Structural Features of GPI-specific Phospholipase D Revealed by Proteolytic Fragmentation and Ca\(^{2+}\) Binding Studies*

(Received for publication, April 22, 1994, and in revised form, September 7, 1994)

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Glycosylphosphatidylinositol-specific phospholipase D (GPI-PLD) is abundant in plasma and is potentially capable of degrading the anchor utilized by many cell surface proteins. The goal of this work was to study structural features of the GPI-PLD that might be involved in regulation of its activity. Trypsin cleaved the 100--110-kDa GPI-PLD polypeptide into three major fragments (two of ~40 kDa and a carboxy-terminal fragment of 50 kDa) which were relatively resistant to further proteolysis. Pretreatment of the GPI-PLD with chelators resulted in complete degradation. During the cleavage process the GPI-PLD enzymatic activity increased ~3--4-fold but no other major change in its properties (e.g., inhibition by chelators and lipids, thermal stability, oligomerization, etc.) was observed. Intact or trypsinized GPI-PLD bound ~5.5 ions/molecule GPI-PLD; \(K_d \approx 16.1\ \mu\text{m}\) as determined by equilibrium dialysis which could not be blocked by the addition of other divalent metal ions. However, inhibition of enzymatic activity by divalent cation chelators appeared to involve removal of bound Zn\(^{2+}\) rather than Ca\(^{2+}\). A metal analysis of GPI-PLD revealed ~6 and 10 atom/molecule of calcium and zinc, respectively. The data suggest that the predicted integrin E-F hand-like sites in GPI-PLD are functional but not directly involved in enzymatic activity.

Glycosylphosphatidylinositol-specific phospholipase D (GPI-PLD), hydrolyzes the covalently attached GPI moiety used as a membrane anchor by a large number of cell surface proteins. GPI-PLD, a 100--110-kDa protein, is present in large amounts in the plasma (1--5) and is secreted by some cell types in culture (6--8). It is therefore potentially capable of releasing GPI-anchored proteins from the cell surface in vivo (9, 10). However, even though crude and purified serum GPI-PLD are highly active toward detergent-solubilized GPI-anchored proteins they are unable to hydrolyze these substrates when located in membranes (11, 12). The structural features of the membrane which restrict the ability of GPI-PLD to hydrolyze its substrate are currently unknown but they are, nevertheless, of considerable physiological importance. In the absence of any restriction on plasma GPI-PLD, rapid release of GPI-anchored proteins from endothelial and blood cells would occur with potentially deleterious consequences. In addition to the secreted form, several cell types appear to retain substantial amounts of GPI-PLD at an intracellular location (8, 13). Since the membrane environments experienced by GPI-anchored proteins early in the secretory pathway are believed to be very different from those on the cell surface it is possible that GPI anchor cleavage only takes place at an intracellular location on proteins which are in transit to the cell surface (14--16). Studies in which GPI-anchored proteins and GPI-PLD have been cotransfected into COS cells or CHO fibroblasts provides some support for this hypothesis (17, 18).

The sequence of GPI-PLD offers no obvious clues as to the location of the active site, the mechanism of GPI hydrolysis, or how this activity might be regulated in vivo. However, the available peptide and cDNA sequences do reveal four repeats with a high degree of similarity to metal ion-binding domains in the \(\alpha\) subunits of proteins belonging to the integrin family of cell surface adhesion molecules (4, 17, 19). These repeats (in both GPI-PLD and integrins) contain within them sequences which exhibit some similarity with the E-F hand found in a number of soluble calcium-binding proteins such as parvalbumin, troponin C, and calmodulin. The E-F hand is known from x-ray crystallography to be responsible for metal ion binding by the latter group of proteins (20). Since interaction of integrins with their natural protein ligands usually requires Ca\(^{2+}\) ions, the E-F hand-like regions were also predicted to be involved in Ca\(^{2+}\)-binding by integrins. Subsequent experimental and theoretical studies have provided additional support for this prediction although the exact mechanism is not known since crystal structures are not yet available (19, 21--24). However, the role of these metal ion-binding sites in GPI-PLD is less obvious since our work suggests that Ca\(^{2+}\) is not necessary for enzyme action. An alternative possibility is that the integrin E-F hand-like sites participate in Ca\(^{2+}\)-dependent binding of protein ligands by GPI-PLD. Ligand binding might play an important role in vivo in the transport and regulation of GPI-PLD as a result of interaction with plasma high density lipoproteins or proteins in extracellular matrix or intracellular membranes (5, 25). To determine whether these possibilities are likely or not, we have used proteolytic fragmentation and metal ion binding to explore the structure of GPI-PLD with the eventual aim of identifying catalytic and Ca\(^{2+}\)-dependent ligand-binding/regulatory domains.

*This work was supported by National Institutes of Health Grants GM-40083 and GM-35873, a Grant-in-Aid from the American Heart Association (National Center), and a gift from Samuel W. Rover. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate that costs of publication were defrayed in this manner.

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†The abbreviations used are: GPI-PLD, glycosylphosphatidylinositol-specific phospholipase D; PI-PLC, phosphatidylinositol-specific phospholipase C; PAGE, polyacrylamide gel electrophoresis; CHAPS, (3-[cholamidopropy]dimethylammonio-1-propanesulfonate); TPCK, L-tosylamide-2-phenylchloromethyl ketone; TEMED, N,N,N',N'-tetramethylethylenediamine.

‡M. G. Low, unpublished work.
EXPERIMENTAL PROCEDURES

Materials—Bovine serum was from Pel-Freez Biologicals. Trypsin, trypsinogen, trypsin, subtilisin, protease K, papain, soybean trypsin inhibitor, cattle pancreatin, trypsin inhibitor sold by Sigma, 2-mercaptoethanol, tri-chloroacetic acid, 7-amino-2- heptanone, Nonidet P-40, magnesium chloride, calcium chloride, Trizma base, sodium chloride, zinc chloride, N-acetylglucosamine, phosphatidic acid, lipid A, 1,10-phenanthroline, EDTA, EGTA, phenylmethylsulfonyl fluoride, HEPES, and (3-cholamidopropyl)-dimethylammonio-1-propanesulfonate (CHAPS) were purchased from Sigma. Tween 20, SDS, acrylamide, bis-acrylamide, gelatin, TEMED, ammonium per sulfate, nitrocellulose membrane, and protein standards were obtained from Bio-Rad or Amersham Corp. Dulbecco's modified Eagle's medium, Earle's balanced salt solution, trypsin, glutamine, penicillin, and spec- tionomyein were from Life Technologies, Inc. "Ca" and "Mg" were obtained from DuPont NEN. 

Structural Features of GPI-specific Phospholipase D

PURIFICATION OF GPI-PLD

GPI-PLD activity was eluted in a broad peak at ~0.3 M NaCl associated with a relatively large A_{280} peak (4). However, this second peak of GPI-PLD activity was extremely variable in composition sometimes containing more of a broad band at 80 kDa than the 100-110 kDa component; lower molecular mass components in the range 50-90 kDa were also present. Other work suggests that many of these fragments were derived from GPI-PLD as a result of proteolysis by endogenous proteases.6 Unless specified otherwise GPI-PLD from the first mono Q peak was used in these studies. For some experiments trypsinized bovine serum GPI-PLD was repurified by chromatography on the mono Q column in order to remove trypsin. The second form of native GPI-PLD was isolated from the conditioned medium of CHO fibroblasts transfected with the bovine liver GPI-PLD cDNA (27) and purchased from Dr. J. P. Kochan, Hoffman-La Roche.

Metal Analysis of GPI-PLD—Serum GPI-PLD was prepared for analysis of bound metal ions by the standard purification procedure (4) except that the buffers used for chromatography on mono Q and subsequent steps were pretreated with Chelex 100 to remove contaminating divalent metal ions. Pooled fractions were subjected to four cycles of 4-fold concentration/dilution using a Centricon-10 ultrafilter and mono Q column buffer previously diluted 10-fold. A sample (0.48 ml, 2.9 mmol) of the final concentrate was then analyzed for trace metals by ICM Laboratories (Randolph, NJ) using an inductively coupled argon plasma spectrometer.

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that the three fragments originated with very different kinetics. At the lowest concentration of trypsin used (0.04 μg/ml) the 100–110 kDa band, corresponding to native GPI-PLD, was markedly reduced and was replaced by fragments of ~80 (Fragment I), 50 (Fragment IV), 40 (Fragment III), and 30 kDa (Fragment II). Analysis at intermediate trypsin concentrations (0.4 and 4 μg/ml) suggested that Fragment II was not further reduced in size or amount (data not shown) and was therefore similar to Fragment II’ in the limit digest. It appeared that Fragment I was cleaved first into Fragments III and IV. Fragment IV was then cleaved to produce Fragment V. However, there was some variability between experiments in the relative amounts of Fragments I, III, and IV observable at the different trypsin concentrations. Minor proteolytic products (~25 and 45 kDa) were also observed at low trypsin concentration, but these were variable between experiments and they were not characterized further. Fragments II/II’, III/III’, and V appear to be relatively resistant to further degradation because there was no significant decrease in their size or amount when the trypsin concentration was increased from 4.0 to 40 μg/ml (Fig. 1, compare lanes 4 and 6).

It was noticed in some experiments that there was a substantial amount of an 80-kDa fragment (and smaller amounts of an 50-kDa fragment) in the sample that was incubated in the absence of trypsin (Fig. 1, lane 1). In control experiments the amount of this 80-kDa fragment increased when the sample was incubated in the absence of trypsin suggesting that it was produced by trace amounts of a contaminating protease activity.

Fragments comparable in size to II/II’, III/III’, and V were obtained when native bovine serum GPI-PLD was incubated with increasing concentrations (0.04–40 μg/ml) of TPCK-trypsin, subtilisin, or proteinase K instead of trypsin (data not shown). Similar-sized fragments were also produced when recombinant GPI-PLD secreted from CHO fibroblasts transfected with the bovine liver cDNA was digested with trypsin or TPCK-trypsin (data not shown).

Trypsinized GPI-PLD was repurified by anion-exchange chromatography on Mono Q (see “Experimental Procedures”) followed by gel filtration chromatography on a Superose 6 column (in 50 mM Tris-HCl, pH 7.5, containing either 2 mM NaCl, 0.1% Nonidet P-40, or 0.1% SDS) in order to determine if the trypptic fragments were associated or not. SDS-PAGE analysis showed that the active fractions contained the same relative amounts of Fragments II/II’, III’, and IV. Furthermore, none of these fragments appeared to be enriched in fractions which were devoid of GPI-PLD activity (data not shown). Although the molecular mass of the enzyme activity peak was dependent on the additions to the elution buffer (400–500, 300–400, and ~200 kDa for NaCl, Nonidet P-40, and SDS, respectively), it was not reduced by tryptic treatment. However, when chromatographed in the presence of SDS, the trypsinized form gave relatively low recovery of GPI-PLD activity in column fractions. Collectively these results suggest that oligomerization might stabilize the GPI-PLD after proteolytic cleavage.

Identification of the Tryptic Cleavage Sites—To identify the location of the cleavage sites, trypsinized GPI-PLD was purified by anion-exchange chromatography on Mono Q to remove trypsin, GPI-PLD fragments were separated by SDS-PAGE, transferred to PVDF membranes, and subjected to amino-terminal sequence analysis (Table I). These analyses showed that the two 40-kDa fragments (i.e. III’ and V) which accumulate after treatment with 40 μg/ml trypsin had quite different amino-terminal sequences. Fragment V had the same amino-terminal sequence as native GPI-PLD whereas that of Fragment III’ exhibited a sequence starting at residue 332 as derived from the bovine liver cDNA (17). This result is of particular interest because it eliminates the possibility that the two 40-kDa fragments are essentially conterminous peptides with their slight mobility differences resulting from minor variations in post-translational modification. Fragment II exhibited a major sequence starting at residue 591 and a minor sequence starting at residue 593 (Table I). The sequence data indicated that the three fragments which accumulate in the limit digest are arranged in the linear sequence: NH2-V-III’-II/II’-COOH (Fig. 2).

These conclusions were confirmed by analysis of the peptides generated after treatment with low concentrations of trypsin (0.04 μg/ml). Fragments I and IV had the same amino terminus as the native GPI-PLD (Table I). Furthermore, a comparison of the amino-terminal sequences of Fragments II and III generated at high or low trypsin concentrations indicated that the initial cleavage products were susceptible to further cleavage in the presence of large amounts of trypsin (Table I). Collectively, the data suggest the following sequence of events as the trypsin concentration was increased (Fig. 2): (i) native GPI-PLD was cleaved between residues 590 and 591 to generate the Fragments I and II. (ii) Fragment I was cleaved between residues 325 and 326 to generate Fragments III and IV. (iii) Fragment IV was cleaved close to its carboxyl terminus to generate Fragment V. (iv) A hexapeptide was cleaved from the amino terminus of Fragment III presumably by chymotrypsin contaminating the trypsin) to generate Fragment III’. (v) A dipeptide was partially cleaved from the amino terminus of Fragment II to generate Fragment II’. We cannot exclude the possibility that additional cleavages took place at the carboxyl termini of Fragments II/II’, III/III’, and V; however, these would necessarily be quite small since essentially all of the original molecular mass was accounted for.

Effect of Proteolytic Cleavage on GPI-PLD Activity—Although treatment of native GPI-PLD with trypsin (and other proteases) resulted in multiple cleavages in two or more regions of the molecule the NH2-terminal sequencing, gel filtration, and SDS-PAGE analyses described above indicate that the majority of the polypeptide chain remained intact (Fig. 2). It was therefore of interest to determine if proteolytic cleavage had any effect on the enzymatic activity of GPI-PLD using detergent-solubilized [3H]myristate-labeled VSG as substrate. As shown in Fig. 3, there was little if any change in activity at the lowest trypsin concentration used (0.04 μg/ml). However, there was a progressive, 3-4-fold increase in the GPI-PLD activity as the trypsin concentration was increased to 40 μg/ml. Samples of the digests were also analyzed by SDS-PAGE (procedure as...
Described in Fig. 1) in an attempt to correlate directly changes in activity with the changes in GPI-PLD primary structure already established. The results of several such experiments indicated that the increase in activity corresponded to the appearance of Fragments I/II', IV, and IV rather than the conversion of native GPI-PLD to Fragments I and II (data not shown). Control incubations containing trypsin alone indicated that the increase in GPI-degrading activity was not due to contaminants in the trypsin (Fig. 3). Furthermore, the increase in GPI-PLD activity could not be due to an indirect effect of trypsin on the GPI-anchored protein substrate (i.e. VSG) because the reaction was quenched by diluting in buffers containing protease inhibitors. Trypsinized GPI-PLD exhibited similar increases in activity when purified, detergent-solubilized alkaline phosphatase was used as the substrate instead of VSG (data not shown). Increases in activity (~2-3-fold) were also obtained when bovine serum GPI-PLD was treated with other proteases (0.04–40 µg/ml) such as TPCK-trypsin, chymotrypsin, subtilisin, and proteinase K or when recombinant bovine GPI-PLD was treated with trypsin or TPCK-trypsin (data not shown).

Previous studies indicated that alkaline phosphatase, a GPI-anchored protein, was resistant to the action of GPI-PLD when located in a cell membrane (11). However, resistance was abolished when the membrane was solubilized in detergent. The assays described above were done with detergent-solubilized substrates and consequently would not have been capable of detecting any changes in the ability of trypsized GPI-PLD to act on membranes. ROS cells were incubated with mono Q-purified trypsized GPI-PLD but there was relatively little increase (i.e. <5%) in the release of alkaline phosphatase into the supernatant (Fig. 4). The amount released was indistinguishable from that obtained with native GPI-PLD purified from bovine serum or from the medium of CHO fibroblasts transfected with the bovine liver cDNA. Control incubations using bacterial PI-PLC indicated that at least 90% of GPI-anchored alkaline phosphatase was accessible at the surface of the ROS cells (Fig. 4). These data suggest that trypsination is unable to overcome the restriction on GPI-PLD activity encountered with GPI substrates located in a membrane. It is possible that trypsination produces intermediate forms of GPI-PLD (e.g. the 80-kDa fragment) which are only transiently active on membrane substrates but then lose this property due to further cleavage and/or slow conformational changes. Neither of these possibilities would be detected in the experiment described in Fig. 4 which used purified, fully trypsized GPI-PLD. However, attempts to address both of these issues by adding unpurified GPI-PLD digests (in which the trypsin concentration and incubation time were varied) directly to ROS cells were confounded by the effects of trypsin on the cells which were difficult to control.

<table>
<thead>
<tr>
<th>Approximate molecular mass of band on gel</th>
<th>Fragment no.</th>
<th>Amino-terminal sequence</th>
<th>Location in intact protein sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>kDa</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>80’</td>
<td>I</td>
<td>XGISTHIEI...</td>
<td>Amino terminus</td>
</tr>
<tr>
<td>50’</td>
<td>IV</td>
<td>XGISTHIEI...</td>
<td>Amino terminus</td>
</tr>
<tr>
<td>40’—</td>
<td>V</td>
<td>XGISTH...</td>
<td>Amino terminus</td>
</tr>
<tr>
<td>40’</td>
<td>III</td>
<td>SIREMPIGSS...</td>
<td>326–335</td>
</tr>
<tr>
<td>30’</td>
<td>III’</td>
<td>IGSSQPLT...</td>
<td>322–337</td>
</tr>
<tr>
<td>30’</td>
<td>II</td>
<td>TRDEKQS...</td>
<td>591–597</td>
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<td>30’</td>
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<td>TRDEKQS...</td>
<td>591–597</td>
</tr>
<tr>
<td>30’</td>
<td>II’</td>
<td>DEKQS...</td>
<td>593–597</td>
</tr>
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</table>

*a 0.04 µg/ml trypsin.
*b Predicted amino-terminal residue (cysteine) could not be assigned definitively and is designated as X.
*c 40 µg/ml trypsin.
*d Upper band in 40-kDa doublet.
*e Minor sequence; ~33% of total.

Fig. 2. Location of the GPI-PLD proteolytic cleavage sites. Upper part of diagram gives the approximate location of structural features of GPI-PLD as predicted from the bovine liver cDNA sequence; potential N-linked glycosylation sites (solid circles), integrin E-F hand-like sites (solid rectangles). The center part of diagram describes a proposed scheme for generation of fragments with increasing trypsin concentrations (for additional information see Table I). Carboxyl-terminal sequences were not determined so the approximate location of C termini were estimated from relative sizes of fragments and are represented by dotted lines. The lower part of the diagram gives the approximate location of regions containing monoclonal antibody epitopes (hatched rectangles).

Fig. 3. Effect of trypsin digestion on GPI-PLD activity. GPI-PLD activity was assayed in duplicate by hydrolysis of 3H-labeled VSG as described under "Experimental Procedures." The activity is expressed as a percentage of that of native GPI-PLD. The mean (± S.D.) of three separate experiments is shown at each trypsin concentration. Controls containing no GPI-PLD demonstrated that the increased activity was not contributed by trypsin itself.

<table>
<thead>
<tr>
<th>TRYSIN (µg/ml)</th>
<th>GPI-PLD ACTIVITY (% of control)</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>200</td>
</tr>
<tr>
<td>0.04</td>
<td>300</td>
</tr>
<tr>
<td>0.4</td>
<td>400</td>
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<tr>
<td>4</td>
<td>500</td>
</tr>
<tr>
<td>40</td>
<td>600</td>
</tr>
<tr>
<td>NO GPI-PLD</td>
<td></td>
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</table>
The effect of trypsinization on the ability of a variety of agents to influence GPI-PLD activity was also tested. These agents included inhibitory lipids (34) such as dilauryl PA (IC_{50} \sim 0.5 \mu M for intact GPI-PLD) and lipid A from Shigella flexneri (IC_{50} \sim 0.5 \mu M) as well as chelators such as 1,10-phenanthroline (IC_{50} \sim 10–20 \mu M), EDTA (IC_{50} \sim 100–200 \mu M) and EGTA (IC_{50} \sim 200–500 \mu M). However, there were no major (i.e., <2-fold) increases or decreases in the sensitivity to these agents after trypsinization (data not shown). The ability of Zn^{2+} to block the inhibitory effect of EDTA (see below) was also unchanged by trypsinization. Finally, the enzyme activity of intact and trypsinized forms of GPI-PLD both showed a similar response to changes in the pH or detergent concentration in the assay or to thermal denaturation at 65 °C (data not shown).

Effect of Proteolytic Cleavage on Immunoreactivity of GPI-PLD—The fully trypsinized GPI-PLD was analyzed by immunoblotting with monoclonal antibodies previously shown to bind to intact, denatured GPI-PLD (data not shown). These studies revealed that antibody 193 (and 196) bound exclusively to Fragment III, while antibody 194 bound to Fragment II’. Unexpectedly, antibody 191 did not bind to any of the fragments suggesting that the epitope was cleaved by trypsin. Further analysis with lower trypsin concentrations showed that 191 was able to bind Fragments I and IV, but immunoreactivity was lost when Fragment IV was converted to Fragment V by cleavage at its carboxyl terminus. The complete loss of immunoreactivity suggests that the 191 epitope contained a tryptic cleavage site. However, it is also possible that the epitope was localized entirely on a small fragment that was unable to bind to nitrocellulose and thereby escaped detection. The immunoblotting results are consistent with the sequence analyses already described indicating that Fragments II, III/III’, and IV/V are derived from non-overlapping regions of the GPI-PLD polypeptide and are summarized in Fig. 2.

Immunoreactivity of trypsinized GPI-PLD was also determined under non-denaturing conditions using an enzymatic immunocapture assay. Monoclonal antibody 191 was unable to bind significant amounts of trypsinized GPI-PLD under non-denaturing conditions consistent with the suggestion that its epitope is either close to or contains a tryptic cleavage site (data not shown). By contrast the ability of antibodies 193 and 196 to bind GPI-PLD activity was not substantially decreased by tryptic cleavage suggesting that the gross conformation as well as the accessibility of these epitopes was similar in the intact and trypsinized enzyme. Unfortunately, a similar analysis was not possible with the carboxyl-terminal reactive antibody, 194, because it does not bind native GPI-PLD.

Calcium Binding by Native and Trypsinized GPI-PLD—Previous work has shown that GPI-PLD possesses four repeats (see Fig. 2) of a region with a high degree of similarity to the metal ion-binding domains of integrins (17). It was therefore of interest to determine if the predicted metal ion-binding sites in GPI-PLD were functional. Using a slot-blot procedure, it was shown that EDTA-treated intact and trypsinized GPI-PLD both bind ⁴⁵Ca^{2+} in amounts comparable to that obtained with parvalbumin (Fig. 5A). Given the difference in the relative molecular masses and number of metal ion-binding sites between the two proteins this result suggests that the E-F hand-like sites in GPI-PLD are able to bind Ca^{2+} effectively. Comparable amounts of ⁴⁵Ca^{2+} binding were observed with recombinant GPI-PLD purified from CHO fibroblasts confirming that this was a property of the GPI-PLD rather than a minor contaminant (data not shown). ⁴⁵Ca^{2+} binding by GPI-PLD appeared to be specific in that it was not observed with equal amounts of bovine serum albumin (Fig. 5A), and ⁸⁶Mn^{2+} did not bind to GPI-PLD under these conditions (data not shown). GPI-PLD was unable to bind ⁴⁵Ca^{2+} when the enzyme was previously boiled for 2 min (Fig. 5A) or if it was first denatured with SDS, electrophoresed on SDS-PAGE, and then electroblotted onto nitrocellulose (data not shown). Binding of ⁴⁵Ca^{2+} was displaced by 0.5 mM of unlabeled CaCl_{2} and partially by CdCl_{2} but not by CoCl_{2}, MgCl_{2}, MnCl_{2}, NiCl_{2}, or ZnCl_{2} (Fig. 5B) suggesting that these metal ion-binding sites were relatively specific for Ca^{2+} or Cd^{2+}. Furthermore, ⁴⁵Ca^{2+} binding was not prevented by pretreatment of the GPI-PLD with the transition metal chelator, 1,10-phenanthroline (data not shown). Ca^{2+} binding by intact bovine serum GPI-PLD was further investigated by equilibrium dialysis. Although these experiments were limited by the availability of purified GPI-PLD, they confirmed that Ca^{2+} binding occurred in the micromolar range. Scatchard analysis of the high affinity component of the binding data (i.e., in the range 1–10 \mu M) indicated that 5.5 Ca^{2+} ions were bound to GPI-PLD with an average K_{d} of \sim 16.1 \mu M (Fig. 6).

Role of Ca^{2+} and Transition Metal Ions in GPI-PLD Activity—Several previous studies have shown that GPI-PLD is inhibited by EGTA or EDTA (1–4, 36, 37). Since this effect can be blocked by the addition of Ca^{2+} it has been proposed that GPI-PLD is Ca^{2+}-regulated (3, 37). Other interpretations of the blocking effect are possible, but the ability of GPI-PLD to bind Ca^{2+}, described above, seems consistent with this idea. However, the observation that 1,10-phenanthroline (IC_{50} \sim 10–20 \mu M) is a more effective inhibitor of GPI-PLD than EGTA (IC_{50} \sim 200–500 \mu M) suggested a requirement for transition metals as well (1, 4). These considerations prompted us to investigate the inhibition of GPI-PLD by chelators in more detail. We first compared the ability of Ca^{2+} ions and several transition metal ions to block the inhibitory effect of EGTA and 1,10-phenanthroline. As shown in Fig. 7A, Ca^{2+} is relatively ineffective at blocking EGTA compared with Zn^{2+}. Thus, the metal ion/chelator ratio required to retain 50% of the activity was –0.1
repurified trypsinized GPI-PLD samples were also denatured by heating at 100 °C for as positive and negative controls respectively. Similar results were done in the absence (no metal) or presence of CaCl₂, CdCl₂, CoCl₂, MgCl₂, MnCl₂, NiCl₂, and ZnCl₂. Equal amounts of parvalbumin and BSA were included as controls in the binding experiments.

We investigated the effect of preincubating GPI-PLD for 30 min with the chelator prior to addition of the metal ions. These experiments showed that all the metal ions tested were as effective at blocking the inhibition after 30 min exposure to EGTA (Fig. 7C) compared with simultaneous addition with the chelator. Zn²⁺ and Co²⁺ were also able to reactivate chelator-treated GPI-PLD after it is already inhibited by the chelator. Zn²⁺ and Co²⁺ were able to restore the activity after 30 min of preincubation with 1,10-phenanthroline whereas Ni²⁺, Cd²⁺, and Mn²⁺ were less effective (Fig. 7B). These data indicate that the addition of metal ions can reactivate GPI-PLD after it is already inhibited by the chelator. Similar results were obtained when the preincubation period with EGTA or phenanthroline was extended up to 2 h, but with longer preincubation periods irreversible losses of activity occurred (data not shown). This may, in part, explain why attempts to reactivate chelator-treated GPI-PLD with transition metal ions after removal of the chelate (by dialysis, gel filtration, or ultrafiltration) have so far been unsuccessful.

We also investigated the possibility that binding of transition metal ions by GPI-PLD might play a role in stabilizing the native conformation of the enzyme. The ability of trypsin to degrade GPI-PLD depleted of bound metal ions was tested by including EDTA or 1,10-phenanthroline in the digestion reactions. Neither EDTA nor 1,10-phenanthroline had a major effect on the degradation of GPI-PLD with 0.04 μg/ml trypsin (Fig. 8, compare lane 1 with lanes 3 and 5). A similar result was obtained after 0.4 μg/ml trypsin treatment (data not shown). However, when GPI-PLD was treated with a high concentration of trypsin (40 μg/ml) in the presence of either chelator it was completely degraded and Fragments II/II', III', and V did not accumulate (Fig. 8, compare lane 2 with lanes 4 and 6).

Collectively, these data indicate that GPI-PLD contains bound Zn²⁺ and that this ion is necessary for maintaining its conformation and catalytic activity. This idea was confirmed by a metal analysis of GPI-PLD which indicated the presence of...
FIG. 6. Determination of the number and affinity of Ca\(^{2+}\)-binding sites on GPI-PLD. The amount of \(^{45}\)Ca\(^{2+}\) binding to intact bovine serum GPI-PLD was determined by equilibrium dialysis at Ca\(^{2+}\) concentrations in the range of 1-400 \(\mu\)M as described under "Experimental Procedures." Combined data from two separate experiments are shown. The number of high affinity binding sites was determined from a Scatchard plot using the data collected at 1-10 Ca\(^{2+}\) (\(\bullet\)). Insufficient GPI-PLD was available to make an accurate analysis of the low affinity binding observed at higher Ca\(^{2+}\) concentrations.

FIG. 7. Metal ion dependence of GPI-PLD. A and B, GPI-PLD (0.5 unit) was preincubated at 0 °C for 30 min with 1.25 mM EGTA (A) or 125 \(\mu\)M 1,10-phenanthroline (B) and Zn\(^{2+}\) (\(\bullet\)), Co\(^{2+}\) (\(\triangle\)), Mn\(^{2+}\) (\(\triangledown\)), or Ca\(^{2+}\) (\(\bullet\)) at the concentrations indicated in the figure and then assayed for GPI-PLD activity. C and D: GPI-PLD (0.5 unit) was preincubated at 0 °C for 30 min with 1.25 mM EGTA (C) or 125 \(\mu\)M 1,10-phenanthroline (D). Metal ions were added either at the beginning (solid bars) or at the end (cross-hatched bars) of the preincubation period. The samples were then incubated for a further 30 min at 0 °C before assay for GPI-PLD activity. In C, metal ion concentrations were 1.25 mM except for Zn\(^{2+}\) (0.375 mM) and Ca\(^{2+}\) (5 mM). In D, all metal ions were added at 625 \(\mu\)M. GPI-PLD activity was determined and expressed as a percentage of control activity containing no added chelator or metal ions. Metal ion and chelator concentrations refer to those finally present in the GPI-PLD assay.

DISCUSSION

The goal of these studies was to identify structural features of GPI-PLD which might help us to understand its mechanism of regulation. Our approach was guided by two rather disparate pieces of information. First, the observation that GPI-PLD is inhibited by divalent cation chelators and therefore might be Ca\(^{2+}\)-regulated (1-4, 36, 37). Second, the prediction from sequence similarity that GPI-PLD contains four E-F hand-like Ca\(^{2+}\)-binding sites that are also found in integrins (4, 17). However, our preliminary studies did not provide convincing evidence that Ca\(^{2+}\) had any direct effects on GPI-PLD activity and suggested instead that the inhibitory effect of chelators might be to remove transition metal ions bound to the enzyme. This raised the possibility that GPI-PLD contains two different types of metal ion-binding site located in structurally and functionally distinct domains of the protein. In order to evaluate this model further we have used the following two approaches: (i) proteolytic fragmentation with the goal of identifying structurally distinct domains in GPI-PLD and possibly separating them, and (ii) \(^{45}\)Ca\(^{2+}\) binding and chelator inhibition studies to establish that GPI-PLD does in fact contain two distinct types of metal ion-binding site.
The results indicate that GPI-PLD can be cleaved by trypsin into three major fragments which are resistant to further proteolytic degradation. Although each fragment is predicted to contain at least 2 cysteine residues (17), they do not appear to be involved in interfragment disulfide linkages raising the possibility that the fragments may form two or three distinct and separable domains. The existence of distinct domains would readily account for the fact that proteolytic cleavage does not have a large effect on activity (see below) or any of the other properties examined (e.g. thermal stability, sensitivity to inhibitors, ability to bind Ca$^{2+}$ or form aggregates, etc.). However, attempts to localize the enzymatic activity to one of these fragments have so far been unsuccessful because conditions which allow their physical separation also inactivate the enzyme. This could indicate that GPI-PLD consists of a single compact domain with two polypeptide loops exposed at the surface providing sites for proteolytic cleavage. With this type of structure, separation of the fragments could only occur under conditions where extensive, and possibly irreversible, denaturation had taken place. However, since both native and trypsinized GPI-PLD appear to exist as oligomeric aggregates under non-denaturing conditions it is difficult to preclude the alternative possibility that the GPI-PLD monomer does consist of distinct domains, but these are prevented from dissociating after cleavage because of pre-existing interactions with other monomers in the aggregate.

The activity increases ~3-4-fold during the fragmentation process. The mechanism for this increase in activity is not known but is unlikely to be due to the removal of specific sequences or residues since proteases with different bond specificities, such as chymotrypsin and subtilisin, gave similar increases in activity. There is also no obvious change in the ability of GPI-PLD to form oligomers or its sensitivity to dissociation by detergents. Furthermore, proteolytically cleaved GPI-PLD is still unable to overcome the restriction on its activity provided by the membrane of the intact cell (11). It therefore seems unlikely that proteolysis per se is an important physiological mechanism for regulating GPI-PLD activity. In spite of this, proteolytic cleavage does seem to be occurring in vivo. We have identified (by NH$_2$-terminal sequencing and immunoblotting) similar-sized fragments in GPI-PLD isolated from bovine serum or from the transfected CHO fibroblasts. The identity and cellular location of the protease(s) involved in cleavage of GPI-PLD in vivo is currently under investigation.

One goal of these studies was to determine if GPI-PLD contains the Ca$^{2+}$-binding sites predicted from their sequence similarities with integrins (4, 17). We have now shown that both bovine serum and recombinant GPI-PLD bind $^{45}$Ca$^{2+}$ with reasonably high affinity and specificity. In contrast to the E-F hand and integrin metal ion-binding sites, the Ca$^{2+}$-binding sites in GPI-PLD are irreversibly disrupted by denaturation. Furthermore, the sites seem to be more specific than the integrins because GPI-PLD does not bind $^{54}$Mn$^{2+}$, and $^{45}$Ca$^{2+}$ binding was not prevented by the inclusion of divalent cations such as Mg$^{2+}$, Mn$^{2+}$, and Co$^{2+}$ which are known to compete with Ca$^{2+}$ for the metal ion-binding sites in some integrins (23, 24, 38). Although insufficient GPI-PLD was available to deduce the exact number of Ca$^{2+}$-binding sites, the data demonstrate that GPI-PLD can bind several Ca$^{2+}$ ions over the 1-10 $\mu$M range. The observed affinity is thus intermediate between that found for integrins ($K_d = 10-100$ $\mu$M) and many of the E-F hand, Ca$^{2+}$-binding proteins ($K_d = 10^{-9}-10^{-6}$ M (20)). These results strongly suggest that the predicted integrin-E-F hand-like sites are functional in GPI-PLD and likely to be occupied under physiological conditions. Definitive proof that the integrin-E-F hand-like sites are responsible for Ca$^{2+}$ binding by GPI-PLD will, however, require mutagenesis studies.

The role of the bound Ca$^{2+}$ was not revealed by our studies. Although E-F hands are found in several enzymes which require Ca$^{2+}$ either for substrate binding or catalysis (including at least one phospholipase (PLC-$\delta$) (39)), they are not found in most other Ca$^{2+}$-dependent phospholipases (i.e. the extracellular phospholipases $A_2$ and the other inositol phospholipid-specific phospholipase C isozymes, PLC-$\beta$ and PLC-$\gamma$). Furthermore, integrin-E-F hand-like sites have not been reported previously in any enzyme (22). We therefore propose that the integrin-E-F hand-like sites in GPI-PLD serve a non-catalytic, regulatory role independent of substrate binding or catalysis which involves a Ca$^{2+}$-dependent interaction with protein ligands. In this regard it is of interest to note that the sequence similarity between GPI-PLD and the integrin sites extends on either side of the E-F hand core region (17). In addition the E-F hands in both GPI-PLD and integrin $\alpha$ subunits are missing a calcium coordinating group at position 12. In integrins this group was proposed to be supplied by an aspartate residue either in its protein ligand or on the integrin $\beta$ subunit (21). More recently it has been shown that a recombinant fragment of the integrin $\alpha_\text{IIb}$ subunit not only binds Ca$^{2+}$ but also RGD-containing ligands (23). However, in preliminary experiments we have been unable to demonstrate the binding of GPI-PLD to immobilized RGD-containing proteins such as collagen, fibrinogen.

![FIG. 8. Effect of EDTA and 1,10-phenanthroline on tryptic digestion of GPI-PLD. Bovine serum GPI-PLD (30-40 $\mu$g/ml) and 40 $\mu$g/ml (lanes 2, 4, and 6) 10 mM EDTA (lanes 3 and 4) or 1 mM 1,10-phenanthroline (lanes 5 and 6) were included in some of the incubations. Samples were analyzed by SDS-PAGE on a 10% gel followed by silver-staining as described under "Experimental Procedures." The migration position of Fragments I (80 kDa), IV (50 kDa), HIV (40 kDa), and II (30 kDa) are indicated by arrowheads along the left side of the figure.](image-url)

<table>
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<tr>
<th>Table II</th>
<th>Metal analysis of purified bovine serum GPI-PLD</th>
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<tr>
<td></td>
<td>Ca</td>
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<tr>
<td>Total metal in sample (nmol)$^a$</td>
<td>15.09</td>
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<tr>
<td>Free metal in solution (nmol)$^b$</td>
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<tr>
<td>Metal bound to GPI-PLD (nmol)</td>
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<tr>
<td>Metal bound to GPI-PLD (atoms/molecule)</td>
<td>4.89</td>
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$^a$ Values for 2.9 nmol of purified GPI-PLD.

$^b$ Values for same volume of ultrafiltrate.

$^c$ denotes value is below detection limit specified.
gen, and fibronectin. Further studies will be necessary to identify the protein ligands predicted by the present and previous work on GPI-PLD.

GPI-PLD catalytic activity was inhibited, and the protein became more sensitive to proteolysis after treatment with the calcium chelators, EDTA and EGTA. However, similar effects were also produced by 1,10-phenanthroline which is unable to bind Ca\(^{2+}\) ions. Our data indicate that removal of bound transition metal rather than Ca\(^{2+}\) ions was responsible for the changes in GPI-PLD activity and proteolytic sensitivity. In stark contrast to the effect of chelators on these properties of GPI-PLD, it is relevant to recall that \(^{40}\)Ca\(^{2+}\) binding was not affected by 1,10-phenanthroline emphasizing the structural independence of the two types of metal ion-binding. We have so far been unable to identify the transition metal required for GPI-PLD activity because reactivation after removal of the chelate was unsuccessful. However, the ability of Zn\(^{2+}\) to block the inhibitory effects of EGTA at substoichiometric levels and the participation in phosphate ester binding hydrolysis by enzymes such as the P1 nuclease, alkaline phosphatase, and phosphatidylcholine phospholipase C (40). However, the amount of zinc described by purified GPI-PLD suggests that this ion is a good candidate. Active site Zn\(^{2+}\) is well known for its catalytic role in the lipid modification of proteins (40).

Acknowledgments—We are grateful to Pat Andreola for excellent technical assistance. We also thank Dr. J. P. Kochan for providing CHO fibroblasts transfected with the GPI-PLD cDNA and Dr. Y.-C. E. Pan for providing sequencing facilities.

REFERENCES