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Mutations affecting conserved cysteine residues within the extracellular domain of Neu promote receptor dimerization and activation

(receptor tyrosine kinase/transformation)

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ABSTRACT **Overexpression of the Neu/ErbB-2 receptor tyrosine kinase has been implicated in the genesis of human breast cancer. Indeed, expression of either activated or wild-type *neu* in the mammary epithelium of transgenic mice results in the induction of mammary tumors. Previously, we have shown that many of the mammary tumors arising in transgenic mice expressing wild-type *neu* occur through somatic activating mutations within the *neu* transgene itself. Here we demonstrate that these mutations promote dimerization of the Neu receptor through the formation of disulfide bonds, resulting in its constitutive activation. To explore the role of conserved cysteine residues within the region deleted in these altered Neu proteins, we examined the transforming potential of a series of Neu receptors in which the individual cysteine residues were mutated. These analyses indicated that mutation of certain cysteine residues resulted in the oncogenic activation of Neu. The increased transforming activity displayed by the altered receptors correlated with constitutive dimerization that occurred in a disulfide bond-dependent manner. We further demonstrate that addition of 2-mercaptoethanol to the culture medium interfered with the specific transforming activity of the mutant Neu receptors. These observations suggest that oncogenic activation of Neu results from constitutive disulfide bond-dependent dimerization.**

Oncogenic activation of growth factor receptors can be mediated by the overexpression of either the ligand or receptor, as well as mutations that promote ligand-independent dimerization or catalytic activation. The Neu receptor tyrosine kinase is one such growth factor receptor that has been shown to be activated by several of these mechanisms (1). Neu is a member of the epidermal growth factor receptor (EGFR) family, which includes the EGFR (ErbB-1), Neu (ErbB-2), ErbB-3, and ErbB-4 receptor tyrosine kinases (2–7). Although a specific ligand that binds directly to Neu has yet to be cloned, several reports suggest that a biochemical activity known as the Neu activating factor may be one such candidate (8, 9). Activation of Neu can occur through the formation of heterodimers with other members of the EGFR family following stimulation with ligands specific for EGFR, ErbB-3, and ErbB-4 (10–14). Overexpression of the wild-type Neu receptor has also been reported to overcome the need for ligand activation and increases the transforming ability of the receptor (15). Finally, mutational activation of Neu has been described; both point mutation (16) or deletion of the entire extracellular domain constitutively activates the receptor (17). The best characterized of these mutations is the transmembrane point mutation that converts a valine residue to glutamic acid at amino acid position 664 (16). This particular mutation has been shown to increase the kinase activity of Neu (18–20) by promoting receptor dimerization (21).

More recently, we have described the occurrence of novel mutations within the extracellular domain of Neu in mammary tumors derived from transgenic mice expressing a mouse mammary tumor virus (MMTV)/wild-type *neu* fusion gene (22, 23). Although these experiments demonstrated that the mutations occurring within the *neu* transgene were capable of increasing the transforming ability of Neu, the precise molecular mechanism by which this occurs has yet to be addressed. Because the well-characterized point mutation in the transmembrane domain (16) has been shown to induce receptor dimerization (21), we examined the possibility that these mutations within the extracellular domain could function in a similar manner. Here we demonstrate that dimers of these altered Neu receptors can be detected when immunoprecipitates are separated under nonreducing conditions but are undetectable when a reducing agent is present. Because many of the deletions affect specific conserved cysteine residues in the juxtatransmembrane region of Neu, we investigated the importance of these cysteine residues by creating a series of point mutations and measuring their specific transforming activity in Rat-1 cells. Like deletions, certain mutations affecting these cysteine residues were capable of activating the transforming ability of Neu by promoting receptor dimerization. The importance of disulfide bonding in receptor dimerization was further reinforced by the observation that the transforming activity of these mutant Neu molecules was impaired by the addition of reducing agents. Taken together, our observations suggest that oncogenic activation of these Neu mutants involves constitutive dimerization of the receptor through the formation of cysteine disulfide bonds.

MATERIALS AND METHODS

DNA Constructs. Generation of the pJ4Ω plasmids containing various forms of the rat *neu* cDNA have been described (22). The cysteine mutations were created by polymerase chain reaction (PCR) following previously described methods (22, 24). The resulting PCR products containing the various cysteine mutations were cloned into the wild-type *neu* cDNA as *Bst*1107I/*Eag*I fragments. The amplified regions were sequenced to ensure that only the desired mutations were present. A plasmid (PGK-puro) containing the puromycin resistance gene under the transcriptional control of the phosphoglycerate kinase promoter was a generous gift of Michael A. Rudnicki (McMaster University, Hamilton, ON, Canada). The plasmid pJ4ΩmT was kindly provided by Marc A. Webster (McMaster University) and was constructed by releasing the polyomavirus middle T antigen cDNA from the plasmid

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Abbreviations: EGFR, epidermal growth factor receptor; MMTV, mouse mammary tumor virus.

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pMMTV MT (25) as a *Hin*dIII-*Eco*RI fragment and inserting it into the corresponding sites of pJ4Ω.

Cell Lines and Focus Assays. Rat-1 fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin, streptomycin, and fungizone. Stable cell lines expressing the Neu cysteine mutants were derived by electroporation as described (22). DNA was introduced into the Rat-1 cells at a 10:1 ratio of expression plasmid to puromycin resistance plasmid (PGK-puro) and resistant colonies were selected in media containing 3.0 μg of puromycin per ml. The focus assays were performed as previously outlined (22). When 2-mercaptoethanol was included, the cells were allowed to reach a monolayer prior to adding the reducing agent. The monolayer was maintained for 12 days in DMEM supplemented with 5% fetal bovine serum, penicillin, streptomycin, and fungizone.

Immunoprecipitations and Immunoblotting. Detection of dimer formation between Neu receptors harboring either deletions or cysteine point mutations was performed as follows. Confluent 100-mm plates of Rat-1 cells expressing the various forms of Neu were washed twice with ice cold 1× PBS containing 10 mM iodoacetamide. The cells were lysed for 20 min on ice in TGP buffer (1% Triton X-100/10% glycerol/50 mM Hepes, pH 7.4/1 mM sodium orthovanadate/10 μg of aprotinin per ml/10 μg of leupeptin per ml) containing 10 mM iodoacetamide. Lysates were cleared by centrifugation at 4°C for 10 min, and protein concentrations were determined by the Bradford assay (Bio-Rad). Immunoprecipitations were carried out in duplicate by using 200 μg of total protein that was incubated with anti-Neu antibodies (7.16.4) conjugated to protein G-Sepharose beads (Pharmacia). Immunoprecipitations were carried out at 4°C for 3–4 h with gentle agitation. Neu immunoprecipitates were washed three times in Ripa lysis buffer (1% Triton X-100/0.1% SDS/1% sodium deoxycholate/10 mM $NaPO_4$/150 mM NaCl/2 mM EDTA/50 mM NaF/1 mM sodium orthovanadate/10 μg of aprotinin per ml/10 μg of leupeptin per ml) and one set was resuspended in SDS gel loading buffer (62.5 mM Tris·HCl, pH 6.8/2% SDS/5% glycerol/0.25% bromophenol blue) containing 0.8 M 2-mercaptoethanol while the duplicate set was resuspended in SDS gel loading buffer lacking any reducing agent. Immunoprecipitates that were subjected to either reducing or nonreducing conditions were boiled for 10 min, and the proteins were separated by electrophoresis through 4–12% gradient SDS polyacrylamide gels. The proteins were transferred and the membranes probed as described (26).

RESULTS

Mutations Within the *neu* Protooncogene Are Clustered Within a Cysteine Rich Region of the Extracellular Domain That Is Conserved Among the Four EGFR Family Members. The mutations initially discovered in tumors arising in MMTV/wild-type *neu* transgenic mice reside in the extracellular domain of the Neu receptor itself (22). These altered receptors are capable of mediating transformation as measured by focus forming assays in Rat-1 fibroblasts (ref. 22; Fig. 1*A*). Alignment of these deletions revealed that they affect three of five cysteine residues located in the juxtatransmembrane region of Neu. In the one case where all five cysteine residues were retained (NeuX3, Fig. 1*A*), the deletion removed a conserved proline residue immediately adjacent to the cysteine residue proximal to the transmembrane domain. We have also isolated an example of an insertion mutation involving the in-frame insertion of 3 amino acids (Neu8564). Significantly, one of the inserted amino acids in this transforming Neu mutant was a cysteine residue (Fig. 1*A*). The importance of these cysteine residues is further reinforced by the observation that all five cysteine residues located within this region are conserved among the other three EGFR family members (Fig. 1*B*). Taken together, these observations suggest that alteration of these cysteine residues, either by deletion or mutation, may be involved in the activation of the Neu receptor tyrosine kinase.

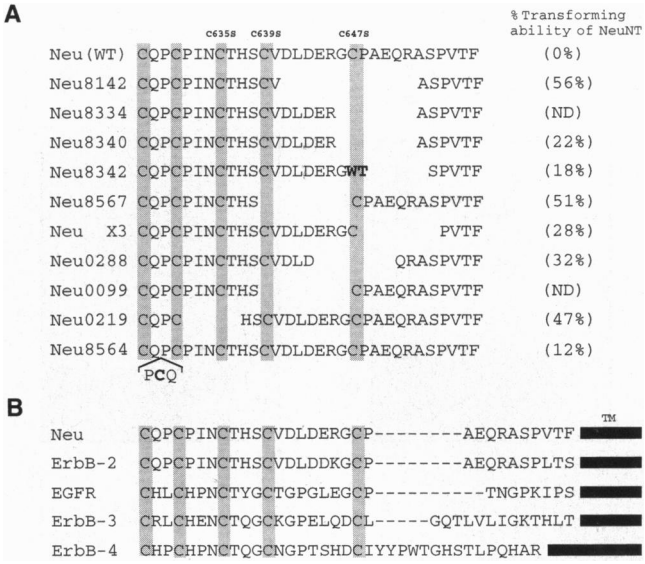

FIG. 1. The region of Neu containing the identified deletions displays limited homology among the other members of the EGFR family. (*A*) Protein sequence alignment of the deletions within Neu that have been cloned and sequenced (22). The transforming abilities of the various altered receptors were assessed by focus forming assays in Rat-1 cells and are expressed as percentage transformation compared with a well-characterized transforming mutant of Neu (NeuNT; 16). The values listed represent the average obtained from three independent focus assays. The transforming activities for Neu8142, Neu8340, Neu8342, and Neu8567 have been reported (22). ND, not determined. (*B*) Protein sequence alignment of the four members of the EGFR family. Both the rat (Neu) and human (ErbB-2) sequence is included for Neu/ErbB-2, while the human sequence alone is presented for EGFR, ErbB-3, and ErbB-4. The dashes have been included for the purposes of this alignment (adapted from ref. 7). The black box represents the position of the transmembrane domain (TM).

The Presence of Deletion Mutations Within *neu* Promotes Receptor Dimerization. Because previous studies have demonstrated that activated Neu receptors bearing the single point mutation in the transmembrane domain were activated due to constitutive dimerization (21), we were interested in assessing whether the altered Neu receptors harboring these deletions were also capable of dimer formation. To accomplish this, stable cell lines expressing selected mutant receptors were generated (22). Neu was immunoprecipitated from these cell lines and separated on SDS/polyacrylamide gels under both nonreducing and reducing conditions. As shown in Fig. 2*A*, Neu immunoprecipitates separated under nonreducing conditions revealed the presence of Neu dimers in those cell lines expressing mutant receptors (lanes 3–6). In contrast, stable dimer formation could not be detected in a cell line overexpressing the wild-type Neu receptor (N17; lane 2) The absence of dimer formation in wild-type Neu-expressing cells was not the result of decreased protein levels since both mutant and wild-type Neu-expressing cells possessed comparable levels of Neu (Fig. 2*B*). Interestingly, the extent of dimer formation observed for the different Neu deletion mutants was directly correlated with their transforming activity (compare Fig. 1 and Fig. 2). For example, a larger percentage of the higher transforming mutant, 8567, was in the form of dimers when compared with the weaker transforming mutant, 8342, despite the fact that both cell lines expressed similar levels of Neu protein (Fig. 2*A*, lanes 5 and 6). To explore whether receptor dimerization required disulfide bonding, duplicate immuno-

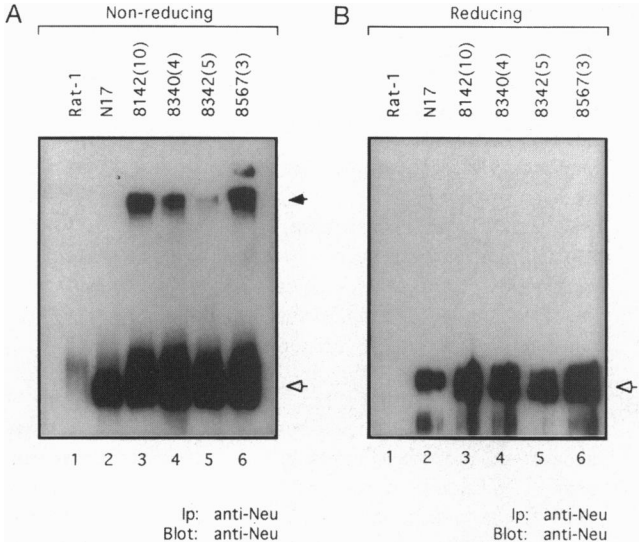

FIG. 2. Neu receptors harboring deletions undergo constitutive dimerization. Immunoprecipitates (IP) from stable Rat-1 cell lines expressing either wild-type Neu (N17) or selected altered forms of Neu (8142, 8340, 8342, 8567) were electrophoresed through 4–12% gradient SDS/polyacrylamide gels under nonreducing (*A*) or reducing (*B*) conditions. Proteins were transferred to polyvinylidene difluoride membranes and subjected to immunoblot analysis (blot) with antibodies specific to Neu (anti-Neu). (*A*) Neu dimers are indicated by the solid arrowhead, while the position of the monomer is marked by the open arrowhead. (*B*) The position of monomeric Neu is indicated by the open arrowhead. In both *A* and *B*, the lane marked Rat-1 indicates the parental cell line that does not contain an expression vector.

precipitates were subjected to immunoblot analysis for Neu after separation under reducing conditions (Fig. 2*B*). The results revealed that under these reducing conditions only the monomeric form of Neu was detected (Fig. 2*B*). These results provide evidence that deletions within this region of Neu promote the formation of disulfide-linked dimers.

Point Mutations Affecting Particular Conserved Cysteine Residues Can Induce Neu-Mediated Transformation. To directly test the importance of the conserved cysteine residues in activating the transforming potential of Neu, we mutated individual cysteine residues located at positions 635, 639, and 647. These residues were selected for mutagenesis because each of these cysteines was removed by at least one of the deletions (Fig. 1*A*). Initially we constructed expression vectors containing mutant *neu* molecules in which these individual cysteine residues were converted to serine residues (C635S, C639S, and C647S; Fig. 3*A*). Analysis of the transforming activity of these altered Neu receptor tyrosine kinases in Rat-1 cells revealed that conversion of cysteine 635 or 647 to a serine residue resulted in activation of the transforming potential of Neu (15% and 12% of NeuNT, respectively; Table 1). However, mutation of cysteine 639 to a serine residue (C639S) resulted in only a weak stimulation of transforming activity (2% of NeuNT; Table 1). To ensure that it was the loss of the cysteine residues that was critical for receptor activation, the cysteine at amino acid position 635 was also converted to either a glycine or a methionine (C635G and C635M; Fig. 3*A*). The substitution of this cysteine with either residue resulted in transformation of Rat-1 cells to ≈14% and 13% of NeuNT respectively (Table 1). To confirm that the altered Neu receptors promote transformation, stable cell lines individually expressing the four deletion mutants and three cysteine mutants described in this study were tested for their ability to grow in soft agar. In contrast to the parental Rat-1 line or cells overexpressing normal Neu (N17), all of the cell lines harboring mutant versions of Neu formed colonies in soft agar with

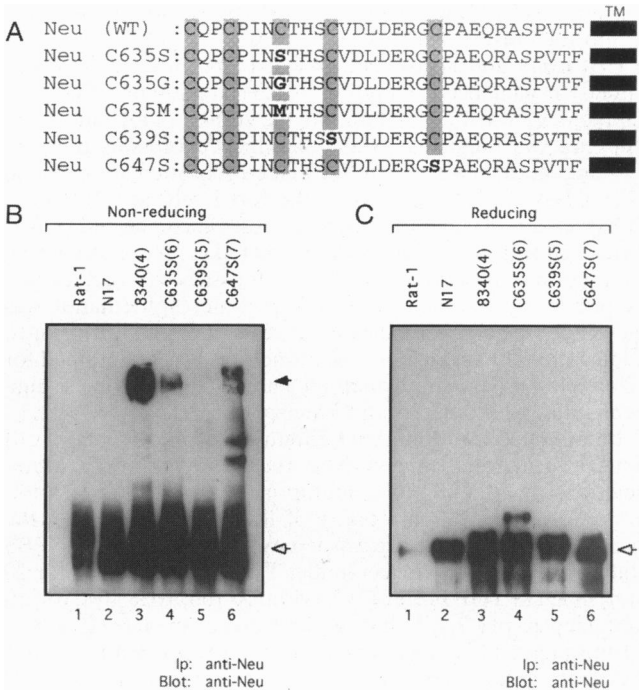

FIG. 3. Individual alteration of conserved cysteine residues in Neu promotes receptor dimerization. (*A*) Protein sequence alignment of the wild-type Neu sequence and each of the five cysteine mutations. The residues outlined in gray highlight the conserved cysteine residues in this region of Neu. The amino acids that have been substituted for the indicated cysteine residues are denoted by boldface type. The black box following the sequence in each case represents the start of the transmembrane domain (TM). (*B* and *C*) Neu was immunoprecipitated (IP) from stable Rat-1 cell lines expressing either wild-type Neu (N17) or cell lines expressing Neu cysteine mutants (C635S, C639S, C647S). The immunoprecipitates were separated on 4–12% gradient SDS/polyacrylamide gels under nonreducing (*B*) or reducing (*C*) conditions. Proteins were transferred to polyvinylidene difluoride membranes and subjected to immunoblot analysis with Neu-specific antibodies (anti-Neu). Dimers of the Neu receptor are indicated by the filled in arrowhead while the position of the monomer is marked by an open arrowhead. The lanes designated Rat-1 indicate immunoprecipitates from the parental cell line that does not contain an expression vector.

varying efficiencies (data not shown). Finally, transgenic mice have been derived which overexpress either the 8142 or 8342 deletion (Fig. 1*A*) in the mammary gland under the control of the MMTV promoter. Both strains of transgenic mice develop mammary tumors that appear with a shorter latency period when compared with animals expressing a MMTV/wild-type *neu* transgene (data not shown). These observations argue that mutations affecting conserved cysteine residues in this region of Neu can enhance transformation mediated by this receptor both *in vitro* and *in vivo*.

Like the cells expressing the Neu deletion mutants, stable cell lines expressing these point mutants of Neu exhibited elevated levels of tyrosine phosphorylated Neu which correlated with their ability to transform Rat-1 cells (ref. 22; data not shown). To explore whether the elevated levels of tyrosine phosphorylated Neu observed in these cells was due to enhanced receptor dimerization, Neu immunoprecipitates from cell lines expressing the three cysteine to serine mutations (Fig. 3*A*) were separated under both nonreducing and reducing conditions (Fig. 3 *B* and *C*). The results revealed that, like the Neu deletion mutants, mutation of cysteine 635 or 647 to serine resulted in the formation of Neu dimers under nonreducing conditions (Fig. 3*B*, lanes 4 and 6) which could be converted to the monomeric form by addition of reducing agents (Fig. 3*C*, lanes 4 and 6). In contrast, a majority of the

Table 1. Transformation of Rat-1 cells by Neu receptors containing cysteine mutations

Expression plasmid	Focus assay 1		Focus assay 2		Focus assay 3		Relative transforming ability‡
	Average no. of foci/μg*	% transformation of pJ4ΩneuNT†	Average no. of foci/μg*	% transformation of pJ4ΩneuNT†	Average no. of foci/μg*	% transformation of pJ4ΩneuNT†	
pJ4ΩneuN	0	0	0	0	0	0	0
pJ4ΩneuNT	109 ± 5	100	219 ± 11§	100	144 ± 9	100	100 ± 0
pJ4Ωneu(C635S)	15 ± 3	14	38 ± 3	17	20 ± 2	14	15 ± 2
pJ4Ωneu(C639S)	3 ± 3	3	4 ± 1	2	2 ± 1	1	2 ± 1
pJ4Ωneu(C647S)	NA	NA	31 ± 2	14	14 ± 1	10	12 ± 3
pJ4ΩneuN	0	0	0	0	0	0	0
pJ4ΩneuNT	156 ± 5	100	160 ± 13	100	215 ± 19	100	100 ± 0
pJ4Ωneu(C635G)	21 ± 2	14	23 ± 1	15	31 ± 2	14	14 ± 1
pJ4Ωneu(C635M)	14 ± 2	9	20 ± 4	12	37 ± 4	17	13 ± 4

Three independent focus assays were performed with Rat-1 fibroblasts. Each of the *neu* cDNAs were placed under the control of the Moloney murine leukemia virus long terminal repeat. The first two experiments were performed with the same large scale preparation of DNA while the third experiment was performed with DNA from an independent preparation. NA, data not available.

*Values listed represent the mean number of foci counted on six plates ± SD.

†Values represent the ratio of the mean number of foci obtained for each construct with respect to the activated form of *neu* (neuNT).

‡Values listed represent the mean transforming abilities from all three experiments ± SD.

§Values listed represent the mean number of foci counted on five plates ± SD.

Neu protein derived from cells expressing the weakly transforming C639S mutant Neu protein was found as a monomer under nonreducing conditions (Fig. 3*B*, lane 5). The receptors harboring these cysteine mutations display a reduced ability to dimerize when compared with the deletion mutant, 8340, which transforms at 22% of NeuNT (compare lanes 3 to 4–6). Taken together, these data argue that alteration of these conserved cysteine residues plays a crucial role in the oncogenic activation of Neu due to stimulation of disulfide bond mediated receptor dimerization.

The Disruption of Disulfide-Linked Dimers Results in Decreased Transformation of Rat-1 Cells by Altered Neu Receptors. Given that the presence of a reducing agent (2-mercaptoethanol) was capable of disrupting Neu dimers (Figs. 2*B* and 3*C*), we examined whether the addition of 2-mercaptoethanol could interfere with the ability of a Neu deletion mutant (8142) and a point mutant (C635S) to transform Rat-1 cells. Indeed, reducing agents have been used previously to interfere with signaling from activated versions of the thrombopoietin receptor (27). As shown in Fig. 4, addition of increasing concentrations of 2-mercaptoethanol resulted in a dose-dependent decrease in the ability of both Neu mutants to transform cells. Addition of 500 μM of 2-mercaptoethanol virtually abolished the ability of both mutants to transform Rat-1 cells. To ensure that the decrease in the number of foci did not reflect a toxic effect of the reducing agent on the Rat-1 cells, we also tested the effect of 2-mercaptoethanol on the ability of polyomavirus middle T antigen to transform Rat-1 cells. Because polyomavirus middle T is primarily localized on the inner face of the cytoplasmic membrane (28), its transforming activity should not be affected by 2-mercaptoethanol. In contrast to the Neu mutants, addition of the same concentrations of 2-mercaptoethanol had no effect on the ability of polyomavirus middle T to induce focus formation (Fig. 4). These data demonstrate that disruption of receptor dimerization with reducing agents can dramatically interfere with the capacity of these Neu mutants to induce malignant transformation *in vivo*.

DISCUSSION

We have demonstrated that mutations affecting a highly conserved cysteine-rich region within the extracellular domain of Neu can activate its oncogenic potential. Our observations further suggest that the transforming activity of these mutant receptors is the result of the formation of disulfide-linked dimers. The ability of these mutants to transform cells correlates with their ability to induce receptor dimerization. Furthermore, we have shown that addition of a reducing agent to the culture medium can dramatically interfere with the ability of these mutant Neu receptors to induce transformed foci of Rat-1 cells. Taken together, these observations suggest that oncogenic activation of Neu in these mutant receptors occurs through a mechanism involving the constitutive formation of disulfide bond-mediated receptor dimers. It should be noted that the parental Rat-1 cells used in this study express both the EGFR and Neu (ref. 10; personal observations), albeit at low levels (10). Given that the mutant versions of Neu are expressed to much higher levels than the endogenous protein (Figs. 2*B* and 3*C*), we believe our data results from the formation of homodimers between the altered Neu receptors.

One potential explanation for our results is that alteration of the balance of cysteine residues within this region may disrupt the normal cysteine pairing that occurs in the unactivated receptor. Such a mutation would leave an unpaired cysteine residue that could participate in an intermolecular interaction with another altered receptor, resulting in a disulfide-linked dimer. Indeed, a similar mechanism has been proposed to account for the oncogenic activation of the Ret protooncogene in inherited forms of endocrine neoplasia type 2A (29). Although mutation of individual cysteine residues within this region of Neu resulted in its oncogenic activation, many of the Neu deletion mutations that removed these residues exhibited increased transforming activity. For example, five of the deletion mutants that remove or alter the cysteine at position 647 displayed elevated transforming activities compared with that of the C647S point mutation (22–56% compared with 12%). One potential explanation for this observation is that the spatial arrangement between the remaining cysteine residues also plays an important role in mediating receptor dimerization. For example, certain deletions may result in the free cysteine being exposed at the surface of the receptor where it is more accessible to the formation of an intermolecular disulfide bond.

The observation that deletion, insertion, or point mutation of cysteine residues within the juxtatransmembrane region of Neu results in receptor dimerization has important implications in understanding the mechanism of receptor tyrosine kinase activation. Significantly, this region displays homology among the remaining EGFR family members, particularly with respect to the position of the five conserved cysteine residues. Future experiments involving the generation of comparable mutations in the other EGFR family members will provide insights into whether these cysteine residues function in a

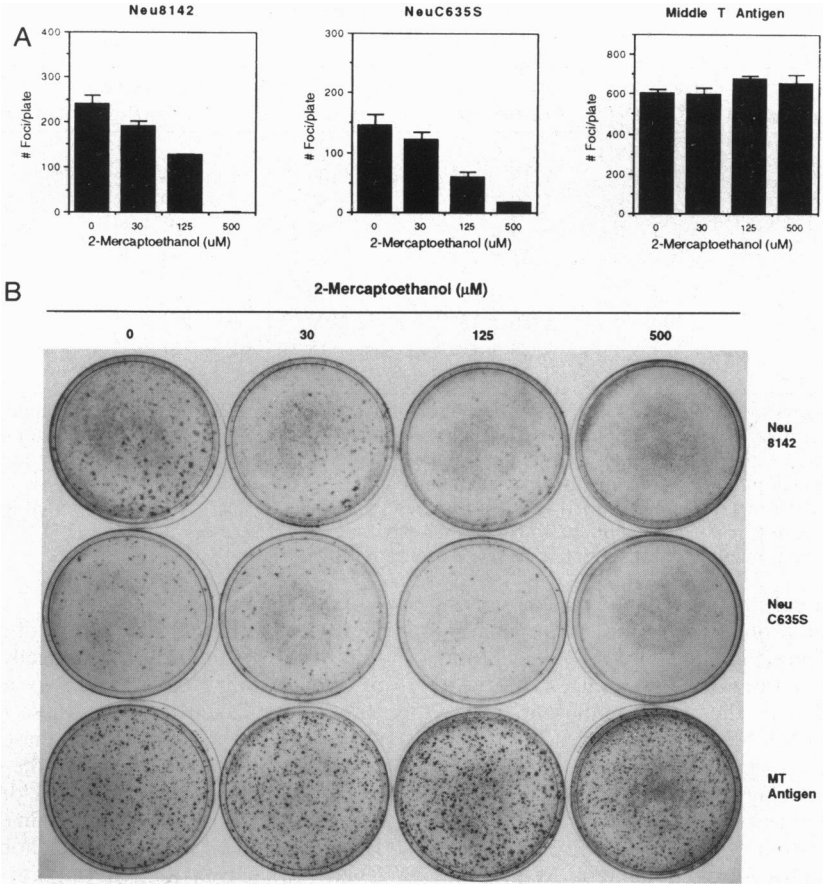

FIG. 4. Transformation mediated by mutant Neu receptors is impaired in the presence of reducing agents. (*A*) Focus assays were performed with a representative deletion (Neu8142) and point mutant (C635S) in the presence of increasing concentrations of 2-mercaptoethanol. Foci were scored over four plates for each concentration. The polyomavirus middle T antigen was used as a positive control. (*B*) Representative plates from the focus assay shown in *A* illustrating the decrease in transformed foci as a consequence of increasing 2-mercaptoethanol concentrations.

similar manner. Given the capacity for Neu/ErbB-2 to form heterodimers (10–14), it is conceivable that these cysteine residues are also involved in heterodimerization between these closely related family members.

Further evidence implicating cysteine residues in the process of receptor dimerization stems from observations that the introduction of an extra cysteine residue proximal to the transmembrane domain of either the EGFR or Neu results in the formation of disulfide-linked dimers (30, 31). In contrast to our observations, the introduction of an extra cysteine residue in Neu did not activate the transforming ability of the altered receptor (31). The involvement of cysteine residues in receptor dimerization is further reinforced by the germline mutations within the *ret* protooncogene that occur in Men2A patients (32). These mutations affect one of five cysteine residues located in the extracellular domain of the Ret receptor. Subsequent analyses of these mutations revealed that they function by promoting receptor dimerization of the mutant receptors (29, 33). Finally, studies of receptors belonging to the haemopoietin/cytokine superfamily also indicate the importance of cysteine residues in receptor dimerization. The substitution of cysteine residues in place of specific residues in the extracellular domain of both the erythropoietin receptor (34, 35) and the thrombopoietin receptor, c-Mpl, (27) results in the formation of disulfide-linked dimers that constitutively activate these receptors. Furthermore, it has recently been demonstrated that disruption of disulfide bonding by the addition of reducing agents can interfere with signaling from activated thrombopoietin receptors (27). Consistent with this result, we have demonstrated that the addition of 2-mercaptoethanol can impair Neu-mediated transformation.

The observation that the transforming activity of these mutant Neu receptors can be severely impaired by the addition of reducing agents has potentially important therapeutic implications in the treatment of cancers overexpressing Neu. For example it may be possible to design peptide inhibitors that can effectively interfere with dimerization of the receptor by complexing with the critical cysteine residues within this region. Future studies directed toward elucidating the mechanism of disulfide-mediated receptor dimerization should provide important insights into the feasibility of this approach.

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