Glycosyl-phosphatidylinositol anchor attachment in a yeast *in vitro* system

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The yeast mating pheromone precursor prepro-alpha factor was fused to C-terminal signals for glycosyl-phosphatidylinositol (GPI) anchor attachment, based on the sequence of the *Saccharomyces cerevisiae* protein Gas1p. Maturation of fusion proteins expressed *in vivo* required the presence of both a functional GPI attachment site and the synthesis of GPI precursors. Constructs were translated *in vitro* for use in cell-free studies of glycolipid attachment. The radiolabelled polypeptides were post-translationally translocated into yeast microsomes, where at least one third of the molecules received a GPI anchor. This approach offers distinct advantages over anchor attachment reactions that require co-translational translocation of secretory peptide substrates.

INTRODUCTION

Of the variety of lipid structures that tether proteins to cell surface membranes, the most elaborate is glycosyl-phosphatidylinositol (GPI). Polypeptides destined to receive GPI anchors are synthesized at the endoplasmic reticulum (ER) and then rapidly modified by the removal of an N-terminal signal sequence and the attachment of a preformed glycolipid at the C-terminus (for reviews see [1,2]). The latter process links an ethanolamine at the non-reducing end of the glycan core to the C-terminal amino acid residue of the protein *via* an amide bond [3] and displaces a short peptide [4], presumably through a transamidation reaction.

Studies in mammalian cells [5,6] and in *Saccharomyces cerevisiae* [7] have used variants of C-terminal sequences to define the elements that are necessary and sufficient for GPI attachment (reviewed in [8]). In order from the C-terminus, these consist of a stretch of 8–20 hydrophobic residues, a short region of amino acids rich in charged residues and proline, and a domain of three small amino acids, the most N-terminal of which is the attachment or ω site [9]. The ω and $\omega+2$ residues are restricted to a subset of amino acids; otherwise anchor attachment does not occur. These constraints are similar but not identical in species examined in detail, including trypanosomes, yeast and mammals [7,10]. Interestingly, many characteristics of the C-terminal anchor attachment site mirror the pattern of residues that constitutes the N-terminal signal sequence [11].

A tantalizing question in the field of GPI modification involves the nature of the critical enzyme that attaches the anchor to the protein C-terminus. No biochemical purification of an anchor attachment activity has been achieved to date, but two gene products of *S. cerevisiae* may participate in the reaction. One is encoded by *GAA1*, an essential gene required for the addition of GPI precursors to substrate proteins [12]. When temperature-sensitive *gaa1* mutants are grown under restrictive conditions complete anchors are produced but not attached to protein. *GAA1* overexpression increases the efficiency of anchor attachment to polypeptides with mutant acceptor sites [12], suggesting a function for protein Gaa1p in anchoring. *GPI8* encodes a heterogeneously glycosylated type I transmembrane protein localized to the ER, with its main portion in the lumen [13]. This protein is an exciting candidate for an attachment enzyme because of its appropriate cellular location and its homology to a class of vacuolar plant endopeptidases, one of which exhibits transamidating activity *in vitro* [13].

Speculations on the specific reactions involved in anchor attachment have been included in the literature for a decade, but only recently have experiments in mammalian cells provided hints as to a mechanism of action [14]. These studies use a modified version of the GPI-anchored protein placental alkaline phosphatase (mini-PLAP [15]), which is cotranslationally translocated into membranes and modified by GPI moieties *in vitro* [16]. The putative transamidase is believed to act through an enzyme-bound activated-substrate carbonyl intermediate, which is resolved upon nucleophilic attack by the GPI precursor, water, or exogenously added nucleophiles such as hydrazine and hydroxylamine [17,18].

We are interested in studying GPI anchor attachment in an *in vitro* system in yeast. Our initial aim was to design a substrate that could be post-translationally added to membranes, allowing the preparation of large amounts of precursor for convenient and standardized studies. *S. cerevisiae* has been used extensively to study intracellular transport of proteins *in vitro* [19]. Numerous yeast strains are available with mutations in proteins involved in anchor synthesis [20,21] and attachment [12,13], ER chaperones implicated in GPI addition [22–24], mediators of intracellular traffic [19], and other polypeptides of potential importance in the transport of GPI-anchored species [e.g. Emp24p [25] and serine palmitoyl transferase [25a]. Establishing a yeast *in vitro* system to study GPI anchoring would allow the exploitation of these powerful experimental tools.

For a marker protein, we chose the yeast mating pheromone precursor prepro-alpha factor (ppαf), encoded by *MFα1* [26]. The processing of this polypeptide has been thoroughly characterized (reviewed in [27]). ppαf is synthesized as an 18.6 kDa species, which is modified in the ER by the removal of an N-terminal signal sequence and the addition of core N-glycans to three sites in its pro-region. The resulting polypeptide, glycopro-alpha factor (gpαf, 23 kDa) proceeds through the secretory pathway to the Golgi apparatus, where the glycans are elongated and the four αf peptide repeats are released by endoproteolytic cleavage and trimming. MATα cells secrete the mature αf

Abbreviations used: B88, buffer 88; ER, endoplasmic reticulum; gpαf, glycopro-alpha factor; GPI, glycosyl-phosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C; PLAP, placental alkaline phosphatase; ppαf, prepro-alpha factor; TX-100, Triton X-100; YPD, 1% bacto yeast extract/2% bacto peptone/2% dextrose.

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pheromone (13 residues) into the medium, where it acts in initiation of yeast mating. ppαf has been used as a substrate for detailed *in vitro* studies of translocation and of ER to Golgi transport [28,29]. When ppαf translated *in vitro* is added to yeast microsomes, it is post-translationally translocated into the membranes, glycosylated and packaged into transport vesicles competent to fuse with the Golgi apparatus [30]. *In vitro* assays exploiting this model protein have been critical in current understanding of yeast intracellular transport [31,32].

We chose to link ppαf to the C-terminal signal sequence of Gas1p [33], a cell cycle regulated *S. cerevisiae* protein required for normal mother–bud separation and β1,3-glucan synthesis [34,35]. Gas1p is the most abundant GPI-anchored protein in yeast [36], and has been well studied with respect to its anchor structure [37] and anchoring sequence requirements [7]. We hoped that a construct composed of both αf and Gas1p elements would efficiently enter membranes and acquire a GPI anchor, so that the useful properties of ppαf and yeast membranes as investigational tools could be directed towards greater understanding of C-terminal glycolipid attachment.

MATERIALS AND METHODS

Strains and growth conditions

The following *S. cerevisiae* strains were grown at 30 °C with agitation (250 rev./min): RSY1155 (*ino1-13, ino2-8, his4-59, leu2, suc2*Δ*9, MAT*α [38]); RSY1156 (*ino1-13, ino2-8, his4-59, leu2, suc2*Δ*9, MATa* [38]); RSY255 (*ura3-52, leu2-3,112, MAT*α); and SNY9 (*ura3, lys2, ade2, trp1, his3, leu2, bar1::HIS3, mf*α*1::ADE2, mf*α*2::TRP1, MAT*α; generously provided by Dr. A. Nakano, Department of Biology, University of Tokyo; [39]). Unless indicated otherwise, the medium was YPD [1 % bacto yeast extract/2 % bacto peptone (Difco Laboratories, Inc., Detroit, MI, U.S.A.)/2 % dextrose] and growth was monitored by spectrophotometer readings (600 nm). If not specified, materials were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Plasmids

Plasmid pDJ100 directs SP6 transcription of MFα1. Two fusions of ppαf linked to C-terminal sequences of Gas1p were constructed. First, primers TDGas9 and TDGas10 (Table 1) were used to amplify a portion of *GAS1* from plasmid pCNYCG, kindly provided by Dr. H. Riezman (University of Basel, Basel, Switzerland; [7]) to yield a *Bam*H1 site, followed by a sequence encoding the myc epitope, the *GAS1* sequence from bp 1960 to bp 2148 (initial ATG is at 412; stop at 2089), and an *Xba*I site. A modified αf sequence with upstream *Eco*RI and *Nde*I sites and a 3′ *Bam*HI site (TACTAA of *MF*α1 replaced by TGTTCTGGATCC) was amplified from pDQ2.4 (M. Pilon, D. Quach and R. Schekman, unpublished work) using primers TDaf11 and TDaf12 (Table 1). The two PCR products were subcloned into *Eco*RI–*Xba*I-digested pAS56 [40], a modification of plasmid pSP65 (Promega Biotech, Madison, WI, U.S.A.) that includes A_{58} between the *Sal*I and *Pst*I sites of the polylinker (kindly provided by Dr. A. Sachs, University of California, Berkeley, CA, U.S.A.), yielding pTD31. A slightly longer portion of *GAS1* was amplified from pCNYCG using primers TDGas9 and TDGas19 and was used to replace the *Bam*HI–*Xba*I fragment of pTD31, forming pTD42. pTD42Q is a variant of pTD42 where the triplet encoding amino acid 528 is changed from AAT to CAG (to encode Q instead of N).

For expression in yeast, the *Eco*RI–*Xba*I fragment of pTD42 was ligated into the *Eco*RI and *Avr*II sites of pYX112 (an *ARS/CEN*, *URA3* vector with a triosephosphate isomerase promotor upstream of a multi-cloning site; R & D Systems, Inc., Minneapolis, MN, U.S.A.) to form pTD52 (Figure 1). pTD52Q contains the coding region of pTD42Q in pYX112. pTD71 is the same as pTD52, except that the *MF*α*1* sequence encoding amino acids 26–88, inclusive, has been deleted. Further construction details are available from T. L. D. All constructs were verified by DNA sequencing (Barker Hall Sequencing Facility, University of California, Berkeley, CA, U.S.A.).

Radiolabelling and immunoprecipitation

Yeast cells were transformed by electroporation and grown in minimal medium (with 2 % dextrose, without uracil) to an A_{600} of about 1.0. Cells were washed twice and resuspended in the same medium lacking methionine, and incubated at 30 °C for 15–30 min before addition of [^{35}S]Promix (1200 Ci/mmol; Amersham, Arlington Heights, IL, U.S.A.). To initiate chase, unlabelled methionine and cysteine were added, each at 0.6 mg/ml. At the indicated times, labelling was terminated by the addition of an equal volume of ice-cold 15 mM NaF/15 mM NaN_3, cells were lysed with glass beads in buffer containing 1 % SDS, and the extract was processed for immunoprecipitation and SDS/PAGE analysis as before [38]. All gels were 12.5 % acrylamide except for those used to analyse anti-Gas1 antibody immunoprecipitates (7.5 %). Proteins were detected and quantification was performed on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, U.S.A.). Polyclonal antiserum to Gas1p was raised in rabbits against a His-tagged fusion protein expressed in *Escherichia coli* (from plasmid pQE9, a gift from Dr. H. Riezman, University of Basel, Switzerland [41]) and purified by nickel affinity chromatography and SDS/PAGE. Polyclonal antiserum to ppαf was generated by A. Eun (R. Schekman laboratory) against a *lacZ–MF*α*1* fusion, and ascites fluid containing monoclonal anti-myc antibody was prepared by S. Grell (laboratory of W. Allison, University of California at Berkeley).

Table 1 Primers used in plasmid construction

Sequences corresponding to restriction-enzyme cleavage sites are italicized. Underlined sequences in TDGas9 and TDGas19 indicate myc epitope sequences; the underlined triplet in TDaf11 encodes starting methionine.

Primer name	Primer sequence (5′ to 3′)
TDGas9	CGC*GGATCC*GAGCAAAAGCTCATTTCTGAAGAGGACTTGTCTTCAGCTTCATCTTCATCTTCTAGCAAG
TDGas10	GC*TCTAGA*CCACAATGAAATAAGATACCATACCTTATC
TDaf11	CCG*GAATTC*AAA*CATATG*AGATTTCCTTCAATTTTT
TDaf12	CCGTTCTTGCCAATCCCCATATTTTGG
TDGas19	CGC*GGATCC*GAGCAAAAGCTCATTTCTGAAGAGGACTTGTCGGGGTCTTCTTCCAAGTCTAACTCCGGC

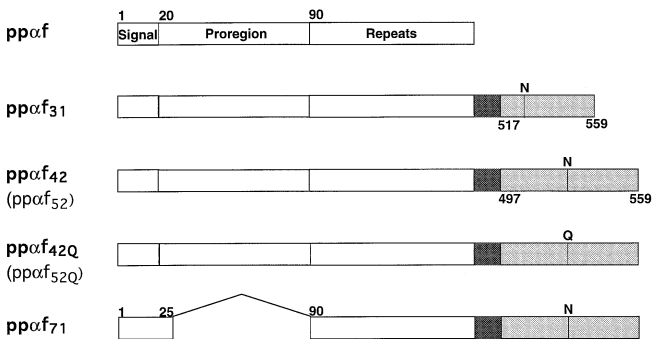

Figure 1 Schematic representation of fusion proteins

Constructs used in these studies are designated ppαf_x, where x is the plasmid number. Names in parentheses indicate constructs with the identical coding sequence cloned into a yeast expression vector (see the Materials and methods section for details). Open bars, ppαf sequence; black portion, myc epitope; gray region, Gas1 sequence. Numbers on diagrams indicate amino acid position in the wild-type protein sequence; where not shown these are the same as in the construct above. N or Q indicates the amino acid present at the ω position of the Gas1 sequence.

Halo assays

Sst1 strain JTY625 (*ade2, his6, leu2-3,112, lys2-333, trp1-289, ura3-52, MATa*; generously provided by Dr. J. Thorner, University of California at Berkeley) was grown to A_{600} of ~ 0.5, diluted 15-fold with melted YPD agar and poured onto YPD plates. Strains to be tested were spotted onto cooled test plates and incubated at 30 °C for 2 days.

RNA production and translation *in vitro*

Plasmids were linearized with *Xba*I (pDJ100) or *Pvu*II (others) and transcribed according to the manufacturer's instructions for synthesis of capped RNA (Ampliscribe SP6 transcription kit; Epicentre Technologies, Madison, WI, U.S.A.). RNA was translated in a nuclease-treated yeast S100 fraction in the presence of [^{35}S]methionine (translation grade, $>$ 1000 Ci/mmol; NEN Dupont, Wilmington, DE, U.S.A.) [29]. The radiolabelled translation reaction was desalted on a Sephadex G-25 column equilibrated in buffer 88 (B88; 20 mM Hepes/0.25 M sorbitol/0.15 M KOAc/1 mM MgOAc, pH 6.8) to remove unincorporated radiolabel, and peak fractions were pooled and stored in aliquots (15–20 portions from a 1 mCi labelling reaction) at -70 °C.

In vitro assays

Yeast microsomes (~ 0.4 μg of protein, prepared as in [42]) were thawed at 10 °C and incubated (30 min, 10 °C) in B88 containing $(1–5) \times 10^6$ c.p.m. of radiolabelled translation product, 1 mM ATP, 40 mM creatine phosphate, 200 μg/ml creatine phosphokinase and 50 μM GDP-mannose. Reactions were chilled on ice (5 min) and washed twice with B88 (TOMY refrigerated microcentrifuge, 12000 ***g*** for 4 min at 4 °C). For protease protection studies, washed membranes were resuspended in B88, divided into thirds and incubated on ice with trypsin (0.25 mg/ml, 10 min) in the presence or absence of 1% Triton X-100 (TX-100) before trichloroacetic acid precipitation and SDS/PAGE analysis.

Phospholipase treatment and TX-114 partition

Membranes containing translocated ppαf (or fusion proteins) were washed in B88, then resuspended in ice-cold 100 mM Tris (pH 7.5), 10 mM EDTA, 1 μg/ml pepstatin, 2 μg/ml antipain and 2 μg/ml leupeptin, and extracted on ice with precondensed TX-114 (final concentration 1%; [43]) for 30 min with occasional vortex mixing. Each sample was subjected to centrifugation (TOMY microcentrifuge, 12000 ***g*** for 10 min at 4 °C) and the resulting supernatant fraction was removed to a new tube. The extract was warmed to 32 °C to allow detergent condensation, centrifuged (Fisher microfuge, top speed for 30 s at room temperature), and the aqueous phase was discarded. The detergent phase was diluted 10-fold, divided in two, and aliquots were incubated (3 h at 30 °C) with or without 0.5 units of phosphatidylinositol-specific phospholipase C (PI-PLC; from *Bacillus cereus*; Boehringer Mannheim, Indianapolis, IN, U.S.A.). After incubation, phases were separated as above, and each phase was back-extracted, diluted with cold buffer to the original volume and precipitated with trichloroacetic acid for SDS/PAGE analysis.

RESULTS

Studies *in vivo*

We constructed plasmids containing *MF*α1 and a GPI attachment sequence from *S. cerevisiae GAS*1, separated by a sequence encoding the myc epitope [44], to aid in analysis or localization (Figure 1). Surprisingly, an initial attempt to direct GPI attachment using only the final 43 residues of proGas1p (ppαf_{31}, Figure 1) was unsuccessful, although by sequence analysis this region was expected to signal GPI addition [11]. Polypeptide was synthesized, but there was no evidence of anchor attachment, either *in vivo* or *in vitro* (results not shown). We next included in the construct a larger portion of the C-terminus of the Gas1p sequence encoding an additional 20 residues. When cells transformed with this plasmid (pTD52) were metabolically radiolabelled and immunoprecipitated with anti-ppαf serum, precipi-

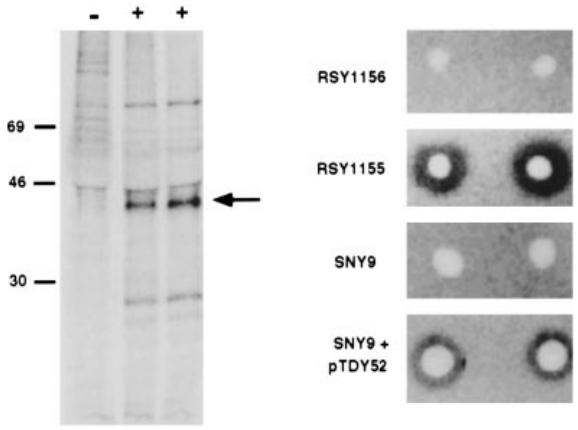

Figure 2 Expression of ppαf_{52} in intact cells

Left: immunoprecipitation. RSY1156 cells transformed with pTD52 (two independent transformants; marked +) or control cells (−) were radiolabelled as described in the Materials and methods section (1 A_{600} unit/ml; 20 μCi [^{35}S]Promix/ml; 20 min pulse) and material precipitated with anti-ppαf serum was resolved by SDS/PAGE. Molecular mass values (kDa) of standards are indicated on the left; arrow indicates the position of migration of pTD52 product. Right: halo assays. Duplicate samples of each strain are shown. RSY1156 (MATa) and RSY1155 (MATα) are negative and positive controls respectively.

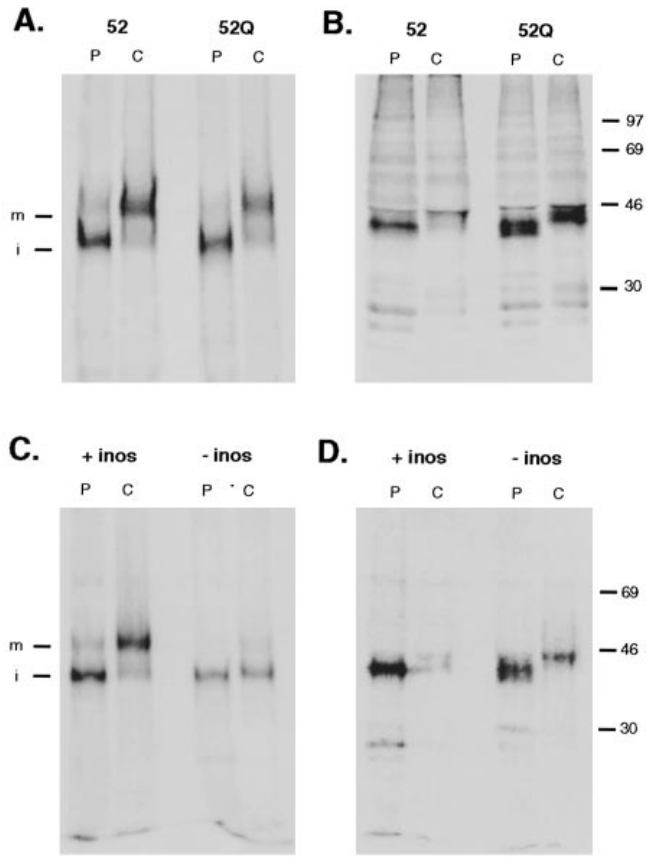

Figure 3 Pulse–chase analysis of fusion proteins

Cells were radiolabelled for 10 min (1 A_{600} unit/ml; 20 μCi [^{35}S]Promix/ml) without (P) or with (C) a 10 min chase. Immunoprecipitation was with antibody to Gas1p (**A**, **C**, 7.5% SDS/PAGE) or to ppαf (**B**, **D**, 12.5% SDS/PAGE). m and i show positions of migration of mature and immature Gas1p [7,48]; molecular mass values (kDa) of standards are indicated on the right (kDa). (**A**, **B**) Cells expressing either ppαf_{52} or ppαf_{52Q} were radiolabelled in minimal media as described in the text. (**C**, **D**) Cells expressing ppαf_{52} were incubated for 90 min in minimal medium with (+ inos) or without (— inos) inositol [38] before radiolabel addition.

tates contained a new protein species of about 42 kDa (Figure 2, left, indicated by an arrow). The polypeptide portion of this fusion should have a molecular mass of about 26 kDa, modified by three N-glycans (which normally add 7.4 kDa to the α-factor [45]) to give a minimum expected size of $\sim$ 33 kDa. The additional 9 kDa are probably contributed by the extensive O-glycosylation known to occur at a serine-rich region of Gas1 [46], most of which is included in ppαf_{52}. Nearly every serine in this stretch is thought to be modified by a 3–4-residue oligosaccharide [47], which would easily alter the protein mobility to the extent observed. A similar product was found in SNY9 cells transformed with the same plasmid (results not shown). Faint signals were also detected with anti-Gas1 antibody, which were stronger in fusion proteins containing larger portions of Gas1p (results not shown). The less prominent bands migrating ahead of the 30 kDa standard may represent processing intermediates, as they disappear with chase (see below).

We tested whether the fusion protein was processed and whether pheromone was secreted from the transformed cells by halo assays, which detect αf by its property of inhibiting growth of *MATa* cells, thus creating a 'halo' when secreting cells are grown on an *MATa* lawn (Figure 2, right). Constructs were expressed in SNY9 (from Dr. A. Nakano, University of Tokyo, Tokyo, Japan), a *MAT*α strain in which both *MF*α genes have been disrupted so it does not ordinarily produce αf. Transformed cells produced a halo in this assay, indicating αf secretion.

We used two methods to assess whether the constructs expressed *in vivo* were anchored by GPI structures. One approach was to use mutated anchoring sequences, based on the fact that alteration of the Gas1p ω site N residue to a Q prevents anchor attachment and protein exit from the ER [7,38]. We compared cells expressing either a fusion protein with that mutation (ppαf_{52Q}) or normal ppαf_{52} in a radiolabelling experiment (Figures 3A and 3B). As shown in Figure 3(A), both cell types were able to perform normal GPI processing of endogenous Gas1p [7,48]. However, we observed a difference in processing of the two ppαf–GPI fusion proteins (Figure 3B). ppαf_{52}, with a normal attachment site, disappeared during chase. This is probably due to protein processing leading to release of αf peptide. In contrast, the mutant protein experienced a small decrease in gel migration, with no reduction in total radiolabelled protein. The observed decrease in mobility is presumably due to glycosylation; ppαf_{52} also exhibited this shift during early chase points in a time course experiment (results not shown).

To confirm that this difference in behaviour reflects anchor attachment, we used inositol starvation, which we have previously showed interferes with GPI synthesis and, consequently, anchorage and maturation of Gas1p [38]. Inositol starvation prevents normal processing of Gas1 protein, as evidenced by the inefficient protein maturation in starved cells compared with unstarved cells (Figure 3C). Aberrant processing of the αf-GPI construct (αf_{52}) was also evident in the inositol-starved cells (Figure 3D). The fusion product matured and disappeared during chase in unstarved cells, whereas in starved cells there was slight processing of the expressed fusion protein, but the material remained intact. Under the inositol starvation conditions used, the trafficking of proteins that are not GPI-modified is unchanged [38]. Together with the anchor-site mutation results, these experiments suggest that the fusion protein is indeed anchored: two alterations that specifically affect GPI-anchored proteins through distinct mechanisms each alter protein processing.

Studies *in vitro*

We were satisfied that pTD52 encoded a protein with the ability to become GPI-anchored *in vivo*, and therefore proceeded to *in vitro* studies. RNA transcripts of pTD42, which contain the same coding region, were translated *in vitro* in a yeast S100 fraction. The radiolabelled product, ppαf_{42}, could be immunoprecipitated by antibody to both ppαf and the myc epitope, as expected (Figure 4, top, indicated by an arrow). The gel migration position was consistent with that predicted for the primary translation product of this sequence; the lack of glycosylation and signal processing account for the difference in apparent molecular mass between this species and the equivalent species expressed *in vivo*. Anti-myc antibody did not precipitate wild-type ppαf (Figure 4A).

We next addressed whether the GPI-anchored construct could be post-translationally translocated into yeast microsomes. Like wild-type ppαf, a portion of the fusion protein molecules was converted into a reduced electrophoretic-mobility species contained within a protease-protected compartment upon incubation with membranes in the presence of ATP and GDP mannose (gpαf_{42}, Figure 4B). The size changes observed were consistent with the addition of the expected N-glycan core structures, and none were seen in the absence of ATP (results not shown).

Finally, we investigated whether anchor addition occurred in

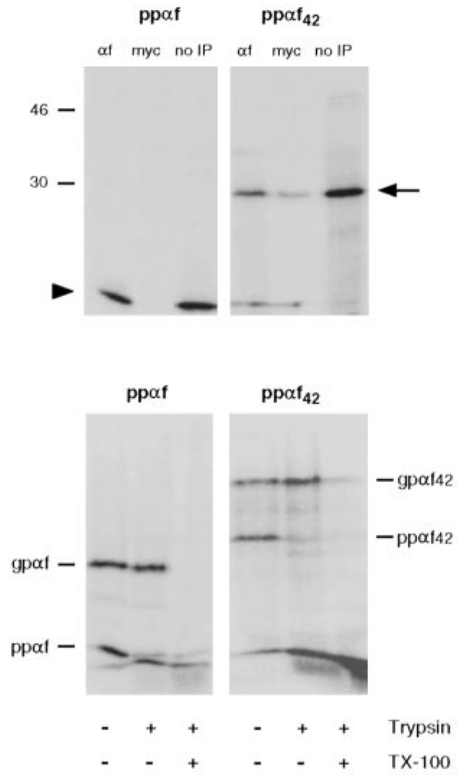

Figure 4 Translation and translocation of ppαf and ppαf$_{42}$

Top: products translated *in vitro*, ppαf (arrowhead) or ppαf$_{42}$ (arrow), were immunoprecipitated with either anti-ppαf or anti-myc antibodies as indicated. The third lane (from the left) of each panel is total translation product (not immunoprecipitated). Molecular mass standards are indicated on the left. Bottom: translocation of ppαf and ppαf$_{42}$ was performed as described in the Materials and methods section. Conditions for assessment of protease protection are indicated below lanes.

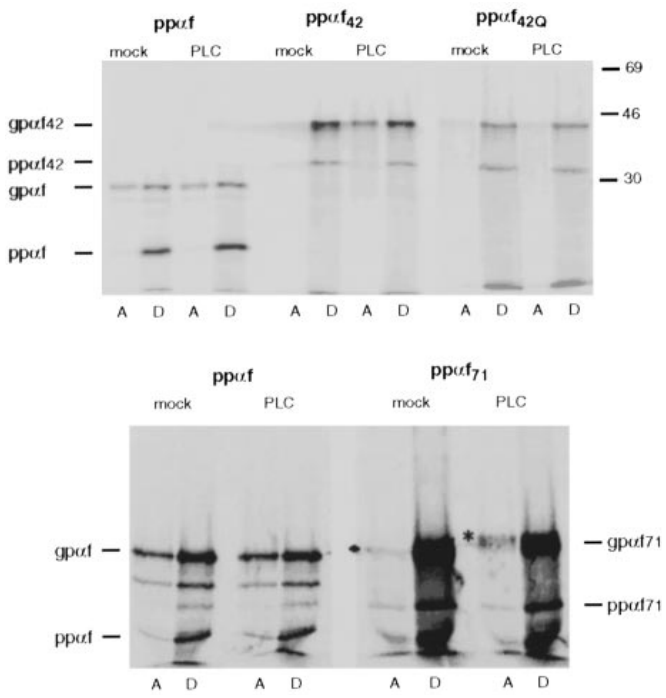

Figure 5 Phospholipase C analysis of translocated products

Translocation, lipase treatment (mock or enzyme treatment indicated), and TX114 partition (A, aqueous phase; D, detergent phase) were as described in the Materials and methods section. The version of ppαf used is indicated over each set of lanes. Symbols on the gel indicate bands mentioned in the text.

the yeast membrane system *in vitro*. We first examined the partitioning of translocated material between the aqueous (A) and detergent (D) phases of a TX-114 extract, as an indication of the hydrophobicity of these species (Figure 5, top, mock lanes). In the presence of membranes, even wild-type ppαf partitioned predominantly ($> 95\%$) into the detergent phase (Figure 5, top, first two lanes), although this was not as striking in their absence (only 40 % in detergent phase; results not shown). Upon glycosylation, however, there was a shift in distribution (33 % of gpαf in aqueous, compared with $< 5\%$ of ppαf), as expected with the addition of hydrophilic oligosacharides. We noted that glycosylation of the ppαf$_{42}$ did not induce an alteration in partitioning behaviour; for both ppαf$_{42}$ and gpαf$_{42}$ about 5 % was in the aqueous phase. This could be due either to the presence of a GPI anchor or to the additional hydrophobic sequence present in the fusion protein.

To distinguish between these possibilities, we treated translocated material with PI-PLC, which should specifically cleave anchored material and release it into the aqueous phase of a TX-114 partition (Figure 5, top, PLC). For gpαf$_{42}$, treatment caused 30–35 % of the material to shift to the aqueous phase, indicating cleavage of a GPI structure. In contrast, gpαf$_{42Q}$, which is not capable of accepting an anchor, showed no comparable change in partitioning behaviour. Longer treatments or increased enzyme did not alter the amount released (results not shown; see the Discussion section).

We detected no difference in gel migration between gpαf$_{42}$ and gpαf$_{42Q}$. This was not surprising, as the removal of the C-terminal sequence (3.2 kDa) and concomitant addition of GPI (about 2 kDa) would not appreciably alter the migration pattern in this region of the gel, especially since hydrophobic regions and GPI moieties do not predictably alter gel migration behaviour.

In order to detect altered migration, we examined a truncated construct. ppαf$_{71}$ is the same as ppαf$_{42}$, except that the pro-region of the latter has largely been deleted, leaving only the first five residues (Figure 1). The repeat regions of αf were not deleted because they are required for efficient translocation of this polypeptide [49]. Translocation and glycosylation of ppαf$_{71}$ were as expected, but gpαf$_{71}$ exhibited a smeared appearance on gel analysis (Figure 5, bottom). PI-PLC treatment consistently released material from the upper part of this smear into the aqueous phase (Figure 5, bottom, band marked with an asterisk), which suggested altered mobility of anchored material compared with the trace unanchored material normally present in the aqueous phase upon partition (Figure 5, bottom, faint band indicated with a diamond). These experiments show that the yeast membrane *in vitro* system is competent to add GPIs to post-translationally translocated precursor polypeptides.

DISCUSSION

We have characterized the behaviour of proteins where a yeast pheromone precursor, ppαf, is fused to a sequence directing GPI anchor attachment. Maturation of these hybrid polypeptides in intact yeast cells depends on the presence of inositol and on a normal anchor-attachment site (Figure 3). These results are consistent with previous observations that polypeptides with

GPI anchoring sequences are not properly transported or processed under conditions where anchoring cannot occur because of attachment-site mutations [7] or absent or insufficient glycolipid substrate [38,50–53].

We noted that more of the C-terminal region of Gas1p was required to direct anchor attachment than we anticipated by sequence analysis [11]. It is possible that the additional region, a highly O-glycosylated serine-rich stretch, thought to exist in an extended conformation [47], is required to allow access by the anchoring machinery. These results suggest that care should be taken when predicting minimum sequences required to signal attachment.

Experiments with anchored constructs *in vitro* showed that they can be post-translationally translocated (Figure 4) and receive GPI modification in yeast microsomes. The evidence for anchoring is the acquisition of susceptibility to PI-PLC action, which consistently reached $\sim 30\,\%$ (Figure 5). The observed PI-PLC cleavage is clearly specific to fusions that contain anchoring sequences, as neither wild-type gpαf nor gpαf_{42Q} were altered by enzyme digestion. The partial cleavage observed could be due to incomplete anchoring, for example if GPI precursors are limiting in the microsomes. Alternatively, this could reflect problems of accessibility or structural features of the precursor. GPI anchors modified by inositol acylation cannot be cleaved by PI-PLC; yeast anchors do undergo acylation during biosynthesis [54], although this modification is not generally present in mature proteins [37].

The ability to achieve anchoring of αf-based fusion proteins in yeast membranes *in vitro* offers several experimental advantages. Firstly, because the fusions may be post-translationally translocated, large quantities of radioactive substrate may be prepared in advance, simplifying the assay procedure. Mammalian studies have been quite successful, using a co-translational step to 'load' membranes with prepro-miniPLAP, followed by a second incubation to allow anchor attachment [16], but this does require translation to accompany each experiment. The efficiency of the yeast *in vitro* reaction also compares favourably with the results seen in mammalian studies. Based on average efficiencies of translation ($30\,\%$), translocation ($50\,\%$) and anchoring ($30\,\%$), approximately 1 pmol of product is produced in a typical experiment, and this could easily be scaled up; the estimated yield of GPI-linked product in mammalian experiments is 20–100 fmol [8].

A further strength of the yeast system is that transport in these membranes has been well characterized, and thus experiments could be extended to examine the properties of ER exit (budding reaction) and further transport (i.e. Golgi fusion) of GPI-anchored species through the secretory pathway. This could be a valuable tool for stepwise dissection of GPI-protein transport, which is likely to differ from that of other proteins in terms of ER exit [38], ceramide dependence [41] and, possibly, reliance on accessory proteins for budding [25]. Lastly, use of the yeast system allows analysis of mutant cells to characterize the requirements of anchor addition *in vitro*. A number of mutants are already known that participate in synthesis of the GPI anchor [20,21], and others are thought to be involved in the process of anchor addition, either in the catalysis step [12,13] or by acting as chaperones [22–24]. Using GPI-anchored fusion constructs, it will be possible to explore the roles of the proteins altered in these mutants, and possibly others, in GPI anchor attachment.

Biochemical characterization of the anchor addition step will require purification of the putative transamidase. Again, the ability to grow large quantities of yeast for membrane preparation would be an advantage in this endeavour, and the use of this *in vitro* approach as a starting point for an assay should be feasible. It will also prove fruitful to compare studies in yeast membranes with those in mammalian systems. Although similarities between anchoring components in various organisms are expected, any differences may be quite important in terms of developing toxic agents against pathogenic organisms in which GPIs are abundant, such as parasites and pathogenic fungi [55].

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