MOLECULAR AND CELLULAR BIOLOGY, Oct. 1996, p. 5726–5736 Vol. 16, No. 10
0270-7306/96/$04.00+0

Synergistic Interaction of the Neu Proto-Oncogene Product and Transforming Growth Factor α in the Mammary Epithelium of Transgenic Mice

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Received 8 May 1996/Returned for modification 24 June 1996/Accepted 15 July 1996

Transgenic mice expressing either the *neu* proto-oncogene or transforming growth factor (TGF-α) in the mammary epithelium develop spontaneous focal mammary tumors that occur after a long latency. Since the epidermal growth factor receptor (EGFR) and Neu are capable of forming heterodimers that are responsive to EGFR ligands such as TGF-α, we examined whether coexpression of TGF-α and Neu in mammary epithelium could cooperate to accelerate the onset of mammary tumors. To test this hypothesis, we interbred separate transgenic strains harboring either a mouse mammary tumor virus/TGF-α or a mouse mammary tumor virus/*neu* transgene to generate bitransgenic mice that coexpress TGF-α and *neu* in the mammary epithelium. Female mice coexpressing TGF-α and *neu* developed multifocal mammary tumors which arose after a significantly shorter latency period than either parental strain alone. The development of these mammary tumors was correlated with the tyrosine phosphorylation of Neu and the recruitment of c-Src to the Neu complex. Immunoprecipitation and immunoblot analyses with EGFR- and Neu-specific antisera, however, failed to detect physical complexes of these two receptors. Taken together, these observations suggest that Neu and TGF-α cooperate in mammary tumorigenesis through a mechanism involving Neu and EGFR transactivation.

The epidermal growth factor receptor (EGFR) family comprises four closely related type 1 receptor tyrosine kinases (RTKs) (EGFR, Neu [erbB-2, HER2], erbB-3 [HER3], and erbB-4 [HER4]) that are receptors for a variety of mitogenic growth factors (36). Enhanced expression of the EGFR family has been implicated in the genesis of human breast cancers. For example, amplification and consequent overexpression of *neu* have been observed in a significant proportion of human breast cancers and appear to be inversely correlated with patient survival (9, 12, 25, 32, 33, 37). More recently, overexpression of the other members of the EGFR family, including EGFR, erbB-3, and erbB-4, has also been implicated in the pathogenesis of human breast cancer (15, 16, 26, 27).

The activity of these EGFR family members can also be affected by expression of a variety of specific ligands for these RTKs. For example, Neu is a substrate of the activated EGFR following stimulation of cells with EGF or transforming growth factor α (TGF-α) (1, 8, 13, 35). Although Neu shares homology with the EGFR, Neu does not bind these EGF ligands. Rather, the observed tyrosine phosphorylation of Neu by the EGFR is thought to be mediated by heterodimerization and/or transactivation between Neu and EGFR, resulting in a high-affinity receptor for these EGFR ligands (8, 39). Consistent with these observations, coexpression of Neu and EGFR results in efficient transformation of fibroblasts in vitro (14). Moreover, elevated expression of both Neu and EGFR can be detected in primary human breast cancers (16). Taken together, these observations suggest that these two closely related RTKs may collaborate in mammary tumorigenesis.

Direct evidence of the involvement of EGFR family members in the induction of mammary tumors derives from observations made with transgenic mice expressing *neu* in the mammary epithelium (4, 10, 21). High-level expression of a constitutively active form of *neu* bearing a point mutation in the transmembrane domain (3) resulted in the development of nonstochastic, multifocal mammary tumors that affected every female carrier (21). In contrast, expression of the wild-type *neu* proto-oncogene in the mammary epithelium of transgenic mice resulted in the focal development of mammary tumors that arose after long latency (10). Interestingly, induction of mammary tumors in wild-type *neu* transgenic mice correlated with the frequent occurrence of activating mutations in the *neu* transgene (31). Thus, activation of the Neu RTK appears to be a pivotal step in the induction of mammary tumors in these mice.

Additional evidence implicating the EGFR family in mammary tumorigenesis derives from observations made with transgenic strains expressing an EGFR-specific ligand, TGF-α, in the mammary epithelium. Mammary gland-targeted expression of TGF-α in various transgenic strains results in the development of mammary epithelial hyperplasias that progress to focal mammary tumors after a long latency, as in wild-type *neu* transgenic mice (11, 18, 29). In mouse mammary tumor virus (MMTV)/TGF-α transgenic mice, increased expression of EGFR was observed in mammary tumors compared with adjacent, histologically normal tissue (18).

Given the potential of Neu and TGF-α (along with enhanced EGFR expression) to cooperate through a mechanism

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of receptor transactivation, we were interested in determining whether coexpression of *neu* and TGF-α could accelerate the induction of mammary tumors in vivo. To accomplish this, separate strains of transgenic mice carrying either an MMTV/*neu* or an MMTV/TGF-α transgene were interbred to generate dual carriers that coexpressed both *neu* and TGF-α in the mammary epithelium. The bigenic animals developed multifocal mammary tumors within a significantly shorter latency period than either parental strain alone. The induction of mammary tumors in these strains was further correlated to tyrosine phosphorylation of Neu and the recruitment of the c-Src tyrosine kinase to this complex, even in the absence of *neu* mutations. Cross-linking studies and reciprocal EGFR and Neu immunoprecipitations, however, did not demonstrate a physical association between EGFR and Neu in bigenic mice. Although these negative data do not disprove a transient physical association between these two receptors, the evidence favors receptor transactivation as the mechanism responsible for the observed cooperativity between EGFR and Neu in mammary tumorigenesis. Taken together, these data argue that coexpression of TGF-α and *neu* can act synergistically to transform the mammary epithelium.

MATERIALS AND METHODS

DNA constructs and generation of transgenic mice. The plasmid used to generate the antisense *neu* riboprobe was constructed by inserting an *Sma*I-*Xba*I fragment (nucleotides 1684 to 2332) into pSL301 (Invitrogen) (31). The phosphoglycerate kinase 1 (PGK-1) internal control plasmid was obtained from M. Rudnicki and was generated by inserting an *Acc*I-*Pst*I fragment (nucleotides 939 to 1633 of the PGK-1 cDNA) (20) into the *Pst*I site of pSP64 (Promega). The TGF-α riboprobe was constructed by inserting the 632-bp *Nco*I fragment into the corresponding site in PSL301. The generation and characterization of both MMTV/wild-type *neu* and MMTV/TGF-α mice have been described previously (10, 18).

RNase protection assays. Total RNA was isolated from tissues by guanidinium thiocynate extraction, followed by CsCl gradient fractionation (5). The RNA yield was determined, after resuspension in sterile H_2O, by measuring the UV A_{260}. To generate the antisense *neu* probe, the template plasmids described above were linearized with *Sma*I and then subjected to an in vitro transcription reaction with T7 RNA polymerase (19). The PGK-1 internal control probe was produced by digesting the template plasmid with *Eco*NI and transcribing the product with SP6 RNA polymerase. The TGF-α riboprobe was generated by cleavage of the template plasmid with *Xho*I, followed by in vitro transcription with the T3 RNA polymerase. The RNase protection assays were performed by hybridizing the above-mentioned probes to 20 μg of total RNA as previously described (19). The protected fragments were separated on a sodium dodecyl sulfate (SDS)–6% polyacrylamide gel and subjected to autoradiography.

Immunoprecipitation and immunoblotting. Tissue samples were ground to powder under liquid nitrogen and lysed for 20 min on ice in TNE lysis buffer (50 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% Nonidet P-40, 2 mM EDTA, 1 mM sodium orthovanadate, 2 mM dithiothreitol, 10 μg of leupeptin per ml, 10 μg of aprotinin per ml). The lysates were cleared by centrifugation at 12,000 × *g* for 10 min at 4°C. Immunoprecipitations were performed by incubating 2.0 mg of the protein lysate with either 300 ng of anti-Neu monoclonal antibody 7.16.4 (6) or 300 ng of an anti-EGFR antibody (Transduction Laboratories catalog no. E12020) for 30 min at 4°C. Following incubation with protein G-Sepharose beads (Pharmacia) on a rotating platform at 4°C for 30 min, the precipitates were washed four times with TNE. The Neu and EGFR immunoprecipitates were resuspended in SDS-gel loading buffer, and the proteins were resolved on an SDS–9% polyacrylamide gel. The proteins were transferred onto a polyvinylidene difluoride membrane (Millipore) with an immunoblot transfer apparatus (Bio-Rad). Following overnight incubation in 3% bovine serum albumin (Sigma) in Tris-buffered saline (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM KCl), the membrane was probed for 2 h with antiphosphotyrosine antibodies (1:500; Upstate Biotechnology, Inc.) in bovine serum albumin in Tris-buffered saline. After being washed in Tris-buffered saline–0.05% Tween 20, the blots were incubated in 3% milk in Tris-buffered saline for 1 h. The membrane was incubated with goat anti-mouse immunoglobulin G, and the proteins were visualized by the enhanced-chemiluminescence detection system (Amersham).

For studies demonstrating the in vivo association of Neu with c-Src, tumor lysates were prepared and cleared as described previously (24). Proteins were immunoprecipitated by incubating 1.0 to 2.0 mg of total cell lysate with 2 μg of anti-c-Src antibody 7D10 (Quality Biotech) for 3 h at 4°C and subsequently washed five times with lysis buffer. The samples were resolved on an SDS–8% polyacrylamide gel and blotted onto a polyvinylidene difluoride membrane. The membrane was probed with anti-Neu antibody AB.3 (1:1,000; Oncogene Science) as previously described (24).

For studies examining receptor association, tumor membranes were prepared from 0.3- to 0.6-g tissue aliquots by homogenization in a detergent-free hypotonic buffer as described previously (2). Following 45 min of ultracentrifugation at 100,000 × *g* and 4°C, pellets were solubilized for 45 min in 3-[(3-cholamidopropyl)-dimethyl-amonio]-1-propanesulfonate (CHAPS) buffer (50 mM Tris [pH 8.0], 0.7% CHAPS, 50 mM NaCl, 1 mM sodium orthovandate, 10 μg of aprotinin per ml, 10 μg of leupeptin per ml). Nonidet P-40-insoluble material was removed by centrifugation at 14,000 × *g* for 15 min. Equivalent amounts of membrane protein were immunoprecipitated with either Neu polyclonal antiserum 21N (30) or anti-EGFR polyclonal antiserum 986 (30) and Staph A cells (Calbiochem) for 1 to 2 h. After washes, both Neu and EGFR immunoprecipitates were subjected to immunoblot analyses with the 21N polyclonal antiserum or an anti-EGFR monoclonal antibody (Transduction Laboratories). For detection, horseradish peroxidase-linked sheep anti-rabbit or anti-mouse antibodies were utilized. Direct binding assays were performed primarily as described previously (23).

Histological evaluation. Complete autopsies were performed. Tissues were fixed in 4% paraformaldehyde, sectioned at 4 μm, routinely stained with hematoxylin and eosin, and examined as indicated in the legend to Fig. 4. Whole-mount analyses were performed as previously described (11).

RESULTS

Detection of TGF-α and *neu* transcripts in mammary epithelium of transgenic mice carrying both MMTV/*neu* and MMTV/TGF-α transgenes. To determine if coexpression of TGF-α and *neu* could cooperate in mammary tumorigenesis, separate strains of MMTV/*neu* and MMTV/TGF-α were interbred to generate offspring carrying both transgenes. The MMTV/TGF-α strain is derived from line 29 and originates from a C57BL × DBA genetic background (18), whereas the MMTV/*neu* strain is derived from the N#202 founder animal and is derived from an inbred FVB genetic background (10). Because TGF-α-expressing females are unable to nurse their young, the F_1 progeny from this cross were generated by crossing MMTV/TGF-α males with MMTV/*neu* females. Females derived from this cross were segregated into breeding and virgin female groups. Analyses of the multiparous animals derived from this cross revealed that dual carriers possessing both the TGF-α and *neu* transgenes were incapable of nursing their young, like TGF-α-expressing females. In addition to the apparent lactation defect, multiparous female transgenic mice bearing the TGF-α and *neu* transgenes exhibited uniform hypertrophy of the mammary glands (22).

To assess whether the mammary epithelium derived from the various genotypes expressed the appropriate transgenes, RNAs derived from the mammary tumors derived from *neu*/TGF-α or *neu*/+ virgin transgenic mice were subjected to RNase protection analyses with riboprobes specific for *neu* (Fig. 1A) or TGF-α (Fig. 1B). To ensure equal loading of RNA, a PGK-1 antisense probe (20) was also included in the hybridization reaction mixtures. Examination of RNA samples derived from the tumors of eight MMTV/*neu* female mice revealed increased levels of *neu* transcripts. Interestingly, several of these tumor samples demonstrated evidence of altered transcripts (Fig. 1A, lanes 9, 10, and 13 to 15). Indeed, previous studies have demonstrated that these altered transcripts invariably encode in-frame deletions in the extracellular domain of Neu which result in its oncogenic activation (31). As expected, elevated *neu* transcript levels were observed in mammary tumors derived from bitransgenic animals harboring both transgenes. Significantly, the mammary tumor RNA samples obtained from seven dual transgene carriers did not exhibit evidence of altered transcripts that were observed in the *neu*-induced tumors (Fig. 1A, lanes 1 to 7).

An identical RNase protection analysis was performed on these RNA samples to assess the levels of TGF-α (Fig. 1B). These analyses revealed that TGF-α could be detected in

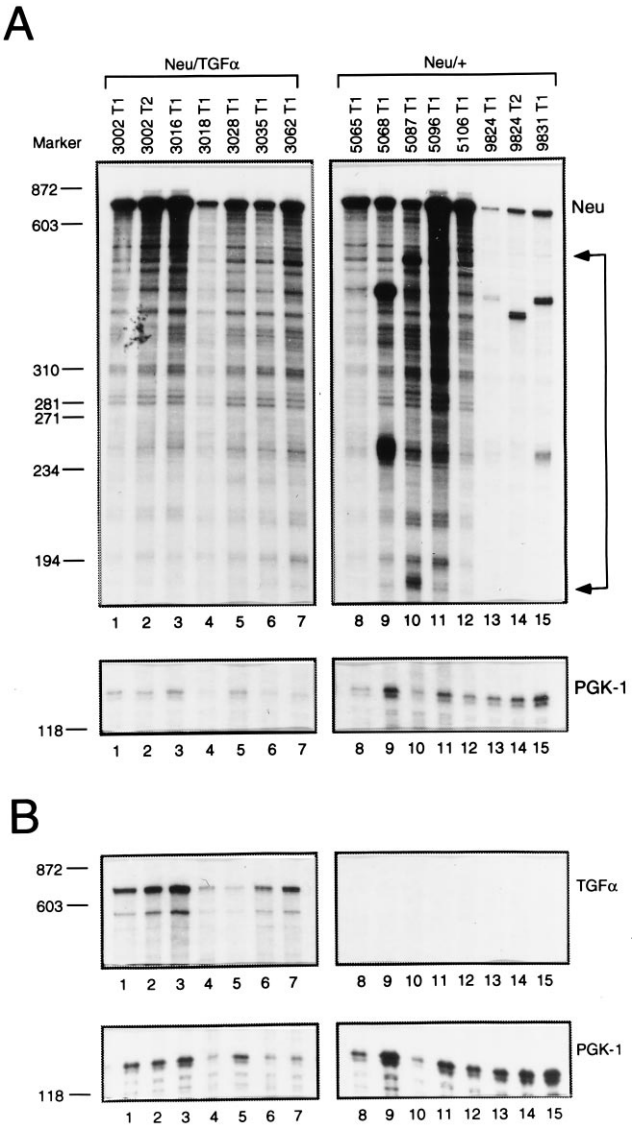

FIG. 1. Expression of Neu and TGF-α transgenes in mammary tissue of transgenic mice. (A) Neu transgene expression in mammary tissues of mice carrying the MMTV/*neu* transgene (*neu*/+) and both transgenes (Neu/TGF-α). RNA samples derived from tumor tissue (T) were subjected to RNase protection analyses. The protected wild-type *neu* transcript is 640 nucleotides long. Protected fragments corresponding to the altered *neu* transcript are indicated by arrows. Tumor RNA samples were derived from virgin female animals. An antisense riboprobe directed against the mouse PGK-1 gene was used to control for equal loading of RNA on the gel. The PGK-1 probe protects a 124-nucleotide fragment, as indicated in the lower panels. (B) The identical RNA tissue samples were hybridized with an antisense probe directed against the mouse TGF-α gene. The TGF-α antisense probe protects a 632-nucleotide fragment. The PGK-1 probe protects a 124-nucleotide fragment, as indicated in the lower panels. The numbers on the left are molecular sizes in nucleotides.

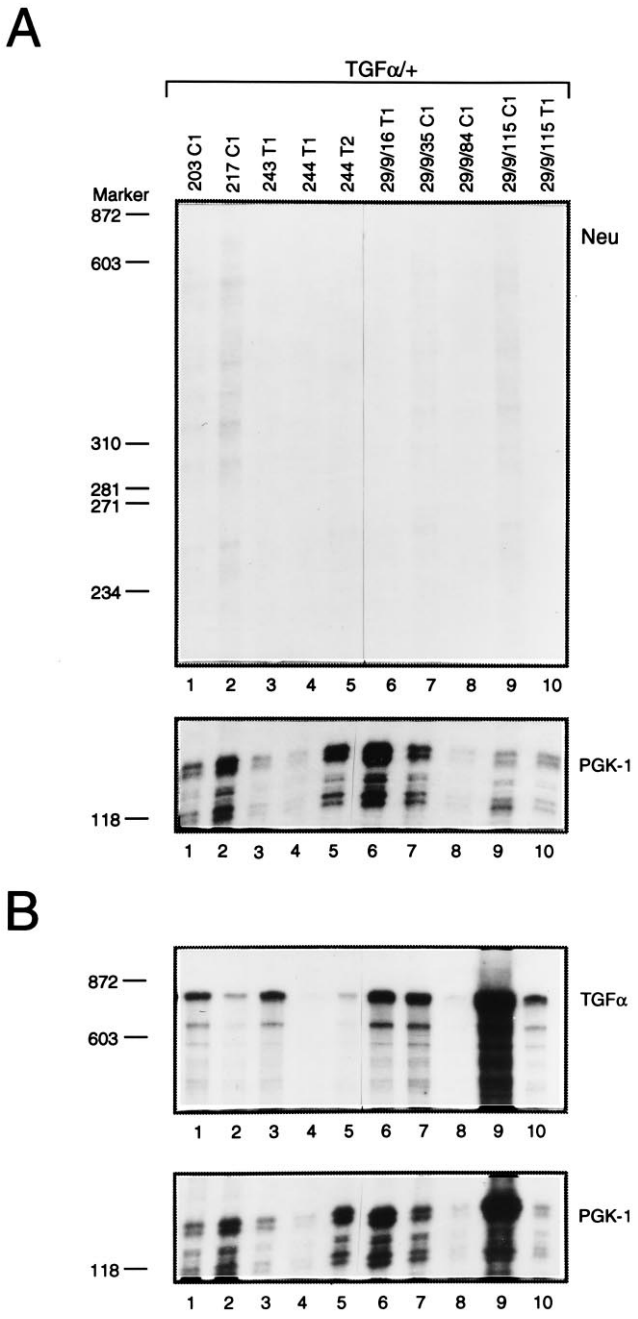

FIG. 2. Expression of Neu and TGF-α transcripts in tumors and hyperplasias derived from MMTV/TGF-α transgenic mice. (A) Endogenous Neu expression in mammary tissues of mice expressing the TGF-α transgene (TGF-α/+). Tumor (T) and cystic hyperplastic (C) tissue RNA samples from virgin female TGF-α carriers were subjected to RNase protection analyses with a *neu* riboprobe. The protected *neu* transcript is 640 nucleotides long. An antisense riboprobe directed against the mouse PGK-1 gene was used to control for equal loading of RNA on the gel. The PGK-1 probe protects a 124-nucleotide fragment, as indicated in the lower panel. (B) RNA tissue samples identical to those in panel A were probed with an antisense probe directed against the mouse TGF-α gene. The TGF-α antisense probe protects a 632-nucleotide fragment. The numbers on the left are molecular sizes in nucleotides.

mammary tumor RNA samples derived from both MMTV/TGF-α mice (Fig. 2B, lanes 1 to 10) and mice carrying both the *neu* and TGF-α transgenes (Fig. 1B, lanes 1 to 7). In contrast, no detectable transcripts corresponding to the TGF-α transgene were detected in mammary tumor RNA samples from transgenic mice carrying the *neu* transgene alone (Fig. 1B, lanes 8 to 15). Analyses of a representative sample of tumors and cystic hyperplasias derived from virgin females carrying the MMTV/TGF-α transgene alone with the identical riboprobes revealed no evidence of expression of *neu* (Fig. 2A, lanes 1 to 10); however, high TGF-α transcript levels were detected in these tissues (Fig. 2B, lanes 1 to 10). Seven MMTV/TGF-α tumor samples and five cysts were analyzed by

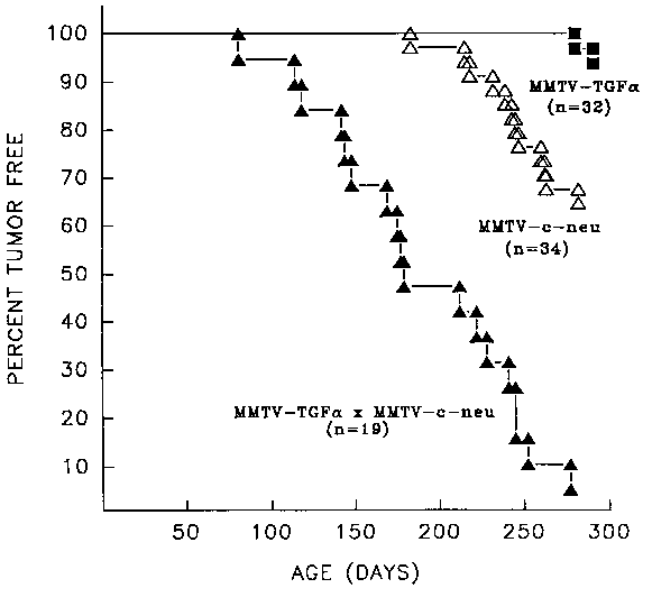

FIG. 3. Kinetics of tumor occurrence in monogenic and bigenic animals harboring the MMTV/TGF-α and MMTV/*neu* transgenes. Comparison of the kinetics of tumor formation between virgin female carriers bearing the MMTV/TGF-α, MMTV/*neu*, and both transgenes. The numbers of mice examined are indicated.

RNase protection. Taken together, these observations indicate that the dual carriers coexpress elevated levels of TGF-α and *neu* in the mammary epithelium.

Coexpression of TGF-α and *neu* in mammary epithelium results in rapid generation of multifocal mammary tumors. To test whether *neu* and TGF-α could collaborate in mammary tumorigenesis, virgin female mice carrying the *neu*, TGF-α, or both transgenes were monitored for the physical appearance of mammary tumors by palpation weekly. As shown in Fig. 3, the appearance of mammary tumors in transgenic mice expressing TGF-α or *neu* alone occurred only after long latency. Only 6% of the MMTV/TGF-α and 35% of the MMTV/*neu* mice developed palpable mammary tumors by 250 days (Fig. 3). In contrast, 95% of the bigenic mice developed palpable tumors by this time (Fig. 3), and 50% of these dual carriers had tumors by 175 days, at which time neither the TGF-α- nor the *neu*-expressing mice exhibited tumors (Fig. 3). In addition to the accelerated onset of mammary tumors, the mammary tumors in the bigenic mice developed were multifocal and encompassed the entire mammary epithelium, whereas transgenic mice expressing either TGF-α or *neu* alone developed mammary tumors that were generally focal in origin.

To explore the phenotypic differences between the various transgenic mice, mammary fat pads derived from age-matched virgin female mice were subjected to whole-mount analyses (Fig. 4). The results showed that mice carrying the *neu* transgene possessed mammary trees indistinguishable from those of virgin FVB female mice (Fig. 4B); however, the mammary trees of either the TGF-α or the *neu*/TGF-α mice were grossly abnormal (Fig. 4C and D). In fact, both the TGF-α and *neu*/TGF-α mice displayed extensive lobuloalveolar development resembling that of a normal FVB lactating female mouse (Fig. 4A). Careful examination of whole-mount preparations derived from TGF-α and *neu*/TGF-α mice revealed clear differences between the two. The alveoli present in the *neu*/TGF-α mice contained a denser cell lining in the walls (Fig. 4D) than did the large, cystically dilated alveoli found in the TGF-α mice (Fig. 4C). Consistent with these whole-mount findings, histological examination of these mammary hyperplasias derived from the MMTV/TGF-α mice had extensive lobular development (Fig. 5C) which resembled that of a lactating nontransgenic animal (Fig. 5A), except that the alveoli were irregular and dilated (Fig. 5C). Further, the interstitial stroma was edematous and had a modest increase in mononuclear and polymorphonuclear leukocytes. The mammary gland of the nulliparous female had minimal lobular development and no evidence of a stromal response (Fig. 5B). In contrast, the mammary gland of the bitransgenic animal frequently showed epithelial hyperplasia and dysplasia along with stromal inflammation (Fig. 5D). Therefore, the presence of inflammatory stroma tissue in the mammary gland is closely associated with expression of the MMTV/TGF-α transgene. The mammary tumors arising from the monogenic animals were typical of those previously reported: TGF-α-expressing animals demonstrated tubular adenocarcinomas, whereas *neu*-expressing mice typically developed nodular tumors (10, 18). The tumors arising in the bigenic *neu*/TGF-α animals were interesting in that they were either nodular (Fig. 6A) or tubular (Fig. 6B) but did not demonstrate a mixed or different morphological pattern; however, both categories of tumors coexpressed *neu* and TGF-α (Fig. 1). Together with the histological observations, these findings suggest that coexpression of *neu* and TGF-α is associated with induction of widespread morphological abnormalities in the mammary gland.

Constitutive tyrosine phosphorylation of Neu in mammary tissues coexpressing TGF-α and *neu*. The results above strongly suggest that coexpressed TGF-α and *neu* are highly synergistic in their capacity to transform the mammary epithelium. A probable biochemical explanation for this observation is that TGF-α activates Neu-associated tyrosine kinase activity by transphosphorylation through the activated EGFR, especially in light of the lack of activating mutations of *neu* in the bigenic tumors. To explore this possibility, protein extracts obtained from the mammary glands of MMTV/*neu* mice, MMTV/TGF-α mice, and dual carriers were subjected to immunoprecipitation analyses with either EGFR- or Neu-specific antiserum and immunoblotted with phosphotyrosine-specific antiserum (Fig. 7B and D, respectively). To control for the amount of protein, the same immunoprecipitates were also immunoblotted with either EGFR- or Neu-specific antiserum (Fig. 7A and C, respectively). Low but detectable quantities of EGFR protein were found in tumor samples from transgenic animals expressing either Neu or TGF-α alone (Fig. 7A, lanes 1 to 6); tumors coexpressing TGF-α and Neu expressed variable levels of EGFR (Fig. 7A, lanes 7 to 10). The levels of tyrosine-phosphorylated EGFR in these tumor samples directly correlated with the results of the immunoblot analyses (Fig. 7B). In contrast to the variable expression of EGFR, extremely high levels of tyrosine-phosphorylated Neu were detected in mammary tumors induced by the *neu* transgene alone or by both transgenes (Fig. 7D, lanes 1 to 3 and 7 to 10), which correlated with the total levels of Neu protein immunoprecipitated (Fig. 7C, lanes 1 to 3 and 7 to 10). No detectable tyrosine-phosphorylated Neu was observed in mammary protein samples from mice expressing TGF-α alone (Fig. 7C and D, lanes 4 to 6).

To further explore the mechanism by which Neu was transactivated in mammary tumors coexpressing TGF-α and *neu*, protein lysates derived from *neu*/TGF-α-, *neu*-, or TGF-α-expressing mice were subjected to reciprocal immunoprecipitation and immunoblot analyses with antisera specific to EGFR and Neu (Fig. 8). Although immunoprecipitation of these protein lysates with either Neu- or EGFR-specific anti-

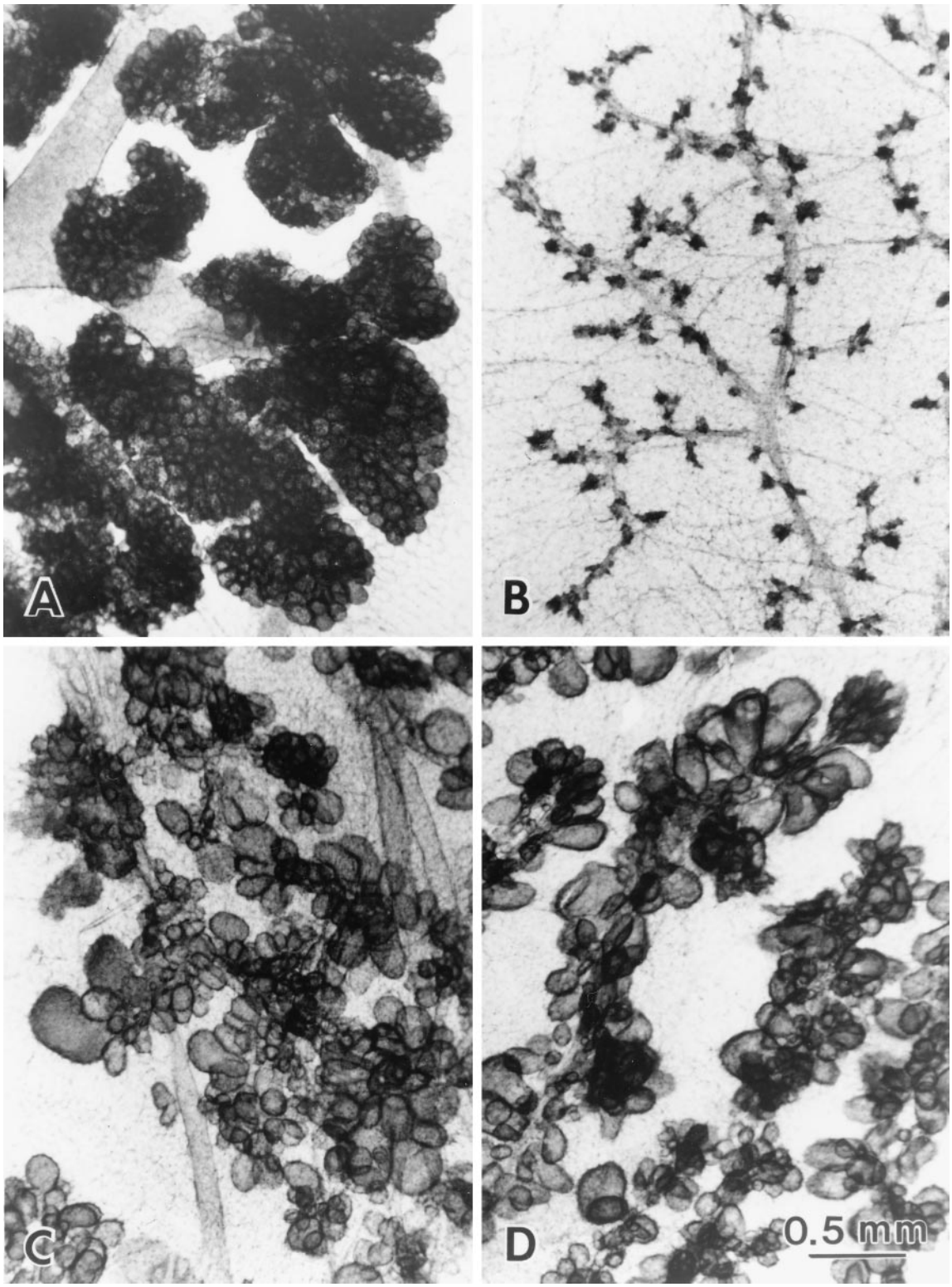

FIG. 4. Whole-mount analyses of mammary fat pads derived from monogenic and bigenic female mice. Shown are whole-mount preparations illustrating the appearance of mammary trees from a lactating FVB female (A), a virgin female with the *neu* transgene (note the numerous side buds which give the mammary tree a spiculated appearance) (B), a virgin female with the TGF-α transgene (note the well developed, cystically dilated alveoli) (C), and a virgin female with both the TGF-α and *neu* transgenes (note the larger cystic alveoli with darker walls, indicating a denser cell lining in the walls) (D). Compare these preparations with the comparable histologic preparations in Fig. 5. Magnification, ×31.5.

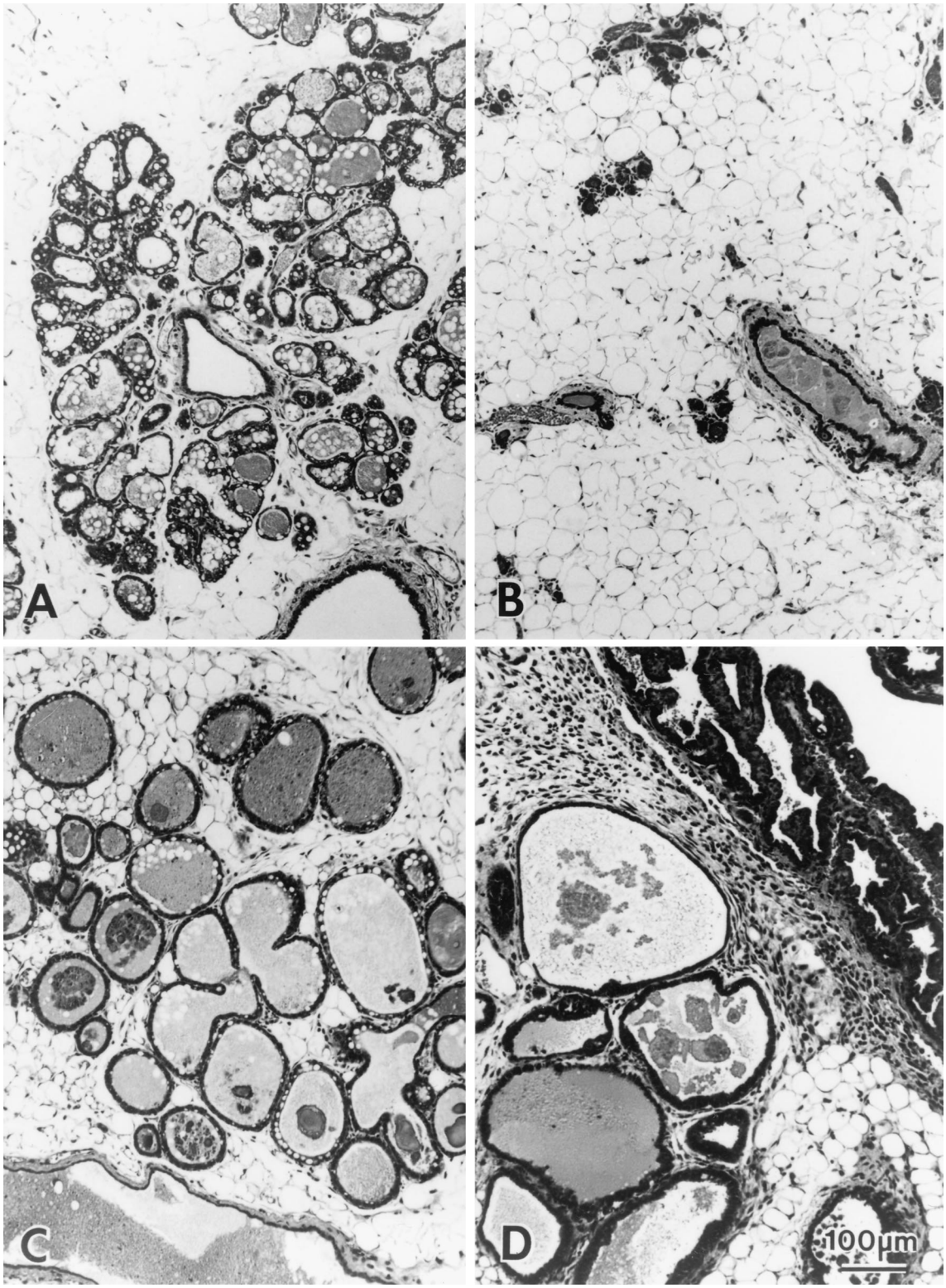

FIG. 5. Histopathology of mammary tissues derived from virgin monogenic and bigenic transgenic animals. (A) Normal FVB lactating female mouse showing lobuloalveolar development and milk production. (B) Transgenic *neu* virgin female mouse illustrating rudimentary mammary acinar development without significant luminal secretions. (C) Transgenic TGF-α virgin female mouse illustrating extensive alveolar development in comparison with a lactating mammary gland (A). Note that the alveoli are much more distended with secretory products than the FVB lactating tissue but contain fewer clear lipid vacuoles. (D) Transgenic *neu*/TGF-α virgin female mouse illustrating areas of alveolar development with papillary hyperplasia in the upper right corner. The virgin *neu*, TGF-α, and *neu*/TGF-α mice were age matched (139 days) and identical to those described in Fig. 4.

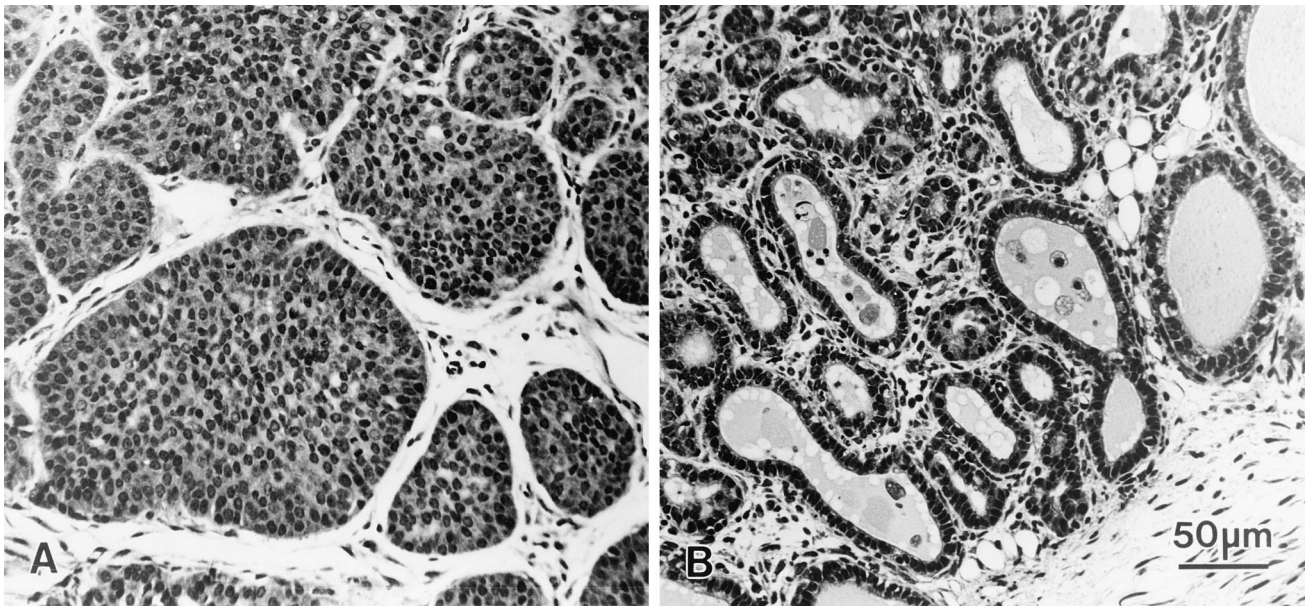

FIG. 6. Histology of the two types of mammary tumors observed in *neu*/TGF-α bigenic mice. (A) Nodular tumor typical of the Neu phenotype. (B) Tubular type typical of the TGF-α phenotype. Magnification, about ×147.

bodies, followed by immunoblot analyses with Neu-specific antiserum, revealed abundant Neu protein in the immunoprecipitates from the *neu*/TGF-α- and *neu*-expressing tumors, no detectable Neu was found in the EGFR immunoprecipitates (Fig. 8A). Conversely, immunoprecipitation with EGFR-specific antibodies, followed by immunoblot analyses with Neu-specific antiserum, failed to demonstrate the presence of EGFR in these complexes (Fig. 8B). Thus, under these experimental conditions, we could not detect a physical interaction between the EGFR and Neu in bigenic mice, and these findings indicate that transactivation of Neu by TGF-α does not involve the formation of stable Neu-EGFR heterodimers.

Induction of mammary tumors by *neu* and *neu*–TGF-α transgenes correlates with the capacity of Neu to associate with the c-Src tyrosine kinase in vitro and in vivo. One possible explanation for the observed synergy between Neu and TGF-α/EGFR is that these closely related type 1 receptors recruit distinct but complementary pathways. Indeed, we have previously demonstrated that activation of c-Src by the activated EGFR in fibroblasts is mediated by direct and specific association of c-Src with Neu (23). To test whether the c-Src signaling pathway was also recruited to Neu in *neu*/TGF-α-expressing tumors, Neu immunoprecipitates derived from *neu*, TGF-α, and *neu*/TGF-α tumor samples were resolved on an SDS-polyacrylamide gel, transferred onto a polyvinylidene difluoride membrane, and probed with a radiolabeled glutathione *S*-transferase (GST) fusion protein containing the SH2 domain of c-Src (GSTag–c-Src–SH2) (Fig. 9A). Consistent with previous observations (23), the radiolabeled GSTag–c-Src–SH2 probe bound to the Neu immunoprecipitates derived from mammary tumors expressing Neu alone (Fig. 9A, lanes 1 to 3). By contrast, the radiolabeled fusion protein failed to bind the Neu immunoprecipitates derived from the TGF-α-induced tumors (Fig. 9A, lanes 4 to 6). An identical analysis of the Neu immunoprecipitates derived from tumors coexpressing both Neu and TGF-α revealed that they also bound strongly to the radiolabeled GSTag–c-Src–SH2 probe (Fig. 9A, lanes 7 to 10). The observed binding of the GSTag–c-Src–SH2 probe is likely specific to tyrosine-phosphorylated Neu, since previous studies have demonstrated that the radiolabeled GSTag–c-Src–SH2 probe cannot bind comparable levels of tyrosine-phosphorylated EGFR (23). Thus, Neu derived from *neu*/TGF-α tumors is capable of interacting with c-Src in a direct manner in vitro.

To confirm that c-Src could interact with Neu in vivo, the same protein lysates were immunoprecipitated with c-Src-specific antiserum and subjected to immunoblot analyses with Neu-specific antiserum (Fig. 9B). Consistent with the in vitro binding data, Neu protein was found in c-Src immunoprecipitates derived from tumors expressing *neu* alone or coexpressing both *neu* and TGF-α (Fig. 9B, lanes 1, 2, and 5 and 7) but was absent from tumors expressing TGF-α alone since the latter fail to express detectable levels of Neu (Fig. 9B, lanes 3 and 4). Taken together, these observations suggest that transactivation of nonmutated Neu by the activated EGFR results in recruitment of the c-Src signaling pathway.

DISCUSSION

Our results show that coexpression of *neu* and TGF-α in the mammary epithelium of transgenic mice results in the induction of multiple growth disturbances in the mammary epithelium, leading to tumor formation. We also present evidence that the occurrence of these growth disturbances correlates with the constitutive activation of the tyrosine kinase activity of Neu. These observations suggest that TGF-α and *neu* cooperate in mammary tumorigenesis, possibly through transactivation of Neu by the EGFR.

The phenotype exhibited by transgenic mice coexpressing TGF-α and *neu* provides important insight into the interaction of EGFR family members in mammary tumorigenesis. Virgin female mice coexpressing TGF-α and *neu* demonstrated dramatic and distinct mammary morphological differences in comparison with either parental strain (Fig. 4 and 5). In addition, dual transgene carriers developed mammary tumors with greater penetrance and shorter latency than either *neu* or TGF-α animals alone (Fig. 3). One of the most striking features of the mammary tumor tissue derived from bigenic animals is the hyperproliferation of the stromal tissue adjacent to

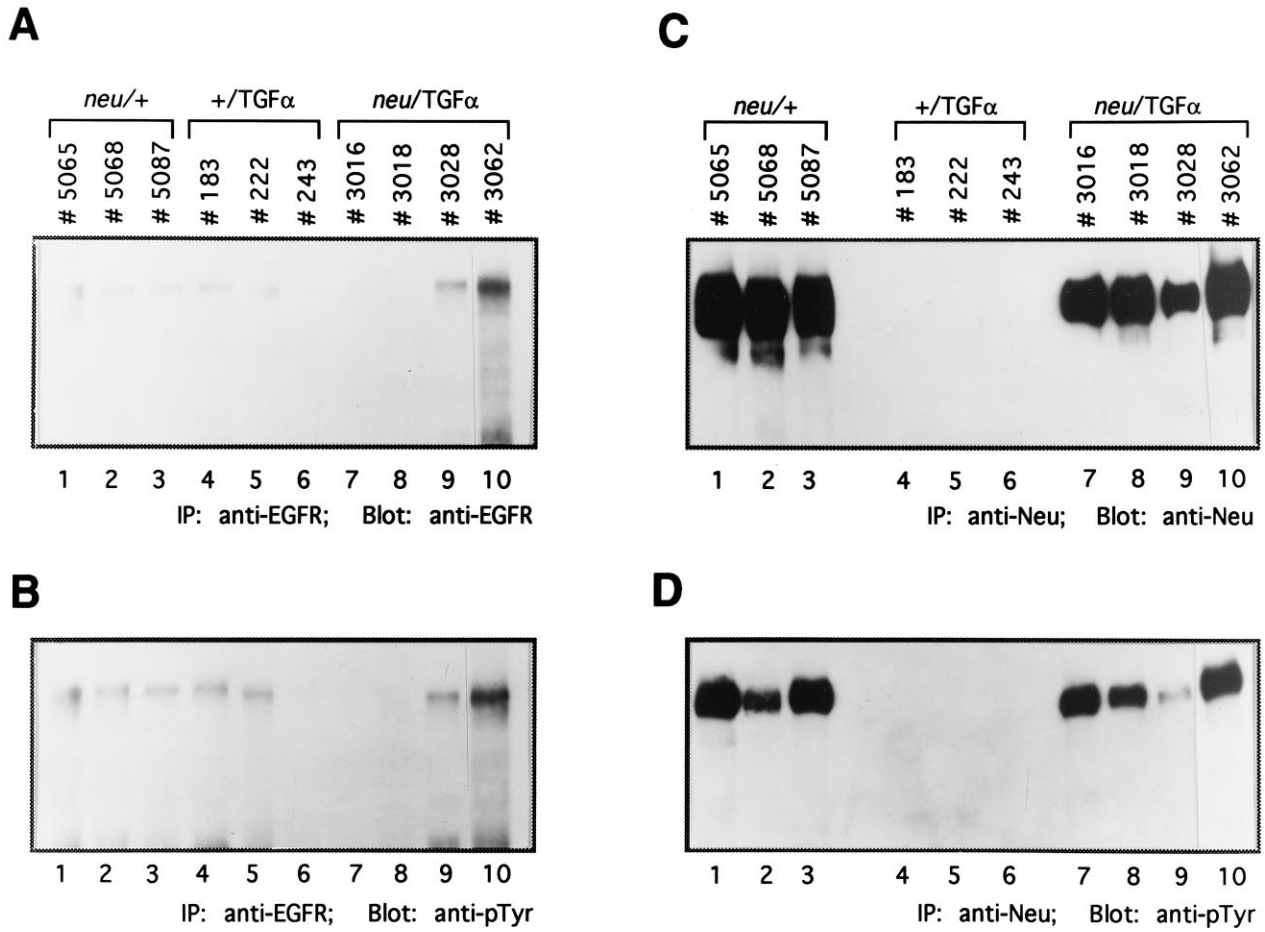

FIG. 7. Mammary tumor tissue from bigenic *neu*/TGF-α mice possess constitutively activated Neu. (A) Protein lysates from tumor tissue carrying either the MMTV/*neu* transgene (*neu*/+), the MMTV/TGF-α transgene (TGF-α/+), or both transgenes (*neu*/TGF-α) were immunoprecipitated (IP) with an anti-EGFR antibody and then subjected to immunoblot analysis with the same antiserum. (B) Tissue lysates identical to those in panel A were immunoprecipitated with anti-EGFR serum and subjected to immunoblot analyses with antiphosphotyrosine antibody 4G10. (C) Protein lysates identical to those in panel A were immunoprecipitated with the 7.16.4 monoclonal (anti-Neu) antibody and then subjected to immunoblot analysis with anti-Neu polyclonal antibody AB.3 (Oncogene Sciences). (D) Tissue lysates identical to those in panel A were immunoprecipitated with the 7.16.4 monoclonal (anti-Neu) antibody and subjected to immunoblot analysis with antiphosphotyrosine antibody 4G10.

the neoplastic mammary epithelium. The occurrence of inflammatory stroma in these tumors was due to the expression of TGF-α, since the MMTV/TGF-α mice also developed inflammatory stroma adjacent to the mammary epithelial hyperplasias. Because the MMTV promoter-enhancer is normally not active in the adjacent stromal tissue (21), the stromal hyperplasias observed in these animals were likely the consequence of local paracrine stimulation of the adjacent stromal cells by the adjacent TGF-α-expressing epithelial cells. Coexpression of TGF-α and *neu* in the mammary epithelia results in the epithelial dysplasias which frequently progressed to mammary adenocarcinomas (Fig. 4, 5, and 6). These observations strongly suggest that TGF-α and Neu can cooperate during mammary tumorigenesis in vivo.

The rapid induction of mammary tumors in the dual bigenic female mice correlates with elevated expression of both the TGF-α and *neu* transgenes. Interestingly, the mammary tumors induced by *neu* alone displayed evidence of altered transcripts (Fig. 1A). Indeed, previous studies have demonstrated that these altered transcripts encode mutant Neu proteins which possess constitutive tyrosine kinase activity (31). Consistent with these data, the levels of tyrosine-phosphorylated Neu in tumors derived from MMTV/*neu* mice were greatly elevated (Fig. 7D). In contrast to these observations, altered *neu* transcripts were not detected in mammary tumors coexpressing TGF-α and *neu* (Fig. 1A). Nonetheless, tyrosine phosphorylation of nonmutated Neu was detected in these tumor tissues, thus supporting EGFR-mediated transactivation of the *neu* proto-oncogene product (Fig. 7D). In addition to tyrosine-phosphorylated Neu, various levels of tyrosine-phosphorylated EGFR were also detected in mammary tumors from transgenic mice coexpressing TGF-α and *neu* (Fig. 7B). The reason for the highly variable EGFR levels in these tumors does not appear to be sampling error, since the same samples possessed elevated Neu. It is conceivable that the different ratios of the *neu* transgene to the endogenous EGFR may influence the phenotype exhibited by the tumors arising in these dual carriers. In this regard, it is interesting that the tumors arising in these bigenic mice exhibited a tubular or nodular phenotype (Fig. 6). However, determination of whether the ratio of the *neu* transgene to the endogenous EGFR influences these phenotypes awaits further analyses.

Unlike the tumors arising in parental MMTV/*neu* mice, which frequently possess activating mutations in the transgene

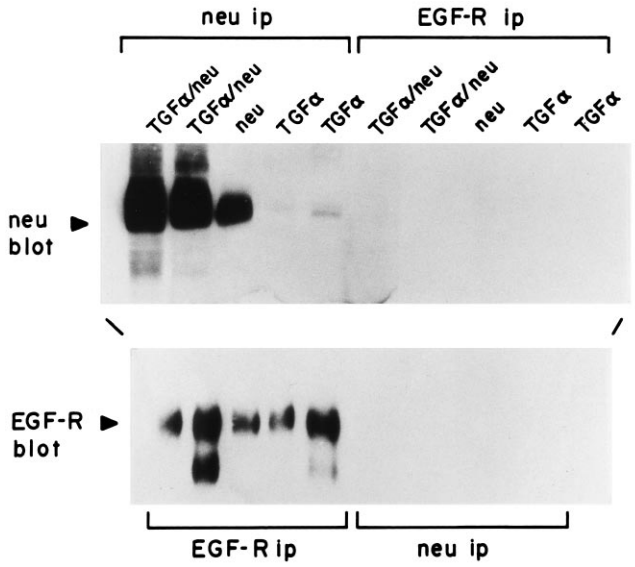

FIG. 8. Lack of detectable EGFR-Neu association in transgenic mammary tumors. Tumor membranes were prepared as described in Materials and Methods. Four hundred micrograms of membrane protein from each tumor was immunoprecipitated (ip) for 2 h with anti-Neu polyclonal antibody 21N or 986 anti-EGFR serum and Staph A cells. Precipitates were then subjected to immunoblot analysis with Neu (top panel)- or EGFR (bottom panel)-specific antiserum. Despite a detectable level of precipitable EGFRs in all of the tumors, EGFR was undetectable in all of the Neu immunoprecipitates.

(31), comparable *neu* mutations were not required for mammary tumorigenesis in bigenic *neu*/TGF-α mice (Fig. 1A, lanes 1 to 7). One possible explanation for the lack of activating *neu* mutations in these tumors is that Neu is activated through association with the EGFR. Consistent with this hypothesis, several groups have demonstrated in both fibroblasts and mammary epithelial cell lines that Neu can be transphosphorylated by the activated EGFR following EGF stimulation (1, 8, 13, 35). In fact, transphosphorylation of Neu can be mediated through the formation of a Neu-EGFR heterodimer (14). Moreover, EGFR-Neu heterodimers exhibit a 10-fold greater affinity for EGFR ligands (39). However, in tumors derived from *neu*/TGF-α mice, stable Neu-EGFR heterodimers were not detected (Fig. 8). Therefore, if heterodimerization between EGFR and Neu is involved in the synergistic induction of mammary tumors, the formation of these complexes is likely transient. This possibility cannot be ruled out by our experimental methods. On the other hand, these heterodimers have been reported only in cells with $>10^5$ EGF-binding sites per cell and after the addition of >10 nM exogenous EGF (8, 39). A lower level of EGFR in *neu*/TGF-α breast tumors may not allow adequate stoichiometric interactions between both RTKs and thus explain our inability to detect receptor heterodimerization. The ability of Neu to cooperate with the activated EGFR is consistent with a number of previous studies. For example, it has been demonstrated that EGFR and Neu can cooperate to transform cell lines in vitro (14). Conversely, it has been shown that administration of antibodies directed against either the EGFR or Neu reverses the transformed phenotype of cells coexpressing both Neu and the EGFR (38).

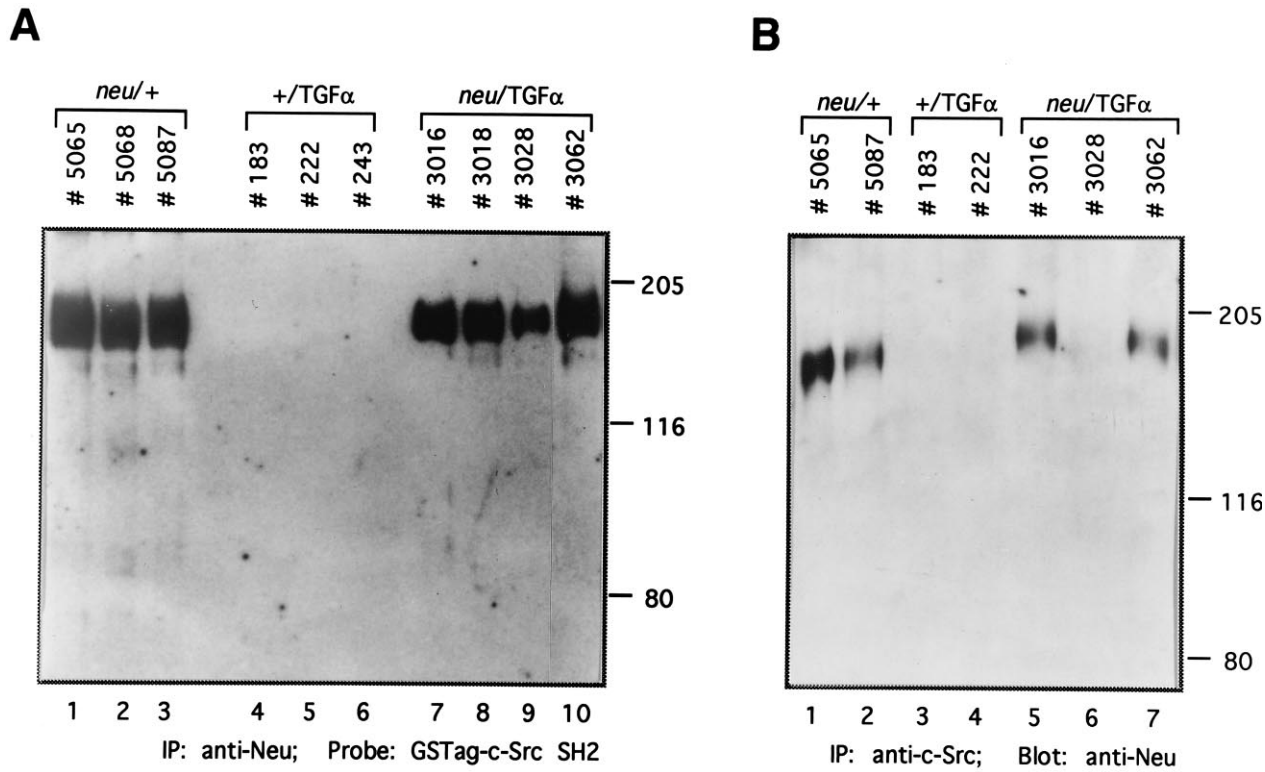

FIG. 9. c-Src is complexed with tyrosine-phosphorylated Neu in *neu*/TGF-α tumors in vitro (A) and in vivo (B). (A) Anti-Neu immunoprecipitates (Anti-Neu) from *neu*/+-, +/TGF-α-, and *neu*/TGF-α-expressing tumors were resolved in an SDS-polyacrylamide gel, blotted onto a polyvinylidene difluoride membrane, and probed with a radiolabeled GSTag–c-Src–SH2 fusion protein. (B) c-Src was immunoprecipitated (IP) (anti-c-Src) from the identical set of lysates and probed with anti-Neu serum.

Although it is clear from these results, as well as other observations, that activation of the Neu RTK by TGF-α results in synergistic transformation of mammary epithelial cells, the molecular basis for this cooperation is unclear. It is conceivable that activation of these closely related RTKs results in the recruitment of distinct but complementary signaling pathways to each of these receptors that then cooperate to transform the mammary epithelial cells. This hypothesis implies that each of these type 1 RTKs is coupled to distinct signaling pathways. In fact, several studies have suggested that coupling of the EGFR to the phosphatidylinositol 3′-kinase requires participation of the c-*erbB*-3 RTK (28, 34). We have demonstrated that the direct and specific interaction of c-Src with Neu is involved in signaling by the activated EGFR (23). Consistent with these earlier observations, we have shown that in mammary tumors induced by coexpression of *neu* and TGF-α, c-Src is complexed both in vitro and in vivo with tyrosine-phosphorylated Neu (Fig. 9). Although preliminary analyses suggested that c-Src activity was elevated in these *neu*- and TGF-α-coexpressing tumors, precise quantitation of the specific activity of c-Src in these tumors was problematic because of the extensive inflammatory stroma present in these tumors (Fig. 6).

Although these studies strongly suggest that TGF-α cooperates with Neu through the activated EGFR, it is unclear whether activation of EGFR is necessary for the induction of mammary tumors by Neu. However, several recent studies suggest that the activity of the EGFR is required for normal mammary epithelial proliferation. For example, a naturally occurring mouse mutant known as waved-2, which possesses a mutation in the EGFR catalytic domain that renders the EGFR functionally inactive (17), exhibits a severe lactation defect (7). Crosses between MMTV/*neu* transgenic mice and waved-2 mice should allow this question to be addressed.

ACKNOWLEDGMENTS

We appreciate the excellent photographic support of Robert Munn and the technical assistance of Monica Graham.

This work was supported by research grants awarded to W.J.M. by the Medical Research Council of Canada, the U.S. Army (DAMD17-94-J-4300), and the Canadian Breast Cancer Initiative. This work was also supported by operating grants to R.J.C. and R.D.C. from the National Cancer Institute (NCI CA 46413 and RO1-CA S4285). W.J.M. is a recipient of a Medical Research Council of Canada Scientist award, M.A.W. was supported by a studentship provided by the Cancer Research Society, P.M.S. was supported by a studentship from the Medical Research Council of Canada, and S.K.M. was a Canadian International Development scholar. R.J.C. and C.L.A. are supported by a Clinical Investigator Career Development award and Merit Review grants from the Department of Veterans Affairs. R.J.C. acknowledges the generous support of the Joseph and Mary Keller Foundation.

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