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Biochemical and Biophysical Research Communications 316 (2004) 226–232
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Nuclear export signal in CDC25B

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Received 31 January 2004

Abstract

CDC25B is a dual-specificity phosphatase that activates CDK1/cyclin B. The nuclear exclusion of CDC25B is controlled by the binding of 14-3-3 to the nuclear export signal (NES) of CDC25B, which was reported to be amino acids H28 to L40 in the N-terminal region of CDC25B. In studying the subcellular localization of CDC25B, we found a functional NES at V52 to L65, the sequence of which is **V**TT**L**TQT**MH**D**L**AG**L**, where bold letters are leucine or hydrophobic amino acids frequently seen in an NES. The deletion of this NES sequence caused the mutant protein to locate exclusively in nuclei, while NES-fused GFP was detected in the cytoplasm. Moreover, the introduction of point mutations at some of the critical amino acids impaired cytoplasmic localization. Treatment with leptomycin B, a potent inhibitor of CRM1/exportin1, disrupted the cytoplasmic localization of both Flag-tagged CDC25B and NES-fused GFP. From these results, we concluded that the sequence we found is a bona fide NES of CDC25B.

Keywords: CDC25B; Nuclear export signal; Subcellular localization; GFP; 14-3-3; Leptomycin B

The activities of CDK (cyclin-dependent kinase) family proteins are regulated by associations with cyclin proteins and the phosphorylation–dephosphorylation cycle of CDK [1]. For instance, CDK1, which is necessary for the onset and maintenance of mitosis, is phosphorylated by Wee1/Myt1 kinases at threonine 14 and tyrosine 15 and is dephosphorylated by CDC25 family dual-specificity phosphatases. In higher eukaryotes, CDC25 phosphatases consist of three members, CDC25A, CDC25B, and CDC25C [2], and CDC25B is reported to have three isoforms, CDC25B1, -B2, and -B3, produced by alternative splicing [3]. Recent reports indicate that CDC25A plays a central role in cell cycle progression not only by regulating the G1 to S transition, but also by functioning in the G2 to M transition and M phase maintenance [4–7]. Furthermore, recent results of studies with CDC25B/CDC25C knock-out mice indicate that these proteins are not essential for development [8,9], supporting the idea that CDC25A is a prototype of the CDC25 family proteins. Unlike CDC25A, the functions of CDC25B and C are restricted to activating the CDK1/cyclin B1 complex, contributing to G2 to M traverse [2].

In spite of the non-essentiality of CDC25B in the mouse model, evidence has been accumulating that

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doi:10.1016/j.bbrc.2004.02.039

CDC25B may have a positive function in tumorigenesis. First, CDC25B has an oncogenic role in cellular transformation [10]. Second, the over-expression of CDC25B has been reported in many primary human tumors and is correlated with a poor prognosis [11–15]. Third, transgenic mice over-expressing CDC25B in the mammary gland exhibited hyperplasia and susceptibility to carcinogen-induced mammary tumors [16,17]. Furthermore, the over-expression of CDC25B abrogated the G2/M checkpoint induced by DNA damage [6,18,19].

Recent reports indicate that the subcellular localization of CDC25B is controlled by the nuclear localization signal (NLS), the nuclear export signal (NES), and the association of 14-3-3 with Ser323, a specific site on CDC25B2 and B3 that corresponds to Ser309 of CDC25B1 [20,21]. The treatment of CDC25B-transfected cells with leptomycin B (LMB), a specific inhibitor of CRM1/exportin1 [22,23], abolishes its cytoplasmic localization, which indicates the presence of a functional NES in CDC25B [20].

During analyses of the control of cytoplasmic localization of CDC25B, we identified a functional NES on CDC25B at a site different from the published site [20]. In our study, deletion of the published NES region, from His28 to Leu40, did not influence the cytoplasmic localization of CDC25B; instead, a further deletion beyond Val52 impaired its cytoplasmic localization.

Materials and methods

Cell culture and transfection. HEK293 cells (ATCC No. CRL-1573) were cultured in Dulbecco's modified Eagle's medium (DMEM: Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Invitrogen, USA), 100 U/ml penicillin, and 10 μg/ml streptomycin. Transient transfections were performed with FuGENE6 (Roche Diagnostics, Germany). For the indirect immunofluorescence experiments, cells were plated at the density of 2.0×10^5/well on a six-well plate 48 h before transfection, transfected with 3 μg FLAG-tagged CDC25B and then processed for immunostaining 24 h after transfection.

Plasmids. Human CDC25B (CDC25B1 subtype) cDNA was obtained from H. Okayama (Laboratory of Molecular Biology, Graduate School of Medicine, University of Tokyo, Japan). Venus/pCS2, which expresses a modified GFP, was provided by A. Miyawaki (Laboratory of Cell Function and Dynamics, Advanced Technology Development Center, Brain Science Institute, RIKEN, Japan). To express proteins as N-terminal FLAG-tagged forms, the pEF6/myc-HisB vector (Invitrogen, USA) was modified to contain two tandem arrays of the FLAG sequence between *Acc*65I and *Bam*HI sites. In this vector construct, the original *Acc*65I site was deleted and a new *Acc*65I site followed by an *Nde*I site was created in front of the *Bam*HI site. This modified vector was named pEF6/2× FLAG.

Constructing cDNA clones and site-directed mutagenesis. The coding sequence of CDC25B1 was amplified by PCR using primers B1-forward (5′- GGCCCCGGTACCATGGAGGTGCCCCAGCCG-3′, *Acc*65I site underlined) and B1-reverse (5′-GGCGCAGATATCTCACTGGT CCTGCAGCCG-3′, *Eco*RV site underlined).

After PCR amplification, DNA was digested with *Acc*65I and *Eco*RV and inserted into the pEF6/2× FLAG vector to be expressed in an N-terminal FLAG-tagged form. To introduce mutations into the human CDC25B cDNA (CDC25B1), the oligonucleotides listed below (and their complements) were used.

L32AAA: 5′-GGCCACCTCCCGGGCGCCGCAGCTGGATCT CATGGCCTC-3′
L74, 76A: 5′-AGCCGCCTGACGCAC GCATCCGCTTCTCG ACGGG CATCC-3′
L239AAA: 5′-AGCCCCCTGGCCGCAGGTCGCGCCTCTGCC ACCCCTGCAGAG-3′
V52A: 5′-GCTTCCTCGCCGGCCACCACCCTCACC-3′
V55A: 5′-CCGGTCACCACCGCC ACCCAGACCATG-3′
H60AAA: 5′-ACCCAGACCATGGCTGACGCAGCCGGGGCT GGCAGCCGCAGC-3′
L71A: 5′-AGCCGCAGCCGCGCAACGCACCTATCC-3′

N-terminal deletion mutants were produced by PCR using the following oligonucleotides as forward primers and the B1-reverse primer as the reverse primer (*Acc*65I site underlined):

Δ46: 5′-GGGTCCCCGGGTACCATGGCCGCTTCCTCGCCG GTCACC-3′
Δ51: 5′-GCCGCTGGTACCATGGTCACCACCCTCACCCAG ACC-3′
Δ54: 5′-ATAATAGGTACCATGCTCACCCAGACCATGCACG AC-3′
Δ56: 5′-ATAATAGGTACCATGCAGACCATGCACGACCTCG CCGGG-3′
Δ76: 5′-CTGACGCACGGTACCATGTCTCGACGGGCATCC GAATCC-3′

All the CDC25B mutant cDNAs were inserted between the *Acc*65I and *Eco*RV sites in the pEF6/2× FLAG vector for expression as N-terminal FLAG-tagged forms, as was done with the wild-type CDC25B1. The mutations were confirmed by sequencing.

The Venus-GFP coding sequence was excised from Venus/pCS2 with *Bam*HI and *Eco*RI and subcloned into the pEF6 vector to express the N-terminal 2× FLAG form of Venus-GFP. To make oligonucleotide-fused Venus-GFP cDNA, the oligonucleotides listed below (and their complements), each with an *Acc*65I sequence at the 5′-end and a *Bam*HI sequence at the 3′-end, were introduced between the FLAG sequence and Venus-GFP using *Acc*65I and *Bam*HI restriction sites. The sequences of these oligonucleotides were confirmed.

52–63: 5′-GTCACCACCCTCACCCAGACCATGCACGACCTC GCC-3′
52–66: 5′-GTCACCACCCTCACCCAGACCATGCACGACCTC GCCGGGCTCGGC-3′
52–73: 5′-GTCACCACCCTCACCCAGACCATGCACGACCTC GCCGGGCTCGGCAGCCGCAGCCGCCTGACGCAC-3′

Indirect immunofluorescence microscopy. Transfected HEK293 cells grown on glass coverslips were fixed in 3.7% formaldehyde in PBS and then permeabilized with 0.5% Triton X-100 in PBS. Venus-GFP and its fused proteins were detected following fixation. FLAG-tagged CDC25B and mutants were detected with rabbit polyclonal anti-FLAG antibody [24] and Alexa Fluor 594-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR). DNA was visualized using 0.1 μg/ml of 4′,6-diamidino-2-phenylindole (DAPI; Sigma, St. Louis, MO).

Results and discussion

Mutations in a possible NES of CDC25B1 did not induce nuclear localization

On the analysis of subcellular localization of CD C25B, we found CDC25B in nucleus or cytoplasm, which indicates that human CDC25B contains NES and NLS. Studies on the role of 14-3-3 subcellular localization of CDC25B have enabled us to conclude that

binding of 14-3-3 to CDC25B mobilizes CDC25B from nucleus to cytoplasm ([25] and our data will be published elsewhere). Experiments with *Xenopus* CDC25C, which has both NES and NLS, strongly suggest that the binding of 14-3-3 masks the NLS located downstream from the binding region, which would make nuclear export more active than import [26]. To assess 14-3-3 binding to CDC25B and its role in subcellular localization in detail, we examined spatial relationship between NES, NLS, and 14-3-3 binding site. CDC25B possesses typical bipartite NLS starting from Lys320. Contrary to NLS, it does not have typical NES such as [$Lx_{(1-3)}Lx_{(2-3)}LxL$], where L stands for leucine or hydrophobic amino acids and x is any amino acids.

In order to identify NES in CDC25B, we first chose three leucine-rich, potential NES sequences: 28-H***L***PG***LLL***GS***H***G***LL***-40, 67-SRSR***L***T***HL***S***L***SRR-79, and 231-***V***EE***L***SP***L***A***L***GR***F***S***L***TP-246 (the bold italic letters are hydrophobic amino acids frequently observed in NES, Fig. 1A). One of the three sites, His28 to Leu40, was previously reported to be a functional NES [20]. Note that all three CDC25B sequences are conserved in the other two isotypes, CDC25B2 and -B3, although the numbering of the amino acids differs [3]. We introduced mutations at the critical leucine residues in these sequences. The mutation of Leu to Ala usually abolishes the function of a bona fide NES, and mutant proteins should be observed in nuclei. Contrary to this expectation, transiently expressed mutant clones, represented as L32AAA, L74/76A, and L239AAA (refer to Fig. 1A and its legend), did not exhibit specific nuclear localization (Figs. 1B and C). Moreover, these CDC25B1 mutant proteins localized in nuclei LMB-dependently. These results imply that CDC25B contains an NES in a region of other than those listed above, although His28 to Leu40 has been designated as the NES of CD C25B [20].

The functional NES of CDC25B1 starts at Val52

These results prompted us to find the functionally active NES in CDC25B. Therefore, we made a series of deletion mutants starting downstream from Leu40, since point mutations upstream from this had no effects on localization. First, we made a mutant with a deletion of the N-terminal 76 amino acids and expressed it as N-terminally FLAG-tagged CDC25B1. Surprisingly, the mutant localized exclusively in nuclei (Fig. 1D). The results strongly suggested that the functional NES of CDC25B1 occurs within the first 76 amino acids and that His28 to Leu40 is not relevant to the NES. Therefore, we created the following mutants with N-terminal deletions that started between Leu40 and Leu76: starting from Ala47 (Δ46 in Fig. 2), Val52 (Δ51), Leu55 (Δ54), and Gln57 (Δ56) (see Fig. 2A). These mutants were expressed as N-terminally FLAG-tagged forms to facilitate detection. On transfection with these mutants, the localization of proteins was observed, and their distribution was determined. As shown in Figs. 2B and C, the deletion of the first 51 amino acids did not affect cytoplasmic localization, but the deletion of the next three amino acids completely abolished the cytoplasmic localization of CDC25B1. These results imply that amino acid 52, 53, or 54 is crucial for cytoplasmic localization. Consulting the sequence, we tentatively concluded that Val52 was most likely the critical residue because the other two residues are threonine, which does not usually fulfill a function in an NES.

The NES of CDC25B1 localizes between Val52 and Leu66 and possesses LMB sensitivity

To determine the C-terminal end of the NES, several oligonucleotides encompassing Val52 to Leu71 were designed and attached to the 5′-end of Venus cDNA, a modified EGFP [27]. The N-terminal part of the construct was tagged with the 2× FLAG sequence for alternative detection of the protein. We made plasmids that contained Val52 to Ala63, Val52 to Gly66, and Val52 to His 73, designated 52–63, 52–66, and 52–73, respectively (Fig. 3A). The oligonucleotides contained Leu, which is usually critical to the function of an NES, although there is no typical NES sequence in this region at first glance.

These plasmids and FLAG-NES, which does not contain an insert and is referred to as Vector in Fig. 3, were transfected into HEK293 cells, and the localization of Venus-GFP protein was detected. As shown in Fig. 3B, GFP signals were detected uniformly in cells expressing FLAG-Venus and FLAG-Venus with amino acids Val52 to Ala63, called Vector and 52–63, respectively. Plasmid 52–66, in which the insert was extended by three C-terminal amino acids, gave rise to a GFP protein that localized in the cytoplasm. A further extended clone, 52–73, was also detected in the cytoplasm (Fig. 3B, 52–73). Therefore, we speculated that the functional NES of CDC25B1 is located between Val52 and Gly66 and that Leu65 is a critical C-terminal amino acid.

Next, we considered whether the NES we detected is LMB-sensitive. Transfectants of clone Venus (52–66) were treated with 20 ng/ml LMB for 3 h, and the localization of Venus-GFP was determined. As Fig. 3C shows, the cytoplasmic localization of Venus (52–66) is completely disturbed by LMB and it is evenly distributed throughout the cells. Therefore, we tentatively concluded that the amino acid sequence identified here is a new LMB-sensitive NES of CDC25B1.

Point mutations of hydrophobic amino acids in the NES abolish cytoplasmic localization

The LMB-sensitive NES contains functionally important hydrophobic amino acids, frequently Leu or

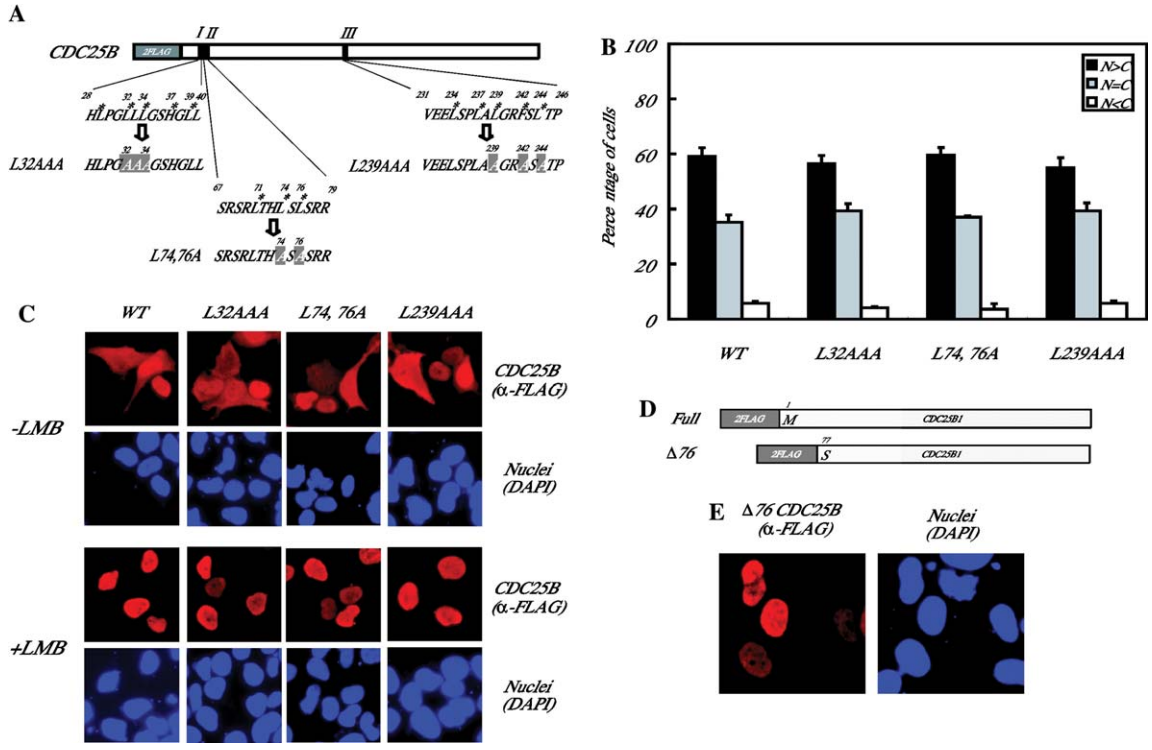

Fig. 1. Detection of NES activity in CDC25B1. (A) Schematic figures of CDC25B1 and the three possible NES regions (I, II, and III) are shown. Region I was previously reported to be an NES of CDC25B3. Sequences of the additional possible NES regions are shown. The amino acids indicated by asterisks are hydrophobic amino acids frequently observed in an NES. We constructed three mutants with Leu to Ala mutations (highlighted) that should abrogate the NES activity of each possible NES region. The numbers in each possible NES sequence represent the amino acid number from the N-terminus of CDC25B1 itself and not FLAG-tagged CDC25B1. CDC25B1 and the NES mutants were expressed as N-terminal 2× FLAG-tagged proteins. (B) HEK293 cells expressing CDC25B1 protein after transfection were detected and quantified in three groups as follows. N > C: cells with CDC25B detected specifically primarily in nuclei. N < C: cells with CDC25B detected specifically primarily in cytoplasm. N = C: cells with CDC25B detected non-specifically and evenly throughout cells. Standard errors, indicated as bars along the *y*-axis, result from three independent determinations that counted more than 200 cells in each experiment. (C) The effects of LMB (20 ng/ml for 3 h) on the localization of possible-NES mutants were determined. Expressed wild-type or mutant CDC25B1 was fixed and detected with anti-FLAG antibody and then treated with anti-rabbit Alexa Fluor 549 antibody as described in Materials and methods. Nuclei were identified by staining with DAPI. (D) The schematic figure of the 2× FLAG-CDC25B1Δ76 mutant. (E) The cytoplasmic localization of CDC25B1 is abolished by the deletion of the N-terminal 76 amino acids.

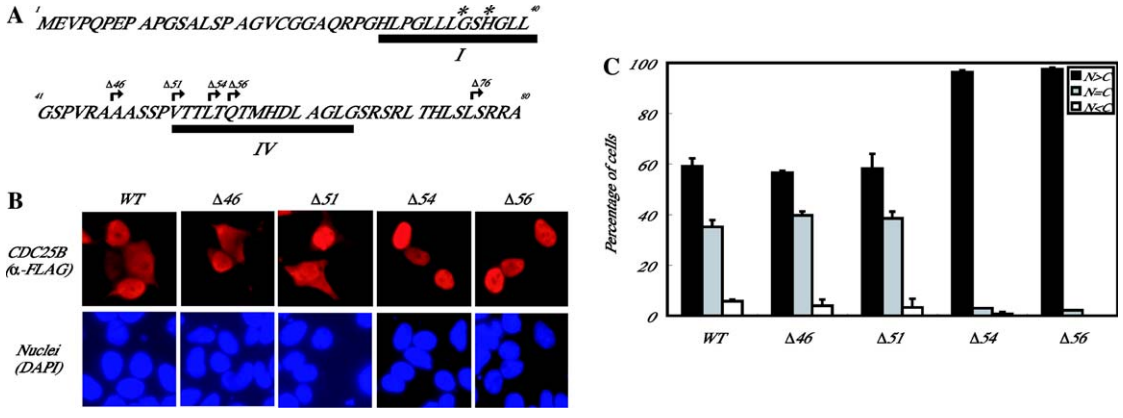

Fig. 2. Determination of the N-terminal boundary of the NES. (A) The sequence of the first N-terminal 80 amino acids is indicated. Underline I indicates the possible NES sequence previously reported. The amino acids marked with asterisks are those determined in a previous report to be essential for this NES activity. Underline IV is the NES sequence identified in this report. Δ46, Δ51, Δ54, Δ56, and Δ76 with arrows indicate the N-terminal ends of the N-terminal deletion mutants used in this experiment. These N-terminal deletion mutants were also expressed as N-terminal 2× FLAG-tagged proteins, as shown in Fig. 1A. (B) The subcellular localization of wild type or N-terminal deletion mutants was determined by transfection and detection of expressed proteins by indirect immunofluorescence as described in Materials and methods. (C) Percentages of cells expressing CDC25B1 protein primarily in nuclei (N > C), primarily in cytoplasm (N < C), or evenly distributed between both compartments (N = C) were quantified as described in the legend for Fig. 1D.

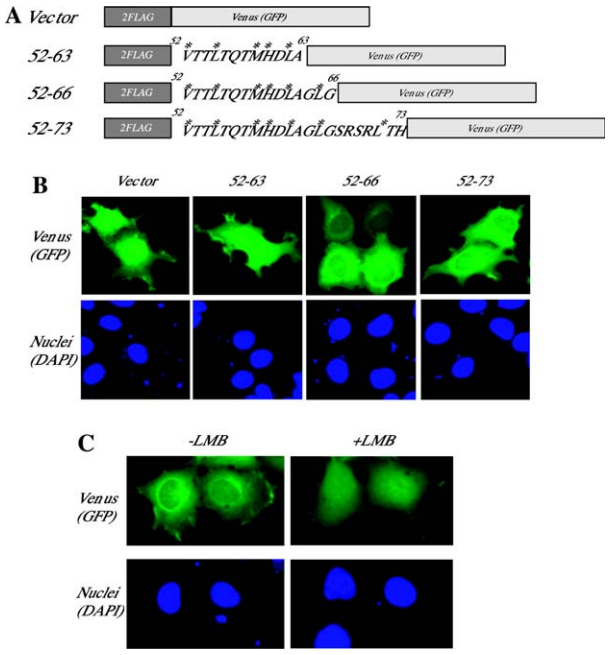

Fig. 3. Establishment of the minimum NES sequence of CDC25B. To determine the minimum NES sequence, oligonucleotides of several lengths starting at Val52 were designed and fused to Venus-GFP protein. (A) Schematic figures of the constructs are shown. Amino acids marked with asterisks are hydrophobic amino acids observed frequently in an NES. (B) Each plasmid was transfected into HEK293 cells and the localization of each GFP protein was determined. (C) To assess the effects of LMB on the GFP-fused NES, transfected cells were treated with 20 ng/ml LMB for 3 h and fixed, and the localization was determined. +LMB and −LMB indicate the results from cells with or without LMB treatment, respectively.

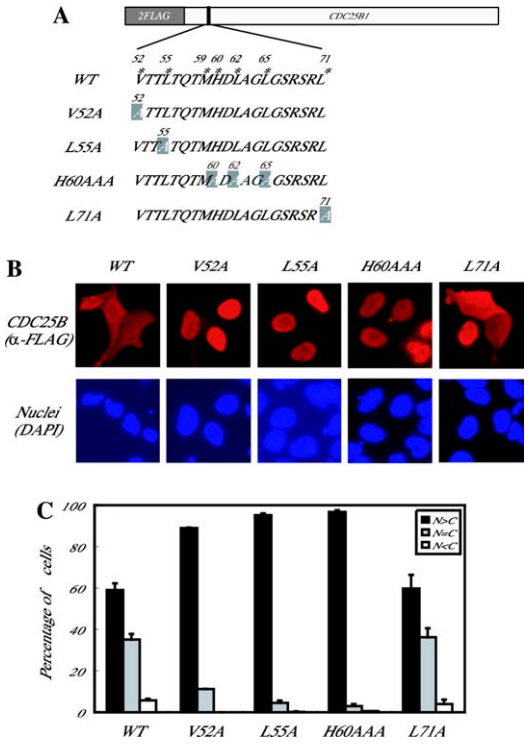

Fig. 4. Ablation of cytoplasmic localization of CDC25B1 by the introduction of mutations in the NES. Mutations were introduced at the possible critical amino acids in the NES region of N-terminally FLAG-tagged CDC25B1. (A) Schematic figures of mutated sequences of each mutant are indicated. Hydrophobic amino acids are marked with asterisks, and mutated amino acids, Ala, are highlighted. (B) Wild-type or mutant CDC25B1 plasmids were transfected into HEK293, and the expressed CDC25B1 proteins were detected by indirect immunofluorescence with anti-FLAG antibody and anti-rabbit Alexa Fluor 549. (C) The percentages of cells expressing CDC25B with a nuclear, diffused, or cytoplasmic distribution were determined as shown in Fig. 1D.

structurally similar residues such as Ile, Val, or Phe, that abort NES function when mutated [28]. The NES in CDC25B1 reporting here seems to include several potentially critical hydrophobic amino acids, Val52, Leu55, His60, Leu62, Leu65, and, less likely, Leu71.

It is important to determine if the amino acids between Val52 and Leu65 are essential to the NES of CDC25B1. To address this, the following CDC25B1 clones with different mutations in the suspected NES sequence were constructed: Val52 to Ala (denoted V52A), Leu55 to Ala (L55A), His60, Leu62, and Leu65 to Ala (H60AAA), and Leu71 to Ala (L71A) (Fig. 4A). The mutant CDC25B1 clones were expressed with FLAG-tags at the N-termini and their localizations were examined. Dramatic differences in the localization of the mutants were observed as shown in Fig. 4B. The mutation at Val52 abolished cytoplasmic localization, while that at Leu71 did not (Figs. 4B and C). Therefore, the NES of CDC25B1 starts at Val52 and ends before Leu71. Thus, the most important amino acid near the C-terminal end of the NES is Leu65. In addition, other mutants such as L55A and H60AAA with mutations in internal hydrophobic residues exhibited clear nuclear localization (Figs. 4B and C). Essentially the same results were obtained with Venus-GFP-fused fragments although NES-defective constructs did not exhibit specific localization in cells because of the lack of NLS sequence in Venus-GFP (data not shown). Therefore, we concluded that the amino acid sequence from Val52 to Leu65 of CDC25B1 is a functional NES in CDC25B1.

The subcellular localization of CDC25B is determined by the balance between NES and NLS activities and their modifiers, such as NES phosphorylation or 14-3-3 binding. On roles of 14-3-3 binding to CDC25B, there is much evidence that the binding masks the NLS located just downstream from the 14-3-3 binding site, tipping the balance in favor of the NES overriding the NLS. The proper cytoplasmic localization of target proteins with NES sequences seems to require both NES and 14-3-3 binding for *Xenopus* CDC25C and FKLRL1 [26,29]. CDC25B also seems to be the case based on our results. The loss of 14-3-3 binding due to the mutation of a specific serine residue to alanine resulted in nuclear localization in CDC25B. Since a single NES domain from CDC25B can translocate GFP to the cytoplasm in

the absence of an NLS, the NLS in CDC25B would have to be stronger than the NES to keep the protein in the nucleus. Therefore, it is reasonable to conclude that both NES and 14-3-3 binding must be necessary to allow CDC25B to be exported from the nucleus to the cytoplasm. This is in clear contrast to human CDC25C in which the mutation of Ser216 to Ala at the 14-3-3 binding site does not completely abolish its cytoplasmic localization [30,31].

The significance of the cytoplasmic retention of CDC25 at the G2/M checkpoint requires a more thorough examination. In *Xenopus* or fission yeast, the ablation of 14-3-3 binding accelerates mitotic entry or erases cell cycle arrest due to DNA damage [32–34]. It is also reported that Ser309 of human CDC25B must be kept phosphorylated in order to exert proper G2 checkpoint [21,35]. These results strongly support the idea that 14-3-3 binding is necessary for G2 arrest following DNA damage. They do not, however, directly indicate that the cytoplasmic localization of CDC25 is essential for G2 arrest. In experiments with fission yeast, the necessity for the cytoplasmic retention of CDC25 at the DNA damage checkpoint was negated [36]. In addition, there are reports that phosphorylation of the 14-3-3 binding site in CDC25 directly inhibits its phosphatase activity [37] and that 14-3-3 binding inhibits the phosphatase activity of CDC25B [21,38]. Therefore, more experiments to assess the role of the cytoplasmic localization of CDC25B at the G2 DNA-damage checkpoint.

Acknowledgments

We thank H. Okayama (University of Tokyo) and A. Miyawaki (RIKEN) for the generous gifts of CDC25B1 cDNA and Venus, a modified GFP expression plasmid, respectively. This work was supported in part by Grants-in-Aid for Scientific Research (to K.Y. and Y.I.) and for the Second Term of the Comprehensive 10-Year Strategy for Cancer Control (to H.N.) from the Ministry of Health, Labor, and Welfare and by Grants-in-Aid of Scientific Research from the Japan Society for the Promotion of Science, the Ministry of Education, Science, Sports and Culture of Japan (to M.H. and T.M.).

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