Localization and Targeting of the \textit{Saccharomyces cerevisiae} Kre2p/Mnt1p \(\alpha_{1,2}\)-Mannosyltransferase to a medial-Golgi Compartment

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Abstract. The yeast \(\text{Kre2p/Mnt1p}\ \alpha_{1,2}\)-mannosyltransferase is a type II membrane protein with a short cytoplasmic amino terminus, a membrane-spanning region, and a large catalytic luminal domain containing one N-glycosylation site. Anti-Kre2p/Mnt1p antibodies identify a 60-kD integral membrane protein that is progressively \(\mathit{N}\)-glycosylated in an \(\text{MNNL}\)-dependent manner. Kre2p/Mnt1p is localized in a Golgi compartment that overlaps with that containing the \textit{medial-Golgi} mannosyltransferase \(\text{Mnn1p}\), and distinct from that including the late Golgi protein \(\text{Kex1p}\). To determine which regions of Kre2p/Mnt1p are required for Golgi localization, Kre2p/Mnt1p mutant proteins were assembled by substitution of Kre2p domains with equivalent sequences from the vacuolar proteins \(\text{DPAP B}\) and \(\text{Pho8p}\). Chimeric proteins were tested for correct topology, in vitro and in vivo activity, and were localized intracellularly by indirect immunofluorescence. The results demonstrate that the \(\mathit{NH}_2\)-terminal cytoplasmic domain is necessary for correct Kre2p Golgi localization whereas, the membrane-spanning and stem domains are dispensable. However, in a test of targeting sufficiency, the presence of the entire Kre2p cytoplasmic tail, plus the transmembrane domain and a 36-amino acid residue luminal stem region was required to localize a Pho8p reporter protein to the yeast Golgi.

The Golgi apparatus plays a fundamental role in glycan processing and sorting of newly synthesized proteins in the secretory pathway of eukaryotic cells. The Golgi apparatus of a typical mammalian cell is composed of a polarized stack of membranous sacculles that are differentiated into functionally distinct subcompartments. After the addition of core \(\mathit{N}\)-linked sugar moieties in the endoplasmic reticulum, glycoproteins proceed through the \textit{cis-Golgi} network, the \textit{cis-}, \textit{medial-}, and \textit{trans-}cisternae, and the \textit{trans-}Golgi network, where further modifications take place. These include the addition of \(\mathit{O}\)-linked sugars and the elaboration, in a protein-specific manner, of complex \(\mathit{N}\)-linked carbohydrate structures. The Golgi apparatus also constitutes a major organelle responsible for protein trafficking where particular proteins are directed to precise cellular locations. Two specific Golgi subcompartments have been found to be involved in protein sorting. The \textit{cis-Golgi} network constitutes the site from which many resident \(\mathit{ER}\) proteins are retrieved, and the main function of the \textit{TGN} is to direct glycoproteins exiting the Golgi complex to either the lysosome or the cell surface (Pelham and Munro, 1993; Gleeson et al., 1994; Low and Hong, 1994).

In the yeast \textit{Saccharomyces cerevisiae}, Golgi-related processes have been studied from genetic and biochemical perspectives (Kukuruzinska et al., 1987; Pryer et al., 1992) resulting in a functionally defined Golgi apparatus that has been subdivided into several subcompartments on the basis of \(\alpha_{1,2}\)-linked \(\mathit{N}\)-linked oligosaccharide modifications (Franzusoff and Schekman, 1989; Graham and Emr, 1991; Wilsbach and Payne, 1993; Gaynor et al., 1994). In yeast, secretory pathway glycoproteins can acquire two types of \(\mathit{N}\)-linked oligosaccharides, either a simple core carbohydrate or one extended by an outer-chain glycan structure. The \(\mathit{N}\)-linked core oligosaccharide elaborated in the \(\mathit{ER}\) is mainly constituted of \(\text{Man}_9\text{GlcNAc}_2\) and may undergo Golgi maturation resulting in \(\text{Man}_{9,13}\text{GlcNAc}_2\). In other cases, glycoproteins traversing the Golgi apparatus have their core oligosaccharide extended by outer chains (see Fig. 2; Ballou, 1990; Herscovics and Orlean, 1993). The initial step in outer-chain synthesis takes place and defines an early Golgi compartment in which a backbone of \(\alpha_{1,6}\)-linked mannose residues is attached to the core oligosaccharide. This Golgi compartment has been likened to the mammalian \textit{cis-Golgi} network (Wilsbach and Payne, 1993). Outer chain elaboration is then brought to completion: the \(\alpha_{1,6}\)-linked mannose structure is enlarged in a sequential fashion by \(\alpha_{1,2}\) and \(\alpha_{1,3}\)-mannosyltransferases in a subsequent distinct compartment.

1. Abbreviations used in this paper: DAPI, 4',6-diamidino-2-phenyl-indole; Kre2p, the product of the \textit{KRE2/MNT7} gene; \(\mathit{N}\)-linked, asparagine-linked; \(\mathit{O}\)-linked, serine/threonine-linked; \(\text{TMD}\), transmembrane domain.
partment defining the yeast medial-Golgi compartment. Mature glycosylated proteins are finally transported to a late Golgi compartment where proteolytic processing of secreted protein precursors occurs. Some evidence suggests that this Golgi subcompartment is also involved in vacuolar protein sorting, making this late Golgi compartment comparable to the mammalian TGN (Wilsbach and Payne, 1993).

In *S. cerevisiae*, the various Golgi compartments have not been morphologically well characterized. The yeast Golgi complex is not structurally similar to the perinuclear stacked cisternal subcompartments characteristic of the Golgi apparatus of mammalian cells. Immuno-electron microscopy revealed that the yeast Golgi is composed of as many as 30 concave, disikelike membranous cisternae scattered in the cytoplasm and most often not organized in parallel stacks (Preuss et al., 1992). In indirect immunofluorescence using antibodies to different yeast Golgi proteins, the isolated cisternae are visualized as dispersed punctate spots (Franzusoff et al., 1991; Redding et al., 1991; Antebi and Fink, 1992; Cooper and Bussey, 1992; Roberts et al., 1992).

The establishment and maintenance of the polarized organization of the Golgi apparatus relies on the existence of complex sorting and transport mechanisms permitting specific Golgi proteins to be delivered to their precise cisternal destinations. Resident proteins of the secretory pathway of eukaryotic cells require particular targeting signals that specify their final location (Pelham and Munro, 1993; Gleeson et al., 1994; Low and Hong, 1994). Mammalian membrane glycoproteins not possessing positive sorting sequences are transported to the cell surface through a bulk flow of proteins and lipids (Pelham and Munro, 1993). In yeast, secretory pathway membrane glycoproteins lacking targeting sequences are brought by default to the vacuole (Cooper and Bussey, 1992; Roberts et al., 1992; Wilcox et al., 1992; Nothwehr et al., 1993; Gaynor et al., 1994; Hill and Stevens, 1994). Proteins associated with glycan modifications constitute a major class of resident Golgi proteins. Enzymes belonging to different mammalian glycosyltransferase families share a similar type II structural organization, but lack amino acid sequence homology or discernible targeting motifs even if situated in the same Golgi compartment (Shaper and Shaper, 1992; Kleene and Berger, 1993). It has been established that the membrane-spanning domain of animal glycosyltransferases plays a central role in Golgi localization (Munro, 1991; Nilsson et al., 1991; Swift and Machamer, 1991; Aoki et al., 1992; Burke et al., 1992; Colley et al., 1992; Tang et al., 1992; Teasdale et al., 1992; Wong et al., 1992; Gleeson et al., 1994; Low and Hong, 1994). To define more precisely the *S. cerevisiae* Golgi complex and to better understand how posttranslational modifications occur, we have studied the glycosyltransferase, Kre2p/Mntlp.

Kre2p/Mntlp possesses a type II orientation, is progressively N-glycosylated in an MNN1-dependent fashion, and is localized in a Golgi subcompartment that overlaps with the Mntlp medial-Golgi compartment. In addition, we show that the short NH2-terminal cytoplasmic tail domain of Kre2p/Mntlp is required for correct Golgi localization, whereas the membrane-spanning and stem domains are dispensable. However, localization of a reporter protein to the yeast Golgi requires a region of Kre2p/Mntlp encompassing the cytoplasmic tail, the transmembrane domain (TMD), and a segment of the luminal stem domain.

Materials and Methods

Yeasts, Strains, Culture Conditions, and Procedures

All yeast constructions used strains SEY6210 (MATa, leu2-3, ura3-52, his3-D200, lys2-801, trp1-D801, succ2-A901; or cAABYS68 [S86] (MATa, Dura3-2, leu2 his3 pral pb2 prl1 cps)). Yeast cells were grown under standard conditions, (yeast extract peptone dextrose, yeast nitrogen base [YNB] buffered with Halvorson medium, when required) as previously described (Boone et al., 1990). Strains were transformed using the lithium acetate procedure using sheared, denatured carrier DNA (Gietz et al., 1995). Transformants were selected on synthetic minimal medium with auxotrophic supplements. Levels of sensitivity to K1 killer toxin were evaluated in SEY6210 by a seeded plate assay using a modified medium consisting of 0.67% YNB, 0.0025% required amino acids, 1.0% bacto agar, 0.001% methylene blue, 2% glucose, and buffered to pH 4.7 with Halvorson minimal medium (Lusier et al., 1993, 1995a; Brown et al., 1994).

DNA Procedures and Chimeric Constructions

All DNA manipulations were carried out as described in Sambrook et al. (1989), unless otherwise stated. A deletional disruption of the *KRE2/MNT1* locus in strain SEY 6210 was made with the *TRP1* gene by the single-step gene replacement procedure of Rothstein (1991) using the pAH11 plasmid (kindly provided by Drs. A. Hausler and P. W. Robbins, Massachusetts Institute of Technology, Cambridge, MA). Briefly, the 1329-bp *KRE2* gene was disrupted by replacement of the region from bases 78 to 1315 with a 1.2-kb fragment encoding *TRP1*. A linearized DNA fragment comprising the *kre2::TRP1* allele was used for integration. Disruption of *KRE2* in strain S86 was made by replacing a 627-bp coding fragment (position 245 to 872 from ATG) with the *HIS3* gene. All gene disruptions were confirmed by Southern analysis (data not shown).

Oligonucleotide-mediated mutagenesis of sequences corresponding to the NH2-terminal region of Kre2p and leading to the production of the different chimeric proteins was performed according to Kunkel et al. (1987). A 1,922-bp SstI-XbaI DNA fragment containing the 1329 Kre2p open reading frame was subcloned in pBluescript KS+ (Stratagene Inc., La Jolla, CA) and transformed in strain CJ236 from which single-stranded DNA was produced and used for subsequent mutagenesis.

**KDKK** (see Fig. 6) was made by a domain substitution in which recombination of the Kre2p transmembrane domain with the of DPAP B (Roberts et al., 1989) was obtained after mutagenesis using oligonucleotide 5'-CTCAAGAAGACGTGGTACGCGATATGGCGGTTT-GTTTGGTCTGGTATAATTGGTGACACTCCTGATATGGCGGTTTCCAGCTACACAGTACATTTCCCGG-3' resulting in sequence MALFLSKRLLR-- result in the underline region corresponds to the DPAP B TMD region of the PPKK was obtained similarly with the Kre2p TMD replaced with that of the Pho8p vacuolar alkaline phosphatase (Kaneko et al., 1987) using oligonucleotide 5'-CTCAGTAAAGACGTGGTACGCGATATGGCGGTTT-GTTTGGTCTGGTATAATTGGTGACACTCCTGATATGGCGGTTTCCAGCTACACAGTACATTTCCCGG-3'.
The chimeric protein sequence MALFLSKRLLRFVIVLLLTLNSNSRTQQYIP... DAKKTRASHKKKNV... corresponds to the Pho8p luminal domain. The 27-bp KRE2 DNA fragment corresponding to all amino acid residues of the cytoplasmic NH2 terminus of Kre2p with the exception of the initiation codon and Arg residue lying directly adjacent to the membrane-spanning domain resulting in sequence MRFTVIAGAVIVLLLTLNSNSRTQQYIP... where the underlined region corresponds to the Kre2p TMD (bold). Chimeric protein KKKP contains the NH2 terminus and TMD of DPAP B and lacks the first 36 amino acid residues of the Kre2p stem domain. KD-K was obtained by inserting a MluI site directly after the TMD of KKKK and a second MluI site 105 bp downstream from this region. The mutagenized KKKK DNA was digested with the restriction enzyme MluI, religated, and the sequence encoded by the resulting construct was MALFLSKRLLRVGHIL- V LiLVWTVLLTTEQSAI... . where the underlined region corresponds to the DPAP B membrane-spanning domain and the bold region represents the altered luminal domain.

Chimeric construct KKP contains the NH2 terminus and TMD of Kre2p and the catalytic domain of Pho8p. KKP was obtained by insertion of a BsiWI restriction site immediately after the membrane-spanning domain of both Kre2p and Pho8p, followed by replacement of the Kre2p luminal domain with that of Pho8p, resulting in the sequence MALFLSKRLL-RFTVIAGAVIVLLLTLNSNSRTASHKKKNV... where the Kre2p NH2 terminus and TMD (bold) are fused to the Pho8p luminal domain (underlined). KKKK comprises the NH2 terminus, TMD and partial stem domain (36 amino acid residues) of Kre2p and the luminal domain of Pho8p. It was assembled by inserting a MluI site in KREE site 108 bp downstream from the region corresponding to the Kre2p TMD, and a second MluI site in PHO8 at the start of the luminal region immediately after the membrane-spanning domain. The MluI-digested DNA fragment corresponding to the Pho8p luminal domain was then joined to the Kre2p region encompassing the entire NH2 terminus, TMD, and partial stem domain. The amino acid sequence of KPPK is MALFLSKRLLRFVITAGAVIVLLLTLNSNSRTASHKKKNV... where the Kre2p NH2 terminus and TMD (bold) are fused to the Pho8p luminal domain (underlined). Finally, KKKK was obtained by replacing the Kre2p TMD in KKKK with that of Pho8p using the same oligonucleotide that was used to obtain KPPK.

All chimeric protein constructions were verified by DNA sequencing using the dideoxy chain termination procedure (Sanger et al., 1977) with the Sequenase enzyme (United States Biochemical Corp., Cleveland, OH), α-32P-DATP and specific DNA primers.

Preparation and Affinity Purification of Antisera

Kre2p antibodies were raised in rabbits against a BSA-coupled synthetic peptide corresponding to the last 10 amino acid residues of the protein (NH2-KPKNWKKFRE-COOH; 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 wk intervals. The conjugated peptide was coupled to Sepharose CL-6B (Pharmacia LKB Biotechnology, Inc., Montréal, Canada) and used in a column to affinity purify the antiseraum as described by Raymond et al. (1990).

Yeast Cell Extracts, Membrane Preparation, and Immunoblotting

Yeast total-cell protein extracts were prepared from cultures growing exponentially in yeast nitrogen base selective medium by cell lysis with glass beads in the presence of protease inhibitors. Membrane fractions were prepared as described (Nakayama et al., 1992) by centrifuging cell lysates at 10,000 g for 20 min and by centrifuging the resulting supernatant at 100,000 g for 1 h. The high speed pellet contained the insoluble membrane fraction. Yeast proteins were separated by SDS-PAGE, and immunoblots were carried out mainly as described (Lussier et al., 1995a). Briefly, 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 with affinity-purified anti-Kre2p antibodies in the same buffer. After antibody binding, membranes were washed in TBST and a second antibody directed against rabbit immunoglobulins and conjugated with alkaline phosphatase, was then added. The blots were again washed and proteins detected using an enhanced chemiluminescence procedure (Amersham Canada, Oakville, Ontario).

Radiolabeling of Yeast Cells and Immunoprecipitations

Analysis of [35S]methionine-labeled proteins was as described (Cooper and Bussey, 1989). Briefly, cells were grown at an OD600 of 0.4–0.45 in selective medium (YNB) and then labeled with [35S] methionine (100 μCi; ICN Biochemicals Inc., Montréal, Canada) for 10 min at 30°C. Yeast cells treated with tunicamycin (10 μg/ml) were precultivated before radiolabeling for 30 min at 30°C. Tunicamycin is a hydrophobic analogue of UDP-N-acetylglucosamine that blocks the addition of N-acetylgalactosamine to dolichol phosphate, the first step in N-linked oligosaccharide formation. Chase conditions were achieved by addition of 1-methionine and L-cysteine to a final concentration of 1 mM.

Assay of Mannosyltransferase Activity

α1,2-mannosyltransferase activity assays were performed essentially as described (Lewis and Ballou, 1991; Häusler and Robbins, 1992). S. cerevisiae cells (S86 background) were grown in selective medium to an OD600 of 0.8–0.9 and lysed with glass beads in the presence of protease inhibitors. High speed pellet fractions including Golgi and vacuolar membranes were prepared by centrifuging cell lysates at 1,130 g for 20 min and by centrifuging the resulting supernatant at 100,000 g for 1 h at 4°C. Incubation mixtures contained 50 mM Hepes, pH 7.2, 10 mM MgCl2, 0.1% Triton X-100, 0.2 mM GDP-[3H]mannose, and 10 mM α-methylmanno-pyranoside as an acceptor (Sigma Chemical Co.) and 10 μl of membrane fraction in a total volume of 20 μl. Reaction mixtures were incubated for 30–60 min at 30°C and then passed through a resin (AGI-X4; Bio-Rad Laboratories, Hercules, CA) column to remove unreacted GDP-mannose. Neutral products were eluted with 1 ml of water and radioactivity was measured. Control assays were conducted in which the saccharide acceptor was omitted and counts obtained in these assays were subtracted from values obtained in assays made with the sugar acceptor. Enzymatic activities (see Fig. 9) are expressed as percentages of specific activity (nmol/h/mg of membrane protein) for each chimeric protein.

Immunofluorescence

Yeast cells were grown in selective medium to an OD600 of 0.5–0.7 and immediately fixed by the addition to cultures of potassium-phosphate (pH 6.5) to 0.1 M, and formaldehyde to 3.7%. After gentle agitation for 30 min, cells were pelleted and resuspended in a fixation buffer containing 0.1 M potassium-phosphate (pH 6.5) and 4.5% formaldehyde. Cells were further fixed for another 30 min. Formaldehyde-treated cells were washed with 0.1 M potassium-phosphate (pH 6.5) buffer, resuspended in a solution of 0.1 M Tris-HCl, pH 8.5, 25 mM EDTA, and 1.2 M sorbitol and incubated for 10 min at 30°C with gentle agitation. After washing of the fixed cells, cell walls were removed by treatment with Zymolyase 100T (ICN Biochemicals) at a final concentration of 200 μg/ml in 0.1 M potassium phosphate (pH 6.5) 1.2 M sorbitol for 20–30 min at 30°C. Fixed spheroplasted cells were washed in 0.1 M potassium-phosphate (pH 7.4) 1.2 M sorbitol and resuspended in the same buffer. Cells were subsequently adsorbed to poly-L-lysine coated microscope slides, permitted to stand for 10 min and washed with PBS. Slides were then immersed in 20°C methanol for 1–6 min, and then for 20–60 s in −20°C acetone, depending on yeast strains. Treated slides were air-dried and stained immediately or stored at 4°C until needed. Fixed mounted cells were incubated with primary antibodies diluted in PBS containing 0.5% mg/ml BSA for 1 h at 25°C or overnight at 4°C. Anti-Kre2p Ab was used at dilutions of 1:25–1:100.

For dual-labeling experiments involving Kre2p and medial or late Golgi markers, the influenza hemagglutinin virus epitope (sequence YPYDVPDYA) was inserted by oligonucleotide-directed mutagenesis directly
Results

Identification of Kre2p as an Integral Membrane Protein

The product encoded by the KRE2 gene is a predicted type II membrane protein of 442 amino acid residues with a short cytoplasmic NH2 terminus, a hydrophobic transmembrane region, and a large luminal enzymatic domain containing one potential N-glycosylation site (Fig. 1A).

To identify and subsequently analyze Kre2p, a specific rabbit antiserum was raised, affinity purified, and used to detect antigen–antibody complexes by Western blotting of total-cell protein extracts of yeast strains harboring a KRE2 disruption or containing a KRE2 multicopy plasmid to facilitate immunological detection. The affinity-purified Ab detected Kre2p as a 59–60 kD protein in the KRE2-overexpressing strain that was absent from the kref2::TRP1 strain (Fig. 1B), and not detected by preimmune antiserum (data not shown). The in vivo–produced Kre2p is ~8.0 kD larger than the 51.5-kD molecular mass predicted from the DNA sequence. The possible integral membrane protein nature of Kre2p was examined by using reagents extracting cytoplasmic, vesicle-enclosed, and peripheral membrane proteins but leaving intact tightly associated membrane proteins. Cells expressing KRE2 at high levels were lysed with glass beads and treated with sodium carbonate or urea. After high-speed centrifugation of the treated cell lysates, the distribution of Kre2p in the pellet and supernatant fractions was assessed by Western immunoblotting. As can be seen in Fig. 1C, Kre2p was found only in membrane pellet fractions.

Kre2p Acquires Posttranslational Modifications within the ER and Golgi Apparatus

The difference between the expected (51.5 kD) and observed molecular mass of Kre2p (Fig. 1B) is likely to be at least partly due to N-glycosylation, since Kre2p is expected to act in the secretory pathway and the protein possesses a single site for N-glycosyl attachment in its predicted luminal domain (Asn197-Gln-Thr) (see Fig. 6). To test for Kre2p N-glycosylation, yeast cells were [35S]methionine labeled in the presence or absence of tunicamycin, an inhibitor of N-glycosylation. Immunoprecipitation and SDS-PAGE analysis of labeled cell lysates revealed that Kre2p was N-glycosylated (see Fig. 3, lanes 1 and 2), with the position of the sole N-glycosylation attachment site at Asn197 being consistent with a type II topology for Kre2p. The molecular mass of Kre2p in the presence of tunicamycin is about 54 kD, still 2.5 kD larger than its pre-
dicted molecular mass. The membrane nature of Kre2p or other posttranslational modifications could explain this discrepancy.

To establish possible Golgi modifications that Kre2p might acquire, and to attempt to assess to which Golgi sub-compartments it had been exposed, the size of the Kre2p-linked oligosaccharide chains was examined in specific N-linked glycosylation mutants. The mnnl strains carry mutations at various loci resulting in glycosylation defects (Fig. 2; Ballou, 1990; Ballou et al., 1990). The N-linked carbohydrates from an mnnl strain lack the outer chain. The mnnl strain produces glycoproteins with N-linked chains lacking terminal α,1,3-mannosyl residues (Fig. 2).

Wild-type, mnnl, and mnn9 mutant yeast strains carrying KRE2 were [35S]methionine labeled for 10 min and chased for 45 min, and the extent of glycosylation of immunoprecipitated Kre2p was examined (Fig. 3). The size of Kre2p produced in a wild-type strain after a 10-min radiolabeling is ~57 kD (major band, lane 2). After an additional 45-min chase, an apparent increase of molecular mass from 57 to ~59–60 kD was seen (Fig. 3, lanes 2 and 5). This apparent 2–3 kD increase in mass suggests that Kre2p may undergo a post-ER modification not involving extensive outer chain elaboration. The molecular mass of Kre2p produced in an mnn9 strain after a 10-min pulse (Fig. 3, lanes 2 and 4) and a 45 min chase (lanes 5 and 7) was identical to the mass of Kre2p from a wild-type cell, indicating that Kre2p does not receive an outer chain oligosaccharide. We tested whether the time-dependent additional carbohydrate modification required the MNN1-encoded Golgi α,1,3-mannosyltransferase, an enzyme that acts on both core and outer chains (Fig. 2; Ballou et al., 1990; Graham and Emr, 1991; Graham et al., 1994; Yip et al., 1994). After a short pulse, Kre2p synthesized in a mnnl strain is approximately of wild-type size (Fig. 3, lane 3). After a 45-min chase, however, the mnnl-produced Kre2p was ~1–2 kD smaller than the wild-type protein (lanes 5–7) indicating that Kre2p is normally exposed to a Golgi compartment where the Mnnlp α,1,3-mannosyltransferase adds at least three mannose residues to the Kre2p N-glycosyl core. Mnnlp therefore contributes to most of the observed Kre2p post-ER modifications (Fig. 2).

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**Figure 2.** Elaboration of N-linked carbohydrate modifications and putative compartmental organization of the yeast Golgi complex. Yeast glycoproteins can acquire two different types of N-linked modifications after acquiring a ManGlcNAc2 core in the ER. The core oligosaccharide can undergo maturation in the Golgi resulting in a Manα1,3 GlcNAc2 carbohydrate structure. In other cases, core structures may be extended by an outer chain of variable size (up to 200 mannose residues) that is composed of a backbone of α,1,6-mannosyl residues to which are attached branched α,1,2- and α,1,3-mannosyl side chains. Recent evidence suggests that the initiating Ochlp α,1,6-mannosyltransferase (Nakayama et al., 1992) defines a very early Golgi compartment that appears to be distinct from a subsequent early Golgi compartment responsible for α,1,6-mannosyl side chains elongation (Gaynor et al., 1994). The α,1,2- and α,1,3-mannosyl side chain modifications are predicted to occur in the medial-Golgi. The possible S. cerevisiae N-linked oligosaccharide structures are shown (adapted from Ballou, 1990 and Ballou et al., 1990). Arrows depict β,1,4, α,1,6, α,1,2, and α,1,3 linkages between mannoses of the core and outer chain. X = 10 on average. The mannose units not present in the mnn9 mutation are indicated. The Mnnlp-terminal α,1,3-mannosyltransferase is responsible for the addition of all α,1,3-mannosyl residues in a medial-Golgi compartment (Ballou, 1990; Graham et al., 1994; Yip et al., 1994). M represents mannose residues that are thought to be added to the Kre2p core oligosaccharide in a MNN1-dependent manner (see Fig. 3). Fully glycosylated proteins are then transported to a late Golgi compartment which includes the proteolytic enzymes (Kex2p, Kex1p, and DPAP A) responsible for maturation of secreted protein precursors. Glycoproteins that are not retained in the Golgi complex can be subsequently directed to the vacuole through an endosomal compartment or to the cell surface where they can be (a) incorporated into the plasma membrane, (b) secreted and retained in the periplasmic space/cell wall, or (c) secreted extracellularly.
Kre2p Is in a Golgi Location that Overlaps with the medial-Golgi Mannosyltransferase Mnnlp Compartment, a Location Distinct from that Containing the Late Golgi Protein Kex1p

Both the function of Kre2p as a O1,2-mannosyltransferase and its slow receipt of MNN1-dependent modifications imply localization to a Golgi compartment. Kre2p subcellular localization was examined by indirect immunofluorescence and showed a punctate pattern of fluorescent signals indicative of a Golgi association (Fig. 4 C). Between 3 and 14 structures per cell can be seen, depending on individual cells and the plane of focus. Punctiform fluorescence was never seen with the anti-Kre2p antibody in kre2:: TRP1 cells (Fig. 4 A). The Kre2p signal distribution did not overlap with nuclei or mitochondria viewed by DNA staining with DAPI (Fig. 4 D).

To attempt to define in which Golgi compartment Kre2p is localized, its intracellular localization was compared with that of a known medial-Golgi protein, the mannosyltransferase Mnnlp (Bailou, 1990; Graham et al., 1994; Yip et al., 1994), by double-label immunofluorescence (Fig. 5). Quantitative evaluation of punctate spot distribution (described in Materials and Methods) revealed that 75% of the Kre2p corresponding Texas red-labeled structures coincided with the FITC-labeled Mnnlp fluorescence spots. The location of Kre2p was also compared with that of a late Golgi protein, the carboxypeptidase Kex1p (see Figs. 2 and 5; Dmochowska et al., 1987; Cooper and Bussey, 1989, 1992). A quantitative scoring of punctiform fluorescence distribution indicated that 65% of the Kre2p corresponding structures did not coincide with the Kex1p fluorescence patches.

Short Cytoplasmic NH2-terminal Domain of Kre2p Is Required for Correct Golgi Localization Whereas the Membrane-spanning and Stem Domains Are Dispensable

To examine the basis of localization of a yeast Golgi glycosyltransferase, an analysis of Kre2p noncatalytic domains was made. The roles of the cytoplasmic NH2 terminus, TMD, and luminal stem region of Kre2p in Golgi targeting were tested by constructing chimeric proteins in which Kre2p-specific segments were substituted with the corresponding domains of the DAP2 or PHO8 gene products (Fig. 6). DAP2 encodes the vacuolar dipeptidyl aminopeptidase B (Roberts et al., 1989), DPAP B, and is a type II integral membrane glycoprotein that lacks apparent vacuolar targeting information. No individual domain of DPAP B was shown to be required for its transport to the vacuole besides a nonspecific hydrophobic TMD (Roberts et al., 1992). PHO8 encodes a vacuolar alkaline phosphatase. It is also a type II membrane protein (Kaneko et al., 1987; Klionsky and Emr, 1989; Nothwehr et al., 1993) that is thought to be transported to the vacuole by default (Nothwehr et al., 1993). Substitutions designed with vacuolar proteins were used to avoid potential problems with cryptic Golgi–targeting sequences. Indirect immunofluorescence detection of chimeric proteins was undertaken to determine their intracellular location(s).

Initially, the role of the membrane-spanning domain of Kre2p was assessed with fusion protein KDKK (Fig. 6), in which the Kre2p TMD was removed and substituted with that of DPAP B. KDKK was found, like wild-type Kre2p, to be localized to small punctate structures of the yeast Golgi (Fig. 7). To exclude the possibility that the DPAP B TMD is able to function as a Kre2p TMD in the context of chimeric protein KDKK by sharing some unknown common features not apparent at the amino acid level, a chimeric protein consisting of Kre2p with the Pho8p membrane-spanning domain was made. As was the case with KDKK, KP KKK was also found to be localized to the Golgi.
Figure 5. The Kre2p Golgi localization overlaps with that of the medial-Golgi mannosyltransferase Mnn1p, but is distinct from the late Golgi protein Kex1p. Double-immunofluorescence labeling of the Golgi proteins Kre2p and Mnn1p or Kex1p was performed as described in Materials and Methods. Diploid yeast cells (SEY 6210) containing KRE2 on multicopy plasmid YEp351 (LEU2; Hill et al., 1986) and the influenza hemagglutinin virus epitope-tagged MNN1 on multicopy plasmid YEp352 (URA3; Hill et al., 1986) or epitope-tagged KEX1 gene (see Materials and Methods) under an ADH1 gene promoter on centromeric-based plasmid pRS316 (URA3; Sikorski and Hieter, 1989) were fixed, spheroplasted, attached to polylysine-coated glass slides, and then incubated with affinity-purified anti-Kre2p and 12CA5 mAb (Kolodziej and Young, 1991). Fluorescence signals were obtained by subsequent incubation of treated cells with Texas red-conjugated goat anti-rabbit IgG and FITC-conjugated goat anti-mouse IgG which were used as secondary antibodies. Arrows indicate colocalization of punctiform fluorescence.

apparatus (Fig. 7) indicating that the Kre2p TMD is dispensable for Golgi retention.

The function of the cytoplasmic domain of Kre2p was examined with chimeric protein DKKK which possesses the cytoplasmic NH2-terminus of Kre2p replaced by that of DPAP B. DKKK could not be visualized in SEY6210 (data not shown) suggesting possible degradation of this protein in the yeast vacuole as found with other mistargeted Golgi proteins (Cooper and Bussey, 1992; Roberts et al., 1992; Wilcox et al., 1992; Nothwehr et al., 1993). To test this possibility, DKKK was expressed in strain S86 which contains mutations in the major vacuolar hydrolases and was found to be exclusively mislocalized to the yeast vacuole as shown by colocalization with a vacuolar membrane marker (Fig. 8). In contrast, when wild-type Kre2p was expressed from a 2μ-based plasmid in strain S86, all positive cells showed a punctate pattern of fluorescence. However, 15% of these stained cells also showed fluorescence associated with the vacuole (data not shown). These vacuolar stained cells appeared to be expressing high levels of Kre2p as they displayed high fluorescence levels. The Kre2p cytoplasmic tail NH2-terminus was deleted to further explore its role in targeting. Chimeric construct MR/KKK lacks the Kre2p cytoplasmic tail, with the exception of the initiating methionine codon and the Arg residue lying directly adjacent to the TMD. MR/KKK was
found to be mislocalized to the vacuole (Fig. 8), again demonstrating that the cytoplasmic NH$_2$-terminal domain of Kre2p is required for correct Golgi localization. Some ER retention of this MR/KKK protein was seen in ~10% of positive cells, indicative of an increased transit time through this organelle.

The role of the luminal amino acid residues flanking the Kre2p TMD was examined with fusion protein KD-K. It comprises the Kre2p NH$_2$-terminus, the membrane-spanning domain of DPAP B, the Kre2p stem region lacking the first 36 amino acid residues following the TMD, and the catalytic domain of Kre2p. KD-K was found to be localized in the Golgi complex (Fig. 7) suggesting that neither the deleted stem region nor the TMD is necessary for Golgi retention in the context of a Kre2p catalytic domain.

In parallel with the localization studies, all chimeric proteins were assessed functionally. They were verified to be membrane associated and properly glycosylated (data not shown), showing that they possess a type II membrane orientation (see Fig. 6). In addition, their in vitro and in vivo enzymatic activities were assayed since low levels of mannosyltransferase activity would imply abnormal secondary structure of a particular chimera and possible mislocalization in the secretory pathway. The in vitro α1,2-mannosyl-transferase activity was assayed in cell-membrane fractions. The specific activity of each chimeric protein is displayed in Fig. 9, where KRE2 expressed in a kre2Δ strain was arbitrarily determined to be 100%, a value just slightly lower than that of wild-type mannosyltransferase activity from a genomic copy of KRE2. KDKK, KPKK, DKKK, as well as MR/KKK appear to be fully active. KD-K displays intermediate levels of mannosyltransferase activity suggesting that the stem region from position 31 to 66 is necessary for folding or proper catalytic activity.

The capacity of chimeric proteins to function in vivo in Golgi-localized mannosylation was assessed by using a K1 killer toxin sensitivity assay (Fig. 9). K1 killer yeast strains secrete a small pore-forming toxin that requires a cell-wall receptor for function (Bussey, 1991). KRE2 null mutations lead to shorter mannose chains on cell-wall mannoproteins disrupting the toxin receptor and leading to resistance (Hill et al., 1992; Häusler et al., 1992). Yeast cells containing different chimeric proteins were assayed for killer toxin sensitivity and comparisons were made between a sensitive wild-type strain, a resistant kre2 null strain, and the null strain containing plasmids bearing wild-type KRE2 or different chimeric constructions. As can be seen in Fig. 9, the kre2 null mutant is toxin resistant, showing no killing zone, but the same strain containing the KRE2 gene, or expressing KDKK or KPKK displays a large clear killing zone of ~20 mm similar to wild-type cells consistent with correct Golgi localization. Chimeric protein KD-K has also been shown to be localized to the Golgi complex but has a reduced zone size (15 mm) likely as a consequence of its reduced mannosyltransferase activity. The reduced killing zone (10 mm) of cells expressing DKKK which has wild-type enzymatic activity in vitro indicates that it is not correctly retained in the Kre2p Golgi subcompartment. MR/KKK has also been shown to be localized to the vacuole, but displays an almost wild-type zone size (17.5 mm). Its catalytic domain appears to have a normal conformation as it possesses wild-type mannosyltransferase activity, but it is partially retained in the ER (see above). Thus, MR/KKK while slowly passing through the secretory pathway en route to the vacuole, is likely able to correctly mannosylate cell-wall proteins.

Finally, these results indicate that for Kre2p the default compartment for mislocalization is the vacuole, a conclusion previously reached for late Golgi membrane proteins (Cooper and Bussey, 1992; Roberts et al., 1992; Wilcox et al., 1992; Nothwehr et al., 1993) and some ER membrane proteins (Gaynor et al., 1994; Hill and Stevens, 1994).

**A Region of Kre2p Encompassing the Cytoplasmic Tail, the TMD, and a Partial Stem Region Is Necessary to Localize a Reporter Protein to the Yeast Golgi Complex**

To determine which regions of Kre2p are sufficient to target a reporter protein to the Golgi complex, chimeric proteins KKK and KKKK were constructed (see Fig. 6 and Materials and Methods for details). KKK consists of the first 36 amino acid residues following the TMD, and a segment encompassing the first 36 amino acid residues of the stem domain fused to...
the Pho8p luminal domain. Both chimeric proteins were first verified to be membrane associated and properly glycosylated (data not shown), indicative of a correct type II membrane orientation (Fig. 6). Chimeric protein KKKP was found, like wild-type Kre2p, to be typically localized to punctiform Golgi structures (Fig. 10). However, when the partial stem domain was removed, the majority of the resulting chimeric protein (KKP) was found to be localized in the vacuole. The intracellular localization of chimeric protein KKP was quantitatively scored (see Materials and Methods); 70% of positive cells displayed a vacuolar signal and 26% of the cells had vacuolar and punctate fluorescent signals (typical results are shown in Fig. 10), whereas 4% showed exclusively punctiform fluorescence. Thus, the Kre2 protein region encompassing the NH2 terminus, TMD, and partial stem domain is sufficient for full Golgi retention of a vacuolar protein. These results were extended biochemically by examining the processing kinetics of both chimeric proteins. The Pho8p alkaline phosphatase is activated in the vacuole (Klionsky and Emr, 1989; Nothwehr et al., 1993) where the catalytic luminal domain is processed by the proteinase A (Ammerer et al., 1986; Woolford et al., 1986). KKKP was found not to be cleaved, whereas in contrast, the majority of KKP was processed in accord with its mostly vacuolar location (data not shown).

Lastly, to assess if the Kre2p TMD is required for proper Golgi retention in the context of an heterologous luminal domain, we constructed fusion protein KPKP in which the Kre2p TMD of KKKP was removed and substituted with that of Pho8p (see Fig. 6). Golgi retention of this fusion protein would indicate that the Kre2p NH2 terminus cytoplasmic and stem domains are sufficient to achieve correct Golgi intracellular localization. Immunofluorescence studies of KPKP revealed that this chimeric protein was retained in the ER (data not shown), and thus uninformative.

Discussion

Kre2p/Mntlp Is a medial-Golgi Protein

Kre2p is part of the yeast glycosylation machinery and was anticipated to be localized in a medial-Golgi compartment where N- and O-modified proteins receive α1,2-linked mannose residues (Fig. 2). Evidence presented here is consistent with this expectation. We have demonstrated that in situ detection of Kre2p by indirect immunofluorescence reveals intracellular punctiform staining typical of the yeast Golgi, in agreement with Chapman and Munro (1994). Kre2p is an α1,2-mannosyltransferase that receives an Mnnlp-dependent α1,3 modification, the next sequential step in the orderly glycosylation of yeast proteins. Thus, Kre2p must reach a Golgi compartment harboring the Mnnlp α1,3-mannosyltransferase (Fig. 2). These α1,2- and α1,3-linked activities could be colocalized, or, if they are situated in two distinct and consecutive cisternae, Kre2p would have to reach the Mnnlp compartment and then be retrieved to its own preceding organelle. Kre2p and Mnnlp have been shown to colocalize to a considerable extent, placing Kre2p in or close to the Mnnlp medial-Golgi compartment.

Consistent with this assignment, previous biochemical studies provide evidence that Kre2p is localized in a compartment distinct from the late Golgi. Immunoprecipitated late Golgi organelles containing the endoproteinases Kex1p, Kex2p, and DPAP α were shown to be devoid of Kre2p (Bryant and Boyd, 1993). Our immunocytochemical colocalization results also indicate that Kre2p is not in the same compartment as Kex1p. The 35% of Kre2p and Kex1p punctiform fluorescence signals that do colocalize could be due to two or more stacked cisternae that are seen in ~40% of all cisternae in a given yeast cell (Preuss et al., 1992). However, the possibility remains that proteins from the medial-Golgi (Kre2p) and from the late
The apparent double role of Kre2p in O- and N-linked glycosylation (Häusler et al., 1992; Hill et al., 1992; Lussier et al., 1995b) is most simply explained if both types of modification are carried out in the same Golgi compartments. The observations that Mnn1p also acts on O- and N-linked chains (Ballou, 1990; Herscovics and Orlean, 1993), and that the O-glycosyltransferase Kre2p is itself N-glycosylated, are consistent with a common set of compartments for these modifications. However, more complex scenarios could be envisaged with specific subcompartments for O- and N-glycosylation.

Kre2p is predicted to act in a functionally and genetically defined compartment where various α1,2-mannosyl residues are added to oligosaccharide chains. This compartment(s) is also likely to include other α1,2-mannosyltransferases responsible for N-linked outer chain elaboration (Fig. 2). A family of putative Kre2p-like mannosyltransferases has been identified (Hill et al., 1992; Bussereau et al., 1993; Lussier et al., 1993; Mallet et al., 1994), and some members have been shown biochemically to be Golgi mannosyltransferases (Lussier et al., 1995b).

### Mannosyltransferase Targeted?

Immunolocalization results from the different chimeric proteins demonstrate that the Kre2p NH2-terminal cytoplasmic domain was required for localization to the Golgi complex. Chimeric proteins lacking the 11-amino acid residue cytoplasmic domain are fully active as a mannosyltransferase in vitro but fail to be properly retained and are mislocalized to the vacuole. A trivial explanation for the localization of DKKK to the vacuole is that the cytoplasmic domain of DPAP B is not necessary for vacuolar targeting (Roberts et al., 1992; Nothwehr et al., 1993). To ensure that mislocalization of DKKK was not caused by some previously unrecognized vacuolar targeting signal of the NH2-terminal domain of DPAP B, chimeric protein MR/KKK was constructed in which the Kre2p cytoplasmic tail was deleted. As with DKKK, MR/KKK was mislocalized to the vacuole, showing that the NH2-terminal domain of Kre2p is necessary for proper Golgi targeting.

Chimeric proteins KDKK and KPKK could not be distinguished from wild-type Kre2p, permitting the conclusion that the Kre2p TMD in the context of a native protein is not necessary for correct Golgi localization. Chimeric construct KD-K which comprises the Kre2p cytoplasmic tail, the DPAP B membrane-spanning domain, a Kre2p partial stem region, and the Kre2 protein mannosyltransferase domain was correctly localized to the Golgi complex. This indicates that the first 36 amino acid residues of the stem region of Kre2p are dispensable for Golgi targeting. The results obtained with this fusion protein again show that the Kre2p TMD is not required for Golgi retention. The fact that in the KD-K construct, the Kre2p TMD and part of the stem region are not present, argues that in the context of a protein containing a Kre2p luminal catalytic domain the cytoplasmic tail of Kre2p is sufficient to correctly target such a chimera to the Golgi complex.

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**Table:**

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**Figure 9.** Activity of chimeric proteins. Enzymatic activity was measured in vitro by α1,2-mannosyltransferase assays of membrane preparations, and in vivo mannosylation of cell-wall proteins was assessed by verifying K1 killer toxin sensitivity in a wild-type strain and a kre2 null mutant containing KRE2 and chimeric constructs expressed from the centromeric-based plasmid pRS316 (Sikorski and Hieter, 1989). Enzymatic activity is expressed as percentages of specific activity (mmol/h/mg) for each chimeric protein. A kre2 null strain expressing KRE2 from pRS316 arbitrarily represents 100% specific activity. Concentrated K1 killer toxin was spotted on a lawn of ~10^6/ml cells from a fresh culture of each strain (see Materials and Methods).
Figure 10. Localization of chimeric proteins KKKP and KKP. Yeast cells carrying a PHO8 disruption (pho8::TRP1) in a SEY6210 background and containing the different chimeric constructions on YEp352 were fixed, spheroplasted, attached to polylysine-coated glass slides, and incubated with anti-Pho8p antibodies followed by a subsequent incubation with a FITC-coupled anti-rabbit secondary Ab and DAPI. Colocalization of vacuolar localized chimeric proteins with the yeast vacuolar membrane H+-ATPase was achieved by coincubation of fixed cells along with mAb 13D11 (Kane et al., 1992) followed by incubation of treated cells with Texas red-conjugated anti-mouse secondary antibodies. Arrows point to Golgi punctiform structures.

less some remaining luminal sequence also plays some role in retention.

To analyze further the targeting role of the three nonenzymatic domains of Kre2p and to remove the possible additional complexity of a targeting mechanism involving the mannosyltransferase portion of Kre2p, combinations of noncatalytic domains of Kre2p were tested to assess what region of Kre2p was sufficient to target a Pho8p reporter protein to the Golgi complex. As opposed to the results obtained with fusion protein KD-K, all three noncatalytic domains of the Kre2 protein were found to be required for full Golgi retention of the Pho8p luminal region (KKKP). These results are in agreement with those of Chapman and Munro (1994) who found that a fusion protein containing the NH2 terminus, the membrane-spanning domain, and a partial stem region of Kre2p linked to a reporter protein was retained in the Golgi complex. The mostly vacuolar localization of KKP demonstrated that the first part of the Kre2p stem region which is not required for retention in the context of a Kre2p catalytic domain (KD-K), is necessary, in combination with the Kre2p cytoplasmic tail and TMD for the targeting of the Pho8p catalytic portion to the Golgi complex (KKKP). Taken together, the intracellular localizations of chimeric proteins KD-K, KKP, and KKKP suggest that the Kre2p luminal domain does play a role in Golgi localization.

Data obtained with chimeric proteins KDKK, KP KK, KD-K, and KKP clearly show that the Kre2p TMD is dispensable for Golgi retention and not sufficient to retain a reporter protein in the yeast Golgi complex. Our results are, however, at variance with conclusions reached in a recent study of Chapman and Munro (1994) who found that a fusion protein containing the NH2 terminus, the membrane-spanning domain, and a partial stem region of Kre2p linked to a reporter protein was retained in the Golgi complex. The mostly vacuolar localization of KKP was inferred to be in the Golgi complex as no invertase was secreted from the cell. When the TMD of Kre2p was replaced with that of Pho8p (MPMI) in this protein, it appeared to be mislocalized to some extent, as some invertase was secreted from the cell. This result was interpreted to indicate a role for the Kre2p TMD in Golgi retention. However, the intracellular localization of this chimeric protein was not visualized by immunofluorescence. We replaced the Kre2p TMD in the native protein with two different nonrelated TMD’s, and showed that both proteins (KDKK and KP KK) were Golgi localized by using indirect immunofluorescence microscopy. In addition, we tested that the proteins had the right conformation by measuring their glycosylation in vivo, and their mannosyltransferase activity in vitro and in vivo. These proteins (KDKK and KP KK) behaved phenotypically as wild-type Kre2p in our tests with no indication that the Kre2p TMD in the context of a native protein is necessary for proper retention. A possible explanation of the Chapman and Munro (1994) results is that the Kre2p TMD is somehow necessary for Golgi retention in the context of those large heterologous proteins perhaps because some retention property of the luminal domain of Kre2p has been disrupted in the invertase fusion. Unfortunately, the ER retention of chimeric protein KPK did not permit resolution of this question.

Second, Chapman and Munro (1994) reported that a Pho8 chimeric protein containing a Kre2p TMD (chimeric protein PKP) slowed vacuolar processing of the luminal portion of Pho8p. They inferred from their results that PKP was at least partially localized in the Golgi complex and was retained by the Kre2p TMD. Again, the PKP chimeric protein was not localized intracellularly. This is clearly different from our results with Pho8p chimeras,
which show that the Kre2p cytoplasmic NH$_2$-terminal and transmembrane domains (KKP) are not sufficient for proper Golgi retention and the presence of the Kre2p cytoplasmic tail, TMD, and stem region is required for full retention of a Pho8p reporter protein (KKKP) in the yeast Golgi. To resolve this apparent inconsistency, we obtained the PKP construct and, on examination of its cellular location, found that it was exclusively localized to the vacuole displaying no punctiform fluorescence (data not shown). This finding is entirely consistent with our localization results with KKP, and offers no support for the Chapman and Munro conclusion that the Kre2p TMD will at least partially retain a heterologous protein in the yeast Golgi.

The membrane-spanning domain plays a crucial role in the localization of resident animal Golgi glycosyltransferases. In contrast to Kre2p, alterations of the TMD of a certain number of mammalian glycosyltransferases results in some mislocalization of the modified protein (Munro, 1991; Aoki et al., 1992; Teasdale et al., 1992; Burke et al., 1994; Gleeson et al., 1994). The hydrophobic TMD of several mammalian glycosyltransferases appears to be both necessary and sufficient to confer complete Golgi retention (Nilsson et al., 1991; Aoki et al., 1992; Shaper and Shaper, 1992; Teasdale et al., 1992; Gleeson et al., 1994; Low and Hong, 1994) whereas in other cases, some additional flanking sequences are required to achieve effective Golgi targeting (Munro, 1991; Burke et al., 1992; Shaper and Shaper, 1992; Dahdal et al., 1993; Burke et al., 1994; Gleeson et al., 1994; Low and Hong, 1994). The exact nature of these NH$_2$-terminal and stem sequences remains the be precisely determined but do not appear to involve the whole of each domain but mainly the short stretches of hydrophilic residues directly flanking the lipid-embedded TMD (Munro, 1991; Burke et al., 1992; Shaper and Shaper, 1992; Dahdal et al., 1993; Gleeson et al., 1994; Low and Hong, 1994).

It has been postulated that the targeting of glycosyltransferases carrying a TMD sorting signal could be due to interactions between the membrane-spanning domain and compartment specific membrane lipids (Pelham and Munro, 1993). There is no evidence that Kre2p is retained by such a mechanism, since in the series of constructs we have devised, the Kre2p TMD is not implicated in retention. In this lipid interaction model, the length of the membrane-spanning domain is important for the proper sorting of animal glycosyltransferases (Munro, 1991; Pelham and Munro, 1993). In the case of KDKK the TMD of DPAP B is three amino acid residues shorter than the Kre2p TMD, and in the case of KPKK the TMD of Pho8p is seven amino acid residues longer than the Kre2p TMD, yet both are retained in the Golgi complex.

None of the studied animal glycosylation enzymes appear to need an intact cytoplasmic domain to achieve Golgi retention (Munro, 1991; Aoki et al., 1992; Colley et al., 1992; Shaper and Shaper, 1992; Teasdale et al., 1992; Dahdal et al., 1993; Burke et al., 1994; Gleeson et al., 1994; Low and Hong, 1994). Kre2p, thus, constitutes the first demonstration of an eukaryotic glycosyltransferase requiring a short cytoplasmic amino-terminal domain for correct intracellular localization. The 11–amino acid residue-targeting domain of Kre2p seemingly parallels that of the late yeast Golgi enzymes Kex1p, Kex2p and DPAP A (Cooper and Bussey, 1992; Wilcox et al., 1992; Nothwehr et al., 1993). It was shown for DPAP A that a 10–amino acid region within the cytoplasmic domain was both required and sufficient for proper retention (Nothwehr et al., 1993), and for Kex2p, a 27–amino acid region was found to be essential (Wilcox et al., 1992). The accurate Golgi targeting of Kex2p and DPAP A is clathrin dependent and aromatic residues are thought necessary for this retention process (Wilcox et al., 1992; Nothwehr et al., 1993; Wilzbach and Payne, 1993). While unrelated at a sequence level with Kex2p and DPAP A, the amino-terminal domain of Kre2p contains a single aromatic phenylalanine residue (MALFLSKRLLR) which was mutated to an alanine, an alteration showing no effect on targeting and, therefore, providing no evidence for clathrin-based retention of Kre2p (Lus-sier, M., A.-M. Sdicu, T. Ketela, and H. Bussey, unpublished results).

The Mnn1p α1,3-mannosyltransferase is the only other yeast glycosyltransferase where targeting has been examined. Mnn1p targeting appears different from that of Kre2p since a mutant Mnn1p lacking its NH$_2$-terminal cytoplasmic tail is properly localized to the Golgi complex (Graham et al., 1994). Correct retention of Mnn1p is clathrin dependent but, contrary to Kex2p and DPAP A, does not seem to be mediated by a direct interaction through its cytoplasmic tail. The reason for the Kre2p requirement for its cytoplasmic tail remains unclear. It could be clathrin dependent but not involving an aromatic residue, or through some other process. The Gdalp type II Golgi membrane protein (Abeijon et al., 1989; Abeijon et al., 1993) has been shown to be properly localized in a strain lacking clathrin heavy chains (Seeger and Payne, 1992; Wilzbach and Payne, 1993). The Mnn1p, Kre2p, and Gdalp enzymes appear to be in close proximity or even in the same Golgi compartment (this work; Abeijon et al., 1989; Graham et al., 1994). This raises the possibility that specific Golgi membrane proteins showing a similar compartmental distribution may be retained by more than one mechanism.

Our results indicate that although only the cytoplasmic NH$_2$ terminus has been shown to be required for Golgi retention, no single domain is able to specify correct Golgi localization. A chimeric protein including a Kre2p cytoplasmic tail and enzymatic domain (KD-K) was properly targeted. In contrast, a luminal reporter protein could only be properly retained in the Golgi complex by the Kre2p cytoplasmic tail, plus the membrane-spanning and a 36–amino acid residue stem domain (KKKP). The entire Kre2p cytoplasmic tail and membrane-spanning domain were not sufficient to retain Pho8p in the Golgi complex (KKP). Therefore, it appears that a combination of Kre2p topological domains is needed to achieve proper Golgi localization.

The presence or requirement of more than one specific targeting signal in a given secretory pathway membrane–associated protein has been hinted at in a few cases recently. The TMD might not be the sole targeting motif present in particular animal glycosyltransferases as some cytoplasmic and luminal sequences appear to be involved in retention (Munro, 1991; Burke et al., 1992; Shaper and Shaper, 1992; Dahdal et al., 1993; Burke et al., 1994; Gleeson et al., 1994; Low and Hong, 1994). The trans-Golgi
network protein TGN38 appears to achieve proper Golgi localization using two nonoverlapping targeting domains: a tyrosine-based cytoplasmic retrieval signal and a retention signal found in its membrane spanning domain (Bos et al., 1993; Humphrey et al., 1993; Wong and Hong, 1993; Ponnambalam et al., 1994). For the CGN Sed5p syntaxin, the cytoplasmic domain appears to be involved (Banfield et al., 1994). Targeting studies on the p63 type II membrane protein indicate that the cytoplasmic, membrane-spanning and luminal domains are all necessary for proper ER–Golgi intermediate compartment localization (Hauri and Schweizer, 1992; Schweizer et al., 1993, 1994). Finally, the Wbp1 type I membrane protein of the yeast ER possesses a cytoplasmic KKKK Golgi-to-ER retrieval motif. When this targeting motif is inactivated, Wbp1 is still ER retained indicating that another targeting signal is present in the protein (Gaynor et al., 1994).

The fact that Kre2p requires more than one topological segment to achieve proper Golgi localization, emphasizes that there may be distinct, but not necessarily mutually exclusive, mechanisms of retention. Golgi proteins that possess a cytoplasmic targeting signal have been proposed to be sorted via a receptor-mediated mechanism (Wilsbach and Payne, 1993; Gleeson et al., 1994; Low and Hong, 1994). Overexpression of the late Golgi yeast proteins Kex1p, Kex2p, and DPAP A leads to some vacuolar mis-localization, indicating saturation of the capacity of a receptor-based sorting process (Cooper and Bussey, 1992; Wilcox et al., 1992; Nothwehr et al., 1993; Wilsbach and Payne, 1993). Kre2p overexpression also results in some mistargeting to the vacuole, and could be similarly explained. When wild-type Kre2p is expressed from a 2μ-based multicopy vector in a background with mutations in the major vacuolar hydrolases, all positive cells display a punctiform pattern of fluorescence with 15% of the stained cells also showing vacuolar fluorescence. This small percentage of doubly stained cells are likely expressing high levels of Kre2p as determined by the intensity of the fluorescence. In contrast, overexpression of animal glycosyltransferases does not bring about saturation of the retention mechanism (Munro, 1991; Aoki et al., 1992; Burke et al., 1992; Colley et al., 1992; Teasdale et al., 1992; Gleeson et al., 1994). Interpreting our data in the simplest way, the Kre2p segment encompassing the NH2 terminus along with the TMD and stem region could interact directly with a medial-Golgi localized receptor spanning both sides of the Golgi lipid bilayer. For Kre2p to be retained in the Golgi, an interaction between the putative receptor and the NH2-terminal domain is essential. Part of the presumed Kre2p receptor complex could be part of the Golgi extracisternal space matrix which in mammals was recently shown to bind medial-Golgi enzymes presumably through their cytoplasmic tails (Siusarewicz et al., 1994). Interestingly, examination of the cytoplasmic NH2-terminal domains of the six members of the KRE2 mannosyltransferase family (Lussier et al., 1993; Mallet et al., 1994) reveals that the Kre2p (MALFSLRLLR) sequence resembles only that of Ktr4p (MRFLSKRLK; Mallet et al., 1994) where the sequence FLSKR(1)/L(K/R) is conserved in both enzymes. This may imply a common receptor for the two proteins. Interaction of the presumed receptor protein with a chimeric construct lacking the Kre2p stem domain but including a reporter luminal domain (KKP) may be partial, and insufficient to retain the fusion protein in the Golgi complex. To achieve full Golgi retention of a reporter luminal protein, interaction of the postulated receptor with the three nonenzymic domains would be required.

In the case of KD-K, only partial interaction would occur with the putative receptor and the observed Golgi retention may now reveal an additional mechanism involving a segment of the Kre2p luminal region possibly by oligomerization/kin recognition that has been implicated in the retention of certain mammalian Golgi membrane proteins. It has been proposed that protein oligomers are assembled through their TMD and/or luminal domains in a particular Golgi cisternae and because of their high order structure are consequently excluded from entering forward-moving secretory vesicles (Weisz et al., 1993; Gleeson et al., 1994; Low and Hong, 1994; Nilsson et al., 1994; Schweizer et al., 1994; Yamaguchi and Fukuda, 1995). Applying such a model to a functionally active Kre2 protein involves self-association or formation of heterooligomers between Kre2p and other medial-Golgi protein(s) either through the catalytic domain or possibly with some contiguous stem sequences retained in construct KD-K.

Overall, our results suggest that proper targeting of Kre2p in the yeast medial-Golgi may involve two different mechanisms. The saturability of the Kre2p retention system implies a receptor-based retention mechanism that could involve protein–protein interactions over a tail/TMD/partial stem region. In addition, a second mechanism that may involve oligomerization would operate in some way through the Kre2p luminal domain. In conclusion, our observations on Kre2p targeting may point to differences in Golgi localization mechanisms between yeast and animal glycosyltransferases or could indicate that multiple retention or retrieval mechanisms are used to varying extents in both systems.

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