We have isolated a soluble 35-kDa protein from A-431 cells that in the presence of calcium can serve as a substrate for the epidermal growth factor (EGF)-receptor/kinase. The purification procedure exploits the reversible, Ca"+-dependent binding of the 35-kDa protein to the A-431 total particulate fraction. The 35-kDa protein was purified by 1) Ca"+-dependent adsorption to A-431 particulate fractions, 2) release by chelation of Ca"+ with ethyleneglycolbis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, and 3) chromatography on Sephadex G-100, DEAE-cellulose, and CM-cellulose columns.

When a plasma membrane preparation from A-431 cells is used as a source of the EGF-receptor/kinase, the phosphorylation of the 35-kDa protein occurs on tyrosine, is greatly enhanced in the presence of EGF, and occurs only when Ca"+ is added to the standard reaction mixture for phosphorylation. Autophosphorylation of the receptor does not require Ca"+. We have postulated that one of the roles of Ca"+ is to facilitate the interaction of the 35-kDa protein with cellular membranes. Ca"+ enhances, but apparently is not essential for, the direct phosphorylation of the 35-kDa protein by the Triton X-100-solubilized, purified EGF-receptor/kinase.

Incubation of intact A-431 cells with EGF at 37 °C (but not 0 °C) enhances the ability of the particulate fraction prepared from these cells to bind and/or phosphorylate the 35-kDa protein. We suggest that this enhancement in the phosphorylation of the 35-kDa protein, a presumed physiological substrate, is associated with the clustering and internalization of the EGF receptor/kinase complex.

One of the initial biochemical events that occurs in target cells subsequent to the binding of EGF to its plasma membrane receptor is the activation of a tyrosine-specific protein kinase (1, 2). We have presented evidence that the tyrosine kinase is an integral part of the EGF receptor (3) and that the EGF-receptor/kinase itself can serve as a substrate for this tyrosine-specific protein kinase (autophosphorylation) (1, 2, 4). Thus, it appears that the native 170-kDa EGF-receptor/kinase consists minimally of 1) an EGF-binding domain, 2) a tyrosine-specific protein kinase domain, and 3) a phosphate acceptor site(s) for the autophosphorylation. The EGF-receptor/kinase also appears to catalyze the phosphorylation of a limited number of exogenous substrates, such as tubulin, histones, the heavy chain of anti-pp60"c", synthetic peptides related to pp60"c", and some naturally occurring peptides and proteins (2, 5-12).

Tyrosine-specific kinase activity, first detected in the src gene product (pp60"c") and then in the receptor for EGF, has recently been shown also to be associated with membrane receptors for both insulin and platelet-derived growth factor (13-16). Although there appears to be a relationship between tyrosine kinase activity and the regulation of cell proliferation, the specific metabolic consequences of the activation of tyrosine kinases have not yet been established.

It is clear that the isolation and identification of cellular substrates for tyrosine-specific protein kinase activities will aid in understanding the functions of this class of enzymes. To this end, we examined the possibility of using the EGF-receptor/kinase, present in crude particulate fractions prepared from A-431 carcinoma cells, as an affinity matrix to adsorb intracellular substrates for the EGF-receptor/kinase. In addition, we examined whether the particulate matrices prepared from control and EGF-treated cells differed with respect to their abilities to phosphorylate adsorbed proteins.

This approach has led to the isolation of soluble 35-kDa protein from A-431 cells that can serve as a substrate for the EGF-receptor/kinase in plasma membrane preparations, but only in the presence of Ca"+. The initial step in the purification of this substrate takes advantage of the reversible, Ca"+-dependent adsorption of the 35-kDa protein to the A-431 total particulate fraction.

EXPERIMENTAL PROCEDURES

Materials—Mouse EGF was isolated by published procedures (17). [γ-32P]ATP was purchased from ICN. DE52 and CM52 cellulose were purchased from Whatman. Protein molecular weight standards for SDS-gel electrophoresis were purchased from Bio-Rad and for gel chromatography, from Pharmacia. The 34-kDa substrate for pp60"c" isolated from A-431 cells and cross-reacting antiserum to this protein were kindly provided by Dr. R. Erikson, University of Colorado.

Growth of A-431 Cells and Preparation of Shed Membrane Vesicles—The A-431 cells were grown in 100-mm dishes or roller bottles (Falcon) containing Dulbecco's modified Eagle's medium (Gibco) supplemented with 20 mM Hepes, 5% calf serum (Flow Laboratories), and gentamycin (Schering Corp.). The shed membrane vesicle preparation was obtained as described (2).

Experimental Protocol and Preparation of Total Particulate Fraction—Immediately prior to an experiment, the medium in each 100-mm dish was aspirated and the monolayers were washed five times.
with 4.0 ml of Hank's balanced salt solution at room temperature. The cells were then incubated with 4 ml of Dulbecco's MEM in the absence or presence of EGF (200 ng/ml) at 37 °C for the indicated times. Incubations of 10 min or less were performed by floating the 100-mm culture dishes on the surface of a water bath maintained at 37°C.

After incubation, the medium was aspirated and the cell monolayers were washed four times with 4.0 ml of Dulbecco's phosphate-buffered saline (Ca²⁺ and Mg²⁺ free). The cell monolayer was drained for 5 s and 750 μl of the homogenization buffer was added. The standard homogenization buffer, designated Buffer H, consisted of Hepes (20 mM, pH 7.4), KCl (150 mM), MgCl₂ (2 mM), and iodoacetic acid (4 mM). The monolayer was scraped with a plastic spatula and the suspension of partially disrupted cells was transferred to a ground glass homogenizer. Each dish was rinsed with an additional 750 μl of Buffer H which was combined with the initial cell suspension. The cell suspension was immediately homogenized with 30 strokes of a motorized Teflon pestle tissue homogenizer. (The harvest buffer contained KCl (150 mM), MgCl₂ (2 mM), and iodoacetic acid (4 mM).) The homogenate was centrifuged at 125,000 × g, 10 min and the supernatant was resuspended into 150 μl of a buffer composed of Hepes (20 mM, pH 7.4), KCl (150 mM), and MgCl₂ (2 mM) by rapid vortexing and passage several times through a fine pipette tip.

**Standard Phosphorylation Assay**—The reaction mixtures contained: aliquots of the total particulate fraction or A-431 membrane vesicle preparation (5-40 μg of protein), Hepes (20 mM, pH 7.4), KCl (150 mM), MgCl₂ (50 mM), CaCl₂ (1 mM) unless otherwise stated, Na vasopressin (10 μM), and [γ-32P]ATP (15 μM, 2-3 × 10⁶ cpm in a final volume of 60 μl). The reaction tubes containing all components except the ATP were placed on ice and preincubated for 10 min at 0°C, in the absence and the presence of EGF (100 ng). The phosphorylation reaction was initiated by the addition of [γ-32P]ATP and incubation continued for 10 min. The reaction was terminated by the addition of 30 μl of SDS sample buffer (18) and heating for 2 min at 100°C.

**SDS-Gel Electrophoresis and Autoradiography**—The procedures for SDS-gel electrophoresis and autoradiography were carried out as previously described (19). SDS-gels were composed of 9% acrylamide unless otherwise noted.

Hydrolysis of 32P-labeled 35-kDa Protein and Phosphoamino Acid Determination—The band corresponding to the 32P-labeled protein was identified by the presence of EGF and the phosphoamino acid destination. The gel slice was hydrolyzed in 1.0 ml of 6 N HCl for 1.5 h at 100°C, exactly as described (11). The hydrolysate was performed using a pyridine-acetic acid buffer (pH 3.5) by a previously published method (11).

Isolation of 35-kDa Protein from A-431 Cells—Roller bottles (Falcon No. 3027) were harvested at a concentration of 3-4 × 10⁶ cells/10 cm. The cells were washed three times with phosphate-buffered saline (Ca²⁺ and Mg²⁺ free). The cells were resuspended in 40 ml of 0.25 M sucrose containing 10 mM EGTA and 1 mM iodoacetic acid. The suspension was then homogenized in a Polytron homogenizer. The homogenate was centrifuged (125,000 × g, 10 min) and the supernatant was discarded. The pellets were washed three times with 80 ml of wash buffer, centrifuging after each wash. (The wash buffer contained: KCl (150 mM), MgCl₂ (2 mM), Hapes (20 mM, pH 7.4), leupeptin (1 μg/ml), aprotinin (1 μg/ml), and CaCl₂ (1 mM)).

The final pellet was resuspended in 2 ml of elution buffer. (The elution buffer contained: KCl (150 mM), MgCl₂ (2 mM), Hapes (20 mM, pH 7.5), and EGTA (1 mM).) The suspension was allowed to stand on ice for 5 min and centrifuged (125,000 × g, 10 min). The EGTA elution was repeated and the supernatants (4 ml total) were pooled and stored frozen.

The EGTA eluate (32 ml from eight roller bottles) was concentrated to 2 ml by pressure filtration through an Amicon YM-5 membrane. The concentrate was chromatographed on a column (1.5 × 77 cm) of Sephadex G-100 equilibrated with ammonium acetate (0.06 M, pH 8.3) at a flow rate of 0.2 ml/min; 2-ml fractions were collected. Fractions 23-26 (see "Results"), that contained the 35-kDa protein were pooled, diluted with 2 volumes of water (to adjust the buffer to 0.02 M ammonium acetate), and concentrated to 2 ml by pressure filtration. The pH of the concentrate was adjusted to 8.6 with dilute NH₄OH and passed through a column (0.7 × 2 cm) of DE-52 cellulose, equilibrated with ammonium acetate buffer (0.02 M, pH 8.6), at a flow rate of 0.1 ml/min. Fractions (0.8 ml) were collected and those containing the 35-kDa protein (see "Results") were pooled and stored frozen. Protein concentrations were estimated by the Bradford procedure (20) with γ-globulin as a standard.

**RESULTS**

Detection of the 35-kDa Substrate for the EGFR-Receptor/Kinase—In initial experiments, we compared particulate fractions prepared from control and EGF-treated cells with regard to their abilities to phosphorylate endogenous proteins. A-431 cells were incubated for 30 min at 37°C, in the absence and the presence of EGF, in Dulbecco's MEM. The cells were washed, homogenized, and centrifuged to obtain a high speed particulate fraction. Each of the two protocols, A and B, described under "Experimental Procedures," was used. The particulate fractions were subjected to the standard phosphorylation procedure, with and without added EGF. When protocol A was used, the standard phosphorylation assay was carried out in the absence of Ca²⁺. The endogenous phosphorylated proteins were examined by SDS-gel electrophoresis and autoradiography.

The results of this experiment are shown in Fig. 1. In the absence of Ca²⁺ (lanes E-H), the major phosphorylated protein detected had an Mr = 170,000. This protein had previously been identified as the EGF-receptor/kinase in membrane preparations from A-431 cells (2). Incubation of cells with EGF did not result in the phosphorylation of additional proteins. As expected, the addition of EGF to the assay mixture containing pellets from EGF-treated cells had little effect on its phosphorylation. The enhanced autophosphorylation observed in the presence of Ca²⁺, an additional phosphorylated protein, with Mr = 35,000, was detected. The 35-kDa protein was phosphorylated to a much greater extent in the particulate fraction prepared from cells that had been incubated with EGF.
incubation with EGF for 10 min resulted in only a slight increase; the maximal increase was observed after 30–60 min of incubation with EGF and this increase persisted for at least 120 min (data not shown).

In order to separate the variables of 1) the time required for EGF binding to cell surface receptors and 2) the time required for other possible cellular processes to occur that could lead to the observed increased extent of phosphorylation of the 35-kDa protein, a different protocol was employed to determine the time course. Cultures of A-431 cells were washed, chilled to 0 °C, and incubated for 60 min at 0 °C in Dulbecco’s MEM, in the absence and the presence of EGF (200 ng/ml), to allow binding of EGF to its receptors. The medium was aspirated and unbound EGF was removed from the cells by washing at 0 °C. The cultures were then warmed by the addition of fresh Dulbecco’s MEM at 37 °C, and incubation at 37 °C was continued for various lengths of time. At the times indicated (Fig. 2), the cells were washed again, and total particulate fractions were prepared by protocol B exactly as described under “Experimental Procedures.” Aliquots of the particulate fraction were subjected to the standard phosphorylation procedure, in the presence of Ca++,

and the phosphorylated products were examined by SDS-gel electrophoresis and autoradiography. The results of this time course experiment are illustrated in Fig. 2. After 2 min of incubation at 37 °C (lanes A–D), the particulate fractions from both control cells and cells that had been preincubated with EGF were identical, with respect to the extent of phosphorylation of the 35-kDa protein. However, after 30 min of incubation at 37 °C, and persisting for at least 60 min, an enhanced extent of phosphorylation of the 35-kDa protein was detected in cells that had bound EGF (lanes E–L). We concluded that the extent of phosphorylation of the 35-kDa protein was not due simply to occupation of the EGF receptor by EGF (lanes E–L).

than in the identical fraction prepared from control cells (lanes C and D). The pattern of autophosphorylation of the EGF-receptor/kinase (Mr = 170,000), was not affected by the presence of Ca++. Some enhancement of the phosphorylation of the 35-kDa protein was observed when EGF was added directly to the phosphorylation assay of the control cell particulate fraction (lanes C and D). However, it is apparent from a comparison of lanes A and C that the addition of EGF to the phosphorylation assay is not as effective as prior exposure of intact cells to EGF, with respect to enhancement of the phosphorylation of the 35-kDa protein.

Further, when the particulate fraction from cells that had been incubated with EGF was prepared in the absence of Ca++ (as described in Fig. 1, lanes E–H), where no phosphorylation of the 35-kDa protein was detectable, the addition of Ca++ to the phosphorylation assay mixtures did not permit the detection of the phosphorylation of the 35-kDa protein (data not shown). Thus, the phosphorylation of this 35-kDa protein was detectable only when Ca++ was added to the homogenate prior to centrifugation.

**Time Course of EGF-induced Changes in the Phosphorylation of Endogenous Proteins of the Total Particulate Fraction**—An experiment was designed to define the time course for the increase in the extent of phosphorylation of endogenous 35-kDa protein due to the incubation of intact cells with EGF. In preliminary experiments, EGF was added to replicate cultures and cells were incubated at 37 °C for various periods of time ranging from 5 min to 2 h. Total particulate fractions were prepared by protocol B (Ca++ present); each was phosphorylated in the presence of Ca++ (1 mM) and analyzed as in Fig. 1. Incubation of the cells with EGF for 5 min did not affect the extent of phosphorylation of the 35-kDa protein;
A-D) and that incubation of intact cells at 37 °C for periods longer than 2 min, subsequent to the binding of EGF to its cell surface receptor, is required.

The Role of Ca\(^{2+}\) in the Detection of Phosphorylated 35-kDa Protein—The phosphorylation of the 35-kDa protein (described in Figs. 1 and 2) was detected only when Ca\(^{2+}\) was added to homogenates prior to centrifugation. We therefore considered the possibility that one of the roles of Ca\(^{2+}\) might be to facilitate binding of the 35-kDa protein to a component of the particulate fraction of the homogenate, thus permitting the 35-kDa protein to be pelleted by centrifugation. We addressed this question in the following experiments.

A series of replicates homogenates from cells incubated with EGF for 1 h at 37 °C were prepared in buffer H, Ca\(^{2+}\) was added to the series (protocol B), and, after incubations of 5, 30, and 60 min at 0 °C, a 2-fold molar excess of EGTA was added to chelate Ca\(^{2+}\). As positive controls, EGTA was not added to an identical series of replicate homogenates. The homogenates were then centrifuged and the pellets were subjected to the standard phosphorylation procedure in the presence of Ca\(^{2+}\) (1 mM). The addition of EGTA at any time prior to the centrifugation resulted in a preparation with no detectable phosphorylated 35-kDa protein even though excess Ca\(^{2+}\) was present in the phosphorylation assay (data not shown). Thus, the effect of Ca\(^{2+}\) in the homogenate was fully reversible by chelation prior to centrifugation; this result is consistent with the postulated role of Ca\(^{2+}\) in facilitating binding of the 35-kDa protein to the particulate fraction.

A second experiment described below demonstrates that the presence of the 35-kDa protein in the phosphorylation reaction mixture is not sufficient to detect its phosphorylation; Ca\(^{2+}\) must also be present. Total particulate fractions were prepared from EGF-treated cells by protocol B, exactly as described in Fig. 1, lanes A and B. Replicate aliquots of the resuspended preparation were subjected to the standard phosphorylation under two assay conditions: 1) Ca\(^{2+}\) present at 1 mM (standard), and 2) EGTA added to 2 mM final concentration. The results are shown in the autoradiograph in Fig. 3. In the presence of Ca\(^{2+}\), as expected, the 35-kDa protein was phosphorylated (Fig. 3, lane A). No phosphorylation of the 35-kDa protein was detected in the presence of EGTA (Fig. 3, lane B). The 170-kDa EGF-receptor/kinase was autophosphorylated equally under either condition. We concluded from this observation that Ca\(^{2+}\) is required in the reaction mixture for phosphorylation of the 35-kDa protein to occur.

We also were interested in whether an intact membrane was required for detection of the 35-kDa phosphorylation. It can be seen in Fig. 3, lane C, that addition of 0.5% Triton X-100 to the standard phosphorylation assay greatly reduced the \(^{32}\)P-labeling of the 35-kDa protein although phosphorylation of the 170-kDa receptor was not affected. The identical result was obtained with Nonidet P40 (data not shown). The residual phosphorylation of the 35-kDa protein probably is not simply a consequence of residual kinase activity of incompletely solubilized membrane, since it is possible to demonstrate phosphorylation of the 35-kDa protein by the EGF receptor/kinase purified from solubilized membranes (see later section).

Effects of EGF on Particulate and Soluble Fractions—Making use of a reconstitution protocol, we addressed the question of whether exposure of intact cells to EGF during a 37 °C incubation altered the abilities of the particulate fraction or of the 35-kDa protein to interact in the presence of Ca\(^{2+}\). Cells were incubated at 37 °C for 30 min in the absence and the presence of EGF. Replicate homogenates (1.5 ml buffer/100-mm dish) were prepared in buffer H (Ca\(^{2+}\) free). The homog-
constituted particulate fractions. Washed pellets, were subjected to the standard phosphorylation assay to each reconstituted "homogenate," to final concentration of 1 mM, from individual 100-mm dishes and the homogenates were centrifuged to give particulate fractions (pellets) and high speed supernatants. The supernatants were pooled. Aliquots of each reconstituted "homogenate," to final concentration of 1 mM, and the mixtures were allowed to stand for 15 min at 0°C. The reaction mixtures were analyzed by SDS-gel electrophoresis and autoradiography as described in the text. Lanes A, B, G, and H, original washed pellets from control (A and B) and EGF treated (G and H) cells; C-F and I-L, reconstituted pellets. The sources of the original washed pellets and the supernatants used for each reconstitution are indicated at the top.

The reaction mixtures were analyzed by SDS-gel electrophoresis and autoradiography as described in the text. Lanes A, B, G, and H, original washed pellets from control (A and B) and EGF treated (G and H) cells; C-F and I-L, reconstituted pellets. The sources of the original washed pellets and the supernatants used for each reconstitution are indicated at the top.

We concluded that treatment of intact cells with EGF had altered the particulate fraction, such that increased phosphorylation of the 35-kDa protein was detected. From the data presented, it is not possible to clearly differentiate between an enhancement of the capacity of the particulate fraction to bind the 35-kDa protein or to phosphorylate bound 35-kDa protein. However, since the particulate fraction from cells not incubated with EGF contains bound 35-kDa protein when Ca²⁺ is present (see next section), we favor the latter possibility.

**Isolation of 35-kDa Substrate**—As a first step toward identifying the 35-kDa substrate, we have purified this protein. The presence of the 35-kDa protein throughout the fractionation procedure was monitored by SDS-gel electrophoresis and Coomassie blue staining as well as by its ability to be phosphorylated in the presence of Ca²⁺, using the A-431 plasma membrane vesicle preparation as the source of the EGF-receptor/kinase. In the first step of the purification, advantage was taken of the Ca²⁺-dependent binding of the 35-kDa protein to the particulate fraction of A-431 cells and its elution in the presence of EGTA, as detailed under "Experimental Procedures." In Fig. 5 are illustrated the patterns of the proteins eluted from the washed particulate fraction by a sequential extraction first with a buffer containing 1 mM Ca²⁺ (Fig. 5, lane A) and then the same buffer containing 2 mM EGTA in place of the Ca²⁺ (Fig. 5, lane B). In the presence of EGTA, a number of proteins are eluted, the major one having an M₈ of 35,000.

Aliquots of the EGTA eluate were phosphorylated in the presence and absence of EGTA and the products analyzed (Fig. 5, lanes E and F). The EGF-dependent phosphorylation of this 35-kDa protein is readily detectable.

The EGTA eluate was concentrated and subjected to gel filtration through a calibrated column of Sephadex G-100 (see "Experimental Procedures"). The fractions isolated in this manner (Fig. 6) were subjected to phosphorylation, SDS-gel electrophoresis, and autoradiography. The phosphorylated component detected was the 35-kDa protein visible in fractions 23-26 of Fig. 6 (data not shown). These fractions were pooled.

From a comparison of the elution volume of the 35-kDa component with that of protein standards (see arrows, Fig. 6), the molecular weight of the native substrate was calculated to be between 35,000 and 37,000, in agreement with the SDS-electrophoresis data. We conclude that under these conditions (0.06 M ammonium acetate, pH 8.3) the 35-kDa protein exists as a monomer.

Aliquots of the pooled Sephadex fraction (fractions 23-26) were examined directly (Fig. 7, lane A) and after phosphorylation (Fig. 7, lanes C and D) by SDS-gel electrophoresis, Coomassie blue staining, and autoradiography. The purification and phosphorylation of the 35-kDa protein may be noted (compare with Fig. 5, lanes B, E, and F).

The pooled Sephadex fraction was passed through DE52 cellulose at pH 8.6 as described under "Experimental Procedures." Under these conditions, the 35-kDa protein was not
adsorbed. The purification and phosphorylation of the 35-kDa protein in the DE52 flow-through fraction are shown in Fig. 7, lanes B, E, and F.

The DE52 flow-through fraction was then subjected to ion-exchange chromatography using CM52 cellulose with a linear ammonium acetate gradient as described under "Experimental Procedures." Aliquots (10 μl) of the fractions isolated in this manner were again directly examined by SDS-gel electrophoresis (10% acrylamide gels) and Coomassie blue staining (Fig. 8A) or examined after phosphorylation by SDS-gel electrophoresis and autoradiography (Fig. 8B).

It may be seen in Fig. 8B that the major extent of phosphorylation occurred on the 35-kDa protein isolated in fractions 6–9. A lesser extent of phosphorylation was detected in a protein of slightly lower Mr in fractions 13–17. Fractions 6–9 were pooled and the material was considered "pure" since no other protein bands were detected after Coomassie blue staining (Fig. 8A, fractions 6–9). Occasionally, the SDS-gel system resolved the 35-kDa band into a very close doublet band containing a major and a minor component. This may be a reflection of the state of phosphorylation of the 35-kDa protein (see "Discussion"). In typical preparations, 300 to 600 μg of the pure 35-kDa protein were recovered from 1.4 g of cell protein.

Phosphoamino Acid Analysis of 32P-labeled 35-kDa Protein—The 32P-labeled phosphoamino acid composition of the 35-kDa protein was determined (Fig. 9). 32P-labeled phosphotyrosine was the only labeled amino acid detected.

Kinetics of 35-kDa Substrate Phosphorylation by A-431 Plasma Membrane Vesicle Preparation—Some of the kinetic parameters of the phosphorylation of the 35-kDa protein by the A-431 vesicle preparation were examined. Under the standard phosphorylation conditions (see "Experimental Procedures") with 5 μg of plasma membrane protein, 100 ng of EGF and either 3 or 0.5 μg of the 35-kDa protein in a total volume of 40 μl, the reaction was linear for at least 20 min (data not shown). Under these conditions, the half-maximal rate of phosphorylation of the 35-kDa protein occurred at a substrate concentration of approximately 0.8 μM (Fig. 10). It is of interest that the half-maximal rates of phosphorylation of the synthetic peptides related to pp60src occur in the millimolar range (7, 8).

In a separate experiment, the maximal extent of phosphorylation of the 35-kDa protein was estimated by using increasing quantities of the vesicle preparation (5–100 μg of protein) as a source of EGF-receptor/kinase and 2.3 μM substrate (35-kDa protein) under conditions identical with those shown in Fig. 10. We calculated that a maximum of 0.15 pmol of phosphate/μmol of 35-kDa protein could be incorporated. Since the extent and nature of any pre-existing phosphate on the 35-kDa protein as isolated is not known, and protein phosphatase activity is known to be present in the membrane vesicle preparation used, it is not possible to clearly interpret the significance of the observed amount of phosphate incorporated.

Ca2+ Requirement for Phosphorylation of the 35-kDa Protein by the Plasma Membrane Vesicle Preparation and by Purified EGF-Receptor/Kinase—The phosphorylation of the 35-kDa protein by either the total particulate fraction of A-431 cells, or the membrane vesicle preparation, required the presence of Ca2+ in the reaction mixture. The concentration of Ca2+ required to detect the phosphorylation of the 35-kDa protein by the vesicle preparation was determined, in the presence and the absence of EGF. It may be seen in Fig. 11 that: 1) in...
Chromatography was adjusted to pH 6.0 and applied to a CM52 cellulose column. Elution was carried out with a linear ammonium acetate gradient as described under "Experimental Procedures." Aliquots of the fractions (indicated at the bottom) were analyzed, either directly or after phosphorylation, by SDS-gel electrophoresis, Coomassie blue staining, and autoradiography exactly as described in Fig. 5. EGF (100 ng) was included in all phosphorylations.

Although it is clear from Fig. 11 that EGF enhances the observed phosphorylations, the possibility exists that the EGF-receptor/kinase in the membrane vesicle preparation phosphorylates and activates a second membrane associated protein kinase which requires Ca\(^{2+}\) for the phosphorylation of the 35-kDa protein. This possibility is rendered unlikely, but not disproved, by the observation that the soluble, purified EGF-receptor/kinase is able to directly phosphorylate the purified 35-kDa protein (Fig. 12). Under the assay condition employed, phosphorylation of the 35-kDa protein was detected even in the absence of Ca\(^{2+}\) (lane A); however, the addition of Ca\(^{2+}\) clearly enhanced its phosphorylation with little effect on the 170-kDa receptor/kinase. It should be noted that the reaction mixture employed in Fig. 12 contained Mn\(^{2+}\) (1 mM) in place of Mg\(^{2+}\) (20 mM). Mn\(^{2+}\) was more effective than Mg\(^{2+}\) in catalyzing the phosphorylation of the 35-kDa protein (data not shown); the addition of Ca\(^{2+}\) increased the extent of phosphorylation of the 35-kDa protein in both reaction mixtures.

### Relationship of the 35-kDa Protein to the 34-kDa Erikson

Since some phosphorylation of the 35-kDa protein by the purified EGF-receptor/kinase was detected in the absence of Ca\(^{2+}\) when Mn\(^{2+}\) was used as the sole divalent cation present during the phosphorylation, a similar experiment was carried out using the membrane vesicle preparation as a source of the kinase. With Mn\(^{2+}\) as the sole divalent cation, concentrations between 10 and 1000 \(\mu\)M resulted in an EGF-stimulated phosphorylation of both the EGF-receptor/kinase and the 35-kDa protein.

Although it is clear from Fig. 11 that EGF enhances the observed phosphorylations, the possibility exists that the EGF-receptor/kinase in the membrane vesicle preparation phosphorylates and activates a second membrane associated protein kinase which requires Ca\(^{2+}\) for the phosphorylation of the 35-kDa protein. This possibility is rendered unlikely, but not disproved, by the observation that the soluble, purified EGF-receptor/kinase is able to directly phosphorylate the purified 35-kDa protein (Fig. 12). Under the assay condition employed, phosphorylation of the 35-kDa protein was detected even in the absence of Ca\(^{2+}\) (lane A); however, the addition of Ca\(^{2+}\) clearly enhanced its phosphorylation with little effect on the 170-kDa receptor/kinase. It should be noted that the reaction mixture employed in Fig. 12 contained Mn\(^{2+}\) (1 mM) in place of Mg\(^{2+}\) (20 mM). Mn\(^{2+}\) was more effective than Mg\(^{2+}\) in catalyzing the phosphorylation of the 35-kDa protein (data not shown); the addition of Ca\(^{2+}\) increased the extent of phosphorylation of the 35-kDa protein in both reaction mixtures.

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Calcium-dependent 35-kDa Substrate for EGF-Receptor/Kinase

32P
- + + + + EGF
-170 K
-35 K

A B C D E F G H I J

Fig. 11. Electrophoresis and autoradiography of purified 35-kDa protein, phosphorylated in the presence of various Ca\(^{2+}\) concentrations. Purified 35-kDa protein was phosphorylated, using A-431 plasma membrane vesicles as a source of the EGF receptor/kinase, by the standard procedure described under "Experimental Procedures" except that the concentration of Ca\(^{2+}\) present during the phosphorylation was varied from 0-1 mM. The reaction mixtures were subjected to SDS-gel electrophoresis, Coomassie blue staining, and \(^{32}\)P autoradiography as described in the text. EGF (100 ng) was included during the phosphorylation reaction as indicated at the top. Lanes A and B, Ca\(^{2+}\) free; C and D, 25 \(\mu\)M Ca\(^{2+}\); E and F, 100 \(\mu\)M Ca\(^{2+}\); G and H, 300 \(\mu\)M Ca\(^{2+}\); I and J, 1 mM Ca\(^{2+}\).

32P-Autoradiography

-170 K
-35 K

A B

Fig. 12. Phosphorylation of the 35-kDa protein by the solubilized, purified EGF-receptor/kinase. The reaction mixtures contained: affinity purified EGF-receptor/kinase (2 \(\mu\)g) (2); 35-kDa protein (3 \(\mu\)g); Hepes (10 mM, pH 7.4); MnCl\(_2\) (1 mM); CaCl\(_2\) (when added, 500 \(\mu\)M); Triton X-100 (0.25%); \([\gamma-^{32}\text{P}]\)ATP (15 \(\mu\)M, 2-3 \(\mu\)Ci); and EGF (100 ng); in a final volume of 60 \(\mu\)l. The reaction tubes were preincubated for 10 min at room temperature prior to the addition of \(^{32}\)P-labeled ATP. Incubation at room temperature was continued for 10 min and the reaction mixtures were analyzed by SDS-gel electrophoresis, Coomassie blue staining, and autoradiography as described under "Experimental Procedures." Lane A, Ca\(^{2+}\) free; lane B, Ca\(^{2+}\) (500 \(\mu\)M).

Substrate (24)—A concern throughout this study has been whether we had isolated, by a different approach, a previously described substrate for tyrosine phosphorylation. A protein (or proteins) designated in various laboratories as 34 (24-27), 36 (28-31), or 39 kDa (32, 33) has been detected in \(^{32}\)P-labeled cells after infection with Rous sarcoma virus and has been partially purified. It has also been reported that incubation of intact A-431 cells or cell homogenates with EGF enhances the phosphorylation of the 34-39-kDa protein (31, 34-36). It is not yet completely clear whether the proteins described by each of these laboratories are identical. However, the 34-kDa substrate described by both Greenberg and Edelman (26, 27) and Ghosh-Dastidar and Fox (36) appears to be closely related to the 34-kDa protein described by Erikson and Erikson (24) on the basis of immunoreactivity with an anti-34-kDa antiserum provided by Dr. R. Erikson. In addition, the 36-kDa protein described by Courtneidge et al. (30) was isolated by the Erikson procedure.

The data we have thus far obtained indicate that the 35-kDa protein, whose EGF-enhanced phosphorylation we report in this paper, differs in significant ways from the 34-kDa protein described by Erikson and Erikson (24). We have been unable to precipitate the phosphorylated 35-kDa protein with an anti-34-kDa antiserum provided by Dr. Erikson (data not shown). This antiserum has been shown to cross-react with the 34-kDa protein isolated from A-431 cells by Erikson et al. (34). Furthermore, we have been unable to phosphorylate authentic 34-kDa protein from A-431 cells, under conditions where the phosphorylation of the 35-kDa protein, isolated by our procedure was readily discernible. The purified 34-kDa protein from A-401 cells was kindly provided by Dr. Erikson. These results are shown in Fig. 13, in which phosphorylation of the Erikson 34-kDa protein (lane b) is compared with the phosphorylation of increasing concentrations of the 35-kDa protein (lanes c-e). The Coomassie blue-staining patterns of the reaction mixtures are shown in the corresponding lanes A-E, as an indication of the relative amounts of the proteins used.

DISCUSSION

We have been able to isolate a substrate for the plasma membrane EGF receptor/kinase by virtue of its Ca\(^{2+}\)-dependent adsorption to the particulate fraction of A-431 cells. It is
of interest to note that a number of other proteins have been described that also exhibit the property of Ca²⁺-dependent binding to particulate subcellular fractions. These include synexin (37) and "chromobindins" (38), as well as protein kinase C (39). Although we have not identified the specific site(s) of adsorption, we suggest that binding of the 35-kDa protein occurs to a component of the plasma membrane, because we can detect Ca²⁺-dependent binding to and phosphorylation of this protein by A-431 cell plasma membrane vesicle preparations. Since rapid phosphorylation of the 35-kDa protein clearly requires the presence of EGF, it appears that at least one site of adsorption is in close proximity to the EGF-receptor/kinase. The 35-kDa protein thus appears to be a soluble, cytoplasmic protein that displays an affinity for membranous structure only in the presence of Ca²⁺; Mg²⁺ will not substitute for Ca²⁺. It is of interest that one of the early effects of EGF on A-431 cells is a stimulation of Ca²⁺ influx (40).

The native 35-kDa protein appears to exist as a monomer in Ca²⁺-free buffers, based upon its behavior during molecular sieve chromatography. When subjected to two-dimensional gel electrophoresis, the 35-kDa protein, as isolated, is found to exist in two forms, a major species with a PI 3.5 and a minor species with a pI 6.45 (data not shown). This difference may be a reflection of the state of phosphorylation of the 35-kDa protein. It is interesting to note that this protein also serves as a good substrate for the protein kinase pp60⁵⁺ of the Rous sarcoma virus.⁴

Enhancement of the binding of the 35-kDa protein to membranes may not be the sole explanation for the Ca²⁺ requirement during the phosphorylation reaction. The observation that Ca²⁺, although not absolutely required, enhances the phosphorylation of the 35-kDa protein by the purified EGF-receptor/kinase suggests that Ca²⁺ may bind directly to the 35-kDa protein and alter its conformation in a manner that increases its susceptibility to phosphorylation. The observation that, upon heat denaturation (2 min at 100 °C), the 35-kDa protein can no longer be phosphorylated by the vesicle preparation (data not shown) supports the conjecture that a specific tertiary structure of the 35-kDa protein is required.

A major question, to which we have no unequivocal answer, is whether or not the 35-kDa protein is a physiological substrate for the EGF-receptor/kinase. We have shown that incubation of intact cells with EGF at 0 °C, to permit binding, is not sufficient to induce the enhancement of the phosphorylation of the 35-kDa protein; a subsequent incubation of the cells at 37 °C is required. The time and temperature dependence of the enhancement of the phosphorylation of the 35-kDa protein may be related to the time and temperature dependence of EGF-induced clustering and internalization of the EGF-receptor/kinase (41). In preliminary experiments, we have detected an increased capacity of a presumptive endosome fraction isolated from A-431 cells after incubation with EGF, to phosphorylate the 35-kDa protein.

It is of interest that a 35-kDa protein was found to be present in an EGTA eluate of a normal mouse liver particulate fraction that was prepared essentially as has been described herein for A-431 cells. This protein could be phosphorylated in an EGF-dependent manner by the A-431 vesicle preparation (data not shown).

The 35-kDa protein, whose EGF-enhanced phosphorylation we report in this paper, differs in several significant ways from the 34–39-kDa protein described by others. An anti-34-kDa protein antiserum did not immunoprecipitate our purified 35-kDa protein and we have been unable to phosphorylate authentic 34-kDa protein from A-431 cells.

Several additional characteristics appear to differentiate our 35-kDa protein from the substrate(s) described by others: 1) the 35-kDa protein appears to be a soluble cytoplasmic component in the absence of Ca²⁺; 2) Ca²⁺ is required for its association with particulate fractions or membranes; and 3) Ca²⁺ is required during the membrane-catalyzed phosphorylation reaction. In contrast, the 34–39-kDa substrate described by other laboratories appears to be associated with the plasma membrane or cytoskeletal structures in the absence of Ca²⁺ (25, 27, 30, 42).

We have tentatively concluded that the 35-kDa protein that we have isolated is not identical with the previously described tyrosine kinase substrate(s). A final determination awaits identification and more rigorous characterization of these substrates.

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