Identification of the MNN3 gene of *Saccharomyces cerevisiae* †

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The MNN3 gene of *Saccharomyces cerevisiae* has been identified as a synonym of VPS74. We have compared phenotype characteristics of the original mnn3 mutant, including low dye binding phenotype, size of external inverrate, clump formation, and sodium orthovanadate resistance and found these to be identical to those shown by vps74Δ. Mating of both haploid strains resulted in non-complementation of mutant phenotypes. Finally, a vector containing wild-type VPS74 complemented the defects of both vps74Δ and mnn3. This work completes the identification of the entire collection of genes that are defective in *mnn* mutants. In addition, we have identified the mnn3 mutation by sequencing the gene from the original mnn3 strain. We found a single amino acid change of Arg97 to Cys. This unique alteration seems to be sufficient to account for the phenotype of mnn3.

Keywords: glycosylation/mannosyltransferase localization/MNN3/Saccharomyces cerevisiae/VPS74

Introduction

The mnn (mannan-defective) mutants of *Saccharomyces cerevisiae* were isolated by Ballou’s group as a tool for the study of the structure and biosynthesis of mannoprotein-linked oligosaccharides (reviewed in Ballou 1990). Following mutagenesis with ethylmethane sulfonate, the mutants were selected for the loss of agglutinability with anti-wild-type antisera. In consequence, they showed alterations in the cell surface, and some of them also showed a low dye-binding (ldb) phenotype since they lost the ability to bind alcian blue dye (Mañas et al. 1997). The phenotype of the mnn mutants was extensively studied by Ballou and co-workers, resulting in the elucidation of the final structure of the N-linked oligosaccharides present in the manno-proteins of *S. cerevisiae* (Hernandez et al. 1989; Ballou et al. 1990). Since then, most MNN genes have been identified. MNN1 encodes an integral membrane glycoprotein of the Golgi complex, required for addition of terminal alpha(1,3)-linked mannoses to N-linked and O-linked oligosaccharides (Yip et al. 1994). The MNN2 gene encodes a protein with alpha(1,2) mannosyltransferase activity (Rayner and Munro 1998), which catalyzes the transfer of the first manno in the branches of the outer chain. Mnn5p has a similar activity and is responsible for the transfer of the second alpha(1, 2)-linked mannoside in the branches (Rayner and Munro 1998). The mnn4 and mnn6 were described as defective in the incorporation of mannosyl phosphate units into the N-linked carbohydrate chains (Ballou et al. 1973; Karson and Ballou 1978; Odani et al. 1996). Jigami and co-workers identified the genes MNN4 (Odani et al. 1996) and MNN6 (Wang et al. 1997). They postulated that MNN6 encoded a mannosyl phosphate transferase, while MNN4p seemed to have a regulatory role.

The mnn7 and mnn8 were found to be allelic (Ballou et al. 1989), and they showed defects in the construction of the outer chain, a similar phenotype to that shown by mnn9 and mnn10 (reviewed in Ballou 1990). The genes encode subunits of the Golgi-located mannan polymerases (M-Pol) I and II involved in elongation of the outer chain of N-linked oligosaccharides (Jungmann and Munro 1998; Jungmann et al. 1999). In that work, Munro and co-workers identified a new subunit of M-Pol II encoded by an open reading frame (ORF) that they named MNN11.

To the best of our knowledge, MNN3 is the only MNN gene not yet identified. The mnn3 mutant was initially described as defective in the incorporation of alpha(1, 2)-linked mannoses to the branches in the outer chain of N-linked oligosaccharides (Ballou et al. 1973; Raschke et al. 1973; Cohen et al. 1980). It was found later that mannan isolated from mnn3 mutant strains was phosphate-deficient (Karson and Ballou 1978). The conclusion of these and other later studies was that the mnn3 defect could not be explained by the loss of a single mannosyl transferase since it led to a general shortening of the carbohydrate chains of the mannoproteins, affecting both N-linked and O-linked chains. Actually, it was suggested that the Mnn3p could have a regulatory function that simultaneously alters the activities of several Golgi-located mannosyltransferases (Cohen et al. 1980; Ballou 1990).

In this work, we have found that the mnn3 mutant is allelic to vps74 and that the mnn3 defect is complemented by VPS74. In consequence, MNN3 must be considered a synonym of VPS74. In a genomic screen, the Vps74p was involved in vacuolar protein sorting (Bonangelino et al. 2002). Later, it was found that Vps74p belongs to a family of cytosolic Golgi-associated...
proteins, suggesting that it may play a role in secretion (Huh et al. 2003). Finally, more recent studies (Schmitz et al. 2008; Tu et al. 2008) demonstrate the function of Vps74p in Golgi retention of glycosyltransferases.

In addition, the mnn3 mutation has been characterized by sequencing the VPS74 gene from the original mnn3 strain.

Results and discussion
Identification of the MNN3 gene

The ldb phenotype is a consequence of a reduction in the amount of mannosylphosphate groups attached to N-linked oligosaccharides (Mañas et al. 1997). The mnn3 mutant has a severe ldb phenotype as revealed by alcian blue staining. To identify the defective gene in the mutant, we followed the same method as previously used in the identification of other ldb mutants (Corbacho et al. 2004). The strain S. cerevisiae mnn3 ura3 was transformed with the CEN BANK genomic library, and the non-ldb transformants were selected by adsorption onto QAE beads. The insert of the complementing plasmid was sequenced and identified by checking the sequence in the Saccharomyces Genome Database. Figure 1 shows that it corresponds to a fragment of chromosome IV with two incomplete and eight complete ORFs.

Since mnn3 is a non-conditional mutant, to identify the ORF responsible for complementation, we first discarded the essential FRQ1 and ARH1, as well as the incomplete CTS2 and RGA2. Then we checked the phenotype of single deletion strains on each of the remaining six ORFs. Figure 2 shows that vps74A showed a severe ldb phenotype (panel A) and a heterogeneous invertase migration pathway (panel B). Both characteristics were indistinguishable from those shown by mnn3.

The strains with deletions in the remaining five ORFs showed both invertase migration pattern and alcian blue staining intensity similar to the wild type (result not shown). In addition, vps74A showed a slightly clumsy phenotype, also identical to mnn3 (result not shown) and an extra sensitivity to the presence of sodium orthovanadate in the culture medium. It was previously described that mutants with defects in protein glycosylation also show a resistance to sodium orthovanadate causing a general shortening of carbohydrate chains, both O- and N-linked, and a reduced mannosylphosphorylation (Karson and Ballou 1978; Ballou 1990). These multiple phenotypes could not be explained by the lack of a single transerase (Cohen et al. 1980; Ballou 1990). In the light of the function assigned to Vps74p (Schmitz et al. 2008; Tu et al. 2008), the previously described mnn3 phenotype is entirely compatible with a defect in VPS74.

Identification of the mnn3 mutation

The crystal structure of Vps74p reveals a single globular domain, predominantly alpha-helical (Schmitz et al. 2008). It forms a central core with four helices (alpha 1, alpha 2, alpha 3, and alpha 12), which is surrounded by solvent-exposed loops and eight amphipathic helices. An important feature is the existence of a beta-hairpin projected away from the helical domain, which is critical for the formation of the biologically active tetramer. In contrast, a variant lacking the first 59 amino acids crystallized in the same crystal form as the complete pro-
tein, suggesting that they are in some way not essential for the protein function.

Bearing this in mind, we sequenced the VPS74 gene of the original mnn3 strain to localize and define the mutation responsible for the mnn3 phenotype. This information might be of interest in order to establish a more precise structure–function relationship for the Vps74p.

The VPS74 was PCR-amplified from genomic DNA of the mnn3 strain. The PCR-generated fragments were integrated in pGEM-T, which was used to transform competent E. coli cells. The inserts of pGEM-T, purified from several E. coli colonies, were sequenced (see Materials and methods). All analyzed samples showed a single base change in position 289 of the coding sequence of VPS74, consisting of substitution of the original C in that position by T, resulting in the codon TGT instead of CGT. In the amino acid sequence, this mutation resulted in the substitution of Arg97 by a Cys.

The isolation and phenotype analysis of mnn mutants by Ballou’s group in the 1970s and 1980s made an essential contribution to the elucidation of the structure and biosynthesis of protein-linked oligosaccharides in S. cerevisiae. The later identification of mutated MNN genes largely contributed to the advance in the elucidation of the complete glycosylation pathway of the yeast. However, the molecular basis of the mnn3 phenotype remained elusive for decades, with mnn3 being the only mutation not identified among the collection of mnn mutants. The work presented here fills this gap in the literature and gives the link between the defective mannan phenotype of the mnn3 mutant and the function of the VPS74 gene in retaining Golgi-localized glycosyltransferases.

Materials and methods

Strains and growth conditions
Saccharomyces cerevisiae mnn3 MAT a (LB54-3A) was a generous gift of Lun and Clint Ballou during a postdoctoral stay of L.M.H. in their laboratory at the University of California in Berkeley. S. cerevisiae BY4741 (MAT a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0), BY4742 (MAT α; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0), and the complete collection of haploid deletion strains (MATα and MATα) were obtained from EUROSCARF.
Cells were grown at 30°C in solid or liquid YPD (1% yeast extract, 2% peptone, and 2% d-glucose). To de-repress external invertase synthesis, the amount of glucose was lowered to 0.1%. When indicated, YPD was supplemented, after autoclaving, with filter-sterilized sodium orthovanadate (Sigma).

Drop-test/spotting assay: Growth phenotypes of strains incubated in the presence of sodium orthovanadate were tested on solid media as in Cottier et al. (2006). Briefly, yeast strains were grown overnight in standard liquid YPD to the early exponential phase of growth. Then, cells were washed and resuspended in fresh YPD to an OD$_{600}$ of 1. Tenfold serial dilutions were prepared, and 10 μl aliquots were spotted onto YPD plates containing increasing concentrations of sodium orthovanadate (3, 4, 5, and 6 mM). Plates were incubated at 30°C for 3 to 4 days.

Sporulation was done following standard published protocols (Kassir and Simchen 1991). To identify strains with the deleted gene (kanMX module), YPD was supplemented with G418 (Sigma) at a concentration of 200 μg/mL.

Escherichia coli DH5α competent cells were from Invitrogen.

Plasmids

The CEN BANK genomic library, constructed in YCp50, was used (Rose et al. 1987). Clones had an insert size ranging from 10 to 20 kb. YCp50 carried Amp$^R$ and URA3 markers for selection in E. coli and S. cerevisiae. pRS426-VPS74 was constructed by T-A cloning in the pRS426 (Christianson et al. 1992) Smal site, of a polymerase chain reaction (PCR) fragment containing the coding sequence plus 1 kb up- and downstream of VPS74. The sequence of VPS74 in the original mnn3 mutant was cloned using the same strategy but in pGEM-T vector (Promega). Sequencing was done by StabVida (Caparica, Portugal).

Transformation and selection

Transformation was done by the lithium acetate method (Gietz and Woods 2006). Selection of mnn3 transformants that have recovered the wild-type phenotype was done as described previously (Mañas et al. 1997; Corbacho et al. 2004). Briefly, the transformants were mixed with QAE-Sephadex beads at pH 3. Cells that have recovered the negative charge on the cell surface have also recovered the ability to bind to the beads. The unbound cells were discarded, and the bound cells were released by washing the beads with 2 M NaCl. The released cells were washed with 0.9% NaCl and spread onto SD–ura plates to obtain individual colonies. Finally, the colonies were stained with alcian blue to assess their recovery of the wild-type phenotype.

Other methods

To determine the extent of mannosylphosphorylation, we used alcian blue staining, as described previously (Mañas et al. 1997; Corbacho et al. 2004, 2005). To check growth at permissive and restrictive temperatures, all strains were grown on YPD plates in a spotting assay. Native gel electrophoresis of secreted invertase (Ballou 1990; Mañas et al. 1997) was used to analyze the degree of underglycosylation.

Micromanipulation and dissection of yeast asci was done with the aid of a Tetrad dissection system from Micro Video Instruments (Avon, MA, USA).

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Conflict of interest statement

None declared.

Abbreviations

ldb, low dye-binding; M-Pol, mannan polymerases; ORF, open reading frame; PCR, polymerase chain reaction

Note added in proof

A recent study (Dippold HC, Ng MM, Farber-Katz SE, Lee SK, Kerr ML, Peterman MC, Sim R, Wiharto PA, Galbraith KA, Madhavarapu S, Fuchs GJ, Meerloo T, Farquhar MG, Zhou HL, Field SJ, 2009. GOLPH3 bridges phosphatidylinositol-4-phosphate and actomyosin to stretch and shape the Golgi to promote budding. Cell 139:337-351) reported that Vps74p (and the Golgi protein GOLPH3 in higher organisms) binds to PtdIns(4)P. The mutation we identified in mnn3 (R97C) is in the same residue required to ligate the 4-phosphate in PtdIns(4)P (Arg97 in yeast, Arg90 in the human GOLPH3 protein). When that residue was mutated in the GOLPH3 gene (R90L) it abolished the interaction of GOLPH3 protein with PtdIns(4)P and disrupted localization of GOLPH3 to the Golgi. The data imply that the mnn3 mutant phenotype may be due to the mislocalization of mutated Vps74p because of its lack of interaction with PtdIns(4)P.

References


