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Epidermal Growth Factor (EGF) Stimulates EGF Receptor Synthesis*

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Epidermal growth factor (EGF) binds to the extracellular domain of a specific 170,000-dalton transmembrane glycoprotein; this results in rapid removal of both ligand and receptor from the cell surface. In WB cells, a rat hepatic epithelial cell line, ligand-directed receptor internalization leads to receptor degradation. We tested whether the EGF receptor was replenished at a constitutive or enhanced rate following EGF binding by immunoprecipitating biosynthetically labeled EGF receptor from cells cultured with [^{35}S]methionine. EGF stimulated receptor synthesis within 2 h in a dose-dependent manner; this was particularly evident when examining the nascent form of the receptor. To determine the site of EGF action, total WB cell RNA was transferred to nitrocellulose paper after electrophoresis and was hybridized to cDNA probes from both the external and cytoplasmic coding regions of the human EGF receptor. EGF increased receptor mRNA by 3–5-fold. Therefore, at least in some cells, the surface action of EGF that leads to EGF receptor degradation is counterbalanced by a positive effect on receptor synthesis.

The cell surface complement of hormone and growth factor receptors is determined by their rates of synthesis and degradation as well as by a regulated flux of receptors between surface and intracellular compartments. Ligand binding rapidly accelerates ligand and receptor internalization, a process referred to as "down regulation" (1, 2). Experiments using radiolabeled EGF[1] have demonstrated that down regulation drastically reduces cell surface EGF binding capacity and leads, in most instances, to ligand degradation (1–5). Down regulation may also lead to EGF receptor degradation (6). Initial studies of EGF receptor synthesis using anti-receptor antibodies have shown that receptor protein is cotranslationally glycosylated so that the nascent receptor migrates at an estimated M_r of 160,000. Within 15–45 min it matures to the 170,000-dalton form and eventually assumes its position in the plasma membrane (7–11).

During our investigations of EGF receptor metabolism during rat liver regeneration (12, 13) we raised an antiserum to the rat EGF receptor. In the present study we have used this antiserum to investigate receptor synthesis in a continuous line of nontransformed rat liver epithelial cells (WB). These cells were initially isolated from a primary culture of Fisher 344 rat hepatocytes (14). WB cells respond to EGF with enhanced DNA synthesis and alteration of the activity of several glycolytic enzymes (15). EGF activates the WB cell EGF receptor tyrosine-specific protein kinase both *in vitro* (15) and *in vivo*.[2] As expected, the addition of EGF to WB cells led to receptor internalization and degradation. However, when the rates of EGF receptor protein and mRNA synthesis were examined after EGF stimulation, an unexpected result was obtained; EGF increased EGF receptor synthesis.

EXPERIMENTAL PROCEDURES

Cell Culture and Immunoprecipitation—WB cells were grown to confluence at 37 °C in a 95% air, 5% CO_2 atmosphere on 35-mm Corning plastic tissue culture dishes with Richter's improved minimal essential medium (Irvine Scientific) plus 0.1 μM insulin (Irvine Scientific) and 10% fetal bovine serum. To label WB cell proteins, the medium was changed to minimal essential medium supplemented with glutamine and containing only 2% of the normal methionine concentration and 50 μCi of [^{35}S]methionine (New England Nuclear). At the indicated times, EGF purified from mouse salivary glands (16) was added to the cultures either before, during, or after the labeling period.

Prior to immunoprecipitation the cells were washed with normal saline, and 0.5 ml of lysis buffer was added to the plates. Lysis buffer contained 1% Nonidet P-40, 50 mM Tris-HCl, pH 8.5, 0.15 M NaCl, 5 mM EDTA, 0.1% bovine serum albumin, 10 μg/ml leupeptin, 25 mM benzamidine, and 50 μg/ml phenylmethylsulfonyl fluoride. Cells were scraped, the plates were washed with another 0.5 ml of lysis buffer, and the lysates were transferred to microfuge tubes where 50 μl of 5 M NaCl was added. The samples were sonicated and the residue was pelleted for 5 min in an Eppendorf microfuge. The supernatants were transferred to new microfuge tubes and incubated for 10 min at 21 °C with 20 μl of a 1:1 slurry of Pansorbin (Calbiochem)/lysis buffer and 5 μl of normal rabbit serum. After pelleting the Pansorbin, the supernatants were again transferred and tumbled with a saturating amount (5 μl) of the polyclonal antiserum, 799, or normal rabbit serum for 30 min at 21 °C. The rabbit polyclonal antiserum was raised against native rat liver EGF receptor purified by EGF affinity chromatography (17). Pansorbin/lysis buffer (20 μl of a 1:1 slurry) was added for an additional 45 min. Immunoprecipitates were pelleted in the microfuge and washed sequentially with 1 ml of the following buffers: 1) 50 mM Tris-HCl, pH 8.5, 0.5 M NaCl, 1 mM EDTA, 0.5% Nonidet P-40; 2) 50 mM Tris-HCl, pH 8.5, 0.15 M NaCl, 1 mM EDTA, 0.5% Nonidet P-40; and 3) 10 mM Tris-HCl, pH 8.5, 0.1% Nonidet P-40. The immunoprecipitates were boiled for 5 min after the addition of 60 μl of sodium dodecyl sulfate containing sample buffer with 50 mM β-mercaptoethanol and then pelleted. Supernatants were subjected to 6 or 8% polyacrylamide gel electrophoresis. Gels were stained, destained, and treated for 1 h with 1 M sodium salicylate. After drying, gels were exposed (Kodak XAR film) for 1–3 days using a Kronex Intensifier. In some cases, the p170 band was identified and

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[1] The abbreviations used are: EGF, epidermal growth factor; SSC, saline sodium citrate; kb, kilobases.

[2] W. R. Rackoff and H. S. Earp, unpublished results.

cut from dried gels. The gel pieces were treated for 24 h at 37 °C with 2 ml of 30% H_2O_2, 1% NH_4OH. The extract was analyzed by liquid scintillation counting using 5 ml of Scintiverse II and 5 ml of H_2O.

RNA Preparation and Hybridization—RNA was prepared by the method of Chirgwin *et al.* (18) from WB cells grown as indicated above. Cells from 8 confluent 100-mm tissue culture dishes were scraped directly into 4 M guanidinium isothiocyanate (1 ml/plate) and the plates were washed with 1 ml of the same buffer. The 16-ml sample was homogenized with a polytron apparatus for 10 s (set 7) and centrifuged before initial precipitation with acetic acid and ethanol. The precipitates were resuspended and reprecipitated twice in guanidine hydrochloride before water extraction and final ethanol precipitation. The ratio of 260/280 absorbance was 1.95–2.00. Total RNA (40 μg) was electrophoresed in 1% agarose, 2.2 M formaldehyde (19). λ *Hin*dIII digests were also electrophoresed on the same gel. The RNA was transferred to nitrocellulose and the filter was vacuum-dried at 80 °C. After prehybridization overnight, hybridization was performed with the indicated probes in 50% formamide at 42 °C for 24 h (20). Two cDNA probes cloned by Ullrich *et al.* (21) were used, 64-1 (an 1838-base pair sequence from the coding region of the human EGF receptor external domain) and 64-3 (a 768-base pair sequence from the cytoplasmic domain in pUC12) Insert 64-1, cDNA 64-3 in plasmid pUC12, and λ*Hin*dIII digests were nick-translated using a kit from Worthington and 75 μCi of [^{32}P]dATP. The blots were washed with 4 changes of 2 × SSC at 42 °C and 2 changes of 0.2 × SSC, 0.1% sodium dodecyl sulfate at 55 °C. They were air-dried and exposed using Kodak XAR film and Dupont Kronex Intensifier for 2 days. Densitometry was performed using a Kontes densitometer and an Hewlett-Packard Integrator.

RESULTS AND DISCUSSION

WB cell proteins were labeled for 18 h with [^{35}S]methionine. Labeling was stopped by changing the medium and EGF was then added to the cells. At 37 °C, EGF treatment resulted in a time-dependent loss of prelabeled immunoprecipitable p170. (Fig. 1). We then tested whether EGF influenced nascent receptor synthesis at the same time it was directing degradation of pre-existing surface receptors. Confluent WB cells were labeled for 4 h with [^{35}S]methionine, with or without added EGF. EGF increased [^{35}S]methionine incorporation into immunoprecipitated receptor in the 160,000–170,000-dalton region of the gel (p160–p170) (Fig. 2, *lanes 3–6*). *Lane 1* shows that little or no ^{35}S-labeled p160–p170 was precipitated by normal rabbit serum.

Antiserum 799 specifically immunoprecipitates either phosphorylated or metabolically labeled EGF receptor from rodent and human cells. This antiserum also precipitates [^{35}S]methionine-labeled 135,000 dalton EGF receptor protein (p135) from tunicamycin-treated WB cells and rat hepatocytes. Thus, the antiserum contains at least some anti-protein determinants that will detect nascent nonglycosylated forms of the receptor. The lower molecular weight form (p160) has been demonstrated to be cotranslationally glycosylated receptor protein (7–11). In WB cells, a shorter labeling period (1 h) clearly distinguished the 160- and 170-kDa forms and revealed that 10 ng/ml EGF increased 160-kDa synthesis within 2 h (Fig. 3 and Table I). The effect of EGF on receptor protein synthesis was also observed in the absence of glycosylation, *i.e.* EGF increased p135 synthesis in WB cells pretreated for 30 min with 10 μg/ml tumicamycin (not shown).

Since EGF alters amino acid transport (see Ref. 1), we quantitated the increase in immunoprecipitable ^{35}S-labeled p170 and compared it to the increase in [^{35}S]methionine incorporation into total cellular protein (Table I). Trichloro-

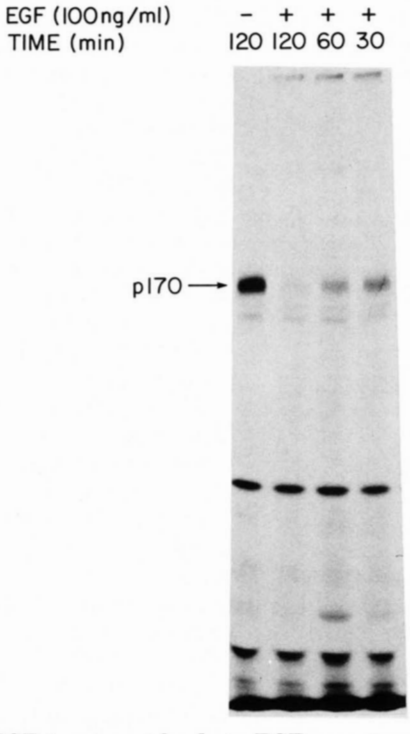

FIG. 1. **EGF treatment leads to EGF receptor degradation in WB cells.** Cells were labeled with [^{35}S]methionine for 18 h. The cells were changed to medium containing unlabeled methionine and EGF at the indicated concentration. Immunoprecipitation, as described under "Experimental Procedures" was performed at 30, 60, and 120 min after EGF addition and was followed by electrophoresis and fluorography.

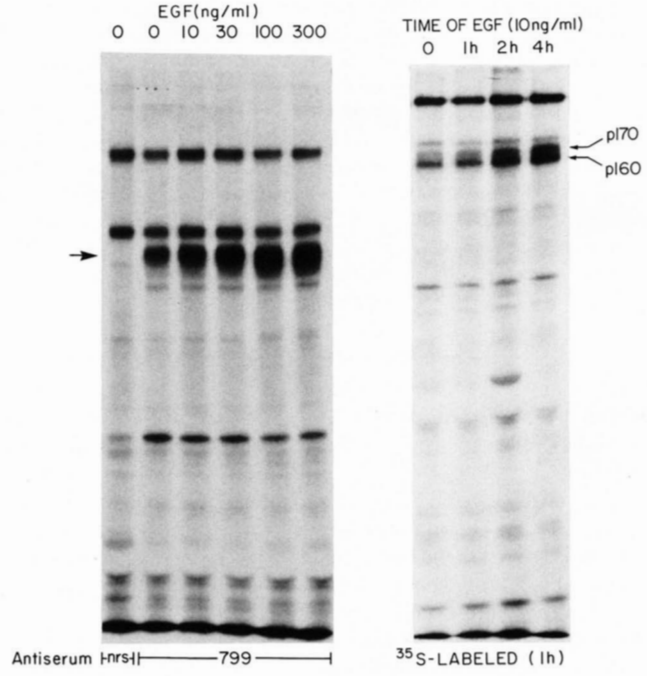

FIG. 2 (*left*). **EGF treatment stimulates EGF receptor synthesis.** Cells were incubated for 4 h with [^{35}S]methionine with or without EGF. Immunoprecipitation with normal rabbit sera (*nrs*) (*lane 1*) or antisera 799 (*lanes 2–6*), 6% polyacrylamide electrophoresis, and fluorography were performed as described under "Experimental Procedures." WB cells were exposed to the following EGF concentrations (ng/ml): *lanes 1* and *2*, 0; *lane 3*, 10; *lane 4*, 30; *lane 5*, 100; and *lane 6*, 300. The *arrow* denotes p170.

FIG. 3 (*right*). **Time course of EGF-induced EGF receptor synthesis.** Confluent WB cells were incubated in 2% methionine-containing medium for 4 h. EGF (10 ng/ml) was added to the cells for the indicated time (0 represents a 4-h incubation without EGF); 50 μCi of [^{35}S]methionine was present for the last 1 h in all samples. Immunoprecipitation was followed by 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis and fluorography as described under "Experimental Procedures."

TABLE I

Time and dose dependence of EGF-stimulated EGF receptor synthesis

In experiment A, cells were incubated with 2% methionine-containing medium and EGF for 2 h. 50 μCi of [^{35}S] methionine was then added to each plate and the incubation continued for another 2 h. After immunoprecipitation electrophoresis and fluorography, the p170 band was cut from dried gels and counted as described under "Experimental Procedures." Duplicate cultures were washed and 0.5 ml of 10% trichloroacetic acid was added. The trichloroacetic acid precipitates were scraped and the plates were washed with an additional 0.5 ml of trichloroacetic acid. The samples were centrifuged and the pellets were treated with NCS Tissue Solubilizer (Amersham Corp.) and aliquots were counted. In experiment B, cells were incubated for 1–4 h in the presence of absence of 10 ng/ml EGF in 2% methionine-containing medium. During the last 1 h, 50 Ci [^{35}S]methionine was added. Following immunoprecipitation, the ^{35}S-labeled p170 was quantitated as above.

Experiment A					Experiment B		
EGF	[^{35}S]Methionine p170	Control		Total ^{35}S incorporated	Time after 10 ng/ml EGF	[^{35}S]Methionine p170	Control
ng/ml	*cpm*	%		*cpm* × 10^6	*h*	*cpm*	%
0	393	100	100	7.8	0	502	100
3	737	188	113	8.8	1	400	80
10	937	238	122	9.5	2	728	145
30	1102	280	126	9.9	3	1623	323
100	885	225	121	9.4	4	1695	338

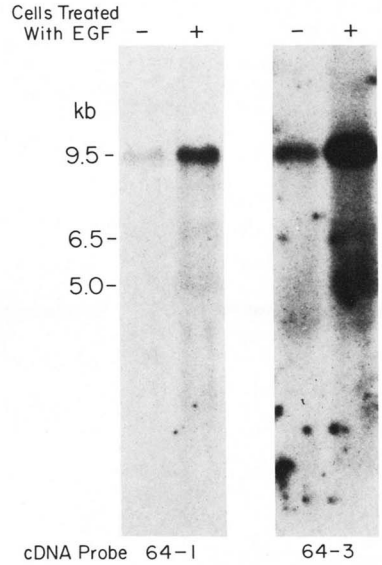

FIG. 4. **EGF treatment increases cellular EGF receptor mRNA content.** Total RNA was isolated from WB cells cultured for 4 h in the presence (*lane 2* and *4*) or absence (*lane 1* and *3*) of 100 ng/ml EGF. After electrophoresis and transfer to nitrocellulose, hybridization was performed. In experiment 1 (*lanes 1* and *2*) cDNA probe 64-1, (ba sequence from the coding region of the human EGF receptor extracellular domain) was used. In experiment 2 (*lanes 3* and *4*) cDNA 64-3, a sequence from the cytoplasmic coding region in pUC12 was used.

acetic acid precipitation (10%) of duplicate plates showed that 3–30 ng/ml EGF increased total ^{35}S incorporation by 13–26%. Gel slice counting of ^{35}S-labeled p170 showed a near doubling of receptor synthesis at 3 ng/ml EGF and a tripling at 30 ng/ml EGF. In addition, cells were lysed in electrophoresis stop solution, and nuclear, cytosolic, and particulate fractions were subjected to polyacrylamide gel electrophoresis and fluorography. No obvious increase in overall protein synthesis was observed after treatment with EGF (not shown). Lastly EGF did not increase the labeling of other ^{35}S-labeled proteins precipitated by normal rabbit serum or antiserum 799 (Figs. 2 and 3).

To determine whether the mechanism of EGF action was pretranslational, RNA was isolated (18) from WB cells cultured for 4 h with or without 100 ng/ml EGF. Fig. 4 shows Northern blots of total RNA from two experiments. Hybridization was performed with two cDNA probes (21) (generously provided by Axel Ullrich), 64-1, a sequence complementary to a portion of the external domain of the human EGF receptor (*lanes 1* and *2*) and 64-3, a sequence complementary to the cytoplasmic domain (*lanes 3* and *4*). The predominant transcript in WB cells was 9.5 kb, slightly smaller than the largest transcript from human cells and tissues (21–23). The two lower molecular weight RNAs, more readily apparent on darker exposures, were 6.5 and 5.0 kb, again slightly different from the human counterparts. RNA prepared from rat liver demonstrated the same 3 transcripts observed in WB cells, again with a predominance of the 9.5-kb transcript. EGF (*lanes 2* and *4*) increased receptor mRNA content when either cDNA was used for hybridization. Densitometry of autoradiographs revealed a 3–5-fold increase in the 9.5-kb transcript. Equal amounts of RNA were transferred from the gel lanes as evidenced by equivalent hybridization of an 18 S RNA probe (not shown). Preliminary results show that injection of EGF into the portal vein of a rat also resulted in an increase in hepatic EGF receptor mRNA synthesis.[3]

Until the recent availability of antireceptor antibodies and cDNAs complementary to receptor mRNA, analysis of receptor synthesis has been difficult. This has led to an emphasis on the role of ligand in speeding internalization, a process that rapidly clears the cell surface of 70–95% of its receptors. That some functional EGF receptor remains at the cell surface in spite of saturating doses of EGF is widely accepted, because it is known that extracellular EGF is necessary for 6–8 h to trigger mitogenesis (24–26). This might be due in part to a class of receptors that are immobile or uncoupled from the down regulation process (27). Alternatively, EGF induction of EGF receptor synthesis could provide a supply of new surface receptor. This may explain the ability of EGF to continue a surface signaling function for 6–8 h.

The data presented here clearly establish that receptor synthesis is not a constitutive process which responds only passively to down regulation. Rather, receptor synthesis is a regulated process. The literature contains examples of changes in EGF surface receptor number at different cell densities, during the cell cycle and following retinoic acid-induced differentiation (28–31). Since the receptor is an inducible protein, altered receptor synthesis may explain some of the above-noted findings. In fact, we have evidence that

[3] H. S. Earp, K. S. Austin, J. Blaisdell, M.-S. Tsao, L. W. Lee, and J. W. Grisham, unpublished results.

density-dependent and retinoic acid-induced changes in hepatic epithelial cell EGF receptor number are due in part to altered EGF receptor synthesis.[3] EGF has been previously demonstrated to stimulate transcription of several specific nucleic acid sequences (32, 33). The EGF receptor appears to be yet another transcript regulated by EGF, although definitive studies of nuclear transcription rates are needed, because changes in mRNA stability are not excluded by these experiments.

Is this a general phenomenon, homeostatic autoregulation of receptor mRNA synthesis by ligand action? With respect to EGF, the phenomenon has also been observed in human skin fibroblasts by Yarden and Ullrich.[4] The answer remains to be determined for other ligand-receptor pairs, but ligand-directed increases in receptor number, "up regulation," have been noted and, in one instance, studied in molecular detail. Interleukin 2 increases IL2 receptor synthesis in T lymphocytes, resulting in a 2–10-fold increase in surface IL2 receptor (34, 35). This appears to be part of a complex process that may require additional factors, the combination of which result in a slightly altered T cell phenotype. Similarly, insulin greatly enhances insulin binding during the differentiation of preadipocytes (36); and prolactin appears to increase prolactin receptors (37).

EGF receptor synthesis has also been investigated in the human A431 cell, using heavy amino acid (^{15}N ^{13}C ^{2}H) labeling techniques and immunoprecipitation; receptor synthesis did not appear to be regulated by EGF (38, 39). However, the A431 cell, which has 2–5 × 10^6 receptors/cell, is not a normal cell. It vastly overproduces receptor and has amplified and rearranged receptor genes (21–23). In fact, several neoplastic cell lines and tumors (40, 41) exhibit increased EGF receptor number and/or receptor gene rearrangements. In addition many neoplastic cells including A431 secrete transforming growth factor α, a EGF-like peptide that binds to the EGF receptor (42–44). These two observations raise an interesting question: do some of these cells, during the transformation process, begin to secrete TGFα, occupy the EGF receptor, and in so doing send a constant stimulus to produce more receptors? If so, could persistent stimulation lead to aberrant receptor expression in a clone of cells during the evolution of the neoplastic state?

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[4] Y. Yarden and A. Ullrich, unpublished results.

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