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Comparative analysis of two murine CDC25B isoforms

Min Kook Kang, Aera Bang, Hwa Ok Choi and Seung Jin Han*

Department of Biological Sciences, Inje University, 197 Inje-ro, Gimhae, Gyeongnam, 50834, Korea

***Corresponding author:** hansjin@inje.ac.kr

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Abstract: CDC25B phosphatase plays a pivotal role in the cell cycle process by dephosphorylating and activating the CDC2 kinase of maturation-promoting factor (MPF). In mice, two transcripts of *Cdc25B* are generated by the alternative splicing of one gene. We compared the properties of these two forms of CDC25B. When the expression pattern of *Cdc25B* was examined using RT-PCR, both forms were detected in almost all mouse tissues tested. The expression of two forms of the CDC25B protein in various mouse tissues was confirmed using Western blotting with generated isoform specific antibodies. CDC25B1 tends to accumulate more in the cytosol than CDC25B2 does, and they have different binding capacity for 14-3-3 proteins. CDC25B1 was more effective in dephosphorylating *in vitro* substrate para-nitrophenyl phosphate and showed higher activity in the modified histone H1 kinase assay than CDC25B2. These results suggest that the two forms of CDC25B play different roles in cell cycle regulation.

Key words: Cdc25B; 14-3-3; localization; phosphatase activity; isoform

INTRODUCTION

Cell cycle progression is orchestrated by several cyclin-dependent kinases (CDKs). Activation of the CDKs requires both dephosphorylation of the inhibitory phosphates on Thr14 and Tyr15 by dual-specificity phosphatase CDC25 [1] and association with cyclins [2]. As a cell cycle regulator, CDC25 is involved in mitosis, meiosis [3], DNA damage and replication checkpoint release [4, 5]. In mammals, three members of the CDC25 family, CDC25A, CDC25B and CDC25C, are expressed differently in diverse tissues and during different developmental stages [6]. Although they have partially functional redundancy, they are involved in different cell cycle processes. CDC25A has a role in the G1/S phase transition, and CDC25C is involved in the G2/M transition [2, 7]. CDC25A-null mice are lethal during the peri-implantation period (embryonic days 5-7) [8], but CDC25C-null mice display no appreciable phenotype, with normal fertility in both males and females [9]. In CDC25B-null female mice, the oocyte cannot undergo resumption of the meiotic progression [10]. The accumulation of CDC25B in mitosis triggers centrosomal microtubule nucleation and activates cyclin B/CDC2 in the centrosome for mitotic entry [11]. Therefore, CDC25B is essential for the G2/M phase transition in the cells.

The human *CDC25B* gene consists of 15 exons, whereas the mouse *Cdc25b* gene is composed of 16 exons in chromosome 2. To date, more than four different types of human CDC25B transcripts generated by the alternative splicing of their amino termini have been found; however, some of the human CDC25B transcripts are not translated into protein [12, 13]. The expression patterns of translated CDC25B isoforms are different in human HeLa cells [14]. The variants of CDC25B have different phosphatase activity when expressed in fission yeast, indicating the amino terminus of CDC25B is responsible for the regulation of its activity toward cyclin B/CDC2 [15].

Endogenous human CDC25B shuttles between nucleus and cytoplasm [16] and human CDC25B subtypes have different subcellular distribution [17]. The phosphorylation of the CDC25B amino terminus by some kinases leads to a change in its localization as well as activity. The CDC25B phosphorylated by Aurora A kinase on S353 at the G2/M phase translocates to the centrosome [18], while the phosphorylation of the same site by PKB/AKT at the interphase stimulates

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its accumulation in cytoplasm [19]. Some CDC25B phosphorylation sites are involved in 14-3-3 binding to modulate the localization of CDC25B and its activity in the mitosis, G2 checkpoint and meiosis processes [20-25]. For instance, p38MAPK phosphorylates the S323 site upon ultraviolet (UV) irradiation and stabilizes 14-3-3 binding to block entry into mitosis [24, 26]. The subcellular localization of CDC25B is dependent on the combined effects of a nuclear localization signal, a nuclear export signal, as well as the interaction with 14-3-3 proteins. It is believed that phosphorylated Ser-323 of human CDC25B is a major 14-3-3 dimer-binding site and pSer-151 and pSer-230 act as low-affinity 14-3-3 binding sites to cooperatively bind with the 14-3-3 dimer by creating an intramolecular bridge [21]. In the mouse GV oocyte arrested in the prophase of meiosis I, PKA phosphorylates CDC25B on S321 (human S323 corresponding site), leading to enhanced binding with 14-3-3 [27] and the localization of CDC25B in the cytosol [25].

Given that the mouse is a major experimental animal model, it is important to understand the similarities and differences in CDC25B protein between mice and humans. However, almost all experiments on the properties of CDC25B protein have mainly been performed with human CDC25B proteins; studies of the mouse ortholog are uncommon. In addition, there is little information about the differences between CDC25B isotypes. In this study, we provide some evidence for the expression of two splicing variants of mouse CDC25B and the properties of these proteins in the cell cycle.

MATERIALS AND METHODS

Reverse transcription and polymerase chain reaction

Full-length mouse *Cdc25b* cDNAs were cloned with mouse lung mRNA using Superscript III reverse transcription (Thermo Fisher Scientific, Inc. Waltham, MA). Primers for mouse *Ccd25b* genes were 5' gcc*ggatcc*gccacgatggaggtacccctg 3' and 5' agg*ctcgag*atcactggtcttgcagcctgc 3' with generated *BamH* I and *Xho* I restriction enzyme sites (italics in the sequence). To check the expression of *Cdc25b* genes in various tissues, cDNAs were amplified with specific primers: *Cdc25b1* forward, 5' cctcattccagctctgccca 3', *Cdc25b2* forward, 5' ccagagaccaagatggaggagc 3', and *Cdc25b* reverse, 5' ccgggctcagctcctctac 3'.

Cell culture

Hek 293, HeLa or Cos7 cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (Thermo Fisher Scientific, Inc.), 30 mg/ml penicillin and 100 mg/mL streptomycin at 37°C under 5% CO_2. For the expression of *Cdc25B* constructs, cells were transfected using JetPEI transfection reagent (Polyplus Transfection Inc., Illkirch-Grafenstaden, France).

Western blot analysis

The various mouse tissues were washed with PBS and then homogenized in RIPA buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP40, 0.1% SDS) using a motor-driven homogenizer (IKA, Guangzhou, China). The lysates were incubated overnight with 0.1 μg of CDC25B antibody (sc326, Santa Cruz Biotech, Inc. Dallas, TX) at 4°C. After immunoprecipitation for 2 h using 30 μL of 50% protein G sepharose (GE Healthcare Life Sciences, Piscataway, NJ), the precipitates were washed with RIPA buffer and subjected to 8% SDS-PAGE. After transfer to PVDF membranes (Millipore Corp., Bedford, MA), immunoblotting was performed by incubating with a 1:1000 dilution of CDC25B1- or CDC25B2-specific antibody. The antibodies that can respectively recognize only CDC25B1 or the B2 specific region (Fig. 1A) were generated using synthesized peptides (AbFrontier, Seoul, Korea).

Subcellular localization of mouse Cdc25B in Cos-7 cells

Cos-7 cells were transfected with HA-tagged wild type, S229A or S321A mutant Cdc25b1. After 24 h, mitochondria in the cells were stained with MitoTracker (Thermo Fisher Scientific) for 1 h. After fixation with 4% paraformaldehyde, cells were stained with HA specific antibody for 2 h. The cells were incubated with FITC conjugated anti mouse IgG specific antibody followed by DAPI containing mounting solution addition.

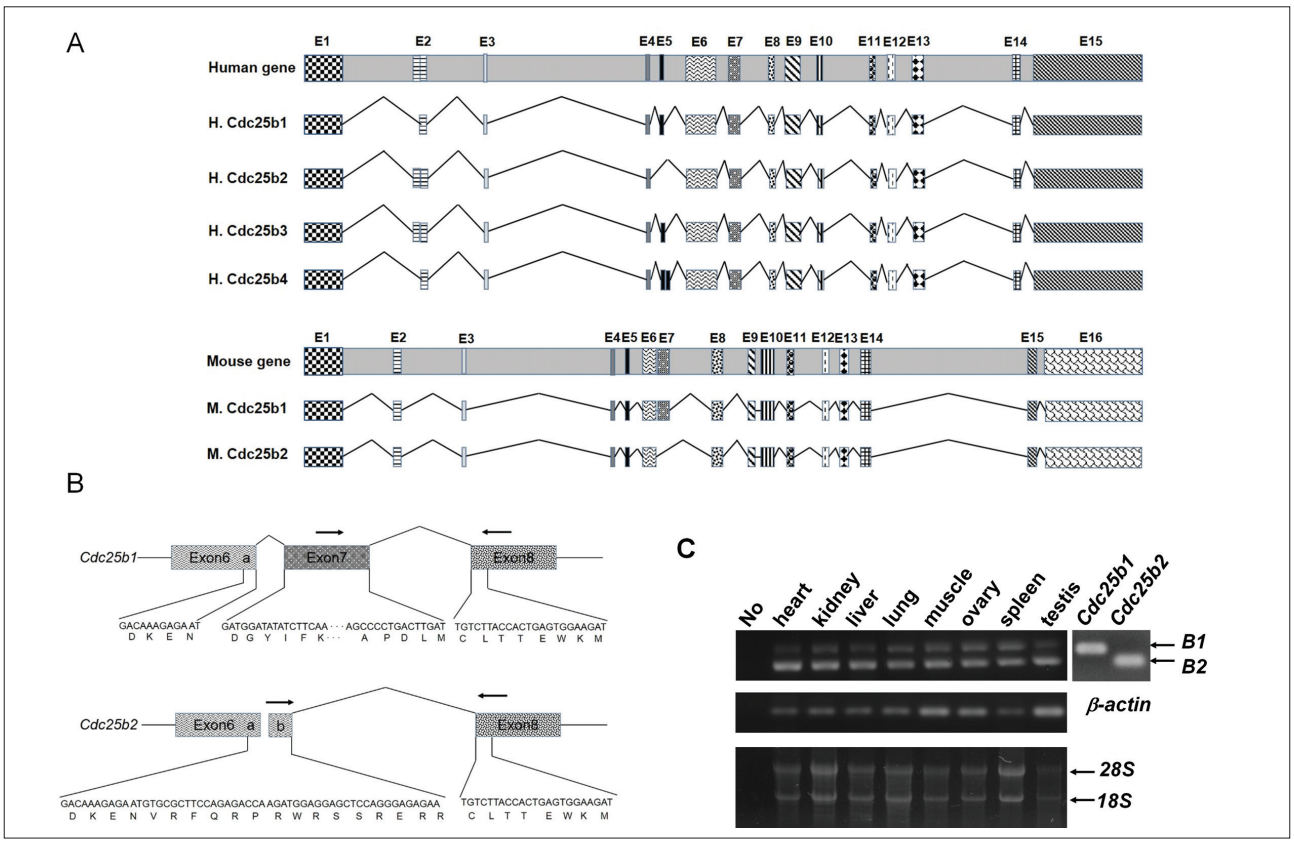

Fig. 1. Two forms of *Cdc25b* transcript are expressed in mouse tissues. A – genomic structure of human and mouse *CDC25B* genes. Exons are alternatively spliced into at least four variants from the human *CDC25B* gene, whereas only two variants are generated from the mouse *CDC25B* gene. B – the arrangement of mouse *Cdc25b* exons and introns around exons 6 and 7. Exons 6, 7 and 8 only are shown of the 16 exons of the *CDC25B* gene in chromosome 2. Exon 6 is divided into a and b, which are contiguous without introns. Two transcripts are generated by the alternative splice donor site in exon 6. *Cdc25b1* is generated by connecting exon 6a with exon 7, whereas *Cdc25b2* is generated by connecting exon 6ab with exon 8. The positions of primers used for RT-PCR in (C) are indicated by arrows. C – expression of two *Cdc25b* transcripts in various mouse tissues. When RT-PCR was performed with a common reverse primer for both transcripts (in exon 8) and two different forward primers specific to one of the transcripts (in exon 7 or exon 6b), two PCR products were detected in the mouse tissues (product size: *Cdc25B1*, 136 bp; *Cdc25B2*, 83 bp). RT-PCR was performed with mRNA from different tissues, including heart, kidney, liver, lung, muscle, ovary, spleen and testis. *Cdc25b1* and *Cdc25b2* cDNA were used as PCR templates in the last two lanes as positive controls. The first lane (No) indicates a PCR product without RT reaction. β-actin was used as a RT-PCR control, and isolated mRNAs are shown as an RNA purification control (bottom panel).

14-3-3 co-immunoprecipitation

To investigate the ability of CDC25B to bind the 14-3-3 proteins, one of the HA-tagged *Cdc25b* genes and one of FLAG-tagged *14-3-3* genes were co-transfected into Hek293 cells at a 1:1 ratio. Cell lysate (500 μg) was immunoprecipitated with HA antibody (HA 1.1, Sigma-Aldrich, St. Louis, MO) at 4°C overnight. After the addition of protein G sepharose, the washed immune complexes were separated by SDS-PAGE. Co-precipitated 14-3-3 was detected with the FLAG antibody (F1804, Sigma-Aldrich).

Modified histone H1 kinase assay

To purify the inactive cyclin B/CDC2 complex, HeLa cells were treated with 10 mM of hydroxyurea for 18 h and immunoprecipitated with cyclin B antibody (H-85, Santa Cruz Biotech). After transfecting various mutants of *Cdc25b* into the HEK293 cells, the CDC25Bs were immunoprecipitated with HA antibody and mixed with the cyclin B/CDC2 complex. The mixture was incubated for 30 min at 30°C in the phosphatase buffer (40 mM Tris-HCl, pH 8.0, 300 mM NaCl, 10 mM EDTA, 10 mM DTT). After being

spun down, the pellet was incubated at 30°C for 15 min with 50 μL of kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 50 μM ATP, 5 μCi/mL [γ-^{32}P]ATP) with the substrate (10 μg histone H1). The reaction was stopped by adding 4X Laemmli buffer, and the samples were loaded on a 12.5% SDS-PAGE gel. The density of ^{32}P incorporation was compensated with the amount of added immunoprecipitated protein after scanning and digitizing the image using the Image J program.

Expression and purification of GST-fused Cdc25b and phosphatase assay

Mouse *Cdc25b1* and *Cdc25b2* were respectively cloned in the pGEX4T-2 vector and induced the expression of a fusion protein by adding 0.2 mM of isopropyl β-D-thiogalactoside (IPTG) in soluBL21 cells (Genlantis, Inc. San Diego, CA). For phosphatase assays, purified GST, GST-CDC5B1 and GST-CDC25B2 were incubated with 20 mM of para-nitrophenyl phosphate (pNPP, Sigma-Aldrich), 0.1% β-mercaptoethanol, 1 mM EDTA and 50 mM Tris-HCl (pH8.2) for 20 min at 30°C. The reaction was terminated by the addition of 160 μL 0.2 M NaOH, and absorbance was measured at 410 nm using an MQX200R Powerwave XS (BioTek, Winooski, VT).

RESULTS

Two forms of Cdc25b transcripts expressed in various mouse tissues

It appears mice have only two forms of protein coding *Cdc25b* transcripts according to the Ensemble genome browser search result (ENSMUSG00000027330.9, Fig. 1A). We isolated two forms of mouse *Cdc25b* cDNA by RT-PCR and called the longer form *Cdc25b1* and the shorter form *Cdc25b2* (Fig. 1A and 1B, NCBI accession numbers NP_001104545.1 and NP_075606.1 for proteins). These were generated by the alternative splicing of one *Cdc25b* gene. The 123 nucleotides of exon 7 in the *Cdc25b1* transcript were replaced by 45 other nucleotides in the *Cdc25b2* transcript by connecting an alternative splice donor site in exon 6 with a splice acceptor site in exon 8 without exon 7 (Fig. 1B). The expression of two different transcripts in the various mouse tissues was confirmed by RT-PCR with different sets of primers designed to distinguish two mRNAs (Fig. 1B). RT-PCR with primers for a specific region of *Cdc25b1* yielded an amplicon with 136 base pairs (bp), while an 83 bp fragment was amplified with the *Cdc25b2* specific primers. All the tested tissues expressed *Cdc25b1* and *Cdc25b2* transcripts (Fig. 1C).

Two forms of CDC25B protein expressed in mice

The deduced mouse CDC25Bs, which were translated from two alternatively generated transcripts, had a common catalytic domain. The 41 amino acids in the CDC25B1 were replaced by 15 other amino acids in CDC25B2 in the amino terminus (Fig. 2A). Therefore, we investigated whether two mouse *Cdc25b* transcripts could be translated into the CDC25B proteins in various mouse tissues. Two antibodies that can respectively recognize only a CDC25B1 or a B2 specific region were produced with two synthesized peptides corresponding to the distinct sites between two proteins (Fig. 2A). The specificity of the generated antibodies was monitored after the transfection of HA-*Cdc25b1* or HA-*Cdc25b2* into the Hek293T cells. The anti-CDC25B1 antibody could specifically detect overexpressed CDC25B1 but not CDC25B2 and *vice versa* (Fig. 2B). To confirm the transfection and expression of HA-tagged proteins, the HA antibody was used after stripping (Fig. 2B, second and fourth panels). The expression of two proteins in the various mouse tissues was examined with these antibodies. Because the amount of CDC25B proteins is below the detectable range with these antibodies, the endogenous CDC25B proteins were immunoprecipitated with the pan-specific CDC25B antibody, and immunoprecipitated CDC25B proteins were checked using the CDC25B1- or CDC25B2-specific antibody. In agreement with the RT-PCR results, CDC25B2 protein (~62.9 kDa) was detected in all the mouse tissues checked. Cdc25B1 protein (~65.5 kDa) was also expressed in several organs, especially in the heart and muscle (Fig. 2C).

Two forms of CDC25B protein have different localization preferences in cells

We checked the difference in the subcellular distribution of two forms of mouse *Cdc25B*. After GFP-tagged

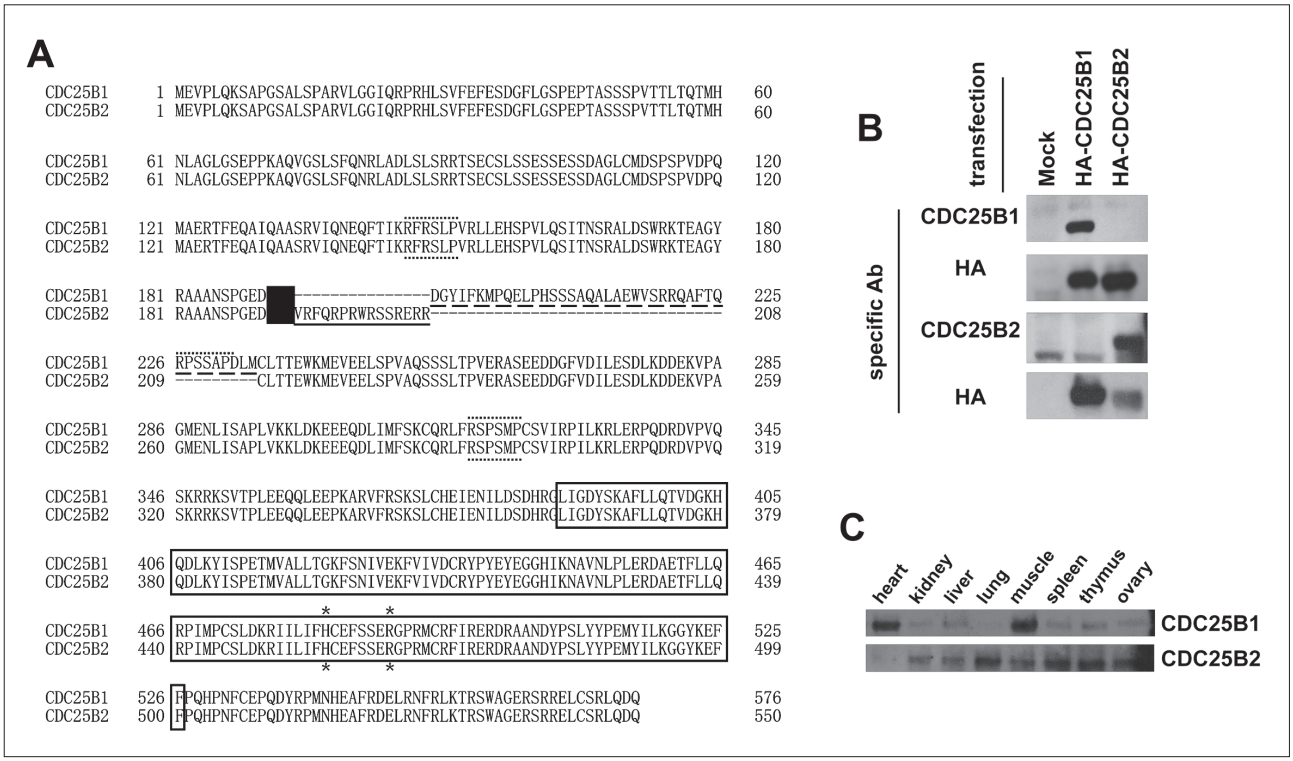

Fig. 2. The expression of two forms of mouse CDC25B proteins. A – alignment of two isoforms of mouse CDC25B proteins. Putative 14-3-3 binding sites are indicated with dotted lines, and the amino acid sequences used for CDC25B1- and CDC25B2-specific antibodies are marked with dashed and solid lines, respectively. KEN boxes are shaded and catalytic domains are boxed. The Cys and Arg residues important for CDC25 activity that were mutated to Ser and Lys in the substrate-trapping method are indicated by an asterisk. B – the antibodies that can distinguish two isoforms were generated. After the transfection of each form of HA-tagged *Cdc25b* in the Hek293T cells, the specificity of the antibodies was examined. CDC25B1 and CDC25B2 antibodies specifically detect HA-CDC25B1 and HA-CDC25B2, respectively, without the cross-reactivity of another form. To confirm the expression of transfected genes, the HA antibody was used after stripping the blot. C – CDC25B protein expression was examined in the various mouse tissues including heart, kidney, liver, lung, muscle, spleen, thymus and ovary. The extracts from different mouse tissues (500 μg) were immunoprecipitated with the pan-specific CDC25B antibody (sc-326), which can recognize both CDC25Bs. The immune complex was subjected to 10% SDS-PAGE, and two forms of CDC25B were detected by immunoblotting with anti-CDC25B1 or anti-CDC25B2 serum.

Cdc25b1 and *Cdc25b2* were transfected into the Cos7 cells, the major position of the tagged proteins in the cells was checked and counted. Both fused proteins were mainly found in the cytosol, and some proteins were detected only in the nucleus or in both areas. When the distribution of each CDC25B was examined, the tendency ratio of cytosol-localized CDC25B1 protein was significantly higher than that of CDC25B2 (Fig. 3A). This result suggests CDC25B1 and CDC25B2 proteins have different localization preferences.

Two forms of CDC25B protein bind 14-3-3 proteins

Mouse CDC25B1 contains a human CDC25B Ser-230 corresponding site (Ser-229), but this residue does not exist in mouse CDC25B2 (Fig. 2A). To elucidate the importance of the Ser-229 site of CDC25B1 as a localization determinant, the localization of CDC25B1 was checked after the mutation of Ser-229 to alanine, which is not phosphorylated by any kinase(s). The S229A mutant tended to accumulate more in the nucleus than the wild-type CDC25B1 protein does (Fig. 3B). Most of the S321A protein was present in the nucleus, indicating Ser-321 residue is the major site to regulate the localization of CDC25B (Fig. 3B).

We assessed the difference between CDC25B1 and CDC25B2 to interact with each 14-3-3 protein of the seven isoforms of 14-3-3 [28]. One of the FLAG-tagged 14-3-3 proteins was co-transfected with one of the HA-tagged CDC25B genes, and immunoprecipi-

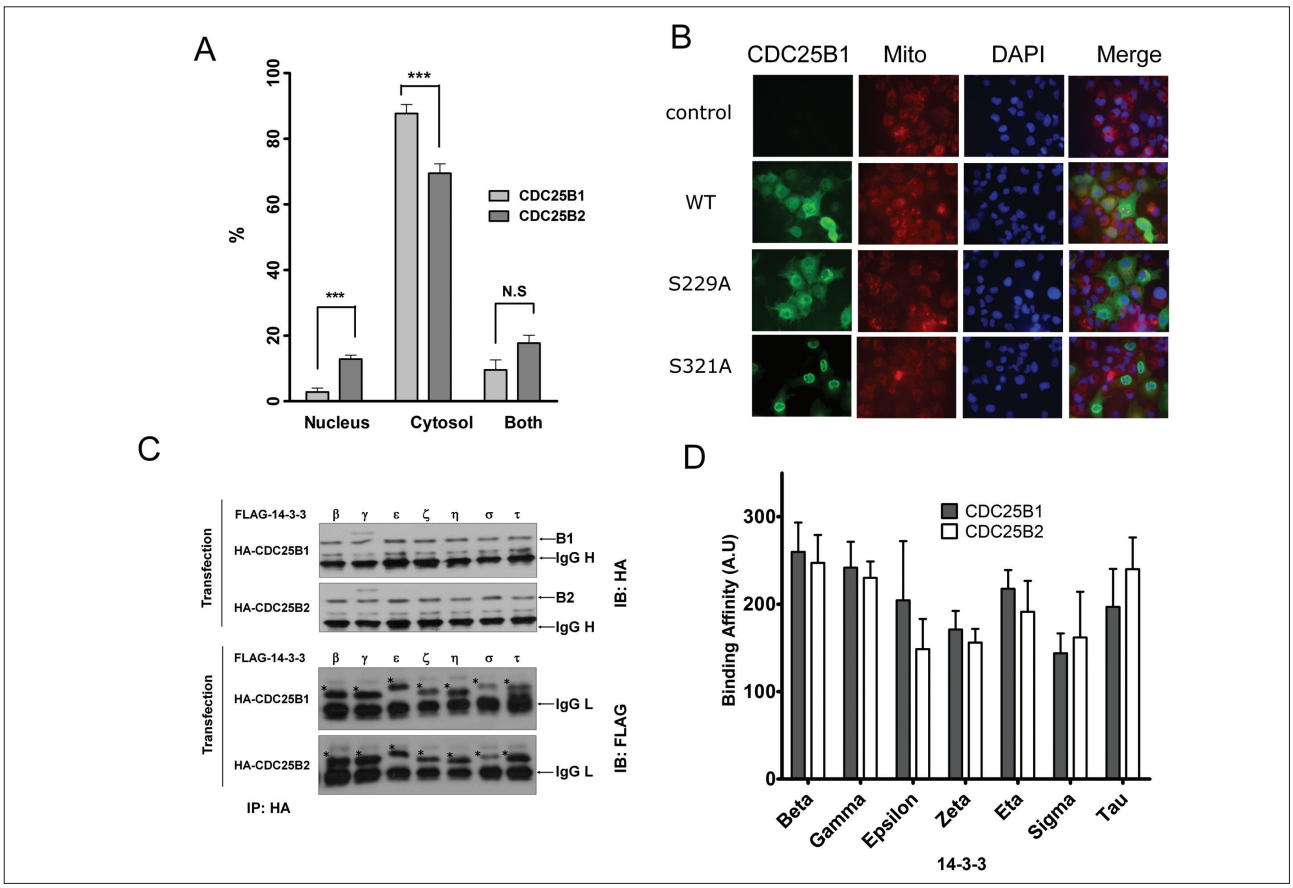

Fig. 3. Localization of Cdc25 in the cells. A – the difference in the localization of CDC25B1 and Cdc25B2. Cos7 cells were transfected with EGFP-tagged *Cdc25b1* or *Cdc25b2* and cultured for 24 h. After taking photographs of images observed under a microscope, the localization of fluorescence protein was counted independently by three people. *** represents a P value <0.001 using a paired t-test. This result is representative of three independent experiments. (N.S. – non-significant). B – Ser-321 and Ser-229 of CDC25B1 are involved in the cellular localization of CDC25B1. Green fluorescence represents HA-tagged CDC25B protein expression, red represents mitochondria and blue represents nuclei. More S229A mutants are distributed in the nucleus compared to wild-type CDC25B1s, and the majority of S321A mutant CDC25B1s move to the nucleus. C, D – two forms of CDC25B protein interact with 14-3-3s. The binding affinities of two mouse CDC25Bs to various 14-3-3s were compared by immunoprecipitation. After transfection of HA-tagged *Cdc25b1* or *Cdc25b2* into the Hek293 cells, expressed CDC25Bs were precipitated with HA antibody. The co-immunoprecipitated 14-3-3 was checked with a FLAG-specific antibody. The amount of immunoprecipitated CDC25B was confirmed by Western blot analysis. The results are representative of three independent experiments. Ig G H: Ig G heavy chain; Ig G L: Ig G light chain; IP: immunoprecipitation; IB: immunoblot. D – the co-immunoprecipitated 14-3-3 in (C) was scanned and the density was digitalized. The signal intensities were plotted against the amount of immunoprecipitated CDC25Bs.

tation was performed with the HA antibody. When the co-precipitated 14-3-3 were monitored with the FLAG-specific antibody, all forms of 14-3-3 proteins were detected (Fig. 3C). The β, γ and τ forms of 14-3-3 interacted with CDC25Bs more strongly than the ζ and σ forms (Fig. 3C, 3D), indicating the affinity of 14-3-3 to CDC25Bs is different from the 14-3-3 protein isoforms. It appears that there is some difference in binding affinity between CDC25B1 and CDCB2 for each 14-3-3 isoform, but it is not statistically significant (Fig. 3D).

Two forms of CDC25B protein have different phosphatase activity

To better determine the difference between two forms of mouse CDC25B, we investigated the affinity of two CDC25B forms to the substrate cyclin B/CDC2. We conducted a substrate-trapping experiment [21] using a phosphatase-dead mutant for each form of CDC25B. The wild-type CDC25B dephosphorylated immediately and dissociated from its substrate, tyrosine 15 residue phosphorylated Cdc2 (Fig. 4A, first and fifth

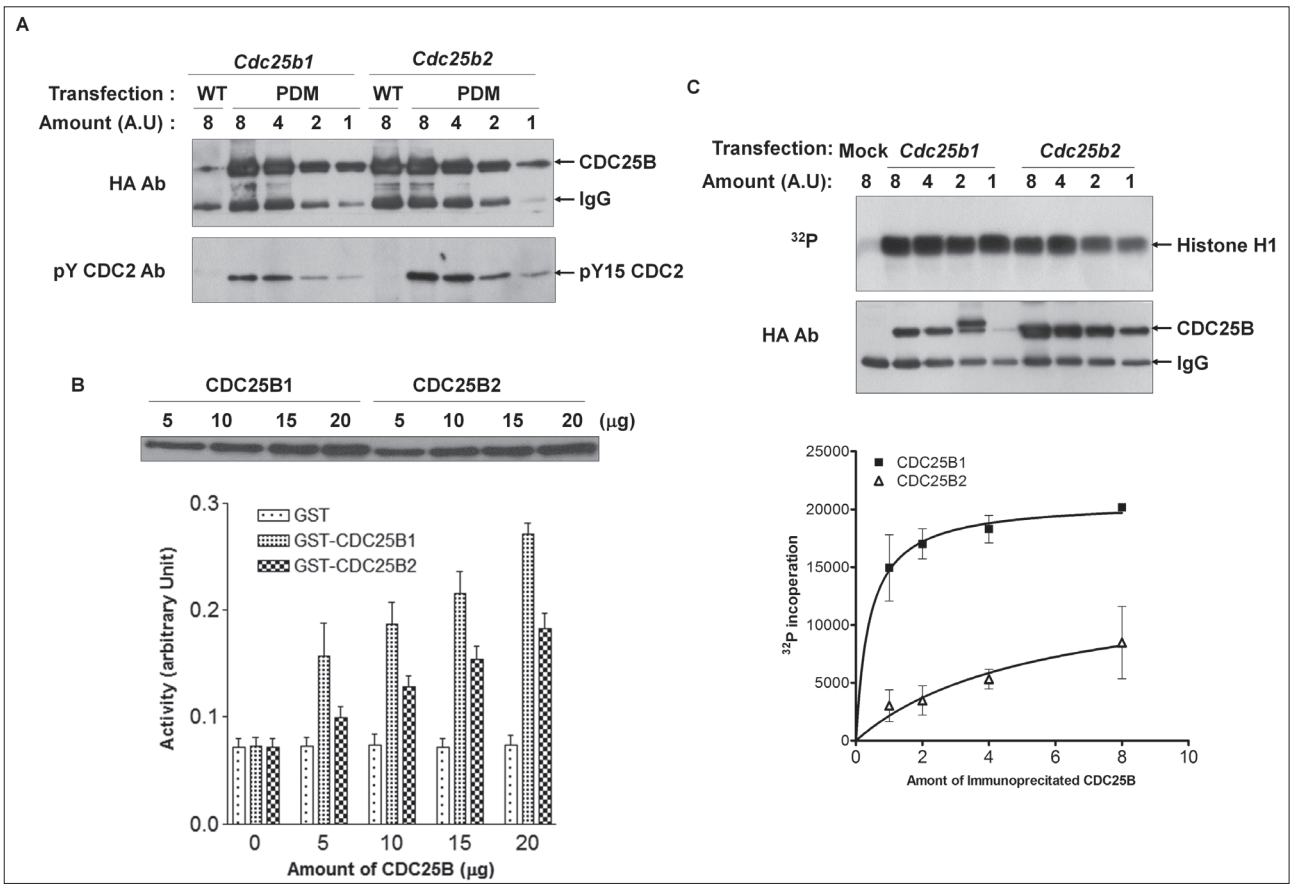

Fig. 4. The difference between Cdc25B1 and Cdc25B2 in the phosphatase activity. **A** –affinity of CDC25Bs were tested with substrate-trapping method. After transfection and immunoprecipitation of phosphatase-mutated CDC25Bs, the indicated amount of immunocomplexes were separated by SDS-PAGE and detected with HA antibody (upper panel). Co-immunoprecipitated phosphorylated CDC2 was monitored with a specific antibody (lower panel). A.U: arbitrary unit; PDM: phosphatase-dead mutant. **B** – the difference in phosphatase activity between two forms of CDC25B. The purified GST, GST-CDC25B1 and GST-CDC25B2 were incubated with pNPP and the phosphatase activity was examined. The upper panel indicates the amount of added fusion proteins. **C** – indicated amounts of immunoprecipitated CDC25B1 or CDC25B2 were incubated with immunoprecipitated cyclin B/CDC2 complex, followed by the addition of histone H1 and 5 μCi [γ-^{32}P]ATP for an additional 30 min. The phosphorylation state of histone H1 was detected by autoradiography (upper panel), and the amount of added CDC25B was checked by Western analysis with HA antibodies (lower panel). Representative results of the three experiments are presented. The graph shows the ratio of [^{32}P] incorporation into histone H1 (lower graph).

lane). Because the mutant has no dephosphorylation activity for the phosphate residue in the substrate, it binds stably and is easily co-immunoprecipitated with the phosphorylated form of CDC2 [29]. Therefore, the method well reflected the affinity of CDC25 phosphatase enzymes for the substrate. The HA-tagged CDC25B was transfected, followed by immunoprecipitation. The different amounts of the immune complexes were resolved by SDS-PAGE, and the co-immunoprecipitated endogenous phosphorylated CDC2 was checked. Surprisingly, CDC25B2 had a stronger affinity to its substrate than CDCB1 (Fig. 4A).

Next, we examined the difference in activity between two forms of mouse CDC25B using an *in vitro* phosphatase activity assay. The activity was checked by incubating the purified GST-fused CDC25B1 or CDC25B2 with pNPP as a substrate [30]. The ability of GST-CDC25B1 to dephosphorylate pNPP was greater than that of GST-CDC25B2 (Fig. 4B). To confirm the result, we used a modified histone H1 kinase assay. Histone H1 is a well-known substrate for the cyclin B/CDC2 complex activated by CDC25B phosphatase. Therefore, if histone H1 is incubated with the cyclin B/CDC2 complex after treatment with Cdc25B,

the phosphorylation degree of histone H1 could reflect the ability of Cdc25B to activate the cyclin B/CDC2 complex. When the phosphorylation state of histone H1 was checked, a lower amount of CDC25B1 induced more ^{32}P incorporation into histone H1 compared to CDC25B2. Altogether, these results indicate CDC25B1 has a greater ability than CDC25B2 to activate the cyclin B/CDC2 complex.

DISCUSSION

CDC25B is implicated in cell cycle regulation in germ cells and somatic cells [27, 31], but its regulation mechanism has not been completely elucidated. As most of the experiments to elucidate the properties of CDC25B have been performed with human CDC25B, we tried to better understand the common roles of CDC25B in cell cycle regulation by monitoring the characteristics of mouse CDC25B isoforms and comparing them with human ones. In this study, we isolated two spliced forms of mouse *Cdc25b-Cdc25b1* and *Cdc25b2*. The human *CDC25B* gene is transcribed into at least four transcripts [14]; however, only *CDC25B2* and *CDC25B3* proteins have been biochemically detected in human cells [13]. It is likely two transcripts are transcribed from one mouse *Cdc25b* gene and both are translated into proteins in various mouse tissues (Fig. 1, 2). There are significant differences in the expression pattern of *Cdc25B1* as assessed by Western blot analysis and RT-PCR (Figs 1C and 2C, respectively). However, several reports demonstrated that the correlation between the expression levels of mRNA and protein can be poor, showing that the correlation is less than 40% [32, 33]. We believe that the expression of the CDC25B1 isoform might be a plausible example of that poor correlation. Recently, a truncated nuclear form of CDC25B missing the amino terminus was identified from human spermatozoa, and it was found to be specifically involved in G2/M checkpoint recovery [34]. We could not rule out the possible existence of a mouse counterpart for the shorter human CDC25B form, as several commercial antibodies have been able to recognize the small-sized proteins in Western blot using mouse tissue extracts (data not shown).

While yeast has only one CDC25 [35] and *Drosophila melanogaster* has two [36], most other animals have three (CDC25A, B and C). Three types of CDC25 proteins share common features in the phosphatase catalytic domain in their carboxyl half [37]. The cysteine in the HCXXXXXR motif plays a central role in human CDC25B [38], as is the case for the corresponding cysteine site (Cys483) of mouse CDC25Bs (Fig. 2A, 4A). The comparatively diverse amino-terminal domain of the CDC25s may be implicated in the regulation of catalytic activity or in substrate recognition [13]. Therefore, CDC25Bs that have different amino termini would have different affinities to substrate and phosphatase activity. As shown in Fig. 4, mouse CDC25B1 and CDC25B2, which have different amino termini, possess different affinities and different phosphatase activity compared to the *in vivo* substrate cyclin B/Cdc2 and the *in vitro* substrate pNPP. Several isoforms can also be generated by alternative splicing, not only in CDC25B but also in other types of CDC25, such as CDC25C [39].

The two isoforms have different binding affinity with different 14-3-3s, which likely results in the distinction in their localization and activity in the cells (Fig. 3). It is known that Ser-151 and Ser-230 in human CDC25B3 has weak affinity to 14-3-3, whereas the Ser-323 site binds more tightly. It is believed one subunit of the 14-3-3 dimer binds Ser-323 and the other flip-flops either on Ser-151 or Ser-230 to form an intramolecular bridge. The binding prevents CDC25B from accessing its substrate, the cyclin B/CDC2 complex [21]. In mouse CDC25B2, the Ser-230 corresponding site is missing; therefore, it is likely the CDC25B2 isoform has a more rigid conformation to access its substrate, resulting in less activity.

Alternative splicing might also be an important mechanism in regulating stability. Human CDC25B is an unstable protein and degrades in a proteasome-dependent manner upon phosphorylation by the cyclin A/Cdk1 complex [40]. The ubiquitination and subsequent degradation of a number of substrates has been shown to depend on the presence of a KEN box degron motif recognized by the APC/C^{cdh1} E3 ligase [41]. In mouse Cdc25B, the KEN box exists at the end of exon 6a. Therefore, the adjacent amino acid sequences are different between CDC25B1 and B2 (Fig. 1B), suggesting the two isoforms may be differently regulated by degradation. This difference should be investigated.

Why two different forms of Cdc25B are expressed in mouse tissue is still under investigation. One plausible explanation is that they are separately involved in the fine tuning of the cell cycle progression. It is necessary to knock down each transcript to elucidate their exact roles in the cell cycle.

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Authors' contribution: MKK and AB contributed equally to this work. MKK, AB and HOC carried out the experiment and analyzed the data. SJH conceived the study, and participated in its design, data analyses and coordination. All authors read and approved the final manuscript.

Conflict of interest disclosure: No potential conflict of interest was reported by the authors.

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