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FAST TRACK

IGF-1 Induces Pin1 Expression in Promoting Cell Cycle S-Phase Entry

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Abstract Insulin-like growth factor I (IGF-1) is a well-established mitogen to many different cell types and is implicated in progression of a number of human cancers, notably breast cancer. The prolyl isomerase Pin1 plays an important role in cell cycle regulation through its specific interaction with proteins that are phosphorylated at Ser/Thr-Pro motifs. Pin1 knockout mice appear to have relatively normal development yet the Pin1$^{-/-}$mouse embryo fibroblast (MEF) cells are defective in re-entering cell cycle in response to serum stimulation after G0 arrest. Here, we report that Pin1$^{-/-}$ MEF cells display a delayed cell cycle S-phase entry in response to IGF stimulation and that IGF-1 induces Pin1 protein expression which correlates with the induction of cyclin D1 and RB phosphorylation in human breast cancer cells. The induction of Pin1 by IGF-1 is mediated via the phosphatidylinositol 3-kinase as well as the MAP kinase pathways. Treatment of PI3K inhibitor LY294002 and the MAP kinase inhibitor PD098059, but not p38 inhibitor SB203580, effectively blocks IGF-1-induced upregulation of Pin1, cyclin D1 and RB phosphorylation. Furthermore, we found that Cyclin D1 expression and RB phosphorylation are dramatically decreased in Pin1$^{-/-}$ MEF cells. Reintroducing a recombinant adenovirus encoding Pin1 into Pin1$^{-/-}$ MEF cells restores the expression of cyclin D1 and RB phosphorylation. Thus, these data suggest that the mitogenic function of IGF-1 is at least partially linked to the induction of Pin1, which in turn stimulates cyclin D1 expression and RB phosphorylation, therefore contributing to G0/G1-S transition. J. Cell. Biochem. 84: 211–216, 2002.

Key words: IGF-1; Pin-1; cyclin D; cell cycle

The effectiveness of insulin-like growth factor I (IGF-1) as a mitogen of the human breast epithelial breast cancer cell line MCF-7 is well established. Serum-starved MCF-7 cells enter the cell cycle by the sole action of IGF-1, which leads to upregulation of cyclin D1 [Karey and Sirbasku, 1988; van der Burg et al., 1988]. It has been well documented that D-type cyclins (D1, D2, and D3) bind to Cdk4 and Cdk6 and thereby form an active kinase to phosphorylate retinoblastoma protein (RB) [Sherr, 1996]. Phosphorylated RB releases the E2F transcription factors, which in turn stimulate a set of S-phase and cell cycle promoting genes [Sherr, 1996]. Since cyclin D2 and D3 are not expressed in MCF-7 cells [Gorospe et al., 1996], cyclin D1 is the essential D-type cyclin.

It has become clear that cyclin D1 is a rate-limiting factor for the transition of cell cycle G0 to S phase. Inhibition of cyclin D1 by microinjection of antibodies or by antisense expression cause cell growth arrest [Sherr, 1996]. Furthermore, overexpression of cyclin D1 prevents cells from entering G0 in low-serum condition, suggesting that cyclin D1 is a critical player in cell cycle exit [Zwijsen et al., 1996]. Moreover, the pathological significance of cyclin D1 is supported by the observations that cyclin D1 is overexpressed in 50% of breast cancer patients [Bartkova et al., 1994; Gillet et al., 1994]. Given the role of IGF-1 in breast cancer, the IGF-1

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mediated activation of cyclin D1 may play an important role in breast tumorigenesis.

The Pin1 prolyl *cis–trans* isomerase is an evolutionarily conserved enzyme that promotes the *cis–trans* isomerization of the peptide bond at the amino side of the proline residue [Lu, 2000]. Pin1 plays an important role in cell cycle regulation through its specific interaction with proteins that are phosphorylated at Ser/Thr-Pro motifs [Lu et al., 1996; Yaffe et al., 1997; Shen et al., 1998]. Pin1 is essential for cell cycle progression in yeast and in mammalian cells [Lu et al., 1996]. Overexpression of Pin1 leads to G2 arrest, whereas inhibition of Pin1 causes mitotic arrest [Lu et al., 1996]. Interestingly, Pin1 knockout mice appear to have relatively normal development yet the $Pin1^{-/-}$ mouse embryo fibroblast (MEF) cells are defective in re-entering cell cycle in response to serum stimulation after G0 arrest [Fujimori et al., 1999].

Pin1 has been implicated in many important biological processes. Pin1 has been shown to bind to Cdc25c and Tau in facilitating dephosphorylation of both proteins [Zhou et al., 2000]. More recently, the *Drosophila* Pin1 binds to and promotes the degradation of the transcriptional factor CF2 in responding to the MAP kinase signal pathway during *Drosophila* oogenesis [Hsu et al., 2001]. Furthermore, increasing evidence indicates that Pin1 may play a role in breast tumorigenesis. Pin1 has been identified as a gene target repressed by the breast and ovarian cancer susceptibility gene (BRCA1) [MacLachlan et al., 2000].

In this study, we report that IGF-1 induces Pin1 expression which is important in cyclin D1 expression and RB phosphorylation and that IGF-1 mediated induction of Pin1 is via PI3 kinase and MAP kinase pathways. Thus, the mitogenic function of IGF-1 may be at least partially linked to the induction of Pin1, which in turn stimulates cyclin D1 expression and RB phosphorylation, therefore contributing to G0/G1-S transition.

MATERIALS AND METHODS

Cell Culture and Reagents

MCF-7 cells were cultured in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), L-glutamine (2 mM), penicillin (100 IU/ml), and streptomycin (100 μg/ml). For serum starvation, MCF-7 cells at 60% confluence were incubated in DMEM free of serum for 48 h. Primary MEFs (passages 4–8) derived from the wild-type, or $Pin1^{-/-}$ mice were cultured in DMEM medium containing 15% FBS. MEF cells were starved in DMEM containing 0.1% FBS for 48 h. IGF-1 was purchased from Sigma (St. Louis, MO). LY294002 and SB203580 were purchased from Calbiochem (San Diego, CA). Anti-Pin1 polyclonal antibody was a gift (from Dr. K.P. Lu, Department of Medicine, Harvard Medical School). Anti-cyclin D1 (HD-11), anti-cyclin E (M20), anti-actin (C11) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-RB (G3-245) antibody was purchased from PharMingen (San Diego, CA).

Adenovirus Infection

HA-Pin1 recombinant adenovirus were generated as described [He et al., 1998]. MEF cells at 60% confluence were seeded into 60 mm dishes and infected with the Pin1 recombinant adenovirus or the virus-vector at the MOI of 200. After 24 h infection, cells were incubated in DMEM medium containing 0.1% FBS for 48 h prior to the treatment with IGF-1 (50 ng/ml).

Western Blot Analysis and Flow Cytometry

Cells were washed with PBS and suspended in EBC250 lysis buffer (50 mM Tris pH 8.0, 250 mM NaCl, 0.5% (w/v) NP-40, 50 mM NaF, 0.5 mM Na3VO4, 0.1 mM phenylmethlsulfonyl fluoride, 10 μg/ml leupeptin, 2 μg/ml aprotinin) and brief sonication. Cell debris was removed by centrifugation at 14,000 rpm for 20 min at 4°C. Protein concentration was determined by the Bradford assay. Equal amounts of protein (10–15 μg) were separated in 10% SDS-PAGE gels and transferred to PVDF membrane for subsequent detection by ECL. For analysis of RB phosphorylation, 6.5% of SDS-PAGE gels were used. For FACS analysis, MEF cells were collected by trypsinization and stained with propidium iodine. Flow cytometric analysis was performed on a Becton Dickinson FACScan. Data were analyzed by CellQuest software (Becton Dickinson).

RESULTS AND DISCUSSION

Delayed Cell Cycle S-Phase Entry of Serum-Deprived $Pin1^{-/-}$ Cells in Response to IGF-1

Mice lacking Pin1 appear to undergo normal development. However, $Pin1^{-/-}$ MEF cells are

defective in re-entering cell cycle after serum starvation, suggesting that the cell cycle G0/G1-S progression is impaired [Fujimori et al., 1999]. Therefore, we examined the cell cycle S-phase entry resulting from the IGF-1 stimulation on the serum-deprived wild-type and $Pin1^{-/-}$ MEF cells. Wild-type MEF cells started to enter the S-phase at 12 h and reached peak at 16 h after IGF-1 treatment, whereas $Pin1^{-/-}$ cells did not enter S-phase at all at 12 h, began to enter S-phase only at 16 h, and reached peak at 20 h after IGF-1 treatment (Fig. 1). These data suggest that the cell cycle progression is impaired in the absence of Pin1 in response to growth stimulatory signals.

IGF-1 Upregulates Pin1 and Cyclin D1 in PI3K Pathway-Dependent Manner

The effectiveness of IGF-1 as a mitogen of the human breast epithelial breast cancer cell line MCF-7 is well established. Serum-starved MCF-7 cells enter the cell cycle by the sole action of IGF-1 which leads to upregulation of cyclin D1 [Karey and Sirbasku, 1988; van der Burg et al., 1988]. Since Pin1 is implicated in cell cycle G0/G1 transition, we examined the effect of IGF-1 on the expression of Pin1 in serum-starved MCF-7 cells. IGF-1 progressively upregulated Pin1 with more than four-fold increase in the protein expression in comparison to the control (Fig. 2A). Induction of Pin1 by IGF-1 was also observed in human osteosarcoma U2-OS cells (data not shown). Similarly, EGF also induced Pin1 expression (Fig. 2B). It has been shown that IGF-1 and EGF transduce signals primarily through the PI3K/Akt pathway [Vercoutter-Edouart et al., 2000]. Therefore, we examined the effects on Pin1 expression by specific kinase inhibitors. IGF-1 upregulation of Pin1 was completely abolished by PI3K inhibitor LY294002 but not by p38 kinase inhibitor SB203580 and less effectively by the MAP kinase inhibitor PD098059 (Fig. 3).

Next, we analyzed the IGF-1 effects on cyclin D1 expression and phosphorylation of RB in serum starved MCF-7 cells. IGF-1 significantly induced cyclin D1 protein expression and RB hyperphosphorylation, which correlated well with the IGF-1 induction on Pin1 (Fig. 3). In addition, the induction of cyclin D1 expression

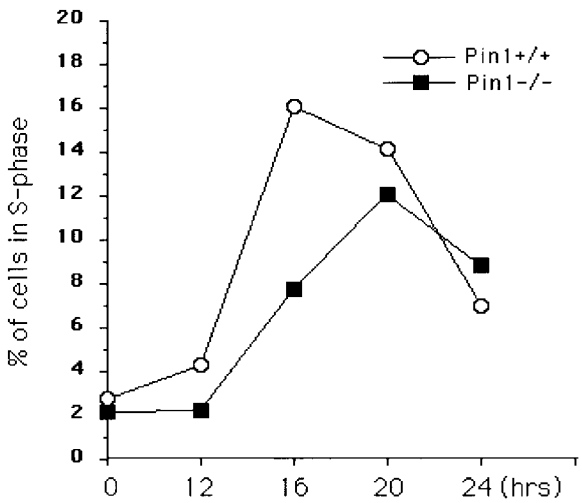

Fig. 1. Cell cycle S-phase entry is delayed in serum-deprived $Pin1^{-/-}$ MEF cells in response to IGF-1. $Pin1^{-/-}$ or $Pin1^{+/+}$ MEF cells were serum starved (0.1% FBS) for 48 h prior to the treatment with IGF-1 (50 ng/ml) for an additional time as indicated. Cells were harvested, stained with propidium iodine, and subjected to FACS analysis. A representative of two independent experiments is shown as the percentage of cells in S-phase at each time point.

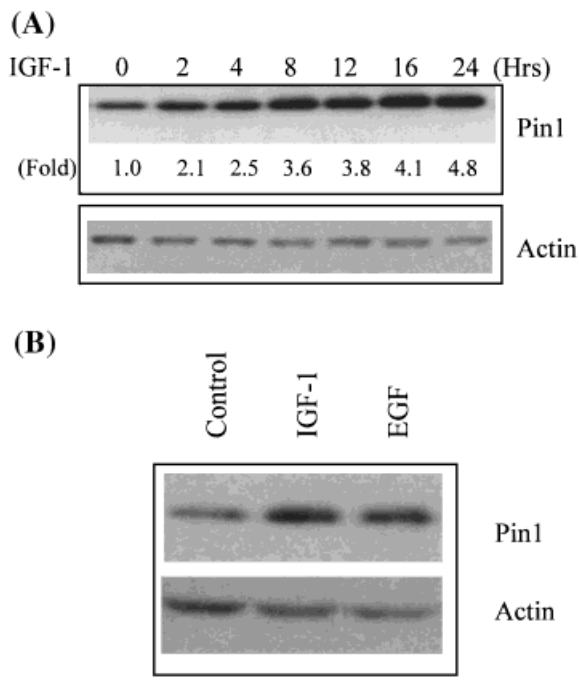

Fig. 2. **A**: Induction of Pin1 by IGF-1 in serum-starved MCF-7 cells. MCF-7 cells were starved in DMEM medium free of serum for 48 h and were treated with IGF-1 (50 ng/ml). Cells were harvested at indicated times, and equal amounts of cell lysates (10–15 μg of total proteins) were subjected to Western blot analysis using antibodies specific for Pin1. Blots were re-probed for actin expression as a loading control. The expression of Pin1 is normalized to actin by densitometry with the fold expression normalized to the control (at 0 h IGF-1 treatment). **B**: Induction of Pin1 by IGF-1 and EGF. Serum-starved MCF-7 cells were treated with IGF-1 (50 ng/ml) or EGF (25 ng/ml) for 4 h. Equal amounts of cell lysates were subjected to Western blot analysis for Pin1 and actin.

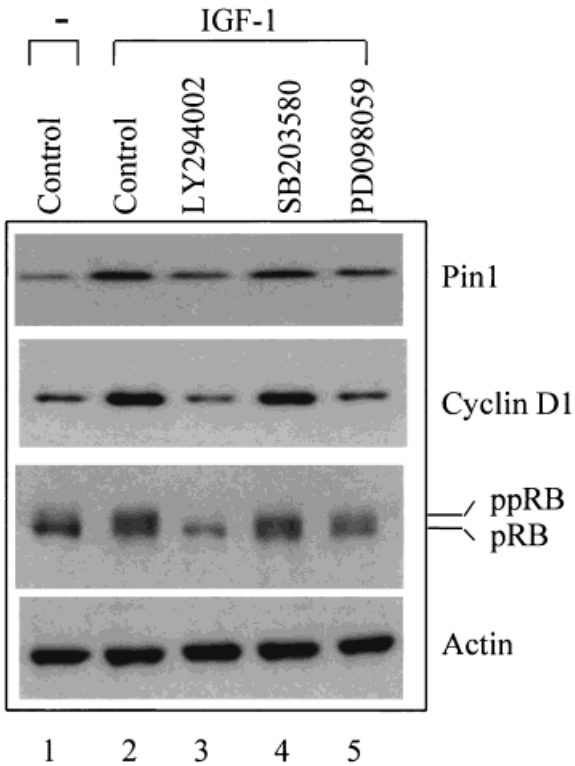

Fig. 3. Induction of Pin1, cyclin D1, and RB phosphorylation by IGF-1. Serum-starved MCF-7 cells were pretreated with LY294002 (20 μm), SB203580 (10 μm), or PD098059 (50 μm) for 2 h prior to the treatment of IGF-1 (50 ng/ml). Cells were harvested 4 h after IGF-1 stimulation. Equal amounts of cell lysates were subjected to Western blot analysis for Pin1, cyclin D1, and RB. Actin was probed for loading control.

and RB phosphorylation were effectively inhibited by PI3K inhibitor LY204002, partially inhibited by the MAP kinase inhibitor PD098059, but not by p38 kinase inhibitor SB203580, which again correlated well with the inhibitory effects on Pin1 expression by the kinase inhibitors (Fig. 3). These data indicate that IGF-1 effect on Pin1 is primarily via PI3 kinase pathway whereas the MAP kinase pathway is also involved, similar to the observation that IGF-1 effect on cyclin D1 is primarily through PI3K/Akt pathway and the MAP kinase pathway [Dufourny et al., 1997, 2000].

Pin1 is Important for the Expression of Cyclin D1 and Phosphorylation of RB in Response to IGF-1

Given the close correlation between the induction of Pin1 and cyclin D1 as well as the RB phosphorylation, we examined cyclin D1 expression in exponentially growing wild-type and $Pin1^{-/-}$ cells at early passages. As shown in Figure 4A, cyclin D1 expression (indicated by an arrow) and the RB phosphorylation were dramatically reduced in $Pin1^{-/-}$ cells. These data are consistent with the observation that $Pin1^{-/-}$ cells grow slower in comparison to the wild-type MEF cells in DMEM containing 15% FBS (data not shown) [Fujimori et al., 1999]. However, cyclin E expression was not apparently affected regardless of Pin1 status (Fig. 4A). Ectopic expression of HA-tagged Pin1 by infection of a recombinant adenovirus into $Pin1^{-/-}$ MEF cells effectively restored cyclin D1 expression (Fig. 4B), indicating that Pin1 is critical in cyclin D1 expression. The expression of HA-Pin1 was confirmed by Western blot analysis (Fig. 4B).

Next, we further examined effects of the Pin-1 adenovirus infection in $Pin1^{-/-}$ MEF cells on the time course of cyclin D1 induction and RB phosphorylation in response to IGF-1. In the absence of Pin1, cyclin D1 induction was not apparent until after 16 h of IGF-1 treatment. Most strikingly, no significant RB phosphorylation was induced even in the presence of cyclin1 protein accumulation (Fig. 4C, the left panel). In contrast, induction of cyclin D1 by Pin1 was evident even in the absence of IGF-1. RB phosphorylation was progressively induced in response to IGF-1 (Fig. 4C, the right panel). These data indicate that Pin1 is critical for cyclin D1 induction and RB phosphorylation in response to IGF-1.

Taken together, these data suggest that IGF-1 induces Pin1 in promoting cell cycle progression likely through the induction of cyclin D1 and RB phosphorylation. It is interesting that the PI3 kinase pathway, as well the MAP kinase pathway, is involved in this process. It has been established that IGF-1 binds to its receptor (IGF-1R) to activate the IGF-1R tyrosine kinase activity which, in turn, phosphorylates the insulin receptor substrate-1 (IRS-1). Activated IRS-1 serves as a docking site for several SH2-containing proteins such as p85, the regulatory subunit of PI3 kinase, and the guanine-nucleotide exchange factor Grb2/Sos. PI3 kinase activates Akt, whereas Grb2/Sos leads to activation of ras-raf-MAP kinase pathway [Yee and Lee, 2000]. However, the molecular mechanism of IGF-1 induction of Pin1 remains to be determined.

Most recently, it has been reported that overexpression of Pin1 is found in breast cancers which correlates with activation of cyclin D1

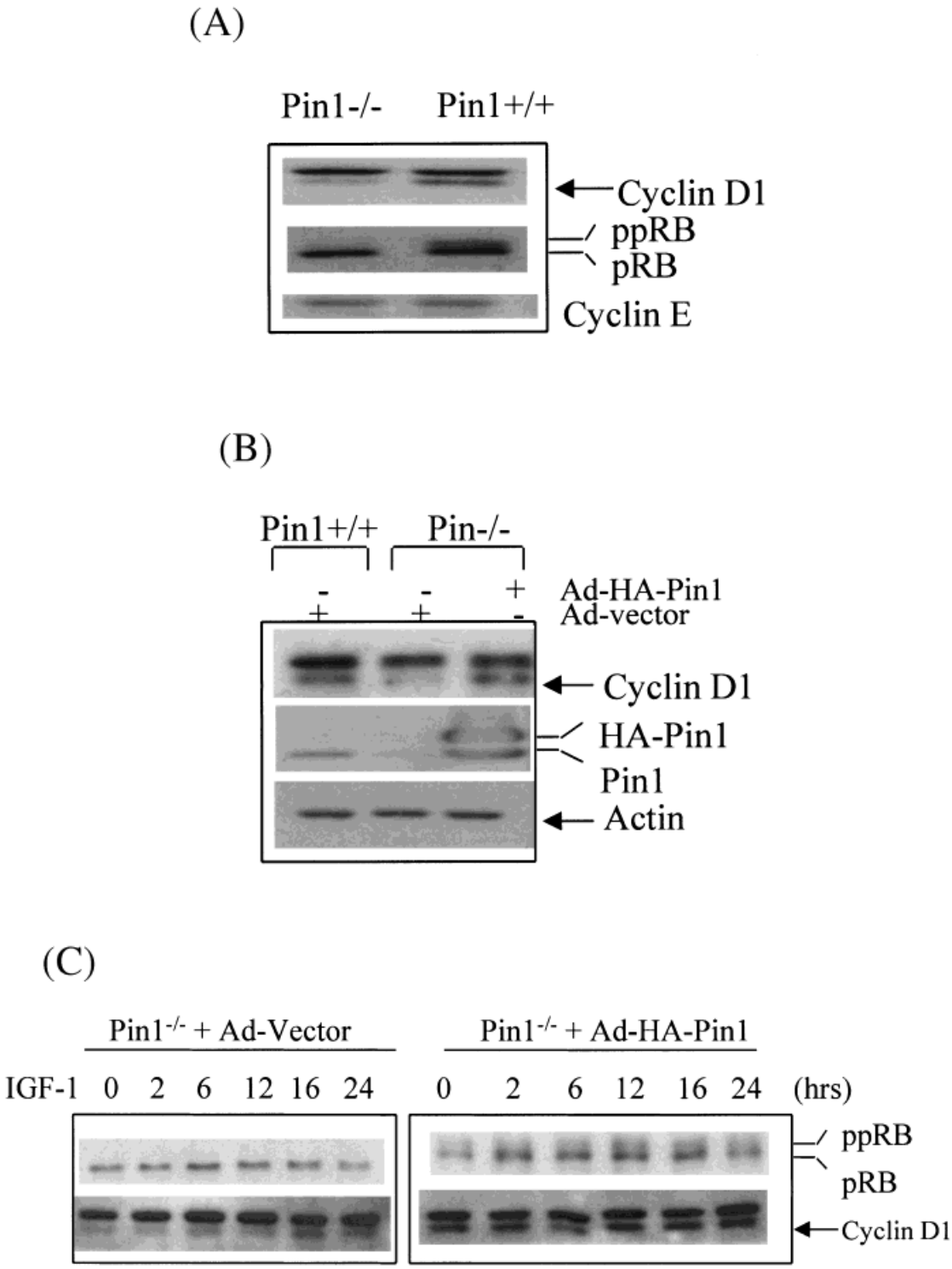

Fig. 4. Pin1 is required for induction of cycling D1 and for timely RB phosphorylation in response to IGF-1. **A**: $Pin1^{-/-}$ and $Pin1^{+/+}$ MEF cells were grown in DMEM medium containing 15% FBS. Equal amounts of cell lysates were subjected to Western Blot analysis for cyclin D1, cyclin E, and RB. **B**: $Pin1^{-/-}$ or $Pin1^{+/+}$ MEF cells at 60–70% confluence in DMEM medium containing 15% FBS were infected with a Pin1 recombinant adenovirus or the virus-vector at the same MOI (200). Twenty-four hours post-infection, cells were harvested and subjected to Western blot analysis for the expression of cyclin D1, Pin1, and actin. **C**: $Pin1^{-/-}$ MEF cells grown in DMEM containing 15% at 60% confluence were infected with a Pin1 recombinant adenovirus or the adenovirus-Vector. Twenty-four hours post-infection, cells were serum-starved in DEME medium containing 0.1% FBS for 48 h prior to the addition of IGF-1 (50 ng/ml). Cells were harvested at the indicated time points and lysed. Cell lysates were subjected to Western blot analysis for RB and cyclin D1.

[Wulf et al., 2001]. Moreover, Pin1 is shown to stimulate cyclin D1 transcription through C-Jun in cooperation with the Ras signaling pathway [Wulf et al., 2001]. It is possible, therefore, that Pin1 may directly activate cyclin D1 transcription in response to IGF-1. Alternatively, Pin1 may activate cyclin D1 at the post-transcriptional levels, such as through regulation of phosphorylation or protein stability.

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