Use of Molecular Simulation for Mapping Conformational CYP2E1 Epitopes*

Received for publication, June 30, 2004, and in revised form, September 3, 2004. Published, JBC Papers in Press, September 28, 2004, DOI 10.1074/jbc.M407329200

Matteo Vidalì‡, Mats Hidestrand§, Erik Eliasson§, Elisa Mottaran‡, Emanuela Reale‡, Roberta Rolla‡, Giuseppa Occhino‡, Emanuele Albano‡, and Magnus Ingelman-Sundberg§

From the ‡Department of Medical Science, University “Amedeo Avogadro” of East Piedmont and Interdipartimental Research Center for Autoimmune Diseases (IRCAD), 28100 Novara Italy, the §Division of Molecular Toxicology, Institute of Environmental Medicine, Karolinska Institutet, Stockholm SE-17177, Sweden, and the ¶Division of Clinical Pharmacology, Department of Laboratory Sciences, Karolinska Institutet, Karolinska University Hospital, Huddinge SE-14186, Sweden

The identification of the epitopes recognized by autoantibodies against cytochrome P450s (CYPs) associated with drug-induced hepatotoxicity is difficult because of their conformational nature. In the present investigation, we used a novel approach based on the analysis of the whole molecule antigenic capacity following single amino acid substitutions to identify the conformational epitopes on CYP2E1. A molecular model of CYP2E1 was generated based on the CYP2C5 crystal structure, and potential motifs for amino acid exchanges were selected by computer simulation in the surface of α helices and β sheets. Fourteen modified, apparently correctly folded CYP2E1 variants were produced in Escherichia coli and evaluated in immunoprecipitation experiments using sera with anti-CYP2E1 autoreactivity from 10 patients with halothane hepatitis and 12 patients with alcoholic liver disease. Ala substitution of Glu-248 and Lys-251 as well as of Lys-324, Lys-342, Lys-420, and Phe-421 severely decreased or abolished CYP2E1 recognition by the majority of both the halothane hepatitis and alcoholic liver disease sera, whereas the other substitutions had only minor effects. Based on the structural model, these substitutions identified two distinct epitopes on the CYP2E1 surface corresponding to the G-helix and an area formed by juxta-position of the J’ and K’ helices, respectively. The combined use of molecular modeling and single amino acid mutagenesis is thus a useful approach for the characterization of conformational epitopes recognized by autoantibodies.

Cytochrome P450 2E1 (CYP2E1)1 is a hemoprotein belonging to the cytochrome P450 family that is responsible for the biotransformation of a variety of low molecular weight xenobiotics including halogenated hydrocarbons, benzene, acetaldehyde, and ethanol as well as for the oxidation of endogenous ketone bodies (1). Studies by Bourdi and et al. (2) and Eliasson and Kenna (3) have shown that patients suffering from halothane hepatitis develop autoantibodies specifically targeting CYP2E1. Similar autoantibodies are also detectable in anesthesiologists exposed to halogenated anesthetic gases (4). We have reported that chronic intragastric alcohol-fed rats develop IgG directed toward CYP2E1, and their titers correlate with the extent of hepatic injury (5). These observations have been confirmed in humans, showing that high titers of anti-CYP2E1 autoantibodies are present in about 40% of patients with advanced alcoholic liver disease but not in heavy drinkers without liver damage (6). In the former, the presence of anti-CYP2E1 IgG correlated with the extent of lymphocyte infiltration in liver biopsies (7), suggesting a possible contribution of autoimmune mechanisms in the pathogenesis of alcohol liver injury.

Anti-CYP autoreactivity is not uncommon in liver diseases, and antibodies against different CYP isoforms can be detected in the case of dihydralazine- (anti-CYP1A2) or tienilic acid- (anti-CYP2C9) induced hepatitis as well as during hypersensitivity reactions to the aromatic anti-convulsants (anti-CYP3A) or in children treated with immunosuppressive drugs (CYP3A4, CYP2C9) (8–11). Furthermore, CYP2D6 is a target of anti-liver kidney microsome type I (LKM-1), present in type II autoimmune hepatitis and in virus C hepatitis (12). Epitopes in CYP11A (cholesterol side-chain cleavage enzyme), CYP17 (steroid-17α hydroxylase), and CYP21A2 (steroid-21α hydroxylase) are also recognized by autoantibodies associated with autoimmune polyendocrine syndrome and autoimmune Addison’s disease (13, 14). For some of these autoantibodies, extensive epitope mapping studies have been performed to get a better understanding of the mechanisms leading to autoimmunity (15–21). The data so far obtained regarding anti-CYP2D6, anti-CYP2C9, and anti-CYP3A1 autoantibodies show that several linear and conformational epitopes are recognized in the different CYPs (15–21).

These observations, along with the evidence indicating that CYP2E1 autoantibodies target functionally active CYP2E1 present on the outer layer of hepatocyte plasma membrane (3), prompted us to investigate the epitope specificity of CYP2E1 autoantibodies associated to halothane hepatitis or alcoholic liver disease to understand more about their formation and to get information of value for the development of more specific diagnostic tests. For this purpose, we used a novel approach by analyzing the whole molecular antigenic capacity following single amino acid substitutions designed using a structural model of CYP2E1 generated with the crystal CYP2C5 structure as a template.
**CYP2E1 conformational epitopes**

**EXPERIMENTAL PROCEDURES**

**Patients**—For this study, the sera of 10 patients with unexplained hepatitis following multiple halothane anesthesia and negative for markers of hepatitis viruses or evidence of exposure to hepatotoxic drugs or alcohol were used along with 12 sera from patients with severe alcoholic liver disease (ALD). Sera from 10 patients with halothane hepatitis were generously provided by Dr. J. G. Kenna, Imperial College School of Medicine, London, UK. Their properties have been described in previously published studies (22, 23). In brief, halothane hepatitis was defined clinically as severe, otherwise unexplained, histologically confirmed hepatitis occurring within 4 weeks after halothane exposure in patients with normal preoperative liver function.

The diagnosis of ALD was based on clinical, ecological, and laboratory criteria. Liver biopsies were available for all the ALD patients and showed the classical features of micronodular cirrhosis, hepatocyte ballooning degeneration with Mallory’s bodies, and inflammatory infiltrates. The patients with alcohol abuse were negative for serum markers for hepatitis virus and for the presence of hepatitis C virus RNA. The reactivity of the sera with CYP2E1 was preliminarily assessed by enzyme-linked immunosorbent assay using as antigen recombinant human CYP2E1 (Oxford Biochemicals Inc., Oxford, MI) (500 ng/well) as reported previously (6) and was at least three times higher (1:100 dilution) than those of 50 control sera.

**Immunoprecipitation of [35S]Methionine-labeled CYP2E1**—[35S]Methionine-labeled wild-type CYP2E1 and the N-terminal and C-terminal deleted forms were produced from the respective pGEM4z plasmid cDNA (1 μg) using a rabbit reticulocyte "in vitro" translation/transcription mixture. The translation/transcription mixture was diluted (1:20) with RIPA buffer (50 mmol/liter Tris/HCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mmol/liter NaCl, and 1 mmol/liter EDTA, pH 7.4) and preincubated 1 h at 4 °C with 50 μl of protein A-Sepharose CL4B beads (50% w/v suspension in phosphate-buffered saline) (Amersham Biosciences). After centrifugation at 5,000 rpm for 5 min, 95 μl of the supernatant were added to 5 μl of human sera (dilution 1:20 in RIPA buffer) and first incubated 12 h at 4 °C in an orbital shaker then 2 h at 4 °C in the presence of protein A-Sepharose CL4B beads (50 μl of 50% w/v suspension in phosphate-buffered saline). As a positive control, a polyclonal rabbit anti-CYP2E1 (dilution 1:100 in RIPA buffer) was used. Immunocomplexes bound to protein A-Sepharose were recovered by centrifugation, washed three times with 1.5 ml of phosphate-buffered saline, and solubilized in 40 μl of SDS buffer, pH 6.8 (4% w/v sodium dodecyl sulfate, 0.02% w/v mercaptoethanol, 0.2% w/v glycerol). One aliquot containing 1.5 μl of protein was added to scintillation fluid and used for radioactivity determination. The other was boiled 5 min, centrifuged, and used for SDS-PAGE electrophoresis. The recovery of radioactive CYP2E1 was calculated as (dpm sample – dpm background)/dpm positive control – dpm background) × 1,000, where dpm background was the radioactivity recovered in the absence of added serum. SDS-PAGE electrophoresis was performed for 45 min at 200 V using 4% stacking and 10% resolving gels. The proteins were transferred to Hybond-C Extra nitrocellulose gel (Amersham Biosciences) and exposed to autoradiography using x-ray films (Eastman Kodak Co.).

**CYP2E1 Computer Simulation**—A structural model of human CYP2E1 was generated by the first approach mode using the SWISS-MODEL automated comparative protein modeling server (www.expasy.ch/swissmod/SWISS-MODEL.html) (24) and the template coordinates of CYP2C5 (code ExPDB 1DT6A) (25). Energy minimization of the model was performed using GROMOS96 force field algorithm. Root mean square errors and atomic distances were calculated using DeepView-SwissPdbViewer software (www.expasy.org/spdbv/) (24). Graphical representation was made by the use of Rastop (version 2.0.3) by Philippe Valadon (La Jolla, CA).

**Subcellular localization of CYP2E1**—The CYP2E1 was cloned into the expression plasmid pCWori1* (26) between the restriction sites NdeI and HindIII. Additional bases encoding 6 C-terminal histidine tags were added, and nucleotides encoding the first 18 amino acids were removed to optimize the expression (27). The mutant CYP2E1s were generated by using the QuikChange™ XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. The forward and reverse DNA primers used for each mutation are listed in Table I. XL-1 Blue supercompetent Escherichia coli strain was transformed with the different plasmids by heat shock and selected on LB agar plates containing ampicillin. Single colonies were further expanded by overnight culture at 37 °C in 5 ml of LB medium plus ampicillin (50 μg/ml). Plasmid DNA was isolated using QiAprep spin miniprep columns (Qiagen Inc., Valencia, CA), and plasmid concentration was evaluated spectrophotometrically. The correct sequence of the insert was confirmed by automated DNA sequencing with the ABI PRISM dye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA).

**Prokaryote Expression of Mutated and Wild-type CYP2E1**—For the expression of wild-type and mutated CYP2E1 variants, 70 μl of XL1 Blue supercompetent E. coli cells were transfected with 1 μl of the respective plasmid solution as described above, and colonies were selected on LB agar plates with ampicillin. Single colonies were further grown for 16 h at 37 °C and 10 h at 4 °C on LB medium ampicillin. Two ml of these cultures were used to seed 198 ml of LB agar ampicillin medium supplemented with 1 mmol/liter thiamine, 0.5 mmol/liter δ-aminolevulinic acid, 25 μmol/liter FeCl3, and incubated at 30 °C under continuous shaking (25). Bacterial growth was monitored spectrophotometrically until reaching 0.7 OD units before adding 0.5 mmol/liter imidazole and 1 mmol/liter isopropyl β-D-thiogalactoside. After a further 24 h of culture at 30 °C, CYP expression was monitored in whole cells by recording absorption spectrum at 450 nm in the presence of reducing agent and CO (28).

**Purification of Recombinant CYPs**—Bacteria were separated from the incubation medium by centrifugation (2,800 × g for 12 min at 4 °C) and resuspended in cold 50 mmol/liter Tris-HCl buffer, pH 7.4, plus 250 mmol/liter sucrose and 0.25 mmol/liter EDTA and 0.25 mg/ml lysozyme. After a 30–60-min incubation in ice, spheroplasts were recovered by centrifugation (2,800 × g for 12 min at 4 °C), resuspended in 0.1 M phosphate buffer, pH 7.6, containing 6 mmol/liter magnesium acetate, 20% (v/v) glycerol, and 25 μM protease inhibitor mixture (Hoffmann-La Roche) and lysed by sonication. The bacterial lysate was centrifuged at 4 °C (12,000 × g for 12 min), and the membrane fraction was recovered from the supernatant by centrifugation at 100,000 × g for 60 min. The pellet was resuspended in 2 ml of 50 mmol/liter sodium phosphate buffer, pH 7.4, supplemented with 300 mmol/liter NaCl, 5 mmol/liter imidazole 20% (v/v) glycerol, 1% (w/v) sodium deoxycholate 1 mmol/liter phenylmethylsulfonyl fluoride and further centrifuged at 100,000 × g for 60 min. The supernatant was added to 0.5 ml of nickel-charged polypropylene-agarose columns (Qiagen Inc.). Following a 1-h incubation at 4 °C under shaking, the mixture was loaded to nickel-nitrilotriacetic acid-agarose columns (Qiagen Inc.). The columns were first washed four times with 4 ml of 50 mmol/liter sodium phosphate buffer, pH 7.4, containing 300 mmol/liter NaCl, 10 mmol/liter imidazole 20% (v/v) glycerol, 0.1% (w/v) sodium deoxycholate, and the CYP2E1-
containing fractions were eluted by the subsequent addition of 0.5 ml of 50 mmol/liter Tris-HCl buffer, pH 7.4, plus 100 mmol/liter NaCl, 500 mmol/liter imidazole 20% (v/v) glycerol, 0.1% (w/v) sodium deoxycholate. CYP2E1 recovery was estimated spectrophotometrically at 450 nm according to Omura and Sato (28).

**Results**

**Reaction of Human Sera with Mutated CYP2E1s—**The recognition of mutated and wild-type CYP2E1 by the human sera was estimated in immunoprecipitation experiments using 10 pmol of CYP2E1 solubilized in RIPA buffer and 5 µl of the different sera (1:20 dilution in RIPA buffer) as described above. Immunocomplexes bound to protein A-Sepharose beads were solubilized in 40 µl of SDS buffer, pH 6.8 (4% w/v sodium dodecyl sulfate, 0.2 mol/liter Tris/HCl, 0.2 mol/liter imidazole 20% (v/v) glycerol, 0.1% (w/v) sodium deoxycholate). CYP2E1 recovery was estimated spectrophotometrically at 450 nm according to Omura and Sato (28).

**Results**

Initial experiments confirmed that the sera from patients with halothane-induced hepatitis (HAL) and ALD recognizing recombinant human CYP2E1 in enzyme-linked immunosorbent assays immunoprecipitated [35S]methionine-labeled CYP2E1 translated in vitro using the rabbit reticulocyte system. To discriminate between conformational and linear epitopes, we prepared CYP2E1 constructs with the N-terminal (222 amino acids) or C-terminal (271 amino acids) moieties. However, the reactivity of these human sera against these two truncated forms were undetectable (not shown), indicating that the anti-CYP2E1 autoantibodies in both HAL and ALD are directed toward conformational epitopes. This conclusion was strengthened by the observation that the human sera failed to recognize CYP2E1 in Western blots (not shown).

To characterize these conformational epitopes, we decided to study the effects of single amino acid substitutions on the antigenic capacity of the whole molecule. A computer-simulated structure of CYP2E1 was generated using the SWISS-MODEL automated comparative protein modeling server (wwp.expasy.ch/swissmod/SWISS-MODEL.html) (24) and the crystal structure of rabbit CYP2C5, which share 59% amino acid sequence homology with CYP2E1 (29). The three-dimensional model of CYP2E1 obtained was quite similar to the crystal structure of rabbit CYP2C5, which share 59% amino acid sequence homology with CYP2E1 (29). The three-dimensional model of CYP2E1 obtained was quite similar to the crystal structure of rabbit CYP2C5, which share 59% amino acid sequence homology with CYP2E1 (29).
CYP2C5 and bacterial CYPs (joneslab.wsu.edu). Using the CYP2E1 structural model, sequences containing charged amino acids facing the outer surface of the protein were selected in the /H9251 helices and /H9252 sheets located on the external portions of the molecule. We focused our attention to protruding areas suitable for antibody binding, whereas sequences that were in troughs or not apparently accessible were excluded. Since most of the epitopes so far identified in CYPs largely involve the C-terminal portion of the molecule (15–21), the sequence between amino acids 200 and 493 was investigated. Preliminary experiments using purified rat or rabbit CYP2E1 showed that human anti-CYP2E1 IgG cross-reacted with the enzyme from these species in enzyme-linked immunosorbent assays (not shown). Therefore, amino acid sequences of human CYP2E1 not in common with the rat and rabbit CYP2E1 orthologues were excluded. The potential antigenic capability of the selected sequences was then confirmed by computer assessment of the antigenic index according to Jameson and Wolf (30) (GCG Wisconsin package; Accelrys Inc.). To produce major changes in the configuration of the possible epitopes without disrupting the tertiary structure of the molecule, we decided to insert a neutral amino acid having a low steric hindrance such as alanine in place of charged residues of lysine, arginine, and glutamic acid. The effects of these mutations on CYP2E1 antigenic potential was evaluated using the GCG Wisconsin software. As a result, 20 single amino acid substitutions were selected (Table I).

Site-directed mutagenesis was used to introduce the mutations into cDNA, and the modified CYP2E1s were then expressed in E. coli as His6-tagged proteins. The capacity to retain the heme iron on the reduced state, as measured by the spectral absorption at 450 nm of the reduced CYPs (28), was used as an index of the correct protein folding. Fourteen out of 20 mutated CYP2E1s (Table I) showing spectral features comparable with the wild-type protein were used for further experiments. The capacity of the human sera to recognize these CYP2E1 variants was assayed by immunoprecipitation followed by Western blotting. As shown in Fig. 2, about half of the substitutions impaired significantly the ability of the sera to immunoprecipitate CYP2E1. Alanine substitutions of Glu-272, Lys-342, and Lys-420 affected the antibody binding to the highest extent. The effects of Lys-324, Arg-374, Phe-421, and Glu-440 substitutions were less consistent, being evident with 2–3 of the different sera (Fig. 2). Conversely, the Ala substitution of Lys-234, Glu-320, Arg-331, Arg-344, and Glu-346 did not interfere with the antibody recognition or had scattered effects with a single serum (Fig. 2). Although the mutations were able to reduce CYP2E1 antigenicity involved amino acids located far away from each other in the J helix and the /-sheet between K and L helices, respectively, computer simulations revealed that the positions of Lys-342, and Lys-420 on the tertiary structure were rather close (about 2.5–3.0 nm) and identified an area in CYP2E1 surface compatible with the presence of a distinct conformational epitope (Fig. 3). This interpretation was supported by the observation that the substitutions of Lys-324 and Phe-421 (Figs. 2 and 3) also decreased the antigen recognition of some sera.

| Lys-234 | Glu-272 | Glu-320 | Lys-324 | Arg-331 | Lys-342 | Arg-344 | Glu-346 | Arg-374 | Lys-420 | Phe-421 | Glu-440 |
|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|
| HAL1    | 80%     | 100%    | 80%     | 83%     | 96%     | 50%     | 90%     | 84%     | 77%     | 46%     | 100%    |
| HAL2    | 80%     | 60%     | 79%     | 95%     | 91%     | 100%    | 79%     | 100%    | 64%     | 100%    | 52%     |
| HAL3    | 80%     | 100%    | 100%    | 100%    | 53%     | 100%    | 52%     | 100%    | 100%    | 55%     | 96%     |
| HAL4    | 100%    | 44%     | 68%     | 73%     | 85%     | 50%     | 78%     | 65%     | 53%     | 95%     | 100%    |
| HAL5    | 100%    | 26%     | 78%     | 31%     | 100%    | 74%     | 100%    | 100%    | 100%    | 0%      | 100%    |
| ALD1    | 100%    | 92%     | 100%    | 69%     | 83%     | 56%     | 70%     | 100%    | 44%     | 92%     | 50%     |
| ALD2    | 100%    | 92%     | 100%    | 51%     | 92%     | 62%     | 100%    | 99%     | 94%     | 23%     | 22%     |
| ALD3    | 100%    | 66%     | 55%     | 79%     | 100%    | 7%      | 96%     | 42%     | 74%     | 45%     | 91%     |
| ALD4    | 100%    | 75%     | 45%     | 26%     | 91%     | 29%     | 89%     | 78%     | 82%     | 48%     | 100%    |
| ALD5    | 43%     | 44%     | 99%     | 62%     | 93%     | 67%     | 62%     | 100%    | 59%     | 56%     | 71%     |

**Fig. 2.** Effect of single amino acid substitutions on the recognition of recombinant human CYP2E1 by the sera of five patients with ALD and five patients with HAL. Ten pmol of wild-type (WT) or mutated CYP2E1s were incubated with 5 μl of the different human sera (1:20 dilution), and the immunocomplexes recovered by protein A-Sepharose beads were estimated by Western blotting using a monoclonal mouse IgG targeting the His6 tag tail of the CYP2E1s. The intensities of the bands were measured by videodensitometry, and the results were expressed as the percentage of the recovery of modified CYP2E1s as compared with the wild-type protein included in each blot. The black squares indicate a reduction in the antibody recognition ≥ 50%, and the gray squares indicate a reduction between 40 and 50%.
The poor immunoreactivity of three HAL and one ALD sera with CYP2E1 modified by the substitution of Glu-272 in the /H9252 sheets between the G and H helices (Fig. 2) prompted us to extend the investigation to include substitutions encompassing the G helix between Lys-243 and Lys-251. Fig. 4 shows that the substitution of Lys-243, Glu-244 did not significantly affect CYP2E1 recognition. However, a further change of Glu-248 and Lys-251 for Ala appreciably reduced the capacity of three out of five of HAL and in four out of five of the ALD sera to immunoprecipitate CYP2E1 (Fig. 4). Structural simulation revealed that the mutated amino acids were closely associated with a distinct area on the enzyme surface (Fig. 3).

These results indicated that anti-CYP2E1 autoantibodies associated to both halothane hepatitis and alcoholic liver disease recognized similar conformational epitopes and that two of these epitopes were located in, respectively, the G helix and between J/H11032 and K/H11033 helices in the C-terminal portion of the molecule. This conclusion was supported by further analysis of additional five HAL and seven ALD sera revealing that these two epitopes were recognized by four out of the five HAL sera and five out of the seven ALD sera (Table II). Altogether, the epitopes identified account for the antigen specificity of 19 out of all 22 (86%) human sera investigated.

**DISCUSSION**

In recent years, several investigators have evaluated the epitope specificity of LKM-1 autoantibodies on CYP2D6 by comparing the cross reactivity of human sera with prokaryotic expressed peptides spanning the entire protein sequence. This approach has allowed the identification of several linear
epitopes with different specificity for LKM-1 associated with type II autoimmune hepatitis and chronic hepatitis C (17–21). Furthermore, it has been shown that some of these epitopes share structural homologies with protein sequences present in type 1 herpes simplex virus and hepatitis C virus (17, 21). The use of deleted CYP mutants has also provided important information concerning the structure of different epitopes present in CYP2C9, CYP3A1, and CYP1A2 (15–17). However, it is well known that B-cell epitopes associated to autoimmune disease are often conformational. For instance, conformational epitopes in CYP2D6 and CYP2C9 are implicated in the autoreactivity of, respectively, LKM-1 and LKM-2 antibodies (15, 31). According to Bourdi et al. (2), the sera of patients with either halothane hepatitis or alcoholic liver disease does not recognize denaturated human CYP2E1. The same sera also have low reactivity toward truncated forms of CYP2E1, not allowing the use of deletion mutants for the identification of the epitopes. We have, therefore, devised a new approach based on the analysis of the whole molecule antigenic capacity following single amino acid substitutions that modify the charge distribution on the CYP2E1 surface without affecting the molecule conformation. Hereby, the data obtained demonstrate that Ala substitutions of Lys-324, Lys-342, Lys-420, and Phe-421 identify a conformational epitope formed by the juxtaposition of the J’ and K’ helices. The distance between the single amino acids confirms the compatibility with an antigen-antibody binding area. The J’ K, and L helices have been implicated as sites of conformational epitopes recognized in CYP2C9 by LKM2 autoantibodies associated with tienilic acid-induced autoimmune hepatitis (15), whereas the K helix of CYP3A4 is considered a major epitope recognized by autoantibodies of patients with hypersensitivity reactions to aromatic anti-convulsing drugs (16). It is interesting to note that both Lys-324 and Lys-342 are comprised in a sequence with good homology to the CYP2D6 C321–351 epitope (19), whereas Lys-324 is conserved in the CYP2C9 C314–322 epitope (15). Lys-324 is also close to the CYP2D6 C316–327 sequence that, according to Ma et al. (20), represents a key target for autoantibodies on CYP2D6 surface. Similarly, the CYP2D6 C410–429 epitope (19) has good homology with the sequence containing Lys-420 and Phe-421. This suggests the possibility that the J’ K, and L helices might represent an important antigenic area in CYPs, able to give rise to antibodies against both linear and conformational epitopes. A further conformational epitope corresponding to Glu-248 and Lys-251 is also evident in a distinct area of the G helix that is located on the opposite side of the CYP2E1 surface. This epitope is recognized by 50% of the 22 sera tested, five of them in combination with the epitope in J’-K’ helices. The C-terminal portion of CYP3A4 spanning up to Thr-208 and the region between Thr-208 and Ser-281, which comprises the G helix, is the target of autoantibodies present in a subset of alcoholics (5). However, it is unlikely that anti-CYP3A4 autoantibodies might account for the recognition of CYP2E1 conformational epitopes because they are directed against linear structures, and there is no sequence homology between G helices of CYP2E1 and CYP3A4.

We have recently reported (32) that the N-terminal portion of CYP2E1 and particularly the amphipathic amino acids in the B-helix are responsible for electrostatic interactions with negatively charged phospholipids that anchor CYP2E1 to the cell membranes. The orientation of CYP2E1 in relation to the membrane (Fig. 5) shows that the epitopes formed by J’ and K’ helices and G helix are both on the outer portion of the molecule and well accessible to antibody binding. This is consistent with previous observations showing that functionally active CYP2E1 is present on the outer layer of hepatocyte plasma membrane, where it is targeted by specific antibodies (3, 33, 34). Thus, the recognition of these conformational epitopes on the portion of CYP2E1 that faces the extracellular spaces can be involved in triggering antibody-mediated hepatocyte killing.

In conclusion, the results presented demonstrate that anti-CYP2E1 autoantibodies recognize at least two distinct conformational epitopes present in the G-helix and in an area formed by the juxtaposition of J’ and K’ helices on the C-terminal portion of the molecule surface. Moreover, we propose the combined use of molecular modeling and site-directed mutagenesis as a novel method to investigate the epitope specificity of conformational anti-CYP autoantibodies associated to liver diseases.

REFERENCES
1. Ronis, M. J. J., Lindros, K. O., and Ingelman-Sundberg, M. (1996) in Cytochromes P450: Metabolic and Toxicological Aspects (Ioannides, C., ed) pp. 211–239, CRC Press, Boca Raton.
2. Bourdi, M., Chen, W., Peter, R. M., Martin, J. L., Buters, J. T. M., Nelson, S. D., and Pohl, L. R. (1996) Chem. Res. Toxicol. 9, 1159–1166.
3. Eliasson, E., and Kenna, J. G. (1996) Mol. Pharmacol. 50, 573–582.
4. Njoku, D. B., Greenberg, R. S., Bourdi, M., Borkowf, C. B., Dake, E. M., Martin, J. L., and Pohl, L. R. (2002) Anesth. Analg. 94, 243–249.
5. Lytton, S. D., Hellander, A., Zhang-Gouillon, Z. Q., Stokkeland, K., Bordone, R., Arici, S., Albano, E., French, S. W., and Ingelman-Sundberg, M. (1999)
CYP2E1 conformational epitopes

6. Vidali, M., Stewart, S. F., Rolla, R., Daly, A. K., Chen, Y., Mottaran, E., Jones, D. E., Leathart, J. B., Day, C. P., and Albano, E. (2003) *Hepatology* **37**, 277–285

7. Stewart, S. F., Jones, D. E., Vidali, M., Haugk, B., Burt, A. D., Albano, E., and Day, C. P. (2004) *J. Hepatol.* **40**, Suppl. 1, 176

8. Bourdi, M., Larrey, D., Nataf, J., Bernuau, J., Pessayre, D., Iwasaki, M., Guengerich, F. P., and Beaune, P. H. (1990) *J. Clin. Investig.* **85**, 1967–1973

9. Beaune, P. H., Dansette, P. M., Mansuy, D., Kiffel, L., Finck, M., Amar, C., Leroux, J. P., and Homberg, J. C. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 551–555

10. Leeder, J., Riley, S. R. J., Cook, V. A., and Spielberg, S. P. (1992) *J. Pharmacol. Exp. Ther.* **263**, 360–367

11. Lecoeur, S., Andre, C., and Beaune, P. H. (1996) *Mol. Pharmacol.* **50**, 326–333

12. Manns, M. P., and Obermayer-Straub, P. (1997) *Hepatology* **26**, 1054–1066

13. Leeder, J. S., Gaedigk, A., Lu, X., and Cook, V. A. (1996) *Mol. Pharmacol.* **49**, 234–243

14. Belloc, C., Gauffre, A., Andre´, C., and Beaune, P. H. (1997) *Pharmacogenetics* **7**, 181–186

15. Manns, M. P., Griffin, K. J., Sullivan, K. F., and Johnson, E. F. (1991) *J. Clin. Investig.* **88**, 1370–1378

16. Yamamoto, A. M., Cresteil, D., Homberg, J. C., and Alvarez, F. (1993) *Gastroenterology* **104**, 1702–1707

17. Ma, Y., Thomas, M. G., Okamoto, M., Bogdamos, D. P., Nagl, S., Kerkar, N., Lopes, A. R., Muratori, L., Bianchi, F. B., Mieli-Vergani, G., and Vergani, D. (2002) *J. Immunol.* **169**, 277–285

18. Kerkar, N., Choudhuri, K., Ma, Y., Mahnoud, A., Bogdamos, D. P., Muratori, L., Bianchi, F. B., Williams, R., Mieli-Vergani, G., and Vergani, D. (2003) *J. Immunol.* **170**, 1481–1489

19. Kenna, J. G., Neuberger, J., and Williams, R. (1998) *Hepatology* **8**, 1635–1641

20. Kenna, J. G., Neuberger, J., and Williams, R. (1998) *Hepatology* **8**, 1635–1641

21. Smith, C. M., Kenna, J. G., Harrison, D. J., Tew, D., and Wolf, C. R. (1993) *Lancer* **342**, 963–964

22. Schwede, T., Koop, J., Guex, N., and Peitsch, M. C. *Nucleic Acids Res.* **31**, 3381–3385

23. Williams, P. A., Cosme, J., Sridhar, V., Johnson, E. F., and McBee, D. E. (2000) *Mol. Cell* **5**, 131–131

24. Barnes, H. J. (1996) *Methods Enzymol.* **272**, 3–14

25. Gillam, E. M., Guo, Z., and Guengerich, F. P. (1994) *Arch. Biochem. Biophys.* **312**, 59–66

26. Omura, T., and Sato, R. (1964) *J. Biol. Chem.* **239**, 2370–2378

27. Lewis, D. F. V., Lake, B. G., Bird, M. G., Loizou, G. D., Dickens, M., and Goldfarb, P. S. (2003) *Toxicol. In Vitro* **17**, 93–105

28. Jameson, B. A., and Wolf, H. (1988) *Comput. Appl. Biosci.* **4**, 181–186

29. Lewis, D. F. V., Lake, B. G., Bird, M. G., Loizou, G. D., Dickens, M., and Goldfarb, P. S. (2003) *Toxicol. In Vitro* **17**, 93–105

30. Neve, E. P. A., Hidestrand, M., and Ingelman-Sundberg, M. (2003) *Biochemistry* **42**, 14566–14575

31. Wu, D. F., and Cederbaum, A. I. (1992) *Hepatology* **15**, 515–524

32. Clot, P., Parola, M., Bellomo, G., Bianchi, F., Cederbaum, A. I., Ingelman-Sundberg, M., and Albano, E. (1997) *Gastroenterology* **113**, 265–276