Effects of interphase and mitotic phosphorylation on the mobility and location of nucleolar protein B23

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Summary

B23 (or nucleophosmin, NPM) is a multifunctional protein involved in ribosome biogenesis, control of centrosome duplication and in sensing cellular stress. It is phosphorylated during interphase by casein kinase 2 (CK2) and during mitosis by cyclin-dependent kinase (CDK). In this study we have addressed the role of these phosphorylation events in the dynamics and location of protein B23. Mutation of the CK2 phosphorylation site to alanine results in slower recovery of the mutant compared with the wild-type protein as measured by fluorescence recovery after photobleaching (FRAP). Immunofluorescence studies using an antibody against phosphorylated Thr199 revealed that B23 is phosphorylated at this CDK1 site at the start of mitosis and is dephosphorylated during anaphase. The CDK1-type phosphorylation sites are in the nucleic acid binding region of B23 and may contribute to its dissociation from the nucleolus during mitosis. A Thr to Glu mutant of the CDK1-type sites as well as other members of the nucleoplasm family that lack the C-terminal nucleic-acid-binding region showed a greater mobility and/or faster recovery than wild-type B23.1, the longer variant. These results provide evidence that phosphorylation at these sites reduces the affinity of B23 for nucleolar components and might be a factor in regulating its location during the cell cycle.

Introduction

The nucleolus is the most prominent subnuclear structure in the eukaryotic nucleus, whose primary responsibility has traditionally been ribosome biogenesis (Olson, 2004a). Apart from this conventional role, the nucleolus has been implicated in such diverse functions as a stress sensor (Rubbi and Milner, 2003; Olson, 2004b), signal recognition particle biogenesis (Grosshans et al., 2001; Politz et al., 2002), mRNA transport (Ideue et al., 2004), controlling telomerase activity (Olson et al., 2002) and regulating cellular aging (Yu et al., 1997; Yankiwski et al., 2000). Two different, but related studies on the nucleolar proteome revealed that a number of proteins involved in cellular functions not related to ribosome biogenesis reside in the nucleolus (Anderson et al., 2002; Scherl et al., 2002). A recent study by Anderson et al. (Anderson et al., 2005) also showed that the nucleolar proteome changes over time in response to alterations in cell growth conditions. All these studies suggest that the nucleolus is a highly dynamic entity whose protein composition adapts to the metabolic state of the cell.

During interphase, nucleoli are relatively stable structures that can be isolated for functional studies. However, at the onset of mitosis, the nucleolus as well as the nuclear envelope begins to disassemble. Many of these events result from the phosphorylation of proteins by cyclin-dependent kinases (CDKs). The two most abundant proteins in the nucleolus, B23 and nucleolin, are also phosphorylated by CDKs during early mitosis (Peter et al., 1990). At this time the nucleolar components begin to distribute among several locations. Most of the proteins involved in rRNA transcription remain associated with the nucleolar organizer regions (NORs) (Sirri et al., 1999) whereas those involved in rRNA processing are dispersed throughout the cell as well as associated with the chromosome periphery. Some of these factors accumulate during anaphase to form nucleolus-derived foci (NDF) in the cytoplasm and prenucleolar bodies (PNBs) in the newly forming nucleus during telophase. The NDF contain both processing components and partially processed rRNA (Dundr et al., 1997; Dundr and Olson, 1998). Their constituents include protein B23, nucleolin, fibrillarin, hPop1 subunit of RNase P, U3, U8 and U14 snoRNAs. PNBs are similar to NDF in composition, but they are formed only during telophase when the nuclear envelope starts to form. Reassembly of the nucleolus involves the incorporation of the material from PNBs into the reforming nucleoli as the RNA pol I transcription is reactivated during telophase (Dundr et al., 2000; Benavente et al., 1987). Reactivation of transcription at the end of mitosis is accomplished by dephosphorylation of cdc2/cyclin-B-type sites in transcription factors (Heix et al., 1998; Klein and Grummt, 1999). Similar phosphorylation sites are present in some of the PNB constituents including B23 and nucleolin.

Proteins involved in different steps of ribosome biogenesis have varying dynamics in living cells. Fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP) analyses demonstrate that factors...
involved in rRNA transcription (upstream-binding factor, UBF), processing (nucleolin, fibrillarin and RNase MRP subunit, Rpp29), and ribosome assembly (B23) exchange rapidly between the nucleolus and the nucleoplasm (Chen and Huang, 2001). By contrast, ribosomal proteins S5 and L9 showed relatively slower mobility in the nucleolus. These results support the notion that assembly of ribosomal subunits is a slower process than rRNA transcription and processing. Though the molecular switch governing the rapid association and dissociation of these nucleolar processing components is not known, phosphorylation would be a good candidate because many of the nucleolar components analyzed in the above study are reversibly phosphorylated during the cell cycle by different kinases (Chan et al., 1986; Peter et al., 1990; Heix et al., 1998; Klein and Grummt, 1999).

In the current work we have used B23 to study the effects of phosphorylation on the dynamics and localization of a well-characterized nucleolar protein. Protein B23, also known as nucleophosmin (NPM) or NO38, is one of the most abundant proteins in the nucleolus (Orrick et al., 1973; Schmidt-Zachmann et al., 1987; Schmidt-Zachmann and Franke, 1988). This multifunctional protein is implicated in ribosome biogenesis (Itahana et al., 2003; Huang et al., 2005), control of centrosome duplication (Okuda et al., 2000; Tokuyama et al., 2001) and sensing cellular stress (Wu and Yung, 2002; Kurki et al., 2004). Recently it was shown that protein B23 is necessary for nucleolar disassembly by protein YB1/FRG2a (Gonda et al., 2006). It has also been shown that protein B23 is a component of pre-ribosomal ribonucleoprotein (RNP) complexes (Zirwes et al., 1997; Pinol-Roma, 1999). It is expressed as two isoforms, B23.1 and B23.2, which differ only at the C-terminal end. B23.1 is primarily located in the nucleolar granular component, which contains the maturing pre-ribosomal RNP particles (Spector et al., 1984). B23.2 is present in the nucleoplasm as well as in the nucleolus (Okuwa et al., 2002). Protein B23.1 has nucleic-acid-binding, ribonuclease and molecular chaperone activities (Hingorani et al., 2000). It is also an oligomer that probably exists as a pentamer, which dimerizes to form a decamer (Chan and Chan, 1995; Namboodiri et al., 2004). The segment containing the molecular chaperone activity overlaps with the oligomerization region.

The linear sequence of B23.1 shows two acidic regions in the center of the molecule (Fig. 1). Protein B23 has several sites that become phosphorylated at various times during the cell cycle. The first acidic segment contains a major protein kinase casein kinase 2 (CK2) site (Ser125) (Chan et al., 1986). Phosphorylation by CK2 takes place during interphase and it might be responsible for regulating B23 function in the nucleolus. Protein B23 interacts with many proteins, including nuclear as well as viral and bacterial proteins (Olson et al., 2002). Recently, a novel mechanism was proposed in which phosphorylation of B23 by protein kinase CK2 leads to the release of the substrate (Szebeni et al., 2003). The potential sites of CK2 phosphorylation on protein B23 (T199, T219, T234, T237) are in the C-terminal end, and are in or near the nucleic-acid-binding region (Okuwa et al., 2002). It seems likely that phosphorylation at the CK2 sites promotes the dissociation of B23 from the nucleolus and dephosphorylation facilitates its reassociation with NDF, PNBs and newly forming nucleoli, thereby accounting for its location during various phases of mitosis. In prophase, most of it is dispersed throughout the cell but some of it stays with the nucleolar remnants. During metaphase and anaphase it is present at the chromosomal periphery and in the interzone of the mitotic spindle (Ochs et al., 1983). It also appears in the NDF during anaphase and in PNBs in telophase (Dundr et al., 1997; Dundr and Olson, 1998; Dundr et al., 2000).

In this paper we analyze the effects of interphase and mitotic phosphorylation of protein B23 on its mobility in HeLa cell nucleoli. It was found that the phosphorylation enhances its mobility, which supports the idea that it reduces the affinity of protein B23 for other nucleolar components. It was also shown that protein B23 is phosphorylated at Thr199 and Thr234/237 by CK1-type kinase during mitosis and dephosphorylated by a calycin-A-sensitive phosphatase. These phosphorylation and dephosphorylation events correlate with its location in the cell.

**Results**

**Cellular location of the GFP-tagged wild type and CK2 site mutant proteins**

Previous studies have shown that many nucleolar components involved in ribosome biogenesis exchange rapidly between the nucleolus and nucleoplasm (Chen and Huang, 2001). Protein B23 is phosphorylated at Ser125 (Ser125-P) in a highly acidic region by casein kinase 2 (CK2) during interphase (Fig. 1A,B). In vitro studies have suggested that phosphorylation at this site can modulate the interaction of B23 with some proteins (Szebeni et al., 2003). To determine the effect of CK2 phosphorylation on the dynamics of B23 we used the fluorescence recovery after photobleaching (FRAP) technique to monitor the mobility of protein B23 inside the cell. For this we prepared green fluorescent protein (GFP)-tagged constructs of the wild-type B23 and its mutants. The mutants were generated by overlap extension PCR by converting Ser125 to Ala or Glu. On the one hand, the S125A mutant cannot be phosphorylated by CK2 and should behave like a dephosphorylated protein B23. On the other hand, the S125E mutant should mimic the phosphorylated B23 because we have introduced a negative charge at this position. The localization of the wild-type protein tagged with GFP was followed during...
mitosis. The GFP-tagged protein had patterns of localization nearly identical to those of the endogenous protein as detected by immunofluorescence during different phases of mitosis (Fig. 2A). Also, the cells were transiently transfected with these GFP-tagged constructs and analyzed for their location and expression. Western blot analysis using anti-GFP antibody showed the expected size band for the fusion proteins in the transfected cells (Fig. 2B). The GFP-tagged wild-type and mutant proteins localized to the nucleolus as does the endogenous protein B23 (Fig. 2C). These results show that the cellular locations of the GFP-tagged proteins are similar to those of the endogenous protein B23.

A CK2 mutant has slower mobility in the nucleolus than the wild-type protein B23

HeLa cells were transfected with GFP-B23, the -S125A mutant or the -S125E mutant. Twenty four to 36 hours post transfection, FRAP analyses were performed using the laser-scanning confocal microscope. Either a portion of, or an entire nucleolus, was bleached using the 488-nm laser line. Images were collected before bleaching, immediately after bleaching and subsequently every 5 seconds for at least 160 seconds. The half time \( t_{1/2} \) for the fluorescence recovery of the wild-type B23 in nucleoli was 22 seconds. A similar recovery rate was reported for B23 by Chen and Huang (Chen and Huang, 2001). The \( t_{1/2} \) of recovery of the S125A mutant was considerably longer (44 seconds) than the wild type in the HeLa cells (Fig. 3). One interpretation of this longer recovery time is that the mutant exchanges more slowly with the nucleoplasmic pool of free GFP-tagged mutant protein than the wild-type protein and implies that proteins not phosphorylated at this site have a higher affinity for nucleolar components. FRAP studies on the phosphorylation-mimicking mutant (S125E) showed no significant difference in the mobility compared with the wild type. Since this mutant is negatively charged at position 125, this might suggest that endogenous B23 is phosphorylated at Ser125 during interphase.

Members of nucleoplasmin family proteins lacking the C-terminal nucleic-acid-binding region have faster mobility than wild-type B23.1

The family consists of the proteins nucleoplasmin, NPM/B23.1, NPM2/B23.2 and NPM3. Proteins B23.2 and NPM3 lack a portion or all of C-terminal nucleic acid-binding region of B23.1 (Fig. 4A). Protein B23.1 is mostly localized in the nucleolus whereas nucleoplasmin, a histone chaperone, is

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**Fig. 2.** Location of protein B23-GFP and mutants during interphase and mitosis. (A) Localization of GFP-tagged wild-type and endogenous protein B23 during different phases of mitosis. Both the proteins showed similar localization during various phases of mitosis. Upper panels, GFP-tagged expressed protein; lower panels, endogenous protein as detected by immunofluorescence using a monoclonal antibody. Arrows indicate the position of NDF in an anaphase cell and arrowheads indicate the position of PNBs in the newly forming nucleus at telophase. (B) Expression of the wild type protein and the mutants tagged with GFP. Immunoblot was developed with anti-GFP mAb. B23-GFP (lane 1), S125A-GFP (lane 2), S125E-GFP (lane 3) and untransfected cell lysate (lane 4). (C) Location of (a) wild-type B23-GFP, (b) S125A-GFP, (c) S125E-GFP and (d) endogenous B23 detected by immunofluorescence using a monoclonal antibody in interphase HeLa cells. All the fusion proteins localized to the nucleolus as did the endogenous B23 protein. Corresponding images of Hoechst 33342-stained DNA are shown in (a’), (b’), (c’) and (d’). Bars, 10 μm.
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almost completely nucleoplasmic (Zirwes et al., 1997) and the family members lacking the C-terminal end of B23.1 (B23.2 and NPM3) are distributed both in the nucleolus and nucleoplasm (Fig. 4B) (Huang et al., 2005). It seems likely that this C-terminal region of B23.1 contributes to the retention of the protein in the nucleolus by binding to RNA (Okuwaki et al., 2002). To test this possibility, HeLa cells were transfected with GFP-B23.1, GFP-B23.2 or GFP-NPM3 and subjected to FRAP analysis. The $t_{1/2}$s for the fluorescence recovery of B23.2 and NPM3 were 14 seconds and 9 seconds, respectively compared to 22 seconds for wild-type GFP-B23.1 (Fig. 4C).

Thus, these proteins that lack the C-terminal nucleic-acid-binding region recover faster than the wild-type protein. These results support our hypothesis that interaction of the nucleic-acid-binding portion of B23.1 with nucleolar components, possibly with RNA, contributes to the retention of the protein in the nucleolus.

Inhibition studies of protein B23 phosphorylation and dephosphorylation during mitosis

At the onset of mitosis, transcription is shut down and translation is suppressed (Prescott and Bender, 1962; Hartl et al., 1993; Qin and Sarnow, 2004) and the nucleolus, as well as the nuclear envelope, begins to disassemble. Most of these events result from the phosphorylation of proteins by CDKs. Previous studies have shown that B23 is phosphorylated during mitosis by CDK1-type kinases near the C-terminal end (Peter et al., 1990). Studies were initiated to determine the timing of the phosphorylation of these sites during the cell cycle of synchronized HeLa cells. Fluorescence-activated cell sorter (FACS) analysis at different time points after double thymidine block showed that the maximum mitotic population was achieved at 8 hours after the release from thymidine block (Fig. 5A). The availability of antibodies to phosphorylated Thr199 (Thr199-P) and Thr234/237-P allowed us to estimate when...
It was shown previously that the sites in the C-terminal end are phosphorylated by CDK1 in vitro (Okuwaya et al., 2002). Another report has suggested that T199 is phosphorylated by CDK2 in vivo at the G1-S border (Tokuyama et al., 2001). To confirm whether protein B23 is phosphorylated by CDK1 at Thr199 and Thr234/237 in vivo, we blocked the cells in mitosis by nocodazole treatment followed by incubation in 75 μM roscovitine. Roscovitine is a selective inhibitor of cyclin-dependent kinases (Meijer et al., 1997). Several cyclin-dependent kinases including CDK1-cyclin B, CDK2-cyclin A or E and CDK5 are very sensitive to roscovitine. Because CDK5 is only active during neuronal differentiation (Tsai et al., 1993) and CDK2 is inactive during mitosis, CDK1-cyclin B is probably the only kinase inhibited by roscovitine when mitotic cells are treated with this inhibitor. Western blotting of the samples showed that the Thr199-P and Thr234/237-P bands were not present in the roscovitine-treated sample (Fig. 5C, lane 2). This result shows that CDK1 is inhibited in the roscovitine-treated sample and therefore is unable to phosphorylate B23 at these sites.

Because the nocodazole-arrested mitotic cells were treated with roscovitine for only 30 minutes, the B23 molecules that were phosphorylated before this treatment should remain phosphorylated. Therefore, appearance of the unphosphorylated form of B23 after roscovitine treatment implies that a phosphatase is present in these cells, is active after CDK1 inhibition and dephosphorylates B23 to prevent its re-phosphorylation in nocodazole-arrested mitotic cells. To identify the phosphatase responsible for this effect, we treated the nocodazole-arrested mitotic cells with okadaic acid and calycin A before roscovitine treatment. Both compounds are potent inhibitors of protein phosphatase type 2A (PP2A) with an IC50 value of ~1 nM, but calycin A is a more effective inhibitor of protein phosphatase type 1 (PP1) than okadaic acid, with IC50 values of 2 nM and 60-500 nM respectively (Ishihara et al., 1989). Phosphorylated B23 remained in cells after treatment with 500 nM okadaic acid 30 minutes before roscovitine treatment (Fig. 5C, lane 3). The same effect was observed with 5 nM calycin A, but not with 5 nM okadaic acid, suggesting the involvement of PP1 in B23 dephosphorylation during mitosis. These results strongly suggest that B23 phosphorylation during mitosis depends upon the balance between the relative activities of CDK1 and PP1.

A site at the C-terminal end of protein B23 is phosphorylated between prophase and mid anaphase

Previous results from FACS analysis and kinase inhibitor treatment gave us an idea about the approximate timing of B23 phosphorylation during mitosis. To determine more precisely the mitotic stage at which B23 is phosphorylated and dephosphorylated, we carried out indirect-immunofluorescence studies using antibody against Thr199-P (Fig. 6). This study revealed that this site becomes phosphorylated very early in prophase, probably before complete nuclear envelope breakdown, because most of the fluorescence was inside the nucleus and not diffused in the cytoplasm, and remains phosphorylated through metaphase (Fig. 6A). It remains phosphorylated during early anaphase but the signal for Thr199-P disappeared as the cell progressed further in anaphase (Fig. 6B). The dephosphorylation coincides with the appearance of NDF during anaphase. No signal for
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Thr199-P was detected in the NDF or the PNBs (Fig. 6C). Little or no signal was seen in the interphase cells with this antibody. This result further supports the hypothesis that phosphorylation promotes the dispersal of protein B23 throughout the cell during mitosis possibly by decreasing the affinity of protein B23 towards its interphase partners. However, the protein begins to appear in complexes with other nucleolar components, in the form of NDF and PNBs in anaphase and telophase when the CDK activity is diminished (Dundr et al., 1997; Dundr and Olson, 1998; Dundr et al., 2000).

A phosphomimetic mutant of protein B23 has increased mobility

Results from the phosphorylation studies on B23 and the FRAP analysis of the nucleoplasm in family members lacking the C-terminal end suggest that the CDK1 phosphorylation (at sites near the C-terminal nucleic-acid-binding region) is responsible for lowering the affinity of protein B23 towards other nucleolar components. Previous in vitro studies showed that the phosphorylation of B23 at the C-terminal CDK1 sites reduces its affinity for the RNA (Okuwaki et al., 2002). We made a phosphorylation-mimicking mutant of protein B23 by changing all four CDK1 sites to glutamate. The mutant (T4E-GFP) was transiently transfected into HeLa cells and its localization and expression were analyzed. Although this mutant localizes to the nucleolus, a considerable amount of fluorescence can be seen in the nucleoplasm as with protein B23.2 and NPM3 (Fig. 7A). A western blot of the cells transfected with this mutant shows a single band with anti-GFP antibody (Fig. 7B). The diffuse localization pattern of this mutant, like B23.2 and NPM3, suggests that the mutant might have reduced affinity for the nucleolar components and therefore exchange rapidly with the free pool of the protein. The mutant was transfected into HeLa cells and subjected to photobleaching studies. It showed recovery rates similar to those of protein B23.2 and NPM3 (Fig. 7C). Faster recovery of this mutant in the nucleolus than the wild type suggests that phosphorylation in the C-terminal region reduces the binding affinity of B23 for the nucleolar components. We also made phosphorylation-mimicking mutants of protein B23 by changing amino acids Thr199, Thr219 and Thr234/237 to glutamate. The recovery of these mutants was similar to the wild-type protein suggesting that the phosphorylation of all these sites exert a synergistic effect on the mobility of protein B23 (supplementary material Fig. S1).

Discussion

Most proteins involved in ribosome biogenesis are not stationary in the nucleolus, but they rapidly exchange between the nucleolus and the nucleoplasm (Chen and Huang, 2001). As a consequence, the structure of a nucleolus in a living cell is the sum total of molecules entering and leaving a defined subnuclear space, with association and dissociation rates governing its overall organization (Misteli, 2001; Olson and Dundr, 2005). Thus, small changes in dissociation constants could facilitate the assembly or disassembly of the nucleolus. Because the assembly, disassembly and maintenance of a functional nucleolus depends on the activities of different protein kinases (Sirri et al., 2002), phosphorylation is likely to...
be a switch controlling the association and dissociation constants of components in the nucleolus, thereby regulating its structural function. We have used the abundant nucleolar phosphoprotein, B23, to study how phosphorylation events affect its dynamics and location. This protein is phosphorylated during interphase by CK2 and by CDK during mitosis (Chan et al., 1986; Peter et al., 1990). In these studies we showed that phosphorylation events modulate the rate of exchange of protein B23 between the nucleolus and the nucleoplasm. We also showed the correlation between the phosphorylation of protein B23 and its subcellular location during mitosis.

In the case of CK2 phosphorylation, the S125A mutant has a longer residence time in the nucleolus than the wild-type protein. This is consistent with previous work from this laboratory showing that CK2 phosphorylation of B23 reduces its affinity for test substrates in chaperone assays (Szebeni et al., 2003). A model was proposed where phosphorylation-dephosphorylation cycles modulate the binding of the substrate to protein B23. The current report shows that CK2 phosphorylation of B23 enhances its mobility in the nucleolus, which supports the idea that phosphorylation at the CK2 site reduces its affinity for other nucleolar components. Protein B23 exists as an oligomer (Chan, 1989) and the phosphorylation effect could be due to a change in the oligomerization state. However in gel filtration experiments we did not observe any differences in the molecular size of the unphosphorylated versus phosphorylated protein (results not shown). Therefore, it is unlikely that phosphorylation at the CK2 site changes oligomerization properties. Although the in vivo role of CK2 phosphorylation is not known, it seems likely that it regulates the interaction of B23 with other proteins.

The nucleoplasmin family consists of four proteins: nucleoplasmin, B23.1, B23.2 and NPM3. All the members show high homology to each other, especially in the N-terminal half of the molecule. Nucleoplasmin, a histone chaperone, is 50% identical to B23.1 in the N-terminal half (Schmidt-Zachmann et al., 1987), but it is not a nucleolar protein. B23.2 is a product of alternative splicing of B23 mRNA and lacks 35 amino acids at the C-terminal end (Chan and Olson, 1989). The N-terminal sequence of protein B23.1 is 42% identical to that of NPM3. Both B23.2 and NPM3 lack the C-terminal tail. This segment, which is rich in aromatic and basic amino acid residues and is unique to B23.1, is essential for nucleic acid-binding activity (Wang et al., 1994; Hingorani et al., 2000). The tagged forms of B23.2 and NPM3 have diffuse staining in the nucleolus with a significant amount of signal seen in the nucleoplasm, whereas B23.1 is mostly nucleolar with very little nucleoplasmatic staining. The predominantly nucleolar presence of B23.1 is attributed to its ability to bind rRNA. It can be dispersed from nucleolus to nucleoplasm by actinomycin D treatment and is a part of a RNP complex (Pinol-Roma, 1999). A recent report from this lab showed that protein B23 co-immunoprecipitates with 28S rRNA (Huang et al., 2005). By contrast, B23.2 and NPM3 show weak or no affinity towards rRNA (Okuwaki et al., 2002; Huang et al., 2005). Therefore, the greater nucleoplasmic localization of B23.2 and NPM3 compared with B23.1 might be due to the lack of the nucleic-acid-binding tail. Consistent with this, the current study shows that the nucleoplasmin family members lacking the C-terminal tail recover faster in the nucleolus, thereby underlining the importance of this region. It is likely that both of these proteins have higher mobility in the nucleolus because of their inability to bind the nucleic acids present there.

At the onset of mitosis, many nucleolar components involved in rRNA processing are dissociated from the NOR and dispersed in the cytoplasm. These events overlap with the increase in CDK1 activity at the start of mitosis (Moreno et al., 1989). A study on the timing of nucleolar breakdown during mitosis showed that B23 dissociation from the nucleolus occurs before complete breakdown of the nuclear envelope (Leung et al., 2004). Our results from western blotting and the indirect immunofluorescence studies show that B23 is phosphorylated at Thr199 and Thr234/237 at the onset of mitosis, probably before complete nuclear envelope breakdown as determined by the lack of cytoplasmic fluorescence and that the protein dissociates from the nucleolus during the time of
its mitotic phosphorylation. This may be due to the reduced affinity of phosphorylated B23 for the nucleolar component during mitosis.

Treatment of cells with roscovitine, a potent inhibitor of CDK1 during mitosis, resulted in the inhibition of B23 phosphorylation at Thr199 and Thr234/237, indicating that CDK1 is responsible for phosphorylating these sites. As the cell progresses into anaphase, CDK1 activity decreases, which allows phosphatases to remove phosphoryl groups. This coincides with B23 dephosphorylations at site Thr199 and Thr234/237 indicating that some form of mitotic phosphatase is responsible for this dephosphorylation. In fact, prior treatment of mitotic cells with the phosphatase inhibitors okadaic acid or calyculin A followed by roscovitine treatment preserves the phosphorylation of these sites. Treatment of mitotic cells with 5 nM calyculin A, but not 5 nM okadaic acid, preserved the B23 phosphorylation. These results implicate PP1 in mitotic dephosphorylation of B23, which itself is regulated by CDK1 during mitosis (Kwon et al., 1997). Our results suggest that the B23 phosphorylation and dephosphorylation cycle during mitosis depends upon the balance between the CDK1 and PP1 activity. This might be a general mechanism used by the cell to sequester or inactivate the components of ribosome assembly or other essential cellular processes that need to be regulated during mitosis.

The dissociation of protein B23 from the nucleolus early in mitosis and its reappearance in NDF and PNBs during anaphase and telophase correlate with the activity of CDK1 and PP1 (Moreno et al., 1989; Kwon et al., 1997). This is supported by a study showing that CDK1 phosphorylation lowers the RNA-binding affinity of B23, which would promote dissociation of the protein from the nucleolus (Okuwa, et al., 2002). As the cell progresses through anaphase Thr199 and Thr234/237 are completely dephosphorylated. This event approximately coincides with the appearance of NDF during anaphase and telophase and may signal the beginning of nucleolar reassembly. These NDF are positive for many nucleolar-processing components including B23. It seems likely that the incorporation of B23 into the processing complexes contained in the NDF and PNBs is due to the re-association of the dephosphorylated B23 with RNA present in them. Also, NDF formation can be prematurely induced in metaphase cells by using roscovitine to inhibit CDK1, further supporting the notion that reassociation of B23 with nucleolar components is through its dephosphorylated state (Zatsepin, unpublished results). We hypothesize that the mitotic phosphorylation changes B23 structure and increases its negative charge, especially in the C-terminal nucleic-acid-binding region so that its affinity for RNA in the nucleolus is lowered. This could shift the equilibrium toward the dissociated state.

In addition to proteins associated with the pre-rRNA transcription apparatus, the major nucleolar proteins B23 and nucleolin have been shown to be phosphorylated by CDK1 early in mitosis (Peter et al., 1990). The highly phosphorylated nucleolar protein NopP140 also has potential sites for phosphorylation by this kinase complex (Meier and Blobel, 1992; Waggener and DiMario, 2002). A systematic search for nucleolar proteins that are phosphorylated during the first half of mitosis is needed to clarify the general applicability of this mechanism to nucleolar disassembly.

### Materials and Methods

**Cell culture, transfection and synchronization**

HeLa cells were maintained in DMEM (Invitrogen Corp.) supplemented with 10% FBS and 100U/ml penicillin and streptomycin at 37°C and 5% CO2. Transfection was performed using FuGENE 6 (Roche Applied Science) according to the manufacturer’s instructions. For cell synchronization, HeLa cells were treated with 2 mM thymidine for 16 hours. Cells were washed and allowed to grow for 9 hours in fresh medium. Thymidine (2 mM) was again added for 16 hours to accumulate cells at the G1-S boundary. Cells were released from the thymidine block and harvested at different times to determine the percentage of cells in G1, S and G2-M phase by FACS. Mitotic populations were also achieved by single thymidine block followed by incubation in nocodazole (0.05 μg/ml) containing medium for 12-14 hours. Mitotic cells were harvested by mechanical shock.

**Plasmid construction**

GFP-NPM3 was constructed as described by Huang et al. (Huang et al., 2005). Full-length human B23 cDNA was amplified from pEG-B23 vector using the primer 5'-AAATGGAAGACTCTGATGGCACATG-3' and 5'-AGAGACTTCTCCTCAC- GCCAG-3', and cloned into the GFP vector (Clontech). All the mutants were constructed using overlap extension polymerase chain reaction using Pfu polymerase (Stratagene). For generating multiple mutations (in case of T4E), first Thr234/237 to Gln was mutated followed by mutating Thr199 to Gln. Then, using this as the template the Thr219 to Gln mutation was added by mutating Thr219 to Gln. The following primers were used for the synthesis of each construct. Primers for S125A were 5’-GGAGATGTCAGGCAAGAAGTGG-3’ and 5’-CATCCTTCTGCTCCTG- CATCCTCC-3’. For S125E, 5'-GGAGATGTCAGGCAAGAAGTGG-3’ and 5’-CATCCTTCTTCCCTGCATCCTCC-3’. For T196E, 5’-GGACCTACCATGACGC-3’ and 5’-GCTGATGATGG-3’. For T219E, 5’-AACCATATCAAGAAGATGTCAATC-3’ and 5’-ATGATCTGGAATTCGATAGTTCC-3’. For T219E, 5’-AACCATATCAAGAAGATGTCAATC-3’ and 5’-ATGATCTGGAATTCGATAGTTCC-3’.

**Polymerase chain reaction products** were excised from the gel and ligated into pCR-TOPO II vector (Invitrogen). All constructs were sequenced and subsequently subcloned into EGFPN2 (Clontech) vector at the EcoR1 site.

**Kinase and phosphatase inhibitor treatment**

Synchronized mitotic cells were treated with 75 μM roscovitine (Calbiochem) for 30 minutes. Control cells were treated with DMSO for the same period. For phosphatase treatment, cells were blocked in mitosis with nocodazole followed by okadaic acid (500 nM or 5 nM) (Calbiochem) or calyculin A (5 μM) (Calbiochem) treatment for 30 minutes. This was followed by 75 μM roscovitine treatment for 30 minutes. Cells were harvested at this point, lysed in SDS sample buffer and subjected to western blotting.

**Photobleaching and live cell microscopy**

Cells were grown on LabTek II chambered cover glasses (NalgeNunc). 24-36 hours after transfection, new medium was added to the cells supplemented with 25 nM HEPE. Cells were maintained at 37°C using a professional hot-air blower. FRAP was performed on a Leica microscope (DM-IRE2) (Leica Microsystems). The 488 nm laser and a 63X plan Apo lens with 1.4 NA was used in the photobleaching experiments. The region of interest (ROI) was bleached with the 488 nm laser at full power and subsequent scans were taken at 2% of full power. Five images were taken before bleaching and then images were acquired every 5 seconds for at least 160 seconds. At least eight datasets were analyzed for each result.

**Quantification of relative fluorescence intensity**

Fluorescence intensity was measured using Leica image-processing software. The average intensity in the ROI before bleaching, immediately after bleaching and post bleaching was measured. Fluorescence intensity of the nucleus was also measured. Background fluorescence was measured in a field outside the cell and subtracted from the nucleolar and nuclear fluorescence values. The relative fluorescence intensity increase, ΔF/F0, was calculated as follows: ΔF/F0=(F(t)/F0−1), where ΔF=the average fluorescence intensity of the photobleached region at various time points after photobleaching, F0=the average fluorescence intensity of the entire nucleus at the corresponding time point, F(t)=the average fluorescence intensity of the photobleached region before photobleaching, and TN=the average fluorescence intensity of the entire nucleus before photobleaching. Non-linear curve fitting of the recovery data was carried out using ORIGIN (Microcal Software). The fluorescence recovery was plotted against time and t1/2 was calculated using the equation: F(t)=F∞+(F0−F∞)/(1+e-(t−t0)/τ), where τ=the intensity value immediately after bleaching, F∞=the maximum intensity value, and t0=the time delay.

**Indirect immunofluorescence analysis**

HeLa cells grown on coverslips were transiently transfected with the GFP-tagged construct using FuGENE 6 reagent (Roche Applied Science). 24-36 hours post transfection, cells were fixed with 2% paraformaldehyde for 20 minutes at room temperature (RT) and permeabilized with 0.5% Triton X-100 at RT for 5 minutes.
Cells were incubated with primary antibody for either 2 hours at RT or overnight at 4°C followed by incubation in Texas Red or FITC conjugated secondary antibody. DNA was stained with Hoechst 33342. Fluorescence microscopy was performed using a Nikon epi-fluorescence microscope with 30× objective and images were acquired using Metamorph software (Universal Imaging).

Western blotting

For protein analysis, cells were lysed in either SDS sample buffer or cell lytic M buffer. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed, and proteins were transferred to a nitrocellulose membrane. Membranes were blotted with antibodies against B23 (Zymed), Thr199-phosphorylated B23 (Cell Signal Technology) and Thr234/237-phosphorylated B23 (Cell Signaling). Membranes were stained with horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences) or horseradish peroxidase-conjugated secondary antibodies (Cell Signal Technology) and visualized using SuperSignal West Pico (Pierce). Protein bands were visualized by chemiluminescence.

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References


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Fluorescence Recovery

\text{Time (seconds)}

$t_{1/2}$ in seconds

- T199E: 24.0 +/- 1.3
- T219E: 22.2 +/- 1.2
- T234/237E: 23.6 +/- 2.2