Evidence against a Role for Serine 129 in Determining Murine Cytochrome P450 Cyp2e-1 Protein Levels

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ABSTRACT: The cytochrome P450 CYP2E subfamily plays a central role in drug and carcinogen metabolism. The cellular content of this protein is regulated at both the transcriptional and posttranslational levels. CYP2E1 is degraded by both rapid and slow acting proteolytic systems. In the presence of a substrate, CYP2E1 becomes stabilized, and the contribution of the rapid actinig proteolytic pathway to its destruction decreases. It has been suggested that phosphorylation at serine 129 acts as a switch to initiate the fast acting degradative pathway. Phosphorylation at serine 129 has also been suggested to be the point at which hormones, such as insulin, exert actions on the stability of this protein. In order to investigate the role of phosphorylation in determining murine Cyp2e-1 levels, serine 129 was changed by site-directed mutagenesis to amino acids that could not be phosphorylated and the recombinant proteins expressed in COS 7 cells. Replacement of serine 129 with alanine and glycine does not lead to Cyp2e-1 accumulation. In the presence of insulin, although Cyp2e-1 levels increase slightly, specific stabilization of the wild-type protein relative to the two mutant forms is not observed. These observations provide evidence that insulin can act by stabilization of Cyp2e-1 protein but suggest that the phosphorylation of serine 129 is not the molecular basis of stabilization observed.

The cytochrome P450 multigene family encodes enzymes which function as terminal electron acceptors, catalyzing the reductive activation of molecular oxygen and the subsequent insertion of one molecule of oxygen into a substrate. Mammalian P450[1] can be divided broadly into two classes: first, enzymes which play a critical role in normal homeostasis, such as steroid and bile acid biogenesis, and enzymes localized in the endoplasmic reticulum involved in foreign compound metabolism. The capacity of this latter group of "xenobiotic metabolizing" P450s to generate genotoxic products has been linked to chemical carcinogenesis [see Wolf (1990)].

The activities of many of the xenobiotic metabolizing P450s are induced by the compounds which they metabolize, and this capacity may represent part of an adaptive response to toxic lipophilic chemicals (Wolf, 1986). The nomenclature (Nelson et al., 1993), catalytic activities, regulation, and evolution of the P450 superfamily have been reviewed (Nelson & Strobel, 1989; Ryan & Levin, 1990; Nelson et al., 1993).

The cytochrome P450 CYP2E subfamily is highly conserved and encodes a protein with a high degree sequence identity between mammalian species. The subfamily is represented by a single gene in the human, rat, and mouse but by two recently duplicated genes in the rabbit [see Nelson et al. (1993)]. Interest in the CYP2E subfamily has centered mainly around its role in the metabolism and activation of a series of commonly occurring carcinogenic nitrosamines, such as *N*-nitrosodimethylamine (NDMA). An understanding of the regulation of the CYP2E subfamily will provide an insight into situations that may be associated with a higher carcinogenic risk, following exposure to those compounds which are CYP2E substrates (Yang et al., 1990). In addition to nitrosamines, CYP2E1 also catalyzes the metabolism of numerous small solvent molecules, such as ethanol, isopropanol, acetone, acetol, benzene, *p*-nitrophenol, aniline, carbon tetrachloride, chloroform, and several ether molecules. The CYP2E subfamily has also been shown to catalyze the metabolism of several common drugs, for example, acetominophen [see Yang et al. (1990)].

Cellular levels of CYP2E1 are regulated by solvents such as ethanol and acetone in all species studied to date (Freeman et al., 1992; Henderson & Wolf, 1992). This induction appears to be by a posttranslational mechanism, as no associated increase in CYP2E1 mRNA is observed. In addition to this mechanism of regulation, CYP2E1 levels are increased in starved or diabetic rats, mice, or humans (Song et al., 1989). In these latter situations, in contrast to solvent induction, the elevated level of CYP2E1 is accompanied by increased CYP2E1 mRNA levels. It was suggested that acetone may mediate the endogenous responses regulating CYP2E1 levels in fasting, starvation, and diabetes where levels rise with the increased β-oxidation of fatty-acids (Koop & Cassaza, 1986).

In vivo studies demonstrated that the rat CYP2E1 population is degraded biphasically by both rapid and slow removal system. Following treatment with acetone, the contribution of the rapid pathway is reduced leading to elevated CYP2E1 levels by protein stabilization (Song et al., 1989). The rapid CYP2E1 degradation system was suggested to be endoplasmic reticulum-associated whereas the slow removal resulted from a bulk flow, autolysomal pathway [see Ingelmann-Sundberg et al. (1992)]. However, the molecular basis for the induction of the CYP2E1 proteins is unclear and may involve a posttranslational modification of the protein, making it more resistant to the rapid degradation pathway.

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[1] Abbreviations: P450, cytochrome P450; PK-A, protein kinase A.

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Rat CYP2E1 can be phosphorylated by cAMP-dependent protein kinase (PK-A) at serine 129. Phosphorylation at this site has been observed for a large number of other CYP2 family members (Pyerin & Taniguchi, 1989; Koch & Waxman, 1989). The role of phosphorylation of CYP2E1 on its turnover in the presence of substrates has been investigated in primary hepatocytes. The inclusion of CYP2E1 substrates increased the level of CYP2E1 protein whereas elevation of PK-A activity, by the addition of membrane permeable cAMP analogues, or hormones, such as glucagon, increased the CYP2E1 phosphorylation and increased the degradation rate. The addition of insulin, however, which reduces PK-A activity, led to increased CYP2E1 retention (Johansson et al., 1991; Eliasson et al., 1992). These data support a role of PK-A and protein phosphorylation at serine 129 in the rapid degradation of CYP2E1 and suggest that phosphorylation at this site acts as a switch to enable rapid degradation. In order to establish whether serine 129 is indeed involved, Cyp2e-1 was mutated at this site, and the effects of these mutations on Cyp2e-1 stability in the presence and absence of insulin were determined.

EXPERIMENTAL PROCEDURES

Nomenclature. The nomenclature used throughout is that recommended for the P450 multigene family by Nelson et al. (1993).

Chemicals and Reagents. All chemicals used were of analytical grade or purer, and were obtained from either Sigma (Poole, U.K.) or BDH (Glasgow, U.K.). Restriction endonucleases, T4 DNA ligase, T7 polynucleotide kinase, and the large fragment of *Escherichia coli* DNA polymerase I ("Klenow") were obtained from Boehringer Mannheim (Lewes, U.K.). [α-^{35}S]Thio-dATP (600 Ci/mmol) and ^{125}I-conjugated protein A (40 mCi/mg) were obtained from Amersham (Aylesbury, U.K.). Oligonucleotides were prepared using an Applied Biosystems 380A DNA synthesizer, and autoradiography employed Kodak X-OMAT AR-5 X-ray film.

Recombinant DNA Manipulation and the Generation of Mutant Cyp2e-1 cDNAs. A Cyp2e-1 cDNA was isolated from a BALB/c male mouse liver library constructed in λZAPII as described previously (Freeman et al., 1992); M13 mp18 RF DNA was obtained from Boehringer Mannheim (Lewes, U.K.). Plasmid and recombinant M13 mp18 RF DNA were isolated using Qiagen columns according to the manufacturer's instructions (Qiagen Inc., Hilden, Germany). Manipulations leading to the generation and isolation of recombinant constructs were performed according to standard protocols as described in Sambrook et al. (1989).

The serine 129 residue within a mouse Cyp2e-1 cDNA was modified by site-directed mutagenesis to alanine and glycine in two separate constructs using the *dut*$^-$ *ung*$^-$ *E. coli* strain BW313. Alanine and glycine were selected to replace serine as they most closely resemble it in size, charge, and structure and so would not be expected to introduce perturbations in the Cyp2e-1 protein folding.

A full-length mouse Cyp2e-1 cDNA (Freeman et al., 1992) was subcloned into the *Xba*I and *Sal*I restriction endonuclease sites of M13 mp18. Single-stranded DNA was isolated from this construct and oligonucleotide-directed mutagenesis performed. The two mutant Cyp2e-1 cDNA sequences

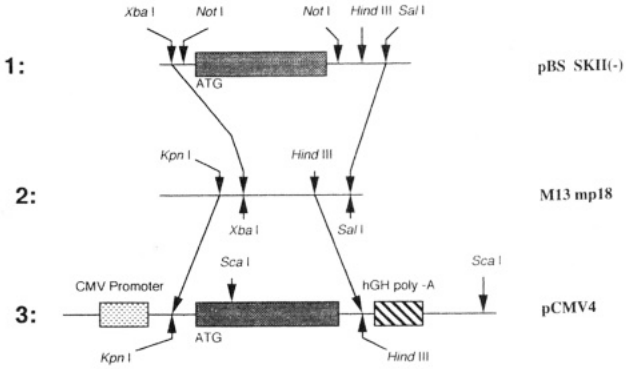

FIGURE 1: Generation of two Cyp2e-1 cDNA sequences with directed mutations at serine 129. (1 and 2) A Cyp2e-1 cDNA contained in PBS SKII(−) was directionally cloned into M13mp18 where mutagenesis was performed using the oligonucleotides 5′-GGATACTTAGGGCAAACCTCCGC-3′ and 5′-GGATACTTAG-GCCAAACCTCCGC-3′ for alanine and glycine, respectively. (3) The two mutant Cyp2e-1 cDNA sequences were directionally cloned into the pCMV4 transient expression vector. ATG, the Cyp2e-1 initiation codon; CMV promoter, the human cytomegalovirus major intermediate early gene promoter; hGH poly(A), the poly(A) addition site of the human growth hormone gene.

generated were then subcloned into the *Kpn*I and *Hin*dIII restriction endonuclease sites of the transient eukaryotic expression vector pCMV4. Expression of P450 proteins from CMV driven constructs in this manner has proved a powerful tool in the analysis of active proteins in this multigene family (Clark & Waterman, 1991). Both mutants clones were sequenced in the pCMV4 vector to confirm the presence of the mutation and the lack of any additional changes in the Cyp2e-1 sequence using Sequenase according to the manufacturer's instructions (USB, Cleveland, OH). The introduction into the pCMV4 vector of the Cyp2e-1 cDNA associated *Not*I/*Eco*RI restriction endonuclease sites (Freeman et al., 1992) allowed the use of the *Not*I restriction endonuclease site to clone in the unmodified Cyp2e-1 cDNA. pCMV4 vectors containing wild type Cyp2e-1 cDNA in both orientations were thus generated and confirmed through the use of a diagnostic *Sca*I restriction endonuclease site. The correctly orientated wild-type Cyp2e-1 pCMV4 construct was sequenced to confirm the presence of the serine 129 codon. The oligonucleotides employed, the determination of codon mutations, and the strategy used in the preparation of the wild-type and mutant Cyp2e-1 constructs are shown in Figure 1.

Maintenance and Manipulation of the COS 7 Cell Line. Cultures of COS 7 were grown as a monolayer at 37 °C in a 5% CO_2 incubator in Dulbecco's modified Eagle's medium (Gibco BRL, Paisley, U.K.) supplemented with 10% fetal calf serum (Gibco BRL, Paisley, U.K.), 20 mM HEPES (pH 7.4), 100 units of penicillin/mL, and 100 mg/mL streptomycin; insulin (Sigma, Poole, UK) was added to the cultures to a final concentration of 1×10^{-7} M, where stated. For DNA transfections, cells were plated at a density of 1×10^6 cells per 25 cm^2 flask and transfected with 20 μg of DNA using the calcium phosphate method, (Sambrook et al., 1989). The viability of the cells was assessed by nigrosin dye exclusion.

Analysis of Cyp2e-1 Expressed in COS 7 Cells. Following transfection, cells were harvested by trypsinization, pelleted, and resuspended in 10 mM sodium phosphate buffer containing, 2 mM $MgCl_2$, 2 mM DTT, and 1 mM EDTA

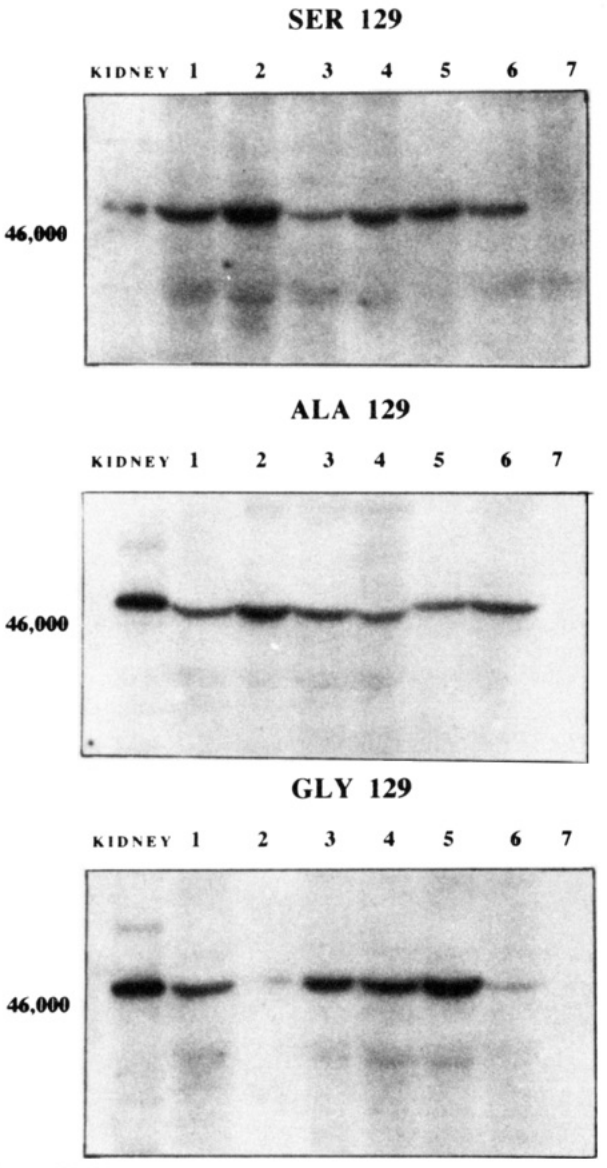

FIGURE 2: Expression of Cyp2e-1 cDNAs in COS 7 cells. COS 7 cells were transfected with the parental Cyp2e-1 construct (Ser 129) and the two mutant Cyp2e-1 cDNAs (Ala 129 and Gly 129). One to seven days (1−7) following transfections, total COS 7 cell protein was isolated and 100 μg of each sample separated by SDS−PAGE on 12% gels with 10 μg of DBA2/N male mouse kidney microsomes as standard. Protein was transferred to nitrocellulose and probed with polyclonal antisera raised to purified rat CYP2E1 protein. The Cyp2e-1 protein migrated with a M_r of approximately 53 000, and no bands other than those shown were detected.

(pH 7.4). The cells were disrupted by sonication (Soniprep 150 sonicator, MSE) and the protein concentrations assessed according to the method of Lowry et al. (1951). The protein samples were separated on denaturing gels and electrophoretically transferred to nitrocellulose membranes, and immunoblotting was performed as previously described (Freeman et al., 1992). Cyp2e-1 activity was measured in the cell extracts using *p*-nitrophenol as a substrate (Koop et al., 1989). The polyclonal antisera employed was raised to purified rat CYP2E1 protein (kind gift, Prof. C. S. Yang), and the reactivity of this antisera with mouse Cyp2e-1 protein has been demonstrated previously (Freeman et al., 1992). A donkey anti-rabbit horseradish peroxidase-labeled second antibody was used to reveal immune complexes using 4-chloronaphthol as the substrate, and the complex was radiolabeled using ^{125}I-conjugated protein A.

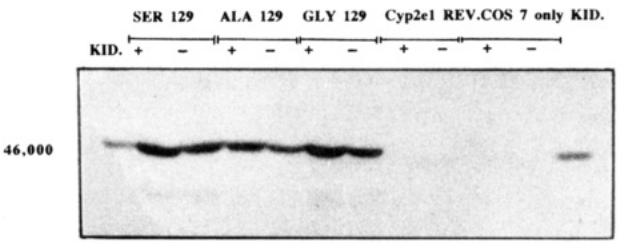

FIGURE 3: Effect of insulin on Cyp2e-1 levels. COS 7 cells were transfected with the parental Cyp2e-1 (Ser 129) and the two mutant Cyp2e-1 cDNAs (Ala 129 and Gly 129) in the presence (+) or absence (−) of 1×10^{-7} M insulin in the medium. Controls were either transfected with a vector containing parental Cyp2e-1 cDNA in the reverse orientation (Cyp2e-1 REV) or were mock transfected (COS 7 only). Cells were harvested 2 days after transfection and analyzed as described in the legend to Figure 2.

RESULTS

Following transfection with the expression constructs into COS 7 cells, Cyp2e-1 protein was produced from the correctly orientated Cyp2e-1 construct, as well as the two mutants. No endogenous CYP2E1 protein was detected. All three proteins were active in *p*-nitrophenol hydroxylation, producing 0.06 nmol of 4-nitrocatechol/(min·mg of protein). No activity could be measured in control cells. Cyp2e-1 was expressed at detectable levels until day 6 following transfection. Through the use of a nigerosin dye exclusion assay, the viability of the COS 7 cells was seen to be little changed over this period and at days 6 and 7 following transfection were 85−90% viable. Immunoblot analysis of protein extracts prepared from these cells showed that all three Cyp2e-1 proteins were expressed. Although some variability was seen in the level of Cyp2e-1 protein, which is probably a factor of the transfection efficiency, they were all expressed at essentially the same level over the 7 day period; i.e., there was no accumulation of the mutant Cyp2e-1 forms (Figure 2). These observations were confirmed in three separate experiments.

In primary rat hepatocytes, insulin has been reported to increase the retention of CYP2E1 protein (Johansson et al., 1991). If serine 129 is involved, then the mutant proteins would be predicted to be nonresponsive to the presence of this hormone. COS 7 cells were transfected with the three Cyp2e-1 constructs in the presence or absence of insulin (1×10^{-7} M) and harvested 2 days later. The COS 7 cells demonstrated insulin responsiveness, manifested by a characteristic increase in cell density and protein concentration (Stumpo et al., 1988). Following normalization of protein concentrations, it was seen that the presence of insulin in the media led to a slight increase in the levels of both native and the mutant proteins (Figure 3). No difference between the different transfectants was observed.

DISCUSSION

It has been suggested that protein phosphorylation at serine 129 plays a central role in regulating cellular levels of cytochrome P450 CYP2E1. In this report we have shown that removal of this site has no effect on the steady-state concentration of this protein expressed in COS 7 cells, which questions the role of phosphorylation at this site as a factor in determining Cyp2e-1 levels. It is unlikely that the absence of an effect is a consequence of the recipient cells used as

COS 7 cells are derived from the kidney, a tissue where Cyp2e-1 regulation by insulin and acetone can be observed (Freeman et al., 1992; J. E. Freeman, unpublished results). Also, these cells contain protein kinase A, are responsive to insulin, and have been used to study the turnover of other proteins (Tsuneoka & Meka, 1992; Juratka et al., 1993).

One explanation for the difference observed here and previous reports is that Cyp2e-1 phosphorylation is only casually linked to the insulin effects on protein levels, i.e., phosphorylation may represent a marker for a confirmational change produced elsewhere in the molecule and is not directly involved in initiating the degradation process. For example, it has been noted in the purification of several P450 enzymes that addition of substrate leads to the stabilization of the protein [see Ryan and Levin (1990)]. In many of these cases a phosphorylation site is absent in the C-helix suggesting that the substrate alone is responsible for this phenomenon. In addition, the inclusion of a CYP2E1 substrate in the purification of CYP2E1 heterologously expressed in *E. coli* also improved its stability (Larson et al., 1991). These observations suggest that the presence of a substrate through binding to the active site is sufficient to generate a more stable P450 conformation.

Nearly all cytochrome P450 family 2 members can be phosphorylated by PK-A both *in vitro* and *in vivo* (Parkinson et al., 1988; Koch & Waxman, 1989); however, only CYP2E1 and CYP2A1 are degraded in a biphasic manner. Proteins such as CYP2B1 and CYP2B2 are removed by the slower degradation mechanism (Parkinson et al., 1988; Song et al., 1989). It has been argued that P450 family 2 isoforms have similar tertiary structures and most members retain the serine 129 phosphorylation site (Nelson & Strobel, 1989; Zvelebil et al., 1991). Phosphorylation of this site would therefore be predicted to generate similar structural modifications in all family 2 proteins. If serine 129 phosphorylation is to be viewed as a biologically relevant control mechanism, it is not easy to rationalize how such an event can produce accelerated degradation of one subfamily 2 protein isoform relative to others.

The effects of insulin on Cyp2e-1 protein levels could be explained by the nonspecific global changes in the rate of translation of a variety of transcripts due to translational machinery modification and increased translational efficiency (Marino et al., 1992). The cellular response to insulin also leads to a slowing of membrane bulk-flow generating a nonspecific decrease in the level of protein degradation (James et al., 1988). The insulin associated elevation of Cyp2e-1 may therefore result from a general increase in translational efficiency or decreases in the activity of a bulk-flow, nonspecific, degradative system. These observations clearly underline the problems associated with any attempts to assign specific roles for factors like insulin in the control of the CYP2E subfamily against the background of global modifications in the cellular translation and degradation machinery. Similar observations of global elevations in P450 activities have been made in studies on the effect of insulin on steroid hormone metabolizing P450 which do not possess a phosphorylatable serine (Gulati & Skett, 1989). We are currently testing the wild-type and mutant proteins in other cell systems to attempt to establish another role of serine 129 phosphorylation in the function of this protein.

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