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Characterization of Proline-Serine Rich Carboxyl Terminus in Human Sulfotransferase 2B1b: Immunogenicity, Subcellular Localization, Kinetic Properties and Phosphorylation

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Running title: Characterization of Human SULT2B1b

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Abbreviations: SULT, sulfotransferase; PSC, proline and serine-rich carboxyl; DHEA, dehydroepiandrosterone; CK II, casein kinase II; PAPS, 3'-phosphoadenosine 5'-phosphosulfate; 2-D, two dimension; MALDI-TOF MS, matrix-assisted laser desorption/ionization-time of flight mass spectrometry; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; cAMP, cycling adenosine, 5'monophosphate; kD, kilodalton

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Abstract

The human sulfotransferase (SULT) 2B1 gene is a member of the SULT2 gene family and encodes two isoforms, SULT2B1a and SULT2B1b. Although messages for both SULT2B1a and SULT2B1b are detectable in human tissues, only SULT2B1b has been identified immunologically. Compared with other human SULTs, SULT2B1b has an extension at the proline and serine-rich carboxyl (PSC)-end of about 53 amino acids. The structure and function of this unique PSC-extension was investigated. Constructs of full-length SULT2B1b as well as truncated SULT2B1b without the PSC-extension were expressed in *E. coli*. Removal of the PSC-extension significantly decreased the thermostability of the expressed enzyme as well as decreasing the rate of dehydroepiandrosterone (DHEA) sulfation. Rabbit polyclonal antibodies were raised against both the full-length and truncated SULT2B1b proteins. Immunoblot analysis showed that antibodies raised to full-length SULT2B1b immunoreact only with full-length SULT2B1b, whereas antibodies raised to truncated SULT2B1b react with both full-length and truncated SULT2B1b. Unlike full-length SULT2B1b, truncated SULT2B1b was incapable of translocation to nuclei in transfected human BeWo choriocarcinoma cells. Phosphorylated serines were detected in the PSC-extension of full-length SULT2B1b expressed in BeWo cells but not in truncated SULT2B1b. At least, one phosphorylated serine was detected in expressed SULT2B1b via 2D gel electrophoresis, immunoblot analysis and mass spectroscopic analysis. Bacterially expressed full-length SULT2B1b but not truncated SULT2B1b was phosphorylated by casein kinase or Cdc2 protein kinase *in vitro*. This study suggests that the PSC-extension of SULT2B1b is important site in the immunogenicity, nuclear translocation, kinetic activity and thermostability of this SULT isoform.

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Introduction

Human cytosolic sulfotransferases (SULTs) comprise a family of enzymes responsible for the Phase II sulfoconjugation of numerous exogenous and endogenous compounds (Falany, 1997). Conjugation with the charged sulfonate group generally decreases the biological activity and increases water solubility and excretion of most acceptor compounds (Glatt et al., 2001). At least twelve isoforms of cytosolic SULT have been identified in human tissues and cells (Falany et al., 2004) and eight of these isoforms have been demonstrated to conjugate hydroxysteroids and/or estrogens (He et al., 2004; Falany, 2005).

In the human SULT gene family, the isoforms that catalyze the sulfation of hydroxysteroids are primarily members of the SULT2 subfamily. The two major isoforms are SULT2A1 and SULT2B1. SULT2A1 is expressed predominantly in the liver and the reticular layer of the adrenal gland (Comer and Falany, 1992; Parker et al., 1995). In addition to sulfation of bile acids in liver (Radominska et al., 1990), SULT2A1 is involved in the synthesis and secretion of dehydroepiandrosterone (DHEA)-sulfate from the reticular layer of the adrenal gland (Falany, 2005). SULT2A1 is capable of conjugating both 3α- and 3ß-hydroxysteroids, the 3-phenolic group of steroids as well as aliphatic hydroxyls in therapeutic drugs (Falany et al., 2006) and other xenobiotics (Falany et al., 1995; Falany et al., 2004).

The SULT2B1 gene encodes two isoforms SULT2B1a and SULT2B1b, utilizing different start sites of transcription. SULT2B1a and SULT2B1b have different amino-terminal sequences resulting from the incorporation of different initial exons. Other than different initial sequences, the sequences are identical resulting in 94% identity in amino acid sequence (Her et al., 1998). Although both SULT2B1a and SULT2B1b messages have been detected in several human tissues, but to date only SULT2B1b protein has been immunologically detected (He et al., 2004). SULT2B1b immunoreactive protein is expressed in prostate, placenta, breast, lung, brain and skin as well as human prostate and breast cancer cell lines (He et al., 2004; Higashi et al., 2004; Meloche and Falany, 2001). In general, tissues that express SULT2B1b do not demonstrate high levels of SULT2A1 expression suggesting different functional roles in hydroxysteroid sulfation.

SULT2B1b is selective for the sulfation of 3ß-hydroxysteroids such as DHEA, pregnenolone, androstenediol and 5α-androstane-3ß,17ß-diol. No sulfation is detectable with 3α-hydroxysteroids or estrogens as substrates (Meloche and Falany, 2001). SULT2B1b also conjugates selected phytoestrogens and drugs such as genistein, tibolone and raloxifene (Falany et al., 2004; Falany et al., 2006; Meloche and Falany, 2001). Cholesterol is an important substrate for SULT2B1b and the enzyme has been suggested to have a role in cholesterol sulfation in skin (Higashi et al., 2004). The subcellular localization of SULT2B1b differs between human tissues. In term placenta, SULT2B1b is localized primarily in nuclei of synchiotrophoblasts. In both prostate and lung, SULT2B1b is found in only the cytosol of epithelial cells whereas in normal breast and breast cancer tissues the enzyme is present in both cytosol and nuclei (Falany, 2004; He et al., 2005).

The SULT2B1 isoforms are unique in having a 53 amino acid PSC-extension that possesses seventeen prolines and ten serines residues (Meloche and Falany, 2001). The PSC-extension is not present in other human SULTs and its function in the regulation and localization of SULT2B1b is not known. He *et al.* (He et al., 2004) have reported that forskolin stimulates the movement of SULT2B1b into the nuclei of BeWo cells. Forskolin is generally associated with an increase in cAMP followed by an increase in phosphorylation activity. Sequence analysis indicates the presence of putative kinase targeting sites in the PSC-extension sequence suggesting a role for phosphorylation in the translocation mechanism. In this report, the unique properties of the PSC-terminus of SULT2B1b are evaluated to ascertain its role in the immunoreactivity, stability, kinetic activity and nuclear localization of SULT2B1b.

Materials and Methods

Materials

The bacterial expression vector pProEX HTa, the mammalian expression vector pcDNA3.1(-), Lipofectin, Opti-MEM and OptiPrep were purchased from Invitrogen (Carlsbad, CA). Protein A Sepharose-4B was obtained from Amersham Biosciences (Piscataway, NJ). Mouse monoclonal anti-phosphoserine antibody, phosphatase inhibitor cocktail 1, complete Freund's adjuvant and incomplete Freund's adjuvant were from Sigma-Aldrich, Inc. (Saint Louis, MO). 2D eletrophoresis reagents and apparatus were purchased from BioRad Laboratories, Inc. (Hercules, CA). [1,2,6,7-^{3}H(N)]-DHEA (80 Ci/mmol) was from Perkin-Elmer Life Sciences, Inc. (Boston MA). 3'-Phosphoadenosine 5'-phosphosulfate (PAPS) was purchased from Dr. Sanford Singer (University of Dayton, Dayton, OH). Casein kinase (CK II) and Cdc2 protein kinase were obtained from New England Biolabs Inc. (Beverly, MA). The BeWo placental choriocarcinoma cell line was from American Type Culture Collection (Rockville, MD).

Methods

Expression of SULT2B1b isoforms

The cDNAs encoding amino acids 1-365 (full-length) SULT2B1b and amino acids 1-312 (truncated) SULT2B1b were cloned into the pProEX HTa expression vector via ligation at the *Nco* I and *Hind* III sites. The 6-His tagged SULT2B1b proteins were expressed in *E. coli* DH5α and purified by Ni-NTA affinity chromatography. After cleavage with rTEV protease, the purified SULT2B1b proteins were recovered by passage through the Ni-NTA affinity column to remove the cleaved 6-His tags. Enzymatically active SULT2B1b was stored at -80° C.

BeWo human placental choriocarcinoma cells, which do not express SULT2B1s (Meloche and Falany, 2003) were chosen for the expression of SULT2B1b to explore the function of the PSC-extension in mammalian cells. The full-length SULT2B1b and the truncated SULT2B1b cDNAs were cloned into the mammalian expression vector pcDNA3.1(-) at the *EcoR* I and *Hind* III sites (He et al., 2004). BeWo cells were transformed with the pcDNA3.1/SULT2B1b plasmids using Lipofectin at a DNA/Lipofectin ratio of 1:3 in Opti-MEM. Opti-MEM was replaced with minimal essential medium (MEM) containing 10% FBS at 6 h post-transformation. After 48 h, the transformed BeWo cells were maintained in 10% FBS/MEM with 400 μg/ml geneticin for selection of geneticin-resistant colonies. Medium was changed daily until colonies appeared then colonies were isolated and expanded for analysis.

Production of polyclonal rabbit anti-SULT2B1b antibody

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Our laboratory has previously raised and characterized a rabbit anti-human SULT2B1b polyclonal antibodies to the full-length SULT2B1b protein (Meloche and Falany, 2001). Preliminary studies indicated that the rabbit anti-SULT2B1b antibody did not react with the truncated form of SULT2B1b lacking the PSC-extension. Therefore, a rabbit anti-truncated SULT2B1b polyclonal antibody was generated. The His-tagged truncated SULT2B1b protein was expressed in *E. coli* XL1-Blue cells, purified by Ni-NTA chromatography, resolved by SDS-polyacrylamide gel electrophoresis and isolated from gel pieces by electroelution in a Bio-Rad electroeluter. Pure truncated SULT2B1b (100 μg) was mixed with Freund's complete adjuvant and subcutaneously injected into a New Zealand white rabbit. The immunized rabbit was boosted with 100 μg of truncated SULT2B1b protein mixed with Freund's incomplete adjuvant at two weeks and four weeks after the initial injection. Antiserum was obtained from the rabbit two weeks after the third injection. The IgG fraction of the rabbit anti-SULT2B1b antiserum was purified by Protein A-Sepharose 4B chromatography then immunoaffinity-purified using pure truncated SULT2B1b covalently attached to cyanogen bromide activated Sepharose 4B (Lane, 1988).

Immunogenicity of the SULT2B1b proline-serine-rich carboxy (PSC) terminus

To evaluate the immunoreactivity of the SULT2B1b PSC-terminus, aliquots of expressed full-length SULT2B1b (45, 90 and 225 ng) and truncated SULT2B1b (1, 5, 10 μg) were resolved by 12% SDS-polyacrylamide gel electrophoresis (PAGE) and electrotransferred to nitrocellulose membranes for analysis with the rabbit anti-full length SULT2B1b antibody. For analysis of anti-truncated SULT2B1b immunoreactivity, aliquots of expressed full-length SULT2B1b (45, 90 and 225 ng) and truncated SULT2B1b (45, 90 and 225 ng) were utilized. Membranes were blocked with 5% non-fat milk then incubated with either the anti-full-length SULT2B1b or anti-truncated SULT2B1b antibody (1:1000) for 1 h. Goat anti-rabbit IgG horseradish peroxidase conjugate (1:60,000) was used as secondary antibody and visualization of bound proteins was carried out using the Supersignal West Pico System (Pierce).

Kinetic study of SULT2B1b

Purified full-length and truncated SULT2B1b activities were analyzed using radioactively ^{3}H-labeled dehydroepiandrosterone (DHEA) as described previously (Meloche and Falany, 2001). Briefly, purified full-length (0.15 μg/μl) and truncated (0.17 μg/μl) were incubated with 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 4 μM DHEA, [^{3}H]-DHEA (approx. 40,000 dpm/reaction) and 25 μM PAPS. The reactions were incubated at 37°C then terminated by the addition of 0.25 M Tris-HCl, pH 8.6 and chloroform. The formation of DHEA-sulfate was quantified by scintillation counting of an aliquot of the aqueous phase. Specific activities were expressed as pmol/mg/min and calculated as means of triplicate assays. The

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kinetic parameter K_m and V_{max} were determined using the Enzymekinetics 1.5 enzyme kinetics software (Trinity Software, Campton NH).

Thermostability of SULT2B1b

Our previous studies demonstrated that different SULT2B1b recombinant isoforms might have different enzymatic thermostabilities (He, 2005). Therefore the effect of the SULT2B1b PSC-extension on enzymatic thermostability was examined. The purified full-length and truncated forms of SULT2B1b were incubated at 42°C for 0, 15, 30, 45, 60 and 120 min then placed on ice. The activity of each SULT2B1b enzyme preparation was then examined for DHEA sulfation activity (Meloche and Falany, 2001).

Influence of the PSC-extension of SULT2B1b on subcellular translocation

Our laboratory has previously reported the localization of SULT2B1b in placental nuclei and in the nuclei of transfected placental BeWo cells (He et al., 2004). Analysis of human SULT2B1b amino acid sequences using the PSORT II subcellular localization program indicates that SULT2B1b has a unique amino acid composition in its carboxyl end indicative of a tendency for nuclear localization. To investigate the role of the PSC-extension on the nuclear translocation of SULT2B1b, stably transfected BeWo cells were prepared with either full-length or truncated SULT2B1b. Transfected BeWo cells were collected from 20 confluent T-75 culture flasks (a total of approximately 10^9 cells) and homogenized in a Teflon-glass homogenizer with homogenization medium (0.25 M sucrose, 25 mM KCl, 5 mM $MgCl_2$, 20 mM Tris-KOH, pH 7.8). The homogenate was centrifuged at 10,000 x g for 10 min at 4° C to recover the crude nuclear pellet. The supernatant fraction was further centrifuged at 100,000 x g to collect the cytosolic supernatant fraction and microsomal pellet. The purified nuclear fraction was prepared from the crude nuclear pellet. The pellet was resuspended in 2 ml of homogenization medium then mixed with equal volumes of Optiprep Solution C (mixture of 5 volumes of Solution A (Optiprep) + 1 volume Solution B (150 mM KCl, 30 mM $MgCl_2$, 120 mM Tricine-NaOH, pH 7.8). The solution was layered on top of 10 ml of a 30% Optiprep solution with an underlayer of 10 ml of a 35% Optiprep solution in a Beckman Ultra-Clear centrifuge tube (25 x 89 mm). After centrifugation in a Beckman SW28 swinging-bucket rotor at 6,000 rpm for 40 min at 4° C, BeWo cellular nuclei were collected at the 30-35% Optiprep interface. SULT2B1b was identified in the isolated fractions of cytosolic protein, microsomal protein and Optiprep purified nuclei by immunoblot analysis as described above. Nuclei were identified by immunoblot analysis with a mouse anti-histone monoclonal antibody (He et al., 2004).

2-D electrophoresis analysis of SULT2B1b modification

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For 2D-electrophoresis, immobilized pH gradient (IPG) strips (pH 3-10) were rehydrated with 150 µg of full-length SULT2B1b transfected BeWo cell lysate in rehydration buffer, placed in a rehydration tray and covered with the mineral oil overnight according to the manufacturer's procedure (BioRad). The sample was focused using the manufacturer's recommendations. The strips were re-equilibrated, placed into an SDS-PAGE gel well and sealed with agarose sealing solution. Electrophoresis in the second dimension was run at 150 V until the bromophenol blue dye ran off the end of the gel. One gel was subjected to Sypro staining and the other gel was electrotransferred to a nitrocellulose membrane. The membrane was immunoblotted with the rabbit anti-full-length SULT2B1b antibody (1:1000) as described above.

Purification and mass spectroscopic analysis of SULT2B1b in transfected BeWo cells

Full-length SULT2B1b cDNA with a 6-His tag incorporated into the N-terminus was cloned into pcDNA 3.1(-). The recombinant pcDNA3.1-SULT2B1b-6-His cDNA was then stably transfected into BeWo cells. Approximately 2×10^9 tranfected BeWo cells were collected from 40 confluent T-75 culture flasks and lysed in 50 mM Tris-HCl, 5 mM β-mercaptoethanol and 1 mM PMSF (with or without phosphatase inhibitor cocktail 1) followed by four 10 sec sonications with 30 sec cooling between bursts. The supernatant fraction was obtained after centrifugation at 100,000 × g for 50 min then subjected to Ni-NTA affinity chromatography according to the manufacturer's protocol (Qiagen). Purified SULT2B1b without phosphatase inhibitor cocktail 1 protection was incubated with calf intestinal alkaline phosphatase (Sigma) at 37° C for 3 h. Dephosphorylated SULT2B1b and non-dephosphorylated SULT2B1b as well as purified bacteria expressed SULT2B1b were then resolved by SDS-PAGE and visualized by Coomassie blue staining. The protein band that migrated analogous to bacterially expressed SULT2B1b was excised from the gel and destained with three consecutive washes of 50% 25 mM ammonium bicarbonate/ 50% acetonitrile for 30 min. Samples were then washed with 25 mM ammonium bicarbonate for 10 min and enzymatic digestion with modified sequencing grade Glu C protease (Roche) was carried out at 37° C for 16 h. The peptide-containing solution was then extracted with 5% formic acid for 30 min, desalted using C18 ZipTips (Millipore) and eluted directly onto the MALDI target using the MALDI matrix (α-cyano-4-hydroxycinnamic acid, 10 mg/ml in 70% acetonitrile, 0.03% trifluoroacetic acid). MALDI-TOF analysis was performed in the reflection positive ion mode in the Mass Spectroscopy Core Facility of the UAB Comprehensive Cancer Center. The resulting monoisotopic masses were searched against the NCBI database using the Mascot program.

In vitro SULT2B1b carboxyl-end phosphorylation by serine protein kinases

To examine the potential phosphorylation of serines present within several consensus kinase targeting motifs in the carboxyl-end of SULT2B1b, 2 µg of purified full-length or truncated SULT2B1b was incubated with CK II or Cdc2 protein kinase at 30° C for 6 h. The CK II reaction included 20 mM Tris-HCl,

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50 mM KCl_2, 10 mM $MgCl_2$, 4 mM ATP and 100 units casein kinase II. The Cdc2 protein kinase reaction contained 50 mM Tris-HCl, 10 mM $MgCl_2$, 1mM EGTA, 2mM dithiothretol, 0.01%Brij 35, 4 mM ATP and 2,000 units Cdc2 protein kinase. Reaction products were then incubated with rabbit anti-truncated SULT2B1b IgG bound to protein A Sepharose beads. Immunoprecipitates were resolved by 12% SDS-PAGE and subsequently detected with the mouse monoclonal anti-phosphoserine antibody.

Immunoreactive phosphorylated serine in the SULT2B1b PSC-terminus

To explore serine phosphorylation in the C-terminus of SULT2B1b in mammalian cells, a monoclonal mouse anti-phosphoserine antibody was utilized to detect phosphorylated serines in SULT2B1b isolated from transfected BeWo cells. BeWo cells were homogenized in homogenization medium supplemented with phosphatase inhibitor cocktail 1. Nuclear, microsomal and cytosolic fractions were then isolated from BeWo cell homogenate using an Optiprep gradient and 600 µg of each fraction was immunoprecipitated with rabbit anti-truncated SULT2B1b IgG bound to protein A Sepharose beads (Lane, 1988). The immunoprecipitated fractions, and aliquots of the nuclear (20 µg), microsomal (100 µg) and cytosolic (100 µg) fractions were analyzed by immunoblot analysis using mouse anti-phosphoserine monoclonal antibody and rabbit anti-truncated SULT2B1b IgG.

Results

Immunogenicity of the SULT2B1b PSC-terminus

The presence of the PSC-extension sequence significantly affects the immunogenicity of SULT2B1b. Figure 1A shows the immunoblot analysis of expressed full-length SULT2B1b (45, 90 and 225 ng) and truncated SULT2B1b (1, 5, 10 µg) using the rabbit anti-full-length SULT2B1b antibody. The anti-full-length SULT2B1b antibody readily detects 45 ng of full-length SULT2B1b whereas reactivity with 10 µg of truncated SULT2B1b is not apparent. In contrast, Figure 1B shows that the rabbit anti-truncated SULT2B1b antibody can recognize equivalent amounts of both full-length SULT2B1b and truncated SULT2B1b. These results suggest that the antibody generated by full-length SULT2B1b was induced primarily by the PSC-terminus indicating that this region is highly immunogenic.

Enzymatic activity of full-length and truncated SULT2B1b

The effect of the presence of the PSC-extension on the sulfation of DHEA was investigated using the bacterially expressed forms of full-length and truncated SULT2B1b. The different forms of SULT2B1b

were expressed in *E. coli* DH5α with amino-terminal His-tags and the His-tags were removed with rTEV protease. Figure 2 shows the effect of increasing concentrations of DHEA on its sulfation by both full-length and truncated SULT2B1b. The Km values for DHEA sulfation were 3.8 μM and 0.6 μM for full-length and truncated SULT2B1b, respectively. The Vmax values for the full-length and truncated forms of SULT2B1b were 1752 and 176 pmol/min/mg protein, respectively. Removal of the PSC-extension therefore decreased Kcat approximately 91% indicating that removal of the PSC-extension has a significant role in decreasing the ability of SULT2B1b to conjugate DHEA.

Enzymatic thermostability of SULT2B1b

Thermostability has been used to characterize the effects of sequence differences on the activity of several SULT isoforms (Falany et al., 1994). To examine the effects of the PSC-extension on the activity of SULT2B1b, aliquots of either full-length or truncated SULT2B1b were incubated at 42 °C for varying lengths of time and then DHEA sulfation activity was evaluated. Figure 3 demonstrates that while both full-length and truncated SULT2B1b are thermolabile, truncated SULT2B1b was more sensitive to thermal inactivation than the full-length enzyme. After incubation at 42° C for 30 min, DHEA sulfation activity with truncated SULT2B1b was decreased 3.5-fold more than that of full-length SULT2B1b. After 45 min of incubation at 42° C, truncated SULT2B1b activity was completely eliminated while full-length SULT2B1b still maintained about 70% of its original activity. These results indicate that removal of the PSC-extension significantly destabilizes the enzyme to thermal inactivation.

Subcellular localization of SULT2B1b in BeWo cells

Our laboratory has previously reported that SULT2B1b can be localized in both cytosol and nuclei of human cells (He et al., 2004). To investigate the role of the PSC-extension in subcellular localization, cytosol, microsomes and nuclei were isolated from BeWo cells transformed with either full-length or truncated SULT2B1b. Cell fractions were evaluated by immunoblot analysis using rabbit anti-truncated SULT2B1b IgG and a mouse anti-histone monoclonal antibody. Figure 4 shows that in full-length SULT2B1b transfected BeWo cells, SULT2B1b can be detected in nuclei as well as in cytosol and microsomes. However, in truncated SULT2B1b transfected BeWo cells, there is no detectable immunoreactive SULT2B1b in nuclei although it is readily detectable in both the cytosolic and microsomal fractions at approximately the same levels found in cells transfected with the full-length enzyme. As a nuclear marker, histones were specifically detected in nuclei isolated from both full-length and truncated SULT2B1b transfected BeWo cells in approximately equal amounts. These results indicate that full-length SULT2B1b can be localized to the nucleus whereas the truncated form of SULT2B1b is not.

Evaluation SULT2B1b modification by 2D electrophoresis

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Using bioinformatic tools (Phosite 1.0 and Netphos 2.0), analysis of the PSC-extension of SULT2B1b indicated putative sites for serine phosphorylation. The identification of two charged forms of SULT2B1b isolated from transfected BeWo cell lysate by 2D electrophoresis is shown in Figure 5. Subsequent to electrophoresis, identical 2D-gels were analyzed separately. One gel was stained with Sypro to demonstrate the migration of all proteins in the lysate (Fig. 5A) while the second gel was utilized for immunoblot analysis with rabbit anti-SULT2B1b antibodies (Fig. 5B). The immunoblot for SULT2B1b shows two spots at the same apparent molecular weight. This indicates that a SULT2B1b protein modification occurs via charge alteration. Upon comparison with the Sypro stained gel, there was no apparent visible spot that correlated with the SULT2B1b spots detected on the immunoblot.

Mass spectroscopy analysis of SULT2B1b phosphorylation

Mass spectroscopy was used to investigate the possible site of phosphorylation in the PSC-extension of SULT2B1b. SULT2B1b purified from BeWo cells was treated with alkaline phosphatase and compared to an identical untreated control sample as well as to bacterially expressed SULT2B1b. The samples of SULT2B1b were then enzymatically digested with modified sequencing grade Glu C protease, processed and analyzed by MALDI-TOF mass spectroscopy. The resulting monoisotopic masses were searched in the NCBI database. As shown in Figure 6A, digestion of bacterially expressed SULT2B1b with Glu C protease generated a peptide anion with an average m/z of 2652.3 that corresponded to the theoretical peptide anion containing amino acids 343-367 (*REPRPNSSPSPSPGQASETPHPRPS*). In SULT2B1b purified from BeWo cells, there was a 2652 m/z peak as well as an additional peptide with a m/z of 2733. This peptide correlated with the predicted 343-367 peptide anion containing one phosphorylated amino acid (Fig. 6B). After treatment with alkaline phosphatase, the SULT2B1b spectrum contained a peptide peak with the average m/z of 2652, but was lacking the 2733 m/z peptide peak (Fig. 6C). Thus, SULT2B1b expressed in BeWo cells exists at least partially in a phosphorylated form that is not apparent in the enzyme generated by bacterial expression.

Phosphorylation of SULT2B1b by serine protein kinase in vitro

To investigate whether the change in the mass of the 343-367 peptide was a phosphorylation event, purified full-length or truncated SULT2B1b was treated with CK II or Cdc2 protein kinase then immmunoprecipitated with anti-truncated SULT2B1b IgG. The two SULT2B1b proteins were analyzed by immunoblotting with a monoclonal anti-phosphoserine antibody. As shown in Figure 7A, full-length SULT2B1b contained an immunoreactive phosphorylated serine residue after incubation with either CK II or Cdc2 protein kinase, whereas no phosphorylated serine residues were identified in the truncated

SULT2B1b protein (Fig. 7B). These results demonstrate that the PSC-terminus of the full-length SULT2B1b protein is the region capable of undergoing serine phosphorylation.

Serine phosphorylation of SULT2B1b in transfected BeWo cells

Nuclear, microsomal and cytosolic fractions were isolated from both full-length and truncated SULT2B1b transfected BeWo cells and immunoprecipitated with rabbit anti-truncated SULT2B1b IgG. The precipitated proteins were analyzed by immunoblotting with the mouse anti-phosphoserine antibody (Fig. 8A) and rabbit anti-truncated SULT2B1b IgG (Fig. 8B). Phosphorylated serine signals were detected in all subcellular fractions from cells transformed with full-length SULT2B1b. Moreover, nuclear fractions possessed relatively higher phosphoserine levels than either the microsomal or cytosolic fractions from these cells (Fig. 8C). In contrast, there was no detectable phosphoserine in truncated SULT2Bb1 transfected BeWo cellular fractions, as would be expected if the site of phosphorylation was in the PSC-extension.

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DISCUSSION

SULT2B1b is a member of the hydroxysteriod SULT gene family (Her et al., 1998). Expressed SULT2B1b demonstrates a high catalytic affinity for sulfation with 3ß-hydroxysteroids such as DHEA, pregnenolone and androstenediol, and generally low reactivity with 3α-hydroxysteroids and the phenolic hydroxyl of estrogens (Geese and Raftogianis, 2001; Meloche and Falany, 2001). SULT2B1b is 61% similar and 48% identical in amino acid sequence to the other member of this gene family, SULT2A1 (Meloche and Falany, 2001). Comparison of these SULT isoforms shows that SULT2B1b has a carboxyl-end extension of 53 amino acids. Therefore, SULT2B1b has an apparent SDS-PAGE molecular mass of approximately 48 kD as compared to a 34 kD mass for SULT2A1 (Falany et al., 1989; Meloche and Falany, 2001).

The 53 amino acid PSC-extension of SULT2B1b possesses 17 proline residues. Proline has several unique features including the unusual shape of its pyrrolidine ring, conformational constraints on its dihedral angles imposed by its cyclic side chain, its secondary structural preferences, its substituted amide nitrogen and the relative stability of the cis isomer in a peptide bond (Zarrinpar et al., 2003). Thus, proline is structurally unique and, correspondingly, proline-rich motifs are capable of conferring distinct properties to proteins. Protein crystallization studies to ascertain the structure of human SULT2B1b were unsuccessful due to difficulties in crystallization of the PSC-extension, presumably due to the rigid peptide structure conferred by the multiple proline residues (Lee et al., 2003).

The role of the unique PSC-extension in the activity SULT2B1b is not well studied. Fuda et al. (Fuda et al., 2002) reported that removal of the PSC-extension does not affect the sulfation of cholesterol by SULT2B1b. However, the removal of the PSC-extension significantly increased the affinity of SULT2B1b for DHEA as well as decreased the Vmax for DHEA sulfation. Overall, there was a decreased Kcat resulting from removal of the PSC-extension. The thermostability studies indicate that the PSC-extension also has a role in stabilizing the enzyme, possibly via protein-protein interactions. These studies were carried out with the bacterially expressed enzyme and do not account for possible effects from phosphorylation of the PSC-extension.

In proteins, proline generally occurs at solvent-exposed sites in such as loops, turns, N-terminal first turn of helix and random coils (Tchernychev et al., 1997). Therefore, peptides containing proline repeats usually exist on the surface of the protein structure. The peptide structure with proline repeats can consequently serve as a conformation-dependent common “public” epitope for natural antibodies (Tchernychev et al., 1997). Examination of the specificity of the rabbit polyclonal antibody raised to full-length SULT2B1b demonstrated that, when utilized for immunoblot analysis, this antibody readily detected full-length SULT2B1b but did not immunoreact with truncated SULT2B1b. In contrast, the antibody generated to truncated SULT2B1b detected equivalent amounts of truncated and full-length

SULT2B1b (Fig 1). These results suggest that the antibody raised to full-length SULT2B1b was induced primarily by the PSC-extension.

Our previous studies of the subcellular localization of SULT2B1b revealed that SULT2B1b was able to translocate to placental nuclei as well as the nuclei of transformed BeWo cells (He et al., 2004). Analysis of the human SULT2B1b amino acid sequence using the PSORT II subcellular localization program indicates that the composition and sequence of the PSC-extension is indicative of a tendency for nuclear localization. In the present study, when the PSC-extension is removed and truncated SULT2B1b is expressed in BeWo cells the truncated protein was not found in isolated nuclei while it was present in both the cytosolic and microsomal fractions (Fig. 4). This is in contrast to full-length SULT2B1b that is found in the nuclear fraction as well as in cytosol and microsomes. Thus, as predicted by PSORT II, the PSC-extension of SULT2B1b is involved in determining its subcellular localization.

The mechanism by which the PSC-extension of SULT2B1b confers the ability to translocate to the nucleus in placental cells remains unclear. Proline, along with other amino acids like serine or tyrosine, can readily form active phosphorylation sites to enhance protein function (Cornell et al., 1995; Kay et al., 2000; Kitazawa et al., 2000). In addition to proline, the PSC-extension of SULT2B1b is rich in serine. A wide variety of post-translational modifications, such as phosphorylation, glycosylation, methylation and acetylation, are known to play key roles in regulating function, localization, binding specificity and stability of target proteins (Kaufmann et al., 2001). Because 10 of the 53 amino acid residues in the PSC-extension of SULT2B1b are serines, we anticipated that post-translational phosphorylation might be occurring.

He et al. (He et al., 2004) have reported that forskolin treatment increases translocation of SULT2B1b to nuclei in transfected BeWo cells. Forskolin was demonstrated to promote certain protein functions via cAMP-inducible protein kinase enhancement (Kobierski et al., 1999; Li et al., 2000). Thus, it suggests that SULT2B1b nuclear localization is potentially associated with SULT2B1b modification by certain protein kinases. When lysate of SULT2B1b transfected BeWo cells was analyzed by 2D electrophoresis, two different charged forms of SULT2B1b were resolved by isoelectric focusing (Fig. 5). This result implies that expressed SULT2B1b in BeWo cells is modified via charge alteration. MALDI-TOF-MS was used to demonstrate the presence of a phosphate group in the peptide fragment encompassing residues 343-367 (*REPRPNSSPSPSPGQASETPHPRPS*) of the PSC-extension (Fig. 6). The putative phosphorylation site was identified as a phosphoserine residue using an anti-phosphoserine antibody. Analysis of the PSC-extension amino acid sequence using bioinformatic analysis tools (MacVector, Phosite 1.0, Netphos 2.0) indicated that there are several protein kinase targeting sites within PSC-extension, such as CK II and Cdc2 targeting sites. Purified full-length SULT2B1b incubated with either CK II or Cdc2 protein kinase generated a phosphorylated protein signals while truncated SULT2B1b did not (Fig. 7). Also, phosphorylated SULT2B1b was detected in the three cellular compartments of SULT2B1b transfected BeWo cell lysate and the relative levels of the phosphorylated enzyme were highest in nuclei (Fig. 8).

SULT2B1b is the first cytosolic SULT isoform to be reliably localized to nuclei in human tissues as well as also the first to be characterized as undergoing post-translational modification. These characteristics are also associated with the presence of the unique PSC-extension that is not present in other human SULTs. Also, the mouse and rat homologs of SULT2B1 possess only short carboxy-extensions as compared to human SULT2B1b and these sequences lack putative phosphorylation sites (Kohjitani et al., 2006). Whether the mouse or rat homologs of SULT2B1 localize to the nuclei in any tissue has not been reported (Shimizu et al., 2003). The possible functions involved with the nuclear localization of SULT2B1 are not known, although preliminary studies demonstrating the role of SULT2B1b in the regulation of the ability of DHEA to regulate estrogen receptor-ß expression in human LNCaP prostate adenocarcinoma cells has been reported (He et al., 2006).

In summary, SULT2B1b has a unique carboxy-terminal sequence rich in proline and serine residues that is not found in any of the other human SULTs. Our studies demonstrates that the PSC-extension of SULT2B1b is the preferred site for eliciting an immune response, and is involved in nuclear translocation and enzymatic thermostability. It is known that proline residues are important in conferring both immunogenicity and stability; additionally, the serine phosphorylation that occurs in this PSC-extension most likely plays a role in altering protein-protein interactions that may be involved in cellular translocation. Further investigation to determine whether SULT2B1b can interact with other proteins or partners at its carboxyl terminus that promote SULT2B1b to translocate into nuclei of placental cells is underway and should lead to further biochemical characterization of SULT2B1b.

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FOOTNOTES

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Figure Legends

Figure 1. Immunogenicity of SULT2B1b PSC-terminus. Antibodies raised to full-length or truncated SULT2B1b was used in immunoblot analysis of purified full-length and truncated SULT2B1b. (A) Immunoblot with full-length SULT2B1b antibody of full-length SULT2B1b (lanes 1-3; 45, 90 and 225 ng) and truncated SULT2B1b (lanes 4-6; 1, 5 and 10 μg) and (B) immunoblot with truncated SULT2B1b antibody of full-length SULT2B1b (lanes 1-3; 45, 90 and 225 ng) and truncated SULT2B1b (lanes 4-6; 45, 90 and 225 ng).

Figure 2. DHEA sulfation by full-length and truncated SULT2B1b. Purified full-length and truncated bacterially expressed SULT2B1b were used to sulfate increasing concentrations of DHEA. Full-length and truncated SULT2B1b sulfation activities were examined with varying concentrations of DHEA ranged from 0.05 - 5 μM.

Figure 3. Enzymatic thermostability of SULT2B1b. Purified full-length and truncated bacterially expressed SULT2B1b were incubated at 42°C for various time intervals and evaluated for DHEA sulfation activity. Relative activity was expressed as percentage of the enzymatic activity with no 42°C incubation (100%). Squares denote full-length SULT2B1b activity; circles denote truncated SULT2B1b activity.

Figure 4. Subcellular localization of SULT2B1b in BeWo cells. Cytosol, microsomes and nuclei were isolated from BeWo cells transformed with either full-length or truncated SULT2B1b using an Optiprep gradient. Subcellular fractions on duplicate immunoblots were analyzed with either the anti-truncated SULT2B1b IgG or mouse anti-histone monoclonal antibody. (A) Immunoblot with anti-truncated SULT2B1b IgG. Lanes 1, 3 and 5; cytosol (120 μg), microsomes (80 μg) and nuclei (12 μg), respectively, from BeWo cells transformed with full-length SULT2B1b. Lanes 2, 4 and 6 contain cytosol (120 μg), microsomes (80 μg) and nuclei (12 μg), respectively, from BeWo cells transformed with truncated SULT2B1b. (B) Immunoblot of the same fractions using a mouse monoclonal anti-histone IgG.

Figure 5. 2D electrophoresis of SULT2B1b.
SULT2B1b transfected BeWo cell lysate was evaluated by 2D electrophoresis. The 2D gels were run as described in methods. (A) Immunoblot of the 2D gel with anti-SULT2B1b antibody. Horizontal direction, left to right, represents pH gradient 3-10. The vertical direction with protein molecular weight markers on the left demonstrates SDS-PAGE. Arrowheads indicate the two protein spots with differing charges. (B) 2D gel stained with Sypro to show total protein migration pattern.

Figure 6. Mass spectrometry analysis of SULT2B1b phosphorylation.

Bacterially expressed SUL2B1, and SULT2B1b from BeWo cells treated alkaline phosphatase were enzymatically digested with modified sequencing grade Glu C and subjected to MALDI-TOF analysis. (A) Mass spectrum of peptides from bacterially expressed SULT2B1b. (B) Mass spectrum of peptides from BeWo cell expressed SULT2B1b. (C) Mass spectrum of peptides from BeWo cell expressed SULT2B1b treated with alkaline phosphatase. Regions of the spectra containing only the peptides of interest are shown.

Figure 7. Phosphorylation of SULT2B1b by serine protein kinase *in vitro*.

Purified full-length or truncated SULT2B1b (2 μg) was incubated with CK II or Cdc2 protein kinase. Reaction products were subjected to immunoblot analysis with monoclonal anti-phosphoserine antibody. (A) Full-length SULT2B1b. Lane 1, untreated CKII control; lane 2, CKII treatment; lane 3, untreated Cdc2 control; lane 4, Cdc 2 treatment. (B) Truncated SULT2B1b. Lane 1, untreated CKII control; lane 2, CKII treatment; lane 3, untreated Cdc2 control; lane 4, Cdc 2 treatment. SULT2B1b phosphoserine bands are indicated on the right side.

Figure 8. Immunoblot analysis of serine phosphorylation of SULT2B1b in transfected BeWo cells.

Nuclear, microsomal and cytosolic fractions (600 μg) isolated from full-length or truncated SULT2B1b transformed BeWo cells were immunoprecipitated with rabbit anti-truncated SULT2B1b IgG. The precipitated proteins were analyzed by immunoblot using mouse anti-phosphoserine monoclonal antibody or rabbit anti-truncated SULT2B1b IgG. (A) Immunoreactive phosphoserine protein in subcellular fractions. Left, full-length SULT2B1b; right, truncated SULT2B1b. (B) Immunoreactive SULT2B1b. Left, full-length SULT2B1b; right, truncated SULT2B1b. The different subcellular fractions are labeled appropriately. (C) Relative optical density of phosphoserine bands compared to SULT2B1b bands for full-length SULT2B1b.

Figure 1.

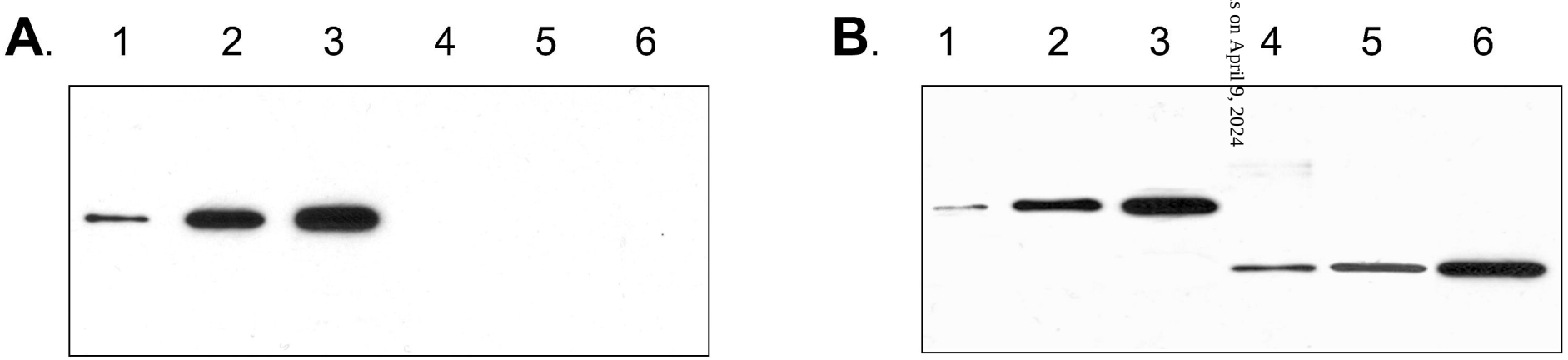

Figure 2

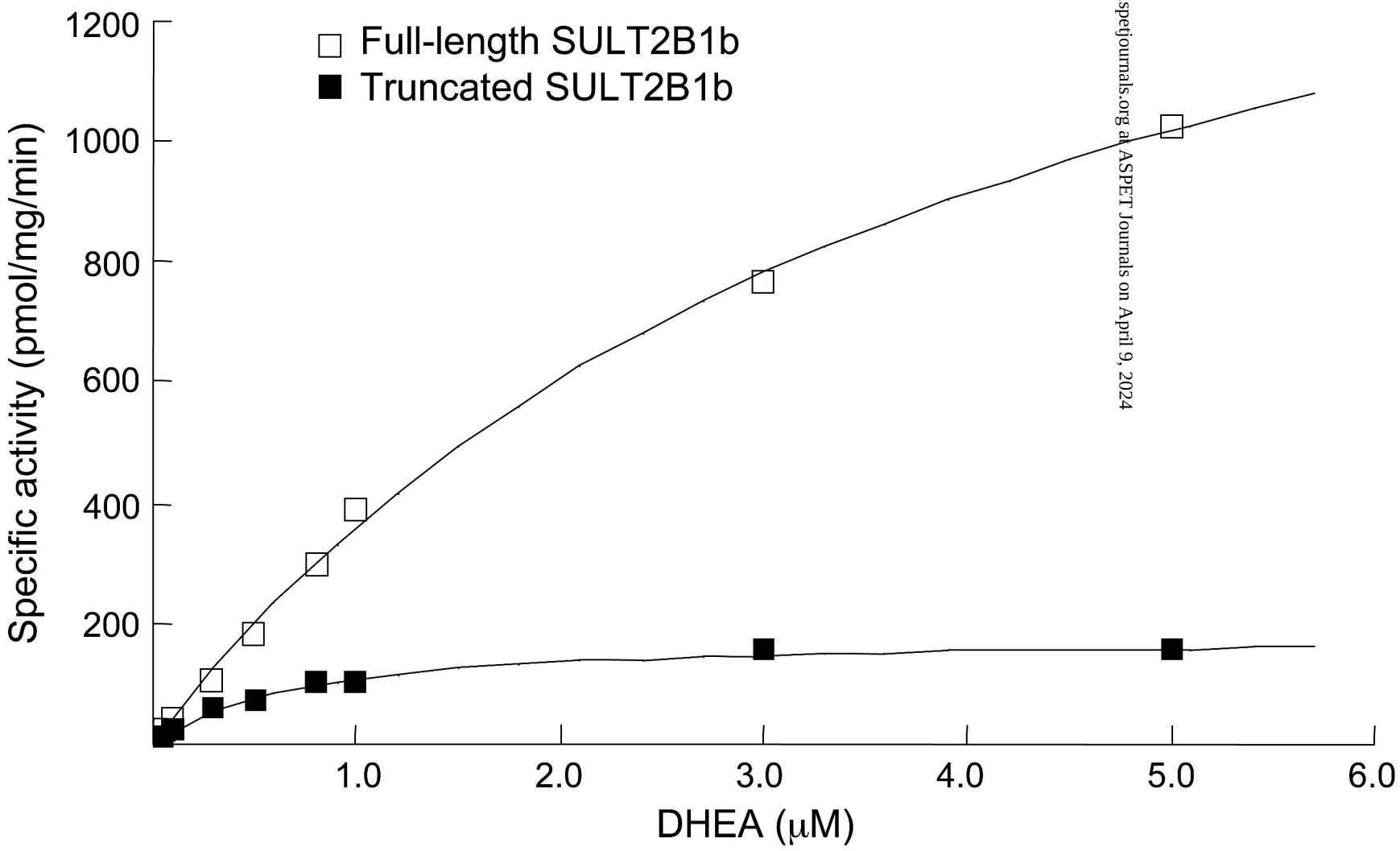

Figure 3

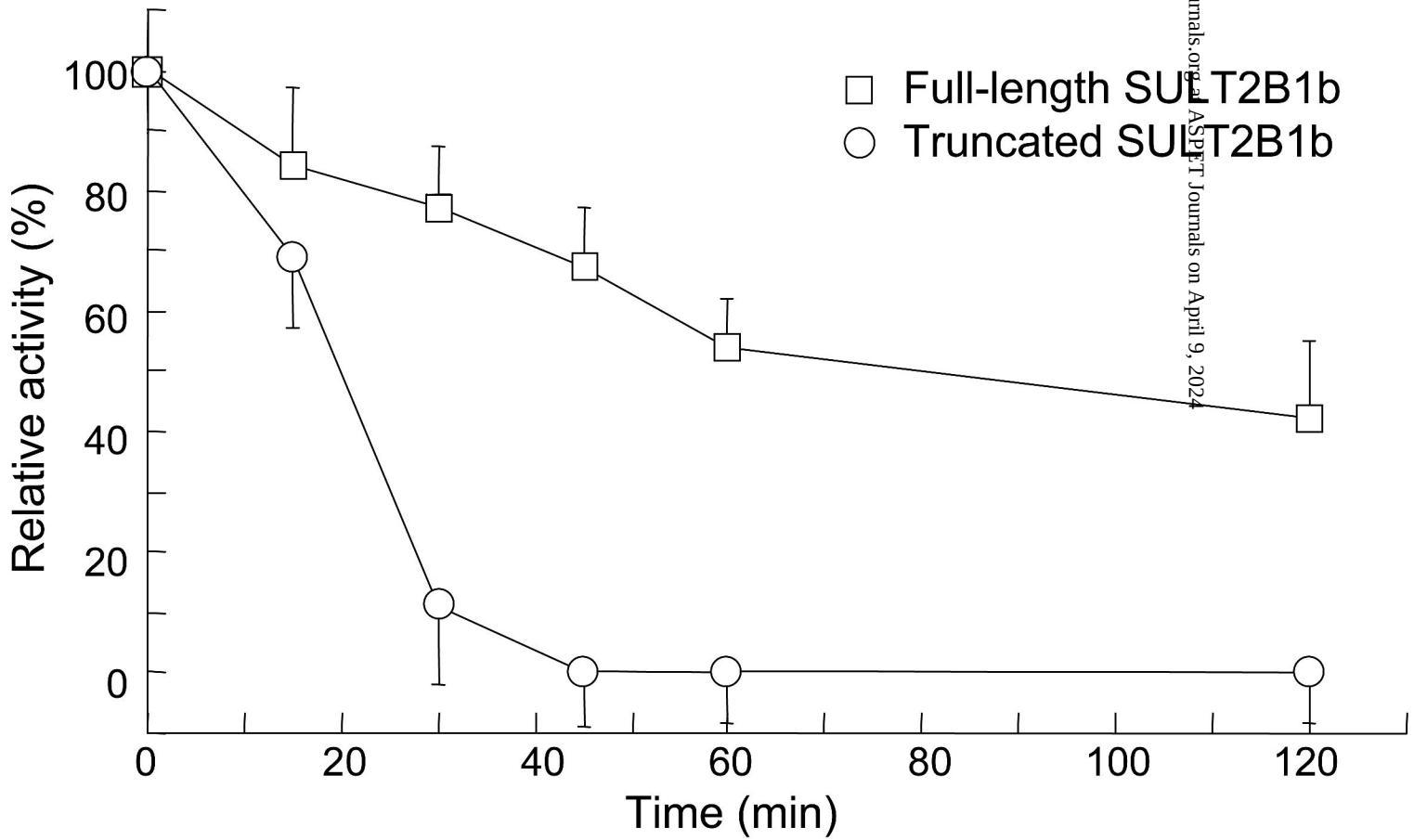

Figure 4

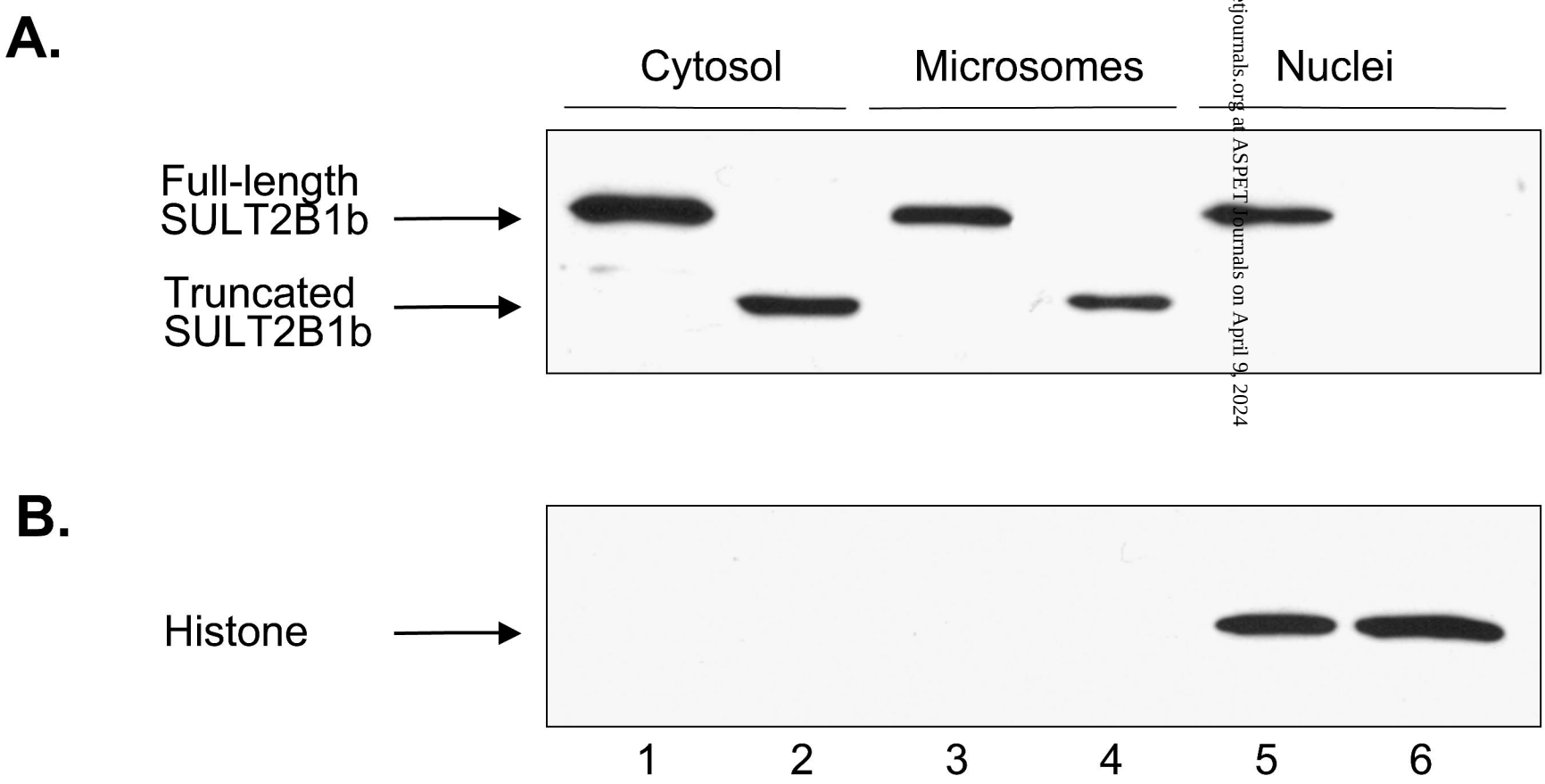

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Figure 5

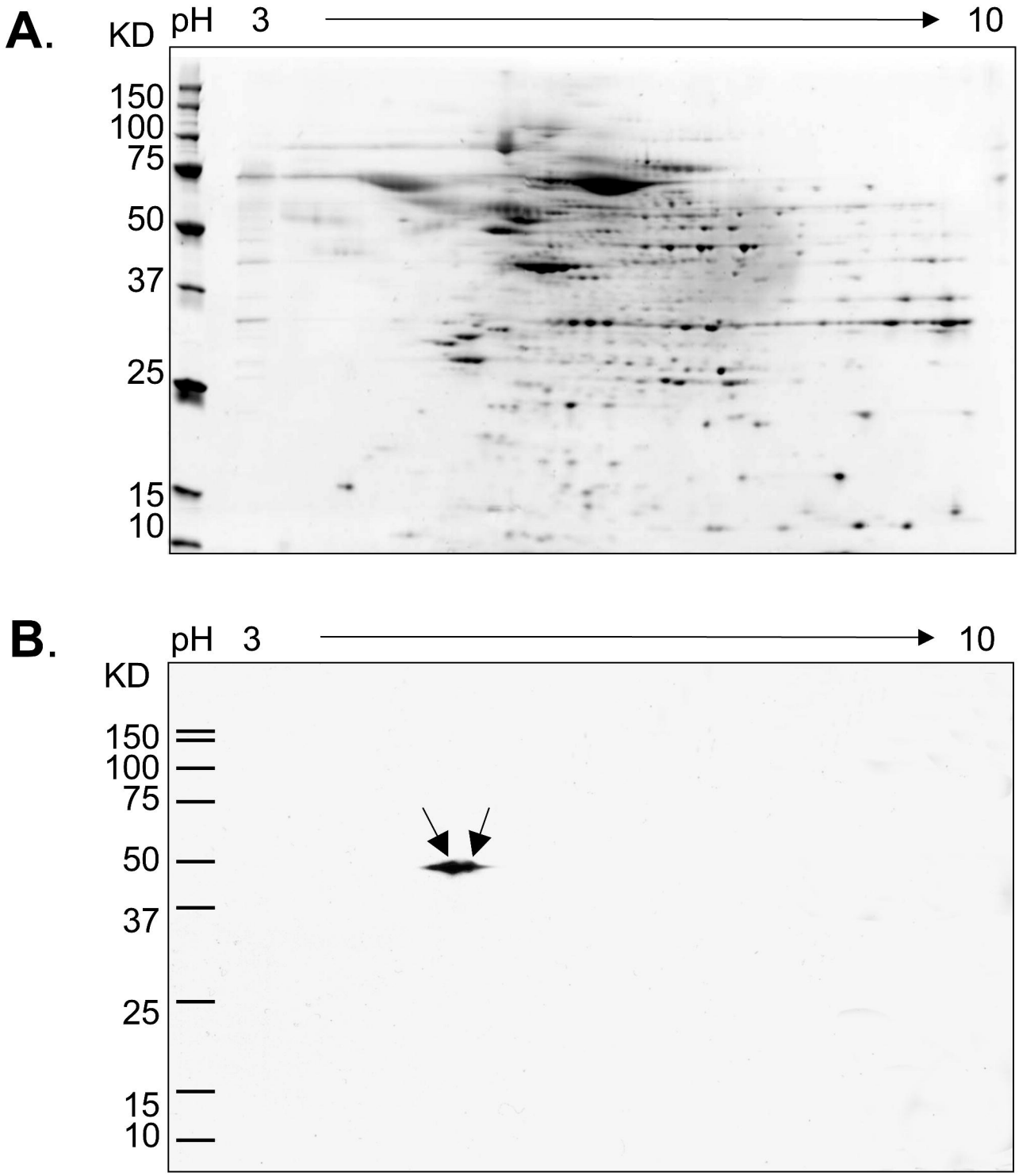

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Figure 6

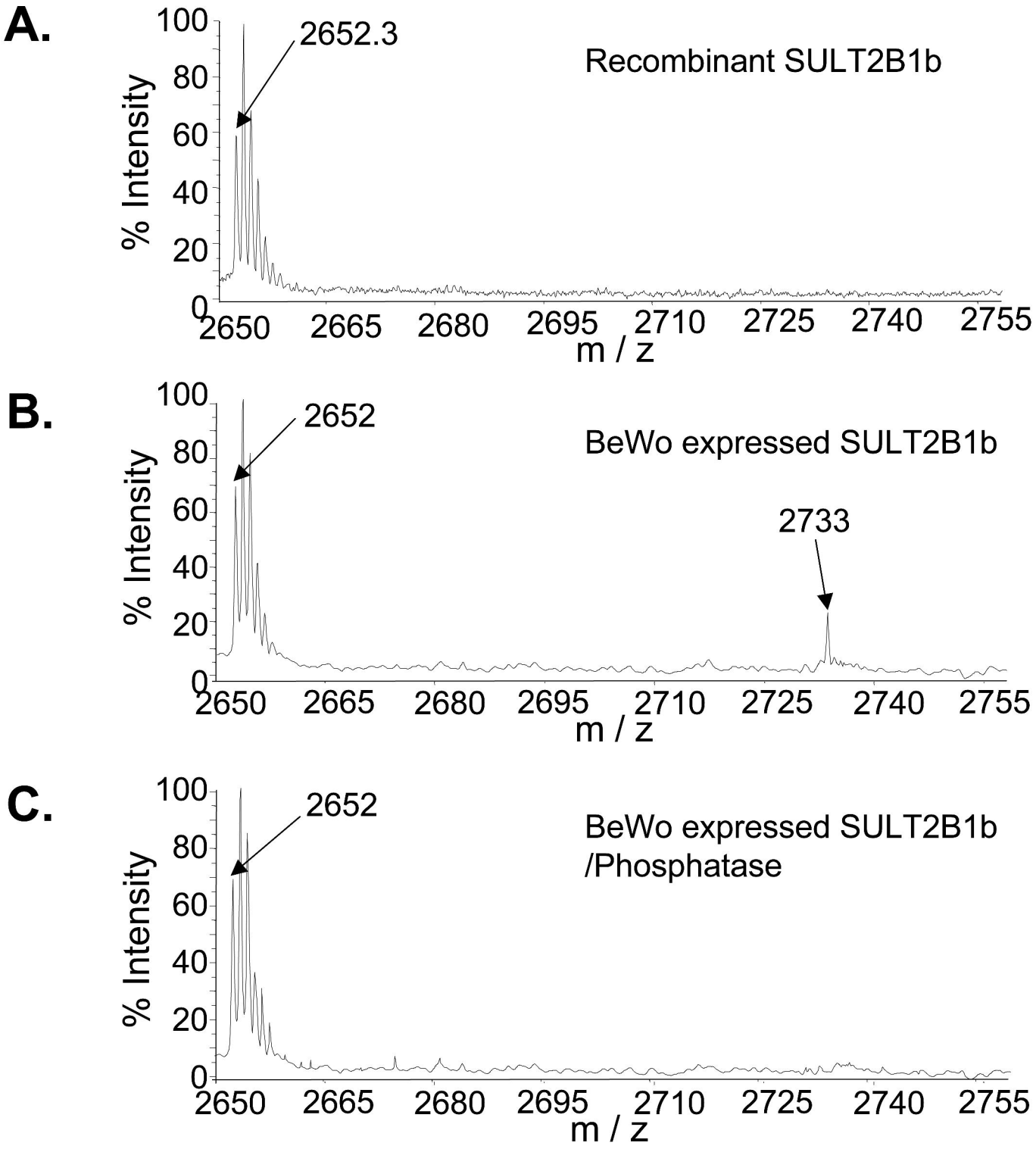

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Figure 7

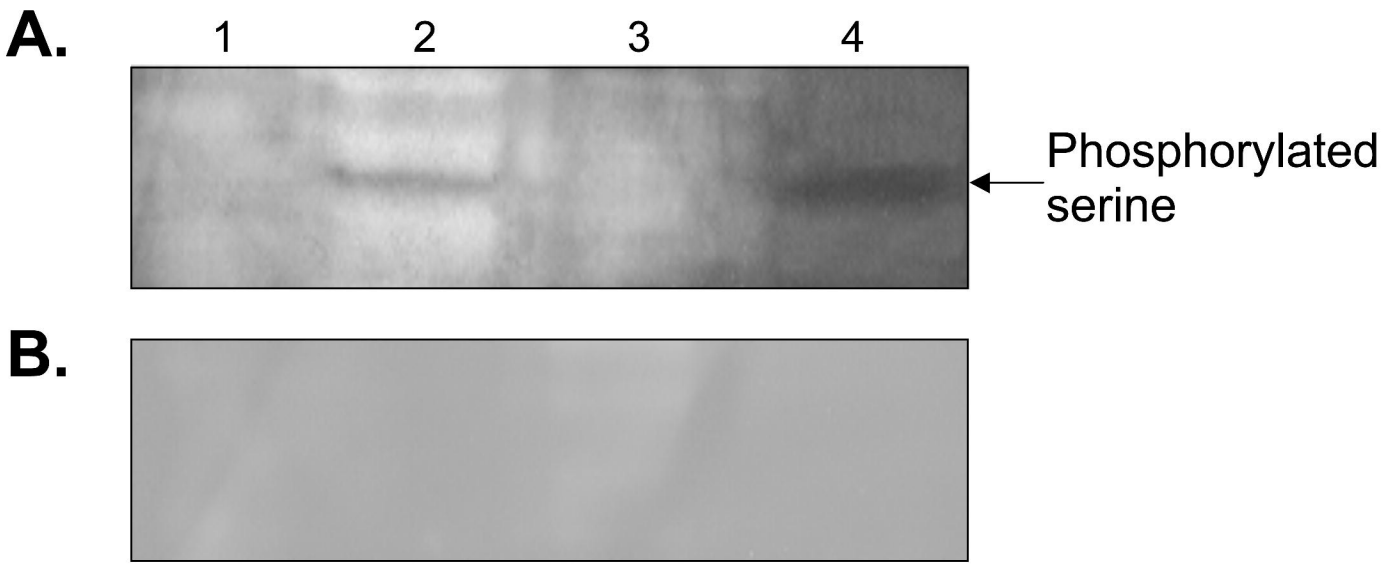

Figure 8