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# Identification and characterization of a novel and functional murine Pin1 isoform

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**Abstract**

Pin1, a phosphorylation-dependent peptidyl-prolyl *cis*/*trans* isomerase (PPIase), regulates the activity of a number of cell cycle regulators, transcription factors, and microtubule-associated tau. Aberrant expression of Pin1 is implicated in carcinogenesis and neurodegenerative diseases. Yet, there are discrepancies regarding its biological significance in different organisms. Pin1 was essential in HeLa cells, while Pin1-deficient mice showed no lethal phenotypes. We here identified a novel murine Pin1 isoform (mPin1L) consisting of the WW domain and the PPIase domain. Murine Pin1L shares 92% sequence identity with the wild-type Pin1 and shows wide tissue distribution with highest levels in mouse testis. The recombinant mPin1L is enzymatically active, but is approximately three times less efficient than Pin1 in catalyzing the *cis*/*trans* isomerization. These data suggest that mPin1L may serve as a surrogate for Pin1. The finding provides insights into phenotypic consequences for Pin1-null mice and may facilitate future biological study and pharmacological development in mice.


*Keywords:* Pin1 isoform; Pin1L; Pin1; Peptidyl-prolyl *cis*/*trans* isomerase; Pin1 surrogate; Pin1-knockout

There are three families of peptidyl-prolyl isomerases: cyclophilin, FK506-binding protein (FKBP), and parvulin. Pin1 belongs to the highly conserved parvulin family, and was initially identified as the human homolog of Ess1, an NIMA-interacting kinase essential for cell cycle progression in budding yeast [1]. Pin1 specifically catalyzes the *cis*/*trans* isomerization of phosphorylated Ser/Thr-Pro peptide bonds in various phosphoproteins. The conversion mediated by Pin1 modulates protein structure, stability or activity, and thus represents a novel post-translational regulatory mechanism [2]. Pin1 was shown to regulate many cell cycle regulators such as Cdc25, c-Myc, Cyclin D, Cyclin E, and Emi 1 [3–6], and transcriptional factors such as c-Fos, c-Jun, NF-κB, and p53 [7–10].

Deregulation of Pin1 is implicated in pathological consequences. Overexpression of Pin1 was postulated to activate multiple oncogenic pathways. Elevated levels of Pin1 were found in breast, ovary, prostate, and lung cancers [8,11], and were also shown to transform mammary epithelial cells [12]. Conversely, depletion of Pin1 displayed apoptotic phenotypes and led to mitotic arrest in HeLa cells [1,13]. Stable expression of Pin1-specific small interfering RNA was shown to sensitize anticancer agents in prostate cancer cell lines and inhibit tumor growth in xenograft mice [14]. On the other hand, Pin1 was shown to regulate and restore the function of hyperphosphorylated tau isolated from neurofibrillary tangles in Alzheimer's disease brains [15,16]. Pin1 expression levels were inversely correlated with the secreted levels of insoluble amyloid beta

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peptides (Aβ42) from amyloid precursor proteins (APP) in Chinese hamster ovary cell lines transfected with both *Pin1* and *APP*. Pin1-null mice had significantly higher insoluble Aβ42 than wild-type mice [17]. These data suggest that modulation of Pin1 activity may serve as a potential therapeutic approach for the intervention of oncogenic or neurodegenerative diseases.

Pin1 orthologs are highly conserved consisting of both the WW domain and the PPIase domain [18]. Nevertheless, ablation of *Pin1* in different organisms showed conflicting phenotypes. Pin1 was essential for cell cycle progression in HeLa cells, *Xenopus laevis* egg extracts, and budding yeast (Ess 1) [1,3,19]. However, *Pin1*-null mice as well as *dodo*-deficient *Drosophila* displayed no lethal consequences [20,21], suggesting that undisclosed Pin1 isoforms may play a role for Pin1. Indeed, a nonfunctional human Pin1 isoform (Pin1L) was previously identified though this human isoform encodes only the WW domain due to a shift in the reading frame [22]. In this communication, we described molecular cloning and biochemical characterization of a functional Pin1 isoform in mice.

## Materials and methods

Mouse Marathon-Ready cDNA from mouse testis and Mouse Multiple Tissue cDNA Panel I were purchased from Clontech (Palo Alto, CA). *Tag* DNA polymerase, *Escherichia coli* BL21(DE3) competent cell, pCR2.1-Topo, pProEXHTb, and rTEV protease were purchased from Invitrogen (Carlsbad, CA). Protease inhibitor cocktail, subtilisin Carlsberg, trifluoroethanol, and LiCl was purchased from Sigma (St. Louis, MO). The fluorescence peptide (FL-WFYpSPFLE) (Pintide) was synthesized at Pfizer La Jolla Laboratories. All other peptides were bought from Bachem Bioscience (King of Prussia, PA). All chromatography media were from GE Bioscience.

*Construction of murine Pin1 isoform.* Murine Pin1 cDNA coding sequence (GenBank Accession No. NM_023371) was used to query DNA fragments obtained by shotgun sequencing of the mouse genome (Celera) prior to assembly of the full mouse genome. The assembly of the resulting DNA fragments created two clusters using ContigExpress (Invitrogen). One cluster is identical to mouse *Pin1* and the other cluster contains an open reading frame (ORF) encoding the newly identified mouse Pin1 isoform (m*Pin1L*) (Fig. 1).

*Primer design and cloning of murine Pin1 isoform.* Three 5′ primers and one 3′ primer were designed for PCR amplification based on the assembled murine Pin1L sequence (Figs. 1 and 2A). Pin1L-F1 (293–329): 5′-ACCCTCATTTTATAGGTTACTCCTGAGCGAAGATGGC-3′; Pin1L-F2 (141–176): 5′-GTTTGCTGAGCCATCTAGGCATCCCAGG

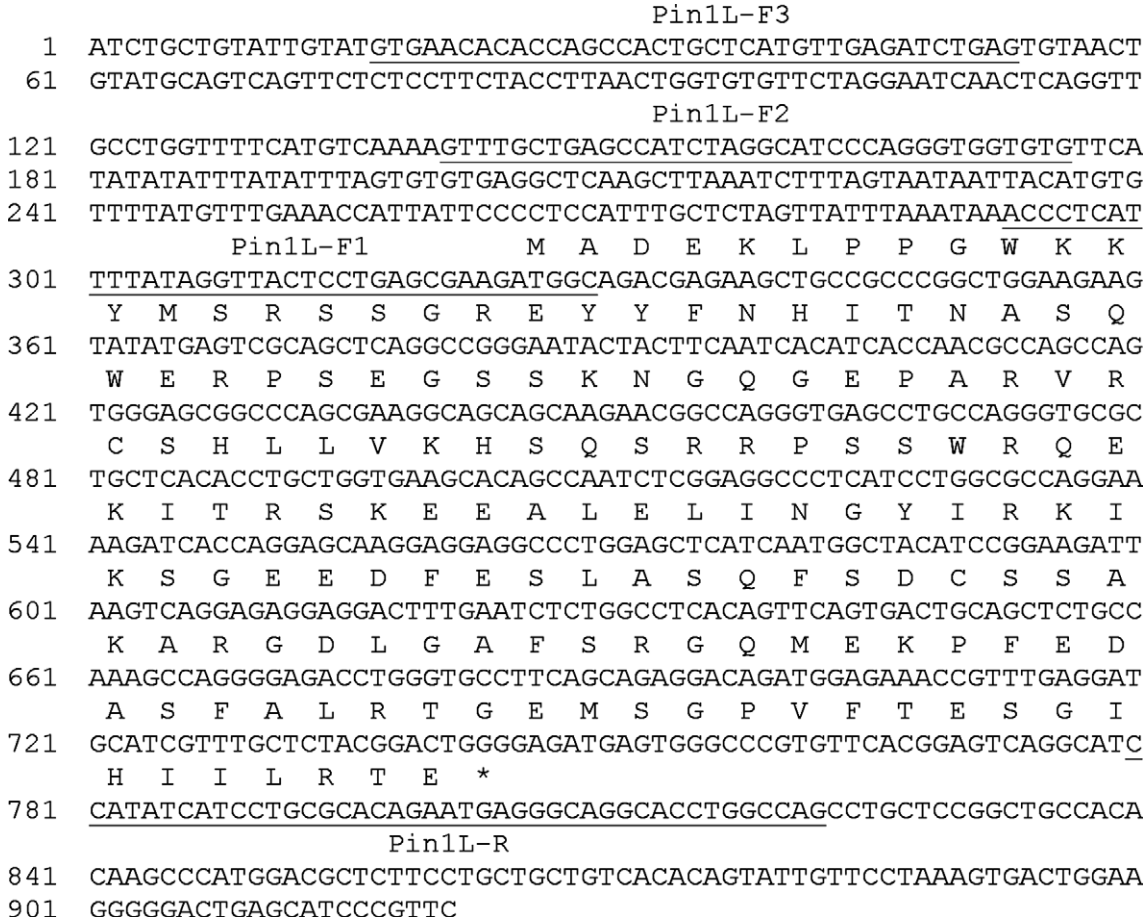


Fig. 1. Nucleotide and deduced amino acid sequences of murine Pin1L. The sequence cluster was initially assembled from mouse genomic DNA fragments. Pin1L-F1, F2, F3, and Pin1L-R (the sequence complementary to the underlined) were used to amplify the murine Pin1L from cDNA pools. The coding sequence (325–801) codes for full length Pin1L of 159 amino acid residues. The asterisk (*) indicates the termination codon.

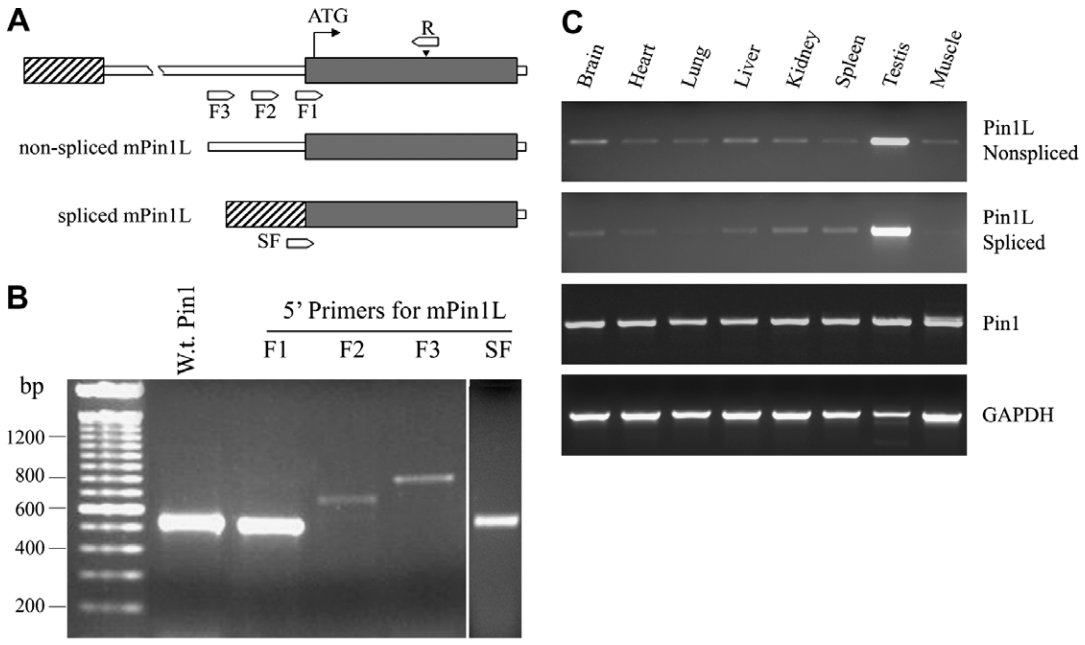


Fig. 2. PCR-amplification of mPin1L and its splice variant from mouse cDNA pools. (A) Schematic representation of mPin1L gene structure. Primer Pin1L-F1, F2, F3, and R were designed as shown in Fig. 1. Pin1L-SF is specific for the spliced variant. The hatched box is the first exon containing the 5′ untranslated sequence. The black box is the second exon containing the ORF (from the translation initiation codon ATG to the stop codon (filled triangle)). (B) PCR amplification of mPin1L and mPin1 from murine testis cDNA pools using indicated 5′ primers and a common 3′ primer. (C) Relative tissue expression of Pin1L variants and Pin1 by PCR using Mouse Multiple Tissue cDNA Panel I that was normalized with GAPDH.

GTGGTGTG-3′; Pin1L-F3 (17–53): 5′-GTGAACACACCAGCCACTG CTCATGTTGAGATCTGAG-3′; Pin1L-R (822–780): 5′-CTGGCCAG GTGCCTGCCCTCATTCTGTGCGCAGGATGATATGG-3′. The amplified Pin1L transcripts from mouse cDNA pools using the above primers were designated as nonspliced mPin1L. Subsequent to the cloning of the nonspliced mPin1L, a mouse Pin1-like transcript was reported (GenBank Accession No. NM_001033768). This mouse Pin1L transcript (designated as spliced mPin1L) was generated from the splicing of two exons: 1–207 and 208–1186 with the coding sequence at 225–704.

Since both the nonspliced mPin1L and spliced mPin1L contain the identical ORF located in the second exon, two 5′ primers were designed to differentiate these two splice variants. Pin1L-SF is specific for the spliced mRNA (−34 to −4 upstream from the initiation codon in NM_001033768, 5′-GCTCACTTTATCTCAAGGTTACTCCTGAG CG-3′). Pin1L-GF (5′-CTCCATTTGCTCTAGTTATTTAAATAAACC CT C-3′) is specific for the nonspliced mRNA (−59 to −27 in Fig. 1). Pin1-F (5′-GGAGCAGGCGCTGCGGCA-3′) is specific for murine Pin1 and 3′ primer Pin1(L)-R (5′-TCATTCTGTGCGCAGGATGATA-TG-3′) was used for both Pin1 and Pin1L.

mPin1L (or Pin1) was PCR-amplified in a 20 μl reaction containing 2 μl 10× PCR buffer, 1 μl mouse testis cDNA pool, 1.5 mM $MgCl_2$, 500 μM dNTP, 0.5 μM 5′ primer, 0.5 μM 3′ primer, and 0.5 μl Tag DNA polymerase (5 U/μl). The reaction was carried out in PTC-200 Peltier Thermal Cycler using the Touchdown PCR in which the annealing temperature decreased 1 °C every two cycles from 65 °C to 55 °C followed by 15 additional cycles at 55 °C. The PCR products were cloned into pCR 2.1-Topo for sequence verification, and then subcloned into pProExHTb between NcoI and HindIII for protein expression.

*Tissue distribution.* The relative abundance of mPin1L variants and Pin1 in different tissues was determined by RT-PCR. Mouse cDNA pools were normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). A typical PCR reaction contained 0.2 μM 5′ specific primer (Pin1L-SF, Pin1L-GF, or Pin1-F) and 3′ common primer (Pin1(L)-R), 2.5 μl cDNA, 0.5 μl *Tag* DNA polymerase. The PCR products were amplified for 35 cycles in the following PCR program: 94 °C for 30 s, 55 °C for 30 s, 68 °C for 40 s, and analyzed in the 1% agarose gel.

*Expression and purification.* mPin1L (or Pin1) was expressed from *E. coli* BL21(DE3) cells in 2xYT media containing 100 μg/ml ampicillin, 0.3 mM IPTG at 37 °C for 3 h. Purification was performed at 4 °C. Briefly, cell pellets were resuspended in Buffer A (50 mM Tris–Cl, pH 7.5, 300 mM NaCl, 20 mM imidazole, 5 mM β-mercaptoethanol). The cell suspension was lyzed, centrifuged, and loaded into a Q-sepharose column. The flowthrough was then loaded into a Ni-NTA column (Qiagen). 6xHis-Pin1L was eluted by running a linear gradient from 100% Buffer A to 100% Buffer A containing 300 mM imidazole, and 6xHis-tags were cleaved by adding 6xHis-rTEV protease. The cleaved solution was passed through another Ni-NTA column. The flowthrough was collected, concentrated, and injected into a Superdex 75 column equilibrated in 25 mM Tris–Cl, pH 7.5, 5 mM β-mercaptoethanol, 150 mM NaCl. The purified protein was stored at −80 °C.

*Analytical gel filtration.* About 1.5 mg of proteins (100 μl) were injected into the Superdex 200 FPLC (10 × 30) column equilibrated in 25 mM Tris–Cl, pH 7.5, 5 mM DTT, 150 mM NaCl at 0.3 ml/min. The column was calibrated with the gel filtration standard from Bio-Rad.

*Protein concentration.* The concentration was determined in Coomassie Plus Assay (Pierce) using BSA as the standard.

*PPIase activity.* The determination of the isomerase activity is a modification of Kofron's method [23]. The modified method substituted subtilisin for α-chymotrypsin. The *cis*/*trans* conversion of *cis*-Suc-AEPF-pNA peptide by the PPIase led to the cleavage of *para*-nitroaniline by subtilisin which was monitored at 390 nm. The assay solution, consisting of 25 mM MOPS, pH 7.5, 33 nM Pin1L (Pin1), 1.25 μg/μl subtilisin, 0.1% CHAPS, 0.5 mM TCEP, and 2% DMSO, was initiated by adding 50 μM peptide substrate at 15 °C. The reaction was monitored continuously at 390 nm using Beckman DU-7400 diode array spectrophotometer with a peltier temperature controller.

*Binding constant.* A fluorescence polarization assay was used to determine dissociation constants between Pin1 isoforms and the fluorescence Pintide (FL-WFYpSPFLE) complex [24,25]. Fluorescence anisotropy was measured in the solution containing 25 mM MOPS pH 7.5, 0.05 mM TCEP, 0.4 μM peptide and various Pin1L (Pin1) concentrations (0–60 μM) on Analyst HT with Fluorscein 505 filter. Binding constants were calculated as described by Vinson et al. [25].

## Results

### *Cloning of murine Pin1 isoform (mPin1L)*

By querying shotgun fragments of the mouse genome with murine Pin1 coding sequence, we identified a novel murine Pin1 isoform (nonspliced mPin1L) containing an ORF (325–801) encoding 159 amino acids (Fig. 1). This putative isoform shares 94% sequence identity to murine Pin1 at the nucleotide sequence level and 92% sequence identity at the amino acid sequence level. The 3′ untranslated regions are highly homologous between Pin1 and Pin1L, but little homology was observed in the 5′ untranslated regions.

Next, we cloned Pin1L from mouse testis cDNA pools using three 5′ primers located upstream of the ORF and one 3′ primer complementary to the sequence containing the translation termination codon (Figs. 1 and 2A). Murine Pin1L was readily amplified by PCR (Fig. 2B), and the cloned sequence was completely identical to that derived from the assembled cluster.

Subsequently, a Pin1-like transcript (GenBank Accession No. NM_001033768) was derived from mouse transcriptome [26]. This mPin1L transcript (spliced mPin1L) can be mapped to mouse chromosome 2, and is generated by splicing two exons: 1–206, and 1489–2365 from the genome sequence (GenBank Accession No. NC_000068 region: complement (104514765 to 104517129)) (Fig. 2A). Since the ORF is located in the second exon, the spliced Pin1L encodes the identical protein as the nonspliced Pin1L.

The amplicon using Pin1L-F1 is much stronger than those using Pin1L-F2 and Pin1L-F3 in Fig. 2B. The difference is partly due to the fact that the primer Pin1L-F1 overlaps the spliced Pin1L, suggesting that the amplification from the spliced Pin1L also contributes to the signal. This was further confirmed by PCR using the splice-specific primer Pin1L-SF (Fig. 2B). These data indicate that mouse Pin1L has two splice variants encoding the same protein but with distinctive 5′ flanking sequences. For comparison, murine Pin1 was also amplified and showed the similar expression level as Pin1L in mouse testis.

### *Wide tissue distribution and high sequence conservation*

To determine tissue distribution, both mPin1L variants were PCR-amplified from mouse cDNA pools using splicing-specific primers in mouse tissue samples (Fig. 2C). Both Pin1L variants were ubiquitously expressed in different organs but at lower levels compared to Pin1 which showed high and constant expression levels. Interestingly, the expression of Pin1L in murine testis was much higher and comparable with Pin1, suggesting that mPin1L may play a more important role in mouse testis.

Although human Pin1L contains only the WW domain [23], murine Pin1L is highly homologous to Pin1 consisting of the WW domain and the PPIase domain based on the sequence alignment analysis (Fig. 3). The WW domain has four point aberrations from Pin1 to Pin1L (E12 to K11, R14 to Y13, V22 to E21, and G39 to E38), and the PPIase domain has five point aberrations (K56 to R50, Q96 to R90, P126 to A120, Q133 to E127, and D155 to E149). mPin1L also has the deletion of five residues (TVGGS) located in the flexible region between two domains. All key residues implicated in substrate recognition, catalysis, and nuclear localization are conserved [18].

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Pin1_Human     (1) MADEEKLPPGWEKRMSRSSGRVYYFNHITNASQWERPSGNSSSG--GKNG
Pin1L_Human    (1) MADEEKLPPGWEKRMSRPSGRGYYFNHITNPSQWERPSGNSSSG--GKIW
Pin1_Mouse     (1) MADEEKLPPGWEKRMSRSSGRVYYFNHITNASQWERPSGGSTVGGSSKNG
Pin1L_Mouse    (1) MAD-EKLPPGWKKYMSRSSGREYYFNHITNASQWERPSEGS-----SKNG
                       Δ       * *       *                 *  ΔΔΔΔΔ

Pin1_Human    (49) QGEPARVRCSHLLVKHSQSRRPSSWRQEKITRTKEEALELINGYIQKIKS
Pin1L_Human   (49) QGEPARVRRSHLLVKPVKAALDLAAGNHPDQGGGPGADQRLHPEDQGRRE
Pin1_Mouse    (51) QGEPAKVRCSHLLVKHSQSRRPSSWRQEKITRSKEEALELINGYIQKIKS
Pin1L_Mouse   (45) QGEPARVRCSHLLVKHSQSRRPSSWRQEKITRSKEEALELINGYIRKIKS
                         *                                       *

Pin1_Human    (99) GEEDFESLASQFSDCSSAKARGDLGAFSRGQMQKPFEDASFALRTGEMSG
Pin1L_Human   (99) GL-------------------------------------------------
Pin1_Mouse   (101) GEEDFESLASQFSDCSSAKARGDLGPFSRGQMQKPFEDASFALRTGEMSG
Pin1L_Mouse   (95) GEEDFESLASQFSDCSSAKARGDLGAFSRGQMEKPFEDASFALRTGEMSG
                                          *      *

Pin1_Human   (149) PVFTDSGIHIILRTE
Pin1L_Human  (101) ---------------
Pin1_Mouse   (151) PVFTDSGIHIILRTE
Pin1L_Mouse  (145) PVFTESGIHIILRTE
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Fig. 3. Sequence alignment of Pin1 isoforms. mPin1L contains both the WW domain (5–39) and the PPIase domain (54–165). The difference between mPin1L and mPin1 was indicated as ∗: aberration; Δ: deletion. The underlined indicates the changed amino acids of human Pin1L due to the frame shift.

*Isomerase activity and specificity*

To evaluate the isomerization activity, recombinant mPin1L and mPin1 were expressed and purified to homogeneity (Fig. 4A). Both mPin1 and mPin1L existed as monomers in the solution, but eluted at different volumes (15.9 and 17.5 ml, respectively) in the analytical gel filtration (Fig. 4B), suggesting these two isoforms differs structurally to some extent. Mass spectrometry data indicated that there was no modification for both proteins (data not shown).

In the isomerization assay using the Pin1 substrate (*cis*-Suc-AEPF-pNA) [27], mPin1L was enzymatically active but less efficient than Pin1 (Fig. 4C). Like Pin1, mPin1L also showed high substrate specificity ($k_{cat}/K_m$) for the peptide containing the negatively charged residue (Glu) immediately preceding Pro (Table 1). By comparison, Pin1 showed nearly three times higher $k_{cat}/K_m$ than Pin1L and thus is a more efficient PPIase. In the fluorescence polarization assay, mPin1 and mPin1L showed dissociation constants of 1.4 and 5.4 μM, respectively, indicating that mPin1L had 3.8 times lower affinity for the peptide Pintide compared to Pin1 (Table 1). These data suggest that murine Pin1L may serve as a less efficient substitute for the wild-type Pin1.

Table 1
Catalytic specificity and binding dissociation constants of murine Pin1 and Pin1L with peptide substrates

| PPIase | Substrate | $k_{cat}/K_m$ ($mM^{-1}\ S^{-1}$) | $K_d$[a] (μM) |
|---|---|---|---|
| Pin1 | Suc-AEPF-pNA | 6210 | — |
| Pin1L | Suc-AEPF-pNA | 2126 | — |
| Pin1 | Suc-AAPF-pNA | 25 | — |
| Pin1L | Suc-AAPF-pNA | 14 | — |
| Pin1 | FL-WFYpSPFLE | — | 1.4 ± 0.29 |
| Pin1L | FL-WFYpSPFLE | — | 5.4 ± 1.1 |

—, no data was determined.
[a] Errors: deviations from theoretical binding isotherms.

## Discussion

Pin1 catalyzes the isomerization of pSer/Thr-Pro—containing proteins and is essential for cell cycle progression and mitosis. Lack of lethal phenotypes in Pin1-null mice prompted us to search for murine Pin1 isoforms. We here provided direct evidence that mice contain a closely-related Pin1 isoform that was transcribed as both spliced and nonspliced variants encoding the same protein. These two variants show similar expression profiles in various mouse tissues, but may be regulated differently due to the distinctive 5′ flanking regions. The wide tissue distribution and high sequence homology with Pin1 suggest that mPin1L may serve as a surrogate for Pin1, which was further supported by the isomerization activity observed in the enzymatic assay. The highest expression level of mPin1L in mouse testis that is comparable with Pin1 suggests that mPin1L may play an important role during germ cell development and spermatogenesis.

By querying various genome databases with Pin1 sequences, we found that chimpanzee and rat also contain the highly homologous WW domain as human Pin1L (Table 2). Interestingly, murine Pin1L is the only ortholog consisting of both the WW and the PPIase domains. Although the WW domain is responsible for the interaction with the pSer/Thr-Pro motif in proteins [28], the biological significance of these WW-containing alleles remains to be elucidated.

Kinetic study of recombinant murine Pin1 and Pin1L indicates that Pin1L is nearly three times less efficient than Pin1 in the isomerization of pSer/Thr-Pro-containing peptides, and 3.9 times weaker than Pin1 in binding Pintide. The lower efficiency may be associated with the difference of several amino acid residues proximal to the catalytic site. Two residues in mPin1L: E127 (Q133 in mPin1, Q131 in human) and E149 (D155 in mPin1, D153 in human) may have a significant effect on the enzymatic activity. In the human Pin1 crystal structure (PDB Accession No. 1PIN),

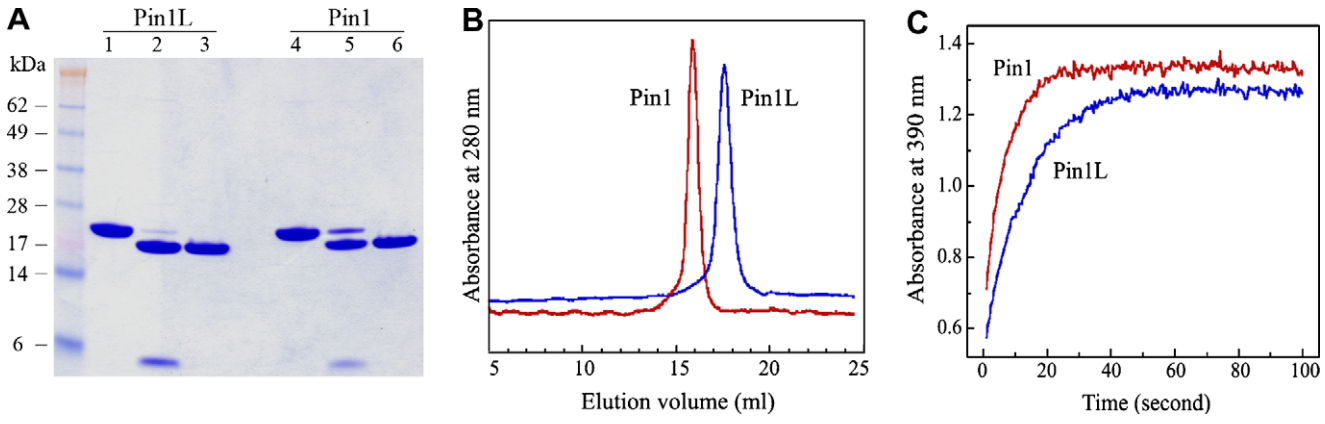


Fig. 4. (A) Purification of recombinant murine Pin1L and Pin1 from *E. coli*. Lanes 1 and 4: purified 6xHis tagged proteins. Lanes 2 and 5: cleavage of 6xHis tags by rTEV protease. Lane 3 and 6: final purification after a Superdex 75 column. (B) Elution profiles of purified proteins in the analytical Superdex 200 FPLC column (10 × 30). (C) Kinetic curves of mPin1 and mPin1L-mediated isomerization of *cis*-Suc-AEPF-pNA in the enzyme-coupled spectrophotometric assay at 15 °C.

Table 2
Summary of chromosome location and functional domains for Pin1 isoforms

| Organism | Location in chromosome | | Pin1L domains |
|---|---|---|---|
| | Pin1 | Pin1L | |
| *H. sapiens* | 19 | 1 | WW |
| *P. troglodytes* | 19 | 1 | WW |
| *Bos taurus* | 7 | — | — |
| *C. familiaris* | 20 | — | — |
| *M. musculus* | 9 | 2 | WW, PPIase |
| *R. norvegicus* | 8 | 16 | WW |
| *D. melanogaster* | X | — | — |

both Q131 and D153 are in very close proximity to the active site key residue R68 (R64 in mPin1L) [18]. D153 has H-bonding with the backbone amide of R68, and Q131 is involved in an H-bonding network with Ser154, R68, and the substrate. It is conceivable that Q/E and D/E substitutions may rearrange the H-bonding network or introduce a new salt bridge, thus influencing the catalytic efficiency. In the mPin1L WW domain, all aberrations except E21 (V22 in Pin1) are solvent-exposed and away from the hydrophobic cluster, a cavity proposed to mediate protein to protein interaction [18]. Nevertheless, Pin1 and Pin1L had slightly different gel filtration profiles and 3.9-fold difference in binding dissociation constants, suggesting that the small difference in these amino acid residues may influence the packing geometry of the WW domain and the PPIase domain. Further structural and enzymatic studies may delineate the effect of these substitutions.

Although Pin1-knockout mice developed normally, they displayed some proliferation abnormalities including decreased body weight, testicular atrophy, retinal degeneration, and impaired mammary gland development [29]. Deletion of Pin1 in mice resulted in significantly impaired proliferation of primordial germ cells (PGC) and the progressive degeneration of spermatogenic cells which eventually led to the complete loss of germ cells in adult mice [30,31]. The identification of murine functional Pin1L may provide an explanation for these observations. First, it further underlines the biological significance of Pin1 in cell cycle progression as demonstrated in HeLa cells. Second, mPin1L may partially compensate for the absence of mPin1, but it is not a complete surrogate for Pin1 due to its lower catalytic efficiency and lower expression levels. Alternatively, the PPIase activity might be compensated by remotely-related homologs, for example in *Drosophila* where deletion of *dodo* has no effect on viability and there is no closely-related *dodo* paralog in the *Drosophila* genome [20]. However, a recent study suggests that other isomerases including cyclophilin and FKBP could not compensate for Pin1 activity in Pin1-knockout mice [32], implying that mPin1L partially offsets for the loss of Pin1. Taken together, the discovery of the functional murine Pin1L sheds some light on Pin1-deficient phenotypes and may open a novel avenue for future biological study and pharmacological characterization of Pin1 isoforms in mice.

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