A Dual-Specificity Phosphatase Cdc25B Is an Unstable Protein and Triggers p34<sup>cdc2</sup>/Cyclin B Activation in Hamster BHK21 Cells Arrested with Hydroxyurea

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Abstract. By incubating at 30°C in the presence of an energy source, p34<sup>cdc2</sup>/cyclin B was activated in the extract prepared from a temperature-sensitive mutant, tsBN2, which prematurely enters mitosis at 40°C, the nonpermissive temperature (Nishimoto, T., E. Eilen, and C. Basilio. 1978. Cell. 15:475–483), and wild-type cells of the hamster BHK21 cell line arrested in S phase, without protein synthesis. Such an in vitro activation of p34<sup>cdc2</sup>/cyclin B, however, did not occur in the extract prepared from cells pretreated with protein synthesis inhibitor cycloheximide, although this extract still retained the ability to inhibit p34<sup>cdc2</sup>/cyclin B activation. When tsBN2 cells arrested in S phase were incubated at 40°C in the presence of cycloheximide, Cdc25B, but not Cdc25A and C, among a family of dual-specificity phosphatases, Cdc25, was lost coincidentally with the lack of the activation of p34<sup>cdc2</sup>/cyclin B. Consistently, the immunodepletion of Cdc25B from the extract inhibited the activation of p34<sup>cdc2</sup>/cyclin B. Cdc25B was found to be unstable (half-life < 30 min). Cdc25B, but not Cdc25C, immunoprecipitated from the extract directly activated the p34<sup>cdc2</sup>/cyclin B of cycloheximide-treated cells as well as that of nontreated cells, although Cdc25C immunoprecipitated from the extract of mitotic cells activated the p34<sup>cdc2</sup>/cyclin B within the extract of cycloheximide-treated cells. Our data suggest that Cdc25B made an initial activation of p34<sup>cdc2</sup>/cyclin B, which initiates mitosis through the activation of Cdc25C.
Lee et al., 1991, 1994. It is supposed to be carried out by the stoichiometric inhibitors of the p34\textsuperscript{cdcl2}/cyclin B complex, which titrates out the protein(s) other than MOS appears to be required to activate the p34\textsuperscript{cdcl2}/cyclin B. Wasserman and Masui (1975) originally found that protein synthesis inhibitors block the Xenopus oocyte maturation induced by progesterone. As a protein that is newly synthesized by progesterone treatment, cyclin B is plausibly, since it is synthesized from S phase onwards and is essential for the activation of p34\textsuperscript{cdcl2}/cyclin B (Evans et al., 1983). However, cyclin B already exists in Xenopus oocytes (Minshull et al., 1991) and also in BHK21 cells arrested in S phase (Nishitani et al., 1991). Another candidate for a newly synthesized activator is a c-mos proto- oncogene product (MOS)-like protein. In Xenopus oocyte, MOS that is synthesized just before meiosis I is essential for the activation of p34\textsuperscript{cdcl2}/cyclin B (Sagata et al., 1988). Microinjected MOS protein can induce meiosis I, but not meiosis II, without protein synthesis (Yew et al., 1992; Furuno et al., 1994). Thus, the synthesis of protein(s) other than MOS appears to be required to complete Xenopus oocyte maturation. Alternatively, if Xenopus oocytes and BHK21 cells arrested in S phase contain all the molecules required for p34\textsuperscript{cdcl2}/cyclin B activation, the inhibition of p34\textsuperscript{cdcl2}/cyclin B activation caused by blocking protein synthesis may reflect the loss of endogenous activators of p34\textsuperscript{cdcl2}/cyclin B that are rapidly turning over.

tsBN2 mutation causes the loss of RCC1 function, resulting in either G1 arrest or premature activation of p34\textsuperscript{cdcl2}/cyclin B depending on the cell cycle stage (Nishimoto et al., 1978; Nishitani et al., 1991). RCC1 is localized on chromatin (Frash, 1991) and functions as a GDP/GTP exchanging factor on Ran, a Ras-like nuclear G protein (Bischoff and Ponstingle, 1991). GTP hydrolysis of Ran is essential for the nuclear import of karyophilic proteins (Melchior et al., 1993; Moore and Blobel, 1993). It can be argued, therefore, that all of the phenotypes of rcl\textsuperscript{–} are indirect consequences of the role of Ran in nuclear transport. However, PCC induced by the loss of RCC1 can be inhibited by microinjection of both GTP- and GTP\textsubscript{S}-bound Ran (Ohba et al., 1996), indicating that GTP-Ran plays some role in the nucleus. This finding is consistent with the fact that Yrb2p that possesses a Ran-binding domain similar to RanBP1/Yrb1p (Dingwall et al., 1995), thereby specifically binding to the GTP-bound Gsp1p S. cerevisiae Ran homologue, is localized in the nucleus (Noguchi et al., 1997). The disruptant of the YRB2 gene, which is cold sensitive, has no defect in the nucleus/cytosol exchange of macromolecules (Noguchi et al., 1997).

When tsBN2 cells are incubated in S phase at 40°C, the nonpermissive temperature, Cdc25C enters the nucleus parallel with PCC induction (Seki et al., 1992). This is inconsistent with the findings that the loss of RCC1 function retards nuclear protein import (Tachibana et al., 1994; Dickmanns et al., 1996). We noticed the fact that the inhibition of protein synthesis blocks the nuclear import of Cdc25C as well as the activation of p34\textsuperscript{cdcl2}/cyclin B (Seki et al., 1992), which suggests that loss of RCC1 function induces a new protein synthesis required either for nuclear entrance of Cdc25C or for the activation of p34\textsuperscript{cdcl2}/cyclin B. To clarify the relationship between the loss of RCC1 and the p34\textsuperscript{cdcl2}/cyclin B activation, we began to identify the protein(s) required for p34\textsuperscript{cdcl2}/cyclin B activation induced by the loss of RCC1 (Nishitani et al., 1991). To do this, we have first established a method to activate the p34\textsuperscript{cdcl2}/cyclin B complexes in vitro by using the extract prepared from BHK21 cells arrested in S phase (Seki et al., 1992). In this paper, we confirmed our previous results by using tsBN2 cells. We found that the in vitro activation of p34\textsuperscript{cdcl2}/cyclin B occurred without new protein synthesis and that p34\textsuperscript{cdcl2}/cyclin B was not activated in the extract of cells pretreated with protein synthesis inhibitor. Instead of a new synthesis of p34\textsuperscript{cdcl2}/cyclin B activators, therefore, an endogenous activator(s) of p34\textsuperscript{cdcl2}/cyclin B was suggested to be lost by blocking protein synthesis. Cdc25B, but not Cdc25C or Cdc25A, was identified as such an unstable activator of p34\textsuperscript{cdcl2}/cyclin B.

### Materials and Methods

#### Cells and Cell Culture

The BHK21 cell line derived from golden hamsters and its ts mutant tsBN2 cell line (Nishimoto et al., 1978) and human HeLa cell line were grown in DME containing 10% calf or FCS in a humidified atmosphere containing 10% CO\textsubscript{2}. HeLa cells were cultured at 37.5°C, and BHK21 and tsBN2 cells were cultured at 33.5°C. As the nonpermissive temperature, 40°C was used for tsBN2 cells.

#### Synchronization of Cells

Wild-type BHK21 and tsBN2 cells were cultured in isoleucine-free medium for 24–30 h (G1/G0 arrest) and then in DME containing 10% FCS and 2.5 mM hydroxyurea (HU) for 15.5 h (S phase arrest) as described (Nishitani et al., 1991). To synchronize in mitosis, after the release from HU block, cells were incubated in DME medium containing 10% FCS and 0.4 μg/ml Nocodazole for 8 h.
HeLa cells were cultured in medium containing thymidine (2 mM) for 14 h, in a normal growth medium for 13 h, and finally in medium containing 2.5 mM HU for 14 h (S phase arrest).

Preparation of Cell Extracts

Cells were collected with a scraper and then sequentially washed, first with TDB buffer containing 136.8 mM NaCl, 5 mM KCl, 0.7 mM Na$_2$HPO$_4$, and 25 mM Tris-HCl (pH 7.4), with the EB buffer, and finally with the 0.5 × EB buffer as described (Seki et al., 1992). The EB buffer contained 20 mM Hepes-KOH (pH 7.4), 50 mM KCl, 50 mM 2-glycerophosphate, 15 mM EGTA, and 10 mM MgCl$_2$. Washed cells were lysed by pipetting and vortexing in the buffer (0.5 × EB–1), 0.5 × EB buffer supplemented with 5 μg/ml cytochalasin B, 5 mM DTT, a mixture of protease inhibitors, 1 mM ATP–S, and 0.5% NP-40. After being kept on ice for 30 min, the extract was centrifuged at 2,000 × _g_ for 30 min. The supernatant was used as the cytosolic extracts. The total cell extract was prepared with the same procedure, except that the cells were lysed directly in the 0.5 × EB-1 buffer supplemented with 500 mM NaCl. The mixture of protease inhibitors contained 2% dihydrochloride, leupeptin, and pepstatin A, and 100 μM _p_-aminophenyl methansulfonyl fluoride (pAPMSF).

The cell extracts were desalted, concentrated by centrifuge (Amicon Corp., Danvers, MA), and then subjected to use as the cell extracts for the in vitro activation assay of p34$^{cd2}$x protein kinase.

Preparation of _E_. _coli_-Produced Hamster Cdc25C

_E_. _coli_ BL21 (DE3)LyS3/pGEX-KG-hamster Cdc25C (a gift of K. Yamashita, Kyushu University, Fukuoka, Japan) strains were cultured at 30°C up to an OD 600 of 0.4, treated with IPTG (final concentration, 1.0 mM) for 3 h, and then collected by centrifugation. Cell-pellet was lysed by SDS and glutathione-S-transferase (GST)-Cdc25C was purified using glutathione Sepharose 4B beads (Pharmacia Fine Chemicals, Piscataway, NJ) as described previously (Noguchi et al., 1997). Eluted GST-Cdc25C was further purified by gel filtration using Sephacryl S-100 HR column (Pharmacia Fine Chemicals).

Immunodepletion of cyclin B, cdc25B, and cdc25C from Cytoplasmic Extract

The antibody was mixed with protein G Sepharose (Pharmacia Fine Chemicals), rotated at 4°C for 2 h. Beads were pulled down by centrifugation, washed with 0.5 × EB-1 buffer, and then suspended in the cytosolic extracts. After rotation for 2 h at 4°C, the beads were pulled down by centrifugation. The resulting supernatant was used as immunodepleted extracts.

In Vitro Activation of p34$^{cd2}$/cyclin H Histone H1 Kinase and Assay of Histone H1 Kinase Activity

The extract of 5–15 μl (protein concentration: 3 mg/ml) was incubated at 30°C in the presence of 1 mM ATP, 10 mM creatine phosphate, and 2.5 μg/ml creatine kinase as an energy source (Kumagai and Dunphy, 1992). The reaction was stopped by a buffer containing 40 mM Hepes-NaOH (pH 7.5), 60 mM 2-glycerophosphate, 20 mM _p_-nitrophenyl phosphate, 0.5 mM Na$_2$VO$_4$, 250 mM NaCl, 15 mM MgCl$_2$, 1% Triton X-100, 1 mM DTT, and a mixture of protease inhibitors. p34$^{cd2}$/cyclin B complexes were precipitated with the anti-cyclin B antibody or the p13suc1 beads (Nishitani et al., 1991), as indicated. The histone H1 kinase activity of precipitates was assayed by using the histone H1 as the substrate, as described (Nishitani et al., 1991).

The reaction was carried out more than twice by using different extracts.

Immunoblotting

Antibodies used were the anti-cdc2 antibodies against PSTAIR peptide (Nishitani et al., 1991), the anti-phosphotyrosine antibody, pTyr (a gift of Dr. J.Y.J. Wang, University of California, San Diego, CA; Morla and Wang, 1986), and the antibodies against cdc25A, cdc25B and cdc25C antibodies (Seki et al., 1992), cyclin B, and cdk2 (all from Santa Cruz Biotechnology, Santa Cruz, CA). The procedure for immunoblotting was carried out as described elsewhere (Nishitani et al., 1990), except that the detection was achieved using an ECL detection system (Amer sham).
protein synthesis inhibitor on the activation of p34<sup>cdc2</sup>/cyclin B, we prepared the extracts from S phase-arrested wild-type BHK21 cells that had been incubated in the presence of protein synthesis inhibitor cycloheximide (10 μg/ml) for 1 h before cell harvest (henceforth designated cycloheximide treated). When the extract of cycloheximide-treated cells was incubated at 30°C for 1 h, there was no activation of p34<sup>cdc2</sup>/cyclin B (Fig. 2, columns 1 and 2), consistent with the finding that p34<sup>cdc2</sup> was not dephosphorylated (Fig. 3, lanes 1 and 2). Therefore, cycloheximide-treated cells had no ability to activate the p34<sup>cdc2</sup>/cyclin B complexes, both in vivo and in vitro.

To exclude the possibility that cycloheximide carried over from cycloheximide-treated cells inhibited the activation of p34<sup>cdc2</sup>/cyclin B, the extract was prepared from wild-type BHK21 cells that had not been treated with cycloheximide, and then incubated in vitro either in the presence or absence of 10 μg/ml of cycloheximide. In both cases, p34<sup>cdc2</sup>/cyclin B was activated (Fig. 2, compare columns 4 and 6), indicating that BHK21 cells that were arrested in S phase possessed all molecules required for the observed in vitro activation of p34<sup>cdc2</sup>/cyclin B. The lack of activation of p34<sup>cdc2</sup>/cyclin B in the extract prepared from
cycloheximide-treated cells, therefore, suggested that some molecules or protein modifications required for the activation of p34cdc2/cyclin B were lost during the blocking of protein synthesis.

**Activator(s), but Not Inhibitor(s) of p34cdc2/cyclin B Was Lost by Blocking Protein Synthesis**

To investigate the possibility that the activator(s) of p34cdc2/cyclin B was lost by blocking protein synthesis, the extract was prepared from S phase-arrested BHK21 cells that had been either treated with cycloheximide or not treated with cycloheximide (henceforth designated nontreated) as described in Materials and Methods. To exclude the possibility that p34cdc2/cyclin B is involved in the activation step, the p34cdc2/cyclin B complexes were depleted using the anti-cyclin B antibodies, from these extracts. The cyclin B-depleted extracts were then mixed and incubated at 30°C, with the extract prepared either from cycloheximide-treated or from nontreated cells, respectively.

The cyclin B-depleted extract prepared from nontreated cells, while it had no activity of p34cdc2/cyclin B (Fig. 4 C), possessed the ability to activate the p34cdc2/cyclin B within the extract prepared from cycloheximide-treated cells (Fig. 4 A). On the other hand, the cyclin B-depleted extract prepared from cycloheximide-treated cells had no ability to activate the p34cdc2/cyclin B within the extract prepared from cycloheximide-treated cells. To the contrary, the cyclin B-depleted extract of cycloheximide-treated cells was found to have the ability to inhibit the in vitro activation of p34cdc2/cyclin B (Fig. 4 B). These findings indicated that wild-type BHK21 cells that were arrested in S phase possessed both the activator(s) and the inhibitor(s) of p34cdc2/cyclin B. By blocking protein synthesis, the activator(s), which was not the endogenous p34cdc2/cyclin B complex, was lost, but the inhibitor(s) remained. The inhibitory activity for p34cdc2/cyclin B of the cycloheximide-treated cell extracts may reflect either the activation of p34cdc2/cyclin B inhibitor(s) or the loss of balance between the inhibitor and the activator of p34cdc2/cyclin B.

**Cdc25B Was Lost by Blocking Protein Synthesis**

To identify proteins that were required for p34cdc2/cyclin B activation and disappeared during treatment of cells with protein synthesis inhibitors, cultures of tsBN2 cells were synchronized in S phase at 33.5°C and then incubated at 40°C, either in the absence or presence of cycloheximide (10 μg/ml). Every hour, cells were harvested and assayed for the presence of p34cdc2/cyclin B activators, such as Cdc25A, B, and C, and as controls, p34cdc2, p34cdc1, and cyclin B, by immunoblotting analysis (Fig. 5).

While p34cdc2/cyclin B was activated in tsBN2 cells without cycloheximide, no activation of p34cdc2/cyclin B occurred in the presence of cycloheximide as previously reported (Nishitani et al., 1991; Fig. 5 A). Parallel with the activation of p34cdc2/cyclin B, the molecular mass of p34cdc2 was shifted down to the faster-migration form, while that of Cdc25C was shifted up to the retarded-migration form, particularly of Cdc25A and C and p34cdc2 seemed to be constant during treatment with cycloheximide. However, Cdc25B became undetectable after inhibiting protein synthesis for 1 h. Loss of Cdc25B was also observed in cycloheximide-treated wild-type BHK21 cells (Fig. 6 A), indicating that Cdc25B was an unstable protein in BHK21 cells.

Interestingly, even in the absence of cycloheximide, the amount of Cdc25B was gradually reduced and finally lost in parallel with the reduction in histone H1 kinase activity of p34cdc2/cyclin B, similar to cyclin B (Fig. 5, compare A and B).

**Cdc25B Is an In Vitro Activator of p34cdc2/cyclin B**

To confirm that Cdc25B is an activator required for the in vitro activation of p34cdc2/cyclin B, Cdc25B, and as a control, Cdc25C, which is essential for p34cdc2/cyclin B activa-

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**Figure 4.** Both activator(s) and inhibitor(s) of p34cdc2/cyclin B are present in HU-arrested BHK21 cells. Cultures of wild-type BHK21 cells synchronized in S phase with HU were incubated in the absence (nontreated) or presence (CHX-treated) of cycloheximide (10 μg/ml) for 1 h and then lysed to prepare the cytosolic extracts. When indicated as cyclin B-depleted, the p34cdc2/cyclin B complexes were immunoprecipitated using the anti-cyclin B antibody and the supernatant was used as the cyclin B-depleted extracts as described in Materials and Methods. 8 μl of the total cytosolic extracts prepared from CHX-treated cells (A), from nontreated cells (B), and as a control of the buffer (0.5× EB-1; C) were mixed with 0, 4, 8, 16, and 32 μl of the cyclin B-depleted extracts prepared from nontreated (– ● –) or CHX-treated cells (– ○ –). In each case, the final volume was adjusted to 40 μl with the buffer (0.5× EB-1). After incubation at 30°C for 1 h, the p34cdc2/cyclin B complexes were immunoprecipitated using the anti-cyclin B antibodies and assayed for histone H1 kinase activities.
Cdc25B Is a Short-Life Protein in Mammalian Cells

Our present results suggest that Cdc25B was lost in tsBN2 cells during the passage of Cdc25B-depleted extract contained the ability to activate the p34cdc2/cyclin B complex of the extract prepared from cycloheximide-treated cells, whereas the cyclin B- and Cdc25B-depleted extract did not.

To further confirm the involvement of Cdc25B in the in vitro activation of p34cdc2/cyclin B, proteins were immunoprecipitated from the extracts of cycloheximide-treated and nontreated cells, using the anti-Cdc25B antibody and assayed for their abilities to activate the p34cdc2/cyclin B complex within the extract of cycloheximide-treated cells. Proteins that were immunoprecipitated from the extracts of nontreated cells activated p34cdc2/cyclin B, but those precipitated from cycloheximide-treated cells did not (Fig. 7 A). In both cases, immunoprecipitated proteins alone had no activity of histone H1 kinase. As a control, Cdc25C was immunoprecipitated from the extract of BHK21 cells arrested either in S phase or in mitosis. While Cdc25C derived from mitotic cells had an ability to activate the p34cdc2/cyclin B complex within the extract of cycloheximide-treated cells, Cdc25C derived from S phase-arrested cells did not (Fig. 7 B). These findings indicated that Cdc25B was an activator of the p34cdc2/cyclin B complex, which was lost by cycloheximide treatment.

Cdc25B Directly Activated p34cdc2/cyclin B

To address the question of whether Cdc25B directly activates p34cdc2/cyclin B, Cdc25B was immunoprecipitated from the extract of nontreated cells, and as a control, from the extract of cycloheximide-treated cells. On the other hand, the p34cdc2/cyclin B complexes were immunoprecipitated using the anti-cyclin B antibody, from the extracts of cycloheximide-treated and nontreated cells. Proteins immunoprecipitated with the anti-Cdc25B antibody alone had no activity of histone H1 kinase (Fig. 8, base line). By mixing with the p34cdc2/cyclin B, which had no histone H1 kinase activity by itself (Fig. 8, zero point), Cdc25B of nontreated cells activated p34cdc2/cyclin B complexes isolated from both the extracts of nontreated and cycloheximide-treated cells, with a similar kinetics (Fig. 8, A and B). As a control, mock-precipitated Cdc25B from cycloheximide-treated cells had no ability to activate p34cdc2/cyclin B.

Compared to the p34cdc2/cyclin B retained within the extract, the isolated p34cdc2/cyclin B complexes were highly activated by Cdc25B (compare Fig. 7 and 8). This finding may suggest that the inhibitors for p34cdc2/cyclin B activation retained within the extracts were removed from the p34cdc2/cyclin B complexes by immunoprecipitation. With these results taken together, we concluded that Cdc25B directly activated the p34cdc2/cyclin B complex, while we could not exclude the possibility that some proteins that were co-precipitated with p34cdc2/cyclin B or with Cdc25B, activated Cdc25B before the activation of p34cdc2/cyclin B (Galaktionov and Beach, 1991). These findings also indicated that the protein modification required for the activation of p34cdc2/cyclin B was not lost by blocking protein synthesis.

Cdc25B Is a Short-Life Protein in Mammalian Cells

Our present results suggest that Cdc25B was lost in tsBN2 and wild-type BHK21 cells treated with protein synthesis induction induced by loss of RCC1 (Seki et al., 1992), were depleted from the cytosolic extract prepared from nontreated, wild-type BHK21 cells, using the anti-Cdc25B and Cdc25C antibodies, respectively. Depletion of Cdc25B and Cdc25C was confirmed by immunoblotting analysis (Fig. 6 A). In both cases, Cdc25A remained in the extracts.

Cdc25B- and Cdc25C-depleted extracts were then incubated at 30°C for 1 h. The p34cdc2/cyclin B complex was activated in the Cdc25C-depleted extract, but not in the Cdc25B-depleted extract (Fig. 6 B, cdc25B and cdc25C). The effect of Cdc25B depletion was further confirmed using the extracts from which p34cdc2/cyclin B had been depleted, in addition to Cdc25B or Cdc25C. These extracts alone possessed no activity of p34cdc2/cyclin B. However, as shown in Fig. 6 C, the cyclin B- and Cdc25C-depleted extract contained the ability to activate the p34cdc2/cyclin B complex of the extract prepared from cycloheximide-treated cells, whereas the cyclin B- and Cdc25B-depleted extract did not.

Figure 5. Cdc25B was lost by treatment with cycloheximide. A series of cultures of tsBN2 cells synchronized in S phase with HU was incubated at 40°C in the absence (nontreated) or presence (CHX-treated) of cycloheximide (10 µg/ml). Every hour, cells were harvested to prepare the total cell extracts. (A) p34cdc2/cyclin B complexes of total cell extracts prepared from nontreated (–CHX) or cycloheximide-treated cells were immunoprecipitated using the anti-cyclin B antibody and assayed for histone H1 kinase activities. (B) Proteins of total cell extracts prepared from nontreated (–CHX) and CHX-treated (+CHX) cells were electrophoresed in SDS-10% polyacrylamide gel and analyzed by immunoblotting using as a probe the antibodies against the proteins indicated.
inhibitor cycloheximide. Since the accumulation of truncated; abnormal proteins may enhance ubiquitin-dependent proteolysis (Hershko, 1983), it is possible that Cdc25B, which is stable in a normal growth condition, becomes unstable by blocking protein synthesis. To address this question, Cdc25B was pulse labeled with [35S]methionine and chased in the presence of an excess amount of cold methionine in wild-type BHK21 cells that had been arrested in S phase. As shown in Fig. 9 A, Cdc25B was rapidly lost even in the absence of cycloheximide. To further confirm the instability of Cdc25B, human Cdc25B (a gift of H. Okayama) was transfected into wild-type BHK21 cells, and the expressed human Cdc25B was pulse–chased in randomly growing wild-type BHK21 cells. Exogenously expressed human Cdc25B was rapidly lost with a half-life of <30 min, similar to the endogenous Cdc25B (Fig. 9, A and B). In the same cultures of wild-type BHK21 cells expressing human Cdc25B, the endogenous hamster Cdc25B was lost with the kinetics similar to that of human Cdc25B (data not shown). Thus, Cdc25B was unstable in BHK21 cells, and the stability of Cdc25B was not changed by arresting cells in S phase with hydroxyurea. Even in HeLa cells, Cdc25B, but not Cdc25A and Cdc25C, was lost during treatment with cycloheximide (Fig. 9 C). Taking these results together, we concluded that Cdc25B was rapidly turning over in mammalian cells.

All of p34cdc2 in the Extract Was Not Activated by Cdc25B

To address the question whether the observed in vitro activation of p34cdc2/cyclin B complex reflects a full activation of p34cdc2 within the extract, we added the E. coli-produced Cdc25C to the extract of BHK21 cells. By the addition of E. coli-produced Cdc25C, the histone H1 kinase activity increased compared to the extract alone (Fig. 10). This finding indicated that all of p34cdc2 in the extract was not activated by Cdc25B. Probably, in our reaction condition, the activity of p34cdc2/cyclin B complexes obtained by the in vitro incubation was not enough to overcome the threshold for mitotic entrance, which is supposed to be carried out by forming a positive feedback mutual activation loop between Cdc25C and p34cdc2/cyclin B complexes (Kumagai and Dunphy, 1992; Hoffmann et al., 1993; Izumi and Maller, 1993). To address this issue, we added an increasing amount of p34cdc2/cyclin B complexes that had been activated by Cdc25B, to the extract of S phase-arrested BHK21 cells. However, the high activation of the p34cdc2 that was obtained by the addition of E. coli-produced Cdc25C, did not occur by the addition of extra p34cdc2/cyclin B complexes (data not shown). The similar result has been reported by Lee et al. (1994a) using Xenopus egg extract.
Over-Expression of Cdc25B Enhanced Chromatin Condensation

To address whether Cdc25B is rate limiting for entry into mitosis, increasing doses of Cdc25B cDNA were introduced into wild-type BHK21 cells using lipofection. Transfected cells were synchronized at G1 by isoleucine deprivation and then at S phase with HU. After incubation for 15.5 h in the presence of HU, cells were stained with Hoechst 33342, and cells showing PCC were scanned with photomicroscope. There were no cells showing PCC (<0.1%). Next we examined whether over-expressed Cdc25B can enhance...
chromatin condensation induced by addition of caffeine. HU-arrested cells were given 5 mM of caffeine along with 0.2 μg/ml of nocodazole and every hour, cells were scanned for cells showing PCC by photomicroscope. As shown in Fig. 11, the frequency of cells showing PCC was increased in a dose-dependent manner of transfected Cdc25B cDNA. When > 6 μg/35-mm dish of Cdc25B cDNA was introduced into cells, cells rapidly died as previously reported (Makishima et al., 1997). Thus, the question whether the amount of Cdc25B is rate limiting for entry into mitosis still remains to be answered. But our present finding indicates that Cdc25B enhanced chromatin condensation. Alternatively, it is possible that over-expressed Cdc25B could not stimulate cells to enter mitosis due to the tight cellular regulation of Cdc25B activity, since no cells showing PCC appeared until 2 h after caffeine addition, even in the case of a higher dose of Cdc25B cDNA.

**Discussion**

**Cdc25B Is a Short-Life Protein**

The activation of p34cdc2/cyclin B occurs in the extract prepared from S phase-arrested hamster BHK21 cells without protein synthesis, but not in the extract of cells pretreated with protein synthesis inhibitor cycloheximide, which inhibits the in vivo activation of p34cdc2/cyclin B as well. Taking these results together, it can be deduced that S phase-arrested hamster BHK21 cells possess all the molecules required for the observed activation of p34cdc2/cyclin B and that either some molecules or protein modifications that are required for the activation of p34cdc2/cyclin B were lost by blocking protein synthesis. Since the p34cdc2/cyclin B complexes of cycloheximide-treated cells can be directly activated by immunoprecipitated Cdc25B, the modification of p34cdc2/cyclin B may not be lost. It is, therefore, rational to consider that some molecule(s) essential for the activation of p34cdc2/cyclin B is lost by blocking protein synthesis.

Cyclins are most important proteins for the mitotic activation of p34cdc2/cyclin B, and their cellular contents change depending on the cell cycle (Norbury and Nurse, 1992). In hamster BHK21 cells, cyclin B is present in S phase-arrested cells as reported (Nishitani et al., 1991). The amount of cyclin B, however, is not reduced by cycloheximide treatment. The other protein that is essential for the activation of p34cdc2/cyclin B is a family of dual-specificity phosphatase Cdc25 that perform dephosphorylation of Thr-14 and Tyr-15 (Dumphry, 1994). Among the family of Cdc25 phosphatases, we have found that Cdc25B, while Cdc25A and Cdc25C are stable, is lost by blocking protein synthesis. Indeed, the half-life of Cdc25B is <30 min in hamster BHK21, and also in HeLa cells. In this context, Cdc25B is unique among the Cdc25 family. The finding that human Cdc25B expressed in hamster wild-type BHK21 has a short life similar to hamster Cdc25B suggests that mammalian Cdc25B possesses a character of rapid turn over. Consistent with the loss of Cdc25B by cycloheximide treatment, the immunodepletion of Cdc25B from the extract abrogates the in vitro activation of p34cdc2/cyclin B.

**Cdc25B Functions as a Trigger for Mitosis**

Previously we have found that Cdc25C is essential for p34cdc2/cyclin B activation induced by the loss of RCC1 (Seki et al., 1992). However, Cdc25C is not required for the observed in vitro activation of p34cdc2/cyclin B. Cdc25C from mitotic cells activates p34cdc2/cyclin B within the ex-
Cyclin B, p34cdc2 is dephosphorylated and Cdc25C is phosphorylated and thereby activated, parallel with the activation of p34cdc2/cyclin B (Kumagai and Dunphy, 1992), as found in tsBN2 mutant and in Xenopus extract (Kumagai and Dunphy, 1992; Izumi and Maller, 1993). We think that Cdc25C has a low activity of tyrosine phosphatase that restrains an interaction between an enzyme and its substrate may play a role in the regulation of p34cdc2/cyclin B activation. In this context, the in vitro spontaneous activation of p34cdc2/cyclin B in the cell extract suggests that the cell disruption that may mimic loss of the cellular compartment may play a role in the regulation of p34cdc2/cyclin B activation.

Cellular Regulation of Cdc25B Function

While Cdc25C is activated by phosphorylation (Izumi et al., 1992; Kumagai and Dunphy, 1992, 1996), it is not yet clear how Cdc25B is activated. The finding that Cdc25B immunoprecipitated before the in vitro incubation activates the p34cdc2/cyclin B complexes that were isolated from cycloheximide-treated cells indicates that the Cdc25B of S phase-arrested BHK21 cells is active. If Cdc25B is already active in cells, there must be some regulatory system that restrains an interaction between an enzyme and its substrate may play a role in the regulation of p34cdc2/cyclin B activation. In this context, the in vitro spontaneous activation of p34cdc2/cyclin B in the cell extract suggests that the cell disruption that may mimic loss of the cellular compartment causes an activation of p34cdc2/cyclin B. In the interphase, therefore, we assume that the compartment mechanism that restrains an interaction between an enzyme and its substrate may play a role in the regulation of p34cdc2/cyclin B activation. In human HeLa cells, cyclin B1 is anchored in the cytoplasm during the interphase and enters the nucleus at the beginning of mitosis (Pines and Hunter, 1991, 1994). Recently, Nim1, which phosphorylates and thereby inhibits Wee1, is found to be localized in the cytoplasm.
Unstable Triggers and Stable Inhibitors for the Initiation of Mitosis

Although we could not exclude the possibility that the other unstable proteins like Cdc25B are also required for the initiation of mitosis, our findings indicate that the activator(s) is rapidly turning over, but the inhibitor(s) is stable. In S phase-arrested BHK21 cells, the activator(s) and the inhibitor(s) for p34<sup>cdc2/cyclin B</sup> seem to be balanced, and thereby, cells do not enter mitosis. The instability of the p34<sup>cdc2/cyclin B</sup> activator(s) may reflect an adaptation of cells to stresses, including the inhibition of protein synthesis. In such circumstances, it is better for cells to remain in a less active state of p34<sup>cdc2/cyclin B</sup>, or otherwise cells will suffer further damage from proceeding through the cell cycle. In this context, it seems to be reasonable that the ‘starter’ for the mitotic entrance is labile.

By loss of the activator(s), the presence of p34<sup>cdc2/cyclin B</sup> inhibitor(s) became obvious. It is also possible that the inhibitor(s) was activated by cycloheximide treatment. In mammalian cells, the inhibitory kinases for p34<sup>cdc2/cyclin B</sup> like Wee1 and Myt1 (Parker and Piwnica-Worms, 1992; Mueller et al., 1995a,b), the phosphatase INH/Pp2A (Cytet and Kirschner, 1988; Lee et al., 1991, 1994a) and the stoichiometric inhibitors (Kumagai and Dunphy, 1995; Lee and Kirschner, 1996) have been reported to prevent the activation of p34<sup>cdc2/cyclin B</sup> in the interphase. The finding that the addition of p34<sup>cdc2/cyclin B</sup> complexes pre-activated by Cdc25B could not induce a full activation of p34<sup>cdc2/cyclin B</sup> in the extract, suggests that our extracts contain the inhibitory kinase of p34<sup>cdc2/cyclin B</sup> or the phosphatase INH/Pp2A.

In summary, our results indicate that Cdc25B functions as a trigger for mitosis. Our finding that Cdc25B is an unstable trigger for the entrance of mitosis will provide a new aspect regarding the checkpoint control. In S. pombe, the level of Cdc25 is regulated by ubiquitin ligase Pub1 (Nefsky and Beach, 1996). Probably it is also true in mammalian cells. The stabilization of Cdc25B, therefore, may disturb the cell cycle control at the G2/M transition.

We thank J.Y.J. Wang for the anti-phosphotyrosine antibody, H. Okayama for human Cdc25B, K. Yamashita for pGEX-KG-hamster Cdc25C, and Miss K. Miller (Royal English Language Centre, Fukuoka, Japan) for proofreading the English used here.

This work was supported by Grants in Aid for Specially Promoted Research and for Cancer Research from the Ministry of Education, Science and Culture and from the Science and Technology Agency and by Human Frontier Science Program.

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