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Colorectal Cancer-Specific Cytochrome P450 2W1: Intracellular Localization, Glycosylation, and Catalytic Activity[S]

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ABSTRACT

Cytochrome P450 2W1 (CYP2W1) is expressed at high levels in colorectal cancer cells. Moreover, we have shown previously that a higher tumor expression is associated with less survival. In this study, we characterize post-translational modification, inverted endoplasmic reticulum (ER) topology, and catalytic activity of CYP2W1. The analysis of colorectal normal and cancer tissues and CYP2W1 overexpressing human embryonic kidney (HEK) 293 cells showed that a fraction of CYP2W1 is modified by *N*-glycosylation. Bioinformatic analysis identified Asn177 as the only possible glycosylation site of CYP2W1, which was supported by the inability of an N177A mutant to be glycosylated in HEK 293 cells. Analysis of the membrane topology indicated that unlike other cytochromes P450, CYP2W1 in HEK 293-transfected cells and in nontransfected Caco2TC7 and HepG2 cells is oriented toward the lumen of the ER, a topology making CYP2W1 available to the ER glycosylation machinery. Immunofluorescence microscopy and cell surface biotinylation experiments revealed approximately 8% of the CYP2W1 on the cell surface. Despite the reverse orientation of CYP2W1 in the ER membrane, apparently making functional interactions with NADPH-cytochrome P450 reductase impossible, CYP2W1 in HEK 293 cells was active in the metabolism of indoline substrates and was able to activate aflatoxin B1 into cytotoxic products. The study identifies for the first time a cytochrome P450 enzyme with a luminal ER orientation and still retaining catalytic activity. Together, these results suggest the possibility of using CYP2W1 as a drug target in the treatment of colon cancer using antibodies and/or specific CYP2W1 activated prodrugs.

Introduction

Cytochrome P450 (P450) is a very large and diverse superfamily of hemoproteins present in all domains of life that use a plethora of both exogenous and endogenous compounds as substrates in enzymatic reactions that include oxidation, hydroxylation, reduction, and hydrolysis. CYP2W1, one of the novel members of this family of enzymes, was first cloned in our laboratory (Karlgren et al., 2005, 2006). We found that it is expressed at relatively low levels (mRNA) in the human adult nontransformed tissues, whereas the expression in the tumors and in colorectal cancer tissues in particular was significantly higher (both at mRNA and protein levels) (Karlgren et al., 2006; Gomez et al., 2007). We have also shown its expression in the fetal rat colon at the mRNA level (Karlgren et al., 2006), whereas others showed mRNA expression in some mouse tissues (Choudhary et al., 2005). Approximately 30% of human colorectal specimens have been found to express high amounts of CYP2W1 (Karlgren et al., 2006; Gomez et al., 2007; Edler et al., 2009), and indeed, a recent study suggests that the extent of CYP2W1 expression in colorectal cancers might be a prognostic marker for malignancy and survival (Edler et al., 2009). The expression of CYP2W1 is also regulated by DNA methylation at a CpG island in the exon1/intron1 junction (Gomez et al., 2007). Several groups have reported metabolism of various exogenous and endogenous substrates by CYP2W1 in microsomes and bacterial membranes, although at a very low rate (Karlgren et al., 2006; Wu et al., 2006; Yoshioka et al., 2006).

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ABBREVIATIONS: P450, cytochrome P450; ER, endoplasmic reticulum; Endo H, endoglycosidase H; PNGase F, *N*-glycosidase F; HEK, human embryonic kidney; β2-AR, β2-adrenergic receptor; HPLC, high-performance liquid chromatography; A4QN, 1,4-bis{[2-(dimethylamino-*N*-oxide) ethyl]amino}-5,8-dihydroxyanthracene-9,10-dione.

Using truncated forms expressed in *Escherichia coli*, it seems that indoles and aflatoxin B1 might constitute substrates (Wu et al., 2006; Yoshioka et al., 2006), although the activity of the reconstituted system consisting of bacterially expressed CYP2W1 and NADPH-cytochrome P450 reductase toward 17 different fluorescent P450 class substrates has been found negligible, with the exception of weak catalysis of pargyline and aminopyrine (M. Tachibana and M. Ingelman-Sundberg, unpublished observations).

Protein glycosylation represents one of the most common but complex post-translational protein modifications (Walsh and Jefferis, 2006) and has hitherto only been described for CYP19A1 (Cepa et al., 2008) among the human P450 enzymes. Because microsomal forms of P450 are oriented toward the cytoplasm, it is evident that they cannot be exposed to the glycosylation machinery residing in the luminal compartment of the ER. Not surprisingly, such modification has not been described for any of the members in the CYP1 to CYP4 families, although a purified preparation of CYP2B from rabbit contains some sugar moieties (Aguiar et al., 2005). We have earlier reported the appearance of several CYP2W1 immunoreactive bands detected by immunoblotting upon transient transfections in HEK 293 cells (Karlgren et al., 2006), which were suggested to represent post-translationally modified form(s) of CYP2W1 (Gomez et al., 2007). Furthermore, multiple bands of CYP2W1, indicative of putative glycosylation, were detected in Western blot using subcellular fractions from human colorectal tumors (Gomez et al., 2007).

In the present study, we describe the *N*-linked glycosylation of CYP2W1 in vitro, upon its overexpression in HEK-293 cells, and in vivo, in the normal colon tissue and in colorectal cancer specimens, which provides the first case to our knowledge of glycosylation of a human drug-metabolizing P450 enzyme. We found that CYP2W1 has an inverted ER membrane topology, becoming therefore available to glycosyltransferases in the ER lumen but unavailable for functional interactions with cytosol-oriented P450 reductase. A fraction of both glycosylated and nonmodified CYP2W1 is located on the cellular surface. In intact CYP2W1 containing HEK 293 cells, we found that CYP2W1 was catalytically active in the transformation of aflatoxin B1 to cytotoxic products and in the metabolism of indolines, indicating functional intracellular electron transfer. The cell surface localization of the enzyme and the ability of CYP2W1 to activate chemicals to cytotoxic products indicate that CYP2W1 can be used as a target in the treatment of colorectal cancers using either antibodies or prodrugs.

Materials and Methods

Cell Lines and Human Tissue Samples. The HEK 293, HepG2, and Caco2TC7 cell lines were grown as described previously (Karlgren et al., 2006; Gomez et al., 2007), and the human tissue samples were obtained from sources described earlier (Gomez et al., 2007). These samples include tumor tissues and surrounding nontumor tissues, which are properly dissected and examined for homogeneity by a pathologist.

***N*-Glycosylation.** *N*-glycosylation of CYP2W1 was examined using either the endoglycosidase H (Endo H; Roche Diagnostics, Indianapolis, IN) or the PNGase F (New England Biolabs, Ipswich, MA) enzymes. Thirty micrograms of microsomal protein was incubated with either of the enzymes based on the manufacturers' suggestions with some modifications (i.e., longer incubation time was used for Endo H). After treatments, samples were immediately prepared for gel electrophoresis.

DNA Constructs and Mutagenesis. The cDNA of CYP2W1 cloned into the pCMV4 plasmid (Karlgren et al., 2006) and was used for transfection experiments. The glycosylation site of the CYP2W1 protein at $N^{177}IT$, as predicted by the NetNGlyc software (http://www.cbs.dtu.dk/services/NetNGlyc/), was mutated using the Gene Tailor Site-Directed Mutagenesis System (Invitrogen, Carlsbad, CA) to $A^{177}IT$ (i.e., N177A) using the manufacturer's recommendations.

Cell Transfections. Transient transfections were done using the Lipofectamine 2000 reagent (Invitrogen). HEK 293 cells were transfected with a mixture of plasmid DNA (Karlgren et al., 2006) and Lipofectamine 2000 in Dulbecco's modified Eagle's medium. Complete growth medium was reintroduced to the cells after washing off the transfection mixture, and the cells were incubated further until 48 h after transfection.

The Flp-In system (Invitrogen) was used for the construction of the stable cell line expressing CYP2W1. Before transfections, Zeocin (Invitrogen) was added to the Dulbecco's modified Eagle's medium cell culture medium at a final concentration of 100 μg/ml. CYP2W1 cDNA was inserted into the pcDNA5/FRT plasmid and used in transfections of Flp-In-293 cells. The CYP2W1-pcDNA5/FRT plasmid was cotransfected with the Flp recombinase expression plasmid pOG44 using Lipofectamine 2000 (Invitrogen) according to manufacturer's protocol. Single colonies resistant to Hygromycin B (Invitrogen) were selected and subcultured with the addition of 75 μg/ml Hygromycin B. Mock cells were produced by the transfection of HEK 293 cells with pcDNA5/FRT and pOG44 plasmids and prepared as described above.

Western Blot. Immunoblot analysis was performed as described earlier (Nickson et al., 2007). In brief, 30 μg of protein samples were electrophoresed in denaturing polyacrylamide gels and then transferred onto Hybond-C nitrocellulose membranes (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Membranes were incubated with primary antibodies specific for the COOH-terminal region of CYP2W1 (Karlgren et al., 2006), ERp29 (Mkrtchian et al., 1998), calnexin, β2-adrenergic receptor (β2-AR; Santa Cruz Biotechnology, Santa Cruz, CA), or cytochrome b_5 (Santa Cruz Biotechnology), followed by incubation with horseradish peroxidase-conjugated secondary antibodies. Protein bands were visualized using the SuperSignal chemiluminescence system (Pierce Chemical, Rockford, IL). To detect minor amounts of CYP2W1 in the tumor samples (Fig. 1), we applied a more sensitive Western blot development method using the near-infrared fluorescence detection in which the horseradish peroxidase-conjugated secondary antibodies were replaced with the antibodies labeled with IRDye, and protein bands were visualized by the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE).

Protease Protection Assay. Membrane topology of the CYP2W1 protein was determined in the microsomal fractions obtained after differential centrifugation of the postnuclear fraction (Neve and Ingelman-Sundberg, 2000; Karlgren et al., 2006). In brief, microsomal fractions isolated from HEK 293 cells transfected with CYP2W1 or untransfected Caco2TC7 and HepG2 cells were incubated with proteinase K in a buffer consisting of 50 mM Tris-HCl, pH 8.0, and 10 mM $CaCl_2$. Incubations were performed in the presence or absence of 1% Triton X-100. After incubation at 37°C for 30 min, the reaction was stopped by the addition of 10 mM (final concentration) phenylmethylsulfonyl fluoride. After the incubation of proteins on ice for 30 min, samples were prepared immediately for gel electrophoresis.

Mass Spectrometry. Microsomes obtained from transfected HEK 293 cells were treated or not with Endo H. Samples were then electrophoresed in 10% SDS polyacrylamide gel, Coomassie-stained, and the portion of the gel that corresponded to the molecular weight of the glycosylated species (as identified by identical samples applied to the same gel and immunoblotted) was sliced out, trypsin-digested, and analyzed by Voyager DE-PRO matrix-assisted laser desorption

ionization/time of flight mass spectrometer (Protein Analysis Centre, Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Stockholm, Sweden), and proteins were identified using the MS-Fit database (http://prospector.ucsf.edu/).

Immunocytochemistry. Immunocytochemistry methods were based on techniques described previously with some modifications (Karlgren et al., 2006). In brief, HEK 293 cells were grown on poly(L-lysine)-coated glass coverslips. Transfection of HEK 293 cells was performed as mentioned above. Cells were then fixed with 3.7% formaldehyde for 10 min and then either permeabilized or not with 0.5% Triton X-100 for 5 min. Incubation for 1 h in blocking buffer (1% bovine serum albumin in 1× phosphate-buffered saline) was done before probing with primary and secondary antibodies in blocking buffer. Coverslips were finally mounted, and the images were analyzed by the LSM TPMT confocal microscope (Carl Zeiss, Thornwood, NY).

Cell Surface Protein Identification. Detection of CYP2W1 at the cell surface was performed by a Cell Surface Protein Isolation kit (Pierce Chemical) according to the manufacturer's procedure.

Metabolism by CYP2W1. CYP2W1 stably expressing HEK 293 and mock cells were incubated with 150 μM 5-bromoindoline or 100 μM 2-methyl-5-nitroindoline (Sigma-Aldrich, St. Louis, MO) for 2 and 24 h, respectively. The cells were harvested, washed with phosphate-buffered saline, and centrifuged at 2000 rpm for 10 min. Cell pellet was frozen to −70°C for 2 h and thawed thereafter with the addition of 100 μl of water. Suspension was mixed with 100% ice-cold acetonitrile, vortexed, and centrifuged at 13,300 rpm for 20 min. Supernatant was collected and analyzed by HPLC. In brief, the separation was carried out on the reverse-phase C18 column (150 × 2 mm, 5 μm; Phenomenex Inc., Torrance, CA) using the gradient system consisting of acetonitrile and 1 mM ammonium acetate as described by Sun et al. (2007). The metabolite peaks were monitored at 250 nm using a Varian UV detector (Varian Inc., Palo Alto, CA).

Cell Viability. HEK 293 cells with stable expression of CYP2W1 and corresponding mock-transfected cells were seeded on 48-well plates in 300 μl of medium. Aflatoxin B1 was dissolved in acetonitrile/dimethyl sulfoxide (final concentration of solvents was 0.25%). Viability of cells was determined with EZ4U assay (Biomedica, Vienna, Austria) according to the manufacturer's protocol with some minor modifications. In brief, 6 μl of 1 M HEPES, pH 7.4, and 50 μg/ml reagent were added to each well (300 μl of medium) and incubated at 37°C for 20 min.

Results

CYP2W1 Is Glycosylated In Vivo and in Transfected Cells. Transient overexpression of the CYP2W1 cDNA in HEK 293 cells generates CYP2W1 proteins of different molecular sizes as represented by the multiple CYP2W1-specific immunoreactive bands suggestive of post-translational modification (e.g., by glycosylation) (Karlgren et al., 2006). Microsomes from HEK 293 cells overexpressing CYP2W1 and from the human normal and tumor colon tissues were treated with PNGase, an enzyme that cleaves off all types of *N*-linked oligosaccharide moieties (Medzihradszky, 2008), and immunoblotted. Figure 1A demonstrates glycosylation of a fraction of CYP2W1 in the transfected HEK 293 cells as judged by the disappearance of the upper immunoreactive band. The situation in the colon tissues is more complex: CYP2W1 in the sample of the normal colon and in the three of tested cancer samples (CT-11, 13, 14) is represented by one band, which is shifted down after the treatment, indicating glycosylation of the total protein pool. In the rest of colon cancer samples (CT-5, 9), only a fraction of CYP2W1 (top band) was glycosylated similar to the situation in the transfected cells. This upper band in HEK 293 cells is sensitive to yet another deglycosylating enzyme, Endo H, which removes only high mannose and some hybrid types of *N*-linked carbohydrates (Fig. 1B). It is of note that the glycosylated form of CYP2W1 is not present in detectable amounts in HepG2 and Caco2TC7 cells that constitutively express CYP2W1, which is suggested by the absence of band shift after the endoglycosidase treatment (Fig. 1C). Taken together, these data support the hypothesis of complete or partial glycosylation of CYP2W1 in vitro and in vivo.

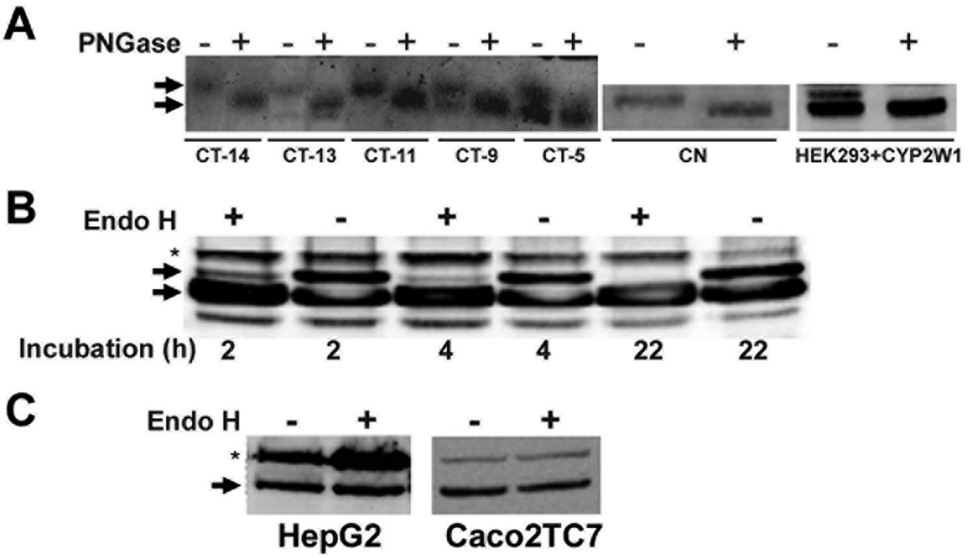


Fig. 1. Glycosylation of CYP2W1 in vivo and in transfected cells. A, microsomal fractions from normal (CN), colon tumor (CT) tissues, and HEK 293 cells transiently transfected with CYP2W1 were treated with the enzyme PNGase. Immunoblotting was done using CYP2W1 antibodies, and protein bands were visualized using near-infrared fluorescence detection system for the increased sensitivity (colon tissues) and conventional chemiluminescent kit (cells). Arrows indicate glycosylated (top band) and nonglycosylated (bottom arrow) CYP2W1 species. B, microsomal fractions from CYP2W1 transfected HEK 293 cells were incubated at varying times at 37°C with or without Endo H. Treatment shows the disappearance of one of the CYP2W1 bands (top arrow). C, microsomal fractions obtained from HepG2 and Caco2TC7 cells were treated with or without Endo H, and immunoblotting was performed using CYP2W1 antibody. Arrow refers to nonglycosylated species, and asterisk refers to an unspecific band.

CYP2W1 Is Glycosylated at Asn177. To determine the precise glycosylation site of CYP2W1, we analyzed its primary structure using NetNGlyc software (http://www.cbs.dtu.dk/services/NetNGlyc/). The consensus *N*-linked glycosylation motif is defined as NXS or NXT sequence, in which X can be any amino acid except for proline. Thus, in silico analysis predicted a putative glycosylation site at Asn177 of CYP2W1 within an NIT tripeptide motif (Fig. 2A). To test the functional importance of this site, we mutated Asn177 to alanine and expressed the ensuing mutant CYP2W1 N177A in HEK 293 cells. The expressed mutant lacked the top glycosylated band and consequently was insensitive to Endo H treatment, suggesting the essential role of this residue for CYP2W1 glycosylation (Fig. 2B). To confirm the identity of the glycosylated protein band, the microsomal fraction of HEK 293 cells overexpressing CYP2W1 was separated electrophoretically, the gel was stained by Coomassie blue, and the region corresponding to the top glycosylated band was cut, sliced, and subjected to mass spectrometry. Identification of the peptides produced after tryptic digestion showed that the region in question was substantially enriched in CYP2W1 in addition to several other proteins with similar molecular masses (Fig. 2C and Supplemental Fig. 1S) (Neve and Ingelman-Sundberg, 2000, 2010).

CYP2W1 ER Membrane Topology. ER resident P450 proteins are typically oriented toward the cytoplasm, making them unavailable for the glycosyltransferases that are present in the luminal space of the endoplasmic reticulum. Determining the membrane topology of CYP2W1 may provide important information concerning its availability to the glycosylation enzymes in the ER. For this purpose, we used the protease protection assay to analyze the CYP2W1 topology in the ER membrane. In the HEK 293 cells transfected with CYP2W1 in the absence of detergent, both fractions of CYP2W1 were protected from digestion by Proteinase K (similar to the ER luminal marker ERp29), whereas a combination of detergent and protease led to the complete degradation of the enzyme (similar to the ER membrane marker cytochrome b_5) (Fig. 3A). These data suggest an inverted (compared with other P450 enzymes) membrane topology of CYP2W1 in which the major portion of the protein molecule is oriented toward the lumen of ER, thus colocalizing with the luminal glycosyltransferases. Identical topology was found in the cell lines with the constitutive expression of CYP2W1, Caco2TC7 and HepG2 (Fig. 3, B and C).

Plasma Membrane Localization of CYP2W1. It has been shown previously that glycosylation facilitates the selective transport of proteins to the cell surface (Gladysheva et

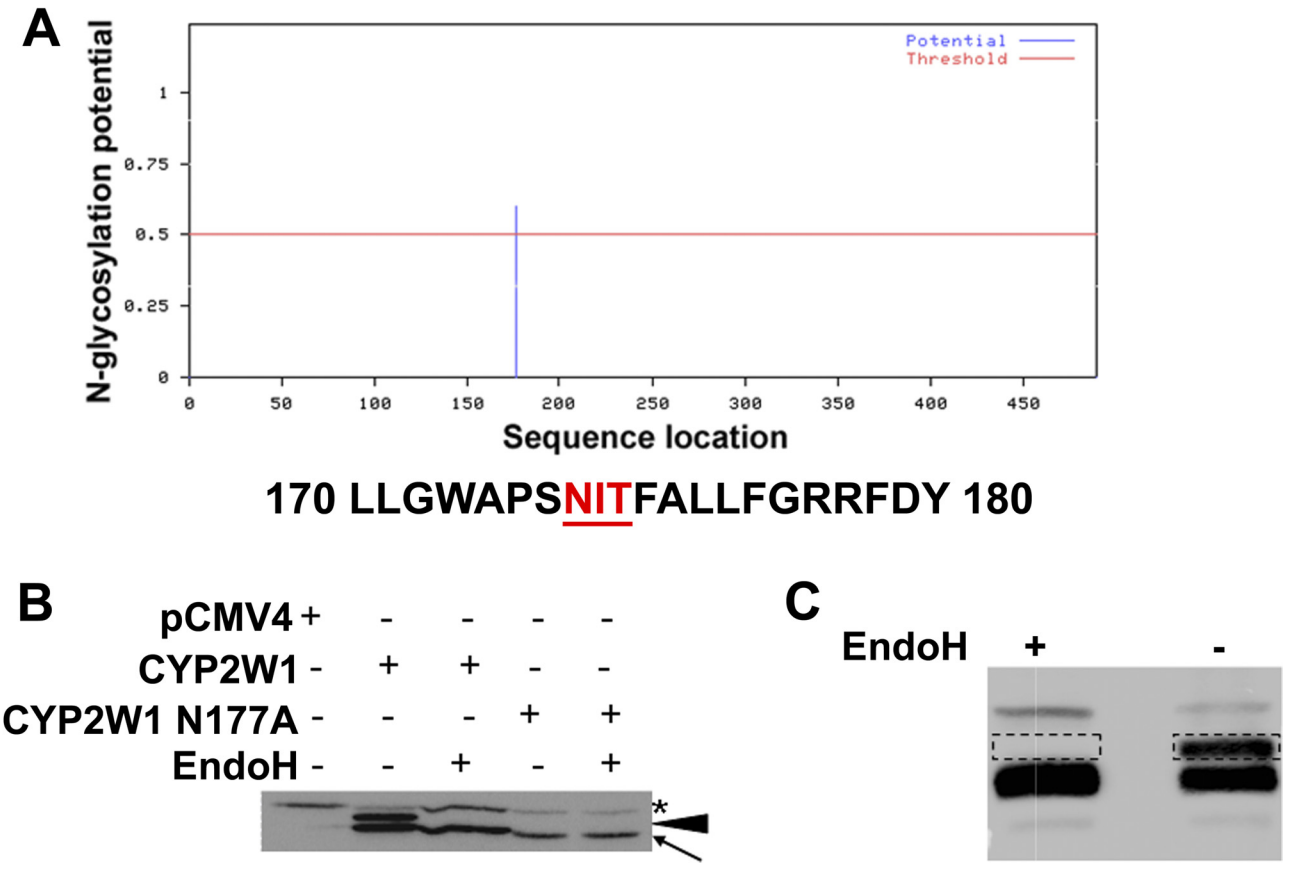


Fig. 2. Glycosylation site of CYP2W1. A, analysis of CYP2W1 peptide sequence using the NetNGlyc software (http://www.cbs.dtu.dk/services/NetNGlyc/) reveals putative glycosylation site at Asn177. B, site-directed mutation of the predicted glycosylation site in CYP2W1 (N177A) inhibited the expression of the Endo H-sensitive (glycosylated) band. Arrow refers to nonglycosylated species, arrowhead refers to the glycosylated species, and asterisk refers to an unspecific band. C, microsomes isolated from HEK 293 cells transiently transfected with the CYP2W1 construct were treated or not with Endo H. Subsequent to electrophoresis in SDS-polyacrylamide gel electrophoresis and Coomassie staining, the regions corresponding to the glycosylated CYP2W1 (boxed in the shown immunoblot, see *Materials and Methods* for details) were sliced out, trypsin-digested, and analyzed by mass spectrometry. CYP2W1 was present only in the non-Endo H-treated sample (for the complete list of identified proteins, see Supplemental data, Fig. 1S).

al., 2008) and that some members of the P450 family localize to the plasma membrane (Neve and Ingelman-Sundberg, 2000, 2010). Immunofluorescent confocal microscopy of permeabilized HEK 293 cells overexpressing wild-type and mutant CYP2W1 detected the majority of intracellular CYP2W1 colocalizing with an ER resident molecular chaperone BiP (Fig. 4A) similar to most cytochromes P450. As expected, BiP staining was absent in nonpermeabilized cells because this protein is not known to exhibit cell surface expression. However, a relatively small fraction of CYP2W1 appears on the surface of HEK 293 cells forming typical patch-like structures (Fig. 4A). It is noteworthy that both forms of CYP2W1 can be seen on the cell surface. To further describe this phenomenon, cell surface proteins were labeled with biotin before cell lysis, isolated with streptavidin agarose beads, and immunoblotted using antibodies against CYP2W1, β2-adrenergic receptor (β2-AR), a cell surface protein, as a positive control, and calnexin, an ER-resident membrane protein, as a negative control. CYP2W1 was detected in the streptavidin-isolated fraction of biotinylated surface proteins, suggesting that certain amounts of CYP2W1 are indeed localized on the cell surface (Fig. 4B). Again, glycosylation of CYP2W1 did not give any preferential advantage for plasma membrane targeting. To support this conclusion and to evaluate quantitatively the extent of CYP2W1 cell surface expression, we carried out densitometric analysis of CYP2W1 immunoreactive bands representing biotinylated as well as intracellular and total CYP2W1 pools. The data shown in Fig. 5 indicate that 8.1% of total CYP2W1 in the transfected HEK 293 cells is localized on the cell surface. The fact that the extent of CYP2W1 glycosylation remains constant (~3–4%) in the intracellular and cell surface fractions supports the above-mentioned observation, suggesting that glycosylation is not a prerequisite for the cell surface targeting.

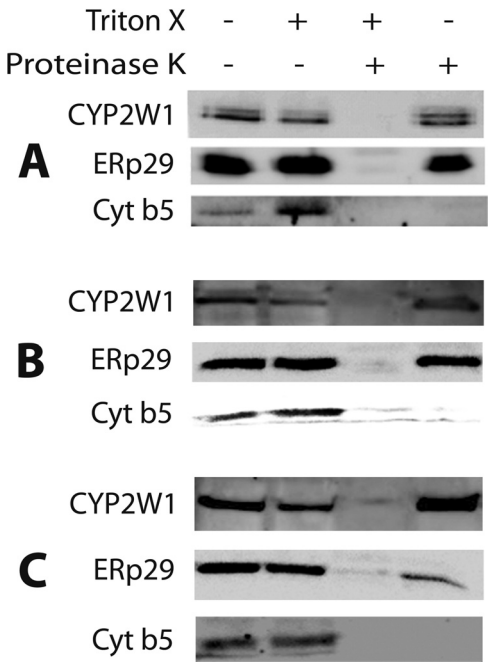


Fig. 3. Topological orientation of CYP2W1. Protease protection assay was performed on microsomal fractions obtained from HEK 293 transfected with CYP2W1 (A), Caco2TC7 (B), and HepG2 cells (C). The microsomal fraction was incubated with or without proteinase K in the presence or absence of 0.5% Triton X-100. Western blot analysis also included ERp29 as the luminal protein control and cytochrome b_5 as the membrane protein control. Asterisk refers to an unspecific band.

Catalytic Activity and Prodrug Activation by CYP2W1. The inverted membrane topology of CYP2W1 questions its interaction with the other members of the P450 electron transport chain and consequently the biological activity per se. The endogenous substrates of CYP2W1 are unknown; however, some xenobiotics, such as indoles and aflatoxin B1, were shown to be catalyzed by the truncated form of the enzyme in bacterial systems, albeit at a very low rate (Wu et al., 2006; Yoshioka et al., 2006). When CYP2W1 stably ex-

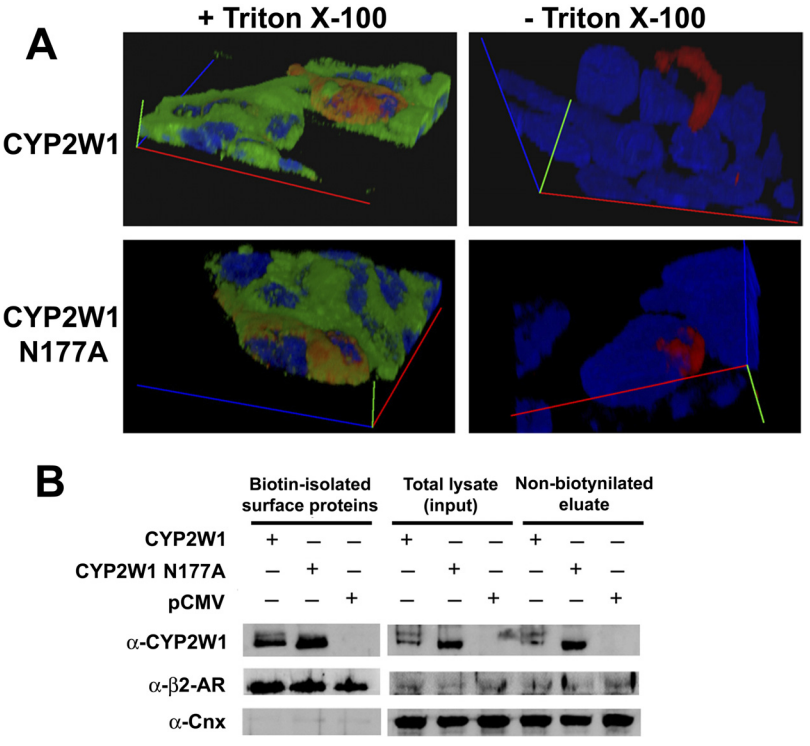


Fig. 4. Plasma membrane localization of CYP2W1. A, three-dimensional confocal microscopy analysis of HEK 293 cells identifies CYP2W1 on the cell surface of nonpermeabilized (i.e., non–Triton X-100-treated) cells expressing wild-type or glycosylation site-mutated CYP2W1. Red stain, CYP2W1; Green stain, BiP, an abundant ER chaperone serving as a negative control; Blue stain, nuclear staining by 4,6-diamidino-2-phenylindole. B, proteins on the surface of nonpermeabilized HEK 293 cells overexpressing wild-type and glycosylation mutant CYP2W1 were labeled by biotin and isolated with streptavidin agarose beads. CYP2W1 seems to be labeled by biotin, indicating its presence on the cell surface similar to β2-AR, which was used as a positive cell membrane protein control. The ER protein calnexin (Cnx) was used as a negative cell membrane protein control.

pressing HEK 293 cells were incubated with 2-methyl-5-nitroindoline or 5-bromoindoline, the subsequent HPLC assay revealed two 5-bromoindoline (M1, M2) and one 2-methyl-5-nitroindoline (M1) derivatives that were not formed upon the incubations with mock cells (Fig. 6). This indicates catalytic conversion of the indolines to the corresponding metabolites by CYP2W1.

It might be suggested that cytochromes P450 with a specific expression in the cancer cells and capable of activation of prodrugs to cytotoxic metabolites can be used as drug targets for anticancer therapeutics. Aflatoxin B1 was shown to induce bacterial revertants in CYP2W1-expressing bacteria (Wu et al., 2006). Incubation of aflatoxin B1 with the HEK 293-CYP2W1 stable cells resulted in the significant dose-dependent loss of cell viability in a marked contrast to the weak toxic effect in the mock-transfected cells (Fig. 7). This indicates the ability of the intracellular CYP2W1 to convert chemicals into cytotoxic products, which is of interest with respect to its use as a drug target for anticancer therapy.

Discussion

In this study, we have characterized the ER membrane topology, post-translational modification, intracellular localization, and catalytic activity of the colorectal cancer-specific cytochrome P450 2W1. CYP2W1 is partially (in the transfected cells and some tumor and normal tissues) or completely (in the tumor tissues) *N*-glycosylated at Asn177. Such unusual for cytochromes P450 modification is allowed by its inverse (luminal) membrane orientation. Furthermore, both CYP2W1 forms are present on the cell surface at a relatively high extent (8%). Intracellular CYP2W1 is able to activate chemicals to cytotoxic end products despite its atypical membrane topology.

Although the majority of cytochromes P450 are generally considered to be membrane-integrated ER resident proteins, previous studies have shown localization of members of the P450 family to the plasma membrane and other cellular organelles (Ronis et al., 1991; Loeper et al., 1993; Eliasson and Kenna, 1996; Pahan et al., 1997; Szczesna-Skorupa et al., 1998; Omura, 2006). With respect to the plasma membrane-bound CYP2E1, it was calculated by biotin labeling that approximately 2% of the total hepatocyte CYP2E1 resides in the plasma membrane (Neve and Ingelman-Sundberg, 2000). It was concluded from this study that the NH_2-terminal transmembrane domain of CYP2E1 plays a critical role in directing the protein to the cell surface (Neve and Ingelman-Sundberg, 2000). We show here that both the wild-type and the glycosylation site mutant CYP2W1 are detected on the plasma membrane of HEK 293 cells, and moreover, the extent of the wild-type CYP2W1 glycosylation remains constant in both intracellular and cell surface-localized pools. This suggests that glycosylation is not a prerequisite for the plasma membrane-targeting of CYP2W1, and therefore the molecular mechanism that brings CYP2W1 to the cell surface is yet to be determined. With other P450 proteins, it has been apparent that a fraction of the translated protein is inversely incorporated into the ER membrane dependent on the NH_2-terminal sequence, explaining the plasma membrane appearance of 2 to 3% of the common drug-metabolizing P450 enzymes (Neve and Ingelman-Sundberg, 2000, 2010). It cannot be excluded that a small fraction of CYP2W1 indeed has a cytosolic orientation, although our data indicate that the majority localized toward the lumen. The amount of plasma

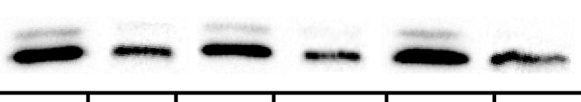
	Total lysate (input)		Non-biotinylated eluate		Biotin-isolated surface proteins	
CYP2W1						
Amount loaded/ total volume (μl)	1/500	0.5/500	1/500	0.5/500	20/400	10/400
Total CYP2W1 %	100		90.2 ± 10.3		8.1 ± 2.1	
Glycosylated CYP2W1 %	3.8 ± 1.1		4.4 ± 1.3		3.4 ± 0.6	

Fig. 5. Quantitative analysis of the cellular distribution and glycosylation of CYP2W1. HEK 293 cells expressing wild-type CYP2W1 were biotinylated as described in Fig. 4. CYP2W1 was identified by immunoblotting, and the bands were analyzed by densitometry using ScienceLab (Fujifilm, Tokyo, Japan) software. Densitometry of the bands representing two times-diluted fractions verified the linear range of the analysis (results not shown).

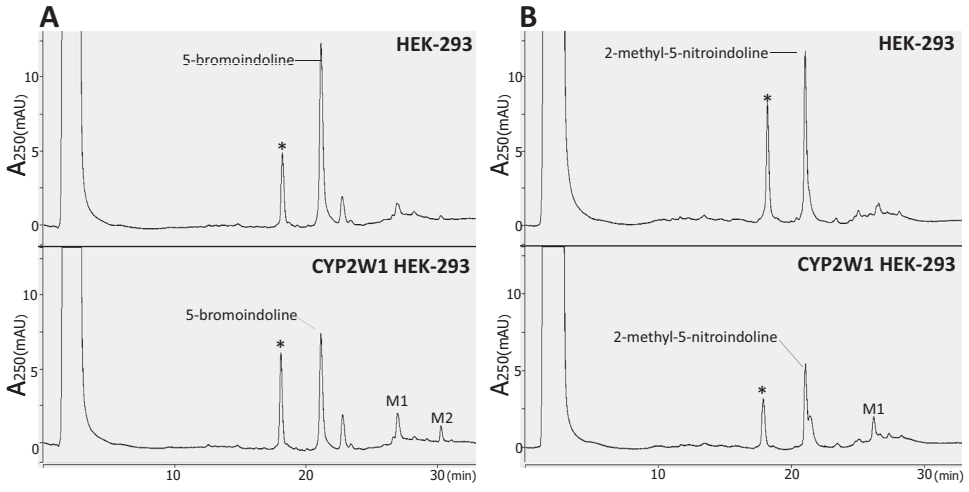


Fig. 6. Metabolic activity of CYP2W1. HPLC profiles of 5-bromoindoline (A) and 2-methyl-5-nitroindoline (B). Mock (top) and CYP2W1 HEK 293 (bottom) cells were incubated with 5-bromoindoline and 2-methyl-5-nitroindoline as described under *Materials and Methods.*. M1 (retention time, 26.9 min in A and 26.1 min in B), M2 (29.1 min), and M3 (30.1 min) are putative metabolites of corresponding parental indolines. HPLC analysis of the substrates alone produced chromatograms identical with the ones obtained upon the incubation of the substrates with the mock cells (results not shown). *, nonspecific peak from HEK 293 cells.

membrane-localized CYP2W1 of 8% identified in current study (Fig. 5) is the highest amount of microsomal P450 detected on the cell surface. This raises the possibility of using colorectal tumor-specific CYP2W1 for antibody-mediated cancer therapy, an interesting aspect for further drug development.

The extent of glycosylation of the CYP2W1 protein in transfected HEK 293 cells is 4% of the total cellular CYP2W1 (Fig. 5). In vivo (i.e., in colorectal tumors), it seems, based on the Western blot analysis, that the extent of glycosylation varies extensively between different tumors (Fig. 5) (Gomez et al., 2007). In some tumors, the entire amount of CYP2W1 is glycosylated, whereas in others, this fraction is much smaller, thus identifying two major phenotypes of CYP2W1 in the tumors. The question remains as to whether the glycosylated forms of CYP2W1 in the tumors are catalytically active.

The bacterially expressed CYP2W1, when reconstituted with NADPH-cytochrome P450 reductase, has been a very silent enzyme toward all of the tested substrates, including benzphetamine, 17 different fluorescent P450 substrates (BD Chemical, Greenwood Village, CO), pargyline, and aminopyrine (M. Tachibana and M. Ingelman-Sundberg, unpublished observations), whereas a low reduction capacity was found toward the anticancer prodrug 1,4-bis{[2-(dimethylamino-*N*-oxide)ethyl]amino}-5,8-dihydroxyanthracene-9,10-dione (A4QN) (Nishida et al., 2010). Wu et al. (2006) did not show true direct catalytic activity of CYP2W1. Yoshioka et al. (2006) demonstrated that CYP2W1 catalyzed conversion of indole, albeit with very high K_m and relatively low K_{cat}. Because of the inverted orientation of CYP2W1, we decided to test catalytic activity in intact cells under the assumption that a separate electron transfer system would provide an electron donor to CYP2W1. Indeed, the results using indolines and aflatoxin B1 as substrates indicate the presence of the functional electron transport chain that makes possible the substrate conversion on CYP2W1, although we cannot at the moment exclude also the contribution of a possible fraction of the enzyme that is oriented toward cytoplasm. Further experiments are necessary to identify the individual components of such chain.

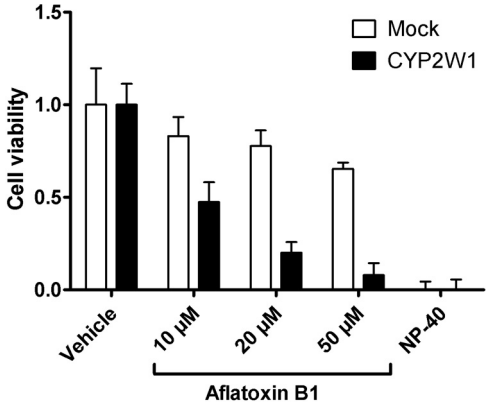


Fig. 7. CYP2W1-mediated cytotoxicity of aflatoxin B1. HEK 293 cells stably expressing wild-type CYP2W1 and corresponding mock-transfected cells were seeded on 48-well plates, and aflatoxin B1 was added when cells had reached 70 to 90% confluence. Cell viability was analyzed with EZ4U assay after 3 days of treatment. NP-40-treated cells were used as a negative control.

In a recent publication, Nishida et al. (2010) found that using recombinant P450 reductase and CYP2S1 and CYP2W1, both P450 enzymes were reduced by NADPH. This result is in contrast to the absence of functional interactions between P450 reductase and CYP2S1 described by Bui and Hankinson (2009). The basis for this discrepancy might be due to the fact that Bui and Hankinson, as well as ourselves, have used aerobic conditions, whereas Nishida et al. (2010) conducted experiments under strictly anaerobic conditions when studying the electron transfer to CYP2S1 and CYP2W1. Under such conditions, dioxygen reduction and the ubiquitous hydrogen peroxide production seen in such systems (Ingelman-Sundberg and Johansson, 1984) do not occur, forcing the reduction of P450 heme instead. It remains to be studied whether any P450 reductase-mediated reduction of CYP2S1 and CYP2W1 occurs under more aerobic conditions. Nishida et al. (2010) did also find that at low oxygen concentrations (<0.5%), CYP2W1 and CYP2S1 were able to reduce A4QN to the active anticancer metabolite AQ4 at efficiency higher than registered previously with CYP3A4, which would indicate a possible use of this reaction in the anaerobic tumors. However, they did not show any activity of CYP2W1 in conventional P450 catalysis, and yet it cannot be excluded that P450 reductase, under the anaerobic conditions used, serves as the terminal electron donor to A4QN, influenced by the presence of the P450 in question, although this might be less likely.

Colorectal cancer is the third most common form of cancer in the Western world with approximately one million new cases and a half million deaths annually (Parkin et al., 2005). Gene-directed enzyme prodrug therapy is an approach by which the anticancer prodrugs can be activated by specific enzymes and CYP2W1 is an interesting target in this respect for the treatment of colorectal cancer. Previous use of cytochromes P450 as drug targets include aromatase inhibitors (CYP19A1) in breast cancer and CYP1B1 in the treatment of several cancer forms (Bruno and Njar, 2007; Rodriguez-Antona et al., 2010). Thus, CYP1B1 has been evaluated as drug target for aryl oxime prodrugs in tumor xenografts, and a number of compounds, including isoflavonoids, stilbenes, pyrrolo-indole, and pyrrolo-quinoline derivatives have been patented and are under investigation as CYP1B1-targeted prodrugs (McFadyen and Murray, 2008). The other example of gene-directed enzyme prodrug therapy approach is the introduction of exogenous *CYP2B6* gene into the tumor cells followed by the treatment with phosphamides (Chen et al., 2005). However, using CYP1B1 and CYP2B6 as drug targets is problematic because of their expression in many tissues, which is prone toward unwanted toxic side effects. The use of CYP2W1 for the same purpose has an advantage of a specific enzyme expression in the tumor cells, thus avoiding the unnecessary toxicity in other organs.

In conclusion, the colorectal tumor-specific CYP2W1 with an inverse ER orientation can bioactivate chemicals to cytotoxic products, is glycosylated on Asn177, and, to a relatively large extent, is localized at the cell surface. These properties make the enzyme an interesting candidate for a drug target in anti-colon cancer therapy both using prodrugs bioactivated selectively by the enzyme and for immune-based therapy.

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