Genetic Alterations Disrupting the Nuclear Localization of the Retinoblastoma-related Gene RB2/p130 in Human Tumor Cell Lines and Primary Tumors

Caterina Cinti, Pier Paolo Claudio, Candace M. Howard, Luca Maria Neri, Yan Fu, Lorenzo Leoncini, Gian Marco Tosi, Nadir Mario Malardi, and Antonio Giordano

ABSTRACT

The prototypic tumor suppressor gene, the retinoblastoma gene (RB/p105), is mutated in a variety of human tumors. However, to date, mutational data on retinoblastoma family members p107 and RB2/p130 in tumors is lacking. We studied the expression of pRB2/p130 by immunocytochemistry and Western blot analysis in a panel of human osteosarcomas and lymphoid cell lines. Only the lymphoid cell lines showed an abnormally cytoplasmic localization of pRB2/p130, suggesting possible alterations within the region of nuclear localization signaling. We screened these cell lines for genetic alterations of the RB2/p130 gene in the region of the putative bipartite nuclear localization signal (NLS). This region is highly homologous with that of the RB/p105 gene. In addition, we screened four primary Burkitt’s lymphomas for genetic alterations in the RB2/p130 gene. Naturally occurring mutations, which disrupt the putative bipartite NLS, were found in lymphoma cell lines and primary tumors, but not in the osteosarcoma cell lines, where normal nuclear localization of the protein was detectable. Site-directed mutagenesis and transfection assay using NLS mutants displayed markedly reduced biological activity as measured by flow cytometric analysis. This study clearly describes RB2/p130 as an important target for mutations and subsequent inactivation in lymphoma pathogenesis, thus validating that RB2/p130 is a classical tumor suppressor gene.

INTRODUCTION

According to Knudson’s “two hit” hypothesis, many types of human cancers are thought to develop by genetic alterations of putative tumor suppressor genes that have not yet been identified (1). The retinoblastoma gene (RB/p105), whose inactivation is related to neoplastic transformation, is the prototypic tumor suppressor gene (2). The product of the retinoblastoma gene (pRB/p105) is a nuclear phosphoprotein expressed ubiquitously in vertebrates that plays a key role in the negative regulation of cellular proliferation (3). The inhibition of cell growth by pRB/p105 is dependent on the sequences necessary for interaction with the transcription factor E2F as well as with a number of oncoproteins from human DNA tumor viruses such as E1A, T antigen, and E7 (4–7). These interactions also require the biological active form of pRB/p105 in a hypophosphorylated state (8). The phosphorylation status of pRB/p105 oscillates regularly throughout the cell cycle (9, 10). In the G0 and early G1 phases of the cell cycle, hypophosphorylated pRB/p105 sequesters the transcriptional activity of E2F. As the cell cycle continues, pRB/p105 becomes hyperphosphorylated, resulting in the dissociation of pRB/p105 complexes from specific transcription factors, thus allowing for the expression of genes required for progression through the cell cycle (11). A bipartite NLS3 in the COOH terminus of the RB/p105 gene is necessary for pRB/p105 nuclear transport. Its disruption abrogates the interaction of pRB/p105 with the E2F transcription factor as well as the oncoproteins E1A and large T antigen, demonstrating that the NLS present in pRB/p105 is important for its biological activity (12). Based on structural and functional similarity to pRB/p105, the p107 and pRB/p130 proteins form the retinoblastoma protein family (13–16). Maximal identity among the three proteins is found in the conserved pocket region, by which pRB/p105 interacts with E2F and the viral oncoproteins (3). Accordingly, the three nuclear proteins display a phosphorylation status that is cell cycle regulated, reaching a peak of phosphorylation at the G1–S-phase transition of the cell cycle (17). Like pRB/p105, p107 and pRB/p130 also form complexes with the E2F family of transcription factors. However, the temporal order of complex formation varies (18–21). Additionally, p107 and pRB/p130, as well as pRB/p105, act as negative regulators of cell cycle progression, blocking the cells in the G1 phase (22–25). However, the three proteins exhibit unique growth-suppressive properties in a cell type-specific manner, suggesting that although the different members of the retinoblastoma protein family may complement each other, they are not fully functionally redundant (26). Because p107 and pRB/p130 display functional properties similar to pRB/p105, they too may act as tumor suppressor genes. However, to date, there are no examples of naturally occurring mutations of p107. Additionally, p107 maps to a chromosome region that is not frequently found to be cytogenetically altered in human neoplasia (13). On the other hand, RB2/p130 maps to human chromosome 16q12.2, an area in which loss of heterozygosity is found in several human neoplasias (27). Moreover, previous results show a tight inverse correlation between tumor malignancy and pRB/p130 expression in lung cancer, suggesting a direct involvement of pRB2/p130 in the course of this disease (28). Furthermore, induction of pRB/p130 expression suppresses tumor growth in vivo (29). Starting from this background, and taking advantage of the knowledge of the complete genomic structure of the RB2/p130 gene (30), we tested two clusters of tumor lymphoid and osteosarcoma cell lines, together with primary human tumors, for the expression and genomic organization of the RB2/p130 gene.

In this report, we describe a prevailing nuclear exclusion of pRB2/p130 in the lymphoid tumor cell lines that is dependent on the presence of mutations that affect the NLS of pRB2/p130. Neither abhorrent cytoplasmic localization nor NLS-specific mutations were found in the osteosarcoma cell lines.
Materials and Methods

Cell Culture and Transfection. The cell lines were obtained from the ATCC (Manassas, VA) and the European Collection of Animals Cell Cultures. The four human osteosarcoma cell lines (Saos-2, HOS, MG-63, and U2OS from ATCC) were grown at 37°C in DMEM supplemented with 15% fetal bovine serum, whereas CCRF-CEM (acute T lymphoblastic leukemia), Molt-4 (acute T lymphoblastic leukemia), and Duadi (B lymphoblast Burkitt's lymphoma) from the ATCC and Jurkat (leukemia T-cell lymphoblast) from the European Collection of Animals Cell Cultures were grown in RPMI 1640 plus 10% fetal bovine serum.

Saos-2 cells were plated at a concentration of 1 x 10^6 cells/plate in triplicate. After 24 h, the cells were transfected by the standard calcium phosphate precipitation method (25).

Immunofluorescence and Confocal Microscopy Analysis. All cell lines were fixed in 4% paraformaldehyde in PBS (immunoreaction buffer) for 30 min at 37°C, reacted with the primary monoclonal anti-Rb2/p130 antibody (clone 10; Transduction Laboratories, Lexington, KY) diluted in immunoreaction buffer 1:50 for 3 h at 37°C, and then reacted with secondary FITC-conjugated rabbit antimouse IgG (Sigma, St. Louis, MO) for 1 h at 37°C. DNA was counterstained with 4',6-diamidino-2-phenylindole (Sigma) to assess the nuclear domains. The samples were analyzed by a Zeiss LSM410 (Carl Zeiss) confocal laser scanning microscope equipped with a 100x oil immersion lens (numerical aperture = 1.4) and a 488/514 nm argon laser. Image acquisition, recording, and filtering were performed on the z-series of confocal data (stacks) by an Indy 4600 graphic workstation (Silicon Graphics) as described previously (31).

Western Blot Analysis. Whole cell lysates were prepared by resuspending cell pellets in 200 μl of lysis buffer (50 mM Tris- HCl, 5 mM EDTA, 250 mM NaCl, 50 mM NaF, 0.1% Triton, 0.1 mM Na3VO4, and protease inhibitors 1 mM phenylmethylsulfonyl fluoride and 1 μg/ml aprotinin and leupeptin). The lysates were cleared by centrifugation for 15 min at 13,000 g at 4°C, and total protein extracts were determined (25). The protein (40 μg) was denatured by boiling in 2x Laemmli buffer [62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and bromophenol blue] and size-fractionated by electrophoresis in 6% SDS-polyacrylamide gel. The electrophoretic transfer of the protein to a polyvinylidene difluoride membrane (Millipore) was performed in 3-(cyclohexylamino)propanesulfonic acid and 2% methanol (pH 11). The membrane was blocked with 5% fat-free dried milk in TBS-T buffer [2 mM Tris, 13.7 mM NaCl, and 0.1% Tween-20 (pH 7.6)] and incubated with the primary monoclonal antibody (Transduction Laboratories) at a dilution of 1:500 in 3% milk. After several washes, the membrane was incubated with antimeasure antibody coupled with horseradish peroxidase (Amersham) for 1 h and detected using the ECL detection system (Amersham). The immunoreactive bands were detected under exposure to Kodak X-OMAT film. DNA was stained with 0.1 μg/ml of ethidium bromide, and DNA bands were quantified by densitometry. The bands were cut from the gels. DNA was purified using the QIAquick gel extraction kit (Qiagen, Santa Clarita, CA) and used for automated DNA forward and reverse sequencing using dye terminator reaction chemistry for sequence analysis on the Applied Biosystem Model 373A DNA sequencer. The forward and reverse sequences were repeated more times for each PCR product.

PCR and SSCP Analysis. The PCR reaction mixture (50 μl) contained genomic DNA at a final concentration of 4 ng/μl, 0.2 mM of each of the four deoxynucleotide triphosphates, 2 units of Klen Taq (Ab Peptides), and the intron primer pairs (exon 19, 5'-AGGCTCATACACGACAGGTGT-3', exon 19 reverse primer, 5'-GCTTAGTACCTCTTGAAAGGC-3'; exon 20, 5'-GAGGTAGATTATATCCATCAGG-3'; exon 20 reverse primer, 5'-GTCGAAATCTGCTCTATCAG-3'; exon 21, 5'-GGTTGAGAAGCAGTAC-3'; exon 22, 5'-GGTTGAGAAGCAGTAC-3'; exon 23, 5'-GTCGGCTAGTGTTTGTTTATG-3'; exon 24, 5'-GTCGGCTAGTGTTTGTTTATG-3'; exon 25, 5'-GTCGGCTAGTGTTTGTTTATG-3'; exon 26, 5'-GTCGGCTAGTGTTTGTTTATG-3'; and two adenosine residues just before the BanHI site to keep the heterologous fusion protein between the bipartite NLS and the NH2 terminus of the EGFP protein in frame. The oligonucleotides were annealed and ligated into the HindIII and BanHI restriction sites of the pEGFP-N1 expression construct to form the pEGFP-N1-NLS construct. pEGFP-N1-NLS-NQ1, pEGFP-N1-NLS-NQ2, and pEGFP-N1-NLS-NQ1&2 were constructed as described previously, except that the oligonucleotides synthesized encoded point mutations that altered amino acids Lys1090 to Asn and Arg1093 to Gln in the first site and Lys1090 to Asn and Arg1093 to Gln in the second site, and the Lys-to-Asn and Arg-to-Gln mutations were combined in both sites, separately. The point mutations in the bipartite NLS of full-length Rb2/p130 cDNA were performed by a combination of elongation PCR technique developed in our laboratory that allows site-specific mutagenesis (33). Wild-type Rb2/p130 cDNA with an exogenous HA epitope at the COOH terminus (pcDNA3-Rb2/p130-HA) as well as with the HA epitope and the c-myc major NLS (HAN) at the COOH terminus (pcDNA3-Rb2/p130-HAN) were used as the PCR template. The pcDNA3-Rb2/p130-3 constructs contained the equivalent Lys-to-Asn and Arg-to-Gln mutations in the bipartite NLS as described above in the first site (NLS-NQ1), the second site (NLS-NQ2), or both (NLS-NQ1&2), with either the HA or HAN epitope, as indicated. The pcDNA3-Rb2/p130-3-WT and pcCMV-CD20 constructs, which express the wild-type Rb2/p130 without any epitope tags and the interleukin 2 receptor, respectively, were described previously (25).

Flow Cytometry Analysis and Colony Formation Assays. Flow cytometry analysis (FACS) was carried out according to the procedure described previously (25). Ten μg of DNA (pcDNA3, pcDNA3-Rb2/p130-WT, pcDNA3-Rb2/p130-HA, pcDNA3-Rb2/p130-HAN, pcDNA3-Rb2/p130-NLS-NQ1-HA, pcDNA3-Rb2/p130-NLS-NQ2-HA, pcDNA3-Rb2/p130-NLS-NQ2-HAN, pcDNA3-Rb2/p130-NLS-NQ2-HAN, and pcDNA3-Rb2/p130-NLS-NQ2-HAN) were cotransfected with 2 μg of pcMV-CD20. Eighteen h after transfection, the cells were washed twice with 1× PBS and once with culture medium and incubated with fresh medium at 37°C. The cells were collected with 1× PBS and 0.1% EDTA at 48 h, processed for FACS analysis by incubation with the FITC-conjugated anti-CD20 monoclonal antibody (Becton Dickinson, Franklin Lakes, NJ), fixed in 70% ethanol, stained with propidium iodide, and treated with RNase A, as described previously (25). FACS analysis was performed on a Coulter Elite apparatus, and data from 1 × 10^6 CD20-positive cells were used to determine the cell cycle distribution of the selected cells. The colony formation assay was performed by transfecting triplicate dishes of Saos-2 cells with 10 μg of the indicated DNAs according to the procedure described previously (25). Saos-2 cells were selected with 800 μg/ml G418 for 3 weeks and then stained with 1% methylene blue in 50% ethanol.

Detection of EGFP and EGFP-Fusion Proteins. Sterile glass coverslips were placed into 10-cm tissue culture dishes, and Saos-2 cells were plated 1 × 10^6 cells/dish. Forty-eight h after transfection with the indicated pEGFP-N1 (Clontech, Palo Alto, CA) constructs and fusion proteins, cells were washed three times with 1× PBS and fixed directly on coverslips in freshly made 4% paraformaldehyde for 30 min at room temperature. Cells were then washed twice with 1× PBS, counterstained with 0.04 mg/ml propidium iodide in 1× PBS, mounted onto a glass microscope slide with a drop of PBS, and sealed with rubber cement. Slides were examined with a
Leitz Wetzlar fluorescence-equipped microscope. Images for illustration purposes were obtained using a cooled charge-coupled device camera (Princeton Instruments, Trenton, NJ). The images of the EGFP and EGFP-fusion proteins were superimposed on those of the propidium iodide staining.

RESULTS

Expression of pRb2/p130 in Tumor Cell Lines. To study the status of pRb2/p130, the expression of the protein was determined by immunocytochemistry in four osteosarcoma cell lines and four lymphoid tumor cell lines as well as in normal peripheral blood cells using a monoclonal antibody against the NH2-terminal region of pRb2/p130.

As depicted in Fig. 1, all of the lymphoid tumor cell lines show a prevailing localization of the protein at the cytoplasmic level (Fig. 1, a–d), whereas normal human lymphocytes (Fig. 1e) used as a control and the osteosarcoma cell lines (Fig. 1, f–i) all exhibit an exclusive nuclear localization of pRb2/p130.

Because mutations within the bipartite NLS in the COOH terminus of RB/p105 abrogate nuclear localization in certain tumors, we hypothesize that the cell lines that showed a cytoplasmic localization of the pRb2/p130 protein may harbor mutations disrupting the RB2/p130 NLS.

Mutational Screening. In pRb2/p130, like pRb/p105 (12), a putative NLS consisting of two clusters of basic residues separated by a stretch of amino acids is present in the COOH terminus of the protein. This sequence is highly homologous with the pRb/p105 NLS (Table 1) (in bold). In addition, the majority of the point mutations in RB/p105 are located in the B domain and COOH-terminal region (34). To verify whether or not the intracellular distribution of the pRb2/p130 protein depends on mutations that affect NLS motifs differently, we studied the structure of exons 19–22 of the RB2/p130 gene that encode the B domain and the COOH terminus of the protein, where the putative NLS is located (30). Genomic DNA sequences from coding exons 19–22 were amplified and screened for mutation by SSCP analysis. The direct PCR products were sequenced to identify the actual mutations. To reduce artifactual misincorporation generated by Taq polymerase, we used high-fidelity Taq, and forward and reverse sequences were repeated three times for each sample using PCR products derived from different amplifications. Human placental DNA and DNA from normal peripheral blood lymphocytes were used as controls for PCR reactions, SSCP analysis, and sequences. CCRF-CEM cells showed a homozygous insertion in exon 21, and Daudi and Molt-4 cell lines showed a heterozygous insertion that caused the loss of the bipartite NLS resulting from a shift in the coding frame with a consequent stop codon upstream of the NLS present in exon 22 (Table 2). The Jurkat cell line showed two heterozygous mutations located at the end of exon 21 that caused the loss of the two serines upstream of the NLS. Moreover, two other heterozygous mutations inside the first and second regions of the bipartite NLS were present in this cell line (Table 2). These compound mutations caused the loss of nuclear localization of the protein as shown by immunocytochemistry (Fig. 1).

None of the osteosarcoma cell lines demonstrated any insertions or mutations that could alter the putative NLS, even if point mutations in exons 19, 20, and 21 were identified (Table 2).

To rule out the hypothesis that mutations of the RB2/p130 gene are a product of cell growth in culture, we performed the same genetic analysis on primary Burkitt’s lymphomas positive for EBV. The mutations and insertions present in four primary lymphomas were the same as those found in the Daudi cell line, which is derived from a Burkitt’s lymphoma (Table 2).

Because some of the mutations found in the lymphoid cell lines caused a premature stop codon, predicting a shorter transcription product with respect to wild type, we performed a Western blot analysis to detect the molecular weight of the pRb2/p130 protein in these cell lines.
Western Blot Analysis. Western blot analysis of the cell lysates revealed the wild-type phosphorylated and hypophosphorylated forms (M, 130,000/M, 120,000) of the protein in the osteosarcoma cell lines (Saos and Hos) and in three of the lymphoid cell lines examined (Jurkat, Daudi, and Molt-4). One band at a different molecular weight with respect to the wild type is detectable in the same lymphoid cell lines (Fig. 2). In the Jurkat cell line, only the unphosphorylated form of M, 120,000 was present because two heterozygous mutations upstream of the NLS changes two serines into two arginines, altering two putative sites of phosphorylation (see Table 2). Therefore, mutations resulting in a persistent hypophosphorylated form of the protein should result in an enhanced growth suppressive activity, but the concomitant presence in this cell line of two point mutations in the bipartite NLS prevents this activity, confining the protein to the cytoplasm. An abnormal band at M, 116,000 was present in CCRF-CEM, Daudi, and Molt-4 cells, resulting from a guanosine or cytosine insertion, respectively, that caused a frameshift in the sequence and a premature stop codon with a protein of predicted molecular weight of 116,000. In addition, the presence of the M, 130,000/M, 116,000 and M, 130,000/M, 120,000/M, 116,000 bands in Daudi and Molt-4 cells, respectively, was the result of different heterozygous mutations in these cell lines that disrupt the phosphorylation status in a different way (see Table 2). In fact, these mutations also changed the amount of serines and threonines that could be phosphorylated.

Identification of the pRB2/p130 NLS. To determine the functional consequences of disruptions in the putative NLS of pRB2/p130, we first determined whether or not this region can serve in and of itself as a NLS. The putative NLS of pRB2/p130 from amino acids 1082–1102 was fused to the NH2 terminus of EGFP in the pEGFP-N1-NLS expression vector, which expresses a human codon-optimized, red-shifted green fluorescent protein that can be fused to heterologous proteins serving as a fluorescent tag (Clontech). EGFP is a low molecular weight protein that lacks any localization signal and is equally distributed in the nuclear and cytoplasmic compartments (Fig. 3B). Point mutations were also constructed in the first region of the putative bipartite NLS, resulting in a change of amino acids Lys1082 to Asn and Arg1083 to Gln (pEGFP-N1-NLS-NQ1), and in the second site, resulting in a change of amino acids Lys1100 to Asn and Arg1102 to Gln (pEGFP-N1-NLS-NQ2), as well as the combination mutations in both bipartite sites (pEGFP-N1-NLS-NQ1&2). This was done because similar point mutations in the downstream sequences of the bipartite NLS of pRB/p105 (12) and nucleoplasmin disrupted nuclear localization (35). The plasmids were transfected into Saos-2 cells, and the locations of the ectopically expressed proteins were determined by fluorescence microscopy. Saos-2 cells were chosen because pRB2/p130 is found to be exclusively nuclear in these cells, confirming that they do not harbor any mutations in other proteins that may affect nuclear shuttling directed by this region. The cells were counterstained with propidium iodide. As shown in Fig. 3, the level of background green fluorescence was determined to be nonsignificant in mock-transfected cells (Fig. 3A). In pEGFP-N1-transfected cells, EGFP was expressed ubiquitously in the cell (Fig. 3B). Fusion of the wild-type putative bipartite NLS to EGFP localized expression exclusively in the nucleus (Fig. 3C). EGFP fused to point mutations in either the upstream region (Fig. 3D) or downstream region of the bipartite NLS (Fig. 3E) resulted in primarily nuclear expression. Mutations in both bipartite sites resulted in an expression pattern

Table 2 Mutations in lymphoid cell lines, primary Burkitt’s lymphomas and osteosarcoma cell lines determined by DNA sequence analysis

<table>
<thead>
<tr>
<th>Protein</th>
<th>Mutation</th>
<th>Coding effect</th>
<th>Localization (bp)</th>
<th>Codon</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human pRB/p105</td>
<td>C→G</td>
<td>Pro→Arg</td>
<td>3029 (exon 20)</td>
<td>1010</td>
<td>Homozygous</td>
</tr>
<tr>
<td>Human pRB/p130</td>
<td>1082-KRSLREINMIRGEFTKRR-1102</td>
<td>14–16</td>
<td></td>
<td></td>
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![Fig. 2. Western blot analysis of whole cell lysates using a monoclonal anti-pRB/p130 antibody that recognizes the NH2-terminal region of pRB/p130. The M, 130,000 and M, 120,000 wild-type bands of the hyperphosphorylated and hypophosphorylated forms are shown. The M, 116,000 mutant form derived from the frameshift insertion induced by the insertion of guanosine (G) or cytosine (C) is also present.](image)
without any nuclear targeting, and the fusion protein equilibrated between the nuclear and cytoplasmic compartments (Fig. 3F) because it was of such low molecular weight. This was the same expression pattern seen with EGFP alone (compare Fig. 3, B and F). Therefore, this region of pRb2/p130 serves as a bipartite NLS where both the upstream and downstream signals can independently dictate nuclear expression. Only combined mutations in both the bipartite sites resulted in a complete loss of exclusive nuclear expression. This is consistent with the mutational data from the lymphoid cell lines, where both sites of the bipartite NLS were disrupted by insertions or by point mutations.

**Functional Consequences of NLS Disruption.** The effects of mutations on the growth-suppressive function of pRb2/p130 were analyzed by recreating the NQ point mutations by PCR-based site-directed mutagenesis in the full-length pRb2/p130 protein in the pcDNA3 mammalian expression vector that drives gene expression by the constitutive CMV promoter. Each of the constructs was tagged at the COOH terminus with a single HA epitope (HA tag from *Hemophilus influenzae*) so that exogenous expression could be distinguished from that of the endogenous protein. Each of the mutants and wild-type plasmids was expressed at approximately the same level, as determined by immunoprecipitation and Western blot analysis with the HA epitope (data not shown). Saos-2 cells were transfected with the mutant plasmids as well as with vector alone and with plasmids expressing the wild-type pRb2/p130 protein as controls, and their effects on cellular proliferation were measured by FACS analysis.

The wild-type Rb2/p130 cDNA (pcDNA3-Rb2/p130) with or without an exogenous HA epitope at the COOH terminus (pcDNA3-Rb2/p130-HA) as well as with the HA epitope and the c-myc major NLS (HAN) at the COOH-terminus (pcDNA3-Rb2/p130-HAN) in the pcDNA3 vector were used. Additionally, the NLS mutants (NLS-NQ1-HA, NLS-NQ2-HA, or NLS-NQ1&2-HA) and the NLS mutants with the HA epitope and the c-myc major NLS (HAN) at the COOH terminus (NLS-NQ1-HAN, NLS-NQ2-HAN, or NLS-NQ1&2-HAN) were also used. Transient transfections using 10 μg of either plasmid were performed along with 2 μg of pCMV-CD20 encoding for the interleukin 2 receptor. Data from 1 × 10^4 CD20-sorted cells were used to determine the cell cycle distribution of the selected cells. With respect to mock-transfected cells, in which 42% of cells were in G0-G1, 32% in S phase, and 26% in G2-M phase, the amount of G0-G1-blocked cells was increased to about 60% in either pRb2/p130 WT-, pRb2/p130 HA-, and pRb2/p130 HAN-transfected cells. Mutations within either the upstream or downstream region of the bipartite NLS had no significant effect (G0-G1 about 60%) on the growth-suppressive activity of pRb2/p130 (pRb2/p130 NQ1-HA, pRb2/p130 NQ1-HAN, pRb2/p130 NQ2-HA, and pRb2/p130 NQ2-HAN). Their expression...
still led to a G₀-G₁ phase growth arrest that was consistent with the localization data, demonstrating that each of the bipartite signals can independently direct nuclear transport (Fig. 3, D and E). On the contrary, the combined mutations in both bipartite sites (pRb2/p130 NQ1&2-HA) almost completely abolished the growth-suppressive activity of pRb2/p130 because the amount of cells in G₀-G₁ was identical to that of mock-transfected cells (G₀-G₁ about 42%). Addi-

Table 3 Colony assay: average number of G418-selected colonies with respect to the vector, ±SD

<table>
<thead>
<tr>
<th>Vector</th>
<th>pRb2/p130 WT</th>
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<tbody>
<tr>
<td>pRb2/p130-HA</td>
<td>108.3 ± 2.5</td>
</tr>
<tr>
<td>pRb2/p130-HA</td>
<td>114.6 ± 10.4</td>
</tr>
<tr>
<td>NLS-NQ1-HA</td>
<td>140.6 ± 9.3</td>
</tr>
<tr>
<td>NLS-NQ2-HA</td>
<td>140.6 ± 10.4</td>
</tr>
<tr>
<td>NLS-NQ1&amp;2-HA</td>
<td>145.6 ± 7.0</td>
</tr>
<tr>
<td>NLS-NQ1&amp;2-HAN</td>
<td>131.3 ± 5.8</td>
</tr>
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</table>

The results of this investigation indicate the presence of naturally occurring mutations in the COOH-terminal region of the RB2/p130 gene that may inactivate the pRb2/p130 protein and lead to tumor formation. An altered intracellular distribution of pRb2/p130 was found in lymphoid cell lines that depended on the presence of mutations that disrupted the bipartite NLS. The insertions located upstream of the bipartite NLS and the point mutations of the serines immediately upstream of the first region of NLS and inside the two regions of NLS abolished nuclear localization of the protein in these cells, as confirmed by immunofluorescence in confocal laser scanning microscopy and by in vitro site-directed mutagenesis. Transfections of wild-type full-length (open reading frame) RB2/p130 and of a series of NLS mutants showed that the bipartite NLS is necessary for exclusive nuclear expression and growth-suppressive activity of pRb2/p130. Similar mutants of RB/p105 have been shown to display reduced growth inhibition in vitro, most likely due insufficient levels of the protein in the nucleus (12). A similar phenomenon may also occur for the pRb2/p130 cytoplasmic mutants that may predispose the cells to neoplastic transformation and/or give the cells a more aggressive or enhanced malignant phenotype. This is supported by immunohistochemical studies in lung cancer in which the grade of the tumor inversely correlates with the expression level of pRb2/p130 (28).

The functional significance of the other mutations we found in osteosarcoma cell lines remains to be determined, even if they do not involve the NLS. Moreover, some mutations of the RB2/p130 gene were found in primary Burkitt’s lymphomas as well as in tumor-derived cell lines. These results demonstrate that the genetic alterations observed in tumor cell lines are unlikely to be purely a result of cell growth in culture.

While this work was in progress, the loss of pRb2/p130 in a small cell lung carcinoma cell line was reported (36). This result supports the possible involvement of RB2/p130 gene alterations in lung cancer formation and/or progression (28) and strengthens the data on the genetic alterations of the retinoblastoma-related gene RB2/p130 that we found in different human tumors. In conclusion, these data clearly demonstrate genetic alterations in the RB2/p130 gene in tumor cell lines as well as in primary tumors, suggesting that alterations not only in the RB/p105 gene but in the RB2/p130 gene as well may contribute directly to the initiation and/or progression of the development of human lymphomas and leukemias. pRb2/p130 acts as negative regulator of cell cycle progression, blocking the cell in the G₁ phase, and pRb2/p130 induction suppresses tumor growth (29), as does pRb/ p105, and its inactivation is fundamental for tumor progression. The members of the retinoblastoma protein family exhibit different growth-suppressive properties in specific cell lines and are not functionally redundant. Each member may play a unique and complementatory role necessary to suppress neoplastic transformation.

ACKNOWLEDGMENTS

We thank Dr. Alfredo Ciccodicola for assistance in DNA sequencing, Dr. Erminia Mariani for providing lymphoid cell lines, Dr. Alfonso Baldi for the characterization of the genomic structure of RB2/p130, and Dr. Alessandro Matteucci for the Western blot analysis.

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