

BrdU. After 12 hours, cells were infected with adenoviruses in fresh medium containing 1  $\mu$ M BrdU. Cells were collected onto cover slips at 24 hours after infection, fixed for 1 hour at  $-20^{\circ}\text{C}$  in 70% ethanol and 50 mM glycine (pH 2.5), and processed for TUNEL labeling. Subsequently, BrdU incorporation was visualized with a Boehringer BrdU Labeling and Detection Kit #1 with a TRITC-coupled secondary antibody (Jackson). Based on the BrdU incorporation, infection with AdTRF2 and AdTRF2 $\Delta$ BM resulted in 55% and 83% inhibition of S phase, respectively.

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## Function of WW Domains as Phosphoserine- or Phosphothreonine-Binding Modules

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Protein-interacting modules help determine the specificity of signal transduction events, and protein phosphorylation can modulate the assembly of such modules into specific signaling complexes. Although phosphotyrosine-binding modules have been well-characterized, phosphoserine- or phosphothreonine-binding modules have not been described. WW domains are small protein modules found in various proteins that participate in cell signaling or regulation. WW domains of the essential mitotic prolyl isomerase Pin1 and the ubiquitin ligase Nedd4 bound to phosphoproteins, including physiological substrates of enzymes, in a phosphorylation-dependent manner. The Pin1 WW domain functioned as a phosphoserine- or phosphothreonine-binding module, with properties similar to those of SRC homology 2 domains. Phosphoserine- or phosphothreonine-binding activity was required for Pin1 to interact with its substrates *in vitro* and to perform its essential function *in vivo*.

Interactions through specific protein modules help determine the specificity of signal transduction events (1, 2). These modules, such as SH2 domains (3), are small conserved domains that bind specific sequences in target proteins and recruit proteins into signaling complexes. Serine or threonine phosphorylation appears to regulate the formation of protein complexes (2) and acts as a signal to trigger ubiquitination and subsequent degradation of a wide range of regulatory proteins (4). With the exception of phosphoserine (pSer)- or phosphothreonine (pThr)-binding proteins 14-3-3 (5), small independent pSer- or pThr-binding modules reminiscent of SH2 domains have not been described. Similarly, it is not known whether phosphoproteins directly interact with ubiquitin-protein ligases (E3), enzymes that mediate substrate recognition in the ubiquitin-mediated pathway (6).

WW domains contain 38 to 40 amino acid

residues in a triple-stranded  $\beta$  sheet (7–10). These small modules are found in proteins that participate in cell signaling or regulation, including the peptidyl-prolyl isomerase (PPIase) Pin1 and the ubiquitin ligase Nedd4 (7–10). WW domains have been implicated in mediating protein-protein interactions by binding to Pro-rich motifs (10). However, bona fide Pro-rich motifs are not found in many potential WW domain targets, such as Nedd4 substrates (11) and Pin1-binding proteins (12–14). Pin1 is an essential regulator at mitotic entry that binds a defined subset of mitosis-specific phospho-

proteins, including the phosphatase Cdc25C, kinases Myt1 and Plk1, and the small guanosine 5'-triphosphate-binding protein Rab4 (12–14). Thus, the role of WW domains remains to be determined.

We examined whether WW domains might mediate protein-protein interactions by binding pSer or pThr. Glutathione S-transferase (GST)-fusion proteins containing Pin1, its NH<sub>2</sub>-terminal WW domain (amino acids 1 to 54), or COOH-terminal PPIase domain (47 to 163) were incubated with extracts from interphase or mitotic HeLa cells. Bound proteins were detected by the monoclonal antibody MPM-2 that recognizes the overlapping subset of Pin1-binding proteins, or by antibodies to specific proteins (15). Although no specific binding was detected for the PPIase domain or control GST, WW domains of Pin1 and its yeast homolog Ess1 bound almost all of Pin1-binding proteins present in mitotic extracts (Fig. 1, A and B).

To test whether WW domain binding is phosphorylation-dependent and whether it is mediated by specific pSer or pThr residues in Pin1 target proteins, we analyzed binding to Cdc25C (16) because Pin1 interacts with Cdc25C just before entry into mitosis and regulates its activity (13). Both Pin1 and its WW domain bound the mitotically phosphorylated, but not the interphase phosphorylated, form of Cdc25C (Fig. 2A). Furthermore, they failed to bind Cdc25C if the mitotically phosphorylated Cdc25C was dephosphorylated (Fig. 2A). These results demonstrate a phosphorylation-dependent interaction between the WW domain and Cdc25C. Pin1 and its WW domain were found to bind two conserved pThr-Pro sites (Thr<sup>48</sup> and Thr<sup>67</sup>) in Cdc25C, as identified by

**Table 1.** Binding constants of WW domains and peptides. The NH<sub>2</sub>-terminus of peptides was labeled with fluorescein and purified by thin-layer chromatography. Various concentrations of GST-WW domains and control GST were incubated with the labeled peptides, and dissociation constants were measured by fluorescence anisotropy assay. Each value (in micromolar) represents the average of the three independent experiments. No binding was detected between GST and any peptides. NB, no binding detected.

WW domain	Peptide		Cdc25 peptide		Pro-rich peptide
	WFYpSPFLE	WFYSPFLE	EQLPtPVTDL	EQPLTPVTDL	IPGTPPPNYD
Pin1	1.0	NB	2.2	NB*	NB
Nedd4	10.0	NB	20.0	NB*	>40† (47–118‡)

\*No binding detected by incubating the GST-WW domain with the peptide immobilized on a membrane, followed by immunoblotting analysis with antibody to GST (17). †An estimated  $K_d$  because binding did not reach the plateau even at 100  $\mu$ M WW domain. ‡Previously reported  $K_d$ 's for the interaction between the Yap WW domain and various Pro-rich peptides (9). Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

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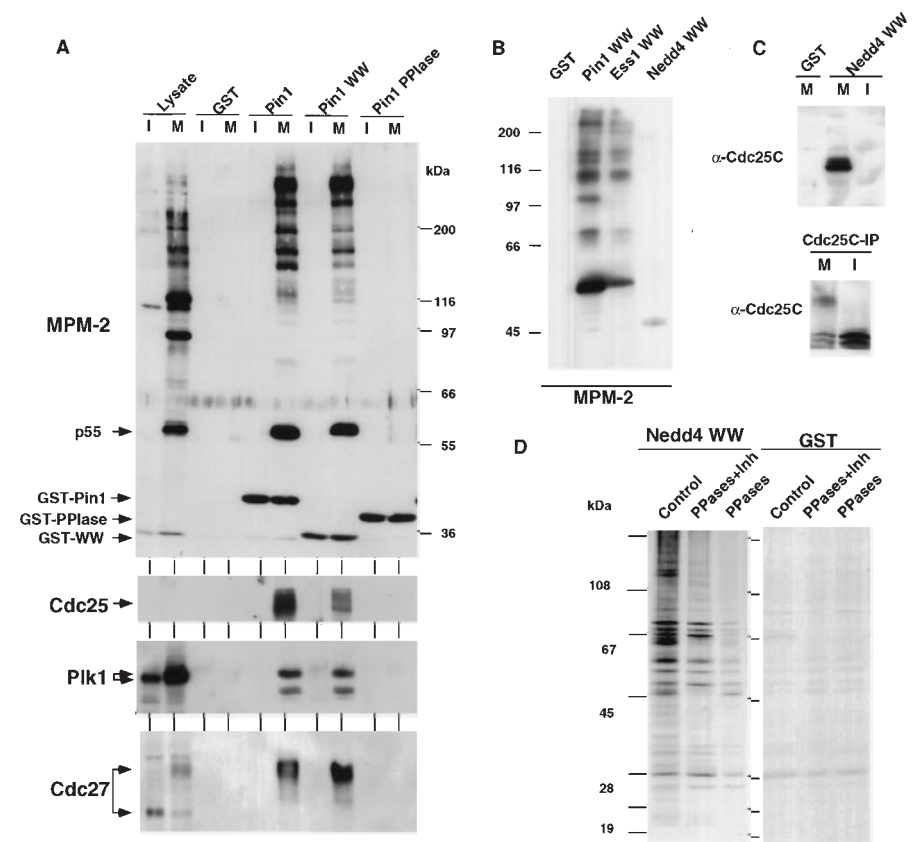
**Table 2.** Functional properties of the WW domain mutants. The phosphoprotein-binding activity was assayed as described in the legend to Fig. 3. ++, normal binding; +/-, reduced binding; -, no binding. PPlase activity was assayed with the peptide substrate as described (12); activity of the wild-type protein was defined as 100%. The in vivo function of the mutants was assessed by their ability to rescue the temperature-sensitive *ptf1* mutant in yeast (Fig. 4).

Pin1 protein	Phospho-protein-binding activity	PPlase activity (%)	In vivo function
Pin1	++	100	+
WW domain	++	0	-
PPlase domain	-	90	-
Pin1 <sup>R14A</sup>	++	92	+
Pin1 <sup>S16A</sup>	++	96	+
Pin1 <sup>W34A</sup>	-	94	-
Pin1 <sup>Y23A</sup>	-	85	-
Pin1 <sup>Y23F</sup>	+/-	94	-

peptide scan (17). This is consistent with the finding that Pin1 cannot bind a Cdc25C triple Thr mutant (T<sup>48</sup>A,T<sup>67</sup>A,T<sup>138</sup>A) (13). Furthermore, phosphorylated Thr<sup>48</sup> peptide (EQPLpT-PVTDL) competed with Cdc25C for binding to the WW domain, whereas the nonphosphorylated control had no effect (Fig. 2C). The phosphorylated Thr<sup>48</sup> peptide also directly bound the WW domain (Table 1). Thus, the Pin1 WW domain binds specific pThr-Pro sequences present in Cdc25C.

We next investigated whether the WW domain binds other phosphoproteins through the same pSer- or pThr-binding pocket. To answer this question, Pintide (WFYpSPRLKK), which was identified to be the optimal Pin1-binding peptide (12), was used to compete with phosphoproteins for binding (18). The control peptides were the nonphosphorylated Pintide and an epithelial Na<sup>+</sup> channel-derived Pro-rich peptide (IPGTPPPNYD), which was thought to be a Nedd4 WW domain-binding site (10). Binding of Pin1 and its WW domain to either <sup>32</sup>P-labeled proteins or MPM-2 antigens was reduced by Pintide in a concentration-dependent manner, whereas neither the nonphosphorylated Pintide nor the Pro-rich peptide had any effect (Fig. 2, D to F). Thus, the WW domain appears to bind phosphoproteins through the same pSer- or pThr-binding pocket.

We measured binding constants of the WW domain and phosphopeptides with a quantitative fluorescence polarization assay (18). To prevent nonspecific labeling, we used a Pintide analog (WFYpSPFLE), which was predicted to bind Pin1 with a high affinity (12). Although no binding was detected between Pin1 or its two domains and the nonphosphorylated peptide or the Pro-rich peptide, binding with the Pintide analog was readily detected (Table 1). Pin1 displayed two binding sites for Pintide with affinities (dissociation constants, *K<sub>d</sub>*'s) of 1.2



**Fig. 1.** Interaction between WW domains and subsets of phosphoproteins. (A) Interaction of the Pin1 WW domain and mitotic phosphoproteins. Interphase (I) and mitotic (M) HeLa cell lysates were incubated with glutathione beads containing control GST, GST-Pin1, its NH<sub>2</sub>-terminal WW domain, or COOH-terminal PPlase domain. After washing, proteins associated with the beads were subjected to immunoblotting analysis with MPM-2 antibody, or antibodies to specific proteins. Total lysates were used as control. (B) Interaction of WW domains and MPM2 antigens. GST or GST-WW domains of Pin1, Ess1, or Nedd4 were incubated with I or M HeLa extracts and washed. Bound proteins were detected with MPM-2. (C) Interaction between the Nedd4 WW domain and the mitotic Cdc25C in HeLa cells. (Top) Glutathione beads containing GST or GST-Nedd4 WW domain 2 were incubated with I or M HeLa cell extracts and washed. Immunoblotting analysis was done with antibodies to Cdc25C. (Bottom) Cdc25C immunoprecipitation (IP) and immunoblotting with the same antibodies as control. (D) Phosphorylation-dependent interaction between the Nedd4 WW domain and cellular proteins. Growing HeLa cells were incubated with [<sup>32</sup>P]orthophosphate or <sup>35</sup>S-Met, and soluble proteins were subjected to GST-WW domain 2 pull-down assay before (control) or after treatment with three Ser-Thr phosphatases (PPases) in the absence or presence (Inh) of phosphatase inhibitors (19).

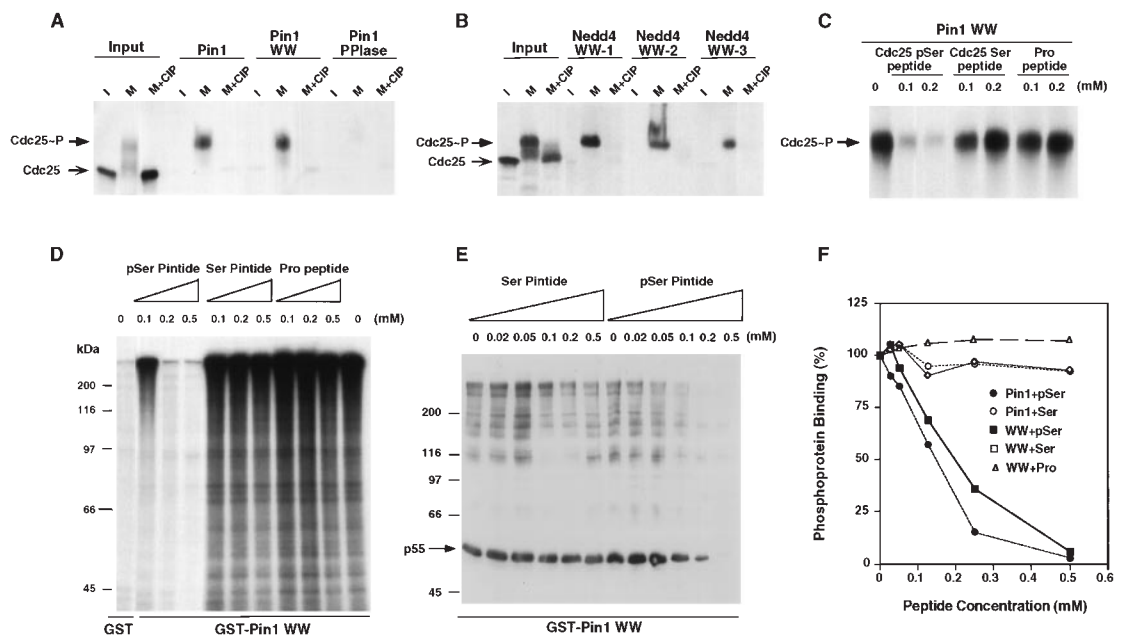
and 11.0 μM, which corresponded to those of the isolated WW domain and PPlase domain, respectively (Table 1). Similar binding constants were obtained between the WW domain and the phosphorylated Cdc25C peptide (Table 1). Collectively, the above results demonstrate that the Pin1 WW domain binds phosphopeptides and a subset of mitotic phosphoproteins through interacting with pSer or pThr.

To examine whether other WW domains also bind phosphoproteins, we chose WW domains of Nedd4, because it is an enzyme that ubiquitinates phosphoproteins, such as uracil permease and Cdc25C, that do not contain the typical Pro-rich motif (7, 11). Mouse Nedd4 contains three WW domains. In contrast to the Pin1 WW domain, the Nedd4 WW domain 2 only bound a few MPM-2 antigens (Fig. 1B). To detect interactions with other phosphoproteins, we incubated the Nedd4 WW domain 2

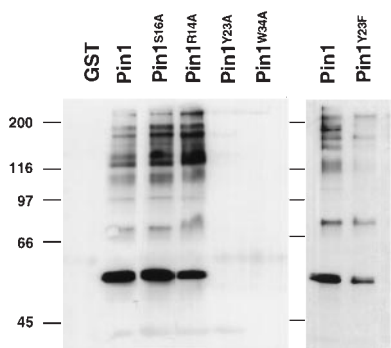
with <sup>32</sup>P- or <sup>35</sup>S-labeled proteins from cell lysates (19) and found binding to a subset of proteins (Fig. 1D). If lysates were treated with Ser phosphatases, binding of the WW domain to cellular proteins was reduced to one-tenth that in untreated extracts. However, binding was reduced only to about half that in control extracts if phosphatase inhibitors were included (Fig. 1D). These results indicate that Pin1 and Nedd4 WW domains interact with distinct subsets of cellular phosphoproteins in a phosphorylation-dependent manner. All three Nedd4 WW domains bound, to some extent, the mitotically phosphorylated form of synthesized Cdc25C, but not its interphase or dephosphorylated form (Fig. 2B). The Nedd4 WW domain 2 also bound the mitotic form of Cdc25C in HeLa cells (Fig. 1C) and showed phosphorylation-dependent binding to the Cdc25C peptide and Pintide (Table 1). The *K<sub>d</sub>* values for the

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**Fig. 2.** Phosphorylation-dependent interactions between WW domains and phosphoproteins. (A and B) The phosphorylation-dependent interaction between WW domains and Cdc25C. Cdc25C protein were synthesized by in vitro transcription and translation in the presence of <sup>35</sup>S-Met. The labeled protein was added into *Xenopus* I or M extracts or first mitotic extracts and then dephosphorylated with calf intestine phosphatase (M+CIP). Proteins were separated on SDS-containing gels either directly (input) or after GST pull-down experiments with various domains of Pin1 (A) or various WW domains of Nedd4 (B). (C to F) Competition of WW domain binding by phosphopeptides, but not by Pro-rich peptide. Pin1 or its WW domain was incubated with various concentrations of peptides as indicated, and then incubated with mitotically phosphorylated Cdc25C, <sup>32</sup>P-labeled mitotic extracts, or regular mitotic extracts. WW domain-binding proteins were separated on SDS-containing gels and detected by autoradiography or immunoblotting analysis with MPM-2. (C) Effects of phosphorylated or nonphosphorylated Cdc25C Thr<sup>48</sup> peptides or Pro-rich peptide on Cdc25C



binding; (D) effects of Pintide, its nonphosphorylated control, or Pro-rich peptide on cellular phosphoprotein binding; (E) effects of Pintide or its nonphosphorylated control on MPM-2 antigen binding; (F) a semi-quantitative presentation of the effects of Pintide, its nonphosphorylated control, or Pro-rich peptide on MPM-2 antigen binding, as determined by analyzing the density of p55 with Imagequant.

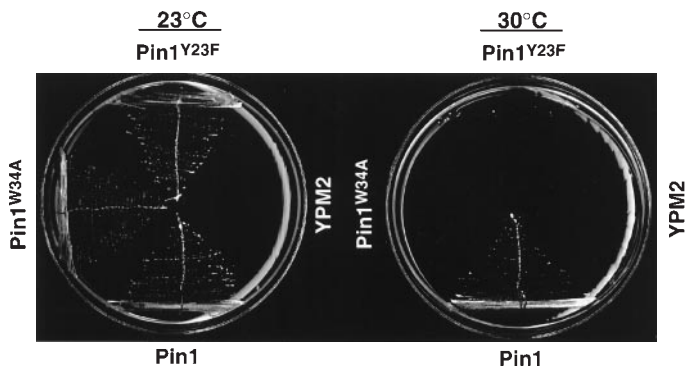


**Fig. 3.** Identification of the pSer- or pThr-binding pocket in the Pin1 WW domain. Glutathione agarose beads containing Pin1 or its various point mutants were incubated with mitotic extracts. The beads were washed and proteins associated were subjected to immunoblotting analysis with MPM-2. Although the intensity of MPM-2 staining varied in different lots of lysates, overall patterns were similar. Equal amounts of GST proteins were used in each lane.

phosphopeptides were lower than those for the Pro-rich peptide that was thought to be a Nedd4 WW domain-binding site (Table 1) (10). Thus, Nedd4 WW domains also bind pSer- or pThr-containing sequences.

In the Pin1 crystal structure, the WW domain contains a hydrophobic cluster sequestering a PEG molecule, which forms close contacts with Ser<sup>16</sup>, Tyr<sup>23</sup>, and Trp<sup>34</sup> located on three different strands of the  $\beta$  sheet (9). A hydrophobic patch at the molecular surface is

**Fig. 4.** Requirement of the phosphoprotein-binding activity of the WW domain for Pin1 function in vivo. Pin1 and its mutant cDNAs were subcloned in a yeast expression vector and transformed into the temperature-sensitive *PTF1* mutant strain YPM2. Those yeast strains expressing similar levels of transgenes, as detected by immunoblotting analysis with 12CA5 antibody, were transferred onto minimal media and incubated for 2 to 3 days at 23°C (the permissive temperature) or 30°C (the restrictive temperature).



usually conserved for protein-protein interaction, and a statistical analysis ranks the propensity of Tyr to bind the phosphate group of pSer or pThr next only to that of Arg (20). The hydrophobic cluster at WW domains may be a pSer- or pThr-binding pocket. We therefore constructed Pin1 proteins containing mutations at the above three residues and Arg<sup>14</sup>, a residue close to Tyr<sup>23</sup> in the structure (9), and tested their PPIase activity and binding to phosphoproteins or peptides. All the mutations in the WW domain did not affect PPIase activity of Pin1 (Table 2). A substitution of Arg<sup>14</sup> or Ser<sup>16</sup> with Ala did not alter binding (Fig. 3). In contrast, a single Ala point mutation of either Tyr<sup>23</sup> or Trp<sup>34</sup> completely abolished binding of Pin1 to phosphoproteins (Fig. 3). Similar effects were observed on binding of Pin1 to Pintide

(21). Substitution of Phe for Tyr<sup>23</sup> reduced binding of Pin1 to phosphoproteins (Fig. 3), demonstrating the importance of the Tyr hydroxyl group in mediating pSer or pThr binding. Similar Tyr-mediated pSer binding has been observed in the complex of phosphorylated CREB and CBP (22).

To address the function of the WW domain of Pin1 in vivo, we took advantage of the ability of human Pin1 to perform the essential function of *ESS1/PTF1* in yeast (8). Various Pin1 mutant cDNAs were expressed in similar amounts in a temperature-sensitive *ptf1* mutant strain (23). In contrast to Pin1, neither its NH<sub>2</sub>-terminal WW domain nor its COOH-terminal PPIase domain rescued the phenotype (Table 2). Furthermore, although those mutants that bound phosphoproteins completely rescued the *ptf1*

phenotype (Table 2), all Pin1 mutants, including Y23F, that did not bind phosphoproteins failed to support cell growth (Fig. 4). These results indicate that pSer- or pThr-binding activity of the Pin1 WW domain is required for the protein function in vivo.

We have demonstrated a function of WW domains as pSer- or pThr-binding modules and an essential role for WW domains in mediating protein-protein interactions. Serine or Thr phosphorylation, often on PEST sequences (rich in Pro, Glu, Ser, and Thr), controls the timing of ubiquitination of various proteins, and ubiquitin-protein ligases are responsible for substrate recognition (4). Our results indicate that WW domains of ubiquitin ligases may bind pSer- or pThr-containing sequences, thus targeting their catalytic domains to phosphorylated substrates to initiate protein degradation. Proline residues can put kinks into polypeptide chains because they undergo cis or trans isomerization catalyzed by PPLases (24). Phosphorylation reduces the isomerization rate of the pSer- or pThr-Pro bond, and Pin1 is a unique enzyme that isomerizes the pSer- or pThr-Pro bond and regulates activity of phosphoproteins (12, 13). The Pin1 catalytic domain neither interacts with protein substrates in vitro, nor performs the essential function of the protein in vivo. The WW domain binds pSer- or pThr-Pro-containing peptides and mediates Pin1 interactions with most substrates. One of its biological functions may be to facilitate the processive isomerization of Pin1 substrates. SH2 domains have similar functions. Some SH2 domains in Tyr kinases preferentially bind pTyr residues phosphorylated by the catalytic domain, thereby increasing the local kinase concentration so that substrates are processively phosphorylated on multiple sites (25). Because Pin1 substrates are regulated by phosphorylation of multiple Ser or Thr residues clustered at the regulatory domains (12–14, 26), isomerization of these sites may provide a means to generate coordinate “all-or-none” activity of heavily phosphorylated phosphoproteins.

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15. Pin1 and its mutants were produced, and their binding to phosphoproteins was assayed, as described (12, 13).
16. Cdc25C was synthesized by in vitro transcription and translation in the presence of <sup>35</sup>S-Met and phosphorylated by *Xenopus* interphase or mitotic extracts, and some mitotically phosphorylated samples were dephosphorylated with calf intestine phosphatase (CIP) before binding, as described (13).
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18. For peptide competition experiments, various peptides were incubated with GST-Pin1 or -WW domain in a binding buffer (13). After 1 hour of incubation, <sup>32</sup>P-labeled (21) or nonlabeled mitotic cell extracts were added. Proteins associated with glutathione beads were detected by autoradiography or immunoblotting with MPM-2. To obtain semi-quantitative data, we scanned films of immunoblots at the region of 55 kD, the major Pin1-binding protein, and analyzed data with ImageQuan (ScanJet II CX). Peptide dissociation constants were measured with a fluorescence polarization assay as described (22). Peptides were labeled at the NH<sub>2</sub>-terminus and purified by thin-layer chromatography. Various concentrations of WW domain proteins were incubated with 0.1 μM of the labeled peptides in a binding buffer containing 50 mM Hepes (pH 7.4), 100 mM NaCl, and 2% glycerol, and fluorescence polarization values were obtained with a PanVera 2000 system.
19. HeLa cells were labeled overnight with <sup>32</sup>P-orthophosphate or <sup>35</sup>S-Met as described (27). Cells were lysed in lysis buffer with or without phosphatase inhibitors (40 mM glycerol phosphate, 50 mM NaF, 10 mM NaVO<sub>4</sub>, and 2 μM okadaic acid) (12, 13). For dephosphorylation experiments, three Ser phosphatases (CIP, PP1, and PP2A) were added to lysates for 30 min at 30°C in the absence or presence of the above phosphatase inhibitors as described (27).
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23. To determine the function of Pin1 mutants in vivo, cDNAs of PIN1 or its mutants were subcloned into the yeast expression vector YEp451 and transformed into a temperature-sensitive *ptf1* strain YMP2, as described (28). Those strains expressing similar amounts of Pin1 proteins were selected, as detected by immunoblotting with the 12CA5 antibody to the inserted NH<sub>2</sub>-terminal hemagglutinin A epitope tag (21). Three to four independent strains were tested for each transformation.
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## Facilitation of Signal Onset and Termination by Adenylyl Cyclase

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The α subunit (G<sub>sq</sub>) of the stimulatory heterotrimeric guanosine triphosphate binding protein (G protein) G<sub>s</sub> activates all isoforms of mammalian adenylyl cyclase. Adenylyl cyclase (Type V) and its subdomains, which interact with G<sub>sq</sub>, promoted inactivation of the G protein by increasing its guanosine triphosphatase (GTPase) activity. Adenylyl cyclase and its subdomains also augmented the receptor-mediated activation of heterotrimeric G<sub>s</sub> and thereby facilitated the rapid onset of signaling. These findings demonstrate that adenylyl cyclase functions as a GTPase activating protein (GAP) for the monomeric G<sub>sq</sub> and enhances the GTP/GDP exchange factor (GEF) activity of receptors.

Regulators of G protein signaling (RGS proteins) increase the GTPase activity of α subunits of heterotrimeric G proteins and play an important role in the termination

and onset of signals mediated by the G<sub>i</sub> and G<sub>q</sub> families of G proteins (1–8). To date, however, no protein that acts as a specific GAP for G<sub>sq</sub> has been identified. Because all