Subcellular Localization of the Retinoblastoma Protein

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Abstract

The subcellular localization of the retinoblastoma (RB) protein has been studied in primate cell lines by immunofluorescence staining using different monoclonal and polyclonal antibodies. The protein appeared as granules of heterogeneous size over the interphase nuclei. Computer assisted digital overlap analysis indicated that it was predominantly localized in euchromatic areas with low DNA density. The largest RB positive grains lined up on the heterochromatin/euchromatin boundary. During mitosis, the RB protein dissociated from the condensing chromosomes. It was dispersed throughout the cytoplasm during metaphase and anaphase, and it reassocciated with the decondensing chromatin during telophase. A new monoclonal antibody, designated aRB1C1, was raised against a bacterial TrpE/human retinoblastoma protein. It specifically recognized the nonphosphorylated and differentially phosphorylated forms of the RB protein in immunoprecipitation experiments. A collection of RB expressing cell lines gave a positive staining reaction with the antibody, whereas the RB negative Weri-RB-27 retinoblastoma and OHS osteosarcoma cells failed to react. Wild-type RB complementary DNA was introduced into Weri-RB-27 by retrovirus mediated gene transfer. Similar experiments were performed with the DU145 prostatic carcinoma cell line that expresses a mutant RB protein. Reconstituted cells of both lines expressed the normal size RB protein and gave a positive immunofluorescence reaction with the aRB1C1 and other anti-RB antibodies. The new monoclonal antibody, however, showed cell type dependent differences of the staining pattern compared to other anti-RB antibodies, suggesting differentiation dependent accessibility to its epitope.

Introduction

The RB gene is located on chromosome 13q14 and consists of 27 exons spread over more than 200 kilobases. The 4.7-kilobase RB mRNA has the capacity to code for a 928-amino-acid-long polypeptide (1, 2). Its product has been identified as a 105–110 kD phosphoprotein that migrates as a cluster of bands on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (3). The multiple bands are different phosphorylated forms of the same protein. The RB protein is ubiquitously expressed in all normal cell types examined, suggesting that it is a household gene. This is consistent with its promoter structure, which has a high C + G content and lacks CCAAT and TATA motifs (4).

Loss of the functional RB protein is an essential step in the development of both familial and sporadic retinoblastoma (5). Bone and soft tissue sarcomas that arise as a late complication in persons with a familial RB deletion express no functional RB protein either. RB protein losses have also been found in a substantial portion of other sarcomas (6–8), breast carcinomas, (9, 10), and small cell lung carcinomas (11–13).

The tumorigenesis of RB negative retinoblastoma and osteosarcoma cells can be suppressed by the introduction of a wild-type RB gene carried by an appropriate retroviral vector (14, 15). Phosphorylation of the RB protein showed cell cycle dependent changes (16–19). It has been suggested that the RB protein plays an important role in cell cycle regulation. According to this hypothesis (20), the nonphosphorylated form of the wild-type protein prevents the entry of G<sub>1</sub> cells into the S phase. Only the nonphosphorylated form is detectable in G<sub>0</sub> and G<sub>1</sub> cells. The protein is phosphorylated at the G<sub>1</sub>–S transition, leading to the appearance of multiple phosphorylated forms during S and G<sub>2</sub>–M.

The RB protein forms complexes with the transforming proteins of small DNA tumor viruses like SV40 large T (18, 21, 22), adenovirus E1A (23), and papilloma virus E7 (24, 25). This interaction is believed to play an important role in transformation (26–28).

For the further functional characterization of the RB gene, it is important to determine the subcellular localization of the protein during different phases of the cell cycle and its relationship to other nuclear structures. Earlier studies have shown that the protein binds to DNA and localizes in the nucleus during interphase (3, 29, 30). The present study describes its distribution in the interphase nucleus during mitosis and its relationship to the DNA, as assessed by digital analysis.

Results

Detection of the RB Protein in Different Cell Types. A set of bacterial TrpE fusion proteins, containing different fragments of the human RB protein, was produced in Escherichia coli. A mouse monoclonal antibody (aRB1C1) was generated against the purified TrpE/RB fusion protein containing the RB sequences between amino acid positions 300 and 928. Using other TrpE/RB fusion proteins, containing RB sequences coded by the first and second BglII-BglII cDNA fragments, respectively, the epitope of
the antibody was mapped between amino acid positions 330 and 612. It precipitated the multiple 105-110 kD bands of the native RB protein (Fig. 1) from \(^{35}\)S\)methionine labeled RB positive Ramos cells but not from RB negative Weri-RB-27 cells. Immunoprecipitation from metabolically labeled or unlabeled cells followed by Western blotting showed that aRB1C1 detected the unphosphorylated and differentially phosphorylated forms of the same protein as the unrelated pMG3-245 mouse monoclonal and anti-RB rabbit polyclonal antibodies (obtained from Dr. Wen-Hwa Lee).

The specificity of the immunofluorescent staining was examined by comparing Weri-RB-27 cells reconstituted with the Moloney murine leukemia virus long terminal repeat driven normal human RB cDNA retroviral construct (14, 15) with the original cell line (Fig. 2). Only the reconstituted cells were stained. All three antibodies stained RB positive cells. Nuclear staining was also obtained with the following RB positive cells: CV1 and COS monkey kidney epithelial cells; human embryo fibroblasts (Fig. 3A); 293 adenovirus infected human kidney cells; COLO 320 human colon carcinoma cells; A549

![Fig. 1. Immunoprecipitation of the RB protein from \(^{35}\)S\)methionine labeled RB positive Ramos and RB negative Weri-RB-27 cells with two unrelated mouse monoclonal antibodies, aRB1C1 and pMG3-245.](image1)

![Fig. 2. Detection of the RB protein in human RB cDNA carrying retrovirus infected cells. Methanol-acetone fixed, noninfected (A, C) and infected (B, D) Weri-RB-27 cells stained with aRB1C1 (A, B) or with pMG3-245 (C, D) and noninfected (E) and infected (F) DU 145 prostatic carcinoma cells stained with pMG3-245.](image2)
Fig. 3. RB protein in paraformaldehyde fixed human cells: human lung embryo fibroblasts (A); Raji (B); IARC 171 (C); Ramos (D); phytohemagglutinin stimulated peripheral lymphocytes (F) [note the exclusively nuclear staining in all cells except in a mitotic form (up and left from the center)]; leukocytes from the peripheral blood with double fluorescent layer enhanced staining (F) (arrowhead, a granulocyte with the characteristic cleaved nucleus).

Fig. 4. Fixation, antibody, and cell type dependent staining pattern of the RB protein. CV1 nuclei with paraformaldehyde fixation/Triton X-100 permeabilization, using pMG3-245 (A) or aRB1C1 (B) antibodies or after methanol-acetone fixation with pMG3-245 (C) or aRB1C1 (D). RHEK-1 nuclei after methanol-acetone fixation with pMG3-245 (E) or aRB1C1 (F). The high resolution distribution of the RB protein in the single interphase nuclei shows size heterogeneity of granules and avoidance of the nucleoli. The large distinct granules are more easily detectable after methanol-acetone fixation. While there are no big differences between the structures detected by the two antibodies in CV1 cells with either fixation, in RHEK-1 cells, the aRB1C1 stains mainly the large granules and much less efficiently the small ones. The pMG3-245 gives a more homogeneous staining.
human lung squamous cell carcinoma cells; RHEK-1 immortalized human keratinocytes; EBV carrying human lymphoblastoid lines designated IARC 171 (Fig. 3C), ÅSA 17, NAD 20, and RBLCL-MAG; the human B-cell lymphoma line BJAB; the EBV negative Burkitt’s lymphoma BL line Ramos (Fig. 3D); and the EBV positive BL line Raji (Fig. 3B), an adherent variant of the B95–8 EBV transformed marmoset BL. Weak nuclear staining was detectable in DU 145, which contains an aberrant, truncated RB protein (Fig. 2E). After introduction of wild-type RB protein, expressed from a retroviral vector, a fraction of the cells became strongly positive (Fig. 2F). No staining was detected in the RB negative OH5 cells. In human peripheral white blood cells, the RB protein was not detectable by regular indirect immunofluorescence (mouse monoclonal IgG with FITC conjugated rabbit anti-mouse IgG), but a second FITC conjugated swine anti-rabbit IgG gave clear nuclear staining in the granulocytes and monocytes. Lymphocytes stained less intensively (Fig. 3F). RB negative Weri-RB-27 cells, used as parallel controls, remained unstained. Phytomhemagglutinin stimulated lymphocytes showed strong nuclear staining 72 h after stimulation, even by ordinary indirect immunofluorescence (Fig. 3E). Increased expression of the RB protein in blast transformed lymphocytes is in agreement with a previous report (31).

**Distribution of the RB Protein in Interphase Cells.** All RB positive cell lines tested gave a positive nuclear staining with RB antibodies, although the intensity varied both within and between lines. The cytoplasm showed no significant staining. Methanol-acetone and paraformaldehyde fixation gave slightly different results. A similar distribution of fine and somewhat larger granules, sparing the nucleoli, was obtained by both methods. Methanol-acetone fixed cells (Fig. 4, C and D) showed a greater granule size variation than paraformaldehyde fixed cells (Fig. 4, A and B). The two monoclonal antibodies aRB1C1 and pMG3-245 gave an almost identical staining pattern on CV1 cells but showed differences with some other cell lines. In RHEK-1 (Fig. 4F) and COLO 320 cells, aRB1C1 stained large nuclear granules very intensely, leaving the rest of the nucleus essentially unstained, whereas pMG3-245 gave more uniform, diffuse, fine granular staining (Fig. 4E).
Visual comparison of the DNA and RB staining patterns suggested a possible negative correlation. This was examined by computer assisted image analysis. CV1 cells were double stained for DNA and RB protein, using Hoechst 33258 and rabbit polyclonal anti-RB serum, respectively. The two fluorescent signals were recorded directly by a highly sensitive camera, digitalized, and transferred to the computer, and areas of maximal fluorescence intensity were calculated (Fig. 5). Superimposition of the two images (Fig. 5G) showed minimal overlap, suggesting that RB is localized in nuclear compartments with low DNA density. The distinct granular staining obtained with the aR81C1 antibody on methanol-acetone fixed CV1 cells permitted further assessment of the relationship between the stained structures and DNA distribution. Visual comparisons suggested that the largest granules lined up on the border of the low and high DNA density areas. This was confirmed by computer assisted localization of the most intensively stained granules. From the total RB specific fluorescent signals (Fig. 6, middle), the computer selected the strongest dots, eliminating the more homogeneous weaker signals below a subjective threshold level. Superimposition of the selected dots over the Hoechst 33258 detected DNA pattern (Fig. 6, bottom) showed that the biggest granules were not randomly scattered in the low DNA density regions but localized on the heterochromatin/euchromatin boundary (Fig. 6, top).

In order to examine the changes of the RB staining pattern during the cell cycle, logarithmically grown, EBV transformed, near euploid, IARC 171 human lymphoblastoid cells were stained for RB and DNA and separated by FACScan according their DNA content, corresponding to the G1, G2, or S phase, respectively. Cells in G1 showed a somewhat stronger RB staining, but there was no detectable difference in the nuclear distribution of the RB protein between the three phases (Fig. 7).

**RB Protein in Mitotic Cells.** RB positive granules appeared in the cytoplasm during mitosis, in parallel with chromosome condensation and breakdown of the nuclear membrane. The protein was still largely concentrated in the area of the dissolving nucleus in prophase cells, but the newly condensed chromosomes were negative (Fig. 8, A and B). In metaphase cells, the positive grains were scattered throughout the entire cytoplasm (Fig. 8, C and D). The granules were somewhat more concentrated in the mitotic spindle pole regions during metaphase and anaphase (Fig. 8, D and F). The size distribution of the grains was similar in interphase and mitotic cells. The protein relocalized to the nucleus in parallel with the decondensation of the chromosomes at telophase (Fig. 8, G and H). RB positive granules could be detected in the cytoplasm as long as the chromosomes were intact. In parallel with the appearance of the daughter nuclei, the RB positive grains disappeared totally from the cytoplasm (Fig. 8, I and J). The same pattern of RB distribution during mitosis was detected in all cell types studied, namely human keratinocytes, fibroblasts, lymphoblastoid lines, immunoblasts, Burkitt’s lymphoma cells, colon and lung carcinoma lines, and monkey kidney epithelial cells.

**Discussion**

Phosphorylation of the RB protein at the transition between G1 and 5 is believed to play a major role in permitting the entry of the cells into the S phase (20, 21). We have not found any substantial difference in the staining pattern or distribution of the RB protein in G1, S, and G2, indicating that the functional change of the protein is not associated with major redistribution. During mitosis, the RB protein dissociates from the condensing chromosomes. It localizes in the cytoplasm during metaphase and anaphase and reassociates with the chromatin of the daughter nuclei. Interphase nuclei show fine, speckled, granular distribution, avoiding the nucleoli. The RB protein behaves similarly to other DNA binding proteins in this respect, including c-myc (32), c-myb; Ki-67 (33), DNA polymerase α (34), SV40 large T (35), and c-fos (36).

The staining patterns of interphase nuclei and the computer assisted comparisons have suggested that the RB protein localizes to at least two different nuclear compartments. The diffusely distributed small granular form is spread over the entire euchromatin, whereas a more concentrated form appears as large, well circumscribed nuclear granules. The relative preponderance of the two types varies with the fixation, the antibody, and the cell type, suggesting that the accessibility of the
relevant epitopes is not uniform. Differentiation dependent differences in compartmentalization appear likely. The application of the aRB1C1 monoclonal antibody may help the in situ discrimination between these compartments.

It was suggested that the RB protein regulates the transcription of specific genes, like c-los, by direct or indirect interaction with their promoter (37). The localization of the RB protein in the transcriptionally active regions of the nucleus is consistent with this idea.

The preferential localization of the coarse granules on the euchromatin/heterochromatin borderline, however, suggests that the protein may influence DNA condensation. It has been proposed that the RB protein regulates DNA replication (38). In this connection, it is interesting to mention that the heterochromatin/euchromatin border is an active site of replication in the middle S phase (39).

The distribution of the protein expressed from a retroviral construct was similar to the corresponding normal distribution in RB positive cells. No aberrant nuclear inclusions that sometimes accompany the overexpression were detectable (40).

Materials and Methods
Cell Culture. All of the cell lines were grown at 37°C in 5% CO₂, in Iscove's medium supplemented with 10% heat inactivated fetal calf serum, with 1000 units/ml of penicillin, 100 µg/ml streptomycin, and 500 µg/ml of gentamycin. Peripheral blood leukocytes were obtained from healthy donors. To isolate granulocytes, lymphocytes, and monocytes, 2 ml of blood were diluted with an equal amount of PBS, carefully layered on Hystopaque 1119 (Sigma), and spun at 800 × g for 10 min. The buffy coat was then washed three times with PBS to get rid of the platelets. Lymphocytes were isolated with the same procedure using Lymphoprep (Nycomed AS) gradient centrifugation. To induce blast transformation of the quiescent lymphocytes, 10⁷ cells were resuspended in 25 ml medium; after 24 h, the cells were stimulated with 20 µg/ml phytohemagglutinin (Wellcome Research Laboratories, England) for 72 h.

Production and Purification of Anti-RB Monoclonal Antibody. The EcoRI-HindIII fragment from the human RB cDNA was inserted in the pATH-3 bacterial expression vector, and an E. coli TrpE/human RB fusion protein was produced. The bacteria carrying the recombinant plasmid were induced by β-indolacrylic acid, and insoluble inclusion bodies were released by lysozyme, detergent, and DNase treatment. The inclusion bodies containing the fusion protein were lysed in Laemml buffer, separated on a 7.5% preparative sodium dodecyl sulfate-polyacrylamide gel, and transferred to a Schleicher & Schuell nitrocellulose filter. The band of the fusion protein was cut out and used to immunize mice after homogenization. Ten µg fusion protein were injected s.c., followed by two boosting injections at 3-week intervals. The mouse producing the highest immunoglobulin titer against the fusion protein received a final boost directly.
into the spleen (41), and after 3 days, the splenic lymphocytes were fused with Sp2/0 myeloma cells. The screening of the hybridomas resulted in a clone producing an IgG1 antibody (aRB1C1) which specifically reacted with the human RB protein. The antibody was purified from ascites on a Protein G affinity column (Pharmacia), eluted with 2.5 M MgCl₂, dialyzed against PBS, and concentrated by ultrafiltration.
Immunofluorescent Staining. Monolayer cells were grown on a sterile coverslip. Cells growing in suspension were washed 2 times with PBS and spun on slides with a Cytospin cytocentrifuge at 900 rpm for 10 min. The cells were fixed either in PBS containing 4% paraformaldehyde for 10 min at room temperature and permeabilized with 0.2% Triton X-100 for 5 min or with methanol-acetone (1:1) at −20°C for 5 min and rehydrated with PBS for 30 min. The nonspecific binding sites were blocked by incubating the cells in 3% bovine serum albumin and 0.2% Tween 20 in PBS for 30 min. The first antibody was diluted in this blocking solution (the rabbit polyclonal serum 1:500, the pMG3–245 mouse monoclonal antibody 1:500 (both were generous gifts of Dr. Wen-Hwa Lee), and the aRB1Cl 1:100) and incubated for 30 min, washed three times with PBS, and incubated with FITC conjugated rabbit anti-mouse IgG (Dakopatts), which was preabsorbed on acetone fixed cells and diluted 1:20 in the blocking solution for 30 min.

For double layer fluorescent staining, 1:200 diluted, FITC conjugated swine anti-rabbit IgG (Dakopatts) was added for an additional 30 min. All of the incubations were carried out at room temperature. The second or third antibody solution always contained 0.4 μg/ml Hoechst 33258 (Farbwerke Hoechst AG, FRG) for DNA staining and to monitor the absence of Mycoplasma contamination. Finally, the cells were washed five times with PBS and mounted with balanced salt solution-glycerin (1:1) containing 2.5% 1,4-diazabicyclo-(2.2.2) octane (Sigma) antifading material. The edges of the coverslips were sealed with Universal synthetic glue.

Microscopy, Photo and Image Analysis. The fluorescence was visualized using a Leitz microscope with Wild Leitz MPS 46 photoautomat. For comparison of digital images and fluorescence patterns, the two-color stained preparations were examined in a Zeiss Universal fluorescence microscope equipped with a silicon intensified target video camera. The images transferred from the silicon intensified target camera were digitized, stored and processed in a Zeiss/Kontron IBAS 2000 interactive image analysis computer.

Reconstitution of Weri-Rb-27 and DU 145 cells, the detection of RB protein by immunoprecipitation, and Western blotting were carried out essentially as described earlier (15). The metabolic labeling with [35]Smethionine and immunoprecipitation were carried out according to W-H. Lee (3).

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References


