Modulation of a $M_r$ 175,000 c-neu Receptor Isoform in G8/DHFR Cells by Serum Starvation*

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The transforming neu oncogene was originally isolated from chemically induced rat neuroectodermal tumor cells and subsequently found to be homologous to a nontransforming cellular gene structurally similar to that encoding the epidermal growth factor receptor (EGFR) (1). This nontransforming neu gene proved to be the rodent counterpart of the human HER2 gene (2) which is amplified in 20-30% of advanced breast cancers (3, 4). Amino acid homology between the tyrosine kinase domains of EGFR and HER2 exceeds 80%, and all 50 cysteine residues are identically positioned within the extracellular domains of the two proteins (5, 6). This marked structural homology has suggested that the neu/HER2 gene product may, like EGFR, prove to be a cell surface receptor with ligand-dependent tyrosine kinase activity.

The steps involved in EGFR activation have been well characterized. Two distinct ligands for this receptor, epidermal growth factor (EGF) and transforming growth factor-α (TGF-α), are initially synthesized as large membrane-bound precursor molecules (7, 8), and proteolytic cleavage yields mature soluble ligands which bind to the extracellular domain of EGF. Ligand binding induces EGFR dimerization and tyrosine kinase activation (9) followed by endocytosis and degradation of both ligand and receptor (10). Receptor activation is not exclusively dependent on ligand binding since antibody binding also induces EGFR kinase activity, possibly by inducing EGFR dimerization (11); conversely, ligand availability does not suffice for receptor activation since EGFR exists in both high and low affinity ligand-binding states (12). Reduction of both ligand-binding affinity and tyrosine kinase activity occurs following EGFR phosphorylation by a variety of heterologous transmodulating ligands known to activate protein kinase C, including platelet-derived growth factor (PDGF) (13), bombesin (14), and phorbol ester (15). How do these findings compare with current knowledge regarding neu? Like EGFR, phosphorylation of neu serine/threonine residues is inducible by phorbol ester, though in this case without demonstrable effect on receptor tyrosine kinase activity (16). Similarly, EGF exposure may cause rapid tyrosine phosphorylation of neu, but this takes place without apparent biological or biochemical consequences and seems most likely to reflect cellular serine/threonine kinase transactivation by EGFR (16, 17). Since extensive analyses of neu activation in other proliferating cell systems have yielded only limited information, we decided to investigate the response of the normal neu gene product to growth arrest in G8/DHFR fibroblasts. This is an NIH 3T3-derived cell line expressing a methotrexate-amplified dicistronic rat c-neu/dihydrofolate reductase gene construct (18). Our findings indicate that neu-associated tyrosine kinase activity of G8/DHFR fibroblasts is increased following serum starvation, and that this process is accompanied by conversion of the 185-kilodalton receptor to a low molecular weight form. We also show that a human breast cancer cell line characterised by neu/HER2 gene amplification exhibits no effect of serum starvation on receptor size and that the phosphotyrosine content of these receptors is unaffected by serum conditions.

EXPERIMENTAL PROCEDURES

Cell Culture and Manipulation—Stock cultures of G8/DHFR murine fibroblasts containing an amplified dicistronic rat c-neu/dihydrofolate reductase construct (a gift of Dr Robert Weinberg, Whitehead Institute, Cambridge MA) were maintained in Dulbecco’s minimal essential medium (DME) supplemented with 10% bovine calf serum, glutamine, antibiotics (hereafter referred to as “growth medium”) and 0.3 μM methotrexate. Unless stated otherwise, experi-
mental cell samples were seeded into 10-cm tissue culture dishes, grown to confluence over 3 days, and then maintained for a further 3 days without serum replenishment prior to lysis; the cultured supernatant from these samples is hereafter referred to as "conditioned medium." Cell stimulation was performed by adding the appropriate dilutions of bovine calf serum (HyClone), v-sis PDGF (Angen), EGF (Sigma), TGF-a (Calbiochem), suramin (Mobay), and/or tetradecanoylphorbol ester acetate directly to the conditioned medium unless otherwise specified.

**Immunoblotting and Immunoprecipitation**—The pAb-1 rabbit polyclonal antibody to a cytoplasmic peptide sequence of the neu gene (Triton Biosciences, Alameda CA) was reconstituted in water and diluted 1:100 in TBST buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) for immunoblotting or 10 mM Tris-HCl, pH 7.4, 1 mM ethylenediaminetetraacetate (EDTA) buffer (10 mM Na, HPO4, 7H2O, 10 mM NaH2PO4, H2O, 150 mM NaCl, 1% Nonidet P-40 (v/v), 10% glycerol (v/v), 50 mM sodium fluoride, 10 mM sodium pyrophosphate) plus protease inhibitors (1 mM sodium orthovanadate, 40 mM leupeptin, 10 μg/ml aprotinin, 1 mM phosphomethylsulfonyl fluoride) at a 1:50 concentration for immunoprecipitations. Monoclonal anti-phosphotyrosine antibody was purified over a Staphylococcus aureus protein A affinity column and used at a 1:50 concentration for both immunoblotting and immunoprecipitation. Polyclonal rabbit antibody to murine PDGF receptor (type B) was generously provided by Dr. Mark Mercola, while monoclonal antibody to EGF receptor was a kind gift of Dr. Lam-Bo Chen. Protein lysates were performed by washing monolayer cultures twice with ice-cold phosphate-buffered saline (PBS) and then adding 1 ml of lysis buffer plus inhibitors for 15 min at 4 °C with gentle rocking. Following centrifugation to remove cell debris, lysates were either incubated with antibody overnight followed by a further 60-min incubation with 40 μl of protein-A-Sepharose CL-4B beads (Pharmacia LKB Biotechnology Inc.) for immunoprecipitations, or else immediately boiled for 5 min in sample buffer (6.7% sodium dodecyl sulfate (w/v), 30% glycerol (v/v), 62.5 mM Tris base, pH 6.8, 0.01% bromphenol blue (w/v), or with 0.3 M dithiothreitol in reducing or nonreducing samples, respectively) for immunoblotting. Following boiling, samples were loaded onto a 6% denaturing polyacrylamide gel and electrophoresed for 1 h.

For radioimmunoprecipitations, cell monolayers were washed once with PBS, rinsed with methionine-free DME, then incubated with 3 ml of methionine-free DME plus glutamine, 0.2% platelet-poor plasma, and 350 μCi of [35S]methionine (Du Pont-New England Nuclear) for 4 h at 37 °C. Samples were then washed with PBS and overlaid with the appropriate medium prior to lysis as described above. Following electrophoresis the gel was fixed, dried, and exposed to x-ray film at -70 °C for approximately 72 h. For immunoprecipitations not involving 35S-labeling and for immunoblotting, samples were electrophoresed as above and then transferred onto nitrocellulose as described (19). Membranes were blocked using 2% gelatin for 1 h at 37 °C and then incubated with primary antibody overnight at 4 °C. Following a wash with gentle shaking, membranes with TBST, membranes were incubated with secondary antibody, either 125I-labeled (Du Pont-New England Nuclear) or alkaline phosphatase-conjugated (Promega) polyclonal anti-rabbit/anti-mouse IgG, for at least 2 h. 125I-labeled membranes were exposed to x-ray film for approximately 12 h at room temperature, while alkaline phosphatase-conjugated membranes were developed using chromogenic reagents.

**In Vitro Kinase and Phosphatase—**For tyrosine autophosphorylation reactions, immunoprecipitates were washed once with PBS plus 0.5% Nonidet P-40, twice with 0.5 M LiCl, 50 mM Tris, pH 7.4, and once with kinase buffer (10 mM Tris, pH 7.4, 50 mM NaCl, 10 mM MgCl2, 1% Nonidet P-40) at 4 °C. The beads were then drained, resuspended in 40 μl of kinase buffer containing 2 μl of [γ-32P]ATP (specific activity 3000 Ci/mM), and incubated at room temperature for 20 min. The reaction was stopped by addition of sample buffer followed by boiling for 5 min. Tyrosine phosphorylation of exogenous substrate was assessed using rabbit anti-phosphotyrosine antibody (Promega) and detected out of 100 μl of kinase buffer containing 10 μM ATP and 2 μl of [γ-32P]ATP. The samples were then incubated for 15 min at room temperature prior to addition of sample buffer and boiling for 5 min. Phosphatase reactions were carried out as described previously (20). Briefly, immunoprecipitates were washed as above then resuspended in 100 μl of buffer containing 100 mM Tris-HCl, pH 8.0, 5 mM MgCl2, 100 mM NaCl, plus leupeptin, aprotinin, and phenylmethylsulfonyl fluoride. A further 40 μl of this buffer containing 24 units of calf intestinal alkaline phosphatase (Boehringer Mannheim) was then added for 15 min at 37 °C. The beads were washed once, resuspended in sample buffer, and boiled for 5 min prior to gel loading. Parallel controls containing additional phosphatase inhibitors (100 mM NaF, 200 μM sodium orthovanadate) were also performed to exclude proteolysis.

**In Vivo Phosphorylation and Phosphoamino Acid Analysis—**Confluent cell monolayers were washed free of medium using PBS, rinsed once with phosphate-free DME, and then incubated for 4 h with 3 ml of phosphate-free DME containing glutamine, 0.2% platelet-poor plasma, and 4 μCi of [32P]orthophosphate (Du Pont-New England Nuclear) per 10-cm plate. Samples were subsequently washed twice with PBS and overlaid with 5 ml of conditioned medium plus or minus 10% bovine calf serum for 15 min at 37 °C. The cells were then lysed, immunoprecipitated using neo antibody, washed, boiled, and loaded onto a denaturing 0.1% SDS, 6% acrylamide gel. Following exposure of the dried gel, the gel slices of interest were excised, cleaned, fixed in 30% methanol overnight, then trypsinized for 24 h. The samples were then dried, washed free of trypsin, resuspended in 100 μl of H2O, relyophilized, and then incubated in 90 μl of distilled 6 n HCl at 100 °C for 2 h. HCl was removed by 4 cycles of vacuum centrifugation with serial H2O washes. These dried samples were Cerenkov-counted and then dissolved in buffer containing 0.3% each of xylene cyanol, orange G, and acid fuchsin dye stocks. Following correction for total Cerenkov counts, approximately 0.5 μl of each sample was spotted onto a 20 × 20-cm plastic-backed cellulose thin layer chromatography plate (EM Laboratories). Electrophoresis was then performed at 800 V for 75 min, following which the plate was exposed to x-ray film for 3 weeks.

**RESULTS**

**Serum-starved G8/DHFR Cells Express a Low Molecular Weight c-neu Receptor Which Is Tyrosine-phosphorylated and Associated with Increased Tyrosine Kinase Activity—**Immunoblotting of serum-starved and serum-fed G8/DHFR cell lysates reveals increased electrophoretic mobility of the neu receptor in starved samples, corresponding to a 10-kilodalton reduction in molecular mass (Fig. 1A); this difference is seen in both reduced and nonreduced samples, with nonreduced samples exhibiting marginally greater electrophoretic mobility (data not shown). Immunoprecipitation of the neu receptor followed by immunoblotting for phosphotyrosine indicates that the low molecular mass form of neu (p175) seen in serum-starved samples is associated with a tyrosine-phosphorylated form (p185) present in serum-replenished samples is not (Fig. 1B). When neu immunoprecipitates are allowed to autophosphorylate under conditions favoring tyrosine kinase activity (Fig. 1C) we observe a 2-fold increase in phosphate content of the neu isoform in serum-starved versus serum-stimulated samples, as determined by either densitometry or scintillation counting. Tyrosine phosphorylation of enolase, an exogenous substrate for tyrosine kinases, is also twice as efficient in starved neu immunoprecipitates when compared to serum-stimulated samples by scintillation counting (Fig. 1D). Control experiments confirm that anti-phosphotyrosine immunoprecipitates yield neu bands on immunoblotting only in serum-starved cells (Fig. 1E), whereas the phosphotyrosine content of anti-PDGF-receptor immunoprecipitates is exclusively associated with serum-stimulated G8/DHFR cell lysates (Fig. 1F). These data exclude PDGF receptor as a confounding source of tyrosine kinase activation in serum-starved G8/DHFR cell samples. Compared with neu and PDGF receptor expression, few EGF receptor sites are able to be immunoprecipitated from this cell line (Fig. 1G), tending similarly to exclude EGF receptor as a confounding factor in these experiments.

**Concurrence of p175 to p185 Occurs Within Minutes of Serum
Serum Starvation Modulates c-neu Activity

FIG. 2. Immunoblotting of the c-neu gene product (A) and phosphotyrosine (B) at different times after serum replenishment of serum-starved G8/DHFR cells. 1st lane, serum-starved cells; 2nd lane, cells re-fed for 2 min prior to lysis; 3rd lane, cells re-fed for 10 min; 4th lane, cells re-fed for 30 min; 5th lane, cells re-fed for 4 h; 6th lane, cells re-fed for 24 h. Immunoblotting was performed as described under “Experimental Procedures” except that lysates were electrophoresed on a 7.5% SDS-polyacrylamide gel. Blots were probed using alkaline phosphatase-conjugated second antibodies followed by colorimetric analysis. For discussion of findings, see “Results.”

Replenishment—Two forms of the c-neu receptor can be distinguished when using an alkaline phosphatase immunoblotting technique. Starved confluent G8/DHFR cell samples, and those re-fed with serum for 2 min only, express p175 as the predominant receptor form, while samples re-fed for between 10 min and 4 h express mainly p185; 24 h following refueling p175 is again predominant (Fig. 2A), suggesting either that serum exhaustion has occurred or that the effect of serum is transient in density-arrested cell samples. Anti-phosphotyrosine immunoblotting shows that attenuation of tyrosine phosphorylation in p175 begins as early as 2 min following serum replenishment (Fig. 2B). Phosphotyrosine reappears as early as 4 h after refueling of confluent cultures, and the process is completed within 24 h (Fig. 2B).

The Serum-induced Increase in Receptor Size Is Associated with Increased Phosphoamino Acid Content—In vivo phosphoamino acid labeling of G8/DHFR cells followed by neu immunoprecipitation (Fig. 3A) indicates that 15-min exposure to fresh serum results in at least 2-fold greater incorporation of orthophosphate by p185 when compared to p175 by densitometry. Phosphoamino acid analysis reveals that this increase is associated with phosphorylation of serine and threonine residues in serum-stimulated samples, and confirms that serum-starved cells incur an increase in phosphotyrosine (Fig. 3B). The effects of serum on neu phosphoamino acid composition were mimicked by PDGF and phorbol ester (data not shown). Phosphatase treatment of neu immunoprecipitates indicates that the serum-induced increase in molecular weight is phosphatase-sensitive (Fig. 3C). The phosphatase-induced increase in p175 electrophoretic mobility probably reflects the removal of known phosphate residues from this protein, whereas the difference in mobility between phosphatase-treated p175 and p185 could reflect availability of phosphate moieties or an additional serum-stimulated modification of p185. Tunicamycin, an inhibitor of O-glycosylation, does not affect conversion of p175 to p185 (data not shown).
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FIG. 3. A, in vivo phospholabeling of serum-starved and serum-replenished G8/DHFR cells immunoprecipitated using neu antibody (see “Experimental Procedures” for methods). B, phosphoamino acid analysis of cell samples treated with conditioned medium alone, 10% bovine calf serum, or PDGF. The positions of ninhydrin-treated control samples are indicated at left. C, in vitro phosphatase treatment of 32P-prelabeled starved and serum-stimulated neu immunoprecipitates. 1st and 3rd lanes, starved and fed samples, respectively; 2nd and 4th lanes, phosphatase-treated starved and fed samples, respectively; 5th and 6th lanes, starved and fed samples treated with phosphatase plus phosphatase inhibitors. See “Experimental Procedures” for methods. D, effect of growth factors and phorbol ester on p175. Cell samples represented in the 1st through 6th lanes were treated for 15 min with the appropriate reagent, lysed, and then immunoblotted using neu antibody electrophoresed, and exposed to x-ray film for 1 week. D, effect of varying serum concentrations on c-neu receptor in semiconfluent G8/DHFR cells during log-phase growth. Growth medium was replaced with the indicated concentration of bovine calf serum in DME for 16 h, following which cell samples were lysed and immunoblotted using either neu or phosphotyrosine antibody. PPP, platelet-poor plasma.

Conversion of p175 to p185 Is Inducible by Platelet-derived Growth Factor or Phorbol Ester, But Not by Epidermal Growth Factor or Transforming Growth Factor-α—To investigate whether the effect of serum is attributable to one of its known constituents, serum-starved cell samples were treated with various serum components. Exposure of starved cells to fresh platelet-poor plasma induces no change in p175 (Fig. 3D, lane 2). Similarly, neither EGF nor TGF-α exposure affect p175 electrophoretic mobility (lanes 4 and 5, respectively) or phosphotyrosine content (data not shown). Treatment with either PDGF or phorbol ester (both agonists of protein kinase C) mimics the effect of serum, inducing conversion of p175 to p185 (lanes 3 and 6, respectively). Long-term (24 h) phorbol
ester addition to down-regulate protein kinase C is associated with similar attenuation of the response to both phorbol ester itself (lane 7) and to PDGF (lane 8), indicating that the p175-to-p185 conversion is at least partly dependent upon a protein kinase C pathway. Cycloheximide does not inhibit serum- or PDGF-induced conversion of p175 to p185, suggesting that this rapid effect does not depend upon new protein synthesis, while addition of suramin antagonizes the serum-induced conversion of p175 to p185 but does not affect the electrophoretic mobility or phosphotyrosine content of p175 (data not shown); the latter finding is consistent with the known effect of suramin on PDGF binding to its receptor but does not provide positive support for the existence of a soluble ligand in this experimental system.

Actuation of p175 Takes Place in the Apparent Absence of Soluble Ligand—While it is clear from the above that at least one soluble factor in serum (PDGF) can induce conversion of p175 to p185, it remains uncertain whether activation of the c-neu receptor seen in serum-starved G8/DHFR cells reflects release of a soluble peptide into the conditioned medium. Conversion of p175 to p185 occurs with equal rapidity irrespective of whether serum is added directly to the conditioned medium or whether the conditioned medium is replaced by serum in fresh medium (Fig. 4A). In contrast, replacement of conditioned medium with fresh serum-free medium for 15 min affects neither the size nor the phosphotyrosine content of p175 (Fig. 4A, lane 4). These findings suggest either that in serum-starved G8/DHFR cells c-neu receptor activation occurs in the absence of soluble ligand or, less plausibly, that such ligand is very tightly bound to the receptor for long periods.

Control experiments confirmed that conditioned medium promptly induces growth retardation in G8/DHFR cells, with cell doubling time increasing from 16 to 28 h within 1 day (data not shown); interpretation of these experiments is hindered by the confounding effects of nutrient depletion and toxin accumulation. Unlike the rapid conversion of p175 to p185 seen with serum addition, however, exposure to conditioned medium for up to 15 min has little if any effect on p185 expressed by confluent G8/DHFR cells (Fig. 4B); reconversion of p185 to the tyrosine-phosphorylated p175 requires several hours (see Fig. 2 and below), similar to the reported 6–8-h duration of PDGF-induced EGFR inactivation (13). This is again consistent with the absence of soluble ligand, but could also indicate that serum stimulation induces a nonligand-binding form of the receptor which persists for several hours following serum removal.

To assess whether the effect of prolonged incubation with conditioned medium is reproducible by fresh medium containing low serum alone, confluent G8/DHFR monolayers were fed for 1 h with fresh growth medium then washed and re-fed for 8 h with fresh medium containing varying concentrations of serum. Fig. 4C shows that low serum induces the appearance of p175; this finding tends to implicate serum starvation, rather than the existence of a soluble ligand found exclusively in conditioned medium, as the basic prerequisite for activation of the c-neu receptor in G8/DHFR cells. In addition, this finding excludes toxin accumulation (such as might be induced in experiments involving long-term incubation of cells in conditioned medium) as a confounding variable in the interpretation of these data. In a variation of this experiment, semiconfluent G8/DHFR cells in exponential growth phase were washed free of growth medium and re-fed for 16 h with medium containing varying serum concentrations. Once again, low serum is associated with the appearance of tyrosine-phosphorylated p175 (Fig. 4D), indicating that density-de-

dependent growth arrest is not essential for c-neu receptor activation. This does not exclude the possibility that 

activation.

Serum Starvation Affects Neither Electrophoretic Mobility Nor Phosphotyrosine Content of SK-Br-3 Human Breast Cancer Cells—To investigate whether serum- and PDGF-induced regulation of c-neu receptor activity also occurs in human tumor cells, we examined SK-Br-3 human breast adenocarcinoma cells which are known to overexpress the homologous (and apparently nonmutated) human gene. Fig. 5 shows that serum starvation induces no measurable change in either the size of the 190-kilodalton HER2 receptor or the phosphotyrosine content thereof. Moreover, the absolute amount of measurable phosphotyrosine in both starved and fed cell samples appears small by comparison with that seen in the nontransformed G8/DHFR cell line despite approximately equal receptor contents. Another breast cancer cell line, BT-474, expressed HER2 receptor which was similarly unaffected by serum with respect to its electrophoretic mobility and phosphotyrosine content; unlike SK-Br-3 cells, however, phosphotyrosine associated with the BT-474 HER2 receptor was readily detectable in both the presence and absence of conditioned medium (data not shown). These findings suggest that neither of these two cell lines may secrete a soluble ligand for HER2; the invariant tyrosine phosphorylation found in BT-474 cells could indicate constitutive receptor activation consistent with structural abnormalities present in the extracellular domain of the receptor in this cell line.2

DISCUSSION

The central findings of this study are that c-neu-associated kinase activity is negatively regulated by PDGF (or serum that contains PDGF) and that this negative regulation is reversible. There are two caveats which accompany these conclusions. First, the modulation of tyrosine kinase activity we observed was only 2-fold in magnitude; full activation of c-neu by its ligand may trigger much larger changes in tyrosine kinase activity which may or may not be modulated by serum starvation. Second, our data were generated with a cell line engineered to overexpress the c-neu protein. We have yet to

2 M. Page, personal communication.
show that the effects of serum starvation described here can be documented in cells expressing c-neu at normal abundance levels; however, there is no a priori reason for expecting a qualitative change in the biochemistry of c-neu to accompany a quantitative change in abundance of the protein. Furthermore, the data shown here tie into a broader body of literature on c-neu and its close structural homologue, the EGFR receptor.

The c-neu receptor has previously been reported to be tyrosine-phosphorylated via EGF-induced activation of EGFR in Rat-1 fibroblasts (17, 22), MKN-7 human gastric cancer cells (16), KB human epidermal carcinoma cells (23), and SK-BR-3 human breast cancer cells (21); under these circumstances, however, the neu receptor’s tyrosine kinase activity remains unchanged (16) and the receptor is not down-regulated (21). Such findings indicate that in these cell systems EGF-induced neu tyrosine phosphorylation is not associated with detectable biological consequences. In contrast, the abolition of p175-neu tyrosine phosphorylation in G8/DHFR cells by serum, PDGF, or phorbol ester is clearly associated with receptor inactivation, thus suggesting a paradigm of c-neu regulation in which receptor inactivity cannot automatically be interpreted as signifying ligand absence. The apparent failure of EGF to affect p175-neu in serum-starved G8/DHFR cells is in accord with the results of others using this cell line (24), perhaps reflecting an inability of EGF to bind EGFR in the serum-starved state. Our data also suggest that the transmodulating effects of PDGF and serum are mediated at least in part by protein kinase C, although recent reports caution that such heterologous transmodulation may occur to some extent via non-protein kinase C pathways (16, 25, 26).

Few genes or gene products are known to become activated during cell progression to quiescence, yet the demonstration here of starvation-induced c-neu tyrosine kinase activation (Fig. 1) indicates that this proto-oncogene product may be such a molecule. Indirect evidence has recently suggested that NIH 3T3 cells synthesise the neu/HER2 ligand, albeit at low levels (27), and our findings are consistent with low level ligand binding mediating starvation-induced receptor activation. An alternative interpretation is that removal of transmodulating serum growth factors may play a purely permissive role in receptor activation. However, the reappearance of phosphotyrosine within 4 h of refeeding density-arrested cultures (Fig. 2B) indicates that receptor activation can occur even in the presence of growth factors, thus raising the possibility that quiescence per se (rather than growth factor deprivation) is responsible for p175 activation. Our study provides no firm data as to the availability of the putative activating ligand, although the apparent absence of soluble ligand in the context of receptor activation is intriguing.

The virtual absence of detectable neu/HER2 receptor activation in serum-starved SK-BR-3 breast cancer cells (Fig. 5) could reflect any one of several possible explanations, including failure of serum starvation to induce quiescence, constitutive transmodulation of the receptor preventing ligand binding, or absent ligand production by the carcinoma cells. It is therefore conceivable that the reported amplification of the neu/HER2 proto-oncogene in human tumors could reflect a compensatory rather than a pathogenetic process, a possibility consistent with the occurrence of such gene amplification in advanced rather than early stage disease (3) and with the related finding of other regulatory gene anomalies in human breast tumors (28, 29). However, the finding of abundant HER2 tyrosine phosphorylation in another breast cancer cell line suggests that a variety of mechanisms may regulate the activation of this receptor. Resolution of these and other uncertainties obscuring the function of neu/HER2 will almost certainly require identification of the binding ligand; our findings here suggest that experimental strategies toward this end may need to incorporate the notion that such binding may be prevented by receptor transmodulation.

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