Structural Aspects of the Epidermal Growth Factor Receptor Required for Transmodulation of erbB-2/neu*

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The epidermal growth factor receptor (EGF-R) is known to transmodulate the activity and level of the erbB-2/neu protein in several epithelial cell lines. We therefore determined which structural features of the EGF-R were important in transmodulating erbB-2. We found that the addition of EGF to nontransformed epithelial cells resulted in down-regulation of erbB-2 with the same kinetics and similar extent as the EGF-R. By using cells expressing a series of EGF-R modified by site-directed mutagenesis, we found that EGF-R tyrosine kinase activity was not necessary for down-regulation of erbB-2, but receptor sequences between 899 and 958 in the EGF-R were required. To determine whether transmodulation was associated with activation of erbB-2, tyrosine phosphorylation of erbB-2 was determined following addition of EGF. Again, phosphorylation of erbB-2 following EGF addition did not require the intrinsic tyrosine kinase activity of the EGF-R, but did require sequences between 899 and 958. To determine the localization of EGF-R and erbB-2 following EGF addition, the relative distribution of the two receptors was evaluated by fluorescence microscopy. Surprisingly, the majority of erbB-2 was found in small cytoplasmic vesicles, whereas the EGF-R was predominantly found on the cell surface. Addition of EGF resulted in a redistribution and consequent colocalization of both receptors in endosomal and lysosomal structures. We conclude that activation and transmodulation of erbB-2 does not require intrinsic tyrosine kinase activity of the EGF-R, but does require sequences in the EGF-R which regulate its trafficking.

The human epidermal growth factor receptor (EGF-R) is the prototype for a family of four related cell surface receptors: EGF-R, erbB-2, erbB-3, and erbB-4. Each receptor has a single membrane-spanning domain, a ligand-activated tyrosine kinase domain, and a carboxy-terminal regulatory domain harboring several tyrosine residues which can be phosphorylated and serve as SH2 domain docking sites (1, 2). Comparison of amino acids conserved in all four receptors reveals a highly conserved tyrosine kinase domain (44% identical, 63% similar) and a highly divergent carboxyl-terminal signaling domain (3% identical, 15% similar (3–7)). Significantly, none of the tyrosine residues known to serve as autophosphorylation sites in EGF-R are conserved in the other erbB family members.

Signaling through EGF-R homologs is known to play a key role in the development of Drosophila, Caenorhabditis elegans, and mouse embryos. Studies of Drosophila embryos demonstrate the requirement for the Drosophila EGF-R in the establishment of both anterior/posterior and dorsal/ventral polarities (8, 9). The C. elegans homolog of the EGF-R (let-23) is necessary for normal vulval development (10). Recently, mice lacking functional EGF-R have been generated, resulting in many developmental defects in epithelial and neural tissues (11–14). Members of the EGF-R family have been implicated in the development of cancer. Rearrangements in the EGF-R is common in glioblastomas and have been observed in a number of other cancers (15). The majority of malignancies that correlate with EGF-R or erbB-2 abnormalities, however, involve overexpression of an intact receptor (7, 16–20).

Ligand activation of EGF-R leads to heterodimer formation between EGF-R and erbB-2, which is thought to result in activation of erbB-2 by transphosphorylation (21, 22). Transactivation of erbB-2 also occurs upon the addition of heregulin, a ligand for erbB-3 and erbB-4 (23–26). As observed for EGF, heregulin induces the formation of heterodimers between erbB-3 or erbB-4 with erbB-2, resulting in erbB-2 becoming tyrosine phosphorylated (4, 7, 24, 27, 28). There is also evidence to suggest that EGF-R interacts with erbB-3 and erbB-4 (29). Thus, activation of any member of the EGF-R family results in signaling through multiple receptor types. Understanding how these receptors interact with each other is therefore essential to knowing how they work.

The pattern of tyrosine phosphorylation of EGF-R and erbB-2 is important in signal transduction. Specific phosphorylated tyrosines residues serve as docking sites for proteins containing SH2 domains, such as the phosphatidylinositol 3-kinase p85 subunit and GRB2 (27). The assembly of signaling complexes dictates the subsequent pattern of signal transduction. Intracellular trafficking of the activated EGF-R also regulates receptor activity by controlling the availability of substrates and signaling partners (30). Although ligand binding induces rapid internalization and subsequent lysosomal targeting of EGF-R, it is uncertain whether erbB-2 trafficking is influenced by the EGF-R. It has been suggested that all members of the erbB family are “internalization defective” except for the EGF-R (31), but those studies were done by direct activation of erbB family members or by using chimeric receptors. It has been reported that EGF treatment can stimulate the degradation of erbB-2 in some epithelial cells, but the mechanism of this transmodulation is unclear (32). If EGF-R activation

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1 The abbreviations used are: EGF-R, epidermal growth factor receptor; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.
does alter the trafficking of erbB-2, this could be an important mechanism for regulating both the activity and distribution of these signaling molecules in cells.

We have previously investigated the mechanisms that regulate the activity and distribution of EGF-R in cells. We have now extended our studies to examine the activity of the EGF-R to transmodulate erbB-2. We report here that activation of the EGF-R does result in the tyrosine phosphorylation of erbB-2 and a subsequent loss in the cellular mass of erbB-2. Surprisingly, this transmodulation does not require either the intrinsic tyrosine kinase activity of the EGF-R or sequences in its regulatory carboxy tail. Sequences containing the lysosomal targeting domain of the EGF-R were required, however, suggesting that correct intracellular trafficking of the EGF-R is necessary for transmodulation of other members of the erbB family.

EXPERIMENTAL PROCEDURES

General—Polyclonal rabbit antibody N-13 directed against a peptide corresponding to residues 1–13 in human EGF-R was a gift of Dr. Debora Cadena. Polyclonal rabbit antibody 1917 to erbB-2 directed against a peptide corresponding to the 15 carboxy-terminal residues of human erbB-2, were provided by Dr. Gordon Gill. Rabbit polyclonal antibody C18 against amino acids 1169–1186 of neu/erbB-2 was obtained from Santa Cruz Biotechnology. Polyclonal rabbit antibodies specific for phosphotyrosine were generated and affinity-purified as described (33). Monoclonal antibodies 528, 579, and 225 against the EGF-R (34) were purified from hybridomas obtained from the American Type Culture Collection and grown in McCoys 5A supplement. Monoclonal antibody 13A9 against the human EGF-R was a generous gift from Genentech. Ab5 against the extracellular domain of human erbB-2 was obtained from Oncogene Sciences. Secondary antibodies labeled with either Texas Red or fluorescein were obtained from Cappel Laboratories.

Cell Culture—B2B mouse L cells, which contain no endogenous EGF receptors (35), and B22 cells transfected with normal (WT) or mutated (M721,c967g, c958, and M721,c958) human EGF receptors were a generous gift of Dr. Gordon Gill. Their construction was described previously (36). B22 cells were grown in Dulbecco’s modified Eagle’s medium (Flow Laboratories) containing dialyzed 10% calf serum (HyClone). 5 μM Methotrexate was added to the medium for those cells transfected with human EGF receptor. The human mammary epithelial cell line 184A1L5 (37) was obtained from Dr. Martha Stampfer and was cultured in medium DFCI-1 as described (38). The human mammary epithelial cell line HB2 was a gift from Joyce Taylor-Papadimitriou and cultured in medium DFCI-1 as described (38). The concentrations of and incubation times with and were determined with 10% fetal calf serum (HyClone).

Quantification of EGF-R, erbB-2, and Phosphotyrosine Levels—Confluent cultures of cells were rinsed and lysed in an Nonidet P-40 buffer (150 mM NaCl, 1% Nonident P-40, 50 mM Tris, pH 8.0) and debris removed by centrifugation. Samples were brought to 2% SDS, 1% β-mercaptoethanol and heated to 100 °C for 5 min. Equal amounts of total cellular protein from each sample were separated on 5–7.5% gradient gels and transferred to nitrocellulose. EGF-R and erbB-2 were detected by N-13 and 1917 polyclonal antisera, respectively, using 125I-labeled protein A as described (41). The concentrations of and incubation times with 125I-labeled protein A were chosen to be in the linear range of the protein load of the gels. The blots were analyzed by storage phosphor plates using the Bio-Rad G250 Molecular Imager. The Bio-Rad Molecular Analyst package was used to quantify the amount of radioactivity associated with each band. Quantification of EGF-R mRNA Levels—Total cellular RNA was isolated using Tri-Reagent (Molecular Research Center, Inc.) following the manufacturer’s directions. cDNA was synthesized using Moloney murine leukemia virus-Reverse Transcriptase (Promega) and specific primers were amplified in a polymerase chain reaction using the Idaho Technology air cyer with the following primer pairs: erbB2, 5′-AATGCTGACCACCTCTCGGTG-3′ and 5′-CAGGAGATCACGATGCCTTG-3′ at 63°C, annealing, 94°C polymerization for 15 s for 30 cycles. Glyceraldehyde–3-phosphate dehydrogenase, 5′-GAGCTTGACAAAGTGTGCTTGAGG-3′ and 5′-CCACAGCTGCTCCACTG-GCCAC-3′ at 60°C annealing, 94°C polymerization for 15 s for 24 cycles. The number of cycles was chosen empirically to keep product amplification in the linear range. Reaction products were separated on 1.8% agarose gels and stained with ethidium bromide.

Fluorescence Microscopy—Cells were plated on fibronectin-coated coverslips 48 h before the experiment. Cells treated either without or with EGF were fixed with 10% with freshly prepared 3.6% paraformaldehyde and 0.024% saponin in Ca2+/Mg2+-free phosphate-buffered saline. Free aldehyde groups were quenched with 0.1% NaN3 for 10 min. Cells were incubated simultaneously with a mixture of anti-EGF-R monoclonals 528, 579, 225, and 13A9 (10 μg/ml each) and anti-erbB-2 C18 (1:100; Santa Cruz Biotechnology, Inc.) in 0.012% saponin for 45 min following by staining with fluorescein isothiocyanate-labeled goat anti-mouse and Texas Red-labeled goat anti-rabbit IgG antibodies (1:100) for 45 min. The coverslips were mounted in ProLong antifade medium (Molecular Probes, Inc.) and viewed with a Nikon inverted fluorescence microscope with a × 40 oil immersion objective. Images (512 × 512) were acquired using a Photometrics cooled CCD camera with a Macintosh workstation running OncorImage software. For confocal microscopy, samples were viewed with a Bio-Rad MRC 600 laser scanning confocal imaging system attached to a Zeiss Axioplan microscope with a × 60 objective. Excitation was achieved with a Krypton/Argon laser using the 488 and 568 nm lines. Paired images (384 × 512 pixels each) were individually scaled to 256 gray levels using Adobe Photoshop 3.0 on the Macintosh before output to a film recorder.

Flow Cytometry—Cells were removed from plates by a brief trypsinization, which did not alter the measurable number of either EGF-R or erbB-2 at the cell surface. Cells were fixed for 10 min at 22°C in freshly prepared 3.6% paraformaldehyde, rinsed, and incubated for 1 h in either anti-EGF-R monoclonal antibody 225 or anti-erbB-2 monoclonal antibody Ab5 followed by fluorescein isothiocyanate-labeled goat anti-mouse antibody for 1 h. Samples were analyzed on a FACSscan flow cytometry instrument (Becton-Dickinson, Mountain View, CA) and the data analyzed using Cell Quest software.

RESULTS

EGF Treatment Reduces erbB-2 Levels in Human Mammary Epithelial Cells—It is known that binding of EGF to its receptor leads to rapid internalization and reduction of EGF-R levels as a result of lysosomal targeting (43). It is also well established that activated EGF-R can form heterodimers with erbB-2, resulting in erbB-2 transactivation (44). Studies using a normal mouse mammary cell line, HC11, showed that EGF treatment resulted in a loss of erbB-2 cell surface expression due to accelerated degradation (32). Other studies, however, using either Rat-1 cells (45) or transformed human cell lines showed that EGF had no effect on erbB-2 levels (40). Our first goal, therefore, was to determine whether erbB-2 expression or trafficking was affected by EGF in a non-transformed human mammary epithelial cell line, 184A1L5 (37). This line is mitogenically responsive to EGF and expresses both EGF-R and erbB-2.

Cells were treated with 100 ng/ml EGF for 1 h at 37°C. The levels of cell surface EGF-R and erbB-2 were then measured by flow cytometry. As shown in Fig. 1, EGF treatment reduced the surface expression of EGF-R in 184A1L5 cells by approximately 50%. Although cell surface erbB-2 levels were not as
cell type is the mouse B82 cell line which expresses the murine forms of the EGF-R introduced by gene transfection. A suitable endogenous EGF-R. This allows the use of different mutant which expressed normal levels of erbB-2, but does not have transmodulation of erbB-2, it is preferable to use a cell type define the structural features of the EGF-R responsible for the responsible structural features of the EGF-R.

If this "transmodulation" between the two molecules, then it should be possible to define of erbB-2 by the EGF-R requires a direct physical interaction similar mechanism may be responsible. If this "transmodulation" of both proteins were remarkably similar, suggesting that a sim- milar mechanism could be responsible. Immunofluorescence visualization is a sensitive method for determining en-

high as EGF-R levels, EGF treatment resulted in an approxi-
mately 3-fold reduction in their levels. These data demonstrate that activation of the EGF-R in mammary epithelial cells not only down-regulates the EGF-R, but also reduces the surface levels of erbB-2.

To determine whether the reduction of surface erbB-2 levels was accompanied by a reduction in total cellular erbB-2 mass, we treated cells with EGF for varying periods of time and then determined total erbB-2 levels by Western blot analysis. EGF-R levels were simultaneously measured by using a specific enzyme-linked immunosorbent assay (42). EGF treatment resulted in a progressive loss of both proteins over a 24-h time period (Fig. 2, left panel). These data show that EGF can down-regulate erbB-2 levels in cells in a similar manner as the EGF-R, confirming results previously obtained with mouse mammary epithelial cells (32). The kinetics and extent of loss of both proteins were remarkably similar, suggesting that a sim-
ilar mechanism may be responsible. If this "transmodulation" of erbB-2 by the EGF-R requires a direct physical interaction between the two molecules, then it should be possible to define the responsible structural features of the EGF-R.

**Model System for Analyzing erbB-2 Transmodulation**—To define the structural features of the EGF-R responsible for transmodulation of erbB-2, it is preferable to use a cell type which expressed normal levels of erbB-2, but does not have endogenous EGF-R. This allows the use of different mutant forms of the EGF-R introduced by gene transfection. A suitable cell type is the mouse B82 cell line which expresses the murine homolog of erbB-2, but lacks endogenous EGF-R (35). To deter-

To check for this possibility, both 184A1L5 and B82 cells were treated without or with 100 ng of EGF for 4 h at 37 °C. Total RNA was extracted and the levels of erbB-2 mRNA were determined by reverse transcriptase-polymerase chain reaction. As shown in Fig. 3, treatment of either cell type with EGF had no discernible effect of erbB-2 mRNA levels. This result is consistent with a post-translational mechanism for the reduction of erbB-2 levels by EGF treatment.

**Decreased Levels of erbB-2 in Response to EGF Is Due to Enhanced Lysosomal Targeting**—It is known that the decrease in EGF-R protein levels in response to EGF addition is due to a degradative mechanism. It remained a formal possibility, however, that a reduction in erbB-2 mRNA levels could also be involved. To check for this possibility, both 184A1L5 and B82 cells were treated without or with 100 ng of EGF for 4 h at 37 °C. Total RNA was extracted and the levels of erbB-2 mRNA were determined by reverse transcriptase-polymerase chain reaction. As shown in Fig. 3, treatment of either cell type with EGF had no discernible effect of erbB-2 mRNA levels. This result is consistent with a post-translational mechanism for the reduction of erbB-2 levels by EGF treatment.
There was little overlap between the EGF-R and erbB-2 staining pattern. The addition of peptide specific for the anti-erbB-2 antibody abolished the erbB-2 immunofluorescence, but had no effect on the EGF-R staining pattern (Fig. 4, C and D), indicating the observed localization was specific for erbB-2. The addition of EGF resulted in reduced levels of surface EGF-R and a corresponding increase in intracellular EGF-R, particularly in perinuclear and lysosomal structures (Fig. 4E). Significantly, EGF also caused a redistribution of erbB-2 into lysosomal structures (Fig. 4F). Although EGF treatment increased the overlap between intracellular EGF-R and erbB-2, there was still a large number of vesicles that contained either EGF-R or erbB-2, but not both. We conclude that EGF treatment accelerates lysosomal targeting of both the EGF-R and erbB-2, but that the normal cellular distribution of the two proteins is different.

We were surprised at the predominant intracellular localization of erbB-2 because this receptor has previously been described as being cell surface-associated (45). To determine whether the intracellular localization of erbB-2 in B82 cells was atypical, we used confocal microscopy to examine the localization of erbB-2 in various cell lines (Fig. 5). In the case of both B82 cells and the nontransformed human mammary epithelial cell line HB2, there was a high level of intracellular erbB-2 (top panels) and ErbB-2 (right panels) using monoclonal and polyclonal antibodies, respectively. Optical sections 0.5 microns thick were taken through the lower one-third of the cells. The two images were collected separately using barrier filters to prevent spillover between the fluorescein isothiocyanate images (left) and Texas Red images (right). Top panels are mouse B82 fibroblasts. Middle panels are human HB2 mammary epithelial cells and bottom panels are human breast carcinoma line SKBR3.

**FIG. 3. Levels of erbB-2 mRNA do not change after EGF treatment.** Monolayers of either HMEC 184A1L5 (left) or B82 mouse fibroblast expressing human EGF-R (right) were treated without or with 100 ng/ml EGF for 4 h at 37 °C. Total RNA was extracted, reverse transcribed, and specific erbB-2 transcripts (top panel) and control glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were amplified by polymerase chain reaction as described under “Experimental Procedures” using the indicated amounts of cDNA. Shown is a scanned image of the reaction products run on 1.8% agarose gels and stained with ethidium bromide.

**FIG. 4. Distribution of EGF-R and erbB-2 in mouse B82 fibroblasts.** Cells were fixed and permeabilized and the distributions of EGF-R (left panels) and erbB-2 (right panels) were determined by use of mouse monoclonal antibody 528 and rabbit polyclonal antibody C18, respectively. Panels A and B are untreated cells, panels B and C are also untreated cells, but incubation with primary antibodies was done in the presence of competing erbB-2 peptide C18. Panels E and F are cells following treatment for 4 h at 37 °C with 100 ng/ml EGF. Arrows indicate identical vesicles. Images were acquired with a Photometrics cooled CCD camera as described under “Experimental Procedures.” Exposures times and scaling of all images were identical for each receptor type.

**FIG. 5. Distribution of EGF-R and erbB-2 in different cell types as determined by confocal microscopy.** Cells grown on coverslips were fixed, permeabilized, and simultaneously stained for EGF-R (left panels) and erbB-2 (right panels) using monoclonal and polyclonal antibodies, respectively. Optical sections 0.5 microns thick were taken through the lower one-third of the cells. The two images were collected separately using barrier filters to prevent spillover between the fluorescein isothiocyanate images (left) and Texas Red images (right). Top panels are mouse B82 fibroblasts. Middle panels are human HB2 mammary epithelial cells and bottom panels are human breast carcinoma line SKBR3.
Bio-Rad Molecular Imager. The EGF-R types used were wild type (●, n = 7), kinase-inactive M721 (■, n = 5), c'958 (○, n = 4), c'958 M721 (□, n = 3), c'958 (●, n = 2), and c'647 (○, n = 2). The error bars represent the S.E. of the indicated number of independent experiments.

The cell surface distribution of erbB-2 previously described may either be a result of cell transformation or overexpression of erbB-2.

**Structural Aspects of EGF-R Required for erbB-2 Transmodulation**—Several models could explain the ability of activated EGF-R to target erbB-2 to lysosomes. One is that the EGF-R activates erbB-2 by direct tyrosine transphosphorylation. The activated erbB-2 would then enter the lysosomal targeting pathway by a similar mechanism as the EGF-R. We tested this possibility by determining the effect of EGF on erbB-2 levels using cells expressing kinase inactive EGF-R. As shown in Fig. 6, full-length kinase inactive EGF-R still reduce erbB-2 levels in response to EGF, although not as rapidly as the wild type EGF-R (Fig. 6, A versus B). This suggests that direct tyrosine phosphorylation of erbB-2 by the EGF-R is not necessary for erbB-2 transmodulation.

Heterodimerization between EGF-R and erbB-2 is thought to occur through their extracellular domains, but transactivation may also require cytoplasmic sequences of the EGF-R (46, 47). These sequences fall into three main domains: the regulatory cytoplasmic tail (residues 958–1186), the conserved kinase domain (688–958), and the submembrane region (residues 645–688). In an effort to determine which domain may be involved in transmodulation of erbB-2, we used B82 cells expressing EGF-R that lacked various regions. As shown in Fig. 6D, receptors lacking all cytoplasmic sequences (c'647) or sequences distal to the submembrane region (c'688) did not reduce erbB-2 levels following EGF addition. Receptors having the conserved kinase domain (c'958) were able to efficiently reduce erbB-2 levels in response to EGF as effectively as full-length receptors (Fig. 6A). Eliminating the intrinsic kinase activity of the c'958 receptor through a point mutation in its ATP-binding site (M721) also did not eliminate its ability to reduce erbB-2 levels (Fig. 6B). We conclude that although the kinase domain of the EGF-R is required for transmodulation of erbB-2, the kinase activity of this domain is not required. This suggests that direct phosphorylation of erbB-2 by the EGF-R is not necessary.

**The Ability of Mutant EGF-Rs to Induce Tyrosine Phosphorylation of erbB-2 Correlates with Its Ability to Transmodulate erbB-2 Levels**—Although direct phosphorylation of erbB-2 by occupied EGF-R is clearly not involved in transmodulation, it seemed possible that heterodimerization of kinase inactive EGF-R with erbB-2 could result in activation of erbB-2 through interaction of their cytoplasmic regions. It has been previously shown that EGF-R lacking intrinsic kinase activity can activate erbB-2 (48). To test this possibility, we tested cells expressing various EGF-R mutants for 15 and 120 min with EGF. erbB-2 was then immunoprecipitated and probed for phosphorysyrine by Western blot analysis. As shown in Fig. 7, both the full-length and c'958 mutants were able to induce tyrosine phosphorylation of erbB-2. Phosphorylation was highest at 15 min and fell by 2 h. The c'958 EGF-R mutant was able to induce a higher stimulated level of erbB-2 phosphorylation, although the basal level of erbB-2 phosphorylation was higher as well. Interestingly, kinase-inactive versions of the full-length and c'958 EGF-R were also able to induce tyrosine phosphorylation of erbB-2, albeit to a lower degree than the kinase active EGF-R. The kinetics of erbB-2 phosphorylation were also similar.

In contrast to the results obtained with the full-length and c'958 EGF-R, receptors truncated to residue 688 were not able to induce tyrosine phosphorylation of erbB-2 (Fig. 8). Thus the ability of EGF-R to mediate phosphorylation of erbB-2 is correlated with its ability to reduce erbB-2 levels. This is consistent with a model in which erbB-2 is transactivated by heterodimerization with the EGF-R. The transactivated erbB-2 would then be targeted to lysosomes in an analogous fashion as ligand-activated EGF-R.

**The Distal Region of the EGF-R Tyrosine Kinase Domain Is Required for Transmodulation of the EGF-R**—Although the region between 688 and 958 of the EGF-R contains the conserved tyrosine kinase domain, another activity that has been mapped to the distal region of this domain is endosomal retention/lysosomal targeting (between 899 and 958) (41). To determine whether this region is required for transmodulation of erbB-2, we prepared an EGF-R truncated at residue 899. As
Discussed that EGF-R kinase activity was not required for EGF-induced erbB-2 signaling is also occurring. It was surprising, however, that the kinase domain of the EGF-R eliminated transmodulation of erbB-2. We examined changes in erbB-2 phosphorylation and protein levels because these parameters are fairly direct measurements of the status of erbB-2 itself. In addition, these parameters reflect the status of the entire cellular pool of erbB-2.

We found that transmodulation of erbB-2 by EGF is fairly rapid, occurring with similar kinetics and extent to that observed for down-regulation of the EGF-R. Analysis of EGF-R mutants demonstrated that neither intrinsic tyrosine kinase activity nor the regulatory carboxyl terminus distal to the kinase domain were required for reduction of erbB-2 levels. Further truncation of 59 amino acids from the carboxy end of the kinase domain were required for reduction of erbB-2 levels. Over the last several years, it has become clear that members of the EGF receptor family interact extensively. These interactions are thought to be important in generating the pattern of intracellular signals triggered by ligand binding (27). Receptor activity is regulated by diverse dynamic processes, such as phosphorylation, endocytosis, and lysosomal targeting and these processes require specific receptor sequences that interact with regulatory machinery of the cell. Previously, we have identified EGF receptor sequences that are required for its endocytosis and lysosomal targeting (41). In the current study, we sought to define EGF receptor domains that are responsible for interacting with erbB-2. We examined changes in erbB-2 phosphorylation and protein levels because these parameters are fairly direct measurements of the status of erbB-2 itself. In addition, these parameters reflect the status of the entire cellular pool of erbB-2.

There is no obvious direct relationship between the degree of erbB-2 phosphorylation and its down-regulation (Figs. 6 and 7). However, this does not mean the phosphorylation per se is not required for erbB-2 down-regulation. The degree of phosphorylation seen in our experiments may reflect modification of multiple tyrosine residues in erbB-2, not all of which may be involved in receptor trafficking. This is especially plausible if the different EGF-R mutants which transmodulate erbB-2 vary in their ability to induce phosphorylation by transphosphorylation versus transactivation. However, levels of erbB-2 phosphorylation drop from a peak at 15 min to a lower, more uniform value for all EGF-R mutants tested (Fig. 7). Because
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erbB-2 down-regulation requires prolonged incubation with EGF, it is difficult to know the relevant time frame to compare these two processes. Finally, there may actually be no direct relationship between phosphorylation and down-regulation. Both processes may simply reflect the consequence of EGF-R and erbB-2 interactions. Further studies will be required to discriminate between these possibilities.

Although the extracellular domain of the EGF-R has been reported to be sufficient for heterodimer formation, it is clearly not sufficient to either transactivate or transmodulate erbB-2 (46, 47). Because removal of sequences distal to 899 in the conserved kinase domain abolishes erbB-2 transmodulation, an additional cytoplasmic interaction between the two receptors may be required to efficiently heterodimerize or cause an activating conformational change. Alternately, trafficking of the EGF-R into intracellular compartments containing high levels of erbB-2 may be required for heterodimerization, activation, and down-regulation of erbB-2.

Consistent with a role for EGF-R trafficking in erbB-2 activation is our observation that there are significant intracellular pools of erbB-2 in cells which display EGF-R-mediated transmodulation. In the absence of ligand, there was little overlap of EGF-R and erbB-2 distribution. In the presence of EGF, however, there was significant overlap between the distribution of EGF-R and erbB-2. In addition, the region of the EGF-R required for transmodulation of erbB-2 (between 899 and 958) maps to the region necessary for lysosomal targeting of the EGF-R. Recently, it has been shown that this domain of the EGF-R binds to the protein SNX1 which mediates receptor transfer to late endosomes and lysosomes (50). Alternately, the region between 899 and 958 could simply allow hetrodimerization of EGF-R and erbB-2. In addition, the region of the EGF-R that overlaps with the cytoplasmic region between 899 and 958 maps to the region necessary for lysosomal targeting. Furthermore, loss of sequences distal to 899 in the conserved kinase domain abolishes erbB-2 transmodulation, an additional cytoplasmic interaction between the two receptors may be required to efficiently heterodimerize or cause an activation of both EGF-R and erbB-2.


does not display EGF-induced down-regulation of erbB-2 (Ref. 40; also data not shown). A correlation between high erbB-2 levels and low transmodulation by the EGF-R has been observed by other investigators as well (54). It is possible that overexpression of erbB-2 saturates the intracellular trafficking machinery, preventing down-regulation of erbB-2 following activation. This may enable the cell to exhibit prolonged signaling from erbB-2 and confer a growth advantage, analogous to the situation postulated for internalization-defective EGF-R (55). Alternatively, a modified distribution of EGF-R and erbB-2 may facilitate heterodimerization in compartments that normally would not contain such activated complexes. This in turn may allow access to a different set of substrates or restrict the influence of negative regulatory elements, such as phosphatases or kinases. Further work is obviously necessary to determine the mechanisms and importance of transmodulation of erbB-2 by the EGF-R. It is clear, however, that normal regulation of both EGF-R and erbB-2 requires their correct intracellular localization.

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