Human Cancer Cells Exhibit Protein Kinase C-dependent c-erbB-2 Transmodulation That Correlates with Phosphatase Sensitivity and Kinase Activity*

Xiaomei Ouyang‡§, Tim Gulliford‡¶, Hongyi Zhang‡¶, Guo Cai Huang‡§, and Richard Epstein**‡‡

From the ‡Division of Cell, Molecular and Oncology Research, Charing Cross and Westminster Medical School, University of London and ¶‡‡Cancer Research Campaign Laboratories, Department of Medical Oncology and (Department of Biochemistry, Charing Cross Hospital, Fulham Palace Road, London W6 8RF, United Kingdom

The c-erbB-2 receptor tyrosine kinase is often overexpressed in human tumors, but the functional implications of this phenotype remain unclear. We previously used phosphorylation-specific antibodies to define major differences in c-erbB-2 tyrosine kinase activity between overexpressing human tumor cell lines (Epstein, R. J., Druker, B. J., Roberts, T. M., and Stiles, C. D. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10435–10439). Here we extend this approach to define the relationship between c-erbB-2 tyrosine phosphorylation and protein kinase C (PKC)-dependent transmodulation. Phosphorylation-specific antibodies to the juxtamembrane PKC site Thr686 recognize tyrosine-dephosphorylated wild-type c-erbB-2 following G8/DHFR 3T3 cell treatment with PKC agonists. B104-1-1 cells transformed by activated c-erbB-2 express a subset of tyrosine-phosphorylated receptors that are homologously phosphorylated on Thr686, indicating that Thr686 phosphorylation alone is insufficient to abrogate receptor tyrosine phosphorylation. Similarly, the c-erbB-2 overexpressing human cancer cell lines SK-Ov-3 and BT-474 express constitutively Thr686-phosphorylated receptors. SK-Ov-3 cells express predominantly kinaseinactive c-erbB-2 that is heavily Thr686-phosphorylated, indicating that Thr686 phosphorylation in this line is heterologous in origin. In contrast, BT-474 cells express constitutively auto-phosphorylated c-erbB-2 despite Thr686 phosphorylation. These results indicate that Thr686 phosphorylation does not directly abolish c-erbB-2 activity and suggest that such phosphorylation reflects constitutive PKC activity induced by either receptor-activating mutations or heterologous growth factors. The latter possibility suggests in turn that c-erbB-2 interacts in an as yet undefined way with heterologous growth factor receptors in human tumor cells.

The c-erbB-2 (neu, HER-2) receptor is a type I receptor tyrosine kinase commonly overexpressed in human tumors such as breast cancer (1). A plausible explanation for this phenotype is that functional kinase activity predisposes to the clonal outgrowth of a neoplastic population. This hypothesis has been supported by transgenic studies of mutant neu overexpression, which have confirmed in vivo tumorigenesis (2, 3). Extrapolating from such studies to the clinic has proven problematic, however, for various reasons. First, tyrosine phosphorylation of the wild-type receptor in vitro has been associated with growth arrest (4) and differentiation (5) as well as with mitogenesis. Second, transforming c-erbB-2 mutations are readily inducible by point mutation (6), yet few if any such mutations have been documented in sporadic tumors (7). Third, correlation of functional c-erbB-2 activity with clinical outcome is still awaited, whereas certain c-erbB-2 antibodies used in such studies preferentially detect catalytically inactive receptors (8). Finally, overexpression of c-erbB-2 occurs more commonly in subtypes of preinvasive (in situ) disease than in established breast cancer (9), casting doubt on the evolutionary role of this molecule in tumor growth.

How c-erbB-2 is functionally regulated in human tumor cells therefore remains an open question with potentially important therapeutic implications. Unlike its homologue, the epidermal growth factor receptor (EGFR), c-erbB-2 has no known homodimerizing ligand and remains largely uncharacterized with respect to autoregulatory homeostatic mechanisms. Catalytic activation of c-erbB-2 appears inducible by either mutation-driven homodimerization (10) or perhaps by membrane overexpression alone. However, the latter mechanism does not explain the variability in net receptor autophosphorylation between cell lines with similar c-erbB-2 expression (11), suggesting that other variables, such as heterodimer formation, paracrine ligand expression, or signaling crosstalk, are likely to influence in vivo receptor function.

The ability of protein kinase C (PKC)1-dependent signaling pathways to induce homologous desensitisation and/or heterologous transmodulation of receptor tyrosine kinases has been recognized for over a decade (12–18). In recent years, much of this work has focused on PKC-dependent phosphorylation of threonine-654 (Thr654) in the juxtamembrane domain of EGFR (19–22). The importance of this Thr654 site for negative EGFR regulation has now been established by site-directed mutagenesis (23–26). This regulatory domain is approximately 70% homologous to the Thr686-containing region of the c-erbB-2 receptor (27, 28). We and others have previously used in vivo labeling and phosphoamino acid analysis to show that threonine phosphorylation of c-erbB-2 occurs in response to PKC agonists such as phorbol esters (11, 29, 30) and heterologous growth factors (4, 29), and have confirmed that such exposures are tightly linked to c-erbB-2 tyrosine dephosphorylation (4, 31). Because oncogene-inhibitory signaling pathways are potentially attractive targets for anticancer drug development, clarification of PKC-dependent c-erbB-2 transmodulation could

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††To whom correspondence should be addressed. R. E. is supported by the Cancer Research Campaign.

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1 The abbreviations used are: PKC, protein kinase C; EGFR, epidermal growth factor receptor; PdBU, phorbol dibutyrate.
prove useful in developing novel therapies.

In an earlier report, we demonstrated the utility of phosphorylation-specific c-erbB-2 antibodies for identifying intrinsic differences in receptor catalytic activity between human tumor cell lines (8, 11). Here we use phosphothreonine-specific c-erbB-2 antibodies to analyze regulatory patterns of PKC-inducible receptor desensitisation in human and rodent cell lines expressing different forms of the receptor. The results support the paradigm that juxtamembrane threonine phosphorylation is linked to negative c-erbB-2 regulation in some cell systems and confirm differences in phosphorylation patterns between wild-type and mutant receptors and between malignant and nonmalignant cell lines. They also indicate, however, that the neoplastic phenotype may be affected by cell signaling alterations distinct from (though not necessarily independent of) c-erbB-2 overexpression.

EXPERIMENTAL PROCEDURES

Cell Culture—GBDHFR cells (NIH 3T3-derived murine fibroblasts) expressing a transfected and methotrexate-amplified diestradiol rat c-neu/dihydrofolate reductase clone (32) were a gift of Dr. Robert Weinberg (Whitehead Institute, Cambridge, MA). The cell lines BT-474, SK-Ov-3, and B104-1 were obtained from the American Type Culture Collection. GBDHFR cells were maintained in a humidified 5% CO$_2$ incubator at 37°C in Dulbecco’s minimal essential medium supplemented with 10% fetal calf serum, glutamine, and antibiotics; for GBDHFR stock cultures, 0.3 mM thioglycollic acid was added to the medium. SK-Ov-3 cells were maintained in RPMI plus 10% fetal calf serum, glutamine, and antibiotics. Experimental cell samples were seeded into tissue culture dishes and treated by adding either phorbol dibutyrate (PdBu; Sigma; 100 ng/ml) or sodium orthovanadate (Sigma; 1 mM) directly into the medium.

Immunological Reagents—The pAb-1 rabbit polyclonal antibody to the tyrosine 1248-containing C-terminal peptide sequence of c-erbB-2 (anti-Tyr$^{1248}$, Triton Biosciences, Alameda, CA) was reconstituted in water and diluted 1:1000 in TBST buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) for immunoblotting. The Ab-3 mouse monoclonal antibody against the same peptide sequence (Oncogene Science) was used at 1:1000 for immunoprecipitations. Monoclonal 4G10 antiphosphotyrosine antibody (a kind gift of B. Druker and T. Roberts) was purified over a Staph aureus Protein A affinity column, diluted in TBST buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) and used at 1:1000 for immunoblotting.

Phosphorylation-specific c-erbB-2 Antibodies—These were developed as described previously (13). Briefly, phosphothreonine-specific c-erbB-2 antibodies were developed by immunoabsorbing rabbits with a threonine-phosphorylated peptide KIRKYTMRLRL (Calbiochem-Novabiochem) corresponding to the juxtamembrane Thr$^{668}$-containing erbB-2 sequence (28). The resulting antiserum was screened by immunoblotting (1:10,000) using control and PdBu-treated GBDHFR cell lysates. Immunoprecipitation and immunocytochemical studies were performed at 1:5000 dilution. Adsorption of crude antiserum to the unphosphorylated peptide to eliminate contaminating non-phosphorylation-specific antibodies was carried out with Lysates.

In Vitro Protein Kinase C Phosphorylation—PKC was purchased from Promega. The sequence of the control Ser$^{1113}$-containing peptide was PLQRYSEDP. The assay was performed by incubating 25 ng of PKC with 200 ng of peptide substrate at 37°C for 20 min in PKC assay buffer (20 mM HEPES, pH 7.4, 1.5 mM CaCl$_2$, 1 mM diithiothreitol, 10 mM MgCl$_2$, 1 mM ATP, 100 mM glycerol) containing 10 μCi ($^{32}$P)ATP in a total volume of 25 μl. An identical assay without PKC was used as a negative control. The reaction was terminated by adding 25 μl of 1.5% H$_2$O$_2$, and the reaction mix was spotted onto a Whatman P-81 filter. After washing in 0.5% H$_2$PO$_4$ for 4 min each filter, the air was dried and radioactivity determined by scintillation.

Protein Lysis, Immunoblotting, and Immunoprecipitation—Protein lysis was performed by washing monolayer cultures twice with ice-cold PBS, then adding lysis buffer (10 mM Na$_2$HPO$_4$, 7H$_2$O, 10 mM NaH$_2$PO$_4$, 150 mM NaCl, 1% Nonidet P-40 (v/v), 0.1% glycerol (v/v), 50 mM sodium fluoride, 10 mM sodium pyrophosphate) plus protease inhibitors (1 mM sodium orthovanadate, 40 μM leupeptin, 10 μM aprotinin, 1 mM phenylmethylsulfonyl fluoride) for 15 min at 4°C with gentle rocking. After being centrifuged to remove cell debris, lysates for immunoblotting studies were immediately boiled for 5 min in sample buffer (6.7% sodium dodecyl sulfate (w/v), 30% glycerol (w/v), 62.5 mM Tris base, pH 6.8, 0.01% bromphenol blue) then loaded onto a 7.5% polyacrylamide gel. For immunoprecipitations, 100 μl lysates were incubated with antibody overnight followed by a further 30-min incubation with 40 μl protein A-Sepharose CL4B beads (Pharmacia Biotech Inc.) prior to washing three times in ice-cold TBS, addition of sample buffer, and boiling. Samples were electrophoresed as above and then blottedted onto nitrocellulose as described (33). Membranes were blocked for 2 h at 37°C, then incubated with primary antibody overnight at 4°C with gentle shaking. Following three washes with TBS, membranes were incubated with alkaline-phosphatase-conjugated (Promega) polyclonal anti-rabbit/anti-mouse IgG for at least 2 h, washed, then developed immediately using colorimetric reagents.

In Vivo Phospholabeling and Phosphoaminoacid Analysis—Cell monolayers were washed free of medium using PBS, rinsed once with phosphate-free Dulbecco’s minimal essential medium, and incubated for 4 h with 3 ml of phosphate-free Dulbecco’s minimal essential medium containing glutamine, 0.2% bovine calf serum, and 4mCi $^{32}$P-orthophosphate per 10-cm plate. Samples were subsequently washed twice with PBS and overlaid with 5 ml of medium plus or minus PdBu for 15 min at 37°C. The cells were then lysed, immunoprecipitated using c-erbB-2 monodonal antibody, washed, boiled, and loaded onto a denaturing 0.1% SDS 7.5% acrylamide gel. After exposure, the gel slices of interest were excised, cleaned, fixed in 30% methanol overnight, and then trypsinised for 24 h. The samples were dried, washed free of trypsin, resuspended in 100 μl of H$_2$O$_2$, rehyphosphilated, and then incubated in 90 μl of distilled 6% HCl at 100°C for 2 h. HCl was removed by four cycles of vacuum centrifugation with serial H$_2$O washes. Dried samples were Cerenkov counted and then dissolved in buffer containing 0.3% each of xylene cyanol, orange G, and acid fuchsin dye stocks. After 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. Electrophoresis was then performed at 800 V for 75 min, and the plate was exposed to x-ray film for 2 weeks.

RESULTS

The Thr$^{668}$-containing juxtamembrane peptide sequence of c-erbB-2 is efficiently phosphorylated by PKC in vitro. As mentioned earlier, the juxtamembrane Thr$^{668}$-containing peptide sequence of EGFR is the main protein kinase C site of the receptor (20, 23, 34) and exhibits major homology to the Thr$^{686}$-containing sequence in c-erbB-2, which has been characterized as a PKC consensus site (27, 28). To confirm that this region is indeed a PKC substrate, we compared the PKC-inducible phosphorylation of this peptide sequence in vitro with that of a second c-erbB-2 peptide sequence homologous to a second EGFR desensitization domain believed to represent a CaM kinase II site (35). The results of these experiments, presented in Table I, confirm that the Thr$^{668}$-containing peptide is phosphorylated by PKC almost 100-fold more efficiently than the Ser$^{1113}$-containing peptide, or a peptide sequence from the extracellular domain of c-erbB-2 (data not shown). The explanation for the phosphorylation detected on the peptide incubated with isoforme but without PKC is not clear (see Table I), though we note that this accounts for less than 2% of the signal seen with PKC. We therefore conclude that the Thr$^{668}$-containing peptide is an excellent PKC substrate in vitro.

Polycyclic Antibodies to a Synthetic Thr(P)$^{668}$-containing Peptide Display Selective Immunoreactivity for the c-erbB-2
Receptor in Phorbol- and Vanadate-treated G8/DHFR Cells—

Given the foregoing, the Thr<sup>686</sup>-containing peptide sequence of c-erbB-2 seemed an appropriate immunogen for raising antibodies against the PKC-modified receptor, with the resulting antisera capable of being screened using protein lysates obtained from cells treated with known PKC agonists and antagonists. The former include PdBU (36) as well as the tyrosine phosphatase inhibitor sodium orthovanadate (because tyrosine phosphorylation is an activator of PKC), whereas the latter include calphostin C (38). Fig. 1A illustrates the screening of crude antisera to the Thr<sup>686</sup> immunogen and shows the inverse pattern of immunoreactivity when compared with an antiphosphotyrosine antibody. The anti-Thr<sup>686</sup> antisera also selectively recognizes the native form of PKC-modified c-erbB-2 as demonstrated by immunoprecipitation (Fig. 1B).

Binding of anti-Thr<sup>686</sup> to c-erbB-2 Is Phosphorylation-specific, Receptor-specific, and PKC-dependent—The phosphorylation status of synthetic phosphopeptides used for immunization was confirmed using mass spectrometry (data not shown). These phosphopeptides were then used in conjunction with unphosphorylated peptides of identical sequence to establish the phosphorylation specificity of anti-Thr<sup>686</sup> binding. Immunoreactivity of control and PdBU-treated G8/DHFR lysates is abolished by preincubation of anti-Thr<sup>686</sup> for 30 min with the immunizing phosphopeptide KIRKYTMRRLL (+Thr<sup>10</sup>; 10 μg/ml; Fig. 2A, lanes 3 and 4). In contrast, incubation with the unphosphorylated peptide (+Thr, 10 μg/ml) has only a minor effect on immunoreactivity, abolishing nonphosphorylation-specific “background” antisera binding from control cells (Fig. 2A, lane 5) but sparing anti-Thr<sup>686</sup> immunorecognition of the PdBU-treated sample (Fig. 2A, lane 6). This indicates that the crude anti-Thr<sup>686</sup> antiserum binds preferentially to the threonine-phosphorylated peptide and that a minor degree of non-phosphorylation-specific background binding of the polyclonal can be eliminated by competing with the unphosphorylated peptide, as demonstrated in a previous report (8). A 10-fold molar excess (w/v) of soluble phosphothreonine fails to abrogate anti-Thr<sup>686</sup> immunoactivity when compared with the phosphopeptide (data not shown), indicating that the antisera does not recognize phosphothreonine alone but specifically detects the threonine-phosphorylated c-erbB-2 juxtamembrane sequence. Similarly, immunoreactivity of anti-Thr<sup>686</sup> for PKC-modified c-erbB-2 is not attenuated by preincubating the antibody with tyrosine- or serine-phosphorylated peptides (data not shown). Phosphoamino acid analysis confirms phorbol-inducible enhancement of c-erbB-2 threonine (and serine) phosphorylation associated with c-erbB-2 tyrosine dephosphorylation (Fig. 2B).

Because the phosphopeptide immunogen used to raise anti-Thr<sup>686</sup> exhibits significant amino acid homology to the corresponding EGFR sequence, we next sought to exclude cross-reactivity of the antisera with protein lysates from ligand- and phorbol-treated EGFR-expressing cells. Fig. 3 shows that neither high concentrations of EGF (100 ng/ml) nor high concentrations of PdBU (100 ng/ml) induce anti-Thr<sup>686</sup> immunorecognition in EGFR-expressing chimeric 293 cell transfectants, thus confirming the receptor specificity of this reagent.

The PKC dependence of anti-Thr<sup>686</sup> immunoreactivity was tested by pretreating G8/DHFR cells with the fluorescence-activated protein kinase C antagonist calphostin C (38) prior to PdBU or vanadate exposure. This inhibitor reduces anti-Thr<sup>686</sup> immunoreactivity induced by these agonists (Fig. 4A), consistent with the PKC dependence of these effects. In contrast, treatment of unstimulated G8/DHFR cells with the serine-threonine phosphatase inhibitor okadaic acid does not affect anti-Thr<sup>686</sup> immunoreactivity (Fig. 4B), consistent...
with the findings of other groups studying EGFR. However, pretreatment of cells with PdBU is associated with enhancement of anti-Thr(P)686 binding by okadaic acid exposure (Fig. 4B), indicating that a phosphatase acting at this site is itself dependent upon PKC for activation.

Thr686 Phosphorylation of Wild-type c-erbB-2 Is Heterologously Induced in G8/DHFR Cells, Whereas Mutant Tyrosine-phosphorylated Receptors in B104–1–1 Cells Are Homologously Thr686-phosphorylated—Experimental use of phorbol ester provides an artificial means of manipulating PKC such that enzyme in all cell sites is nonselectively activated; in other words, this treatment represents a way of effecting heterologous receptor transmodulation. In contrast, ligand-inducible receptor activation might be expected to induce homologous receptor desensitization via phosphorylation events localized to activatable G8/DHFR cells, whereas mutant receptor subsets from B104–1–1 cells are activated (10) and cell transformation (40). Characterization of anti-Thr(P)686 immunoreactivity using kinase and phosphatase inhibitors. A, effects of the protein kinase C inhibitor calphostin C (CC) on PdBU- and vanadate-induced anti-Thr(P)686 (aPT-686) binding. Indicated cell samples were pretreated with calphostin C under fluorescent light (38) (2 \mu M, 1 h) prior to treatment with either PdBU (10 min, 100 ng/ml) or sodium orthovanadate (10 min, 30 min). Protein lysis and immunoblotting were then carried out as described above. B, effect of the serine-threonine phosphatase (PP1, PP2A) inhibitor okadaic acid (OA) on anti-Thr(P)686 immunoreactivity in PdBU-treated and control G8/DHFR cells. Cells were treated with 1 \mu M OA for 5 min prior to treatment (PdBU, 100 ng/ml, 10 min) followed by lysis and immunoblotting.

nonselective process occurring in trans, rather than one that is restricted to tyrosine-phosphorylated receptor subsets. Hence, this is consistent with vanadate-inducible phospholipase C-\gamma activation (37).

To simulate the effects of ligand stimulation, analysis of transmembrane mutant receptors from B104–1–1 cells was undertaken. This mutation, originally induced by the carcinogen ethylnitrosourea, causes constitutive c-erbB-2 homodimerization and cell transformation (40). In Fig. 6, most anti-Thr(P)686 immunoreactivity is found in the trophoblastic c-erbB-2 receptor subset, even though this subset comprises only a small proportion of total cellular receptor expression. The coexistence of this relatively heavy trophoblastic phosphorylation within the trophoblastic phosphorylated receptor subset suggests either that receptor autophosphorylation is not efficiently abolished by PKC-dependent c-erbB-2 modification or else that trophoblastic phosphotyrosine mutant receptors are not efficiently down-regulated by Thr686 phosphorylation.

Thr686 Is Constitutively Phosphorylated in SK-Ov-3 Human Ovarian Cancer Cells and BT-474 Human Breast Cancer Cells—We previously showed that G8/DHFR cells overexpress c-erbB-2, which is constitutively tyrosine-phosphorylated (i.e. independent of medium conditioning or cell contact), but that receptor tyrosine dephosphorylation occurs rapidly following exposure to PKC agonists, such as calf serum, platelet-derived...
growth factor, or phorbol ester (4, 31). In addition, we showed that a human ovarian cancer cell line (SK-Ov-3) that overexpresses c-erbB-2 to a similar extent, on the other hand, expresses c-erbB-2 receptors that are minimally kinase-active by comparison with some other cell lines, whereas BT-474 cells express receptors that are both strongly tyrosine-phosphorylated and kinase-active (11). To determine the relationship between c-erbB-2 threonine and tyrosine phosphorylation events in these cell lines, patterns of receptor tyrosine and threonine phosphorylation were analyzed in control, vanadate-treated, and phorbol-treated samples. Exposure to the tyrosine phosphatase inhibitor orthovanadate increases tyrosine phosphorylation in G8/DHFR and SK-Ov-3 cells but not in BT-474 cells; the latter cells express strong tyrosine phosphorylation at 185 kDa without treatment (Fig. 7), raising the possibility that a defect in phosphatase action may contribute to the constitutive kinase activity of this receptor (11).

Fig. 8 shows further that both tumor cell lines exhibit anti-Thr(P)686 immunoreactivity (though more pronounced in SK-Ov-3 than in BT-474 cells) whereas control G8/DHFR cells express little detectable threonine-phosphorylated c-erbB-2. Moreover, unlike G8/DHFR cells, minimal enhancement of anti-Thr(P)686 binding in SK-Ov-3 and BT-474 cells is induced by either vanadate or PdBU, indicative of constitutive receptor threonine phosphorylation; the intensity of this phosphorylation varies with serum batches (data not shown), consistent with mediation in part by heterologous growth factors. We note also that the greater Thr686 phosphorylation of SK-Ov-3 relative to BT-474 cells correlates with the respective sensitivity of c-erbB-2 in these cell lines to vanadate.

As in B104–1-1 cells, the coexistence of Thr686 phosphorylation and c-erbB-2 autophosphorylation in BT-474 cells indicates that the former is not sufficient to eliminate the latter. The apparent resistance of BT-474 cells to vanadate (Fig. 7) may be consistent with a receptor-activating mutation; however, we cannot exclude the possibility that primary or secondary reductions in tyrosine phosphatase action may contribute to both the constitutive autophosphorylation and Thr686 phosphorylation in these cells. A similar pattern of coexisting c-erbB-2 tyrosine and Thr686 phosphorylation is seen in SK-Br-3 human breast cancer cells (data not shown).

**DISCUSSION**

Receptor tyrosine kinases are among the most potent transforming molecules known, making them prime targets for antineoplastic medical therapies. An obstacle to rational drug development of this kind has been the limited understanding of mechanisms controlling the kinase activity of these molecules. PKC-dependent transmodulation of the EGF receptor is a case...
in point: although many studies have suggested a critical role for Thr686 phosphorylation in this process (23–25, 41), others have cast doubt upon this conclusion (42). Loss of high affinity ligand binding (17, 25, 43) has been associated with phorbol-inducible receptor transmodulation, though less specifically with Thr686 phosphorylation per se (24, 41). Such PKC-dependent reductions in ligand binding may in turn reflect induction of receptor internalization (44), either with (45) or without receptor degradation (18, 23, 26). The complexity and interdependence of the signaling pathways involved in these phenomena is suggested here by the PKC-dependent enhancements of Thr686 phosphorylation induced by inhibitors of tyrosine and serine-threonine phosphatases (Fig. 4).

As previously reported, GB/DHFR cells exhibit a minor degree of constitutive c-erbB-2 tyrosine autophosphorylation that is rapidly abolished by a variety of PKC agonists (4, 31). In contrast, SK-Ov-3 ovarian cancer cells express c-erbB-2 receptors that virtually lack autokatalytic activity in vitro (8, 11). The present study provides a potential explanation for the differential tyrosine phosphorylation of these two cell lines by revealing reciprocal differences in anti-Thr(P)686 immunoreactivity.

Of the latter in GB/DHFR cells is associated with moderate c-erbB-2 catalytic activity (4), whereas selective induction of threonine phosphorylation by phorbol ester is tightly associated with tyrosine dephosphorylation; conversely, constitutive receptor threonine phosphorylation is associated with absent receptor autokatalytic activity in SK-Ov-3 cells (11), with receptor tyrosine autophosphorylation exclusively demonstrable in these cells by inhibiting tyrosine phosphatases (Figs. 7 and 8). BT-474 cells, on the other hand, appear constitutively phosphorylated on both tyrosine and threonine residues, with this lack of inducibility suggesting a mutational basis for receptor activation. Our own sequencing studies, however, indicate that no mutation is present in either the transmembrane or juxtamembrane c-erbB-2 region in these cells (data not shown).

Our data thus far do not explain the well-documented relationship between PKC activity and tyrosine phosphatase function. A coherent hypothesis is that inducible c-erbB-2 threonine phosphorylation is linked to inducible receptor interaction with phosphatases in GB/DHFR cells. Similarly, constitutive threonine phosphorylation may be linked to constitutive c-erbB-2 tyrosine dephosphorylation in SK-Ov-3 cells expressing the wild-type receptor (again consistent with a preferential interaction of tyrosine phosphatases with the Thr686-phosphorylated receptor subset), whereas the less intense Thr686 phosphorylation seen in the BT-474 cell line may be related to the vanadate-resistant phenotype of these cells. Interestingly, sequencing of the c-erbB-2-associated PTP1B tyrosine phosphatase (46, 47) has revealed numerous N-terminal mutations in this and other tumor cell lines (data not shown).

Mutation studies are planned to clarify the mechanisms by which juxtamembrane threonine phosphorylation may facilitate c-erbB-2 interaction with phosphatases.

To our knowledge, this is one of the first published studies to use a high specificity antiphosphothreonine antibody raised against synthetic phosphopeptides. The immunogenicity of phosphotyrosine is long established (48), consistent with the major structural consequences now known to be induced by this critical posttranslational modification (49). However, the structural changes induced by serine/threonine phosphorylation remain unclear, and high quality antibodies selectively detecting such modifications have proven difficult to obtain. The findings of this study confirm that sensitive detection of threonine phosphorylation events is indeed possible, and we are currently extending our use of this approach to the analysis of c-erbB-2-overexpressing tumor specimens.

In conclusion, our findings suggest that c-erbB-2 Thr686 phosphorylation may be constitutively induced in human tumor cell lines, and we speculate that the etiology of this constitutive event may be either heterologous or mutational. A third possibility is that primary dysregulation of cellular PKC activity could play a role in modulating the neoplastic phenotype in vivo, a possibility consistent with studies suggesting a role for PKC in cell transformation (50), tumorigenesis (51), and metastasis (52). Perhaps the most intriguing of these possibilities, however, is that c-erbB-2 acts as a heterologous integration point for growth factor signaling, because this possibility has additional implications for the physiological role of c-erbB-2. We predict that more extensive use of phosphothreonine-specific antibodies will permit functional clarification of these signaling interactions both in vitro and in vivo.
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