The Ktr1p, Ktr3p, and Kre2p/Mnt1p Mannosyltransferases Participate in the Elaboration of Yeast O- and N-linked Carbohydrate Chains*

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We have determined a role for Ktr1p and Ktr3p as mannosyltransferases in the synthesis of the carbohydrate chains attached to Saccharomyces cerevisiae O- and N-modified proteins. KTR1 and KTR3 encode related proteins that are highly similar to the Kre2p/Mnt1p mannosyltransferase (Lussier, M., Camirand, A., Sdicu, A.-M., and Bussey, H. (1993) Yeast 9, 1057–1063; Mallet, L., Busserreau, F., and Jacquet, M. (1994) Yeast 10, 819–831). Examination of the electrophoretic mobility of a specifically O-linked protein from mutants and an analysis of their total N-linked mannosyl chains demonstrates that Ktr1p, Ktr3p, and Kre2p/Mnt1p have overlapping roles and collectively add most of the second and the third α1,2-mannose residues to O-linked glycosylation. Determination of the mobility of the specifically N-linked glycoprotein in vitro in different null strains indicates that Ktr1p, Ktr3p, and Kre2p are also jointly involved in N-linked glycosylation, possibly in establishing some of the outer chain α1,2-linkages.

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Yeast Ktr1p and Ktr3p Mannosyltransferases

**Fig. 1. Detection of Kre9p synthesized in different yeast null mutants.** Kre9p was overexpressed from the 2μ-based plasmid YEp351 (28) in different yeast strains and concentrated from exponentially growing cultures. Yeast extracellular protein extracts were immunoblotted with affinity-purified anti-Kre9p polyclonal antibodies. The molecular mass standard is shown in kilodaltons.

and the third α1,2-linked mannose residues on O-linked carbohydrate chains and that also participate in N-linked outer chain elaboration.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains, Culture Conditions, and Methods**—All yeast strains used were based on SEY6210 (MATα, leu2-3,112, ura3-52, his3-D200, lys2-801, trpl-D00, suc2-D9) and were grown under standard conditions (Yeast extract, Peptone, Dextrose, Yeast Nitrogen Base) as described previously (18).

**Gene Disruptions**—Deletional disruptions of KRE2 and KTR1 are described elsewhere (8, 10). Disruption of the KTR3 locus was made by a single-step gene replacement procedure (19). First, the KTR3 gene was synthesized in vitro by the polymerase chain reaction (20) using the pfu DNA polymerase (Stratagene, La Jolla, CA) and CCACCACTTC-TCAGCATTG and GAAACCAAGAGGCACTAG as 5′ and 3′ primers, with yeast genomic DNA as a template. The 2-kilobase fragment obtained was then subcloned directly in the pT2/PC vector as described previously (14). A 294-base pair EcoRV coding sequence fragment was removed and replaced by a 1.8-kilobase NcoI fragment containing the complete HIS3 gene. A linear 3.5-kilobase NcoI fragment containing the complete HIS3 gene as well as the flanking regions plus the coding sequences of KTR3 gene was gel purified and integrated into the isogenic diploid SEY6210 strain. Integrants were verified by Southern analysis (data not shown).

**Immunoblotting**—The extracellular proteins Kre9p and invertase were expressed at high levels from a 2μ-based multicycop vector in yeast strains bearing different disruptions and concentrated from cultures growing exponentially in YNB selective medium containing 5% (v/v) glycerol and 2% glucose using Amicon centriprep concentrators (W. R. Grace & Co. Danvers, MA). Immunoblots were carried out as described (28) in different yeast strains and concentrated from exponentially growing cultures. Yeast extracellular protein extracts were immunoblotted with affinity-purified anti-Kre9p polyclonal antibodies. The molecular mass standard is shown in kilodaltons.

**Mannose Labeling and β-Elimination**—Yeast cells were labeled using [3H]mannose, and β-eliminations were performed as described previously (10, 14, 21).

**RESULTS**

**Functional Characterization of Three Members of the KTR Gene Family**—The possible roles of members of the KTR family in O- and N-linked glycosylation remain to be determined. A testable assumption is that those proteins in the family that are most closely related are most likely to possess functional similarities. A relational homology tree constructed using the catalytic domains of each protein clarifies such relationships and has permitted an attempt to functionally group the different enzymes (16). One conclusion drawn from this analysis was that Ktr2p is most related to Ktr1p and Ktr9p, indicating that these three proteins form a subfamily with possible related functions (16).

Recent in vitro studies using O-linked type substrates demonstrated that Ktr1p is a α1,2-mannosyltransferase with enzymatic properties highly similar to those of Ktr2p, suggesting that these two enzymes act in similar ways (23).

**Fig. 2. β-Elimination profiles.** Paper chromatograms of mannose-containing oligosaccharides released by β-elimination from bulk yeast glycoproteins of wild type cells (SEY 6210), and of the same strain in which different genes were disrupted. The peaks designated M1 to M5 represent carbohydrate chains bearing one to five mannose residues. M1, M2, and M3 co-migrate with mannose, maltose, and raffinose standards.

The relationshhips and examine the role of Ktr3p and reassess the role of Ktr1p and Kre2p in the synthesis of O-linked chains, the extent of O-glycosylation in yeast strains bearing mutations in these genes was investigated. Deletional disruptions of KRE2 and KTR1 were previously obtained (8, 10), and a disruption of the KTR3 locus was made (see “Experimental Procedures”). Double and triple disruptions were subsequently constructed using standard genetic techniques. The single and double null mutants showed no growth defects when compared with the wild type strain. However, the haploid kre2 ktr1 ktr3 triple null mutant had a slow growth phenotype, indicating a genetic interaction between the three genes.2

Ktr1p, Ktr3p, and Kre2p Participate in Adding the Second and Third α1,2-linked Mannose Residues on O-linked Oligosaccharides—O-glycosylation was examined in yeast strains bearing different disruptions by following the mobility of Kre9p (Fig. 1), a protein involved in cell wall assembly that is highly O-mannosylated but receives no N-linked modifications (22). Kre9p produced in a wild type strain migrates at an apparent mass of 55 kDa (10, 21, 22). As previously observed, Kre9p isolated from a krc2 null strain migrated more quickly than did the wild type Kre9p, with an apparent molecular mass of approximately 47 kDa (10, 21), whereas Kre9p produced by ktr1 (10) or ktr3” single null disruptants was indistinguishable from that produced by a wild type strain. Kre9p produced in a ktr1

2 A.-M. Sdicu, M. Lussier, and H. Bussey, unpublished observations.
Yeast Ktr1p and Ktr3p Mannosyltransferases

**Scheme 1. Assembly of the S. cerevisiae O-linked oligosaccharide structures.** Arrows depict α1,2- and α1,3-linkages between mannose residues. The various enzymes attaching the different mannose residues are indicated. The Pmnp family of protein O-mannosyltransferases adds the first mannose on the Ser/Thr residues in the ER (4). Ktr1p and Ktr3p along with the Kre2p/Mnt1p α1,2-mannosyltransferase participate in the addition of the second mannose residue onto O-linked chains. Kre2p has been known to be the main enzyme responsible for the addition of the third mannose on O-glycans (7, 8). Ktr1p and Ktr3p are also able to add this particular mannose, although to a lesser extent than Kre2p (see Fig. 2). The Mnn1p α1,3-mannosyltransferase attaches the fourth mannose residue in the linear chain of up to five mannose residues (11–13).

FIG. 3. Detection of invertase synthesized in wild type and different null mutants. Invertase was overexpressed from the 2μ-based plasmid YEp352 (28) in different yeast strains and concentrated from exponentially growing cultures. Extracellular protein extracts were immunoblotted with anti-invertase polyclonal antibodies (see “Experimental Procedures”). The molecular mass standard is shown in kilodaltons. The possible S. cerevisiae N-linked oligosaccharide structures are also shown, and X equals 10 on average (11).

ktr3 double null mutant showed a small increase in mobility, whereas Kre9p produced in a krc2 ktr3 mutant was similar to Kre9p produced in a krc2 single mutant. However, a ktr1 mutant exacerbated the O-mannosylation defects of a krc2 mutant as the apparent molecular mass of Kre9p was approximately 5 kDa smaller (42 kDa) than when produced in a krc2 mutant alone. Kre9p was smallest (~38 kDa) when produced in a triple ktr1 ktr3 krc2 mutant, indicating a cumulative involvement of all three proteins in O-linked modifications.

To determine the extent of this involvement, an analysis was made of total O-linked carbohydrate chains present on glycoproteins of mutants carrying different combinations of the disrupted genes (Fig. 2). O-linked carbohydrate chains were specifically released from the total glycoprotein fraction of in vivo [3H]mannose-labeled yeast cells by β-elimination and resolved by chromatography (10, 14, 21; see “Experimental Procedures”). The wild type strain (Fig. 2A) and the ktr1 and ktr3 single null mutants showed the normal profile of five oligosaccharide peaks, as was the case with the ktr1 ktr3 double null mutant (Fig. 2B). As expected, the pattern obtained from the krc2 null strain gave two major peaks (Man1-Man2; Fig. 2C), consistent with failure to add the third α1,2-linked mannose residue and a minor peak indicating that a small proportion of total glycoproteins received a third mannose, an effect previously seen (7). Other yeast enzymes are, therefore, able to carry out this particular reaction to a limited extent.

A significant reduction in mannosylation is evident in both the ktr1 krc2 and ktr3 krc2 double null mutants, each of which gave two peaks (Man1-Man2) but with a decreased proportion of glycoproteins receiving 2 mannose residues when compared with a krc2 disruptant alone (Fig. 2, D and E), suggesting an involvement of both Ktr1p and Ktr3p in adding this mannose to O-modified chains. In those two double mutants, the proportion of O-glycoproteins receiving a third mannose is less than in a krc2 single null, suggesting that both Ktr1p and Ktr3p have a limited capacity to add a third mannose on O-linked chains. In a ktr1 ktr3 krc2 triple mutant, the predominant O-linked oligosaccharides assembled were composed of a single mannose (Fig. 2F), indicating that collectively Ktr1p, Ktr3p, and Kre2p are responsible for adding the third mannose and most of the second on O-linked glycans. At least one other enzyme, still unidentified but possibly encoded by a member of the KTR gene family (16), is responsible for the residual level of attachment of a second mannose (Fig. 2F). All the enzymes now known to be responsible for the assembly of yeast O-linked sugars are outlined in Scheme 1.

Ktr1p, Ktr3p, and Kre2p Jointly Participate in N-Glycosylation—The Kre2p mannosyltransferase is presumably involved in the elaboration of N-linked outer chain glycans (9, 10), but the precise mannoses added by this enzyme remain to be determined. In view of the cumulative role of KTR1, KTR3, and KRE2 in O-glycosylation, the effect on N-glycosylation of inactivating different combination of these genes was assessed. The carbohydrate chains of invertase, a specifically N-modified protein, were analyzed by measuring the mobility of the secreted form of this protein (Fig. 3), which is extensively modified with core oligosaccharides extended with outer chain glycans (24, 25).

The extracellular wild type protein, as is the case when it is synthesized in ktr1 and ktr3 single null mutants,2 has a molecular mass of around 150 kDa (Fig. 3). As seen before (10), invertase produced in a krc2 null mutant receives less N-modification (~143 kDa). The oligosaccharides attached to invertase synthesized in a ktr1 ktr3 double null mutant appear unaffected because the approximate molecular mass of the polypeptide chains made in this particular strain equals that of wild type proteins. The oligosaccharidic defects of invertase produced in a ktr1 krc2 double null mutant were similar to those observed in a krc2 single null mutant, whereas a disruption in KTR3 slightly exacerbates the N-mannosylation defects.
Yeast Ktr1p and Ktr3p Mannosyltransferases

seen in a krc2 mutant because invertase synthesized in a ktr3 krc2 mutant has a molecular mass of ~137 kDa. However, invertase secreted from a ktr1 ktr3 krc2 triple null mutant was found to have a molecular mass of ~129 kDa demonstrating that, as was the case with O-glycosylation, all three proteins are collectively involved in N-linked modifications.

DISCUSSION

Ktr1p and Ktr3p are involved in the elaboration of O-linked glycans by adding the second mannose in the linear five mannose carbohydrate chain. This was not initially seen because yeast strains bearing ktr1 and ktr3 single or double disruptions possess a normal O-glycosylation pattern. Only in a ktr1 ktr3 krc2 triple mutant is the full effect of the absence of these enzymes apparent, where the third mannose is absent and a severely reduced level of mannose 2 is observed. In the absence of Ktr1p and Ktr3p, Kre2p is able to add both the second and third α1,2-mannose residue in the linear five mannose carbohydrate chains. Ktr1p and Ktr3p are to a limited extent also capable of attaching the third α1,2-mannose onto O-linked chains. All 3 enzymes, therefore, appear to have overlapping roles in the addition of both the second and third α1,2-linked mannose residues of O-glycosyl chains in yeast (see Scheme 1).

The exact in vivo contribution of each enzyme to the synthesis of the second and third mannose linkages in wild type cells remains to be determined.

The results presented here demonstrate that Ktr1p, Ktr3p, and Kre2p are also implicated in N-linked outer chain elaboration. These transferases do not participate in N-linked core glycan synthesis because the molecular mass of the core modified oligosaccharide received by the late Golgi protein Kex1p (26) is the same in the ktr1 ktr3 krc2 triple null mutant and in a wild type strain. Therefore, the marked reduction in the sugar chains received by invertase in the ktr1 ktr3 krc2 triple mutant indicates that Ktr1p, Ktr3p, and Kre2p act in the Golgi apparatus to elaborate outer chain glycans of N-linked oligosaccharides by making some of the branched mannose side chains that are attached to the α1,6-mannosyl residues backbone (see Fig. 3). Of the five mannoses constituting this backbone sugar chain, all are substituted by at least one α1,2-linked mannose residue, and three are modified by at least two α1,2-linked mannoses. Because the structure of these N-linked branched mannose side chains is reminiscent of O-linked oligosaccharides, it is a reasonable speculation that Ktr1p, Ktr3p, and Kre2p collectively participate in establishing some of these outer chain α1,2-linkages. Recent in vitro enzymatic studies are consistent with this (23).

Both Kre2p and Ktr1p utilize the N-glycan type oligosaccharidic substrate, Man15-30GleNAc, which contains the α1,6-mannose outer chain backbone attached to the core sugar but lacks the α1,2-mannose containing branches (see Fig. 3), indicating that both enzymes have the ability to add a first α1,2-linked mannose residue to the outer chain backbone. Moreover, the mannosyltransferase reaction involving Kre2p exhibited biphasic kinetics, with an increase in mannose 2 being observed. In the absence of Kre2p, Ktr1p, and Ktr3p, the second O-linked mannose residue is absent. Individual enzymes can also participate in more than one biosynthetic step. Kre2p, Ktr1p, and Ktr3p, which can add the second mannose residue to O-linked chains, are also involved together in adding a third mannose residue, though to differing extents (see Scheme 1.).

The level of apparent redundancy observed between the enzymes can be explained in several ways. It is possible that each enzyme possesses a high affinity for a limited set of amino acid sequence contexts around the Ser/Thr and Asn residues at which mannosylation occurs. By having a number of enzymes of varying specificity, the cell is able to extend the range of mannosylated residues on glycoproteins. There is strong evidence for this notion with the first enzyme required in the O-mannosylation pathway, the protein mannosyltransferase, where some of the seven PMT encoded enzymes have been shown to have differing specificities for the mannosylation of peptide substrates of different sequence (4, 21, 27). Although there is no direct evidence for this conjecture in the KTR family, variable sequence specificities can be hinted at when comparing Kre9p O-mannosylation patterns with those seen through β-elimination in the total O-mannoprotein fractions of the krc2 ktr1 and krc2 ktr3 double null mutants. The mannosylation deficiencies of Kre9p are smallest in a krc2 ktr3 mutant, whereas the defects of the bulk of O-modified proteins are far more pronounced in this strain (see Figs. 1 and 2, D and E). This is consistent with these enzymes having different specificities with Kre9p sites being atypical.

The redundancy observed here can also be explained by a sequential action through intracellular compartmentalization. If related enzymes have broadly similar or overlapping substrate affinities but are in a distinct Golgi compartments, then those mannosylation sites on proteins that fail to be mannosylated in one compartment have subsequent glycosylation opportunities as they move through the later mannosyltransferase-containing cisternae. If this were the case, Ktr1p and Ktr3p that have less ability to add the third mannose in O-glycosylation might be found in cisternae before Kre2p, the enzyme most responsible for Man3 addition. This would build a level of redundant function into the processive extension of mannose chains in successive Golgi compartments and increase O-mannosylation efficiency. The Golgi targeting regions of Kre2p, Ktr1p, and Ktr3p are unrelated, consistent with them having different intracellular locations (8, 10, 16).

The addition of O- and N-mannose oligosaccharides in S. cerevisiae requires the action of many related mannosyltransferases. Studying the roles of gene families such as KTR will be informative both for the analysis of protein glycosylation and to offer insights into the biological reasons that allow such diversity of related gene products to occur.

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