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Cell cycle-dependent phosphorylation of Disabled-2 by cdc2

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Disabled-2 (Dab2; also known as p96 and DOC-2) is a signal transduction protein that has been implicated in the control of cell growth. Dab2 is known to be a phosphoprotein, but little is known about the kinases that phosphorylate Dab2. We have found that Dab2 phosphorylation is markedly increased during the mitosis phase of the cell cycle. This phosphorylation is blocked by roscovitine, a selective inhibitor of cyclin-dependent kinases. Dab2 robustly coimmunoprecipitates from cells with the cyclin-dependent kinase cdc2, and purified cdc2 can phosphorylate purified Dab2 fusion proteins *in vitro* on multiple sites. Cellular phosphorylation of Dab2 by cdc2 promotes the association of Dab2 with Pin1, a peptidylprolyl isomerase that regulates the rate of Dab2 dephosphorylation. These findings reveal that Dab2 is differentially phosphorylated during the cell cycle by cdc2 and provide a potential feedback mechanism by which Dab2 inhibition of cell growth and proliferation may be regulated.
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Introduction

The *Drosophila* protein Disabled (Dab) was originally identified as the product of a gene with a key role in neural development (Gertler *et al*., 1989). Mammals possess two Dab orthologs: Dab1, which is almost exclusively expressed in the brain (Howell *et al*., 1997a), and Dab2 (also called p96 and DOC-2), which is expressed in many tissues (Mok *et al*., 1994; Xu *et al*., 1995; Albertsen *et al*., 1996). Mutations to Dab1 underlie the abnormal brain development observed in the mutant strains of mice known as *scrambler* and *yotari* (Howell *et al*., 1997b; Sheldon *et al*., 1997; Ware *et al*., 1997), suggesting a role for Dab1 in neural development similar to that of *Drosophila* Dab. In contrast, Dab2 plays no known role in neural development, but rather is considered a candidate tumor suppressor because it is downregulated in various types of tumor (Mok *et al*., 1994; Fulop *et al*., 1998; Fazili *et al*., 1999) and is capable of strongly inhibiting cell growth and proliferation in many cell types (Fulop *et al*., 1998; Mok *et al*., 1998; Tseng *et al*., 1999; Sheng *et al*., 2000; He *et al*., 2001b; Smith *et al*., 2001; Wang *et al*., 2001).

The structure of Dab2 suggests that it may act as an adapter protein. Dab2 contains an amino-terminal phosphotyrosine-binding (PTB) domain, which has been shown to associate with transmembrane proteins such as the low-density lipoprotein receptor (Morris and Cooper, 2001) and transforming growth factor β receptors (Hocevar *et al*., 2001), as well as with the Ras GAP DIP1/2 (Wang *et al*., 2002). Additionally, Dab2 contains a carboxy-terminal proline-rich domain (PRD), which can associate with SH3 domain-containing proteins such as Grb2 (Xu *et al*., 1998; Zhou and Hsieh, 2001). It has also recently been reported that the Dab2 carboxyl terminus can associate with myosin VI (Inoue *et al*., 2002; Morris *et al*., 2002). The association of Dab2 with multiple signaling proteins and the lack of intrinsic Dab2 enzymatic activity point to a role for Dab2 as an adapter protein involved in organizing cellular signaling pathways involved in the regulation of cell growth.

Dab2 is known to be heavily phosphorylated in cells (Xu *et al*., 1995; Tseng *et al*., 1999). This phosphorylation is known to occur exclusively on serine residues (Xu *et al*., 1995), but the kinases mediating this phosphorylation have only been partially identified. One kinase that acts on Dab2 is protein kinase C (PKC), which can phosphorylate Dab2 on Ser24 (Tseng *et al*., 1999). It is known that there are multiple other sites of Dab2 phosphorylation (Tseng *et al*., 1999), but the kinase(s) mediating phosphorylation of these other sites have not been elucidated. Since Dab2 plays a key role in regulating cell growth and proliferation, we examined the state of Dab2 phosphorylation during the cell cycle. Our data indicate that Dab2 phosphorylation is profoundly enhanced during mitosis phase due to phosphorylation on multiple sites by the cyclin-dependent kinase cdc2.

Experimental procedures

Cell culture

All tissue culture media and related reagents were purchased from Gibco/Life Technologies. HeLa and

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COS-7 cells were maintained in complete medium (double minimal essential medium plus 10% fetal bovine serum and 1% penicillin/streptomycin) in a 37°C/5% CO_2 incubator. HeLa cells were synchronized at the G1/S phase boundary using the thymidine–aphidicolin double-block method, as described previously (He *et al*., 2001a). Cells in S phase were obtained 3 h after release from the G1/S phase block. Mitosis and G2 phase cells were obtained following a 14–18 h treatment with nocodazole (50 ng/ml, Sigma), as described previously (He *et al*., 2001a). The cell cycle state of the cells harvested following these various protocols was confirmed using flow cytometry, as described previously (He *et al*., 2001a).

Preparation of fusion proteins

Three distinct regions of Dab2 were expressed as glutathione-*S*-transferase (GST) fusion proteins using the pGEX-2TK vector (Pharmacia). The phosphotyrosine interacting domain (PID, amino acids #1–233), middle region (M15, amino acids #335–610), and PRD (amino acids #600–730) of Dab2 were created by PCR using cDNA as a template and inserted into the pGEX-2TK vector. All sequences were confirmed by ABI sequencing. The construct for expressing GST-Pin1 was a generous gift from Michael Yaffe (Massachusetts Institute of Technology).

SDS–PAGE and Western blotting

Samples (5 μg/lane) were run on 4–20% SDS–polyacrylamide gels (SDS–PAGE) (Novex) for 1 h at 150 V, and then transferred to nitrocellulose. The blots were blocked in blot buffer (2% nonfat dry milk, 0.1% Tween 20, 50 mM NaCl, and 10 mM HEPES, pH 7.4) for at least 30 min and then incubated with primary antibody in blot buffer for 1 h at room temperature. The blots were washed three times with 10 ml of blot buffer and incubated for 1 h more at room temperature with a horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech) in blot buffer. Finally, the blots were washed three more times with 10 ml of blot buffer and visualized via enzyme-linked chemiluminescence (ECL) using the ECL kit from Amersham Pharmacia Biotech.

Pull-down assays

GST–PIN1 fusion proteins were purified on glutathione agarose beads, as described previously (He *et al*., 2001a). Aliquots of the fusion protein/bead mixture in 1.5 ml microcentrifuge tubes were blocked for 1 h with 1 ml of a 3% BSA buffer (also containing 10 mM HEPES, 50 mM NaCl, and 0.1% Tween 20), and then incubated with cell lysates for 2 h. The beads were washed five times with ice-cold 3% BSA blocking buffer and washed once with harvest buffer. The proteins were eluted from the beads with 1 × SDS–PAGE sample buffer, resolved via SDS–PAGE and transferred to nitrocellulose. Dab2 was detected via Western blotting with the anti-p96 antibody from Transduction Laboratories (1 : 2000), AP-2 was detected with the anti-AP-2 antibody from Transduction Laboratories (1 : 1000), and bands were visualized via chemiluminescence as described above.

Kinase assays

In vitro phosphorylation experiments were carried out at room temperature in a final volume of 25 μl with 10 mM HEPES, pH 7.4, 10 mM $MgCl_2$, and 2 mM EDTA. In this buffer, purified fusion proteins (2 μg), either GST–PID, GST–M15 or GST–PRD, were incubated with purified recombinant human Cdc2/cyclin B (10 units, Calbiochem). Reactions were initiated via the addition of [γ-^{32}P] ATP (1 μCi; Amersham Pharmacia Biotech) and were allowed to proceed for 30 min at room temperature before being stopped with SDS–PAGE sample buffer.

Phosphorylated samples were run on 4–20% SDS–PAGE, fixed, dried, and exposed to film. The extent of phosphorylation was quantified via the scanning of films followed by densitometric analysis of bands.

Immunoprecipitation

Cells were harvested and lysed in 500 μl of ice-cold lysis buffer (10 mM HEPES, 50 mM NaCl, 5 mM EDTA, 1 mM benzamidine, 0.5% Triton X-100). Lysates were solubilized via end-over-end rotation at 4°C for 30 min and clarified via centrifugation at 14 000 r.p.m. for 15 min. A small fraction of the supernatant was taken at this point and incubated with SDS–PAGE sample buffer in order to examine expression of proteins in the whole cell extract. The remaining supernatant was incubated with 15 μl of anti-Cdc2 (Santa Cruz), anti-CDK5 (Santa Cruz) or anti-CDK2 (Upstate Biotechnology) for 2 h with end-over-end rotation at 4°C before being incubated with Protein A/G agarose beads (Sigma) for 1 h. For the mock-immunoprecipitated sample, Protein A/G agarose beads were added in the absence of any antibody. Following the 1 h incubation, the beads were washed five times with 1.0 ml of lysis buffer, and immunoprecipitated proteins were eluted from the beads with 1 × SDS–PAGE sample buffer, resolved by SDS–PAGE, and subjected to Western blot analysis with anti-Dab2 antibodies (Transduction Labs) or anti-AP-2 antibodies (Transduction Labs).

Results

Dab2 is hyperphosphorylated during mitosis phase

Phosphorylation of Dab2 is known to correlate with reduced mobility of Dab2 on SDS–PAGE (Xu *et al*., 1995; Tseng *et al*., 1999). We performed Western blot analyses of Dab2 in HeLa cells that were synchronized and harvested at various points during the cell cycle. The SDS–PAGE mobility of Dab2 was consistent across all of the cell cycle phases except for mitosis phase, where the Dab2 polypeptide exhibited a striking decrease in electrophoretic mobility (Figure 1a). This

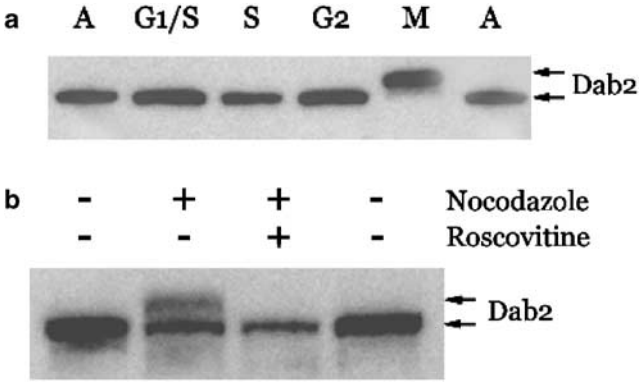

Figure 1 Dab2 exhibits a cell cycle-dependent mobility shift on SDS–PAGE. (**a**) Dab2 mobility shifts during mitosis phase. Lysates of asynchronous HeLa cells (**a**) or HeLa cells synchronized at G1/S phase (G1/S), S phase (S), G2 phase (G2) or mitosis phase (M) were separated on 4–20% SDS–PAGE gels and then transferred to nitrocellulose. The blots were probed with an anti-Dab2 antibody. Almost all Dab2 protein shifted to a larger apparent size during mitosis phase. The blot shown is representative of five independent experiments. (**b**) The mitosis phase Dab2 mobility shift is due to phosphorylation by cyclin-dependent kinases. HeLa cells were arrested in mitosis phase with nocodazole (50 μM, 14–16 h), and mitosis-phase Dab2 was shifted to a larger apparent size as shown in the previous panel. However, in the presence of roscovitine (20 nM), a selective inhibitor of cyclin-dependent kinases, the Dab2 SDS–PAGE mobility shift was completely blocked. This blot is representative of three independent experiments

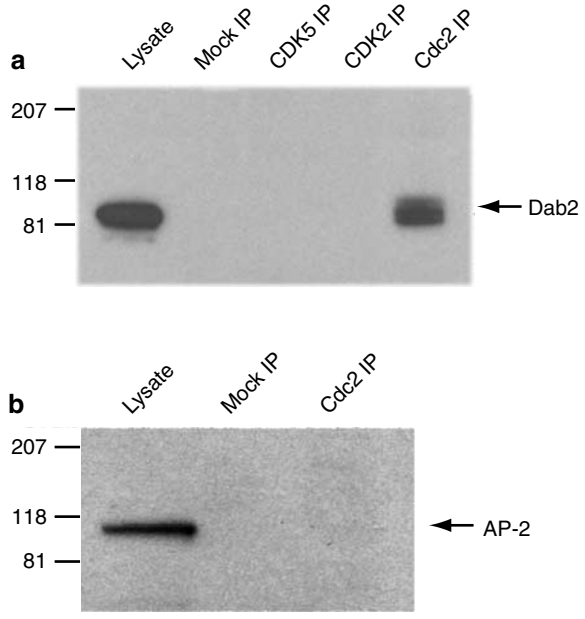

Figure 2 Dab2 associates with cdc2 in cells. (**a**) Mitosis-phase lysates were immunoprecipitated with anti-cdc2, anti-CDK2 or anti-CDK5 antibodies. The immunoprecipitates were fractionated along with a mock-immunoprecipitated sample ('Mock IP') on 4–20% SDS–PAGE gels, transferred to nitrocellulose and Western blotted with an anti-Dab2 antibody. Coimmunoprecipitation of Dab2 was observed only in the anti-cdc2 immunoprecipitates, revealing that cdc2 and Dab2 form a complex in cells during mitosis phase. (**b**) Lack of interaction between AP-2 and cdc2. Anti-cdc2 immunoprecipitates were also probed via Western blot with an antibody specific for AP-2. No AP-2 immunoreactivity could be detected in the immunoprecipitates, demonstrating the specificity of the coimmunoprecipitation between Dab2 and cdc2. The blots shown in both panels of this figure are representative of at least three independent experiments each. The sizes (in kDa) and positions of molecular mass standards are shown on the left side of each panel

shift in the apparent size of Dab2 was reversed via treatment of the harvested lysates with alkaline phosphatase (data not shown), indicating that the shift is due to phosphorylation of Dab2. Moreover, the mitosis-phase-dependent shift in Dab2 electrophoretic mobility was completely blocked by incubation of the cells with roscovitine, a specific inhibitor of cyclin-dependent kinases (Figure 1b). Similar observations of the increased apparent size of Dab2 during mitosis phase and the blockade of this effect by roscovitine were made in COS-7 cells (data not shown). These data indicate that Dab2 is phosphorylated by cyclin-dependent kinases during mitosis phase.

Dab2 associates with and is phosphorylated by cdc2

In order to examine the specific cyclin-dependent kinase that might be responsible for the observed mitosis-phase phosphorylation of Dab2, coimmunoprecipitation experiments were performed. Interactions between cyclin-dependent kinases and their substrates are typically quite stable and can frequently be detected via coimmunoprecipitation (Endicott *et al*., 1999). We immunoprecipitated three cyclin-dependent kinases from mitosis-phase HeLa cell lysates: cdc2 (CDK1), CDK2, and CDK5. Dab2 specifically coimmunoprecipitated with cdc2 but was not detectably coimmunoprecipitated with the other two kinases (Figure 2a). Anti-cdc2 immunoprecipitates were also probed for AP-2, an unrelated protein that is abundant in HeLa cells and similar in size to Dab2. No AP-2 immunoreactivity could be detected in the cdc2 immunoprecipitates (Figure 2b), demonstrating the specificity of the coimmunoprecipitation between Dab2 and cdc2. These data reveal that cdc2 specifically associates with Dab2 and suggest that cdc2 may be the kinase responsible for the observed phosphorylation of Dab2 during mitosis phase.

In vitro phosphorylation experiments with purified cdc2 and purified Dab2 fusion proteins were performed next to see if Dab2 is in fact a substrate for cdc2. As shown in Figure 3, GST fusion proteins corresponding to the PTB and PRD domains of Dab2 were robustly phosphorylated by purified cdc2. In contrast, neither control GST nor a GST fusion protein corresponding to the middle portion of the Dab2 polypeptide, referred to as the M15 region (Xu *et al*., 1995) were detectably phosphorylated in these experiments. These findings indicate that cdc2 can phosphorylate Dab2 on multiple sites in two distinct domains of the Dab2 protein.

cdc2-phosphorylated Dab2 associates with Pin1

A number of signaling proteins are known to interact with the peptidylprolyl isomerase Pin1, following cdc2-

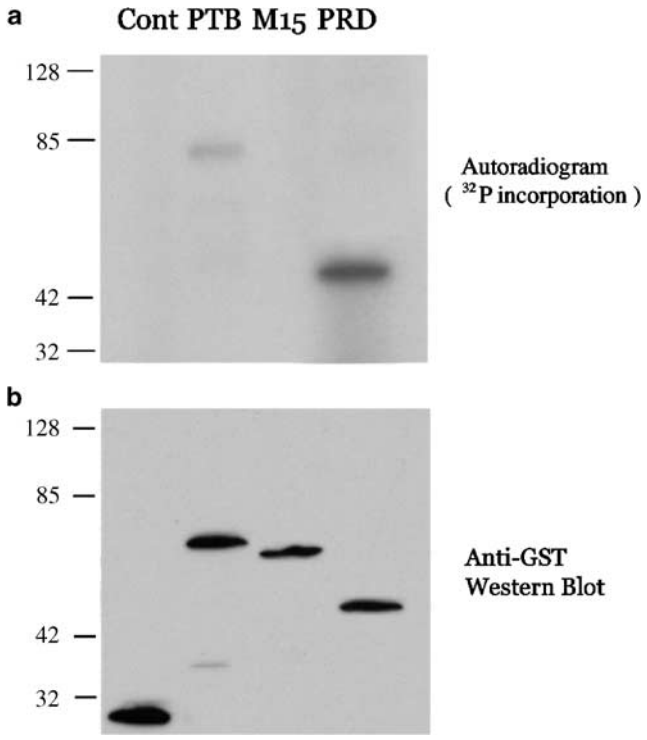

Figure 3 Dab2 is phosphorylated by purified cdc2. (**a**) The PTB, M15, and PRD domains of Dab2 were expressed as GST fusion proteins, and 2 μg of each was incubated with purified cdc2/cyclin B (10 U) for 30 min in the presence of [^{32}P]-ATP. The resultant phosphorylation is shown in this autoradiogram, which is representative of three independent experiments. The PTB and PRD domains of Dab2 were phosphorylated by purified cdc2 *in vitro*, while M15 was not phosphorylated at all. (**b**) The relative loading and size of the fusion proteins examined for cdc2 phosphorylation in the experiment shown in panel a is shown in an anti-GST Western blot. The sizes (in kDa) and positions of molecular mass standards are shown on the left side of each panel

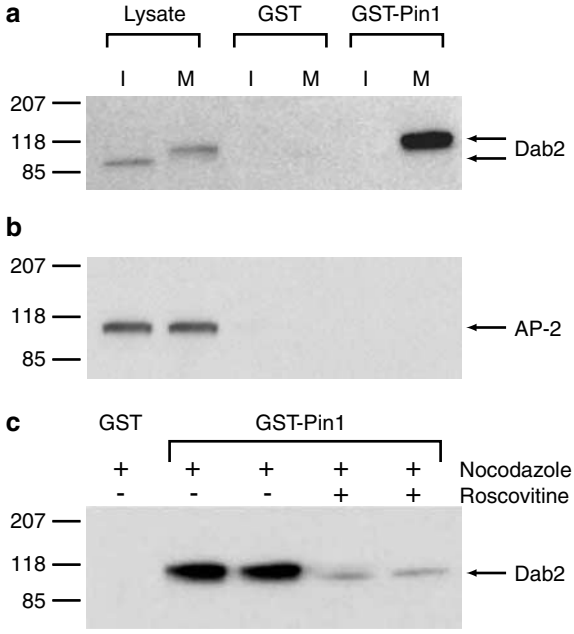

Figure 4 Interaction of mitosis-phase Dab2 with Pin1. (**a**) HeLa cell lysates prepared from interphase (I) or mitosis phase (M) were incubated with glutathione agarose beads loaded with GST or GST-Pin1. After washing, proteins associated with the beads were subjected to Western blotting with an anti-Dab2 antibody. No binding of Dab2 to control GST was detectable under any condition. In contrast, the GST-Pin1 beads robustly pulled down Dab2 from mitosis-phase lysates but not from interphase lysates, revealing an association between Pin1 and Dab2 that is hyperphosphorylated in mitotic cells. (**b**) The same samples from the experiments illustrated in panel a were also probed via Western blot for AP-2 immunoreactivity. AP-2 was robustly detected in the cell lysates but could not be detected in any of the GST or GST-Pin1 pull-down samples, demonstrating the specificity of the interaction between Dab2 and Pin1. (**c**) Blocking Dab2 phosphorylation inhibits Dab2 interaction with Pin1. HeLa cells were stopped in mitosis phase via treatment with nocodazole (50 μM, 14–16 h), and Dab2 was robustly pulled down from these lysates with GST-Pin1, as shown in the previous panel. However, treatment of the cells with the cyclin-dependent kinase inhibitor roscovitine (20 nM, 14–16 h) sharply reduced the amount of Dab2 that could be pulled down with GST-Pin1, indicating that the Dab2/Pin1 association requires phosphorylation by cyclin-dependent kinases such as cdc2. The data shown are representative of four independent experiments. The sizes (in kDa) and positions of molecular mass standards are shown on the left side of each panel

mediated phosphorylation (Lu *et al*., 2002). We therefore examined the potential association of Dab2 with Pin1. Lysates from HeLa cells harvested during either interphase or mitosis were incubated with beads loaded with either GST or GST-Pin1. No Dab2 association with control GST was detected, nor was any Dab2 association with GST-Pin1 evident in the lysates derived from interphase HeLa cells. In contrast, Dab2 from mitosis-phase lysates was robustly pulled down by GST-Pin1 (Figure 4a). GST-Pin1 failed to pull down any detectable AP-2 from the cell lysates (Figure 4b), demonstrating the specificity of the interaction between Dab2 and Pin1. The association of Dab2 with Pin1 was blocked by treatment of mitosis-phase cells with roscovitine (Figure 4c), suggesting that the Dab2/Pin1 interaction is dependent upon cdc2-mediated phosphorylation.

Dephosphorylation of Dab2 is dependent on Pin1 and PP1/PP2A

We next examined the dephosphorylation of Dab2. HeLa cells were reseeded after mitosis phase and examined via Western blot at various time points. Dab2 was progressively dephosphorylated over several hours, with 4 h being the time point at which full dephosphorylation was consistently observed (Figure 5a). The half-life for the return of Dab2 to the dephosphorylated state was approximately 1.5 h, as shown in the quantification of the data presented in Figure 5b. This time frame corresponded well with the time it took for the cells to completely proceed through mitosis and exit mitosis phase, according to our own flow cytometry studies (data not shown) and the previous work of others on the exit of HeLa cells from nocodazole-induced blockade of mitosis (Zieve *et al*., 1980). The dephosphorylation of Dab2

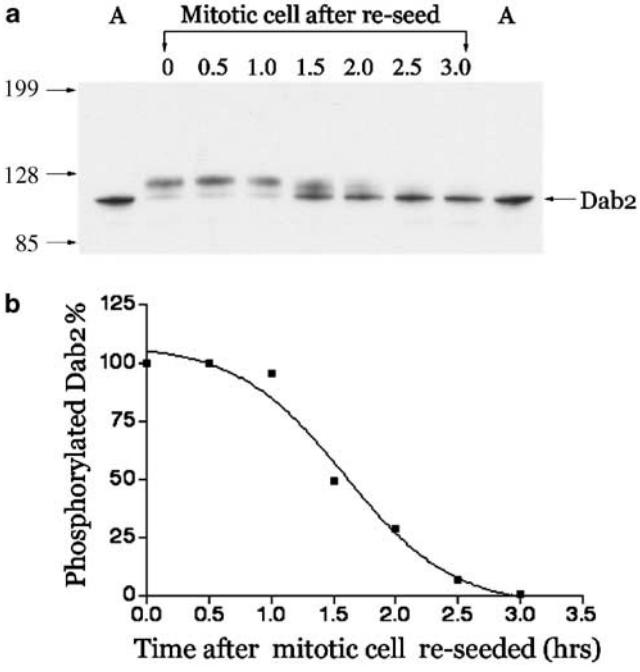

Figure 5 Dab2 is dephosphorylated following cell exit from mitosis phase. Mitotic HeLa cells were washed three times with DMEM medium and then reseeded in six-well plates. The cells were harvested at different time points and run on 4–12% SDS–PAGE gels. The time course of Dab2 dephosphorylation is shown in panel a. Dab2 from asynchronous HeLa cells ('A') is shown in lane 1, while Dab2 from mitosis phase cells is shown in lane 2. The mitotic Dab2 exhibited a mobility shift on SDS–PAGE, due to Dab2 phosphorylation by cyclin-dependent kinases (as shown in previous figures). The mitosis phase-dependent mobility shift disappeared over a period of 2–3 h. The bands corresponding to hyperphosphorylated Dab2 were quantified via densitometry, and a graphical representation of the rate of Dab2 dephosphorylation is shown in panel b. These data represent averaged values from three independent experiments

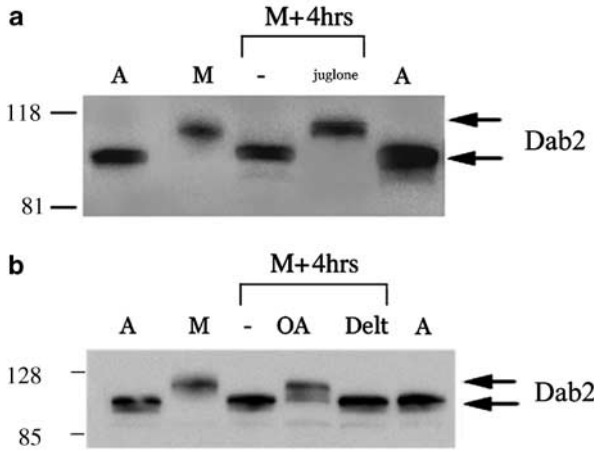

Figure 6 Pin1 and PP1/PP2A play a role in Dab2 dephosphorylation. (**a**) Dab2 dephosphorylation is blocked by Pin1 inhibition. Mitotic HeLa cells were washed three times with DMEM medium and reseeded in six-well plates. The cells were then harvested after a 4 h incubation in the absence or presence of the Pin1 inhibitor juglone (20 μg/ml). Dab2 dephosphorylation was blocked by the juglone treatment, indicating that Pin1 prolyl isomerase activity promotes Dab2 dephosphorylation. (**b**) Dab2 dephosphorylation is mediated by PP1and/or PP2A. Mitotic HeLa cells were reseeded in six-well plates and then harvested after 4 h in the absence or presence of the PP1/PP2A-selective phosphatase inhibitor okadaic acid ('OA': 1 μM) or the PP2B-selective inhibitor deltamethrin ('Delt': 10 μM). Treatment with okadaic acid blocked Dab2 dephosphorylation, while deltamethrin had no effect, indicating that PP1 and/or PP2A are the phosphatases responsible for the observed dephosphorylation of Dab2. Molecular mass standards are shown on the left. All data shown in this figure are representative of at least three independent experiments

following exit from mitosis phase was completely blocked by incubation of the cells with juglone, an inhibitor of Pin1 peptidylprolyl isomerase activity (Figure 6a). These data reveal a key role for Pin1 in regulating the dephosphorylation of cdc2-phosphorylated Dab2.

Many substrates for cyclin-dependent kinases are dephosphorylated by protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) (Veeranna *et al*., 1995; Ajiro *et al*., 1996; Kwon *et al*., 1997; He *et al*., 2001a), which explains in part why inhibitors of PP1 and PP2A are potent tumor promoters (Haystead *et al*., 1989; Suganuma *et al*., 1990). We examined the abilities of the PP1/PP2A-selective inhibitor okadaic acid and the PP2B-selective inhibitor deltamethrin to block dephosphorylation of Dab2. As shown in Figure 6b, okadaic acid strongly inhibited Dab2 dephosphorylation following the end of mitosis phase, while deltamethrin had no significant effect on Dab2 postmitotic dephosphorylation. These data indicate that Dab2 is dephosphorylated following the end of mitosis phase by PP1 and/or PP2A, but not by PP2B.

Discussion

We have found that Dab2 is hyperphosphorylated in mitosis phase. We have also found that this phosphorylation is mediated by cdc2, a cyclin-dependent kinase that is known to be most active in mitosis phase (Kishimoto and Okumura, 1997). The motif preferred for phosphorylation by cdc2 is a serine or threonine followed by a proline, with flanking residues playing a modulatory role (Holmes and Solomon, 1996; Endicott *et al*., 1999). Analysis of the Dab2 polypeptide sequence reveals a number of serines that conform to the preferred cdc2 phosphorylation motif. Indeed, our data demonstrate that Dab2 can be phosphorylated on multiple sites by cdc2, since both the Dab2 PTB and PRD domains are excellent substrates for phosphorylation by purified cdc2.

The only kinase that has been previously reported to phosphorylate Dab2 is PKC, which phosphorylates Ser24 (Tseng *et al*., 1999). This phosphorylation enhances the association of Dab2 with the Ras GAP Dip1/2 (Wang *et al*., 2002). Interestingly, phosphorylation of Dab2 by cdc2 also promotes Dab2 association with another protein, the peptidylprolyl isomerase Pin1. A number of proteins that are phosphorylated by cdc2 have been shown to associate with Pin1 following phosphorylation (Lu *et al*., 2002). Pin1 contains a WW domain that binds with high affinity to particular motifs

containing proline residues and phosphorylated serines (Verdecia *et al*., 2000). The association of some phosphoproteins with Pin1 has been found to regulate their rate of dephosphorylation, perhaps through Pin1-induced conformational changes that alter the accessibility of phosphorylated residues to phosphatases (Lu *et al*., 2002). Our data reveal that the interaction between Dab2 and Pin1 facilitates Dab2 dephosphorylation. Moreover, this interaction may have other functional implications for Dab2, given the large number of proteins involved in the regulation of cell growth that are known to associate with Pin1.

Dab2 shares significant sequence homology with Dab1 (Howell *et al*., 1997a). Mammalian Dab1 is known to be phosphorylated by the tyrosine kinase Src (Keshvara *et al*., 2001) and by the cyclin-dependent kinase cdk5 (Keshvara *et al*., 2002). None of the identified Dab1 phosphorylation sites, however, are conserved in Dab2. Indeed, we found no evidence in our studies for Dab2 interaction with cdk5, although we did detect a robust physical association between Dab2 and cdc2. These findings suggest that Dab1 and Dab2 are phosphorylated by distinct cyclin-dependent kinases, perhaps reflecting the distinct roles of these two signaling proteins in regulating cellular processes.

Since Dab2 is a candidate tumor-suppressor gene product (Mok *et al*., 1994; Fulop *et al*., 1998; Fazili *et al*., 1999) and is known to inhibit cell growth and proliferation in various cell types (Fulop *et al*., 1998; Mok *et al*., 1998; Tseng *et al*., 1999; Sheng *et al*., 2000; He *et al*., 2001b; Smith *et al*., 2001; Wang *et al*., 2001), the observation that its phosphorylation state and interactions with other proteins can change during the cell cycle is of interest. Cyclin-dependent kinases are known to phosphorylate many proteins that regulate the rate of progression through the cell cycle and exert reciprocal effects on cyclin-dependent kinase activity (Ferrell, 2002). Along these lines, the cdc2-mediated phosphorylation of Dab2 described here may represent a feedback mechanism by which the effects of Dab2 on cell growth may be regulated during the cell cycle.

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