Nuclear export of cyclin B1 and its possible role in the DNA damage-induced G₂ checkpoint

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M-phase-promoting factor (MPF), a complex of cdc2 and a B-type cyclin, is a key regulator of the G₂/M cell cycle transition. Cyclin B1 accumulates in the cytoplasm through S and G₂ phases and translocates to the nucleus during prophase. We show here that cytoplasmic localization of cyclin B1 during interphase is directed by its nuclear export signal (NES)-dependent transport mechanism. Treatment of HeLa cells with leptomycin B (LMB), a specific inhibitor of the NES-dependent transport, resulted in nuclear accumulation of cyclin B1 in G₂ phase. Disruption of an NES which has been identified in cyclin B1 here abolished the nuclear export of this protein, and consequently the NES-disrupted cyclin B1 when expressed in cells accumulated in the nucleus. Moreover, we show that expression of the NES-disrupted cyclin B1 or LMB treatment of the cells is able to override the DNA damage-induced G₂ checkpoint when combined with caffeine treatment. These results suggest a role of nuclear exclusion of cyclin B1 in the DNA damage-induced G₂ checkpoint.
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Introduction

In eukaryotic cell cycles, the initiation of mitosis requires the activity of M-phase-promoting factor (MPF), a complex of a cyclin-dependent kinase cdc2 and a B-type cyclin (Nurse, 1990; Hunt, 1991; Dunphy, 1994; King *et al.*, 1994; Morgan, 1995). The activity of MPF is regulated by phosphorylation/dephosphorylation of cdc2 and accumulation of cyclin B protein. During S and G₂ phases, the B-type cyclins accumulate and bind to cdc2 to form heterodimers. Cyclin B facilitates the inhibitory phosphorylation of cdc2 at Thr14 and Tyr15 (Meijer *et al.*, 1991; Parker *et al.*, 1991), which is catalysed by Wee1, Mik1 and Myt1 kinases (Nurse, 1990; Dunphy, 1994; King *et al.*, 1994; Mueller *et al.*, 1995; Liu *et al.*, 1997). At the end of G₂, abrupt dephosphorylation of these sites by the phosphatase cdc25 triggers the activation of cdc2–cyclin B (Dunphy, 1994; King *et al.*, 1994; Morgan, 1995). There is a universal checkpoint mechanism that prevents cells from entering lethal mitosis when their DNA is incompletely replicated, or is damaged (Hartwell and Weinert, 1989; Elledge, 1996; Nurse, 1997). Although the

detailed molecular mechanisms controlling the signalling pathways have not been fully defined, the checkpoint signals are suggested to be transduced to the cdc2–cyclin B complex.

In fission yeast *Schizosaccharomyces pombe*, defects that reduce Tyr15 phosphorylation lead to premature mitosis (Gould and Nurse, 1989). In addition, recent studies demonstrated that the DNA damage checkpoint system of *S.pombe* works by maintaining phosphorylation of cdc2 at Tyr15 through regulation of wee1 or cdc25 activities (Furnari *et al.*, 1997; O'Connell *et al.*, 1997; Rhind *et al.*, 1997). Thus, cdc2 tyrosine dephosphorylation determines the timing of mitosis in *S.pombe*. In mammalian cells, however, the phosphorylation state of cdc2 does not seem to be a sole factor that determines mitotic timing. Expression of cdc2AF, non-phosphorylatable Thr14 and Tyr15 mutant of cdc2, in mammalian cells induces premature mitosis to only a limited extent (Heald *et al.*, 1993). Moreover, cells expressing cdc2AF have a reduced but still significant G₂ delay in the DNA damage-induced checkpoint mechanism (Jin *et al.*, 1996). These results suggest the possibility that mechanisms other than the inhibitory phosphorylation of cdc2 may operate in the DNA damage G₂ checkpoint in higher eukaryotic cells.

Cyclin B1, which accumulates in the cytoplasm during S and G₂ phases, translocates to the nucleus before nuclear envelope breakdown during prophase (Pines and Hunter, 1991; Ookata *et al.*, 1992), as does avian cyclin B2 (Gallant and Nigg, 1992). Cytoplasmic localization of cyclin B1 in interphase is shown to be determined by its short amino acid sequence (~50 amino acid residues) region called the cytoplasmic retention signal (CRS) (Pines and Hunter, 1994). However, the detailed molecular mechanisms and biological significance of the cytoplasmic localization of cyclin B1 have not been well understood.

Nuclear export signal (NES), a short leucine-rich sequence motif, is a recently identified transport signal which is necessary and sufficient to mediate nuclear export of large carrier proteins (Gorlich and Mattaj, 1996; Nigg, 1997). Many proteins have recently been reported to be spatially controlled by their NES, including HIV-Rev (Fischer *et al.*, 1995), PKI (Wen *et al.*, 1995), RanBP1 (Richards *et al.*, 1996) and MAP kinase kinase (Fukuda *et al.*, 1996). Most recently, CRM1, which was first identified in fission yeast (Adachi and Yanagida, 1989), has been shown to be a receptor for NES (Fornierod *et al.*, 1997; Fukuda *et al.*, 1997a; Ossareh-Nazari *et al.*, 1997; Stade *et al.*, 1997). Thus, the NES-mediated intracellular transport system is a universal and conserved mechanism to control subcellular localization of proteins in cells.

In this report, we show that the cytoplasmic localization of cyclin B1 in interphase is directed by its NES-dependent transport mechanism. We have identified an NES in the CRS region, and found that disruption of this NES induces

Fig. 1. (A) Cell cycle-dependent accumulation and localization of cyclin B1 in HeLa cells. HeLa cells were synchronized by a double-thymidine block, and then released and incubated for 0, 8 and 9 h to obtain cells in G₁/S phase, G₂ phase and M phase, respectively. Cells were fixed and stained with anti-human cyclin B1 antibody and DAPI. (B) Left panel: cyclin B1 accumulates in the etoposide-induced G₂-arrested cells as in normal metaphase cells. HeLa cells were synchronized at early S phase by a double-thymidine block. At 6 h after release, cells were treated with (lane 3) or without (lanes 1 and 2) etoposide (20 μ g/ml) and incubated for 3 h (lanes 1 and 3) or 5 h (lane 2). Cell extracts were prepared and subjected to immunoblotting with anti-cyclin B1 antibody (upper panel). Histone H1 kinase activities in each extract are shown in a lower panel. Right panel: effect of LMB on the subcellular distribution of endogenous cyclin B1 in G₂-arrested HeLa cells. HeLa cells were synchronized by a double-thymidine block and arrested in G₂ phase with etoposide as described in Materials and methods. Cells were then treated with or without LMB (2 ng/ml) for 1.5 h, fixed and stained with anti-human cyclin B1 antibody and DAPI.

nuclear localization of this protein. Expression of the NES-disrupted cyclin B1 in mammalian cells is able to override the DNA damage-induced G₂ checkpoint in combination with caffeine. These results suggest a possible role for the NES-dependent cytoplasmic localization of cyclin B1 in the DNA damage-induced checkpoint.

Results

Leptomycin B disrupts the cytoplasmic localization of cyclin B1 in G₂ cells

In agreement with previous reports (Pines and Hunter, 1991; Ookata *et al.*, 1992), we observed in HeLa cells by using a specific antibody against cyclin B1 (Figure 1B, left) that cyclin B1 accumulates in the cytoplasm during the G₂ phase of the cell cycle and translocates to the nucleus during prophase before nuclear envelope breakdown (Figure 1A). The staining of cyclin B1 was most intense during metaphase (Figure 1A) and cyclin B1 almost disappeared at anaphase (Figure 1A and B, left). We considered the possibility that the cytoplasmic localization of cyclin B1 in interphase is ensured by the NES-mediated nuclear export system. To test this possibility, we used leptomycin B (LMB), a specific inhibitor of the NES-dependent intracellular transport, that is shown to inhibit interactions of the NES receptor (CRM1 = exportin 1) with NES (Fornerod *et al.*, 1997; Fukuda *et al.*, 1997a; Ossareh-Nazari *et al.*, 1997).

To arrest cells in G₂, HeLa cells were first synchronized by a double-thymidine block. At 6 h after release from the block, the cells were incubated for 4 h with etoposide, an inhibitor of topoisomerase II. Indirect immunofluorescence staining of HeLa cells and immunoblotting of the extracts showed that the etoposide-induced G₂-arrested cells accumulated cyclin B1 in the cytoplasm (Figure 1B, left, lane 3 and right, upper panels). When treated with LMB, endogenous cyclin B1 in these G₂-arrested cells entered and accumulated in the nucleus (Figure 1B, right, lower panels). These data suggest that in the G₂-arrested cells, cyclin B1 is continuously exported from the nucleus by an NES-dependent mechanism and thus is confined to the cytoplasm.

Nuclear export of cyclin B1

Our data presented above suggest that the NES-mediated transport system contributes to maintaining the cytoplasmic localization of cyclin B1. To test this possibility further, we purified a bacterially produced GST fusion form of cyclin B1 and injected this recombinant cyclin B1 into the nucleus of S-phase-arrested HeLa cells. At 3 h after injection, the injected recombinant cyclin B1 was completely exported from the nucleus to the cytoplasm (Figure 2, WT in lower panels, cyclin B1, -LMB) whereas co-injected RITC-BSA remained in the nucleus (Figure 2, WT in lower panels, BSA, -LMB). In the presence of LMB, the nuclear export of recombinant cyclin B1 was

Fig. 2. Nuclear export of cyclin B1. Recombinant wild-type (WT) cyclin B1 protein or a mutant (3A) cyclin B1 protein (Val149, Leu151 and Val153 are replaced by Ala) (2 mg/ml) was co-injected with RITC-BSA into the nucleus of HeLa cells presynchronized at early S phase by a double-thymidine block. The cells were incubated for 3 h in the presence or absence of LMB (2 ng/ml), and then fixed and stained with anti-cyclin B1 antibody. LMB was added 30 min before injection.

almost completely suppressed (Figure 2, WT in lower panels, cyclin B1, +LMB). Thus, cyclin B1 is actually exported from the nucleus by a mechanism sensitive to LMB.

NES in cyclin B1

We have identified an NES sequence in cyclin B1. Pines and Hunter (1994) previously identified the CRS in the N-terminal portion of cyclin B1 comprising ~50 amino acid residues that is responsible for its cytoplasmic localization. By examining the amino acid sequence of CRS, we found one putative NES sequence. We therefore focused on this sequence which is conserved in vertebrates (Figure 3A) and contains four hydrophobic residues present at a typical, characteristic spacing, like the well-established NES (Figure 3A).

To test whether this sequence functions as an NES, we synthesized a corresponding peptide, conjugated it to ovalbumin, and injected the resulting conjugate (NES-OVA) into the nucleus of HeLa cells. The injected conjugate was exported from the nucleus almost completely within 30 min (Figure 3B, upper, NES-OVA) whereas co-injected RITC-BSA remained in the nucleus (Figure 3B, upper panels, RITC-BSA). Importantly, the nuclear export of NES-OVA was completely inhibited by LMB (Figure 3B, lower panels, NES-OVA). These results indicate that the putative NES sequence of cyclin B1 in the CRS can function potentially as an LMB-sensitive NES.

Nuclear export of cyclin B1 is mediated by NES in the region of CRS

To show that the nuclear export of cyclin B1 is mediated by its NES in the region of the CRS, we constructed a mutant form of cyclin B1 (3A-cyclin B1) in which

Fig. 3. A putative NES sequence in cyclin B1. (A) Schematic representation of the primary structure of cyclin B1 (human) (Pines and Hunter, 1989) showing a putative NES sequence in CRS (residues 142–151, 139–148, 132–141 and 107–116 in human, mouse, rat and *Xenopus* cyclin B1, respectively). The NES sequences of MAPKK, PKI and Rev are aligned at the bottom. Important hydrophobic residues in the sequences are boxed. DB, destruction box; CRS, cytoplasmic retention signal. (B) A putative NES sequence in human cyclin B1 can function as NES. Ovalbumin conjugated with a putative NES peptide shown in (A) (NES-OVA) was co-injected with RITC-BSA into the nuclei of HeLa cells in the presence or absence of LMB (2 ng/ml). At 30 min after injection, cells were fixed and stained with anti-OVA antibody. LMB was added 30 min before injection.

presumably critical hydrophobic residues (Val149 and Leu151; Figure 3A) and one additional hydrophobic residue (Val153) were replaced by alanines to disrupt NES (3A in Figure 2). It has been established before that this type of mutation makes NES non-functional (Fischer *et al.*, 1995; Wen *et al.*, 1995; Gorlich and Mattaj, 1996; Nigg, 1997). We purified a recombinant 3A-cyclin B1 mutant protein, and injected it into the nucleus of S-phase-arrested HeLa cells. Unlike wild-type protein, 3A-cyclin B1 was not exported from the nucleus (Figure 2, 3A in lower panels).

We then expressed the HA-tagged forms of WT- or 3A-cyclin B1 in HeLa cells and examined their subcellular distribution by staining with anti-HA antibody. WT-cyclin B1 localized exclusively to the cytoplasm in the absence of LMB (Figure 4, WT, -LMB), while it accumulated in the nucleus in the presence of LMB (Figure 4, WT, +LMB). In contrast, 3A-cyclin B1 localized primarily to the nucleus even in the absence of LMB (Figure 4, 3A).

Fig. 4. Expression of WT- or 3A-cyclin B1 in HeLa cells. HeLa cells were transiently transfected with plasmids encoding a haemagglutinin (HA)-tagged form of WT-cyclin B1 or 3A-cyclin B1. At 20 h after transfection, cells were treated with or without LMB (2 ng/ml) for 2 h, then fixed and stained with anti-HA antibody.

Thus, the putative NES identified in the region of CRS mediates the cytoplasmic localization of cyclin B1.

These results taken together indicate that cytoplasmic localization, or nuclear exclusion, of cyclin B1 is achieved and maintained by its NES-dependent transport mechanism.

LMB treatment overrides the DNA damage-induced G₂ arrest in combination with caffeine treatment

It has been reported that expression and accumulation of cyclin B1 during S and G₂ phases is delayed after gamma-irradiation in HeLa cells (Muschel *et al.*, 1991; Bernhard *et al.*, 1994; Maity *et al.*, 1995) and that the protein level of cyclin B1 can be a rate-limiting component of the radiation-induced G₂ delay in HeLa cells (Kao *et al.*, 1997). We have found, however, that in HeLa cells arrested in G₂ by treatment with etoposide, a topoisomerase II inhibitor that inflicts DNA damage (Downes *et al.*, 1994), a normal level of cyclin B1, comparable with that at normal metaphase, accumulates in the cytoplasm (Figure 1B, left and right). These results are consistent with previous reports demonstrating that cyclin B1 protein accumulates in cells arrested in G₂ by etoposide treatment or by UV irradiation (Maity *et al.*, 1996; Gabrielli *et al.*, 1997). Thus, mechanisms other than the down-regulation of the expression of cyclin B1 may exist to arrest cells in G₂ in response to etoposide-induced DNA damage.

As shown in Figure 5A (first column), in the etoposide-induced G₂-arrested cells, endogenous cyclin B1 localized to the cytoplasm. Because Heald *et al.* (1993) demonstrated previously that the activation of cdc2 in the nucleus may be necessary for the initiation of mitosis, we considered the possibility that the exclusion of cyclin B1 from the nucleus prevents cells from entering into premature mitosis in the etoposide-induced G₂-arrested cells. To test this idea, we treated such cells with LMB, which resulted in the nuclear accumulation of cyclin B1. As shown in Figure 5A (second column), cyclin B1 accumulated in the nucleus in the LMB-treated cells. However, we found no mitotic phenotypes such as premature chromosomal condensation and rounding of the cells (Figure 5A, second column), most of which were still arrested in G₂. The histone H1

kinase activity, a measure of MPF, was not activated in LMB-treated cells (Figure 5B, compare lane 2 with lane 1). These results are consistent with the previous reports demonstrating that localizing human cyclin B1 or chicken cyclin B2 to the nucleus failed to elicit premature mitosis (Gallant and Nigg, 1992; Pines and Hunter, 1994; Gallant *et al.*, 1995). Neither did expression of 3A-cyclin B1 (the NES-disrupted cyclin B1), which accumulates in the nucleus (see Figure 4), induce premature mitosis (Figure 4 and data not shown). Thus, the nuclear accumulation of cyclin B1 is not sufficient to override the G₂ arrest induced by etoposide.

It has been reported that caffeine, a drug known to induce premature mitosis in a certain cell type under some conditions (Schlegel and Pardee, 1986; Steinmann *et al.*, 1991), cannot override the G₂ arrest induced by etoposide (Downes *et al.*, 1994). In agreement with this previous report, we also observed that the etoposide-treated cells were still arrested in G₂ 6 h after incubation with caffeine (Figure 5A, third column). It should be noted that a normal level of cyclin B1 was expressed and present in the cytoplasm of the caffeine-treated cell (Figure 5A, third column). Surprisingly, treatment of etoposide-induced G₂-arrested cells with LMB together with caffeine induced typical premature mitotic phenomena, including abnormal chromosomal condensation and rounding of the cell (Figure 5A, fourth column). Thus, etoposide-treated cells were released from the G₂ arrest by treatment with LMB plus caffeine. The H1 kinase activity in cells treated with caffeine was as high as that in cells treated with caffeine plus LMB (Figure 5B, lanes 3 and 4), suggesting that the high H1 kinase activity is not sufficient to override the etoposide-induced G₂ arrest. These results suggest the possibility that the etoposide-induced G₂ arrest is ensured by at least two different mechanisms, a caffeine-sensitive mechanism and an LMB-sensitive, NES-mediated transport mechanism.

Overexpression of 3A-cyclin B1 overrides the DNA damage-induced G₂ arrest in combination with caffeine treatment

To determine whether NES-mediated nuclear export of cyclin B1 is involved in the etoposide-induced G₂ arrest, we transiently transfected HeLa cells with WT- or 3A-cyclin B1, incubated the cells with etoposide for 7 h to arrest them in G₂, and examined the state of chromosomes in the presence or absence of caffeine. Caffeine treatment alone or expression of WT- or 3A-cyclin B1 alone caused abnormal chromosomal condensation in ~25–30% of the cells under these conditions (Figure 6B). Expression of 3A-cyclin B1, but not that of WT-cyclin B1, together with caffeine treatment induced abnormal chromosomal condensation with a significantly higher frequency (~48%) (Figure 6A and B). Abnormal chromosomes of cells transfected with 3A-cyclin B1 in the presence of caffeine were more densely condensed (Figure 6A) than those of non-transfected cells treated with LMB and caffeine (Figure 5A, fourth column). This might result from a longer incubation with etoposide in the transfected cells than in the non-transfected cells. These results thus suggest that the NES-mediated nuclear export of cyclin B1 may play a role in the DNA damage-induced G₂ checkpoint.

Fig. 5. (A) Effect of LMB on the etoposide-induced G₂ arrest. HeLa cells were presynchronized at early S phase by a double-thymidine block. At 6 h after release, cells were treated with etoposide (20 µg/ml) for 1 h, and then treated with LMB (2 ng/ml), caffeine (5 mM) or both, as indicated. Cells were incubated for an additional 7 h, then fixed and stained with anti-cyclin B1 antibody and DAPI. Phase-contrast images were obtained before fixation, therefore identifying cells different from those in the second and third rows that show double staining. (B) Effect of LMB and caffeine on histone H1 kinase activity. HeLa cells were treated as in (A). Cell extracts were prepared, and the histone H1 kinase activities in each extract were determined.

Discussion

Nuclear export signal in cyclin B1

We have shown here that human cyclin B1 has a functional NES sequence which ensures cytoplasmic localization, or nuclear exclusion, of cyclin B1 during interphase. The identified NES contains four hydrophobic residues at a characteristic spacing, typical of the well-established NES, and thus is sensitive to LMB, which was recently shown to be a specific inhibitor of the NES-dependent transport (Fornerod *et al.*, 1997; Fukuda *et al.*, 1997a; Ossareh-Nazari *et al.*, 1997; Wolff *et al.*, 1997). Disruption of this NES sequence alone by alanine substitutions abolished the cytoplasmic localization of cyclin B1 and induced its nuclear accumulation, suggesting that there is no other functional NES in cyclin B1.

It has been reported that human cyclin B1 has a CRS which is responsible for the cytoplasmic localization of cyclin B1 (Pines and Hunter, 1994). Since the NES sequence of cyclin B1 localizes in the region of CRS, it seems that CRS and NES are functionally similar. However, several reports demonstrated that cyclin B1 is associated with detergent-resistant structures, microtubules and centrosomes in human cells (Bailly *et al.*, 1992; Jackman *et al.*, 1995) and in starfish oocytes (Ookata *et al.*, 1992,

1993). Moreover, although human cyclin B2 also has a functional CRS which is responsible for the cytoplasmic localization of cyclin B2 (Pines and Hunter, 1994), its sequence corresponding to the NES in CRS of cyclin B1 is not a typical NES sequence; the third hydrophobic residue (valine in cyclin B1) is alanine in cyclin B2. Thus, it is possible that the CRS has some other functions which directly target cyclin B1 to particular structures in the cytoplasm.

The possible role of nuclear export of cyclin B1 in the etoposide-induced G₂ arrest

It has been reported that a rather high concentration (10 ng/ml) of LMB causes cell cycle arrest in G₁ or G₂ phases in rat fibroblastic 3Y1 cells when added to the synchronized cells at G₀ or early S phase (Yoshida *et al.*, 1990). However, we observed that LMB at 2 ng/ml does not affect the progression from S to M phase or the mitotic timing of HeLa cells when added to the synchronized cells at the S/G₂ phase boundary (data not shown), and that LMB rather promotes transition from G₂ to M phase of the cell cycle in combination with caffeine (Figure 5A). The difference between the previous observation and our present observations may result from the difference

Fig. 6. Effect of expression of 3A-cyclin B1 on the etoposide-induced G₂ arrest in combination with caffeine. HeLa cells were co-transfected with pEGFP-C1 (Clontech Laboratories Inc.) (0.5 μ g) and SR α HA or SR α HA-WT-cyclin B1 or SR α HA-3A-cyclin B1 (1.0 μ g). At 20 h after incubation, cells were treated with etoposide (20 μ g/ml) for 7 h to arrest cells in G₂. Cells were then treated with caffeine (5 mM) for an additional 6 h, fixed and stained with DAPI. Transfected cells were recognized by fluorescence of green fluorescent protein (GFP). (A) Typical images of cells transfected with SR α -3A-cyclin B1 and pEGFP-C1 after treatment with etoposide and caffeine. (B) Percentages of cells with condensed chromosomes in transfected cells with each constructs. '-' represents a control vector (SR α HA). The data were obtained from two independent experiments.

in the timing of the addition of LMB and/or the differences in the concentration of LMB and the cell lines used. There may be a number of proteins affecting the cell cycle whose subcellular localization is regulated by an LMB-sensitive transport mechanism, besides cyclin B1 which was identified in this study (see below). For example, a direct activator for MAP kinase, MAP kinase kinase (MAPKK, also known as MEK), whose activity is required for G₀/G₁ transition, has a typical NES in its N-terminal region which regulates cytoplasmic localization and function of MAPKK (Fukuda *et al.*, 1996, 1997b,c).

We have found that LMB is able to override the etoposide-induced G₂ arrest in combination with caffeine. Because accumulated cyclin B1 in the etoposide-induced G₂-arrested cells is confined to the cytoplasm, LMB-

induced nuclear translocation of cyclin B1 may be one of the triggers that induce mitosis in the presence of caffeine. This idea is supported by the observation that, when combined with caffeine treatment, expression of 3A-cyclin B1—a NES-disrupted cyclin B1 mutant which accumulates in the nucleus—induces abnormal chromosomal condensation more efficiently than expression of WT-cyclin B1 in etoposide-treated cells (Figure 6). However, the ability of LMB to induce mitotic phenotypes is stronger than that of 3A-cyclin B1, suggesting that there might be other components in the DNA damage-induced G₂ checkpoint pathway whose subcellular distribution is also regulated by an LMB-sensitive, NES-dependent mechanism.

It is well established that the initiation of mitosis is regulated by the activation of cdc2–cyclin B complex (Nurse, 1990; Hunt, 1991; Dunphy, 1994; King *et al.*, 1994). Although we were unable to reveal the direct target of caffeine, we showed that caffeine treatment alone induced activation of the H1 kinase activity in G₂-arrested cells (Figure 5B), whereas it did not induce premature mitosis (Figure 5A). Moreover, we revealed that LMB did not induce the activation of the H1 kinase activity (Figure 5B) or premature mitosis (Figure 5A, second column) in the etoposide-induced G₂-arrested cells in the absence of caffeine, but overrode the G₂ arrest in the presence of caffeine (Figure 5A, fourth column). These results suggest that an LMB-sensitive, NES-mediated transport system may work in the DNA damage-induced G₂ checkpoint in conjunction with the caffeine-sensitive regulatory pathway for cdc2. In summary, our results in this study suggest that in higher eukaryotic cells, cyclin B1 is excluded from the nucleus by its NES-dependent transport mechanism during interphase and that NES-mediated export of proteins from the nucleus, including cyclin B1, plays a role in the DNA damage-induced G₂ checkpoint.

It has recently been reported that phosphorylation of cyclin B1 is able to regulate its nuclear translocation (Li *et al.*, 1997). One of the possible phosphorylation sites in cyclin B1 localizes to the NES region (Li *et al.*, 1995), suggesting the possibility that the phosphorylation of cyclin B1 disrupts the function of NES, inducing the accumulation of cyclin B1 in the nucleus. Identification and characterization of a kinase(s) acting on cyclin B1 should shed light on not only regulatory mechanisms of nuclear export of cyclin B1 but also its role in the DNA damage-induced G₂ checkpoint.

Materials and methods

Cell culture, synchronization, reagents and cell staining

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% bovine calf serum. HeLa cells were synchronized with a double-thymidine block. Exponentially growing cells were arrested in S phase by treatment with thymidine (2 mM) for 17 h, and were released from the arrest by washing twice with fresh medium. Cells were grown in fresh medium for 9 h and then re-treated with thymidine (2 mM) for 15 h. More than 40% of cells had a rounded mitotic phenotype 9 h after release from a double-thymidine block. Etoposide-induced G₂-arrested cells were obtained by treating synchronized HeLa cells with etoposide (Sigma) (20 μ g/ml) 6 h after release from a double-thymidine block, and incubating for more than 4 h. Indirect immunofluorescent staining for cyclin B1 was performed as follows: HeLa cells were fixed in 3.7% formalin solution for 10 min at 37°C, permeabilized in 0.5% Triton X-100 in phosphate-buffered saline (PBS)

for 10 min, rinsed in a blocking solution (PBS) containing 3% bovine serum albumin (Sigma) and 0.1% globulin (Sigma) for 1 h at 37°C, and then stained with rabbit anti-human cyclin B1 antibody (Santa Cruz Biotechnology Inc.) overnight at 4°C. After washing three times with PBS, cells were stained with DAPI and FITC-labelled anti-rabbit IgG antibody (Cappel) for 1 h at room temperature. Caffeine was purchased from Nacalai Tesque Inc. (Japan). LMB was a kind gift of M.Yoshida (University of Tokyo).

Conjugation of a synthetic peptide to ovalbumin

The NES peptide corresponding to the sequence of residues 141–154 (DLQAFSDVILAVN) of human cyclin B1 was synthesized and conjugated to ovalbumin (OVA) with the bifunctional cross-linking reagent sulfo-SMCC (Calbiochem) as previously described (Fukuda *et al.*, 1996).

Mutagenesis

The mutagenic primers (5'-TTGCCGCGGCAATTGCATCAGAGAA-AGC-3' and 5'-ATTGCCGCGGCAATGATGTGGAT-3') were used to obtain 3A-cyclin B1 in which Val149, Leu151 and Val153 are replaced by Ala. The mutations were confirmed by DNA sequencing.

Recombinant proteins

GST-WT-cyclin B1 and GST-3A-cyclin B1 were prepared by using the expression vector pGEX-2T (Pharmacia Biotech Inc.), expressed in *Escherichia coli*, and purified by affinity chromatography on glutathione-Sepharose 4B (Pharmacia Biotech Inc.) followed by loading onto a HiTrap SP (Pharmacia Biotech Inc.).

Microinjection

Microinjection of NES-OVA was performed as previously described (Fukuda *et al.*, 1996). At 30 min after injection, cells were fixed and stained with anti-ovalbumin antibody (Cappel) as previously described (Fukuda *et al.*, 1996). GST-WT-cyclin B1 and GST-3A-cyclin B1 were injected into the nucleus of HeLa cells synchronized at early S phase by a double-thymidine block. After injection, cells were fixed and stained with rabbit anti-human cyclin B1 antibody as described above.

Transfection

WT-cyclin B1 and 3A-cyclin B1 were subcloned into pcDL-SRαHA1 as described (Shirakabe *et al.*, 1997). HeLa cells were transiently transfected by the use of Lipofectoamine (Gibco-BRL) as described (Moriguchi *et al.*, 1997). After 24 h, cells were fixed and stained with rabbit anti-HA antibody (Santa Cruz Biotechnology Inc.) and DAPI.

H1 kinase assays

The cell lysate was prepared as described (Moriguchi *et al.*, 1997). In brief, HeLa cells were lysed in lysis buffer (20 mM HEPES pH 7.35, 12.5 mM 2-glycerophosphate, 150 mM NaCl, 1.5 mM MgCl₂, 2 mM EGTA, 10 mM NaF, 0.5% Triton X-100, 2 mM DTT, 1 mM sodium vanadate, 1 mM PMSF and 20 µg/ml aprotinin) and then centrifuged at 15 000 r.p.m. for 15 min. The supernatant was mixed with 5 µg of histone H1, 100 µM ATP, 15 mM MgCl₂, 0.5 µCi of [γ -³²P]ATP in a final volume of 15 µl and incubated for 10 min at 30°C. The reaction was stopped by addition of Laemmli's sample buffer and boiling.

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