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Biochemistry

Characterization of the phosphatidylinositol-glycan membrane anchor of human placental alkaline phosphatase

(inositol-specific phospholipase C)

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ABSTRACT **Placental alkaline phosphatase [orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1] is a member of a diverse group of membrane proteins whose attachment to the lipid bilayer is mediated by a phosphatidylinositol-glycan. To investigate structural aspects of the glycolipid anchor, cultured WISH cells were used because we found that they produce the enzyme in abundant quantities. When cell suspensions were incubated with purified phosphatidylinositol-specific phospholipase C, most of the placental alkaline phosphatase was released from membranes in a hydrophilic form. On incubation of the cells with [^{14}C]ethanolamine, [^{14}C]myristic acid, or *myo*-[^{3}H]inositol, each was incorporated into the phosphatase near the carboxyl terminus, showing that these components, which are found in other phosphatidylinositol membrane-linked proteins, are also present in placental alkaline phosphatase.**

Alkaline phosphatases [AP; orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1] are a ubiquitous group of cell surface glycoproteins whose characteristic enzymatic activity is to hydrolyze phosphate monoesters. Three isozymic forms of AP are found in man and higher primates and are classified in accordance with their predominant tissue distribution—intestinal, placental, and the tissue-unspecific form present in liver, bone, kidney, and most other tissues (1, 2). The APs are highly glycosylated homodimers that have subunit molecular masses ranging from 70 to 80 kDa. The recent molecular cloning of the cDNAs for all three human isozymes (3–9) has provided primary structural data on this protein family.

The APs can be classified as integral membrane proteins since membrane-disrupting agents are necessary to remove them from the lipid bilayer. However, under some conditions of purification, AP isolated from different sources can be essentially hydrophilic and is water soluble when freed from the lipid bilayer (10). Recent evidence suggests that AP is anchored to the plasma membrane by a phosphatidylinositol-glycan (PI-G) moiety (11, 12). It has recently been suggested that proteins attached by their carboxyl terminus to a PI-G be termed PI-G-tailed proteins (42). A rapidly growing and functionally diverse group of proteins that utilize this mechanism of membrane attachment includes the variant surface glycoproteins of trypanosomes, Thy-1 antigen, T-cell activating protein, the globular form of acetylcholinesterase, the neural cell adhesion molecule 120, and the decay-accelerating factor of complement (for reviews, see refs. 13–15). The major criterion used initially to classify these proteins was their specific release from cellular membranes by phosphatidylinositol-specific phospholipase C (inositol phospholipase). Consistent with this requirement, all three AP isoenzymes are released from intact cells by this phospholipase (11, 12, 16, 17). In both the variant surface glycoprotein and Thy-1, for which direct chemical data are available on the structure of the PI-G moiety, this moiety is attached to the carboxyl terminus of the protein by an amide-linked ethanolamine (18, 21). It also contains a glycan of variable structure, unacetylated glucosamine, inositol, phosphate, fatty acids, and glycerol.

The biogenesis and transfer of the unique PI-G structure to certain membrane proteins to form PI-G-tailed proteins remains a matter of speculation. Largely based on data obtained from variant surface glycoprotein biosynthesis, it appears that co- or posttranslationally a hydrophobic, carboxyl-terminal extension of the nascent protein is removed, the glycolipid moiety is added to the mature protein, and then the conjugated protein is transported to the cell surface. This putative condensation, or PI-G-tailing step [also termed "glypiation" by Cross (14)], occurs rapidly, is independent of N-linked glycosylation, and probably occurs in the endoplasmic reticulum (20, 22–24). In the case of mammalian Thy-1, direct chemical analysis of the conjugated protein has also documented the absence of the hydrophobic, carboxyl-terminal peptide sequence predicted from the cDNA structure (21).

Although AP was one of the first membrane proteins to be classified as a PI-G-tailed protein, all that is known is that it contains inositol (25), can be cleaved from membranes specifically by inositol phospholipase, and following treatment with inositol phospholipase, loses its hydrophobic character (10). To investigate other aspects of the PI-G moiety, we used WISH cells, a human amniotic cell line that synthesizes the placental AP (PLAP) in abundant quantities. Several of the radiolabeled components of the putative PI-G moiety, when incubated with WISH cells, were incorporated into PLAP in covalent linkages near the carboxyl terminus of the mature protein.

MATERIALS AND METHODS

Cell Culture. The WISH cell line (ATCC-CCL 25), originally derived from normal amniotic tissue, was grown on 60-mm culture dishes containing F-11 medium supplemented with 10% fetal calf serum and antibiotics. Cells were seeded at a density of 1.5×10^5 cells per ml 48 hr prior to the initiation of all experiments.

AP Extraction and Assay. Confluent cell monolayers (10^6 cells) were rinsed twice with Dulbecco's phosphate-buffered saline (PBS) and twice with extraction buffer (10 mM Tris·HCl, pH 7.4/1 mM $MgCl_2$/20 μM $ZnSO_4$) and then were scraped into extraction buffer. The cell suspension was centrifuged at 2000 rpm ($1000 \times g$) for 5 min at 25°C, and the pellet was homogenized in extraction buffer (5 vol) using a Potter–Elvehjem tissue grinder. 1-Butanol was then added to a final concentration of 30% (vol/vol), and the mixture was

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Abbreviations: AP, alkaline phosphatase; PLAP, placental alkaline phosphatase; PI-G, phosphatidylinositol-glycan.

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incubated, with mixing, for 2 hr at 25°C. The homogenate was centrifuged (12,000 × *g* for 10 min at 25°C), the butanol and interface were discarded, and the aqueous layer was retained for further analysis. Approximately 80% of the total AP activity was extracted by using this procedure. Enzyme activity was determined as described in the Sigma catalog with *p*-nitrophenyl phosphate as substrate. One unit of AP corresponds to 1 μmol of substrate hydrolyzed per min at 37°C. The effects of the specific inhibitors L-phenylalanine (20 mM) and L-homoarginine (20 mM) were determined by preincubation with the extract for 10 min at 37°C. Heat inactivation was performed at 56°C or 65°C for 10 min. Calculation of the quantity of a specific AP isoenzyme type relative to the total AP activity was determined as described by Mulivor *et al.* (26). A crude particulate fraction from human placenta was prepared as described previously (10).

NaDodSO$_4$/PAGE and Immunological Blot Analysis. NaDodSO$_4$/PAGE was performed using the discontinuous system of Laemmli (27) with 8 or 10% acrylamide separating gels. Electroblotting of proteins to nitrocellulose and subsequent processing of filters was carried out as previously described (28). The rabbit antiserum to human PLAP is directed against the first nine amino acids of the mature enzyme (29) and is highly specific for this isozyme (unpublished observations). Blotted proteins were visualized using goat anti-rabbit IgG horseradish peroxidase (Bio-Rad) with 3-amino-1-ethylcarbazole (30) (Sigma) as substrate. In order to visualize gels from metabolic radiolabeling experiments, they were fixed in 40% methanol/10% acetic acid, fluorographed with EN^{3}HANCE (New England Nuclear), dried, and exposed to Kodak XAR-5 film at −70°C.

RNA Blot-Hybridization Analysis. Total cellular RNA was extracted by the guanidine thiocyanate procedure followed by CsCl centrifugation (31). The RNA was electrophoresed in a 1.1% agarose gel after denaturation with glyoxal and dimethylsulfoxide and was electroblotted onto a nylon membrane (Zeta-Probe; Bio-Rad). The filter was probed according to Thomas (32) utilizing a ^{32}P-labeled *Kpn* I/*Taq* I PLAP cDNA fragment consisting of nucleotides 1928–2305 (5). This fragment, from the 3′ untranslated region, yields a probe that is highly specific for the PLAP transcript (unpublished results).

Biosynthetic Radiolabeling and Immunoprecipitation. Prior to addition of radiolabeled compounds, confluent cell monolayers (≈1 × 10^6 cells) were rinsed twice in PBS and twice in the appropriate labeling medium. For [^{35}S]methionine labeling (1400 Ci/mmol; 50 μCi; 1 Ci = 37 GBq), cells were preincubated for 1 hr in methionine-free Eagle's minimal essential medium (MEM)/5% dialyzed fetal calf serum. Fresh medium containing [^{35}S]methionine was then added, and labeling was conducted for 12 hr. Labeling with [1-^{14}C]myristic acid (54 mCi/mmol; 400 μCi; Amersham) was performed for 3 hr in serum-free MEM/5 mM sodium pyruvate to prevent conversion to amino acids. Incubation with *myo*-[D-^{3}H]inositol (110 Ci/mmol; 200 μCi) or [2-^{14}C]ethanolamine (50 mCi/mmol; 200 μCi) was conducted for 5 hr in vitamin-free MEM/5% dialyzed fetal calf serum. Following incubation with the radiolabeled metabolites, cells were washed three times in PBS and either suspended in phospholipase assay buffer (10 mM Tris·HCl, pH 7.4/0.25 M sucrose/10 mM glucose) or solubilized in RIPA buffer (50 mM Tris·HCl, pH 7.5/1% Triton X-100/0.5% sodium deoxycholate/0.2% NaDodSO$_4$/100 mM NaCl/1 mM EDTA). RIPA cell lysates were clarified by centrifugation (12,000 × *g* for 15 min at 4°C), and specific immunoprecipitation of PLAP was conducted using affinity-purified rabbit anti-human PLAP antiserum (Dakopatts, Copenhagen) (at a final dilution of 1:500 for 1.5 hr at 25°C) followed by incubation for 15 min at 25°C with 100 μl of formalin-treated *Staphylococcus aureus* cells bearing protein A (IgGsorb, The Enzyme Center, Malden, MA). Immunoprecipitates were washed three times in RIPA buffer, and bound immune complexes were eluted by boiling for 5 min in sample buffer (3% NaDodSO$_4$/100 mM dithiothreitol/10% glycerol/0.001% bromophenol blue/62.5 mM Tris·HCl, pH 6.8). When immunoprecipitates were to be subjected to HPLC analysis (except for [^{35}S]methionine), prior to dissociation from IgGsorb, noncovalently bound radioactivity was removed by extensive washing in RIPA buffer and chloroform/methanol extraction (2:1, vol/vol). Immune complexes were then eluted from the IgGsorb by boiling in 50 mM Tris·HCl, pH 7.4/1% NaDodSO$_4$.

Treatment of Cells with Phospholipases. Purification and assay of inositol phospholipase and phosphatidylcholine-specific phospholipase C (choline phospholipase) from *Bacillus cereus* were carried out as described by Sundler *et al.* (33). The purified enzymes had activities of ≈3000 units per mg of protein, where 1 unit is defined as the amount of enzyme catalyzing the release of 0.1 μmol of inositol or choline in 2 hr at 25°C. Purified inositol phospholipase exhibited only slight (<5%) activity towards phosphatidylcholine and had no detectable protease activity when casein or PLAP was used as substrate and trypsin was used as a standard.

Release of PLAP from intact cells by the phospholipases was monitored in the following manner. Cells were washed three times in PBS and three times in lipase assay buffer and were then suspended in the same buffer at a concentration of ≈5 × 10^6 cells per ml. Duplicate 30-μl aliquots of the cell suspension were incubated with 1 unit of inositol phospholipase, 1 unit of choline phospholipase, or buffer for 1.5 hr at 37°C. For treatment with bromelain, cell suspensions were incubated with 0.1 unit of the protease for 45 min at 37°C. Following incubation, the cells were pelleted by centrifugation at 12,000 × *g* for 2 min, and the supernatants and pellets were first assayed for PLAP enzyme activity and then were immunoprecipitated using PLAP-specific antiserum. For deamination, immunoprecipitates of *myo*-[D-^{3}H]inositol-labeled PLAP were treated with nitrous acid in sodium acetate (pH 4) for 4 hr at 37°C as described by Medof *et al.* (24).

HPLC Analysis of Radiolabeled PLAP. PLAP immunoprecipitates absorbed to IgGsorb were first freed of noncovalently bound radioactivity as described above. To remove any residual noncovalently bound radioactivity released during elution of the immune complexes from IgGsorb, each eluted immune complex was subjected to repeated extraction in chloroform/methanol (2:1). Any remaining water-soluble label was removed by ultrafiltration in a Centricon 30 (Amicon). For analysis of covalently bound fatty acids, solutions containing extracted immune complex were taken to dryness and hydrolyzed in 100 μl of 6 M HCl/0.2% thioglycolic acid for 24 hr at 110°C *in vacuo*. The hydrolysate was then extracted with benzene until no radioactivity was left in the aqueous phase, and the benzene extracts were pooled and dried under nitrogen. The fatty acids were converted to their methyl esters by treatment with 83% methanol/2 M HCl. After evaporation of the solvent, the fatty acid methyl esters were redissolved in methanol; 400 μg each of unlabeled myristate, methyl myristate, palmitate, and methyl palmitate were added; and the samples were subjected to reverse-phase HPLC on a C-18 column (Lichrosorb ODS; Rainin, Woburn, MA; 4.6 mm × 25 mm). The mobile phase was 85% acetonitrile in 0.1% trifluoroacetic acid/H_2O; the flow rate was 1 ml/min. Three-minute fractions were collected. The elution positions of the unlabeled standards were determined by absorption at 214 nm, and radioactivity was measured in a scintillation counter. Covalently-bound *myo*-[D-^{3}H]inositol was released from the immunoprecipitates by hydrolysis *in vacuo* (6 M HCl/0.2% thioglycolic acid for 8 hr at 110°C). Unlabeled *myo*-inositol (0.1 μmol), added as carrier prior to hydrolysis, was stable to the hydrolysis conditions. The hydrolysates were dried, dissolved in water,

and injected onto a Polypore CA ion-exchange column (100 mm × 4.6 mm; Brownlee) equipped with a Polypore H guard cartridge. The mobile phase was water at a flow rate of 0.3 ml/min, and the column temperature was maintained at 85°C. One-half minute fractions were collected. Several radiolabeled monosaccharides were used for calibration as shown in the corresponding figure legends. Release of covalently bound [2-^{14}C]ethanolamine from acid hydrolysis of immunoprecipitates was monitored by ion-exchange HPLC on an amino acid analyzer equipped with fluorescamine detection (34). The ethanolamine standard eluted with a retention time of 118 min. The flow rate was 6 ml/hr, and 0.5 min fractions were collected. In each case, control experiments were carried out in which the labeled compound was added to the cell culture, which was then harvested immediately without further incubation. These samples were treated as described above.

RESULTS

Source of PLAP. Confluent cultures of WISH cells yielded extracts containing ≈20 units of PLAP per mg of protein, a value as high as any previously reported for cultured cell lines, including HeLa cells (35). Based on established criteria used to distinguish among the isozymes—namely, differential heat inactivation and inhibition by specific amino acids (36) (Table 1)—the PLAP isozyme accounted for over 60% of the total activity, while the remaining activity could be ascribed to the intestinal form. Although the latter shares over 90% homology with PLAP, the two are readily distinguished by the antisera and cDNA reagents that were utilized. The cells were devoid of the tissue-unspecific isozyme. Due to the predominance of PLAP in these cells and the availability of specific probes, this isozyme became the focus of further experiments. To determine the size of the PLAP transcript, blot-hybridization analysis of total RNA was carried out with a highly specific, ≈0.4-kilobase (kb), ^{32}P-labeled cDNA probe from the 3′ untranslated region of PLAP cDNA (see *Materials and Methods*). As shown in Fig. 1*A*, lanes 2 and 3, a single, intense band was observed at about 2.8 kb. This coincides with the PLAP mRNA in extracts of human placenta (Fig. 1*A*, lane 1). Immunological blot analysis using PLAP-specific antiserum detected a single, specific band of protein of ≈68 kDa (Fig. 1*B*, lane 2) identical to that found in butanol extracts of human placenta (Fig. 1*B*, lane 1).

Mode of Membrane Attachment of PLAP. The effect of inositol phospholipase on the release of PLAP from WISH cells was investigated by labeling the cells with [^{35}S]methionine for 12 hr, treating whole cell suspensions with inositol phospholipase, choline phospholipase, or buffer, and isolating the PLAP from cell pellets and supernatants by immunoprecipitation. NaDodSO$_4$/PAGE followed by fluorography was used to analyze the immunoprecipitates. Immunoprecipitates of PLAP from cell pellets that were incubated with buffer alone yielded an intensely stained band of protein at ≈68 kDa (Fig. 2, lane 2). This was not observed when preimmune serum was substituted for the specific antiserum (Fig. 2, lanes 7 and 8). On incubation of cell suspensions with choline phospholipase, only a small amount of PLAP was solubilized (Fig. 2, lane 3). In contrast, inositol phospholipase solubilized a large proportion of immunoprecipitable PLAP (60–70%) without any change in molecular mass (Fig. 2, lane 5). When WISH cells were treated with bromelain, an endoprotease that has been reported to cleave the peptide chain of PLAP near its carboxyl terminus (37), most of the enzyme was released into the medium (data not shown).

Membrane-bound PLAP is considered to be hydrophobic because of its putative PI-G moiety (10). To investigate further the effects of the various enzymes on the cleavage of PI-G from PLAP derived from WISH cells, we utilized the Triton X-114 phase separation method that was previously used by Malik and Low (10). The segregation of PLAP was then measured in the detergent and aqueous phases by enzyme activity as well as by immunoprecipitation of the protein. Following treatment of the cells and placental tissue with buffer or with choline phospholipase, almost all the AP activity was recovered in the hydrophobic phase. In contrast, inositol phospholipase altered the partitioning behavior so that most of the PLAP activity was found in the hydrophilic phase (Fig. 3*B*). Subsequent to treatment with the various enzymes, ^{35}S-labeled PLAP protein partitioning in the aqueous phase was identified by immunoprecipitation followed by NaDodSO$_4$/PAGE (Fig. 3*A*, lane 3). The results obtained by this method coincided with the measurements of enzyme activity. Incubation with buffer or choline phospholipase produced small amounts of hydrophilic PLAP (Fig. 3*A*, lanes

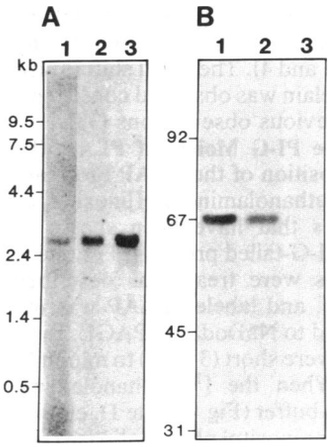


FIG. 1. (*A*) Blot-hybridization analysis. Total RNA was extracted from placenta (lane 1, 10 μg) or from WISH cells (lane 2, 10 μg; lane 3, 20 μg). RNA sizes (in kb) were determined by comparison with RNA size standards (Bethesda Research Laboratories). (*B*) Immunological blot analysis. Extracts of placenta (lane 1, 10 μg) or WISH cells (lanes 2 and 3, 100 μg) were incubated with PLAP antiserum (lanes 1 and 2) or preimmune serum (lane 3). Molecular mass markers (Bethesda Research Laboratories) are given in kDa.

Table 1. Biochemical characterization of AP activity in WISH cell butanol extracts

Treatment	AP activity, % of control
Heat (56°C or 65°C for 10 min)	63
L-Phenylalanine	22
L-Homoarginine	100
Heat + L-phenylalanine	15
Heat + L-homoarginine	70

Ten millimolar L-homoarginine or 10 mM L-phenylalanine was used to selectively inhibit the tissue-unspecific or placental isozyme, respectively. Both the tissue-unspecific and intestinal isozymes are relatively heat labile, whereas PLAP is heat stable (25). Control AP activity was 25 milliunits.

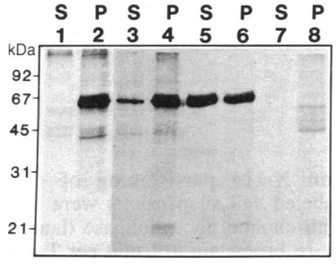


FIG. 2. Release of PLAP from cells by inositol phospholipase. [^{35}S]methionine-labeled cell suspensions were incubated for 1.5 hr at 37°C in buffer (lanes 1, 2, 7, and 8), choline phospholipase (1 unit per 2 × 10^5 cells; lanes 3 and 4), or inositol phospholipase (1 unit per 2 × 10^5 cells; lanes 5 and 6). Soluble (S) and particulate (P) material were separated by centrifugation; PLAP was isolated by immunoprecipitation with antiserum (lanes 1–6) and was analyzed by NaDodSO$_4$/PAGE and fluorography. The preimmune control is shown in lanes 7 and 8.

1 and 2). Digestion with inositol phospholipase or bromelain, however, produced large amounts of PLAP protein in the aqueous phase (Fig. 3*A*, lanes 3 and 4). The small shift in size following treatment with bromelain was observed consistently and is in agreement with previous observations (38).

Radiolabeling of the Putative PI-G Moiety of PLAP. To further characterize the composition of the PLAP PI-G tail, cells were incubated with [^{14}C]ethanolamine, [^{3}H]inositol, or [^{14}C]myristic acid, metabolites that have been shown to compose the anchor of other PI-G-tailed proteins. Following radiolabeling, cell suspensions were treated as described under *Materials and Methods*, and labeled PLAP was immunoprecipitated and subjected to NaDodSO$_4$/PAGE. In all experiments, labeling periods were short (3–5 hr) to minimize metabolic interconversion. When the [^{14}C]ethanolamine-labeled PLAP was treated with buffer (Fig. 4, lane 1), choline phospholipase (Fig. 4, lane 2), or inositol phospholipase (Fig. 4, lane 3), a labeled band of protein was observed at 67 kDa. Following treatment with the endoprotease bromelain, most of the radioactivity incorporated into PLAP was released (Fig. 4, lane 4). The site of incorporation of *myo*-[^{3}H]inositol into PLAP was probed in two ways, by nitrous acid deamination, a method reported to cleave the putative glucosamine-inositol linkage when the amino group of glucosamine is not acetylated (39), and by bromelain treatment. Nitrous acid (Fig. 4, lane 6) and bromelain (Fig. 4, lane 7) released the *myo*-[^{3}H]inositol label completely from PLAP, whereas incubation with sodium citrate buffer (Fig. 4, lane 5) or the phospholipases had no effect (data not shown). Labeling with [^{3}H]myristic acid is shown in Fig. 4, lanes 8–11. Treatment of the [^{3}H]myristate-labeled PLAP with inositol phospholipase (Fig. 4, lane 10) or bromelain (Fig. 4, lane 11) completely removed the label, whereas choline phospholipase (lane 8) was without effect.

Covalent association of all the radiolabeled compounds with PLAP is indicated since in all cases the label remained bound to the immunoprecipitated enzyme after washing of the precipitates in NaDodSO$_4$, Triton X-100, and sodium

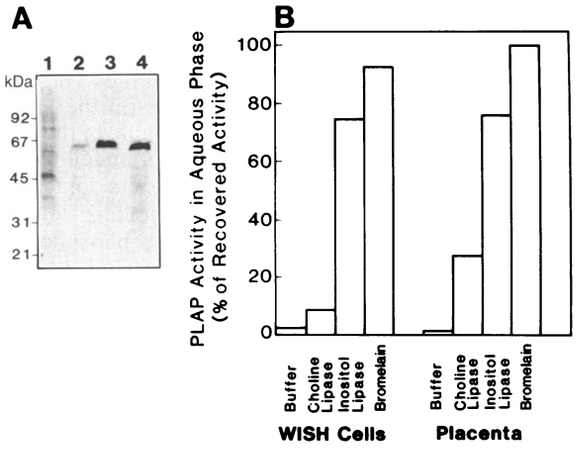


FIG. 3. Triton X-114 partitioning of cell extracts. (*A*) [^{35}S]methionine-labeled cell suspensions were incubated in buffer alone (lane 1), with choline phospholipase (lane 2), inositol phospholipase (lane 3), or bromelain (0.1 unit per 2 × 10^5 cells; lane 4). The cells were then extracted with Triton X-114, aqueous and hydrophobic phases were separated, and PLAP was isolated from both phases by immunoprecipitation. Each lane indicates the labeled PLAP left in the aqueous phase after partitioning. (*B*) Aliquots of the aqueous phase from *A* were assayed for PLAP enzyme activity. An extract of human placenta that received the same treatment as the cell extract is included for comparison. PLAP enzyme activity was 25 milliunits for the cells and 57 milliunits for placental extracts. Recovery of activity in both aqueous and hydrophobic phases was essentially quantitative in all cases.

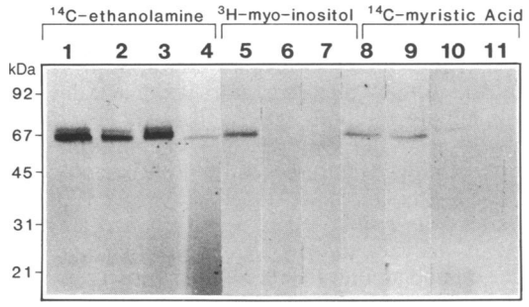
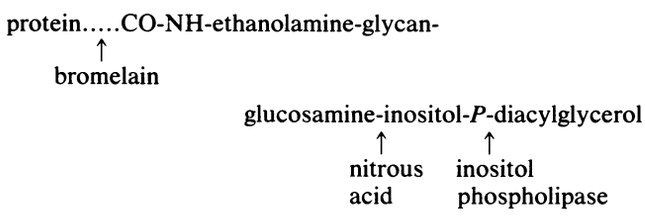


FIG. 4. Incorporation of radiolabeled putative anchor precursors into PLAP. Cells (10^6) were incubated with [^{14}C]ethanolamine (lanes 1–4), *myo*-[^{3}H]inositol (lanes 5–7), or [^{14}C]myristic acid (lanes 8–11), extracted, and then treated with the appropriate buffer (lanes 1, 5, and 8), bromelain (lanes 4, 7, and 11), nitrous acid (lane 6), choline phospholipase (lanes 2 and 9), or inositol phospholipase (lanes 3 and 10). PLAP was then isolated by immunoprecipitation and was subjected to NaDodSO$_4$/PAGE and fluorography.

deoxycholate; delipidation with chloroform/methanol; trichloroacetic acid precipitation; and boiling in NaDodSO$_4$ with subsequent NaDodSO$_4$/PAGE. To determine whether the labeled precursors had been incorporated directly into PLAP or had done so after metabolic transformation, extracted immune complexes (see *Materials and Methods*) were hydrolyzed in acid and subjected to HPLC analysis. [^{14}C]Ethanolamine released from PLAP was detected on an amino acid analyzer as a labeled peak coeluting with the unlabeled ethanolamine standard (Fig. 5*A*). The [^{14}C]-ethanolamine peak accounted for 60% of the radioactivity applied to the column. *myo*-[^{3}H]Inositol was released from PLAP and applied to an ion-exchange HPLC column that resolves monosaccharides. Greater than 90% of the applied radioactivity coeluted with the *myo*-inositol standard (Fig. 5*B*). Fatty acids present in the hydrolysate were extracted with benzene, treated with methanol/HCl, and resolved by reverse-phase HPLC on a C-18 column. Apparently esterification was inefficient, and all of the applied radioactivity was distributed in three peaks (Fig. 5*C*) having the elution characteristics of free myristic acid (48%), free palmitic acid (13%), and methyl palmitate (30%). Interconversion of myristate to palmitate has been reported previously even when short labeling periods were employed (39).

DISCUSSION

Current knowledge of the structure of the nonprotein moiety of PI-G-tailed proteins is based largely on data obtained from just a few isolated proteins (18–24, 40). All PI-G-tailed proteins that have been characterized thus far have the basic structure shown below:



They vary with respect to the glycan and the fatty acids in the diacyl glycerol moiety. Data presented here further characterize the PI-G moiety of PLAP and show its similarity to those found coupled to other proteins in this class (see diagram). Three of the components that are known to be present in the PI-G tail of other proteins (ethanolamine, *myo*-inositol, and myristic acid) were incorporated into PLAP, as demonstrated by chromatography of hydrolyzed

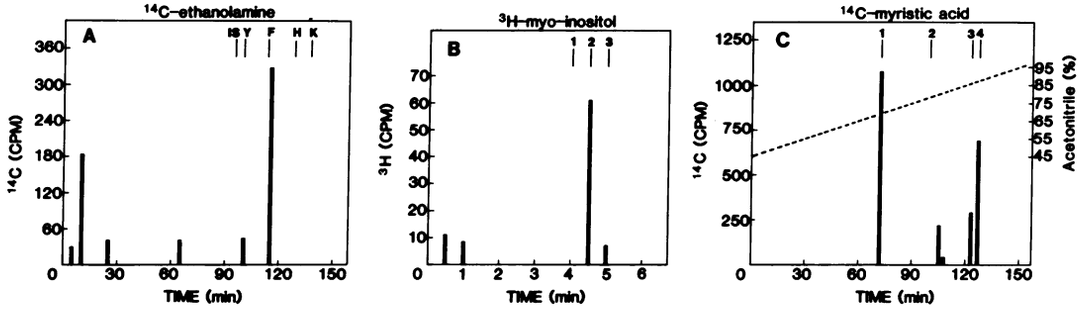


FIG. 5. Identification of the putative anchor components in hydrolysates of PLAP. Immunoprecipitates (Fig. 4) were hydrolyzed in acid and subjected to HPLC. (*A*) Ion-exchange HPLC of [^{14}C]ethanolamine released from PLAP. Calibration standards are amino acids given in the single letter code. IS, isoleucine. (*B*) Ion-exchange HPLC of ^{3}H-*myo*-inositol released from PLAP. Standards: 1, mannose; 2, *myo*-inositol; 3, galactose. (*C*) Reverse phase HPLC of ^{14}C-labeled fatty acids released from PLAP. Standards: 1, myristic acid; 2, palmitic acid; 3, methyl myristate; 4, methyl palmitate.

PLAP. The labeled precursors were localized at the carboxyl terminus of PLAP. Because of their selective release by the reagents shown in the above diagram, they are most probably present in a sequence similar to that found in other PI-G-linked proteins.

In accord with previous studies on tissue-derived PLAP, WISH cell PLAP was shown to be released from membranes in a hydrophilic form by highly purified inositol phospholipase. The [^{3}H]myristic acid that was incorporated into PLAP was specifically released by inositol phospholipase which shows that it was present in a diacyl glycerol moiety. A substantial proportion of myristic acid was converted to palmitic acid during incubation, and both fatty acids were found in PLAP. Whether or not both are normally present in the diacyl glycerol moiety of PLAP remains to be seen. Evidence for a glycosidic bond between *myo*-inositol and a non-N-acetylated amino sugar (possibly glucosamine) was provided by showing that treatment with nitrous acid cleaved the *myo*-[^{3}H]inositol from PLAP. This finding corroborates the recent report by Malik and Low that PLAP from placental tissue contains stoichiometric amounts of *myo*-inositol in a form that is predominantly bound by nitrous acid-labile linkages (10). The exact chemical composition of the PI-G moiety in PLAP remains to be determined.

Just as this paper was being readied for submission, it came to our attention that Jemmerson and Low (41) had made similar and complementary observations on the incorporation of some of the PI-G components into PLAP produced by HeLa cells.

We wish to thank Cynthia Smith for expert assistance with cell culture.

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