Elevated Levels of Epidermal Growth Factor Receptor/c-erbB2 Heterodimers Mediate an Autocrine Growth Regulatory Pathway in Tamoxifen-Resistant MCF-7 Cells

JANICE M. KNOWLDEN*, IAIN R. HUTCHESON*, HELEN E. JONES, TRACIEANN MADDEN,
JULIA M. W. GEE, MAUREEN E. HARPER, DENISE BARROW, ALAN E. WAKELING, AND
ROBERT I. NICHOLSON

Tenovus Centre for Cancer Research (J.M.K., I.R.H., H.E.J., T.M., J.M.W.G., M.E.H., D.B., R.I.N.), Welsh School of Pharmacy, Cardiff University, Cardiff CF10 3XF, United Kingdom; and AstraZeneca (A.E.W.), Mereside, Alderley Park, Macclesfield, Cheshire SK10 4TG, United Kingdom

The development of acquired resistance to antihormonal agents in breast cancer is a major therapeutic problem. We have developed a tamoxifen-resistant (TAM-R) MCF-7 breast cancer cell line to investigate the mechanisms behind this condition. Both epidermal growth factor receptor (EGFR) and c-erbB2 mRNA and protein expression were increased in TAM-R compared with wild-type MCF-7 cells, whereas comparable levels of c-erbB3 mRNA and protein were expressed in both cell lines. Under basal conditions, phosphorylated EGFR/c-erbB2, EGFR/c-erbB3 but not c-erbB2/c-erbB3 receptor heterodimers were detected in TAM-R cells in association with increased levels of phosphorylated extracellular-signal regulated kinase 1/2 (ERK1/2). Both cell lines were capable of generating a range of EGFR-specific ligands and increased

expression of transforming growth factor α was observed in TAM-R cells. Treatment of TAM-R cells with ZD1839 (Iressa) or trastuzumab (Herceptin) blocked c-erbB receptor heterodimer formation and phosphorylation, reduced ERK1/2 activity, and strongly inhibited cell growth. The MAPK kinase inhibitor PD098059 specifically reduced phosphorylated ERK1/2 levels and inhibited TAM-R growth. All three agents abolished ERK1/2 activity in wild-type cells but caused only small reductions in cell proliferation. These results demonstrate that TAM-R MCF-7 cell growth is mediated by the autocrine release and action of an EGFR-specific ligand inducing preferential EGFR/c-erbB2 dimerization and downstream activation of the ERK pathway. (*Endocrinology* 144: 1032–1044, 2003)

ALTHOUGH TAMOXIFEN IS considered by many to be the first-line endocrine agent for the treatment of estrogen receptor (ER)-positive breast cancer, with approximately 50% of patients benefiting from this therapy, almost all responsive tumors eventually relapse due to the development of tamoxifen resistance (1, 2). Acquired resistance to tamoxifen is a serious therapeutic problem, and major efforts are now being made to understand the underlying mechanisms responsible for this condition (1–3). It is unlikely that loss of ER signaling contributes to antiestrogen resistance, as both tamoxifen-resistant tumors and breast cancer cell lines frequently demonstrate sustained ER content and remain responsive to pure antiestrogen therapy (4–7). Therefore, it has been proposed that cells in the presence of antiestrogens use an alternative growth regulatory pathway. It is now clear that breast cancer development and progression involves complex interactions between steroidal and growth factor signaling pathways (8, 9). An important growth regulatory pathway active in a variety of cancer types, including breast, uses the epidermal growth factor (EGF) receptor (EGFR/c-erbB1) and c-erbB2 (10–13), members of the type 1 super family of receptor tyrosine kinases (RTKs) that also includes c-erbB3 and c-erbB4 (13, 14).

An inverse relationship between ER activity and EGFR and c-erbB2 expression has been reported in clinical breast cancer, with overexpression of these RTKs being associated with decreased sensitivity to endocrine therapy and a poorer prognosis (15–17). Transfection studies in hormone-sensitive breast cancer cell lines have also demonstrated that increased EGFR and c-erbB2 expression promotes hormone-independent growth (18–22), whereas cell models of acquired tamoxifen resistance have also suggested that raised levels of EGFR may contribute to increased proliferative activity (23, 24). Indeed, we have recently demonstrated in our laboratory that cells with acquired resistance to the pure antiestrogen fulvestrant (Faslodex) exhibited increased EGFR expression/signaling and an enhanced sensitivity to the EGFR tyrosine kinase inhibitor (EGFR-TKI) ZD1839 (Iressa) (25).

The c-erbB family of receptors regulates cell proliferation through activation of downstream signal transduction cascades. Binding of EGF-related growth factors results in receptor homo and/or heterodimerization and stimulation of the intrinsic RTK activity. This promotes autophosphorylation of specific tyrosine residues within the cytoplasmic domain of the receptor, which provide docking sites for a variety of adaptor proteins involved in the activation of downstream intracellular signaling cascades including the MAPK and phosphoinositide 3-kinase pathways (26). The MAPK (or ERK) pathway is a key mediator of cell proliferation and increased activity of this signal cascade has also been associated with a poorer quality and shorter duration

of response to tamoxifen and decreased survival time in ER-positive breast cancer patients (27, 28). Furthermore, constitutive activation of this pathway contributes to antiestrogen resistance in human breast cancer cells (22, 29, 30), and increased MAPK activity has been demonstrated in long-term estrogen-deprived, nonestrogen-responsive, and antiestrogen-resistant cell lines (25, 31, 32).

In the present study, we have developed a tamoxifen-resistant cell line (TAM-R), derived from endocrine-sensitive wild-type (WT)-MCF-7 human breast cancer cells, to mimic the development of acquired tamoxifen resistance as seen in the clinic. We have investigated the basal expression and activation of all four c-erbB family members and examined the receptor homo- and heterodimerization patterns that exist within the two cell lines. We have also looked at the activation status of the associated downstream signaling components ERK1/2 and MAPK. The effects of the EGFR-TKI ZD1839 (33), the MAPK kinase (MEK) inhibitor PD098059 (34), and the anti-c-erbB2 monoclonal antibody trastuzumab (35) were also studied to delineate the involvement of components of the c-erbB/MAPK signaling pathway in the proliferative response of both cell lines.

Materials and Methods

Cell culture

WT-MCF-7 breast cancer cells, a gift from AstraZeneca (Macclesfield, UK), were seeded at a density of $2 \times 10^5/cm^2$ and routinely cultured in phenol-red-free RPMI medium supplemented with 5% fetal calf serum plus penicillin-streptomycin (10 IU/ml–10 μg/ml) and fungizone (2.5 μg/ml). Cultures were maintained at 37 C in a humidified 5% CO_2 atmosphere. All tissue culture medium and constituents were purchased from Life Technologies Europe Ltd. (Paisley, UK) and tissue culture plasticware was obtained from Nunc (Roskilde, Denmark).

Establishment of TAM-R

The MCF-7 cell monolayers were washed thoroughly with PBS, and transferred to phenol-red-free RPMI medium containing 5% charcoal-stripped steroid-depleted fetal calf serum, antibiotics, glutamine (200 mM), and 4-hydroxytamoxifen (4-OH-TAM, 10^{-7} M in ethanol). Cells were continuously exposed to this treatment regimen for 6 months, during which time the medium was replaced every 4 d and the cell cultures were passaged by trypsinization after 70% confluency was reached. Initially, the MCF-7 cell growth rates were reduced but, after 2 months' exposure to the medium, cell growth gradually increased, indicating the development of a cell line resistant to the growth-inhibitory properties of 4-OH-TAM. This cell line, designated TAM-R, was cultured for a further 4 months in medium containing 4-OH-TAM before characterization studies.

Experimental cell culture

Western blotting and RT-PCR studies. The two cell lines were grown for 4 d before being transferred into phenol-red/steroid-free, serum growth factor-free DCCM medium [Biosynergy (Europe), Cambridge, UK] for 24 h. The cells were then lysed to measure basal protein and mRNA expression. To examine the effects of pharmacological agents, cells were lysed following a further incubation in DCCM medium supplemented with increasing concentrations of either ZD1839 (0.03–1 μM in ethanol; AstraZeneca) for 10 min, trastuzumab (1–100 nM in water; Genentech, Inc., South San Francisco, CA) for 7 d, or PD098059 (1–100 μM in dimethylsulfoxide; Alexis Corp., Nottingham, UK) for 1 h. From these concentration-response studies, 1 μM ZD1839, 100 nM trastuzumab, and 50 μM PD098059 were selected for subsequent investigations. Controls were incubated for the same periods of time with the appropriate vehicle. All experiments were performed at least three times.

Immunocytochemistry studies. Cells were grown on sterile 3-aminopropyltriethoxysilane-coated coverslips at 1×10^4 cells/cm^2 according to the protocol described above. Basal expression of c-erbB receptor expression was assessed by incubation of cell monolayers for 24 h in DCCM before PBS wash and cell fixation according to the immunocytochemical assay being performed. The effects of pharmacological treatments were investigated by incubation of cell monolayers for 7 d in DCCM containing either ZD1839 (1 μM), trastuzumab (100 nM), PD098059 (50 μM), or the appropriate vehicle control before PBS wash and fixation.

Southern blotting. Both cell lines were seeded at a density of 1×10^5 cells/cm^2 and grown for 7 d before being harvested for DNA extraction. Experiments were performed four times.

Growth studies. Cell monolayers were grown for 7 d in serum growth factor-free DCCM medium in the presence of increasing concentrations of either 4-OH-TAM (0.1–1000 nM), ZD1839 (0.01–1 μM), 100 nM trastuzumab (1–100 nM), or PD098059 (1–100 μM). Cell population growth was then evaluated by means of trypsin dispersion of the cell monolayers and cell number was measured using a Beckman Coulter (Luton, UK) counter. All experiments were performed in triplicate.

Protein cell lysis

Cells were washed three times with PBS and lysed using ice-cold lysis buffer (36). The cellular contents were transferred to microfuge tubes, clarified by centrifugation at 13,000 rpm for 15 min at 4 C and supernatant aliquots were stored at −20 C until required. Total protein concentrations were determined using the DC Bio-Rad Laboratories Ltd. protein assay kit (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK).

Immunoprecipitation

Cell lysates containing 1 mg protein were immunoprecipitated using 1 μg specific antibody and incubated on ice for 1 h. Thirty microliters of protein A agarose (Insight Biotechnology Ltd., Wembley, UK) were added to the mixture and the mixture inverted frequently by hand for a further 2 h. The immune complex was centrifuged at 3000 rpm at 4 C for 5 min and washed with ice-cold lysis buffer. This procedure was repeated twice and the resultant pellet resuspended in 20 μl Laemmli sample loading buffer containing 0.01 M dithiothreitol. Samples were heated to 100 C for 5 min to release the bound proteins before gel loading.

Western blotting

Protein samples from total cell lysates (20 μg) and following immunoprecipitation were subjected to electrophoresis separation on a 7.5% polyacrylamide gel and then *trans*-blotted onto a nitrocellulose membrane (Schleicher & Schuell, Inc., Dassel, Germany). Blots were blocked at room temperature for 1 h in 5% skimmed milk powder made up in Tris-buffered saline (TBS)-Tween 20 (0.05%) and then incubated for a minimum of 1 h in primary antibody diluted 1/40,000 for β-actin (reference control) or 1/1000 for EGFR, c-erbB2, c-erbB3, c-erbB4, and ERK1/2 MAPK in 1% marvel/TBS-Tween. The membranes were washed three times in TBS-Tween and then incubated for 1 h with the required secondary IgG horseradish peroxidase-labeled donkey antirabbit or sheep antimouse (Amersham Biosciences, Buckinghamshire, UK), diluted 1/20,000 in 1% marvel/TBS-Tween. Detection was performed using West Dura chemiluminescent detection reagents (Pierce and Warriner Ltd., Chester, UK). Antibodies used were: total EGFR (SC-03), total c-erbB2 (SC-284), total c-erbB3 (SC-285), total c-erbB4 (SC-283), and antiphosphotyrosine (PY-20; SC-508; Insight Biotechnology Ltd., Wembley, UK); anti-phospho-EGFR (Y1173) mouse IgG and anti-phospho-c-erbB2 (Y1248) mouse IgG (Upstate Biotechnology, Inc., Buckingham, UK); total ERK1/2 (T-9102) and phospho-ERK1/2 (A-9101) MAPK rabbit polyclonal IgG (New England Biolabs, Inc., Hertfordshire, UK) and β-actin (AC-15) mouse IgG (Sigma-Aldrich, Poole, UK). These phospho-antibodies were selected as they have been demonstrated to be mono-specific and do not cross-react with other family members. Results were scanned using a Bio-Rad Laboratories Ltd. model GS-690 Imaging Densitometer.

Immunocytochemical assays

Total and phosphorylated c-erbB receptor and phosphorylated ERK1/2. Both cell lines were immunocytochemically assayed for total and phosphorylated EGFR, total and phosphorylated c-erbB2, total c-erbB3, and activated ERK1/2 as described previously (25, 37–41).

Immunostaining analysis. The detection system used for all studies was the diaminobenzidine tetrahydrochloride and hydrogen peroxide chromagen substrate followed by either methyl green or 10% hematoxylin counterstaining. Evaluation of immunostaining was carried out by two people on an Olympus Corp. BH-2 light microscope using a dual-viewing attachment. Membrane and cytoplasmic staining was assessed for total and phosphorylated EGFR, total and phosphorylated c-erbB2, and total c-erbB3. Cytoplasmic and nuclear phosphorylated ERK1/2 staining was also determined. Intensity of immunostaining and percentage of cells exhibiting positive staining was estimated using a minimum evaluation of 2000 cells per coverslip. From these data a field scoring index, H-score, was constructed for each marker, as described previously (42).

RT-PCR

Cell monolayers were lysed and the total RNA was isolated using an RNA isolator kit (Genosys Biotech Inc., Cambridge, UK). Total RNA (1 μg) was reverse transcribed and the resulting cDNA was amplified using specific primers for EGFR, c-erbB2, c-erbB3, c-erbB4. RT-PCR conditions were optimized for each primer set using standard conditions as described previously (39, 43). For the detection of EGFR and c-erbB3, cDNA samples were coamplified for 27 cycles using both primers for the gene of interest and β-actin (internal control). For the other two c-erbB members where a higher cycle number was needed to permit detection, single PCRs were performed alongside β-actin on these cDNA samples using 30, 40, and 27 cycles of amplification for c-erbB2, c-erbB4, and β-actin, respectively. All amplimers were then separated by gel electrophoresis, visualized under UV illumination, photographed, and scanned where necessary using a Bio-Rad Laboratories Ltd. model GS-690 Imaging Densitometer.

Primers used were either as reported previously or designed manually using OLIGO (MedProbe AS, Oslo, Norway) primer pair design software. Primers were designed where possible to span intron/exon borders and specificity was checked using the European Molecular Biology Laboratory (EMBL)-GenBank database software using the BLAST program.

β-Actin 5′-GGA GCA ATG ATC TTG ATC TT and 3′-CCT TCC TGG GCA TGG AGT CCT (204 bp), EGFR 5′-CAA CAT CTC CGA AAG CCA and 3′-CGG AAC TTT GGG CGA CTA T (636 bp), c-erbB2 5′-CCT CTG ACG TCC ATC ATC TC and 3′-ATC TTC TGC TGC CGT CGC TT (98 bp), c-erbB3 5′-GGT GCT GGC CTT GCT TTT and 3′ GGT GGC TGG AGT TGG TGT TA (365 bp), c-erbB4 5′-TGT GAG AAG ATG GAA GAT GCC, and 3′ GTT GTG GTA AAG TGG AAT GGC (265 bp).

Southern blotting

DNA extraction and Southern analyses were performed as outlined in Sambrook *et al.* (44) with the following modifications. The DNA was extracted using 50 mM NaCl, 20 mM Tris (pH 8.0), 5 mM EDTA (pH 8.0), 0.1% wt/vol SDS containing 100 μg/ml proteinase K (Sigma-Aldrich) and digested overnight at 50 C. Contaminating RNA was removed by digestion with 20 μg/ml ribonuclease A (Sigma-Aldrich) for 15 min at 37 C. For Southern analysis 25-μg aliquots of *Eco*R1 (Promega Corp., Southampton, UK) digested DNA were separated on a 0.8% agarose gel and transferred to a Zeta-Probe GT membrane (Bio-Rad Laboratories Ltd.) according to the manufacturer's instructions.

Southern blots were hybridized overnight in Express Hyb (BD Biosciences, CLONTECH UK, Oxford, UK) with c-erB2 and EGF-R RT-PCR products random prime labeled with [α-^{32}P] deoxy-CTP (Amersham Life Sciences) using the Prime-a-Gene Labeling system (Promega Corp.). Autoradiography was performed using Kodak (Hertfordshire, UK) BIOMAX MR film at −80 C with intensifying screens. All Southern blots were re-probed with a commercially available β-actin cDNA probe (CLONTECH UK) as a control for loading efficiency. For quantification the resulting autoradiographs were scanned using densitometry as described above.

Statistics

Overall differences between control and treatment groups were determined by two-way ANOVA. Direct comparisons between WT-MCF-7 and TAM-R cells or between control and treatment effects were assessed using a Student's *t* test with the Bonferroni adjustment factor. Differences were considered significant at the $P < 0.05$ level.

Results

TAM-R cell line

WT-MCF-7 cell growth was inhibited by treatment with 4-OH-TAM (100 nM) by approximately 60% after 2 wk. The growth of this cell population remained static over the following 2 months, after which time cell growth rates gradually increased, reaching levels comparable to WT cells grown in the absence of 4-OH-TAM after a further 3 months. Additionally, compared with the WT-MCF-7 cells, which possessed smooth margins and grew in a well-organized, pavement-like monolayer, the resistant cells were angular in shape with elongated processes and grew in a more disorganized manner. These cells, designated TAM-R, were cultured for another month before any characterization studies. ER-α expression as determined by immunocytochemistry and Western blotting was not different between the WT and TAM-R cell lines (45). Proliferation of WT-MCF-7 and TAM-R cells in the presence of 4-OH-TAM was compared by means of growth curves to confirm the resistant phenotype. In the presence of the antiestrogen, it was seen that growth rate of TAM-R cells was considerably higher than that observed for the WT-MCF-7 cells, by some 200% after 11–13 d proliferation (Fig. 1A). Furthermore, TAM-R cells showed no reduction in growth compared with the control, even at the highest concentration of 1 μM 4-OH-TAM, whereas WT-MCF-7 cell proliferation was reduced in a concentration-dependent manner with up to 50–70% growth inhibition being observed at 0.1–1 μM 4-OH-TAM (Fig. 1B).

Basal expression of c-erbB receptors

Western blotting and RT-PCR analysis revealed a marked increase in the levels of total EGFR and c-erbB2 protein (Fig. 2A, *top panel*) and mRNA expression (Fig. 2A, *bottom panel*) in the TAM-R cells compared with the WT-MCF-7 cells. Densitometry readings revealed that levels of EGFR protein and mRNA were significantly 3-fold and 6-fold higher ($P < 0.05$ and $P < 0.05$), respectively, and that levels of c-erbB2 protein and mRNA were significantly 1.5-fold and 3-fold greater ($P < 0.05$ and $P < 0.05$), respectively, in TAM-R cells (Table 1). Levels of c-erbB3 protein and mRNA were found to be similar for both cell lines (Table 1), whereas c-erbB4 protein or mRNA expression was undetectable in both cell lines under the same conditions (Fig. 2A). Expression levels of β-actin protein and mRNA remained constant for all samples, which verified equivalent sample loading (Fig. 2A). When each c-erbB species was first immunoprecipitated from WT-MCF-7 and TAM-R protein cell extracts, immunoblotted and immunologically detected using specific c-erbB antibodies, EGFR, c-erbB2 and c-erbB3 were again readily detectable showing the same expression profile as observed in Fig 2A. c-erbB4 expression levels, however, were now detectable, but extremely low and showing similar levels in both cell lines (not illustrated).
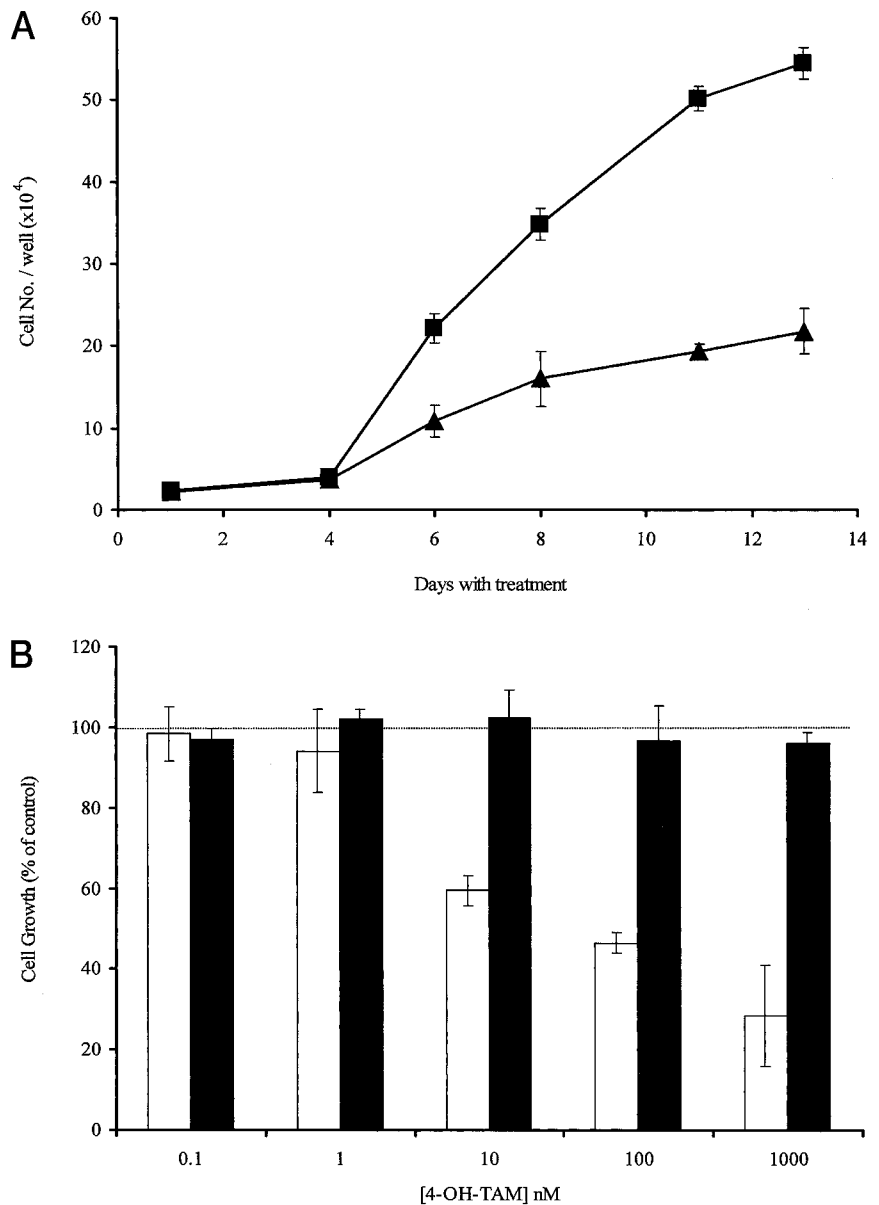
FIG. 1. A, Growth curves of WT-MCF-7 (▲) and TAM-R (■) cells in the presence of 4-OH-TAM (0.1 μM). B, Growth responses of WT-MCF-7 (*empty bar*) and TAM-R cells (*filled bar*) to increasing concentrations of 4-OH-TAM (0.1 nM–1 μM) on d 7 from initial treatment. The results for both graphs are expressed as the mean (SD) of triplicate wells and are representative of three separate experiments.

For the predominant c-erbB receptors, parallel immunocytochemical analysis and subsequent H-score analysis again showed increases in EGFR and c-erbB2 protein. As with Western blotting, significant elevations in EGFR membrane and cytoplasmic staining were observed in the TAM-R cells compared with the WT-MCF-7 ($P < 0.001$ for both, Fig. 2B, Table 2). Immunostaining and subsequent H-score analysis of c-erbB2 protein also revealed a significant increase in membrane and cytoplasmic staining for this protein in the TAM-R cells compared with the WT-MCF-7 cells ($P < 0.001$ for both, Fig. 2B, Table 2). c-erbB3 protein immunocytochemical and H-score analysis revealed that staining was predominantly cytoplasmic, with a small plasma membrane signal (Table 2). Furthermore, there was no significant difference in the c-erbB3 staining intensity between WT-MCF-7 and TAM-R cells, in agreement with the Western blotting results (Fig. 2B).

Gene amplification

Southern blot analysis revealed that, when corrected for loading, there were no differences in EGFR and c-erbB2 copy number between the parental MCF-7 and the tamoxifen-resistant cell lines (Fig. 3). Following densitometry analysis and normalization with β-actin, the WT:TAM-R ratios for the EGFR and the c-erbB2 genes were 1:1.4 and 1:1.1, respectively, after the *Eco*R1 digest.

Basal dimerization and activation profiles of c-erbB receptors

Western blotting studies demonstrated higher levels of basal phosphorylated EGFR and c-erbB2 in TAM-R compared with WT-MCF-7 cells (Fig. 4, A1 and A2). Immunocytochemical analysis of activated EGFR and c-erbB2 confirmed Western blotting data. TAM-R cells expressed
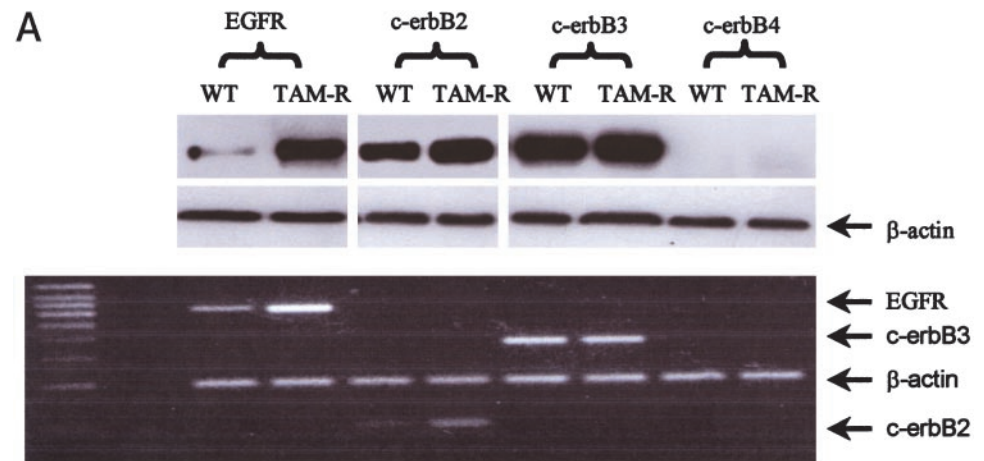
FIG. 2. A, Total c-erbB receptor and β-actin protein (*top panels*) and mRNA (*bottom panel*) expression levels in TAM-R and WT-MCF-7 cells. β-Actin was used as an internal control for both techniques. Levels of protein and mRNA were quantified by densitometry. B, Immunocytochemical localization of nonactivated total EGFR (*top panel*), c-erbB2 (*middle panel*), and c-erbB3 (*bottom panel*) levels in WT-MCF-7 and TAM-R cells. Data shown are representative of at least three separate experiments for each receptor examined. Original magnification, ×40.

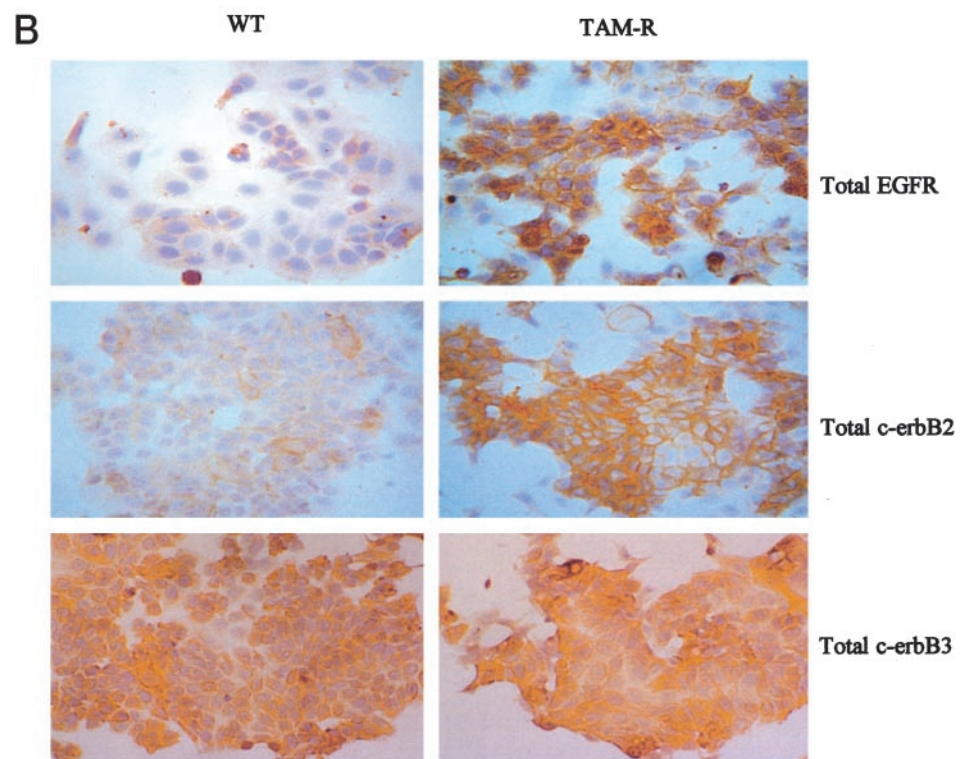**TABLE 1.** RT-PCR and Western blotting analysis of EGFR, c-erbB2, and c-erbB3 expression in WT-MCF-7 and TAM-R cells

	Protein		mRNA	
	WT	TAM-R	WT	TAM-R
EGFR	2.5 (1.1)	7.8 (3.8)^a	0.043 (0.018)	0.26 (0.1)^a
c-erbB2	17.6 (5.3)	25.5 (6.3)^a	0.013 (0.017)	0.044 (0.01)^a
c-erbB3	38.4 (7.3)	41.2 (8.1)	0.15 (0.07)	0.13 (0.09)

Densitometry readings of mRNA and protein levels are expressed as mean (SEM) of at least three separate experiments for each methodology. Densitometry values are in arbitrary units.
^{a} $P < 0.05$ *cf.* WT.

significantly higher levels of cytoplasmic phospho-EGFR and phospho-c-erbB2 staining compared with WT cells ($P < 0.001$ and $P < 0.05$, respectively; Fig. 4B and Table 2). Activated EGFR membrane staining was very low in both cell lines but a significant, increase was observed in TAM-R cells ($P < 0.05$; Table 2). A more striking and highly significant increase in activated c-erbB2 membrane staining was found in the TAM-R cells compared with the WT cells ($P < 0.001$; Table 2). As no specific antiphosphorylated c-erbB3 antibody was available for Western blotting and immunocytochemistry, we immunoprecipitated c-erbB3 and probed with an antibody directed against phosphorylated tyrosine residues. This revealed the presence of phospho-tyrosine residues associated with the c-erbB3 receptor in both cell lines, with WT cells demonstrating a higher level of tyrosine phosphorylation compared with the TAM-R cells (Fig. 4, A3).

Immunoprecipitation with either EGFR, c-erbB2, or c-erbB3 followed by probing for phosphorylated EGFR revealed that TAM-R cells expressed measurable levels of basally active EGFR/c-erbB2, EGFR/c-erbB3 receptor het-

TABLE 2. Comparison of immunocytochemically determined total and phosphorylated c-erbB receptor and ERK1/2 expression in WT-MCF-7 and TAM-R cells

		WT-MCF-7 H-score	TAM-R H-score
Total EGFR	Membrane	4 (1.1)	43 (8.8)[a]
	Cytoplasmic	19 (3.0)	98 (6.5)[a]
Phosphorylated EGFR	Membrane	2.5 (0.85)	13 (4)[b]
	Cytoplasmic	79 (3.3)	129 (3.3)[a]
Total c-erbB2	Membrane	53 (6.2)	98 (5.4)[a]
	Cytoplasmic	66 (4.9)	107 (6.3)[a]
Phosphorylated c-erbB2	Membrane	0.66 (0.4)	23 (4.4)[a]
	Cytoplasmic	12.3 (3.7)	40 (8.1)[b]
Total c-erbB3	Membrane	40 (6.8)	45 (4.8)
	Cytoplasmic	160 (16.9)	170 (18.9)
Phosphorylated ERK1/2	Cytoplasmic	27 (3.2)	103 (5.8)[a]
	Nuclear	1 (0.26)	7 (1.2)[a]

H-score values are expressed as mean (SEM) of the assessment of five fields of view per coverslip in triplicate experiments.
[a] $P < 0.001$, [b] $P < 0.05$ *cf.* WT-MCF-7.

FIG. 3. Southern hybridization analyses of *Eco*R1 restriction endonuclease digests of WT-MCF-7 and TAM-R cellular genomic DNA probed with ^{32}P-labeled (A) EGFR (7.8 kb), (B) c-erbB2 (6.7 kb), and (C) β-actin (12.2 kb) cDNA. The results shown are representative of four independent analyses.

erodimers, as illustrated in Fig. 4C (*first row*), findings not evident in the WT-MCF-7 cells. When these blots were then reprobed for the presence of activated c-erbB2, substantial levels of basally active c-erbB2/EGFR heterodimers could only be measured in TAM-R cells (Fig. 4C, *second row*). No phosphorylated c-erbB2/c-erbB3 receptor dimers were detected in either cell line. Evidence for EGFR/c-erbB2 heterodimerization was corroborated by blotting for total c-erbB2 (in the case of EGFR-IP) and total EGFR (in the case of c-erbB2-IP), not illustrated. Blots were also probed for total c-erbB and β-actin levels (Fig. 4C, *third and fourth rows*), to demonstrate even sample loading. Active c-erbB4 dimers could not be detected in either cell line following immunoprecipitation and probing with the antiphosphotyrosine antibody (not shown).

Basal activation of ERK1/2 MAPK

Increased basal levels of ERK1/2 MAPK activation in the TAM-R cells compared with the WT cells were observed by Western blotting analysis, as illustrated in Fig. 5A; total expression levels were similar. In both cell lines, immunocytochemical assessment demonstrated that basally active MAPK was predominantly localized within the cytoplasm with TAM-R cells expressing significantly higher levels compared with the WT cell line (Figs. 5B, $P < 0.001$, Table 2). Additionally, H-score assessment of the minimal nuclear phosphorylated MAPK staining indicated that TAM-R cells also possessed significantly higher levels of this localized activated protein kinase than the WT cells ($P < 0.001$, Table 2).

Effects of ZD1839, Trastuzumab, and PD098059

A concentration-related decrease in basal phosphorylated ERK1/2 was observed in both cell lines in response to ZD1839; a concentration of 1 μM, virtually abolished the activity (Fig. 6A, *top*). In subsequent studies, the profound reduction in ERK1/2 activity in response to 1 μM ZD1839 in TAM-R cells was associated with a complete loss of phosphorylated EGFR, shown both by Western blotting and immunocytochemically (Fig. 6B, *left column*; Fig. 7A; Table 3), and a small inhibition of c-erbB2 phosphorylation compared
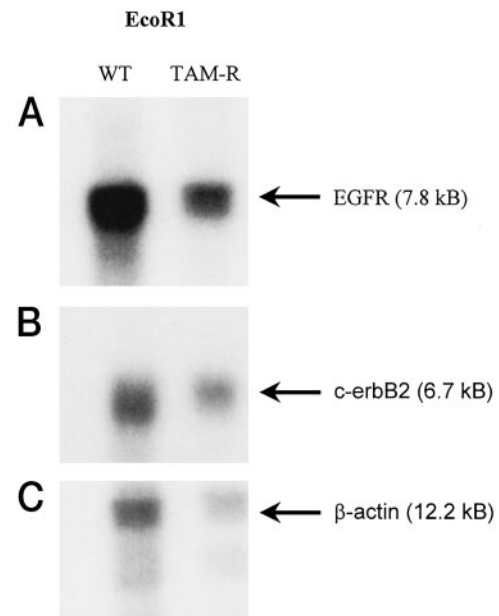
with the controls (Fig. 6B, *left column*). ZD1839 also inhibited the small level of c-erbB2 and ERK1/2 MAPK phosphorylation present in WT-MCF-7 cells but exerted no effect on the total protein expression levels of these proteins in either cell line. ZD1839 induced a concentration-dependent inhibition of TAM-R cell growth with a concentration of 1 μM reducing proliferation significantly by approximately 60% compared with control ($P < 0.001$). However, this agent had only a small effect on WT-MCF-7 cells: approximately 15% reduction in growth compared with control at 1 μM, which was not significant (Fig. 8A). At this concentration, ZD1839 demonstrated no cytotoxic effect on the cells (data not illustrated).

Increasing trastuzumab concentrations induced a concentration-related reduction in total c-erbB2 levels in both cell lines, with the maximal effect being attained at a concentration of 100 nM [Figs. 6, A (*middle*) and B (*middle column*), and 7B; and Table 3]. A significant reduction in total EGFR expression along with a small reduction in c-erbB2 activity was also revealed by Western blotting in both cell lines (Fig. 6B, *middle column*). However, this agent had little or no effect on phosphorylated EGFR expression in either WT or TAM-R cells (Fig. 6B, *middle column*). ERK1/2 phosphorylation was significantly reduced in the TAM-R cells with only a small inhibition being observed in WT-MCF-7 cells following treatment, but total protein expression levels, in contrast, remained unaffected (Fig. 6B, *middle column*). Both WT and TAM-R cell proliferation was significantly decreased in a concentration-related manner by trastuzumab, with growth inhibition significantly reaching 65% of control in the TAM-R cells at 100 nM ($P < 0.001$), whereas growth of WT cells was inhibited by approximately 30% by the same concentration of trastuzumab (Fig. 8B).

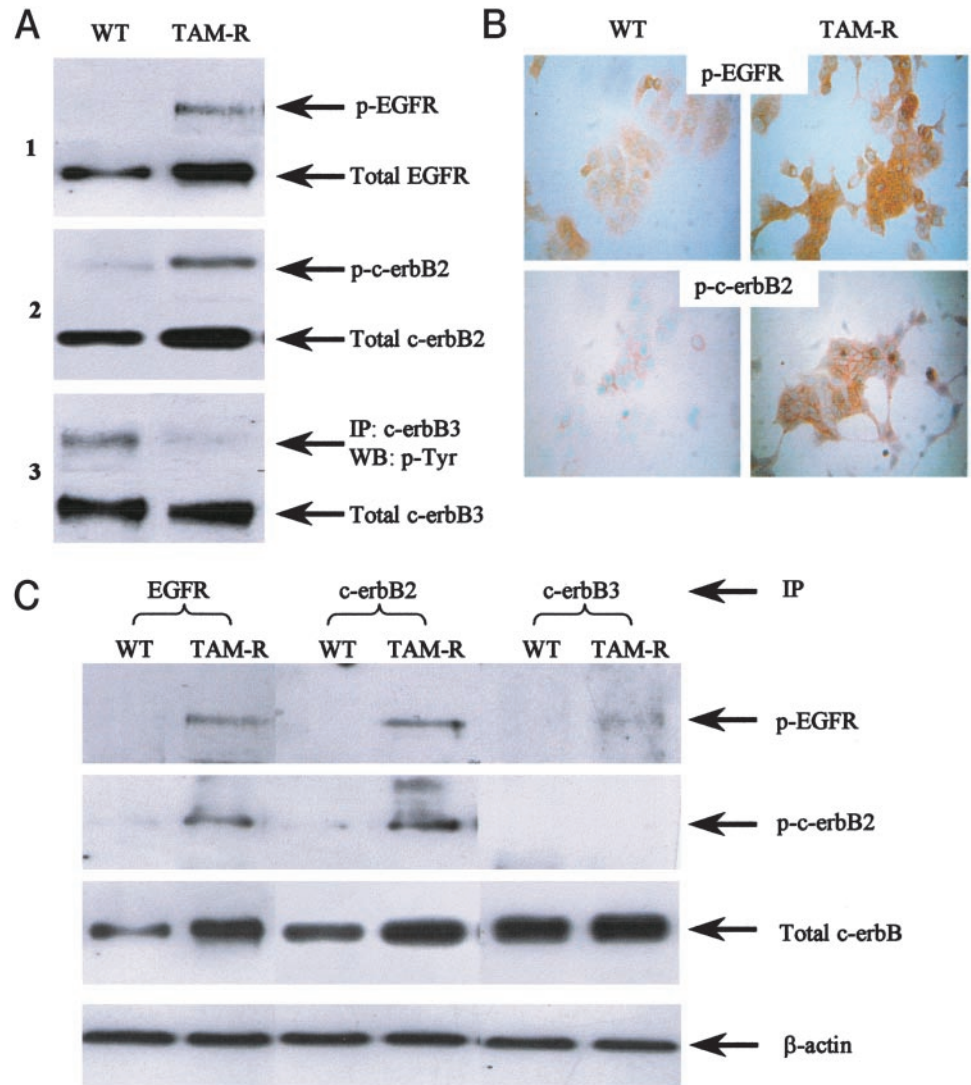The selective MEK inhibitor PD098059 induced a concen-

FIG. 4. A, Measurement of basal activation status of 1) EGFR, 2) c-erbB2, and 3) c-erbB3 in WT-MCF-7 and TAM-R cells by Western blotting analysis. Phospho-specific antibodies for EGFR and c-erbB2 were used, whereas basal c-erbB3 activity was assessed by immunoprecipitation of the receptor with total c-erbB3 antibody followed by detection of phosphorylated tyrosine residues. B, Immunocytochemical analysis of EGFR (*top panel*) and c-erbB2 (*bottom panel*) activity using phospho-specific antibodies in the two cell lines. All data are representative of at least three experiments for each c-erbB receptor. C, Basal activated c-erbB receptor dimerization profiles in WT-MCF-7 and TAM-R cell lines. Immunoprecipitation of EGFR, c-erbB2, and c-erbB3 followed by probing for either activated EGFR or c-erbB2. Blots were also probed for total (nonactivated) EGFR, c-erbB2, c-erbB3, and β-actin protein. All immunoprecipitation studies were performed at least three times for each c-erbB receptor. Original magnification, $\times 40$.

tration-related reduction in phosphorylated ERK1/2 levels with 10–100 μM abolishing activity in both cell lines (Fig. 6A, *bottom*). Subsequent immunocytochemical and Western blotting studies in both cell lines revealed that 50 μM PD098059 specifically inhibited ERK1/2 phosphorylation [Figs. 6B (*right column*) and 7C; and Table 3], whereas it had no effect on basal levels of phosphorylated EGFR or c-erbB2 compared with the control (Fig. 6B, *right column*). This inhibitor had no effect on total protein levels, as illustrated in Fig. 6B (*right column*). PD098059 caused a concentration-related inhibition of growth in both cell lines, growth of TAM-R cells being significantly inhibited by approximately 50% (as a percentage of the control) over the 10- to 100-mm concentration range ($P < 0.001$) compared with 30% in the WT cells ($P < 0.05$) (Fig. 8C).

Discussion

We have derived a TAM-R cell line from endocrine-sensitive WT-MCF-7 breast cancer cells to elucidate the mechanisms responsible for the development of antiestrogen-resistant cell growth. We have previously reported that an

MCF-7 cell line resistant to the pure antiestrogen fulvestrant displayed an increased reliance on elements of the EGFR/MAPK signaling pathway to mediate cell growth (25) and in the present study have investigated whether a similar growth regulatory mechanism is used in our more clinically relevant TAM-R cell model.

These studies indicated that both WT-MCF-7 and TAM-R cells expressed measurable levels of EGFR, c-erbB2, and c-erbB3 mRNA and protein as comprehensively determined by RT-PCR, Western blotting, and immunocytochemistry. EGFR expression was low in WT cells, consistent with the findings of other groups who have reported little or no detectable EGFR in this cell line (46, 47). In the TAM-R cell line, however, both EGFR mRNA and protein levels were considerably elevated compared with WT cells, with immunocytochemical staining demonstrating a significant 10-fold increase in plasma membrane staining for this receptor. Similarly, these methodologies demonstrated that TAM-R cells expressed significantly increased levels of c-erbB2 protein and mRNA with a near doubling of expression compared with WT cells. These data add to the increasing body of
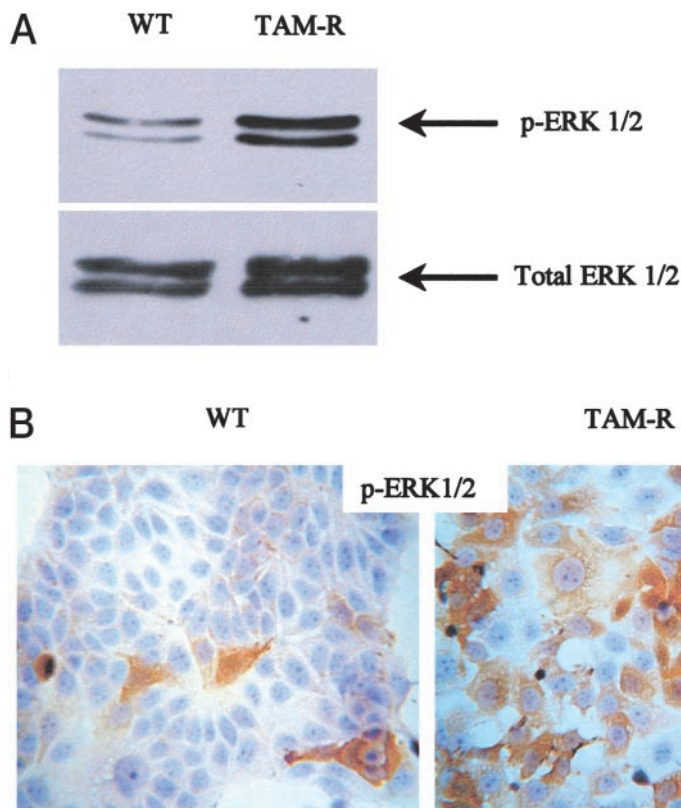
Fɪɢ. 5. Basal ERK1/2 activity in WT-MCF-7 and TAM-R cells by (A) Western blotting and (B) immunocytochemical analysis. Total ERK1/2 expression was assessed by Western blotting to confirm equal loading of sample. Data are representative of at least three experiments for each methodology. Original magnification, ×40.

evidence implicating the increased expression of EGFR and/or c-erbB2 in the development of antiestrogen resistance *in vivo* and *in vitro* (15–17, 25). Overexpression of EGFR and c-erbB2 in breast cancer can be a consequence of gene amplification (48); in the present study, Southern blot analysis indicated that the increases in EGFR and c-erbB2 expression were a consequence of changes in gene transcription and not amplification as genomic levels were similar in the two cell lines. Expression of c-erbB3 mRNA and protein was high in both cell lines but no significant difference in levels or immunocytochemical localization was observed between the two cell lines. Expression of c-erbB4 mRNA was below detectable limits in both cell lines. Protein content could only be detected in Western blotting studies following immunoprecipitation, and these results indicated that c-erbB4 expression levels were also similar in the two cell lines. These findings are consistent with previous reports of c-erbB3/4 expression in antiestrogen-sensitive and -resistant MCF-7 cells (46) and suggest that neither receptor type plays a direct role in the development of this condition.

To investigate whether the c-erbB receptors were functional in the two cell lines, we examined their activation status under basal conditions of growth, *i.e.* in the absence of exogenous ligand. Western blotting studies demonstrated that TAM-R cells expressed higher levels of phosphorylated EGFR and c-erbB2 than WT cells. It remains to be determined whether this is a consequence of either the increased expression of total EGFR and c-erbB2 protein or a specific increase in receptor phosphorylation. Immunocytochemical staining confirmed that the levels of phosphorylated EGFR and c-erbB2 were substantially greater in TAM-R compared with

WT cells, with expression of phosphorylated EGFR being predominantly cytoplasmic, whereas phosphorylated c-erbB2 was observed both at the membrane and within the cytoplasm. This localization probably reflects the different endocytic routing of these two receptor types, as activated EGFR is rapidly internalized via clathrin-coated pits (49), whereas c-erbB2 is endocytosis-impaired and therefore remains at the cell membrane for longer following activation (50, 51). Phosphorylated c-erbB2 was detected in WT cells, but at levels far lower than observed in the TAM-R cell line, activated EGFR was undetectable. The low expression levels of phosphorylated EGFR and c-erbB2 in WT cells was confirmed using immunocytochemistry. In contrast, phosphorylated c-erbB3 expression was present in WT cells but barely detectable in TAM-R cells. The expression of phosphorylated c-erbB3 in WT cells may be a consequence of the autocrine generation of the c-erbB3/4 ligands, heregulin (HRG) α and β. Indeed, a recent study has reported that estradiol can stimulate HRG production in MCF-7 cells (52). In the present study, we were unable to detect expression of HRGs by RT-PCR analysis (data not shown) in agreement with a previous report by DeFazio *et al.* (47); however, it is remains a possibility that WT cells synthesize these ligands but at levels below the detectable limit for our assay.

Activation of EGFR results from ligand binding and dimerization with a member of the c-erbB family, with c-erbB2 being the preferred dimerization partner for all c-erbB receptors (53, 54). Dimerization results in activation of the receptors' intrinsic tyrosine kinase activities and autophosphorylation of tyrosine residues within their cytoplasmic domains. Coexpression of EGFR with c-erbB2, followed by
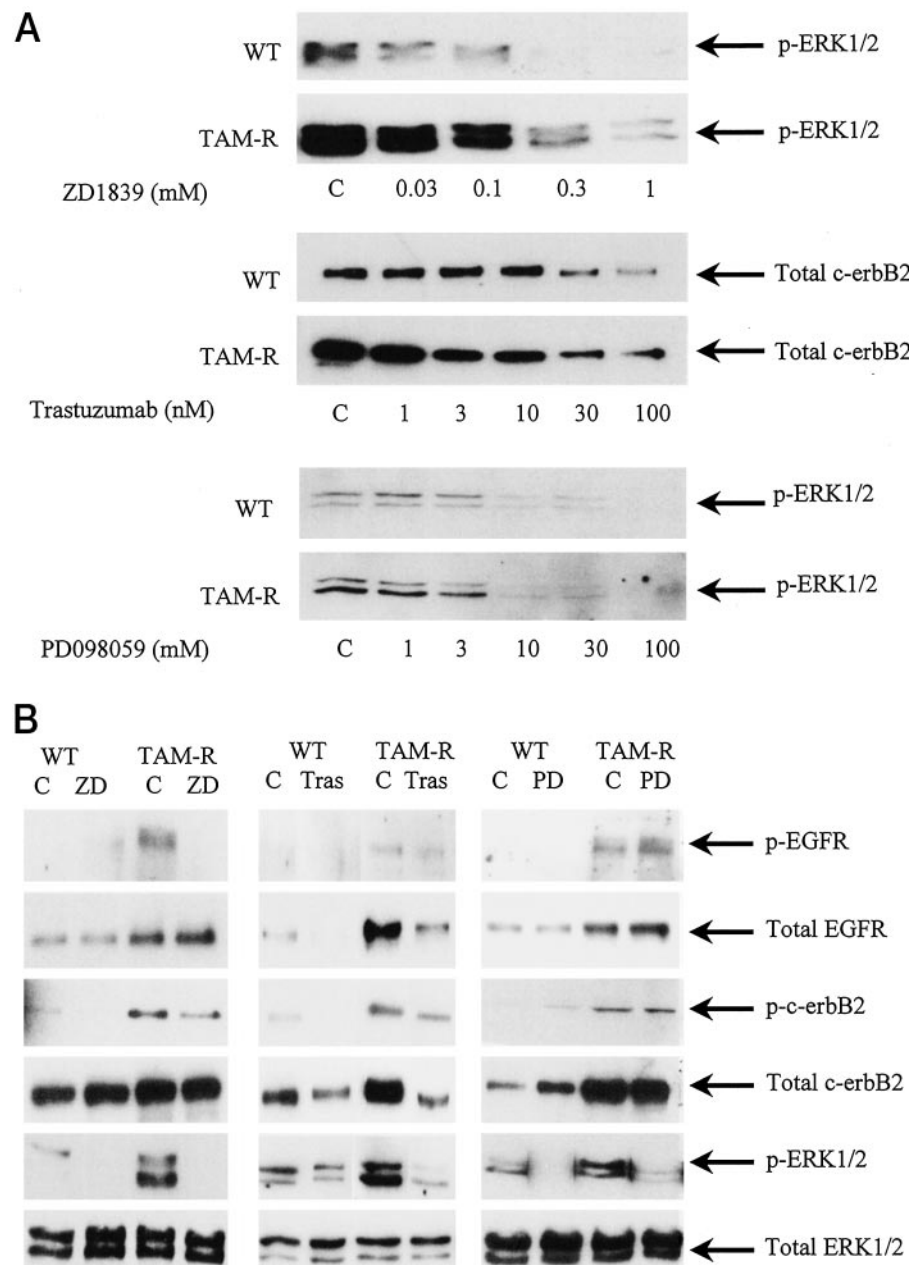
FIG. 6. A, Concentration-response studies investigating the effects of increasing concentrations of ZD1839 (0.03–1 μM, *top panels*) and PD098059 (1–100 μM, *bottom panels*) on ERK1/2 activity and increasing concentrations of trastuzumab (1–100 nM, *middle panels*) on total c-erbB2 expression in the two cell lines. B, Effects of ZD1839 (1 μM, *left panel*), trastuzumab (100 nM, *middle panel*) and PD098059 (50 μM, *right panel*) on basal expression of total and activated EGFR, c-erbB2, and ERK1/2 in the two cell lines. All data are representative of at least three experiments for each treatment.

receptor heterodimerization and activation, results in a synergistic interaction between the two receptors that enhances their mitogenic and transforming activity. Indeed, simultaneous overexpression of EGFR and c-erbB2 has been shown to enhance cellular transformation and aid development of hormone independence *in vitro* (55–57) and to be associated with endocrine insensitivity and a more aggressive tumor phenotype in the clinic (11). To investigate c-erbB receptor interactions in our two cell lines, we assessed their dimerization and activation status. Using phosphorylation site-specific antibodies directed against EGFR and c-erbB2, we demonstrated the presence of active EGFR/c-erbB2 and EGFR/c-erbB3 in TAM-R cells but not in the WT cells. The strongest signal observed was for the phosphorylated EGFR/c-erbB2 heterodimer, indicative of the preference for

EGFR to dimerize with c-erbB2. Such a preference would potentially limit the availability of c-erbB2 receptors in TAM-R cells, which consequently may result in the reduced c-erbB3 transphosphorylation in this cell line.

It is possible that activated EGFR/EGFR and c-erbB2/c-erbB2 homodimers were also present, but this could not be established with any certainty using the immunoprecipitation/Western blotting technique, as under these conditions the detection antibody cannot differentiate between the precipitated receptor and its dimerization partner. The formation of c-erbB3/c-erbB3 homodimers is unlikely as this receptor is kinase impaired and thus requires transphosphorylation by other c-erbB family members (58). We are currently evaluating other technologies that will allow investigation of c-erbB receptor homodimer production in the two
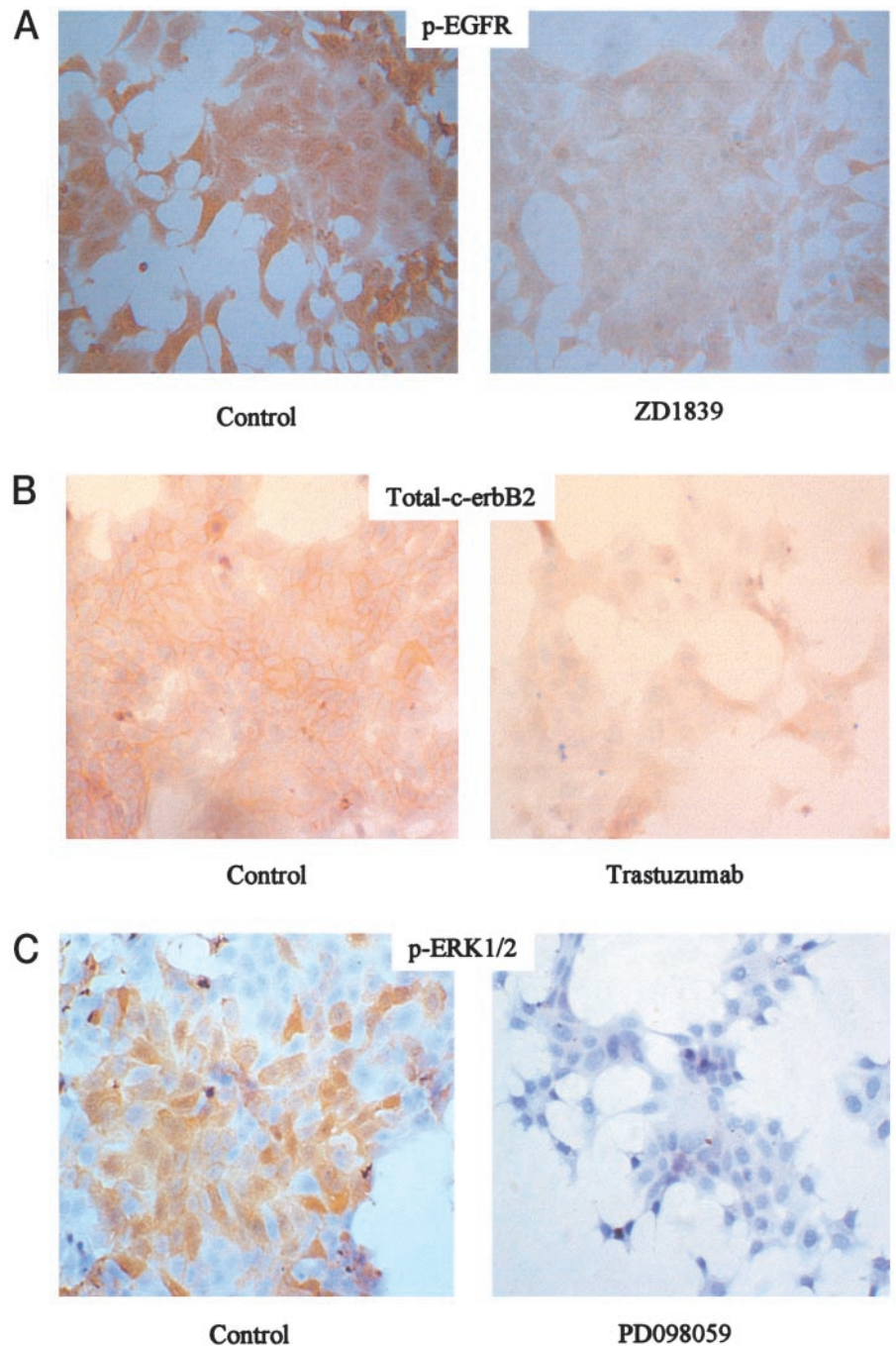
FIG. 7. Immunocytochemical analysis of the effects of A, ZD1839 (1 μM), on basal EGFR activity; B, trastuzumab (100 nM) on total c-erbB2 expression activity; and C, PD098059 (50 μM) on basal levels of phosphorylated ERK1/2 in TAM-R cells. All data are representative of at least three experiments for each treatment. Original magnification, ×40.

cell lines. Active c-erbB2/c-erbB3 receptor heterodimers could not be detected in either WT or TAM-R cells; however, phosphorylated forms of both c-erbB2 and c-erbB3 could be detected in WT cells. Because dimerization is a requirement for c-erbB activation, these data suggest that active c-erbB2/c-erbB3 receptor heterodimers are present in WT cells but at levels below the detectable limit for our immunoprecipitation assay. This low heterodimer activation status would suggest that there is minimal utilization of these receptors in WT cells. The loss of phosphorylated c-erbB3 and the inability to detect active c-erbB2/c-erbB3 heterodimers in TAM-R cells further suggests that HRGs are unlikely to play a major

role in the acquisition of tamoxifen resistance in this cell line. It should be noted, however, that a paracrine action of HRGs, and other ligands capable of activating c-erbB signaling pathways, in clinical tamoxifen-resistant tumors cannot be discounted. Indeed, it has been shown that MCF-7 cells transfected with HRGβ develop estrogen independence and resistance to antiestrogens both *in vivo* and *in vitro* (59).

As EGFR was the only common receptor subtype within the phosphorylated dimers present in the TAM-R cells, it is likely that the action of an EGFR-specific ligand was responsible for this dimerization profile. Furthermore, because all experiments were conducted using serum growth factor-free

TABLE 3. Effects of ZD1839, trastuzumab, and PD098059 on immunocytochemically determined phosphorylated EGFR and ERK1/2 and total c-erbB2 expression in WT-MCF-7 and TAM-R cells

	WT-MCF-7 Membrane	Cytoplasmic	TAM-R Membrane	Cytoplasmic
Activated EGFR				
Basal − ZD1839	2.5 (0.85)	79 (3.3)	13 (4)	129 (3.3)
Basal + ZD1839	3.4 (0.9)	67 (2.5)[a]	3 (0.8)[a]	67 (5)[b]
Total c-erbB2				
Basal − trastuzumab	50 (3.4)	43 (5.7)	132 (7.4)	91 (5.5)
Basal + trastuzumab	6.5 (2.8)[b]	34 (6.1)	22.5 (4.7)[b]	35 (5.6)[b]

	Cytoplasmic	Nuclear	Cytoplasmic	Nuclear
Activated ERK1/2				
Basal − PD098059	27 (3.2)	1 (0.3)	103 (5.8)	7 (1.2)
Basal + PD098059	2 (0.5)[b]	0.3 (0.2)	5 (0.8)[b]	1 (0.3)[b]

The effects of 1 μM ZD1839, 100 nM trastuzumab, and 50 μM PD098059 were assessed over a 7-d period. H-score values are expressed as mean (SEM) of the assessment of five fields of view per coverslip in triplicate experiments.
[a], $P < 0.05$; [b], $P < 0.001$ *cf.*-agent.

medium, the source of this ligand must be the cells themselves. Using RT-PCR, we found that TAM-R cells are capable of expressing a wide range of EGF-related ligands, with comparable expression levels apparent in the two cell lines (data not shown). Increased expression of TGFα has been shown to be related to endocrine insensitivity in ER-positive breast cancer specimens (60) and its coexpression with EGFR has been reported in acquired tamoxifen-resistant disease (8). Preliminary immunocytochemical studies have suggested that TAM-R cells express more TGFα protein under basal growth conditions than WT-MCF-7 cells (data not shown). However, further analysis of this ligand and all other EGF-related ligands will be required before a definitive statement on which ligand is responsible for TAM-R cell proliferation can be made.

To assess how the increased expression and activation of EGFR and c-erbB2 correlated with enhanced proliferative activity in TAM-R cells, we determined the effects of the specific EGFR-TKI ZD1839 and the anti-c-erbB2 monoclonal antibody trastuzumab on cell proliferation. ZD1839 abolished EGFR activity and significantly inhibited TAM-R cell proliferation while having little growth inhibitory activity in WT cells. Furthermore, trastuzumab reduced c-erbB2 protein expression and activation in both cell lines and, like ZD1839, inhibited growth of TAM-R cells to a greater extent than the WT cells. These results support our previous findings in fulvestrant-resistant MCF-7 cells, which demonstrated substantially increased sensitivity to the growth inhibitory action of ZD1839 compared with WT cells (25) and further indicate the importance of coexpression with c-erbB2 in the antiestrogen-resistant growth response in this system. Reduced levels of phosphorylated c-erbB2 levels were also observed following ZD1839 treatment in both cell lines suggesting that this agent can inhibit EGFR/c-erbB2 heterodimer activity. This is probably an EGFR-dependent effect rather than a consequence of direct inhibition of c-erbB2 phosphorylation as it has previously been demonstrated, in a range of c-erbB2 overexpressing breast cancer cell lines, that ZD1839-induced c-erbB2 inhibition occurs only within cells coexpressing EGFR (61). The dominant action of trastuzumab was down-regulation of total levels of both c-erbB2 and EGFR indicating that this agent can also target EGFR/c-erbB2 heterodimers. This effect was most apparent in

TAM-R cells, indicative of the substantial amounts of EGFR/c-erbB2 heterodimers present in this cell line and suggests that overexpression of both receptor types plays an important role, alongside activation status, in promoting tamoxifen-resistant cell proliferation. The ability of trastuzumab to inhibit WT cell growth by approximately 30% also suggests that this cell line uses c-erbB2 in its proliferative response, despite the apparent low activation levels of this receptor type in this cell line. Indeed, it has recently been reported that c-erbB2 may play a significant role in estrogen-mediated signaling in WT-MCF-7 cells with trastuzumab substantially inhibiting estrogen-induced cell proliferation (52). However, the mechanisms by which the c-erbB receptors interact with ER signaling in WT-MCF-7 cells remain unclear and require further investigation.

It is well established that phosphorylation of EGFR results in the activation of the Ras/MEK signal transduction cascade, a key regulatory pathway for cell proliferation (26). In the present study, we demonstrated increased basal levels of activated ERK1/2 in TAM-R compared with WT cells, indicating that this active kinase was targeting substrates localized within the cytoplasm and nuclei of the resistant cell line. Basal ERK1/2 activity was abolished by ZD1839 and substantially reduced by trastuzumab in both cell lines, confirming the link between increased EGFR/c-erbB2 activity and phosphorylation of the downstream ERK1/2 MAPK pathway. The loss of ERK1/2 activity correlated well with inhibition of growth of TAM-R but not WT cells, indicative of the increased reliance of the tamoxifen-resistant cell line on the EGFR/c-erbB2/MAPK signaling pathway to mediate growth. This was further demonstrated by the increased sensitivity of TAM-R cells, compared with their WT counterparts, to the growth inhibitory actions of the specific MEK1/2 inhibitor, PD098059, despite the ability of this agent to abolish ERK1/2 activity in WT cells. These findings provide further evidence for the importance of the MAPK pathway in the development of antiestrogen resistance that has been reported in both clinical tissue and breast cancer cell lines (22, 25, 27–32). The small growth inhibitory action of PD098059 in WT cells, however, does suggest a possible minor growth regulatory role for the MAPK pathway in this cell line. It is possible that such an effect may be a consequence of a nonspecific action of this agent due to the rel-
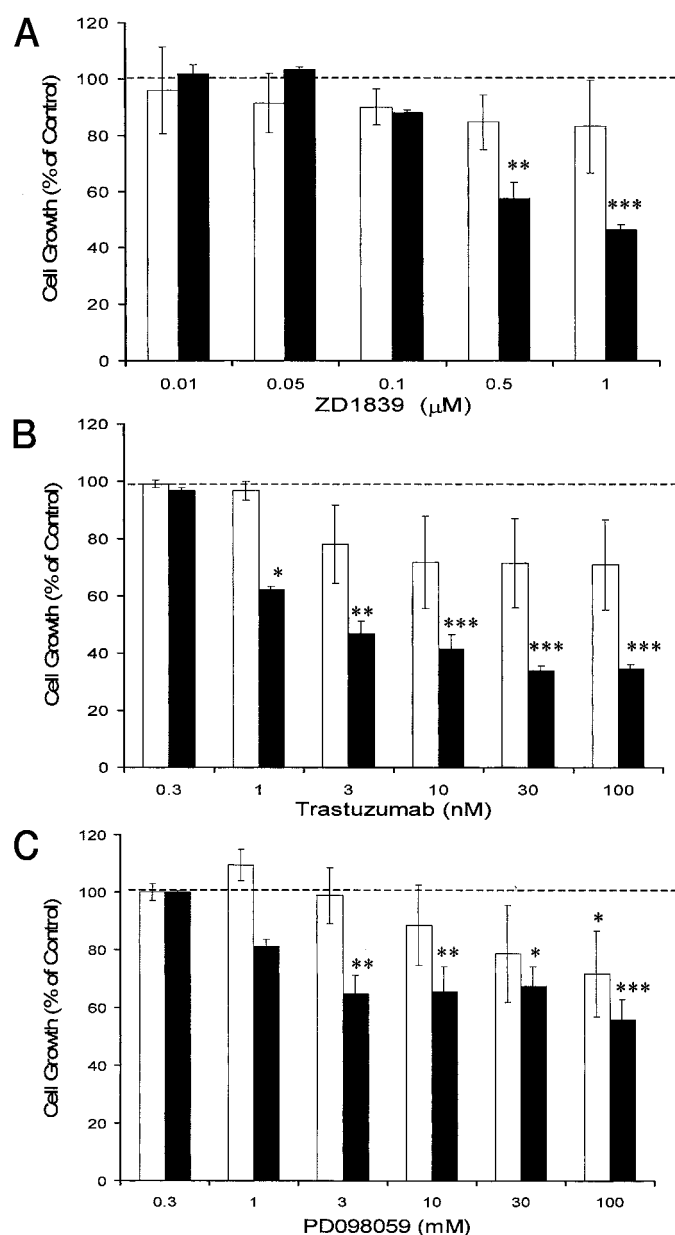
FIG. 8. The effects of increasing concentrations of A, ZD1839 (0.01–1 μM); B, trastuzumab (0.3–100 nM); and C, PD098059 (0.3–100 μM) on the basal growth of WT-MCF-7 (*empty bar*) and TAM-R (*filled bar*) cells on d 7 from initial treatment. The results are expressed as mean (SEM) of triplicate wells and are representative of three separate experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *cf.* control

atively high concentration used in this study. However, the differential effect of PD098059 on the growth of the two cell lines and its lack of inhibitory activity on basal levels of phosphorylated EGFR or c-erbB2 in either cell line would argue against this.

In conclusion, we have demonstrated that, whereas tamoxifen initially reduced proliferation of WT-MCF-7 breast cancer cells, this inhibitory action was not sustained as the cells were capable of establishing an alternative basal growth regulatory pathway through the activation of an EGFR/c-erbB2/MAPK signaling cascade. The involvement of other

receptor tyrosine kinases and signaling pathways in the development of tamoxifen-resistant growth cannot be discounted; however, the high sensitivity of TAM-R cells to the growth inhibitory effects of ZD1839 would suggest that the EGFR/MAPK pathway appears to be the predominant growth regulatory pathway in this cell line. Similar characteristics are displayed by MCF-7 cells resistant to the pure antiestrogen fulvestrant (25), suggesting that increased activity of this growth factor signaling cascade is the common mechanism by which this ER-positive breast cancer cell line develops resistance to antihormonal therapies. The EGFR therefore presents itself as a promising therapeutic target for antihormone-resistant tumors.

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