p130/pRb2 Has Growth Suppressive Properties Similar to yet Distinctive from Those of Retinoblastoma Family Members pRb and p107

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Abstract

The retinoblastoma tumor suppressor gene product, as well as its related protein p107, has been shown clearly to exert its growth suppressive effects in a cell cycle dependent manner. In this study we demonstrate that the introduction of our recently cloned Rb family member p130/pRb2 causes growth arrest in three tumor cell lines. In addition, in the nasopharyngeal carcinoma derived cell line HONE-1, we identified a low level of expression of p130/pRb2, possibly due to gene rearrangement, and a drastic reduction in proliferation upon introduction of a constitutive active p130/pRb2 complementary DNA clone. Furthermore, we were able to dissect distinct properties of the Rb family by demonstrating that p130/pRb2 inhibits proliferation of the glioblastoma cell line T98G, which is resistant to the growth suppressive effects of both pRb and p107. Our studies demonstrate that the Rb family proteins identified to date may complement each other but they are not fully functionally redundant.

Introduction

According to the two-hit hypothesis of Knudson (1), the development of several human cancers is thought to involve loss of heterozygosity of putative tumor suppressor genes, several of which are not yet identified (2). Many forms of malignancies have been linked to mutations in the tumor suppressor gene retinoblastoma RB. The nuclear phosphoprotein pRb serves as a negative regulator of cell cycle progression, at least in some specific cell types. In cell lines which lack a functional pRb protein, restoration of pRb function by either microinjection of the protein in early to mid-G1, or transfection of the cDNA3 arrested cell growth in G1 (3–5). This inhibition of cell growth by pRb is dependent on the sequences necessary for the interaction of pRb with the transcription factor E2F, as well as a number of oncoproteins from human DNA viruses such as E1A, T antigen, and E7 (6–9). Binding of pRb to this set of associated proteins occurs at a specific pocket region in pRb. This pocket region is shared by two additional E1A associated proteins and has lead to the identification of two members of the Rb family, p107, and p130/pRb2 form complexes with E2F (6, 16–18). However, the main form of E2F detected in G0/G1 in primary mouse fibroblasts is E2F1 complexed to p130/pRb2 which is then substituted by p107-E2F1 complexes in late G1 (19). In addition to these differences there is no evidence to support the notion that p107 is normally a tumor suppressor. To date there are no examples of naturally occurring mutations of p107, despite the testing of several hundred human tumor samples and cell lines4; in addition, p107 has been mapped to a chromosome region not commonly found to be cytogenetically altered in human neoplasias (10). Furthermore, the expression of p107 in cell lines derived from retinoblastoma tumors implies that p107 is unable to complement the lack of pRb and to suppress tumor formation (20). p130/pRb2, however, has been mapped to human chromosome 16q12.2, an area in which deletions have been found in several human neoplasias including breast, ovarian, hepatic, and prostatic cancers which is in support of an involvement of the p130/RB2 gene in human cancer as a tumor suppressor gene (21).

To test the hypothesis that pRb2/p130 has tumor suppressive properties and that its lack of expression or function is involved in tumor formation we began to investigate the effects of p130/pRb2 on cellular proliferation and tested several tumor samples and cell lines for the expression and genomic organization of p130/pRb2. In this study we demonstrate that constitutive expression of p130/pRb2 suppresses proliferation of several tumor cell lines. Furthermore, in the nasopharyngeal cell line HONE-1, there is a drastic reduction in the expression level of p130/pRb2, while that of the other Rb family members remain at consistently elevated levels, associated with a potential structural alteration of the p130/RB2 gene locus. This implies a possible involvement of the p130/RB2 gene in nasopharyngeal carcinogenesis which is a rare disease in most parts of the world; however, the disease has a racial and geographical distribution. The people of Southern China are among those who deviate from the low risk profile so much that nasopharyngeal carcinoma is the most common cancer in the city of Canton and constitutes 32% of all cancer (22, 23).

Materials and Methods

Plasmid Constructs. The putative full length clone was inserted in the expression vector pCDNA3 either in the sense (pCMVpRb2-S) or the antisense orientation (pCMVpRb2-AS).

Cell Culture and Transfection. The human tumor cell lines Saos-2 (osteosarcoma), T98G (glioblastoma), and HONE-1 (nasopharyngeal) were used.
Saos-2 cells were purchased from the American Type Culture Collection. T98G cells (24) and HONE-1 cells (25) were described previously. Saos-2 cells were cultured into Dulbecco's modified Eagle's medium supplemented with 10% FBS, l-glutamine, sodium pyruvate, and penicillin-streptomycin. T98G cells were cultured into minimal essential medium with Earl's salt, supplemented with 10% FBS, l-glutamine, basal medium-Eagle's vitamin solution, and penicillin-streptomycin. HONE-1 cells were cultured into E-minimal essential medium with Earle's salt, supplemented with 15% FBS, l-glutamine, and penicillin-streptomycin. All the cell lines were transfected by the standard calcium-phosphate precipitation method (26). DNA precipitates were left on the cells for 12 h. Cells were then washed with phosphate buffered saline and cultured in fresh media.

Colony Formation Assay. The colony formation assay was performed as described (15) and selection was performed using G418. Saos-2 cells were selected with 800 μg/ml, T98G cells with 600 μg/ml, and HONE-1 cells with 1000 μg/ml of the antibiotic for 3 weeks. Colonies were washed in phosphate buffered saline and stained with methylene blue in 50% ethanol for 10 min.

RNA Extraction, Northern Blot Analysis, and RT-PCR. Adult and fetal human tissue blots from Clontech were used. Each lane contained 2 μg of polyadenylated RNA. An α-CTP random primer labeled p130 cDNA probe (nucleotides 1–1032) was used for the blot hybridization following the manufacturer's instructions.

Cytoplasmic RNA was extracted utilizing the RNAzol B method (CINNA/BIOTECH, Friendswood, TX) from the human nasopharyngeal cell line HONE-1 and after homogenization from frozen human normal adenoid tissue. All human tissues used were the kind gift of the Department of Surgical Pathology of the First University of Naples. RNAs were quantitated spectrophotometrically and their integrity was confirmed by fractionation of 1 μg of RNA on 1% agarose gel with ethidium bromide staining. Twenty μg of RNA from each sample were subjected to electrophoresis in triplicate through a 1% denaturing agarose gel containing formaldehyde. RNAs were transferred overnight onto an Immobilon N (Millipore) nylon membrane with 20 × SSC and RNA was UV cross-linked onto the membrane. The membrane was prehybridized at 42°C in a solution containing 5 × standard saline-phosphate-EDTA; 10 × Denhardt's solution; 100 μg/ml of fresh denatured, sheared salmon sperm DNA; 50% formamide; and 2% SDS for 12 h. RNAs were hybridized with α-CTP random primer labeled cDNA probes using 3 × 10⁶ cpm/ml. The probes representing the 5' region of the genes were purified by Gene Clean method (Bio101); the size of the fragments was 1038 base pairs for p105/Rb, 1293 base pairs for p107, and 1032 base pairs for p130/Rb2. Filters were also probed with β-actin to normalize the signal detected. After overnight hybridization, blots were washed in 2 × SSC-0.2% SDS twice at room temperature for 10 min and then in 0.1 × SSC-0.1% SDS three times at 42°C for 20 min and exposed to a Kodak X-ray film at −80°C with the aid of an intensifying screen for different time periods (p105, 3 h; p107, overnight; p130, 10 days; β-actin, 1 h), since the intensity of the signals was different.

RT-PCR analysis was performed as described previously (27).

Results and Discussion

RB2/p130 Expression in Normal Human Tissues. Expression of the p130/ RB2 gene in various human tissues was assessed by Northern blot analysis. A prominent band of 4.85 kilobases and a less abundant band of 7.5 kilobases exhibited ubiquitous expression in normal human tissues (Fig. 1). The faster migrating band of 4.85 kilobases agrees with the length of the cDNAs. The slower band of 7.5 kilobases could represent alternative splicing of the precursor mRNA, unprocessed RNA, or alternative polyadenylation. Alternative mRNA splicings are likely to be an important regulatory mechanism in the control of gene expression. A multitude of protein isoforms may be formed from a single precursor mRNA by various alternate splicing choices such as intron retention, exon skipping, alternative 5' splice sites, alternative 3' splice sites, and mutually exclusive exon combinations (29). This choice of splicing pattern may be influenced by physiological conditions, stage of development, or tissue specificity (29–31). The expression level of p130/RB2 was consistently high in both adult and fetal tissues.

RB Family Expression in Nasopharyngeal Cancer Cell Line. The human nasopharyngeal cell line HONE-1 showed a drastically reduced expression level of p130/RB2 as indicated by Northern blot analysis, compared to normal adenoid tissue, after a very long period of exposure (Fig. 2).
which alters the stability and/or the splicing pattern of the RNA or to abilities are supported by the results from Southern blot analysis. pRB and p107 showed high levels of expression in this cell line. The expected size of 4.85 kilobases; D, normalization by β-actin hybridization.

expression level of p105/Rb with the expected size of 4.7 kilobases; B, expression level of p107 with the expected size of 5.9 kilobases; C, a drastically reduced expression level, detected only after a very long period of exposure, of p130/Rb2 with an expected size of 4.85 kilobases; D, normalization by β-actin hybridization.

This signal could be definitively detected only by the more sensitive RT-PCR technique (data not shown). The other Rb family members, pRb and p107, showed high levels of expression in this cell line. Equal amounts of total RNA were loaded for each Northern blot experiment as evidenced by the level of β-actin mRNA used as control. Normal adenoid tissue, i.e., a tissue which belongs topographically to the nasopharyngeal region, demonstrated a high level of expression of p130/pRb2. The reduced RNA levels of p130/Rb2 in HONE-1 cells could be due to a point mutation in the coding region which alters the stability and/or the splicing pattern of the RNA or to a mutation within the promoter region which affects the binding by RNA polymerases and/or its transcriptional modulators. These possibilities are supported by the results from Southern blot analysis. Genomic DNA isolated from HONE-1 cells and human placenta (control) was digested with several restriction enzymes. Identical band patterns were found in blots of DNA digested with SspI, SmaI, and PvuII hybridized with full length Rb2 cDNA probes. However, upon digestion of the DNA with PstI, we reproducibly saw an extra hybridizing band of 5.3 kilobases in HONE-i genomic DNA (Fig. 3).

Further experiments are directed to characterize this possible rearrangement and its relationship to gene down-regulation.

Western blot analysis of HONE-1, HeLa, 293, and ML1 cells showed a low level of p130/pRb2 protein as well as two isoforms of the protein at Mr 120,000 and 130,000 in HONE-1 cells only. These two isoforms could represent differences in the phosphorylation levels. These results indicate that there may be an alteration in the functional capacity of the p130/pRb2 protein, subcellular localization, and/or the half-life of the protein affecting its relative stability in HONE-1 cells.

Effects of p130/pRb2 on Cellular Proliferation. The proteins pRb and p107 when overexpressed in certain cell lines are able to inhibit cell proliferation in a manner which is both cell type and cell cycle dependent (5, 14, 15, 32–34). The importance of pRb activity in cell transformation was evidenced by the reversion of the transformed phenotype in pRb− cells after restoration of a functional pRb (35, 36). To analyze if p130/pRb2 has similar growth suppressive effects, we took advantage of the colony formation assay used previously to assess growth suppressive effects of pRB and p107. The p130/pRb2 expression constructs pCMV-pRb2(S-AS) contained a neomycin resistance marker. G418 was added to the media 48 h after transfection and the cells were cultured in G418-containing media for approximately 3 weeks in order to allow resistant cells to form colonies. This assay was repeated in triplicate three different times for each cell line and the results were consistent each time. The Saos-2, HONE-1, and T98G cells transfected with the p130/pRb2 expression construct consistently demonstrated a dramatic reduction in colony number and in the size of the residual colonies (Fig. 4). Both pRb and p107 have shown previously to suppress cell growth in Saos-2 cells (5, 9).

The suppressive effect in the T98G cell line was most interesting since this cell line contains apparently normal pRB and is resistant to the

This result indicates a possible p130/Rb2 gene rearrangement, an occurrence which can alter gene expression. This may be the mechanism for the down-regulation of p130/Rb2 in nasopharyngeal cancer. Further experiments are directed to characterize this possible rearrangement and its relationship to gene down-regulation.

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The suppressive effect in the T98G cell line was most interesting since this cell line contains apparently normal pRB and is resistant to the
suppression effects of both pRb and p107 expression (5) but not of p130/pRb2. This suggests that p130/pRb2 may elicit its effects through a signal transduction pathway, different from that of the other family members pRb and p107.

Is p130/pRb2 an Alter Ego of pRb? The E1A associated proteins p130/pRb2, p107, and pRb share many structural features, especially within the pocket region, where pRb interacts with the transcription factor E2F as well as the viral oncoproteins (E1A, T antigen, and E7). The amino acid comparisons of the three protein sequences suggest a closer relationship of p130/pRb2 to p107 (11). However, the E1A mutant pm928 which binds p300 and p107 but fails to bind pRb and p130/pRb2 is able to initiate DNA synthesis, but the transformation property of the oncoprotein is abrogated (37–39). This implies that p130/pRb2 and pRb may elicit repression of cell cycle progression at similar check points. Recently we have shown that p130/pRb2 is able to repress E2F1 activity upon complex formation and that this activity may be fully restored by E1A and E7 from the high risk papillomavirus type 16 but not by E7 from the low risk virus form which is unable to associate with p130/pRb2.6 The results in this paper give the first insight into the function of the newly cloned p130/pRb2 as a negative regulator of cellular proliferation. Just as with pRb and p107 (5, 9), the overexpression of p130/pRb2 in the Saos-2 cell line elicited an inhibition of proliferation. The introduction of p130/pRb2 in HONE-1 cells, a cell line which expresses p130/pRb at a low level and which shows the potential of a gene rearrangement, caused a significant reduction in cell proliferation and a change in morphology. It has also been reported previously and confirmed here that the HONE-1 cell line expresses the RB gene with normal size and abundance and that no point mutation was detected in the common sites for RB mutations, two independent E1A/large T-binding regions (40). Previous evidence has led to the conclusion that nasopharyngeal carcinogenesis shows no detectable retinoblastoma susceptibility gene alterations (40). Our recent findings hint at a possible involvement of p130/pRb2 in nasopharyngeal carcinogenesis.

Most interestingly, the glioblastoma cell line of T98G which is resistant to the suppression effect of both pRb and p107 (5) demonstrated a drastic reduction in cellular proliferation upon overexpression of p130/pRb2. This suggests that p130/pRb2 may function in a completely different pathway than that of p107 or pRb and that certain mutation(s) within the T98G cells predisposes them to be more sensitive to the effects of p130/pRb2 but not to those of p107 or pRb. An alternate explanation may be that the three proteins may share functional properties while p130/pRb2 has an additional property that p107 and pRb2 are unable to complement in this particular cell line. We are currently investigating the involvement of p130/pRb2 in a Chinese/Cantonese population of nasopharyngeal tumors (23) as well as in various solid tumors by analyzing the genomic organization of the p130/pRb2 gene locus.

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