Increased Expression of Epidermal Growth Factor Receptor Induces Sequestration of Extracellular Signal-Related Kinases and Selective Attenuation of Specific Epidermal Growth Factor-Mediated Signal Transduction Pathways

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Abstract

Increased expression of the epidermal growth factor receptor (EGFR) is common in cancer and correlates with neoplastic progression. Although the biology of this receptor has been the subject of intense investigation, surprisingly little is known about how increased expression of the wild-type EGFR affects downstream signal transduction in cells. We show that increasing the expression of the receptor results in dramatic shifts in signaling with attenuation of EGF-induced Ras, extracellular signal-related kinases (ERKs), and Akt activation, as well as amplification of STAT1 and STAT3 signaling. In this study, we focus on the mechanism of attenuated ERK signaling and present evidence suggesting that the mechanism of attenuated ERK signaling in EGFR-overexpressing cells is a sequestration of ERKs at the cell membrane in EGFR-containing complexes. Increased expression of the EGFR results in an aberrant localization of ERKs to the cell membrane. Furthermore, ERKs become associated with the EGFR in a physical complex in EGFR-overexpressing cells but not in control cells. The EGFR-ERK association is detected in unstimulated cells or on exposure to a low concentration of EGF; under these conditions, ERK activation is minimal. Exposure of these cells to saturating concentrations of EGF results in a decreased membrane localization of ERKs, a concomitant dissociation of ERKs from the EGFR, and restores ERK activation. A similar association can be detected between the EGFR and MEK1 in receptor-overexpressing cells, suggesting that multiple components of the ERK signaling pathway may become trapped in complexes with the EGFR. These findings can be demonstrated in cells transfected to express high levels of the EGFR as well as in cancer cells which naturally overexpress the EGFR and, thus, may be representative of altered EGFR signaling in human cancer.

Introduction

The epidermal growth factor receptor (EGFR) is a transmembrane receptor tyrosine kinase that is involved in the regulation of the proliferation, motility, and differentiation of a variety of cell types (1, 2). The engagement of the receptor by its ligand results in receptor dimerization, activation of the intrinsic tyrosine kinase activity of the receptor, and autophosphorylation of the receptor on tyrosine residues (3, 4). This results in the creation of docking sites for proteins bearing SH2 and PTB domains, which associate with the receptor (5). This interaction may result in the tyrosine phosphorylation and/or activation of these proteins generating signaling cascades that culminate in gene transcription and changes in the phenotype of the cell (6).

EGFR activation stimulates the exchange of GTP for GDP on Ras leading to its activation (7). Ras is known to be a key component in mitogenic signaling in certain cells and is also involved in oncogenesis (8). However, in other experimental systems, activated Ras has more complex effects, including growth arrest and modulation of cell death (9, 10).

Activation of Ras leads to the activation of MAP kinases, which are groups of evolutionarily conserved proteins linking cell surface receptors to critical intracellular targets (11, 12). Mitogen-activated protein kinase (MAPK) activity is regulated through the successive activation of a three-tiered cascade of kinases. The extracellular signal-related kinases (ERKs) are activated by the sequential activation of Raf and MEK kinases. A part of the intracellular pool of activated ERK translocates to the nucleus where it phosphorylates and activates transcription factors involved in mitogenic signaling. ERKs are involved in cell proliferation through phosphorylation of carbamoyl phosphate synthetase 2 (13), a rate-limiting enzyme in pyrimidine synthesis. ERKs may also promote cell-cycle progression by inactivating the cell-cycle inhibitory kinase MYT1, and by inducing Cyclin D1 induction (14, 15). ERK activation has also been implicated in protection from apoptosis (16). In certain

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cells, this may be accomplished through activation of RSK that, in turn, inactivates the proapoptotic protein BAD (17). The EGFR also induces the activation of protein kinase B/Akt which plays a key role in cell survival (18).

Another signaling cascade triggered by the activated EGFR is STAT signaling (19). Activation of the EGFR leads to the tyrosine phosphorylation and activation of STATs that translocate to the nucleus and induce transcription of target genes (20). EGFR-induced STAT3 activation has been implicated in growth of human tumors that express high levels of the receptor (21, 22).

Receptor endocytosis and degradation is an important mechanism for terminating signals generated by the activated EGFR (23, 24). Ligand-induced internalization of the EGFR is a saturable process and increases in the level of EGFR expression beyond a certain threshold lead to impaired internalization of the receptor (25, 26). A number of studies have suggested that internalization of the EGFR is not just a mechanism for signal attenuation but may also be required for generation of certain signals (27). For example, it has been reported that expression of a dominant negative dynamin mutant in HeLa cells results in attenuated EGF-induced ERK activation (28). However, subsequent studies have produced conflicting results regarding the importance of internalization of the EGFR in EGF-induced ERK signaling (29, 30). While debate continues regarding the importance of signaling by internalized EGFR, the evidence suggests that mitogenic signaling is generated primarily by activated receptors at the plasma membrane (31).

The EGFR is commonly amplified and expressed at high levels in cancer (32). The types of cancer that exhibit overexpression of the EGFR include glioblastoma multiforme, squamous cell carcinoma of the lung, head and neck carcinomas as well as gynecologic malignancies, among others. Amplification and/or overexpression of the EGFR can be detected in about 50% of anaplastic astrocytoma and glioblastoma multiforme while it is rare in low-grade gliomas (33). The common occurrence of high levels of wild-type and/or mutant EGFR in malignant neoplasms suggests a causal role in tumor progression. Furthermore, studies have also shown that experimentally increasing the level of wild-type EGF receptors in cells can lead to transformation (34, 35).

While it is known that the EGFR can stimulate growth of tumors, the downstream signals driving this growth are not well understood. We demonstrate that overexpression of the wild-type EGFR does not result in a global amplification of signals. We identify defects in the generation of several key downstream signals including activation of ERKs. In this study, we focus on elucidating the mechanism of attenuated ERK signaling in EGFR-overexpressing cells and present evidence suggesting that the mechanism of attenuated ERK signaling is sequestration of ERKs by the overexpressed EGFR at the cell membrane.

Results

Tyrosine Phosphorylation Is Robust and Persistent in Cells Overexpressing the EGFR

We began our study of signal transduction in cells expressing high levels of the EGF receptor by first examining whether overexpression alters EGF-induced tyrosine phosphorylation of the receptor. We compared tyrosine phosphorylation of the EGF receptor in Rat-1 cells to R1hER cells, which have been transfected to express high levels of the human wild-type EGF receptor (36). We used a low concentration of EGF (1 ng/ml) as well as a saturating concentration (100 ng/ml). The experiment was performed by immunoprecipitating the EGFR from cells followed by immunoblotting with a phosphotyrosine antibody. As can be seen in Fig. 1A, there is significant basal tyrosine phosphorylation of the EGFR in R1hER cells in the absence of exogenous ligand. This is consistent with previous studies that have shown constitutive tyrosine phosphorylation of the EGFR in cells expressing high levels of the receptor. Upon addition of low concentration of EGF, a slight increase in tyrosine phosphorylation of the EGFR can be detected in Rat-1 cells, while it is difficult to detect a significant increase in R1hER cells. At a higher EGF concentration (100 ng/ml), a robust increase in EGFR tyrosine phosphorylation can be detected in both types of cells (Fig. 1A). This experiment demonstrates that a significant level of EGF-induced tyrosine phosphorylation of the EGFR is preserved when the receptor is overexpressed. In R1hER cells, we can detect an increase in tyrosine phosphorylation of the EGFR when cells are exposed to an

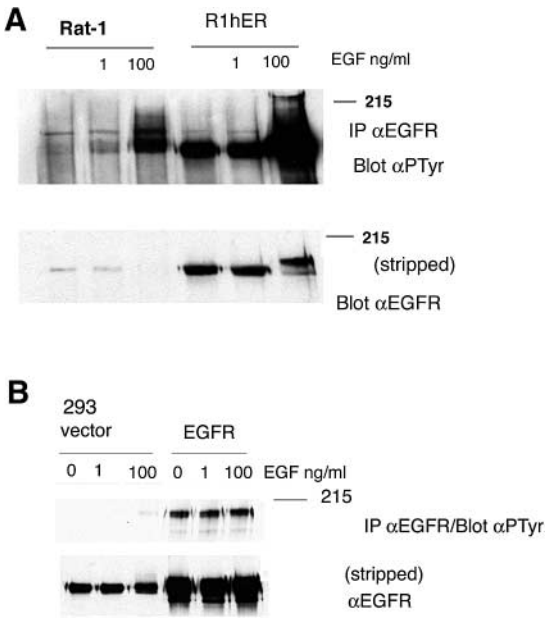

FIGURE 1. **A.** Tyrosine phosphorylation of the EGF receptor in Rat-1 cells and R1hER cells that express high levels of the human EGFR. In **A**, cells were serum starved in 100 mm dishes overnight, followed by EGF stimulation, with either 1 or 100 ng/ml for 5 min, followed by immunoprecipitation of cell lysates with EGFR specific antibodies and immunoblotting with phosphotyrosine antibodies (*upper panel*). The Western blot was stripped and reprobed with EGFR antibodies (*lower panel*). **B.** Tyrosine phosphorylation of the EGFR in 293 cells transfected with either an empty vector or with the human EGFR (10 μg) in 100 mm dishes. Twenty-four hours after transfection, cells were serum starved overnight, stimulated with EGF (1 or 100 ng/ml) for 5 min followed by immunoprecipitation of cell lysates with EGFR specific antibodies and immunoblotting with phosphotyrosine antibodies (*upper panel*). The Western blot was stripped and reprobed with EGFR antibodies (*lower panel*) to show loading and the relative levels of the receptor in EGFR transfected cells compared to cells expressing empty vector.

EGF concentration of 5 ng/ml or greater (data not shown). A time-course shows that tyrosine phosphorylation of the EGFR is quickly down-regulated in Rat-1 cells, whereas in R1hER cells, it persists unabated for prolonged periods of time (data not shown). A persistent tyrosine phosphorylation of the EGFR can also be detected in A431 cells, a widely studied tumor cell line which naturally expresses high levels of the EGFR (data not shown).

Next, we examined tyrosine phosphorylation of transiently expressed EGFR in 293 NX cells. These cells are derived from 293T cells and the transfection efficiency in these cells approaches 90% in our hands (data not shown). Transient overexpression of the EGFR in these cells results in a high level of constitutive tyrosine phosphorylation of the EGFR, and it is difficult to detect an increase in the total phosphotyrosine content of the receptor in response to ligand, whereas in vector-transfected cells, there is an increase in tyrosine phosphorylation is response to EGF (Fig. 1B). The lower panel in Fig. 1B shows relative EGFR levels in these cells. Using immunocytochemical staining with an anti-phospho-EGFR antibody, we find that following EGF stimulation, a significant proportion of the tyrosine-phosphorylated EGFR in cells overexpressing the receptor remains at the cell membrane for prolonged periods of time (data not shown).

The EGFR levels in the three cell types we have studied were similar. A431 cells express about 2×10^6 receptors, a level that has been reported in human cancer *in vivo*. For example, in a study of glioblastoma multiforme, a significant number of tumors were found to express EGFR levels equal to or greater than A431 cells (37). We transfected 293 cells to express an EGFR level that is similar to A431 cells. R1hER cells also express a level of EGFR similar to A431 cells (data not shown).

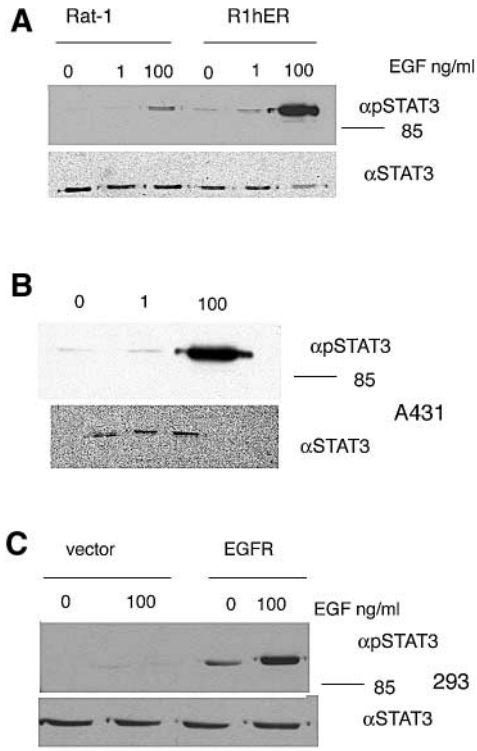

FIGURE 2. Activation of STAT3 assessed by anti-phospho-STAT3 antibodies in Western blots. Cell lysates were analyzed by electrophoresis followed by Western blotting. **A.** Level of activation of STAT3 in Rat-1 cells compared to R1hER cells after 5 min of EGF stimulation following overnight serum starvation. The Western blot was probed with anti-phospho-STAT3 antibodies in the *upper panel* that was stripped and reprobed with STAT3 in the *lower panel*. **B.** Same experiment in A431 cells. **C.** STAT3 activation in 293 cells transfected with either an empty vector or EGFR. The duration of EGF exposure was 5 min.

STAT Signaling Is Amplified in EGFR-Overexpressing Cells

Previous studies have shown that activation of the EGF receptor leads to the tyrosine phosphorylation and activation of STAT1 and STAT3. An increase in STAT3 activation has also been reported in tumor cells that naturally overexpress the EGF receptor, but have also undergone many other genetic alterations (22). We demonstrate that experimentally increasing the EGFR level also results in amplified STAT3 activation, both constitutively as well as in response to EGF. In our study, activation of STATs was examined using anti-phospho-STAT3 antibodies in Western blots. As can be seen in Fig. 2A, EGF-induced STAT3 activation is up-regulated in R1hER cells compared to Rat-1 cells. In R1hER cells, there is a constitutive activation of STAT3. Addition of EGF at a concentration of 1 ng/ml leads to a small additional increase in STAT3 activation, while high concentrations of EGF lead to a dramatic increase in STAT3 activation. In Rat-1 cells, there is no activation of STAT3 either constitutively or at low levels of EGF stimulation, whereas at high EGF concentration, a smaller increase in STAT3 activation can be detected. The activation of STAT3 is persistent in cells that express high levels of the EGFR, whereas it is transient in cells expressing lower levels of the EGFR (data not shown). We also detect STAT3 activation in A431 cells, which as in R1hER cells, is quite dramatic when cells are exposed to a high concentration of EGF (Fig. 2B). Transient overexpression of the EGFR in 293 cells also results in increased STAT3 activation that is constitutive as well as ligand induced (Fig. 2C).

Increased activation of STAT1 is also a feature of EGFR overexpression. As can be seen in Fig. 3A, activation of STAT1 can be detected in R1hER cells, but not in Rat-1 fibroblasts. However, in contrast to STAT3, we do not detect STAT1 activation constitutively or in response to low concentrations of EGF in R1hER cells. STAT1 activation in R1hER and A431 cells appears to require a high concentration of EGF (Fig. 3A and C). In 293 cells, we detect a low level of constitutive STAT1 activation consistent with the maximal constitutive tyrosine phosphorylation of the EGFR when overexpressed in these cells. Addition of EGF to these cells results in a significant further increase in STAT1 activation while STAT1 activation is not detectable in vector-transfected 293 cells (Fig. 3B).

EGF-Induced Activation of Akt Is Attenuated and Delayed in Cells Expressing High Levels of the EGFR

The activation of protein kinase B/Akt is a key survival signal generated by activated growth factor receptors and is dependent on PI 3-kinase activity. We find that Akt activation is

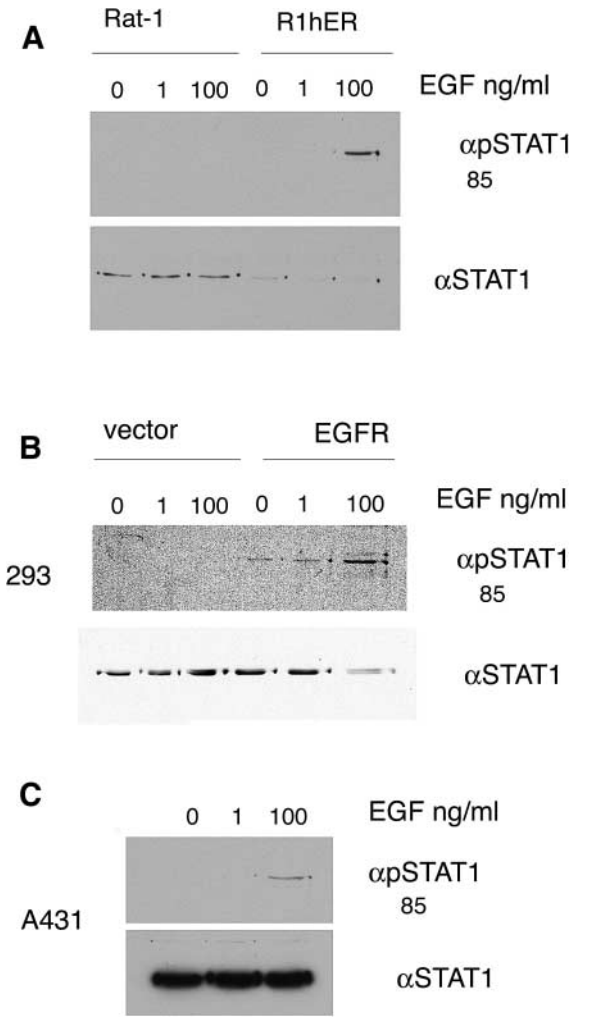

FIGURE 3. Activation of STAT1 assessed by anti-phospho-STAT1 antibodies. **A.** Activation of STAT1 in Rat-1 cells compared to R1hER cells after 5 min of EGF stimulation following overnight serum starvation. Cell lysates were analyzed by electrophoresis followed by Western blotting. The blot was probed with anti-phospho-STAT1 antibodies in the *upper panel* that was stripped and reprobed with STAT1 in the *lower panel*. **B.** STAT1 activation in 293 cells transfected with either an empty vector or EGFR. In 293 cells transiently overexpressing the EGFR, a low level of STAT1 activation is detectable constitutively. This is further increased by high concentrations of EGF. **C.** Same experiment in A431 cells. The duration of EGF exposure was 5 min.

significantly attenuated in cells that express high levels of the EGFR. This was examined using an anti-phospho-Akt antibody (S473) in Western blots. Fig. 4A shows that after a 5-min exposure to EGF, Akt activation is robust in Rat-1 cells, whereas in R1hER cells, it is highly attenuated even when a high concentration of EGF (100 ng/ml) is used. Similarly, EGF-induced Akt activation is highly attenuated in A431 cells (Fig. 4B, Rat-1 fibroblasts are shown as a positive control) and in 293 cells overexpressing the EGFR (Fig. 4C). Fig. 5 shows a time-course experiment comparing Akt activation in Rat-1 cells to R1hER cells. Akt activation is significantly delayed in R1hER cells. When stimulated with a high concentration of EGF (100 ng/ml), the peak of Akt activation is at 60 min in R1hER cells, whereas it is at 1 min in Rat-1 fibroblasts (Fig. 5C). A delayed peak of Akt activation cannot be detected when R1hER cells are stimulated with a low concentration (1 ng/ml) of EGF, with Akt activation remaining highly attenuated at all time points studied (data not shown).

Activation of Ras Is Altered in Cells Expressing Increased Levels of the EGFR

Exposure of cells to growth factors results in a rapid increase in GTP-bound Ras which is the activated form of the protein. Activated Ras binds to a downstream kinase Raf, which, in turn becomes activated. We examined the effect of EGF on Ras

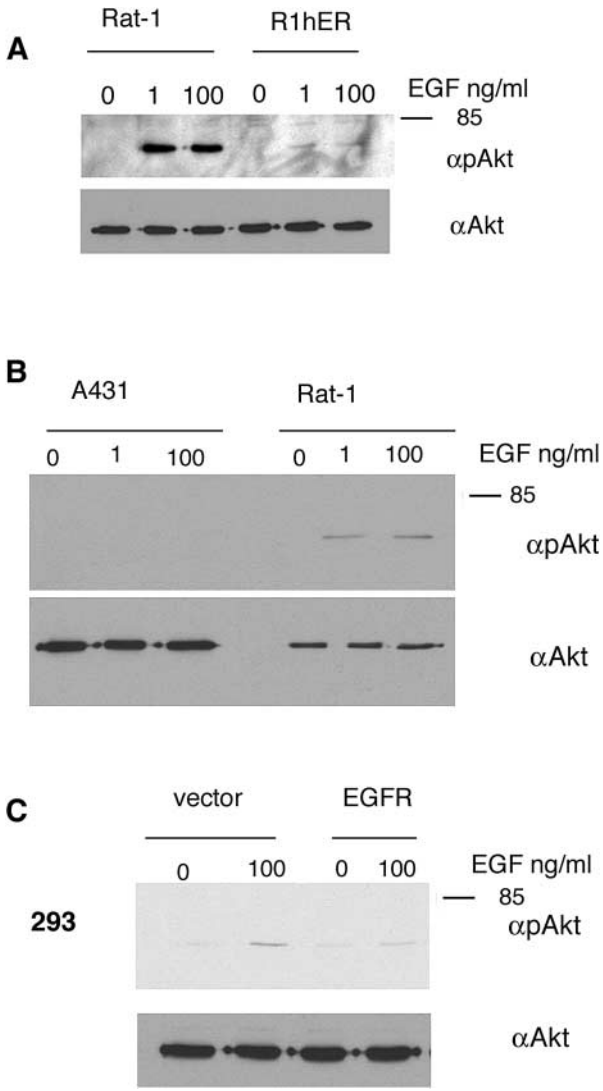

FIGURE 4. Activation of protein kinase B/Akt assessed by anti-phospho-Akt antibodies. **A.** Activation of Akt in Rat-1 cells compared to R1hER cells after 5 min of EGF stimulation following overnight serum starvation. Cell lysates were analyzed by electrophoresis followed by Western blot analysis. The blot was probed with anti-phospho-Akt antibodies in the *upper panel* that was stripped and reprobed with Akt antibodies in the *lower panel*. **B.** Same experiment in A431 cells. Rat-1 fibroblasts were run side by side as a positive control. **C.** Akt activation in 293 cells transfected with either an empty vector or EGFR. The duration of EGF exposure was 5 min. Akt activation is highly attenuated in cells that express high levels of the EGFR.

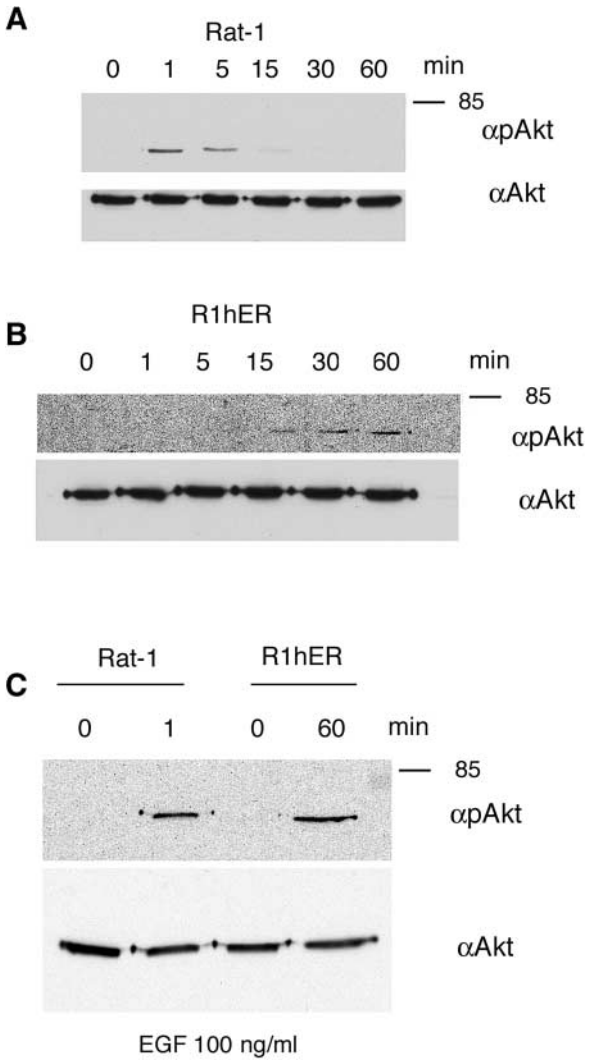

FIGURE 5. Time course of protein kinase B/Akt activation assessed by anti-phospho-Akt antibodies. **A.** Activation of Akt in Rat-1 cells at various time points after EGF stimulation (100 ng/ml) following overnight serum starvation. Cell lysates were analyzed by electrophoresis followed by Western blot analysis. The blot was probed with anti-phospho-Akt antibodies in the *upper panel* that was stripped and reprobed with Akt antibodies in the *lower panel*. **B.** Time course in R1hER cells. **C.** Akt activation is significantly delayed in R1hER cells when cells are stimulated with a high concentration of EGF (100 ng/ml).

loading of GTP in R1hER cells and compared this to Rat-1 fibroblasts. As shown in Fig. 6A, in Rat-1 cells, EGF-induced Ras loading is detectable almost equally at both low and high concentrations of EGF. While Ras loading is efficient at low concentrations of EGF in R1hER cells, surprisingly, stimulation with a high concentration (100 ng/ml) of EGF fails to activate Ras in these cells. Similar to R1hER cells, Ras activation is detectable at low concentrations of EGF in A431 cells and impaired at high EGF concentrations (Fig. 6B). In 293 cells also, transient EGFR overexpression leads to alterations in EGF-induced Ras loading. In these cells, however, activation is maximal at high concentrations of EGF (Fig. 6C), while it is attenuated at low EGF concentrations. The reason for this differential sensitivity to EGF concentration is not known but may reflect differences in cellular context. It is important to note that in all the three cell types where the EGFR is expressed at high levels, efficient activation of Ras is much more sensitive to concentration of EGF.

Fig. 7 shows a time course of Ras activation in R1hER cells. Ras activation is highly attenuated at all time points studied when these cells are stimulated with 100 ng/ml of EGF compared to 1 ng/ml.

ERK Activation Is Attenuated in Cells Expressing High Levels of the EGFR

The activation of the ERKs is an important step in growth factor-induced mitogenic signal transduction. We find that EGF-

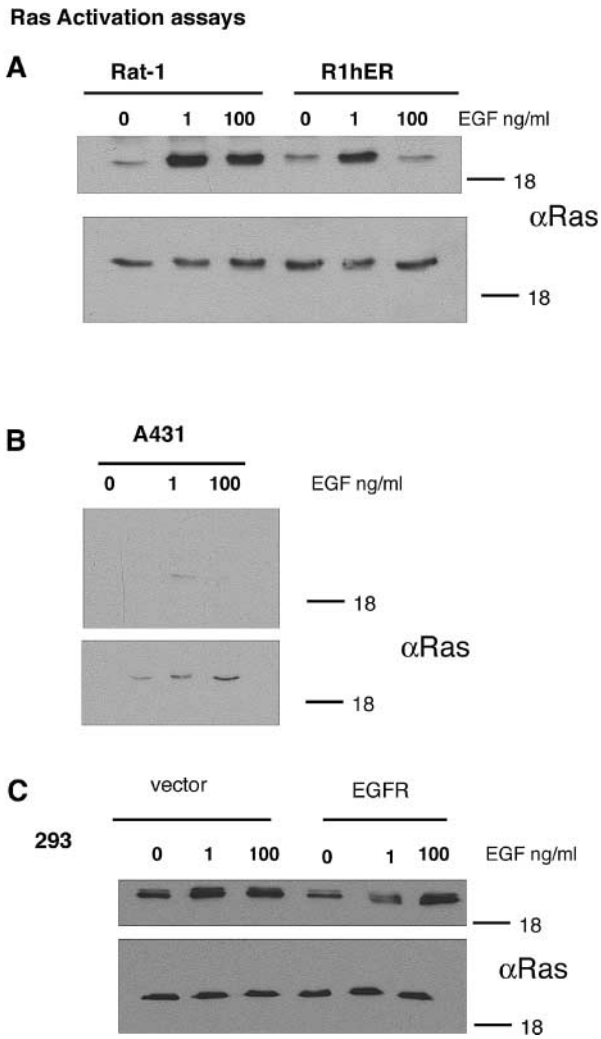

FIGURE 6. Ras activation assays in response to EGF. In **A** (*upper panel*), Rat-1 fibroblasts were compared with R1hER cells. The assay was performed as described in "Materials and Methods." Before incubation with Raf-RBD agarose, an aliquot was removed from the cell lysate and run on a separate gel to assess amounts of Ras in the cell lysates (*lower panel*). Both Western blots were probed with anti-Ras antibody. Ras activation is impaired in R1hER cells at high EGF concentrations (100 ng/ml). Cells were treated with EGF for 10 min. **B.** Ras activation in A431 cells. The experiment was otherwise identical to **A**. **C.** Same experiment in 293 cells transfected either with empty vector or with the EGFR.

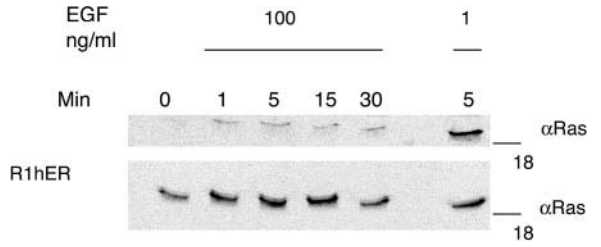

FIGURE 7. Ras activation in R1hER cells at different time points (*upper panel*). The experiment was conducted as described in Fig. 6. The *lower panel* shows an aliquot removed from the cell lysate and run on a separate gel to assess amounts of Ras in the cell lysates.

induced ERK activation is significantly attenuated in cells that overexpress the EGFR. ERK activation was determined by using an anti-phospo-ERK antibody in Western blots. This antibody recognizes phosphorylated forms of both ERK1 and ERK2. In Rat-1 fibroblasts, EGF-induced ERK activation is maximal at low concentrations of EGF (1 ng/ml) and does not increase significantly at a high EGF concentration (100 ng/ml). In R1hER cells, ERK activation is attenuated at all concentrations tested, but in contrast to Rat-1 cells, ERK activation improves significantly with increasing EGF concentration (Fig. 8A). While we do not have a control cell line for A431 cells for comparison, we find an increase in ERK activation with increasing EGF concentration similar to R1hER cells (Fig. 8B). In 293 cells transiently overexpressing the EGFR, ERK activation cannot be detected at low concentrations of EGF. However, a high EGF concentration compensates for this defect (Fig. 8C). These experiments demonstrate again that downstream signal transduction in cells overexpressing the EGF receptor depends critically on the concentration of ligand. Interestingly, while both Ras and ERK activation are optimal at high EGF concentrations in 293 cells transiently overexpressing the EGFR, there is a lack of correlation between Ras and ERK activation in both R1hER and A431 cells. In R1hER cells and A431 cells, Ras activation is optimal at low EGF concentrations while ERK activation is optimal at high EGF concentrations. This discrepancy may be explained by previous observations that EGF-induced ERK activation can be Ras dependent or independent (38).

Fig. 9 shows a time-course experiment of ERK activation in R1hER cells. No early peak of ERK activation can be detected in R1hER cells regardless of the concentration of EGF used (Fig. 9A and C). Rather, ERK activation is delayed in these cells. When a high concentration of EGF is used, ERK activation is more prolonged in R1hER cells compared to Rat-1 cells (Fig. 9B and C).

Increasing the Expression of the EGFR Leads to Aberrant Localization of ERKs

Because previous studies have invoked a role for receptor internalization in EGF-induced ERK activation, we considered the possibility that decreased ERK activation in EGFR-overexpressing cells could result from decreased internalization in response to ligand. While we find that internalization of the EGFR is indeed decreased in EGFR-overexpressing cells when examined as an internalized/surface ratio, the absolute levels of internalized radioactivity are significantly greater in EGFR-overexpressing cells (data not shown). This excludes a simple failure of ligand-induced internalization as the mechanism for diminished ERK activation.

We also considered the possibility that attenuation of ERK activation may result from constitutive activation of signaling pathways resulting from EGFR overexpression. R1hER cells were incubated overnight with a selective EGFR inhibitor (Tyrphostin AG 1478–200 μM) or DMSO. Cells were subsequently washed extensively, followed by stimulation with EGF and immunoblotting with phospho-ERK antibodies (Fig. 10A). Suppression of constitutive signaling did not result in an increase in ERK activation in these cells. A parallel experiment confirmed that treatment with Tyrphostin AG 1478 resulted in a near-complete suppression of the constitutive tyrosine phosphorylation of the EGFR in these cells (Fig. 10B). Thus, constitutive signaling does not appear a likely explanation for the attenuation of EGFR-induced signals in these cells.

Next, we examined the hypothesis that increased expression of the EGFR may lead to an altered localization of ERKs. Because effective signal transduction is critically dependent on a precise spatial localization of substrate molecules, aberrant localization could explain the defect in ERK activation noted in

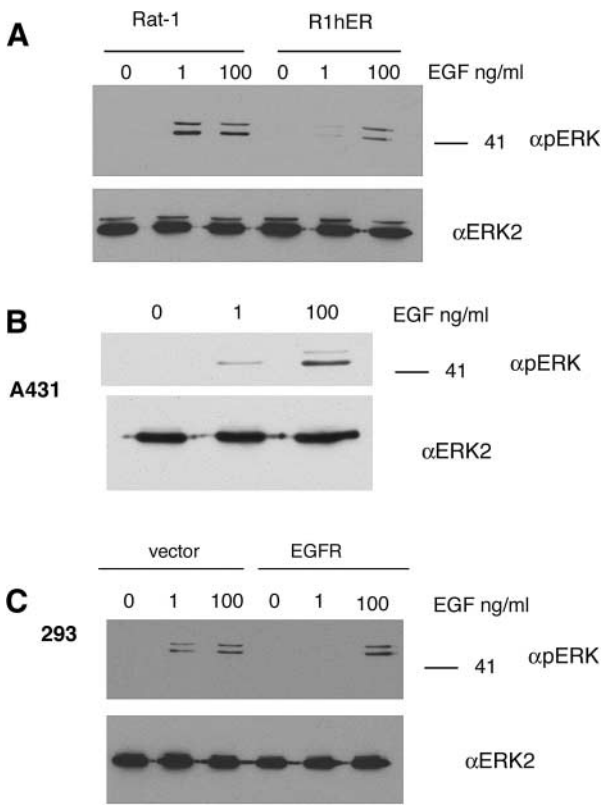

FIGURE 8. ERK activation in EGFR-overexpressing cells. In **A**, ERK activation in Rat-1 fibroblasts is compared to R1hER cells. Cells were cultured in 100 mm dishes, serum starved overnight, and treated with the indicated EGF concentrations for 5 min. Cells were lysed and analyzed by electrophoresis followed by Western blotting. The Western blot was probed with anti-phospho-ERK antibodies (*upper panel*). The blot was stripped and reprobed with ERK2 antibodies to show ERK levels. **B** shows the same experiment in A431 cells and **C** shows the same experiment in 293 cells transfected with either an empty vector or the EGFR.

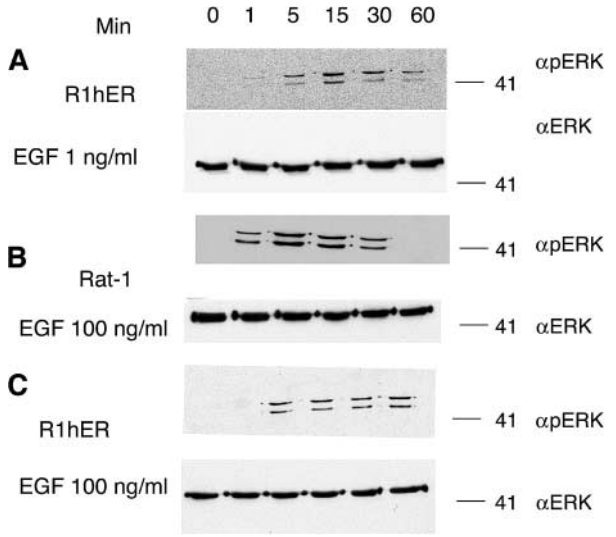

FIGURE 9. Activation of ERKs at different time points in R1hER cells in response to EGF at a concentration of 1 ng/ml (**A**) or 100 ng/ml (**C**). The experiment was conducted as described for Fig. 8. **B.** Time course for Rat-1 cells exposed to EGF 100 ng/ml. *Lower panels* show protein loading.

EGFR-overexpressing cells. A total of 293 cells was transiently transfected with Myc-tagged ERK1 along with either empty vector or EGFR, and the distribution of ERK was examined by immunocytochemical staining with anti-Myc antibodies. As can be seen in Fig. 11, Myc-ERK distribution is diffuse and cytoplasmic when Myc-ERK is co-expressed with the empty vector and we do not detect a significant cell membrane localization of Myc-ERK in these cells. In vector-transfected cells, it is difficult to detect the low level of endogenous EGFR by immunocytochemical staining. However, as shown earlier (Fig. 1B, *lower panel*), Western blotting can readily detect expression of the EGFR in these cells. In contrast to vector-transfected cells, increasing the expression of EGFR leads to a striking alteration in the localization of Myc-ERK (Fig. 11). While Myc-ERK is also present in the cytoplasm, there is a clear increase in membrane localization of Myc-ERK in these cells compared to vector controls. The membrane localization of ERK is detected both in unstimulated cells and also in cells stimulated with a low concentration of EGF (1 ng/ml). The merged images show that the EGFR and Myc-ERK co-localize to the cell membrane in EGFR-overexpressing cells under these conditions. As shown in Fig. 8C, ERK activation cannot be detected under these conditions in EGFR-overexpressing 293 cells, whereas there is substantial ERK activation in vector-transfected cells when a low concentration of EGF is used. Exposing EGFR-overexpressing cells to saturating concentrations of EGF (100 ng/ml) for 5 min results in a rapid decrease in the membrane localization of Myc-ERK as shown in Fig. 11, and restores ERK activation in these cells (Fig. 8C). Using immunocytochemical staining with an anti-phospho-ERK antibody, we find that the pattern of ERK activation is also abnormal in EGFR-overexpressing 293 cells (Fig. 12). In vector-transfected cells, exposure to EGF (100 ng/ml) leads to a diffuse cytoplasmic pattern of ERK activation. In contrast, in EGFR-transfected cells, a significant level of phospho-ERK staining is initially detected at the cell membrane. The membrane localization of phospho-ERK is rapidly lost on continued exposure to EGF, as shown in the time-course experiment.

Next, we examined the pattern of ERK activation in cells that stably express high levels of the EGFR. Fig. 13 shows the pattern of phospo-ERK staining in Rat-1 fibroblasts exposed to EGF (100 ng/ml). ERK activation is diffuse and cytoplasmic in Rat-1 cells. In contrast, in R1hER cells, phospho-ERK staining is initially localized to the membrane as well as the cytoplasm, and becomes mostly cytoplasmic with continued exposure to EGF. Stimulation of R1hER cells with a low concentration of EGF (1 ng/ml) results in a low level of phospho-ERK staining also along the cell membrane (data not shown).

Finally, we examined the pattern of ERK activation in A431 cells, a widely studied cancer cell line that naturally expresses high levels of the EGFR. As can be seen in Fig. 14, an aberrant pattern of phospho-ERK activation along the cell membrane can also be detected in these cells and is subsequently lost on continued exposure to EGF.

Thus, we find that increased expression of the EGFR leads to an aberrant membrane localization of ERKs. This localization is present under conditions where ERK activation is absent, *i.e.*, in unstimulated cells and on exposure to a low EGF concentration. The membrane localization of ERKs is lost and ERK activation is restored when a high concentration is used.

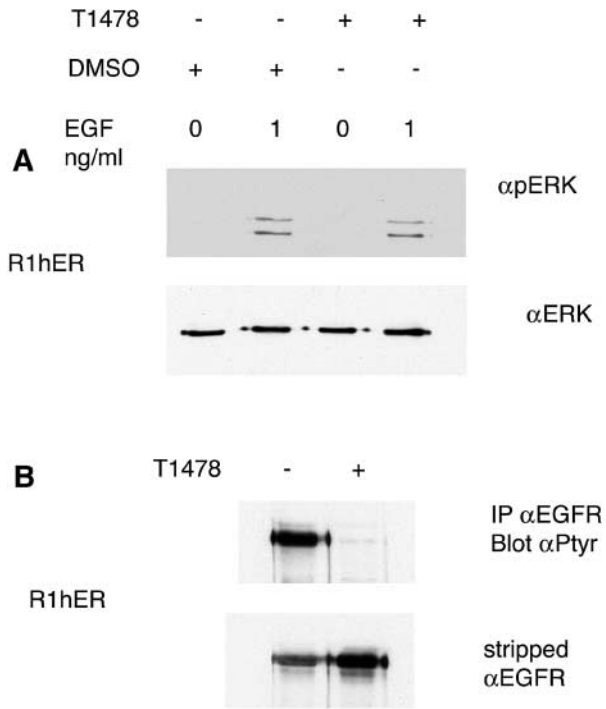

FIGURE 10. ERK activation in R1hER cells pretreated with Tyrphostin AG 1478 or vehicle (*DMSO*). **A.** Cells were preincubated overnight with Tyrphostin followed by three washes in PBS and EGF stimulation and immunoblotting of cell lysates with anti-phospho-ERK antibodies. **B.** Tyrosine phosphorylation of the EGFR in cells incubated with Tyrphostin AG 1478 or DMSO. Cell lysates were immunoprecipitated with anti-EGFR antibodies followed by immunoblotting with phosphotyrosine antibodies (*upper panel*). The *lower panel* shows EGFR loading.

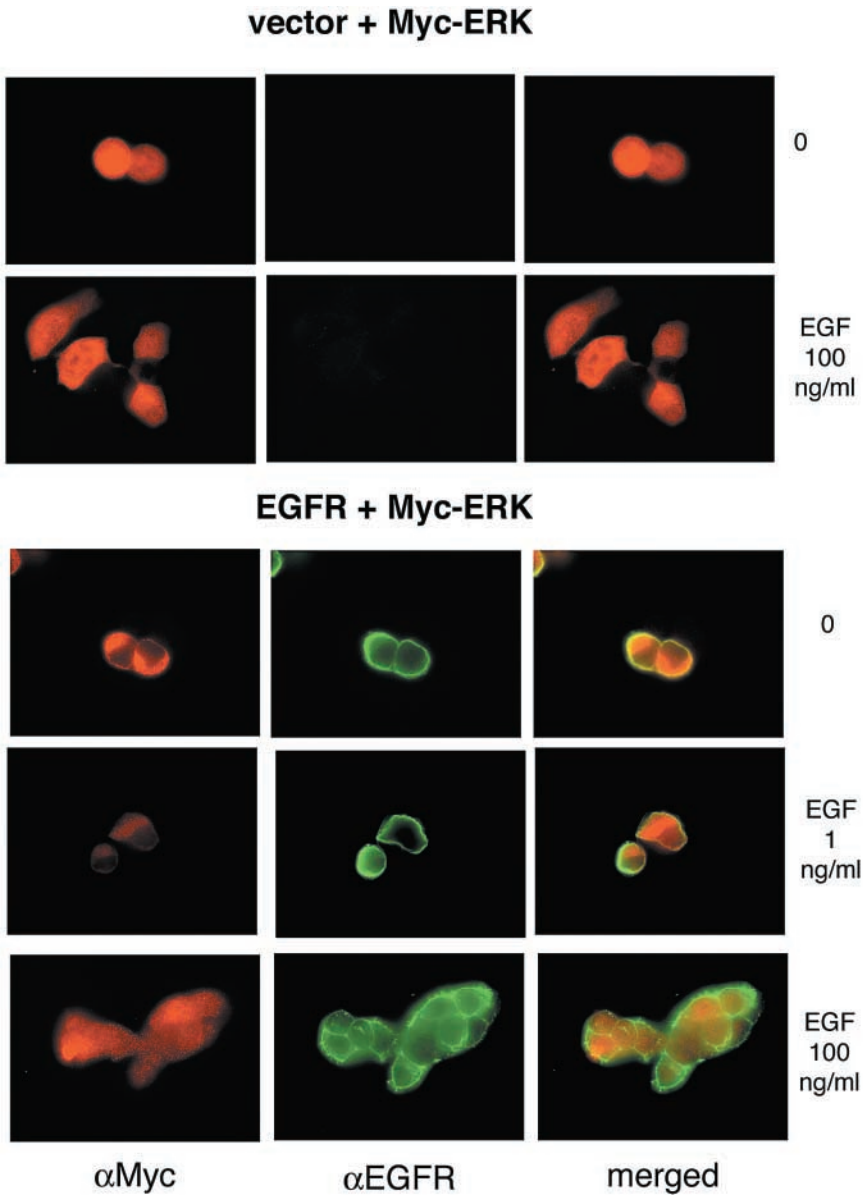

FIGURE 11. Localization of Myc-tagged ERK in 293 cells expressing either an empty vector or the EGFR. Cells were transfected with Myc-tagged ERK1 with either empty vector or the EGFR. Cells were serum starved overnight, followed by EGF stimulation and immunocytochemical staining as described in "Materials and Methods." The *left panel* was stained with anti-Myc antibodies and the *middle panel* with anti-EGFR antibodies. The *right panel* shows merged images.

ERKs Become Associated in a Physical Complex with the EGFR When the Receptor Is Expressed at High Levels: Saturating Concentrations of EGF Result in Dissociation of ERKs from the EGFR

When the EGFR is expressed at high levels, most of the receptor is localized to the cell membrane (Fig. 11). Because ERKs become localized to the cell membrane in cells overexpressing the EGFR, and exposing these cells to saturating concentrations of EGF results in both a loss of membrane localization of ERKs and restores ERK activation, we considered the possibility that the overexpressed EGFR may sequester ERKs in a physical complex at the cell membrane. Fig. 15A shows 293 cells that have been transiently transfected with either an empty vector or EGFR. Cell lysates were immunoprecipitated with an ERK2 antibody and the Western blot was probed with an EGFR antibody. As can be seen in Fig. 15A, the EGFR coimmunoprecipitates with ERK2 in cells that express high levels of the EGFR but not in vector-transfected cells. Importantly, the association is decreased when cells are treated with a high concentration of EGF. In 293 cells overexpressing the high levels of the EGFR, there may be a slight increase in EGFR-ERK association when cells are treated with a low concentration of EGF (1 ng/ml). It should be noted that in these cells, no ERK activation could be detected at this EGF concentration (Fig. 8C).

Next, this experiment was performed in R1hER cells. As demonstrated in Fig. 15B, the EGFR exists in a complex with ERK2 in R1hER cells but not in Rat-1 cells. The EGFR-ERK complex can be detected in unstimulated cells. When stimulated with a low level of EGF (1 ng/ml), there may be a small decrease

in EGFR-ERK association. As shown earlier in Fig. 8A, a low level of ERK activation can be detected in R1hER cells exposed to EGF at a concentration of 1 ng/ml. Exposure of cells to saturating concentrations of EGF results in a near-complete dissociation of EGFR-ERK (Fig. 15B), and substantial increases in ERK activation as shown earlier in Fig. 8A. As noted for R1hER cells, in A431 cells, a high concentration of EGF results in increased ERK activation (Fig. 8B), as well as dissociation of ERKs from the EGFR (Fig. 15C). The EGFR-ERK association in EGFR-overexpressing cells may be mediated directly or indirectly through other proteins.

MEK1 Becomes Associated with the EGFR in Overexpressing Cells

MEK1 and MEK2 are kinases that lie upstream and activate the ERKs by dual phosphorylation on tyrosine and threonine residues. To check if overexpression of the EGFR results in complex formation between the receptor and additional components of the ERK signaling pathway, we transfected 293 cells with the EGFR or empty vector. Cell lysates were then immunoprecipitated with a MEK1 antibody and the Western blot was probed with EGFR specific antibodies. As can be seen in Fig. 16, the EGFR coimmunoprecipitates with MEK1 in cells that express high levels of the EGFR but not in vector-transfected cells. Importantly, the association is decreased when cells are treated with a high concentration of EGF.

We do not detect alterations in the localization of MEK1 in EGFR-transfected cells. The experiment was conducted in 293 cells by transfecting HA-tagged MEK1 along with either empty vector or the EGFR followed by immunocytochemical staining with anti-HA antibodies. We find that MEK1 is localized both to the cell membrane as well as the cytoplasm and do not detect differences in the gross distribution of MEK1 as a result of EGFR expression (data not shown).

Discussion

A better understanding of signal transduction in cells expressing high levels of EGFRs is central to understanding how EGFR overexpression influences the biology of tumors. It is also critical for efforts to selectively inhibit signaling pathways as treatment for cancer. In this study, we show that increasing the expression of the EGFR does not result in a uniform alteration or amplification of downstream signals. Rather, increased EGFR expression appears to influence downstream signaling in a way that is specific and unique for each signal (or group of signals). For example, even among signals that are attenuated, differences can be detected. Ras activation is attenuated at high EGF concentrations while ERK activation is attenuated at low EGF concentrations. Akt activation is attenuated at low EGF concentration, while at high EGF concentrations, Akt activation is significantly delayed but not attenuated. These observations suggest that the mechanisms underlying these signaling changes are likely to be complex and multiple.

Increasing the level of wild-type EGFR results in a constitutive tyrosine phosphorylation of the receptor, presumably secondary to dimerization in the absence of exogenous ligand. Addition of EGF results in further increases in tyrosine phosphorylation of the EGFR. Trafficking of the EGFR is also altered in overexpressing cells with decreased internalization, increased recycling and prolonged membrane persistence of the activated receptor (23). In addition, increased expression of the receptors overwhelms mechanisms for dephosphorylation of receptors. All of these changes favor a state of unattenuated and persistent signaling. However, as noted, a number of downstream signals are actually attenuated and amplification of downstream signals is restricted to specific pathways.

ERK activation, a key component of mitogenic signaling in cells, is significantly attenuated when the EGFR is overexpressed in cells. In all the three cell types we have studied that overexpress the EGFR (293, R1hER, and A431), EGF-induced ERK activation is optimal when saturating concentrations of EGF are used. In R1hER cells and in 293 cells transiently overexpressing the EGFR, ERK activation is absent or highly attenuated compared to control cells when a low EGF concentration (1 ng/ml) is used. In A431 cells, for which we do not have a control cell type, ERK activation is low at low EGF concentrations and increases significantly when a high EGF concentration is used.

Efficient signal transduction is critically dependent on a

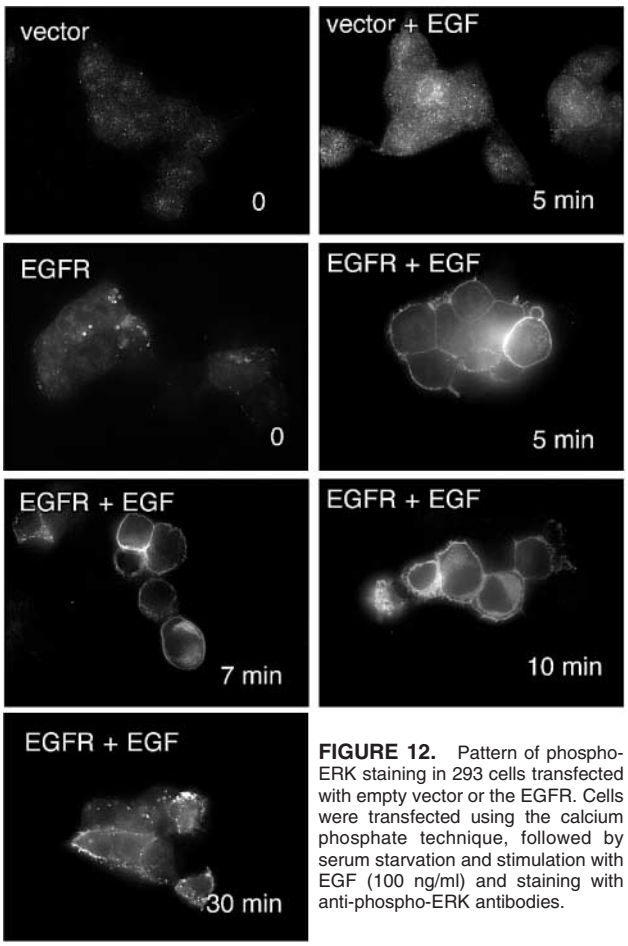

FIGURE 12. Pattern of phospho-ERK staining in 293 cells transfected with empty vector or the EGFR. Cells were transfected using the calcium phosphate technique, followed by serum starvation and stimulation with EGF (100 ng/ml) and staining with anti-phospho-ERK antibodies.

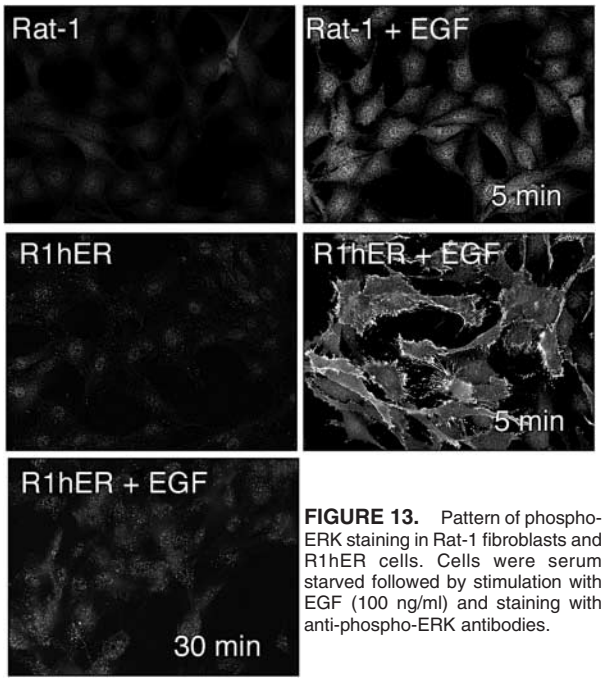

FIGURE 13. Pattern of phospho-ERK staining in Rat-1 fibroblasts and R1hER cells. Cells were serum starved followed by stimulation with EGF (100 ng/ml) and staining with anti-phospho-ERK antibodies.

precise spatial localization of signaling substrates. We demonstrate that increasing the expression of the EGFR results in an altered localization of ERKs to the cell membrane, whereas in normal cells, the distribution of ERK is diffuse and cytoplasmic. We show that addition of saturating concentrations of EGF results in a rapid decrease in membrane localization of ERKs. We also show that the pattern of ERK activation in these cells is initially localized to the cell membrane.

When the EGFR is expressed at high levels, most receptors are localized to the cell membrane (Fig. 11). ERKs also localize, in part, to the cell membrane in cells overexpressing the EGFR. Exposing these cells to saturating concentrations of EGF results in both a loss of membrane localization and restored ERK activation. Therefore, it is possible that the overexpressed EGFR may trap ERKs at the cell membrane in a physical complex, sequestering ERKs from signaling partners and thus preventing ERK activation. The EGFR-ERK association is likely to occur at the cell membrane because most of the EGFR is localized to the membrane, and we detect co-localization of the two proteins only at the cell membrane under conditions of low EGF stimulation. The sequestration hypothesis predicts that the EGFR exists in a physical complex with ERKs under conditions where ERK activation is low, *i.e.*, when ligand is absent or low. A second prediction is that when saturating concentrations of EGF that restore ERK activation are used, ERKs would dissociate from the EGFR. We find that these predictions are fulfilled in cells overexpressing the EGFR. In all the three cell types overexpressing the EGFR, the ERKs associate in a physical complex with the EGFR and in all cell types, ERKs dissociate from the EGFR when cells are exposed to high concentrations of EGF. Under conditions of absent or low EGF, ERKs remain associated with the EGFR, localized to the cell membrane, and ERK activation is attenuated. When exposed to saturating EGF concentrations, ERKs are released from the EGFR, there is a loss of membrane localization of ERKs, and ERK activation is restored. A similar association between EGFR and MEK1 can also be demonstrated in EGFR-overexpressing cells, suggesting that multiple components of the ERK signaling pathway could become sequestered in EGFR-containing complexes in these cells.

It could be argued that while Myc-ERK localizes to the cell membrane when co-expressed with the EGFR, a significant amount of Myc-ERK remains cytoplasmic and potentially available for activation. We propose that activation of cytoplasmic ERKs is impaired firstly, because the membrane-localized and -sequestered ERKs may exercise a dominant negative effect on activation of cytoplasmic ERKs. This hypothesis is supported by results of a previous study showing that membrane-targeted ERK chimeras exert a dominant negative effect on the activity of endogenous ERKs (39). Secondly, multiple components of the ERK signaling pathway may become trapped in complexes with the EGFR in these cells, as demonstrated by the EGFR-MEK1 association. However, not all components of the ERK signaling pathway form complexes with the EGFR in these cells. For example, we do not detect an association between Ras and the EGFR in these cells (data not shown).

In contrast, to the activation of ERK and Akt that are attenuated, STAT3 activation is significantly amplified in EGFR-overexpressing cells. EGFR-induced STAT3 activation may be a key factor driving the growth of tumors that express high levels of the wild-type EGFR because previous studies have shown that blocking STAT3 activation by antisense mechanisms, or by expression of a dominant negative STAT3 mutant, results in a decreased proliferation in certain EGFR-overexpressing cancer cells (22). STAT1 activation is also amplified on EGFR overexpression. However, STAT1 activation is detected only with high EGF concentrations. This argues against a role for STAT1 in tumor growth in these cells, assuming that the stromal content of EGF is low. Moreover, previous studies have suggested that STAT1 activation is not important in EGF-mediated

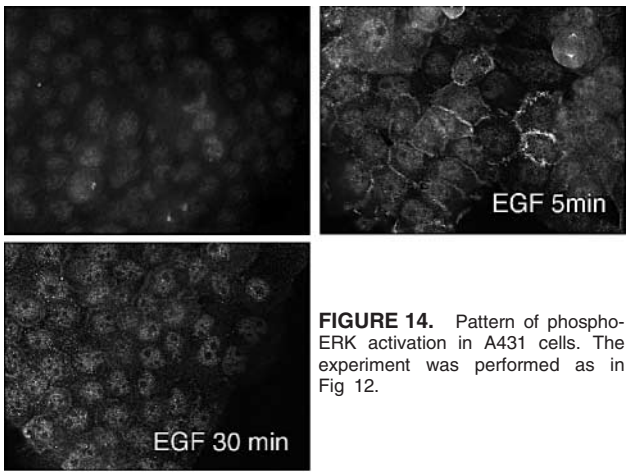

FIGURE 14. Pattern of phospho-ERK activation in A431 cells. The experiment was performed as in Fig 12.

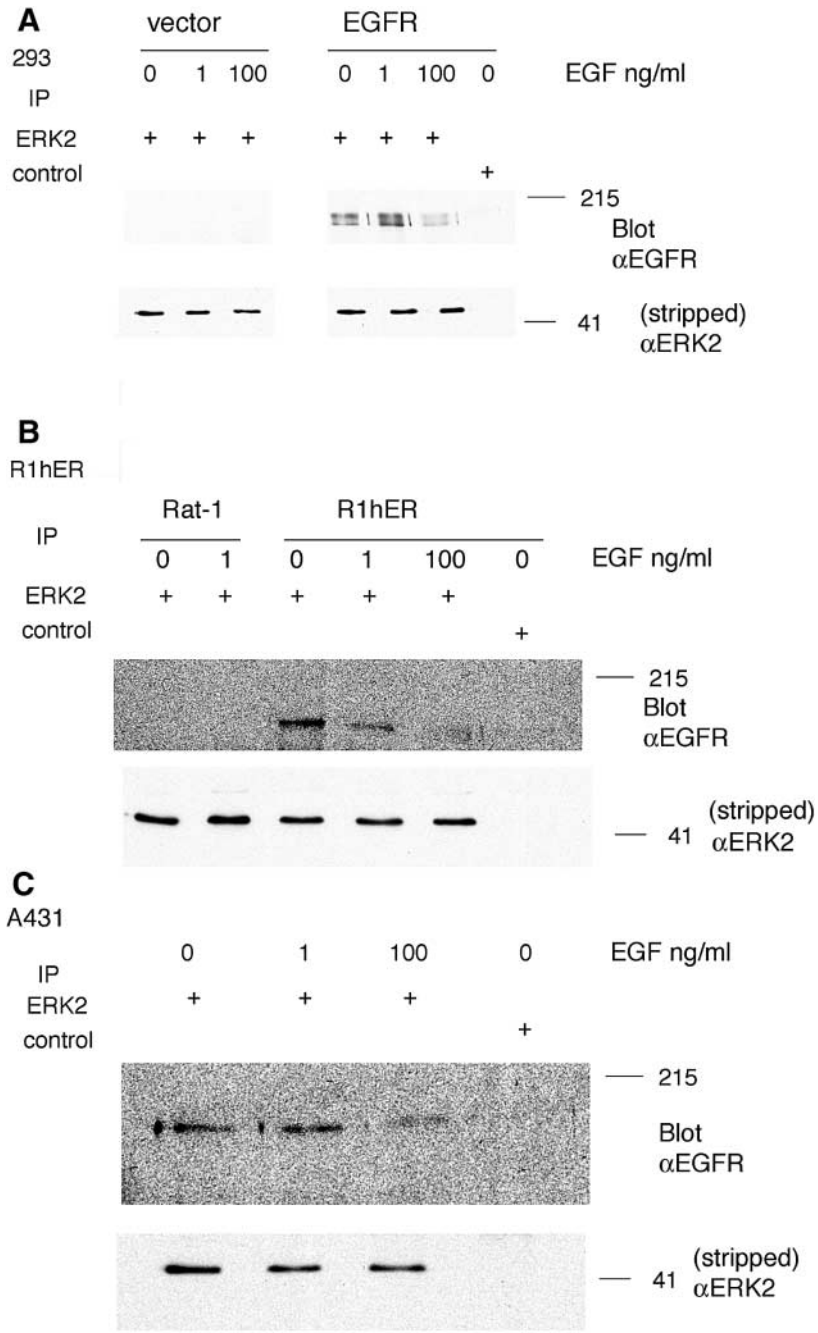

FIGURE 15. EGFR coimmunoprecipitates with ERK2 in cells which express high levels of the receptor. **A.** A total of 293 cells was transiently transfected with either the empty vector or the EGFR and treated with EGF (1 or 100 ng/ml) for 5 min. Cell lysates were subsequently immunoprecipitated with either ERK2 antibodies or isotype-matched control antibodies as indicated. This was followed by Western blotting followed by staining with anti-EGFR antibodies (*upper panel*) followed by stripping and reprobing with ERK2 antibodies (*lower panel*). **B.** Same experiment in Rat-1 cells compared to R1hER cells. The EGFR coimmunoprecipitates with ERK2 only in R1hER cells which are unstimulated or when stimulated with a low concentration of EGF (although the association is weaker). Exposure to high concentrations of EGF results in a dissociation of EGFR from ERKs. EGFR-ERK association is not detectable in Rat-1 cells that are unstimulated or stimulated with a low concentration of EGF. We have not found an EGFR-ERK association in Rat-1 fibroblasts treated with a high concentration of EGF (data not shown). **C.** EGFR-ERK association in A431 cells. The experiment was performed as described above for other cell types.

growth (22). Previous studies have also shown that overexpression of the EGFR also favors activation of the transcription factor NF-κB that may also influence survival and proliferation of cells (40, 41).

An interesting general principle that emerges from these studies is that in cells expressing a high level of the EGFR, downstream signaling is strongly influenced by the concentration of EGF in the medium. In contrast, in cells where the EGFR is expressed at lower levels, signal transduction tends to be more uniform over a broad range of EGF concentration. The different signals generated in response to varying concentrations of ligand may help to explain the "paradoxical" effects of EGF stimulation in cells overexpressing the EGFR (42). While expression of high levels of EGFR in cells is known to result in increased growth, a number of studies have also reported that these cells undergo apoptosis in response to EGF (43–46). Previous *in vitro* studies have also indicated that a low level of EGF stimulation leads to mitogenesis in EGFR-overexpressing cells while a high concentration leads to growth suppression (47). We have also found a dose-dependent effect

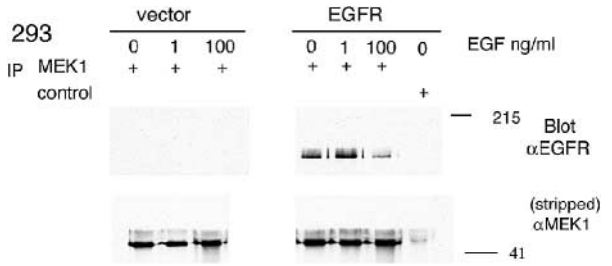

FIGURE 16. EGFR coimmunoprecipitates with MEK1 in cells that express high levels of the receptor. A total of 293 cells was transiently transfected with either the empty vector or the EGFR and treated with EGF as indicated for 5 min. Cell lysates were subsequently immunoprecipitated with either MEK1 antibodies or with isotype-matched control antibodies as indicated. This was followed by Western blotting followed by staining with anti-EGFR antibodies (*upper panel*) followed by stripping and reprobing with MEK1 antibodies (*lower panel*). Exposure to high concentrations of EGF results in a dissociation of EGFR from MEK1.

of EGF in inducing apoptosis in R1hER cells.[1] The present study provides clues to understanding the signaling mechanisms that may mediate these divergent biological outcomes. While the stromal concentration of ligand in EGFR-overexpressing tumors is difficult to determine, the plasma concentration of EGF tends to be low, around 1 ng/ml (48). A low concentration of EGF in the stroma would lead to activation of STAT3 and Ras, signals that would favor growth of tumors. Under these conditions, use of a specific antibody which blocks the activity of the EGF receptor would inhibit growth of tumors (49). When cells are exposed to high concentrations of EGF, signals that favor cell death may become activated. For example, STAT1 may become activated only on exposure to a high concentration of EGF (Fig. 4). Previous studies have postulated a role for STAT1 in EGFR-induced as well as other types of apoptosis (44, 50, 51). Another key factor favoring apoptosis may be the lack of Ras activation at high EGF concentrations in EGFR-overexpressing cells. We have previously shown that blocking Ras activation in R1hER cells using a dominant negative Ras mutant leads to a dramatic potentiation of EGF-induced apoptosis (46). The attenuation and delay of Akt activation is also likely to render these cells susceptible to apoptosis, because Akt is a known key anti-apoptotic signal. Similarly, ERK activation, which is also attenuated and aberrant in EGFR-overexpressing cells, is antiapoptotic. Thus, when cells are exposed to high concentrations of EGF, STAT1 activation combined with attenuation of Ras, ERK, and Akt signaling may tilt the balance toward apoptosis. It could be argued that in 293 cells transiently overexpressing the EGFR, STAT1 activation is constitutive, although it increases when cells are exposed to a high concentration of EGF. The constitutive STAT1 activation in these cells could be explained by the maximal constitutive tyrosine phosphorylation of the EGFR on transient EGFR overexpression in these cells. The constitutive activation of STAT1 is also consistent with our previous finding that transient overexpression of the EGFR results in a ligand-independent apoptosis in 293 cells (46).

[1]A. A. Habib, unpublished observations.

The EGFR (ErbB1) is part of the ErbB family of receptor tyrosine kinases which also includes ErbB2, ErbB3, and ErbB4 (52). These receptors can homodimerize or heterodimerize in response to ligand and the downstream signal transduction events that result depend on the particular homodimer or heterodimer formed. Among inter-receptor interactions, ErbB2-containing heterodimers may be formed preferentially (53, 54). Increased expression of the EGFR could result in alterations in the levels of other ErbB receptors, or result in altered patterns of heterodimer formation that influence downstream signaling.

Previous studies have reported that ErbB2 and ErbB3 are not expressed in 293 cells (55, 56). We have not found significant expression of either ErbB3 or ErbB4 in 293 cells by Western blotting, while we can detect a low level of ErbB2 expression (data not shown). Similarly, we do not detect ErbB3 or ErbB4 in Rat-1/R1hER cells, and detect a low level of ErbB2 expression. However, increased expression of the EGFR does not influence ErbB2 levels in either cell type. A431 cells have been reported to express ErbB2 and ErbB3 but not ErbB4 (57). The formation of certain heterodimers may favor specific downstream signals. For example, PI 3-kinase couples directly with ErbB3 and ErbB4, but indirectly with ErbB1 and ErbB2 (58). It is (to our knowledge) unknown if a particular ErbB receptor heterodimer favors STAT activation. It is also unclear whether formation of a particular heterodimer results in attenuation of specific signals (compared to homodimer formation). Previous studies have also documented the production of TGFα by A431 cells, suggesting that some of the constitutive activation of the EGFR in overexpressing cells may result from autocrine production of ligand (59). In addition, mRNA for Amphiregulin, another EGFR ligand, has also been detected in A431 cells (60). To summarize, it does not appear that the alteration in signal transduction resulting from increased expression of the EGFR can be readily explained by an influence on other members of the ErbB receptor family. However, further studies are necessary to elucidate this question.

Although we have started to identify signaling alterations that result from overexpression of the wild-type EGFR, a number of questions require further investigation. For example, it is unknown whether the EGFR-ERK interaction is direct or mediated indirectly via other proteins. It is also unknown how a saturating concentration of EGF results in a dissociation of ERKs from the EGFR. This could be related to conformational changes in the EGFR on maximal stimulation, or it could be mediated through recruitment and/or tyrosine phosphorylation of other proteins. Further studies are also needed to determine the biological consequences of attenuated ERK signaling in EGFR-overexpressing tumors.

Materials and Methods

Cell Lines, Reagents, and Transfection

A431 cells were obtained from American Type Culture Collection. R1hER cells were obtained from Dr. Michael Weber (Charlottesville, VA). 293 NX cells were obtained from Dr. Garry P. Nolan (Stanford, CA). These cells were used for all transient transfection experiments in this study. These cells are derived from 293T cells and are highly transfectable. A human EGFR construct cloned into pcDNA 3.1 has been described

previously (61). A wt MEK1 cDNA was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). An anti-phospho-ERK antibody was obtained from Promega, Madison, WI (Cat. No. V8031) and used for Western blotting. An anti-phospho-ERK antibody (Cat. No. 9101) was obtained from Cell Signaling Technology (Beverly, MA) and used for immunocytochemical staining. Anti-phosphotyrosine (PY20) and MEK1 antibodies were obtained from BD Transduction Labs, Lexington, KY. Sheep polyclonal anti-EGFR antibodies (06-129), anti-phospho-STAT3 (05-485), anti-STAT3 (06-596), anti-phospho-Akt antibodies (Ser 473, 06-801), anti-STAT1 (06-501), and anti-phospho-EGFR antibodies (05-483), anti-ErbB2 (06-562), anti ErbB3 (05-390), ErbB4 (06-572) were obtained from Upstate Biotechnology. Anti-Myc (9E10 sc-40), anti-phospho-STAT1 (sc-8394), anti-Akt (sc-8312), and anti-ERK2 (sc-154 and sc-1647), ErbB3 (sc-7390) and ErbB4 (sc-283) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-HA antibodies were purchased from Roche Molecular Biochemicals (Indianapolis, IN). Tyrphostin AG 1478 was purchased from Calbiochem (San Diego, CA).

Transfections were done using the calcium phosphate technique using standard protocols (62) and expression of transfected genes was confirmed by Western blotting. For transient transfection experiments, cells were harvested 48 h after transfection.

Western Blotting and Immunoprecipitation

Standard protocols were used for immunoprecipitation and Western blotting (61, 62). A Bio-Rad detergent-compatible protein assay kit (Bio-Rad, Hercules, CA) was used for quantitation of proteins. For immunoprecipitation, cells were lysed in a lysis buffer containing 150 mM NaCl, 1% Nonidet P-40, 0.25% deoxycholate, 1 mM EGTA, 1 mM NaF, 50 mM Tris-HCl, 1 mM phenylmethylsulfonyl fluoride, and 2 mM orthovanadate, and equal amounts of protein were incubated with the primary antibody for 90 min. Protein A-agarose or Protein G-agarose beads were subsequently added to the lysates and incubated overnight at 4°C. The beads were subsequently washed and solubilized in SDS sample buffer and then boiled and analyzed by SDS-PAGE followed by transfer to nitrocellulose. Western blots were developed with enhanced chemiluminescence reagents (Amersham, Piscataway, NJ). In experiments where cell lysates were examined directly, cells were lysed in lysis buffer and spun down. Subsequently, SDS sample buffer was added to the samples, followed by boiling and the samples were subjected to SDS-PAGE, followed by Western blotting.

Ras Activation Assays

A Ras Activation Assay kit was obtained from Upstate Biotechnology, and the experiment was conducted according to the manufacturer's protocol. Briefly, cells were serum starved overnight, followed by growth factor stimulation. Cells were lysed followed by addition of Raf-1 Ras-binding domain (RBD) agarose to cell lysates followed by incubation at 30 min at 4°C. Beads were washed three times and the bound Ras was detected by incubation in SDS sample buffer and boiling followed by electrophoresis and Western blotting with an anti-Ras antibody (clone RAS 10).

Immunocytochemical Staining

Cells growing on coverslips coated with poly-L-ornithine (Sigma, St. Louis, MO) were fixed with 4% paraformaldehyde for 5 min, at room temperature. Coverslips were rinsed three times with PBS and then permeabilized in 0.1% Triton X-100-containing PBS for 10 min. Cells were incubated with primary antibodies in 0.1% Triton X-100 and 1% BSA containing PBS for 1 h at room temperature. After being washed with PBS three times, the coverslips were incubated with FITC or Cy 3-labeled anti-mouse or anti-rabbit antibody for 1 h at room temperature. The cells were rinsed three times with PBS, mounted on the glass slides using mounting media and viewed on a Nikon Eclipse microscope.

Internalization of I^{125} EGF

Internalization of I^{125} EGF was performed essentially as described previously (63). I^{125} EGF was purchased from Amersham. Cells were cultured in 24-well dishes and serum starved overnight. Subsequently, I^{125} EGF (1 ng/ml) was added to cells in binding medium (DMEM, 0.1% BSA, 20 mM HEPES) for the times indicated. Cells were rapidly washed three times in cold DMEM to remove unbound ligand and then incubated for 5 min with 0.2 M acetic acid (pH 2.8) containing 0.5 M NaCl at 4°C. Following the acid wash, cells were lysed in 1N NaOH. The ratio of internalized and surface radioactivity was plotted against time. Nonspecific binding was measured for each time point in the presence of 100-fold molar excess of unlabeled EGF and did not exceed 5% of the total counts.

Acknowledgments

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