Accelerated Publication

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 4, Issue of January 25, pp. 2381–2384, 2002
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Printed in U.S.A.

Critical Role of WW Domain Phosphorylation in Regulating Phosphoserine Binding Activity and Pin1 Function*

Received for publication, May 4, 2001,
and in revised form, November 21, 2001
Published, JBC Papers in Press, November 26, 2001,
DOI 10.1074/jbc.C100228200

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Phosphoserine-binding modules help determine the specificity of signal transduction events. One such module, the group IV WW domain, plays an essential role in targeting the phosphorylation-specific prolyl isomerase Pin1 to its substrates. These modules require Ser/Thr phosphorylation of their ligands for binding activity. However, phosphorylation of these modules and its functional significance have not been described, nor is it known whether the function of Pin1 is regulated. Here we show that Pin1 WW domain is phosphorylated on Ser^{16} both *in vitro* and *in vivo*. Further, this phosphorylation regulates the ability of the WW domain to mediate Pin1 substrate interaction and cellular localization. Moreover, both Pin1 and WW domain mutants refractory to Ser^{16} phosphorylation act as dominant-negative mutants to induce mitotic block and apoptosis and increase multinucleated cells with 8 N DNA content. Thus, phosphorylation is a new mechanism critical for regulating WW domain phosphoserine binding activity and Pin1 function.

Compelling evidence indicates that phosphorylation on Ser or Thr residues (pSer/Thr) also promotes the formation of protein-protein (1–3). Small pSer/Thr-binding protein modules reminiscent of SH2[1] domains involved in recognition of phosphorylated Tyr have been recently described, which include group IV WW domains and FHA domains (2–7). However, the role of phosphorylation of these modules themselves has not been described.

The best characterized pSer/Thr-binding WW domain is one in the peptidyl-prolyl isomerase (PPIase) Pin1 (4, 7). Pin1 specifically isomerizes the pSer/Thr–Pro bond (8–10). Pin1 is essential for mitotic progression (11–13) and is required for the DNA replication checkpoint (14). Pin1 substrates are a defined subset of phosphorylated proteins, including many MPM-2 antigens (8, 13, 15–20). Pin1-catalyzed prolyl isomerization regulates the conformation and function of phosphoproteins (15, 17) and also facilitates dephosphorylation (12, 15, 17). Thus, Pin1-dependent peptide bond isomerization is a critical post-phosphorylation regulatory mechanism (12). However, it is likely that Pin1 is regulated by a post-translational modification.

The first and essential step in Pin1-dependent regulation of targets is the substrate binding activity mediated by its WW domain (10). The WW domain functions as a pSer/Thr-Pro-binding module, with the binding pocket including the side chains of Ser^{16}, Arg^{17}, Tyr^{23}, and Trp^{34} (4, 7). Interestingly, Ser^{16} is located at the center of the shadow pSer/Thr-Pro-binding pocket. Here we show an essential role for phosphorylation of Ser^{16} in the regulation of the WW domain pSer/Thr binding activity and subsequent Pin1 cell cycle function.

MATERIALS AND METHODS

Interaction of Pin1 Mutants and MPM-2 Antigens or Phosphopeptides—GST fusion proteins were expressed and purified, and their ability to bind mitotic phosphoproteins was assayed as described (8, 15). Peptide binding was measured as described (4).

Localization Analysis of GFP-Pin1 and Its Mutant Proteins— Pin1 or its mutants were subcloned into pEGFP-C1 or pERFP-N1 (CLONTECH), respectively. HeLa cells were transfected with pGFP- or pRFP-Pin1 or its mutants using Superfect (Qiagen). After 6–24 h, cells were fixed and examined under a fluorescence microscope (Nikon).

In Vivo and in Vitro Phosphorylation of Pin1—To detect Pin1 phosphorylation *in vivo*, HeLa cells were labeled with [^{32}P]orthophosphate, and Pin1 was immunoprecipitated with anti-Pin1 or anti-HA antibodies, followed by SDS-PAGE and autoradiography (4). ^{32}P-labeled Pin1 proteins were extracted and digested with trypsin followed by two-dimensional chromatography (23). *In vitro* phosphorylation of Pin1 and its mutants were carried out as described (4). To detect the mobility shift of Pin1 during the cell cycle, HeLa cells were arrested at the G_1/S boundary and released to enter the cell cycle as described (15). Cell lysates were subjected to low bis-SDS-PAGE, followed by immunoblot with anti-Pin1 or anti-cyclin B1 antibodies.

Analysis of Cell Cycle Phenotypes—HeLa cells were transfected with various Pin1 constructs, and cells were observed live or after fixation. To detect the cell cycle profile of GFP-Pin1-transfected cells, cells were fixed and subjected to flow cytometry (Becton Dickinson), as described (11).

RESULTS AND DISCUSSION

WW Domain Phosphorylation Regulates Its Ability to Mediate the Pin1 Substrate Interactions in Vitro—To examine whether phosphorylation affects the binding activity of the Pin1 WW domain, we first incubated Pin1 and its WW domain with purified kinases and found that PKA and PKC readily phosphorylated Pin1 and its WW domain (Fig. 1*A*, data not shown). Furthermore, phosphorylation by PKA, but not PKC, completely abolished the interactions between Pin1 or its WW domain and MPM-2 antigens (Fig. 1*B*). Even after Pin1 had already bound to MPM-2 antigens, PKA treatments resulted in disassociation of

* This work was supported by National Institutes of Health Grants R01GM56230 and GM58556 (to K. P. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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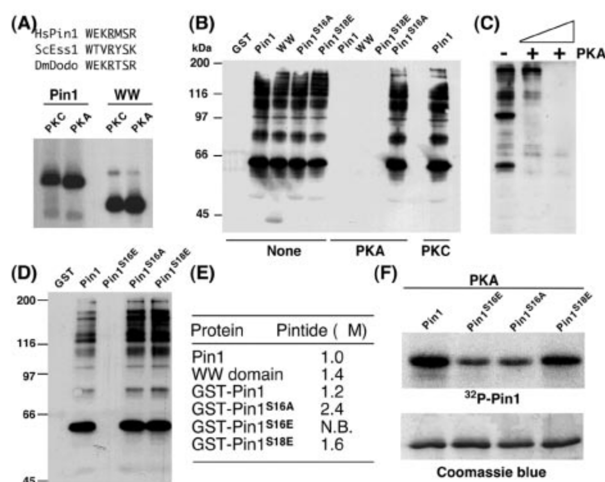
[1] The abbreviations used are: SH2, Src homology 2; WW, Trp-Trp; PPIase, peptidyl-prolyl isomerase; MPM-2, mitotic phosphoprotein monoclonal-2; GST, glutathione *S*-transferase; GFP, green fluorescent protein; RFP, red fluorescent protein; DAPI, 4–6-diamidino-2-phenylindole; PKA, protein kinase A; PKC, protein kinase C; pSer/Thr, phosphorylated serine or threonine residue.



Protein	Pintide (M)
Pin1	1.0
WW domain	1.4
GST-Pin1	1.2
GST-Pin1S16A	2.4
GST-Pin1S16E	N.B.
GST-Pin1S18E	1.6

FIG. 1. **Phosphorylation on Ser_{16} is both necessary and sufficient to regulate the Pin1 substrate interaction *in vitro*.** *A*, alignment of the Ser^{16} region of selected Pin1 proteins and phosphorylation by PKA or PKC. Pin1 and its WW domain were phosphorylated followed by SDS-PAGE and autoradiography. *B*, effect of phosphorylation on Pin1 binding. GST fusion proteins were phosphorylated with PKA overnight, purified, and then incubated with mitotic HeLa extracts followed by immunoblotting with MPM-2. *C*, dissociation of Pin1 substrate binding by PKA. Mitotic extracts were bound to GST-Pin1 beads and then incubated with PKA followed by immunoblotting with MPM-2 after washing. *D*, phosphoprotein-binding activity of Pin1 mutants. Mitotic extracts were bound to GST-Pin1, or its mutants were bound to glutathione beads and washed followed by immunoblotting with MPM-2. *E*, phosphopeptide binding activity of Pin1 mutants. Different mutant proteins were incubated with the fluorescent Pintide, and binding affinity was measured by fluorescence anisotropy assay. *N.B.*, no binding. *F*, phosphorylation of Pin1 mutants by PKA. After phosphorylation Pin1 and its mutants were separated on SDS gels followed by autoradiography (*top*) or Coomassie stain (*bottom*).

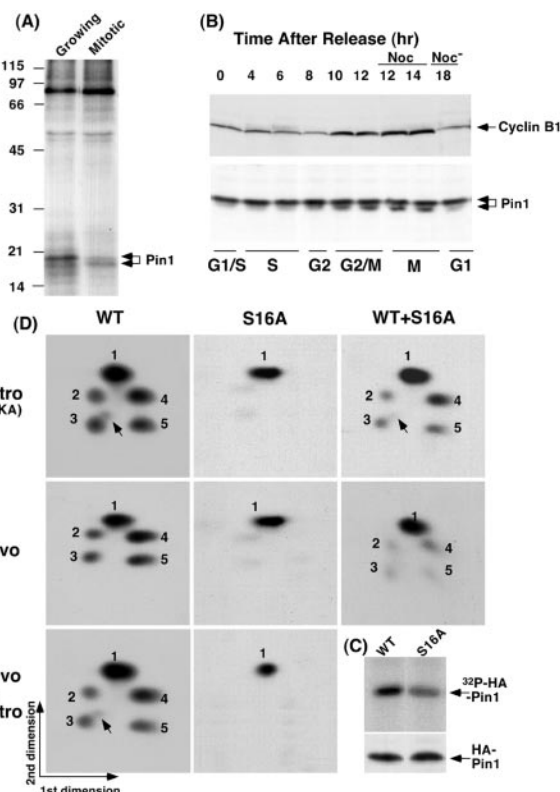


FIG. 2. **Cell cycle-regulated phosphorylation of Pin1 on Ser_{16} in cells.** *A*, phosphorylation of endogenous Pin1. Growing or mitotic HeLa cells were labeled with ^{32}P, and Pin1 was immunoprecipitated followed by SDS-PAGE and autoradiography. *B*, cell cycle-dependent Pin1 mobility shift. HeLa cells synchronized at G_1/S (time 0) were released to enter the cell cycle. To obtain mitotic cells, nocodazole was added to cells at 8 h and incubated for another 4 h (12+Noc) or 6 h (14+Noc). To obtain G_1 cells, mitosis-arrested cells were plated in fresh media for another 4 h (18+Noc$^-$). The cell cycle status was determined by flow cytometry. Cell lysates were subjected to immunoblot with anti-cyclin B1 or anti-Pin1 antibodies. *C*, phosphorylation of wild-type Pin1 and its S16A mutant. After transfection, HeLa cells were labeled with ^{32}P and soluble proteins subjected to immunoprecipitation with the anti-HA antibody, followed by autoradiography (*upper panel*) or immunoblotting with anti-HA antibody (*lower panel*). *D*, phosphopeptide analysis. ^{32}P-labeled Pin1 proteins were recovered from gels and digested with trypsin followed by separation on two-dimensional TLC plates and autoradiography. *Arrows* point to a minor phosphopeptide present only upon *in vitro* phosphorylation.

Pin1 from MPM-2 antigens (Fig. 1*C*). These results indicate that phosphorylation of Pin1 at the WW domain, possibly on Ser^{16}, can prevent Pin1 from interacting with its substrates.

To examine the importance of Ser^{16} of Pin1, it was mutated to Glu to mimic pSer, and the mutant $Pin1^{S16E}$ failed to bind mitotic phosphoproteins (Fig. 1*D*). Similar effects were observed on binding to Pintide (WFYpSFLE) (Fig. 1*E*), an optimal peptide ligand for the Pin1 WW domain (8), as determined by the fluorescence polarization assay (4). As a control, a Glu substitution was made at Ser^{18} outside of the binding pocket (9); this mutation had no effect (Fig. 1, *B, D*, and *E*). These results confirm that the S16E mutation completely abolishes the ability of the Pin1 WW domain to bind mitotic phosphoproteins.

We examined whether Ser^{16} is the only phosphorylation site that regulates Pin1 binding activity. To address this question, Ser^{16} was substituted with Ala. Like Pin1, $Pin1^{S16A}$ bound to most, if not all, Pin1 substrates (Fig. 1, *B* and *D*) and to Pintide with a compatible binding affinity (Fig. 1*E*). Furthermore, $Pin1^{S16A}$ binding to phosphoproteins was not affected by PKA (Fig. 1*B*), although $Pin1^{S16A}$ was still phosphorylated on another site by PKA (Figs. 1*F* and 2*C*). These results indicate that Ser^{16} phosphorylation is both essential and sufficient to regulate the ability of the WW domain to mediate phosphorylation-dependent ligand recognition.

WW Domain Phosphorylation Regulates Its Ability to Mediate the Pin1 Substrate Interaction and subcellular Location in Vivo—To examine whether Pin1 is phosphorylated in cells and whether Pin1 phosphorylation is regulated during the cell cycle, we first performed *in vivo* ^{32}P labeling experiments and showed that Pin1 was phosphorylated in growing HeLa cells, exhibited as a single slow migrating species on SDS gels. Moreover, this species was dephosphorylated upon mitotic arrest, with the appearance of a faster migrating species (Fig. 2*A*). To determine the kinetics of Pin1 dephosphorylation during the cell cycle, lysates were collected at various times after release from G_1/S arrest and separated on modified SDS gels followed by immunoblotting with Pin1 antibodies. As shown by Shen *et al.* (15), Pin1 levels did not significantly fluctuate, but two different forms of Pin1 were detected (Fig. 2*B*). The appearance of the faster migrating form of Pin1 was cell cycle-dependent (Fig. 2*B*). Thus, Pin1 is phosphorylated in a cell cycle-regulated manner. Interestingly, the dephosphorylated form of Pin1 is detected in breast cancer tissues overexpressing Pin1 (22).

Next, to examine whether Ser^{16} of Pin1 is phosphorylated *in vivo*, we transfected HeLa cells with HA-Pin1, $Pin1^{S16A}$, or $Pin1^{S16E}$ and labeled them with ^{32}P. The majority of these expressed proteins was not phosphorylated in cells (Fig. 2, *A* and *C*, data not shown). A fraction of proteins was phosphorylated to allow us to analyze the phosphorylation site. Although Pin1 and its mutant proteins were expressed at similar levels, the intensity of ^{32}P-labeled $Pin1^{S16A}$ or $Pin1^{S16E}$ was slightly less than half of that observed in wild-type Pin1 (Fig. 2*C*, data not shown). To confirm that Ser^{16} is indeed phosphorylated *in vivo*, ^{32}P-labeled proteins were extracted from the gels and digested with trypsin followed by two-dimensional phos-

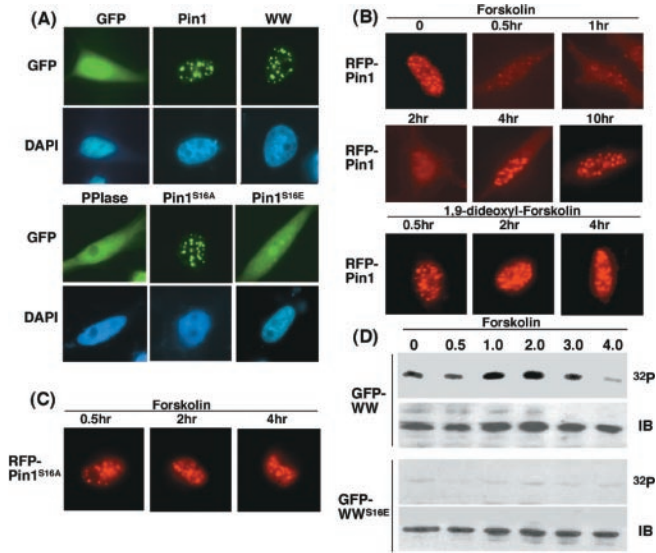


FIG. 3. **Phosphorylation on Ser_{16} regulates the subcellular location of Pin1.** *A*, subcellular localization of GFP-Pin1 and mutants. HeLa cells were transfected with GFP or various GFP-Pin1 constructs followed by fluorescence microscopy. *B*, translocation of Pin1 induced by activation of PKA. HeLa cells were transfected with RFP-Pin1 and treated with forskolin or 1,9-dideoxyl-forskolin followed by determination of the subcellular localization of RFP-Pin1 at the times indicated. A representative cell is shown for each time point. Neither forskolin nor 1,9-dideoxyl-forskolin affected RFP localization (not shown). *C*, failure of PKA activation to affect localization of $Pin1^{S16A}$. HeLa cells were transfected with RFP-$Pin1^{S16A}$ and treated with forskolin followed by fluorescence microscopy. *D*, phosphorylation of Ser^{16} in cells after forskolin treatment. Cells were transfected with GFP-WW domain or GFP-WW^{S16E} and labeled with ^{32}P followed by treating cells with forskolin for various times. GFP WW domain and its mutant protein were then immunoprecipitated using anti-GFP antibodies and then subjected to SDS-PAGE followed by autoradiography, to detect phosphorylation of WW domain proteins, or immunoblotting analysis with anti-GFP antibodies, to detect protein levels.

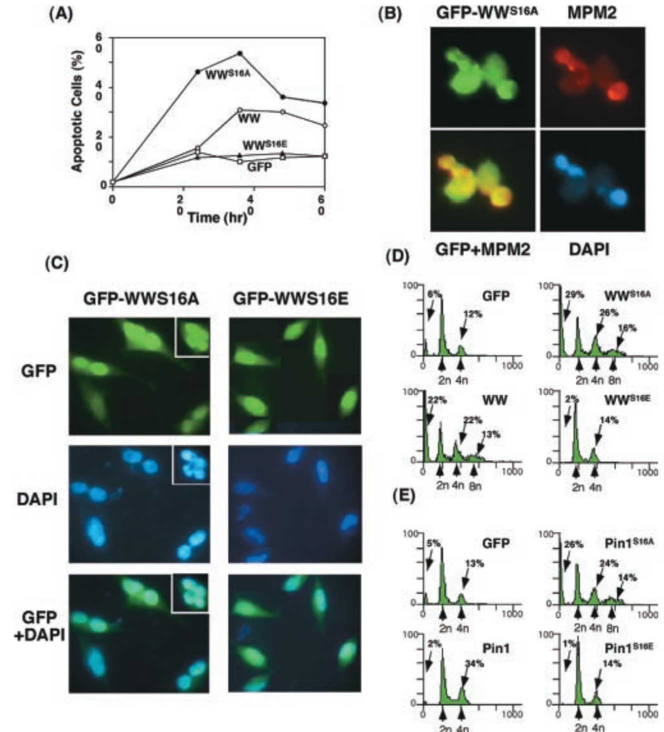


FIG. 4. **WW domain S16A but not S16E mutant affects the cell cycle *in vivo.*** *A*, apoptosis induced by WW domain and WW^{S16A} mutant. HeLa cells were transfected for the times indicated, fixed, and examined by fluorescence microscopy. Apoptotic cells with highly condensed or fragmented DNA or with micronuclei were scored. *Solid circles*, WW^{S16A}; *open circle*, WW domain; *solid triangles*, WW^{S16E}; *open squares*, control GFP. *B*, mitotic block and apoptosis induced by the WW^{S16A} mutant. After transfection with GFP-WW^{S16A} for 24 h, cells were fixed and stained with both MPM-2 and DAPI followed by fluorescence microscopy. The pictures show two transfected cells with colocalization of MPM-2 and GFP-WW ^{S16A} indicated by the *yellow color* in the merged image. *C*, multinucleated cells induced by WW^{S16A} mutant. After transfection for 36 h, HeLa cells were stained with DAPI followed by fluorescence microscopy. Pictures are selected to show mitotic cells that contained separating two or sometimes four nuclei (*inserts*). *D* and *E*, flow cytometry analysis. Cells were transfected for 36 h with GFP-WW domain or its mutants (*D*) or with GFP-Pin1 or its mutants (*E*). Cells were fixed and subjected to flow cytometry.

phopeptide mapping (23). As a control, we used Pin1 and $Pin1^{S16A}$ phosphorylated with PKA *in vitro* (Fig. 1*F*). PKA phosphorylated Pin1 and produced 5 major tryptic peptides, No1–5, and a minor species (Fig. 2*D*). Interestingly, *in vivo* labeled Pin1 also produced the same five tryptic peptides, as indicated by mixing experiments, and phosphorylation of peptide No1 was not affected by the S16A mutation either *in vivo* or *in vitro* (Fig. 2*D*). These results indicate that PKA phosphorylates Pin1 on the same sites as those observed *in vivo* and that peptide No1 is independent of Ser^{16}. Future work is required to determine the phosphorylation site of the No peptide. In contrast, the appearance of peptides No2–5 was completely abolished by the Ser^{16} mutations both *in vitro* and *in vivo* (Fig. 2*D*). Although based on the sequence around Ser^{16}, one would expect two related phosphopeptides to be generated from this sequence upon tryptic digestion, *i.e.* RMpSR and MpSR. The presence of two pairs of spots might be explained by incomplete oxidation of the Met residue, leading to spot separation in the chromatography dimension. Therefore, these results show that Ser^{16} in Pin1 is phosphorylated by PKA *in vitro* and in cells.

Expressed Pin1 has been shown to be localized at a nuclear substructure called the nuclear speckle during interphase (11), Because the Pin1 WW domain mediates the substrate interaction of Pin1, it may determine the localization of Pin1 in the cell given that Pin1 is a 16-kDa protein small enough to diffuse freely within a cell. To examine this possibility, we expressed various GFP-Pin1 fusion proteins in HeLa cells. Although GFP was observed in both the nucleus and the cytoplasm, GFP-Pin1 was localized at the nuclear speckle (Fig. 3*A*) as shown for HA-Pin1 (11). Interestingly, the GFP-WW domain exhibited the same pattern as GFP-Pin1, whereas the GFP-PPIase domain was observed as a diffuse pattern in whole cells (Fig. 3*A*). These results indicate that the WW domain determines the subcellular localization of Pin1, consistent with a recent report (24).

We then asked whether Pin1 localization is driven by the WW binding to its target proteins and whether this localization can be regulated by phosphorylation on Ser^{16}. Therefore, questions, we examined the localization of GFP-Pin1 Ser^{16} mutants. Although $Pin1^{S16A}$ remained in the nuclear speckle, $Pin1^{S16E}$ was distributed diffusely in the whole cell, the patterns similar to those of the WW domain and the PPIase domain, respectively (Fig. 3*A*). These results show that the pSer-mimicking S16E mutation disrupts the Pin1 subcellular localization.

To confirm that Ser^{16} phosphorylation can regulate the Pin1 localization, we tried to alter the phosphorylation status of Ser^{16} in cells. If PKA can phosphorylates Ser^{16} *in vivo*, PKA activation would disrupt the Pin1 WW domain binding to its binding proteins in the nuclear speckle and abolish its nuclear speckle localization. To examine this possibility, we transfected HeLa cells with a red RFP-Pin1 construct and treated them with forskolin and the inactive forskolin analog 1,9-dideoxylforskolin (25). Upon forskolin treatment, the translocation of RFP-Pin1 occurred immediately and reached a maximum after 30 min in the majority of cells (Fig. 3*B*). The relocation of Pin1

back to the nuclear speckle also occurred at a similar rate for most cells (Fig. 3*B*). In contrast, this Pin1 relocation activity was not observed with 1,9-dideoxyl-forskolin (Fig. 3*C*). Furthermore, the localization of Pin1^{S16A} and Pin1^{S16E} was not affected by forskolin treatment (Fig. 3*C*, data not shown). These results demonstrate shown that activation of PKA abolishes the preferential localization of Pin1 to the nuclear speckle. In addition, PKA-mediated phosphorylation can lead to dissociation of Pin1 from its substrates *in vitro* (Fig. 1*C*). We also compared *in vivo* phosphorylation of GFP-WW and GFP-WW ^{S16E} at different times after forskolin treatment. A significant fraction of GFP-WW domain, but not GFP-WW ^{S16E}, was phosphorylated at time 0 (Fig. 3*D*), consistent with phosphorylation of Pin1 on Ser^{16}. Importantly, forskolin treatment increased phosphorylation of GFP-WW domain in a time-dependent manner, reaching the maximal level between 1.0 and 2.0 h and then decreasing (Fig. 3*D*). Interestingly, the kinetics of phosphorylation was similar to that of dispersal and then re-speckling. These results indicate that PKA activation can disrupt the substrate interaction and subcellular localization of Pin1 via Ser^{16} phosphorylation. If PKA phosphorylates Pin1 *in vivo*, it would be consistent with previous reports that both cAMP and PKA activity oscillate during the cell cycle, reaching their lowest levels at the G_2/M transition and the highest levels upon exit from mitosis (26, 27).

WW Domain S16A but Not S16E Mutation Affects the Cell Cycle in Vivo—Given the essential role of WW domain phosphorylation in abrogating Pin1 substrate interactions and its subcellular localization, we would expect that the S16A mutations would affect Pin1 function. To examine this possibility, we determined the effects of transfected GFP-WW domain or GFP-Pin1 proteins on cell and nuclear morphology in HeLa cells. Expression of either vector GFP or the WW^{S16E} domain had no significant effect (Fig. 4). In contrast, a significant number of cells expressing WW^{S16A} were rounded up and their chromatin condensed at 24 h after transfection. By 36 h, 54% of the cells expressing the GFP-WW^{S16A} and 34% of the cells expressing the GFP-WW domain were rounded up, with condensed chromatin and/or fragmented nuclei, which were often present in two dividing mitotic cells (Fig. 4, *A* and *B*), similar to phenotypes induced by depletion of Pin1 (11). These results suggest that WW domain and WW^{S16A}, but not WW^{S16E}, induce mitotic block and apoptosis.

To examine whether WW^{S16A} induced apoptosis from interphase or mitosis, we used MPM-2 antibodies to mitosis-specific phosphoproteins in apoptotic cells. WW^{S16A}- but not WW^{S16E}-expressing cells were strongly stained with MPM-2 after a 24-h transfection. This staining was observed in apoptotic cells even at 36 h, as shown in Fig. 4*B*. Moreover, GFP-WW^{S16A} and MPM-2 antigens were co-localized at certain substructures in mitotic and apoptotic cells (Fig. 4*B*). The nuclear lamina in WW^{S16A}-expressing cells was also dissembled (data not shown). These results indicate that expression of WW^{S16A} or WW domain induces mitotic block and apoptosis. In addition, after a 36-h transfection, two nuclei or sometimes even four nuclei were observed in the cells expressing the WW^{S16A} but not the WW^{S16E} mutant (Fig. 4*C*).

To confirm the morphological phenotypes, we used flow cytometry. Expression of the GFP vector or WW^{S16E} had no detectable effect (Fig. 4*D*). However, expression of either WW^{S16A} or WW domain resulted in a significant increase in cells with 4 N DNA content and with the sub-G_1 DNA content (Fig. 4*D*), supporting the theory that WW^{S16A} or WW domain induces mitotic block and apoptosis. Furthermore, 16% of WW^{S16A}- and 13% of WW-expressing cells contained the 8 N DNA content (Fig. 4*D*), confirming the appearance of multinucleated cells (Fig. 4*C*). These results indicate that expression of WW^{S16A} or WW domain induces mitotic block and apoptosis and increases multinucleated cells.

To ensure that similar phenotypes are also observed in full-length Pin1, Pin1 and its point mutants Pin1^{S16A} and Pin1^{S16E} were transfected into cells. Overexpression of Pin1 delays the G_2/M transition in HeLa cells as shown previously (11, 15, 16). Importantly, although Pin1^{S16E} had no effect, GFP-Pin1^{S16A} induced mitotic block and apoptosis and formation of multinucleated cells with the 8 N DNA content (Fig. 4*E*), the same phenotype as induced by WW^{S16A} (Fig. 4*D*). These results demonstrate that expression of the S16A Pin1 mutant either as the isolated WW domain or as full-length Pin1 results in the same phenotypes.

In summary, we have demonstrated that phosphorylation of the Pin1 WW domain on Ser^{16} regulates its ability to function as a pSer/Thr-binding module. The biological significance of the phosphorylation is demonstrated by the findings that the mutant Pin1^{S16A} or WW^{S16A}, but not Pin1^{S16E} or WW^{S16E}, acts as a dominant-negative mutant to induce mitotic block and apoptosis and increase multinucleated cells. These results suggest a new mechanism for regulation of the phosphorylation-specific isomerase Pin1 and constitute the first demonstration that a pSer/Thr-binding module is subjected to post-translational modification and resultant functional modification. Because Ser^{16} of the Pin1 WW domain is highly conserved in many other WW domains, some of these latter WW domains may also be subjected to phosphorylation-mediated *in vivo* regulation.

Acknowledgments—We thank L. Cantley, B. Neel, and T. Hunter for constructive suggestions.

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