Phosphorylation of Native and Heme-Modified CYP3A4 by Protein Kinase C: A Mass Spectrometric Characterization of the Phosphorylated Peptides†

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ABSTRACT: As an initial approach toward the characterization of the phosphorylation of cumene hydroperoxide (CuOOH)-inactivated cytochrome P450 (CYP3A4, the major human liver drug-metabolizing enzyme) and its role in the degradation of the inactivated protein, we have identified one of the major participating cytosolic kinase(s) as rat liver cytosolic protein kinase C (PKC) with the use of specific and general kinase inhibitors. Accordingly, we employed a model phosphorylation system consisting of purified PKC, γ-S-[^{32}P]ATP, and either native or CuOOH-inactivated purified recombinant His_6-tagged CYP3A4. Lysylendoprotease (Lys)-C digestion of the phosphorylated CuOOH-inactivated CYP3A4$(His)_6$ followed by HPLC-peptide mapping and mass spectrometric (LC/MS/MS) analyses led to the isolation and the unambiguous identification of two PKC-phosphorylated CYP3A4 peptides: E_{258}SRLEDT(p)QK_{266} and F_{414}LPERFS(p)K_{421}. Similar analyses of the PKC-phosphorylated native enzyme predominantly yielded E_{258}SRLEDT(p)QK_{266} as the phosphorylated peptide. Studies are currently in progress to determine whether phosphorylation of any or both of these peptides is required for the Ub-dependent 26S proteasomal degradation of CuOOH-inactivated CYP3A4.

The family of hepatic hemoproteins collectively termed cytochromes P450 (P450s; CYPs)[1] are monooxygenases engaged in the metabolism of a chemically diverse array of endo- and xenobiotics (*1*−*3*). The enzyme levels are intracellularly regulated by control of their synthesis both at the transcriptional and at the translational steps, and/or of their degradation by posttranslational stabilization (*4*−*12*). Several of the rat liver enzymes (CYPs 2E1, 2B1, 3A1) are also known to be posttranslationally modified by phosphorylation in vivo or in hepatocytes in suspension and/or culture by dibutyryl cyclic AMP (cAMP) or 8-bromoadenosine 3′,5′-cAMP (cell-permeable analogues of cAMP), or by agents such as glucagon and epinephrine that enhance the intracellular adenyl cyclase activity (*13*−*29*). Such phosphorylation is believed to inactivate the P450s by causing the loss of their prosthetic heme and to trigger their cellular degradation (*14*, *15*, *20*−*29*). This process is inhibited by stabilization of the individual P450 enzyme by binding of selective substrates/ligands (*23*, *24*, *28*). Rat liver P450s of the subfamily 2 such as CYPs 2B1, 2C11, and 2E1 contain a well-recognized consensus sequence (RRX$S_{128/129}$) for protein kinase A (PKA) and have been shown to be particularly susceptible to phosphorylation by the cAMP-dependent PKA in vitro. P450s from the other subfamilies (i.e., CYP3A, CYP17) also contain secondary PKA consensus sites and/or sites for phosphorylation by protein kinase C (PKC) and Ca^{2+}/calmodulin-dependent kinase II (CaM kinase II) (*28*, *29*). Indeed, rat liver CYP3A1 has been shown to be phosphorylated by a microsomal cAMP-dependent kinase at a secondary PKA consensus site (KGS_{393}) (*28*). We have shown that CYP3A4, the major human liver drug-metabolizing enzyme, is also phosphorylated by an as yet unidentified rat liver cytosolic kinase in an in vitro system (*30*). This phosphorylation apparently is enhanced by cumene hydroperoxide (CuOOH)-mediated inactivation via heme modification of the protein within the active site, a process that targets the protein to proteolytic degradation by the ubiquitin (Ub)-dependent 26S proteasome system (*30*). Because the inactivated CYP3A4 protein is considerably more susceptible to both phosphorylation and ubiquitination than its native counterpart, and the phosphorylation precedes its ubiquitination (*30*), it is conceivable that phosphorylation is required for ubiquitination and/or degradation. Well-known precedents for such a causal association exist in the literature, particularly for the Ub-dependent 26S proteasomal degradation of

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[1] Abbreviations: CaM kinase II, Ca^{2+}/calmodulin-dependent kinase II; CID, collision-induced dissociation; CPK, creatine kinase; CuOOH, cumene hydroperoxide; CYPs, P450s, cytochromes P450; cAMP, cyclic AMP; GSH, glutathione (reduced form); HPLC, high-performance liquid chromatography; IPP, inorganic pyrophosphatase; IDA, information-dependent acquisition; Lys-C, lysylendoprotease-C; LC/MS/MS, liquid chromatography/tandem mass spectrometric analyses; Ni-NTA, nickel-nitriloacetate; OA, okadaic acid; PKA, protein kinase A; PKC, protein kinase C; PKG, protein kinase G; QoaTOF, quadrupole-orthogonal-acceleration-time-of-flight; Ub, ubiquitin; Ubal, Ub aldehyde.

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several short-lived cell regulatory proteins such as IκBα, $p58^{Ctf13}$, Sic1p, and G-cyclins and other cell-cycle-regulatory proteins (*31*−*38*). Thus, our overall research goal is to determine whether, in common with the degradation of these cell regulatory proteins, the phosphorylation of inactivated CYP3A4 is also a required prelude for its Ub-dependent 26S proteasomal degradation, and, if so, to identify the responsible cellular kinase from its characteristic signature motif branded within the phosphorylated CYP3A4 domain(s) (*39*).

As an initial approach toward the characterization of CYP3A4 phosphorylation and its role in the degradation of the inactivated protein, we sought to identify the cytosolic kinase(s) involved in this process. Studies with specific and general kinase inhibitors indicated that rat liver cytosolic PKC may be one of the kinases largely responsible for the phosphorylation of the CuOOH-inactivated CYP3A4 (see below). However, structural analyses of inactivated P450s are notoriously difficult, because unlike their native counterparts not only do the inactivated proteins tend to aggregate, but also their modified, highly hydrophobic active site cores are relatively recalcitrant to the commonly used approaches such as proteolytic digestion coupled with HPLC-peptide mapping and mass spectrometric (MS) analysis of the relevant peptides (*40*). Previous identifications of the P450 peptide sequences phosphorylated by specific kinases have been construed largely from compositional amino acid and/or Edman degradation analyses of phosphopeptides isolated from tryptic digests of the phosphorylated P450s (*16*, *20*, *23*, *28*). While such an exercise is feasible with the phosphorylated native P450s, extensive experience in the characterization of suicidally inactivated P450s, either via heme-modification or drug-adduction of the protein (*40*−*42*), leads us to believe that trypsin is incapable of digesting the relatively hydrophobic active site regions of inactivated P450s relevant to the identification and characterization of the modified P450 peptides. Thus, while tryptic digestion may release the hydrophilic peptide regions of CuOOH-inactivated CYP3A4 phosphorylated by the cytosolic kinase(s), it would be incapable of releasing those possibly buried within the hydrophobic heme-modified active sites that may become accessible to the kinases after enzyme inactivation. For these reasons, as a first step in the elucidation of the role of phosphorylation in the overall degradation of CYP3A4 by the Ub-dependent proteasomal system, we have used a model system to gain some insight into the methodological approaches required for the structural analyses of such recalcitrant P450 active site peptides potentially phosphorylated by cytosolic kinases. In this system, purified PKC is used to phosphorylate either native or CuOOH-inactivated purified recombinant His_6-tagged CYP3A4, which is then subjected to lysylendoprotease (Lys)-C digestion and HPLC-peptide mapping coupled with LC/MS/MS analyses. Using this approach, we now describe the isolation and the unambiguous identification of two PKC-phosphorylated peptides of CuOOH-inactivated CYP3A4. Corresponding analyses of the PKC-phosphorylated native enzyme revealed that only one of these peptides is predominantly phosphorylated.

MATERIALS AND METHODS

Materials. Staurosporine was purchased from Alexis (San Diego, CA). Calpain inhibitor I, bisindolylmaleimide III HCl, Calphostin C, Go 6976, H-89, and KN-62 were obtained from Calbiochem (La Jolla, CA), while okadaic acid (OA) was obtained from Gibco, BRL (Rockville, MD). Aprotinin, α-macroglobulin, leupeptin, creatine phosphate, creatine kinase (CPK), inorganic pyrophosphatase (IPP), calyculin A, ATP, ubiquitin (Ub), cumene hydroperoxide (CuOOH), DTT, and glutathione (GSH, reduced form) were obtained from Sigma-Aldrich (St. Louis, MO). Ub aldehyde (Ubal) was synthesized as described previously (*30*, *43*) by carboxypeptidase digestion of Ub, conjugation with 2,3-aminopropanediol, and subsequent periodate oxidation of the Ub diol. Its authenticity was ascertained by mass spectrometric analyses. Ni-NTA superflow resin was obtained from Qiagen (Valencia, CA). γ-S-[^{32}P]ATP (specific activity, 3000 mCi/mmol) was obtained from New England Nuclear (Boston, MA). All other buffers and reagents were of the highest commercial grade.

Enzymes. A CYP3A4 cDNA, engineered to code for deletions of the N-terminal residues 3−12 and substitution of residue Ser18 (with Phe) of the full-length protein, and incorporated into the pCW vector [a gift from Dr. R. Estabrook (SW Medical School, Dallas, TX)], was tagged at its C-terminus with a poly($His)_6$ tail. The CYP3A4($His)_6$ protein was heterologously expressed in *E. coli* DH5αF′ cells as described (*44*) and purified to homogeneity by Ni-NTA affinity chromatography. Protein kinase C (PKC), catalytic subunit purified from rat brain, was obtained from Calbiochem. One unit is defined as the amount of the enzyme that transfers 1.0 nmol of phosphate from ATP to histone III-S per minute at 30 °C. Fraction II, a subfraction that contains the yet to be identified CYP3A4-phosphorylating kinase along with the enzymes required for Ub activation and conjugation as well as the 26S proteasome, was prepared from rat liver cytosol as described (*30*, *45*, *46*). Lysylendoprotease-C (Lys-C) was obtained from Wako Chemicals USA, Inc. (Richmond, VA).

Inactivation of CYP3A4(His)$_6$. Purified CYP3A4($His)_6$ (1.5 nmol) was inactivated with CuOOH (0.5 mM) at 37 °C for 15 min in HEPES buffer (50 mM, pH 7.4) containing 20% glycerol, EDTA (1 mM), and GSH (1 mM). The reactions were terminated by the addition of DTT (5 mM, final concentration) and incubated at room temperature for a further 5 min. Comparable aliquots of the purified CYP3A4-($His)_6$ were incubated in parallel in the absence of CuOOH and served as the native controls.

Phosphorylation of CYP3A4(His)$_6$ by Rat Liver Cytosolic Fraction II. Heme-modified or native CYP3A4($His)_6$ (1.5 nmol) was incubated, in a final volume of 1 mL, with rat liver cytosolic Fraction II (4 mg of protein/mL) at 30 °C for 30 min in HEPES buffer (50 mM, pH 7.4) containing 20% glycerol, $MgCl_2$ (10 mM), EGTA (1 mM), Ub (1 mg/mL), Ubal (3 μM), creatine phosphate (10 mM), CPK (10 units/mL), IPP (10 units/mL), OA (3 μg/mL), calyculin A (30.5 μM), α-macroglobulin (1 μg/mL), aprotinin (2.4 μg/mL), leupeptin (0.5 μg/mL), calpain inhibitor I (176 μM), ATP (0.5 mM), and γ-S-[^{32}P]ATP (∼15−20 μCi). To reisolate CYP3A4($His)_6$, the phosphorylation/ubiquitination mixtures were then mixed with Ni-NTA superflow resin (50% slurry, 0.6 mL) and incubated at room temperature for 30 min with gentle rotation. The Ni-NTA column was washed extensively with HEPES buffer (50 mM, pH 7.4) containing 20% glycerol and 20 mM imidazole. CYP3A4($His)_6$ was then

eluted with 200 mM imidazole in HEPES buffer (50 mM, pH 7.4; 20% glycerol), and aliquots were subjected to SDS−PAGE analyses on 9% gels as described (*30*). Autoradiographs were scanned with a Storm 860 PhosphorImager (Molecular Dynamics, Sunnyvale, CA) equipped with ImageQuant software for quantitation.

In the kinase inhibition studies, rat liver cytosolic Fraction II was preincubated with either DMSO (a vehicle control) or individual inhibitors [dissolved in DMSO at the final concentrations indicated (Results)] at 30 °C for 15 min in HEPES buffer (50 mM, pH 7.4) containing 20% glycerol, α-macroglobulin (1 μg/mL), aprotinin (2.4 μg/mL), leupeptin (0.5 μg/mL), and calpain inhibitor I (176 μM), before phosphorylation/ubiquitination of native or CuOOH-inactivated CYP3A4(His)$_6$, and aliquots were analyzed by SDS−PAGE/PhosphorImager analyses as described above.

Phosphorylation of CYP3A4(His)$_6$ by PKC. Native or heme-modified CYP3A4(His)$_6$ (1.5 nmol) was phosphorylated by incubation with the catalytic subunit of protein kinase C (PKC, 0.02 unit) in HEPES buffer (50 mM, pH 7.4; 20% glycerol) containing $MgCl_2$ (10 mM), EGTA (0.5 mM), EDTA (0.5 mM), ATP (0.5 mM), and γ-S-[^{32}P]ATP (∼10 μCi). The reaction mixtures were incubated at 37 °C for 30 min, then CYP3A4(His)$_6$ was reisolated by Ni-NTA chromatography, and aliquots were subjected to SDS−PAGE/PhosphorImager analyses as described above.

Lys-C Digestion and HPLC-Peptide Mapping of the Phosphorylated CYP3A4. The ^{32}P-phosphorylated native or CuOOH-inactivated CYP3A4(His)$_6$ eluted from the Ni-NTA column was dialyzed overnight against 100 mM Tris-HCl buffer (pH 8.7) containing 20% glycerol. The enzyme was then denatured at 60 °C for 20 min in the presence of 8 M urea. Following the dilution of urea to 2 M final concentration with Tris-HCl buffer (pH 9.1 containing 20% glycerol), Lys-C was added (at a Lys-C:P450 molar ratio of 1:20) and incubated at 37 °C for 12−16 h. The solution was acidified and subjected to HPLC chromatography (Varian, C-18 column, 5 μM, 0.4 × 25 cm). The peptides[2] were eluted using a linear gradient of 0−70% (v/v) acetonitrile/H_2O containing 0.1% TFA at a flow rate of 1 mL/min over 70 min. One milliliter fractions were collected, and aliquots were analyzed for radioactivity by scintillation counting. The peak radioactive peptide fractions were dried down to 10 μL and the phosphopeptides analyzed by LC/MS/MS.

LC/MS/MS Analysis of the Phosphorylated CYP3A4 Peptides. Phosphopeptide-containing fractions from either the native or the heme-modified CYP3A4(His)$_6$ were fractionated by reversed-phase HPLC on an UltiMate HPLC system, equipped with a FAMOS autoinjector (LC Packings, San Francisco, CA). Experimental conditions were as follows: 1 μL injection; 75 μm × 150 mm PepMap column; solvent A, H_2O with 0.1% formic acid; solvent B, acetonitrile with 0.1% formic acid; gradient, 0−40% B over 40 min at a flow rate of ∼250 nL/min. Mass spectrometric analysis was performed with a QSTAR quadrupole-orthogonal-acceleration-time-of-flight tandem mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA) in the information-dependent acquisition (IDA) mode: 2 s survey acquisitions were followed by 5 s collision-induced dissociation (CID) acquisitions, in which the most abundant ion of each survey scan was selected as the precursor, unless specified differently in the inclusion list. The collision energy was preset, optimized for the doubly charged ion of a standard peptide. The mass range recorded in survey acquisitions was *m*/*z* 300−1400. For CID experiments, the lower mass limit was changed to *m*/*z* 60. All the data presented here were measured using a two-point external calibration. The instrument affords ∼8000 resolution and 30 ppm mass accuracy with external calibration in both the MS and the CID mode.

[2] Complete Lys-C digestion of native CYP3A4 theoretically results in 34 peptides, 26 of which contain a potentially phosphorylatable residue (Thr, Ser, or Tyr).

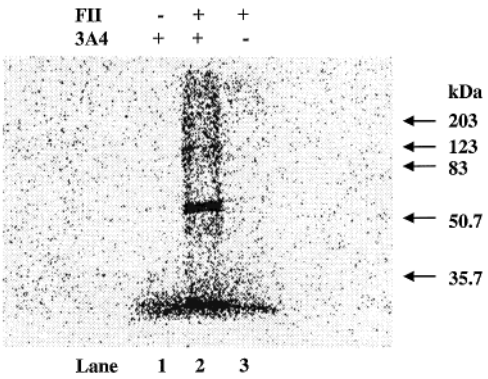

FIGURE 1: Phosphorylation of CuOOH-inactivated CYP3A4(His)$_6$ by rat liver cytosolic Fraction II. CuOOH-inactivated CYP3A4-(His)$_6$ was incubated with or without Fraction II (FII) and γ-S-[^{32}P]ATP in an ATP-regenerating system as described (Materials and Methods). Phosphorylated CYP3A4(His)$_6$ was reisolated by Ni-NTA affinity chromatography, and equivalent aliquots were subjected to SDS−PAGE PhosphorImager analyses.

RESULTS AND DISCUSSION

CYP3A4 Phosphorylation by Cytosolic Kinases. SDS−PAGE/PhosphorImager analyses of CuOOH-inactivated purified recombinant CYP3A4 incubated with rat liver cytosolic Fraction II in the presence of γ-S-[^{32}P]ATP and an ATP-regenerating system, at 37 °C for 30 min, predominantly reveal the phosphorylation of the 55 kDa parent protein, along with a ladder of phosphorylated CYP3A4 protein bands extending between 55 and >203 kDa that presumably correspond to their polyubiquitinated species (Figure 1), as previously documented (*30*). This phosphorylation profile is not observed if Fraction II or CYP3A4 is excluded from the incubations (Figure 1). Inclusion of either general or selective kinase inhibitors (*47*) in the incubation system led in some cases to a substantial attenuation of this profile (Figure 2A). Accordingly, a 50% inhibition was observed with staurosporine, a general inhibitor of PKA, PKC, and PKG (*47*), at 0.1 μM, and this was only slightly enhanced at 1 μM. On the other hand, bisindoleylmaleimide III, a relatively selective inhibitor of PKC (*47*), caused a concentration-dependent inhibition of this phosphorylation, reaching a maximum of ≈50% at 5 μM (Figure 2A). Other relatively selective PKC inhibitors, Calphostin and Go 6976 (*47*), also led to a similar level of inhibition, as did the relatively selective PKA inhibitor H-89 (Figure 2A). In contrast, no inhibition of CYP3A4 phosphorylation was observed by KN-62, a relatively selective inhibitor of CaMKII [(*47*); Figure 2A]. These studies thus suggested that cytosolic PKC and PKA might be involved in the phosphorylation of CuOOH-inactivated CYP3A4.

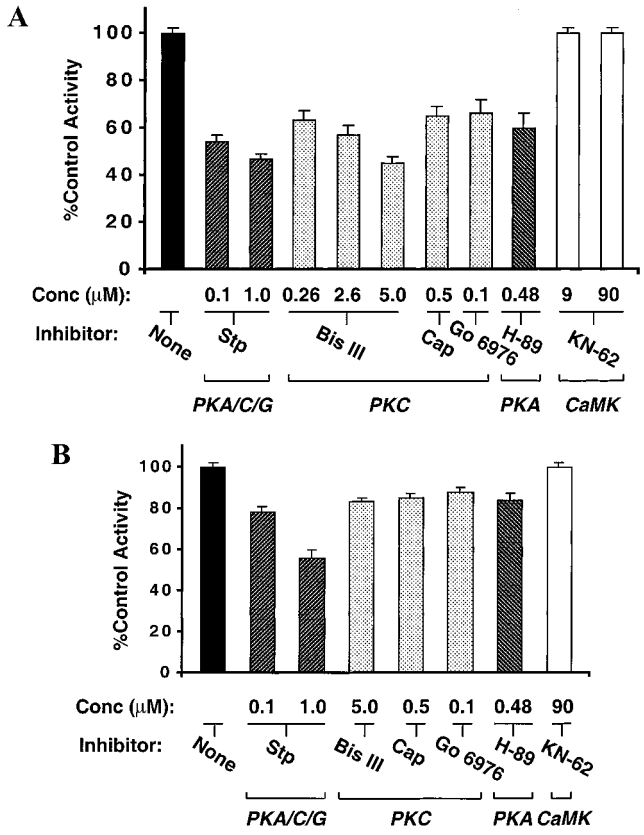

FIGURE 2: Relative effect of various kinase inhibitors on Fraction II-mediated phosphorylation of CuOOH-inactivated (A) or native (B) CYP3A4(His)$_6$. CuOOH-inactivated (A) or native (B) CYP3A4-(His)$_6$ was incubated in a γ-S-[^{32}P]ATP-dependent phosphorylating mixture with Fraction II that had been preincubated with the vehicle DMSO alone (None) or with each of the general or selective kinase inhibitors at the concentrations shown. Phosphorylated CYP3A4-(His)$_6$ was reisolated by Ni-NTA affinity chromatography, and equivalent aliquots were subjected to SDS−PAGE PhosphorImager analyses. For details, see Materials and Methods. Bis III, bisindolylmaleimide III HCl; Stp, staurosporine; Cap, Calphostin. 100% control activity corresponds to 31 000 and 14 000 cpm of ^{32}P phosphorylation (mg of CYP3A4 protein)$^{-1}$ (30 min)$^{-1}$ in panels A and B, respectively.

Similar studies with the native CYP3A4 (Figure 2B) indicated a considerably lower extent (≈45%) of basal phosphorylation of the enzyme by liver cytosolic Fraction II. Indeed, less than 20% of this CYP3A4 phosphorylation was inhibited by the PKC-selective inhibitors bisindoleylmaleimide III, Calphostin, and Go 6976, at the highest concentrations used (see above). A comparable level of inhibition was also observed with the relatively selective PKA inhibitor H-89 (Figure 2B). On the other hand, staurosporine (1 μM), a general inhibitor of PKA, PKC, and PKG, inhibited the phosphorylation of the native CYP3A4 to an almost comparable extent to that of the heme-modified enzyme, consistent with an additive effect due to the inhibition of all three kinases (Figure 2B). Collectively these findings suggest that the phosphorylation of the native enzyme is relatively less susceptible to selective PKC inhibitors than that of the inactivated enzyme.

PKC-Mediated Phosphorylation of Native and CuOOH-Inactivated CYP3A4. SDS−PAGE/PhosphorImager analyses of native CYP3A4 incubated with PKC in a γ-S-[^{32}P]ATP-supplemented phosphorylation system led to some extent of CYP3A4 phosphorylation, which was augmented when the native enzyme was replaced with the CuOOH-inactivated CYP3A4 (Figure 3; ≈55 kDa band). No corresponding ^{32}P-phosphorylated bands were detected if CYP3A4 was omitted from the incubates. Lys-C digestion of the ^{32}P-phosphorylated CuOOH-inactivated CYP3A4,[2] followed by HPLC-peptide mapping of the digests (Figure 4A), yielded two prominent ^{32}P-phosphorylated peptide peaks, one eluting at ≈20 min and the other at ≈32 min (Figure 4B; solid line). Parallel HPLC-peptide mapping of Lys-C digests from ^{32}P-phosphorylated native CYP3A4 predominantly yielded only one peptide peak eluting at ≈20 min, whose radioactivity was found to be comparable to that of its inactivated CYP3A4 counterpart (Figure 4B; dashed line). These peaks were individually collected and purified by further HPLC and

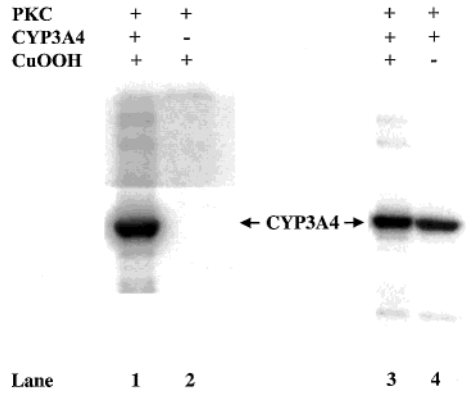

FIGURE 3: Phosphorylation of CYP3A4(His)$_6$ by PKC. CuOOH-inactivated or native CYP3A4(His)$_6$ was incubated in a γ-S-[^{32}P]-ATP-dependent phosphorylating mixture with PKC as detailed under Materials and Methods. Phosphorylated CYP3A4(His)$_6$ was reisolated by Ni-NTA affinity chromatography, and equivalent aliquots were subjected to SDS−PAGE PhosphorImager analyses.

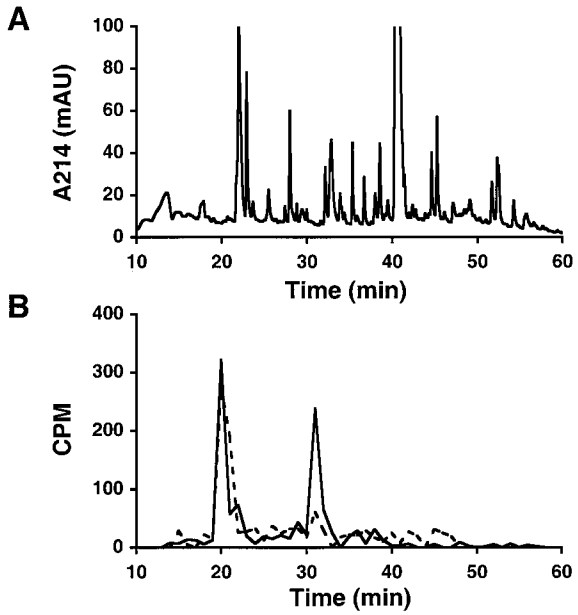

FIGURE 4: HPLC-peptide mapping of the Lys-C digests of the ^{32}P-phosphorylated CuOOH-inactivated and native CYP3A4. For details, see Materials and Methods. (A) UV profile of HPLC-peptide elution monitored at 214 nm. (B) Corresponding ^{32}P-radioactivity profile of the peptides as determined by scintillation counting of aliquots of consecutive peptide eluates. Profiles from the ^{32}P-phosphorylated CuOOH-inactivated CYP3A4 Lys-C digests are depicted by the solid line, and those from the native enzyme by the dashed line.

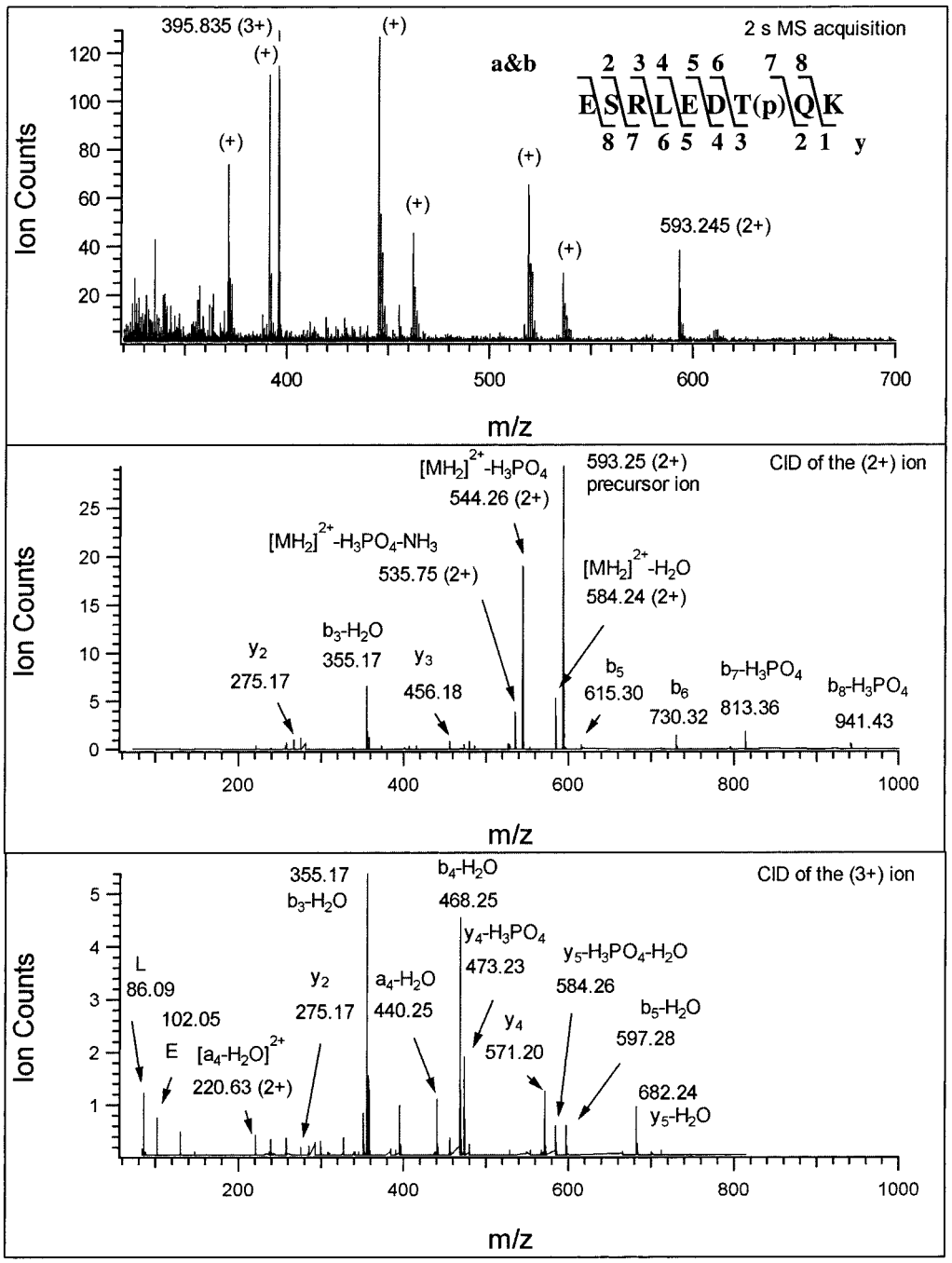

FIGURE 5: IDA LC/MS/MS data of the phosphopeptide eluting at 20 min. The upper panel shows the MS survey acquisition data obtained for the peptide peak (eluting at 20 min) from Lys-C digests of CuOOH-inactivated CYP3A4 that revealed the presence of phosphorylated E_{258}SRLEDTQK_{266}. The middle and lower panels show CID spectra acquired from the doubly and the triply charged ions, respectively [nomenclature according to K. Biemann (*56*)].

subjected to LC/MS/MS analyses. It is noteworthy that only a minor radioactive peak[3] was observed eluting at ≈32 min in this native enzyme profile, too small for accurate structural analyses.

LC/MS/MS Analysis of the ^{32}P-Phosphorylated CYP3A4 Peptides. Phosphopeptide-containing HPLC fractions from Lys-C digests of ^{32}P-phosphorylated CuOOH-inactivated CYP3A4 (Figure 4B) were further fractionated on a reversed-phase nanocolumn, and the eluent was analyzed on-line by a QSTAR quadrupole-orthogonal-acceleration-time-of-flight (QoaTOF) tandem mass spectrometer operated in the IDA mode. During this analysis, 2 s survey acquisitions were followed by 5 s CID experiments for which the computer selected the most abundant ion in the survey as the precursor. The earlier eluting fraction yielded a series of different unmodified P450 peptides as well as the phosphorylated E_{258}-SRLEDTQK_{266} peptide. This peptide was represented by *m*/*z* 395.835 (3+) and 593.245 (2+) ions (Figure 5, upper panel).

[3] The relative radioactive content of this peak was found to be ≈20% of that of its counterpart in the CuOOH-inactivated Lys-C digests. We believe that this peak may arise from the PKC-mediated phosphorylation of a small fraction of the native enzyme that incurs thermal denaturation and consequent structural damage when it is consecutively incubated in parallel during the CuOOH inactivation (15 min) and phosphorylation (30 min) reactions.

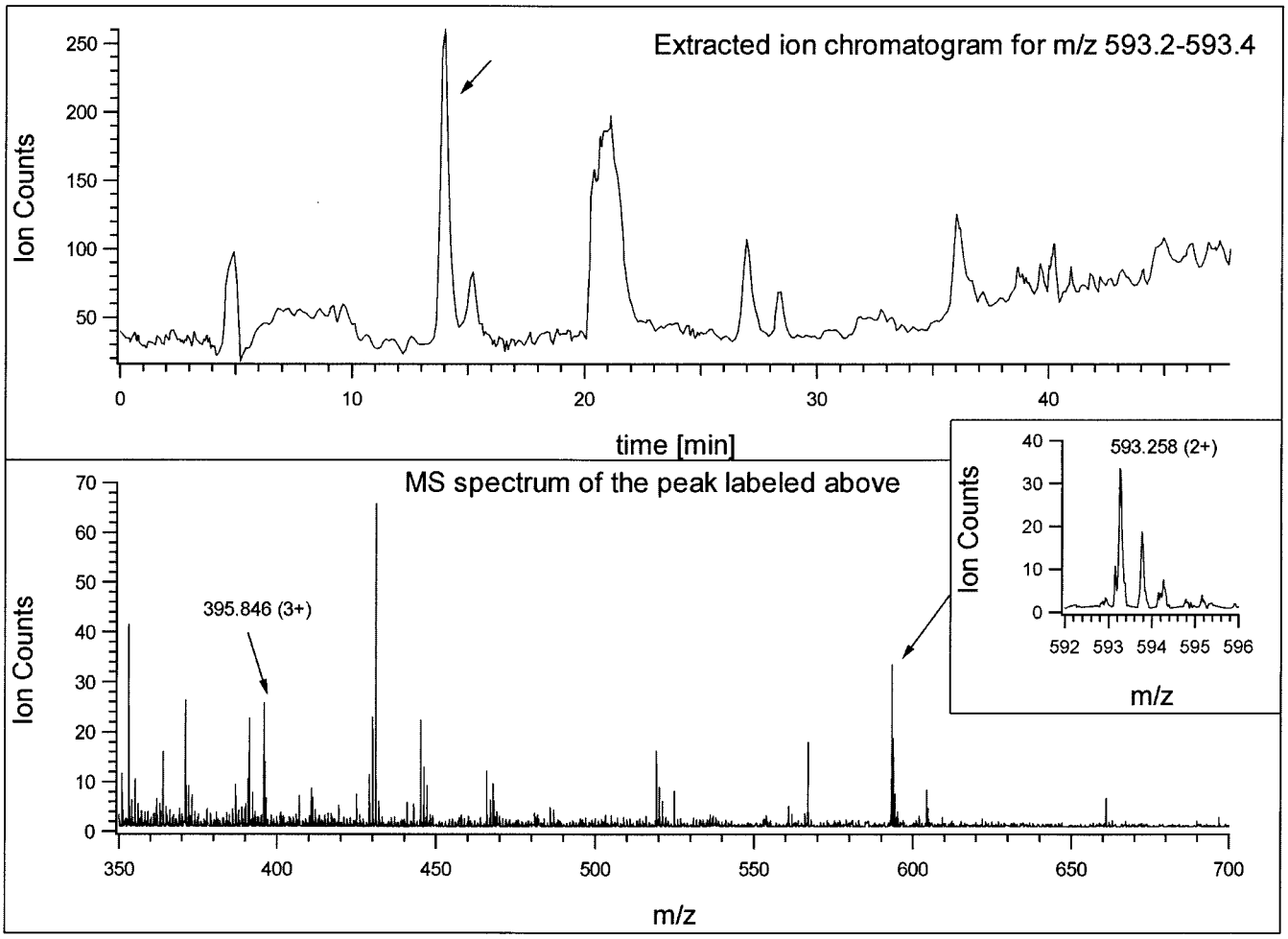

FIGURE 6: Extracted ion chromatogram (upper panel) and corresponding mass spectrum (lower panel) illustrating the presence of phosphorylated E_{258}SRLEDTQK_{266} in the native CYP3A4(His)$_6$ phosphorylated by PKC. Corresponding nano LC/MS data of the ^{32}P-phosphopeptide (eluting at ~20 min) from Lys-C digests of the native CYP3A4 phosphorylated by PKC are shown (upper panel). This peptide was represented by *m*/*z* 395.846 (3+) and 593.258 (2+) (lower panel with inset illustrating the resolution). MS/MS data (not shown) confirmed the identity of this peptide.

The MH^+ value calculated from these masses is 1185.4858 Da, that is within 25 ppm of the calculated 1185.5153 Da for this sequence. In the IDA analysis, the triply charged ion was automatically selected as the precursor ion for CID analysis. The CID data obtained confirmed the identity of the peptide, but did not provide sufficient information to assign the phosphorylation site (data not shown). Thus, the LC/MS/MS experiment was repeated using lower collision energy for the CID experiments and setting up the IDA acquisition so that only the ions representing the phosphopeptide would be selected for CID analysis. As shown in Figure 5 (middle panel), the N-terminal ions b_5 and b_6 were observed at the predicted masses for the unmodified sequence, indicating that Ser_{259} is not phosphorylated. Meanwhile, the masses of C-terminal fragment ions y_3 (middle panel) and y_4 (lower panel) show the 80 Da shift due to phosphate incorporation. Normally, both y and b fragments may undergo further fragmentation to lose H_2O or NH_3. In the case of phosphopeptides, β-elimination of the phosphate is usually favored, as observed for b_7 and b_8 (Figure 5, middle panel), as well as y_4 fragment ions (Figure 5, lower panel). Thus, CID spectra of both the doubly and triply charged ions provided sufficient information to unambiguously assign Thr_{264} as the site of phosphorylation.

Corresponding nano LC/MS data of the ^{32}P-phosphopeptide (eluting at ~20 min) from the native CYP3A4 phosphorylated by PKC are shown in Figure 6 (top panel). This peptide was represented by *m*/*z* 395.846 (3+) and 593.258 (2+) (Figure 6, lower panel with inset illustrating the resolution). MS/MS data (not shown), together with its relative HPLC coelution profile, confirmed the identity of this peptide as E_{258}SRLEDT(p)QK_{266}.

The first IDA LC/MS/MS analysis of the second HPLC fraction (elution time ~32 min) from Lys-C digests of ^{32}P-phosphorylated CuOOH-inactivated CYP3A4 did not yield any phosphopeptide CID spectra. All the abundant ions in this fraction represented unmodified P450 peptides. However, in survey acquisitions a minor component was detected at *m*/*z* 552.270 (2+). The corresponding $MH^+ = 1103.5322$ is within 3 ppm of the calculated value for phosphorylated F_{414}-LPERFSK_{421} peptide. Interestingly, the unmodified peptide was the major component of this fraction and eluted a few minutes earlier than its tentatively identified phosphorylated counterpart. The IDA experiment was repeated to select the doubly charged ions of the unmodified and phosphorylated species as precursors for CID experiments (Figure 7). The CID spectra acquired unambiguously confirmed the identity of the peptides with the detection of a few C-terminal ions

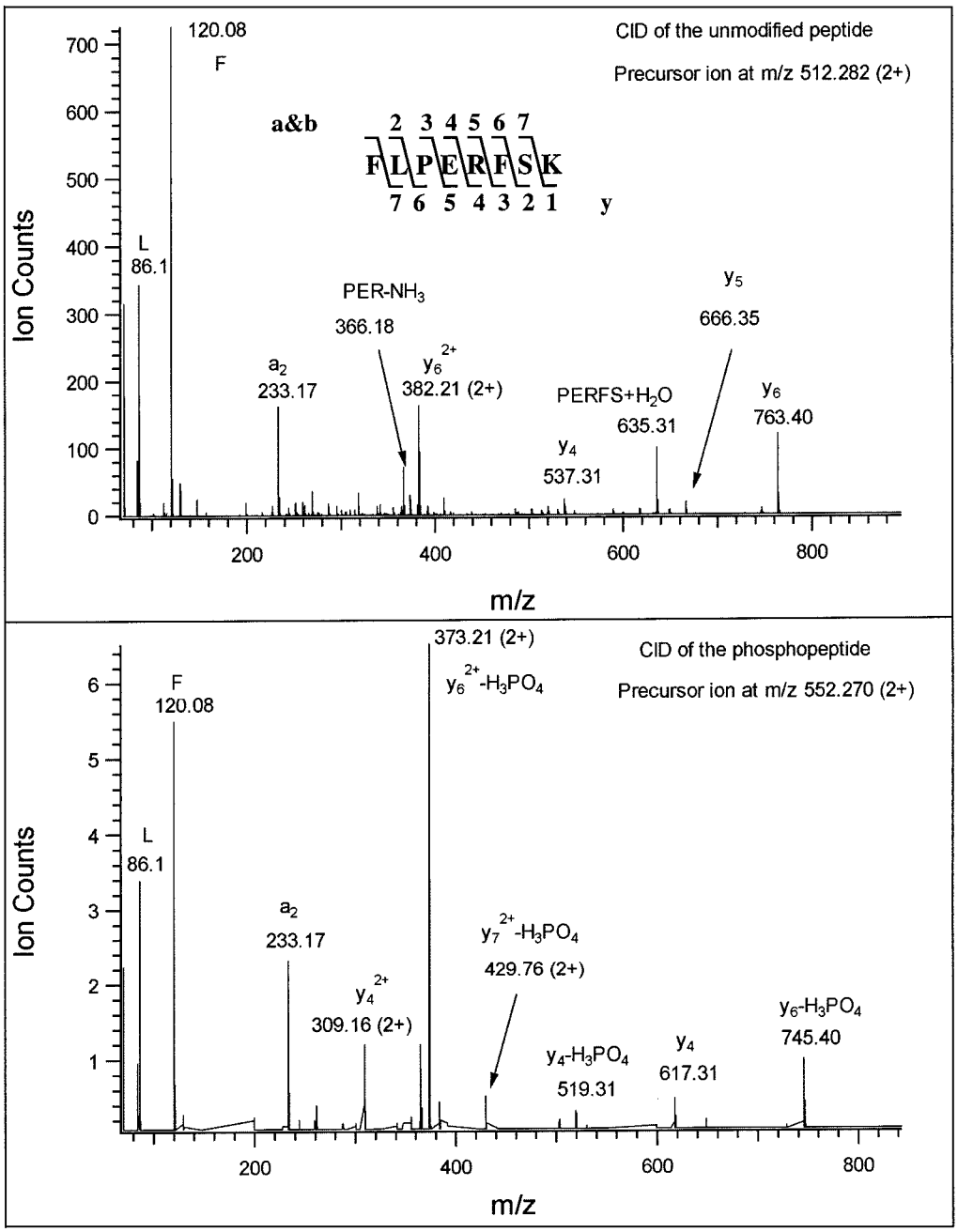

FIGURE 7: CID spectra acquired during an IDA LC/MS/MS analysis of the peptide eluting at ≈32 min. Data for the unmodified (upper panel) and phosphorylated (lower panel) peptide F_{414}LPERFSK$_{421}$ obtained from Lys-C digests of CuOOH-inactivated CYP3A4 are shown. See the text for details.

(y_4, y_5, and y_6), as well as the N-terminal ion a_2, which is formed via CO loss from its corresponding b ion. The site of phosphorylation is obviously Ser_{420}. Fragment y_4 (Figure 7, lower panel) indeed shows the 80 Da mass shift.

Summary and Conclusions. Kinase consensus sequence analysis of the entire CYP3A4 amino acid sequence revealed multiple putative sites for its phosphorylation by PKA, PKC, PKG, CaMKII, and casein kinase I and even a site for tyrosine (EGF-receptor) kinase (*48*). IDA-LC/MS/MS analyses of the PKC-^{32}P-phosphorylated CuOOH-inactivated or native CYP3A4 peptides identified the peptide eluting at 20 min as E_{258}SRLEDT(p)QK$_{266}$ and that eluting at 32 min as F_{414}LPERFS(p)K$_{421}$. This constitutes the first ever direct evidence of CYP3A4 phosphopeptides characterized by mass spectrometry. Phosphorylation of both these peptides is entirely consistent with the predicted PKC-recognition sequences. Sequence alignment of CYP3A4 with those of bacterial P450s of known crystal structures places the first sequence in a region linking helices G and H, whereas the second sequence lies in the meander region, between helix K″ and the Cys region containing the conserved heme-ligating Cys_{442} residue (Figure 8A). Examination of a homology model of CYP3A4 (*49*) reveals that the targeting of Thr_{264} by PKC is entirely feasible, given that it lies in an outwardly projecting loop that would be quite accessible to kinases in the cytosol or PKC in the medium, as the case may be. Thus, not surprisingly, this Thr_{264} residue is found to be predominantly phosphorylated not only in the inactivated CYP3A4 but also in the native enzyme (Figures 4−6). Interestingly, this exposed loop is also observed in the crystal structure of CYP2C5, the first membrane-bound enzyme to be structurally analyzed after crystallization (*50*, *51*). How-

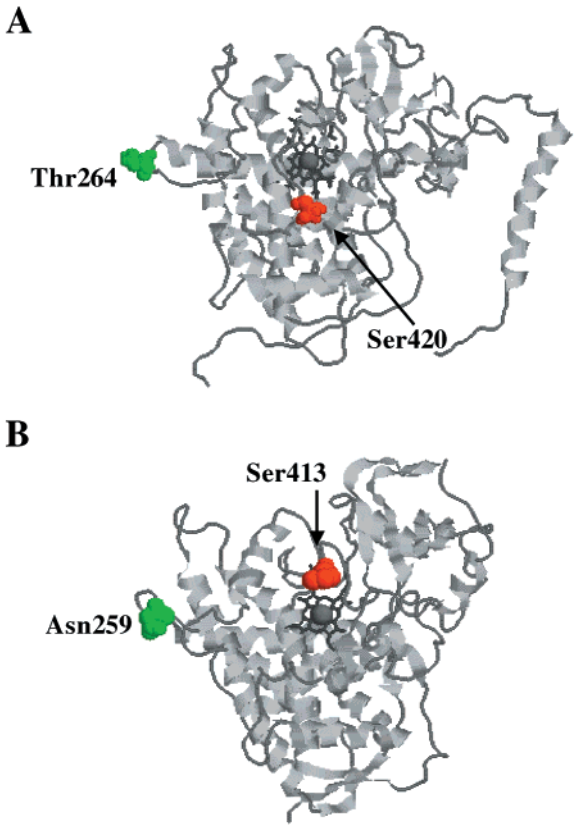

FIGURE 8: Structure depictions of CYP3A4 and CYP2C5. (A) Homology model of CYP3A4 structure based on a homology model (*49*) is shown. Thr_{264} is shown in green, and Ser_{420} in red. (B) Depiction of CYP2C5 based on the reported crystal structure (*50*), with the residue Asn_{259} (corresponding to Thr_{264} of CYP3A4) shown in green and Ser_{413} (corresponding to Ser_{420} of CYP3A4) in red.

ever, the alignment of the CYP3A4 and CYP2C5 sequences suggests that the residue corresponding to Thr_{264} of CYP3A4 is Asn_{259}, and no phosphorylatable Ser/Thr or Tyr residues are found in its immediate vicinity (Figure 8B). Other members of the CYP3A subfamily (CYPs 3A1, 3A23, and 3A5) lack the Thr residue corresponding to the CYP3A4 Thr_{264} residue phosphorylated by PKC, but contain either a Ser and/or a Thr (corresponding to CYP3A4 Ser_{259}) in this particular region (*52*).

Phosphorylation of Ser_{420} in the CYP3A4 meander region, on the other hand, is quite instructive. The residue corresponding to Ser_{420} of CYP3A4 is highly conserved throughout the 3A subfamily (*52*), and a Ser residue (Ser_{413}), although not directly aligned with Ser_{420} of CYP3A4, is also present in the meander region of CYP2C5 [(*50*); Figure 8B]. The corresponding meander region in the CYP2C5 crystal structure is believed to mimic CYP101 more closely than CYP102, but then apparently diverges from all bacterial structures by exhibiting a turn that harbors a sulfate or phosphate ion (*50*). Intriguingly, the Pro residue in this meander sequence, corresponding to Pro_{416} of CYP3A4, is highly conserved in numerous mammalian sequences and could account for the turn. However, this domain appears quite buried in the CYP3A4 structure, and it is unclear whether CuOOH-mediated inactivation of the enzyme results in structural damage that exposes Ser_{420} and other normally concealed phosphorylatable targets for PKC attack. This possibility is further strengthened by the finding that PKC predominantly phosphorylated the apparently more accessible Thr_{264}-containing peptide domain over that containing the relatively concealed Ser_{420} in the largely structurally intact native enzyme. We also find it noteworthy that this Ser_{420}, although not truly within a phosphorylation-prone PEST sequence (*53*), is bracketed between two pseudo-PEST sequences, and immediately precedes a potentially ubiquitinable Lys-Lys motif (*54*, *55*).

In summary, we have provided unambiguous mass spectrometric evidence for two peptides phosphorylated by PKC after CuOOH-mediated inactivation of CYP3A4, only one of which is phosphorylated in the native enzyme. The relevance of these CYP3A4 phosphopeptides to the Ub-dependent 26S proteasomal degradation of the enzyme remains to be determined. However, the experimental approach described herein, i.e., HPLC-peptide mapping coupled with LC/MS/MS, will undoubtedly be critical in our ongoing characterization of the posttranslational modifications of both the native and inactivated CYP3A4. Using this approach, studies are currently in progress to identify the corresponding peptides phosphorylated by an as yet to be identified cytosolic kinase, and to determine whether they are identical to the ones phosphorylated by PKC (and/or PKA) and whether such phosphorylation actually targets the enzyme for degradation via the Ub-26S proteasomal system.

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