Regulated Phosphorylation of a Major UDP-glucuronosyltransferase Isozyme by Tyrosine Kinases Dictates Endogenous Substrate Selection for Detoxification

Whereas UDP-glucuronosyltransferase-2B7 is widely distributed in different tissues, it preferentially detoxifies genotoxic 4-OH-estradiol and 4-OH-estrone (4-OHE1) with barely detectable 17β-estradiol (E2) conversion following expression in COS-1 cells. Consistent with the UDP-glucuronosyltransferase requirement for regulated phosphorylation, we discovered that 2B7 requires Src-dependent tyrosine phosphorylation. Y323F-2B7 and Y438F-2B7 mutants were null and 90% inactive, respectively, when expressed in COS-1. We demonstrated that 2B7 incorporated immunoprecipitable [33P]orthophosphate and that 2B7His, previously expressed in SYF-(Src,Yes,Fyn)−/− cells, was Src-supported or phosphorylated under in vitro conditions. Unexpectedly, 2B7 expressed in SYF−/− and SYF+/− cells metabolized 4-OHE1 at 10- and 3-fold higher rates, respectively, than that expressed in COS-1, and similar analysis showed that E2 metabolism was 16- and 9-fold higher than in COS-1. Because anti-Tyr(P)-438–2B7 detected Tyr(P)-438–2B7 in each cell line, results indicated that unidentified tyrosine kinase(s) (TKs) phosphorylated 2B7 in SYF−/−. 2B7-transfected COS-1 treated with increasing concentrations of the Src-specific inhibitor PP2 down-regulated 4-OHE1 glucuronidation reaching 60% maximum while simultaneously increasing E2 metabolism linearly. This finding indicated that increasing PP2 inhibition of Src allows increasing E2 metabolism caused by 2B7 phosphorylation by unidentified TK(s). Importantly, 2B7 expressed in SYF−/− is more competent at metabolizing E2 in cellulo than 2B7 expressed in COS-1. To confirm Src-controlled 2B7 prevents toxicity, we showed that 2B7-transfected COS-1 efficiently protected against 4-OH-E1-mediated depurination. Finally, our results indicate that Src-dependent phosphorylation of 2B7 allows metabolism of 4-OHE1, but not E2, in COS-1, whereas non-Src-phosphorylated 2B7 metabolizes both chemicals. Importantly, we determined that 2B7 substrate selection is not fixed but varies depending upon the TK(s) that carry out its required phosphorylation.

Mammalian UDP-glucuronosyltransferase (UGT) isoforms constitute a chemical defense system that detoxifies an enormous number of lipophilic substrates. Each isoform links an acceptor substrate to glucuronic acid to produce glucuronides with greatly increased water solubility and excretable. The endoplasmic reticulum-bound superfamily of UGT isoforms protects biological systems from toxic metabolites and ingested chemicals (1) encountered on a regular basis in dietary plants and environmental contaminants (2). To a detriment, UGTs clear many medications prematurely (3, 4). Isoforms are distributed primarily in hepatic tissues and differentially in a wide range of other tissues (2, 5). In the case of impaired UGT isozymes, lipophiles accumulate in tissues causing deleterious effects that include hepatotoxicities, developmental defects, mutagenesis, as well as initiation and promotion of cancer (6). Inheritance of defective bilirubin-conjugating isozyme (7) in Crigler-Najjar (UGT1A1−/−) children cause high serum bilirubin levels that deposit in the central nervous system, causing kernicterus.

Except for UGT1A10, studies reveal that UGT’s poorly metabolize primary estrogens (8, 9), E1 and E2. To the contrary, cytochromes 1B1 and 1A1/1A2 primarily generate the catechol-estrogen (CE) metabolites (for review see Ref. 10). 4-OHE/E1 and 2-OHE/E1, respectively, that undergo extensive glucuronidation (11, 12). Highly genotoxic 4-OHE1 and 4-OHE2 are preferred 2B7 substrates, and minimally toxic 2-OHE2 and 2-OHE1 are also O-methylated (10, 13). Genotoxic 4-OHE2 and 4-OHE1 are associated with the initiation of breast cancer (14). Without detoxification, 4-OHE2 and 4-OHE1 compared with 2-OHE2 and 2-OHE1 undergo extensive redox cycling as semiquinone(s)-quinone(s) (14) producing superoxide anions (O2−) that depurinate DNA-forming adducts that undergo excision, leaving behind abasic sites (14). Often an error-prone base excision repair process ensues with departed adenines rapidly replaced with fixed mutations,
rendering the metabolites prime tumor initiators in breast (14, 15) and other tissues (14).

Moreover, breast tissue contains high concentrations of E2 and E1 (16) and moderately high levels of the monoxygenases that oxidize E2 and E1 to CEs as well as non-CE derivatives (for reviews see Refs. 10 and 14). The non-CE metabolite, 16α-OHE1, is positively associated with breast cancer patient survival (17). Generally, the least toxic 2-OH-CEs are synthesized in the highest amount (10, 14). When in vivo conditions generate imbalances in cytochrome P450 activities that increase 4-OH-CE levels in combination with low levels of antioxidant reactions and detoxifying enzymes, cellular environments favor carcinogenesis (14). 4-OH-CE-generated superoxide anions (14) are also suspected of promoting cancer invasiveness and metastases by activating matrix metalloproteinases that degrade the extracellular matrix, which is the barrier to tumor passage (18). Because chemical reactivity of 4-OH-CE metabolites can both initiate and promote cancer invasiveness, CE-detoxifying enzymes (8, 9, 11, 12) are critical to the normal health of mammary gland and other estrogen-responsive tissues.

Whereas 2B7 was considered a pivotal isozyme for detoxifying toxic estrogen metabolites, its distribution in both estrogen-responsive and other tissues (2, 5) suggested that the isozyme is critical to protection against mutagenizing estrogen metabolites without altering E2 levels. An immunocytochemical study (19) detailed 2B7 distribution in normal breast tissue, its elevation in in situ lesions within some carcinomas, and its dramatic reduction in invasive carcinomas that showed disorganized basement membranes. Separately, an immunohistochemical study showed 2B7 is located in normal breast ductal and endometrial epithelial cells (20). Contrariwise, family A UGT1A10, primarily distributed throughout gastrointestinal tissues (2, 5, 8) and bladder (5) without detection in mammary gland and liver (2, 5), avidly metabolizes primary estrogens, CEs, and phytoestrogens (8). Hence, 2B7 is likely the relevant UGT that detoxifies 4-OHE2 and 4-OHE1 in estrogen-responsive tissues and not 1A10 isozyme, which converts E1, E2, and their metabolites at robust rates (8).

Consistent with the recent discovery that UGTs require phosphorylation (3, 8, 21–24), 2B7 contains two tyrosine kinase (TK) sites, Tyr-236 and Tyr-438, that require phosphorylation (24). Despite our confirmation that 2B7 requires Src-supported phosphorylation in COS-1 cells, unexpectedly we demonstrate that phosphorylation of 2B7 in Src-free (SYF/−/−) cells enhanced metabolism of 4-OHE1, 10-fold over that in Src-containing COS-1 cells. Moreover, barely detectable E2 metabolism in COS-1 cells was increased 16-fold in SYF/−/− cells. Although Src phosphorylation of 2B7 expressed in COS-1 cells is required, its overall rate of 4-OHE1 glucuronidation is greatly inferior to that in SYF/−/− cells, with barely detectable turnover of E2. This finding indicates that UGT2B7 substrate selection is not fixed but is differentially controlled by TKs phosphorylation similar to our observations that differential PKCε phosphorylation of sites in UGT1A7 controlled its substrate selections (22).

**EXPERIMENTAL PROCEDURES**

**Materials**—COS-1, SYF/−/− (Src, Yes, and Fyn), and SYF/+/+ cells were from ATCC (Manassas, VA); serum was from Intergen Company (Purchase, NY). BCA protein assay kit was from Pierce. PP2 and anti-v-Src were from Calbiochem (La Jolla, CA), and restriction enzymes were from New England Biolab. Lipofectamine 2000 was from Invitrogen. [33P]Orthophosphate and UDP-[14C]glucuronic acid were from PerkinElmer Life Sciences; the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide kit, UGT’s acceptor substrates, and anti-β-actin were from Sigma. COS-1 and mouse SYF fibroblast cells were grown in DMEM with 4 and 10% fetal bovine serum, respectively. Antibodies against 2B7 were as follows: anti-UGT-1168 (25) detected protein backbone, and anti-Tyr(P)-438–2B7, generated by Syn-Pep (Dublin, CA), detected only Tyr(P)-438–2B7. Anti-Fgr was from Cell Signaling Technology. The reagents used in cell culture were solubilized in fresh Me3SO to allow 0.5% final concentration. The viability of cells treated with curcumin was monitored with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

**Mutation of PKC and TK Phosphorylation Sites in UGT2B7**—Because 2B7 contains three PKC and two tyrosine kinase phosphorylation sites, each site was changed independently and in various combinations as already described (24).

**Transfection of COS-1 and SYF Cells with pSVL-based UGT2B7, 2B7 Mutant, or 2B7-His-tagged Construct**—The 2B7 expression unit, pSVL-UGT2B7, was cloned (11). All of the pSVLUGT2B7 mutants (24) and the pSVLUGT2B7His construct (24) were generated as described. 80% confluent COS-1 cells or 50% confluent SYF cells were transfected with pSVL-based 2B7-cDNA expression unit using Lipofectamine 2000 (according to the manufacturer’s protocol); COS-1 cells involving radiolabeling were initiated 72 h after transfection.

**Glucuronidation Assay**—Reactions contained the common donor substrate UDP-[14C]glucuronic acid (1.40 mM, 1.4 μCi/μmol), 200 μM unlabeled acceptor substrate, 4-OHE1, or E2 with 150 μg of cellular homogenate in 40 mM phosphate (pH 6.4) or triethanolamine (pH 7.6) buffer as previously described (2). Homogenates were treated with 0.5 mg of CHAPS/mg of protein before addition to a reaction, which was incubated at 25 °C for 16 h or at 37 °C for 2 h. Following standardization of the exposure process, radioactivity contained in [14C]glucuronides resolved in TLC plates was stored in phosphor-based film over 1–2 h in preparation for transferring the same to the PerkinElmer Cyclone Storage Phosphor Imaging for quantitation.

**Production of Anti-Tyr(P)-438–2B7 and Anti-UGT-1168**—Phosphorylated Tyr-438 peptide (CKRVINDPSY(P0)KENV) derived from 2B7 was used to generate rabbit antibody (Syn-Pep), which was preabsorbed against nonphosphorylated peptide and then positively purified over phosphorylated peptide-containing resin. Anti-UGT-1168 was generated against highly purified mouse Ugt2b5 as described (25).

**Western Blot Analysis**—COS-1 or SYF cells were washed, harvested in cold PBS, solubilized in loading buffer, resolved in a 4–15% SDS-PAGE system, and subjected to Western blot
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analysis as described (8, 22, 23). Western blots exposed to antibody directed against phospho-groups were blocked with 5% bovine serum albumin (8, 23); others were blocked with 5% milk as described (23). Anti-Tyr(P)-438–2B7, anti-UGT-1168, anti-v-Src, and anti-β-actin were used as needed with different blots.

**Incorporation of [33P]Orthophosphate into UGT2B7 and Its Mutants**—Sixty hours after transfection with UGT2B7 or its mutants, the cells were processed and treated with [33P]orthophosphate to establish incorporation of immuno-precipitable label into specific protein as previously demonstrated (22).

**Preparation of Microsomes from UGT2B7His-transfected SYF−/− Cells**—Microsomes isolated from cultured cells were prepared as described (2) with modifications. The cells were harvested in PBS containing 100 mM KCl, and protease mixture inhibitor (Sigma; catalog number P8849) before homogenization in 0.25 M sucrose, 0.15 M KCl adjusted to pH 7.4 containing 100 mM sodium orthovanadate, 5 mM NaF, and protease mixture inhibitor using a Dounce system. Cellular material was further disrupted by spraying through a 29-gauge needle before centrifugation at 10,000 g for 10 min to remove mitochondria. The resulting supernatant was centrifuged at 100,000 g for 10 min to remove ribosomes. The resulting supernatant was suspended in 0.15 M sodium orthovanadate containing 5 mM NaF, and protease mixture inhibitor using a Dounce system. Cellular material was further disrupted by spraying through a 29-gauge needle before centrifugation at 10,000 g for 10 min to remove mitochondria. The resulting supernatant was centrifuged at 100,000 g for 10 min to remove ribosomes before microsomes were pelleted at 60,000 g for 30 min and resuspended in 0.25 M sucrose, 0.15 M KCl.

**In Vitro Phosphorylation of 2B7 by Src**—To confirm Src phosphorylates 2B7 in vitro, microsomes isolated from non-transfected and 2B7His-transfected SYF−/− cells were solubilized (23, 24), affinity-purified, and subjected to [γ-33P]ATP-dependent phosphorylation with and without SrcTK. The 2B7His-transfected sample was divided into two equal aliquots, and [γ-33P]ATP was added to the two aliquots and to control sample; Src kinase, added to equal amounts of transfected and nontransfected sample, incubated 30 min before affinity purification. Parallel duplicate systems containing nonlabeled ATP were similarly prepared. All of the samples were electrophoresed in a 4−15% SDS-PAGE system (23) and quantitated as described. The resolved gel was exposed to film for autoradiography, and parallel nonradiolabeled samples were Western blotted with anti-Tyr(P)-438 and anti-UGT-1168.

**Glucuronidation of 17β-Estradiol in Cellulo by 2B7-transfected SYF−/− and COS-1**—Thirty-four hours after 2B7 transfection, SYF−/− and COS-1 cells were subjected to condition-ing in Dulbecco’s modified Eagle’s medium containing 4% charcoal-stripped fetal bovine serum (8) for 8 h and exposed to 50 μM 17β[13C]E2 (2.5 μCi/μmol) in fresh Dulbecco’s modi-fied Eagle’s medium charcoal-stripped fetal bovine serum for 16 h. For analysis of in cellulo 17β[14C]E2 glucuronide formed during exposure to 17β[14C]E2, 500 μl of medium were utilized in triplicate and diluted 1:2 with cold ethanol before cen-trifugation to remove protein. Recovered supernatant was spun dry and re suspended in phosphate-buffered saline. One-half of each sample was treated with buffer or buffer containing 50 units of β-glucuronidase in 50 μmol phosphate buffer (pH 6.8) for 4 h at 37°C. The samples were vacuum-dried, re-
ence between the two treatment groups of cells was evaluated for significance using Student’s *t* test. The results were highly significant at *p* = 0.001 with a 95% confidence interval.

**RESULTS**

**UGT2B7 Requires Tyrosine Kinase Phosphorylation**—Previously, we demonstrated that treatment of constitutive UGTs in human colon cell lines (2, 21) and recombinant UGT1A isozymes expressed in COS-1 cells (3, 23) is reversibly down-regulated by appropriate concentrations of dietary antioxidant, curcumin. Its inhibition of PKC isozymes led to the discovery that UGTs require phosphorylation. According to co-immunoprecipitation of signaling partners in complexes (22, 23) that are also distinguished by differential sedimentation profiles during centrifugation,5 each of three UGT1A

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5 N. K. Basu, M. Basu, and I. S. Owens, manuscript in preparation.
isoenzymes studied requires phosphorylation by different PKC isozyme(s). To the contrary, the three PKC sites in 2B7 undergo phosphorylation (Fig. 1), but mutant PKC sites have little effect on activity (24). Because combined treatment with cycloheximide and curcumin was not different from curcumin alone, the evidence indicates that curcumin also down-regulates tyrosine kinase(s) as previously shown (29, 30). Hence, we have demonstrated that the agent inhibits phosphorylation of TK sites in 2B7, which completely down-regulated activity within 15 min with detectable recovery between 20 and 24 h (supplemental Fig. S1). As shown in Fig. 1A, site-directed mutagenesis of tyrosine residues, Tyr-236-2B7 and Tyr-438-2B7 (Fig. 1A, M4 and M5), caused null and 90% loss of activity, respectively, indicating that both residues require phosphorylation (Fig. 1B, left panel).

Development and Characterization of Anti-Tyr(P)-438-UGT2B7—To monitor the status of Tyr(P)-438-2B7 residue, we carried out Western blot analysis with our custom developed anti-Tyr(P)-438-2B7 preparation that was purified as described (see “Experimental Procedures”). Anti-Tyr(P)-438-2B7 recognized family A UGTs: 1A1, 1A3, and 1A10, phosphorylated residues in the SH2 domain uncovered by software (31, 32), it should be pointed out that the Src-interacting pro-

Identification of the Tyrosine Kinase Responsible for Phosphorylation of UGT2B7 Using [33P]Orthophosphate—Finding that phosphorylation of TK sites (24), but not PKC sites (24), in 2B7 is critical for activity led us to compare [33P]orthophosphate incorporation into 2B7 wild-type with its mutants at predicted PKC and TK phosphorylation sites. Results show the highest level of immunodetectable label in wild-type 2B7 with 5 kinase sites with progressively less in M2, M1, and M3, representing 3, 2, and 0 phosphorylation sites, respectively (Fig. 1C). Because all Src-interacting proteins contain Src homology binding domains with phosphoryrosine sequences (31, 32), it should be pointed out that the radiolabel in the 2B7 penta-mutant is likely due to phosphorylated residues in the SH2 domain uncovered by software available on-line.

Whereas the triple PKC site mutant in 2B7 had little effect on estriol and 17-epiestriol turnover (24), the results in Fig. 1C indicate that the mutant metabolizes 4-OHE1 at 63% of the rate of wild-type 2B7 (Fig. 1C, bottom row) following normalization. Wild-type 2B7 and its triple mutant, M1, failed to show detectable turnover of E2 in a 2-h incubation (Fig. 1C, bottom row). Typically, recombinant UGT2B7 expressed in cell culture has shown low or no detectable turnover of E2 (9, 11).

Identification of the Tyrosine Kinase Responsible for Phosphorylation in COS-1 Cells—To identify the TK responsible for phosphorylation of 2B7, we examined effects of different TK effector molecules on constitutive 2B7 activity in LS-180 cells using 2B7-specific substrate, hydroxyecholic acid. Treatment of the cells with Src activator (33), vitamin D, led to an increase in 2B7 activity, which suggested Src affected activity. We also demonstrated that the Src-specific inhibitor (34) PP2 inhibited 2B7 activity ~60% maximally under conditions of equal specific protein by anti-UGT-1168 analysis (data shown in Fig. 4).

In Vitro Phosphorylation of UGT2B7 by Src Tyrosine Kinase—To gain definitive evidence that Src supports or phosphorylates 2B7, we expressed the 2B7His construct or its vector in SYF−/− cells and isolated microsomes for solubilization as described under “Experimental Procedures.” To solubilized 2B7His in microsomal preparations from 2B7His-transfected SYF−/− cells, we added Src-TK and [γ-33P]ATP versus [γ-33P]ATP alone for 30-min incubations as described under “Experimental Procedures.” Following affinity purification of [γ-33P]2B7His, we detected 6-fold greater labeling following Src-TK and [γ-33P]ATP treatment versus [γ-33P]ATP alone (Fig. 2A). Also, its affinity-purified Src-supported [γ-33P]2B7His showed 17-fold greater incorporation of labeled phosphate at the 55–58-kDa position in the 4–15% SDS-PAGE system (Fig. 2B), considering 458 versus 26 cpm (background corrected) with no visible band for 2B7His treated with [γ-33P]ATP alone (lane 2). In parallel samples, 2B7His treated with Src and unlabeled ATP generated markedly higher Tyr(P)-438-2B7 content than ATP treatment alone (Fig. 2A, second panel, lanes 3 and 2); reactions in lanes 3 and 2 contained equal microsomal 2B7 by anti-UGT-1168 analysis (third panel). The finding that Src and ATP incubation led to more than 17-fold greater labeling than ATP alone indicates that Src avidly supports 2B7 phosphorylation or phosphorylates the protein as already described (24).

Different Substrate Selections by UGT2B7 Expressed in SYF−/−, SYF+/−, and COS-1 Cells—Despite finding that 2B7 expressed in COS-1 cells is dependent upon Src-TK for required phosphorylation, we unexpectedly uncovered 10-fold greater 4-OHE1 conversion by 2B7 following its expression in Src-free cells (SYF−/−) than that expressed in COS-1 (Fig. 3, second panel). For comparison, 2B7 expressed in SYF+/− cells allowed only a 3-fold increase in 4-OHE1 conversion above that expressed in COS-1 cells. The fact that expression of 2B7 in cells that lacked both or one Src allele led to 10- and 3-fold higher 4-OHE1 conversion, respectively, than 2B7 expressed in COS-1 demonstrated unexpectedly that Src had markedly reduced 4-OHE1 turnover by 2B7, despite its required phosphorylation or support for phosphorylating 2B7.

Considering the fact that 2B7 expressed in COS-1 (11) and HEK293 cells (9) generated nondetectable and barely detectable E2 conversion, respectively, the highly favorable 4-OHE1 conversion by 2B7 expressed in SYF−/− cells suggested that this isozyme preparation possibly increased E2 turnover. Conversion of E2 by 2B7 expressed in SYF−/− cells increased 16-fold, and that expressed in SYF+/− increased 9-fold over that in COS-1 following normalization. Because anti-Tyr(P)-438-2B7 and anti-UGT-1168 Western blot analysis showed that 2B7 was phosphorylated in the three cell lines despite far less 2B7 protein in SYF−/− cells (Fig. 3, first panel), the results...
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A

In Vitro Src phosphorylation of UGT2B7

B

In Vitro Kinase assay

FIGURE 2. In vitro Src phosphorylation of UGT2B7His expressed in SYF−/− mouse fibroblast cells. A, microsomes isolated from vector- or 2B7His-transfected SYF−/− cells after 60 h of incubation were solubilized and subjected to phosphorylation using 100 μM [γ-32P] ATP (3000 Ci/mmol) without or with Src kinase in a 30-min incubation at 30 °C. All of the reactions were stopped by adding 10 mM EDTA (final concentration). Duplicate reactions were carried out with unlabeled ATP. 2B7His affinity-purified samples were resolved in duplicate 4–15% SDS-PAGE with labeled or unlabeled reaction. Gel I with 32P labeling was fixed, dried, and exposed to x-ray film (lane 1); gel II was transferred and processed for Western blot analysis using antibodies shown (lanes 2 and 3). B, 2B7His-specific protein was affinity-purified and counted by liquid scintillation.

indicate that unidentified tyrosine kinase(s) in SYF−/− cells can also phosphorylate or support phosphorylation of 2B7 that is far more productive than that by Src, as shown after normalization. Western blot analysis (Fig. 3, first panel) confirmed that SYF−/− and SYF+/− lacked Src or contained less active Src (top band), respectively, than COS-1. Hence, the data indicate that the presence of Src reduced both 4-OHE1 and E2 turnover, in parallel, in COS-1 such that 2B7 barely converted the primary estrogen. It is noted that E2 was also metabolized by constitutive activity in SYF cells that decreased with the addition of a Src allele. Most importantly, different substrate selections by 2B7 following its phosphorylation by different TKs indicate that the isozyme substrate pattern is not fixed but varies according to factors that determine the particular TK(s) carrying out its phosphorylation. Finding that 2B7 depends upon Src phosphorylation in COS-1, which actually reduces its potential maximum activity, is a conundrum that controls substrate selection.

Effects of PP2 inhibition of 2B7 on 4-OHE1 and 17β-Estradiol Conversions—Because the absence of Src in SYF−/− cells allowed unidentified TK enzymes to phosphorylate 2B7 in a manner that enabled it to support E2 turnover, the question arose as to whether inhibition of Src in COS-1 permits other TK(s) to phosphorylate 2B7, operationally, so as to metabolize E2. Hence, we examined the effect of the Src-specific inhibitor (34) PP2 on E2 turnover by 2B7 expressed in COS-1. Without PP2 treatment, 2B7 metabolized 4-OHE1 during a 2-h incubation at 37 °C, whereas E2 requires 16-h incubation at 25 °C. Treatment with increasing concentrations of PP2 led to a maximum of 60% inhibition of 4-OHE1 metabolism, whereas E2 turnover was simultaneously increasing linearly with concentration (Fig. 4). Western blot analysis did not detect changes in the 2B7 level, whereas its phosphorylation status and the level of Src decreased. Hence, it remains to be seen whether the level of Tyr(P)-438-2B7 controls differential substrate selections for 4-OHE1 and E2. Nevertheless, counts describing preferential turnover of 4-OHE1 over E2 remain high at a ratio of 2500 to 850 or 2.9-fold (Fig. 4, 10 μM PP2). The results strongly suggest that at least two different TK systems can phosphorylate 2B7 and that Src has dominance over the unidentified TK(s) uncovered in the SYF−/− cells.

In Cellulo Conversion of 17β-Estradiol by 2B7-transfected SYF−/−—To determine whether 2B7-transfected SYF−/− cells were also competent to metabolize E2, we compared its conversion in both 2B7-transfected SYF−/− and COS-1 cells. Normalized results for 2B7-transfected cells show SYF−/− generated 17-fold greater counts, 2680 versus 151, than COS-1 after 16 h of in cellulo metabolism (Fig. 5A, top panel). Sensitivity of the product to β-glucuronidase treatment confirmed that 2B7 generated E2 glucuronide product in both cells (Fig. 5A, bottom panel). Simultaneously, harvested cells were homogenized and assayed in vitro using nonlabeled E2 in the regular glucuronidation assay with co-substrate [14C]UDP-glucuronic acid. After normalization by Western blot analysis using anti-1168, anti-438, and anti-β-actin, the results show that 2B7 expressed in SYF−/− generated 3-fold more E2 glucuronide than 2B7 expressed in COS-1 cells in 16 h. Hence, it is evident that SYF−/− cells are more competent to metabolize E2 compared with COS-1 cells. Because of variability in SYF−/− cells growth and, hence, transfection efficiency, we observed a range between 3- and 20-fold higher efficacy of those cells to support 2B7 turnover of E2 than its expression in COS-1.
Lack of Fgr Kinase in SYF/H11002 Cells—To determine whether the companion Src family Fgr kinase is a candidate for the unknown TK(s) that phosphorylate 2B7 expressed in SYF/H11002 cells, we compared Western blot analysis of SYF/H11002 cells and COS-1 cells expressing 2B7 using anti-Fgr. Although Fgr kinase has been reintroduced into SYF cells, it is not present in SYF/H11002 cells (supplemental Fig. S2). Hence, Fgr is not involved in 2B7 phosphorylation in SYF/H11002 cells.

Demonstration 2B7 Transfection Protects COS-1 Cells against 4-OHE1 Depurination—We have demonstrated that Src-supported phosphorylation of 2B7 in COS-1 cells detoxifies 4-OHE1 between 10 and 30% the rate 2B7 expressed in SYF/H11002 cells, it was of interest to determine whether that level of activity provides measurable protection against 4-OHE1 toxicity. Hence, we adapted the comet assay to score depurination following 4-OHE1 treatment as described under “Experimental Procedures” (26–28). To capture images to demonstrate DNA damage, we first treated nontransfected and 2B7-transfected COS-1 cells with sufficient 4-OHE1, to cause nearly all DNA to redistribute from the head into the comet tail due to damage revealed by SYBR green staining (Fig. 6A, left bottom panel) versus no major DNA redistribution following 4-OHE1 treatment of 2B7-transfected cells (Fig. 6A, right bottom panel). Also, results showed that Me2SO (vehicle) treatment of either nontransfected (Fig. 6A, top left panel) or 2B7-transfected cells (Fig. 6A, top right panel) caused no major change in DNA shift from the head.

For the purpose of scoring depurinated DNA redistribution to the comet tail, treatment of nontransfected COS-1 cells

![Figure 3](image-url)  
**Figure 3.** *In vitro* glucuronidation of 4-OHE1 or 17β-estradiol by 2B7 expressed in SYF/H11002, SYF/H11546, and COS-1 cells. Cells transfected with vector or 2B7 that had been incubated for 72 h were harvested, and homogenates were used for 4-OHE1 or E2 glucuronidation assays at 25 °C for 16 h and for resolution in 4–15% gradient gels. Aliquots of corresponding homogenates were resolved in 4–15% gradient gels, transblotted, and processed for Western blot analysis using the antibody preparations shown. Note: anti-SrcTK detects regular Src between 56 and 60 kDa in cultured cells.

![Figure 4](image-url)  
**Figure 4.** Effects of *in cellulo* treatment of 2B7-transfected COS-1 cells with increasing concentrations of PP2. Increasing concentrations of PP2 were added to 2B7-transfected COS-1 cells during the final 4-h of a 72-h culture period. The cells were harvested; homogenates were used for 4-OHE1 or E2 glucuronidation assays at 25 °C for 16 h and for resolution in 4–15% gradient gels that were processed for Western blot analysis using antibodies shown as described under “Experimental Procedures.”
with 100 μM 4-OHE₁ for 6 h underwent linear accumulation of DNA fragments in tails that averaged 50–60% protection, whereas similar treatment of 2B7-transfected cells and Me₂SO treatment of nontransfected or 2B7-transfected cells scored by the percentage of damaged DNA in comet tail (Fig. 6B, left panel) or by tail moment (Fig. 6B, right panel) showed 92% protection against DNA redistribution of damaged DNA into comet tails. Computer analysis was carried out with software (see “Experimental Procedures”). The results demonstrate that 2B7 glucuronidation of 4-OHE₁ expressed in COS-1 had a significant protective effect against depurination of DNA.

**DISCUSSION**

Previously, we demonstrated that UGT2B7 transfected into COS-1 cells preferentially metabolized genotoxic 4-OHE₂ and 4-OHE₁ at rates 10–100-fold higher than minimally toxic 2-OHE₂ and 2-OHE₁ (11, 12, 14). More recently, we showed that overexpression of Src kinase in co-transfection studies with the 2B7-cDNA construct in COS-1 cells yielded a 1.5-fold maximum increase in 2B7 activity (24) with a constant 2B7 protein level but increased levels of anti-Tyr(P)-438-2B7 and anti-regular Src signals. Presently, we have confirmed that 2B7 requires tyrosine phosphorylation at residues Tyr-236 and Tyr-438 as suggested by site-directed mutagenesis (24). Moreover, we monitored the phosphorylation status of Tyr-438 with our custom-developed and highly specific anti-Tyr(P)-438-2B7 that recognizes only Tyr(P)-438-2B7. Similar to results seen for three different UGT1A isozymes shown to require regulated phosphorylation at PKC sites (21–23), 2B7 activity is reversibly down-regulated by curcumin treatment following expression in COS-1 (supplemental Fig. S1), indicating that it also requires regulated phosphorylation but at tyrosine residues. Although 2B7 is not only down-regulated by general TK inhibitors (24), it is down-regulated by Src-specific PP2 inhibitor (34), which suggests that the isozyme requires Src-dependent phosphorylation (Fig. 4, top panel). Consistent with Src kinase-supported phosphorylation of 2B7 in COS-1 cells, the two proteins also showed co-localization that was partially disrupted by PP2 pretreatment (24).

To establish that Src supports 2B7 phosphorylation, we demonstrated that Src kinase activity was 6-fold more effective at generating [γ³²P]2B7His using [γ³²P]ATP compared with [γ³²P]ATP alone (Fig. 2B). Migration of the in vitro Src-generated product to a 55–56-kDa position in the SDS gradient gel verified that the tyrosine kinase supports 2B7 phosphorylation; furthermore, 9-fold greater label at that position generated by Src compared with ATP alone is further evidence that Src supports 2B7 activity. Moreover, 2B7His contained 10–12-fold greater anti-Tyr(P)-438-2B7 signal with unlabeled ATP and Src than ATP alone, which also verified its effects on 2B7His (Fig. 2A, second panel).

The fact that 2B7 phosphorylated in Src⁻/⁻ cells was 8-fold more active toward 4-OHE₁ and 16-fold more active toward E₂ than when expressed in Src-containing COS-1 cells represented the first indication that 2B7 phosphorylation could be distinguished operationally by different TKs altering its active site so as to change substrate selection (Fig. 3). Hence, the capacity of 2B7 to glucuronidate E₂ following PP2 inhibition...
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**A. COS-1 cells**

|          | NT      | UGT2B7 |
|----------|---------|--------|
| DMSO     |         |        |
| Treated with 4-OHE Estrone |         |        |
| NT      | = Non Transfected |        |
| UGT2B7 = UGT2B7 transfected |        |        |

**B. Comet Assay**

|          | % of Damaged DNA | Tail Moment (A.U.) |
|----------|------------------|--------------------|
| COS-1    | NT UGT2B7        |                   |
| DMSO     | Treated with 4-OHE Estrone |       |
| COS-1    | NT UGT2B7        |                   |

*P < 0.001

FIGURE 6. Effect of 2B7 transfection on 4-OHE-1-damaged DNA in comet tails. A, depiction of 2B7 protection against 4-OHE-1-based depurination. B, quantitation of protection against 4-OHE-1-dependent depurination of COS-1 cells. The cells were treated with vehicle or 100 μM 4-OHE-1 for 6 h, processed, and stained before evaluation of damaged DNA using Comet Score v1.5 software as described under “Experimental Procedures.” The results for 200 cells/group were randomly and cumulatively evaluated from three experiments, using two different parameters as shown: percentage of damaged DNA in tail and tail moment (arbitrary units). Student’s t test generated a p value of 0.001.

of Src in COS-1 was evidently due to 2B7 phosphorylation by unidentified TK(s), enabling it to glucuronidate E2, as seen in SYF−/− cells. Our finding demonstrates that 2B7 undergoes Src-dependent regulated phosphorylation allowing “alternative active site” utilization with enhanced discrimination between substrates. This allows 4-OHE1 conversion with E2 exclusion. Such action is consistent with 1A7-regulated phosphorylation and substrate selection (see below) (22, 23). Here it appears that the 2B7 “maximum rate” of 4-OHE1 glucuronidation following its phosphorylation by unidentified TKs in SYF−/− is significantly reduced by Src phosphorylation, which on a relative scale prevents E2 glucuronidation, an essential physiological benefit regarding estrogen-responsive tissues.

Enzymatically, there are 19 human UGT isozymes, and each covalently links a lipophilic chemical to common donor-substrate glucuronic acid (1), generating inactive excretable metabolites and environmental contaminants. The mechanism utilized by a finite number of UGT isozymes to metabolize an indeterminate number of toxic, as well as nontoxic acceptor substrates, is unknown. Whereas preliminary evidence predicts that 13 UGTs undergo regulated phosphorylation (21–24), detailed evidence demonstrates an association of regulated phosphorylation and substrate expansion for 1A7 (Ref. 22 and see below), and here we demonstrate an association of regulated phosphorylation and substrate selection for 2B7. Hence cumulative results support the concept that substrate signaling is possibly the underpinning of ongoing refolding and dynamic conformational changes of active site(s) for 2B7 and 1A7 carried out by an appropriate assembly of select proteins including UGT(s), kinase(s), phosphatase(s), and adapter/scaffold protein(s) (23). Such an adaptable mechanism of refolding at the active site(s) could provide an enormous substrate potential that is susceptible to molecular probes that affect diseases and cellular homeostasis. To date, each UGT is associated with a different kinase(s) (22, 23, 35), which is likely based on the over-arching role of each isozyme.

Earlier COS-1 cells expressing UGT1A7-S432G or UGT1A7 treated with antagonist PKCε-specific peptide that blocked phosphorylation of Ser-432 (22) (Figs. 4 and 5) revealed that dephosphorylation at Ser-432 in 1A7 enabled the isozyme to gain new substrates simultaneous with a catalytic pH shift from 8.0 to 6.5 (22). Hence, ongoing refolding of the active site(s) in the isozymes mediated by phosphate signaling is a plausible explanation of our data.

The model dietary agent and UGT substrate curcumin reversibly down-regulates both PKC- and TK-regulated UGT isozymes. Hence, phosphate signaling enables cells to invoke appropriate kinase(s) and confers different substrate specificity(s) to respond appropriately to new chemical pressures similar to the myriad of other cellular pressures and phosphate signaling that maintain cellular homeostasis. Moreover, evidence indicates that substrate selections can be manipulated by appropriate agents. This was shown by PP2 inhibition of Src kinase that enhanced 2B7 metabolism of E2 while reducing 4-OHE-1 in this study and by PKCε-specific peptides that increased E2 glucuronidation (22), demonstrating that regulated phosphorylation is a target for manipulating UGT substrate activities. Hence, our findings suggest the possibility of advancements in drug development that could lead to specific and targeted treatments for chemically mediated diseases including breast cancer initiation (14).

Emerging evidence continues to demonstrate unexpected UGT flexibility controlled by regulated phosphorylation. Earlier we showed that the UGT system in tissues of the gastrointestinal tracts of mice could be temporarily down-regulated (3) by ingesting the antioxidant curcumin to allow enhanced uptake and efficacy of immunosuppressant mycophenolic acid, which otherwise undergoes extensive glucuronidation by gastrointestinally distributed UGTs. Although Src-regulated phosphorylation of 2B7 reduces 4-OHE1 glucuronidation evidently to prevent E2 metabolism, PKCε-regulated phosphorylation of 1A7 appears to expand its substrate selection (22). Hence, accumulating evidence indicates that UGT substrate
selections are not fixed and that selections can be manipulated by treatment with appropriate agents that modulate select kinase(s) activities. Detection of UGT substrate changes noted here were made possible, no doubt, by en masse shifts away from basal ongoing signaling equilibria to a single equilibrium by chemical treatments, kinase-specific disruptions, or mutant UGT isozymes as cited.

Concerning a possible mechanism to explain the variation in substrate selection following differential phosphorylation of Tyr-438-2B7 by Src kinase versus unidentified TK(s), it is noted that PP2 treatment caused concurrent decreases in both detectable Tyr(P)-438 in 2B7 and its turnover of 4-OHE1, simultaneously with its increase in E2 turnover (Fig. 4). Hence, the possibility exists that the high level of detectable phosphorylation of 2B7 by Src kinase restricts access of 4-OHE1 to the catalytic cavity(s), whereas less phosphorylation of 2B7 by unidentified TK(s) allows a more accessible 2B7 catalytic cavity(s), and entry of both 4OHE1 and E2 molecules. The apparent inverse relationships between 2B7 phosphorylation status and turnover of two different substrates suggest that two different catalytic cavities exist that can interchange. Highly charged but variably formed salt bridges involving phosphate moieties could possibly participate in establishing such changing 2B7 active sites. If changing active site(s) are involved, then analysis of a single enzyme preparation to describe catalytic activity(s) may not describe all of the possibilities. Hence, it may be necessary to use a Src-phosphorylated 2B7 preparation and an "unidentified TK" phosphorylated preparation expressed in SYF/H11002 cells for eventual x-ray analysis.

In the case of mammary gland-distributed 2B7, it should be pointed out that Src is critical to its development based on rudimentary mammary gland structures in a Src knock-out mouse (36). Whereas Src is known to promote differentiation, controlling 2B7 specific substrate turnover via signaling is noted here were made possible, no doubt, by en masse shifts away from basal ongoing signaling equilibria to a single equilibrium by chemical treatments, kinase-specific disruptions, or mutant UGT isozymes as cited.

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