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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.

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The discovery [1,2] that ER-bound UDP-glucuronosyltransferase (UGT)-2B7 detoxifies catechol metabolites of primary estrogens, as well as biliary-based hyodeoxycholic 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 excretability. 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 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 17β-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 superoxide anions ($O_2^{\cdot -}$) 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-OHE_2(E_1)-1-N7Gua], which undergo depurination. 4-OHE_1(E_2)-1-N3Ade and

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4-OHE$_1$(E_2)-1-N7Gua are excised spontaneously and over 3 h, 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-OHE$_1$(E_2)-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, 16α-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 immunohistocytochemical 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 and 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 60 kDa species in tissue samples.

Mutagenesis of PKC phosphorylation sites in 2B7. As 2B7 contains 3 predicted PKC- and 2 tyrosine kinase phosphorylations 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 hours 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 h 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 h 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 h 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($P0_3$)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 μM. Whereas classical inhibitors did not affect activity, 10 μM curcumin inhibited activity between 25 and 75%; 25 μM 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 and 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 and B). Whereas specific 2B7 content did not vary with concentration of TK inhibitors, the level of TK-site(s) phos-