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

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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.

Many eukaryotic cell surface proteins are tethered to the plasma membrane via glycosylphosphatidylinositol (GPI). The GPI moiety is synthesized in the endoplasmic reticulum (ER) by the sequential addition of sugars and ethanolamine phosphate to phosphatidylinositol (1, 2). In addition to the amino-terminal signal peptide required for entry into the ER, precursor proteins destined to be GPI-anchored have carboxyl-terminal GPI attachment signals that direct GPI modification. GPI transamidase mediates cleavage of the GPI attachment signal peptide and en bloc transfer of a preassembled GPI to newly exposed carboxyl terminus of precursor proteins. During this transamidation reaction, GPI transamidase forms a carboxyl intermediate with substrate proteins (1).

GPI anchoring is essential for mammalian development and many specific cellular functions (3–6) but not for cell survival itself. In Saccharomyces cerevisiae, GPI is essential for growth (7). The requirement of GPI in protozoan parasites depends on the species and stage of life cycle (8, 9). For example, the bloodstream form of Trypanosoma brucei requires GPI; in contrast the GPI-deficient procyclic form (insect stage) of this parasite is viable but has decreased infectivity toward its vector, tsetse fly (9, 10). Thus selective inhibitors of GPI synthesis could be potent therapeutic drugs for diseases caused by these microorganisms (11), and such drugs could utilize the differential substrate specificity of enzymes mediating GPI synthesis and anchoring. It is, therefore, important to characterize human and parasitic GPI transamidases and investigate molecular mechanisms conferring specificity.

Human GPI transamidase is a complex consisting of at least five components, GAA1, GPI8, PIG-S, PIG-T, and PIG-U (12, 13). S. cerevisiae transamidase is composed of the respective orthologues Gaa1p, Gpi8p, Gpi17p, Gpi16p, and Cde91p (12–14). All these components are essential for the formation of carboxyl intermediates (12, 13, 15). Several lines of evidence indicate that GPI8/Gpi8p are catalytic components. (i) They belong to the C13 cysteine peptidase family (16), one of which, Canavalia ensiformis asparaginyl endopeptidase, catalyzes a transamination reaction in vitro (17). (ii) Mutation of cysteine and histidine residues proposed to form a catalytic dyad resulted in complete loss of function (18, 19) and sensitivity to sulphydryl-alkylating reagents (20, 21). (iii) The recombinant GPI8 of T. brucei cleaved a small peptide-based fluorescent substrate (22). (iv) Finally in vitro translated model substrate proteins can be cross-linked to GPI8 indicating that they are situated in close proximity to each other during the transamidation reaction (23, 24). GPI8 homologues are divided into two groups, type I transmembrane proteins (human, S. cerevisiae, and Schizosaccharomyces pombe) and soluble proteins lacking a corresponding transmembrane region (T. brucei, Leishmania mexicana, Drosophila melanogaster, and Caenorhabditis elegans). A human GPI8 mutant lacking its transmembrane region seemed to be integrated into the complex correctly because it fully rescued GPI8 mutant cells and was co-precipi-
tated with GAA1 (18). We have proposed that PIG-T/Gpi16p has a central role in the formation of the transamidase complex because stable expression of GPI8/Gpi8p exclusively depends on PIG-T/Gpi16p (12, 14).

Here we demonstrate that GPI8 and PIG-T form a disulfide bridge that is required for normal transamidase activity via conserved cysteine residues not only in mammalian cells but also in *T. brucei* and provide direct evidence that a transamidase complex consisting of five components associates with translocated substrate proteins.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—K562, class K (15), PIG-T knockout F9 (12), and HeLa cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Chinese hamster ovary (CHO) cells were cultured in Ham’s F-12 supplemented with 10% fetal calf serum.

**Mammalian Expression Plasmids and Selection of Transfectants**—All expression plasmids were constructed on the pME18Sf vector, a gift from Dr. K. Maruyama (Tokyo Medical and Dental University, Tokyo, Japan). Phosphoglycerokinase-driven puromycin, hygromycin, and blasticidin resistance gene cassettes (18) were cloned into a unique HindIII site of the pME18Sf vector (designated as pMEpuro, pMEhyg, and pMEbsd, respectively) and used to establish stable transfectants. Plasmids for FLAG-GAA1, GST-GPI8, HA-PIG-S, and Myc-PIG-T were used previously (12). The amino- and carboxyl-terminal FLAG and GST tags used were described previously (12) with the addition of a single and triple flexible linker (Gly-Gly-Gly-Gly-Ser) between FLAG and GST as well as between GST and the tagged protein, respectively, to improve the yield of affinity purification. Cysteine-to-serine mutants of GPI8 and PIG-T were made with a QuickChange site-di- rected mutagenesis kit (Stratagene). Sequences of all primers used in this study are available upon request. K562 and class K cells were transfected with pMEpuro-derived plasmids and selected at 2 μg/ml puromycin. PIG-T knockout F9 cells were transfected with pMEbsd-CD59. After selection at 4 μg/ml blasticidin, a clone with high transfection efficiency and high PIG-T/Pig-T-dependent surface expression of CD59 was isolated. This clone was further transfected with pMEhyg-PIG-T plasmids and selected at 500 μg/ml hygromycin.

**Purification of Transamidase Complex and Identification of Associated Proteins**—Affinity purification of the transamidase complex with anti-FLAG beads (Sigma) and glutathione beads (Amersham Biosciences) was performed as reported previously (12) except that SDS-PAGE sample buffer contained 8 M urea to improve denaturation of PIG-U (13) and 10 mM iodoacetamide for non-reducing conditions to prevent disulfide rearrangements. The amino-terminal sequence was determined from Coomasie-stained bands transferred to a polyvinylidenefluoride membrane and analyzed by liquid chromatography and tandem mass spectrometry with MAGIC 2002 (Michrom Bioresources) and Q-ToF2 (Micromass), and identified by Mascot search (Matrix Science).

**Analysis of Protein Complexes**—To analyze the formation of the disulfide bond between GPI8 and PIG-T, cells were pretreated in phosphate-buffered saline containing 20 mM N-ethylmaleimide (NEM) for 30 min on ice and lysed in Nonidet P-40 lysis buffer (20 mM Tris, 140 mM NaCl, 1% Nonidet P40, pH 7.4) containing 20 mM NEM. CHO cells were electrophoresed on a 6% gel each of the immobilized plasmids as reported previously (12). GST-tagged GPI8 was precipitated with glutathione beads. The precipitates were divided into four aliquots and Western blotted with anti-FLAG M2 (Sigma), anti-GST (Amersham Biosciences), anti-Myc (Oncogene Research Products), anti-HA (Roche Molecular Biochemicals), and horse radish peroxidase-conjugated protein G (Amersham Biosciences) antibodies. Western blots of fractions of class K cell lysate transfected with FLAG- and Myc-PIG-T constructs and PIG-T knockout cells transfected with Myc-PIG-T constructs were subjected to precipitation with glutathione beads and anti-Myc antibody plus protein G beads (Amersham Biosciences), respectively. Whole cell lysates were prepared as follows. After treatment with NEM, cell pellets were solubilized in lysis buffer with NEM at 2 × 107 cells/ml and HeLa or 4 × 107 cells/ml for class K cells. Postnuclear lysates were mixed with an equal volume of SDS sample buffer with iodoacetamide.

**In Vitro Translation**—For in vitro translation of human GPI8, 1.2-kb Neo-1-Xho1 fragments of full-length wild-type and C92S mutant GPI8 cDNA were cloned in Neo and XhoI sites of pSPUTK (Stratagene). Plasmids were linearized with EcoRI and used as templates for in vitro transcription. Capped mRNA synthesized using the AmpliScribe SP6 High Yield Transcription Kit (Epititrex) and RNA cap structure analog (New England Biolabs) was in vitro translated using Flexi rabbit reticulocyte lysate (Promega) and class K microsomal membrane for 90 min in the presence or absence of 2 nM dithiothreitol in 25 μl according to the manufacturer’s protocol. After incubation, 20 μl of the reaction mixture was diluted 10-fold with a buffer (50 mM triethanolamine, 250 mM sucrose, 20 mM NEM, pH 7.5), layered on, and sedimented through 200 μl of a cushion (50 mM triethanolamine, 500 mM sucrose, 20 mM NEM, pH 7.5) by ultracentrifugation with a Beckman TL-100.2 rotor at 80,000 rpm for 15 min. Membrane pellets were washed with phosphate-buffered saline containing 20 mM NEM and solubilized in sample buffer with iodoacetamide. To reduce the sample, one-fifth volume of 2-mercaptoethanol was added. For direct analysis by SDS-PAGE, 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 (placental 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 5H8 followed by phycoerythrin-conjugated streptavidin (Biomedics) and analyzed on a FACScaliber (BD Biosciences).

**Polynuclear Antibodies against Human GPI8 and PIG-T Proteins**—We immunized 1-day-old Balb/c mice with affinity-purified, carboxyl-terminally His-tagged human GPI8 (Ser100–Ser164) and human PIG-T (Ile308–Leu379). Human GPI8 (Ser26–Ser26) and human PIG-T (Leu22–Leu279), respectively, were used as mouse-specific 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 Protein 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 (TbGPI8) gene in procyclic form of *T. brucei* as described previously (9). An epistomal plasmid, pTMCszeo, was constructed by replacing the neo/zeocin drug resistance gene with an expression cassette carrying a multiple cloning site (nine restriction enzyme sites) flanked by aldolase splice acceptor signal and polyadenylation signal into a unique *Sma*I site of *T. brucei* (26). A 1.1-kb fragment encoding TAG-tagged wild-type or C76S mutant TbGPI8 was amplified by PCR and cloned into *Kpn*I and *Bam*III sites of *T. brucei* TbGPI8 knockout precursors were transfected with the resulting plasmids by electroporation and selected at 2–10 μg/ml phleomycin. Transfectants were stained with monoclonal anti-EP-procyclin (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 a 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. 1). 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 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. 1C). 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 Cys89 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 Cys89 of GPI8 is used for the disulfide bond.

Cys139 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 C139S mutant formed the covalent complex (Fig. 2C). Therefore, Cys89 of GPI8 and Cys182 of PIG-T are involved in formation of the disulfide bond. Notably the disulfide linkage is not essential for
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 when 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 activities 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 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, 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 procyclin 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 procyclin 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
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 microsome 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. Asterisks indicate nonspecific bands.

**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.
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 procyclin (18). 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 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 160-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 exchange that might be caused by protein denaturation at high pH or that endogenous TbGPI8 binds its partner protein mainly co-新たに，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 or 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 NKG2D (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 proform 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 uncleanable 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. 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 FG-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 variable 

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