An uncleaved glycosylphosphatidylinositol signal mediates Ca^{2+} -sensitive protein degradation

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Inv-gp80 is a chimeric protein which contains a signal for the attachment of a glycosylphosphatidylinositol (GPI) anchor. When expressed in *Dictyostelium discoideum*, this protein fails to become GPI anchored and is retained within the cell as an integral membrane protein. We have compared the subcellular localization and degradation of Inv-gp80 with that of its intracellular but soluble counterpart, Inv-gp80_{sc}. Inv-gp80_{sc} lacks the hydrophobic C-terminal 22 amino acids of Inv-gp80. The N-linked oligosaccharides of both Inv-gp80 and Inv-gp80_{sc} remained sensitive to endoglycosidase H, and both proteins co-fractionated with endoplasmic reticulum marker enzymes on Percoll gradients. Under normal conditions, Inv-gp80 displayed a half-life ($t_{1/2}$) of 90 min, while Inv-gp80_{sc} displayed a $t_{1/2}$ of

120 min. The degradation of both proteins required ATP, was inhibited by tosyl phenylalanylchloromethane (Tos-Phe-CH₂Cl) and was insensitive to inhibitors of lysosomal function. While depletion of Ca^{2+} from the endoplasmic reticulum had no effect on the degradation of Inv-gp80_{sc}, it stimulated the degradation of Inv-gp80. When the GPI anchor signal sequence of Inv-gp80 was replaced with the transmembrane domain of the interleukin-2 receptor, the degradation of the protein was no longer influenced by Ca^{2+} fluxes. The data suggest that while the GPI anchor sequence of Inv-gp80 does not contain determinants regulating the degradation of the protein under basal conditions, it targets Inv-gp80 for rapid degradation under conditions where Ca^{2+} is depleted from the endoplasmic reticulum.

INTRODUCTION

In response to the removal of their nutrient source, *Dictyostelium discoideum* amoebae undertake a complex developmental programme. The initial phase of this programme is characterized by the migration of individual cells towards one another in response to the chemotactic signal, cAMP pulses [1]. During their aggregation, cells form two distinct types of contacts. Associations that can be readily disrupted by incubating cells with EDTA, referred to as EDTA-sensitive contacts, form during the earlier hours of aggregation and are believed to be mediated by gp24, a glycoprotein of approx. 24 kDa [2]. EDTA-resistant intercellular contacts are established later in the aggregation programme and apparently reflect the homophilic interaction mediated by gp80 [3]. gp80 is an 80 kDa glycoprotein which is first detected on cells at the onset of their migration. Expression of gp80 becomes maximal as cells advance in their aggregation and then decreases rapidly following the formation of the multicellular aggregates and their organization into more advanced structures. gp80 is re-expressed at a later developmental stage, early culmination, suggesting a role for this protein in the formation of the fruiting body, the final structure of the developmental programme [4].

gp80 is biochemically, as well as developmentally, a relatively complex molecule. It is extensively modified by both N-linked and O-linked oligosaccharides [5,6]. The former do not appear to influence the adhesion properties of the protein while the latter may protect the protein from proteolytic cleavage [6,7]. The membrane association of gp80 is mediated by a glycosylphosphatidylinositol (GPI) anchor [8,9]. Evidence has been

presented that the GPI anchor is responsible for the targeting of this protein to the plasma membrane [9]. It may also be important in the release of the protein from the cell surface by an endogenous GPI-phospholipase C-like activity during the formation of the multicellular aggregate [10].

Work from a number of laboratories has elucidated a variety of features necessary for the addition of a GPI anchor to a protein. These include the presence of a C-terminal hydrophobic domain preceded by a proper cleavage site to which the GPI anchor is attached [11–13]. In the case of gp80, the C-terminal 25 amino acids of the protein are sufficient to confer upon a reporter protein the ability to be GPI anchored [14]. In our studies of GPI anchoring in *D. discoideum*, we constructed a chimeric protein, Inv-gp80, in which sequences encoding the C-terminal 136 amino acids of yeast invertase were replaced with sequences encoding the C-terminal 110 amino acids of gp80. This region of gp80 contains all the sequence information necessary to direct GPI anchoring and the gp80 sequences thought to be O-glycosylated. In creating this chimera, we expected to confer upon invertase the ability to be GPI anchored. Additionally, we expected this fusion protein to be O-glycosylated, thus allowing us to monitor its transition through the Golgi. However, when expressed in *D. discoideum*, Inv-gp80 failed to become GPI anchored. The lack of GPI anchoring of Inv-gp80 was attributed to the rapid oligomerization of this fusion protein, a normal part of the biogenesis of invertase [15]. This result suggested that in addition to the appropriate sequence elements, proteins that are to be GPI anchored must also remain monomeric prior to the addition of the GPI anchor.

Abbreviations used: GPI, glycosylphosphatidylinositol; PLC, phospholipase C; TBS, Tris-buffered saline; Endo H, endoglycosidase H; ER, endoplasmic reticulum; CCCP, carbonyl cyanide *m*-chlorophenylhydrazide; Tos-Lys-CH₂Cl, *N*-tosyl-L-lysylchloromethane; Tos-Phe-CH₂Cl, *N*-tosyl-L-phenylalanylchloromethane; BHQ, 2,5-di-(*t*-butyl)-1,4-hydroquinone; hGH, human growth hormone; DAF, decay-accelerating factor; IL-2, interleukin 2; $t_{1/2}$, half-life.

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Independently of the exact mechanism, the lack of GPI anchoring provided us with the opportunity to evaluate the fate of a GPI-anchored protein when the addition of the anchor was prevented. Several investigators have examined that problem using chimeric proteins mutated in the GPI-anchoring signal sequences while, in some cases, GPI anchoring was blocked by expressing the protein in LTMK⁻ cells, which are defective in anchor biosynthesis [16–20]. Generally, the improperly processed proteins were retained within a pre-Golgi compartment. In the two cases where it was examined, the precursor proteins were not membrane-associated, even though they retained the C-terminal hydrophobic amino acids normally removed during the anchoring process [19,20]. This is a surprising finding since these C-terminal amino acids are generally thought to be indistinguishable from other transmembrane domains. Inv-gp80 expressed in *D. discoideum* was also retained in a pre-Golgi compartment but, in contrast to the aforementioned reports, was present as a membrane-bound protein. Retention of Inv-gp80 was shown to be mediated by its C-terminal 22 amino acids since removal of those sequences produced a protein that was secreted [15]. These observations raise the question as to why the failure to anchor Inv-gp80 results in its retention as an integral membrane protein. Do the C-terminal hydrophobic domains of proteins destined to be GPI anchored have unique properties? If so, do they contain specific information concerning the anchoring process itself or the fate of those proteins should that process be inhibited? Such questions are of increasing importance in light of the growing body of evidence indicating that transmembrane domains are more than just innocuous stretches of amino acids that serve only to position proteins in the membrane. Transmembrane domains have in fact been shown to confer unique qualities to proteins in terms of their localization, oligomerization and degradation [21–23]. To begin to address some of these questions, we have evaluated how the C-terminal amino acids influence the fate of Inv-gp80 in terms of its subcellular localization and degradation. The results are reported in this paper.

MATERIALS AND METHODS

Cell culture and transformation

D. discoideum Ax-2 [24] cells were grown axenically in HL-5 medium [25]. Cells were transformed as described by Nellen et al. [26]. Stable transformants were selected for G418 resistance and maintained under constant selection. The construction of the plasmids encoding Inv-gp80 and the protein lacking the C-terminal 22 amino acids has been described previously [15]. To generate Inv-gp80IL-2, a *Pst*I site was introduced into the Inv-gp80 cDNA at position 1543. Sequences encoding the C-terminal 25 amino acids were removed by digestion with *Pst*I and *Sac*I, and replaced with a PCR-derived fragment encoding nucleotides 870–996 of the human interleukin-2 (IL-2) receptor α -chain [27].

SDS/PAGE and Western blotting

SDS/PAGE was performed using 10% (w/v) acrylamide gels according to the method of Laemmli [28]. Gels were transferred to nitrocellulose (Schleicher and Schuell) by the method of Towbin et al. [29]. Membranes were blocked by incubation in Tris-buffered saline (TBS: 10 mM Tris, pH 7.4, 150 mM NaCl) containing 5% (w/v) non-fat dry milk and incubated overnight at room temperature with anti-invertase antibody. Membranes were then washed in TBS and incubated for 2 h with ¹²⁵I-labelled Protein A. Membranes were washed with TBS, dried and autoradiographed at –70 °C with intensifying screens. Linear-range autoradiograms were quantified by scanning densitometry

using a Bio-Rad densitometer and the one-dimensional Analyst program.

Sodium carbonate extraction and endoglycosidase H (Endo H) analysis

Sodium carbonate extraction was performed by the method of Fujiki et al. [30]. In brief, cells were harvested, washed and resuspended at 2×10^8 cells/ml in TED buffer (10 mM Tris, pH 8.0, 1 mM EDTA, 1 mM EGTA, 1 μ M dithiothreitol, 40 mM Na₄P₂O₇·10H₂O and a mixture of protease inhibitors) [8]. Cells were lysed by passage through a 5 μ m-pore-size polycarbonate filter (Nucleopore Corp) [31]. Unbroken cells and nuclei were removed by centrifugation at 3000 *g* for 10 min. The resulting supernatant was centrifuged at 15000 *g* for 20 min at 4 °C to obtain crude membrane and supernatant fractions. The crude membrane fraction was resuspended in TED containing 100 mM sodium carbonate, pH 11.5, such that the final protein concentration, determined by a modification of the method of Lowry [32], was 1 mg/ml. Samples were mixed thoroughly, left on ice for 1 h, and centrifuged at 145 000 *g* for 2 h at 4 °C. The resulting pellet was solubilized in TED containing 1% Triton X-100. Pellet and supernatant fractions were then analysed by Western blotting.

For Endo H analysis, cells were solubilized in TBS containing 1% (w/v) SDS and boiled for 5 min. To examine the secreted protein, an aliquot of the medium was adjusted to 1% (w/v) SDS and boiled for 5 min. After boiling, all samples were cleared by centrifugation at 15000 *g* for 10 min. The resulting supernatants were adjusted to 5% (v/v) with Triton X-100 and then diluted with 100 mM sodium citrate, pH 5.5. Endo H (1 m-unit; Boehringer Mannheim) was added to the appropriate samples which were then incubated at 37 °C for 24 h. Reactions were stopped by the addition of SDS/PAGE sample buffer, and analysed by SDS/PAGE and Western blotting.

Half-life (*t*_{1/2}) determinations

To determine the *t*_{1/2} of the chimeric proteins, logarithmically growing *D. discoideum* expressing each of these proteins was incubated in the presence of 300 μ g/ml cycloheximide to totally inhibit new protein synthesis [33]. At the specified times, aliquots were harvested and analysed by SDS/PAGE and Western blotting. The resulting autoradiographs were quantified by scanning densitometry. The data were plotted using the Prism program and the *t*_{1/2} of the protein estimated from the initial slope of the curve. The various compounds used to characterize the degradation of these proteins were added at the time of cycloheximide addition, with the exception of 2,5-di-(*t*-butyl)-1,4-hydroquinone (BHQ), where cells were preincubated for 15 min prior to the addition of cycloheximide. Inhibitors were added from stock solutions to the final concentrations indicated in the Figure legends. In each of the experiments, cell viability after treatment was judged by monitoring the ability of the cells to undertake their developmental programme after being washed free of medium and resuspended in phosphate buffer. To verify that the presence of cycloheximide did not alter the rate of protein degradation, we also determined that rate using pulse-chase analysis. To do so, cells were incubated at 1×10^8 cells/ml in HL-5 medium with 0.5 mCi/ml [³⁵S]methionine for 5 min. After labelling, cells were harvested and resuspended at 1×10^7 cells/ml in HL-5 medium containing 10 mM methionine. At the indicated times 4×10^7 cells were lysed and the protein immunoprecipitated with an anti-invertase antibody as described previously [15].

Cell fractionation and marker enzyme assays

The fractionation of cell lysates over Percoll gradients was performed by a modification of the method of Wood and Kaplan [34]. Logarithmically growing cells $[(2-4) \times 10^6 \text{ cells/ml}]$ were harvested by centrifugation, washed twice with 10 mM Tris, pH 7.4, and resuspended at $3 \times 10^8 \text{ cells/ml}$ in STKM (0.25 M sucrose, 50 mM Tris, pH 7.5, 12.5 mM KCl, 5 mM MgCl_2) containing a mixture of nine protease inhibitors [8]. Cells were lysed, and the unbroken cells and nuclei removed as described above. The resulting supernatant was layered over 9 ml of 30% Percoll (Pharmacia) in STKM, and centrifuged at $66000 g$ for 30 min. Fractions (1 ml) were collected from the top and transferred to tubes containing 20 μl of 10% (v/v) Triton X-100, and placed on ice. Fractions were assayed for acid phosphatase, a marker enzyme for lysosomes, as described by Loomis and Kuspa [35]. They were also assayed for α -glucosidase II, a marker for the endoplasmic reticulum (ER), by the method of Borts and Dimond [36], except that the buffer used was 50 mM Tris, pH 7.5, and for total protein concentration [32]. Coloured density-marker beads (Pharmacia) were included in a control gradient centrifuged in parallel to calibrate the density.

RESULTS

Localization of Inv-gp80 and Inv-gp80_{sc}

We previously reported the creation of a truncated form of Inv-gp80 which lacks the GPI signal sequences of Inv-gp80. When expressed in *D. discoideum*, the protein was predominantly soluble and secreted into the medium [15]. We will refer to this secreted protein as Inv-gp80_{ss}. A population of the protein, however, remained cell-associated. As shown in Figure 1, the retained form of the protein, which we will refer to in this manuscript as Inv-gp80_{sc}, co-fractionated with the crude membrane pellet (Figure 1, lane 1), but was then quantitatively released from the membrane fraction after treatment with sodium carbonate (Figure 1, lane 4). Treatment of membranes with sodium carbonate strips peripherally associated proteins from membranes and converts membrane vesicles into sheets, releasing trapped proteins from within [30]. We have previously demonstrated that Inv-gp80, which is quantitatively retained by cells, remains associated with the membrane fraction when treated in this manner, indicative of its presence as an integral membrane protein [15]. Since Inv-gp80_{sc} lacks the C-terminal GPI signal sequence, we undertook comparative studies with Inv-gp80 to examine the role of the GPI anchor signal sequence in determining the fate of GPI precursor proteins.

Figure 1 Inv-gp80_{sc} is a soluble protein

Cells were transformed with the Inv-gp80 cDNA in which the C-terminal 22 amino acids had been deleted. Cells were lysed and crude membrane (lane 1) and supernatant (lane 2) fractions were obtained as described in the Materials and methods section. Crude membranes were then treated with 100 mM sodium carbonate and centrifuged at $145000 g$ to obtain pellet (lane 3) and supernatant (lane 4) fractions. Samples were analysed by Western blotting using anti-invertase serum.

Figure 2 Endo H sensitivity of the chimeric proteins

Cells transformed to express Inv-gp80 (lanes 1 and 2) or Inv-gp80 lacking the C-terminal 22 amino acids (lanes 3 and 4) were lysed and boiled in buffer containing 1% SDS. In the latter case, an aliquot of the medium in which the cells had been growing was made 1% in SDS and boiled (lanes 5 and 6). Samples were incubated in the absence (lanes 1, 3 and 5) or presence (lanes 2, 4 and 6) of Endo H at 37 °C for 24 h, and analysed by Western blotting. Medium from non-transformed cells is shown in lane 7. The positions of molecular-mass markers are shown to the right.

We first evaluated the intracellular location of Inv-gp80_{sc} and compared it with that of Inv-gp80. Inv-gp80 is retained within the cell in a pre-Golgi compartment, as shown by the lack of any post-translational modifications (O-linked oligosaccharides) and the sensitivity of the N-linked sugars to Endo H digestion [15]. As a means of defining the subcellular localization of Inv-gp80_{sc}, we also analysed the possible oligosaccharide modifications of this protein (Figure 2). Treatment of Inv-gp80 with Endo H results in an approximate 20 kDa decrease in the apparent molecular mass of the protein (Figure 2, compare lanes 1 and 2). A similar decrease in apparent molecular mass was seen when Inv-gp80_{sc} was Endo H-treated (compare lanes 3 and 4), indicating that most of the N-linked oligosaccharides of this protein are also Endo H-sensitive. The secreted form of this protein, Inv-gp80_{ss}, displayed a higher molecular mass on SDS/PAGE than its cellular counterpart (Figure 2, compare lanes 3 and 5), and migrated as a 96 kDa protein. This reflects the post-translational modification of the C-terminal gp80 sequences of the protein as it migrated along the secretory pathway. The molecular mass of Inv-gp80_{ss} was only slightly altered by Endo H digestion (Figure 2, lane 6), indicating that the majority of the N-linked oligosaccharides of the secreted protein had been converted into an Endo H-resistant form. The 58 kDa cross-reacting band visible in lanes 5 and 6 is not related to either Inv-gp80_{sc} or Inv-gp80_{ss} since a similar band was detected in media from non-transformed cells. That Inv-gp80_{ss} displayed some Endo H sensitivity was not unexpected since partial Endo H sensitivity of the N-linked oligosaccharides of invertase expressed in a variety of systems, including *D. discoideum*, has been observed [37–39]. This is believed to reflect the proper folding of the protein, protecting specific N-linked oligosaccharide chains from additional processing [40]. That Inv-gp80_{ss} was appropriately modified indicates that the lack of O-glycosylation of both Inv-gp80 and Inv-gp80_{sc}, and the Endo H sensitivity of their N-linked oligosaccharide chains, did not reflect an unusual protein conformation, conferred by the gp80 sequences, that would have somehow rendered them inaccessible to the Golgi enzymes. The sum of the data suggests that Inv-gp80 and Inv-gp80_{sc} are both localized to a pre-Golgi compartment.

Another means of identifying the subcellular location of Inv-gp80 and Inv-gp80_{sc} was to fractionate *D. discoideum* lysates from cells expressing each of these proteins on Percoll gradients (Figure 3). Since biochemical analyses already indicated that these proteins were not located on the plasma membrane or in the Golgi, gradient fractions were assayed for the ER marker enzyme α -glucosidase II and the lysosomal marker enzyme acid phosphatase (Figure 3a). Those markers were chosen since ER and lysosomes contain independent protein degradation ma-

Figure 3 Percoll gradient fractionation of Inv-gp80 and Inv-gp80_{sc}

Transformants were harvested, lysed and the post-nuclear supernatants fractionated on 30% Percoll gradients as described in the Materials and methods section. (a) Fractions were collected from the top and assayed for the marker enzymes acid phosphatase (AP; ▲) and α -glucosidase II (Glu; ■). (b and c) Fractions were also analysed by SDS/PAGE and Western blotting for Inv-gp80 (b) and Inv-gp80_{sc} (c). Fractions containing the peak of AP and Glu activity are indicated.

chinery (see below). Fractions were also analysed by SDS/PAGE and Western blotting for the presence of Inv-gp80 and/or Inv-gp80_{sc}. As shown in Figures 3(b) and 3(c), neither Inv-gp80 nor Inv-gp80_{sc} was found in the fractions containing the peak of acid phosphatase activity, indicating that these proteins were not localized to the lysosomes. Instead, both proteins co-fractionated with the peak of α -glucosidase II activity, the marker for the ER. Unfortunately, since the anti-invertase antibody recognizes only the denatured protein, we were unable to use confocal microscopy as an additional approach to evaluate the location of these proteins.

Degradation of Inv-gp80 and Inv-gp80_{sc} is non-lysosomal

To further characterize the subcellular compartment in which Inv-gp80 and Inv-gp80_{sc} were localized, we analysed the degradation of the proteins. Degradation of proteins along the secretory pathway has been shown to occur in one of two compartments; in lysosomes [41] and in a pre-Golgi compartment generally believed to be located within the ER [42]. In characterizing the degradation of Inv-gp80 and Inv-gp80_{sc} we would not only obtain additional evidence for the subcellular location of these proteins, but also information concerning the role of the transmembrane domain of Inv-gp80 in determining its fate. The $t_{1/2}$ values of Inv-gp80 and Inv-gp80_{sc} were determined by pulse-chase analysis and immunoprecipitation from [³⁵S]methionine-labelled cells. The $t_{1/2}$ of each protein was also determined by treating cells with the protein synthesis inhibitor, cycloheximide, and at various times determining the levels of residual protein by Western blotting. Since the $t_{1/2}$ obtained for each of the proteins was the same using both methods we performed all subsequent experiments using Western blot analysis. As shown in Figure 4(A), Inv-gp80 displayed a $t_{1/2}$ of approx. 80–90 min, while the $t_{1/2}$ of Inv-gp80_{sc} (Figure 4B) was slightly longer (110–120 min). We also monitored the cell medium to confirm that the loss of Inv-gp80_{sc} reflected its degradation as opposed to its eventual secretion. Only a small amount of the protein was detected in the medium and it appeared within the first few minutes of in-

Figure 4 Effects of ammonium chloride and methylamine degradation of Inv-gp80 and Inv-gp80_{sc}

Cells expressing Inv-gp80 (A) or Inv-gp80_{sc} (B) were harvested and resuspended in medium containing 300 μ g/ml cycloheximide (■), or in medium containing cycloheximide and either 100 mM ammonium chloride (▲), or 2.5 mM methylamine (●). At the indicated times, aliquots were analysed by Western blotting. The resulting autoradiographs were quantified by scanning densitometry. The level of protein remaining at each time point is expressed as a percentage of the amount present at the start of the experiment. Results are the mean $\pm$ S.D. of three separate experiments.

cubation. Such a result would be expected if cycloheximide did not inhibit the secretion of the protein that was in transit at the time the drug was added.

Protein degradation within lysosomes exhibits a number of hallmark features, in particular a sensitivity to acidomorph agents such as ammonium chloride and methylamine [43]. Such treatments, however, did not inhibit the degradation of either Inv-gp80 (Figure 4A) or Inv-gp80_{sc} (Figure 4B), indicating that the degradation of these proteins did not occur within lysosomes. That ATP was required for the degradation of both Inv-gp80 and Inv-gp80_{sc} was demonstrated by incubating cells with 1 μ M carbonyl cyanide *m*-chlorophenylhydrazine (CCCP), a treatment previously shown to rapidly deplete cellular ATP levels in *D. discoideum* [44]. Incubation of cells with CCCP completely inhibited the degradation of both Inv-gp80 and Inv-gp80_{sc} (Table 1).

Effects of protease inhibitors on the degradation of Inv-gp80 and Inv-gp80_{sc}

The identities of the proteases involved in the degradation of proteins in the ER are not yet known. They have been charac-

Table 1 Effects of various inhibitors on the degradation of Inv-gp80 and Inv-gp80_{sc}

Cells were incubated in the presence of the various inhibitors listed. Levels of Inv-gp80 and Inv-gp80_{sc} were monitored at 20 min intervals by Western blotting as described in the Materials and methods section. The results represent the means of at least three determinations. Abbreviation: TLCK, Tos-Lys-CH₂Cl.

Treatment	$t_{1/2}$	
	Inv-gp80	Inv-gp80 _{sc}
Control	90 min	120 min
20 μ M TLCK	105 min	120 min
15 μ M 1,10-Phenanthroline	100 min	115 min
10 μ M CCCP	> 4 h	> 4 h
100 μ M BHQ	50 min	120 min

Figure 5 Effect of TPCK on the degradation of Inv-gp80 and Inv-gp80_{sc}

Transformants were incubated in medium with 300 μ g/ml cycloheximide in the absence (■) or presence (▲) of 10 μ M TPCK. At the indicated times, the level of Inv-gp80 (A) and Inv-gp80_{sc} (B) was determined by Western blotting. Each value is the mean $\pm$ S.D. of three experiments. Abbreviation: TPCK, Tos-Phe-CH₂Cl.

terized, however, in a number of different systems on the basis of inhibition studies [45–47]. In an effort to characterize the protease(s) involved in the degradation of Inv-gp80 and Inv-gp80_{sc}, cells expressing each of these proteins were treated with the membrane-permeant protease inhibitors tosyl-

lysylchloromethane (Tos-Lys-CH₂Cl), tosylphenylalanylchloromethane (Tos-Phe-CH₂Cl) or 1,10-phenanthroline. Tos-Lys-CH₂Cl (20 μ M) or 1,10-phenanthroline (15 μ M) had little or no effect on the degradation of either Inv-gp80 or Inv-gp80_{sc} (Table 1). Higher concentrations of these inhibitors proved to be toxic to these cells as shown by their inability to aggregate after treatment. The results suggest that trypsin-like serine proteases and metalloproteases are not involved in the degradation of either Inv-gp80 or Inv-gp80_{sc}. In contrast, incubation of cells with 10 μ M Tos-Phe-CH₂Cl significantly altered $t_{1/2}$ of both Inv-gp80 and Inv-gp80_{sc} (Figure 5). In the presence of Tos-Phe-CH₂Cl, only 20–25 % of Inv-gp80 was degraded in a 2-h period, while 60–70 % of the protein was degraded in the absence of such treatment (Figure 5A). The same general picture emerged when examining the effect of Tos-Phe-CH₂Cl on Inv-gp80_{sc} (Figure 5B). Tos-Phe-CH₂Cl is a relatively specific inhibitor of cysteine and chymotrypsin-like serine proteases [48]. Our results suggest that a similar protease(s) is involved in the degradation of both Inv-gp80 and Inv-gp80_{sc}.

Depletion of cellular calcium selectively increases the degradation rate of Inv-gp80

Depletion of Ca²⁺ from the lumen of the ER has been shown to have profound effects on the rate at which some proteins are degraded within this organelle. Depletion of Ca²⁺ can either stimulate or inhibit the degradation of proteins within this organelle depending upon the substrate being measured [47,49]. Depletion of ER Ca²⁺ can be achieved by incubating cells with the Ca²⁺ ionophore A23187, or through the use of inhibitors of the ER Ca²⁺-ATPase. To investigate the role played by Ca²⁺ in the degradation of Inv-gp80 and Inv-gp80_{sc}, we incubated cells with 1 μ M A23187 in the presence of 0.9 μ M EDTA, conditions that result in the rapid depletion of cellular Ca²⁺ from *D. discoideum* [50]. Incubation of cells under such conditions had no apparent effect on the rate of degradation of Inv-gp80_{sc} (Figure 6A). In contrast to that result, however, cell treatment with A23187/EDTA significantly increased the rate at which Inv-gp80 was degraded. Only 35–40 min was required to obtain a 50 % loss of protein compared with the 90 min required in untreated cells (Figure 6B). That this enhanced degradation was not a reflection of a transient increase in the concentration of cytosolic Ca²⁺ was shown by replacing the EDTA in the medium with 2 mM CaCl₂. Under those conditions the ionophore was less effective in stimulating the degradation of Inv-gp80. We also treated cells with BHQ, an inhibitor of the ER Ca²⁺-ATPase, to evoke a depletion of Ca²⁺ from this organelle [51]. As shown in Table 1, incubation of cells expressing Inv-gp80 in the presence of BHQ also stimulated the degradation rate of this protein. The $t_{1/2}$ of Inv-gp80 in the presence of BHQ was approx. 50 min, again suggesting that depletion of Ca²⁺ from the ER stimulates the degradation of Inv-gp80. BHQ had no effect on the degradation of Inv-gp80_{sc}, confirming that ER Ca²⁺ fluxes do not affect the degradation of this protein.

The experiments described above demonstrate that the uncleaved GPI anchor signal sequence of gp80 targets Inv-gp80 for rapid degradation under conditions where Ca²⁺ is depleted from the ER. It remained unclear if this reflected the presence of specific determinants within the GPI signal sequence or if the retention of Inv-gp80 as a transmembrane protein was sufficient to render the protein susceptible to a Ca²⁺-regulated degradation. To distinguish between these possibilities we replaced the uncleaved GPI signal of Inv-gp80 with sequences encoding the transmembrane and cytosolic domains of the human IL-2 receptor α -chain. The resulting protein will be referred to as Inv-

Figure 6 Effect of A23187 on the degradation of Inv-gp80 and Inv-gp80_{sc}.

(A) Cells expressing Inv-gp80_{sc} were incubated with 300 μ g/ml cycloheximide (■), or cycloheximide plus 1 μ M A23187 and 0.9 mM EDTA (▲). At the indicated times, levels of Inv-gp80_{sc} were determined by Western blotting. (B) Cells expressing Inv-gp80 were incubated with cycloheximide (■), cycloheximide plus 1 μ M A23187 and 0.9 mM EDTA (▲), or cycloheximide plus 1 μ M A23187 and 2 mM CaCl₂ (●). Aliquots were analysed by Western blotting at the indicated times. The results are the mean $\pm$ S.D. of three determinations.

gp80IL-2. If being transmembrane is sufficient to render the degradation of Inv-gp80 sensitive to Ca²⁺ fluxes, then the degradation of Inv-gp80IL-2 should, like Inv-gp80, be accelerated in the presence of the ionophore. If, however, the determinants contained in the uncleaved GPI anchor signal are required for accelerated degradation, then replacement of this region with a heterologous transmembrane domain should render the degradation of Inv-gp80 insensitive to cell treatment with ionophore.

Transformed *D. discoideum* expressed Inv-gp80IL-2 at levels comparable with those of Inv-gp80. Inv-gp80IL-2, like Inv-gp80 and Inv-gp80_{sc}, was retained in a pre-Golgi compartment as indicated by the lack of O-glycosylation and the Endo H sensitivity of its N-linked oligosaccharides. Experiments similar to those in Figure 1 verified that the protein behaved as an integral membrane protein (results not shown). We then determined the $t_{1/2}$ of Inv-gp80IL-2 in the presence and absence of the Ca²⁺ ionophore. As shown in Figure 7, the $t_{1/2}$ of Inv-gp80IL-2 was approx. 80–90 min whether or not cells had been treated with A23187/EDTA. The data support the hypothesis that the uncleaved GPI signal of Inv-gp80 contains information necessary for the rapid degradation of the protein under conditions where Ca²⁺ is depleted from the ER.

Figure 7 Effect of A23187 on the degradation of Inv-gp80IL-2.

Cells expressing Inv-gp80IL-2 were incubated with 300 μ g/ml cycloheximide in the presence (▲) or absence (■) of 1 μ M A23187 and 0.9 mM EDTA. At the indicated times, cellular levels of Inv-gp80IL-2 were determined by Western blotting. The results are the mean $\pm$ S.D. of three separate experiments.

DISCUSSION

The cellular retention of mutant cell-surface and secretory proteins has been reported to occur in a number of human diseases, including cystic fibrosis [52], genetic atherosclerosis [53], and paroxysmal nocturnal haemoglobinuria (PNH) [54]. Efforts have been directed towards understanding the mechanism(s) by which mutant precursor proteins are retained and degraded within the cells, to eventually develop specific treatments for these diseases. Reports from a number of different laboratories have provided conflicting evidence concerning the localization of GPI-anchored proteins that fail to become modified by the addition of the anchor. Using confocal microscopy, Field et al. [20] demonstrated that when human growth hormone (hGH)-decay-accelerating factor (DAF) fusion proteins are mutated to prevent GPI anchoring, they co-localize with p53, a marker for a novel pre-Golgi compartment distinct from the ER. A pre-Golgi localization for such proteins was proposed in earlier reports showing that these proteins remained sensitive to Endo H digestion [18]. Singh et al. [17], using electron microscopic cytochemistry, reported that expression of alkaline phosphatase in LMTK⁻ cells, a line defective in anchor biosynthesis, resulted in the production of a non-GPI-anchored protein present in cytosolic vacuoles that resembled lysosomes. The localization of the precursor alkaline phosphatase within lysosomes was confirmed by the observation that its degradation was inhibited when cells were treated with acidomorphogenic agents. In contrast, studies of the Endo H sensitivity of Q7b expressed in LMTK⁻ cells indicated that the protein was retained in a pre-Golgi compartment which, by immunoelectron microscopy, was identified as the cisternae of the ER [19]. The underlying reasons for these differences are not clear but may reflect intrinsic properties of the particular proteins being examined, or the mechanisms underlying the anchoring defect. In either case, too few proteins that fail to become GPI-anchored have been examined to gain a general picture of the ultimate fate of those proteins and the events involved in regulating that fate.

In the case of Inv-gp80, our analyses suggest that failure to GPI anchor the protein in *D. discoideum* results in its retention in a pre-Golgi compartment likely to be the ER. This conclusion is based partly on biochemical analyses of the oligosaccharide modifications of Inv-gp80. The protein was not O-glycosylated,

even though the acceptor sites for O-linked oligosaccharides were present and accessible for modification, as shown by the post-translational modification of the secreted form of the protein, Inv-gp80_{ss}. The N-linked oligosaccharides of Inv-gp80 remained sensitive to Endo H digestion. Again this did not reflect an altered conformation or inaccessibility of the residues to the modifying enzymes since most of the residues on the secreted form of the protein became Endo H resistant. Those results argue for a pre-Golgi localization of Inv-gp80, a premise supported by the co-fractionation of Inv-gp80 with the ER. Unfortunately, we were unable to monitor the protein by confocal microscopy since the antisera against invertase recognize only the denatured form of the protein. However, experiments designed to characterize the degradation of the protein also indicated that the protein was retained in the ER: the insensitivity of Inv-gp80 degradation to ammonium chloride and methylamine, two inhibitors of lysosomal function, confirmed that the protein was not degraded in lysosomes, while the alternative possibility, that the ER was the site for Inv-gp80 degradation, was supported by the observations that the degradation of the protein was stimulated by treatments resulting in an efflux of Ca²⁺ from the ER. Incubating cells with the Ca²⁺ ionophore, A23187, in the presence of EDTA resulted in an enhanced degradation of Inv-gp80. The stimulation of Inv-gp80 degradation did not reflect a corresponding increase in cytosolic Ca²⁺ since cell incubation with the ionophore in the presence of Ca²⁺ (to favour an influx of the cation) was significantly less effective. BHQ, an inhibitor of the Ca²⁺-ATPase of the ER, also enhanced the degradation of Inv-gp80. The sum of the data argue that in *D. discoideum* proteins that fail to become GPI anchored are retained in the ER for degradation. Although the precursor Inv-gp80 is a transmembrane protein, it would appear that the ER retention of GPI precursor proteins is not dictated by its membrane association. This statement is based upon the finding that the Q7b precursor, like Inv-gp80, is retained in the ER but is present as a soluble protein [19].

The question arises as to why Inv-gp80 remains membrane-bound while precursors Q7b and hGH-DAF are soluble. Subtle differences in the C-terminal hydrophobic domains of these proteins must account for their varied abilities to function as transmembrane domains. Of particular interest are the singly charged residues present in the C-terminal hydrophobic domains of both DAF and Q7b. Delahunty et al. [19] have reported that replacing the charged residue in the Q7b C-terminus with a hydrophobic amino acid renders Q7b membrane-bound when it is expressed in LTMK⁻ cells, i.e. when it is not GPI anchored. Perhaps, as suggested by Delahunty et al. [19], the charged residue affects the overall hydrophobicity of the region, eliminating its ability to function as a transmembrane domain. In this light, it should be noted that the C-terminus of Inv-gp80 does not contain any charged residues and is extremely hydrophobic as determined by Kyte and Doolittle analysis.

Little information is presently available concerning the degradation of proteins that fail to become GPI anchored. Our report represents a detailed analysis of the processes involved in their removal from the secretory pathway. Additionally our analyses of both the soluble and membrane-bound forms of Inv-gp80 provide insights into the functional significance of the C-terminal hydrophobic domain in regulating that process. In examining the degradation of Inv-gp80 and Inv-gp80_{sc}, we observed that the basic processes involved were similar. The basal rates of degradation of both proteins, and their sensitivities to a variety of protease inhibitors, were comparable, suggesting that the same protease(s) could be involved. The degradation of both proteins was also unaffected by inhibitors of protein synthesis, indicating

that neither situation involved a labile protease. The turnover of both proteins was completely inhibited by the depletion of cellular ATP levels. An ATP requirement for protein degradation in the ER has been interpreted in a number of different ways. In many cases, it has overlapped with a requirement for protein synthesis, suggesting that the need for ATP is actually secondary to that process. This would not apply to the situation for Inv-gp80 and Inv-gp80_{sc}. Alternatively, an ATP requirement for protein degradation might reflect, for example, the involvement of an ATP-dependent protease or the need to release the substrate from a chaperone.

The main difference between the degradation of Inv-gp80 and Inv-gp80_{sc} was the response to a depletion of Ca²⁺ from the ER. While the degradation of Inv-gp80 was stimulated when cells were incubated with the Ca²⁺ ionophore A23187 or with BHQ to cause an efflux of Ca²⁺ from the ER, those same conditions did not affect the degradation of Inv-gp80_{sc}. Replacement of the GPI anchor signal sequence of Inv-gp80 with the transmembrane domain from the IL-2 receptor did not alter the basal rate of degradation of the protein but it did eliminate its sensitivity to changes in luminal Ca²⁺ concentration. Those results argue that being transmembrane is not sufficient to render Inv-gp80 sensitive to a Ca²⁺-regulated degradation and that features of the GPI signal sequence are required.

The selective response of Inv-gp80 to ER Ca²⁺ fluxes could alternatively be interpreted to indicate that the protein is localized to a different compartment to Inv-gp80_{sc} and Inv-gp80IL-2. This would still argue that the GPI anchor signal sequence possesses specific features such that Inv-gp80, as opposed to either Inv-gp80IL-2 or Inv-gp80_{sc}, is selectively targeted to the ER. Given the pre-Golgi localization of the three proteins, their co-fractionation, and similarities of their degradation under basal conditions, we feel that this scenario is less likely. In either case, it would be of interest to characterize the degradation of the precursors of Q7b or hGH-DAF to determine if uncleaved GPI anchor sequences, when present in soluble proteins, can still render their degradation sensitive to Ca²⁺ fluxes.

Few reports have dealt with the degradation of soluble and transmembrane forms of the same protein within the ER. Most of the information available comes from studies of the α - and β -subunits of the T-cell receptor [55–57]. When expressed in CHO cells in the absence of the other subunits of this receptor complex, the α - and β -chains are retained within the ER and rapidly degraded [55,57]. Truncation of these proteins such that their transmembrane domains are removed results in the expression of soluble proteins that are also retained (for as yet undefined reasons) within the lumen of the ER. The soluble α - and β -chains display half-lives approx. 5 times longer than that of their transmembrane counterparts under normal conditions [55,56]. Thus, determinants for the basal rate of degradation of these proteins appear to be contained within their transmembrane domains. Inv-gp80, Inv-gp80_{sc} and Inv-gp80IL-2, however, showed comparable turnover rates under normal conditions, indicating that the determinants for the basal rate of degradation for these proteins lie outside the C-terminal sequences of Inv-gp80 that would signal membrane association.

In the case of T-cell receptor β -subunit, treatment of cells with the Ca²⁺ ionophore stimulated the degradation of both the soluble (truncated) and membrane forms approx. 3-fold, demonstrating that the determinants necessary for accelerated degradation of the β -chain reside outside the membrane domain [49]. This result contrasts sharply with the selective response of Inv-gp80 to Ca²⁺ fluxes. Our data, therefore, represent a novel situation wherein determinants for Ca²⁺-stimulated degradation in the ER have been localized within the transmembrane domain

of a protein. More specifically, these determinants have been localized within a GPI anchor signalling sequence.

Depletion of Ca^{2+} from the ER may activate a specific protease or it may disrupt interactions between Inv-gp80 and a chaperone that would otherwise protect it from degradation. The latter is an intriguing possibility given the current information concerning ER chaperones like calnexin and calreticulin, whose associations with select nascent polypeptides require Ca^{2+} [58,59]. Independently of the underlying mechanism, the presence of a Ca^{2+} -regulated degradation process in *D. discoideum* provides an enhanced quality control system functional whenever cells undergo changes in ER Ca^{2+} levels. A well-studied example would be when cells, as part of their aggregation programme, produce and respond to cAMP pulses. Periodic activation of the cell-surface cAMP receptor stimulates the expression of gp80, which participates in the formation of specific cell-cell contacts during aggregation [3]. Binding of cAMP to its cell-surface receptor also results in the production of inositol-1,4,5-trisphosphate [60] and subsequent release of Ca^{2+} from the ER. Such a scenario would activate the ER proteolytic machinery enhancing the removal of any improperly processed gp80 from this organelle. It is also likely that this Ca^{2+} -regulated proteolytic machinery plays a role in the ER degradation of other proteins whose transmembrane domains simulate that of the uncleaved GPI signal of gp80.

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