Isolation and Characterization of a Folate Receptor-directed Metalloprotease from Human Placenta*

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Glycosyl-phosphatidylinositol-anchored hydrophobic placental folate receptors (PFRs), which have an important functional role in maternal-to-fetal transplacental folate transport, can be converted to soluble hydrophilic forms by a placental metalloprotease. Using a Triton X-114 temperature-induced phase separation assay to monitor enzyme-mediated conversion of radiolabeled hydrophobic PFR into hydrophilic PFR, a metalloenzyme was isolated to apparent homogeneity from Triton X-114-solubilized human placenta using concanavalin A-Sepharose and reverse-phase high performance liquid chromatography (HPLC) as major purification steps. The purified hydrophobic enzyme eluted as a single protein peak on reverse-phase HPLC and SDS-polyacrylamide gel electrophoresis revealed a single 63,000-Mr protein, which was reduced to 58,000-Mr, following de-glycosylation, findings comparable with amino acid analysis (46,000–59,000). The metalloenzyme was activated by Mg²⁺, Zn²⁺, Mn²⁺, and Ca²⁺, optimally at physiologic pH; it also exhibited EDTA-sensitive endoproteolytic cleavage of [³²P]leucine-labeled full-length nascent PFR polypeptide generated in vitro in the absence of microsomes. Rabbit polyclonal anti-metalloprotease antiserum specifically immunoprecipitated ¹²⁵I-metalloprotease and recognized cross-reacting moieties on plasma membranes of normal human hematopoietic progenitor cells and human cervical carcinoma cells, both of which also express FR.

Folate receptors (FR) are cell surface glycoproteins that bind physiologic serum 5-methyltetrahydrofolate with high affinity and transport the vitamin into cells (reviewed in Ref. 1). Human placental FR (PFR) have recently been shown to have a major functional role as modulators of maternal-to-fetal transplacental folate transport (2). Native hydrophobic PFR are glycosyl-phosphatidylinositol (GPI)-anchored proteins, have a Mr of 35,000, and require detergent for solubilization out of membranes (3). Because of the GPI anchor, PFR are susceptible to cleavage to soluble forms by GPI anchor-specific phospholipases C and D (3). However, through studies on detergent-solubilized crude placenta (containing cytosolic, nuclear, and membrane proteins), we also identified a specific Mg²⁺-dependent enzyme that converted hydrophobic PFR to hydrophilic forms that retained ligand binding capacity (4). The functional nature of this metalloenzyme in chorionic villi cultured under serum-free conditions has been demonstrated (5). Because hydrophilic and GPI-anchored FR from nasopharyngeal carcinoma (KB) cells (6) have similar amino-terminal amino acid sequences (7), the locus of cleavage by the placental metalloenzyme has been provisionally assigned to the carboxy-terminal domain of the native PFR species (1, 5). Furthermore, isolation of hydrophilic PFR released into the growth medium of chorionic villi identified a species that was much smaller on amino acid analysis (22.5 kDa) when compared with native PFR but was of comparable Mr, with the soluble folate binding protein isolated from human milk (8) and the growth medium of KB cells (9). This lent further support to the conclusion that the placental metalloenzyme was a protease; however, such data were indirectly generated, and a direct demonstration of endoproteolytic cleavage of the native PFR by the metalloenzyme was lacking. In addition, all studies that demonstrated metalloenzyme activity have relied on gel filtration in Triton X-100, which reliably separates the hydrophobic (apparent 160 kDa) FR substrate from its hydrophilic (40 kDa) product (4, 5, 10). However, gel filtration is a cumbersome assay that is not easily adaptable to analysis of multiple variables required in isolation of proteins. This may explain why additional information on physico-chemical characteristics of this metalloprotease or its localization has not been generated.

Although FR in KB cells are GPI-anchored (6), in the membranes of another KB cell line, the biosynthetically labeled [³²P]leucine-hydrophobic FR fortuitously contained a full-length FR polypeptide that is leucine-rich in its COOH-terminal end (10). Therefore, when these investigators identified an activity in washed, Triton X-100-solubilized KB and placental membranes that converted [³²P]leucine-labeled hydrophobic FR to hydrophilic forms, the assignment of this enzyme as a metalloprotease (as opposed to a GPI-specific phospholipase) was direct. This unique KB cell FR differed considerably from PFR, which, like other GPI-anchored proteins, have lost their COOH-terminal hydrophobic polypeptide during post-translational addition of the preformed GPI anchor (11, 12). Thus, it is still unclear whether the putative placental metalloprotease, which endoproteolytically cleaved the FR polypeptide substrate from KB cells (10), is the same enzyme that converts mature GPI-linked PFR (3, 5) to soluble forms.

The biological significance of the conversion of placental hydrophobic to hydrophilic FR is unclear. In the case of FR on malignant and normal cells, the conversion of hydrophobic FR to hydrophilic FR as mediated by a metalloprotease or GPI-specific phospholipase can potentially be an important mechanism for post-translational regulation of the expression of FR on the cell surface. Although a major role for the metallopro-
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tease was not identified in the placenta at term (2), its activity could easily determine the extent of acquisition of folates by FR on trophoblasts, thereby influencing placental growth and development. However, no probes (antibodies or cDNA) are as yet available to study such PFR-directed enzymes. Because the GPI-linked hydrophobic PFR sequesters in the micellar phase at the cloud point of Triton X-114 (3), whereas the hydrophilic PFR product sequesters in the aqueous phase, and the EDTA-sensitive enzyme in solubilized human placenta can completely cleave all native hydrophobic GPI-anchored FR into hydrophilic forms (3, 4, 13), we developed a rapid assay for the metalloenzyme using Triton X-114 and focused on developing a purification strategy. We now report on the isolation and characterization of this enzyme and show that moieties that share epitopes with this metalloprotease are present on the surface of malignant and normal cells that co-express the FR substrate.

**EXPERIMENTAL PROCEDURES**

**Assay for the Placental Metalloenzyme**

The assay, which was carried out in Beckman 96-well flat bottom immunoassay plates, relied on the ability of solubilized placental hydrophobic metalloenzyme to cleave mature $^{125}$I-hydrophobic PFR to hydrophilic forms. Both enzyme and substrate were retained in the micellar phase at the cloud point of Triton X-114 (3), whereas the hydrophobic PFR was recovered in the aqueous phase. Briefly, 350 fmol of $^{125}$I-hydrophobic PFR (2 × 10$^9$ cpm in 20 μl contained ~12.25 ng) was added to 100 μl of metalloenzyme in the absence or the presence of 60 mM EDTA or 60 mM EGTA in 10 mM potassium phosphate, pH 7.5, and 20 mM MgCl$_2$ in a final reaction volume of 250 μl. Each assay was routinely determined in duplicate and judged valid only when there was <5% variation from the mean. After incubation at 37°C overnight, 50 μl of 2% preconcentrated Triton X-114 was added, and the mixture was incubated at 4°C for 15 min to disperse micelles (14). After re-incubation at 37°C for 30 min, the regenerated micelles were sequestered in a detergent-rich micellar phase of Triton X-114 in the absence or presence of 15 mM Tris-HCl, pH 7.4, and centrifugation at 4°C for 15 min in a microplate carrier designed for a Beckman GH 3.7 rotor in a Beckman GPR table-top centrifuge. Following centrifugation, 130 μl of the aqueous phase from each well that contained $^{125}$I-hydrophilic PFR was counted for radioactivity, and values were normalized for a total volume of 250 μl. One unit of metalloenzyme activity was defined as the amount required to convert 2 fmol of $^{125}$I-hydrophobic PFR to hydrophilic forms in 1 min at 37°C.

**Purification of the Placental Hydrophobic PFR-directed Metalloprotease**

Preparation of Crude Solubilized Metalloenzyme—A full-term normal placenta following uncomplicated vaginal delivery was homogenized in 1 h with 2 volumes of 14 mM potassium phosphate, pH 7.5, at 4°C and centrifuged (30,000 × g for 30 min) (3). The pellet was washed with another cycle of homogenization and centrifugation before solubilization with 10 mM potassium phosphate, pH 7.5, containing 1.5% Triton X-114 and 20 mM MgCl$_2$ for 16 h at 4°C (13). After centrifugation (30,000 × g for 30 min), the metalloenzyme-rich supernatant was collected.

Temperature-induced Phase Separation in Triton X-114—Triton X-114 was then added to achieve a final concentration of 2%, and the mixture was incubated at 37°C overnight to generate a detergent-rich micellar phase and an aqueous supernatant (14). To capture residual metalloenzyme from the supernatant, the mixture was loaded onto a packed bed of Sepharose, which was pre-equilibrated with the equilibration buffer consisting of 80% buffer A (0.1% trifluoroacetic acid in water) at a flow rate of 5 ml/min for 5 min, the column was eluted using a gentle “convex-up” (program #2) gradient increase of buffer B (75% isopropanol/20% acetonitrile/5% water containing 0.1% trifluoroacetic acid, final pH 3.2) from 20 to 60% over 25 min. The fraction containing metalloenzyme eluting to a peak of 51% was collected in 100 μl of 1 M Tris-HCl, pH 8.0, containing 100 mM MgCl$_2$, and aliquots were assayed for activity and protein and analyzed by SDS-PAGE (5).

Characterization of the Metalloenzyme

**Gel Filtration Analysis of Metalloenzyme Activity—**$^{125}$I-hydrophobic PFR (30,000 cpm) was incubated with ConA-Sepharose eluate (40 μg) and HPLC-purified metalloenzyme (6–20 μg) in a final volume of 200 μl of buffer consisting of 10 mM potassium phosphate, pH 7.5, 20 mM MgCl$_2$, 0.1% Triton X-114 in the presence or the absence of 100 μl of EDTA, and conversion to hydrophilic PFR was analyzed by Sephacyr S-200 gel filtration in Triton X-100 (2). Radial immunoblotting—Purified metalloenzyme (20 μg) and hydrophobic PFR (10 μg) (3) were iodinated (5), and free $^{125}$I-NaI was removed by passage of the reaction mixture over a NucTrap push column (Stratagene, La Jolla, CA). Assuming 100% recovery, the specific activities of $^{125}$I-metalloprotease and $^{125}$I-hydrophobic PFR were 0.13 and 1.6 μCi/μg, respectively. Hydrophobic PFR (3) was also covalently labeled within its ligand-binding site with the N-hydroxysuccinimide ester of $[^{3}H]IptGlu (16). Based on a 1:1 molar stoichiometry and assuming 100% recovery, the specific activity of $[^{3}H]IptGlu$-labeled hydrophobic PFR was 2.87 Ci/mmol.

Verification of Hydrophobicity of Purified Metalloenzyme—The lyophilized HPLC-purified 63-kDa metalloenzyme was resuspended in water and analyzed as follows: one aliquot was subjected to temperature-induced phase separation, and after the aqueous and micellar phase fractions were brought up to the same volume and final Triton X-114 concentration, samples derived from these phases were assayed for metalloenzyme activity. Another aliquot was iodinated, and after removal of unincorporated $[^{125}$I], Triton X-114 was added to a final concentration of 5%. Following three cycles of temperature-induced phase separation with separate pooling of all three supernatants and all three pellets, aliquots of these pooled fractions were counted for radioactivity.

**Amino Acid Analysis—**Analysis of purified metalloenzyme was carried out after vapor hydrolysis and preclusion phenylisothiocyanate derivative derivatization (17). Briefly, 10 μg of HPLC-purified metalloenzyme (6–20 μg) in a final volume of 6 μl HCl. Phenylthiocarbamyl amino acids were analyzed on an Applied Biosystems 130A separation system using a 2.1 × 220-mm Brownlee C$_{18}$ column (Foster City, CA). Phenylthiocarbamyl amino acid peaks were calibrated with the Pierce amino acid standard H.

Endoproteinolytic Cleavage of In Vitro Translated Nascent PFR by Metalloprotease—The 1.1 kilo-base pair PDR DNA generously provided by Dr. P.C. Elwood (Medical Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD) (18) was cloned into the EcoRI site of the transcription factor vector, pSPT18 (Pharmacia Biotech Inc.). Recombinant plasmid DNA was then expanded and purified, and PDR DNA was recovered by restriction with Bgl II (19). The Sp6T7 transcription kit (Pharmacia, Piscataway, NJ) was used to generate the PDR mRNA. Nascent PFR polypeptide was labeled with $[^{3}H]$leucine in vitro during translation of PDR RNA in the absence of microsomes. Briefly, 2 μg of PDR mRNA was translated in vitro in the presence of 8 μCi of $[^{3}H]$leucine and 40 μl reticulocyte lysate (19). $[^{3}H]$leucine-labeled nascent polypeptide was analyzed as follows: one aliquot of the translation mixture (50 μl of metalloenzyme-purified ConA-Sepharose eluate) in the absence of the presence of 60 μl EDTA in a final volume of 220 μl for 16 h at 37°C. The samples were subsequently loaded onto the reverse-phase HPLC (Vydac) column, which was equilibrated with 95% buffer A (0.1% trifluoroacetic acid in water) and 5% buffer C (0.1% trifluoroacetic acid in water, 95% acetonitrile). After basal conditions of perfusion were continued for 5 min, a linear gradient (from 5 to 100% buffer C over 55 min) was established. Fractions collected at a flow rate of 5 ml/min/fraction were counted for radioactivity. A second aliquot was analyzed by Sephacryl S-200 gel filtration...
RESULTS

Assay for the Solubilized Placental Metalloenzyme

In preliminary studies (data not shown), the specific conversion of GPI-anchored 125I-hydrophobic PFR (which sequesters in the detergent-rich, Triton X-114 micellar phase) to 125I-hydrophilic PFR (released into the aqueous phase) using the temperature-induced phase separation assay was verified by the gel filtration assay in Triton X-100 (4). In addition, when the ligand-binding site of hydrophobic PFR was covalently labeled with N-hydroxysuccinimide ester of [3H]PteGlu, metalloenzyme-mediated cleavage of this substrate also led to release of the [3H]PteGlu-bound hydrophobic PFR into the aqueous phase (4). Thus, this phase separation assay appropriately reflected the conversion of hydrophobic PFR to hydrophilic forms consistent with the activity of the putative FR-directed metalloprotease.

Identification of Distinct EDTA-sensitive and EDTA-insensitive PFR-directed Enzymes

Dose-response studies using a fixed amount of [3H]PteGlu-labeled hydrophobic FR incubated with increasing concentrations of crude enzyme revealed that ~50% conversion to hydrophilic PFR was achieved with 62.4 μg of crude enzyme, and 90% conversion was achieved with 400 μg (Fig. 1A). Although a major portion of the crude metalloenzyme at low concentrations (~40 μg of protein) was EDTA-sensitive, at higher concentrations (~200 μg of protein), nearly equivalent amounts of conversion were accounted for by an EDTA-insensitive activity, suggesting the presence of another enzyme that cleaved hydrophobic PFR. Alternatively, this could be a function of reversal of the chelating effect of EDTA (present in fixed concentrations) by the addition of increasing amounts of Mg2+ (extant in the crude enzyme preparation) to the reaction mixture; these possibilities were further investigated. From five different crude metalloenzyme preparations, on average 74% of substrate was converted under basal conditions, of which 36% was EDTA-insensitive. When this preparation was phase separated at the cloud point of Triton X-114 and the micellar and aqueous phases were individually assayed, the latter was enriched for the EDTA-insensitive enzyme, i.e. 48% of total conversion was not inhibited by EDTA. More importantly, however, the micellar phase was significantly enriched for the EDTA-sensitive enzyme because only 5% of converting activity was EDTA-insensitive. Although these data confirmed the presence of two distinct placental enzymes that converted hydrophobic PFR to hydrophilic forms, they also supported the conclusion that the EDTA-sensitive enzyme (metalloenzyme) was hydrophobic, whereas the EDTA-insensitive enzyme was hydrophilic.

The optimal duration of incubation at 37°C was determined for two levels of crude metalloenzyme (Fig. 1B). The basal conversion recorded for the higher level of metalloenzyme likely occurred during the incubation at 37°C to effect temperature-induced phase separation. Following incubation for 2 h, there was 60 and 90% conversion of hydrophobic PFR to hydrophilic PFR at 400 and 2000 μg of metalloenzyme, respectively. Thus, 2 h was optimum, with no additional gain even after incubation for 16–24 h, consistent with earlier data (4).

In preliminary studies (data not shown), an activity that bound to and was eluted from ConA-Sepharose was noted to be almost (>95%) entirely sensitive to EDTA; SDS-PAGE of this preparation revealed a major protein that migrated at 63,000 M(r). When the ConA-Sepharose eluate was subjected to HPLC gel filtration in Triton X-114 and each eluted fraction was individually tested for metalloenzyme activity, there was a major protein peak with activity that also migrated on SDS-PAGE at 63 kDa. Thus, this species was the likely metalloenzyme.

Isolation of the Hydrophobic Metalloenzyme

Table 1 reveals the purification scheme used to isolate the metalloenzyme 267-fold with a final yield of 16% from human placenta. The isolated metalloenzyme eluted as a single protein peak at a concentration of 51% buffer B on reverse-phase HPLC (Fig. 2A). SDS-PAGE of this preparation revealed a single protein band of 63,000 M(r), which reduced to 58,000 M(r) after deglycosylation with recombinant glycopeptidase F (Fig. 2B). In addition, temperature-induced phase separation assay of
this sample revealed a dose-dependent release of $^{125}$I-hydrophobic PFR into the aqueous phase (Fig. 2 C). Furthermore, the $^{125}$I-species recovered from the aqueous phase (following incubation of $^{125}$I-hydrophobic PFR with HPLC-purified metalloenzyme and temperature-induced phase separation) exhibited an elution profile consistent with that of hydrophilic PFR on gel filtration analysis in Triton X-100 (2) (data not shown). Together, these data indicated that the HPLC-purified metalloenzyme was functionally active. When 20 mg of this preparation was iodinated, a single iodinated peak was identified on SW300 HPLC gel filtration, which also migrated as a 63-kDa species on SDS-PAGE (data not shown). Interestingly, although both crude and ConA-Sepharose-eluted metalloenzyme exhibited $^{125}$I-hydrophobic PFR-directed activity on Sephacryl S-200 gel filtration in Triton X-100 (2), similar activity could not be clearly documented using the isolated 63-kDa metalloenzyme. Instead, the products of the reaction mixture consistently yielded a single $^{125}$I peak, which eluted at the same position as $^{125}$I-hydrophobic PFR (data not shown). Because this was not due to the lack of activity of the HPLC-purified metalloenzyme, we did not investigate the basis for these observations. It is possible that the harsh reverse-phase HPLC conditions modified the metalloenzyme and led to protein-protein interactions with the substrate under conditions of gel filtration. Finally, the apparently homogeneous protein was hydrophobic because 80% of HPLC-purified metalloenzyme and 87% of $^{125}$I-metalloenzyme were recovered in the micellar phase following temperature-induced phase separation analysis (data not shown).

Characterization of the Metalloenzyme

The partially purified ConA-Sepharose eluate was used to characterize the enzyme's activity under controlled conditions.

Rate of Conversion—Fig. 3 A shows the conversion of $^{125}$I-hydrophobic PFR to hydrophilic PFR by a fixed amount of metalloenzyme as a function of increasing incubation times. Although relatively less active when compared with crude enzyme (Fig. 1 B), the rate of conversion was linear as a function of time; by 8 h, 91% conversion to hydrophilic PFR was achieved.

**Table 1**

Summary of purification of metalloprotease from human placenta

| Step                        | Volume | Protein | Total units | Specific activity | Recovery % | Purification |
|-----------------------------|--------|---------|-------------|-------------------|------------|--------------|
| Solubilized pellet          | 815    | 3261    | 8153        | 2.5               | 100        | 100          |
| Triton X-114 phase-separation | 250   | 1400    | 4375        | 3.1               | 54         | 1.2          |
| Concanavalin A-Sepharose eluate | 250   | 200     | 3125        | 15.6              | 38         | 6            |
| Ultrafiltration             | 50     | 56      | 1333        | 667.0             | 16         | 267          |
| HPLC peak fraction          | 2      | 2       | 1333        | 667.0             | 16         | 267          |

* Percentage of recovery = total units/8153.

**Fig. 2.** Reverse-phase HPLC, SDS-PAGE, and functional analysis of the purified metalloprotease. A, the HPLC-purified metalloenzyme (eluted by 51% buffer B) was reanalyzed under similar conditions by reverse-phase HPLC, and each fraction was spectrophotometrically analyzed for protein. B, SDS-PAGE (7.5%) of reverse-phase HPLC-purified metalloenzyme before (lane 1) and after (lane 2) deglycosylation with recombinant glycopeptidase F. Each well was loaded with 15 μg of protein and stained with Coomassie Blue. C, dose-response curve of the purified sample using the temperature-induced phase separation assay in Triton X-114.

**Fig. 3.** Analysis of various parameters of metalloprotease activity. A, rate of conversion of $^{125}$I-hydrophobic PFR to hydrophilic PFR as a function of time. 60 μg of metalloenzyme in 100 μl of 10 mM potassium phosphate, pH 7.5, containing 20 mM MgCl$_2$ was incubated with $^{125}$I-hydrophobic PFR (350 fmol) for various times indicated in the absence or the presence of 60 mM EDTA. Each data point represents the mean of experiments carried out in duplicate; there was <5% variation from the mean in more than three comparable experiments carried out with different preparations. B, dose-response curves using a fixed concentration of $^{125}$I-hydrophobic PFR (350 fmol) and increasing concentrations of purified metalloenzyme in the absence or the presence of EDTA. C, determination of pH optimum for metalloenzyme activity in the absence or the presence of EDTA. D-F, characteristics of inhibition with 1,10-phenanthroline (D), and reactivation of metalloenzyme (50 μg) with increasing concentrations of various cations (MgCl$_2$, MnCl$_2$, CaCl$_2$, and ZnCl$_2$) after inhibition with 60 mM EDTA (E) and 60 mM EGTA (F).
Dose-Response Studies—Using a fixed amount of $^{125}$I-hydrophobic PFR, conversion as a function of dose of metalloenzyme was determined. Thus, 50% conversion was achieved by 10 $\mu$g of metalloenzyme and maximal conversion with 25 $\mu$g (Fig. 3B).

Determination of the pH Optimum—Conversion of $^{129}$I-hydrophobic PFR at pH 4, 6, 7.5, 8, and 10 was 56, 74, 100, 80, and 68%, respectively (Fig. 3C). When EDTA sensitivity was also tested at these pH levels, there was significant inhibition of conversion at pH 7.5. Thus, the EDTA-sensitive metalloenzyme was maximally active at physiological pH.

Effect of Inhibitors and Reactivation by Divalent Cations—The metalloenzyme was inhibited by 1,10-phenanthroline in a dose-dependent manner (Fig. 3D). In addition, after incubation with EDTA, which inhibited enzyme activity ~95%, increasing concentrations of Mg$^{2+}$, Ca$^{2+}$, Mn$^{2+}$, and Zn$^{2+}$ overcame the inhibition in a dose-dependent manner (Fig. 3E). Although inhibition was also achieved with EGTA and the pattern of re-activation with Ca$^{2+}$ or Mn$^{2+}$ was similar to that using EDTA, there was no major recovery despite addition of 400 $\mu$M of Mg$^{2+}$ (Fig. 3F). In addition, the data with Zn$^{2+}$ indicated that although progressively increasing concentrations up to 4 $\mu$M led to reactivation of the enzyme at a lower dose compared with EDTA, reactivation in the presence of EDTA, concentrations > 4 $\mu$M led to progressive inhibition of enzyme activity.

Amino Acid Analysis—Table II shows the number of amino acid residues/mol of HPLC-purified metalloenzyme (based on 63,000 M$_r$). There were a total of 483 amino acid residues (minus tyrosine residues) that predicted a total M$_r$ of 59,179, comparable with that of deglycosylated metalloenzyme. The amino acids that were hydrophobic, hydrophilic, and with ionizable side chains constituted 57, 17, and 26% of the protein, respectively.

Evidence That the Purified Metalloenzyme Is a Protease—Because neither gel filtration nor temperature-induced phase separation assays distinguished EDTA-sensitive protease from phospholipase activity, an important question was whether the hydrophobic 63-kDa human placental metalloenzyme was a protease or GPI-specific phospholipase. Prompted by an earlier report (10), we hypothesized that if PFR was labeled with $[^{3}H]$leucine during in vitro translation of PFR mRNA in the absence of microsomes, all 22 leucine residues in the nascent PFR polypeptide (18) could be replaced by radiolabel, and the full-length $[^{3}H]$leucine-labeled nascent PFR polypeptide (deduced M$_r$ of 29,817) would also contain no post-translational modifications, including GPI anchor addition (11, 12). Therefore, we reasoned that EDTA-sensitive cleavage of this nascent polypeptide (especially a loss of the COOH-terminal $[^{3}H]$leucine-rich fragment) would result in a net reduction in counts from the major fragment of $[^{3}H]$leucine-labeled PFR polypeptide and an alteration in its hydrophobicity leading to an altered reverse-phase HPLC elution profile.

Following in vitro translation of full-length 1-kilobase pair nascent PFR mRNA, ~5–10% of total $[^{3}H]$leucine integrated into nascent PFR polypeptide. The M$_r$ of this species was ~30,000 on SDS-PAGE, and it eluted at apparent M$_r$ of ~80,000 on Sephadryl S-200 gel filtration analysis in Triton X-100, findings distinctly different from mature GPI-anchored PFR, which migrated on SDS-PAGE at 44,000 M$_r$, and eluted at apparent 160,000 M$_r$, respectively (3). Furthermore, although ~1 $\mu$l of anti-PFR antiserum immunoprecipitated >90% of mature $[^{125}]$PteGlu (histamine derivative)-labeled FR (3, 20, 21), at least 20 $\mu$l of this antiserum was required to specifically immunoprecipitate $[^{3}H]$leucine-labeled nascent PFR polypeptide (data not shown). Thus, not unexpectedly, $[^{3}H]$leucine-labeled nascent FR polypeptide had distinct differences on SDS-PAGE, gel filtration profile in Triton X-100, and antigenic determinants when compared with mature PFR.

The reverse-phase HPLC elution profile of in vitro translated and $[^{3}H]$leucine-labeled nascent PFR polypeptide generated in the absence of microsomes indicated a single sharp peak of radioactivity that eluted at 5 min. When this species was incubated with 50 $\mu$g of metalloenzyme, there was a clear-cut shift of the peak to a retention time of 10 min (data not shown). This conversion was EDTA-sensitive because the reaction was completely inhibited in its presence. In addition, the total counts in the major peak eluting at 10 min (i.e. the product) constituted 73% of the total counts in the EDTA-inhibited control (precursor), confirming that the alteration in profile was a function of loss of net radioactivity from the nascent PFR polypeptide. We could not locate the putative cleaved $[^{3}H]$leucine-labeled COOH-terminal fragment released from the nascent polypeptide. Because the in vitro translation mixture itself contained several crude proteins, some of which could be nonspecific proteases, it is possible that this smaller hydrophobic species was degraded shortly after it was released from the major body of the nascent PFR polypeptide by the metalloenzyme. Therefore, because it cleaved and shifted the elution profile of $[^{3}H]$leucine-labeled nascent PFR polypeptide and reduced net cpm in the major fragment, these data supported the conclusion that the metalloenzyme was a protease (referred to hereafter as a metalloprotease).

Studies with Anti-metalloprotease Antiserum

An immunoprecipitation curve involving the reaction of $^{125}$I-metalloprotease with increasing concentrations of anti-metalloprotease antisera revealed a dose-dependent immunoprecipitation of $^{125}$I-metalloprotease with antisera under conditions where nonimmune serum led to (nonspecific) precipitation of <10% of the radioactivity (data not shown). Thus, this antiserum contained antibodies that recognized epitopes on the isolated placental metalloprotease. HeLa-IU1 cells contain GAP-anchored FR on plasma membranes (23) and an activity that was consistent with a membrane-associated metalloenzyme (data not shown). Therefore, we determined whether the putative metalloenzyme in these cells would be recognized by anti-metalloprotease antiserum using fluorescence-activated cell sorting/fluorescence microscopy. As shown in Fig. 4, when compared with nonimmune serum, two levels of anti-metalloprotease antiserum led to a progressive shift of the fluorescence to the right by more than 10- and 100-fold, respec-

**Table II**

| Item          | Residues/mol of Metalloenzyme | Mols percent |
|---------------|------------------------------|--------------|
| Aspartic acid | 20                           | 4.14         |
| Glutamic acid | 14                           | 2.90         |
| Serine        | 26                           | 5.38         |
| Glycine       | 103                          | 21.33        |
| Histidine     | 27                           | 5.59         |
| Arginine      | 48                           | 9.94         |
| Threonine     | 55                           | 11.39        |
| Alanine       | 18                           | 3.73         |
| Proline       | 12                           | 2.48         |
| Tyrosine      | 31                           | 6.42         |
| Valine        | 31                           | 6.42         |
| Methionine    | 33                           | 6.83         |
| Cysteine      | 3                            | 0.62         |
| Isoleucine    | 34                           | 7.04         |
| Leucine       | 25                           | 5.18         |
| Phenylalanine | 18                           | 3.73         |
| Lysine        | 16                           | 3.31         |
| Total amino acids | 483                      | 25.80         |
| Total molecular weight | 59,179                 | 100.00      |
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**DISCUSSION**

GPI-anchored hydrophobic FR can be cleaved within its GPI anchor by GPI-specific phospholipases C and/or D (3, 6, 25, 26), as well as endoproteolytically by EDTA-sensitive enzymes (4, 5, 10) and an EDTA-insensitive enzyme(s) (this report). Thus, cleavage of the GPI-anchored FR substrate into hydrophilic forms as monitored by our assay could represent activity of any or all of these enzymes. Furthermore, in the initial purification steps, there was some contamination of the crude solubilized metalloprotease with endogenous hydrophobic PFR. These would reduce the specific activity of \(^{125}\)I-hydrophobic PFR (the substrate) and result in underestimation of the amount of metalloprotease. Thus, except for the final purified preparation, the calculations to determine yield during the earlier purification steps can only be viewed as general estimates of relative improvement in the purity of the metalloprotease.

The reverse-phase HPLC-isolated metalloprotease, which exhibited biological activity in converting hydrophobic PFR to hydrophilic forms, met several criteria for purity: it exhibited a single protein peak on reverse-phase HPLC and a single band of protein staining on SDS-PAGE at 63,000 M\(_r\). Furthermore, when this preparation was iodinated and similarly analyzed, there was only a single iodinated species. Moreover, amino acid analysis of the purified protein revealed a net M\(_r\) of 59,000, which closely approximated the M\(_r\) estimated following deglycosylation of the metalloprotease and SDS-PAGE (58,000 M\(_r\)). Whereas these data supported the conclusion that the metalloprotease was apparently homogeneous, immunofluorescence data indicated that moieties with shared epitopes with placental metalloprotease were localized on plasma membranes of normal and malignant cells in a similar distribution as the hydrophobic FR substrate.

Major differences between the KB cell FR-directed metalloprotease (10) and the PFR-directed metalloprotease (this report) were characteristics related to cation dependence: thus, KB cell metalloprotease was activated by Mn\(^{2+}\), Ca\(^{2+}\), and Zn\(^{2+}\) but not by Mg\(^{2+}\), whereas placental metalloenzyme was responsive to Mg\(^{2+}\) in addition to the above cations. Although we had earlier tested whether the placental metalloenzyme was activated by Zn\(^{2+}\) and Mn\(^{2+}\), our results failed to assign these cations as activators (4). Retrospective review of those results within the context of the present data has identified the most likely reason: earlier, because \(^{125}\)I-hydrophilic PFR was unavailable, we used the property of reversible \(^{[3H]}\)PteGlu binding to hydrophobic PFR (as opposed to covalent binding of the radioligand to hydrophobic PFR in the present studies). These two cations (Zn\(^{2+}\) and Mn\(^{2+}\)) chemically precipitate free \(^{[3H]}\)PteGlu but do not have any such effect on \(^{[3H]}\)PteGlu that is covalently bound to hydrophobic PFR. Thus, when these cations were added to activate the metalloenzyme, most of the free radioligand would likely precipitate and thereby be unavailable for noncovalent, albeit high affinity, interaction with PFR. This would severely limit our capacity to detect conversion of hydrophobic to hydrophilic forms of PFR. In fact, review of our original "raw" data (4) revealed that the net amount of soluble (nonprecipitated) radioligand applied to the gel filtration column was reduced to less than 10% of the total radioactivity added to the reaction mixture. However, the use of \(^{[3H]}\)PteGlu covalently labeled to hydrophobic PFR and \(^{125}\)I, hydrophobic PFR circumvented these issues and allowed reassessment of these cations as activators of the enzyme. Nevertheless, the fact that Mg\(^{2+}\) activates the placental (but not KB cell) metalloprotease suggests that these enzymes may not be identical.

The strategy employed to prove that the isolated metalloenzyme was a protease and not a GPI-specific phospholipase was to determine if it cleaved in vitro synthesized \(^{[3H]}\)leucine-labeled nascent PFR substrate, which was generated in the absence of microsomes. As pointed out earlier (10), based on the deduced amino acid sequence from PFR cDNA (18), there are 257 amino acid residues among which there are a total of 22 leucine residues: five are within the signal peptide (amino acids 1–25), and eight are in the hydrophobic COOH-terminal domain (between amino acids 227 and 257), whereas the other nine are distributed between amino acids 26 and 226. The nascent PFR polypeptide translated in vitro in the absence of microsomes would be full-length and not be truncated in its hydrophobic COOH-terminal domain during addition of the GPI-anchor (11, 12). Therefore, \(^{[3H]}\)leucine would be biosynthetically incorporated proportionately to its distribution in the nascent polypeptide, i.e. 23% in the signal peptide, 36% in the COOH-terminal domain, and 41% in other regions of the polypeptide. Because the COOH-terminal domain is hydrophobic, if EDTA-sensitive endoproteolytic cleavage occurred either within or proximal to this region, the PFR polypeptide would be expected to be converted to a relatively hydrophilic form with loss of specific radioactivity in the major fragment by up to 36% of the original value. In fact, the metalloprotease did endoproteolytically alter the substrate in an EDTA-sensitive manner, and the net recovered radioactivity was –30% less than the original substrate.

What is unexplained is the paradoxical conversion of the
Several metalloproteases have been reported in various tissues with different biological functions (reviewed in Ref. 29). Among two distinct metalloproteases with M, that are comparable with that estimated for hydrophobic PFR-directed metalloprotease, one from human cartilage (M, of 62,000) has proteolytic activity against elastin and requires Zn for optimum activity (30). Another from Leishmania major promastigotes (M, of 63,000) is a membrane-associated glycoprotein that degrades azoscein in a pH range 7.0–9.0 (31). Human placenta is also rich in a number of metalloproteases such as collagenase (32). Therefore, it will be of significant interest to determine if additional substrates exist for this newly isolated placental metalloprotease.

REFERENCES

1. Antony, A. C. (1992) Blood 79, 2807–2820
2. Henderson, G. I., Perez, T., Schenker, S., Mackins, J., and Antony, A. C. (1995) J. Lab. Clin. Med. 126, 184–203
3. Verma, R. S., Gullapalli, S., and Antony, A. C. (1992) J. Biol. Chem. 267, 4119–4127
4. Antony, A. C., Verma, R. S., Unune, A. R., and Laftosa, J. A. (1989) J. Biol. Chem. 264, 1911–1914
5. Verma, R. S., and Antony, A. C. (1991) J. Biol. Chem. 266, 12522–12535
6. Luhrs, C. A., and Slomiany, B. L. (1989) J. Biol. Chem. 264, 21446–21449
7. Luhrs, C. A., Pittirangon, P., da Costa, M., Rothenberg, S. P., Slomiany, B. L., Brink, L., Tous, G. I., and Stein, S. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 6546–6549
8. Antony, A. C., Utley, C. S., Marcell, P. D., and Kohlhouse, J. F. (1982) J. Biol. Chem. 257, 10081–10089
9. Elwood, P. C., Kane, M. A., Portillo, R. M., and Kohlhouse, J. F. (1986) J. Biol. Chem. 261, 15416–15423
10. Elwood, P. C., Deutsch, J. C., and Kohlhouse, J. F. (1991) J. Biol. Chem. 266, 2346–2353
11. Antony, A. C., and Miller, M. E. (1994) Biochem. J. 296, 9–16
12. Uldenfriind, S., and Kodukula, K. (1995) Annu. Rev. Biochem. 64, 563–591
13. Antony, A. C., Utley, C., Van Horne, K. C., and Kohlhouse, J. F. (1991) J. Biol. Chem. 266, 9684–9692
14. Bordier, C. (1981) J. Biol. Chem. 256, 1604–1607
15. Zieglerbauer, K., and Overath, P. (1992) J. Biol. Chem. 267, 10791–10796
16. Henderson, G. B., and Zoely, E. M. (1984) J. Biol. Chem. 259, 4538–4562
17. Moore, S., and Stein, W. H. (1963) Methods Enzymol. 6, 819–831
18. Elwood, P. C. (1989) J. Biol. Chem. 264, 14893–14901
19. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
20. Antony, A. C., Verma, R. S., and Kincade, R. S. (1987) Anal. Biochem. 162, 224–235
21. Antony, A. C., Kincade, R. S., Verma, R. S., and Krishnan, S. R. (1987) J. Clin. Invest. 80, 711–723
22. Antony, A. C., Briddel, R. A., Brandt, J. E., Straneva, J. E., Verma, R. S., Miller, M. E., Kalasinski, L. A., and Hoffman, R. (1991) J. Clin. Invest. 87, 313–325
23. Sun, X. L., Murphy, B. R., Li, Q. J., Gullapalli, S., Mackins, J., Jayaram, H. N., Srivastava, A., and Antony, A. C. (1995) J. Clin. Invest. 96, 1535–1547
24. Antony, A. C., Bruno, E., Briddel, R. A., Brandt, J. E., Verma, R. S., and Hoffman, R. (1987) J. Clin. Invest. 80, 1618–1623
25. Lee, H. C., Shoda, R., Kralj, J. A., Foster, J. D., Selhub, J., and Rosenberg, T. L. (1992) Biochemistry 31, 3236–3243
26. Hansen, S. I., and Holm, J. (1992) Biosci. Rep. 12, 87–93
27. Zhou, N. E., Mant, C. T., and Hodges, R. S. (1990) Pept. Res. 3, 8–20
28. Kane, M. A., Elwood, P. C., Portillo, R. M., Antony, A. C., Najfeld, V., Finley, A., Waxman, S., and Kohlhouse, J. F. (1988) J. Clin. Invest. 81, 1386–1406
29. Ehlers, M. R., and Riordan, J. F. (1991) Biochemistry 30, 10065–10074
30. Granda, J. L., Lande, M. A., and Karvonen, R. L. (1990) Connect. Tissue Res. 24, 249–263
31. Tziniia, A. K., and Soteriades, K. P. (1991) Mol. Biochem. Parasitol. 47, 83–89
32. Lala, P. K., and Graham, C. H. (1990) Cancer Metastasis Rev. 9, 369–379

2 Q.-J. Li, X.-L. Sun, and A. C. Antony, unpublished data.
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