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Evidence for phosphorylation requirement for human bilirubin UDP-glucuronosyltransferase (UGT1A1) activity

Nikhil K. Basu, Labanyamoy Kole, and Ida S. Owens*

Heritable Disorders Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892-1830, USA

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Abstract

Our discovery of rapid down-regulation of human bilirubin UDP-glucuronosyltransferase (UGT) in colon cell lines that was transient and irreversible following curcumin- and calphostin-C-treatment, respectively, suggested phosphorylation event(s) were involved in activity. Likewise, bilirubin-UGT1A1 expressed in COS-1 cells was inhibited by curcumin and calphostin-C. Because calphostin-C is a highly specific protein kinase C (PKC) inhibitor, we examined and found 4 to 5 predicted PKC phosphorylation sites in 11 UGTs examined. UGT1A1 incorporated [^{33}P]orthophosphate, which was inhibited by calphostin-C. Also triple mutant, T75A/T112A/S435G-UGT1A1, at predicted PKC sites failed to incorporate [^{33}P]orthophosphate. Individual or double mutants exhibited dominant-negative, additive, or no effect, while the triple mutant retained 10–15% activity towards bilirubin and two xenobiotics. Compared to wild-type, S435G and T112A/S435G shifted pH-optimum for eugenol, but not for bilirubin or anthraflavic acid, toward alkaline and acid conditions, respectively. This represents the first evidence that a UGT isozyme requires phosphorylation for activity.

Keywords: UDP-glucuronosyltransferase; Bilirubin; Phosphorylation; PKC; Curcumin; Calphostin-C

Although endoplasmic reticulum (ER)-bound UDP-glucuronosyltransferase (UGT) isozymes collectively detoxify a very broad range of metabolites, toxins, drugs, and environmental carcinogens by conjugation to glucuronic acid, the UGT1A1 isozyme plays an even more critical role because of its unique capacity to metabolize the lethal neurotoxin, bilirubin. Lipophilic-behaving bilirubin IXα requires conversion to water-soluble glucuronides for clearance from the body to prevent neonatal jaundice and neurotoxicity [1]. While other bilirubin isomers appear not to require conjugation for clearance when administered to animal models, the endogenously generated bilirubin 1Xα isomer undergoes internally hydrogen-bonding to form a lipophilic-behaving structure. Following transport from its site of synthesis in spleen [1], many early studies demonstrated the liver as the organ responsible for metabolizing bilirubin for clearance through the hepato-biliary system. Furthermore, substantial amounts of the heme derivative are synthesized daily, primarily, from salvaged heme recovered from the normal turnover of senescent red blood cells and less from inactive cytochrome P450 creating a continuous demand on UGT1A1 [1].

Upon cloning UGT1A1, deleterious defects in the isozyme of Crigler–Najjar (CN) Type I patients confirmed its critical role in severe hyperbilirubinemia [2]; less deleterious changes were shown to cause moderate and mild phenotypes associated with CN II disease and Gilbert's syndrome [3], respectively.

Although recent Northern blot and RT-PCR analyses [4,5] demonstrated the isozyme is also present in gastrointestinal (GI) tissues, we found UGT1A1 in a wide range of tissues and that, by in situ analysis, it is strategically located in the mucosa layers of GI and at very high levels in duodenum with less in the remainder of the GI tract (N.K. Basu et al., submitted). Also, UGT1A1 was found to glucuronidate not only bilirubin, but a vast selection of dietary chemicals, environmental carcinogens, and toxins [6–8]. While four different types

* Corresponding author. Fax: 1-301-480-8042.
E-mail address: owens@helix.nih.gov (I.S. Owens).

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of dietary chemicals, such as flavonoids, phytoestrogens, phenols, and anthraquinones (N.K. Basu et al, submitted) were found to be substrates, carcinogenic chemicals derived from the pyrolysis of cigarettes, wood, and petroleum [8] were also avidly metabolized by UGT1A1. Studies showing UGT1A1 is distributed in mucosal layers of the gastrointestines combined with its vast substrate selection suggest the isozyme also plays an important role in detoxifying and controlling uptake of potentially toxic polyphenols that are ingested on a regular basis. Hence, UGT1A1 is the most critical UGT isozyme because it plays a critical role in both the detoxification of neurotoxic bilirubin in the liver and a wide range of potentially harmful chemicals ingested and handled at the level of the GI tract.

Here, we provide evidence for the first time that a UGT isozyme, UGT1A1, requires phosphorylation for activity. While examining the effects of newly uncovered UGT1A1 substrates on activity in LS180 cells, we observed that curcumin rapidly and transiently down-regulated activity toward bilirubin and anthraflavic acid when measured in vitro. Furthermore, when UGT1A1-cDNA was expressed in transfected COS-1 cells, curcumin also rapidly down-regulated bilirubin activity. Also, we observed that the highly specific protein kinase C (PKC) inhibitor, calphostin-C [9], irreversibly inhibited activity. Among many other effects, curcumin was previously shown to be a general kinase inhibitor [10]. As suggested by calphostin-C inhibition, we carried out computer-based searches for predicted PKC phosphorylation sites in UGT1A1, as well as in 10 other UGTs, and found multiple consensus sites in each UGT. Further, we demonstrated that UGT1A1 expressed in COS-1 cells incorporated [^{33}P]orthophosphate, which was inhibited by calphostin-C treatment in parallel with inhibition of bilirubin and anthraflavic acid glucuronidation. The triple mutant, T75A/T112A/S435G, at predicted PKC sites did not incorporate detectable label and expressed only 10–15% normal activity. Single and double mutants expressed either dominant-negative, additive, or no effect on activity. Although UGT1A1 activity toward eugenol at pH 6.4 and 7.6 did not differ from those for bilirubin and anthraflavic acid, the overall pH profiles of T112A/S435G and the S435G-mutant when metabolizing eugenol, but not bilirubin and anthraflavic acid, showed a significant shift toward acid and basic conditions, respectively. The results indicate that UGT1A1 requires phosphorylation that enhances activity as much as sixfold and that it can affect pH optimum for catalysis for certain substrates.

Materials and methods

Cell culture. Human colon LS180 and HT29 cells, obtained from the ATCC (Manassas, VA), were grown in DMEM and McCoy's medium, respectively, with 10% fetal calf serum. Tissue culture medium was from Cellgro (Rockville, MD); bilirubin, anthraflavic acid, calphostin-C, cycloheximide, curcumin, and the MTT assay kit were from Sigma Chemicals (St. Louis, MO); and [^{33}P]orthophosphate was from NEN (Rockford, IL).

Treatment of cells with curcumin and calphostin-C. LS180 and HT-29 colon cells were treated with either curcumin or calphostin-C solubilized in newly opened dimethyl sulfoxide that did not exceed 0.5% in 80% confluent cells.

The glucuronidation assay. Conditions of the glucuronidating assay system have been described [11,12]. The common donor substrate, UDP-[^{14}C]glucuronic acid (1.41 mM, 1.4 μCi/μmol), was used in all reactions with an unlabeled acceptor substrate. Reactions were incubated for 1 or 1.5 h at 37 °C. UGT1A1 pH profiles used 0.04 M sodium phosphate from 5.0 to 7.0 and 0.04 M triethanolamine from 7.0 to 9.0. All products were resolved by thin-layer-chromatography [11] and quantified as previously described [12]. For protein estimations, we used the BCA kit (Pierce, Rockford, IL).

Western blot analysis of UGT expressed in LS180 or COS-1 cells. Either curcumin- or calphostin-C-treated LS180 or UGT1A1-transfected COS-1 cells were analyzed for relative UGT levels using an antiUGT preparation previously described [13]. Twenty-five μg of total cellular protein, solubilized in SDS-sample buffer and electrophoresed in a 7.5 or 12% polyacrylamide (PAGE)–SDS gel system, was electrotransblotted onto a nitrocellulose membrane and blocked with reconstituted nonfat dried milk. Following serial exposure of membranes to antiUGT, antirabbit-IgG-HRP, and X-ray film [13], each was scanned on a UMAX system and quantified using Adobe Photoshop software. Toxicity of curcumin and calphostin-C was monitored using the MTT assay.

Labeling of recombinant UGT1A1 in COS-1 cells with [^{33}P]orthophosphate. Sixty hours after UGT1A1 transfection [11], COS-1 cells were conditioned in phosphate-free and serum-free media as described [14] before exposure to [^{33}P]orthophosphate (5 mCi/ml) for 8 h. Radiolabeled UGT1A1 in solubilized cellular extracts was immunocomplexed with antiUGT immunoglobulin/protein-A–Sepharose, washed, and resolved in a SDS-7.5% polyacrylamide gel [15], which was fixed and exposed to X-ray film for development. Parallel preparations of unlabeled cells were harvested to determine activity toward bilirubin and anthraflavic acid.

Site-directed mutagenesis. Mutagenesis at amino acids 75, 112, and 435 in UGT1A1 was carried out using UGT1A1-specific oligonucleotides as described [12].For position 75, primers were: sense, 1A1PKC75S (5′-tac**g**ccttgaagacgtaccctgtgcc-3′) and antisense, 1A1PKC75AS (5′-ggc acagggtacgtcttcaagg**c**gta3′); for position 112, primers were: sense, 1A1PKC112S (5′cctgcagcgtgtgatcaaa**g**catacaaga-3′) and antisense, 1A1PKC112AS (tcttgtat**g**ctttgatcacacgctgcagg-3′); and primers for position 435 were: sense, 435S (5′-caaa**g**gttacaaggaga acatcatgcgcc-3′) and antisense, 435AS (5′-ggcgcatgatgttctccttgtaac **c**tttg-3′). All altered nucleotides were sequenced to verify specificity of mutations; normal and mutated cDNAs were inserted into the pSVL vector (Amersham Pharmacia Biotech, Piscataway, NJ) for expression in COS-1 cells for enzyme and other analyses.

Results and discussion

Typically, treatment of cell culture systems with various xenobiotics often elicits an increase in UGT activity over time. As UGT1A1 was shown to reside also in mucosal epithelia of GI, we treated colon cell lines with dietary chemicals for short-term to determine any immediate impact on bilirubin glucuronidating activity when analyzed in an in vitro assay. The dramatic down-regulation of bilirubin UGT activity in LS180 cells by

50 μM curcumin, a non-specific kinase inhibitor [10], that culminated at nearly 100 and 70% in LS180 and HT29 colon cell lines within 1 h with complete recovery by 24 h (Fig. 1B) was unexpected. Importantly, re-exposure of cells to curcumin caused the cycle to repeat (data not shown). Moreover, in vitro control experiments indicated there was no inhibitory effect on the glucuronidation assay; recombinant UGT1A1 showed a typical increase in activity with increasing concentrations up to at least 400 μM and the K_m (curcumin) was approximately 100 μM (data not shown). Also, Western blot analysis of extracts from the curcumin-treated LS180 cells over the 24 h period showed no detectable effects on UGT protein level (Fig. 1A). Additionally, inhibition of protein synthesis with cycloheximide treatment had no effect on UGT1A1 activity (Fig. 1A), indicating a substantial half-life. Among a number of other substrates examined, we also observed 50% inhibitory effects of nordihydroquaiaretic acid by 1 h in LS180 cells.

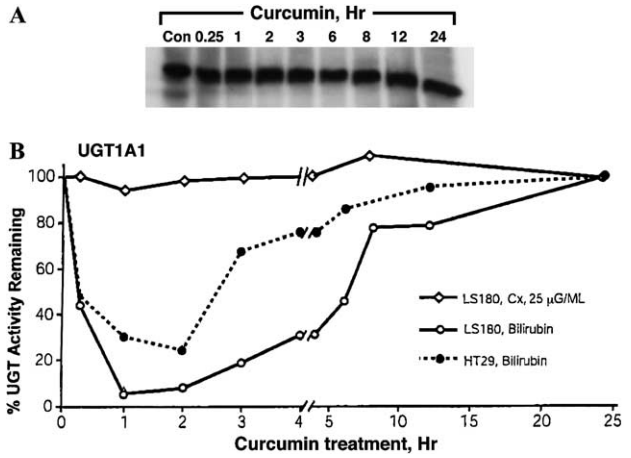

Fig. 1. Curcumin treatment of LS180 and HT-29 colon cell cultures. Western blot analysis of extracts from curcumin (50 μM)-treated LS180 cells over 24 h was resolved on a SDS–12% polyacrylamide gel electrophoresis system as described under Materials and methods (A). In-vitro bilirubin glucuronidating activity using 80% confluent LS180 and HT29 cells treated with 50 μM curcumin or 25 μg/ml cycloheximide over 24 h (B). Analysis was measured as described under Materials and methods. The experiment was repeated three times. Control bilirubin activity was 384 pmol/mg prot/1.5 h; standard errors ranged from 2% to 4%.

To determine whether curcumin affected *recombinant* UGT1A1, we examined concentration-dependent effects on UGT1A1-transfected COS-1 cells after 1 h of treatment using bilirubin and anthraflavic acid as substrates. The results showed concentration-dependent inhibition of both activities (Figs. 2B and C). Also, we found no effects of curcumin on specific protein level at the highest concentration (0.2 mM) used (Fig. 2A). Due to the cyclic nature of the inhibition by curcumin and the fact that the chemical was shown to be a non-specific kinase inhibitor [10], we examined the action of a number of kinase inhibitors on UGT activity. We observed that calphostin-C also caused concentration-dependent inhibition of recombinant UGT1A1 in COS-1 cells after 1 h of treatment in in vitro assays for each substrate (Figs. 2B and C). The effect of calphostin-C was significant because it is a highly specific PKC inhibitor [9]

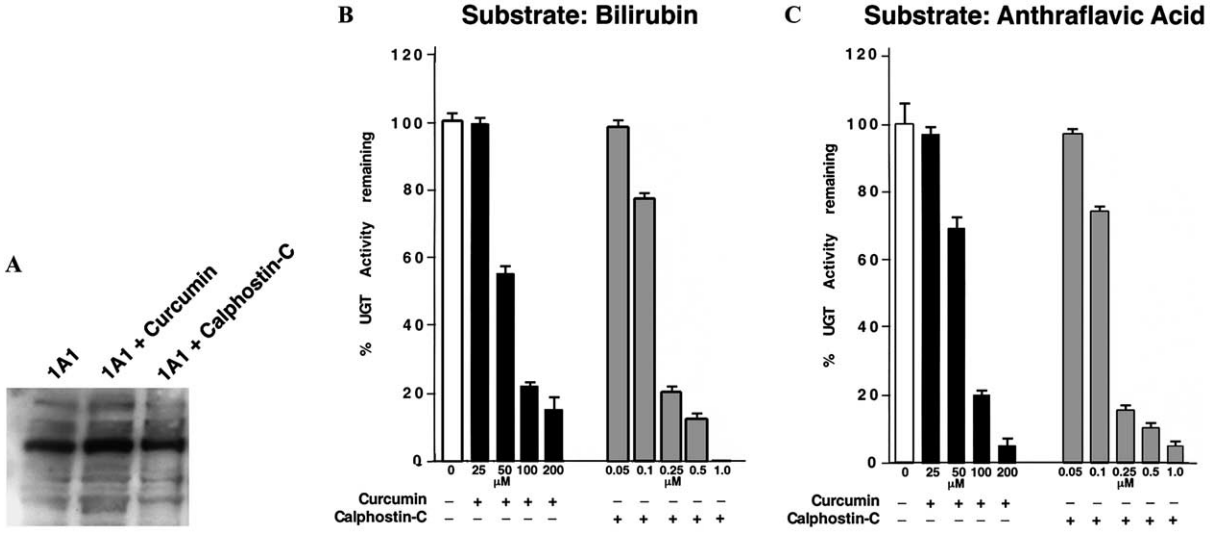

Fig. 2. Concentration-dependent inhibition of recombinant UGT1A1 in COS-1 cells by curcumin and calphostin-C. Western blot analysis of extracts of UGT1A1-transfected COS-1 cells that were incubated for 72 h and treated the last hour with 0.2 mM curcumin or 1.0 μM calphostin-C as described under Materials and methods (A). UGT1A1-transfected COS-1 cells, incubated as described in (A), were treated with different concentrations of curcumin or calphostin-C for 1 h and analyzed for both bilirubin (B) and anthraflavic acid (C) as described under Materials and methods. The experiments were repeated three times. Control bilirubin activity was 758 ± 22 pmol/mg protein/1.5 h; similar activity for anthraflavic acid was 3673 ± 212 pmol/mg protein/h. The experiment was repeated three times.

and the inhibition was irreversible. Simultaneously, Western blot showed no detectable change in UGT protein level by calphostin-C (1.0 μM) (Fig. 2A). Activity was much more sensitive to calphostin-C than curcumin, requiring only 1.0 μM versus 200 μM to achieve nearly 95% inhibition. By comparison LS180 cells required 50 μM of this dietary constituent to achieve 90% inhibition of bilirubin UGT activity (Fig. 1A). Although curcumin inhibition of bilirubin activity could be associated with non-specific kinase effects, calphostin-C-inhibition suggested phosphorylation event(s) involving PKC are essential for activity. The lower sensitivity of UGT1A1-transfected COS-1cells to curcumin, compared to LS180 cells, is possibly related to differences in sensitivity of PKC isozymes or to the presence of relatively higher levels of UGT1A1 in transfected-COS-1 cells versus that in LS180 colon cells. [Results of the bilirubin substrate activity in Fig. 2B {see legend (C): 758 pmol/mg prot/1.5 h} and Fig. 1B {legend: 384 pmol/mg prot/1.5 h} generated a 2:1 ratio for that in transfected COS-1 versus LS180 cells.] Also, it is possible that some other curcumin-insensitive and unidentified enzyme(s) are also involved in the phosphorylation process.

Under our experimental conditions, we did not detect any toxicity by curcumin or calphostin-C using the MTT assay kit. The lack of cellular toxicity by curcumin is in agreement with the lack of toxicity of this dietary agent seen in many other studies. Moreover, the agent has highly beneficial effects when used at moderately high [16] and high concentrations [17,18].

Because the highly specific PKC inhibitor, calphostin-C, inhibited UGT1A1 activity, we examined the proteins for consensus sequences for predicted PKC phosphorylation sites. A computer search using the Prosite program uncovered consensus sequences for predicting PKC phosphorylation sites in all nine UGTs encoded at the *UGT1* locus and in two isozymes examined which are encoded by *UGT2* genes (Fig. 3). Among the 35 cloned UGT cDNAs (www.unisa.edu.au/pharm_medsci/Gluc_trans/table1.htm), we examined 11 isozymes. Phosphorylation sites (S/TXK/R) for PKC isozymes

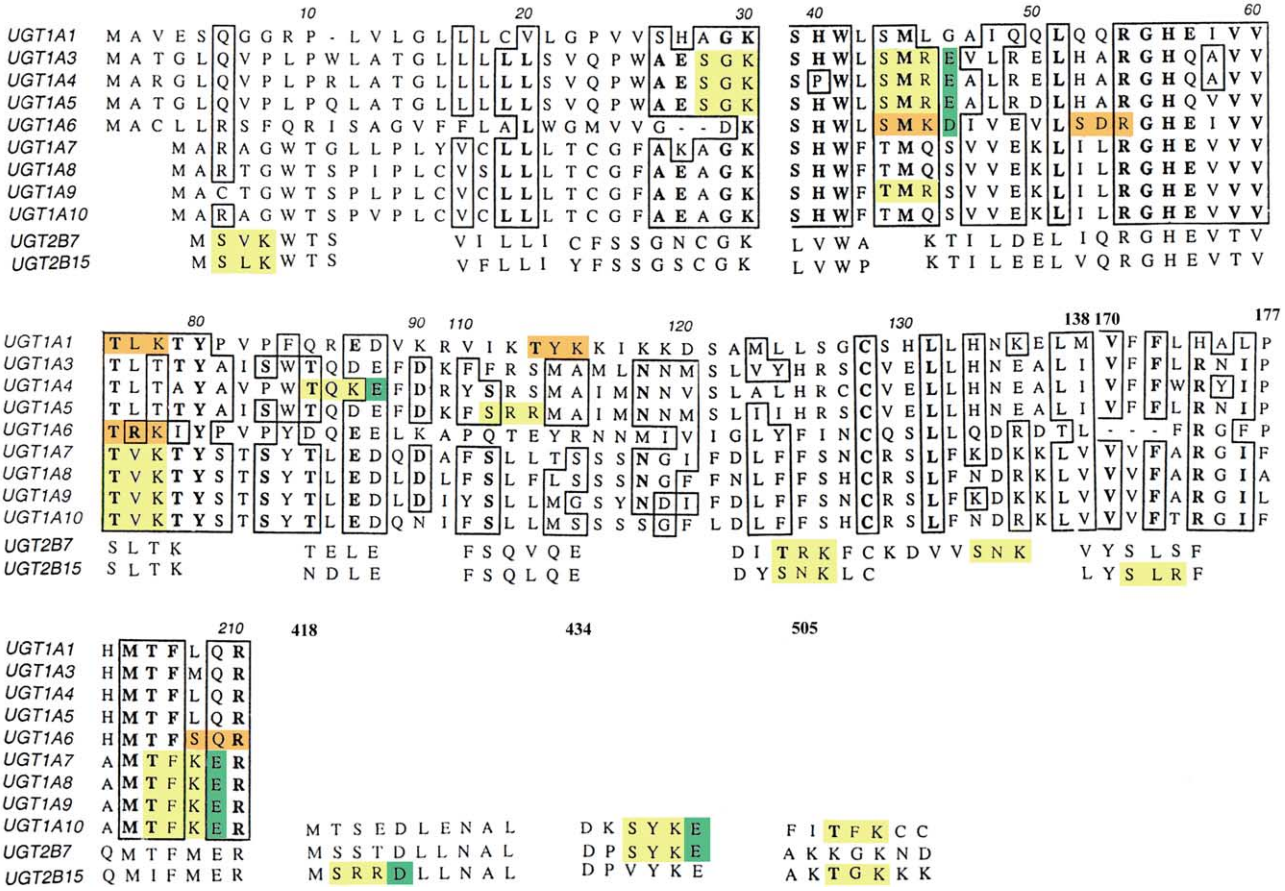

Fig. 3. Predicted phosphorylation sites in the nine UGT1-encoded and two UGT2B isozymes. Using the Prosite program, we carried out computer searches for phosphorylation sites on the nine *UGT1*-encoded isozymes (UGT1A1 and UGT1A3 through UGT1A10) and on two *UGT2B* encoded members, UGT2B7 and UGT2B15. The *UGT1*-encoded isozymes share a 246-residue common carboxyl end and, therefore, share the sites labeled 434 and 505. The colored consensus sequences, S/TXK/R and S/TXK/RD/E, are predicted phosphorylation sites for PKC isozymes and overlapping PKC/casein kinase, respectively.

exist in the common-end of the *UGT1*-gene family A at positions 432/434/435/436 and 503/505/506/507 out of 530–533 amino acid residues. In the unique amino terminus of the isoforms, potential sites are at: 75 and 112 for UGT1A1; 28 and 43 for UGT1A3 through UGT1A5 with an additional site at 85 for UGT1A4 and at 111 for UGT1A5; 41, 50, 74, and 206 for UGT1A6; and 73 and 202 for UGT1A7 through UGT1A10 with an additional site at 40 for UGT1A9. In the UGT2B family, similarly sequences are located at: 2, 123, 132, and 437 for UGT2B7 and at 2, 124, 172, 422, and 523 for UGT2B15. Each member of the UGT1 family, except for UGT1A1, has predicted overlapping PKC/casein kinase sites in S/TXK/RE/D. UGT1A6 has one predicted tyrosine phosphorylation site at position 191 (not shown); UGT2B7 and UGT2B15 have two such sites at positions 236/438 and 99/237 (not shown), respectively. Since a short signal peptide exists at the amino terminus of immature UGTs for targeting the proteins to the ER that undergoes cleavage to form the mature proteins [19], it is predicted that phosphorylation sites between 1 and 28 amino acids are unlikely to exist in the mature UGT. Further, the 90%-amino segment that is extramembranous and contains the catalytic domain of mature UGT is predicted to project into the lumen of ER. Because the catalytic domain is consecutive with a single membrane-anchoring domain, it is predicted that phosphorylation sites in the carboxyl-terminus beyond position 498 project into the cytoplasm and may not affect activity.

Because UGT1A1 is sensitive to both curcumin and calphostin-C suggesting the protein undergoes phosphorylation, we attempted to incorporate [^{33}P]orthophosphate into the recombinant isozyme in COS-1 cells. [^{33}P]Orthophosphate label was detected in the 52-kDa UGT1A1 resolved in an SDS–PAGE system after it was immunocomplexed with antiUGT [11] (Fig. 4A, panel 2). Furthermore, incorporation of label was blocked by calphostin-C treatment. The inhibition of labeling by calphostin-C that paralleled the loss of UGT1A1 activity for both bilirubin (Fig. 4A, panel 3) and anthraflavic acid (Fig. 4A, panel 4) suggests phosphorylation is required for catalytic activity.

To determine whether specific PKC phosphorylation sites in UGT1A1 affect bilirubin activity, we carried out site-directed mutagenesis at three predicted phosphory-

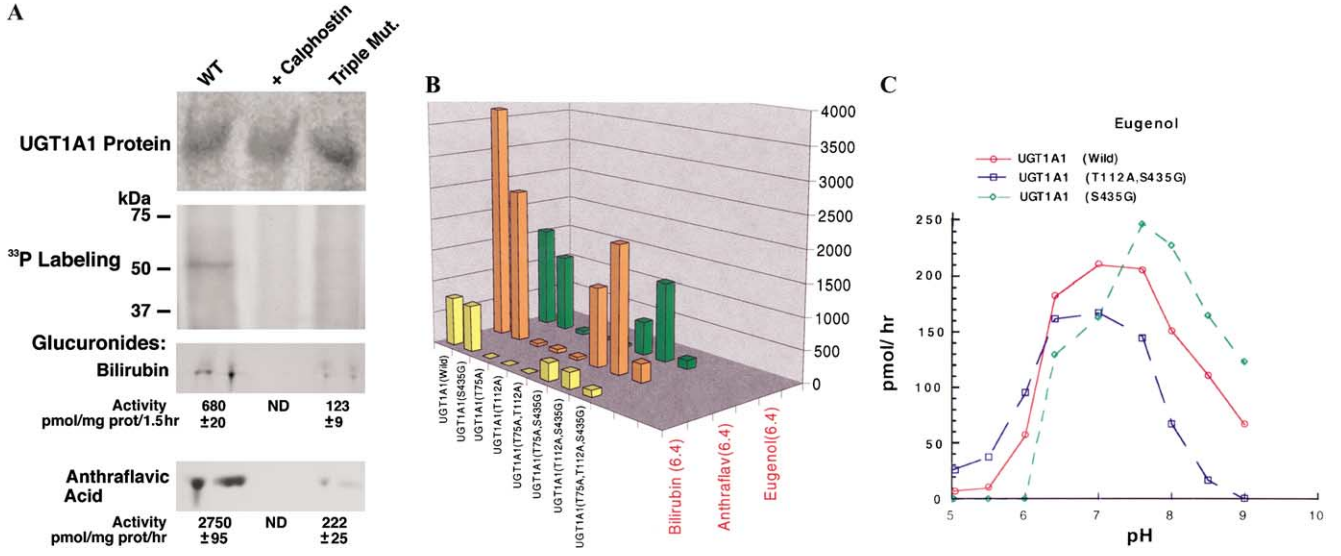

Fig. 4. Incorporation of [^{33}P]orthophosphate into UGT1A1 and its triple mutant and effects of calphostin-C (A). Sixty hours after transfection with UGT1A1 or its triple T75A/T112A/S435G-mutant, COS-1 cells were conditioned in phosphate-free and serum-free media before exposure to [^{33}P]orthophosphate (5 mCi/ml) for 8 h [14]. Additionally, 8-h-labeled UGT1A1-transfected cells were treated the last hour with 1.0 μM calphostin-C. Supernatants from solubilized cellular extracts containing equal protein were immunocomplexed with antiUGT/protein-A–Sepharose, washed, and resolved in a SDS–7.5% polyacrylamide gel [15] before staining with GELCODE blue. The stained gel was photographed (A, top panel) before drying as described under Materials and methods and exposing to X-ray film for 48 h to develop the image of different ^{33}P-labeled UGT1A1 proteins (A, second panel). Bilirubin and anthraflavic acid activity at pH 6.4 was carried out on parallel unlabeled cultures (A, bottom two panels) ND designates not detectable. The experiment was repeated three times using triplicate deteminations. Activity toward bilirubin, anthraflavic acid, and eugenol using single, double, and triple mutants of UGT1A1 that underwent site-directed mutagenesis at predicted PKC phosphorylation as described under Materials and methods (B). UGT1A1-cDNA and its mutants were transfected into COS-1 cells as described under Materials and methods. Cells were harvested after 72 h, normalized by Western blot for specific protein amount, and activity toward bilirubin, anthraflavic acid, and eugenol activity was determined. Activity is expressed as pmol/mg protein/h, except bilirubin is pmol/mg protein/1.5 h. The experiments were repeated three times using triplicate deteminations. Comparison of the pH profiles for eugenol glucuronidation by UGT1A1, S435G-UGT1A1, and T112A/S435G-UGT1A1 (C). Seventy-two hours after transfection with wild-type, S435G-UGT1A1, or T112A/S435G-UGT1A1 construct, COS-1 cellular extracts (100 μg protein) were used to analyze pH profiles for eugenol glucuronidation. Sodium phosphate (0.04 M) was used from pH 5.0 to 7.0 and triethanolamine (0.04 mM) was used from pH 7.0 to 9.0. The experiment was repeated three times. The standard errors ranged between 2 and 4% on normalized protein.

lation sites: T75A, T112A, and S435G. Although the S435G alteration had no detectable effect on bilirubin activity, either T75A or T112A alone or a combination of both mutations completely abolished glucuronidation of the heme metabolite, anthraflavic acid, and eugenol (Fig. 4B). Combinations of either T75A or T112A with S435G caused additive activity for the individual mutants for each substrate.

Finally, the triple mutant, T75A/T112A/S435G-UGT1A1, which failed to show detectable labeling with [^{33}P]orthophosphate (Fig. 4A, third lane), had only 10–15% of wild-type activity (Fig. 4A, bottom two panels and B). Hence, phosphorylation of UGT1A1 is critical for full expression of activity. Since our results demonstrate complete inactivation of UGT1A1 occurred with either T75A or T112A or a combination of both and that S435G had no detectable effect on catalysis, one can conclude that both phosphorylated threonines are equally critical to activity and that the phosphorylation status of serine-435 has less effect on the two xenobiotic substrate activities. Although S435G mutant alone did not affect activity, it is interesting that its activity as a double mutant with either T75A or T112A was additive with the dominant-negative effect expressed by the single mutant at either threonine site. Because of the apparent normalcy of activity for the S435G single mutant, one would expect dominant-negative effects for the double mutants of T75A or T112A and S435G. The additivity represents a detectable effect of S435G and indicates that the phosphate(s) on UGT1A1 are utilized in a hierarchical and/or combinatorial manner in controlling activity. The low activity for the triple mutant which exceeds either of the two dominant-negative single mutants—T75A and T112A—also supports a hierarchical and/or combinatorial role for the different phosphate groups.

Because a comprehensive study of substrate selections by five different UGT1 isozymes versus pH optima showed major variations that were isozyme-specific and substrate-dependent (N.K. Basu et al., submitted), we routinely analyze mutated isozymes to assess whether there are changes in pH optima. When we compared the pH profile of S435G-UGT1A1 with that for the wild-type for eugenol, we observed a significant shift of the entire profile to more alkaline conditions (Fig. 4C). In contrast, the lower activity by T112A/S435G-UGT1A1 showed a substantial shift towards more acid conditions. The pH optimum for conversion of bilirubin or anthraflavic acid by the mutants did not differ from that of the wild-type. Although the significance of pH shifts is not clear, the results suggest phosphate(s) play a role in controlling pH for catalysis in a substrate-dependent manner.

In conclusion, we have shown for the first time that a recombinant UGT, the bilirubin metabolizing UGT1-A1, undergoes phosphorylation and that this modification appears to be required for catalysis. Furthermore, the highly specific PKC inhibitor rapidly inhibits this event simultaneously with inhibition of activity. Calphostin-C inhibition of activity, along with the presence of predicted PKC phosphorylation sites in UGT1A1 that when mutated abolish or alter activity, also strongly indicates PKC is involved in this process. While we have preliminary evidence to suggest that other UGTs also require phosphorylation, studies are underway to determine whether PKC directly phosphorylates UGT and to determine whether signaling is involved in regulating the phosphorylation event(s). Future analysis on the effects of phosphorylation in other isozymes should more clearly demonstrate the role phosphate group(s) play in the catalytic process.

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