A phospholipase D specific for the phosphatidylinositol anchor of cell-surface proteins is abundant in plasma
(glycosyl-phosphatidylinositol/membranes/phospholipase C/alkaline phosphatase/5'-nucleotidase)

MARTIN G. LOW*† AND A. R. S. PRASAD‡
Oklahoma Medical Research Foundation, 825 North East 13th Street, Oklahoma City, OK 73104

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ABSTRACT An enzyme activity capable of degrading the glycosyl-phosphatidylinositol membrane anchor of cell-surface proteins has previously been reported in a number of mammalian tissues. The experiments reported here demonstrate that this anchor-degrading activity is also abundant in mammalian plasma. The activity was inhibited by EGTA or 1,10-phenanthroline. It was capable of removing the anchor from alkaline phosphatase, 5'-nucleotidase, and variant surface glycoprotein but had little or no activity toward phosphatidylinositol or phosphatidylcholine. Phosphatidic acid was the only [3H]myristate-labeled variant surface glycoprotein. It could be distinguished from the Ca++-dependent inositol phospholipid-specific phospholipase C activity in several rat tissues on the basis of its molecular size and its sensitivity to 1,10-phenanthroline. The data therefore suggest that this activity is due to a phospholipase D with specificity for glycosylphosphatidylinositol structures. Although the precise physiological function of this anchor-specific phospholipase D remains to be determined, these findings indicate that it could play an important role in regulating the expression and release of cell-surface proteins in vivo.

A number of cell-surface proteins are attached to the membrane by covalent linkage to a glycosyl-phosphatidylinositol anchor (for reviews, see refs. 1 and 2). The observation that this unusual and complex anchoring structure is present in an evolutionary and functionally diverse group of proteins has suggested that it might serve novel functions in addition to retaining proteins at the cell surface. One possibility is that the glycosyl-phosphatidylinositol-anchoring domain is the site for specific enzymic cleavage allowing the protein to be released from the cell surface (1, 2). Anchor-degrading activities have been described in several mammalian tissues but these were originally suggested to be due to the action of an inositol phospholipid-specific phospholipase C since enzymes of this specificity are widely distributed in mammalian tissues (3–10). However, recent studies showed that much of the inositol phospholipid-specific phospholipase C activity in human placenta could be inhibited without affecting anchor-degrading activity, indicating that this enzyme was not responsible (11). It also appeared to be distinct from the anchor-specific phospholipase C purified from Trypanosoma brucei (12–14) and rat liver (15) since it was sensitive to divalent cation chelators. Furthermore, the ability of the human placental anchor-degrading activity to produce [3H]phosphatidic acid from [3H]myristate-labeled variant surface glycoprotein (VSG) suggested that it was due to a highly specific phospholipase D that had not been described previously (11). In the present study we show that this anchor-specific phospholipase D is abundant in mammalian plasma. We also provide additional evidence distinguishing it from the inositol phospholipid-specific phospholipases C. The observation of this enzyme in an extracellular rather than an intracellular location indicates that it could play a role in releasing glycosyl-phosphatidylinositol-anchored proteins from cell surfaces in vivo.

MATERIALS AND METHODS

Purification of Human Placental Alkaline Phosphatase. Alkaline phosphatase, with the phosphatidylinositol anchor intact, was purified from human placenta by a procedure based on our previous studies of anchor degradation in human placenta (11). Briefly, this involves (i) extraction of alkaline phosphatase from human placenta with aqueous butanol at pH 8.0, (ii) phase separation with Triton X-114, (iii) removal of Triton X-114 by chromatography on SM-2 Bio-Beads, and (iv) isolation of the hydrophobic aggregated form of alkaline phosphatase by gel filtration on Sephacryl S-300. NaDodSO4/polyacrylamide gel electrophoresis of this material shows it to be ≈50% pure, with alkaline phosphatase being the single major protein species present.

Assay for Anchor-Degrading Activity. Unless specified otherwise, anchor-degrading activity was assayed by using human placental alkaline phosphatase purified as described above. The alkaline phosphatase substrate detergent mixture (0.05 ml), containing 1 vol of purified alkaline phosphatase (0.5 unit), 2 vol of 1% (wt/vol) Nonidet P-40, and 2 vol of 200 mM Tris maleate (pH 7.0), was incubated with aliquots of plasma, supernatant fractions, etc., in a total volume of 0.2 ml for 10–60 min at 37°C. The incubation mixture was then diluted with 0.8 ml of ice-cold 150 mM NaCl/0.1 mM MgCl2/0.01 mM zinc acetate/10 mM Hepes/NaOH, pH 7.0, and a 0.05-ml aliquot was removed and mixed with 0.2 ml of the same buffer and 0.25 ml of a 2% solution of precondensed Triton X-114 (11). After sampling a 0.1-ml aliquot for assay of total alkaline phosphatase activity the mixture was incubated at 37°C for 10 min and centrifuged at 1500 × g for 2 min to separate the phases, and a 0.1-ml aliquot of the upper (detergent-poor) phase was sampled. Alkaline phosphatase activity in these samples was determined as described (11) except that 0.2% Triton X-100 was included in the alkaline phosphatase assay incubation. Anchor degradation was measured by comparing the activity in the upper phase (i.e., the degraded form) with that in the total incubation mixture before phase separation at 37°C.

In some experiments VSG biosynthetically labeled with [3H]myristate (15) was used as substrate. [3H]Myristate-

Abbreviation: VSG, variant surface glycoprotein.
*Present address: Department of Physiology and Cellular Biophysics, College of Physicians and Surgeons of Columbia University, 630 West 120th Street, New York, NY 10032.
†To whom reprint requests should be addressed.
‡Present address: Department of Pathology, University of Texas Health Science Center, San Antonio, TX 73104.

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labeled VSG (8000 cpm) was incubated with plasma in the presence of 20 mM Tris maleate/75 mM NaCl/0.1% Nonidet P-40 (final assay volume, 0.4 ml) for 10 min at 37°C. The reaction was stopped by addition of 1.5 ml of chloroform/methanol, 1:2 (vol/vol), and the phases were separated by centrifugation after addition of 0.5 ml each of chloroform and 0.1 M HCl. The radioactivity in a 0.9-ml aliquot of the chloroform phase was determined (after drying) by liquid scintillation spectrometry.

**Assay for Phospholipid Hydrolysis.** Phospholipase activity was assayed in supernatants and column fractions (i.e., Table 2 and Fig. 4) by determining hydrolysis of [3H]inositol-labeled phosphatidylinositol as described (16). Under these incubation conditions [5 mM CaCl₂/0.1% (wt/vol) sodium deoxycholate, pH 7.0] the major species of cytosolic inositol phospholipid-specific phospholipase C can be detected by using phosphatidylinositol as substrate (16-19). Phosphatidylinositol and phosphatidylcholine hydrolysis by plasma was determined by using a similar procedure except that the incubation conditions were as described for the assay of alkaline phosphatase anchor degradation.

**RESULTS**

**Degradation of the Glycosyl-Phosphatidylinositol Anchor of Human Placental Alkaline Phosphatase by an Enzyme Activity in Plasma.** Incubation of human placental alkaline phosphatase with plasma from several mammalian species resulted in extensive degradation of its anchoring domain (Fig. 1). Under the conditions employed here, enzyme activity was readily detected after a 60-min incubation with rat or rabbit plasma diluted 1:10,000 (data not shown). The activity present in human plasma (Fig. 1) was substantially lower (~30% of that observed for rat); a similar result was obtained with plasma from a total of four human donors, but less than half of this activity was observed in plasma from a fifth donor. A comparison of plasma and serum (from rabbit or human) revealed no major differences in the levels of activity. Levels of activity in this same range (i.e., as in Fig. 1) were also observed in dog plasma and fetal bovine, chicken, and horse serum (data not shown). Similar amounts of activity were present before and after ultracentrifugation (150,000 × g; 40 min) of plasma samples, indicating that it was not associated with membrane fragments.

**Gel-Filtration Analysis of the Plasma Anchor-Degrading Activity.** Gel-filtration chromatography of rabbit plasma on Sephacryl S-300 showed that the activity that degrades the human placental alkaline phosphatase anchor was eluted as a single peak with an approximate Mₐ of 500,000 (Fig. 2). Anchor-degrading activity in rat and dog plasma exhibited similar elution behavior (data not shown). Whether this relatively large molecular weight is due to formation of multimers or association with other proteins or lipids (e.g., plasma lipoproteins) is unknown at present. When anchor-degrading activity in the eluates from rabbit (Fig. 2) and rat plasma (data not shown) was measured by using other glycosyl-phosphatidylinositol-anchored proteins—i.e., rat liver 5'-nucleotidase, the VSG of *T. brucei*, and a genetically distinct type of alkaline phosphatase from pig kidney—a peak of activity with the same molecular weight was observed. The peak fractions were pooled and this partially purified enzyme preparation was utilized for the experiments described in Fig. 3 and Table 1. This gel-filtration procedure resulted in a 15-fold increase in specific activity with

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**Fig. 1.** Degradation of the hydrophobic anchor of human placental alkaline phosphatase by mammalian plasma. Purified human placental alkaline phosphatase was incubated at 37°C with 200 ml of rat (○), rabbit (●), or human (△) plasma for the time period indicated. A control with no added plasma is also shown (■). Heparinized plasma was pooled from five animals each for the experiments with rat and rabbit; human plasma was from a single donor (see text).

**Fig. 2.** Copurification of plasma anchor-degrading activity acting on different glycosyl-phosphatidylinositol-anchored proteins. Twenty milliliters of rabbit plasma was centrifuged at 150,000 × g for 40 min and applied to a column (two 2.5 × 120 cm columns connected in series) of Sephacryl S-300 equilibrated in 150 mM NaCl/10 mM Hepes, pH 7. The column was eluted at 12 ml/hr and 18- to 20-ml fractions were collected. Fractions were monitored for protein by A₂₈₀ (●) and anchor-degrading activity measured against human placental alkaline phosphatase (■), rat liver 5'-nucleotidase (○), and [3H]myristate-labeled VSG (△). Fractions were also assayed by using pig kidney alkaline phosphatase as substrate, but for clarity these data were not included in the figure. The approximate elution volumes of the activity peak were 551 ml and 552 ml for the rat and rabbit enzymes, respectively. Approximate elution volumes and molecular weights of standard proteins were as follows: thyroglobulin (502 ml; M₀, 669,000), ferritin (563 ml; M₀, 440,000), catalase (637 ml; M₀, 232,000), and aldolase (652 ml; M₀, 158,000). The void volume determined with blue dextran was ~430 ml.
75–100% recovery of the anchor-degrading activity in rat and rabbit plasma.

Inhibitor Sensitivity. Characteristics of the anchor-degrading enzyme in plasma were further investigated to determine its relationship to the enzyme activity that we observed previously in human placenta (11). The enzyme in human, rat, and rabbit plasma was inhibited by EGTA or the thiol-blocking agent p-hydroxymercuriphenylsulfonic acid (Table 1). The inhibitory effects of EGTA were partially blocked by addition of excess Ca\(^{2+}\) ions. However, 1,10-phenanthroline, a chelator of transition metal ions, was a more effective inhibitor than EGTA, suggesting that Ca\(^{2+}\) ions are not directly required for activity. The inhibitors used had similar effects on degradation of placental alkaline phosphatase and VSG (Table 1) and also on rat liver 5'-nucleotidase (data not shown). Protease inhibitors were without effect (Table 1). The same general pattern of inhibitor sensitivity was exhibited by unfraccionated plasma and plasma after partial purification by gel filtration (Table 1). The activity observed previously in human placenta (11) was stimulated by butanol at pH 5.0 and was relatively heat stable; these properties were also exhibited by the activity in plasma (M.G.L., unpublished data).

Specificity. To determine the bond specificity of the anchor-degrading enzyme the products of \(^{3}H\)myristate-labeled VSG hydrolysis by rabbit plasma were analyzed by thin-layer chromatography. Approximately 95% of the recovered \(^{3}H\)-radioactivity comigrated with a dimystylphosphatidic acid standard (Fig. 3). Significant increases in radioactivity near the solvent front (presumably due to \(^{3}H\)-labeled fatty acids, mono- or diacylglycerol) and just ahead of the origin were also observed. However, these individually accounted for <2% of the \(^{3}H\) radioactivity recovered from the thin-layer chromatography plate. A similar result was obtained for the other samples tested—i.e., rabbit and rat plasma after purification by gel filtration. Substrate specificity was further defined by determining the ability of the phospholipase D to act on free phospholipids. Weak phospholipase activity was detected toward \(^{3}H\)-inositol-labeled phosphatidylinositol or \(^{3}H\)choline-labeled phosphatidylcholine using the enzyme purified from rat and rabbit plasma by gel filtration. However, this amounted to <5% substrate hydrolysis with a combined incubation time and an amount of enzyme at least 1000 times greater than that required to give 50% hydrolysis of VSG or placental alkaline phosphatase (data not shown).

Activity in Cytosolic Fractions of Rat Tissues Can Be Distinguished from Inositol Phospholipid-Specific Phospholipase C. Anchor-degrading activities have been reported in the cytosolic fraction of heart and platelets (4, 8, 10) that were attributed to the inositol phospholipid-specific phospholipase C that is widely distributed in mammalian tissues (16–19). In preliminary experiments it was shown that rat liver and heart contained approximately one-sixth the concentration of anchor-degrading activity that was present in rat plasma (data not shown), suggesting that a significant proportion of the activity observed in these tissues could be due to contamination by the plasma phospholipase D. It was therefore of interest to determine the relationship of this anchor-degrading activity to that observed in plasma. Gel filtration of supernatant fractions of rat heart and brain on Sephacryl S-300 revealed that the anchor-degrading activity had a similar size to the plasma enzyme (compare Figs. 2 and 4). Furthermore, it was well separated from phospholipase C activity measured by using \(^{3}H\)phosphatidylinositol as substrate (Fig. 4). The anchor-degrading activity in pooled peak fractions was inhibited by p-hydroxymercuriphenylsulfonic acid, EGTA, and 1,10-phenanthroline but was insensitive to protease inhibitors (data not shown), as found for this activity in plasma (see Table 1) or from human placenta (11).

Further evidence distinguishing the anchor-degrading and phospholipase C activities in supernatants was obtained by exploiting its sensitivity to 1,10-phenanthroline. As shown in Table 2, phospholipase C activity in supernatant fractions from rat heart, liver, and brain was insensitive to 1,10-phenanthroline at concentrations 25-fold higher than those required to give ≈95% inhibition of anchor-degrading activity.

**DISCUSSION**

Some previous studies have noted the existence of an enzyme activity capable of degrading the glycosyl-phosphatidylinositol membrane anchor of alkaline phosphatase, acetylcholinesterase, or a 130-kDa rat hepatoma glycoprotein in the soluble or membrane fractions prepared from several mammalian tissues (3–11). The results presented here support the proposition (11) that the intracellular inosi-

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**Table 1. Inhibitor sensitivity of plasma anchor-degrading enzyme**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Alkaline phosphatase degradation, % of control</th>
<th>VSG degradation, % of control</th>
<th>Purified enzyme</th>
<th>Purified enzyme (purified enzyme)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rabbit</td>
<td>Rat</td>
<td>Human</td>
<td>Rabbit</td>
</tr>
<tr>
<td>p-Hydroxymercuriphenylsulfonic acid (2.5 mM)</td>
<td>25.6</td>
<td>16.6</td>
<td>17.7</td>
<td>19.5</td>
</tr>
<tr>
<td>EGTA (0.2 mM)</td>
<td>33.3</td>
<td>29.3</td>
<td>31.9</td>
<td>31.4</td>
</tr>
<tr>
<td>EGTA (2.0 mM)</td>
<td>1.6</td>
<td>4.6</td>
<td>1.7</td>
<td>2.5</td>
</tr>
<tr>
<td>+ CaCl(_2) (2.1 mM)</td>
<td>67.3</td>
<td>49.1</td>
<td>48.3</td>
<td>52.1</td>
</tr>
<tr>
<td>1,10-Phenanthroline (4.0 (\mu)M)</td>
<td>79.4</td>
<td>65.1</td>
<td>68.4</td>
<td>59.9</td>
</tr>
<tr>
<td>1,10-Phenanthroline (20.0 (\mu)M)</td>
<td>4.2</td>
<td>4.2</td>
<td>7.9</td>
<td>7.6</td>
</tr>
<tr>
<td>Protease inhibitor mixture</td>
<td>105.3</td>
<td>110.0</td>
<td>112.0</td>
<td>96.0</td>
</tr>
<tr>
<td>Leupeptin (1 mM)</td>
<td>98.9</td>
<td>94.5</td>
<td>91.9</td>
<td>109.0</td>
</tr>
<tr>
<td>Phenylmethylsulfonyl fluoride (0.25 mM)</td>
<td>101.5</td>
<td>94.6</td>
<td>105.0</td>
<td>93.8</td>
</tr>
</tbody>
</table>

Samples of rat, rabbit, and human plasma or the anchor-degrading enzyme partially purified from rat and rabbit plasma by gel filtration (see Fig. 2) were preincubated for 30 min at 0°C in a total volume of 0.15 ml with the inhibitors shown. Placental alkaline phosphatase or \(^{3}H\)myristate-labeled VSG substrates (0.05 ml) were added and anchor degradation was determined after incubation at 37°C for 10 min. The inhibitor concentrations refer to those present during the incubation with substrate. Activities are expressed relative to those observed in control incubations without inhibitors. The protease inhibitor mixture included pepstatin A (1 mg/ml final concentration), benzamidine (0.1 mg/ml), aprotinin (0.1 mg/ml), soybean trypsin inhibitor (0.1 mg/ml), and bacitracin (0.4 mg/ml).
Fig. 3. Products of hydrolysis of the glycosyl-phosphatidylinositol anchor of VSG. [\(^{3}H\)]Myristate-labeled VSG (8000 cpm) was incubated with 20 \(\mu\)l of rabbit plasma (i.e., a 10-fold excess activity over that required to give complete hydrolysis of VSG). A 0.9-ml sample of the chloroform phase was mixed with 100 \(\mu\)g of dimyristoyl phosphatidic acid and 50 \(\mu\)g each of pig brain phosphatidylcholine and 1,2-diolein dried at 37°C under nitrogen and applied to a silica gel 60 thin-layer chromatography plate. The plate was developed in chloroform/pyridine/70% formic acid, 50:30:7 (vol/vol), and dried overnight at 55°C; the lipids were visualized after spraying with a 1 M solution of 2-\((p\)-toluidinyl)naphthalene-6-sulfonate dissolved in 50% ethanol. Scrapings were eluted with four 1-ml aliquots of chloroform/methanol/H\(_2\)O, 10:10:3 (vol/vol), the eluates were dried at 55°C, and \(^{3}H\) radioactivity was determined by liquid scintillation spectrometry. Approximately 60–70% of the applied radioactivity was recovered from the plate. Control incubations (without added enzyme) done in parallel contained <30 cpm in each region of the thin-layer chromatography plate and are therefore not shown. The mobility of various lipids in this solvent system is indicated: phosphatidylinositol (PI), phosphatidylethanolamine (PE), dimyristoylphosphatidic acid (dmPA), phosphatidic acid derived from egg yolk phosphatidylethanolamine (PA), and 1,2-diolein (DAG). Phosphatidylethanolamine had a mobility similar to phosphatidylinositol, and oleic acid, 1-monolein, 1,3-diolein, myristic acid, 1-monomyristin, 1,2-dimyristin, and 1,3-dimyristin had mobilities similar to 1,2-diolein (not shown in figure).

Fig. 4. Resolution of anchor-degrading and phosphatidylinositol-hydrolyzing activities in rat brain and heart by gel filtration. Supernatant fractions (prepared as in ref. 18) from rat brain (Upper) and heart (Lower) were applied to a column of Sephacryl S-300 (two 2.5 \(\times\) 120 cm columns connected together) and eluted as described in the legend to Fig. 2. The void volume of this column was \(\approx\)430 ml. Fractions were assayed for alkaline phosphatase anchor-degrading activity and phosphatidylinositol (PI) hydrolysis.

Identified an EGTA-sensitive phospholipase D in human and bovine serum that degrades the anchor of decay-accelerating factor and \(^{3}H\)myristate-labeled VSG. Although a definitive assignment of the bond and substrate specificity of this enzyme activity awaits final purification, the similarities between the present and previous studies (3-11) tend to support the general conclusion that the plasma and tissue activities are due to a phospholipase D enzyme with specificity for glycosyl-phosphatidylinositol.

The physiological role of the anchor-specific phospholipase D is uncertain. However, the present observation of this activity in an extracellular rather than an intracellular location increases the likelihood that it is involved in protein

Table 2. 1,10-Phenanthroline inhibits anchor degradation but not phosphatidylinositol hydrolysis by supernatants from rat tissues

<table>
<thead>
<tr>
<th>1,10-Phenanthroline, (\mu)M</th>
<th>Anchor degradation</th>
<th>Phosphatidylinositol hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>Liver</td>
<td>Brain</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>5.3</td>
<td>79.5</td>
<td>89.7</td>
</tr>
<tr>
<td>26.7</td>
<td>4.1</td>
<td>3.4</td>
</tr>
<tr>
<td>133.3</td>
<td>4.7</td>
<td>0</td>
</tr>
<tr>
<td>666.7</td>
<td>10.4</td>
<td>0</td>
</tr>
</tbody>
</table>

Supernatant fractions from rat heart, brain, and liver (prepared as described in ref. 20) were preincubated with 1,10-phenanthroline for 30 min at 0°C in a total volume of 0.15 ml. Anchor-degrading and phosphatidylinositol-hydrolyzing activities were then assayed. Since the final volumes of the two assays were different (i.e., 0.2 and 0.4 ml, respectively), the concentrations given refer to those present during preincubation. Activities are expressed relative to those obtained with control incubations without added phenanthroline.
release from the cell surface in vivo (reviewed in refs. 1 and 2). One additional consequence of the release of proteins from the cell surface by the anchor-specific phospholipase D would be the generation of phosphatidic acid in the outer leaflet of the lipid bilayer. Addition of this lipid (or a bacterial phospholipase D) to various mammalian cells has been shown to result in Ca\(^{2+}\) mobilization, decreased cellular cAMP, increased DNA synthesis, and oncogene induction possibly as a result of increased inositol phospholipid hydrolysis resulting from the direct activation of the intracellular phospholipase C (refs. 21 and 22 and references therein). Whether similar effects on cell physiology would be produced by the phosphatidic acid molecules liberated during the release of cell-surface proteins by the anchor-specific phospholipase D is not known. However, it is of interest to note that, in one of these studies, addition of serum instead of phosphatidic acid produced very similar responses (21). Production of phosphatidic acid as a result of phospholipase D-mediated anchor degradation would certainly be a useful mechanism for coordinating release and expression of some cell-surface proteins with intracellular metabolism.

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