Eukaryotic glycan structures are progressively elaborated in the secretory pathway. Following the addition of a core N-linked carbohydrate in the endoplasmic reticulum, glycoproteins move to the Golgi complex where the elongation of O-linked sugar chains and processing of complex N-linked oligosaccharide structures take place. In order to better define how such post-translational modifications occur, we have been studying a yeast gene family in which at least one member, KRE2/MNT1, is involved in protein glycosylation. The family currently contains five other members: YUR1, KTR1, KTR2, KTR3 and KTR4 (Mallet, L., Bussereau, F., and Jacquet, M. (1994) Yeast 10, 819–831). All encode putative type II membrane proteins with a short cytoplasmic N terminus, a membrane-spanning region, and a highly conserved catalytic lumenal domain.

Kre2p/Mnt1p is a α1,2-mannosyltransferase involved in O- and N-linked glycosylation (Häusler, A., Ballou, L., Ballou, C. E., and Robbins, P. W. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 6846–6850); however, the role of the other proteins has not yet been established. We have carried out a functional analysis of Ktr1p, Ktr2p, and Yur1p. By in vitro assays, Ktr1p, Ktr2p, and Yur1p have been shown to be mannosyltransferases but, in vivo, do not appear to be involved in O-glycosylation. Examination of the electrophoretic mobility of the N-linked modified protein invertease in null mutant strains indicates that Ktr1p, Ktr2p, and Yur1p are involved in N-linked glycosylation, possibly as redundant enzymes. As found with Kre2p (Hill, K., Boone, C., Goebi, M., Puccia, R., Sdicu, A.-M., and Bussey, H. (1992) Genetics 130, 273–283), Ktr1p, Ktr2p, and Yur1p also seem to be implicated in the glycosylation of cell wall mannanproteins, since yeast cells containing different gene disruptions become K1 killer toxin-resistant. Immunofluorescence microscopy reveals that like Kre2p; Ktr1p, Ktr2p and Yur1p are localized in the Golgi complex.

The covalent addition of glycans to secretory and membrane proteins constitutes one of the major post-translational modifications known to occur in eukaryotes. The biosynthetic pathway leading to N-glycosylation has been studied in considerable detail and involves the ordered assembly of a core oligosaccharide on the lipid carrier dolichol phosphate, which is embedded in the ER I membrane. Once this oligosaccharide has been completed, it is transferred onto specific asparagine residues of proteins and subsequently altered by specific glycosidases and glycosyltransferases. The elaboration and initial processing of N-linked oligosaccharides in the ER are similar in all eukaryotes, but subsequent phases of glycosylation are different in a broad range of organisms (Tanner and Lehle, 1987; Herscovic and Orlean, 1993; Knauer and Lehle, 1994; Lehle and Tanner, 1995).

In the yeast Saccharomyces cerevisiae, the N-linked core oligosaccharide is mainly constituted of Man₆GlcNAC₂ and may undergo Golgi maturation resulting in Man₈GlcNAC₂. In other cases, glycoproteins traversing the Golgi have their core oligosaccharide extended by outer chains containing up to 200 mannose residues (Ballou, 1990; Herscovic and Orlean, 1993; Lehle and Tanner, 1995). Protein N-glycosylation appears essential for cell function since mutants of S. cerevisiae lacking protein subunits of the core oligosaccharyltransferase or mutants defective in the synthesis of the dolichol pyrophosphate-oligosaccharyl precursor are not viable (Huffaker and Robbins, 1982; teHeesen et al., 1992, 1993; Stagljar et al., 1994; Keléher and Gilmore, 1994), although the biochemical basis of this lethality remains unclear (Tanner and Lehle, 1987; Lehle and Tanner, 1995).

The structure and biosynthesis of O-linked carbohydrate chains attached to serine and threonine show considerable evolutionary diversity. The primary reaction in the modification of mammalian O-linked proteins involves the attachment of a GalNAc that has been transferred from UDP-GalNAc within the Golgi (Roth, 1984). The carbohydrate chains of mammalian O-linked modified proteins are variable in length and composition and include galactose, sialic acid, fucose, GalNAc, and GlcNAc (Elhammer and Kornfeld, 1984; Roussel et al., 1988; Jen et al., 1990; Krijnse Locker et al., 1992). In contrast, it has been demonstrated that in S. cerevisiae O-modified proteins possess a linear carbohydrate chain consisting of up to 5 mannose residues (Tanner and Lehle, 1987; Herscovic and Orlean, 1993; Lehle and Tanner, 1995).

Some of the structural genes coding for yeast mannosyltransferases have been isolated. OCH1 encodes the first α1,6-mannosyltransferase involved in initiating outer chain elaboration (Nakayama et al., 1992; Nakaniishi-Shindo et al., 1993). KRE2/MNT1 is the only known α1,2-mannosyltransferase gene isolated to date (Häusler and Robbins, 1992) and is implicated in

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The abbreviations used are: ER, endoplasmic reticulum; bp, base pair(s); kb, kilobase pair(s); CTAB, cetyltrimethyl ammonium bromide; DAPI, 4',6-diamidino-2-phenylindole; Ab, antibody; GPI, glycosyl phosphatidylinositol.
N-linked outer chain oligosaccharide synthesis (Hill et al., 1992) and is also responsible for the addition of the third mannosic residue of O-linked carbohydrate chains (Häusler et al., 1992). Outer chain and core modified oligosaccharides are brought to completion by the action of a terminal α(1,3)-mannosyltransferase encoded by the MNN1 gene and similarly to KRE2p/Mnt1p, Mnn1p also mannosylates O-linked glycans (Ballou, 1990; Yip et al., 1994).

To examine how post-translational modifications occur in Saccharomyces cerevisiae and to further define the responsible enzymes, we have functionally characterized three members of the KRE2/MNT1 putative mannosyltransferase gene family. This growing gene family was known to contain KRE2/MNT1, YUR1, KTR1, KTR2 (Häusler and Robbins, 1992; Lussier et al., 1993), and recently two other homologues, KTR3 and KTR4, have been found by the yeast genome project (Mallet et al., 1994). These genes are predicted to encode type II membrane proteins with a short cytoplasmic N terminus, a membrane-spanning region, and a highly conserved catalytic lumenal domain. While the precise role of KRE2p/Mnt1p as a mannosyltransferase in O-glycosylation has been established (Häusler and Robbins, 1992; Häusler et al., 1992), the role of the other genes remains to be determined. We have carried out a functional analysis of Yur1p, Ktr1p, and Ktr2p and demonstrate that they are mannosyltransferases involved in N-linked glycosylation.

Experimental Procedures

Yeast Strains, Culture Conditions, and Methods—All yeast constructions used strain SEY6210 (MATa, leu2-3, 112, his3-A200, lys2-801, trp1-901, suc2-3). Yeast cells were grown under standard conditions (yeast extract peptone dextrose, Yeast Nitrogen Base selective media containing 5% (v/v) glycerol, 2% glucose using Amicon Centriprep concentrators (W. R. Grace & Co., Danvers, MA). Proteins were then separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Schleicher & Schuell). Blots were treated in TBST buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween 20, 5% nonfat dried milk powder) and subsequently incubated in TBST buffer with affinity purified anti-Kre9p or anti-invertase antibodies. After antibody binding, membranes were washed in TBST buffer and a second antibody directed against rabbit immunoglobulins and conjugated with horseradish peroxidase, was then added. The blots were again washed and proteins detected using an enhanced chemiluminescence procedure (Amersham Canada, Oakville, Ontario).

Mannan Acceptor Preparation—The mannan protein acceptors were prepared from yeast cells according to a modification of the method of Ballou (1990). Pelleted cells from a 200-ml culture of quadruple mutant yeast strains lacking invertase and the KRE2, YUR1, and MNT1 genes grown in Yeast Nitrogen Base media were washed once with 0.9% NaCl, then with water, and the paste was resuspended in 50 ml of 0.02 M sodium citrate buffer, pH 6.8, and autoclaved for 90 min. After cooling and low speed centrifugation, the supernatant was kept, and the gelatinous solid pellet was re-extracted with citrate buffer. The supernatants were combined and concentrated by means of a 0.45-mm filter, and 2 ml of 10% trichloroacetic acid in water was added. The pH of the solution was then adjusted to 8.8 with KOH, and precipitation of the mannan-protein-CTAB complex was left to proceed overnight at room temperature. The solution was then centrifuged, the pellet was washed with 0.5% sodium borate buffer at pH 8.8 and dissolved in 12 ml of 2% acetic acid to dissociate mannan-proteins from CTAB. Finally, the mannan was precipitated in three volumes of ethanol, washed once with 2% acetic acid in ethanol, then once in ethanol, and dissolved in water. The solution was dialyzed overnight at room temperature against water, then lyophilized. For use as acceptor in enzyme reactions, the mannan fraction was resuspended at a concentration of 10 mg/ml in 50 mM Hepes, pH 7.2, 10 mM MnCl2, and 0.1% Triton X-100.

Preparation of Membranes and Assay of Mannosyltransferase Activity in Vitro—Mannosyltransferase activity assays were performed essentially as described (Lewis and Ballou, 1991; Häusler and Robbins, 1992; Lussier et al., 1995b). Briefly, reactions were carried out in a solution containing yeast membranes and consisting of 50 mM Hepes, pH 7.2, 10 mM MnCl2, and 0.1% Triton X-100. Values of specific mannosyltransferase activities (see Fig. 6) are expressed in dpm for a 10-min reaction and for 10 μg of membrane proteins. Results represent the average of three independent determinations.

Preparation of Antibodies—Invertase antisera was obtained as described (Hill and Ballou, 1991). Rabbit antisera against a recombinant protein corresponding to the N-terminal half of invertase, Briefly, a DNA fragment was excised with SmaI and XbaI from pSEY304 (kindly provided by Dr. T. H. Stevens, Institute of Molecular Biology, Eugene, OR), cloned into plasmid pEXP2, and expressed in Escherichia coli. Ktr1p antibodies were raised in rabbits against a synthetic peptide, the first 14 amino acid residues of the protein (NH2-NKLPKPGWNHIGCOOH; obtained from the Sheldon Biotechnology Centre, McGill University, Montréal, Québec, Canada). Initially, rabbits were injected with 500 μg of conjugated peptide in Freund's complete adjuvant, followed by three subsequent injections with equivalent amounts of peptide in Freund's incomplete adjuvant at 3–4-week intervals. The conjugated peptides were coupled to cyanogen bromide-activated Sepharose CL-6B (Pharmacia Biotech Inc., Montréal, Québec, Canada) and used in a column to affinity-purify the antisera as described by
Kre2p, the possible role of the 37–38% identity at the amino acid level. However, both proteins are the most diverged from Kre2p, with just 62% identity. How-
uniquetoeachprotein(forsequencecomparison,seeLussier 1992; Kleene and Berger, 1993; Lussier 1993). The conserved region in the Kre2p family encompasses a
domain to the membrane-spanning region (Shaper and Shaper, 1995b) (see Fig.
1). The observed region in the Kre2p family encompasses a large central region, with the N- and C-terminal portions being unique to each protein (for sequence comparison, see Lussier et al. (1993) and Mallet et al. (1994)). Yur1p and Ktr2p are the most similar members of the family, with 62% identity. How-
ever, both proteins are the most diverged from Kre2p, with just 37–38% identity at the amino acid level.

In view of their sequence and structural similarities with Kre2p, the possible role of the KTR1, KTR2, and YUR1 gene products as protein mannosyltransferases was analyzed. One-
step gene replacements were carried out using different marker genes (see “Experimental Procedures” and Fig. 2). KRE2 and KTR2 single gene disruptions were previously shown to have no growth phenotypes at 30 °C (Häusler et al., 1992; Lussier et al., 1993). Analysis of spore progeny derived from SEY6210 ktr1::LYS2 or yur1::HIS3 heterozygotes showed that neither gene was essential for cell viability nor were they required for normal vegetative cell growth. To assess whether a haploid strain carrying deletions in several of these genes possessed a more severe phenotype, double, triple, and quadruple disruptions were sequentially constructed using standard genetic techniques. Meiotic tetrads segregating combinations of these disrupted genes were dissected and haploid spore progeny grown at 30 °C. Haploid strains harboring ktr1::LYS2 ktr2::URA3 yur1::HIS3 triple null mutations or haploids carrying a set of four disruptions were viable and did not grow noticeably slower than wild type cells. 2

Ktr1p, Ktr2p, and Yur1p Have No Apparent Role in O-Man-
nosylation—To examine whether Ktr1p, Ktr2p, and Yur1p are involved in O-linked glycosylation, an analysis of O-modified glycoproteins was made from yeast strains carrying different null mutations. O-Linked carbohydrate chains were specifi-
cally released from the glycoprotein fraction of in vivo [3H]-mannose-labeled yeast cells by β-elimination and resolved by chromatography. The wild type strain showed the normal profile of five oligosaccharide peaks (Fig. 3, Man1-Man5). The pattern obtained from the ktr2 null strain gave two peaks (Man1 and Man2), consistent with failure to add the third α1,2-linked mannose residues in this mannosyltransferase-defective mutant. A ktr2 null mutation was previously shown to possess a wild type pattern of five oligosaccharide peaks (Lussier et al., 1993). The ktr1 and yur1 single null mutant strains also gave a wild type pattern of five mannose peaks as did the ktr1 ktr2 yur1 triple null mutant (Fig. 3), providing no evidence for their involvement in O-linked chain elaboration.

The extent of O-glycosylation in yeast strains with mutations in these genes was also analyzed by measuring the mobility of a yeast O-glycoprotein, Kre9p. Kre9p is an extracellular matrix protein involved in cell wall assembly that is extensively O-
mannosylated but lacks N-linked modifications (Brown et al., 1993). When synthesized in a wild type strain, Kre9p migrates at an apparent mass of 55 kDa. As expected, Kre9p isolated from a ktr2 null strain migrated more quickly than did the wild type Kre9p, with an apparent molecular mass of approximately 47 kDa (Fig. 4). However, Kre9p produced by ktr1, ktr2, or yur1 single null disruptants or by a triple null mutant strain was indistinguishable from that produced by a wild type strain.

Role of Ktr1p, Ktr2p, and Yur1p in N-Glycosylation—The Ktr2p O-mannosyltransferase is also involved in the elaboration of N-linked carbohydrate chains (Hill et al., 1992); conse-
sequently, the effect of KTR1, KTR2 and YUR1 gene disruptions on invertase N-glycosyl modifications was analyzed. The prod-
The product of the SUC2 gene, invertase, is a specifically N-modified protein, which is extensively glycosylated (Orlean, 1991; Ziegler, 1988). Three different classes of invertase can be distinguished: 1) a cytoplasmic form (60 kDa), which lacks a signal sequence and is therefore not glycosylated; 2) a transient ER form, which is heterogeneous in size (80–90 kDa), as a consequence of the number of core oligosaccharide chains that are attached to the protein; and 3) a secreted form, which constitutes an array of differently modified proteins (>100 kDa) resulting in elongation from the core oligosaccharide of outer chain glycans in the Golgi complex (see Fig. 5).

As found previously (Hill et al., 1992), invertase synthesized in a kre2 null mutant has a molecular mass (~137 kDa) that is smaller than the secreted wild type protein (~145 kDa). In contrast, the carbohydrate chains of invertase produced in ktr1, ktr2, or yur1 single null mutants appear to be intact, as the molecular mass of the protein made in these strains is wild type (~145 kDa). Similarly, in ktr1 ktr2, ktr1 yur1, or ktr2 yur1 double null mutants, no obvious reduction in size of invertase was apparent. However, invertase synthesized in a ktr1 ktr2 yur1 triple null mutant possessed a molecular mass of approximately ~127 kDa. Invertase was smallest (~120 kDa) when produced in a quadruple kre2 ktr1 ktr2 yur1 mutant, indicating a cumulative involvement of all four proteins in N-linked modifications.

Mannosyltransferase Activity of Ktr1p, Ktr2p, and Yur1p—In parallel to the in vivo glycosylation studies, an analysis of the in vitro enzymatic activity of Ktr1p, Ktr2p, and Yur1p in carbohydrate chain elaboration was performed by measuring the transfer of [14C]Man residues from GDP-[14C]Man to a specific acceptor. To reduce the possible background mannosyltransferase activity, the enzymatic source for the in vitro assays consisted of membrane preparations from a kre2 ktr1 ktr2 yur1 quadruple null strain in which each of the genes was individually overexpressed (Fig. 6). In one assay, α-methylmannoside was used as an acceptor. A second assay used mannoprotein...
prepared from a kre2 ktr1 ktr2 yur1 quadruple null strain as an acceptor. If these mutant acceptor proteins are incompletely mannosylated, they may allow detection of an expanded range of mannosyltransferase activities. Using ω-methylmannoside, a ktr2 disruptant possesses about 38% residual activity when compared to that of wild type, an activity consistent with that found previously (Häusler et al., 1992). A strain carrying deletions of all four genes showed diminished enzymatic activity corresponding to about 18% of that found in a wild type, indicating these deleted genes contribute to the total mannosyltransferase activity. The enzymatic activity of YUR1, KTR1, and KTR2 was subsequently assessed (Fig. 6). Using ω-methylmannoside as an acceptor, an extract from the quadruple null strain overexpressing Kre2p showed an elevated (6.2-fold) enzymatic activity compared with an extract from the quadruple null strain, YUR1, when overexpressed, also displayed increased enzymatic activity (5.5-fold), demonstrating that Yur1p is a mannosyltransferase. However, using this acceptor, extracts from strain overexpressing KTR1 and KTR2 displayed activity levels equivalent to that of background. To attempt to detect mannosyltransferase activities using an alternative assay, a mannanoprotein preparation from the quadruple null strain was used as an acceptor. Again a net increase in activity was seen with KRE2 (2.8-fold) and YUR1 (2.1-fold) when these genes were overexpressed singly in the quadruple null background. Activity higher than background was also detected with the KTR1 and KTR2 genes, although at lower levels. Strains overexpressing KTR2 or KTR1 had mannosyltransferase activities that were 1.8-fold higher and 1.5-fold higher than background, respectively.

Multiple Disruptions of KTR1, KTR2, and YUR1 Lead to K1 Killer Toxic Resistance—The role of the Ktr1p, Ktr2p, and Yur1p proteins as mannosyltransferases was also assessed in vivo by using a K1 killer toxin sensitivity assay (Fig. 7). K1 killer yeast strains secrete a small pore-forming toxin that requires a cell wall receptor for function (Bussey, 1991). This receptor appears to consist of the glycosyl moieties of cell wall glucomannoproteins. Killer-resistant mutants have been found to be defective in β1,6-glucan and in O-mannosylation, suggesting that the in vivo receptor includes these polymers, which are cross-linked in cell wall glucomannoproteins (Montijn et al., 1994; Lu et al., 1995).

Yeast strains harboring single and double mutations, as well as a triple null mutation, of KTR1, KTR2 and YUR1 were assayed for killer toxicity (Fig. 7). When evaluated by seeded plate assays, the wild type toxin-sensitive SEY6210 strain displayed a large killing zone (15 mm), whereas the ktr2 mutant was completely toxin-resistant. A strain bearing a single KTR1 disruption showed no phenotypic resistance to K1 killer toxin. When compared to the wild type strain, yur1 (10 mm) and ktr2 (12.5 mm) single null disruptions were both partially resistant to the killer toxin, yur1 being more resistant. Yeast cells carrying ktr1 ktr2, ktr1 yur1, or ktr2 yur1 double disruptions all displayed pronounced levels of resistance. The ktr1 ktr2 (10 mm) double null strain showed a stronger phenotype than either the ktr1 or ktr2 single null. Both ktr1 yur1 (8 mm; clear) and ktr2 yur1 (7 mm; fuzzy) double nulls are more resistant than a strain carrying a yur1 null mutation indicating that disruption of either KTR1 or KTR2 exacerbates the cell wall defect of a yur1 mutant. Finally, a ktr1 ktr2 yur1
triple null mutant is almost totally resistant, suggesting a cumulative effect on the reduction of carbohydrate chains leading to killer resistance. These results thus appear to also implicate N-linked chains as part of the killer receptor.

The killer phenotype of some single null mutants allowed a test of possible suppression of the loss of one gene by another homologous counterpart. Ktr1p, Ktr2p, and Yur1p could not suppress the killer resistance of a KRE2 null mutant and thus could not functionally substitute for it. Functionality suppression could only be established between the YUR1 and KTR2 genes, with overexpression of KTR2 in a strain carrying a yur1 null mutation completely suppressing the yur1 killer resistance phenotype, indicating that when expressed at very high levels, Ktr2p has the capacity to substitute in vivo for the absence of the Yur1p. These two proteins are 62% identical and constitute the most homologous pair among members of the Kre2p family (Lussier et al., 1993; Mallet et al., 1994).

Ktr1p, Ktr2p, and Yur1p Are Localized in the Yeast Golgi Complex—The Kre2p a1,2-mannosyltransferase has been localized to a medial Golgi compartment (Chapman and Munro, 1994; Lussier et al., 1995b). The apparent role of Ktr1p, Ktr2p, and Yur1p as glycosyltransferases and their similarity to Kre2p make these proteins candidates for Golgi localization and this was examined by indirect immunofluorescence. To identify and analyze the localization of the three proteins, a specific rabbit antiserum was raised against Ktr1p, and the influenza hemagglutinin virus epitope (Kolodziej and Young, 1991) was inserted directly at the C-terminal domain of Ktr2p and Yur1p (see "Experimental Procedures"). An affinity-purified anti-Ktr1p Ab detected Ktr1p in Western blotting of total cell protein extracts as a 55-kDa protein that was absent from the ktr1::LYS2 strain. Immunoblots of the 12CA5 hemagglutinin epitope specific monoclonal antibody detected only the epitope-tagged version of Ktr2p and Yur1p. When the relevant antibodies, plasmids and strains were used for whole cell indirect immunofluorescence, all three proteins showed a punctate pattern of fluorescent signals (Fig. 8) indicative of Golgi localization (Redding et al., 1991; Cooper and Bussey, 1992; Roberts et al., 1992; Graham et al., 1994; Lussier et al., 1995b). In each case, between 3 and 10 structures/cell can be seen, depending on individual cells and the plane of focus. Fluorescence signals were never seen with the anti-Ktr1p antibody in ktr1::LYS2 cells nor with the 12CA5 monoclonal antibody with cells not expressing tagged versions of the Ktr2p and Yur1p proteins. The signal distribution of Ktr1p, Ktr2p, and Yur1p did not overlap with nuclei or mitochondria as viewed by DNA staining with DAPI (Fig. 8).

**DISCUSSION**

Kre2p is an α1,2-mannosyltransferase (Häusler and Robbins, 1992; Häusler et al., 1992), and we present here evidence showing that Ktr1p, Ktr2p, and Yur1p are also involved in protein glycosylation. As Kre2p is a mannosyltransferase adding the third mannose residue on O-linked mannos glycosyl Lamp III, the possible role of Ktr1p, Ktr2p, and Yur1p in O-glycosylation was analyzed. Experiments indicated that neither the O-glycosylation of total yeast mannoprotein nor the O-glycosylation of Kre9p is affected by these proteins, since no differences from wild type were seen in single or triple null mutants (ktr1 ktr2 yur1). The influence of KTR1, KTR2, and YUR1 gene disruptions on protein N-glycosylation was analyzed. The N-glycosylated protein invertase was found to be undergycosylated in the ktr1 ktr2 yur1 triple null mutant compared to a wild type strain but not in single or double disruptants, except in the case of kre2 where, as expected, an effect was seen (Hill et al., 1992). Invertase receives even less glycosylation when synthesized in the quadruple, ktr1 ktr2 yur1 kre2, null strain but is still heavily N-modified since its migration pattern (∼120 kDa) remains considerably larger than the molecular mass of the protein predicted from the DNA sequence (59 kDa). These
results are consistent with these enzymes having redundant functions in N-linked glycosylation.

Possible additional roles for Ktr1p, Ktr2p, Yur1p, and Kre2p were also assessed. S. cerevisiae carries several phosphoinositol-containing sphingolipids, specifically inositol phosphoceramides, mannosylinositol phosphoceramides (which contain a mannose attached to the inositol), and mannosyl(inositol phospho)2 ceramides (which is substituted with one mannose and 2 phosphoinositol groups). Ktr1p, Ktr2p, Yur1p, and Kre2p do not appear to be involved in this lipid mannosylation, as none of the strains containing single or multiple deletions of these genes lacked any of the mannosylated inositol phospho-ceramides. Another possibility is that Ktr1p, Ktr2p, Yur1p, and Kre2p elaborate part of the short α-linked mannose side chains found on protein-bound GPI anchors. None of the enzymes could be solely responsible for a single biosynthetic step in GPI core synthesis, since this is an essential process in yeast (Leidich et al., 1994, 1995), and none have a lethal phenotype when disrupted. Strains carrying single or multiple deletions of these four genes all synthesized normal GPI anchors (Sipos et al., 1995).

Further evidence that Ktr1p, Ktr2p, and Yur1p are mannosyltransferases was obtained by evaluating their in vitro enzymatic activities. Using α-methylmannoside and oligosaccharides found on mannoproteins from a kre2 ktr1 ktr2 yur1 quadruple null strain as acceptors, YUR1 overproducing cells showed a 5.5- and 2.1-fold increase in activity over background respectively. These values are similar to those obtained by overproduction of the known mannosyltransferase encoding gene, KRE2. By comparison, when α-methylmannoside was used as a saccharide acceptor, high levels of expression of KTR1 and KTR2 did not result in activity levels higher than those obtained with the parental quadruple null strain. However, when the mannoprotein fraction was used as an acceptor, increased levels of activity similar to those obtained with YUR1 were reproducibly found with both KTR1 and KTR2, indicating that their gene products also are mannosyltransferases. The activity difference seen between these enzymes in the two assays suggests that these mannosyltransferases differ in substrate specificity.

Further evidence that Ktr1p, Ktr2p, and Yur1p are mannosyltransferases comes from an in vivo analysis of their function. Strains carrying non-functional copies of KTR1, KTR2, and/or YUR1 genes, became to varying extents K1 killer toxin-resistant, the triple null mutant being most resistant. These results indicate that, as is the case with KRE2 null mutations, singly or in combination disruptions of KTR1, KTR2, and YUR1 lead to a reduced amount of N-linked glycans on cell wall mannoproteins perturbing the cell surface toxin receptor and leading to resistance. The fact that functional replacement by overproduction could only be obtained between the most similar gene pair, YUR1 and KTR2, also suggests that these mannosyltransferases likely perform different functions.

From the above results, it can be concluded that Ktr1p, Ktr2p, and Yur1p are implicated as mannosyltransferases in N-linked glycan elaboration. However, these enzymes do not participate in the synthesis of the basic N-linked core oligosaccharide, as they are situated in the Golgi apparatus and the core oligosaccharide is elaborated and transferred to protein in the ER (Herscovic and Orlean, 1993; Lehle and Tanner, 1995). Similarly, Ktr1p, Ktr2p, and Yur1p do not participate in core Golgi modifications (see Fig. 5), as the size of the core modified oligosaccharide received by the late Golgi protein Kex1p (Cooper and Bussey, 1992) is the same in the triple ktr1 ktr2 yur1 null mutant and in wild type. Thus, it is likely that the role of Ktr1p, Ktr2p, and Yur1p is to participate in the elaboration of the outer chain glycans of N-linked oligosaccharides. A number of distinct α1,2-linked mannosylation reactions are required for the synthesis of the outer chain of yeast N-linked modified proteins (see Fig. 5), and we speculate that Ktr1p, Ktr2p, Yur1p, and also Kre2p (Hill et al., 1992) are partially responsible for establishing some of these α1,2-linkages in the Golgi apparatus.

The cumulative effect of multiple gene disruptions seen on the size of the N-linked carbohydrates carried by invertase and on the degree of in vivo killer toxin resistance can be rationalized in distinct ways that are not necessarily mutually exclusive. Ktr1p, Ktr2p, Yur1p, and Kre2p, and other similar mannosyltransferases could function redundantly in the sense of having overlapping specificities. Different forms of functional redundancy can be envisaged in the context of a large family of glycosyltransferases elaborating complex glycans. 1) More than one enzyme could be able to establish one specific class of glycosyl linkage. This could happen in normal vegetative growth or could be achieved by differential regulation under specific conditions. The PMT gene family encoding protein O-mannosyltransferases constitutes an example of this type of redundancy (Strahl-Bolsinger et al., 1993; Lussier et al., 1995a; Immervoll et al., 1995). 2) Conversely, an individual mannosyltransferase may catalyze the assembly of one type of carbohydrate linkage to more than one type of oligosaccharide, as is the case for the Mnn1p terminal α1,3-mannosyltransferase (Bal- lou, 1990; Cooper and Bussey, 1992; Graham et al., 1994; Yip et al., 1994; Lussier et al., 1995b). 3) Added complexity may arise if members of the Kre2p family are not localized in the same Golgi compartment. Some member proteins could possess the same enzymatic specificity, but their Golgi retention signal would be different, targeting them to distinct intracellular locations. A key element in the targeting of Kre2p to the medial Golgi has been shown to lie in the N-terminal region (Lussier et al., 1995b). This interesting region of the sequence of Kre2p, Ktr1p, Ktr2p, and Yur1p is unique to each protein hinting that they may be localized to different Golgi subcompartments.

Taken together, our results indicate that Ktr1p, Ktr2p, Yur1p, and Kre2p are involved in the elaboration of the outer chain N-linked glycans. The specificity of the mannosyltransferase reactions catalyzed by these four enzymes is likely to vary, as each showed a different pattern of activity toward the two acceptors used in our in vitro assays. Multicopy suppression of the phenotype caused by one deleted transferase by another provides an indication of at least some partial overlap of mannosyltransferase specificity. The lack of multicopy suppression, however, provides little information as enzymes with similar specificity may reside in different Golgi compartments or be differentially regulated. A clearer picture of the overall specificity, location, and regulation of the KRE2/MNT1 family awaits the identification and characterization of the entire gene family in S. cerevisiae, a goal likely attainable by the completion of the genome sequence of this organism.

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