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 glycosyltransf erases, and mutations in this motif in Mnn9p or Van1p reveal that both proteins contribute to mannos 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-Man,Glc, structure that is attached during insertion into the endoplasmic reticulum (ER). 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-Man,Glc,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 mannoses 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 mannone 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 mannone to the mannone added by Och1p, followed by terminal α-1,3-linked mannoses from Mnn1p (17, 18). In contrast, on mannoproteins an α-1,6-linked mannone is attached to the mannone 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 mannosides (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, endo-glycosidase H; PIPES, 1,4-piperazinediethanesulfonic acid; FACE, fluorophore-assisted carbohydrate electrophoresis.
main (25–27). Moreover, they both have a well conserved DXD 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 aspartate residues coordinate the Mn$^{2+}$ ion that plays a central role in catalysis (31–34). Moreover, the aspartates have been shown to necessary for the activity of a wide range of glycosyltransferases (29–35). In this paragraph, we determine the stoichiometry of Mnn9p and Van1p in M-Pol I, and use mutations in their DXD 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 trp1–Δ901 lys2–401 trp1–Δ901 leu2–3,112 his3–200 trp1–Δ901 ade2–101 trc259) and SEY8211 (MATα ura3–52 leu2–3,112 his3–200 trp1–Δ901 ade2–101 trc259) 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 histi gene (40). Strains were checked by PCR or Western blotting. MNN1 was deleted using plasmid pDRMNN1, which contains 432 bp of the MNN1 promoter and 542 bp of MNN1 terminator sequences flanking the LEU2 gene in pUC19. Strains containing a tandem repeat of the tandemly cloned 5′-1,6-mannosidase (Aspergillus satoi) were a generous gift from Daniel Tissier (Biotechnology Research Institute, Montreal), and used for large scale purification of lysozyme-G49N. The Δchi1 Δmann1 strain was generated by sporulation of a cross of CYX24–30D (MATα ura3–52 trp1–52 leu2–101 ade2–101 leu2–3,112 mnn11:LEU2, Ref. 25) with a Δchi1 strain (SEY6210 och1::HIS5Sp). The multiple proteosede deficient strain c19-ABYS 86 (MATα pro1–1 prb1–1 pro1–1 eps1–3 ura3Δ5 leu2–3,112 his3; Ref. 42) was used for expression of Och1p-Δ.

**Plasmid Constructs—**A CDA encoding hen egg lysozyme was mutagenized to change the codon for glycine 49 to asparagine (Quick-Change, Stratagene). The CDA was cloned into yeast vectors pVT100-U (43) for Western blot analysis, and pTGI0241 (44) for preparative purification of lysozyme-G49N. The MNN1 gene was tagged at the C terminus with a triple HA epitope tag. To generate a form of lysozyme suitable for modification by M-Pol I, the protein purified from strain DT111 was first mammalized in vitro with Och1p. A soluble, protein A-tagged form of Och1p expressed in yeast from the plasmid Och1p-Δ was described above, was isolated with IgG-Sepharose from 0.1 g (wet weight) of cells, and the beads were incubated with 8 μg of lysozyme-G49N as acceptor protein in 60 μl of 50 mM HEPES, pH 7.2, 10 mM MnCl₂, 0.1% dodecylmaltoside, 10 mM α-1,6-mannobiose (Dextra Laboratories), 0.6 mM GDP-mannose, 62 nCi of GDP-[U-14C]mannose (Amersham Biosciences). Quantitation and fluorophore-assisted carbohydrate electrophoresis (FACE) analysis of reaction products was done essentially as described (23, 46). For mammalid digestions α-1,6-mannobiose (New England Biolabs) and 10 mM of GDP-mannose (Sigma) were added per well 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.

**Mammalidase Transfertase Reaction 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 mammalized in vitro with Och1p. A soluble, protein A-tagged form of Och1p expressed in yeast from the plasmid Och1p-Δ was described above, was isolated with IgG-Sepharose from 0.1 g (wet weight) of cells, and the beads were incubated with 8 μg of lysozyme-G49N as acceptor protein in 60 μl of 50 mM HEPES, pH 7.2, 0.5% Triton-X 100, 1 m M MnCl₂, 0.6 m M unlabeled GDP-mannose for 7.5 h at 30 °C. Reaction products were dialyzed against phosphate-buffered saline to remove unused GDP-mannose and then incubated with M-Pol I complexes immobilized on IgG-Sepharose beads. Reaction conditions contained 50 mM HEPES, pH 7.2, 1 m M MnCl₂, 125 nCi of GDP-[U-14C]mannose, 0.1% dodecylmaltoside and 50 μl of beads containing M-Pol I complexes prepared from 0.5 g (wet weight) of cells. Incubation was performed for 7.5 h at 30 °C, followed by a brief centrifugation to remove the beads. The supernatant was precipitated with trichloroacetic acid, washed with acetone, and boiled in SDS sample buffer for separation in a 15% gel. After Coomassie Blue staining and drying, the gels were exposed to a PhosphorImager screen (Typhoon, Molecular Dynamics).

**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, the manufacturer’s protocol, the protein was used for immunizations of rabbits. The antiserum against hen egg lysozyme (Sigma) was a gift from Mike Lewis, MRC-LMB. Antiserum against lysozyme, Mnn9p and Van1p (23) were affinity purified on antigen coupled to cyanogen bro-
A proportion of Mnn9p was also bound to the beads indicating beads, while the untagged Van1p remained in the supernatant.

The M-Pol I Mannosyltransferase Complex

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 ∆mnn9 and ∆van1 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
that Mnn9p coprecipitates with Van1p-ZZ on IgG-Sepharose beads regardless of whether there is an AXD mutation in MNN9 or VAN1 or both. Thus mnn9-AXD and van1-AXD alleles encode stable proteins that are capable of normal interactions but appear to have defective activity.

Use of Hen Egg Lysozyme as an in Vivo Reporter of Protein Glycosylation—To analyze directly the effect of the AXD mutations in MNN9 and VAN1 on mannan synthesis we used as a reporter protein a glycosylated mutant of hen egg lysozyme. The signal sequence of the avian protein is functional in reporter protein a glycosylated mutant of hen egg lysozyme. and the protein is secreted into the medium from \textit{S. cerevisiae}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Characterization of AXD versions of Mnn9p and Van1p. A, strains that carry disruptions or AXD alleles of MNN9 or VAN1 were spotted on YPD plates containing 10 mM sodium vanadate and incubated for 3 days at 30 °C. Strains containing the HA-tagged versions of both genes were also analyzed. B, anti-Mnn9p protein blots of IgG-Sepharose precipitations from Δmnn9 strains expressing from 2-μ plasmids the indicated combinations of untagged Mnn9p and protein-A tagged Van1p (Van1p-ZZ). Total (t), unbound (f), and bound (b) fractions were analyzed. Due to the binding of rabbit anti-Mnn9p to protein A, Van1p-ZZ is also detected on the blot. In each case some Mnn9p does not bind to the IgG-Sepharose beads, presumably because it is present in the M-Pol II complex.}
\end{figure}

FIG. 2

FIG. 3

Expression of a glycosylated form of hen egg lysozyme in wild-type and glycosylation mutants of \textit{S. cerevisiae}. A, protein blots of the media from wild-type SEY6210 cells expressing wild-type or the G49N version of lysozyme (HEL). Samples were digested with or without endo H prior to gel electrophoresis, as indicated. 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.

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 subunits Anp1p 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
mannose can be attached than occurs when only Van1p is active, indicating that the first step or steps in the extension of the mannan backbone are mediated by Mnn9p rather than Van1p.

**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 AXI 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 mannosyl 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

**Fig. 4. 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 Mnn9pAXD-Van1pAXD 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-aminonaphthalene-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.
complex with only Mnn9p wild-type contained a mixture of α-1,6 and α-1,2 linkages. Quantification revealed that more than 40% of the radioactivity appears in the form of monomeric mannose after digestion with α-1,6-specific mannosidase. This is consistent with previous analysis of the products of the wild-type complex in which only mannose trimers were resistant to α-1,6-specific mannosidase (22). Taken together, these results show that both proteins contribute α-1,6-transferase activity to the complex, but that Mnn9p appears to also have the ability to attach mannose via an α-1,2-linkage to at least a simple substrate, in this case mannobiose.

**In Vitro Mannosyltransferase Activity on a Glycoprotein Substrate**—Many in vitro assays of glycosyltransferase activity rely on small oligosaccharide substrates such as those used in the assays above. However, the in vivo substrates of Golgi glycosyltransferases are of course much larger glycoproteins. In the case of M-Pol I these are proteins that contain ER-derived GlcNAc2Man9 structures that have been then modified in the Golgi by addition of an α-1,6-linked mannose by Och1p (Fig. 1A). Therefore, we examined whether the glycosylated variant of lysozyme could be used as a model glycoprotein substrate for the in vitro reconstitution of outer chain initiation and elongation by M-Pol I.

To obtain lysozyme-G49N with N-glycans in an ER form the protein was expressed in a yeast strain DT111 that lacks MNN1, OCH1, and MNS1, and has been shown to produce glycoproteins with the form GlcNAc2Man9 (41). The glycosylated lysozyme produced by this strain was initially mannosylated by Och1p in vitro. This could not be done in vivo as glycoproteins produced in strains lacking M-Pol I, but containing Och1p, have an additional α-1,2-linked mannose attached to the mannose added by Och1p (13, 52). This linkage apparently precludes any further extension of the mannan backbone and seemed likely to have some effect in vitro. Thus we expressed Och1p as a fusion protein to protein A, isolated it on IgG-Sepharose beads and used it to modify the glycosylated lysozyme-G49N in vitro using GDP-mannose as a donor (see “Experimental Procedures”). Control reactions using radiolabeled GDP-mannose confirmed that the lysozyme-G49N was mannosylated by Och1p in an MnCl2-dependent manner (data not shown).

The Och1p-modified lysozyme-G49N was then used as an acceptor for in vitro mannosyltransferase reactions with isolated M-Pol I complexes immobilized on IgG-Sepharose. The donor was radiolabeled GDP-mannose, and the reaction products were separated on protein gels and analyzed by Coomassie staining and autoradiography (Fig. 5). When M-Pol I containing AXD mutation in either one or both of Mnn9p and Van1p was used, the 16-kDa band corresponding to core-glycosylated lysozyme-G49N received only trace labeling. This appears to be due in part to low level contamination of Mnn1p and Och1p on the IgG-Sepharose beads (data not shown). In contrast, the complex consisting of wild-type Van1p and Mnn9p produced a striking incorporation of radioactivity into the glycoprotein substrate. This leads to an increase in apparent molecular size from 16 kDa to up to 40 kDa. This size is similar to that of the lysozyme-G49N produced by the *anp1* mutant in vivo (Fig. 3A), suggesting that the complex has considerable processivity in vitro. As expected this high level of incorporation was not observed with complexes in which either Mnn9p or Van1p contained the AXD mutation. However, because of the trace labeling observed in all cases on the core-glycosylated lysozyme-G49N it was not possible to determine whether the Mnn9pAXD-Van1pDXD complex was still able to attach an initiating mannose, as predicted from the in vivo results. Nonetheless, the reconstitution of outer chain mannosylation from the purified wild-type proteins suggests that all the proteins that are necessary to initiate the mannan outer chain are identified and that lysozyme-G49N can be directly recognized by the M-Pol I complex.

**DISCUSSION**

The synthesis of the mannan 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 that 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 that 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 vitro until Mnn9p has attached this first mannose (Fig. 6). This model is 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 mannosyl residue, 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-mannobiase in vitro (22, 23). The results above indicate that this activity it mediated by Mnn9p. Glycoproteins that do receive mannose are found to have instead an α-1,2-linked mannosyl residue 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 mannose 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 attaches the first α-1,6-linked mannosyl 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 moity, Mnn9p would add either an α-1,6- or α-1,2-linked mannosyl (Fig. 6). If an α-1,2-linked mannosyl residue is attached, then the mannan backbone will be further extended by M-Pol I in a Van1p-dependent manner. However, if an α-1,2-linked mannose residue has been 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 mannan, 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 mannan (52), perhaps by members of the large MNT1/KRE2 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-requireing 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-acetylglucosamine 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 polymers 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 subset 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.

Acknowledgments—We would like to thank the referees for helpful comments.

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Saccharomyces cerevisiae

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