Epidermal growth factor regulates the expression of its own receptor

(protooncogene/phorbol ester/cycloheximide/nuclear transcription/internalization)

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ABSTRACT The epidermal growth factor (EGF) receptor gene is the cellular homolog of the avian erythroblastosis virus *erbB* oncogene. Control of EGF receptor expression determines cellular responsiveness to EGF and might play an important role in neoplastic development. Using RNA blot hybridization, we have found that exposure of human KB carcinoma cells to EGF results in elevated levels of EGF receptor mRNA. The phorbol ester 4 β -phorbol 12-myristate 13-acetate also stimulates EGF receptor RNA accumulation. Immunoprecipitation of metabolically labeled (30 min) EGF receptor protein revealed that synthesis of new EGF receptor follows the increase in receptor RNA. Addition of cycloheximide together with EGF further enhances EGF receptor RNA accumulation. Results of nuclear runoff-transcription experiments suggest that the stimulatory effects of EGF and cycloheximide are most likely due to a posttranscriptional control mechanism.

Epidermal growth factor (EGF) induces a wide variety of cellular responses, including stimulation of DNA replication and cell division (1). EGF binds to specific cell surface receptors, activating the EGF receptor tyrosine kinase (2). Following the addition of EGF to cells bearing EGF receptors, the receptor and its ligand are internalized, sorted in the Golgi region, and transferred to lysosomes, where both are degraded, inducing a net loss, or down-regulation, of cell surface receptors (1, 3). In human KB cells, which possess $\approx 2 \times 10^5$ EGF receptors per cell (4), the addition of EGF promotes EGF receptor internalization followed by receptor degradation with a half-life of about 40 min (5). The phorbol ester 4 β -phorbol 12-myristate 13-acetate (PMA) induces EGF internalization without degradation (6). We have used KB cells to investigate whether any alteration in expression of the EGF receptor gene follows EGF binding and internalization. Using the cDNA clone pE7, which encodes 2.4 kilobases (kb) of the human EGF receptor mRNA (7), as a hybridization probe, we demonstrate here that receptor activation and internalization in KB cells is followed by enhanced accumulation of EGF receptor RNA.

MATERIALS AND METHODS

Isolation and Analysis of RNA. KB cells (American Type Culture Collection CCL 17, human epidermoid carcinoma line) were plated at a density of 5×10^6 cells per 100-mm dish and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. After 24 hr, 0.4 or 4.0 nM mouse EGF (Collaborative Research, Waltham, MA, or Bethesda Research Laboratories), 100 nM PMA (Sigma), or 10 μ g of cycloheximide/ml was added to the medium. Cells were homogenized in 4 M guanidinium isothiocyanate and RNA was centrifuged through a cesium

chloride gradient (8). RNA samples were quantified by measurement of A_{260} and loaded on nitrocellulose filters. The filters were baked, prehybridized, and hybridized to the nick-translated cDNA insert from pE7, encoding the human EGF receptor, as described (7, 9, 10). The intensity of the autoradiographic bands was quantified by microdensitometry. For estimation of ribosomal RNA content, the same filters were washed in 15 mM NaCl/1.5 mM sodium citrate, pH 7, at 95°C and hybridized to a nick-translated ribosomal DNA probe. For RNA blot analysis, poly(A)⁺ RNA was prepared by oligo(dT)-cellulose chromatography, fractionated by electrophoresis in a 1% agarose/formaldehyde gel (7, 9, 11), and transferred to nitrocellulose as described (12). The resulting filter was hybridized to pE7 and subjected to autoradiography.

Protein Labeling and Immunoprecipitation. KB cells were plated at a density of 2×10^5 cells per 35-mm dish in Dulbecco's modified Eagle's medium with 10% fetal calf serum. After 48 hr, EGF was added to the medium as indicated. The monolayers were washed twice in phosphate-buffered saline, and the cells were incubated for 30 min at 37°C with [³⁵S]methionine (1 mCi/ml; 1 Ci = 37 GBq) in methionine-free medium with 5% fetal calf serum. Cell lysates were prepared and duplicate aliquots of each lysate (2×10^6 cpm as determined by trichloroacetic acid precipitation) were immunoprecipitated with 20 μ l of rabbit anti-EGF receptor antiserum (2913) and 50 μ l of a suspension of *Staphylococcus aureus* as described (10).

Nuclear Runoff Transcription. Nuclei were isolated from KB cells treated with 4 nM EGF and/or 10 μ g of cycloheximide/ml by lysing the cells in 0.25% Nonidet P-40 as described (13, 14). Transcription took place under standard reaction conditions (13, 14) in the presence of 200 mM KCl and of [³²P]GTP. RNA was isolated as described by Groudine *et al.* (14), except that the aqueous mixture of labeled Cl₃CCOOH-precipitable RNA was concentrated by 2-butanol extraction before phenol/chloroform extraction. RNA samples of equal radioactivity were hybridized with immobilized DNA for 3–4 days at 42°C in 50% (vol/vol) formamide. Filters were washed in 30 mM NaCl/3 mM sodium citrate, pH 7/0.1% NaDodSO₄ at 50°C for 1 hr, treated with RNase A, and subjected to autoradiography for 1–2 days (13).

RESULTS

KB cells were incubated with 0.4 or 4.0 nM EGF for various times. Total cellular RNA was isolated, bound to nitrocellulose filters, and hybridized to a nick-translated EGF receptor-specific pE7 plasmid (7, 9) probe. An increase in EGF receptor RNA was detected within a few hours after addition of 0.4 nM EGF, reaching a maximum at ≈ 4 hr (Fig. 1 *Left*). This rise was followed by a marked fall in the EGF receptor

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Abbreviations: EGF, epidermal growth factor; PDGF, platelet-derived growth factor; PMA, 4 β -phorbol 12-myristate 13-acetate; kb, kilobase(s).

FIG. 1. (Left) Time course showing the change in EGF receptor RNA levels after addition of 0.4 nM (○) or 4.0 nM (●) EGF. The hybridization probe was pE7. The intensity of the hybridization signal for each time point is shown relative to the level of zero time. For 0.4 nM EGF, the result of a single experiment is shown, whereas for 4 nM, the mean $\pm$ SEM of three separate experiments is shown (with the exception of the 10-hr point). Aliquots (5 μ g) were applied in triplicate to nitrocellulose using a "slot-blot" apparatus. Ribosomal RNA levels (□) were measured on the same blots as a control for total bound RNA. (Right) Autoradiograph of an RNA blot [7.5 μ g of poly(A)⁺ RNA per well] showing the change in the level of the 10- and 5.6-kb mRNAs encoding the EGF receptor after a 6-hr treatment with 0.4 nM EGF.

RNA level, presumably because 0.4 nM is a nonsaturating concentration of EGF (6) and internalization rapidly depletes the medium of EGF; medium depletion should not be significant at the higher EGF concentration. Addition of 4.0 nM EGF resulted in the elevation of the level of EGF receptor RNA, which plateaued at about 4 hr (Fig. 1 Left). RNA blot analysis (Fig. 1 Right) showed that this rise in RNA level is the result of accumulation of both the 10-kb and the 5.6-kb species of mRNA (7, 9, 15, 16). When the medium was supplemented with additional amounts of either platelet-

derived growth factor (PDGF), fibroblast growth factor, transferrin, or insulin, no effect on the level of EGF receptor RNA was detected (data not shown).

To determine whether the accumulation of EGF receptor RNA was accompanied by an increase in EGF receptor protein, KB cells were pretreated with EGF for various periods of time, then incubated for 30 min with [³⁵S]methionine. Lysates of the labeled cells were immunoprecipitated with an anti-EGF-receptor antibody specific for the C-terminal portion of the receptor (10). Fig. 2 Left shows the

FIG. 2. (Left) Autoradiograph of [³⁵S]methionine-labeled EGF receptor immunoprecipitated from KB cells treated with 0.4 nM or 10 nM EGF for various times as indicated (in hr) above each lane. Cell lysates were prepared and duplicate aliquots of each lysate were immunoprecipitated with rabbit polyclonal anti-EGF receptor antiserum and *S. aureus* (10). The immunoprecipitates were analyzed in 5–15% linear gradient NaDodSO₄ polyacrylamide gels. The arrow indicates *M_r* 160,000. (Right) Time course showing the relative amounts of labeled EGF receptor protein in KB cells treated with 0.4 nM (○) or 4 nM (●) EGF. The EGF receptor bands (represented in Fig. 2 Left) were excised from the gel, solubilized, and assayed for radioactivity by liquid scintillation counting. Background radioactivity was determined by excising another region from the same gel lane. □, Transferrin receptor from cells treated with 10 nM EGF immunoprecipitated by a polyclonal rabbit antiserum (17).

result of immunoprecipitation. The M_r of the radioactive EGF receptor after a 30-min labeling period is 160,000, representing an incompletely processed form; the mature, fully glycosylated M_r 170,000 product is not seen with this short period of labeling (18). Addition of 0.4 nM EGF to the medium resulted in increased incorporation of [35 S]methionine into this M_r 160,000 band, peaking at ≈ 4 hr and then declining in a manner similar to that observed for EGF receptor RNA. When 10 nM EGF was added, EGF receptor synthesis remained elevated over a 10-hr period. The M_r 160,000 bands were excised and their radioactivity was measured (Fig. 2 *Right*). In general, the protein-synthesis pattern mirrors the RNA accumulation data.

Previous work showed that phorbol esters transiently reduce EGF binding at the cell surface by causing receptor internalization (6, 39, 40). In contrast to EGF-induced internalization, however, the receptor internalized in the presence of PMA is not degraded in lysosomes but is recycled to the cell surface within 45 min (6). It was, therefore, of interest to determine whether the transient disappearance of the receptor from the cell surface would be sufficient to trigger new receptor synthesis. Fig. 3 shows that after PMA treatment, EGF receptor RNA levels increased, reaching a peak after about 3 hr. 4 β -Phorbol, an analog that does not activate protein kinase C (41), did not exhibit this effect (Fig. 3). RNA blot hybridization showed that both the 5.6-kb and the 10-kb mRNA species increased after PMA treatment (data not shown).

The protein translation inhibitor cycloheximide has been shown to stimulate the accumulation of many RNA species, including oncogene products (19, 20). This is thought to occur because cycloheximide inhibits the synthesis of either a labile transcription repressor or a species of RNase. When cycloheximide was added to KB cells in serum, EGF receptor RNA was induced about 5-fold (Table 1). When cycloheximide was added together with EGF, a superinduction of about 11-fold was observed (Table 1). That the EGF induction could be potentiated by cycloheximide suggests that the effects are separate and additive.

To gain insight into the mechanism by which these alterations in EGF receptor gene expression occur, nuclear RNA runoff-transcription assays were performed. Cells were incubated with or without EGF for 0–4 hr and nuclei were isolated. Nuclei were incubated with [32 P]GTP, and then RNA was purified and hybridized to EGF receptor cDNA and genomic DNA sequences immobilized on nitrocellulose. Little difference in the initiation of EGF receptor RNA was observed over 4 hr of exposure to EGF (Fig. 4A). Further-

FIG. 3. Time course showing the effect of 100 nM PMA (●) or 100 nM 4 β -phorbol (■) on EGF receptor RNA levels. The hybridization probe was pE7. RNA samples were immobilized on nitrocellulose in a slot blot apparatus. Ribosomal RNA (□) acts as a control for RNA concentrations.

Table 1. Cycloheximide and EGF increase EGF receptor RNA levels

Treatment	Relative RNA level			
	Exp. 1	Exp. 2	Exp. 3	Average
None	1.0	1.0	1.0	1.0
Cycloheximide	4.4 $\pm$ 0.5	6.4 $\pm$ 0.4	4.1 $\pm$ 1.1	4.9 $\pm$ 0.7
EGF	2.7 $\pm$ 0.6	7.4 $\pm$ 0.6	5.9 $\pm$ 2.0	5.3 $\pm$ 1.1
Cycloheximide plus EGF	6.7 $\pm$ 0.7	19.7 $\pm$ 2.0	6.0 $\pm$ 1.6	10.8 $\pm$ 2.7

KB carcinoma cells were incubated with 4.0 nM EGF and/or 10 μ g of cycloheximide/ml for 4 hr. RNA was isolated, spotted on nitrocellulose, and hybridized to EGF receptor cDNA pE7 (7). Number of hybridization slots scanned for each treatment was 6 (Exp. 1), 6 (Exp. 2), and 3 (Exp. 3). All values were normalized to those obtained with untreated KB cells, which were assigned the arbitrary value of 1.0 for each experiment.

more, the effect of cycloheximide on transcriptional initiation of the EGF receptor gene was minimal whether EGF was present or not (Fig. 4B). These results suggest that the majority of the changes elicited by EGF or cycloheximide on the expression of the EGF receptor gene occur via some posttranscriptional control mechanism.

DISCUSSION

We have shown that exposure of KB cells to EGF or PMA, two agents that cause internalization of the EGF receptor (6), stimulates accumulation of EGF receptor mRNA and EGF receptor protein. The newly synthesized receptor is very probably processed and transported to the cell surface. Aharonov *et al.* (22) demonstrated that EGF binding returns to normal within 10–12 hr after down-regulation by EGF in nondividing Swiss 3T3 cells. In dividing cells it was shown that a return of EGF binding occurred 4–5 hr after addition of EGF. In the experiments presented here, the KB cells were 80–90% confluent and rapidly dividing. It is of interest

FIG. 4. (A) Time course of EGF receptor (EGFR) RNA transcriptional initiation as measured by nuclear RNA runoff-transcription analysis. Cells were treated for various times with 4.0 nM EGF, and their nuclei were isolated and incubated with [α - 32 P]GTP. Labeled RNA was then hybridized to insert EGF receptor genomic DNA (0.25 μ g/slot) isolated from both λ ER1 and λ ER7 (21), together with pE7 insert cDNA (7) (0.5 μ g/slot) immobilized on nitrocellulose. Parts per million (ppm) represents the amount of hybridizable radioactivity per 10^6 total dpm. (B) Effect of 2-hr exposure to cycloheximide (CH, 10 μ g/ml) on EGF receptor gene transcription in the presence or absence of 4 nM EGF, as measured by nuclear RNA runoff-transcription analysis; Ppm were not determined in this experiment, but microdensitometric scanning indicated that cycloheximide treatment increased EGF receptor transcription by $<50\%$.

that the kinetics of this compensatory response in these two cell lines are similar. However, by use of a heavy-isotope density-shift method, it has been found that EGF receptor down-regulation is not accompanied by new receptor synthesis in A431 cells (23); this may reflect atypical regulation of the amplified EGF receptor gene in these cells (9, 15, 16, 24). The phenomenon of ligand-dependent induction of specific cell surface receptors has also been shown at the protein level in the case of growth hormone (25), prolactin (26), and interleukin 2 (27, 28).

Both EGF and PMA stimulate transcription of the rat prolactin gene (29, 30). However, comparison of the sequence of the promoter regions of the human EGF receptor gene (21) and the rat prolactin gene (31) does not reveal any regions of striking homology that might suggest binding of a common regulatory molecule. In addition, a nuclear runoff-transcription assay (Fig. 4) revealed no increase in EGF receptor gene transcriptional initiation, resulting from exposure to EGF, that could account for the observed accumulation of RNA. [In contrast, a nuclear runoff-transcription assay has shown that interleukin 2 increases transcription of the interleukin 2 receptor gene (32).] When the EGF receptor promoter region was placed upstream of the bacterial chloramphenicol acetyltransferase gene (33) and introduced into KB cells, no effect of EGF on chloramphenicol acetyltransferase activity was observed in these transfected cells (data not shown). These results are consistent with the hypothesis that EGF receptor gene expression is modulated by EGF at least in part at some posttranscriptional level. Support for this idea has come from some preliminary experiments in which transcription was blocked with actinomycin D; the results (not shown) suggest that EGF receptor mRNA has a rather short half-life. A mechanism is therefore available wherein a small shift in the stability of the EGF receptor RNA could lead to significant changes in the cellular levels of these RNAs and their encoded proteins.

We have found that addition of cycloheximide and EGF to KB cells has an additive stimulatory effect on EGF receptor gene expression. These combined effects have been observed for other genes as well. In BALB/c-3T3 cells, *c-fos* was found to be induced by PDGF and to be superinduced by PDGF plus cycloheximide (19). Also, addition of EGF together with cycloheximide produces a synergistic effect on cellular expression of the mouse actin gene (34). There are examples where cycloheximide causes a change in nuclear runoff transcription, probably by blocking the production of a labile repressor protein (34). Our results suggest that cycloheximide affects the expression of the EGF receptor gene, in the presence or absence of EGF, at least in part by some other mechanism, possibly by preventing the synthesis of some RNase and thereby stabilizing EGF receptor RNA.

The finding that EGF induces accumulation of its own receptor mRNA attains greater significance because the EGF receptor is the cellular homolog of the *v-erbB* oncogene product (35). In tumor cells the EGF receptor gene is often overexpressed, sometimes as a result of gene amplification (9, 15, 16, 36, 37). It now appears that expression of the EGF receptor may also be stimulated as a result of the binding of EGF by the receptor, a finding that is not incompatible with the autocrine theory of oncogenesis (38).

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