the electrode obeyed the Nernst equation at all Ca^{2+} concentrations.

10. $^{45}Ca^{2+}$ release rate is expressed as a percent of the total initial vesicular $^{45}Ca^{2+}$ content released in each fraction (*6*). Calculations normalized to percent of $^{45}Ca^{2+}$ released provided the most reproducible comparison of data between experiments. In Fig. 2, an additional ordinate is included that provides a less precise (±10%) approximation of the release rates as nanomoles of $^{45}Ca^{2+}$ per milligram of protein per second.
11. IP_3-induced $^{45}Ca^{2+}$ release decayed from its maximum rate with a time course that can be approximated by the sum of two first-order processes. The more rapidly decaying component predominated and is the basis for the majority of the analysis in this report.
12. Net IP_3-stimulated $^{45}Ca^{2+}$ release was the difference between $^{45}Ca^{2+}$ release at given IP_3 and Ca_c^{2+} concentrations and $^{45}Ca^{2+}$ release in the presence of the corresponding Ca_c^{2+} concentration alone. We did not use Mg^{2+} to differentiate the two components of release because the Mg^{2+} block was incomplete at higher Ca_c^{2+} concentrations.
13. Time constants for the rapid decay of $^{45}Ca^{2+}$ release were calculated for a range of IP_3 concentrations (100 nM to 10 μM) at a range of Ca_c^{2+} concentrations (300 nM to 10 μM). For a given Ca_c^{2+} concentration, the time constant for the more rapidly decaying component was constant (±10%) and ranged from 190 ms at 300 nM Ca_c^{2+} to ≤70 ms at or above 3 μM Ca_c^{2+}.
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Primary Structure and Functional Activity of a Phosphatidylinositol-Glycan–Specific Phospholipase D

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A phosphatidylinositol-glycan–specific phospholipase D (PI-G PLD) that specifically hydrolyzes the inositol phosphate linkage in proteins anchored by phosphatidylinositol-glycans (PI-Gs) has recently been purified from human and bovine sera. The primary structure of bovine PI-G PLD has now been determined and the functional activity of the enzyme has been studied. Expression of PI-G PLD complementary DNA in COS cells produced a protein that specifically hydrolyzed the inositol phosphate linkage of the PI-G anchor. Cotransfection of PI-G PLD with a PI-G–anchored protein resulted in the secretion of the PI-G–anchored protein. The results suggest that the expression of PI-G PLD may influence the expression and location of PI-G–anchored proteins.

THE ANCHORING OF PROTEINS TO the lipid bilayer of plasma membranes was initially thought to be mediated mainly by hydrophobic amino acid sequences. However, some proteins are covalently linked to a phosphatidylinositol-glycan (PI-G) molecule in the lipid bilayer (Fig. 1) (*1*). This structure has been shown to anchor numerous proteins in species as diverse as trypanosomes, schistosomes, mice, and humans (*1*). An enzyme has been identified that selectively hydrolyzes the inositol phosphate linkage of PI-G–anchored proteins, PI-G lipids, and related molecules (*2–5*). The enzyme, PI-G–specific phospholipase D (PI-G PLD), is abundant in serum and has been purified and characterized (*3, 4*). Purified PI-G PLD from serum can hydrolyze the inositol phosphate linkage of PI-G–anchored proteins in vitro in the presence of detergents (*2–5*). However, for reasons that are not understood, it has not been possible to release proteins with a PI-G anchor from the surface of intact cells using PI-G PLD (*2–4*).

On the basis of the amino acid sequences of eight tryptic fragments from bovine serum PI-G PLD (*3*), four degenerate oligonucleotide probes were made for the purpose of screening bovine DNA libraries for PI-G PLD DNA clones (*6*). A cDNA clone that predicted the exact amino acid sequence of all eight tryptic fragments was obtained. Comparison of the deduced protein sequence to the NH_2-terminal amino acid sequence of intact PI-G PLD revealed that the clone encoded the mature NH_2-terminus of the protein and that the translation product contained a signal peptide of 23 amino acids. A translation stop codon indicated that the gene encoded a mature protein of 817 amino acids (90.2 kD) with eight potential sites of N-linked glycosyla-

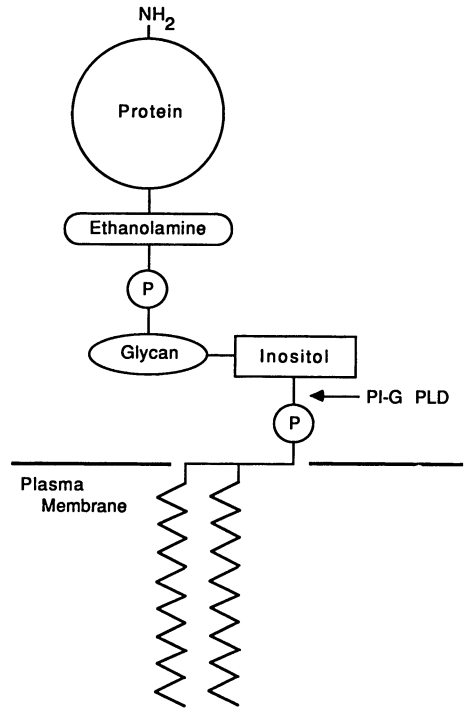

Fig. 1. Structure of the PI-G anchor. The COOH-terminal amino acid of the protein is linked to an ethanolamine residue which in turn is linked by a phosphodiester bond to a complex glycan moiety. In PI-G anchors studied in detail, such as variant surface glycoprotein from trypanosomes (*1*) and Thy-1 antigen from rat brain (*1*), the glycan is comprised of a mannose-mannose-mannose-glucosamine core structure with variable side chains. The glucosamine is linked to a membrane-anchored phosphatidylinositol. The site of PI-G PLD hydrolysis is marked.

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tion (the DNA sequence has the GenBank accession number M60804).

Analysis of the deduced amino acid sequence revealed four regions of internal homology (amino acids 357 to 379, 426 to 448, 489 to 511, and 694 to 716) that showed between 21 and 54% identity (Fig. 2). A computer search of the GenBank database (version 62) revealed significant similarity of these repeats with the metal ion binding domains of the alpha subunits of integrins (Fig. 2). These sequences share an aspartate-rich core flanked by short, conserved segments that occur only in the integrins. Apart from the absence of a glutamate residue and an isoleucine residue, the PI-G PLD core sequence Asp-X-(Asp or Asn)-X-Asp-Gly-X-X-Asp matches a motif characteristic of a number of Ca^{2+} and Mg^{2+} binding proteins such as calmodulin, troponin C, and parvalbumin (*7*). The presence in the deduced protein sequence of domains similar to metal ion binding domains of the integrins is consistent with the observation that PI-G PLD requires calcium for activity. The sequence Arg-Arg-Phe-Ser in PI-G PLD (amino acids 689 to 692) matches the consensus sequence for sites phosphorylated by cyclic adenosine monophosphate–dependent protein kinase (*8*). It is not known whether this site has any functional role. No significant homology was observed between bovine PI-G PLD and the PLD (sphingomyelinase) from the Gram-negative bacterium *Corynebacterium pseudotuberculosis* (*9*).

The cloned cDNA was ligated into the eukaryotic expression plasmid, pBC12BI (*10*), and the resulting plasmid (pBJ1682) was introduced into COS-1 cells. Expression of PI-G PLD was detected by immunofluorescence of permeabilized cells and protein immunoblot analysis using monoclonal antibodies against the purified enzyme (*3, 11*). Culture media and cell lysates of pBJ1682-transfected or mock-transfected COS cells were assayed for activity of PI-G PLD by incubation with ^{3}H-labeled PI-G–anchored variant surface glycoprotein (VSG). The amount of PI-G PLD activity detected in the medium of cells transfected with pBJ1682 increased during the 46-hour period following transfection (Fig. 3). PI-G PLD activity did not increase in medium from mock-transfected cells. The amount of PI-G PLD activity secreted from the pBJ1682-transfected cells reached 65 U/ml [approximately 0.15 μg/ml, provided that the secreted enzyme had the same specific activity as the enzyme purified from bovine serum (*3*)]. Analysis of lysates (*12*) prepared 46 hours after transfection with pBJ1682 revealed that only a small part (10 U/ml) of the PI-G PLD activity was associated with the cells. These results indicate that the cloned gene encodes a phospholipase and that most of the enzymatic activity was secreted from the COS cells.

Analysis of the reaction products resulting from hydrolysis of ^{3}H-labeled VSG confirmed that the phospholipase activity in DNA-transfected cells was that of phospholipase D. Hydrolysis of ^{3}H-labeled VSG by the phospholipase secreted from transfected cells yielded a major product that co-migrated with dimyristoyl phosphatidic acid during thin-layer chromatography (*13*). The same product was obtained when purified PI-G PLD from serum was used in the assay. The cloned PI-G PLD did not exhibit phosphatidylcholine-specific PLD activity when assayed as described (*13, 14*).

To assess PI-G PLD activity in vivo, we transfected COS cells with a gene encoding placental alkaline phosphatase (PLAP) (*15*),

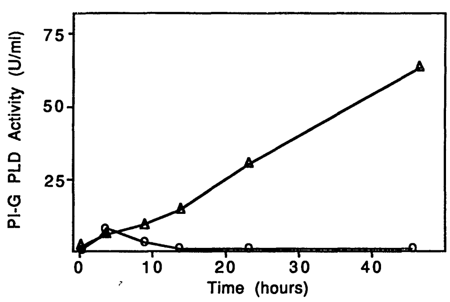

Fig. 3. Demonstration of PI-G PLD activity in transfected COS cells by hydrolysis of ^{3}H-labeled VSG. Mock-transfected or pBJ1682-transfected COS cells were transferred to serum-free Dulbecco's modified Eagle's medium (DMEM) (2 ml) containing 1% Nutridoma (Boehringer Mannheim) 24 hours after DEAE-dextran–mediated transfection (*10*). At various times, portions of medium (10 μl) were withdrawn and assayed for activity. Samples were incubated at 37°C for 30 min with 100 μl of buffer containing 40 μM tris-maleate, pH 7.0, 0.2% NP-40, and ^{3}H-labeled VSG (4000 to 5000 cpm) (*3, 29*). Reactions were terminated with butanol saturated with 1 M NH_4OH (0.5 ml), samples were centrifuged, and portions (0.35 ml) of the upper organic phase were counted in scintillation fluid. One unit of activity was defined as the amount of enzyme hydrolyzing 1% of the [^{3}H]myristate–labeled VSG per minute; △, activity in DNA-transfected cells; ○, activity in mock-transfected cells.

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L G W A M T S A D L N Q D G Y G D - L V V G A P   PI-G PLD (357-379)
F G S A V A V L D F N V D G V P D - L A V G A P   PI-G PLD (426-448)
L G W T L L A A D V N G D S E P D - L V I G S P   PI-G PLD (489-511)
F G G V L H L S D L D N D G L D E - I I V A A P   PI-G PLD (694-716)

F G S V L C S V D V D K D T I T D V L L V G A P   VLA-2      (462-485)
F G A S V C A V D L N A D G F S D - L L V G A P   VLA-4      (273-295)
F G Y A V A A T D V N G D G L D D - L L V G A P   VLA-5      (285-308)
F G H S V A V·T D V N G D G R H D - L L V G A P   GP IIb     (289-311)
F G F S V A A T D I N G D D Y A D V - F I G A P   VN Rc      (276-298)
F G G E L C G V D V D Q D G E T E L L L I G A P   CD 11a     (435-459)
F G A S L C S V D V D S N G S T D L V L I G A P   CD 11b     (441-464)
F G A S L C S V D V D T D G S T D L V L I G A P   CD 11c     (439-462)
F G Y S L A T S D V D G D G L D D - L L I G A P   PS2 Ag     (332-355)

F G - - - - - - D - D - D G - - D - - - I G A P   Conserved
L               N         E       V               amino acids

- - - - - - - - D - D - D G - I D - - E - - - -   EF Hand Consensus
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Fig. 2. Comparison of the four regions of internal homology in PI-G PLD to each other and to the metal ion binding domains of alpha subunits of integrins. The GenBank database was searched for sequences similar to PI-G PLD with NBRF programs Fasta and Search. All sequences are of human origin except the PS2 antigen of *Drosophila*. Conserved amino acids are boxed. Numbers refer to amino acid positions in the protein. The domains compared are as follows: VLA-2 (*19*); VLA-4 (*20*); VLA-5, fibronectin receptor (*21*); GP–IIb, platelet membrane glycoprotein IIb (*22*); VN Rc, vitronectin receptor (*23*); CD 11a, lymphocyte function associated antigen-1 (*24*); CD 11b, Mac-1 (*25*); CD 11c, p150 (*26*); PS2 Ag, *Drosophila* position-specific antigen 2 (*27*); and EF hand consensus sequence (*28*). Abbreviations for the amino acid residues are A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

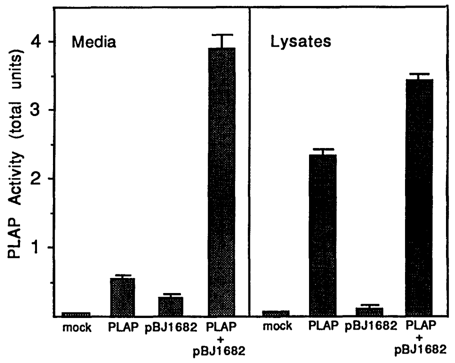

Fig. 4. Demonstration of PI-G PLD activity in COS cells cotransfected with pBJ1682 and a gene encoding PI-G–anchored PLAP. Equal amounts of DNA encoding PLAP and pBJ1682 were either mixed and introduced into COS cells or introduced into COS cells separately. Transfected cells were cultured in DMEM medium containing 10% serum for 68 hours. Cell lysates were prepared as described (*12*). Medium was collected, centrifuged to remove any suspended cells, and PLAP activities were determined (*29*). Before the assays were done, homoarginine (10 mM) was added to each sample to inhibit endogenous COS cell alkaline phosphatase activity (*16*). One unit of PLAP activity was defined as the amount of enzyme hydrolyzing 1 μmol of *p*-nitrophenyl phosphate (Sigma). For each set of data, transfections were carried out in triplicate and PLAP activities for each sample assayed in duplicate. Error bars represent one standard deviation from the mean.

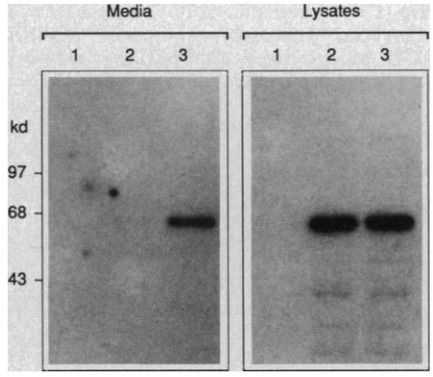

Fig. 5. Immunoprecipitation of PLAP from [^{3}H]ethanolamine-labeled COS cells. COS cells were transfected with no DNA (lane 1), DNA encoding PLAP (lane 2), or DNA encoding PLAP and pBJ1682 (lane 3). Twenty-four hours later, cells were labeled in medium containing [^{3}H]ethanolamine (100 μCi/ml; specific activity, 29.5 Ci/mmol, Amersham) supplemented with dialyzed fetal calf serum (10%). After 24 hours the conditioned medium was collected and clarified by centrifugation at 200*g* for 10 min. Cells were rinsed with phosphate-buffered saline and lysed with 10 mM CHAPS {3-[(3-cholamidopropyl)dimethylammonio] 1-propanesulfonate} in phosphate-buffered saline containing protease inhibitors (*12*). Clarified media and lysates were first incubated with protein G agarose beads (Genex) coated with a nonspecific antibody. PLAP was then immunoprecipitated with protein G agarose beads coated with polyclonal antibodies to PLAP. Immunoprecipitated proteins were fractionated on a 10% SDS-polyacrylamide gel and detected by autoradiography (17 days).

a protein that is PI-G–anchored and therefore a potential substrate for PI-G PLD. When COS cells were transfected with the PLAP cDNA alone, alkaline phosphatase activity was detected primarily in the cell lysate (Fig. 4). When COS cells were cotransfected with both PLAP and pBJ1682, the amount of alkaline phosphatase activity secreted into the medium was much higher than that released from cells transfected with PLAP cDNA alone. In COS cells transfected with pBJ1682 alone, low levels of endogenous alkaline phosphatase activity were detected in the medium or lysates (Fig. 4). The total activity of PLAP detected in the medium and lysates of cotransfected cells was consistently three to four times higher than that from cells transfected with PLAP alone. Berger *et al.* (*16*) have shown that the total activity of PLAP produced in COS cells transfected with secreted forms of PLAP was 5 to 13 times greater than that produced in cells transfected with the PI-G–anchored form. Thus, in cotransfected cells, PLAP may be continuously synthesized and released as a result of PI-G PLD activity.

To demonstrate that the release of PLAP was due to hydrolysis of its PI-G anchor rather than proteolytic cleavage, COS cells were transfected with PLAP alone or with pBJ1682 and labeled with [^{3}H]ethanolamine. PLAP was immunoprecipitated from the medium and checked for ^{3}H label by fluorography after gel electrophoresis. PLAP released into the medium was labeled with [^{3}H]ethanolamine confirming that it had been modified by the addition of a PI-G anchor (or a related precursor) before secretion (Fig. 5).

It was not known whether the PI-G PLD secreted from COS cells would hydrolyze PLAP already anchored to the cell surface. Medium from pBJ1682-transfected cells was incubated with PLAP-transfected cells and assayed for activity of PLAP after 1, 3, 8, and 24 hours. No PLAP activity was released from the cells even though the PI-G PLD in the medium was active when assayed with VSG both before and after the 24-hour incubation period. Furthermore, the ability of PI-G PLD in the medium of cotransfected cells to hydrolyze VSG was inhibited by polyclonal antibodies to PI-G PLD while release of alkaline phosphatase activity into the medium was unaffected by the antibodies (*17*). Together, these results indicate that intracellular PI-G PLD is capable of hydrolyzing intracellularly localized PLAP, but secreted PI-G PLD (and PI-G PLD from serum) cannot hydrolyze PLAP on the cell surface.

Our data demonstrate that the cloned PI-G PLD specifically hydrolyzed a PI-G–anchored protein in vitro and that coexpression with a PI-G–anchored protein resulted in secretion of that protein from transfected cells. The physiological significance of PI-G PLD activity remains to be determined. PI-G–anchored proteins are found in extracellular fluids (*1*), and inositol phosphate–glycan molecules are generated by signal transduction mechanisms (*18*). The potential involvement of PI-G PLD in these phenomena merits further investigation.

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