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Phosphorylation-Dependent Migration of Retinoblastoma Protein into the Nucleolus Triggered by Binding to Nucleophosmin/B23

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Underphosphorylated retinoblastoma (Rb) protein inhibits progression around the cell cycle by binding to transcription factors like E2F; subsequent hyperphosphorylation of Rb protein releases E2F from the complex so that it can then drive the cell into S phase. We immunolocalized Rb protein in human cells during the cell cycle. Rb protein translocated into nucleoli after DNA replication completed, and the nucleolar Rb was shown to be in the hyperphosphorylated form by immunoblotting. This form, but not its underphosphorylated counterpart, interacted with the nucleolar protein nucleophosmin/B23. The two formed a salt-resistant complex *in vitro,* and the two could be immunoprecipitated together from nucleolar extracts. These results suggest that hyperphosphorylated Rb protein is imported into nucleoli late in S or G2 phase with nucleophosmin/B23. Analysis of the nucleolar location of Rb protein using various deletion mutants tagged with the green fluorescent protein implicated pocket A of Rb protein as the region responsible for nucleolar targeting; this region also interacted with nucleophosmin/B23. Nucleolar translocation of Rb mutant was inhibited by introducing nucleophosmin/B23 antisense oligomer. These results suggest that nucleolar translocation of Rb protein is promoted by the binding with nucleophosmin/B23 via the pocket A region.

Key Words: **retinoblastoma protein; Rb; nucleolus; translocation; nucleophosmin; B23; phosphorylation.**

INTRODUCTION

Retinoblastoma (Rb) protein is phosphorylated at multiple sites by the cyclin-dependent kinases (cdks) that are activated by various growth signals [1]. These cdks depend on cyclins D (cdk4, cdk6), E (cdk2), and A (cdk2, cdk1) [2–8]. At the G1/S boundary, cyclin E takes over from cyclin D to drive Rb protein hyperphosphorylation, and this irreversibly commits the cell to enter S phase [9]. The hyperphosphorylated Rb protein then remains nuclear but "inactive" until the end of mitosis when it is rapidly dephosphorylated [10].

Most explanations of the role of Rb protein in controlling the cycle involve a sequestration or "corralling" by hypophosphorylated Rb protein of transcription factors like E2F so that they are prevented from acting; then, Rb protein phosphorylation would lead to the release of these factors so that they could now drive progression around the cycle [11]. Indeed, un- or underphosphorylated Rb protein is tightly associated with the nuclear substructure, and hyperphosphorylated Rb protein is easily solubilized in a hypotonic buffer [12]. More recent work suggests that underphosphorylated Rb protein complexed with E2F might recruit histone deacetylase I to promoters, so reducing access of the transcription machinery to the region [13]. Harbour *et al.* suggest a molecular mechanism for the interaction between Rb protein and E2F, and that Rb protein phosphorylation disrupts the structure of pockets A and B in its E2F-binding site [14].

We have observed that Rb protein interacts with the major nucleolar protein nucleophosmin/B23 [15]. Nucleophosmin/B23 is a multifunctional and conserved phosphoprotein mainly localized in the granular and fibrillar regions of nucleolus where ribosomal RNA synthesis and ribosome assembly take place [16]. It shuttles between nucleoli and other cellular compartments [17], and it binds to proteins (e.g., HIV-1 Rev, HTLV-1 Rex) that are imported into nucleoli [18–20]. It is also phosphorylated by cdk2/cyclin E to initiate centrosome duplication [21], and dephosphorylated by the δ isoform of protein phosphatase I [22].

Here we observe that hyperphosphorylated Rb pro-

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tein is localized in nucleoli late in S or G2 phase, and that the hyperphosphorylated form binds tightly to the nucleolar protein nucleophosmin/B23, through pocket A. As a result, Rb protein is inevitably imported into nucleoli with this nucleolar protein.

MATERIALS AND METHODS

Antibodies and vectors. Monoclonal antibodies were against human Rb protein (3H9, MBL Co. Ltd., Ina, Japan; and 14001A, BD PharMingen, San Diego), unphosphorylated Rb protein (14441A, BD PharMingen), phosphorylated Rb oligopeptides (anti-phospho-Thr373, anti-phospho-Ser780, anti-phospho-Ser795, and anti-phospho-Ser 807/811, New England Biolabs Ltd., Hitchin, UK and Cell Signaling Technology, Inc.), calf DNA polymerase α (CL22, MBL Co. Ltd.), and nucleophosmin/B23 (NB23) [15]. Vector pKK223-3 was a gift of Dr. M. O. J. Olson (University of Mississippi).

Indirect immunofluorescence. Normal human fibroblasts were synchronized by serum starvation. Cells cultured for 48 h in DMEM + 0.5% fetal calf serum (FCS) were transferred into DMEM + 10% FCS; 8, 10, 16, and 22 h later, they were fixed (20 min on ice) in 5% formaldehyde in phosphate-buffered saline (PBS), permeabilized by sequential treatments (1 min on ice) in 10, 30, 50, and 70% ethanol, blocked with blocking buffer (5% normal goat serum in PBS) at room temperature for 20 min, and incubated at 37°C for 1 h with primary antibodies and then with secondary antibodies conjugated with FITC or Texas Red as indicated at 37°C for 1 h. The samples were examined under a confocal laser microscopy MRC-1024 (Bio-Rad Laboratories, Hercules).

Cells were also pulse-labeled (30 min) with 10 μM bromodeoxyuridine (BrdU) at 8, 10, 16, and 22 h after the release by 10% FCS. After fixation and permeabilization, cells were stained with anti-Rb antibody and observed under a confocal microscope as described above. Then, cells were treated (30 min, room temperature) with 4 N HCl, washed in ice-cold PBS five times (5 min each), treated with the blocking buffer at room temperature for 20 min, stained with anti-BrdU (MBL Co. Ltd.) at 37°C for 1 h, washed in ice-cold PBS five times (10 min each), reblocked with blocking buffer at room temperature for 20 min, and stained with FITC-conjugated secondary antibodies at 37°C for 1 h. The samples were examined by confocal laser microscopy (MRC-1024 Bio-Rad Laboratories).

Isolation of the nucleoli. Nucleoli were prepared using the Nonidet P-40 method [23, 24] from synchronized HeLa S-3 cells grown in DMEM + 10% FCS. Purity of nucleoli was monitored by staining with Azure C (Sigma Chemical Co., St. Louis, MO) [23]. Proteins in whole nuclei and nucleoli were resolved using 7.5% sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) [25] and analyzed by immunoblotting.

Immunoprecipitation analysis. Isolated nucleoli were suspended in extraction buffer (5 mM Tris–HCl, pH 7.5, 1 mM EDTA, 40 μg/ml leupeptin, 1 mM PMSF), gently stirred, and pelleted (8000*g*, 10 min), and antibody NB23 was added to the supernatant. After incubation (4°C, 16 h), protein G–Sepharose beads (Amersham-Pharmacia Biotech, Little Chalfont, UK) were added. After incubation for 1 h at room temperature, the beads were pelleted and washed three times in PBS, and the attached proteins were analyzed by 7.5% SDS–PAGE followed by immunoblotting using anti-Rb protein antibodies.

Construction and expression of greenfluorescent protein (GFP)-conjugated Rb proteins. cDNAs encoding wild-type Rb, Rb-del-C (Δ773-928), Rb-del-BC (Δ645-928), Rb-del-SBC (Δ574-928), Rb-del-ASBC (Δ393-928), Rb-del-NAS (Δ1-644), Rb-del-NASC (Δ1-644, Δ773-928), Rb-del-NSBC (Δ1-392, Δ574-928), and Rb-del-NABC (Δ1-573, Δ645-928) were generated by PCR using the wild-type Rb cDNA as a template, 5′-primers complementary to the sequence at *Bam*HI site and 3′-primers complementary to the sequence at *Hin*dIII site, and were inserted into pEGFP-C1 (BD Clontech, Basingstoke, UK) cut with *Bam*HI–*Hin*dIII. After cloning, purified plasmid DNA was transfected to Rb-negative HTB-9 cells (purchased from American Type Culture Collection) using LipofectAMINE PLUS reagent according to the manufacturer's instruction (GIBCO BRL manual, GIBCO BRL, Rockville). After culturing for 2 days in DMEM + 10% FCS, cells expressing GFP-Rb fusion proteins were examined by confocal laser microscopy. Proliferation capacity of the transfected cells were assessed by incorporation of fluorescein-conjugated deoxynucleotides as described previously [26, 27].

Purification of Rb proteins, Rb deletion mutants, and nucleophosmin/B23. Underphosphorylated human Rb protein was purified from baculovirus-infected Sf9 cells [28–31], and hyperphosphorylated Rb protein was from Sf9 cells infected with three baculoviruses encoding Rb protein, cdk2, and cyclin E [31]. Deletion mutant Rb proteins were purified as follows: cDNAs encoding Rb-del-C (Δ773-928), Rb-del-BC (Δ645-928), Rb-del-SBC (Δ574-928), and Rb-del-ASBC (Δ393-928) were introduced into pFastBacHTb (GIBCO BRL) cut with *Bam*HI–*Hin*dIII and expressed in Sf9 cells according to the manufacturer (GIBCO BRL); Rb-del-SBC and Rb-del-ASBC were purified on cobalt columns (TALON metal affinity resin, GIBCO BRL) and wild-type Rb protein, Rb-del-C, and Rb-del-BC on the anti-Rb antibody (3H9)-column [31]. Recombinant rat nucleophosmin/B23 was purified from *E. coli* harbouring a pKK223-3 expressing this protein [20]. Purity of proteins was assessed by SDS–PAGE, run in parallel with molecular mass standards from Bio-Rad Laboratories.

Filter-binding assay. The "first proteins" (0.1 μg each of Rb proteins) were dot-blotted onto PVDF membrane (Millipore, Bedford, MA), blocked with 5% skim milk in PBS overnight, and the "second protein," nucleophosmin/B23 (NB23) (0.1 μg) applied in 0.1, 0.2, or 0.4 M KCl; after washing in PBS, the membrane was incubated with antibody NB23 and stained with horseradish peroxidase-conjugated secondary antibody, and the relative intensities were quantified using NIH Image (W. Rasband, NIH).

Coimmunoprecipitation with purified proteins. Purified Rb proteins were incubated with purified nucleophosmin/B23 in 0.4 M KCl at 4°C for 2 h, and then anti-NB23 antibody was added to the reaction mixtures. After incubation at 4°C for 16 h, protein G–Sepharose (Amersham-Pharmacia Biotech) was added. After incubation for 1 h at room temperature, the beads were pelleted and washed three times in PBS, and attached proteins were analyzed by 7.5% SDS–PAGE followed by immunoblotting using anti-Rb protein antibody 14001A.

Transfection with antisense oligodeoxynucleotides. Oligodeoxynucleotides (oligomers) corresponding to the sequences of $-2 \sim +18$ of the nucleophosmin/B23 cDNA were synthesized in the reverse (sense) and the antisense orientations as described previously [32]. HTB-9 cells expressing Rb-del SBC were cultured as described above and then transfected with oligomers using OligofectAMINE reagent according to the manufacturer's protocol (GIBCO BRL). The culture medium was then replaced with DMEM + 10% FCS containing oligomers (10 or 20 μM). After 2 days, the levels of nucleophosmin/B23 were measured by SDS–PAGE followed by immunoblotting of whole-cell extracts. Next, cells were subjected to confocal laser microscopy (MRC 1024, Bio-Rad Laboratories). Proliferation capacity of the transfected cells was assessed by incorporation of fluorescein-conjugated deoxynucleotides as described previously [26, 27].

RESULTS

Rb protein relocates to nucleoli after the completion of DNA replication. Although Rb protein is always nuclear, its distribution varies from cell to cell. In order to address whether these differences depended on the cell cycle, we immunolabeled normal human fibroblasts in

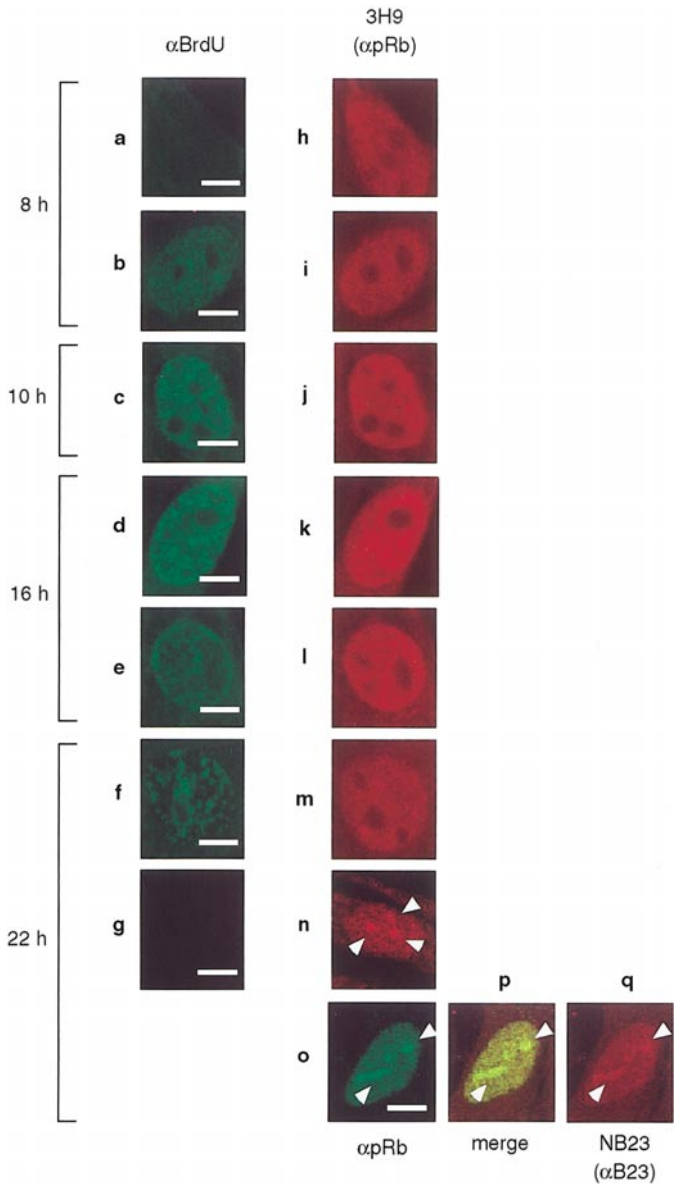

FIG. 1. Rb protein is relocated to nucleoli after DNA replication completed. Normal human fibroblasts were arrested in G0 by serum starvation, restarted by serum addition, and grown for 8, 10, 16, and 22 h. Cells were pulse-labeled for 30 min with BrdU, and were immunostained with antibodies against BrdU (a–g) and Rb protein (3H9 (h–n)). Staining of Rb protein (o) and nucleophosmin/B23 (q) is merged in (p). White arrowheads indicate nucleoli. Bar, 10 μm.

early, middle, and late S or G2 phase using monoclonal antibody 3H9, which detects Rb protein irrespective of its phosphorylation status. The cells were also pulse-labeled with BrdU so that the stage of the cell cycle could be confirmed; cells incorporated this precursor into foci characteristic of the stage of S phase (Figs. 1a–1g) [33]. Rb protein was dispersed throughout nuclei during early and mid S phase (Figs. 1h–1m), but became concentrated in nucleoli in late S or G2 phase (Figs. 1n and 1o). Nucleolar localization was confirmed by the double staining; Rb protein colocalized with the nucleolar marker nucleophosmin/B23 (Figs. 1o–1q).

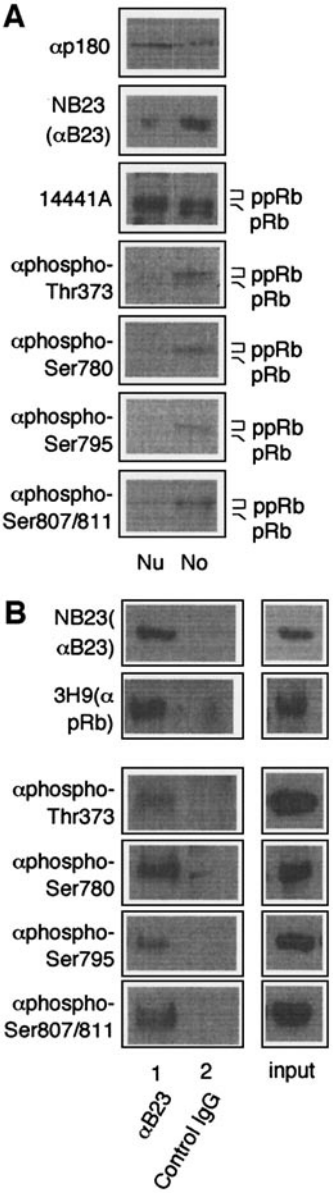

FIG. 2. Analyses of isolated nucleoli. (A) Western blot analyses of isolated nuclei (Nu) and nucleoli (No). (A) Equal amounts of proteins from isolated nuclei and nucleoli were resolved by electrophoresis, blotted, and probed with antibodies against DNA polymerase α (αp180), nucleophosmin/B23 (NB23), unphosphorylated Rb protein (14441A), and four different Rb phosphopeptides. (B) Hyperphosphorylated Rb protein coimmunoprecipitates with nucleophosmin/B23 from nucleolar extracts. (B) Antinucleophosmin/B23 antibody (lane 1) and a control IgG (lane 2) were incubated with an extract from isolated HeLa nucleoli, and any precipitated proteins were resolved by electrophoresis in a 7.5% gel and blotted; then, the filter was probed with antibodies directed against nucleophosmin/B23 (NB23), under- and hyperphosphorylated Rb protein (3H9), and the various Rb phosphopeptides as indicated. Input indicates whole nucleolar extract processed in the same manner.

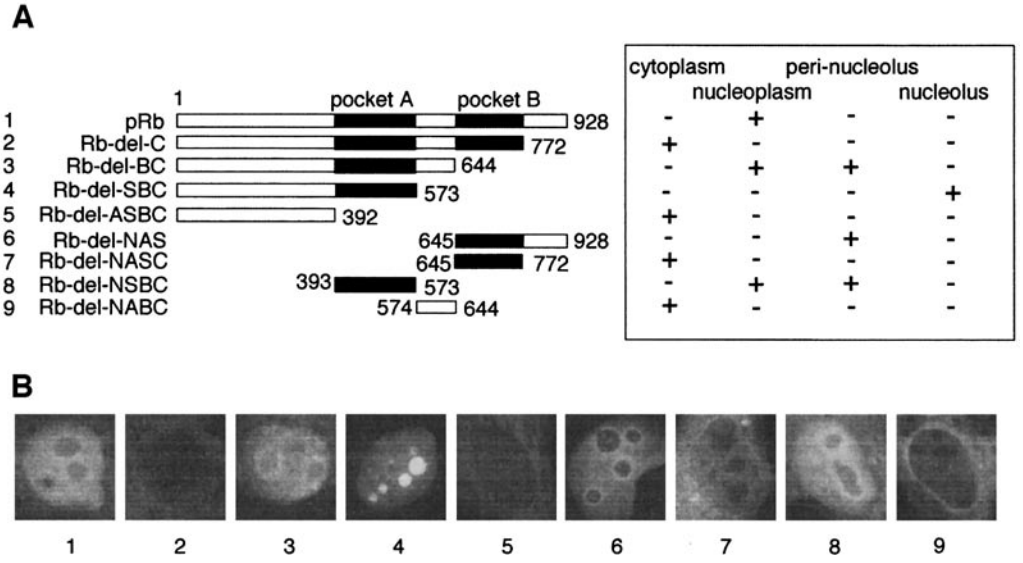

FIG. 3. Intracellular localization of GFP-conjugated Rb protein and Rb deletion mutants. (A) Schematic representation of the deletions of Rb protein; GFP was placed in all constructs at the N terminus. Localizations are summarized in box. (B) Rb protein and its deletion mutants conjugated with GFP were expressed in Rb-negative HTB-9 cells. Expressed GFP-Rb proteins were detected by confocal laser microscopy. The numbers correspond with those in (A).

Hyperphosphorylated Rb protein is found in isolated nucleoli as a complex with nucleophosmin/B23. Since bulk Rb protein is hyperphosphorylated during S phase, we examined whether the nucleolar form is also hyperphosphorylated. We isolated nucleoli from synchronized HeLa cells that were nearly homogeneous as examined by staining with Azure C [23, 24] (data not shown). Immunoblot analyses of nucleolar (No) and nuclear (Nu) fractions were carried out using antibodies raised against four different Rb phosphopeptides bearing phosphates at Thr373, Ser780, Ser795, or Ser807/811 and against nucleophosmin/B23. It was clearly shown that all the corresponding epitopes were concentrated in nucleoli (Fig. 2A). They were also detected in the nuclear fraction, which contained nucleoli, at the lesser concentrations. These results strongly suggest that hyperphosphorylated Rb protein was concentrated in nucleoli at late S or G2 phase cells in the HeLa cell population. In contrast, underphosphorylated Rb protein (detected by 14441A), as well as the catalytic subunit of DNA polymerase α, existed in both nucleoplasm and nucleoli.

We then examined whether the hyperphosphorylated Rb protein formed a complex with nucleophosmin/B23 *in vivo* (Fig. 2B). Proteins extracted from HeLa nucleoli were subjected to immunoprecipitation using an antinucleophosmin/B23 antibody. As shown in Fig. 2B (lane 1), hyperphosphorylated Rb proteins (detected using various phospho-specific antibodies) were coprecipitated with nucleophosmin/B23. A large part of nucleolar Rb protein was coprecipitated with nucleophosmin/B23 (Fig. 2B, lane 1 and input). All these results suggest that hyperphosphorylated Rb proteins bind with nucleophosmin/B23, so that it is imported into nucleoli.

Role of pocket A in the nucleolar localization of Rb protein. Phosphorylation of the C-terminal region of Rb protein induces conformational changes especially in the E2F-binding face composed of pockets A and B. As a result, pockets A and B are separated, leading to the loss of Rb function [14, 34]. Exposure of the separated pockets A and/or B might be related to the nucleolar localization of Rb protein. In this context, we examined whether deleting pockets A or B could affect the nucleolar localization, since such deletions might mimic the conformational changes induced by phosphorylation.

We introduced plasmids encoding GFP-tagged wild-type Rb protein or various deletion mutants (Fig. 3A) into HTB-9, an Rb-negative human cell line. Expressed Rb mutants showed characteristic distributions (Fig. 3B). Full-length Rb protein, Rb-del-BC, Rb-del-NAS, and Rb-del-NSBC were all dispersed throughout nuclei except for nucleoli. Some of them concentrated in perinucleolar regions (Fig. 3B, panels 1, 3, 6, and 8). Rb-del-C, Rb-del-ASBC, Rb-del-NASC, and Rb-del-NABC were mainly cytoplasmic (Fig. 3B, panels 2, 5, 7, and 9). Remarkably, a mutant lacking the spacer and pocket B, Rb-del-SBC, was predominately found in nucleoli, much like the hyperphosphorylated Rb protein (Fig. 3B, panel 4). Deletion of pocket A from Rb-del-SBC (as in Rb-del-ASBC) eliminated this nucleolar localization, implicating pocket A in nucleolar target-

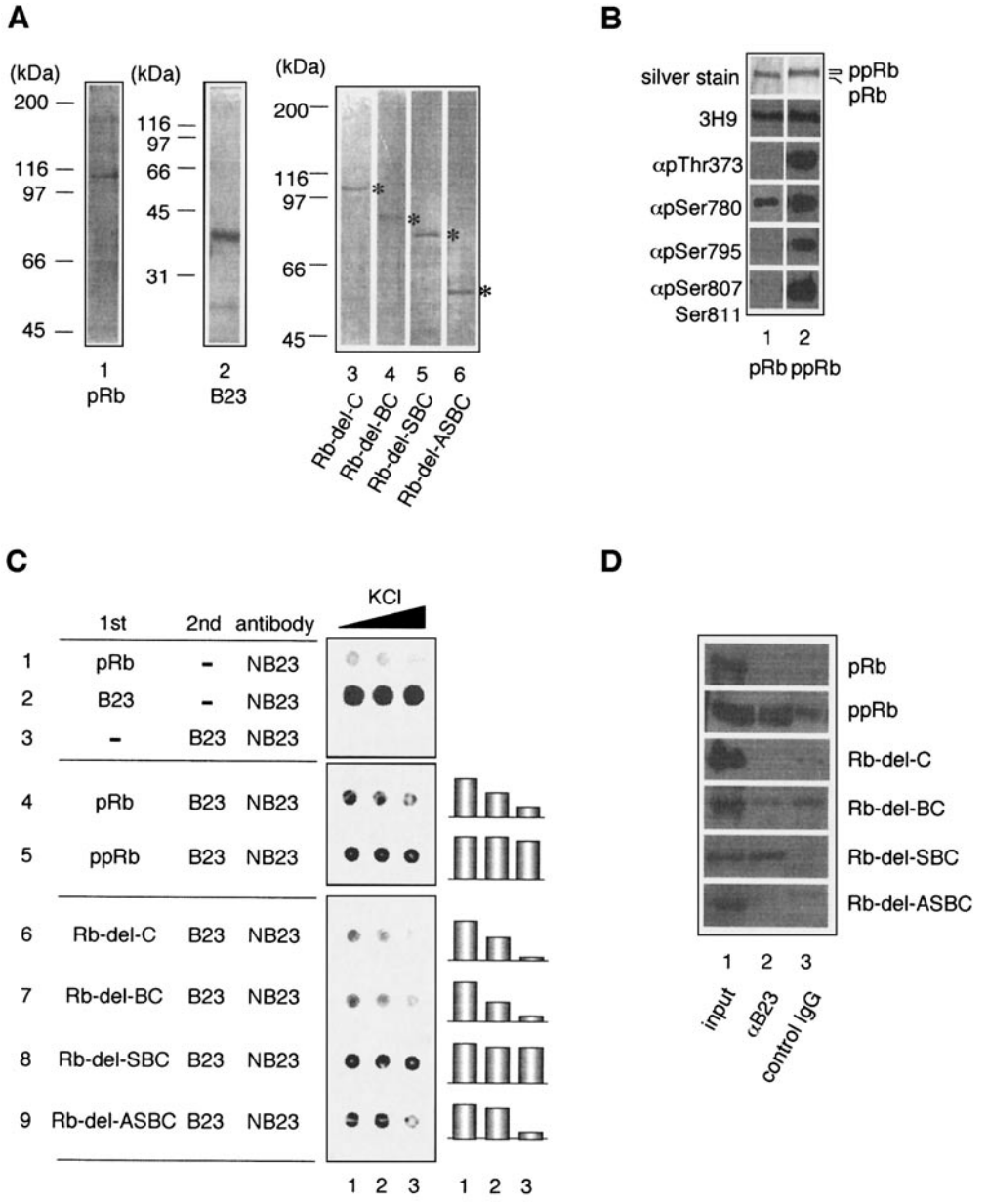

FIG. 4. Nucleophosmin/B23 binds preferentially to hyperphosphorylated Rb protein and Rb-del-SBC. (A) Electrophoretic profiles of purified Rb protein (lane 1; 7.5% gel, silver staining), nucleophosmin/B23 (lane 2; 10% gel, Coomassie staining), and Rb deletions (lanes 3–6; 7.5% gel, silver staining, asterisks). (B) Phosphorylation pattern of purified Rb proteins. Purified samples of under- (lane 1) and hyperphosphorylated (lane 2) Rb protein were subjected to 7.5% SDS–PAGE, blotted, and probed with the antibodies against Rb phosphopeptides indicated. (C) Filter-binding assay. The "first" protein (pRb, underphosphorylated Rb protein; ppRb, hyperphosphorylated Rb protein) was dot-blotted on a filter and incubated with the "second" protein (± B23) in 0.1, 0.2, or 0.4 M KCl (lanes 1–3, respectively). The bound nucleophosmin/B23 was immunolabeled, and the filter was photographed (boxes). The relative amounts of bound nucleophosmin/B23 were shown as intensities quantified by the NIH image, indicated by columns to the right of the panels. (D) Immunoprecipitation. Purified Rb proteins were incubated with purified nucleophosmin/B23 in 0.4 M KCl, and then antinucleophosmin/B23 antibody (NB23) was added to the reaction mixtures (lane 2). After the incubation, protein G–Sepharose beads were added and then pelleted and washed three times in PBS. Attached proteins were analyzed by 7.5% SDS–PAGE followed by immunoblotting using anti-Rb protein monoclonal antibody 14001A.

ing (Fig. 3B, panels 4 and 5). However, Rb-del-NSBC, which is pocket A itself, was not found in nucleoli, but dispersed throughout nuclei. It is suggested, therefore, that the N-terminal half is also necessary to the nucleolar localization in addition to pocket A (Fig. 3B, panels 4 and 8).

Since Rb protein relocalizes into nucleolus only at late S/G2 (Fig. 1), the nucleolar localization of Rb-del-

SBC could be due to cell cycle progression of the transfected cells. In this context, proliferation states of these transfected HTB-9 cells were assessed by the incorporation of fluorescein-conjugated deoxynucleotides [26, 27]. None of them exhibited active DNA synthesis (data not shown), indicating the cells being arrested presumably at G1 by transfection/overexpression. Therefore, difference in the localization of Rb mutants shown in Fig. 3 may not be due to the different phases in cell cycle.

Wild-type Rb protein and Rb-del-NAS were localized in nuclei (Fig. 3B, panels 1 and 6), consistent with the existence of a nuclear localization signal (NLS) in the C terminus [35]. However, Rb-del-BC was also found in nuclei (Fig. 3B, panel 3), suggesting an unknown nuclear localization mechanism/sequence other than the C-terminal NLS.

Hyperphosphorylated Rb protein binds to nucleophosmin/B23. Rb protein binds to nucleophosmin/B23 [15], and so could enter the nucleoli as a complex. Therefore, we compared the binding capacity of under- and hyperphosphorylated Rb protein to nucleophosmin/B23. Under- and hyperphosphorylated Rb protein were purified from insect cells infected either with one baculovirus encoding Rb protein (for the former) or with three different viruses encoding Rb protein, cdk2, and cyclin E (for the latter) [28–31]. Nucleophosmin/B23 was purified from bacteria carrying an expression plasmid [20]. High purity of the resulting preparations was shown by SDS–PAGE (Fig. 4A, lanes 1 and 2). The phosphorylation pattern of the Rb preparation (Fig. 4B, lane 2) was shown to be similar to that of the nucleolar Rb (Fig. 2A, lane No), when probed by antibodies directed against various Rb phosphopeptides.

With these preparations, binding affinities of Rb proteins to nucleophosmin/B23 were measured. A filter-binding assay revealed that, in 0.4 M KCl, the hyperphosphorylated Rb protein bound to nucleophosmin/B23 more tightly than the underphosphorylated one (Fig. 4C, rows 4, and 5). These results suggest that phosphorylation increases the binding affinity of Rb protein to nucleophosmin/B23.

Interaction of pocket A with nucleophosmin/B23. We then examined the binding of the Rb deletion mutants with nucleophosmin/B23, to see which domain was responsible for the interaction. Histidine-tagged Rb-del-C, Rb-del-BC, Rb-del-SBC, and Rb-del-ASBC were expressed in insect cells and proteins were purified on cobalt or anti-Rb protein columns (Fig. 4A, asterisks). A filter-binding assay revealed that progressively less nucleophosmin/B23 bound to three of the mutant proteins as the KCl concentration was raised from 0.2 to 0.4 (Fig. 4C, rows 6, 7, and 9). In contrast, increasing salt levels had little effect on the binding of Rb-del-SBC (Fig. 4C, row 8), as seen with full-length

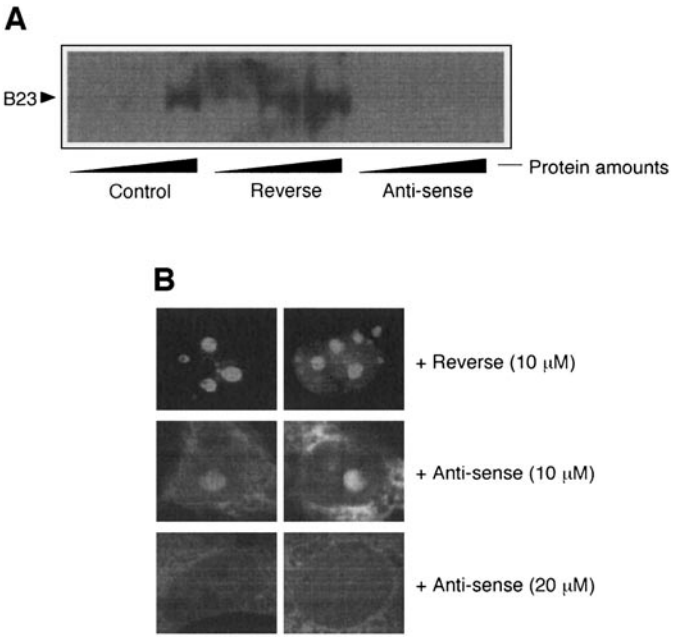

FIG. 5. Inhibition of nucleolar localization of Rb deletion mutant, GFP-Rb-del-SBC, by a nucleophosmin/B23 antisense oligodeoxynucleotide. (A) Inhibition of the expression of nucleophosmin/B23 protein in the cells by antisense oligomer was measured by Western blot analyses of cell extracts using antinucleophosmin/B23 antibody NB23. Reverse (sense) oligomer was used as a control. (B) HTB-9 cells expressing GFP-tagged Rb-del-SBC were treated with nucleophosmin/B23 antisense oligomer (10 and 20 μM) as described under Materials and Methods. Localization of GFP-tagged Rb-del-SBC was detected by the confocal laser microscopy. Reverse (sense) oligomer (10 μM) was used as a control.

hyperphosphorylated Rb protein (Fig. 4C, rows 5 and 8). These results are consistent with the concept that the pocket A of Rb protein mediates the interaction with nucleophosmin/B23, and the interaction is suppressed by the unphosphorylated C-terminus region including spacer and pocket B.

Then we performed an immunoprecipitation assay. Purified samples of Rb proteins and nucleophosmin/B23 were mixed in 0.4 M KCl and then immunoprecipitated with an antinucleophosmin/B23 antibody. As shown in Fig. 4D, Rb-del-SBC, as well as the hyperphosphorylated full-length Rb protein, was coprecipitated with B23/nucleophosmin, while other deletion mutants and the underphosphorylated Rb protein were not. These results indicate the importance of pocket A for the interaction with nucleophosmin/B23, consistent with results of the filter-binding assay (Fig. 4C).

Effects of a nucleophosmin/B23 antisense oligomer. To test whether the nucleolar localization is entirely dependent on the nucleophosmin/B23, we examined the effects of nucleophosmin/B23 antisense oligomer into HTB-9 cells expressing GFP-Rb-del-SBC. As shown in Fig. 5A, the antisense oligomer largely inhib-

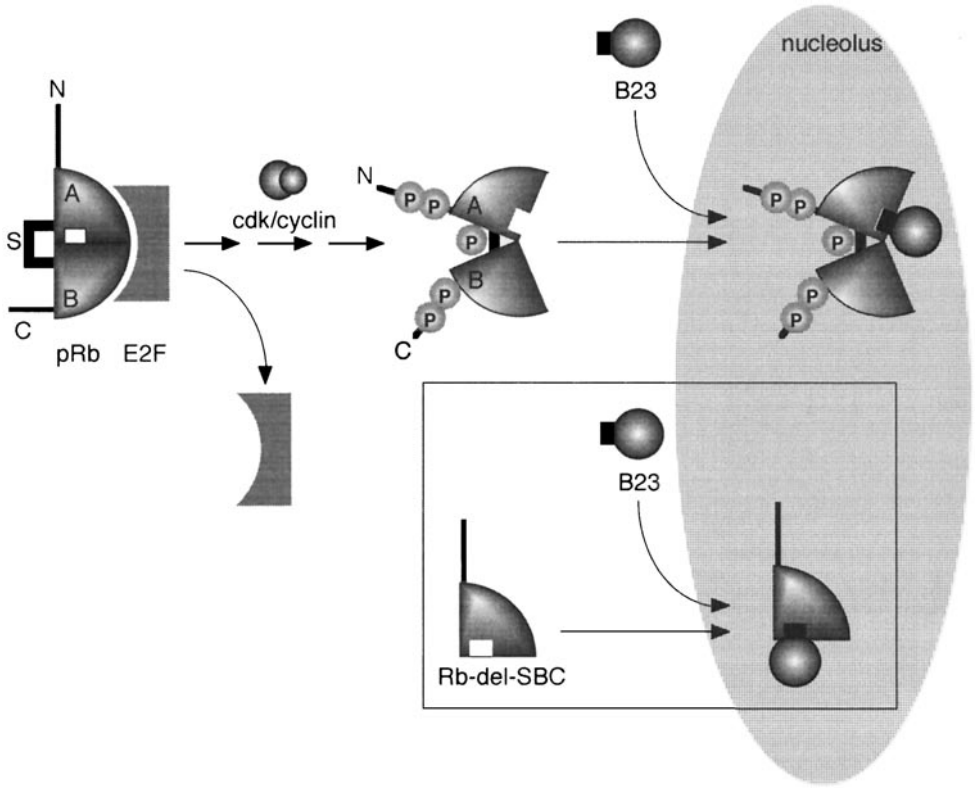

FIG. 6. Hypothetical model for the nucleolar localization of Rb protein. Phosphorylation of Rb protein by multiple cdks/cyclins leads to dissociation of E2F and exposure of a nucleophosmin/B23-binding site. Nucleophosmin/B23 binds only when pocket A is exposed; then the Rb protein:nucleophosmin/B23 complex is imported into the nucleolus. Box: a model for nucleolar import of Rb-del-SBC. pRb, underphosphorylated Rb protein; N, N-terminus region; A, pocket A; S, spacer; B, pocket B; C, C terminus; P, phosphate group.

ited the expression of nucleophosmin/B23 protein, while the reverse oligomer (sense) showed no inhibition (Fig. 5A). The nucleolar localization of Rb-del-SBC was seen in reverse oligomer-transfected cells (Fig. 5B) as in control cells (Fig. 3B, panel 4). In contrast, in the antisense oligomer-transfected HTB-9 cells, Rb-del-SBC was not transported into nucleoli but remained in the cytoplasm. Nucleolar localization was inhibited by the antisense oligomer in a dose-dependent manner (Fig. 5B). Since the transient expression of Rb protein or its deletion mutants resulted in cell cycle arrest of HTB-9 cells as described above, the inhibition of nucleolar localization of Rb-del-SBC may be solely due to the elimination of nucleophosmin/B23 and not due to the different phase in the cell cycle. These results further support the idea that Rb protein is imported into nucleoli in association with nucleophosmin/B23.

DISCUSSION

We immunolocalized hyperphosphorylated Rb protein in human cells and found it translocated into nucleoli after DNA replication completed (Fig. 1). Biochemical fractionation and immunoblotting confirmed that the hyperphosphorylated form was in the nucleolus (Fig. 2). We also found that hyperphosphorylated Rb protein, but not its underphosphorylated counterpart, interacted with the nucleolar protein nucleophosmin/B23. The two formed a salt-resistant complex *in vitro* (Fig. 4), and the two could be immunoprecipitated together from cellular extracts (Fig. 2). Cytological study using GFP-tagged deletion mutants of Rb protein implicated pocket A as the region responsible for nucleolar localization (Fig. 3). Consistently, deletion of pocket A abolished the formation of the salt-resistant complex with nucleophosmin/B23 *in vitro* (Fig. 4). Furthermore, antisense strategy strongly suggested that nucleolar localization of Rb mutant depended on the presence of nucleophosmin/B23 (Fig. 5). Both pockets A and B are known to mediate the formation of the Rb:E2F complex [14]. Intriguingly, the presence of the spacer region between pockets A and B, as well as pocket B and C terminus, inhibited nucleolar localization (Fig. 3). These results, combined with those of other groups [14, 36], lead to a simple model, illustrated in Fig. 6. During G1 phase, Rb protein is underphosphorylated and bound to E2F so that it inactivates

transcription factors. Then, Rb protein phosphorylation by cdks/cyclins leads to release E2F so that it can drive the cell into S phase. Phosphorylation of Rb protein also promotes binding to nucleophosmin/B23, and the resulting complex is imported into the nucleoli (Fig. 6).

Crystallographic analyses have revealed that the surface of pockets A and B are made up of hydrophobic α-helices, i.e., α8, α9, α10, α11, and α13 [36]. Do these bind to a hydrophobic region of nucleophosmin/B23? Rat nucleophosmin/B23 is found in two isoforms generated by alternative splicing, B23.1 and B23.2 [37]. B23.1 possesses an extra 35 amino acids at the C terminus (residues 256–292), and it, but not B23.2, can bind to Rb protein [15]. Since this C terminus contains several hydrophobic clusters [16], these clusters may interact with the hydrophobic face of an exposed pocket A (Fig. 6). The data shown in Fig. 3 suggest that Rb N-terminal region is also necessary for nucleolar translocation.

We have suggested that Rb protein may play a dual role in regulating the cell cycle; when underphosphorylated it suppresses progression around the cell cycle, and when hyperphosphorylated, it promotes entry into S phase [10, 31]. For what reason, then, is hyperphosphorylated Rb imported into nucleoli after DNA replication is completed? Several possibilities can be speculated. First, it may play a role in the replication of nucleolar DNA late in S phase, as hyperphosphorylated Rb protein and nucleophosmin/B23 synergistically stimulate DNA polymerase α, a major replicative polymerase [15, 31, 38–41]. We are currently attempting to localize hyperphosphorylated Rb protein relative to nascent nucleolar DNA, but this is proving difficult, as there is such a high background of nascent DNA immediately around the nucleolus [42]. Second, its entry may lead to the suppression of transcription of 45S ribosomal RNA. When U937 cells are induced to differentiate on treatment with phorbor ester, it enters nucleoli and binds to the transcription factor UBF, and this, in turn, suppresses transcription by RNA polymerase I [43]. The hyperphosphorylated Rb protein might suppress transcription for preparing nucleolar segregation in the mitotic phase. Third, it may be sequestered in the nucleolus once it has finished playing a role in replicating euchromatic DNA. Note that DNA polymerase δ also enters the nucleolus in association with the DNA helicase WRN, which is mutated in Werner's syndrome [44]. Although the biological significance of all these phenomena remains obscure, it seems likely that the cell exploits the changes in location of these key regulators during its proliferation cycle.

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