Two Subunits of Glycosylphosphatidylinositol Transamidase, GPI8 and PIG-T, Form a Functionally Important Intermolecular Disulfide Bridge*

Received for publication, January 19, 2003, and in revised form, February 10, 2003
Published, JBC Papers in Press, February 11, 2003, DOI 10.1074/jbc.M300586200

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Many eukaryotic cell surface proteins are tethered to the plasma membrane via glycosylphosphatidylinositol (GPI). GPI transamidase is localized in the endoplasmic reticulum and mediates post-translational transfer of preformed GPI to proteins bearing a carboxyl-terminal GPI attachment signal. Mammalian GPI transamidase is a multimeric complex consisting of at least five subunits. Here we report that two subunits of mammalian GPI transamidase, GPI8 and PIG-T, form a functionally important disulfide bond between conserved cysteine residues. GPI8 and PIG-T mutants in which relevant cysteines were replaced with serines were unable to fully restore the surface expression of GPI-anchored proteins upon transfection into their respective mutant cells. Microsomal membranes of these transfectants had markedly decreased activities in an in vitro transamidase assay. The formation of this disulfide bond is not essential but required for full transamidase activity. Antibodies against GPI8 and PIG-T revealed that endogenous as well as exogenous proteins formed a disulfide bond. Furthermore, trypanosome GPI8 forms a similar intermolecular disulfide bond via its conserved cysteine residue, suggesting that the trypanosome GPI transamidase is also a multimeric complex likely containing the orthologue of PIG-T. We also demonstrate that an inactive human GPI transamidase complex that consists of non-functional GPI8 and four other components was co-purified with the proform of substrate proteins, indicating that these five components are sufficient to hold the substrate proteins.

* This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan. 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.

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1 The abbreviations used are: GPI, glycosylphosphatidylinositol; ER, endoplasmic reticulum; CHO, Chinese hamster ovary; GST, glutathione S-transferase; HA, hemagglutinin; FG, FLAG and GST tandem; NEM, N-ethylmaleimide; PLAP, placental alkaline phosphatase; MBP, maltose-binding protein; MFI, mean fluorescence intensity; UL16BP2, UL16-binding protein 2.
Plasmids and selected at 500 infection efficiency and high PIG-T-dependent surface expression of CD59.

XbaI fragments of full-length wild-type and C92S mutant

buffer with iodoacetamide. Phosphoglycerokinase-driven puromycin, hygromycin, and from Dr. K. Maruyama (Tokyo Medical and Dental University, Tokyo, Japan). PIG-T

100°C, 1% SDS, and 5% β-mercaptoethanol was added. For direct analysis by SDSPAGE, 5 μl of each reaction was mixed with 15 μl of sample buffer with iodoacetamide or 2-mercaptoethanol immediately after the reaction. The

in vitro GPI transamidase assay using miniFLAP (placentalk alkaline phosphatase) originally developed by Kodukula et al. (25) was performed as described previously (18).

Flow Cytometric Analysis—Cells were stained with biotinylated anti-CD59 5F8 followed by phycoerythrin-conjugated streptavidin (Biomedia) and analyzed on a FACScalibur (BD Biosciences).

Polyclonal Antibodies against Human GPI8 and PIG-T Proteins—We immunized 1 mice with affinity-purified, carboxyl-terminally His-tagged human GPI8 (Ser26–Ser264) and human PIG-T (Leu208–Leu779), respectively, that were expressed as maltose-binding protein (MBP) fusion proteins and coupled to HiTrap N-hydroxysuccinimide-activated HP columns (Amersham Biosciences). An IgG fraction of antisera was isolated with HiTrap rProtein A FF columns (Amersham Biosciences), preadsorbed with MBP-coupled columns, and finally affinity-purified with MBP-GPI8 or MBP-PIG-T columns.

Trypanosome Manipulation—We disrupted trypanosomal GPI8 (ThGPI8) gene in procyclic form of T. brucei as described previously (9).

An episomal plasmid, pTMCszeo, was constructed by replacing the neomycin-resistance gene with hisG, containing pSB10 (Zacharias et al. (20)). pSB10 contains a multiple cloning site (nine restriction enzyme sites) flanked by aldolase splice acceptor signal and polyadenylation signal into a unique SmaI site of pT111s (26). A 1-kb fragment encoding FLAG-tagged wild-type or C76S mutant ThGPI8 was amplified by PCR and cloned into KpnI and BamHI sites of pTMCszeo. ThGPI8 knockout precocies were transfected with the resulting plasmids by electroporation and selected at 2–10 μg/ml phleomycin. Transfectants were stained with monoclonal anti-EP-procytin (Cedarlane Laboratories) plus fluorescein isothiocyanate-labeled secondary antibody and analyzed in a FACScaliber. For immunoprecipitation, cells of a confluent 10-culture (10^6) were pretreated, lysed in 1 ml of lysis buffer with NEM, and subjected to immunoprecipitation with anti-FLAG beads.

RESULTS

GPI8 and PIG-T Are Linked via a Disulfide Bond—We previously reported that GPI transamidase components GAA1, PIG-S, PIG-T, and PIG-U were co-purified with FLAG- and GST-tagged GPI8 (FG-GPI8) by two-step affinity purification using anti-FLAG and glutathione beads. Under reducing conditions, FG-GPI8, GAA1, PIG-S, and PIG-T migrate at 60–70 kDa (12), while PIG-U migrates as a diffuse band at 35 kDa (13) (Fig. 1C, left lane). Under non-reducing conditions, however, most of the FG-GPI8 migrates at about 160 kDa. Western blotting with anti-GST antibody detected an intense 160-kDa band with more slowly migrating minor bands in addition to a faint 70-kDa monomeric FG-GPI8 band (Fig. 1C). These high molecular mass bands disappeared when the sample was analyzed under reducing conditions (data not shown), suggesting that a major fraction of GPI8 is ligated to other proteins through a disulfide bond. To see whether GPI8 is linked to other GPI transamidase components or to unknown proteins, we analyzed the same sample by silver staining. Consistent with the Western blotting results, several bands appeared at and over 160 kDa, and only two intense bands remained at around the 70-kDa position (Fig. 1B), suggesting that a covalent complex was formed between GPI8 and another 60–70-
kDa component of transamidase. To test this, the same sample was analyzed by two-dimensional electrophoresis (Fig. 1). The first dimension of electrophoresis was performed under non-reducing conditions as in Fig. 1B, and then a gel strip was reduced and subjected to a second dimension of electrophoresis under reducing conditions. Two spots derived from the 160-kDa bands in the first dimension ran side by side below the gel diagonal (Fig. 1C), suggesting that formation of several 160-kDa bands may be due to multiple conformations of a single GPI8-containing complex. The two 60–70-kDa bands in the first dimension gave two spots on the diagonal (Fig. 1C), indicating that the 160-kDa bands are intermolecular disulfide-bonded complexes of two 60–70-kDa proteins. While one of these proteins is FG-GPI8, the partner protein could not be identified based on mobility because the difference in molecular weight between GAA1, PIG-S, and PIG-T is quite small and the two dimensional gel was slightly distorted.

To determine the partner protein linked to GPI8, we transfected differentially tagged GPI8, PIG-T, GAA1, and PIG-S into CHO cells. Transfectants were pretreated and lysed in the presence of 20 mM N-ethylmaleimide, a membrane-permeable alkylating reagent, to trap disulfides formed within the cells and to prevent their rearrangement during and after lysis (27, 28). The GPI transamidase complex was precipitated with glutathione beads and Western blotted with anti-tag antibodies (Fig. 2A). Anti-GST and anti-Myc but not anti-FLAG or anti-HA antibodies detected the 160-kDa band, indicating that PIG-T is the partner protein. The anti-GST- and anti-Myc-reactive 160-kDa band was not detected when the samples were reduced (data not shown).

Human GPI8 has five cysteines, but only Cys92 and Cys206 are conserved in other orthologues. To determine the cysteine residue involved in this disulfide linkage, we constructed cysteine-to-serine mutants of GPI8. Cys206 is a catalytic site of GPI transamidase as reported previously (18). Mutant GPI8 proteins were stably expressed in class K cells. Expression of C51S was very low, but this mutant clearly generated the 160-kDa band (Fig. 2B). Only the C92S mutant was incapable of forming the 160-kDa band, indicating that Cys92 of GPI8 is used for the disulfide bond.

Cys129 and Cys182 of human PIG-T are conserved between species. These cysteine residues were mutated to serines and stably expressed in PIG-T knockout cells. The C182S mutant of PIG-T did not form a covalent complex, whereas the C192S mutant formed the covalent complex (Fig. 2C). Therefore, Cys92 of GPI8 and Cys182 of PIG-T are involved in formation of the disulfide bond. Notably the disulfide linkage is not essential for

**Fig. 1.** GPI8 forms an intermolecular disulfide bond with another transamidase component. A and B, a GPI transamidase complex was purified from class K cells expressing FLAG/GST-tagged GPI8 by a two-step affinity purification and electrophoresed under non-reducing conditions. A, Western blotting with anti-GST antibodies; B, silver staining. C, a gel strip as shown in B was excised before silver staining, reduced, and overlaid on another gel for the second dimension electrophoresis under reducing conditions. The gel was then silver-stained. A reduced sample of the purified complex was also loaded in the left lane as reference components. The identities of bands are indicated on the left.

**Fig. 2.** GPI8 and PIG-T form a disulfide-bonded complex through specific cysteine residues. A, CHO cells were transiently transfected with a mixture of GST-GPI8, FLAG-GAA1, HA-PIG-S, and Myc-PIG-T. Two days later cells were lysed, and the transamidase complex was precipitated with glutathione beads. The precipitates were divided into four aliquots, electrophoresed under non-reducing conditions, and Western blotted with the indicated antibodies. B, class K cells were stably transfected with an empty vector, FG-tagged wild-type GPI8, or cysteine-to-serine mutant GPI8 proteins. The transamidase complexes precipitated with glutathione beads were electrophoresed under non-reducing conditions and Western blotted with anti-GST antibodies. C, PIG-T knockout F9 cells expressing CD59 were stably transfected with an empty vector, Myc-tagged wild type, or cysteine-to-serine mutant PIG-T proteins. The transamidase complexes immunoprecipitated with anti-Myc antibody plus protein G beads were electrophoresed under non-reducing conditions and Western blotted with anti-Myc antibody. The asterisk indicates a nonspecific band.
the generation of the tetrameric complex since the C182S mutant of PIG-T, GAA1, and PIG-S were co-precipitated with GPI8, and conversely the C92S mutant of GPI8 co-precipitated GAA1, PIG-T, and PIG-S normally expressed in CHO cells (data not shown).

**Functional Importance of the Disulfide Bond for Transamidase Activity**—To examine whether the disulfide linkage is functionally important for GPI transamidase activity, we determined the abilities of GPI8 and PIG-T mutants to restore the surface expression of GPI-anchored proteins on their respective mutant cells (Fig. 3). Wild-type GPI8 restored the surface CD59 expression on class K cells to a level similar to parental K562 cells (data not shown). The C206S mutant had no complementation activity as previously reported because this cysteine is an active site of GPI transamidase (18). The C51S and C275S/C280S mutants restored CD59 expression at levels similar to wild-type GPI8, whereas the C92S mutant had partially restored CD59 expression even in this overexpression experiment. The C139S mutant (MFI/H11005 = 13962) restored the surface expression of GPI-anchored marker proteins on their respective mutant cells (Fig. 3). Wild-type GPI8 restored the surface CD59 expression on class K cells to a level similar to parental K562 cells (data not shown). The C206S mutant had no complementation activity as previously reported because this cysteine is an active site of GPI transamidase (18). The C51S and C275S/C280S mutants restored CD59 expression at levels similar to wild-type GPI8, whereas the C92S mutant only partially restored CD59 expression even in this overexpression experiment (mean fluorescence intensities (MFIs) for wild type, C51S, C275S/C280S, and C92S were 893, 678, 873, and 187, respectively) (see below for a slightly lower MFI for C51S mutant).

We also assessed the activities of PIG-T mutants in PIG-T knockout F9 cells, which express a high level of CD59 on the cell surface when rescued with PIG-T. In stable transfectants, the difference in MFI between wild type (MFI = 1269), the C139S mutant (MFI = 1222), and the C182S mutant (MFI = 935) transfectants was relatively small, probably due to a compensation of low activity inherent to stable overexpression systems. We therefore tested transient expression of PIG-T proteins using the same cells. While wild type (MFI = 1141) and the C139S mutant (MFI = 1042) restored the surface expression of CD59 at comparable levels, the C182S mutant restored it at a significantly lower level (MFI = 434).

The surface expression of GPI-anchored marker proteins is not a direct measure of the rate of GPI modification but rather measures the accumulation of GPI-anchored proteins on the cell surface. Therefore, we used an in vitro GPI transamidase assay for more direct evaluation (Fig. 4). Again the C206S mutant GPI8 showed no transamidase activity. The C275S/C280S mutant processed miniPLAP at a level similar to wild type. C51S had a significantly lower activity, but this might be due to its low expression. When specific activity was calculated by dividing the intensity of the GPI-anchored and free form bands by the GPI8 protein level, the C51S mutant had 95% of the activity of wild type. The C92S mutant was expressed well; however, it had only 17% of the wild-type activity (Fig. 4, left). Similarly the C182S mutant of PIG-T restored only a trace amount of GPI-anchored miniPLAP in both stable and transient expression systems (Fig. 4, right). Thus, the disulfide linkage between GPI8 and PIG-T is important but not essential for normal GPI transamidase activity.

**Endogenous GPI8 and PIG-T Form a Complex**—To further rule out the possibility that disulfide formation is restricted to overexpression experiments and does not reproduce in an in vivo situation, it is important to demonstrate that endogenous GPI8 and PIG-T form an intermolecular disulfide bond. First we tested disulfide bond formation in an in vitro translation system that can reproduce co- and post-translational modifications occurring in the ER. We have previously shown that a trimeric complex of GAA1, PIG-T, and PIG-S is stably formed in the absence of GPI8; thus class K cells might contain this trimeric complex. Microsomal membranes of class K cells were, therefore, expected to process a disulfide bond formation between in vitro translated GPI8 and pre-existing endogenous PIG-T. As shown in Fig. 5, the proform of wild-type GPI8 was converted to the mature form by cleavage of the signal peptide and was able to form a 160-kDa complex when the translation reaction was performed in the absence of dithiothreitol (lane 1). This allowed synthesis of functional proteins with native disulfide bonds such as major histocompatibility complexes within the ER (29). This complex was destroyed when the sample was reduced (lane 5). Under the same conditions, the C92S mutant failed to form this complex (lane 3). Formation of this disulfide was sensitive to the redox status during translation. When switched to reducing conditions by dithiothreitol addition, complex formation with wild-type GPI8 was abolished (lane 2).

Next rabbit polyclonal antibodies were raised against human GPI8 and PIG-T using bacterially expressed recombinant proteins. To confirm antibody specificities we used purified endogenous proteins. Transamidase complexes purified with FG-GPI8 or FG-PIG-T contained endogenous PIG-T or GPI8, respectively, and FG-PIG-S co-precipitated both proteins (Fig. 6A, compare lane 1 and lanes 2 and 3). Western blotting with anti-GPI8 antibodies detected endogenous GPI8 (lanes 4 and 6) as well as FG-GPI8 (lane 5). Similarly anti-PIG-T antibodies reacted with both endogenous PIG-T (lanes 7 and 8) and FG-PIG-T (lane 9). Neither of these antibodies cross-reacted with other transamidase components. With these antibodies, we tested the in vivo formation of a disulfide bond between endogenous GPI8 and PIG-T (Fig. 6B). Anti-GPI8 antibody detected the 160-kDa band in K562 (lane 1) and HeLa cells (lane 3) but not in class K cells (lane 2), consistent with class K cells being mutated for GPI8. Similarly K562 and HeLa cells (lanes 4 and 6) but not class K cells (lane 5) contained the anti-PIG-T-reactive 160-kDa protein complex. In contrast to the experiment with overexpressed FG-GPI8 (see Figs. 1A and 2B), monomeric GPI8 was not detected in those wild-type cells. In addition, neither K562 nor HeLa cells contained monomeric PIG-T. These results suggest that all of the PIG-T and GPI8 are complexed within the wild-type cells. These results demonstrated that endogenous GPI8 and PIG-T are covalently linked within normal cells.

**Trypanosome GPI8 Forms a Similar High Molecular Weight Complex via a Disulfide Bond—Cys76 of TbGPI8 corresponds to Cys92 of human GPI8. TbGPI8 might, therefore, form an intermolecular disulfide bond via Cys76. Expression plasmids for FLAG-tagged wild type and the C76S mutant were transfected into the TbGPI8 knockout procyclic form of T. brucei. TbGPI8 was immunoprecipitated and analyzed by Western blotting with anti-FLAG antibody under non-reducing conditions. As shown in Fig. 7A, the wild-type transfectant generated a 160-kDa band in addition to a very faint 35-kDa band corresponding to a predicted size of monomeric TbGPI8 (lane 2). In contrast, the C76S mutant produced only the 35-kDa band (lane 3). When the samples were reduced, the 160-kDa band disappeared, and only the 35-kDa band was detected in wild-type transfectants (lane 5).

We next examined the surface expression of EP-procyclin, a major GPI-anchored coat protein of the procyclic form of trypanosome (Fig. 7B). The TbGPI8 knockout trypanosome does not express surface procyclin. Wild-type TbGPI8 rescued procyclin expression on 70% of transfectants at a level comparable to that of wild-type cells, whereas only 30% of transfectants were obtained with the C76S mutant. Therefore, TbGPI8 also forms a functionally important disulfide bond with its conserved cysteine residue.

**GPI Transamidase Consisting of the Five Components Binds the Proform of Substrate Proteins**—Because of a lack of a membrane-free assay of GPI transamidase, it is unclear whether five components are sufficient. We predicted that the noncatalytic C206S mutant of GPI8 should terminate the reaction.
immediately after the recognition of GPI attachment signals and that the five-protein complex with C206S GPI8 would be able to hold the substrate protein. A representative result is presented in Fig. 8. When proteins co-purified with FG-tagged wild-type and C206S mutant GPI8 proteins were compared, two additional bands at 97 kDa (closed arrowhead) and 31 kDa

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**Table 1:**

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<tr>
<th>Class K transfectants</th>
<th>PIG-T KO transfectants</th>
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<tr>
<td>Stable</td>
<td>Stable</td>
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<tr>
<td>Empty vector</td>
<td>Empty vector</td>
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<tr>
<td>Wild-type</td>
<td>Wild-type</td>
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<tr>
<td>C51S</td>
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<td>C92S</td>
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<td>C275/280S</td>
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**Fig. 3.** A disulfide bridge between GPI8 and PIG-T is important for the surface expression of GPI-anchored proteins. Surface expression of CD59 on class K cells and PIG-T knockout (KO) CD59 transfectants was measured by flow cytometry. The stable transfectants of class K and PIG-T knockout cells used in Fig. 2, B and C, were used. For the transient transfection of PIG-T knockout cells, cells were transfected with the same plasmids used to establish stable transfectants except for the absence of a phosphoglycerokinase-hygromycin resistance gene and stained 2 days after transfection. Bold and dotted lines indicate anti-CD59 and control staining, respectively.
were observed in the mutant transfectant (lane 2), and only the 97-kDa band was observed in the wild-type transfectant (lane 1). We identified 97- and 31-kDa bands as calnexin and UL16-binding protein 2 (UL16BP2), respectively, by mass spectrometry. UL16BP2 is a GPI-anchored major histocompatibility complex class I-related protein with N-glycan. Calnexin may have been associated with UL16BP2. Amino-terminal sequencing of UL16BP2 showed that the amino-terminal signal peptide had been cleaved off, therefore it had been translocated into the ER lumen as a proform. Most of the UL16BP2 proteins were eluted by a Nonidet P-40 wash from the complex purified with digitonin (lanes 4 and 6), suggesting that interaction of its GPI attachment signal and the transamidase complex is relatively weak. Consistent with this, UL16BP2 was not co-purified in the presence of Nonidet P-40 (lane 8). These results demonstrate that the affinity-purified transamidase complex comprised of five components was sufficient to hold the proform of substrate GPI-anchored proteins.

**FIG. 4.** GPI8 and PIG-T, which are unable to form a disulfide linkage, have decreased transamidase activities in vitro. Transfectants analyzed for the surface expression of CD59 in Fig. 4 were assayed for in vitro transamidase activity. Top, miniPLAP mRNA was translated in vitro using rabbit reticulocyte lysates and microsomal membranes prepared from the indicated transfectants. Radiolabeled miniPLAP proteins were immunoprecipitated with anti-PLAP antibody, electrophoresed, and visualized by autoradiography. Identities of the bands according to Kodukula et al. (25) are shown on the left. Bottom, transfectants analyzed in the upper panel for transamidase activity were evaluated for expression levels of the proteins. Precipitated proteins were electrophoresed under reducing conditions and Western blotted with the indicated antibodies. KO, knockout.

(open arrowhead) were observed in the mutant transfectant (lane 2), and only the 97-kDa band was observed in the wild-type transfectant (lane 1). We identified 97- and 31-kDa bands as calnexin and UL16-binding protein 2 (UL16BP2), respectively, by mass spectrometry. UL16BP2 is a GPI-anchored major histocompatibility complex class I-related protein with N-glycan. Calnexin may have been associated with UL16BP2. Amino-terminal sequencing of UL16BP2 showed that the amino-terminal signal peptide had been cleaved off, therefore it had been translocated into the ER lumen as a proform. Most of the UL16BP2 proteins were eluted by a Nonidet P-40 wash from the complex purified with digitonin (lanes 4 and 6), suggesting that interaction of its GPI attachment signal and the transamidase complex is relatively weak. Consistent with this, UL16BP2 was not co-purified in the presence of Nonidet P-40 (lane 8). These results demonstrate that the affinity-purified transamidase complex comprised of five components was sufficient to hold the proform of substrate GPI-anchored proteins.

**FIG. 5.** In vitro translated GPI8 forms a disulfide-bonded high molecular weight complex in the presence of class K microsome membranes. Wild-type (WT) or mutant (C92S) GPI8 mRNA was translated in rabbit reticulocyte lysate using class K microsomes in the presence (+) or absence (−) of 2 mM dithiothreitol (DTT). After incubation, microsomes were pelleted by ultracentrifugation through a sucrose cushion, solubilized in a sample buffer, and electrophoresed under non-reducing (left) or reducing (right) conditions. Radiolabeled proteins were visualized by autoradiography. An arrowhead indicates a high molecular weight complex. Note that direct treatment of samples in a sample buffer without isolation of microsome membranes gave a similar result (data not shown).

**FIG. 6.** Endogenous GPI8 and PIG-T form an intermolecular disulfide bond. A, the transamidase complex was purified from K562 cells expressing FG-tagged PIG-S (lanes 1, 4, and 7) or PIG-T (lanes 3, 6, and 9) and class K cells expressing FG-tagged GPI8 (lanes 2, 5, and 8) and electrophoresed under reducing conditions. Proteins were visualized by silver staining (left) or Western blotted with anti-GPI8 (middle) or anti-PIG-T (right) antibodies. Identities of bands on a silver-stained gel are indicated on the right. B, whole cell lysates of K562 (2 × 10⁵), class K (4 × 10⁵), and HeLa (2 × 10⁵) cells were electrophoresed under non-reducing conditions and Western blotted with anti-GPI8 (left) or anti-PIG-T (right) antibodies. Asterisks indicate nonspecific bands.
Fig. 7. Trypanosome GPI8 forms a disulfide-bonded protein complex through a conserved cysteine residue. A, the TbGPI8 knockout procyclic trypanosome was transfected with an empty vector (lanes 1 and 4); FLAG-tagged wild-type TbGPI8 (lanes 2 and 5), or its C76S mutant (lanes 3 and 6) and selected by phleomycin. Cell lysates were prepared and subjected to immunoprecipitation with anti-FLAG antibodies plus protein G beads. Immunoprecipitates were electrophoresed under non-reducing (left) or reducing (right) conditions and analyzed by Western blotting with anti-FLAG antibody. A band at about the 50-kDa position is nonspecific. B, surface expression of GPI-anchored procyclin. Transfectants used in A were stained for procyclin and analyzed by flow cytometry. Bold and dotted lines indicate anti-procyclin and control staining, respectively. KO, knockout.

DISCUSSION

Functional Importance of the Disulfide Bond between GPI8 and PIG-T—We found that the majority of GPI8 is linked to PIG-T via a disulfide bond in the GPI transamidase complexes. Formation of a disulfide linkage between GPI8 and PIG-T requires these proteins to be in close proximity and is therefore consistent with our previous result showing that expression of GPI8 was dependent on and stabilized by PIG-T probably through a direct interaction (12).

The disulfide linkage per se is not required for generation of the tetrameric complex because co-precipitation of GAA1 and PIG-S with GPI8 occurred normally with the C182S mutant of PIG-T. In contrast, the disulfide linkage between GPI8 and PIG-T is important for normal activity of the GPI transamidase. We have previously reported that Cys92 of GPI8 is important for its function; an alanine mutant restored the surface expression of GPI-anchored proteins on class K cells to only 10% of the wild-type level (18). We confirmed this using the C92S mutant that restored only 20% of the surface CD59 expression (Fig. 3). A 10% difference in this activity could be due to different expression levels of GPI8 with higher expression levels in stable transfectants than in transient transfectants. This disulfide bond also affects the activity of PIG-T in the GPI transamidase holoenzyme since the C182S mutant PIG-T has a considerably lower activity (35%) in restoring the surface expression of CD59 on the transient transfectants of PIG-T knockout cells (Fig. 3), although it has 75% of the activity in stable transfectants. Moreover, in an in vitro transamidase assay, the C92S GPI8 mutant had 17% of the wild-type activity; transient and stable transfectants of the C182S PIG-T mutant had 16 and 37% of the wild-type activity, respectively (Fig. 4). It seems, therefore, that the complex formed by non-covalent interaction has a very weak activity.

These analyses were performed using overexpressed proteins. To address how covalent and non-covalent association of GPI8 and PIG-T occurs in the endogenous GPI transamidases, we raised antibodies against GPI8 and PIG-T and demonstrated that the majority of GPI8 and PIG-T are covalently linked in two wild-type cell lines (Fig. 6B). Taken together, these data clearly demonstrate that a disulfide bond between GPI8 and PIG-T is formed within normal cells and is critical for full transamidase activity probably through holding the proper position of components and stabilizing the complex.

Trypanosome GPI Transamidase Complex Has an Architecture Analogous to but Different from Mammalian Enzyme—The disulfide bond is formed between Cys92 of GPI8 and Cys182 of PIG-T (Fig. 2, B and C). Both residues are conserved in GPI8 and PIG-T homologues of S. cerevisiae, S. pombe, C. elegans, D. melanogaster, Anopheles gambiae, and Arabidopsis thaliana. In addition, T. brucei, L. mexicana, and Plasmodium falciparum GPI8 contain a cysteine residue corresponding to Cys92. It is, therefore, possible that a similar disulfide bridge is present in the GPI transamidases of those organisms. Indeed
we demonstrated that trypanosome GPI8 formed an intermolecular disulfide bond through this conserved cysteine because the C76S mutant failed to form a high molecular weight complex (Fig. 7A). Although the partner protein was not identified, PIG-T homologue is a likely candidate because its molecular mass seemed to be similar to human PIG-T (43-kDa human and 35-kDa trypanosome GPI8 proteins formed the 180-kDa disulfide-bonded complex). We recently identified a T. brucei homologue of PIG-T.

It has been reported that GPI anchoring ability is lost when the microsomal membranes of T. brucei are washed at high pH (20), whereas a similar treatment of the mammalian microsomal membranes has no effect (30). Moreover, the GPI anchoring can be restored by adding the high pH extract of trypanosomal membranes or recombinant GPI8 protein of the related protozoan L. mexicana to the washed membranes (20). These results suggest that trypanosomal but not mammalian GPI8 is a soluble protein. In fact, T. brucei and L. mexicana GPI8 lacks the transmembrane domain (8, 10, 22). Our results suggest either that TbGPI8 is released from the complex due to a disulfide change that might be caused by protein denaturation at high pH or that endogenous TbGPI8 binds its partner protein mainly non-covalently, whereas transfected TbGPI8 mainly uses covalent binding. Identification of the partner protein in trypanosome would not only resolve this issue but also lead to an understanding of the molecular mechanisms that determine the specificity of GPI attachment signals.

Requirements for GPI attachment signals are different between mammalian cells and parasitic protozoa. The larger size of amino acids at the ω to ω + 2 sites in parasitic protozoa as compared with those in human suggests that parasite GPI transamidase accommodates and tolerates larger amino acids in its catalytic pocket (31). It would, therefore, be possible to design inhibitors that specifically inhibit parasitic transamidase, leading to potent chemotherapeutics for diseases caused by trypanosomes and potentially other protozoan parasites (11). A candidate gene for T. brucei and Leishmania major GAA1 homologues with a similar hydrophobic structure was suggested, but its function was not demonstrated (22, 32).

Molecular cloning of the partner proteins of GPI8 will reveal whether transamidase complexes of those parasitic protozoa have an analogous architecture with similar components.

**GPI Transamidase Comprising Five Components Captures Substrate Proteins**—We found that a proform of UL16BP2 was co-purified with an inactive transamidase complex. The members of the human UL16BP family, consisting of three molecules, are GPI-anchored ligands for natural killer cell receptor NK2D (33). Although it was reported that UL16BP2 is expressed well in K562 cells, the parent line of class K cells, many other proforms of GPI-anchored proteins were not co-purified. There are some explanations for this. (i) Within the cells a number of proforms proteins bound to the complex, but most of them were lost during affinity purification of the complex. (ii) UL16BP2 predominantly occupied the complex already in the ER due to its higher affinity. (iii) UL16BP2 is extremely abundant in this cell line, and while many other proproteins co-purified with the complex, they are below our detection limit.

The interactions of substrate proteins with transamidase components have been reported previously (23, 24). These studies were carried out with *in vitro* translation of model proteins followed by chemical or photo cross-linking, demonstrating that substrate proteins are positioned in close proximity to GPI8. Co-purification of UL16BP2 with the complex rendered inactive by a mutation of the catalytic site cysteine of GPI8 is expectable and is consistent with a prolonged association of substrate proteins with GPI8 when GPI attachment signal was uncleavable or GPI was not available, which is supported by the successful chemical cross-linking of these proteins (24). Our results clearly demonstrate that the complex consisting of five components is sufficient for physical association with the substrate proteins. In at least one case, release of UL16BP2 from the complex by Nonidet P-40 implies that the interaction between GPI attachment signals and the transamidase complex is relatively weak.

**Composition of GPI Transamidase Subunits**—The difference between the molecular mass of GPI transamidase complex determined experimentally in digitonin extract of HeLa cells and S. cerevisiae (about 460 and 430–650 kDa, respectively) (14, 34) and the mass calculated from the five known human components (280 kDa) has several potential explanations: a non-globular shape of the complex, bound detergent, multiple molecules of components, or unidentified components that are lost during affinity purification of the complex. The association of tubulin and the transamidase complex may partially account for this difference (34).

GPI transamidase contains one copy of GAA1 because endogenous GAA1 is not found in the complex containing FLAG-tagged GAA1 (34). Similarly our results using antibodies against GPI8 and PIG-T suggest that one molecule each of GPI8 and PIG-T are contained within this complex because neither endogenous GPI8 nor PIG-T co-purified with FLAG-tagged GPI8 or FG-PIG-T, respectively (Fig. 6A), although the number of molecules for PIG-S and PIG-U remains to be determined in a similar way. Under the conditions used, five components were consistently purified, but variability of the minor bands accompanying the complex was observed between the individual preparations and conditions used (Fig. 8). Purification of the complex under various conditions including various detergents, ionic strength, and so on could identify such weakly associated proteins. Development of the assay for GPI anchoring using a purified transamidase complex will clarify these issues.

**Acknowledgments**—We thank Fumiko Ishii and Keiko Kinoshita for excellent technical assistance.

**REFERENCES**
