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A defect in glycosylphosphatidylinositol (GPI) transamidase activity in mutant K cells is responsible for their inability to display GPI surface proteins

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ABSTRACT **The final step in the pathway that provides for glycosylphosphatidylinositol (GPI) anchoring of cell-surface proteins occurs in the lumen of the endoplasmic reticulum and consists of a transamidation reaction in which fully assembled GPI anchor donors are substituted for specific COOH-terminal signal peptide sequences contained in nascent polypeptides. In previous studies we described a human K562 cell mutant line, designated class K, which assembles all the known intermediates of the GPI pathway but fails to display GPI-anchored proteins on its surface membrane. In the present study, we used mRNA encoding miniPLAP, a truncated form of placental alkaline phosphatase (PLAP), in *in vitro* assays with rough microsomal membranes (RM) of mutant K cells to further characterize the biosynthetic defect in this line. We found that RM from mutant K cells supported NH_2-terminal processing of the nascent translational product, preprominiPLAP, but failed to show any detectable COOH-terminal processing of the resulting prominiPLAP to GPI-anchored miniPLAP. Proteinase K protection assays verified that NH_2-terminal-processed prominiPLAP was appropriately translocated into the endoplasmic reticulum lumen. The addition of hydrazine or hydroxylamine, which can substitute for GPI donors, to RM from wild-type or mutant cells defective in various intermediate biosynthetic steps in the GPI pathway produced large amounts of the hydrazide or hydroxamate of miniPLAP. In contrast, the addition of these nucleophiles to RM of class K cells yielded neither of these products. These data, taken together, lead us to conclude that mutant K cells are defective in part of the GPI transamidase machinery.**

Posttranslational glycosylphosphatidylinositol (GPI) anchor addition serves as a general mechanism for linking a number of proteins to the outer leaflet of the cell-surface membrane (for review, see refs. 1 and 2). This mechanism is used by all eukaryotic cell types thus far studied and attaches proteins with a wide range of functions. Anchor incorporation into these proteins occurs in a concerted reaction in which preassembled GPI donors are substituted for COOH-terminal signal sequences in their nascent polypeptides following translocation of these polypeptides across the endoplasmic reticulum (ER) membrane.

Most, if not all, of the intermediate steps in the biosynthesis of the GPI moiety have been elucidated (3–7). In the initial phase of the process, GlcN-acyl phosphatidylinositol (PI) is synthesized by assembly, deactylation, and acylation of GlcNAc-PI. In subsequent reactions, mannose (Man) and ethanolamine-phosphate (EthN-P) are added sequentially to this intermediate to produce the glycan core. Mutant cells defective in one or another of these biosynthetic steps do not exhibit GPI proteins on their surfaces (8–10). *In vitro* translation studies have shown that COOH-terminal processing of nascent polypeptides to GPI proteins in rough microsomal membranes (RM) from such mutant cells is markedly diminished (11).

In previous studies we isolated a human K562 mutant cell line that, despite synthesis of all the known intermediates of the GPI anchor assembly sequence (12), fails to express GPI-anchored proteins on its surface membrane. Comparisons of levels of individual intracellular GPI species in this line, designated mutant K, with those in parental K562 cells showed greatly increased amounts of terminal mannolipid products (6, 7, 13) that correspond to the known structures of protein-associated anchors (14–16) and consequently are thought to represent the fully assembled anchor donors. Although these findings suggested that the defect in this mutant resides downstream of the anchor assembly process and could involve anchor transfer to protein, the precise nature of the biochemical lesion in this line remained unclarified.

In the present study, we investigated the ability of RM isolated from mutant K cells to carry out COOH-terminal processing of a nascent protein containing a COOH-terminal signal peptide. For this purpose we used mRNA encoding miniPLAP, a truncated form of placental alkaline phosphatase. This mRNA generates nascent preprominiPLAP and the NH_2-terminal-processed product, prominiPLAP, which is the acceptor of the GPI donor (Fig. 1). We exploited recent findings that prominiPLAP, in the presence of the GPI transamidase can react with the nucleophiles hydrazine or hydroxylamine in place of GPI to yield the hydrazide or hydroxamate of miniPLAP (17).

METHODS

Biosynthetic Labeling and TLC Analyses. Cells (2×10^8) in 100 ml of glucose-free RPMI medium/10% dialyzed fetal bovine serum supplemented with glucose (10 μg/ml) were preincubated for 1 hr with tunicamycin at 10 μg/ml and then labeled at 37°C for 4 hr with 1 mCi of [^{3}H]Man. After incubation, reaction mixtures were extracted with equal volumes of chloroform and methanol calculated to give the final proportions of chloroform/methanol/water, 10:10:3. The solvent mixtures were evaporated to dryness and the dried extracts were partitioned between butanol/water. The butanol-associated lipids were analyzed on TLC silica 60 plates using a Berthold LB285 TLC scanner as described (6, 12).

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Abbreviations: GPI, glycosylphosphatidylinositol; ER, endoplasmic reticulum; PI, phosphatidylinositol; Man, mannose; EthN-P, ethanolamine phosphate; RM, rough microsomal membranes; PLAP, placental alkaline phosphatase; TEA, triethanolamine.

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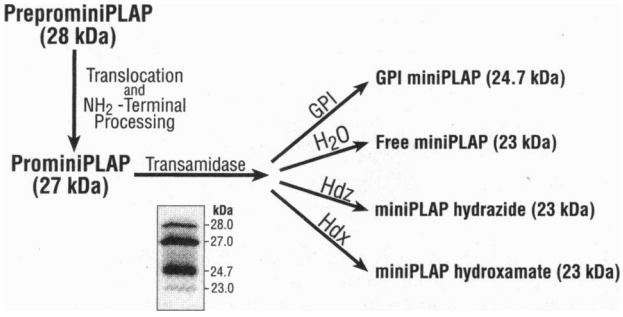

FIG. 1. Schematic of processing to prominiPLAP. Products resulting from coupling of the alternative donors GPI, H_2O, hydrazine (HDZ), or hydroxylamine (HDX) are shown. (*Inset*) Gel with the products (after immunoprecipitation and PAGE) normally formed during cotranslational processing of miniPLAP mRNA in the presence of RM.

Cell Culture and Preparation of RM. CHO cells were grown at 37°C in Iscove's modified medium and HeLa cells in Dulbecco's minimal essential medium (DMEM) both containing 10% newborn calf serum. Parental and mutant murine lymphoma cells were grown in DMEM/10% horse serum (6) and K562 parental cells and mutant K cells in RPMI medium/10% newborn calf serum fortified with 2 mM L-glutamine (12). For the preparation of RM (18), harvested cells were pelleted at 1000 × *g*, washed once with ice-cold phosphate-buffered saline (PBS), and then washed with 10 mM triethanolamine (TEA), pH 7.5. The washed cells were resuspended in 10 mM TEA (3 × the packed-cell volume) and then mixed with an equal volume of 10 mM TEA/0.5 M sucrose. The cell suspension was placed in a cell disruption bomb (Parr Instruments, Moline, IL) that was charged to 1200–1500 psi (1 psi = 6.9 kPa) with nitrogen and placed on ice for 1.5 hr. The cell lysate that resulted was centrifuged at 1000 × *g* for 10 min at 4°C, and the low-speed pellet was resuspended in 10 mM TEA/250 mM sucrose and recentrifuged at 1000 × *g*. The supernatants remaining after the two centrifugations were pooled and clarified by centrifugation at 27,000 × *g* for 12 min at 4°C in a Sorvall SS-34 rotor. The clarified supernatant containing RM was recovered and recentrifuged for 90 min at 78,000 × *g* in a Ti 70 rotor at 4°C. The RM-containing pellet resulting from this centrifugation was resuspended in buffer B (250 mM sucrose/50 mM TEA, pH 7.5) and stored at −70°C.

Cotranslational Processing of MiniPLAP. Cell-free translation of miniPLAP mRNA in the presence of rabbit reticulocyte lysate was carried out as described by Pelham and Jackson (19) and Micanovic *et al.* (20). Typically, reactions were performed in a 25 μl translation mixture containing 1 μl of miniPLAP mRNA at 100 ng/μl, 1.75 μl of [^{35}S]methionine (15 mCi/ml, 1100 Ci/mmol; Ci = 37 GBq; Amersham) and 12.5 μl of (nuclease-treated) rabbit reticulocyte lysate, 0.75 μl of a 1 mM amino acid mixture minus methionine, and 0.5 μl of RNase inhibitor at 40 unit/μl (all supplied by Promega). To this was added 2.5 μl of buffer A (100 mM KAc/4 mM MgAc) containing the protease inhibitors aprotinin, antipain, bestatin, chymostatin, leupeptin, and pepstatin each at 20 μg/ml. RM was added in a volume of 4 μl from a stock containing 50 OD_{280} units per ml. The mixture was then incubated at 30°C for 90 min. Immunoprecipitations were done essentially as described by Bailey *et al.* (21).

Preparation of RM Preloaded with ProminiPLAP. To prepare RM preloaded with miniPLAP for the analysis of COOH-terminal processing independently of translation, multiple translation mixtures (25 μl each) were incubated for 2 min at 30°C before RM addition. The RM then were added, and the mixtures were incubated at 30°C for an additional period of time (as specified in *Results*). The samples then were placed on ice for 5 min to stop the reactions and pooled; 100 or 300 μl was layered over a 100- or 500-μl cushion of 500 mM sucrose/50 mM TEA, pH 7.5. After centrifugation at 267,000 × *g* for 15 min at 4°C in a TLA100 or TLA100.2 rotor, the supernatant containing cotranslational components was removed, and the pelleted RM were rinsed with 500 μl of 250 mM sucrose/50 mM TEA/buffer. The preloaded RM then were resuspended in the same buffer (200 μl of buffer per 600 μl of starting material).

Processing of ProminiPLAP in Preloaded RM. Preloaded RM (12.5 μl) were diluted with an equal volume of buffer containing 20 mM creatine phosphate/creatine phosphate kinase (100 μg/ml)/4 mM dithiothreitol/160 mM KAc/8 mM MgAc/1 mM ATP/1 mM GTP containing the mixture of protease inhibitors specified above for cotranslational processing. For studies with hydrazine and hydroxylamine, 1 μl of each agent or of water was added before incubation for the specified time period.

Protease Protection Assay. After translation and processing, the RM-containing reaction mixture was mixed with 25 μl of 50 mM TEA. pH 7.5/140 mM KAc/2.4 mM MgAc, and proteinase K was added to 100 μg/ml. After incubating the mixture on ice for 30 min, the reaction was stopped by adding two volumes of saturated ammonium sulfate/6 mM phenylmethylsulfonyl fluoride and further incubating the mixture on ice for another 20 min. At this point, the products were spun for 10 min at 4°C in a microcentrifuge at high speed. The pellet (RM) was washed with 5% trichloroacetic acid and resuspended in 25 μl of buffer B.

RESULTS AND DISCUSSION

TLC analyses of [^{3}H]Man-labeled GPI intermediates recovered in the butanol phase after partitioning of chloroform/methanol extracts of mutant K and parental K562 cells following *in vivo* [^{3}H]Man-labeling of the cells are shown in Fig. 2. As reported previously (12), compared to parental cells, mutant K cells accumulate greatly increased amounts of H6 [ManMan(EthN-P→)ManGlcN-acyl PI] and the terminal intermediates H7 [EthN-P-6ManMan(EthN-P→)ManGlcN-acyl PI] and H8 [EthN-P-6Man(EthN-P) 6Man(EthN-P→)ManGlcN-acyl PI], which correspond in their core structures to mature protein-associated GPI anchors (14–16) and presumably represent the fully assembled GPI donor(s). Analysis of the chloroform/methanol extract and of the aqueous phase remaining after butanol partitioning with Sep-Pak resin

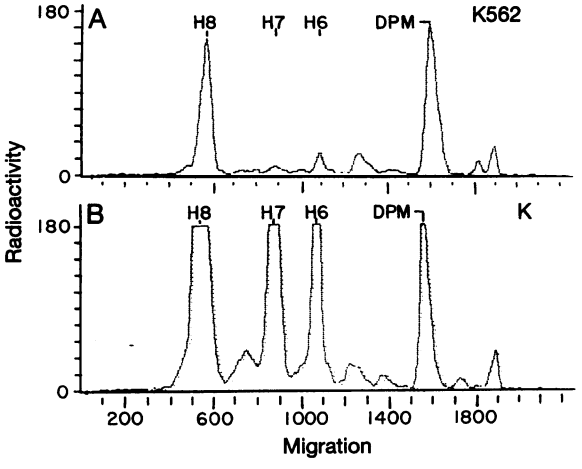

FIG. 2. Intracellular GPI mannolipid intermediates expressed in mutant K and wild-type cells. Mutant K and parental K562 cells were labeled for 4 hr at 37°C with [^{3}H]Man as described. TLC patterns of butanol-extractable ^{3}H-labeled GPI products generated by the two cell types are shown. Identities of the peaks are designated. DPM, Dolichol-phosphomannose.

(Waters–Millipore) showed that even more H8 was present in the nonbutanol extractable phases than was recovered in the butanol phase. Thus, as was shown previously, mutant K cells produce and accumulate GPI end products but do not couple them to protein.

To further investigate the biochemical defect underlying the GPI end-product accumulation in mutant K cells, we exploited the miniPLAP *in vitro* cotranslational processing system (18, 19) to examine GPI anchor transfer to nascent polypeptides. As indicated in the introduction (Fig. 1), in this system products deriving from initial translation of miniPLAP mRNA (28-kDa preprominiPLAP), NH_2-terminal signal peptide cleavage (27-kDa prominiPLAP), COOH-terminal signal cleavage of the latter with concurrent GPI anchor addition (24.7-kDa mature miniPLAP), and COOH-terminal signal cleavage by hydrolysis (17)—i.e., without GPI anchor addition (23-kDa free miniPLAP)—can be distinguished. In initial experiments we compared products generated using RM from GPI-sufficient HeLa cells, parental and classes F and B Thy-1$^-$ murine lymphoma cells defective in incorporating EthN-P and Man 3 into the GPI core (6, 8, 10), and parental K562 and mutant K cells. The results of these studies are shown in Fig. 3. As can be seen, although there was some variability in processing efficiencies of the different RM preparations, HeLa cell RM (lane 2) and RM from each of the parental lines (lanes 3, 5, and 7) supported the production of 24.7-kDa GPI-anchored miniPLAP and showed small amounts of 23-kDa free miniPLAP. In accordance with previous findings using this cotranslational system (11), RM from the two GPI-defective Thy-1$^-$ mutant lines, classes F (lane 6) and B (lane 8), showed trace amounts of the 24.7- and 23-kDa products. In contrast, RM from mutant K cells produced 27-kDa NH_2-terminal-processed prominiPLAP with efficiency equal to or greater than the parental or Thy-1$^-$ mutant lines but showed none of the further COOH-terminal-processed 24.7- or 23-kDa products. Thus, mutant K cells, in addition to elaborating fully assembled intracellular GPI precursors, can generate the GPI-acceptor protein, prominiPLAP, but do not produce GPI-linked miniPLAP.

Although the finding that RM from mutant K cells efficiently supported NH_2-terminal processing of preprominiPLAP argues that their RM are intact, we performed proteinase protection assays to verify that the defect in these cells is not related to improper translocation of prominiPLAP into the ER lumen. After translation and processing, aliquots of the reaction mixture were incubated with proteinase K in the absence or presence of TX-100-detergent. Parallel aliquots were prepared from reaction mixtures of identical experiments performed with CHO and HeLa RM (data not shown) as controls. Fig. 4 shows that in the absence of detergent,

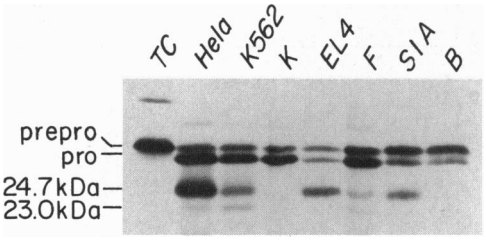

FIG. 3. Comparative processing of preprominiPLAP by RM from mutant K and other cell lines. MiniPLAP mRNA was incubated for 90 min at 30°C in the presence of RM from each of the indicated cell types. Products were immunoprecipitated with rabbit anti-PLAP antibodies and analyzed on 15% SDS/PAGE gels. A translation control (TC) performed in the absence of RM is shown in lane 1. In contrast to RM from wild-type cells (lanes 2, 3, 5, and 7) and from classes F and B Thy-1$^-$ murine lymphomas (lanes 6 and 8), RM from mutant K cells (lane 4) showed prominiPLAP but no detectable 24.7- or 23-kDa C-terminal-processed products (see text).

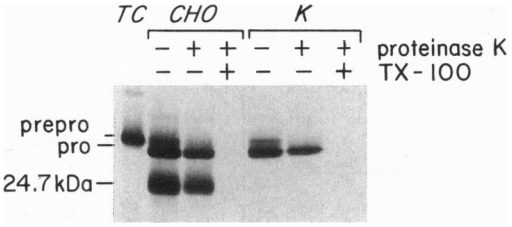

FIG. 4. Resistance of prominiPLAP in mutant K RM to proteinase K digestion. RM from control and mutant K cells, containing miniPLAP products generated during 90 min of cotranslational processing, were incubated for 30 min at 0°C with proteinase K at 100 μg/ml in the absence or presence of 0.5% TX-100 detergent. Digestion products then were immunoprecipitated and analyzed as described in the Fig. 2 legend. As observed with prominiPLAP in RM from the CHO and HeLa control cells (data not shown), prominiPLAP in RM from mutant K cells was digested only when detergent was included in the reaction mixture.

prominiPLAP (27 kDa) in RM from mutant K cells survived proteinase treatment identically to that in RM from the control cells. In contrast, prominiPLAP was completely destroyed in RM from all sources when the membranes were pretreated with detergent. A second set of experiments performed with trypsin in place of proteinase K yielded identical results (data not shown). Thus, in mutant K cells, as in other cells, the NH_2-terminal-processed product prominiPLAP is present within the ER lumen. It therefore should be available for coupling to GPI donors, which in this cell line are present in great amounts (Fig. 2), unless there is a defect in the proper localization of the GPI donor within the ER or in the coupling reaction (i.e., transamidation reaction).

To further assess the nature of the block in COOH-terminal processing in mutant K cells, we conducted experiments with hydrazine, which can function as an alternate nucleophile (17) in the transamidation reaction that couples GPI anchor structures to polypeptides. As can be seen in Fig. 4*A*, when cotranslational processing in the presence of hydrazine was carried out with RM from parental K562 cells, a large amount of the putative 23-kDa hydrazide product that comigrates with free miniPLAP (17) was formed (see Fig. 1). In distinction to this result, cotranslational processing with RM from mutant K cells yielded no such product. As seen in Fig. 5*B*, these results using RM from mutant K cells contrasted with the results of identical experiments done with RM from Thy-1$^-$ lymphoma mutant F or B cells. In the absence of hydrazine, the latter cell lines produced small amounts of 24.7- and 23-kDa products (Fig. 1) and in the presence of the nucleophile generated much larger amounts of the 23-kDa hydrazide product. Similar results previously have been obtained with RM from GPI-defective Ltk$^-$ cells (17) harboring a defect in the initial step in GPI anchor synthesis. The inability of mutant K cells to couple hydrazine to prominiPLAP in place of GPI, even though this nucleophile should be available in high concentrations both outside and within the lumen of the ER, indicates that the defect in these cells is not due to incorrect localization of GPI and consequently must reside in GPI transamidase activity.

Preloading of RM with prominiPLAP allows monitoring of COOH-terminal processing independently of translation. The use of this method permits analysis of the effects of another nucleophilic agent, hydroxylamine, which can serve as second alternative donor to the GPI anchor that otherwise would interfere with translation (17). To determine whether COOH-terminal processing of prominiPLAP by RM from mutant K cells could be achieved with hydroxylamine or otherwise forced under experimental conditions used in preloading, we compared posttranslational studies of this type using RM from HeLa, K562 parental, Thy-1$^-$ lym-

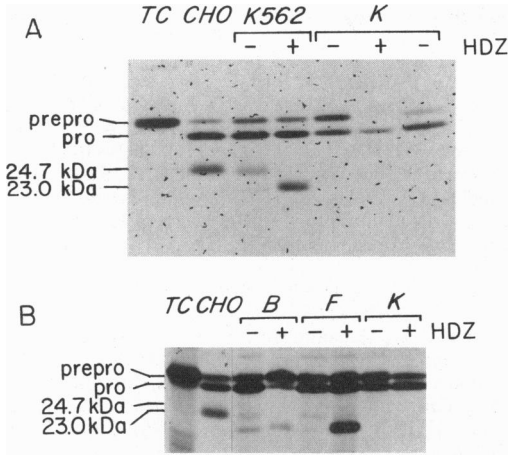

FIG. 5. Comparative effects of hydrazine on COOH-terminal processing of prominiPLAP in RM from mutant K and other cell types. Cotranslational processing of RM from each of the designated cell types was conducted in the absence or presence of 10 mM hydrazine (HDZ). (*A*) Studies with RM from mutant K cells and from parental K562 cells. (*B*) Studies with RM from mutant K cells and from parental (EL-4 and S1A) and Thy-1$^-$ (classes F and B) murine lymphomas. The translation control (TC) and products generated by CHO cell RM are included as controls in *A*, and the products generated by parental K562 cell RM are included in *B*.

phoma mutant F, and mutant K cells. Fig. 6 shows that upon further incubation of preloaded RM from HeLa, parental K562, or Thy-1$^-$ lymphoma mutant F cells, there was virtually complete conversion of prominiPLAP into 23-kDa products representing the putative 23-kDa hydrazide or hydroxamate species, respectively. In marked contrast, when RM from mutant K cells were used under identical conditions, no hydrazide or hydroxamate could be detected. In the above experiments, preloading of RM was carried out for 20 min. When the preloading time was extended to 60 min, RM from the parental K562 cells contained large amounts of GPI-linked miniPLAP (24.7 kDa) in addition to prominiPLAP (27 kDa). In the presence of hydrazine or hydroxylamine, both miniPLAP species were enzymatically converted to the 23-kDa hydrazide or hydroxamate. When RM from K mutant cells were preloaded in an identical fashion and the preloaded RM was incubated with hydrazine or hydroxylamine, prominiPLAP was produced but again no 23-kDa products appeared (data not shown). Since the reaction of the nucleophiles hydrazine and hydroxylamine with prominiPLAP bypasses the requirement for GPI, the above findings provide further evidence that the defect in K mutant cells resides in the transamidase machinery.

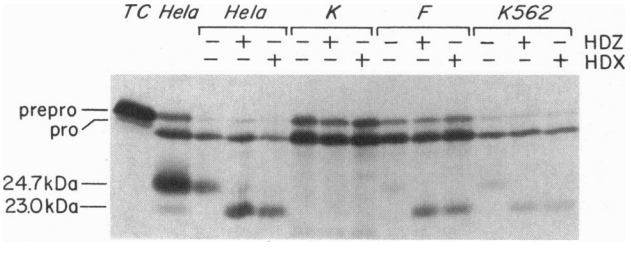

FIG. 6. Comparative effects of hydrazine (HDZ) and hydroxylamine (HDX) on COOH-terminal processing of prominiPLAP preloaded in RM from mutant K and from other cell types. MiniPLAP mRNA in the presence of translation mix was processed for 20 min with RM from each of the designated cell types so as to effect translation and produce NH_2-terminal-processed prominiPLAP. After separation of the RM as described, the isolated RM from each cell type was further incubated at 30°C for 60 min (together with ATP and GTP) in the absence of or presence of 10 mM HDZ or 10 mM HDX.

The original studies with intact K mutant cells (12) indicated that their inability to form GPI-anchored membrane proteins was not due to a lack of mature GPI. These studies also indicated that GPI synthesis proceeds to completion independently of GPI coupling to proteins. That K mutant cells overproduced mature GPIs suggested a defect in GPI utilization—i.e., coupling to protein, a defect in GPI compartmentalization, or a defect in some other essential GPI-processing step preliminary to coupling. To investigate GPI addition to nascent polypeptides in these mutant cells, in the current work we conducted studies with ER, the site of processing to GPI proteins (1, 2). With the use of RM (a crude ER source) and mRNA encoding miniPLAP, the present studies show that the mutant cells, which are rich in mature GPI, cannot couple this glycolipid to the N-terminal-processed acceptor protein, prominiPLAP. ProminiPLAP couples to GPI in RM from all other cells, including K562 cells, the parental cell line of mutant K cells. Even the small amounts of GPI present in the RM of other mutants defective in one or another enzymes or cofactors involved in GPI preassembly undergo coupling to prominiPLAP to yield small amounts of GPI-linked miniPLAP (11). In RM from K mutant cells, not even a trace of GPI-linked miniPLAP was formed. Reaction of prominiPLAP with hydrazine or hydroxylamine, which bypasses the need for GPI in all other cells, did not occur in RM from mutant K cells. These latter experiments rule out the possibility that the forms of mature GPI produced by the mutant cells differ in some uncharacterized way from those in normal cells or that the glycolipid donors are not properly localized either externally or within the ER lumen. Furthermore, the secretory pathway of protein synthesis in RM from K mutant cells functions normally, as shown by the cotranslational production of preprominiPLAP and prominiPLAP and by findings that class I HLA proteins on the cells are identical to those on parental cells (unpublished data). As in RM from control cells, the prominiPLAP is resistant to the action of proteases, indicating that it is translocated within the ER lumen.

The simplest explanation for the deficit in mutant K cells would be a mutation in the gene coding for the GPI transamidase enzyme. However, it is alternatively possible that the defect is not in the transamidase enzyme itself but rather in unidentified auxiliary protein(s) required for its activity. Because the secretory pathway in these cells functions normally, defects in known chaperones are apparently excluded. Nevertheless, the function of the GPI transamidase could require some hitherto unrecognized chaperone. It is also possible that the enzyme could require a low-molecular-weight cofactor and that the defect could reside in the synthesis of this factor. A further possibility is that the GPI transamidase exists as a heteromultimer with the active enzyme covalently attached to one or more other polypeptides that are required for its expression in an active form.

While identification of the protein that is altered or absent in mutant K cells should provide valuable new information, it is important to recognize that the GPI transamidase machinery may consist of a group of factors. Recent complementation studies in yeast have provided evidence for at least two components (22). Even though cDNA encoding the altered factor could rescue intact mutant cells and the corresponding mRNA restore transamidase activity within RM from the mutant, these results would not formally establish that the resulting protein was GPI transamidase. Informative data could come from the use of an *in vitro* assay specific for transamidase function. The ability to bypass the requirement for GPI with small organic nucleophiles as used in this study (17) opens the possibility of purifying the enzyme and setting up such an assay.

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