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Isolation and characterization of cytoplasmic cyclin D1 mutants

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ABSTRACT

To elucidate the mechanism governing the subcellular distribution of cyclin D1 protein, we randomly mutagenized human cyclin D1 cDNA and isolated mutants that encode the protein predominantly located in the cytoplasm. Experiments with Leptomycin B suggested a defect in transportation from the cytoplasm to the nucleus rather than enhanced nuclear exportation. Sequencing revealed that the mutations responsible for the cytoplasmic localization of cyclin D1 resided in the vicinity of the cyclin box, which affected interaction with a catalytic partner, Cdk4. We propose that interaction between cyclin D1 and Cdk4 triggers the mechanism controlling the nuclear transportation of this kinase complex.

Structured summary:
MINT-7033488: *Cdk4* (uniprotkb:P30285) *physically interacts* (MI:0218) with *Cdk1* (uniprotkb:P25322) by *anti bait coimmunoprecipitation* (MI:0006)
MINT-7033511, MINT-7033534: *Cdk4* (uniprotkb:P30285) *physically interacts* (MI:0218) with *Cyclin D1* (uniprotkb:P24385) by *anti bait coimmunoprecipitation* (MI:0006)

1. Introduction

The first gap (G1) phase of the mammalian cell cycle is an unique period when cells respond to environmental signals to determine cell fate such as proliferation, differentiation, cellular senescence, and survival [1]. Important cell cycle regulators, which govern the progression of the G1 phase, include the D-type cyclin–Cdk4, Cdk6 protein kinase complexes [2]. The expression of three D-type cyclins (D1, D2, and D3) fluctuates during the cell cycle and differs in a tissue-specific manner, while Cdk4 and Cdk6 are stably expressed in a cell cycle-independent manner, indicating that the expression of D-type cyclins is key to the control of cyclin D-dependent kinase complexes. The expression of cyclin D1, the major D-type cyclin in fibroblasts, is largely regulated at the transcriptional level by a group of transcriptional activators and repressors and is triggered by extracellular growth factors, rather than controlled by the intracellular "cell cycle clock" mechanism.

In addition to transcriptional control, cyclin D1 is subjected to additional post-translational regulation associated with its subcellular distribution. With the aid of the Cdk inhibitors p27 and p21 [3], the cyclin D1 in the cytoplasm is bound to Cdk4, and then transported into the nucleus in an importin-dependent manner. The mechanism of the nuclear transportation remains ill defined because neither cyclin D1 nor Cdk4 contains a canonical nuclear localization signal sequence. In the nucleus, the cyclin D1–Cdk4 complex is activated through modification by the Cdk-activating kinase (CAK) [4] and phosphorylates several key substrates, including a molecule critical for G1 progression, a retinoblastoma protein (pRb) [5]. After the entry into S phase, cyclin D1 is phosphorylated by GSK3β, which triggers its association with CRM1 and nuclear exportation [6]. In the cytoplasm, phosphorylated cyclin D1 is ubiquitinated by SCF^{Fbx4} [7], resulting in proteolysis by the proteasome.

The expression of cyclin D1 was unexpectedly observed in many growth-arrested cells, such as cardiomyocytes [8], neurons [9], and hematopoietic stem cells [10]. In such cases, cyclin D1 was frequently detected in the cytoplasm, indicating that the cyclin D1 in these cells is functionally inactivated because the major (and the only physiologically significant) substrates for the cyclin D–Cdk complex are the Rb and Rb-related proteins, which are compartmentalized within the nucleus. Thus, besides transcriptional control, additional ill-defined post-translational mechanisms regulate cyclin D–Cdk complexes in mammalian cells. In this study, we isolated cyclin D1 mutants, which occur in the cytoplasm, from among a randomly mutagenized cyclin D1 cDNA library and characterized their properties in terms of cell cycle regulation and subcellular distribution.

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2. Materials and methods

2.1. Random mutagenesis by low fidelity PCR and generation of the mini-library

cDNA corresponding to the entire coding sequence of human cyclin D1 was amplified by PCR (35 cycles of 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 1 min) in a low fidelity PCR buffer containing 0.5 mM $MnCl_2$, twice as much Taq polymerase (5 units/100 μl), an increased concentration of $MgCl_2$ (final concentration, 7 mM), and extra dCTP and dTTP (final concentration, 1 mM) in addition to the standard PCR mixture [11] using a pair of primers specific to the human cyclin D1 coding sequence (5′-TTC GGA TCC GCC CAT GGA ACA CCA-3′ and 5′-ATC TAT GCG GCC GCT CAG AT-3′). The resulting DNA fragments were cloned into the vector pIRES-puro-CAG-GFP in frame with the green fluorescence protein (GFP). The size of the resulting mini-cyclin D1 library (the number of independent clones) was about 10^4. We characterized plasmids derived from several independent colonies picked randomly from the library, and found that the cloning efficiency (the percentage of clones that contain cDNA) was ca 90% and the average mutation rate was 100%. (All six clones chosen from the mini-library contained 1–10 point mutations that contribute to amino acid substitutions. For the detail, see Ref. [11].)

2.2. Cell culture, high efficiency transfection, and selection of the cyclin D1 mutants

NIH3T3 mouse fibroblasts and 293T human embryonic kidney cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 units/ml of penicillin, and 100 μg/ml of streptomycin (GIBCO/BRL). In some experiments, cells were treated with LMB (2 ng/ml, purified and prepared by Yoshida, see Ref. [12]) for 5 h. Expression vectors were introduced into cells by the modified calcium phosphate–DNA precipitation method [13,14]. The transfected cells were selected in the presence of puromycin (5 μg/ml) for 5 days and the distribution of the GFP-fused mutant cyclin D1 in each independent colony was observed under a fluorescence microscope. The images were quantitatively analyzed using Imagequant software. The single cell-derived colonies were expanded and high molecular weight (HMW) DNA was extracted from each colony using standard procedures. Mutant human cyclin D1 cDNA was amplified by PCR as described above using the HMW DNA as a template and recloned into the pIRES-puro-CAG-GFP vector in frame with GFP. Resulting expression vectors were re-screened as above. We repeated these steps three times and the resulting cDNAs were purified and sequenced by the standard protocol.

2.3. Protein analyses

Cell lysis, immunoprecipitation, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and immunoblotting were performed as described [11,14–16]. Developed films were quantitatively analyzed with a densitograph (ATTO, Japan).

2.4. Antibodies

Rabbit polyclonal antibodies to GFP and p21 were generated using bacterially produced polypeptides in our laboratory. Rabbit polyclonal antibodies to Cdk4 were provided by Dr. Charles J. Sherr. Rabbit polyclonal antibodies against p27 (C-19), Cdk2 (M2), Cdk4 (C-22), and Grb2 (C-23) and mouse monoclonal antibody to cyclin D1 (72-13G) were purchased from Santa Cruz Biotechnology. Mouse monoclonal antibodies to γ-tubulin (GTU-88) and an HA epitope (clone 12CA5) were obtained from Sigma and Roche Applied Science, respectively.

Further details on cell cycle analysis and immunofluorescence staining are provided as Supplementary data.

3. Results

3.1. Isolation of the cyclin D1 mutants

We randomly mutagenized the entire coding sequence of human cyclin D1 cDNA by use of the low fidelity PCR method [11] and generated the cyclin D1 mutant library in the pIRES-puro-CAG-GFP vector, which allows the expression of GFP-fused cyclin D1 mutants in mammalian cells under the control of a strong (CAG) promoter together with the puromycin resistance gene from the same transcript owing to the IRES sequence. To isolate the cyclin D1 mutant by expression screening, we introduced the mutant cDNA library into the mammalian cell lines, NIH3T3 and HEK293T. Because the abrupt, strong expression of the wild-type (functional) cyclin D1 protein in mammalian cells inhibits proliferation [17], we expected cells expressing mutant cyclin D1 to have a growth

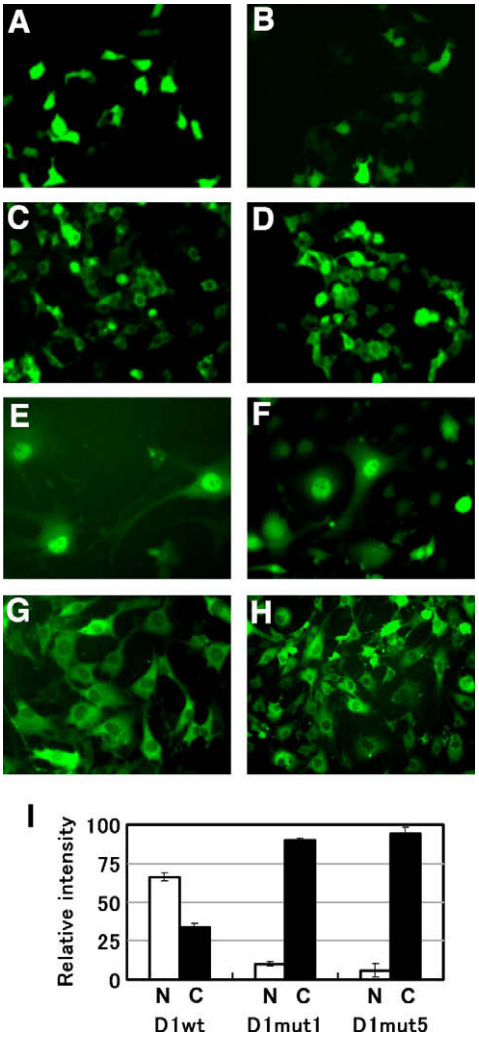

Fig. 1. Cytoplasmic distribution of the cyclin D1 mutants. HEK293T (A–D) and NIH3T3 (E–H) cells were transfected with the pIRES-puro-CAG-GFP expression vectors containing wild-type (A, B, E, and F) and mutant (mut 1 for C and G, and mut 5 for D and H) cyclin D1 cDNAs, and selected in puromycin for 1 week. GFP-positive colonies were photographed under the fluorescence microscope. (I) The nuclear (N) and cytoplasmic (C) signals were quantified and calculated as the percentages of the total signal.

advantage over those expressing the wild-type protein. After selection in the presence of puromycin, a number of colonies expressing the GFP signal were obtained. Among these, we chose those showing the dominantly cytoplasmic GFP signal (data not shown, but see Fig. 1C and D for example). After expansion of the cells, HMW DNA was extracted from individual candidate clones, and the mutant human cyclin D1 cDNA was amplified by PCR, subcloned into the same vector, introduced into 293T cells, and selected by observation of the cytoplasmic GFP signal.

From three rounds of transfection, we eventually established several independent 293T-derived cell lines harboring a cytoplasmic GFP signal, from which we successfully isolated six cyclin D1 mutant cDNAs (designated mut 1–6, see Fig. 2A). Sequencing revealed that each harbored 10–15 missense point mutations (Fig. 2A). For further analysis, we chose two mutant cDNAs, mut 1 and mut 5, which exhibited distinctive cytoplasmic signals in both 293T (Fig. 1C and D, respectively) and NIH3T3 (Fig. 1G and H, respectively) cells compared to cells transfected with wild-type cyclin D1 fused with GFP (Fig. 1A, B, E, and F).

3.2. Cytoplasmic cyclin D1 mutants harbor mutations around the cyclin box domain

To narrow down the site of the mutations responsible for the cytoplasmic localization, we separated cyclin D1 cDNA into three pieces by digestion with Pst I and Stu I, and swapped each piece between the wild-type and mutant cDNAs (Fig. 2B and C). We then reconstituted the entire coding sequence in the pIRES-puro-CAG-GFP vector, transfected it onto 293T and NIH3T3 cells, and observed the cells under the fluorescence microscope. For both mut 1 and mut 5, the mutations responsible for cyclin D1's cytoplasmic localization resided between the Pst I and Stu I sites. In the case of

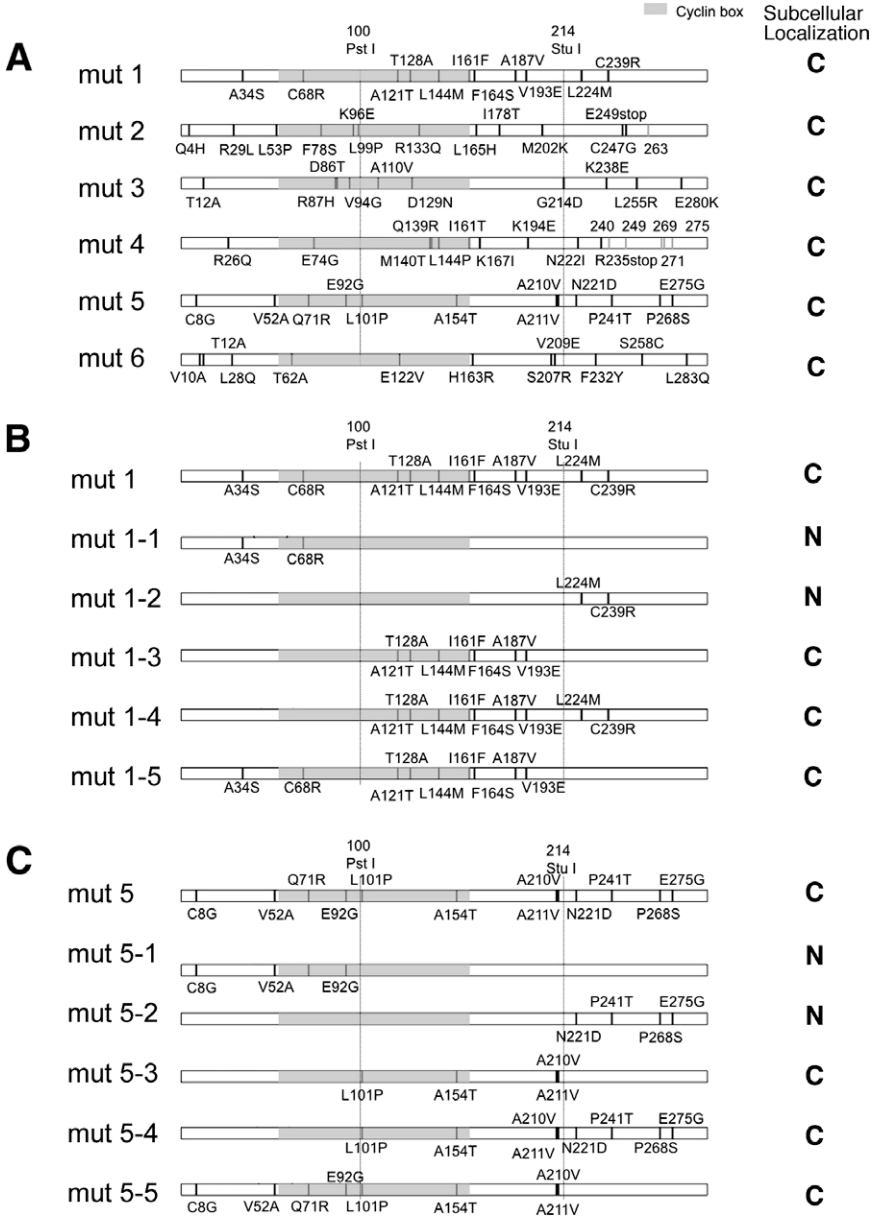

Fig. 2. Summary of the cytoplasmic cyclin D1 mutants. Positions of the amino acid substitution in each cytoplasmic cyclin D1 mutant are shown. The subcellular distribution is summarized on the right of each panel. The cyclin box is shaded with grey. (A) Six independently isolated cytoplasmic cyclin D1 mutants (mut 1–6). (B) Cytoplasmic mutant mut 1 and its derivatives (mut 1–1 to 1–5). (C) Cytoplasmic mutant mut 5 and its derivatives (mut 5–1 to 5–5).

mut 5, we found 4 amino acid substitutions in this region (summarized in the right margin of Fig. 2, and also see Fig. 3 for the mutants 1–3 and 5–3).

To further pinpoint the mutation responsible in mut 5–3, we generated three independent mutants (L101P, A154T, and A210/211V) harboring a single point mutation (in the case of 210/211, two tandem mutations). However, none of the three mutants showed a cytoplasmic localization (negative data not shown), indicating that two or more point mutations function synergistically. Furthermore, considering that none of the amino acid substitutions were shared between the mut 1 and mut 5 mutants, we speculate that conformational change rather than a single amino acid substitution in this region is important to disrupt the nuclear accumulation of the cyclin D1 protein. Therefore, for further analysis, we used mutant mut 1–3 and 5–3, both of which exhibited a clear cytoplasmic localization (see Fig. 3A and B). The cytoplasmic localization of the mutants is not due to the fusion with GFP, because we detected HA-tagged mutant (mut 1–3) protein in the cytoplasm, while wild-type cyclin D1 was predominantly found in the nucleus (Fig. 3C).

3.3. The cytoplasmic distribution of the cyclin D1 mutants is not dependent on nuclear export

During the normal cell cycle, cyclin D1 is excluded from the nucleus after entry into S phase by the nuclear export mechanism. However, cell cycle analysis of the stable transfectants showed that the expression of mutant cyclin D1 had little effect on the cell cycle progression (negative data not shown). Therefore, it is not due to an increase in the number of cells in the S phase that mutant cyclin D1 occurs in the cytoplasm.

To investigate further with a more direct approach, we treated the stable transfectants with an inhibitor of CRM1-dependent nuclear export, leptomycin B (LMB) (Fig. 4). GFP-tagged wild-type cyclin D1 distributed mainly largely in the nucleus though some was detected in the cytoplasm. LMB treatment diminished the cytoplasmic but not nuclear signals. In contrast, GFP-mutant cyclin D1 (mut 1–3) was located in the cytoplasm in the absence and presence of LMB, indicating inefficient nuclear transport rather than enhanced nuclear export.

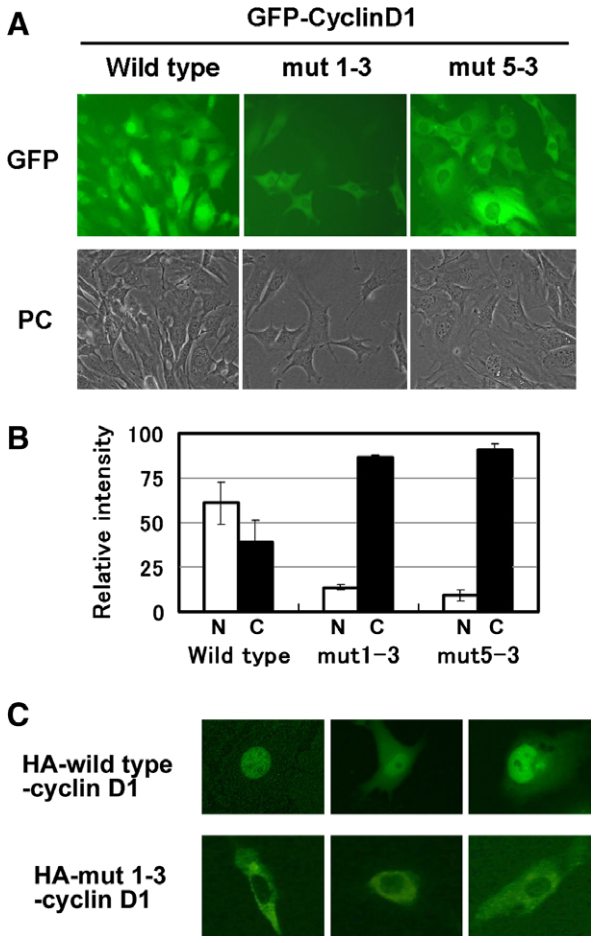

Fig. 3. Subcellular distribution of wild-type and cytoplasmic mutant cyclin D1. (A) NIH3T3 cells were transfected with the pIRES-puro-CAG-GFP expression vectors containing wild-type and mutant (mut 1–3 and 5–3) cyclin D1 cDNAs. After selection in puromycin, cells stably expressing GFP-cyclin D1 were isolated and photographed under the fluorescence microscope. (B) The nuclear (N) and cytoplasmic (C) signals in panel A were quantified and calculated as the percentages of the total signal. (C) NIH3T3 cells were transfected with the expression vectors encoding HA-tagged wild-type (upper panels) and mutant (mut 1–3, lower panels) cyclin D1 cDNAs. Cells were fixed, stained with antibody to an HA tag and FITC-labeled secondary antibody, and photographed under the fluorescent microscopy. Three independent cells from each transfectant are shown.

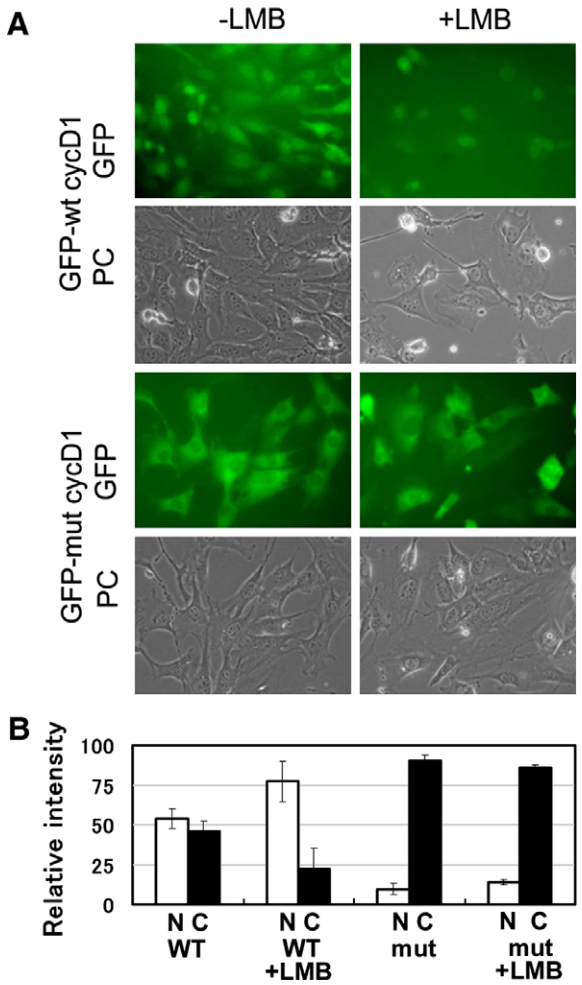

Fig. 4. The cytoplasmic distribution of the cyclin D1 mutant is resistant to LMB. (A) NIH3T3 cells stably expressing GFP-cyclin D1 (wild-type and cytoplasmic mutant, mut 1–3) were cultured in the presence (+LMB) and absence (−LMB) of LMB and photographed under the fluorescence microscope. (B) The nuclear (N) and cytoplasmic (C) signals in panel A were quantified and calculated as the percentages of the total signal.

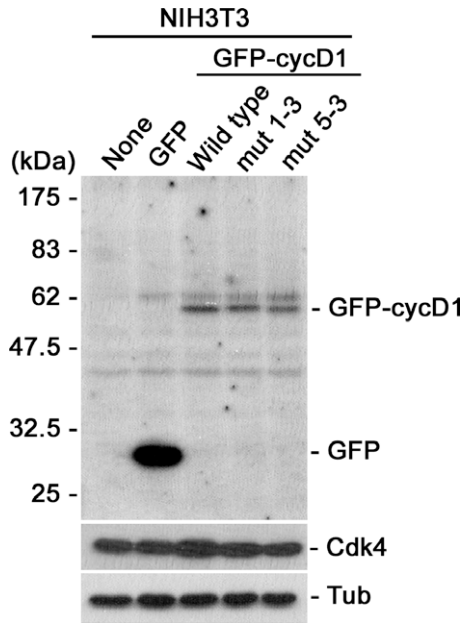

Fig. 5. Stable expression of wild-type and mutant cyclin D1 proteins. The lysates isolated from NIH3T3 cells stably expressing GFP and GFP-cyclin D1 (wild-type and cytoplasmic mutants, mut 1–3 and 5–3) were analyzed by immunoblotting with antibodies specifically recognizing GFP, Cdk4, and γ-tubulin (upper, middle, and lower panels, respectively).

3.4. Cytoplasmic cyclin D1 mutants are inefficient in forming a complex with the catalytic partner, Cdk4

Because the cytoplasmic cyclin D1 mutants harbored missense mutations in the vicinity of the cyclin box, we investigated the possibility that the mutants were unable to interact with the catalytic partner protein Cdk4. First, the proliferating NIH3T3 cells mock-transfected or transfected with GFP, GFP-wild–type cyclin D1, and GFP-mutant cyclin D1 (mut 1-3 and 5-3) were harvested and analyzed by immunoblotting using antibodies to GFP. Fig. 5 shows that the GFP-tagged proteins were expressed less extensively than the parental GFP protein but levels of wild-type and mutant cyclin D1s were almost the same, and we observed almost undetectable levels of degradation intermediates. These results indicate that the GFP signals observed under the fluorescence microscope reflected the full-length GFP-fused proteins and that the expression of the cyclin D1 mutants was stably maintained in the proliferating fibroblasts.

Next, we immunoprecipitated Cdk4 protein from lysates isolated from the proliferating NIH3T3 transfectants and performed immunoblot analyses using antibodies to cyclin D1 and GFP. Because our antibody to cyclin D1 is specific to the mouse protein, and the GFP-fused protein is of human origin, the endogenous and ectopically expressed proteins can be distinguished using these two antibodies [18]. In addition, we used two types of anti-Cdk4 antibodies, Ry and Rz, the former recognizing all types of Cdk4 protein while the latter recognizing only the active form of Cdk4 [18].

Fig. 6 shows that the proliferating NIH3T3 cells contained an active cyclin D1–Cdk4 complex (lane 4) and the amount of the complex increased with the ectopic expression of GFP-wild-type cyclin D1 (lane 8). Because the amount of endogenous cyclin D1 bound to Cdk4 did not decrease (lanes 7 and 8) although the ectopic GFP-cyclin D1 was incorporated in the complex with Cdk4 (lanes 7 and 8), we speculated that the level of cyclin D1 is the rate limiting factor in the proliferating NIH3T3 cells and unbound Cdk4 is pooled in these cells.

In contrast, little mutant cyclin D1 was found in a complex with Cdk4 (lanes 11, 12, 15, and 16). The endogenous cyclin D1–Cdk4 complex remained unchanged (lanes 11, 12, 15, and 16). Thus, we conclude that the cytoplasmic cyclin D1 mutants were unable to associate with the catalytic partner Cdk4 in vivo.

4. Discussion

In this study, we isolated mutant cDNA encoding cyclin D1 predominantly located in the cytoplasm from a pool of randomly mutagenized human cyclin D1 cDNAs. Mutant cDNAs contain amino acid substitutions in the vicinity of the so-called "cyclin box", thereby preventing interaction with the catalytic partner Cdk4. We failed to obtain the mutant with a single amino acid substitution. This could be because cyclin D binds to its catalytic partner Cdk using more than one interaction surface, or alternatively, and most likely, we need to enlarge the size of the mutant library to select out the single point mutant. Mutations in other areas did not affect the subcellular distribution. In addition, the cytoplasmic protein was resistant to the nuclear export inhibitor LMB. Therefore, we concluded that the cyclin box is important for the nuclear import of cyclin D1, presumably by forming a complex with Cdk4.

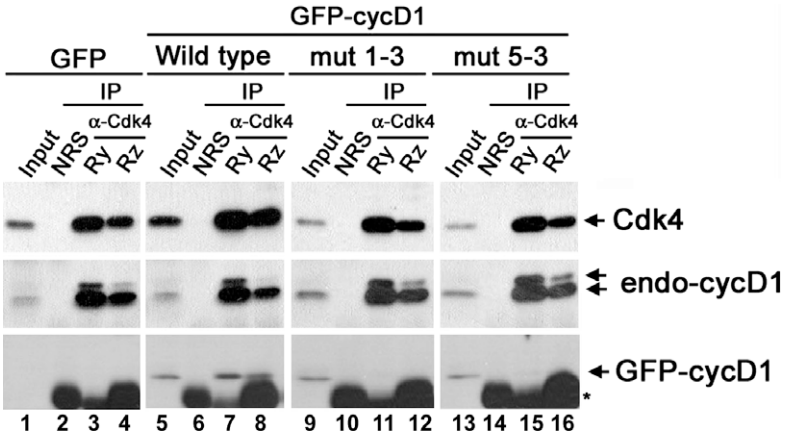

Fig. 6. Cytoplasmic cyclin D1 mutant proteins do not efficiently associate with Cdk4 in proliferating cells. Endogenous mouse Cdk4 was immunoprecipitated with antibodies against Cdk4 (Ry and Rz) from lysates isolated from NIH3T3 cells stably expressing GFP and GFP-cyclin D1 (wild-type and cytoplasmic mutants, mut 1–3 and 5–3). The immunoprecipitates were analyzed by immunoblotting with antibodies specifically recognizing Cdk4, mouse cyclin D1 (endo-cycD1), and GFP (upper, middle, and lower panels, respectively). NRS; normal rabbit serum (negative control). The asterisk indicates the position of the immunoglobulin heavy chain.

Although most experiments were performed in the mouse fibroblasts with human cDNA, we obtained the same results in human 293T cells, suggesting that the conclusions are common between species.

In addition to Cdk4, Cdk6 is also a catalytic partner of cyclin D [2]. The choice of partner remains poorly understood. Furthermore, cyclin D1 is known to form a complex with Cdk2 and Cdk5 [19]. In such cases, cyclin D1 is incapable of activating bound Cdks (Cdk2 and Cdk5) [5,20] so the physiological significance of this interaction remains to be elucidated. Considering our results, the interaction of inactive Cdk subunits may be required for cyclin Ds to enter the nucleus, where they may play a role other than in phosphorylating their substrates, the Rb family [5,21].

In the case of post-mitotic cardiomyocytes [8] and neurons [9], cyclin D1 was found to be associated with Cdk4 in the cytoplasm, showing that the formation of a complex with its catalytic partner may be required but is not sufficient for transportation into the nucleus. Thus, in addition to nuclear export, the mechanism governing the selective nuclear import of cyclin D1 needs to be elucidated, which, in turn, may provide a clue as to how the state of mammalian cells (most differentiated cells and stem cells in the adult body) is controlled.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.04.036.

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