Epidermal Growth Factor Stimulates Tyrosine Phosphorylation of Specific Proteins in Permeabilized Human Fibroblasts

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We have investigated the epidermal growth factor (EGF)-stimulated tyrosine-specific protein kinase activity in quiescent cultures of diploid human fibroblasts that have a well characterized mitogenic response to EGF. We developed a method of permeabilizing cells with digitonin or other agents that permitted the rapid labeling of cellular proteins with exogenously added [γ-32P]ATP while allowing only about 25% of marker cytosolic enzymes to escape from the cells. When phosphatases were inhibited with zinc and vanadate, EGF induced up to 8-fold stimulation of the incorporation of radioactivity from [γ-32P]ATP into a 35-kDa band on sodium dodecyl sulfate gels. Alkali treatment of gels showed that EGF stimulated the phosphorylation of bands with apparent molecular masses of 170, 45, 35, 26, 22, and 21 kDa. Phosphoamino acid analysis was performed on the 170- and 35-kDa bands and revealed that the EGF-stimulated phosphorylation was on tyrosyl residues. The 35-kDa band was resolved into four spots by two-dimensional gel electrophoresis. The most acidic form was the most prominent and it was precipitated by an antiserum against a 35-kDa protein from A-431 cells; heretofore, this protein has only been reported to be phosphorylated in an EGF-dependent manner by A-431 membranes in vitro (Fava, R. A., and Cohen, S. (1984) J. Biol. Chem. 259, 2636-2645). This antiserum also precipitated a 35-kDa phosphoprotein from extracts of intact [32P]orthophosphate-labeled cells which was phosphorylated on tyrosine in an EGF-dependent manner. None of the forms of the 35-kDa phosphoproteins labeled in permeabilized cells were immunologically related to the 34-kDa protein that is a substrate for the tyrosyl kinase encoded by Rous sarcoma virus. Other mitogens (serum, insulin, platelet-derived growth factor, and thrombin) did not detectably stimulate phosphorylation in permeabilized cells.

Cellular tyrosine-specific protein kinase activity is stimulated by EGF1 (1-3) and other growth factors (4, 5). The oncosenes of a number of tumor viruses code for tyrosine-specific protein kinases and these genes were acquired from normal vertebrate cells (6). These and other parallels between EGF-stimulated growth and the rapid growth associated with cells transformed by tumor viruses raise the possibility that EGF stimulates cell replication by stimulating tyrosine-specific protein kinases that phosphorylate and thus modulate the activity of, regulatory proteins. A more complete understanding of these processes requires that these phosphorylated proteins be identified. For the EGF receptor/kinase this task has been approached both in vitro and in intact cells. In vitro experiments involved the incubation of plasma membranes or purified EGF receptor/kinase with [γ-32P]ATP and measurement of the labeling of either endogenous membrane proteins (1, 7, 8), exogenously added proteins (9, 10), cell homogenates (11), or synthetic peptides (12). In vivo experiments involved labeling intact cells with [32P]orthophosphate followed by treatment with the hormone and analysis of the labeled proteins (3, 13). A disadvantage of the in vitro method concerns its disruption of the interaction between the receptor/kinase and other cellular regulatory mechanisms. The in vivo method overcomes this disadvantage but requires extended incubation (4 to 18 h) with high levels of [32P]orthophosphate followed by a relatively brief treatment with the hormone. Thus, the effects of the hormone can be obscured by the incorporation of radiactivity into phosphoproteins before hormone treatment.

Because tyrosine kinase activity is very low in nontransformed cells and transformation by many retroviruses is via transforming proteins that are tyrosine kinases, most searches for cellular substrates have been performed on transformed cells. Two of the more interesting substrates for the EGF receptor/kinase have been identified in a phosphorylated form in a transformed cell line, A-431, and have apparent sizes of 34-39 kDa (3, 14) and 35 kDa (15), respectively. The former substrate also has been identified in numerous retrovirus-transformed cells with estimated size from 34 to 36 kDa (16-18). However, the EGF-dependent phosphorylation of these proteins has not been demonstrated in nontransformed cells that have a mitogenic response to EGF. The 34-kDa protein is not phosphorylated in mitogenically responsive human and mouse fibroblasts following exposure to EGF even when this treatment stimulated the phosphorylation of other proteins on tyrosine (19, 20). Thus, the question arises as to whether the phosphorylated proteins in transformed cells are physiological substrates or are phosphorylated due to an overproduction of tyrosine kinases that lack stringent substrate specificity.

We have attempted to identify cellular substrates for EGF-induced phosphorylation in cultured diploid human fibroblasts that have a well characterized mitogenic response to EGF (21-24). To develop a sensitive and rapid assay for the EGF-stimulated kinase activity while maintaining at least some degree of interaction between various regulatory mech-
anisms, we have studied phosphorylation in cells that were made permeable to exogenously added 32P-ATP by mild treatments. To enhance the incorporation of radioactivity into phosphotyrosine (10), Molecular methods for activation were performed under conditions that are optimal for phosphatases and inhibit tyrosyl phosphatases (26, 27). Under these conditions, EGF markedly increased the incorporation of radioactivity into certain phosphoproteins.

EXPERIMENTAL PROCEDURES

Materials—EGF was isolated (28) from mouse submaxillary glands and iodinated by published procedures (29). [γ-32P]ATP was synthesized from carrier-free [γ32P]P32 orthophosphate (ICN, Irvine, CA) using Gamma Prep Synthesis Systems (Promega Biotec, Madison, WI). Antibody to the 34-kDa substrate for pp60src (16,17) was kindly provided by Dr. Stanley Cohen, Vanderbilt University. Antibody to the 34-kDa substrate for pp60src (16,17) was kindly provided by Dr. Randy Schatzman and Dr. J. Michael Bishop, University of California, San Francisco and Dr. Jonathan Cooper, Irvine.

Cell Culture—Experimental cultures of diploid human foreskin fibroblasts (passage 7-13) were plated in 35-mm cell culture dishes (Falcon) of L929 mouse embryonic cell medium (Gibco) supplemented with 20 mM Hepes, 10% calf serum (Gibco), and penicillin-streptomycin (Gibco). The cells were grown to confluency (4-7 days) and shifted to 1% calf serum 48 h before labeling experiments. Protein content of cell extracts was measured by the method of Lowry (31).

Standard Phosphorylation Assay—Unless otherwise stated, permeabilization and phosphorylation were studied as follows. Monolayer cultures of quiescent human fibroblasts were permeabilized and labeled simultaneously by incubation in 0.2 ml of buffer A (145 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl2, 0.8 mM MgSO4, 20 mM Hepes, pH 7.4) containing MnCl2 (2 mM), ZnCl2 (20 μM), sodium orthovanadate (20 μM), digitonin (75 μg/ml), and [γ-32P]ATP (15 μM, 16-333 Ci/mmol) for 5 min at room temperature. The incubation was carried out in the presence or absence of EGF (500 ng/ml). The reaction was terminated by removing the labeling solution and rinsing twice with buffer A containing ZnCl2 (30 μM) and sodium orthovanadate (39 μM) at 4°C. The labeled proteins were extracted by different methods for the various analysis procedures (see below).

SDS-Gel Electrophoresis and Autoradiography—Cellular proteins were extracted from the culture dish using SDS-gel sample buffer (32) and then were fractionated by SDS-gel electrophoresis by the method of Laemmli (32). Molecular standards used for calibration of the gels were from a prepackaged mix obtained from Sigma. The gels contained the following: rabbit muscle myosin (200 kDa); β-galactosidase (116 kDa); bovine serum albumin (66 kDa); ovalbumin (45 kDa); glyceraldehyde-3-phosphate dehydrogenase (36 kDa); carbonic anhydrase (29 kDa); trypsinogen (24 kDa), and soybean trypsin inhibitor (20 kDa). The gels were stained in 45% methanol, 9% acetic acid, and 0.05% Coomassie R 250; the stained proteins were exposed to film as described above.

RESULTS

Digitonin Permeabilization of Cells—We developed a method for permeabilizing cells with the steroid glycoside digitonin so that intracellular kinases could be exposed to exogenously added [γ-32P]ATP. Conditions were selected such that the plasma membrane was permeable to small molecules such as ATP but remained relatively impermeable to intracellular proteins. Cells were made permeable by incubation with digitonin (25-100 μg/ml) in buffer A for 5 min at room temperature. Incubation with digitonin resulted in the cells becoming permeable to small extracellular molecules as measured by the uptake of trypan blue; 85-90% of the cells that were incubated in 25 μg/ml digitonin for 5 min at room temperature were stained by trypan blue as compared to 1% of control cells (Table I). The digitonin-dependent loss of intracellular proteins was determined by measuring the distribution of cytosolic marker enzyme activities. Treatment with increasing digitonin concentration resulted in an increasing loss of cytosolic enzyme activity (Fig. 1). In most phosphorylation assays (see below) 75 μg/ml digitonin was used to permeabilize cells; at this concentration there was approximately a 35% loss of lactate dehydrogenase and a 20% loss of glucose-6-phosphate dehydrogenase activity from the cytosol, the cellular loss in cytosolic marker enzymes occurred within the first 2 min of exposure to digitonin without any further change occurring over the next 30 min (data not shown).

Digitonin
EGF-dependent Protein Phosphorylation in Permeabilized Cells

TABLE I

| Permeability* | 125I-EGF binding† | Total protein† |
|---------------|-------------------|--------------|
| Control       | 0                 | 127          |
| Digitonin-treated* | 85-90             | 98           |

* Cells were incubated in trypsin blue (0.04% w/v) for 4 min at room temperature. Numbers indicate percentage of cells taking up trypsin blue.
† Cells were incubated with 125I-EGF (25 ng/ml, 17,000 cpm/ng) for 30 min at 37°C, rinsed 5 times with phosphate-buffered saline, solubilized with NaOH, and counted. Radioactivity was normalized to counts/min/240,000 cells.

FIG. 1. The effect of digitonin concentration on the release of cytosolic marker enzymes. Monolayer cultures of fibroblasts were incubated for 5 min at room temperature in buffer A containing increasing concentrations of digitonin. The digitonin solutions were removed and the cells were solubilized in either NaCl (0.2 M), Tritton X-100 (0.5%), NaH2PO4, (0.05 M, pH 7.5), or RSA (0.1%), Tritton X-100 (0.5%), glycine (5 mM, pH 8.0). The former lysate was assayed for lactate dehydrogenase (LDH) and the latter for glucose-6-phosphate dehydrogenase (G6PDH) activity by spectrophotometric measurement of NADH oxidation in the presence of pyruvate (36) or NADP reduction in the presence of glucose-6-phosphate (37), respectively. The lactate dehydrogenase (C) and glucose-6-phosphate dehydrogenase (G6PDE) activities measured in the digitonin wash was also assayed. The error bars represent 95% confidence intervals of triplicate measurements.

In Table I, the effect of digitonin treatment on the release of cytosolic marker enzymes is shown. Digitonin treatment (25 μg/ml) also resulted in an approximate 25% loss of both total cellular protein and EGF-binding capacity (Table I). The overall appearance in the phase-contrast microscope of the digitonin-treated cells was similar to the control cells although there was a slight increase in the number of broken cells.

Assays for Phosphorylation of Celluar Proteins—In an initial attempt to detect changes in protein phosphorylation in cells that are mitogenically responsive to EGF, we labeled human fibroblasts with [32P]orthophosphate by procedures previously used to investigate EGF effects in A-431 carcinoma cells (3). Monolayers of quiescent fibroblasts were preincubated in phosphate-free DMEM supplemented with 1% calf serum for 4 h, then [32P]orthophosphate was added and the cells were incubated for an additional 18 h. EGF was added for the final 15 min of the incubation with [32P]orthophosphate. After labeling, the media was removed, and the cells were rinsed, extracted, and analyzed by either two-dimensional or SDS-gel electrophoresis and autoradiography. Little or no effect of EGF on the incorporation of radioactivity into proteins could be detected on SDS-polyacrylamide gels (Fig. 2, lanes a and b) or two-dimensional gels (data not shown).

As expected, incubating intact cells with exogenously added (72P)ATP did not result in labeling of cellular proteins (Fig. 2, lane c). However, fibroblasts that were permeabilized with digitonin and exposed to (72P)ATP for 5 min in buffer A incorporated radioactivity into cellular protein. Under these conditions EGF had little detectable effect on the incorporation (Fig. 2, lanes d and e). Since permeabilization of the membrane with digitonin allows one to manipulate the ionic environment of the cellular kinases, we attempted to increase the ability to detect tyrosine-specific protein kinase activity by adding ionic components that increase tyrosyl kinase activity and other ionic components to inhibit phosphotyrosyl protein phosphatases. The EGF receptor/kinase activity has been shown to be enhanced relative to serine or threonine kinases when in vitro reactions are performed in the presence of manganese as compared to magnesium (1, 25). Therefore buffer A, which contains low concentration of magnesium (0.8 mM), was supplemented with 2 mM MnCl2 and used as the labeling media. Under these conditions the overall amount of phosphorylation increased slightly in the presence and absence of EGF. In the presence of EGF there also were specific effects; there was a severalfold increase in the radioactivity in a 35-kDa band of phosphoproteins when EGF was added to this labeling media (Fig. 2, lanes f and g).

Other experiments tested the effects of the phosphotyrosine protein phosphatase inhibitors zinc and vanadate (26, 27). When zinc was added to the labeling media containing MnCl2, the EGF-induced phosphorylation of the 35-kDa band was slightly more pronounced (Fig. 2, lanes h and i), but when both zinc and vanadate were added to the same medium the EGF-induced phosphorylation of the 35-kDa band increased markedly (Fig. 2, lanes j and k). The addition of only vanadate to labeling media containing MnCl2 enhances the phosphorylation of the 35-kDa band to the same extent as zinc and vanadate combined (data not shown). Even under optimum ionic conditions (zinc and vanadate) the phosphatases are not inhibited completely. If the cells were labeled for 5 min with radioactively labeled ATP and then chased with excess unlabeled ATP, the amount of 32P incorporation into the 35-kDa band was reduced by 40% at 15 min at 23°C. However, the fold increase in 32P incorporation into the 35-kDa band by incubation with EGF was the same whether measured after the 5-min labeling period or after the 15-min chase.

The possibility that phosphorylated proteins were being released into the labeling medium or into the washes was evaluated by analysis of the fractions by SDS-gel electrophoresis followed by autoradiography. Only a small amount of labeled proteins were in these fractions and EGF did not have an effect on any of the observed bands.

The effects of EGF on phosphorylation are not unique to cells permeabilized with digitonin because effects also were seen when the cells were permeabilized with other agents and labeled with (72P)ATP. Digitonin was replaced with the peptide ionophore alamethicin (750 μg/ml) and cells were labeled as described for Fig. 2, lanes j and k, in the presence and absence of EGF (Fig. 2, lanes 1 and m). Less overall labeling was seen than in the same conditions with digitonin (75 μg/ml) but the pattern of labeling and the effects of EGF were similar. Similar effects on overall protein labeling and EGF-induced increases in radioactivity in the 35-kDa band also were seen if the cells were permeabilized with lysolceithin (250 μg/ml) or saponin (500 μg/ml) under the conditions described for lanes j–m in Fig. 2 (data not shown). However, when phosphorylation was performed following more drastic detergent treatment (e.g., 0.5% Triton X-100) that extracts...
most proteins except the cytoskeletal matrix (38), no effects of EGF were observed (data not shown).

Although, the most striking effect of EGF in phosphorylation of cellular proteins occurred in a radioactive band that migrated with an apparent molecular mass of 35 kDa, there were other bands that showed an EGF dependence when the gels were exposed to film for longer times (data not shown). These and other bands were more obvious in gels that were preincubated for 4 h in phosphate-free DMEM containing 1% calf serum then labeled for 18 h with $[^{32}P]P$ orthophosphate (0.5 mCi in 1 ml) (lanes a and b). EGF was added to one culture (lane b) for the final 15 min of labeling. Lane c is an extract of cells that were incubated with $[^{32}P]P$ATP in buffer A for 5 min at room temperature without digitonin permeabilization. Monolayer cultures of fibroblasts were permeabilized with digitonin (75 µg/ml) (lanes d–k) or alamethicin (750 µg/ml) (lanes l and m) and labeled with $[^{32}P]P$ATP (50 µCi/dish, 16.7 Ci/mmol), as described under “Experimental Procedures,” in buffer A (lanes d and e) or buffer A supplemented with either 2 mM MnCl$_2$ (lanes f and g), 2 mM MnCl$_2$ and 20 µM ZnCl$_2$ (lanes h and i), or 2 mM MnCl$_2$, 20 µM ZnCl$_2$, and 20 µM sodium orthovanadate (lanes j–m). Lanes e, g, i, h, and m contained EGF (500 ng/ml) during the 5-min incubation with $[^{32}P]P$ATP and either digitonin or alamethicin. Cultures were extracted and fractionated on an 8.2% SDS-polyacrylamide gel and processed for autoradiography as described under “Experimental Procedures.”

**FIG. 2 (left). Comparison of assays for EGF-dependent protein phosphorylation.** The effect of EGF on the incorporation of phosphate into cellular proteins was measured either in intact fibroblasts incubated with $[^{32}P]P$ATP or in permeabilized fibroblasts incubated with $[^{32}P]P$ATP. Monolayer cultures of fibroblasts were preincubated for 4 h in phosphate-free DMEM containing 1% calf serum then labeled for 18 h with $[^{32}P]P$ orthophosphate (0.5 mCi in 1 ml) (lanes a and b). EGF was added to one culture (lane b) for the final 15 min of labeling. Lane c is an extract of cells that were incubated with $[^{32}P]P$ATP in buffer A for 5 min at room temperature without digitonin permeabilization. Monolayer cultures of fibroblasts were permeabilized with digitonin (75 µg/ml) (lanes d–k) or alamethicin (750 µg/ml) (lanes l and m) and labeled with $[^{32}P]P$ATP (50 µCi/dish, 16.7 Ci/mmol), as described under “Experimental Procedures,” in buffer A (lanes d and e) or buffer A supplemented with either 2 mM MnCl$_2$ (lanes f and g), 2 mM MnCl$_2$ and 20 µM ZnCl$_2$ (lanes h and i), or 2 mM MnCl$_2$, 20 µM ZnCl$_2$, and 20 µM sodium orthovanadate (lanes j–m). Lanes e, g, i, h, and m contained EGF (500 ng/ml) during the 5-min incubation with $[^{32}P]P$ATP and either digitonin or alamethicin. Cultures were extracted and fractionated on an 8.2% SDS-polyacrylamide gel and processed for autoradiography as described under “Experimental Procedures.”

**FIG. 3 (right). Effect of EGF on alkali-resistant phosphorylation in fibroblasts made permeable with digitonin.** Monolayer cultures of fibroblasts were permeabilized with digitonin and labeled with $[^{32}P]P$ATP (16.7 Ci/mmol) in the presence of MnCl$_2$ (2 mM), ZnCl$_2$ (20 µM), and sodium orthovanadate (20 µM) by the standard phosphorylation assay described under “Experimental Procedures.” The samples were fractionated on a 10% SDS gel, and the gel was treated with alkali and processed for autoradiography as described under “Experimental Procedures.” Lane a, control cells; lane b, 5-min exposure to EGF (500 ng/ml) during labeling.
about 4-fold with longer periods of exposure (15 min) to EGF and [γ-32P]ATP. Although the relative amounts of label in certain bands varied with time, there was no qualitative change in the labeling pattern from 1 to 15 min in the presence or absence of EGF.

Under identical experimental conditions we compared the dose response curves for EGF binding and phosphorylation to determine what relationship exists between receptor occupancy and the phosphorylation of the 35-kDa band. Digitonin-permeabilized cells were exposed to increasing concentrations of 125I-EGF for 15 min at room temperature. The binding curve in digitonin-treated cells (Fig. 4) was very similar to binding in untreated fibroblasts (data not shown), and approximately half-maximal binding occurred at 1.6 nM. The dose response to the EGF-stimulated increase in phosphorylation of the 35-kDa band was measured by labeling digitonin-permeabilized cells in the presence of varying concentrations of EGF for 15 min at room temperature. Cell extracts were fractionated on a SDS gel, and the 35-kDa band was excised, solubilized, and counted. The amount of radioactivity in the 35-kDa band increased in an EGF-dependent manner with maximal stimulation occurring at approximately 2 nM and half-maximal occurring at 0.003 nM EGF (Fig. 4). Thus, the EGF concentration that gives a 50% maximal increase in phosphorylation is about 20-fold less than the concentration required for 50% of maximal binding. Although residual phosphatase activity is present in the phosphorylation reaction, it does not appear to have a major effect on the EGF dose response curve because the same relative effects were seen when the phosphorylation of the 35-kDa band was analyzed immediately after the reaction or when the reaction was analyzed after a 15-min chase with unlabeled ATP.

The effects of EGF on kinase activity also were studied by two-dimensional gel electrophoresis in order to obtain better resolution of the phosphorylated proteins. Digitonin-permeabilized fibroblasts were labeled for 5 min at room temperature in the presence or absence of EGF and then fractionated by two-dimensional gel electrophoresis using isoelectric focusing in the first dimension followed by SDS-gel electrophoresis in the second dimension (33). Radioactive proteins were visualized before and after exposing the gels to KOH to preferentially hydrolyze phosphoserine. As expected from the SDS gels (Figs. 2 and 3) there were EGF-dependent changes in phosphorylation of both total and alkali-stable phosphoproteins in the 35-kDa region of the two-dimensional gels (Fig. 5); however, the 35-kDa band was resolved into at least four distinct species that differed in charge. In both KOH-treated and untreated gels, all four species showed an EGF-induced change in 32P content (Fig. 5). The most acidic of these spots had an apparent pI of 7.75 (Fig. 5, spot a), and EGF stimulated a 7.5-fold increase in its 32P content as measured by excision of the corresponding portion of the gel and determination of the radioactivity in the excised gel by liquid scintillation counting. The next two spots had apparent pI values of 8.10 and 8.17 (Fig. 5, spots b and c) and showed a small EGF-dependent increase, 4.5- and 2-fold respectively, in 32P content in gels showing total phosphoproteins (Fig. 5, A and B); however, they showed a marked EGF dependence in alkali-treated gels (Fig. 5, C and D). The most basic of the labeled spots had an apparent pI of 8.23 (Fig. 5, spot d) and was detected only when the cells were labeled in the presence of EGF. Thus, EGF stimulates the phosphorylation of as many as four 35-kDa proteins in digitonin-permeabilized human fibroblasts.

In addition to the 35-kDa phosphoproteins, a number of other EGF-dependent phosphoproteins were detected on KOH-treated two-dimensional gels (Fig. 5, C and D). Phosphoproteins, with apparent molecular masses of 170, 45, and 26 kDa and with apparent pI values of 7.10, 7.20, and 7.55, respectively, were detected when digitonin-permeabilized cells were labeled in the presence of EGF but not in the absence of EGF (Fig. 5, spots i, h, and e). Two other phosphoproteins, with approximately sizes of 26 kDa and apparent pI values of 7.75 and 8.20, respectively, were barely detected in control cells, but labeling in the presence of EGF resulted in a marked increase in their phosphorylation (Fig. 5, spots f and g).

Phosphoamino Acid Analysis of Labeled 35-kDa Proteins—Quantitative studies of the amino acid specificity of the kinase(s) stimulated by EGF were performed. Digitonin-permeabilized fibroblasts were labeled in buffer A containing manganese, zinc, vanadate, and [γ-32P]ATP in the presence and absence of EGF, and the cellular phosphoproteins were fractionated by SDS-polyacrylamide gel electrophoresis. The 35-kDa band was excised from the gel, eluted, hydrolyzed in HCl, and the phosphoamino acid composition was determined by electrophoresis on thin layer plates (Fig. 6). In the absence of EGF, 60% of the radioactivity that comigrated with the ninyhydrin-stained standards was with phosphoserine, 16% with phosphothreonine, and 22% was with phosphotyrosine. Addition of EGF caused an increase in the amount of phosphorylation of tyrosyl residues but no change in the phosphorylation of seryl or threonyl residues. In EGF-treated cells the acid-stable radioactivity in phosphotyrosine increased 6-fold resulting in the phosphoamino acids migrating as 60% phosphotyrosine, 8% phosphothreonine, and 31% phosphoserine (Table II).

Fig. 5 showed that fractionation of extracts of digitonin-permeabilized, [γ-32P]ATP-labeled cells on two-dimensional gels separated the 35-kDa band into at least 4 phosphoproteins that differ in net charge. Phosphoamino acid analysis of these four 35-kDa phosphoproteins excised from two-dimensional gels showed that labeling in the presence of EGF resulted in an increase in the phosphotyrosine content of each (Fig. 6 and data not shown). Fig. 6 shows that in the absence...
FIG. 5. Two-dimensional gel analysis of the effect of EGF on protein phosphorylation in digitonin-permeabilized fibroblasts. Cultures of fibroblasts were permeabilized with digitonin and labeled with $[^{32}P]ATP$ in the absence (A and C) and presence (B and D) of EGF (500 ng/ml) and the samples were processed for fractionation on two-dimensional gels as described under “Experimental Procedures.” A and B show total phosphoproteins and C and D show phosphoproteins remaining after treatment with 1 N KOH for 2 h as described under “Experimental Procedures.”

Partial Identification of EGF-dependent Phosphoproteins in Fibroblasts—In an attempt to identify the EGF-dependent phosphoproteins that were detected in digitonin-permeabilized fibroblasts, we tested their reactivity with antibodies against various known substrates for the EGF-stimulated kinases. When extracts were incubated with control rabbit serum, no $[^{32}P]P$-labeled bands were detected on the gel (data not shown). Polyclonal antibodies against the EGF receptor (30) precipitated a 170-kDa phosphoprotein from extracts of digitonin-permeabilized cells labeled with $[^{32}P]ATP$ (Fig. 7, lanes a and b). The immunoprecipitates of the EGF receptor show a marked EGF dependence in the amount of incorporated radioactivity. Cerenkov counts of the excised 170-kDa band showed that labeling in the presence of EGF for 5 min stimulated up to a 6-fold increase in the radioactivity incorporated into the immunoprecipitate. Phosphoamino acid analysis of the immunoprecipitated EGF receptor band excised from a SDS gel showed that, in the presence of EGF, phosphotyrosine was the only phosphoamino acid detected; however, because of the low amounts of radioactivity involved, up to about 20% of the counts could have been in another phosphoamino acid and remained undetected.

Extracts from digitonin-permeabilized $[^{32}P]ATP$-labeled cells also were tested for reactivity with antiserum against a 35-kDa protein, isolated from A-431 cells by Fava and Cohen, that is a substrate for the EGF receptor/kinase in vitro (15). SDS gel analysis showed that this antiserum precipitated a 35-kDa $[^{32}P]$-labeled phosphoprotein and its phosphorylation was stimulated about 5-fold in cells treated with EGF for 5 min (Fig. 7, lanes c and d). The 35-kDa phosphoprotein that is precipitated with this antiserum ran with the same mobility as the 35-kDa phosphoprotein in whole cell extract when the two samples were run on adjacent lanes in the same SDS gel. Two-dimensional gel analysis of replicate extracts showed that this antiserum precipitated only the most acidic of the 35-kDa phosphoproteins that are seen on two-dimensional gels (Fig. 7B).

To determine if the 35-kDa protein identified by Fava and Cohen (15) is a substrate for EGF-stimulated kinases in intact cells, antiserum against this protein was incubated with extracts from $[^{32}P]$orthophosphate-labeled cells. Analysis of the immunoprecipitates by SDS-gel electrophoresis revealed a 35-kDa $[^{32}P]$-labeled phosphoprotein and its phosphorylation was stimulated up to 12-fold by a 60-min exposure to EGF (Fig. 7A, lanes g and h). The 35-kDa bands from Fig. 7, lanes g and h, were excised and subjected to phosphoamino acid analysis. In the absence of EGF the amount of radioactivity was too
EGF-dependent Protein Phosphorylation in Permeabilized Cells

The effect of EGF on the phosphorylation of a 35-kDa protein that is immunologically related to the 34-kDa protein isolated from A-431 cells by Fava and Cohen (15). Thus, EGF treatment of digitonin-permeabilized and intact cells (14), extracts from [γ-32P]ATP-labeled digitonin-permeabilized cells and processed as above. The higher molecular weight bands seen in A, lanes g and h, were nonspecific because they were also seen when control serum was used instead of anti-35-kDa protein.

Several laboratories have described the phosphorylation of a 34- to 39-kDa protein that is a substrate for tyrosyl kinases (16–18, 38, 42, 43). A 34-kDa protein, shown to be a substrate for p60src, has been purified and used to raise an antisera to EGF (13, 44). This antisera did not detectably react with proteins labeled with [γ-32P]ATP in digitonin-permeabilized fibroblasts (Fig. 7, lanes e and f). However, as expected from studies in intact cells (14), extracts from [γ-32P]ATP-labeled digitonin-permeabilized A-431 cells did contain a radioactive 34-kDa protein that was precipitated by this antisera (data not shown). Thus, none of the 35-kDa phosphoproteins detected in human fibroblasts are immunologically related to the 34-kDa protein that has been purified by Erikson and Erikson (43).

**Table II**

Phosphoamino acid analysis of 35-kDa phosphoproteins

The acid hydrolysates of the 35-kDa bands from an SDS gel of extracts of [γ-32P]ATP-labeled, digitonin-permeabilized cells were fractionated on TLC plates as described in the legend to Fig. 6. Phosphoamino acid standards were detected by staining with ninhydrin. The areas containing the ninhydrin-stained standards were scraped, and the phosphoamino acids were eluted with water and counted. Cells were labeled for 5 min at room temperature in the presence of EGF (800 ng/ml).

| Control cells | EGF-treated cells |
|---------------|-------------------|
| cpm           | %                 |
| Phosphoserine  | 549 61            |
| Phosphothreonine | 146 16           |
| Phosphotyrosine | 202 23          |

Fig. 6. The phosphoamino acid content of the EGF-dependent 35-kDa phosphoproteins. The effect of EGF on the phosphoamino acid content of the 35-kDa band excised from an SDS gel and for spot a (see Fig. 5) excised from a two-dimensional gel. Cells were permeabilized with digitonin, labeled with [γ-32P]ATP (100 Ci/mmol in A and B, 333 Ci/mmol in C and D), extracted, fractionated either on an 8.2% SDS gel (A and B) or a two-dimensional gel (C and D), and processed for autoradiography as described under “Experimental Procedures.” Following autoradiography, the area of interest was excised from the gel and the protein was eluted according to the method of Beemon and Hunter (39). The eluate was resuspended at 6 N HCl and hydrolyzed at 110 °C for 1 h. The acid hydrolysate was separated on thin layer cellulose plates by electrophoresis at pH 1.9 at 750 volts for 3 h in the first dimension followed by electrophoresis at pH 3.5 at 700 volts for 1 h in the second dimension (40, 41). Phosphoamino acid standards were detected by staining with ninhydrin and the radioactive phosphoamino acids were detected by autoradiography as described under “Experimental Procedures.” A and B were exposed to film for 48 h. D was exposed for one-tenth the exposure time of C (120 h) to normalize for the variation in total counts loaded on each plate. A and C, control cells; B and D, 5-min exposure to EGF (500 ng/ml) during labeling. P-Ser, phosphoserine; P-Thr, phosphothreonine; P-Tyr, phosphotyrosine.

Fig. 7. Immunoprecipitation of substrates for tyrosine-specific kinases. Monolayer cultures of human fibroblasts were either permeabilized with digitonin and labeled with [γ-32P]ATP (106 Ci/mmol) (A, lanes a–f) or labeled with [32P]orthophosphate (1.5 mCi in 1 ml) for 4 h (A, lanes g and h) as described in the legend to Fig. 2. Radiolabeled cells were extracted and immunoprecipitated with either anti-EGF receptor (A, lanes a and b), anti-35-kDa protein (A, lanes c, d, g, and h, and B), or anti-34-kDa protein (A, lanes e and f) serum as described under “Experimental Procedures.” The extracts fractionated on the gel in A, lanes b, d, and f, and B contained EGF (500 ng/ml) during the 5-min incubation with [γ-32P]ATP. One of the [32P]orthophosphate-labeled cultures (A, lane h) was exposed to EGF (100 ng/ml) for the final 1 h of incubation with label. Immunoprecipitates either were boiled in SDS-sample buffer and fractionated on a 9% SDS-polyacrylamide gel (A) or were taken up in two-dimensional gel sample buffer and fractionated on a two-dimensional gel (B) as described under “Experimental Procedures.” No detectable radioactive bands were seen when control serum was incubated with extracts from digitonin-permeabilized cells and processed as above. The higher molecular weight bands seen in A, lanes g and h, were nonspecific because they were also seen when control serum was used instead of anti-35-kDa protein.
In order to fully understand the physiological role of EGF-stimulated tyrosine protein kinases, it is necessary to identify and characterize their cellular substrates. In \[^{32}P\]orthophosphate-labeled transformed cells, EGF has been shown to stimulate incorporation of radioactivity into 21-, 34-, 39-, 42-, 43-, and 45-kDa phosphoproteins (3, 11). In certain cells that are mitogenically responsive to EGF (3T3, rat, and human fibroblasts), the hormone has been shown to stimulate the phosphorylation of tyrosyl residues of 42-, 43-, and 45-kDa proteins (13, 19, 20, 46). The physiological consequences of the phosphorylation of these proteins are not known.

We attempted to use the \[^{32}P\]orthophosphate labeling method (3) to detect EGF stimulation of phosphorylation events in cultures of human diploid fibroblasts but could detect little or no effect when labeled phosphoproteins were fractionated on one- or two-dimensional gels. In an attempt to develop a more sensitive method for detection of EGF-modulated phosphorylation, we developed an assay in which monolayer cultures of diploid human fibroblasts were made permeable to exogenously added \[^{32}P\]ATP by incubation with digitonin. Digitonin permeabilization of cells has been used by others to study a number of processes including gastric proton pumps (47), catecholamine secretion (48, 49), and receptor-mediated endocytosis (50, 51). In both the current study and in previous studies the same general effects of digitonin on cell structure was observed. The cells became permeable to exogenously added compounds while the plasma membrane retained some degree of integrity as evidenced by the fact that soluble cytosolic enzymes are at least partially retained within the cells (Fig. 1). This would suggest that soluble protein substrates could be detected by our assay. Subcellular fractionation of labeled digitonin-permeabilized cells revealed that many phosphoproteins were in the soluble fraction although most of the major EGF-dependent bands were in the particulate fraction. Although it is not as yet clear if digitonin permeabilization assay will reveal growth factor-dependent phosphorylation of soluble substrates, it is clear that the phosphorylation observed in cells following mild digitonin treatment is quite different than that observed following more drastic detergent treatment. Several laboratories have studied phosphorylation in virally transformed cells that were treated by methods described by Burr et al. (38) in which high concentrations of detergent were used to strip away the majority of cellular protein, leaving behind a cytoskeletal matrix. We were unable to detect any effects of EGF in human fibroblasts treated by these methods.2

To enhance the ability to detect tyrosine protein kinase activity in permeabilized cells, the assay was performed in the presence of zinc and vanadate to inhibit cellular phosphotyrosine phosphatase activity. Under these conditions there was a rapid and efficient incorporation of radioactivity into endogenous proteins. EGF, but not other mitogens tested, stimulated the incorporation of radioactivity into alkali-stable phosphoproteins with apparent sizes of 170, 45, 35, 26, 22, and 21 kDa on SDS gels. The greatest effect of EGF was on the 35-kDa band of phosphoproteins. This band could be resolved into at least 4 spots with apparent pl values of from 7.75 to 8.23 (Fig. 5) on two-dimensional gels by the method of Garrels (33). When cellular extracts were prepared and fractionated on two-dimensional gels by the method of O’Farrell (62), the most acidic spot had an apparent pl of about 6.50 and the other three spots were not as clearly resolved (data not shown). The reason for these differences is not yet known. In both gel systems, EGF has the greatest effect (up to about 7-fold stimulation) on the phosphorylation of the most acidic spot (spot a in Fig. 5). Phosphoamino acid analysis of acid hydrolysates showed that spot a contained both phosphoserine and phosphotyrosine, and EGF stimulated the incorporation of radioactivity into only phosphotyrosine (Fig. 5).

Two of the phosphoproteins that were phosphorylated in an EGF-dependent manner have been identified by reactivity with antisera that are specific for purified proteins. The 170-kDa phosphoprotein is the EGF receptor (Fig. 7). The most acidic of the 35-kDa phosphoproteins (Fig. 5, spot a) was immunoprecipitated (Fig. 7) with an antibody against a 35-kDa protein that was isolated from A-431 carcinoma cells by Fava and Cohen (15). They showed that the 35-kDa protein from A-431 cells underwent a reversible, calcium-dependent association with the particulate fraction of cellular extracts and that it was a substrate for the EGF receptor/kinase in vitro (15). In permeabilized cells the identity of the EGF-dependent phosphoproteins other than the EGF receptor and the most acidic of the 35-kDa proteins has not yet been determined. A phosphoprotein of approximately 34 to 39 kDa that is not related to the Fava and Cohen 35-kDa protein (15) has been detected by a number of laboratories in cells that are infected with Rous sarcoma virus (16-18). This protein appears to be related to a protein that is phosphorylated in an EGF-dependent manner in intact A-431 cells (3, 14) or cell homogenates (11). Antiserum against this protein did not react with any of the proteins that were phosphorylated in an EGF-dependent manner in permeabilized human fibroblasts (Fig. 7).

Gerke and Weber (53) recently showed that the 36-kDa subunit of their Protein I appears to be very similar and possibly identical to the 34- to 39-kDa substrate of Rous sarcoma virus tyrosine kinase. They also showed that Fava

\(^2\)T. D. Giugni and H. T. Haigler, unpublished results.

**FIG. 8. Time course of EGF-dependent phosphorylation of a 35-kDa protein in intact human fibroblasts.** Monolayer cultures of human fibroblasts were labeled with \[^{32}P\]orthophosphate (1.5 mcCi/ml) for 4 h as described in the legend to Fig. 2. Labeled cultures were exposed to EGF (100 ng/ml) for the indicated time, then extracted, immunoprecipitated with anti-35-kDa serum (15), and fractionated on a 9% SDS gel as described in the legend to Fig. 7. The autoradiograms of gel were scanned with a Quickscan densitometer (Helena Laboratories) and the peak values corresponding to the 35-kDa protein were integrated. The values were normalized to the control for each experiment. Each point corresponds to the average value of from 2 to 5 determinations. The error bars correspond to 95% confidence intervals.

**DISCUSSION**
and Cohen's protein is not identical to this subunit of Protein I but presented preliminary data which suggested that the two may share certain properties. Additional experiments are required to determine the exact relationship between these proteins and the 35-kDa phosphoprotein substrates identified in the current study. The physiological role of the EGF-dependent phosphorylation of the 35-kDa protein in human fibroblasts is not yet known but it is intriguing to note that a similar EGF effect is observed in other cells that are mitogenically responsive to EGF (Chinese hamster and mouse embryo fibroblasts) but not in GH3 cells that are not mitogenically responsive but do change differentiated cell function in response to EGF. Since only one of the four 35-kDa phosphoproteins was identified, the relationship between them is not known. Differences in phosphate content on a single polypeptide could cause separation of the magnitude seen; however, it seems unlikely that all four species represent different phosphorylation states of the same 35-kDa protein since only the most acidic species was immunoprecipitated with the antibody against the Fava and Cohen 35-kDa protein. In addition, preliminary results suggest that the nonphosphorylated form of the Fava and Cohen 35-kDa protein could be identified by Western blot analysis of two-dimensional gels, and the same spot on the Western blot was slightly more basic than the immunoprecipitated phosphoprotein but more acidic than the three spots that were not immunoprecipitated. It is possible that some of the substrates identified in permeabilized cells will not be phosphorylated in an EGF-dependent manner in intact cells. The tyrosine kinase encoded by Rous sarcoma virus phosphorylates certain proteins in vitro that are not phosphorylated in Rous sarcoma virus-infected cells (54, 55). This could be a result of lost specificity due to conditions of the assay or loss of cellular compartmentalization. In the phosphorylation method described herein for digitation-permeabilized cells, the ionic conditions of the assay do not mimic intracellular conditions so this could lead to a loss of specificity. With regard to compartmentalization in digitonin-permeabilized cells, it has been shown by others under similar conditions that endocytic vesicles remain intact (50) and suggests that many intracellular membranes may retain their integrity. Also the plasma membrane seems to be partially intact because about 75% of the soluble intracellular marker enzymes were retained (Fig. 1).

In isolated A-431 membranes it has been shown that EGF must be bound to the EGF receptor/kinase for kinase activity to be stimulated (56). If this is true in intact cells, one would expect to see an increase in phosphorylation of the EGF receptor/kinase substrates that directly parallels receptor occupancy. However, the dose response data in permeabilized cells showed that the phosphorylation of the 35-kDa substrate was half-maximally stimulated by EGF when binding was only about 5% of saturation (Fig. 4). Although there are many possible interpretations of this result, one interesting speculation is that the EGF receptor/kinase is not directly responsible for phosphorylation of the 35-kDa substrates. Instead, the signal generated by EGF binding may be amplified via the activated EGF receptor/kinase phosphorylating another kinase that in turn phosphorylates the 35-kDa substrate. A cascade mechanism such as this also is consistent with the data of Novak-Hofer and Thomas (57, 58) which showed the presence of a kinase in soluble extracts from EGF-treated cells which had a stimulated capacity for phosphorylating serine residues of the ribosomal protein S6. The S6 kinase may have been activated by phosphorylation of one of its tyrosyl residues by a tyrosine-specific protein kinase because the stimulated level of S6 kinase activity could be demonstrated only when the extracts were prepared in the presence of inhibitors of phosphotyrosyl protein phosphatases (55).

It is of interest to compare the phosphorylation of the 35-kDa protein by labeling in digitonin-permeabilized cells with [32P]ATP by labeling with [32P]orthophosphate in intact cells. Since a specific antiserum was available, it was possible to detect up to a 10-fold increase in the phosphorylation of this protein in intact cells following immunoprecipitation (Figs. 7 and 8) even though no effect of EGF could be observed when entire cell extracts were fractionated on SDS gels. In contrast, EGF stimulation of phosphorylation of this protein could easily be observed when the entire extract of cells labeled by the digitonin-permeabilization method were fractionated on SDS gels. Thus, although it is certainly essential to verify the physiological significance of the event, the digitonin-permeabilization method may provide a very sensitive method to identify novel substrates for growth factor-stimulated kinases such as the unidentified ones reported herein.

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