The Dual Histidine Motif in the Active Site of Pin1 Has a Structural Rather than Catalytic Role[†]

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ABSTRACT: The catalytic domain of the peptidyl-prolyl *cis*/*trans* isomerase Pin1 is a member of the FKBP superfold family. Within its active site are two highly conserved histidine residues, H59 and H157. Despite their sequence conservation in parvulin PPIase domains, the role of these histidine residues remains unclear. Our previous work (Behrsin et al. (2007) *J. Mol. Biol. 365*, 1143−1162.) was consistent with a model where one or both histidines had critical roles in a hydrogen bonding network in the active site. Here, we test this model by looking at the effect of mutations to H59 and H157 on Pin1 function, activity, and protein stability. Using a yeast complementation assay, we show that both H59 and H157 can be mutated to non-hydrogen bonding residues and still support viability. Surprisingly, a nonfunctional H59L mutation can be rescued by a mutation of H157, to leucine. This double mutation (H59L/H157L) also had about 5-fold greater isomerase activity than the H59L mutation with a phosphorylated substrate. Structural analyses suggest that rescue of function and activity results from partial rescue of protein stability. Our findings indicate that H59 and H157 are not required for hydrogen bonding within the active site, and in contrast to the active site C113, they do not participate directly in catalysis. Instead, we suggest these histidines play a key role in domain structure or stability.

Peptidyl-prolyl isomerases (PPIases[1]) are a class of enzymes that catalyze rotation about the peptide bond preceding a proline (*1, 2*). Pin1, a member of the parvulin family of PPIases, is unique among this class of enzymes because of its ability to bind and isomerize phosphorylated sequences suggesting a role for Pin1 in cell signaling (*3*−*7*). Indeed, Pin1 is involved in many cellular processes including cell growth and division (*4, 8*−*12*), transcription (*13*−*16*), DNA damage repair, and apoptosis (*17*−*22*). As such, Pin1 has been shown to interact with a number of phosphoproteins involved in these processes (*5, 10*). Furthermore, the Pin1 homologue, Ess1, is essential in *Saccharomyces cerevisiae* (*23*) and *Candida albicans* (*24*), and Pin1 itself is implicated in Alzheimer's disease (*22, 25*) and cancer (*26*−*29*).

Structurally, Pin1 consists of two domains: an N-terminal WW domain, which binds phosphoserine/threonine-proline (pS/T-P) motifs with high affinity, and a C-terminal PPIase domain responsible for Pin1 isomerase activity that is shared among all the members of the parvulin family (*6, 7, 30*). Although structures of Pin1 and related parvulins are known, its catalytic mechanism remains elusive. Mutational analysis and inhibitor studies involving the active site residue, C113, have shown that this residue plays a key role in catalysis (*31, 32*). As substitution of C113 with aspartic acid results in partially active Pin1, catalysis likely proceeds through a noncovalent mechanism (*31*). However, the roles of other active site residues in Pin1 activity and function are not well understood. Ultimately, a more comprehensive study of residues in the active site of Pin1 is required to better decipher its regulatory role in cells and develop its potential as a therapeutic target.

Previously, our laboratory used a plasmid shuffling assay in *S. cerevisiae* to screen for residues of human Pin1 that were important for function (*31*). Surprisingly, a number of residues, including two active site histidines, H59 and H157, could be mutated without impeding function (*31*). This was unexpected as these two histidine residues are absolutely conserved in parvulin PPIase domains. In this study, we use a mutational approach to define the sequence requirements at positions H59 and H157. Our results reveal that these residues do not seem to be important for hydrogen bonding or direct interactions with the substrate, but rather play a key role in the stability of the active site.

EXPERIMENTAL PROCEDURES

Construction of Plasmids. The LEU2-containing centromeric pY204 constructs for expressing wild-type, H59L and H157L Pin1 alleles in yeast have been described previously (*31*). The double H59L/H157L mutant was made by combining NotI/*Pst*I and *Pst*I/*Eco*RI fragments from H59L and H157L alleles into a digested NotI/*Eco*RI pY204 vector. Oligonucleotides for other histidine mutations are shown in Table 1. Mutations to H59 were made in the pY204-WTPin construct using the Quikchange kit (Stratagene) according

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[1] Abbreviations: PPIase, peptidyl-prolyl isomerase; FKBP, FK506-binding protein; 5FOA, 5-fluoroorotic acid.

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Table 1: Oligonucleotides Used in Site-Directed Mutagenesis in This Study

name	sequence
H59A forward	5′-GTC CGC TGC TCG GCC CTG CTG GTG AAG-3′
H59A reverse	5′-CTT CAC CAG CAG GGC CGA GCA GCG GAC-3′
H59N forward	5′-GTC CGC TGC TCG AAC CTG CTG GTG AAG-3′
H59N reverse	5′-CTT CAC CAG CAG GTT CGA GCA GCG GAC-3′
H59F forward	5′-GTC CGC TGC TCG TTC CTG CTG GTG AAG-3′
H59F reverse	5′-CTT CAC CAG CAG GAA CGA GCA GCG GAC-3′
H59S forward	5′-GTC CGC TGC TCG AGC CTG CTG GTG AAG-3′
H59S reverse	5′-CTT CAC CAG CAG GCT CGA GCA GCG GAC-3′
H157A reverse	5′-GGA ATT CTC TCA CTC AGT CGC GAG GAT GAT GGC GAT GCC GGA-3′
H157N reverse	5′-GGA ATT CTC TCA CTC AGT CGC GAG GAT GAT GTT GAT GCC GGA-3′
H157F reverse	5′-GGA ATT CTC TCA CTC AGT CGC GAG GAT GAT GAA GAT GCC GGA-3′
H157S reverse	5′-GGA ATT CTC TCA CTC AGT CGC GAG GAT GAT GCT GAT GCC GGA-3′

to the manufacturer′s instructions. Mutations to H157 were made through PCR using the Pin1 forward primer: 5′-ATA AGA ATG CGG CCG CCA TGG CGG ACG AGG AGA-3′ and the appropriate reverse primer with either wild-type Pin1 or the H59L construct as template. PCR products were then ligated into PCR blunt (Invitrogen) and subcloned into the pY204 construct using NotI and *Eco*RI. Constructs for the expression of GST fusion proteins were made by subcloning the mutants using NcoI and *Hin*dIII into a pGEX construct with an added TEV cleavage site 5′ to the multiple cloning site.

Yeast Plasmid Shuffling Assay and Yeast Protein Expression. The yeast plasmid shuffling assay to determine functionality of the Pin1 alleles was performed after transformation of Pin1-containing alleles into yeast strain YKH100 (genotype, MAT*a ura3−1 leu2−3*, *112 trp1−1 can1−100 ade2−1 his3−11*, 15[phi+] *ess1*Δ*::TRP1* (*31*)) as described previously (*31*). To evaluate the expression of Pin1 derivatives, yeast were lysed by bead lysis (*31*). Crude protein lysates were separated by SDS−polyacrylamide gel electrophoresis (SDS−PAGE) and Western blotted with anti-FLAG M2 (Sigma).

GST Fusion Protein Purification. GST fusion proteins were expressed from pGEX constructs transformed into *E. coli* strain BL21. Individual colonies were grown in L Broth with 100 μg/mL ampicillin (Roche) at 37 °C, then induced overnight at 18 °C with 0.6 mM isopropylthio-α-D-β-galactoside. Bacteria were pelleted and resuspended in PBS containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg/mL pepstatin A, and 10 μg/mL leupeptin) and then lysed by sonication before mixing with 1% Triton X-100 for 15 min at 4 °C. Bacterial lysates were cleared by centrifugation, and GST fusion proteins were bound to glutathione cross-linked agarose beads (Sigma) and eluted in 10 mM reduced glutathione (Sigma) in PBS. Proteins were analyzed by SDS−PAGE to assess purity and then dialysed into PBS containing 20% glycerol for storage.

Cleavage of GST Fusion Proteins. Proteins used for trypsin and CD experiments were expressed as GST fusions and purified as described above, but without dialysis into storage buffer. Instead, proteins were cleaved with TEV protease for 4 h at room temperature and dialysed overnight into Buffer A (10 mM NaH_2PO_4, 15% glycerol, 5 mM NaN_3, and 20 mM β-mercaptoethanol at pH 6.5). Proteins were loaded onto a cation exchange column (HiTrap SP HP, GE Healthcare), washed, and eluted over a gradient of Buffer B (10 mM NaH_2PO_4 and 15% glycerol at pH 11). Alternatively, for experiments performed in Tris buffer, proteins were loaded onto the cation exchange column and washed with Buffer A, then exchanged into 10 mM Tris-HCl, 10 mM sodium acetate, and 15% glycerol at pH 7.5 and eluted over a gradient of 10 mM Tris-HCl, 10 mM sodium acetate, and 15% glycerol at pH 9. Proteins were analyzed for purity by SDS−PAGE. Pin1-containing fractions were pooled, buffer exchanged to pH 7.5 where necessary, and concentrated for analysis.

In Vitro Isomerase Assays. Isomerase activity was measured as described previously (*31*). Assays were performed at 0 °C in a Cary-100 spectrophotometer. Briefly, the AEPF-*p*NA or Pintide (WFYpSPR-*p*NA) substrate (Bachem) was added to assay buffer (50 mM Hepes, 100 mM NaCl, and 5 mM NaN_3 at pH 7.5) before the addition of chymotrypsin or trypsin (Sigma). Cleavage of the *p*NA group from the substrate was followed at 390 nm, 405 nm, 430 nm, or 445 nm depending on substrate concentration. Once the *trans* form of the substrate was cleaved, the chemical rate of catalysis was recorded before the addition of wild-type or mutant Pin1. Rates of reaction for 4 to 7 different substrate concentrations were recorded, corrected for the rate of chemical isomerization and enzyme concentration, and plotted against substrate concentration to determine the k_{cat}/K_M.

Partial Trypsin Proteolysis. For trypsin digestion, 100 μg of recombinant protein was digested with 0.5 μg of trypsin (Sigma) in 200 μL of 10 mM NaH_2PO_4 and 15% glycerol at pH 7.5 at room temperature. Aliquots of 20 μL were taken at indicated times and the reaction stopped by the addition of SDS sample buffer and boiling. Samples were analyzed by SDS−PAGE and bands visualized after staining with Coomassie Brilliant Blue R-250 (Bio-Rad).

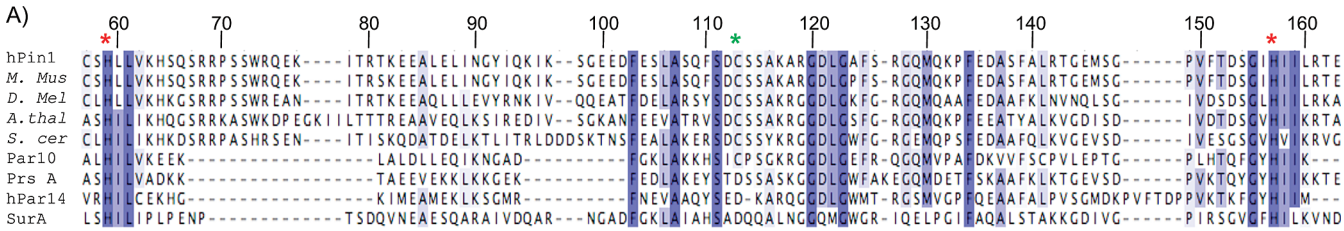
Circular Dichroism Measurements. CD measurements were performed in 10 mM NaH_2PO_4 and 15% glycerol pH ∼7.5 on a Jasco J-810 spectropolarimeter. Scans were taken using a 0.1 mm path length cuvette with protein concentrations between 0.736 mg/mL and 0.934 mg/mL over a temperature range of 20 °C−80 °C.

GST Pull-Downs. Mitotically arrested U2OS lysates were made by treating cells with 0.06 μg/mL of nocodazole (Sigma) for 18 h. Cells were harvested by shake off into NP-40 buffer (50 mM Tris-HCl, 150 mM NaCl, and 1% NP-40) containing 1 mM PMSF, 10 μg/mL pepstatin A, 10 μg/mL leupeptin, and 1 μM okadaic acid, lysed by sonication and cell debris pelleted by centrifugation. For GST pull-downs, 100 μg of GST fusion protein was bound to glutathione cross-linked to agarose beads (Sigma). Beads were washed with PBS buffer then incubated with 1 mg of U2OS lysate for 1 h at 4 °C. Beads were washed with 400 μL NP-40 buffer and bound protein eluted into SDS sample buffer by boiling. Proteins were analyzed by SDS−PAGE and Western blotted with MPM-2 antibody (Upstate Biochemistry).

RESULTS

Mutation of H59 and H157 in Pin1 Support Viability in Yeast. Pin1 contains two histidine residues, H59 and H157, that had been postulated to be important for Pin1 function

A)



B)

X=	H59X	H157X
L	-	+
A	+	+
N	+	+
F	-	+
S	+	+

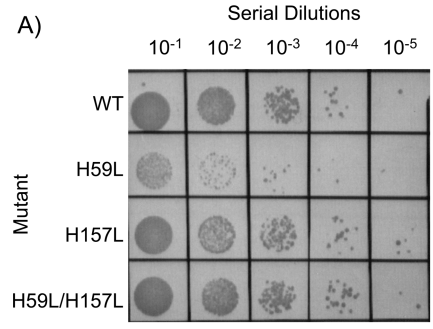
FIGURE 1: (A) Sequence alignment of Pin1 and parvulin PPIase domains from various organisms. The conserved histidine residues H59 and H157 (in human Pin1) are conserved in all species and indicated by red asterisks. C113 is indicated by a green asterisk. Residues are numbered according to human Pin1. Alignment was done using Clustal W. (B) Site-directed mutation of conserved residues H59 and H157. Individual mutants were transformed into yeast, and plasmid shuffling was performed. Transformants were normalized according to cell density, and serial dilutions were spotted onto 5FOA plates to monitor growth. Mutations were classified as either functional with growth comparable to wild-type Pin1 (+) or nonfunctional with significantly reduced growth compared to wild-type Pin1 (−) in rescuing the ess1::TRP1 disruption after being spotted onto 5FOA.

as they are absolutely conserved in parvulin PPIase domains (Figure 1A). Furthermore, these two residues lie side by side in the active site, and H59 specifically is within hydrogen-bonding distance of the catalytic C113 residue of Pin1. Previously, we performed a unigenic evolution analysis of human Pin1 by selecting for mutations that would support viability in the yeast *S. cerevisiae*. As part of this analysis, we found that H59 could be altered to Q without affecting viability; however, alteration to L led to inviability. In contrast, altering H157 to L supported viability. Accordingly, we hypothesized that H59 and H157 are involved in catalysis by stabilizing the transition state through hydrogen bonding to the substrate carbonyl oxygen (*31*).

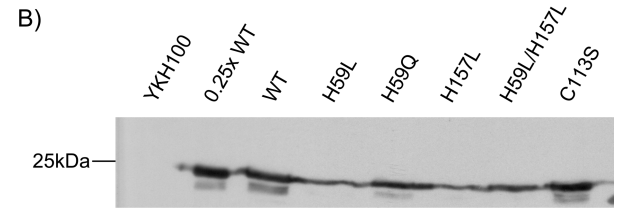
As a logical extension of our earlier work, we tested this hypothesis by performing a more detailed investigation of these two active site histidines. Single mutations to H59 and H157 were generated, and their function in maintaining yeast viability (as determined by their growth on plates containing 5FOA) was evaluated. Although these two histidines are well conserved and it was expected that their mutation would be detrimental to Pin1 function, many mutations were tolerated in yeast at these two positions (Figure 1B). For residue 59, mutations to large hydrophobic residues, such as leucine and phenylalanine, displayed significantly reduced growth in yeast compared to wild-type Pin1. However, substitution of H59 with the smaller hydrophobic residue, alanine, supported viability. Unlike H59 mutations where only some substitutions were tolerated, all of the single mutations to H157 supported viability (Figure 1B), suggesting that H157 is not as important in Pin1 function in yeast as H59. Together, our results indicate that while highly conserved, neither of the histidines are essential for viability.

Double Mutation of Active Site Histidines Restores Viability in Yeast. During the analysis of single mutations, we serendipitously engineered a double mutation containing H59L and H157L. Surprisingly, in contrast to the single H59L mutation, the double mutation supported viability (Figure 2A) suggesting that the H157L mutation rescues Pin1 activity. To examine further how this rescue was occurring,

A)



B)



C)

X=	H59L/157X
L	+
A	+
N	-
F	+
S	+/-

FIGURE 2: Mutation of H157 rescues the inviable H59L mutation in *S. cerevisiae*. (A) Individual mutants were transformed into yeast and plasmid shuffling was performed. Transformants were normalized according to cell density, and serial dilutions were spotted onto 5FOA plates to monitor growth. Those mutants that grew robustly on 5FOA were considered to be functional in yeast cells. (B) Expression of Pin1 plasmids in yeast. Cultures of each mutant were grown in SC-W-L-U media overnight and lysed using glass beads. One hundred twenty-five micrograms of each were run on 12% SDS−PAGE transferred and blotted with anti-FLAG antibody. (C) Site-directed mutations were made to H157 in the presence of H59L and tested in the yeast plasmid shuffling assay as in A. Mutations were classified as functional (+), nonfunctional (−), or partially functional (±) in rescuing the ess1::TRP1 disruption after being spotted onto 5FOA.

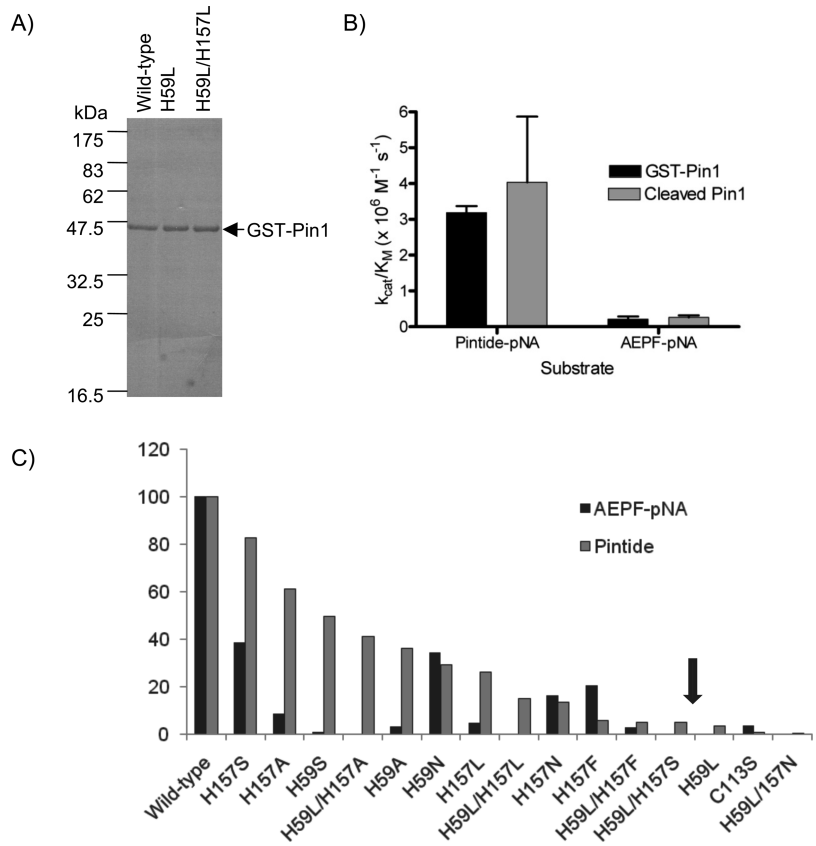


FIGURE 3: *In vitro* isomerase activities of Pin1 recombinant proteins. (A) Coomassie-stained SDS−PAGE of proteins after purification of GST fusion proteins. (B) Comparison of *in vitro* isomerase activities of purified wild-type Pin1 proteins before and after cleavage of the GST tag using APEF-*p*NA and Pintide-*p*NA substrates. (C) Comparison of the *in vitro* isomerase activities of histidine mutants using AEPF-*p*NA and Pintide-*p*NA peptide substrates. Histidine mutations are ordered according to their isomerase activities using Pintide. The division between the functional and nonfunctional mutations is marked with an arrow.

various double mutations where H157 was mutated in the presence of H59L were examined for their ability to support viability in yeast. These studies revealed that other hydrophobic substitutions to H157, including phenylalanine and alanine, can rescue Pin1 function in the presence of H59L (Figure 2C). By comparison, the polar H157S mutation could only partially rescue the H59L mutation, while the mutation of H157N was unable to rescue. Collectively, these results indicate that a combination of hydrophobic residues at positions 59 and 157 will support viability.

H59 and H157 Mutants Have Isomerase Activity against a Phosphorylated Substrate in Vitro. It was unexpected that mutations to H59 and H157 would be so well tolerated in yeast because of their placement within the active site and their conservation in parvulin PPIase domains. To further examine how these residues are involved in Pin1 and their role in the active site, recombinant proteins for each of the mutations were purified (Figure 3A) and their activity compared using an *in vitro* isomerase assay. To enable rapid purification, proteins were expressed as GST fusions. Tests with the wild-type Pin1 GST fusion and a cleaved version of the protein without the tag show that the presence of GST is not detrimental to isomerase activity (Figure 3B). Initial results with an unphosphorylated AEPF peptide substrate found the nonfunctional C113S mutation had 3.4% of wild-type activity and the functional histidine mutations had activities, which were undetectable for H59L/H157L but were as high as 38.5% for H157S (Figure 3C). Clearly, activities using the AEPF substrate did not correlate well with our results in yeast as many of the alleles that supported viability (H59A, H59L/H157L, H59L/H157F, and H59S) had activities lower than the C113S mutation (Figure 3C).

These inconsistencies led us to examine a more physiologically relevant phosphorylated peptide substrate (Pintide) in the assays. As shown in Figure 3C, many of the histidine mutations have much higher activities with Pintide than with AEPF indicating that isomerase activity measurements can be drastically different depending upon the substrate used. For example, the H59L/H157A mutation has less than 0.1% of wild-type activity with AEPF but 41.2% of wild-type activity with Pintide. Also, results from assays using Pintide show that the low activity of the H59L mutation can be increased almost 5-fold when combined with a mutation of H157L (Figure 3C). This is consistent with results in yeast where mutations of H157L could rescue the inviable H59L mutation (Figure 2A). With Pintide as substrate, isomerase activity of the remaining mutations also correlated well with yeast plasmid shuffling results (including C113S, which had an activity of only 0.81% with Pintide). The ordering of mutations by their activity in Figure 3 indicates that at least 5% of wild-type Pin1 activity is needed to maintain viability in yeast in this system. However, the exact amount of activity needed is difficult to determine precisely as expression levels of the mutants in yeast can vary slightly (Figure 2B).

The rank ordering of activities in Figure 3C also reveals a number of general trends about the histidine mutations. First, as with the yeast results, mutations of H157 are not generally as detrimental to activity as mutation of H59 to

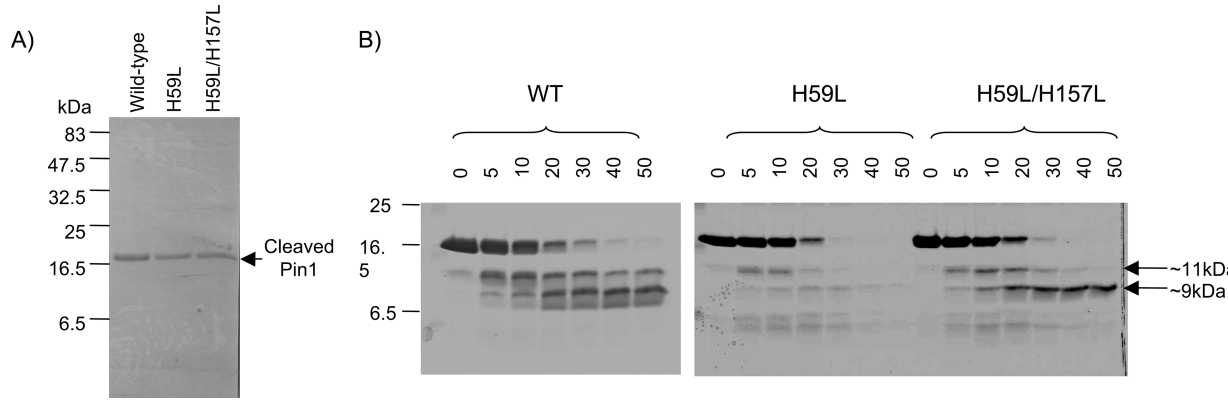


FIGURE 4: (A) Coomassie-stained SDS−PAGE of proteins after cleavage from the GST tag. (B) Partial trypsin proteolysis. Trypsin was added to wild-type or leucine mutant proteins in a 1:200 ratio and incubated at room temperature. Aliquots were taken at stated times analyzed by SDS−PAGE and stained with Coomassie Blue.

the same residue. For example, the H59A mutation had 36.0% of wild-type activity, whereas H157A activity was almost 2-fold higher at 61.0%. This suggests that H157 is less critical for enzyme function than H59. A second observation from Figure 3 is that mutation of either H59 or H157 to small residues (A, N, or S) is less disruptive to Pin1 activity than mutation to larger hydrophobic residues (L or F). In contrast, the low activity of H59L can be increased by mutating H157 to other hydrophobic residues making it likely that there is an interaction between these two spatially close residues. Finally, with the exception of H59L/H157N, all of the histidine mutations, even those to large unconserved residues, had higher activity than C113S. This suggests that although these two histidines are in the active site, they are not as important as C113 for catalysis, at least with a phosphorylated substrate. Since Schutkowski et al. observed dramatic changes in activity by lowering pH suggesting that ionization of the active site histidines influenced catalysis (*33*), we also examined the activities of H157A and H157N at lower pH (pH 6). In these assays, the behavior of the mutants in response to the changes in pH did not differ from the wild-type enzyme (data not shown).

Mutation of Active Site Histidines Affects Pin1 Stability. The double H59L/H157L mutation clearly has more activity than a single H59L. As the rescue of the H59L mutation by alteration of H157 to L is inconsistent with our proposed catalytic mechanism that requires side-chain hydrogen bonding at these positions (*31*), these residues must have another noncatalytic function. NMR data shows that these two histidines have only slight changes in chemical shift upon the addition of substrate (*34*−*36*). Thus, H59 and H157 are likely not involved in substrate binding. However, the work of Tossavainen et al. with another member of the parvulin family, PrsA from *Bacillus subtilis*, shows that the residue corresponding to H59 is essential for the correct folding of the PrsA PPIase domain (*36*). Since our data indicates that smaller residues (whether polar or apolar) appear to have a less severe effect on function and activity than larger residues, we investigated whether the histidine residues are involved in Pin1 protein stability.

We first used partial proteolysis to investigate the stability of wild-type, H59L, and H59L/H157L proteins. We note that for this and all stability experiments, the N-terminal GST was removed and the proteins further purified by ion-exchange chromatography (Figure 4A). Pin1 derivatives were incubated with trypsin and aliquots of the reaction taken at the indicated times and analyzed by SDS−PAGE. Cleavage patterns of the proteins reveal that the highest band at ∼17 kDa (representing full-length Pin1) disappears at a similar rate for each protein. However, the wild-type protein shows more stable cleavage products (at ∼11 kDa and ∼9 kDa) than either of the mutant derivatives indicating that both the single H59L and double H59L/H157L mutations lead to enhanced susceptibility to trypsin cleavage (Figure 4B). Interestingly, the ∼9 kDa band is more stable for the H59L/H157L derivative from 20−50 min than for the H59L derivative (Figure 4B). This suggests that the low activity of H59L may be due in part to a change in the protein that renders it more susceptible to trypsin cleavage and that this defect can be partially rescued by a mutation of the second histidine to leucine.

To further test the stability of Pin1 proteins, we used Far-UV CD to examine the global structures of the proteins at different temperatures. CD spectra of the H59L and H59L/H157L proteins both overlay on the wild-type Pin1 spectrum at 20 °C indicating that these proteins have similar global folds at this temperature (Figure 5A). Spectra collected at higher temperatures, reveal that while wild-type Pin1 has a stable spectrum up to at least 50 °C, both proteins containing histidine mutations have altered spectra and are destabilized at temperatures above 30 °C (Figure 5B). To further examine the thermal denaturation of each of these proteins, their CD signal at 200 nm was monitored over a temperature range from 20 °C−70 °C. The 200 nm wavelength was chosen because the change in CD signal of the WW domain was minimal over this temperature range, while the PPIase domain where we made our mutations showed a significant shift (data not shown). Denaturation of Pin1 proteins was not reversible (data not shown); therfore, CD spectra were used as a comparative assessment between different proteins. These curves revealed that both H59L and H59L/H157L start to decrease in signal at much lower temperatures than wild-type Pin1 (Figure 5C), but the H59L mutation appears to lose signal before H59L/H157L. To evaluate if buffer composition differentially affected the CD profiles of the mutants, thermal denaturation was performed for proteins purified in buffer lacking phosphate or sulfate. While the Pin1 proteins were less stable in the Tris-acetate buffer, the rank order of different mutations were the same in both buffers (Figure 5C). Although the data collected do not allow

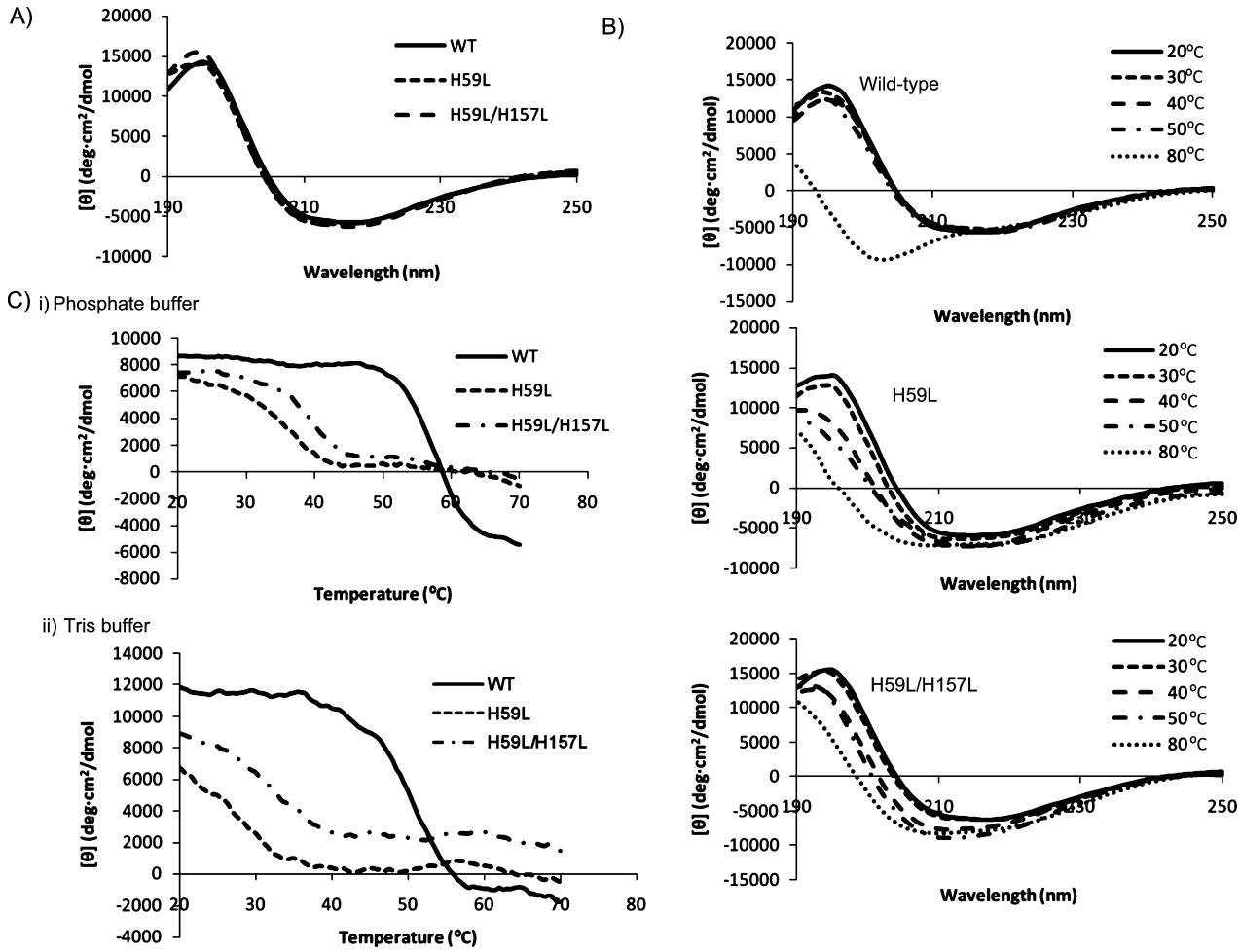


FIGURE 5: Far-UV CD of Pin1 proteins. (A) Far-UV spectra of wild-type, H59L, and H59L/H157L proteins at 20 °C. (B) CD spectra of wild-type, H59L, and H59L/H157L proteins at stated temperatures. (C) Thermal denaturation curves of Pin1 wild-type and mutant proteins taken at 200 nm in (i) phosphate and (ii) Tris buffers.

for an exact interpretation of how histidine mutations affect Pin1 structure, it does suggest that Pin1 containing H59L is unstable at higher temperatures and that alteration of H157L can help stabilize the H59L form of the enzyme. As with the trypsin proteolysis, these results point to a structural role for the conserved histidines. However, the result that the histidine mutations cause increased trypsin proteolysis at room temperature, but do not display changes in CD spectra at 20 °C suggests that these residues may affect the protein dynamics of Pin1 rather than its overall structure (see Discussion).

PPIase Domain Mutations Are Not Critical for Pin1 Target Interactions. To address whether the impaired function of the PPIase domain due to mutations in H59 and H157 affects the ability of the WW domain to bind interacting partners, we performed GST pull-downs. Extracts from mitotically arrested U2OS cells were applied to glutathione beads on which were immobilized GST fusion proteins of Pin1 derivatives. Beads were washed and bound proteins analyzed by Western blotting with anti-MPM-2, an antibody that has been shown to recognize many Pin1-interacting proteins (*4*). As shown in Figure 6, both wild-type and mutant GST-Pin1 proteins bind many more MPM-2 antigens than GST alone (compare Figure 6, lanes 1 and 2); however, these proteins do not bind all of the MPM-2 reactive proteins in the initial input (e.g., compare Figure 6, lane 2 and 7).

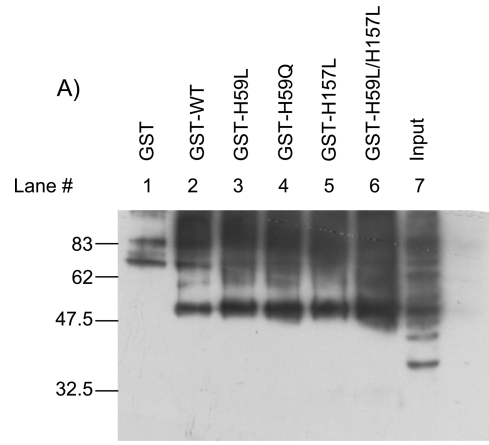


FIGURE 6: Pull-downs with recombinant GST Pin1 proteins. Nocodazole-treated U2OS lysates were incubated with GST-Pin1 wild-type or Leucine mutant proteins bound to glutathione beads. Bounds interactors were blotted with anti-MPM-2.

Furthermore, all Pin1 derivatives bind MPM-2 antigens similar to wild-type. This shows that Pin1 histidine mutations can interact with proteins in a manner similar to wild-type Pin1. As most of these interactions occur through the WW domain of Pin1, Figure 6 demonstrates that the integrity of the WW domain is not affected by the PPIase mutations nor does a nonfunctional mutation in the PPIase domain (e.g.,

H59L) appear to affect the ability of the WW domain to bind interacting partners.

DISCUSSION

Many of the amino acid substitutions to H59 and H157 supported viability in yeast. The finding that these functional mutations had a range of activities *in vitro* is not unexpected as in *S. cerevisiae*, only a small amount of isomerase activity is needed to support viability (*37*). Isomerase activities with the Pintide substrate correlated well with the ability of mutants to support viability demonstrating that phosphorylation is important for Pin1 function *in vivo*. Approximately 5% of wild-type activity was sufficient to maintain viability in yeast. Results show that assays containing AEPF substrate had different activities that did not correlate well with results in yeast (Figure 3). This may be because the more unstable mutant proteins could not bind an E-P motif as well as the preferred pS-T motif. Alternatively, the Pintide substrate is longer than AEPF by an extra two amino acids, which may have allowed it to make more contacts outside of the active site that helped in its binding to the PPIase domain. Another possibility is that either the active site histidines or the phospho-motif of the substrate may be involved in acid/base reactions, which are required for isomerization. Overall, as assays using Pintide show a much better correlation to yeast function than those of AEPF, Pintide is a much more relevant substrate for the interpretation of Pin1 activity.

The C113 residue of Pin1 is thought to be critical for catalysis (*6, 31, 32*). Because of their close proximity, both active site histidines, especially H59, were also thought to play a role in catalysis (*6, 31*). Since most substitutions at H59 and H157 had greater activity than C113S, the histidines cannot be as important for activity. The observation that the alanine set of mutations have significant activity suggests that hydrogen bonding at these positions is not important for isomerization. That double hydrophobic mutations, such as H59L/H157L, had significant isomerase activity and could support viability in yeast eliminates the possibility that there is functional redundancy at these positions.

Other studies in which these residues were mutated found that H157A had 92% of wild-type isomerase activity (*38*), consistent with our results, but H59A had only 5.8% of wild-type activity or lower (*4, 11*). However, the assays by Shen and colleagues did not use the Pintide-*p*NA substrate (*4*) and, as mentioned earlier, our results suggest that Pintide is a more accurate indicator of Pin1 function. The assays by Yaffe and colleagues (*11*) measuring H59A activity also had a slightly different substrate (AApSPR-*p*NA versus WFYpSPR-*p*NA). Other differences between studies may reflect protein purification procedures or buffer conditions, which affect protein instability caused by these mutations.

Partial proteolysis and CD analysis of mutant proteins support a model whereby H59 and H157 are important for the integrity of the Pin1 active site. Histidines H59 and H157 are on two adjacent antiparallel β-strands, central in both the four-strand β-sheet and the structure of the PPIase domain (*6, 30*). Because of their central location, it is possible that these two histidines have a key role in the assembly of the sheet/domain structure, supporting the integrity of the active site. As mutations to H59 seem to be more severe than those of H157, it is likely that H59 assumes a more critical and perhaps a dual role, yet the partial rescue of H59L protein instability with an H157L mutation suggests there is structural interaction between these two residues. Exactly how these mutations cause the instability seen in the trypsin digests and denaturation experiments is unclear. Notably, the CD spectra at 20 °C demonstrate that these proteins have the same global structure at this temperature. However the enhanced susceptibility of the mutant proteins to proteolytic cleavage at room temperature does reveal one or more structural differences. This suggests that the effects of the histidine mutations may more accurately be described as changing the protein dynamics of Pin1 rather than the global structure. Several recent studies have examined Pin1 protein dynamics and indicate that certain regions of Pin1 structure may show a degree of flexibility (*39*−*41*). In this respect, mutations to the central histidines may result in perturbations that cause structural fluctuations that are not reflected in the overall average structure of Pin1. For example, the study by Namanja and colleagues reveals the importance of the flexibility of side chains in the active site of Pin1 in substrate recognition and function (*41*). It is possible that mutations to the dual histidine motif in our work may disrupt the mobility of neighboring side chains and that this could affect both Pin1 stability and catalysis. Alternatively, the phosphospecific-binding loop that contains multiple basic residues (K63, R68, and R69) directly follows the β-strand that contains H59 (*6, 7*). This loop has been crystallized in two conformations, reflecting a certain amount of flexibility. Mutations of H59 to L may increase this flexibility, further exposing the basic residues in the loop and making them more sensitive to trypsin.

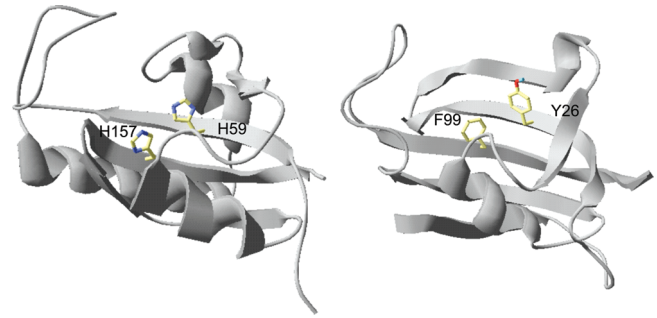


FIGURE 7: Ribbon diagrams of the Pin1 PPIase domain (left; PDB code 1PIN) and FKBP12 (right; PDB code 1FKB) are shown. Residues H59 and H157 of Pin1 lie in the same positions as Y26 and F99 of FKBP12. Y26 and F99 lie in regions of the protein shown to be part of the folding nucleus of FKBP12.

The PPIase domain of Pin1 is part of the FKBP superfold family (*6*). FKBP12, a representative member of the family, has two residues, a tyrosine (Y26) and a phenylalanine (F99), at the same positions as H59 and H157 (Figure 7). Y26 and especially F99 are important in FKBP12 binding and catalysis (*42, 43*). Interestingly, an F99Y mutation has an altered far-UV CD spectrum at 25 °C (*42*). Both F99 and Y26 lie on two β-strands (β2 and β5) that form part of the folding nucleus needed to stabilize the transition state structure during folding (*44*), but as the β-sheet of FKBP12 has an extra strand and its active site is much more hydrophobic than the active site of Pin1, the exact interactions involved at these positions may not be the same as those at H59 and H157. It is also interesting to note that upon superposition of the Pin1 and FKBP12 PPIase domains,

the catalytic C113 residue of Pin1, which can also be an aspartic acid in some parvulin domains (Figure 1A) corresponds to D37 of FKBP12, suggesting that it may have a role similar to C113 in the catalysis of isomerization. By comparison, we propose that H59 and H157 play a role in the folding of the PPIase domain.

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