Activation of ErbB4 by the Bifunctional Epidermal Growth Factor Family Hormone Epiregulin Is Regulated by ErbB2*

(Received for publication, July 2, 1997, and in revised form, January 12, 1998)

David J. Riese, II†‡§, Toshi Komurasaki, Gregory D. Plowman**, and David F. Stern† ‡‡

From the †Department of Pathology, Yale University, New Haven, Connecticut 06520-8023, **Bristol-Myers-Squibb Pharmaceutical Research Institute, Seattle, Washington 98121 and Sugen, Inc., Redwood City, California 94063-4720, and Molecular Biology Laboratory, Taisho Pharmaceutical Research Center, Ohmiya-shi, Saitama 330, Japan, and §Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University, West Lafayette, Indiana 47907-1333

Epiregulin (EPR) is a recently described member of the epidermal growth factor (EGF) family of peptide growth factors. The ever expanding size of the EGF family has made distinguishing the activities of these hormones paramount. We show here that EPR activates two members of the ErbB family of receptor tyrosine kinases, epidermal growth factor receptor (EGFR) and ErbB4. Therefore by these criteria, EPR is qualitatively similar to another EGF family hormone, betacellulin (BTC). Yet, here we also demonstrate quantitative differences between EPR and BTC. EPR stimulates higher levels of EGFR phosphorylation than does BTC, whereas BTC stimulates higher levels of ErbB4 phosphorylation than does EPR. Moreover, the EPR and BTC dose response curves show that although EGFR is more sensitive to EPR than is ErbB4, ErbB4 is more sensitive to BTC than is EGFR. Finally, ErbB2, which is not activated by EPR when expressed on its own, increases the sensitivity of ErbB4 for activation by EPR. Therefore, these results establish that EPR exhibits novel activities and modes of regulation, which may have significant implications for EPR function in vivo.

The continuing discovery of novel members of the epidermal growth factor (EGF) family of peptide growth factors has led to an increased appreciation of the functional differences among these hormones, as well as a realization of the complex hormone-receptor interactions fostered by these peptides. EGF, transforming growth factor α (TGF-α), and amphiregulin all bind exclusively to the EGF receptor (EGFR). Yet, these hormones can also activate in trans (transmodulate) the other three ErbB family receptors (Neu/ErbB2/Her2, ErbB3/Her3, ErbB4/Her4) through ligand-induced receptor heterodimerization with the EGFR (1–8). Other EGF family hormones bind multiple receptors. Neuregulin (NRG) and neuregulin2 (NRG2) bind ErbB3 and ErbB4 and transmodulate EGFR and ErbB2 (9–16). Betacellulin (BTC) combines some of the properties of EGF and NRG by activating EGFR and ErbB4 (7).

EPR was initially purified from the conditioned medium of a tumorigenic clone of NIH3T3 fibroblasts. It competes with EGF for binding to A431 cells, which overexpress EGFR, suggesting that EPR is a ligand for EGFR (17). Since at least one of the EGF family hormones that activates EGFR also activates ErbB4, we wished to evaluate EPR function in a set of cell lines expressing all four ErbB family receptors, both singly and in every pairwise combination.

We demonstrate here that EPR activates not only EGFR, but ErbB4 as well. However, the dose-response curves for BTC and EPR in a cell line expressing both EGFR and ErbB4 are markedly different. Whereas ErbB4 is more responsive to BTC than is EGFR, ErbB4 is less responsive to EPR than is EGFR. Moreover, ErbB2 expression increases saturated ErbB4 phosphorylation in response to EPR and dramatically enhances the sensitivity of ErbB4 for activation by EPR as well. In this respect EPR resembles NRG, which displays a low affinity for ErbB3 that increases in cells where ErbB2 is co-expressed (12).

EXPERIMENTAL PROCEDURES

Cell Lines and Cell Culture—BaF3 is an immortal mouse lymphoblastoid cell line (31). BaF3-derived cell lines expressing combinations of ErbB family receptors have been described previously (14). The ranked order of receptor expression in the double recombinant BaF3 cell lines is as follows. For EGFR expression, BaF3/EGRF+ is higher than BaF3/EGFR+ErbB2, which is higher than BaF3/EGFR+ErbB3. For ErbB2 expression, BaF3/ErbB2+ErbB4 is equivalent to BaF3/EGFR+ErbB2, both of which are markedly higher than BaF3/ErbB2+ErbB3. The levels of ErbB3 expression are similar in the BaF3/EGRF+ErbB3, BaF3/ErbB2+ErbB3, and BaF3/ErbB3+ErbB4 cell lines. The levels of ErbB4 expression are similar in the BaF3/EGRF+ErbB4, BaF3/Neu+ErbB4, and BaF3/ErbB3+ErbB4 cell lines (7, 14).

CEM is an immortal human T-lymphoblastoid cell line that does not endogenously express EGFR, ErbB2, ErbB3, or ErbB4. CEM-derived cell lines expressing ErbB4 have been described previously (10). Cell culture conditions were as described (10, 14).

Growth Factors—Recombinant human EPR was produced in Bacillus brevis.† Recombinant NRGβ was the generous gift of Kerry Russell and Jeffrey Byr (Yale University). We are grateful to Jim Moyer, Brad Guarino, and Glenn Andrews (Pfizer Central Research, Groton, CT) for synthetic NRGβ (32). Recombinant BTC and NRGβ were purchased from R & D Systems (Minneapolis, MN), whereas recom-

† T. Nakazawa et al., in preparation.
EPR stimulation of receptor phosphorylation in Ba/F3 cells expressing a single ErbB family receptor. Ba/F3/EGFR, Ba/F3/ErbB2, and Ba/F3/ErbB4 cells (14) were stimulated with 1000 ng/ml EPR (E), 100 ng/ml BTC (B), 100 ng/ml recombinant NRGβ (N), or were mock stimulated with phosphate-buffered saline (M) as described previously (7, 14). ErbB family receptors were immunoprecipitated from lysed cells using specific antireceptor antibodies and separated by SDS-PAGE as described previously (7, 14). Tyrosine-phosphorylated ErbB family receptors were detected and visualized by immunoblotting using the 4G10 monoclonal antiphosphotyrosine antibody as described previously (7, 14).

Stimulation and Analysis of Receptor Phosphorylation—The conditions for stimulation of ErbB family receptor tyrosine phosphorylation have been described previously (7, 14). The analysis of ErbB family receptor tyrosine phosphorylation by immunoprecipitation and antiphosphotyrosine immunoblotting has been described previously (7, 14). Immunoprecipitating antireceptor antibodies were anti-EGFR mouse monoclonal antibody 528 (33), anti-ErbB2 mouse monoclonal antibody TA-1 (OP-39, Calbiochem), anti-ErbB3 rabbit polyclonal antiserum SC-285 (Santa Cruz Biotechnology), and anti-ErbB4 rabbit polyclonal antiserum SC-285 (Santa Cruz Biotechnology). Specificity of antireceptor antibodies has been verified by testing for cross-reactivity (data not shown).

Immunoblot autoradiographs were digitized using a Hewlett-Packard 3p flatbed scanner set for 600 dpi resolution and controlled by Hewlett-Packard Deskscan II for Macintosh software. Images were cropped using Adobe Photoshop, and the band intensity was quantified using NIH Image software. Net receptor activation was calculated by subtracting the amount of tyrosine phosphorylation observed in samples from mock-stimulated cells.

RESULTS

EPR Activates EGFR—We first sought to identify which ErbB family receptors are activated by EPR when the receptors are expressed individually. We previously developed a panel of cell lines based on the mouse Ba/F3 hematopoietic cell line that expresses the four ErbB family receptors, both singly and in every pairwise combination. Hence, we incubated the Ba/F3 cell lines ectopically expressing EGFR, ErbB2, or ErbB4 with EPR. EPR, like BTC, stimulated EGFR tyrosine phosphorylation, consistent with published results suggesting that EPR binds EGFR (17) (Fig. 1, EGFR panel; compare lanes E and B with M). However, EPR did not stimulate phosphorylation of ErbB2 or ErbB4 (Fig. 1, ErbB2 and ErbB4 panels; compare lanes E and M). In contrast, the positive control NRGβ stimulated ErbB2 tyrosine phosphorylation and BTC stimulated ErbB4 phosphorylation. The ErbB2 phosphorylation observed in Ba/F3/ErbB2 cells stimulated with NRG is the result of transmodulation of ErbB2 by the NRG receptor ErbB3, which is endogenously expressed at low levels in Ba/F3 cells (14). Neither EPR nor any of the other EGF family ligands tested to date stimulated tyrosine phosphorylation in Ba/F3 cells expressing only ErbB3 (data not shown) (7, 8, 14). However, since ErbB3 lacks tyrosine kinase activity (18), these experiments do not rule out EPR binding to ErbB3.

Since EPR activates EGFR, we next determined whether EPR activates the other three ErbB family receptors in trans via EGFR. A panel of Ba/F3 cell lines ectopically expressing EGFR together with one of the other three ErbB family receptors was stimulated with EPR. EPR activated the EGFR in all three cell lines (Fig. 2A; compare E a1 lanes with the M a1 lanes). Both EPR (E lanes) and the positive control TGF-α (T lanes) strongly activated ErbB2 in the cell line co-expressing EGFR+ErbB2 (Fig. 2A, EGFR+ErbB2 panel; α2 lanes). In contrast, neither EPR nor TGF-α activated ErbB3 or ErbB4 (Fig. 2A, EGFR+ErbB3 and EGFR+ErbB4 panels; α3 or α4 lanes). This is consistent with the conclusion that ErbB2 is a preferential target for transmodulation by the EPR compared with the other ErbB family receptors (7, 8, 19–21). However, higher concentrations of EPR than those used for these experiments did stimulate ErbB4 phosphorylation in the EGFR+ErbB4 cell line (see below; Fig. 6A).

Since ErbB3 lacks functional kinase activity, EGFR family hormones can activate ErbB3 only in the presence of another ErbB family receptor, particularly ErbB2, which permits the highest levels of ErbB3 phosphorylation by NRG (11, 12, 14).
Tyrosine-phosphorylated ErbB family receptors were detected and visualized by immunoblotting using the 4G10 monoclonal antiphosphotyrosine antibody as described previously (7, 14).

Therefore, BaF3 cells expressing both ErbB2 and ErbB3 or both ErbB3 and ErbB4 were stimulated with EPR to determine if ErbB3 is a receptor for EPR. In the ErbB2+ErbB3 cell line, the positive control NRGβ activated both receptors (Fig. 2B, ErbB2+ErbB3 panel, compare N lanes with M lanes), whereas in the ErbB3+ErbB4 cell line NRGβ stimulated a marked increase in ErbB3 phosphorylation and a modest increase in ErbB4 phosphorylation (Fig. 2B, ErbB3+ErbB4 panel, compare N lanes with M lanes). In contrast, EPR did not stimulate receptor phosphorylation in either of these cell lines, suggesting that ErbB3 is not a receptor for EPR (Fig. 2B; compare E lanes with M lanes).

**EPR Activates ErbB4 in CEM Cells**—BTC activates both ErbB4 and EGFR when expressed individually (7). We tested whether EPR behaves like BTC and also activates ErbB4 when expressed alone using derivatives of the CEM human T-lymphoblastoid cell line that ectopically expresses ErbB4 alone or both ErbB2 and ErbB4 (10). EPR activated ErbB4 in CEM cells expressing ErbB4 alone and both receptors in CEM cells expressing ErbB2 and ErbB4 together (Fig. 3; compare E lanes with M lanes). In experiments done in parallel using identical growth factor concentrations, EPR did not activate ErbB4 in BaF3 cells expressing ErbB4 alone (also see Fig. 1) but stimulated ErbB2 and ErbB4 phosphorylation in BaF3 cells expressing both ErbB2 and ErbB4 (data not shown). It is unclear why EPR failed to activate ErbB4 in the BaF3 cells expressing ErbB4 alone. Nonetheless, because EPR activates EGFR as well as ErbB4, EPR resembles BTC, which also activates these receptors (7).

**EPR Stimulates EGFR More Than ErbB4 and EGFR Is More Sensitive Than ErbB4 to EPR**—Since BTC and EPR can activate EGFR and ErbB4 when the receptors are expressed individually, we measured BTC and EPR stimulation of EGFR and ErbB4 phosphorylation in BaF3 cells that express both receptors together (Fig. 4). BTC stimulated saturated levels of EGFR phosphorylation at a concentration of 10 ng/ml, whereas BTC stimulated saturated levels of ErbB4 phosphorylation at a concentration of 25 ng/ml. Therefore, in subsequent experiments we have assumed that 100 ng/ml BTC stimulates saturated levels of EGFR and ErbB4 phosphorylation.

At a concentration of 4000 ng/ml, EPR stimulates slightly greater levels of EGFR phosphorylation than does BTC (Fig. 4). In contrast, 4000 ng/ml EPR stimulates much lower levels of ErbB4 phosphorylation than does BTC (Fig. 4). This suggests that EPR stimulates EGFR more than ErbB4 and that EGFR is more sensitive to EPR than ErbB4.

Since BTC and EPR can activate EGFR and ErbB4 when the receptors are expressed individually, we measured BTC and EPR stimulation of EGFR and ErbB4 phosphorylation in BaF3 cells expressing both receptors. BTC activated both receptors (Fig. 2B) as well as BTC or ErbB4 phosphorylation in BaF3 cells expressing both receptors together (Fig. 3; compare N lanes with M lanes). In contrast, EPR did not stimulate receptor phosphorylation in either of these cell lines, suggesting that ErbB3 is not a receptor for EPR (Fig. 2B; compare E lanes with M lanes).

**EPR Activates ErbB4 in CEM Cells**—BTC activates both ErbB4 and EGFR when expressed individually (7). We tested whether EPR behaves like BTC and also activates ErbB4 when expressed alone using derivatives of the CEM human T-lymphoblastoid cell line that ectopically expresses ErbB4 alone or both ErbB2 and ErbB4 (10). EPR activated ErbB4 in CEM cells expressing ErbB4 alone and both receptors in CEM cells expressing ErbB2 and ErbB4 together (Fig. 3; compare E lanes with M lanes). In experiments done in parallel using identical growth factor concentrations, EPR did not activate ErbB4 in BaF3 cells expressing ErbB4 alone (also see Fig. 1) but stimulated ErbB2 and ErbB4 phosphorylation in BaF3 cells expressing both ErbB2 and ErbB4 (data not shown). It is unclear why EPR failed to activate ErbB4 in the BaF3 cells expressing ErbB4 alone. Nonetheless, because EPR activates EGFR as well as ErbB4, EPR resembles BTC, which also activates these receptors (7).

**EPR Activates ErbB4 in CEM Cells**—BTC activates both ErbB4 and EGFR when expressed individually (7). We tested whether EPR behaves like BTC and also activates ErbB4 when expressed alone using derivatives of the CEM human T-lymphoblastoid cell line that ectopically expresses ErbB4 alone or both ErbB2 and ErbB4 (10). EPR activated ErbB4 in CEM cells expressing ErbB4 alone and both receptors in CEM cells expressing ErbB2 and ErbB4 together (Fig. 3; compare E lanes with M lanes). In experiments done in parallel using identical growth factor concentrations, EPR did not activate ErbB4 in BaF3 cells expressing ErbB4 alone (also see Fig. 1) but stimulated ErbB2 and ErbB4 phosphorylation in BaF3 cells expressing both ErbB2 and ErbB4 (data not shown). It is unclear why EPR failed to activate ErbB4 in the BaF3 cells expressing ErbB4 alone. Nonetheless, because EPR activates EGFR as well as ErbB4, EPR resembles BTC, which also activates these receptors (7).

**EPR Stimulates EGFR More Than ErbB4 and EGFR Is More Sensitive Than ErbB4 to EPR**—Since BTC and EPR can activate EGFR and ErbB4 when the receptors are expressed individually, we measured BTC and EPR stimulation of EGFR and ErbB4 phosphorylation in BaF3 cells that express both receptors together (Fig. 4). BTC stimulated saturated levels of EGFR phosphorylation at a concentration of 10 ng/ml, whereas BTC stimulated saturated levels of ErbB4 phosphorylation at a concentration of 25 ng/ml. Therefore, in subsequent experiments we have assumed that 100 ng/ml BTC stimulates saturated levels of EGFR and ErbB4 phosphorylation.

At a concentration of 4000 ng/ml, EPR stimulates slightly greater levels of EGFR phosphorylation than does BTC (Fig. 4). In contrast, 4000 ng/ml EPR stimulates much lower levels of ErbB4 phosphorylation than does BTC (Fig. 4). This suggests that EPR stimulates EGFR more than ErbB4 and that EGFR is more sensitive to EPR than ErbB4.

Since BTC and EPR can activate EGFR and ErbB4 when the receptors are expressed individually, we measured BTC and EPR stimulation of EGFR and ErbB4 phosphorylation in BaF3 cells expressing both receptors together (Fig. 4). BTC stimulated saturated levels of EGFR phosphorylation at a concentration of 10 ng/ml, whereas BTC stimulated saturated levels of ErbB4 phosphorylation at a concentration of 25 ng/ml. Therefore, in subsequent experiments we have assumed that 100 ng/ml BTC stimulates saturated levels of EGFR and ErbB4 phosphorylation.

At a concentration of 4000 ng/ml, EPR stimulates slightly greater levels of EGFR phosphorylation than does BTC (Fig. 4). In contrast, 4000 ng/ml EPR stimulates much lower levels of ErbB4 phosphorylation than does BTC (Fig. 4). This suggests that EPR stimulates EGFR more than ErbB4 and that EGFR is more sensitive to EPR than ErbB4.
ErbB4 homodimers.

The EPR and BTC Dose-Response Curves Are Different in Cells Expressing EGFR and ErbB4—EPR resembles BTC in its ability to activate either EGFR or ErbB4 when expressed individually (7). Yet, at saturation EPR stimulated almost 2-fold more EGFR phosphorylation than BTC, whereas BTC activated about 3-fold more ErbB4 phosphorylation than did EPR (Fig. 5, A and C). This suggested that BTC and EPR are functionally distinct. Hence, we compared EGFR and ErbB4 phosphorylation following stimulation with increasing concentrations of BTC or EPR in a BaF3 cell line that expresses both EGFR and ErbB4 (Fig. 6A).

We first compared the magnitude of receptor phosphorylation stimulated by EPR and BTC by plotting receptor phosphorylation relative to the maximal phosphorylation stimulated by BTC (Fig. 6B; Table I). In contrast, half-maximal EGFR activation occurred at an EPR concentration of approximately 320 ng/ml, whereas half-maximal ErbB4 activation occurred at an EPR concentration of approximately 790 ng/ml (Fig. 6C; Table I).

### Table I
Relative sensitivities of EGFR and ErbB4 to EPR and BTC

| Cell line & receptor | EPR yielding half-maximal receptor activation | BTC yielding half-maximal receptor activation |
|----------------------|---------------------------------------------|---------------------------------------------|
| BaF3/EGFR            | 380 ng/ml                                    | NT*                                         |
| CEM/ErbB4            | 1790 ng/ml                                   | NT                                          |
| BaF3/EGFR+ErbB4      | 320 ng/ml                                    | 35 ng/ml                                    |
| EGFR                 | 790 ng/ml                                    | 5 ng/ml                                     |
| ErbB4                | 400 ng/ml                                    | NT                                          |
| CEM/ErbB2+ErbB4      | 630 ng/ml                                    | NT                                          |
| ErbB2                |                                            |                                             |
| ErbB4                |                                            |                                             |

* NT, not tested.

ErbB4 homodimers.

The EPR and BTC Dose-Response Curves Are Different in Cells Expressing EGFR and ErbB4—EPR resembles BTC in its ability to activate either EGFR or ErbB4 when expressed individually (7). Yet, at saturation EPR stimulated almost 2-fold more EGFR phosphorylation than BTC, whereas BTC activated about 3-fold more ErbB4 phosphorylation than did EPR (Fig. 5, A and C). This suggested that BTC and EPR are functionally distinct. Hence, we compared EGFR and ErbB4 phosphorylation following stimulation with increasing concentrations of BTC or EPR in a BaF3 cell line that expresses both EGFR and ErbB4 (Fig. 6A).

We first compared the magnitude of receptor phosphorylation stimulated by EPR and BTC by plotting receptor phosphorylation relative to the maximal phosphorylation stimulated by BTC (Fig. 6B and C). In agreement with results presented above (Fig. 5, A and C), EPR stimulated higher saturated levels of EGFR phosphorylation than BTC, whereas BTC activated greater ErbB4 phosphorylation than did EPR (Fig. 6A and C). However, the magnitude of these differences was much less in the EGFR+ErbB4 cell line compared with the differences in phosphorylation that we observed between the cell lines expressing EGFR and ErbB4 individually (Fig. 5, A and C).

Next, we compared the sensitivities of EGFR and ErbB4 with BTC and EPR by identifying the growth factor concentrations required for half-maximal receptor phosphorylation. Half-maximal EGFR activation occurred at a BTC concentration of approximately 35 ng/ml, whereas half-maximal ErbB4 activation occurred at a BTC concentration of approximately 5 ng/ml (Fig. 6B; Table I). In contrast, half-maximal EGFR activation occurred at an EPR concentration of approximately 320 ng/ml, whereas half-maximal ErbB4 activation occurred at an EPR concentration of approximately 790 ng/ml (Fig. 6C; Table I).
FIG. 6. EPR and BTC dose response in BaF3 cells expressing both EGFR and ErbB4. A, BaF3/EGFR+ErbB4 cells were stimulated with increasing concentrations of betacellulin or epiregulin or were mock stimulated with phosphate-buffered saline (Mock) as described previously (7, 14). EGFR or ErbB4 was immunoprecipitated as indicated using specific antireceptor antibodies and separated by SDS-PAGE as described previously (7, 14). Tyrosine phosphorylated ErbB family receptors were detected and visualized by immunoblotting using the 4G10 monoclonal antiphosphotyrosine antibody as described previously (7, 14). B and C, antiphosphotyrosine immunoblot images were scanned on a Hewlett-Packard ScanJet 3P flatbed scanner set for 600 dpi optical resolution. Images were cropped using Adobe Photoshop and receptor tyrosine phosphorylation was quantified using NIH Image. Net receptor tyrosine phosphorylation was calculated by subtracting the receptor tyrosine phosphorylation exhibited by mock stimulated cells. Tyrosine phosphorylation stimulated by BTC (B) or EPR (C) was expressed relative to the maximal tyrosine phosphorylation stimulated by BTC, ER, EGFR; □, ErbB4.

This suggests that ErbB4 is 7-fold more sensitive to BTC than is EGFR, whereas EGFR is more than 2-fold more sensitive to EPR than is ErbB4.

Finally, these results illustrate that EGFR expression, like ErbB2 expression, shifts the EPR dose-response curve in cells expressing ErbB4. Half-maximal ErbB4 phosphorylation in a CEM cell line expressing ErbB4 alone occurs at an EPR concentration of 1790 ng/ml (Fig. 5D; Table I). In contrast, half-maximal ErbB4 phosphorylation in BaF3 cells expressing both EGFR and ErbB4 occurs at 790 ng/ml (Fig. 6C; Table I).

EPR Activates ErbB Family Receptor Coupling to IL3 Independence—Although EPR and BTC stimulate qualitatively identical patterns of receptor phosphorylation, these hormones are quantitatively distinct. One possible mechanism is that EPR and BTC stimulate EGFR and ErbB4 tyrosine phosphorylation at different sites. This would account for the higher levels of EGFR activation by EPR compared with BTC and the higher levels of ErbB4 activation by BTC compared with EPR. Moreover, this would also enable these hormones to couple to distinct receptor effectors and physiologic responses. Therefore, we compared EPR and BTC induction of receptor coupling with physiologic responses. BaF3 cells require interleukin-3 (IL3) for survival and for proliferation. Activation of either EGFR or ErbB2 permits survival of BaF3 cells in the absence of IL3 (7, 14). However, ErbB4 activation by either NRG or BTC is not coupled to IL3-independent survival (7, 14). In BaF3 cells expressing both ErbB2 and ErbB4 together, activation by either BTC or NRG induces IL3-independent survival, presumably through ErbB2 transmodulation by ErbB4 (7, 14).

EPR, like BTC (7), induces IL3-independent survival in BaF3 cells expressing EGFR, but not in vector control BaF3 cells or cells expressing ErbB2 (Fig. 7). (The IL3-independent response of BaF3 cells expressing ErbB2 to NRG is the result of ErbB2 transmodulation by endogenous ErbB3 in these cells.) EPR, BTC, and NRG all induced IL3 independence in cells co-expressing ErbB2 and ErbB4 (Fig. 7). This implies that BTC and NRG are functionally equivalent. However, the response to BTC and NRG is greater than the response to EPR, which may reflect subtle functional differences between BTC and EPR.

DISCUSSION

We previously demonstrated that the EGF family of peptide growth factors can be divided into three distinct functional groups (8) (Fig. 8). The first group consists of EGF, TGF-α, and amphiregulin. These hormones bind and activate only the EGFR, but they can activate the other three ErbB family receptors in trans via heterodimerization with the EGFR. The second group consists of NRG and NRG2, which bind ErbB3 and ErbB4 and transmodulate EGFR and Neu via the binding receptors. The third group consists of BTC, which binds and activates both EGFR and ErbB4. Recent data suggests that heparin-binding EGF-like growth factor may also bind and activate EGFR and ErbB4, which would make heparin-binding EGF-like growth factor a member of this group as well (22).

Although EPR activates both EGFR and ErbB4, the interactions of EPR with these two receptors appear to be quite different. Compared with BTC, EPR stimulates higher levels of EGFR phosphorylation and lower levels of ErbB4 phosphorylation. Whereas both EPR and BTC stimulate EGFR and ErbB4 homodimerization and signaling, the geometry of the receptor dimers induced by EPR and BTC may be subtly different. The alignment of the kinase domain of one receptor molecule of a receptor homodimer with the autophosphorylation site of the other receptor molecule following EPR stimulation could be different from this alignment following BTC stimulation, affecting the cross-phosphorylation within receptor dimers. Al-

This would account for the higher levels of EGFR activation by EPR compared with BTC and the higher levels of ErbB4 activation by BTC compared with EPR.
ternatively, ligand-induced changes in the conformation of the receptor kinase domains might be different when the receptors are activated by BTC and EPR. Therefore, BTC and EPR may differentially stimulate receptor kinase activity. In either scenario, BTC and EPR could stimulate receptor autophosphorylation on different tyrosine residues, which could also be reflected in differences in gross levels of receptor phosphorylation. In this manner BTC and EPR could differentially modulate receptor coupling to signaling effectors and physiologic responses.

Another difference between EPR and BTC is that while EGFR is much more sensitive than ErbB4 to EPR, EGFR is less sensitive than ErbB4 to BTC. This suggests that although the affinity of EPR for EGFR is higher than the affinity for ErbB4, the affinity of BTC for EGFR is lower than the affinity for ErbB4. This too suggests that BTC and EPR have distinct biological functions, even in cells with identical patterns of ErbB family receptor expression.

Another important aspect of EPR function is the observation that the sensitivity of ErbB4 for EPR and the magnitude of ErbB4 activation by EPR can be modulated by the expression of other ErbB family receptors. EGFR expression increases the sensitivity of ErbB4 for EPR (Fig. 5, A and D; Fig. 6, A and C; Table I). Of course an alternative explanation is that the increased ErbB4 sensitivity in the presence of EGFR is due solely to EPR-induced transphosphorylation of ErbB4 by EGFR.

ErbB2 also increases the sensitivity of ErbB4 for EPR (Fig. 5, A-D; Table I). Because EPR does not activate ErbB2 in cells devoid of EGFR or ErbB4 (Figs. 1 and 7), the mechanism for the increased sensitivity of ErbB4 for EPR may be that ErbB2-ErbB4 heterodimers have a higher affinity for EPR than ErbB4-ErbB4 homodimers (Fig. 5, A and D; Fig. 6, A and C; Table I). Of course an alternative explanation is that the increased ErbB4 sensitivity in the presence of EGFR is due solely to EPR-induced transphosphorylation of ErbB4 by EPR.

These observations that ErbB4 activation by EPR can be influenced by EGFR or ErbB2 is consistent with existing models for receptor heterodimerization and transmodulation. It has been proposed that receptor heterodimerization is mediated through low affinity hormone-receptor interactions and heterotypic receptor-receptor contacts, after which there is cross-phosphorylation by the receptor kinase domains (23). It is possible that EGFR and ErbB2 are favored over ErbB4 for dimerization with ErbB4 in the presence of EPR. Therefore, there would be greater ErbB4 dimerization in cells expressing EGFR and ErbB4 or Neu and ErbB4 than in cells expressing ErbB4 alone. This may account for the increased sensitivity of ErbB4 for EPR in the presence of EGFR or ErbB2. It is also possible that ErbB2 is a better kinase for ErbB4 than ErbB4 itself. Consequently, ErbB2 may cross-phosphorylate more ErbB4 tyrosine residues in receptor heterodimers than ErbB4 would in receptor homodimers. Similarly, ErbB2 may phospho-
rylate the same tyrosine residues as ErbB4 to a greater extent than does ErbB4. Either of these last two possibilities would account for the increased tyrosine phosphorylation of ErbB4 by EPR in the presence of ErbB2.

As this manuscript was being prepared for submission, it was reported that radiolabeled EPR can be cross-linked to EGFR and ErbB4 in human breast tumor cell lines but not to ErbB2 or ErbB3. Furthermore, EPR stimulated high levels of EGFR and ErbB4 tyrosine phosphorylation and more modest levels of ErbB2 and ErbB3 tyrosine phosphorylation (24). Because the cell lines used in these studies express at least two and in some cases all four ErbB family receptors, some caution must be used in interpreting these results. Nonetheless, these data are entirely consistent with our findings that EGFR and ErbB4 are the receptors for EPR.

To date there have been only a few clues to EPR function. EPR transcripts are not detected in normal adult mouse liver, kidney, brain, spleen, testis, or skeletal muscles. However, low levels of EPR transcripts are detectable in adult mouse lung, smooth muscle, and heart, whereas more robust EPR transcrip-

Additional hints to EPR function arise from our data suggesting that EPR is a ligand for both EGFR and ErbB4. In most contexts EGFR activation is coupled to cellular DNA synthesis and proliferation. In contrast, there is mounting evidence that activated ErbB4 is coupled to growth inhibition, differentiation, and possibly human breast tumor cell survival (26), whereas NGF implants stimulate the differentiation of the mouse mammary epithelium in vivo (27). BTC stimulates the differentiation of pancreatic AR42J cells into insulin-secreting cells, but EGF and TGF-α do not (28). Finally, agonistic anti-

Furthermore, because EPR activation of ErbB4 is regulated by ErbB2 and activated ErbB2 appears to couple to mitogenesis and cell proliferation, the effects of EPR on cells expressing ErbB4 may be tightly linked to a balance of ErbB4 and ErbB2 expression. In cells having relatively low levels of ErbB2, EPR may have little effect because it fails to bind to ErbB4, and in cells having moderate levels of ErbB2 and high levels of ErbB4, EPR may act as a differentiation agent and inhibit cell prolif-

response to EPR will be dictated by relative levels of EGFR, ErbB2, and ErbB4 expression and not just the absolute level of expression of any single ErbB family receptor.

Acknowledgments—We thank Jerry Russell and Jeffrey Bender (Yale University) for recombiant NRGβ and Hideo Masui (Rockefeller University) for anti-EGFR monoclonal antibody 528. We are grateful to Jim Moyer, Brad Guarino, and Glenn Andrews (Pfizer Central Re-

REFERENCES

1. Stern, D. F., Heffernan, P. A., and Weinberg, R. A. (1986) Mol. Cell. Biol. 6, 1729–1740
2. King, C. R., Borrello, I., Bellot, P., Comoglio, P., and Schlessinger, J. (1988) EMBO J. 7, 1647–1651
3. Stern, D. F., and Kamps, M. P. (1988) EMBO J. 7, 995–1001
4. Goldman, R., Ben-Levy, R., Peles, E., and Yarden, Y. (1990) Biochemistry 29, 11924–11929
5. Wada, T., Qian, X., and Greene, M. I. (1990) Cell 61, 1339–1347
6. Johnson, G. R., Kannan, B., Shoyab, M., and Stromberg, K. (1993) J. Biol. Chem. 268, 2924–2931
7. Riese, D. J., II, Kehrel, M. M., van Raaij, T. M., Buckley, S., Plowman, G. D., and Stern, D. F. (1991) Oncogene 12, 345–353
8. Riese, D. J., II, Kim, E. D., Elenius, K., Buckley, S., Klagesberg, M., Plowman, G. D., and Stern, D. F. (1991) J. Biol. Chem. 266, 20947–20952
9. Culousouc, J.-M., Plowman, G. D., Carlton, G. W., Green, J. M., and Shoyab, M. (1993) J. Biol. Chem. 268, 18407–18410
10. Plowman, G. D., Green, J. M., Culousouc, J.-M., Carlton, G. W., Rothwell, V. M., and Buckley, S. (1993) Nature 366, 473–475
11. Carraway, K. L., III, Sliwkowski, M. X., Akita, R., Platko, J. V., Guy, P. M., Nuijens, A., Diamenti, A. J., Van, Rendell, R. L., Cantley, L. C., and Cerione, R. A. (1994) J. Biol. Chem. 269, 14303–14306
12. Sliwkowski, M. X., Schafer, G., Akita, R. W., Lofgren, J. A., Fitzpatrick, V. D., Nuijens, A., Fendly, B. M., Cerione, R. A., Van, Rendell, R. L., and Carraway, K. L., III (1994) J. Biol. Chem. 269, 14651–14655
13. Tzahar, E., Levkovitz, G., Karunagaran, D., Lavi, S., and Yarden, Y. (1990) J. Biol. Chem. 265, 25226–25233
14. Riese, D. J., II, van Raaij, T. M., Plowman, G. D., Andrews, G. C., and, Stern, D. F. (1995) Mol. Cell. Biol. 15, 5770–5776
15. Chang, H., Riese, D. J., II, Gilbert, W., Stern, D. F., and McManus, U. J. (1997) Nature 387, 509–512
16. Carraway, K. L., III, Weber, J. L., Unger, M. J., Lai, C., Andes, M., Yu, Y., Nussmann, M., and Lai, C. (1997) Nature 387, 512–516
17. Toyota, H., Komuraskasi, T., Ichida, T., Takayama, Y., Isobe, T., Okuyama, T., and Hanada, K. (1995) J. Biol. Chem. 270, 7485–7500
18. Guy, P. M., Platko, J. V., Cantley, L. C., Cerione, R. A., and Carraway, K. L., III (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 8132–8136
19. Pinkas-Kramarski, R., Soussan, L., Waterman, H., Levkovitz, G., Alroy, I., Klapper, L., Lavi, S., Seger, R., Ratzkin, B. J., Sela, M., and Yarden, Y. (1999) EMBO J. 15, 2452–2467
20. Tzahar, E., Waterman, H., Chen, X., Levkovitz, G., Karunagaran, D., Lavi, S., Ratzkin, B. J., and Yarden, Y. (1996) Mol. Cell. Biol. 16, 5376–5387
21. Graus-Porta, D., Beerli, R. R., Daly, J. M., and Hynes, N. E. (1997) EMBO J. 16, 1647–1655
22. Eilenius, K., Paul, S., Allinson, G., Sun, J., and Klagsburg, M. (1997) EMBO J. 16, 1268–1278
23. Lemmon, M. A., Bu, Z., Ladbury, J. E., Zhou, M., Pinchasi, D., Lax, I., Engelman, D. M., and Schlessinger, J. (1997) EMBO J. 16, 281–284
24. Komuraskasi, T., Toyota, H., Ichida, T., and Morimoto, S. (1997) Oncogene 15, 2841–2848
25. Toyota, H., Komuraskasi, T., Ikeda, Y., Yoshimoto, M., Morimoto, S. (1995) FEBS Lett. 377, 403–407
26. Peles, E., Bacus, S. S., Koski, R. A., Lu, H. S., Wen, D., Ogden, S. G., Ben, Levy, R., and Yarden, Y. (1996) Cell 69, 205–216
27. Jones, F. E., Berry, D. J., Guarino, B. C., Andrews, G. C., and Stern, D. F. (1996) Cell Growth Differ. 7, 1031–1038
28. Mashima, H., Ohnishi, H., Wakabayashi, K., Mine, T., Miyagawa, J.-I., Hanafusa, T., Seno, M., Yamada, H., and Kojima, I. (1996) J. Clin. Invest. 97, 1647–1654
29. Chen, X., Levkovitz, G., Tzahar, E., Karunagaran, D., Lavi, S., Ben-Baruch, L., Neitner, O., Ratzkin, B. J., Bacus, S. S., and Yarden, Y. (1996) J. Biol. Chem. 271, 7620–7629
30. Bacus, S. S., Chin, D., Yarden, Y., Zelnick, C. R., and Stern, D. F. (1996) Am. J. Path. 148, 549–558
31. Palacios, R., and Steinmetz, M. (1985) Cell 41, 727–734
32. Barbacci, E. G., Guarino, B. C., Streb, J. G., Singleton, D. H., Romack, K. J., Meyer, J. D., and Andrews, G. C. (1995) J. Biol. Chem. 270, 9855–9859
33. Gill, G. N., Kawamoto, T., Cochet, C. Le, A., Sato, J. D., Masui, H., McLeod, C., and Mendelsohn, J. (1984) J. Biol. Chem. 259, 7755–7760

3 T. Komuraskasi, unpublished data.