The EMBO Journal Vol.17 No.2 pp.423–434, 1998

Multi-protein complexes in the *cis* Golgi of *Saccharomyces cerevisiae* with α-1,6-mannosyltransferase activity

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Anp1p, Van1p and Mnn9p constitute a family of membrane proteins required for proper Golgi function in *Saccharomyces cerevisiae*. We demonstrate that these proteins colocalize within the *cis* Golgi, and that they are physically associated in two distinct complexes, both of which contain Mnn9p. Furthermore, we identify two new proteins in the Anp1p–Mnn9p-containing complex which have homology to known glycosyltransferases. Both protein complexes have α-1,6-mannosyltransferase activity, forming a series of poly-mannose structures. These reaction products also contain some α-1,2-linked mannose residues. Our data suggest that these two multi-protein complexes are responsible for the synthesis and initial branching of the long α-1,6-linked backbone of the hypermannose structure attached to many yeast glycoproteins.

Keywords: glycosyltransferase/Golgi apparatus/mannosyltransferase/N-linked glycan/secretory pathway

Introduction

Many eukaryotic proteins are covalently modified by the addition of oligosaccharides. This is especially common for proteins produced in the secretory pathway with a wide range of structures attached to either asparagines (N-linked) or serines and threonines (O-linked). This glycosylation can be crucial for the folding and function of individual proteins, as well as providing a mechanism to alter general surface properties of cells such as adhesiveness, accessibility and immunogenicity (Varki, 1993; Gahmberg and Tolvanen, 1996). The formation of N-linked oligosaccharides on glycoproteins begins in the endoplasmic reticulum (ER) with the attachment of a core oligosaccharide to specific asparagine residues (Kornfeld and Kornfeld, 1985; Silberstein and Gilmore, 1996). This $Glu_3Man_9GlcNAc_2$ core structure is the same in both yeast and mammalian cells, and following removal of all three glucoses and a single mannose, there is no further processing until after the proteins have exited the ER (Moremen *et al.*, 1994). However, upon arrival at the Golgi the further modification of N-linked glycans varies enormously, both between species, and also between individual proteins within a species. In yeasts, no further mannoses are removed but rather the N-linked glycan can undergo further maturation by addition of an outer chain of up to 200 mannose residues. In *Saccharomyces cerevisiae* this 'hypermannose' structure consists of a long backbone of α-1,6-linked residues with α-1,2-linked branches which terminate in α-1,3-linked residues (Peat *et al.*, 1961; Ballou, 1974; Herscovics and Orlean, 1993). The addition of this structure is highly specific in that it is only present on a subset of glycosylated proteins, usually secreted enzymes, such as invertase, and the 'mannan' structural proteins of the cell wall. Proteins of the internal membranes, such as the vacuolar protease CPY, usually have a single α-1,6-linked mannose added which is then terminated by an α-1,2-linked residue (Trimble and Atkinson, 1986; Ballou *et al.*, 1990). As with the protein-specific modification of N-linked glycans observed in higher cells, the structural features of the glycoproteins that are recognized to determine this different outcome of Golgi-processing are not understood.

Although hypermannosylation of N-linked glycans is not observed in mammalian cells, there are several reasons why it will be interesting to understand this process further. First, a long branched α-1,6-linked backbone structure has been found in a wide variety of yeast species including *Candida*, *Schizosaccharomyces*, *Citeromyces* and *Kloeckera* suggesting it is a general feature of most yeasts (Ballou, 1974; Kobyashi *et al.*, 1987; Kobyashi *et al.*, 1989). These chains are usually branched with α-1,2-linked mannoses which are then extended with a variety of species specific linkages. These large oligosaccharide structures are thought to contribute to cell wall integrity, aid in shielding the cell wall from antibodies and digestive enzymes, and in maintaining its resident proteins (Klis, 1994; Cid *et al.*, 1995). Secondly, when mammalian proteins are expressed in the yeast secretory pathway they can acquire these non-mammalian structures (Nakamura *et al.*, 1993; Kniskern *et al.*, 1994). Thirdly, most if not all eukaryotes make carbohydrate polymers in their Golgi, including glycosaminoglycans and mucins in animals, and hemicelluloses and pectins in plants. How Golgi enzymes are organized to facilitate the efficient synthesis of such polymeric structures is at present poorly understood. Finally, studies of transport through the secretory pathway of *S.cerevisiae* have relied on following protein modifications, especially those of N-linked glycans (Pryer *et al.*, 1992). The Golgi in yeast, like that of mammalian cells, is divided into at least three subcompartments, or cisternae, each with a distinct composition of enzymes. These cisternae are termed *cis*, the first compartment after the ER; *medial*; and *trans*, the final compartment from which proteins are sorted to a variety of destinations within the cell. How proteins move from one cisternae to another is a matter of considerable debate. To fully understand protein transport events it is crucial to identify and precisely localize the Golgi modification enzymes used to follow the movement of proteins between compartments.

The Golgi enzymes involved in N-linked sugar modi-

fication have been sought either by screening for mutants in N-linked mannosylation, or by purification of enzymatic activities (Ballou, 1990; Nagasu *et al.*, 1992). Screens for mutants with defects in mannans (*mnn*), and outer chains glycans (*och*), have been augmented by the realization that such defects often result in resistance to orthovanadate (Ballou *et al.*, 1991; Kanik-Ennulat *et al.*, 1995; Poster and Dean, 1996). Additional mutants with defects in Golgi modification have also been identified in a variety of other genetic screens, including those for resistance to carbohydrate-binding toxins or hypersensitivity to various drugs such as hygromycin B (Klis, 1994; Dean, 1995). In a few cases it has been possible to correlate an observed phenotype to a particular enzymatic activity. Thus *MNN1* encodes the terminal α-1,3-mannosyltransferase, and Och1p adds the initiating α-1,6-mannose of the backbone (Graham *et al.*, 1992; Nakayama *et al.*, 1992). Protein purification led to the isolation of *KRE2/MNT1* which encodes an α-1,2-mannosyltransferase involved in synthesis of O-linked glycans (Häusler and Robbins, 1992). However, activities have not so far been assigned to many of the other genes defective in glycosylation mutants, or to the sequence relatives of these few identified enzymes that have been revealed by the completed yeast genome sequence (five of Mnn1p, three of Och1p and eight of Kre2p; Lussier *et al.*, 1997; Neiman *et al.*, 1997).

The enzymes responsible for making the long branched backbone of yeast outer chains have not been identified. In several of the *mnn* mutants the backbone is shorter or absent (Ballou, 1990). However, similar phenotypes have also been observed with mutations in proteins responsible for provision of the correct substrates and co-factors for general mannosylation—these include the enzymes which make GDP–mannose, the putative Golgi transporters for GDP–mannose and for manganese, and the Golgi GDPase (Abeijon *et al.*, 1993; Lapinskas *et al.*, 1995; Poster and Dean, 1996; Hashimoto *et al.*, 1997). In addition, mutations in several proteins thought to have a role in protein trafficking within the Golgi also show glycosylation defects (such as *erd1*, *rer1* and *sft1*), possibly due to defects in delivery of protein substrates to the enzymes, or mislocalization of the Golgi enzymes (Hardwick *et al.*, 1990; Boehm *et al.*, 1994; Banfield *et al.*, 1995).

One family of genes apparently required for synthesis of the polymannose backbone of yeast outer chains is made up of *ANP1*, *MNN9* and *VAN1* (Ballou *et al.*, 1980; McKnight *et al.*, 1981; Kanik-Ennulat *et al.*, 1990). Mutations in any one of these related genes results in the absence (*mnn9* and *van1*) or reduction in size (*anp1*) of the outer chain (Ballou *et al.*, 1980; Tsai *et al.*, 1984; Chapman and Munro, 1994; Kanik-Ennulat *et al.*, 1995; Sipos *et al.*, 1995). All three of these proteins are predicted to be type II transmembrane proteins with a short cytosolic amino-terminal portion (Chapman and Munro, 1994; Yip *et al.*, 1994). The functions of these three proteins are unknown, although it has been suggested that defects in *MNN9* may alter trafficking of proteins through the Golgi, allowing them to bypass the compartment containing the outer chain synthesizing enzymes (Gopal and Ballou, 1987). Although Anp1p was originally identified as conferring drug sensitivity, we also isolated mutations in Anp1p in a genetic screen for proteins involved in retention of the *medial* Golgi enzyme Mnt1p (Chapman and Munro, 1994). The gene was also isolated in a screen for mutants defective in retention of the late Golgi protease DPAP-A (Nothwehr *et al.*, 1996). We were thus interested in identifying the function of Anp1p and its relatives.

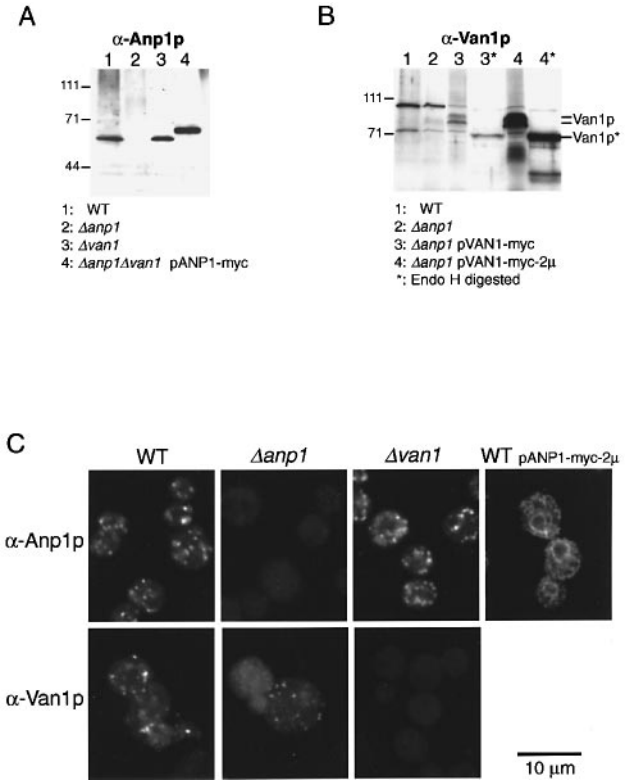


Fig. 1. Immunodetection of Anp1p and Van1p. Total protein isolates from the indicated yeast strains were separated by SDS–PAGE, transferred to nitrocellulose and probed with either α-Anp1p serum (**A**) or affinity-purified α-Van1p serum (**B**). pANP1-myc and pVAN1-myc are *ARS CEN* plasmids whereas pVAN1-myc-2μ is a multi-copy plasmid. These plasmids encode carboxy-terminal myc-tagged versions of Anp1p or Van1p, respectively. An over-exposed image is shown for (B) to allow visualization of Van1p in the Δ*anp1* strain. When over-expressed, Van1p appears as two bands both of which are sensitive to Endo H. Since the upper one migrates with Van1p expressed at native levels (allowing for the addition of the myc-tag), over-expression apparently results in under usage of a site for N-linked glycosylation. (**C**) Confocal microscopic images of yeast cells of different strains analyzed by immunofluorescence using the affinity-purified antisera against Anp1p or Van1p.

In this paper we show that Anp1p, Van1p and Mnn9p colocalize within the *cis* Golgi. Moreover, we show that Mnn9p binds independently to both Anp1p and Van1p, and identify two additional proteins present in the Mnn9p–Anp1p-containing complex, both of which have homology to known glycosyltransferases. Both of the Mnn9p-containing complexes possess α-1,6-mannosyltransferase activity, forming mannose polymers *in vitro*, suggesting that they are responsible for forming the backbone of the long outer chains of yeast N-linked glycans.

Results

Localization of Anp1p, Van1p and Mnn9p

To investigate the intracellular localization of Anp1p and Van1p we generated antisera to the lumenal parts of both proteins. His-tagged fusions were expressed in *Escherichia coli*, and then polyclonal antisera were raised against the purified recombinant proteins. Figure 1 shows that these

antibodies specifically recognize the proteins against which they were raised. The homology between these two proteins does not result in any cross-reactivity. The anti-Anp1p serum enabled detection of Anp1p, which was observed as a single band of the predicted molecular weight even at the native expression level, and expression of a carboxy-terminal myc-tagged version of Anp1p resulted in a corresponding increase in size of the protein (Figure 1A). It was necessary to overexpress Van1p to allow ready detection with the serum in whole cell extracts, and the band seen is converted to a more rapidly migrating form by digestion with endoglycosidase H, indicating usage of at least one of the two potential sites for addition of N-linked sugars in the Van1p lumenal domain (Figure 1B).

In order to determine the intracellular localization of Anp1p and Van1p, the antisera were affinity purified, and cell wall-reactive components removed by absorption to fixed yeast. After this procedure, both antisera specifically recognized native Anp1p or Van1p at wild-type expression level in immunofluorescence studies (Figure 1C). Surprisingly, both proteins were distributed in small patches throughout the cytosol, typical for yeast Golgi proteins. This localization was contrary to the ER localization that we had previously observed with overexpressed, epitope-tagged Anp1p (Chapman and Munro, 1994). Indeed, when the anti-Anp1p sera was used to examine the localization of the previously described myc-tagged version of Anp1p over-expressed from a 2μ multi-copy vector, we observed the same perinuclear ER staining (Figure 1C). Thus it appears that over-expression of Anp1p leads to its accumulation in the ER, but at normal expression levels immunofluorescent staining of Anp1p or Van1p shows cytoplasmic patches characteristic of the Golgi apparatus rather than the ER. Since the subcellular localization of the third member of this family, Mnn9p, has not been reported, we also examined the distribution of this protein. Three copies of the HA (influenza hemagglutinin) epitope were inserted at the carboxy-terminus of the *MNN9* open reading frame by homologous recombination (Baudin *et al.*, 1993). The tagging did not result in the hygromycin B hypersensitivity observed in a *mnn9* deletion mutant, indicating that the tagged version of Mnn9p is functional (Dean, 1995). Figure 2A shows that the tagged Mnn9p was also found in a punctate staining pattern characteristic of Golgi resident proteins. These data suggest that all three members of the Anp1p/Van1p/Mnn9p family localize to the Golgi apparatus.

Colocalization of Anp1p, Van1p and Mnn9p in the cis Golgi

To determine whether the cytoplasmic patches observed above correspond to the Golgi apparatus, and, if so, to which of the Golgi subcompartments, we used double-label immunofluorescence (Figure 2A). Anp1p colocalizes with both Van1p and Mnn9p, indicating that these three proteins are localized to the same compartment. In addition, Anp1p shows extensive colocalization with two markers for the *cis* Golgi. These are the *cis* Golgi t-SNARE Sed5p, and the *cis* Golgi α-1,6-mannosyltransferase, Och1p, which is responsible for the first post-ER modification of N-linked carbohydrate chains (Hardwick and Pelham, 1992; Nakayama *et al.*, 1992). In addition, Figure 2A shows that the punctate staining of Anp1p almost completely matched the staining pattern of Pmr1p, a Golgi protein with homology to Ca^{2+}-ATPases and proposed to transport Mn^{2+} (Antebi and Fink, 1992; Lapinskas *et al.*, 1995). The localization of Pmr1p within the Golgi apparatus has not been previously reported but it also appears to be present in the *cis* Golgi. In contrast, Anp1p does not colocalize with the *medial* Golgi markers Mnt1p (Figure 2A), or Mnn1p (data not shown).

To confirm these localizations, cell fractionations were also performed. Figure 2B shows that on a sucrose velocity gradient the fractions containing Anp1p clearly separate from the ER and vacuolar fractions, although on these gradients the *cis* and *medial* Golgi markers show similar profiles. To compare the localization of Anp1p with the late Golgi marker Kex2p, a continuous density gradient was used and this showed that Anp1p separates from fractions containing Kex2p (Figure 2C).

Thus Anp1p separates from late Golgi markers, but not *cis* and *medial* markers on gradients. The distinction between *cis* and *medial* relies on immunofluorescence, and although double-label immunofluorescence showed considerable overlap between Anp1p and *cis* Golgi markers, some spots did not coincide. A similar phenomenon has been reported in previous studies on proteins in the yeast *medial* Golgi (Lussier *et al.*, 1995), and may reflect the mechanisms of protein sorting in the Golgi. There is already evidence that components of the yeast *cis* Golgi are highly dynamic, with Och1p apparently cycling through the *trans* Golgi whilst Emp47p cycles through the ER (Schroder *et al.*, 1995; Harris and Waters, 1996). In such circumstances it perhaps not surprising that markers do not precisely coincide. Counting the Anp1p-positive spots showed 87% coincidence with an established *cis* marker (Och1p, $n = 210$), whilst only 20% with a *medial* one (Mnt1p, $n = 50$). Taken together these results strongly suggest that Anp1p, Mnn9p and Van1p are all primarily localized to the *cis* Golgi.

Physical interactions of Anp1p, Van1p and Mnn9p

Anp1p is normally localized to the Golgi, raising the question of why it accumulates in the ER upon over-expression. One possibility is that Anp1p is normally bound to another protein, and when Anp1p is synthesized in excess of this binding partner, the extra material is unable to fold and assemble correctly, and so does not exit the ER. Thus immunopreciptation was used to look for proteins interacting with Anp1p, and since Anp1p, Van1p and Mnn9p colocalize we initially looked for interactions between the members of this family.

Yeast, in which the endogenous Mnn9p was triple-HA-tagged, were solubilized in the non-denaturing detergent digitonin, and proteins precipitated with the anti-HA monoclonal antibody, 12CA5. The immunoprecipitates were then probed with specific antisera, and Figure 3 shows that both Anp1p and Van1p co-precipitated with Mnn9p. In both cases the precipitation almost completely depleted the protein from the cell lysate. Immunoprecipitations carried out with deletion strains indicated that the presence of Anp1p is not required for the Mnn9p–Van1p interaction, and Van1p is not required for the Mnn9p–Anp1p interaction. Furthermore, we were unable to detect any interaction between Anp1p and Van1p by precipitation

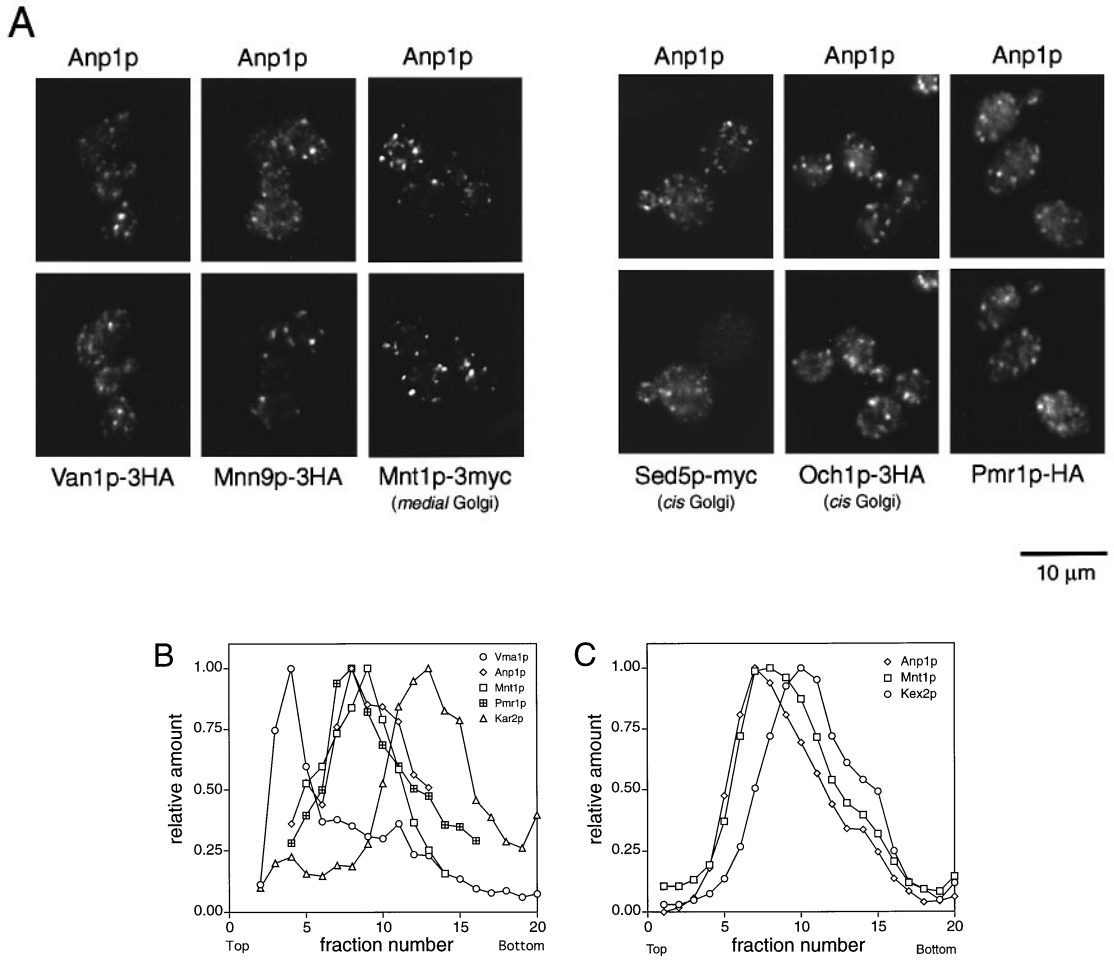


Fig. 2. Anp1p, Van1p and Mnn9p are localized in the *cis* Golgi. (**A**) Confocal micrographs of yeast cells expressing different tagged proteins (as indicated) and analyzed by double-label immunofluorescence using both the affinity-purified antiserum raised against Anp1p, and the monoclonal antibodies 12CA5 or 9E10 which recognize the HA-epitope, or the myc-epitope, respectively. Van1p, Mnn9p and Och1p, were triple-HA tagged at the endogenous locus. Triple-myc-tagged Mnt1p expressed from its own promoter, and myc-tagged Sed5p expressed from the TPI promoter, were in multi-copy plasmids. Pmr1p-HA is expressed from its own promoter integrated in the diploid strain AA662 (Antebi and Fink, 1992). (**B**) Cell lysates from the strain AA662 fractionated on a discontinuous sucrose velocity gradient (22–60% sucrose), or (**C**) by a continuous density gradient (30–65% sorbitol). Mnt1p (*medial* Golgi) and Kex2p (late Golgi) were assayed enzymatically, and Kar2p (ER), Pmr1p-HA (Golgi), Vma1p (vacuole) and Anp1p were quantified by Western blotting using ^{125}I-labeled secondary antibodies and a PhosphorImager.

of triple-HA-tagged Anp1p (Figure 3C). These results suggest there are two distinct Mnn9p-containing complexes in the *cis* Golgi, an Mnn9p–Anp1p-containing complex and an Mnn9p–Van1p-containing complex. In addition it appears that most, if not all, of the Van1p and Anp1p resides in these complexes.

Several experiments were carried out to ascertain that the complexes observed existed *in vivo*. Mnn9p was also co-precipitated with the α-Anp1p serum showing that the precipitation of the protein complexes is not dependent on the antibody used (data not shown). The co-precipitations are specific as co-precipitation of triple-HA-tagged Mnn9p with other membrane proteins such as Sed5p (*cis* Golgi), Mnt1p (*medial* Golgi) or Ufe1p, (ER) (data not shown), or precipitation of Och1p with Van1p (Figure 3C) was not observed. In order to control for complex formation after detergent addition, a wild-type lysate was mixed with a lysate from a Δ*anp1* strain carrying triple-HA-tagged Mnn9p. Following precipitation of Mnn9p we could not detect any Anp1p in the precipitate (data not shown). In addition Anp1p with an HDEL ER retention sequence at the carboxy-terminus was co-expressed in a Δ*anp1* strain with triple-HA-tagged Mnn9p. The Anp1p was ER localized and some, but not all of the Mnn9p was now also relocated to the ER (not shown). Finally in a Δ*anp1* strain Mnn9p is unaffected, whilst in a Δ*mnn9* strain the steady state levels of Anp1p are greatly reduced, indicating that normal accumulation of Anp1p requires the presence of Mnn9p (Figure 3D). Together, these data show that the co-immunoprecipitations reflect specific interactions which occur *in vivo*.

Identification of new proteins in the Mnn9p-containing complexes

The possibility that the two Mnn9p-containing complexes contained additional proteins was investigated by examining the immunoprecipitates from metabolically labeled cells. Yeast expressing Mnn9p–3HA were labeled *in vivo* with ^{35}S-Met/^{35}S-Cys, lysed and the complexes precipitated using the monoclonal antibody 12CA5 covalently linked to protein A beads. Examination of the co-precipitating proteins separated by SDS–PAGE revealed at least two additional proteins, p40 and p43, which were dependent on the presence of the epitope-tagged Mnn9p (Figure 4A).

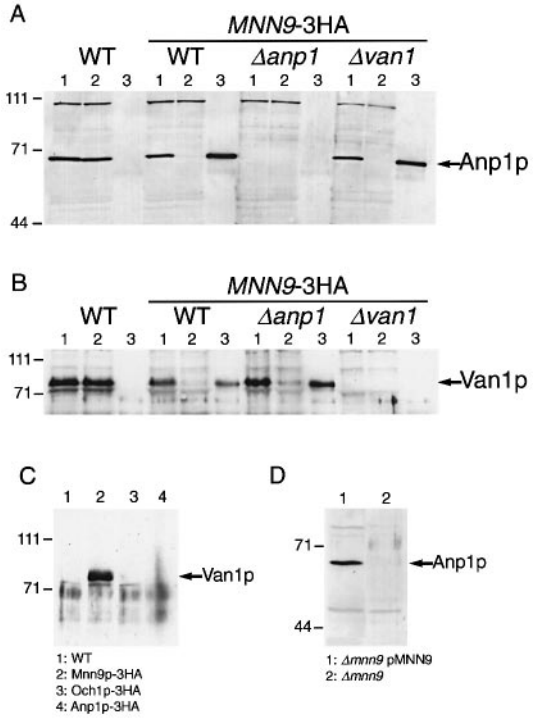


Fig. 3. Binding of Mnn9p to Anp1p and Van1p. Anti-HA monoclonal 12CA5 was used for immunoprecipitation from digitonin lysates of the indicated yeast strains (*MNN9* being triple-HA-tagged at the endogenous locus). The lysate (lane 1), the supernatant after the immunoprecipitation (lane 2) and the precipitate (lane 3) were separated by SDS–PAGE and immuno-blotted using either the α-Anp1p (**A**) or the α-Van1p (**B**) serum. (**C**) Immuno-blot probed with α-Van1p serum of 12CA5 immunoprecipitates from yeast containing the indicated HA-tagged proteins. (**D**) Immuno-blot probed with anti-Anp1p serum of total proteins from SEY6210/Δ*mnn9* containing either CEN plasmid pRS316 or the same plasmid containing Mnn9p-myc expressed from its own promoter (pMNN9).

There were a considerable number of non-specific background bands reflecting the mild detergent conditions used with the aim of preserving protein complexes. Interestingly, Anp1p is required for the co-precipitation of p40 with Mnn9p, suggesting that there is a direct interaction between p40 and the Anp1p in the Mnn9p–Anp1p complex (Figure 4A).

To identify p40 and p43 a large-scale immunoprecipitation was carried out. Proteins were separated by PAGE, blotted onto a PVDF membrane and visualized with Coomassie blue R250. Coomassie staining also revealed that the Mnn9p precipitate specifically contained both p40 and p43 (data not shown). The amino-terminal sequence of both proteins was determined by Edman degradation. The amino-terminal residues of p40 correspond to an uncharacterized protein encoded by ORF YJL183w. The protein has a single potential TMD near the amino-terminus, and is predicted to have a type II topology (TMpred, Hofmann and Stoffel, 1993). The current databases were searched with the sequence of Yjl183p using the BLAST algorithm (Altschul *et al.*, 1990). This revealed clear homologies ($P < 10^{-6}$) to members of a recently described family of proteins including Mnn10p, a *S.cerevisiae* Golgi protein of unknown function; *Schizosaccharomyces pombe* α-1,2-galactosyltransferase; and putative type II membrane proteins of unknown function from *Arabidopsis thaliana* (Chappell *et al.*, 1994; Dean and Poster, 1996) (Figure 4B). In addition, a family of genes in the genome of *Caenorhabditis elegans* encoding putative type II proteins more distantly related to this family was revealed by searching with Psi-BLAST (Altschul *et al.*, 1997) (Figure 4B).

The amino-terminal sequence of p43 matched the amino-terminus of Hoc1p, a known Golgi protein originally identified as a suppressor of the cell lysis phenotype of mutations in protein kinase C (PKC1) (Neiman *et al.*, 1997). The function of Hoc1p is unclear. Neiman *et al.* (1997) noted a sequence homology to Och1p, the well-characterized α-1,6-mannosyltransferase of the *cis* Golgi, but deletion of *HOC1* does not result in any detectable defect in protein glycosylation (Nakayama *et al.*, 1992; Neiman *et al.*, 1997).

Characterization of Yjl183p and Hoc1p

To examine further the interactions between Yjl183p and Hoc1p and the members of the Anp1p/Van1p/Mnn9p family, we constructed triple-HA-tagged alleles of both proteins by integration at the endogenous genes. Then we precipitated these fusion proteins with the monoclonal antibody 12CA5, and found that in both cases Anp1p was co-precipitated, but not Van1p (Figure 4C). Precipitation of either Anp1p or Van1p did not bring down the other protein, whilst precipitation of Mnn9p brought down both as expected. This confirms that Anp1p and Van1p associate separately with Mnn9p, and that both Yjl183p and Hoc1p are associated only with the Mnn9p–Anp1p complex. Double-label immunofluorescence of the strains expressing the triple-HA-tagged version of Yjl183p and Hoc1p revealed that, as expected, the distribution of the triple-HA-tagged proteins was almost identical to that of Anp1p (data not shown).

Mannosyltransferase activity of the Mnn9p-containing complexes

One of the Mnn9p-containing complexes contains proteins with sequence homology to known glycosyltransferases. Moreover, deletion of any of the three genes *ANP1/VAN1/MNN9* results in severe defects in the elaboration of the long outer-chains of N-linked glycans in the Golgi apparatus (Chapman and Munro, 1994; Yip *et al.*, 1994; Kanik-Ennulat *et al.*, 1995). Perhaps these complexes have a direct role in protein glycosylation. This notion was directly tested using a modification of the *in vitro* mannosyltransferase assay of Nakajima and Ballou (1975). This assay was carried out using the immunoprecipitated complexes bound to the protein A beads. Figure 5A shows that protein complexes precipitated from a strain expressing the triple-HA-tagged Mnn9p, clearly have mannosyltransferase activity using GDP–mannose, and α-1,6-mannobiose as an acceptor. Activity could also be detected when the complexes were precipitated from either Δ*anp1* or Δ*van1* deletion strains, although deletion of *ANP1* reproducibly resulted in a reduction of the observed enzymatic activity, whereas deletion of *VAN1* had little effect (Figure 5A). The activities in the Δ*anp1* and Δ*van1* strains thus add up to more than the total recovered from the wild-type, even though Western blotting revealed that in all cases the majority of the Mnn9p was being precipitated (not shown). This excess activity could result from Mnn9p still having activity when Van1p is removed,

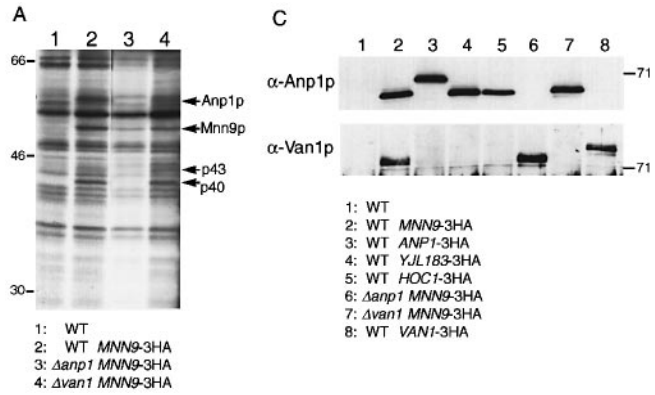
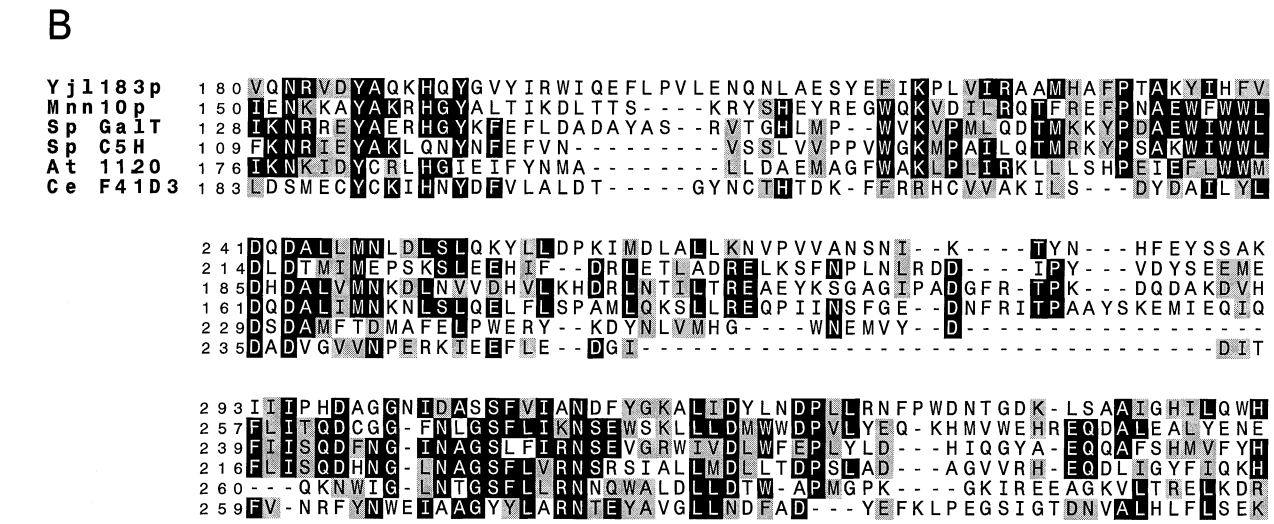


Fig. 4. Identification of additional proteins interacting with Mnn9p. (**A**) Yeast strains with the indicated genetic backgrounds were *in vivo* labeled with ^{35}S-Met/^{35}S-Cys, lysed and the triple-HA-tagged Mnn9p immunoprecipitated. The precipitated proteins were separated by PAGE, and visualized by fluorography. (**B**) Sequence comparison of Yjl183p, Mnn10p, *S.pombe* α-1,2-galactosyltransferase (GalT), a related *S.pombe* ORF, C5H10.11 (C5H), an *A.thaliana* ORF, T10P11.20 (11.20, DDBJ/EMBL/GenBank accession No. AC002330) and a *C.elegans* ORF, F41D3.a (F41D3,DDBJ/EMBL/GenBank accession No. Z81537) which shows clear homology to the Mnn10p family using Psi-Blast with the *C.elegans* sequence. The central portions of the lumenal domains were aligned with CLUSTAL W, and >2/6 identity is indicated by black bars, >2/6 similarity by grey bars. (**C**) Immunoblot of anti-HA precipitates from lysates of yeast strains expressing the indicated triple-HA-tagged proteins, the immunoprecipitates separated by SDS–PAGE and then immunoblotted using the α-Anp1p or α-Van1p sera.



or alternatively the activities of the complexes could be up-regulated in some way when the other is deleted.

In contrast to the above activity, we could not detect any mannosyltransferase activity with α-1,2-mannobiose (an acceptor for Mnn1p). If either Mnn1p, or Och1p were assayed similarly no activity was detected with α-1,6-mannobiose as acceptor (data not shown). Figure 5B shows that mannosyltransferase activity towards α-1,6-mannobiose could also be detected in precipitates prepared using the anti-Anp1p serum. Together these results show that both the Anp1p–Mnn9p- and Van1p–Mnn9p-containing complexes have mannosyltransferase activity, which is distinct from the activity of the previously cloned mannosyltransferases from yeast.

Characterization of the mannosylated products of the two Mnn9p-containing complexes

Synthesis of the outer chain of N-linked glycans in the *S.cerevisiae* Golgi involves the production of a long α-1,6-linked backbone which is attached to an initial mannosyl residue transferred to the core structure by Och1p (Nakayama *et al.*, 1992). To this backbone are attached α-1,2-linked branches which terminate in an α-1,3-linked residue. To investigate the nature of the mannose-containing structures made by the Mnn9p-containing complexes, the reaction products of the *in vitro* assays using ^{14}C-GDP–mannose were isolated and digested using specific exomannosidases. The undigested and digested reaction products were then analyzed using fluorophore-assisted carbohydrate electrophoresis (FACE, Jackson, 1994). Briefly the reaction products were modified by the addition of the charged fluorophore 8-aminonaphthalene-1,3,6,-trisulfonic acid (ANTS), at the reducing end, and then separated by electrophoresis on polyacrylamide gels. The gels were examined using both fluorography and autoradiography. The autoradiogram in Figure 6A shows that the precipitated protein complexes are capable of adding multiple mannosyl residues to the α-1,6-mannobiose acceptor resulting in the formation of a ladder of sugars. The carbohydrate chains produced by the Δ*anp1* strain are shorter than those from the wild-type or Δ*van1* strains (Figure 6A).

Examination of these poly-mannose chains after mannosidase digestion showed that treatment with α1-6 exomannosidase reduced the intensity of the ladder releasing 63% of the labeled mannose as monomer. This partial release is unlikely to be due to insufficient digestion as the unmodified α-1,6-mannobiose acceptor is completely digested (Figure 6B). Treatment with the α1-2,3 exomannosidase released only 7% as monomer, but digestion with both enzymes resulted in the complete conversion of the labeled ladder into monomer (Figure 6C). This suggests that the ladder produced by the Mnn9p-containing complexes consists of an α-1,6-linked backbone with some branches which block the action of the α1-6 exomannosidase. These branches are likely to be α-1,2-linked as the

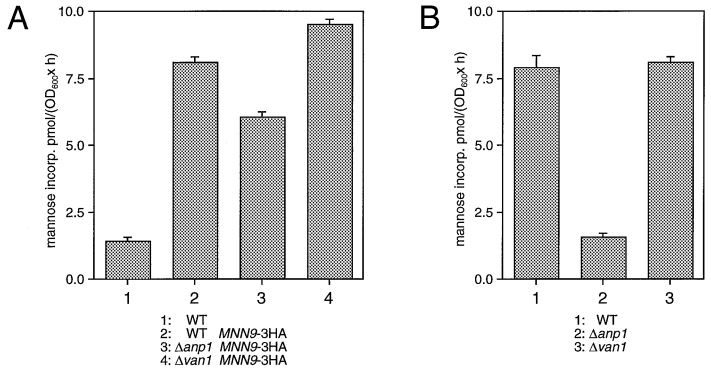


Fig. 5. Mannosyltransferase activity of complexes containing Mnn9p. Protein complexes from the indicated strains were isolated by precipitation of either the triple-HA-tagged Mnn9p with the monoclonal antibody 12CA5 (**A**) or of Anp1p with affinity-purified anti-Anp1p (**B**). Mannosyltransferase activity of complexes precipitated from the indicated strains was determined using ^{14}C-GDP–mannose, and α-1,6-mannobiose as an acceptor as described in Material and methods, with transfer of mannose from GDP–mannose into an uncharged fraction indicated. Values are means of triplicates with the standard deviation indicated by error bars. Data shown are representative of similar results from four independent determinations.

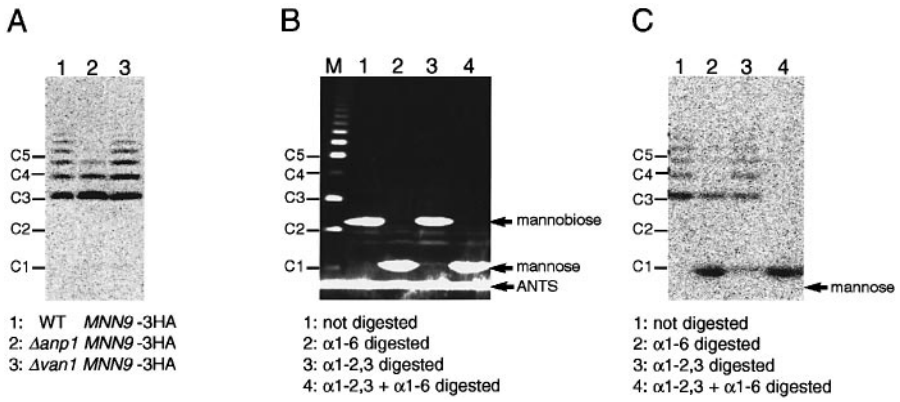


Fig. 6. Characterization of the oligosaccharide chains synthesized by Mnn9p-containing protein complexes. (**A**) Protein complexes from the indicated strains, all expressing the triple-HA-tagged Mnn9p, were immunoprecipitated and a mannosyltransferase reaction performed using ^{14}C-GDP–mannose and α-1,6-mannobiose. The reaction products were labeled with ANTS and separated by FACE. The ^{14}C-labeled oligosaccharides were visualized using a PhosphorImager. The electrophoretic mobility of a standard ladder of glucose oligomers is marked. (**B**) Protein complexes from a strain expressing the triple-HA tagged Mnn9p were immunoprecipitated and mannosyltransferase reactions performed as for (A). The synthesized oligosaccharides were treated with linkage-specific exomannosidases as indicated, labeled with ANTS and separated by FACE, along with a glucose oligomer standard ladder (M), and the fluorescent oligosaccharides visualized using a UV transilluminator. The α-1,6-mannobiose (the substrate of the transferase reaction), the mannose monomer (the product of the mannosidase digestions) and the unreacted ANTS are indicated. (**C**) The same gel as in (B) but the radioactively labeled oligosaccharides were visualized using a PhosphorImager.

MNN1 gene product is required for all detectable α-1,3-mannose addition to N- and O-linked glycans *in vivo*, and for all detectable α-1,3-mannosyltransferase activity *in vitro*, and, as mentioned above, its activity was not detected in these precipitates (Nakajima and Ballou, 1975; Verostek and Trimble, 1995). Digestion of the products produced by the complexes from the two deletion strains revealed that neither were completely digested by the α1-6 exomannosidase with 47 and 70% released as monomer from the Anp1p–Mnn9p complex and the Van1p–Mnn9p complex, respectively. These results demonstrate that both of the Mnn9p-containing complexes in the *cis* Golgi have mannosyltransferase activity, and are apparently capable of making polymeric structures containing both α-1,6- and α-1,2-linked mannoses.

Discussion

With specific antisera against Anp1p and Van1p we have shown that these proteins, and their homologue Mnn9p, are primarily localized to the *cis* Golgi of yeast. Our immunoprecipitation experiments demonstrate that Anp1p and Van1p exist in two distinct protein complexes, both of which contain Mnn9p. In addition, the Anp1p–Mnn9p complex contains two additional proteins Hoc1p and Yjl183p. Both of these complexes have mannosyltransferase activity using α-1,6-mannobiose as an acceptor, and the poly-mannose structures synthesized appear to contain mainly α-1,6 linkages with some additional mannoses, presumably α-1,2-linked.

Examination of yeast glycoproteins has shown that α-1,6 linkages are only added to N-linked glycans, and only as part of the α-1,6-linked polymer which forms the backbone of the hypermannose structure (Herscovics and Orlean, 1993). The first residue of this polymer is added by Och1p (Figure 7). It has been known for some time that in *mnn9* and *van1* mutants there are no further α-1,6-linked residues added *in vivo*, but rather a single α-1,2-linked residue is added so that the structure is now the same as that on proteins which are normally not hypermannosylated (Tsai *et al.*, 1984; Ballou *et al.*, 1991). In *anp1* mutants an α-1,6-linked backbone is present but

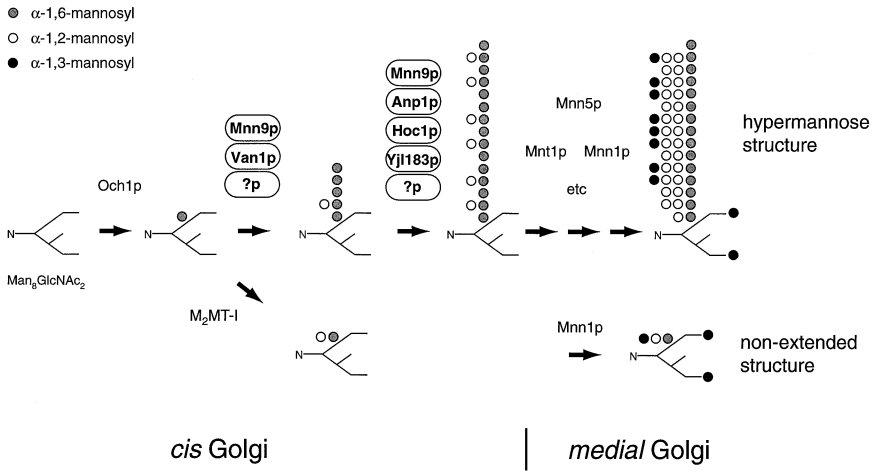


Fig. 7. A model for the synthesis of hypermannose structures in the yeast Golgi. Glycoproteins arriving in the *cis* Golgi from the ER have N-linked $Man_8GlcNAc_2$ structures to which Och1p adds a single α-1,6-linked mannose. We propose that the next step is for the Mnn9p–Van1p complex to synthesise a short α-1,6-linked mannopolymer which receives at least some of its α-1,2-linked branches. This is then elongated to its full length by the Mnn9p–Anp1p complex. It is known that in the final structure the backbone is completely branched, and this could be mediated entirely by the Mnn9p-containing complexes, or only partly, and then the gaps filled in by additional α-1,2-mannosyltransferases as shown here. Yet further α-1,2-mannosyltransferases(s) would extend the branches before Mnn1p adds the terminal α-1,3-mannoses and phosphate groups (not shown) are incorporated. The question marks indicate the possibility that the complexes contain further enzymes in addition to the proteins described here. Those N-linked glycoproteins that are not hypermannosylated may simply not be substrates for the Van1p–Mnn9p complex; alternatively, the action of this complex may be blocked by the addition to a subset of proteins of a single α-1,2-linked mannose by a putative α-1,2-mannosyltransferase, termed M_2MT-I by Lewis and Ballou (1991).

contains only 6–16 residues, instead of the normal 50 (Ballou *et al.*, 1989, *MNN8* being that same as *ANP1*, N.Dean, personal communication).

Ballou and co-workers argued that Mnn9p was not part of an elongating α-1,6-mannosyltransferase as they could still detect α-1,6-transferase activity in lysates from *mnn9* strains. This led them to propose that the defect was due to substrate proteins skipping the α-1,6-mannosyltransferase-containing compartment (Tsai *et al.*, 1984; Gopal and Ballou, 1987; Lewis and Ballou, 1991). In the light of current knowledge of the secretory pathway, and the results described in this paper, it seems more likely that Mnn9p is part of two enzyme complexes directly responsible for synthesizing the backbone of the outer chain of yeast N-linked glycans. The simplest interpretation of our results is that these complexes participate in making, and branching, the backbone in two steps (Figure 7). Thus we suggest that after Och1p has attached the first α-1,6-linked mannose to the core structure, the complex containing Van1p and Mnn9p elongates the chain, adding several mannoses but not all of the c50 residues of the final backbone. Then the Anp1p–Mnn9p complex is able to recognize this extended structure and elongate it further, whilst also attaching α-1,2-linked branches of at least one residue length. The choice of which proteins to hypermannosylate could be determined by the ability of the Van1p–Mnn9p complex to recognize the Och1p product on a subset of proteins. Alternatively, the specificity could lie in the activity of the specialized α-1,2-transferase which has been proposed to block further addition by modifying the residue added by Och1p (Gopal and Ballou, 1987). This latter possibility is unlikely to provide the complete solution as in the absence of Mnn9p or Van1p this α-1,2-transferase also modifies those substrates which would normally have their backbones extended (Tsai *et al.*, 1984; Hernandez *et al.*, 1989). It was previously argued that Mnn9p was not an elongating transferase on the grounds that α-1,6-transferase activity was still detectable in extracts from *mnn9* mutant strains. However, this *in vitro* activity could lie with Van1p, which is still present, or with the residual Anp1p, but the presence of Mnn9p is required *in vivo* to allow efficient recognition of the Och1p product on specific proteins. Such residual activity may also explain why *van1* and *anp1* are apparently lethal in combination (Chapman and Munro, 1994). Some key α-1,6 elongations may continue in the *van1* or *mnn9* mutations, even though the bulk of outer chains are absent. Alternatively one or more of the enzymes in the Mnn9p-containing complexes may have a role in some essential process other than outer chain synthesis.

Whilst we have been able to demonstrate that α-1,6-mannosyltransferase activity is associated with these Mnn9p-containing complexes, the precise role of the individual proteins within the complexes will require further investigation. However, one simple interpretation of our results is that Mnn9p, Anp1p and Van1p are all α-1,6-mannosyltransferases, with the presence of two different enzymes in a single complex facilitating the rapid synthesis of a polymeric structure. However, it also conceivable that these proteins serve as a scaffold to locate, or provide specificity to, the actual transferases, and proof of an enzymatic role will ultimately require reconstitution of the activity from heterologously expressed purified proteins, which we are currently undertaking. Such an approach may also provide a means to understand the function of Hoc1p and Yjl183p, the two additional proteins present in the Mnn9p–Anp1p complex. Hoc1p is related to Och1p, an α-1,6-mannosyltransferase, and although deletion of the protein does not result in a detectable reduction in the length of the outer chains of invertase, it may have a role in further elongating the backbone of particular substrates or under particular conditions (Neiman *et al.*, 1997).

However, the mannosidase digestion results presented

here suggest that the Mnn9p-containing complexes can also incorporate some α-1,2-linked mannose in addition to α-1,6-linked residues, suggesting the presence of at least one α-1,2-mannosyltransferase in the complex. Yjl183p is related to a galactosyltransferase from *S.pombe* and although *S.cerevisiae* does not incorporate galactose into its glycans, it is perhaps significant that this *S.pombe* protein attaches galactose to the long α-1,6-mannose backbone via an α-1,2-linkage (Chappell *et al.*, 1994). We are currently investigating the function of this in more detail, and if the presumed role in outer chain synthesis is confirmed we propose this ORF should be named *MNN11*. It is also interesting to note that there are at least three homologues of Yjl183p p in *Arabidopsis* [the ORF shown in Figure 3, and the two ESTs noted by Dean and Poster (1996)], and several more distant relatives in *C.elegans*. In plants the Golgi is the site of synthesis of many complex oligosaccharides such as hemicelluloses and pectins which consist of long backbones with extensive branching (Carpita and Gibeaut, 1993).

Although it will require further work to be certain which activity resides with which protein in these complexes, or even if there are further components which precipitate sub-stoichiometrically, our results demonstrate that Golgi enzymatic activities can be present in multi-protein complexes, a phenomenon which may well apply to other enzymes and to other species. Indeed it was previously observed that two mammalian Golgi enzymes *N*-acetylglucosaminyltransferase I (NAGT I) and mannosidase II physically interact, leading to the suggestion that this reflects a mechanism of retention in which all the enzymes in a given compartment form large hetero-oligomers (Nilsson *et al.*, 1994). However, the interactions observed here are of a completely different nature, being highly specific interactions between defined subsets of enzymes within the same Golgi compartment. Indeed this could also be the case for the NAGT I–mannosidase II complex as the region of NAGT I that is required for interaction with mannosidase II does not correlate to the region involved in retention (Munro, 1995; Nilsson *et al.*, 1996). Thus it may be that many Golgi enzymes exist in specific complexes which carry out reactions required in successive steps to generate elaborate products—as is indeed seen for many biosynthetic multi-enzyme complexes such as fatty acid synthase. This may be significant for studies of Golgi enzyme retention where individual proteins may be examined when expressed at high levels, when *in vivo* they might only ever exist as components of a large complex, with sorting information also present in the other proteins of the complex.

Our interest in Anp1p arose when we isolated the gene in a screen for mutations which affected the Golgi retention of a *medial* enzyme Mnt1p. However, Mnt1p does not co-precipitate with the Anp1p-containing complex, and indeed the two proteins appear to localize to different cisternae of the Golgi. Thus the effect of *anp1* on Mnt1p is likely to be indirect, perhaps due to some subtle perturbation of Golgi structure or dynamics. This view is strengthened by the fact that loss of Anp1p also affects the Golgi localization of DPAP-A, a protease of the late Golgi (Nothwehr *et al.*, 1996). If the internal trafficking and enzymatic activity of the Golgi were under regulation, then such indirect effects could easily arise. Indeed, there is considerable evidence to suggest that glycan synthesis by the yeast Golgi is under regulation during the cell cycle. First, deletion of either *OCH1* or *MNN10* results in cell cycle defects with blocked, or delayed, bud emergence (Nagasu *et al.*, 1992; Mondesert and Reed, 1996). Secondly, Hoc1p, a component of the Anp1p–Mnn9p complex, was originally isolated as a suppressor of mutations in PKC, a key regulatory protein in cell wall maintenance, mutations in which are also suppressible by over-expression of Golgi enzymes involved in cell wall biosynthesis (Roemer *et al.*, 1994; Neiman *et al.*, 1997). Finally, several genes encoding proteins involved in *N*-glycan synthesis, including Och1p, Mnn1p and GDP–mannose pyrophosphorylase, as well as other proteins involved in cell wall biosynthesis, are induced ~10-fold at the start point of the cell cycle (Benton *et al.*, 1996; Igual *et al.*, 1996). This presumably reflects the need for increased or altered N-linked glycan during the process of initiating, and then expanding, the cell wall of the bud. In addition to this cell cycle regulation, N-linked glycosylation becomes more extensive as cells enter the late-log phase of growth (Valentin *et al.*, 1987). There may also be regulation within the Golgi to ensure that the glycosylation capacity is sufficient for the flow of substrates through the structure, as the Golgi enzymes are required to complete their actions within a relatively short fixed period of time.

Our results suggest that the core structure of outer chains of yeast N-linked glycans are synthesized by at least two complexes each containing multiple enzymes. Such an elaborate situation is consistent with the need to specifically recognize a subset of proteins and then add to them an elaborate structure whose quantity and size is under regulation. Understanding how these complexes are assembled and retained, and how they recognize and modify substrates, could well reveal important principles for complex oligosaccharide synthesis in many other organisms.

Materials and methods

Yeast strains and plasmids

Standard protocols were followed for growth of yeast strains, yeast transformation by the lithium acetate method, preparation of total yeast DNA, crosses, sporulation of diploids and tetrad dissection (Guthrie and Fink, 1991; Ausubel *et al.*, 1993). The wild-type strains used were SEY6210 (*MATα ura3-52 his3Δ200 leu2-3, 112 trp-Δ901 lys2-801 suc2-Δ9*) and SEY6211 (*MATa ura3-52 his3Δ200 leu2-3, 112 trp-Δ901 ade2-101 suc2-Δ9*). AA662 (*MATα/MATa PMR1::HA/PMR1::HA*) was kindly provided by Gerry Fink (Antebi and Fink, 1992). The myc-tagged Anp1p in the plasmids pANP1-myc and pANP1-myc-2μ adds the epitope sequence to the carboxy-terminus, changing the sequence from 'FDPDRN*' to 'FDPDRNSRSMEQKLISEEDLN*'. The myc-tagged Van1p in the plasmids pVAN1-myc and pVAN1-myc-2μ adds the epitope sequence to the carboxy-terminus, changing the sequence from 'KRRQSE*' to 'KRRQSESRSMEQKLISEEDLN*'. Both tagged proteins were expressed under the control of a truncated *PHO5* promotor, either from an ARS CEN plasmid (pANP1-myc, pVAN1-myc) or a multi-copy plasmid (pANP1-myc-2μ, pVAN1-myc-2μ). Plasmids pM3M-426, expressing Mnt1p with a carboxy-terminal triple myc-tag, and pJS209m5, expressing Sed5p with a single amino-terminal myc-tag, are 2μ multi-copy number plasmids.

Gene disruption and epitope tagging of ORFs

In Δ*anp1* mutants the whole *ANP1* ORF was replaced by *S.cerevisiae LEU2*. The disruption of the *VAN1* gene has been described previously (Chapman and Munro, 1994). All other yeast mutants were made by

disrupting, or carboxy-terminal triple-HA epitope tagging, ORFs using PCR-based homologous recombination (Baudin *et al.*, 1993). The selectable marker was the *S.pombe HIS5* gene from pFA6a-HIS3MX6, (Erickson and Hannig, 1995; Wach *et al.*, 1997).

Antibody production and affinity purification

The DNA regions encoding the lumenal parts of either Anp1p or Van1p were amplified by PCR using the primers PJJ11 (CGGGATCCATATCGCTATTCCAGCTGGTAACTTTC) and PJJ12 (CCCAAGCTTTCTATCAGGGTCGAAGTCTAATGG) (for *ANP1*) and PJJ13 (CGGGATCCGGCCTGGGCATTGGTGTATCCACGCAA) and PJJ14 (CCCAAGCTTCTCTGATTGTCTTCTCTTCTCTCTTTC) (for *VAN1*). The PCR products were then cloned into the *Bam*HI and *Hin*dIII sites of the vector pTrcHis A (Invitrogen). The amino-terminal $(His)_6$-tagged proteins were expressed in *E.coli* strain DH5α, and purified on Ni^{2+}-NTA-agarose under denaturing conditions according to the manufacturer's protocol (QIAGEN). After stepwise dialysis against decreasing concentrations of urea and then against PBS, 50–100 μg of the fusion proteins were injected into rabbits, and the sera collected after three boosts with the same amount of fusion protein. The anti-Anp1p serum was purified on a cyanogen bromide-activated Sepharose 4B column (Pharmacia) with the $(His)_6$-Anp1p antigen covalently bound. The anti-Van1p was purified on $(His)_6$-Van1p antigen bound to Western blot strips (Harlow and Lane, 1988). Prior to use for immunofluorescence, the affinity-purified antibodies were incubated with fixed cells (SEY6210) for ~4 h at 4°C in PBS, 2% dried milk, 0.5% Tween-20 to remove antibodies reacting with cell walls.

Indirect immunofluorescence and immunoblotting

Indirect immunofluorescence was performed as described previously (Hardwick and Pelham, 1992). The anti-HA monoclonal antibody 12CA5 and the anti-myc monoclonal antibody 9E10 were used at 1 μg/ml and 10 μg/ml respectively. Fluorescent secondary antibodies were FITC-conjugated donkey anti-rabbit Ig (1/50), Cy3™-conjugated goat anti-rabbit IgG (H+L) and Cy3™-conjugated goat anti-mouse IgG (H+L) (both 1/700) (Amersham International). Images were obtained using a Bio-Rad MRC-600 confocal laser-scanning microscope.

Total yeast protein extracts were prepared by resuspending cells at 1 OD_{600} unit per 20 μl SDS sample buffer, vortexing with glass beads (425–600 microns, Sigma) for 5 min at 4°C, and extracting the proteins for 5 min at 65°C. For immunoblotting, proteins were separated by SDS–PAGE and then electrophoresed onto nitrocellulose (0.45 μm, Schleicher and Schuell). Antibody incubations were carried out in PBS, 2% dried milk, 0.5% Tween-20. The rabbit antisera against Anp1p or Van1p were diluted 1/8000 or 1/4000, respectively, 12CA5 or 9E10 were used at 3 μg/ml or 6 μg/ml, respectively. Peroxidase-conjugated anti-rabbit (Sigma, 1/3000) and anti-mouse (Bio-Rad, 1/3000) secondary antibodies were detected by chemiluminescence (ECL, Amersham).

Subcellular fractionation

Frozen spheroplasts from the strain AA662 were lysed by several passages through a 26G needle. The supernatant from a 5 min 13 000 *g* spin was then fractionated by a stepped sucrose velocity gradient (22–60% sucrose, w/v) (Antebi and Fink, 1992) or a continuous density gradient (30–65% sorbitol). 20× 0.66 ml fractions were removed from the top, and assayed for Mnt1p or Kex2p enzymatic activity (Cunningham and Wickner, 1989; Häusler and Robbins, 1992), or analyzed by quantitative immunoblotting using ^{125}I-labeled secondary antibodies (Amersham) and a PhosporImager (Molecular Dynamics). Vma1p was detected with monoclonal 8B1-F3 (Molecular Probes) and Kar2p with monoclonal 2E7 (Napier *et al.*, 1992).

Protein labeling

Yeast cells were grown in SD minimal medium supplemented by uracil, L-tryptophan, L-histidine, L-leucine and L-lysine (Guthrie and Fink, 1991) to log phase. Cells (5 OD_{600} units) were harvested by centrifugation and resuspended in 2 ml of growth medium containing 150 μCi Tran ^{35}S-label (ICN). After 1 h cell growth was stopped by addition of 10 mM sodium azide, and cells lysed as described below.

Immunoprecipitation

Yeast cell growth was stopped in log phase by addition of 10 mM sodium azide and after washing in 10 mM sodium azide, 10 OD_{600} units of cells were resuspended in 500 μl 100 mM Tris, 10 mM DTT pH 9.4 and incubated at 30°C for 15 min. Cells were then resuspended in spheroplasting solution [1 M sorbitol, 50 mM Tris pH 7.4, 2 mM $MgCl_2$, 10 mM DTT, 10 mM sodium azide, 0.2 mg/ml lyticase (Sigma)] and incubated at 30°C for 90 min. Spheroplasts were then washed in spheroplasting solution without lyticase at 4°C, and lysed at 4°C for 30 min by shaking in 400 μl T/D/I buffer (10 mM triethanolamine pH 7.5, 150 mM NaCl, 1% digitonin, 2 mM EDTA supplemented by protease inhibitors). Cell debris was removed by centrifugation at 4°C (10 min at 14 000 *g*) and the supernatant precleared by incubation with 10 μl of protein A–Sepharose (Pharmacia) at 4°C for 1 h. The antibody was then added (12CA5, 3.8 μg/ml or affinity-purified anti-Anp1p, diluted 1/300) followed after 2–4 h by 12.5 μl of protein A–Sepharose, and complexes precipitated by gentle agitation at 4°C overnight. The precipitated proteins were then washed in T/D/I buffer, and either eluted with a small volume of SDS sample buffer at 65°C for 5 min; or used directly for mannosyltransferase assays.

Mannosyltransferase assay

Mannosyltransferase assays were essentially as described by Nakajima and Ballou (1975) using proteins immunoprecipated on protein A beads. Immunoprecipitates were washed once with T/D/I buffer (4°C, 15 min) and once briefly with wash buffer II (50 mM HEPES, pH 7.2, 0.4% digitonin, 10 mM $MnCl_2$). Then 50 μl of reaction buffer [wash buffer II supplemented by 0.6 mM GDP–D-mannose, 10 mM α-1,6-D-mannobiose (Dextra Labs), 62 nCi GDP–D-[U-^{14}C]mannose (Amersham)] was added to the beads, and the mixture was incubated with continuous gentle agitation for 1 h at 30°C (shown to be within the linear range). The reaction was terminated by passing the supernatant of the reaction mixture through a 0.5×5 cm Dowex 1-X8 column. The neutral reaction products were eluted by 800 μl of water, analyzed by liquid scintillation counting or used for further experiments.

Mannosidase digestion

The carbohydrate products of the mannosyltransferase assays were phenol-extracted and purified on a Sephadex G-10 gel filtration column (Pharmacia) to separate oligosaccharides from the bulk of the unmodified α-1,6-mannobiose substrate. The sugars were then concentrated and digested by either α1-6 exomannosidase or α1-2,3 exomannosidase (New England Biolabs) according to the manufacturer's instructions. The products of these reactions were phenol-extracted prior to analysis by FACE.

Fluorophore-assisted carbohydrate electrophoresis (FACE)

The reducing ends of oligosaccharides were reacted with the negatively charged fluorophore ANTS (Glyko) to form a Schiff base which was then reduced by cyanoborohydride (Glyko) to a secondary amine according to Jackson (1994). The labeled sugars were then separated by electrophoresis on 40% polyacrylamide gels, and detected either by UV light on a transilluminator or, if the sugars were radioactively labeled, the gel was air-dried and exposed using a PhosphorImager.

Acknowledgements

We would like to thank Christine Wiggins for invaluable help with subcellular fractionation, Rob Arkowitz, Gerry Fink and Mike Lewis for providing strains and antibodies, Pat Barker for protein sequencing, Peter Jackson for advice on FACE, Neta Dean and Vivian MacKay for helpful advice on yeast Golgi, and Hugh Pelham and Rob Arkowitz for critical reading of the manuscript and many helpful discussions. J.J. was supported by the Boehringer Ingelheim Fonds.

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Received August 29, 1997; revised October 31, 1997; accepted November 7, 1997