Ability of human CDC25B phosphatase splice variants to replace the function of the fission yeast Cdc25 cell cycle regulator

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Abstract

CDC25 phosphatases are essential and evolutionary-conserved actors of the eukaryotic cell cycle control. To examine and compare the properties of three splicing variants of human CDC25B, recombinant fission yeast strains expressing the human proteins in place of the endogenous Cdc25 were generated and characterized. We report, that the three CDC25B variants: (i) efficiently replace the yeast counterpart in vegetative growth, (ii) partly restore the γ and UV radiation DNA damage-activated checkpoint, (iii) fail to restore the DNA replication checkpoint activated by hydroxyurea. Although these yeast strains do not reveal the specific functions of the human CDC25B variants, they should provide useful screening tools for the identification of new cell cycle regulators and pharmacological inhibitors of CDC25 phosphatase.

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1. Introduction

CDC25 phosphatases are essential actors of the eukaryotic cell cycle control. They dephosphorylate and activate cyclin-dependent kinases which, in association with their cyclin regulatory subunits, are responsible for progression at the key transitions of the cell cycle. For instance, from yeast to mammals, the CDK1 kinase, also known as CDC2, associated with cyclin B is responsible for the phosphorylation of a number of substrates, thus setting up the architectural and biochemical events required for entry into the mitotic process [1]. CDK1 is kept inactive during interphase through WEE1 kinase-dependent phosphorylation on tyrosine 15. CDC25-dependent dephosphorylation is the critical event that triggers the activation of CDK1 at mitosis [1].

There is only one Cdc25 protein in fission yeast [2] that acts as a dose-dependent inducer of mitosis. In contrast, there are three genes encoding CDC25 phosphatases in human cells [3]. CDC25A was initially thought to be involved in the control of the activity of the CDK2-cyclin E complex at the G1/S transition, but has also recently been proposed to play a role at mitosis [4]. CDC25C is the main activator of the CDK1-cyclin B complex at mitosis (see [3] for review), but has also been reported to be active in S-phase [5]. CDC25B accumulates in the cells from S-phase to mitosis [6,7] and has been proposed to be involved in the progression of the cell in S-phase [8] and in the control of the activation of the early centrosomal events leading to entry into mitosis [6].

CDC25 phosphatases are highly-regulated enzymes and phosphorylation of their amino-terminal regulatory
domains by a number of kinases, including the CDK-cyclin complexes themselves, has been reported to modulate their catalytic activity, their localisation, and/or their interactions with regulatory partners. This is indeed true for CDC25B which has been shown to be phosphorylated by various kinases, including protein kinase CK2 [9], CDK-cyclins [10,11], Eg3 kinase [12], PKB/Akt [13] and Aurora-A [14].

A least three CDC25B splice variants have been identified in human cells [7,15]. They differ by the presence or the absence in their amino-terminal regulatory halves of short, 14- and 42-residue peptides that are encoded by alternately spliced exons. The significance and role of this splicing mechanism in the control of the biological activity of CDC25B phosphatase remains unclear. Nonetheless, experimental evidence indicates that these variants are differentially regulated, both in various cell types and during the cell cycle, and hence may participate in specific biological functions [7]. The CDC25B variants differ in their ability to complement upon epistomal expression a cdc25 thermosensitive allele in fission yeast [7], CDC25B2 being a more effective inducer of mitosis than CDC25B3 or CDC25B1. Serine 146, whose phosphorylation by CDK1-cyclin B is essential for mitosis than CDC25B3 or CDC25B1. Serine 146, whose phosphorylation by CDK1-cyclin B is essential for mitosis-inducing activity associated with nuclear retention is not present in the CDC25B2 variant [11]. Finally, overexpression of CDC25B has been observed in a number of cancers (see [16] for review) and, intriguingly, expression of the B2 variant has specifically been shown to be associated with high-grade tumours and poor prognosis [16,17].

Fission yeast has already proven to be a convenient model for the dissection of the function and regulation of several human cell cycle regulatory proteins [18–20]. However, these studies were based on the episomal expression of human proteins at non-physiological levels. We therefore, have developed a strategy of expression at physiological levels, based on the replacement of the yeast coding frame by its human counterpart. This strategy has successfully been used to study the ability of the human CDC25C and CDC25A phosphatases to restore a functional DNA checkpoint [21,22]. These fragments were cloned to flank the ORF of human CDC25B variants leading to the plasmid HsCDC25B1, 2 and 3.

2.2. Strains

Strain cdc25::ura4+ wee1-50 leu1-32 ura4-D18, h− was co-transformed with the BglIII–HindIII fragment of HspCDC25B1-3 and pIRT2 by electroporation and plated out on minimal medium supplemented with adenosine and uracil. Ura-clones were then selected using the 5-fluoro-orotic acid test, and the gene replacement checked by PCR. Selected clones were crossed back to a wild-type strain, to eliminate the wee1 thermosensitive allele.

2.3. DNA damage assays

500 Exponentially growing cells were plated on YES agar plates (yeast extract plus supplements, BIO 101, Carlsbad, CA, USA), then exposed to increasing doses of γ irradiation (60Co source, Shepherd, 2500Ci, 8Gy min−1) (CERT-ONERA, Toulouse, France) or to increasing doses of UV (Crosslinker, Appligen, Paris, France). Colonies were scored after 3–5 d at 30 °C. To examine sensitivity to hydroxyurea, exponentially-growing cells were spotted on YES agar plates supplemented with 0, 1, 3, or 6 mM hydroxyurea (HU) (Sigma H8627). Growth was observed after 3 d. Monitoring of the production of ‘cut’ phenotype was performed after addition of 12 mM HU to the liquid culture.

2.4. Genotypes of strains used in this study

Genotypes are indicated in parentheses. SP200; Wild type (leu1-32 ura4 D18), SP867 (wee1-50 cdc25::ura ade6-210 leu1-32 ura4-D18), T394; Δchk1 Δcds1 (chk1::ura4 cds1::ura4 leu1-32 ura4-D18), T401; Δedc25 (cdc2-3W cdc25::ura4 leu1-32 ura4-D18), T459 CDC25B1, T460 CDC25B2 and T461 CDC25B3.

2.5. Western-blot analyses

Total protein extracts were prepared according to [23] with the addition of Complete Protease Inhibitor Cocktail tablets (Roche), 1 mM sodium orthovanadate, 10 mM β-glycerophosphate and 1 mM DTT to the extraction buffer. 20 μg of total protein extract were run on a 4–12% NuPAGE Bis-Tris gel (Invitrogen), transferred to nitrocellulose and probed with rabbit anti-CDC25B (C20, Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:1500). Goat anti-rabbit conjugated to hors eradish peroxidase (HRP; Cell Signaling, TWC Biosearch, Hong Kong, China) (1:5000) was used as secondary antibody. Mouse anti-Actin (Chemicon, Temecula, CA, USA) (1:10000) monoclonal antibody

2. Materials and methods

2.1. Strains construction

The Schizosaccharomyces pombe cdc25 promoter and terminator were amplified by PCR and assembled as already described [21,22].
and HRP-conjugated horse anti-mouse antibody (HRP; Cell Signaling) (1:10000) as secondary antibody were used to detect Actin as loading control. Immunoblots were developed using the Western Lightning Chemiluminescence Reagent kit (Perkin–Elmer Life Sciences).

3. Results and discussion

3.1. Construction of fission yeast strains expressing human CDC25B splice variants

Episomal expression of the CDC25B variants is unlikely to accurately indicate their respective abilities to replace effectively the different functions of Cdc25 in fission yeast. To overcome this, we constructed three strains expressing the CDC25B1, CDC25B2 and CDC25B3 proteins in place of the S. pombe Cdc25B by replacement of the genomic sequence. A nullizigous strain for CDC25B3 was placed under the control of the S. pombe wee1 promoter and at physiological levels of expression. This was achieved, as previously reported for both CDC25B3 [21] and CDC25A [22], by replacement of the genomic sequence of fission yeast cdc25 by the three human CDC25B open reading frames. In order to enhance the targeting efficiency, the human CDC25B cDNAs were flanked by 5' non-coding regions corresponding to the pombe cdc25 genomic sequence. A nullizigous strain for cdc25 expressing a thermosensitive allele of wee1 was used as the parental strain as it can grow at restrictive temperature in the absence of both wee1 and cdc25; the correct integration restored viability at permissive temperature for wee1 and the ability to grow on 5-FOA containing medium by reversion of the ura prototrophy. Integration of the CDC25B open reading frames at the correct locus was checked by PCR on genomic DNA of the constructed strains and the cells were crossed to wild type to eliminate the wee1 thermosensitive allele. As depicted in Fig. 1, we obtained a set of three fission yeast strains in which the expression of the human CDC25B splice variants (B1, B2 and B3) was placed under the control of the S. pombe cdc25 promoter.

Cells lacking cdc25 are not viable and cdc25 loss of function results in an elongated cdc-phenotype [2]. All three humanized strains being viable, we can conclude that CDC25B1, B2 and B3 are all able to complement the essential features of their S. pombe homologue. Since, as reported previously (see introduction) episomal overexpression CDC25B2 was found to be largely more active than CDC25B1 and CDC25B3, we next investigated in detail the ability of these three proteins to fully replace fission yeast cdc25 when expressed at physiological levels.

3.2. Characterisation of the CDC25B variant humanized strains

As shown in Fig. 1(B), microscopic examination of the cells together with DAPI staining of the nucleus revealed that the CDC25B1 humanized cells displayed a slightly elongated phenotype. The average cell size of CDC25B1 was significantly larger than CDC25B2, CDC25B3 and wild-type strains (18.5, 12.8, 11.3 and 12.4 μm, respectively). This difference in cell size was not dependent on the expression level (Fig. 2), as the three CDC25B proteins were similarly detected by Western blot in total protein extracts from these three strains using an antibody specific to human CDC25B. Furthermore, the generation time of the CDC25B1-expressing strain was found to be slightly larger than that of the wild type strain and to correlate with the observed differences in size (Fig. 1(B)). Finally, analyses of the cell cycle distribution by flow cytometry after propidium iodide staining of DNA indicated that, as for wild type, the humanised cells were predominantly in the G2 phase (not shown).

These observations confirmed that all three variants of human CDC25B were able to replace fission yeast cdc25 phosphatase in cell cycle control during vegetative growth, however with unequal efficiency. CDC25B1 seems to be the least efficient variant, as was already proposed on the basis of overexpression experiments [7].

To further investigate the properties of the three splice variants, we next examined the sensitivity of the CDC25B humanized strains in their response to the activation of the DNA damage checkpoint after exposure to ionizing radiation (IR), to UV radiation and to hydroxyurea (HU) treatment.

3.3. CDC25B splice variants partially replace S. pombe cdc25 in the response to UV irradiation

We first examined and compared the ability of the CDC25B variants to restore the integrity of the checkpoint activated in response to UV irradiation. A wild-type strain and nullizigous strains for both chk1 and cds1 were used as controls in this experiment. As shown in Fig. 3(A) and as already reported [24–26], a checkpoint-deficient strain was unable to survive exposure to a dose of UV radiations of 50 J m⁻². Survival of the three CDC25B splice variants expressing strains to increasing doses of UV radiation was similar to that of the wild type for radiation exposure up to 100 J m⁻². At 200 J m⁻² the CDC25B3 strain was slightly more radiosensitive, although CDC25B1 and B2 remained as efficient as wild-type pombe Cdc25. These results indicate that the function of Cdc25 in the control of the cell cycle control upon UV-induced DNA damage is either efficiently rescued by human CDC25B proteins or, alternately, is not strongly dependent on the integrity of the CDC25 pathway.

3.4. CDC25B variants do not efficiently replace S. pombe cdc25 in the response to γ radiations

To monitor the radiosensitivity of the CDC25B humanised strains, a known number of exponentially-

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growing cells were plated onto YES agar plates at 30 °C, then exposed to increasing doses of γ irradiation (0–500 Gy). Colonies were scored after 3 d of incubation at 30 °C. Wild-type and Δchk1Δcds1 cells were used as positive and negative control, respectively. As shown in Fig. 3(B), checkpoint kinase-deficient cells partly survived exposure to 100 Gy and were unable to survive 200 Gy, whilst in contrast 100% of the wild-type cells survived exposure up to 400 Gy. Cells expressing CDC25B variants similarly survived γ radiation exposure, displaying a slightly enhanced radiosensitivity compared to wild-type cells. These results indicated that the ability of CDC25B variants to recapitulate the G2 checkpoint, after γ irradiation-induced DNA damage, was similar although less efficient than that of the wild type.

3.5. CDC25B inefficiently replaced S. pombe cdc25 in the checkpoint response to hydroxyurea

Finally, we investigated the ability of human CDC25Bs to restore an efficient DNA replication checkpoint in response to hydroxyurea treatment. A given number of cells was spotted onto plates containing increasing concentrations of hydroxyurea. As shown in Fig. 4, wild-type cells were able to grow on plates containing up to 6 mM HU. In similar conditions, checkpoint-deficient cells do not grow even at the lowest HU concentration [21]. CDC25B-expressing cells did not efficiently grow on 3 mM HU. However, CDC25B1-expressing cells were still able to form a few colonies up to 6 mM HU, indicating a slightly higher

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Fig. 1. Characterisation of fission yeast strains expressing human CDC25B splicing variants. (A) Scheme of the construction of the human CDC25B1, B2 and B3 expressing strains. (B) Micrographs of the strains expressing human CDC25B splice variants in place of fission yeast cdc25 after DAPI staining and differential interference contrast (DIC). The generation time (hours ±SD) and the average cell size (±SD) of wild-type and humanised cells are indicated. Size bar: 5 μm.
efficiency in response to the activation of the replication checkpoint.

We also examined the efficiency of these cells to arrest the cell cycle when DNA replication was impaired, by monitoring the percentage of cells undergoing aberrant mitosis and displaying an anucleate or 'cut' phenotype. Cells were grown in liquid medium containing 12 mM HU for 8 h. As shown in Fig. 5(A), wild-type cells elongated and arrested their cell cycle while checkpoint kinase-deficient cells displayed a typical 'cut' phenotype with abnormal nuclear morphology and uneven segregation of nuclear material between daughter cells. CDC25B1, B2 and B3 displayed an unexpected elongated phenotype reminiscent of a cell cycle arrest, however nuclear abnormalities and/or 'cut' phenotype were observed in almost all of the divided cells. As shown in Fig. 5(B), monitoring the accumulation of the 'cut' phenotype after HU treatment indicates that CDC25B-expressing cells are unable to correctly arrest cell cycle progression upon replication checkpoint activation, but that this effect is delayed as it is only observed after 8 h of HU exposure. In contrast, checkpoint kinase-deficient cells already display a very high level of 'cut' phenotype after 4 h of treatment.

Fig. 2. Expression level of CDC25B in the CDC25B humanised strain. Western blot of total lysate from wild-type and human CDC25B1-3 expressing strains using antibodies against the human CDC25B protein. The bottom part of the gel was incubated with an anti-actin antibody as loading control. Molecular weight markers are indicated (kDa).

Fig. 3. CDC25B variants partially restore checkpoint responses to UV and ionizing radiation (IR). (A, B) Wild type, CDC25B variant humanised cells and a checkpoint deficient strain (∆chk1∆cds1) were used to monitor UV and IR sensitivity. (A) UV response: plated cells were exposed to increasing doses of UV radiation (0, 50, 100 and 200 J m⁻²), then grown at 30°C. Survival was determined by colony scoring after 5 d. Results were reproducible and are expressed as percentage survival and are the average of a least three independent experiments. (B) IR response: plated cells were exposed to increasing doses of γ-irradiation (0–400 Gy), then grown at 30°C. Radiation survival was determined by colony scoring after 3 d and is expressed as percentage survival for various IR doses. The results are reproducible and expressed as the average of a least 3 independent experiments.

Fig. 4. CDC25B variants do not restore checkpoint response to hydroxyurea Dilutions of wild-type and CDC25B strains were spotted on YES medium supplemented with increasing concentration of hydroxyurea (0, 1, 3, 6 mM) and incubated for 4 d at 30°C.
4. Conclusion

In conclusion, we have shown here that CDC25B splicing variants are all able to support vegetative growth of fission yeast cells and to function in the UV- and ionizing-radiation-activated cell cycle-checkpoint. The efficiency of this activity is somewhat lower than for wild-type fission yeast cdc25 and there are subtle differences between variants. It is clear that CDC25B1 is the least efficient in providing the mitotic activity of Cdc25, as was suggested previously [7].

Among the human CDC25 family members, it appears that CDC25B and CDC25A are less efficient than CDC25C in replacing fission yeast Cdc25. In particular, CDC25C is able to restore a functional DNA replication checkpoint activated by HU, while CDC25A and CDC25B variants are not. The significance of these findings remains unclear and a relation with the putative role of these phosphatases in human cells is difficult to establish.

The experiments reported here were aimed at providing an alternative approach for the investigation of the involvement of the human CDC25B protein phosphatases in cell cycle control and in checkpoint control after DNA damage. The outcome of this study suggests that the evolutionary conservation of CDC25 family members and their regulators is probably not sufficient to achieve that goal. However, these strains represent powerful tools that might be used to isolate new regulators and actors of the cell cycle control. Finally, as already proposed with CDC5A-humanised cells [22], these strains will be of major interest for the screening and for selectivity studies of new CDC25 pharmacological inhibitors.

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