The *mnn2* mutant of *Saccharomyces cerevisiae* is affected in phosphorylation of *N*-linked oligosaccharides

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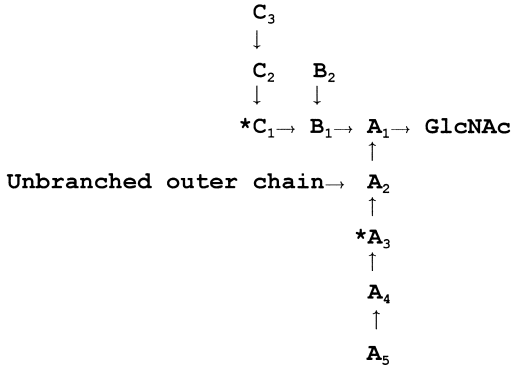
Abstract We studied the phosphorylation of the inner core region of *N*-linked oligosaccharides in the mannan defective mutant *Saccharomyces cerevisiae mnn2* which was described as unable to synthesize branches on the outer chain. We performed structural studies of the *N*-oligosaccharides synthesized by the strains *mnn2*, *mnn1mnn2mnn9* and *mnn1mnn9ldb8*, and the results are compared with previously published structural data of *mnn1mnn2mnn10* and *mnn1mnn9* [Hernández, L.M., Ballou, L., Alvarado, E., Tsai, P.-K. and Ballou, C.E. (1989) J. Biol. Chem. 264, 13648–13659]. We conclude that the *mnn2lldb8* mutation is responsible for the inhibition of incorporation of phosphate to mannose A_3 (see below), a particular phosphorylation site of the inner core, while phosphorylation at the other possible site (mannose C_1) is allowed, although it is also reduced.



***Phosphorylation sites in *mnn1mnn9*.**

Key words: Phosphorylation; *N*-Oligosaccharide; *mnn2*; *Saccharomyces cerevisiae*

1. Introduction

Phosphorylation of *N*-oligosaccharides in *Saccharomyces cerevisiae* is a complex process in which several gene products are involved. The *MNN6* gene, a member of the *KRE2/MNT1* gene family, encodes a mannosylphosphate transferase involved in phosphorylation of *N*-linked oligosaccharides [1]. These authors also suggested the existence of redundant functional genes since the Mnn6p was apparently not the only transferase responsible for phosphorylation. The *MNN4* gene has also been cloned [2]. The Mnn4p is required to mediate phosphorylation in both the core and the outer chain and it was proposed that Mnn4p is the putative positive regulator of *MNN6*, acting at the transcriptional level [3].

Two more mutants of *S. cerevisiae* affected in *N*-linked oligosaccharide phosphorylation have been isolated: *ldb1* and *ldb2* [4]. Both mutants synthesize *N*-oligosaccharides completely depleted of phosphate groups, which suggests that the *LDB1* and *LDB2* gene products are also involved in the phosphorylation process. A more recent paper [5] proposed the existence of mannosylphosphate transferase complexes containing several proteins localized in the Golgi apparatus. At least the *LDB2* gene product might be part of the complexes as well as Mnn4p, Mnn6p and some others. The complexes may contain more than one transferase, each with different specificities.

The *mnn2* mutant of *S. cerevisiae* was initially described as unable to synthesize branches on the outer chain (for a review see [6]). Such a phenotype led to the suggestion that Mnn2p could be the α(1,2) mannosyltransferase that catalyzes the transfer of the first mannose of the branches during branch biosynthesis, which is α(1,2)-linked to the α(1,6) linear backbone of the outer chain. The cloning of the gene has confirmed that MNN2 encodes the mentioned mannosyltransferase [7].

In a previous work, it was observed that the triple mutant *mnn1mnn2mnn10* lacked phosphate groups at one of the two possible phosphorylation sites of the core region found in *mnn1mnn9* [8]. In this paper we demonstrate that the *mnn2* mutation is responsible for the aforementioned phosphorylation defect.

2. Materials and methods

2.1. Strains

S. cerevisiae X2180 1A and *S. cerevisiae* X2180 1B were from the laboratory collection. The mutants: *mnn1*, *mnn2*, *mnn1mnn2*, *mnn1mnn9* and *mnn1 mnn2mnn10* were kindly provided by Lun and Clint Ballou (University of California at Berkeley, CA, USA). The mutant *mnn1ldb8* was from a previous work [4] and the strains *mnn1mnn2mnn9* and *mnn1mnn9ldb8* were constructed for this study.

2.2. Growth and sporulation conditions

Cells were grown at 30°C in solid or liquid YEPD (1% yeast extract, 2% peptone, 2% D-glucose). To induce external invertase synthesis, the amount of D-glucose was lowered to 0.05%. For sporulation, we essentially followed previously published protocols [9].

2.3. Purification of mannoproteins, isolation and fractionation of *N*-oligosaccharides

We followed previously published protocols [4,6,8,10,11]. Manno-

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Abbreviations: endo H, endo β-*N*-acetylglucosaminidase; ^{1}H-NMR, ^{1}H-nuclear magnetic resonance; MAPK, mitogen-activated protein kinase

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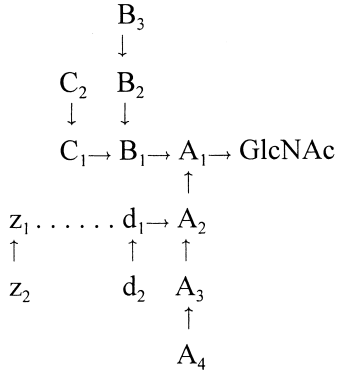
proteins were solubilized by the hot citrate method and purified by precipitation as cetavlon complex and DEAE-Sephacel chromatography. The *N*-linked oligosaccharides were released by treatment of lyophilized mannoproteins with endo β-*N*-acetylglucosaminidase H (endo H) (Sigma). The fractionation of endo H released material was done by gel filtration through Bio-Gel P-4 (400 mesh) (1.5×170 cm) from Bio-Rad laboratories and ion exchange chromatography in QAE-Sephadex (Pharmacia) (1.5×9 cm). Carbohydrate was measured in the fractions by the phenol–sulfuric acid method at 490 nm [12].

2.4. Convention for identifying mannose units in N-linked yeast oligosaccharides

In the nomenclature of individual mannoses we have adopted the convention proposed in Hernández et al. [8]:



Scheme 1.

Upper case letters are assigned to mannoses derived from the lipid-linked oligosaccharide, and mannoses added during processing are shown in lower case.

The ^{1}H-NMR spectra allows the identification of mannoses present in these structures [8,11,13,14]. The anomeric proton chemical shifts are as follows: A_1, δ 4.77; A_2, δ 5.32; A_3, δ 5.29; A_4, δ 5.03; B_1, δ 4.87; B_2, δ 5.379 (δ 5.08–5.11 when B_3 is not present); B_3, δ 5.05; C_1, δ 5.125; C_2, δ 5.04; d_1, δ 5.125; d_2, δ 5.04. If the outer chain is extended to z_1, then the signals for z_1 and z_2 replace those for d_1 and d_2. When a monoesterified phosphate group is attached to A_3, then A_1, A_2 and A_3 change to δ 4.81, δ 5.35 and δ 5.24 respectively. If this phosphate is diesterified with an αMan, the new signal for the anomeric proton of the αM-P appears at δ 5.43–5.45 and A_1, A_2 and A_3 change to δ 4.80, δ 5.31 and δ 5.28. If the phosphate is attached to C_1 the spectrum does not change when the phosphate is monoesterified, but if it is diesterified, then the signal for the αM-P appears at δ 5.43–5.45. Finally, when the α(1,6)-linked mannoses of the outer chain (d_1 to z_1) appear as a linear backbone without branches, their anomeric protons resonate in the region of δ 4.90–4.92.

2.5. Other methods

The multiple mutants were constructed by standard genetic techniques. To make the *mnn1mnn2mnn9* triple mutant, the strains *mnn1mnn2* and *mnn1mnn9* of different sexual type were crossed and the resultant heterozygous diploid was induced to sporulate. The asci were dissected and each of the spores was checked for the following phenotypic characteristics: size of glycosylated invertase in non-denaturing 5% acrylamide gels, staining characteristics with alcian blue, and the size of the colonies formed from a single cell after 48 h of growth in solid medium. The colonies from cells bearing the *mnn9* mutation were smaller in size because of the slower growth rate. The strains bearing the *mnn2*, the *mnn9*, or both mutations showed a detectable reduction in the size of the glycosylated invertase as well as lower affinity for the alcian blue dye [6]. We selected the tetrads that showed two wild type spores with respect to the mentioned characteristics and the other two are assumed to carry both the *mnn2* and *mnn9* mutations. Since *mnn1* was present in both parental strains, it was also present in the four spores from each tetrad, as was also proved by the lack of affinity for anti-α(1-3)-linked mannose antibodies. As a control, the triple mutants were then back-crossed to the wild type and single mutants containing each one of the three mutations were obtained. Since the mutant *ldb8* has been shown to be allelic to *mnn2* [4], the *mnn1mnn9ldb8* triple mutant was constructed in the same way except that *mnn1ldb8* and *mnn1mnn9* were used as parental strains. Micromanipulation and dissection of asci was done with the aid of a Tetrad Dissection system from Micro Video Instruments (Avon, MA, USA).

For digestion with α(1,6) endomannanase, purified mannoproteins were lyophilized and resuspended in 0.1 M citrate–phosphate buffer, pH 6, containing 1 mM $CaCl_2$ at a final concentration of 200 mg of mannoprotein in 1 ml. Then 0.4 U/ml of the enzyme were added and the reaction was incubated at 45°C for 24 h. At this time, 0.4 more U/ml of enzyme were added and the reaction was continued for 24 h more. The reaction was stopped by heat and the digested mannoproteins were dialyzed against distilled water and lyophilized. The α(1,6) endomannanase was a generous gift of Lun Ballou (University of California, Berkeley, CA, USA) [15]. Non-denaturing acrylamide gel electrophoresis of invertase was done as described previously [4,6].

For desalting oligosaccharide samples, we used gel filtration chromatography through a column (1×30 cm) packed with Bio Gel P-4 (Bio-Rad) by elution with water, or ion exchange chromatography through a column (1.5×9 cm) packed with Dowex AG50W X8 (Bio-Rad), also by elution with water, followed by lyophilization.

For ^{1}H-NMR, samples were resuspended in D_2O. Spectra were recorded at 40°C in a 400 MHz Bruker spectrometer, in the Department of Organic Chemistry, University of Extremadura, Badajoz, Spain [8,11,13].

3. Results and discussion

The inner core of *N*-oligosaccharides in *S. cerevisiae* has two potential phosphorylation sites (mannoses C_1 and A_3 in Scheme 1). In the mannan defective mutant *mnn1mnn9*, around 50% of the endo H released molecules are phosphorylated. Of these, 60% are phosphorylated only in mannose C_1, 20% are phosphorylated only in mannose A_3, and both mannoses are phosphorylated in the remaining 20% of the molecules [8]. These data indicated that 40% of the total endo H

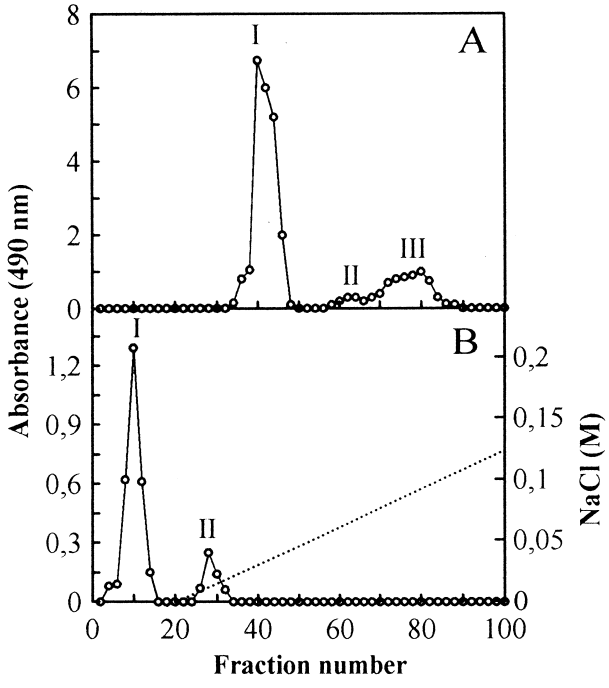


Fig. 1. Fractionation of *N*-linked oligosaccharides from the *mnn2* mutant mannoproteins. A: Biogel P-4 chromatography of endo H digested mannoproteins. B: Ion exchange chromatography through QAE-Sephadex of peaks II and III from A. The carbohydrate (○) was determined by the phenol–sulfuric acid method and the NaCl concentration (...) by conductivity.

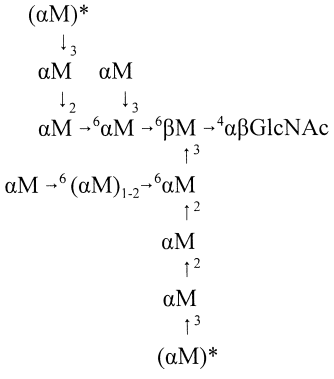
released oligosaccharides had a phosphate attached to mannose C_1 and 20% had the phosphate attached to mannose A_3. However, in the *mnn1mnn2mnn10* triple mutant, diphosphorylated molecules were not detected and all the monophosphorylated ones had the phosphate group attached to mannose C_1 [8]. Such results suggested a specific inhibition of incorporation of phosphate groups to mannose A_3 in the inner core. The total percentage of phosphorylated molecules in the endo H released material was not reported in that study.

The possible reasons for the differences in the phosphorylation pattern of *mnn1mnn9* and *mnn1mnn2mnn10* could be the following: (a) the presence of the *mnn10* mutation in the latter, (b) the presence of the *mnn2* mutation also in the latter, (c) the presence of both mutations simultaneously, and (d) a steric impediment due to the presence of some mannoses of the outer chain in the triple mutant. In order to check which of these possibilities was correct, we have analyzed the phosphorylation pattern in endo H released oligosaccharides from purified mannoproteins of the following mutants: *mnn2*, *mnn1mnn2mnn9*, and *mnn1mnn9ldb8*.

3.1. Analysis of inner core phosphorylation in N-oligosaccharides from the mnn2 mutant

Purified mannoproteins from the *mnn2* strain (0.2 g) were exhaustively digested with α(1,6) endomannanase in order to hydrolyze the unbranched outer chain synthesized by the mutant and get the inner core portion of the *N*-oligosaccharides. After dialysis against distilled water, the sample was lyophilized and digested with endo H. The reaction mixture was chromatographed in the BioGel P-4 (1.5×170 cm) by elution with 0.1 M ammonium acetate. From previous similar experiments [4,8,11] we assume that peaks II and III in Fig. 1A must contain the core-sized oligosaccharides, phosphorylated (II) and neutral (III), that have been released by the endo H, while peak I includes the undigested material (mainly *O*-linked sugars). Since the separation of peaks II and III was not complete, they were collected together, desalted with Dowex AG50W X8, lyophilized, and subjected to ion exchange chromatography through QAE-Sephadex. The neutral oligosaccharides were not retained in the column (Fig. 1B, peak I) while the phosphorylated (Fig. 1B, peak II) eluted after the application of the NaCl gradient in a position expected for a molecule with only one negative charge. This must correspond to oligosaccharides with a single diesterified phosphate group [8].

The suggested structures were confirmed by nuclear magnetic resonance. The fractions from each of the two peaks resolved by QAE-Sephadex chromatography were collected together, desalted by gel filtration chromatography, and subjected to ^{1}H-NMR. Fig. 2A shows the spectrum of the material not adsorbed to the ion-exchange QAE-Sephadex column, included in peak I of Fig. 1B. The signals in this spectrum correspond to a neutral molecule with the following structure (see Section 2):



Structure I. *These terminal α(1,3)-linked mannoses are present in a low proportion of the molecules.

The presence of 1 to 3 mannoses in the outer chain is supported by the presence of the two signals at around δ 4.90.

Fig. 2B shows the spectrum of the material adsorbed to QAE-Sephadex, included in peak II of Fig. 1B. The main differences of this spectrum with respect to the one shown in Fig. 2A are as follows: (a) The presence of a doublet at δ 5.40–5.42 whose integral corresponds to one mannose. This

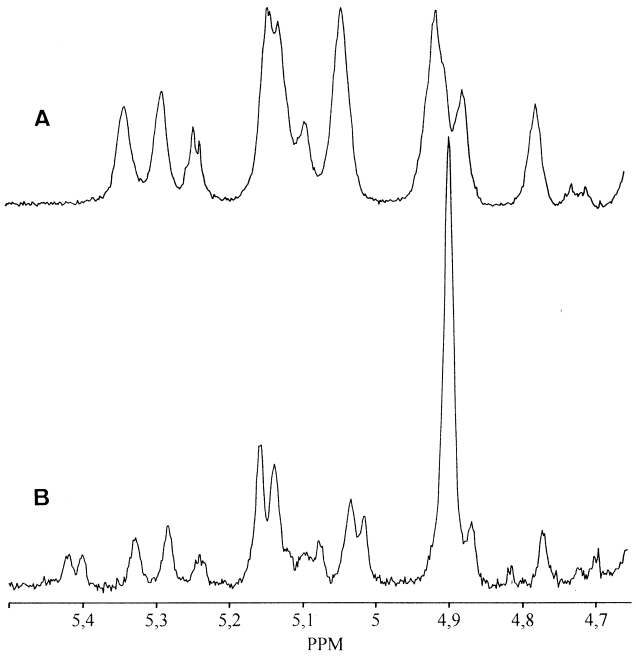


Fig. 2. ^{1}H-NMR spectrum (anomeric proton region) of the inner core region of endo H released oligosaccharides from the *mnn2* mutant mannoproteins. The inner core region was obtained by digestion of the mannoproteins with α(1,6) endomannanase before treatment with endo H (see text). A: Neutral fraction. B: Phosphorylated fraction.

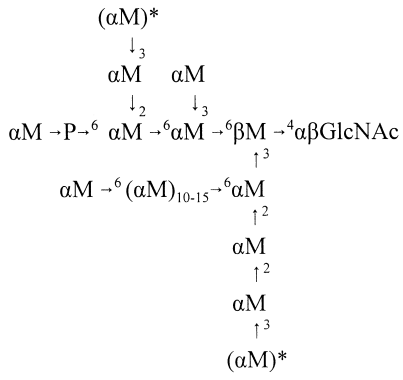
Table 1
Effect of several mutations on the phosphorylation of the inner core of *N*-oligosaccharides in *S. cerevisiae*

Strain	Phosphate in C_1	Phosphate in A_3	Reference
mnn1mnn2mnn10	Yes	Not detected	[8]
mnn1mnn9	Yes	Yes	[8]
mnn2	Yes[a]	Not detected	This study
mnn1mnn2mnn9	Yes[a]	Not detected	This study
mnn1mnn9ldb8	Yes[a]	Not detected	This study
mnn1mnn4mnn9	Yes[b]	Yes[b]	[8]
mnn1mnn6mnn9	Yes[b]	Yes[b]	[8]
mnn1mnn9ldb1	Not detected	Not detected	[4]
mnn1mnn9ldb2	Not detected	Not detected	[4]

[a] Reduced by half as compared to *mnn1mnn9*.
[b] Reduced to less than 20% as compared to *mnn1mnn9*.

doublet indicates the presence in the molecule of one mannose diesterifying a phosphate group. (b) The signal at δ 4.90 is significantly larger in this case. The integral indicates the presence of 10–15 α(1,6)-linked mannoses, by average, in the outer chain.

The spectrum is compatible with the following structure:



Structure II. *These terminal α(1,3)-linked mannoses are present in a low proportion of the molecules.

The phosphate group must be linked to mannose C_1 because the signals of mannoses A_1, A_2 and A_3 are in the same position as in the neutral molecule. As we pointed out in Section 2, phosphorylation on mannose A_3 which is the other possible phosphorylation site of the inner core, leads to some changes in the chemical shifts of the anomeric protons of the mannoses A_1, A_2 and A_3. The presence of only one diesterified phosphate group is supported by the elution position in the QAE-Sephadex chromatography and the doublet at δ 5.40–5.42 which integrates for only one mannose.

The reason for the presence of 10 to 15 mannoses in the outer chain of the phosphorylated molecules might be that the presence of the phosphate group in some way interferes with the hydrolysis by the α(1,6) endomannanase. The enzyme seems to be unable to complete the hydrolysis of the outer chain probably due to steric impediments.

The results shown so far demonstrate that possibilities (a) and (c) mentioned above must be discarded since a strain with the *mnn2* but without the *mnn10* mutations shows the same absence of phosphorylation in A_3 as the *mnn1mnn2mnn10*. The same results suggest that the reason must be either the *mnn2* mutation (b) or a steric impediment due to the presence of the outer chain (d).

3.2. *Analysis of inner core phosphorylation in N-oligosaccharides from the mnn1mnn2mnn9 triple mutant*

In order to elucidate whether the *mnn2* mutation or the outer chain were responsible for the absence of phosphate attached to mannose A_3, we must construct a strain with only one of the two mentioned characteristics and check for the phosphorylation pattern in the inner core of the *N*-linked oligosaccharides. The triple mutant *mnn1mnn2mnn9* does have the *mnn2* mutation but it only synthesizes a single branch of the outer chain because of the presence of the *mnn9* mutation. We also included the *mnn1* mutation because it was previously published that the double mutant *mnn1mnn9* is able to incorporate phosphate at both the C_1 and A_3 phosphorylation sites [8,11]. In addition, the *mnn1* mutation will prevent the addition of the terminal α(1,3)-linked mannoses (asterisks in Structures I and II) to the core, which will make the characterization of the oligosaccharides easier.

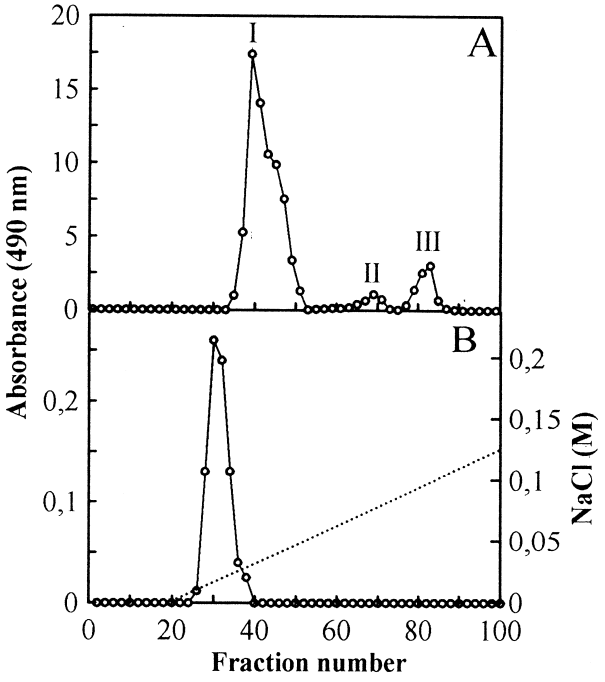


Fig. 3. Fractionation of *N*-linked oligosaccharides from the *mn1mnn2mnn9* mutant mannoproteins. A: Biogel P-4 chromatography of endo H digested mannoproteins. B: Ion exchange chromatography through QAE-Sephadex of peak II from A. The carbohydrate (○) was determined by the phenol-sulfuric acid method and the NaCl concentration (...) by conductivity.

Purified mannoproteins (0.2 g) from the triple mutant were exhaustively digested with endo H and the released oligosaccharides were fractionated in the BioGel P-4 column

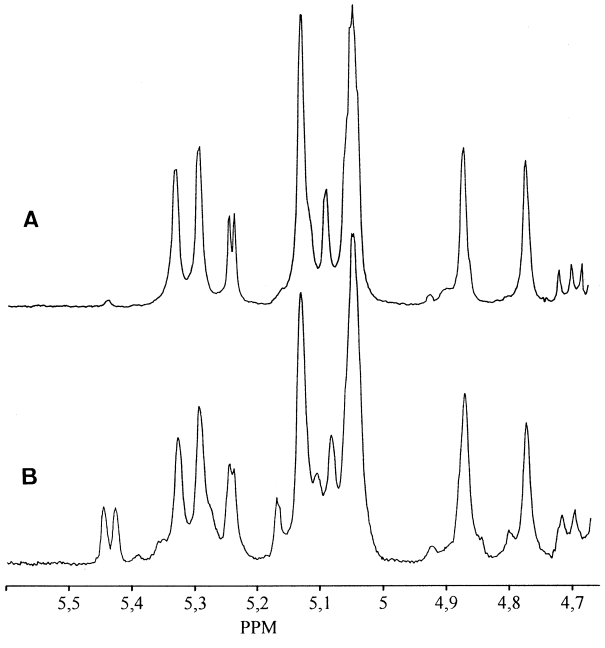
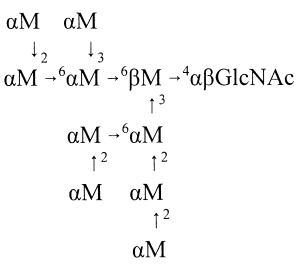


Fig. 4. ^{1}H-NMR spectrum (anomeric proton region) of the endo H released oligosaccharides from the *mnn1mnn2mnn9* mutant mannoproteins. A: Neutral fraction. B: Phosphorylated fraction.

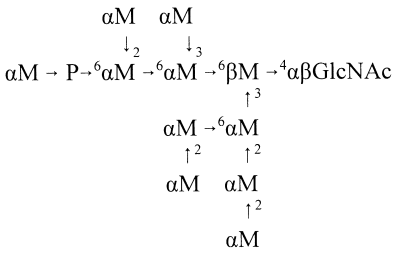
(1.5×170 cm) by elution with 0.1 M ammonium acetate. Fig. 3A shows the results of the chromatography. As in Fig. 1A, peak I was discarded (*O*-linked sugars). The endo H-released material eluted as two well resolved peaks (II and III) that were collected separately, desalted and chromatographed in the QAE-Sephadex column. The reason for the better resolution of these two peaks, as compared with those of Fig. 1A, is that in the case of the triple mutant *mnn1mnn2mnn9* the size of the oligosaccharides is more homogeneous (core-sized) because of the presence of *mnn1* and *mnn9* mutations, and the different elution volume is only determined by the presence of phosphate groups. As expected, the material included in peak II of Fig. 3A was adsorbed to the QAE-Sephadex and eluted as a single peak after application of the NaCl gradient, in the position expected for oligosaccharides with a single diesterified phosphate group (Fig. 3B). The material in peak III of Fig. 3A was not adsorbed to the QAE-Sephadex column which indicates that it includes the neutral oligosaccharides (the chromatography is not shown).

The ^{1}H-NMR spectrum of the unadsorbed oligosaccharides is shown in Fig. 4A. As expected, the spectrum is characteristic of neutral M_{10}-GlcNAc with a structure identical to that found in *mnn1mnn9* [8,11]:



Structure III

The spectrum shown in Fig. 4B confirms that the acidic material that binds to QAE-Sephadex has a single diesterified phosphate group linked to the mannose C_1. The only difference between this spectrum and the one shown in Fig. 4A for the neutral molecule is the presence of the doublet at δ 5.40–5.42 that integrates for one mannose. As we indicated previously (see above) this signal corresponds to the anomeric proton of the mannose that is diesterifying the single phosphate group. The phosphate must be linked to the mannose C_1 because the rest of the signals in the spectrum remain unchanged. The structure is as follows:



Structure IV

The phosphorylated oligosaccharides in *mnn1mnn2mnn9* represent around 20% of the total endo H-released material.

If we compare these results with those previously published for the *mnn1mnn9* double mutant [8] we find two main differences: (a) In the *mnn1mnn9*, the QAE-Sephadex chromatography was able to separate two peaks with one and two diesterified phosphate groups. The presence of the *mnn2* mutation seems to block the synthesis of the diphosphorylated molecules by specifically blocking the transfer of phosphate groups to the mannose A_3 which is the other site for phosphorylation in the core, in addition of mannose C_1 [8] and (b), as we remarked above, in the *mnn1mnn9* the proportion of endo H released molecules with a phosphate group attached to mannose C_1 was close to 40%, while in the case of *mnn1mnn2mnn9* it was only 20%. This difference clearly indicates that the inclusion of the *mnn2* mutation in the *mnn1mnn9* double mutant results in a general decrease of phosphorylation of the inner core. Phosphorylation at C_1 is significantly reduced, while phosphorylation at A_3 is blocked.

It is difficult to explain how a mutation in the gene *MNN2*, that encodes an α(1,2) mannosyltransferase, could affect phosphorylation of the inner core, especially at a particular phosphorylation site. The possibility exists that the defect in phosphorylation could be due to another mutation linked to *mnn2* in the particular strain used for this study. In a previous work [4] we isolated several mutants of *S. cerevisiae* defective in *N*-glycosylation. The mutants were named *ldb* (low dye binding) because they were selected by their reduced affinity for the alcian blue dye as compared to the wild type strain. One of these mutants, *ldb8*, turned out to be allelic to *mnn2*, and we have used it in this study to check for the phosphorylation defect found in the original *mnn2* strain. We constructed the strain *mnn1mnn9ldb8* and analyzed the endo H-released oligosaccharides from purified mannoproteins. The results were indistinguishable from those obtained with the *mnn1mnn2mnn9* (results not shown). From this, we can conclude that the inhibition of phosphorylation at mannose A_3 and the reduction of phosphorylation at mannose C_1 of the inner core of *N*-oligosaccharides is due to the mutation in the *MNN2* gene. We must discard the participation of any other mutation since it is very improbable that the same two mutations (*mnn2-X* or *ldb8-X*) occur in each of the two selected cells in two different mutagenesis experiments. On the other hand, there is nothing strange in single mutations which affect the secretion–glycosylation pathway resulting in more than one detectable phenotypic change [4,16,17]

In other previously described mutants that are affected in the phosphorylation of *N*-oligosaccharides (*mnn4*, *mnn6, ldb1* and *ldb2*), there has been no description of any detectable differential effect of the mutations on the two phosphorylation sites of the inner core [4,8]. In Table 1 we give a summary of data concerning the effect of the mentioned mutations on the addition of mannose-phosphate groups to the mannoses C_1 and A_3 of the inner core of *N*-linked oligosaccharides.

In recent years, the work by Jigami and co-workers (reviewed in [5]) has established the complexity of the system involved in phosphorylation of *N*-linked sugars. Most probably several mannosylphosphate transferases and regulatory proteins form complexes located in the Golgi apparatus that are responsible for the transfer of the phosphate groups. It is possible that different complexes are responsible for the transfer of mannosyl-phosphate groups to the two phosphorylation sites in the inner core, and that the Mnn2p participates in some way mainly in the complex involved in the phosphorylation of mannose A_3. Indeed, it has been definitely proved that the *MNN2* gene encodes an α(1,2) mannosyltransferase

that seems not to function as part of any of the two Golgi complexes of mannosyltranferases that are involved in the elongation of the outer chain [7].

Another possible way to explain the *mnn2* phenotype is related to the fact that in the *N*-linked oligosaccharides of *S. cerevisiae* there are two sequences susceptible of incorporating Man-P groups (in the asterisked mannoses): αMan1→2αM*1→6αM1→ and αMan1→2αM*1→2αM1→ [8]. The former corresponds to mannose C_1 in the inner core and is also present at the non-reducing end of the α(1,6) backbone of the outer chain in the wild type. In the *mnn2* mutant the synthesis of this sequence is not affected, and in both cases the mannose can be phosphorylated. The second sequence, which corresponds to mannose A_3 in the core, in the wild type is also present in most of the branches of the outer chain where it can also be phosphorylated. However, in the *mnn2* mutant, the outer chain is unbranched, which means that the only acceptor sequence present is the small proportion which is in the inner core. The possibility exists that the presence of a large amount of the acceptor sequences in the branches of the wild type exerts a stimulatory effect on the mannosylphosphate transferase, or on the complex responsible for phosphorylation in this particular acceptor sequence. The total absence of branches in the outer chain of the *mnn2* mutant would imply the absence of such a stimulatory effect and could be the reason for the absence of phosphorylation in mannose A_3 of the core but not in mannose C_1. If this were the case, there would be no need for a direct interaction of the Mnn2p in the hypothetical Golgi complexes involved in phosphorylation of the *N*-linked oligosaccharides.

A more plausible explanation for the *mnn2* mutant phenotype comes from the fact that the enzymes involved in the synthesis of the cell wall components seem to interact with signal-transduction pathways in such a way that when a component of the cell wall is defective, the cell tries to compensate for the defect through these pathways by inducing changes in the synthesis of other components [18]. In this context, it would seem reasonable for the cell's economy that if the Mnn2p is not working, the protein(s) involved in further modification of the structures synthesized by Mnn2p (i.e. addition of Man-phosphate groups) do not need to be active or even translated. A direct interaction between the product of the gene *CRV4/TTP1* and the mitogen-activated protein kinase (MAPK)-mediated pathways has been described [19]. A deletion in the gene *CRV4/TTP1* showed synthetic lethality in combination with a deletion in *MPK1*. In a more recent paper [7] the *MNN2* gene was sequenced and turned out to be identical to *CRV4/TTP1*.

Finally, the decrease in phosphorylation of mannose C_1 could also be explained by the relatively low specificity of some enzymes involved in the biosynthesis of protein-linked oligosaccharides of *S. cerevisiae*. A well-known example is the case of the members of the *KTR* gene family (reviewed in [20]). A number of proteins coded by members of this family (Ktr1p, Ktr2p, Ktr3p, Kre2p and Yur1p) participate in various steps of the biosynthesis of both *N*- and *O*-linked oligosaccharides, but the degree of participation of the proteins varies from one step to another. It is possible that the Mnn2p dependent complex, or complexes, involved in the transfer of Man-phosphate groups to mannose A_3, also participates to a lesser degree in the transfer of the same groups to mannose C_1.

Further work will be necessary to fully understand the complexity of the *N*-linked oligosaccharide phosphorylation system in *S. cerevisiae*. The demonstration that the *mnn2* mutation definitely affects the phosphorylation process is a new insight that contributes to increase this complexity even more.

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References

[1] Wang, X.H., Nakayama, K., Shimma, Y., Tanaka, A. and Jigami, Y. (1997) J. Biol. Chem. 272, 18117–18124.
[2] Odani, T., Shimma, Y., Tanaka, A. and Jigami, Y. (1996) Glycobiology 6, 805–810.
[3] Odani, T., Shimma, Y., Wang, X.H. and Jigami, Y. (1997) FEBS Lett. 420, 186–190.
[4] Mañas, P., Olivero, I., Avalos, M. and Hernández, L.M. (1997) Glycobiology 7, 487–497.
[5] Jigami, Y. and Odani, T. (1999) Biochim. Biophys. Acta 1426, 335–345.
[6] Ballou, C.E. (1990) Methods Enzymol. 185, 440–470.
[7] Rayner, J.C. and Munro, S. (1998) J. Biol. Chem. 273, 26836–26843.
[8] Hernández, L.M., Ballou, L., Alvarado, E., Tsai, P-K. and Ballou, C.E. (1989) J. Biol. Chem. 264, 13648–13659.
[9] Kassir, Y. and Simchen, G. (1991) Methods Enzymol. 194, 94–110.
[10] Ballou, L., Alvarado, E., Tsai, P.-K., Dell, A. and Ballou, C.E. (1989) J. Biol. Chem. 264, 11857–11864.
[11] Hernández, L.M., Ballou, L., Alvarado, E., Gillece-Castro, B.L., Burlingame, A.L. and Ballou, C.E. (1989) J. Biol. Chem. 264, 11849–11856.
[12] Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. and Smith, F. (1956) Anal. Chem. 28, 350–356.
[13] Cohen, R.E. and Ballou, C.E. (1980) Biochemistry 19, 4345–4358.
[14] Hernández, L.M., Olivero, I., Alvarado, E. and Larriba, G. (1992) Biochemistry 31, 9823–9831.
[15] Nakajima, T., Maitra, S.K. and Ballou, C.E. (1976) J. Biol. Chem. 251, 174–181.
[16] Ballou, L., Hitzeman, R.A., Lewis, M.S. and Ballou, C.E. (1991) Proc. Natl. Acad. Sci. USA 88, 3209–3212.
[17] Mañas, P., Olivero, I. and Hernández, L.M. (1998) Biochim. Biophys. Acta 1380, 320–328.
[18] Ram, A.F., Kapteyn, J.C., Montijn, R.C., Cano, L.H.P., Douwes, J.E., Baginsky, W., Mazur, P., Van den Ende, H. and Klis, F.M. (1998) J. Bacteriol. 180, 1418–1424.
[19] Nakamura, T., Ohmoto, T., Tsuchiya, E. and Miyakawa, T. (1997) Mol. Gen. Genet. 256, 481–487.
[20] Lussier, M., Sdicu, A.M. and Bussey, H. (1999) Biochim. Biophys. Acta 1426, 323–334.