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Original Article

Cyclin B2/cyclin-dependent kinase1 dissociation precedes CDK1 Thr-161 dephosphorylation upon M-phase promoting factor inactivation in *Xenopus laevis* cell-free extract

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ABSTRACT Cyclin-dependent kinase 1 (CDK1) is the enzymatic subunit of M-phase Promoting Factor (MPF). It is positively regulated by phosphorylation on Thr-161 and association with a cyclin B molecule. The role of Thr-161 dephosphorylation upon MPF inactivation remains unclear; nevertheless, degradation of cyclin B is thought to be a direct cause of MPF inactivation. However, MPF inactivation actually precedes cyclin B degradation in *Xenopus* cell-free extracts. Here we study in details the temporal relationship between histone H1 kinase (reflecting MPF activity) inactivation, Thr-161 dephosphorylation, CDK1-cyclin B2 dissociation and cyclin B2 proteolysis in such extracts. We show an asynchrony between inactivation of histone H1 kinase and degradation of cyclin B2. CDK1 dephosphorylation on Thr 161 is an even later event than cyclin B2 degradation, reinforcing the hypothesis that cyclin B dissociation from CDK1 is the key event inactivating MPF. Cyclins synthesized along with MPF inactivation could deliver shortly living active MPF molecules, potentially increasing the asynchrony between histone H1 kinase inactivation and cyclin B2 degradation. We confirm this by showing that in the absence of protein synthesis, such a tendency is lower, but nevertheless, still detectable. Finally, to characterise better CDK1/cyclin B dissociation, we show that CDK1 begins to dissociate from cyclin B2 before the very beginning of cyclin B2 degradation and that the diminution in CDK1-associated cyclin B2 is faster than the decline of its total pool. Thus, neither cyclin B2 degradation nor Thr-161 dephosphorylation participates directly in CDK1 inactivation as measured by histone H1 kinase decline upon the exit from mitotic M-phase in *Xenopus* embryo extract.

KEY WORDS: *cell cycle, histone H1 kinase, mitosis, protein phosphorylation/dephosphorylation, Thr-161*

Introduction

M-phase Promoting Factor (MPF) is a universal mitotic regulator. It is composed of the enzymatic subunit - CDK1 (Cyclin-Dependent Kinase 1) and the regulatory subunit - cyclin B. MPF activation triggers both mitotic and meiotic M-phase, while its inactivation results in mitotic or meiotic exit. For long time MPF inactivation upon the exit from meiosis or mitosis was thought to be provoked by cyclin B degradation *via* ubiquitin-proteasome pathway since the two phenomena are temporarily tightly linked (Murray *et al*., 1989; Glotzer *et al*., 1991). The proteolysis of mitotic cyclins is coordinated by Anaphase Promoting Complex/Cyclosome (APC/C; Peters, 2002) which has an activity of ubiquitin ligase (Ciechanover, 2005; Hershko, 2005). It polyubiquitinates substrates and targets them to 26S proteasome for degradation.

Cyclin B2 is one of the two major B-type cyclins expressed in *Xenopus laevis* oocytes and early embryos. Upon oocyte activation or in calcium-treated CSF extract made of such oocytes all cyclins B are degraded very fast (up to 10 min post-activation). Cyclins B1 and B4 disappear first and B2 and B5 are degraded slightly later (Hochegger *et al*., 2001). Cyclin B5 seem to be much less abundant than cyclin B2 (ibid.). B2 cyclin is therefore the latest major cyclin B degraded upon M-phase exit in *Xenopus* oocytes. The same sequence of cyclins B degradation is observed upon mitotic exit in cell free embryo extracts (Hochegger *et al*., 2001, Chesnel *et al*., 2005; 2006). The association of cyclin B2 with CDK1 is accompanied by its rapid phosphorylation on multiple N-terminal residues (Meijer *et al*., 1989; Gautier and Maller, 1991; Izumi and Maller, 1991; Li *et al*., 1995; Peter *et al*.,

Abbreviations used in this paper: CDK1, cyclin-dependent kinase 1MPF, M-phase promoting factor.

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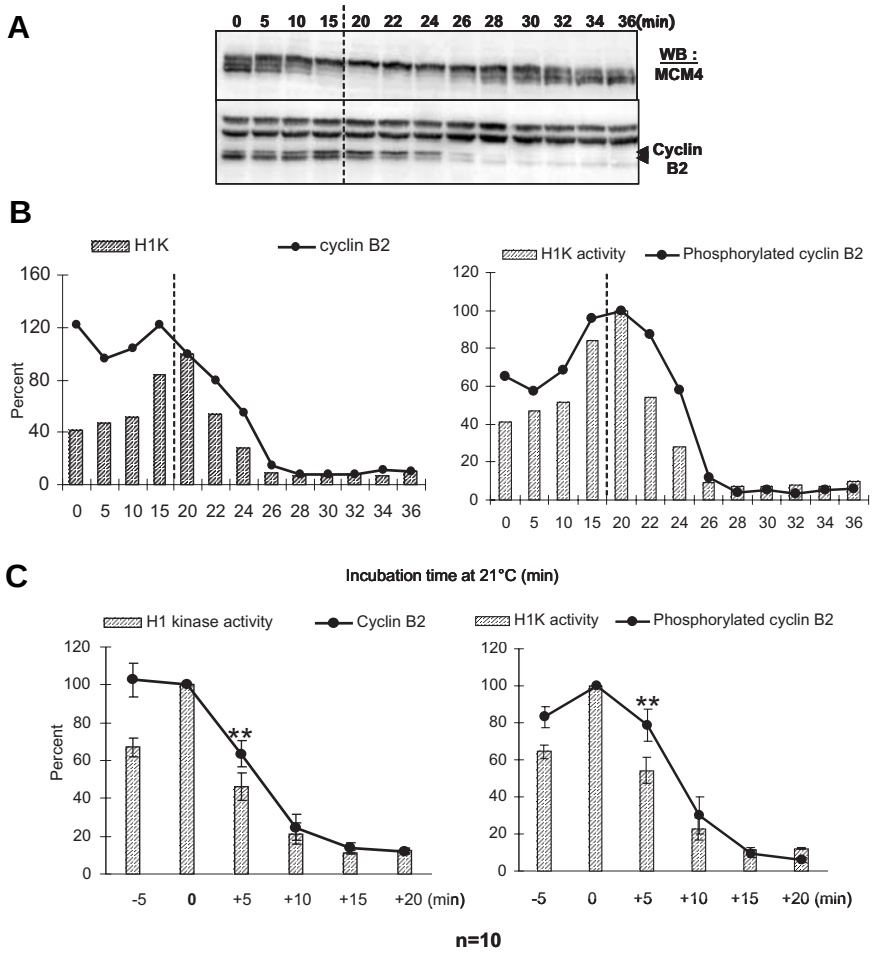

Fig. 1. The dynamics of histone H1 kinase inactivation are faster than cyclin B2 degradation in cell-free mitotic extract. *Samples were collected every 5 min (left from the vertical dotted line) and every 2 min starting from 20 min time point of incubation of the extract at 21°C (right from the dotted line). Western blots of MCM4 and cyclin B2* **(A)** *during the first mitosis in cell-free extract are shown. The relationship between histone H1 kinase inactivation (histograms) and the dynamics of total cyclin B2 degradation and the phosphorylated form of cyclin B2* **(B)** *were quantified and plotted. Note that cyclin B2 levels (total and phosphorylated) fall down clearly slower than histone H1 kinase inactivation at 22 and 24 min time points. Relationship between the dynamics of histone H1 kinase inactivation and the total pool of cyclin B2 degradation and the phosphorylated form of cyclin B2* **(C)** *in 10 separate experiments. The maximum amplitude of histone H1 kinase activity, as well as the corresponding value of the quantity of cyclin B2 obtained by quantification of 10 Western blots, were taken as 100 % in each M-phase. Histone H1 kinase values are presented as histograms, while those of cyclin B2 amount as curves. Note that at +5 min time points (B, left and right) the values of the means for histone H1 kinase and cyclin B2 amount are statistically different (**, p<0.01; paired t-test).*

2002). Due to a clear up-shift on Western blot one can easily distinguish the phosphorylated, certainly associated with CDK1 and non-phosphorylated, not necessarily associated with CDK1 forms of this cyclin B. Cyclin B2 is phosphorylated by different kinases (Mos, Roy *et al*., 1990; cyk, Stevens *et al*., 1999; Plk1, Toyoshima-Morimoto *et al*., 2001; MAP kinase, Walsh *et al*., 2003) including CDK1 itself (Izumi and Maller, 1991). Judging by a very high affinity of cyclins B to CDK1 and the excess of CDK1 in relation to B-type cyclins in *Xenopus laevis* oocytes and embryos, the pool of continuously synthesized cyclins most likely forms a complex with CDK1 almost immediately (Kobayashi *et al*., 1999; Hochegger *et al*., 2001). Therefore, we suppose that the non-shifted form of cyclin B2 cannot be unequivocally considered as CDK1-free, while the shifted one is with no doubt associated with this kinase and forms active MPF (ibid.).

Proteasome inhibition using ALLN or MG132 arrests eukaryotic somatic cells in the mitotic M-phase (Sherwood *et al*., 1993; Genschik *et al*., 1998; Uzbekov *et al*., 1998; Kim *et al*., 2004). This arrest was attributed to the presence of non-degraded cyclins B (Potapova *et al*., 2006). However, Nishiyama and colleagues (2000) and our laboratory (Chesnel *et al*., 2006) observed that MPF inactivation is separated from cyclin B degradation upon meiotic as well as mitotic exit in *Xenopus laevis* cell-free extracts due to a non-proteolytic activity of the proteasomes themselves. Therefore, inactivation of MPF upon inhibition of the proteasome proteolytic activity apparently requires only the physical separation of cyclin B and CDK1 most likely mediated by the proteasome lid and not by cyclin B degradation (Nishiyama *et al*., 2000). Moreover, we have shown recently that such a sequence of events takes place also during the exit from the first embryonic M-phase in *Xenopus* extract without proteasome inhibition (Chesnel *et al*., 2006). It is therefore likely that the mitotic arrest of somatic cells by proteasome inhibitors is rather due to the presence of other non-degraded substrates which act up stream from cyclins B proteolysis (Chesnel *et al*., 2006; reviewed by Fry and Yamano, 2006).

Activity of CDK1 depends also on status of its phosphorylation. Phosphorylation of threonine 161 (Thr-160 in CDK2) located in the protein T-loop is absolutely required for its activity (Solomon *et al*., 1992; De Smedt *et al*., 2002). Despite that the major kinase phosphorylating CDK1 on this amino acid remains constitutively active during the cell cycle (Shuttleworth *et al*., 1990) the levels of Thr-161 phosphorylation changes between interphase and M-phase (Solomon *et al*., 1992) suggesting the major regulatory function of some phosphatases. Type 2C protein phosphatases (PP2Cs) were identified as major CDK1 Thr-161 dephosphorylating enzymes in yeast (Cheng *et al*., 1999) and in *Xenopus* (De Smedt *et al*., 2002). Besides this activating phosphorylation, two other residues, namely Thr-14 and Tyr-15, are subjected to the inhibitory phosphorylation within the CDK1 ATP accepting binding site (Krek and Nigg, 1991; Solomon *et al*., 1992; Rime *et al*., 1995). This phosphorylation enables to keep CDK1 in its inactive state. Dephosphorylation of Thr-14 and Tyr-15 is therefore necessary

for CDK1 activation. In *Xenopus* cell-free extract it precedes slightly the peak of mitotic MPF activation (Chesnel *et al*., 2005). However, it does not seem to play a crucial role during mitotic inactivation of MPF since rephosphorylation of these two sites is a very slow process and concerns only a small proportion of CDK1 following the first embryonic M-phase (ibid.). Therefore, of these two regulatory mechanisms, only Thr-161 phosphorylation seems a potential key M-phase regulator of CDK1 activity.

Here we focus our attention on interactions between cyclin B2 and CDK1 in relation to the dynamics of MPF inactivation and Thr-161 phosphorylation status during mitotic exit in *Xenopus* embryo cell-free extracts. Our data herein confirm that dissociation of cyclin B2 from CDK1 and not cyclin B degradation or Thr-161 dephosphorylation of CDK1 is the earliest event in mitotic MPF inactivation.

Results

Histone H1 kinase activity declines faster than cyclin B2 amount upon mitotic exit

During mitosis in cell-free extract cyclin B2 changes its phosphorylation status, as visualised by the up-shift on Western blot and is degraded upon mitotic exit (Fig. 1A, lower blot). Phosphorylation of other proteins, e.g. MCM4 (Fig. 1A, upper blot) which is a direct substrate of CDK1-cyclin B kinase, also changes accordingly to histone H1 kinase fluctuations which reflects the activity of this kinase (Fig. 1B, histograms).

We compared precisely the dynamics of cyclin B2 diminution and histone H1 kinase activity. Samples were collected every 5 min at the beginning of the experiment and every 2 min starting from 20 min. incubation time, i.e. the time point when we expected the beginning of the mitotic exit. The maximum of histone H1 kinase activity as well as the maximum of cyclin B2 level were arbitrarily expressed as 100 % (Fig. 1 B and C). Inactivation of histone H1 kinase proceeded with clearly faster dynamics than the diminution of cyclin B2 as shown in a representative experiment (Fig. 1B, left and right). The difference in the two dynamics is the highest at 22 and 24 min time points both for the total pool of cyclin B2 (Fig. 1B, left) or the phosphorylated cyclin B2 form (Fig. 1B, right). This suggested that inactivation of histone H1 kinase could precede cyclin B2 degradation.

We then compared the dynamics of histone H1 kinase activity and cyclin B2 diminution in ten separate experiments where samples were collected every 5 min (Fig. 1C). In all experiments, inactivation of histone H1 kinase proceeded with faster dynamics than diminution of cyclin B2. In statistical data, this difference is clear at the 5 min time point following the peaks of both histone H1 kinase activity and cyclin B2 level (Fig. 1C, left and right, "+5 min"). In all but one individual experiments the decline of histone H1 kinase activity was deeper at that time point than the decline of cyclin B2 level. This difference was statistically significant ($p<0.01$; paired t-test). In the experiment with inverted values for histone H1 kinase and cyclin B2 level at that time point, the two processes proceeded with the slowest dynamics of all 10 cases examined. In addition, in this particular experiment during further time points (+10, 15 and 20 min.) histone H1 kinase decline was faster than the decline of cyclin B2 (data not shown). This strengthened our hypothesis that during the mitotic exit the dynamics of inactivation

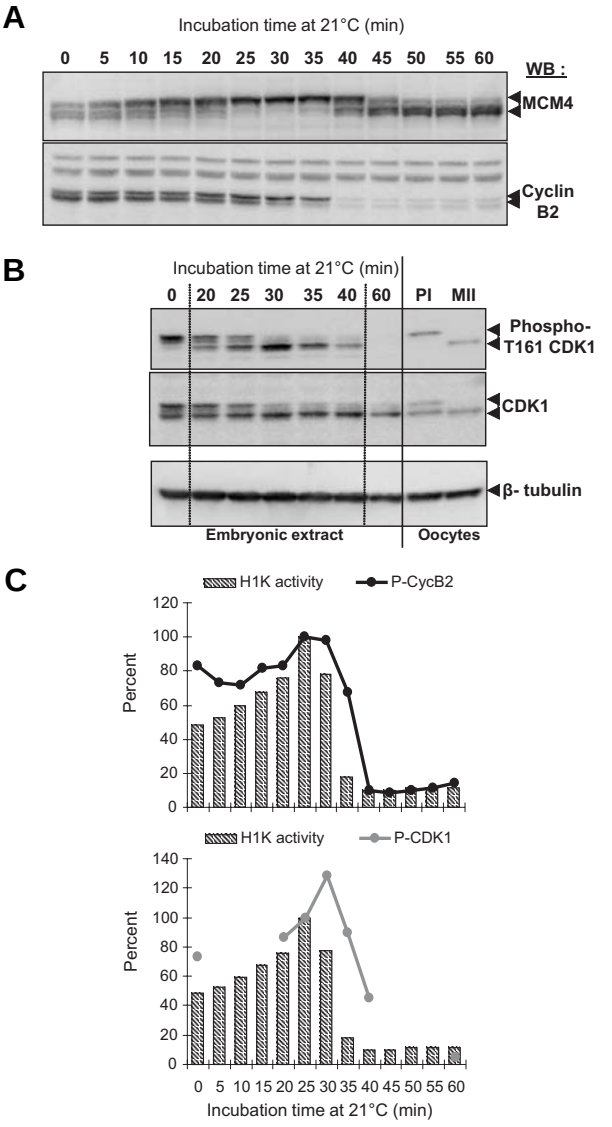

Fig. 2. Dephosphorylation of CDK1 Thr-161 follows MPF inactivation and cyclin B2 degradation in mitotic cell-free extracts. *Samples were collected every 5 min during incubation of the low-speed extract at 21°C and histone H1 kinase activity, MCM4, cyclin B2, β-tubulin (used for quantification as stable standard), total CDK1 and Thr-161-phosphorylated CDK1 levels were examined* **(A,B)**. *CDK1 and Thr-161 CDK1 levels and migration patterns are also compared between prophase I (PI) and metaphase II (MII) oocytes* **(B)**. *Histone H1 kinase , phosphorylated cyclin B2 and Thr-161 CDK1 were quantified and their relationship is compared* **(C)**. *Histone H1 kinase activity decreases the fastest and is followed by cyclin B2 and Thr-161 CDK1 diminution.*

of histone H1 kinase is faster than cyclin B2 degradation.

Thr-161 dephosphorylation of CDK1 follows histone H1 kinase inactivation and cyclin B2 degradation

Thr-161 dephosphorylation could be involved in inactivation of CDK1 upon mitotic exit. Therefore we studied the dynamics of this process in parallel with histone H1 kinase activity and cyclin B2 levels. The comparison of Western blots showing the dynamics of changes of MCM4, cyclin B2, total CDK1 and Thr-161-phospho-

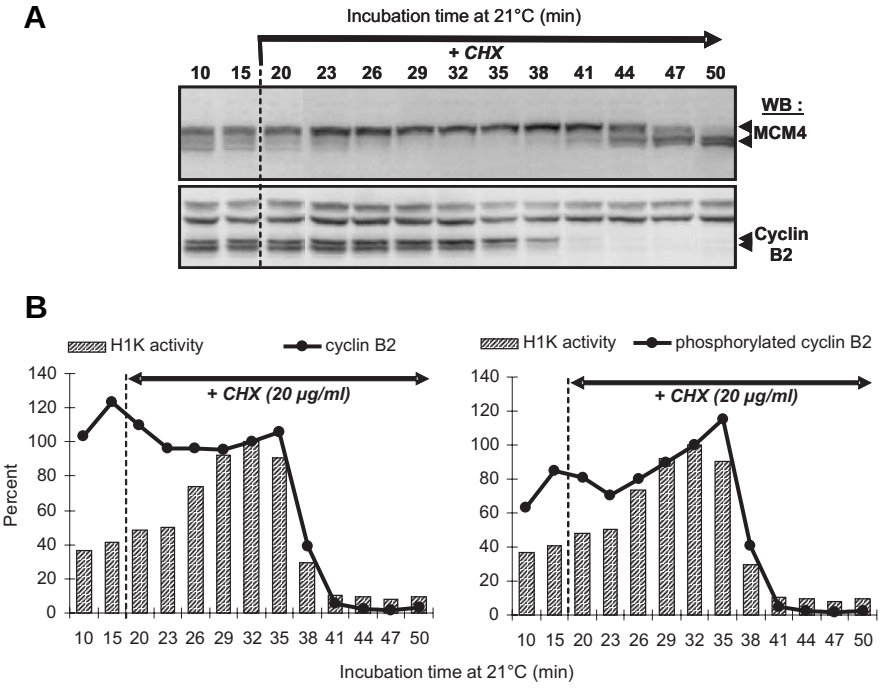

Fig. 3. The dynamics of histone H1 kinase inactivation is faster than cyclin B2 degradation in the cell-free mitotic extract in which protein synthesis was inhibited with 20 µg/ml CHX 14 min before the peak of histone H1 kinase activity. *Low-speed extracts were incubated at 21°C and histone H1 kinase activity, MCM4 and cyclin B2 levels were examined every 3 min starting from 20 min time point (right from the dotted line). MCM4 and cyclin B2 were blotted* **(A)** *and total cyclin B2 and the phosphorylated form of this cyclin were quantified* **(B)** *. These graphs illustrate the relationship between histone H1 kinase inactivation and the dynamics of the total pool of cyclin B2 and phosphorylated form of cyclin B2 degradation upon inhibition of protein synthesis. The maximum amplitude of histone H1 kinase activity observed at 32 min of incubation as well as the level of total and phosphorylated form of cyclin B2 at the same time point obtained by quantification of Western blots were taken as 100 %. Histone H1 kinase values are presented as histograms. Note that at 35 and 38 min time points cyclin B2 levels (total or phosphorylated) fall down slower than histone H1 kinase inactivation.*

rylated CDK1 indicates that the latter is a late mitotic event (Fig. 2A,B,C; see the 40 min time point when no trace of cyclin B2 is present while clear levels of Thr-161 CDK1 are detected indicating that it concerns a pool of cyclin-free pool of CDK1). The comparison of quantifications of histone H1 kinase activity and Western blots shows the differences in these dynamics more clearly (Fig. 2C). Thr-161 phosphorylation increases in parallel with the increase in histone H1 kinase activity and keeps rising during the very beginning of the drop in this activity. Following the sharp peak of Thr-161 phosphorylation at that time point (30 min time point in Fig. 2C), it starts to diminish dramatically. The profile of Thr-161 phosphorylation suggests an important regulatory role of this mechanism during M-phase. However, this dephosphorylation occurs too late to be directly involved in inactivation of histone H1 kinase activity of CDK1. It seems that it rather prevents precocious re-activation of CDK1 in the absence of rapid and efficient re-phosphorylation of CDK1 on Thr-14 and Tyr-15 (Chesnel *et al*., 2005).

Newly synthesized cyclin B2 is only partially responsible for the asynchrony between histone H1 kinase inactivation and cyclin B2 diminution during mitotic exit

The results described so far could be biased by neo-synthesis of cyclin B2 concomitant with its degradation, which would increase the pool of cyclin B2 present during the process of its mitotic degradation. To verify the extent of such a potential bias, we treated cytoplasmic extracts with cycloheximide (CHX; 20 or 50 µg/ml giving the same results) shortly before MPF activity peaks in order to inhibit protein synthesis specifically during the period of MPF inactivation and massive cyclin B degradation (as used before; Chesnel *et al*., 2005). We analysed the profile of histone H1 kinase activity as well as cyclin B2 degradation dynamics (Fig. 3A, lower blot) and MCM4 phosphorylation pattern (Fig. 3A, upper blot) by Western blotting by sampling the extract every 3 min. (starting from 20 min. time point). CHX was added 18 min. after the beginning of incubation, which corresponded in this experiment to the time point preceding by 14 min the peak of histone H1 kinase activity (Fig. 3B). Again, the dynamics of histone H1 kinase decline was faster than that of diminution of phosphorylated cyclin B2 (compare the curve of cyclin B2 level with the histogram of histone H1 kinase activity at 35 and 38 min time points; Fig. 3B, left for the total pool of cyclin B2; right for the phosphorylated forms of cyclin B2). As expected, this difference was smaller than in experiments without CHX (compare with 22 and 24 time points in Fig. 1B), which correlates with the absence of neo-synthesized cyclin B2 upon CHX treatment. This kind of experiments was repeated at least five times with results confirming this tendency. However, the exact timing (18 min of incubation for CHX addition) was repeated twice with very similar results despite that in a sibling experiment the period between CHX addition and histone H1 kinase peak was 5 min. due to more rapid entry into the M-phase (data not shown). We chose to present here a single representative experiment instead of statistical analysis since in each experiment the maximum of histone H1 kinase activity took place at slightly different time (after CHX addition) modifying experimental conditions and making statistics difficult. Indeed, we described recently that cycloheximide modifies both the amplitude and the timing of histone H1 kinase inactivation in a manner strictly dependent on the period of its action in relation to the beginning of the mitotic exit (see Fig. 6 in Chesnel *et al*., 2005).

Histone H1 kinase inactivation is synchronised with cyclin B2 separation from CDK1 and not with cyclin B2 degradation

Since histone H1 kinase activity strictly depends on the quantity of active CDK1 (associated with cyclin B) we asked whether the asynchrony observed above concerns also cyclin B2 separation from CDK1. To distinguish between the dynamics of diminu-

tion of the total cyclin B2 pool and the CDK1-associated one, we immunoprecipitated proteins present in samples obtained during the experiment similar to the one shown in Fig. 3 (Fig. 4A) collected at 23, 26, 29 and 32 min of incubation (i.e. before, during and just after the total histone H1 kinase inactivation and cyclin B2 degradation) with anti-cyclin B2 antibody. Immunoprecipitation was followed by Western blot analysis of the pellet and supernatant with anti-cyclin B2 and anti-CDK1 (Fig. 4B). The cyclin B2 Western blot shows the levels of total cyclin B2 present during the analysed period (Fig. 4B, top panel, "bound"). The CDK1 Western blot shows the levels of CDK1 co-precipitated with this antibody (Fig. 4B, bottom panel, "bound"). The levels of CDK1 visualised in this way are proportional to the levels of cyclin B2 still associated with CDK1 and therefore active as MPF. The Western blots of the pellet were quantified and the dynamics of the total cyclin B2 pool as well as of CDK1 associated with cyclin B2 were compared in parallel with the total activity of histone H1 kinase measured during the period of MPF inactivation (Fig. 4C, left, top graph for cyclin B2 and left, bottom one for CDK1) and with the total pools of cyclin B2 and CDK1 present in the samples before immunoprecipitation (Fig. 4C, right, top graph for total cyclin B2 and right, bottom one for total CDK1). This shows a clear difference in dynamics between the diminution of the total cyclin B2 level and cyclin B2-associated pool of CDK1 (compare the curves in fig. 4C left, top graph for cyclin B2 and left, bottom one for cyclin B2-associated CDK1). This difference is the most striking at the 26 min time point when cyclin B2 remains still on a high level, while cyclin B2-associated CDK1 has diminished already to 60 % of the maximal level. This shows the clear delay of cyclin B2 degradation in relation to its dissociation from CDK1 during MPF inactivation.

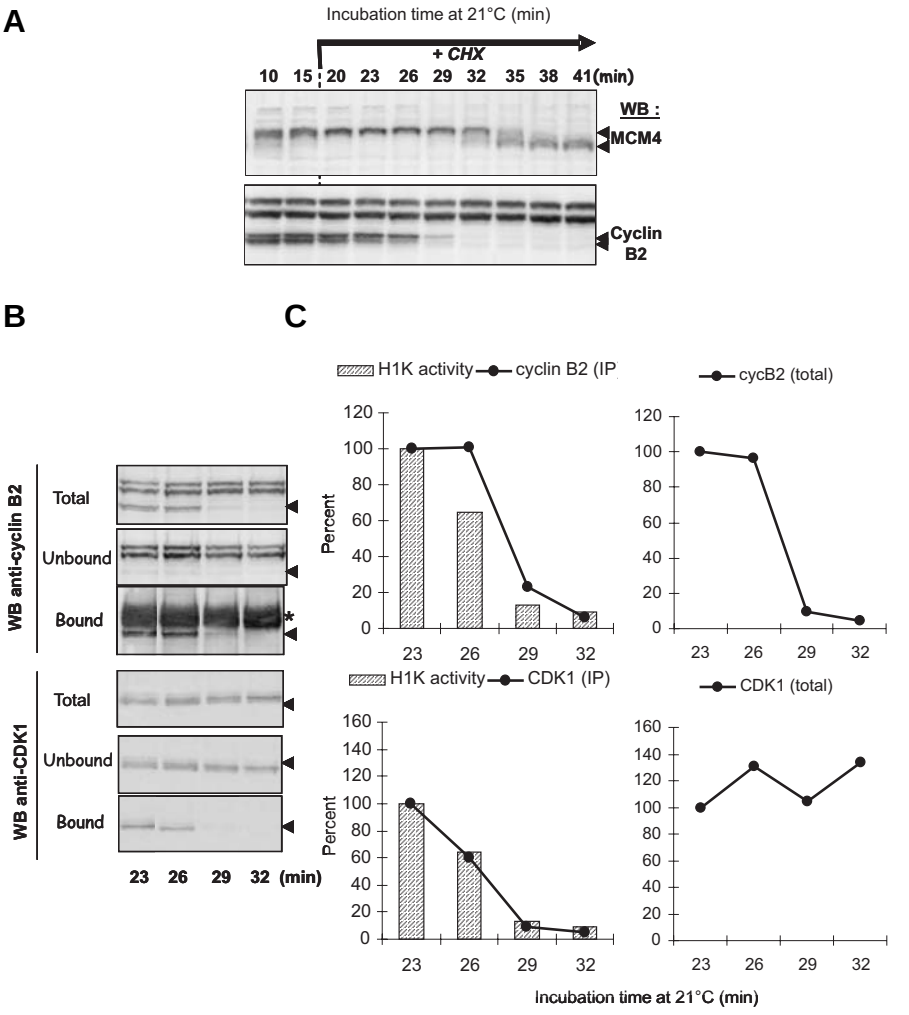

Fig. 4. CDK1 separates from cyclin B2 with faster dynamics than the progression of the proteolysis of this cyclin. *Samples were collected every 5 min (left from the vertical dotted line) and every 3 min starting from 20 min time point of incubation of the extract at 21°C (right from the dotted line). Western blots of MCM4 and cyclin B2 upon mitotic exit during the first mitosis in cell-free extract are shown* **(A)**. *Samples from 23, 26, 29 and 32 min of incubation from this experiment (similar to the one shown in Fig. 3) were divided in two equal parts and either immunoprecipitated with anti-cyclin B2 antibody (Fig. 4B) or precipitated with p9 beads (Fig. 5). Total extract, unbound and bound materials were blotted with anti-cyclin B2 and anti-CDK1* **(B)**. **, IgG heavy chain. Specific signals for the total pool of cyclin B2 and CDK1* **(C)** *present in the same samples of the analysed extract following dilution with IP buffer, but before immunoprecipitation (total) were quantified and plotted. The Western blot of the imunoprecipitaed cyclin B2 was quantified and compared with histone H1 kinase activity. At the 26 min time point, the level of immunoprecipitated cyclin B2 is still high, while the decrease of CDK1 co-precipitated with this cyclin reaches already 60 % of the initial (23 min time point) amount of CDK1 co-precipitated with cyclin B2. Note that the dynamics of the decrease in CDK1 associated with immunoprecipitated cyclin B2 is proportional to the decrease in total histone H1 kinase activity measured during mitotic exit.*

In the same experiment we compared the dynamics in cyclin B2 diminution, CDK1 levels and histone H1 kinase decline in sibling samples after precipitating CDKs with p9 beads and visualising CDK1 (Fig. 5A, bottom panel) as well as the co-precipitated cyclin B2 (Fig. 5A, top panel) with Western blot. We reasoned that we should observe relatively stable levels of CDK1 throughout the mitotic exit since p9 beads do not discriminate between active and inactive CDK1. However, if cyclin B2 indeed dissociates from CDK1 before its degradation, as suggested by the results shown in Fig. 4, we should observe faster histone H1 kinase inactivation than cyclin B2 diminution as in experiments shown in Fig. 1 and 2 and faster diminution of CDK1-associated cyclin B2 than the diminution of cyclin B2 found in the total pool. Quantifications of Western blots shown in fig. 5A confirmed this hypothesis. CDK1 levels found on p9 beads oscillate slightly (Fig. 5B, left, bottom), but the total pool of CDK1 present during this period in the collected samples undergoes very similar oscillations (Fig. 5B, right, bottom). Cyclin B2 associated with CDK1 starts to decline slightly at the 26 min. time point (Fig. 5B, left, top, arrow), while histone H1 kinase activity diminishes already to 60 % of the maximum and no sign of the decline of the total pool of

cyclin B2 can be detected at that time point (Fig. 5B, right, top, arrow). Three minutes later CDK1-associated and total cyclin B2 become very low, as well as the histone H1 kinase activity (Fig. 5B, top left and right graph respectively). This experiment was repeated twice with very similar results. The experiments shown in Figs. 4 and 5 confirm that the dynamics of cyclin B2 dissociation from CDK1 is slower than histone H1 kinase inactivation and shows directly that the latter corresponds to the dynamics of CDK1 inactivation and not to cyclin B2 degradation.

Discussion

In this study, we have investigated the relationship between the dynamics of inactivation of the global MPF activity (reflected by histone H1 kinase) and the timing of diminution of either the total pool or phosphorylated form of cyclin B2 and Thr-161-phosphorylated form of CDK1. Our results show a clear asynchrony between the fast MPF inactivation and slower cyclin B2 degradation and CDK1 dephosphorylation on Thr-161 upon mitotic exit during the first embryonic M-phase in cell-free extract.

Thr-161 dephosphorylation appears the latest of herein studied events potentially involved in MPF inactivation. This enabled us to exclude its role in the immediate mechanism of the switch from active to inactive CDK1. Thr-161 dephosphorylation mediated by 2C protein phosphatases (PP2Cs; Cheng *et al*., 1999; De Smedt *et al*., 2002) plays certainly an important role in the cell cycle regulation of CDK1. However, it seems to act rather on monomeric CDK1 which is already inactivated. This argues in favour of a key inactivating role of conformational changes in CDK1 three-dimensional structure occurring upon its dissociation from cyclin B molecule. For these reasons we will focus further attention on the asynchrony between the MPF inactivation and cyclin B2 association with CDK1 and degradation.

This asynchrony is measurable both in individual as well as in statistically analysed group of such experiments. The newly synthesized, thus yet potentially free of CDK1, cyclin B is only partially responsible for this phenomenon since CHX treatment only diminishes, but not suppresses, the asynchrony. In addition, p9 precipitation experiments enabling to visualise the dynamics of association/dissociation of cyclin B2 from CDK1 also confirmed the above observations as well as they showed that CDK1-associated cyclin B2 starts to diminish before the total pool of cyclin B2. The latter difference suggests the presence in the total pool of cyclin B2 of a portion of this cyclin corresponding to cyclin B2 dissociated from CDK1 and not yet degraded. Immunoprecipitation experiments with anti-cyclin B2 confirmed directly the asynchrony between cyclin B2 separation from CDK1 and its degradation. The separation of CDK1 from cyclin B2 is therefore indeed faster than the proteolysis of this cyclin B upon mitotic inactivation of MPF. The differences in dynamics of biochemical events described in this paper are very subtle and their study required very precise and frequent sampling during mitotic exit. This seems, however, not surprising since we followed here extremely rapid processes. The present data strengthen our hypothesis formulated also in regard to cyclins B1 and B4 (Chesnel *et al*., 2006) that the dissociation of B-type cyclins from CDK1 and not their degradation *per se* is responsible for MPF inactivation during mitotic exit in *Xenopus* embryo cell-free extract.

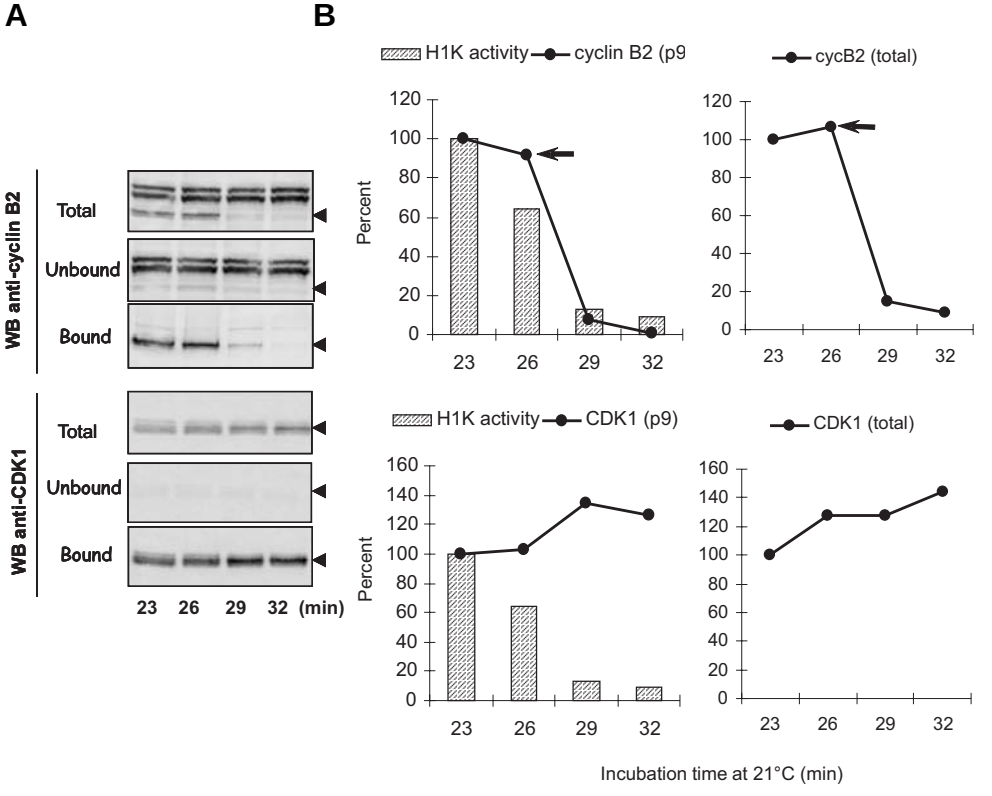

Fig. 5. CDK1-associated cyclin B2 begins to decrease before the diminution of the total pool of this cyclin and its diminution progresses with slower dynamics than inactivation of histone H1 kinase. *Half of the each sample (23, 26, 29 and 32 min of incubation) from the experiment shown in Fig. 4 was precipitated with p9 beads (which have affinity for CDKs) and blotted with anti-cyclin B2 and anti-CDK1 antibodies* **(A)**. *Total extract, unbound and bound materials were blotted with anti-cyclin B2 and with anti-CDK1. Specific signals for the total pool of cyclin B2 and CDK1 present in the same samples following dilution with IP buffer, but before precipitation* **(B)** *(total) were quantified and plotted. The Western blot of the cyclin B2 co-precipitated with CDK1 was quantified and compared with histone H1 kinase activity. At the 26 min time point the level of co-precipitated cyclin B2 begins to fall down while the total cyclin B2 is still high. p9-bead associated cyclin B2 at that time point falls however with slower dynamics than histone H1 kinase activity similarly as in Figs. 1,3 concerning the whole pool of cyclin B2. The quantities of CDK1 precipitated with p9 beads vary slightly similarly to the total pool of CDK1.*

Cyclin B2 is a particularly interesting example of B-type cyclins metabolism. It is a major cyclin B present in early *Xenopus laevis* embryos. Its active and phosphorylated form associated with CDK1 is easily distinguishable in Western blots (contrary to cyclin B1). It is also one of the latest cyclins B degraded upon mitotic exit. Another major, but earlier-degraded B-type cyclin, namely cyclin B1, is present in two isoforms also migrating in PAGE as a doublet, but its phosphorylation does not change the mobility in SDS-PAGE (Hochegger *et al*., 2001). We took the advantages of cyclin B2 as a model for B-type cyclin metabolism to demonstrate straightforwardly the separation of MPF inactivation and B-type cyclins proteolysis.

Cyclins B degradation was considered for a long time as a key event of MPF inactivation (Evans *et al*., 1983; Murray *et al*., 1989; Glotzer *et al*., 1991). A recent study in which the dynamics of mitotic cyclins degradation and histone H1 kinase inactivation were compared showed that cyclins (labelled with S^{35} and precipitated on $p13^{suc1}$ beads, thus associated with CDK1) degradation preceded histone H1 kinase inactivation (measured after precipitation with $p13^{suc1}$ beads; Pomerening *et al*., 2005; see fig. 1V). However, Nishiyama and colleagues (2000) have shown that upon meiotic exit the MPF inactivation in the presence of proteasome inhibitor MG115 takes place in the absence of cyclin B degradation. Since then, the role of the proteolysis of cyclins B in this process was seriously questioned (Josefsberg *et al*., 2001; Michel *et al*., 2001; 2004). Recently, our laboratory has confirmed the results by Nishiyama and colleagues (2000) in mitotic extracts of *Xenopus laevis* one- and two-cell embryos. We have shown that the dissociation of B-type cyclins from CDK1 is sufficient to trigger CDK1 inactivation also during the early mitotic M-phases in such extracts even in the absence of proteasome inhibitors. The direct evidence for that came from our observation that the total pools of cyclins B1, B2 and B4 diminished slower than the pools of respective cyclins B associated with CDK1 (Chesnel *et al*., 2006). Our results focused on cyclin B2 presented in the current paper reinforce this hypothesis by the observation showing that CDK1-cyclin B2 complex dissociation is faster than cyclin B2 degradation. They are in clear contradiction with results by Pomerening and colleagues (2005) quoted above. One can speculate that $p13^{suc1}$ beads used to precipitate kinases able to phosphorylate histone H1 by these authors have unspecific affinity to other histone H1 kinases than CDK1 which are inactivated following cyclins B degradation. ERK2 MAP kinase is a potential candidate for such a contamination since it binds to $p13^{suc1}$ beads (Shibuya *et al*., 1992) and is indeed inactivated lately during the first mitosis (Guadagno and Ferrell, 1998; Chesnel *et al*., 2005).

While inhibition of degradation of endogenous cyclin B does not prevent M-phase exit (Nishiyama *et al*., 2000; Chesnel *et al*., 2006, this paper), N-terminal mutants of these cyclins deprived of their degradation boxes (Murray *et al*., 1989) or N-terminal fragments of cyclins B (Van der Velden and Lohka, 1993) efficiently arrest MPF at the high level. This may appear as a paradox. However, Nishiyama and colleagues (2000) have shown that endogenous cyclin B is sequestered within the proteasome in the CSF extract and our results confirmed this in mitotic extracts (Chesnel *et al*., 2006), which explains why this cyclin B pool is mitotically inert. On the other hand, Holloway and colleagues (1993) have demonstrated basic differences in the way of action of N-terminal mutants and N-terminal fragments of cyclin B. Namely, the first ones participate in formation of their own MPF activity via association with endogenous CDK1 since degradation box deprived, they are not recognized by the ubiquitin-proteasome pathway. They do not prevent endogenous cyclins proteolysis and substitute for the endogenous pool of cyclins, therefore sister chromatid separation occurs without M-phase exit as degradation of other proteasome substrates is not affected. N-terminal fragments possessing degradation box sequences are recognized by the ubiquitin-proteasome pathway, they act as specific competitors for cyclin degradation, induce a delay both in MPF inactivation as well as in sister chromatids separation (Holloway *et al*., 1993). Therefore, differential action of each of these cyclins in *Xenopus* cell-free extracts is comprehensive.

Separation of cyclin B2 from CDK1 before degradation of the cyclin component is not surprising in the light of well documented data concerning a similar fate of other partners present within MPF complex, namely the CDK1 inhibitors (Schwob *et al*., 1994; Verma *et al*., 1997). Hence, the proteasome has apparently a capacity to degrade individual proteins and not the proteinous complexes.

In this paper we do not question the major role of cyclins B and specially cyclin B2, degradation for the normal course of the cell cycle. It should be, however, taken into account that the proteolytic degradation of these proteins is relatively late phenomenon of the mitotic exit and in any way it does not participate in MPF inactivation *per se* contrary to widely accepted model that cyclins B degradation triggers this process.

Material and Methods

Frogs and drugs

Xenopus laevis females were purchased from NASCO (Fort Atkinson, WI, USA). Cycloheximide (CHX) was purchased from Sigma (Irvine, CA, USA). Other chemicals were obtained either from Sigma or ICN (Irvine, CA, USA) unless otherwise stated.

Eggs collection and activation

Females were subcutaneously injected with human chorionic gonadotropin (500-600 IU per female; Organon, Puteaux, France) and kept overnight at 21°C in 110 mM NaCl. Unfertilized eggs collected from "overnight lay" were dejellied with 2% L-cysteine pH 7.81 in XB buffer (Murray, 1991; 100 mM KCl, 1 mM $MgCl_2$, 50 μM $CaCl_2$, 10 mM HEPES, 50 mM sucrose pH 7.6), washed in XB, treated for 1.5 minute with 0.5 μg/ml calcium ionophore A23187 and then extensively washed in XB. Eggs were then incubated in XB at 21°C.

Cell-free extracts

Cytoplasmic extracts from calcium ionophore-activated embryos before the first embryonic mitosis were prepared according to Murray (1991) with modifications previously described in Chesnel *et al*. (2005). These low-speed supernatants were then re-incubated at 21°C for 60 min and every 5 min, aliquots were taken out and either frozen in liquid nitrogen and stored at -70°C (for subsequent H1 kinase activity assays or protein (immuno-precipitations) or mixed with Laemmli sample buffer (Laemmli *et al.*, 1970), heated at 85°C for 5 minutes and stored at -20°C (for Western blot analyses).

Electrophoresis, antibodies and Western blotting

Extracts were subjected to electrophoresis on 8 to 12.5% SDS-PAGE gels (Laemmli *et al.*, 1970). Separated proteins were transferred to nitrocellulose membranes (Hybond C, Amersham Biosciences) accord-

ing to standard procedures and probed either with antibodies against cyclin B2 (gift from Thierry Lorca), MCM4 (gift from Marcel Méchali), CDK1 (gift from Tim Hunt), CDK1 Thr-161 (Cell Signalling Technology, gift from Robert Poulhe) and β-tubuline (Sigma). Antigen–antibody complexes were revealed using alkaline phosphatase conjugated anti-rabbit or anti-mouse secondary antibody (diluted 1:20,000) in combination with Enhanced Chemifluorescence reagent (ECF; Amersham Biosciences). Signal quantification was performed using ImageQuant 5.2 software (Amersham Biosciences).

In vitro *assay for histone H1 kinase activity*

MPF activity in embryos or in cell-free extracts was measured as previously described (Chesnel *et al.,* 1997) with minor modifications: extracts (1 µl) were diluted in 25 µl MPF buffer (80 mM β-glycerophosphate, 50 mM sodium fluoride, 20 mM EGTA, 15 mM $MgCl_2$, 1 mM DTT, 20 mM HEPES, pH 7.4) supplemented with 0.5 mM sodium orthovanadate and 5 µg/µl of leupeptin, aprotinin, pepstatin and chymostatin and containing 0.4 mg/ml H1 histone (type III-S), 1 µCi [γ^{32}P] ATP (specific activity: 3000 Ci/mmol; Amersham Biosciences) and 0.8 mM ATP. After incubation for 30 minutes at 30°C, phosphorylation reactions were stopped by adding Laemmli sample buffer and heating for 5 minutes at 85°C. Histone H1 was separated by SDS-PAGE and incorporated radioactivity was measured by autoradiography of the gel using a STORM phosphorimager (Amersham Biosciences) followed by a data analysis with ImageQuant 5.2 software.

Cyclin B2 immunoprecipitation

For immunoprecipitation, 10 µl of extracts were diluted 20 times in IP buffer (20mM Tris-HCl pH 7.5, 150 mM NaCl, 50 mM β-glycerophosphate, 50 mM sodium fluoride, 2 mM EDTA, 10% glycerol, 0.2% nonidet-P40) extemporaneously supplemented with 0.5 mM AEBSF, aprotinin, leupeptin, pepstatin (5 µg/ml each) and 0,5 mM sodium orthovanadate. A 5µl-aliquot of this diluted extract ("*total*") was collected to be analyzed by Western blotting and purified anti-cyclin B antibody was added to the extract. After a 1hr-incubation on ice, 10 µl protein A Affiprep beads (Bio-Rad) pre-equilibrated in IP buffer were added and the mixture was agitated for 12 h at 4°C. After centrifugation (1,000 g, 3 min, 4°C), the supernatant (*"unbound"*) was collected to be analyzed by Western blotting while the pelleted beads were washed four times with 1 ml of IP buffer and once with 20 mM Tris-HCl pH 7.5; 150 mM NaCl. The beads were then resuspended in 10 µl of 2x Laemmli sample buffer and heated at 85°C for 5 min. Samples ("*total*", "*unbound*" and "*bound*") were then subjected to 12% SDS-PAGE and cyclin B and CDK1 were detected by Western blotting.

CDK1 precipitation with p9 beads

The p9 sepharose beads used for affinity precipitation were kindly provided by Lénaïck Detivaud and Laurent Meijer (Roscoff). Ten µl of extracts were added to 10 µl p9 beads pre-equilibrated with homogeneizing buffer (MOPS pH 7.2, 60 mM β-glycerophosphate, 15 mM EGTA, 15 mM $MgCl_2$, 2 mM dithiothreitol, 1 mM sodium fluoride, 1 mM sodium orthovanadate and 1 mM disodium phenyl phosphate) extemporaneously supplemented with 1% BSA, 1mM AEBSF and aprotinin, leupeptin, pepstatin, chymostatin (10 µg/ml each). The mixtures were agitated for 2.5 h at 4°C. After a brief centrifugation (5,000 g, 1 min, 4°C), the supernatant ("*unbound*") was collected to be analyzed by Western blotting while the pelleted p9 beads ("*bound*") were washed four times with 1 ml of washing buffer (50 mM Tris-HCl pH 7.4, 250 mM NaCl, 5 mM EDTA, 5 mM EGTA, 5mM sodium fluoride and 0.1% nonidet-P 40) containing 0.5 mM AEBSF, aprotinin, leupeptin, pepstatin, chymostatin (10 µg/ml each). The beads were then resuspended in 12 µl of 2x Laemmli sample buffer and heated at 85°C for 5 min. Samples *("total", "unbound"* and *"bound")* were then subjected to 12% SDS-PAGE and cyclin B and CDK1 were detected by Western blotting.

Statistics

The relationship between activity of histone H1 kinase and the level of cyclin B2 was analysed using paired t-test (http://faculty.vassar.edu/lowry/VassarStats.htm).

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