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Transient Epidermal Growth Factor (EGF)-dependent Suppression of EGF Receptor Autophosphorylation during Internalization*

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To study the activity of the epidermal growth factor (EGF) receptor during EGF-directed internalization, liver epithelial cells were exposed to EGF at 37 °C for various periods of time, washed, and homogenized at 0 °C. EGF receptor autophosphorylation was assessed in homogenates using [γ-^{32}P]ATP. Autophosphorylation was stimulated 3- to 6-fold in homogenates of cells incubated with EGF (100 ng/ml) for 15 min but was at or below basal levels in homogenates of cells treated with EGF for 2.5–5 min. This was surprising because immunoblotting revealed that EGF receptor phosphotyrosine (P-Tyr) content in intact cells was near maximal from 30 s to 5 min after EGF treatment. Excess EGF (1 μg/ml), added after homogenization but prior to the assay, increased autophosphorylation in homogenates of cells that had not been treated with EGF, but failed to increase activity in homogenates of cells treated with EGF in culture for 2.5–5 min. Suppression of tyrosine phosphorylation of an exogenous kinase substrate was also observed at times paralleling the suppression of EGF receptor autophosphorylation.

The transient suppression of receptor autophosphorylation in the cell-free assay was not explained by persistent occupation of autophosphorylation sites by phosphate added in the intact cells. The sites were >80% dephosphorylated during the homogenization. Additionally phosphatase inhibition that prevented the normal loss of EGF receptor P-Tyr in intact cells at 15 min did not affect the pattern of early (2.5–5 min) suppression and later (15 min) stimulation of autophosphorylation measured in the cell-free assay. The supression was not explained by activation of protein kinase C in that depletion of >95% of cellular protein kinase C activity by an 18-h incubation of cells with 10 μM 12-*O*-tetradecanoylphorbol 13-acetate (TPA) did not affect the early suppression of autophosphorylation in EGF-treated cells. Moreover, under the conditions tested, activation of protein kinase C by short-term treatment (0.5–10 min) with TPA or angiotensin II did not appreciably alter subsequent autophosphorylation in the cell-free assay. In contrast, a 30 °C preincubation of homogenates from cells with suppressed EGF receptor autophosphorylation led to the recovery of the ability of EGF to stimulate EGF receptor autophosphorylation. These results suggest that a rapid reversible protein kinase C-independent process prevents detection of EGF receptor kinase activity during an early phase of EGF-dependent receptor internalization.

Epidermal growth factor (EGF)[1] alters cellular metabolism and stimulates cell growth by interacting with a 170,000-dalton transmembrane receptor. EGF binding activates the intrinsic tyrosine kinase of the receptor's cytoplasmic domain. Current evidence indicates that regulation of EGF receptor action may involve not only modulation of the rates of receptor synthesis and degradation, but also reversible modification of the receptor protein (1, 2). For example, EGF promotes receptor aggregation and internalization, processes which may influence receptor signaling (3–6). Another example is the phosphorylation of the EGF receptor on threonine 654 by protein kinase C which decreases the affinity of the receptor for EGF and, in some cells, decreases EGF receptor tyrosine kinase activity as measured in cell-free assays (7–10). Protein kinase C also phosphorylates other EGF-R threonine and serine residues, but the significance of these modifications is unknown (9, 11, 12). Thus, hormones and other agents which activate protein kinase C may affect EGF/EGF receptor interactions. Since EGF itself can activate phospholipase C and generate diacylglycerol in some cells (13–15), the effect of EGF on EGF receptor signal transduction may be more complex than simple initiation of receptor endocytosis. In A431 cells, a human tumor cell line which overexpresses the EGF receptor, EGF activates protein kinase C, resulting in phosphorylation of EGF receptor threonine 654 (13, 14). Chinkers and Garbers (10) demonstrated that EGF treatment of intact A431 cells induced a suppression of EGF receptor/kinase activity in subsequently solubilized extracts of A431 cell membranes. Sturani *et al.* (16) used anti-P-Tyr immunoblotting to demonstrate reduced EGF-dependent EGF receptor P-Tyr content in intact A431 cells treated with phorbol esters. Each group suggested that activation of protein kinase C modulated EGF receptor autophosphorylation and kinase activity.

We have shown previously that EGF stimulates phosphoinositide hydrolysis in a nontransformed rat liver epithelial cell line (15), and in the present report we demonstrate a transient suppression of EGF receptor/kinase activity in cell extracts, minutes after the addition of EGF to intact cells.

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[1] The abbreviations used are: EGF, epidermal growth factor; EGF-R, epidermal growth factor receptor; EGF receptor/kinase, epidermal growth factor tyrosine kinase; TPA, tetradecanoylphorbol 13-acetate; P-Tyr, phosphotyrosine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

This raises the possibility of an EGF-stimulated protein kinase C-mediated feedback inhibition of EGF-dependent tyrosine phosphorylation in these cells. In fact we have obtained evidence that activation of protein kinase C inhibits some measures of EGF-dependent tyrosine phosphorylation in intact WB cells.[2] However, the suppression of activity detected in our cell-free assay was not due to protein kinase C activation, since it occurred in cells depleted of protein kinase C. In fact, activation of protein kinase C prior to the addition of EGF had little effect on autophosphorylation of the EGF receptor assayed in cell-free extracts. The data presented in this and another recent report (17) suggest that, in a nontransformed epithelial cell with a normal receptor complement and internalization pattern, there are at least four separable stages through which the EGF-R tyrosine kinase passes upon the binding of EGF. The first stage, kinase activation which occurs within seconds of EGF-binding to cell surface receptor, is responsible for a large proportion of the cellular protein tyrosine phosphorylation (17) and initiates the internalization process (1, 2). The second stage is characterized by a conformation change in the receptor or its association with other cellular constituents. This stage, defined by suppressed autophosphorylation and kinase activity as measured in a cell-free assay, occurs within 2–5 min of exposure of the cell to EGF. The suppression is not mediated by protein kinase C and probably involves only those receptors occupied by EGF. The third stage is characterized by re-establishment of detectable kinase activity in the internalized receptor pool 10–30 min after the initial internalization. During this time period, the EGF receptor/kinase is active in cell-free assays, but becomes effectively inactive in intact cells as shown by progressive loss of receptor P-Tyr content in the intact cell (17). This loss of P-Tyr on autophosphorylation sites may be due to sequestration of the receptor from cellular ATP pools as the receptor moves into complex intracellular vesicles. Last, in the fourth stage, transfer of receptors to lysosomes abolishes kinase activity, presumably due to receptor degradation.

EXPERIMENTAL PROCEDURES

Materials—EGF, purified from male mouse salivary glands (18), was iodinated with ^{125}I (Amersham Corp.) by the chloramine-T method (19). [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—WB-344 cells (20), maintained in Richter's improved minimal essential medium supplemented with 10% fetal bovine serum and 0.1 μM insulin, were used as confluent cultures of passage 19–25. For short-term (up to 2 h) experimental incubations with EGF, the culture medium was changed to serum-free Eagle's minimal essential with 20 mM HEPES, pH 7.4, 15–30 min prior to addition of EGF.

Antibodies and Immunoblotting—Monospecific polyclonal antiphosphotyrosine antibodies were prepared by immunizing rabbits with a random co-polymer of phosphotyrosine, glycine, and alanine coupled to keyhole limpet hemocyanin (Calbiochem) using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Calbiochem) as described by Kamps and Sefton (21). The injection and affinity purification are detailed elsewhere (17). After experimental manipulations, lysates of WB cells were prepared and subjected to immunoblotting with the affinity purified anti-P-Tyr antisera as described (17).

Kinase Assays—To assess EGF-R autophosphorylation activity, monolayers were washed, scraped into 0.5 ml of cold 10 mM Tris-HCl, pH 7.4, 0.25 M sucrose containing 10 μg/ml leupeptin, 20 μg/ml phenylmethylsulfonyl fluoride, and 100 KIU/ml aprotinin, and homogenized on ice in a glass vessel with 30 strokes of a motor-driven Teflon pestle. This resulted in breakage of greater that 95% of the cells as assessed by light microscopic examination. Autophosphorylation activity was assessed at 0 °C in 60 μl containing 50 mM PIPES, pH 7.0, 1 mM $MnCl_2$, and 0.2 mM sodium vanadate (22). Each reaction mixture, containing 20 μl (approximately 40 μg of protein) of homogenate, was preincubated for 10 min on ice with or without 1 μg/ml EGF. The reaction was initiated by adding 20 μl containing 5 μCi of [γ-^{32}P]ATP (final concentration, 1 μM) and Triton X-100 (final concentration 0.1%). After 1 or 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.

To assess EGF-dependent tyrosine-specific kinase activity directed toward exogenous substrates, the sythetic peptide RRLIEDAEYAARG was used in an assay modified from Earp *et al.* (22). Cell homogenate (10 μl) was incubated in a 30-μl reaction mixture containing 20 mM PIPES, pH 7.0, 1.0 mM $MnCl_2$, 0.1% Triton X-100, and 1 mM peptide substrate. An 8-min 30 °C incubation was initiated by adding 20 μM ATP containing 10 μCi of [γ-^{32}P]ATP/assay. The reaction was stopped by adding 15 μl of 1 mM ATP in 0.1% bovine serum albumin followed by 40 μl of 5% trichloroacetic acid. After 20 min at 0 °C, samples were centrifuged in an Eppendorf microfuge for 10 min. Aliquots (30 μl) of supernatant were spotted onto phosphocellulose paper (P-81 Whatman) and washed three times (15 min each) with 30% acetic acid and once with acetone. Dried filters were counted for Cerenkov radiation. Aliquots from each homogenate were assayed in triplicate, and background (assay without peptide) for each homogenate was determined in duplicate and subtracted.

RESULTS

Suppression of EGF Receptor Autophosphorylation in Homogenates of Cells during Endocytosis—Using anti-P-Tyr Western blotting techniques, we have noted previously that EGF stimulated EGF receptor tyrosine phosphorylation within 5 s of addition to intact WB cells. The stimulation was maximal between 30 s and 2.5 min but persisted for 15–30 min (17). When EGF receptor autophosphorylation was examined in cell homogenates, receptor autophosphorylation was stimulated in cells treated with EGF 15 and 30 min prior to homogenization (Figs. 1 and 2). Addition of 1 μg/ml EGF to the homogenate assay did not further increase receptor autophosphorylation in cells pretreated with EGF for 15–30 min. As expected, EGF addition to homogenates of cells that had not been treated with EGF stimulated receptor phosphorylation. In contrast, homogenates from cells treated with EGF in culture for 2.5–5.0 min did not exhibit stimulated

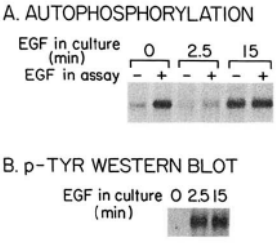

FIG. 1. **Suppression of EGF receptor autophosphorylation by EGF-treatment of cultured cells.** Intact WB cells were incubated without EGF or with EGF (100 ng/ml) for 2.5 or 15 min at 37 °C. At indicated times, cell monolayers were washed, scraped, and homogenized as described under "Experimental Procedures." *A*, aliquots (20 μl) of each homogenate were incubated without or with 1 μg/ml EGF for 10 min at 0 °C and then assayed for EGF receptor autophosphorylation activity as described under "Experimental Procedures." Gel electrophoresis and autoradiography were performed. The ^{32}P-p170 region of the autoradiograph is shown. *B*, aliquots (50 μl) from the same homogenates assayed for autophosphorylation were added to 25 μl of 3 × SDS-PAGE sample buffer (with 6% β-mercaptoethanol) and heated at 100 °C for 3 min. They were electrophoresed and analyzed by immunoblotting with anti-P-Tyr, as described under "Experimental Procedures." The 170-kDa band corresponding to EGF receptor is shown from the autoradiograph detecting ^{125}I-protein A-labeled anti-P-Tyr antibodies. In this experiment the cultured cells were preincubated with 200 μM sodium vanadate; under these conditions p170 retains P-Tyr for greater than 15 min.

[2] W. R. Huckle, J. R. Hepler, S. G. Rhee, T. K. Harden, and H. S. Earp, submitted for publication.

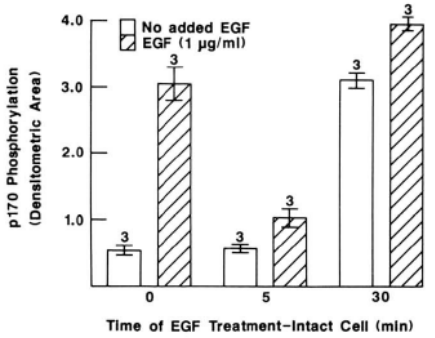

FIG. 2. **Transient suppression of EGF receptor autophosphorylation in homogenates of EGF-treated cells.** Triplicate sets of WB cells were incubated with or without 100 ng/ml EGF in culture at 37 °C for the indicated times. Homogenates were made and incubated with (*hatched bars*) or without (*empty bars*) 1 μg/ml EGF for 10 min at 0 °C and assayed for EGF receptor autophosphorylation. ^{32}P-p170 (EGF receptor) bands on autoradiographs were quantitated by laser densitometry. The *brackets* represent the standard error of the mean.

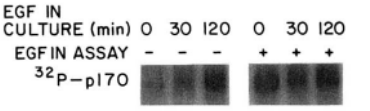

FIG. 3. **Effect of 18 °C incubation on suppression of EGF receptor autophosphorylation.** WB cells were incubated with EGF in culture at 18 °C for the indicated times, homogenized, and analyzed for EGF receptor autophosphorylation. The p170 region of the autoradiograph is shown.

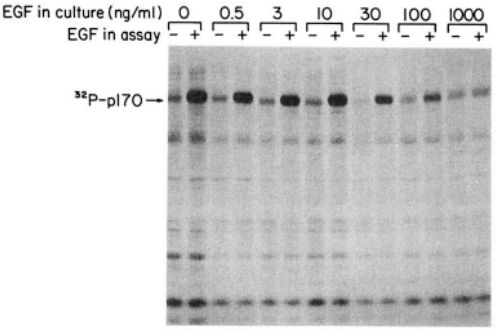

FIG. 4. **Dose response of EGF receptor autophosphorylation suppression.** WB cells were incubated for 2.5 min at 37 °C with the indicated amounts of EGF, homogenized, incubated with or without 1 μg/ml EGF for 10 min at 0 °C, and assayed for EGF receptor autophosphorylation as described under "Experimental procedures."

EGF receptor autophosphorylation nor were they appreciably stimulated by 1 μg/ml EGF added to the assay. Thus, although the receptor kinase had been active in autophosphorylation during some portion of the 2.5 min after EGF addition (as indicated by increased EGF receptor P-Tyr in the immunoblot from the intact cell Fig. 1*B* and Ref. 17), this activity was suppressed by the time of the cell-free assay. Immunoblotting with anti-rat EGF receptor antibody has demonstrated that the amount of immunoidentifiable EGF receptor in the WB cell homogenates was equivalent in control cells and at times up to 15 min after the addition of EGF to intact cells (17). Thus, the differences in autophosphorylation at 2.5–5 min are not due to differential recovery of EGF receptor at various times during internalization.

Suppression of autophosphorylation was not detected in homogenates of cells treated with EGF in culture for only 30 s, *i.e.* preincubation of such homogenates with excess EGF lead to activation in the autophosphorylation assay (data not shown). Therefore a time-dependent yet transient (~1–5 min) suppression of EGF receptor autophosphorylation was observed in homogenates of cells treated with EGF in culture. Maximal suppression typically occurred at 2.5 min. Since ^{125}I-EGF binding studies (after acid stripping) of the intact WB cells showed that at least 40% of the receptors remained accessible at the cell surface after 2.5 min of EGF incubation (17), suppression of receptor autophosphorylation (as defined by this assay) can occur without internalization of the entire receptor population. Although added EGF (1 μg/ml) might not be expected to gain access to the EGF binding site within the endocytotic vesicle, it could not activate (in homogenates) the population of receptors (~40%) that are not yet within an enclosed vesicle at 2.5 min.

Homogenates of cells incubated with EGF in culture for 15–30 min at 18 °C also showed suppressed autophosphorylation activity, indicating that the suppression time course can be slowed at temperatures known to retard receptor internalization (Fig. 3). Homogenates of cells incubated with EGF for 2 h at 18 °C showed stimulated autophosphorylation (Fig. 3). Thus, there appears to be a point in the internalization process at which the autophosphorylation reaction is inhibited. The time required to reach this state (and to exit from it) varies with temperature.

Dose Response of EGF Receptor Autophosphorylation Suppression—Suppression of EGF receptor autophosphorylation occurred in a dose-dependent manner. Homogenates were prepared from WB cells which had been incubated for 2.5 min at 37 °C at EGF concentrations ranging from 0.5 to 1000 ng/ml. Each sample showed only basal levels of EGF receptor autophosphorylation activity without preincubation with excess EGF prior to assay (Fig. 4). Preincubation of these homogenates with 1 μg/ml EGF for 10 min at 0 °C prior to the autophosphorylation assay stimulated EGF receptor autophosphorylation if the EGF dose in culture had been 10 ng/ml or less. From 30 to 1000 ng/ml EGF there was a dose-dependent decrease in the ability to respond to added EGF in the homogenate autophosphorylation assay (Fig. 4). This suggests that only occupied receptors undergoing internalization enter the suppressed state, whereas the unoccupied receptors remaining at the surface behave like those in cells that were not treated with EGF.

The Relationship between EGF Receptor P-Tyr Content and Autophosphorylation Activity—P-Tyr Western blotting demonstrates a dramatic increase in EGF receptor P-Tyr content within 5 s of adding EGF to intact WB cells. Maximal P-Tyr levels persist for 2.5–5 min and decrease by 62% at 15 min (17). We examined whether the transient early suppression could be ascribed to occupancy of receptor autophosphorylation sites by unlabeled phosphate from cellular ATP.

Initially, cells were incubated with EGF under conditions which inhibited the loss of P-Tyr from EGF receptor. Inhibition of tyrosine phosphatases (23) by 200 μM vanadate prevented the loss of EGF receptor P-Tyr between 2.5 and 15 min of EGF treatment, as assessed by P-Tyr Western blots (*e.g.* Fig. 1*B*). However, homogenates of vanadate-treated cells still exhibited the same pattern of transient suppression followed by stimulation of autophosphorylation (Fig. 1*A*). Likewise, incubation of cells in the presence of 10 mM methylamine, which inhibits endosome acidification, inhibited the loss of P-Tyr from EGF receptor assessed in whole cell lysates between 2.5 and 15 min (17). Again, the suppression/activation pattern of EGF receptor autophosphorylation in the homogenates of methylamine-treated cells was maintained (data not shown). Thus, maintenance of EGF receptor P-Tyr in the intact cell for 15 min by vanadate or methylamine did

not prolong the suppressed state to 15 min.

Since the homogenization for the cell-free assay was done in the absence of phosphatase inhibitors, EGF receptor P-Tyr may have been lost prior to the cell-free assay. Comparison of EGF receptor P-Tyr content in homogenates of EGF-treated cells with the content in rapidly prepared radioimmune precipitation buffer lysates (both assessed by anti-P-Tyr immunoblots) showed an 84 ± 4% (n = 3) loss of EGF receptor P-Tyr under the standard homogenizing conditions. Thus, during preparation for the cell-free assay, virtually all the receptor P-Tyr was lost, yet decreased autophosphorylation occurred. This clearly indicates that suppression is not due to occupation of autophosphorylation sites.

The suppressed autophosphorylation in homogenates of cells treated for 2.5 min with EGF could be reversed by an incubation at 30 °C (Fig. 5*A*). If the 30 °C incubation was performed in the absence of vanadate, dephosphorylation of the 15–20% of the receptors that still contained P-Tyr occurred. The 30 °C incubation restored the ability of EGF (1 μg/ml) to stimulate receptor autophosphorylation. Restoration of autophosphorylation did not occur if the 10-min incubation was performed at 0 °C (*first two lanes* in Fig. 5*A*). The addition of 500 μM vanadate to the homogenate prevented loss of P-Tyr from EGF receptor during the 10-min 30 °C preincubation (Fig. 5*B*), but did not prevent the return of the ability of EGF to stimulate autophosphorylation (Fig. 5*A*). Thus, a temperature-dependent event(s) other than EGF receptor tyrosine dephosphorylation was responsible for returning the receptor to an activatable state.

Effect of Protein Kinase C Activation on Suppression of Homogenate EGF Receptor Autophosphorylation—Since others had detected or postulated protein kinase C-dependent decreases in EGF receptor autophosphorylation in A431 cells (10, 16), we investigated the possibility that a protein kinase C-catalyzed event was responsible for the suppression measured in cell-free extracts of WB cells. Cells were preincubated for 18 h with 10 μM TPA. This treatment results in both depletion of >95% of the protein kinase C activity in these cells and abolition of the ability of TPA to inhibit hormone-stimulated activation of phospholipase C (15). Subsequently all cells were treated with EGF for the indicated times, homogenized, and subjected to the cell-free autophosphorylation assay. The temporal pattern of autophosphorylation activity in protein kinase C-depleted cells (Fig. 6, *C* and *D*) was almost identical to that of untreated cells (Fig. 6, *A* and *B*), suggesting that protein kinase C is not involved in the early suppression of autophosphorylation activity measured under these conditions. Peak autophosphorylation activity was slightly greater in TPA-treated cells. This is consistent with increases in surface EGF binding that we and others have noted following long-term TPA treatment (24, 25). Conversely, we tested the direct effect of TPA by incubating WB cells for 2.5–15 min with or without 100 nM TPA. At these early time points (2.5–15 min), TPA did not appreciably alter receptor autophosphorylation measured in the cell-free assay (Fig. 7 and Table I). ^{125}I-EGF binding studies demonstrated that 100 nM TPA

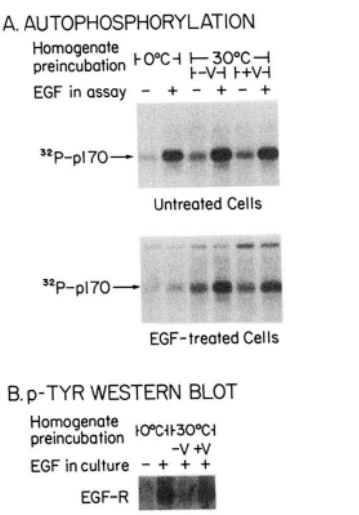

FIG. 5. **Effect of preincubation of homogenates at 30 °C on EGF receptor autophosphorylation suppression and EGF receptor P-Tyr content.** *A*, homogenates were prepared from untreated cells or EGF-treated cells (100 ng/ml for 2.5 min at 37 °C) as described under "Experimental Procedures." Aliquots (20 μl) of each homogenate were incubated for 10 min at 0 °C, or at 30 °C with or without 500 μM sodium vanadate (*V*), as indicated. The aliquots were then incubated with or without 1 μg/ml EGF for 10 min at 0 °C and assayed for EGF receptor autophosphorylation as described under "Experimental Procedures." *B*, an aliquot (50 μl) of the untreated cell homogenate (*left lane*) and aliquots of the EGF-treated cell homogenate (*right three lanes*) described above were incubated for 10 min at 0 or 30 °C without or with 500 μM sodium vanadate (*V*), as indicated. They were then incubated for an additional 10 min at 0 °C. SDS-PAGE sample buffer (25 μl) was added, the samples were heated to 100 °C for 3 min, and the samples were analyzed for tyrosine phosphoproteins by immunoblotting with anti-P-Tyr as described under "Experimental Procedures." The 170-kDa band corresponding to EGF receptor is shown.

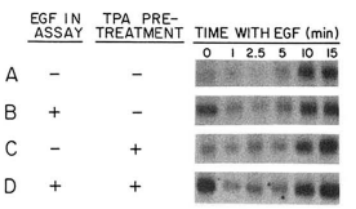

FIG. 6. **Depletion of protein kinase C by overnight TPA pretreatment does not alter EGF-induced suppression of EGF receptor autophosphorylation.** All cells were incubated with 100 ng/ml EGF at 37 °C for the indicated time periods, homogenized, aliquots incubated without (*A* and *C*) or with added EGF (1 μg/ml) (*B* and *D*), and assayed for EGF receptor autophosphorylation. The cells used in *C* and *D* had been preincubated for 18 h with 10 μM TPA in culture, a treatment that depletes >95% of the protein kinase C activity.

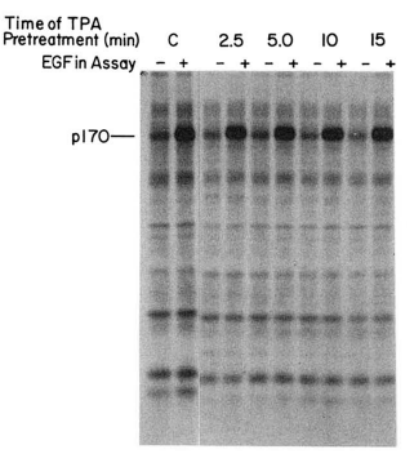

FIG. 7. **Preincubation with TPA does not produce suppression of EGF receptor autophosphorylation.** WB cell were incubated without or with 100 nM TPA at 37 °C for the indicated times prior to homogenization and phosphorylation as described under "Experimental Procedures." The addition of TPA did not significantly alter EGF-dependent homogenate EGF receptor autophosphorylation at times at which EGF results in suppressed autophosphorylation. TPA did alter EGF receptor metabolism within this time frame as shown by decreases in ^{125}I-EGF binding.

TABLE I

Effect of TPA and angiotensin on subsequent EGF-dependent autophosphorylation in homogenates

Intact WB cells were incubated with the indicated concentration of TPA or Angiotensin II for the indicated time at 37 °C. Homogenates were prepared and phosphorylated in the presence or absence of 1 μg/ml EGF and subjected to SDS-PAGE as described under "Experimental Procedures." Densitometric analysis of ^{32}P-p170 was performed and the fold increase obtained by dividing the extent of autophosphorylation in the presence of EGF by the value in the absence of EGF. The values, except for the 5-min TPA time point, are the averages of two separate experiments.

Time of pretreatment	Fold increase in EGF receptor autophosphorylation stimulated by EGF	
	Cells treated with TPA (100 nM)	Cells treated with angiotension II (1 μM)
min		
0 (control)	3.1	4.5
0.75		4.3
1.5		4.5
2.5	4.2	4.5
5	3.0	4.0
10	4.9	3.9
15	4.3	

decreased surface binding within this time frame (data not shown). However, to more precisely mimic the physiologic EGF-dependent activation of protein kinase C, WB cells were pretreated with angiotensin II. This agent activates phospholipase C in WB cells, generating endogenous inositol phosphates and presumably diacylglycerol within 5–30 s (15). Angiotensin decreases ^{125}I-EGF binding within 5 min (data not shown), but EGF receptor autophosphorylation in homogenates was unaffected by pretreatment with angiotensin for 45 s to 10 min (Table I). It should be noted that no steps were taken to preserve EGF receptor Thr-654 phosphorylation, a protein kinase C-dependent phosphorylation known to inhibit EGF-dependent EGF receptor tyrosine kinase activity. Thus, the above only demonstrate that protein kinase C is not responsible for the phenomenon studied in this cell-free assay.

Suppressed Phosphorylation of Exogenous Substrate—To test whether EGF-R/kinase activity toward exogenous substrates showed an EGF-dependent suppression/activation profile similar to that of autophosphorylation, homogenates of EGF-treated cells were assayed for their ability to phosphorylate the synthetic peptide from the region around a major pp60*src* autophosphorylation site, RRLIEDAEYAARG. The cumulative results of three separate experiments are shown in Fig. 8. Peptide phosphorylation was observed over basal levels in homogenates of cells treated for 5 min with EGF. However, the activity increased further in homogenates of cells treated with EGF for 15 min. The activity declined slightly in homogenates of cells treated for 30 min with EGF and dropped to below basal levels in homogenates of cells incubated with EGF for 120 min; this decline correlates with receptor degradation (17). Homogenates of cells incubated with EGF at 18 °C showed a 2-fold increase in peptide phosphorylation at 30 min, increasing to 4-fold by 120 min (Fig. 8). Although there was some increase over basal at the early times, it should be noted that the peptide phosphorylation assay unlike the autophosphorylation assay requires a 30 °C incubation and a 30 °C incubation is capable of reactivating EGF receptor autophosphorylation (see Fig. 5). Thus, although the suppression of exogenous substrates phosphorylation after EGF treatment (5 min at 37 °C or 30 min at 18 °C) is not as complete as that measured

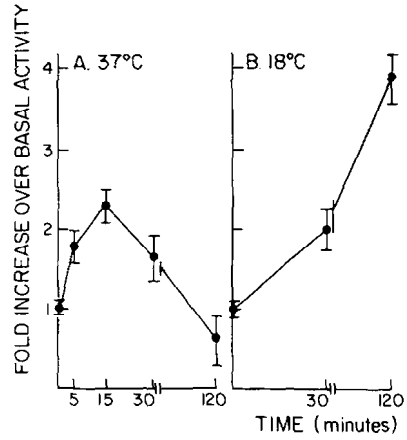

FIG. 8. **EGF-dependent tyrosine phosphorylation of an exogenous peptide substrate.** Homogenates were incubated for 8 min at 30 °C with the peptide substrates as described under "Experimental Procedures." Samples were obtained from untreated cells (zero time) or cells treated for the indicated times with 100 ng/ml EGF at 37 °C (*A*) or at 18 °C (*B*). The mean from three separate experiments at each time point is shown and is compared with the basal activity for that assay. The *brackets* represent the standard errors of the mean of the three experiments. Although the receptor had been fully active in the intact cell (see Fig. 1 and Ref. 17 for P-Tyr immunoblots of cell lysates), the kinase activity of the earlier time points (5 min at 37 °C, 30 min at 18 °C) was below that of late time points. This pattern correlates qualitatively with the pattern of suppression and relief of suppression measured in the EGF receptor autophosphorylation assay.

by the autophosphorylation assay (see Figs. 1–4), the kinase activity is still clearly below that of the later time points. Since nothing was done to these cells other than allowing endocytosis to proceed for prolonged periods, the best explanation for the subsequent increase in kinase activity is the relief of suppression. The timing of the relief of suppression in both assays is parallel at both 37 and 18 °C.

DISCUSSION

EGF receptor autophosphorylation activity in a cell-free assay was suppressed in homogenates of cultured rat liver epithelial cells incubated with EGF for short periods of time (2.5–5 min). This suppression was followed by a 3- to 6-fold increase in autophosphorylation activity after 15–30 min of EGF incubation (*e.g.* Figs. 2 and 6). This suppression in the homogenate assay needs to be interpreted in the light of the fact that EGF-dependent stimulation of substrate tyrosine phosphorylation (assessed by immunoblotting) is maximal within 15–30 s of exposure to EGF and that EGF receptor P-Tyr content remains elevated for 2–5 min and then declines (17). Our initial interpretation was that the lack of [^{32}P]ATP labeling of the receptor in the cell-free assay was due to autophosphorylation site occupancy. However, incubation conditions in culture that inhibited tyrosine dephosphorylation (vanadate or methylamine) failed to maintain the suppressed state of the receptor. In addition, measurement of EGF receptor P-Tyr remaining after preparation for the cell-free autophosphorylation assay demonstrated >85% loss receptor P-Tyr. These and other experiments (Fig. 5) demonstrate that suppression of kinase activity could not be explained by EGF receptor P-Tyr occupancy.

We next investigated the potential role of protein kinase C-catalyzed phosphorylation of the EGF receptor (7–10). EGF stimulates phospholipase C activity in WB and A431 cells and leads to Thr-654 phosphorylation in A431 cells (13, 15). A 5-min treatment of WB cells with TPA is capable of decreasing EGF-dependent phospholipase C γ-tyrosine phos-

phorylation.[2] However, depletion of protein kinase C by prolonged incubation of WB cells with 10 μM TPA had no effect on the suppression of EGF receptor autophosphorylation in the cell-free assay. Moreover, the addition of TPA or angiotensin II prior to homogenization did not suppress autophosphorylation measured under these assay conditions. Thus, protein kinase C is not responsible for the phenomenon under study in this report. These findings are different from those of Chinkers and Garbers (10), who described near-maximal EGF-dependent suppression of EGF receptor/kinase activity in partially purified receptor preparations from A431 cells. Their effect could be seen when only a small portion of available EGF receptors were occupied. (In WB cells high concentrations of EGF were required, Fig. 4.) They suggested that protein kinase C suppressed EGF receptor autophosphorylation. One major difference between our study and those noted above is the cell type used. Receptor internalization is inefficient in A431 cells (16), and EGF receptor autophosphorylation is maintained for up to 6 h, presumably due to the persistence of cell surface receptors (16). In WB cells, at the high dose of EGF at which suppression is observed, the receptor is >90% cleared from the surface by 15 min. In addition our cell-free assay are different from that used by Chinkers and Garbers (10) in A431 cells. Using our methods, neither angiotensin II nor TPA provoke the same type of suppressed autophosphorylation that is induced by EGF in WB cells. This again indicates that protein kinase C is not involved.

The suppressed state in WB cells at 2.5–5 min cannot be overcome by adding EGF to the cell-free assay. This could be due in part to enclosure of EGF binding sites within vesicles. However, this inaccessibility of binding site to added EGF does not obviate the finding of suppression. The EGF receptor internalized after a 15 min EGF treatment is quite active without added EGF. EGF present within the endocytotic vesicle at 15 min is sufficient, under these conditions, to sustain full activity, provided the receptor is in a state in which it can be activated; this is not the case at 2.5–5 min. Since all autophosphorylation assays were initiated in the presence of detergent, it is unlikely that differences in the sealing of the vesicles explains the difference in homogenates from cells 2.5 or 15 min after EGF addition to intact cell (see Ref. 17 for a detailed assessment of the effect of detergent on EGF receptor autophosphorylation in WB cell homogenates).

The question arises as to whether suppression is only seen with analysis of autophosphorylation or whether it can be detected using an exogenous substrate assay. Fig. 8 shows that exogenous substrate phosphorylation is also suppressed at times similar to those in which autophosphorylation is suppressed. EGF-R/kinase activity toward exogenous substrates is somewhat higher when compared with the near total suppression of autophosphorylation. Activity is modestly increased in homogenates of 5-min EGF-treated cells, but progresses to peak activity in homogenates of cells treated with EGF for 15 min. Similarly, in incubations carried out at 18 °C, the increase in exogenous peptide phosphorylation at 30 min (when autophosphorylation is suppressed, see Fig. 3) is only one-third of the increase observed at 120 min (when autophosphorylation is also greatly stimulated), even though receptor internalization is virtually complete by 30 min (17). Thus, exogenous peptide phosphorylation at times of suppressed autophosphorylation is clearly less than the increase observed at times of stimulated autophosphorylation. One particular difference in the autophosphorylation and peptide phosphorylation assays may account for modest activity noted in the peptide phosphorylation at early time points. The peptide assay is carried out at 30 °C, a temperature at which we have observed loss of autophosphorylation suppression. Thus, slightly higher level of peptide phosphorylation (compared with autophosphorylation) may be due to "desuppression" of a receptor activity inherent in the 30 °C assay conditions. In addition, the peptide assay would be sensitive to the activities of other tyrosine kinases activated indirectly by the action of EGF on intact cells; these activities would not be detected in the autophosphorylation assay.

At least three other mechanisms for suppressed autophosphorylation merit consideration: (i) activation of a serine/threonine protein kinase, (ii) a conformational change in the receptor which inhibits autophosphorylation, or (iii) an association with elements of the internalization pathway, *e.g.* clathrin or the cytoskeleton, that inhibit autophosphorylation. EGF-dependent activation of a serine/threonine kinase other than protein kinase C clearly occurs. EGF treatment of intact WB cells resulted in the appearance of a new phosphoprotein, p280, when homogenates are phosphorylated in the presence of [γ-^{32}P]ATP (see Fig. 5*A* in this paper and Fig. 3 in Ref. 17). Phosphoamino acid analysis demonstrated that p280 is phosphorylated on threonine residues.[3] Stimulation of p280 phosphorylation in homogenates is only seen when EGF is added to intact cells at least 30–60 s before homogenization; addition of EGF to control cell homogenates does not stimulate p280 phosphorylation. Moreover, EGF treatment initiates p280 homogenate phosphorylation in cells depleted of >95% of their protein kinase C activity.[3] This indicates that EGF can activate a serine/threonine kinase in these cells in addition to protein kinase C and may indicate that EGF can activate a kinase in WB cells similar to the microtubule-associated protein 2 kinase (26, 27). The most compelling argument against the involvement of a novel serine/threonine kinase is the high dose of EGF (30–1000 ng/ml) required to obtain suppression (Fig. 4). This suggests that suppression is related to individual receptor occupancy. Thus, a novel kinase would seem to be involved only if the EGF receptor had to be occupied by EGF to be a substrate.

A second possibility is that, in intact cells, EGF induces a series of conformational changes in the EGF receptor resulting in kinase activation followed by transient suppression. EGF-induced receptor oligimerization in intact cells has been demonstrated (4, 5) and is a mechanism postulated either to activate EGF receptor/kinase (1–3) or to reduce kinase activity (28). In addition, DiPaolo and Maxfield (29) have presented evidence that the acidic environment of the endosome may cause a conformational change in the EGF receptor. The EGF-dependent suppression of detectable kinase activity during internalization could be due to a transient alteration in the oligomeric state of receptor. As endocytosis proceeds (or during a 30 °C incubation of homogenates, see Fig. 5), the internalized receptor may once again be restored to a state in which autophosphorylation is detectable.

The third possibility is that suppression is related to a time- and dose-dependent association of receptor with structural elements of the endocytotic pathway. Perhaps the mobility of the EGF receptor/kinase is sterically hindered by components of the nascent endosomes such as clathrin polymers or cytoskeletal components which form about the cytoplasmic surface of the plasma membrane and persist around the cytoplasmic surface of early endosomes (30). Movement into the coated vesicles and associated elements in the first 2–5 min could even cause suppression of receptors whose binding domains are still external to the cell but whose cytoplasmic

[3] B. K. McCune, R. C. Dy, C. A. Prokop, and H. S. Earp, unpublished results.

domains are enmeshed in the forming vesicle. An association of the receptor with cytoskeletal elements has been postulated by Landreth and co-workers (31) who detected a more stable association of the EGF receptor with a Triton-insoluble fraction after the addition of EGF to A431 cells. The massive overexpression of EGF receptor in A431 and the attendant inefficient entry into the degradation pathway may explain a more prolonged association with the cytoskeleton in A431 cells. In WB cells, the association with the subsurface network may be terminated as the endosome moves away from the plasma membrane. This movement may relieve the suppression.

Our results do not establish a physiological role for the observed suppression nor do they prove that the kinase undergoes transient suppression in the intact cell. However, our results do clearly indicate that some biochemical change in the receptor's kinase activity occurs at defined times during internalization; this change is reversed as endocytosis proceeds. The phenomenon is not due to occupation of autophosphorylation sites nor is it mediated by protein kinase C. It remains to be determined whether the suppression is causally related to the observation that the majority of substrates detectable by P-Tyr immunoblots of intact WB cells are phosphorylated during the first 5–60 s of EGF action (17), a time prior to the onset of detectable suppression.

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