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The Epidermal Growth Factor Receptor Tyrosine Kinase in Liver Epithelial Cells

THE EFFECT OF LIGAND-DEPENDENT CHANGES IN CELLULAR LOCATION*

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Bryan K. McCune‡§¶ and H. Shelton Earp‡‖**

From the Cell Biology Program, ‡Lineberger Cancer Research Center and the Departments of §Pathology and ‖Medicine and Pharmacology, University of North Carolina, Chapel Hill, North Carolina 27599

Epidermal growth factor (EGF) activates the intrinsic tyrosine-specific protein kinase of its receptor (EGF-R). We studied the effect of EGF-dependent EGF-R internalization on receptor autophosphorylation and on the appearance of tyrosine phosphoproteins in rat liver epithelial cells. Peak receptor autophosphorylation activity (3- to 6-fold over basal) was found in homogenates of EGF-treated cells at times when the majority of receptors (>90%) had been internalized but not yet degraded (15 to 30 min). Stimulated activity persisted for at least 2 h if EGF-R degradation was blocked by methylamine or 18 °C incubation. Detection of stimulated autophosphorylation in homogenates of cells treated with EGF in culture required detergent in the assay. Detergent was not necessary to detect stimulated autophosphorylation when EGF was added directly to homogenates of untreated cells.

Immunoblots using antibodies against phosphotyrosine (p-Tyr) demonstrated that EGF treatment of intact cells increased the p-Tyr content of at least seven proteins (EGF-R, 115, 100, 75, 66, 57, and 52 kDa) within 5 s. Incubation of intact cells with EGF at 0 °C to prevent endocytosis still resulted in tyrosine phosphorylation of these seven proteins. In contrast, several substrates (120, 78, and 38 kDa) showed delayed increases (45–90 s) in tyrosine phosphorylation at 37 °C; their phosphorylation was even slower at 18 °C and did not occur at 0 °C. In cells incubated with EGF at 18 °C or in the presence of methylamine, EGF-R p-Tyr in the intact cell was lost by 2 h even though receptor was not degraded and still exhibited enhanced autophosphorylation in the homogenate assay. These findings suggest that tyrosine phosphorylation in response to EGF occurs predominantly during the initial stages of endocytosis and is mediated for the most part by ligand-receptor complexes at the cell surface. A subset of phosphorylations may require intracellular movement.

The interaction of epidermal growth factor (EGF)[1] with its cell surface receptor results in a number of rapid events. These include the activation of the receptor's intrinsic tyrosine kinase and endocytosis of the receptor-ligand complex (see Refs. 1 and 2 for current reviews). Activated EGF-R/kinase catalyzes self-phosphorylation as well as phosphorylation of other cellular proteins (1, 2). Delayed responses requiring 1 to 24 h occur in a number of cell types. These include increased protein, RNA, and DNA synthesis and enhanced cell proliferation (1–3). The mechanism coupling early and delayed responses is unknown. There is, however, evidence that the tyrosine kinase activity is important in signal generation. Chen *et al.* (4) used site-directed mutagenesis to create an EGF receptor devoid of kinase activity but still able to bind EGF normally. Cells expressing the kinase-defective receptor failed to exhibit the expected early and late responses to EGF, including endocytosis. Honegger *et al.* (5) described a similar kinase-deficient mutant receptor which underwent EGF-dependent endocytosis but did not enter the degradative pathway; this receptor also failed to transduce the EGF signal (6). The precise mechanism of signalling by the kinase, which presumably involves phosphorylation of specific substrates, remains to be determined.

EGF and its receptor traverse a heterogeneous group of intracellular membrane vesicles termed endosomes following interaction at the cell surface (7–11). In most cells studied, the endocytotic pathway ends in lysosomes, where ligand and receptor are degraded (8–11). Recent studies on EGF-R/kinase in endosomes have raised the possibility that intracellular kinase activity may be important for EGF action (12, 13). While these studies demonstrate EGF-dependent translocation of active EGF-R/kinase from the plasma membrane to endosomes, the physiologic significance of the internalized activity remains unclear.

In this study, we have attempted to analyze the time frame and cellular location of physiologically significant EGF-R/kinase activity following ligand-receptor interaction at the cell surface. We have examined the relationships between EGF-R phosphotyrosine content, kinase activity as defined by receptor autophosphorylation, and subcellular location during EGF-dependent receptor endocytosis. Certain incubation conditions (16–20 °C) and pharmacologic agents (*e.g.* methylamine) have been shown to inhibit degradation of internalized receptors by inhibiting endosome-lysosome "fu-

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¶ Present address: Laboratory of Chemoprevention, National Cancer Institute, Bethesda, MD 20892.

** To whom correspondence and reprint requests should be addressed.

[1] The abbreviations used are: EGF, epidermal growth factor; EGF-R, epidermal growth factor receptor; p-Tyr, phosphotyrosine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); KLH, keyhole limpet hemocyanin; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

sion" (14, 15). We have employed such inhibitors to study the characteristics of receptor allowed to accumulate intracellularly in prelysosomal membrane compartments. These studies were carried out using a nontransformed cell line derived from rat liver epithelial cells designated WB 344 (16) which has a surface complement of EGF-R similar to that of hepatocytes (200,000–300,000/cell) (17). EGF induces a variety of responses in these cells, including increased growth rate (17), altered patterns of enzyme activities (17), increased EGF receptor protein synthesis and mRNA levels (18), and increased phosphoinositide turnover (19). Our findings indicate that there are at least two classes of p-Tyr substrates in these cells, one class that must be immediately accessible to surface EGF receptor as these substrates are easily detectable within 5 s of EGF addition and can be phosphorylated in cells maintained at 0 °C. The other group of substrates appears to require time- and temperature-dependent changes in cell metabolism before its p-Tyr content is altered.

EXPERIMENTAL PROCEDURES

Materials—EGF was purified from mature male mouse salivary glands as described by Savage and Cohen (20) and iodinated with ^{125}I (Amersham) by the chloramine-T method of Hunter and Greenwood (21). [γ-^{32}P]ATP, ^{125}I-protein A, and [^{35}S]methionine were purchased from Du Pont-New England Nuclear. Other reagents were purchased from Sigma, unless otherwise indicated.

Cell Culture—The WB 344 cell line, established from a primary culture of Fisher 344 rat hepatocytes, was maintained in tissue culture plates in Richter's improved minimal essential medium supplemented with 10% fetal bovine serum and 0.1 μM insulin, at 37 °C in a humidified 5% CO_2 atmosphere (17). Experiments were performed on confluent cultures of passage 19 to 25. For short term (up to 2 h) experimental incubations with EGF, the culture medium was changed to serum-free Eagle's minimal essential medium with 20 mM HEPES, pH 7.4, 15 to 30 min prior to addition of EGF.

Antibodies—Polyclonal antiphosphotyrosine antisera were prepared by serially injecting rabbits with a preparation of phosphotyrosine, glycine, and alanine coupled to KLH (Calbiochem) using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Calbiochem) as described by Kamps and Sefton (22). Our injection protocol differed slightly from that of Kamps and Sefton (22). Rabbits were initially injected with a total of 1.5 mg of the conjugate in Freund's complete adjuvant at four to six intradermal sites. Intradermal boosts in incomplete adjuvant (also 1.5 mg distributed among four to six sites) were given every 5 weeks for three rounds of injections. All subsequent boosts were given intraperitoneally using a suspension of 125 μg of KLH conjugate in aluminum hydroxide prepared as follows: 1.25 g of alum (purchased at a grocery store) was dissolved in 25 ml of water. Sodium hydroxide was added to 50 mM, and the resultant precipitate ($Al(OH)_3$) was pelleted and washed 3 times with PBS (pH 7.4, without divalent cations), making sure that the last wash was at pH 7.4. The washed precipitate was suspended in 25 ml of PBS. KLH (125 μg) conjugate was added to 1.5 ml of $Al(OH)_3$ suspension. The mixture was centrifuged, and the pellet was resuspended in 0.5 ml of PBS and injected intraperitoneally into lightly anesthetized rabbits. The rabbits were bled 10–14 days after boosting.

Immunoglobulins were precipitated with 33% saturated $(NH_4)_2SO_4$, washed with 40% saturated $(NH_4)_2SO_4$, resuspended in ⅓ volume of 50 mM Tris-HCl, pH 7.2, 150 mM NaCl (TN), and dialyzed for 48 h against this buffer (two changes, 100 volumes each). The dialysates (up to 15 ml) were then affinity-purified essentially as described by Kamps and Sefton (22), except that we used phosphotyrosine in the coupling reaction instead of phosphotyramine. Four-ml columns of phosphotyrosine coupled to a 1:1 mixture of Affi-Gel 10 and 15 (Bio-Rad) were prepared by coupling the Affi-Gel slurry overnight at 4 °C in 1 volume of 75 mM phosphotyrosine, 100 mM MOPS, pH 7.5. Columns were equilibrated with TN. Affinity purification procedures were performed at room temperature. Each dialysate was passed over the column twice. The columns were then washed with 40 ml of TN, followed by 40 ml of 5 mM sodium phosphate, pH 7.2, 5 mM phosphoserine, 5 mM phosphothreonine, 100 mM NaCl, to elute potentially cross-reacting antibodies. Phosphotyrosine antibodies were eluted with 12 ml of 40 mM phenylphosphate, 90 mM NaCl, 30 mM Tris, pH 7.2, and dialyzed for 14 days against 150 volumes of TN containing 0.01% sodium azide, changing the buffer every 48 h. The affinity columns were used twice.

Polyclonal rabbit anti-rat EGF-R antiserum (Ab 1382) was prepared using EGF-R purified from Triton X-100-solubilized rat liver microsomal fractions by sequential affinity chromatography on wheat germ agglutinin-agarose and EGF-Affi-Gel columns as immunogen (18).

Immunoblotting and Immunoprecipitation—Immunoprecipitation of EGF-R from [^{35}S]methionine-prelabeled cells has been described (18). After experimental manipulations, lysates of WB cells for Western blot tyrosine phosphoprotein analysis were prepared as follows: 0.25 ml of radioimmune precipitation buffer was added to a 35-mm plate, and the monolayer was scraped and transferred to a glass test tube. These manipulations were done on ice. Radioimmune precipitation buffer contains 50 mM Tris, pH 7.4, 150 mM NaCl, 0.5 mM sodium vanadate, 5 mM EDTA, 1% Triton X-100, 0.1% SDS, 1% deoxycholic acid, 20 μg/ml leupeptin, 20 μg/ml phenylmethylsulfonyl fluoride, 100 kallikrein inactivating units/ml of aprotinin, and 10 mg/ml *p*-nitrophenyl phosphate. SDS-PAGE stop buffer (0.125 ml of a 3 × concentrated solution containing SDS and 6% β-mercaptoethanol) was added, and the mixture was incubated for 5 min at 100 °C. A fraction of the sample, kept constant within experiments (approximately 100 μg protein), was subjected to SDS-PAGE on 7% gels and transferred to nitrocellulose. The transfer was performed at 200 mA for 3-4 h in a buffer containing 25 mM Tris, pH 8.3, 0.2 M glycine, 20% methanol, 0.075% SDS, and 0.5 mM sodium vanadate. After blocking overnight at 21 °C in 10 mM Tris-HCl, pH 7.2, 150 mM NaCl, 3% bovine serum albumin (Boehringer Mannheim), blots were incubated with 2 μg/ml monospecific anti-phosphotyrosine antibodies in blocking solution for 1 to 2 h and washed. The washing procedure included two 5-min washes in 10 mM Tris-HCl, pH 7.2, 150 mM NaCl (rinsing buffer), one 5-min wash in rinsing buffer containing 0.05% Tween 20 and 0.05% Nonidet P-40, and two additional washes in rinsing buffer without additives. The blot was then incubated with ^{125}I-protein A (150,000 cpm/ml in blocking solution) for 1 h and washed as above. All antibody incubations and washes were done at room temperature. Autoradiography was then performed on the damp blots.

Immunofluorescence—WB cells grown on glass coverslips were fixed for 10 min at room temperature in either 95% ethanol (for surface and intracellular staining) or 1% paraformaldehyde in PBS (for surface-only staining). The coverslips were then incubated sequentially with Ab 1382 (1:50 dilution) and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (1:50, Cappel) at 21 °C for 1 h each, washing in PBS between and after incubations. Nonspecific staining was reduced by preabsorbing 1382 with an acetone-precipitated extract prepared from rat heart and spleen tissue (23). Coverslips were mounted in PBS/glycerol (1:1), viewed, and photographed using a Zeiss fluorescence microscope.

^{125}I-EGF Binding Assay—After experimental manipulations, WB cells on 35-mm plates were acid-washed with cold 50 mM glycine, pH 3.0, 100 mM NaCl to remove any surface-bound unlabeled EGF (24). The monolayers were then washed three times with cold Waymouth's medium containing 30 mM HEPES, pH 7.4, and 0.1% bovine serum albumin, and incubated in 0.6 ml of this medium containing 400,000 cpm of ^{125}I-EGF (approximately 1-2 ng) for 2 h at 4 °C. The monolayers were then washed, solubilized in 1% SDS, 0.1 N NaOH, and counted in an LKB Rackgamma counter. Determinations were performed in duplicate or triplicate, and nonspecific binding (determined by incubation with approximately 200-fold excess unlabeled EGF) was subtracted.

Autophosphorylation Assay—To assess EGF-R autophosphorylation activity, WB cell monolayers were washed and scraped in 0.5 ml of cold 10 mM Tris, pH 7.4, 0.25 M sucrose containing 10 μg/ml leupeptin, 20 μg/ml phenylmethylsulfonyl fluoride, and 100 kallikrein inactivating units of aprotinin. The cell suspensions were then homogenized in a glass vessel with 30 strokes of a Teflon pestle attached to a motorized tissue grinder assembly, while kept on ice. This resulted in breakage of greater than 95% of the cells as assessed by microscopic examination. The homogenates were then analyzed for EGF-R autophosphorylation activity at 0 °C in a final volume of 60 μl containing 50 mM PIPES, pH 7.0, 1 mM $MnCl_2$, and 0.1 mM sodium vanadate, using a procedure modified from Earp *et al.* (25). Each reaction mixture contained 20 μl (approximately 40 μg of protein) of homogenate. The reaction mixture was preincubated for 10 min on ice with or without 1 μg/ml EGF (EGF added at this point is hereafter referred to as added "directly to the homogenate" to distinguish it from EGF added in culture). The reaction was initiated by adding 20

μl of a solution containing 5 μCi of [γ-^{32}P]ATP (final concentration, 1 μM) and Triton X-100 (final concentration, 0.1%). When indicated, water or 0.2% Nonidet P-40 was substituted for Triton X-100. After 2 min on ice, the reaction was terminated by adding 30 μl of SDS-β-mercaptoethanol sample buffer and heating to 100 °C for 3 min. After SDS-PAGE on 7% gels, the gels were stained, dried, and exposed to Kodak XAR film. [^{32}P]p170 bands on the autoradiographs were quantitated using an LKB laser densitometer.

RESULTS

EGF-dependent Internalization and Degradation of EGF-R—In order to relate the receptor's kinase activity to its intracellular localization, we studied the time course of EGF-dependent receptor movement by immunofluorescence. We then assessed the loss of surface EGF binding capacity during EGF-dependent EGF-R internalization. Indirect immunofluorescence of ethanol-fixed cells showed the loss of surface staining with the appearance of brightly staining vesicles in the perinuclear Golgi region within 15 min of EGF addition (Fig. 1). Staining was lost after 2 h in the presence of EGF alone, but persisted in the form of large cytoplasmic vesicles in the presence of 10 mM methylamine (Fig. 1) or when the cells were incubated at 18 °C (data not shown). When the cells were fixed in 1% paraformaldehyde, which does not permit the antibody access to the cell interior, EGF-dependent loss of surface staining was observed but vesicle formation was not seen (data not shown).

Cell surface ^{125}I-EGF binding capacity (assessed after acid washing to remove unlabeled EGF remaining bound at the cell surface) declined steadily from greater than 90% to less than 20% of control levels between 0.5 and 5 min after the addition of EGF to the medium (Fig. 2). Surface EGF binding then further declined to 10% of control levels by 15 min. Binding studies using saturating concentrations of ^{125}I-EGF (60 ng/ml) demonstrated 30% residual surface binding at 5 min and again less than 10% of control after 10 min with EGF in culture (data not shown). Methylamine and 18 °C incubations did not prevent EGF-dependent loss of surface binding, although the decline was slower at the lower temperature (Fig. 2). If EGF was removed from the medium after the first 5 or 15 min of the incubation period, a gradual recovery of a small portion (10–20%) of the lost surface binding was seen over a 2-h incubation period, indicating that substantial recycling does not occur in these cells (data not shown). Immunoprecipitation of EGF-R from [^{35}S]methionine-labeled cells demonstrated EGF-dependent EGF-R degradation. With 100 ng/ml EGF, the EGF-R half-life was about 1 h. Methylamine (10 mM) and 18 °C incubation completely blocked EGF-dependent receptor degradation (data not shown).

EGF Receptor Autophosphorylation Activity in Whole Cell Homogenates—Significant stimulation of EGF-R autophosphorylation was detected in homogenates prepared from WB cells 15 to 60 min after the addition of 100 ng/ml EGF to the medium (Fig. 3). Densitometric analysis of [^{32}P]p170 bands in numerous experiments demonstrated peak increases ranging from 3- to 6-fold over basal levels at 15 to 30 min.

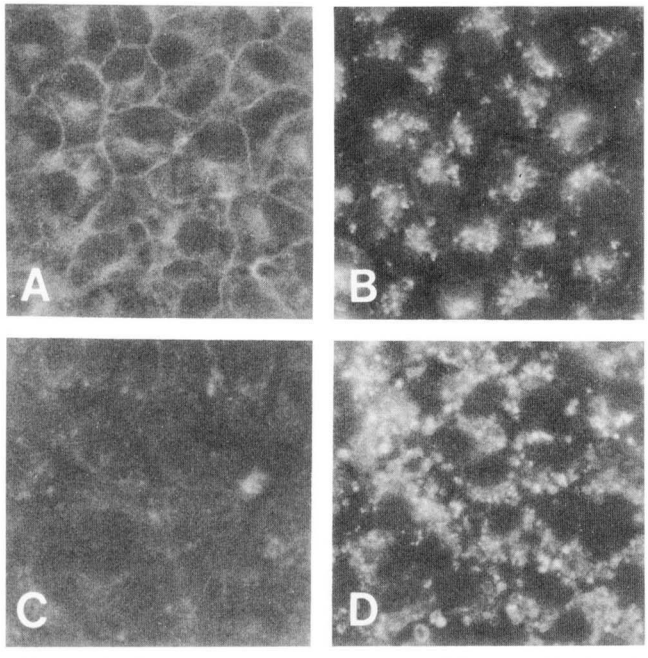

FIG. 1. **Indirect immunofluorescence of EGF-R in WB cells.** Confluent cultures of untreated cells (*A*), cells incubated with 100 ng/ml EGF at 37 °C for 15 min (*B*) or 2 h (*C*), and cells incubated with 100 ng/ml EGF for 2 h in the presence of 10 mM methylamine (*D*) were fixed in 95% ethanol and stained with anti-EGF-R antiserum as described under "Experimental Procedures."

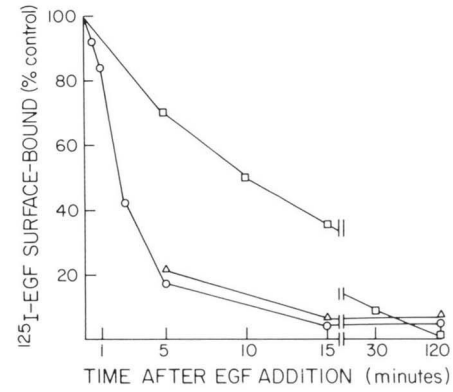

FIG. 2. **^{125}I-EGF surface binding to WB cells.** Confluent cultures were incubated with 100 ng/ml EGF for the indicated time periods, acid-washed to remove unlabeled surface-bound EGF, and incubated with ^{125}I-EGF for 2 h at 4 °C to determine residual surface binding activity, as described under "Experimental Procedures." Each *point* represents the average of three determinations minus nonspecific binding (standard error <10%), expressed as % of control. ○——○, 37 °C; □——□; 18 °C; △——△, 37 °C with 10 mM methylamine.

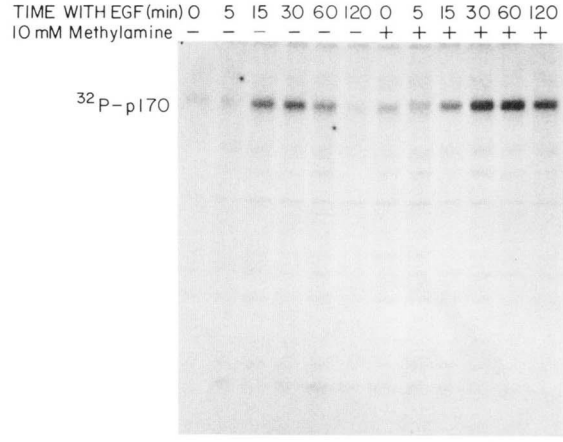

FIG. 3. **Autophosphorylation activity of EGF-R in WB cell homogenates.** Cultures were incubated with 100 ng/ml EGF at 37 °C for the indicated time periods in the absence (*left 6 lanes*) or presence (*right 6 lanes*) of 10 mM methylamine, homogenized, and assayed for EGF-R autophosphorylation activity as described under "Experimental Procedures." The assay mixture included 0.1% Triton X-100 in this experiment.

Autophosphorylation activity declined to below basal levels at 2 h (Fig. 3).

As indicated above, incubation of WB cells with EGF in the presence of 10 mM methylamine or low temperature (18 °C) prevented EGF-R degradation. Stimulated EGF-R autophosphorylation activity was at near peak levels in homogenates of cells incubated for 2 h with EGF and 10 mM methylamine (Fig. 3) or at 18 °C (not shown). The immunofluorescence and surface binding data indicate that the EGF-R assayed under these conditions was intracellular. Moreover, the stimulated autophosphorylation was demonstrated without adding EGF directly to the homogenate. This suggests that the EGF added in culture 2 h previously was responsible for stimulated autophosphorylation.

Effect of Detergent on Detection of EGF-R Autophosphorylation—Once the receptor had been internalized by incubating cells in culture with EGF, homogenate activity could only be observed after the addition of detergent to the assay mixture. In contrast, detergent was not required to detect EGF-R autophosphorylation if EGF was added directly to homogenates of untreated cells whose receptor had not been internalized. Thus, homogenization *per se* did not introduce a requirement for detergent in this assay; internalization did. Fig. 4 shows that autophosphorylation activity was at the same low level in EGF-treated and untreated cells if Triton X-100 was omitted from the assay mixture (Fig. 4, *lanes A* and *C*). EGF added directly to homogenates of control cells stimulated EGF-R autophosphorylation (Fig. 4, *lane B*), but did not affect homogenates of EGF-treated cells in the absence of detergent (Fig. 4, *lane D*). However, if 0.1% Triton X-100 was included in the assay of homogenates of EGF-treated (in culture) cells, autophosphorylation activity was substantially higher than basal levels (Fig. 4, *lane G*). EGF added to homogenates of EGF-treated cells did not further stimulate autophosphorylation in the presence of detergent (Fig. 4, *lane H*). Triton X-100 did not qualitatively alter the results in control cell homogenates. Identical results were obtained if the nonionic detergent, Nonidet P-40 (0.2%) was substituted for Triton X-100 in this assay (data not shown).

The effect of detergent was even more dramatic in EGF-treated cells in which all of the receptor accumulated intracellularly due to the action of methylamine. Stimulated autophosphorylation was not detected in homogenates of cells incubated with EGF for 30 or 120 min with or without

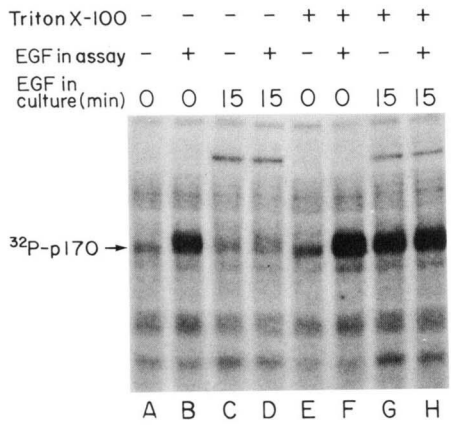

FIG. 4. **Effect of detergent on detection of EGF-R autophosphorylation activity.** Cultures of untreated or EGF-treated (100 ng/ml for 15 min at 37 °C) WB cells were homogenized and assayed for EGF-R autophosphorylation. Additional EGF (1 μg/ml at 0 °C for 10 min prior to addition of [γ-^{32}P]ATP) or 0.1% Triton X-100 (added with the [γ-^{32}P]ATP) was included in the reaction mixture where indicated.

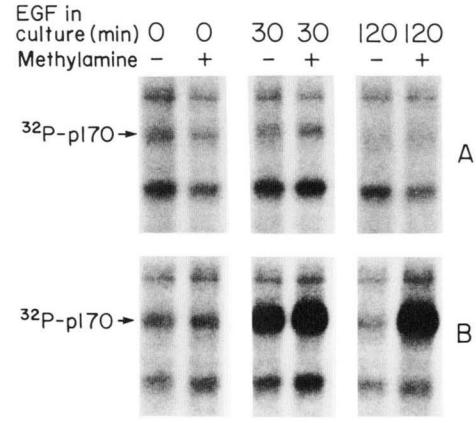

FIG. 5. **Effect of detergent on EGF-R autophosphorylation in homogenates of methylamine- and EGF-treated WB cells.** Cultures were incubated with 100 ng/ml EGF at 37 °C with or without 10 mM methylamine for the indicated time periods, homogenized, and assayed for EGF-R autophosphorylation activity in the absence of 0.1% Triton X-100 (*row A*) or in the presence of 0.1% Triton X-100 (*row B*), as described under "Experimental Procedures." Cells were preincubated with 10 mM methylamine for 15 min prior to the addition of EGF.

methylamine in the absence of Triton X-100 (Fig. 5, *row A*). The inclusion of Triton X-100 in the assay revealed markedly stimulated EGF-R autophosphorylation in homogenates of 30-min EGF-treated cells with or without methylamine and in homogenates of 120-min EGF-treated cells with methylamine (Fig. 5, *row B*). Without methylamine, receptor was degraded by 120 min in EGF-treated cells. Similar results were obtained when receptor degradation was blocked by incubating at 18 °C (data not shown). It is noteworthy that detergent specifically affects EGF-R and not the background pattern of ^{32}P-phosphoproteins which presumably results from the activity of other kinases in this assay. For example, a higher molecular weight substrate of about 280 kDa is phosphorylated in cells that had been treated in culture with EGF, but the demonstration of p280 phosphorylation is not dependent on detergent (Fig. 4). This substrate is phosphorylated on threonine residues presumably by an EGF-activated kinase.[2]

Evaluation of EGF-dependent Cellular Tyrosine Phosphoproteins Using Antiphosphotyrosine Antibodies—EGF-R in unstimulated WB cells contains virtually no detectable p-Tyr (Fig. 6). Incubation of WB cells with EGF at 37 °C produced a dramatic increase in the p-Tyr content of a 170-kDa protein corresponding to EGF-R within 30 s (Fig. 6). Increases in the p-Tyr content of several other proteins also occurred. EGF-R p-Tyr remained at high levels until 5 min and decreased over the remainder of the 2-h incubation period. Densitometric analysis EGF-R p-Tyr bands in three separate experiments showed 72, 47, and 66% (average 62%) decreases in EGF-R p-Tyr content between 2.5 and 15 min of EGF incubation at 37 °C. A parallel immunoblot using anti-receptor antibody did not detect receptor loss during this time period (data not shown). Thus, EGF-R dephosphorylation proceeds more rapidly than does receptor degradation.

We reasoned that substrates dependent on cellular movement, such as receptor internalization, would show delayed increases in p-Tyr content relative to those phosphorylated by EGF-R/kinase at the cell surface. A detailed early time course of cellular protein tyrosine phosphorylation in response to EGF is shown in Fig. 7. Increases in the p-Tyr

[2] B. K. McCune and H. S. Earp, unpublished results.

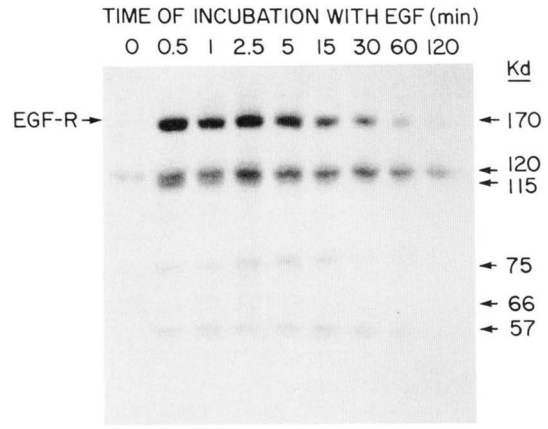

FIG. 6. **Western blot of WB cell lysates using antiphosphotyrosine antibodies.** Cells were incubated with 100 ng/ml EGF in culture at 37 °C for the indicated times and analyzed for p-Tyr phosphoproteins, as described under "Experimental Procedures."

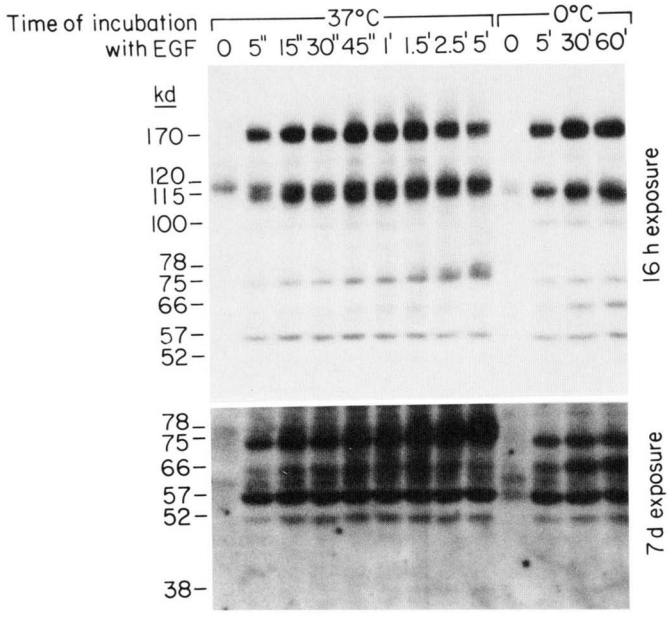

FIG. 7. **The effect of temperature and time on EGF-dependent p-Tyr substrates in WB cells.** Cells were incubated with 100 ng/ml EGF in culture at 37 °C or 0 °C for the indicated time periods and analyzed for p-Tyr phosphoproteins by immunoblotting with anti-p-Tyr as described under "Experimental Procedures." The *bottom panel* shows a longer exposure of the gel. Increased immunoidentifiable p-Tyr was initially observed within 5 s in all substrates except p38, p75, and p120. Increases in p-Tyr in these substrates were first observed at ~1 min. In cells treated with EGF at 0 °C, increased p-Tyr was not detected at times up to 60 min. The lack of increased p-Tyr in p120 and p78 is particularly notable at 0 °C.

content of at least 7 proteins (EGF-R, p115, p100, p75, p66, p57, and p52) were seen at the earliest time point examined, 5 s after the addition of EGF to the culture medium.

Three substrates showed delayed onset of their tyrosine phosphorylation. P120 was the most prominent protein recognized by anti-p-Tyr sera in cells prior to EGF addition. The cells are routinely cultured in 0.1 μM insulin and a prominent 120-kDa substrate for the insulin receptor tyrosine kinase has been described in hepatic cells (26, 27). P120 p-Tyr increased with time after EGF, but a portion of the increased p120 p-Tyr may be due a time-dependent mobility shift in p115 or the tyrosine phosphorylation of another p120 substrate.

A long exposure of the immunoblot revealed a faint 38-kDa tyrosine phosphoprotein appearing at 60–90 s following the addition of EGF to intact cells. Concurrent with the appearance of p38, a somewhat indistinct tyrosine phosphoprotein migrating at about 78 kDa appeared and continued to increase through 5 min at 37 °C (Fig. 7). The total p-Tyr accumulation in the 75-kDa and 78-kDa substrates clearly increased with time. However, it is difficult to determine whether increasing tyrosine phosphorylation of p75 initiates a process that results in retarded electrophoretic mobility and subsequent accumulation of increased p-Tyr p75 in the 78 kDa region of the gel.

To eliminate the contribution of EGF-R internalization to altered patterns of tyrosine phosphorylation, a time course in which cells were incubated with EGF at 0 °C was performed (8, 28, 29). Tyrosine phosphorylation of EGF-R, p115, p100, p75, p66, p57, and p52 occurred within 5 min (Fig. 7). This pattern remained unchanged at incubation times of up to 60 min. In contrast to the findings at 37 °C, there were no significant changes in phosphorylation of p120, p78, and p38 at 0 °C. The findings depicted in Fig. 7 have been observed in three separate experiments. The lack of phosphorylation of certain substrates at 0 °C is not due to a generalized toxic effect of maintaining cells at 0 °C. A 2-h incubation with EGF at 0 °C resulted in a higher p-Tyr content in several substrates (EGF-R and p66) when compared to EGF treatment for 60 s at 37 °C. In addition, when cells were held at 0 °C for 30 min and immediately treated with EGF when returned to 37 °C, phosphorylation of the secondary set of substrates (*e.g.* p78) was again observed (data not shown).

We next examined the response to EGF at 18 °C. Tyrosine phosphorylation of EGF-R, p115, p100, p75, p66, p57, and p52 occurred within 1 min of EGF addition, but increases in the p-Tyr content of p38 and p78 did not occur until 5–15 min. The p-Tyr content of p120 increased gradually over the first 15 min. At 18 °C, most of the p-Tyr was lost from EGF-R by 2 h (Fig. 8). Also shown in Fig. 8 for comparison are the tyrosine phosphoprotein patterns of cells incubated with EGF for 30 min at 0 °C and 2.5 min at 37 °C in the same experi-

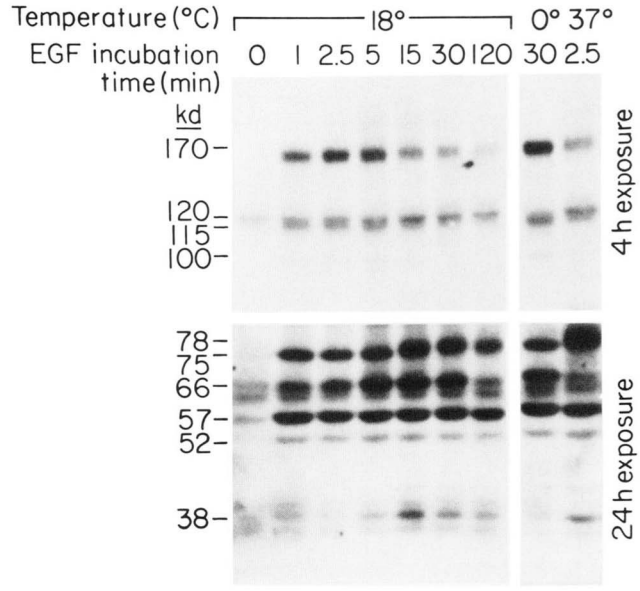

FIG. 8. **Time course of EGF-dependent tyrosine phosphorylation at 18 °C.** WB cells were incubated with 100 ng/ml EGF in culture at 18 °C, 0 °C, or 37 °C for the indicated times and analyzed for p-Tyr containing proteins by immunoblotting with anti-p-Tyr as described under "Experimental Procedures." The *bottom panel* shows a longer exposure of the gel. Again, the differential between p120, p78, and p38 p-Tyr accumulation at 0 °C and 37 °C is noted as is the delayed times course of p38 p-Tyr accumulation at 18 °C.

ment. Again, the lack of p-Tyr at 0 °C in p38, p78, and p120 is demonstrated.

p-Tyr loss from EGF-R was inhibited in the presence of methylamine; densitometric analysis of anti-p-Tyr immunoblot autoradiographs showed no significant difference between EGF-R p-Tyr levels in cells incubated with 100 ng/ml EGF for 2.5 or 15 min in the presence of 10 mM methylamine (absorbance units ± S.E. of triplicate samples at 2.5 min: 1.86 ± 0.23; at 15 min: 1.89 ± 0.39). By 2 h in the presence of EGF and 10 mM methylamine, EGF-R p-Tyr levels were negligible (data not shown). Methylamine alone had no effect on EGF-R p-Tyr levels.

The substrates observed after a 2.5-min exposure of intact cells to EGF at 37 °C were the only ones detected in these experiments, *i.e.* novel tyrosine phosphoproteins were not identified under conditions that inhibited receptor degradation for up to 2 h (18 °C or methylamine). In addition, EGF-R p-Tyr did not persist under these conditions. Therefore, even though the receptor was clearly enzymatically active in cell-free assays, there was no evidence for prolonged activity of the EGF-R/kinase intracellularly.

DISCUSSION

We have examined the tyrosine-specific protein kinase activity of the EGF receptor in cultured rat liver epithelial cells during EGF-dependent receptor endocytosis, by analyzing both endogenous substrate phosphorylation and receptor autophosphorylation. Our results are in agreement with those of Cohen and Fava (12) and Kay *et al.* (13) in that they suggest that EGF-R is capable of enzymatic activity in prelysosomal membrane compartments following EGF-induced internalization. Their studies demonstrated increased EGF-R autophosphorylation and EGF-R/kinase activity toward added substrates in endosome-enriched subcellular fractions from EGF-treated cells. No evidence for internalization-dependent protein tyrosine phosphorylation was described.

We have addressed the issue of physiologic activity of internalized EGF-R/kinase in the present study by relating the location of the receptor to the phosphotyrosine response in EGF-treated intact cells. In addition to increases in EGF-R p-Tyr, increases in the p-Tyr content of a number of other proteins were observed in WB cells within seconds after adding EGF to the culture medium. Most notable were proteins of apparent molecular weights 115,000, 100,000, 75,000, 66,000, 57,000, and 52,000. After 2.5 to 5 min of incubation with EGF, EGF-R p-Tyr content steadily decreased. The other substrates also lost their p-Tyr, but at variably slower rates that EGF-R. Increases in the p-Tyr content of three proteins (p38, p78, and p120) were delayed relative to the others at 37 °C and 18 °C. Delayed phosphorylation of a new substrate was unambiguous in the case of p38. While in most experiments p78 and p120 each appeared to be distinct members of a doublet (p75/p78, p115/p120) that are phosphorylated after 1–2 min of EGF action at 37 °C, we cannot completely exclude the possibility that the increase in the p-Tyr content of p78 and p120 may reflect a time- and temperature-dependent modification of p75 and p115 resulting in decreased mobility. The changes in the p-Tyr content of p38, p78, and p120 did not occur if EGF incubations were carried out at 0 °C, a temperature at which EGF binds to surface EGF-R, but receptor clustering and internalization is prevented (8, 28–30). However, substantial tyrosine phosphorylation of p115, p100, p75, p66, p57, and p52 still occurred at 0 °C, suggesting that these substrates are accessible from the cell surface.

Allowing EGF-R to accumulate in the prelysosomal intracellular compartment by incubating at 18 °C or in the presence of methylamine for up to 2 h resulted in the loss of p-Tyr from EGF-R as well as the substrates. No additional tyrosine phosphoproteins appeared under these conditions. Thus, there was little evidence for continued endogenous tyrosine kinase activity in this prelysosomal compartment even though the receptor was enzymatically active in cell-free assays. These observations suggest that EGF-R/kinase is particularly active during the early stages of endocytosis at or near the cell surface, and many substrates do not depend on receptor internalization. The time and temperature dependence of p38, p78, and p120 phosphorylation raises the possibility that there exist internalization-dependent substrates that are phosphorylated early during the endocytotic process. We cannot presently exclude the possibility, however, that delayed phosphorylation is due to another mechanism, such as: (i) EGF-dependent activation of a different tyrosine kinase, (ii) substrate modification that brings them to surface-active receptors, or (iii) in the case of p78 and p120, continued accumulation of p-Tyr in p75 and p115 which results in their migration in the 78-kDa and 120-kDa region of the gel.

The cellular p-Tyr response to EGF during 0 °C incubations has implications regarding the mechanism of EGF-R activation. Clustering or dimerization of EGF-R following EGF binding has been shown in intact cells and in solubilized EGF-R preparations and has been postulated to be responsible for activation of the receptor's kinase (29–33). A number of studies have demonstrated morphologically and biochemically that no or very little receptor oligermerization occurs when EGF binds to intact cells at 0–4 °C (8, 28–30). Thus, our results suggest that substantial activation of EGF-R/kinase may occur without significant receptor aggregation at the cell surface. In support of this interpretation, EGF-activated EGF-R/kinase monomers have been demonstrated in cell-free EGF-R preparations (34). Against this interpretation is the possibility that some preformed dimers exist on the surface of WB cells. If the latter is true, dimerization alone would not be sufficient to activate the kinase because our studies with anti-p-Tyr sera suggest that basal EGF-R autophosphorylation is extremely low in WB cells at any temperature.

The rapid EGF-R tyrosine phosphorylation/dephosphorylation kinetics observed in WB cells is similar to that described by Sturani *et al.* for EGF-R in human fibroblasts treated with EGF (35). In contrast, these investigators demonstrated persistence of EGF-R p-Tyr in A431 cells incubated with EGF for up to 6 h. A431 is a human epidermoid carcinoma cell line expressing a large number of surface EGF receptors (2×10^6/cell) which do not efficiently internalize EGF-R in response to EGF (12). By acid stripping EGF from the cell surface of A431 cells, this group demonstrated a marked decrease in EGF-R p-Tyr content, suggesting 1) most of the phosphorylated EGF-R was at the cell surface, and 2) binding of EGF was necessary to maintain tyrosine phosphorylation (35). Experiments in WB cells also demonstrated a dramatic decrease in EGF-R p-Tyr after cells incubated with EGF for 30 min at 0 °C were acid-stripped.[2] This suggests that when receptor is held at the cell surface even at low temperatures, continued binding of EGF is necessary to maintain EGF-R in the tyrosine-phosphorylated state.

At later time points during the course of endocytosis, the requirement for detergent to detect increased autophosphorylation activity in homogenates of EGF-treated cells may suggest that the receptor enters an intracellular compartment in which the kinase domain is not exposed to the cytoplasm. The vast majority of receptor apparently accumulates in this compartment in the presence of inhibitors of degradation.

The detergent presumably causes partial permeabilization of the membrane barrier, allowing access of the kinase to the [^{32}P]ATP added in the assay. The multivesicular body, a vesicles-within-a-vesicle structure, is a likely candidate for such a compartment. EGF receptors have been observed to pass through these endosome variants prior to lysosomal degradation in A431 cells and rat hepatocytes (8, 28, 36) and to accumulate in these structures if incubations are carried out at low temperature (36) or in the presence of amines such as methylamine (8). Miller *et al.* (36) showed that the majority of EGF-R in multivesicular bodies was localized to intraluminal vesicles. Detergent is not necessary to detect increased autophosphorylation if EGF is added directly to homogenates of control cells, indicating that the kinase domain is exposed to the cytoplasm at the cell surface. Endocytosis may serve to limit the site and duration of EGF-R/kinase activity by isolating it from ATP and potential substrates before degradation in lysosomes. There may be additional reasons for the need to use detergent to detect EGF receptor autophosphorylation of internalized EGF receptors. The need for detergent is detected at early times in which it is doubtful that all receptor has entered multivesicular bodies. However, it is clear that there is a difference between assay requirements for internalized EGF-R/kinase and surface EGF-R/kinase.

In summary, our results indicate that the cellular phosphotyrosine response to EGF in rat liver epithelial cells occurs predominantly within seconds of exposure to EGF and is complete within the first few minutes of exposure to EGF. It is mediated for the most part by EGF-receptor complexes at the cell surface. Three proteins are identified (p38, p78, p120) which may depend on receptor internalization or some other time- and temperature-dependent process for increased tyrosine phosphorylation. Internalized EGF-R is capable of tyrosine kinase activity, but enters a prelysosomal compartment (?multivesicular body) in which it is functionally inactive due to sequestration from ATP. The finding that EGF-R/kinase is enzymatically intact until it is degraded in lysosomes raises the possibility that altered intracellular routing could result in an altered pattern of protein tyrosine phosphorylation and consequently an altered cellular response to EGF.

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