A Cell-free Assay for Glycosylphosphatidylinositol Anchoring in African Trypanosomes

DEMONSTRATION OF A TRANSMIDATION REACTION MECHANISM*

(Received for publication, December 15, 1998, and in revised form, March 22, 1999)

Deepak K. Sharma§§, Jolanta Vidugiriene¶¶, James D. Bangs**††, and Anant K. Menon‡‡
From the ¶¶Department of Biochemistry, University of Wisconsin, Madison, Wisconsin, the **††Department of Biochemistry, Vilnius University, Ciurlionio 21, Vilnius, Lithuania, and the ‡‡Department of Medical Microbiology and Immunology, University of Wisconsin-Madison Medical School, Madison, Wisconsin 53706

We established an in vitro assay for the addition of glycosyl-phosphatidylinositol (GPI) anchors to proteins using procyclic trypanosomes engineered to express GPI-anchored variant surface glycoprotein (VSG). The assay is based on the premise that small nucleophiles, such as hydrazine, can substitute for the GPI moiety and effect displacement of the membrane anchor of a GPI-anchored protein or pro-protein causing release of the protein into the aqueous medium. Cell membranes containing pulse-radiolabeled VSG were incubated with hydrazine, and the VSG released from the membranes was measured by carbonate extraction, immunoprecipitation, and SDS-polyacrylamide gel electrophoresis/fluorography. Release of VSG was time- and temperature-dependent, was stimulated by hydrazine, and occurred only for VSG molecules situated in early compartments of the secretory pathway. No nucleophile-induced VSG release was seen in membranes prepared from cells expressing a VSG variant with a conventional transmembrane anchor (i.e. a nonfunctional GPI signal sequence). Pro-VSG was shown to be a substrate in the reaction by assaying membranes prepared from cells treated with mannosamine, a GPI biosynthesis inhibitor. When a biotinylated derivative of hydrazine was used instead of hydrazine, the released VSG could be precipitated with streptavidin-agarose, indicating that the biotin moiety was covalently incorporated into the protein. Hydrazine was shown to block the C terminus of the released VSG hydrazide because the released material, unlike a truncated form of VSG lacking a GPI signal sequence, was not susceptible to proteolysis by carboxypeptidases. These results firmly establish that the released material in our assay is VSG hydrazide and strengthen the proof that GPI anchoring proceeds via a transamidation reaction mechanism. The reaction could be inhibited with sulfhydryl alkylating reagents, suggesting that the transamidase enzyme contains a functionally important sulfhydryl residue.

* This work was supported by National Institutes of Health Grants GM55427 (to A. K. M.) and AI35739 (to J. D. B.). 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 this fact.

§ Recipient of a Wellcome Prize Travelling Research Fellowship from the Wellcome Trust. To whom correspondence should be addressed: Dept. of Biochemistry, University of Wisconsin-Madison, 433 Babcock Dr., Madison, WI 53706-1544. Tel.: 608-263-2636; Fax: 608-263-3453; E-mail: dsharma@biochem.wisc.edu.

|| International Scholar of the Howard Hughes Medical Institute.

* The abbreviations used are: GPI, glycosylphosphatidylinositol; GPIT, GPI transamidase; PAS, protein A-Sepharose; pCMB, p-chloromercuriphenylsulfonic acid; pCMPSA, p-chloromercuriphenylsulfonic acid; PLAP, placental alkaline phosphatase; PP1, procyclic GPI anchor precursor; PP3, procyclic GPI (precursor to PP1); VSG, variant surface glycoprotein; PAGE, polyacrylamide gel electrophoresis; ER, endoplasmic reticulum.
chored mini-PLAP. The transition from promini-PLAP to mini-PLAP was found to require ATP, GTP, and ER luminal proteins, consistent with a chaperone-mediated maturation step prior to GPI anchoring (10–12). The less stringent GTP requirement for this process remains enigmatic. However, there appeared to be no requirement for energy in the final conversion from promini-PLAP to mini-PLAP, consistent with a transamidation mechanism for GPI anchoring.

The main product of the reaction in the mammalian translocation-translaction system described above is GPI-anchored mini-PLAP. However, a small amount of free mini-PLAP (lacking the C-terminal signal sequence as well as the GPI anchor) is invariably formed, probably via a nuclease attack by water on the active carbonyl formed as a result of the initial step of the transamidation reaction sequence (13). Maxwell et al. (4) developed this observation further and demonstrated that other nucleophiles such as hydrazine and hydroxylamine could effectively compete with GPI to generate a molecule similar to free mini-PLAP. They presumed that the free mini-PLAP variant generated under these conditions was mini-PLAP hydrazide or mini-PLAP hydroxamate; however, they were unable to demonstrate the nature of these products because of the subpicomole amounts of material generated in their in vitro translation-translocation experiments (4). These data, although incomplete, provide the best published evidence thus far that GPI anchoring proceeds via a transamidation reaction mechanism.

In this paper we describe a convenient assay for GPI anchoring based on a cell-free system from insect stage African trypanosomes engineered to express a well characterized GPI-anchored protein. We use the assay to demonstrate explicitly that the enzyme-mediated, hydrazine-induced cleavage of the C-terminal GPI signal sequence from a pro-protein occurs in early compartments of the secretory pathway, requires a functional GPI signal sequence, and results in the formation of a soluble protein product that is modified by hydrazine at its C terminus. We also demonstrate that sulfhydryl alkylating agents block activity, consistent with a role for a free sulfhydryl residue in catalysis. These data provide proof that GPI anchoring proceeds via a transamidation reaction mechanism.

**EXPERIMENTAL PROCEDURES**

**Materials**—Protein A-Sepharose was from Amersham Pharmacia Biotech, biotin-LC-hydrazide, and sulfo-NHS-biotin were from Pierce, carboxypeptidases P and W were from Calbiochem-Behring Corp. (San Diego, CA), materials for SDS-polyacrylamide gel electrophoresis were from Bio-Rad, 10,000 NMWL Eppendorf filter units were from Millipore (Bedford, MA), materials for SDS-polyacrylamide gel electrophoresis were from Bio-Rad, 10,000 NMWL Eppendorf filter units were from Millipore Corporation (Bedford, MA), cell culture media were from Life Technologies, Inc. and Specialty Media Inc. [3H]Hethanolamine (~30 Ci/mmol) was from American Radiolabeled Chemicals (St. Louis, MO), and EXPRES-SEC cysteine/methionine protein labeling mix (~1,000 Ci/mmol) was from NEN Life Science Products. Autofluor was from National Diagnostics (Atlanta, GA). All other reagents were obtained from Sigma.

**Growth and Metabolic Labeling of Trypanosomes**—The growth and maintenance of procyclic trypanosomes, and the generation of stably transformed procyclic cell lines expressing full-length GPI-anchored proteins, consistent with a chaperone-mediated maturation step prior to GPI anchoring (10–12). The less stringent GTP requirement for this process remains enigmatic. However, there appeared to be no requirement for energy in the final conversion from promini-PLAP to mini-PLAP, consistent with a transamidation mechanism for GPI anchoring.

The main product of the reaction in the mammalian translation-translocation system described above is GPI-anchored mini-PLAP. However, a small amount of free mini-PLAP (lacking the C-terminal signal sequence as well as the GPI anchor) is invariably formed, probably via a nuclease attack by water on the active carbonyl formed as a result of the initial step of the transamidation reaction sequence (13). Maxwell et al. (4) developed this observation further and demonstrated that other nucleophiles such as hydrazine and hydroxylamine could effectively compete with GPI to generate a molecule similar to free mini-PLAP. They presumed that the free mini-PLAP variant generated under these conditions was mini-PLAP hydrazide or mini-PLAP hydroxamate; however, they were unable to demonstrate the nature of these products because of the subpicomole amounts of material generated in their in vitro translation-translocation experiments (4). These data, although incomplete, provide the best published evidence thus far that GPI anchoring proceeds via a transamidation reaction mechanism.

In this paper we describe a convenient assay for GPI anchoring based on a cell-free system from insect stage African trypanosomes engineered to express a well characterized GPI-anchored protein. We use the assay to demonstrate explicitly that the enzyme-mediated, hydrazine-induced cleavage of the C-terminal GPI signal sequence from a pro-protein occurs in early compartments of the secretory pathway, requires a functional GPI signal sequence, and results in the formation of a soluble protein product that is modified by hydrazine at its C terminus. We also demonstrate that sulfhydryl alkylating agents block activity, consistent with a role for a free sulfhydryl residue in catalysis. These data provide proof that GPI anchoring proceeds via a transamidation reaction mechanism as described below.

**Preparation of the Trypanosome Cell-free System**—Trypanosome membranes (trypanosome cell-free system) were prepared from metabolically radiolabeled cells as described previously (7) except that the cells were not preincubated with tunicamycin prior to lysis. Aliquots of membranes (5 × 10^9 cell equivalents/ml) were snap-frozen in liquid nitrogen and stored at −70 °C.

**Trypanosome Cell-free System Incubations and Transamination Assay**—The trypanosome cell-free system was used as the enzyme source. Trypanosome membranes were washed twice in 0.1 M Hepes buffer, pH 7.5, containing 25 mM KCl, 5 mM MgCl_2, 0.1 M aspartate, 2 μg/ml leupeptin and then suspended at 10^9 cell equivalents/ml in 200 mM Hepes, pH 7.5. Aliquots (25 μl) of this lysate were added to tubes containing 25 μl of 20 mM hydrazine (5 mM hydrazine for some experiments), 20 mM Hepes, pH 7.5, or 20 mM Hepes, pH 7.5, only. Protease inhibitors were also added to this solution when they were used. The tubes were incubated at 37 °C (except for the experiment shown in Fig. 1B where temperature dependence of the reaction was investigated) for 45 min. For incubations with biotin-LC-hydrazide, the hydrazide was dissolved in MeSO (50 mM) and 5 μl was added to the incubation. Corresponding controls where just MeSO was added were also carried out. These incubations were for 3 h to maximize the nuclease-induced release of VSG. The reactions were terminated by the addition of 350 μl of 0.1 M sodium carbonate and placed on ice for 30 min. Sodium carbonate extraction allowed for the isolation of membrane bound components from those that are soluble. The solution was then layered on top of a 0.1 M sodium carbonate, 0.5 M sucrose cushion and spun in a Beckman TLA 100.2 rotor at 90,000 rpm for 30 min. 200 μl of the resulting supernatant was transferred to a tube containing 26.2 μl of 1.3 M NaCl, 0.9% SDS, 4.35% deoxycholate, 4.35% Nonidet P-40. The remaining membrane pellet was resuspended in 500 μl of 20 mM Hepes, pH 7.5, 150 mM NaCl, 20 mM EDTA) containing 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS (TEN-detergent or TEN-D). The samples were then subjected to immunoprecipitation as described below.

**Immunoprecipitation and Electrophoresis**—Affinity purified rabbit anti-VSG 117 and rabbit anti-VSG 117 antisera were used in these experiments. Antibody was preadsorbed to protein A-Sepharose (PAS) beads and washed once with 20 mM Hepes, pH 7.5, then resuspended with final volume. Typically 50 μl of PAS-antibody suspension was added to 200 μl of the sodium carbonate washed pellet or supernatant, which was resuspended in TEN-D. Samples were mixed for 2 h at 4 °C and then washed three times with TEN-D and once with TEN. For most of the experiments 25 μl of 2× sample buffer was added to the 25 μl of PAS beads, and the sample was boiled for 10 min. The tubes were then spun in a microcentrifuge, and 22 μl of the supernatant was fractionated by 10% SDS-PAGE (18). Dried gels were analyzed by fluorography.

**Treatment of Purified Membrane Form VSG with Sulfo-NHS-biotin and Biotin-LC-hydrazide**—[3H]Myristic acid-labeled, GPI-anchored VSG 117 was purified from metabolically labeled bloodstream stage trypanosomes as described previously (19). This material was incubated on ice in 100 mM Hepes, pH 8, for 45 min with either sulfo-NHS-biotin (an amine-reactive compound) or biotin-LC-hydrazide. The mixture was then passed through a prewashed Millipore ultrafilter-MC 10,000 NMWL filter (molecular mass cut-off, 10,000 kDa) to wash away any residual biotin-derivatives (biotin-LC-hydrazide or sulfo-NHS-biotin) and precipitated with streptavidin beads or anti-VSG 117 antibody as described below.

**Precipitation Using Streptavidin Beads**—In experiments using biotin derivatized, VSG was analyzed by precipitation with streptavidin-agarose following immunoprecipitation with anti-VSG antibodies. The sodium carbonate extract was passed through a prewashed Millipore ultrafilter-MC 10,000 NMWL filter (molecular mass cut-off, 10,000) to wash away any residual biotin-derivatives (biotin-LC-hydrazide or sulfo-NHS-biotin). The VSG captured on the filter was recovered by
washing the filter twice with 400 µl of TEN buffer and then immunoprecipitating as above. The immunoprecipitated VSG was resuspended with 120 µl of Hepes, pH 7.5, and placed in a 100 °C heating block for 10 min, and the boiled sample was then spun at 14,000 rpm in a microcentrifuge to remove the antibody-coated beads. The supernatant (approximately 100 µl containing immunopurified VSG) was then supplemented with 100 µl of 4× TEN-D buffer and 200 µl of water. Streptavidin coupled to 8% agarose beads was washed twice with TEN-D buffer and resuspended (also in TEN-D) to its original volume. 50 µl of this was added to the immunopurified VSG (in TEN-D) and mixed for 9 h at 4 °C. The samples were then washed three times with TEN-D and once with TEN. Subsequently, 25 µl of 2× sample buffer was added to the streptavidin beads, and the sample was boiled for 15 min and analyzed by SDS-PAGE and fluorography or taken directly for proteinase K (85 µg/ml) treatment to recover the membranes as described (12). The membrane pellet was processed by carbonate extraction to separate water-soluble metabolically pulse-labeled procyclic trypanosome lysates with hydrazine or hydroxylamine. A, crude membranes from [35S]Cys/Met pulse-radioiodinated VSG 117-expressing procyclic trypanosomes were incubated in the absence or presence of hydrazine or hydroxylamine as described under “Experimental Procedures.” Reactions were carried out at 37 °C for 0, 20, or 40 min as indicated. The samples were then analyzed by sodium carbonate extraction, immunoprecipitation with anti-VSG 117 antibodies, and SDS-PAGE and fluorography. B, crude membranes were incubated with hydrazine for 45 min at different temperatures as indicated and processed as described for A. Released VSG was quantitated by densitometry of the SDS-PAGE fluorogram. Hz, hydrazine, Hx, hydroxylamine.

RESULTS

A Cell-free Assay for GPI Anchoring: VSG Is Released from Trypanosome Lysates in a Time- and Temperature-dependent Manner in the Presence of Nucleophiles—We describe a cell-free assay for GPI anchoring based on the premise that the putative GPI transamidase (GPIT) can use small nucleophiles such as hydrazine to effect the displacement of the GPI signal sequence or GPI anchor from a GPI proprotein or GPI-anchored protein, respectively, resulting in the release of a water-soluble derivative of the protein (4). To demonstrate the principle of the assay, we used lysates prepared from procyclic trypanosomes engineered to express VSG 117 (14), a conveniently detectable GPI-anchored protein. The cells were metabolically radiolabeled with [35S]cysteine/methionine for 15 min before being washed, osmotically lysed, and washed again. When the labeled crude membranes (containing membrane-associated, radiolabeled VSG) were incubated with hydrazine and then processed by carbonate extraction to separate water-soluble molecules from membrane-associated material, VSG was detected in the carbonate extract. Analysis of the released material by immunoprecipitation with anti-VSG antibodies, SDS-PAGE, and fluorography confirmed that the released material was a ~58-kDa VSG molecule, comparable in size to an anchorless VSG variant expressed in procyclic trypanosomes (14) (also see Fig. 6). Production of the released material was dependent on hydrazine, time (Fig. 1A, left panel), and temperature (Fig. 1B). As we show later (see Fig. 5), the released material is a hydrazide derivative of VSG, lacking a membrane anchor. VSG release was also observed when the labeled membranes were incubated with hydroxylamine instead of hydrazine (Fig. 1A, right panel, left-hand lane). These results suggest that the production of a soluble derivative of VSG from a
cannot process a transmembrane form of VSG. A, hydrazine-stimulated VSG release was assayed in crude membranes prepared from trypanosomes pulse-labeled with \[^{35}\text{S}\]\text{Cys/Met}\ for 15 min (Pulse) or pulse-labeled for 15 min and chased (in cysteine-methionine-containing medium) for 1 h (Pulse-Chase). Samples were analyzed as in Fig. 1A. B, crude membranes were prepared from \[^{35}\text{S}\]\text{Cys/Met}\-labeled procyclic trypanosomes expressing VSG anchored via a conventional transmembrane domain (see “Experimental Procedures”). The membranes were incubated with or without hydrazine as described under “Experimental Procedures,” and both the sodium carbonate extracted supernatant and pellet from the incubations (as indicated) were immunoprecipitated and analyzed by SDS-PAGE/fluorography. Hz, hydrazine.

Membrane-bound precursor in the presence of nucleophiles is due to temperature-dependent enzymatic activity.

Additional experiments were performed to confirm that nucleophile-induced release of VSG shared some of the characteristics of the GPI anchoring reaction. We specifically set out to show that nucleophile-induced VSG release depended on (i) the cellular location of the VSG molecule and (ii) the presence of either a GPI signal sequence or a GPI anchor at the C terminus of VSG.

**The Enzyme Responsible for Nucleophile-induced VSG Release Is Located Early in the Secretory Pathway—**A pulse-chase analysis revealed that membranes prepared from cells labeled with \[^{35}\text{S}\]\text{Cys/Met}\ for 15 min and then chased for 1 h with complete medium showed very little hydrazine-induced release of VSG when compared with lysates from 15 min pulse-labeled cells (Fig. 2A, compare lanes 1 and 2 with lanes 3 and 4). Samples were prepared from cells metabolically labeled in the presence of 3 mM 1,10-phenanthroline to block the activity of a surface metalloprotease that cleaves VSG molecules when they arrive at the cell surface (14). Based on the half-time (~1.2 h) for export of GPI-anchored VSG to the plasma membrane (22), we would expect that the pulse-labeled samples contain a significant proportion of radiolabeled VSG in the ER, whereas the amount of ER-localized VSG should be greatly reduced in the chase samples (14). We conclude that the hydrazine-induced release of VSG is specific for molecules located in early compartments of the secretory pathway such as the ER, consistent with a wide variety of data indicating that GPI anchoring occurs in an ER-localized event (11, 23).

**VSG 117tm, a VSG Derivative Anchored by a Transmembrane Domain, Cannot Be Enzymatically Processed in the Presence of Hydrazine to Yield a Soluble Product—**In a separate experiment to characterize nucleophile-induced VSG release, procyclic trypanosomes expressing a transmembrane form of VSG 117 (117tm, a VSG molecule with its C-terminal GPI addition sequence replaced with the transmembrane region of p67, a lysosomal protein in *T. brucei* (16)) were pulse-labeled, and lysates were prepared. On incubation of these lysates with hydrazine, no VSG release was seen (Fig. 2B). Thus a VSG variant with a nonfunctional C-terminal GPI signal sequence cannot act as a substrate in the assay. This result suggests that hydrazine-stimulated VSG release requires VSG substrates with either a GPI signal sequence or a GPI anchor. This observation is consistent with the idea that the activity being observed in this assay is the one that usually adds GPI anchors onto proteins.

**VSG Pro-protein Can Be Enzymatically Processed in the Presence of Hydrazine to Yield a Soluble Product—**The pulse-labeled lysates used in the experiments described above contain predominantly GPI-anchored VSG. However, they also likely contain small amounts of unprocessed VSG pro-protein bearing a GPI signal sequence. Both these forms of the protein are potential substrates for the GPI transamidase. To establish whether nucleophile-induced VSG release could originate directly from enzymatic processing of the VSG pro-protein, we established conditions where GPI biosynthesis and GPI anchoring of proteins were abolished. Under these conditions, the only radiolabeled VSG molecules present in the lysates would be pro-forms possessing a GPI signal sequence. To generate a GPI biosynthesis defect we incubated the cells with mannosamine, a compound that when metabolized blocks GPI synthesis prior to the addition of the third mannose residue and phosphoethanolamine cap (17, 24).

Lipid extracts from procyclic trypanosomes metabolically radiolabeled with \[^{3}\text{H}\]ethanolamine contain the mature GPI structure PP1 (ethanolamine-PO\(_4\)-Man\(_1\)-2-Man\(_1\)-6-Man\(_1\)-4-GlcN\(_1\)-6-Fnos(acyl)-PO\(_4\)-monaclylglycerol), as well as the PP1 precursor, PP3 (ethanolamine-PO\(_4\)-Man\(_1\)-2-Man\(_1\)-6-Man\(_1\)-4-GlcN\(_1\)-6-Fnos(acyl)-PO\(_4\)-diacylglycerol) (25, 26) as shown by thin layer chromatographic analysis (Fig. 3A, panel I). Both lipids are susceptible to hydrolysis by GPI-specific phospholipase D (Fig. 3A, compare panels I and II). When cells were labeled in the presence of mannosamine, GPI synthesis was almost completely abolished: no \[^{3}\text{H}\]ethanolamine-labeled PP1 was detected, and only a trace amount of PP3 was synthesized (Fig. 3A, panels III and IV).

To assess the effect of mannosamine on \[^{3}\text{H}\]ethanolamine labeling of proteins, \[^{3}\text{H}\]ethanolamine-labeled cells were analyzed for \[^{3}\text{H}\]ethanolamine-labeled VSG by immunoprecipitation, SDS-PAGE, and fluorography. The fluorogram was quantitated by densitometry. As shown in Fig. 3B, \[^{3}\text{H}\]ethanolamine labeling of VSG was almost completely abolished in mannosamine-treated cells, similar to results obtained by others (17).

The above controls establish that GPI synthesis and the production of GPI-anchored proteins are essentially abolished in mannosamine-treated procyclic trypanosomes, indicating that mannosamine treatment prior to and during pulse-labeling with \[^{35}\text{S}\]\text{Cys/Met}\ would yield cell membranes containing radiolabeled pro-VSG and no GPI-anchored VSG. When membranes prepared from mannosamine-treated cells were incubated with hydrazine, VSG release was detected similar to that seen with control membranes from untreated cells (Fig. 3C, lanes 3 and 4; control samples are shown in lanes 1 and 2). This result indicates that the VSG pro-protein can be directly processed by the GPI transamidase in the presence of hydrazine to yield a soluble product.

**Sulfhydryl Alkylation Reagents Inhibit Nucleophile-induced VSG Release—**It has previously been shown that the GPI anchoring reaction in bloodstream stage trypanosomes is inhibited by the sulfhydryl alkylating reagent *p*-chloromercuriphenyl sulfonyl acid (pCMPSA) (3). We tested the effect of pCMPSA as well as two other reagents (iodoacetamide and *p*-chloromercuribenzoate (pCMB)) on nucleophile-induced VSG...
and the residue was partitioned into the upper phase of an 10:10:3 v/v/v). The lipid extract was dried under a stream of nitrogen, the membranes were extracted into organic solvent (chloroform:methanol:water, 10:10:3 v/v/v). The lipid extract was dried under a stream of nitrogen, and the residue was partitioned into the upper phase of an n-butanol:water two-phase mixture. Lipids recovered in the upper n-butanol-rich phase were dried under nitrogen, resuspended in detergent-containing buffer as described under “Experimental Procedures,” and treated with or without GPI-specific phospholipase D as indicated. Lipids were then re-extracted and analyzed by high performance TLC as described under “Experimental Procedures.” The chromatograms were visualized using a TLC radioscanner. A segment of the chromatogram (corresponding to the region containing the GPIs PP1 (*) and PP3 ()), b) is shown. B, 2.5 x 10^7 cell equivalents of [^3H]ethanolamine-labeled washed trypanosome membranes (prepared from cells labeled in the absence or presence of mannosamine) were re-suspended in buffer for immunoprecipitation (TEN-D) and incubated with anti-VSG 117 antibodies. The immunoprecipitates were analyzed by SDS-PAGE/fluorography, and laser densitometry was used to quantify the amount of [^3H]ethanolamine-labeled proteins. C, crude membranes prepared from cells pulse-labeled with [^35S]Cys/Met for 15 min in the absence or presence of mannosamine were incubated with or without hydrazine for 45 min as in Fig. 1A and analyzed by carbonate extraction, immunoprecipitation, and SDS-PAGE/fluorography. Hz, hydrazine.

release. All three compounds inhibited hydrazine-induced release of VSG (Fig. 4A, compare lane 3 (no inhibitor) with lanes 4 (pCMPSA) and 5 (iodoacetamide)) and compare lane 7 (no inhibitor) with lane 6 (pCMB)). This result is consistent with the proposal that the trypanosome GPI transamidase contains a catalytically important sulfhydryl residue. Another possible but less likely interpretation is that alkylation of a sulfhydryl residue elsewhere in the protein results in inhibition of transamidase activity.

The same inhibitors were tested in a mammalian cell-free system to see if they caused inhibition of the mammalian GPI anchor addition reaction. Messenger RNA corresponding to prepromini-PLAP, a model protein with an N-terminal signal sequence and a C-terminal GPI addition sequence (9), was translated in the presence of thymosa cell microsomes for 30 min at 27 °C, and the incubation was continued for an additional 90 min in the presence or absence of hydrazine and the various sulfhydryl alkylating reagents. The sulfhydryl alkylating reagents could not be introduced at the outset because they inhibit protein translocation (27). The samples were then processed by proteinase K treatment to strip untranslated prepromini-PLAP, immunoprecipitation, SDS-PAGE, and fluorography. Incubations with 4 mM NaOH (used as a solvent for pCMPSA and pCMB) yielded results identical to those shown in lanes 1 and 2.

**Fig. 3. Pro-VSG is a substrate for GPIT.** A, crude membranes were prepared from VSG 117-expressing procyclic trypanosomes metabolically labeled with [^3H]ethanolamine in the absence or presence of mannosamine (ManN). Lipids from 1 x 10^7 cell equivalents of [^3H]ethanolamine-labeled washed trypanosome membranes (prepared from cells labeled in the absence or presence of mannosamine) were re-suspended in buffer as described under “Experimental Procedures,” and treated with or without GPI-specific phospholipase D as indicated. Lipids were then re-extracted and analyzed by high performance TLC as described under “Experimental Procedures.” The chromatograms were visualized using a TLC radioscanner. A segment of the chromatogram (corresponding to the region containing the GPIs PP1 (*) and PP3 ()), b) is shown. B, 2.5 x 10^7 cell equivalents of [^3H]ethanolamine-labeled washed trypanosome membranes (prepared from cells labeled in the absence or presence of mannosamine) were re-suspended in buffer for immunoprecipitation (TEN-D) and incubated with anti-VSG 117 antibodies. The immunoprecipitates were analyzed by SDS-PAGE/fluorography, and laser densitometry was used to quantify the amount of [^3H]ethanolamine-labeled proteins. C, crude membranes prepared from cells pulse-labeled with [^35S]Cys/Met for 15 min in the absence or presence of mannosamine were incubated with or without hydrazine for 45 min as in Fig. 1A and analyzed by carbonate extraction, immunoprecipitation, and SDS-PAGE/fluorography. Hz, hydrazine.

**Fig. 4. Inhibition of trypanosome (but not mammalian) GPIT with sulfhydryl alkylating reagents.** A, hydrazine-stimulated release of VSG was assayed in the presence of sulfhydryl alkylating reagents pCMPSA (lane 4), pCMB (lane 6), and iodoacetamide (lane 5). Both the pCMPSA and the pCMB incubations contained a final concentration of 4 mM NaOH (used to dissolve the reagents); control assays in the presence of 4 mM NaOH are shown in lanes 3 and 7. B, GPI anchoring using mammalian cell microsomes and the prepromini-PLAP reporter was assayed as described under “Experimental Procedures.” Membranes were preloaded with promini-PLAP (lanes 1, 3, 5, and 7) and then incubated with hydrazine (lanes 2, 4, 6, and 8) in the absence (lane 2) or presence (lanes 4, 6, and 8) of sulfhydryl alkylating reagents. Samples were processed by proteinase K treatment (to strip untranslated prepromini-PLAP), immunoprecipitation, SDS-PAGE, and fluorography. Incubations with 4 mM NaOH (used as a solvent for pCMPSA and pCMB) yielded results identical to those shown in lanes 1 and 2.
treatment with sulphydryl alkylating reagents during the “second” incubation is unlikely to have an effect.

Hydrazine Is Covalently Incorporated into VSG as a Consequence of GPIIT Action—A transamidation reaction mechanism predicts that in our assay format an amine containing nucleophile (H₂N-X) becomes covalently attached to the reaction substrate, i.e. the VSG pro-protein (or GPI-anchored VSG), with concomitant displacement of the GPI signal sequence (or GPI anchor) (see Fig. 7). To test this we used a modified hydrazine approach relying on a comparison between VSG hydrazide and another VSG reporter expressed in procyclic trypanosomes. This reporter, 117GPI, is a VSG 117 molecule lacking the C-terminal GPI signal sequence. VSG hydrazide and 117GPI should be similar except for the hydrazine residue covalently incorporated into VSG hydrazide (Fig. 5A). A transamidation reaction mechanism (see Fig. 7) predicts that hydrazine will be incorporated at the C terminus of the released VSG hydrazide molecule rendering it unsusceptible to attack by carboxypeptidases. In contrast, 117GPI with an unblocked C terminus should be susceptible to proteolysis by carboxypeptidases.

Fig. 6 (lanes 1 and 2) shows that VSG hydrazide and 117GPI appear to be of identical molecular mass, consistent with the proposal that VSG hydrazide represents a full-length VSG molecule truncated at or close to the ω site. The results of carboxypeptidase treatment are shown in Fig. 6 (lanes 3 and 4). The data show clearly that although 117GPI can be proteolyzed by carboxypeptidases (compare lanes 2 and 4), VSG hydrazide resists proteolysis (compare lanes 1 and 3). These data are consistent with the proposal that the C terminus of VSG hydrazide is blocked by hydrazine as expected for the product of the transamidation reaction illustrated in Fig. 7.

DISCUSSION

In this paper we describe a convenient assay for GPI anchoring based on a cell-free system from insect stage African
trypanosomes engineered to express a well characterized GPI-anchored protein. We use the assay to demonstrate explicitly that the enzyme-mediated, hydrazine-induced cleavage of the GPI signal sequence from a pro-protein results in the formation of a soluble protein hydrazide product. As illustrated in Fig. 7, the reaction most likely proceeds via activation of the carbonyl group of the ω residue of the pro-protein (or GPI protein) by a hydrophilic group on the transamidase, with concomitant displacement of the GPI signal sequence (or GPI anchor). We provide direct evidence that the pro-protein is indeed a substrate in our assay by using membranes prepared from cells blocked in GPI biosynthesis (Fig. 3). The ability of sulphydryl alkylating reagents to abolish the reaction is consistent with the proposal that the catalytic residue in the enzyme is an -SH group; the enzyme is accordingly depicted as Enz-S⁻ in Fig. 7. Nucleophilic attack on the activated carbonyl group by hydrazine regenerates the enzyme and yields a protein hydrazide product. We show that the protein product of the reaction is a protein hydrazide by using the hydrazide derivative, biotin-LC-hydrazide, and specific precipitation with streptavidin beads (Fig. 5A, lane 8). Characteristics of the assay (Figs. 1 and 2) as well as the molecular mass of the released material and its resistance to proteolysis by carboxypeptidases (Fig. 6) indicate that hydrazine is incorporated into VSG at the ω site (see “Results”). This result strengthens the proof, initiated by the work of Mayor et al. (3) and Maxwell et al. (4), that GPI anchoring proceeds via a transamidation reaction mechanism. Precedent for the type of analysis described above may be found in studies of γ-glutamyl transpeptidase. Here a γ-glutamyl-enzyme intermediate is formed that undergoes nucleophilic attack by an amino acid acceptor (to form a γ-glutamyl-amino acid) or water (to form glutamate) (28). Importantly, for the purposes of the work presented in this paper, γ-glutamyl transpeptidase can catalyze reactions between γ-glutamyl compounds and nucleophiles (e.g. hydroxylamine) to generate the corresponding γ-glutamyl derivatives (e.g. hydroxamates) (29).

In setting up our assay, we chose to work with procyclic stage African trypanosomes because the more commonly used bloodstream stage cells possess a membrane-associated GPI-hydrolyzing phospholipase C activity (30) that would have confounded our experimental readout (3). Due to the nonavailability of adequate immunological reagents and the lack of cysteine/methionine residues for convenient metabolic radiolabeling in procycin the major GPI-anchored protein of procyclic trypanosomes (31, 32), we focused instead on procyclic lines that had been engineered to express the more experimentally accessible bloodstream form VSG (14).

Incubation of pulse-labeled procyclic membranes with hydrazine results in the release of VSG-hydrazide, which is recovered in the supernatant of a carbonate wash of the membranes. Despite the brevity of the labeling pulse during which most of the labeled VSG would be expected to be ER-localized (14), the bulk of the labeled VSG appears not to be accessible to the transamidase, and the amount of released VSG is only a small fraction of the radiolabeled VSG still bound to the membranes (data not shown). This small accessible fraction is eliminated altogether when the labeled cells are “chased” (Fig. 2A), consistent with processing of VSG molecules and export from the ER during the chase period. The small size of the releasable fraction of pulse-radiolabeled VSG can be explained by proposing that VSG pro-protein, not GPI-anchored VSG, is a substrate for the transamidase. Although GPI-anchored VSG could, in principle, be a substrate for the transamidase, we have no data to show that this is the case. The pro-protein pool is expected to be small under normal conditions, and the amount of VSG hydrazide formed is a reflection of the endogenous pro-VSG pool size. The extent to which pro-VSG can be processed may be further restricted if the transamidase is confined to an ER domain, possibly in proximity to ER protein

![Diagram 6](Image)

**Fig. 6.** Hydrazine is incorporated at or near the C terminus of GPI-released VSG hydrazide. VSG 117 hydrazine (Hz) (obtained as in Fig. 1A) and 117ΔGPI (isolated from procyclic trypanosomes expressing this construct) were purified by immunoprecipitation and analyzed by SDS/PAGE fluorography before (lanes 1 and 2) or after (lanes 3 and 4) carboxypeptidase treatment. Both molecules run identically before carboxypeptidase treatment (lanes 1 and 2), but only 117ΔGPI is proteolyzed after incubation with a mixture of carboxypeptidases P and W.

![Diagram 7](Image)

**Fig. 7.** Proposed mechanism for the transamidase-mediated, nucleophile-induced release of VSG (modified from Ref. 1). The carbonyl group of the ω amino acid (aspartic acid) of pro-VSG is activated by a sulphydryl group in the transamidase (Enz-S⁻) resulting in the formation of an enzyme-substrate complex and cleavage of the amide bond between aspartic acid and serine (ω + 1 in the c-terminal signal sequence of pro-VSG). Nucleophilic attack by H₂N-X results in release of VSG-NH-X and regeneration of the active site sulphydryl in the transamidase.

| Reagent | Reaction |
|---------|----------|
| Hydrazine | -NH₂⁻ |
| Hydroxylamine | -OH⁻ |
| Biotin-LC-hydrazide | -NH₂⁻ | -OH⁻ | -COOH | -CH₂OH | -NH⁻ | -C(=CH₂)₆ | -N⁺ | -C(=CH₂)₄ | -O⁻ | -S⁻ |
| VSG-Hz | -NH₂⁻ | -OH⁻ | -COOH | -NH⁻ | -C(=CH₂)₆ | -N⁺ | -C(=CH₂)₄ | -O⁻ | -S⁻ |
translocons (33).

Our overall objective in initiating these studies was to provide biochemical proof of the anchoring mechanism and also to set the stage for subsequent attempts to purify the enzyme. The GPIT has not been purified, and its precise polypeptide make-up is unknown. Genetic approaches in yeast and mammalian cells have led to the identification of two distinct gene products (Gaa1p and Gpi8p) that are required for GPIT activity, possibly implying that GPIT is a complex of at least two polypeptides (34–36). Gpi8p is homologous to a jack bean endopeptidase that is involved in a transpeptidation reaction required for the post-translational processing of concanavalin A (35, 37), and evidence from the mammalian system suggests that Gpi8p may be responsible for the transamidase activity of GPIT (36). However, it is unclear whether Gpi8p is the transamidase itself (35) or whether it requires co-factors such as Gaa1p for activity. The function of Gaa1p is unknown, although it may act to anchor the putative GPIT complex in the ER (34). In analogy with other ER enzymes involved in the co- and post-translational modifications of translocated proteins (38, 39), GPIT may well be a complex of several polypeptides, including Gaa1p and Gpi8p. Assignment of GPIT activity to a particular ER polypeptide (or protein complex) would ultimately require purification of the enzyme and reconstitution of the enzymatic activity.

Acknowledgments—We thank Dawn Ransom and Vivian Fu for help with trypanosome cultures, Venera Bouriaikova for carrying out preliminary experiments, Angela Mehliert for help with the mass spectrometric analyses, Andreas Conzelmann, Terry Smith, Mike Ferguson, Jitu Mayor, and Peter Bütkofer for helpful discussions, and Dave Rancour, Niki Baumann, and Cedric Simonot for comments on the manuscript.

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