Epidermal Growth Factor Stimulates the Synthesis of Its Own Receptor in a Human Breast Cancer Cell Line*

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The MDA 468 human breast carcinoma cell line was examined for changes in epidermal growth factor (EGF) receptor synthesis and degradation under the influence of EGF. This cell line was used because it overexpresses the EGF receptor such that each cell has 10^9 receptors, but unlike the well-studied A431 cell, its receptor gene is amplified but is not rearranged. On exposure to EGF, total cellular receptor protein, measured by immunoprecipitation with monoclonal antibody B1D8, is reduced. The half-life of receptor metabolically labeled with L-[35S]methionine is 24 h in the absence of EGF and is reduced to 12 h in the presence of 10^{-9} M EGF. To measure the effect of EGF on synthesis of the receptor, pulse labeling conditions were selected in which the rate of synthesis of the receptor-precursor were followed. EGF had no significant effect on the rate of general protein synthesis in these cells, yet stimulated the synthesis of the EGF receptor 1.8-fold over the unstimulated rate. This increase in receptor-precursor synthesis showed time and dose dependence. Stimulation could be detected after 3 h exposure to EGF with a maximum at 6-8 h. A concentration of 10^{-11} M EGF gave detectable stimulation with maximal stimulation occurring at 10^{-9} M. Longer times and higher concentrations gave submaximal stimulation. A similar dose-response relationship was observed when the rate of mature 170-kDa receptor protein synthesis was measured. These studies demonstrate that EGF stimulates the synthesis of its own receptor. Down-regulation of the receptor by EGF results from an increased rate of receptor degradation and not decreased synthesis.

The cell surface receptor for EGF and related TGF plays an essential role in signal transduction between the exterior and interior of the cell. The receptor is a 170-kDa protein and consists of an external, heavily glycosylated domain capable of recognizing the growth factor and an internal domain with tyrosine-specific protein kinase activity linked by a 23-amino acid transmembrane bridge (1, 2). The activation of the protein kinase by growth factor is thought to initiate the mitogenic signal. Shortly after binding growth factor, the receptor clusters on the cell surface and then is internalized (3, 4). After internalization, the receptor is degraded in lysosomes (5-7) and it is this degradation that is believed to result in the down-regulation of cell surface receptors.

Recently, we found that a melanoma cell line that secretes an EGF-like TGF had no detectable receptor protein nor receptor mRNA. One explanation for this observation might be that ligand binding to the receptor through an autocrine route ultimately suppresses receptor expression. In addition to increased degradation of receptor following EGF binding, decreased synthesis might be another mode of down-regulation.

The effect of EGF on the synthesis of its receptor in A431 cells has previously been studied employing a heavy isotope method (8) or L-[35S]methionine labeling of the receptor (9, 10). Results showed no change in receptor synthesis rate upon exposure to EGF. However, recent studies on the mechanism of overexpression of the EGF receptor in A431 cells have implicated both gene rearrangement and amplification (2, 11, 12). The genetic rearrangement in A431 cells might result in altered transcriptional control.

Recently, a human breast carcinoma cell line, MDA 468, has been described in which the EGF receptor is also overexpressed to levels nearly as high as in the A431 cell (13). Restriction enzyme mapping of the receptor gene in these cells suggests that the mechanism of overexpression is through gene amplification without rearrangement. Because it is likely that the amplification of the receptor gene occurred along with its normal promotor sequences, this cell line might be ideal for studies on the control of EGF receptor gene expression.

To test our hypothesis that decreased synthesis of receptor might contribute to down-regulation, we measured receptor synthesis in the MDA 468 cell line. Using a monoclonal antibody, B1D8, to immunoprecipitate metabolically labeled EGF receptor and its precursor, we determined the effect of EGF on the synthesis of receptor in these cells. Contrary to our hypothesis, we have shown stimulation of receptor synthesis by EGF. These studies suggest that stimulation by EGF sensitizes the cell to further stimulation by augmenting EGF receptor synthesis.

EXPERIMENTAL PROCEDURES

Materials—Culture media and serum were from Gibco. Mouse EGF was purified from male Swiss Webster mouse (Simonsen) submaxillary glands (14). Purity was verified by amino acid analysis showing

1 The abbreviations used are: EGF, epidermal growth factor; TGF, transforming growth factor; SDS, sodium dodecyl sulfate.


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the absence of phenylalanine, alanine, and lysine. L-[35S]methionine was from Amersham Corp. Formaldehyde-fixed Staphylococcus aureus (Pansorbin) was from Calbiochem-Behring. Mouse monoclonal antibody B1D8 against the EGF receptor was prepared as previously described (15). The human breast tumor cell line (MDA 468) was kindly provided by Dr. Buick (Ontario Cancer Institute, Toronto, Canada).

Cell Cultures—MDA 468 cells were maintained in a CO2 incubator on 10-cm Falcon tissue culture plates in Dulbecco’s modified Eagle’s medium containing 10% calf serum plus penicillin and gentamycin.

Metabolic Labeling—Confluent MDA 468 cells in 3.5-cm plates were metabolically labeled by washing the cells once with methionine-free medium and incubating them for the stated amount of time in 1 ml of methionine-free F-12 medium containing 1% fetal bovine serum and 50 μCi of L-[35S]methionine. When indicated, EGF was added to the labeling medium.

Immunoprecipitation—Cells were washed twice with 20 mM EDTA in Ca2+- and Mg2+-free phosphate-buffered saline to remove culture medium and to inhibit calcium-dependent proteases. They were then solubilized with 250 μl of RIPA buffer containing EDTA (0.15 M NaCl, 50 mM Tris-HCl, pH 7.2, 5 mM EDTA, 1% Triton X-100, and 0.1% SDS). The extract was then centrifuged at 12,000 × g for 15 min. Immunoprecipitation was carried out immediately or the sample was stored at −70°C for processing at a later time. The immunoprecipitation was performed on 35S-labeled extracts adjusted to contain equal amounts of trichloroacetic acid-precipitable radioactivity. Monoclonal antibody B1D8 was added at a final concentration of 10−6 M and incubated for 1 h at room temperature followed by the addition of 100 μl of 10% suspension of fixed Staphylococcus aureus for another 30 min. The immunoprecipitation protocol had been previously optimized to be quantitative. The suspension was centrifuged over a 1 M sucrose cushion and the bacterial pellet washed three times with RIPA buffer. The pellet was then boiled for 5 min in 50 μl of Laemmlli buffer, and the supernatant was electrophoresed on 7.5% SDS-polyacrylamide gel. The gel was fixed and dried, and autoradiography was carried out at −70°C over 48–72 h using Kodak X-Omat AR film.

The bands were cut out of the gel with the help of radioactive ink alignment markers on the autoradiographs. They were then solubilized with 0.4 ml of 20% hydrogen peroxide plus 40 μl of ammonium hydroxide at 37°C overnight. Liquid scintillation counting was done after the addition of 2 g of Triton X-100 and 5 ml of toluene scintillation fluid.

RESULTS

Using B1D8, a monoclonal antibody directed against the external domain of the EGF receptor, the EGF receptor was immunoprecipitated from MDA 468 cells at varying times after exposure to EGF. The immunoprecipitated material was analyzed by gel electrophoresis. The silver-stained gel shown in Fig. 1 reveals that after exposure to 10−9 M EGF, the quantity of receptor protein is diminished in a time-dependent manner, but still present. Since the density of silver staining is not strictly quantitatively linked to the amount of protein, the absolute amount of receptor cannot be quantitated by this technique. Nevertheless, qualitatively, the total amount of receptor protein is diminished by EGF, agreeing with the result obtained in other cells using 125I-EGF binding. The decreased amount of receptor protein in the cells is most apparent after an incubation time of 24–48 h. At concentrations of EGF less than 10−9 M, no decrease in total receptor protein was evident and at concentrations greater than 10−9, no further rate of receptor protein decrease was observed (data not shown).

Experiments were undertaken to separate the rate of synthesis of new receptors from the rate of degradation of receptors under the influence of EGF. To determine the effect of EGF on the rate of receptor degradation, MDA 468 cells were metabolically labeled with L-[35S]methionine. The culture medium containing the labeled methionine was replaced with medium containing unlabeled methionine with and without 10−9 M EGF. At various times after the addition of EGF, the cell monolayers were solubilized and the EGF receptor was quantitatively immunoprecipitated and analyzed by gel electrophoresis. Fig. 2a shows an autoradiogram of the gel in which the amount of receptor labeled prior to exposure to EGF is seen to diminish. The amount of labeled receptor remaining at various times of exposure was quantitated by determining the amount of radioactivity in the receptor band. Fig. 2b shows the receptor to have a half-life of 24 h in the absence of EGF and a half-life of 12 h in the presence of EGF. The rate of decay of labeled receptor is about 2-fold more rapid after exposure to 10−9 M EGF. This change in receptor half-life upon exposure to EGF is similar to half-life changes reported for the A431 cell line (9, 17), where half-lives were reported to change from 20 to 5 h and 20 to 9 h, respectively.

In order to determine the rate of receptor synthesis, an appropriate time for pulse-labeling the EGF receptor was determined. Cells were labeled using L-[35S]methionine for varying times and the receptor protein was immunoprecipitated from the solubilized cells. After 1 h of labeling, only a 160-kDa protein was labeled (Fig. 3). At longer incubation times, the usual, mature 170-kDa protein was seen, indicating that the monoclonal antibody B1D8 also recognized the immature form of the receptor.

Evidence has been presented that the 160-kDa immature form of the receptor is not accessible to ligand on the outer surface of the cell (10). We reasoned, therefore, that this form of the receptor would not be subject to down-regulation in the presence of EGF. This allowed us to pulse label cells for 1 h after a prior incubation with EGF and measure the rate of synthesis of the 160-kDa precursor without having to account for simultaneous degradation of receptor caused by EGF.

First, the effect of various doses of EGF on receptor synthesis was determined. A series of concentrations was chosen to include potentially mitogenic and nonmitogenic levels. Synthesis was stimulated maximally 1.8-fold with the maximal effect observed at a dose of 10−6 M EGF as shown in Fig. 4. The lesser amount of stimulation seen at 10−8 M EGF may reflect the growth inhibitory effect of EGF on these cells at high EGF concentrations. However, very little change in general protein synthesis was observed over the entire EGF dose range (results not shown).

The time course of this stimulatory effect on receptor synthesis by EGF was studied. Fig. 5 shows the result of
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**FIG. 2.** Effect of EGF on the degradation rate of the receptor. MDA 468 cells were metabolically labeled overnight as described under “Experimental Procedures” in the absence of EGF. The cell monolayers were washed and the label was chased in regular growth medium without label in the absence or presence of $10^{-9}$ M EGF. At the indicated times following exposure to EGF, the cells were lysed and the receptor was immunoprecipitated. The immunoprecipitates were run on SDS-polyacrylamide gel electrophoresis, and the labeled proteins were detected by autoradiography (a). Labeled bands corresponding to the EGF receptor were cut out and the radioactivity was quantitated by scintillation counting (b). EGF absent, O; EGF present, •.

incubation of cells with $10^{-9}$ M EGF for varying durations. A maximal stimulation by 1.7-fold in the synthesis of the 160-kDa EGF receptor precursor occurred at 6–8 h after the addition of EGF. By 24 h, the synthetic rate had almost returned to pre-treatment levels (data not shown).

To determine whether stimulation of receptor precursor synthesis was followed by stimulation of mature receptor protein, cells were incubated for 5 h in the presence of various doses of EGF. The EGF-containing medium was removed, the cell monolayers were washed to remove unbound EGF, and the cells were labeled with L-$^{35}$S]methionine for 3 h to allow receptor maturation. Fig. 6 shows that the same dose-response relationship was observed for the synthesis of mature receptor as for the precursor.

Figs. 4, 5, and 6 result from three separate experiments in which maximal stimulations in receptor synthesis of 1.7–2.1-fold were observed. These experiments are representative of a larger series of similar experiments in which stimulations from 1.5–2.1-fold were reproducibly observed (mean 1.72 ± 0.17, n = 15). In addition, experiments were done to test the effect of cell density on the stimulation of receptor synthesis. The synthesis of receptor was stimulated equally in confluent and 20% confluent monolayers (data not shown).

**DISCUSSION**

In this paper, we have shown that EGF stimulates the synthesis of its own receptor in the MDA 468 human breast cancer cell line. Maximal stimulation of approximately 1.8-fold was observed after 6 h of EGF treatment and at a concentration of $10^{-9}$ M EGF. The stimulation of receptor synthesis was seen in the absence of a stimulation of general protein synthesis in the cells, implying that the effect on receptor synthesis was relatively specific. Receptor degradation was also increased by EGF, as has been reported in other cell types (9, 17) such that the half-life of pre-labeled receptor was decreased from 24 to 12 h. Under the influence of EGF,
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Fig. 4. Effect of EGF concentration on the EGF receptor precursor synthetic rate. MDA 468 cells were incubated for 6 h in the presence of various concentrations of EGF. The cells were then washed and metabolically labeled for 1 h under the same EGF concentrations, after which the cells were lysed and the receptor was immunoprecipitated. The immunoprecipitates were processed as in Fig. 2, and autoradiography (a) and quantitation of the 160-kDa precursor (b) are shown.

Fig. 5. Effect of incubation time with EGF on the rate of synthesis of EGF receptor precursor. MDA 468 cells were incubated for varying times in the presence of 10^{-8} M EGF. The cells were washed and metabolically labeled in the presence of 10^{-8} M EGF as described under “Experimental Procedures.” Labeling was continued for 1 h, after which it was stopped by detergent lysing the cells and immunoprecipitation. The immunoprecipitates were processed as in Fig. 2 and quantitation of the 160-kDa precursor is shown. The autoradiograms used to detect the 160-kDa bands appeared essentially as in Fig. 4a.

Fig. 6. Effect of EGF concentration on the receptor synthetic rate. MDA 468 cells were incubated for 6 h in the presence of various concentrations of EGF. The cells were washed to remove EGF and metabolically labeled for 3 h in the absence of EGF. The cells were lysed and the receptor was immunoprecipitated. The immunoprecipitates were processed as in Fig. 2, and autoradiography (a) and quantitation (b) of the mature receptor are shown.

total receptor protein in these cells is decreased, indicating that the stimulation of degradation exceeds the stimulation of receptor synthesis. Therefore, as in other cell lines, EGF stimulation does lead to a net down-regulation of receptors in MDA 468 cells.

The MDA 468 cell line was chosen for these studies because it expresses large amounts of the EGF receptor protein, making measurements of EGF receptor synthesis easier. The overexpression in MDA 468 cells appears to result from gene amplification. By restriction analysis with several enzymes, the flanking sequences of the receptor gene appear to be the same as found in normal cells (13). This contrasts with the A431 cell, in which gene rearrangement has been observed (2, 11, 12). It is therefore more likely that the genetic control of transcription is preserved in MDA 468 cells than in A431 cells. No effect of EGF on EGF receptor synthesis was seen in A431 cells (8-10) which contrasts with our finding that EGF stimulates nearly a doubling of the rate of receptor synthesis in MDA 468 cells. We believe that the stimulatory response observed in these breast cancer cells may be normal and the lack of response observed in A431 cells abnormal.
The effects of a number of agents on EGF receptor number and affinity have been examined, including phorbol esters (18, 19), retinoids (20), platelet-derived growth factor (21), vasopressin (22), bombesin (23), fibroblast stimulating factor (24), vitamin K (25), thyroid hormones (26), glucocorticoids (27), and estrogen (28). Recently, it has been observed that TGF type II stimulation of NRK fibroblasts results in an increase in EGF binding to the cells (29, 30). This increase in binding can be blocked by inhibitors of protein synthesis and glycosylation, suggesting that new receptor synthesis is stimulated by this growth factor. This stimulation may be responsible for the ability of TGF type II to enhance EGF action. Our finding that EGF itself stimulates EGF receptor synthesis, suggests that the stimulation of receptor synthesis may be a more general response to growth factor stimulation rather than a specific response to TGF. Stimulation of receptor synthesis suggests that exposure to EGF may serve to maintain cells in an EGF-sensitive state despite the concurrent increase in receptor degradation rate.

It has been known for some time that new protein synthesis is required for growth factors to cause cells to enter the S-phase of the cell cycle. Insight into the nature of some of these proteins was provided by experiments examining the family of mRNA transcripts induced by platelet-derived growth factor (31). Among the genes whose transcription was stimulated were the oncogenes myc (32) and fos (33). These proteins seem to act in concert to complete the signal that eventually results in mitogenesis.

EGF is already known to increase the synthesis of a number of specific proteins, depending upon the cell line examined (34–37). In addition, as with platelet-derived growth factor, it has also been shown that the proto-oncogenes c-myc and c-fos are rapidly induced by EGF (38). The inductions of c-myc and c-fos by EGF are early events triggered by binding of EGF to the receptor but their relationship to mitogenesis is not clear.

Thus, an impression is emerging that mitogenic stimulation of cells may in part act through the stimulation of transcription of genes for proteins important in the transmission of the mitogenic signal from the external face of the plasma membrane to the cell nucleus. Clearly, the EGF receptor is an important member of the proteins involved in mitogenic signal transduction, and stimulation of its synthesis may reflect part of the pleiotropic response of cells to growth factors.

The mechanism by which EGF stimulates the synthesis of its receptor is not known. EGF was found to stimulate the synthesis of prolactin in pituitary tumor cells and this stimulation was at the translational level (39). The stimulation of EGF receptor synthesis by EGF may also occur at the translational level, but the small magnitude of stimulation seen in our system would have made measurements of transcriptional stimulation difficult, assuming a parallel stimulation of protein and mRNA synthesis.

REFERENCES