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Genetics

A single amino acid substitution results in a retinoblastoma protein defective in phosphorylation and oncoprotein binding

(recessive oncogene/small-cell carcinoma/DNA tumor virus/gene transfection)

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ABSTRACT **We have previously identified a small-cell lung cancer cell line (NCI-H209) that expresses an aberrant, underphosphorylated form of the retinoblastoma protein RB1. Molecular analysis of RB1 mRNA from this cell line revealed a single point mutation within exon 21 that resulted in a nonconservative amino acid substitution (cysteine to phenylalanine) at codon 706. Stable expression of this mutant RB1 cDNA in a human cell line lacking endogenous RB1 demonstrated that this amino acid change was sufficient to inhibit phosphorylation. In addition, this cysteine-to-phenylalanine substitution also resulted in loss of RB1 binding to the simian virus 40 large tumor and adenovirus E1A transforming proteins. These results confirm the importance of exon 21 coding sequences and suggest that the cysteine residue at codon 706 may play a role in achieving a specific protein conformation essential for protein–protein interactions.**

The retinoblastoma susceptibility gene (*RB1*) encodes a nuclear phosphoprotein that is expressed in all normal human and mouse cells examined to date (1–4). Mutational inactivation of *RB1* has been tightly linked with susceptibility to developing the pediatric tumor retinoblastoma and has also been associated with a limited group of nonfamilial tumors, including small-cell lung cancer (SCLC) and several types of sarcomas and carcinomas (5–13). Given that loss of RB1 function is associated with neoplastic proliferation, *RB1* has been variously termed an antioncogene, recessive oncogene, or tumor-suppressor gene. To support this hypothesis, introduction of *RB1* into *RB1*$^-$ cells has been reported to suppress some of the parameters of malignant transformation (13, 14).

Another distinct mechanism of *RB1* inactivation has been inferred from studies of neoplastic transformation mediated by a group of DNA tumor viruses including the adenovirus, papovavirus, and papillomaviruses. The transforming proteins of these tumor viruses (E1A, large tumor, and E7 protein, respectively) form specific complexes with a set of host cellular proteins that has recently been shown to include RB1 (15–19). The formation of these viral oncoprotein–RB1 complexes has been proposed to result in loss or attenuation of RB1 activity, thus rendering virus-infected cells devoid of the growth regulatory activity imposed by a fully functional RB1.

In view of the observation that the *RB1* product is constitutively expressed and that both mRNA and protein are long-lived, phosphorylation of RB1 has been suggested as a mechanism to regulate its activity. In support of this model, changes in the phosphorylation state of RB1 have been closely correlated with specific stages of the cell cycle (20–23). Cells arrested in the G_1 phase of the cell cycle or synchronized postmitotic cells were noted to contain predominantly un- or underphosphorylated RB1 (designated $RB1^{dephos}$), whereas gradual phosphorylation of RB1 ($RB1^{phos}$) occurred in synchronized cells just before entry of S phase. Recently, $RB1^{phos}$ molecules have been shown to be dephosphorylated during passage through mitosis, thus replenishing the intranuclear store of $RB1^{dephos}$ before the next G_1 phase (24). Because RB1 is dephosphorylated in resting cells and becomes phosphorylated before S phase, it has been argued that the active (growth repressor) fraction of RB1 is constituted by the subset of dephosphorylated RB1 molecules. This hypothesis is supported by experiments demonstrating that simian virus 40 large tumor antigen specifically complexes with the dephosphorylated members of the RB1 family for binding (25). To date, however, no other changes in RB1 functional activity have been directly correlated with the state of RB1 phosphorylation.

A common approach to the investigation of protein structure–function has been to study the phenotype of *in vivo* mutant products. For familial or sporadic retinoblastoma tumors, the inactivation of the gene (whether by large or subtle structural mutations) results in the complete absence of detectable RB1 (1, 2). In contrast, stable mutant RB1s have been found in a human bladder carcinoma cell line (10), a prostate carcinoma line (13), and several SCLC samples (2, 26). Molecular analysis of mRNA encoding the truncated RB1s in these samples revealed that single exons (either exons 21, 22, or 16) had been deleted from mature RB1 mRNA. With the exception of the prostate carcinoma line DU145, point mutations with the potential for altering splicing signals were identified in the *RB1* genes encoding these aberrant proteins and were presumed to deregulate exon splicing. Characterization of these truncated proteins showed they were defective both in their ability to be phosphorylated as well as to bind to simian virus 40 large tumor and adenovirus E1A transforming proteins. These, and other studies, have begun to delineate regions along the RB1, such as sequences corresponding to exon 16 and especially exons 21 and 22, which appear to be required for its normal activity.

We have now demonstrated in a well characterized SCLC cell line that a single point mutation near the beginning of exon 21, resulting in a cysteine-to-phenylalanine substitution at codon 706, is sufficient to generate an RB1 defective in phosphorylation and oncoprotein binding. This analysis directs attention to a discrete region of the encoded protein and suggests that Cys-706, or immediately adjacent residues, play an important role in normal activity of the RB1.

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Abbreviations: RB1, protein encoded by the retinoblastoma susceptibility gene; SCLC, small-cell lung cancer; PCR, polymerase chain reaction.

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MATERIALS AND METHODS

Cell Lines. Derivation and propagation of the lung cancer cell lines were as described (27).

Western (Immunologic) Blotting. Cellular protein was extracted by lysing ≈5 × 10^6 cells in 1 ml of 50 mM Tris·HCl, pH 7.5/250 mM NaCl/5 mM EDTA/0.1% Nonidet P-40/50 mM NaF/1 mM phenylmethylsulfonyl fluoride (PMSF) containing leupeptin at 10 μg/ml and aprotinin at 50 μg/ml, followed by quick-freezing of the supernatant. Seventy-five micrograms of cellular protein was electroblotted to nitrocellulose paper after separation on 7.5% SDS/PAGE and then allowed to incubate overnight at 4°C with a 1:100 dilution of monoclonal anti-RB1 antibody at 336 μg/ml (PharMingen, San Diego). The immunoblot was subsequently incubated for 4 hr with a 1:100 dilution of polyclonal rabbit anti-mouse antibody (PharMingen) at 1.89 mg/ml, followed by a 2-hr incubation with 2.5 × 10^6 cpm of ^{125}I-labeled protein A and autoradiography.

Immunoprecipitation. Equivalent numbers of lung carcinoma cells were radiolabeled with either [^{35}S]methionine or [^{32}P]orthophosphate, and lysates were prepared as described (2). Immunoprecipitations were performed with antiserum 147 (15). Immunoprecipitates were then collected on protein A–agarose beads (BRL-Life Technologies, Gaithersburg, MD) and subjected to SDS/PAGE, fluorography, and autoradiography.

RNase Protection Analysis. The plasmid construct, pJD18, containing a 1.5-kilobase (kb) *RB1* insert from nucleotide coordinates 1039–2537 in the vector pBluescript KS (Stratagene) was a gift from J. Dunn and B. Gallie (University of Toronto, Ontario, Canada). The *RB1* nucleotide coordinates presented here are as published (1). The vector was linearized with the restriction enzyme *Nco* I (*RB1* coordinate 1681) to generate a uniformly [^{32}P]UTP-labeled antisense RNA probe. Overnight hybridization at 55°C was performed by using 1 × 10^5 cpm of the RNA probe, and 15 μg of total RNA was extracted from tumor cell lines. The hybridization mix was digested with RNase A, extracted with organic solvents, and precipitated with ethanol. The samples were then separated on a 6% urea/acrylamide gel and autoradiographed.

DNA Sequencing of Polymerase Chain Reaction (PCR)-Amplified cDNA. First-strand cDNA was synthesized from 3 μg of total cellular RNA by using a 3′ oligonucleotide primer from *RB1* coordinates 2395–2371 and avian myeloblastosis virus reverse transcriptase under conditions specified by the manufacturer (BRL-Life Technologies). This was followed by the addition of a 5′ oligonucleotide from coordinates 1910–1935 and PCR amplification using *Thermus aquaticus* (*Taq*) DNA polymerase as specified by the manufacturer (Perkin–Elmer/Cetus). Amplified inserts were cloned into pGem4 vectors (Promega), and nucleotide sequence was determined by the dideoxy nucleotide chain-termination method (28). Nucleotide sequence was obtained from three independent PCR-amplification reactions.

Construction of Mutant *RB1*-Containing Plasmid Vectors. PCR-amplified cDNA was obtained from 3 μg of total RNA from cell line H209 by using primers spanning *RB1* coordinate 1910 at the 5′ end and *RB1* coordinate 2960 at the 3′ end. The amplified fragment containing the point mutation was then digested with the unique, internal restriction enzymes *Mlu* I and *Bsm* I (New England Biolabs), and the resulting fragment was purified on an acrylamide gel and ligated into a wild-type *RB1*-containing vector from which the *Mlu* I–*Bsm* I insert had been previously removed. The entire PCR-generated region was subjected to nucleotide sequence analysis to confirm the presence of the specific point mutation and to assure that the encoded open reading frame had not been altered by *Taq* polymerase incorporation errors.

Stable Expression in *RB1*$^-$ Cancer Cell Line. Eukaryotic expression vectors containing either the wild-type or mutant RB1 open reading frame were transfected via lipofection as described by the manufacturer (BRL-Life Technologies) into the *RB1*$^-$ SCLC cell line H187 (unpublished work). G418-resistant single clones were expanded, and whole-cell protein extracts were harvested and subjected to immunoblotting with an anti-RB1 monoclonal antibody as outlined above.

DNA Tumor Virus Oncoprotein Binding. pGem4 vectors containing either the wild-type or mutant RB1 open reading frame were subjected to *in vitro* transcription and translation in the presence of [^{35}S]methionine, as described by the manufacturer (Promega). Immunoprecipitations were performed as described (2) by using either polyclonal anti-RB1 antiserum 147 (15) or monoclonal antibodies against adenovirus E1A M73 (29) or simian virus 40 large tumor antigen pAb416 (30).

RESULTS

In our original report of *RB1* inactivation in SCLC, we observed detectable levels of apparently normal-sized RB1 mRNA in ≈40% of the SCLC cell lines examined (7). Most of the SCLC lines producing RB1 transcripts, however, either lacked or expressed aberrant forms of RB1 (2). These observations have been confirmed (8) and strongly implicate inactivation of *RB1* as an event common to most SCLC tumors.

In the course of our analysis of SCLC tumors, an example of an aberrant RB1 was noted in one tumor culture, NCI-H209 (2). Cell line NCI-H209 has been characterized as a "classic" SCLC line, exhibiting features of neuroendocrine differentiation (27) and also expressing elevated levels of L-MYC mRNA without *MYC* gene amplification (31). We prepared an immunoblot containing denatured cell lysates from H209 and control cells, and incubated the immobilized proteins with a monoclonal anti-RB1 antiserum (Fig. 1*A*). In contrast to the normal dephosphorylated, RB1^{dephos}, and phosphorylated, RB1^{phos}, migration pattern of ≈110–115 kDa seen in protein extracts from normal cells and most tumor samples, H209 cells demonstrated only a single band comigrating with the unphosphorylated, 110-kDa RB1 species. Immunoprecipitation of [^{35}S]methionine-labeled H209 cell lysates with a polyclonal anti-RB1 synthetic peptide serum also resulted in a single discrete species of RB1 that comi-

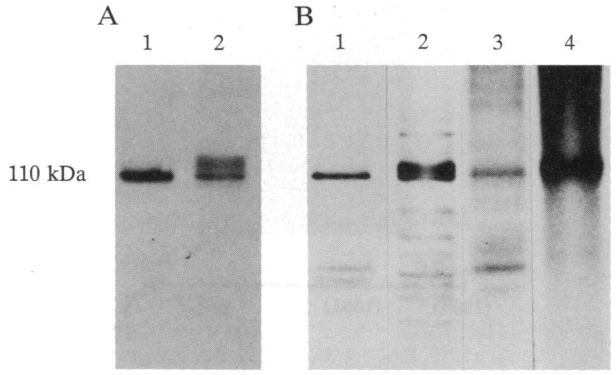

FIG. 1. (*A*) Immunoblot analysis. Seventy-five micrograms of cellular protein extracted from SCLC cell line NCI-H209 (lane 1) and from a control non-SCLC cell line NCI-H522 (lane 2) were immunoblotted to nitrocellulose after separation on 7.5% SDS/PAGE and incubated with an anti-RB1 monoclonal antibody, as described. (*B*) Immunoprecipitation analysis. Anti-RB1 immunoprecipitates of NCI-H209 cells (lanes 1 and 3) or a control lung carcinoma cell line (lanes 2 and 4) were radiolabeled with [^{35}S]methionine (lanes 1 and 2) or [^{32}P]orthophosphate (lanes 3 and 4).

grated on one-dimensional SDS/PAGE with the unphosphorylated form of wild-type RB1 (2) (Fig. 1*B*; lanes 1 and 2). In addition, immunoprecipitations performed on parallel H209 cultures incubated with [^{32}P]orthophosphate showed a marked decrease in detectable phosphorylated RB1 (Fig. 1*B*; lanes 3 and 4).

To determine whether the anomalous gel migration of RB1 in H209 cells was due to a structural alteration of its coding sequence, we performed RNase protection analysis. Using an *in vitro* transcription vector derived from the plasmid p0.9r (32), containing an insert from the 5′ end of the *RB1*-encoded open reading frame (exons 1–9), we observed full-length protection of the antisense RNA probe (consistent with wild-type sequence) in mRNA extracted from H209 (data not shown). In contrast, we found evidence for a potential mutation in H209 cells with the plasmid pJD18. This vector, containing an *RB1* insert spanning from exon 9 to exon 23 (nucleotide coordinate 1039–2537), was linearized at an internal *Nco* I site (coordinate 1681), and a [^{32}P]UTP uniformly labeled antisense RNA probe was generated (Fig. 2). Full-length protection (consistent with a wild-type sequence) was observed using RNA extracted from a non-small cell-lung cancer line (lane 1) and absence of probe protection (consistent with the absence of detectable RB1 transcripts) was observed with RNA obtained from an unrelated SCLC line (lane 2). In contrast, RNase analysis of cytoplasmic RNA from H209 showed two smaller, protected bands (lane 4) mapping the approximate location of a potential nucleotide mismatch. RNA extracted from a lymphoblastoid cell line derived from the normal peripheral lymphocytes of the patient from whom the H209 SCLC tumor originated demonstrated full-length protection consistent with wild-type sequence in the germ-line-transcribed *RB1* (lane 3). The two closely spaced bands sized at ≈400 base pairs (bp) seen in all lanes are "background bands" intrinsic to full-length protection of the probe, as they are also seen when the probe was hybridized to wild-type cloned *RB1* and subjected to a similar analysis (data not shown).

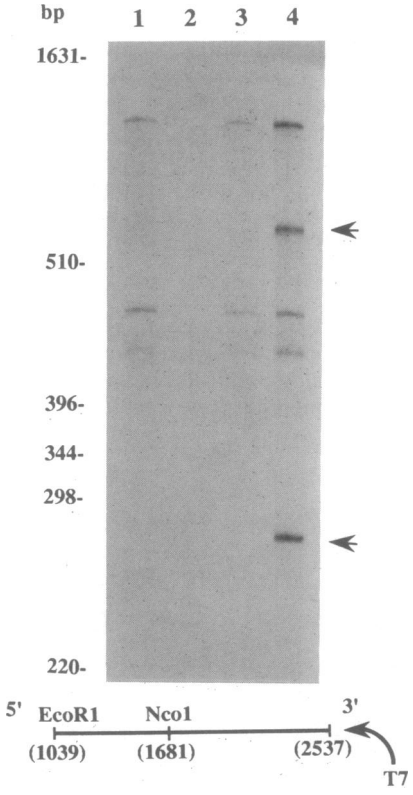

FIG. 2. RNase protection assay. Twenty micrograms of total cellular RNA was extracted from the non-SCLC cell line H522 (lane 1), SCLC lines H187 (lane 2), H209 (lane 4), and a lymphoblastoid cell line (designated in our laboratory as BL-2) derived from the normal peripheral blood of the patient who developed the SCLC tumor H209 (lane 3). The plasmid was linearized at the internal *Nco* I site, and the antisense RNA probe generated spanned *RB1* nucleotide coordinates 2537–1681. Size markers represent pBR322 digested with the restriction enzyme *Hinf*I. Arrows, smaller protected bands mapping the approximate location of a potential nucleotide mismatch.

To identify the specific mutation predicted by the RNase protection analysis, we conducted PCR amplification of first-strand cDNA synthesized from cytoplasmic RNA from H209 cells. Nucleotide sequence analysis revealed a single G → T point mutation at position 2255, corresponding to the location predicted by the RNase protection studies (Fig. 3*A*). The same mutation was seen in seven different *RB1*-containing subclones of H209 cells generated from three independent PCR reactions by using RNA extracted at different cell passages. This confirms that the mutation observed was not a result of an incorporation artifact and also suggests that only the mutated allele is expressed in this cell line. The full-length protection band seen on the RNase protection study (lane 4), therefore, is probably due to incomplete digestion of a single nucleotide mismatch by RNase A. To exclude the possibility of additional somatic mutations, we examined the entire open reading frame of the transcribed *RB1* in cell line H209 by nucleotide sequencing of PCR-amplified cDNA and RNase protection analysis and did not find any other mutations. We then examined the effect this point mutation would have on the predicted encoded amino acid sequence and found a nonconservative amino acid substitution of cysteine by phenylalanine at codon 706 near the beginning of exon 21 (Fig. 3*B*).

To investigate whether this amino acid change is directly responsible for the phosphorylation defect and not the result

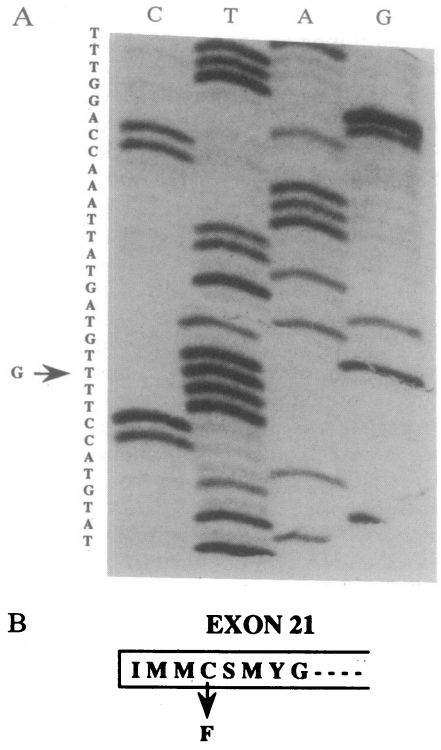

FIG. 3. (*A*) Partial nucleotide sequence of *RB1* transcribed in cell line H209 demonstrating the G → T point mutation. No other mutation was observed through the rest of the encoded open reading frame. (*B*) Partial amino acid sequence of exon 21 in cell line H209 demonstrating the Cys → Phe substitution.

of an RB1 kinase system abnormality in H209 cells, we constructed eukaryotic expression vectors that contained either the open reading frame of the wild-type *RB1* sequence or the mutant sequence as determined in cell line H209. An unrelated SCLC cell line, H187, which had previously been shown to be *RB1*$^-$ secondary to a homozygous structural alteration (7) was transfected with either the wild-type or mutant expression vector. After selection in G418 and expansion of single cell clones, cellular extracts were assayed on immunoblots for the presence of exogenous RB1 (Fig. 4). As predicted, the wild-type *RB1*-containing expression vector generated exogenous RB1 with the expected migration on SDS/PAGE of a normal phosphorylation pattern seen with endogenous RB1. In contrast, the mutant vector resulted in the expression of only the underphosphorylated 110-kDa form as seen *in vivo* with the H209 cells. We have concluded from these results that substitution of Cys-706 → Phe is sufficient to disrupt the phosphorylation of RB1.

Given that the amino acid substitution in H209 was localized to a region of the RB1 thought important for protein–protein interactions (exon 21), we sought to determine whether the H209 RB1 was impaired in its ability to associate with simian virus 40 large tumor antigen or adenovirus E1A. As we have previously shown, association of wild-type RB1 with either oncoprotein will result in coprecipitation of RB1 using antibodies directed against the viral oncoproteins (2, 10, 15). To perform these experiments, wild-type and the H209 mutant RB1 cloned DNA were subjected to *in vitro* transcription and translation in the presence of [^{35}S]methionine. Radiolabeled translation products were then incubated with buffer alone or nonradioactive cell lysates containing either E1A (human 293 cells) or simian virus 40 large tumor antigen (monkey COS cells). After incubation, lysates were challenged with antibodies directed against RB or one of the two viral oncoproteins. As shown in Fig. 5, anti-RB1 antiserum produced identical patterns of immunoprecipitated RB1 from both wild-type and mutant RB1 translation reactions. However, whereas the wild-type protein was capable of noncovalently binding to the transforming proteins of these DNA tumor viruses, the single amino acid change seen in line H209 completely abolishes this protein–protein interaction.

DISCUSSION

Inactivation of *RB1* appears to play a critical role in the genesis of pediatric retinoblastoma tumors and may also participate in the development of other common, adult malignancies. Although the model of the RB1 as a regulator of cell growth is widely accepted, our understanding of the functional role of this protein product is still limited. Previous experiments have shown that the protein is localized to the nucleus, has a half-life of >6 hr, and appears to bind deoxynucleic acids in a nonspecific manner (1, 10). Other features of RB1 are that it can bind to the oncoproteins of at least three DNA tumor viruses (15–19) and that the catalysis of RB1 phosphorylation and dephosphorylation modifications is tightly associated with specific stages of the cell cycle (20–24).

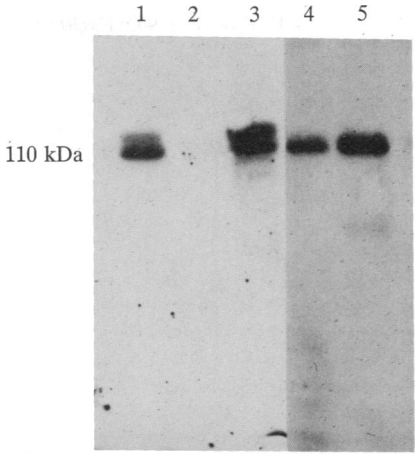

FIG. 4. G418-resistant clones transfected with wild-type and H209 mutant *RB1*-containing expression vectors. Cellular protein (75 μg) was extracted from transfected clones and immunoblotted with an anti-RB1 monoclonal antiserum after electrophoretic separation by SDS/PAGE. Lanes: 1, H187 cells after transfection with wild-type *RB1*-containing expression vector; 2, H187 cells; 3, normal control (non-SCLC line NCI-H522); 4, H187 cells after transfection with H209 mutant RB1 expression vector; 5, H209 cells.

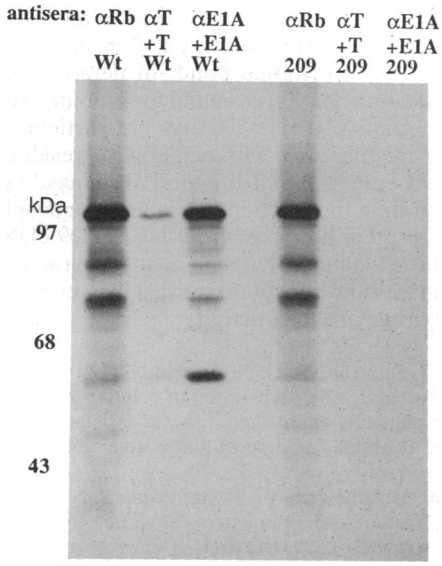

FIG. 5. Immunoprecipitation of RB1 incubated in the presence of large-tumor (T) and adenovirus E1A transforming proteins. Plasmids with the wild-type (Wt) or mutant (209) *RB1* open reading frame were *in vitro* transcribed and translated as described. The labeled RB1 was then incubated with nonradioactive cell lysates containing either SV40 large tumor (+T) or adenovirus E1A (+E1A) proteins followed by immunoprecipitation with anti-RB1 (αRb), anti-large T (αT), or anti-E1A (αE1A) antisera and electrophoretic separation by SDS/PAGE.

One approach to help elucidate more detailed information about the structure–function of this anti-oncogene is to characterize *in vivo* mutant protein products. NCI-H209 tumor cells, producing an aberrant RB1 due to a missense mutation, represents a cell line with a unique type of defective RB1. Previous analyses of truncated RB1s have revealed several mutants lacking specific segments of the encoded open reading frame due to exon-splicing mutations. Although it has been suggested that such aberrant molecules are functionally inactivated through loss of critical residues within the 73 amino acids encoded by exons 21 and 22, it was still conceivable that this region had a propensity, via unknown mechanisms, for mutations inducing splicing errors. The missense mutation identified near the beginning of exon 21 in H209 cells suggests, instead, a functional role for this region of RB1, particularly in the vicinity of codon 706. This residue also lies within an RB1 domain (amino acids 646–772) identified recently by *in vitro* deletion mutants to be required for oncoprotein binding (33). The Cys-706 → Phe substitution appears to simultaneously abrogate RB1 phosphorylation and affinity for viral oncoproteins. As such, we might also conclude that the *RB1* product expressed in these tumor cells is also defective in performing "normal" cellular functions as well. Lack of RB1 phosphorylation alone cannot cause the loss of simian virus 40 large tumor and adenovirus E1A binding because both viral oncoproteins complex with dephosphorylated RB1 (15, 16, 25). That this single amino acid

substitution within exon 21 has such profound effects is consistent with a model where Cys-706 plays a key role, perhaps through a disulfide bond, in determining a three-dimensional conformation essential for protein–protein interactions. Alternatively, Cys-706 may not participate in disulfide bonding and instead, with neighboring residues, may be an important contact point for the RB1 kinase system and RB1–oncoprotein interactions. Given its phosphorylation deficiency, protein produced from NCI-H209 cDNA should prove to be a valuable reagent in the characterization of putative kinases and phosphatases that may interact with and presumably regulate RB1 activity.

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