Effect of Epidermal Growth Factor and 12-0-Tetradecanoylphorbol-13-acetate on the Phosphorylation of Soluble Acidic Proteins in A431 Epidermoid Carcinoma Cells

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ABSTRACT

Treatment of [32P]orthophosphate prelabeled intact human A431 epidermoid carcinoma cells with epidermal growth factor (EGF, 100 ng/ml) or 12-O-tetradecanoylphorbol-13-acetate (TPA, 10⁻⁷M) resulted in a selective enhancement in the phosphorylation of the following soluble acidic proteins: a phosphoprotein with a molecular weight of 17,000 (pp17; similar notation used throughout) pI ~5.5; pp27 (pI ~5.5); pp34 (pI ~6.2); and pp80 (pI ~4.5) as detected by two-dimensional gel electrophoresis. EGF or TPA induced a 4- to 6-fold increase in the phosphorylation of pp17 and a 2- to 4-fold increase in the phosphorylation of pp27, pp34, and pp80 within 15 min after treatment of subconfluent A431 cells. Alkaline treatment of the gels removed most of the incorporated [32P]orthophosphate from the protein. Treatment of A431 cells with dibutyryl cyclic adenosine 3':5'-monophosphate (1 mM) also increased the phosphorylation of pp17, pp27, and pp34 but not of pp80. Activation of endogenous calcium- and phospholipid-dependent protein kinase C in the cytosol of A431 cells in a cell-free system resulted in the enhanced phosphorylation of pp27, pp34, and pp80 but not of pp17 while exogenous addition of the catalytic subunit of cyclic adenosine 3':5'-monophosphate-dependent protein kinase C and/or a cyclic adenosine 3':5'-monophosphate-dependent protein kinase C.

INTRODUCTION

EGF is a potent mitogen for a variety of epithelial and mesenchymal cells in vitro (1, 2); however, nanomolar concentrations of EGF can also inhibit the proliferation of such cells as A431 human epidermoid carcinoma cells, GH, rat pituitary tumor cells, and certain human breast cancer cells (3-5). Although EGF is known to induce a transient increase in diacylglycerol production (3), which inhibit A431 cell growth can induce or stimulate the phosphorylation of at least four distinct acidic cytosol proteins (pp17, pp27, pp34, and pp80), implying that the responses of A431 cells to a physiological growth regulator (EGF) and to a tumor promoter (TPA) involve a shared biochemical pathway. In addition, some of these same proteins are also phosphorylated in intact A431 cells treated with dbcAMP as well as in a cell-free system with exogenously added catalytic subunit of cyclic AMP-dependent protein kinase C and/or a cyclic adenosine 3':5'-monophosphate-dependent protein kinase C.

MATERIALS AND METHODS

Materials. Mouse EGF (receptor grade) was purchased from Collaborative Research, Waltham, MA. TPA was obtained from P-L Biochemicals, Milwaukee, WI and stored at a concentration of 10 mM in absolute ethanol at −20°C. [32P]Orthophosphate (specific activity, 500 mCi/mmol) was purchased from New England Nuclear, Boston, MA and [γ-32P]ATP (specific activity, 25 Ci/mmol) was from ICN Pharmaceuticals, Irvine, CA. Tissue culture medium and fetal bovine serum were obtained from Grand Island Biological Co., Grand Island, NY. Phospholipids, dbcAMP, and other reagents were purchased from Sigma Chemical Co., St. Louis, MO. Type I bovine brain cyclic AMP-dependent protein kinase catalytic subunit was generously supplied by Dr. C. L. Kapoor, National Eye Institute, Bethesda, MD.

Cell Culture. A431 human epidermoid carcinoma cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 4 mM glutamine, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4), streptomycin (100 μg/ml), penicillin (100 units/ml) and 10% fetal bovine serum (v/v) in a humidified atmosphere of 95% air.

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5% CO₂ at 37°C as previously described (3, 11). Cells were routinely subcultured in 75-cm² tissue culture flasks (Falcon Plastics, Oxnard, CA).

Labeling of Cells with ³²P, and Preparation of Cytosols. Cultures of A431 cells were grown in 100-mm Falcon plastic dishes. Subconfluent cultures (4 × 10⁶ cells/dish) were first washed 2 to 3 times in phosphate and serum-free Dulbecco's modified Eagle's medium and incubated with carrier-free ³²Porthophosphate (250 µCi/ml) for 2 h at 37°C. Cultures then were treated with either EGF (100 ng/ml) and/or TPA (10⁻⁷ m) or dbcAMP (1 mM) for the indicated times. At the end of each treatment, cells were washed twice with cold (phosphate-buffered saline (0.15 M NaCl-5.6 mM Na₂HPO₄-1.06 mM KH₂PO₄, pH 7.4), scraped from the dish, and homogenized in 2 ml of 20 mM Tris-HCl (pH 7.5) containing 0.25 M sucrose, 25 mM 2-mercaptoethanol, 2 mM ethylene glycol bis(β-aminoethyl ether) N,N',N'-tetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, and 10 µM sodium fluoride. Homogenates were centrifuged at 600 × g for 10 min at 4°C to remove nuclei and other cellular debris. The 600 × g supernatant then was centrifuged at 100,000 × g for 1 h at 4°C. The proteins in the soluble cytosol fractions were precipitated by the addition of 5 volumes of ice-cold aceton: NH₄OH (30:1.7, v/v) for 10 min at 4°C and centrifuged at 10,000 × g for 10 min at 4°C. Precipitated proteins from the cytosol were dissolved in isoelectrofocusing lysing solution (9.5 mM urea, 2% Nonidet P-40, 5% 2-mercaptoethanol, and 2% LKB ampholytes, pH 3.5–10) and stored at −80°C until analyzed by 2-dimensional polyacrylamide gel electrophoresis.

Two-Dimensional Polyacrylamide Gel Electrophoresis. Samples containing equivalent amounts of radioactivity, approximately 5 × 10⁵ cpm of ³²P, or the equivalent of approximately 60 µg of protein were analyzed by 2-dimensional polyacrylamide gel electrophoresis by the method of O'Farrell (28). First dimension isoelectrofocusing gels containing 2% LKB ampholytes (pH 3.5–10) and second dimension gels were 12% sodium dodecyl sulfate-polyacrylamide (29). These gels were fixed in a solution of 25% 2-propanol and 10% acetic acid for 1 h, stained with Coomassie blue, and dried under vacuum as described (29). For autoradiography, dried gels were exposed at −70°C for 12–24 h to Agfa Gevaert film between Kodak X-Omatic intensifying screens. ¹⁴C-Labeled molecular weight standards (M, 14,000–200,000) were purchased from Bethesda Research Laboratories, Bethesda, MD. Protein standards (M, 14,000–92,000) were obtained from Bio-Rad Laboratories, Rockville Center, NY.

Alkali Treatment of Two-Dimensional Polyacrylamide Gels. Two-dimensional polyacrylamide gels from control and EGF-treated cultures were first fixed and dried. These dried gels were then treated with alkali as described by Feuerstein and Cooper (29). Briefly, gels were soaked in 1 M NaOH for 30 min at room temperature with constant shaking. Thereafter, they were transferred to a second 1 M NaOH bath at 37°C for 1 h, washed, and reextracted at 37°C with 1 M NaOH for 1 h. The gels then were incubated in 7% acetic acid:7% methanol (v/v) for 2 h at room temperature. Dried gels then were exposed to Agfa Gevaert film at −70°C between Kodak X-Omatic intensifying screens.

Quantitation of ³²P in Specific Proteins. Phosphoproteins such as pp17, pp27, pp34, and pp80 were located on the gels by autoradiography. These proteins were then marked, excised from the two-dimensional gels, and the ³²P associated with these samples counted in 10 ml of Aquasol (New England Nuclear) in a liquid scintillation spectrometer as previously described (29). For each labeled protein excised from the gel, a corresponding region of the gel of identical size was also excised from an adjacent area in the gel where no radioactivity was detected by autoradiography, counted, and used as an internal blank. This blank value was subtracted from each of the corresponding labeled protein regions.

Cell-free Protein Phosphorylation by Endogenous Protein Kinase C. Subconfluent cultures of A431 cells were washed, homogenized, and centrifuged at 100,000 × g to obtain cytosol supernatant fractions as described above. Incorporation of ³²P, into endogenous cytosolic proteins from [γ-³²P]ATP (1 × 10⁶ cpm–reaction) was measured under previously optimized conditions for analysis of protein kinase C activity in this cell line (11). Briefly, the cell-free phosphorylation reactions were conducted in a total volume of 250 µl containing 20 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 1 mM CaCl₂, and 50–100 µg of cytosol protein as a source of endogenous protein kinase C as well as endogenous substrate proteins in the absence or presence of 5 µg of PS and 1 µg of 1,2-diolein (diacylglycerol). The reaction mixture was incubated for 5 min at 30°C and terminated by adding 2 ml of ice-cold 20% trichloroacetic acid. Trichloroacetic acid-precipitable material was centrifuged and the pellet was washed three to four times with chloroform and prepared for two-dimensional gel electrophoresis as previously described.

Cell-free Protein Phosphorylation by Exogenous Cyclic-AMP-dependent Protein Kinase. The in vitro phosphorylation reaction mixture (250 µl) contained 20 mM Tris-HCl (pH 7.5):10 mM MgCl₂-1 µM cyclic AMP:50–100 µg of cytosol proteins in the absence or presence of 100 nM of the purified bovine type 1 cyclic AMP-dependent protein kinase catalytic subunit. The reaction was initiated by the addition of 100 µM γ-[³²P]ATP (containing 1–1.5 × 10⁶ cpm). The reaction mixture was incubated for 5 min at 30°C and was terminated by adding 2 ml of cold 20% trichloroacetic acid. Trichloroacetic acid-precipitable material was centrifuged and washed with chloroform three to four times. Proteins were dissolved in isoelectrofocusing lysing solution and analyzed by two-dimensional gel electrophoresis.

Protein Determination. All protein determinations were made by the method of Bradford (30) using bovine serum albumin as a standard.

RESULTS

Effect of EGF on the Phosphorylation of Acidic Cytosol Proteins in Vivo. A431 human epidermoid carcinoma cells were routinely labeled with carrier-free ³²P, for 2 h in phosphate- and serum-free medium. While the absolute levels of total protein phosphorylation cannot be accurately determined under such short labeling conditions, relative changes in phosphorylation among specific proteins as resolved by 2-dimensional electrophoresis can be detected with a high degree of reliability as previously described (29). Equivalent amounts of radioactivity from either control or experimental samples were analyzed in all cases. Changes in the phosphorylation of a specific protein in response to EGF, TPA, or dbcAMP were considered to be significant when visual observation of the labeling intensity on the fluorograms was compared to the labeling intensity of other major proteins such as pp60 (p1 ~4.5), whose phosphorylation remained relatively constant following any of the treatments (see Figs. 1, and 3–5). In addition, relative increases in the phosphorylation of discrete proteins on the fluorograms from the experimental samples could be accurately assessed when the labeling intensity of these proteins was compared to the labeling intensity of the same proteins on the fluorograms from the nontreated control cultures at any time point.

A431 cells were prelabeled with ³²P, and treated with EGF (100 ng/ml) for 15, 30, or 60 min. The cells were then lysed by homogenization, cytosols prepared, and the proteins were separated by 2-dimensional gel electrophoresis on gels containing ampholytes in the pH range of 3.5–10. Under these conditions, proteins with isolectric points between 4.5 and 7.0 could be sufficiently resolved following isoelectric focusing and sodium dodecyl sulfate-gel electrophoresis. Fig. 1 illustrates representative fluorograms of polyacrylamide gels from cells which had been treated with EGF for these time intervals and from the corresponding control cultures. EGF treatment clearly enhanced the relative phosphorylation of at least 4 distinct acidic cytosol proteins within 15 min after addition to subconfluent A431 cells. Proteins in which phosphorylation was elevated in response to EGF, TPA, or dbcAMP were considered to be significant.
EGF AND TPA STIMULATED PROTEIN PHOSPHORYLATION

ACID BASE

Min of pH

9.7

6.3

25.7

18.4

CONTROL (15 min.)

A

pp17

B

pp18

pp27

pp34

pp80

CONTROL (30 min.)

C

CONTROL (60 min.)

D

EGF (15 min.)

E

EGF (30 min.)

Fig. 1. Effect of EGF on the phosphorylation of acidic cytosol proteins in A431 cells. Subconfluent cultures of A431 cells were prelabeled with [32P]phosphate (250 μCi/ml) for 2 h in serum and phosphate-free Dulbecco’s modified Eagle’s medium and then treated in the absence (control) or presence of EGF (100 ng/ml) for 15, 30, and 60 min. The cells were then lysed and the cytosol proteins were subsequently analyzed by two-dimensional gel electrophoresis. A, C, and E, control cultures; B, D, and F, EGF-treated cultures. Arrows, positions of M, 17,000, 18,000, 27,000, 34,000, and 80,000 proteins as well as pp60 (reference protein).

either EGF or TPA (see Fig. 3) since no major increase was observed in other soluble proteins such as pp60. Fig. 2 indicates the absolute amount of radioactivity associated with each of these proteins following the addition of EGF to [32P]phosphate (250 μCi/ml) for 2 h in serum and phosphate-free Dulbecco’s modified Eagle’s medium and then treated in the absence (control) or presence of EGF (100 ng/ml) for 15, 30, and 60 min. The cells were then lysed and the cytosol proteins were subsequently analyzed by two-dimensional gel electrophoresis. A, C, and E, control cultures; B, D, and F, EGF-treated cultures. Arrows, positions of M, 17,000, 18,000, 27,000, 34,000, and 80,000 proteins as well as pp60 (reference protein).

Fig. 2. Effect of EGF or TPA on the kinetics of [32P] incorporation into pp17, pp27, pp34, and pp80. A431 cells were prelabeled with [32P]phosphate (250 μCi/ml) for 2 h and treated with either EGF (100 ng/ml) or TPA (10−7 M) for 30 min. The cells were then lysed and the cytosol proteins resolved by two-dimensional gel electrophoresis. The proteins pp17, pp27, pp34, and pp80 were located on the gels by autoradiography. These proteins were then excised from the gels and counted in a liquid scintillation spectrometer. Zero time point represents the time at which EGF or TPA was added. ♦, EGF; △, TPA; ○, control. Each value is the average of four separate determinations.

Effect of TPA on the Phosphorylation of Acidic Cytosol Proteins In Vivo. Biologically active phorbol esters such as TPA inhibit the proliferation of subconfluent cultures of A431 cells in a manner similar to EGF and can synergistically enhance the growth inhibitory effect of EGF in these cells (3). It was of interest, therefore, to determine whether an optimum growth inhibitory concentration of TPA (10−7 M) could also affect the phosphorylation of the same proteins modified in response to EGF. Cells were prelabeled with [32P]P, for 2 h and treated with EGF (100 ng/ml) and/or TPA (10−7 M) for 30 min. Two-dimensional gel electrophoresis of the phosphorylated cytosol proteins demonstrates that cells which were exposed to TPA also showed an increase in the relative phosphorylation of pp17, pp27, pp34, and pp80 (Fig. 3C) to approximately the same extent as cells which had been treated with EGF for the same period of time (Fig. 3B). TPA induced a 4- to 5-fold increase in the phosphorylation of pp17 and a 2- to 3-fold increase in the phosphorylation of pp27, pp34, and pp80 after a 30-min treatment. Addition of optimum growth inhibitory concentrations of EGF and TPA to A431 cells produced no further change in the phosphorylation of pp17, pp27, pp34, and pp80 beyond that which was produced by either agent alone (Fig. 3D). Fig. 2 illustrates the kinetics of [32P] incorporation into...
pp17, pp27, pp34, and pp80 in intact cells in response to TPA. TPA, like EGF, produces a quantitative increase in total phosphate incorporation into these four acidic proteins. In fact, the magnitude and time course of the response to TPA for each of these proteins is almost identical to that produced in response to EGF treatment.

Alkalai Lability of Phosphoester Bonds in pp27, pp34, and pp80 and Alkalai Stability of pp17. Alkalai hydrolysis of polyacrylamide gels has been routinely used as a preliminary screening method to identify proteins that may be phosphorylated at threonine and tyrosine residues (8, 10, 29, 31). Specifically, the phosphoester bond in proteins containing phosphoserine residues are found to be more sensitive to alkaline hydrolysis than are proteins containing phosphoesters of tyrosine and threonine (8, 10, 29, 31). Fig. 4 demonstrates the 2-dimensional polyacrylamide gels of 32P-labeled A431 cytosol proteins which were obtained from cells treated with EGF for 30 min before and after treatment of the gels with 1 M NaOH. Alkali treatment removed a majority of radioactivity from most of the more pronounced phosphorylated proteins including pp27, pp34, and pp80 (Fig. 4C) suggesting serine phosphoesters in these proteins; however, the radioactivity associated with pp17 was stable to this treatment. The significance of this alkalai resistance will be discussed.

Effect of dbcAMP on the Phosphorylation of Acidic Cytosol Proteins in Vivo. Cyclic AMP has been demonstrated to stimulate the growth of a number of cell types and to synergistically enhance the growth promoting effects of such mitogens as EGF and insulin (32–34). In A431 cells dbcAMP (1 mm) can induce a 50% inhibition in cell growth in subconfluent cultures after 3–4 days treatment (data not shown); moreover, in HL-60 human promyelocytic leukemia cells, dbcAMP, similar to TPA, produces a growth arrest of these cells and induces the phosphorylation of a M, 17,000 protein which has been shown to be identical to pp17 in the present study (29, 35, 36). To determine whether cyclic AMP could affect the phosphorylation of acidic cytosol proteins in A431 cells, 32P-labeled subconfluent A431 cultures were treated with dbcAMP (1 mm) for 30 min and the cytosol proteins were subsequently analyzed by 2-dimensional gel electrophoresis (Fig. 5). The results (Fig. 5B) demonstrate that dbcAMP produced a modest increase in the phosphorylation of pp17 and pp27 and a more pronounced effect on pp34. dbcAMP had no effect upon the phosphorylation of pp80 but did enhance the phosphorylation of another distinct protein, pp18 (pi ~6.2), which was not affected by either EGF or TPA (see Figs. 1 and 3). In addition, the radioactivity associated with pp18 was sensitive to alkaline hydrolysis (see Fig. 4C).

Calcium and Phospholipid-dependent Phosphorylation of Endogenous A431 Cytosol Proteins in Vitro. A431 cells contain a soluble calcium- and phospholipid-dependent protein kinase C (11) which can be directly activated by 1-oleyl-2-acetyl diacylglycerol and TPA (12). To ascertain whether this endogenous protein kinase could serve to phosphorylate any of the proteins whose phosphorylation was enhanced in vivo in response to EGF or TPA, cytosols which were prepared from subconfluent A431 cells were incubated with [γ-32P]ATP in a cell-free assay optimized to measure endogenous protein kinase C activity in
A431 cells (11). Evidence of protein kinase C activity in the cytosol fractions was determined by the ability of PS and 1,2-diolein to enhance overall protein phosphorylation in the presence of calcium as measured in trichloroacetic acid-precipitable proteins. Under these assay conditions, PS and 1,2-diolein produced a 2- to 3-fold increase in the in vitro phosphorylation of endogenous proteins associated with the cytosol fractions. In these experiments, equivalent amounts of protein from the basal and PS/diolein-treated samples were analyzed by two-dimensional gel electrophoresis. Fig. 6B demonstrates that in the presence of PS and diolein, phosphorylation of pp34 and pp80 is strongly enhanced, whereas a modest increase in the phosphorylation of pp27 occurs. A phosphorylated protein comparable to pp17 could not be detected under these in vitro conditions. In addition, several other phosphorylated proteins having molecular weights of 20–22,000 (pI ~5.2–5.5), 25,000 (pI ~5.7), and 30,000 (pI ~5.2) could also be detected. The nature of these proteins is unknown since they are not detected in cells which have been labeled in vivo with $^{32}$P$_{o}$.

Phosphorylation of Endogenous A431 Cytosol Proteins by Cyclic AMP-dependent Protein Kinase in Vitro. Since treatment of intact A431 cells with dbcAMP produced an enhanced phosphorylation of pp17, pp18, pp27, and pp34, the phosphorylation of these proteins might be mediated through a cyclic AMP-dependent protein kinase(s). To determine whether these proteins could act as substrates for this protein kinase, cytosols were incubated in the absence or presence of the catalytic subunit of bovine type I cyclic AMP-dependent protein kinase with [$\gamma$-$^{32}$P]ATP. Two-dimensional gel electrophoresis of the phosphorylated cytosol proteins demonstrated that pp17 and pp34 and to a lesser extent pp27 but not pp80 can be directly phosphorylated by exogenous cyclic AMP-dependent protein kinase (Fig. 7B). Other proteins at molecular weights of 20,000 (pI ~5.1), 21,000 (pI ~5.3), 22,000 (pI ~5.3), and 30,000 (pI ~6.4) were also phosphorylated by exogenous cyclic AMP-dependent protein kinase. The identity of these proteins in
which phosphorylation is enhanced in vitro but not in intact cells has not been ascertained; furthermore, in the absence of any exogenously added cyclic AMP-dependent protein kinase, none of these proteins was significantly phosphorylated (Fig. 7A) in assays designed to measure endogenous cyclic AMP-dependent protein kinase activity.

**DISCUSSION**

Treatment of intact A431 cells with EGF can rapidly stimulate the tyrosine-specific phosphorylation of several membrane-associated proteins such as the EGF receptor and other soluble cytosol proteins (8, 10). In addition, serine- and threonine-dependent phosphorylation of the EGF receptor and other cellular proteins also has been observed in intact A431 cells following treatment with EGF in vivo (8, 17), suggesting that a cascade of different protein kinases may be indirectly augmented by EGF through its initial interaction with and subsequent activation of the EGF receptor-associated, tyrosine-specific protein kinase (8, 12, 13, 16, 17). One such protein kinase which may be involved in this regulatory chain is protein kinase C (25). Protein kinase C is found in A431 cells (11, 12) and can be directly activated in A431 cells by tumor-promoting phorbol esters, by a synthetic diacylglycerol analogue, 1-oleyl-2-acetyl diacylglycerol, and by diacylglycerol (12), the latter being generated by the transient turnover of phosphoinositides in these cells in response to EGF (3, 23). Protein kinase C has been demonstrated to phosphorylate the EGF receptor at a distinct threonine residue in vitro and in vivo in A431 cells (12–14, 16–18); however, other potential cellular substrates for protein kinase C as well as the presence of other serine- and threonine-dependent protein kinases in A431 cells have not yet been identified.

In this report we demonstrate that exposure of intact A431 cells to EGF or TPA induces a selective enhancement in the phosphorylation of at least four distinct proteins as detected by two-dimensional gel electrophoresis. These proteins are acidic (pI < 7.0) and are found to a major extent in the soluble cytosol fraction of these cells. Although comparable in vivo studies with EGF have been performed by Hunter and Cooper (8) in intact A431 cells, these authors were focusing on those more neutral and basic proteins in which phosphorylation on tyrosine residues could be rapidly modified by EGF. The soluble proteins identified in this study are clearly distinct from any of the proteins previously identified by those authors (8, 10) since the proteins in this study are acidic and probably contain serine residues as the major amino acid which is modified by phosphorylation in vivo. Phosphorylation of four proteins, pp17, pp27, pp34, and pp80, is increased in 32P-labeled cells in response to either EGF or TPA. Although the exact biological function or identity of these proteins is presently unknown, the ability of a growth factor, EGF, and a tumor promoter, TPA, to stimulate the phosphorylation of four identical proteins suggests that these two agents may share a common biochemical pathway in cells which are growth arrested by EGF and TPA. Experiments have not yet been performed to determine whether EGF or TPA might affect the dephosphorylation of these proteins by potentially modulating the activity of a specific protein phosphatase(s); nevertheless, the enhanced phosphorylation of pp17 and pp27 may be one of several events which occur during the early biochemical responses produced in A431 cells following exposure to EGF or TPA since 2 identical proteins have been shown to be rapidly phosphorylated in human HL-60 promyelocytic leukemia cells in response to TPA which can also growth arrest these cells (29, 35, 36). The pp17 of HL-60 cells also exhibits unusual resistance to alkali treatment, despite the fact that only phosphoserine is found in this protein (29, 35). The pp17 (pI ~ 5.5) described in this study exhibited similar alkali resistance (29), supporting the conclusion of identity between it and the previously described HL-60 protein (36).

A protein of molecular weight comparable to pp34 (M, 34,000–36,000) but possessing a higher isoelectric point (pI ~ 7.45 instead of 6.2 for pp34) has been reported to be rapidly phosphorylated at tyrosine residues in A431 cells which have been previously treated with EGF (8–10). The isoelectric focusing conditions used in our study did not resolve proteins with isoelectric points above ~ 7.2. This protein has been shown to be phosphorylated by the EGF receptor-associated protein kinase on tyrosine residues in cell-free assays with cytosols prepared from A431 cells (37). It is apparently homologous to the M, 34,000 protein substrate of the pp60src tyrrosine protein kinase (31, 37). Protein pp34 observed in the present study may be distinct from this M, 34,000 protein not only by a difference in charge but also because alkaline treatment of the polyacrylamide gels removed the radioactivity from pp34, indicating that...
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this protein is probably not phosphorylated on tyrosine residues. EGF or TPA but not dbcAMP enhanced the phosphorylation of pp80 (pl ~4.5) in intact A431 cells. A protein of comparable molecular weight and pl (~4.4) has been reported to be rapidly phosphorylated in response to a number of agents including TPA, vasopressin, bombesin, platelet-derived and fibroblast growth factors, and to a lesser extent by EGF in Swiss mouse 3T3-L1 fibroblasts (20, 38). The phosphorylation of this protein is also not affected by treating mouse Swiss 3T3 cells with agents which elevate intracellular levels of cyclic AMP or with dbcAMP (20, 39). We have recently demonstrated that TPA induces the phosphorylation of an analogous protein in mouse NIH-3T3 cells and in mouse JB-6 epidermal cells but not in human HL-60 cells as analyzed by 2-dimensional gel electrophoresis (36). Although the exact identity of this protein is unknown, it has been demonstrated to be a major substrate for protein kinase C in vitro and in vivo in several different types of cells (38, 40); likewise, in the present study we have shown that protein kinase C is involved in mediating the phosphorylation of pp80 in vitro (Fig. 6). The phosphorylation of this protein is not modified by dbcAMP in vivo (Fig. 5) or by exogenous cyclic AMP-dependent protein kinase in vitro (Fig. 7).

The mechanism(s) by which EGF can inhibit A431 cell growth is still unclear; however, the results presented in this study demonstrate that either EGF or TPA induces the selective phosphorylation of a comparable set of specific acidic, soluble proteins, some of which (i.e., pp17 and pp27) are phosphorylated in other cell types such as HL-60 cells which are also growth arrested by TPA (36). Thus, in cells where EGF or TPA can inhibit cell proliferation, EGF may exert part of its effect through a common pathway which is also activated by TPA. Since the phosphorylations which we have described here are not on tyrosine residues, it appears that EGF treatment of intact A431 cells can rapidly enhance the phosphorylation of some cytosol proteins on tyrosine residues (6–10) and the same or other distinct proteins on serine or threonine residues (8, 10, 16–18). The proteins identified in this study can be phosphorylated in vitro by endogenous protein kinase C (i.e., pp80), by exogenous cyclic AMP-dependent protein kinase (i.e., pp17), or by both protein kinases (i.e., pp27 and pp34). Whether these or other serine- and threonine-specific protein kinases are responsible for the phosphorylation of the four proteins which are observed in vivo in response to EGF or TPA has not been determined; moreover, it is not known whether the same amino acid residue(s) are phosphorylated in vivo as in vitro and following treatment of A431 cells with EGF as with TPA. For example, we have previously demonstrated that pp17 in HL-60 cells can be phosphorylated in vitro by an exogenous cyclic AMP-dependent protein kinase and exhibits two distinct sites of phosphorylation, one of which is phosphorylated in response to TPA in vivo and a second site which is not phosphorylated in vivo following TPA treatment (39). Until additional peptide mapping experiments are conducted on these phosphoproteins under various experimental conditions, these questions cannot be adequately addressed; furthermore, it is not known whether comparable changes in these same proteins occur in other cell types where EGF or TPA stimulates rather than inhibits cell growth (1, 2, 21); nevertheless, Blackshear et al. (38) have recently shown that several growth factors including platelet-derived and fibroblast growth factors and EGF are capable of stimulating the phosphorylation of several common soluble acidic proteins in Swiss 3T3-L1 cells. The phosphorylation of these same proteins is also enhanced by TPA suggesting that in these cells as in A431 cells certain growth factors may also exert part of their growth-promoting effects through a pathway of protein phosphorylation which is also shared by the phorbol ester tumor promoters.

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