4-23-2014 12:00 AM

Characterizing the domain- and phosphorylation-requirements of the interaction between peptidyl prolyl isomerase Pin1 and mitotic phosphatase CDC25C

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Biochemistry

CHARACTERIZING THE DOMAIN- AND PHOSPHORYLATION-REQUIREMENTS OF THE INTERACTION BETWEEN PEPTIDYL PROLYL ISOMERASE PIN1 AND MITOTIC PHOSPHATASE CDC25C

(Thesis format: Monograph)

by

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Graduate Program in Biochemistry

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

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Abstract

The enzyme Pin1 is a peptidyl-prolyl *cis-trans* isomerase consisting structurally of two domains, an N-terminal WW protein interaction domain and a C-terminal PPIase catalytic domain. Both domains bind a phosphorylated serine/threonine-proline motif, however, a precise mechanism regarding how binding to interactors is coordinated by both domains has not yet been determined. Although multiple models exist to explain this process, it appears that the interactions may be substrate-specific. With regards to a well–studied Pin1 interactor, CDC25C, we hypothesize that binding occurs via the simultaneous model. This model suggests that two binding sites, each having low affinity, may bind in concert producing a higher affinity interaction. To investigate this we chose to employ a peptide-based approach, using human CDC25C-derived peptides which contained the two identified Pin1 binding sites in phosphorylated and non-phosphorylated combinations. These peptides were utilized in two independent assays, surface plasmon resonance and fluorescence polarization, to elucidate the domain- and phosphorylation-requirements of the Pin1-CDC25C interaction. We showed that the interaction is phosphorylation-dependent, and is optimal when full-length, wild-type Pin1 binds to a doubly-phosphorylated peptide. Collectively, our results support our hypothesis that the Pin1-CDC25C interaction occurs via the simultaneous model, and requires both domains.

Keywords

Pin1, peptidyl-prolyl isomerase, CDC25C, phosphorylated serine/threonine-proline motif, models of binding, simultaneous model, peptide, surface plasmon resonance, fluorescence polarization

Co-Authorship Statement

Dr. Melanie L. Bailey provided all Pin1 plasmids used in this thesis.

Dr. Michael Strong provided all Tau plasmids used in this thesis.

Kristina Jurcic and Christina Booker of the Department of Biochemistry performed MALDI mass spectrometry.

Acknowledgments

I would like to start by thanking my supervisor, Dr. Dave Litchfield, for taking a chance on a high school teacher and giving me an opportunity to work in his lab. I am grateful for his understanding, patience, and continued encouragement to ask questions. I would also like to thank my committee members, Dr. Chris Brandl and Dr. Brian Shilton, for their assistance and constructive criticism. Additionally, various aspects of this work would not have been possible without the assistance of Dr. Shawn Li (Courtney Voss), Dr. Michael Strong (Kathryn Volkening), Lee-Ann Briere (BICF), and both Kristina Jurcic and Christina Booker (MALDI MS facility).

To members of the Litchfield lab whose time overlapped with mine, thanks for your advice and support. Specifically, thanks to Kathryn Garside, who at one point had taught me everything I knew about lab work. Thanks also to Brendan Innes, my fellow Pin-head, for listening while I complained and for making days in the lab entertaining; I accidentally the thesis.

Thanks to Sarah Attridge and Dr. Laurie Turner for supporting me in this endeavour from the very beginning. Also, thanks to Miranda Hunter for superior editing (and comma removal) skills.

Finally, thanks to my family for your unwavering support and encouragement. To my mom and dad, thanks for listening even when you didn't fully understand, and for instilling in me the value of education. And to my sister Tania, feel free to match me in number of degrees, but I'm officially waving the white flag, throwing in the towel, whatever! I'm done! Thank you for being my thesis-writing coach/nazi, and for your perspective and understanding.

Now, time for that bonfire.

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List of Abbreviations

2xYT	2x Yeast Extract Typtone
°C	degrees Celsius
APP	amyloid precursor protein
BCA	bicinchoninic acid
CDC25C	cell division cycle 25 (isoform C)
CDK	cyclin-dependent kinase
cm	centimeter
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DSC	differential scanning calorimetry
DTT	dithiothreitol
EDC	N-ethyl-N'-(dimethylaminopropyl)carbodiimide
EDTA	ethylenediaminetetraacetic acid
EGFP	enhanced green fluorescent protein
FBS	fetal bovine serum
FP	fluorescence polarization
g	relative centrifugal force
GST	glutathione-s-transferase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HBS-E	HEPES buffered saline with EDTA
HPLC	high-performance liquid chromatography
IPTG	isopropylthio-α-D-β-galactoside
MALDI	matrix-assisted laser desorption/ionization
MAPK	mitogen-activated protein kinase
mg	milligram
min	minute
mL	millilitre
mM	millimolar
µM	micromolar
µg	microgram

NHS	N-hydroxysuccinimide
nm	nanometer
NMR	nuclear magnetic resonance
PBS	phosphate buffered saline
PDEA	2-(2-pyridinyldithio)ethaneamine
PEI	polyethylenimine
Pin1	protein interacting with NIMA-1
PMSF	phenylmethlysulfonyl fluoride
RNA	ribonucleic acid
RU	response units
s	second
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SPR	surface plasmon resonance
TEV	Tobacco etch virus
V	volts

1.0 Introduction

Protein phosphorylation is a key mechanism in cellular signalling which allows for precise spatial and temporal control over diverse and complex events. The conformational changes that occur as a result of phosphorylation play a role in signal transduction, for example, by driving protein-protein interactions or activating enzymatic activity (1). Although this is true for phosphorylation of tyrosine, serine, or threonine residues, the common phosphorylation motif of serine/threonine-proline allows for an additional opportunity for post-phosphorylation regulation. This is due to the distinct *cis* and *trans* conformations of proline residues, and although the intrinsic switch between conformations is slow, it can be catalyzed by peptidyl-prolyl isomerases. These enzymes play an important role in protein folding, however the discovery of a unique phosphorylation-dependent peptidyl-prolyl isomerase, Pin1, added a new twist to understanding the importance of proline-directed phosphorylation and resulting conformational changes in cell signalling. Specifically, the catalytic isomerization following phosphorylation will induce a conformational change which can subsequently regulate protein function (2). This has since been shown to be true for a number of Pin1 interactors, proteins which have diverse functions in important cellular events including mitosis, transcription, and DNA repair. This places Pin1 in an important role, as a key regulator of many cellular processes, and emphasizes the significance of post-phosphorylation regulatory mechanisms in signal transduction.

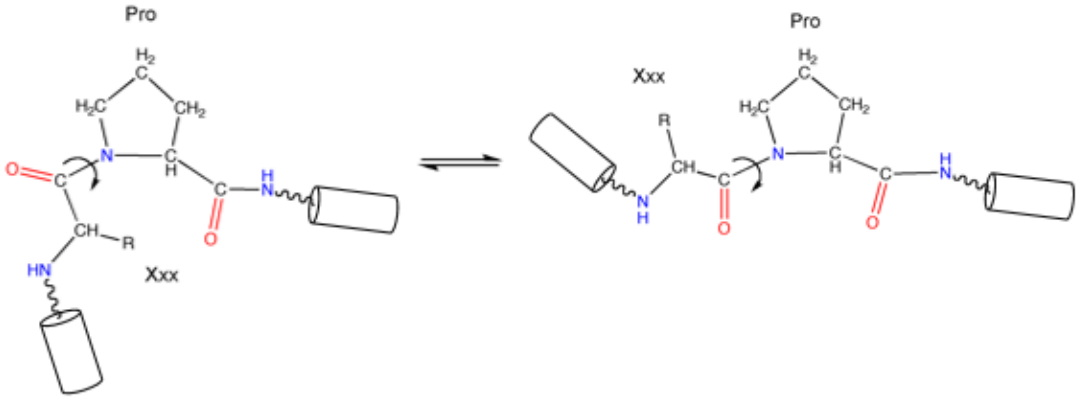
1.1 Historical background

Pin1 is a phosphorylation-dependent peptidyl-prolyl isomerase that was first isolated by a yeast two-hybrid screen designed to identify human proteins which interact with the "Never in Mitosis" gene A (NIMA) (3). Sequence analysis revealed that human Pin1 exhibits approximately 45% sequence identity with the product of the ESS1 gene that was previously identified as essential for growth in the budding yeast *Saccharomyces cerevisiae* (4). It has subsequently been found that Pin1-like proteins are highly conserved, found in both eukaryotes and prokaryotes (5). While it was initially implicated as a regulator of mitosis, it is evident that Pin1 has roles in a number of biological processes.

Pin1 is classified as a peptidyl-prolyl isomerase (PPIase) catalyzing the *cis-trans* conversion of the peptide bond between a proline and the preceding amino acid (Figure 1). It is part of a large superfamily of PPIases, which is divided into three families: the cyclophilins, the FK506 binding proteins (FKBPs), and the parvulins (6). One distinguishing characteristic among the three families of PPIases is their substrate specificities, particularly concerning the residue directly preceding the proline. Like all members of the parvulin family, Pin1 has a preference for hydrophobic residues. However, unique to Pin1 is its phosphorylation-dependence, requiring that the preceding residue be a phosphorylated serine or phosphorylated threonine (7). With phosphorylation playing a pivotal role in cell signalling, one can infer that an isomerase with phosphorylated residues in its specificity determinants would add an additional layer of complexity to signalling pathways. This has been shown in multiple cellular processes

Figure 1 **Peptidyl-prolyl isomerization.**

Cis-trans isomerization of the peptide bond (arrow) preceding the proline. Xxx represents any amino acid.



cis *trans*

where Pin1-catalyzed isomerisation regulates the conformation of key cellular proteins (8).

1.2 Structural and enzymatic features of Pin1

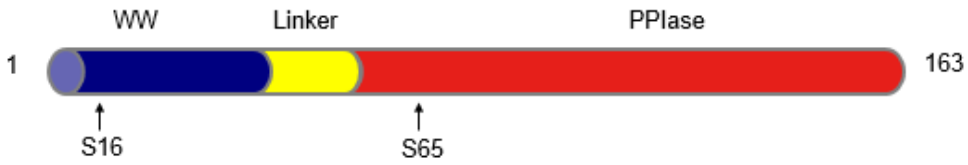
High resolution structures of Pin1 determined by x-ray crystallography (9, 10) revealed that it consists of two structural domains connected by a relatively short linker, which had originally been predicted from its primary sequence (Figure 2). The N-terminal WW domain is named for two conserved tryptophan residues and comprises residues 1-39. It consists of a triple stranded anti-parallel β-sheet, with a hydrophobic patch in the surface (11). Generally described as a protein-protein interaction domain, the WW domain of Pin1 binds pSer/Thr-Pro motifs, and thus is thought to facilitate interactions between Pin1 and its substrates (12). The 118 amino acid catalytic PPIase domain (residues 45-163) is found on the C-terminal end of the protein, and consists of four antiparallel β-sheets, and four α-helices. Within this domain are two relatively well described regions, the proline binding pocket and the phosphate binding loop, which lie on opposite sides of the active site. The hydrophobic proline binding pocket contains three highly conserved residues, Leu122, Met130, and Phe134, which are thought to be responsible for holding the proline in place during catalysis (9). The phosphate binding loop contains two positively charged arginine residues at positions 68 and 69, as well as another positively charged amino acid, lysine at position 63, conferring upon Pin1 its preference for phosphorylated residues preceding the proline (11). A short linker connects the WW and PPIase domains, whose flexibility may contribute to the broad substrate specificity of Pin1 (13). Interestingly, although both domains of Pin1 bind the pSer/Thr-Pro motif, it appears that they may bind differently since the WW domain typically has a higher

Figure 2 **Pin1 structure**

A. Linear representation of Pin1. Phosphorylation sites are indicated.

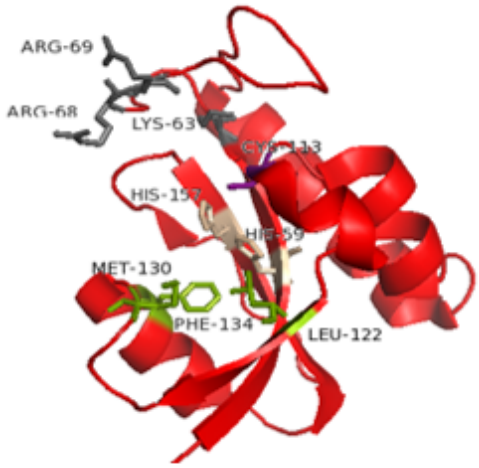
B. and C. High resolution structure of Pin1 determined by NMR (PDB: 1NMV). **B.** The WW domain is shown in blue, the linker region is yellow, and the PPIase domain is shown in red. **C.** Key residues of the PPIase domain are highlighted. See text for more details.

A.


WW
Linker
PPIase
1
163
S16
S65

B.

C.


ARG-69
ARG-68
LYS-63
CYS-113
HIS-157
HIS-59
MET-130
PHE-134
LEU-122

binding affinity for peptides than the PPIase domain (12). This has lead to the generation of multiple models to explain how Pin1 binds to its substrates, which are reviewed in section 1.8.

In spite of the evidence confirming the ability of Pin1 to catalyze *cis-trans* isomerisation, there are still questions concerning its precise catalytic mechanism. On the basis of a crystal structure of Pin1, Ranganathan *et al*. (9), initially proposed a mechanism that involved the formation of a covalent enzyme-substrate intermediate with Cys113, His59, and His157 being key residues involved in catalysis. Since then, however, additional evidence has argued instead for a non-covalent mechanism. In this respect, Lippens *et al.* (14) proposed that the role of Cys113 is to destabilize the peptide prolyl bond to allow for its rotation. This hypothesis is supported by data from Behrsin *et al.* (15) showing that a Cys113/Asp substitution did not abolish Pin1 function. Additionally, with regards to the histidine residues, it has been shown that they do not directly participate in catalysis, suggesting they instead act structurally to support the integrity of the active site (16).

1.3 Physiological regulation of Pin1

It appears that Pin1 is subject to regulation at a number of levels (8). For example, its expression is upregulated in response to growth factors through E2F-mediated transcription, an observation consistent with its role in the cell cycle (17, 18). Pin1 is also regulated through post-translational modifications, including phosphorylation and possibly oxidation. Phosphorylation on Ser16 and Ser65 has opposing effects: the former prevents interactions with substrates (19, 20), while the latter reduces ubiquitylation, thus increasing stability of Pin1 (21). Oxidation of Pin1 may have a relationship to pathologies such as Alzheimer's disease, rather than as a part of normal cell regulation (22, 23).

1.4 Cellular functions of Pin1

Pin1 is primarily localized in the nucleus (3), however, it can also be detected in the cytoplasm (24-26). This pervasive distribution of Pin1 is consistent with its extensive list of target proteins that are localized throughout the cell (27). As previously noted, Pin1 was first identified due to its interaction with NIMA, a protein kinase involved in mitotic regulation. This relationship was the first of many which suggested that Pin1 plays an integral role in regulation of the cell cycle and growth. Since then, Pin1 has been shown to be involved in a variety of additional cellular processes by interacting with a numerous substrates (28-39), emphasizing its diversity and importance (Table 1 provides a selection of such processes and substrates). Loss of function mutations or deletions of Pin1 in yeast and mammalian cells provides striking evidence for its role in the cell cycle, as these cells undergo mitotic arrest and apoptosis (3, 4, 40-43). Furthermore, Pin1 has a lengthy list of substrates which are known to be involved in the cell cycle, including a number of mitotic regulatory proteins (e.g. CDC25 and WEE1) which are targets of proline-directed protein kinases, such as CDKs and MAPKs (8). Pin1-catalyzed isomerisation of these phosphorylated sites may be responsible for coordinating the activity of mitotic proteins, thus allowing for progression through the cell cycle (8). Pin1 has also been shown to coordinate duplication of centrosomes, DNA synthesis (44), and to assist in chromosome condensation (45) further emphasizing its role in the cell cycle.

Similar to its actions in the cell cycle, Pin1 has been shown to interact with proteins involved in cell signalling events and pathways involving proline-directed protein kinases. One such example is the MAPK pathway, where, following proline-directed

Table 1 **Selected Pin1 substrates and consequences of interaction.**

	SUBSTRATE	**CONSEQUENCE OF PIN1 INTERACTION**
Cell Cycle Regulation	CDC25	Dephosphorylation
	WEE1	Inhibition of activity
	Cyclin D1	Stabilization
Apoptosis	p53	Stabilization
Neuro-degeneration	Tau	Dephosphorylation
	APP	APP processing

phosphorylation by MAPK, the proteins c-Jun and c-Fos are acted upon by Pin1 (18, 24, 26, 46, 47).

Briefly adding to the growing list of functions, Pin1 has also been shown to regulate expression of some genes through regulation of their transcription factors (24, 25, 33-35, 47-50), to assist in the maintenance of telomeres through interactions with TRF1 (51), to facilitate DNA repair through interactions with p53 (52), and finally, to support breast development (53).

Additionally, Pin1 has been shown to have specific roles in the immune and nervous systems. These additional functions provide links to the implication of Pin1 in various pathogenic conditions, which will be discussed in the following section. In short however, it has been shown that Pin1 is important for regulating transcription of cytokines in T cells, as well as for survival of eosinophils (54). The importance of Pin1 in the brain is evident in Pin1 knockout mice, which have progressive and age-related neurodegeneration (55). This is directly related to the ability of Pin1 to promote normal neuronal cell functioning and survival through the interaction with proteins such as Tau and amyloid precursor protein (APP) (30, 36-39).

1.5 Pin1 in pathogenesis

Considering the diversity of its roles and importance as a key regulator of many cellular and biological processes, it is not unexpected that Pin1 appears to be involved in various pathological conditions, including cancer, Alzheimer's disease, and asthma. In this respect, Pin1 has been implicated in a variety of cancers, including breast, lung, colon and prostate cancer (8). This is not surprising given its role as a regulator of the cell

cycle. However, the precise role Pin1 plays in cancer is controversial, as levels of Pin1 have been shown to be either positively or negatively related to cancer (56). One of the better understood pathways in which overexpression of Pin1 appears to participate in cancer involves cyclin D1. Not only can Pin1 increase expression of cyclin D1 (24, 25, 48), Pin1 can also directly bind and stabilize cyclin D1 to enhance cyclin D1/CDK activity (26). Conversely, loss of Pin1 can suppress transformation by Neu or Ras (18). Additionally, Pin1 has been shown to stabilize p53, an important tumour suppressor which promotes apoptosis in response to genotoxic stresses (33-35).

With regards to Alzheimer's disease, the precise role of Pin1 in pathogenesis remains uncertain, although evidence suggests that various mechanisms in Alzheimer's disease downregulate and/or inactivate Pin1 (for example, through oxidation (22)), suggesting it has a neuroprotective role (57). The loss of Pin1 function has impacts on two proteins, namely APP and Tau, both found in senile plaques and neurofibrillary tangles. A current model suggests that without Pin1, the pThr668-Pro motif of APP remains in the *cis* form and accumulates in plaques (38). Similarly, the Tau pThr231-Pro motif is also found mostly in the *cis* form, leading to its hyperphosphorylation and subsequent accumulation (30, 55).

The association between Pin1 and asthma can be traced back to the role Pin1 plays in immune cell function. By regulating the release of cytokines from eosinophils, and participating in the apoptotic decision of both T-cells and eosinophils, activated Pin1 modulates the allergic inflammatory response in the lungs associated with asthma (58).

1.6 Emergence of Pin1 as a candidate for molecular-targeted therapy

The prevalence of Pin1 in various human diseases, cancer in particular, makes it an obvious candidate for therapies. Additionally, the fact that other PPIase proteins, specifically cyclophilin and FKBP, have been shown to be good therapeutic targets lends support to attempts to achieve the same success with Pin1. The first general inhibitor of parvulins was juglone, and although it has the ability to irreversibly inhibit Pin1, its use as an anticancer therapy is limited by its non-specificity (59). More recently, work has been focused on structure-based design of Pin1 inhibitors. Features that have been targeted by these rationally-designed Pin1 inhibitors include its hydrophobic binding pocket, the phosphate binding loop (60), or Cys113 within its active site (61). Thus far, these Pin1 inhibitors have had varying degrees of specificity, as well as issues with potency, degradation, and cell permeability. In addition to these inhibitors, there have also been efforts to isolate inhibitors in the form of cyclic peptides which are less likely to be subject to proteolysis and may bind Pin1 with a higher affinity due to their reduced flexibility (62, 63). Although some inhibitors are able to inhibit Pin1 at nanomolar concentrations, their usage currently appears to be more appropriate for further investigations regarding the cellular functions of Pin1, rather than as therapeutic agents. However, this does not preclude the notion of using them as models to guide the design of novel, potentially therapeutic inhibitors of Pin1.

1.7 Pin1 and CDC25C

The progression of determining functions of Pin1, its pathogenic implications, and subsequently investigating the potential for targeted therapy began with the identification

of Pin1 interacting proteins. In 1998 human CDC25C (and its *Xenopus* homologue) was among the first proteins to be identified as a Pin1 interactor (28, 29) and since then details of this interaction and its implications have been further elucidated.

CDC25C is a dual-specificity phosphatase, often referred to as a mitotic trigger due to its importance in the initiation of mitosis (64). It is not surprising therefore, that CDC25C is subject to multiple post-translational modifications which tightly regulate its function. One such modification is hyperphosphorylation, which results in activation of CDC25C (65), and the resultant rapid initiation of mitosis (66). Of equal importance is the subsequent activity of phosphatases, which act to counter the activating effects of phosphorylation. However, the resulting dephosphorylation is dependent upon Pin1 binding to pT48 and pT67 (12, 30). This result provided evidence for multi-step regulation in mitosis (phosphorylation of specific Ser-Thr/Pro motifs followed by Pin1-catalyzed isomerization) and emphasized the role of Pin1 as a mitotic regulator capable of synchronizing events. CDC25C (both human and *Xenopus*) and peptides derived from it have since been used to further investigate the interaction with Pin1, as well as the structure and function of both the WW and PPIase domains with respect to their interaction with CDC25C (12, 19, 67-70). This has led to the discovery that the interaction between Pin1 and CDC25C requires full-length Pin1, as individual WW and PPIase domains failed to interact with the phosphatase (71). Interestingly, this is not the case for all interactors of Pin1 (71), and suggests that interactions are substrate-specific. This adds yet another twist to the role Pin1 plays in post-phosphorylation mechanisms.

1.8 Models of binding

As an enzyme with binding capabilities in both domains, one of the key outstanding questions with regards to Pin1 is the mechanism with which it binds its interactors. To this end, multiple models to explain the binding mechanism have been proposed, as summarized in Figure 3. The first of four models to be reviewed here is the catalysis-first binding model (Figure 3A), suggested by Wintjens *et al.* (68). Given that the WW domain of Pin1 has been shown to bind targets in the *trans* conformation (10, 19, 68), this model suggests that the PPIase domain binds to a pSer/Thr-Pro site in the *cis* conformation, and subsequently catalyses the *cis-trans* isomerization, thus creating a WW-domain binding site. The multimeric model (Figure 3B) suggests that Pin1 exists within a multi-protein complex, bound by its WW domain to another protein. This would then put the PPIase domain in proximity to the Pin1 target, and thus allow for isomerization (72). The third model is the sequential model (Figure 3C), put forward by Zhou *et al.* (11). This model suggests that the WW domain of Pin1 binds pSer/Thr-Pro targets first. The PPIase domain then either binds to the same target (once the WW domain has released it), or binds to an adjacent target to perform isomerization. This model relies on data showing differences in affinity for target sequences, with the WW domain having higher affinity than the PPIase domain (12). Additionally, in cases where the PPIase domain may bind a second pSer/Thr-Pro motif, this model is supported by Pin1 interactors which have multiple (identified) Pin1 binding sites, for example, CDC25C (12, 30), protein kinase CK2 (73), RNA binding protein $p54^{nrb}$ (74), and microtubule binding protein Tau (30, 36, 37). Recently, a fourth model has been proposed by Innes *et al.* (71) (Figure 3D). This simultaneous model is similar in one

Figure 3 **Schematic representation of models of Pin1 binding**

A. The catalysis-first model suggests that the PPIase domain of Pin1 binds to a pS-T-P site in the *cis* conformation. Following subsequent catalysis by the PPIase domain a WW-domain binding site is generated.

B. The multimeric model suggests that Pin1 exists within a multi-protein complex, bound by its WW domain to another protein. The PPIase domain would theoretically be in proximity to the Pin1 target, and thus, catalyze isomerization.

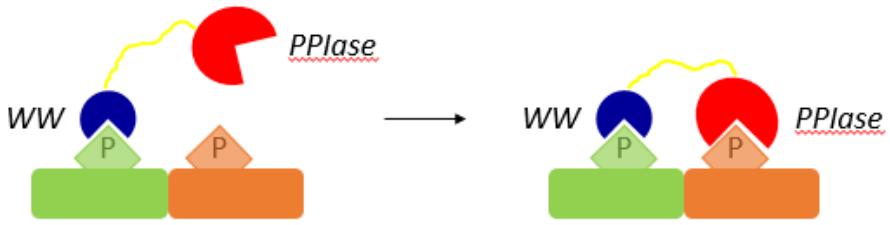
C. The sequential model suggests that the WW domain of Pin1 binds pS/T-P targets first. The PPIase domain will then either bind to the same target (once the WW domain has released it), or bind to an adjacent target to perform isomerization.

D. The simultaneous model suggests that Pin1 binds a multi-phosphorylated target with both the WW domain and the PPIase domain, as each individual site may have low affinity. Binding by both domains at the same time produces a higher affinity interaction.

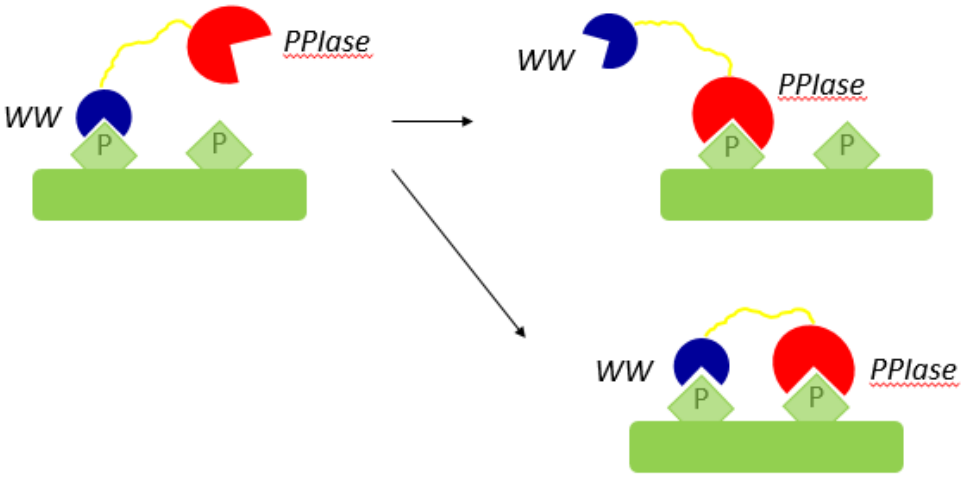
A.


WW
PPIase
P
cis
WW
PPIase
P
trans

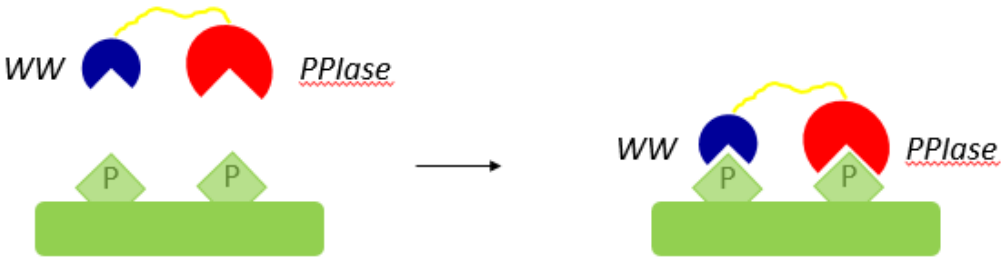
B.


PPIase
WW
P
P
WW
PPIase
P
P

C.


PPIase
WW
P
P
WW
PPIase
P
P
WW
PPIase
P
P

D.


WW
PPIase
P
P
WW
PPIase
P
P

aspect to the sequential model in that it suggests that Pin1 binds a multi-phosphorylated target with both the WW domain and the PPIase domain. It differs however, in how that interaction begins. While the sequential model suggests that a high-affinity WW domain site binds first, the simultaneous model suggests that some sites may have low affinity for the WW domain, and therefore require binding by both domains at the same time in order to produce a higher affinity interaction. Given the data to support each suggested model, it would appear that Pin1 may have more than one way to interact with its various targets.

1.9 Rational, objective, and hypothesis

Since its discovery in 1996, much has been learned regarding the structure, function, and regulation of Pin1. In comparison to other PPIases, one particularly intriguing feature of Pin1 is its phosphorylation dependence which enables Pin1 to introduce an additional level of control in pathways involving proline-directed protein kinases such as CDKs that are central drivers of cell cycle progression. While Pin1 was initially implicated as a key regulator of mitosis, it has subsequently been shown to be important in a diverse array of cellular processes. In concert with its participation in a broad spectrum of biological events, it is noteworthy that Pin1 has been implicated in a variety of diseases including cancer, neurological disorders such as Alzheimer's disease and asthma. Pin1 has thus emerged as a potential candidate for molecular-targeted therapy. Consequently, it can be anticipated that ongoing efforts to understand its regulation and functions and to elucidate its precise catalytic mechanism will foster efforts to develop new approaches that will harness its promise as a therapeutic target. Furthermore, the design of inhibitors of Pin1 will likely be aided by knowledge of the ways in which Pin1 interacts with its targets. However, the dual-domain structure of Pin1 complicates this process, and it appears that

the interactions may vary, depending on the substrate. This represents a rather large gap in the knowledge regarding Pin1, and as a result, the goal of this work was to further elucidate the mechanism through which Pin1 interacts with a key mitotic initiator, CDC25C.

The interaction between Pin1 and CDC25C provided evidence for the role of Pin1 as regulator of mitosis, using a post-phosphorylation mechanism. Specifically, CDC25C is dephosphorylated, and thus inhibited, in response to Pin1 catalyzed isomerization, thus preventing entry into mitosis. Since the interaction between these two proteins requires the presence of full-length Pin1, I hypothesized that this requirement is a result of Pin1 binding using both domains simultaneously. To address this hypothesis, I chose to perform a thorough investigation of the domain and phosphorylation requirements that facilitate the interaction between Pin1 and CDC25C. To this end, I employed a peptide-based strategy, using two independent yet complementary assays to promote precision and accuracy.

The first assay employs fluorescence polarization, enabling for the detection of the interaction between an analyte and ligand, both of which are in solution. Specifically, following excitation the fluorescently labelled ligand will emit light in all planes as a result of its mobility in solution, however, binding of the ligand to an analyte will decrease its mobility thereby increasing the amount of emitted light which is polarized (75). Polarization of emitted light is therefore an indicator of the interaction between the ligand and analyte, and can be analyzed quantitatively and qualitatively to study their affinity (75).

The second peptide-based assay utilizes the phenomena of surface plasmon resonance (SPR) to detect the binding between a ligand and an analyte. Specifically, interactions between a free-flowing analyte and an immobilized ligand can be detected as a result of changes in mass on the immobilization surface. As the mass changes, so to will the angle at which a reduced amount of polarized light is reflected (due to changes in SPR), which is reported in the form of response units (RU) (76). Monitoring of the RU over the course of the interaction produces a sensorgram which can subsequently be analyzed, both qualitatively and quantitatively, to study the affinity between the analyte and ligand (76).

To summarize, although the above methodologies have been used with singly phosphorylated peptides to identify and characterize interaction sites, a comprehensive report which combines an investigation of the domain requirements with the impact of multiple phosphorylation sites with regards to human CDC25C has not been published. As a result, the present approach is unique in its use of a multiphosphorylated human CDC25C-derived peptide.

2.0 Materials and Methods

2.1 GST fusion protein purification

GST fusion proteins were expressed from pGEX constructs (courtesy of Melanie Bailey, Litchfield lab, University of Western Ontario (77)) transformed into *E. coli* strain BL21. Individual colonies were grown in 2xYT broth with 100 µg/mL ampicillin (Roche) at 37 °C until an optical density of 0.6 at 600 nm had been reached. Protein expression was then induced with 0.6 mM IPTG (Roche) for 2 hours. Bacteria were pelleted by centrifugation for 15 min at 4420 xg, resuspended in cold PBS containing protease inhibitors (1 mM PMSF (Sigma), 10 µg/mL pepstatin A (Sigma) and 10 µg/mL leupeptin (Sigma)) and then lysed by sonicating six times 1 min each on ice. Triton X-100 (Sigma) was added to 1% and the mixture was rotated for 15 min at 4°C.

Cell debris was pelleted by centrifugation for 20 min at 23 300 xg and the supernatant was incubated with glutathione cross-linked agarose beads (Sigma) for 1 hour at 4 °C with rotation. After washing beads with 2 column volumes of cold PBS, protein was eluted in five steps with 10 mM reduced glutathione (Sigma) in PBS, followed by 3 steps with 30 mM reduced glutathione (Sigma) in PBS. Aliquots of washes and eluates were analysed by 10-15% SDS-PAGE gels in SDS-PAGE buffer (192 mM glycine (Bioshop), 25 mM Tris-base (Bioshop), 0.1% SDS (Bioshop)) at a constant voltage of 180 V for 1 hour. New England Biolabs broad range prestained molecular marker was used for reference. Protein purity was assessed by staining with Coomassie Blue (Bio-Rad). Fractions containing pure protein were then dialysed at 4 °C for 16-18 hours into PBS

containing 20% glycerol (Caledon) for storage at -80 °C. Protein concentrations were determined using the Bradford Protein Assay (Bio-Rad).

2.2 Cleavage of GST fusion proteins

Proteins used for isomerase assays, and both Biacore and Fluoresence Polarization (FP) experiments were expressed as GST fusions and purified as above, but without dialysis into storage buffer. Instead, TEV protease was added to proteins in a mass ratio of 1:100 for 4 hours at room temperature with 5 mM DTT and 0.5 M NaCl. Proteins were dialysed at 4 °C for 16-18 hours into Buffer A (150 mM NaCl, 50 mM Na_2HPO_4, 5 mM NaN_3, pH 7.8) followed by loading onto a 120 mL HiPrep 16/60 Sephacryl S-100 High Resolution filtration column (GE Healthcare). Proteins were eluted with Buffer A in ninety-six 1.5 mL fractions. As before, aliquots of fractions were analysed by 10-15% SDS-PAGE gels and staining with Coomassie Blue to assess purity and those containing pure protein were pooled and dialysed at 4 °C for 16-18 hours into HBS-E (10 mM HEPES pH 7.4, 150 mM NaCl, 3.4 mM EDTA). Protein concentrations were determined using the Bradford Protein Assay (Bio-Rad).

2.3 Cell culture and transfection

HeLa cells were maintained at 37 °C and 5% CO_2 in DMEM (Invitrogen) supplemented with 10% FBS (Thermo Scientific) and 1% penicillin/streptomycin (Invitrogen). Transfections were performed on cells at ~50% confluence on 10 cm tissue culture plates following the addition of 5 mL of fresh media. All transfected plasmid constructs (courtesy of Kathryn Volkening, Strong Lab, University of Western Ontario) were purified by cesium chloride purification methods. For transfections, 60 µL of 1 mg/mL

PEI (Polysciences Inc.) was added to 440 µL of 150 mM filter sterilized NaCl and 10 µg plasmid DNA. Following vortexing for 10 s, complexes were allowed to form for 10 minutes, before addition to cells. After 16-18 hours, transfected cells were washed with PBS (Invitrogen), fresh media was added, and cells were allowed to grow for an additional 16-18 hours. To arrest cells in mitosis, cells at ~75% confluency were treated with 0.25 µg/mL nocodazole (Sigma) for 18 hours before harvest.

2.4 Lysate preparation

Cells were harvested by shake off and pelleted at 300 xg for 3 min at 4 °C. Cell pellets were washed and re-pelleted twice, with cold PBS containing protease and phosphatase inhibitors (1 mM PMSF (Sigma), 10 µg/mL pepstatin A, 10 µg/mL leupeptin, 1 µM microcystin-LR (Cayman Chemical), 1 µM oakadaic acid (Bioshop), and 1 mM sodium orthovanadate (Aldrich Chemical)). Cells were resuspended in cold lysis buffer (50 mM Tris-HCl, pH 8, 200 mM NaCl, 10% glycerol, 1% Triton X-100) with added protease inhibitors as listed above. Cells were allowed to lyse at 4 °C for 2-3 hours with rotation. Cell debris was then spun down by centrifugation first at 24 100 xg for 15 min and then at again for 30 min. Protein concentration was determined using the BCA protein assay (Thermo Scientific). Cell lysates were either used immediately in pull-downs, or frozen in aliquots at -80 °C.

2.5 GST pull-downs

100 µg of GST fusion protein was incubated with 20 µL of a 1:1 slurry of glutathione cross-linked agarose beads (Sigma) to PBS for 30 min at 4 °C with rotation. The beads were then washed 3 times with cold PBS, and 1 mg of cell extract (prepared as described

in section 2.4) was added and allowed to incubate for 1 hour at 4 °C with rotation. The beads were then washed 3 times with cold PBS. Following removal of the last wash, proteins were eluted into 50 µL of 2X Laemmli sample buffer (4% SDS, 20% glycerol, 120 mM Tris-HCl, pH 6.8, 10% β-mercaptoethanol) by boiling at 100 °C for 3 min.

2.6 SDS-PAGE and western blotting of pull-downs

All pull-downs were run on 10% SDS-PAGE gels in SDS-PAGE buffer (192 mM glycine, 25 mM Tris-base, 0.1% SDS) at a constant voltage of 180 V for 1 hour. New England Biolabs broad range prestained molecular marker was used for reference. Proteins were then transferred to polyvinyl difluoride membrane (Millipore) over 1 hour at 15 V, using a semi-dry transfer unit (Biorad) in blotting buffer (20% methanol, 10 mM Tris-base, 767 mM glycine). Membranes were blocked for 1 hour at room temperature in Odyssey Li-Cor blocking buffer (Li-Cor Biosciences) as per manufacturers' specifications, followed by three 5 min washes with TBS-T (200 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.1% Tween 20). All antibody dilutions were done in TBS-T containing 1% bovine serum albumin (Sigma). Primary antibodies used are as follows: MPM-2 (2 µg/mL; Millipore), Cdc25C (C20) (1/100; Santa Cruz), Tau (T14/T16) (1 µg/mL; Invitrogen), EGFP (0.2 µg.mL, Invitrogen), and NonO (1/2000, Abcam). After incubation with primary antibodies at 4 °C for 16-18 hours, membranes were washed three times with TBS-T, for 5 min each time. Membranes were then incubated with 1:10 000 dilutions of either GAM or GAR secondary antibodies for 1 hour at room temperature. After three 5 min washes with TBS-T, and one 5min wash with TBS, membranes were visualized on a Li-Cor near-infrared fluorescent scanner and quantifications were performed using Odyssey software (Version 3.0).

2.7 CDC25C peptides

Human CDC25C-derived peptides were synthesized by EZBiolab (USA). Peptides were prepared to 95-96% purity, as determined by HPLC (by EZBiolab). Peptide sequences were as follows: PDVPRpTPVGKFLGDSANLSISGGpTPKRSLDW-beta-A-beta-A-beta-A-C, PDVPRpTPVGKFLGDSANLSISGGTPKRSLDW-beta-A-beta-A-beta-A-C, PDVPRTPVGKFLGDSANLSISGGpTPKRSLDW-beta-A-beta-A-beta-A-C (herein referred to as 2xP, 1xP #1, and 1xP #2 respectively). Dephosphorylated peptide (herein referred to as De-P) was obtained following phosphatase treatment of phosphorylated peptide. Phosphorylated peptide at 100 µM in HBS (10 mM HEPES, 10 mM NaCl, pH 7.0) was incubated with 400 units of λ-protein phosphatase (New England Biolabs), with the addition of λ-phosphatase buffer (New England Biolabs) and 1 mM $MnCl_2$. The reaction was allowed to proceed for 60 min at 30 °C, followed by dialysis of peptide into HBS (10 mM HEPES, 10 mM NaCl, pH 7.0). Dephosphorylation was confirmed with MALDI Mass Spectrometry (within the MALDI Mass Spectrometry Facility that is part of the London Regional Proteomics Centre).

2.8 Biacore surface plasmon resonance binding measurements

All measurements were performed on a BIAcore X instrument (GE Health Sciences) equipped with CM5 sensor chips maintained at 25 °C. All buffer solutions were filtered and de-gassed prior to use. Peptide immobilization was performed using the ligand thiol method, described as follows. A continuous buffer flow consisting of HBS-E (10 mM HEPES pH 7.4, 150 mM NaCl, 3.4 mM EDTA) was maintained at 5 µL/min. The carboxylated dextran matrix of one flow cell in each CM5 sensor chip (GE Healthcare) was activated by a 10 µL injection of a solution containing equal volumes of 100 mM N-

hydroxysuccinimide and 400 mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, followed by a 20 µL injection of 80 mM 2-(2-pyridinyldithio)ethaneamine in 0.1 M sodium borate pH 8.5. 35 µL of peptide to be immobilized at 20-30 µM in 10 mM sodium borate, pH 6.0 was then injected, followed by a 20 µL injection of a solution of 0.1 M sodium acetate, pH 4.0, containing 50 mM cysteine and 1 M NaCl. Immobilization was indicated by a change in baseline of 500-900 RU. The second flow cell of each chip was treated identically, without the injection of peptide solution.

Protein samples (10 µL of 5-20 µM) were injected over the chip surface at a flow rate of 10 µL/min, and the peptide-protein complex was allowed to dissociate for 1-2 min. Complete dissociation of protein was achieved following a 5 µL injection of 10 mM NaOH. Analysis was performed using Biaevaluation Software 4.1.1 (GE Health Sciences), following subtraction of background as measured in the non-immobilized flow cell.

Whenever possible, assays with different peptides were performed at the same time. Otherwise, binding ability (or lack thereof) was confirmed with single injections of protein on the sensor chips immobilized with doubly- or de-phosphorylated peptide.

2.9 Fluorescence polarization measurements

All peptides were fluorescently labelled with fluorescein-5-maleimide (Life Technologies). Peptides were dissolved at 75-120 µM in HBS (10 mM HEPES, 10 mM NaCl, pH 7.0) and DTT was added in 10-fold molar excess. Following overnight dialysis to remove excess DTT, fluorescein-5-maleimide (10 mM in DMSO) was added in 10-20-fold molar excess. The reaction was incubated overnight at 4°C, and excess fluorescent

reagent was consumed with 10-fold molar excess β-mercaptoethanol. Peptides were dialyzed into HBS as before, and labelling was confirmed with MALDI. At all times during and following labelling, peptides were stored in the dark.

All fluorescence polarization assays were read using an Envision 2103 multiplate reader (PerkinElmer). Optimal dilutions for each peptide were determined and employed in all future experiments. Individual reactions were carried out in duplicate, in a total volume of 35 µL per well in a 384-well black plate (Corning). A serial dilution of the protein sample was prepared, and 30 µL was added to each well, followed by 5 µL of peptide. Following 1 min incubation with agitation (500 rpm) at room temperature, the plate was spun at 100 xg for 1 min, and fluorescence polarization was read. Analysis was performed using Microsoft Excel (Microsoft) by subtracting reference (buffer) polarization, as well as polarization associated with non-specific interactions between protein and the fluorescein-5-maleimide tag.

2.10 *In vitro* isomerase assays

Assays were performed at 0 °C in a Cary-100 spectrophotometer. The Suc-AEPF-*p*NA substrate (Bachem) was dissolved trifluoroethanol containing 0.3 M LiCl, and the chymotrypsin (Type II; Sigma-Aldrich) was dissolved to a concentration of 50 mg/mL in 1 mM HCl. To assay isomerase activity of Pin1 constructs, substrate at the appropriate concentration was added to 2 mL of assay buffer (100 mM NaCl, 50 mM HEPES, 5 mM NaN_3, pH 7.4) and allowed to incubate for approximately 30 s. Chymotrypsin (50 µL) was then added and following consumption of peptide containing *trans*-proline, the rate of chemical isomerization was measured for approximately 30 s. Pin1 was then added to

the system, and the rate of Pin1-catalyzed prolyl isomerization together with chemical isomerization was measured. Absorbance measurements were made at 405 nm, 430 nm, or 445 nm, depending on the substrate concentration, to ensure that optical density did not exceed 2.0 absorbance units. Rates of reaction for 5 different substrate concentrations were recorded, corrected for the rate of chemical isomerisation and enzyme concentration, and plotted against substrate concentration to determine the k_{cat}/K_M.

2.11 Differential scanning calorimetry

Differential scanning calorimetry was performed on a MicroCal VP-DSC Differential Scanning Calorimeter (GE Healthcare). All solutions were degassed prior to DSC runs. The equipment was first calibrated using 10-20 scans of buffer only, followed by a single scan with sample. Scans were performed by heating from 10 °C -110 °C at 1 °C/min, with pressures of between 23.5-25 psi. Analysis was performed using Origin software (Version 7.0) provided by the manufacturer. Reference (buffer) runs were subtracted, and data was normalized to concentration of sample in order to determine the transition temperature. For samples to be used in additional experiments, sample was removed immediately after the transition temperature was reached to prevent precipitation.

3.0 Results

3.1 Protein purification

As a first step towards testing the determinants of the interaction between Pin1 and CDC25C, recombinant Pin1 proteins were purified. GST fusion proteins were selected for ease of purification, as well as to allow for GST-pullown experiments. Additionally, GST fusion constructs had previously been designed (71, 77) to incorporate a TEV cleavage site between the GST tag and the protein. This allowed for the generation of tag-free proteins for additional assays where tags may have confounded results. Both GST and all GST-Pin1 fusion proteins (Figure 4) were produced in bacteria and purified using glutathione agarose beads. The expressed proteins displayed the expected molecular weights (Figure 5). For proteins used in isomerase assays, and both SPR and fluorescence polarization experiments, TEV cleavage followed by gel filtration also produced proteins of expected molecular weights (Figure 6). Proteins were purified in relatively high concentrations (2-15 mg/ml).

3.2 Pin1 interacts with CDC25C

The interaction between Pin1 and CDC25C has been show to require full-length Pin1, implying that binding occurs via a simultaneous model (71). As a first step towards testing this hypothesis, we sought to confirm the interaction with GST pulldowns using mitotic HeLa cell lysates. Proteins that bound to GST or GST-Pin1 fusion proteins were examined by immunoblotting with CDC25C antibody (Figure 7). Multiple bands representing CDC25C were readily detected in the mitotic cell extract. Full length Pin1 showed interaction with CDC25C, while both the R68/69A mutant Pin1 and the Pin1

Figure 4. **Illustration of GST-Pin1 fusion constructs used in this study.**

Several GST-Pin1 constructs were used. GST-Pin1 is a fusion protein consisting of the full length Pin1 (residues 1 to 163) with the GST protein attached to the N-terminal. GST-Pin1 Y23A is full length Pin1 with a single substitution within the WW domain that decreases interactions between Pin1 and its interactors. GST-Pin1 R68/69A is full length GST-Pin1 with two point mutations that dramatically decrease the isomerisation activity of Pin1. Truncated GST-Pin1 constructs were also used, each consisting of only one Pin1 domain, the WW domain (residues 1 to 40) or the isomerase (PPIase) domain (residues 48 to 163).

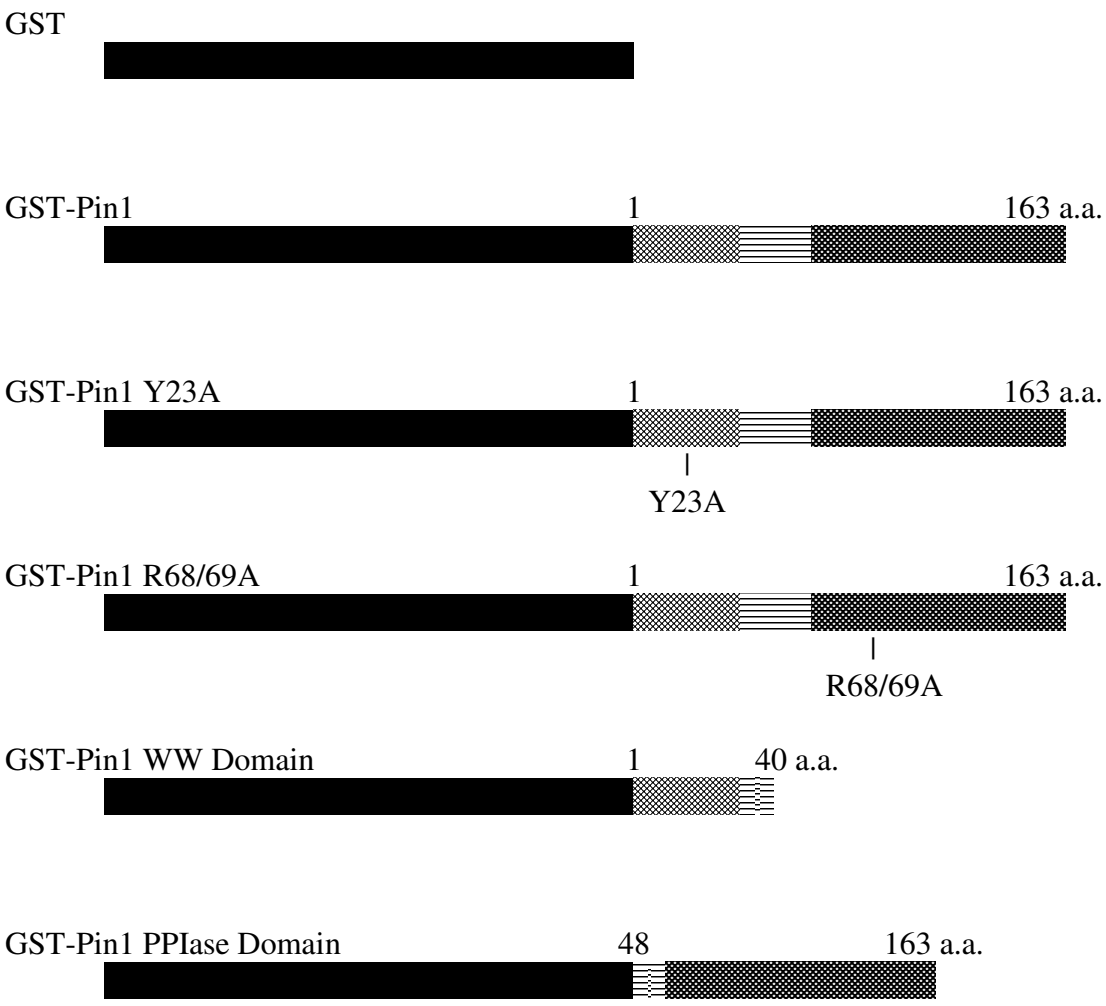
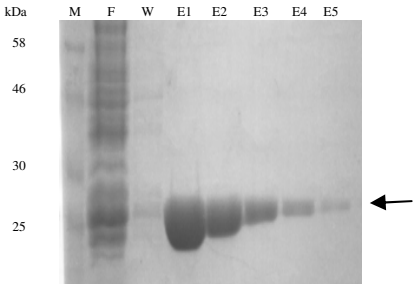

GST
GST-Pin1
1
163 a.a.
GST-Pin1 Y23A
1
163 a.a.
Y23A
GST-Pin1 R68/69A
1
163 a.a.
R68/69A
GST-Pin1 WW Domain
1
40 a.a.
GST-Pin1 PPIase Domain
48
163 a.a.

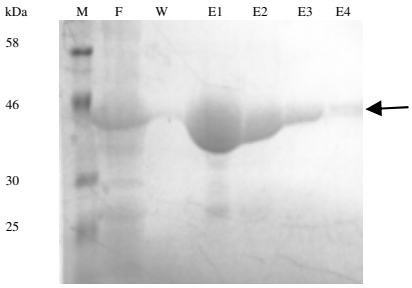
Figure 5. **GST and GST-fusion protein purification.**

GST and GST fusion proteins were isolated from bacterial lysate following induction with IPTG by affinity chromatography using glutathione agarose beads. Samples were run on 10-15% SDS-PAGE gel and proteins visualized with Coomassie Blue. The expected molecular weight for each protein in indicated as follows: GST 26kDa, GST-Pin1 (including full length mutants) 44kDa, GST-Pin1 WW Domain 32kDa, GST-Pin1 PPIase Domain 39kDa. Arrows indicate bands containing protein construct. Lane inputs are as follows: M-marker, F-flowthrough, W-wash(es), E-elution(s), +/- IPTG-pre/post induction with IPTG.

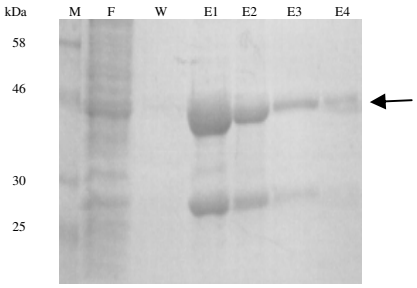
GST



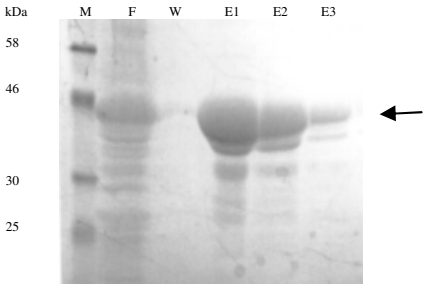
GST-Pin1



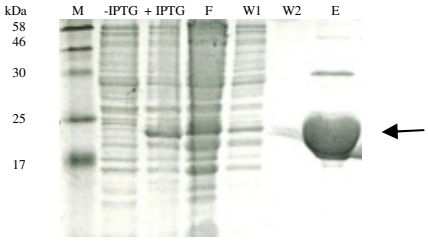
GST- Pin1 Y23A



GST-Pin1 R68/69A



GST-Pin1 WW Domain



GST-Pin1 PPIase Domain

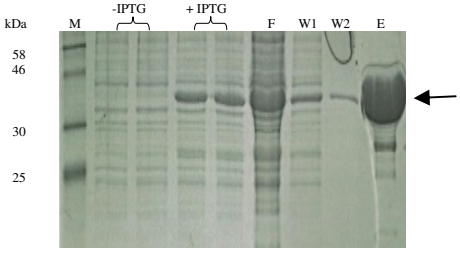
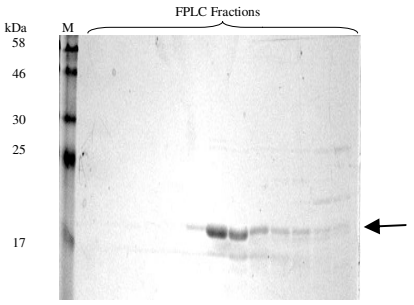


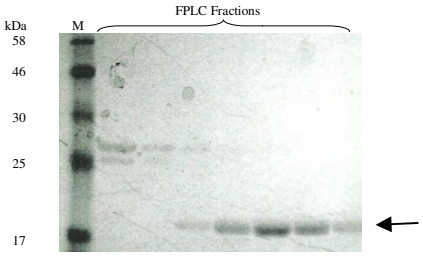
Figure 6. **Untagged protein purification.**

GST fusion proteins were isolated as previously described. Following TEV cleavage, proteins were separated by gel filtration chromatography using a HiPrep 16/60 Sephacryl S-100 High Resolution filtration column. Samples of fractions were run on 10-15% SDS-PAGE gel and proteins visualized with Coomassie Blue. The expected molecular weight for each protein in indicated as follows: Pin1 (including full length mutants) 18kDa, Pin1 WW Domain 6kDa, Pin1 PPIase Domain 13kDa. Arrows indicate bands containing protein construct. Lane inputs are as follows: M-marker.

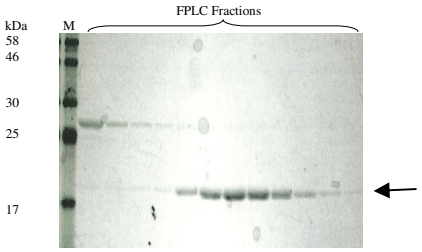
Pin1



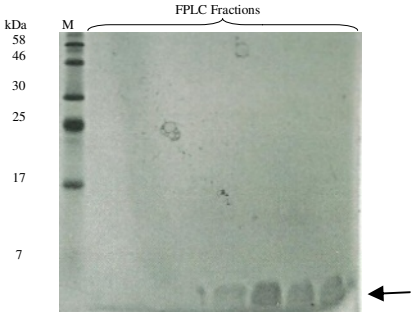
Pin1 Y23A



Pin1 R68/69A



Pin1 WW Domain



Pin1 PPIase Domain

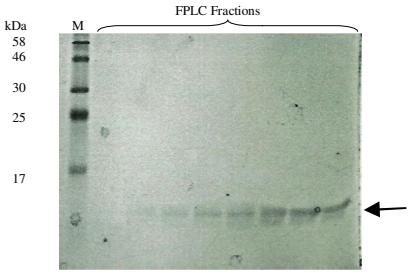
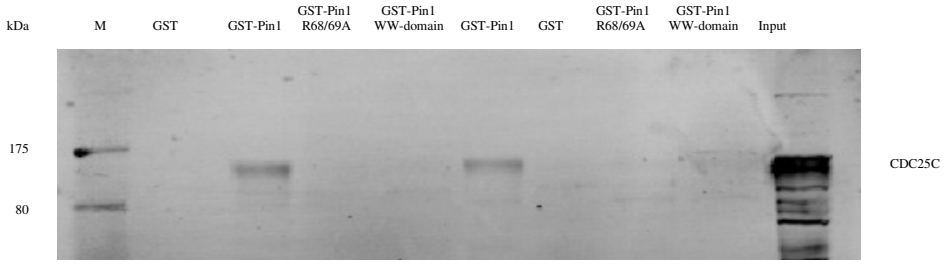


Figure 7. **GST-Pulldown confirms the interaction between Pin1 and CDC25C.**

Mitotic HeLa cell lysates were used in GST and GST-Pin1 fusion protein binding assays. Proteins bound to the GST and GST-Pin1 beads were run on 10% SDS-PAGE gel and transferred to membranes. Input represents 2 % of mitotic HeLa cell lysate used. Blots were probed with antibody for CDC25C.


kDa
M
GST
GST-Pin1
GST-Pin1 R68/69A
GST-Pin1 WW-domain
GST-Pin1
GST
GST-Pin1 R68/69A
GST-Pin1 WW-domain
Input
175
80
CDC25C

WW domain, did not. No CDC25C was detected in the GST binding assay indicating that the Pin1 portion of the GST-Pin1 fusion proteins is that which is responsible for interactions with CDC25C. Overall, these results confirmed that full length Pin1 interacts with CDC25C, in a manner that appears to require the presence of both, intact domains.

3.3 The interaction between Pin1 and CDC25C peptide is phosphorylation-dependent

Pin1 is known to interact preferentially with phosphorylated serine or phosphorylated threonine residues that precede a proline residue (7). As a fairly well characterized interactor of Pin1, CDC25C has two described Pin1 binding sites, T48 and T67 (12, 30) (Figure 8A). To determine the importance of phosphorylation of these binding sites with regards to Pin1 binding we employed two independent assays, using SPR and fluorescence polarization, to test the interaction between Pin1 and peptides derived from human CDC25C.

Peptides were synthesized to contain both phosphorylation sites, as well as upstream and downstream residues (Figure 8B). Three beta-alanine residues were added to the C-terminal end of the peptide to act as a spacer between the residues of the sequence and the surface of the chip used for SPR. Also, since peptide immobilization on chip surfaces was performed using a ligand thiol method (Figure 9A), a cysteine residue was added to the C-terminal. To ensure peptide immobilization was uniform, C71 was substituted with a serine residue to maintain similar amino acid size and structure.

Figure 8. **Human CDC25C-derived peptides used in this study.**

A. Human CDC25 protein sequence is shown, with Pin1 binding sites, T48 and T67 identified in bold (UniProt P30307.2). Amino acid numbers are indicated on the left.

B. Peptide sequences and associated shorthand names of peptides used in this study.

A.

1	mstelfsstr	eegssgsgps	frsnqrkmln	lllerdtsft	vcpdvpr**tp**v	gkflgdsanl
61	silsgg**tp**kr	cldlsnlssg	eitatqltts	adldetghld	ssglqevhla	gmnhdqhlmk
121	cspaqllcst	pngldrghrk	rdamcsssan	kendngnlvd	semkylgspi	ttvpkldknp
181	nlgedqaeei	sdelmefslk	dqeakvsrsg	lyrspsmpen	lnrprlkqve	kfkdntipdk
241	vkkkyfsgqg	klrkglclkk	tvslcditit	qmleedsnqg	hligdfskvc	alptvsgkhq
301	dlkyvnpetv	aallsgkfqg	liekfyvidc	rypyeylggh	iqgalnlysq	eelfnfflkk
361	pivpldtqkr	iiivfhcefs	sergprmcrc	lreedrslnq	ypalyypely	ilkggyrdff
421	peymelcepq	sycpmhhqdh	ktellrcrsq	skvqegerql	reqiallvkd	msp

B.

Peptide Sequence	**Peptide Shorthand Name**
PDVPR**pT**PVGKFLGDSANLSILSGG**pT**PKRSLDW-AAAC	2xP
PDVPR**pT**PVGKFLGDSANLSILSGGTPKRSLDW-AAAC	1xP #1
PDVPRTPVGKFLGDSANLSILSGG**pT**PKRSLDW-AAAC	1xP #2
PDVPRTPVGKFLGDSANLSILSGGTPKRSLDW-AAAC	De-P

CDC25C derived peptides containing either both phosphorylated residues, or no phosphorylated residues, were immobilized onto one flow cell of individual CM5 chips following the ligand thiol method (Figure 9A). A typical sensorgram illustrating immobilization is shown in Figure 9B. The immobilization of 2xP, as well as other peptides, followed this typical pattern as shown in Figure 9C. Immobilization was indicated by a change in baseline response, from that following the injection of PDEA, to the final response after injection of cysteine/NaCl. Differences in response units in this study ranged from 500 RU to 900 RU.

Following immobilization, Pin1 was injected over the surfaces of both flow cells and dissociation was monitored. A typical sensorgram illustrating analyte-ligand binding is shown in Figure 10A, while the results of the Pin1-2xP interaction are shown in Figure 10B. Pin1 showed a direct interaction with 2xP, having distinct association, equilibrium, and dissociation phases. In contrast, the steady baseline level in Figure 10C indicates that there is no detectable binding of De-P by Pin1. The binding results are specific to interactions between Pin1 and peptide, as non-specific interactions between Pin1 and the carboxylated dextran matrix of the chip have been accounted for in the non-immobilized flow cell, and subsequently subtracted. The spikes seen in the Pin1-De-P sensorgram are a result of the small time difference between analyte injection over each flow cell.

The phosphorylation-dependence of Pin1 was also evaluated by fluorescence polarization assays. For these experiments, Pin1 was incubated independently with fluorescein-5-maleimide-labelled CDC25C-derived peptides, and fluorescence polarization was measured. Background fluorescence polarization as a result of labelled peptide in buffer alone was subtracted. The results for Pin1 binding to 2xP mimicked a classic binding

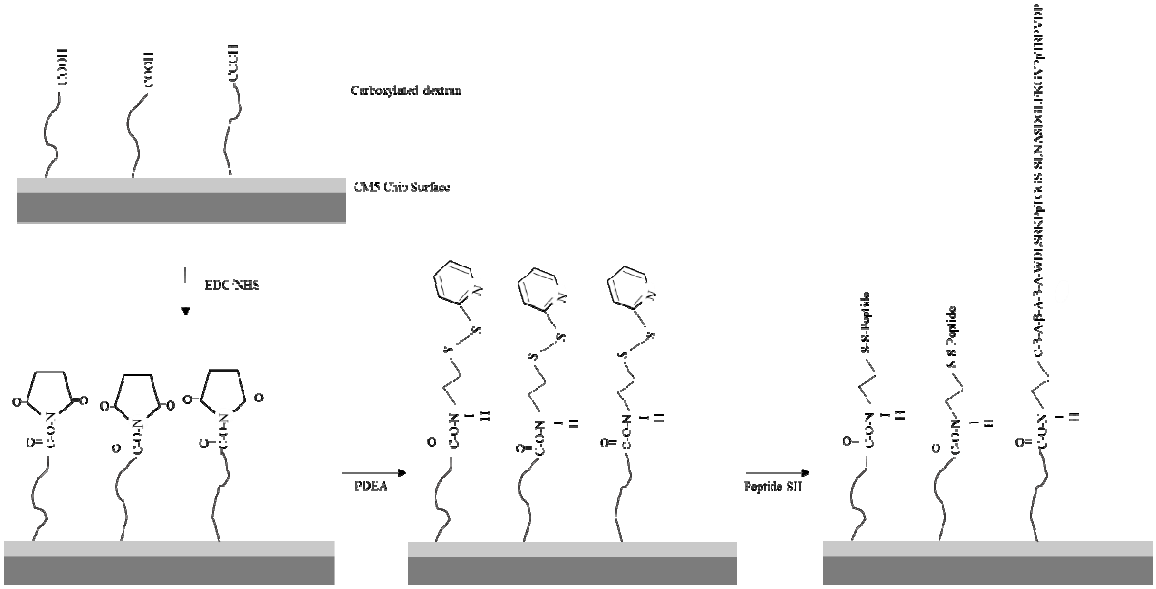
Figure 9. **Peptide immobilization on sensor chip CM5.**

A. Schematic illustrating immobilization chemistry via ligand thiol method. For simplicity, the peptide sequence is represented by 'Peptide' except for the last section, where 2xP sequence is used for representative purposes only.

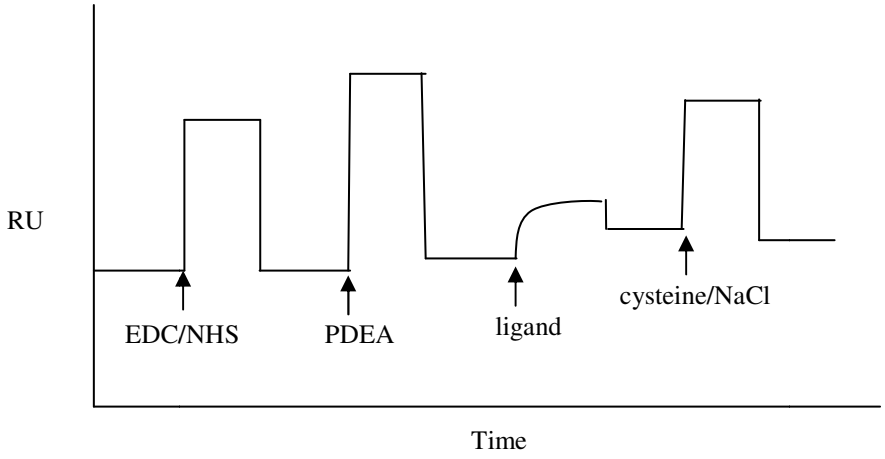
B. Schematic sensorgram showing typical immobilization sequence associated with the ligand thiol method. Injections of EDC/NHS, PDEA, ligand, and cysteine/NaCl are marked. Immobilization is confirmed by the change in baseline response units, pre- and post-injections.

C. Representative sensorgram of SPR analyses for peptide immobilization. The curve shows the specific signal obtained during immobilization of 1xP #1 peptide and is representative of other peptide immobilizations.

A.


Carboxylated dextran
CM5 Chip Surface
EDC/NHS
PDEA
Peptide SH

B.


RU
EDC/NHS
PDEA
ligand
cysteine/NaCl
Time

C.

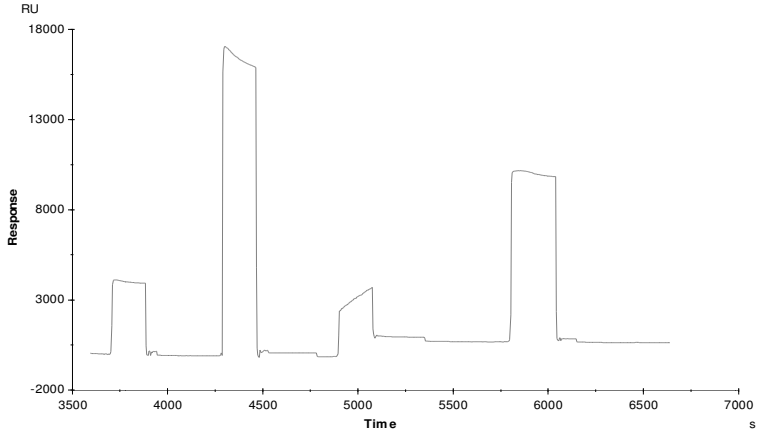
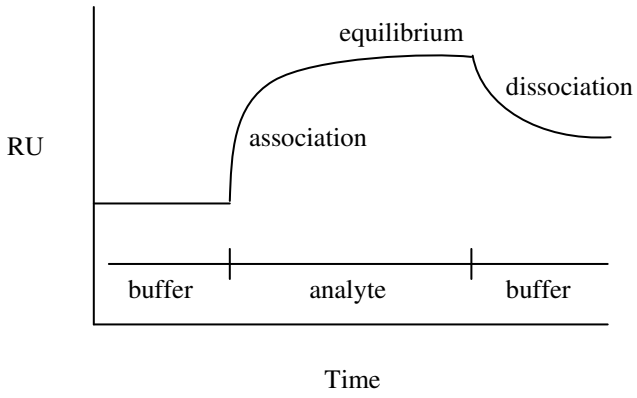

RU
18000
13000
8000
3000
-2000
Response
3500
4000
4500
5000
5500
6000
6500
7000
Time
s

Figure 10. **SPR results indicate that binding of Pin1 to CDC25C-derived peptide is phosphorylation-dependent.**

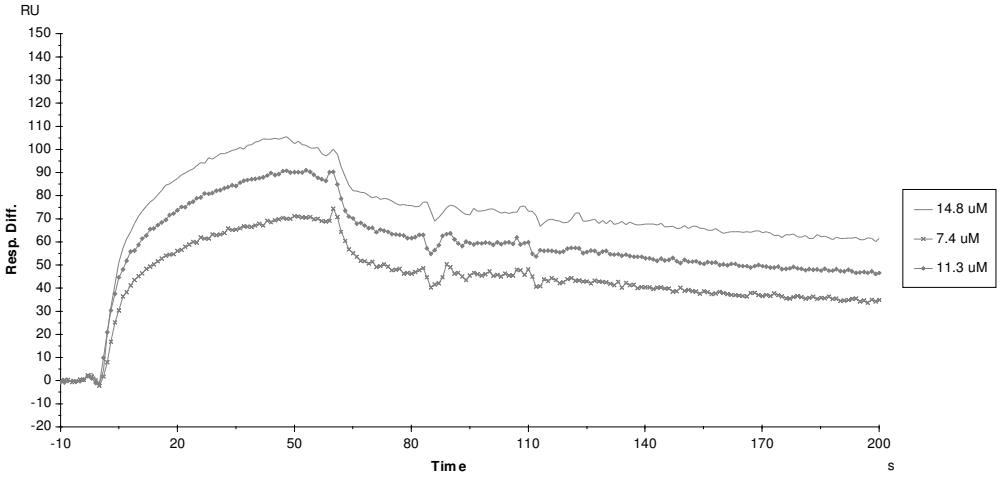
A. Schematic sensorgram, showing association, equilibrium and dissociation phases typical of the interaction between an analyte and its immobilized ligand. Association of analyte with ligand occurs during sample injection. Equilibrium, or steady state, is reached as analyte is continually supplied and removed by sample flow. Return to buffer flow results in dissociation of analyte from surface.

B. and C. Sensorgrams of SPR analyses of the interaction between Pin1 and 2xP peptide (B.), and Pin1 and De-P peptide (C.). Indicated concentrations of Pin1 were injected through both flow cells for 1 minute. The curves show the specific signal obtained after subtraction of background.

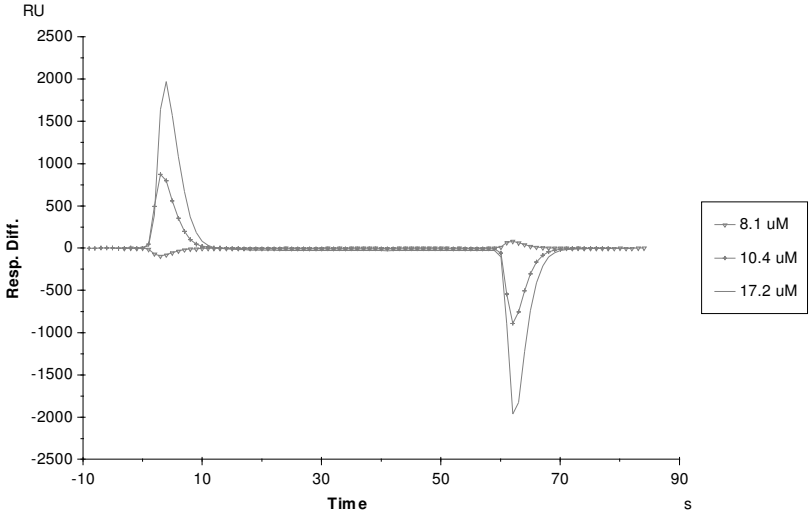
A.


equilibrium
dissociation
RU
association
buffer
analyte
buffer
Time

B.


RU
Resp. Diff.
Time
s
14.8 uM
7.4 uM
11.3 uM

C.


RU
Resp. Diff.
Time
s
8.1 uM
10.4 uM
17.2 uM

curve, with a horizontal asymptote corresponding to 100% saturation (Figure 11A). This is contrary to the results seen with De-P, where no measureable binding was detected (Figure 11A). To determine if the interaction between Pin1 and the labelled peptide was specific, additional assays were run using Pin1 and fluorescein-5-maleimide only. The results (Figure 11B) with low polarization values were indicative of non-specific interactions. These values were subtracted from all fluorescence polarization assays to account for non-specific interactions between Pin1 and the fluorescein tag.

Results from the SPR and fluorescence polarization assays were used to estimate the equilibrium dissociation constant between Pin1 and 2xP to be 1.5 μM and 1.7 μM respectively. The similar values obtained suggest a relatively high degree of precision between the two assays. Additionally, this implies that in SPR assays binding of Pin1 to peptides on the surface of the chip is neither hindered nor enhanced by the chip surface itself.

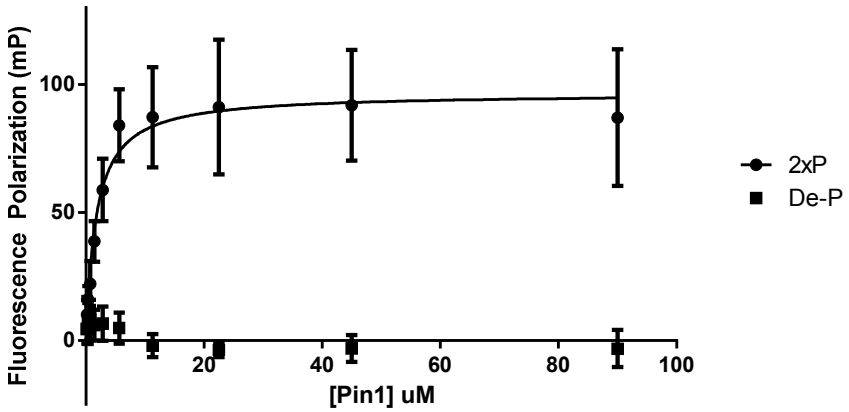
Collectively, this data demonstrates the importance of phosphorylation for Pin1 interaction with our CDC25C-derived peptide. A target with two phosphorylation sites is bound by Pin1 with a relatively high affinity, while a non-phosphorylated target seems to exhibit no interaction with Pin1. These results are not unexpected, as the binding determinants of Pin1 (pSer/Thr-Pro) have been described, however, it was imperative to establish a positive and negative binding result in both assays to facilitate the remainder of this study. Additionally, these results confirmed the use of our CDC25C peptides based on the sequence of human protein as a suitable Pin1 target to further assay binding determinants.

Figure 11. **Fluorescence polarization results indicate that binding of Pin1 to CDC25C-derived peptide is phosphorylation-dependent, and not a result of non-specific binding to fluorescein tag.**

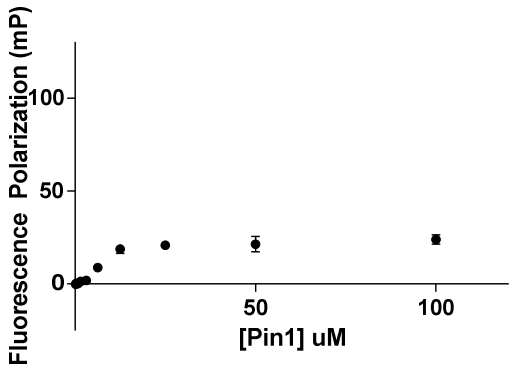
A. Fluorescence polarization assay for Pin1 binding to 2xP and De-P. Labelled peptide was incubated with indicated concentrations of full length Pin1 prior to measuring fluorescence polarization. Each data point represents the mean of 2 (De-P) or 6 (2xP) independent experiments, with error bars representing the standard deviation.

B. Fluorescence polarization assay for Pin1 binding to fluorescein-5-maneimide tag. Fluorescein was incubated with indicated concentration of full length Pin1 prior to measuring fluorescence polarization. Each data point represent the mean of 3 independent experiments, with error bars representing the standard deviation.

A.


Fluorescence Polarization (mP)
100
50
0
20
40
60
80
100
[Pin1] uM
2xP
De-P

B.


Fluorescence Polarization (mP)
100
50
0
50
100
[Pin1] uM

3.4 Effect of number of phosphorylation sites on Pin1-CDC25C peptide interaction

With the knowledge that the interaction between Pin1 and its peptide target is phosphorylation-dependent, and the confirmation that a doubly-phosphorylated peptide was a suitable binding target, we next sought to determine the effect on binding when only a single amino acid is phosphorylated.

As previously described, both CDC25C derived peptides containing a single phosphorylated residue were immobilized onto one flow cell of individual CM5 chips using the ligand thiol method. Following Pin1 injections, dissociation was monitored. The SPR results indicated that Pin1 has similar binding to both singly-phosphorylated peptides (Figure 12A, B). The spike seen in the sensorgram for 1xP #1 (Figure 12A), in which 14.8 μM Pin1 was injected can be attributed to a bubble present in the system. Similar spikes in response units were seen elsewhere (see Appendix 6.1).

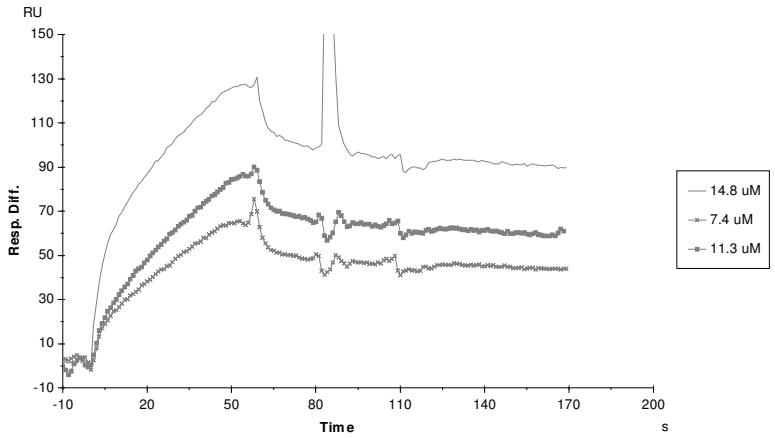
Although it appears that binding to either single-site phosphorylated peptide is similar to binding to the doubly-phosphorylated peptide (compare Figure 10B with Figures 12A, B), close examination of the qualitative characteristics of the sensorgrams highlights some important differences. With regards to the association phase, the curvature appears to be greater in Figure 10B than either of Figures 12A or B. Also, Figures 12A and B do not reach equilibrium, and their dissociation phases appear to be quite pronounced. This is in contrast with the more apparent equilibrium phase seen in Figure 10, as well as the more gradual change seen in the dissociation phase which is indicative of a slower release of analyte. Taken together, these differences make it difficult to generate accurate kinetic data for the singly-phosphorylated peptides to compare with the dissociation constant

Figure 12. **SPR and fluorescence polarization results indicate that binding of Pin1 is reduced on a single-site phosphorylated CDC25-derived peptide.**

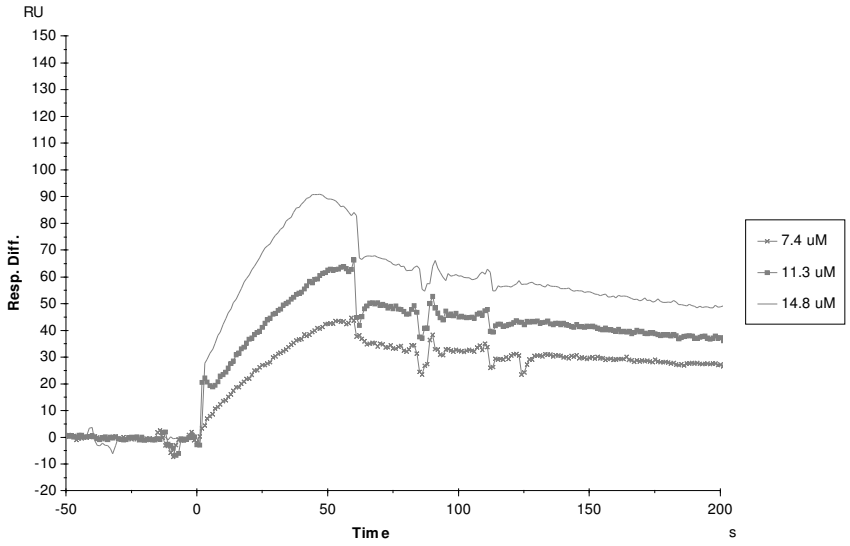
A. and B. Sensorgrams of SPR analyses of the interaction between Pin1 and 1xP #1 peptide (A.), and Pin1 and 1xP #2 peptide (B.). Indicated concentrations of Pin1 were injected through both flow cells for 1 minute. The curves show the specific signal obtained after subtraction of background.

C. Fluorescence polarization assay for Pin1 binding to 1xP #1 and 1xP #2. Independently, labelled peptide was incubated with indicated concentrations of full length Pin1 prior to measuring fluorescence polarization. Each data point represents the mean of 3 independent experiments, with error bars representing the standard deviation.

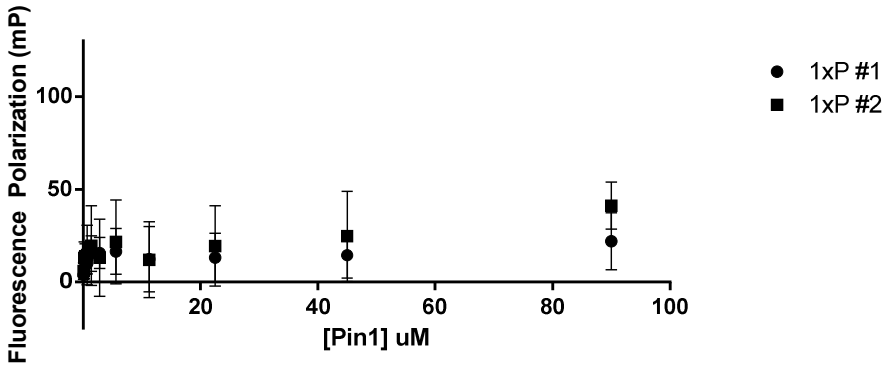
A.


RU
Resp. Diff.
Time
s
14.8 uM
7.4 uM
11.3 uM

B.


RU
Resp. Diff.
Time
s
7.4 uM
11.3 uM
14.8 uM

C.


Fluorescence Polarization (mP)
[Pin1] uM
1xP #1
1xP #2

generated for the doubly-phosphorylated peptide. However, the sensorgrams do suggest that binding affinity to either of the single-site phosphorylated peptides is decreased in comparison to the binding to the doubly-phosphorylated peptide.

Binding data from fluorescence polarization between Pin1 and each of the single-site phosphorylated peptides seemed to suggest a lack of binding to 1xP #1 and 1xP #2 (Figure 12C). The low levels of fluorescence polarization were indicative of a very weak, or non-specific, interaction between the peptides and Pin1, similar to that which was seen in the interaction between Pin1 and the fluorescein tag alone (Figure 11B). These results support our interpretation of SPR results as showing decreased binding between Pin1 and a single-site phosphorylated target.

The difficulty in interpreting the SPR results obtained here may be attributed to the limitation of a fixed target in the SPR assays. As the density of immobilized peptide is difficult to determine, it is conceivable that full length Pin1 is using both domains to bind to two phosphorylated sites on separate neighbouring peptides at the same time. This could produce a false or overestimated binding result. Alternatively, it is also possible that length of the tagged peptide may have affected the results in the fluorescence polarization assays. Specifically, even if Pin1 bound to the phosphorylated site, the C-terminal tag may still have retained sufficient mobility to prevent polarization of light as a result of the peptides length. This would have underestimated the binding between Pin1 and the peptide.

In general, however, the results presented thus far appear to suggest that Pin1 binds preferentially to our doubly phosphorylated peptide (compare Figures 10B and 11A with

Figure 12).

Given that Pin1 has two binding domains, these results may support a hypothesis that Pin1 binds CDC25C using both domains simultaneously. To test this hypothesis, and attempt to clarify the results seen when Pin1 binds to singly phosphorylated targets, we performed the following experiments.

3.5 Binding of Pin1 to CDC25C peptide requires both domains

Given the differing results generated with regards to Pin1 binding to singly-phosphorylated targets, we chose to address the same question using a different approach. If Pin1 has the ability to bind to a single phosphorylated site on a target, it may be binding with only one of its domains. If this were the case, mutants of Pin1 with binding deficiencies in either the WW or PPIase domains should still maintain the ability to bind to a phosphorylated target. Therefore, we utilized full-length Pin1 constructs with mutations in the WW (Y23A) and PPIase domains (R68/69A), which have been shown previously to have binding deficiencies (12, 28, 71). These proteins were used in SPR and fluorescence polarization assays with 2xP, identical to those previously described. We chose to conduct these experiments with the doubly-phosphorylated peptide in case either of the phosphorylated sites was inadvertently targeted by one domain or the other. Use of the doubly-phosphorylated peptide allows both sites to be available for binding by the functional Pin1 domain, and thus ensures that binding is neither over- nor under-estimated.

Similar to the results obtained in SPR and fluorescence polarization analyses with regards to Pin1 and singly-phosphorylated targets, SPR data here indicated that Pin1 mutants

bind to 2xP, while fluorescence polarization indicates a lack of binding (Figure 13). Considering the binding deficiencies of each mutant, as a result of a mutant WW (Y23A) or PPIase domain (R68/69A), it is unlikely that Pin1 binding was being over estimated in the SPR assay as a result of binding multiple peptides simultaneously. It may be more likely that binding measured through fluorescence polarization was being underestimated, as a result of a peptide that was still mobile enough to prevent polarization of light.

Generally speaking, given that there is a close correlation between the SPR response (measured in RU) and the amount of surface bound protein (76), we can compare the results generated from the same chip when proteins of the same molecular weight are used. With regards to the doubly-phosphorylated peptide, binding by wild-type Pin1 resulted in a greater value of RU than did binding by either of the domain mutants, R68/69A or Y23A (compare Figure 10B with Figure 13A, B). We can therefore infer that binding by wild-type Pin1 was stronger than either of the domain mutants.

Overall, the results from both SPR and fluorescence polarization appear to suggest that binding of Pin1 to a doubly-phosphorylated CDC25C-derived peptide was greater when both Pin1 domains are intact. This would appear to suggest that optimal binding of Pin1 to CDC25C involves both domains.

3.6 Individual Pin1 domains are not sufficient to bind CDC25C peptide

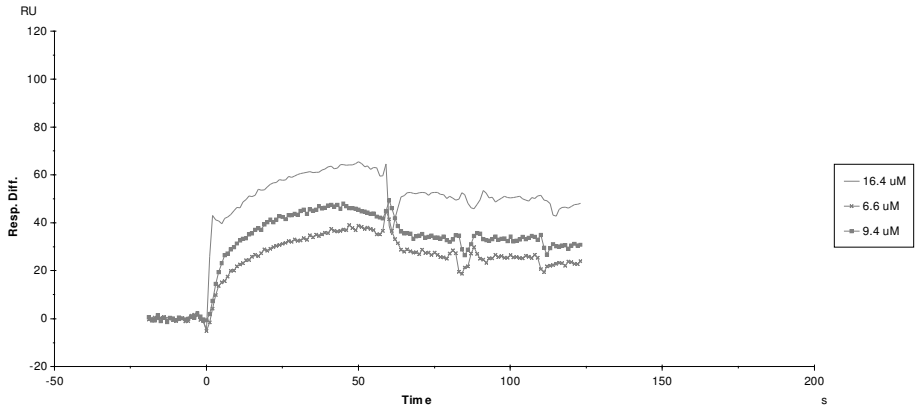
As a dual-domain isomerase, it has been hypothesized that the WW domain of Pin1 acts as a protein-targeting domain, while the PPIase domain performs isomerization, consistent with the sequential model of interaction (11). However, thus far, and consistent

Figure 13. **Binding of Pin1 to CDC25C-derived peptide requires both the WW and PPIase domains.**

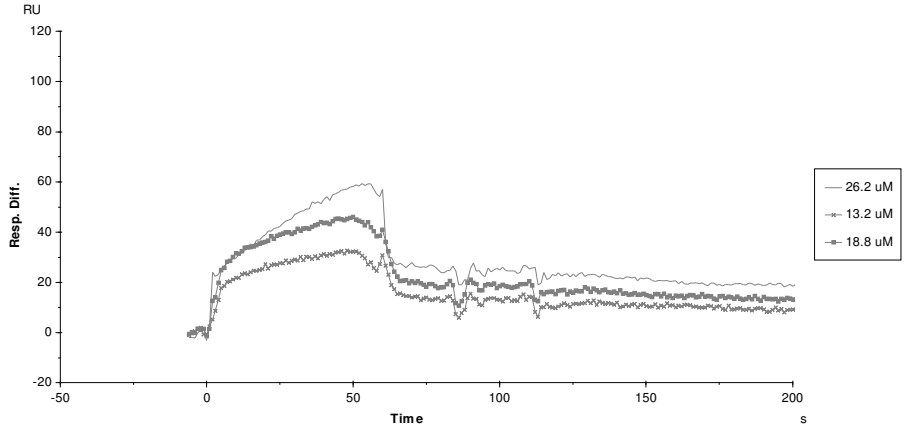
A. and B. Sensorgrams of SPR analyses of the interaction between mutant Pin1 (Y23A (A.) and R68/69A (B.)) and 2xP. Indicated concentrations of mutant Pin1 were injected through both flow cells for 1 minute. The curves show the specific signal obtained after subtraction of background.

C. Fluorescence polarization assay for indicated Pin1 mutants binding to 2xP. Independently, labelled peptide was incubated with indicated concentrations of full length Pin1mutants prior to measuring fluorescence polarization. Each data point represents the mean of 3 independent experiments, with error bars representing standard deviation.

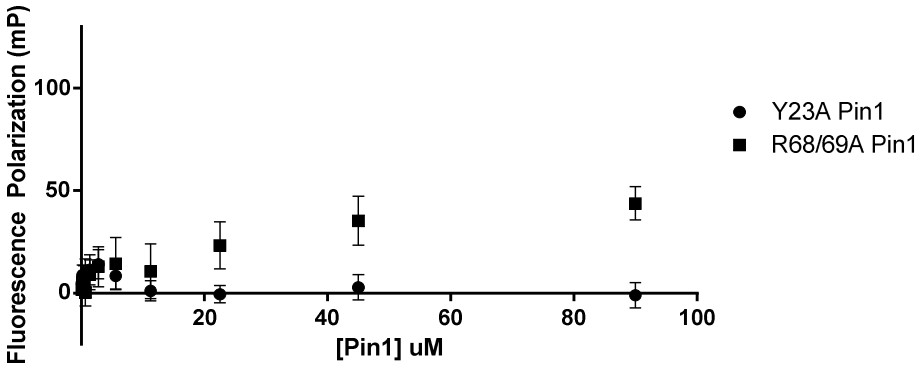
A.


RU
Resp. Diff.
Time
s
16.4 uM
6.6 uM
9.4 uM

B.


RU
Resp. Diff.
Time
s
26.2 uM
13.2 uM
18.8 uM

C.


Fluorescence Polarization (mP)
[Pin1] uM
Y23A Pin1
R68/69A Pin1

with previous research (71) our results suggest that for CDC25C the sequential model does not apply. To further test this, we investigated the binding of individual Pin1 domains with our doubly phosphorylated target. As previously described in section 3.5, the doubly phosphorylated peptide was chosen to ensure both phosphorylation sites were available, in case one inadvertently targeted one domain preferentially. The results from both SPR and fluorescence polarization assays indicated that individual domains of Pin1 were insufficient to bind to our phosphorylated target (Figure 14). This data is in congruence with data from previous sections, and further supports the hypothesis that binding of Pin1 to CDC25C requires the presence of both domains.

3.7 Lack of binding is not due to misfolded proteins

To ensure that any lack of protein binding in either SPR or fluorescence polarization assays was not due to the presence of misfolded proteins, we carried out a series of additional experiments to ensure that all proteins exhibited normal and expected results.

To test for function in PPIase domain-containing constructs, we carried out *in vitro* isomerase assays to ensure that activities were similar to those previously described. A comparison of the relative activities reported as a percent are displayed in Figure 15. A catalytic site mutant of Pin1, C113S, shown previously to have minimal (2 % of wild type) catalytic activity (77) was used as a negative control. Collectively, these results indicated that the PPIase-containing constructs used in this study retained activity, and thus were in the correct conformation to bind substrates, as well as our target peptides.

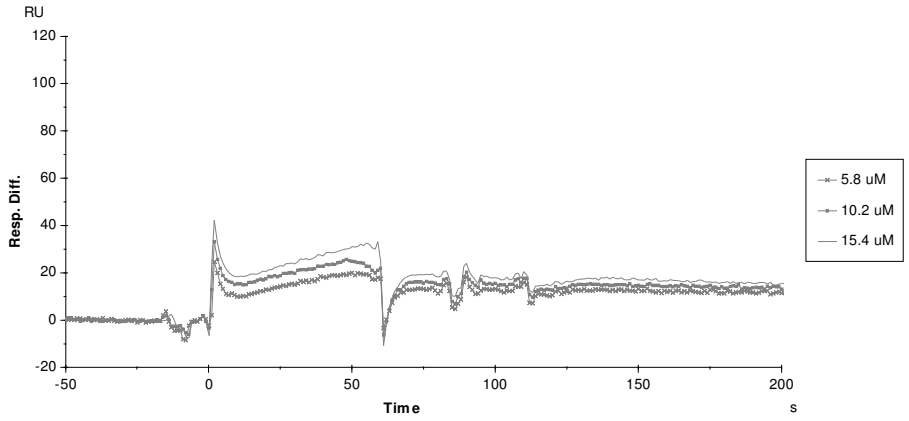
To further investigate the notion that lack of binding may be attributed to misfolded protein, we employed DSC to perform a controlled heat-denature of full length Pin1.

Figure 14. **Individual Pin1 domains are not sufficient to bind to CDC25C-derived peptide.**

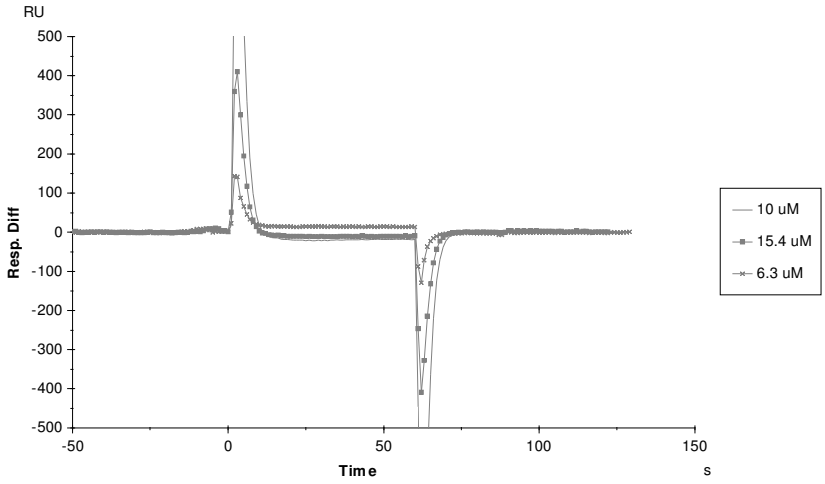
A. and B. Sensorgrams of SPR analyses of the interaction between Pin1domains (PPIase (A.) and WW (B.)) and 2xP. Indicated concentrations of Pin1 domains were injected through both flow cells for 1 minute. The traces show the specific signal obtained after subtraction of background.

C. Fluorescence polarization assay for indicated Pin1 domains binding to 2xP. Independently, labelled peptide was incubated with indicated concentrations of Pin1 domains prior to measuring fluorescence polarization. Each data point represents the mean of 2 independent experiments with error bars representing the standard deviation.

A.


RU
120
100
80
60
40
20
0
-20
Resp. Diff.
-50
0
50
100
150
200
Time
s
5.8 uM
10.2 uM
15.4 uM

B.


RU
500
400
300
200
100
0
-100
-200
-300
-400
-500
Resp. Diff
-50
0
50
100
150
Time
s
10 uM
15.4 uM
6.3 uM

C.

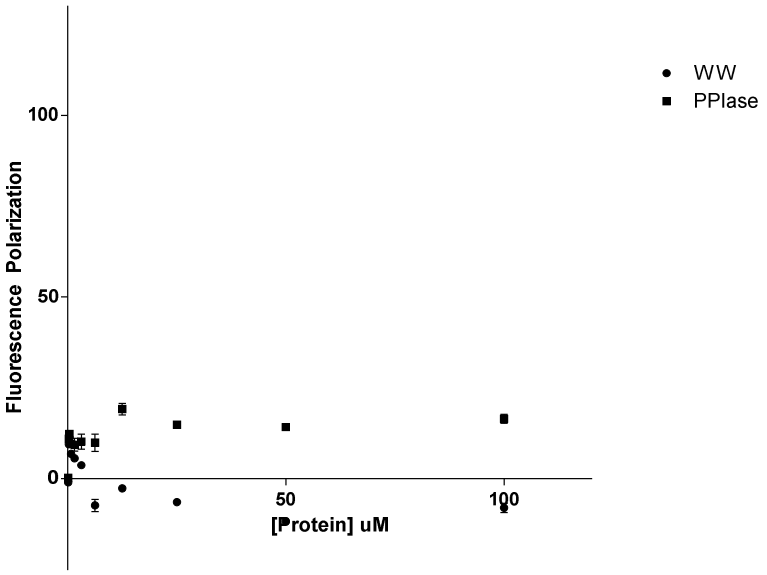
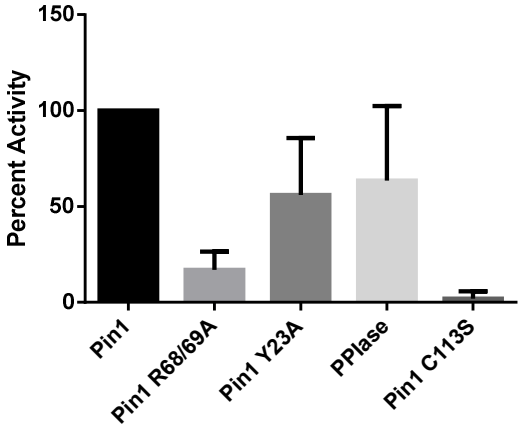

WW
PPIase
100
50
0
Fluorescence Polarization
50
100
[Protein] uM

Figure 15. ***In vitro*** **isomerase activities of PPIase domain-containing constructs used in this study.**

A comparison of the in vitro isomerase activities of all PPIase containing constructs used in this study. Activities are reported as a percent. Pin1 C113S, a catalytic-site mutant of Pin1, known to have no isomerase activity, was used as a negative control. Results are based on the mean of 3 independent experiments, with error bars indicating the standard deviation.


150
100
50
0
Percent Activity
Pin1
Pin1 R68/69A
Pin1 Y23A
PPIase
Pin1 C113S

Protein unfolding was monitored as a function of heat capacity (Figure 16A), and protein sample was extracted from the apparatus prior to precipitation and aggregation. This sample was then immediately used for SPR and fluorescence polarization assays. Results of these assays (Figure 16B and C) were indicative of a lack of binding.

The WW domain construct of Pin1 presented a different problem with regards to testing for proper folding, as an in vitro isomerase assay would not be of any use. Instead, we assayed binding via SPR using our GST-tagged Pin1 WW domain, and upon seeing no binding (Figure 17A), we then confirmed the binding capacity of the construct by utilizing it in a GST-pulldown assay (Figure 17B). The binding results were visualized by blotting with the MPM2 antibody, and showed a characteristic decrease in binding by the WW domain, but not a lack of binding (71). Additionaly, the blot was also probed using antibody for p54nrb, a protein which has previously been shown to bind both full-length Pin1, and the WW domain (71). These binding results indicated that the GST-tagged Pin1 WW domain had the ability to bind targets from a mitotic lysate, however, the same protein did not bind to our immobilized peptide.

Collectively, these results suggest that a lack of binding seen in SPR and/or fluorescence polarization assays cannot be attributed to proteins that are misfolded, and lack function.

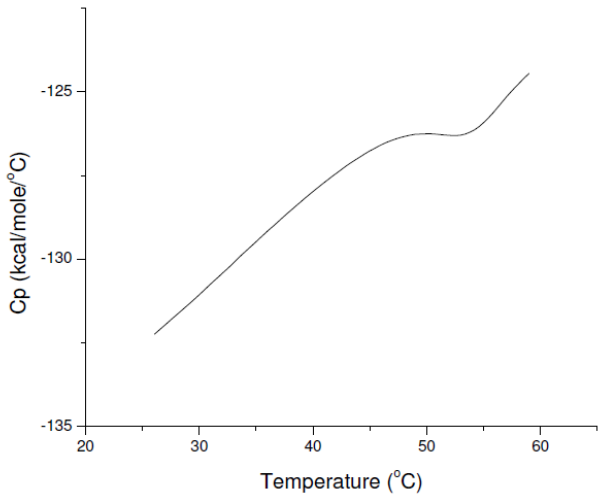
Figure 16. **Heat-denatured full length Pin1 loses ability to bind CDC25C-derived peptide.**

A. Normalized data for denaturing of full-length Pin1 using DSC. Protein sample was removed just prior to 60°C, to prevent protein precipitation.

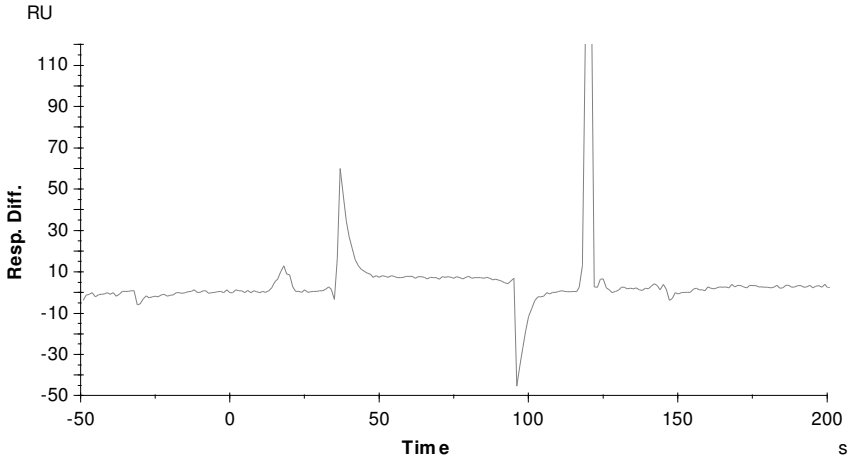
B. Sensorgram of SPR analyses of the interaction between heat-denatured Pin1 and 2xP. Heat-denatured Pin1 was injected through both flow cells for 1 minute. The trace shows the specific signal obtained after subtraction of background.

C. Fluorescence polarization assay for heat-denatured Pin1 binding to 2xP. Labelled peptide was incubated with indicated concentrations of Pin1 prior to measuring fluorescence polarization Data points represent the mean of 2 independent experiment with error bars indicating the standard deviation.

A.


Cp (kcal/mole/°C)
-125
-130
-135
20
30
40
50
60
Temperature (°C)

B.


RU
Resp. Diff.
110
90
70
50
30
10
-10
-30
-50
-50
0
50
100
150
200
Time
s

C.

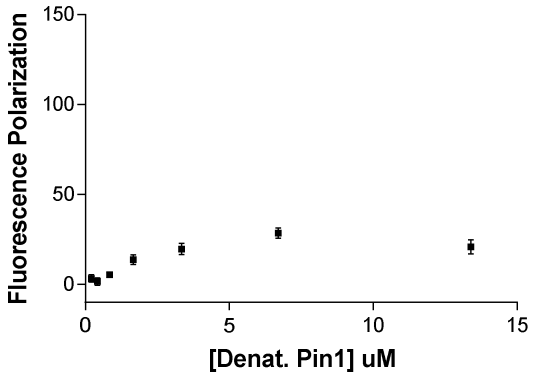
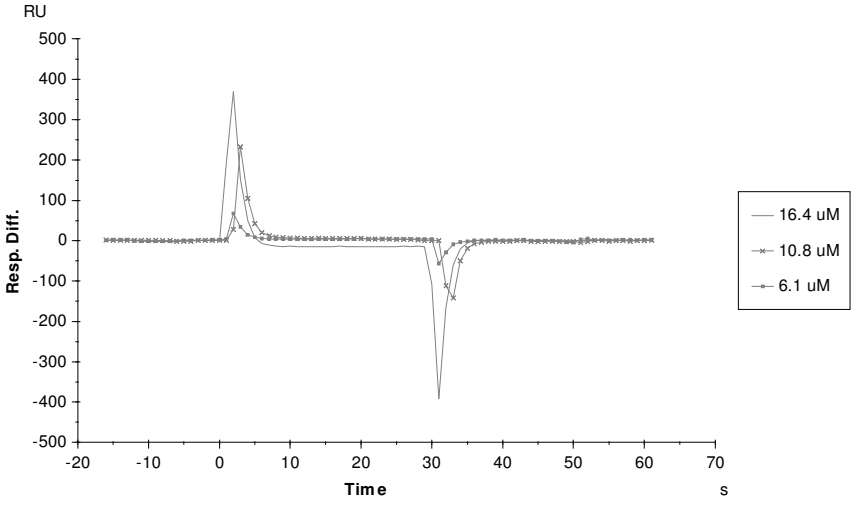

Fluorescence Polarization
150
100
50
0
0
5
10
15
[Denat. Pin1] uM

Figure 17. **GST-tagged Pin1 WW domain has binding capabilities, but will not bind in SPR assay.**

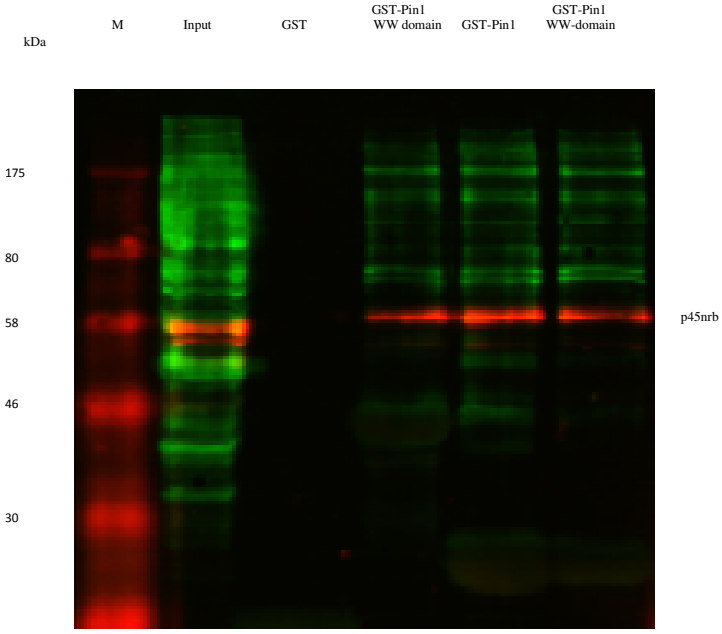
A. Sensorgram of SPR analyses of the interaction between GST-tagged Pin1 WW domain and 2xP. Indicated concentration of protein were injected through both flow cells for 1 minute. The trace shows the specific signal obtained after subtraction of background.

B. Mitotic HeLa cell lysates were used in GST and GST-Pin1 fusion protein binding assays. Proteins bound to the GST and GST-Pin1 beads were run on 10% SDS-PAGE gel and transferred to membranes. Blots were probed with antibody for MPM2 and NonO.

A.


RU
500
400
300
200
100
0
-100
-200
-300
-400
-500
Resp. Diff.
-20
-10
0
10
20
30
40
50
60
70
Time
s
16.4 uM
10.8 uM
6.1 uM

B.


M
Input
GST
GST-Pin1 WW domain
GST-Pin1
GST-Pin1 WW-domain
kDa
175
80
58
46
30
p45nrb

4.0 Discussion

The interaction between Pin1 and CDC25C was first identified from a screen of a subset of many mitotic phosphoproteins (28) and has since been characterized more extensively (12, 19, 28-30, 65-71). The interest in these two enzymes is no doubt fueled by the implications of their interaction; Pin1-catalyzed isomerization of CDC25C regulates its dephosphorylation and thus inhibition, preventing entry into mitosis (28, 30). This was an important discovery which identified Pin1 as an important mitotic regulator, and provided evidence of mechanisms for post-phosphorylation regulation. One of the key features in understanding the interaction between Pin1 and CDC25C stems from the structure of Pin1 as a dual-domain isomerase; having a WW domain and a PPIase catalytic domain which can both bind to the pSer/Thr-Pro motif (9, 12), raises the question of how Pin1 uses each domain when binding to, and subsequently catalyzing isomerization of, its interactors. With regards to CDC25C, the suggestion that full-length Pin1 was required for the interaction (71) led to the hypothesis that binding was being facilitated by both domains, simultaneously.

4.1 Pin1 interacts with CDC25C

As a first step towards elucidating the mechanism of binding between Pin1 and CDC25C, we sought to confirm their known interactions. To this end, and for subsequent assays, protein purifications were performed and sufficient amounts of GST-tagged and untagged versions of Pin1 were obtained. Following GST-pulldown assays, and consistent with previous research (28, 71), we found that CDC25C interacts with Pin1 in a manner that appears to require the presence of both intact domains. To further investigate both the

domain and phosphorylation requirements that facilitate this interaction, we choose to employ a peptide-based strategy, utilizing two independent assays. Such peptide-based approaches to investigate the interaction between Pin1 and CDC25C (human-, and *Xenopus*-derived) are found widely in the literature (12, 19, 67-70). In addition, a bivalent peptide target has previously been shown to have far greater affinity for Pin1 when compared to a monovalent target (78). However, a study that combines an investigation of the domain requirements of Pin1 with an analysis of the impact of the number of phosphorylated binding sites has not been published. As a result, our use of a multiphosphorylated human CDC25C-derived peptide is unique.

4.2 The interaction between Pin1 and CDC25C peptide is phosphorylation-dependent

Despite having multiple sites which would, upon phosphorylation, meet the requirements for the pSer/Thr-Pro Pin1 binding motif, T48 and T67 have been identified as the Pin1 binding sites (12, 30). Our binding results with SPR and fluorescence polarization show that the interaction between Pin1 and our peptide is phosphorylation-dependent. The loss of binding to a non-phosphorylated target is comparable to that seen in studies with full length protein, whereby mutations of T48 and T67 to non-phosphorylatable residues results in loss (or near loss) of detectable binding (30, 77).

The dissociation constants calculated as a result of our SPR and fluorescence polarization assays with Pin1 and 2xP are 1.5 μM and 1.7 μM respectively. These values are not dramatically different from those found by Daum *et al.* (78) whose bivalent ligands had dissociation constants of 0.4-0.8 μM when measured with isothermal titration calorimetry. Perhaps this is an unfair comparison however, given that the peptides used in

the Daum *et al.* study (78) were designed to specifically target both the Pin1 WW and PPIase domains, and thus, bound in a specific orientation. Additionally, those peptides contained only a single phosphorylated residue, and did not resemble a natural Pin1 target sequence. It would therefore be expected that such engineered peptides would bind with somewhat higher affinity than those used in the present study.

Similar to our study, previous work has used multiphosphorylated peptide approaches to investigate the binding affinity between Pin1 and physiologically relevant targets. Verdecia *et al.* (19) used florescence polarization to determine the binding affinity between Pin1 and a peptide derived from the c-terminal domain of RNA polymerase II (a known Pin1 interactor). Their peptide sequence, containing two phosphorylation sites, was significantly shorter than ours, and had phosphorylation sites which were separated by only two amino acid residues (Table 2). These factors may account for their higher dissociation constant of 10 μM. A systematic approach to test this hypothesis would be necessary, as it may be a contributing factor to the differences in binding affinities between our results and those previously published. An additional point of interest lies in the fact that the phosphorylated residues in the Verdecia *et al.* (19) peptides were serine residues. It has been previously shown that the pSer-Pro motif binds with lower affinity compared to the pThr-Pro motif, possibly a result of subtle conformational differences between the two sequences (79, 80). Similarly, Smet *et al.* (37, 81) characterized the interaction between Pin1 and multiphosphorylated peptides derived from Tau and also obtained high dissociation constants. One of their shorter peptides, with 14 residues and only two amino acids between each of two phosphorylated residues (Table 2) had a high dissociation constant of 160 μM (81). However, with a much longer

Table 2. **Comparison of peptides used in the literature, with one used in the present study.**

Peptide Sequence	Peptide Length (number of residues)	Number of intervening residues between phosphorylation sites	Citation
Y**pS**PT**pS**PS	7	2	(19)
SRSR**pT**P**pS**LPTPPTR	14	1	(81)
GSPGTPGSRSR**pT**P**pS**LPTPPTREPKKVAVVR**pT**PPKSPSSAK	40	1, 16	
PDVPR**pT**PVGKFLGDSANLSILSGG**pT**PKRSLDWAAAC	36	18	Current study

peptide (comprising 40 residues, 3 of which were phosphorylated) (Table 2) a dissociation constant of 70 μM was determined. Interestingly, two of the three phosphorylated residues in this longer peptide were spaced 16 amino acids apart, suggesting that the greater distance between phosphorylated residues may have contributed to enhanced binding by Pin1. This hypothesis of cooperativity between phosphorylated sites leading to enhanced binding has previously been proposed (37, 74), as a result of the identification of Pin1 interactors with multiple pSer/Thr-Pro sites, which are often spaced 18-22 amino acids apart. As our peptide meets this criterion, it is possible that such spacing contributed to our relatively strong binding. Taken together, the data presented above suggests that the determinants of Pin1 binding lie in optimal number and positioning of phosphorylated residues. Future work could include sequence analyses of interactors containing multiple Pin1 binding sites to determine if a pattern of recognition exists beyond a single pSer/Thr-Pro site, to include both pSer/Thr-Pro sites and the intervening residues. This knowledge may help identify additional Pin1 interactors, and will likely be valuable for the design of inhibitors of Pin1.

4.3 Effect of number of phosphorylation sites on Pin1-CDC25C peptide interaction

To further investigate the impact of number of phosphorylation sites on Pin1 binding, we designed singly-phosphorylated peptide variants of our original peptide, and tested them for interaction with full length Pin1. Although these peptides appeared to show some binding when using SPR, these results could not be confirmed using fluorescence polarization. As discussed previously (section 3.4) there are confounding factors with both assays which could have contributed to the results obtained, and a systematic

approach to assessing these factors would be an appropriate next step. This would be of great significance, given that singly-phosphorylated peptides derived from CDC25C have been previously shown to have varying binding affinities for Pin1 (19). Of those peptides, one included the same phosphorylated residue as our 1xP#1 (T48), and had a dissociation constant of 4.9 μM, measured using fluorescence polarization (19). That peptide, however, was significantly shorter (six residues) than the ones used in our study. Given that the WW domain of Pin1 recognizes up to five consecutive residues in a peptide (19), and that the PPIase domain has been shown to optimally bind peptides that are five residues in length (10, 82), a short (six residue) peptide would likely bind either the WW or PPIase domain of Pin1 with relatively high affinity. When used in a fluorescence polarization assay, this short, tightly bound peptide, would likely retain little mobility, and polarization of light would be greater. In contrast, if either the WW or PPIase domain of Pin1 were to bind to the single phosphorylated residue on 1xP #1 or 1xP #2, including two residues on either side, it may not stabilize the remainder of the peptide sufficiently to cause polarization of light. This may have contributed to the apparent low binding we saw with Pin1 and either of the singly phosphorylated peptides when assaying fluorescence polarization.

Collectively, our data show that singly-phosphorylated targets appear to bind with lower affinity than our doubly-phosphorylated peptide. This finding is consistent with previous research conducted with peptides derived from other Pin1 interactors. Results using peptides derived from the c-terminal domain of RNA polymerase II show that a doubly-phosphorylated peptide has the greatest binding affinity when compared to singly-phosphorylated versions (19). Similarly, using Tau-derived peptides, Smet *et al.* (37, 81)

found that a second phosphorylated residue increased the affinity of Pin1 for the peptide. Interestingly, they also found that the additional phosphate also decreased the isomerase activity of Pin1 (81). It would be interesting to test the impact of the second phosphorylated residue using our CDC25C-derived peptides, to determine if the inhibitory effect seen with Tau applies to other Pin1 substrates as well.

4.4 Binding of Pin1 to CDC25C targets requires both domains

Given the somewhat inconclusive results obtained with Pin1 and the singly-phosphorylated peptides, we next chose to investigate the possibility of single-domain binding from an alternative perspective, using binding-deficient domain-mutants of Pin1. We hypothesized that binding of wild-type Pin1 to singly-phosphorylated targets should be able to be replicated using domain-mutants of Pin1, given that binding to a singly-phosphorylated peptide may be occurring using a single domain only. To this end, we used binding-deficient mutants of Pin1, with Y23A and R68/69A mutations in the WW and PPIase domain respectively, and measured their binding to the doubly phosphorylated peptide. Similar to the data we presented in section 3.4, we observed a discrepancy in the results between SPR and fluorescence polarization. While results from SPR suggest that both domain mutants bind the peptide, albeit with lower affinity than wild-type, fluorescence polarization data indicates a weak, possibly non-specific, interaction. Given that the mutants would likely be binding the doubly-phosphorylated peptide at only one site, we hypothesize that this may be insufficient to reduce the mobility of the lengthy peptide, and therefore polarization of light would not occur.

Overall, although there are unresolved discrepancies between our data generated with SPR and fluorescence polarization, it seems that full length Pin1 has the greatest binding

affinity when both domains are intact, and when the target peptide sequence is doubly-phosphorylated.

4.5 Individual Pin1 domains are not sufficient to bind to CDC25C target peptide

Our final step in assaying the interactions between Pin1 and our CDC25C-derived peptide was to test the binding ability of the individual domains of Pin1. The results we obtained indicate a lack of binding between either of the domains and the doubly-phosphorylated peptide. This supports our hypothesis that both intact domains are required for binding. However, previous studies have shown that both domains of Pin1 have the ability to bind to target peptides with varying affinities. More specifically, although structures determined using x-ray crystallography have been solved with peptides bound to the PPIase domain of Pin1 (9, 10), a peptide-based binding analysis similar to ours determined that the PPIase domain exhibited no detectable binding to multiple peptides (19). The few peptides which did bind the PPIase domain exhibited binding affinities in the range of 85 to over 500 μM (19). These results, indicating relatively poor binding by the PPIase domain, are consistent with ours, and are also in agreement with *in vitro* studies which show that the PPIase domain of Pin1 is not able to bind phosphoproteins (12, 71). The catalytic function of the PPIase domain suggests that it must have some binding affinity towards substrates, however, it appears to be difficult to measure these interactions. This may be a result of substrates having only transient interactions with this domain (82), given the active site appears to be "primed" for catalysis, even without bound substrate (83). With regards to the WW domain of Pin1, peptides derived from various known Pin1 interactors have been shown to bind with

dissociation constants ranging from 2 to 125 μM (12, 19, 67, 68). The relatively large range of dissociation constants (similar to those obtained for interactions with the PPIase domain) suggests that binding affinities are dependent upon both peptide sequence and length (both of which varied in the sampled studies), and overall, that in some cases individual domains may not be sufficient for binding. Innes *et al.* (71) presented data to suggest that the domain requirements for binding differ depending on the interactor; some interactors (for example, RNA binding protein $p54^{nrb}$) require only the WW domain, while others (for example, protein kinase PLK1), appear to require both domains of Pin1. There have been multiple other interactors which have been shown to require both domains of Pin1 for interaction and subsequent function, including the transcription factor c-Jun (24), tumor suppressor protein p53 (34), transcription activator β-catenin (25), and protein kinase CK2 (73). Collectively, our results suggest that CDC25C is another protein which requires, and binds optimally to, both domains of Pin1.

4.6 Lack of binding is not due to misfolded proteins

Given the somewhat contradictory results we generated, we sought to ensure that the proteins we used in SPR and fluorescence polarization assays were functional in alternative assays. Constructs containing the PPIase domain were assessed based on their isomerase activity, and were found to have activities within the range of data previously published (12, 15, 77). If our constructs were in the correct conformation to bind and isomerize a substrate in an *in vitro* isomerisation assay, we conclude that they must also be in the correct conformation to facilitate binding to our peptides. As a result, we conclude that any lack of binding by any of our constructs is not due to a misfolded

protein. To further this point, our heat-denatured Pin1 showed no binding in either SPR or fluorescence polarization assays.

Without an activity assay to test the function of the Pin1 WW domain, we chose to test its binding capacity as a fusion construct with GST. Our GST-WW domain had the ability to bind varying interacting partners in a GST pulldown assay, similar to those previously published (12, 71). However, the same GST fusion construct failed to bind our peptide via SPR. We propose that this difference in binding capacity is a result of our specific CDC25C-derived peptide; as explained in section 4.5, binding appears to require the presence of both domains of Pin1.

Overall, the results of these assays demonstrate that our proteins retain function in various alternative assays, and a lack of binding to our peptides is not a result of a misfolded construct.

4.7 Model of Pin1 binding to CDC25C

As reviewed in Section 1.6, there are multiple models to describe the interactions between Pin1 and its binding partners. Given the results presented here, that interactions between Pin1 and CDC25C appear to require the presence of both, intact domains, we suggest that neither the multimeric nor catalysis-first models adequately fit the data. Additionally, given that the individual domains of Pin1 lack the ability to bind both our CDC25C-derived peptides and full-length CDC25C (71), we propose that the sequential binding model is similarly a poor fit, as this model would require initial targeting by the WW domain. As a result, the simultaneous binding model appears to be best suited to explain the interactions seen. As previously discussed, this model suggests that although

individually, two pSer/Thr-Pro sites may be poor binding sites for either domain of Pin1, the simultaneous binding of both sites by both domains results in a higher affinity interaction (71). As reviewed in Section 4.3 there are additional examples to demonstrate that binding affinity is increased with a doubly-phosphorylated peptide. These example peptides may or may not be interacting with Pin1 in a simultaneous manner, and an examination of the affinity each has with the WW and PPIase domains would help to determine this. However, given that our results show relatively weak affinity for each of the singly-phosphorylated peptides, yet relatively high affinity with the doubly-phosphorylated peptide, it appears that interactions with CDC25C are occurring in a simultaneous manner. These results support the suggestion made by Innes *et al.* (71) that CDC25C is an interactor that binds according to the simultaneous model.

Considering that there is evidence that Pin1 may not interact with all its binding partners in the same way, or by the same model (71), a next step would be to investigate what determines and/or regulates these differences. Although it is beyond the scope of this work to speculate on the specifics of this, previous work by Jacobs *et al.* (72) showed that interactions between the domains of Pin1 changed depending on the sequence of the bound peptide. These inter-domain interactions may serve to coordinate binding of multiple sites simultaneously, which would otherwise not be bound with high affinity. Additionally, as there may be sequence determinants that facilitate each model of binding, a thorough investigation to compare interactors would be appropriate.

4.8 Future directions

As noted previously, there are still outstanding issues to address, as well as additional experiments that can further the present study. Firstly, with regards to our suggestion that

the length of our peptides could have been a confounding factor in our fluorescence polarization assays, shorter peptides should be used in additional experiments. These shorter peptides may have less mobility once bound by Pin1, and as such, may provide a more accurate representation of binding.

Given the suggestion that peptide length, as well as the number of residues between the phosphorylation sites, may have contributed to differences between our data and those previously published, an investigation into how these factors contribute to binding by Pin1 would be of interest. To this end, a sequence analysis of residues between binding sites may reveal a pattern of residues, or an optimal length, both of which may help to further classify Pin1 interactors based on the modes of binding.

To confirm the data generated in this study, additional assays using our peptides could be performed. Isothermal titration calorimetry may provide more precise binding data, and thus resolve some of the discrepancies with the current data. In addition, sedimentation equilibrium assays would ensure that interactions between Pin1 and our peptides were occurring in a 1:1 ratio.

Finally, as mentioned previously, CDC25C is one of multiple Pin1 interacting proteins that have more than one identified Pin1 binding site, and these additional interactors may bind to Pin1 in a similar fashion to CDC25C, using the simultaneous model. One such protein of interest is the microtubule binding protein Tau. The interaction between Pin1 and Tau has already been a subject of research (30, 36, 37, 81, 84), given the implications their interaction has with regards to contributing to Alzheimer's disease. Similar to CDC25C, Tau has two identified Pin1 binding sites, which are separated by nineteen

amino acids (T212 and T231) (36, 37), and based on the similarities between previous Tau peptide work discussed in sections 4.2 and 4.3 and our own data, it may be possible that Tau is another interactor to which Pin1 binds via the simultaneous model. As peptide studies have already been performed, *in vitro* studies would be a next step to confirm the interaction seen. To this end, we performed preliminary GST-pulldown experiments to test the interactions between full-length Pin1 and Tau. These experiments and their results are presented in Appendix 1. Although these pulldown results should be confirmed with additional assays, it appears that the binding requirements of Tau are similar to those of CDC25C. Taken together with previously published Tau-derived peptide studies, the data suggest that Tau may another interactor to which Pin1 binds via the simultaneous model.

4.9 General summary

The objective of this study was to elucidate the mechanism with which Pin1 interacts with a key mitotic trigger, CDC25C. Despite the caveat that our results display some discrepancies, some important conclusions can still be made. Firstly, the binding of Pin1 to our peptides was phosphorylation-dependent. Additionally, Pin1 bound to a doubly-phosphorylated peptide with higher affinity than either of the singly-phosphorylated peptides. This result suggested that binding to our doubly-phosphorylated peptide was facilitated using both domains of Pin1 simultaneously, each binding a single phosphorylation site. An investigation to determine the binding ability of the individual domains resulted in lack of binding. Furthermore, mutant versions of Pin1 with domain-specific binding deficiencies bound with lower affinity than wild-type Pin1. These results provide additional evidence that interactions with human CDC25C appears to require

both, intact domains of Pin1. Finally, to ensure that our binding results were not being affected by misfolded proteins, additional assays were performed, and ensured all proteins were functional in other (binding-dependent) capacities. Collectively, these results are consistent with the simultaneous model of binding, in which Pin1 binds CDC25C with both domains at the same time.

The existence of multiple models describing the interaction between Pin1 and its targets is perhaps not surprising given the structure of Pin1 as a dual-domain binding protein. And although our results suggest that Pin1 binds CDC25C via the simultaneous model, evidence in support of other models still exists. It is becoming more evident that Pin1 interacts with different proteins in different ways. This dynamic nature of Pin1 is intriguing from many perspectives, and will likely serve to guide further work with respect to the structure and function and Pin1, and its potential as a therapeutic target.

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Appendices

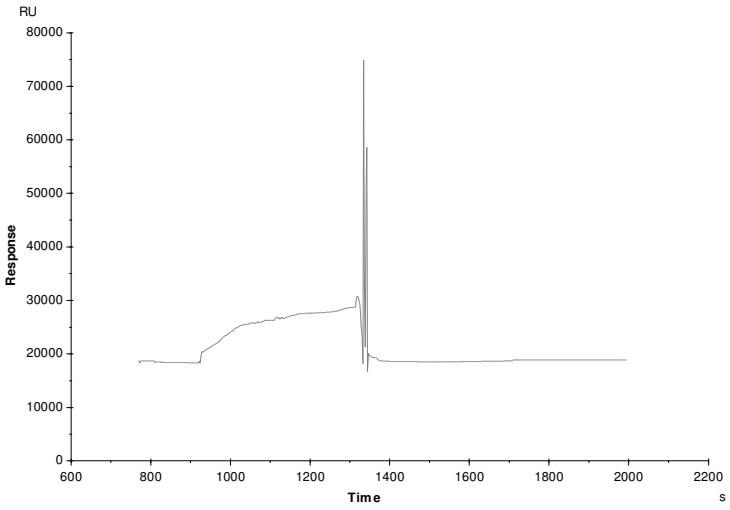
Appendix A. Spikes in sensorgrams are relatively common, and can be attributed to bubbles

The sensitivity of the Biacore instrument makes it susceptible to even the smallest of impurities in buffer and sample solutions. As a result, filtering and de-gassing of all buffer solutions is necessary. However, the introduction of bubbles during the injection process can still occur. Bubbles in the system display as large spikes in the resulting sensorgram as shown in Figure A1.

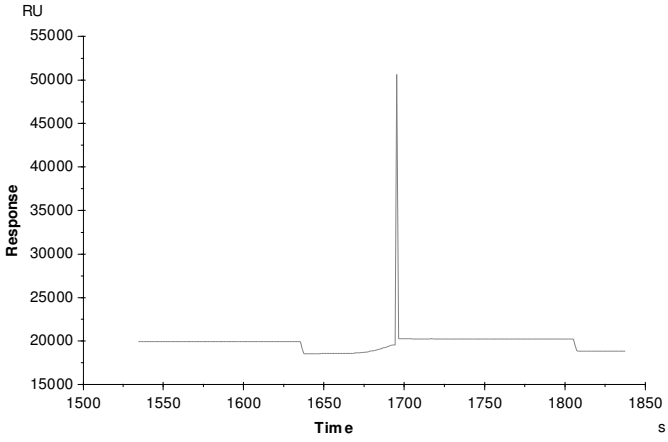
Figure A1. **Spikes in SPR sensorgrams can be attributed to bubbles in the system.**

A. B. and C. Sensorgrams of SPR analyses showing spikes in responses, attributed to the presence of bubbles in the system.

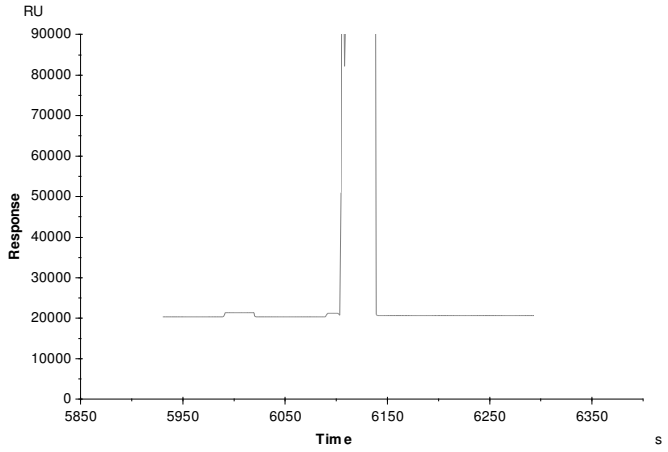
A.


RU
80000
70000
60000
50000
40000
30000
20000
10000
0
Response
600
800
1000
1200
1400
1600
1800
2000
2200
Time
s

B.


RU
55000
50000
45000
40000
35000
30000
25000
20000
15000
Response
1500
1550
1600
1650
1700
1750
1800
1850
Time
s

C.


RU
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
Response
5850
5950
6050
6150
6250
6350
Time
s

Appendix B. Preliminary data to characterize interactions between Pin1 and Tau

As previously discussed, we chose to begin investigating the interaction between Pin1 and Tau. Given the similarities between our peptide work and those previously published using peptides derived from Tau (34, 78), we hypothesized that Tau could be another interactor to which Pin1 binds via the simultaneous model.

Tau is endogenously expressed in multiple isoforms, which differ in length as a result of the presence or absence of (sometimes multiple) binding domains (see Figure A2A). In addition to the two identified Pin1 binding sites, Tau contains 13 sites which, when phosphorylated, match the Pin1 binding sequence, pSer/Thr-Pro (Figure A2B). Some of these sites are located within the repeated domains of Tau, and as a result, we chose to use a long isoform of Tau, 2N4R, so as not to preclude binding by Pin1 to other sites.

To investigate the interaction, we performed GST pulldowns (as previously described) using mitotic HeLa cell lysates. Cells had been transiently transfected with wild-type EGFP-Tau, or either single or double phosphorylation site mutants (T212A, T231A, or T212/231A respectively). Proteins that bound to GST or GST-Pin1 fusion proteins were examined by immunoblotting with GFP antibody. The results shown in Figure A3 demonstrate that Tau follows the same pattern of binding to Pin1 as endogenous CDC25C, with the R68/69A mutant of Pin1 showing a decreased interaction. Mutations of either of the Pin1 binding sites resulted in significantly decreased binding, as did the double mutant.

These results are similar to those seen for CDC25C (compare Figure A3 with Figure 7), suggesting that Tau may be another interactor of Pin1 which binds via the simultaneous model.

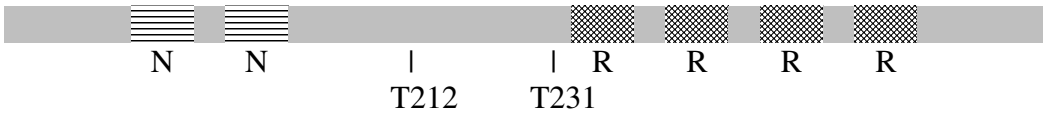
Figure A2. **Illustration of Tau isoform used in this study.**

A. Representation of Tau isoform 2N4R used in this study. This isoform of Tau is a 441 amino acid protein, consisting of 2 repeats of the N domain, and 4 repeats of the R domain. The approximate locations of identified Pin1 binding sites are shown.

B. Human Tau isoform 2N4R protein sequence is shown, with identified Pin1 binding sites shown in bold with underlining. Additional sites which could (upon phosphorylation) meet Pin1 binding requirements are shown in bold only. Amino acid numbers are indicated on the left. (UniProt P10636-8)

A.

Tau – 2N4R 441 a.a.



B.

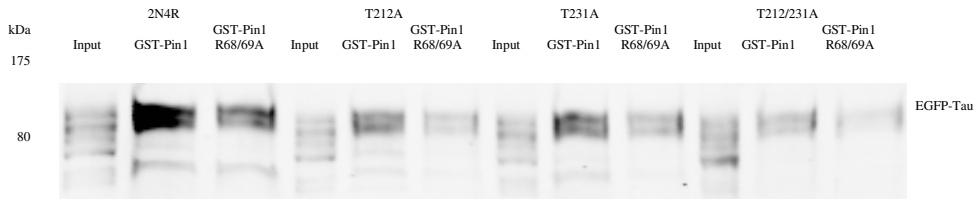
1	maeprqefev	medhagtygl	gdrkdqggyt	mhqdqegdtd	aglke**sp**lq**t**	**p**tedgseepg
61	setsdaks**tp**	taedvtaplv	degapgkqaa	aqphteipeg	ttaeeagigd	**tp**sledeaag
121	hvtqarmvsk	skdgtgsddk	kakgadgktk	ia**tp**rgaapp	gqkgqanatr	ipak**tp**papk
181	**tp**pssgeppk	sgdrsgys**sp**	g**sp**gtpgsrs	r**tp**slp**tp**pt	repkkvavvr	**tp**pk**sp**ssak
241	srlqtapvpm	pdlknvkski	gstenlkhqp	gggkvqiink	kldlsnvqsk	cgskdnikhv
301	pgggsvqivy	kpvdlskvts	kcgslgnihh	kpgggqvevk	sekldfkdrv	qskigsldni
361	thvpgggnkk	iethkltfre	nakaktdhga	eivyk**sp**vvs	gdt**sp**rhlsn	vsstgsidmv
421	d**sp**qlatlad	evsaslakqg	l			

Figure A3. **GST-pulldowns with wild-type and phosphorylation site mutants of Tau 2N4R**

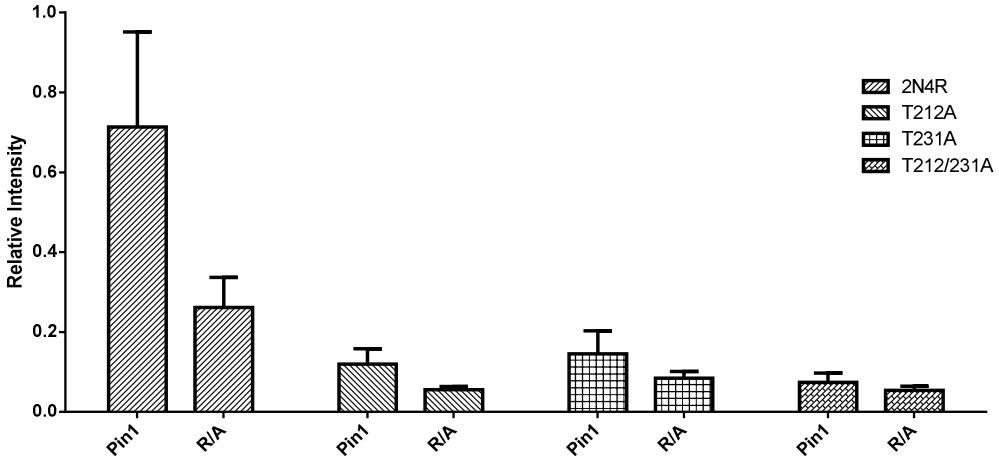
A. Mitotic HeLa cell lysates were used in GST-Pin1 fusion protein binding assays. Cells were transfected with EGFP-Tau constructs, expressing wild-type Tau (isoform 2N4R) or phosphorylation site mutants, T212A, T231A, or T212/231A. Proteins bound to the GST-Pin1 beads were run on 10% SDS-PAGE gel and transferred to membranes. Blots were probed with antibody for EGFP.

B. Quantification of blots shown in figure A. Results are the mean of 3 independent pulldown experiments, with error bars indicating the standard deviation.

A.


kDa
2N4R
T212A
T231A
T212/231A
Input
GST-Pin1
GST-Pin1 R68/69A
175
80
EGFP-Tau

B.


Relative Intensity
1.0
0.8
0.6
0.4
0.2
0.0
2N4R
T212A
T231A
T212/231A
Pin1
R/A

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