Protein Kinase Cα and Src Kinase Support Human Prostate-distributed Dihydrotosterone-metabolizing UDP-glucuronosyltransferase 2B15 Activity*\&\f

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Background: UDP-glucuronosyltransferase 2B15 (UGT2B15) is the only 5α-dihydrotosterone (DHT)-metabolizing enzyme in prostate luminal cells. UGT2B15 requires regulated phosphorylation to metabolize DHT. PKCα and Src support phosphorylation of UGT2B15 at five sites involving two signaling pathways and cross-talk. Strategically controlled DHT synthesis and complex UGT2B15 phosphorylation impose low DHT turnover and presumably homeostatic levels of DHT-occupied androgen receptor for prostate-specific functions.

Results: UGT2B15 requires regulated phosphorylation to metabolize DHT. PKCα and Src support phosphorylation of UGT2B15 at five sites involving two signaling pathways and cross-talk.

Conclusion: PKCα and Src support phosphorylation of UGT2B15 at five sites involving two signaling pathways and cross-talk.

Significance: Strategically controlled DHT synthesis and complex UGT2B15 phosphorylation impose low DHT turnover and presumably homeostatic levels of DHT-occupied androgen receptor for prostate-specific functions.

Because human prostate-distributed UDP-glucuronosyltransferase (UGT) 2B15 metabolizes 5α-dihydrotosterone (DHT) and 3α-androstane-5α,17β-diol metabolite, we sought to determine whether 2B15 requires regulated phosphorylation similar to UGTs already analyzed. Reversible down-regulation of 2B15-transfected COS-1 cells following curcumin treatment raised the issue of whether basal cells import or synthesize testosterone for transport to luminal cells for reduction to DHT by 5α-steroid reductase 2, comparatively low-activity luminal cell 2B15 undergoes a complex pattern of regulated phosphorylation necessary to maintain homeostatic DHT levels to support occupation of the androgen receptor for prostate-specific functions.

Several factors demonstrate that importance of 5α-dihydrotosterone (DHT) homeostasis to prostate health (1). Findings indicate adequate levels of DHT are essential for prostate growth, development, and differentiation, and elevated levels are associated with prostate diseases. Insufficient DHT levels cause developmental defects in the prostate, and conditions that lead to abnormally high DHT levels cause serious virilization syndromes, indicating that regulation of DHT levels is essential. To initiate prostate organogenesis, the urogenital sinus mesenchyme and urogenital sinus epithelium interact upon receiving signals from DHT-occupied androgen receptors in urogenital sinus mesenchyme/stromal cells, giving rise to basal and luminal epithelia (2). In preparation for DHT to reach prostate luminal cells (3) to support androgen-dependent functions, gonad-synthesized testosterone (4) or adrenal-synthesized dehydroepiandrosterone (DHEA) steroid precursor (4) is transported to prostate basal cells via blood (see Fig. 1A) (5). The fact that human basal cells contain appropriate steroidogenic enzymes detected by in situ hybridization analysis (6) suggests that the cells convert DHEA to testosterone. The one-to-one stratification of basal and luminal cells with intercellular gap-junction connections/structures seen in electron micrographs by El-Alfy et al. (6) (see Fig. 1A) provides indirect evidence that small-molecule exchanges occur between the two cell types and not between blood and luminal cells (6). More-

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The abbreviations used are: DHT, 5α-dihydrotosterone; DHEA, dehydroepiandrosterone; 5α-SR-2, 5α-steroid reductase 2; BPH, benign prostate hyperplasia; UGT, UDP-glucuronosyltransferase; TK, tyrosine kinase; PMA, phorbol 12-myristate 13-acetate.
over, the presence of 5α-steroid reductase 2 (5α-SR-2) primarily in luminal cells (7) suggests that testosterone is largely converted to DHT in luminal cells evidently following its movement from basal epithelia (4, 8) and that in situ synthesized DHT is the primary androgen source for occupation of the receptor. The DHT-occupied androgen receptor in luminal cells mediates all prostate-specific functions. The importance of testosterone reduction to DHT by 5α-SR-2 has been confirmed by malformation of prostate external genitalia during development due to type 2 enzyme deficiencies or in animals treated with 5α-SR-2 inhibitors (1) that lead to insufficient DHT levels.

For the first time, studies have described an alternative DHT biosynthetic pathway that bypasses testosterone in prostate cell lines (9) and intratumoral prostate tissues (10). Because 4-androstenedione derived from DHEA can be reduced at the 17-keto position to testosterone by a 17β-hydroxysteroid dehydrogenase or at the 5α-position to 5α-androstanediol by a 5α-SR, two consecutive reductive steps starting with 5α-androstanediol reduction by 5α-SR-1 over 5α-SR-2 and 17β-hydroxysteroid dehydrogenase generate DHT (see Fig. 1B). Analyses of intratumoral prostate tissues that ranged from hormone-naïve prostate cancer to castration-resistant prostate cancers of intratumoral prostate tissues that ranged from hormone-naïve prostate cancer to castration-resistant prostate cancer (9, 10). This finding strongly indicates that the emergence of this alternative pathway is causative in the development of case- tion-resistant prostate cancer (10). Hence, questions arise as to whether prostate DHT regulation is necessary complex and is interdependent upon the two epithelial cell types.

Concerning DHT dysregulation, any negative role DHT plays in prostate diseases was posed in recent studies to inquire whether prostate cancer and/or benign prostate hyperplasia (BPH) is reduced by preventive treatment with 5α-SR-2 inhibitors (11–13). Treatment with the specific inhibitor dutasteride led to a 23% reduction in DHT levels over 4 years (11) that was accompanied by improvement in the incidence of BPH with significant reductions in confirmed prostate cancer upon biopsy. These results indicate that reduced DHT levels in males, on average, positively affect both diseases. In addition, reduced androgen receptor levels (11) are consistent with the preventive efficacy of the 5α-SR-2 inhibitor. Combined, the studies have provided significant evidence that dysregulation of DHT levels is associated with BPH and prostate cancer (11–13).

The fact that prostate basal cells and other peripheral androgen target tissues (5) convert DHEA to DHT provides indirect evidence that enzymes dedicated to its synthesis are distributed locally. Moreover, studies have shown the UDP-glucuronosyltransferase (UGT) isozymes (14–16) that convert DHT and its 5α-androstan-3α,17β-diol metabolite to water-soluble derivatives are also locally distributed. UGT attaches either DHT or 5α-androstan-3α,17β-diol to glucuronic acid to produce glucuronides that undergo facilitated excretion. Cloning studies identified two human prostate-distributed UGTs, UGT2B15 (14) and UGT2B17 (15), that carry out this function. Immunohistochemical studies demonstrated that the 2B15 and 2B17 isozymes are distributed in prostate luminal and basal cells, respectively (16). COS-1 cells transiently expressing 2B15 (14) versus HEK293 cells stably expressing 2B17 (15) metabolize DHT and 5α-androstan-3α,17β-diol but at significantly different rates. A more recent quantitative PCR study showed that 2B15 is also widely distributed in other tissues, such as liver, bladder, breast, ovary, testis, and most gastrointestinal organs, compared with 2B17, which is more narrowly distributed in a few other tissues (stomach, colon, and testis) (17). The previously described 2B7 (18), not detected in prostate (19), was also shown to glucuronidate these same androgens (20). Distribution of low-activity DHT-metabolizing 2B15 in luminal cells, which require DHT-occupied androgen receptor to carry out prostate-specific functions (1–3), and distribution of robust DHT-metabolizing 2B17 activity in basal cells, without clearly defined function(s), necessarily prompt speculation that 2B15 has evolved such activity to maintain DHT-occupied androgen receptor complexes for this vital action. Moreover, electron micrographs that show a one-to-one stratified arrangement of basal/luminal cells attached to basement membrane in human prostate (Fig. 1) (6) support a model of exclusive movement of small molecules from blood to basal cells and, in turn, to luminal cells. These considerations indicate that prostate DHT regulation is necessary complex and is interdependent upon the two epithelial cell types.

Because ongoing in vitro studies (21–25), as well as evidence from an in vivo UGT model (26), have demonstrated each UGT requires regulated phosphorylation carried out by a different kinase, we sought to determine whether UGT2B15 is also dependent upon phosphorylation given that it has five phosphorylation sites: three PKC and two tyrosine kinase (TK) sites. Studies concerning the regulation of prostate-distributed UGTs that hasten DHT excretion from the body take on great importance because these preventive studies have indicated that elevated DHT is associated with prostate cancer and BPH development (11–13). Hence, questions arise as to whether phosphorylation of 2B15 likely impacts DHT clearance.

**EXPERIMENTAL PROCEDURES**

**Materials and Cell Lines**—The UGT2B15 cDNA was cloned and inserted into the pSVL vector (14). The His tag affinity ligand from the pCNA3.1 vector (Invitrogen) was fused in-frame at the 3′-end of pSVL-UGT2B15 cDNA (21, 22–25). Clones were expressed in COS-1 and Src/Yes/Fyn (SYF−/−) cells (American Type Culture Collection, Manassas, VA). Sources for all reagents used in tissue culture studies and the BCA protein assay kit were obtained as described (25). UGT substrates, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide kit, phorbol 12-myristate 13-acetate (PMA), anti-β-actin antibody, 1,25-dihydroxyvitamin D3, PP2, and kinase inhibitors (curcumin, calphostin C, bisindolylmaleimide, Gö 6976, and röttlerin) were from either Sigma or Calbiochem. PKCα and Src siRNAs were from Dharmacon. Anti-His antibody was from BIOSOURCE (Camarillo, CA). The development of the highly specific anti-UGT-1168 antibody has been described (21, 25). UDP-[14C]glucuronic acid (PerkinElmer Life Sciences) generated radiolabeled products in the glucuronidation assay, which were quantitated using the Cyclone storage phosphor imager (PerkinElmer Life Sciences) (21).
**Growth, Transfection, and Treatment of Cells**—COS-1 monkey kidney epithelia and SYF−/− mouse fibroblast cells were grown in DMEM with 4 and 10% fetal bovine serum, respectively. Transfection of cells was carried out with Lipofectamine 2000 at 16 h after plating as described (21). Reagents used in cell culture were solubilized in fresh dimethyl sulfoxide to allow a 0.5% final concentration. COS-1 cells were treated with curcumin, PKC inhibitors or activators, PP2, or Src siRNA at 70 or 24 h, respectively, after transfection, which continued as described in the figure legends. The viability of cells treated with curcumin, PKC, or Src effector agents was monitored using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay kit.

**Glucuronidation Assay**—Cellular extracts were analyzed for glucuronidation as described (25). The common donor substrate UDP-[14C]glucuronic acid (1.4 mM, 1.4 μCi/μmol) for all UGT isozymes was used in all reactions in conjunction with unlabeled dihydrotestosterone or its metabolite, 5α-androstan-3α,17β-diol, as the acceptor substrate. Incubation conditions are described in the figure legends or in the supplemental figure legends. Because of the low activity for 2B15, reactions were continued for 6 h at 37°C.

**Site-directed Mutagenesis of PKC and TK Phosphorylation Sites in UGT2B15**—2B15(S124A) mutations were inserted by DNA amplification using PCR and appropriate primers as follows: primers XhoI(7)S(5'-CTC GAG TCT AGA GAC CAG GAT GTC-3') and 2B15 364AS (5'-CAG AGC TTG TTA GCG TAG TC-3') for fragment 1 and primers 2B15 383AS (5'-CGT AAG CTC TG-3') and 2B15(SacI) 928AS (5'-CCA TTT CCA GAG TCC TGC ACA AAC T-3') for

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**Figure 1.** A, distribution of normal prostate steroidogenic and UGT isozymes according to the publications cited. Based on the studies cited, prostate DHT synthesis and its metabolism are summarized in a schematic. Electron micrographs show 1:1 stratification of human prostate basal/luminal cells with intervening gap junction structures that likely allow movement of small molecules between the two cells (6). Structural integrities allow the transport of the steroid precursor DHEA (5) from blood to basal cells, and not to luminal cells. Appropriate steroidogenic enzymes for DHEA conversion to testosterone are in basal cells (5, 6). The fact that 5α-SR-2 is detected primarily in luminal cells (7) suggests that testosterone is transported to luminal cells via gap junctions to undergo reduction to DHT to support DHT occupation of the androgen receptor to carry out prostate-specific functions. Whereas 2B15 is distributed in luminal cells (15) and it metabolizes the active DHT (14), it is likely that 2B15 generates both DHT- and 5α-androstane-3α, 17β-diol-glucuronides (ADT DIOL-G), which exit via gap junctions to basal cells for excretion into blood for facilitated excretion. Moreover, the entry of luminal cell-reduced testosterone (DHT) to basal cells allows 2B17 (15, 16) to participate in DHT metabolism, B, a recently described testosterone bypass pathway in cell lines (9) and intratumoral tissue (10) shows that 4-androstenedione (4-AD) is reduced consecutively by 5α-SR-1 and 17β-hydroxysteroid dehydrogenase (17BHSD) to DHT. Both 5α-androstanedione and DHT are interconvertible by 3α-hydroxysteroid dehydrogenases (3αHSD) to androsterone (ADT) and 5α-androstane-3α, 17β-diol (ADT DIOL), respectively. 5α-Androstanedione and DHT, as well as androsterone and 5α-androstane-3α, 17β-diol, are also interconvertible by 17β-hydroxysteroid dehydrogenases 2 and 3, respectively. CRPC, castration-resistant prostate cancer.
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fragment 2. The two fragments were joined in a third PCR using outer primers Xhol(−7) S and 2B15(Sacl) 928AS, which produced an ~940-bp fragment with mutation S124A. A fourth PCR was simultaneously carried out to generate the second part of the full-length cDNA (730 bp) using primers 2B15(Sacl) 901S (5′-AGT TTG TGC AGA GCT GTG GAG AAA ATG G-3′) and 2B15(Smal) 1624AS (5′-CCC CGG GGG CTG TTG ATA TAA CTA ATC TC-3′). The 940- and 730-bp fragments were joined by combination in a fifth PCR with the Xhol(−7) S and 2B15(Smal) 1624AS primers, which generated the 1670-bp full-length cDNA was digested with XhoI and SmaI and inserted into the XhoI- and SmaI-digested pSVL vector.

The second and third fragments were combined by joint PCR to introduce Ser422 in both fragments 1 and 2 again with the 2B15(SacI) 901S and 2B15(SmaI) 1624AS outside primers. The third PCR completed the synthesis of the 2B15- His construct was generated using pUGT1A7-His as a template, 2B15-His insertion of His Tag into UGT2B15 cDNA—The 2B15-His construct was made using WT 2B15 and pUGT1A7-His as templates. Initially, a PCR fragment was generated using primers XhoI(−7) S and 2B15(Sacl) 928AS. Then, a PCR fragment was synthesized using primers 2B15(Sacl) 901S and 2B15-thrombin-His AS (5′-CTC GGA TCA ATC TCT TTT C-3′). Subsequently, a third PCR fragment was generated using pUGT1A7-His as a template, 2B15-thrombin-His S (5′-GAA AAG AGA TCT GGC TTT TGC GGT GGC AAT ATG G-3′) and PMel-Smal AS (5′-GGT TTA AAC CCC GGG TCA ATG ATG ATG ATG-3′) with the His tag and a thrombin cleavage site after the 2B15 complete encoded message. This combined product was joined with fragment 1 using primers XhoI(−7) S and PMel-Smal AS. This 1730-bp full-length cDNA containing the His-coding sequence was digested with XhoI and SmaI before ligation into the pSVL vector previously digested with XhoI and SmaI to generate pSVL-UGT2B15-His with a His tag. 2B15(S124A)-His, 2B15(S172A)-His, 2B15(S422A)-His, 2B15(Y99F)-His, 2B15(Y237F)-His, and 2B15(S124A/S422A)-His constructs were also made simultaneously.

Western Blot Analysis of UGT2B15-expressing COS-1 Cells—Relative amounts of UGT in transfected COS-1 cells were established by Western blotting as described (21, 23–25). Twenty-five μg of solubilized cell homogenate was added to SDS sample buffer, applied to SDS-polyacrylamide gel, and subjected to electrophoresis. Following electrotransblotting of proteins onto nitrocellulose membrane, blots were processed as described previously (25) for exposure to x-ray films. In order, blots were exposed to antibody specific for PKCα and other PKC isozymes according to the suppliers' instructions, followed by the appropriate HRP-conjugated secondary antibody before visualizing the reactive pattern as described for anti-UGT antibody (21).

Co-immunoprecipitation of UGT2B15, Its Mutants, and PKCα—To carry out co-immunoprecipitation studies, 2B15-His- or mutant 2B15-His-expressing COS-1 cells were harvested and processed for immunoprecipitation using anti-UGT-1168 antibody as described previously (23–25). Immunoprecipitates were resolved in 4–15% gradient SDS-PAGE systems and transblotted as described for Western blot analysis using the antibodies indicated in the figure legends.

Insertion of His Tag into UGT2B15 cDNA—The 2B15-His construct was made using WT 2B15 and pUGT1A7-His as templates. Initially, a PCR fragment was generated using primers XhoI(−7) S and 2B15(Sacl) 928AS. Then, a PCR fragment was synthesized using primers 2B15(Sacl) 901S and 2B15-thrombin-His AS (5′-CTC GGA TGA TGG ATA TTA GGT G-3′) and 2B15(Y99F) 301AS (5′-TCG AAC GTA CAG GAA GGG-3′) to introduce the mutation at Ser72 substitution of the alanine codon in both fragments 1 and 2, respectively, with the XhoI(−7) S and 2B15(Sacl) 928AS outer primers. Fragments 1 and 2 were again combined in the third reaction using the outside primers, and the full-length cDNA was generated in the fourth and fifth reactions as described for 2B15(S124A). Similarly, 2B15(S422A) was generated using primers 2B15(Sacl) 1254S (5′-CAG GAC CAT GGG AAG TAG-3′) and 2B15(SacI) 1271AS (5′-CTA CTT CCC ATC ATG GTC CGT-3′) to introduce Ser422 in both fragments 1 and 2 again with the 2B15(Sacl) 901S and 2B15(Smal) 1624AS outside primers. Again, the third PCR completed the synthesis of the 2B15 cDNA with 2B15(SacI) 901S XhoI(−7) S and 2B15(Smal) 1624AS and the wild-type fragment (730 bp) as described for 2B15(S124A).

Insertion of His Tag into UGT2B15 cDNA—The 2B15-His construct was made using WT 2B15 and pUGT1A7-His as templates. Initially, a PCR fragment was generated using primers XhoI(−7) S and 2B15(Sacl) 928AS. Then, a PCR fragment was synthesized using primers 2B15(Sacl) 901S and 2B15-thrombin-His AS (5′-CTC GGA TCA ATC TCT TTT C-3′). Subsequently, a third PCR fragment was generated using pUGT1A7-His as a template, 2B15-thrombin-His S (5′-GAA AAG AGA TCT GGC TTT TGC GGT GGC AAT ATG G-3′) and PMel-Smal AS (5′-GGT TTA AAC CCC GGG TCA ATG ATG ATG ATG-3′) with the His tag and a thrombin cleavage site after the 2B15 complete encoded message. This combined product was joined with fragment 1 using primers XhoI(−7) S and PMel-Smal AS. This 1730-bp full-length cDNA containing the His-coding sequence was digested with XhoI and SmaI before ligation into the pSVL vector previously digested with XhoI and SmaI to generate pSVL-UGT2B15-His with a His tag. 2B15(S124A)-His, 2B15(S172A)-His, 2B15(S422A)-His, 2B15(Y99F)-His, 2B15(Y237F)-His, and 2B15(S124A/S422A)-His constructs were also made simultaneously.

In Vitro Phosphorylation of UGT2B15-His Expressed in Src-free Cells—To confirm that PKCα and/or Src phosphorylates 2B15, non-transfected and pSVL-2B15-His-transfected SYF/− fibroblast cells were cultured for 60 h before harvesting in PBS buffer as described previously (21). Cells were solubilized in 5 mM CHAPS, 100 μM Na2VO4, 5 mM NaF, and protease inhibitor mixture (Sigma) in 50 mM phosphate buffer (pH 7.8) as described previously (21, 25). Equal aliquots of Src-TK alone, PKCα alone, Src-TK and PKCα combined, or the appropriate buffer were distributed in reaction systems from a kit (Millipore) to which had been added either solubilized non-transfected or UGT2B15-transfected cellular protein and
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RESULTS

Relative Activity for UGT2B15(Asp85) and UGT2B15(Tyr85)—Considering that two different UGT2B15 alleles exist in the population containing either Asp85 or Tyr85 due to a SNP (29), UGT2B15(Asp85) (14) was analyzed in this study.

Effect of Curcumin Treatment on UGT2B15 Activity—Whereas curcumin treatment of 10 different recombinant UGT isoforms expressed in cell culture led to the observation that each was differentially inhibited (25), further analysis of UGT1A1 (23), UGT1A7 (24, 25), UGT1A10 (24, 25), and UGT2B7 (21) revealed that each required regulated phosphorylation carried out by a different kinase. Hence, it was of interest to extend the curcumin studies by focusing on the predicted phosphorylation sites in luminal cell-distributed 2B15, which is critical for DHT homeostasis in this key cell, which carries out regulated phosphorylation sites in luminal cell-distributed 2B15, which is critical for DHT homeostasis in this key cell, which carries out regulated phosphorylation carried out by a different kinase. Hence, it was of interest to extend the curcumin studies by focusing on the predicted phosphorylation sites in luminal cell-distributed 2B15, which is critical for DHT homeostasis in this key cell, which carries out regulated phosphorylation.

Effect of PKC Agonist PMA on UGT2B15 Activity—Because earlier studies demonstrated that UGT2B7 has three nonessential PKC sites (33) but two required TK phosphorylation sites, which undergo reversible down-regulation by curcumin, we examined whether the classical PKC agonist PMA (25) activates 2B15. Similar to other PKC-dependent systems (25), 2B15 was stimulated optimally by 100 nM PMA.

Effect of Mutations at Predicted Phosphorylation Sites in 2B15—To establish whether any of the three PKC sites or two TK sites in 2B15 is required, we carried out site-directed mutagenesis of the PKC and TK phosphorylation sites in 2B15. Ala was substituted for Ser at positions 124, 172, and 422, and Phe replaced Tyr at positions 99 and 237. Examination of the mutants generated a range of effects that were highly to barely significant. The S172A mutant was null, whereas the S124A mutant was 60 and 80% inactive toward DHT and 5α-androstan-3α,17β-diol, respectively (Fig. 3). In contrast, the 2B15(S422A) mutant activity hardly changed or was ~20% more active than wild-type 2B15 (Fig. 3). Tyrosine mutants 2B15(Y99F) and 2B15(Y237F) were 80 and 90% inactive, respectively (Fig. 3), providing the first evidence these sites in 2B15 require regulated phosphorylation, based on the reversibility of curcumin inhibition that was independent of protein synthesis. All mutants and wild-type 2B15 were under conditions of equal protein for 2B15 and β-actin as shown by Western blot analysis (Fig. 3, lower).

Co-immunoprecipitation of PKCα with UGT2B15-His Its Mutants—To establish the PKC isozyme(s) involved in 2B15-Dependent phosphorylation, we carried out screens with antibodies against different PKC isoforms on a Western blot that contained co-immunoprecipitates of wild-type 2B15-His and 2B15-His mutated at different PKC sites (S124A, S172A, or S422A) and the partially inactive double mutant S124A/S422A. Immunoprecipitates were captured with anti-UGT-1168 anti-
body (Fig. 4A) (21, 24) under conditions of equal UGT-specific protein (Fig. 4B). The results show that 2B15-His migrated to 55–58 kDa (Fig. 4A, lower) and that PKCα migrated to 82 kDa (Fig. 4A, upper) in the gel, whereas WT 2B15-His or its mutant co-migrated as a complex with PKCα to the 130–140- and >150-kDa positions following expression of the 2B15 protein in COS-1 cells. Immunoprecipitation of PKCα-containing complexes using anti-UGT-1168 antibody indicated that PKCα was initially trapped as a complex that disassociated during electrophoresis. Hence, the results indicate that less than half of PKCα trapped with anti-UGT-1168 antibody by the mutants remained associated with the complexes during electrophoresis. Although a substantial amount of PKCα and 2B15(S124A)-His co-migrated to the 130–140-kDa position, the majority of PKCα trapped by the completely inactive mutant dissociated and remained at the 82-kDa position (Fig. 4A). In contrast, the 2B15(S124A) mutant, which was more active than 2B15(S172A), was the least effective at trapping complexes. The double mutant construct 2B15(S124A/S422A) showed some recovery in the amount of the 130–140-kDa complex compared with the single 2B15(S124A) mutant (Fig. 4A). All comparisons of complex formation and stability were made under conditions of equal specific proteins as shown in Fig. 4B. Based on the results of the activity and amount/quality of UGT2B15 and PKCα complexes that were trapped by co-immunoprecipitation and that remained complexed during electrophoresis, our findings indicate that 2B15 phospho-Ser124 is the most critical for the stability of both the 130–140- and >150-kDa complexes.

**Effect of PKCα siRNA on UGT2B15-His Activity**—The finding of only PKCα bound to 2B15-containing complexes in Fig. 4A out of the five different PKC isozymes examined provides strong evidence that PKCα is primarily responsible for phosphorylating PKC site(s) in 2B15. Furthermore, the dramatic reduction in 2B15 activity following treatment with increasing concentrations of PKCα siRNA (Fig. 5, upper), which mimicked the decreases in PKCα protein levels (Fig. 5, lower), is consistent with PKCα carrying out the required phosphorylation of PKC sites in 2B15. Hence, the reductions in 2B15 activity following PKCα siRNA treatment confirm that PKCα plays an important role in 2B15 activity.

**Effects of Src Kinase Effector Agents 1,25-Dihydroxyvitamin D₃, Src-specific PP2 Inhibitor, and Src Kinase siRNA on UGT2B15 Expressed in COS-1 Cells**—To assess whether Src has a positive effect on 2B15 activity, we treated 2B15-expressing COS-1 cells with the Src activator (27, 28) 1,25-dihy-
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droxyvitamin D$_3$. The results show that the agent (2 and 5 nM) caused the typical rapid increase in 2B15 activity, reaching 1.5- and 2.0-fold between 2 and 5 min, without affecting 2B15, Src, or β-actin protein levels (supplemental Fig. S3). Using other typical Src effector agents, we determined that the Src-specific inhibitor PP2 inhibited 2B15 activity in a concentration-dependent manner that reached 50% at 10 μM (Fig. 6A, upper) without affecting general or specific protein levels (Fig. 6A, lower). Finally, treatment of 2B15-transfected COS-1 cells with increasing concentrations of Src siRNA versus non-target siRNA caused a 50–70% decrease in 2B15 activity (Fig. 6B, upper) in parallel with diminution of Src protein but without affecting 2B15 or β-actin protein (Fig. 6B, lower). These combined results clearly demonstrate that Src kinase has positive effect(s) on 2B15 activity.

In Vitro Evidence That PKCα and Src Phosphorylate UGT2B15-His—To assess whether PKCα and/or Src directly phosphorylates 2B15-His, we solubilized 2B15-His-transfected or non-transfected SYF$^{−/−}$ cell homogenates and subjected them to phosphorylation by adding [$γ$-32P]ATP or buffer as described under “Experimental Procedures” and previously (21, 25). Following phosphorylation and His affinity purification as described previously (21, 25), samples were resolved in a 4–15% gradient SDS-PAGE system. PKCα incorporated >2-fold greater phosphate in 2B15-His compared with Src kinase (Fig. 7). Moreover, the combination of the two kinases incorporated additive levels of [$γ$-32P]ATP-dependent label into 2B15-His (Fig. 7). There was very little phosphorylation without the addition of either kinase to the cell homogenate (Fig. 7, lane 2). Hence, the pattern indicates that the two kinases bring about additive phosphorylation of 2B15 predictably involving the phosphorylation sites already identified. The fact that the four mutants at Tyr$^{99}$, Ser$^{124}$, Ser$^{172}$, and Tyr$^{237}$ lost activity suggests that each of the phosphorylated sites positively affects activity compared with the S422A mutant, which was unchanged or was barely up-regulated, suggesting that it moderately down-regulates 2B15 activity.

DISCUSSION

For the first time, we have evidence to indicate that a UGT isozyme undergoes regulated phosphorylation by two different kinases. Both PKCα and Src kinase phosphorylate UGT2B15, which is consistent with the fact that the isozyme contains three PKC and two TK phosphorylation sites by computer analysis. Inhibition of 2B15 activity by typical PKC inhibitors (curcumin, calphostin C, bisindolylmaleimide, Gö 6976, and röttlerin) and activation by PMA support the claim that PKC phosphorylates the isozyme. The fact that individual mutations in the 2B15 isozyme at two of the three predicted PKC phosphorylation sites or at two of the two TK sites ranged from null to 30% active provides strong evidence that 2B15 requires both PKC and TK phosphorylation. Moreover, the reversible inhibition by curcumin that was unaffected by cycloheximide co-treatment indicates that 2B15 undergoes regulated phosphorylation that involves signaling.

Concerning the PKC isozyme(s) responsible for phosphorylating 2B15, the fact that anti-UGT-1168 antibody co-immuno-precipitated wild-type 55–58-kDa 2B15-His and 82-kDa PKCα in 130–140- and >150-kDa complexes in solubilized 2B15-His-transfected COS-1 cells, but not in non-transfected cells, provides strong evidence that PKCα phosphorylates 2B15. In addition, the major decreases in 2B15 activity upon PKCα siRNA treatment support our claim that PKCα phosphorylates 2B15. Contrariwise, finding that 1,25-dihydroxyvitamin D$_3$ treatment of 2B15-expressing COS-1 cells elicited the typical rapid activation of Src-TK (27, 28) manifested as increases in 2B15-dependent DHT metabolism provides the first evidence that Src also phosphorylates 2B15. Notably, the 1,25-dihydroxyvitamin D$_3$ activation of 2B15 is similar to the results in our earlier study in which treatment of LS180 cells with 1,25-dihydroxyvitamin D$_3$ elicited a rapid activation of 2B7, which represents the first indication that 2B7 requires regulated phosphorylation by Src. (The study showed that Src participates in dictating 2B7 endogenous substrate selections (21).) Again considering the predicted 2B15 TK phosphorylation sites, inhibition or down-regulation of 2B15 activity by Src-specific effector agents (PP2 and Src siRNA) provides the first strong evidence that 2B15 also undergoes TK phosphorylation by Src. Hence, the fact that 2B15 undergoes inhibition by both sets of inhibitors and that it is down-regulated by both PKCα and Src

5 S. K. Chakraborty, N. K. Basu, S. Jana, M. Basu, A. Raychoudhuri, and I. S. Owens, unpublished data.
Phosphorylation of Dihydrotestosterone-metabolizing UGT2B15

Control of 2B15 activity by phosphorylation at both PKCa and Src TK sites suggests that the requirement signifies a complex signaling pathway(s) that involves activation and/or inhibition coordinated via cross-talk (34). Whereas the major 2B15 substrate, DHT, is the active steroid that drives prostate specific functions, it is necessary that 2B15 is subjected to careful regulation to avoid DHT insufficiencies or deleterious excesses. Hence, it is possible that complex 2B15 regulation directly anchors metabolism of the active androgen so as to prevent rapid deleterious changes in DHT levels in either direction. This is reminiscent of pathways involving both PKC and Src in relationship to DHT control of prostate cell proliferation (35). Although it is not possible to determine the contribution of each phosphorylated site in 2B15, we have demonstrated that phospho-Ser124 is involved in the stability of 2B15.6

Considering the publications cited (5–7) concerning DHT synthesis in the prostate (see Fig. 1A), the distribution of 5α-SR-2 (7) in luminal cells to reduce testosterone to DHT for occupation of the androgen receptor to carry out prostate-specific functions is likely made possible by 2B15-regulated phosphorylation that imposes low turnover to make DHT homeostasis possible. Providing transport is not encumbered, it is possible that excess DHT is transported via gap junctions into basal cells to undergo rapid glucuronidation by 2B17.7 Importantly, 5α-SR-2 reduction of testosterone to generate DHT appears necessary to prevent castration-resistant prostate cancer development compared with 4-androstenedione reduction by 5α-SR-1 in the recently described testosterone bypass pathway (Fig. 1B) (9, 10).

6 S. Jana, N. K. Basu, A. Raychoudhuri, and I. S. Owens, manuscript in preparation.
7 M. Basu, S. Jana, N. K. Basu, and I. S. Owens, manuscript in preparation.
Also, we recall the preventive treatment studies cited in the Introduction (11–13) that demonstrated that lowering DHT levels in males by lowering the “rate” of testosterone reduction to DHT by inhibitors of 5α-SR-2 significantly reduced the incidences of prostate malignancies, as well as BPH. The finding that luminal cell-distributed 2B15, which is evidently relevant in maintaining constitutive DHT levels for prostate-specific functions, is controlled by an evident complex set of factors involving two different kinases suggests that it is essential to determine the pivotal regulatory cross-talk that dictates the rate of DHT glucuronidation. Moreover, previous studies (6, 7) provide evidence that the stratified prostate epithelial cell types are structurally interconnected, enabling participation of the robust basal cell-distributed 2B17 activity. Whether this connectivity is viable is also relevant.

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