

# Proteasomal Degradation of Human CYP1B1: Effect of the Asn453Ser Polymorphism on the Post-Translational Regulation of CYP1B1 Expression

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## ABSTRACT

Allelic variations in CYP1B1 are reported to modulate the incidence of several types of cancer. To provide a mechanistic basis for this association, we investigated the impact of nonsilent allelic changes on the intracellular levels and post-translational regulation of CYP1B1 protein. When transiently expressed in COS-1 cells, either in the presence or absence of recombinant cytochrome P450 reductase, the cellular level of the CYP1B1.4 allelic variant (containing a Ser at the amino acid position 453; Ser453) was 2-fold lower compared with the other four allelic CYP1B1 proteins (containing Asn453), as analyzed by both immunoblotting and ethoxyresorufin O-deethylase activity. This difference was caused by post-translational regulation; as in the presence of cycloheximide, the rate of degradation of immunodetectable and enzymatically active CYP1B1.4 was distinctly faster than that of CYP1B1.1. Pulse-chase anal-

ysis revealed that the half-life of CYP1B1.4 was a mere 1.6 h compared with 4.8 h for CYP1B1.1. The presence of the proteasome inhibitor MG132 [*N*-benzoyloxycarbonyl (*Z*)-Leu-Leu-leucinal] increased the stability not only of immunodetectable CYP1B1, but also—unexpectedly given the size of the proteasome access channel—increased the stability of enzymatically active CYP1B1. The data presented herein also demonstrate that CYP1B1 is targeted for its polymorphism-dependent degradation by polyubiquitination but not phosphorylation. Our results importantly provide a mechanism to explain the recently reported lower incidence of endometrial cancer in individuals carrying the *CYP1B1*\*4 compared with the *CYP1B1*\*1 haplotype. In addition, the mechanistic paradigms revealed herein may explain the strong overexpression of CYP1B1 in tumors compared with nondiseased tissues.

Cytochromes P450 (P450s) catalyze the metabolism of a plethora of endogenous and exogenous compounds (Guengerich and Shimada, 1998; Waxman, 1999). Whereas most P450 isoforms are expressed mainly in the liver, the isoform CYP1B1 is found in extrahepatic tissues and is highly overexpressed in a variety of tumors compared with nondiseased tissue (Guengerich et al., 2003). CYP1B1 has been implicated in chemical carcinogenesis. For example, mice that were nulled for CYP1B1 displayed a strongly reduced incidence of lymphomas after treatment with 7,12-dimethylbenz[*a*]anthracene (Buters et al., 2003). More im-

portant for the role of CYP1B1 in the development of hormone-dependent cancers, such as endometrial cancer, could be its ability to preferentially catalyze the metabolism of 17 $\beta$ -estradiol to 4-hydroxyestradiol (Spink et al., 1997). Whereas 2-hydroxyestradiol (main 17 $\beta$ -estradiol derivative formed by CYP1A1 and CYP3A4) has little or no carcinogenic activity, 4-hydroxyestradiol has been shown to be a potent carcinogen in rodents (Zhu and Conney, 1998).

Epidemiological data and mechanistic studies suggest that polymorphisms in some cytochrome P450 isoforms contribute to interindividual differences in cancer susceptibility (Wormhoudt et al., 1999; Friedberg, 2001). It is interesting that rare allelic variants of CYP1B1 that are catalytically inactive have been shown to be associated with familial glaucoma (Stoilov et al., 1998). Seven common alleles of CYP1B1 (termed *CYP1B1*\*1 to *CYP1B1*\*7) that encode functional pro-

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**ABBREVIATIONS:** P450, cytochrome P450; OR, odds ratio; CI, confidence interval; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; CPR, cytochrome P450 oxidoreductase; MG132, *N*-benzoyloxycarbonyl (*Z*)-Leu-Leu-leucinal; PAGE, polyacrylamide gel electrophoresis; LDH, lactate dehydrogenase; 2-ME, 2-mercaptoethanol; EROD, ethoxyresorufin O-deethylase.

teins (termed CYP1B1.1 to CYP1B1.7) have been described. These variants show either single or multiple allelic changes in their primary structure at amino acid residues (designation of genotype in parentheses) 48 (m1), 119 (m2), 432 (m3), 443 (m5), and 453 (m4) (Aklillu et al., 2002; Rylander-Rudqvist et al., 2003). Epidemiological data on the impact of the allelic changes on cancer incidence have reported an association of the m3 genotype with the incidence of head and neck squamous cancer (Ko et al., 2001) and intestinal cancer (Fritsche et al., 1999). It is interesting that the etiology of these types of cancers probably involve chemical carcinogens, such as polycyclic aromatic hydrocarbons and heterocyclic aromatic amines that are metabolically activated by CYP1B1. Allelic changes in CYP1B1 have also been correlated to the incidence of hormone-dependent cancers (Friedberg, 2001), however with the biological relevance being more equivocal (Bailey et al., 1998; Watanabe et al., 2000; De Vivo et al., 2002; Tanaka et al., 2002; Rylander-Rudqvist et al., 2003; Sasaki et al., 2003). It is interesting that a very recent report noted that carriers of at least one CYP1B1 (Ser453) allele showed a significantly reduced incidence (OR = 0.62; 95% CI = 0.43–0.91) of endometrial cancer compared with homozygous carriers of the genotype encoding Asn at this position (McGrath et al., 2004).

The significance of the epidemiological data on the association between CYP1B1 polymorphisms and cancer incidence would clearly benefit from mechanistic studies that demonstrate the functional impact of these allelic changes on cellular CYP1B1 enzyme activity in cells. This parameter, like any other enzyme activity, is determined by the protein expression level of the P450 as well as its specific catalytic properties. The catalytic properties of the CYP1B1 variants have been studied by several groups, including ours (Shimada et al., 1999; Hanna et al., 2000; Li et al., 2000), and some evidence of an effect of allelic changes on the CYP1B1-mediated activation of carcinogens has been found. However, it should be noted that the differences in some studies were relatively modest, and there is no agreement on whether allelic changes, either alone or in combination, can actually modulate the catalytic activity of CYP1B1.

Given that in addition to the catalytic properties of CYP1B1 its cellular levels are also important in modulating the metabolism of endogenous and exogenous substrates, we set out to elucidate the post-translational mechanisms that govern CYP1B1 expression levels. Furthermore, we investigated whether the expression level and degradation of CYP1B1 is modulated by allelic changes.

## Materials and Methods

### Cloning of CYP1B1 Alleles into Mammalian Expression Vector.

The bona fide coding region of the CYP1B1 cDNA was isolated by reverse transcription-PCR and inserted into the holding vector pCW as described previously (Li et al., 2000). The cDNA insert was found by sequencing to encode the CYP1B1\*3 allele (GenBank accession number U03688). The vector was subsequently linearized with NdeI and blunt-ended. The linearized DNA was digested with XbaI to release the CYP1B1 cDNA containing a blunt 5'-end and an XbaI-compatible 3'-end. This fragment was inserted into the pDHFR mammalian expression vector (Ding et al., 2001), which had been digested with EcoRV/XbaI. The resulting vector, which contains the CYP1B1\*3 cDNA under the control of the cytomegalovirus promoter,

was used to generate the expression vectors containing the other CYP1B1 alleles.

Vectors with the CYP1B1\*1 and CYP1B1\*4 inserts were isolated by employing an exchange of 3'-restriction fragments between the CYP1B1\*3 template in the pDHFR vector and the cDNAs encoding CYP1B1\*1 and CYP1B1\*4, which had been previously modified at their 5'-end for bacterial expression (Li et al., 2000). By this strategy, the modified 5'-end was replaced with the native 5'-end of CYP1B1. A cDNA fragment encoding the N terminus of CYP1B1\*6 was isolated by PCR by employing templates that encoded this allele but which had been previously modified for bacterial expression at their 5'-end. PCR was performed to remove this modification. The 5'-primer (forward primer, 5'TGGAATTCGAGATTATGGGCAC-CAGCCTCAGCCCGAACGACCCTTGCCGCTAAACCC-3') was chosen to replace the modified 5'-end with a sequence encoding the native N terminus of CYP1B1 and generate an EcoRI site (underlined), whereas the reverse primer (5'-TTGCGGTTGAGCTGCTC-GAATTCGCGGAAA-3') encompassed a unique EcoRI (underlined) in the CYP1B1 cDNA. The PCR fragment was cloned into the pCR-blunt vector (Invitrogen, Paisley, UK) according to the manufacturer's instructions, subsequently released by EcoRI digestion, and inserted into the EcoRI-digested mammalian expression vector pDHFR containing the CYP1B1\*3 or the CYP1B1\*1 templates, thereby obtaining the templates encoding unmodified CYP1B1\*6 and CYP1B1\*2, respectively.

### Expression of CYP1B1 Alleles in Mammalian COS-1 Cells.

COS-1 cells were maintained at 37°C/5% CO<sub>2</sub> in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 units/ml penicillin, 50 µg/ml streptomycin (all medium components obtained from Invitrogen). This medium is termed general growth medium.

For the transient expression of CYP1B1, COS-1 cells were seeded at a density of  $1.5 \times 10^5$  cells/well in six-well multiwell plates. Twenty-four hours later, they were cotransfected with 0.8 µg of the different CYP1B1 alleles in the vector pDHFR and, to assess the transfection efficiency, 0.2 µg of the pSV-β-galactosidase vector (Promega, Southampton, UK) by using LipofectAMINE reagent (Invitrogen) according to the manufacturer's instructions. In some experiments (as indicated in the figure legends), cells were cotransfected with 0.2 µg of β-galactosidase, 0.4 µg of CYP1B1, and 0.4 µg of cytochrome P450 oxidoreductase (CPR) encoding vectors.

After transfection (24 h), the medium was replaced with general growth medium (see above) and incubated at 37°C/5% CO<sub>2</sub> for a further 24 h. The cells were subsequently analyzed by immunoblotting and enzymatic measurements as indicated below.

**7-Ethoxyresorufin O-Deethylation Assay.** After transfection (48 h), cells were incubated with a 400 nM solution of 7-ethoxyresorufin (Sigma-Aldrich, Poole, Dorset, UK) in phosphate-buffered saline at 37°C/5% CO<sub>2</sub>. After various time points, duplicate aliquots of supernatant were transferred into a 96-well plate for the fluorometric measurement of resorufin production (excitation = 530 nm; emission = 584 nm). The amount of product formed was calculated by comparison with a standard of resorufin (Li et al., 2000).

**Determination of Protein Stability Employing Cycloheximide.** After transfection (48 h), cells were exposed to 10 µg/ml cycloheximide (Sigma-Aldrich) in growth medium, both in the presence or absence of 20 µM of the proteasome inhibitor MG132 (Sigma-Aldrich). Cells were then incubated at 37°C/5% CO<sub>2</sub> for different time points and subsequently harvested for immunoblotting and determination of the transfection efficiency.

**Quantification of Protein Expression.** After incubation with ethoxyresorufin (for enzymatic analysis), the cells were scraped off into lysis buffer supplemented with protease inhibitors (lysis reagent as supplied for the β-Gal Reporter Gene Assay chemiluminescent kit; Roche Molecular Biochemicals, Mannheim, Germany). Cell debris was removed by centrifugation at 10,000 rpm for 10 min at 4°C. Protein concentration of supernatants was determined using a Bio-Rad protein assay reagent (Bio-Rad, Hemel Hempstead, Hertford-

shire, UK). Equal amounts of protein were separated by SDS-PAGE in 10% acrylamide gels (Laemmli, 1970) and then transferred to a nitrocellulose membrane and probed with rabbit anti-human CYP1B1 (BD, Gentest, Woburn, MA) and rabbit anti-human lactate dehydrogenase (LDH; Prof. J. D. Hayes, University of Dundee) as a loading control. In some experiments (as indicated in the figure legends), a mouse monoclonal anti-human CYP1B1 [generous gift by G. Murray, University of Aberdeen, Aberdeen, UK (McFadyen et al., 1999)] was used. After washing, the blot was incubated with protein G peroxidase (Sigma-Aldrich) followed by detection employing an enhanced chemiluminescence kit (Amersham Biosciences UK, Little Chalfont, Buckinghamshire, UK). Signals were scanned using the UMAX Astra 5400 scanner (UMAX Systems GmbH, Willich, Germany) and quantified densitometrically using Molecular Analyst software (Bio-Rad). Signals were normalized to the transfection efficiency, which was determined by employing the  $\beta$ -Gal Reporter Gene Assay chemiluminescent kit (Roche Molecular Biochemicals) according to the manufacturer's instructions.

**Analysis of Protein Stability by  $^{35}\text{S}$  Pulse-Chase.**  $^{35}\text{S}$  pulse-chase was performed as described previously with minor modifications (McLaughlin et al., 1999). In brief, COS-1 cells were transfected in duplicate with the various alleles and vector controls as indicated in the figure legends. After transfection (48 h), the cells were washed and preincubated for 1 h with warm methionine-, cysteine-, and glutamine-deficient DMEM (Sigma-Aldrich), to which 4 mM glutamine, 5% dialyzed FBS, 50 units/ml penicillin, and 50  $\mu\text{g}/\text{ml}$  streptomycin (serum and antibiotics obtained from Invitrogen) had been added back. Cells were subsequently incubated for 3 h with the same medium containing 100  $\mu\text{Ci}/\text{ml}$  of a mixture of [ $^{35}\text{S}$ ]methionine and [ $^{35}\text{S}$ ]cysteine (14.3 mCi/ml Pro-mix; Amersham Biosciences UK). After this incubation, cells were chased for different time points in general growth medium and lysed with phosphate-buffered saline containing 1% Triton X-100, 1 mM EDTA, 1  $\mu\text{g}/\text{ml}$  aprotinin, 1  $\mu\text{g}/\text{ml}$  leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Lysates were stored at  $-70^\circ\text{C}$ . Immunoprecipitation was performed from an equal volume of each lysate as described previously (McLaughlin et al., 1999). The immunoprecipitates were analyzed by SDS-PAGE, and the signals were visualized and quantitated using the Molecular Imager FX Pro Plus MultiImager System with the Quantity One software (Bio-Rad).

**Cellular Ubiquitination Assay.** The *in vivo* ubiquitination assay was carried out as described previously (McMahon et al., 2003) with minor modifications. COS-1 cells were cotransfected in triplicate with the mammalian expression vector containing either the *CYP1B1\*1* or *CYP1B1\*4* cDNA along with a pHisUb plasmid (provided by Prof. J. D. Hayes) that expresses a cDNA-encoding hexahistidine-tagged octameric ubiquitin precursor protein from a cytomegalovirus promoter. As controls, cells were also cotransfected with either pDHFR empty vector and pHisUb or pDHFR empty vector together with pDHFR carrying the *CYP1B1\*1* cDNA. After transfection (48 h), cells were treated with 20  $\mu\text{M}$  MG132 for 3 h before the monolayers were washed with prewarmed phosphate-buffered saline and scraped off by the addition of 200  $\mu\text{l}/\text{well}$  of buffer A (6 M guanidine, 10 mM Tris, and 0.1 M phosphate buffer, pH 8) supplemented with 5 mM imidazole. Lysates from triplicate transfections were pooled and sonicated to reduce viscosity. Fifty microliters of nickel affinity gel (Sigma-Aldrich) was added to each pooled lysate, and the mixtures were rotated overnight at  $4^\circ\text{C}$ . Thereafter, the beads were washed sequentially with buffer A supplemented with 10 mM 2-mercaptoethanol (2-ME); buffer B (8 M urea, 10 mM Tris, and 0.1 M phosphate buffer, pH 8) supplemented with 10 mM 2-ME; buffer C (8 M urea, 10 mM Tris, and 0.1 M phosphate buffer, pH 6.5) supplemented with 10 mM 2-ME and 0.2% (v/v) Triton X-100; and, finally, buffer C supplemented with 10 mM 2-ME and 0.1% (v/v) Triton X-100. Proteins subsequently purified by the  $\text{Ni}^{2+}$  resin were analyzed by immunoblotting employing an anti-CYP1B1 monoclonal primary antibody that had been previously preabsorbed with rabbit serum.

**Cellular Phosphorylation Assay.**  $^{32}\text{P}$  cell labeling was performed as described previously (Meek and Milne, 2000) with minor modifications. In brief, COS-1 cells expressing either *CYP1B1\*1* or *CYP1B1\*4* were incubated for 3 h with phosphate-free DMEM (Sigma-Aldrich) supplemented with 5% dialyzed FBS, 50 units/ml penicillin, and 50  $\mu\text{g}/\text{ml}$  streptomycin (antibiotics obtained from Invitrogen) containing 1.5 mCi/ml [ $^{32}\text{P}$ ]orthophosphate (MP Biomedicals, Basingstoke, UK) in the presence or absence of 20  $\mu\text{M}$  MG132 and 1  $\mu\text{M}$  okadaic acid (the latter was added 30 min before the end of incubation; both products obtained from Sigma-Aldrich). Cells were subsequently washed with Tris-buffered saline (25 mM Tris, pH 8.0, 140 mM NaCl, and 3 mM KCl) and lysed with Nonidet P-40 lysis buffer (10 mM sodium phosphate, pH 7.0, 0.15 M NaCl, 1% Igepal CA-630, 2 mM EDTA, 50 mM NaF, 1 mM benzamide, 1  $\mu\text{g}/\text{ml}$  aprotinin, 1  $\mu\text{g}/\text{ml}$  leupeptin, and 1 mM phenylmethylsulfonyl fluoride). Immunoprecipitation was performed from two equal volumes of each lysate as described previously (McLaughlin et al., 1999), using either rabbit anti-human CYP1B1 (BD Gentest) or mouse anti-human p53 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The immunoprecipitates were analyzed by SDS-PAGE, and the signals were visualized and quantitated using filmless autoradiographic analysis (Bio-Rad). After the detection of signals, the polyacrylamide gel used for the electroporation of the anti-CYP1B1 immunoprecipitates was rehydrated following a procedure modified from what has been reported elsewhere (Goncalves and Rodnight, 1993). Proteins from the rehydrated gel were then transferred to a nitrocellulose membrane and probed with monoclonal mouse anti-human CYP1B1 (McFadyen et al., 1999), which had been preincubated with rabbit serum.

**Statistical Analysis.** Statistics were based on the nonparametric, two-tailed, unpaired *t* test with 95% confidence intervals, using the GraphPad Prism software version 3 (GraphPad Software Inc., San Diego, CA).

## Results

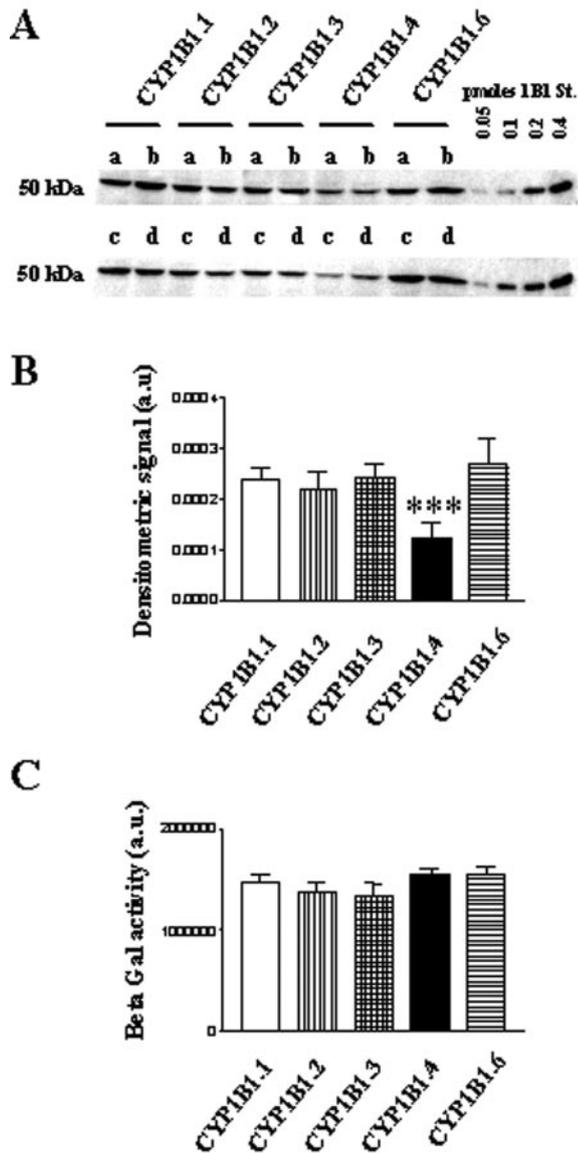
**Influence of Allelic Changes on CYP1B1 Expression Levels.** The following CYP1B1 alleles were isolated [amino acid residues encoded by the alleles at positions 48 (m1), 119 (m2), 432 (m3), and 453 (m4) indicated in parentheses]: *CYP1B1\*1* (RALN), *CYP1B1\*2* (GSLN), *CYP1B1\*3* (RAVN), *CYP1B1\*4* (RALS), and *CYP1B1\*6* (GSVN). The various CYP1B1 alleles were transiently expressed in COS-1 cells and analyzed 48 h after transfection for the expression of CYP1B1 by immunoblotting (Fig. 1). Nontransfected cells did not show any signal in the immunoblot (data not shown; Fig. 3). A distinct signal was obtained with the anti-CYP1B1 polyclonal antibody in cells that had been transfected with the various CYP1B1 alleles. Visual inspection of the blots revealed that the expression level of CYP1B1.4 (RALS) was lower than that of the other variants. This was confirmed by densitometric analysis of the blots (Fig. 1B). The expression level of CYP1B1.4 normalized to the transfection efficiency was significantly ( $p < 0.001$ ) reduced by a factor of two compared with the expression of CYP1B1.1 (RALN). Levels of the other allelic variants tested were not significantly different from that of *CYP1B1\*1*. This result was reproduced in a second experiment (data not shown) in which we had also analyzed the expression of an additional variant (RAVS). Similar to CYP1B1.4, the level of this variant, which has been found only recently in the human population (Rylander-Rudqvist et al., 2003) but is still without allele nomenclature, was reduced compared with the other alleles.

The reduced expression of CYP1B1.4 was also reflected by the ethoxyresorufin *O*-deethylase activity, which was lower

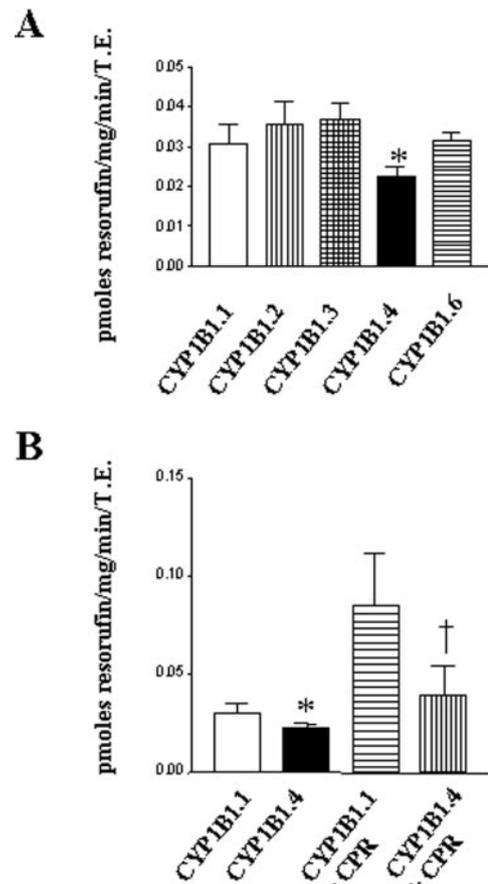
in intact cells expressing this allele compared with cells transfected with the other alleles (Fig. 2A). Cells that had been transfected with the empty expression plasmid did not display any detectable enzyme activity (data not shown). We also investigated the effects of different levels of NADPH CPR on the 7-ethoxyresorufin *O*-deethylation (EROD) activity of CYP1B1.1 and CYP1B1.4 (Fig. 2B). Upon coexpression of CPR, a 2- to 3-fold increased ethoxyresorufin *O*-deethylase activity compared with cells that had only been transfected

with either CYP1B1 allele was noted. As seen already in the absence of recombinant CPR, the activity of cells expressing the CYP1B1.4 protein together with CPR was significantly reduced by a factor of 2-fold ( $p < 0.01$ ) compared with cells containing the CYP1B1.1 protein.

**Influence of Allelic Changes on CYP1B1 Stability.** To investigate whether the reduced expression level of CYP1B1.4 compared with that of the other variants was caused by post-translational mechanisms, cells that heterologously expressed either CYP1B1.1, CYP1B1.3, or CYP1B1.4 were exposed to cycloheximide for various time points. The cellular protein was subsequently analyzed by immunoblotting that employed polyclonal anti-CYP1B1 antibodies (Fig. 3). Cells that were transfected with the empty expression plasmid did not yield any signal (lane U in Fig. 3A). By comparison, with a standard of recombinant CYP1B1, the



**Fig. 1.** Influence of allelic changes on CYP1B1 protein expression. A, COS-1 cells cotransfected as described under *Materials and Methods* in quadruplicate (a, b, c, and d) with expression vectors carrying the cDNAs of the various CYP1B1 variants as indicated (\*1, \*2, \*3, etc.) and a vector encoding  $\beta$ -galactosidase. This activity was employed to determine the transfection efficiency. Cells were lysed 48 h after transfection, and lysates (25  $\mu$ g/lane) were subjected to immunoblot analysis employing a polyclonal anti-CYP1B1 primary antibody. B, blot displayed in A analyzed densitometrically. Densitometric signals are expressed as arbitrary units normalized to the transfection efficiency and as means  $\pm$  S.D. ( $n = 4$ ). \*\*\*, value ( $p < 0.001$ ) significantly different from the value obtained for cells transfected with the CYP1B1\*1 cDNA. C, transfection efficiency values for each allele measured as reported under *Materials and Methods* and expressed as arbitrary units of  $\beta$ -galactosidase activity per milligram of protein (means  $\pm$  S.D.,  $n = 4$ ).



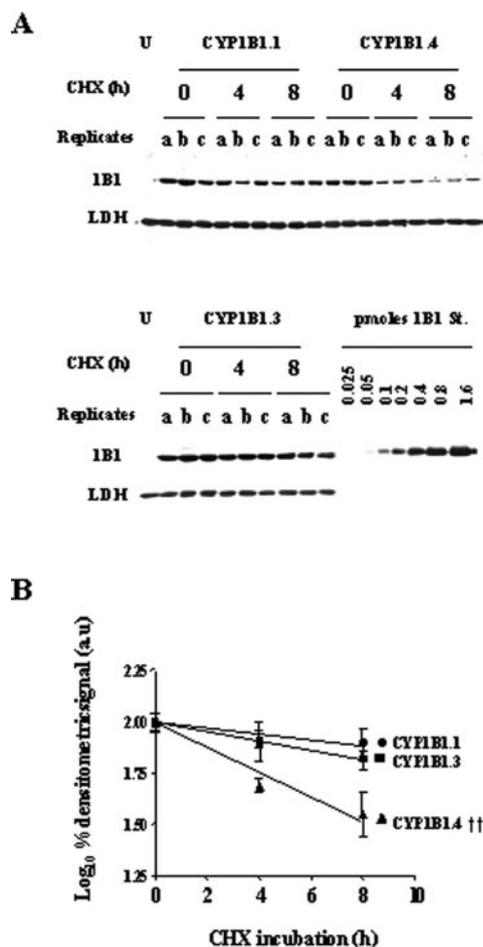
**Fig. 2.** Effect of CYP1B1 and CPR expression on CYP1B1 catalytic activity. Cells that had been analyzed by immunoblotting as described in Fig. 1 were also analyzed for EROD activity. This activity was detected in cultured cells 48 h post-transfection as described under *Materials and Methods*. The EROD activity is expressed as picomoles of resorufin per milligram of protein per minute and normalized to the total  $\beta$ -galactosidase activity (transfection efficiency, TE), with the latter activity and protein concentration having been determined in lysates. Values are means  $\pm$  S.D. ( $n = 4$ ). \*, value ( $p < 0.05$ ) significantly different from the value obtained for CYP1B1\*1. B, cells cotransfected in quadruplicate with vectors encoding CYP1B1\*1 or CYP1B1\*4 together with vectors encoding P450 reductase and  $\beta$ -galactosidase. EROD activity of the cells was determined and is reported as in A. For comparison, the EROD activity of cells in the absence of CPR (see A) is displayed again for the two allelic variants. Values are means  $\pm$  S.D. ( $n = 4$ ). \*, value ( $p < 0.05$ ) significantly different from the value obtained for CYP1B1\*1 in the absence of CPR. †, value ( $p < 0.05$ ) significantly different from the value obtained for CYP1B1\*1 in the presence of CPR.

expression level of CYP1B1.3 in COS-1 cells was estimated to be 17 pmol of P450/mg of total cellular protein (Fig. 3, last blot from top). The level of CYP1B1 decreased upon exposure to cycloheximide. Whereas the velocity of degradation was similar for CYP1B1.1 ( $t_{1/2} = 17$  h) and CYP1B1.3 ( $t_{1/2} = 14$  h), the degradation of CYP1B1.4 ( $t_{1/2} = 5$  h) was significantly more pronounced compared with CYP1B1.1 ( $p \leq 0.01$ ).

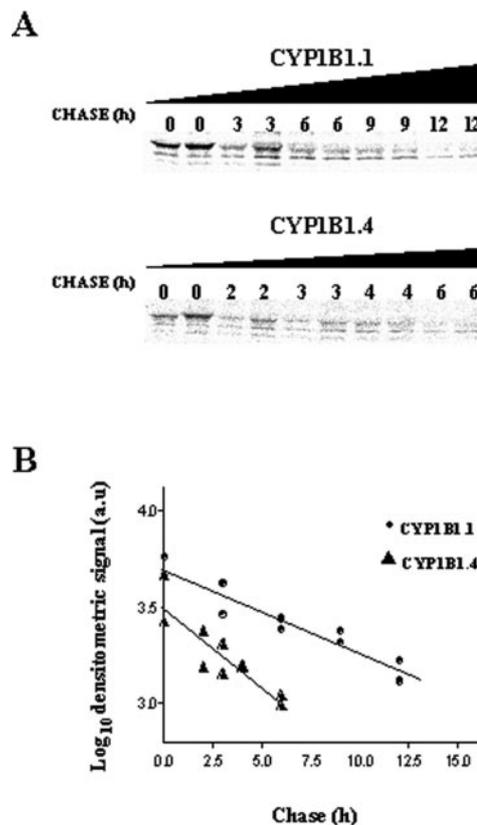
Based on the experiment employing cycloheximide for the analysis of CYP1B1 degradation, we decided to verify differences in the half-lives of CYP1B1.1 and CYP1B1.4 by  $^{35}\text{S}$  pulse-chase analysis (Fig. 4). The duration of the chase period was estimated according to the results displayed in Fig. 3 and chosen to be up to 12 h for CYP1B1.1 and only up to 6 h for CYP1B1.4. In the absence of any chase, the SDS-PAGE

fluorography of the immunoprecipitates formed by the CYP1B1 antibody revealed one major band and two minor bands with a faster mobility than CYP1B1 (Fig. 4A, 0 time points). The intensity of the major band decreased during the chase. Radiometric quantitation of this signal for CYP1B1.1 and CYP1B1.4 (Fig. 4B) clearly demonstrated that the latter allelic protein was degraded at a faster rate ( $t_{1/2} = 1.6$  h) compared with the former allelic variant ( $t_{1/2} = 4.8$  h). It is interesting that these values were distinctly shorter than those determined in the presence of cycloheximide, so clearly suggesting the involvement of short-lived proteases, such as the proteasome, in the degradation of CYP1B1 (see *Discussion*).

**Effects of Allelic Changes on Post-Translational Mechanism that Govern CYP1B1 Levels.** To investigate the involvement of the proteasome pathway in the proteolytic degradation of CYP1B1, COS-1 cells transiently expressing this P450 were exposed to cycloheximide either in the presence or absence of the well characterized proteasome inhibitor MG132 (Fig. 5). As previously found in the presence of cycloheximide (Fig. 3), the rate of degradation of CYP1B1.4



**Fig. 3.** Influence of allelic changes on CYP1B1 stability. A, COS-1 cells cotransfected in triplicate (a, b, and c) with vectors encoding the CYP1B1 alleles as indicated (\*1, \*3, and \*4) and  $\beta$ -galactosidase (transfection control). Cells were exposed to cycloheximide (CHX) 48 h later as reported under *Materials and Methods* for various time points, as indicated. Lysates (35  $\mu\text{g}/\text{lane}$ ) were electrophoresed and probed with anti-CYP1B1 and anti-LDH (loading control). To test for the linearity of the signal, different amounts of recombinant CYP1B1, expressed in *Escherichia coli*, were also analyzed (1B1 St). Lane U, untransfected cells. B, densitometric values determined from each blot as described under *Materials and Methods* and normalized to the  $\beta$ -galactosidase activity (transfection efficiency, TE). For each variant, the mean of the signals obtained at time 0 was set at 100%. Values are expressed as the  $\log_{10}$  percentage of the time 0 value of each variant. The error bars indicate the  $\pm$  S.D. ( $n = 3$ ). The symbols for the various polymorphic forms are displayed on the right of B. ††, slope calculated for CYP1B1\*4 compared with slope calculated for CYP1B1\*1 significantly different ( $p < 0.01$ ), as calculated by the method given under *Materials and Methods*.



**Fig. 4.** Pulse-chase analysis of CYP1B1.1 and CYP1B1.4 stability. After transfection (48 h), COS-1 cells expressing either CYP1B1\*1 or CYP1B1\*4 were grown for 3 h in medium containing [ $^{35}\text{S}$ ]methionine and [ $^{35}\text{S}$ ]cysteine as described under *Materials and Methods*. One set of cells was subsequently harvested and kept as the time 0 point. The other sets of cells were chased in standard growth medium for the indicated time points. Two sets of cells were analyzed per time point per CYP1B1 variant. Cells were lysed and incubated with anti-CYP1B1 antibody. Immunocomplexes were isolated using protein A Sepharose and separated by SDS-PAGE. Visualization (A) and quantitation (B) of signals of the top band were performed using the Molecular Imager FX Pro Plus MultiImager System with the Quantity One software. The values on the ordinate in the graph (B) are expressed as the  $\log_{10}$  arbitrary unit (au) of the radiometric signal. Note that only the top protein band had a molecular weight that was consistent with CYP1B1.

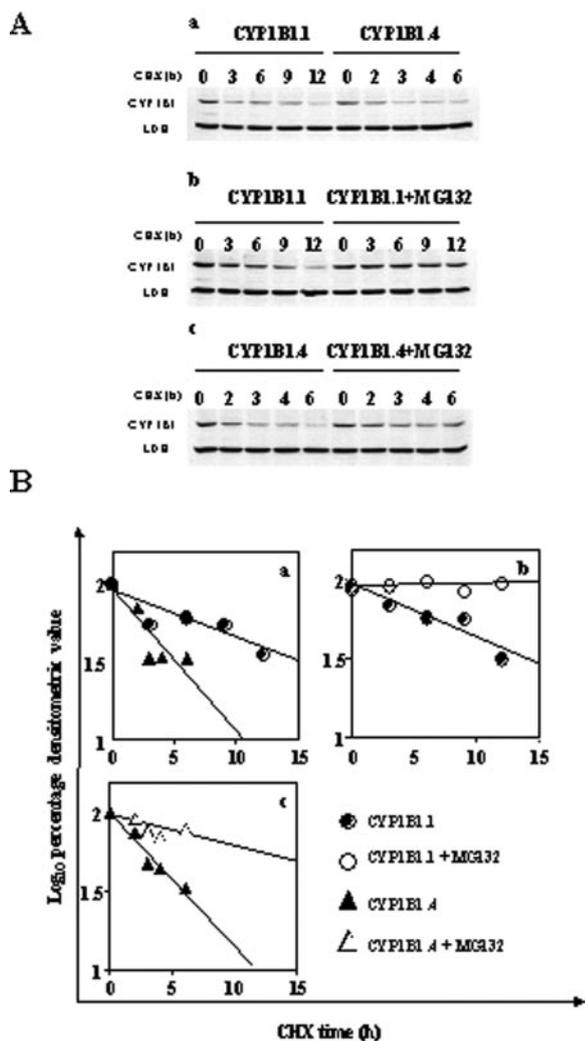
was faster than that of CYP1B1.1, as detected by immunoblotting. The presence of MG132 profoundly inhibited the degradation of CYP1B1.1, whereas the degradation of CYP1B1.4 was inhibited to a lesser degree. This suggests that CYP1B1.1 is degraded entirely via proteasomal pathways, whereas additional proteases may be involved in the degradation of CYP1B1.4.

We were surprised to find that the proteasome is not only involved in the degradation of immunodetectable CYP1B1 (Fig. 5), as had already been shown for some other P450 isoforms, but also in the proteolysis of enzymatically active P450. This is indicated by the data presented in Table 1. As already shown previously (Fig. 1B), the cellular ethoxyresorufin *O*-deethylase activity of CYP1B1.4 was lower than that of CYP1B1.1. It is noteworthy that treatment with cycloheximide reduced the level of catalytically active

CYP1B1.4 by about 60%; however, in the presence of both cycloheximide and MG132, this reduction was only 30%. A similar but less pronounced effect was seen for CYP1B1.1. The results presented in Table 1 also demonstrate that differences seen in the level of the two CYP1B1 variants were not caused by pretranslational regulation (e.g., different mRNA stabilities).

Next we investigated the mechanisms by which CYP1B1 is tagged for degradation by the proteasome. Because a neural network (NetPhos Server; <http://www.cbs.dtu.dk/services/NetPhos/>) trained to recognize potential protein phosphorylation sites had predicted a change in the CYP1B1 phosphorylation pattern upon the allelic change from Asn453 to Ser453 (with the latter residue only being found in CYP1B1.4), we investigated the phosphorylation status of CYP1B1.1 (RALN) and CYP1B1.4 (RALS). In this experiment (Fig. 6), cells that transiently expressed CYP1B1 were incubated with [<sup>32</sup>P]orthophosphate in the presence or absence of both the phosphatase inhibitor okadaic acid and MG132. In this experiment, the CYP1B1 antibody failed to precipitate any <sup>32</sup>P-labeled protein from the various lysates (Fig. 6A). In contrast, the tumor-suppressor protein p-53, which was chosen as a suitable control because it is phosphorylated by a plethora of kinases, yielded a single strong signal of the correct size (Fig. 6B). The addition of MG132 and okadaic acid to the culture medium to prevent degradation or dephosphorylation, respectively, failed to reveal any phosphorylated protein in the precipitates obtained with the CYP1B1 antibody. To verify that the immunoprecipitation of CYP1B1 and its transient expression had been carried out successfully, the Western blot of the immunoprecipitates was incubated with a monoclonal antibody (raised in mouse) and developed by employing a secondary antibody that was specific for mouse and which would not recognize the rabbit antibodies in the immunoprecipitates. As expected, the monoclonal anti-CYP1B1 antibody detected a signal in cells transfected with the CYP1B1 expression plasmids but not in mock-transfected cells. Again, the presence of MG132 increased the level of immunodetectable CYP1B1 (Fig. 6C), with a stronger effect for CYP1B1.1 compared with CYP1B1.4. Thus, it can be concluded that the immunoprecipitated CYP1B1 as visualized in Fig. 6C was not phosphorylated, because no corresponding signal was obtained in the autoradiography displayed in Fig. 6A.

To investigate whether the proteasome-mediated degradation of CYP1B1 was initiated by ubiquitination, the P450 was transiently expressed together with a recombinant His-tagged ubiquitin. Proteins modified by the tagged ubiquitin were subsequently precipitated from cell lysates with Ni<sup>2+</sup>-Sepharose and analyzed by immunoblotting employing an anti-CYP1B1 antibody. No signal was obtained in the analysis of cell lysates isolated from cells that had been transfected with the plasmid encoding the His-ubiquitin together with the empty expression plasmid pDHFR or cotransfected with the latter plasmid plus the vector carrying the CYP1B1\*1 cDNA (Fig. 7). However, analysis of cells that had been cotransfected with the plasmid encoding His-ubiquitin together with either CYP1B1.1 or CYP1B1.4 yielded a typical ubiquitination stepladder. Equal patterns of ubiquitination were detected for CYP1B1 of either form.



**Fig. 5.** Role of the proteasome for CYP1B1 stability. After transfection (48 h), COS-1 cells expressing either *CYP1B1\*1* or *CYP1B1\*4* were exposed for different time points to cycloheximide in the presence or absence of the proteasome inhibitor MG132. Cell lysates were prepared at the indicated time points, and  $\beta$ -galactosidase activity (as a measure of transfection efficiency) was determined as reported under *Materials and Methods*. A, lysates (50  $\mu$ g/lane) electrophoresed and probed with anti-CYP1B1 and anti-LDH as loading control. B, densitometric values detected from each blot as described under *Materials and Methods*. Values were normalized to the  $\beta$ -galactosidase activity and are expressed as  $\log_{10}$  percentage of the values obtained at time 0 normalized to the transfection efficiency.

## Discussion

To improve the mechanistic basis for explaining the association between polymorphisms in CYP1B1 and the incidence of several cancers (Fritsche et al., 1999; Ko et al., 2001; Rylander-Rudqvist et al., 2003; Sasaki et al., 2003), we investigated the impact of allelic changes in this P450 isoform with respect to its protein expression levels and the post-translational mechanisms that are involved in their regulation. Several groups have clearly demonstrated that different P450 isoforms are degraded either via the lysosomal or proteasome pathway often dependent on the presence of substrate (Correia, 2003). However, even though allelic changes in some P450 isoforms have been shown to alter expression levels, relatively little is known about the proteolytic degradation pathways that determine these differences.

To assess the impact of CYP1B1 allelic variations on the expression level of five common CYP1B1 polymorphic variants, we have employed transient expression in mammalian COS-1 cells, which have been extensively employed for the characterization of P450s (Hanioka et al., 2003; Jinno et al., 2003; Li et al., 2003; Szczesna-Skorupa et al., 2003), including CYP1A1 (Meyer et al., 2002), CYP1A2 (Black et al., 1997), and CYP1B1 itself (McLellan et al., 2000). The data represented in Fig. 1 clearly indicated that the level of immunologically detectable CYP1B1.4 was by a factor of two lower than the level of the other allelic proteins. However, it could be argued that this analysis not only detected functional holoenzyme, but also apoenzyme and misfolded CYP1B1. The level of the latter two states can be artificially high upon heterologous expression of enzymes, thus increasing the likelihood of misfolded, rapidly degradable protein (Wickner et al., 1999). It was therefore important to determine the consequences of allelic variations for the level of functional CYP1B1 enzyme, i.e., correctly folded protein capable of coupling with CPR into the endoplasmic reticulum. Data displayed in Fig. 2A revealed that the Asn453Ser exchange in CYP1B1 decreased the cellular ethoxyresorufin *O*-deethylase activity to a similar extent as the decline seen by immunoblotting. In the presence of recombinant CPR, the difference between the cellular enzyme activities of both variants became even more pronounced (Fig. 2B). Based on these results and according to previous investigations reporting highly similar catalytic properties (i.e.,  $K_m$  and  $V_{max}$ ) for CYP1B1.1 and CYP1B1.4 toward the dealkylation of ethoxyresorufin, it can be concluded that the thermal stability and/or rate of proteolytic degradation differs between CYP1B1.1 and CYP1B1.4 holoenzymes. It may be that in

different tissues, differences in the expression level of both variants are distinct from the value reported herein, because the level of proteins is determined by the rate of biosynthesis versus the rate of degradation or thermal denaturation, which may be tissue- and cell type-specific. In this respect, it should be noted that the CYP1B1 content in transiently transfected COS-1 cells was approximately 17 pmol/mg of total cellular protein (see *Results*), which is 1000-fold higher than the level of this P450 in mammary adenocarcinoma as estimated from the estradiol 4-hydroxylase activity found in these tissues (Liehr and Ricci, 1996) and the known turnover number of CYP1B1 for this reaction (Li et al., 2000). Thus, it cannot be excluded that the rate of degradation of CYP1B1 found in COS-1 cells is different from that in tumors. However, differences in the rate of degradation of CYP1B1.1 and CYP1B1.4 *in vitro* will probably also be seen *in vivo*.

Next we investigated the mechanisms involved in the post-translational regulation of CYP1B1. Cycloheximide had been previously used to determine the rate of degradation of murine CYP1B1 (Savas and Jefcoate, 1994) and human CYP1B1.1 and CYP1B1.2; this rate is comparable for both variants when expressed in COS-1 cells (McLellan et al., 2000). In our experiments, comparable degradation rates were also found for CYP1B1.1 and CYP1B1.3. However, as demonstrated by the experiments displayed in Figs. 3 and 4, the half-life of CYP1B1.4 was distinctly shorter than that of the other two variants. It is notable that differences were seen between half-lives determined by exposure to cycloheximide and pulse-chase analysis. For the former approach, the half-lives of CYP1B1.1 and CYP1B1.4 were 17 and 5 h, respectively, whereas in the latter analysis, these values turned out to be 4.8 and 1.6 h, respectively. Differences in the half-lives obtained by both methods are probably caused by cycloheximide inhibiting the biosynthesis of short-lived proteases involved in the degradation of CYP1B1. It is interesting that this "cycloheximide-sensitive" protein degradation has been described by others as a hallmark of nonlysosomal degradation pathways (Eliasson et al., 1992). The data presented in Fig. 5 clearly demonstrate that CYP1B1, similar to CYP3A4 or CYP2E1 (Korsmeyer et al., 1999) but unlike CYP1A1 (Roberts, 1997), is degraded via the proteasomal pathway, because the presence of the well established proteasome inhibitor MG132 considerably decreased the degradation of immunodetectable CYP1B1.1 and CYP1B1.4. Data presented in Table 1 also indicate that the proteasome not only degrades immunodetectable CYP1B1 apoprotein, but also the CYP1B1 holoenzyme, because MG132 rescued the

TABLE 1

Effect of cycloheximide and MG132 on CYP1B1-mediated EROD activity

COS-1 cells were cotransfected in triplicate with vectors encoding *CYP1B1*\*1 or *CYP1B1*\*4, plus CPR, plus  $\beta$ -galactosidase. After transfection (48 h), cells were treated for 5 h with dimethyl sulfoxide (control), cycloheximide, or cycloheximide plus MG132. EROD activity/mg of protein was determined and normalized to  $\beta$ -galactosidase activity. Activity is expressed as percentage of CYP1B1.1-dimethyl sulfoxide control ( $\pm$  S.D. of triplicate values). Asterisks indicate significance of values obtained for the different treatment groups compared with control within each allelic form. Daggers indicate significance of values obtained for CYP1B1.1 vs. CYP1B1.4 within the same treatment group.

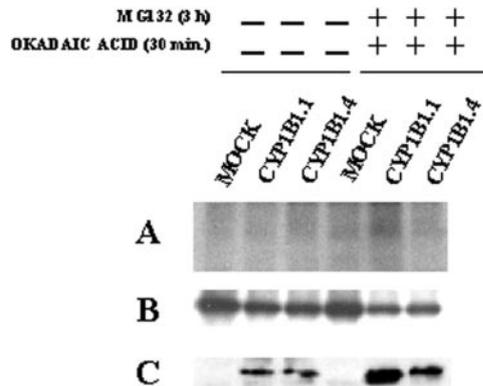
CYP1B1 Variant	Treatment		
	Control	Cycloheximide	Cycloheximide + MG132
CYP1B1.1 (RALN)	100 $\pm$ 9	64 $\pm$ 9**	75 $\pm$ 6*
CYP1B1.4 (RALS)	67 $\pm$ 4 <sup>††</sup>	27 $\pm$ 1***,††	48 $\pm$ 4***,††
CYP1B1.4/CYP1B1.1	0.67	0.42	0.64

\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

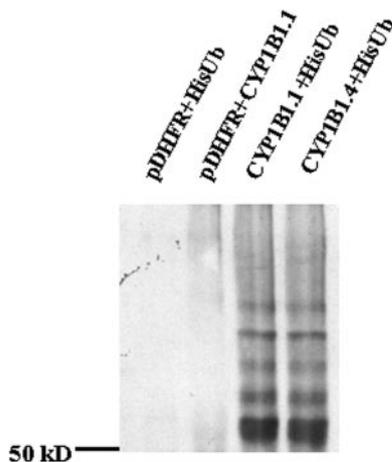
†† $p < 0.01$ .

loss of CYP1B1-mediated EROD activity associated with the exposure to cycloheximide. This was particularly evident for CYP1B1.4. However, we cannot conclude from these experiments whether the proteasome degrades the free CYP1B1 holoenzyme or the P450 complexed with P450 reductase.

Proteins have been shown to be targeted for proteasome-



**Fig. 6.** Analysis of CYP1B1 phosphorylation. After transfection (48 h), COS-1 cells expressing either *CYP1B1\*1* or *CYP1B1\*4* were incubated with [<sup>32</sup>P]orthophosphate in the presence or absence of MG132 and okadaic acid as described under *Materials and Methods*. After 3 h of incubation, cellular protein was subjected to immunoprecipitation using either rabbit polyclonal CYP1B1 (A) or anti-p53 (B) antibody. The immunoprecipitates were analyzed by SDS-PAGE, and the signals were visualized using the Molecular Imager FX Pro Plus MultiImager System with the Quantity One software. To control for successful immunoprecipitation of CYP1B1, the gel was rehydrated, and the proteins were transferred onto a nitrocellulose membrane that was subsequently incubated with mouse monoclonal anti-CYP1B1 antibodies and a horseradish peroxidase-coupled anti-mouse IgG secondary antibody. C, signal developed using the enhanced chemiluminescence system. p-53 served as a control, because it is known to be phosphorylated by a plethora of kinases.



**Fig. 7.** Analysis of CYP1B1.1 and CYP1B1.4 ubiquitination. COS-1 cells were cotransfected in triplicate with the plasmids encoding either CYP1B1.1 or CYP1B1.4 or the empty expression plasmid pDHFR together with the plasmid encoding His-tagged ubiquitin (His-Ub). One set of cells was also cotransfected with the empty expression plasmid pDHFR together with the plasmid encoding CYP1B1.1 (pDHFR + CYP1B1.1). One microgram of total DNA was transfected that contained 0.5  $\mu$ g of the CYP1B1 plasmid where indicated. Therefore, in the experiment labeled (pDHFR + CYP1B1), the pDHFR plasmid was added as filler DNA. After transfection (48 h), cells were treated with MG132 for 3 h. Affinity-purified His-tagged protein fractions were prepared from lysates of the pooled triplicates and analyzed by immunoblotting employing an anti-CYP1B1 monoclonal antibody as primary antibody, as described under *Materials and Methods*. Note that no step-ladder was detected in cells that expressed CYP1B1 alone (pDHFR + CYP1B1.1). Thus, the step-ladder was not a result of the aggregation of CYP1B1.

mediated degradation either by phosphorylation and/or ubiquitination. The data presented in Figs. 6 and 7 demonstrate that CYP1B1 is targeted via the latter modification. However, it should be noted that the intensity of the ubiquitination step-ladder was similar for CYP1B1.1 and CYP1B1.4, which may indicate that differences in the half-lives of both proteins are not determined by their different rates of ubiquitination.

At present, we do not know which structural alterations are responsible for the increased rate of CYP1B1 degradation caused by the allelic change Asn453Ser. This residue is located in the large meander region between the K- and L-helix and probably highly accessible to proteases. Even though allelic changes in P450s and other proteins have been known to affect expression levels mainly caused by differences in thermal stability (Ariyoshi et al., 2001; Nakamura et al., 2002), allele-specific differences in proteasome-mediated degradation have only been described for one other protein, NADPH quinone oxido-reductase 1. It is interesting in this case the Ser187 variant (NQO1\*2) is degraded faster than the Pro187 variant (NQO1\*1), with the 187 residue also located in an exposed meander region of the enzyme (Siegel et al., 2001). It is important to note that even though our study demonstrates an involvement of the proteasome in the degradation of CYP1B1, additional processing of CYP1B1 by other proteolytic pathways cannot be excluded.

The results presented herein demonstrate that CYP1B1.4 protein displays lower intracellular protein levels and is degraded more rapidly than the other CYP1B1 variants. It is therefore probable that individuals with the *CYP1B1\*4* haplotype will display reduced metabolic activation of some endogenous and exogenous carcinogens. This prediction is nicely corroborated by a very recent study that demonstrates that carriers of the CYP1B1 (m4) genotype that encodes Ser at amino acid residue 453 showed a significantly reduced incidence (OR = 0.62; 95% CI = 0.43–0.91) of endometrial cancer compared with homozygotes that carry Asn at this position (McGrath et al., 2004). Likewise, we found that persons who were homozygotes for *CYP1B1\*4* tended to have a lower risk for head and neck squamous cell carcinoma than those who carried at least one *CYP1B1\*1* allele (V. Marth, P. Broede, and Y. K., unpublished observations); however, this difference was not statistically significant (controls,  $n = 213$ ; cases,  $n = 247$ ; OR = 0.48; 95% CI = 0.17–1.34). It should be noted that the allele frequency of *CYP1B1\*4* shows marked interethnic variations (Bailey et al., 1998; Aklillu et al., 2002), making it probable that some of the interethnic variations in the incidence of certain types of cancer may be caused by this CYP1B1 polymorphism.

The results of this work may also contribute to our understanding of the mechanisms that are responsible for the high levels of CYP1B1 protein found in various tumor tissues compared with nondiseased tissues, most of the latter tissues being devoid of this protein, as evidenced by immunohistochemistry (Murray et al., 1997). This difference is also surprising given that CYP1B1 is readily detectable in many nondiseased tissues at the mRNA level (Murray et al., 2001). It is therefore probable that the tumor-specific expression of CYP1B1 as well as the lack of detectable protein in nondiseased tissues is at least partially regulated via proteasome-mediated degradation of the enzyme. Indeed, cancer lesions have been reportedly characterized by an imbalance of pro-

liferation and apoptosis, partly because of the deregulation of protein degradation (Naujokat and Hoffmann, 2002).

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