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Topology Inversion of CYP2D6 in the Endoplasmic Reticulum Is Not Required for Plasma Membrane Transport

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ABSTRACT

The presence of CYP2D6 at the surface of isolated rat and human hepatocytes and its recognition by autoantibodies were reported recently. We wondered whether the unexpected outside orientation at the plasma membrane could be related to topological inversion (luminal-oriented form) of cytochrome P450 in the endoplasmic reticulum. To examine the potential role of cDNA polymorphism, a CYP2D6 variant carrying three positive charges at the amino terminus (2D6ext) was constructed and expressed in yeast. Immunoblotting, flow cytometry, and electron microscopy showed that wild-type CYP2D6 expressed in yeast was present on the outer face of the cell plasma membrane in addition to the regular microsomal location. This location reproduces the hepatocyte situation. 2D6ext expressed in yeast and COS7 cells seemed to be partially *N*-glycosylated and was located at the plasma membrane surface. Nevertheless, the glycosylated form was not enriched in the plasma membranes compared with microsomes. The relationship between CYP2D6 and 2D6ext topologies and catalytic competence was tested. Cumene hydroperoxide-dependent dextromethorphan demethylation was performed on microsomal vesicles after combined proteolysis and immunoinhibition experiments. CYP2D6 activity was completely abolished, whereas the glycosylated and luminal-oriented fraction of 2D6ext remained active. This suggests that a luminal-oriented glycosylated form is not involved in cytochrome P450 transport to the plasma membrane. Yeast thus reproduces the unusual CYP2D6 plasma membrane location and orientation, which do not require sequence alteration, glycosylation, or even an inverted endoluminal orientation.

P450s are xenobiotic metabolizing enzymes involved in the pathogenesis of some kinds of autoimmune hepatitis. These intracellular enzymes are the target of autoantibodies developed in drug-induced hepatitis, such as tienilic acid, dihydralazine, anticonvulsant, and halothane hepatitis (Eliasson and Kenna, 1996). Furthermore, CYP2D6 is also recognized by other autoantibodies (anti-LKM1) in non–drug-induced hepatitis as type-2 autoimmune hepatitis (Zanger *et al.*, 1988; Kiffel *et al.*, 1989; Manns *et al.*, 1989) and hepatitis C infection (Parez *et al.*, 1996). The immunogenic sites map to the catalytic domains of P450s 2C9, 3A, and 2D6 (Manns *et al.*, 1991; Yamamoto *et al.*, 1993; Lecoeur *et al.*, 1996). Surprisingly, immunolabeling of the surface of isolated rat or human hepatocytes, rabbit pulmonary cells, and mammalian transfected cells, demonstrated the presence of P450s from 1A, 2B, 2C, 2D, 2E1, and 3A families on the external face of the cell PM (Eliasson and Kenna, 1996; Loeper *et al.*, 1990, 1993; Robin *et al.*, 1995). Because of the ability of anti-CYP2D6 (anti-LKM1) antibodies to recognize a major epitope (WDPAQPPRD) of the CYP2D6 catalytic domain (Manns *et al.*, 1991; Yamamoto *et al.*, 1993) and the presence of CYP2D6 at the human hepatocyte surface (Loeper *et al.*, 1993), this isoform was chosen as a model.

To further investigate the unusual exposure of P450 epitopes at the surface of the PM, we decided to determine if such phenomenon could be related to the presence of two topologies in the ER. Dual topologies have been suggested for P450s (Cooper *et al.*, 1980) and described for other ER proteins (Alves *et al.*, 1993). P450s in their major cytosol-oriented forms would be restricted to the ER by a stop-transfer signal. In contrast, the luminal-oriented molecules would not be retained in the ER and would follow a vesicular flow to the external face of PM. The charge balance on the amino-terminal sequence is critical in defining the membrane topology of ER proteins and could be a determining factor for protein transport toward the PM (Sato *et al.*, 1990).

Expression in yeast constitutes a useful model system because the mechanisms of translocation and secretory protein transport in this eukaryotic organism are fundamentally the same as in mammalian cells (Stirling *et al.*, 1992; Schwientek *et al.*, 1995). Although heterologous P450s can be efficiently expressed in yeast in functional forms (Guengerich *et al.*,

ABBREVIATIONS: P450, cytochrome P450; CumOOH, cumene hydroperoxide; ER, endoplasmic reticulum; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PM, cell plasma membrane; SDS sodium dodecyl sulfate; MES, 2-(*N*-morpholino)ethanesulfonic acid; TE, Tris/EDTA.

1991; Gautier *et al.*, 1996), it was unknown if this host could reproduce the routing pathways observed in hepatocytes. To determine whether the unexpected presence of P450 on the PM surface originates from a minor population exhibiting an inverted luminal orientation in the ER, we expressed CYP2D6 and a variant (2D6ext) that includes two additional arginines at the amino terminus. Expression in yeast and COS7 cells and subcellular locations were analyzed by immunoblot, electron microscopy, and flow cytometry using anti-CYP2D6 autoantibodies. Endoglycosidase, limited proteolysis, and CumOOH-supported dextromethorphan demethylation were used to probe topology and function.

Materials and Methods

Saccharomyces cerevisiae* strain and growth conditions.** W303-1B (*MATa**, *leu2-3, 112 his3-11 ade2-1 trp1-1 ura3-1 can*^{R} *cyr*$^+$) was used. W(R) was derived from W303-1B by insertion of the *GAL10-CYC1* galactose-inducible promoter immediately upstream of the 5′-end of the open reading frame of the yeast P450 reductase gene *YRED* (Gautier *et al.*, 1996). This strain was transformed by the pYeDP60-derived plasmids (see below) and the transformants selected for adenine prototrophy. Transformants were grown in SLI synthetic medium (Pompon *et al.*, 1996).

Plasmids. The yeast expression vector pYeDP60 (designated V60) was described previously (Pompon *et al.*, 1996). pCYP2D6/V60 (pCYP2D6) was constructed by insertion of the human CYP2D6 open reading frame between the *Bam*HI and *Eco*RI sites of V60 (Gautier *et al.*, 1996). The expression plasmid p2D6ext encoding the CYP2D6 variant was constructed by PCR amplification using pCYP2D6 as the matrix. The sequence 5′-AGAAGA-3′ (encoding two arginines) was introduced after the second codon of the CYP2D6 open reading frame, and the GAA codon encoding the residue Glu-4 was replaced by an AGA arginine codon. The direct PCR primer was thus 5′-gcgaattcATGGGGAGAAGACTAAGAGCACTGGTGCCCCTGGCCG-TGATAG-3′, in which the underlined sequence represents the changes compared with the wild type cDNA. Lower case letters indicate a restriction site. PCR products encompassing the full CYP2D6 or 2D6ext open reading frame were cloned into PCR script giving pSC2D6 and pSC2D6ext, respectively, and then sequenced. The *Bam*HI-*Eco*RI fragment was isolated and inserted into V60. For COS7 transient expression the *Bam*HI fragment of pSC2D6ext encompassing the 2D6ext open reading frame was cloned in the appropriate orientation into the *Bam*HI site of pCB6 (Brewer and Roth, 1991).

Subcellular fractionation. Yeast cells transformed with pCYP2D6 and p2D6ext were exponentially grown in SLI synthetic medium to a density of 1×10^7 cells/ml (A_{600} = 1.5). Harvested cells were disrupted as described previously (Pompon *et al.*, 1996), and microsomal fractions were prepared by ultracentrifugation. PM fractions were prepared using the attachment of spheroplasts on cationic silica microbeads, kindly provided by Dr. Bruce S. Jacobson, (Schmidt *et al.*, 1983). To restore PM integrity after the enzymatic treatment, cells were incubated for 1 hr in SLI medium containing 1.2 M sorbitol. When measurement of P450 activity was required, transformed cells were grown in a buffered SLI medium containing 50 mM MES, pH 6.3, to avoid medium acidification that could inactivate externally exposed P450. Coating buffer, pH 7.8, was used for PM preparations (Schmidt *et al.*, 1983). At the end of the procedure, the PM-coated beads were resuspended in TE buffer (50 mM Tris/HCl, pH 7.4, 1 mM EDTA) and stored at −80°.

Electron microscopy and flow cytometry. Anti-CYP2D6, control human IgG, and secondary antibodies were adsorbed overnight at 4° against control whole yeast cells and spheroplasts for all assays. For peroxidase staining, transformed or control (plasmid with no insert) cells were converted to spheroplasts, fixed with 4% paraformaldehyde, and incubated with anti-CYP2D6 and peroxidase-conjugated antibodies. Postembedding immunoelectron microscopy was performed as described previously (Loeper *et al.*, 1990, 1993; Robin *et al.*, 1995). For immunogold staining, paraformaldehyde (4%)- and glutaraldehyde (0.5%)-fixed cells were embedded in LR White resin at 4°. Ultrathin sections were cut and labeled with anti-CYP2D6 antibodies and protein-A gold particles (10 nm) at a 1:100 dilution. To increase contrast, staining with uranyl acetate and lead citrate was performed. In control experiments postembedded spheroplasts and sections were incubated with control human IgG or directly with protein A-gold. Alternatively, control cells were used. For immunofluorescence, transformed and control fixed spheroplasts were incubated with anti-CYP2D6 and fluorescein isothiocyanate-conjugated antibodies (Pasteur production, Marnes-la-Coquette, France) (Loeper *et al.*, 1990, 1993; Robin *et al.*, 1995). The flow cytometry analysis was performed as described (Robin *et al.*, 1995) on a Coulter Elite ESP (Coultronics, Margency, France) apparatus equipped with an argon ion laser (Coherent, Saclay, France) tuned to 488 nm and 15 mW output power.

Transient transfection of COS7 cells. COS7 cells were grown at 37° in 5% CO_2 in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY) containing 4 mM glutamine, 0.1 mg/ml penicillin, 0.1 mg/ml streptomycin, and 10% fetal calf serum (GIBCO). At 50% confluence, cells were transfected by electroporation (950 μF, 200 v) with 10 μg of p2D6extCB6. After 48 hr, cells were washed with cold phosphate-buffered saline, scraped, and homogenized in 1 ml of TE buffer. Homogenate fractions were treated with 1 × loading buffer for SDS-PAGE and analyzed by immunoblotting using anti-CYP2D6 antibodies (Loeper *et al.*, 1990, 1993).

Enzymatic assays. CumOOH-dependent dextromethorphan demethylation was performed at 24° using subcellular fractions (0.1 mg of microsomal protein in 0.2 ml or 0.1 mg of PM protein bound on silica beads in 0.4 ml) in TE buffer, 50 μM dextromethorphan (Roche, Switzerland), and 0.5 mM purified CumOOH. Aliquots were withdrawn each 30 sec up to 7 min to determine the kinetics of demethylation. The reaction was stopped by addition of 1 M sodium carbonate (1:4 v/v) and extracted with an equal volume of ethyl acetate. The upper phase was collected and air-dried, and products were analyzed by reverse phase high performance liquid chromatography using fluorescence detection (emission, 312 nm; excitation, 270 nm). The initial demethylation rate was calculated from least squares nonlinear regression assuming a first order enzyme inactivation law. Digestions with peptide *N*-glycosidase F were performed according to the instructions of the manufacturer (New England Biolabs, Beverly, MA). Protein concentrations were determined using the Pierce bicinchoninic acid assay with bovine serum albumin as standard (Pierce Chemical, Rockford, IL).

Results

Experimental system and variant design. To determine if yeast can reproduce the double (ER and PM) subcellular location observed for CYP2D6 in rat and human hepatocytes (Loeper *et al.*, 1990, 1993), *S. cerevisiae* strain W(R) was transformed with a galactose inducible expression vector (pCYP2D6/V60) encoding CYP2D6. Insertion of positive charges upstream of the apolar domain of the signal sequence could reverse P450 orientation in the ER membrane and convert this sequence into an export signal (Szczesna-Skorupa and Kemper, 1993). A variant (2D6ext) was built by changing the charge of the amino-terminal extremity of the signal sequence from −1 to +3 (Fig. 1). This was performed by the introduction of two arginine residues at position −3 and by the E6R mutation (see Materials and Methods).

Subcellular location of human CYP2D6 and its amino-terminal variant in transformed yeast. With the exception for the presence of an additional slow migrating faint

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                ++ +                    -   ++ +   +
2D6ext      MGRRLR ALVPLAVIVAIFLLLV DLMHRRQRWAAR YPPGP
                -                       -   ++ +   +
CYP2D6      MGLE ALVPLAVIVAIFLLLV DLMHRRQRWAAR YPPGP
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Fig. 1. Amino acid sequence alignment of the amino-terminal segment of CYP2D6 and 2D6ext. The sequence blocks on the left represent the amino-terminal charged segments of the signal sequence. *Boldface,* residues changed between CYP2D6 and 2D6ext.

band in the 2D6ext sample (Fig. 2, *lane 2*, *band b*), the expressed CYP2D6 and 2D6ext were present at similar levels in yeast microsomes, as demonstrated by Western blot analysis (Fig. 2, *lanes 2* and *4*). This observation suggests that charge balance change in 2D6ext might have caused some topological inversion of the P450 in the ER membrane, resulting in glycosylation and consequently lower electrophoretic mobility.

To study CYP2D6 and its variant in yeast PM, we used the purification method developed by Jacobson (Schmidt *et al.*, 1983). This method involves binding the external face of PM to cationic silica microbeads before cell lysis. The immunoblot PM pattern (Fig. 2, *lanes 1* and *3*) for CYP2D6 and 2D6ext is very similar to the one observed for microsomal proteins. Protein staining of the SDS-PAGE from PM and ER fractions (Fig. 3A) clearly reveals different band patterns. In addition, antibodies to Sec61p, a yeast integral ER protein migrating at 32 kDa (Stirling *et al.*, 1992) recognize only the microsomal fractions (Fig. 3B). These results show that the PM are not contaminated by ER structures.

Analysis of the presence of CYP2D6 and 2D6ext on the plasma membrane by electron microscopy and flow cytometry. To further demonstrate the presence of the P450s at the cell surface, transformed or control spheroplasts were examined by electron microscopy. Immunolabeling was first performed on unpermeabilized spheroplasts (see Materials and Methods). Cells expressing the 2D6ext and CYP2D6 exhibited clear immunostaining only at the surface of the cells (Fig. 4, A and B); no signal was observed with control cells transfected with a void plasmid (Fig. 4C) or with transformed cells treated with control human IgG. The staining pattern was discontinuous, as previously observed with human hepatocytes (Loeper *et al.*, 1993), but is clearly associated with the PM; no staining was observed in the ER present around the nucleus and under the PM (Fig. 4, A and B, *arrows*). In contrast, when immunolabeling was performed on LR White embedded sections (see Materials and Methods), staining was observed on the ER structures close to the nuclear envelope and underneath the PM (Fig. 4, D and E). The vacuoles were mostly devoid of labeling, as in control experiments using cells with void plasmid or transformed cells incubated with control human IgG (Fig. 4F). Under our culture conditions, the expression level of CYP2D6 and 2D6ext corresponds to about 0.2% of the total microsomal proteins, a value similar to the CYP2D6 expression level in hepatocytes. Furthermore, no labeled karmellae structures were detected. Taken together, these results demonstrate that CYP2D6 and 2D6ext are present on the external surface of the PM in addition to their usual localization.

To quantify the expression of CYP2D6 and the variant at the cell surface, flow cytometry analysis was performed (see

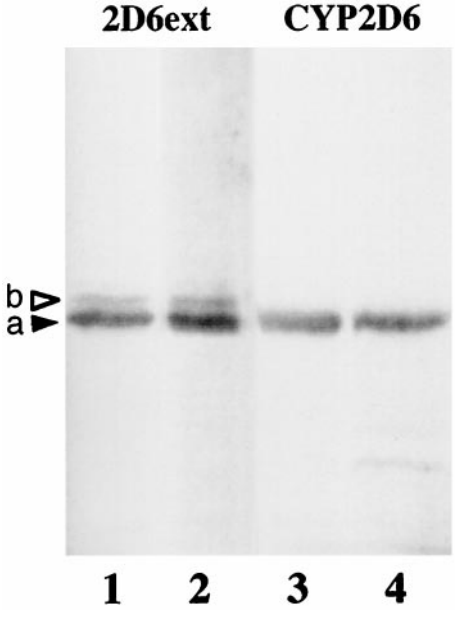

Fig. 2. SDS-PAGE immunoblot analysis of ER and PM fractions from CYP2D6 and 2D6ext expressing yeast. Polyacrylamide gels (9%) were loaded with 40 μg (*lanes 1* and *3*) of PM proteins, and 10 μg (*lanes 2* and *4*) of ER proteins. After SDS-PAGE and electrophoretic transfer to nitrocellulose, the blots were incubated with anti-CYP2D6 autoantibodies at 1:200 dilution. ▶, the position of unmodified P450s; ▷, position of glycosylated form.

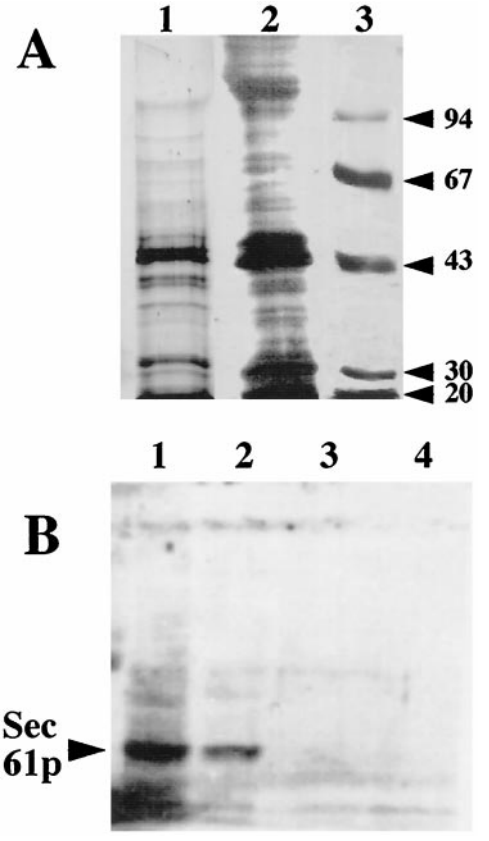

Fig. 3. Yeast subcellular fraction characterization by SDS-PAGE. A, Silver staining of yeast subcellular fractions separated on a 9% gel. PM and ER fractions were prepared as described under Materials and Methods. *Lane 1*, 10 μg of PM proteins; *lane 2*, 5 μg of ER proteins; *lane 3*, 1 μg of low molecular weight range standards (Amersham, Arlington Heights, IL). B, SDS-PAGE immunoblot of two purified ER fractions (*lane 1*, 10 μg; *lane 2*, 5 μg) or PM fractions (*lane 3*, 10 μg; *lane 4*, 5 μg) revealed by electrochemiluminescence (Amersham) using anti-Sec61p antibodies (diluted 1:5000).

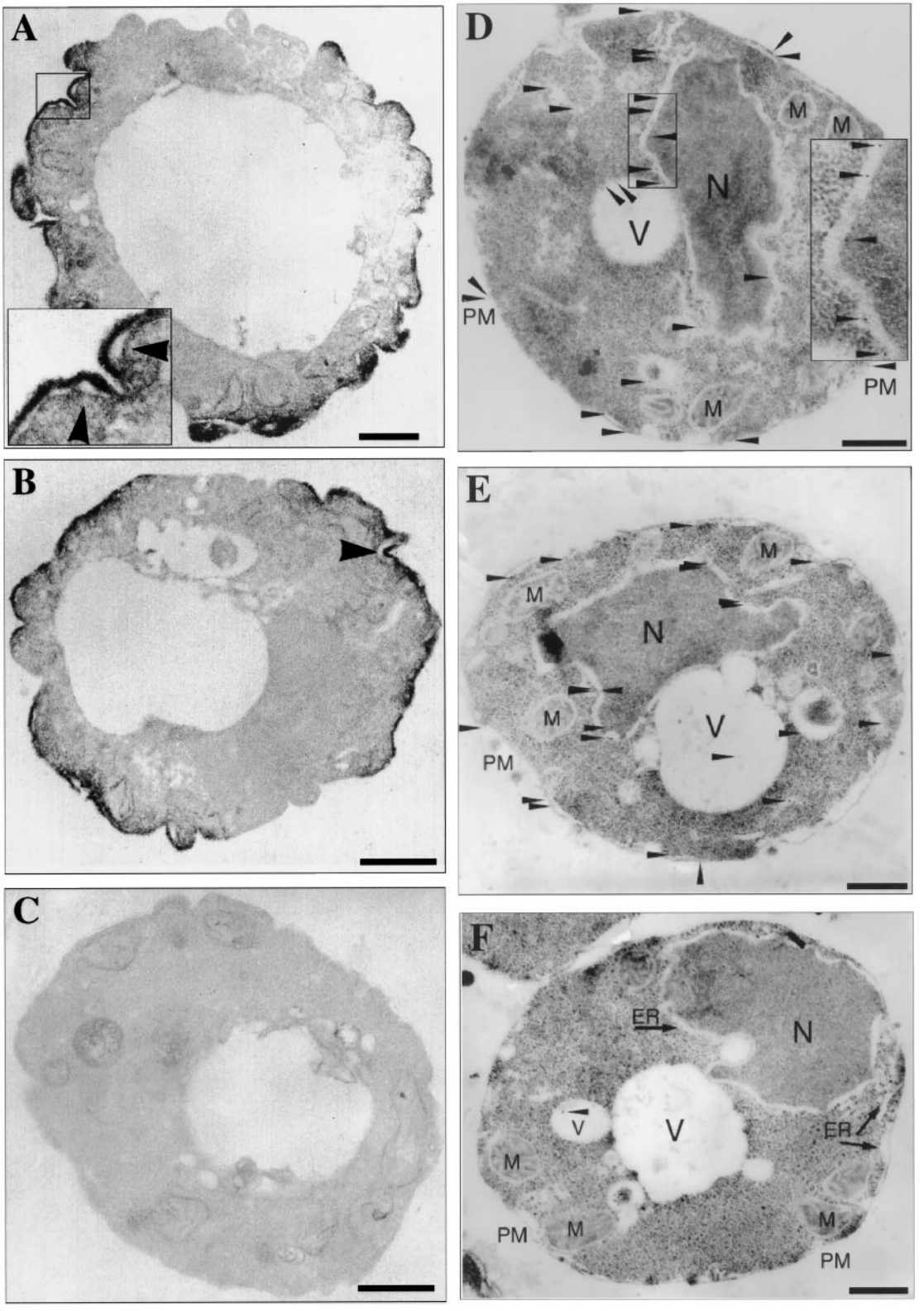

Fig. 4. Electron micrographs of spheroplasts from CYP2D6 or 2D6ext expressing or control cells exposed to anti-CYP2D6 antibodies. A–C, Transformed yeast were converted to spheroplasts, fixed, incubated with anti-CYP2D6 antibodies followed by incubation with peroxidase-conjugated anti-immunoglobulins, and then visualized with diaminobenzidine. The electron microscopy procedure was as described previously (Loeper *et al.*, 1990). *Inset arrowheads*, ER underneath PM. D–F, Fixed spheroplasts were embedded in LR White. Ultrathin sections were cut and immunolabeled with anti-CYP2D6 antibodies and protein A-gold particles then used for microscopy as described in Materials and Methods. A and D, Cell expressing 2D6ext; B and E, cell expressing CYP2D6; C, control cell (void plasmid). F, same as E but using control human IgG. *N*, nucleus; *V*, vacuole; *M*, mitochondrion. *Arrowheads,* protein A-gold particles. *Scale bars*, 0.5 μm.

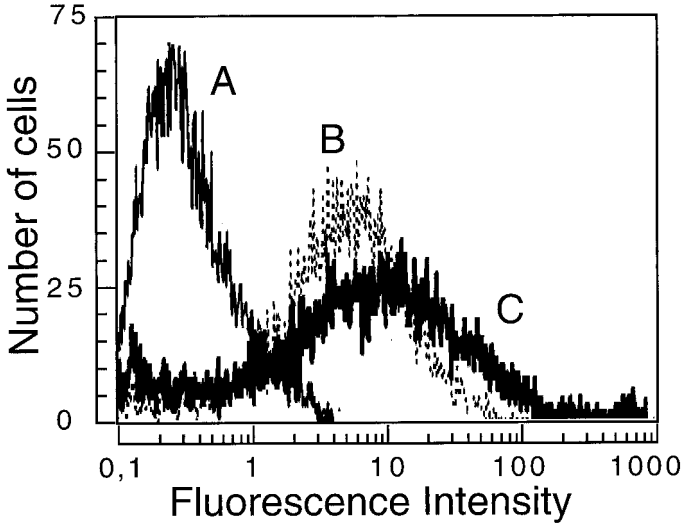

Fig. 5. Flow cytometry analysis of immunolabeled plasma membrane expressing CYP2D6 (*B*), 2D6ext (*C*), and control cells (*A*). Spheroplasts were incubated successively with adsorbed anti-CYP2D6 autoantibodies and adsorbed fluorescein isothiocyanate-anti-human IgG (Loeper *et al.*, 1990). *Curves* show the cell count (arithmetic scale) on the *ordinate* and the fluorescence intensity (log scale) on the *abscissa*.

Materials and Methods). Clear expression of CYP2D6 and 2D6ext (Fig. 5, B and C) on the PM of transformed cells was demonstrated by the rightward shift (i.e., higher fluorescence) of the histogram compared with the one found for control cells (void plasmid) (Fig. 5A). Interestingly, the signal was significantly increased (about a doubled mode value) for 2D6ext (Fig. 5C) when compared with CYP2D6 cells (Fig. 5B). This indicates that the modification of the charge balance on the signal sequence increases the transport efficiency to the PM.

Topology of CYP2D6 and 2D6ext in yeast ER and COS7 cells

CYP2D6 has two potential *N*-glycosylation sites at residues 166 and 398. These sites cannot normally be glycosylated because of the cytosolic orientation of the P450 on the ER surface. In contrast to CYP2D6, 2D6ext appears as two bands after immunoblotting. The slow migrating band could represent 2D6ext *N*-glycosylated molecules formed after topological inversion of the enzyme in the ER lumen. To confirm this hypothesis, microsomal fractions were incubated with peptide *N*-glycosidase F. The slower migrating band of 2D6ext disappeared, whereas no change in migration was observed for the major bands of 2D6ext and CYP2D6 (Fig. 6). To determine whether the observed endoluminal *N*-glycosylation is host-specific, expression of the 2D6ext variant in mammalian COS7 cells was carried out. In whole COS7 cell homogenates, as in yeast, two immunoreactive products were observed, and the low mobility band disappeared upon treatment with peptide *N*-glycosidase F (Fig. 6). This indicates that in both yeast and mammalian cells a fraction of the expressed 2D6ext variant is glycosylated.

To investigate the catalytic competence of the luminal-oriented variant, enzymatic activity was tested. The effects of trypsin digestion on the CumOOH-dependent dextromethorphan demethylase activity of 2D6ext in microsomes was determined (Fig. 7A). This assay does not require P450 reductase, thus avoiding possible interference because of tryptic inactivation of this enzyme. The residual P450 activity of digested samples was measured either after incubation with anti-CYP2D6 or control antibodies. The difference is indicative of the protease-protected P450 fraction on the luminal side of the ER (Fig. 7). Without trypsin, more than 60% of CYP2D6 and 2D6ext activity can be inhibited by anti-CYP2D6 antibodies. After 30 min of digestion, the residual 2D6ext activity (20% of initial) was almost insensitive to anti-CYP2D6 antibodies (Fig. 7A). In contrast, nearly all residual CYP2D6 activity remained inhibited by the antibodies (Fig. 7B). These results suggest that, in contrast to CYP2D6, a significant portion (at least 20%) of 2D6ext is protected because of a topological inversion into the ER lumen. Surprisingly, this inverted enzyme is functionally competent. Translocation of P450 catalytic domain through the ER seems contradictory with the presence of an internal stop transfer sequence (Sato *et al.*, 1990; Szczesna-Skorupa and Kemper, 1993) that would impair transport or at least alter enzyme folding leading to inactivation.

CYP2D6 and 2D6ext are catalytically competent when located on the plasma membrane. Purified PMs from CYP2D6 and 2D6ext expressing cells support CumOOH-dependent dextromethorphan demethylation. To account for the poor linearity of CumOOH-supported P450

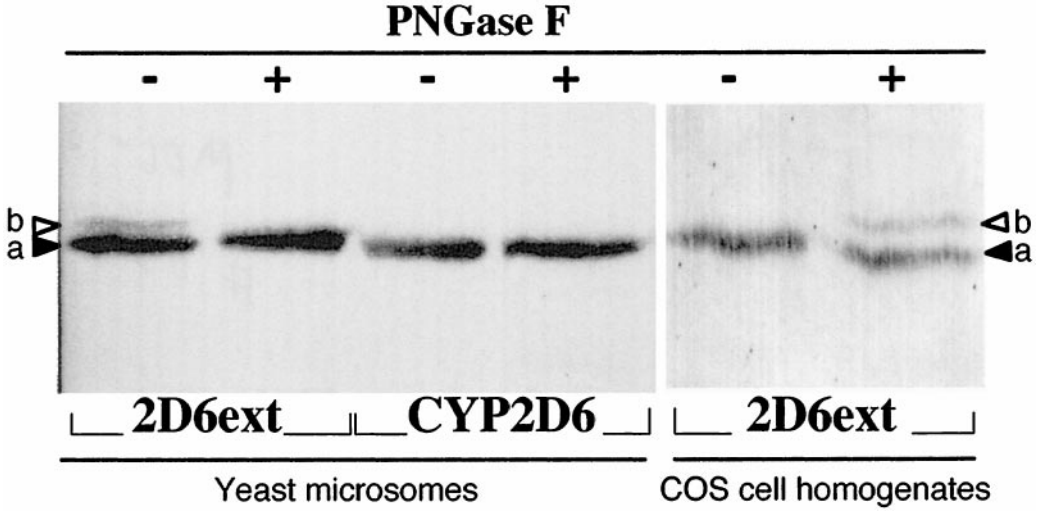

Fig. 6. Peptide *N*-glycosidase F digestion of the fractions of yeast or COS7 cells expressing CYP2D6 or 2D6ext. Ten micrograms of yeast microsomal proteins (*left*) or of whole COS7 cell homogenates (*right*) expressing 2D6ext and 10 μg of yeast microsomal proteins from cells expressing CYP2D6 (*left*) were treated with (+) or without (−) PNGase F before immunoblot analysis (9% acrylamide SDS-PAGE). *Left filled arrowhead (a)*, unglycosylated P450s; *left open arrowhead (b)*, *N*-glycosylated forms were treated with (+) or without (−) PNGase F before immunoblot analysis (9% acrylamide SDS-PAGE). *Right filled arrowhead (a)*, unglycosylated 2D6ext; *right open arrowhead (b)*, *N*-glycosylated form.

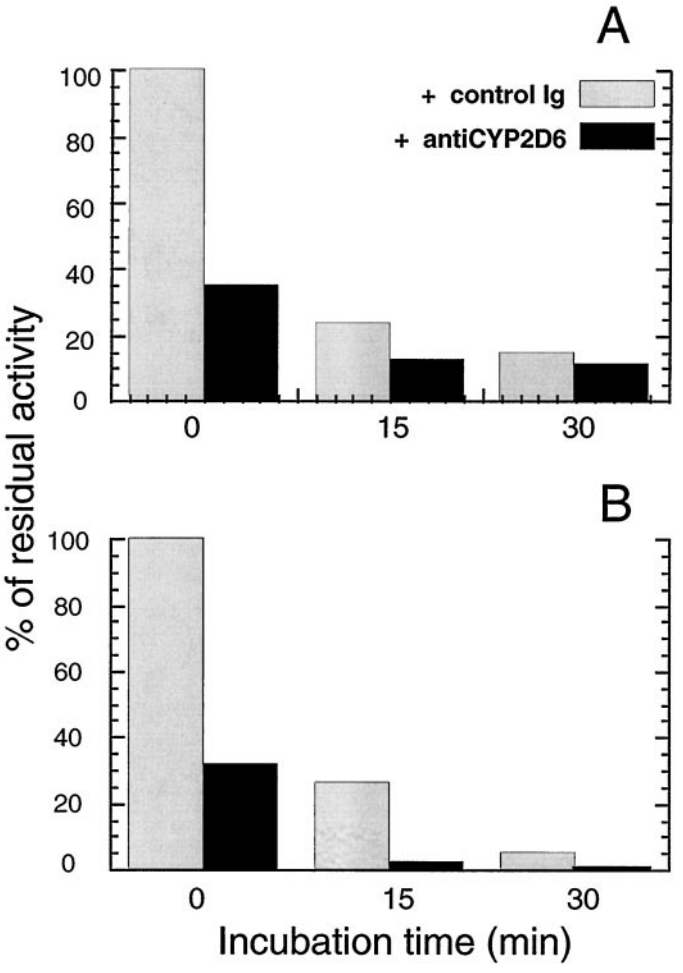

Fig. 7. Course of trypsin digestion of microsomal vesicles from yeast expressing CYP2D6 and 2D6ext. Microsomal vesicles (100 μg of protein in 0.1 ml of TE buffer), containing 2D6ext (A) or CYP2D6 (B) were digested for 0, 15, and 30 min at 30° with TPCK-treated trypsin (25 mg/100 mg of protein) (Sigma, St. Louis, MO). Trypsin digestion was stopped by trypsin-inhibitor (Sigma, 50 mg/100 mg proteins) before incubation with 3 μl of anti-CYP2D6 antibodies (■) or 3 μl of control human sera (control IgG ▨) for 30 min on ice. Control samples (*time 0*) were incubated for 30 min at 30° without trypsin. Samples were tested for CumOOH-dependent CYP2D6 dextromethorphan demethylase activity. All activities are expressed as percent of initial activity (time 0 treated with the control serum). The 100% value corresponds to 8% and 5% dextromethorphan (5 mM initial concentration) conversion per milligrams of protein for 2D6ext and CYP2D6, respectively. Values shown represent the mean of four determinations.

activities, initial velocities were calculated (see Materials and Methods). The specific activities of CYP2D6 and 2D6ext in the PM (4 ± 1 and 7 ± 1% dextromethorphan conversion per mg of proteins, respectively) were half of the values found in microsomal fractions (8 ± 1 and 13 ± 2%, respectively) although the P450 content was at least 4-fold lower based on Western blotting (Fig. 2). The high CYP2D6 and 2D6ext activities in PMs compared with microsomes confirm that they cannot be related to microsomal contamination. Moreover, the 2D6ext CumOOH-dependent activities were found to be about 2-fold higher than for that for CYP2D6, in both PM and ER fractions, even though Western blot signals were similar for these two forms (Fig. 2). The increased PM activity of 2D6ext compared with CYP2D6 is in good agreement with the increased expression of this form at the cell surface.

Discussion

As first demonstrated in hepatocytes (Loeper *et al.*, 1990, 1993), CYP2D6 expressed in yeast localizes both in the ER membrane and at the outer face of the PM. A similar situation is observed with 2D6ext in which the charge at the amino-terminal end of the hydrophobic core is inverted. In hepatocytes, pulmonary cells, and transfected mammalian cells, the presence of surface-exposed P450s was established by immunolabeling (Loeper *et al.*, 1990, 1993; Robin *et al.*, 1995; Eliasson and Kenna, 1996). Based on conventional mechanisms, protein transport to the outer face of the PM is expected to involve a luminal orientation of the protein in the ER (where *N*-glycosylation occurs) followed by transit via the Golgi apparatus. Most mammalian P450s possess potential *N*-glycosylation sites, which are not functional because of the cytosolic orientation of the bulk of the protein. However, topological inversion was reported for P450s containing additional positive charges at their amino terminus (Sato *et al.*, 1990; Szczesna-Skorupa and Kemper, 1993). In our study, partial *N*-glycosylation of the 2D6ext construction in both yeast and mammalian COS7 cells also illustrates this phenomenon. In contrast, no detectable glycosylation was observed for wild type CYP2D6. Our data suggest that P450 PM targeting does not originate from minor and luminal-oriented molecules, because PM and Golgi P450s would be glycosylated and electrophoretically shifted. Such modification in electrophoretic mobility was not detected in human or rat hepatocyte PMs (Loeper *et al.*, 1990, 1993; Robin *et al.*, 1995), or in rat liver Golgi fractions (Neve *et al.*, 1996).

Compared with ER, no enrichment for glycosylated 2D6ext molecules was observed in PM. This suggests no direct relationship between glycosylation and transport to the PM. Consistent with this, CYP1A2, which does not contain *N*-glycosylation sites, was similarly transported to PM in yeast (data not shown). Several hepatic P450s have been detected in the Golgi apparatus (Neve *et al.*, 1996) after a microtubule-dependent vesicular flow toward the hepatocyte plasma membrane (Robin *et al.*, 1995). The Golgi complex, the main site of *O*-glycosylation, is also involved in the remodeling of *N*-oligosaccharide chains in yeast and mammalian cells. For CYP2D6, no electrophoretic mobility shift was detected for P450 between ER and Golgi fractions (Neve *et al.*, 1996) or between ER and PM fractions (Loeper *et al.*, 1990, 1993; current study). Similar results were found for 2D6ext. Thus, one may speculate that P450s enter the Golgi apparatus, but never contact the galactosyltransferases inside the vesicles. A 200-kDa Golgi-associated cytoplasmic protein (related to yeast Sec7p) has been reported to accumulate at the surface of *trans*-Golgi network vesicles that are distinct from the conventional transport vesicles (Narula *et al.*, 1992). Our findings suggest that transported P450 exits from the cytosolic face of the ER and that some topology inversion mechanism must operate between the *trans*-Golgi and PM. Such an inversion has already been suggested for the PE2 portion of the Sindbis alphavirus polyprotein (Liu and Brown, 1993).

In conclusion, this study illustrates that the still controversial and yet unexplained PM localization and topology of P450s observed in mammalian cells also occurs in yeast. Our results strengthen the evidence that a PM transport of xenobiotic metabolizing P450s exists. Finally, yeast provides a useful model system for elucidating non conventional paral-

lel pathways (Saucan and Palade, 1994; Harsay and Bretscher, 1995) to the cell surface, also found in hepatocytes.

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