Epidermal Growth Factor Dependent Phosphorylation of a 35-Kilodalton Protein in Placental Membranes†

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ABSTRACT: In human placental membranes isolated in the presence of ethylenediaminetetraacetic acid (EDTA), epidermal growth factor (EGF) stimulated the [γ-^{32}P]ATP-dependent phosphorylation of tyrosine residues on the 170-kilodalton (kDa) EGF receptor and on a 35-kDa protein. The initial rate of phosphorylation of these proteins in the presence of EGF was 5.2 and 3.5 nmol of phosphate min^{-1} (mg of receptor protein)$^{-1}$, and this was approximately 10- and 6-fold higher than the basal rate, respectively. Half-maximal phosphorylation of both proteins occurred at about 2.5 nM EGF. In the presence of *p*-nitrophenyl phosphate, EGF stimulated the phosphorylation of the 35-kDa protein but not the EGF receptor, suggesting that hormone-stimulated autophosphorylation of the receptor/kinase was not required for kinase activation. The 35-kDa protein exists in two forms: (1) 35K^{eluate}, which was associated with the membrane in the presence of Ca^{2+} but was eluted with EDTA, and (2) 35K^{memb}, which was not eluted from membranes with EDTA. Both forms were immunologically related to a 35-kDa protein previously isolated from A431 cells. Antiserum against the 35-kDa protein also reacted with a protein with an apparent size of 66 kDa that was phosphorylated in an EGF-dependent manner. In phosphorylation reactions performed in the presence of Mg^{2+}, Ca^{2+} was required for phosphorylation of the 35K^{eluate} form, but Ca^{2+} was not required for phosphorylation of the 35K^{memb} form. Phosphorylation appears to change the membrane-binding properties of the 35K^{memb} form because ^{32}P-labeled 35K^{memb} could be eluted from the membrane by EDTA. These results suggest that the nonphosphorylated form of the 35-kDa protein associates with the membrane in a Ca^{2+}-independent manner, while the phosphorylated form requires Ca^{2+} for membrane association. The phosphorylated form associated with phospholipid liposomes in a Ca^{2+}-dependent manner with half-maximum association occurring at approximately 10 μM Ca^{2+}.

Several growth factors stimulate protein tyrosine kinase activity in target cells, and kinase activity is intrinsic to certain receptors including the epidermal growth factor (EGF)[1] receptor (Hunter & Cooper, 1985). Indirect evidence implicates these activities in the regulation of cellular replication (Bishop, 1985; Hunter & Cooper, 1985). An essential step in understanding the molecular mechanism by which mitogenesis occurs is identification of the substrates for these kinases. Most searches for substrates have used transformed cells because they contain elevated protein tyrosine kinase activity. Several substrates have been identified in transformed cells, but only a few have been identified in normal cells (Hunter & Cooper, 1985). However, none of these substrates has been directly connected to the mitogenic signaling process, and there is reason to believe that many of the known substrates are not involved (Hunter & Cooper, 1985; Kamps et al., 1985).

Placenta is a highly proliferative tissue that has a relatively large number of EGF receptors (Hock & Hollenberg, 1980; O'Keefe et al., 1974; Rao et al., 1985). In addition to its presumed role in growth regulation, EGF modulates a number of differentiated cellular functions in cultures of placenta and of placental tumors [e.g., Benveniste et al. (1978) and Hout et al. (1981)]. The characteristics of the EGF receptor/kinase autophosphorylation, and of phosphorylation of exogenously added substrates, have been determined in intact (Avruch et al., 1982; Carpenter et al., 1980) and detergent-solubilized placental membranes (Avruch et al., 1982; Navarro et al., 1982; Pike et al., 1984). In this paper, the phosphorylation of substrates endogenous to placental membranes was investigated. We identified two froms of a 35-kDa protein that are phosphorylated on tyrosine in an EGF-dependent manner. Both forms were structurally related to the 35-kDa substrates previously identified in A431 cells (Fava & Cohen, 1984; Sawyer & Cohen, 1985) and fibroblasts (Giugni et al., 1985). One form of the placental substrate was similar to the A431 substrate (Fava & Cohen, 1984) in that both membrane binding and phosphorylation were Ca^{2+}-dependent. However, the other form of the placental substrate associated with membranes and was phosphorylated in a Ca^{2+}-independent manner.

Materials and Methods

Materials. EGF was isolated (Savage & Cohen, 1972) from mouse submaxillary glands and iodinated by published procedures (Wiley & Cunningham, 1982). [γ-^{32}P]ATP was synthesized from carrier-free [^{32}P]orthophosphate (ICN, Irvine, CA) by Gamma-Prep Synthesis Systems (Promega Biotec, Madison, WI). Antibody to the 35-kDa substrate of the EGF receptor/kinase in A431 cells (Fava & Cohen, 1984) was kindly provided by Dr. Stanley Cohen, Vanderbilt University. Antibody to the 34-kDa substrate for pp60^{src}

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[1] Abbreviations: 35K^{eluate}, the 35-kDa substrate eluted by EDTA from placental membranes prepared in the presence of Ca^{2+}; 35K^{memb}, the 35-kDa substrate in placental membranes isolated in the presence of EDTA; EDTA, ethylenediaminetetraacetic acid; EGF, epidermal growth factor; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; kDa, kilodalton; PNPP, *p*-nitrophenyl phosphate; SDS, sodium dodecyl sulfate.

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(Erickson et al., 1979; Radke & Martin, 1979) was kindly provided by Dr. Randy Schatzman and Dr. J. Michael Bishop, University of California, San Francisco, and Dr. Jonathan Cooper, Salk Institute. Antibody to the EGF receptor/kinase (Stoscheck & Carpenter, 1983) was a generous gift from Dr. Graham Carpenter, Vanderbilt University.

Membrane Isolation. The amnion, chorion, and large blood vessels were removed from fresh term human placenta, and the remaining tissue was rinsed in cold NaCl (0.15 M). Unless otherwise indicated, the washed tissue was homogenized in a Waring blender in 2 volumes (w/v) of Hepes (20 mM, pH 7.4) containing EDTA (0.1 mM) and sucrose (0.25 M) and centrifuged at 600*g* for 10 min. The supernatant was filtered through cheesecloth, and the membranes were pelleted by centrifugation at 120000*g* for 1 h. The pellet was resuspended in the same buffer and centrifuged as before. The pellet then was suspended (10–20 mg of protein/mL) in Hepes buffer (20 mM, pH 7.4) containing glycerol (10%), frozen in small aliquots, and stored at −70 °C.

In certain experiments, the tissue was homogenized as above but in the absence of EDTA and in the presence of iodoacetic acid (4 mM); the iodoacetic acid was added to inactivate certain Ca^{2+}-dependent proteases (Cassel & Glaser, 1982; Gates & King, 1982; Yeaton et al., 1983). After being allowed to sit on ice for 15 min, $CaCl_2$ was added to a final concentration of 1 mM, and the isolation was continued exactly as above but EDTA was replaced with $CaCl_2$ (1 mM).

Membranes were isolated from cultured A431 cells without pretreatment of the cultures with EGF by "protocol A" as previously described (Fava & Cohen, 1984) except 1 mM EDTA was added to the homogenization solution and the membranes were washed by resuspension and centrifugation.

Phosphorylation Assay. Unless otherwise indicated, the phosphorylation reaction mixtures contained placenta plasma membranes isolated in the presence of EDTA (20 μg of protein), Hepes buffer (20 mM, pH 7.4), glycerol (5% w/v), $MnCl_2$ (0.75 mM), [γ-^{32}P]ATP (10 μM, 6 Ci/mmol), zinc chloride (10 μM), sodium orthovanadate (20 μM), and EGF (120 ng) in a final volume of 40 μL. The reaction tubes were incubated on ice in the presence or absence of EGF for 10 min. The reaction was initiated by the addition of [γ-^{32}P]ATP and incubated on ice for 2 min. The reaction was terminated by the addition of a concentrated stock of either Laemmli sample buffer (Laemmli, 1970) or two-dimensional gel sample buffer (see below).

Gel Electrophoresis. SDS gel electrophoresis was by the method of Laemmli (1970). Gels were stained, destained, dried, and exposed to Kodak X-OMAT AR-5 film with intensifying screens at −70 °C as described (Giugni et al., 1985). Gels of the standard phosphorylation assay required a 6-h exposure time. The SDS–polyacrylamide gels were calibrated by molecular weight standards ("high" and "low") from Bio-Rad (Richmond, CA) and by lactic dehydrogenase (35 kDa), malic dehydrogenase (35 kDa), and glyceraldehyde-3-phosphate dehydrogenase (36 kDa).

^{125}I-EGF Binding. Placental plasma membranes (20 μg) were incubated with increasing concentrations of ^{125}I-EGF (17000 cpm/ng) for 30 min at 0 °C in 40 μL of the same solution used for the phosphorylation reaction except [γ-^{32}P]ATP was omitted and bovine serum albumin (0.1 mg/mL) was added. Membrane-bound hormone was separated from unbound hormone by filtration as described (Haigler et al., 1985). Nonspecific binding was determined by measuring the binding in the presence of 100-fold excess unlabeled EGF, and all results are reported as specific binding. Nonspecific binding varied from 5% of total at low hormone concentrations to 18% at the highest hormone concentration.

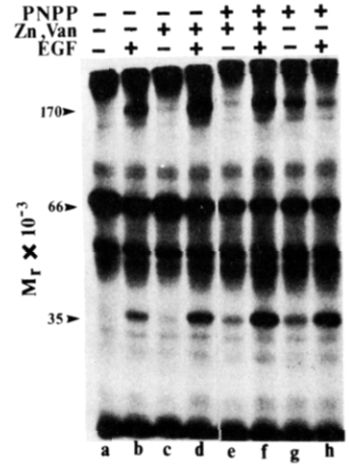

FIGURE 1: Effects of EGF and phosphatase inhibitors on phosphorylation in placental membranes. Placental membranes (20 μg) were reacted with [γ-^{32}P]ATP in the standard phosphorylation assay (see Materials and Methods) in the absence or presence of EGF (500 nM). The indicated reactions also contained zinc chloride (10 μM), sodium orthovanadate (20 μM), and/or PNPP (2 mM). The reaction mixtures were fractionated by SDS gel (8.5%) electrophoresis, and the radioactivity in the dried gel was visualized by autoradiography as described under Materials and Methods.

Liposome Binding Assay. Liposomes containing phosphatidylserine and phosphatidylcholine in a 2:1 molar ratio were prepared in the presence of 240 mM sucrose by the method of Reeves and Dowben (1969). Each assay contained 180 μL of Ca^{2+}-containing buffer [Hepes (20 mM, pH 7.4), KCl (100 mM), $MgCl_2$ (2.0 mM), sodium azide (2.0 mM), and a 1.1-fold concentrated amount of the specified concentration of free Ca^{2+}], 10 μL of ^{32}P-labeled 35-kDa protein in Hepes buffer containing PNPP (10 mM), and 10 μL of liposomes (50 μg) in Hepes/KCl buffer. Parallel control reactions were performed in the absence of liposomes. The free Ca^{2+} concentration in the stock solutions was determined by a Ca^{2+}-sensitive electrode that was calibrated as suggested by the manufacturer (Orion). After incubation at room temperature for 5 min, the liposomes were pelleted by centrifugation (10 min, 20000*g*, 0 °C), and the pellets were suspended in Laemmli sample buffer.

RESULTS

EGF Stimulates Phosphorylation of Endogenous Proteins in Placental Membranes Isolated in the Presence of EDTA. The incubation of placental membranes with [γ-^{32}P]ATP at 0 °C resulted in the incorporation of radioactivity into a number of endogenous proteins, and incubation with EGF stimulated the incorporation into bands with apparent size of 35 and 170 kDa (Figure 1, lanes a and b). As expected from previous studies [e.g., Pike et al. (1984)], the 170-kDa band was identified as the EGF receptor/kinase by immunoprecipitation with a specific antiserum (data not shown). The identity of the 35-kDa band is determined below.

Since zinc, vanadate, and *p*-nitrophenyl phosphate (PNPP) have been reported to inhibit phosphotyrosine protein phosphatases (Brautigan et al., 1981; Swarup et al., 1982), their effects on the reaction were determined. Under various reaction conditions, zinc and vanadate increased the EGF-stimulated incorporation of radioactivity into the 35- and 170-kDa bands 2–5-fold (Figure 1, lanes c and d). The addition of PNPP to reactions containing zinc and vanadate caused an additional 2–4-fold stimulation of the phosphorylation of the

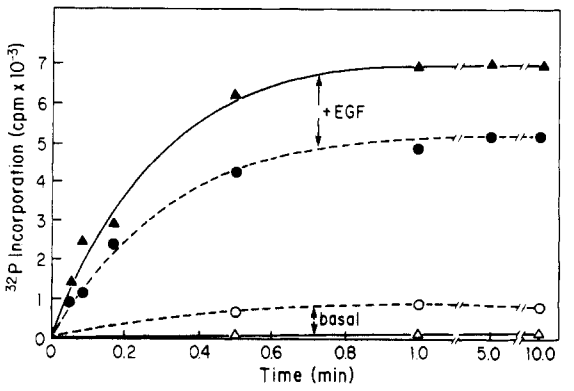

FIGURE 2: Time course of phosphorylation of EGF receptor and 35-kDa protein. Placental membranes (20 μg) were reacted with [γ-^{32}P]ATP (100 Ci/mmol) in the standard phosphorylation assay containing zinc chloride (10 μM), sodium orthovanadate (20 μM), and PNPP (10 mM) in the presence (closed symbols) or absence (open symbols) of EGF (500 nM). The reactions were terminated at the indicated time and fractionated by SDS–polyacrylamide gel (8.0%) electrophoresis. The radioactivity in the dried gel was visualized by autoradiography as described under Materials and Methods, and the radioactive bands at 35 (circles) and 170 (triangles) kDa were excised, digested in 90% Protosol, and counted by liquid scintillation. an equal size piece of gel was excised from directly above each band and treated similarly as a background sample to be subtracted. The results of duplicate determinations are plotted.

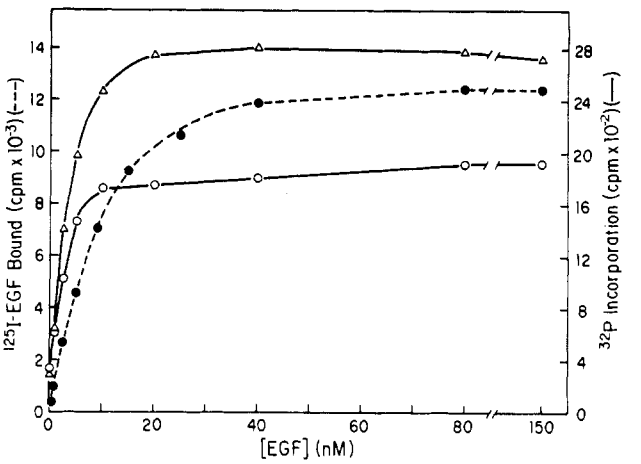

FIGURE 3: Comparison of dose response for ^{125}I-EGF binding and EGF-stimulated phosphorylation. Placental membranes (20 μg) were labeled with [γ-^{32}P]ATP (40 Ci/mmol) as described in Figure 2 except the reaction mixtures were preincubated with or without the indicated concentration of EGF for 29 min prior to a 1-min reaction after addition of [γ-^{32}P]ATP. The reaction solutions contained bovine serum albumin (0.1 mg/mL) to reduce nonspecific binding of the hormone. In the absence of bovine serum albumin, the binding and phosphorylation reactions required about 10-fold more hormone for equivalent effects. The ^{32}P incorporated into the 35- (○) and 170- (△) kDa bands was determined as in Figure 2. The binding of increasing concentrations of ^{125}I-EGF to placental membranes was determined in reactions exactly like the phosphorylation reaction except the [γ-^{32}P]ATP was omitted. Specific binding (●) was measured in duplicate reactions as described under Materials and Methods.

35-kDa band but had little effect on the 170-kDa band (Figure 1, lanes e and f). The effect of PNPP on the phosphorylation of the 35-kDa band was half-maximal at about 1 mM. An effect similar to that of PNPP was seen with sodium phosphate (0.5 mM), ammonium sulfate (5 mM), and sodium arsenate (0.25 mM) (data not shown).

Reactions that contained PNPP but no zinc and vanadate showed an EGF-enhanced phosphorylation of the 35-kDa band (Figure 1, lanes g and h). Curiously, under these conditions the basal level of phosphorylation of the 170-kDa band increased relative to controls, and there was no further increase in the EGF-stimulated reaction (Figure 1, lanes g and h). These effects on both the 35- and 170-kDa bands were obtained when PNPP was replaced with sodium phosphate (0.5 mM) (data not shown). The reaction time in these experiments was 2 min. We considered the possibility that there may have been an EGF-stimulated phosphorylation of the EGF receptor/kinase in the initial part of the 2-min reaction followed by removal of the phosphate by phosphatases. This did not seem to be the case because there was no effect of EGF on the phosphorylation of the EGF receptor/kinase with reaction times of either 5 or 20 s, yet EGF-stimulated phosphorylation of the 35-kDa band still was observed in the brief reactions (data not shown). Since EGF stimulated the phosphorylation of the 35-kDa band under conditions where there was no stimulation of EGF receptor/kinase phosphorylation, it appears that autophosphorylation of the EGF receptor/kinase is not involved in the mechanism by which EGF stimulates the phosphorylation of the 35-kDa band.

The phosphorylation of an unidentified band at 66 kDa was decreased by EGF (Figure 1, lanes a and b) and by PNPP (Figure 1, lanes e–h). Its phosphorylation was decreased in a dose-dependent manner by PNPP with a 10-fold reduction occurring at approximately 10 mM (not shown). The amino acid on which phosphorylation occurred was not determined but it was noted that the radioactivity in this band was sensitive to hydrolysis by KOH under conditions described by Cooper and Hunter (1981).

Characterization of Phosphorylation of EGF Receptor/Kinase and 35-kDa Protein. The time course of phosphorylation of the 35- and 170-kDa bands in the presence and absence of EGF is shown in Figure 2. The EGF-stimulated rate and extent of phosphate incorporation into both proteins were similar. Following a preincubation with EGF, the initial rate of EGF receptor phosphorylation was 0.11 pmol of phosphate/min with a maximum of 32 fmol of phosphate incorporated. From the ^{125}I-EGF binding data (see below), it was estimated that each reaction contains 122 fmol of EGF receptor so the initial reaction rate was 5.2 nmol of phosphate min^{-1} (mg of receptor)$^{-1}$. The EGF-stimulated initial rate of phosphorylation of the 35-kDa band was 3.3 nmol of phosphate min^{-1} (mg of receptor)$^{-1}$. The phosphorylation of the EGF receptor was stimulated greater than 10-fold by EGF. The EGF-stimulated phosphorylation of the 35-kDa protein was about 6-fold higher than the basal rate.

The dose dependence of ^{125}I-EGF binding and the EGF stimulation of phosphorylation were measured in placental membranes under the same experimental conditions (Figure 3). Half-maximal binding of ^{125}I-EGF occurred at about 7.5 nM hormone. This value is higher than most previous reports (Hock & Hollenberg, 1980; Rao et al., 1985) and is, at least in part, a reflection of the relatively brief incubation times at low temperature. The maximum binding capacity was 6.1 pmol/mg of protein and indicates the EGF receptor was about 0.1% of the protein in the preparation. The effects of EGF on phosphorylation of both the 35- and 170-kDa bands were maximal at about 15 nM and half-maximal at about 2.5 nM hormone. The 3-fold difference in half-maximal binding of hormone and half-maximal stimulation of phosphorylation was observed in each of several experiments. The nature of the difference is unknown. An even more pronounced difference in the binding and hormonal stimulation of phosphorylation curves was observed in digitonin-permeabilized fibroblasts (Giugni et al., 1985). Insulin and human chorionic gonadotropin did not have any detectable effects on the phosphorylation of either the 35- or 170-kDa bands (data not shown).

Quantitative studies of the amino acid specificity of the

Table I: Phosphoamino Acid Analysis[a]

	EGF receptor		35-kDa protein	
	control (%)	EGF stimulated (%)	control (%)	EGF stimulated (%)
phosphothreonine	20	2	24	9
phosphoserine	8	6	7	7
phosphotyrosine	72	92	69	84

[a] Placental membranes were reacted for 2 min with [γ-^{32}P]ATP (100 Ci/mmol) in the presence and absence of EGF and fractionated on a 8.0% SDS gel as described in the legend to Figure 2. The radioactive bands at 35 and 170 kDa were excised from the dried gels, and the proteins were eluted according to the method of Beemon and Hunter (1978). The eluate was hydrolyzed in acid, and the phosphoamino acids were separated by thin-layer electrophoresis followed by visualization by ninhydrin staining of standards and by autoradiography (Cooper et al., 1983; Hunter & Sefton, 1980). The spots corresponding to phosphoamino acids were scraped from the plates, and the radioactivity was determined by liquid scintillation counting.

kinase(s) stimulated by EGF were performed. Placental membranes were incubated with [γ-^{32}P]ATP in the standard phosphorylation reaction in the presence and absence of EGF followed by fractionation by SDS gel electrophoresis. The 35- and 170-kDa bands were excised, and the phosphoamino acid composition was determined. In the absence of EGF, approximately 70% of the radioactivity that comigrated with the ninhydrin-stained standards comigrated with phosphotyrosine in the extracts of either band (Table I). Addition of EGF to the reaction caused an increase in the phosphorylation of tyrosyl residues with little or no change in the phosphorylation of seryl or threonyl residues. In the EGF-stimulated reaction, 84 and 92% of the radioactivity comigrated with phosphotyrosine in acid hydrolysates of the 35- and 170-kDa bands, respectively (Table I).

Effects of Calcium on Phosphorylation of the 35-kDa Protein. Protein tyrosine kinase substrates of between 34 and 39 kDa have been purified from various sources, and these proteins undergo Ca^{2+}-dependent association with cellular membranes and Ca^{2+}-dependent phosphorylation in vitro (see Discussion). To determine the Ca^{2+} dependency of phosphorylation for the 35-kDa protein in placenta, membranes were isolated in the presence of either Ca^{2+} or EDTA and then were reacted with [γ-^{32}P]ATP in the presence or absence of Ca^{2+}. When membranes isolated in the presence of Ca^{2+} were reacted with [γ-^{32}P]ATP in the standard phosphorylation assay containing Mn^{2+}, approximately twice as much radioactivity was detected in the 35-kDa band (Figure 4A, lanes e–h) as compared to membranes isolated in the presence of EDTA (Figure 4A, lanes a–d). The addition of Ca^{2+} to the reaction was without effect when either type of membrane preparation was used (Figure 4A; compare lanes a and b with lanes c and d and lanes e and f with lanes g and h). When membranes isolated in the presence of Ca^{2+} were washed with EDTA and then reacted with [γ-^{32}P]ATP, the amount of radioactivity in the 35-kDa band was reduced by about 2-fold (not shown).

The 35-kDa protein also was phosphorylated, although at a somewhat lower level, when the standard phosphorylation assay was modified to contain Mg^{2+} instead of Mn^{2+} (Figure 4B). The major difference in the reaction containing Mg^{2+} was that the 35-kDa protein phosphorylation was stimulated about 2-fold by the addition of Ca^{2+} to the reaction when membranes isolated in the presence of EDTA were used (Figure 4B, lanes a–d). However, even in the reaction containing Mg^{2+}, significant phosphorylation of the 35-kDa protein occurred without added Ca^{2+} (Figure 4B, lanes a and b). The 35-kDa protein also was phosphorylated in reactions in which the Ca^{2+} concentration was reduced to less than 1

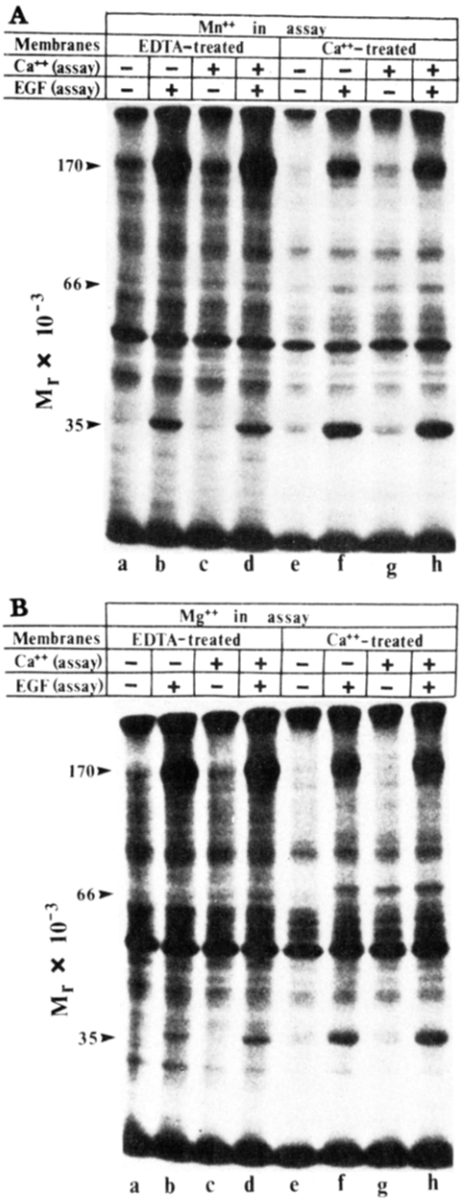

FIGURE 4: Effect of calcium in isolation or phosphorylation buffer on phosphorylation of 35-kDa protein in placental membranes. Placental membranes were isolated in the presence of either EDTA (lanes a–d) or Ca^{2+} (lanes e–h) as described under Materials and Methods. The membranes (20 μg) were reacted with [γ-^{32}P]ATP in the standard phosphorylation assay (see Materials and Methods) containing zinc chloride (10 μM), sodium orthovanadate (20 μm), PNPP (10 mM), and either manganese chloride (0.75 mM) (panel A) or magnesium chloride (20 mM) (panel B). Reactions were performed in the absence (lanes a, c, e, and g) or presence (lanes b, d, f, and h) of EGF (500 nM). Certain reactions (lanes c, d, g, and h) also contained $CaCl_2$ (0.3 mM). The reaction mixtures were fractionated by SDS–polyacrylamide gel (8.5%) electrophoresis, and the radioactivity in the dried gel was visualized by autoradiography as described under Materials and Methods. Panel A was exposed to X-ray film for 7 h, and panel B was exposed for 14 h.

μM by the addition of EGTA to the reaction (not shown).

A small increase in the phosphorylation of the EGF receptor was observed in the membranes prepared in the presence of EDTA (Figure 4). This probably is due to the fact that the receptor is present in higher concentrations relative to other proteins in membranes that had been stripped of peripheral proteins by EDTA.

Because membranes isolated in the presence of Ca^{2+} incorporated more radioactivity into the 35-kDa band in the phosphorylation assay (Figure 4), the possibility that a soluble form of the substrate could be eluted with EGTA was con-

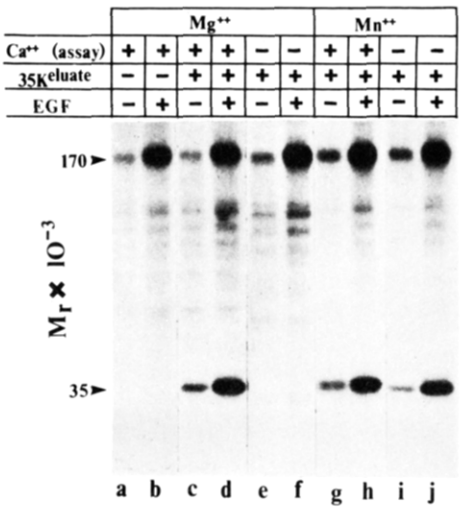

FIGURE 5: Phosphorylation of EDTA-eluted 35-kDa protein by the particulate fraction from A431 membranes. An A431 membrane particulate fraction (5 μg of protein) was incubated with [γ-^{32}P]ATP in the standard phosphorylation conditions plus PNPP (10 mM) as described under Materials and Methods except the reaction time was 20 min and certain reactions contained 20 mM $MgCl_2$ (lanes a–f) and others contained 2 mM $MnCl_2$ (lanes g and h). The indicated reactions contained EGF (500 nM) and $CaCl_2$ (500 μM). Certain reactions contained 400 ng of 35K^{eluate} that was purified from the EGTA eluate of placental membranes (H. T. Haigler, D. D. Schlaepfer, and Burgess, unpublished results). The reaction mixtures were fractionated by SDS–polyacrylamide gel (8.5%) electrophoresis, and the radioactivity in the dried gel was visualized by autoradiography.

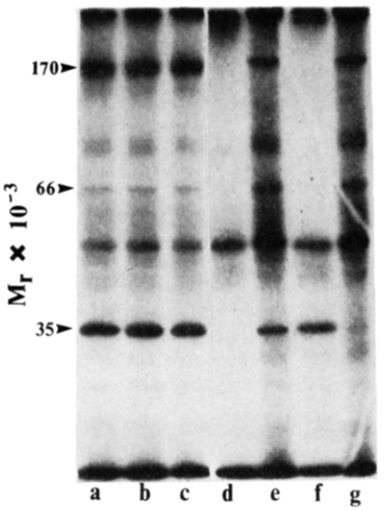

FIGURE 6: Effect of calcium on association of ^{32}P-labeled 35-kDa protein with placental membranes. The standard phosphorylation reaction in the presence of zinc chloride (10 μM), sodium orthovanadate (20 μM), PNPP (10 mM), and EGF (500 nM) was performed on either placental membranes (20 μg) that had been isolated in EDTA as described under Materials and Methods (lane a) or replicate aliquots of placenta membranes that were washed once (lane b) or twice (lane c) by suspension in Hepes buffer (20 mM, pH 7.4) containing glycerol (10%) and EDTA (1 mM), followed by centrifugation at 120000g. Replicate aliquots of placental membranes that were washed twice in EDTA were phosphorylated as above and then the ^{32}P incorporation was stopped by adding unlabeled ATP (final concentration 1 mM). Subsequently, either $CaCl_2$ (1 mM final concentration; lanes d and e) or EDTA (5 mM final concentration; lanes f and g was added to the reaction solution. The reaction solution was centrifuged for 1 min in a Beckman airfuge, and the supernatants (lanes d and f) and pellets (lanes e and g) were solubilized in SDS sample buffer and fractionated on a 8.5% SDS–polyacrylamide gel fol

sidered. The EGTA eluate of membranes isolated in the presence of Ca^{2+} contained a number of proteins including substrates for the EGF-stimulated protein tyrosine kinase. The major substrate in the EGTA eluate has been purified, and the amino acid sequence was determined for approximately 25% of the protein (Haigler et al., 1987). The sequence could be exactly aligned with the sequence of lipocortin derived from the cDNA from a human lymphoma (Wallner, 1986). The form of the 35-kDa placental substrate that eluted from placental membranes with EDTA or EGTA will be called 35K^{eluate}, and the form that remained membrane associated will be called 35K^{memb}. Figure 5 shows that incubating the purified 35K^{eluate} protein with a particulate fraction from A431 cells in the presence of [γ-^{32}P]ATP and appropriate divalent cations resulted in the EGF-dependent phosphorylation of a protein with an apparent size of 35 kDa. The particulate fraction from A431 cells was used as the source of the kinase activity because the kinase is present in high levels and, in contrast to placental membranes, the A431 preparation did not contain any endogenous 35-kDa substrates (Figure 5, lanes a and b). When the reaction was performed in the presence of Mg^{2+}, the phosphorylation of 35K^{eluate} was absolutely dependent on the presence of Ca^{2+} in the reaction (Figure 5, lanes c–f). However, the phosphorylation of 35K^{eluate} occurred equally well in the presence and absence of Ca^{2+} when Mg^{2+} was replaced with Mn^{2+} (Figure 5, lanes g–j). A similar Ca^{2+} dependency of the phosphorylation of added lipocortin was observed when placental membranes isolated in the presence of EDTA were used as the source of the kinase activity (data not shown); however, the analysis of the data was complicated by the presence of the endogenous form of the 35-kDa substrate.

From the above studies, it is clear that there are two forms of the 35-kDa substrate: one form, 35K^{eluate}, could be eluted with EDTA and another, 35K^{memb}, could not. A study of the EDTA-eluted form will be reported elsewhere (Schlaepfer & Haigler, 1987) while the 35K^{memb} form is studied below.

The question arises as to why part of the substrate remained associated with the membrane in the presence of EDTA and why part of it eluted. To determine if it was due to inadequate washing of the membrane preparation, membranes prepared in the presence of EDTA were washed sequentially by suspension in EDTA-containing buffer followed by ultracentrifugation. The standard phosphorylation assay was performed on aliquots of the membranes after each wash, and the total reaction was fractionated by SDS gel electrophoresis. No detectable difference was observed in phosphorylation of the 35-kDa protein after three sequential washes (Figure 6, lanes a–c). The following experiments were performed to determine the membrane-binding properties of the 35K^{memb} form following phosphorylation. Replicate aliquots of the washed membranes were subjected to the standard phosphorylation assay. ^{32}P incorporation was stopped by adding a large excess of unlabeled ATP, and then Ca^{2+} or EDTA was added. The reaction solution was centrifuged to pellet the membranes. The supernatant was separated from the pellet, and both were subjected to SDS gel electrophoresis. In the presence of Ca^{2+}, essentially all of the ^{32}P-labeled 35K^{memb} protein was associated with the membrane fraction (Figure 6, lanes d and e) while in the presence of EDTA most of it was in the soluble fraction (Figure 6, lanes f and g). These results suggest that the nonphosphorylated form of the 35K^{memb} protein associated with the placental membranes in a Ca^{2+}-independent manner and that the phosphorylated form associated with the placental membranes in a Ca^{2+}-dependent manner.

Since the ^{32}P-labeled 35K^{memb} protein could be eluted from the membrane, the following experiments were performed to determine if it would associate with the phospholipid liposomes in a Ca^{2+}-dependent manner. Placental membranes were

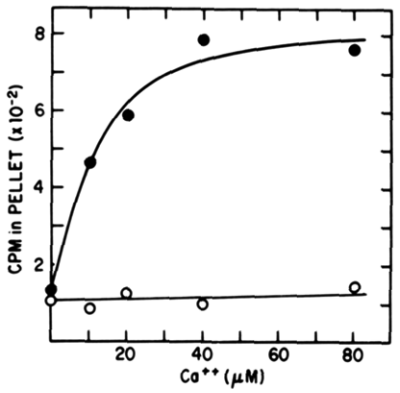

FIGURE 7: Binding of ^{32}P-labeled 35-kDa protein to liposomes. Placental membranes (60 μg) that were isolated in the presence of EDTA were reacted for 1 min with [γ-^{32}P]ATP (200 Ci/mmol) in the presence of EGF as described in the legend of Figure 2. The reaction was stopped by adding EGTA (final concentration 3 mM) followed by centrifugation for 1 min in a Beckman airfuge. The supernatant was applied to a Sephadex G-25 column (0.5 × 6.2 cm) that was equilibrated with Hepes buffer (20 mM, pH 7.4) containing 10 mM PNPP. The column was eluted with equilibration buffer, and the void-volume fractions containing the ^{32}P-labeled protein were pooled. Aliquots of the pooled fractions were tested for calcium-dependent binding to liposomes in the presence of increasing concentrations of Ca^{2+} as described under Materials and Methods. Liposomes were pelleted by centrifugation, and the pellets were fractionated by SDS–polyacrylamide gel (8.5%) electrophoresis. The 35-kDa band of the dried gel was excised, digested in 90% Protosol, and counted by liquid scintillation. The results of duplicate determinations are plotted: (●) reactions containing liposomes; (○) control reactions without liposomes. The point without added Ca^{2+} contained 1 mM EGTA. The total amount of ^{32}P-labeled 35-kDa protein added to each reaction was 874 ± 77 cpm. All of the solutions used in this experiment were prepared from H_2O that was treated with Chelex resin (Bio-Rad) to remove traces of Ca^{2+}.

isolated in the presence of EDTA, reacted with [γ-^{32}P]ATP, treated with EDTA, and centrifuged to separate membranes from the eluted proteins. The ^{32}P-labeled 35K^{memb}, which now was in a soluble form, was separated from low molecular weight components of the reaction by chromatography on Sephadex 25. The ^{32}P-labeled 35K^{memb} was incubated with liposomes and increasing concentrations of Ca^{2+}. Interaction with the liposomes was evaluated by pelleting the liposomes, subjecting the pellet to SDS gel electrophoresis, and determining the amount of radioactivity associated with the 35-kDa band (Figure 7). In the absence of liposomes or in the presence of liposomes in the absence of Ca^{2+}, only 10–15% of the added ^{32}P-labeled 35K^{memb} was observed in the pellet (Figure 7). However in the presence of Ca^{2+} and liposomes, up to 90% of the added radioactive substrate associated with the liposomes. Half-maximal association with liposomes occurred at a Ca^{2+} concentration of approximately 10 μM.

Relationship of 35-kDa Phosphorylation Substrate to Previously Identified Substrates. In an attempt to identify the 35-kDa protein that was phosphorylated in placental membranes, we tested its reactivity with antibodies against known proteins of similar size that are substrates for the EGF-stimulated kinases. An antiserum raised against a 34-kDa protein initially identified as a substrate for p60^{src} (Erickson et al., 1979; Radke & Martin, 1979) and later shown to be phosphorylated in an EGF-dependent manner in A431 cells (Erickson et al., 1981; Hunter & Cooper, 1985) was tested. This antiserum did not precipitate any detectable radioactive protein from placental membranes that were labeled with [γ-^{32}P]ATP in the standard phosphorylation assay (data not shown). As a positive control, it was shown that the antiserum would precipitate a 34-kDa phosphoprotein from extracts of labeled A431 cells.

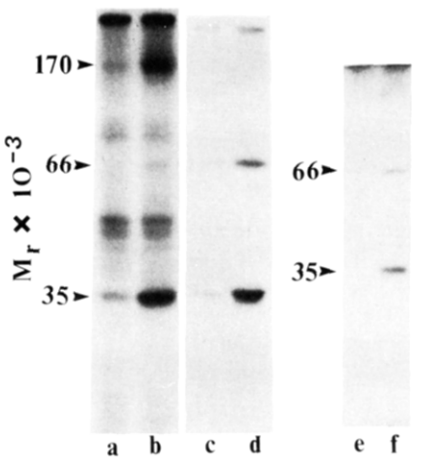

FIGURE 8: Immunoprecipitation of 35-kDa phosphorylation substrate. Placental membranes were reacted with [γ-^{32}P]ATP for 2 min exactly as described in Figure 2 in the absence (lanes a, c, and e) or presence (lane b, d, and f) of EGF (500 nM). Certain reactions (lanes a and b) were solubilized in SDS sample buffer, and an aliquot (20% of total) of the reaction mixture was fractionated on an 8.5% SDS–polyacrylamide gel. Other reaction mixtures were extracted by the method of Cooper and Hunter (1983) and immunoprecipitated as previously described (Giugni et al., 1985) by adding antiserum (2 μL) against the A431 35-kDa protein (Fava & Cohen, 1984) (lanes e and f) or antiserum (10 μL) against the EDTA-eluted 35-kDa protein from placenta (lanes c and d). Immunoprecipitates were boiled in SDS sample buffer and fractionated on a 8.5% (lanes b and c) or a 9% (lanes d and e) SDS–polyacrylamide gel. No bands were detected when control serum was incubated with extracts and processes as above.

Another antiserum specific for a 35-kDa substrate isolated from A431 cells by Fava and Cohen (1984) on the basis of reversible Ca^{2+} binding to membranes was tested. They showed the A431 protein associated with membranes in a Ca^{2+}-dependent manner, and its in vitro phosphorylation was dependent on the presence of Ca^{2+} in the reaction. Placental membranes were labeled with [γ-^{32}P]ATP in the standard phosphorylation assy in the presence and absence of EGF, extracted with detergent, and reacted with this antiserum, and the immunoprecipitated material was fractionated by SDS gel electrophoresis. A radioactive 35-kDa protein that was phosphorylated in an EGF-dependent manner was specifically precipitated (Figure 8, lanes e and f). A ^{32}P-labeled 35-kDa protein also was immunoprecipitated from the reaction mixture by an antiserum against the 35K^{eluate} form of the placental 35-kDa protein (Figure 8, lanes c and d). Control experiments showed that essentially all of the radioactive 35-kDa protein was removed by this antiserum. Both antisera also reacted with a phosphoprotein with an apparent molecular size of about 66 kDa, whose phosphorylation was stimulated by EGF (Figure 8, lanes c–f). This band was not seen when control serum was used. In phosphorylation reactions that contained no PNPP, a major phosphorylation band was seen at approximately 66 kDa (Figure 1). This major band (Figure 1) is not related to the immunoprecipitated 66-kDa band that was phosphorylated in an EGF-dependent manner (Figure 8) because the amount of immunoprecipitated radioactivity in the 66-kDa band was the same when the reaction was performed in the presence and absence of PNPP. EGF-dependent phosphorylation of the 66-kDa band could be detected in autoradiograms of gels in which the entire reaction solution was fractionated if high concentrations of PNPP were present in the reaction (Figure 8, lanes a and b).

Discussion

When placental membranes isolated in the presence of EDTA were incubated with [γ-^{32}P]ATP, we detected phos-

phorylation of 35- and 170-kDa proteins in an EGF-dependent manner. Although there was some variation between preparations, it usually was difficult to detect phosphorylation of the 35-kDa protein without adding substances such as zinc, vanadate, and PNPP (Figure 1). On the basis of previous reports that show these compounds inhibit phosphotyrosine phosphatase activity (Brautigan et al., 1981; Pallen et al., 1985; Swarup et al., 1982), we assume that the effects of these compounds in the placental membrane phosphorylation reaction were due to inhibition of phosphatases, but this was not formally established. The lack of detection of the 35-kDa protein phosphorylation in some of the previous studies (Avruch et al., 1982; Carpenter et al., 1980) probably was due to the lack of phosphatase inhibitors in the reaction.

The initial rate of phosphorylation (Figure 2) and the fold-stimulation by EGF (Figure 3) of both the EGF receptor/kinase and the $35K^{memb}$ substrate were similar. The stoichiometry of phosphorylation of the EGF receptor in placental membranes was approximately 0.26 mol of phosphate/mol of receptor (Figure 3). This is lower than one might expect because four tyrosine autophosphorylation sites have been identified in the receptor (Downward et al., 1984) and high stoichiometry of phosphorylation of these sites has been observed in vitro with A431 membranes (Bertics & Gill, 1985; Downward et al., 1985). Placental membranes contain a much higher phosphatase activity than A431 membranes so the low stoichiometry may be due to incomplete inhibition of these enzymes. Another possibility is that the EGF receptor is highly phosphorylated as isolated from the placenta.

It is interesting to note that when the phosphorylation reaction was performed in the presence of sodium phosphate or PNPP, and in the absence of zinc and vanadate, the 35-kDa protein was phosphorylated in an EGF-dependent manner while the EGF receptor was not (Figure 1). Thus, hormone-dependent phosphorylation of the EGF receptor/kinase is not required to stimulate phosphorylation of the 35-kDa substrate. Recent reports have presented data suggesting that phosphorylation of tyrosine residues on the EGF receptor is (Bertics & Gill, 1985) and is not (Downward et al., 1985) involved in activation of the intrinsic kinase activity. In placental membranes, phosphorylation of the EGF receptor clearly is not involved in stimulation of the phosphorylation of the $35K^{memb}$ substrate. However, it should be noted that there is no evidence the 35-kDa protein was directly phosphorylated by the EGF receptor/kinase.

Two forms of 35-kDa phosphorylation substrate were found in placenta. One form, $35K^{memb}$, was associated with membranes isolated in the presence of EDTA and the other form, $35K^{eluate}$, associated with membranes in the presence of Ca^{2+} but could be eluted with EDTA or EGTA (Figures 4 and 5). A number of experiments were performed to determine if the $35K^{memb}$ form was associated with the membrane due to an artifact of the membrane isolation procedure. The following methods of membrane preparation in the presence of EDTA yielded membranes that phosphorylated an endogenous 35-kDa protein at levels comparable to those shown in Figure 4: (1) as described under Materials and Methods but in the absence of sucrose and at a higher (1 mM) concentration of EDTA, (2) as described under Materials and Methods but in the presence of protease inhibitors (leupeptin, aprotonin, and benzamidine), (3) as described under Materials and Methods followed by centrifugation in a discontinuous sucrose gradient and collection of the material at the 10–37% (w/v) sucrose interface, and (4) the microsomal fraction described by Kellis and Vickery (1984). Western blot analysis experiments with antiserum against the $35K^{eluate}$ also support the idea that two distinct forms of the 35-kDa substrate exist (D. D. Schlaepfer and H. T. Haigler, unpublished results). Only about half of the immunoreactive material could be eluted from membranes prepared in the presence of Ca^{2+} by a single EDTA wash. Additional washes did not remove detectable amounts of the 35-kDa protein. Another possible artifact would be that the membrane preparation was a mixture of inside-out and outside-out vesicles with protein being trapped within the latter. However, this does not seem to be the case because of the following experiment. Placental membranes isolated in the presence of EDTA were suspended in buffer containing EDTA, subjected to sonication, and then pelleted by ultracentrifugation. No phosphorylation substrate was detected in the supernatant, and the pelleted membranes still contained 35-kDa protein that could be detected as radiolabeled material in the standard phosphorylation assay and by Western blot assay using antiserum against $35K^{eluate}$ (D. D. Schlaepfer and H. T. Haigler, unpublished results).

Valentine-Braun et al. (1986) recently reported that a 35-kDa protein is phosphorylated in an EGF-dependent manner in placental membranes prepared in the presence of Ca^{2+} but not in the presence of EDTA. It is possible that the phosphorylated protein they observed in their phosphorylation reaction in the absence of zinc and PNPP is different from the one detected under our reaction conditions. They reported that the 35-kDa phosphorylation substrate that they detected was related to the β-subunit of the GTP-binding proteins known as G or N proteins. No structural relatedness was detected between the sequence of the $35K^{eluate}$ placental substrate (Haigler et al., 1987) and the sequence of the β-subunit of a bovine GTP-binding protein (Sugimoto et al., 1985).

Fava and Cohen (1984) purified a 35-kDa protein from A341 carcinoma cells by exploiting its ability to undergo a reversible, Ca^{2+}-dependent association with the particulate fraction of these cells. Several lines of evidence suggest the A431 35-kDa protein is structurally related to both the $35K^{memb}$ and $35K^{eluate}$ forms of the placental protein. Antiserum against both the A431 protein and the $35K^{eluate}$ protein cross-reacted with the ^{32}P-labeled $35K^{memb}$ protein (Figure 8). The structural relatedness of these proteins also was demonstrated by peptide mapping. Both forms of the placental 35-kDa protein and the A431 protein gave indistinguishable fragmentation patterns of radioactive peptides in Cleveland digests of the ^{32}P-labeled proteins (T. D. Giugni, D. D. Schlaepfer, and H. T. Haigler, unpublished results). The same pattern was seen for the radioactive ^{32}P-labeled peptides of $35K^{eluate}$ when the source of the kinase was A431 or placental membranes. The sequence of approximately 25% of the $35K^{eluate}$ protein has been determined (H. T. Haigler, D. D. Schlaepfer, and Burgess, unpublished results), and the sequence can be exactly aligned with the sequence of recombinant human lipocortin (Wallner et al., 1986). The 35-kDa protein from A431 cells also appears to be structurally related to human lipocortin (Stanley Cohen, personal communication), and a form with a very similar sequence has been isolated from pig lung (De et al., 1986).

Lipocortin is an inhibitor of phospholipase A_2 activity, and its ability to inhibit this enzyme is reported to be inactivated by phosphorylation (Hirata, 1981). The possibility that lipocortin, phospholipase A_2, and the ensuing prostaglandin biosynthesis play a role in the cellular response to EGF opens an exciting new area of research. Previous reports of EGF-dependent phosphorylation of a 35-kDa protein in intact A431 (Sawyer & Cohen, 1985) and in intact diploid human fi-

broblasts (Giugni et al., 1985) now can be reinterpreted to indicate that a lipocortin-like protein is phosphorylated.

After this paper was submitted, it was reported that recombinant lipocortin was phosphorylated in an EGF-dependent manner (Pepinsky & Sinclair, 1986). It also was shown that lipocortin could be extracted from placental membranes (Huang et al., 1986). These studies showed that the 35-kDa substrate in A431 cells was related to human lipocortin I. These proteins also have been named calpactin II (Glenney, 1986b).

A number of Ca^{2+}-binding proteins with apparent sizes of approximately 35 kDa have been purified from intracellular vesicles (Kretsinger & Creutz, 1986). These proteins are postulated to be involved in Ca^{2+}-dependent membrane fusion events. A consensus amino acid sequence repeat recently has been detected in this class of proteins (Geisow et al., 1986), and this sequence is found in lipocortin. This raises the possibility that these proteins promote membrane fusion by modulating the phospholipase A_2 catalyzed production of lysophosphoglycerides. It is interesting to note that inhibitors of phospholipase A_2 activity inhibit the internalization of EGF (Haigler et al., 1980).

There are clear functional differences in the Ca^{2+} dependency of phosphorylation of the $35K^{eluate}$ and $35K^{memb}$ forms of the 35-kDa protein (Figures 4 and 5). The $35K^{eluate}$ form was phosphorylated in a Ca^{2+}-dependent manner in the presence of Mg^{2+} and in a Ca^{2+}-independent manner in the presence of Mn^{2+} (Figure 5). Other experiments (Schlaepfer & Haigler, 1987) show that the $35K^{eluate}$ form will associate with phosphatidylserine-containing liposomes in a Ca^{2+} or Mn^{2+}-dependent manner but Mg^{2+} will not promote this association. We propose that, in the presence of Mg^{2+}, Ca^{2+} is not required for the phosphorylation reaction per se, but it is required to promote association of the 35-kDa substrate with the lipid bilayer and thus position it near the kinase. Furthermore, we propose that the phosphorylation of the $35K^{memb}$ form was Ca^{2+}-independent because this form of the substrate associated with the membrane by a divalent cation independent mechanism. Because these proteins appear to be structurally related, it is assumed that the different forms of the 35-kDa proteins are due to posttranslational modifications of the same gene product and that these modifications result in the observed differences in functional characteristics. In other studies, the 35-kDa substrate from A431 cells was phosphorylated in a Ca^{2+}-dependent manner (Fava & Cohen, 1984), but recombinant lipocortin was reported to be phosphorylated in a reaction containing Mg^{2+} and no Ca^{2+} (Pepinsky & Sinclair, 1986).

Since the membrane-binding properties of ^{32}P-labeled $35K^{memb}$ and nonradioactive $35K^{eluate}$ are similar, the possibility must be considered that $35K^{eluate}$ is phosphorylated endogenously and isolated in a phosphorylated form. Although no direct information is yet available, the similarity of peptide maps of the ^{32}P-labeled forms (T. D. Giugni, D. D. Schlaepfer, and H. T. Haigler, unpublished results) suggests that the site phosphorylated in the in vitro assay is not involved in the in vivo conversion of $35K^{memb}$ to $35K^{eluate}$.

Although the structural differences between the two forms of the 35-kDa proteins are not known, it appears that phosphorylation of the membrane-bound form may change its membrane-binding properties. Multiple washes of the membranes in solutions containing EDTA did not lead to diminished phosphorylation of the 35-kDa protein in subsequent phosphorylation assays (Figure 6). However, following the phosphorylation reaction, the ^{32}P-labeled $35K^{memb}$ phosphoprotein associated with the membranes in the presence of Ca^{2+} but not in the presence of EDTA (Figure 6). These results suggest that the protein was associated with the membrane in a Ca^{2+}-independent manner prior to phosphorylation but in a Ca^{2+}-dependent manner following phosphorylation. The ^{32}P-labeled $35K^{memb}$ form of the protein associated with phosphatidylserine-containing liposomes in a Ca^{2+}-dependent manner with half-maximal association occurring at approximately 10 μM Ca^{2+} (Figure 7). Thus, the phosphorylated form appears to undergo Ca^{2+}-dependent binding to the lipid bilayer rather than to a specific component in the membrane. It has recently been shown that the 36-kDa tyrosine kinase substrate (calpactin I) associates with phospholipid in a Ca^{2+}-dependent manner (Glenney, 1985, 1986a) but the effects of phosphorylation have not been reported.

A complete understanding of the membrane-binding properties of the different forms of the 35-kDa protein will require purification and detailed structural analysis. It is nonetheless interesting to speculate on the potential for EGF exerting a dual regulation on the function of the $35K^{memb}$: a direct effect by tyrosine phosphorylation to render it Ca^{2+} sensitive and an indirect effect by hormonal modulation of Ca^{2+} flux (Sawyer & Cohen, 1981).

Registry No. EGF, 62229-50-9; PNPP, 330-13-2; Ca, 7440-70-2; Zn, 7440-66-6; VO_4^{3-}, 14333-18-7.

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