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Effects of Epidermal Growth Factor and 12-*O*-Tetradecanoylphorbol-13-Acetate on Metabolism of the Epidermal Growth Factor Receptor in Normal Human Fibroblasts

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The biosynthesis, phosphorylation, and degradation of the epidermal growth factor (EGF) receptor were examined in normal human fibroblasts. The receptor was initially synthesized as an M_r = 160,000 immature form which matured to an M_r = 170,000 form in a monensin-sensitive manner. Tunicamycin treatment led to the accumulation of an M_r = 130,000 protein. The receptor was phosphorylated on serine and threonine residues in normally growing and quiescent cells, and treatment with EGF or the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) resulted in a two- to threefold increase in receptor-bound phosphate. EGF increased the amount of phosphoserine and phosphothreonine and caused the appearance of a minor amount of phosphotyrosine. TPA increased the levels of phosphoserine and phosphothreonine exclusively. Prior treatment with TPA inhibited the EGF-dependent appearance of phosphotyrosine in the receptor. Analysis of tryptic phosphopeptides revealed that six of the seven major peptides were common to the receptor from cells treated with EGF or TPA. EGF strongly stimulated [^{3}H]thymidine incorporation in confluent cells, increased final saturation density three to fourfold, and increased whole-cell levels of phosphotyrosine about threefold. Treatment of cells with TPA before addition of EGF inhibited all three of these EGF-dependent responses. EGF also decreased the receptor half-life from 15 h to 1 h, but this was not inhibited by TPA. TPA alone had no detectable effect on the receptor half-life.

Epidermal growth factor (EGF) binds to a specific plasma membrane receptor protein which functions in mediating EGF-induced mitogenesis. Responses to EGF binding to its receptor include activation of a receptor-associated, tyrosine-specific protein kinase (9, 10), increased phosphorylation of the receptor protein (15, 26) and of other unidentified proteins (11, 21), increased nutrient transport (1, 24), transient influx of Ca^{2+} and Na^{2+} ions (38, 45), changes in membrane phospholipid metabolism (40, 43), and ultimately cell division. The primary event is probably an activation of the receptor protein kinase, which could induce the other phenomena through phosphorylation of appropriate protein substrates. EGF-dependent stimulation of the EGF receptor-associated protein kinase has been demonstrated in vitro in membrane preparations from many different cell types (4, 13, 18) and in vivo for the A431 epidermoid carcinoma cell line (26) and 3T3 cells (11). This unusual tyrosine-specific protein kinase activity has also been found associated with the receptors for platelet-derived growth factor (PDGF) (17), insulin (30), and somatomedin C (28) and with the transforming gene products of a number of oncogenic viruses (2). In vivo phosphorylation of the EGF receptor from A431 cells is of a complex nature; phosphate has been found on serine, threonine, and tyrosine residues (12, 15). The insulin receptor has also been shown to contain these three phosphoamino acids (31). The functional significance of receptor phosphorylation is unknown.

EGF binding also results in rapid internalization and degradation of the EGF receptor (23, 41). Receptors aggregate, are internalized in coated vesicles, and are degraded presumedly in lysosomes. Whether mitogenic signals are generated after internalization or whether internalization is required for ligand catabolism has not been established.

Tumor promoters such as the phorbol diester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and teleocidin have been shown to modify cellular responses to EGF (29, 35, 42) and may either block (29, 34) or enhance (16, 19) the mitogenic capacity of EGF, depending on the cell type and growth conditions. Since phorbol esters stimulate the activity of Ca^{2+}-dependent, phospholipid-activated protein kinase C (6), modulation of the EGF system by tumor promoters may involve phosphorylation of the EGF receptor. Phorbol esters have previously been shown to increase phosphorylation of the membrane receptors for insulin and somatomedin C (27).

In this paper, metabolism of the EGF receptor from human foreskin fibroblasts is examined. Data concerning the biosynthesis, processing, regulation of phosphorylation, and regulation of degradation of the EGF receptor are presented. The effects of the tumor-promoting phorbol ester TPA on these parameters are also investigated.

MATERIALS AND METHODS

Cell culture. Early passage human foreskin fibroblasts (kindly provided by P. Ross, The Rockefeller University) were grown in Dulbecco modified Eagle medium (DME) containing 10% calf serum (Flow Laboratories).

Materials. Receptor-grade EGF was from Collaborative Research, Inc., monensin was from Calbiochem-Behring, and tunicamycin, TPA, phorbol 12,13-dibutyrate, and 4α-phorbol were from Sigma Chemical Co. L-[^{35}S]methionine (1,000 Ci/mmol) was from New England Nuclear Corp., [γ-^{32}P]ATP (3,000 Ci/mmol) was from Amersham Corp., *methyl*-[^{3}H]thymidine was from Schwartz/Mann, and carrier free ^{32}P was from ICN Pharmaceuticals, Inc.

Labeling of cells and immunoprecipitation of cell extracts. For all [^{35}S]methionine-labeling experiments, nearly confluent cells in 35-mm culture dishes were washed twice with 1 ml of methionine-free DME and incubated for the specified times with 100 μCi of [^{35}S]methionine in 1 ml of methionine-free DME with 10% dialyzed calf serum. For ^{32}P-labeling in immunoprecipitation experiments, cells were grown in 90-mm culture dishes, washed twice with 4 ml of phosphate-free

DME, and incubated for the specified times with 1 mCi of ^{32}P, per ml in 4 ml of phosphate-free DME containing 1 or 10% dialyzed calf serum. Labeled cells were lysed directly in hot 2% sodium dodecyle sulfate, heated at 100°C for 5 min, and immunoprecipitated with polyclonal antiserum against the EGF receptor as previously described (14). Cells were ^{32}P-labeled for whole-cell analysis of phosphoamino acids essentially as above, except that cells were grown in 35-mm dishes and ^{32}P was added at 0.3 mCi/ml. Monensin and tunicamycin treatments were performed as previously described (15).

Other methods. Analysis of phosphoamino acids, preparation of sodium dodecyl sulfate-polyacrylamide gels, and determination of protein were performed as previously described by Decker (13). For some experiments, gels were prepared for fluorography according to the method of Chamberlain (7). Two-dimensional tryptic peptide maps were done according to the method of Gates and King (20), and [^{3}H]thymidine incorporation was done according to the method of Carpenter and Cohen (3).

RESULTS

We have previously investigated properties of the EGF receptor from the A431 human epidermoid cell line (15); however, these cells are somewhat atypical in that they possess an unusually high number of EGF receptors and that addition of EGF to these cells does not elicit a mitogenic response.

Consequently, we have chosen to further examine EGF receptor metabolism in low-passage normal human foreskin fibroblasts which show a strong proliferative response to EGF (3).

Initial experiments revealed that the biosynthesis of the EGF receptor in human fibroblasts was similar to that found in A431 cells. The mature receptor was a monomeric glycoprotein with an M_r of 170,000 (Fig. 1, lane B) resulting from terminal glycosylation of an M_r = 160,000 precursor (Fig. 1, lanes C and E). Monensin, which impairs Golgi apparatus function, inhibited terminal glycosylation of the M_r = 160,000 precursor (Fig. 1, lane C), and treatment of cells with tunicamycin, which inhibits synthesis of precursor oligosaccarides, resulted in accumulation of an M_r = 130,000 nonglycolyated form (Fig. 1, lane D).

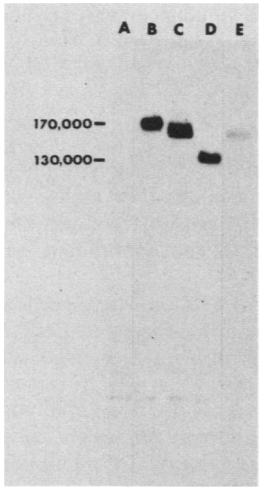

FIG. 1. Immunoprecipitation of the EGF receptor from normal human fibroblasts. Cells were labeled with [^{35}S]methionine for 12 h (lanes A through D) or 45 min (lane E) as described in the text. Lysates of 2×10^5 to 4×10^5 cells were immunoprecipitated with 2 μl of preimmune rabbit serum (lane A) or 2 μl of rabbit anti-EGF receptor serum (lanes B through E). For lanes C and D, respectively, 0.5 μM monensin or 10 μg of tunicamycin per ml was included during the labeling period. The M_r = 130,000 and M_r = 170,000 regions of the gels are indicated.

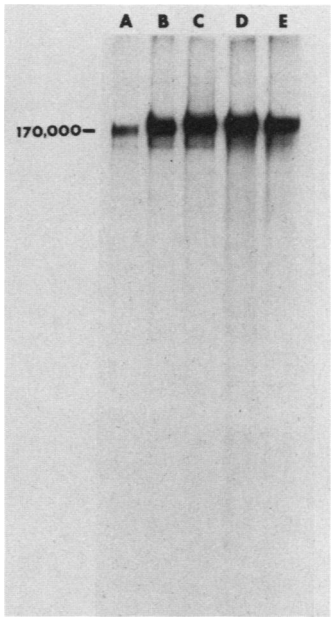

FIG. 2. Effects of EGF and TPA on phosphorylation of the EGF receptor. Human fibroblasts were labeled for 14 h with ^{32}P and immunoprecipitated as described in the text. The cells received the following: no addition (lane A), 5 ng of EGF per ml (lane B), 100 ng of EGF per ml (lane C), 100 ng of TPA per ml (lane D), or 100 ng of TPA per ml and 100 ng of EGF per ml (lane E) for the final 5 min of labeling before lysis.

Phosphorylation of the EGF receptor from normal human fibroblasts. The EGF receptor from normally growing cells was readily labeled with ^{32}P (Fig. 2, lane A). Addition of EGF or TPA caused a rapid two- to threefold increase in ^{32}P incorporation. The stimulatory effects of EGF and TPA on receptor phosphorylation were not additive (Fig. 2E). After EGF or TPA treatment, an increase in the apparent molecular weight of immunoprecipitated ^{32}P-labeled EGF-receptor to an M_r of 175,000 was seen (perhaps due to increased phosphorylation). Similar shifts in molecular weight were found in the EGF receptor from [^{35}S]methionine-labeled cells, indicating that a large proportion of the receptor population was affected by addition of EGF or TPA (as shown in Fig. 5 for TPA). Treatment of cells with 4α-phorbol, a phorbol derivative which is inactive as a tumor promoter and does not activate protein kinase C (6), did not affect phosphorylation of the EGF receptor (data not shown), whereas the active phorbol ester phorbol 12,13-dibutyrate acted similarly to TPA (data not shown).

The acid-stable phosphoamino acid compositions of the EGF receptor from subconfluent cells in DME with 10% calf serum, from these same cells treated with EGF or TPA, or from quiescent cells (confluent cultures incubated for 48 h in DME containing 1% calf serum) are shown in Fig. 3A. For the receptor from subconfluent cells in DME with 10% calf serum, only phosphoserine and phosphothreonine were found. Addition of 5 ng of EGF per ml for 5 min resulted in increased amounts of phosphoserine and phosphothreonine

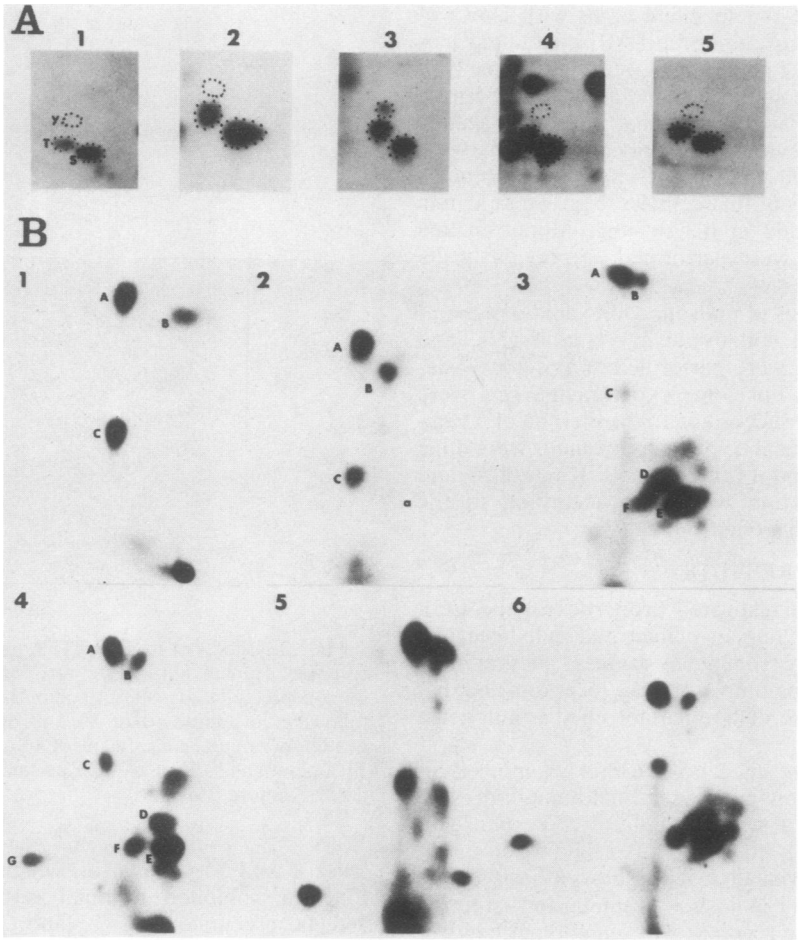

FIG. 3. Phosphoamino acid (A) and tryptic phosphopeptide analysis (B) of the EGF receptor from human fibroblasts. The EGF receptor was ^{32}P-labeled and immunoprecipitated as described in the text. (A) Acid-stable phosphoamino acids from quiescent cells (panel 1), cells growing in DME with 10% calf serum (panel 2), and similar cells treated for 5 min with 5 ng of EGF per ml (gel 3), 100 ng of TPA per ml (panel 4), and 100 ng of TPA per ml for 5 min and then 5 ng of EGF per ml for 5 min (panel 5) are shown. Dashed circles indicate the positions of phosphoserine (S), phosphothreonine (T), and phosphotyrosine (Y) standards. (B) Two-dimensional chromatograms of tryptic phosphopeptides of the EGF receptor from cells treated as described for section A, panel 1 through 5, are presented. For section B, panel 6, peptides from the receptor from EGF-treated (as for section A, panel 3) and TPA-treated (as for section A, panel 4) cells were mixed. Methods are as described in the text. Electrophoresis was from left (−) to right (+), and chromatography was from bottom to top.

and the appearance of a minor phosphotyrosine spot (similar patterns were found with 100 ng of EGF per ml (data not shown). TPA treatment increased only the phosphoserine and phosphothreonine levels; no phosphotyrosine was detected. TPA treatment before the addition of EGF greatly reduced the intensity of the phosphotyrosine spot. The EGF receptor from quiescent cells contained phosphoserine and phosphothreonine. EGF and TPA treatment of quiescent cells gave phosphoamino patterns similar to those obtained with EGF and TPA treatment of subconfluent cells in DME with 10% calf serum (data not shown). Phosphorylation of the EGF receptor was further examined by two-dimensional tryptic peptide analysis (Fig. 3B). The receptor from quiescent cells or subconfluent cells growing in DME with 10% calf serum yielded three major phosphopeptides (A, B, and C) and additional minor peptides. One minor peptide (designated a) was only present in growing cells and seemed to correspond to one of the additional major peptides seen after EGF or TPA treatment (see below). EGF treatment resulted in the appearance of six major phosphopeptides; A, B, and C, as in the quiescent and serum-grown cells, and three other peptides (D, E, and F). TPA also stimulated phosphorylation of D, E, and F and of another major phosphopeptide (G). Treatment with TPA before the addition of EGF produced maps very similar to those obtained with TPA treatment alone.

Effects of EGF and TPA on whole-cell levels of phosphotyrosine and on [^{3}H]thymidine incorporation. Addition of EGF to growing human fibroblasts increased whole-cell levels of phosphotyrosine (Fig. 4). An EGF concentration of 2.5 ng/ml was sufficient to cause maximal stimulation (two- to threefold) of phosphotyrosine levels, and the concentration dependence for stimulation of phosphotyrosine was very similar to the concentration dependence for increased [^{3}H]thymidine incorporation (Fig. 4). The EGF-dependent increase in whole-cell levels of phosphotyrosine reached a maximum 5 min after the addition of EGF and remained elevated over control levels even after 24 h in the presence of EGF (Table 1). The addition of TPA caused a slight (20 to 40%) but reproducible increase in phosphotyrosine levels, but inhibited the larger EGF-induced increase. TPA also inhibited EGF-dependent stimulation of [^{3}H]thymidine

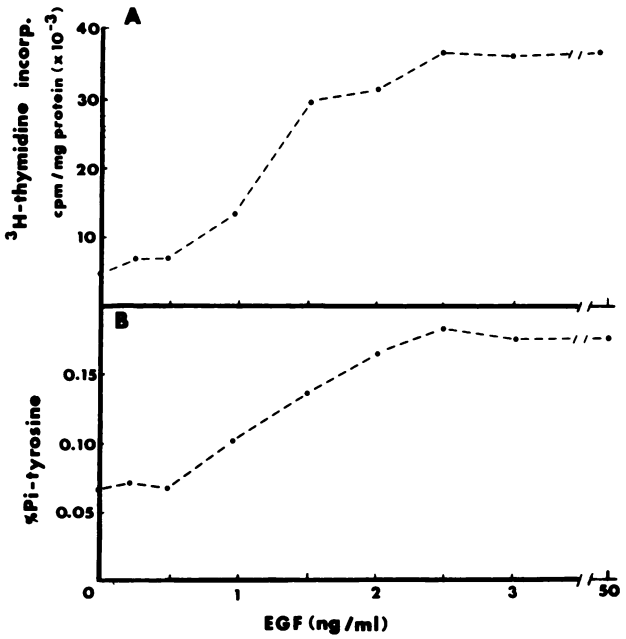

FIG. 4. Effect of EGF concentration on acid-stable phosphotyrosine levels and [^{3}H]thymidine incorporation in human fibroblasts. Whole-cell phosphoamino acid analysis and measurement of [^{3}H]thymidine incorporation were performed as described in the text. For phosphoamino acid determination, various concentrations of EGF were added for 5 min before lysis. For [^{3}H]thymidine incorporation, confluent cells were incubated for 16 h in DME containing 1% calf serum before a further 24-h incubation with additions. [^{3}H]thymidine (1 μCi/ml) was then added for 2 h.

TABLE 1. Effects of EGF and TPA on whole-cell levels of acid-stable phosphotyrosine and [^{3}H]thymidine incorporation in human fibroblasts.

Addition per ml (time after addition)	% Phosphotyrosine[a]	[^{3}H]thymidine incorporation[b] (cpm/mg of protein) (10^3)
None	0.068	
5 ng of EGF (5 min)	0.187	
5 ng of EGF (90 min)	0.124	
5 ng of EGF (24 h)	0.103	
100 ng of TPA (5 min)	0.093	
100 ng of TPA (24 h)	0.095	
100 ng of TPA (5 min), then 5 ng of EGF (5 min)	0.097	
100 ng of TPA (24 h), then 5 ng of EGF (5 min)	0.092	
None		3.6
10 ng of EGF		48.2
100 ng of TPA		3.3
100 ng of TPA and 10 ng of EGF		3.5
100 ng of 4α-phorbol and 10 ng of EGF		46.7

[a] Percent phosphotyrosine presented as percent total acid-stable phosphoamino acids. In these experiments, percents phosphothreonine and phosphoserine were constant at ca. 9 and 91%, respectively. Experimental procedures were performed as described in the legend to Fig. 4.

[b] [^{3}H]thymidine incorporation was performed as described in the text. Cells were incubated for 24 h in DME containing 1% calf serum and the specified additions before assay.

incorporation in quiescent cells (Table 1) and inhibited the EGF-dependent increase in saturation density which occurs in these cells (3) (data not shown).

Half-life of the EGF receptor from human fibroblasts. The half-life of the EGF receptor was determined in pulse-chase experiments with normal cells and cells treated with EGF and TPA (Fig. 5). For the calculation of the receptor half-life from the data in Fig. 5, immunoprecipitated bands were excised from the gels and counts per minute of [^{35}S]methionine per band were determined and plotted. In normally growing cells, the half-life of the receptor was ca. 15 h. Inclusion of 5 or 100 ng of EGF per ml during the chase reduced the half-life of the receptor to ca. 1 and 2 h, respectively. By the first chase point no increase in molecular weight was seen. TPA treatment had little effect on the half-life of the receptor, although the TPA-induced increase in molecular weight to an M_r of 175,000 was apparent 1 h into the chase. As the chase was continued, the molecular weight of the receptor gradually returned to an M_r of 170,000. Since TPA is known to decrease EGF binding to many cell types (25) and also inhibits ^{125}I-EGF binding to human fibroblasts (unpublished data), the effects of prior TPA treatment on EGF-stimulated turnover of the EGF receptor was examined. TPA treatment had no obvious effect on the EGF-dependent increase in the rate of receptor degradation (Fig. 5; panels E and F). As shown previously for A431 cells (15), EGF treatment did not affect the half-lives of the majority of the [^{35}S]methionine-labeled cellular proteins (data not shown). Neither 100 ng of EGF per ml nor 100 ng of TPA per ml altered the rate of synthesis of the EGF receptor, as judged by the amount of [^{35}S]methionine incorporation into the M_r = 160,000 immature form of the receptor during a 45-min labeling period (data not shown).

DISCUSSION

The biosynthesis of the EGF receptor from normal human fibroblasts was found to occur much as previously reported for the EGF receptor from A431 cells (15). Immunoprecipitable receptor was initially present as an immature M_r = 160,000 form which matured to an M_r = 170,000 form in a monensin-sensitive fashion. When the receptor was immun-

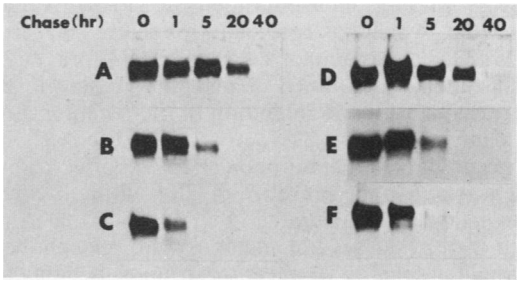

FIG. 5. Effects of EGF and TPA on the half-life of the EGF receptor. The EGF receptor was immunoprecipitated from nearly confluent 35-mm dishes of human fibroblasts labeled with 100 μCi of [^{35}S]methionine per ml as described in the text. After 12 h of labeling, cells were lysed as zero time controls or washed once with normal DME and incubated for the indicated chase periods with normal DME plus 10% calf serum containing the following: no addition (panel A), 5 ng of EGF per ml (panel B), 100 ng of EGF per ml (panel C), 100 ng of TPA per ml (panel D), 100 ng of TPA per ml and 5 ng of EGF per ml (panel E), and 100 ng of TPA per ml and 100 ng of EGF per ml (panel F). The EGF receptor was immunoprecipitated as described in the legend to Fig. 1.

oprecipitated from cells incubated with tunicamycin, only an M_r = 130,000 presumedly unglycosylated form was found. The M_r = 130,000 protein immunoprecipitated from A431 cells, which was not related to the EGF receptor by partial V8 protease mapping (15), was not immunoprecipitated from normal fibroblasts. Increased synthesis of this protein may relate somehow to the aberrant synthesis of the EGF receptor in A431 cells.

EGF caused a two- to threefold increase in receptor-bound ^{32}P in human fibroblasts. This increase is similar to that found for the insulin receptor after insulin treatment of rat hepatoma cells (30). The phosphoamino acid compositions of the EGF and insulin receptors were also similar. (i) both contained phosphoserine and phosphothreonine in the absence of ligand, and (ii) addition of ligand increased phosphoserine and phosphothreonine levels and caused the appearance of a very minor amount of phosphotyrosine (31). In contrast, the EGF receptor from A431 cells contain readily detectable phosphotyrosine in the absence of EGF, and phosphotyrosine levels are enhanced by the addition of EGF (15, 26). The addition of EGF also resulted in an apparent increase in the molecular weight of the EGF receptor from ca. M_r = 170,000 to M_r = 175,000, perhaps due to increased phosphorylation. For 5 and 100 ng of EGF per ml, all the ^{32}P- and [^{35}S]methionine-labeled receptor was present in the M_r = 175,000 form, indicating that the majority of the receptor molecules had undergone this modification. This is surprising for 5 ng of EGF per ml, since only ca. 10 to 15% receptor occupancy is achieved at this EGF concentration (5). An EGF-induced increase in the apparent molecular weight of the EGF receptor from A431 cells has been previously reported (15).

TPA and phorbol dibutyrate, but not 4α-phorbol, also caused a two- to threefold stimulation of EGF receptor phosphorylation and inhibited binding of ^{125}I-EGF (unpublished data). TPA and phorbol dibutyrate are biologically active phorbol esters which function as tumor promoters and which activate Ca^{2+}-sensitive, phospholipid-dependent protein kinase C (6). 4α-phorbol does not possess these activities.

Increased phosphorylation of the EGF receptor from human fibroblasts by TPA is due to increased levels of phosphoserine and phosphothreonine; no phosphotyrosine could be detected. This result is not unexpected if the EGF receptor is directly phosphorylated by TPA-activated protein kinase C, which has specificity for serine and threonine residues (32). Treatment of cells with TPA prevented the EGF-dependent appearance of phosphotyrosine in the receptor, possibly through inhibition of the receptor-associated, tyrosine-specific kinase activity, as discussed below. The majority of the receptor population was affected by TPA treatment, since in the presence of TPA all the [^{35}S]methionine-labeled receptor migrated at an M_r of 175,000.

Three major and several minor tryptic phosphopeptides were found in the EGF receptor from subconfluent and quiescent cells. The EGF receptor from EGF- or TPA-treated cells yielded similar peptide maps, except for one extra TPA-induced major peptide not seen with EGF. The three peptides found in the receptor from serum-grown or quiescent cells remained after EGF or TPA treatment, although the intensity of peptide C seemed to be diminished after the addition of EGF. The similar tryptic maps suggest that EGF or TPA may activate the same, or some of the same, protein kinase(s) responsible for phosphorylating the receptor. Protein kinase C is an obvious candidate since it is directly activated by TPA (6), and in A431 cells EGF stimulates the production of diacylglycerol (43), which acts as a physiological activator of protein kinase C (44).

The inhibition of EGF-stimulated receptor tyrosine phosphorylation by TPA parallels the inhibition of the EGF-stimulated increase in total cellular phosphotyrosine by TPA, suggesting that TPA generally inhibits the EGF-activated, tyrosine-specific kinase activity which is associated with the receptor. In turn, inhibition of the receptor protein kinase correlates with TPA-dependent inhibition of EGF-stimulated [^{3}H]thymidine incorporation and increased saturation density. TPA-dependent phosphorylation of the EGF receptor may inhibit the receptor-associated, tyrosine-specific protein kinase which is responsible for transmitting some or all of the mitogenic signals involved in EGF-dependent cellular proliferation. The TPA-dependent phosphorylation of the receptor could inhibit receptor protein kinase activity directly or indirectly through altering the receptor binding affinity for EGF (33). TPA also stimulates phosphorylation of the insulin and somatomidin C receptors (27), suggesting that protein kinase C activation may be involved in regulation of the activity of several growth factor systems. In this regard, PDGF, thought to sensitize certain cells to the mitogenic effects of EGF (36), somatomedin C (36), and other plasma components (46), strongly stimulates phospholipid breakdown and diacylglycerol production (22) and inhibits ^{125}I-EGF binding (47). PDGF and TPA stimulate phosphorylation of the same M_r = 80,000 protein in 3T3 cells (39), indicating that some of the effects of PDGF may be mediated by protein kinase C. It is possible that TPA mimics some of the physiological effects of PDGF, i.e., regulation of other growth factor systems through phosphorylation. Perhaps inhibition of the EGF system by PDGF would be transient due to down-regulation of PDGF receptors and might involve coordination of growth factor systems during the cell cycle. Whether the EGF-induced phosphorylation of regions of the receptor which are also phosphorylated in response to TPA might be involved in regulation of receptor-associated tyrosine kinase activity is not addressed in this study.

Tumor promotors have been shown to act both synergistically and antagonistically in combination with EGF. Tumor promoters inhibit EGF-induced mitogenesis in human fibroblasts (34), rat hepatoma cells (29), and human hepatoma cells (37) and block EGF-induced cellular adhesion in PC12 phaeochromocytoma cells (8) and EGF-induced production of diacylglycerol in A431 cells (43). We confirm that TPA can inhibit EGF-induced mitogenesis in human fibroblasts and present a possible mechanism. TPA enhances the mitogenic effects of EGF in 3T3 cells (16, 19); the basis of this discrepancy is not known.

The addition of EGF greatly reduced the half-life of the EGF receptor from human fibroblasts. The effect was concentration dependent, since 100 ng of EGF per ml reduced the half-life to a greater degree that 5 ng of EGF per ml. In A431 cells 100 ng of EGF per ml decreases the half-life of the receptor from 20 h to ca. 5 h (15). TPA had no detectable effect on the receptor half-life, although the molecular weight of the receptor increased and then decreased during the chase period, perhaps due to phosphorylation and dephosphorylation of the receptor. TPA treatment does not seem to alter the rate of EGF-induced down-regulation of the receptor, indicating that EGF still interacts significantly with the receptor in the presence of TPA even though ^{125}I-EGF binding over the range of 1 to 50 ng of EGF per ml is inhibited 40 to 60% (unpublished data). This shows that down-regulation of the EGF receptor can occur without

mitogenesis. It seems possible that phosphorylation of the receptor is not involved in receptor internalization and degradation, since both EGF and TPA stimulated phosphorylation of many of the same regions of the receptor.

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