FEBS 16585 FEBS Letters 379 (1996) 217–221

Characterisation of human cdc2 lysine 33 mutations expressed in the fission yeast *Schizosaccharomyces pombe*

Dorothée Leroy[a], Catherine Birck[b], Paolo Brambilla[c], Jean-Pierre Samama[b], Bernard Ducommun[a,*]

[a]*Laboratoire de Pharmacologie et de Toxicologie Fondamentales du CNRS, Cell cycle group, Université Paul Sabatier, 205 route de Narbonne, 31077 Toulouse, France*
[b]*Structural biology group, Université Paul Sabatier, 205 route de Narbonne, 31077 Toulouse, France*
[c]*USSL 63, Ospedale di Desio, 20033 Desio, Italy*

Received 7 November 1995

Abstract The mammalian p34^{cdc2} protein kinase, a universal cell cycle regulator, complements *cdc2/CDC28* temperature-sensitive mutations in yeasts. We report the biochemical characterisation of two substitutions of human cdc2 at lysine 33, a residue involved in nucleotide binding, that differently alter the fission yeast cell cycle. K33A-hscdc2 and K33R-hscdc2 mutants are both catalytically inactive, but overexpression of K33R-cdc2 is lethal while K33A-cdc2 is not. We show that human K33R-cdc2 acts as a dominant negative allele that associates yeast cdc13/cyclinB and therefore renders endogeneous *Schizosaccharomyces pombe* cdc2 unactivatable. These results are discussed on the light of the molecular modeling of the mutants in the cdc2 model structure.

Key words: Cyclin-dependent kinase; cdc2; Cyclin; *Schizosaccharomyces pombe*; Fission yeast

1. Introduction

The cdc2 protein kinase is the prototype of an expanding family of enzymes called cyclin-dependent kinases (cdk) that play essential regulatory functions at the major control points of the cell cycle. Originally identified in yeasts as an essential gene required for both G1/S and G2/M transitions, cdc2/CDC28 is present in every eukaryotic organism examined [1]. In vertebrates, the cdc2 protein kinase is involved in the regulation of entry into mitosis, whereas the control of cell proliferation at G1/S is regulated by the related cyclin-dependent kinases. The periodic activation of cyclin-dependent kinases is tightly regulated by at least four highly conserved biochemical mechanisms (reviewed in [2]). Binding to the cyclin regulatory subunit is required for the activation of the catalytic subunit. However, tight interaction and complete activation requires phosphorylation on a conserved threonine residue (T161 in human) [2,3]. Activation of cdc2 is also coincident with dephosphorylation on tyrosine 15, a residue located within the nucleotide binding site. Finally, inhibitors of cyclin-dependent kinases have also recently been identified and are likely to play essential regulatory functions in the control of cell proliferation [4]. Mutational analysis of cdc2 is providing some important clues toward the understanding of the molecular basis of cyclin interaction with cdk [5–8] and a number of residues implicated in that interaction have been identified [6,9]. Their involvement in the regulation of cdc2 activity has been strenghtened from the modeling of cdc2 based on the structure of cAMP dependent protein kinase [6]. Comparison of the X-ray structures of free cdk2 [10] and of the cyclinA-bound cdk2 complex [11] showed the large conformational changes and the realigning of the active site residues that occurs in the kinase upon cyclin A binding. Here, we report the differential effects of overexpression of human cdc2 lysine 33 mutants on the fission yeast cell cycle. We discuss these data in the context of their predicted effects on cdc2 structure and function.

2. Materials and methods

2.1. Yeast strains, cultures, microscopy and flow cytometry

The *Schizosaccharomyces pombe* strain used in this study is SP199 (h$^+$ leu1-32 ura4 ade210). Cells were cultured and transformed as described [12] with pREP41 plasmids [13] encoding the wild type or the mutant human cdc2 sequences. The promoter was kept under repressed conditions by addition of 4 μM thiamine to the growth medium and derepressed as described [13–15]. Cytological observations were performed as described [16]. Flow cytometry was carried out on a Facscan (Becton Dickinson) following the protocol described in [16].

2.2. Mutagenesis

Oligonucleotide-directed mutagenesis of the human cdc2 coding sequence was performed as described [17]. Mutations were screened and checked by DNA sequencing.

2.3. Kinase assays

Yeast cell extracts and immunoprecipitations were performed exactly as described [12]. Polyclonal antibodies raised against human cdc2 carboxy-terminal sequence (G6), recombinant cdc13/cyclin B (a gift from J. Hyams) and a monoclonal anti-human cdc2 (a gift from J. Lukas) were used. The kinase assay reactions were carried out in the presence of 2 μg histone H1 (Boehringer Mannheim), 10 μM cold ATP and 2.5 μCi of [γ-^{32}P]ATP for 5 min at 30°C. Histone H1 phosphorylation was quantified after electrophoresis, excision from the gel and counting.

2.4. Immunoblot analyses

Cell lysates or immunoprecipitates were electrophoresed, transfered onto Hybond C membrane as described [18]. Immunodetections were performed using the above mentioned antibodies with specific HRP conjugated antibodies (Bio-Rad) and enhanced chemiluminescence detection (NEN).

2.5. Molecular modeling

The cdc2 coordinates were kindly provided to us by S. Taylor and E. Radzio-Andzelm (UCSD, La Jolla). The structure was displayed on an Evans and Sutherland ESV/30-33. Molecular modeling was done using the program O [21] and minimization by X-PLOR (Brunger, A.T., X-PLOR version 3.1, Yale University Press, New-Haven, CT) was run on a digital DEC 3000/400 Alpha workstation (96 Mb RAM).

*Corresponding author. Fax: (33) 61 17 59 94.
E-mail: ducommun@IPBS.fr

SSDI 0014-5793(95)01514-0

3. Results

3.1. Effect of human cdc2 lysine 33 mutant overexpression on cell growth

The human cdc2 (hscdc2) cDNA was mutated using site-directed mutagenesis in order to change lysine 33, a residue conserved in every kinase that has been shown to be essential for the catalytic activity, into alanine or arginine. The effects of these two mutations on the biochemical properties of hscdc2 were investigated in vivo after transfection into wild type fission yeast. Regulated expression of wild type and mutant cdc2 proteins was achieved using the nmt1 regulatable promoter that is derepressed within 15 to 18 h after removal of thiamine from the culture medium.

As shown in Fig. 1A, overexpression of both K33A and K33R hscdc2 mutants proteins impaired yeast proliferation rate. However, while the proliferation of cell overexpressing K33R mutation was totally inhibited, K33A mutation only led to a 50% inhibitory effect on fission yeast growth as compared to the strain overexpressing wild-type human cdc2.

The phenotypic effects of hscdc2 lysine 33 mutants and wild type overexpression were then examined microscopically (Fig. 1B) after 30 h of thiamine removal, i.e. about 12 h of cdc2 protein overexpression. Cells overexpressing wild type hscdc2 were slightly smaller than untransformed cells, displaying a 'semi-wee' phenotype (Fig. 1B, left panel). This effect is likely

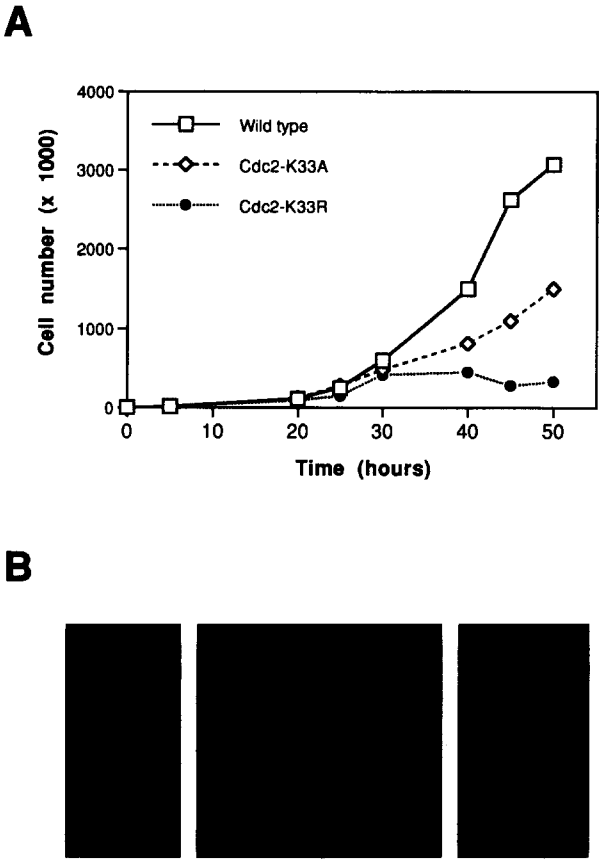

Fig. 1. (A) Effects of cdc2 lysine 33 mutations on fission yeast growth rate. Fission yeast strains carrying plasmids encoding wild-type human cdc2 (open squares) and mutants K33A-cdc2 and K33R-cdc2 (open triangles and open circles, respectively) were grown under promoter repressing conditions (with 4 μM thiamine), then resuspended in thiamine-free media at time 0. Cells were counted at intervals. (B) Phenotypes of *S. pombe* cells overexpressing lysine 33 cdc2 mutants. Cells transformed with plasmids encoding wild type hscdc2 or mutants were cultured in the absence of thiamine for 30 h. (Left panel) wild-type human cdc2, (middle panel) K33R-cdc2, (right panel) K33A-cdc2. Fixed cells are stained with 4'-6-diamidino-2-phenylindole (DAPI).

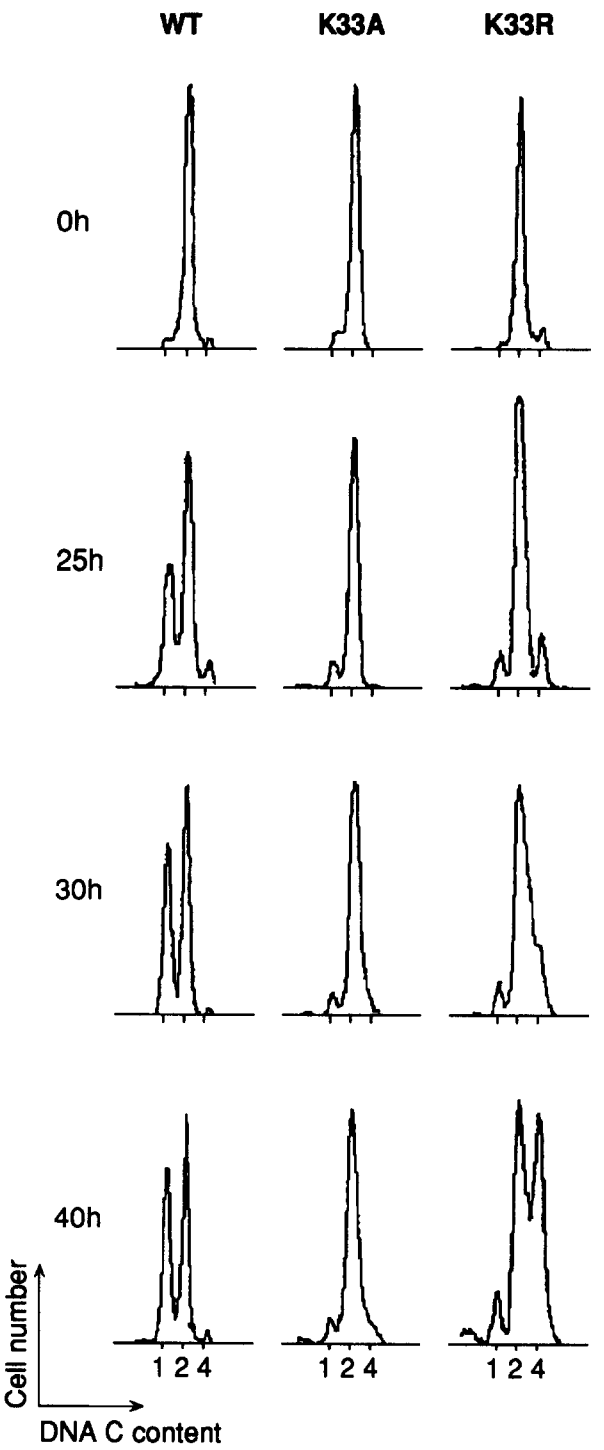

Fig. 2. DNA content analysis of the cells measured by flow cytometry. Cells were cultured in the absence of thiamine for 0, 25, 30 and 40 h. DNA content was determined by flow cytometry after propidium iodide staining. In cells grown with thiamine (time 0) the major peak correspond to cells having a 2C DNA content.

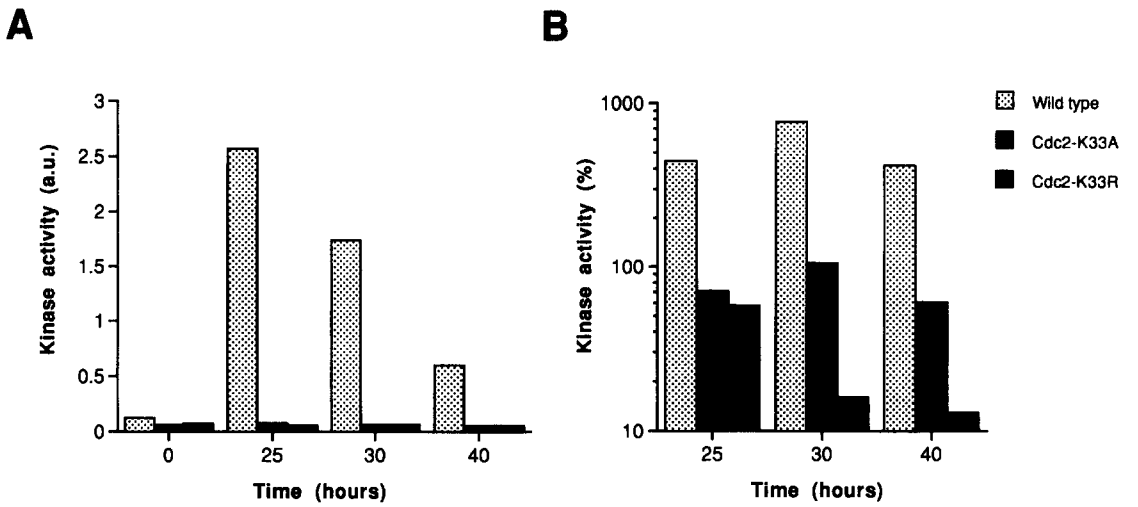

Fig. 3. Protein kinase activity associated with human cdc2 and yeast cdc13-cyclin B. Strains transformed with plasmids allowing the overexpression of wild-type human cdc2 (light gray bars) and mutants K33A-cdc2 and K33R-cdc2 (gray and black bars respectively) were grown under promoter repressing conditions, then resuspended in media without thiamine at time 0. At intervals, samples were collected and cell lysates were processed to an immunoprecipitation. (A) Human cdc2 was immunoprecipitated with an antibody specific to the carboxy terminal human cdc2 (a gift from G. Draetta) and the associated kinase activity toward histone H1 was determined (a.u. arbitrary units). (B) *S. pombe* cdc13-cyclin B was immunoprecipitated with a polyclonal serum raised against the cdc13 recombinant protein (a gift from J. Hyams). The associated kinase activity toward histone H1 was quantified and is shown as a percentage of the activity before induction of the human cdc2 protein (at time 0).

to be due to the overall increase in cdc2 kinase activity (see below). Cells overexpressing K33R and K33A mutant proteins displayed an elongated phenotype as compared to wild type. K33R-hscdc2 and K33A-hscdc2 average cell sizes after 12 h of protein overexpression were 16 μm and 30 μm respectively. While the overexpression of K33R-hscdc2 led to a typical 'cdc'-cell cycle arrest phenotype (Fig. 1B, middle panel), in the case of K33A the cells continuated to divide although at longer size (Fig. 1B, right panel). A flow cytometry analysis of the cellular DNA content was performed in order to investigate the cell cycle distribution of the cells overexpressing wild type and K33 hscdc2 mutants (Fig. 2). In exponentially growing untransformed *S. pombe* cells, G1 phase is usually not detectable because it represents less than 10% of the cell cycle duration. Overexpression of wild-type hscdc2 led to a progressive increase in the percentage of cell displaying a 1C DNA content (44% after 40 h). This reflects the shortening of the G2-phase and the subsequent relative increase in G1-phase length that is necessary to reach the minimum size required to enter S-phase. The small peak (about 5% of the cells) that was also detected after the 2C peak is likely to be due to the cells that are already undergoing S-phase while cytokinesis is still unfinished, a normal feature of exponentially growing fission yeast cell. K33A-hscdc2 overexpression had no noticeable effect on the distribution of the cells in G1- and G2-phases even after a long induction time. However, in the case of K33R-hscdc2, thirty h after thiamine removal, a 4C DNA content peak was clearly visible. After 40 h three peaks representing 8%, 50% and 42% of the cells, and corresponding to 1C, 2C and 4C DNA content respectively, were detected.

The consequences of K33R-hscdc2 overexpression on the cell cycle distribution with the occurrence of a 4C DNA content peak in mononucleated cells (see Fig. 1B) were reminiscent of the effect of cyclin B disruption on the unscheduled entry in S-phase [19], and prompted us to examine in details the biochemical properties of this mutant.

3.2. *Effect of human cdc2 lysine 33 mutants on cdc2 kinase activities*

The kinase activities associated with the overexpressed hscdc2 and with the endogenous fission yeast cdc2 were both examined. Overexpression of wild type or of the two hscdc2 mutants was induced by thiamine removal and yeast cells were collected at intervals. In the three strains, the level of induction of the human cdc2 protein kinase was very similar (see below, Fig. 4B). The kinases activity of the human cdc2 was assayed after immunoprecipitation with a specific anti peptide antibody. As shown in Fig. 3A, the activity of overexpressed wild

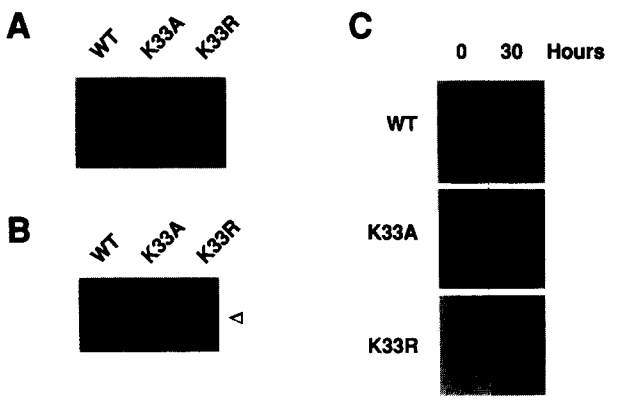

Fig. 4. Interaction of cdc2 lysine 33 mutants with cdc13/cyclin B. Strains transformed with plasmids allowing the overexpression of wild-type human cdc2 and mutants K33A-cdc2 and K33R-cdc2 were grown under promoter repressing conditions, resuspended in media without thiamine (time 0), and samples were collected at 25 h and 30 h. (A and B) 100 μg of total lysates of cells collected at 30 h were subjected to the immunodetection of human cdc2 (A) and *S. pombe* cdc13 (B) with a monoclonal antibody raised against human cdc2 (a gift from J. Lukas) and a with a polyclonal antibody (a gift from J. Hyams), respectively. (C) Cell lysates (2 mg) were subjected to immunoprecipitation with anti-cdc13 antibody and then blotted with monoclonal anti human cdc2.

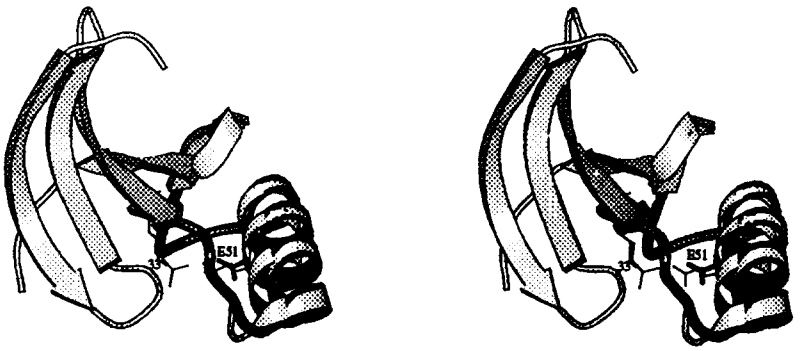

Fig. 5. Structural effect of the K33R mutation in the cdc2 model structure. Stereo view of the part of the N-terminal lobe of the cdc2 kinase in the active conformation. The steric hindrance created by the arginine side-chain at position 33 may be released by a 1.1 Å rigid body movement of the PSTAIRE helix α1 (thick ribbon). This movement affects the loop region at the N-terminal part of helix α1 that bears residues implicated in cyclin binding. A salt-bridge interaction is formed between R33 and E51 (in thick line in the mutant model).

type human cdc2 was high at about 25 h following thiamine removal. It thereafter declined to about 25% of the maximal level. Since this progressive inhibition paralleled the increase in the level of the overexpressed cdc2 protein (data not shown), this indicates that the amount of active cdc2/cdc13 (cyclin B) complex was decreasing in the immunoprecipitate. This effect might be explained by the fact that cyclin B was becoming progressively limiting as regard to the amount of available cdc2. No kinase activity toward histone H1 was detected in the anti-human cdc2 immunoprecipitates performed from lysates of the strains overexpressing K33A- and K33R-hscdc2 mutants, indicating that these two mutants were catalytically inactive. The kinase activity associated with cdc13-cyclin B immunoprecipitates was also measured in these strains at different time after promoter release and was examined as a percentage of the activity before the induction of the human cdc2 protein overexpression (Fig. 3B). In the strain overexpressing wild type human cdc2, a 5- to 8-fold increase of the total kinase activity associated with cdc13 was detected and might explain the 'semi-wee' phenotype of these cells. In the strain overexpressing K33A-hscdc2, the level of cdc13-associated kinase was fairly constant confirming that K33A mutant protein had no activity and was unable to interfere with the activity of endogenous *S. pompe* cdc2-cdc13 complexes. In the case of K33R-hscdc2 mutant overexpression, we observed a progressive decrease in the total level of cdc13-associated kinase, that dropped to about 15% and 10% of the initial activity after 30 and 40 h of overexpression, respectively. This last result suggested that this catalytically inactive mutant was also able to negatively interfere with the activity of the endogenous cdc2/cdc13 kinase complexes.

3.3. K33R-hscdc2 mutant associates cdc13-cyclin B in a inactive complex

To analyse the molecular basis of the differential effects of the K33-hscdc2 mutations, we examined the in vivo interaction of these mutant proteins with endogenous fission yeast cyclin B (the cdc13 gene product). After 30 h without thiamine, the level of induction of wild type, K33A and K33R hscdc2 were similar (Fig. 4A) as was the level of endogenous cdc13-cyclin B (Fig. 4B). At this time point, the association of K33A-hscdc2 protein with cdc13 was comparable to the wild-type, whereas the K33R-hscdc2 mutant protein was at least 3.5-fold (as judged by scanning densitometry) more abundant (Fig. 4C). From this experiment it can be suggested that the K33R-hscdc2 mutant protein binds strongly to the cdc13/cyclin B in an inactive and possibly stabilised complex, making the regulatory subunit unaccessible to *S. pombe* cdc2.

3.4. Molecular modeling of the K33R-cdc2 mutant

The interactions observed for the catalytic triad (Lys33, Glu51, Asp145) in the crystal structure of the cyclinA-bound cdk2 complex [11] were found to be essentially identical to those observed in the catalytically active PKA structure from which a cdc2 model was derived [6]. This model, kindly provided by S.S. Taylor and E. Radzio-Andzelm, was therefore used to analyse the possible effects of the K33R substitution.

Mutation of lysine 33 to an arginine residue lead to steric conflicts which may, obviously, be overcome in the mutant protein as overexpression in fission yeast was successful. From the structural observations, it seemed that the steric constraints may be released in two different ways. The guanidinium group of R33 could move towards D145, inducing a sequential rearrangement of D145, N132 and D127 that would strongly affect ATP binding and catalysis. However, the positional shifts would be restricted to this area and, if they may explain the lack of activity, would not suggest how cyclin B binding is affected. On the other hand, the steric constraints introduced by the K33R substitution could be released through a rigid body displacement of helix α1 (the PSTAIRE helix) (Fig. 5). This would maintain and strengthen the salt-bridge interaction between residue 33 and glutamic 51 when the lysine is substituted for an arginine. The movement of this helix is of small amplitude (1.1 Å) and will also likely affect the conformation of the loop 38–42 whose residues are implicated in cyclin binding to cdc2. The K33R-cdc2 mutant protein would thus adopt a slightly different local conformation in an area that seems critical with respect to both the properties of the catalytic triad and to cyclin binding. Introduction of an arginine residue at position 33, by promoting the formation of a strong R33-E51 salt-bridge interaction, may lock the cdc2 protein in a conformation similar to that found in the cdk2-cyclinA complex.

4. Discussion

We report here the differential effects of overexpression of

two human cdc2 lysine 33 mutant proteins in the fission yeast *S. pombe*. We show that both K33A-cdc2 and K33R-cdc2 are catalytically inactive and inhibit cell proliferation, however, the latter mutant behaves as a dominant negative allele. Three experimental sets of data suggest that K33R-cdc2 might act through the sequestration of the mitotic B-cyclin. First, whereas the cells were mononucleate, the cell cycle distribution after K33R-hscdc2 overexpression desplayed a 4C DNA peak that might reflect the occurrence of a round of DNA replication in the absence of cell division. A similar observation was recently made in cells in which the cyclin B gene was disrupted [19]. Second, we found that overexpressed K33R-cdc2 was associated with cdc13/cyclinB to a higher level than was wild type hscdc2. Third, and probably as a consequence of the binding of cdc13/cyclinB to K33R-cdc2, the overall cellular cdc13/cyclin B associated kinase activity was inhibited. We propose that the K33R mutant of cdc2 is titrating cdc13/cyclinB away from *S. pombe* cdc2, therefore mimicking the loss of cdc13 function.

What are the molecular effects of these mutations that could explain the enhanced interaction with cyclin and the different behavior of the K33A and K33R mutants of cdc2 in vivo? Lysine 33 is a nearly invariant amino acid across all the protein kinase family [20] and is involved in ATP binding by anchoring its α- and β-phosphate groups. The structure of the cyclinA-bound cdk2 complex [11] revealed a significant translation and rotation of the cdk2 PSTAIRE helix (helix α1, residues 44–55) when compared to free cdk2 [10], leading to the formation of the K33-R51 interaction. This movement is accompanied by a conformational change of the preceding loop (residues 38–42) which bears residues implicated in cyclin A binding to cdc2. Charged-to-alanine scanning mutagenesis of human cdc2 has shown that cyclin A binding is inhibited by mutations in several clusters of residues in the N-terminal lobe [6,9]. Residues E38, E40, E41 and E42, play essential roles in the specificity of cdk and cyclin interactions (our unpublished results). Assuming that the conformational changes in cdc2 upon cyclin B binding are essentially similar to those observed in cdk2 in the cdk2-cyclin A complex [11], molecular modelling suggests that the K33R mutation may, through the R33–E51 salt-bridge, lock the cdc2 molecule in a conformation where the association with cdc13/cyclin B is favored. This appealing hypothesis is in good agreement with the immunoprecipitation experiments where it was found that the amount of K33R-hscdc2 mutant associated with cdc13/cyclinB was most abundant. An inactive complex with cdc13/cyclin B would therefore be stabilised, arresting the cell cycle and rendering the cell functionally identical to cyclin B disruptant. The mutation of lysine 33 by an alanine residue has no such structural effect. Removal of a functional group considered to be essential in catalysis will lead to an inactive enzyme, in which one of the key molecular events (the formation of the K33–E51 interaction) linked to cyclin binding cannot occur. There would be therefore no consequence on the affinity of cyclin B to the K33A-cdc2 mutant protein, related to the fact that this mutant protein do not interfere with the activity of endogeneous *S. pombe* cdc2-cyclin complexes. Structure determination of both cdc2 and lysine 33 mutant protein monomeric and in complex with cyclin B would be required to investigate further these proposal.

Acknowledgements: We thank J. Hyams, J. Lukas, D. Beach, G. Draetta, G. Basi and K. Maundrell for providing yeast strains, yeast vectors and antibodies. A critical reading of the manuscript by J. Hyams is also appreciated. D.L. and C.B. were supported by fellowship from the Association pour la Recherche sur le Cancer. This work was supported by grants from l'Association pour la Recherche sur le Cancer, la Ligue Nationale Contre le Cancer and l'INSERM (CRE 930503).

References

[1] Nurse, P. (1985) Trends in Genetics 1, 51–55.
[2] Morgan, D.O. (1995) Nature 374, 131–134.
[3] Draetta, G. (1993) Trends in Cell Biology 3, 287–289.
[4] Elledge, S.J. and Harper, J.W. (1994) Curr. Op. Cell. Biol. 6, 847–852.
[5] MacNeill, S.A. and Nurse, P. (1993) Mol. Gen. Genet. 236, 415–426.
[6] Marcote, M., Knighton, D., Basi, G., Sowadski, J., Brambilla, P., Draetta, G. and Taylor, S. (1993) Mol. Cell. Biol. 13, 5122–5131.
[7] Endicott, J.A., Nurse, P. and Johson, L.N. (1994) Prot. Eng. 7, 243–253.
[8] Brambilla, P., Ducommun, B. and Draetta, G. (1992) Cold Spring Harbor Symp. Quant. Biol. LVI, 515–521.
[9] Ducommun, B., Brambilla, P., Felix, M.-A., Franza, B.R.J., Karsenti, E. and Draetta, G. (1991) EMBO J. 10, 3311–3319.
[10] De Bondt, H., Rosenblatt, J., Jancarik, J., Jones, H., Morgan, D. and Kim, S. (1993) Nature 363, 595–602.
[11] Jeffrey, P.D., Russo, A.A., Polyack, K., Gibbs, E., Hurwitz, J., Massagué, J. and Pavletich, N. (1995) Nature 376, 313–320.
[12] Leroy, D., Baldin, V. and Ducommun, B. (1994) Yeast 10, 1631–1638.
[13] Basi, G., Schmid, E. and Maundrell, K. (1993) Gene 123, 131–136.
[14] Maundrell, K. (1990) J. Biol. Chem. 265, 10857–10864.
[15] Maundrell, K. (1993) Gene 123, 127–130.
[16] Moreno, S., Klar, A. and Nurse, P. (1991) in: Guide to Yeast Genetics and Molecular Biology (Gutrie, C. and Fink, G., Eds.) Vol. 194, pp. 795–826, Academic Press, New York.
[17] Zoller, M.J. and Smith, M. (1987) Methods Enzymol. 154, 329–.
[18] Harlow, E. and Lane, D. (1988) Antibodies. A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
[19] Hayles, J., Fisher, D., Wollard, A. and Nurse, P. (1994) Cell 78, 813–822.
[20] Hanks, S.K., Quinn, A.M. and Hunter, T. (1988) Science 241, 42–52.
[21] Jones, T.A., Zou, J.Y., Cowan, S.W. and Kjeldgaard, M. (1991) Acta Crystallogr. Sec. A47, 110–119.