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Cytosolic Aryl Sulfotransferase 4A1 Interacts with the Peptidyl Prolyl *Cis-Trans* Isomerase Pin1

Deanne J. Mitchell and Rodney F. Minchin

School of Biomedical Sciences, University of Queensland, St. Lucia, Queensland, Australia

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ABSTRACT

Sulfonation by cytosolic sulfotransferases plays an important role in the metabolism of both endogenous and exogenous compounds. Sulfotransferase 4A1 (SULT4A1) is a novel sulfotransferase found primarily in neurons in the brain. It is highly conserved between species, but no substantial enzyme activity has been identified for the protein. Consequently, little is known about the role of this enzyme in the brain. We performed a yeast two-hybrid screen of a human brain library to isolate potential SULT4A1-interacting proteins that might identify the role or regulation of the sulfotransferase in humans. The screen isolated the peptidyl-prolyl *cis-trans* isomerase Pin1. Its interaction with SULT4A1 was confirmed by coimmunoprecipitation studies in HeLa cells and by in vitro pull-down of expressed proteins. Moreover, Pin1 binding was dependent on phosphorylation of the SULT4A1 protein. Pin1 destabilized SULT4A1, decreasing its half-life from more than 8 h to approximately 4.5 h. This effect was dependent on the isomerase activity of Pin1 and was inhibited by okadaic acid, suggesting a role for the phosphatase PP2A. Pin1-mediated SULT4A1 degradation did not involve the proteosomes or macroautophagy, but it was inhibited by the calpain antagonists *N*-acetyl-Leu-Leu-Nle-CHO and Z-Val-Phe-CHO. Finally, Pin1 binding was mapped to two threonine-proline motifs (Thr^{8} and Thr^{11}) that are not present in any of the other human cytosolic sulfotransferases. Our findings suggest that SULT4A1 is subject to post-translational modification that alters its stability in the cell. These modifications may also be important for enzyme activity, which explains why specific substrates for SULT4A1 have not yet been identified.

The cytosolic sulfotransferases catalyze the transfer of a sulfonyl moiety from the cofactor 5′-phosphoadenosine-3′-phosphosulfate (PAPS) to various acceptor molecules, which results in a more hydrophilic metabolite prone to rapid renal excretion (Gamage et al., 2006; Nowell and Falany, 2006; Hempel et al., 2007). To date, 13 human cytosolic sulfotransferase (SULT) genes have been identified, which have been grouped into four families based on amino acid sequence homology: *SULT1*, *SULT2*, *SULT4*, and *SULT6* (Blanchard et al., 2004; Freimuth et al., 2004). The members of each family differ considerably in their substrate specificity and tissue distribution (Coughtrie, 2002). The essential role of some SULTs has been demonstrated in knockout mice models and by the analysis of genetic polymorphisms in human populations (Coughtrie, 2002; Tong et al., 2005; Nowell and Falany, 2006).

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The SULT4 family contains only one member, SULT4A1, which is found almost exclusively in selected regions of the brain (Liyou et al., 2003). The human gene was first cloned from brain cDNA in 2000 (Falany et al., 2000). However, specific substrates for the enzyme have not been identified. SULT4A1 shares less than 36% amino acid sequence homology with other known cytosolic sulfotransferases; unlike many other human SULTs, it is not located in a gene cluster. Nevertheless, it is highly conserved between species, with the human, mouse, and rat SULT4A1 isoforms sharing 97% sequence homology (Falany et al., 2000; Sakakibara et al., 2002; Blanchard et al., 2004). SULT4A1 is highly unusual in that it exhibits the lowest sequence polymorphism of any of the humans SULTs (Hildebrandt et al., 2007) with no synonymous or nonsynonymous base changes in the exonic regions of the gene (Lewis and Minchin, 2009).

Most neurotransmitters, such as the catecholamines, dopamine, and serotonin, are sulfonated by one or more of the cytosolic sulfotransferases. This metabolic pathway leads to their inactivation and eventual excretion, primarily via the

ABBREVIATIONS: PAPS, 5′-phosphoadenosine-3′-phosphosulfate; SULT, sulfotransferase; 3-MA, 3-methyladenine; PP2A, protein phosphatase 2A; HA, hemagglutinin A; BD, binding domain; GST, glutathione transferase; CIP, calf intestinal alkaline phosphatase; ALLN, *N*-acetyl-Leu-Leu-Nle-CHO; MDL28170, *Z*-Val-Phe-CHO; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; MG132, *N*-benzoyloxycarbonyl (*Z*)-Leu-Leu-leucinal; NP40, Nonidet P-40.

kidneys. Thus, the sulfotransferases have been proposed as critical enzymes in neurotransmitter metabolism (Gamage et al., 2006). Although SULT4A1 is capable of binding both epinephrine and norepinephrine (Allali-Hassani et al., 2007), it does not seem to sulfonate these substrates. This may be because the active site of the enzyme is usually smaller than that found in other sulfotransferases, which may inhibit efficient binding of the cofactor PAPS (Allali-Hassani et al., 2007).

The high level of conservation across species and the restricted tissue expression of SULT4A1 suggest an important neuronal function for this protein. However, apart from a recent genetic link to schizophrenia susceptibility (Brennan and Condra, 2005; Condra et al., 2007), the exact physiological role of SULT4A1 has remained elusive. We performed a yeast two-hybrid screen using SULT4A1 as bait and a human brain cDNA library as prey in an attempt to identify interacting proteins involved in SULT4A1 regulation and/or function. Here we report the interaction of SULT4A1 with the essential peptidyl-prolyl *cis-trans* isomerase Pin1.

Materials and Methods

Molecular Cloning. The SULT4A1 coding region was inserted into the yeast expression vector pGBKT7 (Clontech, Mountain View, CA) using the primers GATCCATATGATGGCGGAGAGCGAGGCC and GATCGGATCCTTATAAATAAAAGTCAAACGTGAGGTC. The SULT4A1 coding region was excised from pEF-SULT4A1 and inserted in-frame into the mammalian expression vector pFLAG-CMV-7.1 (Sigma-Aldrich, St. Louis, MO) using the Kpn1 and Xba1 restriction sites. To clone SULT4A1 into the C-terminal FLAG tag vector pFLAG-CMV-14, the coding region was PCR-amplified using the primers GGTACCGGTACCAATGGCGGAGAGCGAGGCC and GATCTCTAGATAAATAAAAGTCAAACGTGAG and cloned using the Xba1 and Kpn1 restriction sites. For generation of the FLAG-Pin1 and pEF-Pin1 constructs, the entire Pin1 coding region was amplified from the pGADT7 library construct isolated from the yeast two-hybrid library screen and cloned in-frame into pFLAG-CMV-7.1 using the primers GATCGAATTCAATGGCGGACGAGGAGAAGCTG and GATCGGTACCCCACTCAGTG-CGGAGGATGATG. The FLAG-Pin1($C^{113}A$) mutant was generated using the GeneTailor Site-Directed Mutagenesis kit (Invitrogen, Carlsbad, CA) and the primers CTGGCCTCACAGTTCAGCGACGCCAGCTCAGCCAAG and GTCGCTGAACTGTGAGGCCAGAGACTCAAAGTC. To generate the FLAG-tagged SULT4A1 mutants SULT4A1-$T^{8}A$ and SULT4A1-$T^{11}A$, the SULT4A1 coding region was PCR-amplified using the forward primers GGGGTACCACATATGGCGGAGAGCGAGGCCGAG**G**CCCCCAGC and GGGGTACCACATATGGCGGAGAGCGAGGCCGAGACCCCCAGC**G**CCCCGGGGGAG, respectively, and the reverse primer GATCGGATCCTTATAAATAAAAGTCAAACGTGAGGTC. The SULT4A1 mutant primers contain a nucleic acid residue change to a **G** (shown in boldface type) at bases 24 or 33 to create the amino acid mutation of the threonine to an alanine at residues 8 or 11, respectively. The SULT4A1-mutated PCR products were then cloned into the pFLAG-CMV-7.1 vector using the Kpn1 and Xba1 restriction sites. The SULT4A1-$S^{104}A$ construct was generated using the GeneTailor Site-Directed Mutagenesis kit (Invitrogen). The primers CATCATCAAGGAA-CTGACC**G**CTCCCCGCCTCATC and GGTCAGTTCCTTGATGATGTCCAGGCCCGG and FLAG-SULT4A1 template were used to change the nucleic acid at 310 to a **G** (in boldface type), altering the serine at position 104 into an alanine.

The HA-tagged SULT4A1 wild type and mutants SULT4A1($T^{8}A$), SULT4A1($T^{11}A$) and SULT4A1$S^{104}A$ were generated by PCR amplification of the coding sequence from the corresponding pFLAG-CMV-7.1-SULT4A1 constructs using the forward and reverse primers GATCGGTACCATGGCGGAGAGCGAGGCCGAG and GATCGATC-GCGGCCGCTTATAAATAAAAGTCAAACGTGAGGTC, respectively. The resulting fragments were then cloned in frame into the respective Kpn1 and Not1 restriction sites of pHM6 (Roche, Indianapolis, IN). All constructs generated using PCR were verified by DNA sequencing.

Yeast Two-Hybrid Library Screen. The full-length human SULT4A1 was cloned into pGBKT7, the DNA binding domain (BD) fusion vector and was used as the bait in a yeast two-hybrid screen. A human brain cDNA library generated in the activating domain fusion vector pGADT7 and pretransformed into the 187 yeast strain was used in this study (Clontech). The bait was transformed into the AH109 yeast strain and mated with the pretransformed library per the manufacturer's instructions. Protein interactions were selected for on Leu^{-}, Trp^{-}, His^{-}, and Ade^{-} solid media. Putative positive clones were further analyzed and validated as follows. The library plasmid was isolated from yeast and after purification from *Escherichia coli* was retransformed into yeast expressing the SULT4A1 bait or Lamin to confirm reporter activation specificity. To investigate the involvement of flanking sequences and fusion proteins in reporter activation, library plasmids were sequenced, and the coding region of the putative interacting protein was cloned into the DNA BD-containing vector and tested with the activating domain plasmid containing SULT4A1 cDNA.

Cell Lines and Transfections. HeLa cells (initially from American Type Culture Collection, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% turbo calf serum and incubated at 37°C and 5% CO_2. HeLa cells were plated at 10^6 cells/well in a six-well plate. Cells were transfected with 2 μg of pFLAG-Pin1 or 2 μg of pFLAG-SULT4A1 or pHM6-SULT4A1 for 24 h before treatment. Total transfected DNA was held constant at 4 μg by the addition of empty control plasmid. For ubiquitin studies, 1 μg of pcDNA3-HA-ubiquitin was also added. Plasmid DNA was transfected using LipofectAMINE 2000 reagent (Invitrogen) according to the manufacturer's instructions.

Coimmunoprecipitation and GST Pull-Down Experiments. Transfected cells were washed in phosphate-buffered saline, and whole-cell extracts were prepared using NP-40 lysis buffer (1% Nonidet P-40, 50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM NaF, 1 mM Na_3VO_4 10 mM β-glycerophosphate, 1 mM EDTA, and protease inhibitor cocktail; Roche, Basel, Switzerland). Lysates were cleared by centrifugation at 14,000*g* for 10 min at 4°C after incubation in lysis buffer for 15 min on ice. Coimmunoprecipitation samples were incubated with or without rabbit anti-HA antibody (Sigma-Aldrich) for 2 h at 4°C, after which protein A Sepharose beads (Sigma-Aldrich) were added, and the samples were incubated for a further 1 h at 4°C. For GST pull-down assays, recombinant GST-tagged proteins were expressed in *E. coli* strain BL21-Codon Plus (DE3)-RIL (Stratagene, La Jolla, CA) with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (Sigma-Aldrich) at 37°C. Bacterial cells were harvested and lysed in NP-40 lysis buffer on ice for 15 min. Lysate was cleared by centrifugation at 14,000*g* for 10 min at 4°C. Expressed GST or GST-Pin1 was purified using glutathione Sepharose 4B (GE Healthcare, Rydalmere, NSW, Australia). Cell lysates were incubated with 25 μg of GST or GST-Pin1 on glutathione Sepharose for 4 h at 4°C. For both coimmunoprecipitation and GST pull-down samples, beads were washed three times with wash buffer (0.5% Nonidet P-40, 50 mM Tris, pH 8.0, and 150 mM NaCl) followed by suspension in SDS loading buffer. Samples were separated using SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with mouse anti-FLAG M2 horseradish peroxidase-conjugated primary antibody (Sigma-Aldrich) to detect FLAG-Pin1 binding. To validate HA-protein pull-down, blots were incubated with mouse anti-HA antibody followed by anti-mouse horseradish peroxidase-conjugated secondary IgG (GE Healthcare). Protein bands were visualized with ImmunoStar (Bio-Rad Laboratories, Hercules, CA) as per the manufacturer's instructions. In the case of phosphatase treatment, cellular lysates were prepared in NP-40 lysis buffer without phosphatase

inhibitors. Cleared lysate was divided, and 5 mM NaF, 1 mM Na_3VO_4, and 10 mM β-glycerophosphate were added to one sample, and 20 U/ml calf intestinal alkaline phosphatase (CIP; New England Biolabs, Ipswich, MA) was added to the other. Both samples were incubated for 30 min at 30°C.

Protein Half-Life. Transfected cells were incubated with 10 μg/ml cycloheximide (Sigma-Aldrich) 24 h after transfection and collected at indicated time points. For inhibitor treatment, cells were also incubated with okadaic acid (100 nM), MG132 (20 μM), ionomycin (1 μM), ALLN (50 μM), MDL 28170 (30 μM), 3-methyladenine (3-MA; 5 mM), or delivery vehicle for the appropriate time. Transfected cells were washed in phosphate-buffered saline and removed from wells by scraping in NP-40 lysis buffer containing protease inhibitor cocktail (Roche, Basel, Switzerland). Lysates were cleared by centrifugation at 14,000*g* for 10 min at 4°C after incubation in lysis buffer for 15 min on ice. Total protein was determined by the Bradford method (Bio-Rad Laboratories) using bovine serum albumin as a standard. An equal quantity of protein from each sample was subjected to SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with mouse anti-FLAG M2 horseradish peroxidase-conjugated primary antibody (Sigma-Aldrich) and mouse antitubulin primary antibody (Calbiochem, San Diego, CA) followed by anti-mouse horseradish peroxidase-conjugated secondary IgG (GE Healthcare). Protein bands were visualized with ImmunoStar (Bio-Rad Laboratories) as per manufacturer's instructions. Densitometry was performed using Quantity One version 4.5.

In Vivo Ubiquitination. Transfected HeLa cells were incubated with MG132 (20 μM) for 8 h, after which HeLa cell lysates were prepared as described previously and then incubated with anti-FLAG M2 monoclonal antibody (Sigma-Aldrich) and continued as described in the coimmunoprecipitation section. SULT4A1 protein ubiquitination was detected using rabbit anti-HA primary antibody (Sigma-Aldrich) followed by anti-rabbit horseradish peroxidase-conjugated secondary IgG (GE Healthcare).

Data Analysis. Data are presented as the mean ± S.E.M. All comparisons were performed using a Student's *t* test assuming a level of significance at $p < 0.05$.

Results

Pin1 Is a Binding Partner of SULT4A1. To identify novel binding partners of SULT4A1, the full-length protein was used in a yeast two-hybrid screen of a human brain cDNA library. A number of independent clones were identified, and their binding to SULT4A1 was confirmed. One set of clones was SULT4A1 itself, which is expected given that the cytosolic sulfotransferases, including SULT4A1, contain a dimerization site in their C termini (Minchin et al., 2008). A second set of clones contained the complete open-reading frame for Pin1, a ubiquitously expressed protein that belongs to the parvulin family of peptidyl-prolyl *cis-trans* isomerases. Pin1 specifically interacts with phosphorylated serine/threonine-proline motifs and catalyzes the *cis*-to-*trans* isomerization of prolines (Zhou et al., 1999). The interaction of SULT4A1 and Pin1 was confirmed by testing GAL4AD-Pin1 with GAL4DNA-BD-Lamin for nonspecific reporter gene activation and by inverse transformation of yeast cells with GAL4AD-SULT4A1 and GAL4DNA-BD-Pin1. Negative reporter gene activation with Lamin and positive activation seen with inverse transformations suggested that Pin1 is a potential SULT4A1 binding protein. When other human SULTs (SULT1A1, SULT1B1, SULT1C1, SULT1E1, or SULT2A1) were used as bait in the yeast two-hybrid assay, no positive interaction with Pin1 was observed. This suggested that the SULT4A1/Pin1 interaction was highly specific.

To determine whether SULT4A1 and Pin1 interacted in a mammalian cell system, we carried out coimmunoprecipitation experiments in HeLa cells transiently transfected with FLAG-Pin1 and HA-SULT4A1. Pin1 coimmunoprecipitated with SULT4A1 in the presence but not in the absence of HA antibody (Fig. 1A). Pin1 did not pull down in cells transfected with the Pin1 construct alone. To confirm this interaction in an in vitro assay, bacterially expressed GST-Pin1 was incubated with cytosolic extract from HeLa cells transfected with HA-SULT4A1 (Fig. 1B). SULT4A1 was detected after pull down with GST-Pin1 but not with GST alone (Fig. 1B). These data show the binding of SULT4A1 and Pin1 both in vitro and in vivo. Finally, Pin1 is known to bind preferentially to phosphorylated serine/threonine-proline motifs (Yaffe et al., 1997). To verify that Pin1 bound to phosphorylated SULT4A1, cell lysate was pretreated with CIP to eliminate the phosphorylation of serine, threonine, and tyrosine residues. Pin1 coimmunoprecipitated with SULT4A1 from untreated cell lysates but not from CIP-treated lysates, indicat-

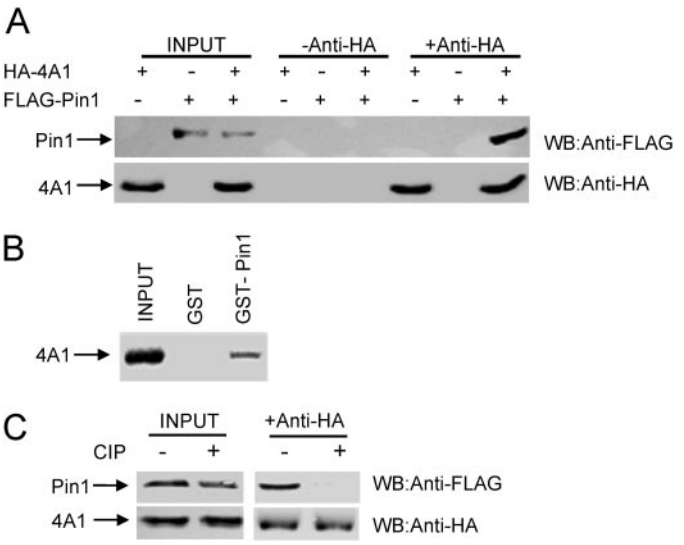

Fig. 1. Interaction of SULT4A1 and Pin1 in HeLa cells. A, coimmunoprecipitation of SULT4A1 and Pin1 in HeLa cells. Cells were transfected with HA-SULT4A1 and FLAG-Pin1 as indicated. Cell lysates were collected in lysis buffer 24 h after transfection, cleared by centrifugation at 15,000*g* for 10 min, immunoprecipitated with anti-HA antibody, and then analyzed by Western blotting (WB) with anti-FLAG and anti-HA antibodies. Columns under Input demonstrate the expression of each protein in the cell extracts. In the absence of anti-HA antibody, no immunoprecipitation was observed. In the presence of anti-HA antibody, both SULT4A1 (bottom blot) and Pin1 (top blot) were pulled down. As controls, cells were transfected with only HA-SULT4A1 or FLAG-Pin1. No coimmunoprecipitation was observed when the proteins were expressed alone. B, in vitro pull-down of SULT4A1 and Pin1. Cell lysates prepared from FLAG-4A1-transfected HeLa cells were incubated with 25 μg of GST (lane 2) or GST-Pin1 (lane 3) bound to GSH-Sepharose beads for 4 h at 4°C. After washing, the beads were suspended in SDS loading buffer, and bound proteins were analyzed by Western blotting with anti-FLAG antibody. Input (lane 1) demonstrates SULT4A1 expression in transfected HeLa cells. SULT4A1 bound to Pin1 but not to the GST alone. C, effect of phosphatase treatment on SULT4A1 binding to Pin1. FLAG-SULT4A1 and FLAG-Pin1 transfected HeLa cells were collected in lysis buffer 24 h after transfection and treated with either phosphatase inhibitors (5 mM NaF, 1 mM Na_3VO_4, and 10 mM β-glycerophosphate) or with 20 U/ml CIP. Samples were immunoprecipitated with anti-HA antibody, and the resulting pulled-down proteins were analyzed by Western blotting using anti-FLAG and anti-HA antibodies. Input lanes demonstrate the expression of each protein in the HeLa cell lysates. Pin1 coimmunoprecipitated with SULT4A1 in the absence of CIP treatment (lane 3) but not after CIP treatment (lane 4). Bottom Western blot shows that SULT4A1 was immunoprecipitated in each condition.

ing that Pin1 interacts with SULT4A1 in a phosphorylation-dependent manner (Fig. 1C).

Pin1 Facilitates the Instability of SULT4A1. Pin1 interaction has previously been shown to regulate the stability of several proteins such as c-Jun, nuclear factor-κB, p53, c-Myc, steroid receptor coactivator-3, and Pim-1 (Wulf et al., 2001; Ryo et al., 2003; Yeh et al., 2004; Yi et al., 2005; Ma et al., 2007). We explored the effect of Pin1 expression on SULT4A1 protein stability in HeLa cells transfected with FLAG-Pin1, or the empty vector control, and FLAG-SULT4A1 in the presence of cycloheximide. In the absence of Pin1, SULT4A1 half-life was greater than 8 h, whereas in the presence of the isomerase, it was less than 5 h (Fig. 2A). To ensure that these results were not due simply to a loss of epitope, SULT4A1 was quantified using a SULT4A1-specific polyclonal antibody (Liyou et al., 2003), and similar results were seen. In addition, introduction of the FLAG tag on the C terminus of the protein also resulted in a similar Pin1-dependent destabilization of the SULT4A1 (data not shown).

We next tested whether isomerization activity of Pin1 was required for SULT4A1 destabilization by mutating the critical cysteine at the active site of the enzyme (Cys^{113}) to an Ala. The resulting enzyme is devoid of catalytic activity but can still bind target proteins via its WW domain (Lu et al., 1999; Winkler et al., 2000; Zhou et al., 2000; Liu et al., 2001; Messenger et al., 2002). The Pin1($C^{113}A$) mutant did not destabilize SULT4A1 (Fig. 2B), as seen with the wild-type protein (Fig. 2A). A coimmunoprecipitation of SULT4A1 and Pin1($C^{113}A$) was performed that showed the mutant Pin1 still interacted with the sulfotransferase (Fig. 2C). Taken together, these results suggest that SULT4A1 can undergo Pin1-dependent post-translational modification and that one outcome of this modification is an increase in SULT4A1 degradation.

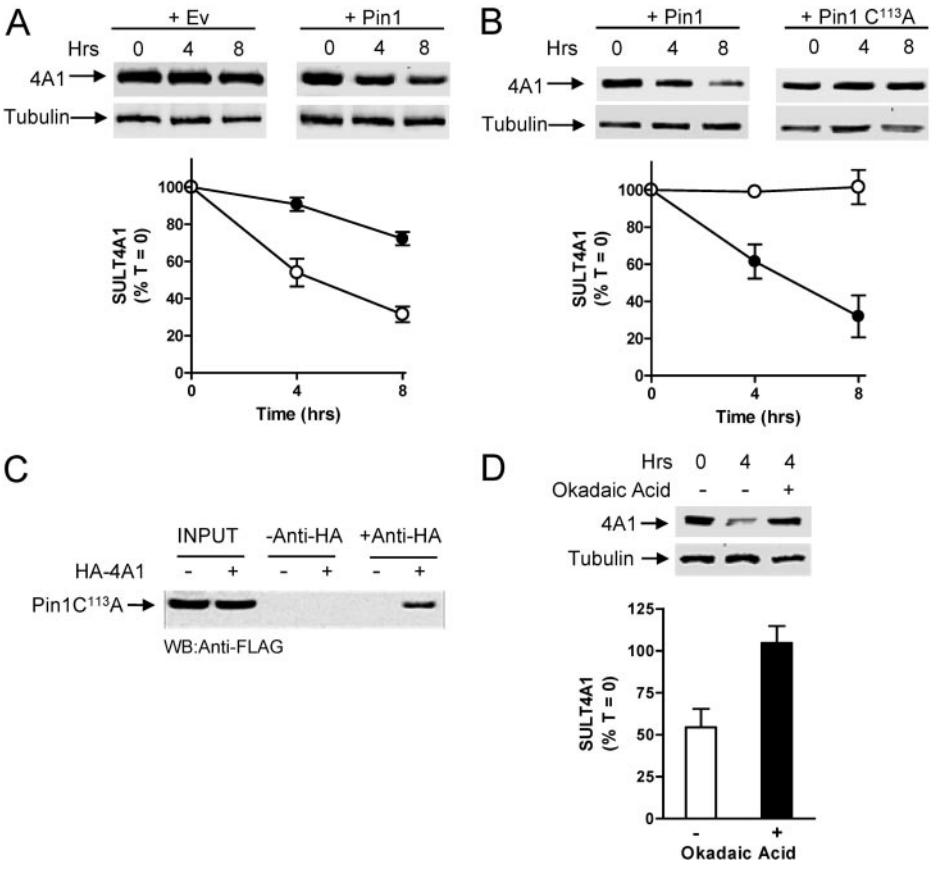

Fig. 2. Pin1 destabilizes SULT4A1. A, SULT4A1 half-life decreases in the presence of Pin1. FLAG-SULT4A1 was expressed in HeLa cells with either empty vector (left) or FLAG-Pin1 (right) for 24 h, after which the cells were treated with cycloheximide (10 μg/ml). Cell lysates were collected at 4 and 8 h, and SULT4A1 levels were quantified by Western blotting using an anti-FLAG antibody. Protein loading was monitored using anti-tubulin antibody (bottom bands). The graph below the blots show SULT4A1 protein levels after expression with FLAG-Pin1 (○) or empty vector control (●) during the course of cycloheximide treatment and normalized to tubulin levels. Data represent the mean ± S.E.M. of three independent experiments. B, isomerase-inactive Pin1 did not destabilize SULT4A1. FLAG-SULT4A1 was expressed with Pin1 (●) or the isomerase-inactive Pin1($C^{113}A$) (○) in HeLa cells and then treated with cycloheximide as described in A. Although the half-life of SULT4A1 in the presence of Pin1 was approximately 4 to 5 h, there was no evidence of SULT4A1 degradation in the presence of the isomerase-inactive protein. Data shown in the graph represent the mean ± S.E.M. of three independent experiments for SULT4A1 levels normalized to tubulin levels. C, SULT4A1 coimmunoprecipitates with Pin1($C^{113}A$). HA-SULT4A1 was expressed with FLAG-Pin1($C^{113}A$) in HeLa cells, and cell lysates were collected 24 h later. SULT4A1 was immunoprecipitated with anti-HA antibody, and bound proteins were then detected by Western blotting using anti-FLAG antibody. The Input lanes show the expression of Pin1($C^{113}A$) in each lysate. In the absence of anti-HA antibody, no coimmunoprecipitation of Pin1($C^{113}A$) was evident (lanes 3 and 4). Lane 6 shows that Pin1($C^{113}A$) coimmunoprecipitated with SULT4A1. D, destabilization of SULT4A1 by Pin1 was phosphatase PP2A-dependent. FLAG-SULT4A1- and FLAG-Pin1-transfected HeLa cells were treated with 10 μg/ml cycloheximide and 100 nM concentration of the PP2A-selective inhibitor okadaic acid for 4 h. Cell lysates were collected and subjected to Western blot analysis using an anti-FLAG antibody. Tubulin was also monitored for protein loading using an anti-tubulin antibody. The quantification of SULT4A1 protein levels normalized to tubulin levels is shown below the Western blots and represents the mean ± S.E.M., $n = 3$. Okadaic acid prevented the degradation of SULT4A1 in the presence of Pin1.

The degradation of some proteins such as c-Myc and Pim-1 is enhanced by Pin1 in a PP2A-dependent manner (Yeh et al., 2004; Brondani et al., 2005; Ma et al., 2007). PP2A may preferentially dephosphorylate phospho-serine/threonine-proline motifs in the *trans* conformation (Fila et al., 2008). It has been suggested that Pin1-catalyzed *cis-trans* isomerization of target proteins enhances dephosphorylation by PP2A and subsequent degradation (Yeh et al., 2004). To test whether PP2A may be involved in the destabilization of SULT4A1, we treated cells transfected with Pin1 and SULT4A1 with okadaic acid and then determined protein stability. Inhibition of the phosphatase completely protected SULT4A1 from Pin1-dependent destabilization (Fig. 2D), suggesting that SULT4A1 may be processed intracellularly in a manner similar to that proposed for c-Myc and Pim-1.

Pin1 Does Not Enhance Polyubiquitination-Dependent SULT4A1 Degradation. Pin1-regulated degradation of many proteins involves polyubiquitination and subsequent degradation in the proteosomes (Yeh et al., 2004; Brondani et al., 2005; Ma et al., 2007). Therefore, we explored the degradation pathway for SULT4A1 in the presence of Pin1 by treating cells with the proteosome inhibitor MG132. After 8 h of cycloheximide treatment to block new protein synthesis, SULT4A1 protein levels were approximately 35% of that in control cells (Fig. 3A, top). MG132 did not affect the disappearance of SULT4A1. By contrast, the degradation of Pin1, which is proteosome-dependent (Eckerdt et al., 2005), was inhibited by MG132 (Fig. 3A, middle). Pin1 was detectable after transfection because it was also FLAG-tagged and migrated separately because of its lower molecular mass (18

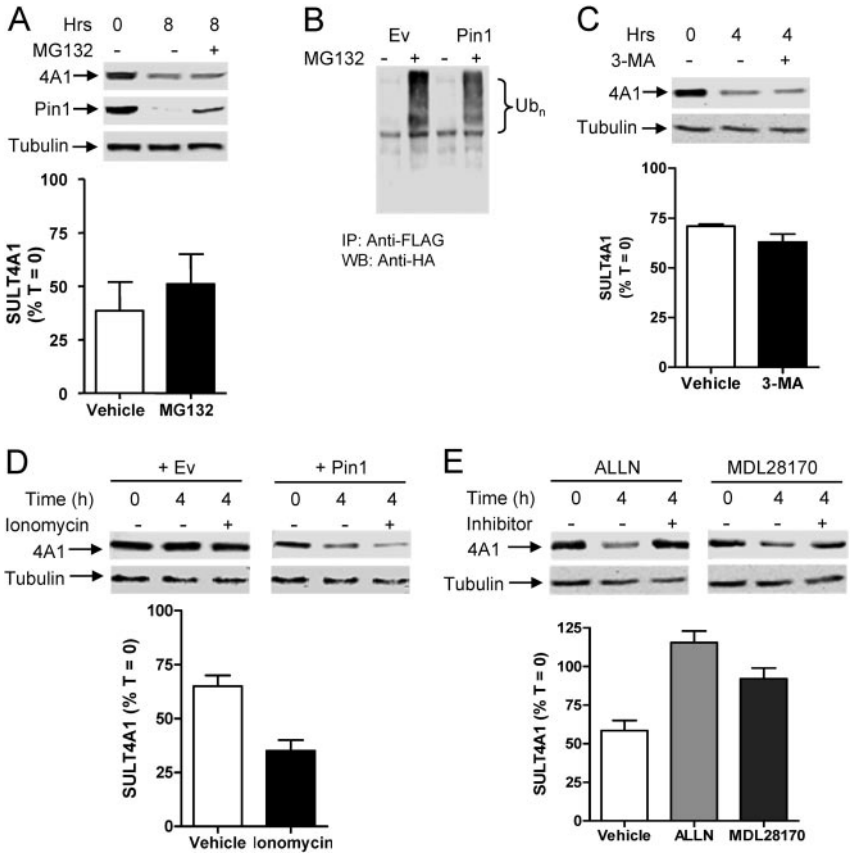

Fig. 3. Degradation pathway for SULT4A1. A, Pin1 did not enhance proteosomal degradation of SULT4A1. Cells transfected with FLAG-SULT4A1 and FLAG-Pin1 were treated with 20 μM MG132 for 8 h, and then both SULT4A1 (top blot) and Pin1 (middle blot) were analyzed by Western blots. The two proteins migrated separately on SDS-PAGE because their different sizes (18 kDa for Pin1 compared with 34 kDa for SULT4A1). Tubulin was also monitored for protein loading using an anti-tubulin antibody. These data, normalized to tubulin, are quantified in the graph below the blots (mean ± S.E.M., $n = 3$). MG132 did not affect SULT4A1 degradation, but it inhibited Pin1 degradation, which is known to be polyubiquitinated and degraded in the proteosomes (Eckerdt et al., 2005). B, Pin1 did not enhance the polyubiquitination of SULT4A1. FLAG-SULT4A1, pEF-Pin1 (or empty vector, Ev), and HA-ubiquitin were coexpressed in HeLa cells. After 24 h, the cells were treated with 10 μg/ml cycloheximide and 20 μM MG132 for 8 h. Cell lysates were then collected and immunoprecipitated with anti-FLAG antibody. SULT4A1 protein ubiquitination was analyzed by Western blotting using anti-HA antibody. Lanes 1 and 2 show that SULT4A1 was polyubiquitinated in the absence of Pin1, whereas lanes 3 and 4 show that Pin1 did not increase the polyubiquitinated products. C, Pin1 did not enhance macroautophagy of SULT4A1. FLAG-SULT4A1- and FLAG-Pin1-transfected HeLa cells were treated 24 h after transfection with 10 μg/ml cycloheximide and 5 mM 3-MA for 4 h. SULT4A1 protein levels were determined as described in A and are quantified in the graph below the Western blots (mean ± S.E.M., $n = 3$, normalized to tubulin). 3-MA did not affect SULT4A1 degradation. D, SULT4A1 degradation was increased by the calcium ionophore ionomycin. HeLa cells were transfected with FLAG-SULT4A1 and Pin1 or empty vector (Ev), and then, after 24 h, they were treated with 10 μg/ml cycloheximide for 4 h in the presence or absence of 1 μM ionomycin. SULT4A1 was then quantified by Western blots and normalized to tubulin (lower graph, mean ± S.E.M., $n = 3$). Ionomycin did not affect SULT4A1 degradation in the absence of Pin1 (left), whereas it increased degradation in the presence of Pin1 (right). E, SULT4A1 degradation is inhibited by calpain inhibitors. FLAG-SULT4A1 and FLAG-Pin1 were cotransfected in HeLa cells for 24 h and then treated with 10 μg/ml cycloheximide in the presence or absence of 50 μM ALLN (left) or 30 μM MDL28170 (right) for 4 h. SULT4A1 protein levels was determined as described in A and are quantified in the graph below the Western blots (mean ± S.E.M., $n = 3$, normalized to tubulin). Both calpain inhibitors prevented the enhanced degradation of SULT4A1 by Pin1.

kDa for Pin1 compared with 34 kDa for SULT4A1). To further investigate whether polyubiquitination was involved in SULT4A1 degradation, cells were cotransfected with SULT4A1, Pin1, and HA-tagged ubiquitin, after which SULT4A1 was immunoprecipitated, and polyubiquitinated products were identified by Western blotting using an anti-HA antibody. Polyubiquitination was detectable in control cells (Fig. 3B, lanes 1 and 2) but this was not enhanced by Pin1 (Fig. 3C, lanes 3 and 4), confirming the results with MG132 and suggesting that SULT4A1 was degraded by a pathway that did not involve polyubiqitination or the proteosomes.

A second major mechanism for protein degradation in cells is the lysosomal-associated macroautophagy pathway, which is selectively inhibited by the purine analog 3-MA (Stroikin et al., 2004). Cells expressing both SULT4A1 and Pin1 were treated with 3-MA, and protein stability was determined (Fig. 3C). However, there was no significant effect of the drug on SULT4A1 stability, suggesting a minor role, if any, for this pathway in SULT4A1 degradation.

Finally, we investigated the possible involvement of calcium-dependent proteases by treating cells with the calcium ionophore ionomycin. Ionomycin did not affect SULT4A1 stability in the absence of Pin1 (Fig. 3D, left) but enhanced degradation in the presence of Pin1 (Fig. 3D, right, and graph). Furthermore, when cells were treated with the calpain inhibitors *N*-acetyl-Leu-Leu-Nle-CHO (ALLN) or Z-Val-Phe-CHO (MDL28170), SULT4A1 degradation was significantly inhibited (Fig. 3E). Taken together, these results suggest that the calcium-dependent calpains may contribute to the regulation of SULT4A1 protein levels in the presence of Pin1.

SULT4A1 Contains Unique Threonine-Proline Motifs That Are Targeted by Pin1. The SULT4A1 protein contains three serine/threonine-proline motifs that are potential Pin1 binding sites (Fig. 4A). Two of these motifs are in close proximity to one another at amino acid positions 8 and 11. No other known SULTs contain Pin1 binding motifs at these corresponding positions. Another potential Pin1 binding site is located at position 104 and consists of a serine rather than a threonine. This site is found in several other human SULT proteins, including SULT1B1, SULT1C1, SULT1C2, SULT1E1, SULT2A1, and SULT2B1. To identify which of these motifs may be involved in the interaction with Pin1, we sequentially mutated each of the threonine or serine residues to an alanine. The resulting SULT4A1 mutants, SULT4A1(T^8A), SULT4A1(T^{11}A), and SULT4A1(S^{104}A), were coexpressed with Pin1 and then immunoprecipitated to identify Pin1 binding. All proteins were detectable after expression in HeLa cells, and all were immunoprecipitated (Fig. 4B, bottom). However, neither the T^8A nor the T^{11}A mutants interacted with Pin1 (Fig. 4B, top). By contrast, the S^{104}A mutant coimmunoprecipitated with Pin1, similar to wild-type protein.

We next investigated whether mutations in the putative Pin1 binding sites affected the ability for Pin1 to destabilize SULT4A1. Unlike the wild-type protein, SULT4A1(T^8A) and SULT4A1(T^{11}A) did not show increased degradation in the presence of Pin1 (Fig. 4C, middle), whereas SULT4A1(S^{104}A) was much less stable (Fig. 4B, bottom). These results are consistent with the binding data shown in Fig. 4B and indicate that the threonine-proline motifs at amino acids 8 and 11 are required for SULT4A1 interaction with Pin1. In addition, the data suggest that the destabilization of SULT4A1 is the consequence of direct Pin1 binding because mutations that altered binding also altered degradation.

Discussion

The present work has led to the identification of Pin1 as an interacting protein with the brain-specific sulfotransferase SULT4A1. The interaction seems to be the result of two closely aligned threonine-proline motifs located in the N terminus of the SULT4A1 protein. Pin1 preferentially recognizes the phosphorylated serine/threonine-proline motif. Trinidad et al. (2008) reported that SULT4A1 isolated from mouse brain was phosphorylated at Thr^8 and Thr^{11}, which is consistent with the site(s) of Pin1 binding identified in the present study. Dephosphorylation of SULT4A1 followed by

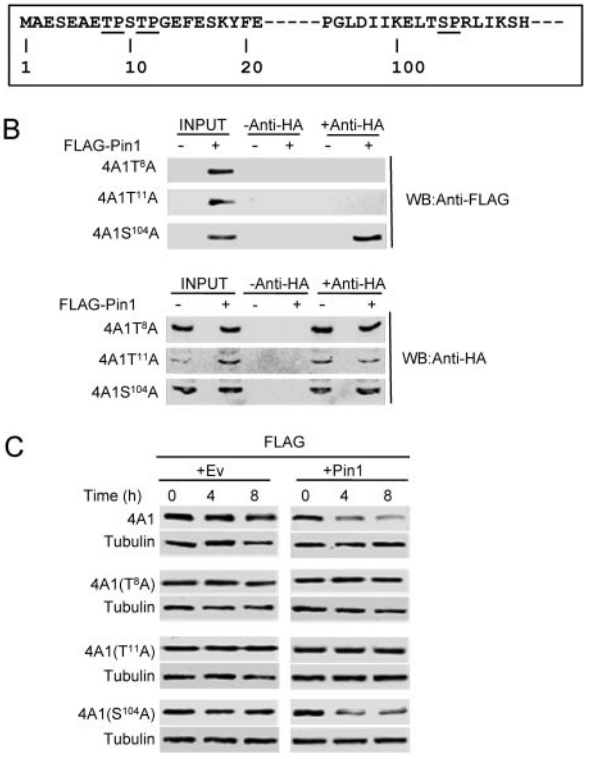

Fig. 4. Pin1 binds to motifs in the N terminus of SULT4A1. A, the SULT4A1 protein sequence contains three Pin1 motifs: Thr^8-Pro^9, Thr^{11}-Pro^{12}, and Ser^{104}-Pro^{105} (underlined). The two motifs close to the N terminus are unique to SULT4A1 and are not found in other mammalian SULTs. The motif at Ser^{104} is common to a number of other SULTs. B, mapping of the Pin1 binding site of SULT4A1 by mutagenesis. HeLa cells were cotransfected with wild-type HA-SULT4A1 or HA-SULT4A1 mutants in which Thr^8, Thr^{11}, or Ser^{104} was separately mutated to alanine. After 24 h, cell lysates were prepared, immunoprecipitated with anti-HA antibody, and analyzed by Western blots using anti-FLAG (top) or anti-HA antibodies (bottom). The Input lanes demonstrate the expression of each protein in HeLa cells. Top, mutation of either Thr^8 or Thr^{11} inhibited binding to Pin1, whereas mutation of Ser^{102} did not. Bottom, each SULT4A1 protein was pulled down in the immunoprecipitation. C, mutations of the N terminus Pin motifs prevented Pin-dependent destabilization of SULT4A1. HeLa cells were transfected with each SULT4A1 construct along with Pin1 (right) or empty vector (Ev, left). After 24 h, the cells were treated with 10 μg/ml cycloheximide, and SULT4A1 protein was analyzed by Western blots after 4 and 8 h. In the absence of Pin1, minimal degradation of each SULT4A1 protein was evident of this time. However, in the presence of Pin1, only the wild-type (SULT4A1) and the Ser^{102} mutants were destabilized. Tubulin levels were monitored using an anti-tubulin antibody for protein loading.

binding studies demonstrated that Pin1 binding was dependent on the phosphorylation state of SULT4A1. Pin1 binding leads to instability of SULT4A1, which seems to be PP2A-dependent. These results imply that SULT4A1 is post-translationally modified. The phosphorylation of SULT2B1b has been reported (He and Falany, 2006). Phosphorylation of SULT2B1 seems to regulate the subcellular localization of the enzyme (Falany et al., 2006). It is noteworthy that SULT2B1 contains several potential Pin1 binding motifs located toward the carboxyl terminus of the protein (He and Falany, 2006). Although not directly determined in this study, SULT4A1 seems to be phosphorylated, which may be important both for the function of the enzyme and its turnover in the cell.

Pin1 is a sequence-specific and phosphorylation-dependent prolyl *cis-trans* isomerase that is widely expressed and has been shown by immunostaining to be localized in both the cytoplasm and nuclei of neuronal cells in normal human brain (Lu et al., 1996; Yaffe et al., 1997). It is known to regulate the conformation and function of many phosphorylated proteins and plays an important role in cell cycle regulation, oncogenesis, and Alzheimer's disease (Lu et al., 1996; Ramakrishnan et al., 2003; Lu, 2004; Segat et al., 2007). Pin1 seems to facilitate protein dephosphorylation by the conformation-sensitive serine/threonine phosphatase PP2A, presumably by increasing the available targets in the *trans* conformation (Zhou et al., 2000; Janssens and Goris, 2001). Our results suggest that Pin1 elicits a similar influence on SULT4A1. When Pin1 was coexpressed with SULT4A1, we observed an enhanced destabilization of SULT4A1, which was not observed with the isomerase inactive Pin1($C^{113}A$). Moreover, studies inhibiting the serine/threonine protein phosphatases with okadaic acid showed a stabilization of the SULT4A1 protein levels in the presence of Pin1. Together, these results suggest that the isomerase activity of Pin1 elicits a conformational change in the structure of SULT4A1, resulting in the PP2A-mediated dephosphorylation and subsequent destabilization of SULT4A1.

We did not observe an increase in SULT4A1 polyubiquitination after Pin1-mediated degradation, which differs from reports for several other proteins (Yeh et al., 2004; Ma et al., 2007). Instead, our data suggest that SULT4A1 was degraded via a pathway involving calcium-dependent proteases, possibly calpains, because degradation was inhibited by both ALLN and MDL28170. A similar pathway has been suggested for Pin1-dependent destabilization of inducible nitric-oxide synthase (Liu et al., 2008). In that study, it was also proposed that Pin1 may down-regulate calpastatin, an endogenous inhibitor of the calpains. We attempted to detect the proteolytic products of SULT4A1 after Pin1-dependent degradation but were unsuccessful (data not shown). Although calpains usually cleave proteins at a limited number of sites and produce large polypeptide fragments rather than small peptides or amino acids (Goll et al., 1992a,b), the resulting polypeptides are often further degraded by other proteases in vivo. Consequently, protein fragmentation after calpain activity is often not observed by Western blotting (Kubbutat and Vousden, 1997; Zamorano et al., 2005; Hill et al., 2008).

The crystal structure of SULT4A1 suggests that the active site is too small to accommodate the cofactor PAPS (Allali-Hassani et al., 2007), although smaller potential sulfate donors are known, and a recently discovered bacterial sulfotransferase has been described that sulfonates substrates in a PAPS-independent manner (Malojcic et al., 2008). An alternative view may be that the SULT4A1 crystal structure does not represent the post-translationally modified structure present in mammalian cells. Moreover, post-translational modification of SULT4A1 may explain why specific substrates for the enzyme have not yet been identified.

It is noteworthy that the Pin1 binding site in SULT4A1 is very close to the N terminus of the protein in an area of low tertiary structure. In such unstructured regions, a high proportion of the proline residues already exist in the *trans* conformation because this is a more favored energetic state (Nelson et al., 2006). Although Pin1 can bind to phosphoserine/threonine-proline motifs in the *trans* conformation, as demonstrated by cocrystallization studies (Verdecia et al., 2000), the requirement for *cis-trans* isomerization in this region of SULT4A1 seems low. Smet et al. (2005) have shown that multiple Pin1 sites, similar to that in the N terminus of SULT4A1, increase binding affinity but decrease isomerase efficiency (Smet et al., 2005), suggesting that SULT4A1 may not be directly isomerized by Pin1 after binding. However, we showed using an enzymatically inactive mutant Pin1 that destabilization of SULT4A1 was dependent on isomerase activity (Fig. 2B). At this stage, we do not know whether the isomerase activity of Pin1 is directed toward SULT4A1 itself or other proteins essential for SULT4A1 degradation are targets of Pin1.

The results from our present study provide an important lead for further investigation. Identification of the various phosphorylation sites on SULT4A1 and what kinases may be responsible for these modifications may reveal information about the activation and regulation of the enzyme. In addition, although both SULT4A1 and Pin1 have been reported in separate studies to be expressed highest in neuronal cells (Liou et al., 2003; Liyou et al., 2003), their colocalization in regions of the brain warrants detailed study. Finally, murine Pin1 knockout animals are available, and the expression of SULT4A1 in the brain of these animals may assist in defining the role of SULT4A1 in vivo.

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Address correspondence to: Dr. Deanne Mitchell, School of Biomedical Sciences, University of Queensland, St. Lucia, QLD, Australia 4072. E-mail: d.mitchell@uq.edu.au