Lack of glycosyl-phosphatidylinositol anchoring leads to precursor retention by a unique mechanism in *Dictyostelium discoideum*

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Gp80, a cell-adhesion molecule in *Dictyostelium discoideum*, is modified by N- and O-linked oligosaccharides, and a glycosyl-phosphatidylinositol (GPI) anchor. To identify sequences important for the addition of these modifications to gp80, we created a hybrid protein in which the C-terminal 136 amino acids of yeast invertase were replaced by the C-terminal 110 amino acids of gp80. When expressed in *D. discoideum*, this protein (Inv-gp80) was not GPI-anchored and was retained in a pre-Golgi compartment. Inv-gp80 did, however, display characteristics of a transmembrane protein, suggesting a novel mechanism for its retention. We also expressed a truncated version of the hybrid protein in which the C-terminal 22 amino acids of the Inv-gp80 were deleted. The truncated protein (Inv-gp80stop) was O-glycosylated and secreted. These observations indicate that the hybrid protein is not abnormally folded and demonstrate the importance of the C-terminal 22 amino acids in the retention of Inv-gp80. Together, the data suggest that oligomerization of the protein blocks its GPI anchoring.

INTRODUCTION

The life cycle of the cellular slime mould *Dictyostelium discoideum* provides a model system for the study of a number of developmental processes. In the presence of a food source, the amoebae live as single cells. Upon starvation, individual cells amass to form aggregates which follow a complex series of morphogenetic cell movements to form a fruiting body [1]. This is accompanied by the expression of new cell-surface components that mediate the formation of specific intercellular contacts at varied stages in the developmental cycle. One of the best characterized adhesion molecules is gp80. It is a cell-surface glycoprotein with an apparent molecular mass of 80 kDa [2], and is thought to be responsible for the formation of the EDTA-resistant cell contacts that form during aggregation [3]. Gp80 is first expressed at the onset of cell migration and accumulates during aggregation. Expression of gp80 drops off rapidly after aggregation, but the protein is later re-expressed for a short period during culmination [4]. The latter refers to a late stage in the developmental cycle where the cell mass emerges from the substratum to form a stem of stalk cells topped by a mass of spore cells, otherwise known as the fruiting body.

The gp80 cDNA encodes a protein of 514 amino acids including a 19-amino-acid signal leader [5,6]. The protein is extensively modified by both N-linked and O-linked oligosaccharides [7,8]. The latter, referred to as type II oligosaccharides, are reflected by the post-translational addition of oligosaccharides that contribute $\sim$ 10–12 kDa to the apparent molecular mass of the protein. Neither type of glycosylation appears necessary for gp80 to function as an adhesion molecule [9,10]. However, the O-linked oligosaccharides may protect the protein from cell-surface degradation by external proteases [8].

In addition, gp80 is modified at its C-terminus by a glycosyl-phosphatidylinositol (GPI) anchor which serves to target the protein to the plasma membrane [11,12]. The presence of an endogenous phosphatidylinositol-specific phospholipase C (PI-PLC)-like enzyme that releases gp80 into the medium under physiological conditions could suggest that the anchor is also important in regulating cell-surface levels of the protein [13]. Little else is known about the function of the anchor.

Also lacking is any information concerning how anchor attachment is signalled in this system. In recent years numerous studies have provided insights regarding necessary structural requirements for the addition of GPI anchors to proteins in mammalian systems. These requirements include an extreme C-terminal hydrophobic domain [14] and a proper cleavage attachment site [15] (called the ω site in studies by Gerber et al. [16]). Only small amino acids are tolerated at the ω site, and substitution of large amino acids at this position blocks GPI anchoring [17,18]. Additionally, amino acids with small side chains seem to be required at positions immediately C-terminal to the cleavage attachment site (termed the $\omega+1$ and $\omega+2$ positions) [16–18]. The positioning of the ω site has also been shown to be important, with the optimum placement being 10–12 amino acids from the start of the C-terminal hydrophobic domain [19].

It is currently unknown whether these requirements, as defined by mammalian systems, are universal. This issue becomes more pertinent in light of the recent report that expression of the variant surface glycoprotein (VSG) from *Trypanasoma brucei* in COS (African green monkey CV-1) cells resulted in efficient synthesis of VSG protein, but only a small percentage of the protein was GPI-anchored [20]. Mutations in the amino acids comprising the VSG cleavage attachment site converted the VSG signal into a form efficiently recognized by the mammalian anchoring apparatus. These data suggest that the degree of specificity in the signals for anchoring is greater than was previously recognized.

The GPI anchor of gp80 has at least two unique features. First, the lipid-bearing constituent of this anchor appears to be a ceramide, rather than a phosphatidylinositol [12]. In this regard, the anchor of gp80 resembles the anchor of proteins produced in yeast and in the lipopeptidophosphoglycans of *Trypanosoma cruzi* [21,22]. Second, the anchor is resistant to cleavage by added PI-PLC [12,13]. Few examples of such resistance have been documented. In the case of human acetylcholine esterase, this reflects the acylation of the inositol ring [23]. The reason for the

Abbreviations used: COS, African green monkey CV-1; Endo H, endoglycosidase H; ER, endoplasmic reticulum; GPI, glycosyl-phosphatidylinositol; Inv, invertase; NP40, Nonidet P-40; PI-PLC, phosphatidylinositol-specific phospholipase C; VSG, variant surface glycoprotein.

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insensitivity of gp80 to added PI-PLC is unclear, but does not appear to reflect an acylation of the inositol ring or the use of a ceramide backbone [24]. As part of our studies of the biogenesis of gp80, we became interested in determining the signal(s) for the addition of this unique GPI anchor to the protein and the basis for its resistance to PI-PLC. Comparisons between the requirements for such events in lower eukaryotes with those previously defined in mammalian systems may help to clarify additional or alternative rules for GPI-anchoring and/or PI-PLC resistance.

To address these questions, we created a hybrid protein in which the C-terminal sequences of a reporter molecule were replaced with the C-terminal sequences of gp80. This hybrid protein when expressed in *D. discoideum* was not GPI anchored, although it contained more than the minimal sequences deemed sufficient to signal GPI anchoring. The hybrid protein was instead retained within the cell and displayed some unique characteristics compared with proteins whose GPI anchoring is blocked in mammalian cells. Our results suggest that additional requirements are required for GPI anchoring to occur, and raise the possibility that alternative signals and mechanisms exist for the retention and degradation of normally GPI-anchored proteins which fail to be modified.

MATERIALS AND METHODS

Plasmid construction

pDNeo67Hex$_{22}$Suc, which contains the full-length invertase cDNA in the R1 site of the pDNeo67 vector, was the gift of Dr. T. Graham and Dr. A. Kaplan (University of Arkansas). The construction of this plasmid, and that used for overexpression of gp80, is described elsewhere [25,26]. To create pDNeo67Hex$_{22}$Suc117, pDNeo67Hex$_{22}$Suc was digested with *Hpa*I and *Sac*I (New England Biolabs) to release a fragment encoding the C-terminal 136 amino acids of invertase. This was replaced with the fragment obtained by digesting the gp80 cDNA with *Hinc*II and *Sac*I. That fragment contains the sequences encoding the C-terminal 110 amino acids of gp80. pDNeo67Hex$_{22}$Suc117stop was generated by using PCR to introduce a stop codon at Ser^{494}. Other recombinant procedures were performed according to Maniatis and colleagues [27].

Cell culture conditions

Ax-2 amoebae [28] were grown axenically in HL-5 media [29]. Cells were transformed as described by Nellen et al. [30]. Stable transformants were selected for G418 (20 μg/ml) resistance and maintained under constant selection. For treatment with varied proteases, cells were harvested by centrifugation (3000 *g* for 10 min), washed with 10 mM Tris, pH 7.4, and either resuspended in 10 mM Tris, pH 7.4, or solubilized with 0.5% (v/v) Nonidet P-40 (NP40)/NET (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA). The latter were left on ice for 10 min, and then cleared of insoluble material by centrifugation at 15000 *g* for 10 min. Intact cells or cell lysates were incubated with 25 μg of either bromelain (Boehringer Mannheim) or trypsin (Sigma) for 2 h at room temperature. Reactions were stopped by boiling in sample buffer and analysed by Western blots. Tunicamycin treatment was carried out by growing cells in media containing 5 μg/ml tunicamycin for 4 h (Inv-gp80) or 16 h (Inv).

SDS/PAGE and Western blotting

SDS/PAGE was performed by the method of Laemmli [31] using 10% (w/v) gels. For immunoblotting, gels were transferred to nitrocellulose (Schleicher and Schuell) by the method of Towbin et al. [32]. After transfer, filters were blocked by incubation in TBS (10 mM Tris, pH 7.4, 150 mM NaCl) containing 5% (w/v) non-fat dry milk. Filters were incubated overnight at room temperature with polyclonal antibodies directed against invertase (gifts from A. Kaplan, University of Arkansas, and T. Stevens, University of Oregon) diluted 1:200 in blocking buffer, or a polyclonal antibody against gp80 diluted 1:800 in blocking buffer. Filters were washed in TBS, and incubated with ^{125}I-labelled Protein A (ICN) in blocking buffer for 2 h. The filters were again washed with TBS, dried and autoradiographed at −70 °C with intensifying screens.

Labelling and endoglycosidase H analyses

For pulse-chase analyses, cells were labelled at 1×10^8 cells/ml in HL-5 with 0.5 mCi/ml [^{35}S]methionine (ICN) for 5 min. After labelling, cells were centrifuged at 15000 *g* for 1 min and resuspended at 1×10^7 cells/ml in fresh HL-5 containing 10 mM methionine. At the indicated times, 4×10^7 cells were harvested and lysed in TBS containing 1% (w/v) SDS. Cell lysates were boiled for 5 min and cleared by centrifugation at 15000 *g* for 10 min. Cleared lysates were diluted fivefold with TBS containing 1% (v/v) Triton X-100 and immunoprecipitated with an anti-invertase antibody (the gift of Dr. P. Silver, Princeton University).

For fatty-acid labelling, cells were incubated with [9,10-^{3}H]palmitate under the exact conditions used to label the GPI-anchor of gp80 [26]. Samples were lysed as described above and immunoprecipitated with anti-invertase antibody. Samples were analysed by SDS/PAGE and autofluorography. To monitor endoglycosidase H (Endo H) sensitivity, cells expressing Hex$_{22}$Suc117, or media containing Hex$_{22}$Suc, were diluted with 100 mM sodium citrate buffer, pH 5.5, containing 1% SDS. Samples were denatured by boiling, cleared by centrifugation at 15000 *g* for 10 min and diluted with 100 mM sodium citrate buffer, pH 5.5, containing 1% Triton X-100. Endo H was added and the samples incubated at 37 °C for 24 h. Reactions were stopped by the addition of sample buffer and boiling, and analysed by Western blotting.

Additional protein characterization

To assess the membrane association of Inv-gp80, 1.5×10^8 vegetatively growing cells were lysed by freeze/thaw in TED buffer (10 mM Tris, pH 8.0, 1 mM EDTA, 1 mM EGTA, 1 μM dithiothreitol, 40 mM $Na_4P_2O_7$,10H_2O and a mixture of protease inhibitors [11]). Cell lysates were centrifuged at 24000 *g* for 20 min at 4 °C to obtain a crude membrane fraction. Membranes were resuspended in TED containing 100 mM sodium carbonate, pH 11.5, such that the total protein concentration, as determined by the method of Lowry [33], was 1 mg/ml [34]. Samples were mixed thoroughly, left on ice for 1 h, and then centrifuged at 145000 *g* for 2 h at 4 °C. The pellet was resuspended in TED buffer and aliquots were incubated in the presence or absence of 0.4 M NaCl or 0.6% (v/v) Tween 20 for 30 min on ice. Samples were then centrifuged at 100000 *g* for 1 h at 4 °C. The different fractions were analysed by Western blotting as described above. Triton X-114 phase separation was performed by the method of Bordier [35]. Size-exclusion chromatography was performed using a 1.6 cm × 45 cm Sephadex G-200 (Sigma) column equilibrated with PBS, pH 7.2. Medium from cells expressing Inv-gp80stop was concentrated ~ 50-fold by Amicon filtration using XM-50 filters. Concentrated medium was then diluted 1:4 with PBS and applied to the column. The flow rate was 0.3 ml/min. Fractions (0.5 ml) were collected and precipitated using trichloroacetic acid with 25 μg of BSA added as a carrier. Precipitates were then analysed by SDS/PAGE and Western blotting. The

resulting autoradiograms were quantified by scanning densitometry using a BioRad densitometer and the 1-D analyst program.

RESULTS

Two approaches are generally used to identify sequences that signal the addition of a GPI anchor to proteins. One is to mutate the C-terminus of the GPI-anchored protein to identify specific amino acids important to the addition of the modification. An alternative approach involves adding the C-terminal sequences of a GPI-anchored protein to a reporter molecule and evaluating whether they confer upon the protein the ability to be GPI-anchored. We have chosen the second approach in our attempts to identify the sequences that signal the addition of a GPI anchor to the *D. discoideum* protein gp80. Yeast invertase was used as the reporter molecule.

Schematic representations of the constructs studied are shown in Figure 1. $Hex_{22}Suc$ contains sequences encoding the N-terminal 22 amino acids of *D. discoideum* β-hexosaminidase fused in-frame to sequences encoding full-length yeast invertase. The 22 amino acids of β-hexosaminidase serve as a signal leader, directing the protein into the lumen of the endoplasmic reticulum (ER) [25]. $Hex_{22}Suc$ encodes a protein (Inv) of 512 amino acids with a minimum predicted molecular mass of 58 kDa. The actual molecular mass of the protein is expected to be influenced by the addition of N-linked oligosaccharides, as the protein contains 13 potential sites for the addition of that modification [36].

For $Hex_{22}Suc$-gp80, the sequences encoding the C-terminal 136 amino acids of invertase were replaced by those encoding the C-terminal 110 amino acids of gp80. This segment of gp80 contains more than the sequences shown to be necessary to signal the addition of a GPI anchor in other systems [14–19]. Additionally, this region of gp80 is rich in serine and threonine residues and thus is believed to contain the putative sites modified by O-linked (type II) oligosaccharides [8]. The post-translational modification of the hybrid protein, Inv-gp80, could then serve as a marker for passage of this protein through the Golgi network.

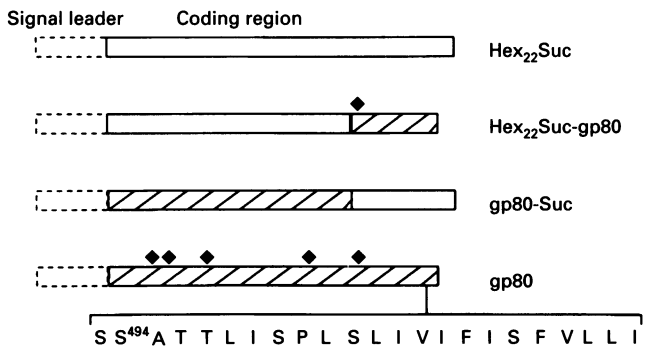

Figure 1 Schematic presentations of hybrid proteins

This Figure presents schematic diagrams of the expected translation products from the cDNA constructs (named on the right) used in this study. Amino acids encoded by the *Suc* gene are shown as clear boxes, and those encoded by gp80 are indicated by the hatched areas. The N-terminal signal leader of each protein is defined by the dashed box. Potential sites for the modification of gp80 by N-linked oligosaccharides are indicated by the diamonds. The sequence (one-letter code) of the C-terminal 23 amino acids of gp80, including Ser^{494}, the amino acid adjacent to the putative site for attachment of the GPI anchor, is given at the bottom of the Figure.

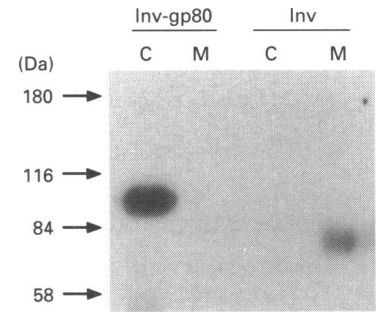

Figure 2 Expression of Inv and Inv-gp80 in *D. discoideum*

Cells (C) and media (M) from transformants expressing Inv-gp80 or Inv were harvested and analysed by SDS/PAGE and Western blotting as described in the Materials and methods section. Western blots were probed with a polyclonal antibody directed against invertase. The positions of the molecular-mass markers are shown to the left.

The predicted molecular mass of the unmodified protein is ~ 56 kDa. The protein contains 11 potential sites for the addition of N-linked oligosaccharides.

For gp80-Suc, the C-terminal 110 amino acids of gp80 were replaced with the C-terminal 136 amino acids of invertase. The unmodified protein encoded by this construct has a predicted molecular mass of ~ 50 kDa and has six potential N-glycosylation sites. It is not expected to be modified by either O-linked oligosaccharides or by a GPI anchor. gp80 encodes a full-length gp80 protein which migrates upon SDS/PAGE with a molecular mass of ~ 80 kDa.

All constructs were expressed in the pDNeo67 vector [26] which allows for expression the these proteins in vegetative cells.

Cells and media from each of the transformants were analysed by SDS/PAGE and Western blotting. We have previously shown that vegetative *D. discoideum* transformed with the gp80 cDNA expresses a protein indistinguishable from the endogenous protein expressed during starvation [26]. Specifically, the extent to which the protein undergoes N- and O-glycosylation is unaltered, as is its GPI anchoring. And, as with starved cells, the anchor of gp80 synthesized in growing cells is resistant to added PI-PLC [26]. Cells transformed with the gp80-Suc construct did not produce detectable protein. Because the gp80-Suc mRNA was present, it is likely that any protein synthesized was rapidly degraded. No additional experiments were performed with this transformant.

Figure 2 shows the results obtained when cells were transformed with the cDNA for $Hex_{22}Suc$. In this case, they secreted a protein (Inv) recognized by the invertase antiserum. No Inv was found associated with the cells. Similar results have been reported by Lacoste, Graham and Kaplan [25]. A different picture emerged with cells transformed to express the $Hex_{22}Sucgp80$ construct. The protein product (Inv-gp80) in this case was cell-associated and none was detected in the medium. The data indicate that the presence of the C-terminal sequences of gp80 can render invertase a cell-associated protein.

Localization of Inv-gp80 in *D. discoideum*

We have previously shown that the GPI anchor of gp80 is sufficient to target this protein to the plasma membrane [11]. Thus, if Inv-gp80 were modified by a GPI anchor, it should also localize to the plasma membrane. This possibility was examined by determining the protease sensitivity of Inv-gp80 using intact cells. If expressed on the plasma membrane, the protein should

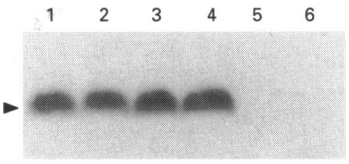

Figure 3 Protease sensitivity of Inv-gp80 in intact cells and cell lysates

Cells were harvested and resuspended in 10 mM Tris, pH 7.4, (lane 1) or with buffer containing 25 μg of bromelain (lane 2) or 25 μg of trypsin (lane 3). Alternatively, cells were lysed with 0.5% (v/v) NP40–NET and lysates were incubated in the absence (lane 4) or presence of bromelain (lane 5) or trypsin (lane 6) for 2 h at room temperature. Reactions were stopped by the addition of SDS/PAGE sample buffer and the products were analysed by Western blotting. The position of Inv-gp80 is indicated by the arrowhead.

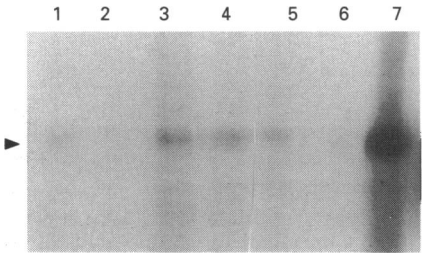

Figure 4 Pulse-chase analysis of Inv-gp80

Vegatatively growing cells expressing Inv-gp80 were labelled in HL-5 with [^{35}S]methionine for 5 min. Cells were then incubated in media containing 10 mM methionine for 2 h. Inv-gp80 was immunoprecipitated after the initial radiolabelling (lane 1) and after 10 min (lane 3), 30 min (lane 4) and 2 h (Lane 5) of chase. Control immunoprecipitations (no antibody added) performed after the initial labelling and after 2 h of chase are shown in lanes 2 and 6 respectively. Inv-gp80 immunoprecipitated from cells labelled continuously for 2 h is shown in lane 7. The position of Inv-gp80 is indicated by the arrowhead.

be susceptible to digestion. Protection from digestion under these conditions, but hydrolysis in corresponding cell extracts, would suggest that Inv-gp80 is not expressed on the cell surface. Intact cells and cell lysates were incubated with trypsin or bromelain.

As shown in Figure 3, Inv-gp80 was readily digested when cell lysates were incubated with each of the proteases. However, the protein was protected from digestion when intact cells were treated with the same proteases. Digestion of Inv-gp80 was dependent upon the presence of the protease as mock-treated cells or lysates showed no apparent degradation. These data indicate that Inv-gp80 is not expressed on the cell surface.

Additional information concerning the localization of Inv-gp80 was obtained in pulse-chase experiments. As previously mentioned, the construct was designed to detect the passage of the protein through the Golgi as reflected by its post-translational modification. Cells were incubated with [^{35}S]methionine for 10 min, washed, and chased for 2 h in the presence of excess unlabelled methionine. Inv-gp80 was immunoprecipitated after the initial radiolabelling and at various times during the 2 h chase. As shown in Figure 4, no change in the apparent molecular mass of the protein occurred during the chase period, indicating that Inv-gp80 was not post-translationally modified. This result suggests that Inv-gp80 does not pass through the Golgi, the site where O-glycosylation is thought to occur [37].

Further insight into the subcellular localization of Inv-gp80 was obtained by examining its Endo H sensitivity compared with that of Inv. Conversion of N-linked oligosaccharides from an Endo-H-sensitive form into one that is Endo-H-resistant marks

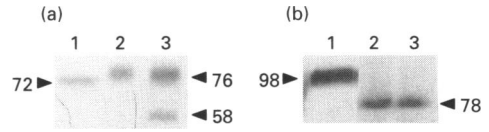

Figure 5 Endo H sensitivity of Inv and Inv-gp80

(**a**) Medium from Inv-expressing cells was denatured by boiling as described in the Materials and methods section. Samples were then incubated in the presence (lane 1) or absence (lane 2) of Endo H and analysed by Western blotting. Inv produced in cells treated with tunicamycin is shown in lane 3. (**b**) Cells expressing Inv-gp80 were lysed in SDS as described in the Materials and methods section. Lysates were incubated in the presence (lane 2) or absence (lane 1) of Endo H. Inv-gp80 produced from cells grown in the presence of tunicamycin is shown in lane 3. The apparent M_r ($\times 10^{-3}$) of each product is indicated by the arrowhead.

the passage of a protein through the medial Golgi [38]. We first evaluated the degrees to which Inv and Inv-gp80 were modified by N-linked oligosaccharides by treating cells expressing each of these proteins with tunicamycin to inhibit N-linked glycosylation. As shown in Figure 5, both Inv and Inv-gp80 produced in cells treated with tunicamycin displayed lower apparent molecular masses upon SDS/PAGE compared with the proteins synthesized in untreated cells. That of Inv was 58 kDa, a size consistent with the molecular mass of the unmodified protein as predicted from its amino acid sequence. In this experiment the effects of tunicamycin appeared to wear off during the 16 h treatment, resulting in the expression of the fully modified form of Inv. On the basis of this result, shorter incubation times (4 h) were chosen for treatment of cells expressing Inv-gp80. In cells treated with tunicamycin, synthesized Inv-gp80 migrated upon SDS/PAGE as a 78 kDa protein, compared with the 98 kDa protein synthesized in untreated cells. Thus, as in the case of Inv, N-linked oligosaccharides contribute ~ 20 kDa to the apparent molecular mass of Inv-gp80.

We next assessed the Endo H sensitivity of the oligosaccharides on Inv and Inv-gp80. As shown in Figure 5(a), the oligosaccharides of Inv were largely insensitive to Endo H, although some digestion was suggested by a small increase in the mobility of the protein (from 76 to 72 kDa) after digestion. Partial Endo H sensitivity of the N-linked oligosaccharides of invertase has been previously reported [39,40] and may reflect the inaccessibility of specific chains to the processing enzymes as the protein is transported through the Golgi [41]. In contrast to Inv, however, the N-linked oligosaccharides of Inv-gp80 were completely sensitive to Endo H. Inv-gp80 digested with Endo H showed the same mobility upon SDS/PAGE as Inv-gp80 obtained from cells grown in the presence of tunicamycin. The lack of any Endo H resistance indicated that Inv-gp80 was located in a pre-Golgi compartment.

Inv-gp80 is membrane-bound but not GPI-anchored

Given the abnormal localization of Inv-gp80, we next determined the basis for the observed cellular association of the protein. Preliminary results were obtained by fractionating cells into crude membrane and cytosolic fractions. In those experiments, Inv-gp80 co-fractionated with the crude membrane pellet. Membranes were then treated with 100 mM sodium carbonate, pH 11.5, to release soluble proteins trapped within membrane vesicles, and to strip peripherally associated proteins from the membrane sheets [34]. Membranes from gp80-expressing cells

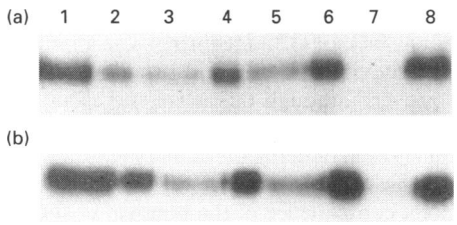

Figure 6 Membrane association of Inv-gp80

Cells expressing Inv-gp80 (**a**) or gp80 (**b**) were lysed, and crude membrane fractions obtained by centrifugation as described in the Materials and methods section. Membranes were treated with 100 mM sodium carbonate, pH 11.5, and then centrifuged at 145000 ***g*** to obtain membrane (lane 8) and supernatant (lane 7) fractions. The membrane fraction was then divided into three aliquots and incubated with 0.6% Tween 20 (lanes 1 and 2), 0.4 M NaCl (lanes 3 and 4) or no additions (lanes 5 and 6). After incubation for 30 min, samples were centrifuged to obtain membrane (lanes 2, 4 and 6) and supernatant (lanes 1, 3 and 5) fractions. Samples were analysed by Western blotting using anti-Inv antiserum (**a**) or anti-gp80 (**b**) antiserum.

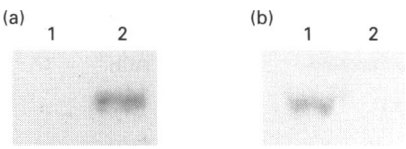

Figure 7 Triton X-114 partitioning of Inv-gp80.

Cells expressing Inv-gp80 (**a**) and gp80 (**b**) were solubilized in ice-cold 10 mM Tris, pH 7.4, 150 mM NaCl containing 1% (v/v) Triton X-114. The resulting lysates were cleared by centrifugation at 4 °C and layered over a sucrose cushion. Samples were heated to 37 °C and the aqueous and detergent phases were separated by centrifugation. The phases were separated and treated for analysis by SDS/PAGE and Western blotting as described in the Materials and methods section. Lane 1, detergent phase; lane 2, aqueous phase.

were also monitored in these experiments to verify the behavior of an integral membrane protein.

As shown in Figures 6(a) and 6(b), both Inv-gp80 and gp80 remained associated with the membrane fraction after treatment with sodium carbonate. As a further test of their membrane association, membranes were subsequently treated with either 0.4 M NaCl or 0.6 % (v/v) Tween 20. The membrane association of each of the proteins was unaffected by the NaCl treatment. A small amount of protein did not centrifuge at 100000 ***g***, but similar results were seen with mock-treated membranes. In contrast, both gp80 and Inv-gp80 were effectively released when membranes were treated with detergent. It appears, therefore, that substitution of the C-terminal amino acids of invertase with those of gp80 rendered Inv-gp80 membrane bound.

To evaluate whether Inv-gp80 was GPI-anchored, we examined whether the protein could be radiolabelled by a GPI-anchor constituent. Cells were incubated with [^{3}H]palmitate under the same conditions that label the GPI anchor of gp80 [26], and Inv-gp80 was immunoprecipitated as described in the Materials and methods section. The lack of radiolabelling suggested that Inv-gp80 was not GPI-anchored (results not shown). This was confirmed by examining the partitioning of the protein in Triton X-114. GPI-anchored proteins are known to partition into the detergent phase of Triton X-114. This reflects the presence of the extremely hydrophobic anchor, because these proteins will partition into the aqueous phase of Triton X-114 when their anchoring is blocked [42,43]. The partitioning of Inv-gp80 was compared with that of gp80 to verify the behavior of an anchored protein.

As expected, gp80 partitioned into the detergent phase when extracted with TritonX-114 (Figure 7). In contrast, Inv-gp80 partitioned into the aqueous phase, indicating that it does not have the same hydrophobicity as a GPI-anchored protein. On the basis of these observations, it would appear that Inv-gp80 is not GPI-anchored, and that its membrane attachment reflects the retention of the C-terminal amino acids that are normally removed during the anchoring process.

Additional evidence that the presence of the extreme C-terminal amino acids of gp80, normally removed during GPI anchoring, are responsible for the membrane association and cellular retention of Inv-gp80 was obtained by transforming cells with the Hex_{22}Suc–gp80stop construct. In this construct, Ser^{494} was changed to a stop codon (see Figure 1). Ser^{494} is immediately C-terminal to the amino acid believed to be the site for attachment of the GPI anchor. The protein (Inv-gp80stop) encoded by this construct would thus lack the C-terminal hydrophobic domain and therefore should produce a soluble form of Inv-gp80 if the aforementioned premise is correct. In addition, if the resulting protein is able to fold properly, it should be secreted.

As shown in Figure 8, Inv-gp80stop was secreted into the culture medium. It was noted, however, that the secretion of Inv-gp80stop was not as complete as the secretion of Inv, in that some cell-associated Inv-gp80stop was detectable. Technical difficulties, e.g. significant differences in the stability of the cellular and secreted forms of the protein, currently limit our ability to quantify the percentage of the protein that is retained. The molecular mass of the cell-associated Inv-gp80stop was ~ 10 kDa smaller than that of the secreted protein. This difference in molecular mass reflects the post-translational modification of the protein signalled by the remaining sequences of gp80 as it passes through the Golgi network. O-linked oligosaccharides contribute a similar increase in the apparent molecular mass of gp80. The fact that a population of Inv-gp80stop was properly processed and secreted suggested that Inv-gp80 could also achieve a relatively normal conformation. These data also indicate that complete retention of Inv-gp80 in a pre-Golgi compartment is dependent upon the presence of the C-terminal hydrophobic amino acids.

Because Inv-gp80 contains all the signals thus far defined in mammalian cells as being necessary for GPI anchoring, the question arises why are the C-terminal hydrophobic sequences retained in this construct? Invertase exists in its native state as a multimer [44]. Initially synthesized as dimers, it can assemble into tetramers and octamers under proper conditions of pH and protein concentration [45]. It is possible that oligomerization, a normal part of the biogenesis of invertase, blocks the GPI

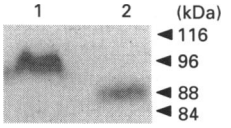

Figure 8 Expression of Inv-gp80stop in *D. discoideum*

The Figure shows an autoradiogram of a Western blot of media (lane 1) and cells (lane 2) from transformants expressing Inv-gp80stop. The apparent molecular masses of the proteins (96 kDa for the secreted form, and 88 kDa for the cellular form) estimated from molecular-mass markers are shown on the right. The positions of two of the markers are indicated.

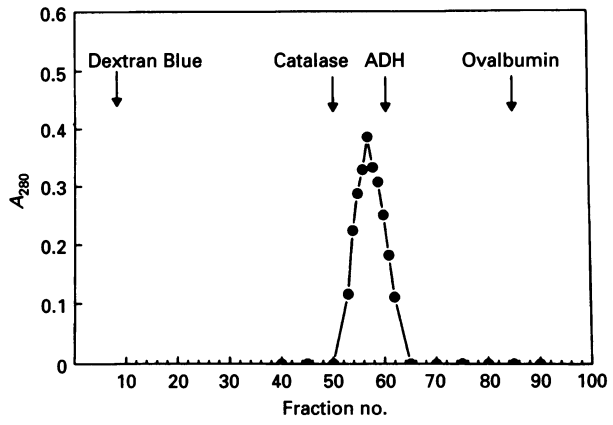

Figure 9 Size-exclusion chromatography of Inv-gp80stop

Medium from cells expressing Inv-gp80stop was chromatographed over Sephadex G-200. The column was developed with PBS, pH 7.2. Fractions (0.5 ml) were collected and analysed by Western blotting. The resulting autoradiograms were quantifed by densitometry. Molecular-mass markers are catalase (232 kDa), alcohol dehydrogenase (150 kDa) and ovalbumin (43 kDa).

anchoring of Inv-gp80. To evaluate the validity of this hypothesis, we determined the multimeric state of the hybrid proteins. Medium from cells expressing Inv-gp80stop was chromatographed over a Sephadex G-200 column. Individual fractions were collected and analysed by SDS/PAGE and Western blotting. As shown in Figure 9, Inv-gp80stop eluted in fractions with a corresponding molecular mass of ~ 190 kDa. We attempted a similar analysis of Inv-gp80 but found variable results, most likely attributable to the need for detergent solubilization of the protein and the resulting formation of micelles. The results obtained with Inv-gp80stop, however, indicate that the gp80 sequences do not alter the ability of the invertase moiety to form dimers.

DISCUSSION

In this study we attempted to define the signal for GPI anchoring of the *D. discoideum* protein gp80. To do so we generated a fusion protein in which the C-terminal amino acids of invertase were replaced by the C-terminal amino acids of gp80. When expressed in *D. discoideum* the fusion protein, Inv-gp80, was not GPI-anchored, as determined by the partitioning of the protein into the aqueous phase of Triton X-114 and the lack of labelling with anchor precursors, i.e. fatty acid. This was rather unexpected because our fusion protein contained more than the necessary sequences required to signal GPI anchoring, as defined in other systems.

While this work was in progress a report by Barth et al. [46] demonstrated that the C-terminal 25 amino acids of gp80 were sufficient to confer GPI anchoring upon a different reporter molecule expressed in *D. discoideum*, indicating that our fusion protein did indeed contain the signal necessary for its anchoring. It is possible that overexpression of Inv-gp80 saturated the cellular machinery responsible for the anchoring process. In that case, however, we would have expected some percentage of the protein to have been appropriately anchored and transported to the cell surface. No evidence of that occurring was obtained. In addition, the observation that overexpression of gp80 does not result in its abnormal processing [26] indicates that cells can readily accommodate higher levels of such proteins and appropriately modify them.

We also considered the possibility that misfolding of Inv-gp80 could explain its lack of GPI anchoring. The absence of measurable invertase activity of the hybrid protein could support this premise. However, although the active-site aspartate is located near the N-terminus of invertase [47], the contribution of the C-terminal sequences in defining the active site is not known. Thus, it is also possible that the absence of invertase activity of Inv-gp80 is a direct consequence of the removal of those sequences. The finding that Inv-gp80 displays an aberrant molecular mass upon SDS/PAGE could be considered as another argument that this protein is abnormally folded. The protein migrates as a 98 kDa protein, as opposed to the 76–78 kDa protein predicted from its amino acid sequence and N-glycosylation. However, a similar disparity is also seen for gp80. Its predicted molecular mass, based on its amino acid sequence, is 49 kDa. The unglycosylated protein, however, migrates with an apparent molecular mass of 54 kDa. The underlying reasons for this disparity are not known, but it appears that all proteins bearing the C-terminal sequences of gp80 display this property. This includes not only Inv-gp80 and gp80 but also Inv-gp80stop, whose conformation is sufficiently normal to render the protein secretable, and a cytosolic protein that we have modified to contain these sequences (P. Pauly, unpublished work). The C-terminal domain of gp80 is a serine/threonine-rich region thought to form an extended structure because of the presence of numerous proline residues. This extended structure may have limited binding capacity for SDS or otherwise resist denaturation by the detergent, resulting in the anomalous migration of the proteins upon SDS/PAGE.

Independent of the underlying mechanism, the aforementioned examples illustrate that aberrant migration upon SDS/PAGE of Inv-gp80 does not necessarily indicate an abnormally folded protein. Indeed, the finding that Inv-gp80stop attains a secretion-competent conformation would argue that the presence of the C-terminal gp80 sequences does not result in the misfolding of the hybrid protein. This premise is further strengthened by the observation that secreted Inv-gp80stop protein was post-translationally modified. The contribution of the O-linked oligosaccharides to the molecular mass of Inv-gp80stop is similar to the contribution that O-linked oligosaccharides make to the molecular mass of gp80 [7]. This indicates that the sites for modification are exposed equally on both proteins, supporting the premise that the gp80 sequences have assumed their correct conformation. (The data also confirm the proposal that the C-terminus of gp80 is the region that is O-glycosylated.)

In addition, one may consider that the conformation of the hybrid protein is sufficiently normal to undergo the oligomerization characteristic of invertase. Another observation that would support the premise that the Inv-gp80 fusion protein is not grossly misfolded is that the addition of N-linked oligosaccharides appears to be unaffected by the substitution of the sequences of gp80. This modification contributes ~ 18–20 kDa to the apparent molecular mass of both Inv and Inv-gp80. It has been suggested that the appropriate addition of N-linked oligosaccharides to a protein reflects its proper folding [48]. Inv-gp80 does differ from Inv-gp80stop in that it contains the extreme C-terminal hydrophobic amino acids normally removed during the anchoring process. Thus, it is possible that this hydrophobic domain imposes a misfolded structure upon Inv-gp80, rendering it incapable of being GPI-anchored. Such a scenario would suggest that the C-terminal 22 amino acids affect the folding of the invertase moiety through the serine/threonine-rich region, which, although possible, appears unlikely.

We would like to propose, as an alternative explanation for the defect in GPI anchoring of Inv-gp80, that it is dimerization of the protein that blocks this process. At first glance, this hypothesis

may seem contradictory to the observation that some GPI-anchored proteins and GPI-anchored chimeras have been shown to exist as oligomers [49,50]. Studies concerning the biogenesis of these proteins indicate, however, that the formation of such oligomers is preceded by a folding step in which the protein exists as a monomer [51,52]. GPI anchoring has been shown to occur very rapidly during the biogenesis of a protein, most probably within the ER membrane during the translocation of the protein [53]. Anchoring, therefore, would precede the oligomerization of these proteins. In contrast, dimers of invertase are the first products observed which, under optimal conditions, may further associate into tetramers and octamers [54]. The monomeric form of the protein has been detected only under denaturing conditions [45]. The rapid formation of the Inv-gp80 homodimer could precede the initiation of the anchoring process and thereby sterically hinder it from occurring. We suggest therefore that in addition to possessing the appropriate signalling sequences, proteins destined to be GPI-anchored must avoid the rapid formation of such protein complexes. Evidence in support of this model comes from studies of membrane-bound IgD. Membrane-bound IgD has been shown in some cell lines to exist as either a transmembrane protein or a GPI-anchored protein [55]. Expression of the transmembrane form of the protein is dependent upon the co-expression of two other proteins, Ig-α and Ig-β. These form a disulphide-linked heterodimer which complexes with IgD in the lumen of the ER. Absence of the α/β heterodimer results in the expression of a GPI-anchored IgD [56]. This implies that the formation of oligomeric structures can block the GPI-anchoring process. Thus one can propose that the dimerization of Inv-gp80 blocks the GPI anchoring of this protein in a manner similar to that seen for the IgD–Ig-α–Ig-β heterotrimer.

Inv-gp80 was not GPI-anchored and was not transported to the cell surface. On the basis of the pulse-chase analysis, indicating a lack of any post-translational modifications, and the sensitivity of its N-linked oligosaccharides to Endo H, we conclude that Inv-gp80 was retained in a pre-Golgi compartment. Such a localization of Inv-gp80 is consistent with reports from other researchers examining the fate of proteins whose GPI anchoring was artificially prevented, either by expressing the protein in cells defective in the anchoring process, or by mutating the GPI-anchoring signal to render it non-functional [57,58]. Our experimental design was distinct, however, in that Inv-gp80 was expressed in a system capable of anchoring the expressed protein, and that the C-terminal sequence of the fusion protein was not altered in a manner that would affect its ability to signal anchoring. Our findings are novel in that Inv-gp80 behaved as a transmembrane protein, i.e. it remained membrane-associated after treatment with sodium carbonate and NaCl. Because Inv-gp80stop is a soluble protein, the C-terminal hydrophobic domain of Inv-gp80, normally removed during the anchoring process, is now serving as a transmembrane domain. This contrasts with several other reports which have suggested that proteins whose GPI anchoring is prevented are soluble [59,60]. This was most rigorously examined by Delahunty et al. [59]. Q7b, normally a GPI-anchored protein, was expressed in LM-TK$^-$ cells, a line defective in anchor biosynthesis. Like Inv-gp80, Q7b was not GPI-anchored and retained its hydrophobic C-terminus. Unlike Inv-gp80, however, Q7b was released from membranes by treatment with sodium carbonate. It is not clear whether additional experiments will indicate that proteins whose anchoring is prevented exist predominantly as integral membrane proteins or as soluble proteins. Our data do, however, raise the question as to the reason for the different behaviours of Inv-gp80 and Q7b. The C-terminal sequences of both proteins contain the general features attributed to GPI-anchor signalling sequences. The one notable difference between them is the presence of a single charged residue, Asp^{316}, in the C-terminus of Q7b which is lacking in gp80. The presence of this charged residue could influence the hydrophobicity of the C-terminal region of Q7b, thereby limiting its ability to act as a transmembrane domain. Consistent with this hypothesis is the observation that conversion of Asp^{316} to a valine resulted in the cell-surface expression of the protein in LM-TK$^-$ cells [59].

Neither Inv-gp80 or Q7b (or other proteins unable to undergo appropriate anchoring) are transported to the cell surface. As mentioned earlier, constructs other than Inv-gp80 that are not anchored appear to be soluble, forming high-molecular-mass homoaggregates [59,60]. The formation of such micelles is attributed to the presence of the C-terminal hydrophobic domain and proposed to result in the retention of protein [59,60]. Because integral membrane proteins could not form these three-dimensional micellar structures, another mechanism must exist by which these proteins are prevented from being transported to the Golgi. It is possible that proteins such as Inv-gp80 contain an as yet unrecognized ER-retention signal encoded by its transmembrane domain. More likely, however, is the possibility that such proteins lack a component necessary for continued transport to the Golgi. The C-terminus of gp80 contains no apparent cytoplasmic tail, the absence of which may be responsible for the inability of Inv-gp80 to translocate to the Golgi. It is interesting to note that the only apparent difference between the C-terminus of Inv-gp80 and the Q7b protein containing the $Asp^{316} \rightarrow Val$ mutation (which can be transported to the cell surface) is that the latter contains a three-amino-acid cytoplasmic tail [59]. Also of interest is the observation that deletion of the three-amino-acid cytoplasmic tail of IgM results in the expression of a GPI-anchored form of this normally transmembrane protein [61]. Such observations highlight the importance of the cytoplasmic tail in determining the fate of specific proteins.

That some proteins whose anchoring is inhibited are soluble whereas others remain transmembrane indicates that more than one mechanism exists by which they are excluded from the normal transport process. This also raises the question as to whether or not the fates of all such proteins are identical. By existing criteria, they both appear to reside in a pre-Golgi compartment. For those proteins that form micellular aggregates, it has been reported that they localize to cytoplasmic vesicles distinct from the ER, and also perhaps from lysosomes, although the data presented have been somewhat contradictory [57,59,60]. Studies aimed at elucidating the fate of Inv-gp80 should indicate whether the nature of the membrane association influences either the subcellular localization or the means by which proteins unable to be anchored are targeted in those compartments.

We thank Dr. T. Graham and Dr. A Kaplan (University of Arkansas) for providing the pDNeo67Hex_{22}Suc construct and invertase antiserum. We also thank Dr. T. Stevens (University of Oregon) and Dr. P. Silver (Princeton University) for their gifts of invertase antiserum. We are grateful to Jim Murray for expert technical assistance and Margaret Klevorn for help in the preparation of this manuscript. This work is supported by NIH grant GM34561. P.P. is supported by NIH Training Grant HL 07050.

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Received 1 July 1994/17 October 1994; accepted 10 November 1994