SUPPLEMENTARY APPENDIX

Structural and cellular mechanisms of peptidyl-prolyl isomerase Pin1-mediated enhancement of Tissue Factor gene expression, protein half-life, and pro-coagulant activity

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SUPPLEMENTAL METHODS

Cell culture

HUVECs were used at passages 1-3 and cultured in M199 medium (Invitrogen) supplemented with 20% fetal calf serum (FCS; Gibco), endothelial cell growth supplement (Sigma), 100U/mL penicillin, 100µg/mL streptomycin, 2mM L-glutamine (all Gibco), and heparin (Invitrogen). Human SMCs were cultured in M199 medium supplemented with 10% FCS, 100U/mL penicillin, and 100µg/mL streptomycin. Mouse aortic SMCs from wild-type mice and $TF^{\Delta CD}$ mice were obtained after collagenase digestion and cultured in DMEM/F12 medium supplemented with penicillin/streptomycin and 20% FCS. Human SMCs were used at passages 5-7 and mouse SMCs at passages 8-10. SMCs were characterized by SM α-actin (1A4; DAKO) showing uniform fibrillar staining. HEK293T and EC-RF24 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 20mM glucose and supplemented with 10% FCS, 100U/mL penicillin, and 100µg/mL streptomycin.

Reverse transcription-quantitative PCR

RNA was isolated using the Aurum Total RNA kit (BioRad). RNA was reverse transcribed into cDNA using the iScript cDNA synthesis kit (BioRad). Quantitative polymerase chain reaction (qPCR) was performed using SYBR Green master mix (BioRad) and the MyIQ system (BioRad). Sequences of primers used are shown in Table S2.

Western blotting

Cells were lysed in NP-40 lysis buffer (50mM Tris-HCl, pH7.4, 100mM NaCl, 10% glycerol, 1% NP-40) supplemented with phosphatase inhibitors (10mM NaF, 1mM Na3VO4) and Complete protease inhibitor cocktail (Roche). Protein samples were separated on 12% SDS-PAGE gels and transferred onto Nitrocellulose membranes (Millipore). Membranes were blocked using LicorBlock (Licor Biosciences) and incubated with appropriate primary antibodies and near-infrared dye conjugated secondary antibodies, followed by scanning using the Odyssey Imaging System (Licor Biosciences). Antibodies used in this study include Mouse-anti-HA (Roche; cat#11583816001), Rabbit-anti-human Tissue Factor (American Diagnostica; cat#4502), Rabbit-anti-Pin1 (Cell Signaling; cat#3722), Mouse-anti-GFP (Santa Cruz; cat#sc-9996), and Mouse-anti-α-Tubulin (Cedarlane; cat#CLT9002).

Co-immunoprecipitation assays

HEK293T cells were co-transfected with plasmids encoding HA-tagged Pin1, full-length Tissue Factor, or mutant Tissue Factor constructs and incubated for 48h. Cells were lysed in NP-40 lysis buffer and lysates were pre-cleared for 1h at 4°C with protein A-Sepharose (GE Healthcare), followed by incubation overnight with 2µg of the indicated pulldown antibody and protein A-Sepharose. Immunoprecipitates were washed three times in NP-40 lysis buffer, eluted by boiling in Laemmli sample buffer, and detected by Western blotting.

TFCD peptide pull-down assays

HEK293T cells were transfected with plasmids encoding either Pin1, Pin1 mutants, or GFP. After 24 h, transfected cells were washed with PBS and lysed in NP-40 lysis buffer supplemented with phosphatase inhibitors (10mM NaF, 1mM Na_3VO_4) and Complete protease inhibitor cocktail. Pull-downs were performed using either biotinylated peptides encoding the human TFCD from Ser241 to Ser263 (SLHKCRKAGVGQSWKENSPLNVS; PepScan), or cysteine-coupled peptides encoding the human TFCD from Cys245 to Ser263 (CRKAGVGQSWKENSPLNVS; synthesized at the core facility of The Scripps Research Institute, La Jolla, California). For biotinylated peptide pull-downs, 20µg of peptide was added to Avidin Agarose pre-cleared whole cell lysates and samples were incubated overnight at 4°C on a rotor. The next day, peptide-protein complexes were pulled down by incubating samples for 2h at 4°C with 20 µL 50% Avidin Agarose slurry that was pre-blocked overnight at 4°C with 5%(w/v) BSA. Precipitates were washed three times with NP-40 lysis buffer and bound proteins were eluted by boiling in Laemmli sample buffer. For cysteine-coupled TFCD peptide pull-downs, peptides dissolved in Coupling Buffer (50mM Tris-HCl, pH 8.0, 5mM EDTA) were reduced by incubating for 10 minutes with 25mM tris(2-carboxyethyl)phosphine (TCEP; Pierce) and subsequently coupled to SulfoLink resin (Pierce) by incubating for 45 minutes under gentle agitation. Resin-peptide complexes were washed three times with Coupling Buffer to remove unbound peptide and subsequently blocked by incubating overnight at 4°C with 5%(w/v) BSA. The next day, 1 mg of whole

cell lysate was pre-cleared by adding 20 µL 50% SulfoLink resin and incubating for 1h at 4°C on a rotor. Blocked resin-peptide complexes were added to pre-cleared whole cell lysates and incubated overnight at 4°C. Precipitates were washed three times with 0.1% Triton X-100 in Coupling Buffer and bound proteins were eluted by boiling in Laemmli sample buffer. Pulled down proteins were detected by Western blotting. Biotinylated peptides were detected using IRDye 800CW-conjugated Streptavidin (Licor Biosciences).

Nuclear magnetic resonance (NMR) spectroscopy

Pin1 WW-domain protein and TFCD peptide preparation

A cDNA construct encoding the WW-domain of human Pin1 fused to an N-terminal glutathione S-transferase (GST) tag was kindly provided by Dr. Jeffrey Kelly (The Scripps Research Institute). The construct was transformed into BL21 (DE3) cells cultured in M9 minimal media containing 1 g/L $(^{15}NH_4)_2SO_4$ and $^{15}NH_4Cl$ and 2.5 g/L ^{13}C-D-glucose. Cell cultures were grown in a high-speed shaker at 37°C to an OD_{600} of 0.5 and GST-Pin1 WW-domain expression was induced with 0.5mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Cells were lysed in PBS, centrifuged at 10,000 x *g* for 30 minutes at 4°C, and passed through a 0.45 µm filter. Filtered lysate was loaded onto a GST column at ambient temperature. After washing the GST-column with PBS, GST-fused protein was eluted using 10mM reduced glutathione in PBS. 1 mg of GST-Pin1 WW-domain was cleaved by 1 unit of thrombin protease at ambient temperature for 16 hours. The cleaved Pin1 WW-domain was purified away from the GST-tag by a reverse-phase HPLC C4-column using increasing concentrations of acetonitrile in the mobile phase and then freeze-dried. Peptides corresponding to the human TFCD from residues Cys245 to Ser263 in either unphosphorylated, Ser253 or Ser258 phosphorylated, or double Ser253/Ser258 phosphorylated states were synthesized at the core facility of The Scripps Research Institute, La Jolla, California.

NMR titration of pS253/pS258 TFCD against Pin1 WW-domain

NMR experiments were performed on Bruker DRX 600 MHz and 800 MHz spectrometers equipped with triple resonance ^{1}H/^{13}C/^{15}N optimized for proton detection at 300K. Acquired data were converted from Bruker XWINNMR format to NMRVIEW version 5.3 format using NMRpipe software[1-2]. All spectra were analyzed using NMRVIEW. All experiments were performed at the UH NMR Facility at the University of Houston.

Pin1 WW-domain/TFCD titrations were performed by step-wise increases in the amount of double phosphorylated TFCD. TFCD phosphorylated peptide with an initial concentration of 21mM was added into a 250 µM sample of ^{1}H/^{15}N labeled Pin1 WW-domain in 10mM Tris (pH 6.3), 100mM NaCl, 5%(v/v) D_2O/H_2O to give molar ratios ranging from 1:0 to 1:10. For each titration point, the pH of the sample was adjusted to 6.3 if required. Two dimensional ^{1}H/^{15}N heteronuclear single-quantum correlation (HSQC) spectra were acquired with 2048 and 256 complex points in t_1 and t_2 respectively with 16 transient numbers of scans per increment. The binding constant for the double phosphorylated TFCD was calculated by non-linear regression using the following equation:

$$2\times\frac{\Delta\delta}{\Delta\delta_{\max}}=\left(1+\frac{[TFCD]}{[WW]}+\frac{K_d}{[WW]}\right)-\left[\left(1+\frac{[TFCD]}{[WW]}+\frac{K_d}{[WW]}\right)^2-4\times\frac{[TFCD]}{[WW]}\right]^{1/2}$$

Where Δδ is the observed chemical shift, $\Delta\delta_{max}$ is the maximum chemical shift, K_d is the dissociation constant, [TFCD] is concentration of double phosphorylated TFCD, and [WW] is the concentration of the Pin1 WW-domain.

NMR structure determination

A 5 mm Shigemi microcell was used for each NMR sample. Backbone assignments were obtained by through-bond triple resonance methods of HNCA, HN(CO)CA, HNCACB, CBCA(CO)NH, HNCO and HN(CA)CO[3]. Chemical shift index, the CN-NOESY, HCCH-TOCSY, HCCH-COSY, and aromatic ^{13}C-edited HSQC were used to determine the interproton distances as well as ψ, φ and χ1 torsion angles[4-7]. Based on the peak volumes, the nuclear Overhauser effects (NOEs) were categorized into four distances - 2.7 Å, 3.5 Å, 5.0 Å, and 6 Å, respectively. Structure calculations were performed using the simulated annealing program, AMBER. In the final round of calculation, the 20 lowest energy structures were selected. Statistics for the accepted structures and structural restraints used for

calculation are listed in Table S1. PROCHECK_NMR was used to analyze the quality of the refined structures[8].

Pin1 WW–pS253/pS258 TFCD structure energy minimization

Energy minimization was performed on each model of the *Pin1 WW–pS253/pS258 TFCD* complex with Amber force field with the GROMACS 4.6.7 program[9-10] to remove possible steric clashes and minimize the potential energy of the system on the UH CACDS super-computing cluster. An Amber99sb force field was compiled to add the parameters for phosphorylated Ser253 and Ser258 residue types. Each conformer was solvated inside a cubic box of explicit solvent model (TIP3P) with at least 10 Å from the water box edge. Three sodium ions were added to neutralize the system. Each system contained about 32,000 atoms and Periodic Boundary Conditions were employed. All structures of *Pin1 WW–pS253/pS258 TFC*D complex underwent a series of step-wise energy minimization using the steepest descent method. Step-wise energy minimization was performed as follows: (1) *Pin1 WW–pS253/pS258 TFC*D complex was constrained while water and ions moved freely; (2) heavy atoms of complex were constrained, while water, ions, and hydrogens moved freely; (3) atoms of the complex main chain were constrained, while the rest of the system moved freely; (4) the entire system was subjected to an unconstrained energy minimization. Total energy minimization was carried out until the system converged to the maximum atomic force <100 kJ mol^{-1} nm^{-1}.

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Figure S1

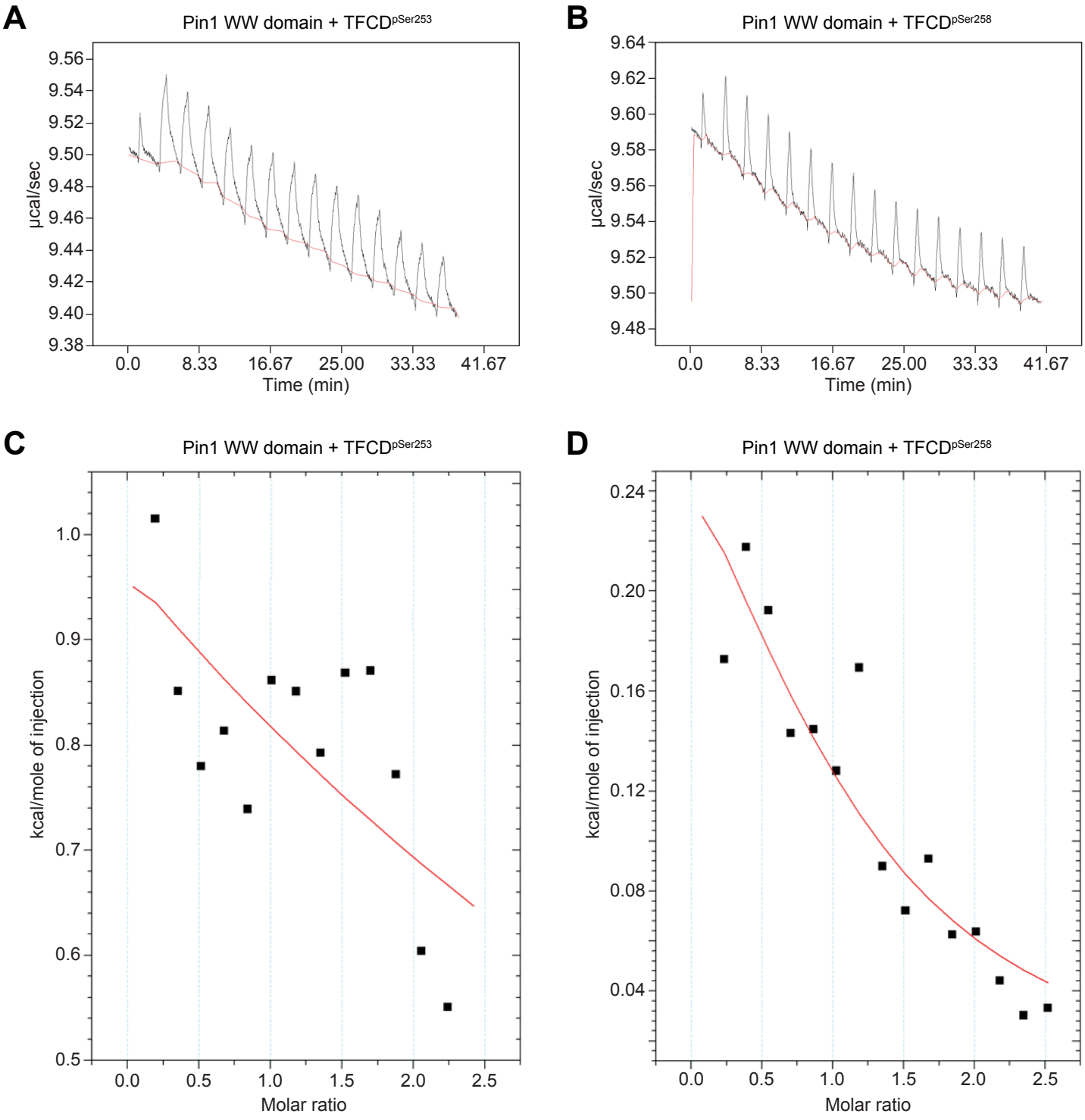
A
Pin1 WW domain + TFCDpSer253
µcal/sec
Time (min)
B
Pin1 WW domain + TFCDpSer258
µcal/sec
Time (min)
C
Pin1 WW domain + TFCDpSer253
kcal/mole of injection
Molar ratio
D
Pin1 WW domain + TFCDpSer258
kcal/mole of injection
Molar ratio

Figure S2

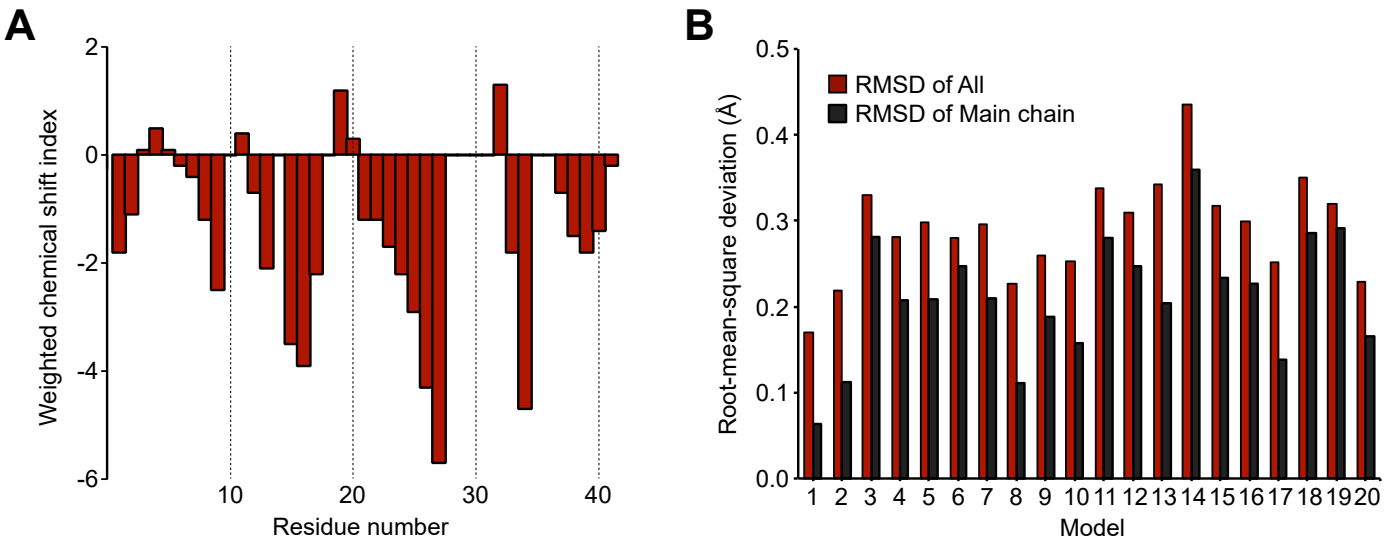
A
Weighted chemical shift index
2
0
-2
-4
-6
10
20
30
40
Residue number
B
Root-mean-square deviation (Å)
0.5
0.4
0.3
0.2
0.1
0.0
RMSD of All
RMSD of Main chain
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Model

Table S1: Summary of the Number of Restraints Used in the Calculations and Structural Statistics for the 20 Final NMR Models.

Restraint Classification	
Total NOE	203
Sequential	56
Medium Range	92
Long Range	111
Intermolecular	7
Total dihedral angle restraints	26
PHI (φ)	11
PSI (ψ)	9
CHI (χ)	8
Amber Structural Statistics	
Maximum distance restraint violation	0.355
Mean constraint violation energy	-1977.61
Maximum dihedral angle violation	13
Mean dihedral angle violation	3.9
Average pairwise RMSD of NMR ensembles (Å)	
All atoms (residues 8-41 [WW]+phosphoSer-Pro motif)	1.94
Backbone (residues)	1.06
Average pairwise RMSD of the minimized complex (Å)	
All atoms (residues 8-41 [WW]+phosphoSer-Pro motif)	0.45
Backbone (residues)	0.3
PROCHECK statistics	
Most favored (%)	60.5
Allowed favored (%)	20.9
Generously allowed (%)	14.0
Disallowed (%)	4.7
MolProbity statistics	
Protein Geometry Poor rotamers	68 (7.56%)
Favored rotamers	765 (85.00%)
Ramachandran outliers	21 (4.57%)
Ramachandran favored	391 (85.00%)
Cβ deviations >0.25Å	11 (1.12%)
Bad bonds	52 / 8652 (0.60%)
Bad angles	58 / 11120 (0.52%)
Peptide Omegas *Cis* Prolines	0 / 60 (0.00%)

Table S2: Sequences of primers used for RT-qPCR.

Primer	**Sequence (5'-3')**
human *F3* for	CTGCTCGGCTGGGTCTTC
human *F3* rev	AATTATATGCTGCCACAGTATTTGTAGTG
mouse *F3* for	CGAGACACAAACCTTGGACAGC
mouse *F3* rev	CGCTTCAGCCTTTCCTCTATGC
human *RPLP0* for	TCGACAATGGCAGCATCTAC
human *RPLP0* rev	ATCCGTCTCCACAGACAAGG
mouse *Rplp0* for	GGACCCGAGAAGACCTCCTT
mouse *Rplp0* rev	GCACATCACTCAGAATTTCAATGG