[25] M.A. Vodicka, Determinants for lentiviral infection of non-dividing cells, Somat. Cell Mol. Genet. 26 (2001) 35–49.
[26] S. Popov, M. Rexach, L. Ratner, G. Blobel, M. Bukrinsky, Viral protein R regulates docking of the HIV-1 preintegration complex to the nuclear pore complex, J. Biol. Chem. 273 (1998) 13347–13352.
[27] R.A.M. Fouchier, B.E. Meyer, J.H.M. Simon, U. Fischer, A.V. Albright, F. Gonzalez-Scarano, M.H. Malim, Interaction of the human immunodeficiency virus type 1 Vpr protein with the nuclear pore complex, J. Virol. 72 (1998) 6004–6013.
[28] E. Le Rouzic, A. Mousnier, C. Rustum, F. Stutz, E. Hallberg, C. Dargemont, S. Benichou, Docking of HIV-1 Vpr to the nuclear envelope is mediated by the interaction with the nucleoporin hCG1, J. Biol. Chem. 277 (2002) 45091–45098.
[29] F. Galimi, I.M. Verma, Opportunities for the use of lentiviral vectors in human gene therapy, Curr. Top. Microbiol. Immunol. 261 (2002) 245–254.
[30] E. Marshall, Gene therapy. Second child in French trial is found to have leukemia, Science 299 (2003) 320.

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 316 (2004) 226–232
BBRC
www.elsevier.com/locate/ybbrc

Nuclear export signal in CDC25B

Sanae Uchida,[a] Motoaki Ohtsubo,[b,1] Mari Shimura,[c] Masato Hirata,[d] Hitoshi Nakagama,[e] Tsukasa Matsunaga,[f] Minoru Yoshida,[g,h] Yukihito Ishizaka,[c] and Katsumi Yamashita[a,*]

[a] *Division of Life Science, Graduate School of Natural Science and Technology, Kanazawa University, General Education Hall, Kakuma-machi, Kanazawa 920-1192, Japan*
[b] *Institute of Life Science, Kurume University, Aikawa 2432-3, Kurume 839-0861, Japan*
[c] *Division of Intractable Disease, International Medical Center of Japan, 21-1, Toyama 1-chome, Shinjyuku-ku, Tokyo 162-8655, Japan*
[d] *Laboratory of Molecular and Cellular Biochemistry, Faculty of Dental Science, and Station for Collaborative Research, Kyushu University, Maidashi, Fukuoka 812-8582, Japan*
[e] *Biochemistry Division, National Cancer Center Research Institute, 1-1, Tsukiji 5-chome, Chuo-ku, Tokyo 104-0045, Japan*
[f] *Laboratory of Molecular Human Genetics, Faculty of Pharmaceutical Sciences, Kanazawa University, 13-1, Takara-machi, Kanazawa 920-0934, Japan*
[g] *Chemical Genetics Laboratory, RIKEN, Wako, Saitama 351-0198, Japan*
[h] *CREST Research Project, Japan Science and Technology Corporation, Saitama 332-0012, Japan*

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 **VTTLTQTMHDLAGL**, 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.
© 2004 Elsevier Inc. All rights reserved.

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

* Corresponding author. Fax: +81-76-264-5989.
E-mail address: katsumi@kenroku.kanazawa-u.ac.jp (K. Yamashita).
[1] Present address: Research Institute for Radiation Biology and Medicine, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553, Japan.

0006-291X/S - see front matter © 2004 Elsevier Inc. All rights reserved.
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*LAL*GR*FSL*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

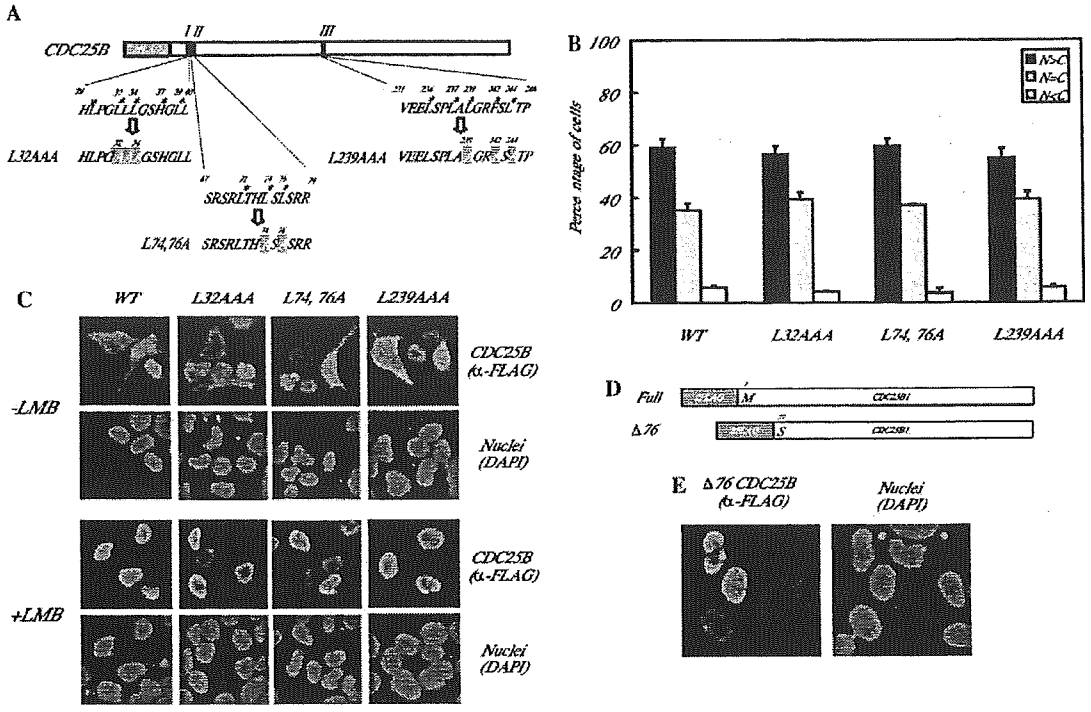


Fig. 1. Detection of NES activity in CDC25B1. (A) Schematic figures of CDC25B1 and the three possible NES regions (I, II, and III) are shown. Region I was previously reported to be an NES of CDC25B3. Sequences of the additional possible NES regions are shown. The amino acids indicated by asterisks are 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.

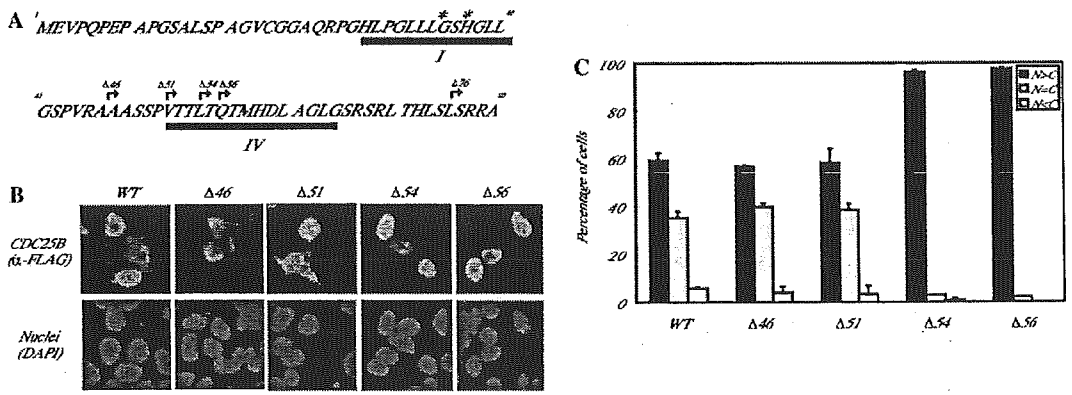


Fig. 2. Determination of the N-terminal boundary of the NES. (A) The sequence of the first N-terminal 80 amino acids is indicated. Underline I indicates the possible NES sequence previously reported. The amino acids marked with asterisks are those determined in a previous report to be essential for this 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.

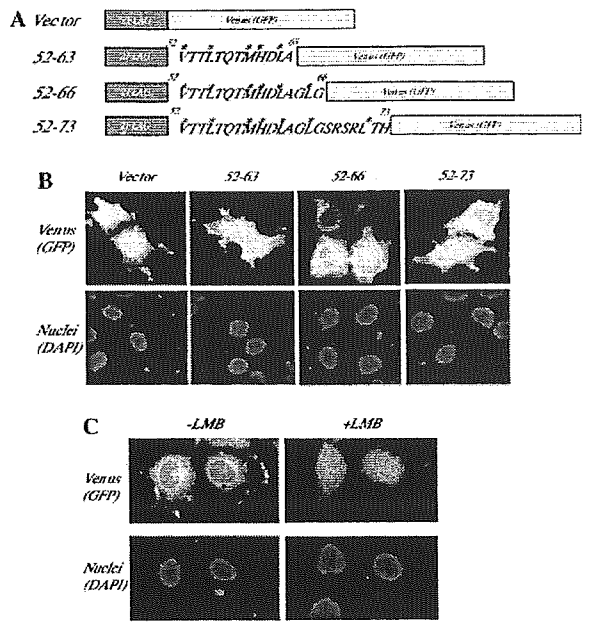


Fig. 3. Establishment of the minimum NES sequence of CDC25B. To determine the minimum NES sequence, oligonucleotides of several lengths starting at Val52 were designed and fused to Venus-GFP protein. (A) Schematic figures of the constructs are shown. Amino acids marked with asterisks are hydrophobic 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.

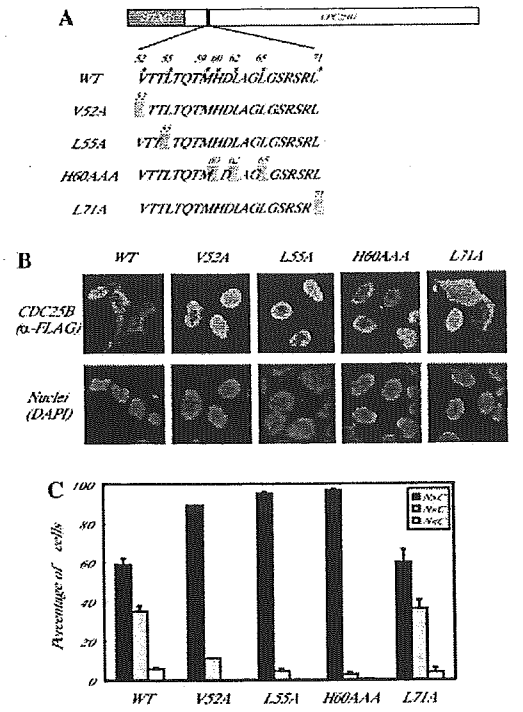


Fig. 4. Ablation of cytoplasmic localization of CDC25B1 by the introduction of mutations in the NES. Mutations were introduced at the possible critical amino acids in the NES region of N-terminally FLAG-tagged CDC25B1. (A) Schematic figures of mutated sequences of each mutant are indicated. Hydrophobic 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.).

References

[1] D.O. Morgan, Cyclin-dependent kinases: engines, clocks, and microprocessors, Annu. Rev. Cell. Dev. Biol. 13 (1997) 261–291.
[2] I. Nilsson, I. Hoffmann, Cell cycle regulation by the Cdc25 phosphatase family, Prog. Cell Cycle Res. 4 (2000) 107–114.
[3] V. Baldin, C. Cans, G. Superti-Furga, B. Ducommun, Alternative splicing of the human CDC25B tyrosine phosphatase. Possible implications for growth control?, Oncogene 14 (1997) 2485–2495.
[4] I. Hoffmann, G. Draetta, E. Karsenti, Activation of the phosphatase activity of human cdc25A by a cdk2–cyclin E dependent phosphorylation at the G1/S transition, EMBO J. 13 (1994) 4302–4310.
[5] N. Mailand, A.V. Podtelejnikov, A. Groth, M. Mann, J. Bartek, J. Lukas, Regulation of G(2)/M events by Cdc25A through phosphorylation-dependent modulation of its stability, EMBO J. 21 (2002) 5911–5920.
[6] J.P. Chow, W.Y. Siu, H.T. Ho, K.H. Ma, C.C. Ho, R.Y. Poon, Differential contribution of inhibitory phosphorylation of CDC2 and CDK2 for unperturbed cell cycle control and DNA integrity checkpoints, J. Biol. Chem. 278 (2003) 40815–40828.
[7] M. Donzelli, G.F. Draetta, Regulating mammalian checkpoints through Cdc25 inactivation, EMBO Rep. 4 (2003) 671–677.
[8] M.S. Chen, J. Hurov, L.S. White, T. Woodford-Thomas, H. Piwnica-Worms, Absence of apparent phenotype in mice lacking Cdc25C protein phosphatase, Mol. Cell. Biol. 21 (2001) 3853–3861.
[9] A.J. Lincoln, D. Wickramasinghe, P. Stein, R.M. Schultz, M.E. Palko, M.P. De Miguel, L. Tessarollo, P.J. Donovan, Cdc25b phosphatase is required for resumption of meiosis during oocyte maturation, Nat. Genet. 30 (2002) 446–449.
[10] K. Galaktionov, A.K. Lee, J. Eckstein, G. Draetta, J. Meckler, M. Loda, D. Beach, CDC25 phosphatases as potential human oncogenes, Science 269 (1995) 1575–1577.
[11] D. Gasparotto, R. Maestro, S. Piccinin, T. Vukosavljevic, L. Barzan, S. Sulfaro, M. Boiocchi, Overexpression of CDC25A and CDC25B in head and neck cancers, Cancer Res. 57 (1997) 2366–2368.
[12] S. Hernandez, L. Hernandez, S. Bea, M. Cazorla, P.L. Fernandez, A. Nadal, J. Muntane, C. Mallofre, E. Montserrat, A. Cardesa, E. Campo, cdc25 cell cycle-activating phosphatases and c-myc expression in human non-Hodgkin's lymphomas, Cancer Res. 58 (1998) 1762–1767.
[13] W. Wu, Y.H. Fan, B.L. Kemp, G. Walsh, L. Mao, Overexpression of cdc25A and cdc25B is frequent in primary non-small cell lung cancer but is not associated with overexpression of c-myc, Cancer Res. 58 (1998) 4082–4085.
[14] I. Takemasa, H. Yamamoto, M. Sekimoto, M. Ohue, S. Noura, Y. Miyake, T. Matsumoto, T. Aihara, N. Tomita, Y. Tamaki, I. Sakita, N. Kikkawa, N. Matsuura, H. Shiozaki, M. Monden, Overexpression of CDC25B phosphatase as a novel marker of poor prognosis of human colorectal carcinoma, Cancer Res. 60 (2000) 3043–3050.
[15] H. Sasaki, H. Yukiue, Y. Kobayashi, M. Tanahashi, S. Moriyama, Y. Nakashima, I. Fukai, M. Kiriyama, Y. Yamakawa, Y. Fujii, Expression of the cdc25B gene as a prognosis marker in non-small cell lung cancer, Cancer Lett. 173 (2001) 187–192.
[16] Z.Q. Ma, S.S. Chua, F.J. DeMayo, S.Y. Tsai, Induction of mammary gland hyperplasia in transgenic mice over-expressing human Cdc25B, Oncogene 18 (1999) 4564–4576.
[17] Y. Yao, E.D. Slosberg, L. Wang, H. Hibshoosh, Y.J. Zhang, W.Q. Xing, R.M. Santella, I.B. Weinstein, Increased susceptibility to carcinogen-induced mammary tumors in MMTV-Cdc25B transgenic mice, Oncogene 18 (1999) 5159–5166.
[18] C. Karlsson, S. Katich, A. Hagting, I. Hoffmann, J. Pines, Cdc25B and Cdc25C differ markedly in their properties as initiators of mitosis, J. Cell Biol. 146 (1999) 573–584.
[19] H. Miyata, Y. Doki, H. Yamamoto, K. Kishi, H. Takemoto, Y. Fujiwara, T. Yasuda, M. Yano, M. Inoue, H. Shiozaki, I.B. Weinstein, M. Monden, Overexpression of CDC25B overrides radiation-induced G2-M arrest and results in increased apoptosis in esophageal cancer cells, Cancer Res. 61 (2001) 3188–3193.
[20] N. Davezac, V. Baldin, B. Gabrielli, A. Forrest, N. Theis-Febvre, M. Yashida, B. Ducommun, Regulation of CDC25B phosphatases subcellular localization, Oncogene 19 (2000) 2179–2185.
[21] A. Forrest, B. Gabrielli, Cdc25B activity is regulated by 14-3-3, Oncogene 20 (2001) 4393–4401.
[22] K. Nishi, M. Yoshida, D. Fujiwara, M. Nishikawa, S. Horinouchi, T. Beppu, Leptomycin B targets a regulatory cascade of crm1, a fission yeast nuclear protein, involved in control of higher order chromosome structure and gene expression, J. Biol. Chem. 269 (1994) 6320–6324.

[23] N. Kudo, B. Wolff, T. Sekimoto, E.P. Schreiner, Y. Yoneda, M. Yanagida, S. Horinouchi, M. Yoshida, Leptomycin B inhibition of signal-mediated nuclear export by direct binding to CRM1, Exp. Cell. Res. 242 (1998) 540–547.
[24] Y. Wang, C. Jacobs, K.E. Hook, H. Duan, R.N. Booher, Y. Sun, Binding of 14-3-3beta to the carboxyl terminus of Wee1 increases Wee1 stability, kinase activity, and G2-M cell population, Cell. Growth Differ. 11 (2000) 211–219.
[25] V. Mils, V. Baldin, F. Goubin, I. Pinta, C. Papin, M. Waye, A. Eychene, B. Ducommun, Specific interaction between 14-3-3 isoforms and the human CDC25B phosphatase, Oncogene 19 (2000) 1257–1265.
[26] A. Kumagai, W.G. Dunphy, Binding of 14-3-3 proteins and nuclear export control the intracellular localization of the mitotic inducer Cdc25, Genes Dev. 13 (1999) 1067–1072.
[27] T. Nagai, K. Ibata, E.S. Park, M. Kubota, K. Mikoshiba, A. Miyawaki, A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications, Nat. Biotechnol. 20 (2002) 87–90.
[28] M. Watanabe, N. Masuyama, M. Fukuda, E. Nishida, Regulation of intracellular dynamics of Smad4 by its leucine-rich nuclear export signal, EMBO Rep. 1 (2000) 176–182.
[29] A. Brunet, F. Kanai, J. Stehn, J. Xu, D. Sarbassova, J.V. Frangioni, S.N. Dalal, J.A. DeCaprio, M.E. Greenberg, M.B. Yaffe, 14-3-3 transits to the nucleus and participates in dynamic nucleocytoplasmic transport, J. Cell Biol. 156 (2002) 817–828.
[30] S.N. Dalal, C.M. Schweitzer, J. Gan, J.A. DeCaprio, Cytoplasmic localization of human cdc25C during interphase requires an intact 14-3-3 binding site, Mol. Cell. Biol. 19 (1999) 4465–4479.
[31] P.R. Graves, C.M. Lovly, G.L. Uy, H. Piwnica-Worms, Localization of human Cdc25C is regulated both by nuclear export and 14-3-3 protein binding, Oncogene 20 (2001) 1839–1851.
[32] A. Kumagai, P.S. Yakowec, W.G. Dunphy, 14-3-3 proteins act as negative regulators of the mitotic inducer Cdc25 in *Xenopus* egg extracts, Mol. Biol. Cell 9 (1998) 345–354.
[33] J. Yang, K. Winkler, M. Yoshida, S. Kornbluth, Maintenance of G2 arrest in the Xenopus oocyte: a role for 14-3-3-mediated inhibition of Cdc25 nuclear import, EMBO J. 18 (1999) 2174–2183.
[34] Y. Zeng, H. Piwnica-Worms, DNA damage and replication checkpoints in fission yeast require nuclear exclusion of the Cdc25 phosphatase via 14-3-3 binding, Mol. Cell. Biol. 19 (1999) 7410–7419.
[35] D.V. Bulavin, Y. Higashimoto, I.J. Popoff, W.A. Gaarde, V. Basrur, O. Potapova, E. Appella, A.J. Fornace Jr., Initiation of a G2/M checkpoint after ultraviolet radiation requires p38 kinase, Nature 411 (2001) 102–107.
[36] A. Lopez-Girona, B. Furnari, O. Mondesert, P. Russell, Nuclear localization of Cdc25 is regulated by DNA damage and a 14-3-3 protein, Nature 397 (1999) 172–175.
[37] B. Furnari, A. Blasina, M.N. Boddy, C.H. McGowan, P. Russell, Cdc25 inhibited in vivo and in vitro by checkpoint kinases Cds1 and Chk1, Mol. Biol. Cell 10 (1999) 833–845.
[38] N. Giles, A. Forrest, B. Gabrielli, 14-3-3 acts as an intramolecular bridge to regulate cdc25B localization and activity, J. Biol. Chem. 278 (2003) 28580–28587.

Binding of 14-3-3β but not 14-3-3σ controls the cytoplasmic localization of CDC25B: binding site preferences of 14-3-3 subtypes and the subcellular localization of CDC25B

Sanae Uchida[1], Akiko Kuma[1,*], Motoaki Ohtsubo[2], Mari Shimura[3], Masato Hirata[4], Hitoshi Nakagama[5], Tsukasa Matsunaga[6], Yukihito Ishizaka[3] and Katsumi Yamashita[1,‡]

[1]Division of Life Science, Graduate School of Natural Science and Technology, Kanazawa University, Kakuma-machi, Kanazawa, 920-1192, Japan
[2]Institute of Life Science, Kurume University, Aikawa 2432-3, Kurume, 839-0861, Japan
[3]Division of Intractable Disease, International Medical Center of Japan, 21-1 Toyama 1-chome, Shinjuku-ku, Tokyo, 162-8655, Japan
[4]Laboratory of Molecular and Cellular Biochemistry, Faculty of Dental Science, and Station for Collaborative Research, Kyushu University, Maidashi, Fukuoka, 812-8582, Japan
[5]Biochemistry Division, National Cancer Center Research Institute, 1-1 Tsukiji 5-chome, Chuo-ku, Tokyo, 104-0045, Japan
[6]Laboratory of Molecular Human Genetics, Faculty of Pharmaceutical Sciences, Kanazawa University, 13-1 Takara-machi, Kanazawa, 920-0934, Japan

*Present address: Department of Cell Biology, National Institute for Basic Biology, 38 Nishigonaka, Myodaiji, Okazaki, 444-8585, Japan
‡Author for correspondence (e-mail: katsumi@kenroku.kanazawa-u.ac.jp)

Accepted 7 January 2004
Journal of Cell Science 117, 3011-3020 Published by The Company of Biologists 2004
doi:10.1242/jcs.01086

Summary

The dual specificity phosphatase CDC25B positively controls the G2-M transition by activating CDK1/cyclin B. The binding of 14-3-3 to CDC25B has been shown to regulate the subcellular redistribution of CDC25B from the nucleus to the cytoplasm and may be correlated with the G2 checkpoint. We used a FLAG-tagged version of CDC25B to study the differences among the binding sites for the 14-3-3 subtypes, 14-3-3β, 14-3-3ε and 14-3-3σ, and the relationship between subtype binding and the subcellular localization of CDC25B. All three subtypes were found to bind to CDC25B. Site-directed mutagenesis studies revealed that 14-3-3β bound exclusively near serine-309 of CDC25B1, which is within a potential consensus motif for 14-3-3 binding. By contrast, 14-3-3σ bound preferentially to a site around serine-216, and the presence of serine-137 and -309 enhanced the binding. In addition to these binding-site differences, we found that the binding of 14-3-3β drove CDC25B to the cytoplasm and that mutation of serine-309 to alanine completely abolished the cytoplasmic localization of CDC25B. However, co-expression of 14-3-3σ and CDC25B did not affect the subcellular localization of CDC25B. Furthermore, serine-309 of CDC25B was sufficient to produce its cytoplasmic distribution with co-expression of 14-3-3β, even when other putative 14-3-3 binding sites were mutated. 14-3-3ε resembled 14-3-3β with regard to its binding to CDC25B and the control of CDC25B subcellular localization. The results of the present study indicate that two 14-3-3 subtypes can control the subcellular localization of CDC25B by binding to a specific site and that 14-3-3σ has effects on CDC25B other than the control of its subcellular localization.

Key words: CDC25B, 14-3-3β, 14-3-3σ, Subcellular localization, G2 checkpoint

Introduction

The CDK (cyclin-dependent kinase) family of proteins controls the eukaryotic cell cycle, and one of these proteins, CDK1, is required for the onset and maintenance of mitosis. The activities of CDK family proteins related to cell cycle control are regulated by associations with cyclin proteins, interactions with cyclin-dependent kinase inhibitors, such as p21 and p27, and the phosphorylation-dephosphorylation cycle of CDK (Morgan, 1997). For instance, the phosphorylation of CDK1 at threonine-14 and tyrosine-15 by Wee1 and/or Myt1 kinases negatively controls CDK1 activity, whereas the dephosphorylation of CDK1 by the CDC25 family phosphatases activates CDK1, an essential step in the transition from G2 to M phase. The CDC25 family of dual protein phosphatases consists of three members, CDC25A, CDC25B, and CDC25C (Nilsson and Hoffman, 2000). CDC25A is thought to regulate the G1 to S transition, and CDC25B and C have been proposed to activate the CDK1/cyclin B1 complex to advance the cell cycle from G2 to M. Recent reports strongly suggest that CDC25A also has a function that is essential for the entry into and maintenance of M phase (Mailand et al., 2002).

The 14-3-3 family of proteins consists of small, acidic, highly conserved proteins that are present in all eukaryotic cells from yeast to mammals. There are seven isotypes present in mammalian cells. The 14-3-3 proteins are involved in numerous cellular processes related to signal transduction (Muslin and Xing, 2000; Tzivion et al., 2001; Yaffe, 2002).

These molecules bind to phosphoproteins at specific sequence motifs, which contain phosphoserine/threonine residues three amino acids downstream of an arginine (RxxS/T), and thereby regulate extracellular signaling or stress response pathways (Muslin et al., 1996; Yaffe et al., 1997). Emerging evidence suggests that 14-3-3 proteins are key regulators of cell cycle control, especially at cell cycle checkpoints, where they might function as negative regulators of DNA damage checkpoints. For example, one canonical 14-3-3 binding motif, which contains a phosphorylated serine residue, is similar to the consensus substrate motif of the checkpoint kinase Chk1 (Sanchez et al., 1997; Hutchins et al., 2000). In fission yeast, the 14-3-3 proteins Rad24/25 are required for checkpoint responses and are essential for cell survival (Ford et al., 1994). One of the 14-3-3 isotype proteins, 14-3-3σ is strongly up-regulated following genotoxic stress and is a downstream target of the tumor suppressor p53 (Hermeking et al., 1997).

The involvement of 14-3-3 in the progression from G2 to M was first suggested by the interactions of isolated 14-3-3β and ε with CDC25B (and CDC25A) and of isolated 14-3-3ζ with Wee1 (Conklin et al., 1995; Honda et al., 1997). Accumulated circumstantial evidence indicates that 14-3-3 negatively controls the G2-M transition by binding to these regulators. An association of 14-3-3 with human CDC25C was detected in G1, S and G2 phases, but not in M phase (Peng et al., 1997). The binding of 14-3-3 requires the Ser216 of CDC25C, and mutating this residue to Ala abolishes the interaction. This site is present in the potential recognition motif for 14-3-3 and is phosphorylated *in vitro* by checkpoint kinases, such as Chk1 and Chk2 (Sanchez et al., 1997; Peng et al., 1998; Matsuoka et al., 1998; O'Neill et al., 2002). Studies of the interaction between *Xenopus* CDC25C and 14-3-3 clearly demonstrated that the binding of 14-3-3 masks the nuclear localization signal of CDC25C, thereby causing nuclear exclusion of the protein without affecting its phosphatase activity (Kumagai et al., 1998; Kumagai and Dunphy, 1999; Yang et al., 1999). By contrast, the binding of 14-3-3 to *Xenopus* Wee1, after Chk1 activation by DNA damage or by stalled replication, augments Wee1 tyrosine kinase activity for CDK1 (Wang et al., 2000; Lee et al., 2001; Rothblum-Oviatt et al., 2001). Thus, the association of 14-3-3 with target proteins could modulate cell cycle progression through different mechanisms such as subcellular localization and enzyme activity, depending on cellular signaling.

In the normal cell cycle, CDC25B accumulates only at G2 phase and is degraded when cells exit M phase (Nagata et al., 1991; Galaktionov and Beach, 1991; Sebastian et al., 1993; Lammer et al., 1998). Interestingly, the overexpression of CDC25B induces a mitotic catastrophe by prematurely activating CDK1/cyclin B1, indicating that CDC25B induces mitosis more efficiently than CDC25C (Karlsson et al., 1999). In addition, the exogenous expression of CDC25B can override the G2 DNA damage checkpoint, and CDC25B is expressed in certain tumors (Miyata et al., 2001). Therefore, CDC25B has been proposed to be a potential oncogene acting to abrogate the DNA damage checkpoint (Galaktionov et al., 1995; Ma et al., 1999; Yao et al., 1999). Subcellular localization of CDC25B can be controlled by its association with 14-3-3 at a specific site on CDC25B2 or B3, Ser323 and might contribute to stall the cell cycle at the G2 phase following DNA damage (Mils et al., 2000; Davezac et al., 2000; Forrest and Babrielli, 2001). Ser323 of CDC25B2 or CDC25B3 (the equivalent to Ser309 of CDC25B1) is a crucial residue in the consensus 14-3-3 binding motif, where it is phosphorylated by the stress kinase p38 (Bulavin et al., 2001).

In the present study, we have analyzed the binding site specificity of three 14-3-3 subtypes, 14-3-3β, ε, and σ. Our results indicate that the binding site of 14-3-3σ differs markedly from those of 14-3-3β and 14-3-3ε. Moreover, the interaction of 14-3-3β or 14-3-3ε, but not of 14-3-3σ with CDC25B drives CDC25B from the nucleus into the cytoplasm. The biological significance of our results is discussed.

Materials and Methods

Cell culture and transfection

HEK293 cells (ATCC number CRL-1573) and U2OS cells (ATCC number HTB-96) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, USA), 100 units/ml penicillin and 10 μg/ml streptomycin. Transient transfections were performed with FuGENE6 (Roche Diagnostics, Germany). For immunoprecipitation, cells were typically seeded at 1.3×10^6 per well. After 24 hours, cells were co-transfected with 2.5 μg of FLAG-tagged CDC25B and 1.0 μg of myc-tagged 14-3-3 DNA. For the indirect immunofluorescence experiments, cells were plated at a lower density, 2.0×10^5 per well and transfected after 24 hours with 3.0 μg of CDC25B DNA and 1.5 μg 14-3-3 of DNA. Transfected cells were processed for immunoblotting, immunoprecipitation, or immunostaining 24 hours after transfection. Leptomycin B, an inhibitor of CRM1 (exportin1), was obtained from Minoru Yoshida (RIKEN, Wako, Japan) and was administered to cells at a dose of 20 ng/ml to induce the nuclear accumulation of CDC25B.

Plasmids and site-directed mutagenesis

The cDNA of human CDC25B (CDC25B1 subtype), a kind gift from H. Okayama (University of Tokyo, Japan), was subcloned into the pEF6B vector (Invitrogen, USA) and expressed in transfected cells with a C-terminal FLAG tag. For point mutations at putative 14-3-3 binding sites, the following oligonucleotides (and their complements) were used to change serine to alanine (SA) in human CDC25B cDNA (CDC25B1). Clones with multiple mutations were generated by exchanging restriction fragments. The mutations were confirmed by sequencing.

S81A: 5′-CTGTCTCGACGGGCAGCCGAATCCTCCCTG-3′,
S137A: 5′-ATCAGACGCTTCCAGGCTATGCCGGTGAGG-3′,
S216A: 5′-GCCCAGAGACCCAGCGCGGCCCCCGACCTG-3′,
S309A: 5′-CTCTTCCGCTCTCCGGCCATGCCCTGCAGC-3′,
S361A: 5′-GTCCTCCGCTCAAAAGCACTGTGTCACGAT-3′.

The cDNAs of human 14-3-3β, ε, and σ were obtained by PCR amplification with the following oligonucleotides:

14-3-3β forward: 5′-ACTTGGAGTCAGCATATGACAATGGAT-3′,
14-3-3β reverse: 5′-CACTGGACGGATCCCAAAGCACGAGAA-3′,
14-3-3ε forward: 5′-GCCGCTGCCCATATGGATGATCGAGAG-3′,
14-3-3ε reverse: 5′-CTCTTGTGGGCGGATCCCTCACTGATT-3′,
14-3-3σ forward: 5′-GTCCCCAGACATATGGAGAGAGCCAGT-3′,
14-3-3σ reverse: 5′-GGTGGCGGGCAAGCTTCAGCTCTGGGG-CTC-3′.

PCR products were subcloned into the pEF6 vector. Each 14-3-3 cDNA was expressed in transfected cells in an N-terminal myc-tagged form.

Antibodies

Anti-FLAG M2 agarose was obtained from Sigma (USA). The rabbit

anti-FLAG antibody was described previously (Wang et al., 2001). Rabbit polyclonal and mouse monoclonal anti-myc-tag antibodies were purchased from Cell Signaling (USA). Antibodies to 14-3-3β (C-20), 14-3-3ε (T-16), and 14-3-3σ (N-14) were purchased from Santa Cruz Biotechnology (USA).

Preparation of crude cell extracts, immunoprecipitation and immunoblotting

Transfected cells were lysed in immunoprecipitation (IP) buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% NP-40, 5 mM EGTA, 1 mM EDTA) supplemented with a protease inhibitor mix and a phosphatase inhibitor mix. The protease inhibitor mix contained a 1:100 dilution of FOCUS protease arrest (Calbiochem, USA), 5 μg/ml E64 (Roche Diagnostics, Germany), 0.4 μM cathepsin inhibitor III (Sigma, USA), 10 μM MG132 (Calbiochem, USA), 20 μM N-acetyl-leu-leu-norleu-ala (Sigma, USA) and 1 mg/ml Pefabloc®SC (Roche Diagnostics, Germany). The phosphatase inhibitor mix consisted of a 1:100 dilution of Phosphatase inhibitor cocktail II (Sigma, USA), 20 mM *p*-nitrophenyl phosphate, 20 mM NaF, 20 mM β-glycerophosphate, 0.2 μM microcystin-LR (Calbiochem, USA), 0.2 μM calyculin A (Wako, Japan), 0.2 μM okadaic acid (Wako, Japan), 0.1 μM phenylarsin (Sigma, USA), and 0.2 μM cantharidin (Sigma, USA). FLAG-tagged CDC25B and mutant proteins were immunoprecipitated using FLAG M2-agarose; myc-tagged 14-3-3 proteins were immunoprecipitated with mouse monoclonal anti-myc tag antibody followed by protein G-Sepharose (Amersham Bioscience, USA). Cell lysates and immunoprecipitates were analyzed on western blots using rabbit polyclonal anti-FLAG (for CDC25B) or anti-myc antibodies (for exogenous 14-3-3), or 14-3-3 subtype-specific antibodies (for endogenous 14-3-3).

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. FLAG-tagged CDC25B and mutants were detected with rabbit polyclonal anti-FLAG antibody and Alexa-594-conjugated goat anti-rabbit IgG (Molecular Probes, USA). Alternatively, myc-tagged 14-3-3 proteins were detected with mouse monoclonal anti-myc-tag antibody and Alexa-488-conjugated goat anti-mouse IgG (Molecular Probes, USA). In all samples, DNA was visualized with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma, USA) at 0.1 μg/ml. To quantify the subcellular localization of CDC25B, more than 200 transfectant cells were counted and classified as having nuclear, diffuse or cytoplasmic localization.

Results

Binding of 14-3-3β, ε, and σ to CDC25B

Several groups have reported the interaction of 14-3-3 isotypes, such as 14-3-3β, ε, η, and ζ, with CDC25B (Mils et al., 2000; Forrest and Gabrielli, 2001). We have isolated 14-3-3β and ε as proteins that interact with CDC25B in yeast two-hybrid screening (S.U., A.K., M.O., M.S., M.H., H.N., T.M., Y.I. and K.Y., unpublished data), obtaining the same results as those previously reported (Conklin et al., 1995). Apart from these two 14-3-3 proteins (β and ε), 14-3-3σ was also reported to be possibly involved in a DNA damage checkpoint (Hermeking et al., 1997; Chan et al., 1999, 2000), which prompted us to isolate its cDNA and analyze its interaction with CDC25B.

We expressed FLAG-tagged CDC25B with myc-tagged 14-3-3β, ε or σ in HEK293 or U2OS cells and examined their interaction (Fig. 1). Expression of these proteins was confirmed in cell extracts prepared from transfected cells, as shown in Fig. 1 (Lysate). CDC25B was immunoprecipitated with anti-FLAG beads followed by western blotting and detection with either anti-FLAG or anti-myc antibody to detect CDC25B bound to 14-3-3. The results in Fig. 1 (IP: CDC25B) clearly indicate that all three 14-3-3 proteins can bind to CDC25B in co-transfected cells. To further confirm these results, reciprocal immunoprecipitation and western blot experiments were conducted in which CDC25B was detected in anti-myc immunoprecipitates of 14-3-3β, ε, or σ (Fig. 1, IP: 14-3-3). Thus, 14-3-3σ was most probably a new CDC25B-interacting protein.

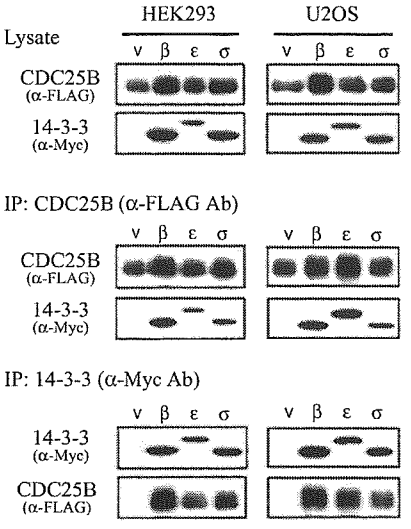


Fig. 1. 14-3-3β, 14-3-3ε, and 14-3-3σ bind to CDC25B in transfected cells. HEK293 (left panels) or U2OS (right panels) cells were transfected with FLAG-tagged CDC25B together with either empty vector or one of the myc-tagged 14-3-3 subtypes as described in Materials and Methods. (Top row) Lysate. Expression of CDC25B and 14-3-3- subtypes was confirmed in cell lysates with anti-FLAG antibody against CDC25B or anti-myc antibody against 14-3-3, respectively. (Middle row) IP: CDC25B (α-FLAG Ab). CDC25B was immunoprecipitated with anti-FLAG beads followed by western blotting and detection with anti-FLAG antibody to detect CDC25B and anti-myc antibody to detect CDC25B-bound 14-3-3. (Bottom row) IP: 14-3-3 (α-myc Ab. Reciprocal immunoprecipitation; CDC25B was detected in anti-myc immunoprecipitates. Protein 14-3-3 subtypes were immunoprecipitated with anti-myc antibody; the collected 14-3-3 or 14-3-3-bound CDC25B was detected by immunoblotting. v, empty vector; β, 14-3-3β, ε; 14-3-3ε, σ; 14-3-3σ.

Binding site specificity of 14-3-3 subtypes

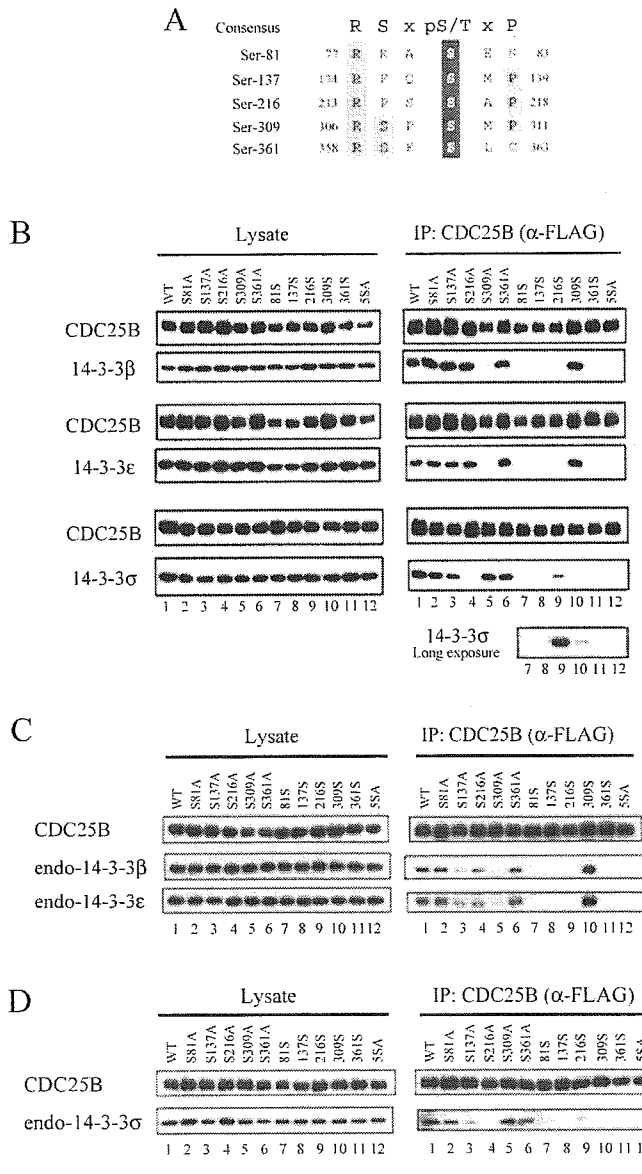
The binding of 14-3-3 proteins to target proteins requires the specific motif RSxS/T(P)xP, where S/T(P) and x represent phosphoserine or phosphothreonine, and any amino acid, respectively (Muslin et al., 1996; Yaffe et al., 1997). The arginine (R) at position –3 from the phosphorylatable serine (or threonine) is a minimal requirement. In *Xenopus* for instance, after phosphorylation of CDC25 or Wee1 by Chk1 or other kinases, 14-3-3ε binds to the phosphorylated Ser287 in the RSPSMP sequence of CDC25 (Kumagai et al., 1998; Yang et al., 1999) and to the phosphorylated Ser549 in the RSVSFT sequence of Wee1 (Wang et al., 2000; Lee et al., 2001). There are several RxxS sites in CDC25B (or in our case, CDC25B1),

of which we chose the following five: 78-RRAS-81, 134-RFQS-137, 213-RPSS-216, 306-RSPS-309, and 358-RSKS-361, as shown in Fig. 2A. Of the relevant serine residues, Ser309 and Ser361 were phosphorylated by p38 in vitro and Ser309 was reported to be crucial for 14-3-3 binding after phosphorylation (Bulavin et al., 2001).

To analyze binding site specificity, we constructed three different groups of mutants in respect to the five above mentioned phosphorylatable serine sites of CDC25B1. Members of the first group have only a single mutation that changed one phosphorylatable serine to a non-phosphorylatable alanine; these mutants were namend CDC25B-S81A, S137A, etc. Members of the second group only remain a single phosphorylatable serine residue and contain mutations that changed the four serine residues to alanines; these mutants were named CDC25B-81S, 137S, etc. The only member of the last group is CDC25B-5SA in which all five serine residues were mutated to alanines. Using these mutants and the wild-type CDC25B, we determined the binding site specificity of 14-3-3β, ε, and σ.

Wild-type or mutant CDC25B were co-transfected with 14-3-3β, ε, or σ. Crude cell extracts were prepared, and expression of CDC25B and 14-3-3 was confirmed. Protein extracts were immunoprecipitated with anti-FLAG or anti-myc antibody, transferred for western blotting and detected with anti-myc or anti-FLAG antibody, respectively, to assess binding. We observed similar expression levels of CDC25B and 14-3-3 in transfected cells (Fig. 2B, Lysate), although lower levels of CDC25B mutants that failed to interact with 14-3-3, such as 81S and 5SA mutants, were occasionally detected (S.U., A.K., M.O., M.S., M.H., H.N., T.M., Y.I. and K.Y., unpublished data).

Interestingly, each 14-3-3 protein bound to a specific site on CDC25B (Fig. 2B, IP: CDC25B). These results clearly indicate that the CDC25B point mutation that changed Ser309 to Ala309, completely abolished 14-3-3β binding and that mutations of the other putative binding sites had essentially no effect on binding when compared with wild-type CDC25B. Also, experiments with the CDC25B mutant containing a single phosphorylatable serine revealed that Ser309 was the



E

14-3-3	CDC25B mutants											
	WT	S81A	S137A	S216A	S309A	S361A	81S	137S	216S	309S	361S	5SA
14-3-3β	++	++	++	++	±	++	–	–	–	++	–	–
endo-14-3-3β	++	++	+	++	–	++	–	–	–	++	–	–
14-3-3ε	++	++	++	++	±	++	–	–	–	++	–	–
endo-14-3-3ε	++	++	+	+	±	++	–	–	–	++	–	–
14-3-3σ	++	++	++	–	++	++	–	–	+	–	–	–
endo-14-3-3σ	++	++	+	–	++	++	–	–	+	–	–	–

Fig. 2. Binding of 14-3-3 subtypes to CDC25B is site specific. (A) Putative 14-3-3 consensus binding sites in CDC25B. (B-D) Mutants of CDC25B were transfected into HEK293 or U2OS cells either alone or together with 14-3-3 subtypes as indicated. Recovered CDC25B proteins are indicated (upper panel of each set of figures). The letters at the top and numbers at the bottom of each blot represent the CDC25B mutants: wild-type (1); S81A (2); S137A (3); S216A (4); S309A (5); S361A (6); 81S (7); 137S (8); 216S (9); 309S (10); 361S (11); 5SA (12). The definitions of the abbreviations for each mutant are described in the text. (B) Mutants of CDC25B were co-transfected into HEK293 cells with 14-3-3 subtypes β, ε or σ . Protein expression was determined by immunoblot. Wild-type or mutant CDC25B proteins were immunoprecipitated with anti-FLAG beads, and CDC25B-bound 14-3-3 was determined in the lysate (Lysate) and the immunoprecipitate [IP: CDC25B (α-FLAG Ab)]. Separate panel 'long exposure' shows 14-3-3 subtype σ after an exposure for 1 hour. (C) Mutants of CDC25B were transfected into HEK293 cells. Recovered CDC25B proteins and CDC25B-bound endogenous 14-3-3β (endo-14-3-3β) or endogenous 14-3-3ε (endo-14-3-3ε) were detected with specific antibodies in the lysate (Lysate) and the immunoprecipitate [IP: CDC25B (α-FLAG Ab]. (D) Mutants of CDC25B were transfected into U2OS cells. Recovered CDC25B and CDC25B-bound endogenous 14-3-3σ (endo-14-3-3σ) were detected with specific antibodies in the lysate (Lysate) and the immunoprecipitate [IP: CDC25B (α-FLAG Ab].(E) Binding of endogenous and transfected 14-3-3 subtypes to CDC25B mutants. ++, well bound; +, detectably bound; ±, faintly bound (could be detected only after long exposure); –, no binding.

sole site responsible for 14-3-3β binding. A faint signal was detected with the CDC25B mutants containing Ser137 or Ser216, but only after a long exposure time (S.U., A.K., M.O., M.S., M.H., H.N., T.M., Y.I. and K.Y., unpublished data). Exactly the same results were obtained for 14-3-3ε binding (Fig. 2B), i.e. the intact Ser309 fulfills the binding requirement. Surprisingly, entirely different results were obtained when 14-3-3σ was co-expressed with CDC25B. As shown in Fig. 2B, the mutation of Ser309 to Ala309 had little effect on 14-3-3σ binding. Instead, a single mutation changing Ser216 to Ala216 apparently abrogated the binding of 14-3-3σ. Experiments with single-serine constructs of CDC25B provided complementary results, indicating that only Ser216 is responsible for 14-3-3σ binding. Notice, that the amount of 14-3-3σ that bound to the CDC25B-S216 mutant was roughly half the amount of 14-3-3β or ε that bound to the CDC25B-S309 mutant. Therefore, the affinity of 14-3-3σ for Ser216 seems to be lower than those of 14-3-3β and ε for Ser309. Furthermore, 14-3-3σ bound to two other binding sites, Ser137 and Ser309, although with a lower affinity than the binding to Ser216 (Fig. 2B, Long exposure).

Binding of endogenous 14-3-3 to CDC25B

Next, we addressed the question of whether endogenous 14-3-3 binds to transfected CDC25B. After transfection of wild-type or mutant CDC25B, CDC25B was recovered and CDC25-bound 14-3-3β, ε, or σ was detected with subtype-specific antibodies. CDC25B was transfected to HEK293 cells to investigate binding of 14-3-3β and ε. U2OS cells were used to determine 14-3-3σ binding because no expression of 14-3-3σ was detected in HEK293 cells. Binding of endogenous 14-3-3β and ε is shown in Fig. 2C and that of 14-3-3σ in Fig. 2D. As illustrated, the results were essentially the same as those for the exogenously expressed ones. 14-3-3β and ε preferentially bound to Ser309 and a mutation to Ala at this site impaired 14-3-3 binding. Unlike 14-3-3β and ε, a Ser to Ala mutation at Ser216 eliminated 14-3-3σ binding (summarized in Fig. 2E). As clearly indicated, both endogenous and exogenous 14-3-3β and ε preferentially bind to Ser309, whereas 14-3-3σ prefers Ser216. Besides these two sites, Ser137 seems to be a favored binding site for the three 14-3-3 subtypes tested here because the binding signals are reduced by mutation at Ser137 (Fig. 2C and D). In respect to the other putative binding sites, we found no evidence that the 14-3-3 subtypes bind to either Ser81 or Ser361.

Multiple binding sites for 14-3-3σ on CDC25B

The results shown in Fig. 2 suggest that 14-3-3σ binds to CDC25B at multiple sites and possibly requires two sites to stably bind the protein. To explore this further, we constructed a series of mutants in which two serine residues were changed to alanine, and examined the binding of the 14-3-3 subtypes (Fig. 3). Compared with the single SA mutant (i.e. S216A), binding of 14-3-3σ to double SA mutants, such as S216/309A, was weaker or absent. Further work with the double mutants indicated that either of two sites, Ser137 or Ser309, seem to work cooperatively with Ser216. These results strongly suggest that 14-3-3σ requires two sites, Ser216 and Ser137 or Ser216 and Ser309, to interact effectively with CDC25B, and that 14-3-3σ might function as a dimer.

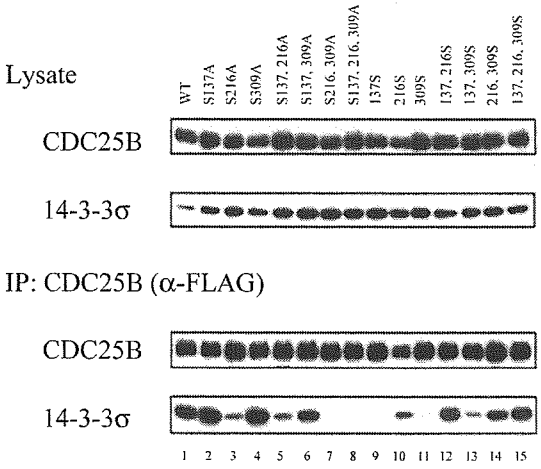


Fig. 3. Efficient binding of 14-3-3σ to CDC25B requires two independent sites. HEK293 cells were co-transfected with 14-3-3σ and a series of CDC25B mutants. Protein expression (Lysate) and protein binding (IP: CDC25B (α-FLAG Ab)) were detected. The letters in the upper panel of Lysate indicate CDC25B wild type and respective mutants. The definitions of the abbreviations for each mutant are described in the text.

14-3-3 binding sites and subcellular localization of CDC25B

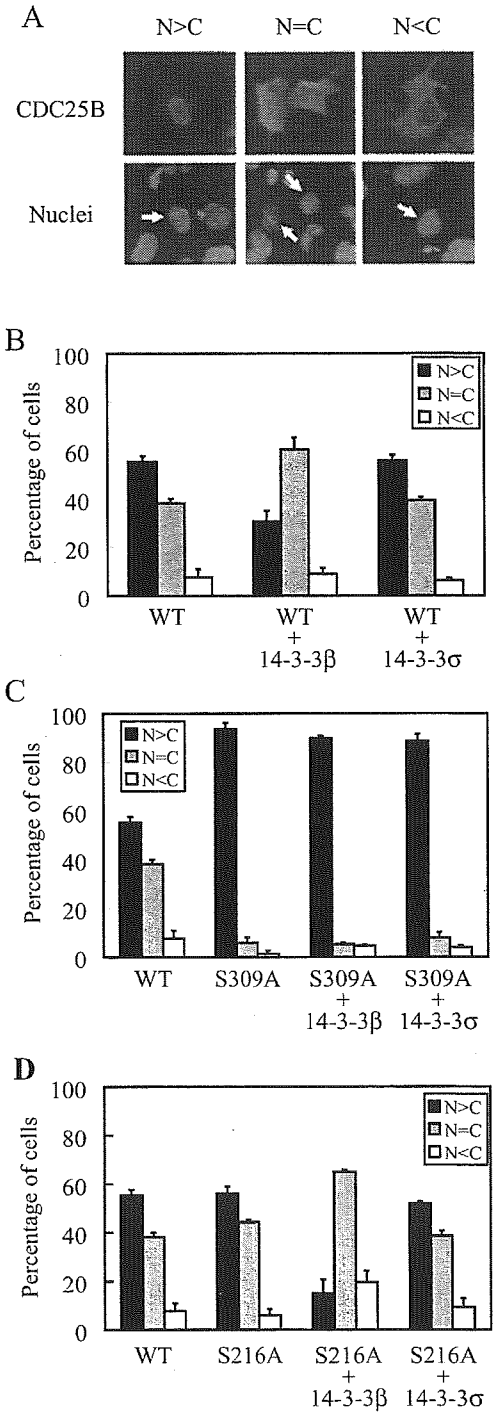
Binding of 14-3-3 to CDC25B was previously reported to induce the redistribution of CDC25B from the nucleus to the cytoplasm; the amino acid residue essential for this effect was shown to be Ser323 of CDC25B3 (or CDC25B2), which corresponds to Ser309 of CDC25B1 in our experiments (Davezac et al., 2000; Forrest and Gabrielli, 2001). Therefore, we analyzed the subcellular localization of CDC25B mutants expressed in combination with 14-3-3 subtypes that possess different binding site preferences. To assess the effects of co-transfection on the subcellular localization of CDC25B, we distinguished three different distributions [nuclear (N), diffuse (N=C) and cytoplasmic (C)] of CDC25B (Fig. 4A). The localization of exogenously expressed CDC25B was mainly nuclear (Fig. 4B), transfected 14-3-3β or σ was detected in the cytoplasm (S.U., A.K., M.O., M.S., M.H., H.N., T.M., Y.I. and K.Y., unpublished data). Upon co-transfection with 14-3-3β, CDC25B exhibited a diffuse distribution (Fig. 4B). Quantitatively, the percentage of cells with nuclear CDC25B was reduced from 55% to 30% and that of cells with a diffuse distribution increased from 38% to 60% when co-expressed with 14-3-3β. Based on our results, it is possible that nuclear localization is disturbed by 14-3-3 binding. Interestingly, the expression of 14-3-3σ had no effect on the localization of CDC25B. These results led us to hypothesize that when 14-3-3β binds to Ser309 of CDC25B, it can drive CDC25B from the nucleus to the cytoplasm, but that 14-3-3σ, which does not bind primarily to Ser309, has no ability to do so.

Effects of mutations at 14-3-3 binding sites on the localization of CDC25B

The primary 14-3-3β binding site on CDC25B was Ser309, and a point mutation at this site that changed serine to alanine abolished the interaction. If the binding of 14-3-3β is correlated

with the cytoplasmic localization of CDC25B, 14-3-3β could not drive the CDC25B mutant out of the nucleus. The results shown in Fig. 4C indicate that the mutation Ser309 to Ala309 in CDC25B completely disrupted its cytoplasmic localization with more than 90% of the mutant protein being located in the nuclei. In contrast to the wild type, this CDC25B mutant was not diffused into the cytoplasm by co-expression of 14-3-3β or 14-3-3σ. However, mutant S216A behaved like the wild type, i.e. its subcellular localization was effectively changed from nuclear to diffuse when co-expressed with 14-3-3β (Fig. 4D). Moreover, introduction of 14-3-3σ did not cause any change in the distribution of CDC25B. Collectively, these results show that Ser309 is essential for the cytoplasmic distribution of CDC25B and that Ser216 does not have any influence on the subcellular localization of CDC25B, even when 14-3-3σ binds to it.

To confirm that the subcellular distribution of CDC25B by 14-3-3β depends on Ser309, we made mutants in which serine was changed to alanine at four of the five sites that have a single phosphorylatable serine residue. The mutants were denoted as CDC25B-81S, CDC25B-137S, CDC25B-216S, CDC25B-309S and CDC25B-361S (as mentioned in Fig. 2). These CDC25B mutants were transfected with or without 14-3-3 and their localizations analyzed. Only CDC25B-309S behaved like the wild type (Fig. 5B); the other mutants exhibited nuclear localizations, probably because they possessed the S309A mutation and could not bind to 14-3-3β (Fig. 5A). Wild-type CDC25B and the CDC25B-309S mutant exhibited nuclear localization in about 60% of the cells (Fig. 5B). As was the case with wild-type CDC25B (see Fig. 4B), the expression of 14-3-3β antagonized the nuclear localization of CDC25B-309S and led to a diffuse distribution (Fig. 5B). In contrast to 14-3-3β, 14-3-3σ did not bind to the mutant and had no effect on the nuclear localization of CDC25B-309S or wild-type CDC25B (Fig. 5B). These results strongly suggest that only Ser309 of CDC25B is required for the control of the subcellular localization of CDC25B by 14-3-3β.



Effects of 14-3-3ε on the nuclear localization of CDC25B

The results shown in Fig. 2 indicate that Ser309 of CDC25B is the specific binding site for 14-3-3ε. We examined the effects

Fig. 4. 14-3-3β but not 14-3-3σ efficiently redistributes CDC25B from the nucleus to the cytoplasm. HEK293 cells were transfected with FLAG-tagged CDC25B in combination with empty vector, myc-tagged 14-3-3β or myc-tagged 14-3-3σ, followed by immunostaining with anti-FLAG antibodies to detect the subcellular localization of CDC25B and with anti-myc antibodies to detect co-transfected 14-3-3 proteins. Analyses showed that more than 95% of the cells that expressed CDC25B also expressed the co-transfected 14-3-3 proteins. (A) Exemplary images, showing how the subcellular distribution of CDC25B was evaluated: N>C, predominantly nuclear; N=C diffuse; N<C, predominantly cytoplasmic. (B) Wild-type CDC25B was co-transfected with empty vector (WT), myc-tagged 14-3-3β (WT+14-3-3β) or myc-tagged 14-3-3σ (WT+14-3-3σ) to quantify the subcellular distribution of CDC25B. Over 200 cells expressing CDC25B were counted to determine the percentage of cells that express CDC25B with nuclear, diffuse and cytoplasmic distribution. Error bars in graphs represent the means±s.d. of three independent experiments. (C) Transfection with wild-type CDC25B alone (WT), S309A mutant of CDC25B alone (S309A) and mutant S309A in combination with myc-tagged 14-3-3β (S309A+14-3-3β) or myc-tagged 14-3-3σ (S309A+14-3-3σ). Over 200 cells expressing CDC25B were counted to determine the percentage of cells that express CDC25B with nuclear, diffuse and cytoplasmic distribution. Error bars in graphs represent the means±s.d. of three independent experiments. (D) Transfection with wild-type CDC25B alone (WT), S216A mutant of CDC25B alone (S216A) and mutant S216A in combination with myc-tagged 14-3-3β (S216A+14-3-3β) or myc-tagged 14-3-3σ (S216A+14-3-3σ). Over 200 cells expressing CDC25B were counted to determine the percentage of cells that express CDC25B with nuclear, diffuse and cytoplasmic distribution. Error bars in graphs represent the means±s.d. of three independent experiments.

of 14-3-3ε on the subcellular localization of CDC25B in three sets of experiments. First, 14-3-3ε was co-transfected with wild-type CDC25B and CDC25B-distribution (as defined above and in Fig. 4A) was analyzed by counting the cells. Co-expression of 14-3-3ε reduced the percentage of cells with nuclear localization of CDC25B from 55% to 47% and concomitantly increased the percentage of cells displaying a diffuse pattern from 40% to 55% (Fig. 6A). Second, 14-3-3ε was co-transfected with the CDC25B-309S mutant. Here, the nuclear localization of CDC25B decreased from 60% to 37%, whereas its diffuse distribution increased from 35% to 55% (Fig. 6B). We found no effects of the co-expression of 14-3-3ε on the subcellular localization of the CDC25B-S309A mutant (Fig. 6C). In summary, the results with 14-3-3ε were exactly the same as those obtained with 14-3-3β and different from those with 14-3-3σ.

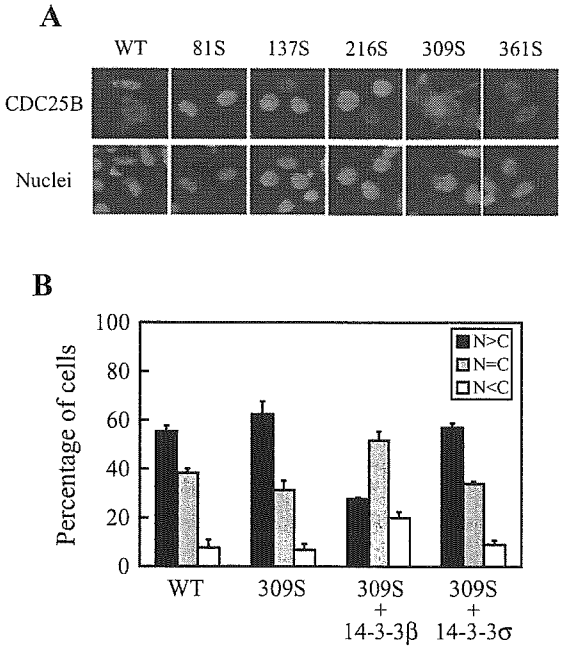


Fig. 5. Only the 309S mutant of CDC25B was distributed diffusely with co-transfection of 14-3-3β. (A) Wild type CDC25B or different CDC25B mutants with a single phosphorylatable serine were co-transfected with 14-3-3β into HEK293 cells. (Upper panels) Subcellular localization of CDC25B wild type and mutants. (Lower panel) Corresponding images of nuclei. (B) Percentage of cells transfected with mutant CDC25B 309S (shown in A) that express CDC25B with nuclear, diffuse and cytoplasmic distribution. Over 200 cells expressing CDC25B were counted to determine the percentage of cells that express CDC25B. Transfection with wild-type CDC25B alone (WT), 309S mutant of CDC25B alone (309S) and mutant 309S in combination with myc-tagged 14-3-3β (309S +14-3-3β) or myc-tagged 14-3-3σ (309S +14-3-3σ). Error bars in graphs represent the means±s.d. of three independent experiments. Subcellular distribution of CDC25B: N>C, predominantly nuclear; N=C diffuse; N<C, predominantly cytoplasmic (C).

Effects of 14-3-3β binding on the nuclear import of CDC25B

Several previous studies demonstrated that treating cells with leptomycin B (LMB), a CRM1 (exportin1) inhibitor, disrupts the cytoplasmic localization of CDC25B (Nishi et al., 1994; Kudo et al., 1998; Karlsson et al., 1999; Davezac et al., 2000) (Fig. 7A). Therefore, it might be that 14-3-3β-binding slows down the nuclear import of CDC25B by LMB. After transfecting CDC25B with or without 14-3-3β, cells were treated with LMB and the nuclear accumulation of CDC25B was measured. As shown in Fig. 7B, co-expression of exogenous 14-3-3β efficiently inhibited the nuclear import of CDC25B. Notice that this effect was not observed when 14-3-3σ was co-transfected with CDC25B. These results suggest that 14-3-3β masks the nuclear localizing signal (NLS) of CDC25B, which is located about 30 amino acids downstream of Ser309.

Discussion

It has long been believed that higher eukaryotic cells have two dual specificity phosphatases, CDC25B and CDC25C, which activate CDK1/cyclin B to initiate mitosis. Recent reports indicate that another dual specificity phosphatase, CDC25A, plays a crucial role in G2-M events (Mailand et al., 2002). CDC25A can bind and activate CDK1/cyclin B, and downregulation by RNAi delays mitotic entry. In addition, the overexpression of CDC25A abrogates the G2 DNA-damage checkpoint (Mailand et al., 2002; Chow et al., 2003). Therefore, it is possible to regard CDC25A as a master activator of CDK/cyclin in the cell cycle, and the roles of

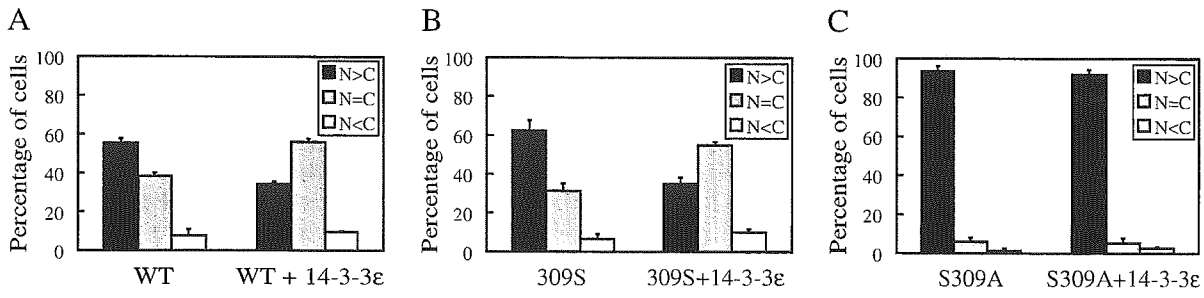


Fig. 6. 14-3-3ε had effects similar to those of 14-3-3β on the subcellular localization of CDC25B. HE293 cells were transfected with (A) wild-type CDC25B, (B) the 309S mutant or (C) the S309A mutant with or without 14-3-3ε. Over 200 cells expressing CDC25B were counted to determine the percentage of cells that express CDC25B with nuclear, diffuse and cytoplasmic distribution. Error bars in graphs represent the means±s.d. of three independent experiments. Subcellular distribution of CDC25B: N>C, predominantly nuclear; N=C diffuse; N<C, predominantly cytoplasmic (C).

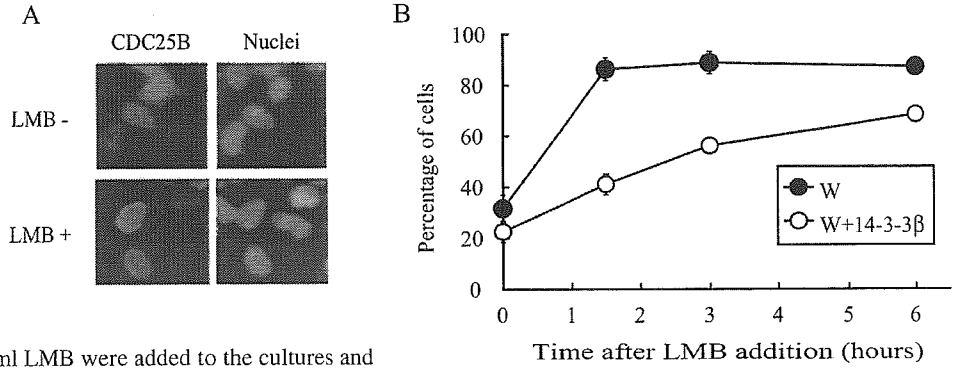


Fig. 7. Binding of 14-3-3β to CDC25B efficiently slowed down the nuclear import of CDC25B induced by leptomycin B (LMB). (A) HEK293 cells transfected with wild-type CDC25B and treated with 20 ng/ml LMB at for 3 hours. Transfected CDC25B was detected with anti-FLAG antibody. The upper and lower panels show the results without or with LMB treatment, respectively. (B) HEK293 cells transfected with wild-type CDC25B alone or with 14-3-3β. Twenty-four hours after transfection, 20 ng/ml LMB were added to the cultures and the percentage of cells exhibiting nuclear-specific localization of CDC25B was determined at the indicated time-points: 0 (before addition of LMB), 1.5, 3 or 6 hours after the addition. Over 200 cells expressing CDC25B were counted to determine the percentage of cells that express CDC25B. The percentage of cells with a nuclear localization (as shown in Fig. 4) was determined from three independent experiments. ●, CDC25B; ○,CDC25B with 14-3-3β.

CDC25B and CDC25C as being restricted to G2-M events to activate CDK1/cyclin B.

It has been proposed that CDC25C inhibits human CDC25C, by downregulating its phosphatase activity or by binding 14-3-3 after the phosphorylation of Ser216 (Peng et al., 1997; Blasina et al., 1999; Furnari et al., 1999; Graves et al., 2001). The amount of cellular CDC25C is essentially kept constant. Therefore, a qualitative regulation of its functions, i.e. enzyme activity and subcellular localization, is required to control cell cycle progression. In the case of CDC25B, the protein accumulates as the cell cycle progresses, reaching a maximum at G2-M phase. Thus, controlling the expression of CDC25B is an effective means of regulating its function. However, at G2 phase, when the CDC25B level is at its peak, an alternate way of keeping it inactive is needed when its activation is inappropriate. Recently, several groups have reported that the binding of 14-3-3, specifically at Ser309 of CDC25B1 or Ser323 of CDC25B2 or CDC25B3, results in the cytoplasmic localization of CDC25B, supporting the theory of its redistribution from the nucleus to the cytoplasm as a critical G2-M checkpoint (Davezac et al., 2000; Forrest and Gabrielli, 2001).

In agreement with these reports, we found that 14-3-3β and 14-3-3ε bound specifically at Ser309 of CDC25B1 and that the binding effectively redistributed CDC25B, decreasing its amount in the nuclei. We consistently detected nuclear localization in about 50% of the CDC25B-transfected cells. Endogenous 14-3-3, detected with a pan-14-3-3 antibody, was recovered as a complex with exogenous CDC25B. The co-expression of 14-3-3β or 14-3-3ε reduced the nuclear localization of exogenous CDC25B by about 20%, but endogenous 14-3-3 was recovered with exogenous CDC25B. More than 95% of the introduced CDC25B was localized in nuclei when the binding of 14-3-3 was abolished by a CDC25B point mutation. Thus, it is reasonable to conclude that the binding of 14-3-3 at Ser309 of CDC25B is essential for the exclusion of CDC25B from the nucleus. We also presented evidence that binding of 14-3-3β to CDC25B slowed down the nuclear import induced by LMB treatment. Since 14-3-3β specifically binds to Ser309, bound 14-3-3 should impair the access of nuclear import cargos, such as importin, to the NLS.

In *Xenopus*, 14-3-3-binding to CDC25C was suggested to mask its NLS, making its nuclear exclusion signal (NES) available for the transfer of CDC25C to the cytoplasm (Kumagai and Dunphy, 1999). The NLS in human CDC25B is located at the same position relative to the 14-3-3-binding site of CDC25C in Xenopus, i.e., about 30 amino acids downstream of Ser309 (Davezac et al., 2000) (S.U., A.K., M.O., M.S., M.H., H.N., T.M., Y.I. and K.Y., unpublished data). Therefore, the binding of 14-3-3β or 14-3-3ε at Ser309 could inactivate the NLS, which in turn would make the N-terminal NES dominant. This idea is further supported by the observation that the preferential binding of 14-3-3σ to Ser216 does not cause cytoplasmic redistribution of CDC25B because Ser216 is too far away to allow 14-3-3σ to mask the NLS. We also conclude from this result that the NES-like sequence present in the C-terminus of all 14-3-3 subtypes does not function as an NES. Thus, our results agree well with the recently presented hypothesis that the binding of 14-3-3 does not add an 'attachable NES' that targets proteins (Rittinger et al., 1999; Brunet et al., 2002). Instead, it might serve other functions, such as providing scaffolding or a cover that hides specific motifs, such as NLS or NES (Muslin and Xing, 2000; Tzivion et al., 2001; Yaffe, 2002).

Ser309 was shown to be phosphorylated by p38 MAP kinase, and the kinase activity was necessary to maintain cell cycle arrest at G2 in response to DNA damage caused by UV light (Bulavin et al., 2001). One of the checkpoint kinases, Chk1, can phosphorylate Ser309 to enhance 14-3-3-binding in vitro (Forrest and Gabrielli, 2001) (S.U., A.K., M.O., M.S., M.H., H.N., T.M., Y.I. and K.Y., unpublished data). Although co-expression of MKK6, Chk1 or Chk2 with CDC25B and 14-3-3β enhanced the binding of 14-3-3β to CDC25B, these effects were not significant (S.U., A.K., M.O., M.S., M.H., H.N., T.M., Y.I. and K.Y., unpublished data). Therefore, Ser309 seems to be constitutively phosphorylated, possibly by p38 or C-TAK1. If phosphorylation of this serine is crucial for the induction and/or maintenance of G2 arrest, the inactivation of the phosphatase responsible for the dephosphorylation might also occur, although enhanced checkpoint kinase activity is usually thought to maintain the phosphorylation state. The significance of the cytoplasmic localization of CDC25B in terms of cell cycle regulation, especially at the G2 checkpoint, is not clear. However, abrogation of the 14-3-3 binding site

abolished G2-arrest and thus caused localization of CDC25B to the nucleus. The overexpression of CDC25B is sufficient to override the G2 DNA damage checkpoint (Miyata et al., 2001), but in this case, Ser309 of the overexpressed CDC25B would be phosphorylated as is the endogenous residue. In addition, the amount of cellular 14-3-3 is obviously in excess of the amount of CDC25B, and thus the equilibrium between 14-3-3-bound CDC25B and unbound CDC25B should be the same in transfected cells and in normal cells. If overexpression enhances the probability of the localization of CDC25B in the nucleus, CDC25B could counteract the inhibitory effects of Wee1 kinase, leading to the activation of CDK1/cyclin B and abrogation of the G2 checkpoint. Phosphorylation of Ser309 should be necessary to inhibit premature mitosis, but it is too early to attribute maintained phosphorylation at the G2 checkpoint to the checkpoint kinases or to p38. So, if Ser309 is constantly phosphorylated, then its phosphorylation level could never be enhanced because of DNA damage (Bulavin et al., 2001). Indeed, no reports indicate a higher than normal phosphorylation level of Ser309 or Ser323 in CDC25B2 or CDC25B3 at the G2 checkpoint, although it is possible to postulate a change from the maintenance kinases to the checkpoint kinases at the checkpoint state to keep the phosphorylation level constant (Bulavin et al., 2002). Thus, the significance of the phosphorylation of Ser309, in combination with the binding of 14-3-3 at the site, must await further conclusions about the G2 checkpoint.

We have reported here the binding of 14-3-3σ at Ser216 of CDC25B, which has not been reported previously. We have also described another site, Ser137, that seems to provide support for the binding of 14-3-3σ. The subtypes 14-3-3β and ε have little preference for either of these sites, although both serine residues partly satisfy the consensus-binding motif of 14-3-3 (RxxS). It is rare to find binding-preference differences among 14-3-3 subtypes and it should be noticed that 14-3-3σ does not prefer Ser309 for binding even though it is in one of the typical 14-3-3 binding motifs. Interestingly, 14-3-3σ also does not bind to CDC25C where Ser216 is located in a typical 14-3-3 binding motif (RSxSMP) (Chan et al., 1999) (S.U., A.K., M.O., M.S., M.H., H.N., T.M., Y.I. and K.Y., unpublished data). Two sites on CDC25B are required for the efficient binding of 14-3-3σ, which means that 14-3-3σ must be a dimer to bind efficiently to the two different sites on CDC25B.

During the preparation of this manuscript an on-line report was published, describing two sites, other than Ser323 of CDC25B2, necessary for 14-3-3 binding (Giles et al., 2003). Those two sites in CDC25B2, Ser151 and Ser230, are exactly the same as Ser137 and Ser216 of CDC25B1 that have been discussed here. We have demonstrated that 14-3-3σ binds to these sites. It is well known that 14-3-3σ is one of the downstream transcriptional targets of p53 (Hermeking et al., 1997). There have been several reports that 14-3-3σ can downregulate CDK activity by binding to it or that 14-3-3σ can move the CDK1/cyclin B complex to the cytoplasm (Bulavin et al., 2002). Here, we suggest that 14-3-3σ downregulates the function of CDC25B and thereby acts as a G2-checkpoint regulator. Our preliminary experiments indicate that the co-expression of CDC25B and Chk1, but not MKK6 (that activates p38), enhances phosphorylation at Ser137 and Ser216 (S.U., A.K., M.O., M.S., M.H., H.N., T.M., Y.I. and K.Y., unpublished data). Further studies are required to determine whether the phosphorylation of both sites leads to the binding of 14-3-3σ and to establish the consequences for CDC25B.

We thank Hiroto Okayama (University of Tokyo) and Minoru Yoshida (RIKEN) for the generous gift of CDC25B1 cDNA and leptomycin B, 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 of Japan.

References

Blasina, A., de Weyer, I. V., Laus, M. C., Luyten, W. H., Parker, A. E. and McGowan, C. H. (1999). A human homologue of the checkpoint kinase Cds1 directly inhibits Cdc25 phosphatase. *Curr. Biol.* **9**, 1-10.

Brunet, A., Kanai, F., Stehn, J., Xu, J., Sarbassova, D., Frangioni, J. V., Dalal, S. N., DeCaprio, J. A., Greenberg, M. E. and Yaffe, M. B. (2002). 14-3-3 transits to the nucleus and participates in dynamic nucleocytoplasmic transport. *J. Cell Biol.* **156**, 817-828.

Bulavin, D. V., Higashimoto, Y., Popoff, I. J., Gaarde, W. A., Basrur, V., Potapova, O., Appella, E. and Fornace, A. J. Jr (2001). Initiation of a G2/M checkpoint after ultraviolet radiation requires p38 kinase. *Nature* **411**, 102-107.

Bulavin, D. V., Amundson, S. A. and Fornace, A. J. (2002). p38 and Chk1 kinases: different conductors for the G(2)/M checkpoint symphony. *Curr. Opin. Genet. Dev.* **12**, 92-97.

Chan, T. A., Hermeking, H., Lengauer, C., Kinzler, K. W. and Vogelstein, B. (1999). 14-3-3Sigma is required to prevent mitotic catastrophe after DNA damage. *Nature* **401**, 616-620.

Chan, T. A., Hwang, P. M., Hermeking, H., Kinzler, K. W. and Vogelstein, B. (2000). Cooperative effects of genes controlling the G(2)/M checkpoint. *Genes Dev.* **14**, 1584-1588.

Chow, J. P., Siu, W. Y., Fung, T. K., Chan, W. M., Lau, A., Arooz, T., Ng, C. P., Yamashita, K. and Poon, R. Y. (2003). DNA damage during the spindle-assembly checkpoint degrades CDC25A, inhibits cyclin-CDC2 complexes, and reverses cells to interphase. *Mol. Biol. Cell* **14**, 3989-4002.

Conklin, D. S., Galaktionov, K. and Beach, D. (1995). 14-3-3 proteins associate with cdc25 phosphatases. *Proc. Natl. Acad. Sci. USA* **92**, 7892-7896.

Davezac, N., Baldin, V., Gabrielli, B., Forrest, A., Theis-Febvre, N., Yashida, M. and Ducommun, B. (2000). Regulation of CDC25B phosphatases subcellular localization. *Oncogene* **19**, 2179-2185.

Ford, J. C., al-Khodairy, F., Fotou, E., Sheldrick, K. S., Griffiths, D. J. and Carr, A. M. (1994). 14-3-3 protein homologs required for the DNA damage checkpoint in fission yeast. *Science* **265**, 533-535.

Forrest, A. and Gabrielli, B. (2001). Cdc25B activity is regulated by 14-3-3. *Oncogene* **20**, 4393-4401.

Furnari, B., Blasina, A., Boddy, M. N., McGowan, C. H. and Russell, P. (1999). Cdc25 inhibited in vivo and in vitro by checkpoint kinases Cds1 and Chk1. *Mol. Biol. Cell.* **10**, 833-845.

Galaktionov, K. and Beach, D. (1991). Specific activation of cdc25 tyrosine phosphatases by B-type cyclins: evidence for multiple roles of mitotic cyclins. *Cell* **67**, 1181-1194.

Galaktionov, K., Lee, A. K., Eckstein, J., Draetta, G., Meckler, J., Loda, M. and Beach, D. (1995). CDC25 phosphatases as potential human oncogenes. *Science* **269**, 1575-1577.

Giles, N., Forrest, A. and Gabrielli, B. (2003). 14-3-3 acts as an intramolecular bridge to regulate cdc25B localization and activity. *J. Biol. Chem.* **278**, 28580-28587.

Graves, P. R., Lovly, C. M., Uy, G. L. and Piwnica-Worms, H. (2001). Localization of human Cdc25C is regulated both by nuclear export and 14-3-3 protein binding. *Oncogene* **20**, 1839-1851.

Hermeking, H., Lengauer, C., Polyak, K., He, T. C., Zhang, L., Thiagalingam, S., Kinzler, K. W. and Vogelstein, B. (1997). 14-3-3 sigma is a p53-regulated inhibitor of G2/M progression. *Mol. Cell* **1**, 3-11.

Honda, R., Ohba, Y. and Yasuda, H. (1997). 14-3-3 zeta protein binds to the carboxyl half of mouse wee1 kinase. *Biochem. Biophys. Res. Commun.* **230**, 262-265.

Hutchins, J. R., Hughes, M. and Clarke, P. R. (2000). Substrate specificity determinants of the checkpoint protein kinase Chk1. *FEBS Lett.* **466**, 91-95.

Karlsson, C., Katich, S., Hagting, A., Hoffmann, I. and Pines, J. (1999). Cdc25B and Cdc25C differ markedly in their properties as initiators of mitosis. *J. Cell. Biol.* **146**, 573-584.

Kudo, N., Wolff, B., Sekimoto, T., Schreiner, E. P., Yoneda, Y., Yanagida, M., Horinouchi, S. and Yoshida, M. (1998). Leptomycin B inhibition of signal-mediated nuclear export by direct binding to CRM1. *Exp. Cell Res.* **242**, 540-547.

Kumagai, A. and Dunphy, W. G. (1999). Binding of 14-3-3 proteins and nuclear export control the intracellular localization of the mitotic inducer Cdc25. *Genes Dev.* **13**, 1067-1072.

Kumagai, A., Yakowec, P. S. and Dunphy, W. G. (1998). 14-3-3 proteins act as negative regulators of the mitotic inducer Cdc25 in Xenopus egg extracts. *Mol. Biol. Cell* **9**, 345-354.

Lammer, C., Wagerer, S., Saffrich, R., Mertens, D., Ansorge, W. and Hoffmann, I. (1998). The cdc25B phosphatase is essential for the G2/M phase transition in human cells. *J. Cell Sci.* **111**, 2445-2453.

Lee, J., Kumagai, A. and Dunphy, W. G. (2001). Positive regulation of Wee1 by Chk1 and 14-3-3 proteins. *Mol. Biol. Cell* **12**, 551-563.

Ma, Z. Q., Chua, S. S., DeMayo, F. J. and Tsai, S. Y. (1999). Induction of mammary gland hyperplasia in transgenic mice over-expressing human Cdc25B. *Oncogene* **18**, 4564-4576.

Mailand, N., Podtelejnikov, A. V., Groth, A., Mann, M., Bartek, J. and Lukas, J. (2002). Regulation of G2/M events by Cdc25A through phosphorylation-dependent modulation of its stability. *EMBO J.* **21**, 5911-5920.

Matsuoka, S., Huang, M. and Elledge, S. J. (1998). Linkage of ATM to cell cycle regulation by the Chk2 protein kinase. *Science* **282**, 1893-1897.

Mils, V., Baldin, V., Goubin, F., Pinta, I., Papin, C., Waye, M., Eychene, A. and Ducommun, B. (2000). Specific interaction between 14-3-3 isoforms and the human CDC25B phosphatase. *Oncogene* **19**, 1257-1265.

Miyata, H., Doki, Y., Yamamoto, H., Kishi, K., Takemoto, H., Fujiwara, Y., Yasuda, T., Yano, M., Inoue, M., Shiozaki, H. et al. (2001). Overexpression of CDC25B overrides radiation-induced G2-M arrest and results in increased apoptosis in esophageal cancer cells. *Cancer Res.* **61**, 3188-3193.

Morgan, D. O. (1997). Cyclin-dependent kinases: engines, clocks, and microprocessors. *Annu. Rev. Cell Dev. Biol.* **13**, 261-291.

Muslin, A. J. and Xing, H. (2000). 14-3-3 proteins: regulation of subcellular localization by molecular interference. *Cell. Signal.* **12**, 703-709.

Muslin, A. J., Tanner, J. W., Allen, P. M. and Shaw, A. S. (1996). Interaction of 14-3-3 with signaling proteins is mediated by the recognition of phosphoserine. *Cell* **84**, 889-897.

Nagata, A., Igarashi, M., Jinno, S., Suto, K. and Okayama, H. (1991). An additional homolog of the fission yeast cdc25+ gene occurs in humans and is highly expressed in some cancer cells. *New Biol.* **3**, 959-968.

Nilsson, I. and Hoffmann, I. (2000). Cell cycle regulation by the Cdc25 phosphatase family. *Prog. Cell Cycle Res.* **4**, 107-114.

Nishi, K., Yoshida, M., Fujiwara, D., Nishikawa, M., Horinouchi, S. and Beppu, T. (1994). Leptomycin B targets a regulatory cascade of crm1, a fission yeast nuclear protein, involved in control of higher order chromosome structure and gene expression. *J. Biol. Chem.* **269**, 6320-6324.

O'Neill, T., Giarratani, L., Chen, P., Iyer, L., Lee, C. H., Bobiak, M., Kanai, F., Zhou, B. B., Chung, J. H. and Rathbun, G. A. (2002). Determination of substrate motifs for human Chk1 and hCds1/Chk2 by the oriented peptide library approach. *J. Biol. Chem.* **277**, 16102-16115.

Peng, C. Y., Graves, P. R., Thoma, R. S., Wu, Z., Shaw, A. S. and Piwnica-Worms, H. (1997). Mitotic and G2 checkpoint control: regulation of 14-3-3 protein binding by phosphorylation of Cdc25C on serine-216. *Science* **277**, 1501-1505.

Peng, C. Y., Graves, P. R., Ogg, S., Thoma, R. S., Byrnes, M. J. 3rd, Wu, Z., Stephenson, M. T. and Piwnica-Worms, H. (1998). C-TAK1 protein kinase phosphorylates human Cdc25C on serine 216 and promotes 14-3-3 protein binding. *Cell Growth Differ.* **9**, 197-208.

Rittinger, K., Budman, J., Xu, J., Volinia, S., Cantley, L. C., Smerdon, S. J., Gamblin, S. J. and Yaffe, M. B. (1999). Structural analysis of 14-3-3 phosphopeptide complexes identifies a dual role for the nuclear export signal of 14-3-3 in ligand binding. *Mol. Cell* **4**, 153-166.

Rothblum-Oviatt, C. J., Ryan, C. E. and Piwnica-Worms, H. (2001). 14-3-3 binding regulates catalytic activity of human Wee1 kinase. *Cell Growth Differ.* **12**, 581-589.

Sanchez, Y., Wong, C., Thoma, R. S., Richman, R., Wu, Z., Piwnica-Worms, H. and Elledge, S. J. (1997). Conservation of the Chk1 checkpoint pathway in mammals: linkage of DNA damage to Cdk regulation through Cdc25. *Science* **277**, 1497-1501.

Sebastian, B., Kakizuka, A. and Hunter, T. (1993). Cdc25M2 activation of cyclin-dependent kinases by dephosphorylation of threonine-14 and tyrosine-15. *Proc. Natl. Acad. Sci. USA* **90**, 3521-3524.

Tzivion, G., Shen, Y. H. and Zhu, J. (2001). 14-3-3 proteins; bringing new definitions to scaffolding. *Oncogene* **20**, 6331-6338.

Wang, Y., Jacobs, C., Hook, K. E., Duan, H., Booher, R. N. and Sun, Y. (2000). Binding of 14-3-3beta to the carboxyl terminus of Wee1 increases Wee1 stability, kinase activity, and G2-M cell population. *Cell Growth Differ.* **11**, 211-219.

Wang, X., Arooz, T., Siu, W. Y., Chiu, C. H., Lau, A., Yamashita, K. and Poon, R. Y. (2001). MDM2 and MDMX can interact differently with ARF and members of the p53 family. *FEBS Lett.* **490**, 202-208.

Yaffe, M. B. (2002). How do 14-3-3 proteins work? Gatekeeper phosphorylation and the molecular anvil hypothesis. *FEBS Lett.* **513**, 53-57.

Yaffe, M. B., Rittinger, K., Volinia, S., Caron, P. R., Aitken, A., Leffers, H., Gamblin, S. J., Smerdon, S. J. and Cantley, L. C. (1997). The structural basis for 14-3-3: phosphopeptide binding specificity. *Cell* **91**, 961-971.

Yang, J., Winkler, K., Yoshida, M. and Kornbluth, S. (1999). Maintenance of G2 arrest in the Xenopus oocyte: a role for 14-3-3-mediated inhibition of Cdc25 nuclear import. *EMBO J.* **18**, 2174-2183.

Yao, Y., Slosberg, E. D., Wang, L., Hibshoosh, H., Zhang, Y. J., Xing, W. Q., Santella, R. M. and Weinstein, I. B. (1999). Increased susceptibility to carcinogen-induced mammary tumors in MMTV-Cdc25B transgenic mice. *Oncogene* **18**, 5159-5166.

ACADEMIC PRESS

Available online at www.sciencedirect.com

SCIENCE DIRECT®

Archives of Biochemistry and Biophysics 412 (2003) 13–19

ABB

www.elsevier.com/locate/yabbi

Loss of p53 induces M-phase retardation following G2 DNA damage checkpoint abrogation

Yuzuru Minemoto,[a,1] Sanae Uchida,[a] Motoaki Ohtsubo,[b] Mari Shimura,[c] Toshiyuki Sasagawa,[d] Masato Hirata,[e] Hitoshi Nakagama,[f] Yukihito Ishizaka,[c] and Katsumi Yamashita[a,*]

[a] *Department of Life Science, Graduate School of Natural Science and Technology, General Education Hall, Kanazawa University, Kakuma-machi, Kanazawa 920-1192, Ishikawa, Japan*

[b] *Institute of Life Science, Kurume University, Aikawa 2432-3, Kurume, Fukuoka 839-0861, Japan*

[c] *Faculty of Health Science, Kanazawa University, 11-80, Kodatsuno 5-chome, 11-80, Kanazawa 920-0942, Ishikawa, Japan*

[d] *Division of Intractable Diseases, International Medical Center of Japan, 21-1, Toyama 1-chome, Shinjyuku-ku, Tokyo 162-8655, Japan*

[e] *Laboratory of Molecular and Cellular Biochemistry, Faculty of Dental Science, and Station for Collaborative Research, Kyushu University, Fukuoka 812-8582, Japan*

[f] *Biochemistry Division, National Cancer Center Research Institute, 1-1, Tsukiji 5-chome, Chuo-ku, Tokyo 104-0045, Japan*

Received 1 November 2002, and in revised form 2 January 2003

Abstract

Most cell lines that lack functional p53 protein are arrested in the G2 phase of the cell cycle due to DNA damage. When the G2 checkpoint is abrogated, these cells are forced into mitotic catastrophe. A549 lung adenocarcinoma cells, in which p53 was eliminated with the HPV16 E6 gene, exhibited efficient arrest in the G2 phase when treated with adriamycin. Administration of caffeine to G2-arrested cells induced a drastic change in cell phenotype, the nature of which depended on the status of p53. Flow cytometric and microscopic observations revealed that cells that either contained or lacked p53 resumed their cell cycles and entered mitosis upon caffeine treatment. However, transit to the M phase was slower in p53-negative cells than in p53-positive cells. Consistent with these observations, CDK1 activity was maintained at high levels, along with stable cyclin B1, in p53-negative cells. The addition of butyrolactone I, which is an inhibitor of CDK1 and CDK2, to the p53-negative cells reduced the floating round cell population and induced the disappearance of cyclin B1. These results suggest a relationship between the p53 pathway and the ubiquitin-mediated degradation of mitotic cyclins and possible cross-talk between the G2-DNA damage checkpoint and the mitotic checkpoint.
© 2003 Elsevier Science (USA). All rights reserved.

Keywords: HPV16E6 gene; G2 checkpoint; Caffeine; p53; CDK1; Cyclin B

Eukaryotic cells respond to genome-damaging agents by activating cell cycle checkpoints that reversibly halt the cell cycle or, in certain cases, induce cell death. Cell cycle arrest at the G1 and G2 checkpoints allows the cells time to repair damaged DNA before replication and mitosis [1]. It is well known that p53 is up-regulated by genome stress and maintains genomic stability through the induction of cell cycle arrest, which is accompanied by either the activation of DNA repair processes or, in cases involving irreparable DNA damage, the induction of cellular apoptosis [2–5].

Although cells with normal levels of p53 arrest in G1 and G2 in response to genotoxic stress, cells that have lost p53 activity as a result of mutation, the introduction of viral genes, or gene rearrangement arrest exclusively in G2 [6]. The stabilization of p53 and the transcrip-

* Corresponding author. Fax: +81-76-264-5989.

E-mail address: katsumi@kenroku.kanazawa-u.ac.jp (K. Yamashita).

[1] Present address: Ben May Institute for Cancer Research, Committee on Cancer Biology, University of Chicago, 5841 South Maryland Avenue, MC 6027, Chicago, IL 60637, USA.

0003-9861/03/$ - see front matter © 2003 Elsevier Science (USA). All rights reserved.
doi:10.1016/S0003-9861(03)00010-9

tional induction of p21, a cyclin-dependent kinase (CDK)[2] inhibitor that inactivates CDK4/cyclin D and CDK2/cyclin E, are key factors in G1 arrest [7].

In contrast to its role in G1-phase arrest, the role of p53 in G2 arrest that is induced by DNA damage is controversial. Although cells that lack functional p53 can arrest in the G2 phase, several lines of evidence implicate p53 in G2 arrest. Some reports have suggested that the effect of p53 on the G2 checkpoint is linked to downstream transcriptional effects of the CDK inhibitor p21 [8,9]. An alternative hypothesis has been put forward recently, i.e., that p53 affects the G2 checkpoint during DNA damage by transcriptionally repressing the activity of genes that control the onset of mitosis, such as CDK1 and cyclin B1 [10–12]. Therefore, it seems likely that p53-dependent and p53-independent G2-checkpoint pathways coexist in cells [10,13].

In this report, we evaluated the roles of the p53 in the process of G2-checkpoint abrogation by comparing the biological and biochemical features of p53-positive and p53-negative cells. We found dramatic differences between these two cell types: the p53-positive cells traversed the M phase efficiently, but the p53-negative cells exhibited delayed progression of the M phase with high CDK1 activity. These results suggest the possible link between the G2-DNA damage checkpoint and the mitotic checkpoint through the p53 pathway.

Materials and methods

Cells and cell cultures

The human lung adenocarcinoma cell line A549 was obtained from Touho Yoshida (Institute for Fermentation, Osaka, Japan). Cells were grown in DMEM with 10% fetal bovine serum and antibiotics (both from Invitrogen, USA). Introduction of the HPV16 E6 gene into A549 cells was accomplished as described previously [14]. Briefly, cells were transfected with pZip-E67B [15] and selected with 400 μg/ml G418 (Invitrogen) to isolate single colonies. These cells also contained either the PG13 or the pWWP luciferase-p53 reporter plasmids [16,17] and were treated with 500 ng/ml adriamycin (Sigma, USA) in order to measure p53 levels under conditions of DNA damage. The control cell line (A549-neo) contained an empty vector with the *neo* marker, but lacked the HPV16 E6 gene. Synchronization at the G2 phase was performed by treating the cells with 2.5 mM aphidicolin (Wako, Japan) for 24 h to briefly synchronize the population in the S phase, followed by administration of 100 ng/ml adriamycin for 15 h, since direct administration of 100 ng/ml to p53-positive A549 cells causes mainly G1 arrest [14]. G2-arrested cells were treated with 2.5 mM caffeine to abrogate the G2 checkpoint in the presence of adriamycin. In order to trap cells at pseudo-metaphase after treatment with caffeine, nocodazole (final concentration at 0.1 μg/ml: Sigma) was added to the culture medium. Butyrolactone I, which is a CDK1 and CDK2 inhibitor, was purchased from Funakoshi (Japan) and added to the culture medium (final concentration at 25 μM). Changes in cell morphology following chemical treatments were monitored by conventional phase-contrast microscopy and photography (Olympus, Japan).

Antibodies and immunological experiments

Rabbit anti-CDK1 serum was used as described [14]. The anti-p53 (DO-1) and anti-p21 monoclonal antibodies (Mabs) were purchased from Santa Cruz Biotechnology (USA) and BD Biosciences (USA), respectively. Anti-hamster MAbs against cyclin A (E23 or E67) and cyclin B1 (V152 and V92) were gifts from J. Gannon (Cancer Research UK, UK). These MAbs recognized human proteins and were used for Western blotting and immunoprecipitation experiments [14]. Rabbit anti-human CDK2 was used as described [18]. The histone H1 kinase assay using an immunoprecipitate is described below, and immunoblotting was carried out according to the method described previously [19]. For Western blotting experiments, horseradish peroxidase-labeled anti-mouse or anti-rabbit secondary antibodies were added, and the signals were detected with SuperSignal West Pico chemiluminescent reagents (Pierce, USA).

Histone H1 kinase assay

For the CDK1 and CDK2 assays, cell extracts were prepared as described previously [19], and the proteins were immunoprecipitated by adding 1 μl of rabbit anti-CDK1 or anti-CDK2 antibody to 200 μl of cell extract that contained 1 μg/μl protein. The kinase activity of the immunoprecipitate was measured and determined as described previously [19].

Cell cycle analysis

To analyze the cell cycle, essentially the same method was used as described [19]. Briefly, cells were collected using trypsin treatment, washed once with PBS, and fixed with 70% methanol. After treatment with 40 μg/ml RNase A (Sigma), the DNA was stained with 40 μg/ml propidium iodide (PI; Wako). The cell cycle stages were analyzed using the EPICS-XL flow cytometer (Beckman-Coulter, USA) and the Multicycler program.

[2] *Abbreviations used:* CDK, cyclin-dependent kinase; DMEM, Dulbecco's modified essential medium; Mabs, monoclonal antibodies; PI, propidium iodide; HPV 16, human papillomavirus type 16; RT-PCR, reverse transcriptase-polymerase chain reaction; SDS, sodium dodecyl sulfate.