Heregulin (HRG)-induced Mitogenic Signaling and Cytotoxic Activity of a HRG/PE40 Ligand Toxin in Human Breast Cancer Cells

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Abstract

The heregulins (HRGs) are a family of growth factors that bind directly to erbB3 and erbB4 and induce tyrosine phosphorylation of erbB2 via receptor heterodimerization. Since erbB2, erbB3, and erbB4 (erbB2–4) are often overexpressed in human breast cancer cells, we produced recombinant HRGs and a HRG-based ligand toxin to investigate the signaling events triggered by HRGs and the ability of these ligands to specifically target such cells. Recombinant HRGβ2 stimulated the tyrosine phosphorylation of erbB2–4 in ZR-75-1 human breast cancer cells. This was accompanied by the tyrosine phosphorylation of Shc and the formation of complexes between Shc and the adapter protein Grb2. Complexes were also detected between Shc and erbB2–4. However, Grb2 was detected in erbB2 and erbB4 but not erbB3 immunoprecipitates. Thus, these receptors exhibit mechanistic differences in their coupling to Ras signaling, and HRGβ2 administration triggers multiple inputs into the Ras signaling pathway, involving receptor-Grb2, receptor-Shc, and Shc-Grb2 complexes. HRGβ2 addition also stimulated the association of erbB3 with phosphatidylinositol-3-kinase. In accordance with the activation of key mitogenic signaling pathways, HRGβ2 stimulated the proliferation of MCF-7 and T-47D human breast cancer cells. Moreover, when tested for the ability to stimulate cell cycle re-entry of T-47D cells arrested under serum-free conditions, HRGβ2 was more effective than insulin, previously the most potent mitogen identified using this system. Finally, a HRGβ2/PE40 ligand toxin was constructed and found to exhibit cytotoxic activity against human breast cancer cells overexpressing erbB3 alone or in combination with erbB4 and/or erbB2.

Introduction

Currently, the erbB family of RTKs consists of four members: the EGFR (or erbB1), erbB2, erbB3, and erbB4 (erbB2–4). All of these receptors are monomeric in structure and belong to the class I family of RTKs. Several lines of evidence support a role for the erbB family of RTKs in the growth and progression of human breast cancer. For example, several EGFR ligands, including EGF, transforming growth factor α, and amphiregulin, are mitogenic for human breast cancer cells in vitro and are thought to function in an autocrine and/or paracrine mode to stimulate the growth of such cells in vivo. In addition, high expression of the EGFR in breast tumors is inversely related to estrogen receptor status and has been proposed as a predictor of early recurrence and death. Furthermore, amplification and/or overexpression of the erbB2 gene occurs in approximately 20% of human breast cancers and is associated with decreased patient survival, whereas high expression of the erbB3 and erbB4 genes has been detected in human breast cancer cell lines. Finally, transgenic models of mouse mammary tumorigenesis support the involvement of the erbB family of RTKs and their ligands in the pathogenesis of breast cancer.

Although related in structure, members of the erbB family exhibit differences in their coupling to specific intracellular signaling pathways. For example, the EGFR and erbB2 recruit Ras GTase-activating protein, phospholipase C-γ1, and Grb2 but exhibit only weak activation of PI3-kinase (11–14). However, despite triggering these pathways to similar extents, the erbB2 receptor exhibits a transforming potential 100-fold higher than the EGFR, and only the EGFR can abrogate interleukin 3 dependence in 32D hematopoietic cells. In the case of erbB3, qualitative differences in signaling potential have been identified. This receptor couples 10 times more efficiently to PI3-kinase than the EGFR, binds Shc, but fails to bind or tyrosine phosphorylate phospholipase C-γ1 or Ras GTase-activating protein (17). These findings are further complicated by the observation that certain members of the erbB family can heterodimerize and transphosphorylate upon activation, leading to receptor "cross-talk." Hence, in the human breast cancer cell line SK-BR-3, activation of the EGFR leads to tyrosine phosphorylation of erbB2 (18), and in cell lines co-expressing the EGFR and erbB3, the EGFR can transphosphorylate the latter receptor, promoting the recruitment of PI3-kinase (19, 20).

Until recently, mechanistic studies on the role of erbB2–4 in human breast cancer have been hampered by the incomplete characterization of ligands for these recep-

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3 The abbreviations used are: RTKs, receptor tyrosine kinases; EGFR, epidermal growth factor receptor; erbB2–4, erbB2, erbB3, and erbB4; PI3-kinase, phosphatidylinositol-3-kinase; HRG, heregulin; PE, Pseudomonas exotoxin; PE40, M, 40,000 modified PE; MBP, myelin basic protein.
tors. However, the recent cloning of the HRG/neu differentiation factor family of polypeptides (21–24) has provided the opportunity to investigate the signaling events triggered by these receptors. The HRGs are the products of a single gene and are initially synthesized as transmembrane precursors, with the extracellular region containing an immunoglobulin homology unit and an EGF-like domain, the latter being responsible for biological activity. The four HRG isoforms originally identified (HRGα, β1, β2, and β3) differ in the COOH-terminal portion of the EGF-like domain and the adjacent juxtamembrane stretch. Although erbB2 was originally proposed to represent the natural receptor for these ligands, the HRGs instead bind directly to erbB3 and erbB4, the observed tyrosine phosphorylation of erbB2 following HRG binding occurring as a consequence of heterodimerization with these receptors (25–28).

The biological effects of the HRGs on human breast cancer cells remain controversial. Several reports have described HRG-induced phenotypic differentiation of human breast cancer cells overexpressing erbB2, including altered morphology and increased synthesis of milk components including casein and lipids. These changes were accompanied by growth inhibition and induction of DNA polyploidy (21, 29, 30). However, others have observed only stimulation of proliferation of such cells (24). The reason behind these discrepancies is unclear. One problem inherent in the use of cell lines overexpressing erbB2 in the study of cellular events initiated by the HRGs is the constitutive activation of this receptor and key signaling pathways downstream of this receptor in such cells (12). Since erbB3 and erbB4 represent the primary receptors for the HRGs, an alternative approach is to use cell lines expressing more moderate levels of erbB2 in combination with these receptors, for example, MCF-7, T-47D, and ZR-75-1 human breast cancer cells. The lower baseline of receptor activation in these cell lines allows the interaction between erbB2–4 in the stimulation of key signaling pathways to be analyzed.

We have, therefore, used recombinant HRGs to examine erbB receptor activation in cell lines expressing erbB2–4 and to delineate the mechanisms underlying coupling to the Ras and PI3-kinase signaling pathways. The potent mitogenic effects of the HRGs on these cell lines are also described. Finally, we have investigated whether the levels of expression of erbB3 and erbB4 found in human breast cancer cells that overexpress these receptors, as opposed to erbB2, are sufficient to confer sensitivity to a chimeric HRG-based ligand toxin.

Results
Production of Recombinant HRG. cDNA fragments encoding the EGF-like domains of each of the four isoforms of HRG were amplified by reverse transcription-PCR and subsequently expressed in Escherichia coli using the pFLAG expression system (31). A ligand toxin construct encoding a fusion protein where the M, 23,000 cell binding domain of PE (32) was replaced with HRGβ2 to produce HRGβ2/PE40 was also assembled and expressed using this system. Recombinant protein was detected in the cell fractions isolated for each HRG isoform (α, β1, β2, β3) and HRGβ2/PE40 at the predicted relative molecular weights of approximately M, 7,000 and M, 52,000 (the PE40 portion of the molecule actually resolves at M, 45,000 upon SDS-PAGE), respectively (Fig. 1). Using an M1 anti-FLAG antibody affinity column, recombinant protein expressed in the periplasmic space was purified to a single band by SDS-PAGE analysis (Fig. 1C; only HRGβ2 and HRGβ2/PE40 shown). Yields of 0.1–1 mg/liter of culture were obtained for soluble protein purified from the periplasmic space. Upon affinity purification, the highest yield was consistently obtained with HRGβ2, and this isoform was used for the subsequent signaling and proliferation studies, as well as the production of a ligand toxin.

HRG Activation of erbB Receptors in Human Breast Cancer Cells. The ability of the HRG isoforms and the ligand toxin HRGβ2/PE40 to activate erbB2–4 was measured by the increase in tyrosine phosphorylation of a M, 180,000–185,000 band (p180–185) detected in MCF-7 cell lysates using an antiphosphotyrosine monoclonal antibody (Fig. 2). The mobility of this band corresponds to that of erbB2–4; see for example Fig. 3. MCF-7 cells were used because they exhibit a low basal level of p180–185 tyrosine phosphorylation. The induction of p180–185 tyrosine phosphorylation exhibited a similar dose response over the concentration range 5 pm to 10 nm for each HRG isoform tested, with the addition of 10 nm HRG resulting in an approximately 10-fold increase over basal levels (Fig. 2A). Further assays demonstrated that the recombinant HRGs also induced tyrosine phosphorylation of p180–185 in SK-BR-3 and MDA-MB-453 human breast cancer cells at concentrations of 20 pm or greater (data not shown). EC50 of 100–250 pm were observed in each cell type tested. These results compare favorably with the EC50 found in a previous study (EC50 = 40 pm) for MCF-7 cells (24). The ability of HRGβ2/PE40 to stimulate tyrosine phosphorylation of p180–185 was indistinguishable from that of HRGβ2 in MCF-7 cells (Fig. 2B). This demonstrates that fusion of PE to the HRGs does not affect the activity of these ligands as judged by their ability to stimulate p180–185 tyrosine phosphorylation.

To ascertain which erbB receptors were tyrosine phosphorylated in response to HRG addition, ZR-75-1 human breast cancer cells were used, since these cells express readily detectable levels of erbB2–4. Western blotting of erbB2 and erbB3 immunoprecipitates from ZR-75-1 cells with an antiphosphotyrosine monoclonal antibody revealed a marked increase in tyrosine phosphorylation of these receptors upon HRGβ2 (10 nm) administration (Fig. 3). Since the anti-erbB4 antibody used in these studies is only suitable for Western blotting, the reciprocal experiment was performed in which antiphosphotyrosine immunoprecipitates from control and HRG-stimulated cells were blotted with the anti-erbB4 antibody. HRG addition clearly resulted in increased recruitment of erbB4 into the antiphosphotyrosine immunoprecipitate (Fig. 3). With prolonged exposure of ECL autoradiographs, it was evident that there was a low basal level of tyrosine phosphorylation on each of erbB2–4. Therefore, these results demonstrate that erbB2–4 become tyrosine phosphorylated in ZR-75-1 human breast cancer cells upon HRG treatment. Immunoprecipitation of erbB2 and erbB3 and blotting with the same antibody served as controls (Fig. 3; only data for erbB2 shown). The same experiments were performed using cell lysates of MCF-7 breast cancer cells. Similar results were obtained except that insufficient levels of erbB4 precluded detection of activation by HRGβ2 (data not shown).

Activation of the Ras Signaling Pathway in Response to HRG-induced erbB Receptor Activation. Activation of the Ras signaling pathway by the EGFR involves recruitment of a complex between the Grb2 adapter protein and the Sos
GDP-GTP exchange factor to the tyrosine-phosphorylated receptor (33). An additional binding site for this complex is present on the tyrosine-phosphorylated Shc proteins, which can also be recruited by activated growth factor receptors (34–36). Therefore, we investigated the involvement of Grb2 and Shc in HRG-induced mitogenic signaling in ZR-75–1 cells.

Western blotting of Shc immunoprecipitates from control and HRG-stimulated cells with an antiphosphotyrosine antibody revealed increased tyrosine phosphorylation of the p52 Shc protein upon HRG addition (Fig. 4A). Also present in the Shc immunoprecipitates was a tyrosine-phosphorylated band of M, 180,000–185,000. To identify the erbB receptors contributing to this band, the immunoprecipitates were Western blotted with specific anti-erbB receptor antibodies (Fig. 4A). These experiments demonstrated that the Shc proteins form complexes with erbB2–4 following HRG-induced receptor activation. Furthermore, Western blotting of the Shc immunoprecipitates with an anti-Grb2 monoclonal antibody revealed that tyrosine phosphorylation of the Shc proteins resulted in increased Shc-Grb2 complex formation (Fig. 4A). Also noteworthy was the detectable basal level of Shc association with erbB2 and erbB4 in the absence of HRG stimulation, presumably due to the basal level of tyrosine phosphorylation of these receptors (Fig. 4A).

To investigate the recruitment of Grb2 by erbB receptors following HRG administration, anti-Grb2 immunoprecipitates were blotted with specific anti-erbB receptor antibodies (Fig. 4B). Increased levels of both erbB2 and erbB4, but not erbB3, were detected in Grb2 immunoprecipitates following HRG addition. To confirm this finding, lysates from control and HRG-stimulated cells were incubated with a GST-Grb2 fusion protein coupled to agarose beads (11). Following washing, bound proteins were Western blotted with either anti-erbB3 or anti-erbB4 antibodies. This experiment demonstrated that HRG-activated erbB4, but not erbB3, bound to the Grb2 fusion protein (data not shown). Furthermore, blotting of erbB3 receptor immunoprecipitates with a Grb2 monoclonal antibody also did not detect any association of Grb2 with erbB3 (Fig. 4B). Immunoprecipitation and blotting of Shc, Grb2, and erbB3 served as controls (Fig. 4, A and B; only data for Shc and Grb2 shown).

To confirm activation of the Ras pathway in response to HRG administration, the activity of MAP kinase, which functions downstream of Ras, was investigated. MAP kinase p44 (ERK1) was immunoprecipitated from ZR-75–1 cells before and after HRGβ2 addition and then examined for its ability to phosphorylate an exogenous substrate, MBP. Densitometric analysis indicated that a 4–5-fold increase in MAP kinase activity was detected 15 min after HRGβ2 addition (Fig. 4C).

Thus, HRG addition to ZR-75–1 human breast cancer cells results in the association of erbB2–4 with Shc proteins, the tyrosine phosphorylation of p52 Shc, and the formation of interaction complexes.
of Shc-Grb2 complexes. Grb2 recruitment by erbB2 and erbB4 was also evident, which could be direct or mediated through the Shc proteins; clearly, these mechanisms are not mutually exclusive. However, erbB3 differs from erbB2 and erbB4 in associating with Shc proteins, but not Grb2. Thus, erbB2-4 use different mechanisms to couple to the Ras signaling pathway.

**HRG-stimulated Association of PI3-kinase with erbB3.**

Other researchers have reported the association of PI3-kinase with activated erbB3 receptors (17, 19, 20, 37, 38). PI3-kinase is a cytosolic enzyme that is recruited to specific protein tyrosine kinases due to binding of the p85 subunit to tyrosine-phosphorylated YXXM motifs. Six such motifs are present in the cytoplasmic domain of erbB3, whereas this motif is not evident in erbB2 or erbB4 (39). Hence, we looked at whether PI3-kinase is associated with erbB3 in HRG-treated human breast cancer cells. In ZR-75-1 cells, HRGβ2 stimulated the tyrosine phosphorylation of erbB3 and increased dramatically the PI3-kinase activity associated with this receptor (Fig. 5). HRG addition also increased the co-immunoprecipitation of the p85 subunit of PI3-kinase with erbB3 (data not shown).

**Effects of HRGβ2 on Human Breast Cancer Cell Proliferation.** MCF-7 and T-47D human breast cancer cells, SK-OV-3 human ovarian cancer cells, and 184B5 immortalized normal breast epithelial cells were used for proliferation assays. 184B5 cells express low or undetectable levels of erbB2 and erbB3, whereas MCF-7 and T-47D cells express similar low levels of erbB2 but overexpress erbB3. The latter two cell lines also express detectable levels of erbB4, as observed by PCR analysis (7) or cell-surface staining using an erbB4 antibody different to that used in this report (40). SK-OV-3 cells express both erbB2 and erbB3 receptors, but the HRGs fail to induce the tyrosine phosphorylation of erbB2 in these cells (40, 41).

The addition of HRGβ2 (0.5 pm to 5 nm) to T-47D or MCF-7 cells maintained in 5% FCS resulted in increased cell numbers relative to untreated controls with one-half maximum responses (EC50) at approximately 30 pm and 300 pm, respectively (Fig. 6A). At 10 pm, cell numbers increased only marginally at 3, 5, and 7 days after treatment, whereas a maximum response was generally achieved at a dose of 1 nm. The effect of an optimal concentration (1 nm) of HRGβ2 on the proliferation rate of MCF-7 and T-47D cells is shown in Fig. 7. In this experiment, HRG addition to MCF-7 cells resulted in a doubling of cell numbers relative to controls after 5 days, whereas for T-47D cells, cell numbers increased by 30–40% over controls after 3–5 days (Fig. 7). Proliferation rates in HRG-treated cultures generally slowed after day 5 as the cells reached confluence. In contrast, both the SK-OV-3 and 184B5 cells showed no proliferative

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Effects of HRGβ2 on Cell Cycle Progression in T-47D Breast Cancer Cells. Since the HRG-induced increase in cell proliferation rate occurred even in the presence of 5% FCS, we investigated the mitogenic properties of HRGβ2 under serum-free conditions. T-47D cells arrested in G₁ were treated with HRGβ2 (5 nM), and entry into S phase was monitored by DNA flow cytometry. After an approximate 12-h delay, a semi-synchronous entry of cells into S phase was observed (Fig. 6B), a result similar to that seen previously with other growth factor mitogens (42). In addition, the relative potencies of HRGβ2, FCS, and insulin were compared for their ability to stimulate cell cycle progression in this model. As evident from Fig. 6C, HRGβ2 was a more potent mitogen than either insulin or FCS.

Cytotoxicity of a HRGβ2/PE40 Ligand Toxin. Identical studies to the proliferation assays described above were performed using the ligand toxin, HRGβ2/PE40, or PE40 alone. In addition, SK-BR-3 and MDA-MB-453 human breast cancer cells were used. These cell lines overexpress (relative to normal breast epithelial cells) erbB2 and erbB3, whereas MDA-MB-453 cells also overexpress erbB4 (7, 40). Each of the breast cancer cell lines treated with HRGβ2/PE40 showed a dramatic decrease in cell number, both relative to nontreated control cultures and the initial plating density (Fig. 7: only MCF-7 and T-47D data shown). Ligand toxin concentrations of greater than 2 pm generally resulted in cytotoxicity with maximum cell death seen at the highest concentration used (5 nM). After 7 days of exposure to the ligand toxin, less than 10% (relative to control cultures) of T-47D, SK-BR-3, and MDA-MB-453 cells were viable (18% for MCF-7). MDA-MB-453 breast cancer cells were the most sensitive to HRGβ2/PE40, with an IC₅₀ of 7–20 pm, whereas T-47D, SK-BR-3, and MCF-7 cells were less sensitive (Table 1). When 184B5 or SK-OV-3 cells were exposed to the HRGβ2/PE40 or PE40, no significant effect on cell number was seen at any dose. At only the highest concentration of PE40 used (5 nM) was proliferation of

Fig. 4. Stimulation of the Ras signaling pathway by HRGβ2 in ZR-75-1 human breast cancer cells. A and B, HRG-stimulated tyrosine phosphorylation of Shc proteins and association of Shc and Grb2 (respectively) with activated erbB receptors. Monolayer cultures of cells were treated either with vehicle alone (–) or with HRGβ2 (+) as described in Fig. 3. Immunoprecipitation (i.p.) experiments were performed as indicated and analyzed by Western blot (blot) using anti-Shc, antiphosphotyrosine (PY20), anti-Grb2, or anti-erbB2-4 antibodies as indicated above the figure. Other bands present in the erbB3 and erbB4 blots of Grb2 immunoprecipitations are due to cross-reactivity of the secondary blotting antibody with the immunoprecipitating antibody. C: activation of MAP kinase in response to HRGβ2 administration. MAP kinase p44 (ERK1) was immunoprecipitated from ZR-75-1 cell lysates before (0) and 15 min after (+ HRG) HRGβ2 addition (final concentration, 10 nM) and then assayed for its ability to phosphorylate an exogenous substrate, MBP. Following SDS-PAGE, radioactive bands representing phosphorylated MBP were revealed by autoradiography, and band intensities were quantified by densitometry. MAP kinase activity is expressed relative to controls following normalization for protein levels (determined by Western blotting MAP kinase immunoprecipitates for ERK1). Bars, SD.

Fig. 5. HRGβ2 stimulation of erbB3-associated PI3-kinase activity in ZR-75-1 breast cancer cells. PI3-kinase activity was assayed in erbB3 immunoprecipitates from HRGβ2-treated (+) or control (–) ZR-75-1 cells, as described in “Materials and Methods.” Left panel, autoradiograph of the reaction products following separation by TLC. Right panel, the result of Western blotting the immunoprecipitates with either anti-erbB3 or antiphosphotyrosine (PY20) antibodies.

responses (Fig. 6A); at all concentrations tested (0.5 pm to 5 nM) and at three different plating densities (200 to 1000 cells/well), there was no difference in relative cell numbers between HRGβ2-treated or untreated cells over the 7 days of the experiment.
Fig. 6. Regulation of cell proliferation and cell cycle progression by HRGβ2. A. Regulation of cell proliferation by the HRGs. MCF-7 (□) and T-47D (■) human breast cancer cells, SK-OV-3 ovarian cancer cells (○), and 184B5 immortalized breast epithelial cells (▲) were plated into 96-well plates. HRGβ2 (0.5 pm-5 nM) was added at day 0, and cell numbers were evaluated using an indirect 3-(4,5dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay at day 3. Shown are cell numbers expressed as a percentage of control untreated cells (mean of three experiments; bars, SD). B. Regulation of cell cycle progression by HRGβ2. T-47D breast cancer cells were arrested in G1 phase by serum starvation and then exposed to HRGβ2 (5 nM). Duplicate flasks of cells were then harvested at 12, 18, 24, 36, and 60 h, and DNA content was evaluated by flow cytometry. The bar graph shows the percentage of cells in S phase versus time for cells exposed to HRGβ2 (●) or vehicle alone (▲). C. T-47D cells were growth arrested as above and then treated with either HRGβ2 (5 nM), insulin (10 μg/ml), or FCS (1%). The graph shows the percentage of cells in S phase 24 h after exposure to the different treatments.

MCF-7 and T-47D cells slightly retarded (Fig. 7); however, IC₅₀ for PE40 were 20-1000 fold higher than those for HRGβ2/PE40 in the breast cancer cell lines studied (Table 1).

Discussion

We describe here the production of recombinant HRG isoforms comprising the EGF-like domain that can be purified from transformed E. coli extracts in a single-step affinity procedure to an essentially homogenous preparation. The four HRG isoforms exhibited similar activities when assayed for their ability to stimulate p180-185 tyrosine phosphorylation in human breast cancer cells, displaying EC₅₀ of 100-250 pm. These values are comparable to those determined for recombinant HRGα₁₋₋₋₋ and HRGβ₁₋₋₋₋ using a similar assay in MCF-7 cells; both proteins showed similar activities, exhibiting EC₅₀ of approximately 40 pm (24). Interestingly, it was observed that the β isoforms of the rat homologue of HRG, neu differentiates factor, demonstrated a 10-fold higher affinity for endogenous binding sites on T-47D cells than the α isoforms (43), and soluble extracellular domains of erbB3 and erbB4 bound the β isoforms of HRG with a higher affinity than HRGα (28). Moreover, HRGβ isoforms were more potent than HRGα in stimulating [³H]thymidine incorporation and proliferation of T-47D breast cancer cells (44) and mouse keratinocytes (45). The reason for this apparent discrepancy is unclear. Recently, several reports have shown that HRGs bind to erbB3 and erbB4, with the observed tyrosine phosphorylation of erbB2 occurring as a consequence of heterodimerization (25-28). In accordance with these findings, HRGβ2 stimulated the tyrosine phosphorylation of erbB2-4 in ZR-75-1 human breast cancer cells (Fig. 3). Similar results were found in MCF-7 cells, except that the low expression of erbB4 precluded the detection of tyrosine phosphorylation of this receptor.

Ligand stimulation and subsequent activation of RTKs trigger several intracellular signaling pathways that have recently been delineated at the molecular level. Two pathways important in growth factor-stimulated mitogenesis are those leading to the activation of Ras (46) and P13-kinase (47). SH2 domain-containing proteins, which play pivotal roles in the regulation of Ras by RTKs, are the adapter proteins Grb2 (11) and Shc (34). The SH2 domain of Grb2 binds activated RTKs containing the appropriate binding site (a strong preference is exhibited for autophosphorylation sites with asparagine at the +2 position relative to the tyrosine), whereas the SH3 domains recruit the Sor Ras GDP-GTP exchange factor. The juxtaposition of Sos next to membrane-anchored Ras is thought to lead to Ras activation (33). Both the EGFR and erbB2 form complexes with Grb2 upon activation (11, 12, 48). The Shc proteins are tyrosine phosphorylated in response to activation of many RTKs, and this phosphorylation occurs on a consensus Grb2 binding site; complex formation with Grb2 subsequently ensues (36). Although the exact function of Shc remains obscure, recent studies have highlighted the importance of the Shc-Grb2-Sos complex in Ras activation (49-51). erbB family RTKs possess two types of binding sites for Shc; NPXY motifs which, when tyrosine phosphorylated as a recently identified NH₂-terminal domain of Shc, the PTB, PI, or SAIN domain (Refs. 52-54) and binding sites for the SH2 domain (48, 55).

Since they express readily detectable levels of erbB2-4, ZR-75-1 human breast cancer cells provide a particularly interesting model in which to study the participation of these receptors in activation of the Ras pathway; in particular, the association of key SH2 domain-containing signaling molecules with erbB4 has not been reported previously. HRGβ2 administration resulted in the tyrosine phosphorylation of Shc and the association of this protein with erbB2-4 and Grb2. However, when Grb2 complex formation with erbB receptors was examined after HRGβ2 addition, co-immunoprecipitation with erbB2 and erbB4, but not erbB3, could be demonstrated. Our results, using native receptors, confirms that reported by Prigent and Gullick (37) using a recombinant EGFR-erbB3 chimera. This suggests that the consensus Grb2 binding sites on erbB3 (36) are either not phosphorylated, not active in binding Grb2, or inaccessible, and also, that Grb2 cannot be recruited to erbB3 via association with Shc. Our demonstration of co-immunoprecipitation of Grb2 and Shc with erbB4 is in
agreement with the presence of predicted binding sites for these molecules on this receptor (39, 54).

PI3-kinase consists of an Mr 85,000 adaptor subunit (p85) and a Mr 110,000 catalytic subunit; recruitment by activated RTKs is mediated by binding of the p85 SH2 domains to tyrosine phosphorylated YXXM motifs. Interestingly, erbB3, but not the other erbB proteins, possesses six COOH-terminal copies of this motif (39). Several groups have reported association of PI3-kinase activity with erbB3 using either EGFR-erbB3 chimeras (17, 37) or erbB3 receptors stimulated with HRGs (38) or transphosphorylated either by the EGFR (19, 20) or erbB2 (57). Our data demonstrate that HRGβ2 addition to ZR-75-1 cells leads to association of PI3-kinase activity with tyrosine-phosphorylated erbB3 (Fig. 5); this was accompanied by recruitment of the p85 subunit of the enzyme (data not shown). In addition, there was no detectable increase in PI3-kinase activity in erbB2 immunoprecipitates following HRG administration (data not shown).

Thus, the activation of erbB2–4 by HRG addition not only leads to the triggering of multiple potential inputs into the Ras pathway (i.e., the formation of receptor-Grb2, receptor-Shc, and Shc-Grb2 complexes) but also recruitment of PI3-kinase to activated erbB3. We were, therefore, interested in examining the mitogenic effects of the HRGs in human breast cancer cell lines. We chose for this study two cell lines, T-47D and MCF-7, which have been well characterized for their proliferative responses to growth factors in our laboratory (42). These cell lines express similar levels of erbB2 to normal breast epithelial cells but over-express erbB3. Although MCF-7 and T-47D cells were reported to exhibit moderate and high levels of erbB4 mRNA expression, respectively (7), only low levels of this receptor were detected in these cell lines by Western and Northern blotting.4

In the presence of 5% FCS, MCF-7 and T-47D cell numbers increased in a dose-dependent manner, with maximum stimulation 3–5 days after HRGβ2 addition. These results are in agreement with Marte et al. (44), who also reported a mitogenic effect of the HRGs on T-47D cells in the presence of serum. To further define HRG-regulated cell proliferation, we studied the effects of HRGβ2 on T-47D cell cycle progression in a well-defined, serum-free model (42). In common with other breast cancer cell mitogens (for example, insulin, insulin-like growth factors, transforming growth factor α, EGF, and fibroblast growth factor), HRGβ2 stimulated cell cycle progression in growth-arrested cells, with cells entering S phase between 12 and 18 h after addition. These temporal changes were similar to those observed with the other growth factors but, more importantly, HRGβ2 represents the most potent mitogen tested in this system. The data in Fig. 6C demonstrate that the proportion of cells in S phase at 24 h was greater in HRG-treated cells than in cells treated with either insulin, the previously most potent individual mitogen identified, or 5% FCS. This effect on cell cycle progression is consistent with the demonstration (Figs. 6 and 7) that HRGβ2 could further increase the proliferation rate of cells growing in 5% FCS. Our data demonstrating that HRGβ2 is a potent mitogen for MCF-7 and T-47D breast cancer cells is in marked contrast to the reported effects of this polypeptide on breast cancer cells that overexpress erbB2. In such cells (AU-565, SK-BR-3, and MDA-MB-453), HRGs apparently cause growth inhibition and induce cellular differentiation (21, 29, 30), although these data remain controversial, and in another study, only proliferative effects were observed in

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4 E. A. Musgrove, unpublished data.
SK-BR-3 cells (24). A potential explanation for these effects is that since breast cancer cells overexpressing erbB2 exhibit high levels of erbB2 tyrosine phosphorylation and MAP kinase activity (12), HRG activation of erbB3 and/or erbB4 may induce the further activation of MAP kinase or related enzymes above a threshold required for nuclear localization and triggering of a differentiation signal (58). Certainly, activation of MAP kinase and p70/p85 S6 kinase occurs in cells stimulated to either proliferate (T-47D cells) or differentiate (AU565 cells) by HRG (44). Consequently, since the HRGs are expressed by a subset of human breast cancers and breast cancer-derived cell lines (59, 60), these polypeptides are likely to function in an autocrine and/or paracrine mode to regulate breast cancer cell proliferation; however, the nature of the cellular response may depend on the relative expression levels of erbB2–4.

Finally, we have shown in vitro that HRGβ2, when coupled to PE40 toxin, exhibits a cytotoxic effect against human breast cancer cells that overexpress, relative to normal breast epithelial cells, erbB3 alone (T-47D and MCF-7) or in combination with erbB2 (SK-BR-3) or erbB2 and erbB4 (MDA-MB-453). These findings are in general agreement with recent publications describing similar HRG-based ligand toxins and their activity against cancer cell lines, as measured by inhibition of protein synthesis (61) or cell killing (40, 62). Significantly, we found that immortalized breast epithelial cells (184B5) were insensitive to the ligand toxin. Therefore, the HRGs represent a potent tool in the development of therapeutic strategies specifically targeting cancers that overexpress erbB3 and/or erbB4.

Materials and Methods

Antibodies. Antibodies were as follows: monoclonal anti-erbB2, Novoceastra; polyclonal anti-erbB3, a gift from Dr. M. H. Kraus (NIH, Bethesda, MD), which was used for immunoprecipitations; monoclonal anti-erbB3 2F12 (19), a gift from Dr. J. G. Koland (University of Iowa, Iowa City, IA), which was used for Western blots; polyclonal anti-erbB4, Santa Cruz Biotechnology; monoclonal antiphosphotyrosine PY20, Transduction Laboratories; polyclonal anti-Grb2 Ab50 (for immunoprecipitation) (11) a gift from Dr. J. Schlessinger (New York University Medical Center); monoclonal anti-Grb2 (for Western blottings), Transduction Laboratories; polyclonal anti-Shc, UBI; and polyclonal anti-ERK antibodies K23 (for Western blottings) and 956/837 (for immunoprecipitation), Santa Cruz Biotechnology.

Cell Lines. The sources and maintenance of the human breast cancer cell lines used in this study were as described previously (63). SK-OV-3 human ovarian cancer cells were obtained from American Type Culture Collection and maintained in the same manner. 184B5 immortalized human breast epithelial cells were the kind gift of Dr Martha Stamper (University of California, Berkeley, CA) and were maintained in mammary epithelial growth medium (Clonetics).

Amplification of cDNAs Encoding HRG Isoforms. The guanidinium isothiocyanate-cesium chloride procedure was used to isolate total RNA from the human breast cancer cell line MDA-MB-231, which expresses high levels of the HRGs. Following first-strand DNA synthesis, HRG cDNA fragments were amplified by PCR using a forward primer common to each HRG isoform (5'-GATCAAGCTTGCAGCTGTAAAATGTGCGG-3') containing a four-nucleotide leader sequence and a HindIII site (underlined) to facilitate subcloning, and reverse primers common to isoforms α, β1, and β2 (5'-GATCAAGCTTCTIACGGTA- CAGCTCTCGCCG-3') or β3 (5'-GATCAAGCTTATCAGCCAGACAAGAAGCGG-3') containing a leader sequence, a HindIII site (underlined), and an in-frame termination codon (double underlined). To clone cDNA suitable for the production of the HRGβ2/PE40 ligand toxin, the following reverse primer was used in which the stop codon was deleted: 5'-AGATCAACGCTCTCGCGTAAGCTTCTCGCGTGCGG-3'. These cDNA fragments encode the EGF-like domain of the HRGs, which has been shown previously to be the biologically active domain (24), consisting of 64, 69, 61 and 65 amino acids for isoforms α (amino acids 177–240), β1 (177–245), β2 (177–237), and β3 (177–241), respectively.

Recombinant HRG and HRG-Toxin Expression. PCR products encoding the HRGs were digested with HindIII and subcloned into the pFLAG-1 expression vector (International Biotechnologies). After transformation into E. coli DH5α, the HRG-coding domains of insert-containing constructs were sequenced (Sequenase version 2.0; USB), and colonies containing cDNA clones coding for each HRG isoform were stored as glycerol stocks. For expression of the ligand toxin, a 1184-bp HindIII/EcoRI cDNA fragment encoding the Pseudomonas exotoxin PE40 was subcloned into pFLAG. This initial construct, when expressed in E. coli, produces recombinant toxin that was used as a ligand toxin control. The HindIII cDNA fragment encoding HRGβ2 was then inserted into this toxin construct, which will result in the expression of the HRG-toxin fusion protein (HRGβ2/PE40). The pFLAG-1 prokaryotic expression vector is designed to produce a soluble form of recombinant protein, expressed in the periplasmic space of transformed bacteria, incorporating an 8-amino acid "FLAG" peptide and a 21-amino acid leader sequence (ompA), which allows translocation of the fusion protein to the periplasmic space. The ompA sequence is cleaved during this process. The recombinant FLAG fusion protein can then be purified by affinity chromatography using the M1 anti-FLAG monoclonal antibody.

Cultures of transformed E. coli were grown at 37°C with shaking until they reached an absorbance (OD600) of 0.8. Isopropyl-1-thio-β-D-galactopyranoside was then added to 500 μM and cultures incubated for a further 2 h. In an initial experiment to determine the level of expression in each E. coli cell fraction, isopropyl-1-thio-β-D-galactopyranoside-induced cultures (100 ml) were divided into three, and cells were initially collected by centrifugation. The whole-cell fraction was isolated by resuspension of one cell pellet in SDS-PAGE sample buffer. The second pellet was used for fractionation of the whole-cell pellet into soluble or insoluble fractions by the addition of 5 ml of extraction buffer A [50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 0.25 mg/ml lysozyme, 50 μg/ml NaNO3] followed by 0.5 ml of extraction buffer B [1.5 mM NaCl, 0.1 mM CaCl2, 0.1 mM MgCl2, 0.02 μg/ml DNase I, 0.2 mM NaVO4, 0.2 mM phenylmethylsulfonyl fluoride, 0.2 mM leupeptin, 0.2 mM aprotinin]. The resulting suspension was centrifuged at 18,000 x g for 1 h, giving the soluble and insoluble cell fractions. The insoluble cell fraction was resuspended in SDS-PAGE sample buffer while an aliquot of the soluble fraction was mixed with an equal volume of 2X sample buffer. Finally, the third cell pellet was prepared for osmotic shock by resuspension in 8 ml of OS buffer [0.5 mM sucrose, 0.03 mM Tris-HCl (pH 8.0), and 1 mM EDTA] and centrifugation at 3500 x g for 10 min at
10°C. Cells were resuspended rapidly in 5 ml of ice-cold water to release periplasmic proteins. Following centrifugation at 3500 x g for 10 min at 4°C, the supernatant was collected, and a 10-μl aliquot was mixed with an equal volume of SDS-PAGE sample buffer. After electrophoresis and transfer of proteins to nitrocellulose membranes (Bio-Rad), FLAG proteins were detected with the anti-FLAG M2 monoclonal antibody (10 μg/ml) and enhanced chemiluminescence (ECL; Amersham). The M2 antibody detects FLAG proteins consisting of both ompA-cleaved and non-cleaved forms.

**Purification of Recombinant Proteins.** For each HRG isoform and the ligand toxin, protein expressed in the periplasmic space was isolated and purified by affinity chromatography using the anti-FLAG M1 monoclonal antibody that binds only ompA-cleaved FLAG fusion proteins. This antibody binds to the FLAG peptide only in the presence of calcium; hence, the periplasmic cell fractions were made up to 2 mM CaCl2 in TBS (150 mM NaCl and 50 mM Tris-HCl, pH 7.5) and passed through the column. Bound proteins were eluted in TBS containing 2 mM EDTA and then analyzed for size and purity by Western blot using the M2 anti-FLAG antibody and by Coomassie staining of SDS-PAGE gels.

**HRG Stimulation of Tyrosine Phosphorylation of erbB Receptors Expressed in Breast Cancer Cells.** MCF-7 human breast cancer cells were grown to near confluence in 6-well tissue culture plates. The cells were then starved for 18 h in medium containing 0.5% FCS. Recombinant HRG (one of the four isoforms) was then added in 100 μl of TBS/0.05% BSA (final concentration, 20 μM to 10 nM) to individual wells. Control wells received vehicle alone. Following a 20-min incubation at 37°C, the medium was removed by aspiration, and cells were collected in 200 μl of lysis buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.2 mM Na3VO4, 0.2 mM phenylmethylsulfonyl fluoride, 0.2 mM leupeptin, and 0.2 mM aprotonin). Following incubation on ice for 5 min, cellular debris was removed by centrifugation.

To determine the tyrosine phosphorylation levels of erbB-2–4 following HRG administration, samples of the resulting cell lysates were separated by SDS-PAGE, transferred to nitrocellulose, and Western blotted using a monoclonal antibody against phosphorylated tyrosine residues (PY20). Detection of bound antibody was by ECL (Amersham).

For immunoprecipitation experiments, ZR-75–1 or MCF-7 breast cancer cells were grown to near confluence in T150 tissue culture flasks. After serum starvation, HRG2 (10 nM) was added for 15 min. The cells were then lysed in 1 ml of lysis buffer as described above. Antibodies (1–2 μg) were incubated with lysates (100 μl of lystate for all reactions, except for the detection of erbB4 in PY20 immunoprecipitates, where 250 μl was used) for at least 2 h at 4°C. The immunocomplexes were then collected by incubation with goat antimouse IgG-Sepharose or protein A-Sepharose beads (40 μl) for at least 1 h at 4°C. Sepharose beads were collected by centrifugation and washed three times in cold lysis buffer, resuspended in SDS-PAGE sample buffer, and subjected to SDS-PAGE. After transfer to nitrocellulose, the samples were Western blotted with the desired antibody.

**MAP Kinase Assay.** MAP kinase p44 (ERK1) was immunoprecipitated from ZR-75–1 cell lysates (250 μl) before and 15 min after HRG2 addition and then examined for its ability to phosphorylate an exogenous substrate, MBP (5 μg), in the presence of ATP (20 μM) and [γ-32P]ATP (4 μCi) for 10 min at 30°C. Radioactive bands representing phosphorylated MBP were revealed by autoradiography following SDS-PAGE of the reaction products and band intensities quantified by densitometry. To control for loading differences, aliquots of the ERK1 immunoprecipitates were Western blotted using a different antibody to ERK1 (K23; Santa Cruz Biotechnology).

**P13-kinase Activity Assay.** erbB3 protein was immunoprecipitated from 500 μl of cell lysates from HRG2-treated or control ZR-75–1 cells using anti-erbB3 monoclonal antibody (2F12). The immunoprecipitates were collected by centrifugation and divided into two aliquots to enable both determination of P13-kinase activity and analysis of the immunoprecipitates by Western blotting with anti-erbB3 and antiphosphotyroline antibodies.

P13-kinase activity was assayed using a method described previously (64). Immunoprecipitates were washed twice with 1% NP40 in PBS, twice in 0.5 M LiCl, 0.1% Trit (pH 7.6), and twice in TNE buffer (10 mM Tris (pH 7.6), 100 mM NaCl, and 1 mM EDTA). The reaction was started by the addition of 50 μl of kinase buffer containing 20 mM Tris (pH 7.6), 75 mM NaCl, 10 mM MgCl2, 200 μM adenosine, 20 μM ATP, 10 μCi [γ-32P]ATP, and PI resuspended by sonication in 20 μl HEPES and added to a final concentration of 0.2 mg/ml. After 15 min at room temperature, the reaction was stopped with 100 μl of 1 M HCl, and lipids were extracted with 200 μl of CHCl3:methanol (1:1). The organic phase was recovered, dried under nitrogen gas, and resuspended in 10 μl of CHCl3:methanol (2:1) containing 0.1% HCl. Phospholipid products were separated by TLC on a silica gel 60 plate (Merck), developed in CHCl3:methanol:4 mM NH4OH (9:7:2), and then exposed to autoradiography. Unlabeled phospholipid standards (PI and phosphatidyl inositol 4-phosphate) were included and visualized by exposure to iodine vapor.

**Proliferation Assays.** Breast cancer cells MCF-7, T-47D (in RPMI 1640 containing 5% FCS), ovarian cancer cells (in RPMI 1640 containing 5% FCS and 100 units/ml of insulin), and normal breast epithelial cells 184B5 (in mammary epithelial growth medium) were dispensed into individual wells of 96-well plates at an initial concentration of 103 cells/well in 50 μl of growth media, 3 days before (day – 3) the addition of recombinant HRG2, HRG2/PE4O, or PE40. At day 0, recombinant proteins were added in 50 μl of growth media to final concentrations ranging from 0.5 μM to 5 nM. Controls received either an equivalent volume of vehicle (0.5 μl of 0.1% BSA) in 50 μl of growth media or growth media alone. Three plates of identical treatments were prepared, and one plate was analyzed for cell growth on each of days 3, 5, and 7 after drug addition. Relative cell numbers (6 wells for each treatment) were measured indirectly using a nonradioactive cell proliferation assay (Promega) by absorbance readings (A955°A630°) using a Dynatech MR7000 Spectrophotometer.

**Flow Cytometry.** T-47D human breast cancer cells were maintained under serum-free conditions in T25 flasks, and the effects of 5% FCS, 10 μg/ml insulin, and 5 nM HRG2 on cell cycle progression were determined as described previously (42).

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