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Dephosphorylation of human p34^{cdc2} kinase on both Thr-14 and Tyr-15 by human cdc25B phosphatase

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In mammalian cells, p34^{cdc2} kinase undergoes phosphorylation at threonine-14, tyrosine-15 and threonine-161 in the S and G_2 phases of the cell cycle. At the onset of mitosis, the kinase becomes dephosphorylated at threonine-14 and tyrosine-15, resulting in activation. Cdc25 phosphatase has been shown to dephosphorylate tyrosine-15 in vitro, but whether it also does at threonine-14 remains unclear. In this study, we have found that human cdc25B phosphatase dephosphorylates both threonine-14 and tyrosine-15 but not threonine-161.

cdc2 kinase; cdc25; Protein phosphorylation; Phosphatase

1. INTRODUCTION

p34^{cdc2} kinase plays a key role in the initiation of mitosis [1]. The activity of this kinase is regulated by its phosphorylation and dephosphorylation. In yeast, phosphorylation occurs at the tyrosine-15 and threonine-167 residues. By contrast, in mammals, phosphorylation takes place mainly at tyrosine-15, threonine-161 (corresponding to threonine-167 in the yeast cdc2 kinase) and threonine-14. Phosphorylation at the threonine-161 residue is essential for the kinase activity [2], whereas phosphorylation at tyrosine-15 and/or threonine-14 is the major mechanism regulating this kinase [3,4]. The enzyme that phosphorylates tyrosine-15 is human p50^{wee1} kinase [5–7], but those that phosphorylate threonine-14 and/or threonine-161 are unknown. The p34^{cdc2} kinase is activated following dephosphorylation by cdc25 phosphatase [8–10]. Unlike yeast, human cells contain three cdc25 phosphatases (A, B, C) [11–13]. However, the role of each remained unclear. Since the human p34^{cdc2} kinase is phosphorylated at tyrosine-15 and threonine-14, it is important to determine whether cdc25 phosphatases dephosphorylate not only tyrosine-15 but also threonine- 14, or whether an unknown phosphatase dephosphorylates threonine-14.

Here, we report that human cdc25B phosphatase, the major species expressed at the G2/M boundary, activates the human p34^{cdc2} kinase by dephosphorylating both tyrosine-15 and threonine-14.

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2. MATERIALS AND METHODS

2.1. *Plasmid construction and expression of cdc25 protein in* E. coli

The 2.1 kb *Sma*I fragment of the human cdc25B cDNA was inserted into the *Bam*HI site of the pET3a plasmid by blunt end ligation after filling the recessed 3′ termini by Klenow fragment. The plasmid was transfected into *E. coli* BL21 (pLysS) strain, and the cdc25B protein produced was extracted from the inclusion body according to the published procedure [14]. After purification by mono S column chromatography, the cdc25B protein was renatured as previously described [5].

2.2. *Labeling of cdc2 kinase*

HeLa S3 cells were cultured in ES medium (Nissui Co., Japan) supplemented with 10% newborn calf serum. Logarithmically growing cells were synchronized at G_1/S phase by adding 1 μg/ml aphidicolin (Sigma, Co.). The synchronized cells were stored frozen until use. 5×10^6 cells were thawed and the cytosolic fraction was prepared as described previously [5]. In the presence of 1 μg human cyclin B1, the cytosolic fraction was incubated for 20 min at 30°C in buffer A containing 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 1 mM EGTA and 0.1 mM [γ-^{32}P]ATP (4,000 cpm/pmol). After incubation, the cdc2 kinase was immunoprecipitated by anti-cdc2 antibody (anti-C-terminal peptide) as described previously [15].

To label cdc2 kinase in vivo, the logarithmically growing cells were washed twice with phosphate free Eagle's MEM and incubated at 37°C for 6 h with the medium supplemented with 10% dialyzed calf serum containing 3.7 MBq/ml orthophosphate. The cells were harvested and washed twice with Mg^{2+},Ca^{2+}-free phosphate-buffered saline (PBS−). ^{32}P-Labeled cdc2 kinase was immunoprecipitated as described above.

2.3. *Phosphatase digestion*

The ^{32}P-labeled cdc2 kinase was incubated at 37°C for 30 min in the presence of 1 μg human cdc25B phosphatase in 50 μl buffer A. After termination of the reaction by the addition of SDS-PAGE sample buffer, the product was separated on SDS–12.5% PAGE. The phosphatase activity of cdc25B was assayed with *p*-nitro-phenyl-phosphate (pNPP) as a substrate as reported [10].

2.4. *Tryptic phosphopeptide mapping and phosphoamino acid analysis*

Radioactive bands corresponding to p34^{cdc2} kinase in SDS-poly-

acrylamide gel were excised, and the proteins in each band were recovered by electro-elution. The recovered proteins were digested with TPCK-treated trypsin (Worthington, Co.) at 37°C overnight. The resulting peptides were applied on thin layer cellulose plates (Merck 5716) by electrophoresis at pH 1.9 (H_2O/acetic acid/formic acid (88%) = 1794:156:50) in the first dimension, followed by chromatography in the second dimension (H_2O:*n*-butanol/pyridine/acetic acid = 60:75:50:15) according to the published procedures [16]. The separated peptides were partially hydrolyzed in constantly boiling 6 N HCl at 110°C for 1.5 h. Phosphoamino acid analyses were performed as described previously [5].

3. RESULTS AND DISCUSSION

The human cdc25B protein produced in *E. coli* was purified almost to homogeneity. It had a molecular weight of 68 kDa (Fig. 1A). Since 30 amino acid residues at the amino-terminus were deleted and 14 amino acids derived from the vector were added to the original cdc25 protein in the process of insertion into the expression vector, cdc25B expressed in *E. coli* was slightly smaller than the original protein. When detected using pNPP as a substrate, cdc25B had phosphatase activity, which was in linear proportion to the protein amount (Fig. 1B). Cdc25A and cdc25C, expressed in *E. coli*, have been shown to efficiently hydrolyze pNPP [10,11].

The cytosolic fraction of HeLa cells was incubated with human cyclin B protein. During the incubation, the monomeric cdc2 kinase in the cytosolic fraction was bound to cyclin B and subsequently phosphorylated [5]. When the [^{32}P]phosphorylated cdc2 kinase was immunoprecipitated and separated by SDS-PAGE, two ^{32}P-labeled bands were detectable. These bands did not change when incubated at 37°C for 30 min in the reaction buffer (Fig. 2A). However, treatment of the phosphorylated cdc2 with cdc25B protein resulted in disappearance of the two bands and concomitant emergence of a new radio-labeled band migrating slightly faster (Fig. 2B). The radioactivity in the new band (L) was much lower than the sum of that in the two bands (U and M) before treatment. Simultaneously, the cdc2 kinase activity (H1 kinase activity) markedly increased (Fig. 2C). The vertebrate or mammalian p34^{cdc2} kinase is phosphorylated at Thr-14, Tyr-15 and Thr-161 [2–4]. The phosphopeptide map of in vivo labeled p34^{cdc2} kinase was shown in Fig. 3D. The phosphoamino acid analyses of these phosphopeptides showed that peptide 1 contained both phospho-Thr and phospho-Tyr and that peptide 2 contained only phospho- Tyr. Peptides 3, 4 and 5 contained only phospho-Thr (data not shown). These results are identical to the previous report [2].

Next, we investigated phosphorylation sites of each band by peptide mapping. The most slowly migrating band (U in Fig. 2A,B) mainly consisted of three phospho-peptides 1, 4 and 5 (Fig. 3A). The phosphopeptides that were present in the middle band (M in Fig. 2A,B) were peptides 2, 3, 4 and 5 (Fig. 3B). Phosphopeptide 1, which was present in the upper band, was not contained in the middle band. Phosphopeptides 2 and 3 were faintly detected in the upper most band (U), presumably due to contamination of the middle band (M). The phosphopeptides contained in the fastest migrating band (L in Fig. 2B) that emerged after cdc25B treatment were peptides 4 and 5 (Fig. 3C). The phosphorylated residue in peptides 4 and 5 was phospho-Thr-161. The peptide 1 contained both phospho-Thr-14 and phospho-Tyr-15. The peptide 2 and peptide 3 contained only phospho-Tyr-15 and only phospho-Thr-14, respectively. The peptides 1, 2 and 3 that disappeared after cdc25 treatment contained phospho-Tyr-15 and/or phospho-Thr-14. These results strongly suggest that the human cdc25B phosphatase dephosphorylated not only phospho-Tyr-15 but also phospho-Thr-14, but not phospho-Thr-161. Since the two phosphorylated bands (U and M) in the immunoprecipitated cdc2 kinase did not change at all by the incubation (Fig. 2A), the possibility was excluded that cdc25B dephosphorylated the

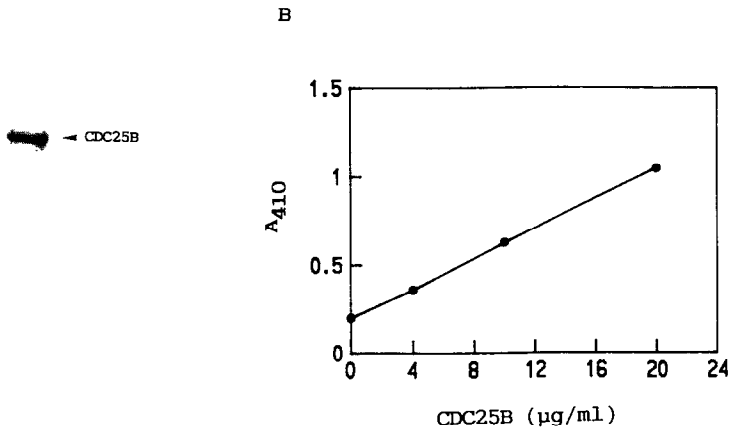

Fig. 1. Phosphatase activity of human cdc25B expressed in *E. coli*. Human cdc25B was produced in *E. coli* and purified by mono S column chromatography described in Materials and Methods. (A) SDS-12.5% PAGE of purified human cdc25B. The proteins were stained with Coomassie brilliant blue R-250. (B) The phosphatase activity of cdc25B. The cdc25B in (A) was used as the enzyme. Phosphatase activity was assayed with pNPP as the substrate.

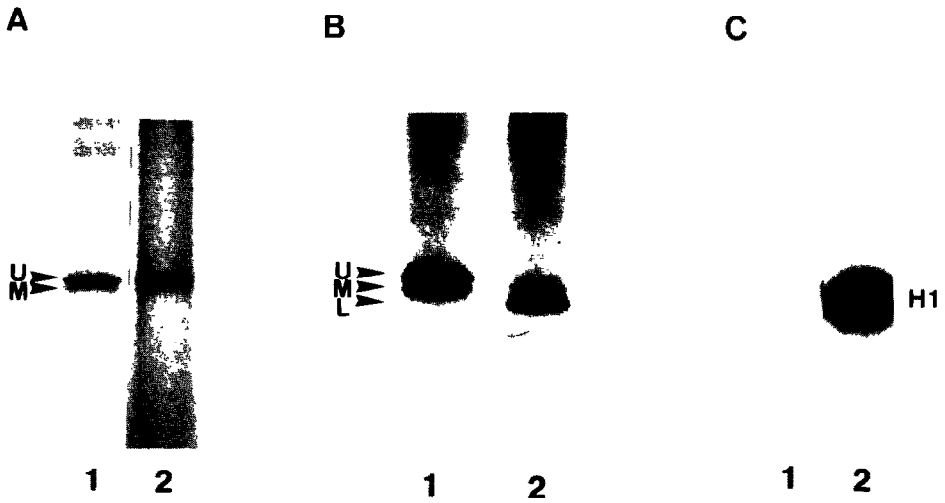

Fig. 2. Dephosphorylation and activation of p34^{cdc2} kinase by cdc25B. (A) Incubation of p34^{cdc2} kinase without cdc25B. Lane 1, before incubation; Lane 2, incubation at 37°C for 30 min with buffer. (B) Dephosphorylation of p34^{cdc2} kinase by human cdc25B. Lane 1, without cdc25B (with buffer); lane 2, with cdc25B. (C) H1 histone kinase activity of p34^{cdc2} kinase with (lane 2) or without (lane 1) treatment with cdc25B. The kinase activity was assayed in the presence of [γ-32P]ATP and the radioactivity incorported into H1 histone was detected by autoradiography.

cdc2 kinase only at Tyr-15, and that the partially dephosphorylated cdc2 kinase was further dephosphorylated at Thr-14 by a specific phosphatase which was contaminated in the cdc2 kinase preparation.

The vertebrate or mammalian cdc2 kinase is inactivated by phosphorylation of both Thr-14 and Tyr-15 and activated following dephosphorylation at both sites [3,4]. It has been shown that, in analogy to the yeast system, cdc25 phosphatase mediates dephosphorylation of phospho-Tyr-15 [8–10], but the enzyme responsible

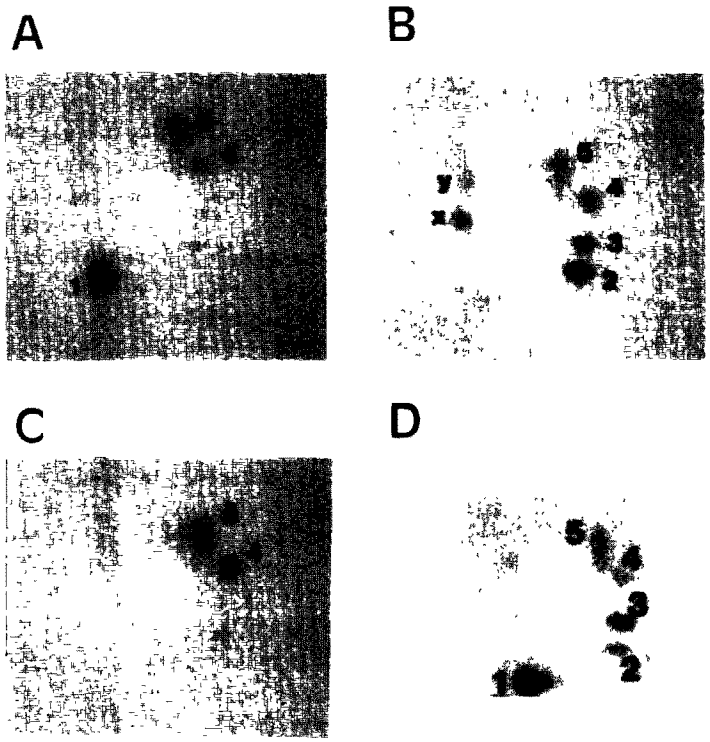

Fig. 3. Phosphopeptide mapping of phosphorylated p34^{cdc2} kinase. The phosphopeptides of three phosphorylated bands (U, M, L) of p34^{cdc2} kinase in Fig. 2B were mapped on cellulose plates. The phosphopeptides were detected by imaging plate using a Fuji BA100. Electrophoresis was run from left to right (anode to cathode) and chromatography was from the bottom to the top. (A) Uppermost band (U) without cdc25B. (B) Middle band (M) without cdc25B. (C) The fastest migrating band (L) resulting from cdc25B treatment. (D) The p34^{cdc2} kinase labeled in vivo. The peptides labeled x or y in (B) have not yet been characterized.

for the dephosphorylation of phospho-Thr-14 has remained unclear. In this report, we have shown that human cdc25B phosphatase, the major mitotic inducer, activates the $p34^{cdc2}$ kinase by dephosphorylating both phospho-Tyr-15 and phospho-Thr-14.

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