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Molecular Basis for an Ancient Partnership between Prolyl Isomerase Pin1 and Phosphatase Inhibitor-2

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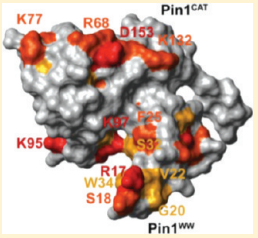
ABSTRACT: Pin1 is a prolyl isomerase that recognizes phosphorylated Ser/Thr-Pro sites, and phosphatase inhibitor-2 (I-2) is phosphorylated during mitosis at a PSpTP site that is expected to be a Pin1 substrate. However, we previously discovered I-2, but not phospho-I-2, bound to Pin1 as an allosteric modifier of Pin1 substrate specificity [Li, M., et al. (2008) *Biochemistry 47*, 292]. Here, we use binding assays and NMR spectroscopy to map the interactions on Pin1 and I-2 to elucidate the organization of this complex. Despite having sequences that are ~50% identical, human, *Xenopus*, and *Drosophila* I-2 proteins all exhibited identical, saturable binding to GST-Pin1 with $K_{0.5}$ values of 0.3 μM. The $^{1}H-^{15}N$ heteronuclear single-quantum coherence spectra for both the WW domain and isomerase domain of Pin1 showed distinctive shifts upon addition of I-2. Conversely, as shown by NMR spectroscopy, specific regions of I-2 were affected by addition of Pin1. A single-residue I68A substitution in I-2 weakened binding to Pin1 by half and essentially eliminated binding to the isolated WW domain. On the other hand, truncation of I-2 to residue 152 had a minimal effect on binding to the WW domain but eliminated binding to the isomerase domain. Size exclusion chromatography revealed that wild-type I-2 and Pin1 formed a large (>300 kDa) complex and I-2(I68A) formed a complex of half the size that we propose are a heterotetramer and a heterodimer, respectively. Pin1 and I-2 are conserved among eukaryotes from yeast to humans, and we propose they make up an ancient partnership that provides a means for regulating Pin1 specificity and function.



Pin1 is the most widely studied member of the parvulin family of peptidyl-prolyl *cis*−*trans* isomerase proteins, with the unique feature of selectively recognizing the phosphorylated Ser/Thr-Pro motif in protein.[1,2] First characterized in 1996 as a regulator of the mitotic kinase NIMA in the fungus *Aspergillus nidulans*,[3] Pin1 is homologous to the previously reported *essential* gene in *Saccharomyces cerevisiae* (Ess1)[4] and to the *Drosophila* gene *dodo*.[5] The Pin1 protein comprises 163 amino acids in two distinct domains, an N-terminal WW domain ($Pin1_{ww}$, residues 1−39) and a C-terminal catalytic domain ($Pin1_{cat}$, residues 45−163), and three-dimensional structures have been determined by crystallography[6] and NMR spectroscopy.[7] Whereas the crystal structure shows the domains compressed together in a single, overall globular shape, the solution structure determined by NMR spectroscopy depicts the domains separated from one another in a more extended configuration. Isomerization of phosphoproteins by Pin1 is considered to modulate many biological processes, especially those involving key proteins such as cyclin D1,[8] c-jun,[9] c-Myc,[10] p53,[11,12] and tau.[13−15] The overexpression of Pin1 in various human tumors[9,16−20] and the recognition that its depletion from cells induces mitotic arrest have made Pin1 an attractive therapeutic target for drug development.[21−23] A first Pin1 inhibitor is juglone, which covalently modifies the Cys in the active site of the isomerase domain[24] but as a reactive compound lacks selectivity. Other Pin1 inhibitors have been described[25,26] but, as far as we know, have not yet entered clinical trials. Because both WW and isomerase domains can bind phospho sites, it has been proposed that Pin1 uses simultaneous interaction with two different phospho sites in a particular substrate.[27] However, Ser16 in the WW domain is phosphorylated by PKA,[28] which then occupies the site where a sulfate ion binds in the three-dimensional (3D) structure.[6] Moreover, dynamic measurements by NMR spectroscopy indicate that dually phosphorylated peptides tend to interact only with the isomerase domain in Pin1.[29] The presumption has been that Pin1 acts as a monomer to isomerize phosphorylated sites in many different proteins, yet the basis for Pin1 substrate specificity is poorly understood.[29]

Inhibitor-2 (I-2) was discovered in 1976[30] as a thermostable protein that inhibited protein phosphatase activity and later was used to distinguish type 1 (I-2 sensitive) from type 2 (I-2 insensitive) protein Ser/Thr phosphatases.[31] I-2 is the most ancient of the more than 200 PP1 binding proteins, with recognized homologues in yeast (Glc8), *Drosophila*, *Xenopus*, and all mammals.[32] The most conserved feature of eukaryotic I-2 proteins is a Pro-X-Thr-Pro (PXTP) sequence motif. The heterodimer of I-2 with PP1 was studied as an "MgATP-dependent phosphatase", wherein the phosphorylation and

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dephosphorylation of Thr73 in the PXTP motif cause conformational activation of the bound PP1.[33] GSK3, MAPK, and CDK kinases phosphorylate this motif in biochemical assays.[34−37] We discovered a >25-fold increase in the level of PXTP phosphorylation during mitosis,[38] catalyzed by CDK1:cyclinB1[36] in a reaction enhanced by Suc1,[32] making this site a potential Pin1 substrate. However, T73-phosphorylated I-2 was not a substrate and did not bind to Pin1, but the negative controls in this assay, using unphosphorylated or T73A I-2, showed formation of a Pin1−I-2 complex.[39] In the presence of I-2, compared to serum albumin as a control, GST-Pin1 binding to a panel of known mitotic phosphoprotein substrates was allosterically modified, with evidence of both enhanced and restricted binding.[39] These results showed that association of Pin1 with I-2 does not occlude its phosphopeptide binding sites but does alter substrate binding specificity. Our hypothesis is that functions of Pin1 and I-2 are interdependent.

Recent results have shown that I-2 acts as a critical regulator of cellular events related to mitosis. The protein is localized at centrosomes and serves to activate Nek2 kinase by inhibition of associated PP1.[40] Centrosomes lie at the base of the primary cilium, and I-2 is concentrated in the cilium, as seen by immunofluorescence microscopy.[41] Knockdown of I-2 prevents formation of the cilium and reduces the level of acetylation of tubulin in the cilium.[41] I-2 is a maternal gene in *Drosophila*, and the I-2 protein is concentrated in oocytes and is required for the proper execution of early mitosis in the syncytial embryo.[42] Hypomorphic embryos exhibit abnormal mitotic spindles and lagging chromosomes, with lethal consequences. The defects are rescued by dose-dependent transgenic expression of DI-2.[42] Likewise, in human epithelial cells, knockdown of I-2 caused defects in mitosis, with the appearance of lagging chromosomes and failed cytokinesis, resulting in multinucleated cells, phenotypes that were rescued by coexpression of I-2.[43] Direct binding of I-2 that activates Aurora A kinase[44] reduces the threshold for CDK activation at entry into mitosis.[45] The biological functions of I-2 can in part be attributed to inhibition of PP1, but that alone seems to be inadequate to account for these phenotypes.

In this study, we have explored the binding of I-2 to Pin1 at atomic resolution. We found that the binding of I-2 to Pin1 is conserved among species, despite the sequences of the proteins being only ∼50% identical. We used NMR spectroscopy and reciprocal labeling to titrate and map the interactions on the surfaces of these two proteins. When we found that a single residue substitution in I-2 gave 50% binding at saturation compared to that of the wild type, we realized that multimers must be involved and used size exclusion chromatography to demonstrate that Pin1 and I-2 form a tetramer in solution. We used truncated and mutated proteins in binding assays to assign which regions of I-2 interact with the WW and isomerase domains in Pin1 to generate a model for this complex. We propose that Pin1 and I-2 functions are, like the proteins themselves, inextricably intertwined, and this opens new directions for future research.

MATERIALS AND METHODS

Restriction enzymes and reagents for polymerase chain reaction were purchased from New England BioLabs. Oligonucleotides were synthesized by Integrated DNA Technologies. Affinity-purified sheep anti-HI-2, anti-GST, anti-XI-2, and anti-DI-2 antibodies have been described previously.[42] Buffers and chemicals were purchased from Thermo Fisher Scientific. Phospho-S16Pin1 antibodies were from Cell Signaling Technologies. Alexa Fluor 680 goat anti-rabbit IgG was purchased from Invitrogen, and donkey anti-rabbit IRDye 800CW was purchased from LI-COR Biosciences (Lincoln, NE).

Cloning and Bacterial Expression of Proteins. Cloning of all I-2 proteins used in this study was performed according to the methods described previously.[32] Human Pin1, the WW domain (residues 1−44), and the isomerase domain (residues 46−163) were cloned in pGEX-4T-1 (Amersham Pharmacia Biotech, Freiburg, Germany). In vitro phosphorylation of purified recombinant GST-Pin1 and GST-WW domain was performed using the pure PKA catalytic subunit with a previously described method.[46]

Escherichia coli strain BL21-CodonPlus (DE3)-*RIPL* (Agilent Technologies) was transformed with pET-I-2 vectors or pGEX-4T-1(Pin1) bacterial expression vectors and grown overnight at 37 °C in 10 mL of TB medium (1.2% tryptone, 2.4% yeast extract, 2% glucose, 0.017 M KH_2PO_4, and 0.072 M K_2HPO_4), 30 μg/mL kanamycin (for pET vectors), 30 μg/mL ampicillin (for pGEX vectors), and 20 μg/mL chloramphenicol. The culture was inoculated into 1.0 L of TB medium, including 30 μg/mL kanamycin or ampicillin and 20 mL of ethanol. The transformed cells were grown to an A_{600} of 0.6 at 37 °C, and the expression of I-2 proteins was induced by addition of isopropyl 1-thio-β-D-galactopyranoside to the culture at a final concentration of 1 mM for 12−16 h at 18 °C. The bacteria were collected by centrifugation at 5000*g* for 10 min, and the cell pellet was washed by suspension in ice-cold PBS, centrifuged at 5000*g* for 10 min, and frozen at −80 °C.

Purification of His6-Tagged I-2 Proteins. The pellet was suspended by a buffer containing 50 mM MOPS-NaOH (pH 7.4), 300 mM NaCl, 10 mM imidazole, 10% glycerol, 1 mM DDT, one Complete EDTA-free tablet (Roche Applied Science) per 30 mL of buffer, and 1.0 mg/mL lysozyme and frozen at −80 °C. After being completely frozen, the pellet was thawed for 10 min in a 37 °C bath and sonicated for 30 s pulses eight times to lyse the cells. The homogenates were centrifuged at 20000*g* for 1 h at 4 °C, and the supernatant was heated for 10 min in boiling water and centrifuged for 20 min at 20000*g*. The supernatant was transferred into a 50 mL tube containing 3 mL of prewashed TALON His-Tag resin (Clontech) to adsorb protein for 1 h at 4 °C. The beads were packed into a column. The column was washed extensively with lysis buffer, and bound proteins eluted with buffer containing 150 mM imidazole hydrochloride. The eluted proteins were dialyzed against 10 mM Tris-HCl (pH 7.4), and a Complete EDTA-free protease inhibitor tablet was added to the dialyzed proteins that were stored at −80 °C.

Purification of GST and GST-Tagged Proteins. Pelleted bacteria were suspended in a buffer containing 100 mM Tris-HCl (pH 8.0), 200 mM NaCl, 10% glycerol, one Complete EDTA-free tablet per 30 mL of buffer, and 1.0 mg/mL lysozyme and frozen at −80 °C. The frozen pellet was thawed for 10 min in a 37 °C bath and sonicated for 30 s eight times for lysis of the cells. The homogenate was centrifuged at 20000*g* for 1 h at 4 °C, and the supernatant was transferred into a 50 mL tube containing 3 mL of prewashed Glutathione Sepharose 4B resin (GE Healthcare Life Sciences and Laboratories). After 1 h at 4 °C, the resin was packed into a column and washed with lysis buffer, and the bound proteins eluted using 10 mM reduced glutathione (GSH, Sigma Aldrich) in the same buffer. Eluted proteins were dialyzed against 10 mM Tris-HCl (pH 7.4), and a Complete

EDTA-free tablet was added to the dialyzed proteins that were stored at −80 °C.

Site-Directed Mutagenesis. Site-directed mutagenesis of HI-2 was performed using the Phusion Site Directed Mutagenesis Kit (New England Biolabs) according to the manufacturer's protocol. Mutagenic primers were designed using Primer X software available online. The mutations were all verified by automated DNA sequencing in the core facility of the University of Virginia School of Medicine.

Protein−Protein Binding Assays. The binding of purified I-2 to GST-Pin1 was performed using pull-down assays, quantified by fluorescence immunoblotting. A key way to reduce background was saturation of beads, accomplished by incubating 10 μL of prewashed glutathione Sepharose beads with excess recombinant GST-Pin1 in binding buffer containing 50 mM HEPES (pH 7.4), 100 mM NaCl, 10 mM $MgCl_2$, 0.5 mM $MnCl_2$, 5 mM EGTA, 1 mg/mL BSA, 0.5 mM DDT, and 1 mM Pefabloc (Roche Applied Sciences). The GST-Pin1 beads were pelleted by centrifugation and washed once with the same buffer. Various concentrations of recombinant I-2 proteins were added to the GST-Pin1 beads in a total volume of 1 mL with binding buffer and incubated for 1 h at room temperature. The beads were pelleted and washed three times by centrifugation with 1 mL of the same buffer, without BSA. Bound proteins were eluted with 2× SDS sample buffer and subjected to 12% sodium dodecyl sulfate−polyacrylamide gel electrophoresis SDS−PAGE and Western blotting as described previously.[47]

Expression and Purification of Pin1 for NMR Spectroscopy Experiments. The BL21(DE3) *E. coli* strain was transformed with the pET15b plasmid (Merck) carrying *pin1*. The recombinant strain was grown at 37 °C in LB medium until A_{600} reached ∼0.8, and then the induction phase was started by addition of 0.5 mM IPTG, for 3 h at 31 °C. The cells were harvested by centrifugation, and the pellet was resuspended in lysis buffer [50 mM Na_2HPO_4/NaH_2PO_4 (pH 8.0) and 300 mM NaCl (buffer A)], with 10 mM imidazole, 1 mM DTT, 0.1% NP40, and a protease inhibitor cocktail (Roche). Cell lysis was performed by incubation with 10 mg of lysozyme and 0.5 mg of DNase I per liter of culture, followed by a brief sonication, and an extract prepared by centrifugation. The soluble extract was loaded on a nickel affinity column (Chelating Sepharose Fast Flow 5 mL, GE Healthcare). Unbound proteins were washed away with 20 mM imidazole in buffer A, and the protein of interest was eluted with 250 mM imidazole in buffer A. The same protocol was performed for the production of ^{15}N-labeled Pin1, with the use of M9 minimal medium complemented with 1 g of $^{15}NH_4Cl$ (Cambridge Isotope Laboratories, Cambridge, MA) as a nitrogen source, 4 g/L glucose, 1 mM $MgSO_4$, 100 mg/L ampicillin, and MEM vitamin cocktail (Sigma). Homogeneous fractions were buffer-exchanged on a PD10 G-25 column (GE Healthcare) equilibrated in the NMR spectroscopy sample buffer [50 mM Tris-d_{11} (pH 6.4), 25 mM NaCl, 1 mM DTT, and 1 mM EDTA].

Expression and Purification of I-2 for NMR Spectroscopy Experiments. The BL21(DE3) *E. coli* strain was transformed with the pET28a plasmid (Merck) encoding HI-2 and grown at 37 °C in LB medium until A_{600} reached ∼0.8, and then 0.5 mM IPTG was added for 16 h at 20 °C. The cells were harvested by centrifugation, and the pellet was resuspended in lysis buffer [50 mM Na_2HPO_4/NaH_2PO_4 (pH 8.0) and 300 mM NaCl (buffer A)], with added 10 mM imidazole, 1 mM DTT, 0.1% NP40, and a protease inhibitor cocktail (Roche). Cell lysis was performed by incubation with 5 mg of lysozyme per liter of culture followed by sonication and centrifugation. The soluble extract was incubated at 75 °C for 15 min and centrifuged at 15000*g*. The protein in the supernatant was purified on a 1 mL column of Chelating Sepharose Fast Flow (GE Healthcare) following the same procedure that was used for Pin1. The same protocol was performed for the production of ^{15}N-labeled or ^{15}N- and ^{13}C-labeled I-2 with the use of M9 minimal medium complemented with 1 g of $^{15}NH_4Cl$ (Cambridge Isotope Laboratories, Cambridge, MA) as the nitrogen source, 4 g/L glucose or 2 g of [$^{13}C_6$]glucose, respectively, 1 mM $MgSO_4$, 20 mg/L kanamycin, and MEM vitamin cocktail (Sigma). The same ^{1}H−^{15}N HSQC spectrum was obtained for I-2, regardless of whether the heating step of the soluble extract at 75 °C was performed during the purification, indicating that heating did not modify the natively unfolded conformation of the I-2 protein. The eluted fractions containing most of the I-2 were pooled and purified by gel filtration (HiLoad 16/60 Superdex75 prep grade, GE Healthcare) in 50 mM ammonium bicarbonate. Fractions were checked on a 12% SDS−polyacrylamide gel, pooled, and lyophilized before being redissolved in NMR spectroscopy sample buffer [50 mM Tris-d_{11} (pH 6.4), 25 mM NaCl, 1 mM DTT, and 1 mM EDTA] or directly in a Pin1 solution for titration.

NMR Spectroscopy. Interactions between I-2 and Pin1 were mapped either with ^{15}N-labeled I-2 at 100 μM and unlabeled Pin1 added to reach 0.5, 1, 2, or 12 molar equiv of Pin1 or ^{15}N-labeled Pin1 at 62.5 μM and unlabeled I-2 as lyophilized aliquots to reach 0.5 or 1 molar equiv of I-2. Spectra were recorded at 293 or 277 K on a 600 or 900 MHz spectrometer equipped with a cryogenic probehead. All ^{1}H spectra were calibrated with 1 mM sodium 3-trimethylsilyl-3,3′,2,2′-d_4-propionate as a reference. ^{1}H−^{15}N HSQC spectra were recorded with at least 2048 points in the proton and 256 points in the nitrogen dimensions. The chemical shift perturbations of individual amide resonances in I-2 protein were calculated with eq 1, taking into account the relative dispersion of the proton and nitrogen chemical shifts (1 and 20 ppm, respectively). Instead, a correction coefficient of 0.2 was applied to the nitrogen dimension for the mapping of Pin1 resonances.

$$\Delta\delta(\text{ppm}) = \sqrt{[\Delta\delta(^1\text{H})]^2 + 0.05[\Delta\delta(^{15}\text{N})]^2} \quad (1)$$

Superose 12 Size Exclusion Chromatography. Chromatography on a Superose 12 HR 10/300 column was performed to study formation of the Pin1−I-2 complex. The GST tag was cleaved from Pin1 by on-column thrombin digestion using the previously described method.[5] Pin1 was concentrated on a centrifugal ultrafilter (10 kDa molecular mass cutoff, Millipore), mixed with a final HI-2 concentration of 1.25 μM, and incubated for 1 h at room temperature. An aliquot (200 μL) was applied to the column and eluted with 50 mM HEPES (pH 7.4), 100 mM NaCl, 10 mM $MgCl_2$, 0.5 mM $MnCl_2$, 5 mM EGTA, 1 mg/mL BSA, 0.5 mM dithiothreitol, and 1 mM Pefabloc at a flow rate of 0.6 mL/min. Fractions of 1 min (0.6 mL) were collected and elution profiles developed by dot-blot immunoblotting. Samples (0.2 mL) of individual fractions were adsorbed on nitrocellulose using a 96-well vacuum manifold and developed as immunoblots for I-2 and Pin1. The staining intensities were quantified by fluorescence scanning and plotted as an elution profile.

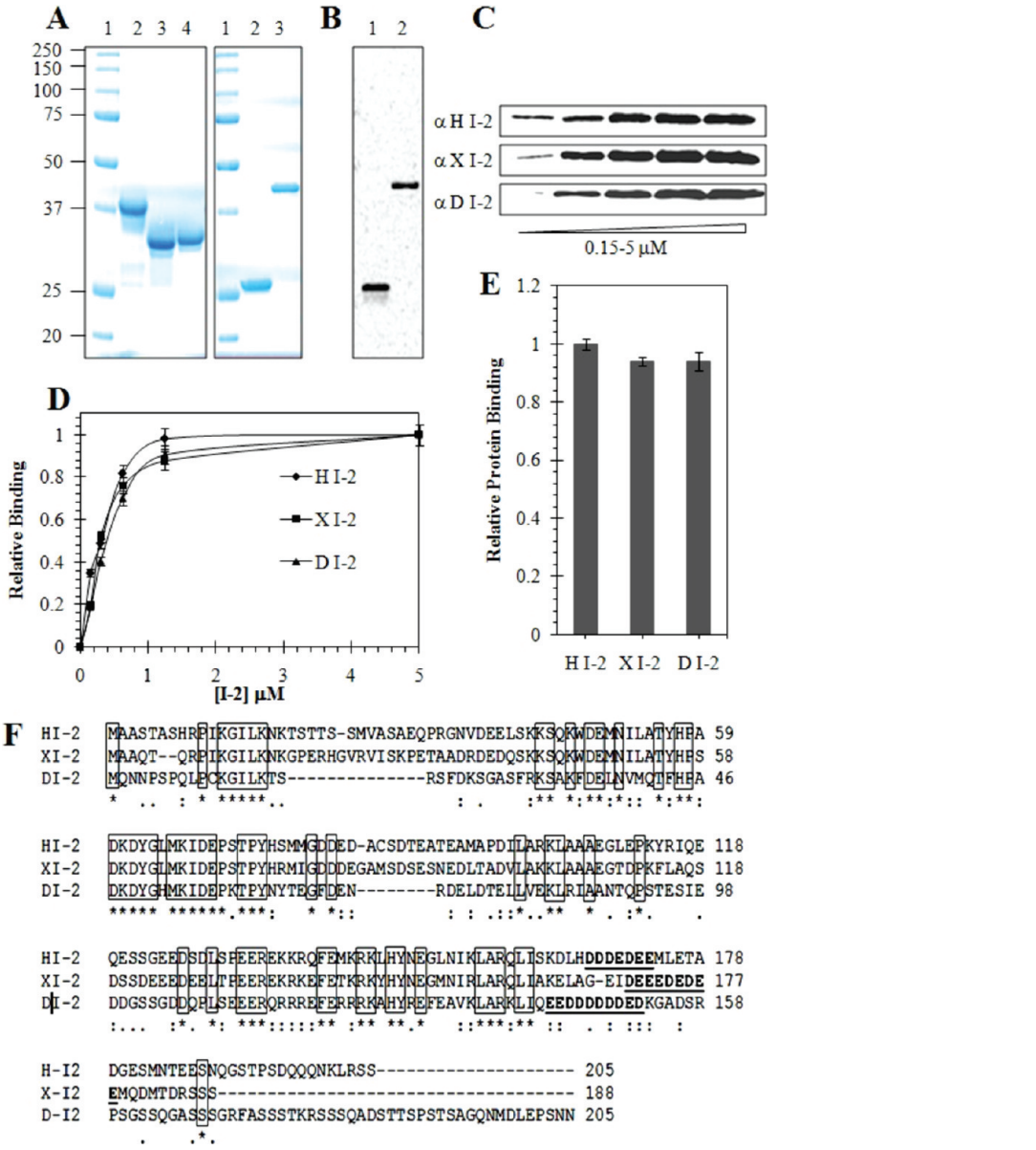

Figure 1. Binding of human, *Xenopus*, and *Drosophila* I-2 proteins to GST-Pin1. (A) Coomassie staining of purified proteins used in binding assays after SDS−PAGE. Left panel: lane 1, molecular size standard proteins; lane 2, human I-2 (HI-2); lane 3, *Xenopus* I-2 (XI-2); and lane 4, *Drosophila* I-2 (DI-2). Right panel: lane 1, molecular size standards; lane 2, GST control protein; and lane 3, GST-Pin1. (B) Immunoblotting of GST protein and GST-Pin1 using the anti-GST antibody. (C) Immunoblotting of different I-2 forms using species-specific antibodies for an assay of direct binding of recombinant His_6-I-2 proteins to GST-Pin1 in a pull-down assay, as described in Materials and Methods. (D) Concentration dependence for I-2 from different species: (◆) HI-2, (■) XI-2, and (▲) DI-2. (E) Quantification of the amounts of different I-2 proteins bound to GST-Pin1, relative to recombinant protein standards. (F) Sequence alignment for human I-2 (HI-2), *Xenopus* I-2 (XI-2), and *Drosophila* I-2 (DI-2) using ClustalW 2.1. Conserved residues are marked with an asterisk and a box drawn around them. The polyacidic region is shown in boldface and underlined. HI-2 is 60% identical to XI-2 and 40% identical to DI-2.

RESULTS

Binding of Inhibitor-2 Proteins with Pin1. To define the structural requirements for interaction, we compared binding of I-2 proteins from different species [human (HI-2), *Xenopus* (XI-2), and *Drosophila* (DI-2)] to human GST-Pin1 in a pull-down assay. The recombinant proteins were expressed in bacteria and extensively purified by affinity chromatography, and the final products were analyzed by Coomassie staining following SDS−PAGE (Figure 1A). GST and GST-Pin1 were detected as single bands by anti-GST immunoblotting (Figure 1B). The binding assay used a range of concentrations of added I-2 proteins (0.1−5 μM) mixed with GST-Pin1 that was bound to glutathione Sepharose beads. The bound proteins were quantified by fluorescence immunoblotting with species-specific anti-I-2 antibodies. Because the I-2 sequences are so divergent, no single anti-I-2 antibody reacts with these three proteins; therefore, we prepared and affinity purified species-specific anti-HI-2, anti-XI-2, and anti-DI-2 antibodies for immunoblotting detection (Figure 1C). Staining intensities on immunoblots were

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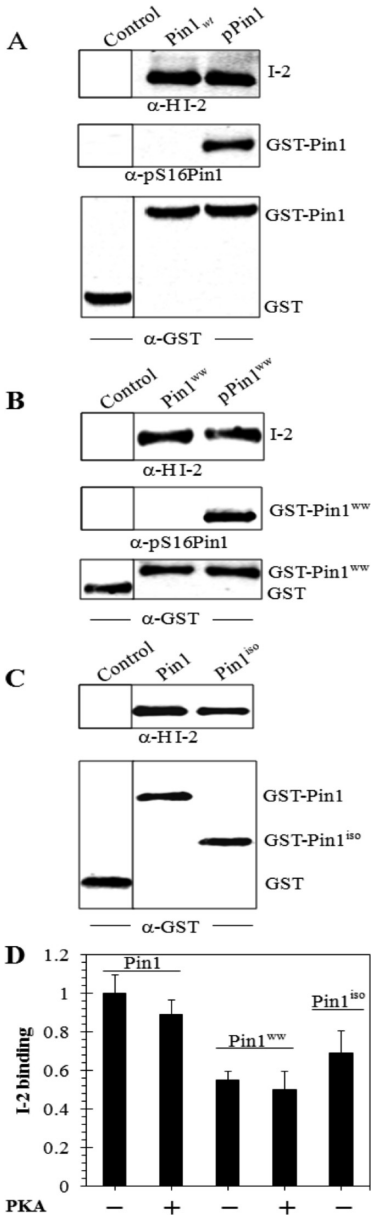

Figure 2. Comparison of I-2 binding to Pin1, phospho-Pin1, and separate WW and isomerase domains. GST-Pin and GST-Pin_{ww} and their PKA-phosphorylated forms, and GST-$Pin1_{iso}$, were prepared as described in Materials and Methods. (A) Glutathione Sepharose beads were saturated with GST (Control), wild-type GST-Pin1 ($Pin1_{wt}$), or phosphorylated GST-Pin1 (pPin1) and subjected to a pull-down assay with human I-2 protein (1.25 μM) as described in Materials and Methods. Proteins were eluted and immunoblotted with anti-human I-2 (top), anti-phospho-S16Pin1 (middle), and anti-GST (bottom) antibodies. (B) GST (Control), GST-$Pin1_{ww}$ ($Pin1^{ww}$), and PKA-phosphorylated GST-$Pin1_{ww}$ ($pPin1^{ww}$) were incubated for 1 h in with HI-2 protein and processed as described for panel A. (C) Direct binding of HI-2 to GST (Control, left lane), GST-Pin1 (center lane), and the GST-Pin1 isomerase domain (right lane). The top panel shows immunoblots for bound HI-2 and the bottom panel immunoblots for GST as a loading control. (D) Quantification of pull-down assays depicted in panels A−C, showing average values and the standard deviation for three independent experiments.

quantified and calibrated over a range of concentrations, using recombinant I-2 standards with the absolute amount of the proteins determined by A_{280}. The binding of HI-2, XI-2, and DI-2 to GST-Pin1 was indistinguishable, with a $K_{0.5}$ of ~0.3 μM, and all showed saturation at 1.25−5 μM (Figure 1D), which is well below the estimated cellular concentration of I-2.[43] The total amount of each I-2 protein bound to GST-Pin1 at saturation was identical (Figure 1E). GST was used as the negative control, and the assay was optimized so there was essentially no I-2 binding to GST beads. This showed that the I-2 proteins were binding to the Pin1 portion of the fusion protein, not to GST itself. These results demonstrated that stable, saturable association with Pin1 is a conserved function of the I-2 family of proteins. Multiple-sequence alignment generated for these I-2 proteins revealed the location and clustering of identical residues (boxed in Figure 1F) that are the most probable regions for interaction with human Pin1.

Association of HI-2 with Phospho-Pin1 and Separate Pin1 Domains. Binding of HI-2 to Pin1 used proteins expressed in bacteria; therefore, they were both devoid of phosphorylations that occur in live animal cells, such as phosphorylation of Ser16 in the Pin1 WW domain.[28] We reacted full-length GST-Pin1 with purified PKA and showed phosphorylation of Ser16 by immunoblotting with a phospho site-specific antibody (Figure 2A). Binding of HI-2 to the PKA-phosphorylated GST-Pin1 was weakened only slightly compared to the binding of HI-2 with nonphosphorylated GST-Pin1 (Figure 2A,D). These results show that phosphorylation of Pin1 by PKA, involving at least Ser16, did not affect the formation of a protein−protein complex with HI-2.

We expressed the N-terminal WW domain of Pin1 ($Pin1_{ww}$) and C-terminal isomerase catalytic domain ($Pin1_{iso}$) fused to GST and used them in pull-down assays with purified HI-2. The amount of HI-2 bound to $Pin1_{ww}$ was approximately half of that bound to Pin1 (Figure 2B,D). Phosphorylation of $Pin1_{ww}$ by PKA was demonstrated by immunoblotting with the pSer16 phospho site antibody (Figure 2B). Phosphorylation of the $Pin1_{ww}$ domain resulted in no difference in binding of HI-2 in the pull-down assay, reinforcing the results with full-length Pin1. These results showed that the WW domain of Pin1 alone was sufficient for interaction with HI-2, albeit at approximately half the level relative to that of full-length Pin1. Assays with the isomerase domain of Pin1 (Pin_{iso}) revealed that the amount of HI-2 bound was ~30% lower than the amount of full-length Pin1 (Figure 2C,D), indicating that the isomerase domain also was sufficient for association with I-2. The results indicate that I-2 independently associates with both the WW domain and the isomerase domain of Pin1.

NMR Spectroscopy Analysis of Association of I-2 with Pin1. Pin1 and I-2 proteins were separately labeled with ^{15}N by expression in bacteria and purified, and their interaction in solution was investigated by NMR spectroscopy. The two-dimensional $^{15}N-^{1}H$ heteronuclear single-quantum coherence (HSQC) spectra of 100 μM ^{15}N-labeled Pin1 alone and mixed with purified unlabeled HI-2 were recorded and superimposed. The spectrum of Pin1 alone (black) and that after the addition of 0.5 (red) and 1.0 (blue) molar equiv of HI-2 showed weak but reproducible shifts of selected resonances in Pin1 (Figure 3A), indicative of physical contacts with HI-2. To delineate the HI-2 binding sites on Pin1, the residues with induced chemical shift perturbations were mapped on the Pin1 3D structure from the Protein Data Bank (PDB) (entry 1PIN), depicted front and back in Figure 3B. Cross-peak movements that correspond to residues with combined chemical shift variations

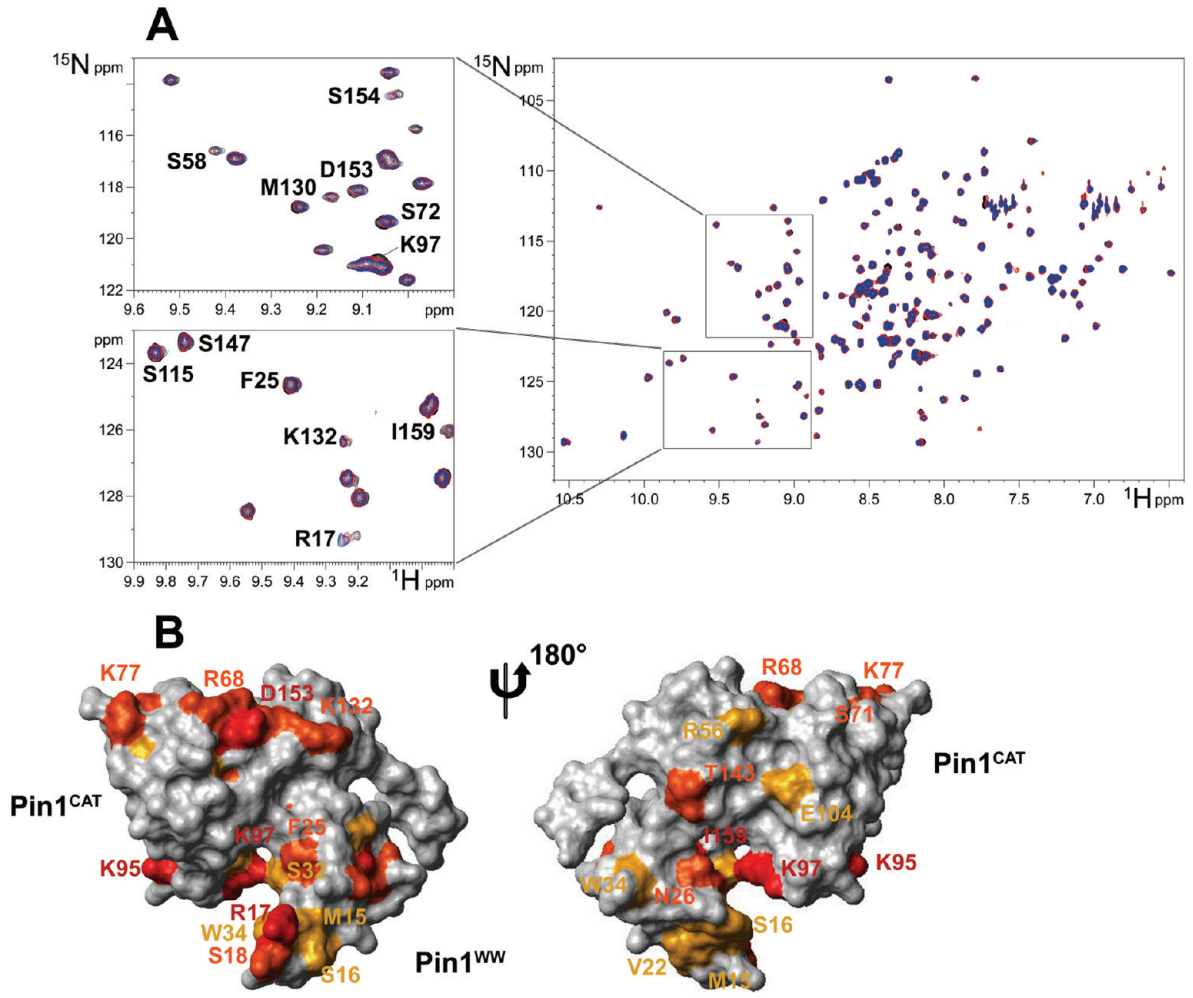

Figure 3. NMR spectroscopy mapping of surface residues in Pin1 that contact I-2. (A) $^{1}H-^{15}N$ HSQC spectrum of ^{15}N-labeled Pin1 alone (black) overlaid with spectra after addition of 0.5 (red) and 1.0 molar equiv (blue) of unlabeled I-2 showing the shifts of resonances, marked with single-letter code and residue number. The magnified areas are enlarged to the left side for closer examination. (B) Mapping of the I-2 interaction sites on the Pin1 structure (PDB entry 1PIN). The isomerase domain ($Pin1^{CAT}$) is shown at the top left and the WW domain ($Pin1^{WW}$) at the bottom right. The structure on the right is rotated 180° to show the reverse side. Residues with combined chemical shift variations (calculated as described in Materials and Methods) greater than 0.04 ppm are colored red, those in the 0.02−0.04 ppm range orange, and those in the 0.015−0.02 ppm range yellow.

greater than 0.04 ppm (red), in the 0.02−0.04 ppm range (orange), and in the 0.015−0.02 ppm range (yellow) mapped residues in contiguous areas on the surfaces of both the WW and catalytic domains of Pin1. The structure is rotated 180° to view the opposite side, where relatively fewer interactions appeared. These images support our results in binding assays in which I-2 interacts with the separate, isolated $Pin1_{ww}$ and $Pin1_{iso}$ domains. The contacts of I-2 with the WW domain involved R17, S18, and S32, and several hydrophobic side chains, including V22, F25, and W34. On the other hand, interactions with the isomerase catalytic domain were dominated by charged side chains, K77, R68, D153, and K132. The I-2 contact surface on the Pin1 catalytic domain includes the putative active site residue R68 but does not appear to occlude the active site to interfere with substrate binding. This is consistent with our previous report that I-2 allosterically modifies Pin1 specificity.

Reciprocal labeling experiments involved the titration of purified ^{15}N-labeled HI-2 with unlabeled Pin1 and were conducted under different conditions (both 4 and 20 °C) and different molar ratios of the proteins (from 0.5 to 12). The HSQC spectra of HI-2 (Figure 4A) in the presence of increasing amounts of Pin1 were superimposed over the spectrum of HI-2 alone (black) to show significant perturbations that correspond to residues in HI-2 in physical contact with Pin1. HI-2 is known to be an extended polypeptide in solution and other than one short helical region is devoid of secondary or tertiary structure, so the results could not be mapped on a three-dimensional structure as for Pin1 (see above); instead, we plotted shifts as a function of sequence position in HI-2 (Figure 4B). This analysis reveals multiple regions in I-2 appear to be in contact with Pin1. These include the N- and C-termini, residues 85−95, and residues 105−125. The region of residues 60−85 was so broadened by addition of excess Pin1 the delta could not be calculated. These relatively small variations could be reproduced under different conditions, and our interpretation was that the extreme flexibility of I-2 did not allow for larger changes. These NMR spectroscopy results with labeled I-2 show binding with Pin1 involved multiple sites of protein−protein interaction.

Defining Regions in HI-2 Involved in Binding to Pin1. To further dissect the structure−function relationship for I-2, we

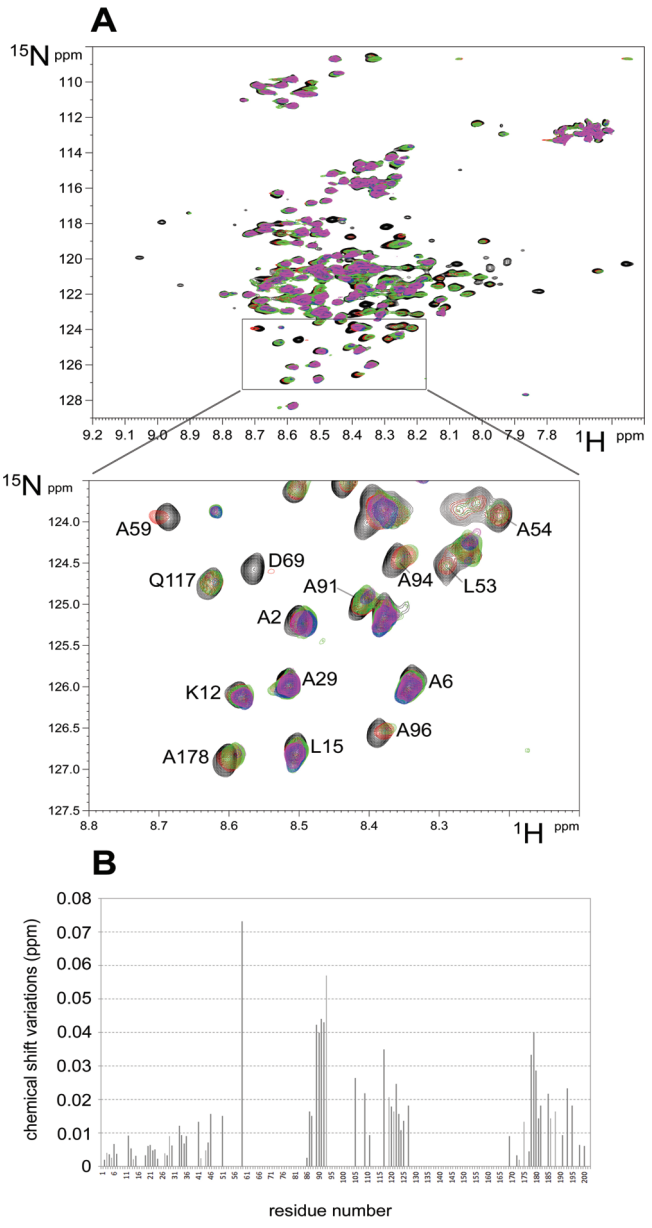

Figure 4. NMR spectroscopy mapping of I-2 residues that contact Pin1. (A) Complete (top) and magnified (bottom) $^{1}H-^{15}N$ HSQC spectrum of ^{15}N-labeled I-2 alone (black) overlaid with spectra recorded after addition of 0.5 (red), 1.0 (green), 2.0 (blue), and 12 (magenta) molar equiv of unlabeled Pin1, recorded at 4 °C showing the shifts of specific residues. (B) Graphical representation of the chemical shift variations in the HDSQ spectrum of I-2 resonances ($\Delta\delta$) upon addition of excess Pin1, as done for panel A.

produced truncated and mutated forms of HI-2 and assayed binding to GST-Pin1 (Figure 5). The following HI-2 variants were used: full-length wild type I-2 (WT), C-terminally truncated I-2 (1−197), substitution of a conserved aliphatic side chain, I-2 (I68A), deletion of both N- and C-terminal regions (14−197), and larger deletions of the C-terminus, (1−152 and 1−119). Recombinant I-2 proteins were affinity purified and assayed at a final concentration of 1.25 μM. The results (Figure 5A) showed no significant difference between the binding of I-2(205) and I-2(197), showing that the extreme C-terminal region was not required for association of HI-2 with GST-Pin1. In contrast, truncation of the 13 N-terminal residues that includes the SILK motif common to many PP1 binding proteins reduced by half the amount of binding of I-2 to Pin1. Further truncation of I-2 from the C-terminus to residue 152, or residue 119, resulted in a significant 75% loss of binding to GST-Pin1. These results reinforced the NMR spectroscopy assignment of a site in I-2 encompassing residues 170−200 as strongly interacting with Pin1. We suspect involvement of an uninterrupted stretch of acidic residues, which is a conserved feature among I-2 proteins that does not align exactly in the same residue positions. It is important to note here that 1−152 was tested because its inhibitory potency for PP1C is identical to that of full-length I-2.[48]

We had reported association of I-2 with Pin1 was exceedingly sensitive to disruption by detergents,[39] presumably because of hydrophobic interaction between the proteins. The highly conserved region of residues 60−85 of I-2 is likely engaged with Pin1 on the basis of results of NMR spectroscopy. Within this region, most of the conserved residues are charged, with the exception of Ile68, which is a strictly conserved, aliphatic residue that we suspected could be involved in a hydrophobic protein−protein interface. Therefore, we produced an I68A substituted form of I-2 and found its level of binding with Pin1 was reduced to half of that of wild-type HI-2 when both proteins were compared at 1.25 μM (Figure 5A). We assayed for binding to Pin1 over a range of concentrations and found a reduced slope in the initial portion (<1 μM) of the saturation curve (Figure 5B). However, more provocative was the observation that when binding of I-2(I68A) reached saturation at approximately the same concentration as wild-type I-2, there was only 50% of the amount of I-2 protein bound. This suggested to us that there were two binding sites for wild-type I-2 binding to Pin1, and I-2(I68A) bound to only one of them.

We compared binding of I-2, I-2(I68A), and I-2(152) to full-length Pin1, $Pin1_{ww}$, and $Pin1_{iso}$ in pull-down assays (Figure 5C, D). Mutation I68A resulted in essentially a complete loss of binding to the WW domain, whereas the binding to the Pin1 isomerase domain was the same as binding to full-length Pin1. Our conclusion was that I68 was critical for binding to the WW domain but was not required for and did not much affect binding of I-2 to the isomerase domain of Pin1. On the other hand, truncation of I-2 to 152 residues essentially eliminated binding to the isomerase domain, while I-2(152) bound to full-length Pin1 and $Pin1_{ww}$. Our hypothesis was that each domain of Pin1 bound separate regions of I-2, and Pin1 bound to two I-2 molecules, one to each domain.

Analysis of HI-2−Pin1 Complexes by Size Exclusion Chromatography. We tested our hypothesis using Superose 12 column chromatography to analyze the complexes formed between His_6-HI-2 and Pin1 (Figure 6). This was a homogeneous solution assay that involved simple mixing of soluble components, compared to the heterogeneous assay that used a GST fusion protein immobilized on beads. Pin1 protein was recovered free of GST by on-column thrombin cleavage of the GST-Pin1 fusion protein purified from bacteria (see the inset of Figure 6D). This preparation of Pin1 eluted as a symmetrical peak centered at 15.6 mL (dashed line in Figure 6D), corresponding to monomeric Pin1 (Figure 6A−D). Purified I-2 eluted from Superose 12 as a peak centered at 13.2 mL (dotted line in Figure 6A−D). Because of its extended and unstructured conformation, I-2 has been known to behave as a larger molecule in solution, and indeed, I-2 eluted before ovalbumin [43 kDa,

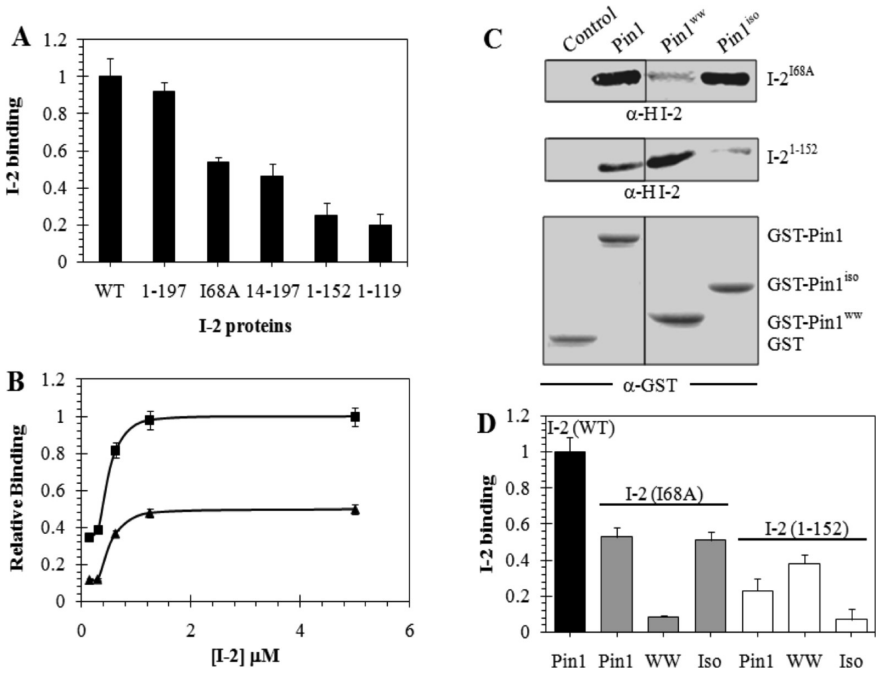

Figure 5. Binding of I-2 variants to GST-Pin1. (A) Binding of wild-type (WT) and truncated and substituted HI-2 to GST-Pin1 in a pull-down assay as described in Materials and Methods. The recovery of bound I-2 was quantitated using Odyssey version 1.2 by immunoblotting, with a correction for amount of GST-Pin1 by simultaneous dual-wavelength scanning of two different secondary antibodies on the same filter (Odyssey, Li-Cor Industries). Values from three independent experiments were normalized to WT, averaged, and plotted with the standard deviation. (B) Dose−response curve for binding of wild-type HI-2 (■) and I-2(I68A) (▲) to GST-Pin1 in a pull-down assay ($n = 3$). Immunoblotting analyzed as described for panel A and data plotted with the standard deviation using Sigma plot 10.0. (C and D) Pull-down assays for binding of 1.25 μM HI-2(I68A) ($I\text{-}2^{I68A}$) (top gel in panel C, gray bars in panel D) or HI-2(1−152) ($I\text{-}2^{1-152}$) (center gel in panels C, white bars in panel D) with GST, GST-Pin1 (Pin1), $GST\text{-}Pin1_{ww}$ ($Pin1^{ww}$ and WW), or $GST\text{-}Pin1_{iso}$ ($Pin1^{iso}$ and Iso). Immunoblotting of GST and fusion proteins is shown in the bottom gel of panel C. For control and normalization of other samples, wild type (WT) HI-2 was pulled down with GST-Pin1. Data ($n = 3$) were analyzed as described for panel A.

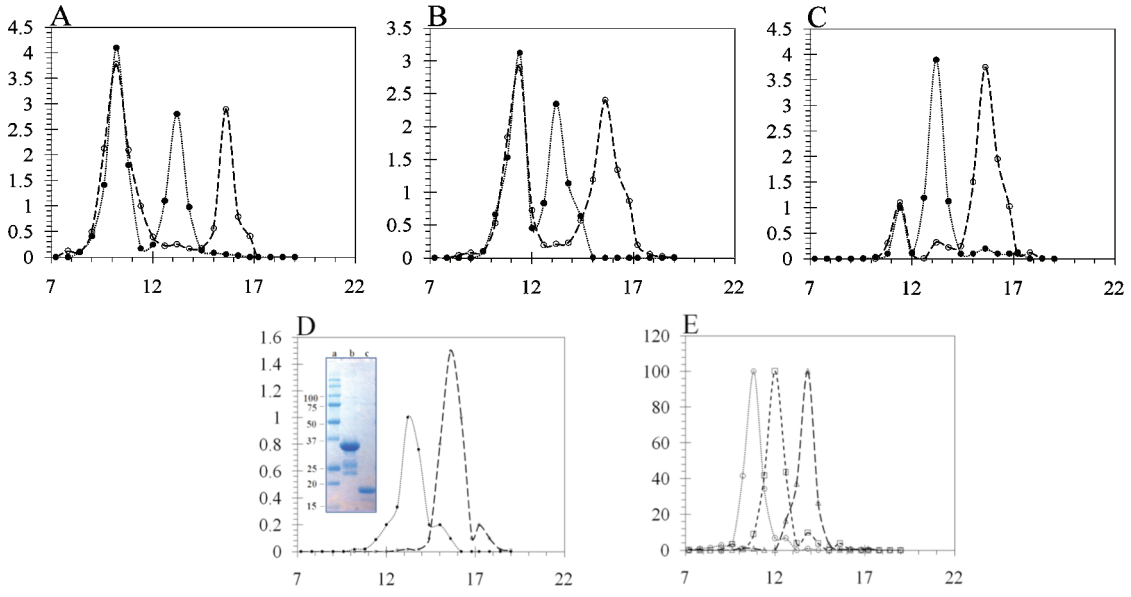

Figure 6. Size exclusion chromatography analysis of Pin1 and I-2 complexes. Recombinant GST-Pin1 was cleaved by thrombin, and the Pin1 protein was concentrated, incubated with recombinant HI-2, and applied to a Superose 12 HR 10/300 column, as described in Materials and Methods. Aliquots of the eluted fractions were immunoblotted with anti-Pin1 (dashed lines) and anti-HI-2 (dotted lines) antibodies using the dot blot technique with a 96-well vacuum manifold. (A) Elution profile of a mixture of Pin1 and wild-type HI-2. (B) Elution profile of a mixture of Pin1 and the I68A mutant form of HI-2. (C) Elution profile of a mixture of Pin1 and the truncated form of HI-2(1−152). (D) Superimposition of separate elution profiles for Pin1 and wild-type HI-2. The inset is a Coomassie-stained SDS−PAGE gel: lane 1, molecular size standards; lane 2, purified Pin1; and lane 3, purified HI-2 protein used in these experiments. (E) Superimposition of separate elution profiles for purified proteins used as molecular size standards: catalase (260 kDa, 10.8 mL), immunoglobulin (150 kDa, 12.0 mL), and ovalbumin (43 kDa, 13.8 mL).

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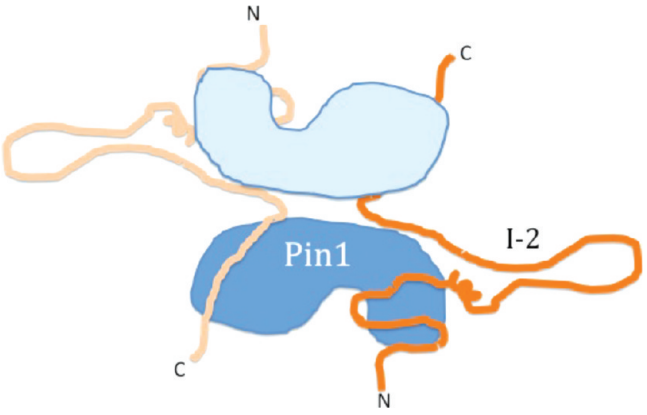

Figure 7. Model of the Pin1−I-2 complex. Cartoon showing the arrangement of two molecules of Pin1 with two molecules of I-2 in a complex formed in solution. Pin1 is colored light and dark blue as two lobes, composed of a smaller WW domain and a larger isomerase catalytic domain. I-2 is colored orange as an unstructured polypeptide with N- and C-termini labeled, using multiple regions for binding to separate domains of Pin1.

13.8 mL (Figure 6E)] in size exclusion chromatography. When recombinant I-2 was mixed with Pin1 and the sample resolved on Superose 12, immunoblotting of the fractions for I-2 revealed two prominent peaks: a major symmetrical peak centered at 10.2 mL that contained ∼60% of the total I-2 protein and a second peak at 11.4 mL corresponding to unbound monomeric I-2 (Figure 6A). Immunoblotting of the fractions for Pin1 showed exact co-elution of Pin1 with I-2 in the peak at 10.2 mL and also a second peak at 15.6 mL, corresponding to free monomeric Pin1. These results showed the formation of a stable, large complex between purified Pin1 and I-2 that eluted at a size larger than that of catalase [260 kDa, 10.8 mL (Figure 6E)]. On the other hand, when the I68A mutant of I-2 was mixed with Pin1, the complex that formed eluted at 11.4 mL (Figure 6B). This complex is smaller than catalase but still larger than immunoglobulin [150 kDa, 12.0 mL (Figure 6E)] and corresponds to approximately half the relative size of the wild-type I-2−Pin1 complex. The distribution of I-2 protein between the Pin1 complexes and the free monomer was approximately the same for I-2(I68A) and wild-type I-2 (Figure 6A,B), and our interpretation was that these different I-2 proteins had approximately the same affinity for Pin1. Analysis of I-2(152) binding to Pin1 showed a very low yield of the complex between the proteins in solution, with most of the proteins eluting separately as monomers (Figure 6C). What little complex was formed eluted at 11.4 mL, not 10.2 mL. The excluded volume (V_0) of this column is 9 mL; thus, the complexes formed were discrete molecular species and not simply polymers. Because the complexes eluting at 10.2 and 11.4 mL both contained equivalent amounts of I-2 and Pin1, we concluded that these peaks represented tetrameric $(Pin1)_2(I\text{-}2)_2$ and dimeric $(Pin1)(I\text{-}2)$ complexes, respectively.

DISCUSSION

The results of this study reveal molecular interactions between two ancient and essential proteins, Pin1 and I-2. Purified recombinant I-2 proteins from divergent species exhibited saturable binding to Pin1 with submicromolar affinity. Estimates for the intracellular concentrations of Pin1 and I-2 are micromolar,[43,49] which would predict that the proteins form complexes together in living cells and tissues. Our results argue that binding to Pin1 is an evolutionarily conserved function, and our hypothesis was that interactions involved regions of the I-2 protein identical in sequence among the different species. Indeed, NMR spectroscopy of reciprocally labeled proteins dynamically interacting in solution showed that multiple conserved sequence regions in I-2 were in contact with surface sites on Pin1, in both the WW and isomerase domains. One key conclusion from the results of NMR spectroscopy is that the I-2 interactions mapped on the surfaces of Pin1 do no occlude the active site. This supports the concept that I-2 does not interfere with substrate binding but acts as an allosteric modulator of Pin1 substrate specificity, as we previously demonstrated using a panel of mitotic phosphoproteins.[39]

Mapping the sites of I-2 interaction on Pin1 by NMR spectroscopy revealed surface regions on the WW domain and on the isomerase domain. Residues in the WW domain that contacted I-2 did not include Ser16, consistent with unimpeded binding of I-2 to PKA-phosphorylated Pin1. Phosphorylation by PKA involves at least Ser16, and this phosphoryl group is thought to preclude the binding of phosphorylated substrates to the WW domain.[28] Binding of I-2 was insensitive to PKA phosphorylation of either the full-length Pin1 protein or the WW domain, so the phosphoryl group at Ser16 did not exert steric or electrostatic constraints upon association with I-2. These results are important in devising a model for the complex between Pin1 and I-2 and potentially will influence our understanding of how signaling events regulate Pin1 function. The NMR spectroscopy analysis also identified several hydrophobic residues in the Pin1 WW domain that made contact with I-2. Pin1 was already known to elaborate a hydrophobic surface on the WW domain that faces the isomerase domain. This forms a hydrophobic cleft that binds polyethylene glycol during crystallization, as visualized in the 3D structure determined by X-ray crystallography.[6] There are relatively few conserved hydrophobic residues in I-2 that would be available for generating an aliphatic protein−protein interface with Pin1. Searching among the conserved sequence regions in I-2 that NMR spectroscopy identified as Pin1 contact sites, we selected Ile68 as a likely candidate. The I68A substitution was a relatively minor modification in the entire 205-residue protein but had profound effects on binding to Pin1. The level of binding was reduced ∼50%, but more importantly, the amount of I-2(I68A) bound at saturating concentrations was only half as much as the amount of wild type. This was unexpected and forced us to consider the possibility that there were two binding sites for I-2 on Pin1, only one of which bound I-2(I68A).

This idea was validated by an assay of I-2 binding to Pin1 and the separate WW and isomerase domains. The I68A mutation essentially eliminated binding of I-2 to the isolated WW domain but did not equally impair binding to the Pin1 isomerase domain. On this basis, we assign the I68 and surrounding region of I-2 as the part of the protein engaged with the WW domain of Pin1. On the other hand, truncation of I-2 to residue 152 eliminates the Pin1 interacting site at residue 170−190 seen by NMR spectroscopy, which we suspect involves a long uninterrupted stretch of acidic residues that appears as a conserved feature among I-2 forms from different species (boldface and underlined in Figure 1). I-2(1−152) bound more weakly to Pin1; however, the level of binding to Pin1 and its isolated WW domain was approximately the same, whereas there was almost no binding to the isomerase domain. Our interpretation of these

results is that the polyacidic site in I-2 probably interacts with the multiple adjacent basic residues across the surface of the isomerase domain in Pin1. Assays with mutated I-2 and Pin1 separate domains were consistent with the results of mapping with NMR spectroscopy and allowed us to assign interactions of different I-2 regions to individual Pin1 domains.

The results led us to consider the idea that if two molecules of I-2 were bound to a single Pin1, at different sites, in the two domains, then it is possible that a second molecule of Pin1 could contact these same two I-2 proteins to form a sandwich, with the Pin1 proteins positioned opposite one another bridged by two molecules of I-2 that are predominantly unstructured and extended (Figure 7). The $[Pin1]_2[I\text{-}2]_2$ heterotetramer is proposed as the native active form of Pin1. Bringing two molecules of Pin1 together positions two phosphoSer/Thr binding sites in the proximity, and the involvement of I-2 in the complex generates a composite protein surface surrounding the Pin1 isomerase domain, which is predicted to dictate the substrate specificity. This tetramer represents a quite different target for drug development compared to monomeric Pin1.

Furthermore, our model accommodates phosphorylation as a means of Pin1 regulation. While PKA phosphorylation of S16 in Pin1 does not affect association with I-2, this phosphorylation of two sites on the surface of the $[Pin1]_2[I\text{-}2]_2$ complex could serve to alter binding to different substrates. Phosphorylation of Thr73 in I-2, which is catalyzed by CDK/cyclinB during mitosis,[36] prevents I-2 from binding to Pin1.[39] Therefore, we predict upon entry into mitosis phosphorylation of I-2 in the $[Pin1]_2[I\text{-}2]_2$ complex will cause dissociation into separate components, releasing monomeric Pin1 to interact with mitotic phosphoproteins. Mitotic exit will involve dephosphorylation of I-2 and reassembly with Pin1 into the heterotetramer. This model implies dynamic regulation of Pin1 complexes that would affect substrate specificity at different stages of mitosis.

We note that I-2 residues 85−95 and 105−125 are engaged by Pin1. For I-2 bound to PP1C, the segment of residues 95−105 is looped out and does not form contacts with PP1C, and NMR spectroscopy shows some tendency for this region to be in an α helix.[50] In a recent publication, the authors speculated that this putative helix could possibly contact another I-2 partner, such as Aurora A or Pin1.[51] This would fit with our results. The region of residues 105−125 we see affected by Pin1 does not overlap with the region of residues 130−142 of I-2 that is 70% populated in a helix conformation, as observed by NMR spectroscopy.[50] The segment of residues 130−142 of I-2 was visualized as a helix bound across the PP1C active site in the cocrystal structure, with only 25% of I-2 showing discernible electron density.[52] Even when in complex with its primary partner, PP1C, most of I-2 is thought to remain as flexible as it is when it is alone in solution. Overall, we are impressed that our NMR spectroscopy results and those of Peti and collaborators map different I-2 residues interacting with Pin1 compared to those that contact the PP1C monomer, or PP1C complexed to neurabin.[51] Although different regions of I-2 are used in these various complexes, we do not imagine this implies assembly of supercomplexes with multiple partners, but rather assembly of I-2 into multiple complexes with different partners. Our previous research has revealed that, in addition to PP1C, I-2 forms complexes with neurabin,[53] KPI-2[54] (also known as LMKT2 and BREK), Aurora A,[44] and, of course, Pin1.[39] This emphasizes the possibilities available to an unstructured protein to employ various sequence segments to engage different protein partners. The ancient and conserved function of I-2 involves a web of protein−protein interactions, and we speculate that binding to Pin1 may be as ancient, and fundamentally important, as binding to PP1, the property for which I-2 was originally named.

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ABBREVIATIONS

GST, glutathione *S*-transferase; HI-2, human phosphatase inhibitor-2; XI-2, *Xenopus* phosphatase inhibitor-2; DI-2, *Drosophila* phosphatase inhibitor-2; $Pin1_{ww}$, human Pin1 WW domain (residues 1−44); $Pin1_{iso}$ or $Pin1_{cat}$, Pin1 isomerase domain (residues 46−163); HSQC, heteronuclear single-quantum coherence.

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