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Evidence that the putative COOH-terminal signal transamidase involved in glycosylphosphatidylinositol protein synthesis is present in the endoplasmic reticulum

(transamidase/Golgi/brefeldin A)

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ABSTRACT **Nascent proteins destined to be processed to a glycosylphosphatidylinositol (GPI)-anchored membrane form contain NH_2-terminal and COOH-terminal signal peptides. The first directs a nascent protein into the endoplasmic reticulum; the second peptide targets the protein to a putative COOH-terminal signal transamidase where cleavage of the peptide and addition of the GPI anchor occur. We recently showed that ATP hydrolysis is required for maturation of GPI proteins at a stage prior to transamidation. Here we show that one of the ATP-requiring proteins involved in processing of GPI-anchored proteins in the endoplasmic reticulum is the immunoglobulin heavy chain binding protein (BiP; GRP 78). This and related findings indicate that GPI transamidase is localized in the endoplasmic reticulum.**

Nascent proteins destined to be processed to a glycosylphosphatidylinositol (GPI)-anchored form contain two hydrophobic signal peptides, one at the NH_2 terminus and another at the COOH terminus (Fig. 1). NH_2-terminal signal peptides direct nascent proteins into the endoplasmic reticulum (ER) and COOH-terminal signal peptides target the partially processed proteins to the site of a putative COOH-terminal signal transamidase. The latter catalyzes concomitant cleavage of the signal peptide and addition of the GPI anchor (1–4). The nascent form of a GPI-anchored protein undergoes two chemical modifications (other than glycosylation) prior to maturation, as shown in Fig. 1. It remains to be established whether the transamidase is located in the ER or Golgi. In recent studies in a cell-free system, we showed that ATP hydrolysis is required for maturation at a stage prior to transamidation (5). We have now found that one of the ATP-requiring entities involved in maturation of GPI-anchored proteins in the ER is the immunoglobulin heavy chain binding protein (BiP; GRP 78) (6, 7). BiP has been shown to bind to metabolic intermediates involved in the biosynthesis of three different GPI proteins as well as to a mutant of one of these proteins that cannot accept the GPI moiety and whose synthesis is aborted at an intermediate stage. These and additional studies with brefeldin A, a compound that leads to disagregation of the Golgi, indicate that the transamidase is located in the ER.

MATERIALS AND METHODS

Materials. Detergents, enzymes, culture media, inhibitors, and buffers were from commercial sources; radiolabeled compounds were from Amersham; brefeldin A was from Epicenter Technologies (Madison, WI). Phosphatidylinositol-specific phospholipase C (PI-PLC) was purified from *Bacillus thuringiensis* (8). Recombinant BiP (hamster) and chicken anti-BiP IgG were kindly provided by Nathan Brot and Herbert Weissbach of our institute. The cDNA construct for CA-IV (human lung) (9) and rabbit antiserum to CA-IV were gifts from William S. Sly (St. Louis University).

Cell Culture and cDNA Transfections. COS7 cells were maintained in culture and transfections were carried out with 300–350 ng of cDNA per well in 35-mm well culture plates by the DEAE-dextran method (10, 11). Mock transfections substituted phosphate-buffered saline (PBS) for cDNA.

Metabolic Labeling. Labeling with [^{35}S]methionine was carried out 48–60 hr after transfection. For steady-state labeling, cells were starved of methionine by preincubating for 1 hr at 37°C in 2.0 ml of methionine-free Dulbecco's minimal essential medium (DMEM) supplemented with 5% dialyzed fetal bovine serum (GIBCO). One milliliter of fresh medium containing [^{35}S]methionine (≈150 μCi per 35-mm well; 1.0 Ci = 37.0 GBq) was then added and incubation was continued for 2 hr. In pulse–chase experiments, unlabeled methionine was added for 2 hr after labeling.

Preparation of Cell Lysates. Following labeling, the medium was aspirated and cells were washed once with PBS. Cells were lysed by adding 500 μl of lysis buffer (0.2% Triton X-100/25 mM Tris·HCl, pH 7.5/2 mM $CaCl_2$/150 mM NaCl) and then scraped from the wells and homogenized in a Dounce homogenizer. The homogenate was then centrifuged for 10 min in a Microfuge and the supernatant was saved.

Preparation of Rough Microsomal Membranes (RM). Typically, transfected cells from ten 35-mm wells were scraped into 5 ml of PBS and RM were prepared (12). The final membrane pellet was resuspended in 200 μl of lysis buffer and samples were used immediately or stored at 4°C.

Immunoprecipitation and SDS/PAGE. Previously we used a buffer containing a mixture of ionic and anionic detergents that dissociate protein complexes during immunoprecipitation. To maintain associations between proteins we used Triton X-100 alone in the lysis buffer. An aliquot (250 μl) of cell lysate or an aliquot (50 μl) of RM suspension was diluted to 500 μl with lysis buffer. For immunoprecipitation of PLAP or miniPLAP-related proteins an anti-PLAP polyclonal antibody (Accurate Chemical, Westbury, NY) (1:500) was added. For CA-IV-related proteins a rabbit anti-CA-IV antiserum at 1:500 was added. Incubations with antibodies were for 12–16 hr at 4°C, after which protein A-Sepharose CL-4B (Sigma) was added and incubation was continued for 1 hr on a rotator at room temperature. The Sepharose beads were washed twice with 750 μl of lysis buffer and extracted with 2× SDS/PAGE sample buffer (20–25 μl), and electrophoresis was carried out on 7.5% or 10% polyacrylamide gels. Gels

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Abbreviations: GPI, glycosylphosphatidylinositol; BiP, binding protein; ER, endoplasmic reticulum; PLAP, placental alkaline phosphatase; RM, rough microsomal membranes; PI-PLC, phosphatidylinositol-specific phospholipase C.

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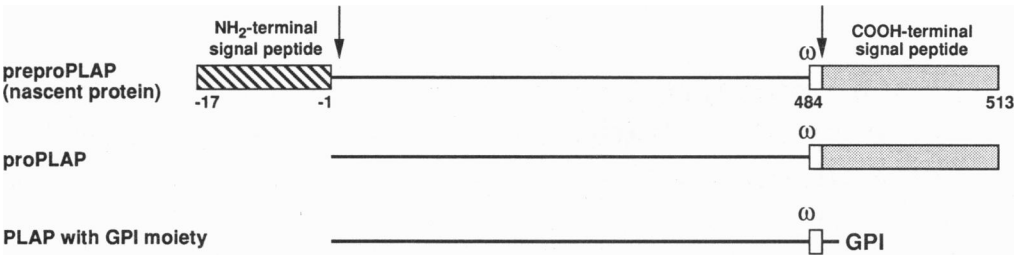

FIG. 1. Intermediates and mature product during processing of the GPI-anchored membrane protein PLAP (placental alkaline phosphatase). The prepro protein is the cDNA deduced amino acid sequence; the pro protein has lost its NH_2-terminal signal peptide (▨); in the mature protein, GPI moiety replaces the COOH-terminal signal peptide (▭). The ω site is the amino acid residue to which the GPI moiety is ultimately attached, residue 484 of nascent PLAP.

were fixed, treated with Amplify (Amersham), dried, and exposed to X-Omat-AR (Kodak) film.

Western Blot Analysis. The immunoprecipitated pellet was suspended in 20 μl of lysis buffer and ATP was added to a final concentration of 5 mM. After incubation at room temperature for 15 min, the sample was centrifuged for 5 min. Supernatants from six such reaction mixtures were pooled on a Spin-X (Costar) filter and the filtrate was concentrated and washed three times with 400 μl of 0.1% SDS on a Centricon-30 unit (Amicon). Finally, the sample was lyophilized, resuspended in 20 μl of 2× SDS/PAGE buffer, and incubated at 70°C for 15 min with occasional spinning in a Vortex. Following SDS/PAGE, proteins were electrotransferred to nitrocellulose membranes (enhanced chemiluminescence grade, Amersham) (13). Membranes were blocked by incubation in TBS/Tween/milk (25 mM Tris·HCl, pH 7.5/150 mM NaCl/0.05% Tween 20/5% nonfat dry milk) for 1 hr at room temperature. Blocked membranes were incubated for 2 hr with chicken anti-bovine BiP IgG diluted 1:1000 in TBS/Tween/1% milk, washed three times (10 min each) with TBS/Tween/milk, and incubated for 1 hr with horseradish peroxidase-conjugated rabbit anti-chicken IgG (United States Biochemical), diluted 1:10,000 in TBS/Tween/1% milk. Finally membranes were washed three times with TBS and peroxidase activity was detected using enhanced chemiluminescence.

Treatment of Cells with Brefeldin A. Transfected COS cells were incubated with brefeldin A (5 μg/ml) for a total of 3 hr, including a methionine starvation period of 1 hr and labeling with [^{35}S]methionine for 2 hr. PLAP was released by PI-PLC treatment (11) with brefeldin A (5 μg/ml) included in the release buffer.

Translation and Processing of MiniPLAP in the Presence of Brefeldin A-Treated RM. To prepare RM, COS cells were incubated for 2 hr in the presence or absence of brefeldin A (5 μg/ml). Brefeldin A was also included in the homogenization buffer at the same concentration. Cell-free translation and processing were carried out and products were analyzed as described (12).

RESULTS

Association of BiP with GPI Proteins During Biosynthesis. The cDNAs encoding PLAP, miniPLAP, the ω proline mutant of PLAP, and CA-IV were transiently expressed in COS cells. Following transfection (48–60 hr), the cells were incubated with [^{35}S]methionine for 2 hr and lysed; proteins were then immunoprecipitated and subsequently analyzed by SDS/PAGE. Each protein species (Fig. 2, cell lysates, lanes 1, 3, and 4) yielded a band corresponding to its mature GPI-anchored form as well as to intermediates that were mostly unresolved. The ω proline mutant (lane 2) was not processed to the GPI-anchored form (14). In all cases, additional bands unrelated to the transfecting cDNA were observed. One was a protein that appeared at 78 kDa, coinciding with the mobility of BiP (6). In another experiment, transfected cells were labeled with [^{35}S]methionine, after which RM were prepared. Again, proteins were immunoprecipitated with the corresponding antibodies and the precipitates were subjected to PAGE. As shown (Fig. 2, RM) some, but not all, proteins that coprecipitated with PLAP from the cells also appeared with precipitates from the RM. The 78-kDa protein appeared in the immunoprecipitates of all four proteins, again showing that a protein with a mass identical to that of BiP was associated with each of the GPI proteins during synthesis and is apparently located in the ER. It should be noted that more of the 78-kDa protein was associated with the ω proline mutant of PLAP than with the other proteins. Neither BiP nor GPI-anchored proteins and intermediates were observed in mock transfected cells (data not shown).

Identification of the 78-kDa Protein as BiP. Dissociation of BiP from a protein that it has chaperoned requires hydrolysis of ATP. When ATP was added prior to immunoprecipitation of PLAP from [^{35}S]methionine-labeled cells, many proteins coprecipitated with PLAP antibody. However, the addition of ATP specifically dissociated the 78-kDa protein from PLAP precipitates. Neither the nonhydrolyzable analog of ATP, adenosine 5′-[β,γ-imido]triphosphate (6), nor GTP affected the amount of BiP that was immunoprecipitated with

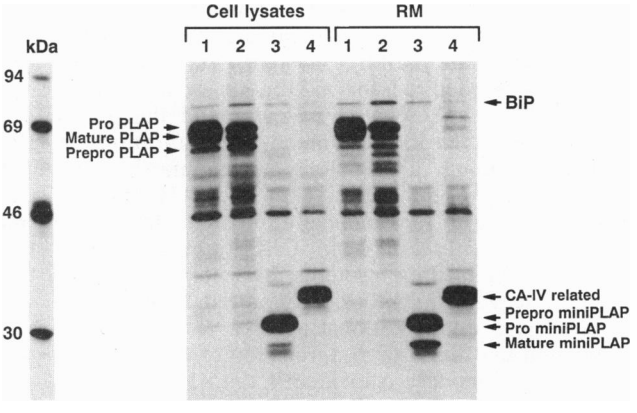

FIG. 2. Immunoprecipitation of different GPI-anchored proteins from cell lysates and RM. COS cells were transfected with cDNAs of wild-type PLAP, ω proline mutant of PLAP, miniPLAP, and CA-IV. [^{35}S]Methionine labeling was carried out 48–60 hr after transfection. Cell lysates and RM for each protein were immunoprecipitated with the respective antibodies and resolved on SDS/PAGE followed by autoradiography. Lanes 1, immunoprecipitate of wild-type PLAP-related proteins; lanes 2, ω proline mutant of PLAP; lanes 3, miniPLAP; lanes 4, CA-IV. BiP coprecipitates with all of the above GPI proteins and migrates at an estimated molecular mass of 78 kDa. In addition to BiP, several other proteins coprecipitated with different GPI proteins; one of them was a 45-kDa protein identified as actin, a common contaminant during immunoprecipitations.

PLAP (data not shown). The following procedure was devised to obtain sufficient amounts of enriched 78-kDa protein for identification. COS cells were transfected with cDNA encoding PLAP and labeled with [^{35}S]methionine, which was followed by extraction and immunoprecipitation. Immunoprecipitates from three wells were either treated with ATP or buffer alone and centrifuged. The supernatants were pooled, concentrated, and subjected to PAGE followed by Western analysis. Fig. 3*A* is an autoradiogram of the ATP-released material. A small amount of the 78-kDa protein was released from the PLAP immunoprecipitates in the absence of ATP (Fig. 3*B*, lane 1). This could be due to the presence of small amounts of endogenous ATP. Addition of ATP caused a massive release of the 78-kDa protein (Fig. 3*B*, lane 2). All three bands comigrated with the BiP standard (Fig. 3*B*, lane 3). A similar experiment carried out with lysates from CA-IV transfected cells also identified the 78-kDa protein that immunoprecipitated with the pro form of CA-IV as BiP (data not shown).

To Which of the Metabolic Intermediates Does BiP Bind? In an initial report on the ATP requirement, we showed that nucleotide hydrolysis was required for conversion of the pro form of miniPLAP to the mature form (5). The following studies indicate that BiP is associated mainly with the pro form. We had previously reported several site-directed mutants of PLAP (14) and miniPLAP (15) that can be converted from their prepro forms to their pro forms but cannot be processed further and apparently accumulate in the ER. The cDNA of one such mutant of PLAP, the ω proline mutant, was transfected into COS cells and labeled with [^{35}S]methionine. The expressed proteins were immunoprecipitated and analyzed by SDS/PAGE. Immunoprecipitates of the ω proline mutant (Fig. 4) contained at least as much BiP as was obtained with wild-type PLAP (Figs. 2 and 3). Since all forms of the ω proline mutant (Fig. 4) interacted with an antibody directed to the COOH-terminal peptide (data not shown), none had lost the COOH-terminal signal peptide nor represent mature forms of PLAP. The band indicated by an asterisk in Fig. 4 migrates like mature PLAP (16). However, it still contains the COOH-terminal signal peptide and probably represents a partially glycosylated pro form of the ω proline PLAP mutant. After a 2-hr chase, the pro form remained and the other two bands diminished, indicating that the latter two are precursors of the pro form (Fig. 4, lane 3). BiP also precipitated with the pro form that remained following the chase and again could be dissociated from the latter by ATP (Fig. 4, lane 4).

Studies with Brefeldin A. Brefeldin A causes disaggregation of the Golgi apparatus resulting in an inhibition of protein secretion (17). Takami *et al.* (18) showed that JEG-3 cells, which normally produce GPI-anchored PLAP, do not do so when pretreated with brefeldin A. The following studies were carried out to determine the effects of brefeldin A in intact cells and in a cell-free system. As shown in Fig. 5*A*, PLAP

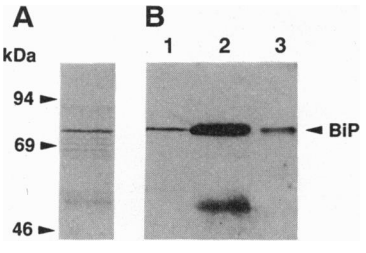

FIG. 3. Immunoblotting of the 78-kDa protein released from PLAP immunoprecipitates by ATP. (*A*) Autoradiography of material released from PLAP immunoprecipitates by ATP. (*B*) Immunoreaction with chicken anti-BiP antibodies and detection by enhanced chemiluminescence. Lane 1, protein released in the absence of ATP; lane 2, protein released by ATP; lane 3, 200 ng of BiP standard.

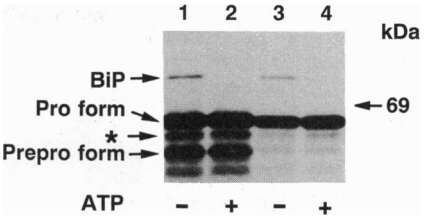

FIG. 4. Association of BiP with the pro form of the ω proline PLAP mutant. COS cells were transfected, labeled for 2 hr with [^{35}S]methionine, and incubated with (lanes 2 and 4) or without (lanes 1 and 3) 2 mM ATP for 15 min at room temperature prior to immunoprecipitation. Lanes 3 and 4 represent the radioactivity remaining after a 2-hr chase. The protein band indicated by an asterisk represents a partially glycosylated pro form of the ω proline mutant.

cDNA transfected COS cells express PLAP (lane 1), some of which is GPI anchored and released by PI-PLC (lane 2). Although treatment with brefeldin A did not alter the amount of PLAP-related protein within the cell (lane 3), no PI-PLC-releasable PLAP appeared on the cell surface (lane 4). However, one cannot determine from whole cell experiments whether brefeldin A blocks cleavage and GPI addition or merely prevents transport of the GPI-linked protein to the cell surface. To investigate the effects of brefeldin A in a cell-free system, we turned to miniPLAP (15). RM were prepared from control and brefeldin A-treated cells and incorporated in a cell-free translation processing system utilizing rabbit reticulocyte lysate and miniPLAP mRNA. As shown in Fig. 5*B*, GPI-linked miniPLAP was produced to the same extent by control and brefeldin A-treated RM. Thus, although brefeldin A-treated cells do not express GPI-linked proteins on their cell surface, the GPI transamidase activity is unaffected.

DISCUSSION

Nascent proteins destined to be processed to a GPI-anchored membrane form contain an additional and unique signal, the COOH-terminal signal peptide, that targets them to a site in the ER or Golgi where the GPI anchor can be added, presumably by a specific transamidase (1–5). The intermediate between the nascent protein (prepro protein) and the mature protein, the pro form of the protein, is thus the substrate for the putative transamidase (Fig. 1). In previous studies we showed that ATP hydrolysis is required for maturation of a GPI protein (5). Here, we show that BiP is bound to the pro form, from which it can be dissociated by ATP. It is likely that BiP begins to interact with the elongating nascent protein after removal of the NH_2-terminal signal peptide and helps in its further translocation into the ER (19). BiP would then continue to interact with the pro protein to catalyze proper folding (19) and assist in translocation to the transamidase (5). Fig. 6*A* depicts sequential steps in the biosynthesis of a GPI-anchored protein starting with the proPLAP–transamidase complex. Fig. 6*A* I shows the pro form attached through its COOH-terminal signal peptide to its binding site on the transamidase, presumably in the membrane of the ER. Two molecules of BiP are arbitrarily shown binding to the pro protein—one in proximity to the small amino acid domain of proPLAP, its function presumably to fold that sequence in an appropriate conformation to bind to the ω and $\omega + 2$ sites in the transamidase (Fig. 6*A* II and III). BiP is shown as being released at each of the two intermediate steps accompanied by ATP hydrolysis. The carbonyl group on the ω residue of proPLAP in the complex with the transamidase (Fig. 6*A* III) is apparently activated. Nucleophilic attack of the activated carbonyl by GPI, through its ethanolamine substituent (4), would yield GPI-

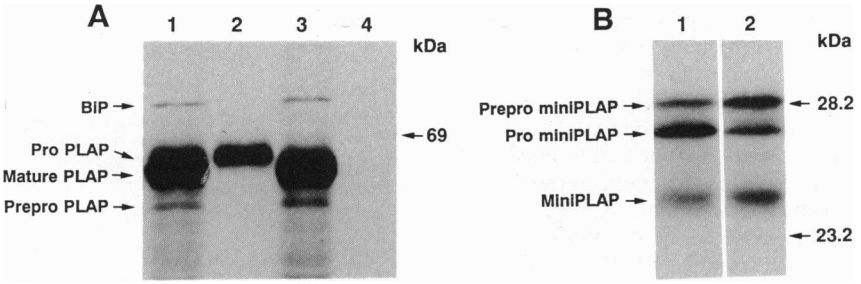

FIG. 5. Effect of brefeldin A on surface expression of PLAP and the COOH-terminal processing and GPI linkage formation *in vitro*. (*A*) COS cells were transfected with PLAP cDNA, treated with (lanes 3 and 4) or without (lanes 1 and 2) brefeldin A, labeled for 2 hr with [^{35}S]methionine, and treated with PI-PLC for 1 hr at 37°C. Lanes 1 and 3 represent the material remaining in the cells after PI-PLC and lanes 2 and 4 represent the PLAP released by PI-PLC. (*B*) Translation and processing of prepro miniPLAP was carried out in the presence of normal RM (lane 1) or RM prepared from brefeldin A-treated cells (lane 2). Samples were resolved in a 15% gel.

PLAP and the free COOH-terminal peptide (Fig. 6*A* IV). Fig. 6*B* shows the fate of an unallowed mutant form of proPLAP. In Fig. 6*B* I, the mutant pro form is attached through the COOH-terminal signal peptide to its binding site on the transamidase. Here again, we show BiP, associated with the pro form in the small amino acid domain, as carrying out a conformational change that normally permits binding at the ω and $\omega + 2$ sites. However, although BiP may bring about conformational change, the unallowed amino acid at the ω site cannot fit into the corresponding site on the transamidase (Fig. 6*B* II). Experimentally, ATP hydrolysis can dissociate BiP from the ω mutant proPLAP (Fig. 5). However, since such mutants are not substrates, they apparently remain bound to the transamidase and accumulate in the ER. Since proteins hung up in the ER are found associated with BiP (20), we consider the final product in this aborted synthesis as the pro form of the mutant bound to the transamidase along with BiP (Fig. 6*B* III).

Thus far, we have assumed that the transamidase is in the ER. However, if it were in the Golgi, then removal of BiP might be required to permit GTP-associated vesicular transport into the Golgi, since BiP is presumably excluded from

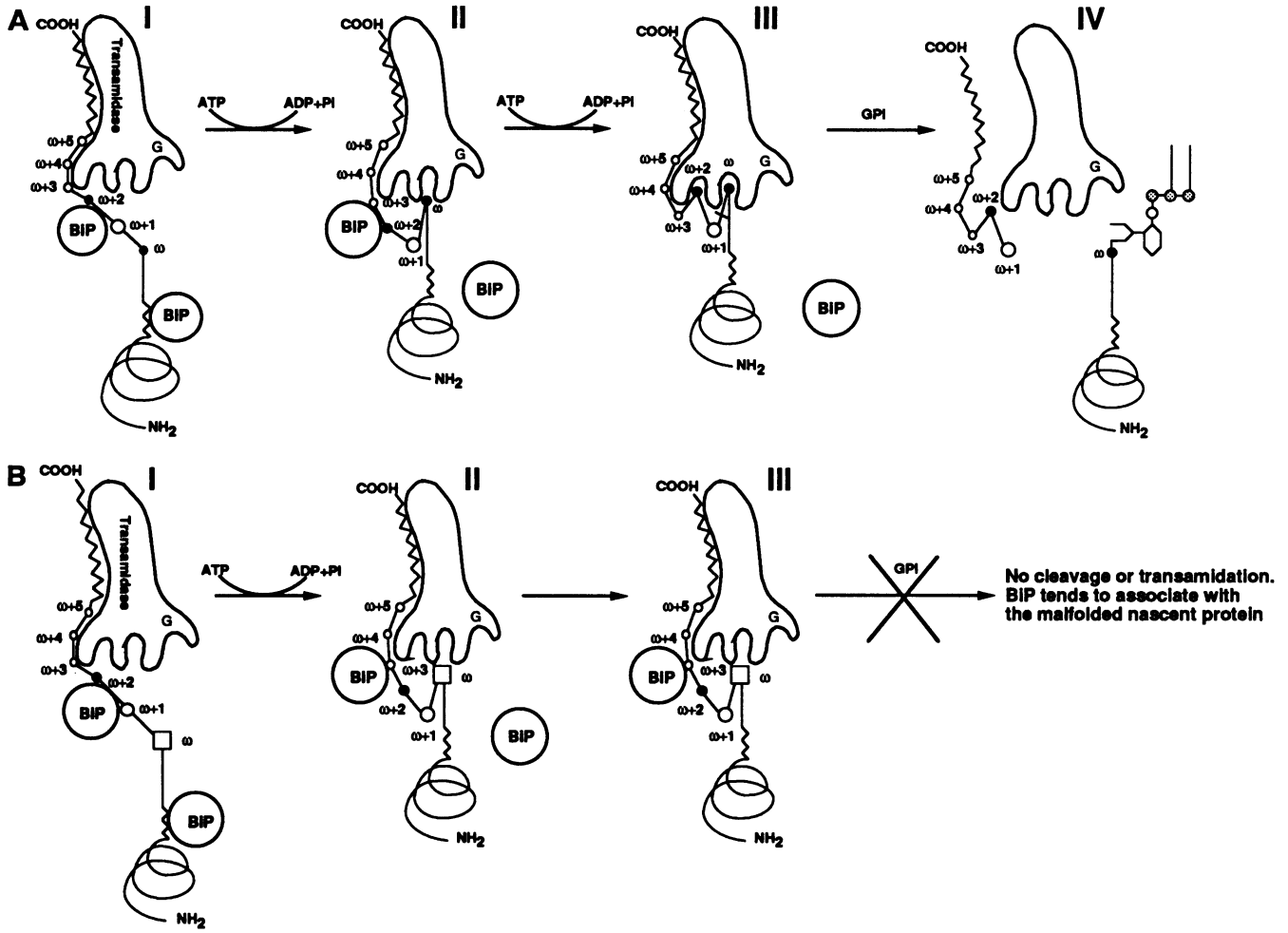

FIG. 6. Sequential steps in biosynthesis of a GPI-anchored protein. ω, Site of cleavage and GPI attachment; G, GPI binding site. Transamidase is probably associated with GPI. The region $\omega + 1$ to the COOH terminus represents the COOH-terminal signal peptide. ●, Wild-type amino acid Asp at ω. The line between ω and $\omega + 1$ residues (*A* III) indicates the site of cleavage. □ (in *B*), an unallowed amino acid such as proline, tryptophan, etc.

the latter compartment (6, 20). Our previous demonstration of a GTP requirement for transamidation (5), in addition to ATP, would be in accord with a Golgi site for transamidation. However, it should be noted that the PLAP ω proline mutant, which cannot undergo transamidation (14) and accumulates as the pro form, bound as much, or more, BiP than did wild-type proPLAP (Fig. 2). Proteins whose transport from the ER is blocked accumulate there in combination with BiP (20). It should be noted (Fig. 6) that the hydrophobic portion of the COOH-terminal signal peptide is probably the initial site of attachment of the pro protein to the transamidase. Since this is the same in wild-type PLAP and in the ω proline mutant, one would expect the mutant to bind to the transamidase but, being unable to undergo transamidation, it would be trapped in the ER. Since BiP has been found only in the ER (6, 20), association of BiP with the apparently trapped pro form of the ω proline mutant suggests that the transamidase is located in the ER rather than in the Golgi and that the observed GTP requirement may be for an as yet unknown function. This conclusion is further strengthened by the fact that disruption of the Golgi by brefeldin A, which interferes with translocation of GPI-linked proteins to the cell surface, has no effect on transamidation. The ω proline mutants of GPI proteins may prove useful as tools for investigating interactions with chaperones or with the putative transamidase.

Heretofore, all of our studies on the biosynthesis of GPI proteins were carried out with PLAP or mutant forms of PLAP (5, 11, 12, 14–16). Here we have utilized an entirely different GPI-anchored protein, CA-IV, and shown that similar intermediates occur during its processing as well as have a similar requirement for ATP and BiP. Thus our previous findings on the biosynthesis of PLAP are probably generally applicable to all GPI proteins.

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