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Distinct Tyrosine Autophosphorylation Sites Negatively and Positively Modulate Neu-Mediated Transformation

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A number of cytoplasmic signaling molecules are thought to mediate mitogenic signaling from the activated Neu receptor tyrosine kinase through binding specific phosphotyrosine residues located within the intracellular portion of Neu/c-ErbB-2. An activated *neu* oncogene containing tyrosine-to-phenylalanine substitutions at each of the known autophosphorylation sites was generated and assessed for its specific transforming potential in Rat1 and NIH 3T3 fibroblasts. Mutation of these sites resulted in a dramatic impairment of the transforming potential of *neu*. To assess the role of these tyrosine phosphorylation sites in cellular transformation, the transforming potential of a series of mutants in which individual tyrosine residues were restored to this transformation-debilitated *neu* mutant was evaluated. Reversion of any one of four mutated sites to tyrosine residues restored wild-type transforming activity. While each of these transforming mutants displayed Ras-dependent signaling, the transforming activity of two of these mutants was correlated with their ability to bind either the GRB2 or SHC adapter molecules that couple receptor tyrosine kinases to the Ras signaling pathway. By contrast, restoration of a tyrosine residue located at position 1028 completely suppressed the basal transforming activity of this mutated *neu* molecule or other transforming *neu* molecules which possessed single tyrosine residues. These data argue that the transforming potential of activated *neu* is mediated both by positive and negative regulatory tyrosine phosphorylation sites.

The *neu*/c-*ErbB*-2 gene encodes a 185-kDa transmembrane receptor tyrosine kinase that is a member of the epidermal growth factor receptor (EGFR) family (4, 15, 76). Elevated expression of the EGFR family members has been implicated in the etiology of human ovarian and breast cancers (reviewed in references 31 and 41). In particular, amplification and consequent overexpression of the *neu* receptor tyrosine kinase have been observed in a large proportion of primary human breast cancers (58, 64, 65). Consistent with these observations, expression of either wild-type *neu* or a constitutively active mutant (V664E, *neu*NT [3, 5]) in the mammary epithelia of transgenic mice results in the induction of metastatic mammary tumors (11, 27, 47). The induction of mammary tumors in the wild-type *neu* transgenic mice correlates with elevated levels of tyrosine-phosphorylated Neu that, in many cases, are due to somatic mutations within the transgene (63).

Although these studies suggest that activation of Neu plays an important role in the induction of mammary carcinoma, the molecular basis for the potent transforming activity of *neu* is poorly understood. Following receptor dimerization, autophosphorylation takes place at several tyrosine residues in the cytoplasmic terminus of Neu (1, 29, 61). Specific phosphotyrosine residues within the receptor provide potential binding sites for cytoplasmic signaling molecules harboring either Src homology 2 (SH2) (51) or phosphotyrosine binding/interacting (PTB/PI) domains (9, 10, 34, 70). A number of these intracellular signaling molecules such as phospholipase Cγ1 (PLCγ1) (22, 32, 60), c-Src (40, 49), and GRB-7 (68) are complexed with Neu through specific phosphotyrosine residues. An additional set of SH2-containing proteins that associate with Neu include the Ras GTPase-activating protein (Ras-GAP) (22, 32), SHC (62), and GRB2 (33). These latter proteins modulate the activity of Ras by either promoting the formation of an active Ras-GTP complex or accelerating the hydrolysis of Ras-GTP to its inactive Ras-GDP state (39). For example, Ras-GAP stimulates the intrinsic GTPase activity of Ras, resulting in the downregulation of Ras activity. By contrast, GRB2 constitutively binds to Sos GDP-GTP exchange proteins through its SH3 domains and promotes the conversion of Ras-GDP into its active Ras-GTP state (12, 13, 21, 23, 37, 56). SHC proteins are thought to promote Ras activation by indirectly recruiting GRB2 to receptor tyrosine kinases (44, 52, 57) through the major phosphorylated tyrosine residue of SHC, corresponding to a consensus binding site for the GRB2 SH2 domain (59, 67).

While the binding sites on Neu for these SH2- and PTB-containing proteins are unclear, deletion or mutation of Neu tyrosine autophosphorylation sites can dramatically affect the transforming activity of *neu* (1, 8, 18, 45, 60). However, these studies differ in their conclusions about the relative importance of these tyrosine autophosphorylation sites in *neu*-mediated transformation. In one set of experiments, alteration of three or five tyrosine phosphorylation sites in Neu resulted in a progressive debilitation of transforming activity (60). In other studies, mutation of tyrosine 1248 in an activated version of the human c-ErbB-2 resulted in a transformation-defective molecule, suggesting that this site was essential for the transforming activity of *neu* (1). More recently, it has been reported that addition of this single tyrosine phosphorylation site to a transformation-defective carboxyl truncation mutant was sufficient to restore the transforming potential of activated *neu* (8). These observations suggested that phosphorylation of this residue in Neu was both sufficient and necessary for its transforming potential. By contrast, alteration of the analogous tyrosine

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residue in the wild-type c-ErbB-2 protein had no effect on its transforming properties (61).

In an attempt to systematically address the role of tyrosine autophosphorylation sites in *neu*-mediated transformation, we used a strategy initially described for the platelet-derived growth factor receptor (PDGFR). In these studies, individual tyrosine residues were restored to a mitogenically inactive mutant PDGFR containing tyrosine-to-phenylalanine substitutions at the known tyrosine phosphorylation sites (add-back mutants [69]). Using a similar strategy, we have generated a catalytically activated Neu molecule (V664E) in which the known tyrosine phosphorylation sites (18, 29) have been similarly altered. This mutant, termed the Neu tyrosine phosphorylation-deficient (NT-NYPD) mutant, was severely impaired in its capacity to transform Rat1 and NIH 3T3 fibroblasts. To elucidate the function of individual tyrosine autophosphorylation sites, we generated a series of add-back mutants in which individual tyrosine phosphorylation sites were restored to the NT-NYPD mutant background. Restoration of any one of four individual tyrosine residues to the NT-NYPD mutant completely restored the transforming activity of this mutant. Interestingly, each of these transforming mutant receptors required Ras activity to induce a proliferative signal.

To elucidate the molecular basis for the functional redundancy of the four tyrosine phosphorylation sites, we analyzed the ability of these mutants to associate with signaling molecules that have been implicated in Neu-mediated transformation. The results showed that two of the add-back mutants containing tyrosine residues at either position 1144 or 1226/1227 were able to independently associate with GRB2 or SHC proteins which are known to couple receptors to the Ras pathway. By contrast to these observations, the addition of tyrosine residue 1028 to NYPD or to any one of three other add-back mutants severely impaired their transformation activities. These differences in transforming activity could not be accounted for by a reduction in the catalytic activities of the mutant receptors but appear to be a result of a reduction in the number of SH2/PTB signaling molecules complexed with these receptors. Taken together, these data argue that autophosphorylation of tyrosine residues within Neu is involved in both the negative and positive regulation of Neu-mediated transformation.

MATERIALS AND METHODS

PCR mutagenesis and DNA constructs. *neu* cDNAs harboring the wild-type gene or the V664E transmembrane mutation were a generous gift of Robert Weinberg (4). Mutations in carboxyl-terminal autophosphorylation sites were generated by standard PCRs for oligonucleotide-directed mutagenesis. Following the introduction of an *Eco*RI site immediately 3′ to the stop codon (nucleotide 3800), the *Nco*I-*Eco*RI fragment (nucleotides 3030 to 3800) was cloned into pSL301 (In vitrogen) to facilitate subsequent sequence analyses. Mutagenic oligonucleotide pairs for each tyrosine were as follows: site A (Y1024) (forward AB 2937, GAAGAGTTTCTAGTGCCCCAGCAGGG, and reverse AB 2938, GGGCACTAGAAACTCTTCAGCGTC); site B (Y1144) (forward AB 2939, CGAGTTTGTTAACCAATCAGAGG, and reverse AB 2940, TTGGTTAACAAACTCGGGCTGGGGGC); site C (Y1201) (forward AB 2941, CTGAATTTTTGGTACCGAGAGAAGGC, and reverse AB 2942, CGGTACCAAAAATTCAGGGTTCTCCAC); site D (Y1225/6) (forward AB 2943, CCTCTTCTTCTGGGACCAGAACTCATCG, and reverse AB 2944, GTCCCAGAAGAAGAGGTTGTCAAAGG); site E (Y1253) (forward AB 2945, TGAGTTTCTAGGCCTGGATGTACC, and reverse AB 2946, CAGGCCTAGAAACTCAGGGTTCTC). Nucleotides which differ from that in the wild-type sequence are underlined. The conditions for amplification were as described previously (63) with the following exceptions. Amplification was performed for 20 cycles of 45 s at 95°C, 1 min 30 s at 37°C, and 2 min 30 s at 73°C. The first amplification products were excised from agarose gels and boiled for 5 min in TE (10 mM Tris [pH 7.6], 1 mM EDTA), and an aliquot was used for a subsequent round of amplification. Amplifications were carried out to create plasmids with single-amino-acid alterations or multiple simultaneous alterations (e.g., tyrosines at sites C and E were simultaneously mutated by initially carrying out three reactions [T7 primer and AB 2942; AB2941 and AB2946; AB2945 and T3 primer], and aliquots of each of the products were mixed and PCR amplified externally with T3 and T7 oligonucleotides). Standard subcloning procedures were used to bring mutations together within the same plasmid. All cloned PCR-amplified regions were sequenced in their entirety by Brian Allore, MOBIX Central Facility of McMaster University.

An *Nco*I site within the 5′-noncoding sequences was destroyed in the *neu*NT cDNA, and an expression plasmid containing this altered *neu*NT cDNA under the transcriptional control of the Moloney murine leukemia virus (MoMuLV) long terminal repeat was created by inserting the cDNA (4) as a *Hin*dIII-*Eco*RI fragment into the corresponding sites of pJ4Ω (46). This plasmid was termed pJNT. Autophosphorylation mutants were subsequently cloned into pJNT from pSL301-derived plasmids as *Nco*I-*Eco*RI fragments to produce expression plasmids with an activated form of *neu* under MoMuLV long terminal repeat control. Autophosphorylation mutants were excised from pJ4Ω-derived plasmids as *Hin*dIII-*Eco*RI fragments and cloned into the corresponding sites of pCDNA3 (In vitrogen) to produce a series of plasmids in which the *neu* mutants are under the transcriptional control of the human cytomegalovirus immediate-early promoter and contain a G418-selectable marker. Two deletion mutants were generated from pJNT by digestion with *Nco*I followed by a fill-in reaction with the Klenow fragment. The generation of an expression plasmid containing wild-type *neu* in pJ4Ω is described elsewhere (63). A plasmid (PGK-puro) containing the puromycin resistance gene under the transcriptional control of the phosphoglyceraldehyde kinase promoter was obtained from M. A. Rundicki.

GSTag-GRB2 encodes the entire GRB2 protein and was generously provided by B. Margolis (68). GSTag-SHC encodes the entire mouse p55-SHC protein (amino acids 1 to 469 [52]) cloned into the *Eco*RI site of GSTag (54) through PCR-engineered restriction sites. The murine SHC cDNA was a gift of T. Pawson.

Cell lines and transformation assays. Rat1 fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin, streptomycin, and amphotericin B. Expression plasmids were introduced into Rat1 cells along with PGK-puro by the method of Graham and van der Eb, as modified by Wigler (75), at a 40:1 ratio of expression plasmid to puromycin resistance plasmid (PGK-puro). Transfected cells were selected in supplemented DMEM containing 3.0 μg of puromycin per ml. In each case, at least 20 clones were isolated and used for further characterization. Focus-forming assays were performed as described previously (63). Briefly, Rat1 fibroblasts (10^7 cells) were electroporated with 50 μg of each mutant pJ4Ω-derived plasmid DNA. Six 100-mm tissue culture dishes were then seeded at 10^6 cells per plate and were maintained in supplemented DMEM for 14 days, with the medium being changed every 3 days. The plates were then stained with Giemsa as specified by the manufacturer.

Growth in soft agar was assessed by plating 1,000 viable (as assessed by trypan blue exclusion) Rat1 and Rat1-derived cells from clonal cell lines in 0.25% agarose in supplemented 1× DMEM containing 5% FBS on top of 0.5% agarose in supplemented 1× DMEM containing 5% FBS on 60-mm plates. The plates were maintained for 21 days, stained overnight with 10 mg of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma no. M2128] per ml in 0.25% agarose–1× DMEM, and counted. In a similar manner, NIH 3T3 fibroblasts were electroporated with pCDNA3 (control) or pCDNA3-derived plasmids containing the indicated Neu add-back mutants. Following 3 weeks of selection with G418 (400 μg/ml), 10^5 cells were plated in 0.25% agarose, and colony formation was assessed 4 weeks later. Colony formation is indicated by (+) and a lack of cell growth is designated (−) in Table 2. Representative colonies were photographed from plates prior to staining (see Fig. 2).

BrdU incorporation. DNA synthesis was assayed after metabolic incorporation of 5-bromo-2′-deoxyuridine (BrdU) (cell proliferation kit; Amersham) essentially as described previously (73). Cells growing on glass coverslips were deprived of serum overnight (~18 h) and then not injected or microinjected with antibodies to Ras (Y13-259, 2 mg/ml) together with biotinylated glutathione-*S*-transferase (1.3 mg/ml). After incubation at 37°C for 2 h, fresh medium containing 10% FBS and BrdU was added and the coverslips were incubated at 37°C for 18 h. Following fixation, BrdU incorporated into DNA was visualized with a BrdU antibody and a rhodamine-labeled secondary antibody to mouse immunoglobulin. Microinjected cells were identified by staining with FITC-labeled avidin (Jackson Immunology Laboratory). No fewer than 100 cells were injected on each coverslip, and DNA synthesis was determined as the percentage of microinjected cells staining positively for BrdU incorporation. Equivalent results were obtained from two independent experiments, and the results from one such experiment are shown. In noninjected cells, the percentage of BrdU-labeled cells was determined for no fewer than 200 cells counted from randomly selected fields of view. The cells were visualized for simultaneous red and green fluorescence with the appropriate filter sets (Carl Zeiss Ltd.).

Immunoprecipitations. Confluent plates were washed twice in ice-cold 1× phosphate-buffered saline, and the cells were then lysed for 20 min either in modified TNE lysis buffer (mTNE) (50 mM Tris HCl [pH 8.0], 150 mM NaCl, 1% Nonidet P-40, 10 mM NaF, 10 mM sodium orthophosphate, 2 mM EDTA), mCHAPS lysis buffer (50 mM Tris HCl [pH 8.0], 50 mM NaCl, 0.7% 3-[(3-cholamidopropyl)-dimethylammnio]-1-propanesulfonate [CHAPS], 10 mM NaF, 10 mM sodium orthophosphate, 2 mM EDTA) or PLC lysis buffer (43) each containing 1 mM sodium orthovanadate, 10 μg of leupeptin per ml, and 10 μg of aprotinin per ml. The lysates were clarified by centrifugation at 4°C for 15 min,

and the protein concentrations were normalized following quantitation by the Bradford assay (Bio-Rad). Equivalent amounts of protein (as indicated in the figure legends) were incubated with the appropriate antibodies and protein A- or G-Sepharose beads (Pharmacia) for 3 h at 4°C. SHC immunoprecipitations were carried out with a mixture of polyclonal antisera (0.1 μg and 0.1 μg/100 μg of lysate [no. S14630, Transduction Labs; and 51-636, Upstate Biotechnology Inc.]) preabsorbed to protein A-Sepharose from lysates prepared with mCHAPS lysis buffer. Neu immunoprecipitates were performed with a mouse monoclonal antibody (MAb 7.16.4; 1 μl/500 μg) (20) in mTNE. The immunoprecipitates were subsequently washed five to seven times with lysis buffer and resuspended in 1× sodium dodecyl sulfate (SDS) gel-loading buffer (63). For GRB2-Neu coimmunoprecipitations, the cells were lysed in PLC lysis buffer and the proteins were immunoprecipitated with MAb 7.16.4 conjugated to protein G-Sepharose (28), since GRB2 comigrates with the antibody light chain. The immunoprecipitates were washed seven times in PLC lysis buffer and boiled in 1× SDS gel-loading buffer lacking reducing agents, and the beads were pelleted by centrifugation. The supernatant was subsequently boiled following the addition of β-mercaptoethanol to 0.8 M.

In vitro kinase assays. Neu was immunoprecipitated as above from cells lysed in mTNE lysis buffer. Following four washes in ice-cold mTNE, the beads were washed twice in ice-cold 1× kinase buffer (100 mM HEPES [pH 7.0], 5 mM $MnCl_2$). All traces of kinase buffer were removed, and 1× kinase buffer containing 10 μCi of [γ-^{32}P]ATP was added. The reactions were carried out at 30°C for 20 min and were terminated by the addition of an equal volume of boiling 2× SDS-polyacrylamide gel electrophoresis (PAGE) loading buffer containing 5 mM EDTA. The products were analyzed by SDS-PAGE (9% polyacrylamide) under reducing Laemmli conditions, and the gels were processed as previously described (49).

Immunoblotting procedures. For immunoblot analyses, immunoprecipitates of 20 to 30 μg of total-cell lysates were electrophoresed on SDS-polyacrylamide gels and the proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore). Phosphotyrosine immunoblots were blocked in Tris-buffered saline–0.05% Tween 20 (TBS-T; 20 mM Tris-HCl [pH 7.5]–3% bovine serum albumin (fraction V; Sigma) overnight at 4°C or at room temperature for 1 h. The membranes were probed with antiphosphotyrosine antibodies (1:1,000, 4G10, Upstate Biotechnology Inc.; or 1:1,000, Py20, Transduction Labs) in blocking buffer for 2 to 3 h at room temperature, washed four times for 15 min in TBS-T, and then incubated at room temperature for 1 h in TBS-T containing 3% skim milk. The membranes were then incubated with horseradish peroxidase-conjugated anti-mouse antibodies (1:5,000; Jackson Laboratories), subjected to four 15-min washes in TBS-T, and visualized by enhanced chemiluminescence (Amersham) as specified by the manufacturer. SHC was detected with a rabbit polyclonal antibody (1:1,000) (S14630; Transduction Labs). GRB2 was detected with either rabbit polyclonal sera (1:400) (C23; Santa Cruz) or a mixture of MAbs (1:1,000 each) (05-226, Upstate Biotechnology Inc.; and g16720, Transduction Labs). Immunoblots for Neu were performed with MAbs AB3 (1:1,000) (Oncogene Science) or for SHC coimmunoprecipitations (1:300) (E19420; Transduction Labs). Antisera made to the epitope of the Neu AB3 MAb were used in the experiment in Fig. 2B to detect Neu. In each case, horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (1:1,000) (Jackson Laboratories) were used.

Direct blot assay. Following SDS-PAGE (8.5% polyacrylamide gel electrophoresis), Neu immunoprecipitates were transferred to PVDF membranes and blocked overnight at 4°C or at room temperature for 1 h in blocking buffer (20 mM HEPES [pH 7.5], 5 mM $MgCl_2$, 1 mM KCl, 5 mM dithiothreitol [DTT], 0.2% sodium azide) containing 5% skim milk. Fusion proteins were purified and immobilized on glutathione-Sepharose beads (Pharmacia) essentially as described previously (65a). Immobilized glutathione-*S*-transferase fusion proteins were washed three times in DK buffer (50 mM KH_2PO_4, 10 mM $MgCl_2$, 5 mM NaF, 4.5 mM DTT). These were incubated at 30°C for 30 min in reaction buffer (50 mM KH_2PO_4, 10 mM $MgCl_2$, 5 mM NaF, 4.7 mM DTT) containing 500 μCi of [γ-^{32}P]ATP (6,000 Ci/mM) and 20 μg of protein kinase A (Sigma no. P-2645), after which time an additional 20 μg of protein kinase A was added. Unincorporated nucleotides were removed by washing six times in ice-cold 1× phosphate-buffered saline containing 5 mM NaF and 5 mM EDTA. Proteins were eluted as described previously (48). The blots were probed for 3 h at room temperature with radiolabeled fusion proteins at 1.5×10^6 cpm in block buffer. The membranes were washed three times in TBS-T and analyzed by autoradiography.

RESULTS

Role of individual Neu tyrosine autophosphorylation sites in cellular transformation. Given the importance of the Neu tyrosine autophosphorylation sites in proliferative signal transduction, we have generated a series of Neu molecules in which the carboxyl-terminal tyrosine phosphorylation sites have been converted to phenylalanine residues (Fig. 1A). Because a ligand for Neu has not been unequivocally described, these autophosphorylation mutations were introduced into a *neu* cDNA which harbors an activating point mutation in the transmembrane domain, rendering Neu constitutively kinase active (V664E [3, 5]) through increased homodimerization of mutant receptors (74). For the purpose of simplicity, we refer to the activated *neu* mutants lacking individual tyrosine autophosphorylation sites as NT-A (tyrosine residue 1028 to phenylalanine), NT-B (Y1144F), NT-C (Y1201F), NT-D (Y1226/7F), and NT-E (Y1253F) (Fig. 1B). Because it was unclear which of two adjacent tyrosine residues (tyrosines 1226 or 1227) was phosphorylated, both tyrosines were simultaneously mutated to phenylalanine residues to create the site D mutant.

To assess the transforming capacities of the various mutant *neu* constructs, we introduced these mutations into a Mo-MuLV-based expression cassette and tested their ability to transform Rat1 cells in culture. Mutation of any single site (NT-A through NT-E) had a minimal effect on its transformation potential compared to the unaltered activated cDNA (NT) (Table 1). For example, single-site mutations in sites B to E resulted in modest impairment of focus-forming activity (75 to 85% of the NT activity). By contrast, removal of site A resulted in a modest but reproducible 1.4- to 2-fold elevation of focus-forming activity compared to that in the activated *neu* construct (NT-A [Table 1]). Similar results were obtained when Rat1-derived cell lines were tested for the ability to grow in an anchorage-independent manner (17). These results suggest that alteration of any single tyrosine phosphorylation site does not drastically interfere with the ability to transform Rat1 cells.

To determine whether the carboxyl region harboring the autophosphorylation sites was required for transformation, *neu* mutants in which all of the known tyrosine phosphorylation sites were either converted to phenylalanine residues (NT-NYPD [Fig. 1C]) or deleted (NT-CT1 and NT-CT2 [Fig. 1B]) were constructed and tested for their capacity to transform Rat1 fibroblasts. As shown in Table 2, conversion of all of these tyrosine residues to phenylalanine residues to create the NT-NYPD mutant resulted in a dramatic impairment in the ability to transform Rat1 fibroblast monolayers (8% of the level of activated *neu*) or to form colonies in soft agar. Deletion of the entire carboxyl terminus completely abolished the ability of *neu* to induce focus formation (NT-CT1 and NT-CT2 [Table 1]). Taken together, these observations suggest that while individual autophosphorylation sites are dispensable for transformation, certain tyrosine phosphorylation sites appear to be required for efficient *neu*-mediated transformation.

To identify whether particular tyrosine residues were sufficient to mediate a transforming signal, individual phenylalanine substitutions in the NT-NYPD mutant were reverted to tyrosine residues to create a series of add-back mutants possessing only one of these sites. For simplicity, we have termed these add-back mutants NT-YA (tyrosine 1028), NT-YB (tyrosine 1144), NT-YC (tyrosine 1201), NT-YD (tyrosines 1226 and 1227), and NT-YE (tyrosine 1253) (Fig. 1C). Restoration of any one of four tyrosine autophosphorylation sites (NT-YB, NT-YC, NT-YD, or NT-YE) resulted in transforming activities comparable to those observed in the parental activated *neu* cDNA as measured by focus-forming assays (Table 2). Similar results were obtained when the transforming activity of these mutants was measured by colony formation in soft agar with stable clonal Rat1-derived cell lines (Table 2) or with pooled Rat1-derived colonies (17). Furthermore, analyses of the transforming activity of these mutants in NIH 3T3 fibroblasts by soft agar colony formation revealed comparable transforming properties (Fig. 2). The results of these experiments suggest that activated *neu* can induce transformation through multiple functionally redundant tyrosine phosphorylation sites by sev-

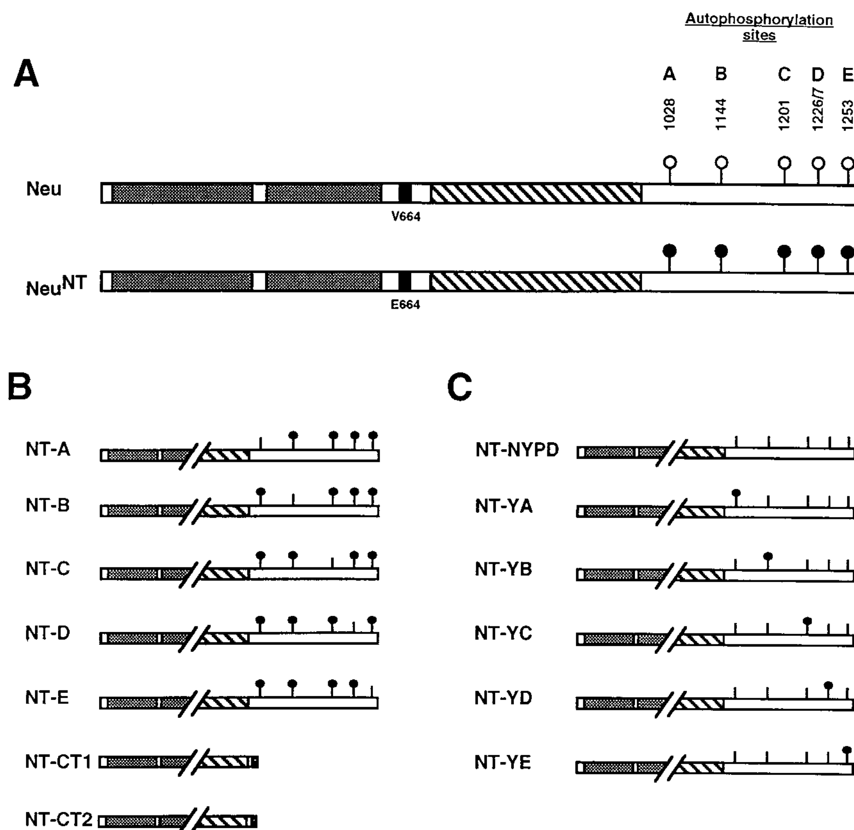


FIG. 1. Schematic representation of Neu receptor kinase mutants. (A) The structures of Neu and activated Neu (Neu^{NT}), containing the transmembrane mutation V664E are depicted. Indicated are two cysteine-rich domains (shaded), a single transmembrane domain (solid), and the tyrosine kinase domain (striped). Autophosphorylation sites at tyrosine residues 1028 (site A), 1144 (site B), 1201 (site C), 1226/1227 (site D), and 1253 (site E) are indicated (♀). (B) The indicated tyrosine residues were converted to phenylalanine residues (❙). NT-CT1 carries a stop codon immediately following the methionine codon at 1005, and NT-CT2 is a frameshift deletion mutant terminating with the sequences ^{1005}M<u>HGQYLLPFTAGR</u>, where the underlined amino acids differ from that of Neu. (C) NT-NYPD contains mutations at each of the indicated autophosphorylation sites. Add-back mutants derived from NT-NYPD contain single autophosphorylation sites and phenylalanine residues at the four remaining sites. All mutants were derived from Neu^{NT}.

eral criteria (Table 2, sites B, C, D, and E). By contrast to these transforming add-back mutants, restoration of tyrosine residue 1028 (site A) to NT-NYPD completely suppressed the basal transforming activity of the NT-NYPD mutant (NT-YA). Conversely, mutation of this tyrosine residue alone to a phenylalanine residue results in a modest but reproducible elevation of transformation activity (Table 1, NT-A). Therefore, unlike tyrosine residues B through E, site A interferes with the transforming activity of *neu*.

The Neu add-back mutants are catalytically active and are tyrosine phosphorylated. To ensure that the observed phenotypes displayed by NT-NYPD and the various add-back mutants were not due to effects on the tyrosine kinase activity or to the stability of the Neu proteins, we established stable Rat1 fibroblasts expressing the various *neu* mutants. Cell lysates from these stable cell lines were subjected to immunoblot analysis with Neu-specific antibodies to determine the amount of Neu expressed in each of these lines (Fig. 3B). By using this screen, representative cell lines for each mutant expressing similar levels of Neu were selected for further biochemical analyses.

To examine whether these mutations affected the catalytic activity of Neu, Neu was immunoprecipitated from cell lysates obtained from these stable transfectants and two-thirds of each immunoprecipitate was subjected to an in vitro autokinase assay. The results revealed that cell lines expressing each of the *neu* mutants (Fig. 3A, lanes 5 to 20) possessed similar levels of tyrosine kinase activity by comparison to the activated Neu-containing cell lines (lanes 2 to 4). As expected, the parental Rat1 cell line harbored no detectable Neu kinase activity (lane 1). To determine whether the levels of Neu kinase activity correlated with its extent of tyrosine phosphorylation, the remaining one-third of each Neu immunoprecipitate was subjected to antiphosphotyrosine immunoblot analysis (Fig. 3C). Consistent with the results of the in vitro kinase assays, our results demonstrated that the NT-NYPD mutant and each add-back mutant possessed significant levels of phosphotyrosine in vivo. To further ensure that the difference in transforming activity between the NT-NYPD and activated Neu was not due to differences in catalytic activity, we performed a series of in vitro kinase assays with an exogenous substrate, myelin basic protein. The results revealed that under conditions of linear incorporation, no significant differences in the specific activities of NT-NYPD mutant and activated Neu could be detected (17). Thus, despite mutation of a number of tyrosine residues in this mutant, the difference in transforming activity of NT-NYPD and the other transforming add-back mutants cannot be attributed to differences in the catalytic activity.

Transformation by activated *neu* occurs through functionally redundant signaling molecules involved in the activation of the Ras pathway. Because the Ras signaling pathway is

TABLE 1. Transformation of Rat-1 cells with *neu* autophosphorylation point mutants[a]

Construct	Focus assay 1		Focus assay 2		Focus assay 3		Relative transforming ability[f]
	Mean no. of foci ± SD[b]	% Transformation of NT[c]	Mean no. of foci ± SD[b]	% Transformation of NT[c]	Mean no. of foci ± SD[d]	% Transformation of NT[e]	
Neu	0	0	0	0	0	0	0
NT	167 ± 13	100	287 ± 23	100	198 ± 20	100	100
NT-A	367 ± 33	220	400 ± 19	140	287 ± 29	145	160 ± 35
					265 ± 21	134	
NT-B	95 ± 13	57	293 ± 21	102	150 ± 15	76	78 ± 18
NT-C	221 ± 21	133	143 ± 10	50	138 ± 11	70	85 ± 31
					172 ± 23	87	
NT-D	111 ± 22	67	213 ± 10	74	166 ± 14	84	75 ± 7
NT-E	134 ± 15	81	248 ± 13	87	161 ± 16	81	83 ± 3
NT-CT1	0 ± 0	0	NA[g]	NA	0 ± 0	0	0 ± 0
NT-CT2	0 ± 0	0	NA	NA	1 ± 1	0	0 ± 0

[a] Three independent focus-forming assays were performed with Rat-1 fibroblasts. The first and second experiments were performed with independent plasmid preparations, while the third experiment was conducted with DNA from focus assay 2. The nomenclature describing the mutants is defined in Fig. 1. All cDNAs were placed under the transcriptional control of the MoMuLV long terminal repeat, with the parental vector being pJ4W. The use of pJ4W did not result in focus formation.
[b] Values represent the mean number of foci per plate counted on six plates ± standard deviation.
[c] Values are the ratios of the mean numbers of foci obtained for each construct with respect to that obtained with NeuNT (NT) multiplied by 100%.
[d] Values are the mean number of foci/plate counted on six plates ± standard deviation. Where two numbers are given, independent DNAs were used in the same focus assay and the values represent the mean number of foci/plate counted on each of six plates ± standard deviation.
[e] Values are the ratios of the mean numbers of foci obtained for each construct with respect to that obtained with NeuNT (NT) multiplied by 100%. Where independent DNAs were used within the same focus assay, the mean of the two ratios is given.
[f] Values are the weighted mean transforming abilities from the three experiments ± standard error.
[g] NA, not available.

thought to play an important role in Neu-mediated transformation (6, 8, 33, 62), we tested the requirement of Ras activity in Neu-mediated proliferation. One means of establishing whether Ras signaling is involved in Neu-mediated cell proliferation is through microinjection of the anti-Ras neutralizing antibody, Y13-259, into Rat1-derived cell lines expressing each of the add-back mutants (19). Y13-259-injected and noninjected cells were then monitored for the induction of DNA synthesis by BrdU incorporation. DNA synthesis was observed in more than 90% of the control cells expressing transforming mutants of Neu compared to 78% of the parental Rat1 cells, while NT-NYPD- and NT-YA-expressing cell lines induced intermediate levels of DNA synthesis (Table 2). Interestingly, microinjection of Ras neutralizing antibodies inhibited BrdU incorporation in all tested cell lines, whereas microinjection of a nonneutralizing anti-Ras antibody (Y13-238) under these conditions does not inhibit DNA synthesis in fibroblasts (73). Inhibition of DNA synthesis in Rat1 cells by Y13-259 is probably a result of an impairment of signaling stimulated by serum factors such as lysophosphatidic acid. These data suggest that Neu-induced proliferation occurs through multiple tyrosine residues, each impinging on Ras.

One possible explanation for these observations is that different add-back mutations remain coupled to the Ras pathway through multiple adapter proteins. For example, activation of Ras by Neu could conceivably occur either through binding

TABLE 2. Transformation and proliferative properties of fibroblasts with add-back mutants[a]

Construct	Focus formation[b] (relative transforming ability)	Soft agar colony formation[c]			% DNA synthesis[d]	
		Mean no. of colonies/10^3 cells	Relative to NT (%)	NIH 3T3[a,e]	Control	Anti-Ras Y13-259 injected
Control[f]	0	0	0	−	78	5.9
NT	100	144 ± 9	100	+	97	8.2
NT-NYPD	8 ± 1	16 ± 7	11	−	76	5.0
NT-YA	0 ± 0	0 ± 0	0	−	83	5.6
NT-YB	117 ± 12	166 ± 32	116	+	98	6.6
NT-YC	108 ± 9	142 ± 10	99	+	95	9.9
NT-YD	122 ± 9	109 ± 31	76	+	97	7.9
NT-YE	114 ± 8	137 ± 28	95	+	92	6.9

[a] All assays were performed with Rat1 fibroblasts unless indicated.
[b] Focus formation assays were performed with Rat1 cells as in Table 1, with at least four independent plasmid preparations. Relative transforming activity was calculated as in Table 1 (± standard error), using the mean values of the %NT transformation of each construct. Seven (control, NT, NT-NYPD, NT-YC, NT-YD, NT-YE) or five (NT-YA, NT-YB) independent experiments were conducted for each mutant.
[c] Colony formation in 0.25% agarose was assessed as in Materials and Methods. The mean number of colonies formed per 10^3 cells (± standard error) was obtained from two independent experiments each with five plates of duplicate cell lines. The cell lines used were Rat1, NT3 and 6, NT-NYPD-6 and -22, NT-YA23 and x4c, NT-YB6 and 7, NT-YC2 and 12, NT-YD6 and 22, and NT-YE2 and 4. The relative soft agar colony formation is the ratio of the mean number of colonies obtained for each mutant relative to NT multiplied by 100%.
[d] Percent DNA synthesis is the percentage of $BrdU^+$-staining cells in noninjected controls or Y13-259-injected cells. The cell lines used were Rat1, NT6, NT-NYPD 22, NT-YA23, NT-YB7, NT-YC2, NT-YD22, and NT-YE2.
[e] NIH 3T3 fibroblasts were electroporated with pCDNA3 (control) or pCDNA3-derived plasmids containing the indicated Neu mutants. After 3 weeks of G418 selection, 10^5 cells were plated in 0.25% agarose, and colony formation was assessed 4 weeks later. +, colony formation; −, lack of cell growth.
[f] Control plasmids pJ4Ω or pJNeu were used for focus-forming assays, and Rat1 cells were used in soft agar colony formation and DNA synthesis assays.

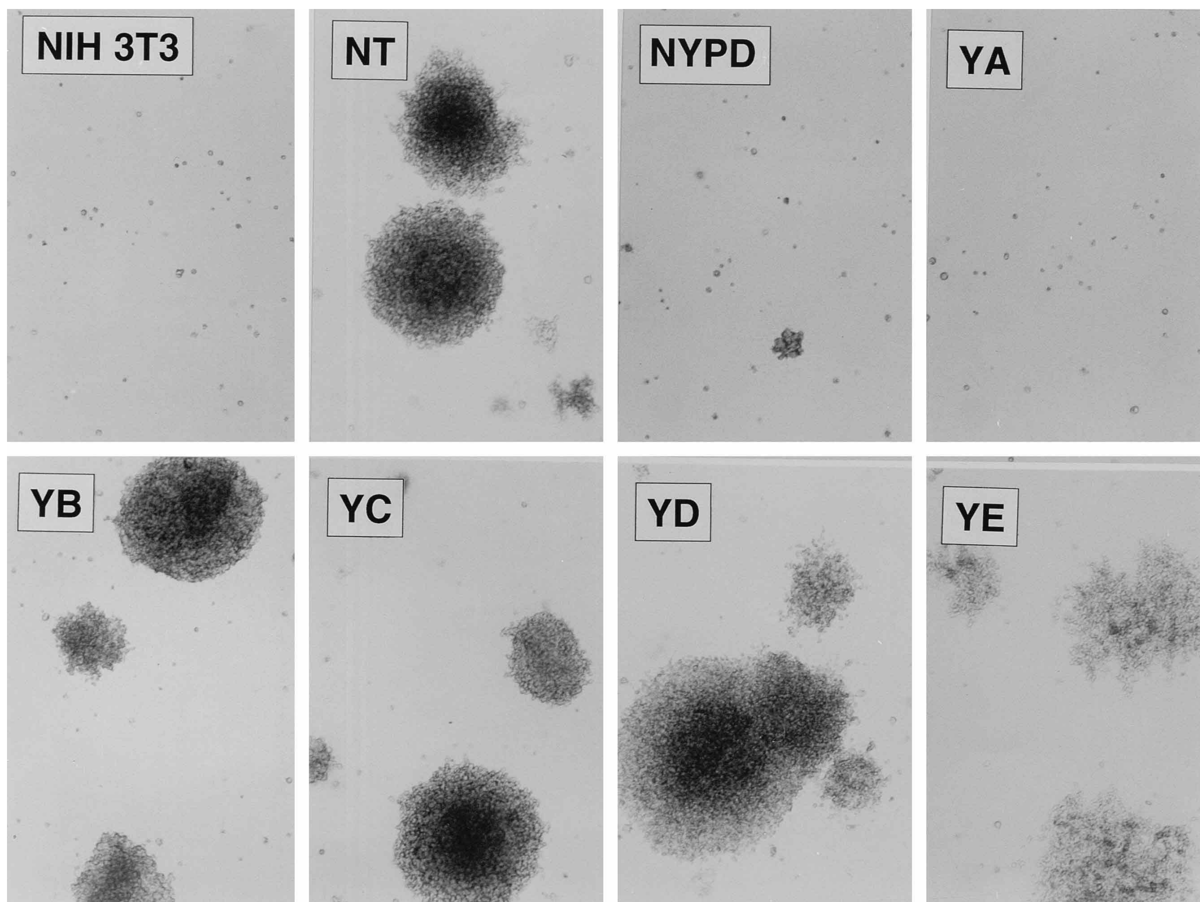


FIG. 2. Morphological transformation of NIH 3T3 fibroblasts expressing different add-back Neu mutants. Stable drug-resistant cells were seeded in soft agarose, maintained in a humidified incubator at 37°C for 21 days, and photographed. Shown are representative colonies of NIH 3T3 cells and derived pooled colonies expressing NT, NT-NYPD, or the indicated NT-derived add-back mutants. Each panel is at the same magnification.

directly to GRB2 or indirectly through the association of GRB2 with SHC proteins. To explore this hypothesis, we examined the capacity of the different add-back mutants to associate with GRB2. Lysates containing activated Neu, add-back mutants (NT-YA through NT-YE), and NT-NYPD were immunoprecipitated with Neu-specific antibodies and were subjected to immunoblot analyses with GRB2-specific antisera. The results showed that GRB2 was associated with Neu in immunoprecipitates from cell lines expressing activated Neu, NT-YB, and NT-YD (Fig. 4A, lanes 2, 5, and 7). By contrast to these observations, GRB2 binding to Neu in lines expressing NYPD, YA, YC, and YE mutants was weak (lanes 3, 4, 6, and 8). The inability to detect strong GRB2 binding to Neu in the NT-YA, NT-YC, NT-YE, and NT-NYPD cell lysates was not due to the lack of tyrosine-phosphorylated Neu, since comparable levels were found within each immunoprecipitate (Fig. 4B). Additionally, this differential binding was not due to differences in the amount of GRB2 in the various cell lines, since equivalent amounts were detected in these lysates (Fig. 4C). These observations demonstrate that the GRB2 adapter protein can physically associate with activated Neu through either tyrosine residues 1144 (B site) or 1226/27 (D site).

Because GRB2 can bind directly (2, 68) or indirectly (38) to receptor tyrosine kinases through its interaction with other adapter proteins, we examined whether the different point and add-back mutants could directly interact with the GRB2 adapter by probing an immunoblot containing Neu immunoprecipitates from these cell lines with a ^{32}P-radiolabeled GST-GRB2 protein. The results of this direct binding assay revealed that Neu derived from lysates containing activated Neu, NT-A, NT-C, NT-D, and NT-E point mutations (Fig. 5A, lanes 2, 3, and 5 to 7) were capable of directly interacting with radiolabeled GST-GRB2. By contrast, Neu immunoprecipitates derived from NT-B cell lysates contained similar levels of tyrosine-phosphorylated Neu (Fig. 5B, lanes 2 to 7) yet failed to bind radiolabeled GST-GRB2 (Fig. 5A, lane 4). These observations argue that tyrosine site B (tyrosine residue 1144) is necessary for the direct interaction of GRB2 with activated Neu in vitro.

To confirm that autophosphorylation site B was not only necessary but also sufficient for the binding of GRB2, immunoblots containing Neu immunoprecipitates of the NT-NYPD and add-back mutant cell lysates were probed with ^{32}P-radiolabeled GST-GRB2. Consistent with the analyses of the point mutations, only Neu immunoprecipitates from the NT-YB cell lines were able to bind efficiently to the GST-GRB2 probe (Fig. 5A, lane 10). The inability to detect binding of the GST-GRB2 probe to Neu from the NT-NYPD mutant or the NT-YA, NT-YC, NT-YD, and NT-YE lysates (lanes 8 and 9 and lanes 11 to 13) was not due to a lack of tyrosine-phosphorylated Neu, because comparable levels of the phosphoprotein could be detected in each immunoprecipitate (Fig. 5B, lanes 8 to 14). To ensure that the binding of the GST-GRB2 to site B was specific, increasing concentrations of protein lysates de-

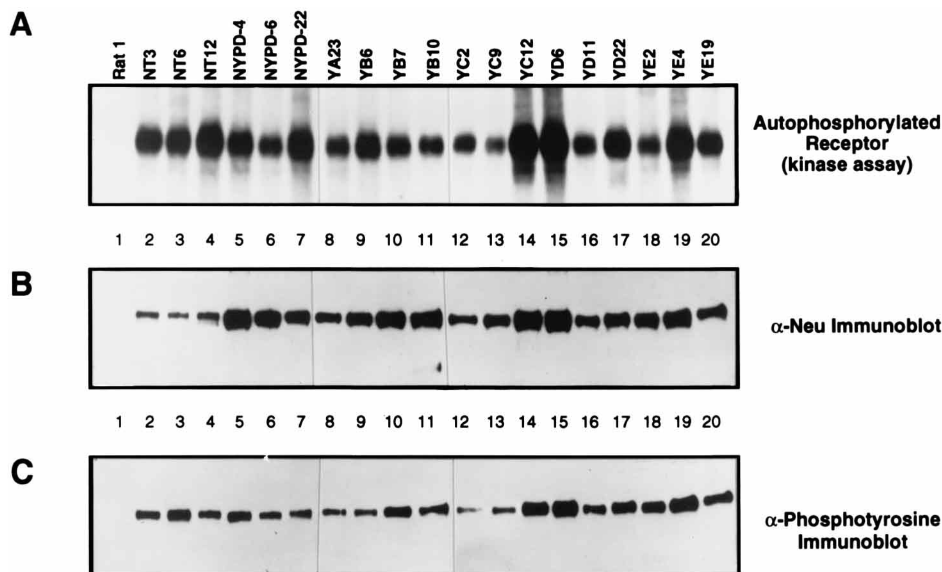


FIG. 3. Autophosphorylation mutants harbor kinase activity and are tyrosine phosphorylated in vivo. Neu was immunoprecipitated from lysates (containing 1.5 mg of protein) obtained from Rat1 or the Rat1-derived stable cell lines expressing the indicated mutant Neu molecules. (A) Two-thirds of each immunoprecipitate was used in an in vitro kinase assay, and the products were electrophoresed on an SDS–8.5% polyacrylamide gel (autophosphorylated receptor). (C) The remaining one-third was electrophoresed as above, transferred to a PVDF membrane, and probed with antiphosphotyrosine MAbs. (B) The secondary antibodies were inactivated, and the membrane was subsequently probed with anti-Neu antiserum.

rived from cell lines expressing wild-type activated Neu, NT-YB, and NT-NYPD proteins were tested for their capacity to bind the radiolabeled GRB2 fusion protein. As shown in Fig. 5C, neither the NT-NYPD nor the parental Rat1 Neu immunoprecipitates bound the GRB2 probe at protein concentrations ranging between 150 and 3,000 μg (lanes 2 and 12 to 14). By contrast, strong binding of the GRB2 probe was observed in Neu immunoprecipitates of extracts derived from the NT and NT-YB cell lines (lanes 4, 5, 8, and 9). As expected, nonspecific control containing p53 immunoprecipitates failed to bind the GRB2 fusion protein (lanes 1, 3, 6, and 11). Taken together, these observations suggest that phosphorylation of tyrosine 1144 is both necessary and sufficient for the direct binding of GRB2 to activated Neu.

Although the results of these direct blot experiments suggested that GRB2 could bind directly to tyrosine 1144 (site B, Fig. 5), the coimmunoprecipitation studies suggested that tyrosines 1226 and 1227 (site D) were also capable of indirectly interacting with GRB2 (Fig. 4, lanes 5 and 7). One potential mechanism by which this might occur is by indirect binding through an adapter protein. For example, GRB2 could couple with activated Neu through its interaction with the SHC adapter protein (57). To explore this possibility, protein extracts from cell lines expressing the various add-back mutants were immunoprecipitated with SHC-specific antisera and subjected to immunoblot analyses with Neu-specific antibodies. The results showed that Neu was found in SHC immunoprecipitates from cell lysates derived from the NT-YD and activated Neu (NT) molecules (Fig. 6A, lanes 2 and 7). By contrast, Neu was not detected in association with SHC immunoprecipitates in cell lysates derived from NT-NYPD-, NT-YA-, NT-YB-, NT-YC-, or NT-YE-expressing cell lines (lanes 3 to 6 and 8). The inability to detect Neu in these immunoprecipitates was not due to variations in the amounts of tyrosine-phosphorylated Neu or to the level of SHC proteins, since equivalent levels of the two proteins were detected in all Neu-expressing cell lines examined (Fig. 6B and D). Interestingly, coimmunoprecipitation analyses revealed that tyrosine-phosphorylated SHC (17) was complexed with GRB2 in cell lines expressing the Neu add-back mutations which were unable to associate with SHC (Fig. 6C, NT-YA, NT-YB, NT-YC, and NT-YE). Therefore, in cell lines expressing a catalytically active Neu, tyrosine phosphorylation of SHC does not appear to require its physical association with Neu. Taken together, these data suggest that the GRB2 found in Neu immunoprecipitates derived from the NT-YD cell line (Fig. 4A) is due to its interaction with SHC and further demonstrate that SHC associates with Neu through the receptor autophosphorylation site D (tyrosines 1226 and 1227).

Although these observations argued that tyrosines 1226 and 1227 were sufficient to bind SHC, it was unclear whether these residues were in fact necessary for this interaction. To explore this possibility, lysates derived from the cell lines expressing the different point mutants were immunoprecipitated with SHC antisera followed by immunoblot analyses with Neu-specific antibodies (Fig. 7A). The results demonstrated that Neu could be detected in SHC immunoprecipitates derived from the NT, NT-A, NT-B, NT-C, and NT-E cells (Fig. 7A, lanes 1, 3 to 5, and 7). By contrast, the SHC immunoprecipitates derived from the NT-D or parental Rat1 cell lines did not contain Neu (lanes 1 and 6). The inability to detect Neu in the SHC immunoprecipitates from the NT-D cell line was not due to reduced levels of tyrosine-phosphorylated Neu or SHC, since comparable levels of these proteins were detected in all cell lines (Fig. 7B and C). Taken together, these observations demonstrate that site D is required for Neu-SHC interactions.

To determine if SHC could directly interact with site D, immunoblots containing Neu immunoprecipitates from cell lines expressing the different add-back mutants were probed with a ^{32}P-radiolabeled GST-SHC fusion protein. Consistent with the coimmunoprecipitation studies, Neu derived from the lysates of NT and NT-YD cell lines strongly bound the radiolabeled GST-SHC protein (Fig. 8A, lanes 2 and 7). On longer exposure of the autoradiograph, weak binding of the radiola-

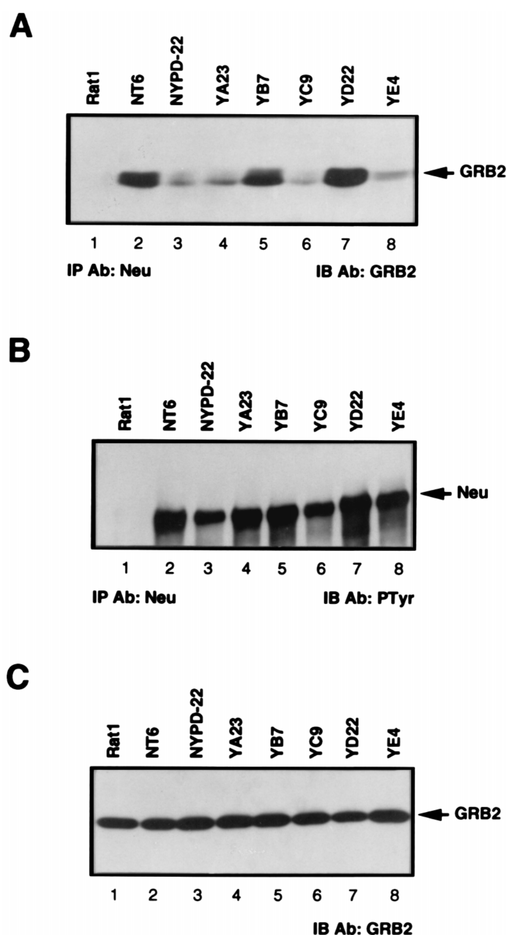


FIG. 4. GRB2 associates with Neu in vivo at distinct autophosphorylation sites. (A) Neu was immunoprecipitated (IP) from 2.5 mg of protein lysates of Rat1 (lane 1) or Rat1-derived cell lines (lanes 2 to 8). The immunoprecipitates were electrophoresed on an SDS–6 to 15% polyacrylamide gradient gel and transferred to a PVDF membrane. The membrane was cut, and the bottom half, containing proteins of 18 to 80 kDa, was probed with GRB2-specific rabbit polyclonal sera. The arrow indicates the position of GRB2. (B) The upper portion of the membrane was probed with antiphosphotyrosine (PTyr) antibodies (Ab), and the arrow indicates the migration of Neu. (C) Equivalent amounts (20 μg) of the same protein lysates were subjected to immunoblot (IB) analyses with GRB2-specific antisera.

beled SHC fusion protein to Neu was also observed in immunoprecipitates derived from the NT-YE cell line. The inability to detect binding to Neu derived from the remaining cell lines was not due to differences in the levels of tyrosine-phosphorylated Neu, since comparable levels could be detected in these immunoprecipitates (Fig. 8B). Taken together, these data demonstrate that in vitro and in vivo the primary site of interaction on Neu for SHC is site D (tyrosine residues 12226 and 1227).

Restoration of tyrosine phosphorylation site A (Y1028) interferes with *neu*-mediated transformation in a specific and *cis*-acting fashion. Although our observations suggest that four of the Neu tyrosine autophosphorylation sites are functionally redundant for *neu*-mediated transformation, restoration of tyrosine phosphorylation site A (tyrosine 1028) to the NYPD mutant abolished the basal transforming properties of this mutant (Table 2). Mutation of site A alone in activated Neu, however, results in a 1.4- to 2-fold activation of transforming activity (Table 1). These observations suggest that this tyrosine residue is involved in the negative regulation of Neu signaling. To further test the specificity by which site A interferes with *neu*-mediated transformation, the tyrosine residue at site A was restored in *cis* to the transforming NT-YB, NT-YC, NT-YD, and NT-YE mutants to create a series of double add-back mutations in which each resulting mutant contains phosphorylation site A and one of the remaining four sites (Fig. 9A). The specific transforming activities of these double add-back mutations were then assessed by using Rat1 fibroblasts. Restoration of site A to the NT-YB, NT-YC, and NT-YE mutants severely impaired the transforming activities of these mutants (Table 3). In particular, addition of site A to the NT-YB mutant (NT-YAB) virtually abolished its transforming activity whereas the NT-YAC and NT-YAE mutants were less severely impaired in their transforming activity (Table 3; Fig. 9B). Additionally, the morphology of foci that were scored in these double add-back mutants was pinpoint, in contrast to the larger foci observed in the parental add-back mutations (Fig.

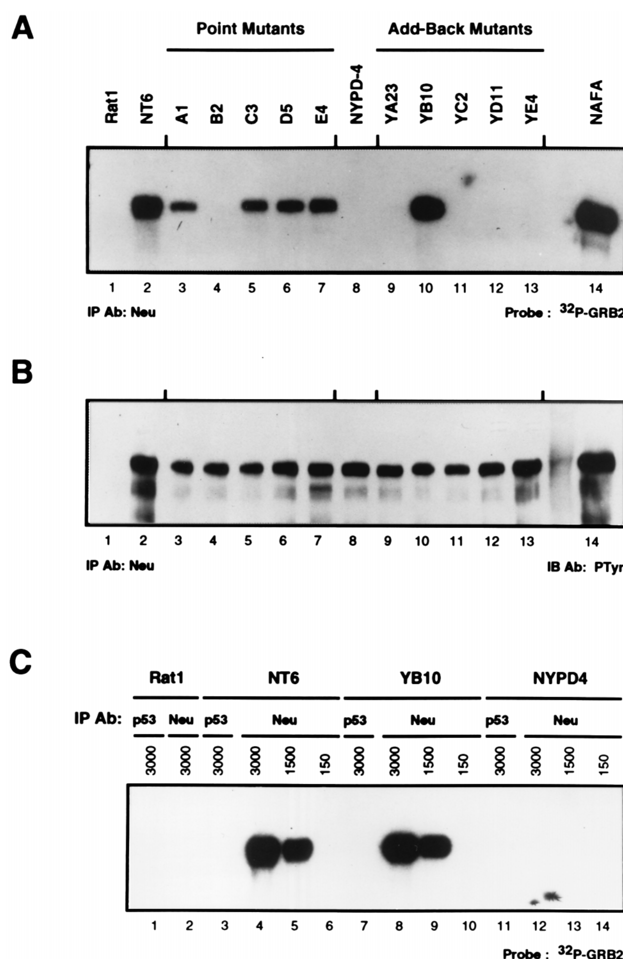


FIG. 5. GRB2 associates with Neu directly and specifically through tyrosine 1144 (site B). Neu was immunoprecipitated (IP) from 1.5 mg of protein lysate derived from the indicated cell lines. (A) Two-thirds of each precipitate was electrophoresed on an SDS-8.5% polyacrylamide gel, transferred to a PVDF membrane, and probed with a ^{32}P-radiolabeled GST-GRB2 fusion protein. (B) The remaining portion of the immunoprecipitate was subjected to immunoblot (IB) analyses with antiphosphotyrosine (PTyr) MAbs as in Fig. 2C. A marker was loaded between lanes 13 and 14. (C) Three or 1.5 mg or 150 μg of protein was immunoprecipitated with either p53 (lanes 1, 7, and 11)- or Neu (lanes 2, 3, 4 to 6, 8 to 10, and 12 to 14)-specific antibodies from the lysates of the indicated cell lines. The immunoprecipitates were subjected to direct blot analyses with ^{32}P-labeled GST-GRB2 as in panel A.

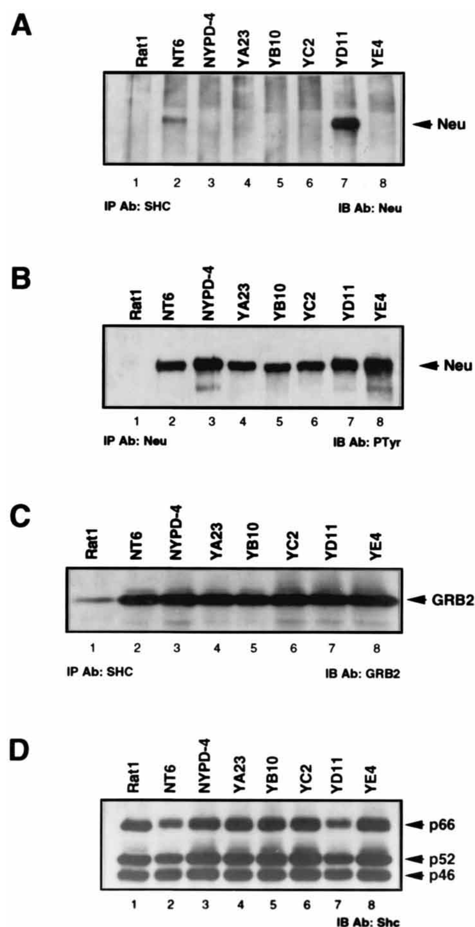


FIG. 6. SHC associates with Neu in vivo at a single autophosphorylation site. (A) SHC proteins were immunoprecipitated (IP) from 1.0 mg of protein lysates derived from the indicated cell lines. The immunoprecipitates were electrophoresed on an SDS–6 to 15% polyacrylamide gradient gel and transferred to a PVDF membrane, which was later cut in half. The top half, containing proteins of 80 kDa and larger, was probed with Neu-specific MAbs. The migration of Neu is indicated. (B) Neu was immunoprecipitated from 500 μg of the same lysates, and the immunoprecipitates were subjected to antiphosphotyrosine (PTyr) immunoblot (IB) analyses as in Fig. 2C. Indicated is the migration of Neu. (C) The lower portion of the membrane in panel A, containing proteins of 18 to 80 kDa, was probed with GRB2-specific MAbs. (D) Equivalent amounts (20 μg) of the same protein lysates were subjected to immunoblot analyses with SHC-specific polyclonal antibodies. Indicated are the 66-, 52-, and 46-kDa forms of SHC.

9B). In contrast to these observations, addition of site A to the NT-YD mutant resulted in a twofold reduction in its specific transforming activity, and, unlike the pinpoint foci detected in the other double add-back mutations, the foci induced by NT-YAD were morphologically comparable to those induced by activated *neu* (Fig. 8B). Thus, unlike for the other add-back mutants, the inclusion of site A has a modest effect on the ability of the NT-YD mutant to transform Rat1 cells. Taken together, these data suggest that site A interferes with Neu-mediated transformation in a *cis*-acting and specific fashion.

The inhibition of transformation induced by the inclusion of tyrosine 1028 (site A) to the transforming add-back mutants (YB) may be a result of a reduced catalytic activity of these double add-back mutants. To address this possibility, we determined the kinetic activities of the NT-YB and NT-YAB mutants in immunoprecipitation kinase assays with an exogenous substrate, myelin basic protein, as an external substrate. The results of these analyses revealed that the NT-YAB mutant displayed activities comparable to activated NT and NT-YB, arguing that differences in transforming activity cannot be accounted for by major differences in catalytic activity (17).

Another possible mechanism by which site A may interfere with Neu-mediated transformation is by interfering with the ability of Neu to couple with its downstream substrates. To explore this possibility, we tested whether double add-back mutants that couple site A to either the GRB2 binding site (NT-YAB) or SHC binding site (NT-YAD) interfered with the capacity of these sites to bind GRB2 or SHC by performing coimmunoprecipitation analyses. As previously determined, GRB2 was absent in Neu immunoprecipitates derived from Rat1 or NT-NYPD-expressing cells but was abundant in those derived from NT, NT-YB, and NT-YD expressors (Fig. 10C). Significantly, GRB2 binding to NT-YAB or NT-YAD appeared to be consistently reduced relative to GRB2 binding to NT-YB and NT-YD (Fig. 10C, compare lanes 4 and 6 to lanes 5 and 7, respectively). These differences cannot be accounted for by differences in the levels of GRB2 expressed in these cell lines (Fig. 10E) or in the amount of tyrosine-phosphorylated Neu found in each immunoprecipitate (Fig. 10A), since comparable amounts were detected. The reduction in the amount of GRB2 bound to the NT-YAD mutant appears to be a result of a decreased amount of SHC bound to the receptor (Fig. 10B) and was not due to differences in the amount of GRB2 bound to SHC, since equivalent amounts of complexed SHC-GRB2 were detected from each of these cell lines (17). Moreover, the differences in binding of SHC between the NT-YD and NT-YAD mutants cannot be attributed to differences in the levels of SHC (Fig. 10D). Taken together, these data dem-

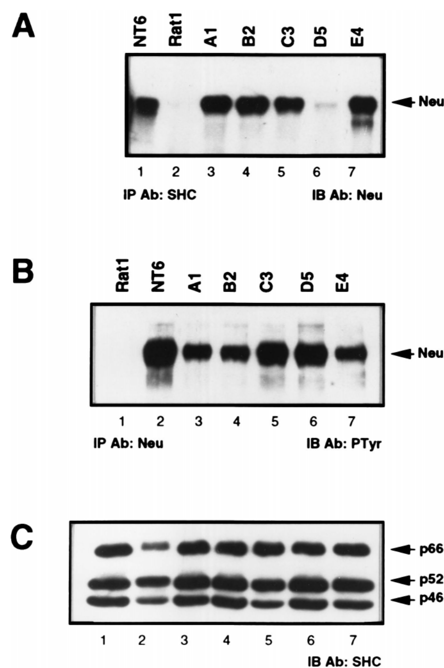


FIG. 7. SHC association with Neu requires a single autophosphorylation site (site D). (A) SHC proteins were immunoprecipitated (IP) and immunoblotted (IB) for Neu as in Fig. 6A. (B) Neu was immunoprecipitated from 500 μg of the same lysates, and the immunoprecipitates were subjected to antiphosphotyrosine (PTyr) immunoblot analyses. Indicated is the migration of Neu. (C) Equivalent amounts (20 μg) of the same protein lysates were subjected to immunoblot analyses with SHC-specific polyclonal antibodies. Indicated are the 66-, 52-, and 46-kDa forms of SHC.

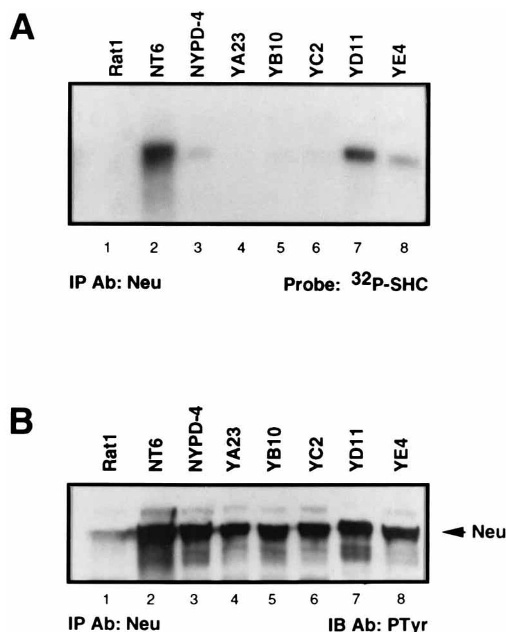
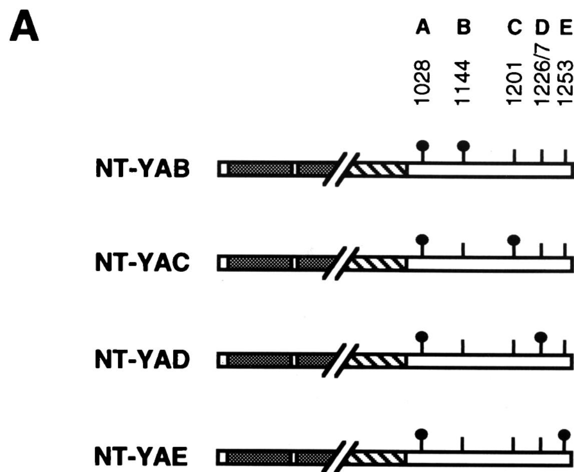


FIG. 8. SHC associates with Neu directly at site D. (A) Neu was immunoprecipitated from 2.0 mg of protein lysate derived from the indicated cell lines. Three-fourths of the immunoprecipitate was electrophoresed on an SDS–8.5% polyacrylamide gel, transferred to a PVDF membrane, and probed with a ^{32}P-radiolabeled GST-SHC fusion protein. (B) The remaining portion of the immunoprecipitate (IP) was subjected to immunoblot (IB) analyses with antiphosphotyrosine (PTyr) MAbs. The arrow indicates the migration of Neu.

onstrate that site A appears to modulate the ability of both SHC and GRB2 to couple to the Neu receptor.

DISCUSSION

The ability of activated receptor tyrosine kinases to signal cellular proliferation and differentiation is dependent on their capacity to couple to a variety of cytoplasmic signaling molecules through mechanisms involving the binding of either SH2 or PTB domain-containing proteins to specific phosphotyrosine residues. To identify the signaling pathways involved in *neu*-mediated transformation, we derived a series of mutants with mutations in the carboxyl terminus of Neu in which the known tyrosine autophosphorylation sites were converted to phenylalanine residues by site-specific mutagenesis. Using this approach, we generated a series of point mutants in which individual tyrosine residues were altered (Fig. 1B). In addition, we constructed add-back mutants in which individual tyrosine residues were reconstituted within a mutant *neu* molecule possessing mutations in all the known tyrosine phosphorylation sites (NT-NYPD [Fig. 1C]). Analyses of the transforming potential of the point mutations revealed that alteration of individual tyrosine residues had a minimal effect on the transforming potential of activated *neu* (Table 1). For example, alteration of sites B (tyrosine 1144), C (tyrosine 1021), D (tyrosines 1226/1227), and E (tyrosine 1253) resulted in transformation efficiencies of 75 to 85% of those of activated *neu* (Table 1). Conversely, alteration of tyrosine phosphorylation site A (tyrosine 1028) resulted in a modest but reproducible 1.4- to 2-fold elevation of the transforming potential of activated *neu*. Although these results suggest that alteration of any single tyrosine residue has a minimal effect on *neu*-mediated transformation, mutation or deletion of all of these sites results in a dramatic reduction in the transforming potential of activated *neu* (NT-NYPD and NT-CT [Tables 1 and 2]). In fact, restoration of tyrosine residues at position B, C, D, or E to NT-NYPD completely restores the transforming potential of activated *neu*, suggesting that Neu could induce cellular transformation through multiple functionally redundant tyrosine phosphorylation sites (Table 2). Kinetic analyses failed to reveal significant differences in the kinase activities of NT and NT-NYPD toward an exogenous substrate, suggesting that the differences in the transforming activities of NT-NYPD and the transforming add-back mutants cannot be attributed to this



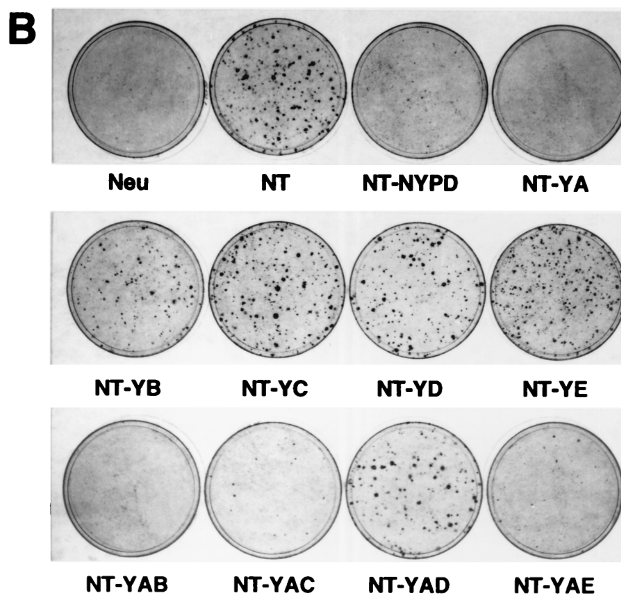


FIG. 9. Transformation of mutant Neu molecules is suppressed by site A. (A) Illustrated are a series of mutant Neu molecules (double add-back mutants), each containing a tyrosine at residue 1028 and a single tyrosine at one of the remaining autophosphorylation sites. A mutant containing tyrosines at 1028 and 1144 and phenylalanines at residues 1201, 1226/1227, and 1253 is termed NT-YAB. Note that all mutants contain the activating transmembrane mutation. (B) Representative plates from focus assay 2 (Table 3) are shown to illustrate the relative transforming abilities and morphologies of the add-back mutants compared to activated Neu (NT) (top and middle panels). The bottom panel illustrates the transformation repression mediated by site A in a series of double add-back mutants.

TABLE 3. Suppression of transformation through site A in Rat-1 fibroblasts[a]

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

[a] Two independent focus-forming assays were performed with Rat-1 fibroblasts. Independent plasmid preparations were used for each experiment.
[b] Values represent the mean number of foci per plate counted on six plates ± standard deviation.
[c] Values are the ratios of the mean numbers of foci obtained for each construct with respect to that obtained with NeuNT (NT) multiplied by 100%.
[d] Values are the weighted mean transforming abilities from the two experiments ± standard deviation.
[e] NA, not available.

property of the kinase. We cannot, however, discount the possibility that there are subtle alterations in the catalytic activities or specificities, which are not revealed by standard analyses. Interestingly, restoration of site A to the same mutant *neu* molecule completely suppressed the basal transforming activity of this mutant molecule (Table 2). Thus, in contrast to the other tyrosine phosphorylation sites, tyrosine phosphorylation at site A (Y1028) appears to play a negative regulatory role in *neu*-mediated transformation (Fig. 11).

Other groups have reported that deletion of the carboxyl terminus of Neu ablates the transforming activity of *neu* (18, 45, 60, 61). More recently, Ben-Levy et al. attempted to identify the important tyrosine residues involved in transformation by grafting oligonucleotides encoding individual tyrosine autophosphorylation sites to a cDNA encoding a transformation-defective *neu* molecule possessing a carboxyl-terminal truncation (8). By using this approach, only the sequence encoding tyrosine phosphorylation site E (tyrosine 1253) was able to restore the transforming activity of activated *neu*. By contrast, we have found that three other tyrosine phosphorylation sites (tyrosine 1144 [site B], tyrosine 1201 [site C], and tyrosines 1226/1227 [site D]) can efficiently mediate a transforming signal in the context of a full-length molecule. There are several possible explanations for these discordant observations. For example, it is conceivable that the inability of the other sites to functionally substitute in this former study reflects the fact they are not efficiently used as phosphorylation sites due to either their sequence or spatial context. Alternatively, if these sites are tyrosine phosphorylated, their inability to induce cellular transformation may reflect an inability to couple properly with their cognate substrates. Furthermore, it is also possible that sequences located outside these phosphorylation sites not included in these grafted mutants contribute to high-efficiency coupling of substrates with activated Neu. Whatever the explanation, our data demonstrate that Neu can induce cellular transformation through multiple functionally redundant tyrosine phosphorylation sites.

The observed functional redundancy between the various autophosphorylation sites in *neu*-mediated transformation can be accounted for in part by their capacity to bind different adapter molecules that allow coupling of the activated Neu receptor to the Ras pathway (8). We have presented evidence that GRB2 can bind directly to activated Neu through tyrosine 1144 (site B) and indirectly through its interaction with the SHC adapter protein through tyrosines 1226 and 1227 (site D). These observations extend earlier data showing that Neu can form physical complexes with both the GRB2 and SHC adapter proteins (33, 34, 62). Consistent with these analyses, it has recently been demonstrated that point mutation of the comparable site to B in c-ErbB-2 (tyrosine 1139) interferes with the capacity of this receptor to couple with GRB2 in vitro (53). Our data further demonstrates that this site not only is required for binding of GRB2 but also is sufficient for its direct association with GRB2 (Fig. 4 and 5). These observations are also consistent with recent studies conducted with the closely related EGFR. In these studies, indirect peptide competition analyses suggested that the principal binding site for GRB2 on the EGFR is tyrosine 1068 (7). Interestingly, the sequence following this phosphotyrosine residue closely resembles those found following tyrosine 1144 in Neu (PVPE^{P}YINQ and PQPE^{P}YVNQ, respectively). Moreover, the GRB2 SH2 domain displays a strong preference for peptides containing an asparagine in the +2 position, relative to the phosphorylated tyrosine (66, 67), as is found at site B. Taken together with our observations, these data suggest that site B is sufficient to direct GRB2 binding and is functionally involved in *neu*-induced cellular transformation.

We have also presented evidence that activated Neu can indirectly recruit GRB2 through its interaction with SHC at site D (tyrosines 1226 and 1227) (Fig. 11). Consistent with these analyses, Kavanaugh et al. have recently provided indirect peptide competition evidence that the site analogous to D (Y1222) in c-ErbB-2 is the primary site of interaction of c-ErbB2 with the PTB domain of SHC (34). Lower-affinity interaction with sites analogous to C and E (Y1196 and Y1248, respectively) were also observed (34). Similar in vitro studies with point mutants of c-ErbB-2 have suggested that sites C, D, and E are the major determinants required for SHC binding (53). We have further demonstrated that, in vivo, physical complexes of activated Neu and SHC require phosphorylation of site D alone (Fig. 6). However, our data strongly suggest that phosphorylation of site D is both sufficient and necessary

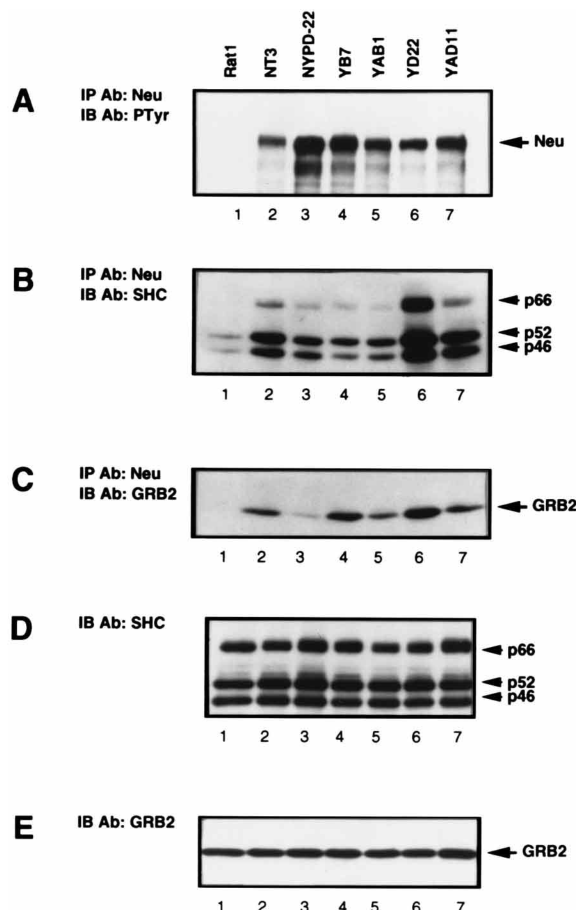


FIG. 10. Site A decreases the binding of GRB2 to sites B and D in vivo. (A) Neu was immunoprecipitated from 2.0 mg of protein lysates of Rat1 (lane 1) or Rat1-derived cell lines (lanes 2 to 7). The immunoprecipitates (IP) were electrophoresed on an SDS–6 to 15% polyacrylamide gradient gel and transferred to a PVDF membrane. The membrane was cut, and the top third, containing proteins larger than 80 kDa, was probed with antiphosphotyrosine (PTyr) antibodies. (B and C) The middle portion of the blot, containing proteins from 32 to 80 kDa, was probed with SHC-specific antisera (B), and the remaining third, containing proteins smaller than 32 kDa, was probed with GRB2-specific rabbit polyclonal sera (C). (D and E) Equivalent amounts (20 μg) of the same protein lysates were subjected to immunoblot (IB) analyses with SHC-specific (D) or GRB2-specific (E) antisera.

to bind SHC (Fig. 6 to 8). Although mutation of site D involves the conversion of two sequential tyrosine residues located at positions 1226 and 1227, it is likely that tyrosine residue 1227 is the tyrosine residue responsible for binding SHC, since this latter residue would conform to the reported SHC-PTB consensus binding site NXXY (6, 34). It should be noted that although early indications suggested that an NPXY motif was required for SHC-PTB binding, a proline-to-leucine alteration at the −2 position allows for the SHC-PTB interaction (36). Consistent with this conclusion, we have recently observed that mutation of the conserved asparagine residue in this motif abolishes SHC binding to Neu and also results in impairment in the transforming potential of the NT-YD mutant (17).

Although our data demonstrate that site D is the primary site of interaction with SHC, mutants incapable of binding SHC are still capable of inducing SHC tyrosine phosphorylation (17) and inducing the formation of SHC-GRB2 complexes (Fig. 6C). One possible explanation for these observations is that these mutant receptors may activate Src family members which then in turn phosphorylate SHC proteins. This notion is supported by the finding that NT-NYPD and each add-back mutant remain capable of binding Src (17). Alternatively, it is theoretically possible that the observed tyrosine phosphorylation of SHC in the absence of direct interaction is due to heterodimerization of these mutant Neu molecules and the other ErbB family members. Regardless of the mechanism involved, tyrosine phosphorylation of SHC and consequent GRB2 association are not sufficient to induce wild-type levels of transforming activity, since the NT-NYPD and NT-YA mutants are severely impaired in their transforming activity (Table 2).

The observation that Neu-mediated transformation can be mediated through two sites that couple to distinct adapter proteins impinging on the Ras pathway has important implications for our understanding of the molecular mechanism by which *neu* transforms mammalian cells. Our observations argue that recruitment of either the SHC or GRB2 adapter proteins to the activated receptor through these distinct sites is functionally equivalent in inducing cellular transformation. Consistent with this hypothesis, it has recently been demonstrated that overexpression of SHC can complement a mutant *BCR-ABL* oncogene defective for GRB2 binding for transformation of hematopoietic and fibroblast cell lines (24). However, because two of the transformation-competent add-back mutants (NT-YC and NT-YE) do not appear to associate with either SHC or GRB2, it is likely that other alternative signaling pathways are also involved in Neu-mediated transformation. Because mitogenesis in cell lines expressing these different add-back mutants also appears to require Ras function (Table 2), these signaling proteins probably impinge on the Ras signaling pathway (Fig. 11). For example, adapter molecules such as NCK and CRK have been implicated in Ras activation and are potential candidates (30, 42).

In addition to the identification of tyrosine phosphorylation sites that are positively involved in transformation, we have identified a tyrosine phosphorylation site (site A, tyrosine 1028) that suppresses the transforming potential of activated *neu*. Evidence supporting this contention stems from the observation that mutation of site A results in a moderate but consistent elevation of the transforming potential of activated *neu* (Table 1). Conversely, restoration of site A to the NT-NYPD mutant or NT-YB, NT-YC, and NT-YE severely suppressed the transforming activities of these molecules (Tables 2 and 3). Although the transforming activities of these add-back mutants are severely affected in *cis* by addition of site A, restoration of site A to the NT-YD mutant resulted in a 50% decrease in its capacity to induce morphological transformation (Table 3; Fig. 9). Biochemical analyses suggest that the transformation defect observed in these double add-back mutants can be explained in part by interference with its capacity to couple with GRB2 and SHC (Fig. 10B and C). Perhaps the relative resistance of the NT-YD mutant to the negative regulatory effects of site A reflects its binding to a PTB domain rather than the SH2-phosphotyrosine interactions that may occur at the other phosphorylation sites. In this regard, it has been reported that the SHC PTB domain has similar affinities but considerably slower kinetics for tyrosine-phosphorylated peptides than does a typical SH2 domain (36), suggesting that PTB domains remain bound, unlike SH2 domains, which are thought to exchange rapidly. Alternatively, SHC may activate other signaling pathways that are refractory to the inhibitory influence of site A. Indeed, evidence suggests that SHC may also function to induce c-*myc* expression independent of its ability to activate the Ras pathway (26). Additional evidence

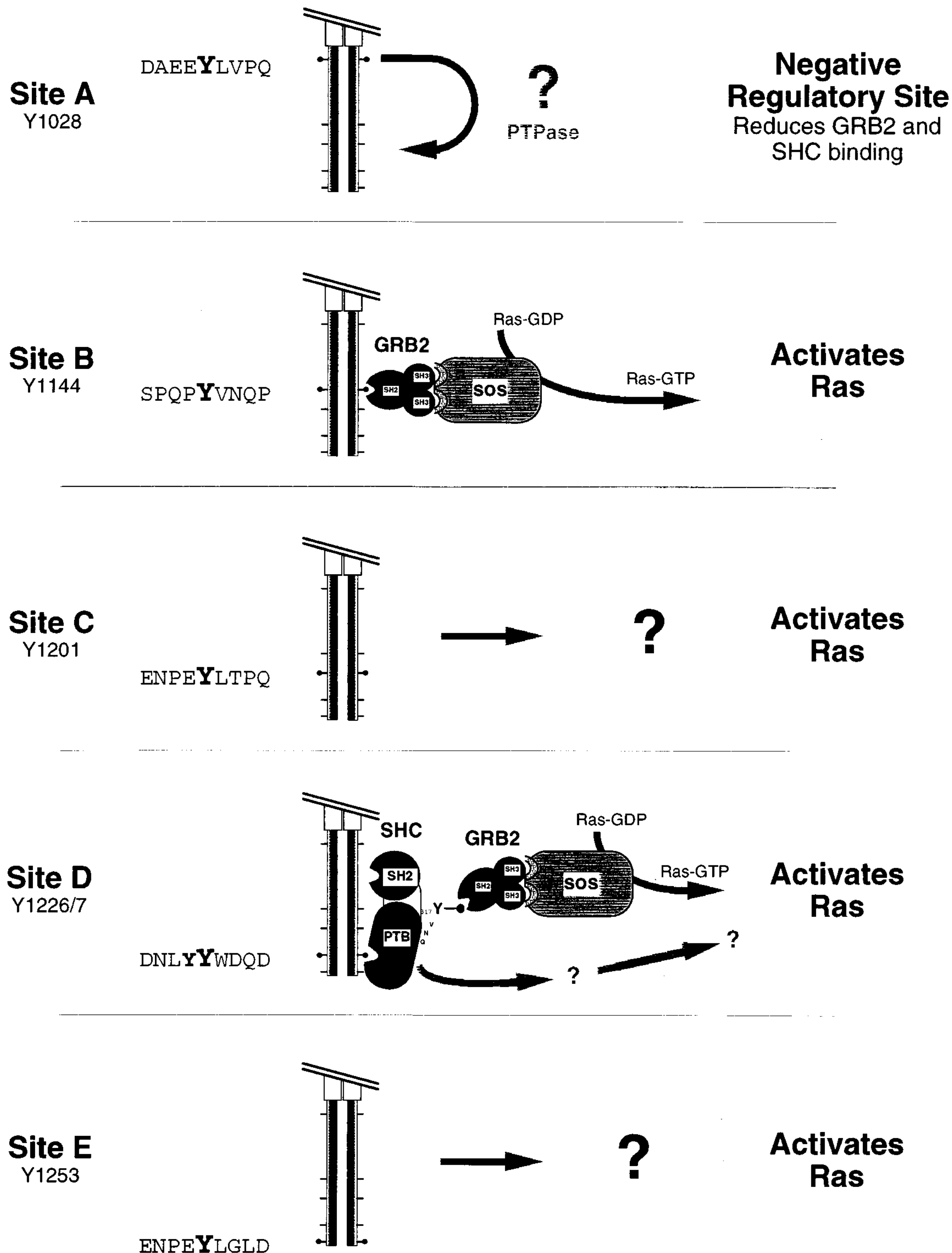


FIG. 11. Schematic representation of both negative and positive signals which emanate from Neu autophosphorylation sites. Indicated are the amino acid sequences surrounding each autophosphorylation site, the proteins identified to bind these sites, and the possible mechanism by which the individual sites function.

supporting a GRB2-independent signal emanating from SHC derives from the observation that while microinjection of a dominant inhibitory GRB2 SH2 domain into NT-YB cells virtually abolishes the induction of DNA synthesis, it has little effect on NT-YD cells (72). Whether this GRB2-independent pathway is refractory to the inhibitory effects of site A awaits further experimentation. Moreover, future studies with these different add-back mutants will provide important insight into the nature and mechanism of the site A inhibitory effect.

The presence of negative regulatory tyrosine autophosphorylation sites in other growth factor receptors has been described. For example, alteration of a tyrosine residue in the carboxyl terminus of the c-*fms* receptor tyrosine kinase can result in its biological activation, although this residue has not

been identified as an autophosphorylation site (55). More recently, mutation of the comparable tyrosine residue to site A in the EGFR (tyrosine 992) appeared to increase EGF-induced mitogenesis from the EGFR (25). Circumstantial evidence indicates a potential mechanism which proposes that increased PLCγ1 binding to this residue through the use of an EGFR-TRK chimeric receptor tyrosine kinase resulted in a decreased ability to induce proliferation. Interestingly, this correlated with an increased mitogen-activated protein kinase activity and receptor dephosphorylation, implicating the existence of a PLCγ1-induced protein tyrosine phosphatase (50). Alternatively, site A may function to down-regulate Ras activity through Ras-GAP. Indeed, activated Neu is known to associate with Ras-GAP (22, 32). A series of studies with autophosphorylation mutants of the *Drosophila torso* receptor tyrosine kinase suggest that one of the tyrosine autophosphorylation sites (Y319) is involved in generating a negative signal that interferes with *torso* function (14). Moreover, these studies suggested that the binding of either mammalian Ras-GAP or PLCγ1 to Torso at this tyrosine autophosphorylation site in vitro correlates with the induction of a negative developmental signal emanating from this site. Interestingly, the sequences surrounding Y319 in Torso (EELYLEPL), Y992 in EGFR (DEYLIPQ) and Y1028 in Neu (EEYLVPQ) are strikingly similar and are consistent with Ras-GAP and PLCγ1 binding these sites (66).

Alternatively, it is conceivable that site A activates a tyrosine phosphatase. In the erythropoietin- and immunoglobulin G-Fc binding (FcgRIIB) receptors, mutation of specific tyrosine phosphorylation sites results in hypersensitivity to ligand stimulation and prolonged receptor-associated tyrosine kinase activity. It is intriguing that in both these receptors, these phosphorylation sites are responsible for recruitment of the PTP1C tyrosine phosphatase (also known as SHP-1 and SH-PTP1) (16, 35). Although there appears to be little sequence similarity between these receptors and Neu, specific complexes of PTP1C and Neu have been detected in certain cell types (71). Moreover, evidence indicates that binding of PTP1D (also known as SH-PTP2 and SHP2) to the PDGFR results in its increased phosphatase activity and that this increase could be reproduced in vitro by incubating purified PTP1D with tyrosine-phosphorylated peptides containing the PTP1D binding site. In this regard, treatment of NT-YAB cells with sodium orthovanadate results in increased GRB2 binding to levels comparable to that found in treated NT-YB cells (17). However, determination of the precise molecular mechanism by which site A functions to down-regulate Neu-mediated transformation will require further eperimentation.

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