Plk1 promotes nuclear translocation of human Cdc25C during prophase

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The nuclear accumulation of active M-phase promoting factor (MPF) during prophase is thought to be essential for coordinating M-phase events in vertebrate cells. The protein phosphatase Cdc25C, an activator of MPF, enters the nucleus to keep MPF active in the nucleus during prophase. However, the molecular mechanisms that control nuclear translocation of Cdc25C during prophase are unknown. We show that phosphorylation of a serine residue (Ser198) in a nuclear export signal sequence of human Cdc25C occurs during prophase and promotes nuclear localization of Cdc25C. We also show that Polo-like kinase 1 (Plk1) is responsible for this phosphorylation and that constitutively active Plk1 promotes nuclear localization of Cdc25C. Remarkably, a mutant Cdc25C in which Ser198 is replaced by alanine remains in the cytoplasm when wild-type Cdc25C accumulates in the nucleus during prophase. These results suggest that Plk1 phosphorylates Cdc25C on Ser198 and regulates nuclear translocation of Cdc25C during prophase.

INTRODUCTION

In eukaryotic cells, initiation of mitosis requires the activation of M-phase promoting factor (MPF), the complex of a cyclin-dependent kinase Cdc2 and the B-type cyclin (for reviews, see Nurse, 1990; Hunt, 1991). The kinase activity of Cdc2/cyclin-B is controlled by phosphorylation of Cdc2 and accumulation of cyclin B protein. During S and G₂ phases, B-type cyclins accumulate and bind to Cdc2 to form heterodimers. Cyclin B facilitates the inhibitory phosphorylation of Cdc2 at Thr14 and Tyr15, which are catalyzed by Wee1, Mik1 and Myt1 kinases. At the end of G₂, abrupt dephosphorylation of these sites by Cdc25 triggers the activation of Cdc2/cyclin-B (for reviews, see Coleman and Dunphy, 1994; Morgan, 1995; Nigg, 2001). In addition to cyclin B accumulation and phosphorylation of Cdc2, intracellular localization of cyclin B1 is also regulated during the progression

of the cell cycle (for reviews, see Pines, 1999; Yang and Kornbluth, 1999). In interphase, cyclin B1 localizes to the cytoplasm due to its NES-dependent nuclear export (Hagting *et al.*, 1998; Toyoshima *et al.*, 1998; Yang *et al.*, 1998). During prophase, cyclin B1 becomes phosphorylated, and the phosphorylation causes nuclear translocation of cyclin B1 (Li *et al.*, 1997; Yang *et al.*, 1998; Hagting *et al.*, 1999; Toyoshima-Morimoto *et al.*, 2001; Yang *et al.*, 2001). The nuclear accumulation of active MPF during prophase is thought to be important for initiating and coordinating M-phase events in vertebrate cells (Heald *et al.*, 1993; Li *et al.*, 1997; Pines, 1999; Yang and Kornbluth, 1999; Takizawa and Morgan, 2000).

However, because Wee1 is constantly nuclear (Heald *et al.*, 1993), the nuclear MPF is inactivated unless the activator phosphatase Cdc25C counteracts the action of Wee1 (Seki *et al.*, 1992; Heald *et al.*, 1993; Dalal *et al.*, 1999). Recent studies have shown that Cdc25C is cytoplasmic during interphase and accumulates in the nucleus during prophase (Seki *et al.*, 1992; Heald *et al.*, 1993; Dalal *et al.*, 1999). The activity of Cdc25C is increased during G₂-M phase, and this activation correlates with its hyperphosphorylation in the N-terminal portion (Izumi *et al.*, 1992; Kumagai and Dunphy, 1992; Hoffmann *et al.*, 1993; Izumi and Maller, 1993), which may in part be attributed to an auto-amplification loop (Hoffmann *et al.*, 1993; Izumi and Maller, 1993; Strausfeld *et al.*, 1994) involving Cdc2/cyclin-B. Therefore, the nuclear accumulation of Cdc25C may play an essential role in maintaining the activity of Cdc2/cyclin-B1 in the nucleus during prophase by counteracting the inhibitory activity of Wee1.

It has been shown that human Cdc25C is phosphorylated on Ser216 during interphase (Peng *et al.*, 1997) and that this phosphorylation creates a binding site for 14-3-3 proteins (Peng *et al.*, 1997; Kumagai *et al.*, 1998a), which are known to bind to a phospho-Ser residue in a number of proteins and regulate their

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functions. C-TAK1 has been shown to be one of the kinases responsible for this phosphorylation (Peng *et al.*, 1998). Check-point kinases Chk1 and Chk2 have also been shown to catalyze this phosphorylation (Furnari *et al.*, 1997; Peng *et al.*, 1997; Sanchez *et al.*, 1997; Kumagai *et al.*, 1998b; Matsuoka *et al.*, 1998; Blasina *et al.*, 1999). Several reports suggested that the cytoplasmic localization of Cdc25C in interphase is maintained by binding to 14-3-3 proteins (Dalal *et al.*, 1999; Kumagai and Dunphy, 1999; Yang *et al.*, 1999; Zeng and Piwnicka-Worms, 1999) and that dephosphorylation of Ser216 occurs during M phase and blocks the interaction between Cdc25C and 14-3-3 proteins (Peng *et al.*, 1997; Kumagai *et al.*, 1998a). Most recently, several groups identified a nuclear export signal (NES) sequence in both *Xenopus* and human Cdc25C and showed that Cdc25C is exported from the nucleus by the NES-dependent transport mechanism (Kumagai and Dunphy, 1999; Yang *et al.*, 1999; Graves *et al.*, 2001). Thus, if Cdc25C is dissociated from 14-3-3 proteins, it should still remain in the cytoplasm. How nuclear translocation of Cdc25C during prophase is caused has not been defined. In this study, we show that phosphorylation of a serine residue (Ser198) in the NES sequence of human Cdc25C occurs during prophase and that this phosphorylation is necessary for the nuclear localization of Cdc25C. We also show that Polo-like kinase 1 (Plk1) (for reviews, see Glover *et al.*, 1998; Nigg, 1998) is responsible for this phosphorylation. As Plk1 has been shown previously to phosphorylate cyclin B1 and induce its nuclear entry during prophase (Toyoshima-Morimoto *et al.*, 2001), it could regulate simultaneity of nuclear entry of Cdc2/cyclin-B1 and its activator Cdc25C and thus act as an important coordinator for intracellular localization of active MPF during G₂-M transition.

RESULTS AND DISCUSSION

To examine the involvement of 14-3-3 proteins in the cytoplasmic localization of Cdc25C in interphase, we examined intracellular localization of a mutant form of Cdc25C, in which Ser216 is replaced by Ala to eliminate the association with 14-3-3 proteins (S216A-Cdc25C) in HeLa cells. The S216A-Cdc25C, like wild-type (WT) Cdc25C, still largely localized to the cytoplasm (Figure 1, S216A). After treatment with leptomycin B (LMB), a specific inhibitor of the NES receptor CRM1, WT-Cdc25C became localized to the nucleus, and S216A-Cdc25C strongly accumulated in the nucleus (data not shown). These results suggest that human Cdc25C, in interphase cells, is exported from the nucleus by an NES-mediated transport mechanism, which is independent of 14-3-3 proteins, and that the 14-3-3 binding may ensure cytoplasmic localization of Cdc25C by inhibiting its nuclear import, as described previously for the *Xenopus* system (Kumagai and Dunphy, 1999; Yang *et al.*, 1999). We found five putative NES sequences (99-LDSSGLQEVHL-109, 190-ISDELMEFSL-199, 256-LCLKTYSL-264, 318-FQGLIEKFYV-327 and 337-LGGHMQGALNL-347) in human Cdc25C. When HA-tagged mutant forms of human Cdc25C, in which two C-terminal hydrophobic residues in each NES sequence, in addition to Ser216, were replaced by Ala (107,109,216A-, 197,199,216A-, 262,264,216A-, 325,327,216A- and 345,347,216A-Cdc25C) were expressed in HeLa cells, only the 197,199,216A-Cdc25C accumulated in the nucleus (Figure 1; data not shown). A mutant 197,199A-Cdc25C, in which Ser216

is not mutated, localized, but did not accumulate, to the nucleus (Figure 1, 197,199A). When a fusion protein between GST and a polypeptide corresponding to residues 173–206 of Cdc25C [GST-Cdc25C (173–206)] was injected into the nucleus, it was exported from the nucleus in an LMB-sensitive manner (data not shown). These results indicate that the amino-acid sequence of residues 190–199 of Cdc25C (see Figure 2A) is a functional NES. A recent report identified, using different approaches, the same portion as an NES of human Cdc25C (Graves *et al.*, 2001).

Cyclin B1 has been shown to be exported from the nucleus by its NES-dependent mechanism in interphase cells (Hagting *et al.*, 1998; Toyoshima *et al.*, 1998; Yang *et al.*, 1998), and phosphorylation of a serine residue (Ser147), which resides in the middle of the NES sequence (see Figure 2A), inactivates the NES and promotes nuclear translocation of cyclin B1 during prophase (Toyoshima-Morimoto *et al.*, 2001; Yang *et al.*, 2001). As two serine residues (191 and 198) reside in the middle of the NES sequence of human Cdc25C, we hypothesized that phosphorylation of these serines might inactivate the NES and promote nuclear localization of Cdc25C. We constructed a mutant form of Cdc25C (S191,198E), in which both serines were replaced by Glu to mimic phosphorylation. This mutant Cdc25C became localized to the nucleus (Figure 1, S191,198E). The nuclear localization was enhanced when Ser216 was further replaced by Ala (Figure 1, S191,198E,S216A). Thus, it is likely that phosphorylation of Ser191 and/or Ser198 may inactivate the NES and promote the nuclear localization of Cdc25C.

Previous reports have shown that Plx1, a *Xenopus* homolog of Plk1, phosphorylates and activates Cdc25C (Kumagai and Dunphy, 1996). However, the phosphorylation sites have not been determined, and whether Plx1 or Plk1 regulates subcellular localization of Cdc25C has not been examined. We reported previously that Plk1 is a kinase that phosphorylates cyclin B1 on Ser147 (Toyoshima-Morimoto *et al.*, 2001) (Figure 2A). The NES sequence of cyclin B1 and that of Cdc25C are both rich in hydrophobic and acidic amino-acid residues (Figure 2A). Our preliminary data suggest that serine residues present in such sequences could be good substrates for Plk1 (H. Nakajima, F. Toyoshima-Morimoto and E. Nishida, unpublished data). We tested whether Plk1 phosphorylates human Cdc25C on Ser191 and/or Ser198. Because Plk1 phosphorylates Cdc25C on multiple sites in the full-length Cdc25C (data not shown), we used GST-Cdc25C(173–206) as a substrate here. Recombinant His-Plk1, which was expressed in, and purified from, bacteria, phosphorylated WT-Cdc25C (173–206) but not S191,198A-Cdc25C (173–206) (Figure 2B, upper). S191A-Cdc25C (173–206) was phosphorylated well, but S198A-Cdc25C (173–206) was scarcely phosphorylated (Figure 2B, upper panel). HA-tagged Plk1, which was expressed in COS cells and purified by immunoprecipitation with anti-HA-antibody, also showed essentially the same substrate specificity (Figure 2B, lower panel). Moreover, His-Plk1 phosphorylated a synthetic peptide corresponding to residues 188–201 (EEISDELMEFSLKD) of Cdc25C (OVA-NES) but not a phosphopeptide (EEISDELMEF-phosphoS198-LKD, OVA-P-NES) (Figure 2C). To examine whether Plk1 phosphorylates full-length Cdc25C on Ser198, we produced anti-phosphoS198 Cdc25C antibody by immunizing rabbits with the phosphoS198-NES peptide (Figure 2C). This antibody reacted with bacterially produced GST-WT-Cdc25C (full length), but not with GST-S198A-Cdc25C (full length), only after pre-incubation with His-Plk1 and ATP

Fig. 1. Serine phosphorylation in the NES sequence of human Cdc25C causes its nuclear localization. Indicated forms of HA-Cdc25C were expressed in HeLa cells. More than 200 cells were examined in each condition, and representative images are shown in the upper panels and DAPI staining shown below. Cells were classified into three categories in terms of location of the transfected HA-tagged Cdc25C, and the percentages of each category are shown in the bottom graph, where N>C shows staining in the nucleus (N) stronger than that in the cytoplasm (C), N=C shows N equal to C, and N<C shows C stronger than N.

(Figure 2D). Taken together, these results show that Plk1 directly phosphorylates Cdc25C on Ser198.

We then examined the activity of endogenous Plk1 in synchronized HeLa cells. The kinase activity of endogenous Plk1, which was immunoprecipitated with anti-Plk1 antibody, toward WT-Cdc25C (173–206) was increased during G₂–M phase, 8–10 h after release from a double thymidine block (Figure 3A, fourth row). Immunoprecipitated Plk1 hardly phosphorylated S198A-Cdc25C (173–206) (Figure 3A, fifth row).

This profile coincided with the kinase activity of total lysates toward WT-Cdc25C and S198A-Cdc25C (173–206) (Figure 3A, first and second rows, respectively). The WT-Cdc25C (173–206)-phosphorylating activity in the extracts from M-phase HeLa cells was decreased markedly by the immunodepletion treatment with increasing amounts of anti-Plk1 antibody, but it was not decreased by the treatment with control IgG (Figure 3B). Bacterially produced His-Plk1 protein restored the activity of the Plk1-immunodepleted extract (see Supplementary figure 1 available

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Fig. 2. Plk1 phosphorylates Ser198 of Cdc25C. (A) NES sequences of cyclin B1 (human) and Cdc25C (human). Important hydrophobic residues in NES are boxed. (B) The kinase activities of recombinant His-tagged Plk1, which was expressed in and purified from bacteria, and HA-tagged Plk1, which was expressed in COS cells and purified by immunoprecipitation with anti-HA, were measured using indicated forms of GST-Cdc25C (173–206). (C) A synthetic peptide corresponding to residues 188–201 (EEISDELMEFSLKD) of human Cdc25C (NES) or a synthetic phosphopeptide [EEISDELMEF-phosphoS198-LKD (phosphoS198-NES)] was conjugated to ovalbumin (OVA). The kinase activities of His-tagged Plk1 toward OVA, OVA-NES and OVA-P-NES are shown. (D) Bacterially produced GST-WT-Cdc25C (full length) and GST-S198A-Cdc25C (full length) were pre-incubated with or without His-Plk1 in the presence of ATP, subjected to SDS-PAGE, transferred to a nylon membrane and then blotted with anti-phosphoS198 Cdc25C antibody (top row). The bottom row shows CBB staining of GST-Cdc25C (full length) on the membrane.

at *EMBO reports* Online). These results suggest that Plk1 is a major kinase that phosphorylates Cdc25C on Ser198 during G_2 –M transition.

To test whether phosphorylation of Ser198 could promote nuclear localization of Cdc25C, we examined the localization of S198E-Cdc25C. As shown in Figure 1, S198E-Cdc25C became localized to the nucleus. The nuclear localization was enhanced when Ser216 was further replaced by Ala (Figure 1, S198E,S216A). Thus, phosphorylation of Ser198 could stimulate nuclear localization of Cdc25C.

Our results suggest that Plk1 phosphorylates Cdc25C on Ser198 during prophase and promotes its nuclear localization. To test this further, we expressed Plk1 with Cdc25C in HeLa cells. When co-expressed with WT-Plk1, both WT-Cdc25C and

Fig. 3. Plk1 phosphorylates Cdc25C on Ser198 during G_2 –M phase in HeLa cells. (A) HeLa cells were arrested at the G_1 –S boundary by a double thymidine block, and cell extracts were prepared at the indicated times after release. The kinase activities in the extracts toward GST-WT-Cdc25C (173–206), S198A-Cdc25C (173–206) and histone H1 are shown in the first, second and third rows, respectively. Endogenous Plk1 was immunoprecipitated from the extracts with anti-Plk1 antibody. The kinase activities of the immunoprecipitates toward GST-WT-Cdc25C (173–206) and S198A-Cdc25C (173–206) are shown in the fourth and fifth rows, respectively. (B) Endogenous Plk1 was immunoprecipitated from the synchronized M-phase extracts with increasing amounts (0, 2.5 and 7.5 μ g) of anti-Plk1 antibody or non-immunized rabbit IgG, and the kinase activity remaining in the supernatant was measured using GST-WT-Cdc25C (173–206) as a substrate (top row). The supernatants were analyzed by SDS-PAGE followed by immunoblotting with anti-Plk1 antibody (bottom row).

S216A-Cdc25C remained in the cytoplasm (Figure 4A, lanes 2 and 5). Co-expression of S216A-Plk1, a constitutively active form of Plk1 (Qian *et al.*, 1999), stimulated nuclear localization of S216A-Cdc25C but not WT-Cdc25C (Figure 4A, lanes 3 and 6). This supports the above idea. However, as the stimulatory effect was not very strong, we then examined whether Cdc2/cyclin-B1, which is able to phosphorylate Cdc25C (Hoffmann *et al.*, 1993; Izumi and Maller, 1993; Strausfeld *et al.*, 1994), affects nuclear localization of Cdc25C, by co-expressing cyclin B1 with Plk1. Because a high-level expression of cyclin B1 together with Cdc25C strongly induced premature mitosis (data not shown), we expressed cyclin B1 at a very low level. Expression of cyclin B1 alone or cyclin B1 plus WT-Plk1 did not induce nuclear translocation of WT-Cdc25C or S216A-Cdc25C (Figure 4A, lanes 7, 8, 10 and 11; B, top panel). However, expression of cyclin B1 plus S216A-Plk1 stimulated significantly nuclear localization of WT-Cdc25C (Figure 4A, lane 9). Expression of S216A-Cdc25C together with this low level of cyclin B1 and S216A-Plk1 still induced a premature mitosis rather strongly under the condition (~15%, data not shown). But, under this condition also, in ~40% of the cells that were not in mitosis, S216A-Cdc25C localized to the nucleus (Figure 4A, lane 12; B, middle panel). This stimulation of the nuclear localization of Cdc25C was significantly reduced when Ser191 and Ser198

Fig. 4. Constitutively active Plk1 causes nuclear translocation of Cdc25C. (A) The indicated forms of Myc-tagged Cdc25C, HA-tagged Plk1 (vector, WT or SDTD) and/or untagged cyclin B1 were expressed in HeLa cells. Fifteen hours after transfection, cells were fixed, followed by staining with anti-Myc and anti-HA antibodies. More than 200 cells were examined in each condition, and the percentages of the cells in which Myc-tagged Cdc25C was accumulated in the nucleus are shown. (B) Typical images of Myc-tagged Cdc25C (middle column) co-expressed with HA-tagged Plk1 (left column) and untagged cyclin B1 are shown with DAPI staining (right column). When S198,216A-Cdc25C was expressed, part of this mutant Cdc25C localized to the nucleus. Expression of SDTD-Plk1 and cyclin B1 did not enhance at all the nuclear localization of this mutant Cdc25C (data not shown).

of Cdc25C were replaced by Ala (Figure 4A, lane 13; B, bottom panel). These results support our idea that Plk1 phosphorylates Cdc25C on Ser198 and stimulates its nuclear translocation during prophase. The protein level of cyclin B1 increases during G₂ phase, and the increased cyclin B1 and/or Cdc2/cyclin-B1 complex may help to stimulate nuclear entry of Cdc25C. The molecular mechanism, however, is not known at present.

To examine whether phosphorylation of Cdc25C on Ser198 occurs *in vivo*, we produced and purified the anti-phosphoSer198 Cdc25C antibody (see Figure 2D). The affinity-purified antibody (α -phosphoS198) reacted with OVA-phosphoS198-NES but not with OVA-NES (Figure 5A). It reacted strongly with a 55-kDa

polypeptide, which co-migrated on SDS-PAGE with a lower, major band of Cdc25C (Figure 5B, arrow), in the M-phase extracts but not in the S-phase extracts from synchronized HeLa cells (Figure 5B, IB; α -phosphoS198). In addition, α -phosphoS198 immunoprecipitated the 55-kDa polypeptide of Cdc25C from M-phase extracts but not from S-phase extracts (Figure 5C), confirming that this antibody recognizes the Ser198-phosphorylated Cdc25C but not the Ser198-unphosphorylated Cdc25C. In synchronized HeLa cells, the phosphoSer198 Cdc25C appeared during G₂-M transition, peaking at M phase, and then disappeared in anaphase/G₁ phase (Figure 5D, IB; α -phosphoS198). This profile correlated with that of the kinase activity of immunoprecipitated endogenous Plk1 toward Cdc25C (173–206) (Figure 5D, lower graph). These results indicate that phosphorylation of Cdc25C on Ser198, which correlates with the activity of Plk1, occurs during G₂-M phase *in vivo*. However, this antibody hardly recognized the slowly migrating form of Cdc25C that appeared during M phase in this type of experiment (Figure 5D, arrowhead). Because the slowly migrating form of Cdc25C appeared during M phase as a minor population due to the inefficiency of the synchronization by a double thymidine block, we expressed Myc-tagged WT-Cdc25C and S198A-Cdc25C in HeLa cells, arrested the cells at M phase by nocodazole treatment and then immunoprecipitated Cdc25C. The anti-phosphoSer198 Cdc25C antibody strongly reacted with the slowly migrating form of WT-Cdc25C (Figure 5E, lane 1) but not with that of S198A-Cdc25C (Figure 5E, lane 3). When the immunoprecipitates were treated with protein phosphatase PP1, they were no longer recognized by the antibody (Figure 5E, lanes 2 and 4, top row). These results suggest that the slowly migrating form of Cdc25C, as well as the unshifted form of Cdc25C, is phosphorylated on Ser198 during M phase and that this phosphorylation does not affect the mobility of Cdc25C on SDS-polyacrylamide gels.

To test whether the phosphorylation of Ser198 of Cdc25C is necessary for its nuclear translocation during prophase, we co-expressed HA-tagged S198A-Cdc25C with Myc-tagged WT-Cdc25C in HeLa cells and examined their localization in prophase cells. S198A-Cdc25C remained in the cytoplasm, whereas WT-Cdc25C localized to the nucleus in prophase cells (Figure 5F). This indicates that the phosphorylation of Ser198 of Cdc25C during G₂-M phase is necessary for its nuclear translocation.

Our results show that Plk1 phosphorylates Cdc25C on Ser198 during G₂-M phase and that this phosphorylation is necessary for nuclear translocation of Cdc25C during prophase. Our previous study showed that Plk1 phosphorylates cyclin B1 and causes its nuclear translocation during prophase (Toyoshima-Morimoto *et al.*, 2001). Therefore, Plk1 regulates the timing of nuclear entry of both Cdc2/cyclin B1 and its activator Cdc25C. Nuclear Cdc25C plays a role in keeping nuclear MPF active by counteracting the inhibitory activity of Wee1, which is constitutively nuclear. However, as a recent report has demonstrated that Cdc25C is not necessarily essential in mice (Chen *et al.*, 2001), Plk1 might also regulate the subcellular localization of Cdc25A and/or Cdc25B, both of which are believed to compensate for loss of Cdc25C in mice. Thus, our results suggest that Plk1 acts as an important coordinator for intracellular localization of active MPF by directing simultaneity of nuclear entry of MPF and its activator, which may be essential for coordinating M-phase events.

METHODS

Mutagenesis, transfections and nocodazole treatment. Mutagenesis was performed by using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). HeLa cells were transfected by using Eugene6 Transfection Reagent (Boehringer Mannheim). To arrest cells in M phase, cells were treated with 250 ng/ml nocodazole for 20 h at 18 h after transfection.

Protein preparation. Recombinant GST-Cdc25C (173–206) and GST-Cdc25C (full length) proteins were prepared by using pGEX-6P1 (Pharmacia), expressed in *Escherichia coli*, and purified by glutathione-Sepharose 4B (Pharmacia). His-tagged Plk1 was prepared as described previously (Toyoshima-Morimoto *et al.*, 2001).

Immunoprecipitation, immunoblotting and cell staining. Cells were lysed in homogenizing buffer (20 mM Tris pH 7.5, 60 mM 2-glycerophosphate, 10 mM MgCl₂, 10 mM NaF, 0.1% NP-40, 1 mM DTT, 1 mM Na₃VO₄, 1 mM PMSF, 2 µg/ml aprotinin, 1 µM okadaic acid) and centrifuged at 20 000 g for 15 min. The cell extracts were subjected to immunoblotting with anti-Plk1 antibody (Zymed) and anti-Cdc25C antibody (Santa Cruz). Endogenous Plk1 or HA-tagged Plk1 was immunoprecipitated with anti-Plk1 (Zymed) or anti-HA antibody (Santa Cruz) coupled to protein A-Sepharose or protein G-Sepharose (Pharmacia). The immunoprecipitates were further washed with homogenizing buffer and subjected to kinase assays as described below. Immunofluorescent cell staining with anti-HA (Santa Cruz or Clontech) and anti-Myc (Santa Cruz) antibodies was performed as described previously (Toyoshima *et al.*, 1998).

Kinase assays. The cell extracts were mixed with 0.5 µg substrate, 100 µM ATP, 15 mM MgCl₂ and 3 µCi [γ -³²P]ATP in a final volume of 15 µl and incubated for 40 min at 25°C. Histone H1 kinase assay was conducted as described previously

Fig. 5. Phosphorylation of Ser198 of Cdc25C occurs during G₂–M phase *in vivo* and is necessary for its nuclear translocation. (A) Indicated amounts of OVA-NES and OVA-phosphoS198-NES were immunoblotted with the affinity-purified anti-phosphoS198 Cdc25C antibody (α -phosphoS198). (B) Cell extracts were prepared from synchronized HeLa cells at 4 h (S phase) and 10 h (M phase) after release from a double thymidine block, analyzed by SDS–PAGE and subjected to immunoblotting with anti-Cdc25C antibody (left panel) or α -phosphoS198 (right panel). α -PhosphoS198 reacted with both a 55-kDa polypeptide in M-phase extracts (arrow), which corresponds to S198-phosphorylated Cdc25C, and an uncharacterized 60-kDa polypeptide (arrowhead). (C) S-phase and M-phase extracts (bottom row) and the immunoprecipitates (top row) were immunoblotted with anti-Cdc25C antibody. (D) HeLa cells were synchronized by a double thymidine block, and cell extracts were prepared at the indicated times after release. The kinase activities in the extracts toward histone H1 are shown in the top graph. Endogenous Plk1 was immunoprecipitated from the extracts with anti-Plk1 antibody, and the kinase activities of the immunoprecipitates toward GST–WT-Cdc25C (173–206) are shown in the bottom graph. The extracts were analyzed by SDS–PAGE followed by immunoblotting with anti-Cdc25C antibody and α -phosphoS198 (bottom panels). (E) Myc-tagged WT-Cdc25C or S198A-Cdc25C was expressed in HeLa cells. The cells were then arrested in M phase by nocodazole treatment. The cell extracts were prepared and subjected to immunoprecipitation with anti-Myc antibody. The immunoprecipitates were treated with or without protein phosphatase PP1 and subjected to immunoblotting with α -phosphoS198S and anti-Myc antibodies. (F) Myc-tagged WT-Cdc25C and HA-tagged S198A-Cdc25C were expressed in HeLa cells. Cells were fixed and stained with anti-Myc and anti-HA antibodies. A typical image is shown. This image was detected in 13 out of 15 prophase cells.

(Toyoshima *et al.*, 1998). In the kinase assays for His-tagged Plk1 or immunoprecipitates, 0.1–1 µg His-tagged Plk1 or immunoprecipitates were mixed with substrate (0.3–1 µg), 50 µM ATP and 15 mM MgCl₂ in a final volume of 15 µl and incubated for 20 min at 30°C in the presence or absence of 3 µCi [γ -³²P]ATP. The reactions were stopped by addition of Laemmli's sample buffer and boiling.

Treatment with protein phosphatase PP1. Myc-tagged Cdc25C was immunoprecipitated with anti-Myc antibody (Santa Cruz) from M-phase-arrested HeLa cells, as described above. Immunoprecipitates were washed with PP1 buffer (50 mM Tris pH 7.0, 0.1 mM EGTA, 5 mM DTT) and then incubated in 30 µl PP1 reaction buffer (50 mM Tris pH 7.0, 1 mM MnCl₂, 0.01% Brij 35, 0.1 mM EDTA, 5 mM DTT) with 1 U PP1 (New England Biolabs) for 20 min at 30°C. The reactions were stopped by addition of Laemmli's sample buffer and boiling.

Production of anti-Ser198-phosphorylated Cdc25C antibody (α -phosphoS198). Anti-phosphoS198 Cdc25C polyclonal antibody was produced in rabbits by immunizing them with a KLH-conjugated synthetic phosphopeptide corresponding to residues 188–201 of human Cdc25C (EEISDELMEF-phosphoS198-LKD). The serum was used at a 1000-fold dilution for the immunoblotting in Figure 5E. The serum was diluted at 100-fold and loaded onto the GST–Cdc25C (full length)-conjugated glutathione–Sepharose 4B, and the unadsorbed fraction was used to detect *in vitro* phosphorylated GST–Cdc25C (full length) in Figure 2D. To affinity purify the antibody, the serum was subjected to affinity chromatography on the phosphopeptide-conjugated cellulose. After several washes with TBS (20 mM Tris pH 7.5, 150 mM NaCl), proteins were eluted with 0.1 M glycine (pH 2.5), immediately neutralized by the addition of 1 M Tris pH 8.5, dialyzed against TBS and loaded onto the non-phosphopeptide (EEISDELMEFSLKD)-conjugated cellulose. The unadsorbed fractions were used for immunoblotting and immunoprecipitation. In immunoprecipitation from the extracts with α -phosphoS198, synchronized HeLa cells were lysed in lysis buffer (20 mM HEPES pH 7.5, 25 mM 2-glycerophosphate, 1.5 mM MgCl₂, 2 mM EGTA, 150 mM NaCl, 50 mM NaF, 0.5% Triton X-100, 2 mM DTT, 1 mM Na₃VO₄, 1 mM PMSF, 2 µg/ml aprotinin) and centrifuged at 20 000 g for 15 min. α -PhosphoS198 coupled to protein A–Sepharose was added to the supernatant, and the immunoprecipitates were further washed three times with lysis buffer and then subjected to immunoblotting with anti-Cdc25C antibody.

Supplementary data. Supplementary data are available at *EMBO reports* Online.

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