The Components of the *Saccharomyces cerevisiae* Mannosyltransferase Complex M-Pol I Have Distinct Functions in Mannan Synthesis*

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The yeast *Saccharomyces cerevisiae* processes *N*-linked glycans in the Golgi apparatus in two different ways. Whereas most of the proteins of internal membranes receive a simple core-type structure, a long branched polymer termed mannan is attached to the glycans of many of the proteins destined for the cell wall. The first step in mannan synthesis is the initiation and extension of an α-1,6-linked polymannose backbone. This requires the sequential action of two enzyme complexes, mannan polymerases (M-Pol) I and II. M-Pol I contains the proteins Mnn9p and Van1p, although the stoichiometry and individual contributions to enzyme action are unclear. We report here that the two proteins are each present as a single copy in the complex. Both proteins contain a DXD motif found in the active site of many glycosyltransferases, and mutations in this motif in Mnn9p or Van1p reveal that both proteins contribute to mannan polymerization. However, the effects of these mutations on both the *in vivo* and *in vitro* activity are distinct, suggesting that the two proteins may have different roles in the complex. Finally, we show that a simple glycoprotein based on hen egg lysozyme can be used as a substrate for modification by purified M-Pol I *in vitro*.

*N*-linked glycans are attached to many of the secreted and membrane proteins made by eukaryotic cells. The structures of *N*-linked glycans vary greatly between species and between individual proteins produced by a single species or cell type. This diversity is generated by differential processing of an invariant GlcNAc<sub>2</sub>Man<sub>9</sub>Glc<sub>3</sub> structure that is attached during insertion into the endoplasmic reticulum (ER).<sup>1</sup> This is initially trimmed by ER-localized glucosidases and mannosidases in a manner that is not protein-specific but rather is linked to protein folding (1). However, when the trimmed GlcNAc<sub>2</sub>Man<sub>9</sub> *N*-glycan arrives in the Golgi it can be trimmed further by mannosidases and then modified by many different glycosyltransferases to generate the observed diversity of glycan structures (2, 3). Golgi glycosyltransferases vary greatly between different species and cell types and can also show substrate selectivity between the glycoprotein substrates expressed by a given cell type. This results in the variation of glycosylation seen between different species and also between the glycoproteins produced by an individual cell.

The yeast *Saccharomyces cerevisiae* has proven useful for identifying features of glycosyltransferase structure, function, and targeting that are well conserved in higher eukaryotes. Yeast generate just two main *N*-glycan structures, a small core-type structure found on many glycoproteins of internal compartments and a large mannan structure found on proteins of the cell wall and the periplasm (4, 5). The mannan structure contains −100–300 mannosines per *N*-glycan and constitutes 40% of the dry weight of the yeast cell wall. Nonetheless mannan is not essential for viability (6, 7). Presumably because of their harsh natural environment yeast have the ability to respond to cell wall damage or perturbation by increasing the synthesis of cell wall components. Thus cells lacking mannan are dependent for their viability on intact stress response pathways, and have elevated levels of chitin and cell wall proteins (8–12). This viability has facilitated the genetic analysis of the steps of mannan synthesis, and combined with the altered chemical sensitivities of glycosylation mutants (13–15), has allowed the cloning of the relevant Golgi glycosyltransferases. The first step of Golgi processing is the addition of a single α-1,6-linked mannose to all *N*-glycans by the mannosyltransferase Och1p (16). The next step is protein-specific (Fig. 1A). For proteins that receive a core-type structure an as yet unidentified enzyme adds an α-1,2-linked mannose to the mannosine added by Och1p, followed by terminal α-1,3-linked mannosines from Mnn1p (17, 18). In contrast, on mannoproteins an α-1,6-linked mannose is attached to the mannosine added by Och1p, and this is then extended to generate a long α-1,6-linked backbone which is then branched by the addition of α-1,2-linked and then α-1,3-linked mannosines (19, 20). The mannan backbone is generated by two multiprotein complexes called mannan polymerase (M-Pol) I and II (21–23).

At present it is not clear why some proteins receive mannan and others do not. This issue is of relevance not only to the general biological question of how glycan diversity is generated in the Golgi, but also to the use of yeast as an expression system for the production of recombinant glycoproteins. The M-Pol I complex is responsible for the first committed step in the generation of mannan structure, and so might be expected to play a role in substrate selection. Indeed, in cells lacking the components of the complex, mannoproteins receive a core-type structure (13, 24). The M-Pol I complex contains two related proteins, Van1p and Mnn9p (22, 23). Both of these appear to be canonical Golgi glycosyltransferases in that they are type II membrane proteins with an N-terminal transmembrane do-

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§ The abbreviations used are: ER, endoplasmic reticulum; HA, hemagglutinin; endo H, endoglycosidase H; PIPES, 1,4-piperazinediethanesulfonic acid; FACE, fluorophore-assisted carbohydrate electrophoresis.
main (25–27). Moreover, they both have a well conserved DXXD motif that is also found to be conserved in many families of nucleoside diphosphate sugar using glycosyltransferases (28–30). Structural studies on a number of enzymes have shown that these asparagine residues coordinate the Mn$^{2+}$ ion that plays a central role in catalysis (31–34). Moreover, the aspartate has been shown to be necessary for the activity of a wide range of glycosyltransferases (28–30). In this paper, we determine the stoichiometry of Mnn9p and Van1p in M-Pol I, and use mutations in their DXXD motifs to investigate in vitro and in vivo the contribution the two proteins make to the activity of the complex.

EXPERIMENTAL PROCEDURES

Yeast Strains—S. cerevisiae SEY6210 (MATα ura3–52 leu2-3,112 his3–200 trpl-Δ901 lys2–801 suc2Δ901) and SEY6211 (MATα ura3–52 leu2-3,112 his3–200 trpl-Δ901 ade2–101 suc2Δ925) were used as wild-type strains (39). Gene disruptions or C-terminal fusions to Van1p or Mnn9p were generated by PCR-mediated homologous recombination using the Schizosaccharomyces pombe his5+ gene (40). Strains were checked by PCR or Western blotting. MNN1 was deleted using plasmid pMRMN1, which contains 432 bp of the MNN1 promoter and 542 bp of MNN1 terminator sequences flanking the LEU2 gene in pUC19. Strains B10C (MATα och1::URA3) and och1::LEU2 mnn1::HIS3 (41) were generous gift from Daniel Tessier (Biotechnology Research Institute, Montreal), and used for large scale purification of lysozyme-G49N. The Δoch1 Δmnn1 strain was generated by sporation of a cross of XCY42–30D (MATα trpl1 trpl2 lys2–101 ade2–101 mnn1::LEU2, Ref. 25) with a och1 strain (SEY6210 och1::HIS350). The multiple protease-deficient strain c13-ABYS 86 (MATα prc1–1 prb1–1 prl1–1 eps1–3 ura3Δ5 leu2–3,112 his3; Ref. 42) was used for expression of Och1p-ZZ.

Plasmid Constructs—A cDNA encoding hen egg lysozyme was mutagenized to change the codon for glycine 49 to asparagine (Quick-Change, Stratagene). The cDNA was cloned into yeast vectors pVT100-U (43) for Western blot analysis, and pTGI0241 (44) for preparative purification of lysozyme-G49N. For co-expression of Mnn9p and Van1p, VAN1-ZZ was cloned by gap repair from an integrative transformant (23) and inserted into pRS424. The MNN9 gene, isolated from a genomic clone as a BamHI fragment, was tagged at the C terminus with a triple HA epitope tag. To generate gene transplacements A::TRP1 and B::TRP1, the strains were grown twice for 24 h in media lacking uracil for 3 days. The medium was diluted 5-fold with 12.5 mM PIPES, pH 6.5, and 5 ml of settled SP Sepharose beads (Amersham Biosciences) were added per ml of medium. After stirring for at least 1 h at 4 °C, the beads were allowed to settle and transferred to a column. After washing with 100 mM NaCl in 10 mM PIPES, pH 6.5, the protein was eluted with 500 mM NaCl in the same buffer, dialyzed against water, concentrated by freeze-drying, dialyzed into PBS, and aliquoted for storage at –20 °C.

Mannosyltransferase Reactions with Lysozyme-G49N as Acceptor—To generate a form of lysozyme suitable for modification by M-Pol I, the protein purified from strain DT111 was first mannosylated in vitro with Och1p. A soluble, protein A-tagged form of Och1p expressed in yeast from the plasmid Och1p-ZZ described above, was isolated with IgG-Sepharose from 0.1 g (wt weight) of cells, and the beads were incubated with 8 µg of lysozyme-G49N as acceptor protein in 50 µl of 20 mM HEPES, pH 7.2, 0.5% Triton-X 100, 1 mM MnCl₂, 0.6 mM unlabeled GDP-mannose for 7.5 h at 4 °C.

RESULTS

Generation of an Antiserum against Mnn9p—The Golgi-luminal domain of Mnn9p was expressed in E. coli, and a polyclonal antiserum was raised in rabbits against the recombinant protein. After affinity purification the serum recognized a protein of 45 kDa in protein blots of total extracts from wild-type cells (Fig. 1B), close to the predicted molecular size of 45.8 kDa for Mnn9p, which has no sites for N-linked glycosylation. This species is drastically increased in abundance when the MNN9 is present on a multicopy plasmid, is absent when MNN9 is deleted, and has reduced mobility when a triple HA epitope tag is inserted at the C terminus of the MNN9 open reading frame. We conclude that the serum specifically recognizes Mnn9p.

M-Pol I Is a Heterodimer of Van1p and Mnn9p—To investigate the stoichiometry of Mnn9p and Van1p in M-Pol I, a diploid strain was constructed in which one allele of the VAN1 gene was tagged at the C terminus with a triple HA epitope tag, and used in yeast two-hybrid assays. The antiserum against lysozyme, Mnn9p and Van1p were affinity purified on antigen coupled to cyanogen bro-
The free Mnn9p is presumably associated with the untagged Van1p, and moreover Mnn9p, unlike Van1p, is also present in the M-Pol II complex. Antiseras against Anp1p, a component of M-Pol II, showed that as expected this complex does not associate with Van1p-HA (Fig. 1C). The absence of association between Van1p-HA and Van1p indicates that the M-Pol I complex contains only a single copy of Van1p.

A similar strategy was used to analyze the stoichiometry of Mnn9p in M-Pol I. Detergent lysates were prepared from a diploid strain in which one copy of MNN9 was tagged with the triple HA epitope. Since Mnn9p is present in both M-Pol I and M-Pol II, the latter complex was initially depleted from the lysate using an anti-Anp1p serum. This treatment removed all of the Anp1p (Fig. 1C) and more than half of the Mnn9p from the lysate (data not shown). When the lysate was then incubated with anti-HA monoclonal and protein A beads, all remaining Mnn9p-HA was precipitated. In contrast, the untagged Mnn9p remained in the supernatant. About half of the Van1p present after removal of M-Pol II coprecipitated with Mnn9p-HA, indicating that the M-Pol I complex had remained intact during the isolation procedure. Taken together these results indicate that M-Pol I is a heterodimeric complex consisting of one copy of Van1p and one copy of Mnn9p.

Interestingly, the situation with M-Pol II appears to be different. If this complex was not removed with anti-Anp1p prior to precipitation of Mnn9p-HA, then untagged Mnn9p is coisolated with Mnn9p-HA (Fig. 1D). A similar immunoprecipitation was performed on a diploid strain carrying one tagged allele of ANP1. After precipitation with 12CA5 and protein A beads, untagged Anp1p co-precipitated with Anp1p-HA, indicating that Anp1p is also present at more than one copy in the M-Pol II complex (Fig. 1D). Together, these results show that the overall architecture of M-Pol I is simpler than the structure of M-Pol II, which involves five subunits, at least two of which are present in multiple copies.

*Inactivating Mutations in the Catalytic Domains Van1p and Mnn9p*—The primary function of M-Pol I is to initiate and extend the α-1,6-linked backbone of the mannan structure, and the complex has mannosyltransferase activity in vitro (22). This raises the question of what contribution the two proteins in the complex make to the synthesis of the α-1,6-linked polymer. This cannot be addressed by simply deleting the individual genes, as it has been found that deletion of MNN9 results in greatly reduced levels of both Van1p and Anp1p, presumably due to destabilization in the absence of their normal binding partner (21, 22). We therefore made use of the DXD motifs in Mnn9p and Van1p, which have been shown in other glycosyltransferases to be present in the catalytic site and essential for activity, but in those cases examined not required for normal folding and assembly (28, 29). Thus mutant versions of MNN9 and VAN1 were generated in which the DXD motif was altered to AXD (mnn9-AXD (D236A); and van1-AXD (D361A)). The mutant alleles were used to substitute the wild-type allele by gene transplacement (47), and protein blotting indicated that the mutant proteins were present at similar levels to those found in wild-type cells (data not shown).

Defects in mannan synthesis are known to result in a resistance to vanadate (13), and Fig. 2A shows that the mnn9-AXD and van1-AXD strains behave like the Δmnn9Δ and Δvan1Δ deletion strains in that they are capable of growing on 10 mM sodium vanadate. To investigate whether the AXD mutations affect the ability of the proteins to associate, the wild-type and mutant forms of the two genes were co-expressed from plasmids in all four possible combinations. In each case Van1p was tagged with protein A (Van1p-ZZ), and IgG-Sepharose was used to isolated the proteins from cell lysates. Fig. 2B shows the primary function of M-Pol I is to initiate and extend the α-1,6-linked backbone of the mannan structure, and the complex has mannosyltransferase activity in vitro (22). This raises the question of what contribution the two proteins in the complex make to the synthesis of the α-1,6-linked polymer. This cannot be addressed by simply deleting the individual genes, as it has been found that deletion of MNN9 results in greatly reduced levels of both Van1p and Anp1p, presumably due to destabilization in the absence of their normal binding partner (21, 22). We therefore made use of the DXD motifs in Mnn9p and Van1p, which have been shown in other glycosyltransferases to be present in the catalytic site and essential for activity, but in those cases examined not required for normal folding and assembly (28, 29). Thus mutant versions of MNN9 and VAN1 were generated in which the DXD motif was altered to AXD (mnn9-AXD (D236A); and van1-AXD (D361A)). The mutant alleles were used to substitute the wild-type allele by gene transplacement (47), and protein blotting indicated that the mutant proteins were present at similar levels to those found in wild-type cells (data not shown).

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The signal sequence of the avian protein is functional in reporter protein a glycosylated mutant of hen egg lysozyme. When the protein is which it can be readily purified by cation-exchange chromatography. When the medium from which it can be readily purified by cation-exchange chromatography. When the protein is N-glycosylated on a single sequon introduced by mutagenesis (G49N), it receives an extensive mannan structure in the Golgi (48). This reporter has the advantage that it is a small protein and so most of its mass is contributed by the saccharide, increasing the ease of detecting changes in glycan addition. Moreover, it appears that when Mnn9p is still active, more mannose is contributed to the product of lysozyme-G49N consistently migrate slightly slower than the wild-type lysozyme, presumably due to the N-acetylglucosamine residue that remains after endo H treatment. The endo H product of lysozyme-G49N consistently migrate slightly slower than the wild-type lysozyme, presumably due to the N-acetylglucosamine residue that remains after endo H treatment. B, as in A, except that the lysozyme-G49N expression plasmid was in the indicated glycosylation mutants.

When expressed in yeast, wild-type lysozyme protein migrates as a 14 kDa protein, and, as previously reported, the G49N form has a greatly reduced mobility, running as a smear of 150–200 kDa on an SDS gel (Fig. 3a and Ref. 48). We did not observe any reporter protein secreted with no N-linked sugars or with only core structures attached, indicating that modification and maturation are very efficient. Treatment with endoglycosidase H (endo H) to release N-linked glycans, restored the mobility of lysozyme-G49N to close to that of the unmodified protein. More lysozyme is detected on the blot after digestion, presumably because the highly glycosylated form of lysozyme is only partially transferred from the gel during blotting.

Having established that lysozyme-G49N behaved as expected in the wild-type strain, we next examined how its mobility was affected by deletion of particular Golgi glycosyltransferases. Fig. 3B shows that loss of the M-Pol II subunit Anp1p resulted in intermediate mobility while cell lacking Och1p and Mnn1p, and hence incapable of any Golgi mannose addition to the ER-derived N-glycan structure, produced a rapidly migrating form (16 kDa). We next examined the effect on glycosylation of the AXD mutations of Mnn9p and Van1p. mnn9-AXD and Δmnn9 cells both produce lysozyme-G49N with the same mobility, close to that of the protein produced in Δoch1 cells, indicating a severe defect in mannan synthesis. Combination of these mutations with a deletion of MNN1 reduces the apparent molecular weight of the lysozyme-G49N. Mnn1p is known to add 2–3 α-1,3-linked mannoses to the core-type structure (6, 18, 51), indicating that the loss of a small number of mannoses leads to a detectable increase in gel mobility. The van1-AXD mutation also results in severe defect in mannan synthesis, with the lysozyme-G49N having a mobility similar to that seen in Δvan1 cells. However, the lysozyme-G49N consistently migrated slightly slower than that produced from Δmnn9 or mnn9-AXD cells, both in the absence of MNN1 (Fig. 3B) and in its presence (data not shown). Taken together, these results indicate that both Mnn9p and Van1p participate directly in the extension of the mannan backbone by the M-Pol I complex. Moreover, it appears that when Mnn9p is still active, more...
Mannosyltransferase activity of wild-type and mutant versions of M-Pol I. A. Mannosyltransferase activity of M-Pol I complexes isolated from the indicated strains. The amount of radioactivity transferred from GDP-[14C]mannose to the acceptor α-1,6-mannobiose was quantified by scintillation counting of the neutral reaction products, and expressed as nmol of mannose transferred per h per mg of starting membrane protein prior to detergent solubilization. The experiment shown is representative of three independent determinations, and the background signal produced by a precipitation from a strain with no tagged protein was indistinguishable from that seen with the Mnn9ΔxD-Van1pΔXΔD complex (data not shown). B, FACE gels of the reaction products from the indicated complexes using the same donor and acceptor as in A. The products were subject to digestion with the indicated mannosidases prior to modification with the negative charged fluorophore 8-aminoanaphthale-1,3,6-trisulfonic acid and gel separation. The resulting gels were exposed to a PhosphorImager screen for 10 days to identify the radioactive products (upper panels) or visualized by fluorescence with ultraviolet light (lower panels). The glucose oligomer size ladder is visible in the latter panels.

Mnn9p Acts as Both an α-1,2- and an α-1,6-Mannosyltransferase—The M-Pol I complex has been previously shown to have mannosyltransferase activity when assayed in vitro (21–23). To correlate the effects of the AXD mutations seen in vivo, with their effects on the activity in vitro, M-Pol I was isolated from cells expressing the 4 possible combinations of Van1p and Mnn9p as the DXD or AXD versions. The various forms of the two proteins were co-expressed from a plasmid in an Δmnn9 mutant, and complexes isolated by use of a protein A tag fused to the C terminus of the plasmid-borne Van1p (Van1p-ZZ). This strategy ensures that the complexes immobilized on the IgG-Sepharose beads were entirely encoded by the plasmid-borne copies of VAN1 and MNN9.

The isolated complexes were assayed for ability to transfer radioactivity from GDP-[14C]mannose to the acceptor α-1,6-mannobiose. Neutral reaction products were quantified by scintillation counting or reacted with a fluorescent dye for analysis by FACE (22, 46). The complexes containing an AXD mutation in just one of the subunits still retained substantial mannosyltransferase activity in vitro, although more remained after mutation of Mnn9p than of Van1p (55 versus 13%, Fig. 4A). If both were mutated then no transferase activity was detected above background. However, FACE analysis of the products of the two single mutant complexes showed that the complex in which only Van1p is wild-type produced a ladder of polymannose products of 3–7 mannose residues in size. In contrast, the complex in which only Mnn9p is wild-type produced only a mannose trimer, consistent with the lower incorporation of radioactive mannose.

Previous analysis of the wild-type M-Pol I complex had shown that it formed both α-1,6- and α-1,2-linkages (22, 23). Thus the products of the two mutant complexes were digested with linkage-specific mannosidases. Fig. 4B shows that the complex with only Van1p wild-type produced almost entirely α-1,6-bonds linkages. In contrast, the trimers produced by the

In vitro mannosyltransferase activity of M-Pol I with a glycoprotein substrate. Protein gel of glycosylated lysozyme-G49N after treatment with GDP-[14C]mannose and M-Pol I containing Mnn9p and Van1p with either AXD mutations (A) or wild-type (D) as indicated. The gel was stained with Coomassie Blue and then dried and exposed to a PhosphorImager screen for 3 days. The substrate lysozyme-G49N was expressed from the strain DT111 and pretreated with cold GDP-mannose and Och1p-ZZ bound to IgG-Sepharose beads. The protein was then used in the mannosyltransferase assays with M-Pol I complexes bound to IgG-Sepharose via a protein A tag on Van1p. Substantial labeling of lysozyme-G49N was only seen if both Mnn9p and Van1p were wild-type, indicating that transfer of mannose to the substrate was M-Pol I-dependent. A small amount of label was apparently transferred even with both proteins being mutant, and this was seen with beads from cells with no tagged protein (data not shown). This background activity is probably caused by contamination of the IgG-Sepharose beads by endogenous mannosyltransferases. Examination of IgG-Sepharose precipitates from appropriate strains indicated that this activity was entirely dependent on Och1p and Mnn1p, and is perhaps due to the presence of glycans on the IgG as at least Och1p has an affinity for large N-glycan structures (16).
The synthesis of the mannann structure attached to a subset of yeast glycoproteins starts with the generation of an α-1,6-linked backbone. Mutations in the related Golgi membrane proteins Mnn9p and Van1p result in the production of glycoproteins lacking this backbone structure, and the two proteins have been found to be physically associated. In this paper we...
report that the Mnn9p-Van1p complex that we term M-Pol I comprises a single copy of each of the proteins. Our previous analysis of the complex by gel filtration in the presence of detergent had indicated an apparent molecular size of ~280 kDa (23). This is clearly larger than the sum of the predicted molecular sizes of Mnn9p and Van1p (107 kDa). However, the complex analyzed by gel filtration will have also included a detergent micelle and the N-linked glycans known to be present on Van1p (22), and so we assume that these, in combination with the shape of the complex, cause the complex to migrate more slowly than its stoichiometry would predict.

We also find from mutation of the putative active sites of Mnn9p and Van1p that both proteins appear to contribute directly to the enzymatic synthesis of the mannan backbone. Glycosylated lysozyme-G49N produced by cells in which the DXD motif in Mnn9p was mutated had a higher mobility than that produced in cells with the corresponding mutation in Van1p. This suggests that Mnn9p is responsible for attaching the first α-1,6-mannose in the backbone, and that the Van1p in the complex, although active in vitro on simple substrates, cannot modify a glycoprotein substrate in vivo until Mnn9p has attached this first mannose (Fig. 6). This model is in accordance with the N-glycan structures previously observed in strains lacking Mnn9p or Van1p. Deletion of Mnn9p destabilizes Van1p (53), and in the effective absence of both proteins no α-1,6-linked mannose is attached to the Och1p product (52). However, in strains lacking Van1p it was found that the N-linked glycans had a single α-1,6-linked mannose attached to the Och1p product to generate a mannan backbone of two residues, a proportion of which were also terminated with an α-1,2-linked residue (13) (vrg1 being allelic to van1, Ref. 4). This implies that in the absence of Van1p there is sufficient residual Mnn9p to add the first residue of the backbone, but that this is not extended further. After Mnn9p has attached the first mannose, the addition of the subsequent 10–15 mannoses to the backbone by M-Pol I could be mediated by Van1p alone, as we find that the complex can still generate α-1,6-linked polymers in vitro when Mnn9p is inactivated by the AXD mutation, or alternatively by both proteins acting in concert.

The mannan backbone contains only α-1,6-linked mannoses, and so its synthesis will require only this transferase activity. However, we have previously found that M-Pol I can also attach an α-1,2-linked mannose to an α-1,6-mannobiose in vitro (22, 23). The results above indicate that this activity it mediated by Mnn9p. Glycoproteins that do receive mannans to have instead an α-1,2-linked mannose attached to the α-1,6-mannose added by Och1p (Fig. 1), and this has been proposed to act as a stop signal to prevent further extension (18, 54). The N-glycan that receives either mannans or this α-1,2-linked residue has the same structure, indicating that the modification fate of glycoproteins must reflect the recognition of features of the proteins themselves. The fact that Mnn9p apparently attatches the first α-1,6-linked mannose in the mannan synthesis pathway, leads us to speculate that this subunit of M-Pol I interacts directly with both glycan and protein. Then, depending on the nature of the interaction with the protein moiety, Mnn9p would add either an α-1,6- or α-1,2-linked mannose (Fig. 6). If an α-1,6-linked mannose is attached, then the mannan backbone would be further extended by M-Pol I in a Van1p-dependent manner. However, if an α-1,2-linked mannose is attached then the substrate would be released and be resistant to further modification by M-Pol I. Of course this is simply one possible model that is consistent with our data. It may be that M-Pol I primarily interacts with only those glycoproteins that receive mannans, and that another enzyme is responsible for attaching the α-1,2-linked residue to the rest. Indeed, it is known that in the absence of Mnn9p, α-1,2-linked mannose is added to even those proteins that would receive mannans (52), perhaps by members of the large MNT/KRE family of α-1,2-mannosyltransferases involved in extension of O-linked glycans (20). Alternatively, more elaborate scenarios are possible such as the involvement of adaptor proteins that present the mannan-requiring glycoproteins to M-Pol I. In any case, it is not at present clear what features of the proteins that receive mannan distinguish them from those that do not. Ultimately, resolution of these issues will require in vitro assays with a number of different glycoprotein substrates, and structural analysis. The observation that lysozyme-G49N can serve as a substrate for modification by purified M-Pol I indicates that this sort of in vitro approach should be feasible.

Proteins closely related to Mnn9p and Van1p are encoded in the genomes of many other yeasts and fungi, including Candida, Histoplasma, S. pombe, and Aspergillus. These organisms contain mannan-like cell wall glycans, although the side branches are highly variable between species, and for Candida it has been shown that an Mnn9p homologue is involved in mannan synthesis (55–58). Although Mnn9p-containing protein complexes in these species have not so far been reported, it seems likely that the mechanisms that determine which proteins receive mannan in S. cerevisiae will be conserved in other yeasts and fungi. Although there are no close relatives of the Mnn9p family in the genomes of higher eukaryotes, a number of mammalian Golgi glycosyltransferases have also been found to be in specific multienzyme complexes. These include exostosin-1 and -2 that are required to generate the polymer of alternating glucuronic acid and N-acetylgalcosamine residues that forms the basis of heparan sulfate. Both proteins have conserved DXD motifs, and form a hetero-oligomer of unknown stoichiometry (59–61). As with Mnn9p and Van1p, mutation in either protein is sufficient to generate a phenotype. It may be that arrangement of transferases into a complex helps to generate polymeric structures more rapidly, and indeed some enzymes that make polysaccharides contain two glycosyltransferase domains in a single polypeptide chain (62). However, it has recently been reported that a complex is formed between two Golgi glycosyltransferases that are not involved in polymer synthesis, but rather catalyze successive steps in a pathway of glycolipid synthesis (63). In this case the physical association of the enzymes may ensure that substrates are handled efficiently, or are channeled down a particular modification pathway. For M-Pol I it is not at present clear whether the association of Mnn9p and Van1p reflects two enzymes acting alternately in several cycles to generate a polymer, or acting sequentially with Mnn9p first selecting substrates, and then Van1p extending the substrate that will receive mannan. In the case of M-Pol II, five putative transferases form a complex that simply extends the mannan backbone on all substrates modified by M-Pol I, and here it seems likely that complex formation is to facilitate the rapid generation of a 50–100-residue polymer. What seems certain though is that the arrangement of Golgi glycosyltransferases into complexes will prove to be an important aspect of the specificity and nature of glycan synthesis.

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