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Ceramide synthesis enhances transport of GPI-anchored proteins to the Golgi apparatus in yeast

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Inhibition of ceramide synthesis by a fungal metabolite, myriocin, leads to a rapid and specific reduction in the rate of transport of glycosylphosphatidylinositol (GPI)-anchored proteins to the Golgi apparatus without affecting transport of soluble or transmembrane proteins. Inhibition of ceramide biosynthesis also quickly blocks remodelling of GPI anchors to their ceramide-containing, mild base-resistant forms. These results suggest that the pool of ceramide is rapidly depleted from early points of the secretory pathway and that its presence at these locations enhances transport of GPI-anchored proteins specifically. A mutant that is resistant to myriocin reverses its effect on GPI-anchored protein transport without reversing its effects on ceramide synthesis and remodelling. Two hypotheses are proposed to explain the role of ceramide in the transport of GPI-anchored proteins.

Key words: GAS1/glycosylphosphatidylinositol/protein sorting/*Saccharomyces cerevisiae*/secretion/sphingolipids

Introduction

A large number of eukaryotic membrane proteins are covalently modified by a glycosylphosphatidylinositol (GPI) moiety that serves to anchor the protein to the membrane. Proteins with this modification were first detected among lower eukaryotes, in particular trypanosomes where this modification is of major importance. It appears that in lower eukaryotes where this modification is most prevalent, it may play among others, a protective, structural role (McConville and Ferguson, 1993). In higher eukaryotes GPI-anchored proteins have been implicated in signal transduction in lymphocytes (Robinson, 1991). In most polarized cell types examined thus far, GPI-anchored proteins have been localized to the apical plasma membrane domain (Brown, 1992), but in Fischer rat thyroid cells some GPI-anchored proteins are targeted to the basolateral surface (Zurzolo *et al.*, 1993). Converting a basolaterally targeted transmembrane protein to a GPI-linked form redirects this protein to the apical surface of MDCK cells (Brown *et al.*, 1989; Lisanti *et al.*, 1991). Therefore, it seems that a GPI anchor can act as a sorting signal to this surface. It has been suggested that GPI-anchored proteins cluster in the plane of the membrane with sphingolipids forming microdomains that are resistant to extraction with some detergents (Brown and Rose, 1992; Fiedler *et al.*, 1993). It is possible that this clustering plays a role in the protein sorting events that direct traffic of GPI-anchored proteins.

Yeast cells have been shown to have both GPI-anchored proteins (Conzelmann *et al.*, 1988, 1990) and sphingolipids (Smith and Lester, 1974). Yeast GPI anchors have a core structure that is identical to that found in trypanosomes and animals (Fankhauser *et al.*, 1993). Gas1p, the best characterized GPI-linked protein (Nuoffer *et al.*, 1991), has a *lyso* or diacylglycerol lipid moiety linked to the inositol phosphate, whereas most of the other GPI-linked proteins have ceramide-containing lipid moieties (Fankhauser *et al.*, 1993). Characteristic of all GPI lipid moieties is a long chain fatty acid (C24–C26). It is most likely that all GPI-anchored proteins first receive a similar *lyso* or diacylglycerol-containing GPI moiety, and that GPI moieties on most of the proteins are then remodelled to ceramides, but relatively little is currently known about the structure of the lipid domain of the GPI precursor glycolipid (Conzelmann *et al.*, 1992). The remodelling of the GPI anchors to ceramide-containing anchors can be monitored by examining the sensitivity of the anchor to cleavage by mild base. Most of the GPI anchors on proteins blocked in the endoplasmic reticulum (ER) using the *sec18* mutant have anchors with their acyl chains in an ester linkage because they are cleaved and rendered hydrophilic by mild base. Upon further transport through the secretory pathway the GPI anchors become resistant to mild base cleavage because the lipid portion of the anchor has been replaced with ceramide, in which the acyl chains are not in an ester linkage (Conzelmann *et al.*, 1992).

Yeast contains three major sphingolipids, inositol-phosphoceramide (IPC), mannose-inositol-phosphoceramide (MIPC) and mannose-(inositol-P)$_2$-ceramide [M(IP)$_2$C] (Smith and Lester, 1974). The ceramides found on GPI anchors are not identical to any of the major ceramides found in yeast (Fankhauser *et al.*, 1993). These phosphoinositol sphingolipids are, like those in animal cells, concentrated at the cell surface (Patton and Lester, 1991) and like GPI moieties, have a long chain fatty acid (Smith and Lester, 1974).

The primary protein sequence requirements of substrates for recognition and attachment of GPI anchors are similar between yeast and man although functional substitution experiments have not been done (Moran and Caras, 1991; Kodukula *et al.*, 1993; Nuoffer *et al.*, 1993). A carboxy-terminal hydrophobic stretch of amino acids must be located ~10–12 amino acids downstream from a suitable anchor attachment site. A suitable site consists of three consecutive amino acids with small side chains. Cleavage

takes place between the first and second of these amino acids and the anchor is attached to the carboxyl group of the first amino acid (Nuoffer *et al.*, 1991; McConville and Ferguson, 1993). Mutant Gas1 proteins that have unacceptable anchor attachment site amino acids, such as Q, do not receive GPI and accumulate as immature proteins, indicating their inefficient transport to the Golgi apparatus (Nuoffer *et al.*, 1993). We took advantage of the difference in mobility on SDS–polyacrylamide gels of the mature and immature forms of Gas1p to screen a number of pure compounds for their ability to inhibit GPI anchoring and/or transport of GPI-anchored proteins. We found one such compound, myriocin, a structural analog of sphingofungins (Kluepfel *et al.*, 1972; Sasek *et al.*, 1989; Horn *et al.*, 1992), that inhibits ceramide synthesis and specifically slows down transport of Gas1p to the Golgi apparatus. Gas1p is GPI-anchored in the presence of myriocin. In presence of myriocin, most GPI anchors remain sensitive to mild base treatment showing that they do not acquire ceramides. We have isolated a myriocin-resistant mutant that restores efficient transport of Gas1p without restoring ceramide biosynthesis or anchor conversion. Our results show that ceramides specifically enhance transport of GPI-anchored proteins to the Golgi apparatus in yeast.

Results

We developed a screen to identify compounds that inhibit anchoring and/or intracellular transport of GPI-linked proteins based on the mobility difference between the immature and mature forms of Gas1p. Maturation of Gas1p, which decreases its mobility in SDS–PAGE due to extensions of N- and O-linked carbohydrate chains, depends upon addition of a GPI anchor (Nuoffer *et al.*, 1993) and upon transport to the Golgi, as carbohydrate chain extension, but not anchoring, is blocked in the *sec18* mutant (Fankhauser and Conzelmann, 1991). Log phase cells were incubated with various compounds for 90 min and total proteins were extracted and separated on SDS–polyacrylamide gels. Gas1p was detected by Western blotting. If anchoring and/or transport was inefficient during the incubation period we would expect to find an accumulation of immature (M_r = 105 kDa) Gas1p. This result was found when cells were incubated with myriocin (Figure 1), a fungal metabolite. As myriocin is structurally similar to another fungal metabolite, sphingofungin C (Figure 5), we also tested this compound. Sphingofungin C also caused accumulation of immature Gas1p (Figure 1).

To determine the specificity and magnitude of the effect of myriocin on Gas1p transport, we performed a pulse–chase labelling experiment. Cells were preincubated with myriocin for 10 min at 30°C, then pulsed with [^{35}S]methionine and [^{35}S]cysteine for 5 min, and chased in the presence of unlabelled amino acids and sulfate for various times. Gas1p was analyzed by immunoprecipitation, SDS–PAGE and fluorography. In the absence of myriocin, maturation of Gas1p was virtually complete after 10 min of chase (Figure 2). In the presence of myriocin, maturation was much slower, but still occurred. Therefore, myriocin had a strong effect on the efficiency of Gas1p transport to the Golgi (site of maturation) even

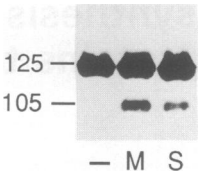

Fig. 1. Accumulation of immature Gas1p during incubation with myriocin. Myriocin (M), sphingofungin C (S) were added to 20 μg/ml to growing yeast cell (RH273-1B) cultures in YPUAD medium and incubated for 90 min at 30°C. The control (–) received 2% methanol as did the experimental samples. Total protein extracts were prepared and Gas1p was detected by Western blotting. The 105 kDa form of Gas1p represents the immature, pre-Golgi form of the protein.

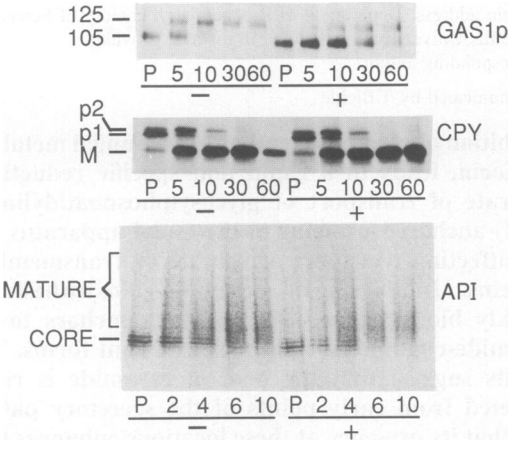

Fig. 2. Kinetics of maturation of Gas1p, CPY and API. For analysis of Gas1p and CPY maturation, yeast cells (RH273-1B) were preincubated with 10 μg/ml myriocin in 2% methanol (+) or with 2% methanol (–) for 10 min, then pulse labelled with [^{35}S]methionine and [^{35}S]cysteine for 5 min at 30°C. Chase was initiated by addition of unlabelled methionine, cysteine and sulfate. At the end of the pulse (P) or at the indicated chase time points NaN_3 and NaF were added and the cells were put on ice. The cells were lysed, and the indicated proteins were immunoprecipitated and analyzed by SDS–PAGE and fluorography. For the analysis of API, strain RH128-1A harboring plasmid pAPI306-67 was used and treated as RH273-1B except that the SDS gel was analyzed using a Molecular Dynamics Phosphorimager. A control experiment with strain RH128-1A showed that the kinetics of Gas1p maturation are affected by myriocin to a similar magnitude as with RH273-1B (data not shown).

after a relatively short incubation period. A slightly smaller effect of myriocin can also be seen when it is added simultaneously with the labelled amino acids. Increasing the preincubation period with myriocin had relatively little additional effect (data not shown).

If the effect of myriocin is specific for GPI-anchored proteins then the maturation of other proteins that follow the secretory pathway should be normal. We analyzed two other proteins, one soluble and the other membrane-bound. Carboxypeptidase Y (CPY), a soluble vacuolar hydrolase, is found in its p1 form in the ER, p2 form in the Golgi and mature form in the vacuole. One can clearly see that the transport and maturation of CPY is unaffected by myriocin treatment (Figure 2). As a transmembrane protein we chose a fusion protein between the first 306 amino acids of alkaline phosphatase (a vacuolar membrane protein) and a secreted protein (invertase) (API), because the maturation of this chimeric protein upon reaching the Golgi is readily detected by a mobility change in SDS–PAGE. API is

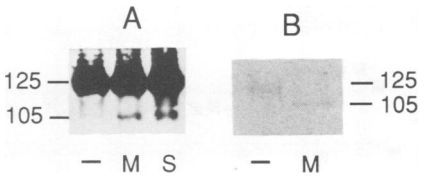

Fig. 3. Gas1p is GPI-anchored in the presence of myriocin. (**A**) Yeast cells (RH273-1B) were treated with myriocin (M) or sphingofungin C (S) and extracts were prepared as for Figure 1. The Western blot was treated with phospholipase C and then with rabbit anti-CRD. The rabbit antibody was revealed using chemiluminescence (ECL). Note that the immature, 105 kDa, Gas1p reacts with rabbit anti-CRD. (**B**) Yeast cells (RH273-1B) were treated for 10 min with 10 μg/ml myriocin (M), or 2% methanol (−), pulse labelled with [^{3}H]*myo*-inositol for 60 min. Extracts were prepared and Gas1p was immunopreciptated, separated on a 7.5% gel, and revealed by fluorography. The film was exposed for 2 months. The long exposure was necessary because Gas1p synthesis is repressed under the conditions required for inositol labelling.

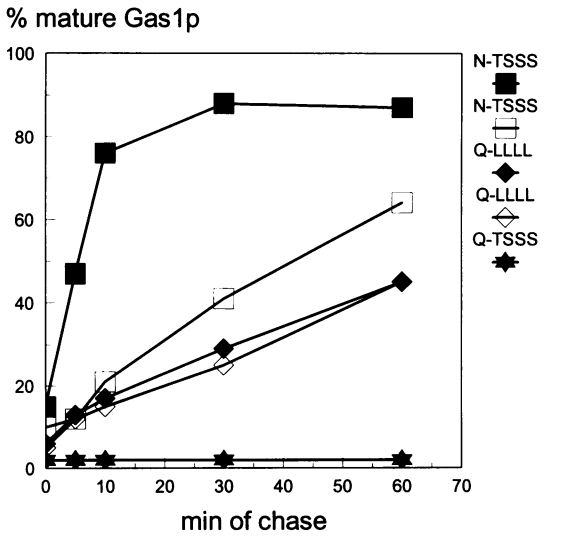

Fig. 4. The effect of myriocin is specific for GPI-anchored Gas1p. Yeast cells (RH273-1A, *gas1::LEU2*) harboring plasmids with wild type *GAS1* (squares; N-TSSS), *gas1-Q*^{506}L^{521,522,525,527} (diamonds; Q-LLLL), or *gas1-Q*^{506} (stars: Q-TSSS), were treated for 5 min with 20 μg/ml myriocin (open symbols: +) or 2% methanol (closed symbols: −), then pulse labelled with [^{35}S]methionine and [^{35}S]cysteine for 5 min at 30°C, and chased for the indicated times. After lysis, immunoprecipitation and SDS−PAGE the percentage of mature Gas1p was measured using a Phosphorimager.

transported from the ER to the Golgi, where it acquires outer chain mannoses, and then on to the vacuole (Klionsky and Emr, 1990). The maturation kinetics and thus transport to the Golgi apparatus of API are also unaffected by myriocin (Figure 2). Therefore, the effect of myriocin on Gas1p transport seems to be specific for this and possibly other GPI-anchored proteins.

A compound that affects transport of Gas1p to the Golgi could affect transport indirectly by inhibiting anchor synthesis and/or attachment. In order to test whether myriocin inhibits these reactions, cells were treated with myriocin or sphingofungin C and then processed for Western blotting. The Western blots were treated with phospholipase C to reveal the cross-reacting determinant (CRD) seen on many GPI anchors from trypanosomes, yeast and animals (Cardoso de Almeida and Turner, 1983; Zamze *et al.*, 1988; Nuoffer *et al.*, 1993). Gas1p is the only GPI-anchored protein in yeast that is detected under normal conditions using this assay (Nuoffer *et al.*, 1993). CRD was then detected using antibodies. The 105 kDa bands of immature Gas1p that accumulate after myriocin or sphingofungin C treatment react with these antibodies (Figure 3A) showing that GPI anchors were added. The signal representing CRD was proportionally lower for the 105 kDa band than for the 125 kDa band. The fact that we do not detect any 105 kDa, anti-CRD-reactive form of Gas1p without inhibitors shows that transport of this precursor to the Golgi is normally very rapid. Since we can detect some with inhibitors, its half time in pre-Golgi compartments must be longer in the presence of inhibitors. The lower reactivity of the 105 kDa with anti-CRD in this assay could be due to a differential susceptibility to phospholipase C, perhaps due to partial acylation of the inositol found in the GPI anchor (Costello and Orlean, 1992). This explanation is in fact quite likely because Gas1p produced in the *sec18* mutant and, therefore, trapped in a pre-Golgi compartment, also reacts weakly with anti-CRD in this assay (data not shown).

To obtain independent evidence that the 105 kDa protein was GPI anchored we labelled myriocin-treated and untreated cells with [^{3}H]*myo*-inositol, extracted proteins and immunoprecipitated Gas1p. As can be seen in Figure 3B, untreated cells had an [^{3}H]inositol-labelled band at 125 kDa, whereas myriocin-treated cells had a band of almost equal intensity at 105 kDa. Densitometric scanning of the two bands showed that in the presence of myriocin, the [^{3}H]inositol incorporated was 80% of the amount incorporated by the control without myriocin. This confirms that myriocin does not inhibit GPI anchoring of Gas1p. Therefore, the effect of myriocin on transport of this protein to the Golgi is not due to an inhibition of GPI anchoring.

The results thus far suggested that the transport delay of Gas1p was due to the fact that the protein is GPI-anchored. To test this more directly we constructed a non-GPI-anchored, membrane-bound form of Gas1p. When the anchor attachment site was mutated from N^{506} to Q^{506}, the protein was no longer GPI-anchored and was found in its ER form even after a 60 min chase period (Figure 4; Q-TSSS, star symbols) (Nuoffer *et al.*, 1993). Preliminary experiments suggested that at least part of the 'ER retention phenotype' of the mutant protein was due to uncharged, polar residues in the carboxy-terminal stretch of hydrophobic amino acids (P.Cosson, personal communication). Therefore, the three S and one T residues in this region were mutated to L and expressed in the context of the Q^{506} mutation yielding construct Gas1p-Q-LLLL. The protein encoded by this construct cannot be GPI-anchored because it did not react with antibodies against CRD (data not shown). When we examined the maturation of Gas1p-Q-LLLL by pulse−chase labelling as described above, we found that the protein was matured more rapidly than the Gas1p-Q-TSSS construct, but with slower kinetics than the GPI-anchored form (Gas1p-N-TSSS) of Gas1p (Figure 4). The maturation kinetics of the non-GPI-anchored protein (Gas1p-QLLLL) were unaffected by myriocin treatment (Figure 4). These results suggested that the reason Gas1p transport to the Golgi is inhibited by myriocin is that Gas1p is a GPI-anchored protein.

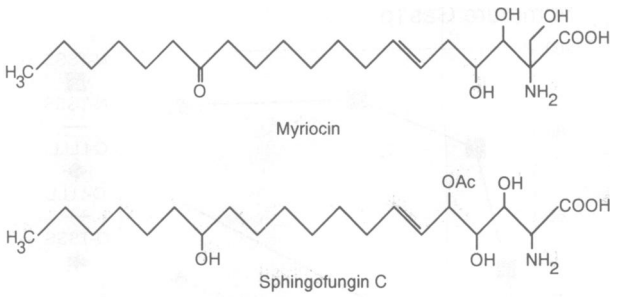

Fig. 5. Structures of myriocin and sphingofungin C.

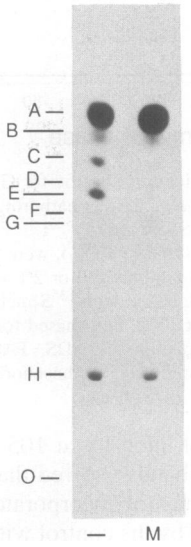

Fig. 6. Myriocin inhibits synthesis of inositol-containing ceramides. Yeast cells (RH273-1B) were preincubated for 5 min with 20 μg/ml myriocin (M) or 2% methanol (−) and pulsed with [^{3}H]*myo*-inositol for 20 min, followed by an 80 min chase. Lipids were extracted and analyzed by TLC. (**A** and **B**) Phosphatidylinositols; (**C** and **D**) inositolphosphoceramides (IPC); (**E**) mannosylinositolphosphoceramide (MIPC); (**F** and **G**) lysophosphatidylinositols; (**H**) mannosyl-(inositolphospho)$_2$-ceramide [M(IP)$_2$C]; O, origin.

Myriocin and sphingofungin C resemble each other (Figure 5) and also resemble dehydrosphinganine, the first product in the synthesis of ceramides. Sphingofungin C has been shown to inhibit the enzyme that produces dehydrosphinganine, serine:palmitoyl CoA transferase (Zweerink *et al.*, 1992). Myriocin also inhibits this enzyme from yeast when tested *in vitro* (M.Schönbächler, personal communication). In order to verify that myriocin also inhibited ceramide biosynthesis, we examined the effects of the compound on sphingolipid synthesis. Yeast cells were labelled in the presence and absence of myriocin with [^{3}H]*myo*-inositol following a pulse−chase protocol. The incorporation of inositol into the major sphingolipids, IPC and MIPC, was inhibited by myriocin (Figure 6C and D, and E, respectively). M(IP)$_2$C synthesis was much less inhibited by myriocin. This is most likely due to the incorporation of [^{3}H]*myo*-inositol into the pre-existing MIPC pool. The MIPC pool was probably not sufficiently depleted by the short preincubation with myriocin. Besides depletion from the ER, MIPC must be depleted from a post-ER compartment where M(IP)$_2$C is formed (Puoti *et al.*, 1991). Metabolic labelling experiments using

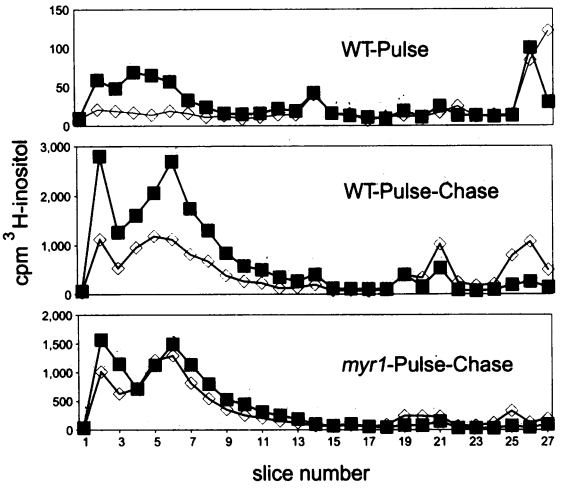

Fig. 7. Influence of myriocin on the incorporation of inositol into glycoproteins. Yeast cells (RH273-1B) were preincubated with 10 μg/ml myriocin (open diamonds) or 2% methanol (closed squares) for 20 min and pulse labelled for 20 min with [^{3}H]*myo*-inositol. Glycoproteins were isolated and separated by SDS−PAGE (10% gel) and the lanes were cut into 4 mm slices. The proteins were digested with pronase and the radioactivity counted (WT-Pulse). Glycoproteins smaller than ~20 kDa were not detected on this gel system. Mutant (myr1-pulse–chase; RH310-1A) cells and their isogenic parent (WT-pulse–chase; MH272-1D) cells were treated as above except that an 80 min chase period was added after the pulse labelling with [^{3}H]*myo*-inositol. Slice number 1 was the stacking gel. These experiments were performed several times and the total incorporation of [^{3}H]*myo*-inositol varied from experiment to experiment. Therefore, only the ratios of high to low molecular weight proteins should be compared for each labelling experiment.

[^{3}H]serine showed that all ceramide synthesis was strongly inhibited by myriocin (M.Schönbächler, personal communication). These experiments, along with the structural similarities and conservation of the critical functional groups (Horn *et al.*, 1992), show that myriocin inhibits ceramide biosynthesis in an analogous fashion to sphingofungin C.

If the effect of myriocin on Gas1p transport is caused by its effect on ceramide biosynthesis, then the two processes should be inhibited by similar doses of myriocin. To test this we performed a dose-inhibition study for the two processes. Both ceramide synthesis and Gas1p transport began to be affected (minimal inhibition concentration) by doses of myriocin between 0.25 and 0.5 μg/ml (0.9–1.8 μM).

Since the effect of myriocin on Gas1p transport is likely to be due to the presence of a GPI anchor on the protein then one would expect to find an effect of the compound on the maturation of other GPI-anchored proteins as well. In order to study this we subjected cells to a pulse or pulse−chase labelling regimen with [^{3}H]*myo*-inositol in the presence and absence of myriocin. [^{3}H]*myo*-inositol can be incorporated into the whole spectrum of GPI-anchored glycoproteins (Conzelmann *et al.*, 1992). Glycoproteins from the labelled extracts were analyzed by SDS−PAGE, the gel lanes were cut into slices and treated with pronase, and the radioactivity was extracted and counted (Figure 7). After pulse labelling with inositol, there was a strong myriocin-dependent decrease in the relative amount of radioactivity found in high molecular weight proteins (slices 2–7) and an increase in the amount

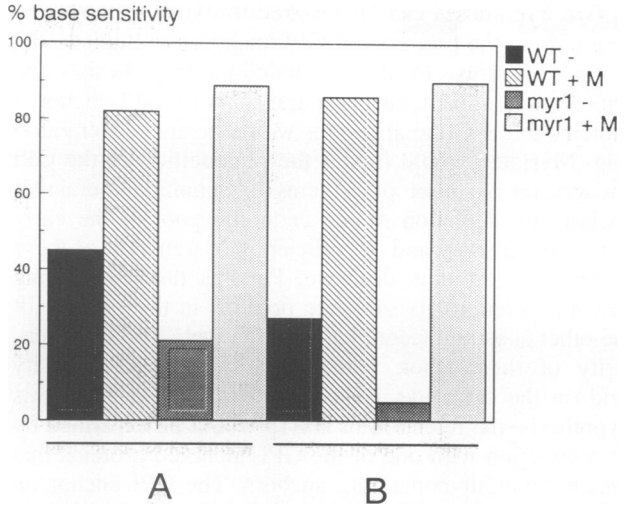

Fig. 8. Myriocin inhibits remodelling of GPI anchors to their mild base-resistant forms. Wild type (WT; MH272-1D) or mutant (myr1; RH310-1A) cells were treated as described in Figure 7 and two pools were created from each sample (**A**, slices 2 and 3; **B**, slices 4–8). The percentage of base-sensitive anchors was determined for each pooled sample as described in Materials and methods. Preincubations were with 20 μg/ml myriocin (M) or 2% methanol (−).

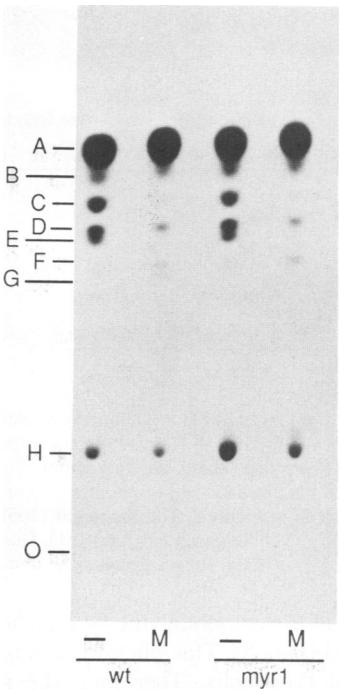

Fig. 9. Ceramide synthesis in the *myr1* mutant is sensitive to myriocin. Wild type (wt; MH272-1D) or mutant (myr1; RH310-1A) cells were treated and analyzed as described in Figure 6.

found at the bottom of the gel. Note that with this gel concentration some of the smaller proteins would have run with the dye front which was not analyzed due to its extremely high amounts of radioactivity due to comigration of phosphatidylinositol and IPCs. The results from this experiment are consistent with an effect of myriocin on the maturation of GPI-anchored proteins in general. In a pulse−chase labelling protocol the effect of myriocin on the relative amount of inositol incorporation into high molecular weight proteins was less than in a pulse labelling, suggesting that maturation of the high molecular weight GPI-anchored proteins was delayed, but not completely blocked, by myriocin.

Many of the GPI-anchored glycoproteins in yeast have IPC-containing anchors. Therefore, we looked for an effect of myriocin on the appearance of this modification. Regions of the gels from the high molecular weight region from the pulse−chase experiment (Figure 7) were digested with pronase and treated with mild base to determine whether the anchors were composed of ceramides. Ceramide-containing anchors are resistant to mild base treatment and still partition into the detergent phase of a Triton X-114 extraction after treatment. In the absence of myriocin, the majority of the anchors in both regions A and B (corresponding to fractions 2–3 and 4–8, respectively, Figure 8) were resistant to mild base treatment, indicating that they contained ceramides. In the presence of myriocin, however, >80% of the anchors remained base-sensitive demonstrating that they had not been converted to ceramide-containing anchors (Figure 8). Therefore, myriocin not only inhibited the transport of Gas1p and most likely that of other GPI-anchored proteins, but also blocked anchor remodelling. This result shows that the eventual maturation of these GPI-anchored proteins was not due to an incomplete effect of myriocin because the matured proteins had anchors that were base-sensitive and thus lacked ceramide.

As a tool to facilitate our understanding of the role of ceramides in protein transport and yeast cell growth, we isolated myriocin-resistant mutants. Since myriocin is toxic for yeast cells, mutant strains were isolated that could grow in the presence of the compound. All of the recessive myriocin-resistant mutants fell into one complementation group, *myr1*. We did not find any associated phenotype, such as temperature-sensitive growth or auxotrophies, except for resistance to sphingofungin C. This mutation did not confer resistance to other growth inhibitors, such as cycloheximide, rapamycin or nystatin, suggesting a specificity towards myriocin. We next investigated which of the ceramide-related phenotypes conferred by myriocin treatment were affected by the mutation.

Wild-type and mutant cells were labelled with [^{3}H]*myo*-inositol in the presence and absence of myriocin, and lipids were extracted and analyzed by TLC. The results of this experiment clearly show that ceramide synthesis in the resistant mutant was still inhibited by myriocin (Figure 9). Therefore, myriocin resistance is most likely not due to destruction or removal of the inhibitor, or to reversal of its effect on ceramide synthesis.

As the ceramides found in GPI anchors are not the major ceramides we have seen on TLC plates it is possible that the synthesis of these minor ceramides was restored in *m*yr1 cells. Therefore, we also analyzed the size of GPI-anchored proteins and the sensitivity to mild base treatment of their anchors after pulse−chase labelling with [^{3}H]*myo*-inositol in the presence or absence of myriocin in wild type and *myr1* cells. The relative amount of [^{3}H]*myo*-inositol incorporation into proteins of high molecular weight was virtually independent of myriocin treatment in the *myr1* mutant suggesting that maturation of GPI-anchored proteins other than Gas1p was restored (Figure 7). In the absence of myriocin, base-resistant anchors were added onto proteins in *myr1* cells, whereas

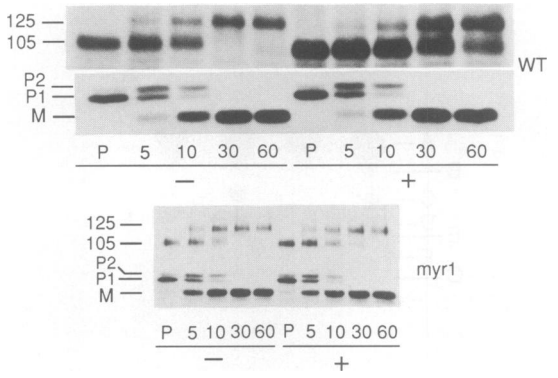

Fig. 10. Maturation of Gas1p in the *myr1* mutant is resistant to myriocin. Wild type (WT; MH272-1D) and mutant (myr1; RH310-1A) cells were analyzed by pulse−chase labelling with [^{35}S]methionine and [^{35}S]cysteine in the presence (+) or absence (−) of myriocin as described in the legend to Figure 2. Top panel, ER (105) and mature (125) forms of Gas1p. Middle panel, ER form (p1), Golgi form (p2) and vacuolar form (M) of CPY. Lower panel, same nomenclature.

in the presence of the compound the GPI anchors remained base-sensitive (Figure 8). This phenotype was the same in *myr1* and wild type cells. Therefore, the resistance to myriocin does not seem to be caused by a restoration of synthesis of ceramide-containing GPI anchors.

We next tested the rate of maturation of Gas1p in wild type and *myr1* cells in the presence or absence of myriocin. Cells were labelled with the same [^{35}S]methionine/[^{35}S]cysteine pulse−chase regimen as above and the rate of protein maturation followed by immunoprecipitation, SDS−PAGE and fluorography. The rate of Gas1p maturation was almost unaffected by myriocin in *myr1* cells, whereas the process was strongly affected in isogenic wild type cells (Figure 10). As expected, the biogenesis of CPY was unaffected by myriocin treatment in either strain. These data suggest that the mutation in the *MYR1* gene can restore rapid transport of Gas1p to the Golgi in the absence of ceramide synthesis.

Discussion

Previous studies in animal cells have shown that GPI-anchored proteins are targeted to specific membranes and seem to associate with sphingolipids, possibly implicating these molecules in the sorting or transport of GPI-anchored protein (Brown, 1992). In this study we showed that myriocin inhibits ceramide biosynthesis in yeast and had a rapid and negative effect on the kinetics of transport of a GPI-anchored protein to the Golgi apparatus. In addition, the remodelling of GPI anchors with ceramides to base-resistant forms was inhibited. Transport of a soluble protein, CPY, and a transmembrane protein to the Golgi were unaffected by myriocin, showing that protein transport to the Golgi proceeded normally for most proteins, but not for GPI-anchored proteins. The rapid action of myriocin suggests a direct effect of ceramides on GPI-anchored protein transport and also shows that ceramides are quickly exported from early stations of the secretory pathway after their synthesis. In normal cells both ceramides and GPI-anchored proteins are transported to the plasma membrane (Nuoffer *et al.*, 1991; Patton and Lester, 1991).

Two hypotheses can be invoked to explain the role of ceramide in the transport of GPI-anchored proteins to the Golgi apparatus. In the 'remodelling hypothesis', the critical determinant enabling transport of GPI-anchored proteins to the Golgi apparatus would be anchor remodelling. Myriocin would inhibit the remodelling of the GPI anchors on a subset of proteins to ceramide-containing anchors by depletion of the ceramide pool in the early secretory pathway and the efficiency of transport of these proteins would thus decrease. Perhaps the remodelling enzyme(s) retains its substrate proteins in the absence of the other substrate, ceramide. Alternatively, some particularity of the anchor, perhaps the length of the fatty acid on the ceramide, is necessary for transport. In this hypothesis, the retention of Gas1p would be explained by an interaction with one of the GPI-anchored proteins that obtain ceramide-containing anchors. The GPI anchor on Gas1p is not remodelled to ceramide (Fankhauser *et al.*, 1993) even though it is possible that it undergoes other remodelling reactions to provide it with a hydroxylated long chain fatty acyl group.

In a second hypothesis, GPI-anchored proteins would be cotransported to the Golgi after aggregation with sphingolipids in the plane of the membrane. In this 'clustering hypothesis', ceramides and GPI-anchored proteins would be cotransported and Gas1p transport would be delayed due to the depletion of ceramide from the ER. The role of sphingolipid would be to stimulate aggregation (assembly) of GPI-anchored proteins in the ER or a pre-Golgi compartment, rendering them competent for transport to the Golgi apparatus. The clustering in the plane of the membrane could function in an analogous manner to protein assembly, which is also thought to play a role in rendering membrane proteins competent for transport to the Golgi apparatus (Rutledge *et al.*, 1992). In both the remodelling and clustering models, the mechanism of vesicular transport to the Golgi apparatus is likely to be the same and similar to that for other proteins because soluble, membrane-bound and GPI-anchored proteins, as well as ceramides, all seem to depend upon the same *SEC* genes for this transport (Kaiser and Schekman, 1990; Fankhauser and Conzelmann, 1991; Puoti *et al.*, 1991).

The current evidence favors the clustering hypothesis for two reasons. First, it is unlikely that GPI anchors need to be remodelled for transport of the corresponding proteins to the Golgi. The *sec18* mutant blocks transport to the Golgi after budding of vesicles from the ER (Kaiser and Schekman, 1990). In this mutant, most of the GPI anchors accumulate in a base-sensitive form (Conzelmann *et al.*, 1992), suggesting that conversion to a base-resistant form usually takes place after leaving the ER. Second, the transport of Gas1p (Gas1p-Q-LLLL) without a GPI anchor is unaffected by myriocin, suggesting that the effect of myriocin on Gas1p transport is mediated through the GPI moiety and not by protein−protein interactions. One problem, however, with this experiment is that the delivery to the Golgi apparatus of the non-GPI-anchored form of Gas1p is inefficient. In both hypotheses, the site of the effect of the block in ceramide biosynthesis on GPI-anchored protein transport must be early in the secretory pathway from where the functional pool of ceramides is therefore quickly depleted.

The clustering hypothesis is very similar to that proposed

for transport of GPI-anchored proteins in animal cells (Simons and van Meer, 1988; Brown, 1992). The evidence in support of this hypothesis in animal cells is mainly circumstantial. GPI-anchored proteins are sorted to specific plasma membrane domains, either the apical or basolateral membrane depending on the cell type (Brown and Rose, 1992; Zurzolo *et al.*, 1993). The sorting event takes place in the Golgi apparatus where the formation of detergent-insoluble complexes containing glycosphingolipids and GPI-anchored proteins also occurs (Brown and Rose, 1992; Zurzolo *et al.*, 1994). Here, we offer another type of evidence consistent with this hypothesis; the introduction of an agent that blocks ceramide biosynthesis perturbs GPI-anchored protein transport. It would be interesting to investigate the effects of myriocin on GPI-anchored protein transport in animal cells.

The main difference between the clustering hypotheses in animals and yeast is the site of formation or function of the sphingolipid-GPI-anchored protein clusters. Our hypothesis proposes that these clusters are formed earlier in the secretory pathway than in animal cells, perhaps in the ER. This difference could be due to the localization of the enzymes that synthesize the sphingolipids. In animal cells, ceramide is made in the ER, but complex sphingolipid synthesis occurs in the Golgi apparatus (van Meer, 1993; Young, 1993). In yeast cells, both ceramide and IPCs seem to be formed in the ER (Puoti *et al.*, 1991; Lester and Dickson, 1993). Therefore, it is possible that the site of formation of these GPI-anchored protein/sphingolipid clusters is dictated solely by the site of synthesis of the relevant sphingolipids.

In animal cells, experiments using an inhibitor of sphingolipid synthesis, PDMP, also revealed effects of inhibition of sphingolipid synthesis on protein transport (Rosenwald *et al.*, 1992). This inhibitor blocked the synthesis of glucosylceramide rather specifically at low concentrations, but affected sphingomyelin as well at higher concentrations. It was found that at high PDMP concentrations transport of a membrane protein through the Golgi was progressively retarded with increasing preincubation times. GPI-anchored proteins were not studied. Therefore, it is difficult to compare the results of this study with ours.

As a first step towards understanding the requirement for ceramides in GPI-anchored protein transport we isolated mutants that were resistant to myriocin. All of the recessive mutants fell into one complementation group. Analysis of the mutant strain showed that ceramide synthesis and the remodelling to base-resistant anchors were still inhibited by myriocin, but the lack of ceramides no longer inhibited protein transport. An understanding of these results and the genetic alteration in the mutant strain should provide insight into the properties of ceramides that stimulate transport. At the present time we do not know how GPI-anchored protein transport is restored by the mutation. The mutation does not affect transport to the Golgi in general, because the transport of other proteins is unaffected. Neither does it inhibit remodelling of GPI anchors because remodelling proceeds normally in the absence of myriocin. It has no major effect on the synthesis of inositol-containing lipids, nor have we detected any other associated phenotypes, such as auxotrophies or growth defects at different temperatures. It is possible that an alternative lipid is synthesized in the mutant strain that functionally replaces ceramides.

The fact that myriocin blocks cell proliferation by inhibiting ceramide synthesis is not surprising. The genes required for expression of serine:palmitoyltransferase activity (*LCB1* and *LCB2*; Pinto *et al.*, 1992), the probable target of myriocin (Horn *et al.*, 1992; Zweerink *et al.*, 1992), are essential for growth on commonly used media. In addition, an extragenic suppressor (*slc1*) of the *lcb1* disruption mutation has been isolated and, like *myr1*, does not restore ceramide biosynthesis (Dickson *et al.*, 1990). In the place of ceramides, this strain produced novel inositolglycerol lipids with long chain fatty acyl chains (Lester *et al.*, 1993). The suppressor mutant was semi-dominant and displayed a growth phenotype (Lester and Dickson, 1993) which is different from that of *myr1*. Nevertheless, even though unlikely, it is not possible to say at the moment that the two mutations are in different genes.

In summary, our results show that inhibition of ceramide synthesis by myriocin leads to a rapid and specific decrease in the rate of transport of GPI-anchored proteins to the Golgi apparatus in *Saccharomyces cerevisiae*. This effect is probably due to a rapid depletion of sphingolipids from the ER. A normal rate of transport of GPI-anchored proteins was seen in a myriocin-resistant mutant without restoration of ceramide synthesis. Further characterization of this mutant may shed some light on the mechanism of transport and provide additional insights into the roles that ceramides play in cell physiology.

Materials and methods

Yeast strains and media

Two sets of isogenic yeast strains were used for most studies: RH273-1B (*MATα his4 leu2 ura3 bar1-1*) and RH273-1A (*MATα his4 leu2 ura3 bar1-1 gas1::LEU2*) (Nuoffer *et al.*, 1991) were used as wild type in the initial studies and host for expression of variant *GAS1* constructs, respectively. The different *GAS1* alleles were constructed and introduced into cells as described previously (Nuoffer *et al.*, 1993). Mutants were isolated from MH272-1D (*MATa his3 leu2 ura3 trp1*) by plating onto YPD plates containing 5 μg/ml myriocin. Several recessive mutants were isolated that all fell into one complementation group, which we called *myr1*. One allele of *myr1* was then further pursued. The mutation conferring myriocin resistance segregated in crosses as a single gene. The backcrossed mutant used for all studies was RH310-1A (*MATa his3 leu2 ura3 trp1 myr1*). The strain RH128-1A (*MATα leu2 ura3 ade2 suc2Δ9*) transformed with plasmid pAPI306-67 (Klionsky and Emr, 1990) was used for expression of the alkaline phosphatase−invertase (API) chimeric protein. Cells containing plasmids were grown under selective conditions on SD drop-out medium (Dulic *et al.*, 1991) to saturation and used to inoculate overnight cultures. Overnight cultures were in rich medium (YPUAD; Dulic *et al.*, 1991) for Western blotting and in semisynthetic medium [0.67% yeast nitrogen base (Difco), 0.2% Bacto yeast extract (Gibco), 2% glucose, 40 μg/ml each uracil, adenine, histidine, leucine and uracil where appropriate] for ^{35}S-labelling or [^{3}H]*myo*-inositol labelling.

Western blotting and immunoprecipitation

Yeast cells (1 OD_{600} unit/ml) were incubated with 10 or 20 μg/ml myriocin (we detected no differences between results with these concentrations) or sphingofungin C added from a stock solution (1 mg/ml in methanol) or with 2% methanol for 90 min. Proteins were extracted from cells and separated on SDS−polyacrylamide gels, and Gas1p was detected with antibodies raised in rabbits, followed by secondary antibodies coupled to horseradish peroxidase, whose reaction product was detected by chemiluminescence using an ECL kit (Amersham) (Nuoffer *et al.*, 1993). Cross reacting determinant (CRD) was detected in a similar manner, but the filter was first treated with phospholipase

C, followed by incubation with rabbit anti-CRD, secondary antibody and chemiluminescence as described (Nuoffer *et al.*, 1993). Anti-CRD was kindly provided by Dr M.-L.Cardoso de Almeida (São Paolo, Brazil).

For immunoprecipitation, cells were broken with 0.2 g glass beads (0.45 mm) in 0.25 ml TEPI (50 mM Tris−HCl pH 8, 5 mM EDTA, 2 mM PMSF, 30 μg/ml each, leupeptin, antipain, pepstatin) by mixing by vortex four times for 1 min each in 1.5 ml Eppendorf tubes. The solution was removed to a fresh tube and the beads were washed with 0.25 ml TEPI which was pooled with the original extract. SDS was added to 1% and the extract was immediately boiled. After centrifugation at top speed in an Eppendorf centrifuge for 15 min, the solution was transferred to 15 ml Falcon tubes and diluted with 10 volumes of TNET (100 mM Tris−HCl pH 8, 100 mM NaCl, 5 mM EDTA, 1% Triton X-100). Immune serum (5–10 μl) was added to the supernatant and incubated at room temperature for 2 h. Protein A−Sepharose beads (50 μl, 30% solution) were added and further incubated for 1 h. After five washes the immune complexes were dissociated with 50–70 μl of 2-fold concentrated sample buffer (Laemmli, 1970) and one-third of this was separated by SDS−PAGE. The gels were treated with 1 M sodium salicylate and exposed against Kodak XOMAT-AR-5 film at −70°C or visualized in a Molecular Dynamics Phosphorimager without prior salicylate treatment.

[^{3}H]inositol labelling and analysis of mild base sensitivity

For lipid extractions cells were grown overnight in semisynthetic medium, were washed twice and concentrated to 4 OD_{600} units/ml with SD medium lacking inositol. After 20 min preincubation with or without myriocin (final concentration 10 μg/ml), 14 μCi [^{3}H]*myo*-inositol/0.5 ml sample were added and incubated at 30°C for 20 min at which time an 80–100 min chase was initiated by adding 4 × concentrated vitamin solution containing inositol (Dulic *et al.*, 1991). After the chase period NaN_3 and NaF were added to 10 mM final concentration each and the cells were chilled on ice. After two washes with H_2O lipids were extracted.

For labelling of proteins with [^{3}H]*myo*-inositol, the labelling procedure was the same except that only 1.5 OD_{600} unit cells/sample were used. In the experiment involving immunoprecipitation of Gas1p, 0.1 mCi [^{3}H]*myo*-inositol was used. The cells were lysed and Gas1p immunoprecipitated as described above. For determination of mild base sensitivity 10 OD_{600} units of cells and 0.1 mCi [^{3}H]*myo*-inositol were used per sample.

Mild base sensitivity (Conzelmann *et al.*, 1992) was determined after inositol labelling and cell disruption with glass beads. The supernatant from the centrifugation of the glass bead lysate was transferred to 15 ml Falcon tubes and diluted with 20 volumes of Con A buffer (50 mM Tris−HCl pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM benzamidine, 1% Triton X-100). Concanavalin A−Sepharose beads (100 μl/sample) were added and shaken overnight at 4°C. The beads were collected by centrifugation in a table top centrifuge and washed five times with Con A buffer. Proteins were eluted with SDS sample buffer, one-fifth of the sample was loaded onto an analytical SDS−polyacrylamide (10%) gel, and four-fifths was loaded onto a preparative SDS−polyacrylamide (10%) gel. The preparative gels (10 cm length) were cut into 4 mm slices and each slice was incubated for 16 h in 0.4 ml pronase buffer (100 mM Tris−HCl pH 8.0, 1 mM $CaCl_2$, 10 mM NaN_3, 0.04% Triton X-100, 20 μg/ml gentamicin, 1 mg/ml pronase). An aliquot (20 μl) was taken for counting and the main peaks were pooled and dried in a Speedvac. Dried samples were resuspended in 0.42 ml of lipid extraction solvent (see above) and shaken for 25 min, then split into four aliquots (labelled 1–4). NaOH was added (0.1 M, final concentration) to samples 1 and 2 and all samples were incubated at 37°C for 30 min. The reaction was stopped by adding 20 μl of 1 M acetic acid to samples 1 and 2 and 20 μl of 1 M sodium acetate pH 5.3 to samples 3 and 4. The samples were dried and resuspended in 0.5 ml of Triton X-114 solution (1% Triton X-114, 10 mM Tris−HCl pH 7.4, 150 mM NaCl) by mixing (30 min), sonication in a bath (10 min) and heating to 37°C (30 min). The samples were spun for 10 min at full speed in an Eppendorf centrifuge and the detergent and aqueous phases separated and counted. The percentage of base-sensitive anchors was calculated in the following manner (average of c.p.m. in aqueous samples 1 and 2 − average of c.p.m. in aqueous samples 3 and 4 = *A*. *A* + average of c.p.m. in detergent phase of samples 3 and 4 = *B*. The percentage of base-sensitive anchors is equal to $A/B \times 100$.

Lipid extraction and chromatography

Lipids were extracted and analyzed with slight modifications from published procedures (Puoti *et al.*, 1991). Labelled cells were collected by centrifugation and resuspended in 0.5 ml of extraction solvent, $CHCl_3$:CH_3OH:H_2O (10:10:3, by vol.), with 0.5 g of glass beads, mixed by vortexing at top speed for 0.5 min, followed by 30 min of shaking on an Eppendorf tube shaker. The supernatant was removed to a new tube and the pellet was further shaken 20 min with 0.3 ml of the same solvent. The extracts were pooled, dried and resuspended in 0.15 ml of *n*-butanol (water-saturated). The butanol phases were extracted with 75 μl of water, the organic phase removed to a fresh tube, and the remaining water phase back-extracted with 75 μl of *n*-butanol. The organic phases were pooled, dried and resuspended in 40 μl of extraction solvent. Samples (4 μl) were loaded onto TLC plates (Kieselgel 60, Merck), dried, then developed with $CHCl_3$:CH_3OH:0.25% KCl (55:45:10, by vol.). The plates were sprayed with EN^{3}HANCE Spray (NEN−Dupont) and exposed to X-ray film at −70°C. *Myo*-[2-^{3}H(*N*)]-inositol (1 Ci/mmol) was from NEN−Dupont.

Maturation of Gas1p

Yeast cells were grown overnight to mid log phase in semisynthetic medium, harvested by centrifugation, washed twice with synthetic medium and resuspended to 2.5×10^7 cells/ml in the same medium. After a 10 min preincubation with or without myriocin (10 μg/ml), [^{35}S]methionine and [^{35}S]cysteine (Expre^{35}S^{35}S; NEN−Dupont) were added to 100 μCi/ml and incubation continued for 5 min. Chase were initiated by adding methionine, cysteine and ammonium sulfate to 30 μg/ml, 30 μg/ml and 3 mM, respectively. At the indicated time points, cells were chilled on ice in the presence of 10 mM NaN_3 and 10 mM NaF. Cells were lysed with glass beads and immunoprecipitations were carried out as described above.

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