Transmembrane Signaling by Epidermal Growth Factor Receptors Lacking Autophosphorylation Sites*  

(Received for publication, March 3, 1993)

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Mutant epidermal growth factor (EGF) receptors in which the five known tyrosine autophosphorylation sites (tyrosines 992, 1068, 1086, 1148, and 1173) were replaced with phenylalanine residues were expressed in NIH-3T3 cells (5F-EGFR) and transmembrane signaling parameters compared with cells expressing wild-type EGF receptor (WT-EGFR). Mutant and wild-type clones were chosen expressing similar numbers of receptors and Scatchard analysis of EGF binding showed high and low affinity binding of equal affinities for both receptor types. EGF stimulated tyrosine phosphorylation of proteins to a much lesser degree in cells expressing 5F-EGFR relative to cells expressing WT-EGFR. Tyrosine phosphorylation of the 5F-EGFR was 2–4% of WT-EGFR. Surprisingly, cells expressing WT-EGFR or 5F-EGFR showed little difference in dose response of EGF-stimulated [3H]thymidine incorporation or EGF stimulation of mitogen-activated protein kinase activity. However, EGF did not induce anchorage-independent growth of cells expressing 5F-EGFR to the same extent as it did for cells expressing WT-EGFR. EGF treatment of 5F-EGFR cells failed to elicit an increase in phosphatidylinositol 3-kinase activity or to stimulate hydrolysis of phosphoinositides or tyrosine phosphorylation of phospholipase C-γ1. These data suggest that a significant proportion of EGF receptor signaling can occur through receptors with altered capacity to interact with src homology 2 domain-containing proteins.

Transmembrane signaling by the epidermal growth factor (EGF)1 receptor has been shown to require the intrinsic tyrosine kinase activity of the receptor (1). A number of proteins are phosphorylated on tyrosine residues following EGF stimulation of cells, including phospholipase C-γ1 (PLC-γ1) (2), the GTPase-activating protein of ras (GAP) and GAP-associated proteins (3), MAP kinase (4, 5), lipocortin I (6), and others (7). It is not known whether tyrosine phosphorylation of these proteins is relevant to signaling by EGF, although tyrosine phosphorylation of PLC-γ1 appears to positively influence PLC-γ1 activity (8).

In many cell types the EGF receptor itself is the major tyrosine-phosphorylated protein found in response to EGF treatment (9). Five autophosphorylation sites have been identified in carboxy-terminal tail of the EGF receptor (10–12) and recent evidence suggests that the src homology 2 (SH2) domains of proteins such as PLC-γ1 and GAP physically associate with these tyrosine-phosphorylated regions of the receptor (13). Similar interactions between SH2 domain-containing proteins and other tyrosine kinase receptors such as the platelet-derived growth factor receptor (14, 15), the colony stimulating factor 1 receptor (16), the fibroblast growth factor receptor (17), and c-kit (18) have been reported. These findings raise the question of whether all signaling by tyrosine kinase receptors occurs through protein-protein interactions between tyrosine-phosphorylated receptor and SH2 domain-containing effector proteins. In order to address this question we have expressed EGF receptors in which all five known tyrosine autophosphorylation sites have been changed to phenylalanines and examined a number of EGF-dependent signaling parameters in cells expressing these mutant EGF receptors.

EXPERIMENTAL PROCEDURES

Materials—[125I]-EGF and receptor-grade EGF were from Collaborative Research. 125I-Protein A was from Amersham Corp.

Cell Culture and Mutagenesis—NIH-3T3 cells expressing normal human EGF receptor or EGF receptor in which tyrosines 992, 1068, 1068, 1148, and 1173 were mutated to phenylalanine residues were grown as previously described (19). Point mutations were made using the Bio-Rad Mutagen kit, and mutations were verified by sequencing. Cells were transfected with pCO12 vector carrying WT-EGFR or 5F-EGFR receptor cDNA as described (19).

DNA Synthesis and Cell Growth Assays—For assay of [3H]thymidine incorporation, cells were grown in 2-cm wells to confluence, then incubated for 24 h in serum-free Dulbecco’s modified Eagle’s medium. Indicated concentrations of EGF or vehicle were added for another 24 h and assay of [3H]thymidine incorporation performed according to Decker (20). Growth in soft agar was performed according to Decker (21).

Assay of EGF-stimulated MAP Kinase Activity—Cells were plated in 35-mm dishes, and the following day medium was changed to serum-free Dulbecco’s modified Eagle’s medium. Indicated concentrations of EGF or vehicle were added for another 24 h and assay of [3H]thymidine incorporation performed according to Decker (20). Growth in soft agar was performed according to Decker (21).

Assay of PI 3-Kinase Activity—Cells were grown to confluence in 100-mm dishes prior to the addition of various concentrations of EGF or vehicle for 5 min. Cells were lysed in 1 ml of 20 mM Tris-HCl, 150 mM NaCl, 1% Triton-X 100, 5 mM EDTA, 100 µM sodium orthovanadate, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride, pH 7.5. Lysates were centrifuged at 12,000 x g for 10 min and immunoprecipitated with anti-phosphotyrosine antibody. Assay of PI 3-kinase activity in immunoprecipitates was performed according to Omichia et al. (22).

Binding of 125I-EGF, anti-phosphotyrosine immunoblotting, and measurement of inositol phosphates were performed according to Decker (19) and immunoprecipitation of PLC-γ1 according to Decker et al. (23).

RESULTS

To evaluate the role of tyrosine phosphorylation of the EGF receptor in receptor signaling, the five known tyrosine autophosphorylation sites in the carboxy-terminal tail of the EGF receptor were changed to phenylalanine residues using site-directed mutagenesis (this mutant receptor was termed 5F-EGFR). 5F-EGFR was expressed in an NIH-3T3 cell strain.
lacking endogenous EGF receptors (20), and WT-EGFR and 5F-EGFR clones were screened in preliminary 125I-EGF binding assays for cells expressing similar numbers of receptors. Representative clones exhibiting similar binding were subjected to Scatchard analysis of 125I-EGF binding (Fig. 1). Both receptor types displayed high affinity binding with dissociation constants of about 1.25 x 10^-10 M (20,000–25,000 sites/cell) and low affinity binding with constants of about 2.5 x 10^-8 M (100,000–125,000 sites/cell). The data presented are from experiments using these matched clones and are representative of results obtained using four other sets of matched clones.

We next examined tyrosine phosphorylation of cellular proteins in the two cell types (Fig. 1A). In anti-phosphotyrosine immunoblots of whole cell lysates from WT-EGFR cells treated with EGF, the EGF receptor was the primary tyrosine-phosphorylated protein with a number of other tyrosine-phosphorylated proteins evident. In EGF-treated 5F-EGFR cells, almost no tyrosine-phosphorylated EGF receptor could be detected, although EGF-stimulated bands of M_e = 150,000, 55,000, and 43,000 were present. EGF had no effect on tyrosine phosphorylation in the parental cells.

To more specifically assess tyrosine phosphorylation of the EGF receptor in these cells, EGF receptors were immunoprecipitated prior to immunoblotting with anti-phosphotyrosine antibody (Fig. 1B). Again, very little tyrosine phosphorylation of the 5F-EGFR was detected (20–40-fold more counts were consistently found in the WT-EGFR than the 5F-EGFR when bands excised and quantitated in a γ counter). This result was confirmed when cells were labeled with [32P]orthophosphate and 32P-labeled EGF receptors subjected to phosphoamino acid analysis (data not shown).

To begin to assess the signaling capacity of the 5F-receptor, EGF-stimulated incorporation of [3H]thymidine was compared between cells expressing WT-EGFR and 5F-EGFR (Fig. 3A). No significant difference was found in the two cell types over a range of EGF concentrations. EGF stimulated 5F-EGFR cells to form colonies in soft agar, although to a lesser extent than for WT-EGFR cells (Table I). In addition,
TABLE I

| Cell type          | Number of colonies per dish^a |
|--------------------|--------------------------------|
|                    | Control | Plus EGF (15 nM) |
| Parental NIH-3T3   | 8 ± 5   | 9 ± 3            |
| WT-EGFR            | 15 ± 6  | 943 ± 54         |
| 5F-EGFR            | 11 ± 4  | 289 ± 32         |

^a Single cell suspensions (2.5 × 10^6 cells/35-mm dish) were plated in 0.3% agar containing vehicle or 15 nM EGF. Cells were incubated for 10 days at which time colonies were counted. Colonies larger than 0.1 mm were scored as positive. Each value represents the mean and standard error from triplicate cultures.

EGF had little effect on the morphology of 5F-EGFR cells, while inducing typical refractile, spindle-shaped cells for WT-EGFR had little effect on the morphology of 5F-EGFR cells, while inducing typical refractile, spindle-shaped cells for WT-EGFR cells (not shown).

Since an increase in MAP kinase activity often correlates with mitogenic signaling and the mechanism of MAP kinase activation is unknown, we examined EGF-stimulated MAP kinase activity in WT-EGFR- and 5F-EGFR-expressing cells (Fig. 3B). A slight shift to the right was seen for the dose-response curve for EGF stimulation of MAP kinase activity for cells expressing 5F-EGFR as compared to WT-EGFR with a somewhat higher maximal response for WT-EGFR cells.

Activation of PI 3-kinase and phospholipase C-γ1 (which contain SH2 domains) is thought to involve interaction with tyrosine-phosphorylated regions of receptors in some cells (3, 23). Experiments were performed to determine whether EGF binding to the 5F-EGFR could still elicit these activities (Fig. 4, A and B). Whereas WT-EGFR cells demonstrated significant EGF-dependent increases in these two assays, EGF was without effect on 5F-EGFR cells. In addition, EGF stimulated tyrosine phosphorylation of M_w = 150,000 PLC-γ1 along with some associated M_w = 170,000 EGFR in cells expressing WT-EGFR, but not in those expressing 5F-EGFR (Fig. 5).

**DISCUSSION**

Experiments were designed to assess the contribution of the known autophosphorylation sites of the EGF receptor to EGF-dependent transmembrane signaling. Recent studies have shown that isolated SH2 domains will bind with relatively high affinity to tyrosine-phosphorylated regions of growth factor receptors and that interactions of SH2 domain-containing proteins with tyrosine-phosphorylated receptors is required for their activation (13, 24). These findings have led to the proposal that large signaling complexes are formed following receptor activation (24) consisting of tyrosine-phosphorylated growth factor receptors and various effector proteins bound to the receptors through their SH2 domains. Such complexes would generate receptor-specific combinations of signals.

In light of these predictions, it was surprising that the 5F-EGFR retained what seemed to be a significant level of transmembrane signaling capability as judged by EGF stimulation of [3H]thymidine incorporation and of soft agar growth. The most straightforward explanation of these results is that a proportion of mitogenic signaling by the EGF receptor can occur through a mechanism not involving binding of SH2 domain-containing proteins to the activated receptors. Since EGF is still capable of stimulating tyrosine phosphorylation of some cellular proteins in cells expressing 5F-EGFR, some aspects of receptor signaling could be mediated through direct phosphorylation of protein substrates by the EGF receptor tyrosine kinase. The fact that mutant EGF receptors truncated at residue 973 (lacking all autophosphorylation sites) are capable of strong EGF-dependent mitogenic signaling (23, 25) supports the contention that in some contexts growth factor receptors and that interactions of SH2 domain-containing proteins will bind with relative high affinity to tyrosine-phosphorylated regions of receptors in some cells (3, 23). Experiments were performed to determine whether EGF binding to the 5F-EGFR could still elicit these activities (Fig. 4, A and B). Whereas WT-EGFR cells demonstrated significant EGF-dependent increases in these two assays, EGF was without effect on 5F-EGFR cells. In addition, EGF stimulated tyrosine phosphorylation of M_w = 150,000 PLC-γ1 along with some associated M_w = 170,000 EGFR in cells expressing WT-EGFR, but not in those expressing 5F-EGFR (Fig. 5).

**FIG. 4. Effects of EGF treatment on phosphatidylinositol 3-kinase activity and hydrolysis of phosphoinositides in cells expressing WT-EGFR or 5F-EGFR.** Two-day-old cultures were treated with vehicle or with indicated concentrations of EGF for 5 min prior to preparation of cells for determination of PI 3-kinase activity (A) or cellular levels of total inositol phosphates (B). Assays of PI 3-kinase and inositol phosphates were performed as described under "Experimental Procedures." Results are expressed as the mean of triplicate determinations ± standard deviation and are representative of three separate experiments.

**FIG. 5. EGF-stimulated tyrosine phosphorylation of PLC-γ1 in cells expressing WT-EGFR or 5F-EGFR.** Cells expressing WT-EGFR (WT) or 5F-EGFR (SF) were treated with EGF for 5 min prior to lysis and immunoprecipitation of lysates with anti-PLC-γ1 serum. Precipitated proteins were resolved on SDS gels, transferred to nitrocellulose paper, and immunoblotted with anti-phosphotyrosine antibody. Analyses were carried out as described under "Experimental Procedures."

EGF receptor kinase activity alone can promote cell proliferation. In addition, oncogenic v-erbB proteins contain various carboxyl-terminal deletions in which autophosphorylation sites are lost (26, 27). It has also been shown that removal of...
tyrosine 766 of the fibroblast growth factor receptor, which, when phosphorylated, binds PLC-γ1, has no effect on the ability of fibroblast growth factor to stimulate mitogenesis (28,29). Reports that kinase negative EGF receptors can activate the MAP kinase pathway also support the idea that the EGF receptor can function through mechanisms not involving binding of SH2 domain-containing proteins (30,31). Alternatively, 5F-EGFR did display some residual EGF-stimulated tyrosine phosphorylation (presumably on as yet unidentified tyrosine residue(s)) which could provide a threshold level of autophosphorylation allowing interaction of some SH2 domain-containing proteins with the receptor. This signaling would appear to include little, if any, of the activities of PLC-γ1 or PI 3-kinase.

Although 5F-EGFR retained substantial signaling ability, EGF failed to stimulate anchorage-independent growth of cells expressing 5F-EGFR to the same extent as in cells expressing WT-EGFR and failed to induce the typical transformed morphology seen with WT-EGFR cells. Previous work (32–35) indicates that triple and quadruple autophosphorylated tyrosine residues of the EGF receptor or on other as yet unknown processes.

In summary, these data suggest that the kinase domain of the EGF receptor may be responsible for generating some components of receptor signaling, whereas other aspects of receptor function may be dependent on interaction of SH2 domain-containing signaling proteins with tyrosine-phosphorylated regions of the EGF receptor or on other as yet unknown processes.

Acknowledgments—I thank Masa Omichi, Long Pang, and Alan Saltiel for helpful discussions and Tania Habib for valuable technical assistance.

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