Localization and Targeting of the *Saccharomyces cerevisiae* Kre2p/Mnt1p α1,2-Mannosyltransferase to a *medial*-Golgi Compartment

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Abstract. The yeast Kre2p/Mnt1p α1,2-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 N-glycosylated in an *MNN1*-dependent manner. Kre2p/Mnt1p is localized in a Golgi compartment that overlaps with that containing the *medial*-Golgi mannosyltransferase Mnn1p, and distinct from that including the late Golgi protein 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 DPAP B and 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 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 saccules that are differentiated into functionally distinct subcompartments. After the addition of core N-linked sugar moieties in the endoplasmic reticulum, glycoproteins proceed through the *cis*-Golgi network, the *cis*-, *medial*-, and *trans*-cisternae, and the *trans*-Golgi network, where further modifications take place. These include the addition of O-linked sugars and the elaboration, in a protein-specific manner, of complex 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 *cis*-Golgi network constitutes the site from which many resident ER proteins are retrieved, and the main function of the 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 *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 asparagine-linked (N-linked)[1] 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 N-linked oligosaccharides, either a simple core carbohydrate or one extended by an outer-chain glycan structure. The N-linked core oligosaccharide elaborated in the ER is mainly constituted of $Man_8GlcNAc_2$ and may undergo Golgi maturation resulting in $Man_{9\text{-}13}GlcNAc_2$. 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 α1,6-linked mannose residues is attached to the core oligosaccharide. This Golgi compartment has been likened to the mammalian *cis*-Golgi network (Wilsbach and Payne, 1993). Outer chain elaboration is then brought to completion: the α1,6-linked mannose structure is enlarged in a sequential fashion by α1,2- and α1,3-mannosyltransferases in a subsequent distinct com-

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1. *Abbreviations used in this paper*: DAPI, 4′,6-diamidino-2-phenyl-indole; Kre2p, the product of the *KRE2/MNT1* gene; N-linked, asparagine-linked; O-linked, serine/threonine-linked; 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. Immunoelectron microscopy revealed that the yeast Golgi is composed of as many as 30 concave, disklike 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/Mnt1p. *KRE2/MNT1* was isolated as a gene implicated in cell-wall assembly conferring K1 killer toxin resistance when mutated (Hill et al., 1992) and found to encode an α1,2-mannosyltransferase (Häusler and Robbins, 1992). *KRE2/MNT1* encodes a 442–amino acid residue predicted type II membrane protein containing a putative transmembrane domain near its NH_2 terminus and one potential luminal N-glycosylation site. Kre2p/Mnt1p specifically adds a third mannose during the linear elongation of O-linked carbohydrate chains that may contain up to five mannose residues (Häusler et al., 1992) and is also apparently involved in asparagine-linked glycosylation (Hill et al., 1992; Lussier et al., 1995*b*).

Here we report further characterization of the *KRE2/MNT1*-encoded mannosyltransferase. We demonstrate that Kre2p/Mnt1p 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 Mnn1p *medial*-Golgi compartment. In addition, we show that the short NH_2-terminal cytoplasmic tail domain of Kre2p/Mnt1p 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/Mnt1p encompassing the cytoplasmic tail, the transmembrane domain (TMD), and a segment of the luminal stem domain.

Materials and Methods

Yeast Strains, Culture Conditions, and Procedures

All yeast constructions used strains SEY6210 (*MATa, leu2-3, ura3-52, his3-Δ200, lys2-801, trp1-Δ901, suc2-Δ9*) or clABYS86 [S86] (*MATa, Δura3-2, leu2 his3 pra1 prb2 prc1 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 (Lussier et al., 1993, 1995*a*; 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 pAHl1 plasmid (kindly provided by Drs. A. Häusler 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 NH_2-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$^+$II (Stratagene Inc., LaJolla, 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 replacement of the Kre2p transmembrane domain with that of DPAP B (Roberts et al., 1989) was obtained after mutagenesis using oligonucleotide 5′-CTCAGTAAGAGACTGTTGAGAGTCGGAATAATCC-TTGTACTCCTGATATGGGGCACTGTTTTGTTGCTAAGAACTC-AGCAATATATTCCG-3′ resulting in sequence MALFLSKRLLRV-GIILVLLIWGTVLLLRTQQYI . . . , where the underlined region corresponds to the DPAP B TMD region. **KPKK** was obtained similarly with the Kre2p TMD replaced with that of the Pho8p vacuolar alkaline phosphatase (Kaneko et al., 1987) using oligonucleotide 5′-CTCAGTAAG-AGACTGTTGAGAATAATAGTATCCACTGTGGTCTGTATTGG-TTTGTTGCTGGTTTTAGTGCAACTGGCATTTCCAACCAGTTTT-

GCTATAAGAACTCAGCAATATATTCCG-3'. The resulting chimeric protein possessed sequence MALFLSKRLLRIIVSTVVCIGLLLVLVQ-LAFPTSFAIRTQQYI . . . , where the underlined region corresponds to the Pho8p TMD.

The **DKKK** chimeric construct was obtained by replacement of the *DAP2* membrane-spanning domain with that of *KRE2* in a fusion protein consisting of the NH_2 terminus and TMD of DPAP B fused to the entire Kre2p luminal region (Lussier, M., unpublished results) after mutagenesis using oligonucleotide 5'-CATTTGTTAGATAAGCTCATAAGGTTT-ACCGTCATTGCAGGTGCGGTTATTGTTCTCCTCCTAACATTG-AATTCCAACAGTCGTACGCAGCAATATATTCCG-3' resulting in amino acid sequence MEGGEEEVERIPDELFDTKKKHLLDKLIRF-**TVIAGAVIVLLLTLNSNS**RTQQYI . . . , where the 29–amino acid NH_2 terminus of DPAP B (*underlined*) are fused to the Kre2p TMD (*bold*). Chimeric protein **MR/KKK** was made by looping out, with oligonucleotide 5'-CAGTTCCACTCGAAAGCATGAGATTTACCGTCATTGC-AGG-3', the 27-bp *KRE2* DNA fragment corresponding to all amino acid residues of the cytoplasmic NH_2 terminus with the exception of the initiation codon and Arg residue lying directly adjacent to the membrane-spanning domain resulting in sequence MR**FTVIAGAVIVLLLTLNSN-SRTQQYI** . . . with the TMD shown in bold. Chimeric protein **KD-K** contains the membrane-spanning domain 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 **KDKK** and a second MluI site 105 bp downstream from this region. The mutagenized **KDKK** DNA was digested with the restriction enzyme MluI, religated, and the sequence encoded by the resulting construct was MALFLSKRLLRVGIIL-VLLIWGTVLLL**TRLEQSALN** . . . , 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 NH_2 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 domains of both Kre2p and Pho8p, followed by replacement of the Kre2p luminal domain with that of Pho8p, resulting in the sequence MALFLSKRLL-R**FTVIAGAVIVLLLTLNSNS**RTASHKKKNV . . . , where the Kre2p NH_2 terminus and TMD (*bold*) are fused to the Pho8p luminal domain (*underlined*). **KKKP** comprises the NH_2 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 *KRE2* 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 *KRE2* region encompassing the entire NH_2 terminus, TMD, and partial stem domain. The amino acid sequence of **KKKP** is MALFLSKRLLR**FTVIAGA-VIVLLLTLNSNS***RTQQYIP* . . . *DAKKTR*ASHKKKNV . . . in which the Kre2p NH_2 terminus, TMD (*bold*), and stem region (*italics*) are fused to the Pho8p luminal domain (*underlined*). Finally, **KPKP** was obtained by replacing the Kre2p TMD in **KKKP** with that of Pho8p using the same oligonucleotide that was used to obtain **KPKK**.

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-^{35}S-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 Kre2p peptide was coupled to cyanogen bromide–activated Sepharose CL-6B (Pharmacia LKB Biotechnology, Inc., Montréal, Canada) and used in a column to affinity purify the antiserum 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., 1995*a*). 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 [^{35}S]methionine-labeled proteins was as described (Cooper and Bussey, 1989). Briefly, cells were grown to an OD_{600} of 0.4–0.45 in selective medium (YNB) and then labeled with Trans^{35}S (100 μCi; ICN Biochemicals Inc., Montréal, Canada) for 10 min at 30°C. Yeast cells treated with tunicamycin (10 μg ml^{-1}) were preincubated before radiolabeling for 30 min at 30°C. Tunicamycin is a hydrophobic analogue of UDP-*N*-acetylglucosamine that blocks the addition of *N*-acetylglucosamine to dolichol phosphate, the first step in N-linked oligosaccharide formation. Chase conditions were achieved by addition of L-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 OD_{600} 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 1,000 *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 $MnCl_2$, 0.1% Triton X-100, 0.2 mM GDP-[^{14}C]mannose, and 10 mM α-D-methylmanno-pyranoside as an acceptor (Sigma Chemical Co.) and 10 μl of membrane fraction in a total vol of 20 μl. Reaction mixtures were incubated for 30–60 min at 30°C and then passed through a resin (AG1-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 (mmol/h/mg of membrane protein) for each chimeric protein.

Immunofluorescence

Yeast cells were grown in selective medium to an OD_{600} 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 100 mM Tris-HCl, pH 8, 25 mM DTT, 5 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 used 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 YPY-DVPDYA) was inserted by oligonucleotide-directed mutagenesis directly

at the COOH-terminal domain of Mnn1p and in the region corresponding to the Kex1p luminal domain. Epitope-tagged Mnn1p and Kex1p were detected with the 12CA5 monoclonal antibody (Kolodziej and Young, 1991). The latter was used at dilutions ranging from 1:250–1:1,000. mAb 13D11 which recognizes the 60-kD subunit of the yeast vacuolar membrane H^+-ATPase (Kane et al., 1992) was used at dilutions of 1:10–1:25 as a vacuolar marker for colocalization studies with Kre2p chimeric proteins. Fluorescence signals were obtained by subsequent incubation of treated cells with rhodamine X sulfonyl chloride (Texas red)–conjugated goat anti–rabbit IgG (1:50–1:200) and FITC-conjugated goat anti–mouse IgG (1:50–1:200) which were used as secondary antibodies. Nuclei and mitochondria were visualized by staining with 4′,6-diamidino-2-phenyl-indole (DAPI). Cells were examined with an epifluorescence microscope (Axiophot; Carl Zeiss, Inc., Thornwood, NY), and photographed with film (T-Max 400; Eastman Kodak Co., Rochester, NY).

The extent of colocalization of Kre2p with Mnn1p or Kex1p was scored by quantitating in a given dual localization experiment 30 cells that contained clearly defined signals representing 200–250 individual fluorescent punctate spots for each antigen. Compiled data revealed that Mnn1p gave rise to approximately the same number of punctate spots per cell as Kre2p and 75% of the punctiform fluorescent spots from Texas red and FITC overlapped. Kex1p gave rise to ~10% fewer punctate spots per cell compared with Kre2p-associated signals, and 65% of the punctiform fluorescent spots emanating from both proteins did not overlap. Finally, the intracellular localization of chimeric protein **KKP** was quantitatively scored by examining 750 individual cells containing clearly defined signals. Punctiform fluorescence different from nucleus, ER, or vacuole was defined as Golgi localization. Vacuolar localization was determined by colocalization with the 60-kD vacuolar membrane H^+-ATPase subunit.

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 NH_2 terminus, a hydrophobic transmembrane region, and a large luminal enzymatic domain containing one potential N-glycosylation site (Fig. 1 *A*). 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 *kre2::TRP1* strain (Fig. 1 *B*), 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. 1 *C*, 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. 1 *B*) 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 (Asn^{197}-Gln-Thr) (see Fig. 6). To test for Kre2p N-glycosylation, yeast cells were [^{35}S]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 Asn^{197} 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-

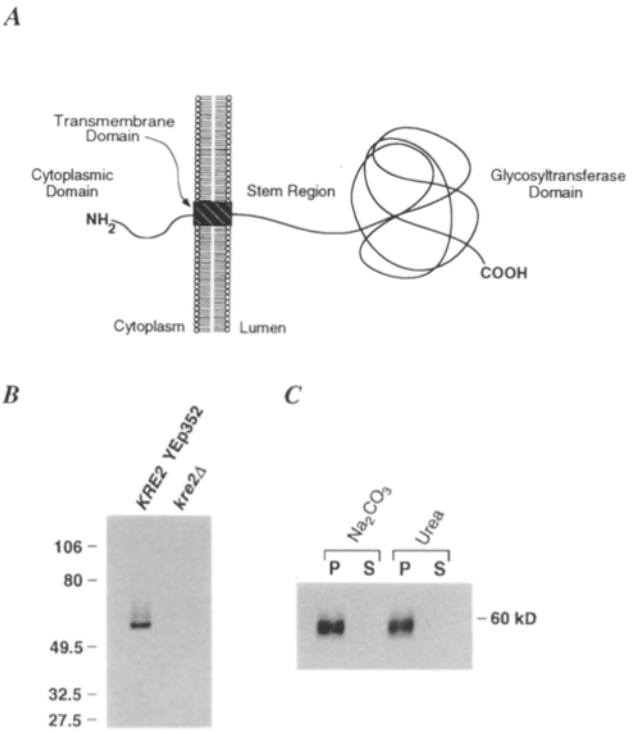

Figure 1. Topologic representation and identification of the Kre2p membrane-associated α1,2-mannosyltransferase. (*A*) Kre2p is oriented as a type II membrane-anchored protein, a topology characteristic of all isolated glycosyltransferases (Shaper and Shaper, 1992; Kleene and Berger, 1993; Gleeson et al., 1994). Kre2p consists of a short amino-terminal cytoplasmic domain, a hydrophobic transmembrane domain, and a large carboxy-terminal luminal catalytic domain. The catalytic domain is linked to the transmembrane domain by a polypeptidic "stem" region. The stem region is generally thought to be devoid of secondary structure. (*B*) Immunological detection of Kre2p. Yeast total-protein extracts from *kre2::TRP1* in SEY6210 and from the same strain expressing *KRE2* from the multicopy plasmid YEp352 (Hill et al., 1986), were immunoblotted with affinity-purified anti-Kre2p polyclonal antibodies (see Materials and Methods). The molecular mass standards are shown in kilodaltons. (*C*) Kre2p is membrane associated. SEY6210 cells overexpressing *KRE2* were lysed, and cell debris was removed by centrifugation. The resulting crude homogenate was aliquoted, and fractions were rendered 0.1 M with Na_2CO_3 or 1.6 M with urea. After 30 min, each fraction was centrifuged at 100,000 *g* for 1 h and after SDS-PAGE, Kre2p was detected by immunoblotting with the anti-Kre2p Ab in pellet (*P*) and supernatant (*S*) fractions.

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 subcompartments it had been exposed, the size of the Kre2p-linked oligosaccharide chains was examined in specific N-linked glycosylation mutants. The *mnn* strains carry mutations at various loci resulting in glycosylation defects (Fig. 2; Ballou, 1990; Ballou et al., 1990). The N-linked carbohydrates from an *mnn9* strain lack the outer chain. The *mnn1* strain produces glycoproteins with N-linked chains lacking terminal α1,3-mannosyl residues (Fig. 2).

Wild-type, *mnn1*, and *mnn9* mutant yeast strains carrying *KRE2* were [^{35}S]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 α1,3-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 *mnn1* strain is approximately of wild-type size (Fig. 3, lane *3*). After a 45-min chase, however, the *mnn1*-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 Mnn1p α1,3-mannosyltransferase adds at least three mannose residues to the Kre2p *N*-glycosyl core. Mnn1p therefore contributes to most of the observed Kre2p post-ER modifications (Fig. 2).

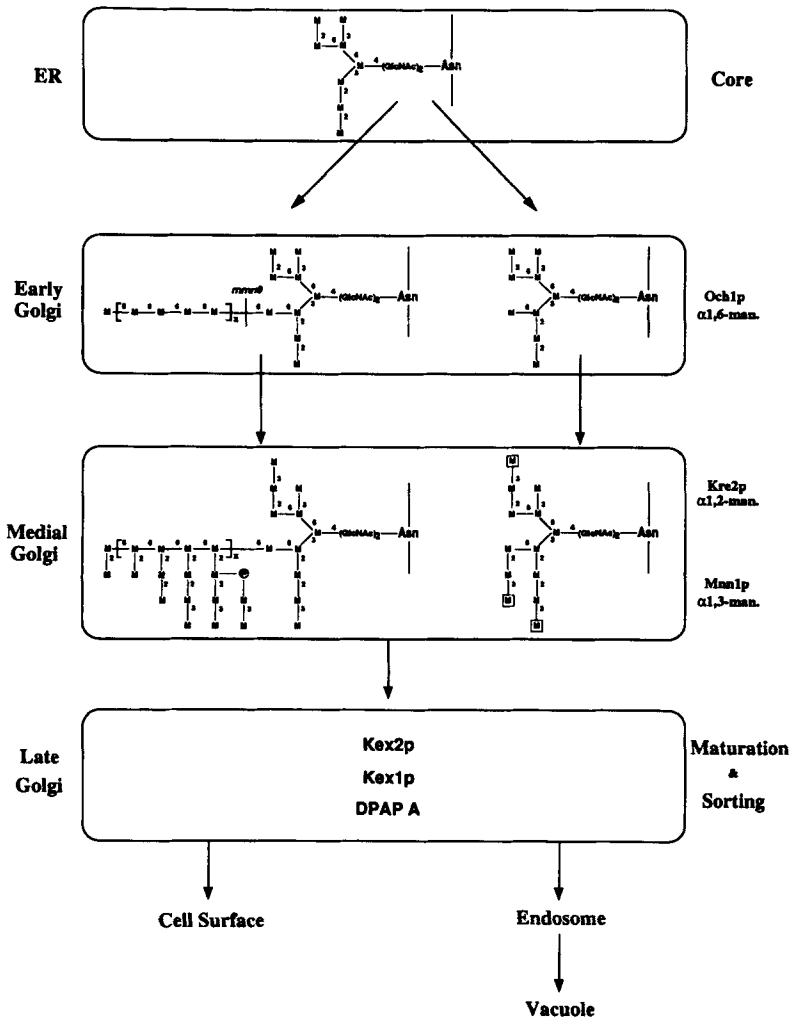

Figure 2. Elaboration of N-linked carbohydrate modifications and putative compartmental organization of the yeast Golgi complex. Yeast glycoproteins can acquire two different types of N-linked modifications after acquiring a $Man_8GlcNAc_2$ core in the ER. The core oligosaccharide can undergo maturation in the Golgi resulting in a $Man_{8\text{-}13}GlcNAc_2$ 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 α1,6-mannosyl residues to which are attached branched α1,2- and α1,3-mannosyl side chains. Recent evidence suggests that the initiating Och1p α1,6-mannosyltransferase (Nakayama et al., 1992) defines a very early Golgi compartment that appears to be distinct from a subsequent early Golgi compartment responsible for α1,6-mannosyl side chains elongation (Gaynor et al., 1994). The α1,2- and α1,3-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 β1,4, α1,6, α1,2, and α1,3 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 Mnn1p-terminal α1,3-mannosyltransferase is responsible for the addition of all α1,3-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.

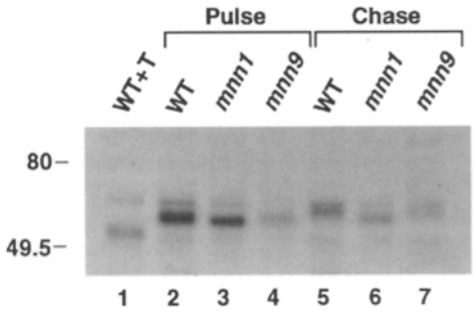

Figure 3. The *N*-glycosyl moiety of Kre2p is modified in the Golgi apparatus in a Mnn1p-dependent manner. Kre2p was immunoprecipitated from wild-type (SEY6210), *mnn1*, and *mnn9* yeast strains containing *KRE2* on a multicopy 2-μm plasmid after [^{35}S]methionine labeling for 10 min and after a 45-min additional chase. Carbohydrate alterations of Kre2p were subsequently examined by SDS-PAGE analysis of immunoprecipitated proteins using the affinity-purified anti-Kre2p Ab. T, a 30 min tunicamycin treatment before radioactive labeling. The molecular mass standards are shown in kilodaltons.

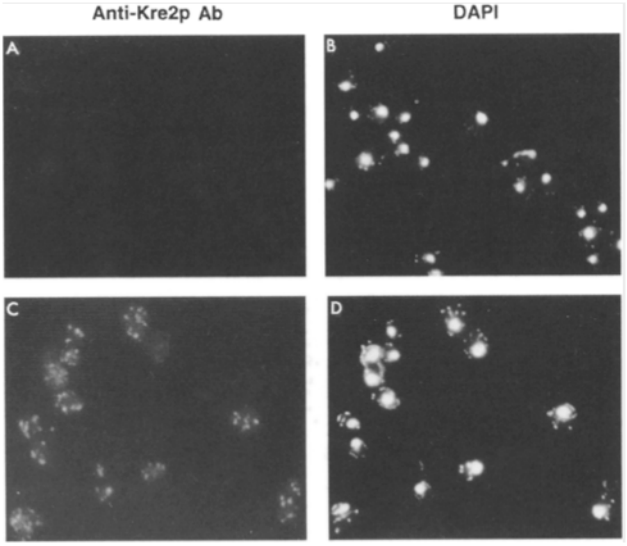

Figure 4. Cellular localization of the Kre2 protein by indirect immunofluorescence. Diploid yeast (SEY6210) containing both copies of *KRE2* gene disrupted (*A* and *B*) or including *KRE2* on a multicopy plasmid YEp352 (*C* and *D*) were fixed, spheroplasted, attached to polylysine-coated glass slides, and then incubated with affinity-purified anti-Kre2p Ab and DAPI. Texas red–coupled secondary Ab was added to detect antigen–immunoglobulin complexes (*A* and *C*). Cellular DAPI staining of nuclear and mitochondrial DNA is shown in *B* and *D*.

Kre2p Is in a Golgi Location that Overlaps with the medial-Golgi Mannosyltransferase Mnn1p Compartment, a Location Distinct from that Containing the Late Golgi Protein Kex1p

Both the function of Kre2p as a α1,2-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 Mnn1p (Ballou, 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 Mnn1p 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 NH_2-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 NH_2 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**, **KPKK** was also found to be localized to the Golgi

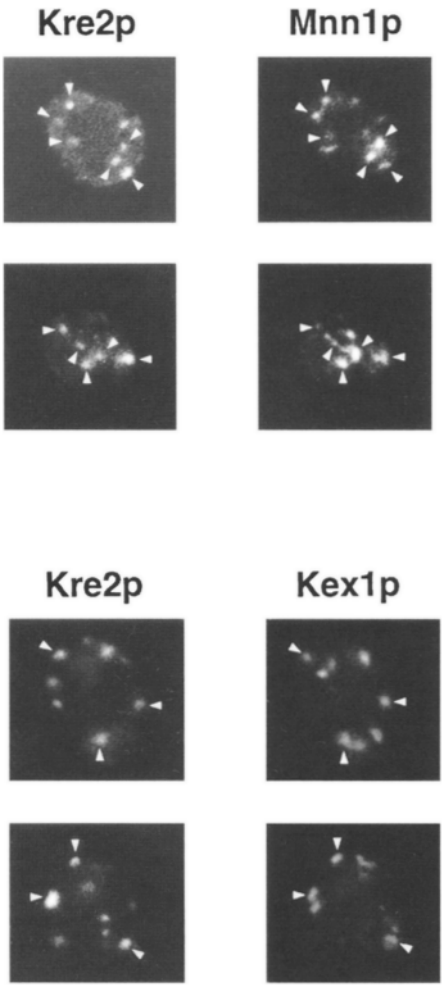

Figure 5. The Kre2p Golgi localization overlaps with that of the *medial*-Golgi mannosyltransferase Mnn1p, but is distinct from the late Golgi protein Kex1p. Double-immunofluorescence labeling of the Golgi proteins Kre2p and Mnn1p or Kex1p was performed as described in Materials and Methods. Diploid 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.

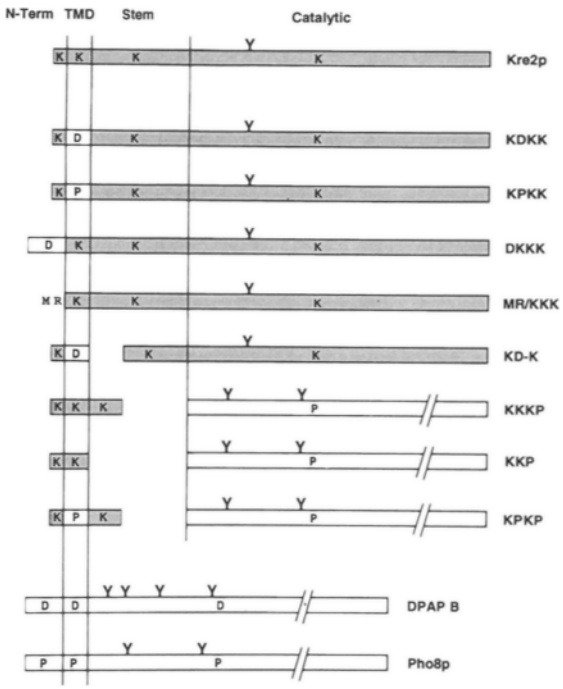

Figure 6. Schematic representation of chimeric proteins used in the analysis of Kre2p Golgi targeting. The Kre2 protein is composed of 442 amino acid residues. The length of the different Kre2p domains are as follows: NH_2 terminus, 11 residues; TMD, 19 residues; stem, ~87 residues and the catalytic domain is ~325 amino acid residues long. DPAP B is an 841–amino acid residue type II vacuolar membrane protein with cytoplasmic NH_2-terminal and membrane-spanning domains of 29 and 16 amino acid residues, respectively, and a luminal domain consisting of 796 amino acid residues. The vacuolar alkaline phosphatase Pho8p is a 566–amino acid residue type II membrane protein. The length of the different Pho8p domains are as follows: NH_2 terminus, 33 residues; TMD, 26 residues; and the luminal domain is 507 amino acid residues long. The different chimeric constructions were made by replacing or fusing specific Kre2p domains with those of vacuolar DPAP B and Pho8p. Chimeric proteins are represented (not to scale) as an assembly of different domains which are distinguished by a letter specifying their origin. *K*, *D*, and *P*, respectively, denote Kre2p, DPAP B, and Pho8p. MR represents the two remaining amino acid residues in the cytoplasmic NH_2 terminus of Kre2p after truncation. Putative N-glycosylation sites are indicated (Y). For details on constructions, see Materials and Methods.

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 NH_2 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 NH_2 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

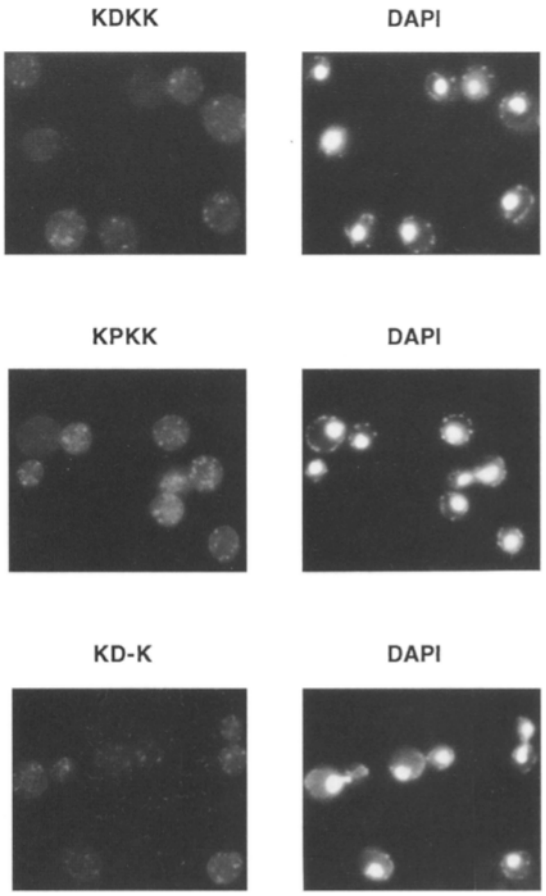

Figure 7. Golgi localization of chimeric proteins *KDKK*, *KPKK*, and *KD-K* by indirect immunofluorescence. Yeast cells (SEY6210) carrying a *KRE2* disruption (*kre2::TRP1*) and containing the different chimeric constructions on YEp352 were fixed, spheroplasted, attached to polylysine-coated glass slides, and incubated with anti-Kre2p antibodies followed by a subsequent incubation with a Texas red–conjugated secondary Ab and DAPI.

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-mannosyltransferase 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 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 **KKP** and **KKKP** were constructed (see Fig. 6 and Materials and Methods for details). **KKP** consists of the Kre2p cytoplasmic and membrane-spanning domains fused to the Pho8p luminal domain. **KKKP** contains the Kre2p NH_2 terminus, TMD, and a segment encompassing the first 36 amino acid residues of the stem domain fused to

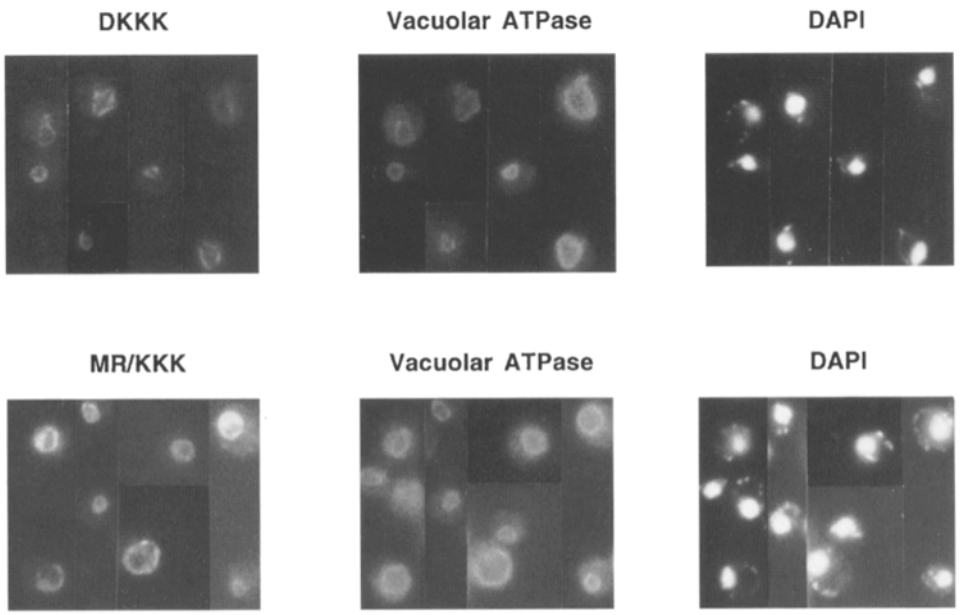

Figure 8. Vacuolar localization of chimeric proteins **DKKK** and **MR/KKK** by indirect immunofluorescence. Yeast cells carrying a *KRE2* disruption (*kre2::TRP1*) in an S86 background and containing the different chimeric constructions on YEp352 were fixed, spheroplasted, attached to polylysine-coated glass slides, and incubated with anti-Kre2p antibodies which was 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 with mAb 13D11 (Kane et al., 1992) with subsequent incubation of treated cells with Texas red-conjugated anti-mouse secondary antibodies.

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 NH_2 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 NH_2 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/Mnt1p 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 Mnn1p-dependent α1,3 modification, the next sequential step in the orderly glycosylation of yeast proteins. Thus, Kre2p must reach a Golgi compartment harboring the Mnn1p 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 Mnn1p compartment and then be retrieved to its own preceding organelle. Kre2p and Mnn1p have been shown to colocalize to a considerable extent, placing Kre2p in or close to the Mnn1p *medial*-Golgi compartment.

Consistent with this assignment, previous biochemical studies provide evidence that Kre2p is localized in a compartment distinct from the late Golgi. Immunoisolated late Golgi organelles containing the endoproteinases Kex1p, Kex2p, and DPAP A 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

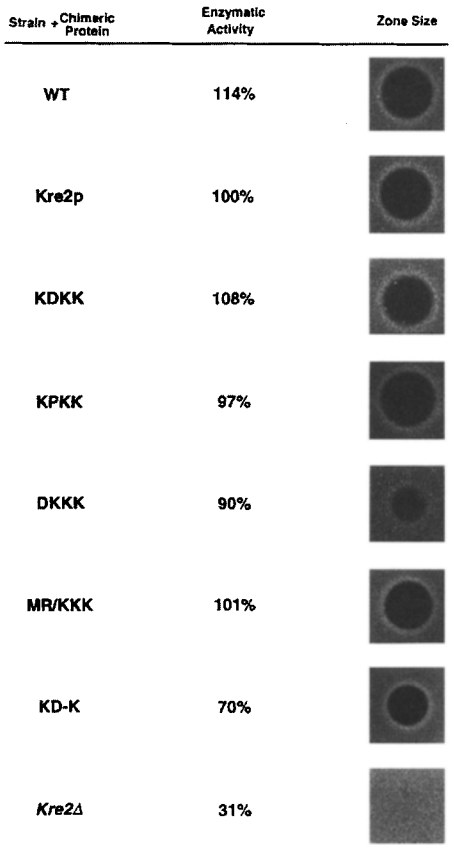

Figure 9. Activity of chimeric proteins. Enzymatic activity was measured in vitro by α1,2-mannosyltransferase assays of membrane preparations, and in vivo mannosylation of cell-wall proteins was assessed by verifying K1 killer toxin sensitivity in a wild-type strain and a *kre2* null mutant containing *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).

Golgi (Kex1p) are not always in mutually exclusive cisternae and their intracellular distribution may partially overlap. This can be the case in mammals where a pair of *trans*-Golgi and *medial*-Golgi glycosyltransferases showed substantial overlap (Nilsson et al., 1993).

The Kre2p/Mnn1p compartment remains to be precisely defined but some evidence suggests it could be identical to, or overlap with, compartments containing the Golgi type II membrane *N*-glycoprotein guanosine diphosphatase, Gda1p (Abeijon et al., 1989, 1993). Subcellular fractionation experiments indicated that Gda1p and Kre2p activities cosediment (Abeijon et al., 1989), whereas Gda1p and the late Golgi marker, Kex2p, are in distinct compartments (Bowser and Novick, 1991). Mnn1p also cosediments with Gda1p and was found in fractions different from those containing Kex2p (Graham et al., 1994). These results are in accord with our immunofluorescence data indicating that Kre2p and Mnn1p are in the same Golgi compartment.

The apparent double role of Kre2p in O- and N-linked glycosylation (Häusler et al., 1992; Hill et al., 1992; Lussier et al., 1995*b*) 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., 1995*b*).

How Is the Kre2p/Mnt1p Mannosyltransferase Targeted?

Immunolocalization results from the different chimeric proteins demonstrate that the Kre2p NH_2-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 contains a cryptic vacuolar targeting signal that overrides the Golgi-targeting sequence of Kre2p. Arguing against this view is the demonstration that the NH_2-terminal 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 NH_2-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 NH_2-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, un-

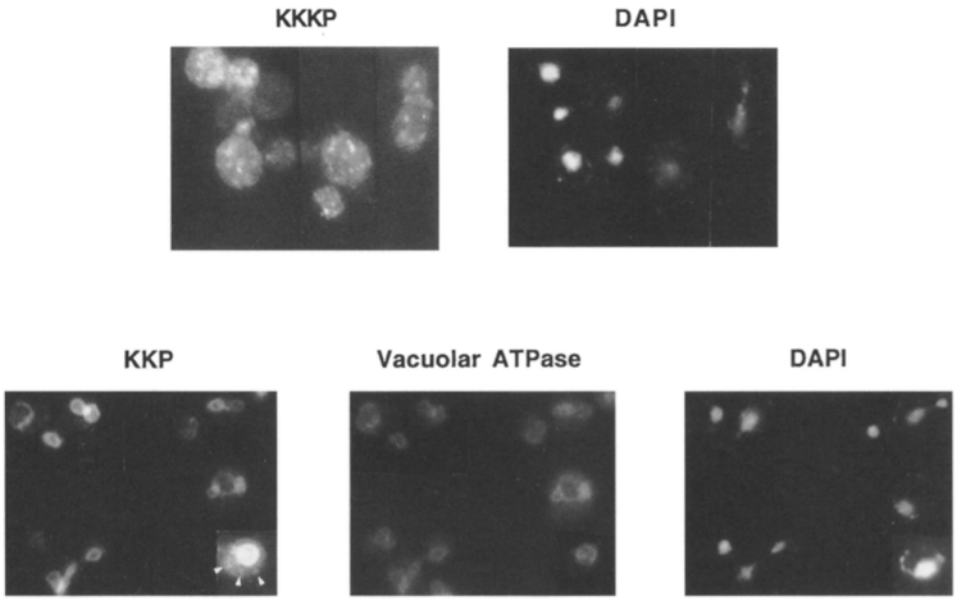

Figure 10. Localization of chimeric proteins **KKKP** and **KKP**. Yeast cells carrying a *PHO8* disruption (*pho8::TRP1*) in a SEY6210 background and containing the different chimeric constructions on YEp352 were fixed, spheroplasted, attached to polylysine-coated glass slides, and incubated with anti-Pho8p 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 NH_2 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**, **KPKK**, **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) and we address this below. In a first set of experiments, Chapman and Munro (1994) tested the role of the Kre2p TMD with a chimeric protein consisting of a full-length fusion of Kre2p/Mnt1p linked to the *SUC2*-encoded invertase by a small region containing a Kex2p-dependent cleavage site (chimeric protein MMMI). The mislocalization of this protein was expected to result in production of soluble invertase when passing through the Kex2p-containing late Golgi compartment. This fusion protein 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 **KPKK**) 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 **KPKK**) 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 **KPKP** 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; Wilsbach 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 (MAL**F**LSKRLLR) which was mutated to an alanine, an alteration showing no effect on targeting and, therefore, providing no evidence for clathrin-based retention of Kre2p (Lussier, 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 Gda1p 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; Wilsbach and Payne, 1993). The Mnn1p, Kre2p, and Gda1p 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 intracellular localization is only partially specified by its TMD and an additional localization signal involving its 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 KKXX 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 mislocalization, 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 NH_2 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 NH_2-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 (Slusarewicz et al., 1994). Interestingly, examination of the cytoplasmic NH_2-terminal domains of the six members of the *KRE2* mannosyltransferase family (Lussier et al., 1993; Mallet et al., 1994) reveals that the Kre2p (MALFLSKRLLR) sequence resembles only that of Ktr4p (MRFLSKRILK; Mallet et al., 1994) where the sequence FLSKR(I/L)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 nonenzymatic 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 hetero-oligomers 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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