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Proprotein Interaction With the GPI Transamidase

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Abstract For characterizing how the glycosylphosphatidylinositol (GPI) transamidase complex functions, we exploited a two-step *miniPLAP* (placental alkaline phosphatase) in vitro translation system. With this system, rough microsomal membranes (RM) containing either [^{35}S]-labeled Gaa1p or epitope-tagged Gpi8p, alternative components of the enzymatic complex, were first prepared. In a second translation, unmodified or mutant *miniPLAP* mRNA was used such that [^{35}S]-labeled native or variant miniPLAP nascent protein was introduced. Following this, the RM were solubilized and anti-PLAP or anti-epitope immunoprecipitates were analyzed. With transamidase competent HeLa cell RM, anti-PLAP or anti-epitope antibody coprecipitated both Gaa1p and Gpi8p consistent with the assembly of the proprotein into a Gaa1p:Gpi8p-containing complex. When RM from K562 mutant K cells which lack Gpi8p were used, anti-PLAP antibody coprecipitated Gaa1p. The proprotein coprecipitation of Gaa1p increased with a nonpermissive GPI anchor addition (ω) site. In contrast, if a miniPLAP mutant devoid of its C-terminal signal was used, no coprecipitation occurred. During the transamidation reaction, a transient high Mr band forms. To definitively characterize this product, RM from K cells transfected with *FLAG*-tagged *GPI8* were employed. Western blots of anti-FLAG bead isolates of solubilized RM from the cells showed that the high Mr band corresponded to Gpi8p covalently bound to miniPLAP. Loss of the band following hydrazinolysis demonstrated that the two components were associated in a thioester linkage. The data indicate that recognition of the proprotein involves Gaa1p, that the interaction with the complex does not depend on a permissive ω site, and that Gpi8p forms a thioester intermediate with the proprotein. The method could be useful for rapid analysis of nascent protein interactions with transamidase components, and possibly for helping to prepare a functional in vitro transamidase system. J. Cell. Biochem. 88: 1025–1037, 2003.

Key words: GPI anchor; transamidase; *GPI8*; *GAA1*; miniplap; thioester

Posttranslational addition of glycosylphosphatidylinositol (GPI) moieties is a ubiquitous mechanism for anchoring cell surface proteins to the plasma membrane [reviewed in Tiede et al., 1999]. It is utilized by all eukaryotic cells for tethering molecules with wide-ranging functions. GPI moieties are built upon phosphatidylinositol in glycosidic linkage to a common core glycan consisting of glucosamine, three mannose residues and a terminal phosphoethanolamine (p-EthN). They are preassembled in the endoplasmic reticulum by stepwise addition of the three core glycan constituents to the inositol-phospholipid.

The final step in GPI anchor processing is the substitution of preassembled GPI donor moieties for hydrophobic C-terminal signal sequences in nascent proproteins [reviewed in Kodukula et al., 1995; Sevlever et al., 2000]. The substitution occurs at a residue termed the ω site which can be one of six small amino acids located 15–30 residues upstream of the C-terminus [Moran et al., 1991; Gerber et al., 1992]. Previous studies have shown that the transfer process consists of a transamidation reaction [Maxwell et al., 1995a] in which the small nucleophiles hydrazine (HDZ) or hydroxylamine (HDX) can substitute for the free amino group of the terminal p-EthN in GPI donors.

Two components of the transamidation machinery that were first characterized are Gaa1p and Gpi8p. In both cases, this initially was

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accomplished by complementation of yeast mutant lines termed *gaa-1* [Hamburger et al., 1995] and *gpi-8* [Benghezal et al., 1995] that accumulated fully assembled GPI donors. The mammalian homologs of the affected genes, i.e., *GAA1* and *GPI8*, subsequently were isolated by (1) 5′ extension of a sequence homologous to the latter (*GPI8*) and use of the sequence to rescue a human K562 cell line designated mutant K [Chen et al., 1996; Yu et al., 1997] with a similar phenotype, and by (2) cloning a gene with sequence homology to the former (*GAA1*) and analyzing the effect of the anti-sense sequence in 3T3 cells [Inoue et al., 1999; Hiroi et al., 2000]. Human (h) Gaa1p is a 621 amino acid long protein that spans the ER membrane six times in its C-terminal region and has a large 328 amino acid—long lumenal domain with two N-linked glycans and a short cytoplasmic oriented N-terminus. [Hamburger et al., 1995; Hiroi et al., 2000]. hGpi8p is a 395 amino acid long type 1 ER protein with a large lumenal domain and a short C-terminal cytoplasmic sequence [Benghezal et al., 1995]. Gpi8p has significant sequence homology with a family of cysteine proteinases including their active site residues, and thus has been proposed to contain the enzymatic site for transamidation.

Although information on the structural organization of the transamidase is beginning to emerge (see Discussion), how it interacts with its proprotein substrates is as yet not understood. Moreover, no direct experimental data are available on how GPI moieties are substituted for C-terminal signal sequences of the proproteins.

The purpose of the present study was to utilize an in vitro translation system to demonstrate directly whether Gaa1p and Gpi8p of the transamidase machinery are involved in interaction with nascent proproteins, investigate the effects of altering the ω site amino acid or the ensuing C-terminal signal sequence, and formally establish whether Gpi8p contains the enzymatic activity and, if so, how it functions.

MATERIALS AND METHODS

Reagents and Cells

The coupled transcription and translation (TNT) kit was obtained from Promega, Inc. (Madison, WI). Anti-FLAG mAB M2, biotinylated M2 mAB, and anti-FLAG M2-coupled agarose beads were purchased from Sigma Chemicals (St. Louis, MO). Horseradish peroxidase (HRP) coupled to streptavidin was bought from Gibco Life Technologies (Grand Island, NY). Anti-PLAP antibodies, rabbit reticulocyte lysate, [^{35}S] M, T7 polymerase, and HDZ were obtained as previously described [Chen et al., 1996].

HeLa cells were obtained from the American Type Culture Collection (ATCC). The K562 K mutant cell line [Mohney et al., 1994; Chen et al., 1996; Yu et al., 1997] was derived as previously described. Each cell line was grown as reported [Mohney et al., 1994; Yu et al., 1997]. Ribophorin cDNA [Harnik-Ort et al., 1987; Fu and Kreibich, 2000] was a gift of Dr. G. Kreibich (New York Univ., NY) and *ER3* and *ER4* [Miyawaki et al., 1997; Miyawaki et al., 1999] cameleon cDNAs (calmodulin linked to green and blue fluorescent proteins) were gifts of Dr. C. Distelhorst (Case Western Reserve Univ., Cleveland, OH).

Constructs

hGAA1 [cloned into the EcoR1 site of pcDNA3 (Invitrogen, Inc., Carlsbad, CA)] was prepared as described [Hiroi et al., 2000]. Chimeric *hGPI8·FLAG* cDNA was prepared by PCR of *hGPI8* sequence in *hGPI8*/pcDNA3 [Yu et al., 1997] with 5′ and 3′ primers containing *Hind*III and *Xho*1 sites. Following this, the digested product was ligated into the *Hind*III and *Xho*1 sites of pFLAG·CTC [*E. coli* carboxy terminal FLAG expression kit (Sigma Chemicals, St. Louis, MO)]. The *hGPI8·FLAG* chimeric cDNA in pFLAG·CTC then was amplified with 5′ and 3′ primers containing *Xho*1 sites and the restricted product cartridged into the *Xho*1 site of pcDNA3. *miniPLAP*, the *miniPLAP* ω mutant W, and the *miniPLAP* ω + 1 stop construct in pGEM3Z were prepared as previously described [Kodukula et al., 1991; Gerber et al., 1992]. *miniPLAP·GAS1* chimeric cDNA was prepared by PCR amplification of nucleotides 1920-2131 (encompassing 161 bp of the *GAS1* 3′ end-coding sequence) of *GAS1* cDNA in pGOZ [Nuoffer et al., 1991]. For this purpose, 5′ and 3′ primers containing *Nae*1 and *Sal*1 sites were used. After digestion, the amplified product was cartridged into the *miniPLAP Nae*1 site and pGEM4Z *Sal*1 site of *miniPLAP*/pGEM4Z.

Transfectants

The *hGPI8·FLAG*/K cell transfectant was prepared by electroporation using previously

described methods [Yu et al., 1997]. *hGPI8·FLAG*/pcDNA3 (50 mg) linearized by *Xho*1 digestion was electroporated into 10^7 mutant K cells in 0.8 ml of HEPES-buffered saline. Transfected cells were cultured for 24 h in complete RPMI 1640 medium and G418 (0.8 mg/μl) was then added. Following selection, surviving neomycin (Neo) resistant cells were labeled with fluorescein isothiocyanate (FITC)-coupled murine IA10 anti-decay accelerating factor (DAF) and phycoerythrin (PE)-coupled murine H19 anti-CD59 antibodies (Pharmingen Inc., La Jolla, CA). The doubly-labeled cells were selected by three cycles of sorting using a Beckman Coulter Elite cell sorter (Beckman, Coulter, Inc.).

In Vitro Translations

Rough microsomal membranes (RM) were prepared as previously described [Kodukula et al., 1992b]. Briefly, pelleted and washed cells, resuspended in 10 mM triethanolamine, pH 7.5 (TEA)/250 mM sucrose, were disrupted for 90 min in a N_2 cavitation bomb charged to 1,200–1,500 psi. The supernatant of the low speed pellet of the resulting cell lysate was recentrifuged for 90 min at 78,000*g* in a Ti70 rotor. The RM-containing pellet was resuspended in 250 mM sucrose, 50 mM TEA, pH 7.5 and stored at 70°C.

The two-step microsomal processing reactions were carried out as follows: In the first step, coupled translation and transcription was performed at 30°C for 30 min using the TNT kit with 0.5 mg of either *hGAA1*/pcDNA3 or *hGPI8-FLAG*/pcDNA3 cDNA in conjunction with T7 polymerase and 2.5 ml of RM from a 50 OD_{280} stock. Following this, the RM were pelleted through sucrose cushions, and the pellets resuspended to three times their original volume in 40 mM HEPES, 350 mM potassium acetate, 2 mM DTT, 1 mM magnesium acetate, pH 7.4 (buffer B) containing 50 mM EDTA. The suspension was then incubated on ice for 15 min. After spinning through sucrose a second time, the RM were rinsed and suspended in buffer B back to their original volume. For the second step, *miniPLAP* mRNA was prepared by priming *Hind*III-linearized pGEM4Z with SP6 RNA polymerase and translating for 90 min at 30°C with rabbit reticulocyte lysate. The mRNA then was incubated with the preloaded and EDTA-treated RM in standard fashion as described [Kodukula et al., 1992b].

Immunoprecipitation/PAGE Gels

For component association studies, membranes were lysed with 1% Deoxy big CHAP detergent (Calbiochem, La Jolla, CA) in buffer B. Immunoprecipitations were performed with rabbit anti-PLAP polyclonal antibody (Accurate Biochemicals) or with murine anti-FLAG M2 mAB followed by protein A- or protein C-Sepharose 4B, respectively (Pharmacia, Uppsala, Sweden). Products were eluted with 1% SDS buffer, 2.5% mercaptoethanol, separated on 15% SDS–PAGE gels and the gels analyzed by autoradiography or phosphorimaging.

Immunoisolation/Western Blotting

For analyses of the high Mr PLAP product, twenty aliquots of 200 ng of *miniPLAP·DAF* [Chen et al., 2001] mRNA (see Results) were individually incubated with 4 μl (50 OD_{280}) of RM from *GPI8·FLAG* / K cell transfectants (prepared in the presence of unlabeled M) and the products combined. After lysis under standard conditions, the lysate was incubated overnight at 4°C with 400 μl of anti-FLAG M2 agarose beads in buffer B, and following three washes with the same buffer, the beads were eluted with nonreducing SDS–PAGE sample buffer. Replicate sets of the eluate and controls were separated on nonreducing 15% gels. The proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA), and the blots blocked with 5% bovine serum albumin (BSA) overnight. The blots were cut in the middle, and the left half of the blot incubated with biotinylated anti-FLAG M2 mAB followed by streptavidin-horseradish peroxidase (HRP). The right half was incubated with rabbit anti-PLAP polyclonal antibody followed by HRP-conjugated goat anti-rabbit Ig (DAKO, Inc., Carpenteria, CA). The blots were then developed with enhanced chemiluminescence (ECL) reagent.

Hydrazinolysis

Following the *miniPLAP·DAF* translations in RM from*GPI8·FLAG*/K cell transfectants and immunoisolation of the products with anti-FLAG M2 agarose beads as described above, the beads were eluted with 50 mM Tris-HCl, pH 7.5 containing 4% SDS. The eluate was incubated for 4 h at 37°C alternatively with 500 mM HDZ or 500 mM NaCl in the same buffer. The products were concentrated with Ultrafree 0.5 ml centrifugal filters (Millipore,

Bedford, MA). Proteins in the concentrates were then separated on 15% SDS–PAGE gels, and the resolved proteins transferred to Immobilon-P membranes (Millipore). After blocking as above, the blots were revealed with anti-PLAP polyclonal antibody followed by HRP-conjugated goat anti-rabbit Ig and ECL reagent.

RESULTS

The miniPLAP in vitro translation system has served as a valuable tool for studying the terminal GPI transfer step in the biosynthesis of GPI-anchored proteins. [Kodukula et al., 1991, 1992a; Gerber et al., 1992; Udenfriend and Kodukula, 1995; Maxwell et al., 1995a; Chen et al., 1996]. It employs a minigene prepared by replacement of the central 305 amino acids of native placental alkaline phosphatase (PLAP) encompassing its glycosylation sites with a short M-rich stretch of nine residues to enhance [^{35}S]-M incorporation and eliminate molecular weight changes due to glycosylation [Kodukula et al., 1991]. In this system, N-terminal processing of 28 kDa preprominiPLAP, the primary translation product, generates 27 kDa prominiPLAP. Subsequent C-terminal processing of this proprotein yields mature 24.7 kDa GPI-anchored miniPLAP. Low level spontaneous hydrolysis or experimental aminolysis with HDZ or HDX yields an alternative 23 kDa product. We used this methodology in conjunction with cDNAs encoding the above-described two components of the GPI transfer machinery and a transamidase-deficient mutant cell line to investigate how proproteins that undergo GPI-anchoring interact with the GPI transferase.

In preliminary experiments carried out to validate the experimental system, we sequentially translated *GAA1* or *GPI8* cDNA followed by *miniPLAP* mRNA (see Materials and Methods) and examined the microsomal products directly, i.e., without detergent extraction and subsequent immunoprecipitation. As seen in Figure 1 (panel A), a larger glycosylated Gaa1p product was generated indicative of its normal

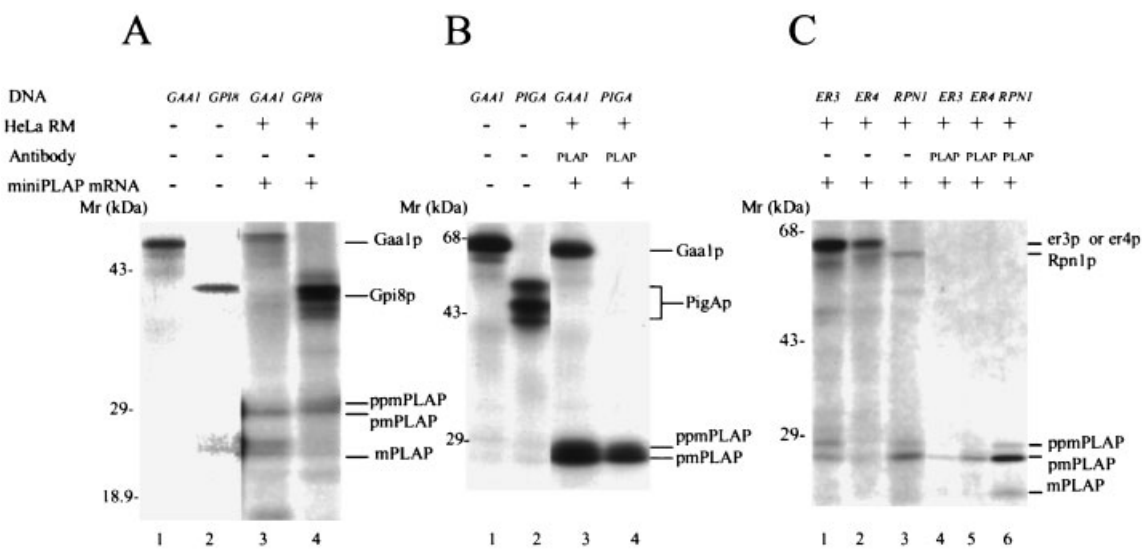

Fig. 1. Overall Experimental System. RM preloaded with [^{35}S]-labeled Gaa1p or epitope-tagged Gpi8p were prepared using the respective cDNAs and the TNT transcription and translation system. Following pelleting and 10 mM EDTA-stripping, the preloaded RM were resuspended in fresh buffer with [^{35}S]M, and *miniPLAP* mRNA was translated. The RM were solubilized with 1% Deoxy big CHAP and products were immunoprecipitated with anti-PLAP or anti-epitope antibody and analyzed on 15% SDS–PAGE gels. **Panel A**: Gaa1p and Gpi8p products generated by themselves or in conjunction with miniPLAP in HeLa cell RM prior to extraction and immunoprecipitation. As seen, Gaa1p was appropriately glycosylated (compare **lane 1** and **lane 3**), Gpi8p N-terminally processed (compare **lane 2** and **lane 4**), and 28 kDa preprominiPLAP (ppmPLAP) and 27 kDa prominiPLAP (mPLAP) (lanes 3, 4) were converted to 24.7 kDa GPI-anchored miniPLAP (mPLAP). The intensities of Gaa1p and Gpi8p bands in different experiments varied (see text). In studies with microsomes in which double translations were done, four volumes of reaction mixtures were used. **Panel B**: Gaa1p or control PigAp microsomal products generated by themselves with Deoxy big CHAP solubilization but without immunoprecipitation (**lanes 1**, **2**) and generated in conjunction with miniPLAP and Deoxy big CHAP solubilization followed by anti-PLAP immunoprecipitation (**lanes 3**, **4**). As seen, the anti-PLAP antibody coprecipitated Gaa1p but not PigAp when *PIGA* cDNA was used as a control. **Panel C**: Additional controls of ribophorin (Rpn1p) or ER cameleon er3p and er4p [generated with their cDNAs (*RPN1*, *ER3* or *ER4*)], in conjunction with *miniPLAP* mRNA and Deoxy big CHAP solubilization without immunoprecipitation (**lanes 1–3**) or with immunoprecipitation (**lanes 4–6**).

N-glycan processing, whereas a Gpi8p product slightly smaller in size than the Gpi8p translation product was produced, consistent with the cleavage of its N-terminal signal peptide (47 AA) and lack of glycosylation sites. In both cases, step-wise conversion of 28 kDa preprominiPLAP to 27 kDa prominiPLAP and then to 24.7 kDa GPI-anchored miniPLAP occurred in accordance with miniPLAP's normal N- and C-terminal processing. Different than usually observed, however, the efficiency of formation of 24.7 kDa GPI-anchored miniPLAP was lower than normally achieved in single step cotranslational assays with untreated HeLa RM (see [Kodukula et al., 1992a] and this study, Fig. 2, panel B) and varied in different experiments (Fig. 1, panel A, lane 3 vs. lane 4). This lesser efficiency is consistent with the two-step protocol in which the *miniPLAP* translation constitutes the second RM reaction using EDTA-washed RM.

Studies next were done with the inclusion of detergent (Deoxy big CHAP) extraction of the RM, followed by immunoprecipitation of solubilized products with anti-PLAP antibodies. As seen in Figure 1 (panel B), the anti-PLAP antibodies coimmunoprecipitated [^{35}S]-labeled Gaa1p but not [^{35}S]-labeled phosphatidylinositol glycan A protein (PigAp) which was identically preloaded in the RM as an ER protein control. As additional controls, three other ER proteins were used. As observed with PigAp, the anti-PLAP antibodies also failed to coimmunoprecipitate preloaded ribophorin, a lumenally-oriented multiple ER membrane-spanning protein, and er3p and er4p, two additional lumenal cameleon ER proteins [Miyawaki et al., 1997, 1999] (Fig. 1, panel C), confirming the specificity of the prominiPLAP interaction. Comparable results were obtained in three independent experiments. In accordance with multiple previous studies in which specific recognition of PLAP has been achieved with the same anti-PLAP antibody [Kodukula et al., 1991, 1992a, 1993; Amthauer et al., 1992; Vidugiriene and Menon, 1995; Maxwell et al., 1995a,b; Ramalingam et al., 1996; Chen et al., 1998; Vidugiriene et al., 1999], studies performed in the absence of *miniPLAP* translation yielded no bands, and the anti-PLAP antibody did not recognize Gaa1p on Western blots in other experiments (see Fig. 5, below) verifying that the recovery of Gaa1p depended on its interaction with prominiPLAP.

Comparative immunoprecipitation experiments next were carried out with RM preloaded with [^{35}S]-Gaa1p or [^{35}S]-Gpi8p. For these analyses, we took advantage of the fact that the *GPI8* construct we used was prepared so as to yield Gpi8p with a FLAG epitope at its C-terminus. As seen in Figure 2 (panel A), both components were coprecipitated by anti-PLAP

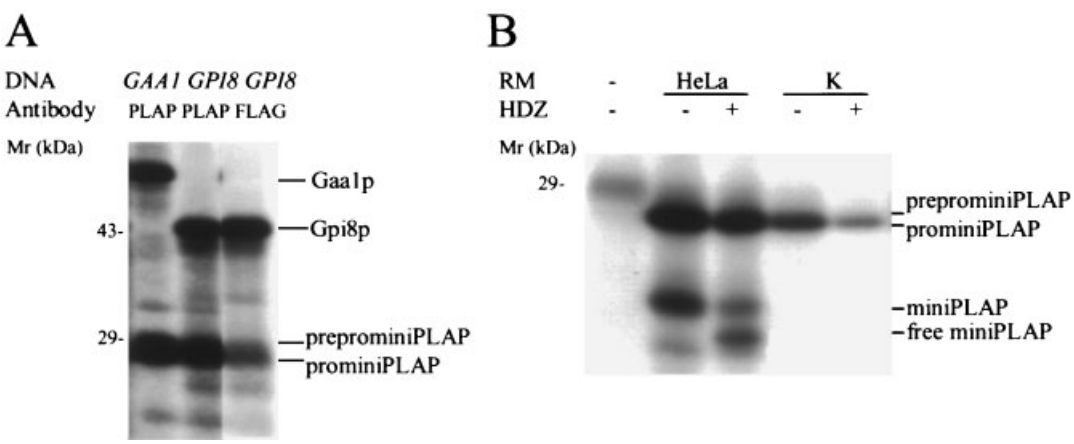

Fig. 2. Panel A: Comparative studies of the interaction of miniPLAP with Gaa1p and Gpi8p. HeLa cell RM were preloaded with [^{35}S]-Gaa1p or [^{35}S]-Gpi8p, the RM stripped, and miniPLAP mRNA then translated as in the legend of Figure 1. When RM from Gpi8p-deficient K cells reconstituted with FLAG-tagged Gpi8p and anti-FLAG antibody were used, similar coimmunoprecipitation occurred. Both Gaa1p and Gpi8p coprecipitated with miniPLAP upon addition of anti-PLAP antibody. **Panel B**: RM from K cells deficient in Gpi8p were verified prior to determination of which component interacts with the nascent miniPLAP proprotein. The products generated following translation of *miniPLAP* mRNA in the presence of (1) HeLa cell RM, or (2) K cell RM without and with added HDZ are shown. K cell RM deficient in Gpi8p were unable to C-terminally process prominiPLAP in the absence or presence of HDZ, verifying that they lacked the respective transamidase component.

antibody. Similar coimmunoprecipitation of Gpi8p occurred if anti-FLAG antibody was used in place of anti-PLAP antibody. In the latter case, less miniPLAP was recovered, presumably due to the fact that prominiPLAP which was constantly being generated was in excess. Consequently not all of the protein was associated with FLAG-Gpi8p.

To distinguish which of the two components interacts with nascent miniPLAP proprotein, studies with our previously described Gpi8p-deficient K562 mutant K line [Chen et al., 1996] lacking the entire length of Gpi8p (see Discussion) [Yu et al., 1997] were undertaken. As previously reported [Chen et al., 1996] validation that the cell in fact is transamidase-defective is shown in Figure 2 (panel B). With HeLa cell RM, processing of prominiPLAP in the absence and presence of HDZ generated large amounts of 24.7 kDa mature GPI-anchored miniPLAP and small amounts of the 23.0 kDa hydrazide, and vise versa, respectively. In contrast, with RM from Gpi8p-deficient mutant K cells, neither product was generated, and consequently, only prominiPLAP was produced.

Using the RM preparation selectively deficient in Gpi8p, sequential translations of *GAA1* and *miniPLAP* followed by immunoprecipitation with anti-PLAP antibody next were repeated. As seen in Figure 3 (panel A), when the Gpi8p-deficient K cell RM preloaded with [^{35}S]-Gaa1p were used and miniPLAP introduced, anti-PLAP antibodies coimmunoprecipitated Gaa1p. Since only Gpi8p sequence corresponding to its cleaved N-terminal signal (see Discussion) is encoded in K mutant cells, the coimmunoprecipitation is indicative of interaction of the proprotein with the Gaa1p-containing transamidase. To establish how the prominiPLAP substrate relates to the two components when both are present, we exploited RM from Gpi8p-deficient K cell mutants reconstituted with FLAG-tagged Gpi8p. The cells were preloaded with Gaa1p and secondarily loaded with prominiPLAP by translating miniPLAP mRNA in standard fashion (as described in the Materials and Methods). The RM then were solubilized with Deoxy big CHAP detergent, and the extract added to agarose beads conjugated with anti-FLAG mAB. Following washing and elution with SDS, the eluate was analyzed on SDS–PAGE gels. As seen in Figure 3 (panel B), both Gaa1p and prominiPLAP were recovered. The results thus in-

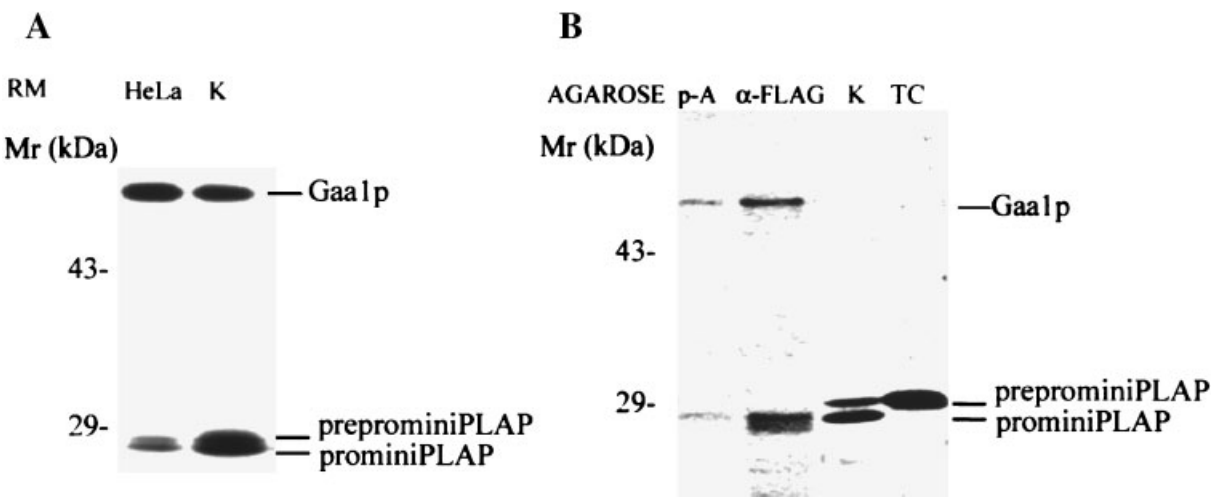

Fig. 3. N-terminally processed prominiPLAP binds to Gaa1p. Gaa1p was preloaded into K cell RM using its cDNA and the TNT system. After washing and resuspension of the RM (see Materials and Methods), *miniPLAP* mRNA was then translated, the RM solubilized, and anti-PLAP immunoprecipitates analyzed. **Panel A**: When K cell RM deficient in Gpi8p were used, coimmunoprecipitation of Gaa1p was observed. **Panel B**: The nascent polypeptide, Gaa1p, and Gpi8p exist in a substrate enzyme complex. Gaa1p was preloaded into RM of K cells reconstituted with FLAG-Gpi8p. Deoxy big CHAP extracts of the RM were incubated with agarose beads conjugated to anti-FLAG mAB or unconjugated agarose control. After elution with SDS, proteins were analyzed on gels. Bands corresponding in size to those of Gaa1p and prominiPLAP were obtained from the anti-FLAG-conjugated beads. Translation controls (TC) for preprominiPLAP and processing controls for N-terminally processed prominiPLAP (K cells) routinely included as markers are shown.

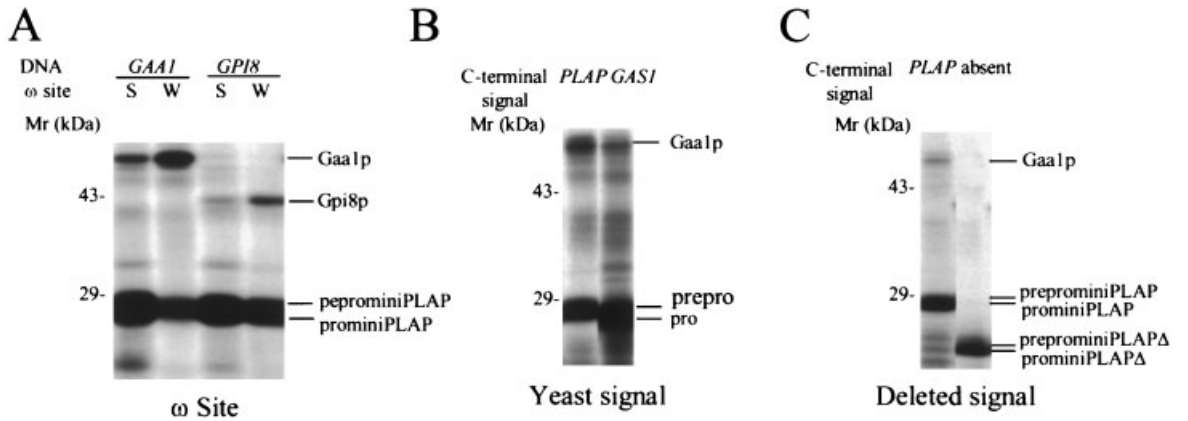

Fig. 4. Effects of varying the ω site amino acid and the ensuing C-terminal signal sequence. HeLa cell RM were preloaded with Gaa1p, and *miniPLAP* mRNA translated in the presence of the EDTA-washed RM as in the legend of Figure 1. **Panel A**: When a mutant *miniPLAP* mRNA encoding a nonpermissive ω site (W) was utilized, an increase in coimmunoprecipitation of Gaa1p and Gpi8p was observed. **Panel B**: When a mutant *miniPLAP* mRNA in which the yeast *GAS1* C-terminal signal sequence was substituted for the *miniPLAP* signal sequence, interaction with Gaa1p was not abolished. **Panel C**: In contrast, when miniPLAP's C-terminal signal was deleted, the binding to Gaa1p was totally abolished. The differences in Gaa1p coprecipitation between panel A in this set of experiments and in Panel A of Figure 2 reflects variability in *GAA1* translation efficiency, but equivalent results were obtained in two additional repeat experiments.

dicated that prominiPLAP associates with both Gaa1p and Gpi8p in a substrate enzyme complex.

To define the requirements for nascent proprotein binding to the complex, we next employed *miniPLAP* cDNA mutants and HeLa RM. As shown in Figure 4 (panel A), when a nonpermissive ω site mutant [Gerber et al., 1992] was utilized, coprecipitation of both Gaa1p and Gpi8p was retained denoting a lack of the requirement of a cleavable ω site for the binding. An increase in coprecipitation was observed, presumably reflective of ineffective subsequent processing of the proprotein by Gpi8p (see below and Discussion). When the signal sequence of yeast GPI-anchored Gas1p surface protein was substituted for that in miniPLAP (Fig. 4, panel B), coprecipitation still occurred. In this case, less coprecipitation was observed possibly due to diminished avidity of the yeast sequence for the hGaa1p/hGpi8p complex. Finally, as seen in Figure 4 (panel C), when the C-terminal signal of miniPLAP was deleted by placing a stop codon at the $\omega+1$ amino acid residue, coimmunoprecipitation was abolished indicative of an essential role of the proprotein's extension peptide for binding to the Gaa1p/Gpi8p complex.

In previous work with the miniPLAP system [Chen and Medof, unpublished], a high molecular weight band has been observed in anti-PLAP precipitates. The band has been noted most prominently (1) with *miniPLAP* in which its ω site amino acid (D) has been replaced by an S residue more efficient in supporting transfer [Gerber et al., 1992] (Fig. 5, panel A, lane 2) and (2) with RM from cells that are most active in the GPI transfer reaction (Fig. 5, panel A, lane 4). It has also been prominently noted in other studies with engineered miniPLAP chimeric constructs [Chen et al., 2001] in which the sequence encoding PLAP's C-terminal signal has been replaced with other C-terminal signal sequences, e.g., that of the decay accelerating factor (DAF) (Fig. 5, panel A, compare lanes 4 and 5) which function more efficiently in conferring GPI-transfer.

Lastly, we performed experiments with RM from Gpi8p-defective K cells transfected with FLAG-tagged Gpi8p to ascertain the nature of the high molecular weight band and determine if it corresponds to the formation of a miniPLAP·Gpi8p reaction intermediate. As seen in Figure 5 (panel B), in accordance with the above results, translation/transcription of *GAA1* cDNA with ([^{35}S]M) followed by immunoprecipitation with anti-FLAG antibodies yielded [^{35}S]Gaa1p consistent with the coexistence of the two transamidase components in a preexisting complex as previously reported [Ohishi et al., 2000]. As a control, no [^{35}S]PigAp was precipitated [despite the production of roughly equivalent amounts of protein (see Fig. 1, panel B)]. Translation of *miniPLAP* mRNA next was carried out followed by immunoisolation of the FLAG-tagged Gpi8p-containing complex with

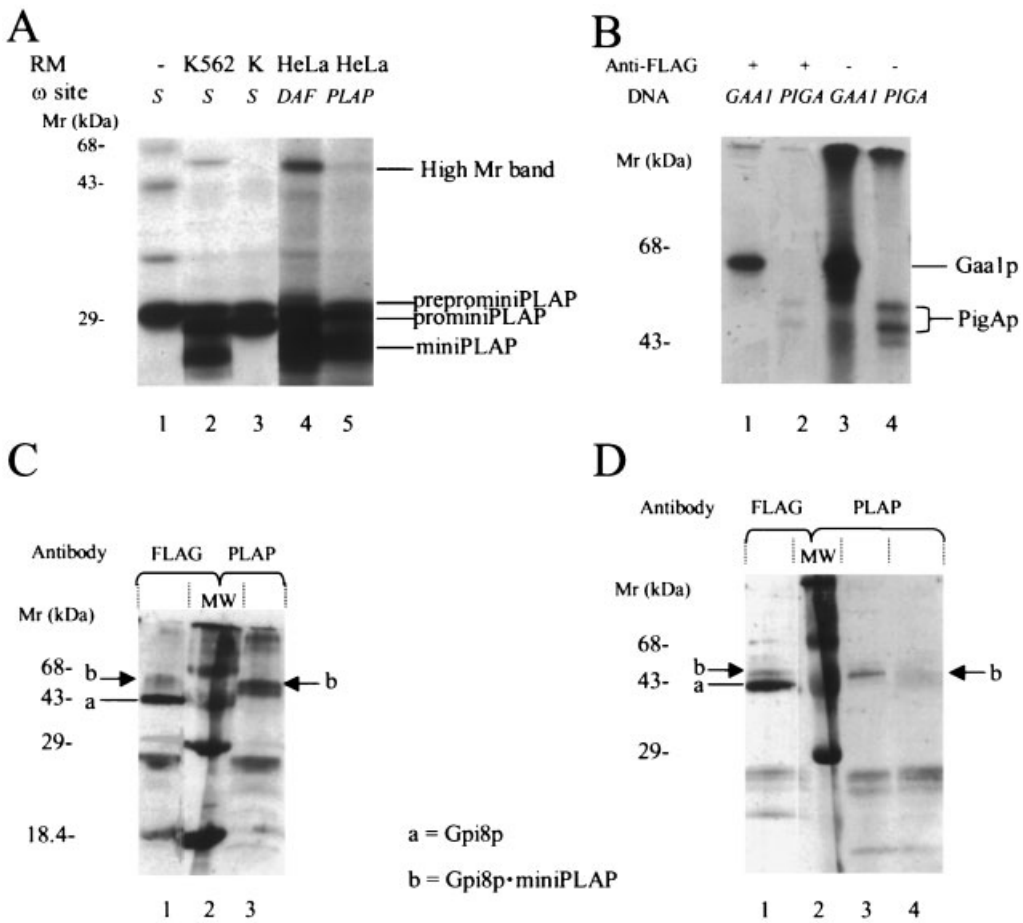

Fig. 5. In all studies, mRNA was translated with RM for 90 min in standard cotranslational assays as previously described [Gerber et al., 1992; Chen et al., 1996]. **Panel A**: High Mr band sometimes seen in in vitro *miniPLAP* translations. **Lanes 1–3**: Translations of *miniPLAP* in which the ω site has been changed to serine in the (1) absence of RM, (2) presence of transamidase-competent K562 RM, and (3) presence of Gpi8p-defective K cell RM. Note the high Mr band generated with K562 cell RM but not K cell RM. **Lanes 4** and **5**: Translation of chimeric *miniPLAP·DAF* [Chen et al., 2001] and *miniPLAP* with its native ω site (D) in the presence of HeLa cell RM which are efficient in transamidation. Note the high Mr band with *miniPLAP·DAF*. **Panel B**: Gaa1p and Gpi8p pre-exist in an enzyme complex. Co-immunoprecipitation of Gaa1p by anti-FLAG antibody from RM of K cells reconstituted with FLAG-tagged Gpi8p. **Lanes 1–2**: Anti-FLAG immunoprecipitates of RM following translation of (1) *GAA1* mRNA, or (2) *PIGA* mRNA. **Lanes 3**, **4**: Nonimmunoprecipitated, (3) *GAA1*, (4) *PIGA* control. **Panel C**: Western blot of protein in eluates of anti-FLAG beads following processing of miniPLAP in *GPI8-FLAG* transfected mutant K cells. **Lane 1** and left half of **lane 2**, blots revealed with biotin-labeled anti-FLAG M2 mAB and streptavidin HRP; right half of **lane 2** and **lane 3**, blot revealed with rabbit anti-PLAP antibody and HRP-conjugated goat anti-rabbit Ig. Two Gpi8p bands are seen (a and b), the higher band (b) containing miniPLAP. The intensity of band b with anti-PLAP antibody is lower presumably because of the transient nature of the transamidation reaction. The overall lesser intensity of the bands as compared to those in panel B presumably reflects the more complex immuno-isolation procedure as well as incomplete recovery at the anti-FLAG bead binding and/or elution steps. **Panel D**: Western blot analyses as in **panel C**. **Lane 1** and left half of **lane 2**, blot revealed with anti-FLAG antibody; right half of lane 2, **lane 3**, and **lane 4**, blot revealed with anti-PLAP antibody. Treatment with HDZ (lane 4) removed band b whereas treatment with NaCl control (lane 3) had no effect. Densitometry showed that the decrease with HDZ was >80%.

anti-FLAG beads. After washing and elution of the beads with SDS, the eluate was analyzed on Western blots employing biotin-labeled anti-FLAG antibodies or anti-PLAP antibodies as alternative revealing reagents. As shown in Figure 5 (panel C), the anti-FLAG antibodies detected a major band comigrating with FLAG-tagged Gpi8p and a less intense band ~25 kDa larger in size. In distinction to this result, the anti-PLAP antibodies selectively reacted with the larger band, establishing that it corresponds to a covalently linked PLAP·Gpi8p product. As seen in Figure 5 (panel D), preincubation of the eluate with HDZ but not an equimolar NaCl control followed by repeat Western blot analyses showed marked diminution of the high

Mr band, consistent with the high Mr band being a thioester-linked intermediate. Quantitative analyses of lanes 3 and 4 indicated that the intensity of the labeled high Mr band was decreased 5-fold.

DISCUSSION

In previous studies [Kodukula et al., 1991; Benghezal et al., 1995; Hamburger et al., 1995; Chen et al., 1996; Yu et al., 1997; Inoue et al., 1999; Hiroi et al., 2000], the GPI transamidase was shown to contain the components, Gaa1p and Gpi8p, and evidence was subsequently presented implicating the presence of a cysteine-proteinase-like active site in Gpi8p [Meyer et al., 2000; Ohishi et al., 2000]. This minimal transamidation complex recently has been augmented by the characterization of PigSp and PigTp, and their yeast orthologs Gaa16p and Gaa17p, as two additional components by both genetic and molecular characterizations [Ohishi et al., 2001]. No direct information is available, however, as yet on the function of Gaa1p or whether it is involved in proprotein interaction with the transamidase complex (see below). In this study, we showed that (1) interaction of nascent proproteins with the transamidase involves Gaa1p, (2) the binding to the complex requires the C-terminal signal peptide but can occur independently of permissible ω site residues or the precise sequence of the C-terminal extension, (3) the nascent proprotein forms an intermediate proprotein enzyme complex containing the substrate, Gaa1p and Gpi8p, (4) once bound to the complex, the nascent protein forms a transient thioester intermediate with Gpi8p, and (5) the substitution of the GPI moiety for the C-terminal peptide occurs by nucleophilic attack of the terminal p-EthN amino group in the GPI on the thioester-linked intermediate. Studies with HDZ showed that this intermediate is prone to aminolysis consistent with the covalent intermediate being released as a GPI-anchored product.

For conducting our studies, we utilized a modified miniPLAP in vitro translation system [reviewed in Kodukula et al., 1995] in which sequential translations of mRNA encoding one of the transamidase components and then of *miniPLAP* were carried out. In the first translation step, we utilized a coupled TNT system. To enhance processing efficiency in the second translation step, we stripped the RM with EDTA. Previous studies have shown that this treatment removes bound ribosomes and unprocessed mRNA [Walter and Blobel, 1983]. To our knowledge, an experimentally useful two-step in vitro translation system for similar purposes has not previously been described.

Our results extend recent studies [Ohishi et al., 2000, 2001] using CHO cells cotransfected with differentially tagged *GAA1*, *GPI8*, *PIG-S*, and *PIG-T* cDNAs which showed that all four proteins are included in the complex and required for the transfer of GPI moieties to proproteins. How the components interact with the GPI proproteins was not investigated. Crosslinking experiments have begun to characterize this interaction. A photocrosslinking study of Vidugiriene et al. [2001] employing lysine deletion variants of miniPLAP with photoreactive lysyl-tRNAs yielded bands consistent with the interaction of the proprotein with Gpi8p and potential additional contacts with Gaa1p as well as other uncharacterized proteins. A study by Spurway et al. [2001] utilizing conventional miniPLAP with a bis-maleimide cross-linker showed interaction of the proprotein with Gpi8p. One caveat in this latter study, however, is that the inherent specificity of the cross-linker for cysteine favored initial modification occurring by virtue of the nucleophilic active site cysteine of Gpi8p cross-linking to proximal cysteine residues in miniPLAP. These residues are limited to the hydrophilic sequence on prominiPLAP's N-terminal side of the ω-site as there are no cysteine residues in the prominiPLAP C-terminal signal sequence. Thus, how the C-terminal processing signal of transamidase substrate proteins interacts with the transamidase complex, and consequently where the specificity of the transamidation reaction is localized, remain largely uncharacterized. Our finding that a thioester is formed between the ω-1 residue of prominiPLAP and the active site cysteine residue of Gpi8p extends the cross-linking study of Spurway et al. [2001] that located the substrate protein near Gpi8p. In accordance with the findings of Vidugiriene et al. [2001], the results suggest that an interaction of the substrate protein with other components of the complex exists prior to the transamidation reaction. Additionally, the coprecipitation of Gaa1p with prominiPLAP in the absence of Gpi8p is consistent with findings of Ohishi et al. [2001] that Gaa1p, PigSp, and

PigTp can form a stable complex without Gpi8p. It further is in accordance with more recent findings by Vainauskas et al. [2002] with Gaa1p mutants showing that deletion of the five most C-terminal lumenal membrane spanning domains does not alter the stability or the ER localization of the Gaa1p: Gpi8p: PigSp: PigTp complex. It, however, abolishes the GPI transamidation reaction, leading to the suggestion by the authors that the deleted region of Gaa1p possibly reacts with the hydrophobic C-terminal sequence of the proprotein.

To ascertain if Gaa1p participates in the association of proproteins with the transamidase complex, we utilized RM from the Gpi8p-deficient K cell line. This line was derived by mutagenesis of K562 cells [Mohney et al., 1994; Chen et al., 1996]. Since previous RT/PCR analyses of *GPI8* mRNA in K cells showed a deletion of $A^{105}T^{106}$ resulting in a stop codon at position 131 [corresponding to amino acid 35 (within the 47 AA-long Gpi8p N-terminal signal which is cleaved)] [Yu et al., 1997], the cell line does not make a significant protein product corresponding to the targeted sequence. The findings with HDZ that the K line is unable to convert prominiPLAP to the miniPLAP hydrazide [Chen et al., 1996 and the present study] confirmed that it is completely defective in transamidase activity. Translated Gaa1p was coimmunoprecipitated by anti-PLAP antibodies from RM of the Gpi8p-deficient K cell line. The simplest explanation would be that Gaa1p is involved in the binding of the proprotein to the transamidase complex. One qualification, however, is that the requirement for the other two components that participate in the complex cannot be excluded.

Our studies with miniPLAP mutants showed that interaction of the proprotein with Gaa1p in

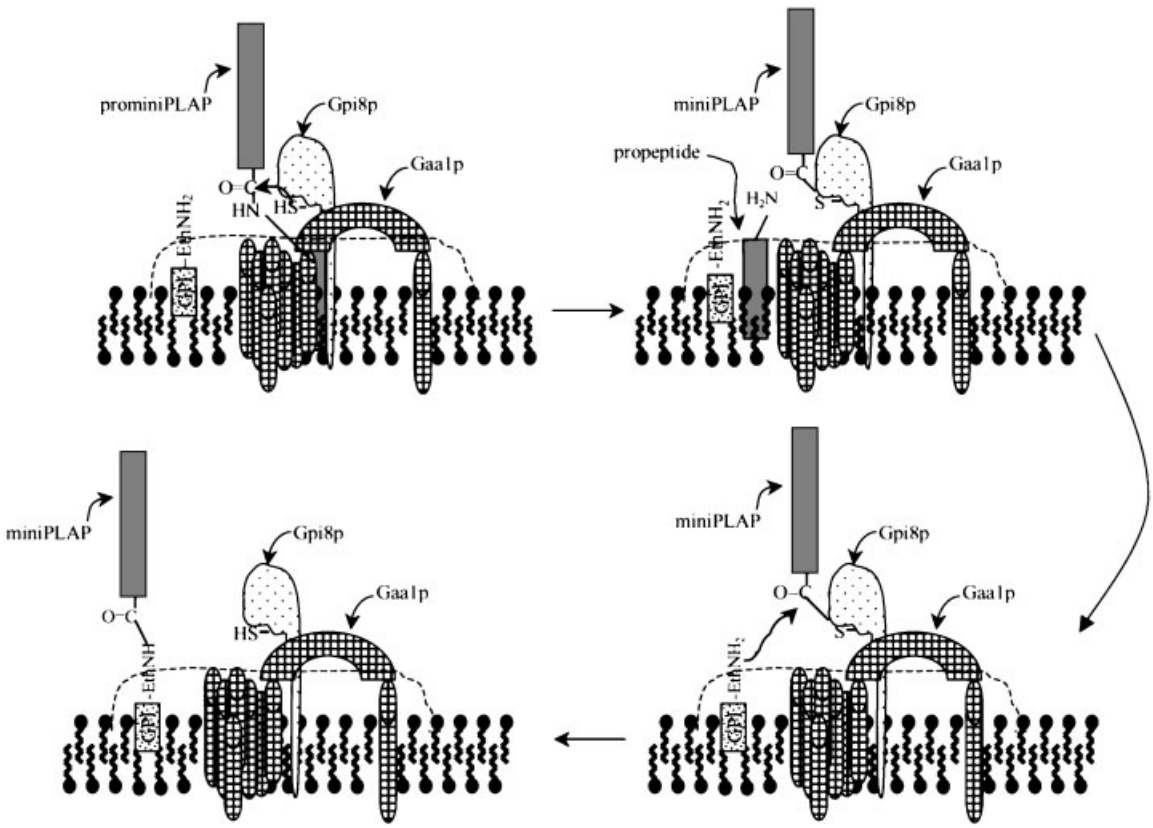

Fig. 6. Proposed mechanism of GPI anchor addition to the proprotein by the transamidase complex. The proprotein, e.g., miniPLAP, binds first to the Gaa1p/Gpi8p complex via its C-terminal signal peptide. The surrounding structure is postulated to be part of Gaa1p, but this is speculative. Once bound, the carbonyl group of the ω site residue of the proprotein is attacked by the active cysteine in the Gpi8p cysteine proteinase to form a covalent thioester-linked intermediate between the proprotein and Gpi8p. The carbonyl in the thioester bond of this covalent intermediate is then attacked by the nitrogen in the terminal p-EthN amine of the GPI to form an amide bond between the C-terminally cleaved proprotein and the GPI anchor. The fate of the C-terminal GPI signal sequence downstream of the ω site that is liberated in the initial reaction is unknown. The dotted line represents the overall transamidase complex containing additional components (see Discussion).

the transamidase complex can occur in the absence of permissible proprotein ω site residues and can occur with widely variant sequences, e.g., the yeast Gas1p C-terminal signal sequence, but is absolutely dependent on the presence of the C-terminal peptide. The latter two results are in keeping with previous characterizations of the requirements of the properties of the C-terminal signal for GPI processing [reviewed in Caras and Weddell, 1989; Caras, 1991; Moran et al., 1991; Moran and Caras, 1991a,b, 1994; Gerber et al., 1992; Udenfriend and Kodukula, 1995]. The precise manner in which amino acid variations in the downstream sequences affect Gaa1p/Gpi8p binding and whether the differences correlate with GPI processing efficiency [Bon et al., 1997; Cross and Boehme, 2000] are not known. Additionally, the site with which the proprotein reacts remains to be defined.

Several studies [Chen and Medof, unpublished] have documented the presence of a high Mr band in anti-PLAP immunoprecipitates. This high Mr band has remained uncharacterized. It sometimes is seen transiently. It was detected as a putative intermediate in the study of Spurway et al. [2001]. In this study, we investigated the precise nature of the band.

Previous analyses of hGpi8p [Ohishi et al., 2000] and yeast Gpi8p [Meyer et al., 2000] have shown that mutagenesis of C^{206} or H^{164}, residues which are conserved in homologous plant cysteine proteinases and in yeast Gpi8p [Benghezal et al., 1995] abolishes its activity, suggesting that they function as catalytic residues in the transamidation reaction. Our experiments with K cells transfected with *FLAG*-tagged *GPI8* in the present study showing that (1) a high molecular band corresponding to miniPLAP covalently bound to Gpi8p can be isolated and (2) treatment of the band with HDZ abolishes it, directly demonstrate that Gpi8p functions as a modified cysteine proteinase and formally establish the mechanism of the reaction. A proposed schematic diagram of the steps which comprise the overall reaction is given in Figure 6.

The experimental system we developed for the present investigation opens up the opportunity for further studies with respect to the transamidase. In addition to mapping important residues in the C-terminal signal, it could allow for mapping of binding or other functional sites in the transamidase components. The double in vitro translation system additionally could constitute a more general method for studies of interactions of other ER proteins.

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