Cell Cycle-controlled Interaction of Nucleolin with the Retinoblastoma Protein and Cancerous Cell Transformation*

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Retinoblastoma protein (Rb) is a multifunctional tumor suppressor, frequently inactivated in certain types of human cancer. Nucleolin is an abundant multifunctional phosphoprotein of proliferating and cancerous cells, recently identified as cell cycle-regulated transcription activator, controlling expression of human papillomavirus type 18 (HPV18) oncogenes in cervical cancer. Here we find that nucleolin is associated with Rb in intact cells in the G1 phase of the cell cycle, and the complex formation is mediated by the growth-inhibitory domain of Rb. Association with Rb inhibits the DNA binding function of nucleolin and in consequence the interaction of nucleolin with the HPV18 enhancer, resulting in Rb-mediated repression of the HPV18 oncogenes. The intracellular distribution of nucleolin in epithelial cells is Rb-dependent, and an altered nucleolin localization in human cancerous tissues results from a loss of Rb. Our findings suggest that deregulated nucleolin activity due to a loss of Rb contributes to tumor development in malignant diseases, thus providing further insights into the molecular network for the Rb-mediated tumor suppression.

Nucleolin is an abundant multifunctional phosphoprotein of proliferating and cancerous cells (1, 2) (reviewed in Ref. 3). High levels of nucleolin expression are related to poor clinical prognosis for certain cancer types (2, 4). A role of nucleolin in activation and repression of gene transcription as well as in regulation of RNA metabolism has been reported (5–7). Nucleolin is a subunit of the transcription factor LR1, which activates expression of the c-myc gene and the EBNA-1 (Epstein-Barr virus nuclear antigen 1) gene in B-cell lymphomas (7–9). Nucleolin is also directly involved in post-transcriptional inhibition of the p53 gene expression (10). As activator of transcription of HPV18\(^2\) oncogenes in cervical cancer cells, nucleolin has oncogenic potential (11). Nucleolin controls the chromatin structure of the HPV18 enhancer in vivo and is directly linked to HPV18-induced cervical cancer formation (11). Nucleolin is a substrate of cyclin-dependent kinases (CDKs), CDK1, CDK4, and CDK2 (12, 13), and its DNA binding activity is most prominent in the S phase of the cell cycle (11). Here we report a functional interaction of nucleolin with Rb, related to cell transformation.

Rb is a prototypical tumor suppressor, frequently inactivated in certain types of human cancer, and it controls cell proliferation, differentiation, and survival (14). Therefore, it is important to work out molecular mechanisms of Rb-mediated tumor suppression to understand the cancer disease process (14, 15). Rb functions to constrain cell proliferation by providing a cell cycle checkpoint between the G1 and S phases (16, 17). Phosphorylation of Rb by cyclin-dependent kinases is initiated in mid-G1 phase, and Rb phosphorylation is associated with G1/S transition and S phase progression (18, 19). Hypophosphorylated Rb restrains cell proliferation, in part by targeting E2F transcription factors (20–23), which are essential for cellular proliferation (24).

We report that nucleolin is as well associated with Rb in intact cells in the G1 phase of the cell cycle, and the complex formation is mediated by the growth inhibitory domain of Rb. Association with Rb inhibits the DNA binding function of nucleolin and in consequence the interaction of nucleolin with the HPV18 enhancer, resulting in Rb-mediated repression of the HPV18 oncogenes. The intracellular distribution of nucleolin in epithelial cells is Rb-dependent, and an altered nucleolin localization in human cancerous tissues results from a loss of Rb. Our findings suggest that deregulated nucleolin activity due to a loss of Rb contributes to tumor development in malignant diseases, thus providing further insights into the molecular network for the Rb-mediated tumor suppression.

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\* This work was supported by research grants from the Research Commission of the Medical Faculty of the University of Düsseldorf and the Ministry of Innovation, Science, Research, and Technology of the State of North Rhine Westphalia (to E. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: HPV, human papillomavirus; Ab, antibody; mAb, monoclonal antibody; CAT, chloramphenicol acetyltransferase; CDK, cyclin-dependent kinase; DAPI, 4′,6-diamidino-2-phenylindole; dl, deletion; DSP, dithiobis-succinimidyl propionate; DTT, 1,4-dithio-dl-threitol; EMSA, electrophoretic mobility shift assay; GST, glutathione S-transferase; Ig, immunoglobulin; Rb, retinoblastoma protein; Tet, tetracycline; URR, upstream regulatory region; WT, wild-type.
**Experimental Interaction between Nucleolin and Rb**

**Experimental Procedures**

**Cell Culture and Synchronization**—The following cell lines were used: human cells HBL100 (25); human HPV18 positive cervical carcinoma-derived cells HeLa; human Rb negative breast carcinoma-derived cells BT549 stably transfected with the pUHD-15-neo plasmid (26), referred to as BT549; BT549 cells stably transfected with the pUHD10-3 plasmid carrying the full-length human Rb cDNA located under tetracycline (Tet)-controlled promoter (26), referred to as BT549-Rb; human epithelial HaCaT/SiHa hybrid cells, containing genomically integrated HPV18 upstream regulatory region (URR) fused to the chloramphenicol acetyltransferase (CAT) reporter gene (27), referred to as HPV18 URR-CAT; and simian kidney cells CV1 and COS7 (28). HBL100 cells were maintained in RPMI 1640 medium, supplemented with 10% fetal calf serum; other cell lines were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Reconstitution of Rb function in BT549-Rb cells was attained by a 48-h culture in the absence of Tet (26).

Detailed cell synchronization conditions and the necessary controls were described (29). In brief, G1 phase cells were prepared by 0.2% lovastatin treatment for 24 h, and S phase cells were prepared by 3 μM aphidicolin treatment for 24 h followed by a 3–3.5 h release in fresh medium.

**In Vitro Binding Assays**—Glutathione S-transferase (GST)-Rb and GST-nucleolin fusion proteins were described (11, 30). GST-nucleolin fusion protein was generated by cloning the cDNA fragment, corresponding to amino acid residues 289–707 of the human nucleolin cDNA, lacking the acidic N terminus, into pGEX2T vector. The fusion proteins were produced in *E. coli* and purified by affinity chromatography using glutathione-Sepharose (Amersham Biosciences) according to the manufacturer’s protocol. For GST pull-down assays, nuclear protein extracts were prepared essentially as described (11). In brief, after cell lysis and isolation of cell nuclei, nuclear proteins were extracted with a buffer containing 10 mM Tris, pH 8.0, 0.9 M NaCl, 1.5 mM MgCl₂, 5% glycerol, 1 mM 1,4-dithio-DL-threitol (DTT), and protease inhibitors and were separated from chromatin DNA by high speed centrifugation at 4 °C. Nuclear extracts were incubated with equivalent amounts of GST fusion proteins (2 μg) bound to glutathione-Sepharose beads, for 1 h at 4 °C in a buffer containing 10 mM Tris, pH 7.5, 100 mM NaCl, 5% glycerol, 1 mM DTT, and protease inhibitors. The beads were washed extensively with the same buffer additionally containing 0.2% Nonidet P-40 and then resuspended in SDS-PAGE sample buffer. Quantification of nucleolin binding with single GST-Rb mutants in Fig. 1C was performed in relation to quantities of the GST-proteins.

**Immunoprecipitation**—Preparation of cell lysates for immunoprecipitation was as described (31). Antibodies (Abs) used were rabbit Ab specific for an N-terminal peptide of nucleolin, affinity-purified on the peptide column (11), together with normal rabbit Ig (sc-2027; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and rabbit Ab specific for epidermal growth factor receptor (sc-03; Santa Cruz Biotechnology) as controls and monoclonal Ab (mAb) specific for Rb (C36; Pharmingen), together with control mouse IgG1 (MOPC-31C; Pharmingen).

Immunoprecipitation experiments were performed with protein A/G PLUS-agarose beads (Santa Cruz Biotechnology), generally following the manufacturer’s protocol. In brief, cell lysates or nuclear extracts prepared as described above were precleared and incubated with Ab for 2 h at 4 °C in a buffer containing 10 mM Tris, pH 7.5, 50 to 100 mM NaCl, 5% glycerol, 1 mM DTT, protease inhibitors, and 0.5% Cohn-fraction-V purified bovine serum albumin (Sigma). The beads were washed extensively with a buffer containing 10 mM Tris, pH 7.5, 50–100 mM NaCl, 0.2% Nonidet P-40, 5% glycerol, 1 mM DTT, and protease inhibitors and then resuspended in SDS-PAGE sample buffer.

Chemical cross-linking was performed with the membrane-permeable, cleavable protein-protein cross-linking reagent dithiobis-succinimidyl propionate (DSP) (Pierce), following the manufacturer’s protocols and published procedures (32). The treatment of intact cells with 0.5 mM DSP for 15 min at room temperature in phosphate-buffered saline was followed by quenching with 10 mM Tris, pH 7.5. Cells were lysed essentially as described (31); the lysis buffer contained 50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, and protease inhibitors. Where indicated, the cross-linker was cleaved by the addition of 0.1 mM DTT and incubation for 45 min at 37 °C.

**Western Blotting, Electrophoretic Mobility Shift Assay (EMSA), and Preparation of Nucleoli**—Immunoblot analysis followed standard procedures. The following are antibodies and their dilutions: affinity-purified Ab specific for nucleolin (11), 1:10000; Ab specific for Rb (C-15; Santa Cruz Biotechnology), 1:2000; mAb specific for Rb (G-34; Pharmingen), 1:1500; mAb specific for unphosphorylated and hypophosphorylated Rb (G-99; 549; Pharmingen), 1:800; Ab specific for Rb dually phosphorylated at serines 807 and 811 (sc-16670; Santa Cruz Biotechnology), 1:2000; Ab specific for Rb dually phosphorylated at threonines 821 and 826 (sc-16669; Santa Cruz Biotechnology), 1:2000; Ab specific for cyclin A (H-432; Santa Cruz Biotechnology), 1:3000; mAb specific for GST (B-14; Santa Cruz Biotechnology), 1:4000. Prestained molecular weight marker proteins (Bio-Rad) were used.

The preparation of nuclear extracts and conditions of EMSA were described (11). In brief, end-labeled double-stranded oligonucleotide from the enhancer of HPV18, corresponding to nucleotide numbers 7634–7671 was used (33), together with 5 μg of poly(dl-dC)(dl-dC) and 5 μg of nuclear extract, in the DNA-binding buffer containing 10 mM Tris, pH 7.5, 50 mM NaCl, 5% glycerol, 1 mM DTT, and protease inhibitors. Affinity-purified GST fusion proteins were preincubated with nuclear extracts for 30 min at 4 °C before processing the reactions for EMSA.

Nucleoli were prepared from purified cell nuclei, following the published method (34); purity of the preparations was monitored by microscopic analysis, after staining with Azure C, as described (34). Cell nuclei were homogeneously pure, and the purity of nucleoli was 95%.

**Transient Transfections and CAT Assays**—For cell transfections, cytomegalovirus promoter-driven expression constructs encoding a full-length human wild-type (WT) Rb cDNA or an Rb cDNA containing a deletion (dl) of amino acid residues
738–775 (35) were used, together with a cytomegalovirus promoter-driven expression construct encoding a full-length nucleolin cDNA and the HPV18 URR-CAT cell line (11, 27). The DNA was purified using a plasmid purification system (Qiagen), and cells were transiently transfected according to standard procedures. HPV18 URR-CAT activity was measured by a CAT enzyme-linked immunosorbent assay kit (Roche Applied Science), and the results were normalized to protein concentration according to the manufacturer’s specifications.

**RESULTS**

**Immunohistochemistry and Detection of HPV**—Immunostaining of paraffin sections of human cervix specimens with the Ab specific for an N-terminal peptide of nucleolin, affinity-purified on the peptide column, was performed as described (11). Detection and typing of HPV DNA followed published procedures (11, 36).

**Immunofluorescence Analysis and Confocal Microscopy**—Cells grown on glass slides were fixed using methanol-acetone, and preincubation for 30 min in blocking solution containing phosphate-buffered saline and 1.5% normal serum (Vector Laboratories) was followed by immunofluorescence analysis according to standard procedures. Antibodies were diluted in blocking solution. Incubation with the rabbit polyclonal Ab specific for the N-terminal peptide of nucleolin, affinity-purified on the peptide column (11), was at a dilution of 1:200 for 30 min at room temperature. The specificity of anti-nucleolin Ab was proved by peptide competition and Western blotting. For staining of cell nuclei, 4,6-diamidino-2-phenylindole (DAPI) was added in the last incubation step. Fluorescence microscopy was performed with a Leica microscope with a ×100 objective lens.

Staining of cells for confocal microscopy was performed as described above. Mouse mAb specific for Rb (G3-245; Pharmingen) was used at a dilution of 1:100, according to the manufacturer’s specifications. Specificity of the Ab was controlled by immunofluorescence staining of Rb-negative cells BT549 and Western blotting. Analysis of subcellular distributions was performed using a MultiProbe 2010 confocal laser-scanning microscope (Molecular Dynamics) equipped with a krypton-argon laser and an Indy work station (Silicon Graphics). Confocal analysis was performed with a resolution (pixel size) of 0.1 × 0.1 μm.

**RESULTS**

**Cell Cycle-dependent Interaction of Nucleolin with Rb**—We analyzed the possibility that nucleolin may interact with Rb. To investigate whether nucleolin and Rb interact in vitro, pull-down experiments using GST-Rb and GST-nucleolin fusion proteins and nuclear extracts from exponentially growing epithelial cells CV1 and HBL100 were performed. The GST-nucleolin fusion protein comprised amino acid residues 289–707, and we refer to this as GST-nucleolin throughout. Interacting proteins were analyzed by immunoblotting with Ab specific for Rb or nucleolin. Fig. 1A, top, shows that the GST-nucleolin fusion protein formed a complex with Rb (lane 2). In contrast, GST backbone protein alone did not interact with Rb (lane 3). The relative quantities of both GST-proteins used were monitored by immunoblotting with Ab specific for GST (Fig. 1A, bottom). The interaction of nucleolin with Rb was also demonstrated using a GST-Rb fusion protein in pull-down experiments (Fig. 1B, top, lane 2). The GST-Rb fusion protein consisted of the large A/B pocket and the C-terminal domain (amino acid residues 379–928) of Rb (30), and we refer to this as GST-Rb throughout. GST backbone protein did not interact with nucleolin (Fig. 1B, top, lane 3). The quantities of both GST-proteins were equivalent, as was confirmed by immunoblotting with Ab specific for GST (Fig. 1B, bottom).

Next, we analyzed Rb/nucleolin interactions by pull-down experiments relying on several mutant GST-Rb proteins (30). The GST-Rb 379–928 dl 738–775 fusion protein, containing a dl of amino acid residues 738–775, a tumor cell-derived mutation within the Rb pocket (37), had a significantly reduced affinity for nucleolin, ~15% as compared with GST-Rb (Fig. 1C, top, lanes 2 and 3). The GST-Rb 379–816 mutant, consisting to a large part of the A/B pocket, interacted with nucleolin to a similar extent as GST-Rb, suggesting that the deleted 112 C-terminal amino acid residues of Rb are not essential for the binding (Fig. 1C, top, lanes 5 and 6). Moreover, the GST-Rb 379–792 dl 573–645 mutant, consisting of the A and B parts of the pocket region, devoid of the spacer separating the A and B parts, interacted with nucleolin, with an affinity of ~35% as compared with GST-Rb (lane 7). GST backbone protein was used as internal control (lanes 4 and 8). The relative quantities of the GST-proteins used were monitored by immunoblotting with Ab specific for GST (Fig. 1C, bottom). Thus, the pocket domain of Rb is involved in the interactions with nucleolin. The pocket domain is important for diverse functions of Rb (23, 30, 35, 38).

To investigate whether nucleolin and Rb are associated in vivo, coimmunoprecipitation assays of endogenous proteins from nuclear extracts of exponentially growing epithelial cells were performed. Fig. 1D (left, lane 2) shows that the Ab specific for a N-terminal nucleolin peptide precipitated Rb from the CV1 cell extract. More than 5% of the endogenous cellular Rb was coimmunoprecipitated with nucleolin in this experiment. The presence of nucleolin in the same immunoprecipitate was demonstrated by an immunoblot with Ab specific for nucleolin (Fig. 1D, lane 6). Furthermore, Rb was neither precipitated with a control Ab specific for epidermal growth factor receptor (lanes 3 and 7) nor with the protein A/G-agarose beads (lanes 4 and 8). Fig. 1E (left, lane 2) shows that mAb specific for Rb precipitated nucleolin from the HBL100 cell extract, as was determined by immunoblotting with the Ab specific for nucleolin. The presence of Rb in the same immunoprecipitate was monitored by an immunoblot with Ab specific for Rb (Fig. 1E, lane 6). Nucleolin was neither precipitated with a control mAb (lanes 3 and 7) nor with the protein A/G-agarose beads (lanes 4 and 8). Thus, nucleolin and Rb are associated with each other in exponentially growing epithelial cells in vivo.

Next, we investigated whether the interaction of nucleolin with Rb is subjected to cell cycle regulation. First, pull-down experiments using GST-nucleolin fusion protein and nuclear extracts from HBL100 cells, biochemically synchronized into cell cycle G1, and S phase populations, respectively, were performed. Fig. 2A shows that Rb from G1 phase cells efficiently bound to the GST-nucleolin fusion protein (Fig. 2A, left, top,
Functional Interaction between Nucleolin and Rb

FIGURE 1. Identification of nucleolin as Rb-binding protein. A, pull-down of Rb with GST-nucleolin fusion protein. Lane 1, nuclear extract used for the binding assay (10%). Binding with GST-nucleolin (lane 2) and with GST backbone protein (lane 3) is shown. Top, Western blotting with Ab specific for Rb (C-15). Endogenous cellular Rb is indicated by an arrow. Bottom, immunoblotting with Ab to GST, showing the relative quantities of GST-proteins used. B, pull-down of nucleolin with GST-Rb fusion protein. Binding with GST-Rb (lane 2), with GST backbone protein (lane 3). Lane 1, nuclear extract used for the binding assay (15%). Top, nucleolin was detected by Western blotting. Endogenous cellular nucleolin is indicated by an arrow. Bottom, immunoblotting with Ab to GST. C, binding of nucleolin with fusion proteins GST-Rb 379–928, dl 738–775 (lane 3), GST-Rb 379–816 (lane 6), and GST-Rb, 379–792, dl 573–645 (lane 7). Lanes 2 and 5, binding with GST-Rb. Lanes 4 and 8, binding with GST backbone protein. Lane 1, nuclear extract used for the binding assay (8%). Top, nucleolin was detected by Western blotting. Endogenous cellular nucleolin is indicated by an arrow. Bottom, immunoblotting with Ab to GST, showing the relative quantities of GST-proteins used. Binding of nucleolin with GST-Rb 379–816 and GST-Rb 379–792 dl 573–645 mutants was analyzed separately and displayed with positive (GST-Rb) and negative (GST) controls. D, endogenous cellular nucleolin associates with Rb. Immunoprecipitations were performed with nucleolin peptide Ab (lanes 2 and 6), with control rabbit Ab (lanes 3 and 7), and with no Ab (lanes 4 and 8). Lanes 1 and 5, nuclear extract used for coimmunoprecipitation assay (8 and 15%, respectively). Lanes 1-4, Western blotting with Ab specific for Rb (C-15). Lanes 6-8, the same immunoprecipitates (IP) were analyzed with Ab to nucleolin. E, immunoprecipitations were performed with mAb specific for Rb (C36) (lanes 2 and 6), with control mAb (lanes 3 and 7), and with no Ab (lanes 4 and 8). Lanes 1 and 5, nuclear extract used for coimmunoprecipitation assay. Lanes 1-4, Western blotting with Ab specific for nucleolin. Lanes 6-8, the same immunoprecipitates were analyzed with Ab specific for Rb (C-15).
The presence of Rb in the cross-linked complex detected was confirmed after cleavage of the cross-linker by a complete reduction of precipitated proteins, followed by SDS-PAGE and immunoblotting with Ab specific for Rb (Fig. 2D, middle, lane 5). Furthermore, the cross-linked Rb complex could not be precipitated with the nucleolin Ab presaturated with the immunizing nucleolin peptide (lane 2) nor with control rabbit Ig (lane 3). No significant amounts of the cross-linked Rb complex were observed in immunoprecipitates from S phase cells (lane 4). The amounts of Rb in both the G1 and S phase cell lysates were similar (not shown). By reducing SDS-PAGE and immunoblotting with Ab specific for nucleolin, it was confirmed that nucleolin was present in identical amounts in the immunoprecipitates from both G1 and S phase cells (Fig. 2D, right, lanes 7 and 9). Moreover, similar cross-linking studies using G1 phase synchronized breast carcinoma derived cells BT549, which lack Rb (41), did not reveal the presence of the cross-linked Rb-nucleolin complex (data not shown). Thus, the interaction of nucleolin with Rb takes place in intact cells and is cell cycle-dependent.

Rb-dependent Subcellular Localization of Nucleolin in Epithelial Cells—We have reported that nucleolin localization is altered in HPV18 positive precancerous and cancerous tissue from the cervix uteri (11). The E7 oncoproteins of high risk HPVs in cervical cancer cells inhibit the function of Rb and cause its degradation (42–44). Here, nucleolin localization in six HPV16-positive carcinomas and high grade intraepithelial lesions and four different normal (HPV-free) tissues of human cervix was analyzed by immunohistochemistry. Fig. 3A shows the altered nucleolin localization in an HPV16-positive cervical carcinoma. We recognize nucleolin localization as a granular pattern in the cell nuclei. In contrast, in the normal, HPV-negative epithelium of the human cervix, analyzed here for comparison, nucleolin was predominantly localized in the cell nuclei, although a diffuse nuclear staining was observed in a subset of cells (Fig. 3B). HPV DNA was detected in tissue specimens by a published method, and typing was performed by reverse line blot hybridization PCR (36). Thus, Rb deficiency might result in an aberrant nucleolin localization in cancerous cells in vivo.

To further analyze this issue, we examined nucleolin localization by immunofluorescence assays, using the cell line BT549 together with the cell line BT549-Rb derived thereof and containing a stably transfected Rb cDNA located under the Tet-controlled promoter. After induction of Rb expression, these cells are reconstituted for Rb function (26), and we refer to this as BT549-Rb throughout. The expression levels of Rb in BT549-Rb cells are similar to those of endogenous Rb in breast carcinoma cells MCF-7 (26). To compare nucleolin localization, BT549 and BT549-Rb cells were grown together, on 2-well chamber slides, in adjacent wells. The cells were synchronized biochemically to the G1 phase of the cell cycle, stained with the Ab specific for nucleolin, and analyzed identically. As shown in Fig. 3C, these cells strikingly differ in their nucleolin localization patterns. In the Rb-negative BT549 cells (Fig. 3C, top, left), nucleolin was present in the nucleoli, and significant amounts of nucleolin were found as a granular pattern in the nucleoplasm and in
Functional Interaction between Nucleolin and Rb

A

GST pull-down assay

GST-Nucleolin

Nuclear extract

Western with Rb

Western with GST

GST-Nucleolin

Cyclin A

Western

B

Coimmunoprecipitation assay

IP

IP

No Ab

Nucleolin Ab

Cell lysate

No Ab

Nucleolin Ab

G1 S

G1 S

Rb

Nucleolin

Western with Rb

Western with nucleolin

Western with Rb

Western with nucleolin

C

Hypo-P-Rb

Rb-P-Ser807/811

Western with hypo-P-Rb

Western with Rb-P-Ser807/811

Coimmunoprecipitation assay

1 2 3 4 5 6

1 2 3 4 5 6

1 2 3 4 5 6

1 2 3 4 5 6

D

Coimmunoprecipitation assay

Cross-linked Rb complex

IP

IP

IP

No Ab

Nucleolin Ab

Control Ab

No Ab

Nucleolin Ab

Control Ab

No Ab

Nucleolin Ab

Control Ab

G1 S

G1 S

G1 S

Western with Rb

Western with Rb

Western with Rb

Western with nucleolin

Western with nucleolin

Western with nucleolin

Coimmunoprecipitation assay

1 2 3 4

5 6

7 8 9 10

125 kD

120 kD

207 kD

120 kD

120 kD

120 kD

210 kD

210 kD
Functional Interaction between Nucleolin and Rb

the cytoplasm. In contrast, in the Rb-reconstituted BT549-Rb cells, nucleolin strongly predominated in nucleoli (Fig. 3C, top, middle). These patterns of nucleolin localization were detected in a large majority of BT549 cells and BT549-Rb cells (>85 and >80%, respectively). The differences in nucleolin localization between BT549 and BT549-Rb cells were observed in several independent experiments. To confirm that nucleolin localization is Rb-dependent in this cell system, BT549-Rb cells in the noninduced state were also examined. In the presence of Tet, these cells express Rb at significantly lower levels (26) (data not shown), and we refer to this as BT549-Rb + Tet. After synchronization of BT549-Rb + Tet cells to the G1 phase, nucleolin localization was analyzed identically as with BT549 and BT549-Rb cells. In BT549-Rb + Tet cells, we recognized an intermediate nucleolin localization phenotype (Fig. 3C, top, right). Significant amounts of nucleolin were present both in the nucleoplasm and in the nucleoli; to a certain extent, nucleolin was also detected in the cytoplasm. The differences in intracellular distribution of nucleolin between BT549 cells and Rb-reconstituted BT549-Rb cells, synchronized to G1 phase, were further confirmed by cell fractionation experiments, followed by Western blotting. Fig. 3C, bottom, shows that, in BT549 cells, ~40% of cellular nucleolin was detected in the cytoplasm (lane 1) and 60% in the nucleus (lane 2). 40% of nuclear nucleolin resided in the nucleolus (lane 3). In BT549-Rb cells, ~5% of nucleolin was in the cytoplasm (lane 4) and 95% in the nucleus (lane 5). 85% of nuclear nucleolin was detected in the nucleolus (lane 6). The preparations of cell nuclei were homogeneously pure, and the purity of nucleoli was 95%, as was determined following the previously described procedure, after staining with Azure C (34). The efficiencies of G1 phase synchronization of BT549 and BT549-Rb cells were controlled by immunoblotting with Ab specific for cyclin A (Fig. 3D, left). Fig. 3D shows that reconstitution of Rb in BT549 cells did not detectably affect cyclin A levels under these conditions (lanes 1 and 2), in line with previous reports (45, 46). Thus, Rb reconstitution in BT549 cells did not significantly influence the efficiencies of G1 phase synchronization, and the observed differences of nucleolin localization are therefore not due to indirect cell cycle effects of Rb expression. The levels of Rb expression in BT549-Rb cells were similar to those of endogenous Rb in MCF-7 cells, as was shown by immunoblotting with Ab specific for Rb (Fig. 3D, right, lanes 5 and 6), consistent with previous observations (26).

Next, nucleolin localization in cervical carcinoma-derived cells HeLa, synchronized biochemically into G1 and S phase populations, respectively, was examined. The expression of the HPV18 E7 oncogene in HeLa cells results in a loss of Rb, and nucleolin was found in the nucleoplasm and in the nucleoli in both G1 and S phase cells, analyzed identically (Fig. 3E). Subcellular localization of nucleolin was very similar in HeLa cells synchronized into G1 and S phase populations, as was observed in several independent experiments. Cell synchronization efficiencies were confirmed by immunofluorescence analysis of cyclin A protein expression (not shown).

Nucleolin localization in COS7 cells, where Rb is blocked by the SV40 large tumor antigen (47), along with CV1 cells, which are closely related to COS7 but lack the large tumor antigen (28), and therefore express a functional Rb, was also examined. In COS7 cells, synchronized biochemically to the G1 phase, nucleolin was detected throughout the cell nuclei (Fig. 3F). In identically synchronized CV1 cells, synchronized biochemically into G1 and S phase populations, respectively, nucleolin strongly predominated in nucleoli in G1 phase cells, whereas in the S phase, nucleolin was detected throughout the cell nuclei (Fig. 3G). These patterns of nucleolin localization were observed in several independent experiments. Cell synchronization efficiencies were confirmed by immunofluorescence staining with Ab specific for cyclin A (not shown). To determine whether nucleolin and Rb localize together in CV1 cells synchronized to the G1 phase, nucleolin was visualized in these cells using the rabbit polyclonal Ab specific for nucleolin and a fluorescein isothiocyanate-labeled second Ab specific for rabbit Ig (green), and Rb was visualized using a mouse mAb specific for Rb and a rhodamine-labeled second Ab specific for mouse Ig (red) (Fig. 3H). Fig. 3H shows detection of nucleolin and Rb by confocal laser-scanning microscopy. We recognize that Rb is present in the nucleus, and significant amounts of Rb are detected in the nucleoli (Fig. 3H, middle). Merging the confocal images of nucleolin and Rb demonstrates that the two proteins colocalize to a significant extent as indicated by the yellow color (Fig. 3H, bottom). This localization pattern of nucleolin and Rb was observed in several independent experiments.

Taken together, these data suggest that subcellular localization of nucleolin in epithelial cells is Rb-dependent, and an
Functional Interaction between Nucleolin and Rb

A

Immunohistochemistry

B

Immunohistochemistry

C

Nucleolin

D

Western

E

Immunofluorescence microscopy

F

Immunofluorescence microscopy

G

Immunofluorescence microscopy

H

Confocal microscopy
Rb regulates DNA binding function of nucleolin. A, top, detection of nucleolin by EMSA with the labeled HPV18 enhancer oligonucleotide RP3. EMSA with nuclear extracts from G1 phase and S phase HBL100 cells (lanes 1 and 2), and from G1 phase and S phase HeLa cells (lanes 3 and 4). Retarded nucleolin-DNA complex is indicated by an arrow. Bottom, Western blot analysis with Ab to cyclin A, B, detection of nucleolin by EMSA with nuclear extracts from G1 phase BT549 cells (lanes 1 and 2), and BT549-Rb cells (lanes 3 and 4). EMSA after preincubation with affinity-purified GST-Rb fusion protein (0.6 and 2 μg); lanes 4 and 5, EMSA after preincubation with affinity-purified GST protein (0.6 and 2 μg); lanes 7 and 8, EMSA after preincubation with affinity-purified GST-Rb protein (2 and 3 μg); lanes 9 and 10, EMSA after preincubation with affinity-purified GST-Rb 379–928, di 738–775 protein (2 and 3 μg). C, top, detection of nucleolin by EMSA with nuclear extracts from G1 phase BT549 cells and BT549-Rb cells (lanes 1 and 2) and the oligonucleotide RP3. Retarded nucleolin-DNA complex is indicated by an arrow. Middle, the same extracts were analyzed by immunoblotting with Ab to Rb. Bottom, analysis by immunoblotting with Ab to Rb (C-15).

FIGURE 3. Rb-dependent subcellular localization of nucleolin in epithelial cells. A, subcellular localization of nucleolin in human HPV16-positive neoplasia. Bound Ab was visualized by avidin-biotin-peroxidase-3,3′-diaminobenzidine tetrachloride and is stained brown. As a control, the Ab specific for nucleolin was presaturated with the immunizing peptide (not shown). Specimens were counterstained with hematoxylin. The counterstained nuclei are light blue. High power view is shown on the right. B, normal, HPV-negative, epithelium of the human cervix. High power view is shown on the right. C, top, after synchronization to G1 phase, BT549 cells, BT549-Rb cells, and BT549-Rb+ Tet cells were stained with Ab specific for nucleolin. Middle, counterstaining of cell nuclei with DAPI. BT549 cells and BT549-Rb cells were grown together, in adjacent wells of a 2-well chamber slide, and BT549-Rb+ Tet cells were grown on a separate slide. The staining was performed identically. Photographs were taken with the same exposure times and magnification (×100 objective lens) and processed identically, thus assuring that the images are representative of the DNA binding function of nucleolin. For this purpose, HBL100 cells, where nucleolin is associated with Rb in G1 phase (Fig. 2), were biochemically synchronized into cell cycle G1, S and phase populations, respectively, and the DNA binding of nucleolin was altered nucleolin localization in cancerous tissues results from a loss of Rb.

Rb Regulates the DNA Binding Function of Nucleolin—Nucleolin specifically binds to the enhancer of HPV18; thereby, it activates transcription of HPV18 oncopgenes in cervical cancer cells and thus the proliferation of these cells (11). The DNA binding activity of nucleolin is cell cycle-regulated and most prominent in S phase. Next, we investigated whether Rb is involved in regulating the DNA binding function of nucleolin. For this purpose, HBL100 cells, where nucleolin is associated with Rb in G1 phase (Fig. 2), were biochemically synchronized into cell cycle G1, S and phase populations, respectively, and the DNA binding of nucleolin was...
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monitored by EMSA using the double-stranded oligonucleotide from the HPV18 enhancer, corresponding to the nucleotide numbers 7634–7671 (33). This oligonucleotide, termed RP3, contains a high affinity nucleolin binding site (11). The DNA binding of nucleolin was present in the S phase but was just detected in G1 (Fig. 4A, top, left). Cell synchronization efficiencies were confirmed by immunoblotting with Ab specific for cyclin A (Fig. 4A, bottom, left). Next, the nucleolin/DNA binding was analyzed with HeLa cells, synchronized into cell cycle G1 and S phase populations, as described above (Fig. 3E). Fig. 4A shows that the formation of retarded nucleolin-RP3 complex was identical in G1 and S phase (Fig. 4A, top, right). The sequence specificity of nucleolin interactions with RP3 oligonucleotide and the presence of nucleolin in the retarded DNA-protein complex was demonstrated previously (11). Cell synchronization efficiencies were confirmed by immunoblotting with Ab specific for cyclin A (Fig. 4A, bottom, right). Thus, the Rb deficiency in HeLa cells is associated with the occurrence of nucleolin/DNA binding in G1 phase. These data suggest a potential involvement of Rb in regulation of nucleolin/DNA interactions.

To directly show an involvement of Rb in regulation of nucleolin/DNA binding, we added increasing amounts of affinity-purified GST-Rb 379–928 fusion protein to nuclear extracts from G1 phase HeLa cells and analyzed these by EMSA with the oligonucleotide RP3. GST-Rb protein inhibited nucleolin/DNA binding in a concentration-dependent fashion (Fig. 4B, lanes 2 and 3). In contrast, GST alone did not detectably affect nucleolin/DNA binding (lanes 4 and 5). Next, the effect of the GST-Rb 379–928 dl 738–775 mutant on the nucleolin/DNA binding was examined. This affinity purified GST-fusion protein was added to the binding reactions, and nucleolin/DNA interactions were monitored by EMSA. It became evident that the GST-Rb 379–928 dl 738–775 mutant did not significantly affect nucleolin-RP3 binding (Fig. 4B, lanes 9 and 10), as compared with GST-Rb (lanes 7 and 8).

Next, BT549 and BT549-Rb cells were synchronized into G1 phase populations, as described above (Fig. 3D), and nuclear extracts were analyzed by EMSA with the RP3 oligonucleotide. The DNA binding of nucleolin was detected in BT549 cells, whereas in BT549-Rb cells no significant nucleolin/DNA binding was observed (Fig. 4C, top). The amounts of nucleolin in the nuclear extracts from BT549 and BT549-Rb cells used in the EMSA

FIGURE 5. Rb controls nucleolin-dependent activation of HPV18 oncogenes. Top, HPV18 URR-CAT cells were transiently co-transfected with a nucleolin cDNA expression construct and the empty Rb expression vector (lane 1), with a nucleolin cDNA expression construct and a WT Rb cDNA expression construct (lane 2), or with a nucleolin cDNA expression construct and an Rb dl 738–775 cDNA expression vector (lane 3). Lane 4, co-transfection of the empty nucleolin expression vector and the empty Rb expression vector. Lane 5, co-transfection of the empty nucleolin expression vector and the WT Rb cDNA expression construct. CAT activity was measured by CAT enzyme-linked immunosorbent assay. The results were normalized to protein concentration. Bottom, HPV18 URR-CAT cells were transiently transfected with expression plasmids containing WT Rb cDNA or Rb dl 738–775 cDNA. The cell extracts were analyzed by Western blotting with Ab to Rb (C-15). 40 μg of each extract were used. Lane 1, mock-transfected cells; lane 2, cells transfected with WT Rb cDNA expression vector; lane 3, cells transfected with Rb dl 738–775 cDNA expression vector. Rb is indicated by an arrow. Note that the polyclonal Ab additionally recognizes endogenous protein of 97 kDa in this experiment.

Western

CAT ELISA

FIGURE 5. Rb controls nucleolin-dependent activation of HPV18 oncogenes. Top, HPV18 URR-CAT cells were transiently co-transfected with a nucleolin cDNA expression construct and the empty Rb expression vector (lane 1), with a nucleolin cDNA expression construct and a WT Rb cDNA expression construct (lane 2), or with a nucleolin cDNA expression construct and an Rb dl 738–775 cDNA expression vector (lane 3). Lane 4, co-transfection of the empty nucleolin expression vector and the empty Rb expression vector. Lane 5, co-transfection of the empty nucleolin expression vector and the WT Rb cDNA expression construct. CAT activity was measured by CAT enzyme-linked immunosorbent assay. The results were normalized to protein concentration. Bottom, HPV18 URR-CAT cells were transiently transfected with expression plasmids containing WT Rb cDNA or Rb dl 738–775 cDNA. The cell extracts were analyzed by Western blotting with Ab to Rb (C-15). 40 μg of each extract were used. Lane 1, mock-transfected cells; lane 2, cells transfected with WT Rb cDNA expression vector; lane 3, cells transfected with Rb dl 738–775 cDNA expression vector. Rb is indicated by an arrow. Note that the polyclonal Ab additionally recognizes endogenous protein of 97 kDa in this experiment.
experiments were similar, as was monitored by immunoblotting with Ab specific for nucleolin (Fig. 4C, middle). The expression of Rb in BT549 and BT549-Rb cells was controlled by immunoblotting with Ab specific for Rb (Fig. 4C, bottom). Taken together, these data demonstrate that Rb acts as a regulator of nucleolin/DNA interactions.

Rb Controls Nucleolin-dependent Activation of HPV18 Oncogenes—Nucleolin is involved in regulation of the chromatin structure of the HPV18 enhancer, and it activates genomically integrated HPV18 URR (11). Thus, we analyzed whether the nucleolin-mediated activation of the HPV18 URR can be regulated by Rb. Co-transfection experiments using a nucleolin expression vector, together with either an expression vector encoding a full-length WT Rb cDNA or an Rb dl 738–775 mutant cDNA (35) and an epithelial cell line harboring a genomically integrated HPV18 URR fused to the CAT reporter gene (HPV18 URR-CAT cells, a HaCaT×SiHa hybrid cell line) (27) were performed. Fig. 5 (top) shows that a co-transfection of the WT Rb expression construct together with nucleolin expression construct significantly inhibited the activation of the HPV18 URR, which was achieved by a transfection of the nucleolin expression construct only (lanes 2 and 1, respectively). In contrast, Rb dl 738–775 mutant did not significantly affect the nucleolin-mediated activation of the HPV18 URR (lane 3). In the absence of overexpressed nucleolin, transfection of the WT Rb expression construct resulted in an inhibition of the HPV18 URR (Fig. 5, top, lane 5). The expression of the WT and mutant Rb proteins in transiently transfected cells was controlled by Western blotting. Fig. 5 (bottom) shows that transfection of the expression vector containing either the WT Rb or Rb dl 738–775 mutant cDNA resulted in efficient expression of each Rb protein at identical levels (lanes 2 and 3). Thus, Rb regulates transcription of HPV18 oncogenes through the functional interaction with nucleolin.

**DISCUSSION**

Our study reveals a functional interaction between nucleolin and Rb, related to cell transformation. Previously, an interaction between nucleolin and the p53 protein has been described, which occurs under certain conditions of cellular stress (48). The interaction of nucleolin with Rb, which we report here, is cell cycle-dependent and takes place in G1 phase, suggesting that the ability of nucleolin to interact with Rb depends on the phosphorylation status of Rb. In early G1, Rb is found in a predominantly underphosphorylated form, and CDK-mediated phosphorylation of Rb, correlated with the disruption of Rb-assembled protein complexes, is observed as cells progress from G1 into S phase (reviewed in Refs. 16, 18, 23, 49, and 50). Thus, we find that hypophosphorylated Rb, unlike certain phosphorylated forms of Rb, Rb phosphorylated at serines 807/811 and at threonines 821/826, interacts with nucleolin (Fig. 2C). The role of Rb phosphorylation at serines 807/811 and threonines 821/826 for regulation of protein binding activities of Rb has been demonstrated previously (39, 40). Both nucleolin and Rb share the property of binding the nuclear matrix (51, 52). Nucleolin is a multifunctional DNA- and RNA-binding protein, which is abundant in proliferating and cancerous cells (2–4). We have reported that nucleolin can act as a cell cycle-regulated transcription activator. It was shown that nucleolin controls the expression of the HPV18 oncogenes in cervical cancer cells and thus the proliferation rates of these cells (11). Cervical cancer represents the second most common malignancy in women, with an annual incidence of ~500,000 new cases (53), and nucleolin is involved in HPV18-induced cervical carcinogenesis. On the other hand, Rb is a prototypical tumor suppressor (14), and its inactivation is a prerequisite for cell proliferation. Rb-mediated repression of transcription and cell growth occurs at multiple levels, and the repression of the E2F family of transcription factors is one prevalent mechanism thereof (15, 23, 50). Here we demonstrate that Rb regulates the function of nucleolin as activator of transcription. Rb controls the interaction of nucleolin with the HPV18 enhancer and thus the nucleolin-dependent activation of transcription of the HPV18 oncogenes. It is recognized that the oncogenes E7 and E6 of high risk HPVs are central to cervical cancer development and are highly expressed in most cervical carcinomas (reviewed in Ref. 54). Oncoproteins E7 and E6 exert their carcinogenic potential by inactivation of the tumor suppressors Rb and p53 (42–44, 55, 56), and overexpression of p16Ink4a, which indicates the Rb deficiency (57), is a specific marker for dysplastic and neoplastic epithelial cells of the cervix uteri (58). Hence, it is tempting to suggest that the targeting of Rb by the E7 oncoprotein contributes to cancer formation via deregulation of nucleolin, which in turn leads to an activation of HPV18 oncogene expression, in HPV18-associated cervical carcinogenesis.

In line with several previous reports, revealing that Rb can accumulate in the nucleolus (59–62), our results obtained with CV1 and BT549-Rb cells (Fig. 3H) (data not shown), suggest that Rb and nucleolin localize together in G1 phase. Of note, Rb immunostaining patterns can be variable (63), and, even in living cells, accumulation of Rb in the nucleolus is cell type-dependent (62).

Nucleolin is a multifunctional protein that is found in the nucleolus, in the nucleus, in the cytoplasm, and at the cell surface (1, 3, 5, 64, 65). A role of nucleolin in activation as well as repression of ribosomal RNA synthesis has been described (1, 66). Thus far, it has not been completely elucidated how the intracellular distribution of nucleolin is controlled. Our data suggest that subcellular localization of nucleolin in epithelial cells is Rb-dependent and correlates with nucleolin/DNA binding activity. Thus, a loss of Rb in cancerous tissues results in altered nucleolin localization, most probably leading to changes in gene expression. It has been shown that nucleolin is detected in complexes, which mediate activation and repression of gene transcription (6, 7, 67, 68). Furthermore, in B-cell lymphomas, nucleolin contributes to activation of the c-myc gene and the EBNA-1 gene (7–9), which are known to promote induction of these malignancies (69, 70). Moreover, nucleolin is directly involved in post-transcriptional inhibition of the p53 gene expression (10) and in activation of the bcl-2 gene (71). It will be important to identify the whole spectrum of genes whose expression is regulated by nucleolin.

Our data and reports from the literature (48) demonstrate that nucleolin is linked to the two major cellular tumor suppres-
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sors, Rb and p53, which are defective in a significant spectrum of malignant diseases. This suggests that nucleolin deregulation directly contributes to the process of tumor development and implicates nucleolin as a novel target molecule for a potential therapy of Rb-defective tumors. Future studies will further address the role of nucleolin in the process of multistage carcinogenesis.

Acknowledgments—We thank Prof. Michael Strauss and Dr. Andreas Heder for discussions, stably transfected BT549 cells, and GST-Rb proteins. We thank Prof. Kristian Helin for Rb expression constructs and Prof. Frank Rösl for HaCaTxSiHa cells. We thank Prof. Ralph Steinman, Prof. Elmar Gren, Dr. Markus Uhrberg, and Prof. Steffen Hauptmann for constructive criticism.

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