Mitosis-Specific Phosphorylation of Nucleolin by p34^{cdc2} Protein Kinase

PASCALE BELENGUER,¹ MICHÈLE CAIZERGUES-FERRER,¹ JEAN-CLAUDE LABBÉ,²
MARCEL DORÉE,² AND FRANÇOIS AMALRIC^{1*}

*Centre de Recherche de Biochimie et de Génétique Cellulaires du Centre National de la Recherche Scientifique,
118 route de Narbonne, 31062 Toulouse Cedex,¹ and Centre National de la Recherche Scientifique
and Institut National de la Santé et de la Recherche Médicale, B.P. 5051,
34033 Montpellier Cedex,² France*

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Nucleolin is a ubiquitous multifunctional protein involved in preribosome assembly and associated with both nucleolar chromatin in interphase and nucleolar organizer regions on metaphasic chromosomes in mitosis. Extensive nucleolin phosphorylation by a casein kinase (CKII) occurs on serine in growing cells. Here we report that while CKII phosphorylation is achieved in interphase, threonine phosphorylation occurs during mitosis. We provide evidence that this type of in vivo phosphorylation involves a mammalian homolog of the cell cycle control Cdc2 kinase. In vitro M-phase H1 kinase from starfish oocytes phosphorylated threonines in a TPXK motif present nine times in the amino-terminal part of the protein. The same sites which matched the p34^{cdc2} consensus phosphorylation sequence were used in vivo during mitosis. We propose that successive Cdc2 and CKII phosphorylation could modulate nucleolin function in controlling cell cycle-dependent nucleolar function and organization. Our results, along with previous studies, suggest that while serine phosphorylation is related to nucleolin function in the control of rDNA transcription, threonine phosphorylation is linked to mitotic reorganization of nucleolar chromatin.

Ribosome biogenesis in eucaryotic cells takes place within the nucleolus (see reference 24 for a review). This fundamental process is directly related to cell growth and modulated during progression through the cell cycle. Fluctuations in ribosomal particle formation are linked to changes in the structural organization of the nucleolus. During interphase, a functional structure in which rDNA transcription occurs is established by the contribution of DNA bearing the ribosomal genes (known as the nucleolar organizer regions [NORs]) from several chromosomes. During prophase, chromosome condensation is accompanied by the disaggregation of the nucleolus. A subset of nucleolar proteins remains associated with the chromosomal NORs (see reference 46 for a review). These proteins are candidates for controlling the switch-off of rRNA synthesis and structural reorganization of the nucleolus following mitosis. Among these, nucleolin (20) is a multifunctional protein (37) that is involved in nucleolar chromatin organization (19, 40) and in pre-rRNA packaging (10, 27).

The idea that nucleolin function is coupled to growth control by phosphorylation is supported by the observation that active rRNA transcription is correlated to highly phosphorylated nucleolin (22, 44, 48, 49). In growing cells, a casein kinase II (CKII) phosphorylates nucleolin on serine during interphase, while in confluent cells, nucleolin is unphosphorylated and rRNA synthesis goes down to 5% of the growing level (4, 12). CKII phosphorylation sites have been mapped in the amino-terminal domain of the molecule next to long acidic domains. Similar stretches are also found in a class of anionic nuclear proteins (see reference 17 for review). A basic motif (TPXK) repeated between the first two acidic domains is reminiscent of sequences present in the amino- and carboxy-terminal domains of histone H1 (52). This motif was recently proposed to determine the consen-

sus phosphorylation sequence (45) of a vertebrate homolog of the yeast cell cycle control Cdc2⁺/CDC28 kinase (see reference 25 for a review). The product of the yeast *cdc2⁺/CDC28* gene, p34^{cdc2} kinase, was shown to be a component of starfish and frog M-phase-specific H1 kinase and M-phase-promoting factor (3, 16, 21, 33-35) and to be homologous to the mammalian growth-associated H1 kinase (36).

As cells enter mitosis, a sharp increase in p34^{cdc2} activity (33), which is also correlated to an interaction with cyclin (43), leads to both hyperphosphorylation of histone H1 and chromatin condensation (6). Following activation of the M-phase-promoting factor, a subset of cyclin has been detected in the nucleolus by immunocytology (53). Although this association is weak, it could suggest that there may be as yet unidentified nucleolar substrates for Cdc2 kinase which could be involved in the mitotic reorganization of the nucleolus. The presence in nucleolin of the consensus TPXK motif, found in histone H1 and used as phosphorylation sites by p34^{cdc2} kinase (38), together with data on its interaction with chromatin (19) and its chromosomal localization during mitosis (20), prompted us to investigate the phosphorylation state of nucleolin in mitosis. We report here that in vivo nucleolin is phosphorylated on threonine in M phase by the p34^{cdc2} kinase, while CKII serine phosphorylation occurs in interphase. In vitro, nucleolin is phosphorylated by p34^{cdc2} and by CKII on the same sites as in vivo. These data provide strong circumstantial evidence that nucleolin function is coupled to cell growth by sequential phosphorylation involving the Cdc2 and the CKII kinases.

MATERIALS AND METHODS

Cell culture and cell labeling. Chinese hamster ovary (CHO) cells were grown as monolayers or in suspension cultures at 37°C in Eagle minimal essential medium (Eurobio) supplemented with 8% fetal calf serum. Cell plates were labeled by incubation with 0.1 mCi of ³²P_i (Amersham) per

* Corresponding author.

FIG. 1. Phosphorylation and purification of nucleolin from exponentially growing cells. (A) Nucleoli of unsynchronized CHO cells that were in vivo ³²P-labeled for 15 h were prepared as detailed previously (55). Nucleolar proteins were analyzed by 10% SDS-PAGE, and the dried gel was exposed for 1 h. Positions of the two major nucleolar phosphoproteins of exponentially growing cells are indicated. Phosphoamino acid analysis of in vivo-labeled nucleolin (100 kDa) was carried out by 6 N HCl hydrolysis at 110°C for 2 h, followed by separation onto a thin-layer plate by high-voltage electrophoresis and autoradiography for 3 days. Positions of phosphoserine (P-Ser) and phosphothreonine (P-Thr) are noted in reference to migration of cold markers. (B) Purification of nucleolin was performed essentially as described by Caizergues-Ferrer et al. (12). Briefly, a low-ionic-strength extract of nuclei (S2, lanes 1 and 4) prepared from exponentially CHO growing cells was applied to a heparin-Sepharose column (HS). Nucleolin and its associated CKII kinase were found to copurify (lanes 2 and 5). An additional Mono Q anion-exchange chromatography step (MQ) was then processed to give pure nucleolin (lanes 3 and 6). Lanes 1 to 3, Coomassie blue (Coo) staining of 12% SDS-PAGE analysis of nucleolin peak fractions. Lanes 4 to 6, autoradiographs showing endogenous phosphorylation activity (12) of the corresponding fractions (exposure time was 30 min).

ml for 2 h before harvesting (11). For the isolation of mitotic cells, nocodazole (methyl[5-(2 thienylcarbonyl)-1H-benzimidazol-2-yl] carbamate; Sigma Chemical Co.) was added to the culture medium to a final concentration of 25 µg/ml. After 15 h of incubation, about 50% of the cells were in mitotic stage as estimated by 4',6'-diamidino-2-phenylindole dihydrochloride staining of DNA. In other experiments, hydroxyurea (Sigma) was added to a final concentration of 2 µM for 15 h.

Purification of in vivo ³²P-labeled nucleolin from interphasic or mitotic cells. In vivo ³²P-labeled untreated cells or hydroxyurea-blocked cells were washed and then harvested by scraping in RIB medium (250 mM sucrose, 40 mM Tris hydrochloride [Tris-HCl, pH 7.4], 25 mM KCl, 4 mM MgCl₂) in the presence of protease inhibitors (10 µg of leupeptin per ml and 0.1 mM phenylmethylsulfonyl fluoride) and phosphatase inhibitors (1 mM *p*-chloromercuriphenyl-sulfonic acid, 5 mM NaF, and 100 mM β-glycerophosphate). When cells were exposed to nocodazole, the loosely attached mitotic cells were detached by gently moving the medium across the plate. Cells were collected by low-speed centrifugation and washed in RIB buffer containing protease and phosphatase inhibitor mixture. Extraction of cellular pellets was carried out in 5 M urea-2 M NaCl-10 mM sodium phosphate (pH 7) for 4 h at 4°C. The supernatant of a high-speed centrifugation (100,000 × *g*, 30 min) was applied

TABLE 1. Target sites for M-phase p34^{cdc2}-associated protein kinase^a

Substrate	Phosphorylated sequences
Nucleolin	
CHO ^b	A ⁵⁸ TPAKK P ⁷⁵ TPAKK V ⁸³ TPGKK A ⁹¹ TPAKK V ⁹⁷ TPAKA A ¹⁰⁵ TPGKK A ¹²⁰ TPGKK V ¹²⁸ TPAKA I ²¹⁹ TPAKG
Chicken ^c	—*TPAKK P ⁹⁴ SPKKA —**TPAKA
Histone H1 ^d	K ¹⁶ TPAKK A ¹³⁶ TPKKP K ¹⁵³ TPKKA K ¹⁸⁰ SPKKA
pp60 ^{c-src}	Q ³⁴ TPNKT R ⁴⁶ TPSRS S ⁷² SPQRA
SV40 T antigen ^f	S ¹²⁴ TPPKK

^a Numbering and asterisks (*, 56, 63, 70, 77, and 85; **, 206 and 211) indicate positions of phosphorylated or putative phosphorylated residues counting from the NH₂ terminus. One asterisk indicates that there are two lysine residues in the motif; two asterisks indicate that there is one lysine residue in the motif. The p34^{cdc2} consensus sequence is X(T/S)PX-Basic, where X may be basic or not.

^b Potential phosphorylation sites on CHO nucleolin sequence (5) were deduced from the p34^{cdc2} consensus.

^c Putative Cdc2 target sites on chicken nucleolin sequence were communicated by A. E. Nigg before publication (42).

^d These data are based on published phosphorylation histone H1 sequences and are reviewed by Hohmann (28) and Matthews and Huebner (38). Growth-associated protein kinase was shown to phosphorylate the amino- and carboxyl-terminal domains of histone H1 in the range of three to six phosphates per molecule depending on H1 subtype. Calf or rabbit thymus H1 is listed.

^e Reference 45.

^f Simian virus 40 (SV40) (39).

FIG. 2. In vitro phosphorylation of nucleolin by Cdc2 and CKII protein kinases. Purified nucleolin and histone H1 were incubated in the presence of [γ-³²P]ATP with Cdc2 or CKII purified protein kinase and loaded onto a 12% SDS-PAGE gel as described in Materials and Methods. Lanes 1 to 6, autoradiographs of dried gels; exposure was for 30 min. Nucleolin (lane 1) or histone H1 (lane 2) was incubated with Cdc2. Lane 3, Cdc2 alone. Nucleolin (lane 4) or histone H1 (lane 5) was incubated with CKII. Lane 6, CKII alone.

TABLE 2. Apparent K_m and V_{max} values for p34^{cdc2} kinase^a

Substrate	K_m (μ M)	V_{max} (arbitrary units)	Reference
Nucleolin	1.2	10	This study
Histone H1	2	100	This study
	1.6		54
	2.3		41
	3		8

^a Conditions are described in the text; values were deduced from Lineweaver-Burke plots.

to a hydroxyapatite column (IBF Biotechnics). The column was washed extensively with 10 mM sodium phosphate buffer containing 10% glycerol. Elution was performed in a 10 mM to 1 M sodium phosphate gradient with 10% glycerol. Fractions were analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by autoradiography and immunodetection of nucleolin by the method of Bugler et al. (9).

Purification of nucleolin and CKII protein kinase from exponentially growing cells. Nuclei were prepared from CHO cell suspensions (55) and lysed in low-ionic-strength buffer. The resulting S2 extract was applied to a heparin-Sepharose column (Pharmacia) as described previously (12). Fractions containing nucleolin and its associated CKII protein kinase were then subjected to anion-exchange chromatography (Mono Q, Pharmacia). CKII activity was eluted (0.28 M KCl [56]) before nucleolin (0.45 M KCl) with a discontinuous linear KCl gradient (0.05 to 2 M) made in 50 mM Tris-HCl (pH 7.9)–0.5 mM EDTA–30% glycerol. CKII kinase assays were performed across the column with exogenously added casein as a substrate in the conditions already detailed by Caizergues-Ferrer et al. (12); 1 U of casein kinase activity corresponds to 1 nmol of ³²P transferred per min at 30°C. Protein-containing fractions were analyzed by 12% SDS-PAGE followed by Coomassie blue staining of gels and autoradiography when endogenous phosphorylation was carried out. Endogenous kinase assays were performed as

FIG. 3. Phosphoamino acid composition of nucleolin phosphorylated in vitro by Cdc2 and CKII protein kinases. In vitro Cdc2- (lane 2) and CKII- (lane 3) ³²P-labeled nucleolin was subjected to 6 N HCl hydrolysis at 110°C for 2 h. Phosphoamino acids were visualized by autoradiography for 10 h after separation by high-voltage electrophoresis on thin-layer plates. Positions of nonradioactive phosphoserine (Ser) and phosphothreonine (Thr) markers visualized by ninhydrin staining are indicated in lane 1. The identity of the extra spot (?) is not known.

FIG. 4. Purification of in vivo ³²P-labeled nucleolin from interphasic or mitotic cells. In vivo ³²P-labeled nucleolin purification was achieved by a chromatography step on a hydroxyapatite column of crude extracts from untreated, hydroxyurea-blocked, and nocodazole-arrested cells. Total trichloroacetic acid-precipitated fractions were subjected to 10% SDS-PAGE and autoradiographed. (A) Only major fractions containing nucleolin are shown; exposure time was 30 min (panel 1), 2 h (panel 2), and 1 h (panel 3). (B) Immunodetection of nucleolin (NUC) by polyclonal antibody (9) on samples of peak fraction containing nucleolin from untreated (lane 1), hydroxyurea-treated (lane 2), and nocodazole-treated (lane 3) cells. Exp. Exponential.

already described without addition of exogenous substrate (12).

p34^{cdc2} protein kinase purification. p34^{cdc2} kinase was highly purified from starfish oocytes arrested at the first meiotic metaphase by using conventional chromatographic steps. The different steps of purification have been described in detail by Labbe et al. (33–35) and included chromatogra-

FIG. 5. Phosphoamino analysis of in vivo ³²P-labeled nucleolin. In vivo ³²P-labeled purified nucleolin was subjected to 6 N HCl hydrolysis at 110°C as detailed in Materials and Methods. Phosphoamino acids were separated by two-dimensional high-voltage electrophoresis on thin-layer plates, visualized by autoradiography, and noted according to migration of cold phosphoserine (S, solid arrow) and phosphothreonine (T, solid arrowhead) markers (panel D). Amino acid composition of nucleolin from untreated (panel A), hydroxyurea-treated (panel B), and nocodazole-treated (panel C) cells is shown. Exposure time was 1 week.

FIG. 6. One-dimensional CNBr phosphopeptide maps of nucleolin phosphorylated in vitro by Cdc2 and CKII protein kinases. (A) Schematic representation of amino-terminal domain of nucleolin primary structure. Solid arrows mark CNBr cleavage sites occurring on methionine residues (M). The 29-kDa and the 33-kDa peptides are phosphorylated (³²P) in vivo in exponentially growing CHO cells as well as in vitro by a CKII protein kinase (12). The 33-kDa ³²P-labeled peptide (residues 17 to 216) results from partial cleavage at methionine 32 and contaminates the carboxyl-unphosphorylated 33-kDa peptide (residues 286 to 546). The four acidic stretches are indicated (A to D). The in vitro CKII-phosphorylated serines (S) are marked by solid arrowheads. Positions of potential threonine acceptor sites for p34^{cdc2} kinase are marked within the basic repeated motifs (TPXK) containing the Cdc2 phosphorylation consensus sequence. (B) Autoradiographs of a 15% SDS-PAGE dried gel showing the ³²P-labeled peptides resulting from CNBr hydrolysis of in vitro Cdc2- (lane 1) or CKII- (lane 2) phosphorylated nucleolin. In vitro Cdc2 (lane 3) or CKII (lane 4) kinase assays were performed on the 29-kDa purified peptide (extracted from the gel, 0.5 μ g) as described in Materials and Methods for nucleolin. Arrowheads indicate the positions of the labeled fragment of 29 kDa and its related peptide of 33 kDa. Exposure times were 4 and 5 h. In vitro Cdc2 (lane 5) and CKII (lane 6) kinase assays were performed on a carboxyl-terminal peptide (2 μ g) noted as 50 kDa and extending from positions 278 to 713 (see panel A). In lanes 7 and 8, purified 29-kDa peptide (0.1 μ g) was added to kinase assay mixes containing purified 50-kDa peptide (2 μ g) as a control for Cdc2 activity. Exposure time was 15 h. The carboxy-terminal 50-kDa peptide was generated by overexpression in *E. coli* (data not shown; L. Ghisolfi, unpublished data) and purified essentially as described previously for nucleolin (12). Lane 9, Coomassie blue staining of purified 50-kDa peptide (0.5 μ g). Lane 10, Immunological detection of the purified peptide performed as for nucleolin.

phy on DEAE-cellulose, hydroxyapatite, TSKG 300 SWG, Mono Q, and Mono S. Kinase activity was monitored on column fractions by incorporation of ³²P into histone H1 which was added exogenously (1 U of H1 kinase activity corresponds to 1 μ mol of ³²P transferred per min at 30°C). An enzyme that was more (Mono Q step) or less (phosphocellulose step) purified was used in Cdc2 kinase assays. Each phosphorylation experiment was carried out at least once

with both Cdc2 protein kinase preparations, and the same results were obtained.

In vitro labeling and kinase assays. Phosphorylation of purified nucleolin (0.3 μ g) or histone H1 (0.1 μ g; Boehringer Mannheim) was assayed in a final volume of 25 μ l in 10 mM Tris-HCl (pH 7.9)–5 mM MgCl₂–10 μ M β -mercaptoethanol–80 mM KCl–10 μ g of leupeptin per ml–10 μ M cold ATP–2.5 μ Ci of [γ -³²P]ATP (Amersham; 3,000 Ci/mmol). 0.1 μ U of

FIG. 7. Two-dimensional tryptic phosphopeptide maps of nucleolin phosphorylated in vitro by Cdc2 and CKII protein kinases. Gel pieces from 10% SDS-PAGE containing in vitro ^{32}P -labeled nucleolin were subjected to trypsin digestion as detailed in Materials and Methods. The resultant tryptic peptides were analyzed by electrophoresis (horizontal direction, pH 4.4, anode to the left) and ascending chromatography. Panels A and B show tryptic fingerprint of nucleolin phosphorylated in vitro by the CKII and Cdc2 kinases, respectively. Tryptic phosphorylated fragments obtained upon

Cdc2 or CKII purified protein kinase was added for nucleolin and histone H1 assays, respectively. When required, heparin (100 $\mu\text{g}/\text{ml}$) was used to inhibit CKII activity. Reactions were performed at 30°C for 15 min, stopped by addition of SDS sample buffer (10 mM Tris hydrochloride [pH 8], 2 mM EDTA, 2% SDS, 0.7 mM β -mercaptoethanol, 10% glycerol), and boiled for 2 min. Samples were loaded onto a 12% SDS-polyacrylamide gel. After Coomassie blue staining, the gel was dried and exposed for autoradiography.

To determine the kinetic parameters (K_m , $V_{\max}$) of $\text{p}34^{\text{cdc}2}$, kinase assays with various substrate concentrations were performed in duplicate, using kinase from the phosphocellulose step, and analyzed both by scintillation counting and densitometric analysis. The nucleolin concentration (Mono Q step of purification), in the range of 0.25 to 1.5 μg , was estimated both by the Bradford protein assay (7) and densitometric analysis as for H1 (0.5 to 2.5 μg). Time course reactions (10 s to 10 min) were stopped either by addition of 15% trichloroacetic acid followed by filtration onto nitrocellulose filters, or by addition of SDS sample buffer followed by analysis on SDS-PAGE gels and autoradiography (linearity was observed for up to 10 min).

In all experiments, autoradiography was carried out with Trimax XM films (3M) at -80°C with an intensifying screen except for the Cdc2 affinity determination, for which the screen was omitted.

In vitro competition experiments. Synthetic peptides derived from nucleolin (P1, [ATPAKKAA] $_2$) and from H1 (P2, [KTPKKAKKP] $_2$) were prepared on an Applied Biosystems 430A. The degree of purity was monitored by mass spectrometry after an RP18 reverse-phase high-pressure liquid chromatography purification step. In vitro phosphorylation competition experiments were assayed in standard conditions for kinase assays followed by analysis on 8 to 20% gradient SDS-PAGE gels. First, 5 μg of P1 and 15 μg of P2 were added to 0.2 and 0.3 μg of H1 and nucleolin, respectively. Then, increasing amounts of peptide competitor P2 (0.25, 0.5, and 1 μg or 0.4, 0.6, 0.8, and 2.4 μg) were added to 0.25 μg of nucleolin or to 0.5 μg of histone H1, respectively. Incorporated radioactivity was estimated by densitometric scanning of autoradiographs and scintillation counting of excised gel pieces after H_2O_2 hydrolysis.

Peptide mapping. For CNBr one-dimensional peptide maps, gel pieces containing in vitro ^{32}P -labeled nucleolin were excised from polyacrylamide gels and treated essentially as described by Zingde et al. (57). Dried gel pieces were incubated at 37°C for 2 h in 80% formic acid in the presence of CNBr (0.3 mg/ml; Sigma) in a final volume of 500 μl . An equal volume of 50 mM ammonium bicarbonate–0.1% β -mercaptoethanol was then added prior to lyophilization. After extensive washing, samples were equilibrated in SDS-PAGE buffer and loaded onto 15% SDS-PAGE gels. The gel was then Coomassie blue stained, dried, and autoradiographed.

For tryptic two-dimensional peptide maps, gel pieces containing ^{32}P -labeled nucleolin were excised from unfixed SDS-polyacrylamide gels and treated as already described (18, 31) with slight modifications. Isopropanol-washed gel

digestion of in vitro Cdc2-labeled purified 29-kDa peptide are shown in panel C. Tryptic digests were loaded onto thin-layer plates at points marked by arrowheads. Highly acidic CKII peptides are referred to as S0 and Cdc2 basic peptides are referred to as S1 to S10. Exposure times were 24 h for A, 72 h for B, and 1 week for C; about 1,000 Cerenkov cpm were loaded.

pieces were digested twice with trypsin (Sigma) to a final concentration of 100 $\mu\text{g/ml}$ for 24 h, in a minimum volume (50 to 100 μl) of 50 mM ammonium bicarbonate (pH 8). Polyacrylamide was discarded by centrifugation, and then extracted peptides were washed, lyophilized, and analyzed on cellulose thin-layer plates (Merck) by electrophoresis at pH 4.4 in pyridine-acetone-acetic acid- H_2O (2:15:4:79) for 2 h at 400 V and then by ascending chromatography in the second dimension for 20 h in pyridine-butanol-acetic acid- H_2O (25:37.5:7.5:30).

Phosphoamino acid analysis. For the phosphoamino acid analysis, gel pieces containing ^{32}P -labeled nucleolin were excised from unfixed gels and digested with trypsin as above. Dried extracted peptides were then hydrolyzed in 50 μl of 6 M HCl at 110°C for 2 h, followed by several cycles of washing and lyophilization. Samples were then suspended in 2 μl of electrophoresis buffer (see below) containing 10 mM cold phosphoserine and phosphothreonine plus 0.3% acidic fuchsin and spotted onto a thin-layer cellulose plate. Electrophoresis was performed in 5% acetic acid–0.5% pyridine (pH 3.5) for 50 min at 1,100 V (30). Migration of unlabeled markers was visualized with ninhydrin staining, and labeled amino acids were detected by autoradiography. To determine the phosphoamino acid content of *in vivo* ^{32}P -labeled nucleolin, two-dimensional thin-layer electrophoresis was performed as described by Cooper et al. (14); migration for 20 min at 1,500 V in formic acid (88%)–acetic acid– H_2O (50:156:1,794) (pH 1.9), followed by 25 min at 1,300 V in pyridine-acetic acid– H_2O (10:100:1,890) (pH 3.5).

RESULTS

In vitro phosphorylation of nucleolin by $\text{p34}^{\text{cdc}2}$ protein kinase. Nucleolin (100 kilodaltons [kDa]) is a major phosphorylated protein present in the nucleolus of exponentially growing cells (Fig. 1A). In unsynchronized cell cultures, nucleolin phosphorylation is prominent on serines, while phosphothreonines are barely detected (Fig. 1A). Phosphoserines have been mapped within amino-terminal clusters of acidic residues that represent ideal CKII phosphorylation sites (12, 32), while no threonine was found in a putative CKII site in rodent nucleolin sequences (5). A search of consensus phosphorylation sites of various protein kinases identified, in nucleolin, an apparent consensus sequence, TPXK, in which threonine could be phosphorylated by $\text{p34}^{\text{cdc}2}$ protein kinase (Table 1). This motif is repeated nine times in the NH_2 part of the molecule and is reminiscent of the growth-associated phosphorylation site present in the amino-terminal domain of histone H1 (Table 1). In nucleolin, a nonpolar residue rather than a basic residue precedes the potential phosphorylated threonines.

We have previously shown (12) that a CKII kinase-like activity copurifies with nucleolin (Fig. 1B, lanes 1 and 4, 2 and 5). The two proteins can, however, be separated by passage through an anion-exchange column. No protein kinase activity was then detected in the fraction containing nucleolin (Fig. 1B, lanes 3 and 6).

To determine whether nucleolin could serve as a substrate for $\text{p34}^{\text{cdc}2}$ protein kinase, the purified proteins were incubated in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. The same experiment was carried out with the protein kinase CKII purified essentially by the method of Caizergues-Ferrer et al. (12). As shown in Fig. 2 (lanes 1 and 4), incorporation of phosphate into nucleolin was efficient with both enzymes ($\text{p34}^{\text{cdc}2}$ was not contaminated by CKII, since the addition of heparin, a potent inhibitor of CKII activity, did not alter nucleolin

FIG. 8. In vitro phosphorylation of synthetic peptides derived from nucleolin and histone H1 sequences. (A) Autoradiographs of 8 to 20% SDS-PAGE showing phosphorylation of peptide models for Cdc2 phosphoacceptor site in histone H1 (P2: [KTPKKAKKP]₂, lane 1) and in nucleolin (NUC) (P1: [ATPAKKAA]₂, lane 4). Kinase assays were performed as described in the text. Inhibition of histone H1 (lane 2) and nucleolin (lane 5) phosphorylation by P2 and P1 are shown in lanes 3 and 6, respectively. (B) Increasing amounts of synthetic H1-like peptide (P2) were added to Cdc2 assay mixes containing nucleolin or histone H1 as the substrate and processed as described in Materials and Methods. Estimation of inhibition of substrate phosphorylation as well as incorporation onto peptide competitor were plotted as a function of competitor concentration (picomoles).

FIG. 9. Comparative two-dimensional tryptic maps of in vivo ^{32}P -labeled nucleolin. Pieces of 10% polyacrylamide gels containing in vivo ^{32}P -labeled nucleolin from untreated (A) or nocodazole-treated (B) cells were subjected to trypsin digestion as described in Materials and Methods. Tryptic phosphopeptides (loaded onto thin-layer plates at points marked by arrowheads) were mapped and numbered as in Fig. 7. Exposure times were 1 week for the mitotic

labeling; data not shown). A control experiment demonstrated that phosphate was efficiently incorporated into histone H1 (a characterized substrate of Cdc2 protein kinase [36]) in the presence of p34^{cdc2} (Fig. 2, lane 2) but not in the presence of the CKII (Fig. 2, lane 5). No labeling was detected without nucleolin with the two kinases (Fig. 2, lanes 3 and 6). Thus, both nucleolin and histone H1 serve as substrates for Cdc2 kinase. While they have similar K_m values (respectively 1.2 and 2 μM), Cdc2 exhibited a 10 times lower V_{max} for nucleolin than for histone H1 (Table 2).

It should be noted that unlike Cdc2-phosphorylated pp60^{c-src} (45) or subunit IIo of RNA polymerase II (13), p34^{cdc2} - and CKII-phosphorylated nucleolin did not exhibit decreased mobility on SDS-PAGE. Unphosphorylated nucleolin presented an abnormal mobility of 100 kDa, probably resulting from the presence in the protein sequence of four long acidic stretches (data not shown).

The amino acid specificity of the p34^{cdc2} and CKII protein kinases was addressed by phosphoamino acid analysis (Fig. 3). p34^{cdc2} induced incorporation of phosphate on threonine (lane 2), while CKII induced incorporation on serine (lane 3). CKII and Cdc2 phosphorylation therefore occurs on distinct sites on nucleolin.

Differential in vivo phosphorylation of nucleolin during the cell cycle. Phosphorylation experiments were carried out with cells blocked by chemical agents to determine whether nucleolin phosphorylation by Cdc2 and CKII kinases occurs in vivo at specific stages of the cell cycle. Mitotic cells were obtained by exposure of monolayer cultures to nocodazole, an agent that disrupts microtubule formation and arrests the majority of cells in a state of pseudo metaphase (15). Hydroxyurea, which interferes with DNA replication, was added to block cells in the G1/S state (1). ^{32}P -labeled proteins purified from normal, hydroxyurea-blocked, and nocodazole-treated cells were subjected to SDS-PAGE followed by autoradiography or by immunodetection of nucleolin (Fig. 4).

The phosphoamino acid composition of purified, in vivo ^{32}P -labeled nucleolin was determined after HCl hydrolysis (Fig. 5). Phosphoserine was found in nucleolin from exponentially growing cells (Fig. 5A) and hydroxyurea-blocked cells (Fig. 5B), while phosphothreonine was prominent in nucleolin from nocodazole-blocked cells (Fig. 5C); in this case, phosphoserine was barely detectable (<1:10, phosphoserine-phosphothreonine).

Mapping of the in vitro Cdc2 phosphorylation sites within the amino-terminal domain of nucleolin. The sequence of nucleolin indicates the presence of Cdc2 consensus phosphorylation sites (TPXK repeat) in the NH_2 -terminal part of the protein. To determine whether this domain is indeed phosphorylated, one-dimensional cyanogen bromide mapping was performed. In vitro-labeled nucleolin was hydrolyzed by CNBr to yield six fragments with apparent mobilities of 33, 29, 13, 10, ($\times 2$), and 5 kDa and reproducible peptides resulting from partial cleavages (47, 33, and 23 kDa; see reference 10 for details). The 29-kDa peptide was localized in the amino-terminal domain of the protein (residues 33

map and 2 days (a) and more than 1 month (b; barely detectable basic phosphopeptide spots are shown by arrows) for interphasic nucleolin (500 to 1,000 Cerenkov cpm loaded). Tryptic fingerprint of in vitro Cdc2-labeled nucleolin is shown in C to allow comparative analysis of in vivo mitotic and in vitro Cdc2 maps (exposure time, 24 h). (D) Comigration experiment of M-phase in vivo- and in vitro Cdc2-labeled materials.

to 216) and contained in vitro CKII-phosphorylated serines (12) (Fig. 6A). The carboxy-terminal 33-kDa peptide (286 to 546) was contaminated by a partial amino-terminal peptide corresponding to residues 17 to 216 (33-kDa, ^{32}P) which included the 29-kDa peptide. As shown in Fig. 6B, CNBr hydrolysis of in vitro Cdc2- (lane 1) and CKII- (lane 2) phosphorylated nucleolin yielded two peptides of 29 and 33 kDa. The 29-kDa peptide must correspond to the amino-terminal peptide containing the TPXK motif described above. Furthermore, the purified 29-kDa peptide was used as a substrate by p34^{cdc2} as well as by CKII protein kinases (Fig. 6B, lanes 3 and 4). Labeling of the 33-kDa peptide probably corresponds to the partial amino-terminal peptide described above, since a carboxy-terminal peptide (residues 278 to 713) was not labeled by either enzyme (Fig. 6B, lanes 5 to 8). This peptide (a gift from L. Ghisolfi) was generated in *Escherichia coli* by genetic engineering and purified (Fig. 6B, lanes 9 and 10) essentially according to the protocol described for nucleolin (data not shown).

Two-dimensional peptide mapping was carried out to locate the phosphorylation sites more precisely. In vitro-labeled nucleolin was digested with trypsin, and the resulting peptides were analyzed by thin-layer electrophoresis and chromatography (Fig. 7). Digestion of CKII-phosphorylated nucleolin yielded highly acidic phosphopeptides that did not run on the thin-layer electrophoresis system (Fig. 7A, spot 0) and which were previously proposed to be localized in acidic regions A and C (12) (Fig. 6A). Tryptic digestion of nucleolin that was labeled in vitro by p34^{cdc2} yielded a distinctive pattern of basic phosphopeptides: five major and two minor spots appeared, and three spots were barely detectable (Fig. 7B). These presumably correspond to the TPXK repeats present in the NH_2 -terminal region of nucleolin, since a similar phosphopeptide map was obtained when the in vitro Cdc2-labeled amino-terminal 29-kDa peptide was subjected to trypsin digestion (Fig. 7C). Furthermore, this suggests that threonine-219 (which does not lie in the 29-kDa peptide) is not a target site for the p34^{cdc2} kinase.

In order to examine the possibility that the phosphorylation sites of the Cdc2 protein kinase on nucleolin are the TPXK motifs, two peptides were synthesized and purified. The nucleolinlike peptide P1 ([ATPAKKAA] $_2$) is representative of the basic repeat present in the amino-terminal

domain of nucleolin. The histone H1-like peptide ([KTPKKAKKKP] $_2$) contains one of the growth-associated H1 kinase phosphorylation sites (38). P1 and P2 were used as substrates by the purified p34^{cdc2} protein kinase (Fig. 8A, lanes 1 and 4). In competition experiments, P2 interfered with both histone H1 and nucleolin phosphorylation, confirming that nucleolin is a substrate of mitotic Cdc2 histone H1 kinase (Fig. 8B). P1 competed efficiently with nucleolin phosphorylation (Fig. 8A, lane 6). Thus, P1 and P2 probably mimic the sequences that serve as in vitro p34^{cdc2} kinase phosphorylation sites in nucleolin and histone H1.

In vivo mitotic and in vitro p34^{cdc2} phosphorylation sites are identical. The in vivo phosphorylation sites in unsynchronous and mitotic cells were localized by phosphopeptide mapping. Tryptic digestion of nucleolin from ^{32}P -labeled growing cells yielded the same highly acidic phosphopeptide (S0) as did nucleolin labeled in vitro by CKII protein kinase (Fig. 9A, a). Several barely detectable basic spots, corresponding to the Cdc2 mitotic labeled spots (see below), were detected in a long exposure (Fig. 9A, b). These were probably generated by digestion of nucleolin extracted from mitotic cells present in low amounts in an unsynchronized culture. Digestion of nucleolin labeled in vivo after nocodazole treatment yielded qualitatively the same phosphopeptide map as did in vitro Cdc2-labeled nucleolin protein kinase (Fig. 9B and C). Thus, in mitotic cells nucleolin phosphorylation occurs at the same sites as in vitro by Cdc2 kinase, as confirmed by comigration experiments with in vivo- and in vitro-labeled materials (Fig. 9D).

DISCUSSION

Nucleolin has previously been shown to be phosphorylated on serines by a CKII protein kinase in exponentially growing cells (12). This pattern of phosphorylation is related to the control of rDNA transcription in interphase (4). In this report we present evidence that nucleolin is readily phosphorylated on threonines in vivo as cells enter mitosis and in vitro by purified starfish p34^{cdc2} kinase. The same sites on nucleolin are used in vivo in M phase and in vitro by the Cdc2 kinase. This provides a strong indication that a mammalian homolog of protein kinase Cdc2 may phosphorylate nucleolin in vivo. The fact that the starfish p34^{cdc2} purified

FIG. 10. Comparison of tryptic fragments generated by cleavage of Cdc2-labeled nucleolin with two phosphopeptides generated by trypsin digestion of Cdc2 model peptides. (A) Potential tryptic cleavages in the amino-terminal repeat (TPXK) containing the Cdc2 consensus sites are indicated by arrows on the nucleolin sequence. Digestion by trypsin between lysine residues is inefficient. Each fragment (a to h) could therefore contain one or more (two or three) lysine residues and generated one or more (two or three) spots when they are separated by electrophoresis in which peptides migrate according to their charge. Minimal sequence (one lysine residue) of tryptic fragments is in boxes. Positions of potential phosphorylated threonines are indicated and numbered from the N terminus. (B) Composition of the different tryptic fragments (restricted to one lysine residue) of nucleolin and their relative hydrophobicity (arbitrary units) as estimated by the method of Hopp and Woops (29). Trypsin digestion of P1 (ATPAKKAAATPAKKAA) and of P3 (AVTPAKKAAATPAKKA, a gift of E. A. Nigg) yielded two fragments, each containing one or two lysine residues. Their composition (one lysine) and hydrophobicity values are listed. P1/2 and P3/1 correspond respectively to fragments d and e in the nucleolin sequence. (C) Comigration experiments between tryptic fragments of Cdc2-labeled nucleolin and Cdc2-labeled P1- and P3-digested peptides. P1 and P3 were digested by trypsin and analyzed as described for nucleolin (see Materials and Methods). This treatment yielded a major spot and a second barely detectable spot (presumably respectively two and one lysine residue) in the first-dimension electrophoretic analysis (horizontal direction, anode to the left) in which peptides are separated according to their charge. The fast-migrating minor form corresponds to undigested Cdc2-phosphorylated peptides; this suggests that only one threonine residue is phosphorylated in P1 and P3 (data not shown). Digestion of nucleolin generated three spots (one to three lysine residues). Each major spot (two lysine residues) was run in a second dimension (ascending chromatography) in which they are separated according to their hydrophobicity. Comigration experiments were performed to compare tryptic fragments generated by cleavage of Cdc2-labeled nucleolin with peptides corresponding to the exact sequence of two sites on its sequence. Nucleolin spots in the second dimension are noted as in Fig. 7 (spots 5 to 8). P1 and P3 tryptic fragments comigrated with the spots 5 and 6 of nucleolin. Comparison of relative hydrophobicity values (see panel B) of the tryptic fragments allowed the localization on the nucleolin sequence of each phosphotryptic peptide. Identical experiments were performed with the slowly migrating form (one lysine residue) with the same result (data not shown). This suggests that spots 1 to 4 could correspond to spots 5 to 8 except for an additional lysine residue. Since additional lysine should reduce the hydrophobicity of a given peptide, spot 1 could correspond to spot 5, 2 to 6, 3 to 7, and 4 to 8 (spots 9 and 10 could correspond respectively to spots 1 through 5 and 2 through 6).

kinase phosphorylates rodent nucleolin is not surprising, since both proteins show a high degree of evolutionary conservation.

The phosphorylation sites of the CKII and p34^{cdc2} kinases (both in vivo and in vitro) lie in the amino-terminal end of nucleolin. This domain reveals highly acidic serine-containing stretches which were previously identified as the CKII target sites (12). Experimental data provide strong evidence that nucleolin Cdc2 phosphorylation occurs on threonine within the basic TPXK (where X is a nonpolar residue) repeats present between the first two acidic stretches. The fact that the peptide P1 can competitively inhibit phosphorylation of nucleolin (which strongly suggests that the phosphorylation is mediated by the Cdc2 kinase) suggests (but does not strictly prove) that sites surrounded by similar flanking sequences are being phosphorylated in nucleolin. Eight threonines out of 10 are in this array in the amino-terminal domain of nucleolin. The TPXK motif, which matches the Cdc2 phosphorylation sequences, is highly conserved in evolution. Nine motifs are present in rodent (5) and human (47) and eight in chicken (42) nucleolin. However, some variation occurs in the phosphorylated target (T or S) as well as in the flanking nonpolar residues. Tryptic mapping of in vitro and in vivo Cdc2-labeled nucleolin led to the separation of 10 phosphopeptides (S1 to S10) according to their charge and hydrophobicity. The partial digest occurring after the lysine doublet which surrounds the putative threonine target site complicates the interpretation of the peptide maps (Fig. 10A). Furthermore, it should be noted that the differences observed in the intensity of each spot could result from higher affinity of Cdc2 for certain sequences or from the superposition of peptides which present the same physicochemical properties (Fig. 10B). A precise assignment of the p34^{cdc2}-phosphorylated threonines will require peptide sequencing or comigration experiments of synthetic peptides corresponding to the exact sequence of potential sites in nucleolin sequence with Cdc2-labeled phosphopeptides. Preliminary comigration experiments carried out with two synthetic peptides (Fig. 10C) allow us to suggest that the threonine residue present in peptides b and g could be phosphorylated. Among peptides a and d and peptides c, e, f, and h, at least one could be phosphorylated. The relatively low intensity of some mitotic spots with respect to the in vitro Cdc2 maps suggests that all the potential sites for Cdc2 kinase are not used with the same efficiency in vivo as in vitro. The increased V_{max} of histone H1 compared with that of nucleolin and the stronger phosphorylation of P2 than of P1 suggest that the sequences flanking the phosphorylated target residue could be important in the recognition of the consensus sequence by Cdc2 kinase. The P2 sequence agrees with the putative Cdc2 phosphoacceptor sites recently proposed: (K/R)(T/S)X(K/R) (see reference 45), while P1 differs somewhat, with a nonpolar residue rather than a basic residue preceding the threonine. This suggests a positive effect of the NH₂-terminal basic residue relative to the phosphoacceptor amino acid.

In the fission yeast *Schizosaccharomyces pombe*, the cell cycle control Cdc2⁺/CDC28 protein kinase has been shown to be essential for both initiation of DNA replication and entry into mitosis (25). Much evidence suggests that p34^{cdc2} protein kinase plays an essential role in regulating mitosis in vertebrate cells. Although recent results indicate the involvement of mammalian Cdc2 kinase activity in the G1/S transition (13, 23, 39), phosphorylation of nucleolin by Cdc2 kinase was only observed in mitosis. In cells arrested in

G1/S, no phosphothreonine, but only CKII phosphoserines were detected as shown by phosphoamino acid analysis and tryptic phosphopeptide mapping (data not shown). Furthermore, the percentage of threonine in unsynchronized cell cultures is low and correlated with the estimated amount of nucleolin present in the subpopulation of mitotic cells. On the other hand, CKII seems to be inactive or less active in mitosis on nucleolin phosphorylation. In vitro serine phosphorylation is not required for achievement of threonine phosphorylation, since serine-dephosphorylated nucleolin is efficiently phosphorylated by Cdc2 kinase (result not shown).

As cells enter mitosis, extensive phosphorylation of histone H1 was shown to occur on Cdc2 consensus sequences, lying on carboxy- and amino-terminal domains of the protein, which were both involved in chromatin condensation (2, 6, 36, 38). These findings lead to the attractive model of involvement of histone H1 phosphorylation by Cdc2 kinase in chromosome condensation, as proposed earlier (6). It is also interesting that yeast topoisomerase II, which is implicated in chromosome condensation, contains a Cdc2 phosphorylation consensus sequence (50, 51) and is readily phosphorylated in mitosis (26). A subclass of nucleolin is associated with chromosomal NORs in metaphase and with chromatin in interphase (19, 20, 40). Furthermore, nucleolin reveals structural analogies with histone H1 around Cdc2-phosphorylated threonine in the TPXK motif. The role of nucleolin Cdc2 phosphorylation on its interaction with chromatin (19) is under investigation. Preliminary experiments suggest that in the vicinity of rDNA genes, nucleolin could modulate the condensation-decondensation process through phosphorylation by CKII or Cdc2.

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