyrosine phosphorylated; the difference in the phosphotyrosine signal between the EGFR precipitated by the polyclonal antibody relative to that precipitated by the GST–SH2 protein was <2-fold. This may reflect the association of highly phosphorylated receptor with the SRC SH2 or protection of phosphotyrosine residues on the receptor from phosphatases present in the cell lysates by the SH2 domain.

In both the AGO3204 and breast carcinomal cell lysates, the SRC SH2 domain precipitated a tyrosyl phosphorylated

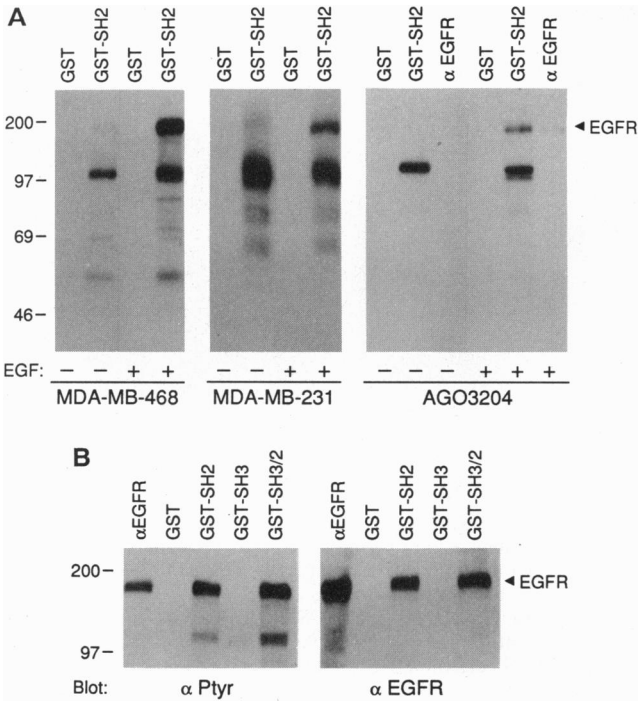

FIG. 1. SH2 domain of pp60$^{c\text{-}src}$ associates with activated EGFR in human cell lines. (*A*) GST or the indicated GST–SRC fusion protein immobilized on glutathione-Sepharose was incubated with 1 mg of protein from lysates of MDA-MB-468, MDA-MB-231, or AGO3204 cells serum starved or stimulated with EGF (30 ng/ml) for 10 min. Complexes were washed and resolved on SDS/7.5% polyacrylamide gels and analyzed by immunoblotting with anti-phosphotyrosine antibody. (*B*) Immobilized GST or the indicated GST–SRC fusion protein was incubated with 1 mg of protein from lysates of MDA-MB-468 cells that had been serum starved or stimulated with EGF as in *A*. EGFR immunoprecipitations were done on 500 μg of protein from lysates using polyclonal anti-EGFR antibody (α EGFR). Samples were resolved by SDS/PAGE and analyzed by immunoblotting with anti-phosphotyrosine antibody (α Ptyr) or α EGFR. Numbers on left are kDa.

protein of 125 kDa. A component of this band is likely to be the focal adhesion kinase, p125^{FAK}, which tightly associates with the SRC SH2 domain in human colon carcinoma cell lysates (unpublished observation).

Phosphopeptide Inhibition of EGFR Binding to SRC SH2. The EGFR has multiple sites of tyrosine phosphorylation in the cell after hormone stimulation (13, 14), and we have not yet determined the phosphotyrosine residue(s) recognized by the SH2 domain. However, since only 10–30% of the EGFR appears to bind the SRC SH2 domain, not all receptors may contain the necessary phosphotyrosine residue. Therefore, we used a baculovirus-expressed, recombinant human EGFR tk partially purified from Sf9 cells to maximize phosphorylation of the receptor C terminus. This construct lacks the N-terminal hormone-binding and transmembrane domains and is an active tyrosine kinase that extensively autophosphorylates *in vitro* (unpublished data). The GST–SH2 fusion protein precipitated virtually all of the truncated EGFR after receptor autophosphorylation (data not shown), unlike the situation in cell lysates. This is likely due to the extent of autophosphorylation of the receptor C terminus.

Precipitation of the autophosphorylated EGFR tk was inhibited by a 13-amino acid phosphotyrosine-containing peptide (TSTEPQY*QPGENL), corresponding to the sequences surrounding tyrosine-530 in the C terminus of pp60$^{c\text{-}src}$ (15–17), with an IC_{50} of ≈44 μM, as shown in Fig. 2. This phosphopeptide binds the SRC SH2 domain and thus acts as a competitive inhibitor for SH2 association (17, 18). No

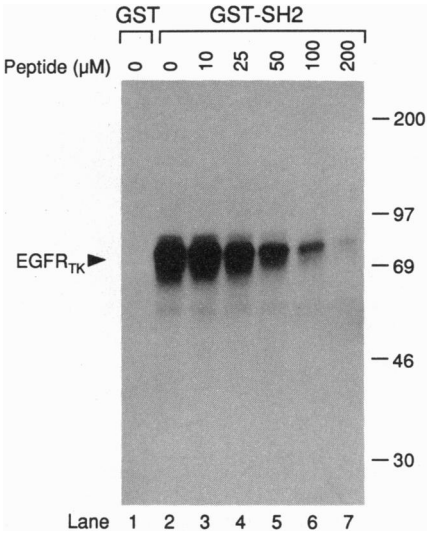

FIG. 2. Inhibition of SRC SH2 binding to the C terminus of EGFR by phosphopeptide. Autophosphorylated EGFR tk was precipitated with either immobilized GST (lane 1) or GST–SH2 (lanes 2–7) in the presence of increasing amounts of phosphopeptide corresponding to the SRC C terminus. Complexes were washed, resolved by SDS/PAGE, and analyzed by immunoblotting with anti-phosphotyrosine antibody. Numbers on right are kDa.

inhibition of this association was observed with the corresponding nonphosphorylated peptide at concentrations up to 10 mM (data not shown). Association of intact EGFR with the SRC SH2 is likely due to direct binding of an unidentified phosphotyrosine residue in the C terminus of the protein with the SH2 domain. To test this hypothesis, several phosphopeptides corresponding to the five major sites of receptor autophosphorylation [Y*-1173, Y*-1148, Y*-1086, Y*-1068, and Y*-992 (13, 14)], as well as phosphopeptides corresponding to several putative autophosphorylation sites in the C terminus of the receptor (Y*-1114, Y*-1101, and Y*-1045), were used to inhibit the association of SRC SH2/SH3 with the EGFR tk recombinant protein in an ELISA. As shown in Table 1, these phosphopeptides were able to inhibit SH2 binding with IC_{50} values ranging from 48 to 340 μM, while the SRC C-terminal peptide inhibited SH2 binding with an IC_{50} of 58 μM. The phosphopeptide corresponding to Y*-992 was the most potent inhibitor with an IC_{50} of 48 μM, slightly better than the SRC C-terminal peptide. Consequently, Y*-992 is a candidate for the high-affinity binding site for the SRC SH2 domain. While these phosphopeptides were able to block SRC SH3/SH2 association with the EGFR tk, they were markedly less effective than the phosphopeptide, Ac-Y*EEIE, which had an

Table 1. ELISA for phosphopeptide inhibition of GST–SRC SH3/SH2 binding to the EGFR tk domain

Tyrosine residue	Peptide sequence	IC_{50}, μM
EGFR Y-992	Ac-VVDADEY*LIPQQG†	48
EGFR Y-1045	Ac-DSFLQRY*SSDPTG†	>1000
EGFR Y-1068	Ac-FLPVPEY*INQSVP†	340
EGFR Y-1086	Ac-SVQNPVY*HNQPLN†	266
EGFR Y-1101	Ac-PSRDPHY*QDPHST†	183
EGFR Y-1114	Ac-AVGNPEY*LNTVQP†	559
EGFR Y-1148	Ac-SLDNPDY*QQDFFP†	126
EGFR Y-1173	Ac-TAENAEY*LRVAPQ†	321
SRC C terminus	TSTEPQY*QPGENL	58
SRC C terminus	TSTEPQYQPGENL	>1000
—	Ac-Y*EEIE	1

Data represent means from three separate experiments done in triplicate.
†Amide.

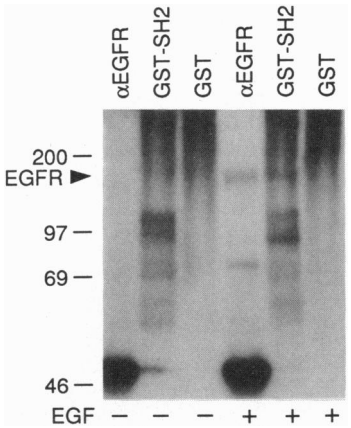

FIG. 3. SRC SH2 directly binds activated EGFR. Cell lysates containing 1 mg of protein from serum-starved or EGF-stimulated MDA-MB-468 cells were incubated with either GST, GST–SH2, or anti-EGFR mAb (αEGFR). Complexes were washed and resolved on SDS/7.5% polyacrylamide gels. Samples were immunoblotted with purified GST–SH3/SH2 and anti-SRC mAb 327, which recognizes an epitope in the SRC SH3 domain. Numbers on left are kDa.

IC_{50} of 1 μM in the assay. The YEEI sequence has been identified as the preferred recognition sequence for the SH2 domains of the SRC family (19). However, with the exception of the hamster polyoma middle-sized tumor antigen, this sequence is not found in any of the proteins with which $pp60^{c\text{-}src}$ is known to associate.

Direct Recognition of EGFR by SRC SH2. Since the SRC SH2 precipitates proteins other than the EGFR from cell lysates, it is possible that the SRC SH2 association with full-length EGFR is indirect, requiring an intermediary protein. To verify that the SRC SH2 domain directly binds to the EGFR, the receptor was precipitated from EGF-stimulated or serum-starved MDA-MB-468 cells using either an EGFR-specific mAb or the GST–SH2 construct. A 170-kDa protein precipitated by EGFR-specific mAb from stimulated cell lysates was detected by blotting with purified GST–SH3/SH2 (Fig. 3). In contrast, the GST–SH3/SH2 fusion protein did not detect a similar protein in immunoprecipitates from unstimulated cell lysates. The purified fusion protein was able to detect a small amount of a similar-sized protein precipitated by the GST–SH2 construct from EGF-stimulated cells. Thus, the SRC SH3/SH2 protein recognizes activated EGFR even under denaturing conditions.

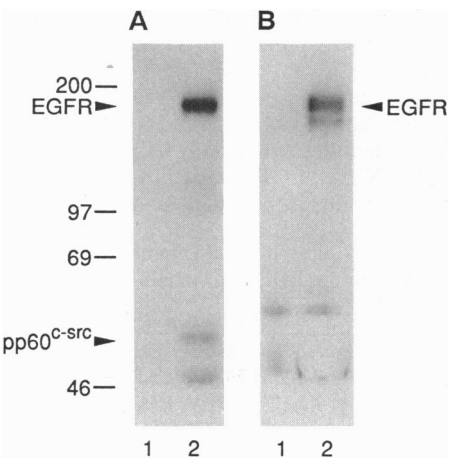

FIG. 4. Association of endogenous $pp60^{c\text{-}src}$ with activated EGFR. (*A*) Cell lysate containing 1 mg of protein from EGF-stimulated MDA-MB-468 cells was immunoprecipitated with anti-SRC mAb 327. Samples were washed, resolved by SDS/PAGE, and immunoblotted with anti-phosphotyrosine antibody and ^{125}I-labeled protein A (16-hr exposure). Lanes: 1, antibody alone; 2, immunoprecipitated cell lysate. (*B*) Nitrocellulose was stripped and reimmunoblotted with polyclonal anti-EGFR antibody. Binding was detected by enzyme-linked chemiluminescence (2-sec exposure). Numbers on left are kDa.

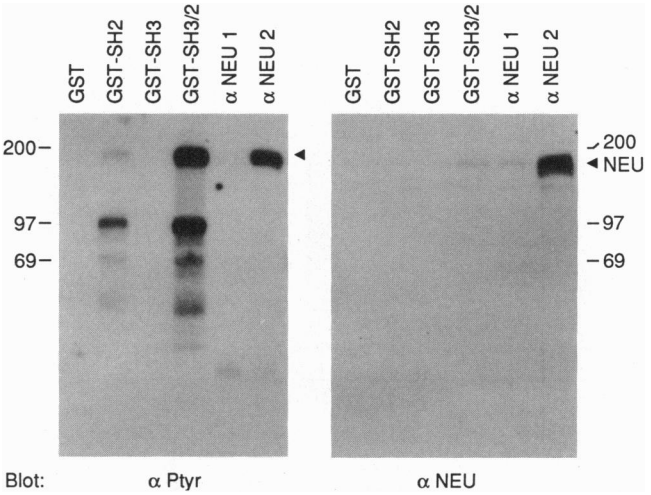

FIG. 5. SRC SH2 domain associates with $p185^{HER2/neu}$. Cell lysates containing 1 mg of protein from logarithmic-phase SKBR3 breast carcinoma cells were incubated with immobilized GST or GST–SRC fusion proteins or two anti-NEU mAbs as described. Samples were washed, resolved by SDS/PAGE, and immunoblotted with either anti-phosphotyrosine (α Ptyr) or anti-NEU2 (α NEU) antibody. Numbers on left and right are kDa.

Endogenous $pp60^{c\text{-}src}$ Binds EGFR. To confirm that the EGFR associates with endogenous SRC protein, we immunoprecipitated $pp60^{c\text{-}src}$ with mAb 327 from lysates of EGF-stimulated MDA-MB-468 cells. As shown in Fig. 4*A*, mAb 327 was able to precipitate $pp60^{c\text{-}src}$ and a tyrosine-phosphorylated 170-kDa protein from stimulated cell lysates. The immunoblot was stripped and reprobed with EGFR-specific antibodies to confirm that the 170-kDa protein was the EGFR (Fig. 4*B*). Thus, consistent with the results observed with the SH2 domain alone, endogenous SRC protein is capable of stable complex formation with the EGFR.

SRC SH2 Recognizes $p185^{HER2/neu}$. Since the protooncogene product $p185^{HER2/neu}$ is a receptor tyrosine kinase that has extensive homology to the EGFR (20, 21), we asked whether the SRC SH2 domain recognized the HER2/NEU protein. As shown in Fig. 5 (*Left*), both the SRC SH2 and SH3/SH2 constructs were able to precipitate a 185-kDa phosphotyrosine-containing protein that comigrated with $p185^{HER2/neu}$ immunoprecipitated from SKBR3 breast carcinoma cells. Immunoblotting duplicate samples with a NEU-specific antibody confirmed that the p185 protein was in fact HER2/NEU. The SH3/SH2 fusion protein was able to detect significantly more $p185^{HER2/neu}$ than the SH2 fusion alone, although the latter fusion protein clearly precipitated p185 on longer exposure of the autoradiograph. It is unclear what role the SH3 domain may play in facilitating the association of the NEU protein with the SH2 domain, since no binding of $p185^{HER2/neu}$ was observed with the fusion containing the SH3 domain alone. As in the case of the EGFR, comparison of the band intensity suggests that $p185^{HER2/neu}$ associated with the SRC SH2 or SH3/SH2 constructs is highly phosphorylated on tyrosine.

DISCUSSION

The interaction of proteins containing SH2 domains with tyrosine-phosphorylated growth factor receptors is a common event following growth factor stimulation. Biochemical and genetic analysis of SH2-mediated protein associa-

tions indicates that the consensus SH2 region defines a domain sufficient to confer high-affinity binding to appropriate target proteins. The ability of isolated SRC SH2 and SH3/SH2 domains expressed as GST fusion proteins to form stable associations *in vitro* with both activated EGFR and $p185^{HER2/neu}$ suggests that the SRC SH2 domain may couple $pp60^{c\text{-}src}$ with signaling pathways mediated by both the EGFR and $p185^{HER2/neu}$ *in vivo*.

Considerable evidence links the EGF signaling pathway to $pp60^{c\text{-}src}$. Overexpression of SRC protein in normal cells results in a significant potentiation of the EGF mitogenic response. This effect of SRC requires expression of membrane-associated, enzymatically active SRC protein that has an intact SH2 domain (7, 8). This requirement for an intact SH2 domain raises the possibility that $pp60^{c\text{-}src}$ may directly associate with activated EGFR *in vivo*. Consistent with this possibility, a recent report indicates that the avian c-*src* domain will bind to the phosphorylated C terminus of EGFR tk (22). We show here that endogenous human $pp60^{c\text{-}src}$ stably binds tyrosine phosphorylated EGFR, supporting the hypothesis that these two proteins functionally interact in signal transduction within the cell. It is quite possible that SRC family members associate with activated EGFR, analogous to the situation observed with the platelet-derived growth factor receptor (PDGFR). The PDGFR will associate with $pp60^{c\text{-}src}$, $pp59^{c\text{-}fyn}$, and $pp62^{c\text{-}yes}$, presumably via the SH2 domains of these proteins, as the SH2 domain of FYN has been shown to bind the PDGFR (23, 24). In the case of $pp60^{c\text{-}src}$, changes in the phosphorylation state and kinase activity of the protein can be detected after stimulation of cells with PDGF, suggesting a role for the protein in PDGF-mediated signal transduction (25). No such association between $pp60^{c\text{-}src}$ and the EGFR has previously been reported, although transient changes in $pp60^{c\text{-}src}$ kinase activity can be detected in cells after EGF stimulation (7). Although we have not yet determined whether $pp60^{c\text{-}src}$ kinase activity is increased as a consequence of complex formation with the EGFR, the specific activity of the SRC protein is elevated 2- to 4-fold within the MDA-MB-468 and MDA-MB-231 cell lines relative to the AGO3204 cells (data not shown). Thus, interaction of SRC family members with receptor tyrosine kinases may be a general feature of signaling mediated through these types of growth factor receptors.

Although transient activation of receptor tyrosine kinases is a necessary event in hormone-induced mitogenesis, constitutive activation of the signaling pathways initiated by these receptors may contribute to human malignancy. Amplification of EGFR or $p185^{HER2/neu}$ is observed in a significant fraction of primary human breast carcinomas and is correlated with poor clinical prognosis. Constitutive activation of the signaling pathways mediated by these tyrosine kinase receptors may contribute to tumor growth rate (3). We propose that association of $pp60^{c\text{-}src}$ with these receptor tyrosine kinases within the cell is an integral part of the signaling events mediated by these receptors.

The authors wish to thank J. Robbins for large scale fermentation of recombinant SRC proteins; D. Ruznak for help with cell culture; G. Shah, D. Baines, A. Hutchinson, and R. Hale (Glaxo Group Research) for the expression and purification of the truncated EGFR; and Drs. L. Hudson, J. Besterman, and T. Tlsty (University of North Carolina) for critical reading of the manuscript.

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