MPF localization is controlled by nuclear export

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In eukaryotes, mitosis is initiated by M phase promoting factor (MPF), composed of B-type cyclins and their partner protein kinase, CDK1. In animal cells, MPF is cytoplasmic in interphase and is translocated into the nucleus after mitosis has begun, after which it associates with the mitotic apparatus until the cyclins are degraded in anaphase. We have used a fusion protein between human cyclin B1 and green fluorescent protein (GFP) to study this dynamic behaviour in real time, in living cells. We found that when we injected cyclin B1–GFP, or cyclin B1–GFP bound to CDK1 (i.e. MPF), into interphase nuclei it is rapidly exported into the cytoplasm. Cyclin B1 nuclear export is blocked by leptomycin B, an inhibitor of the recently identified export factor, exportin 1 (CRM1). The nuclear export of MPF is mediated by a nuclear export sequence in cyclin B1, and an export-defective cyclin B1 accumulates in interphase nuclei. Therefore, during interphase MPF constantly shuttles between the nucleus and the cytoplasm, but the bulk of MPF is retained in the cytoplasm by rapid nuclear export. We found that a cyclin mutant with a defective nuclear export signal does not enhance the premature mitosis caused by interfering with the regulatory phosphorylation of CDK1, but is more sensitive to inhibition by the Wee1 kinase.

Keywords: Cdc2/cyclin/GFP/mitosis/nuclear export

Introduction

To enter mitosis, eukaryotic cells activate MPF, the cyclin B/CDK1 protein kinase (Nurse, 1990). Prior to mitosis, cyclin B accumulates in late S and G₂ phase cells, through inactivation of the cyclin B proteolysis machinery and an increase in the rate of transcription of its mRNA. Cyclin B1 binds to CDK1 and a pool of cyclin B/CDK1 complexes accumulates that is maintained in an inactive form by the Wee1/Myt1 protein kinases (Dunphy, 1994; Morgan, 1995). The Wee1 protein kinase family negatively regulate CDK1 by phosphorylating residues in its ATP-binding site, Tyr15 and Thr14. The Wee1 protein kinase phosphorylates Tyr15 on CDK1 in both yeast and animal cells (Heald *et al.*, 1993; McGowan and Russell, 1995;

Watanabe *et al.*, 1995), and this has been proposed to interfere with the transfer of phosphate from CDK1 to a bound substrate (Atherton Fessler *et al.*, 1993). In fission yeast, phosphorylation on Tyr15 is important for the proper coordination of DNA replication and mitosis. Fission yeast cells with a mutant CDK1 that cannot be phosphorylated on Tyr15 are unable to stop before mitosis in the presence of unreplicated DNA (Gould and Nurse, 1989). In budding yeast, phosphorylation on the analogous Tyr19 is important in the coordination of DNA replication with budding (Lew and Reed, 1995). In animal cells, CDK1 is additionally phosphorylated on Thr14 as well as Tyr15 by the Myt1 protein kinase (Mueller *et al.*, 1995; Liu *et al.*, 1997). Myt1 is a member of the Wee1 protein kinase family but differs markedly from Wee1 in its localization. Whereas Wee1 is a nuclear protein, Myt1 is a cytoplasmic membrane-bound protein, mostly associated with the endoplasmic reticulum and the Golgi apparatus (Kornbluth *et al.*, 1994; Mueller *et al.*, 1995; Liu *et al.*, 1997). Phosphorylation on Thr14 and Tyr15 have both been implicated in preventing mitosis in the presence of damaged DNA (Jin *et al.*, 1996).

The Cdc25 phosphatases activate MPF, and thereby trigger mitosis, by dephosphorylating Thr14 and Tyr15 on CDK1. In animal cells there are three known Cdc25 proteins; Cdc25A, -B and -C. Cdc25A is thought to be involved at the G₁ to S control point, but both Cdc25B and Cdc25C have been implicated in the initiation of mitosis. Like Wee1 and Myt1, Cdc25B and -C differ in their intracellular localization. Cdc25C is a nuclear protein (Millar *et al.*, 1991; Girard *et al.*, 1992), whereas Cdc25B is cytoplasmic (Nagata *et al.*, 1991; Gabrielli *et al.*, 1996; Nishijima *et al.*, 1997). There are three different forms of Cdc25B that arise through alternative splicing, although no functional differences have been observed between them (Baldin *et al.*, 1997). Damaged and unreplicated DNA negatively regulate MPF via T14 and Y15 phosphorylation. Damaged DNA appears to act, at least in part, via the Chk1 protein kinase which phosphorylates Cdc25C on Ser216 and creates a binding site for a 14-3-3 protein (Furnari *et al.*, 1997; Peng *et al.*, 1997; Sanchez *et al.*, 1997). It is not known how binding to a 14-3-3 protein downregulates Cdc25C because its phosphatase activity *in vitro* is unchanged (Peng *et al.*, 1997), but the observation that 14-3-3 σ is a perinuclear protein (Hermeking *et al.*, 1997) led to the suggestion that Cdc25C may be physically sequestered by the 14-3-3 proteins.

It has been unclear how these nuclear proteins are able to downregulate MPF in animal cells, because MPF appeared to be exclusively cytoplasmic throughout interphase (Pines and Hunter, 1991; Gallant and Nigg, 1992; Ookata *et al.*, 1992, 1995). In human and chicken tissue culture cells, and in starfish oocytes, MPF accumulates in the cytoplasm, in association with microtubules

Fig. 1. Cyclin B1-MmGFP behaves in a very similar fashion to endogenous cyclin B1 throughout the cell cycle. HeLa cells were synchronized at G₁/S, released from the block and at specific cell cycle stages either fixed in methanol/acetone and stained with anti-cyclin B1 antibodies (A, red), or microinjected with a cDNA construct expressing cyclin B1-MmGFP from the CMV promoter and the GFP signal visualized in the living cells (B, green). Fixed and living cells were analysed by confocal microscopy. (C) Cyclin B1-MmGFP degradation. Cyclin B1-MmGFP purified from baculovirus-infected Sf9 cells was concentrated to 20 mg/ml and microinjected into the cytoplasm of HeLa cells in G₂. A fluorescence image was captured every 3 min using an exposure of 250 ms. The decrease in intensity is due to degradation, and not bleaching, because no decrease was observed in fluorescence intensity until the cells entered mitosis. Scale bar = 10 μ m.

and centrosomes, until late prophase when it translocates to the nucleus. Soon after this, the nuclear envelope breaks down, implying that the cyclin B/CDK1 complex acts as the nuclear lamin kinase. A clue to understanding this behaviour came from studies showing that the N terminus of cyclin B1 is required to keep the protein in the cytoplasm (Pines and Hunter, 1994), where it may be sequestered by interaction with the microtubule-associated protein, MAP4 (Ookata *et al.*, 1995). Furthermore, phosphorylation sites in the N terminus are essential for cyclin B to trigger meiotic maturation in frog oocytes, and there are some data to suggest that this may be because they are required for cyclin B translocation into the nucleus (Li *et al.*, 1995, 1997). In order to understand the mechanisms behind the dynamic behaviour of MPF we have developed a real-time assay for the localization of cyclin B1 throughout the cell cycle. We have generated fusion proteins between cyclin B1 and MmGFP and characterized its behaviour in living cells. We have found that cyclin B1/CDK1 complexes can shuttle between the nucleus and the cytoplasm throughout interphase. However, we found that a mutant cyclin B1/CDK1 that accumulated in the nucleus did not enhance the number of cells forced into premature mitosis when the phosphorylation of CDK1 was perturbed, although it did affect the

balance between the antagonistic actions of Cdc25C and Wee1 on cyclin B1/CDK1 activation.

Results

To study the dynamics of MPF in real time we have generated a chimera between human cyclin B1 and a modified form of the *Aequorea victoria* green fluorescent protein (MmGFP) (Zernicka-Goetz *et al.*, 1996). We linked MmGFP to the C terminus of cyclin B1 (see Materials and methods), and placed the cDNA under the control of the cytomegalovirus (CMV) promoter to express it in tissue culture cells. For other experiments we expressed and purified the chimera from insect cells using the baculovirus system (see Materials and methods). We injected the cyclin B1-MmGFP chimera as cDNA or protein into HeLa cells and into normal human diploid fibroblasts, and followed its behaviour through the cell cycle by time-lapse fluorescence microscopy using a CCD camera, or by confocal microscopy. We found that the behaviour of cyclin B1-MmGFP was very similar in both types of cell, but only the HeLa cell images are shown here.

To validate the cyclin B1-MmGFP chimera as a live cell marker for endogenous cyclin B1, we compared the behaviour of cyclin B1-MmGFP through the cell cycle in

living cells, with that of endogenous cyclin B1 in fixed cells as revealed by anti-cyclin B1 immunofluorescence (Figure 1A and B). During S and G₂ phases, both endogenous cyclin B1 and cyclin B1–MmGFP localized to the cytoplasm, where a subpopulation associated with microtubules. In late G₂ phase, a fraction of the endogenous cyclin B1 and of cyclin B1–MmGFP was bound to the centrosomes, and migrated to opposite sides of the nucleus. Just after this event, at prophase, the endogenous cyclin B1 and cyclin B1–MmGFP translocated into the nucleus. Live-cell imaging revealed that this process occurred over a period of ~10 min, and ~20 min later we observed nuclear envelope breakdown. Cyclin B1 and cyclin B1–MmGFP then bound to the mitotic apparatus, in particular to the spindle poles and the main spindle fibres. Cyclin B1–MmGFP also transiently associated with condensed chromosomes at the beginning of metaphase. Chromosome association could be seen only occasionally for endogenous cyclin B1 in paraformaldehyde-fixed cells, although it was more consistently observed in methanol/acetone-fixed cells. Once cells entered anaphase, cyclin B1–MmGFP was rapidly degraded (Figure 1C) with similar kinetics to the endogenous cyclin B1 (not shown; Pines and Hunter, 1991).

These results suggested that cyclin B1–MmGFP was a valid marker in living cells for the behaviour of endogenous cyclin B1. We obtained further evidence that cyclin B1–MmGFP behaves in the same way as endogenous cyclin B1 by comparing the pattern of cyclin B1–MmGFP before and after fixing cells for immunofluorescence. We found that there were no marked changes in the appearance of cyclin B1–MmGFP in living compared with fixed cells, although fine detail was lost in the fixed samples (Figure 2A). We eliminated the possibility that the MmGFP moiety caused cyclin B1 to localize to places where the endogenous protein was not present, or conversely that MmGFP prevented cyclin B1 from associating with specific subcellular structures, by staining cells expressing cyclin B1–MmGFP with anti-cyclin B1 antibodies. We found that the GFP and the anti-cyclin B1 stainings were identical (Figure 2B).

To determine whether the cyclin B1–MmGFP fusion protein was capable of binding and activating its partner CDK, we expressed it in baculovirus-infected Sf9 cells co-expressing human CDK1. Cyclin B1–MmGFP efficiently bound and activated human CDK1 in baculovirus cells, and the active complex could subsequently be purified to homogeneity (Figure 2C). In summary, by all the assay criteria used, cyclin B1–MmGFP acted as a proper marker for the behaviour of endogenous cyclin B1.

We wished to use real-time imaging to determine the effect of introducing cyclin B1 into the nucleus at the wrong time in the cell cycle. Therefore, we microinjected purified cyclin B1–MmGFP into the nucleus of S or G₂ phase HeLa cells, and observed the cells by time-lapse fluorescence video microscopy. Unexpectedly, we found that cyclin B1–MmGFP was rapidly and completely exported from the nucleus within 10 min of microinjection (Figure 3A), suggesting that it had a nuclear export signal. Evidence that the cells were undamaged by this treatment, and that the cyclin B1–MmGFP was biologically active, was provided by its subsequent translocation into the nucleus when the cells later entered mitosis. The nuclear

export signal appeared to reside within cyclin B1, because MmGFP alone diffused only gradually out of the nucleus (Figure 3B), and neither cyclin A–MmGFP nor cyclin E–MmGFP, both of which are nuclear cyclins, was exported from the nucleus (data not shown). The cyclin B1 export signal was still recognized when cyclin B1–MmGFP was injected into nuclei in a complex with its partner kinase, CDK1 (Figure 3C), and both cyclin B1–MmGFP and cyclin B1–MmGFP/CDK1 were exported with similar kinetics from the nuclei of both transformed and primary cells (data not shown).

The cytoplasmic localization of cyclin B1 requires a region in its N terminus called the cytoplasmic retention signal (CRS; Pines and Hunter, 1994). Therefore, we tested whether the CRS was responsible for the nuclear export of cyclin B1. We fused the first 157 amino acids of cyclin B1, which includes the CRS, to MmGFP, purified the protein from bacteria, and microinjected it into the nuclei of S or G₂ phase cells. We found that this fusion protein was rapidly exported from the nucleus (Figure 4A), whereas a fusion protein between MmGFP and the first 129 amino acids of cyclin B1, without the CRS, diffused only gradually out of the nucleus (Figure 4B). Nuclear export has recently been shown to be blocked by the Ran T²⁴N mutant (Richards *et al.*, 1997); thus, we tested whether cells expressing Ran T²⁴N were able to export cyclin B1. We found that cyclin B1–MmGFP export was inhibited in these cells (Figure 4C) and, furthermore, that cyclin B1–MmGFP began to accumulate in the nucleus (Figure 4D), showing that nuclear import was not blocked. Overexpressing wild-type Ran had no effect on cyclin B1 export (not shown).

Although the CRS does not have a recognizable leucine-rich nuclear export signal (NES) (Fukuda *et al.*, 1996), other hydrophobic residues can substitute for leucines in the NES of the HTLV Rex protein (Kim *et al.*, 1996). The CRS of cyclin B1 does have a hydrophobic region of 11 amino acids that is highly conserved in all animal B-type cyclins (Figure 5A), except for the nuclear cyclin B3. Therefore, we asked whether this region could act as an NES. The hydrophobic residues within an NES are critical for nuclear export (Fischer *et al.*, 1995; Wen *et al.*, 1995; Fukuda *et al.*, 1996), so we made two mutant versions of the putative NES in cyclin B1, changing the conserved Phe146, or Val149 and Leu151, to alanines (Figure 5A). Fusion proteins between MmGFP and the N terminus of cyclin B1 carrying either the (Phe146Ala), or the (Val149Ala/Leu151Ala) mutation, were injected into the nuclei of S or G₂ phase cells. We found that either of the mutations completely inhibited export of the fusion proteins (Figure 5B and data not shown). Moreover, when expressed from cDNAs, either of these mutations caused the full-length cyclin B1–MmGFP to accumulate gradually in the nucleus (Figure 5C and data not shown), whereas the wild-type cyclin B1–MmGFP remained in the cytoplasm (Figure 5C). Thus, it appears that the cytoplasmic localization of cyclin B1 is due to rapid nuclear export which is required to counteract its constitutive nuclear import.

Recently, nuclear export has been shown to be mediated by at least two different 'export factors': exportin1/CRM1, which is required for the export of the HIV Rev protein and IκBα (Fornerod *et al.*, 1997; Ossareh-Nazari *et al.*, 1997; Stade *et al.*, 1997), and exportin 2/CAS

which is responsible for the export of importin α (Kutay *et al.*, 1997). Nuclear export mediated by exportin 1 is inhibited by the drug leptomycin B (Nishi *et al.*, 1994; Fornerod *et al.*, 1997; Ossareh-Nazari *et al.*, 1997; Wolffe *et al.*, 1997). Therefore we tested whether exportin 1 was involved in the export of cyclin B1 by injecting cyclin B1-MmGFP into the nuclei of S or G₂ phase cells in the presence or absence of leptomycin B (kind gift from M. Yoshida). We found that 2 nM (data not shown) to 20 nM (Figure 6) leptomycin B completely inhibited the export of cyclin B1-MmGFP within 5 min. Furthermore, cyclin B1-MmGFP injected into the cytoplasm accumulated in the nucleus in the presence of leptomycin B (Figure 6).

We found that the export-defective mutant of cyclin B1 had no apparent effect on progress through the cell cycle. This could be because the Wee1 kinase inactivated cyclin B1/CDK1 in the nucleus by phosphorylating CDK1 on Y15. Conversely, keeping cyclin B1/CDK1 in the cytoplasm has been proposed as a mechanism to protect the nucleus from premature mitosis if phosphorylation on CDK1 is compromised (Heald *et al.*, 1993; Jin *et al.*, 1996). For example, only a minority (~12%) of cells prematurely entered mitosis when they expressed phosphorylation site mutants of CDK1 (T14A, Y15F) that cannot be downregulated by Wee1. Similarly, only a minority of cells (~30%) entered prematurely into mitosis when cyclin B1 was overexpressed with Cdc25C, and this effect was suppressed by co-expressing Wee1 (Heald *et al.*, 1993). In both cases, the majority of cells regulated mitosis properly, perhaps because they were able to export active cyclin B1/CDK1 to the cytoplasm.

If MPF export was used to prevent premature mitosis, then cells expressing the nuclear export-defective mutant of cyclin B1 should be more sensitive to CDK1 mutants, and/or to overexpressing Cdc25C. We tested both of these conditions. We synchronized cells in S phase and microinjected cDNAs encoding either wild-type cyclin B1, or the export-defective mutant of cyclin B1, and cDNAs encoding proteins to perturb the regulation of CDK1; either the T14A/Y15F mutant of CDK1, or wild-type Cdc25C or the S216G mutant of Cdc25C—which is unable to be downregulated by the Chk1 protein kinase (Furnari *et al.*, 1997; Peng *et al.*, 1997; Sanchez *et al.*, 1997). Both the wild-type Cdc25 and its S216G mutant were localized to the nucleus as GFP fusion proteins (data not shown). The cDNAs were microinjected within 1–2 h after release from an aphidicolin block, and the cells were followed by time-lapse DIC microscopy. The number of cells that prematurely began mitosis was counted at 30-min intervals throughout G₂. The final percentage of injected cells that prematurely entered mitosis was calculated at 10 h after release from the aphidicolin block. The bulk of normal mitoses began 10–12 h after release from the S phase block. At the end of the experiment we

confirmed that the cells had abnormally condensed chromosomes by staining them with Hoechst 33342 (Figure 7A).

We found that, under our experimental regime, cells expressing wild-type cyclin B1 were equally as sensitive as cells expressing an export-defective cyclin B1 to the effects of the T14A,Y15F mutations in CDK1 (Figure 7B). We also found that wild-type Cdc25C caused a very similar proportion of cells (~40%) to enter mitosis prematurely when co-expressed with either wild-type cyclin B1 or an export-defective cyclin B1. The S216G mutant of Cdc25C caused a greater proportion of cells to enter mitosis (~60%), but again it made little difference whether they were expressing the wild-type or an export-defective cyclin B1 (Figure 7C). However, a much greater percentage of the cells expressing the export-defective cyclin B1 was able to be rescued by co-expressing Wee1 (Figure 7C), and this rescue was dose-dependent (Figure 7C). These results suggested that premature mitosis was caused by changing the balance between the antagonistic actions of Wee1 and Cdc25C on overexpressed cyclin B1/CDK1, and that cyclin B1/CDK1 export can affect this balance.

Fig. 3. Cyclin B1 is exported out of the nucleus. His₆-tagged proteins were expressed in, and purified from, baculovirus-infected Sf9 cells and microinjected into the nuclei of G₂ phase HeLa cells. The fluorescence images were taken at the indicated time points. Arrows indicate cells with injections into the nucleus. Representative results of at least three different experiments for each protein are shown. (A) Cyclin B1-MmGFP, 250 ms exposure. (B) MmGFP, 500 ms exposure. (C) Cyclin B1-MmGFP/CDK1^{K33R}, 750 ms exposure with 1.0 neutral density filter. Scale bar = 10 μ m.

Fig. 2. Cyclin B1-MmGFP is a valid marker for endogenous cyclin B1. (A) HeLa cells were injected with a cDNA construct expressing cyclin B1-MmGFP from the CMV promoter and analysed by confocal microscopy before (top) and after (bottom) fixing the cells in paraformaldehyde and permeabilizing cells in Triton X-100. (B) HeLa cells expressing cyclin B1-MmGFP were fixed in paraformaldehyde and stained with anti-cyclin B1 antibodies. The GFP signal (left panel) and the anti-cyclin B1 signal (middle panel) were analysed by confocal microscopy. The two staining patterns are identical, as shown by absence of either a green or a red signal in the merged image (right panel). (C) Specific activities of baculovirus-expressed and purified cyclin B1/CDK1, cyclin B1-MmGFP/CDK1 and CDK1. Values are given as pmol phosphate transferred to histone H1/min per 15 min incubation at 30°C/ μ g purified protein. The inset shows a silver-stained 15% SDS-PAGE gel of purified cyclin B1-MmGFP/CDK1. The positions of molecular mass markers (kDa) are indicated on the left.

Fig. 4. The CRS region is required for nuclear transport of cyclin B1, and export is inhibited by Ran T²⁴N. His₆-tagged fusion proteins between MmGFP and residues (1–157) or (1–129) of cyclin B1 were expressed in *E.coli*, purified on Ni-agarose, and microinjected into the nuclei of G₂ phase HeLa cells. Fluorescence images were taken at the indicated time points after injection of the protein. **(A)** Cyclin B1 (1–157)–MmGFP, 100 ms exposure. **(B)** Cyclin B1 (1–129)–MmGFP, 50 ms exposure. **(C)** Cyclin B1 (1–157)–MmGFP in cells expressing Ran T²⁴N, 100 ms exposure. Ran T²⁴N cDNA driven by the CMV promoter was microinjected into nuclei 3 h before injection of cyclin B1 (1–157)–MmGFP protein into the nuclei of G₂ phase HeLa cells. To identify cells injected with Ran T²⁴N, the expression construct was co-injected with TRITC–dextran. **(D)** Cyclin B1 (1–157)–MmGFP in the presence or absence of Ran T²⁴N, 100 ms exposure. Filled arrowheads indicate a cell in which Ran T²⁴N cDNA was microinjected into nuclei of HeLa cells and then after 3 h cyclin B1 (1–157)–MmGFP protein was injected into the cytoplasm. Open arrowheads show a control cell not expressing Ran T²⁴N, injected with cyclin B1 (1–157)–MmGFP protein into both the cytoplasm and the nucleus. The third cell (no arrow) is another control cell not expressing Ran T²⁴N, injected with cyclin B1 (1–157)–MmGFP into the cytoplasm. Representative cells of at least three different experiments are shown for all panels. Scale bar = 10 μm.

Discussion

In this paper we have shown that a fusion protein between human cyclin B1 and a modified form of GFP is a valid

live-cell marker for the behaviour of endogenous cyclin B1 in human cells. The fusion protein binds and activates CDK1, localizes correctly throughout the cell cycle, and is degraded when cells enter anaphase. Using this marker,

Fig. 5. Nuclear export of cyclin B1 is blocked by mutations within the putative NES. (A) Alignment of the predicted nuclear export sequences in B-type cyclins with known nuclear export signals. The sequences of B-type cyclins from the sea urchin *Arbacia punctulata* (Ap), the starfish *Asterina pectinifera* (Sf), the clam *Spissula solidissima* (Ss), B1 cyclins from frog (XlB1) and human (HsB1), chicken cyclin B2 (GgB2), and the known nuclear export sequences of the human immunodeficiency virus 1 (REV) and a protein kinase inhibitor (PKI) were obtained from DDBJ/EMBL/GenBank databases and aligned. Conserved hydrophobic residues are shown in bold type. Dots indicate the residues of human cyclin B1 which were mutated to alanines. (B) The His₆-tagged fusion protein between MmGFP and residues (1–157) of cyclin B1 carrying the F¹⁴⁶A mutation was expressed in *E. coli*, purified on Ni-agarose, and microinjected into the nucleus (indicated by the arrow) or cytoplasm of G₂ phase HeLa cells. Fluorescence images were taken at the indicated time points after injection, 250 ms exposure. Representative results of at least three different experiments are shown. (C) Expression constructs of the cDNAs encoding full-length cyclin B1–MmGFP with, or without (wild-type), the F¹⁴⁶A mutation and driven by the CMV promoter, were injected into the nuclei of G₂ phase HeLa cells. Fluorescence images were taken at the indicated time points after injection, 250 ms (F146A) or 500 ms (wild-type) exposures. Representative results of at least three different experiments are shown. Scale bar = 10 μm.

we have determined that human cyclin B1 constantly shuttles between the nucleus and the cytoplasm of interphase cells. However, cyclin B1 appears to accumulate in the cytoplasm because its nuclear import is counteracted by more rapid nuclear export. Cyclin B1 nuclear export is mediated by an atypical hydrophobic NES in the CRS, and requires a functional exportin 1/CRM1 protein. The NES is well conserved in all animal cell B-type cyclins (Figure 3), but absent in the nuclear cyclin B3 (Gallant and Nigg, 1994; Kreutzer *et al.*, 1995; Sigrist *et al.*, 1995). The sequence differs slightly in the cyclin B2 subfamily, where the valine is altered to alanine, but this sequence is still able to confer nuclear export on GFP (not shown). While this paper was under review, Nishida and colleagues identified the same sequence in cyclin B1 as an export signal (Toyoshima *et al.*, 1998), although they differ in concluding that cyclin B1 export makes cells less sensitive to premature mitosis (see below).

Cyclin B1 export is sensitive to leptomycin B (LMB), indicating that it depends upon exportin 1, and that cyclin B1 might interact directly with exportin 1. However, *in vitro* binding assays using human exportin 1 and Ran/TC4 did not reveal an interaction with human cyclin

Fig. 6. Leptomycin B inhibits nuclear export of cyclin B1. G₂ phase HeLa cells were incubated with 20 nM leptomycin B for 5 min, and then purified cyclin B1–MmGFP was microinjected into the nucleus or cytoplasm. Fluorescence images were taken at the indicated time points, 250 ms exposure. The arrowhead indicates a cell with injections into the nucleus. Representative results of at least three different experiments are shown. Scale bar = 10 μm.

B1 (M.Fornerod and I.Mattaj, personal communication; A.Hagting and K.Simpson, data unpublished). Therefore, cyclin B1 may bind with another exportin that is a target

Fig. 7. Accumulating cyclin B1/CDK1 in the nucleus does not make cells more prone to premature mitosis. **(A)** Fluorescence images of the DNA stained with Hoechst 33342 of cells expressing cyclin B1 with the T14A/Y15F mutant of CDK1 (left panel) or with the S216G mutant of Cdc25C (right panel). The abnormally condensed chromatin of cells that prematurely entered mitosis are arrowed. **(B)** S phase HeLa cells were microinjected with cDNAs encoding wild-type CDK1, or a T14A/Y15F mutant of CDK1, with or without wild-type cyclin B1-GFP or a F¹⁴⁶A mutant of cyclin B1, and the number of cells entering prematurely into mitosis was assayed. Approximately 100 fluorescent cells were counted for each sample. The mean values and deviations from the mean from at least two independent experiments are shown. **(C)** S phase HeLa cells were microinjected with cDNAs encoding the S216G mutant of Cdc25C, with or without wild-type cyclin B1-GFP or a F¹⁴⁶A mutant of cyclin B1, and with or without 0.1 μg/ml or 0.02 μg/ml of human Wee1, and the number of cells entering prematurely into mitosis was assayed. Wee1 reproducibly rescued a greater percentage cells with the export-defective cyclin B1, and the mean values and deviations from the mean from at least three independent experiments are shown. Approximately 100 fluorescent cells were counted for each sample.

of, or is indirectly inhibited by, LMB. Alternatively, cyclin B1 may bind to exportin 1, but binding requires a cofactor, or post-translational modification of exportin 1 or cyclin B1. A direct interaction between *Xenopus* cyclin B1 and exportin 1 has been detected in frog egg extracts (Yang *et al.*, 1998).

Cyclin B1/CDK1 is not transported into the nucleus by the classical bipartite or SV40-type NLS-mediated pathway. Neither cyclin B1 nor CDK1 has either of these motifs. Instead, we have recently shown that when a

mitotic cyclin binds to its CDK partner this creates a novel nuclear localization signal, such that purified mitotic cyclin/CDK complexes are recognized and imported into nuclei in an importin/karyopherin α -independent manner (Kubota *et al.*, 1998). Thus, cyclin B1/CDK1 will constantly shuttle between the nucleus and the cytoplasm, and this has a number of implications. First, the constitutive nuclear import of cyclin B1/CDK1 means that there may be no need to invoke a second, cytoplasmic, form of the CDK-activating kinase (CAK). The nuclear cyclin

H/CDK7 complex previously identified as CAK (Solomon *et al.*, 1993; Fisher and Morgan, 1994; Tassan *et al.*, 1994) would be able to phosphorylate newly synthesized cyclin B/CDK1 when it is imported into the nucleus. Secondly, cyclin B1/CDK1 is potentially capable of responding to both nuclear and cytoplasmic signals and coordinate the entry into mitosis of the nucleus and the cytoplasm. Nuclear and cytoplasmic influences on cyclin B1/CDK1 could be executed by the two sets of regulators in animal cells that act on the Thr14 and Tyr15 residues in the ATP-binding site of CDK1. In the nucleus, cyclin B1/CDK1 can be inhibited by the Wee1 protein kinase (Heald *et al.*, 1993; McGowan and Russell, 1995; Watanabe *et al.*, 1995), and activated at mitosis by the protein phosphatase Cdc25C (Millar *et al.*, 1991; Girard *et al.*, 1992), whereas in the cytoplasm, the negative regulator is the Myt1 protein kinase (Mueller *et al.*, 1995; Liu *et al.*, 1997) and the activator appears to be the Cdc25B protein phosphatase (Nagata *et al.*, 1991; Gabrielli *et al.*, 1996). These two sets of regulators could be subject to different signalling pathways and, because they both regulate cyclin B1/CDK1, this might allow the chromosome cycle to be coordinated with cytoplasmic cycles such as the centrosome cycle.

Nuclear-cytoplasmic shuttling may be an important mechanism to coordinate a number of components of the cell cycle machinery. For example, immunofluorescence studies show that in fission yeast the wee1 protein kinase is nuclear, but it is negatively regulated by the nim1 kinase, which is a cytoplasmic protein (Wu and Russell, 1997).

We found that whether cells were overexpressing a nuclear cyclin B1/CDK1 or wild-type cyclin B1, they appeared to be equally as sensitive to a non-phosphorylatable mutant of CDK1. Moreover, we found that, in contrast to other studies (Krek and Nigg, 1991; Jin *et al.*, 1996) overexpressing the T14A/Y15F mutant of CDK1 alone did not cause a significant proportion of cells to enter mitosis prematurely; this required cyclin B1 to be co-expressed. These differences may be due to the amount of protein produced, or to the length of time the proteins are overexpressed. However, even with co-expression of cyclin B1, the non-phosphorylatable mutant of CDK1 still caused only a minority of cells to enter mitosis prematurely, suggesting that there must be some other way for cells to regulate mitosis in addition to phosphorylation on T14 and Y15 of CDK1. This could be explained by a threshold effect, in which cyclin B1/CDK1 gradually titrates out a G₂ phase inhibitor. Candidates for inhibitors of the mitotic kinases are already known in yeast (Sic1) (Donovan *et al.*, 1994) and in *Xenopus* (Lee and Kirschner, 1996). While this paper was under review, Morgan and colleagues (Jin *et al.*, 1998) have reported that, in agreement with our findings, the CDK1AF mutant only caused cells to enter mitosis when co-expressed with cyclin B1. However, in contrast to our results, these authors report that a nuclear-targeted cyclin B1 is more effective than wild-type B1 in causing premature mitosis. This difference may be due to experimental regime, or to a difference between a nuclear-targeted cyclin B1 compared with an export-defective cyclin B1.

We found that there was a difference between cells expressing wild-type cyclin B1 and an export-defective mutant in their response to rescue by Wee1. Although

cells overexpressing Cdc25C with the export-defective mutant of cyclin B1 entered mitosis prematurely, a greater proportion of cells with nuclear cyclin B1 were rescued by exogenous Wee1 than cells with wild-type cyclin B1. One explanation for these results could be that an export-defective cyclin B1/CDK1 would remain in the nucleus to be inactivated by Wee1, whereas some of the wild-type cyclin B1 could be exported to the cytoplasm before it could be phosphorylated by Wee1. Once in the cytoplasm, the active cyclin B1/CDK1 might accelerate entry into mitosis by activating a positive feedback loop between the bulk of the endogenous cyclin B1/CDK1 and the Cdc25B phosphatase. This effect may therefore reflect the role for cyclin B1/CDK1 in coordinating the entry into mitosis of the cytoplasm and the nucleus in the unperturbed cell cycle.

Our results suggest that cyclin B1/CDK1 export can affect its own activation because it influences the balance between the effects of Wee1 and Cdc25C. Cyclin B1/CDK1 that can be exported to the cytoplasm seems to be less sensitive to Wee1, which might be a consequence of its activation in the cytoplasm. There are data to support the idea that M phase is initiated in the cytoplasm. For example, enucleated frog (Hara *et al.*, 1980; Gerhart *et al.*, 1984; Masui and Markert, 1971) and mouse (Balakier and Czolowska, 1977; Balakier and Masui, 1986) eggs will initiate M phase. Furthermore, studies using starfish (Ookata *et al.*, 1992) and *Xenopus* (Iwashita *et al.*, 1998) oocytes have shown that cyclin B1/CDK1 is activated in the cytoplasm prior to its translocation into the germinal vesicle, and during the mammalian somatic cell cycle the cytoplasmic Cdc25B is activated before the nuclear Cdc25C (Gabrielli *et al.*, 1996; Nishijima *et al.*, 1997; I. Hoffmann, personal communication). Cyclin B1/CDK1 itself has a profound effect on the behaviour of cytoplasmic organelles such as centrosomes (Buendia *et al.*, 1992), and it is strongly associated with centrosomes prior to its translocation to the nucleus (Bailly *et al.*, 1992) (Figure 1A and B). However, to prove that cyclin B1 must be cytoplasmic for mitosis to be initiated, the endogenous cyclin B1 will need to be removed, which presents a technical problem because cyclin B1 is an essential gene (Brandeis *et al.*, 1998).

It is likely that cyclin B1 translocation to the nucleus at prophase is caused by phosphorylation of the CRS. Other studies have shown that the sites of phosphorylation in the CRS of cyclin B are important for meiotic maturation, and that the biological activity of a mutant form of cyclin B that cannot be phosphorylated can be restored by the addition of a nuclear localization signal (Li *et al.*, 1995, 1997). We have demonstrated here that simply by stopping export, cyclin B1 will accumulate in the nucleus, and this might be the mechanism by which translocation is triggered at mitosis. Alternatively, phosphorylation could both stop export and induce a positive signal for cyclin B1 nuclear import. Further studies using live cell imaging should allow us to address this and other questions concerning the spatial control of mitosis.

Materials and methods

Cell culture and synchronization

HeLa cells were cultured on plates in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% calf serum at 37°C/10%

CO₂. Cells were synchronized using a thymidine–aphidicolin regime as described previously (Pines and Hunter, 1989). Human primary cells (MHF 181, a kind gift of T.Tilsty, UCSF) were cultured in MEM alpha medium in 10% FCS at 37°C/10% CO₂.

Construction of chimeras and point mutations

The mutations within the MmGFP cDNA and the fluorescent properties of the protein have been described previously (Zernicka-Goetz *et al.*, 1996, 1997). Cyclin–MmGFP fusion proteins and point mutants were constructed by PCR using Vent® polymerase (Biolabs, UK), cloned into baculovirus or bacterial expression vectors, and sequenced on an automated sequencer (Applied Biosystems). The stop codon of cyclin B1 in the pCMX vector was mutated to an *MfeI* site to link cyclin B1 to MmGFP. Cyclin B1 fused to the second amino acid residue of MmGFP by a 5-amino acid linker (AGAQT) was tagged with His₆ at the N terminus and cloned into pVL1393 (Invitrogen) for expression in baculovirus. CDK1 was His₆-tagged at the C terminus and cloned into pAcAB4 (Belyaev and Roy, 1993) for expression in baculovirus. Fusion proteins between the N terminus of cyclin B1 and MmGFP were linked by a 9-amino acid linker (IDPGGFPQF). The cDNAs for the fusion proteins were cloned into the pRSETB vector (Invitrogen) to generate an in-frame His₆ tag at the N terminus and expressed in BL21 DE3 (pLysS) bacteria.

Protein expression and purification

Full-length cyclin–MmGFP fusion proteins and CDK1 protein were expressed in baculovirus-infected Sf9 cells and purified on Ni-agarose followed by gel filtration and ion exchange chromatography as described by Krude *et al.* (1997). Proteins were >90% pure on silver-stained gels. Fusion proteins between the N terminus of cyclin B1 and MmGFP were expressed in *Escherichia coli* BL21 DE3 (pLysS). The *E. coli* cells were grown in 100 ml of 2XTY broth supplemented with 2% (w/v) glucose at 37°C to OD₆₀₀ of 0.5 and then induced for 4 h with 0.1 mM IPTG. The proteins were purified as described previously (Siemering *et al.*, 1996). Proteins were dialysed overnight against PBS in a Slide-a-lyzer 10K dialysis cassette (Pierce), concentrated in a Vivaspin 5000 MW cut-off microconcentrator (Vivascience, UK) and injected into cells using an Eppendorf semi-automatic microinjector attached to a Leica DMIRBE microscope.

Kinase assays

Purified proteins were assayed for kinase activities using Phosphocellulose units (Pierce, IL, USA) as described previously (Krude *et al.*, 1997) and quantified by Cerenkov counting. Protein concentrations were determined using the Coomassie Plus protein assay reagent (Pierce).

Immunofluorescence

Cells were either fixed and permeabilized in cold methanol/acetone, or cross-linked in paraformaldehyde and permeabilized in 0.5% Triton X-100, and stained as described previously (Pines and Hunter, 1991). Affinity-purified anti-cyclin B1 antisera was used at 1:200 dilution. FITC-conjugated goat anti-rabbit secondary antibodies (Cappel) were used at 1:200. Cells were analysed by confocal microscopy as described below but using 10% laser power. When immunofluorescence was performed on cells expressing GFP chimeras, cells were fixed and stained at 3.5 h after microinjection of pCMX/cyclin B1–MmGFP.

Confocal live-cell imaging

HeLa cells or normal diploid fibroblasts were seeded onto metasilicate-coated coverslips. HeLa cells were synchronized as described above and microinjected with the pCMX/cyclin B1–MmGFP construct (0.1 µg/µl) at 9–11 h after release from the aphidicolin block. At 4–6 h after injection, cells were visualized using a Bio-Rad MRC 1024 confocal system on an upright Nikon fluorescence microscope equipped with a 40× NA 0.55 water immersion objective. The argon ion 488 nm line was selected, the laser power was set at 3% and slow scanning mode was used in combination with three times Kalman averaging. Serial sections in the z-axis of the cell were collected at 0.5 µm intervals. For the comparison of cyclin B1–MmGFP localization before and after fixation, cells were subsequently fixed in 3% paraformaldehyde in PBS and permeabilized in 0.5% Triton X-100 as described previously (Pines and Hunter, 1991). Cells were mounted onto slides, injected cells were located, and analysed by confocal microscopy using 1% laser power. Stacks of images were exported to Adobe Photoshop and printed on a dye-sublimation printer (Tektronix, USA).

Time-lapse fluorescence imaging

Cells were incubated in CO₂-independent medium without phenol red (Gibco-BRL, UK), and overlaid with mineral oil for long time courses, at 37°C using a ΔT 0.15 mm dish (Bioprotech, PA). Cells were imaged by time-lapse fluorescence microscopy using a Leica DMIRBE microscope equipped with custom filter sets (Chroma Technology, VE), a PentaMax camera (Princeton Instruments, NJ) and a Lambda 10-2 filter wheel (Sutter, CA) controlled by a PowerWave computer (PowerComputing, TX) running IP Lab Spectrum software (Scanalytics, Inc, VA) as described by Karlsson and Pines (1998). Images were exported into Adobe Photoshop and printed as above.

Premature entry into mitosis

Cells were synchronized in S phase as above, and injected with cDNAs under control of the CMV promoter ~2 h after release from an aphidicolin block. Injected cells were identified by their GFP fluorescence. Cells were followed through G₂ phase and the number of cells that entered mitosis before uninjected cells was assayed at set times after injection and totalled at 10 h after release from the S phase block. At the end of the experiment cells were stained with Hoechst 33342 and scored for abnormally condensed chromatin.

Acknowledgements

We are grateful to Lucy Perkins for excellent technical assistance, Minoru Yoshida for the gift of leptomycin B, Mary Dasso for Ran mutants, Helen Piwnica-Worms for baculovirus expressing CDK1, Tony Hunter for the Wee1 cDNA and for opening our eyes to nuclear export, Sally Kornbluth for communicating results prior to publication, Iain Mattaj for helpful advice, Karen Simpson for helpful discussions, and Magda Zernicka-Goetz for constructive criticisms. A.H. and C.K. are supported by Marie Curie fellowships from the EC, and P.C. is an 1851 Exhibition fellow. This work was made possible by a MRC project grant and supported by a programme grant from the Cancer Research Campaign.

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*Received January 30, 1998; revised May 26, 1998;
accepted June 2, 1998*