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Evidence for Recycling of Cytochrome P450 Sterol 14-Demethylase from the *cis*-Golgi Compartment to the Endoplasmic Reticulum (ER) upon Saturation of the ER-retention Mechanism[1]

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Cytochrome P450 sterol 14-demethylase (P450-CYP51) is the enzyme that catalyzes 14α demethylation of lanosterol, a step in ergosterol biosynthesis, on the cytoplasmic side of the endoplasmic reticulum (ER) in *Saccharomyces cerevisiae*. To investigate its localization and the localization mechanism(s), we constructed a chimera by inserting a 30-residue segment, Leu^{283}–Leu^{312} of P450-CYP51 containing a potential *N*-glycosylation site in the cytoplasmic region, into the N-terminus of the same protein and tagging the C-terminus with three repeats of a hemagglutinin epitope. This chimera complements gene disruption on a single-copy vector and undergoes *N*-glycosylation, showing that it functions normally *in vivo*. Indirect immunofluorescence microscopy revealed that this chimera is localized exclusively to the ER when it is expressed on either a single-copy or multicopy vector. We carried out pulse-chase experiments and found that this chimera, when expressed on a multicopy plasmid, gradually undergoes α1→6 glycosylation, a *cis*-Golgi-specific modification, but not α1→3 glycosylation, a medial Golgi-specific modification. In contrast, a single-copy expression of this chimera does not lead to the *cis*-Golgi-specific modification. These findings suggest that, when expressed on a multicopy plasmid, a fraction of this chimera is transported from the ER to the *cis*-Golgi compartment and subsequently recycled to the ER, but when expressed on a single-copy plasmid, no significant transport of this protein from the ER takes place. We thus suggest the possibility that cytochrome P450 is retained in the ER by a saturable static mechanism.

Key words: cytochrome P450, vesicular transport, yeast *Saccharomyces cerevisiae*.

For proper functioning of eukaryotic cells, proteins need to be localized to specific organelles. Secretory and cell-surface proteins are generally thought to be exported from the endoplasmic reticulum (ER) *via* the Golgi apparatus to the plasma membrane in the absence of specific signals (*1*). Specifically, this requires that proteins that function exclusively in the ER must possess specific motif(s) that make them dynamically recycled from the Golgi apparatus or statically retained.

Many ER luminal proteins possess a KDEL or related signal at their C terminus (*2, 3*) and are known to be recognized by its receptor in the Golgi and recycled to the ER (*4–6*). Some ER membrane proteins have a dilysine motif at the C-terminal end of the cytoplasmic domain (*7*) and are retrieved from the Golgi to the ER through coatomer interaction with the motif (*8, 9*). Others possess an ER retrieval motif (δL) containing a critical aromatic residue in the cytoplasmic domain that interacts with COPI (*10*). In addition, the *RER1* gene product functions in the Golgi membrane of *Saccharomyces cerevisiae* to return to the ER a set of integral membrane proteins including Sec12p, Sec71p, and Sec63p (*11–13*) by recognizing a particular structural motif in the transmembrane domains of these proteins (*14*; Sato, K., Sato, M., and Nakano, A., unpublished results).

Phenobarbital-inducible cytochrome P450 was shown to be exclusively localized to the ER in rat liver cells by immunoelectron microscopy (*15–18*). The same conclusion was reached with the use of a chimeric rabbit cytochrome P450 2C2 protein (*19*) and fusion proteins containing P450 (M1) (*20*). The latter study also found the N-terminal hydrophobic segment sufficient for the ER membrane retention. Moreover, the cytoplasmic domain of Sec12p was discovered to contain the ER retention signal, while the transmembrane domain was found mainly to act as the retrieval signal (*14*).

Microsomal cytochrome P450 in general is a type I integral membrane protein with a short N-terminal segment that serves both as an insertion signal and as a stop-transfer sequence (*21–23*) or as a transmembrane segment right next to the signal-anchor sequence (*24–26*). The signal-

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Abbreviations: ER, endoplasmic reticulum; DAPI, 4′,6′-diamidino-2-phenylindole; HA, hemagglutinin, endo H, endoglycosidase H, SDS, sodium dodecyl sulfate.

anchor function is determined by the charges of the N-terminal amino acid residues and the length of the transmembrane domain (*27–29*). Following the transmembrane domain, a proline-rich region is present in all known microsomal cytochrome P450s, and the proline residues were suggested to be crucial for assuming the correct conformation (*30*). A catalytic, heme-containing domain located C-terminal to the proline-rich domain is peripherally associated with the cytoplasmic surface of the ER membrane (*31*). A protein rotation study revealed a transient association of cytochrome P450 C21 with NADPH-cytochrome P450 reductase (*32*), which serves as an electron transfer enzyme from NADPH to cytochrome P450 species.

Examinations of cytochrome P450 2C2 mobility in the ER membrane indicated that exclusion of this protein from the recycling pathway is not mediated by immobilization in large protein complexes (*33*). The N-terminal amino acids including the transmembrane domain of P450 2C1 and M1 were reported to be sufficient for retention in the ER (*34, 20*). The ER retention of cytochrome P450 2C2, on the other hand, is mediated by its cytoplasmic domain as well as N-terminal hydrophobic domain (*35*).

The demethylation of lanosterol 14α is required for biosynthesis of ergosterol in *S. cerevisiae* (reviewed in Ref. *36*) and of cholesterol in mammals (*37*). The enzyme catalyzing this reaction, cytochrome P450-CYP51, was purified from *S. cerevisiae* (*38–40*) and was found in all yeast strains tested (*41*). The gene encoding the protein, *ERG11/CYP51*, was cloned using as the indicator conferment of resistance to ketoconazole, an inhibitor of the reaction step (*42, 43*), and its expression was shown to be controlled by multiple regulatory elements (*44*). Although the gene was demonstrated to be essential for aerobic growth (*43*), a strain containing null mutations in both *ERG11/CYP51* and *ERG3* is aerobically viable (*45*), because 14-methyl fecosterol is produced instead (*46*). Rat and human cDNAs for P450-CYP51 were subsequently cloned (*47, 48*) and found to be highly homologous to the yeast counterpart.

In this study, we constructed a chimera of cytochrome P450-CYP51 of *S. cerevisiae* which undergoes *N*-linked glycosylation. Indirect immunofluorescence microscopy revealed that this chimera is exclusively localized to the ER even when it is expressed on a multicopy plasmid. When expressed on the multicopy plasmid, however, it was found to undergo *cis*-Golgi–specific but not medial Golgi-specific glycosylation, indicating that the chimera is retrieved from the Golgi apparatus to the ER. In contrast, the chimera expressed on a single-copy plasmid did not exhibit any significant Golgi-specific modifications, suggesting its static retention in the ER. We thus report a possible dosage-dependence of the ER retention of cytochrome P450.

MATERIALS AND METHODS

Strains and Culture Conditions—The *S. cerevisiae* strains used in this study are listed in Table I. The complete, minimal, and sporulation yeast media and procedures of tetrad analysis were as described (*49*). For genetic manipulation, *Escherichia coli* strains DH5α (Bethesda Research Laboratories, Gaitherburg, MD) and SCS1 (Stratagene, La Jolla, CA) were used.

Plasmids and DNA Manipulations—DNA manipulations, including restriction enzyme digestion, ligation, bacterial transformation, and plasmid isolation were carried out by standard methods. For DNA fragment purification, the DNA PREP kit (Asahi Glass, Tokyo) was routinely used. DNA sequencing was performed with an automated DNA sequencer (Model 373A, Applied Biosystems, Foster City, CA). Yeast transformation was carried out by the lithium acetate method (*49*). A yeast multicopy replication plasmid, pYO326 (*URA3*) (*50*), and a single-copy plasmid, pRS316 (*URA3*) (*51*), were previously described.

TABLE I **Strains used in this study.**

Strain	Genotype	Reference
ANY21	*MATa ura3 leu2 trp1 his3 his4 suc2 gal2*	*56*
ANY200	*MATa/MATα ura3/ura3 leu2/leu2 trp1/trp1 his3/his3 his4/his4 suc2/suc2 gal2/gal2*	*12*
KHY02	*MATα ura3 trp1 his3 his4 suc2 gal2 erg11/cyp51::LEU2* [pYO326-*ERG11/CYP51*]	This study
KHY09	*MATα ura3 trp1 his3 his4 suc2 gal2 erg11/cyp51::LEU2* [pYO326-chimera N]	This study
KHY12	*MATa ura3 leu2 trp1 his3 his4 suc2 gal2* [pRS316-chimera N]	This study
KHY13	*MATa ura3 leu2 trp1 his3 his4 suc2 gal2* [pYO326-chimera N]	This study
KHY14	*MATa rer1 .LEU2 ura3 leu2 trp1 his3/his4 gal2* [pYO326-chimera N]	This study
KHY15	*MATα pep4 .ADE2 ura3 trp1 his3/his4 gal2* [pYO326-chimera N]	This study
KHY16	*MATα rer1 LEU2 pep4::ADE2 leu2 ura3 trp1 his3* [pYO326-chimera N]	This study
SNS218-3C	*MATα sec18-1 ura3 lys2 ade2 trp1 his3 leu2 bar1::HIS3 mfα1::ADE2 mfα2..TRP1*	*11*

To facilitate construction of a chimera, we first introduced a *Bam*HI site near the 5′ terminus of the *ERG11/CYP51* gene using PCR. The first PCR fragment was amplified with the oligomers 5′-CAATCTTAAAGGCCGATAATCCAC-3′ and 5′-CTCGGATCCTCCTTGTATTACTCGTTTGTTC-3′ using as template the pBluescript SK- plasmid (Stratagene, La Jolla, CA) containing the *ERG11/CYP51* gene together with the flanking sequences from –404 bp (*Cla*I site) to 2260 bp (*Hin*dIII site). The amplified fragment was digested with *Bam*HI and gel-purified. Another fragment was amplified by PCR with the same template but with 5′-CTCGGATCCTCTGCTACCAAGTCAATCG-3′ and 5′-CGTCAATAGTACCAGTAGT-3′, double-digested with *Bam*HI and *Sty*I, and similarly purified. The two fragments were then ligated to the above-mentioned plasmid digested with *Cla*I and *Sty*I, to produce a plasmid identical with the original one except for a *Bam*HI site introduced at the N-terminus of the *ERG11/CYP51* gene. The sequence of the obtained plasmid was confirmed, and the 2.7-kbp *Sma*I fragment containing the mutated gene was then ligated to pYO326 digested first with *Bam*HI, blunt-ended with T4 DNA polymerase, and then *Cla*I-digested. We next introduced an HA epitope tag with 3 repeats at the 3′ terminus of the *ERG11/CYP51* gene. For this purpose, we first annealed together two pairs of primers: 5′-GATCTACCCATACGATGTTCCGGATTACGCTTACCCATACG-3′ and 5′-GCACATCGTATGGGTAAGCGTAATCCGGAACATCGTATGGGTA-3′; and 5′-ATGTGCCGGATTACGCTTACCCATACGATGTACCGGATTACGCTTA-3′ and 5′-GAT-

CTAAGCGTAATCCGGTACATCGTATGGGTAAGCGTAA-TCCG-3′. Then the two sets of annealed fragments were separately phosphorylated with bacteriophage T4 polynucleotide kinase (New England Biolabs, Beverly, MA), followed by heat-inactivation of the kinase. The two sets of double-stranded DNA were cloned in a three-piece ligation into the above-described vector digested with *Bgl*II (pYO326-*ERG11/CYP51*-3HA), and the direction and sequence of the HA tag were subsequently confirmed. The last construction step was to insert a 30-amino-acid peptide of P450-CYP51, Leu^{283}–Leu^{312}, into the N-terminus. We thus PCR-amplified the portion of the *ERG11/CYP51* gene with 5′-CTCGGATCCATTGATCGATTCCTTGATGAAGA-AC-3′ and 5′-CTCGGATCCTAAGACACCAATTAACAAGT-TAGCG-3′ as primers and *Bam*HI-digested and gel-purified the amplified fragment. The fragment was then inserted in to the *Bam*HI site of pYO326-*ERG11/CYP51*-3HA to produce chimera N on multicopy plasmid (pYO326-chimera N). After sequence confirmation, the *Cla*I–*Sac*I fragment containing chimera N was ligated to the similarly digested pRS316 single-copy vector (pRS316-chimera N). We also inserted the *ERG11/CYP51* gene with the flanking sequence into pYO326 to make pYO326-*ERG11/CYP51* for use as control.

To disrupt the *ERG11/CYP51* gene, we first digested pJJ250 plasmid with *Sal*I, blunted the ends, then digested with *Bam*HI. The *ERG11/CYP51* gene–carrying pBluescript plasmid described above was digested with *Sty*I, blunt-ended, then digested with *Bgl*II. The 2.0-kbp insert and the 4.3-kbp vector were ligated, and the resultant plasmid was triple-digested with *Hin*dIII, *Xho*I, and *Sca*I to produce a 3.3-kbp fragment containing the *ERG11/CYP51* gene with the portion from 195 bp to the 3′ terminus replaced by the *LEU2* gene. Transformation of ANY200 cells with this fragment led to disruption of one copy of the *ERG11/CYP51* gene, which was confirmed by Southern hybridization. The diploid cells were sporulated and subjected to tetrad dissection.

Cytochrome P450 Sterol 14-Demethylase Assay—The biochemical assay of lanosterol 14α demethylase activity was carried out as previously published (*47*).

Antibodies—12CA5 and 16B12, monoclonal antibodies recognizing the HA epitope from human influenza virus hemagglutinin, were from Boehringer Mannheim (Indianapolis, IN) and Berkeley Antibody Company (Richmond, CA), respectively. Fluorescein-conjugated goat anti–mouse IgG was purchased from Organon Teknika (Durham, NC), while $F(ab')_2$ fragment of horseradish peroxidase–linked anti–mouse Ig came from Amersham Life Science (Amersham Place, UK). Mouse anti-P450 polyclonal antibody was raised against a fusion protein of glutathione-S-transferase (Pharmacia, Tokyo) and the Pro^{64}–Lys^{529} peptide of P450-CYP51, and the obtained antiserum was used without purification.

Immunofluorescence Microscopy—Fixed yeast cells were prepared as described (*52*) with the modification that the fixing was done for 1 h at 30°C. After permeabilization with 0.1% Triton X-100, we stained the fixed cells according to the published method (*49*), using the 16B12 antibody at ×100 dilution as the primary antibody and fluorescein-conjugated goat anti-mouse antibody at 10 μg/ml as the secondary antibody, followed by 4′,6′-diamidino-2-phenylindole (DAPI) staining at 0.6 μg/ml. The treated cells were observed and images were recorded using an Olympus BH-2 photo microscope connected to an Apple computer.

Pulse-Chase Experiments—Metabolic labeling of yeast cells and cell extract preparation were carried out as previously described (*52*), except that cells expressing chimera N on the multicopy and the single-copy plasmid were respectively labeled with 1.4 MBq (Tran^{35}S-label, Amersham) for 10 min and 7.4 MBq for 30 min. The extracts were subjected to the immunoprecipitation procedures and endoglycosidase H (endo H) digestion as formerly described (*14*) except that the second immunoprecipitation with anti–α1→3 mannose antibody was omitted for the chimera N extracts expressed on the single-copy plasmid. The samples were then analyzed by use of 11% polyacrylamide sodium dodecyl sulfate (SDS) gels and fluorography as described (*52*). A Fuji Film image analyzer BAS-2000 was used for observation and quantification.

RESULTS

Construction of Chimera N—To analyze the localization of P450, we first introduced potential *N*-glycosylation sites by mutating the N-terminal luminal segment, but found that none of the mutated proteins underwent *N*-glycosylation (data not shown). Following the example of Szczesna-Skorupa and Kemper (*28*), we then constructed chimera N by inserting an HA epitope tag at the C-terminus and by inserting a copy of a cytoplasmic 30-residue segment of P450-CYP51, Leu^{283}–Leu^{312}, which contains a potential *N*-glycosylation site, right after the N-terminal Met residue (Fig. 1). This chimera is comprised of 591 residues and complements *ERG11/CYP51* gene disruption on a single-copy plasmid (data not shown). Moreover, the specific lanosterol 14α demethylase activity of a *Δerg11/cyp51* strain carrying this chimera on a multicopy plasmid (KHY02) turned out to be even larger than that of a *Δerg11/cyp51* strain with the wild-type *ERG11/CYP51* gene on a multicopy plasmid (KHY09) (395 *vs.* 160 pmol/min/nmol P450).

Chimera N Is Glycosylated—We examined whether chimera N is *N*-glycosylated by testing endo H sensitivity of the protein. Chimera N was expressed on a multicopy or a single-copy plasmid, protein extracts were prepared, and portions of the extracts were subjected to endo H digestion. When a wild-type sample was analyzed by Western blotting

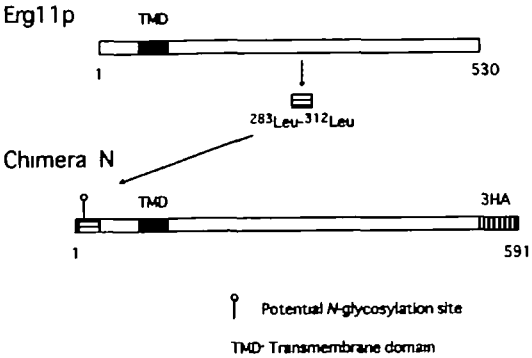

Fig. 1 **Construction of chimera N.** Construction of chimera N is illustrated. P450-CYP51 is an integral membrane protein with its N-terminus in the ER lumen and the catalytic domain exposed to the cytoplasmic side.

using an anti-HA antibody, there was no significant staining (Fig. 2, lane 1). The extracts of chimera N-expressing cells without endo H treatment gave a band at approximately 67 kDa, with higher intensity for the higher expression level (lanes 2 and 4). The corresponding samples with endo H digestion produced a shifted band with the apparent molecular weight of 65 kDa (lanes 3 and 5). The endo H sensitivity indicates that chimera N is *N*-glycosylated. On the other hand, the P450-CYP51 species merely tagged with 3HA at the C-terminus, *i.e.*, chimera N without the N-terminal insertion, did not exhibit endo H sensitivity (data not shown), suggesting that it is the N-terminally inserted segment of chimera N that is *N*-glycosylated. In addition, with the high expression level, a faint band with the same migration distance as that of the endo H–treated samples is visible (lane 4), corresponding to the unglycosylated fraction of chimera N.

Chimera N Is Localized to the ER—We next examined the localization of chimera N by indirect immunofluorescence microscopy using an anti-HA antibody. A strain carrying this chimera on a single-copy plasmid showed fluorescence emanating exclusively from the ER (Fig. 3, panels c and d) (see Ref. *14*), while the corresponding wild-type strain with no plasmid gave no significant fluorescence (panels a and b). Furthermore, the identical strain harboring chimera N on a multicopy plasmid revealed exclusive ER localization of the chimera (panels e and f). Thus chimera N is exclusively localized to the ER even when expressed on a multicopy plasmid.

Chimera N Acquires cis-Golgi–Specific Modification—To investigate whether the ER localization of chimera N is achieved by static retention or recycling from the Golgi, we carried out pulse-chase experiments with a wild-type strain transformed with the multicopy plasmid carrying the chimera. Cells were pulse-labeled with Tran^{35}S-label for 10 min, chased for 0, 1, or 2 h, then subjected to immunoprecipitation analysis. As Fig. 4A shows, chimera N undergoes degradation (compare lanes 1, 4, and 7), while the amount of chimera N with α1→6 mannosyl linkages (*cis*-Golgi specific modification) increased slowly with time (lanes 2, 5, and 8), but that with α1→3 mannosyl linkages (medial Golgi-specific modification) decreased (lanes 3, 6, and 9). Quantification of 10 independent experiments (Fig. 4B) revealed that the fraction of α1→6 glycosylated chimera increased with time, while that of α1→3 glycosylated chimera remained essentially unchanged. A pulse-chase analysis of a *sec18-1* strain SNS218-3C (*11*) transformed with the multicopy plasmid carrying chimera N at 37°C (restrictive temperature) and 24°C (permissive temperature) showed that this chimera did not acquire α1→6 linkages at the restrictive temperature, but did so at the permissive temperature (data not shown). This control experiment verifies that the portion of chimera N that undergoes the *cis*-

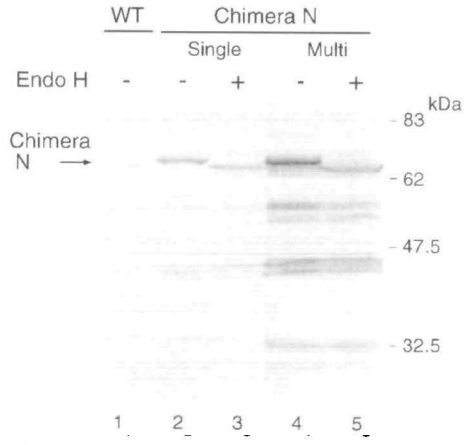

Fig. 2 **Chimera N undergoes *N*-linked glycosylation.** ANY21 (wild-type), KHY12 (ANY21 with chimera N on a single-copy plasmid), and KHY13 (ANY21 with chimera N on a multicopy plasmid) cells were grown in MCD medium with appropriate supplements at 30°C. Protein extracts were prepared from exponentially growing cells by agitation with glass beads in the SDS gel sampling buffer. Aliquots of the KHY12 and KHY13 extracts were subjected to endoH digestion. Proteins (50 μg per lane) were separated on an 11% SDS–polyacrylamide gel and subjected to immunoblotting analysis with the 12CA5 monoclonal antibody against the HA epitope. Chimera N was detected at around the expected molecular weight (arrow). The other bands on lanes 4 and 5 are considered to be its degradation products.

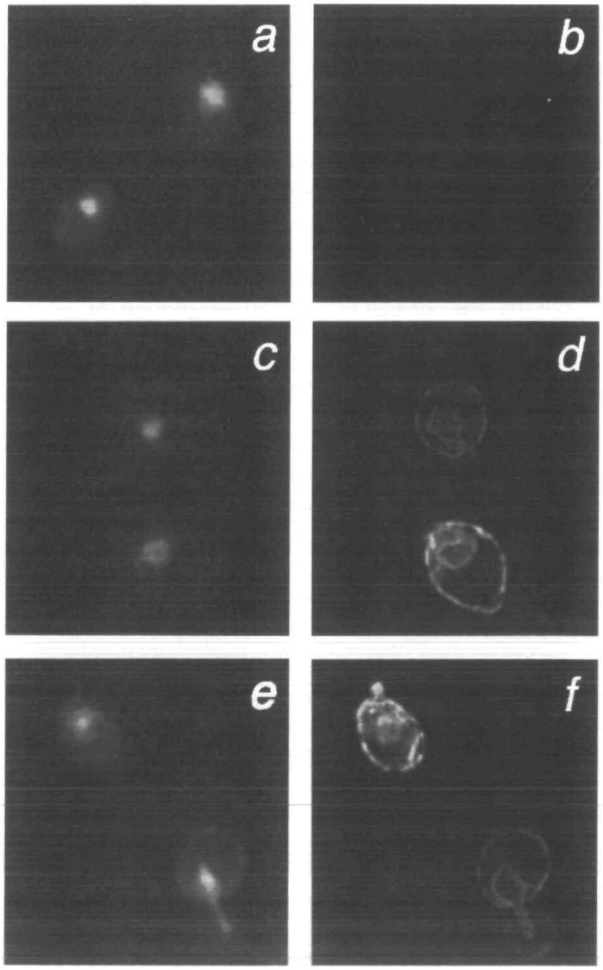

Fig. 3. **Chimera N is localized to the ER.** ANY21 (wild-type) (a and b), KHY12 (ANY21 with chimera N on a single-copy plasmid, c and d), and KHY13 (ANY21 with chimera N on a multicopy plasmid, e and f) cells cultured as in the legend of Fig. 2 were analyzed by immunofluorescence microscopy using the 16B12 anti-HA antibody. Images of the same fields with DAPI DNA staining (a, c, and e) and with the antibody (b, d, and f) are presented.

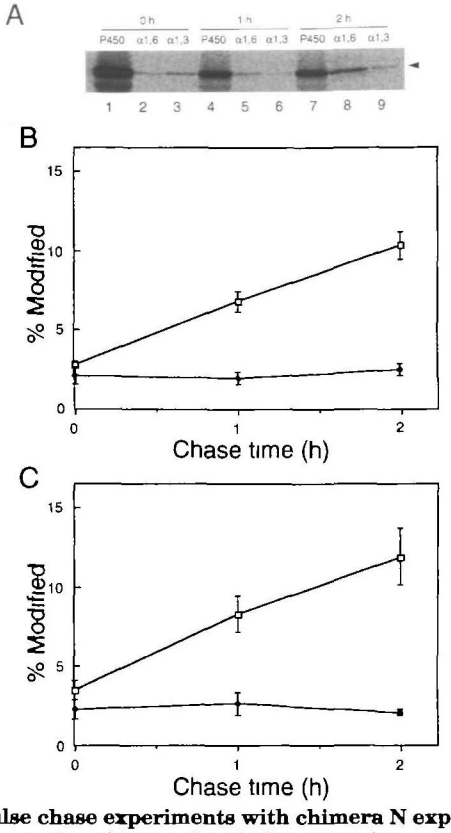

Fig 4. **Pulse chase experiments with chimera N expressed on a multicopy plasmid reveal a *cis*-Golgi specific modification.** A strain harboring chimera N on a multicopy plasmid (KHY13) and an isogenic strain with the *RER1* gene deleted (KHY14) were pulse-labeled for 10 min, chased for the indicated times, immunoprecipitated first with the anti–P450-CYP51 antibody and second with the same antibody (P450), an antibody against α1→6 mannosyl linkage (α1,6), or one against α1→3 mannosyl linkage (α1,3), and endo H digested as in "MATERIALS AND METHODS" (A) An example of radioimages with KHY13 (B) Quantified data from six independent experiments with KHY13 The averages and the standard deviations of the mean are shown Open squares, fraction of α1→6 mannose modified; closed diamonds, fraction of α1→3 mannose modified. (C) Data from six independent experiments with KHY14 are quantified and presented as in (B)

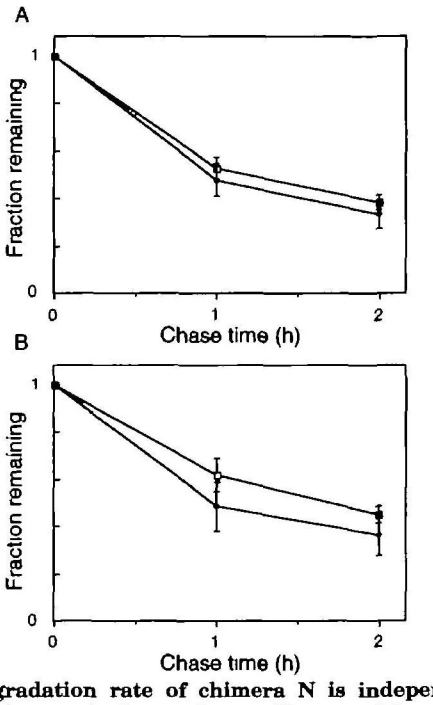

Fig. 5. **Degradation rate of chimera N is independent of Pep4p.** (A) A strain harboring chimera N on a multicopy plasmid (KHY13) and an isogenic strain with the *PEP4* gene deleted (KHY15) were pulse-chased and immunoprecipitated once with the anti-P450 antibody, then endo H-treated as in "MATERIALS AND METHODS" Each sample was subjected to SDS-PAGE and radioimaging and the band corresponding to the chimera was quantified. The presented data are the averages and the standard deviations of the mean of five independent experiments. (B) A *Δrer1* strain expressing chimera N from a multicopy plasmid (KHY14) and the isogenic strain with the *PEP4* gene deleted (KHY16) were examined as in (A), except that experiments were repeated four times. Open squares, *PEP4*, closed diamonds, *Δpep4*

Golgi–specific modification reaches the compartment *via* the secretory pathway, which is blocked in *sec18-1* strain at the restrictive temperature.

To test whether glycosylation depends on Rer1p, we carried out the same pulse-chase experiments with an isogenic *Δrer1* strain carrying chimera N on the same multicopy plasmid. Chimera N in this strain exhibited the same glycosylation trends as in the wild-type strain, as quantified data show (Fig. 4C). That is, the *Δrer1* strain also shows a gradual increase with time in the fraction of α1→6 glycosylated chimera, but no significant change in the fraction of α1→3 glycosylated chimera. Thus the recycling of this chimera is Rer1p-independent.

Degradation of Chimera N Is Pep4p-Independent—As the degradation of this chimera is rapid, we investigated whether it is Pep4p-dependent. The degradation time-course of chimera N in pulse-chase experiments in the wild-type strain was compared with that in an isogenic *Δpep4* strain (Fig. 5A). They did not differ significantly, showing that the degradation was not slowed by disruption of the *PEP4* gene in the *RER1* background. Similarly, the time course of chimera N degradation in *Δrer1* strain was not significantly different from that in an isogenic *Δrer1 Δpep4* strain (Fig. 5B). Thus the degradation of chimera N is independent of the *PEP4* gene both in the *RER1* and *Δrer1* backgrounds.

Recycling of Chimera N Is Not Apparent at a Low Expression Level—Is the apparent leakage of chimera N to the *cis*-Golgi compartment dependent on expression level? To answer this question, we performed pulse-chase experiments with the wild-type strain carrying the chimera on the single-copy plasmid. As Fig. 6A shows, a very small amount of α1→6 glycosylated chimera was observed, which decreased with time. The fraction of the α1→6 glycosylated chimera remained essentially at the background level (Fig. 6B). Hence no significant fraction of chimera N reaches the *cis*-Golgi compartment when it is expressed on the single-copy plasmid.

DISCUSSION

Based on the results, we suggest that chimera N, which contains an *N*-glycosylation site at the N-terminus of P450-CYP51, is localized to the ER by two mechanisms. When it

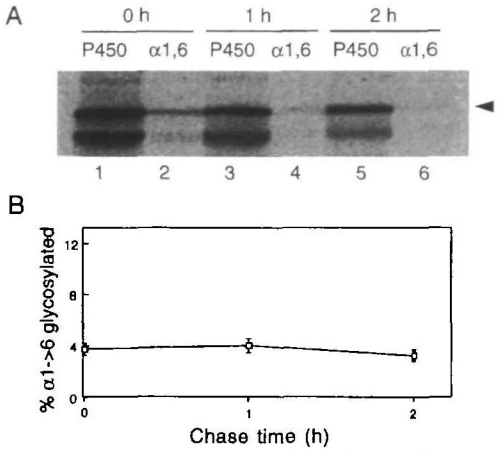

Fig. 6. **Pulse chase experiments with chimera N expressed on a single-copy plasmid reveal no significant *cis*-Golgi specific modification.** The strain harboring chimera N on a single-copy plasmid (KHY12) was pulse-labeled for 30 min, chased for the indicated times, and subjected to immunoprecipitation. The second precipitations were carried out in two aliquots and examined as in the legend of Fig 4. (A) A representative radioimage. (B) Quantified data from triplicates. The averages and the standard deviations of the mean are graphically presented.

is overproduced, a considerable fraction acquires a *cis*-Golgi–specific modification, implying that Golgi-to-ER retrieval takes place. At the low expression level, however, such recycling is not easily detectable and the static retention mechanism seems to dominate. That such a chimera of P450 is recycled from the *cis*-Golgi when overexpressed is in apparent disagreement with the previous reports that P450 2C1 and M1 do not reach the Golgi apparatus in COS cells (*19*, *20*). Although the use of different P450 species and unequal expression levels may have contributed to the discrepancy, our results raise an interesting possibility that even molecules, like P450, that are normally retained statically in the ER can be retrieved from the Golgi when the retention mechanism is saturated. Further study will test the general applicability of the present findings.

We were fully aware of the possibility that such an artificial chimera might assume the wrong topology with respect to the membrane. However, if most of the constructed chimera were inserted into the ER membrane in the reverse orientation, the enzymatic activity would be negligible, in disagreement with the single-copy complementation of an *erg11/cyp51* disruptant by this chimera and the biochemical assay data. Can a small fraction of the chimera be reversely inserted? With three potential *N*-glycosylation sites in the portion corresponding to the cytoplasmic region in the normal orientation, the reversely inserted chimera may well acquire an *N*-glycosylation pattern different from that of the properly oriented one. Therefore, a significant fraction of chimera N reversely inserted would give more than two protein species reacting with an anti-HA antibody before endo H digestion, in contradiction to the Western blotting data (Fig. 2). Thus most of chimera N must be in the proper orientation.

Indirect immunofluorescence microscopy revealed that, even when expressed on the multicopy plasmid, the chimera is exclusively localized to the ER and the expression does not lead to proliferation of the ER, as observed when *Candida maltosa* P-450 forms (Cm1 and Cm2) were expressed in *S. cerevisiae* cells (*53*). The expression level on the multicopy plasmid is not expected to be very high, as only UAS2 in the original *ERG11/CYP51* promoter (*44*) was included in the construction, while the increased ER membrane formation reported was detected when the protein expression was induced by the strong *GAL10* promoter. It is of interest that even at this expression level some of the chimera reaches the *cis*-Golgi compartment.

Although multicopy expression of chimera N leads to a progressive increase in the fraction undergoing α1→6 glycosylation, it leaves the chimera with α1→3 glycosylation at the background level (Fig. 4). These observations indicate that a significant fraction of this chimera reaches the *cis*-Golgi, but not the medial Golgi compartment. As this chimera is exclusively localized to the ER, the portion of chimera N that reaches the *cis*-Golgi is likely to be rapidly recycled to the ER, as is the case with many other ER-resident proteins. That is, short transit time outside the ER must give the appearance of exclusive ER localization. This is not inconsistent with the immunoelectron microscopic observations of cytochrome P450 (PB) (*15–18*).

How is this recycling to the ER achieved then? This chimera does not possess any of the known retrieval signals, namely, HDEL, KKXX δL, or the Sec12p-type transmembrane motif. We additionally found that the chimera retrieval is independent of Rer1p (Fig. 4). Thus the retrograde transport of this chimera must be mediated by a hitherto unknown mechanism.

The chimera is significantly degraded during the chase time (61% is degraded in 2 h when expressed on a multicopy vector in the wild-type strain, Fig. 5A). As 67% of the chimera is degraded within 2 h when expressed on a single-copy plasmid (data not shown), the process is not slowed down with the lower expression level. This means that the degradation is not linked to the transport from and retrieval to the ER, as the *cis*-Golgi–specific modification is evident only when the chimera is expressed on the multicopy plasmid. In addition, the fact that the degradation is not slowed in *Δpep4* strains (Fig. 5) suggests that the chimera is not degraded in the vacuole, because Pep4p is essential for maturation of most vacuolar hydrolases (*54*). Whether wild-type P450-CYP51 is similarly degraded will be answered by future investigation.

If the static retention mechanism of chimera N is indeed saturable, it is interesting to note that cytochrome P450-(PB) and NADPH-cytochrome P450 reductase were suggested to form a monomolecular 1:1 complex in rat liver microsomes (*55*). It is possible that, when overexpressed, some fraction of the chimera fails to form a complex with P450 reductase in the ER, leading to transport to the *cis*-Golgi. It is likely that wild-type P450-CYP51 shows saturability of static retention in the ER, too, because the chimera contains all the wild-type sequences including the critical transmembrane domain and functions as the wild-type species.

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REFERENCES

1 Pfeffer, S.R. and Rothman, J E. (1987) Biosynthetic protein transport and sorting by the endoplasmic reticulum and Golgi. *Annu. Rev. Biochem.* **56**, 829–852
2 Munro, S and Pelham, H R B (1987) A C-terminal signal prevents secretion of luminal ER proteins. *Cell* **48**, 899–907
3 Pelham, H.R.B (1988) Evidence that luminal ER proteins are sorted from secreted proteins in a post-ER compartment. *EMBO J* **7**, 913–918
4. Semenza, J C , Hardwick, K.G , Dean, N., and Pelham, H R.B (1990) *ERD2*, a yeast gene required for the receptor-mediated retrieval of luminal ER proteins from the secretory pathway. *Cell* **61**, 1349–1357
5. Lewis, M.J., Sweet, D J., and Pelham, H R B (1990) The *ERD2* gene determines the specificity of the luminal ER protein retention system. *Cell* **61**, 1359–1363
6 Lewis, M.J. and Pelham, H R B. (1990) A human homologue of the yeast HDEL receptor *Nature* **348**, 162–163
7. Jackson, M R., Nilsson, T , and Peterson, P.A. (1990) Identification of a consensus motif for retention of transmembrane proteins in the endoplasmic reticulum *EMBO J* **9**, 3153–3162
8 Cosson, P. and Letourneur, F. (1994) Coatomer interaction with di-lysine endoplasmic reticulum retention motifs. *Science* **263**, 1629–1631
9. Letourneur, F , Gaynor, E C , Hennecke, S., Démollière, C , Duden, R., Emr, S D , Riezman, H., and Cosson, P. (1994) Coatomer is essential for retrieval of dilysine-tagged proteins to the endoplasmic reticulum. *Cell* **79**, 1199–1207
10. Cosson, P , Lefkir, Y., Démollière, C , and Letourneur, F (1998) New COP1-binding motifs involved in ER retrieval *EMBO J.* **17**, 6863–6870
11 Nishikawa, S. and Nakano, A. (1993) Identification of a gene required for membrane protein retention in the early secretory pathway *Proc. Natl Acad. Sci USA* **90**, 8179–8183
12. Sato, K., Nishikawa, S., and Nakano, A. (1995) Membrane protein retrieval from the Golgi apparatus to the endoplasmic reticulum (ER): characterization of the *RER1* gene product as a component involved in ER localization of Sec12p. *Mol. Biol Cell* **6**, 1459–1477
13. Sato, K., Sato, M., and Nakano, A. (1997) Rer1p as a common machinery for the endoplasmic reticulum localization of membrane proteins. *Proc. Natl Acad. Sci USA* **94**, 9693–9698
14 Sato, M., Sato, K., and Nakano, A. (1996) Endoplasmic reticulum localization of Sec12p is achieved by two mechanisms: Rer1p-dependent retrieval that requires the transmembrane domain and Rer1p-independent retention that involves the cytoplasmic domain. *J Cell Biol* **134**, 279–293
15 Matsuura, S , Fujii-Kuriyama, Y., and Tashiro, Y. (1978) Immunoelectron microscope localization of cytochrome P-450 on microsomes and other membrane structures of rat hepatocytes. *J Cell Biol.* **78**, 503–519
16. Matsuura, S. and Tashiro, Y. (1979) Immunoelectron-microscopic studies of endoplasmic reticulum-Golgi relationships in the intracellular transport process of lipoprotein particles in rat hepatocytes. *J Cell Sci* **39**, 273–290
17 Brands, R., Snider, M.D., Hino, Y., Park, S.S., Gelboin, H.V., and Rothman, J.E. (1985) Retention of membrane proteins by the endoplasmic reticulum. *J. Cell Biol* **101**, 1724–1732
18. Yamamoto, A., Masaki, R , and Tashiro, Y. (1985) Is cytochrome P-450 transported from the endoplasmic reticulum to the Golgi apparatus in rat hepatocytes? *J Cell Biol* **101**, 1733–1740
19 Szczesna-Skorupa, E and Kemper, B (1993) An N-terminal glycosylation signal on cytochrome P450 is restricted to the endoplasmic reticulum in a luminal orientation *J Biol Chem* **268**, 1757–1762
20. Murakami, K., Mihara, K., and Omura, T. (1994) The transmembrane region of microsomal cytochrome P450 identified as the endoplasmic reticulum retention signal. *J Biochem.* **116**, 164–175
21. Bar-Nun, S , Kreibich, G , Adesnik, M., Alterman, L., Negishi, M., and Sabatini, D D (1980) Synthesis and insertion of cytochrome *P*-450 into endoplasmic reticulum membranes. *Proc. Natl. Acad. Sci USA* **77**, 965–969
22 Sakaguchi, M., Mihara, K., and Sato, R. (1987) A short amino-terminal segment of microsomal cytochrome P-450 functions both as an insertion signal and as a stop-transfer sequence. *EMBO J.* **6**, 2425–2431
23. Monier, S , Van Luc, P., Kreibich, G , Sabatini, D D , and Adesnik, M. (1988) Signals for the incorporation and orientation of cytochrome P450 in the endoplasmic reticulum membrane. *J. Cell Biol* **107**, 457–470
24 De Lemos-Chiarandini, C , Frey, A.B., Sabatini, D D., and Kreibich, G (1987) Determination of the membrane topology of the phenobarbital-inducible rat liver cytochrome P-450 isoenzyme PB-4 using site-specific antibodies. *J. Cell Biol* **104**, 209–219
25. Vergères, G , Winterhalter, K.H., and Richter, C. (1989) Identification of the membrane anchor of microsomal rat liver cytochrome P-450. *Biochemistry* **28**, 3650–3655
26 Pernecky, S J , Larson, J.R., Philpot, R.M , and Coon, M J. (1993) Expression of truncated forms of liver microsomal P450 cytochromes 2B4 and 2E1 in *Escherichia coli* Influence of NH_2-terminal region on localization in cytosol and membranes. *Proc. Natl Acad Sci USA* **90**, 2651–2655
27 Szczesna-Skorupa, E., Browne, N., Mead, D , and Kemper, B (1988) Positive charges at the NH_2 terminus convert the membrane-anchor signal peptide of cytochrome P-450 to a secretory peptide *Proc. Natl Acad. Sci USA* **85**, 738–742
28 Szczesna-Skorupa, E. and Kemper, B (1989) NH_2-terminal substitutions of basic amino acids induce translocation across the microsomal membrane and glycosylation of rabbit cytochrome P450IIC2 *J. Cell Biol* **108**, 1237–1243
29 Sato, T., Sakaguchi, M., Mihara, K., and Omura, T (1990) The amino-terminal structures that determine topological orientation of cytochrome P-450 in microsomal membrane. *EMBO J* **9**, 2391–2397
30 Yamazaki, S., Sato, K., Suhara, K., Sakaguchi, M., Mihara, K., and Omura, T. (1993) Importance of the proline-rich region following signal-anchor sequence in the formation of correct conformation of microsomal cytochrome P-450s. *J. Biochem* **114**, 652–657
31 Brown, C.A. and Black, S.D (1989) Membrane topology of mammalian cytochrome P-450 from liver endoplasmic reticulum Determination by trypsinolysis of phenobarbital-treated microsomes *J. Biol Chem* **264**, 4442–4449
32. Ohta, Y , Kawato, S., Tagashira, H., Takemori, S., and Kominami, S. (1992) Dynamic structures of adrenocortical cytochrome P-450 in proteoliposomes and microsomes protein rotation study *Biochemistry* **31**, 12680–12687
33 Szczesna-Skorupa, E , Chen, C -D , Rogers, S , and Kemper, B (1998) Mobility of cytochrome P450 in the endoplasmic reticulum membrane. *Proc. Natl Acad Sci USA* **95**, 14793–14798
34 Ahn, K., Szczesna-Skorupa, E., and Kemper, B. (1993) The amino-terminal 29 amino acids of cytochrome P450 2C1 are sufficient for rentention in the endoplasmic reticulum *J Biol. Chem* **268**, 18726–18733
35 Szczesna-Skorupa, E., Ahn, K., Chen, C.-D , Doray, B., and Kemper, B (1995) The cytoplasmic and N-terminal transmembrane domains of cytochrome P450 contain independent signals for retention in the endoplasmic reticulum *J Biol. Chem.* **270**, 24327–24333
36 Lees, N.D., Skaggs, B., Kirsch, D.R., and Bard, M (1995) Cloning of the late genes in the ergosterol biosynthetic pathway of *Saccharomyces cerevisiae*—A review. *Lipids* **30**, 221–226
37. Trzaskos, J , Kawata, S , and Gaylor, J L (1986) Microsomal enzymes of cholesterol biosynthesis. Purification of lanosterol 14α-methyl demethylase cytochrome P-450 from hepatic microsomes. *J Biol Chem.* **261**, 14651–14657
38. Yoshida, Y , Aoyama, Y , Kumaoka, H., and Kubota, S. (1977) A highly purified preparation of cytochrome P-450 from microsomes of anaerobically grown yeast. *Biochem. Biophys. Res. Commun.* **78**, 1005–1010

39. Yoshida, Y and Aoyama, Y (1984) Yeast cytochrome P-450 catalyzing lanosterol 14α-demethylation I Purification and spectral properties. *J Biol. Chem* **259**, 1655–1660
40. Aoyama, Y, Yoshida, Y., and Sato, R. (1984) Yeast cytochrome P-450 catalyzing lanosterol 14α-demethylation II Lanosterol metabolism by purified P-450$_{14DM}$ and by intact microsomes. *J. Biol. Chem* **259**, 1661–1666
41. Kaergel, E., Aoyama, Y., Schunck, W-H., Mueller, H.-G., and Yoshida, Y. (1990) Comparative study on cytochrome P-450 of yeasts using specific antibodies to cytochrome P-450$_{alk}$ and P-450$_{14DM}$. *Yeast* **6**, 61–67
42. Kalb, V.F., Loper, J C, Dey, C.R., Woods, C.W, and Sutter, T R. (1986) Isolation of a cytochrome P-450 structural gene from *Saccharomyces cerevisiae*. *Gene* **45**, 237–245
43. Kalb, V F., Woods, C W, Turi, T.G, Dey, C.R, Sutter, T.R., and Loper, J.C. (1987) Primary structure of the P450 lanosterol demethylase gene from *Saccharomyces cerevisiae*. *DNA* **6**, 529–537
44. Turi, T.G. and Loper, J.C (1992) Multiple regulatory elements control expression of the gene encoding the *Saccharomyces cerevisiae* cytochrome P450, lanosterol 14α-demethylase (*ERG11*) *J. Biol. Chem.* **267**, 2046–2056
45 Bard, M, Lees, N D., Turi, T, Craft, D., Cofrin, L., Barbuch, R, Koegel, C., and Loper, J.C. (1993) Sterol synthesis and viability of *erg11* (cytochrome P450 lanosterol demethylase) mutations in *Saccharomyces cerevisiae* and *Candida albicans*. *Lipids* **28**, 963–967
46 Watson, P F., Rose, M.E, Ellis, S W, England, H, and Kelly, S.L (1989) Defective sterol C5-6 desaturation and azole resistance: a new hypothesis for the mode of action of azole antifungals. *Biochem. Biophys. Res. Commun.* **164**, 1170–1175
47 Aoyama, Y., Funae, Y., Noshiro, M., Horiuchi, T., and Yoshida, Y. (1994) Occurrence of a P450 showing high homology to yeast lanosterol 14-demethylase (P450$_{14DM}$) in the rat liver *Biochem. Biophys. Res. Commun.* **201**, 1320–1326
48. Aoyama, Y., Noshiro, M., Gotoh, O., Imaoka, S., Funae, Y., Kurosawa, N, Horiuchi, T, and Yoshida, Y. (1996) Sterol 14-demethylase (P45014DM) is one of the most ancient and conserved P450 species. *J. Biochem.* **119**, 926–933
49. Adams, A., Gottschling, D E., Kaiser, C.A., and Stearns, T (1997) *Methods in Yeast Genetics*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
50 Ohya, Y, Goeble, M., Goodman, L E, Petersen-Bjørn, S., Friesen, J.D, Tamanoi, F, and Anraku, Y. (1991) Yeast *CAL1* is a structural and functional homologue to the *DPR1* (*RAM*) gene involved in *ras* processing. *J Biol Chem* **266**, 12356–12360
51. Sikorski, R S. and Hieter, P. (1989) A system of shuffle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* **112**, 19–27
52. Nishikawa, S and Nakano, A. (1991) The GTP-binding Sar1 protein is localized to the early compartment of the yeast secretory pathway *Biochim. Biophys. Acta* **1093**, 135–143
53 Schunck, W-H., Vogel, F., Gross, B., Kärgel, E, Mauersberger, S., Köpke, K., Gengnagel, C., and Müller, H.-G. (1991) Comparison of two cytochromes P-450 from *Candida maltosa*: primary structures, substrate specificities and effects of their expression in *Saccharomyces cerevisiae* on the proliferation of the endoplasmic reticulum *Eur. J. Cell Biol.* **55**, 336–345
54. Jones, E.W, Webb, G.C., and Hiller, M.A. (1997) Biogenesis and function of the yeast vacuole in *Molecular and Cellular Biology of the Yeast Saccharomyces*. Vol. 3 (Pringle, J.R., Broach, J.R, and Jones, E W., eds.) pp. 363–470, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York
55. Gut, J, Richter, C., Cherry, R.J., Winterhalter, K.H, and Kawato, S. (1982) Rotation of chtochrome P-450 II Specific interactions of cytochrome P-450 with NADPH-cytochrome P-450 reductase in phospholipid vesicles. *J Biol Chem.* **257**, 7030–7036
56 Nakano, A., Brada, D., and Schekman, R. (1988) A membrane glycoprotein, Sec12p, required for protein transport from the endoplasmic reticulum to the Golgi apparatus in yeast. *J Cell Biol* **107**, 851–863