Decreased accumulation and dephosphorylation of the mitosis-specific form of nucleophosmin/B23 in staurosporine-induced chromosome decondensation

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Nucleophosmin/B23 is highly phosphorylated by cdc2 kinase during mitosis, and this phosphorylation most probably has a role in initiating and controlling the entry of cells into mitosis [Peter, Nakagawa, Doree, Labbe and Nigg (1990) *Cell* **60**, 791–801]. In the present study, the protein kinase inhibitor staurosporine has been used to examine possible changes in nucleophosmin/B23 at mitosis in HeLa cells. Addition of staurosporine to HeLa cells already arrested at mitosis by nocodazole

causes: (i) decreased accumulation of the mitosis-specific form of nucleophosmin/B23, (ii) dephosphorylation of nucleophosmin/B23, (iii) redistribution of nucleophosmin/B23 to the cytosol, and (iv) concomitant decondensation of chromosomes. These results suggest that the mitosis-specific phosphorylated form of nucleophosmin/B23 may play a role in maintaining mitotic chromosomes in their condensed state.

INTRODUCTION

Staurosporine is a microbial alkaloid known to be a potent inhibitor *in vitro* of a wide range of protein kinases [1], including protein kinase C [2], cAMP-dependent protein kinase [3], protein tyrosine kinase [4], the calcium/calmodulin-dependent protein kinase [2] and p34^{cdc2}/cyclins [5]. Incubation of cultured cells in low concentrations of staurosporine (1–10 ng/ml) selectively arrests non-transformed cells at G₁ but has no effect on the G₁ phase progression of many transformed cells [6]. At higher concentrations of staurosporine (50–100 ng/ml), normal cells arrest in both the G₁ and G₂ phases of the cell cycle, whereas transformed cells arrest only in the G₂ phase [6]. Through the use of synchronized cells, Th'ng et al. [7] showed that staurosporine causes dephosphorylation of histones H1 and H3 and concomitant decondensation of chromosomes.

During mitosis, the nucleoli of higher eukaryotes undergo extensive structural changes. Nucleoli disappear at prophase and reappear at telophase [8], and nucleolar activities cease almost completely during mitosis [9]. Immunocytochemical studies indicate that, as nucleoli disappear in prophase, a specialized area appears around each chromosome which extends a short distance from the chromosomes without encroaching into the surrounding cytoplasm [10]. This chromosome periphery has been shown to consist of closely packed fibrils and unevenly distributed dense granules [11]. Dozens of nuclear proteins such as ribosomal protein S1 [12], fibrillarin [13] and nucleophosmin/B23 [14] are found at the chromosome periphery. The chromosome periphery contains several different classes of proteins and has been attributed various possible roles: (i) in chromosome organization, (ii) as a barrier around the chromosomes, (iii) involvement in compartmentation of the cells in prophase and telophase, and (iv) a binding site for chromosomal passenger proteins necessary for the early process of nuclear assembly [10].

Nucleophosmin/B23, also called protein B23, NO38, or numatrin [15,16], is a major nucleolar phosphoprotein which is significantly more abundant in tumour and proliferating cells

than in normal resting cells [17,18]. Nucleophosmin/B23 is localized in granular regions of the nucleolus [19,20], is associated with pre-ribosomal particles [15,21] and forms hexamers [22] which may be essential for the assembly of ribosomes. Our previous studies have shown that nucleophosmin/B23 is translocated from the nucleoli to the nucleoplasm during the stationary phase of growth [23] or during treatment with certain antitumour drugs, particularly the DNA intercalators [15,24–26]. It was also reported that nucleophosmin/B23 shuttles between the nucleus and the cytoplasm [27], binds to the HIV regulatory protein Rev (which is believed to transport HIV RNA from the nucleoli to the cytoplasm [28]) and also binds to Rex protein, a regulatory factor of the retrovirus HTLV-1 (human T-cell leukaemia virus-1), which is located in the nucleoli of infected cells [29]. Dumbar et al. [30] have shown that nucleophosmin/B23 binds to single- and double-stranded DNA as well as to RNA. Stimulation of calf thymus DNA polymerase α activity [31] and relief of YY-1-induced transcriptional repression [32] by nucleophosmin/B23 have been shown. A recent study by Valdez et al. [33] demonstrated that nucleophosmin/B23 binds to amino acid residues 24–56 of protein p120, a cell-cycle-related protein. Amino acid sequence analysis [34,35] indicates that the N-terminal half of nucleophosmin/B23 has 50 % sequence identity to nucleoplasmin, a nucleosome assembly factor [16]. The middle portion of the nucleophosmin/B23 molecule is highly acidic and contains a stretch of 28 acidic amino acids which may serve as the binding domain for basic ribosomal proteins [36]. The major phosphorylation site has been identified in the acidic region of the molecule [37] and is phosphorylated by nuclear kinase NII [38]. The threonine residues of nucleophosmin/B23 are highly phosphorylated by cdc2 kinase during mitosis [39]. A large amount of evidence points to an important role for phosphorylation in the regulation of the function of nucleophosmin/B23 [38,39]. In the present study, the protein kinase inhibitor staurosporine has been used to examine the changes in nucleophosmin/B23 at mitosis. We show here that staurosporine decreases the accumulation of a mitosis-specific

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; FITC, fluorescein isothiocyanate; MAb, monoclonal antibody; PVDF, poly(vinylidene difluoride); TEMED, *N,N,N',N'*-tetramethylethylenediamine.

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form of nucleophosmin/B23 and causes the dephosphorylation and cytosolic distribution of nucleophosmin/B23, with a concomitant decondensation of chromosomes.

MATERIALS AND METHODS

Cell culture and antibody

HeLa cells were grown as a monolayer in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 0.5% (w/v) antibiotics and 3.7 g/l sodium bicarbonate in a 5% CO₂ humidified incubator at 37 °C. For immunofluorescence studies, cells were subcultured over sterilized coverglasses in 6-well plates. The monoclonal antibody (MAb) to nucleophosmin/B23 (37/5.1) was kindly provided by Dr. P. K. Chan, Department of Pharmacology, Baylor College of Medicine, Houston, TX, U.S.A. Characterization of the anti-nucleophosmin/B23 MAb has been reported previously [19].

Synchronization by double thymidine blockage

Synchronization of HeLa cells was accomplished by growth for 24 h in DMEM containing 2 mM thymidine, followed by growth for 10 h in the absence of thymidine and then for an additional 24 h with thymidine. The G₁/S transition and S phase could then be confined to 0–2 h and 2–6 h respectively after release from the double thymidine block; the G₂ phase was confined to 6–12 h, while the M phase was between 12 and 13 h. Finally, the G₁ phase was confined to 13–20 h after release from the double thymidine block. Flow cytometric analysis confirmed that more than 85% [except for cells in M phase (60%)] of the cells were at each of the defined synchronized phases of the cell cycle.

Electrophoresis

SDS/PAGE was carried out according to the protocol of Laemmli [40]. Alkaline/urea gel electrophoresis was modified from the method of Laemmli [40]. For preparation of the 6% alkaline/urea mini-separating gel, 2.67 ml of 10 M urea, 1.3 ml of 1 M Tris/HCl (pH 8.8), 1 ml of 30% total/3.3% cross-linker acrylamide, 33.4 µl of 10% ammonium persulphate and 5 µl of *N,N,N',N'*-tetramethylethylenediamine (TEMED) were mixed and poured into the gel sandwich. For preparation of the stacking gel, 0.2 ml of 1 M Tris/HCl (pH 6.8), 1.1 ml of 10 M urea, 0.2 ml of 30% total/3.3% cross-linker acrylamide, 16.7 µl of 10% ammonium persulphate and 2 µl of TEMED were mixed and poured into the gel sandwich. Samples were dissolved in an equal volume of sample buffer (31.25 mM Tris/HCl, pH 6.8, 8 M urea, 10% glycerol, 5% β-mercaptoethanol, trace amount of Bromophenol Blue) and 50 µg of cellular protein was loaded into each lane. Electrophoresis was run at 60 V for 2.9 h in electrode buffer (0.1 M Tris base, 0.2 M glycine, pH 8.8).

Mitotic index

The cells were fixed with 3.7% formaldehyde in PBS, stained with 0.1% (w/v) Hoechst 33342 and examined under a Zeiss fluorescent light microscope.

Metabolic labelling

Pulse-chase experiments with [³⁵S]methionine labelling were carried out as follows. HeLa cells arrested in M phase of the cell cycle by nocodazole were incubated for 1 h in 1.5 ml of L-methionine- and L-cysteine-free DMEM (GIBCO BRL) containing 120 µCi/ml [³⁵S]methionine (Amersham) in a 100 mm

tissue culture dish on a rocking platform. The mitotic cells were collected by mitotic shake-off and were replated in fresh medium in the presence of nocodazole and staurosporine for 2 h. The medium was then removed and the cells were washed three times with the culture medium. The cells were further incubated with 10 ml of culture medium supplemented with 10 mM L-methionine. Cells were then washed three times with 10 ml of ice-cold PBS (8.5 mM Na₂HPO₄, 1.6 mM NaH₂PO₄, 0.145 M NaCl, pH 7.2) and lysed in 0.5 ml of phosphate-buffered radioimmune precipitation assay (RIPA) buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 20 mM Na₂HPO₄, 100 mM NaCl, 20 mM NaF, 2 mM Na₃VO₄, 0.2 mM PMSF, 1 mM dithiothreitol, 30 µg/ml DNase and 30 µg/ml RNase).

For [³²P]P_i labelling, HeLa cells in M phase of the cell cycle were incubated in 1 ml of phosphate-free DMEM (GIBCO BRL) containing 1 mCi/ml [³²P]P_i (Amersham) for 1 h. The mitotic cells were collected by mitotic shake-off and were replated in fresh medium in the presence of nocodazole and staurosporine for 2 h. Then the cells were washed twice with 10 ml of PBS and lysed in 0.5 ml of RIPA buffer. All samples were incubated on ice for 20 min before they were centrifuged at 21 000 g for 10 min at 4 °C to remove membrane debris. The supernatants were obtained for immunoprecipitation.

Immunoprecipitation

The solubilized proteins were assayed using the Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, U.S.A.) with BSA as standard. The cell lysate (150 µg of protein) was diluted to 80 µl with RIPA buffer, and was cleared by incubation with washed Protein A-Sepharose beads (Pharmacia). The cleared lysate was incubated with anti-nucleophosmin/B23 MAb (diluted 1:20) for 8 h at 4 °C. The mixture was then incubated with goat anti-mouse IgG antibody (CALTAG) (diluted 1:20) for 8 h at 4 °C. Finally, the immunocomplex was captured with Protein A-Sepharose. The beads were washed twice with RIPA buffer and then twice with PBS, and were finally dissolved in gel electrophoresis sample buffer.

Western blotting

Separated proteins from SDS/PAGE or alkaline/urea gel electrophoresis were electrotransferred to a Hybond poly(vinylidene difluoride) (PVDF) membrane (Amersham). The PVDF membrane was then soaked in a blocking solution [5% (w/v) non-fat milk in PBS] for 1 h at room temperature. The soaked PVDF membrane was then incubated with MAb against nucleophosmin/B23 [diluted 1:2000 in 3% (w/v) non-fat milk in PBS] for 1 h at room temperature, washed with TBS-T buffer [20 mM Tris/HCl, pH 7.5, 0.5 M NaCl, 0.1% (v/v) Tween-20] for 3 × 15 min and incubated at room temperature for 1 h with horseradish peroxidase-conjugated goat anti-(mouse IgG) antibody (diluted 1:2000 in TBS-T buffer). The membrane was washed with TBS-T for 3 × 15 min. Immunobands were identified by chromogenic development and/or the enhanced chemiluminescence reaction (ECL; Amersham).

Phosphoamino acid analysis

Phosphoamino acid analysis were performed as described by Peter et al. [39]. The [³²P]P_i-labelled immunoprecipitates from staurosporine-treated M-phase cells were resolved by alkaline/urea gel electrophoresis (6% gel). The gel slices that contained ³²P-labelled nucleophosmin/B23 were excised from the gel and washed several times in water. The gel slices were then hydrolysed for 2 h in 6 M HCl (Sigma Chemical Co.) at 110 °C in a sealed

Eppendorf tube containing nitrogen. The insoluble material was removed and the sample was dried under vacuum. The dried material was resuspended in pH 3.5 buffer (acetic acid/pyridine/deionized water, 100:10:1890, by vol.) supplemented with 0.5 mM EDTA. The sample was loaded on a thin layer cellulose chromatography plate (Merck) and run at 1.3 kV for 24 min. As marker, 2 mg of each unlabelled phosphoamino acid (Sigma Chemical Co.) was mixed with the samples and loaded. The unlabelled phosphoamino acids were visualized by spraying the plate with 0.2% ninhydrin in acetone. The radioactive phosphoamino acids were determined by autoradiography using a phosphorimager.

Immunofluorescence

An immunofluorescence staining procedure was carried out as described by Yung et al. [26]. HeLa cells grown on coverglasses were fixed in 2% electron microscopy-grade formaldehyde in PBS for 20 min at room temperature. The cells were permeabilized with acetone at -20°C for 3 min. After three washes with PBS, fixed cells were incubated with the anti-nucleophosmin/B23 MAbs (diluted 1:20 in PBS) at 37°C for 1 h. Then cells were washed for 4×15 min in PBS and incubated with fluorescein isothiocyanate (FITC)-conjugated affinity-purified goat anti-mouse IgG (diluted 1:20 in PBS) at 37°C for 35 min. The cells were then washed for 4×15 min with PBS and mounted in 10% glycerol in PBS (pH 9).

RESULTS

Decreased accumulation of the mitosis-specific form of nucleophosmin/B23 after staurosporine treatment

Nucleophosmin/B23 is highly phosphorylated by cdc2 kinase during mitosis, and this phosphorylation most probably has a role in initiating and controlling the entry of cells into mitosis [39]. High levels (50–100 ng/ml) of staurosporine have been shown to block progression of mammalian cells in the G_2 phase of the cell cycle [6]. Upon withdrawal of the inhibitor, mitotic cells begin to appear within 15 min, suggesting that the point of arrest is very late in G_2 or early in M [7]. To elucidate the possible role of nucleophosmin/B23 in mitosis, we have investigated the inhibitory effects of staurosporine on nucleophosmin/B23 in cells that were already in mitosis. HeLa cells were first synchronized at the G_1/S boundary by double thymidine blockage. These cells were then released into the cell cycle in the presence of nocodazole to arrest cells that entered mitosis. The arrested cells were collected by mitotic shake-off and were replated in fresh medium in the presence of nocodazole and staurosporine. Using this protocol, we routinely obtained mitotic indices of between 50 and 75% after a 12–16 h incubation period with 50 ng/ml nocodazole. Microscopic examination revealed that the collected mitotic cells displayed visibly condensed chromosomes and an absence of an intact nuclear membrane (Figure 1A). Exposure of these cells to 75 ng/ml staurosporine for between 45 min and 2 h caused an obvious morphological change to the mitotic cells (Figures 1B and 1C). The chromosomes became diffuse and reassociated with the reformed nuclear membrane. Cells counts did not show a decline in the total number of cells within this period of treatment. Trypan Blue exclusion analyses also showed that there was minimal cell death. Determination of the mitotic index revealed that the number of cells containing mitotic chromosomes fell to less than 10% after 45 min–2 h of exposure to staurosporine. For comparison, cells in G_1/S are illustrated in Figure 1(D), showing a nucleus containing chromatin material that is diffuse.

Figure 1 Effect of staurosporine on mitotic cells

HeLa cells were first synchronized at the G_1/S boundary by double thymidine blockage. These cells were then released into the cell cycle in the presence of 50 ng/ml nocodazole to arrest cells that entered mitosis. The arrested cells were collected by mitotic shake-off (A and E) and were exposed to 75 ng/ml staurosporine for 45 min (B and F) or 2 h (C and G). Untreated interphase (G_1/S) cells are shown in (D) and (H). The cells were fixed with formaldehyde and stained with propidium iodide (A–D) to visualize DNA; (E–H) are the phase-contrast pictures.

On examination by alkaline/urea gel electrophoresis (6% gel) and Western blotting, nucleophosmin/B23 from the collected mitotic cells had an electrophoretic mobility that was slightly greater than that of nucleophosmin/B23 from interphase cells (Figure 2, lanes 1 and 2). Although a single and rather diffuse

Figure 2 Decreased accumulation of mitosis-specific nucleophosmin/B23 in the presence of staurosporine

Nocodazole-arrested mitotic HeLa cells collected by mitotic shake-off were exposed to 75 ng/ml staurosporine. The entire cellular proteins (70 μg) were subjected to alkaline/urea gel electrophoresis (6% gel) and transferred on to a PVDF membrane. The blotted membrane was incubated with anti-nucleophosmin/B23 MAbs. Immunoreactivity was determined using the enhanced chemiluminescence reaction (ECL; Amersham). Lanes 1 and 2 are the untreated interphase (G_1/S) and mitotic cells respectively. Treatment of mitotic cells with staurosporine was for 45 min (lane 3) or 2 h (lane 4). Arrowheads indicate the migration positions of the mitotic form (M) and the interphase form (I) of nucleophosmin/B23.

Figure 3 Localization of nucleophosmin/B23 in nocodazole-arrested HeLa cells in the presence of staurosporine

The nocodazole-arrested cells collected by mitotic shake-off (A) were exposed to 75 ng/ml staurosporine for 45 min (B) or 2 h (C). Untreated interphase (G_1/S) cells are shown in (D). Anti-nucleophosmin/B23 MAb and FITC-conjugated secondary antibody were used to detect nucleophosmin/B23.

band was seen, the band in lane 2 showed reproducibly faster migration than that in lane 1. After 45 min of exposure to 75 ng/ml staurosporine, the accumulation of the mitotic form (M-form) of nucleophosmin/B23 was decreased. Nucleophosmin/B23 in cells that were already arrested at mitosis by nocodazole began to reappear in its interphase-form (I-form) after 45 min–2 h of exposure to staurosporine (Figure 2, lane 3 and 4).

Effect of staurosporine on localization of nucleophosmin/B23 in mitotic cells

The disappearance of mitotic cells was correlated directly with the fall in the accumulation of the mitotic form of nucleophosmin/B23. To provide evidence for a role for mitotic nucleophosmin/B23, the effect of staurosporine on the localization of nucleophosmin/B23 in cells that were already in mitosis was studied by immunofluorescence staining (Figure 3). A MAb to nucleophosmin/B23 and FITC-conjugated secondary antibody were used to detect nucleophosmin/B23. When the synchronized HeLa cells were held at mitosis with nocodazole, nucleophosmin/B23 immunoreactivity was localized in the highly

condensed chromosomes of mitotic cells (Figure 3A). After exposure to 75 ng/ml staurosporine for 45 min–2 h, nucleophosmin/B23 became diffuse and was localized all over the cytosol outside the reformed nuclei (Figures 3B and 3C). For comparison, cells that were in G_1/S are illustrated in Figure 3(D), showing localization of nucleophosmin/B23 in the nucleoli. Table 1 quantifies the proportions of cells displaying condensed or diffuse chromosomes, as well as the proportions of cells in which nucleophosmin/B23 was localized in the condensed chromosomes or the cytosol. More than 90 % of cells displayed diffuse chromosomes and had nucleophosmin/B23 localized in the cytosol after staurosporine treatment of the collected mitotic cells for 45 min–2 h.

Because of the presence of nocodazole, the loss of mitotic cells in response to staurosporine treatment was not due to cells entering G_1 ; this was confirmed by cytofluorimetric analyses (Figure 4). We concluded that the accumulation of the mitosis-specific form of nucleophosmin/B23 was decreased and nucleophosmin/B23 was delocalized from the condensed chromosomes to the cytosol when the mitotic cells underwent the process of decondensation, and the cells were driven back to a G_2 -like state by staurosporine.

Nucleophosmin/B23 in the recovery of cells from staurosporine arrest

The viability of the nocodazole-arrested cells was further confirmed by cytofluorimetric analysis, which showed that they re-entered G_1 within 4 h of the removal of nocodazole (Figure 4A). To investigate further whether the mitotic cells that were driven back to the G_2 -like state by staurosporine could recover from this treatment, nocodazole-arrested HeLa cells, after being exposed to 75 ng/ml staurosporine for 45 min or 2 h, were washed three times with PBS and resuspended in the culture medium. Most of the nocodazole-arrested cells that had been exposed to staurosporine for 45 min or 2 h had completed mitosis and re-entered G_1 by 8 or 12 h respectively after the removal of the drugs (Figures 4B and 4C). The viability of cells as measured by Trypan Blue exclusion remained greater than 90 % during staurosporine treatment as well as during the recovery period. The cell counts also showed about a 2-fold increase after proceeding to G_1 phase. These results indicate that the staurosporine-induced decondensation of the chromosomes is reversible and that the speed of recovery varies depending on the length of exposure to the drug.

In a ^{35}S pulse-chase experiment, nocodazole-arrested cells were labelled with 120 $\mu\text{Ci/ml}$ [^{35}S]methionine for 1 h. The mitotic cells were then collected by mitotic shake-off. Cells were

Table 1 Effect of staurosporine on mitotic cells and on the cellular localization of nucleophosmin/B23

HeLa cells were synchronized at the G_1/S boundary by double thymidine blockage. These cells were then released into the cell cycle in the presence of 50 ng/ml nocodazole to arrest cells that entered mitosis. The arrested cells were collected by mitotic shake-off and were exposed to 75 ng/ml staurosporine for 45 min or 2 h. The cells were fixed with formaldehyde and stained with propidium iodide to visualize DNA. For immunostaining, anti-nucleophosmin/B23 MAb and FITC-conjugated secondary antibody were used to detect nucleophosmin/B23. Triplicate 100-cell counts were performed. The data shown are means $\pm$ S.E.M. and are representative of the results obtained in several (at least three) replications of the experiment.

Length of staurosporine treatment	Cells (%) displaying:		Cells (%) in which nucleophosmin/B23 is localized in:	
	Condensed chromosomes/no intact nuclear membrane	Diffuse chromosomes/Re-formed nuclear membrane	Condensed chromosomes	Cytosol
0	100	0	100	0
45 min	9 $\pm$ 5	91 $\pm$ 4	10 $\pm$ 4	90 $\pm$ 3
2 h	7 $\pm$ 5	93 $\pm$ 4	9 $\pm$ 4	91 $\pm$ 4

Figure 4 Recovery of mitotic cells from staurosporine treatment

Nocodazole-arrested mitotic HeLa cells collected by mitotic shake-off were treated with 75 ng/ml staurosporine for 0 h (A), 45 min (B) or 2 h (C). The cells were then washed three times with PBS and resuspended in the culture medium for incubation periods of 4, 8, 12 and 16 h. Samples removed were analysed by cytofluorimetry.

Figure 5 M-phase-synthesized nucleophosmin/B23 in the recovery from staurosporine treatment

Nocodazole-arrested mitotic cells were pulse-labelled with [35 S]methionine for 1 h. The mitotic cells were then collected by mitotic shake-off (lane 1) and were treated with staurosporine for 2 h, washed with PBS and resuspended in culture medium for incubation periods of 0 h (lane 2), 4 h (lane 3), 8 h (lane 4) and 12 h (lane 5). Nucleophosmin/B23 was immunoprecipitated from the cellular extract (150 μ g), subjected to alkali/urea gel electrophoresis (6% gel) and detected by autoradiography using a phosphorimager.

cultured in medium in the presence of nocodazole and staurosporine for additional 2 h before being washed three times with PBS and replated in fresh medium. At various times (4–12 h) after removal of the drugs, nucleophosmin/B23 was immunoprecipitated. The results showed that [35 S]nucleophosmin/B23 synthesized at M phase was still present after recovery from staurosporine-induced chromosome decondensation (Figure 5).

Induction of nucleophosmin/B23 dephosphorylation in mitotic cells by staurosporine

It has been demonstrated that mitosis is accompanied by extensive phosphorylation of nucleophosmin/B23 [39]. It is likely that the functional role of nucleophosmin/B23 in chromosome condensation is regulated by its phosphorylation. Thus we examined the effect of staurosporine on the phosphorylation of nucleophosmin/B23 in cells that were already in mitosis. In these experiments, nocodazole-arrested cells were labelled with 50 μ Ci/ml [32 P] P_i for 1 h. The mitotic cells were collected by mitotic shake-off. Cells were then cultured in medium in the presence of nocodazole and staurosporine for an additional 2 h before harvest. Nucleophosmin/B23 from mitotic cells with or without exposure to staurosporine was then immunoprecipitated.

Figure 6 Dephosphorylation of mitosis-specific nucleophosmin/B23 in the presence of staurosporine

Nocodazole-arrested mitotic cells were labelled with [32 P] P_i for 1 h. The mitotic cells were collected by mitotic shake-off (lane 1) and treated with staurosporine for 2 h (lane 2). Nucleophosmin/B23 was immunoprecipitated from the cellular extract (150 μ g), subjected to alkali/urea gel electrophoresis (6% gel) and detected by autoradiography using a phosphorimager (A). (B) Immunoprecipitated nucleophosmin/B23 was subjected to SDS/10%-PAGE and transferred on to a PVDF membrane. The blotted membrane was incubated with anti-nucleophosmin/B23 MAb. Immunoreactivity was determined using the enhanced chemiluminescence reaction (ECL; Amersham). (C) Phosphoamino acid analysis of nucleophosmin/B23 from nocodazole-arrested cells without (lane 1) or with (lane 2) exposure to staurosporine, resolved by alkaline/urea gel electrophoresis. The phosphorylated amino acids were separated by one-dimensional electrophoresis as described in the Materials and methods section and autoradiographed using a phosphorimager. The positions of migration of phosphoserine (S), phosphothreonine (T) and phosphotyrosine (Y) standards, detected by ninhydrin staining, are indicated.

Nucleophosmin/B23 from mitotic cells after exposure to staurosporine, resolved by SDS/PAGE, was shown to be phosphorylated to much lower level than that from mitotic cells without exposure to staurosporine (Figure 6A), relative to the amount of nucleophosmin/B23 as detected by Western blotting using anti-nucleophosmin/B23 MAb (Figure 6B). The results indicate that staurosporine induces dephosphorylation of nucleophosmin/B23 in mitotic cells. Figure 6(C) shows the

results of phosphoamino acid analysis of nucleophosmin/B23 from mitotic cells with or without exposure to staurosporine, resolved by alkaline/urea gel electrophoresis (6% gel). Nucleophosmin/B23 from mitotic cells contained substantial amounts of phosphoserine and phosphothreonine, whereas much lower amounts of these phosphoamino acids were detected in nucleophosmin/B23 from cells after exposure to staurosporine. The presence of staurosporine had a more pronounced effect on phosphothreonine than on phosphoserine of nucleophosmin/B23, in that there was almost complete loss of phosphothreonine after exposure to staurosporine. No evidence was obtained for phosphorylation of nucleophosmin/B23 on tyrosine residues.

DISCUSSION

The accumulation of the highly phosphorylated mitosis-specific form of nucleophosmin/B23 is correlated with the condensation of chromatin into mitotic chromosomes. In this study we have shown that, in HeLa cells, the accumulation of the highly phosphorylated mitotic form of nucleophosmin/B23 may play a role in maintaining mitotic chromosomes in their highly condensed state, and that staurosporine inhibition of kinases leads to decreased accumulation and dephosphorylation of the mitotic form of nucleophosmin/B23 and a concomitant decondensation of chromosomes. This linkage of the loss of the mitotic form of nucleophosmin/B23 and its dephosphorylation with the decondensation of mitotic chromosome suggests that the G_2 -M phase chromosome condensation process may involve the phosphorylation of nucleophosmin/B23. Such phosphorylation could play a role in mediating and controlling mitotic reorganization and the activity of nucleoli. The mitotic form of nucleophosmin/B23 may be actively responsible for and implicated in some functional roles in the regulation of mitosis.

As measured by alkaline/urea gel electrophoresis and Western blotting, nucleophosmin/B23 from collected mitotic cells had a greater electrophoretic mobility than that from interphase cells. A single and rather diffuse band of nucleophosmin/B23 is all that is seen. Nevertheless, the band representing mitotic nucleophosmin/B23 migrated more quickly than that representing nucleophosmin/B23 from interphase cells. In studies using two-dimensional gel electrophoresis (isoelectric focusing gel electrophoresis for the first dimension, followed by SDS/PAGE), we recently observed multiple forms of nucleophosmin/B23 during the entry into mitosis (P. S. Jiang and B. Y.-M. Yung, unpublished work). It is possible that the diffuse band of nucleophosmin/B23 at mitosis (Figure 2, lane 2) or during staurosporine treatment (Figure 2, lanes 3 and 4) represents more than one form of the protein. The presence of multiple forms of nucleophosmin/B23 that may display different levels of phosphorylation is currently under investigation.

It has been demonstrated that nucleophosmin/B23 is highly phosphorylated by cdc2 kinase during mitosis [39]. The rapid effects of staurosporine on nucleophosmin/B23 dephosphorylation and chromosome decondensation in mitotic cells suggests that staurosporine is acting directly on cdc2 kinase. These results indicate that the cdc2 kinase responsible for the mitotic phosphorylation of nucleophosmin/B23 in HeLa cells is sensitive to the inhibitory action of staurosporine, and that this kinase is continuously acting in the mitotic cells to maintain a highly phosphorylated state of nucleophosmin/B23. Consistent with the inhibitory effect of staurosporine on cdc2 kinase is the observation that the nuclear membrane re-forms in the presence of the inhibitor. The dissolution of the nuclear envelope during mitosis was shown to result from the phosphorylation of lamins by cdc2 kinase [39]. The inhibitory effects of staurosporine would

thus lead to the re-formation of the nuclear membrane (see Figures 1 and 3). Nucleophosmin/B23 in the mitotic cells that are driven back to a G_2 -like state by staurosporine is distributed in the cytosol even though the nuclear membrane re-forms in the presence of staurosporine. Regardless of the cytosolic localization of nucleophosmin/B23, the cells were still able to recover from staurosporine treatment, completing mitosis and re-entering G_1 .

The greatest difficulty in assessing the role of the cell-cycle-dependent phosphorylation of nucleophosmin/B23 is that the precise function of this protein during interphase remains to be explored. Our recent results [41] show that the alternation of nucleophosmin/B23's nucleolar and nuclear localizations is most frequently observed in cells during the G_1 and G_1/S phases. In parallel to this, the reversible change between the hexameric and monomeric forms of nucleophosmin/B23 [22] also occurs most frequently in cells during the G_1 and G_1/S phases. Nucleophosmin/B23, being possibly involved in rRNA processing and transport, is highly active in the G_1 and G_1/S phases, as demonstrated by the dynamic, reversible, changes in its localization and oligomerization state. It is also conceivable that phosphorylation may modulate interactions between nucleophosmin/B23 and either rRNA or proteins involved in pre-ribosomal particle transport and assembly.

Hyperphosphorylation of histone H1 and phosphorylation of histone H3 also correlate well with the condensation of chromosomes observed during the transition between the G_2 and M phases [7]. It is thus an attractive possibility that histone H1 and H3 phosphorylation by kinases, including cdc2 kinase, may play a role in chromosome condensation. Taken together, the mitotic phosphorylation of histone proteins (H1 and H3) and of a non-histone nucleolar protein (nucleophosmin/B23) may be important in mediating several ultrastructural changes that accompany the entry of cells into mitosis. Specifically, phosphorylation of nucleophosmin/B23 may cause mitotic reorganization of nucleoli and, concomitantly, mitotic arrest of rRNA synthesis and ribosome assembly. Nucleolar disassembly and reassembly are probably controlled by mechanisms fundamentally similar to those implicated in mitotic chromosome condensation.

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