

A Cell-free Assay for Glycosylphosphatidylinositol Anchoring in African Trypanosomes

DEMONSTRATION OF A TRANSAMIDATION REACTION MECHANISM*

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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.

Genes encoding glycosylphosphatidylinositol (GPI)¹-anchored proteins specify two signal sequences in the primary translation product: an N-terminal signal sequence for targeting the protein to the endoplasmic reticulum (ER) and a C-terminal GPI-directing signal sequence directing the attachment of a GPI anchor (1). Both sequences are removed during processing of the preproprotein to the mature GPI-anchored form, but cleavage of the N-terminal signal sequence is not strictly necessary (1, 2). The assembly of GPI-anchored proteins requires translocation of the nascent polypeptide chain across the ER membrane and replacement of the C-terminal signal sequence with a preassembled, ethanolamine-containing GPI moiety attached to the newly exposed carboxyl group. The reaction is presumed to be catalyzed by an ER-localized transamidase enzyme (3, 4).

GPI anchoring can be reproduced in cell-free systems that rely on endogenous, membrane protein acceptors for GPI anchors (3) or, alternatively, that involve an *in vitro* translation system to load microsomal membranes with pro-protein substrates for GPI modification (5, 6). Using such systems, strong, albeit circumstantial evidence was obtained for a transamidation reaction mechanism. In an early report, Mayor *et al.* (3) exploited a trypanosome cell-free system that had been previously used extensively for studies of GPI biosynthesis (7, 8) to show that transfer of *in situ* synthesized or exogenously supplied radiolabeled GPIs to endogenous protein acceptors did not require ATP or GTP. The apparent lack of an energy requirement for GPI anchoring in this system was the first experimental evidence consistent with a transamidation reaction mechanism. Although this result is qualitatively useful, quantitatively only a low level of GPI anchor addition was observed, and the possibility that a preformed energy source or a preactivated GPI anchoring enzyme existed in the lysate could not be ruled out.

Other studies pioneered by Udenfriend and co-workers (1, 6) employed a cell-free system consisting of mammalian cell microsomes capable of processing *in vitro* synthesized preproteins to a GPI-anchored form. Kodukula *et al.* (9) developed a convenient reporter protein for this purpose based on the sequence of placental alkaline phosphatase (PLAP), a GPI-anchored protein. The reporter, prepromini-PLAP was found to be sequentially processed by microsomal enzymes to promini-PLAP (lacking the N-terminal signal sequence) and GPI-anchored

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¹ The abbreviations used are: GPI, glycosylphosphatidylinositol; GPIT, GPI transamidase; PAS, protein A-Sepharose; pCMB, *p*-chloromercuribenzoate; 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 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 nucleophilic 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 reagents 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 Corporation (Bedford, MA), cell culture media were from Life Technologies, Inc. and Specialty Media Inc., [³H]ethanolamine (~30 Ci/mmol) was from American Radiolabeled Chemicals (St. Louis, MO), and EXPRES³⁵S 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 variant surface glycoprotein 117 (VSG 117 or 117wt) and a truncated form lacking the C-terminal GPI signal sequence (117Δgpi) have been described previously (14). Using standard polymerase chain reaction and cloning procedures (15), a peptide transmembrane domain-anchored VSG construct (117tm) was created in which the transmembrane domain of a *Trypanosoma brucei* lysosomal membrane protein (16), was fused to the ω amino acid of VSG 117, thereby replacing the GPI anchor addition signal. The C-terminal amino acid sequence of this construct is: . . . CKDASRSTGIIAVVAALVVGVIAVVLMRPRRStop, where the ω amino acid is in bold and the hydrophobic domain is underlined. A stable procyclic cell line expressing this construct was generated as described for the 117wt reporter. [³⁵S]Cys/Met metabolic radiolabeling of procyclic cell lines was carried out as described previously (14) except that cells were labeled at a density of 10⁹/ml. Cell

viability was monitored throughout the labeling period and was consistently >99%.

Metabolic Radiolabeling of Trypanosomes in the Presence of Mannosamine—Procyclic cells were labeled with [³H]ethanolamine or [³⁵S]Cys/Met in the absence or presence of mannosamine to obtain GPI-anchored VSG or non-GPI-anchored VSG proprotein, respectively. [³H]Ethanolamine labeling of procyclics was carried out as described previously (17). For [³⁵S]Cys/Met labeling, procyclics were cultured in glucose-free RPMI 1640 (supplemented with 10% dialyzed fetal calf serum, 10 mM glycerol, 5.5 mM proline, and 33 mM Hepes, pH 7.4) for 30 min. The cultures were then supplemented with 5 mM mannosamine or water and incubated for a further 90 min. The procyclics were then resuspended in cysteine-, methionine-, and glucose-free RPMI 1640 (supplemented as above) and labeled as described above.

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 × 10⁸ cell equivalents/ml) were snap-frozen in liquid nitrogen and stored at -70 °C.

Trypanosome Cell-free System Incubations and Transamidation 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 MgCl₂, 0.1 mM tosyl-lysine chloromethyl ketone and 2 μg/ml leupeptin and then suspended at 10⁹ 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 Me₂SO (50 mM) and 5 μl was added to the incubation. Corresponding controls where just Me₂SO was added were also carried out. These incubations were for 3 h to maximize the nucleophile-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 TEN buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 5 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 (14) washed once in TEN-D and resuspended to the original 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 ultrafree-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 derivatives, VSG was analyzed by precipitation with streptavidin-agarose following immunoprecipitation with anti-VSG antibodies. The sodium carbonate extract was passed through a prewashed Millipore ultrafree-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 μ l of TEN buffer and then immunoprecipitating as above. The immunoprecipitated VSG was resuspended with 120 μ l 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 μ l containing immunopurified VSG) was then supplemented with 100 μ l of 4 \times TEN-D buffer and 200 μ l of water. Streptavidin coupled to 6% agarose beads was washed twice with TEN-D buffer and resuspended (also in TEN-D) to its original volume. 50 μ l 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 μ l of 2 \times 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 liquid scintillation counting. Precipitation efficiency and sample recovery using streptavidin-agarose was ~4.5% versus ~90% for precipitation with anti-VSG 117 antibodies.

Analysis of the Site of Hydrazide Incorporation into VSG Hydrazide—Metabolically radiolabeled VSG hydrazide was analyzed by carboxypeptidase treatment and SDS-PAGE/fluorography as follows. [³⁵S]Cys/Met-labeled VSG hydrazide was generated as described and immunoprecipitated using anti-VSG antibodies preadsorbed to PAS beads. The beads were repeatedly washed with 0.1 M glycine, pH 2.5, to release the bound material. The immunisolated VSG hydrazide was dialyzed against water (using a dialysis membrane with a 12,000–14,000-Da molecular mass cut-off), dried in a centrifugal evaporator, resuspended in water, boiled, cooled, and then made up to 50 mM NaOAc, pH 4.5. The sample was then incubated with a mixture of carboxypeptidases P and W (140 milliunits and 30 units, respectively) for 18 h at 30 °C. At the end of the incubation period, SDS-PAGE sample buffer was added, and the sample was analyzed by SDS-PAGE/fluorography. Radiolabeled 117 Δ GPI (a VSG molecule lacking the C-terminal GPI signal sequence) was analyzed alongside as a control. Procyclic trypanosomes expressing 117 Δ GPI were metabolically labeled with [³⁵S]Cys/Met as described, and lysates were prepared. The lysates were supplemented with TEN-D buffer (without prior washing) and taken for immunoprecipitation with anti-VSG antibodies preadsorbed to PAS beads as above. The immunoprecipitated 117 Δ GPI was analyzed by carboxypeptidase treatment as described for VSG hydrazide.

Estimate of the Amount of VSG Hydrazide Generated per Assay—Mayor *et al.* (3) estimated that lysates from bloodstream stage trypanosomes contain approximately 400–4,000 VSG molecules per cell equivalent that could act as acceptors in the transamidase reaction; the procyclic lysates used in our experiments have ~5% of the number of acceptors reported for bloodstream cells (22), *i.e.* 20–200 molecules/cell equivalent. Under standard assay conditions using 25 μ l of lysate (2.5×10^7 cell equivalents), this implies release of 5×10^8 to 5×10^9 VSG hydrazide molecules, *i.e.* 0.8–8 fmol (0.05–0.5 ng of protein).

High Performance TLC Analysis—Lipids were extracted from radiolabeled trypanosomes as described previously (Ref. 20; see also legend to Fig. 3), and analyzed by TLC using 10-cm glass-backed silica gel 60 high performance TLC plates (Merck) and chloroform/methanol/1 M ammonium acetate/13 M ammonium hydroxide/water (180:140:9:9:23, v/v/v/v/v) as the solvent system. Radiolabeled components in the chromatogram were visualized using a Berthold LB2842 linear scanner.

GPI-specific Phospholipase D Analysis of [³H]Ethanolamine-labeled Lipid Extract—GPI-specific phospholipase D treatment was performed as described (21), using human serum as the enzyme source.

GPI Anchoring in a Mammalian Cell-free System—Prepromin-PLAP mRNA (prepared from plasmid pGEM-4Z/mini-PLAP ϕ Ser (a gift from Dr. Sidney Udenfriend)) was translated using a nuclease-treated rabbit reticulocyte lysate in the presence of rough microsomes (prepared from mouse thymoma cells (BW5147.3)) according to previously published procedures (12). [³⁵S]Methionine was included in the translation reaction to label the translation product. Translation-translocation was allowed to proceed for 30 min at 27 °C before adding hydrazine, *p*-chloromercuriphenylsulfonic acid (pCMPSA), *p*-chloromercuribenzoate (pCMB), or iodoacetamide as indicated and continuing the incubation for an additional 90 min. The samples were then treated with proteinase K (85 μ g/ml final concentration) for 20 min on ice, diluted with buffer containing phenylmethylsulfonyl fluoride, and centrifuged to recover the membranes as described (12). The membrane pellet was resuspended in SDS-containing sample buffer and analyzed by SDS-PAGE and fluorography.

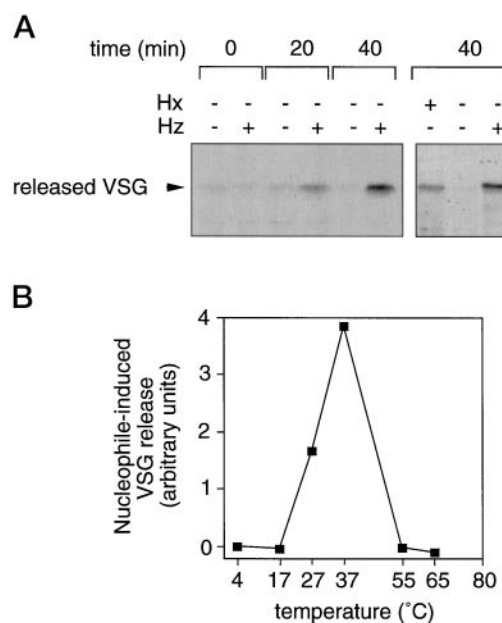


FIG. 1. Production of carbonate-extractable VSG on incubation of metabolically pulse-labeled procyclic trypanosome lysates with hydrazine or hydroxylamine. A, crude membranes from [³⁵S]Cys/Met pulse-radiolabeled VSG 117-expressing procyclic trypanosomes were incubated in the absence or presence of hydrazine 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

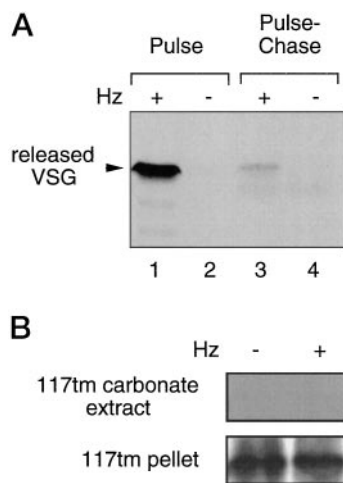


FIG. 2. GPIT is located early in the secretory pathway and cannot process a transmembrane form of VSG. *A*, hydrazine-stimulated VSG release was assayed in crude membranes prepared from trypanosomes pulse-labeled with [³⁵S]Cys/Met for 15 min (*Pulse*) or pulse-labeled for 15 min and chased (in cysteine-methionine-containing medium) for 1 h (*Pulse-Chase*). Samples were analyzed as in Fig. 1*A*. *B*, crude membranes were prepared from [³⁵S]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 [³⁵S]cysteine/methionine 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. 2*A*, 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 is 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. 2*B*). 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 (17, 24).

Lipid extracts from procyclic trypanosomes metabolically radiolabeled with [³H]ethanolamine contain the mature GPI structure PP1 (ethanolamine-PO₄-Man α 1-2Man α 1-6Man α 1-4GlcN α 1-6Inos(acyl)-PO₄-monoacylglycerol), as well as the PP1 precursor, PP3 (ethanolamine-PO₄-Man α 1-2Man α 1-6Man α 1-4GlcN α 1-6Inos(acyl)-PO₄-diacylglycerol) (25, 26) as shown by thin layer chromatographic analysis (Fig. 3*A*, panel *I*). Both lipids are susceptible to hydrolysis by GPI-specific phospholipase D (Fig. 3*A*, compare panels *I* and *II*). When cells were labeled in the presence of mannosamine, GPI synthesis was almost completely abolished: no [³H]ethanolamine-labeled PP1 was detected, and only a trace amount of PP3 was synthesized (Fig. 3*A*, panels *III* and *IV*).

To assess the effect of mannosamine on [³H]ethanolamine labeling of proteins, [³H]ethanolamine-labeled cells were analyzed for [³H]ethanolamine-labeled VSG by immunoprecipitation, SDS-PAGE, and fluorography. The fluorogram was quantitated by densitometry. As shown in Fig. 3*B*, [³H]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 [³⁵S]cysteine/methionine 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. 3*C*, 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 Alkylating 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 sulfonic acid (pCMPSA) (3). We tested the effect of pCMPSA as well as two other reagents (iodoacetamide and *p*-chloromercuribenzoate (pCMB)) on nucleophile-induced VSG

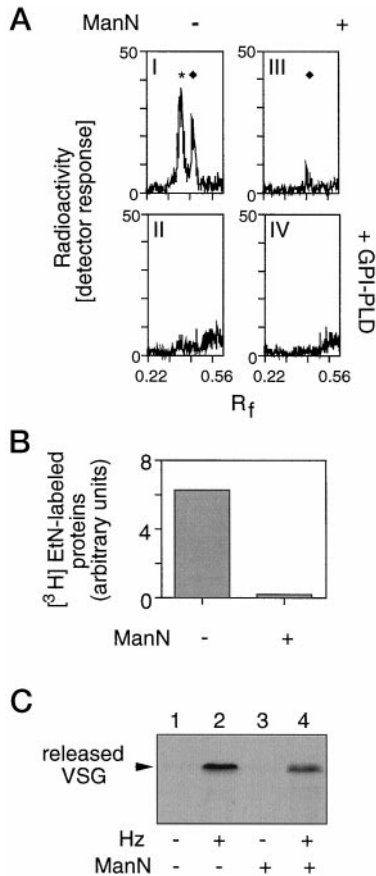


FIG. 3. Pro-VSG is a substrate for GPIT. *A*, crude membranes were prepared from VSG 117-expressing procyclic trypanosomes metabolically labeled with [³H]ethanolamine in the absence or presence of mannosamine (*ManN*). Lipids from 1×10^8 cell equivalents of membranes were extracted into organic solvent (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 (◆) is shown. *B*, 2.5×10^7 cell equivalents of [³H]ethanolamine-labeled washed trypanosome membranes (prepared from cells labeled in the absence or presence of mannosamine) were resuspended 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 quantitate 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. 1*A* and analyzed by carbonate extraction, immunoprecipitation, and SDS-PAGE/fluorography. *Hz*, hydrazine.

release. All three compounds inhibited hydrazine-induced release of VSG (Fig. 4*A*, 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(s) 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 premini-PLAP, a model protein with an N-terminal signal

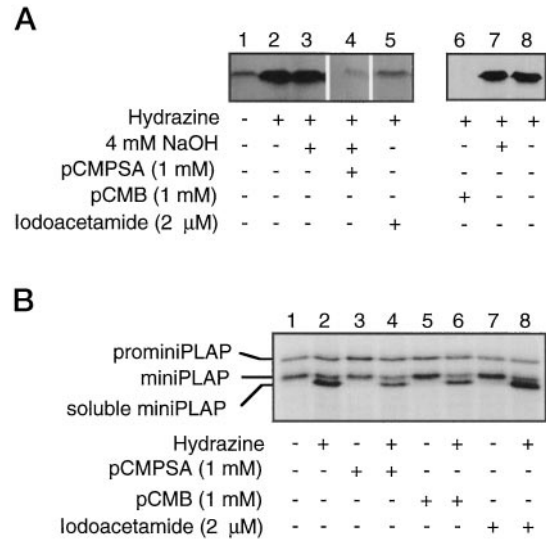


FIG. 4. Inhibition of trypanosome (but not mammalian) GPIT with sulfhydryl alkylating reagents. *A*, hydrazine-stimulated release of VSG was assayed in the presence of sulfhydryl alkylating reagents pCMPSA (lane 4), pCMB (lane 6), and iodoacetamide (lane 5). Both the pCMPSA and the pCMB incubations contained 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 premini-PLAP reporter was assayed as described under "Experimental Procedures." Membranes were preloaded with premini-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 untranslocated premini-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.

sequence and a C-terminal GPI addition sequence (9), was translated in the presence of thymoma 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 eliminate nontranslocated premini-PLAP and analyzed by SDS-PAGE and fluorography. The results of the experiment are shown in Fig. 4*B*. In the absence of inhibitors and hydrazine, two products are seen: premini-PLAP and GPI-anchored mini-PLAP (lane 1). When hydrazine was included in the 90-min incubation, a lower molecular mass product was formed (lane 2) (4, 13). The pattern of bands, particularly the ratio of mini-PLAP-hydrazine to mini-PLAP, seen in lanes 1 and 2 of Fig. 4*B* was unaltered in the presence of pCMPSA (lanes 3 and 4), pCMB (lanes 5 and 6), and iodoacetamide (lanes 7 and 8), indicating that the sulfhydryl alkylating reagents do not affect GPI anchoring in mammalian cells. The lack of effect by pCMPSA may be due to its inability to cross microsomal membranes, but the analogous reagent pCMB, as well as iodoacetamide, are membrane permeant and would be expected to react with luminal targets (40). Although these results appear to suggest that mammalian GPIT has a different inhibition profile from the trypanosome GPIT, an alternative explanation may have to do with the way in which the assay was set-up. Because sulfhydryl alkylating reagents cannot be added at the outset of the assay because of their inhibitory effect on protein translocation (27), it is possible that most of the GPIT is complexed to mini-PLAP (e.g. through a thioester bond between a sulfhydryl residue in GPIT and the ω site in mini-PLAP) during the 30-min loading period and that

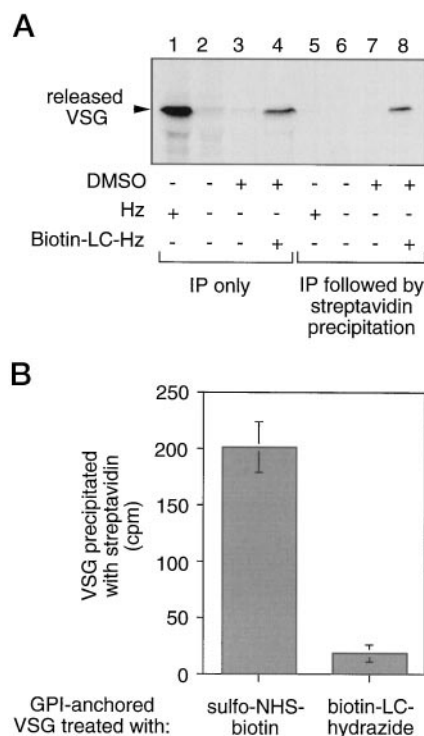


FIG. 5. GPIT-mediated biotinylation of VSG in the presence of biotin-LC-hydrazide. A, 5×10^7 cell equivalents of [35 S]Cys/Met pulse-labeled trypanosomes were used for the incubations in lanes 1–4. Washed membranes were incubated in the absence (lane 2) or presence of hydrazine (lane 1) or biotin-LC-hydrazide (lane 4). The biotin-LC-hydrazide was dissolved in dimethyl sulfoxide (DMSO); a control incubation with just Me₂SO added was also performed (lane 3). The sodium carbonate extract supernatant from these incubations was processed by washing on a Millipore filter, dissolved in buffer, and immunoprecipitated with anti-VSG 117 antibodies. The immunoprecipitates (IP) were analyzed by SDS-PAGE and fluorography (lanes 1–4). An identical set of incubations was also performed on an 8-fold larger scale, and the immunoprecipitates obtained using anti-VSG 117 antibodies were then subjected to precipitation with streptavidin agarose as described under “Experimental Procedures.” The streptavidin-bound material was analyzed by SDS-PAGE/fluorography (lanes 5–8). B, purified [3 H]myristic acid-labeled GPI-anchored VSG 117 (500 cpm) was biotinylated using sulfo-NHS-biotin or biotin-LC-hydrazide as described under “Experimental Procedures.” The treated material was subjected to streptavidin-agarose precipitation, and the precipitates were taken for scintillation counting. The [3 H] cpm recovered are shown as the means \pm error of duplicate determinations. Hz, hydrazine.

treatment with sulfhydryl alkylating reagents during the “second” incubation is unlikely to have an effect.

Hydrazine Is Covalently Incorporated into VSG as a Consequence of GPIT 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 molecule, biotin-LC-hydrazide, containing a biotin moiety amide-linked by a spacer arm to one of the nitrogen atoms in hydrazine (see Fig. 7).

Incubation of procyclic membranes with biotin-LC-hydrazide resulted in VSG release (Fig. 5A, compare lanes 3 and 4), although the efficiency of release was somewhat lower than that seen with hydrazine (Fig. 5B, lanes 1 and 2). The VSG released from the membranes by incubation with either hydrazine or biotin-LC-hydrazide was immunoprecipitated with anti-VSG antibodies, released from the antibody beads by boiling, separated from the beads by centrifugation, and then precipitated with streptavidin-agarose. Control samples using only an

anti-VSG 117 immunoprecipitation step showed the normal profile of VSG release from membranes with both hydrazine and the biotin-LC-hydrazide (Fig. 5A, lanes 1–4). Identically treated samples (containing eight times as much material to compensate for the inefficiency of precipitation and sample recovery using streptavidin beads; see “Experimental Procedures”) subjected to the additional streptavidin precipitation step showed a radioactive band corresponding to VSG 117 only in the sample treated with biotin-LC-hydrazide (Fig. 5A, lane 8); no VSG was recovered in the streptavidin precipitate of the sample treated with hydrazine (Fig. 5A, lane 5), indicating the specificity of the streptavidin precipitation analysis.

We considered the possibility that biotin-LC-hydrazide was reacting with VSG nonenzymatically, resulting in the formation of the biotinylated VSG molecule that we detected by streptavidin precipitation. To address this issue and further verify the specificity of the streptavidin precipitation analysis, we analyzed a sample of purified [3 H]myristic acid-labeled, GPI-anchored VSG 117 incubated with either biotin-LC-hydrazide or sulfo-NHS-biotin (an amine-reactive biotin derivative expected to modify free amines on VSG) in the absence of membranes. After incubation, the samples were washed on low molecular mass cut-off filters to remove unreacted biotin reagents, and the VSG molecules, captured on the filters, were resuspended and treated with streptavidin agarose. VSG exposed to sulfo-NHS-biotin was precipitated with streptavidin, whereas VSG treated with biotin-LC-hydrazide did not bind streptavidin (Fig. 5B). These results, together with the temperature dependence profile (Fig. 1B), strongly indicate that under our usual assay conditions the VSG released from the membranes in the presence of biotin-LC-hydrazide (Fig. 5A, lanes 4 and 8) is an enzymatically generated hydrazide derivative and contains covalently incorporated biotin.

Hydrazine Is Incorporated at or near the C Terminus of GPIT-released VSG Hydrazide—In preliminary attempts to locate the site of hydrazine modification in VSG hydrazide by mass spectrometry we concluded that the amount of starting material required was considerably in excess of what we could hope to purify from our *in vitro* assay system (subnanogram from a single assay; see “Experimental Procedures” for the basis of this estimate). We opted instead for a biochemical approach relying on a comparison between VSG hydrazide and another VSG reporter expressed in procyclic trypanosomes. This reporter, 117ΔGPI, is a VSG 117 molecule lacking the C-terminal GPI signal sequence. VSG hydrazide and 117ΔGPI 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, 117ΔGPI with an unblocked C terminus should be susceptible to proteolysis by carboxypeptidases.

Fig. 6 (lanes 1 and 2) shows that VSG hydrazide and 117ΔGPI 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 117ΔGPI 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 sulfhydryl 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 hydrazine 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 procyclic 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

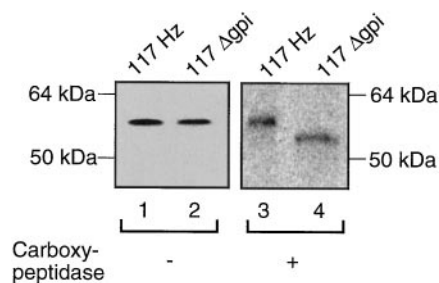
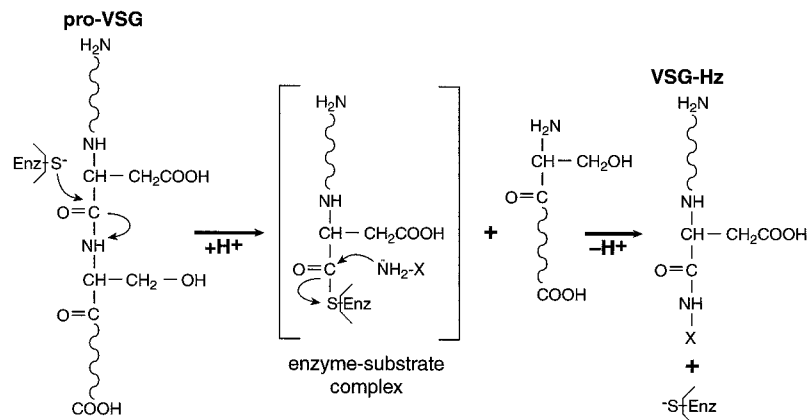


FIG. 6. Hydrazine is incorporated at or near the C terminus of GPIT-released VSG hydrazide. VSG 117 hydrazine (*Hz*) (obtained as in Fig. 1A) and 117 Δ GPI (isolated from procyclic trypanosomes expressing this construct) were purified by immunoprecipitation and analyzed by SDS/PAGE fluorography before (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 117 Δ GPI is proteolyzed after incubation with a mixture of carboxypeptidases P and W.

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 sulfhydryl 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 ($\omega + 1$ in the c-terminal signal sequence of pro-VSG). Nucleophilic attack by $\text{H}_2\text{N-X}$ results in release of VSG-NH-X and regeneration of the active site sulfhydryl in the transamidase.



$\text{NH}_2\text{-X}$	-X
hydrazine	-NH_2
hydroxylamine	-OH
biotin-LC-hydrazide	$\text{-NH-C(=O)-(CH}_2\text{)}_5\text{-NH-C(=O)-(CH}_2\text{)}_4\text{-S}$

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 jackbean 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.

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