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Src supports UDP-glucuronosyltransferase-2B7 detoxification of catechol estrogens associated with breast cancer†

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Abstract

Mammary gland-distributed and ER-bound UDP-glucuronosyltransferase(UGT)-2B7 metabolizes genotoxic catechol-estrogens (CE) associated with breast cancer initiation. Although UGT2B7 has 3 PKC- and 2 tyrosine kinase (TK)-sites, its inhibition by genistein, herbimycin-A and PP2 with parallel losses in phospho-tyrosine and phospho-Y438-2B7 content indicated it requires tyrosine phosphorylation, unlike required PKC phosphorylation of UGT1A isozymes. 2B7 mutants at PKC-sites had essentially normal activity, while its TK-sites mutants, Y236F- and Y438F-2B7, were essentially inactive. Overexpression of regular or active Src, but not dominant-negative Src, in 2B7-transfected COS-1 cells increased 2B7 activity and phospho-Y438-2B7 by 50%. Co-localization of 2B7 and regular SrcTK in COS-1 cells that was dissociated by pretreatment with Src-specific PP2-inhibitor provided strong evidence Src supports 2B7 activity. Consistent with these findings, evidence indicates an appropriate set of ER proteins with Src-homology binding-domains, including 2B7 and well-known multi-functional Src-engaged AKAP12 scaffold, supports Src-dependent phosphorylation of CE-metabolizing 2B7 enabling it to function as a tumor suppressor.

The discovery [1,2] that ER-bound UDP-glucuronosyltransferase (UGT)-2B7 detoxifies catechol metabolites of primary estrogens, as well as biliary-based hydoxycholeic acid, was highly significant, because certain catechol estrogens (CEs) are *genotoxic* and are associated with initiation of breast cancer [3,4]. Whereas select cytochromes P450 form CEs, UGT2B7 preferentially conjugates 4-OH-estrone and -estradiol over 2-OH-estradiol and -estrone [1,2], respectively, leading to their inactivation, increased water-solubility and high excreability. As 4-OH-estrone and -estradiol are the most mutagenizing [3], UGT2B7 substrate-profile suggests it is the critical isozyme protecting estrogen-responsive tissues against mutagenizing estrogen metabolites. Unlike mammary gland-distributed UGT2B7 [5,6] that avidly metabolizes CEs, but show no detectable conversion of primary estrogens [1], UGT1A10, distributed throughout gastrointestinal tissues [7], avidly metabolizes CEs, primary estrogens, and phytoestrogens [8]. Contrariwise, UGT1A10 is not detectable or barely detectable in mammary gland and liver [7]. Evidence indicates UGT1A1 through 1A10 [7,8] have, primarily, a moderate to vast overlapping-substrate activity towards xenobiotics [7,8] that include dietary constituents and environmental contaminants [7,8]. Inextricably, UGT1A isozymes also hasten removal of

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many medicinal chemicals [9,10]. Despite an enormous substrate profile and wide tissue-distribution [7], liver-distributed UGT1A1 uniquely detoxifies bilirubin to prevent CNS accumulation and kernicterus [11]. All UGTs utilize the common donor substrate, UDP-glucuronic acid, to convert lipid-behaving chemicals to excretable glucuronides [12].

Because estrogen responsive tissues have elevated levels of primary estrogens [13,14], along with sulfotransferase and sulfatase activities that interconvert 17 β -estradiol between sulfated and free form [13,14] and select cytochromes P450 [15] that convert estrogens to catechol metabolites, the mammary gland is a particular target for CE toxicity. While more 2-OH-estradiol and -estrone than 4-OH-estradiol and -estrone are typically synthesized by cytochromes P450 [15], 4-hydroxy metabolites are far more mutagenic [3,16].

4-OH-estradiol and -estrone undergo intrinsic oxidative semiquinone-quinone cyclic action [3,16] to form highly reactive free-radical superoxide anions ($O_2^{\bullet-}$) that attack and form DNA adducts, 4-OH-estradiol(-estrone)-1-N3Adenine [4-OHE₂(E₁)-1-N3Ade] and 4-OH-estradiol(-estrone)-1-N7Guanine [4-OHE₂(E₁)-1-N7Gua], which undergo depurination. 4-OHE₁(E₂)-1-N3Ade and 4-OHE₁(E₂)-1-N7Gua are excised spontaneously and over 3 hr, respectively [see review, 16]. The departed adenine leaves apurinic sites that lead to error-prone DNA base-excision repair, which often fixes a mutation at the site [3,16]. 4-OHE₁(E₂)-1-N3Ade is the more damaging adduct and has the highest association with breast cancer initiation [3,16]. Although mutations are found in normal breast tissue extract [17], CE content has ranged from two-fold to higher levels in breast cancers compared to normal tissue with non-catechol metabolite, 16 α -hydroxyestrone, positively associated with breast-cancer survival [18]. Imbalances in cytochromes P450 that generate high levels of 4-OH-estradiol and -estrone in combination with low levels of protective conjugating enzyme(s) are conditions that favor carcinogenesis [3,16].

In addition, highly-reactive oxidized 4-OH-estradiol and -estrone are suspected of promoting cancer invasiveness and metastases by activating matrix metalloproteinases (MMPs) that degrade the extracellular matrix (ECM), which is the barrier to tumor passage [19]. Hence, inactivation and removal of CEs are important to the health of tissues.

Because an immunocytochemical study [5] and, more recently, an immunohistochemical report [6] demonstrated UGT2B7 is distributed in mammary tissue, we questioned whether the CE-metabolizing isozyme also requires phosphorylation similar to family-A UGTs. Previously, we demonstrated that UGT1A1 [20], 1A7 [21,22] and 1A10 [21,22] require PKC-dependent phosphorylation. For the first time here, we provide evidence that 2B7 requires tyrosine phosphorylation that is dependent upon Src tyrosine kinase (SrcTK). While SrcTK is required for normal mammary gland development [23], its role in protecting against estrogen metabolite-based carcinogenesis is of high clinical significance.

Materials and methods

Materials and antibodies used are placed in the Supplement.

Targets of the Src antibodies

For Western blot analysis using Src antibodies, anti-SrcTK detects regular Src between 56–60 kDa in both cultured cells and tissues, whereas anti-activated SrcTK (Clone 28) has greater affinity for activated Src in cultured cells, and it recognizes a distinct 60kDa species in tissue samples.

Mutagenesis of PKC phosphorylation sites in 2B7

As 2B7 contains 3 predicted PKC- and 2 tyrosine kinase phosphorylation sites, we first carried out site-directed mutagenesis at PKC sites, T123, S132 and S437, as previously described [20]. The primers and the method for carrying out site-directed mutagenesis to alter PKC and tyrosine kinase phosphorylation sites are placed in the Supplement.

Transfection of all constructs into COS-1 cells and inhibition of UGT activity

Transfection and treatment with inhibitors are placed in the Supplement.

Co-transfection of COS-1 cells with pUGT2B7 and pSrcTK constructs

For co-transfection studies, we used pSVL-UGT2B7-cDNA and a pUSE-SrcTK-cDNA construct encoding wild-type (W), activated (Ac) or dominant-negative (DN) SrcTK protein or empty pUSE vectors as controls. Twenty-four hrs after seeding and reaching 70 % confluence, cells were cotransfected with 10 µg of pSVL-UGT2B7 [1,2] and either 5 µg of pUSE-w-SrcTK, pUSE-AcSrcTK, pUSE-DN-SrcTK, empty pUSE+ve or empty pUSE-ve using Lipofectamine™ previously optimized at 15 µg DNA per plate. DNA species were precomplexed in DMEM/Plus Reagent without serum or antibiotic for 15 min at 24°C according to the manufacturer's (Life Technologies) direction. After washing cells with PBS, precomplexed DNA was added, and cultures incubated 3 hr at 37°C in the tissue-culture incubator. DNA-containing medium was removed, cultures were washed with PBS, DMEM (5 % FCS) was added and cells continued in culture for 48 hr before Western blot and glucuronidation analyses.

Glucuronidation assay

Details for the glucuronidation assay are placed in the Supplement. For analysis, the TLC plates were exposed to phosphor-based films for 1–2 hr before scanning the same on a Cyclone Storage Phosphor Imager, Perkins-Elmer (Model B431200), which had been previously standardized.

Co-localization of 2B7 and SrcTK in 2B7transfected COS-1 cells

Because our cumulative studies suggested Src phosphorylates 2B7, we inquired whether the two proteins co-localize in 2B7-transfected cells grown and untreated or pre-treated with 10 µM PP2 for 45 min as described [21,22]. 2B7 and regular SrcTK, 56- to 60-kDa, were probed for co-localization by immunofluorescence [21,22] using the following primary antibodies: goat anti-UGT (1168) and mouse anti-v-SrcTK (Calbiochem). Control cells were not treated with primary antibody. 2B7 and Src were visualized with donkey anti-goat-FITC-conjugate (Jackson) and donkey anti-mouse-TRITC-conjugate (Jackson), respectively.

Production of antibody toward phospho-Y438-2B7 and UGT protein

Phosphorylated Y438 peptide [CKRVINDPSY(P0₃)KENV] derived from 2B7 was used to generate rabbit antibody (SynPeP), which was preabsorbed against nonphosphorylated peptide and then positively purified over phosphorylated peptide-containing resin. Anti-UGT-1168 was generated against highly purified Ugt2b5 [24]; it was again examined for specificity (P.S. Mitra/N.K. Basu, K. Chakraborty, and I. S. Owens, Manuscript ready for submission). Again, it showed no signal in nontransfected COS-1 cells (see Fig. 2C). Attempts to produce an antibody with the phosphorylated-Y236 peptide were not successful.

Results

Effect of protein kinase-C inhibitor on 2B7 expressed in COS-1 cells

Because computer searches for kinase-specific sites in UGT2B7 uncovered 3 PKC (T123, S132, and S437) [20] and 2 tyrosine kinase (TK) sites (not previously reported [20]) at Y236 (KKWDQFYSEV) and Y438 (RVINDPSYKEN), we extended studies to determine 2B7 phosphorylation requirement(s). We examined effects of classical PKC inhibitors, BIM, staurosporin or GO6970 (not shown) and putative protein kinase C (PKC)-inhibitor, curcumin, using concentrations ranging between 10 and 100 μ M. Whereas classical inhibitors did not affect activity, 10 μ M curcumin inhibited activity between 25 and 75 %; 25 μ M completely inhibited activity without affecting PKC-sites phosphorylation (not shown) or specific protein level according to Western blot analysis (Fig. 1A). While the result demonstrated PKC site phosphorylation of 2B7 is not required, there was, however, a progressive loss of tyrosine phosphorylation (Fig. 1A, line 2). By contrast, curcumin completely inhibited activity and phosphorylation of PKC sites in family-A isozymes, UGT1A1, UGT1A7 and UGT1A10, following expression in COS-1 cells [20–22].

Because of the unexpected finding that PKC site phosphorylation was not affected by curcumin, we mutated each PKC site in 2B7 independently and in combinations to verify this finding. Activity studies carried out with 4-OH-estrone and 17-epiestriol ranged between a 50% reduction and a 100% increase (Fig. 1B, C).

Whereas curcumin has been shown to inhibit TK phosphorylation by Src, in particular [25], we first examined the effect of tyrosine kinase inhibitors, genistein and herbimycin-A, on 2B7 and its triple PKC-sites mutant (TM). Results show wild type 2B7 was inhibited between 40 and 50 % by herbimycin-A and genistein, whereas the TM of 2B7 was consistently inhibited approximately 10 % less than wild type as concentrations of TK inhibitors increased (Fig. 2A,B). Whereas specific 2B7 content did not vary with concentration of TK inhibitors, the level of TK-site(s) phosphorylation was dramatically reduced for wild-type 2B7, with significantly less effect on its TM (Fig. 2A,B, lines 2). Similar to curcumin effects on PKC phosphorylation of UGT1A isozymes [20,21] and based on the lack of an effect of cycloheximide on curcumin inhibition (P.S. Mitra/N. K. Basu, K. Chakraborty, and I. S. Owens, Manuscript ready for submission), inhibition of 2B7 activity was independent of protein synthesis.

Contrary to the lack of an effect of PKC-sites mutants on 2B7 activity, independent mutation of either TK site, Y236F and Y438F, or their combined mutations in 2B7 completely or nearly completely inhibited activity when expressed in COS-1 cells (Fig. 2C. Our custom-developed anti-phospho-Y438-2B7 preparation, which has been characterized (P.S. Partha/N.K. Basu, K. Chakraborty, and I. S. Owens, Manuscript ready for submission), was shown to detect only 2B7 among 3 family-B and 3 family-A UGTs. For the purpose of establishing the specificity of the anti-phospho-438-2B7 preparation, we carried out Western blot analysis with all combinations of the 3 PKC sites and 2 TK sites. The antibody preparation verified the phosphorylation status of Y438 in 2B7 (Fig. 2C, bottom line 2). Results show that constructs with mutated Y438 (M2, M4 and M5) failed to immunoreact with anti-phospho-Y438-2B7 (Fig. 2C, bottom, line 2), whereas constructs that contain an intact site (Wild type 2B7, M1 and M3) gave a positive signal under conditions of equal specific protein according to anti-UGT-1168 (Fig. 2C, bottom, line 1). Hence, we used this highly specific anti-phospho-Y438-2B7 preparation to assess the phosphorylation status of Y438 in 2B7.

Effect of SrcTK overexpression on 2B7 activity expressed in COS-1 cells

To examine the effect of Src-overexpression on 2B7 activity, we co-transfected regular Src, active Src or dominant-negative Src with UGT2B7 into COS-1 cells. Results show regular or active Src elicited a 1.5 fold increase in 2B7 activity (Fig. 3, bottom, lanes 3 and 4) with a corresponding increase in phospho-Y438-2B7 content (line 3, lanes 3 and 4), evidently reflecting the marked increase in active Src (line 1, lanes 3 and 4, top band). Although Western blot analysis shows *dominant-negative* Src also increased (Fig. 3, line 2, lanes 3, 4 and 5), it did not show, however, an increase in active Src based on immuno-reactivity with anti-active Src (line 1, lanes 3, 4 versus 5). Hence cotransfection of 2B7 with *dominant-negative* Src did not increase 2B7 activity (Fig. 3, bottom, lane 5). Control studies with anti-UGT-1168 and β -actin show there was equal specific protein for all systems (Fig. 3, top panel, lines 4 and 5). It is noted that empty vectors (Fig. 3, lane 1 vs 6) increased the active state of endogenous Src (line 1, top band) some 20 % with similar effects on phospho-Y438-2B7 content (line 3, lane 1) and 2B7 activity (Fig. 3, bottom, lanes 1 vs 6)

Co-localization of 2B7 and SrcTK in 2B7-transfected COS-1 cells

As our accumulated evidence strongly indicates SrcTK phosphorylates 2B7, we examined whether 2B7 and SrcTK co-localize in 2B7-transfected cells. Immunofluorescence with donkey anti-goat UGT-FITC-conjugate and donkey anti-mouse-v-Src-TRITC-conjugate merged to generate yellow fused immunofluorescence following assembly of the green and red images of the conjugates (Fig. 4), which confirmed co-localization of 2B7 and Src. The decrease in fused immunofluorescence indicates pretreatment with Src-specific PP2 inhibitor partially disrupted co-localization, which occurred outside the DAPI-fluorescent nuclei (Fig. 4, top right/bottom right). Immunofluorescent images showing regular Src co-localizes with 2B7 that is partially disrupted by pretreatment with Src-specific PP2 is consistent with its 50 %-maximum inhibition of 2B7 activity and suggest a substantial engagement exists between the two proteins.

Src kinase phosphorylation of 2B7-derived peptides

Consistent with Src phosphorylation of 2B7, custom-synthesized peptides based on computer-derived TK phosphorylation-sites detected in 2B7 were analyzed for in-vitro Src kinase substrate activity. Results show the peptides were highly effective; each normal peptide was 5-fold more active than its mutant where phenylalanine replaced tyrosine (Fig. S1, Supplement).

Discussion

Our earlier demonstration that 2B7 detoxifies catechol metabolites of estrogens [1,2] led us to question 2B7 phosphorylation requirements in order to compare members in the UGT2B and UGT1A families. As our first computer search [20] revealed 2B7 has both PKC and TK (not previously reported) phosphorylation sites, the anti-oxidant, curcumin, led to typical concentration-dependent inhibition following treatment of UGT1A isozymes [20–22]. Partial inhibition by TK-specific inhibitors, genistein, herbimycin and PP2, suggested 2B7 differed significantly from family-A isozymes studied, which were all inhibited by PKC-specific inhibitor, calphostin-C [20–22]. Mutational analysis of 2B7 proved phosphorylation of PKC sites was not required, but that both TK sites, Y236 and Y438, require phosphorylation. The ambiguity of curcumin inhibition of both PKC and TK dependent UGTs may be explained by the fact that both PKC and TK are inhibited by curcumin [21,22,25]. Nevertheless, curcumin inhibition of UGT1A and UGT2B isozymes studied, to date, is reversible [20–22].

In addition, overexpression of regular and active SrcTK, but not the dominant-negative SrcTK mutant, following co-transfection with 2B7 into COS-1 cells caused 50 % increase in both 2B7

activity and in phospho-Y438-2B7 content, which provides strong evidence Src phosphorylates 2B7 (Fig. 3). In addition, disruption of 2B7 and Src co-localization by Src-specific inhibitor, PP2, is also critical evidence SrcTK supports 2B7 activity.

Because previous studies concerning identity of the kinase responsible for phosphorylating a specific UGT1A isozyme have been successfully verified by co-localization of PKC and UGT1A isozyme [21,22], finding 2B7 and SrcTK co-localization outside the nucleus that was partially disrupted by prior treatment with Src-specific inhibitor, PP2, is highly significant evidence Src phosphorylates 2B7.

To address how SrcTK phosphorylates or supports ER-bound 2B7, we examined 2B7 for the typical Src homology binding domains that account for the formation of Src-containing networks to allow interactions between Src and an ER-bound substrate, in particular. Whereas we found ER-bound 2B7 (<http://scansite.mit.edu>), as well as ER-bound PTP1B (protein tyrosine phosphatase1B) [26] and AKAP12 [27] (a kinase anchoring protein-12) scaffold, all contain Src-homology domains, it indicated that appropriate proteins exist to create an ER-anchored protein network to carry out Src phosphorylation of 2B7. Moreover, we have evidence that Src is organized in a network to phosphorylate 2B7 that uses AKAP12 and PTP1B [P.S. Mitra/N.K. Basu, K. Chakraborty and I.S. Owens, Manuscript ready for submission]. Our preliminary evidence suggests this ER-scaffold is analogous to the Src kinase-based signaling network in the mitochondrial outer membrane [28] that includes AKAP121 and PTP1D for phosphorylation that controls PKA signaling. Hence, these data are consistent with Src-based 2B7 glucuronidation of genotoxic CE in mammary gland, in particular. These cumulative findings, along with the multi-functional AKAP12 as a Src-suppressed C kinase substrate with tumor suppressor activity [27] (WWW.breastcancerdatabase.org), suggest a network exists consistent with phosphorylating 2B7 enabling it to also function as a tumor suppressor.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

(A) Effect of different concentrations of curcumin on 2B7 activity and Phospho-Y438-2B7 content. Cells were exposed to varying concentrations of curcumin for 1 hr, and homogenates were assayed for activity towards 4-OH estrone, 17-epiestriol and hyodeoxycholic acid (HDCA). Anti-UGT-1168 and anti-phospho-Y438-2B7 were used for Western blot analysis as described in Methods. The data represent the value for 3 replicate experiments. (B/C) Effect of mutations at PKC sites on 2B7 activity expressed in COS-1 cells. Activity and Western blot analysis for various PKC sites mutants of 2B7 mutants. Wild type and mutant 2B7 constructs were transfected into cells; cellular homogenates were used to glucuronidate 4-OH-estrone,

17-epiestriol (shown), and estriol at pH 7.0 in 2-hr incubations. Western blot was carried out with anti-UGT-1168 as described in Methods. Triplicate experiments were carried out.

Fig. 2.

Effects of (A) genistein and (B) herbimycin on 2B7 and its triple PKC-sites mutant (TM). Wild type 2B7 or its TM mutant was transfected into cells without serum and conditioned between 48 and 72 hr as described in Methods. Cultures were then exposed to different concentrations of genistein or herbimycin-A for 2 hr; glucuronidation reactions with 4-OH-estrone and cellular homogenates incubated 2-hr as already described. For Western blot analysis, anti-UGT-1168, anti-phospho-Y438-2B7 and anti-phospho-tyrosine 4G10 (shown) were used as already described. Results represent triplicate experiments. (C) Effect of TK-sites mutants of 2B7 on activity. Mutations, Y236F-2B7 and Y438F-2B7, as shown, were introduced as described under methods. As results show (below), anti-phospho-Y438-2B7 interacts with only phospho-Y438 in 2B7 (below); moreover, measurements of activity shows null or nearly null activity for Y236F (M1), Y438F (M2), the Y236F/Y438F double mutant (M4), and PKC-/TK-sites penta mutant (M5), but unaffected or higher activity for triple PKC-sites mutant (M3) than wild type 2B7. We used anti-phospho-Y438-2B7 to verify 2B7 phosphorylation at a critical

TK site, as already stated. 2B7 activity was measured with 4-OH estrone (shown), 17-epiestriol and estriol. Triplicate experiments were carried out.

Effect of overexpression of Src on UGT2B7

Fig. 3.

Effect of SrcTK overexpression on 2B7 in COS-1 Cells. The 2B7 construct was cotransfected with wild-type (W), SrcTK, activated (AC) SrcTK, dominant-negative (DN) SrcTK, empty pUSE+ve or empty pUSE-ve into COS-1 cells as described in Methods. Cell homogenates and 4-OH estrone incubated 2 hr at 37°C, and Western blot analysis with antibodies (shown) are as described in Methods. Triplicate experiments were carried out.

Colocalization of UGT2B7 and Src

Fig. 4.

Immunofluorescence of Src and recombinant 2B7. Co-localization of SrcTK and 2B7. Co-localization was processed 72 hr after 2B7-transfection without and with pretreatment with 10 μ M PP2 for 45 min as described in Methods; omission of primary antibody prevented secondary antibody-conjugate binding (not shown). Images of 2B7 and Src immunofluorescence were magnified 63-times, respectively. Scale bar (—) represents 20 μ m.