The functioning of the yeast Golgi apparatus requires an ER protein encoded by *ANP1*, a member of a new family of genes affecting the secretory pathway

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Mnt1p is an α 1,2-mannosyltransferase which resides in an early compartment of the *Saccharomyces cerevisiae* Golgi apparatus. We have shown that the signal-anchor region is sufficient, and the transmembrane domain necessary, for its normal Golgi localization. This is similar to the transmembrane domain-mediated retention of mammalian glycosyltransferases, and distinct from the tail-mediated recycling retention of certain mammalian and yeast trans-Golgi proteins. To examine the mechanism involved in transmembrane domain-mediated retention, we have isolated six classes of mutants which fail to retain Mnt1p–reporter fusions in the early Golgi. These mutants all show additional phenotypes which are consistent with alterations in Golgi function. We have called the mutant classes ‘gem’, for Golgi enzyme maintenance. *GEM3* is identical to the previously cloned gene *ANP1*, and homologous to *VAN1* and *MNN9*. Together, these define a new class of proteins involved in the organization and functioning of the secretory pathway. Interestingly, Anp1p is localized to the endoplasmic reticulum (ER), implying that some function of the ER is required to maintain a functional Golgi apparatus.

Key words: glycosylation/Golgi/protein sorting/transmembrane domain/yeast

Introduction

The secretory pathway of eukaryotes is organized into a series of distinct membrane-bound compartments. Specific vesicular transport maintains a vectorial flow from the endoplasmic reticulum (ER) outwards, via the intermediate compartment and a series of Golgi subcompartments. At the trans-Golgi, the pathway diverges, with some vesicles targeted to the plasma membrane, and others to the lysosomal/vacuolar system. In mammalian cells, specific sorting signals direct both membrane-associated and soluble proteins to lysosomes, with transport to the plasma membrane occurring by default. In yeast, it appears that no sorting signal is needed for transport of membrane proteins to the vacuole (Nothwehr *et al.*, 1993). However, as in mammalian cells, the default path for soluble proteins is secretion. In both yeast and mammalian cells, the components of the earlier compartments need specific sorting signals to ensure that they remain in the appropriate place, rather than continue along the pathway as part of

the bulk flow (reviewed in Pelham and Munro, 1993). Soluble and membrane proteins of the ER are retained by a recycling/retrieval mechanism operating from the early Golgi. Specific sequences are recognized in the Golgi, and the proteins then carried back to the ER (reviewed in Pelham, 1989).

All known Golgi residents are integral membrane proteins. It is becoming clear that in mammalian cells two distinct retention mechanisms are found: one operating throughout the Golgi stack and the other only in the trans-Golgi network (TGN). The retention signal for all of the glycosyltransferases that have been analysed lies in their transmembrane domain, or TMD (Munro, 1991; Nilsson *et al.*, 1991; Machamer, 1993). This is not dependent on particular sequence motifs. However, specific amino acid sequences in the tails of the TGN proteins furin, TGN38 and the mannose 6-phosphate receptor, direct their retention (Johnson and Kornfeld, 1992; Bos *et al.*, 1993; Humphrey *et al.*, 1993; Wong and Hong, 1993; Chapman and Munro, 1994). These, like ER proteins, are retained by a mechanism involving continuous retrieval from a later compartment, in this case from the plasma membrane.

In yeast analyses of DPAP A, Kex2p and Kex1p, enzymes of the most trans-Golgi compartment, show that a sequence-dependent recycling mechanism is in operation (Cooper and Bussey, 1992; Wilcox *et al.*, 1992; Nothwehr *et al.*, 1993). Motifs in their cytoplasmic tails have been shown to be necessary for Golgi localization, and in at least one case recycling via the pre-vacuolar compartment has been demonstrated (Wilsbach and Payne, 1993). Three Golgi mannosyltransferases have also been cloned from yeast: *MNN1*, *OCH1* and *MNT1*. All are predicted to be Type II membrane proteins, with short transmembrane regions and short cytoplasmic tails (Hausler and Robbins, 1992; Hill *et al.*, 1992; Nakayama *et al.*, 1992; Yip *et al.*, 1994). This topology is the same as that of the mammalian glycosyltransferases, and distinct from that of yeast and mammalian TGN proteins.

OCH1 encodes an α 1,6-mannosyltransferase, which is responsible for the first post-ER modification elongating the α 1,6 backbone of N-linked sugars (Nakanishi-Shindo *et al.*, 1993). Functionally, this places it in the earliest Golgi subcompartment. *MNT1* encodes an α 1,2-mannosyltransferase involved in O-glycosylation (Hausler *et al.*, 1992), and *MNN1* the α 1,3-mannosyltransferase responsible for addition of the terminal mannose residues to the outer chain and core of N-linked polysaccharides and to O-linked mannotriose (Raschke *et al.*, 1973; Graham *et al.*, 1992). Biochemical and genetic data place these enzymes in the Golgi, in compartments distinct from that containing the majority of Kex2p (Cunningham and Wickner, 1989; Franzusoff and Scheckman, 1989; Graham and Emr, 1991; Bryant and Boyd, 1993). These enzymes are therefore comparable in localization, and function, to enzymes of the mammalian Golgi stack.

Fig. 1. Schematic illustration of the fusion proteins used in this study. All span the membrane once, with the N-terminus in the cytoplasm. The myc epitope is represented by a black box at the C-terminus of full-length Mnt1p (MMM), and prior to a HA epitope tag at the C-terminus of full-length Och1p (OOO). The myc tag is also present in the cytoplasmic tail of Pho8p (PPP) and at the junction of all the other fusions. All 'MMM' chimeras consist of full-length tagged Mnt1p, with either the 507 luminal amino acids of Pho8p (MMMP), or the 512 C-terminal amino acids of invertase fused to the C-terminus (MMMI). The MMMI fusion also contains a Kex2p-dependent cleavage site from pro- α -factor, in between the epitope tag and the *SUC2*-derived portion. The MPMI construct is equivalent to the MMMI in all but the transmembrane region, where 26 amino acids from Pho8p TMD replace 19 amino acids from Mnt1p. The OOP fusion contains the first 60 amino acids of Och1p fused to the luminal portion of alkaline phosphatase. The PMP protein has the 26 transmembrane amino acids of PPP replaced with the 19 transmembrane amino acids from Mnt1p.

We are interested in the TMD-mediated retention of glycosyltransferases. We have taken a genetic approach to analyse this retention mechanism. We have used two assay systems for measuring Golgi localization; one of these is novel, using activation of alkaline phosphatase fusions to measure mistargeting to the vacuole. Our results indicate that the TMD is involved in the normal localization of Mnt1p. In an attempt to learn more about the mechanism involved, we have isolated mutants which mislocalize Mnt1p-reporter fusions. We have named these 'gem' mutants (Golgi enzyme maintenance). One such gene isolated is identical to *ANP1*, which shows homology to *VAN1* and *MNN9*. Cells lacking any of the proteins encoded by these genes have defects in glycosylation. *ANP1*, *VAN1* and *MNN9* thus define a new class of proteins involved in the organization of the secretory pathway.

Results

Golgi retention is saturable

In order to generate an assay which could distinguish between proteins which are, or are not, localized to the Golgi apparatus, we needed first to characterize the normal distribution of Mnt1p and Och1p. We also wished to test whether Golgi localization was saturable and, if so, where the excess proteins were found.

To localize Mnt1p and Och1p, we constructed epitope-tagged versions of each (Figure 1; MMM and OOO). When expressed at high levels, either on a 2 μ vector or

by multiple integration, both gave immunofluorescent staining in patches throughout the cell, characteristic of the yeast Golgi, and distinct from BiP staining in the ER (Figure 2A). When the distribution of the two proteins was compared, ~70% of the patches showed co-localization (Figure 2B). No signal was detectable when low levels were expressed.

Previously, it has been shown that when overexpressed, the trans-Golgi enzymes Kex2p, Kex1p and DPAP A accumulate in the vacuole (Cooper and Bussey, 1992; Wilcox *et al.*, 1992; Nothwehr *et al.*, 1993). In cells expressing very high levels of tagged Och1p or Mnt1p, we could then see some additional membranes staining (Figure 2A, c and e). These were distinct in form from the Golgi patches (Figure 2A, a). In some cells, we could see co-localization with the ER marker BiP, although in others the membranes were clearly distinct (Figure 2A, c and e). Focusing through the samples showed that these membranes formed part of a larger structure with a morphology similar to that previously described for the vacuole (Roberts *et al.*, 1991). Our samples may underestimate the amount of this staining, as it has been shown that vacuolar proteins are relatively inefficiently fixed by immunofluorescence procedures (Roberts *et al.*, 1991). We therefore used a fractionation procedure to analyse this further. This separates a 100 000 g pellet containing Golgi membranes (P100) from a 13 000 g pellet of bulk cellular membranes (P13) (Horazdovsky and Emr, 1993). In our hands, using densitometry of protein blot signals

Fig. 2. (A) Mnt1p and Och1p are localized to discrete patches throughout the cell. Indirect immunofluorescence of 6210ΔP containing *TPI*-driven MMM integrated (a, e) or *TPI*-driven OOO on a 2μ vector (c). Each is recognized by the anti-myc monoclonal 9E10 and double labelled with rabbit polyclonal anti-BiP antiserum (b, d, f). White arrows indicate vacuolar-type staining, and arrowheads areas of co-localization with BiP. Bar is 2 μm. (B) Partial co-localization of Mnt1p and Och1p. Indirect immunofluorescence of 6210ΔP containing both the MMM and OOO constructs as in (A). Bar is 2 μm. The MMM chimera is recognized by polyclonal anti-myc antiserum (which does not recognize the internal myc tag of the OOO chimera) and the OOO by 12CA5, a monoclonal antibody recognizing the HA epitope. Some of the co-localizing patches are marked with white arrowheads. Despite extensive overlap, there remain patches with distinct staining for either MMM and OOO. (C) Subcellular fractionation of strains expressing the OOO protein. Twenty OD₆₀₀ units of 6210ΔP expressing OOO on 2μ or *CEN* vector were converted to spheroplasts, lysed and differentially centrifuged. Equal amounts of the 13 000 g pellet (P13) and the 100 000 g pellet (P100) were electrophoresed and Western blotted with 9E10 monoclonal, followed by peroxidase anti-mouse antibody. Visualization was by ECL. The position of a molecular weight standard (68 kDa) is marked with an arrow. In our hands, an average of 20% of total BiP, 60% of Kex2p and 20% of mature CPY are found in the P100 by densitometry.

on each fraction, some Kex2p also appears in the P13, but the P100 is relatively free of mature CPY (which is in the vacuole) and BiP, an ER-resident protein. At a very high expression level on a 2μ vector and driven by the *TPI* promoter, the majority of tagged Och1p is found in the P13 (Figure 2C), whereas when expressed at low levels on a *CEN* vector, most is in the P100 (Figure 2C). Similar results were obtained when cells expressing different levels of Mnt1p were examined (data not shown). These fractionation results are consistent with the immunofluorescence data, showing that when overexpressed, some protein accumulates in the ER, but as observed with the trans-Golgi enzymes, Och1p and Mnt1p also reach the vacuole. It appears that the retention of either enzyme in the yeast Golgi is saturable.

Analysis of the signal for Golgi retention

We next asked whether the TMD is involved in the retention of these yeast enzymes. Initially we linked the signal-anchor regions of Mnt1p and Och1p to the luminal portion of alkaline phosphatase, a Type II vacuolar membrane protein encoded by the *PHO8* gene (Figure 1; OOP).

As a control, we used full-length Pho8p (Figure 1; PPP). The signal-anchor region of this protein should not contain targeting information if transport to the vacuole is by default. Fractionation data indicate that ~60% of the OOP fusion is present in the P100 fraction, as compared to 5–7% of the control PPP (Figure 3C). Fractionation of MMP yields similar results (data not shown). Thus, the signal-anchor regions of Och1p and Mnt1p appear sufficient to localize the majority of a heterologous reporter to the yeast Golgi.

To address the question of whether these signal-anchor fusions were correctly localized within the Golgi, we used strains expressing the higher levels needed for an immunofluorescence signal. Figure 3A shows the localization of OOP, detectable in patches characteristic of the yeast Golgi. Double labelling of MMP with anti-Kex2p antiserum (both at high levels of expression) shows some overlapping patches, but many are distinct (Figure 3B). An overlapping immunofluorescence signal could signify targeting to the same compartment, or may simply represent signals from adjacent cisternae which have not been resolved. If these signals are coming from the

Fig. 3. (A) The signal-anchor region of Mnt1p and Och1p is sufficient to localize Pho8p in the Golgi. Double-label immunofluorescence of OOP chimera (integrated under the control of the *TPI* promoter in 6210ΔP) recognized by 9E10, co-stained with anti-BiP antiserum. Bar is 2 μm. (B) Partial overlap of MMP chimera with Kex2p. 6210ΔP cells expressing integrated *TPI*-driven MMP chimera, and plasmid pGA714 (2μ *KEX2*) (gift from Nia Bryant). The 9E10 monoclonal antibody recognizes MMP and polyclonal antiserum from K.Redding recognizes the Kex2p. Bar is 2 μm. (C) Subcellular fractionation of OOP and PPP strains. The strains visualized in (A) and (B) were sphaeroplasted and fractionated as in Figure 2C. The blots were analysed in the linear range by densitometry. The position of a molecular weight standard (68 kDa) is marked with an arrow. (D) The Mnt1p TMD is sufficient to slow cleavage of Pho8p. Equal amounts of log-phase SEY6210 containing PHO5-driven PPP or PMP were vortexed with glass beads in sample buffer. The sample was then processed as in Figure 2C, and analysed in the linear range by densitometry.

same compartment, this could represent a true overlap in distribution. Alternatively, this may be a result of overexpression. However, the non-overlapping patches suggest that the signal-anchor is indeed sufficient to target these fusion proteins to a subcompartment of the Golgi, distinct from that containing Kex2p. This is consistent with the immunoprecipitation data of Bryant and Boyd (1993), where they are able to separate Kex2p-containing compartments from the earlier Mnt1p-containing Golgi compartments.

To address the role of the TMD in retention, we next constructed full-length fusions of Mnt1p to invertase (the product of the *SUC2* gene) with the TMD of Pho8p replacing that of Mnt1p (see Figure 1; MMMI and MPMI). The linking region contains the Kex2p-dependent cleavage site from α-factor. Therefore, if the protein was mislocalized from the early Golgi to the Kex2p-containing compartment, soluble invertase would be produced and secreted from the cell (Figure 4A). Invertase secretion is detectable by a colorimetric assay on plates, and has been used successfully in the past to screen for mislocalization of soluble ER proteins (Pelham *et al.*, 1988). In the fusions containing the Pho8p TMD, more invertase was secreted than in the fusions with the Mnt1p TMD (for example see Figure 5A). Therefore, we would suggest that most of the MPMI protein has been in contact with Kex2p and

is not retained as efficiently in the early Golgi compartment as the MMMI protein.

To establish whether the Mnt1p TMD is sufficient to localize a heterologous reporter to the yeast Golgi, we replaced the TMD of full-length Pho8p with the Mnt1p TMD (Figure 1; PMP). Normally, activation of Pho8p occurs in the vacuole where it is processed by a *PEP4*-encoded protease (Figure 4A) (Klionsky and Emr, 1989). This event is detectable by gel electrophoresis as a shift in mol. wt from 76 to 72 kDa (Klionsky and Emr, 1989). We used this shift to measure the degree of processing of the PMP chimera compared with the PPP protein. Figure 3D clearly shows that in whole cells less of the chimera containing the Mnt1p TMD is processed and quantitation indicates that 90% of the PMP chimera is uncleaved, compared to 25% of the PPP protein. Further, quantitative fractionation indicates that there is a 125% increase in the amount of uncleaved PMP detectable in the P100 compared to the PPP fusion (data not shown). This indicates that the Mnt1p TMD will at least partially retain a heterologous protein in the yeast Golgi. These results are all consistent with the yeast mannosyltransferases being retained in the Golgi by the same mechanism as that used by the mammalian glycosyltransferases, i.e. the signal-anchor region contains information to retain a reporter in the yeast Golgi, with the TMD being necessary but only

Fig. 4. (A) Schematic diagram showing the basis of the assays used in this paper. (B) Immunofluorescent localization of MMMP chimera. 6210ΔP containing integrated *TPI*-driven MMMP. The cells were stained with 9E10 and anti-BiP antiserum as in Figure 2A. Bar is 2 μm.

partially sufficient for this (Munro, 1991; Dahdal and Colley, 1993; Burke *et al.*, 1994).

An assay for Golgi retention

The activation of alkaline phosphatase may be detected by a colorimetric plate assay on lysed cells (Kaneko *et al.*, 1982). We modified this method so that live cells could subsequently be rescued from the colonies, and tested the OOP, MMP, PMP and PPP type chimeras in this assay. PPP gave a stronger assay signal than PMP, which in turn gave a stronger signal than either of the fusions to Golgi proteins. These results are consistent with the data described above, although these signal-anchor constructs gave a slightly stronger signal than the equivalent full-length fusions MMMP and OOP (Figure 1). The reasons for this are unclear, but may reflect a small contribution from the rest of the protein to retention, as has been seen in studies on mammalian glycosyltransferases (Munro, 1991; Dahdal and Colley, 1993; Burke *et al.*, 1994). We decided to use the full-length fusions for all further studies.

Our second method of assaying Golgi localization relied on the fusion of invertase to the C-terminus of Mnt1p and Och1p (described above and in Figure 4A).

These two assays were designed to be complementary, in that one asked for mislocalization to the vacuole, whereas the other asked for a more subtle mixing of the Golgi compartments. In each we expected a background signal due to the leakage and turnover of the fusion proteins, which should vary according to the expression levels. Integration of plasmid DNA into the yeast genome results in strains carrying a varying copy number of the sequence of interest. Thus, we produced strains, in a *suc2 pho8* background, with high (multiple copies) and low (single copy) expression levels of the invertase and alkaline phosphatase fusion constructs. Immunofluorescence of the high-expression strains showed that the chimeras had a patchy distribution, characteristic of the yeast Golgi (Figure 4B).

There was a clear correlation between the steady-state level of expression (detected by whole-cell Western blots)

Fig. 5. (A) The parental MMMI strain used for the screen, a strain expressing *TPI*-driven integrated MPMI chimera and some mutants from the invertase screen were patched out on rich media with fructose instead of glucose and incubated at 25°C overnight. These were removed with a filter paper and overlaid on another filter containing substrates for invertase colorimetric assay. Secreted invertase activity is normally seen as a red ring around the colony. In patches containing slow-growing mutants, the whole patch stains red. (B) Example of complementation from the screen. Invertase activity assay on four haploid mutants and the diploids generated from these by mating.

and the strength of positive signal on the plate assays described (data not shown). Thus, higher expression levels result in a positive signal (i.e. mislocalization) in our assay. This is consistent with the IF and fractionation data already discussed. Taking account of this, these assays can be used on strains with suitably low expression levels, as a sensitive method for monitoring mislocalization from the cis/medial yeast Golgi compartment.

Screen for mutants in Golgi retention

Strains expressing low levels of either the MMMP or the MMMI reporter were constructed in a background deleted for both the *SUC2* and *PHO8* genes. These appeared negative on the plate assays described, but were detectable at low levels on protein blots (data not shown). These strains were then outcrossed to produce strains of the opposite mating type expressing exactly the same amount of fusion protein.

UV-induced mutants of both mating types were detected as colonies giving a positive signal using the appropriate plate assay, and the strongest were picked, streaked out as single colonies twice and re-tested. In total, 19 α and 44 α mutants that contained activated alkaline phosphatase were isolated from 100 000 mutagenized cells, and 47 α and 42 α mutants were selected from 100 000 cells in the invertase secretion screen (for examples see Figure 5A). These were all tested for dominance by backcrossing to the appropriate parent, and the few dominant mutants not characterized further. The rest were crossed with mutants of the opposite mating type which expressed the same construct, and the resulting diploids assayed for a positive signal (for an example, see Figure 5B).

In each screen four complementation groups could be defined, with the rest complementing all mutants of the opposite mating type. Complementation between mutants isolated in each screen shows that the strongest mutants of the two largest groups in each screen are non-complementing, and thus represent the same groups. We term these mutant classes *gem1* and *gem2* (see Table I). In the cross-complementation, the resulting diploid has a single copy of each reporter. Because of the expression level dependence of these assays, the positive signal of non-complementation is weaker than in crosses within an assay. Thus, only the very strongest mutants can be scored. Mutants in the *gem3*–*6* groups were weaker in general; therefore, we were unable to analyse these groups for cross-complementation.

Table I. Summary of *gem* mutants obtained

Class	Number of mutants			
	Invertase secretion		Alkaline phosphatase activation	
	α	α	α	α
<i>gem1</i>	7	13	5	3
<i>gem2</i>	1	1	3	3
<i>gem3</i>		4	6	
<i>gem4</i>	3	3		
<i>gem5</i>	3	2		
<i>gem6</i>	3	3		
Unclassified	33	23	4	29

Additional phenotypes of the *gem* mutants

We tested whether the phenotypes of the *gem* mutants were consistent with defects specifically of the Golgi, or whether they affected retention in the rest of the secretory pathway. Secretion of ER-resident BiP (an *erd*, or ER retention defective, phenotype) and CPY (a *vps*, or vacuolar protein sorting, phenotype) can be easily detected on streak blots. None of the mutants secreted CPY, suggesting that we have not re-isolated any *vps* mutants. A few had a weak *erd* phenotype (for an example, see Figure 6A), which might be expected if the early Golgi is disrupted, as this is where the recycling of ER residents is initiated. The degree of BiP leakage was, however, less than in *erd1* or *erd2* mutants.

Additional phenotypes can be expected for mutant yeast which have a disrupted Golgi. One prediction of reduced retention of mannosyltransferases is an alteration in the overall glycosylation pattern of the cell. Previous screens have identified classes of mutants exhibiting such alterations in their glycosylation profiles, notably the *mn* (mannan defective), *vrg* (vanadate resistant genes) and *alg* (asparagine linked glycosylation) mutants (Ballou *et al.*, 1991; Huffaker and Robbins, 1982, 1983). These classes of mutants have pleiotropic phenotypes including resistance to sodium orthovanadate, enhanced sensitivity to hygromycin B and alterations in cell surface carbohydrate antigenic determinants. Further, deletion strains of *MNT1* have enhanced sensitivity to calcoflur white, a drug which binds to cell wall glucans (Ram *et al.*, 1994), and enhanced resistance to killer toxin which binds to β 1-6 glucan (Hill *et al.*, 1992). It is not clear what these alterations reflect; what is however apparent is the correlation of these with glycosylation defects.

Fig. 6. (A) Streak blot detecting BiP secretion. SEY6210 wild-type, *anp1*, *van1* and *erd1* strains were patched out on rich media, overlaid with nitrocellulose and grown at 30°C overnight. The filter was removed, washed and probed with polyclonal anti-BiP antiserum and peroxidase-conjugated anti-rabbit antiserum. The signal was detected by ECL. **(B)** *van1* and *anp1* cells are more killer toxin resistant than wild type. Equivalent numbers of cells from SEY6210 wild-type, *anp1* and *van1* deletions were diluted into amino acid-supplemented minimal agar buffered to pH 4.7 with succinic acid, and 10 ml were poured into Petri dishes. Equivalent amounts of strain SK-S14a (gift from M.Lussier and H.Bussey), which secretes killer toxin, were pipetted onto the plates, and then incubated at 18°C for 2 h, then for 3 days at 25°C.

We tested the classes of *gem* mutants for alterations in their sensitivity to these drugs, and also to killer toxin (see Table II). Many mutants in each class were more sensitive than wild type to hygromycin and calcoflur, but with the exception of the *gem3* class, very few showed significant resistance to vanadate. Most phenotypes were

Table II. Phenotypes of the *gem* mutants

Strain	Growth phenotype			
	Sensitivity to 100 µg/ml calcoflur ^a	Sensitivity to 100 µg/ml hygromycin ^a	Resistance to 3 mM vanadate	Growth at 37°C
Wild type	—	—	—	+
<i>gem1</i>	20/28	20/28	1/28 ^b	17/28
<i>gem2</i>	6/8	6/8	0/8	4/8
<i>gem3</i>	0/10	0/10	3/10	10/10
<i>gem4</i>	6/6	6/6	0/6	3/6
<i>gem5</i>	3/5	3/5	0/5	4/5
<i>gem6</i>	4/6	4/6	0/6	5/6
<i>gem1.32</i>	—	—	—	—
<i>gem2.41</i>	—	—	—	—
<i>gem3.45</i>	+/-	+/-	+	+

^aAll mutants that were sensitive to calcoflur were also sensitive to hygromycin.

^bThis mutant was also one of those sensitive to calcoflur and hygromycin.

not class specific, but seemed to reflect the severity of the mutation in each case. Thus, within each class the mutants with the strongest missorting phenotype in our assay were also those with the most striking additional phenotypes. As our screen was carried out at 25°C, we also tested for temperature conditional lethality, and 22 strains were inviable at 37°C.

Plasmid rescue of *gem1*, *gem2* and *gem3*

The strongest mutant strains from the *gem1*, *gem2* and *gem3* classes were backcrossed twice to the appropriate parental strains. Each cross produced progeny with a 2:2 segregation of the Golgi mislocalization phenotype and the additional drug sensitivities of the strains chosen (Table II). From this, we deduce that in each case the *gem* phenotype corresponds to mutation at a single locus. After the second backcross, each mutant strain was transformed with a library of yeast genomic DNA in a centromere-containing vector. Colonies were then tested for growth at 37°C in the case of *gem1.32* and *gem2.41*. The rescued strains were also wild type with respect to the additional phenotypes. This rescue was plasmid dependent, as the mutant phenotype was restored when the cells were forced to expel the plasmid. In this way, two separate plasmids rescuing *gem1* and one rescuing *gem2* were isolated. Further analysis of these is in progress.

gem3.45 was the most vanadate-resistant yeast strain isolated from the screen. One plasmid conferring vanadate sensitivity to the mutant was isolated twice from the genomic library. Subcloning showed that this activity was encoded by a 3 kb fragment which also corrected the Golgi mislocalization phenotype.

GEM3 is ANP1

Sequence was obtained from each end of the rescuing 3 kb subclone. Comparison with the sequence database revealed that this was within a region on the left arm of chromosome V which had already been sequenced (Melnick and Sherman, 1993). The major open reading frame (ORF) in this area encodes a gene called *ANP1* (amino nitrophenol propandiol resistance gene), and subcloning showed that this gene was indeed sufficient to

Fig. 7. Invertase glycosylation is altered in *anp1* and *van1* cells. Whole-cell protein from equivalent amounts of SEY6210 wild-type, *anp1* and *van1* strains expressing myc-tagged invertase on a *CEN* vector was extracted and detected as in Figure 2C. The amount of invertase extracted from an equal number of *van1* cells is less than that of the *anp1* and wild-type strains. The reason for this is unclear, although it is possible that the expression level in this strain is lower. The position of a molecular weight standard (97 kDa) is indicated with an arrow.

complement the *gem3.45* mutant. *ANP1* was identified during analysis of a chromosome V deletion which conferred sensitivity to ANP and hypo-osmotic medium, as well as causing flocculation in liquid media (McKnight *et al.*, 1981). The latter phenotypes are consistent with the strain having alterations to the cell wall.

A database search revealed that Anp1p is homologous to Van1p, with 41% identity at the amino acid level (Figure 8). Both are predicted to encode Type II integral membrane proteins, with short cytoplasmic tails and the majority of the protein residing within the lumen of a compartment. *VAN1* was initially isolated as a vanadate-resistant mutant, with alterations in protein phosphorylation and growth control (Kanik-Ennulat and Neff, 1990). Its function, and that of Anp1p, is unknown.

Phenotypes of *anp1* and *van1* disruption mutants

To determine the null phenotypes of *anp1* and *van1*, we made internal deletions of each gene. We used these strains to analyse a variety of different phenotypes (Figure 6A and Table III). *anp1* cells had a strong Golgi mislocalization phenotype by both of our assay criteria, whereas *van1* mutants only had a weak phenotype. This result indicates that *van1* mutants would not have been picked up in either screen, as the null phenotype was not strong enough. Table III shows that *anp1* cells had more pronounced phenotypes than *van1* cells in all tests, except for vanadate resistance.

The phenotypes of *anp1* and *van1* mutants suggest that they have defective cell walls. We tested this more directly by using the killer toxin plate assay developed by Bussey

et al. (1982). Cells lacking Mnt1p are resistant to this toxin (Hill *et al.*, 1992). Therefore, a prediction of the mislocalization of Mnt1p is increased resistance to killer toxin. When grown on minimal media, *anp1* and *van1* cells are more resistant to killer toxin than wild-type cells (Figure 6B). *van1* mutants are totally resistant to killer toxin, with no apparent halo. The *anp1* mutants have a distinct halo, reduced in size in comparison to wild type (Figure 6B). The noticeable stimulation of growth at the halo is probably due to nutrients released into the minimal plates by the dead cells from the zone of killer toxin action.

A strong prediction from the above results is that glycosylation will be altered in *anp1* and *van1* mutants. Not only is this an expectation from the cell wall defects, but also if Golgi enzymes are mislocalized one may expect glycosylation defects. Therefore, we prepared total protein extracts from *anp1* and *van1* mutant strains expressing secreted myc-tagged invertase (Figure 7). In wild-type strains, the invertase runs as a high molecular weight heterogeneously glycosylated smear. The invertase in *anp1* mutants runs as a faster, more compact smear. In *van1* cells the invertase runs even faster, as a compact band. Endoglycosidase H digestion of these samples indicates that the heterogeneity of the invertase mobilities is due to differences in the degree of glycosylation in each sample (data not shown).

Synthetic lethality of *anp1* and *van1* mutants

Disruption of either *VAN1* or *ANP1* is not lethal, although the growth rate of *anp1* cells is reduced relative to wild type. In order to see whether the combination of *anp1* and *van1* mutations was lethal, we constructed a *van1* disruption strain that was rescued by a *URA*⁺ plasmid carrying the wild-type *VAN1* gene. We then disrupted the *ANP1* locus by one-step gene disruption. Strains which had disrupted *anp1* and *van1* genes were unable to lose the *URA*⁺ plasmid when grown on plates containing 5-fluoro-orotic acid. Strains without the second disruption were able to lose the plasmid, with colonies appearing within 2 days at 30°C. From this we deduce that *anp1 van1* double mutants are inviable.

Localization of Anp1p

To localize Anp1p, we constructed a version of the protein which was epitope tagged at the C-terminus. This protein was functional, being able to rescue *anp1* cells, suggesting that the addition of the tag does not prevent the protein folding into a functional conformation (data not shown). When expressed from a 2 μ vector, this gave an immunofluorescent localization around the periphery of the cell and in the nuclear envelope (Figure 9), a distribution characteristic of the ER. Double-label immunofluorescence with the ER protein BiP confirmed that Anp1p was indeed localized to this compartment (data not shown).

Discussion

Retention of Mnt1p and Och1p

With the aim of setting up a screen to look for genes involved in the maintenance of the structure and organization of the Golgi apparatus, we first examined the mechanism by which two of the constituent enzymes of the organelle, Mnt1p and Och1p, were held in place. In this

Fig. 8. Amino acid sequence alignment of Anp1p, Van1p and Mnn9p. Regions of identity are boxed in grey, dashed lines indicate gaps, and the predicted transmembrane domain is underlined. We re-sequenced the last 400 nucleotides of both the predicted *VAN1* and *ANP1* ORFs, and found differences from the previously reported sequence of both genes. There is an additional cytosine at the 1460th nucleotide of the predicted *VAN1* ORF. This would change the protein sequence at this point from 'LAKAKYLET..' to 'LAKAKIFRN..', and extend the region of similarity between the two proteins (see Figure 8). The *ANP1* sequence has an additional cytosine at nucleotide 1414. Addition of this changes the protein sequence at this point from 'DGNPQG' to 'DGNTRR...' and extends the protein by eight amino acids.

paper, we show that the signal-anchor region of these mannosyltransferases is sufficient to localize a reporter enzyme to the early Golgi. Furthermore, we present data indicating that the TMD is necessary for this retention, and will provide information for at least partial Golgi localization when placed into a heterologous protein. This is consistent with what has been previously shown for the mammalian glycosyltransferases (Munro, 1991; Nilsson *et al.*, 1991; Dahdal and Colley, 1993; Machamer, 1993; Burke *et al.*, 1994). Thus, in both yeast and mammalian cells there appears to be a TMD-mediated mechanism that is involved in the retention of glycosyltransferases in the Golgi apparatus. In both systems, there is also a second, cytoplasmic tail-dependent mechanism found to be operating on some late Golgi enzymes—DPAP A, Kex2p and Kex1p in yeast, and TGN38 and furin in mammalian cells. This second system works in both mammalian cells and yeast by directing continuous retrieval of the proteins from a post-Golgi compartment. The mechanism of action of TMD-mediated retention is less clear. Two models have been proposed: either compartment-specific aggregation of enzymes into structures too large to enter transport vesicles (Pfeffer and Rothman, 1987; Nilsson *et al.*, 1993; Weisz *et al.*, 1993), or sorting based on partitioning between lipid domains co-existing within one compartmental bilayer (Bretscher and Munro, 1993). So far there is no evidence, at least in mammalian cells, to indicate

Table III. Phenotypes of *anp1* and *van1* deletion strains

Phenotype	<i>anp1</i>	<i>van1</i>	Wild type
Signal from invertase secretion assay	+++	+	—
Signal from Pho8p activation assay	+++	+	—
Secretion of BiP	+	—	—
Secretion of CPY	—	—	—
Growth on vanadate			
2 mM	+++	+++	+
4 mM	+	+++	—
Colour	yellow	green	
Growth on hygromycin	—	+	+++
Growth on ANP	—	+	+++
Flocculation	+++	++	—
Clumpiness on solid medium	+++	++	—
Resistance to killer toxin	+	+++	—

the involvement of recycling (Machamer *et al.*, 1990; Chapman and Munro, 1994).

Our data also demonstrate that when Mnt1p and Och1p are overexpressed, retention in the Golgi is overwhelmed and the proteins accumulate in the vacuole instead. It has previously been observed that when the trans-Golgi enzymes with retention signals in their cytoplasmic tails were overexpressed, these proteins also accumulated in the vacuole—the same destination being seen when the retention signals were mutated. To explain this, it was

Fig. 9. Anp1p is localized to the ER. Immunofluorescent analysis of C-terminally myc-tagged Anp1p expressed in SEY6210 under the control of the PHO5 promoter on a 2 μ vector. Cells were stained with the anti-myc monoclonal antibody 9E10 to detect Anp1p and DAPI to stain the nuclei.

proposed that overexpression was causing the saturation of a specific receptor for the retention signal, and that the default destination of membrane proteins in the yeast secretory pathway was the vacuole (Cooper and Bussey, 1992; Wilcox *et al.*, 1992; Nothwehr *et al.*, 1993). Our results with the mannosyltransferases are consistent with the latter notion, but they do not necessarily imply that TMD-mediated retention involves a specific saturable receptor. Purified yeast Golgi contains only 0.3% of total cell glycerophospholipid, suggesting that the actual amount of membrane available for residence of enzymes in this organelle is limited (McGee *et al.*, 1994). It does not appear that overexpression of a particular mannosyltransferase expands the Golgi, because by electron microscopy cells overexpressing Mnt1p appear to have a normal amount of intracellular membrane. We did not observe the proliferation of ER, or the appearance of Golgi-derived Berkeley bodies, that has been described previously for cells overexpressing other proteins of the secretory pathway (Hardwick *et al.*, 1992; data not shown). Thus, the observed saturation could reflect there being so much of a Golgi enzyme that it does not have time or space to enter aggregates or lipid domains and so the excess protein flows through the Golgi to the vacuole. Indeed, normal retention could simply represent a slower rate of flow of Golgi enzymes through the pathway compared to bulk retention, rather than a strict localization of each molecule to the Golgi.

ANP1, VAN1 and MNN9 define a new class of proteins involved in secretory pathway function

To look for genes involved in the retention of Mnt1p, we set up screens to detect mislocalization based on the activation of C-terminal fusions. These yielded six groups, which we call 'gem'.

Cloning of *GEM3* revealed that it is identical to a previously isolated gene, *ANP1*. This gene was originally identified by chance as being the gene within a large deletion that was responsible for the observed sensitivity of the strain carrying this deletion to the chloramphenicol

breakdown product amino nitrophenol propandiol (ANP) (McKnight *et al.*, 1981). Otherwise, all that is known about *ANP1* is that the gene encodes a 58 kDa protein which potentially has a single bilayer-spanning domain and a Type II orientation. A database search with the sequence of Anp1p revealed that it was homologous to the protein product of *VAN1* gene (41% identity; Kanik-Ennulat and Neff, 1990) and more distantly related to the product of the recently sequenced *MNN9* gene (20% identity; Yip *et al.*, 1994; see Figure 8). *VAN1* was identified by the fact that mutations in the gene confer resistance to vanadate and *MNN9* was identified as one of several genes required for outer chain glycosylation in yeast. Like Anp1p, the protein products of these two genes have no known function, but both are putative Type II membrane proteins.

The sequence similarities between Anp1p, Van1p and Mnn9p suggest that these proteins have related functions and, consistent with this, we show here that although *anp1* or *van1* cells are viable, combining the mutations is apparently lethal. This raises the question of what the seemingly essential function, or functions, of this family of proteins is and how this relates to the retention of proteins in the Golgi apparatus. It has previously been shown that in *mnn9* mutants most Golgi-specific outer chain glycosylation is missing (Gopal and Ballou, 1987; Ballou *et al.*, 1991). We show here that a similar situation exists in *anp1* and *van1* strains, along with cell wall defects consistent with aberrant Golgi function. Thus, it seems reasonable that either these proteins are mannosyltransferases, or they are involved in maintaining a Golgi apparatus with a full complement of correctly organized and functioning mannosyltransferases. The first possibility seems extremely unlikely for the following three reasons. Firstly, we have clearly demonstrated that Anp1p is an ER-resident protein, whereas it is known that outer chain glycosylation (both N- and O-linked) occurs in a post-ER compartment. Secondly, the truncated sugar structures found on invertase in *mnn9* (and also *van1*, see below) cells are abnormal and do not appear to reflect a simple block in further sugar addition at a particular step, such as is seen if a specific glycosyltransferase such as Och1p, or Mnn1p, is knocked out (Graham *et al.*, 1992; Nakanishi-Shindo, 1993). In fact, it was suggested that an aberrant α 1-2 linkage added to the *mnn9* invertase core sugars actually prevents further enzymes from using it as a substrate, thus producing a severe glycosylation defect (Gopal and Ballou, 1987). This led Ballou and co-workers to suggest that the *mnn9* mutation was either causing transport vesicles to skip particular compartments in the Golgi, or causing the mislocalization of Golgi mannosyltransferases (Lewis and Ballou, 1991). Thirdly, loss of both Anp1p and Van1p is incompatible with viability, and yet it is known that mutant yeast strains that do not make any Golgi-specific additions to their N-linked sugars are still viable, again suggesting that Van1p and Anp1p are not simply sugar transferases (Nakanishi-Shindo *et al.*, 1993). Thus, we suggest that rather than being directly involved in outer chain modification, Anp1p is required in some way for maintaining the organization of the Golgi apparatus. *VAN1* and *MNN9* are unlikely to correspond to any of the other *GEM* mutants as deletion of *VAN1* has only a small effect in the assays used for the screen, and

mn9 is substantially more vanadate resistant than any of the mutants identified here (Ballou *et al.*, 1991). However, it may be that Mnn9p and Van1p are also involved in protein retention, but in other compartments. The underglycosylated invertase in the *anp1* cells appears to acquire more sugar than in the *van1* cells, suggesting that *VAN1*, and *MNN9*, may be required for the organization of earlier compartments in the secretory pathway. Although it is unclear why mutants with defects in glycosylation should show resistance to vanadate, this has been useful in identifying new classes of such mutants. In one such mutant, *vrg7*, the aberrant N-linked sugars that accumulate are not only blocked in early Golgi modifications, but show only partial trimming by the ER α -mannosidase (Ballou *et al.*, 1991). *vrg7* has recently been shown to correspond to *van1*, and is clearly distinct from the ER α -mannosidase itself, raising the interesting possibility that Van1p is involved in the localization of both ER and early Golgi enzymes (Camirand *et al.*, 1991; C.E. Ballou and R.A. Hitzeman, personal communication). An alternative possibility is that Anp1p performs some sort of protein modification, the absence of which exerts a pleiotropic effect on the folding and assembly of many proteins, including Golgi enzymes. We believe that this is unlikely because ANP deletion strains do not show elevated levels of BiP, implying that there are no misfolded, inactive, proteins accumulating in the ER of these yeast (data not shown).

At present we do not know how deletion of Anp1p, an ER-resident protein, might affect the function of the Golgi apparatus. The sensitivity of the mutant to the drug ANP probably just reflects increased uptake of the drug due to an altered Golgi producing defective plasma membrane components, analogous to the Brefeldin A sensitivity of mutants in ergosterol synthesis (Graham *et al.*, 1993; Vogel *et al.*, 1993). Moreover, the effect of the *anp1* mutation on Golgi retention is unlikely to be simply a consequence of the aberrant glycosylation of some other key Golgi component, because *pmr1* and *erd1*, mutations which also block outer chain glycosylation, do not give a positive signal in the assays we used to isolate the *gem* mutants (Rudolph *et al.*, 1989; Hardwick *et al.*, 1990; data not shown). The ER localization makes it unlikely that Anp1p is involved in the tethering of oligomeric structures in the Golgi. It could, however, be involved in the assembly of such structures, or in the maintenance of a compartment-specific lipid composition, or act by some other as yet unanticipated mechanism. At present we cannot distinguish between these possibilities. Nonetheless, it is clear that Anp1p is a member of a family of proteins with at least one essential role in the secretory pathway, and that the intriguing and pleiotropic phenotypes of mutants in this family suggest that they are worth considerable further investigation. Furthermore, we are currently analysing two further genes, *GEM1* and *GEM2*, which may not be in this family, but which appear to encode essential proteins involved in the organization of the Golgi. Analysis of these genes, along with investigation of Anp1p, Van1p and Mnn9p function, should help us to understand the mechanisms by which the Golgi maintains its structure and carries out its central role in the secretory pathway.

Materials and methods

Plasmid construction

All of the cloning was carried out using standard techniques. Anything generated by PCR amplification was checked by sequencing. The myc tag was added in frame on to the '...RE' residues at the C-terminus of Mnt1p using PCR, to produce the sequence 'REPQMEQKLISEEDLN' followed by a stop codon. Bold type indicates the sequence of the tag. This was expressed under the control of the *TPI* promoter in pRS306 (MMM). The same tag, followed by the HA epitope tag, was added on to the 'HK' sequence at the C-terminus of Och1p to produce the sequence 'HKPQMEQ.....LNMAYPYDVPDYA' followed by a stop codon. In this case the HA tag is in bold type. This was expressed under the control of the *TPI* promoter on a 2 μ vector, pZUC13, (OOO). The myc tag was added into the N-terminus of Pho8p, replacing the sequence 'PSEQTRLVPGSDSSS' with the sequence 'PSEQKLISEEDLSSS' (the tag is shown in bold type, PPP). This and the following constructs, except for MMMI, were expressed under the control of part of the *PHO5* promoter, lacking regulatory regions, in the vector pRS306. The MMMP construct was made by adding the 507 luminal amino acids of Pho8p to the myc-tagged MMM protein to produce the sequence F'REPQM....LNSRRSASH'. The MMMI construct was made by adding a Kex2p-dependent cleavage site at the C-terminus, followed by the 512 C-terminal amino acids of invertase. This produces the sequence 'REPQMEQ....LNTTTIASIAAKEEGVSLDKRSMTNET'. The site of Kex2p-dependent cleavage follows the underlined amino acids, and the beginning of the invertase part shown in bold. This was expressed in pRS306 under the control of the *TPI* promoter. The OOP construct was generated by placing the myc tag after the first 60 amino acids of Och1p at the 'HSQ', then fusing this to the 507 luminal amino acids of Pho8p in the same way as in the MMMP construct. This produced the sequence 'HSQMEQ....LNSRRSASH' at the junction. The MPMI construct replaces the 19 transmembrane amino acids of Mnt1p in MMMI with the 26 amino acids of Pho8p. This changes the sequence from 'FLLRFTV...NSRTQ' to 'FLLRLIIVS....FAIRTQ'. The PMP construct replaces the 26 transmembrane amino acids of PPP with the 19 amino acids from Mnt1p, changing the sequence from 'IIVS...FAI' to 'FTV...NS'. To make an internal deletion of *ANP1*, the sequence encoding the 89 amino acids between the *NruI* and *NsiI* sites was replaced with a 2.2 kb fragment containing the *LEU2* gene under the control of its own promoter. To make a deletion of *VAN1*, the sequence coding between the 180th amino acid 'F' and the 405th amino acid 'S' was replaced by a 1 kb fragment containing the *TRP1* gene under the control of its own promoter. The myc-tagged Anp1p adds the epitope sequence to the C-terminus, changing the sequence from 'FDPDRN*' to 'FDPDRNSRSMQKLISEEDLN*'.

Yeast strains

The wild-type strains used for all experiments were SEY6210 (*MAT α* *ura3-52 his3 Δ 200 leu2-3, 112 trp- Δ 901 lys2-801 suc2- Δ 9*) and SEY6211 (*MAT α* *ura3-52 his3 Δ 200 leu2-3, 112 trp- Δ 901 ade2-101 suc2- Δ 9*), both kindly provided by S. Emr. The *PHO8* gene was knocked out in each to produce strains 6210 Δ P and 6211 Δ P, using plasmid pGP10 (a gift from Kathryn Hill). This disruption was checked by Southern blotting analysis.

Integrations and gene disruptions

These were carried out by a one-step strategy. pRCTI- and pRCPI-based constructs were digested with *StuI* and integrated at the *ura3-52* locus of 6210 Δ P. The equivalent 6211 Δ P strains were generated by mating and dissection of the progeny. Those which were then *URA*⁺ contained the equivalent integration. Integration of a 5.2 kb *HindIII* fragment from pLEU Δ ANP disrupted the *ANP1* locus, and integration of the 1.8 kb *BamHI*-*XhoI*-generated fragment from pTRPVAN disrupted the *VAN1* locus. These disruptions were checked by Southern blot analysis.

Plate assays

Secreted invertase was detected as previously described (Pelham *et al.*, 1988). For the alkaline phosphatase assay, yeast strains were grown overnight on solid media. It was important that these were fresh, if they had been kept at 4°C they were replated at least three times before doing this assay. A round filter paper was soaked in 1 ml of reaction solution [1% Triton X-100, 25 mM MgSO₄, 10 mg/ml Fast Red salt from Sigma, 1 mg/ml naphthol as phosphate, 0.5 M Tris (pH 9)]. The wet filter was placed onto the yeast on the agar plate, making sure that no air bubbles were trapped. Colonies expressing active alkaline phosphatase went red underneath the filter. The rate of this increased if the assay was carried

out at higher temperatures or if the amount of active alkaline phosphatase was increased.

The screen for gem mutants

Parental strains expressing the MMMP and MMMI fusions were plated out at a density of 10 000 yeast/plate on rich media. The plates were illuminated with UV, such that ~95% of the yeast were killed. The plates were then incubated at 25°C for 3 days. Plate assays were carried out as described on the mutant colonies. Live yeast could be picked from the colonies which went the deepest red colour in each assay. These were then re-streaked twice as single colonies and re-tested as patches in the plate assays. A total of 200 000 yeast, after mutagenesis, were screened in this way.

Immunofluorescence and immunoblotting

Anti-myc monoclonal 9E10 (2.7 mg/ml) was diluted 1:200 for immunofluorescence and 1:1000 for Western blotting (Munro and Pelham, 1987). Polyclonal anti-BiP antiserum (gift from J. Vogel) was diluted 1:30 000 for immunofluorescence. Affinity-purified polyclonal anti-Kex2 antiserum (gift from K. Redding and B. Fuller) was diluted 1:100 for immunofluorescence. Affinity-purified monoclonal 12CA5 (7.6 mg/ml), which recognizes the HA tag (Field *et al.*, 1988), was used at 1:50 for immunofluorescence. The latter was carried out essentially according to the method described by Hardwick and Pelham (1992). Fixation was carried out for 4 h rather than 90 min. Preparation of whole-cell protein extracts and Western blotting were carried out according to the method of Sweet and Pelham (1993). Cell fractionation was carried out according to the method of Horazdovsky and Emr (1993).

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