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A phospholipid is the membrane-anchoring domain of a protein growth factor of molecular mass 34 kDa in placental trophoblasts

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ABSTRACT **Recently we isolated a protein growth factor of 34 kDa from trophoblastic membranes of human placenta. A fraction (≈50%) of the membrane-associated 34-kDa protein is peripherally associated—i.e., it can be released by high salt treatment. The remainder shows the characteristics of an integral membrane protein—i.e., its release requires detergent treatment. Here we report studies on the structural basis for membrane anchorage of the protein. Phospholipase C was found to release an immunoreactive 34-kDa polypeptide from intact isolated cytotrophoblasts. Studies with isolated trophoblastic membranes showed that phospholipase C specifically released the salt-resistant fraction of the 34-kDa polypeptide. The polypeptide released by phospholipase C showed the same electrophoretic mobility in NaDodSO$_4$/PAGE as the polypeptide prior to phospholipase C treatment. The identity of the released protein with the 34-kDa growth factor has been established by both immunologic and receptor-binding assays. Other studies show that there is biosynthetic incorporation of [^{3}H]myristate into the 34-kDa protein. The myristate label is labile to phospholipase C treatment. These results suggest that some of the 34-kDa protein is anchored to the plasma membrane via a posttranslationally added phospholipid. This mode of anchorage has been observed for some other membrane proteins and raises interesting questions regarding the role of this novel linkage in the mitogenic function of the 34-kDa polypeptide.**

Human placenta contains a growth factor of molecular mass 34,000 that is different from other known placental and nonplacental factors in its antigenic structure, receptor binding specificity, and membrane-association behavior (1–3). Although it is an extracellular protein that exerts its effects upon target cells through a specific surface receptor (1), it is related in its amino acid sequence to the calpactins, a family of cytoplasmic calcium-binding proteins (4). Within placenta the 34-kDa polypeptide is biosynthesized by the embryonally derived trophoblasts. It is expressed on the plasma membranes of both mononuclear and multinuclear trophoblasts. The expression of the protein is developmentally regulated—i.e., trophoblasts from first trimester placenta immunostain far more strongly for the protein than those from term placenta (2). The protein is also expressed in a cell-specific manner in certain adult endocrine and exocrine tissues, such as testis, thyroid, adrenal cortex, pancreas, and stomach, suggesting a role in differentiated cell function (3).

In the trophoblasts, the membrane-association of the 34-kDa protein is mediated through two types of interactions (1): (*i*) a fraction of the membrane-bound protein is only peripherally associated—i.e., it can be released by high-salt treatment (this property has been useful in purification of the protein); and (*ii*) the remainder show the characteristics of an integral membrane protein—i.e., its release requires detergent treatment.

What could be the structural basis for membrane anchorage of the salt-resistant protein? The identical mobilities of the salt-released and salt-resistant polypeptides in NaDodSO$_4$/PAGE discounts the possibility of anchorage to membrane through a protein segment. Also, studies on biosynthesis and turnover of the protein in isolated cytotrophoblasts reveal only one immunoreactive product of 34 kDa in NaDodSO$_4$/PAGE (7). In the light of these findings, we considered the possibility of anchorage through lipids. In recent years a number of membrane proteins have been shown to be anchored to the membrane through posttranslationally added lipids. This mode of anchorage has been confirmed for alkaline phosphatase, Thy-1 antigen, T cell-activating protein, and the surface glycoproteins of trypanosomes (5, 6). We tested whether the 34-kDa growth factor belongs to this category of membrane proteins. The studies described here show that phospholipase C can release the protein from both intact cytotrophoblasts and isolated plasma membranes and that it releases the salt-resistant fraction of the protein. These results suggest that some of the 34-kDa protein is anchored to the plasma membrane via a posttranslationally added phospholipid. This finding raises interesting questions regarding the role of this novel anchorage in the mitogenic function of the 34-kDa protein.

MATERIALS AND METHODS

Antibody. Antibody to the 34-kDa growth factor was generated as described (2). Rabbits were immunized with a pure, homogeneous preparation of the 34-kDa placental protein. The specificity of the antibody for the 34-kDa protein was established by immunoprecipitation, immunoblot analysis, and other analyses (2). The epitopes recognized by this antibody are not carried by other cellular proteins or by a host of other circulating hormones and growth factors.

Isolation of Cytotrophoblasts. Cytotrophoblasts were isolated from term and first-trimester human placenta as described (7). The isolated cells were plated (5×10^5 cells per 16-mm dish) in Dulbecco's modified Eagle's (DME) medium containing 20% fetal bovine serum and were used in experiments 4 hr later.

Labeling of Cytotrophoblasts with [^{35}S]Methionine. Four hours after plating, the cytotrophoblasts were washed with DME medium containing 4 μM methionine and then incubated at 37°C for 16 hr in the same medium containing [^{35}S]methionine (100 μCi/ml; 3.5 mCi/μmol, Amersham; 1 Ci = 37 GBq) and 2.5% dialyzed fetal bovine serum. After incubation, the cells were either immediately lysed and subjected to immunoprecipitation or were first treated with lipases and then subjected to lysis and immunoprecipitation.

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Abbreviations: PtdCho, phosphatidylcholine; PtdIns, phosphatidylinositol.

Treatment of Cytotrophoblasts with Lipases. Cytotrophoblasts (either unlabeled or ^{35}S-labeled) in 16-mm dishes were washed with DME medium and then incubated at 30°C for 1 hr in 100 μl of DME medium containing bovine serum albumin (0.1 mg/ml) with or without one of the following additions: phosphatidylinositol (PtdIns)-specific phospholipase C (10 μg/ml) purified from *S. aureus* (8); phospholipase C (5 units/ml) from *Bacillus cereus* (Sigma); or phospholipase A_2 (5 units/ml) (Sigma).

After the treatment, the medium was carefully taken out, briefly centrifuged to remove any detached cells, and then subjected to immunoprecipitation (in the case of biosynthetically labeled cells) or to immunoblot analyses (in the case of unlabeled cells). The unlabeled medium samples for immunoblot analysis were subjected to trichloroacetic acid precipitation (0.5 ml of 10% trichloroacetic acid and 10 μl of insulin at 1 mg/ml as a carrier protein), followed by solubilization in $NaDodSO_4$ sample buffer prior to analysis.

The cells remaining in the dishes were combined with those detached off the dish and then treated as follows: (*i*) the biosynthetically labeled cells were lysed with 50 μl of a buffer containing 1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, 25 μg of aprotinin per ml, 0.15 M NaCl, and 10 mM sodium phosphate buffer (pH 7.4), and the lysate was subjected to immunoprecipitation as described below; (*ii*) the unlabeled cells were lysed with 50 μl of $NaDodSO_4$ sample buffer and subjected to immunoblot analyses as described below.

Immunoprecipitation. The samples were treated first with 240 μg of rabbit nonimmune IgG at 4°C for 1 hr and then with 10 mg of *Staphylococcus aureus* at 4°C for 30 min. The resultant supernatant was subjected to immunoprecipitation by using 25 μg of rabbit anti-34-kDa peptide immune IgG (or 25 μg of nonimmune rabbit IgG) and 10 mg of washed *S. aureus* as described (2) and then was analyzed by $NaDodSO_4$ gel electrophoresis and fluorography (9).

Electrophoretic Immunoblotting Method. The cell or medium samples were subjected to $NaDodSO_4$/PAGE under reducing conditions (9). The electrophoretically separated proteins were electrophoretically transferred to nitrocellulose sheets. The sheets were treated with anti-34-kDa peptide antibody, a second antibody, and ^{125}I-labeled protein A as described (2) and then were washed, dried, and subjected to autoradiography.

Labeling of Cytotrophoblasts with [^{3}H]Myristic Acid. At 4 hr after plating, the cytotrophoblasts were incubated at 37°C for 16 hr with [^{3}H]myristic acid (100 μCi/ml; 20 Ci/mmol, New England Nuclear) in DME medium containing 2.5% dialyzed fetal bovine serum. After incubation, the cells were lysed, and the lysates from labeled cells were incubated at 30°C for 1 hr with or without 10 units of phospholipase C per ml in a buffer containing 20 mM Hepes (pH 7.4), 0.5% Triton X-100, 2 mM phenylmethylsulfonyl fluoride and 20 μg of leupeptin per ml. The samples were then subjected to immunoprecipitation, $NaDodSO_4$/PAGE and fluorography.

Lipase Treatment of Placental Membranes. Syncytiotrophoblast membranes were isolated from term human placenta as described (1). Membranes (3 μg of protein) were salt-treated by stirring at 4°C for 2 hr with 20 μl of 3 M NaCl/20 mM Hepes, pH 7.4. The salt treatment results in the removal of <2% of total membrane proteins but releases >30% of the membrane-associated 34-kDa protein. The salt-treated membranes were washed once with 1 ml of 20 mM Hepes (pH 7.4) and then incubated at 30°C for 1 hr in 20 μl of 20 mM Hepes containing 0.1 mg of bovine serum albumin per ml (pH 7.4) with or without 5 units of phospholipase C from *B. cereus* (Sigma) per ml. At the end of the incubation, the membrane suspensions were transferred to ice, diluted with 25 μl of 20 mM Hepes (pH 7.4), and then centrifuged. The supernates and pellets obtained were boiled with $NaDodSO_4$ sample buffer and subjected to immunoblot analysis as described above.

RESULTS

Release of the Immunoreactive 34-kDa Protein from Intact Cells by Phospholipase C. Cytotrophoblasts from both term and first-trimester placenta can biosynthesize a 34-kDa polypeptide that is specifically recognized by the anti-34-kDa peptide antibody (Fig. 1). It should be noted that cells from first-trimester placenta are far more active in biosynthesis of the protein than the term placental cells are (Fig. 1). These results agree with our earlier immunohistochemical observations (2, 3) and indicate that the expression of this protein in placenta is developmentally regulated. Since the first trimester cell type is a good system for studies on the 34-kDa protein, it was used for the experiments described here.

To test for the possibility of large posttranslational modifications, pulse–chase experiments were conducted (7). The results indicate that biosynthesis in neither cell type (term or first trimester) is associated with any gross posttranslational modification that would produce a change in mobility during $NaDodSO_4$/PAGE (data not shown).

To test whether the 34-kDa protein in cytotrophoblasts undergoes a lipid addition that could account for its anchorage in the membrane, we tested the effect of phospholipase C and phospholipase A_2. Biosynthetically labeled first-trimester cytotrophoblasts were used in these studies. Fig. 2 *Left* shows that phospholipase C but not phospholipase A_2 can release the immunoreactive 34-kDa protein from intact cells, suggesting that the protein may be anchored to the membrane through a posttranslationally added phospholipid.

The phospholipase C-mediated release of the 34-kDa protein into medium was not accompanied by the release of the transmembrane EGF receptor (not shown), indicating

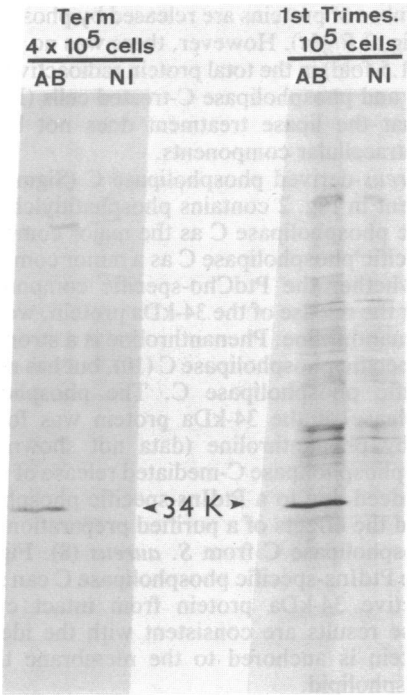

FIG. 1. Synthesis of the 34-kDa polypeptide by purified cytotrophoblasts from term and first-trimester human placenta. The indicated numbers of cytotrophoblasts were labeled for 4 hr with [^{35}S]methionine, and the lysates from labeled cells were subjected to immunoprecipitation with anti-34-kDa antibody (lanes AB) or nonimmune rabbit IgG (lanes NI). K, kDa.

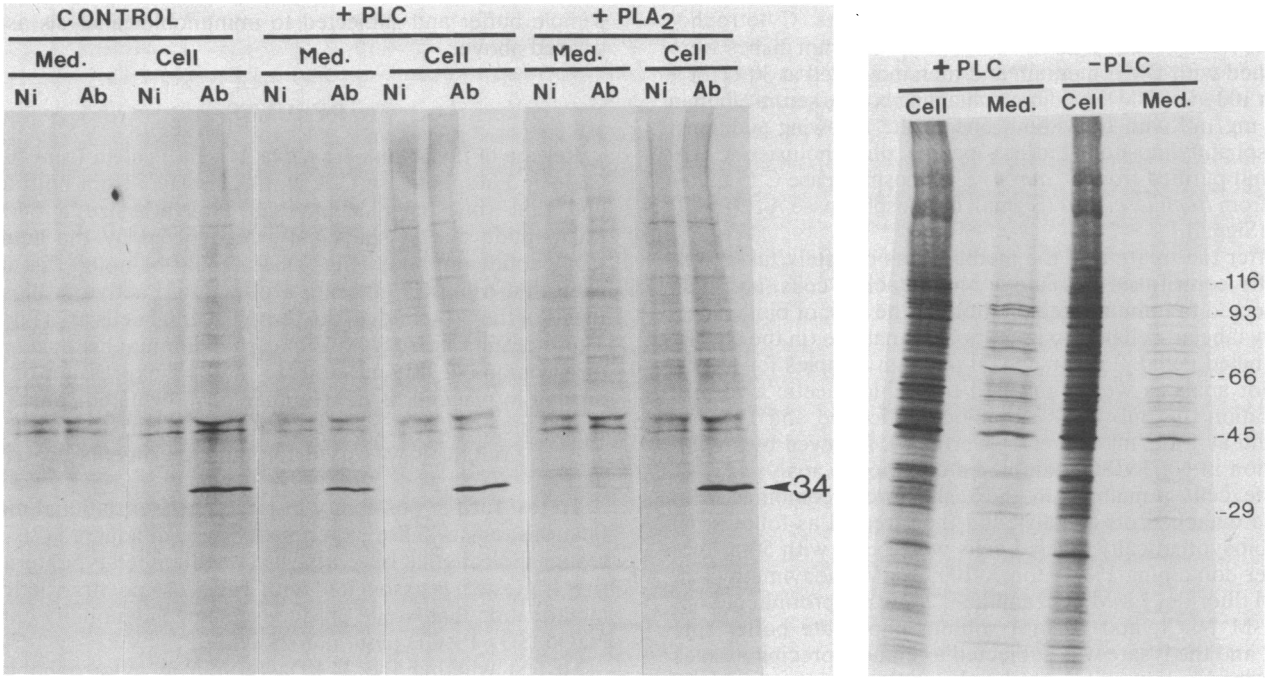

FIG. 2. Release of biosynthetically labeled 34-kDa protein from intact cytotrophoblasts by phospholipase C. [^{35}S]Methionine-labeled first-trimester cytotrophoblasts were treated with phospholipase C (PLC) or phospholipase A_2 (PLA_2). The radioactivity released into the medium (lanes "Med.") and the cell-associated radioactivity (lanes "Cell") were subjected to immunoprecipitation with anti-34-kDa antibody (lanes Ab) or nonimmune rabbit IgG (lanes Ni). (*Left*) Immunoprecipitation analysis of medium-released and cell-associated proteins after treatment of cells with lipases. (*Right*) $NaDodSO_4$/PAGE analysis of total radioactive proteins released into the medium (lanes Med.) by control and phospholipase C-treated cells (no immunoprecipitation was performed). The figure also shows the total protein radioactivity remaining associated with the cells (lanes Cell) after the treatment.

that this lipase-treatment does not release proteins that are anchored to the membrane through hydrophobic protein segments. An examination of the total medium-released ^{35}S-labeled proteins showed some differences in profile, indicating that a number of proteins are released by phospholipase C treatment (Fig. 2 *Right*). However, there was no gross difference (i.e., >1.5-fold) in the total protein radioactivity released from control and phospholipase C-treated cells (Fig. 2 *Left*), indicating that the lipase treatment does not bring about leakage of intracellular components.

The *B. cereus*-derived phospholipase C (Sigma) used for the experiment in Fig. 2 contains phosphatidylcholine (PtdCho)-specific phospholipase C as the major component and a PtdIns-specific phospholipase C as a minor component. To determine whether the PtdCho-specific component is responsible for the release of the 34-kDa protein, we tested the effect of phenanthroline. Phenanthroline is a strong inhibitor of PtdCho-specific phospholipase C (10), but has no effect on PtdIns-specific phospholipase C. The phospholipase C-mediated release of the 34-kDa protein was found to be unaffected by phenanthroline (data not shown). To test whether the phospholipase C-mediated release of the 34-kDa protein is indeed due to a PtdIns-specific phospholipase C, we examined the effects of a purified preparation of PtdIns-specific phospholipase C from *S. aureus* (8). Fig. 3 shows that the pure PtdIns-specific phospholipase C can release the immunoreactive 34-kDa protein from intact cytotrophoblasts. These results are consistent with the idea that the 34-kDa protein is anchored to the membrane through an inositol phospholipid.

We next tested whether *B. cereus*-derived phospholipase C and *B. aureus* derived PtdIns-specific phospholipase C act at similar sites. We found that the *S. aureus* PtdIns-specific phospholipase C treatment of cells that were previously treated with *B. cereus* phospholipase C did not bring about any additional release of the immunoreactive 34-kDa protein, but the reverse treatment (i.e., incubation with *B. cereus* phospholipase C following a treatment with *S. aureus* PtdIns-specific phospholipase C) resulted in release of additional 34-kDa protein (data not shown). These results indicate that a fraction (≈25%) of membrane-anchored 34-kDa protein is released by both *S. aureus* PtdIns-specific phospholipase C and *B. cereus* phospholipase C, but there is an additional fraction (≈10% of the total) that is released only by *B. cereus* phospholipase C and not by *S. aureus* PtdIns-specific phos-

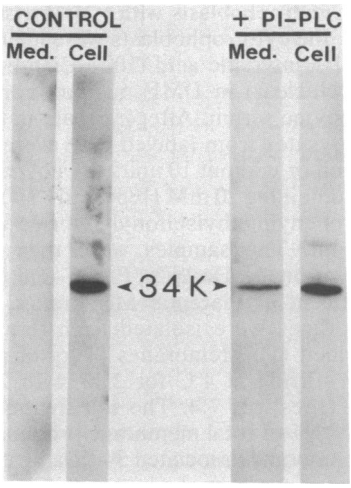

FIG. 3. Release of the 34-kDa protein from intact cytotrophoblasts by PtdIns-specific phospholipase C (PLC). At 16 hr after plating, first-trimester cytotrophoblasts in 16-mm dishes were treated with 10 μg of pure PtdIns-specific phospholipase C per ml. The medium-released (lanes Med.) and cell-associated proteins (lanes Cell) from control and PtdIns-specific phospholipase C-treated plates were subjected to immunoblot analysis as described. K, kDa.

pholipase C. This extra releasing activity of *B. cereus* phospholipase C is unrelated to its PtdCho-specific phospholipase C activity (see the previous paragraph). We do not know whether this difference between *S. aureus* PtdIns-specific phospholipase C and *B. cereus* phospholipase C is due to heterogeneity of the lipid anchor or due to differences in accessibility of the anchoring phospholipid to enzymes from different species.

Acylation of the 34-kDa Protein. We next demonstrated that [^{3}H]myristate is biosynthetically incorporated into the 34-kDa protein (Fig. 4). Identity of the labeled 34-kDa protein was confirmed by immunoprecipitation analysis (Fig. 4). We investigated the nature of the fatty acid–protein attachment. The radioactivity associated with the [^{3}H]myristate-labeled 34-kDa protein was labile to phospholipase C treatment—i.e., the fluorogram of the digested and undigested [^{3}H]myristate-labeled 34-kDa protein shows loss of covalently bound label by phospholipase C digestion. In contrast, similar phospholipase C digestion of the [^{35}S]methionine-labeled 34-kDa protein in solution failed to release radioactivity from the protein (not shown), discounting the likelihood that the observed loss of [^{3}H]myristate label is due to a contaminating protease activity in the phospholipase C preparation. These results indicate that the fatty acid is bound to the 34-kDa protein in the form of phospholipid.

Biological Activity of the 34-kDa Protein Released by Phospholipase C. To test the biological activity of the phospholipase C-released protein, we used purified plasma membranes from placental trophoblasts. The results in Fig. 5 show that a substantial fraction of the salt-resistant 34-kDa protein is released from membrane by phospholipase C. The release showed the following characteristics (data not shown): (*i*) the phospholipase C-mediated release was not inhibited by phenanthroline, a strong inhibitor of PtdCho-specific phospholipase C; (*ii*) the release of the 34-kDa protein was not accompanied by release of the EGF receptor, a prototypical transmembrane protein; and (*iii*) no release was observed after treatment with phospholipase A_2. Thus, the characteristics of phospholipase C-mediated release of the 34-kDa protein from isolated membranes are very similar to those of its release from intact cells.

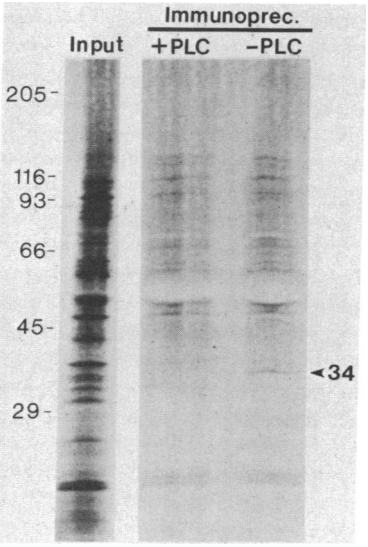

FIG. 4. [^{3}H]Myristic acid labeling of the 34-kDa protein, and loss of label by digestion with phospholipase C (PLC). First-trimester cytotrophoblasts were labeled with [^{3}H]myristic acid. The phospholipase C-induced loss of radiolabel from the 34-kDa protein was specific for the [^{3}H]myristate label. In a parallel experiment in which [^{35}S]methionine-labeled 34-kDa protein was subjected to an identical phospholipase C treatment, no loss of radiolabel was observed.

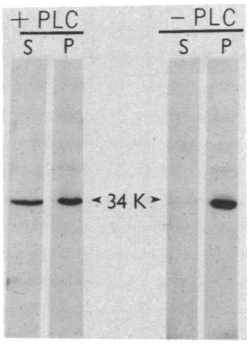

FIG. 5. Phospholipase C (PLC) specifically releases the salt-resistant fraction of the protein from membranes. Syncytiotrophoblast membranes from human term placenta were salt-treated as described. This resulted in the release of about 30% of the immunoreactive 34-kDa protein. The treated membranes were washed and then incubated at 30°C for 1 hr with or without phospholipase C. The supernatant (lanes S) and pellet (lanes P) fractions were subjected to immunoblot analysis. K, kDa.

The results in Fig. 5 show that the salt-resistant 34-kDa protein released by phospholipase C is antigenically identical to the salt-released 34-kDa protein. To test for its biological functionality, we examined the phospholipase C-released protein for its receptor binding activity. The 34-kDa protein released by phospholipase C digestion of placental membranes competes well with authentic ^{125}I-labeled 34-kDa protein for binding to its receptor (Fig. 6). The ability to compete (i.e., affinity of the phospholipase C-released protein for the receptor) is comparable to that of the authentic (i.e., salt-released) 34-kDa protein.

DISCUSSION

The experiments described here have produced the following results. (*i*) Phospholipase C can release the immunoreactive 34-kDa protein from both intact cytotrophoblasts and isolated trophoblastic membranes; the salt-resistant fraction of the protein is specifically released. (*ii*) The identity of the released protein with the 34-kDa growth factor has been established by both immunologic and receptor-binding assays. (*iii*) There is biosynthetic incorporation of [^{3}H]myristic acid into the 34-kDa protein, and the incorporated label is labile with phospholipase C treatment. (*iv*) The involvement of an inositol phospholipid in membrane anchorage is suggested by the fact that pure PtdIns-specific phospholipase C can release the protein.

These results suggest that the 34-kDa protein is anchored to the membrane via a posttranslationally added phospholipid. Recent studies show that anchorage of proteins to membranes is not absolutely dependent upon the presence of a hydrophobic protein segment; anchorage may be mediated through posttranslationally added lipids (5, 6). Fatty acid acylation has been demonstrated in a number of membrane proteins. In some cases, a myristic acid that is amide-linked to the α-amino group on the N-terminal glycine residue is responsible for membrane anchorage (11). In other cases a phospholipid (PtdIns with myristic acid as the acyl group) is covalently attached through an intervening glycan structure to an amino acid in the protein (5, 6). Examples in the latter category include the Thy-1 antigen (12), the T cell-activating protein (13), alkaline phosphatase (14), 5′-nucleotidase (15), acetylcholinesterase (16), decay-accelerating factor (17), 120-kDa neural cell adhesion molecule (18), and the surface proteins of trypanosomes (19) and leishmania (20). These

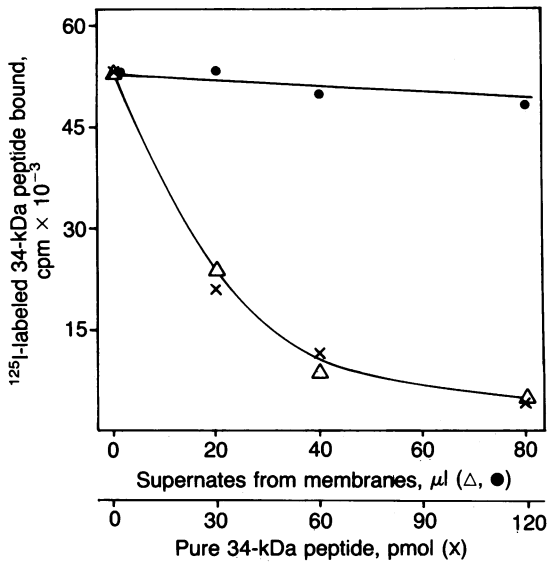

FIG. 6. Receptor-binding activity of the phospholipase C-released 34-kDa protein. Salt-treated placental membranes (1 mg of protein) were incubated at 30°C for 1 hr with or without 10 units of phospholipase C per ml in 250 μl of 50 mM Hepes (pH 7.4) containing 0.2 mM phenylmethylsulfonyl fluoride and 10 μg of leupeptin per ml. The suspension was cooled in ice and centrifuged at 100,000 × *g* for 1 hr. The supernates obtained were subjected to gel-filtration on a TSKG3000SW column (0.8 × 30 cm) in a LKB bioseparation system. Fractions corresponding to elution volumes of 9.5–10.8 ml (the position of the 34-kDa protein) were collected and concentrated by ultrafiltration. This procedure yields a preparation of the 34-kDa protein that is ≈90% pure and free of phospholipase C and major membrane-released protein contaminants. The preparation in 300 μl of Earle's balanced salt solution containing 1 mg of bovine serum albumin per ml (EBSS-BSA) was tested for competitive inhibition of ^{125}I-labeled 34-kDa protein binding to mouse 3T3 cells as described (1). Briefly, the cell monolayers (in 16-mm dishes) were incubated at 4°C for 2 hr in 200 μl of EBSS-BSA containing 5 nM ^{125}I-labeled 34-kDa protein (50,000 cpm/ng) with or without one of the following additions: (*i*) the purified and concentrated supernate from phospholipase C-treated membranes (△), (*ii*) the purified and concentrated supernate from control-treated membranes (●), and (*iii*) pure unlabeled 34-kDa protein isolated from the salt extract of membrane (×). At the end of incubation, cell-associated radioactivity was determined as described (1). The values plotted represent specific binding. Nonspecific binding (≈2200 cpm) was determined in the presence of 2.5 μM unlabeled 34-kDa protein.

PtdIns-anchored proteins are all on the surface. In the case of the 34-kDa growth factor, an extracellular location is indicated by the fact that phospholipase C can release the protein from intact cells (Fig. 2 *Left*). Surface radioiodination studies also indicate an external location for the protein (data not shown).

The studies described here are consistent with the utilization by the 34-kDa protein of a covalently attached PtdIns as the membrane anchor. However it should be noted that PtdIns-specific phospholipase C does not release all of the immunoreactive 34-kDa protein from membranes. We do not know whether this resistance is due to masking of the phospholipid or the presence of other lipid anchors. A similar or higher degree of resistance to PtdIns-specific phospholipase C has been noted for a number of other PtdIns-anchored proteins—i.e., 5′-nucleotidase (15), acetylcholinesterase (16), and decay-accelerating factor (17).

Apart from anchoring proteins to membranes, the functions of this particular anchoring mechanism are unknown. Several questions are raised by this model and remain to be answered. (*i*) Is the ratio of salt-released to salt-resistant 34-kDa protein in trophoblasts dependent upon the state of activation of an anchor-degrading phospholipase (21) similar to that described (22) as participating in insulin action? (*ii*) Is the release of the 34-kDa protein from its membrane anchor necessary for acquisition of receptor-binding activity, and if so, does the release lead to salt-sensitive binding to membrane through specific receptors? (*iii*) Alternatively, if the membrane-anchored protein itself is competent to bind to receptors, could it mediate intercellular adhesion by this mechanism?

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