Biochem. J. (1989) 259, 577–583 (Printed in Great Britain)

Epidermal-growth-factor-stimulated phosphorylation of calpactin II in membrane vesicles shed from cultured A-431 cells

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Membrane vesicles shed from intact A-431 epidermoid carcinoma cells and harvested in the presence of Ca^{2+} contained epidermal-growth-factor (EGF) receptor/kinase substrates of apparent molecular masses 185, 85, 70, 55, 38 and 27 kDa. The 38 kDa substrate (p38) was recognized by an antibody that had been raised against the human placental EGF receptor/kinase substrate calpactin II (lipocortin I). The A-431 and placental substrates, isolated by immunoprecipitation after phosphorylation *in situ*, yielded identical phosphopeptide maps upon limited proteolytic digestion with each of five different enzymes. The A-431-cell vesicular p38 is therefore calpactin II. EGF treatment of the intact A-431 cells before inducing vesiculation was not necessary for the substrate to be present within the vesicles. Our data thus indicate that receptor internalization is not a prerequisite for receptor-mediated phosphorylation of calpactin II. The ability of the protein to function as a substrate for the receptor/kinase depended upon the continued presence of Ca^{2+} during the vesicle-isolation procedure. EGF-stimulated phosphorylation of calpactin II was much less pronounced in vesicles prepared from A-431 cells in the absence of Ca^{2+}, although comparable amounts of the protein were detectable by immunoblotting. Calpactin II therefore appears to be sequestered in a Ca^{2+}-modulated manner within shed vesicles, along with at least four other major targets for the EGF receptor/kinase. The vesicle preparation may be a useful model system in which to study the phosphorylation and function of potentially important membrane-associated substrates for the receptor.

INTRODUCTION

Since the discovery that the receptor for epidermal growth factor (EGF, also termed urogastrone) contains an intrinsic, EGF-activatable tyrosine-protein kinase activity (Cohen *et al.*, 1980; Buhrow *et al.*, 1982, 1983; see also Cohen, 1986), there has been great interest in the ability of EGF to modulate the phosphorylation state of specific cellular proteins. A variety of proteins and peptides can be phosphorylated by the EGF receptor/kinase *in vitro* or in intact cells (see Zendegui & Carpenter, 1984). The substrate that has perhaps attracted the greatest interest is a 35–38 kDa membrane-associated protein first studied in detail by Cohen and his co-workers, initially in A-431-cell particulate membrane fractions (Fava & Cohen, 1984), and subsequently in intact A-431 cells (Sawyer & Cohen, 1985). A similar substrate has been identified in human placental membranes (Huang *et al.*, 1986; Valentine-Braun *et al.*, 1986, 1987; Glenney *et al.*, 1987; Haigler *et al.*, 1987; Sheets *et al.*, 1987), in various rat and porcine tissues (De *et al.*,1986) and in cultured human fibroblasts (Giugni *et al.*, 1985).

Attention was recently focused on this substrate of the EGF receptor/kinase by reports that it is indistinguishable from lipocortin I, a putative glucocorticoid-induced inhibitor of phospholipase A_2 (De *et al.*, 1986; Huang *et al.*, 1986; Pepinsky & Sinclair, 1986). It has also been found that the substrate is a cytoskeleton-associated protein that can, in a Ca^{2+}-dependent manner, bind to phospholipid and actin. Thus it can also be termed a calpactin (see Glenney, 1987). It is referred to as 'calpactin II' (Glenney, 1986), to distinguish it from the substrate of the $pp60^{src}$ tyrosine kinase, calpactin I, which was first detected as a 34–39 kDa phosphoprotein in sarcoma-virus-transformed cells (Radke & Martin, 1979; Erikson & Erikson, 1980; Cooper & Hunter, 1981). Calpactin I can similarly inhibit phospholipase A_2 activity, and hence has also been termed 'lipocortin II' (Huang *et al.*, 1986).

The reason for the great interest in these two tyrosine kinase substrates is the possibility that they may play some role in the action of EGF (calpactin II/lipocortin I) or in the process of cell transformation (calpactin I/lipocortin II). In spite of extensive biochemical information, however, the role of the two molecules in cell regulation is poorly understood. The reported inhibition of phospholipase A_2 by the calpactins raises the possibility that the phosphorylation of these substrates might be an intermediate step in the regulation of production of eicosanoids (see Brugge, 1986). Indeed, early reports suggested that the phospholipase-inhibitory activity of lipocortin (lipomodulin) could be regulated by phosphorylation of the molecule on tyrosine residues (Hirata, 1981; Hirata *et al.*, 1984). However, the phospholipase-inhibitory activity of the calpactins appears to be a consequence of the sequestering of substrate phospholipids rather than the result of a direct interaction with the enzyme (Davidson *et al.*, 1987). Thus the status of the calpactins/lipocortins as regulators of eicosanoid metabolism remains uncertain.

Abbreviations used: EGF, epidermal growth factor; DMEM, Dulbecco's modified Eagle medium containing glucose (4.5 g/l), penicillin (100 units/ml) and streptomycin (100 μg/ml); FCS, fetal-calf serum; PBS, phosphate-buffered saline (140 mM-NaCl/2.7 mM-KCl/8 mM-Na_2HPO_4/1.5 mM-KH_2PO_4, pH 7.2); PAGE, polyacrylamide-gel electrophoresis.

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A different view of the role of the calpactins is that they may participate in membrane vesicular trafficking processes. Calpactin I, in particular, can bind to, and cause aggregation of, secretory-granule membranes in a Ca^{2+}-dependent manner, and may therefore function in exocytotic mechanisms (Creutz *et al.*, 1987; Drust & Creutz, 1988; see also Burgoyne, 1988). The possibility that calpactin II might play a similar role remains an open question. Previous work with A-431 cells suggested that internalization of the occupied EGF receptor into an endosomal compartment preceded the phosphorylation of calpactin II. Thus Fava & Cohen (1984) found that prior exposure of intact A-431 cells to EGF for 30 min at 37 °C (before the preparation of a particulate membrane fraction) was necessary to produce a maximal increase in labelling of the 35 kDa protein in subsequent phosphorylation assays. This implied that certain cellular events which followed binding of the growth factor to its receptor were required for substrate phosphorylation to take place. In subsequent studies using intact cells, a lag time of more than 5 min from the start of EGF treatment was observed before significant phosphorylation of the 35 kDa substrate occurred (Sawyer & Cohen, 1985). Other work (Cohen & Fava, 1985) indicated that an intracellular vesicle fraction isolated from A-431 cells was active in phosphorylating exogenous 35 kDa protein. Such results suggested that internalization of the EGF·receptor/kinase complex might be required for the phosphorylation of calpactin II, which could therefore be involved in the endocytic process.

In an effort to understand the role that receptor internalization might play in the context of EGF-triggered substrate phosphorylation, we have begun to study the receptor-mediated phosphorylation of proteins in membrane vesicles shed from cultured A-431 cells. This approach is an alternative to studying the phosphorylation of receptor/kinase substrates either in the intact cell or in membranes isolated from cell homogenates. The vesicle preparation provides a more defined environment for the EGF receptor/kinase and its substrates than does the intact cell, and avoids some of the problems of protein degradation and phosphatase activation inherent in the use of plasma membranes prepared by more conventional techniques. Shed membrane vesicles have previously been used to advantage in studies of EGF-receptor function (Gates & King, 1985), diacylglycerol kinase activity (Kato *et al.*, 1985) and Na^+/H^+ exchange (Mancuso & Glaser, 1985).

We show here that vesicles shed from A-431 cells contain, along with the receptor, a number of substrates that can be phosphorylated in an EGF-dependent manner in the isolated vesicles. We demonstrate further that one prominent 38 kDa substrate is localized in the vesicles in a Ca^{2+}-dependent manner, cross-reacts with an antibody to calpactin II and yields phosphopeptide maps that are identical with those of intact human placental calpactin II. In addition, we show that prior treatment of the cells with EGF is not necessary for recovery and phosphorylation of calpactin II within the vesicles.

EXPERIMENTAL

Cell culture

A-431 human epidermoid carcinoma cells were maintained in DMEM supplemented with 5% (v/v) FCS. Cultures were passaged every 3–4 days at a 1:4 subculture ratio. Cells for vesicle preparation were grown in 850 cm^2 roller bottles, in DMEM supplemented with 5% FCS and containing 20 mM-Hepes, pH 7.4.

Membrane vesicle preparation

Shed membrane vesicles were prepared from A-431 cell cultures by the method of Cohen *et al.* (1982). Briefly, the cells were first washed with PBS, and were then incubated for 15 min at room temperature with hypo-osmotic (one-twentieth iso-osmotic) PBS to induce swelling. The swollen cells were further incubated in an alkaline (pH 8.5) vesiculation buffer, for 20 min at room temperature, followed by 60 min at 37 °C. The resultant shed vesicles were recovered by centrifugation and resuspended in 20 mM-Hepes pH 7.4 (resuspension buffer). When vesicles were to be prepared in the presence of Ca^{2+}, $CaCl_2$ was added to the following solutions at the indicated concentrations: PBS, 0.9 mM; vesiculation buffer, 0.25 mM; resuspension buffer, 1.0 mM. The vesicles were stored at −70 °C until required. On isolation (and even upon careful thawing of quick-frozen preparations), the vesicles appeared sealed and largely 'outside-out', in that the transfer of labelled phosphate from ATP to vesicle proteins was minimal, and EGF stimulation of phosphorylation was absent. The binding of ^{125}I-EGF by these putatively sealed vesicles was 10 fmol/μg of membrane protein at 3 nM-^{125}I-EGF (non-specific binding 2%). Subsequent successive freezing and thawing (two or three times) was required to render the vesicles permeable and suitable for phosphorylation studies.

Placental membrane preparation

Membranes were prepared from full-term human placentae as previously described (Hock & Hollenberg, 1980; Valentine-Braun *et al.*, 1986, 1987).

Phosphorylation assay

Membranes (30 μg) were routinely phosphorylated in an incubation mixture containing 150 mM-NaCl, 20 mM-Hepes, 1 mM-$CaCl_2$, 20 mM-$MgCl_2$, 1 mM-$MnCl_2$, 20 μM-Na_3VO_4 and 20 μM-$ZnCl_2$, at a final pH of 7.4. The mixture was incubated for 10 min at 4 °C in the absence or presence of 20 nmol of EGF, and then for another 10 min after the further addition of [γ-^{32}P]ATP (12 μCi; final concn. 15 μM). Samples were analysed by SDS/PAGE (Laemmli, 1970) using 6–18%-(w/v)-polyacrylamide linear-gradient gels with 2.7% *NN'*-methylenebisacrylamide cross-linker. The molecular-mass standards used were from Bio-Rad and were myosin (200 kDa), β-galactosidase (116.3 kDa), phosphorylase *b* (92.5 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa) and aprotinin (6.5 kDa). The gels were dried, then autoradiographed with Kodak X-Omat AR film at −70 °C.

Immunoprecipitation

Phosphorylated membranes (150 μg) were solubilized for 20 min at 4 °C in a mixture containing 150 mM-NaCl, 20 mM-Hepes, 1% Nonidet P40, 1% deoxycholate, 0.1% SDS, 2 mM-EGTA, 1 mM-*p*-nitrophenyl phosphate, 100 μM-Na_3VO_4 and 1000 kallikrein-inhibitory units of aprotinin/ml. The extracts were clarified by centrifugation at 100000 *g* for 30 min at 4 °C, and then supplemented with 0.5 vol. of 10% washed immuno-

precipitin (fixed staphylococcal A cells). After incubation for 30 min at 4 °C, the samples were centrifuged further at 8000 *g* for 30 min at 4 °C to remove the immunoprecipitin and non-specifically adsorbed proteins. Aliquots of the resulting supernatant were supplemented with a dilution of non-immune serum or anti-(calpactin II) antiserum (Valentine-Braun *et al.*, 1987), such that the final concentration of detergents was one-fifth of that given above and the final dilution of (anti-)serum was 1:250. After 20 h at 4 °C, 0.1 vol. of 10% washed immunoprecipitin was added to the samples, which were then further incubated for 60 min at 4 °C. The immunoprecipitin was collected (8000 *g*, 60 s) and washed four times in the low-detergent buffer. The adsorbed proteins were eluted by heating at 100 °C for 4 min in electrophoresis sample buffer (Laemmli, 1970) and were analysed by electrophoresis as described above.

Phosphopeptide mapping

Immunoprecipitated phosphoproteins were separated by electrophoresis and localized by autoradiography. The required bands were excised from the wet gel and subjected to enzyme digestion by the 'in-gel' method described by Cleveland *et al.* (1977), followed by SDS/PAGE analysis (15% polyacrylamide gel).

Immunoblotting

Electrophoretic transfer of proteins to nitrocellulose and localization of immunoreactivity with the anti-(calpactin II) antiserum were performed as described previously (Valentine-Braun *et al.*, 1987). Photographic negatives of the immunoblot were scanned with a Bio-Rad model 620 video densitometer linked to a model 3392A integrator.

Materials

DMEM was from Gibco, FCS from Flow Laboratories and roller bottles from Corning. A-431 cells were obtained from the American Type Culture Collection (CRL 1555). EGF was prepared as described by Savage & Cohen (1972). [γ-^{32}P]ATP (10–50 Ci/mmol) was from NEN (du Pont). Preparation of the antibody against placental calpactin II (p35/p38) was described previously (Valentine-Braun *et al.*, 1987). Immunoprecipitin was obtained from BRL. All other reagents were from Sigma.

RESULTS

A-431 cells were induced to vesiculate in the presence of Ca^{2+}, and the shed vesicles were harvested in Ca^{2+}-containing buffers. The resultant membrane preparations contained several components for which the phosphorylation was increased by EGF (Fig. 1). The apparent molecular masses of the major phosphorylated proteins were 185, 85, 70, 55, 38 and 27 kDa. The 185 kDa component was the EGF receptor itself, being immunoprecipitated by receptor-specific antisera (results not shown).

The 38 kDa substrate (p38) was compared with a protein of similar molecular mass that is a substrate of EGF-dependent phosphorylation in human placenta. A 35 kDa form of the latter (p35) has an *N*-terminal amino acid sequence that is indistinguishable from that of calpactin II/lipocortin I, beginning at residue 13 (Valentine-Braun *et al.*, 1987); i.e. p35 is a proteolytic derivative of human placental calpactin II. Phosphorylated p38 from A-431 cell vesicles was recognized by an antibody raised against purified placental p35 (Fig. 2). The EGF receptor was also co-precipitated at low efficiency (Fig. 2). A further minor labelled component

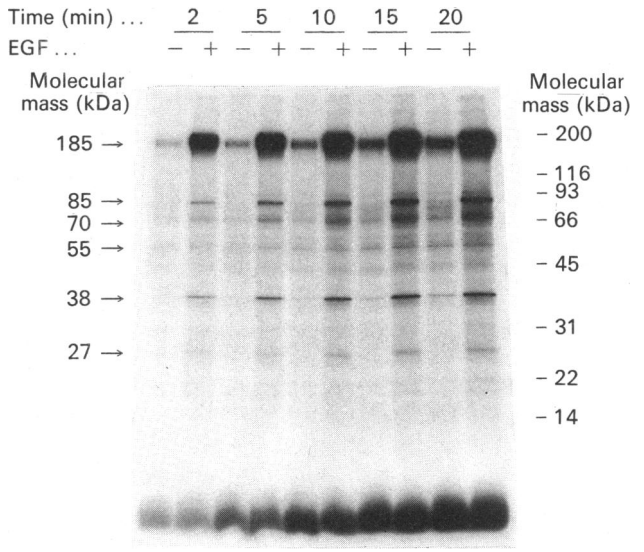

Fig. 1. Time course of EGF-dependent phosphorylation in membrane vesicles

A-431-cell membrane vesicles prepared in the presence of Ca^{2+} were phosphorylated in the absence or presence of EGF for the times indicated. The positions of phosphoproteins that are referred to in the text are marked. In this Figure and Figs. 2 and 3 the molecular masses given on the right refer to markers identified in the Experimental section.

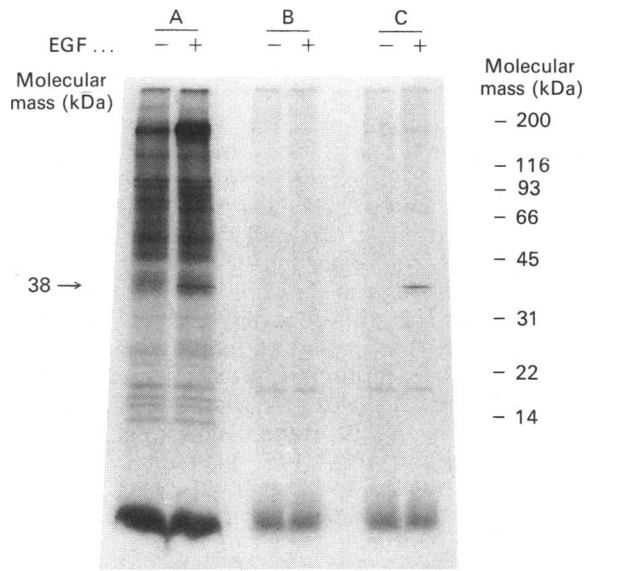

Fig. 2. Immunoreactivity of p38 with anti-(calpactin II) antiserum

A-431-cell membrane vesicles (prepared with Ca^{2+} present) were phosphorylated in the absence or presence of EGF (lanes A). Extracts of total phosphoproteins were then subjected to precipitation with non-immune serum (lanes B) or anti-(calpactin II) antiserum (lanes C).

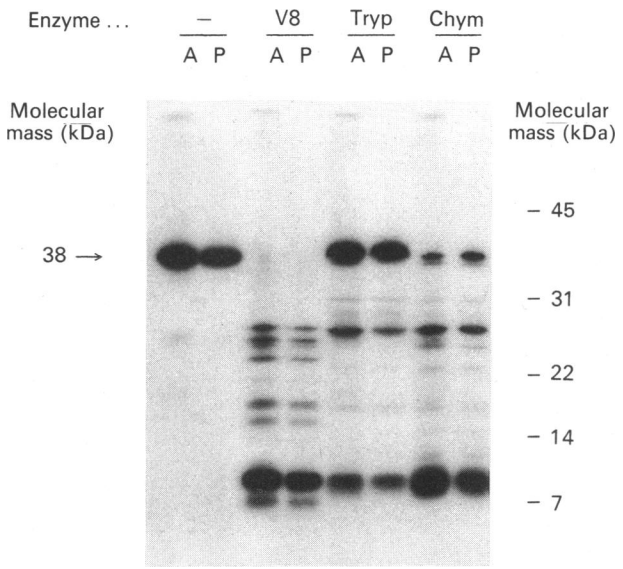

Fig. 3. Proteolytic digestion of phosphorylated 38 kDa substrates from A-431-cell vesicles and placental membranes

Membranes were phosphorylated in the presence of EGF, and the immunoreactive phosphoproteins were precipitated with anti-(calpactin II) antiserum. After separation by electrophoresis, the 38 kDa bands were excised from the gel and subjected to partial proteolysis with staphylococcal V8 proteinase ('V8'; 0.2 μg), trypsin ('Tryp'; 20 μg) or chymotrypsin ('Chym'; 2 μg) as indicated. The resultant phosphopeptide maps for the A-431-cell ('A') and placental ('P') substrates are shown.

of molecular mass ~ 70 kDa was also detected by immunoprecipitation (Fig. 2; see the Discussion section).

When placental membranes were prepared in the presence of Ca^{2+} together with multiple proteinase inhibitors, the major EGF-dependent receptor/kinase substrate in this molecular-mass region also migrated with an apparent molecular mass of 38 kDa; this protein was readily distinguished from the faster-migrating 35 kDa form. The phosphorylated 38 kDa substrates from A-431-cell vesicles and placental membranes were each isolated by immunoprecipitation and were then subjected to partial proteolytic digestion. Phosphopeptide maps obtained with staphylococcal V8 proteinase, trypsin or chymotrypsin (Fig. 3), papain or elastase (results not shown) were identical. Thus A-431-cell vesicular p38 is indistinguishable from human placental calpactin II/lipocortin I.

In vesicles that had been prepared in the absence of Ca^{2+}, EGF-stimulated phosphorylation of calpactin II in vesicular membranes was much less pronounced (Fig. 4*a*; cf. preparations A and C). However, immunoblot analysis of the preparations with anti-(calpactin II) antibody showed the presence of similar amounts of the protein (Fig. 4*b*). Scanning densitometry confirmed that the amount of blottable p38 was only ~ 20% less in vesicles prepared without Ca^{2+} than in those prepared with Ca^{2+} present. The calpactin II in A-431-cell vesicles prepared in the absence of Ca^{2+} was thus somehow less accessible to phosphorylation by the EGF receptor/kinase.

Pretreatment of intact A-431 cells (in Ca^{2+}-containing medium) with EGF before vesicle preparation in the absence of Ca^{2+} did not result in retention of the substrate in a form sensitive to marked phosphorylation (Fig. 4*a*; preparation D). However, if vesicles were prepared in the presence of Ca^{2+} from EGF-treated cells, pronounced phosphorylation of p38 was again observed (Fig. 4*a*; preparation B). Again, the amounts of immunoreactive protein were comparable in vesicles isolated in either the presence or absence of Ca^{2+} (Fig. 4*b*). The phosphorylation of calpactin II in vesicle preparations isolated from EGF-treated cells was not stimulated further by EGF present in the phosphorylation assay, presumably because of prior occupancy and activation of the EGF receptors. A major immunoreactive band of ~ 30 kDa, which was also detected by immunoblotting, did not appear to be phosphorylated by the EGF receptor/kinase (Figs. 4*a* and 4*b*; see the Discussion section).

DISCUSSION

Previous studies of the phosphorylation of the 35–38 kDa EGF receptor/kinase substrate in membranes from A-431 cells (Fava & Cohen, 1984; Cohen & Fava, 1985) showed that EGF-triggered receptor internalization, leading to the formation of 'enzymically-active' endosomes, might be an important process linked to substrate phosphorylation. Those data obtained with membrane preparations were supported by further experiments showing that, in intact A-431 cells prelabelled with [^{32}P]P_i, there was an appreciable time lag (> 5 min) between occupation of the receptor by EGF and a subsequent increase in phosphorylation of the 35 kDa substrate (Sawyer & Cohen, 1985). It was suggested that endosomes containing internalized EGF·receptor/kinase complexes were primarily responsible for the phosphorylation of the 35 kDa substrate (Sawyer & Cohen, 1985). In view of those studies, one main finding of the work we describe here is that neither exposure of intact cells to EGF nor internalization of occupied EGF receptors are obligatory for association of the substrate with the plasma membrane in a form accessible to the EGF receptor/kinase.

Although experimentally elicited membrane vesiculation has received attention as a means of isolating plasma membranes for research (Scott, 1976), vesiculation may also be a physiological/pathological mechanism that operates in such processes as the reaction of the cell to membrane stress (Wagner *et al.*, 1986) and cellular maturation (Johnstone *et al.*, 1987). The biochemistry of membrane vesicles may thus be of wider importance than at first apparent. Shed A-431 vesicle preparations have previously been used for studies of EGF receptor structure and function (Cohen *et al.*, 1982; Gates & King, 1985), but little attention has been paid so far to the endogenous substrates that may be retained within the vesicles.

From our initial studies of phosphorylation with the freshly shed vesicles, it was apparent that they were relatively impermeable to ATP, such that a multiple freeze–thawing treatment was required to observe phosphorylation of the receptor and its substrate. This was an unexpected finding, since early studies had suggested that such vesicles were permeable (Cohen *et al.*, 1982). We found only a low level of EGF-*in*sensitive phosphorylation before freeze–thawing. This is consistent with there being a relatively small proportion of sealed

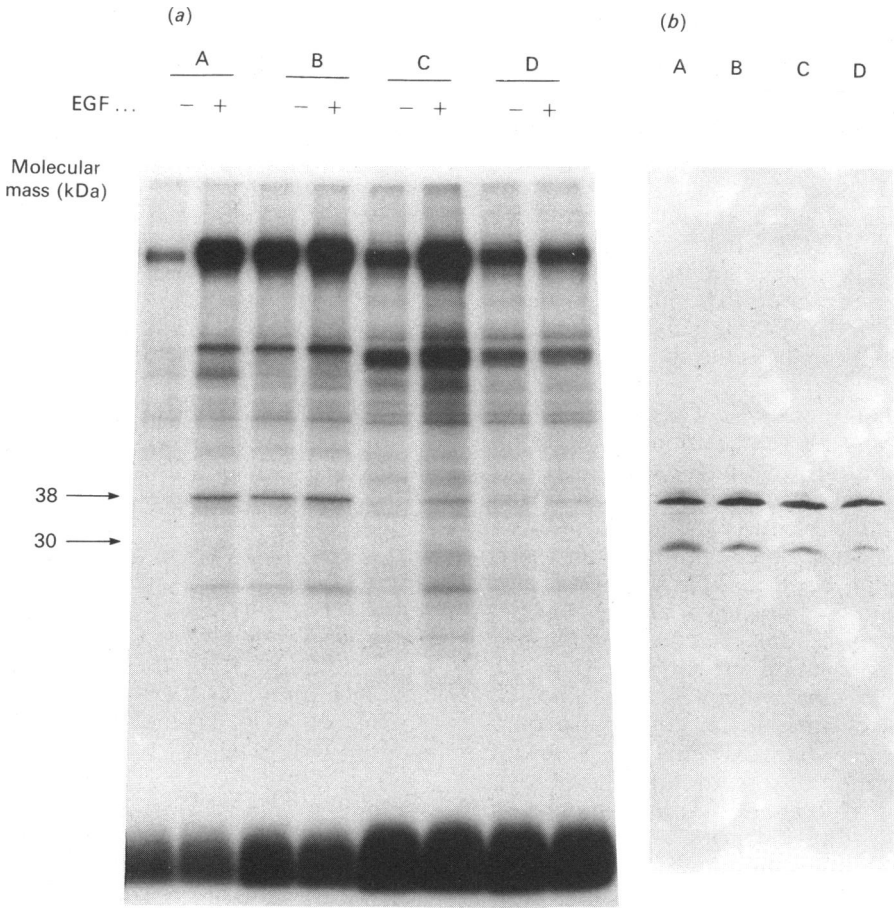

Fig. 4. EGF-dependent phosphorylation (*a*) and calpactin II-immunoreactivity (*b*) of proteins in A-431 membrane vesicles prepared under different conditions

Intact A-431 cells were treated for 4 h in serum-free DMEM supplemented with 33 nM-EGF (lanes B and D) or control vehicle (lanes A and C). Membrane vesicles were then prepared in the presence (lanes A and B) or absence (lanes C and D) of Ca^{2+}. The preparations were then examined for EGF-dependent phosphorylation (*a*) or for immunoreactivity with anti-(calpactin II) antiserum (*b*).

'inside-out' vesicles (having the receptor's kinase domain on the outside and the EGF-binding domain within the vesicle, and thus inaccessible to EGF). These fresh preparations bound appreciable amounts of ^{125}I-EGF (see the Experimental section), so probably contained a reasonably high proportion of 'outside-out' vesicles, with the EGF-binding domain outside and the kinase domain of the receptor and its substrate trapped within. Thus the predominant orientation of the shed vesicles appears to be 'outside-out', the reverse of that of the EGF-triggered endosome.

Another important feature distinguishing the shed vesicles from the previously studied endosomal preparations is that the vesicle preparation minimizes the exposure of the associated proteins to intracellular degradation (cf. Cohen *et al.*, 1982). It is very likely for this reason that we were able to identify the intact 38 kDa form of the receptor/kinase substrate in the vesicle preparation. Our observations are consistent with the view that the 35 kDa form previously observed (Fava & Cohen, 1984; Valentine-Braun *et al.*, 1986) is a proteolytic derivative of the intact 38 kDa substrate.

The molecular mass of one of the major substrates in the vesicles (38 kDa), its immunoreactivity with an antibody that recognizes calpactin II, but not calpactin I (Valentine-Braun *et al.*, 1987), and the identity of its phosphopeptide maps with those of placental calpactin II, leave no doubt in our minds that: (1) the 38 kDa substrate found in the shed vesicles is indeed calpactin II/lipocortin I, and (2) that this represents the same entity described as a 35 kDa EGF receptor/kinase substrate in previous studies involving A-431 cells (Fava & Cohen, 1984; Cohen & Fava, 1985; Sawyer & Cohen, 1985).

The autoradiograms of phosphorylated proteins (Figs. 1 and 4) revealed a number of other vesicle-associated constituents for which phosphorylation was increased in the presence of EGF (apparent molecular masses 85, 70, 55 and 27 kDa). These substrates may be related to minor constituents previously observed to be phosphorylated in an EGF-dependent manner in A-431 membranes (Carpenter *et al.*, 1978; King *et al.*, 1980). In addition, the immunoblots (Fig. 4) revealed the presence of a non-phosphorylated ~30 kDa constituent that corresponds to a calpactin II-related protein observed by us (Valentine-Braun *et al.*, 1987) and by others (Haigler *et al.*, 1987) not to be a substrate for the EGF receptor/kinase. The minor 70 kDa phosphoprotein detected in immunoprecipitation (Fig. 2) likely represents a calpactin II-related EGF receptor/kinase substrate that we have

previously observed in placental membranes (Valentine-Braun *et al.*, 1987). This substrate appears to be distinct from a 68 kDa calpactin-related protein (p68) recently identified by Crompton *et al.* (1988) and said to be a good substrate for tyrosine kinases present in the membrane fraction of A-431 cells. The major 70 kDa protein substrate of the EGF receptor/kinase, purified from human placenta, has a different amino acid sequence from that reported for p68 (D. Michiel, personal communication), suggesting that there are two distinct tyrosine kinase substrates of about that molecular mass. The characterization of the 70 kDa substrate and the other vesicular phosphoproteins is the subject of further work in our laboratory.

It is of interest that there was a requirement for extracellular Ca^{2+} during the vesiculation procedure in order to obtain preparations in which calpactin II was susceptible to EGF-stimulated phosphorylation. From Fig. 4, it is evident that the retention and/or phosphorylation of other vesicle-associated proteins (in particular those migrating in the 70–85 kDa range) was also altered, depending upon the presence or absence of Ca^{2+} during the vesiculation procedure. The greatly diminished EGF-mediated phosphorylation of calpactin II in the vesicles isolated in Ca^{2+}-free buffer (Fig. 4*a*, lanes C) could not be attributed either to an appreciable diminution in EGF receptor/kinase activity *per se* (as reflected by the stimulation of receptor autophosphorylation) or to a marked decrease in the amount of calpactin II associated with the vesicles, as demonstrated by immunoblotting (Fig. 4*b*, lane C). Possible explanations for the apparent diminution in calpactin II phosphorylation in such vesicles are: (1) reduced EGF receptor/kinase activity toward exogenous substrates, without a comparable change in autophosphorylation; (2) a change in the topological distribution of calpactin II mediated by Ca^{2+}; or (3) a functional change in calpactin II itself caused by a Ca^{2+}-triggered process. At present, our data do not distinguish between these three possibilities. However, the finding that membrane vesicles prepared in the absence of Ca^{2+} are capable of supporting EGF-regulated phosphorylation of exogenous purified 35 kDa protein (Fava & Cohen, 1984) would suggest that the EGF receptor/kinase is fully functional in such preparations, and point to a change in calpactin II localization or function as the underlying cause for the observed diminution in phosphorylation.

In summary, our analysis of the vesicle preparation indicates that calpactin II/lipocortin I, along with a number of other protein kinase substrates, is topologically closely associated with the membrane domain in which the EGF receptor/kinase resides. Such vesicle-associated proteins may play an integral role in the action of EGF. Since the formation of membrane vesicles (or endosomes) at the cell surface, by processes of membrane fusion/fission, may involve profound changes in the relationships of cytoskeletal elements with membrane-associated proteins, and since calpactin II is associated with the cytoskeleton (Glenney, 1986; see also Glenney, 1987), it seems reasonable to suggest that this particular receptor/kinase substrate may indeed play some role in the process of membrane fusion/fission. However, there appears to be no requirement for receptor internalization or endosome formation for EGF to be able to stimulate its phosphorylation.

This work was supported by grants from the Canadian Medical Research Council and by a Fellowship Award (to J. B.) from the Alberta Heritage Foundation for Medical Research.

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Received 25 July 1988/23 November 1988; accepted 25 November 1988