Expression of Human Tyrosine Kinase-negative Epidermal Growth Factor Receptor Amplifies Signaling through Endogenous Murine Epidermal Growth Factor Receptor*

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Recent findings have suggested that certain ligand-dependent responses to EGF may be propagated in a manner that is not dependent on the intrinsic tyrosine kinase activity of the epidermal growth factor receptor (EGF-R, Campos-Gonzalez, R., and Glenney, J. R., Jr. (1992) J. Biol. Chem. 267, 14535–14538) or, alternatively, that these responses may occur through the interaction of the human tyrosine kinase-deficient EGF-R with an as yet unidentified kinase (Selvz, E., Raden, D. L., and Davis, R. J. (1993) J. Biol. Chem. 268, 2250–2254). These conclusions represent a significant departure from our current understanding of signal transduction by receptor tyrosine kinases. Therefore we examined the effect of expression of tyrosine kinase-negative human EGF receptor in murine NIH-3T3-2.2 cells on the EGF-dependent phosphorylation of mitogen-activated protein (MAP-2) kinase. In parental cells (NIH-3T3-2.2) that express low levels of endogenous murine EGF-R, there was no demonstrable EGF-dependent coupling to MAP-2 kinase. In NIH-3T3-2.2 cells transfected with tyrosine kinase-negative human EGF-R, there was unexpected EGF-dependent phosphorylation of MAP-2 kinase. Analysis of the tyrosine kinase-negative human EGF-R in these cells revealed significant tyrosine phosphorylation of the EGF-R. A low level of endogenous murine EGF-R present in these cells was also phosphorylated on tyrosine residues and displayed autokinase activity. Similar results were obtained using an unrelated cell line (B82L cells), in which EGF-dependent phosphorylation of MAP-2 kinase was previously attributed to signal propagation through a tyrosine kinase-negative human EGF-R (Campos-Gonzalez, R., and Glenney, J. R., Jr. (1992) J. Biol. Chem. 267, 14535–14538). Taken together, these results suggest that the tyrosine kinase-negative human EGF-R are able to amplify the response to activation of low levels of endogenous murine EGF-R, thus leading to EGF-dependent phosphorylation of MAP-2 kinase in cells expressing tyrosine kinase-negative human EGF-R.

The human epidermal growth factor receptor (EGF-R) is comprised of a single 170-kDa glycosylated polypeptide, which forms an extracellular ligand binding domain, an intracellular tyrosine kinase and autophosphorylation domain, and a connecting transmembrane sequence (1, 2). EGF-R exemplifies a class of ligand-stimulated receptor tyrosine kinases that serve both as activators and as targets for phosphorylation by kinases involved in mitogenic signaling (1–3). The binding of EGF to the extracellular domain results in an increase in tyrosine kinase activity and autophosphorylation by a mechanism involving receptor dimerization (Ref. 1 and references therein). A compendium of evidence has accumulated suggesting that the tyrosine kinase activity of the EGF-R is essential for many of the biochemical events that follow receptor activation (1, 2, 4, 5). Thus, mutations inhibiting the tyrosine kinase activity of the EGF-R have been reported to block signal transduction by the receptor, thereby leading to the hypothesis that the receptor tyrosine kinase directly mediates the process of signaling by the EGF-R (4, 5). However, recent studies have suggested the possibility that certain ligand-dependent responses of receptor tyrosine kinases might occur through a mechanism independent of the intrinsic tyrosine kinase activity of the receptor (6–11).

One such response is phosphorylation and activation of members of the mitogen-activated protein (MAP) kinase family (also known as ERKs; Ref. 12). These are a family of predominantly serine/threonine kinases, which are phosphorylated on both tyrosine and serine/threonine residues and activated in response to occupancy of the EGF-R and other receptor tyrosine kinases, as well as in response to other mitogens (e.g. tumor promoting phorbol esters). In one recent study (6), EGF-dependent phosphorylation of MAP-2 kinase was demonstrated in B82L cells that had been transfected with a human EGF-R defective in intrinsic tyrosine kinase activity by virtue of the substitution of methionine in place of lysine at position 721 in the cytoplasmic ATP binding domain of the receptor. The possibility of a pathway for propagation of a ligand-dependent signal directly through a tyrosine kinase-deficient EGF-R or, alternatively, through an interaction with another kinase potentially has profound implications for our overall understanding of growth factor action and represents a significant departure from previous studies regarding receptor tyrosine kinase signaling. Accordingly, we sought to clarify whether signal propagation resulting in phosphorylation of MAP-2 kinase could result from an

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1The abbreviations used are: EGF-R, epidermal growth factor receptor; ECL, enzyme chemiluminescence; EGF, epidermal growth factor; mAb, monoclonal antibody; MAP, mitogen-activated protein; ERK, extracellular regulated kinase; PAGE, polyacrylamide gel electrophoresis; DMEM, Dulbecco's modified Eagle's medium.
amplification of the ligand-dependent signal emanating from a small population of endogenous kinase-competent murine receptors as a result of the transfection of kinase-negative human EGF-R.

**EXPERIMENTAL PROCEDURES**

**Materials—**EGF (receptor-grade) and anti-phosphotyrosine antibodies 4G10 and 4G10 coupled to agarose were purchased from Upstate Biotechnology (Lake Placid, NY). Protein A coupled to Sepharose CL-4B was from Pharmacia LKB Biotechnology Inc. K2PO4 (1000 mM/cm), (γ-32P)ATP (4500 Ci/mmol), and anti-phosphotyrosine antibodies (PY20) were obtained from ICN. DMEM, calf serum and tissue culture reagents were purchased from Life Technologies, Inc. Antibodies linked to alkaline phosphatase or horseradish peroxidase and prestained molecular weight markers were obtained from Bio-rad. ECL developer was from Amersham Corp.

**Cell Culture and Stimulation—**NIH-3T3 clone 2.2 cells and K721A cells were generously provided by Dr. J. Schlessinger (Department of Pharmacology, New York University Medical Center, New York, NY) and have been described previously (4, 13). NIH-3T3-2.2 cells were shown to express undetectable amounts of endogenous murine EGF-R as assessed by ligand binding (4, 13). K721A cells were derived from NIH-3T3-2.2 cells and were transfected with a mutated construct of the human EGF-R such that lysine 721 in the ATP binding fold was replaced with alanine, thus eliminating its tyrosine kinase activity (4, 13). All cell types were maintained in DMEM containing 25 mM glucose supplemented with 50 units/ml penicillin, 50 μg/ml streptomycin, and 10% calf serum (Life Technologies, Inc.) in 5% CO2 in air at 37 °C. On day 0, cells were pelleted and resuspended in DMEM supplemented with 1 mM sodium orthovanadate and analyzed for phosphotyrosine-containing proteins or MAP-2 kinase as detailed below. Parental B2L cells, B2L cells transfected with wild type human EGF-R, and B2L cells transfected with M721 tyrosine kinase-negative mutant construct of the human EGF-R (M721 cells) were kindly provided by Dr. H. Elsholtz (C. H. Best Institute, Toronto, Canada) from Dr. G. Gill (University of California, San Diego, CA). These cells were grown in DMEM plus 10% calf serum as previously described (5, 17) and were stimulated with EGF (100 nM) or vehicle (50 μM acetic acid). Cells were washed twice, solubilized in SDS sample buffer containing 20 μM sodium orthovanadate, and analyzed for phosphotyrosine-containing proteins or MAP-2 kinase as described below. Parental NIH-3T3 cells, consistent with a deficiency of active endogenous murine EGF-R. In contrast, EGF-dependent phosphorylation of a 42-kDa protein was evident in the K721A cells. Similar results were obtained using several different anti-phosphotyrosine antibodies, including 4G10 and PY20; moreover, the phosphorylation of the 42-kDa protein was also evident following stimulation by phorbol myristate acetate (data not shown). This suggested that the 42-kDa protein might be a MAP-2 kinase. Accordingly, K721A cells were stimulated with vehicle or EGF, the phosphotyrosine-containing proteins were immunoprecipitated from cell lysates using anti-phosphotyrosine antibodies, and the immunoprecipitates were immunoblotted with an antibody to MAP-2 kinase. As shown in Fig. 1b, MAP-2 kinase was phosphorylated on tyrosine residues in an EGF-dependent manner in K721A cells. The faster migrating band corresponds to the 42-kDa band described in Fig. 1a and likely represents ERK2 (12). In addition, a more slowly migrating 45-kDa band was also resolved and was phosphorylated in an
EGF-dependent phosphorylation of the human EGF-R in the K721A cells was indeed evident. The level of EGF-dependent phosphorylation of the tyrosine kinase-deficient EGF-R in the K721A cells was approximately 23% of the EGF-dependent phosphorylation using the same number of HER cells transfected with wild type EGF-R. We also analyzed another murine-derived cell line (M721) expressing tyrosine kinase-negative receptors, in which EGF-dependent phosphorylation of MAP-2 kinase has been described (6). As shown in Fig. 2, this cell line also displayed EGF-dependent phosphorylation of the EGF-R, which was approximately 10% of the EGF-dependent phosphorylation of the corresponding wild type receptor from the same number of cells.

The EGF-dependent phosphorylation of the human EGF-R and of MAP-2 kinase in cells expressing tyrosine kinase-negative human EGF-R was unexpected. Although the parental NIH-3T3-2.2 clone chosen had been reported to be deficient in endogenous murine EGF-R on the basis of ligand binding experiments, the absence of immunodetectable EGF-R by immunostaining of cell lysates, and the absence of EGF-dependent signaling responses, nevertheless we considered the possibility of the presence of a small number of endogenous murine EGF-R that might be capable of participating in signal propagation following transfection with a large number of kinase-negative human EGF-R. Accordingly, lysates from EGF-stimulated NIH-3T3-2.2 cells were immunoprecipitated with anti-EGF-R antibody RK2 (21), which recognizes both the human and murine EGF-R, and the immunoprecipitates were immunoblotted with anti-phosphotyrosine antibodies. As shown in Fig. 3, using this experimental approach, endogenous EGF-R were indeed detected in NIH-3T3-2.2 cells, which are the parental cells for the K721A cell line. Similarly, endogenous EGF-R were also detected (although to a lesser extent) in the B82L cells, which are the parental cell line for M721 cells. There was an EGF-dependent increase in the detection of endogenous phosphorylated EGF-R in both NIH-3T3-2.2 and B82L cells. Thus receptors from NIH-3T3-2.2 cells gave an optical density reading of 0.008/mg of protein (basal) and 0.141/mg of protein (EGF), while the B82L cells gave an optical density reading of 0.020/mg of protein (basal) and 0.034/mg of protein (EGF).

We next sought to ascertain whether in K721A cells, evidence could be obtained for the presence of endogenous murine EGF-R, which could participate in EGF-dependent signal propagation. Lysates from K721A cells were immunoprecipitated with anti-EGF-R antibodies mAb 108 or RK2. Immunoprecipitated proteins were then separated, transferred to Immobilon, and immunostained with RK2 anti-EGF-R antibody. Fig. 4a demonstrates the presence of only one band with an electrophoretic mobility of 170 kDa corresponding to the human EGF-R in the mAb 108 immunoprecipitate, but the presence of two bands, one at 170 kDa and another at a slightly higher electrophoretic mobility, in the RK2 antibody immunoprecipitate. Since RK2 antibody is able to detect both murine and human EGF-R while mAb 108 preferentially detects human EGF-R (23), these results suggested that the slower migrating band in the RK2 antibody immunoprecipitate corresponded to endogenous murine EGF-R.

We next sought to determine the pattern of phosphorylation of the different populations of EGF-R in the K721A cells. Vehicle or EGF-treated cells were lysed and the lysates immunoprecipitated with either antibody RK2 or mAb 108, and then immunoprecipitated proteins immunoblotted with anti-phosphotyrosine antibodies. Fig. 4b shows a difference in the appearance of the phosphotyrosine-containing band corre-
The presence of vehicle (-) or EGF (+) for 3 min at 37 °C. Following cell lysis in buffer containing 1 mM sodium orthovanadate, EGF-R were immunoprecipitated with mAb 108 (monoclonal anti-EGF-R antibody) and analyzed by SDS-PAGE using a 7.5% separating gel. Following transfer to Immobilon, immunoprecipitated proteins were immunostained with the anti-phosphotyrosine antibody (α-P-tyr, 4G10) or a polyclonal anti-EGF-R antibody (RK2) and detected with horseradish peroxidase-conjugated secondary antibody followed by ECL development. 4 and 6 mg of total protein lysate was used for the K721A and M721 immunoprecipitates. The plots shown are representative of four separate experiments.

**Fig. 3. Identification of EGF receptors in NIH-3T3-2.2 and B82L cells.** NIH-3T3-2.2 (NIH2.2) and B82L cells from 15-cm dishes and were stimulated with EGF (100 nM) for 3 min at 37 °C. Following cell lysis in buffer containing 1 mM sodium orthovanadate EGF-R were immunoprecipitated from NIH-3T3-2.2 cell lysates (7 mg of protein) and B82L cell lysates (16 mg of protein) with a polyclonal anti-EGF-R antibody (RK2). The immunoprecipitated proteins were analyzed by SDS-PAGE in a 7.5% gel, transferred to Immobilon, and immunostained with the anti-phosphotyrosine antibody (4G10) and detected with horseradish peroxidase-conjugated secondary antibody followed by ECL development. The immunostained EGF-R was scanned in an LKB Ultrascan and the band intensity normalized with respect to total protein. The NIH-3T3-2.2 cells gave an optical density reading of 0.141/mg of total protein, and the B82L cells gave an optical density reading of 0.034/mg of total protein. The blot shown is representative of three separate experiments.

**Fig. 2.** EGF-induced tyrosine phosphorylation of human EGF receptors in K721A and M721 cells. Cells were stimulated in the presence of vehicle (-) or EGF (+) for 3 min at 37 °C. Following cell lysis in buffer containing 1 mM sodium orthovanadate, EGF-R were immunoprecipitated with mAb 108 (monoclonal anti-EGF-R antibody) and analyzed by SDS-PAGE using a 7.5% separating gel. Following transfer to Immobilon, immunoprecipitated proteins were immunostained with the anti-phosphotyrosine antibody (α-P-tyr, 4G10) or a polyclonal anti-EGF-R antibody (RK2) and detected with horseradish peroxidase-conjugated secondary antibody followed by ECL development. 4 and 6 mg of total protein lysate was used for the K721A and M721 immunoprecipitates. The plots shown are representative of four separate experiments.
Phosphorylation of Tyrosine Kinase-negative-EGF-R and MAP-2 Kinase

In K721A. Cells from 15-cm dishes were stimulated in the presence of vehicle (50 μM acetic acid, lanes 1 and 3) or EGF (100 nM, lanes 2 and 4) for 3 min at 37°C. Following cell lysis in buffer containing 50 mM NaF, 10 μg of protein from each lysate was used to immunoprecipitate the EGF-R with RK2 (polyclonal anti-EGF-R antibody, lanes 1 and 2) or mAb 108 (monoclonal anti-EGF-R antibody, lanes 3 and 4). The immunoprecipitated EGF-R were analyzed by SDS-PAGE in a 6% gel and immunostained with a polyclonal anti-EGF-R antibody (RK2) and detected with horseradish peroxidase-conjugated secondary antibody followed by ECL development. The blot is representative of three separate experiments. Panel b, EGF-induced tyrosine phosphorylation of mouse and human EGF receptors in K721A cells. Cells from 15-cm dishes were stimulated in the presence of vehicle (50 μM acetic acid, lanes 1 and 3) or EGF (100 nM, lanes 2 and 4) for 3 min at 37°C. Following cell lysis in buffer containing 1 mM sodium orthovanadate, 10 μg of protein from each lysate was used to immunoprecipitate the EGF-R with RK2 (polyclonal anti-EGF-R antibody, lanes 1 and 2) or mAb 108 (monoclonal anti-EGF-R antibody, lanes 3 and 4). The immunoprecipitated EGF-R were analyzed by SDS-PAGE in a 6% gel and immunostained with an anti-phosphotyrosine antibody (α-P-tyr, 4G10) and detected with horseradish peroxidase-conjugated secondary antibody followed by ECL development described under “Experimental Procedures.” The blot is shown is representative of three separate experiments. Panel c, in vitro autophosphorylation of the mouse and human EGF receptors in K721A cells. Cells from 15-cm dishes were stimulated in the presence of vehicle (50 μM acetic acid, lanes 1 and 3) or EGF (100 nM, lanes 2 and 4) for 3 min at 37°C. Following cell lysis 10 μg of protein from each lysate was used to immunoprecipitate the EGF-R with RK2 (polycional anti-EGF-R antibody, lanes 1 and 2) or mAb 108 (monoclonal anti-EGF-R antibody, lanes 3 and 4). The immunoprecipitated EGF-R were used in an in vitro autokinase assay, analyzed by SDS-PAGE in a 6% gel, and exposed to x-ray film for 24 h. The autoradiogram shown is representative of three separate experiments. The gel was exposed to storage phosphor plates for 2 h and scanned, and the bands corresponding to the human and mouse EGF-R were quantitated.

In the case of the EGF receptor, the SH2/SH3 domain-containing protein (Grb-2 physically associates through its SH3 domains with the activated EGF-R (28). Further steps in the signaling cascade include the engagement of a guanylnucleotide exchange protein (SOS), through the SH3 domain of Grb-2 followed by activation of Ras and then a downstream signaling cascade including Raf and MAP kinase/ERK kinase among others (29-32). Grb-2 is itself not phosphorylated, but tyrosine phosphorylation of the EGF receptor is required for physical association of Grb-2 and initiation of the signaling cascade (28). Accordingly, in the current study, it would be possible for the tyrosine kinase-negative human EGF-R to propagate a signal resulting in MAP-2 kinase phosphorylation so long as there was tyrosine phosphorylation of the tyrosine kinase-negative human EGF-R itself. Indeed, we found EGF-dependent phosphorylation of EGF-R in both K721A and M721 cells. These findings are not consistent with the previous report by Campos-Gonzalez and Glenney (6) in which it was reported that although two other high molecular mass proteins (170 and 185 kDa) were tyrosine-phosphorylated in M721 cells, they did not represent the EGF-R when analyzed with monoclonal antibodies to the receptor. However, the results in the current findings are consistent with a more recent report by Selva et al. (33) in which EGF-dependent tyrosine phosphorylation of EGF receptors was observed in Chinese hamster ovary cells that had been transfected with tyrosine kinase-negative human EGF receptors. The discrepancy in the ability to detect tyrosine-phosphorylated EGF receptors in an anti-EGF receptor immunoprecipitate from M721 cells in the current study, as compared to the inability to detect tyrosine-phosphorylated EGF receptors in M721 cells in the study of Campos-Gonzales and Glenney, may be attributed to the use of different antibodies to immunoprecipitate the EGF-R in our (mAb 108) versus the latter (mAb 74 and c11) studies. We have found that mAb 74 does not recognize the murine EGF-R and that in K721A cells, it does not immunostain the human tyrosine kinase-negative EGF-R, even though the latter undergoes tyrosine phosphorylation.

It was of interest to address the question of how human EGF-R are phosphorylated in an EGF-dependent manner in the K721 and M721 cells in the current study. Both Campos Gonzalez and Glenney (6) and Selva et al. (33) have suggested that an as yet unidentified kinase may be activated by an EGF-dependent conformational change in the EGF-R and may be the initial step resulting in phosphorylation and activation of MAP-2 kinase. This interpretation is contingent upon the assumption that these cell lines lack endogenous kinase-competent EGF-R. The presence of even a small number of endogenous kinase-competent EGF-R could result in EGF-dependent phosphorylation of the tyrosine kinase-negative human EGF-R and thereby provide a route for amplification of the signal resulting in phosphorylation of MAP-2 kinase. In RT-PCR studies using primers specifically designed to detect murine EGF-R, we found clear evidence for the presence of low but equivalent amounts of murine EGF-R transcript in both NIH-3T3-2.2 as well as K721A cells. This led us to seek evidence for immunodetectable murine EGF-R in these cells as well as in M721 cells. Immunoprecipitation with the polyclonal anti-EGF-R antibody RK2, followed by immunostaining with anti-phosphotyrosine antibodies allowed the detection of even the low levels of endogenous EGF-R present in these cells.

Further studies show that it was possible under appropriate

2 N. Hack, A. Sue-A-Quan, G. B. Mills, and K. L. Skorecki, unpublished observation.

3 H.-U. Simon and G. B. Mills, unpublished observation.
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Experimental conditions to separately identify in K721A cells bands of different electrophoretic mobility in immunoprecipitates using the RK2 anti-EGF-R antibody, which recognizes both murine and human EGF-R, whereas only human EGF-R was detected in immunoprecipitates using mAb 108. This led us to hypothesize that EGF-dependent heterodimerization of mouse-human receptors could occur in K721A cells, resulting in the heterologous tyrosine phosphorylation of the tyrosine kinase-negative human EGF-R. This is in agreement with recent reports providing clear evidence for heterodimerization and heterologous transphosphorylation of tyrosine kinase-negative EGF-R receptors as has been reported for HER-2/neu and the EGF-R (34), and a tyrosine kinase-negative FGR receptor and a tyrosine kinase-negative fibroblast growth factor receptor (35).

The findings in the current study are consistent with the postulate that, in K721A cells, EGF causes heterodimers to form between endogenous murine EGF-R and transfected tyrosine kinase-negative human EGF-R with transphosphorylation of the latter by the former. This results in amplification of the signal by making available a larger number of phosphorylated EGF-R for interaction with Grb-2 and consequent signal propagation. An alternative formulation is that proposed by Selva et al. (33), who postulate the presence of an as yet unidentified kinase, which is activated by an EGF-dependent conformational change in the kinase negative human EGF-R. Such a postulate predicts that if this kinase is cytosolic, then the EGF dependence of its activation requires that it recognize the state of dimerization of the EGF-R, which is itself an EGF-binding protein, and, hence, by definition, another class of EGF-R. We suggest that it is most likely that the results of these studies by Selva et al. might also be explained by the presence of endogenous hamster EGF-R, a possibility that cannot be thoroughly ruled out until studies are done using the appropriate reagents in these cells. Certainly, the absence of the endogenous EGF-R cannot be conclusively inferred simply on the basis of experiments using either radioligand binding, immunostaining of lysates with anti-EGF-R antibodies, studies of EGF-dependent signal propagation, or even analysis of EGF-R mRNA levels using probes or primers derived from the human EGF-R sequence. These approaches would have suggested that NIH-3T3-2.2 and B82L cells are devoid of endogenous EGF-R, but subsequent studies revealed this not to be the case.

The results of the current study suggest an alternative possibility aside from postulating the presence of an as yet unidentified kinase to explain EGF-dependent signal propagation in cells expressing tyrosine kinase-negative human EGF-R receptors. However, it should be noted that it is possible that the endogenous tyrosine kinase-competent EGF-R, even if they are present, do not play a role in MAP-2 kinase phosphorylation by the tyrosine kinase-negative EGF-R. In view of recent developments in our understanding of the early steps involved in EGF-R signal propagation, and the presumed importance of this pathway in mitogenic and other responses, further examination of signal propagation in cells expressing tyrosine kinase-negative human EGF-R in the absence of endogenous EGF-R is warranted.

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