Soluble GPI8 restores glycosylphosphatidylinositol anchoring in a trypanosome cell-free system depleted of lumenal endoplasmic reticulum proteins

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We previously established an *in vitro* assay for glycosylphosphatidylinositol (GPI) anchoring of proteins using trypanosome membranes. We now show that GPI anchoring is lost when the membranes are washed at high pH and restored to physiological pH prior to assay. We show that soluble component(s) of the endoplasmic reticulum that are lost in the high-pH wash are required for GPI anchoring. We reconstituted the high-pH extract with high-pH-treated membranes and demonstrated restoration of activity. Size fractionation of the high-pH extract indicated that the active component(s) was 30–50 kDa in size and was inactivated by iodoacetamide. Activity could also be restored by reconstituting the inactivated membranes with *Escherichia coli*-expressed, polyhistidine-tagged *Leishmania mexicana* GPI8 (GPI8-His; *L. mexicana* GPI8 is a soluble homologue of yeast and mammalian Gpi8p). No activity was seen when iodoacetamide-treated GPI8-His was used; however, GPI8-His could restore activity to iodoacetamide-treated membranes. Antibodies raised against *L. mexicana* GPI8 detected a protein of approx. 38 kDa in an immunoblot of the high-pH extract of trypanosome membranes. Our data indicate (1) that trypanosome GPI8 is a soluble lumenal protein, (2) that the interaction between GPI8 and other putative components of the transamidase may be dynamic, and (3) that GPI anchoring can be biochemically reconstituted using an isolated transamidase component.

Key words: BiP, GPI, *Leishmania*, transamidase.

INTRODUCTION

The assembly of glycosylphosphatidylinositol (GPI)-anchored proteins typically requires translocation of the nascent polypeptide across the endoplasmic reticulum (ER) membrane, cleavage of the N-terminal ER targeting signal sequence and replacement of a C-terminal GPI-directing signal sequence with a pre-assembled, phosphoethanolamine-containing GPI lipid [1]. GPI anchoring proceeds via a transamidation reaction mechanism [2–4], in which the GPI moiety is attached to the newly exposed C-terminus of the protein via an amide linkage between the GPI ethanolamine amine group and the carboxy group of the C-terminal amino acid. Genetic studies indicate that the transamidation reaction is catalysed by a protein complex in the ER, containing the membrane proteins Gaa1p and Gpi8p [5–8]. Analyses of the membrane topology of yeast and mammalian Gaa1p and Gpi8p indicate that Gaa1p contains many transmembrane spans [5,8], whereas Gpi8p, a type I membrane protein, traverses the membrane only once [7]. The Gpi8p protein bears resemblance to a jackbean endopeptidase involved in the post-translational splicing of concanavalin A [7,9], suggesting that it is most probably the catalytic component of the GPI:protein transamidase complex.

The GPI anchoring reaction can be reproduced in a number of differently formatted cell-free systems, some of which take advantage of endogenous protein acceptors as substrates for the anchoring reaction [2,4,10,11]. We previously developed such a system to demonstrate that GPI anchoring involves a transamidation reaction mechanism [4]. The system we used consisted of washed trypanosome membranes containing an ER-localized membrane-associated radiolabelled protein [variant surface glycoprotein (VSG)] capable of acting as a substrate in the GPI anchoring reaction. On incubation of these membranes with hydrazine, a small nucleophile capable of substituting for GPI in the anchoring reaction [3], the membrane-bound VSG was converted into soluble VSG hydrazide, containing hydrazine instead of GPI at the C-terminus of the protein [4]. Production of VSG-hydrazine was monitored by carbonate extraction of the membranes: VSG-hydrazine was released to the supernatant, while unreacted VSG remained membrane-associated.

In order to develop this assay system further so as to be able eventually to introduce membrane-impermeant substrates to the lumenally disposed transamidase enzyme, we investigated the possibility of opening up the membranes using treatment with alkaline pH. This approach has been used successfully in the past to deplete ER vesicles of lumenal content and to reconstitute the resulting lumen-depleted vesicles with specific lumenal proteins [12–14]. Indeed, we previously used this approach with mammalian microsomes [14] to show that processing of freshly translocated proproteins to the GPI-anchored form required the participation of soluble ER proteins, most likely chaperones such as BiP/GRP78 (binding protein/glucose-regulated protein of 78 kDa) [15]. In the same study we also noted that GPI anchoring in microsomes preloaded with proprotein acceptors,

Abbreviations used: BiP/GRP78, binding protein/glucose-regulated protein of 78 kDa; ER, endoplasmic reticulum; GPI, glycosylphosphatidylinositol; GPI8-His, polyhistidine-tagged GPI8; MWCO, molecular mass ('weight') cut-off; PLAP, placental alkaline phosphatase; VSG, variant surface glycoprotein.

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equivalent to the trypanosome cell-free system described above, was unaffected by high-pH treatment, suggesting that soluble ER chaperones are required only at an early stage of nascent chain processing [14].

In the present study we report that, unlike the situation with proprotein-loaded mammalian microsomes, GPI anchoring activity is lost when trypanosome membranes are diluted into high-pH buffers and restored to physiological pH prior to assay. Control experiments showed that loss of activity was not due to inactivation of the transamidase by exposure to high pH, but rather to the loss of critical components from the membrane system during the high-pH wash. Further analyses indicated that GPI anchoring activity could be restored by re-introducing a 30–50 kDa size-selected fraction of the high-pH wash to the lumen of membrane compartments in the cell-free system. The activity of this fraction could be entirely substituted by a bacterially expressed, polyhistidine-tagged variant of *Leishmania mexicana* GPI8 protein, a soluble homologue of yeast and mammalian Gpi8p [21]. Antibodies raised against *L. mexicana* GPI8 detected a protein of approx. 38 kDa in an immunoblot of the high-pH extract of trypanosome membranes. These results show that the GPI anchoring reaction can be reconstituted biochemically by re-introducing an isolated transamidase component, and set the stage for future studies of the organization and enzymology of the transamidase complex.

EXPERIMENTAL

Materials, growth and metabolic labelling of trypanosomes, and preparation of the trypansome cell-free system

Amicon NMWL filter units were from Millipore. The sources of all other materials are given in [4]. Stably transformed procyclic cell lines expressing full-length GPI-anchored VSG 117 (also known as 117 wt) were maintained as described previously [4,16]. The trypanosome cell-free system was prepared from metabolically radiolabelled cells or unlabelled cells as described previously [4,17], except that the cells were not preincubated with tunicamycin prior to labelling and lysis. Aliquots of membranes (5×10^8 cell equivalents/ml) were snap-frozen in liquid nitrogen and stored at -80 °C.

pH dependence of the transamidation reaction

A 700 μl aliquot of the trypanosome cell-free system was washed twice [4], and the pellet was resuspended in 175 μl of 10 mM Hepes (pH 7.5). Aliquots (35 μl) of the washed membranes were diluted with an equal volume of 400 mM buffer (Hepes, pH 7.5; Tris, pH 8.5; Tris, pH 9.0; Caps, pH 9.5; Caps, pH 10.0; or Caps, pH 11.0). The samples were vortexed and placed on ice for 20 min, and then 330 μl of 400 mM Hepes, pH 7.5, was added to each sample. (In some experiments, the volumes of high-pH buffer and the subsequent addition of neutralizing buffer were increased to test the effect of dilution on the recovery of transamidase activity.) The resulting 400 μl samples were each layered on top of separate 100 μl 0.5 M sucrose cushions (prepared in 400 mM Hepes, pH 7.5), and centrifuged in a Beckman TLA 100.2 rotor at 56000 ***g*** for 10 min. The supernatant plus cushion was then removed and the membrane pellets were each resuspended in 70 μl of 20 mM Hepes, pH 7.5. Aliquots (25 μl) of the resuspended membranes were added to an equal amount of 20 mM Hepes, pH 7.5 (in the presence or absence of 20 mM hydrazine) to assay transamidation activity. The samples were incubated for 1 h at 37 °C, and the release of soluble VSG-hydrazine was determined as described previously [4].

Alkaline extraction and molecular mass fractionation of soluble, lumenal ER proteins

A 2 ml sample of unlabelled, washed trypanosome membranes was resuspended in 5 ml of 50 mM Caps, pH 11.0, vortexed and placed on ice for 20 min. The sample was then layered on top of a 1 ml sucrose cushion (0.5 M sucrose in 50 mM Caps, pH 11.0) and centrifuged at 56000 ***g*** for 10 min. The resulting supernatant (load plus cushion) was passed through four pre-washed Microcon 10 kDa molecular mass ('weight') cut-off (MWCO) filters. The material remaining on the filters was resuspended in 400 μl of distilled water. An aliquot of this material was set aside (for use in experiments such as the one shown in Figure 2B, lanes 1 and 2), while the remainder was passed sequentially over four pre-washed Microcon filters in the following order: 100, 50, 30 and 10 kDa MWCO. In each case the size-selected material remaining on the filters (material corresponding to proteins of $>$ 100 kDa, 100–50 kDa, 50–30 kDa and 30–10 kDa respectively) was resuspended in 50 μl of distilled water and used in reconstitution studies (e.g. Figure 2B) or analyses of BiP content (e.g. Figure 2A).

Reconstitution of extracted proteins into transamidation-depleted lysate

A 600 μl sample of radiolabelled washed trypanosome membranes was resuspended in 5 ml of Caps buffer (pH 10.0), vortexed and placed on ice for 20 min. The membranes were then layered on top of 1 ml of a 50 mM Caps/0.5 M sucrose cushion and spun at 56000 ***g*** for 10 min. The pellet (reticuloplasm-depleted membranes) was resuspended in 300 μl of 30 mM Caps, pH 10.0, and 25 μl aliquots of this solution were added to 10 μl samples of the various alkaline-extracted molecular-mass extracts. The samples were then placed on ice for a further 10 min before adding 10 μl of 1 M Hepes (pH 7.5) to re-adjust the pH to 7.5. Transamidation assays were performed on each of the samples as described above.

Construction of expression vector

GPI8 was amplified from pGL 187 [21] by PCR [15 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 120 s; VENT DNA polymerase (New England Biolabs)] using the following oligonucleotide primers: OL437, CGGGATCCGGCAAGGGCCAGAGCAACAAC (the *Bam*HI restriction site is underlined); OL438, CTAGTCGACCTACTCGAGGTCGTAGCGGACGT (the *Sal*I restriction site is underlined).

The amplified fragment was digested with *Bam*HI and *Sal*I, cloned into similarly digested pQE-30 vector (Qiagen) and sequenced to check for mutations. This plasmid, designated pGL184, was used to transform M15[pREP4] *Escherichia coli* for the expression of an N-terminally $6 \times$ histidine-tagged GPI8 (GPI8-His). The N-terminal sequence of the GPI8-His construct is MRGSHHHHHHGS**G**KGQSNNW (where the **G** in bold designates the first *GPI8*-encoded amino acid).

Expression and purification of GPI8-His

GPI8-His was expressed by induction of pGL184-transformed M15[pREP4] with 2 mM isopropyl β-D-thiogalactoside according to the QIAexpress® Expression Kit protocol (Qiagen). GPI8-His was purified in the form of inclusion bodies using the method of Kuhelj et al. [18], and was solubilized in 8 M urea, 100 mM NaH_2PO_4 and 10 mM Tris/HCl, pH 8.0. GPI8-His was purified on a 4 ml Ni^{2+}-nitrilotriacetate Superflow column (Qiagen),

washed at pH 6.3, 5.9 and 4.5, and eluted in 6 M guanidine hydrochloride/0.2 M acetic acid according to the manufacturer's protocol (Qiagen).

Refolding and reconstitution of denatured GPI8-His

An aliquot (50 μl; 2.5 mg/ml) of nickel-purified, denatured GPI8-His protein was diluted with 450 μl of 100 mM Caps (pH 10.0)/1 mM dithiothreitol (containing, additionally, 2 μM iodoacetamide for experiments in which the effect of this reagent on GPI8-His activity was to be tested). The sample was then washed through a 10 kDa MWCO filter, and the protein retained on the filter was dissolved in 500 μl of 100 mM Hepes, pH 7.5. This material was washed again using a fresh 10 kDa MWCO filter, and the filter-retained material was dissolved in 20 μl of distilled water and used for reconstitution studies as described above.

Immunoprecipitation, electrophoresis and immunoblotting

Immunoprecipitation and electrophoresis were done as described previously [4]. For immunoblotting to detect GPI8, samples were solubilized in SDS sample buffer and electrophoresed on a 10 %-polyacrylamide gel in the presence of SDS. The SDS/PAGE-resolved material was transferred to a Hybond-C nitrocellulose membrane (Amersham), and processed for immunoblotting with polyclonal antiserum raised against *E. coli*-expressed GPI8-His [diluted 1:1000 in 10 mM Tris/HCl (pH 8.0), 150 mM NaCl and 0.05 % Tween 20]. Blots were developed using horseradish peroxidase-conjugated anti-rabbit IgG (Promega) and the Supersignal Luminol enhancer kit (Sigma). No reaction was detected when preimmune serum was used.

RESULTS AND DISCUSSION

We previously described an assay for GPI anchoring using a trypanosome cell-free system containing ER-localized, membrane-bound, [^{35}S]methionine/cysteine-labelled VSG [4]. On incubation of the cell-free system with hydrazine, membrane-bound VSG was converted into soluble VSG-hydrazine through the action of the GPI transamidase. [^{35}S]VSG-hydrazine was separated from unreacted membrane-bound VSG by carbonate extraction, then purified by immunoprecipitation and analysed after SDS/PAGE and fluorography (Figure 1A, lanes 1 and 2). Treatment of the cell-free system with thiol alkylating reagents – such as iodoacetamide – abolished VSG-hydrazine production, consistent with the presence of a catalytically important cysteine residue in one of the transamidase subunits.

Very little is known about the enzymology and molecular organization of the GPI transamidase. In order to approach this problem, we wished to extend the assay described above so that it would be possible in the future to deliver exogenously added membrane-impermeant substrates to the lumenally disposed transamidase. In order to do this, we tested a protocol in which alkaline pH is used to open up ER membranes in a manner such that they re-seal when returned to physiological pH.

Washes at alkaline pH deplete trypanosome lysates of transamidation activity in a dilution-dependent manner

Before using alkaline-pH-mediated permeabilization of membranes in the cell-free system, we tested the effect of pH on the transamidation reaction. Previous studies with mammalian microsomes had shown no effect of high pH on the GPI anchoring of a model protein, preprominiPLAP (where PLAP is placental alkaline phosphatase), provided that the membranes were loaded with prominiPLAP prior to high-pH treatment [14]. Since the trypanosome membranes in our cell-free system were already loaded with pro-VSG [4], we anticipated that our tests with trypanosome membranes would simply confirm the data obtained with mammalian membranes. Figure 1(A) shows that this was not the case. When the trypanosome cell-free system was diluted with an equal volume of 400 mM buffer (pH in the range 7.5–11.0), then washed, resuspended at pH 7.5 and assayed for VSG-hydrazine production, we found that the yield of VSG-hydrazine decreased as a function of increasing pH (e.g. compare lane 12 with lane 2 in Figure 1A). The magnitude of the high-pH effect depended on the dilution factor, since membranes diluted 1:1 (v/v) in 400 mM buffer at pH 11.0 retained some ability to generate VSG-hydrazine in the assay (Figure 1B, lane 4 versus lane 2), whereas membranes diluted 1:6.5 (v/v) showed no activity (Figure 1B, lane 6 versus lane 2).

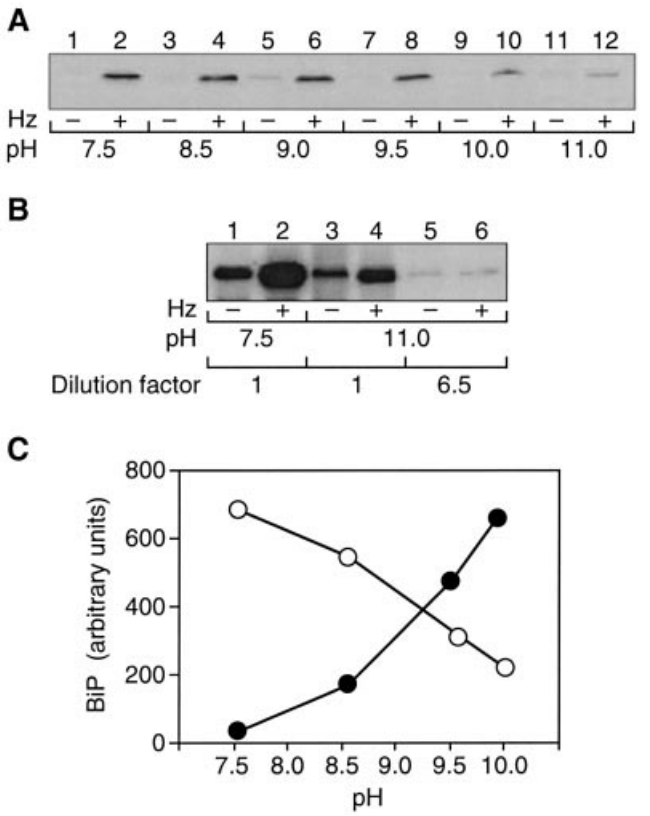

Figure 1 Alkaline washing of the trypanosome cell-free system results in a loss of GPI transamidation activity

(**A**) Aliquots of trypanosome membranes prepared from [^{35}S]Cys/Met pulse-radiolabelled cells were treated with buffered solutions of different pH, as described in the Experimental section [the samples were diluted 1:1 (v/v) into the various buffers]. After treatment, the samples were neutralized and membranes were re-isolated by centrifugation through a sucrose cushion, resuspended in Hepes (pH 7.5) and assayed for transamidation activity by monitoring the hydrazine (Hz)-induced release of [^{35}S]VSG-hydrazine. (**B**) The [^{35}S]Cys/Met-labelled trypanosome membrane preparation was treated with buffer of pH 7.5 or pH 11.0, as described for (**A**), except that the pH 11.0 treatment was carried out by diluting the membranes 1:1 (v/v) (lanes 3 and 4; similar to lanes 11 and 12 in **A**) or 1:6.5 (v/v) (lanes 5 and 6) in pH 11.0 buffer. The samples were processed and tested for transamidation activity as described for (**A**). Film exposure was longer than that used for (**A**), in order to highlight the lack of activity above background in lanes 5 and 6. (**C**) Aliquots of the trypanosome membrane preparation were treated with buffered solutions of different pH exactly as described for (**A**). The samples were neutralized and the membranes were re-isolated by centrifugation as in (**A**), and aliquots of the resuspended membranes (○) and the centrifugation supernatant (●) were analysed by SDS/PAGE and immunoblotting with anti-BiP antibodies.

Since the membranes are resuspended in pH 7.5 buffer after the pH treatment, the pH sensitivity of the transamidation assay cannot be attributed to changes in the ionization of hydrazine, the nucleophile substrate for the transamidase. Furthermore, the dilution effect indicates that the loss of activity that we observed is not a direct effect of high pH on the transamidase. We propose, instead, that membranes exposed to high-pH buffers become permeabilized and release their lumenal content, including factor(s) essential for transamidation, but re-seal once the pH is returned to physiological levels. The efficiency with which high-pH-released material (soluble proteins and other content of the ER lumen) is captured upon re-sealing of the membranes depends on the dilution factor. The efficiency of capture during the re-sealing process decreases with increasing dilution, corresponding to a loss of activity. We confirmed these ideas by monitoring the release of the soluble lumenal protein BiP/GRP78 as a function of pH: as shown previously [13,14], the amount of BiP recovered in the supernatant after pH treatment and re-sealing of the membranes increased with increasing pH (Figure 1C) and increasing dilution (results not shown). Thus, unlike the situation with mammalian microsomes preloaded with proprotein substrates for the transamidase, soluble components of the ER lumen (reticuloplasm) appear to be required for GPI anchoring in the trypanosome cell-free system.

Figure 1(B) (lanes 1, 3 and 5) shows that the amount of VSG recovered in the carbonate wash of the control 'minus hydrazine' samples decreased in the same way as seen for VSG-hydrazine in the 'plus hydrazine' samples. This suggests that VSG recovered in the carbonate extract of samples in the absence of hydrazine results from transamidase-mediated hydrolysis of pro-VSG via a reaction in which water, rather than hydrazine, acts as the nucleophile. Transamidase-mediated hydrolysis of proproteins has been noted previously in mammalian microsomes [1].

A 30–50 kDa, iodoacetamide-sensitive protein(s) in the high-pH extract can restore transamidation activity when added back to reticuloplasm-depleted membranes

Since loss of activity in high-pH-treated membranes appeared to be due to the loss of soluble components of the ER lumen, we attempted to re-introduce the high-pH wash material to the lumen of the depleted membranes, to see whether activity could be restored. The cell-free system was treated with pH 11.0 buffer to release ER lumenal content, and the resulting extract (reticuloplasm) was concentrated using a 10 kDa MWCO filter. The concentrated material was re-introduced to the reticuloplasm-depleted membranes by exposing the membranes to pH 10.0 buffer under low dilution conditions. This procedure causes the membranes to open up, thus permitting capture of some of the concentrated reticuloplasm upon re-sealing. After re-isolating the membranes by centrifugation through a pH 7.5 sucrose cushion, transamidation activity was assayed. Activity was stimulated in membranes reconstituted with reticuloplasm when compared with membranes reconstituted with buffer alone (results not shown; but see Figure 2B, lanes 1 and 2). When the reticuloplasm was treated with 2 μM iodoacetamide before reconstitution, no stimulation of activity was observed (results not shown; but see Figure 3D, lanes 1–4). We conclude that an iodoacetamide-sensitive protein (> 10 kDa) of the high-pH extract is critical for transamidation in the cell-free system.

We fractionated the high-pH extract, using a series of MWCO filters, in an attempt to identify the molecular mass of the active component(s). The extract was passed sequentially over filters with MWCO values of > 100 kDa, 50 kDa, 30 kDa and 10 kDa,

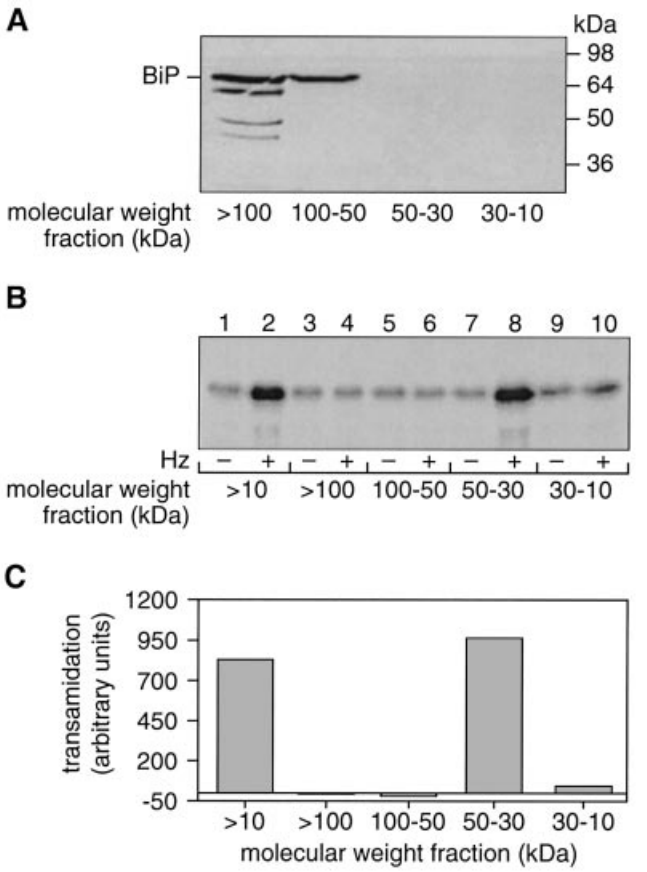

Figure 2 Recombination of the high-pH extract with reticuloplasm-depleted membranes reconstitutes transamidase activity

A high-pH extract of trypanosome membranes was concentrated by passage through a 10 kDa MWCO filter, as described in the Experimental section. The material remaining on the filter was dissolved in water, and then size-fractionated by sequential passage through MWCO filters designed to exclude proteins of > 100 kDa, > 50 kDa, > 30 kDa and > 10 kDa, as described. Aliquots of the size-selected fractions were taken for SDS/PAGE and immunoblotting with anti-BiP antibodies (**A**) and reconstitution with reticuloplasm-depleted [^{35}S]Cys/Met-labelled membranes (**B** and **C**). (**A**) Immunoblot to detect BiP in size-selected fractions of the high-pH wash. (**B**) Transamidation activity in reticuloplasm-depleted membranes reconstituted with concentrated high-pH extract (> 10 kDa) or equivalent amounts of size-selected fractions. Transamidation was assayed as in Figure 1 by monitoring the release of [^{35}S]VSG-hydrazine (Hz is hydrazine). (**C**) Quantification of the data shown in (**B**). The fluorogram in (**B**) was quantified using a densitometer, and the results are plotted in arbitrary densitometry units.

resulting in size-selected fractions corresponding to molecular mass ranges of > 100 kDa, 100–50 kDa, 50–30 kDa and 30–10 kDa. The quality of the fractionation was assessed by immunoblotting for BiP/GRP78: trypanosome BiP is a ~ 72 kDa protein [19] that is expected to fractionate in the 100–50 kDa fraction and, because of its association with other endogenous proteins [20], it is also expected to appear in the > 100 kDa fraction. Figure 2(A) shows that BiP was distributed exactly as expected, confirming the accuracy of the fractionation procedure.

The size-selected reticuloplasm subfractions were reconstituted with reticuloplasm-depleted membranes using the high-pH/low-dilution protocol described above, and the ability of the reconstituted preparations to carry out the transamidation reaction was assessed (Figures 2B and 2C). The results clearly show that the activity present in the reticuloplasm was recovered in a single fraction, representing proteins in the 30–50 kDa range [Figure 2B, lane 2 (starting material) and lane 8 (active fraction);

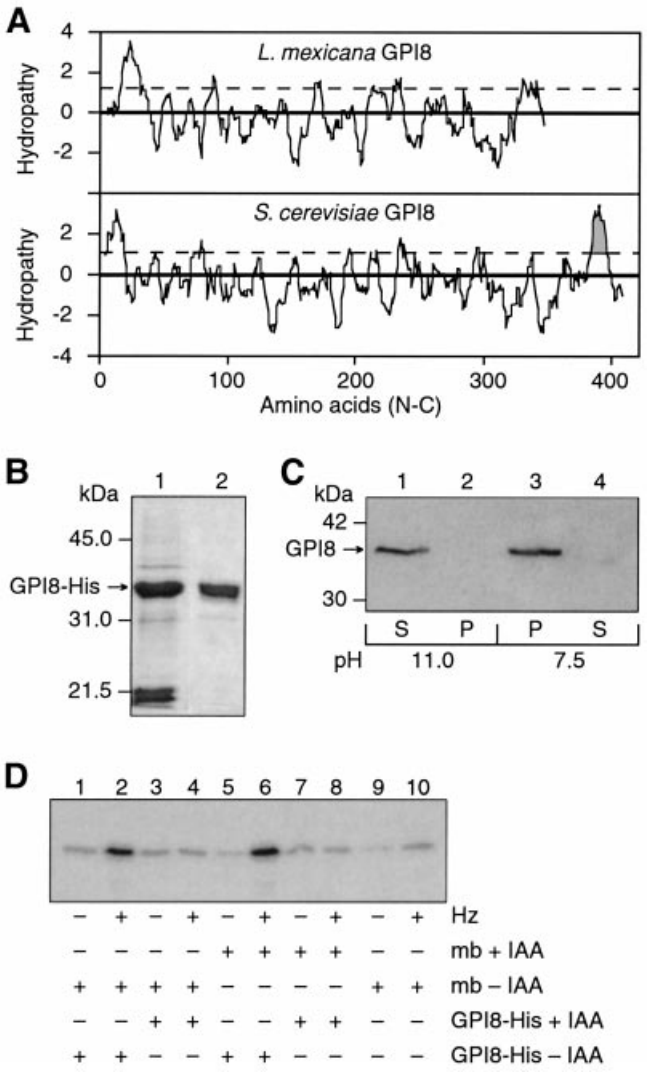

Figure 3 ***Leishmania mexicana*** **GPI8-His can restore transamidase activity to reticuloplasm-depleted membranes in an iodoacetamide-sensitive manner**

(**A**) Comparison of GPI8 proteins. Kyte and Doolitle [25] hydropathy profiles of GPI8 proteins from *L. mexicana* (upper panel) and *Saccharomyces cerevisiae* (lower panel) (nomenclature for the *L. mexicana* protein is according to [26]). Both have a hydrophobic N-terminal ER-directing signal sequence, but only the *S. cerevisiae* protein has a putative type 1 hydrophobic membrane-spanning region (shaded area). (**B**) SDS/PAGE analysis of *E. coli*-expressed GPI8-His. A poly-His-tagged version of *L. mexicana* GPI8 was expressed in *E. coli*. The protein was recovered from inclusion bodies, purified on a nickel column and refolded prior to use in reconstitution experiments. The Coomassie Blue-stained gel shows inclusion body material (lane 1) and purified GPI8-His (lane 2). (**C**) Immunoblot analysis of procyclic trypanosomes using antiserum raised against GPI8-His. Procyclic trypanosomes were hypo-osmotically lysed, pelleted, then treated on ice with pH 7.5 (Hepes) or pH 11.0 (Caps) buffer, as described in the Experimental section. The membranes were recovered by pelleting through a 0.5 M sucrose cushion (pH 7.5), and the membrane pellets and centrifugation supernatants were taken for SDS/PAGE analysis on a 10% (w/v) acrylamide gel and immunoblotting with anti-GPI8-His antiserum. A total of 2.5×10^7 cell equivalents were analysed in the pellet (P) samples (lanes 2 and 3), and 2.5×10^8 cell equivalents were analysed in the supernatant (S) samples (lanes 1 and 4). The migration of molecular-mass markers is indicated. Losses during processing of the supernatant fractions necessitated the use of a 10-fold higher sample loading compared with the pellet fractions. (**D**) A trypanosome cell-free system prepared from [^{35}S]Cys/Met pulse-radiolabelled cells was divided into two portions. One portion [membranes (mb) + iodoacetamide (IAA); lanes 5–8] was treated with iodoacetamide at a final concentration of 2 mM, then washed to remove excess reagent. The other portion (mb — IAA; lanes 1–4, 9 and 10) was processed identically, except that the iodoacetamide treatment was omitted. Both membrane preparations were then treated with high-pH buffer to deplete ER lumenal content, and reconstituted with GPI8-His that had been pretreated or not with iodoacetamide (GPI8-His + IAA, lanes 3, 4, 7 and 8; GPI8-His — IAA; lanes 1, 2, 5 and 6), as described in the Experimental section. The reconstituted preparations were assayed for transamidase activity as in Figure 1. A control set of incubations using reticuloplasm-depleted membranes (no iodoacetamide) reconstituted with buffer alone is shown in lanes 9 and 10.

see also Figure 3D]. No hydrazine-stimulated release of VSG was seen with any of the other fractions.

Leishmania mexicana GPI8 can restore transamidation in reticuloplasm-depleted membranes

Recent work [21] revealed that the protozoan parasite *Leishmania mexicana* possesses a homologue of yeast and mammalian Gpi8p which, based on the predicted amino acid sequence, is expected to be a soluble protein (Figure 3A). Since the molecular mass of the active fraction identified in the reconstitution experiments described above corresponds to the molecular mass range of the Gpi8p family of proteins, we considered the possibility that African trypanosomes, like *L. mexicana*, possess a soluble Gpi8p. This would account for our ability to abolish transamidation competence with an alkaline wash and restore it by re-introducing the alkali-extracted material into the lumen of the reticuloplasm-depleted membranes.

To test this idea, we took advantage of the availability of a preparation of polyhistidine-tagged *L. mexicana* GPI8 that had been expressed in *E. coli*. GPI8-His was purified from inclusion bodies by solubilization in 8 M urea and passage over a nickel colum (Figure 3B). Polyclonal antibodies raised against GPI8-His specifically detected a band of $\sim$ 38 kDa in an immunoblotting analysis of trypanosome proteins. Moreover, this immunoreactive band was detected in the supernatant of a trypanosome membrane sample that had been exposed to pH 11 buffer, but was found in the pellet fraction of a parallel sample that had been exposed to pH 7.5 buffer (Figure 3C). These data clearly indicate that procyclic trypanosomes contain a soluble, lumenal protein in the expected molecular-mass range for GPI8 that cross-reacts with antiserum raised against GPI8-His. The data further suggest that the activity in the 30–50 kDa fraction of a high-pH extract of trypanosome membranes (Figures 2B and 2C) is due to GPI8.

Column-purified GPI8-His was diluted in Caps buffer (pH 10.0) containing dithiothreitol, concentrated on a 10000 Da MWCO filter, washed again in Caps (pH 10.0) to remove dithiothreitol, and resuspended in Hepes buffer, pH 7.5 (in the presence or absence of iodoacetamide, as indicated). The protein was then re-filtered using the 10 kDa MWCO filter and dissolved in distilled water before being reconstituted with the reticuloplasm-depleted cell-free system as described above. (The reticuloplasm-depleted cell-free system was prepared by alkaline washing of membranes treated with or without iodoacetamide as indicated.) Figure 3(D) shows that when GPI8-His was reconstituted with reticuloplasm-depleted membranes, transamidation activity was observed [Figure 3D; compare lanes 1 and 2 (+GPI8-His) with lanes 9 and 10 (+buffer)]. No activity was seen when iodoacetamide-treated GPI8-His was used for reconstitution (Figure 3D, lanes 3 and 4). In contrast, iodoacetamide treatment of the reticuloplasm-depleted membranes had no effect on GPI8-His-mediated restoration of activity provided that untreated GPI8-His was used for reconstitution (Figure 3C; compare lanes 5 and 6 with lanes 7 and 8). These results clearly indicate that *E. coli*-expressed *L. mexicana* GPI8-His can restore transamidation activity to reticuloplasm-depleted trypanosome membranes. The data also indicate that the previously noted sensitivity of the transamidation reaction to thiol alkylating reagents [2,4] is confined to the effect of these reagents on GPI8.

Concluding discussion

The ease with which we were able to extract transamidation activity and functionally reconstitute it with heterologous GPI8 protein indicates that trypanosome GPI8, like *L. mexicana* GPI8, is a soluble protein that can be extracted from the lumen of the ER by high-pH treatment. Data from yeast and mammalian cells indicate that the transamidase enzyme is a complex of at least two proteins, GAA1 and GPI8 [5,7,8]. There may be other subunits in this complex, but none have been identified thus far. If we assume that trypanosomes have a transamidase enzyme that is compositionally similar to the one found in yeast and mammals, then our data indicate that GPI8 can be readily extracted from the transamidase complex, and just as readily recombined with it. This suggests that the transamidase, at least in trypanosomes, may be a dynamic complex *in situ*. Recent reports [22,23] indicating that Gpi8p works as part of a homo- or hetero-polymeric complex in the ER are consistent with this idea.

A homologue of GAA1 is yet to be identified in trypanosomes. Preliminary immunoprecipitation experiments using anti-GPI8 antiserum and extracts prepared from metabolically radiolabelled trypanosomes were insufficiently clean to be able to identify potential interactions between GPI8 and other ER proteins such as a putative GAA1 (results not shown). Nevertheless, it is likely that GAA1, or another transamidase subunit, exists in trypanosomes to anchor GPI8 near the membrane, where it can interact with its membrane-bound substrates. GAA1 (or another protein) may also be required to retain GPI8 in the ER [5], since GPI8 appears not to possess a characteristic ER retention/retrieval signal [19,21]. From a mechanistic perspective, transamidase subunits such as GAA1 may be required to segregate GPI proproteins from transmembrane proteins emerging from the ER translocon, and to transfer these proteins to GPI8. If this is the case, GPI8 need only interact transiently with GAA1 (or other transamidase components) and may exist in a sub-stoichiometric relationship with these components in the ER. This may be a way in which trypanosomes and *Leishmania* cope with the considerable flux through the GPI anchoring pathway that is a characteristic of these parasites [24]. Our results, demonstrating the ability to manipulate GPI8 levels biochemically and to introduce tagged or otherwise altered variants of GPI8 into reticuloplasm-depleted trypanosome membranes, makes it possible to test these ideas directly.

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