Growth Regulation of Human Breast and Ovarian Tumor Cells by Heregulin: Evidence for the Requirement of ErbB2 as a Critical Component in Mediating Heregulin Responsiveness

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ABSTRACT

Alterations in the expression of the epidermal growth factor (EGF) receptor ErbB family are frequently encountered in a number of human cancers. Two of these receptors, ErbB3 and ErbB4, are known to bind a family of related proteins termed heregulins (HRGs) or neu differentiation factors. In biologically relevant systems, interaction of HRG with ErbB3 or ErbB4 results in the transactivation of ErbB2. In this report, we show that ErbB2 is a critical component in mediating HRG responsiveness in a panel of human breast and ovarian tumor cell lines. Because HRGs have been reported to elicit diverse biological effects on cultured cells, including growth stimulation, growth inhibition, and induction of differentiation, we systematically examined the effect of rHRGß1 on tumor cell proliferation. HRG binding studies were performed with a panel of breast and ovarian tumor cell lines expressing a range of levels of ErbB2. The biological responses to HRG were also compared to EGF and to the growth-inhibitory anti-ErbB2 antibody, 4D5. In most cases, HRG stimulation of DNA synthesis correlated with positive effects on cell cycle progression and cell number and with enhancement of colony formation in soft agar. On each cell line tested, the HRG effects were distinguishable from EGF and 4D5. Our findings indicate that HRG induces cell proliferation in a number of tumor cell lines. In addition, we show that methods for measuring cell proliferation, as well as experimental conditions, are critical for determining HRGs effect on tumor cell growth in vitro.

INTRODUCTION

Clinical interest in the ErbB family of receptor tyrosine kinases stems from the observations that these receptors are frequently overexpressed in a number of human cancers (1–5). Five different gene products are known to activate the prototypical member of this family, EGFR. In several different tumor types, the coexpression of EGFR with one of its cognate ligands, transforming growth factor α, has been correlated with greater metastatic potential (6, 7). A second group of ligands, which collectively have been termed neuregulins, are known to arise from alternative splicing of a single gene mapped to human chromosome 8p22–pl 1 (8, 9). This family of proteins includes HRG (10), NDF (11, 12), gp30 (13, 14), acetylcholine receptor-inducing activity (15), glial growth factor (16), and sensory neuron-derived factor (17) and are ligands for ErbB3 and ErbB4 (18). One hallmark of this class of receptors is their propensity for heterodimerization, which upon ligand stimulation can lead to transphosphorylation and ultimately transactivation (19). To date, no ligands have been characterized, to the extent that their cDNA clones have been obtained, that specifically interact with ErbB2. ErbB2 is, however, frequently transactivated by either EGFR ligands or neu-regulins, and in several instances, the activation of ErbB2 has been shown to be essential for the generation of an active receptor signaling complex (20–22).

The importance of ErbB2 as a negative prognostic indicator in breast (23) and ovarian (24) cancer is now well established (25). Since HRG (10) and NDF (11) were originally identified based on their ability to activate ErbB2, it is of interest to determine the biological outcome of this activation on the growth of human breast and ovarian tumor cells. To address this question, we have undertaken a systematic study of HRG responsiveness using a panel of human breast and ovarian tumor cell lines with known ErbB2 levels. These cell lines were then characterized with regard to both their affinities and capacities to bind HRG. Antibodies directed against ErbB2 were used to determine if ErbB2 was essential in mediating HRG interactions with ErbB3 or ErbB4. To determine whether HRG treatment of these tumor cell lines resulted in cell proliferation (10) or growth inhibition (12, 26), we performed these assays under a series of well-defined experimental conditions and using a number of different assay formats. In addition, HRG responses were compared to EGF and a cytostatic monoclonal antibody directed against ErbB2, 4D5 (27, 28). Our conclusions from these studies are that ErbB2 plays a critical role in mediating HRG responsiveness over a wide range of ErbB2 expression levels. Cellular responses to exogenous HRG under carefully controlled experimental conditions are cell specific but, in general, result in the proliferation of human breast and ovarian tumor cells. These studies suggest that development of HRG antagonists or compounds that target HRG receptors may find clinical utility in the treatment of a number of important human cancers.

MATERIALS AND METHODS

Cells and Cell Culture. The following cell lines were obtained from the American Type Culture Collection: MCF7, SK-BR-3, MDA-MB-175-VII, MDA-MB-231, MDA-MB-361, MDA-MB-453, MDA-MB-468, BT-474, HBL-100, T47D, BT-20, BT-483, Caov3, and SK-OV-3. For all experiments, cells were used between passages 5 and 15. All lines were maintained in Ham’s F-12/DMEM (50:50) supplemented with 10% heat-inactivated FBS and 2 mM L-glutamine.

HRG Binding Assays. Tumor cells were plated in 24-well plates at 10⁵ cells/well in F12/DMEM containing 10% FBS. After 48 h, the cultures were washed two times with F12/DMEM. Cells were placed on ice and briefly incubated with binding buffer (0.1% BSA in F12/DMEM). ¹²⁵I-labeled rHRGß1 (5 pm to 2 nm) was then added to each well. Binding reactions were performed for 4 h on ice. Kinetic studies showed that equilibrium was obtained at 4 h, and no significant changes in binding constants were observed if the reactions were conducted up to 16 h. Cells were then washed twice with 0.5 ml binding buffer, and bound radioactivity was determined after solubilization with 0.1% SDS in 0.1 N NaOH or with 5 m urea in 3 M acetic acid. Scatchard analysis was performed as described previously (10, 29). The values reported in Table 1 for these binding experiments are the average of three different binding experiments, each of which consisted of triplicate incubations for each concentration of ¹²⁵I-labeled rHRGß1 (5 pm to 2 nm) tested. Error values for both Kd and sites/cell were on the order of 10% or less for each binding experiment.

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Inhibition of HRG binding to tumor cell lines by anti-ErbB2 antibodies were also performed on ice with monolayer cultures in a 24-well-plate format. Anti-ErbB2 monoclonal antibodies were added to each well and incubated for 30 min. 125I-labeled HRGβ177-244 (25 pm) was added, and the incubation was continued for 4 to 16 h. For several cell lines, incubations were performed for 4 h, since longer incubations at reduced temperatures resulted in cells detaching from the plate.

Tyrosine Phosphorylation Assays. MCF7 cells were plated in 24-well plates as described above for HRG binding experiments. Monoclonal antibodies to ErbB2 were added to each well and incubated for 30 min at room temperature; then HRGβ177-244 was added to each well for a final concentration of 0.2 nm, and the incubation was continued for 8 min. Media was carefully aspirated from each well, and reactions were stopped by the addition of 100-μL lysis sample buffer (5% SDS, 25 mm DTT, and 25 mm Tris-HCl, pH 6.8). Each sample (25 μL) was electrophoresed on a 4–12% gradient gel (Novex) and then electrophoretically transferred to polyvinylidene difluoride membrane. Antiphosphotyrosine (4G10, from Ubi, used at 1 μg/mL) immunoisobots were developed, and the intensity of the predominant reactive band at Mr ~180,000 was quantified by reflectance densitometry, as described previously (10, 29).

Cell Proliferation Determined by [3H]Thymidine Incorporation into DNA. Cells were seeded into 96-well plates at a density of 10^4/well for treatments up to 3 days and 5 × 10^5/well for the 6-day incubation. Following an overnight incubation to allow for cell adherence, the medium was removed and replaced with medium containing 0.1% FBS to growth arrest the cells. After a 48-h incubation, this medium was replaced with medium containing 0.1, 1.0, or 10% FBS and rHRGβ1 (5 pm-10 nm), 3 nm EGFR (Sigma Chemical Co.), or 10 μg/ml (65 nm) of the anti-ErbB2 monoclonal antibody 4D5 (27, 28, 30). The cells were then incubated for 0.5, 1, 3, or 6 days, pulsed with 1 μCi/well [3H]thyminidine (Amersham) for 4 h, and harvested onto Unifilter GF/C plates (Packard Instrument Co.) using a Packard Filtermate 196 harvester. After allowing the filter plates to dry, MicroScint 20 liquid scintillation cocktail (Packard) was added to each well; then the plates were heat-sealed and counted in a Packard TopCount.

Cell Cycle Analysis. Cells were plated at a density of 2 × 10^4 cells/dish in 60 × 15-mm culture dishes and allowed to adhere overnight. The monolayers were then washed with PBS and incubated with medium containing 0.1% FBS for 48 h to arrest cell growth. The medium was removed and replaced with medium supplemented with 0.1, 1.0, or 10% FBS alone or containing 0.3 nm rHRGβ1, 3 nm EGFR, or 65 nm 4D5. After a 1-day incubation, the cells were trypsinized, washed with PBS, fixed in ice-cold methanol, and stored at –20°C. The fixed cells were then washed twice with PBS and incubated with 100 μg/ml RNAse (Worthington Biochemical) for 30 min at 37°C. The RNAse was removed by centrifuging the cells, and the pellet was incubated with 50 μg/ml propidium iodide (Molecular Probes, Inc.) in PBS for DNA staining. The samples were then incubated overnight at 4°C and analyzed on an Epics Elite flow cytometer (Coulter Corporation) using Modfit LT software (Verity Software House).

Cell Proliferation Assay with Crystal Violet. Tumor cells were plated at a density of 2 × 10^4/well in 96-well plates in media containing 0.1% FBS and allowed to adhere for 2 h. Monoclonal antibodies (10 nm) or media alone were added, and the cells were incubated for 2 h at 37°C. HRGβ1 (0.3 nm) was then added, and the cells were incubated for 3 days. Monolayers were then washed with PBS and fixed stained with 0.5% crystal violet. Plates were air dried, the RNAse was removed by centrifuging the cells, and the pellets were incubated with 100 μg/ml RNAse (Worthington Biochemical) for 30 min at 37°C. The RNAse was removed by centrifuging the cells, and the pellets were stained with 250 μg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (Sigma Chemical Co.) and enumerated using an Omnicron 3600 Tumor Colony Image Analysis System (Imaging Products International, Inc.).

RESULTS

Determination of HRG Binding. Using [125I]-labeled rHRGβ177-244 to perform direct binding experiments, we determined both the affinity and the number of HRG receptors by Scatchard analysis on 13 different cell lines of breast and ovarian origin. A summary of these data is shown in Table 1. For each of these cell lines, the binding data could be fit to a single class of high affinity binding sites. Only the immortalized human mammary epithelial cell line, HBL-100, lacked detectable HRG binding. Very low (<10,000 receptors/cell) HRG binding could be detected on the ovarian cell line SK-OV-3 and the breast tumor cell lines MDA-MB-175-VII, MDA-MB-468, and BT-20. The following breast cancer cell lines, MCF7, MDA-MB-361, T47D, and BT-483, and the ovarian cancer cell line Caov3 were found to have between 10,000–22,000 receptors/cell, and these cell lines were arbitrarily characterized as containing intermediate levels of HRG binding sites. Cell lines having high numbers of HRG binding sites (i.e., >25,000 receptors/cell) were SK-BR-3, MDA-MB-453, and BT-474. Previously, we have determined that in the absence of ErbB2, ErbB3 binds HRG with a binding affinity of 0.9–2 nm (31). When COS7 cells or NIH3T3 cells coexpress ErbB2 with ErbB3, a higher affinity binding site (~20 pm) is observed (29, 32). Using a similar COS7 cell system, Tzahar et al. (33) have reported recently that NDF binds to ErbB4 and ErbB3 with Kd of 1.5 and 8 nm, respectively. These authors speculated that the difference in the binding constants may be related to cell-specific determinants or are due to the preparation or radiolabeling of the recombinant ligands. Recently, we have determined that ErbB4 or ErbB3 binds HRG with similar affinity, if either is expressed singly in cells of hematopoietic origin in the absence of any other ErbB receptors. The average Kd for the 12 cell lines shown in Table 1 that exhibit HRG binding is 99 ± 14 pm. The binding constants shown in Table 1 are at least an order of magnitude higher in affinity than what has been reported for ErbB3 or ErbB4 alone. These data suggest that other components besides ErbB3 or ErbB4 are contributing to the formation of a high affinity HRG binding site on these cells.

Monoclonal Antibodies to ErbB2 Block HRG Binding to Breast and Ovarian Tumor Cells. Based on our previous observations with COS7 cells expressing ErbB3 and ErbB2, as well as our experience with human Schwann cells, we hypothesized that the high-affinity HRG receptors present on these tumor cells were the result of heterodimer formation of ErbB3 with ErbB2 or possibly ErbB4 with ErbB2 (21, 29). To evaluate whether ErbB2 contributes to the formation of this high-affinity HRG binding site on human tumor cells, we surveyed a panel of well-characterized anti-ErbB2 antibodies (28) for their ability to inhibit HRG-stimulated tyrosine phosphorylation of proteins in the Mr 180,000 range from whole-cell lysates of MCF7 cells. MCF7 cells are reported to express all known ErbB receptors, but at relatively low levels (34). Since ErbB2, ErbB3, and ErbB4 have similar affinity, if either is expressed singly in cells of hematopoietic origin in the absence of any other ErbB receptors, we surveyed a panel of well-characterized anti-ErbB2 antibodies (28) for their ability to inhibit HRG-stimulated tyrosine phosphorylation of proteins in the Mr 180,000 range from whole-cell lysates of MCF7 cells. MCF7 cells are reported to express all known ErbB receptors, but at relatively low levels (34). Since ErbB2, ErbB3, and ErbB4 have nearly identical molecular sizes (35, 36), it is not possible to discern which protein is becoming tyrosine phosphorylated when whole-cell lysates are evaluated by Western blot analysis. However, these cells are ideal for HRG tyrosine phosphorylation assays because under the assay conditions used, in the absence of exogenously added HRG, they exhibit low to undetectable levels of tyrosine phosphorylation proteins in the Mr 180,000 range. As shown in Fig. 1A, several of these antibodies, including 2C4, 7F3, and 4D5, significantly inhibited

Table 1 HRG receptors on human tumor cell lines

Direct binding of 125I-labeled hHRGβ1,177-244 to tumor cells. Breast and ovarian cancer cell lines were plated in 24-well plates at an initial density of 10^5 cells/well and allowed to grow for 48 h. The cultures were washed twice with F12DMEM and then placed on ice to thermally equilibrate. 125I-labeled hHRGβ1,177-244 (5 pm to 2 nm) was then added to each well. Binding reactions were performed for 4 h on ice. Scatchard analysis was performed as described previously (10, 29). The values reported are the average and SD for three different binding experiments, each of which consisted of triplicate determinations for each concentration of 125I-labeled hHRGβ1,177-244 tested. Error values for both K and sites per cell were <10% for each binding experiment. The cell number was determined by trypan blue exclusion.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Kd (pm)</th>
<th>Sites/cell</th>
<th>ErbB-2 expressiona</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBL-100</td>
<td>NDa</td>
<td>ND</td>
<td>Normal/low</td>
</tr>
<tr>
<td>SK-OV-3</td>
<td>84 ± 18</td>
<td>2500 ± 440</td>
<td>++</td>
</tr>
<tr>
<td>MDA-MB-175</td>
<td>222 ± 103</td>
<td>2900 ± 1700</td>
<td>+</td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td>68 ± 6</td>
<td>4000 ± 500</td>
<td>ND</td>
</tr>
<tr>
<td>BT-20</td>
<td>57 ± 3</td>
<td>7300 ± 400</td>
<td>Normal/low</td>
</tr>
<tr>
<td>MCF7</td>
<td>91 ± 16</td>
<td>12,700 ± 1300</td>
<td>Normal/low</td>
</tr>
<tr>
<td>MDA-MB-361</td>
<td>87 ± 3</td>
<td>14,500 ± 1000</td>
<td>++</td>
</tr>
<tr>
<td>T47D</td>
<td>81 ± 8</td>
<td>15,900 ± 800</td>
<td>+</td>
</tr>
<tr>
<td>BT-483</td>
<td>81 ± 6</td>
<td>20,100 ± 1000</td>
<td>Normal/low</td>
</tr>
<tr>
<td>Caov3</td>
<td>140 ± 3</td>
<td>21,700 ± 1200</td>
<td>Normal/low</td>
</tr>
<tr>
<td>SK-BR-3</td>
<td>54 ± 8</td>
<td>27,300 ± 1500</td>
<td>++</td>
</tr>
<tr>
<td>MDA-MB-453</td>
<td>76 ± 3</td>
<td>29,000 ± 500</td>
<td>++</td>
</tr>
<tr>
<td>BT-474</td>
<td>137 ± 25</td>
<td>34,600 ± 2200</td>
<td>+++</td>
</tr>
</tbody>
</table>

a Values taken from Lewis et al. (30).

b ND, not detected.

The generation of a HRG-induced tyrosine phosphorylation signal at Mr 180,000. In the absence of HRG, none of these antibodies were able to stimulate tyrosine phosphorylation of proteins in the Mr 180,000 range (data not shown). Also, these antibodies do not cross-react with EGFR (28), ErbB3, or ErbB4. Antibodies 2C4 and 7F3 significantly inhibited HRG stimulation of p180 tyrosine phosphorylation to <25% of control. However, 4D5 was able to block HRG stimulation of tyrosine phosphorylation by ~50%. Previously, 2C4 and 7F3 were assigned the same ErbB2 epitope as reported in the original characterization of this antibody panel, and this epitope is different than that recognized by 4D5 (28). Fig. 1B shows dose-response curves for 2C4 or 7F3 inhibition of HRG stimulation of p180 tyrosine phosphorylation as determined by reflectance densitometry. Evaluation of these inhibition curves using a 4-parameter fit yielded an IC50 of 2.8 ± 0.7 nM and 29.0 ± 4.1 nM for 2C4 and 7F3, respectively. Varying concentrations of 2C4 or 7F3 were incubated with MCF7 cells in the presence of 125I-labeled rHRGβ1, and the degree of specific 125I-labeled rHRGβ1 binding was determined. The results from this study are shown in Fig. 2. Binding of 125I-labeled rHRGβ1 could be significantly inhibited by either 2C4 or 7F3 in all cell lines, with the exception of the breast cancer cell line MDA-MB-468, which has been reported to express little (37) or no ErbB2 (30, 38). The remaining cell lines are reported to express ErbB2, with the level of ErbB2 expression varying widely among these cell lines (30, 38). In fact, the range of ErbB2 expression in the cell lines tested varies by more than 2 orders of magnitude. For example, BT-20, MCF7, and Caov3 express ~10^6 ErbB2 receptors/cell, whereas BT-474 and SK-BR-3 express ~10^6 ErbB2 receptors/cell (30). Given the wide range of ErbB2 expression in these cells and the data in Fig. 2, it was concluded that the interaction between ErbB2 and ErbB3 or ErbB4, was itself a high-affinity interaction that takes place on the surface of the plasma membrane. At present, it is unclear whether these complexes involving ErbB2-ErbB3 or ErbB2-ErbB4 are preexisting in the absence of HRG or whether the association with

Fig. 1. Effect of anti-ErbB2 antibodies on rHRGβ1 activation of MCF7 cells. A. effect of the anti-ErbB2 antibodies on the generation of a phosphotyrosine signal at Mr 180,000. Antiphosphotyrosine immunoblots were developed as described in “Materials and Methods.” *, immunoreactive bands that result from the interaction of the murine monoclonal antibody with the secondary antibody detection system. B, dose-response curves for 2C4 or 7F3 inhibition of HRG stimulation of p180 tyrosine phosphorylation as determined by reflectance densitometry. Evaluation of these inhibition curves using a 4-parameter fit yielded an IC50 of 2.8 ± 0.7 nM and 29.0 ± 4.1 nM for 2C4 and 7F3, respectively.

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ErbB2 is driven by the formation of HRG-ErbB3 or HRG-ErbB4 complexes.

Stimulation of Mitogenesis by HRG in Human Tumor Cells. We initially reported that HRG stimulated the growth of SK-BR-3 breast tumor cells as measured by crystal violet staining (10). Having determined the levels of HRG receptors in a number of different human tumor cell lines, we examined the mitogenic response of these cells to rHRGβ1. Dose-response curves consisting of 5 pM-10 nM rHRGβ1 in different serum concentrations were generated for all of the cell lines listed in Table 1. Prior to treatment with rHRGβ1, cells were growth arrested by incubation in medium containing 0.1% FBS for 48 h. Fig. 3 shows typical curves for three breast tumor cell lines after treatment for 3 days. MCF7 responsiveness to rHRGβ1 was dependent on serum concentration in the assay medium. When assayed in 0.1% FBS, MCF7 cells showed a 4–5-fold stimulation of [3H]thymidine incorporation in a serum-dependent manner after 1 day of treatment with rHRGβ1 (data not shown) and after 3 days of treatment (Fig. 3, b and c). Although there was no effect in 0.1% FBS, [3H]thymidine incorporation increased in cells exposed to rHRGβ1 in 1 or 10% FBS.

Comparison of the HRG Response to EGF and 4D5. Since responsiveness of a number of tumor cell lines to either EGF or the anti-ErbB2 monoclonal antibody 4D5 has been extensively studied, we compared treatment of these cell lines with either EGF or 4D5 under identical conditions as a comparison to rHRGβ1. In vitro, EGF inhibits the growth of tumor cell lines such as MDA-MB-468 (43) and A431 (44, 45), both of which overexpress EGFR. Conversely, cells that express normal/low or moderate levels of EGFR are frequently growth-stimulated by exogenous EGF (39). Inhibition of cell growth by 4D5 is dependent on the expression level of ErbB2 (30), i.e., 4D5 is cytostatic only for cells that overexpress ErbB2. Using a 3-day assay, the data in Fig. 4 show that the changes in [3H]thymidine incorporation in these cell lines were distinct, depending on the treatment conditions. rHRGβ1 stimulated thymidine incorporation in MCF7, SK-BR-3, BT-474, MDA-MB-453, MDA-MB-361, T47D, and BT-20 cells at all serum concentrations tested. However the magnitude of the response varied, depending on the serum concentrations present in the assay. In contrast, EGF (3 nM) stimulated [3H]thymidine incorporation in HBL-100, T47D, BT-20, and SK-OV-3 cells under all conditions tested. Both rHRGβ1 and EGF stimulated MCF7 cells in both 0.1% and 1% FBS, although the magnitude of the response was less with EGF than with rHRGβ1. 4D5 had no effect on this cell line, as reported previously (30). DNA synthesis was inhibited by EGF and, more potently, by 4D5 in SK-BR-3 cells at all serum concentrations tested. Similarly, [3H]thymidine incorporation in BT474, MDA-MB-453, and MDA-MB-361 breast tumor cells was also inhibited by 4D5 since these cell lines overexpress ErbB2. rHRGβ1 and 4D5 did not affect [3H]thymidine incorporation in the MDA-MB-231 and MDA-MB-468 breast tumor lines. Consistent with previous reports (43), EGF was inhibitory for MDA-MB-468 cells. No significant changes in MDA-MB-231 cells were detected with either rHRGβ1, EGF, or 4D5 under the conditions tested.
ROLE OF ErbB2 IN HEREGULIN-MEDIATED SIGNALING

Cell Cycle Changes Induced by rHRGβ1, EGF, and Murine Monoclonal Antibody 4D5. To further characterize the effects of rHRGβ1, EGF, and 4D5 on tumor cell growth, analyses of cell cycle phase fraction distributions were performed. MCF7, SK-BR-3, and MDA-MB-453 breast tumor cells were serum starved and then treated for 1 day with 0.3 nm rHRGβ1, 3 nm EGF, or 66 nm 4D5 in 0.1, 1, or 10% FBS. Fig. 5 shows the results presented as the fraction of cells in S phase as an indicator of proliferative status measured at ~30 h following treatment. For both MCF7 and SK-BR-3 cells, the changes in the percentage of S-phase cells after each treatment correlates well with the effects on [³H]thymidine incorporation (Fig. 3). rHRGβ1 caused a 3-fold increase in the percentage of MCF7 cells in S-phase in 0.1% FBS. EGF treatment induced a smaller proportion of cells to enter S phase in 0.1 and 1% FBS (1.6- and 1.3-fold, respectively),

Fig. 3. Stimulation of DNA synthesis by rHRGβ1. MCF7 (A), SK-BR-3 (B), and MDA-MB-453 (C) breast tumor cells were plated at a density of 10⁵ cells/well in 96-well microtiter plates and allowed to adhere overnight. The medium was then replaced with low serum medium (0.1% FBS) to growth arrest the cells. After 48 h, this medium was removed, and the cells were treated with different concentrations of rHRGβ1 in medium supplemented with 0.1 ( ), 1 ( ), or 10% FBS ( ) for 3 days. At the end of each incubation, the cells were radiolabeled with 1 μCi/well [³H]thymidine for 4 h, then harvested onto filter plates for scintillation counting. The data are expressed as the mean cpm of 4–8 replicates; bars, SE.

Fig. 4. Comparison of the response of different breast and ovarian tumor cell lines to rHRGβ1, EGF, and 4D5. Mitogenic assays were performed as described in Fig. 3. Cells were treated with 0.37 nm rHRGβ1, 3 nm EGF, or 66 nm 4D5 in medium containing 0.1% ( ), 1% ( ), or 10% ( ) FBS for 3 days. The data are expressed as the percentage of [³H]thymidine incorporation (mean of 4–8 replicates; bars, SE) as compared to untreated control cells.
while 4D5 had no effect. With SK-BR-3 cells, rHRGβ1 stimulation of the percentage of cells in S phase was enhanced with increasing serum concentration (1.5–1.8-fold). EGF had an inhibitory effect in 0.1 and 1% FBS, and as expected, 4D5 decreased the percentage of S-phase cells at all concentrations of serum. MCF7 and SK-BR-3 cells were also treated for 3 days with rHRGβ1 with similar results. In contrast, the fraction of cells in S phase in the MDA-MB-453 cell line upon exposure to rHRGβ1 did not differ from controls in the three serum concentrations tested at 30 h. These results are different from the data shown in Fig. 4, where rHRGβ1 increased the incorporation of [3H]thymidine in the MDA-MB-453 cells in 1 and 10% FBS. On the other hand, EGF and 4D5 have similar effects on both [3H]thymidine incorporation and cell cycle progression. As expected, EGF-treated MDA-MB-453 cells were not different from control, since this cell line expresses little or no detectable EGFR (30, 38), whereas treatment with 4D5 reduced the number of S-phase cells. In contrast to previous reports (11, 26, 46), upon HRG treatment, there was no suggestion of a G2–M growth arrest nor was there evidence for induction of a ploidy abnormality. Explanations for these discrepancies can likely be attributed to differences in the purity of the NDF preparations or the particular cell lines being used in these earlier studies.

**Effect of rHRGβ1 on Cell Number.** Because of the discrepancy between the MDA-MB-453 [3H]thymidine incorporation and cell cycle experiments, it was necessary to determine actual cell number after rHRGβ1 treatment. Fig. 6 shows that the effects of rHRGβ1 on MCF7 and SK-BR-3 cell number are in agreement with the data in Figs. 3 and 5. The MCF7 breast tumor cells show the greatest increase in cell number to rHRGβ1 after 3 days of treatment in 0.1% FBS, while proliferation of SK-BR-3 cells is increased in higher serum concentrations. There was no change in MDA-MB-453 cell number after exposure to 0.3 nm rHRGβ1 in 0.1, 1, or 10% FBS. Therefore, it appears that the incorporation of [3H]thymidine in this cell line is not an indicator of DNA synthesis and cell proliferation. These results are in agreement with those reported earlier for the response of MCF7 and SK-BR-3 cells to concentrated conditioned medium containing recombinant NDF (25). Our data for the responsiveness of MDA-MB-453 and SK-BR-3 cells differ from those reported earlier for NDFα (12) or purified natural gp30, which has been shown to be related to HRG/NDF (14, 26).

**HRG Stimulation of Tumor Cell Growth Is Inhibited by Anti-ErbB2 Monoclonal Antibodies 2C4 and 7F3.** Since 2C4 and 7F3 are cytostatic for cells that overexpress ErbB2 (30), we could not test the antagonist effect of 2C4 or 7F3 on HRG-induced growth of cells that overexpress ErbB2. The effect of 2C4 or 7F3 on three cell lines that express low/normal levels of ErbB2 is shown in Fig. 7. MCF7 and T47D are breast cancer cell lines that are known to express low to intermediate levels of all ErbB receptors (34, 36, 39). Although ErbB4 is not expressed in the ovarian cancer cell line Caov3 (35), other ErbB receptors are present (30) (data not shown). The effect of 2C4 or 7F3 on rHRGβ1 stimulation of cell growth using a 3-day crystal violet assay format is shown in Fig. 7. In each case, rHRGβ1-mediated growth was inhibited by 2C4 or 7F3 to levels close to those observed without HRG treatment. The results observed with 2C4 or 7F3 are similar to those reported recently by Graus-Porta et al. (22), using a version of T47D cells engineered to sequester ErbB2 in the endoplasmic reticulum with a single-chain anti-ErbB2 antibody. These data are also similar to those obtained when rHRGβ1 was tested in combination with 2C4 on human Schwann cells (21).

**Anchorage-independent Growth in Soft Agar.** For a final determination of the effects of rHRGβ1 on cell proliferation, we studied the anchorage-independent growth of different breast and ovarian tumor cell lines in soft agar and compared the response with EGF. To allow for enhanced proliferation of colonies in soft agar, cells were seeded at densities resulting in minimal colony formation in untreated cells, and all experiments had to be performed in 10% serum. As shown in Fig. 8, MCF7, T47D, SK-BR-3, BT474, and SK-OV-3 cells showed a greater than 2-fold increase in colony formation in response to treatment with 0.3 nm rHRGβ1. Growth in soft agar of the MDA-MB-468 cell line was also slightly enhanced. Since this cell line does not express ErbB2 (30, 38) and presumably low or undetectable levels of ErbB4 but does express ErbB3, this response might be mediated by ErbB3. Since ErbB3 is an impaired kinase (47, 48), these data suggest that ErbB3 may be heterodimerizing with another ErbB family member other than ErbB2. One plausible candidate for this active signaling complex may be EGFR-ErbB3 since MDA-MB-468 cells overexpress EGFR (49). Colony formation by MDA-MB-453 and MDA-MB-361 cells was inhibited by HRG treatment relative to control plates. In comparison, EGF had little effect on MCF7, MDA-MB-453, MDA-MB-361, or MDA-MB-231 cells. Growth in soft agar was stimulated by EGF in SK-OV-3 cells and was slightly enhanced in SK-BR-3 and BT474 cells. Treatment of MDA-MB-468 cells with EGF completely abolished colony formation.
ROLE OF ErbB2 IN HEREGULIN-MEDIATED SIGNALING

Fig. 7. Effect of 2C4 or 7F3 on rHRGβ1 induced proliferation of MCF7 (A), T47D (B), and Caov3 (C) tumor cell lines. Monoclonal antibodies (10 nM) or medium alone were added, and the cells were incubated for 2 h at 37°C. rHRGβ1 (0.3 nM) was then added, and the cells were incubated for 3 days. Monolayers were then washed with PBS and fixed/stained with 0.5% crystal violet; then the absorbance was read at 540 nm to determine cell proliferation (30).

Fig. 8. Colony formation in soft agar by breast and ovarian tumor cell lines. Cells were seeded in 60 × 15-mm dishes onto a bottom layer of 0.5% agar and overlaid with 0.25% agar in Ham’s F12:DMEM (50:50) medium supplemented with 10% heat-inactivated FBS and 2 mM L-glutamine. Treatment groups for each cell line were 0.3 nM rHRG, 3 nM EOF, or an untreated control (designated by horizontal bar). After 2–4 weeks, the colonies were stained with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide and counted using an Omnicon 3600 Image Analysis System. The data are represented as the fold change in the number of colonies per dish (bars, SE) that were greater than 80 μm relative to untreated control dishes. Each treatment group consisted of three replicate dishes.

DISCUSSION

In this study, we describe a systematic analysis of HRG responsiveness in a panel of human breast and ovarian cancer cell lines. High-affinity HRG receptors could be detected on most of these cell lines. However, the range of HRG receptor expression is significantly narrower than that observed for either EGFR or ErbB2. Since HRG binding requires the presence of ErbB3 or ErbB4, these data imply that ErbB3 and ErbB4 expression may be more tightly regulated in breast and ovarian tumors cells than either EGFR or ErbB2. Antibodies directed against ErbB2 inhibited HRG binding in these cell lines, suggesting that the formation of this high-affinity binding site was likely the result of heterodimerization of ErbB2 with ErbB3 or with ErbB4. In addition, the binding affinity of HRG for ErbB4 appears to be considerably lower than the values determined here for this panel of human tumor cell lines (33). Although ErbB4 is expressed in some of these cell lines (34, 35), our analysis of ErbB4 expression in these cells using a panel of ErbB4-specific monoclonal antibodies indicates that the ErbB4 expression level is significantly lower than EGFR, ErbB2, or ErbB3. ErbB4 is a fully functional HRG receptor (35, 50), and it has been demonstrated recently that ErbB4 is required for normal embryonic neural and cardiac development (51). Regardless of the relative expression levels or the activation of particular signal transduction pathways, the abrogation of HRG responsiveness by anti-ErbB2 monoclonal antibodies indicates that ErbB2 is critical in mediating HRG responsiveness. Our conclusion from these studies is that inhibition of HRG response by these blocking anti-ErbB2 antibodies is the result of inhibiting the recruitment of ErbB2 to a high-affinity complex with ErbB3 or ErbB4. Using different experimental approaches similar conclusions have also been drawn regarding the role of ErbB2 in mediating HRG responses in the breast cancer cell lines T47D (22) and MCF7 (52).

At present, more is known about the interaction of ErbB2 with ErbB3 than ErbB2 with ErbB4. Although ErbB3 can function as a HRG-binding protein, it is a unique receptor in that its intrinsic tyrosine kinase activity appears to be impaired (47, 48). When coexpressed with ErbB2, ErbB3 becomes phosphorylated on tyrosine residues in its cytoplasmic domain that generate a signal primarily through the PI3-kinase pathway (32, 53). Thus, a functional HRG receptor may be viewed as a heterodimer of ErbB3 with ErbB2, where ErbB2 modulates the affinity of ErbB3 for HRG binding and contributes an active tyrosine kinase domain. Conversely, ErbB3 is required for HRG binding and upon phosphorylation of its unique tyrosine residues in the cytoplasmic domain, ErbB3 is capable of coupling to PI3-kinase. This latter adapter function is analogous to the interaction of IRS-1 with insulin receptor. IRS-1 is the principal substrate of the activated insulin receptor tyrosine kinase. After becoming tyrosine phosphorylated, IRS-1 couples with PI3-kinase and other SH2-proteins for the propagation of downstream signaling (54). Coopera-
tion between ErbB3 and ErbB2 in neoplastic transformation of NIH3T3 cells has also been demonstrated recently (53, 55).

We also studied the effects of rHRG on the growth of these cell lines using several different assay formats. In a number of these formats, it was found that the presence of serum profoundly influenced the magnitude of the HRG response on a number of these cell lines. In general, the results from these experiments agreed among the formats used. MCF7, Caov3, T47D, BT-20, BT-474, and SK-BR-3 cells were growth stimulated in a number of assays under defined serum concentrations. Our results with the stimulation of growth of these breast tumor cell lines are similar to those recently obtained with the mouse mammary epithelial cell line HC11 (56, 57) and the immortalized human epithelial cell line MCF-10A (41). Two notable exceptions in the present study were MDA-MB-453 and MDA-MB-361 cells. Although these cell lines were stimulated by rHRG treatment in the mitogenic assay, this incorporation of [3H]thymidine did not appear to lead to cell division. Indeed, anchorage-independent growth assays suggested that HRG treatment was growth inhibitory at high serum concentrations. There are several possible explanations for the apparent discrepancy between the stimulation of [3H]thymidine incorporation in the MDA-MB-453 and MDA-MB-361 cells and the negative results obtained with cell cycle, cell count, and soft agar experiments. Incorporation of [3H]thymidine into DNA occurs exclusively via the salvage pathway for DNA synthesis. Differences in rates of transport of labeled nucleotide into the cell, in activity of thymidine kinase or other synthetic or degradative enzymes, or in the size of intracellular nucleotide pools can lead to inconsistent results (58). Extracellular labeling would also lead to enhanced incorporation independent of DNA synthesis. Studies showing increased [3H]thymidine incorporation without subsequent cell division have been reported (59, 60).

We compared the HRG response in these cell lines to 4D5, a cytostatic monoclonal antibody directed against ErbB2. Growth inhibitory response of cell lines to 4D5 is strictly dependent on ErbB2 expression levels (30) and appears to be independent of serum concentrations. The growth of cell lines such as BT-474 and SK-BR-3 is inhibited by 4D5, whereas under the same conditions, they are growth stimulated by HRG. Additionally, HRG-mediated growth responses in cell lines known to express low/normal levels of ErbB2 such as MCF7, T47D, and Caov3 were inhibited by two other anti-ErbB2 antibodies, 2C4 and 7F3. To explain these results with HRG and 4D5, we speculate that ErbB2 heterodimerization with other ErBB family members is itself a high-affinity interaction and is preferred to ErbB2 homodimerization. This hypothesis is supported by the observation that HRG activation of ErbB2 occurs at a wide range of ErbB2 expression levels. In the present study, ErbB2 activation occurs by at least two different mechanisms, heterodimerization or homodimerization. In addition to ErbB2, the heterodimerization pathway requires HRG and a receptor for HRG, i.e., ErbB3 or ErbB4. Monoclonal antibodies directed against ErbB2, such as 2C4 or 7F3 and to a lesser extent 4D5, are capable of disrupting these ErbB2-ErbB3/4 interactions so that HRG activation of ErbB2 is ablated. Constitutive activation of ErbB2, which is likely the result of ErbB2 homodimerization or oligomerization, occurs at high ErbB2 receptor densities and is independent of HRG, ErbB3, and ErbB4 expression. In agreement with this hypothesis is the observation that a threshold of ErbB2 expression must be surpassed to transform rodent fibroblast (61). Moreover, reversion of this transformed phenotype can occur by treatment with anti-ErbB2 monoclonal antibodies (27). Additionally, the growth inhibitory properties of anti-ErbB2 monoclonal antibodies are observed only on human tumor cell lines that overexpress ErbB2 (30). Thus, activation of ErbB2 by HRG or ErbB2 overexpression can result in the proliferation of tumor cell growth. As observed, antibody-


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