The Amino-terminal Region of the Retinoblastoma Gene Product Binds a Novel Nuclear Matrix Protein That Co-Localizes to Centers for RNA Processing

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Abstract. The tumor suppressing capacity of the retinoblastoma protein (p110^{RB}) is dependent on interactions made with cellular proteins through its carboxy-terminal domains. How the p110^{RB} amino-terminal region contributes to this activity is unclear, though evidence now indicates it is important for both growth suppression and regulation of the full-length protein. We have used the yeast two-hybrid system to screen for cellular proteins which bind to the first 300 amino acids of p110^{RB}. The only gene isolated from this screen encodes a novel 84-kD nuclear matrix protein that localizes to subnuclear regions associated with RNA processing. This protein, p84, requires a structurally defined domain in the amino terminus of p110^{RB} for binding. Furthermore, both in vivo and in vitro experiments demonstrate that p84 binds preferentially to the functionally active, hypophosphorylated form of p110^{RB}. Thus, the amino terminus of p110^{RB} may function in part to facilitate the binding of growth promoting factors at subnuclear regions actively involved in RNA metabolism.

MUTATIONAL inactivation of the retinoblastoma (RB)[1] susceptibility gene has been found in a variety of human neoplasms; cells derived from these tumors consistently display reduced tumorigenicity following reintroduction of a wild-type RB allele demonstrating the tumor suppressing ability of the encoded protein (Bookstein and Lee, 1991). Growth suppression assays of cultured cells suggest that the COOH-terminal half of the retinoblastoma protein is sufficient for this function. Specifically, microinjection of an amino-terminally truncated form, p56^{RB}, into cells early in the G$_1$ phase of the cell cycle blocks their progression into S phase (Goodrich et al., 1991). These results were confirmed in an independent growth retardation assay (the "flat cell assay") following transfection of various RB constructs (Qin et al., 1992). While these experiments establish the growth suppressing capabilities of the RB protein, a concise mechanistic picture of how this occurs remains elusive.

The RB gene product (p110^{RB}) is a 110-kD nuclear protein (Lee et al., 1987) that is phosphorylated on both serine and threonine residues (Buchkovich et al., 1989; Ludlow et al., 1989; Shew et al., 1989). These modifications occur in a cell cycle–dependent manner (Buchkovich et al., 1989; Chen et al., 1989; DeCaprio et al., 1989), and are hypothesized to be important in negatively regulating the protein. During the G$_1$ phase of the cycle, p110^{RB} is hypophosphorylated and in an active state. At some point later in G$_1$, it becomes hyperphosphorylated, likely by a member of the CDK family of kinases (Lees et al., 1991; Lin et al., 1991), inactivating its growth suppressing function(s). Dephosphorylation of the RB protein occurs during mid-M phase, activating the protein prior to the next cell cycle. Evidence strongly implicates the type 1 protein phosphatase as being critical for this process (Alberts et al., 1993; Durfee et al., 1993; Ludlow et al., 1993).

The RB protein is also known to bind the oncoproteins of several DNA tumor viruses: the adenovirus E1A protein (Whyte et al., 1988), the SV-40 large T-antigen (T-ag) (DeCaprio et al., 1988), and the HPV E7 protein (Dyson et al., 1989). All three of these viral oncoproteins bind preferentially to the hypophosphorylated isoform of p110^{RB} (Imai et al., 1991; Ludlow et al., 1989; Templeton et al., 1991), and require regions of p110^{RB} for binding which are affected by most known naturally occurring mutations (Hu et al.,

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1. *Abbreviations used in this paper*: BCIP, 5-bromo-4-chloro-3-indolyl-phosphate; CSK, cytoskeletal buffer; GST, glutathione S-transferase; NBT, nitro blue tetrazolium; nt, nucleotide; snRNP, small nuclear RNP; RB, retinoblastoma; SC, synthetic complete; T-ag, T-antigen; 3-AT, 3-amino-triazole.

1990; Huang et al., 1990; Kaelin et al., 1990). These domains, the T/E1A domains, lie near the carboxy-terminal portion of the molecule, and are necessary for the growth suppressing activity of the protein. Although precisely how these domains function is unclear, they are required for interacting with a variety of cellular proteins in complexes believed to be essential for the growth-suppressing activity of the RB protein (for review see Goodrich and Lee, 1993).

Like many other nuclear antigens, p110^{RB} is concentrated in multiple foci in the nuclei of intact cells (Szekely et al., 1991; Mancini, M. A., and W.-H. Lee, unpublished results). The highest concentrations of p110^{RB} exist in regions of low DNA density (actively transcribed euchromatin) (Szekely et al., 1991). This heterogeneous distribution suggests that a structure exists for spatially organizing p110^{RB} within the nucleus. The best candidate for such a structure is the nuclear matrix, biochemically and morphologically defined as the chromatin-free, insoluble framework of the nucleus. It is upon this scaffolding that many functional subdomains of the nucleus are organized, including those involved in replication, RNA transcription and splicing (Jackson and Cook, 1988; Nickerson et al., 1993; Carter et al., 1992, 1993). Hypophosphorylated p110^{RB} has also been shown to "tether to the nuclear structure" (Mittnach and Weinberg, 1991), and in fact, under more rigorous conditions can be shown to associate with the nuclear matrix (Mancini et al., 1994). Interestingly, the three viral oncoproteins which interact with p110^{RB} are also found in association with the nuclear matrix (Chatterjee and Flint, 1986; Greenfield et al., 1991; Schrimbeck and Deppert, 1989). The fact that matrix association is specific for the active isoform of p110^{RB}, and is abolished by naturally occurring RB mutations, argues that the protein may normally function, at least in part, within the architectural framework of the nucleus (Mancini et al., 1994; Mittnach and Weinberg, 1991; Templeton et al., 1991). Currently, the mechanism by which p110^{RB} associates with the nuclear matrix is poorly understood, although anchoring through interaction with matrix proteins is a strong possibility. p110^{RB} has been shown to bind lamins A and C in vitro (Mancini et al., 1994; Shan et al., 1992), which may contribute to the preferential binding of hypophosphorylated p110^{RB} to isolated nuclear shells (Templeton, 1992). However, as the spatial distribution of nuclear matrix–associated p110^{RB} is more widespread than that of the lamins (Mancini et al., 1994), it seems certain that the RB protein will interact with additional nuclear matrix factors, perhaps also in a region-specific manner.

Whereas carboxy-terminal domains of p110^{RB} are clearly important in growth suppression (Goodrich et al., 1991; Qin et al., 1992), evidence pointing to a functional role for the amino-terminal region in this process is only now emerging. First, small deletions affecting the amino-terminal segment of p110^{RB} block the ability of transfected RB to inhibit growth in the flat cell assay (Qian et al., 1992; Hinds et al., 1992). Interestingly, these mutants do not qualitatively block the ability of the resultant RB protein to bind the transcription factor E2F, or the E1A protein (Qian et al., 1992), indicating an additional activity is required. More recently, a RB mutation has been isolated from a retinoblastoma tumor, which is predicted to specifically remove only exon 4 (amino acids 126-166) (Hogg et al., 1993). This is the first example of a clinical specimen with an inactivating mutation affecting only the amino-terminal domain. Structurally, two protease-

resistant domains reside in this region of the protein, some portion of which can physically interact with the carboxy-terminal half of p110^{RB} (Hensey et al., 1994). Deletion studies also indicate that amino-terminal sequences are necessary for the hyperphosphorylation of the full-length molecule (Hamel et al., 1990; Qian et al., 1992; Hinds et al., 1992). It is not clear, however, whether this is due to a structural defect, or the loss of important cdc2 sites located therein (Lees et al., 1991; Lin et al., 1991). The severe functional and structural consequences of amino-terminal mutations clearly demonstrate the importance of this domain in the context of native p110^{RB}, and warrant further investigation into the activity of this part of the molecule.

Evidence now suggests that the RB protein exerts its function through interaction with other cellular proteins. To ascertain whether cellular proteins capable of binding to the p110^{RB} amino terminus exist, we conducted a screen using a modified version of the yeast two-hybrid system (Fields and Song, 1989; Durfee et al., 1993). This and other variations have proven effective in isolation of interacting proteins from cDNA expression libraries in the yeast *S. cerevisiae* (Chien et al., 1991; Dalton and Treisman, 1992; Durfee et al., 1993; Yang et al., 1992). The only gene isolated from this screen encodes a novel nuclear matrix protein which interacts specifically with the amino-terminal region of hypophosphorylated p110^{RB}. This protein, p84, localizes to regions of the nucleus known to be sites of RNA processing. This interaction may provide a link between p110^{RB} and RNA processing centers in the nucleus.

Materials and Methods

Strains and Media

Plasmids

Library Screening and Plasmid Recovery

(Breeden and Nasmyth, 1985; Durfee et al., 1993). The time required for color development ranged from 30 min to overnight. Colonies corresponding to positives in this screen were then patched onto a master plate and further analyzed.

Total DNA from yeast was prepared using the method of Hoffman and Winston (1987), and used to transform JA226 via electroporation using a BioRad GenePulser according to the manufacturer's specifications. Transformation were plated on minimal media lacking leucine and containing ampicillin.

Quantitation of β-Galactosidase Activity in Yeast

2.5 ml cultures were grown in the appropriate selecting media to OD_{600} 1.0–1.2. Cells were then prepared and permeabilized as described (Guarente, 1983). For quantitation the same procedure as in Guarente (1983) was followed except cell pellets were resuspended in 900 ml H buffer (100 mM Hepes, 150 mM NaCl, 2 mM $MgCl_2$, 1% BSA, pH 7.0), 100 ml 50 mM chlorophenyl-red-b-D-galactopyranoside (CPRG; Boehringer Mannheim Biochemicals, Indianapolis, IN) was added following permeabilization, and the amount of liberated CPR was determined by OD_{574}.

Sequence Analysis

Clones were sequenced using dideoxy-NTPs and Sequenase 2.0 according to the manufacturer's specifications (United States Biochemical, Cleveland, OH). Sequence analysis and homology searches were performed using DNASTAR software (DNASTAR, Inc., Madison, WI).

Construction of GST Fusions, Protein Preparation, and In Vitro Binding

To construct GST-N5, the N5 yeast plasmid was digested with BglII and the 0.9-kb insert fragment subcloned into the BamHI site pGEX-3X. One resulting clone had the N5 cDNA insert in reverse orientation with respect to the GST coding sequences, and was designated GST-R5N. GST-Tag has been described previously (Durfee et al., 1993). Expression of proteins in *E. coli*, and in vitro assays were identical to those in Durfee (1993).

Antibody Preparation, Eukaryotic Cell Extracts, and Protein Detection

Antisera were raised in female BALB/c mice injected subcutaneously with 80 μg GST-N5 bound to glutathione beads in 75 μl sterile PBS. Mice were boosted with 100 μg GST-N5/beads after 2 wk and again after 2 mo. An additional boost was given 6 wk later, 3 d before spleenic fusion with NS1 cells (American Type Culture Collection, Rockville, MD) according to standard protocol. Fused cells were screened by ELISA against GST-N5 or GST. After three rounds of screening for N5-specific clones, supernatants were collected, evaluated by immunoblotting and immunostaining, and then frozen at −80°C. 5E10 was the most reactive clone and was subsequently single cell cloned.

For antibody preparations which were preabsorbed before use, 10 μg of the appropriate GST fusion protein was incubated with 1 μg of antibody serum in 250 buffer (50 mM Tris, pH 7.4, 250 mM NaCl, 5 mm EDTA, 0.1% NP-40, 50 mM NaF, 1 mM PMSF, 1 μg leupeptin per ml, 1 μg antipain per ml) for 60 min at 4°C. Glutathione-coated Sepharose beads were added, and the mix incubated for an additional 30 min at 4°C while rotating. Following centrifugation, the supernatant was used for immunoprecipitation.

Yeast cell extracts were prepared by growing 5 ml of cells to stationary phase under the appropriate selecting conditions. Cells were then pelleted by centrifugation, and lysed by boiling in loading buffer for 30 min. Aliquots representing equivalent cell numbers (~5 × 10^6 cells) were then separated on 8% SDS-polyacrylamide gels and transferred to Immobilon membranes. Monkey kidney CV-1 extracts were prepared by lysing approximately 1 × 10^7 cells in lysis 250 buffer, subjecting extracts to three freeze/thaw (liquid nitrogen/37°C) cycles and clearing by centrifugation (14K, 2 min at room temperature). Immunoprecipitates were separated by SDS-PAGE, and Western blotted. Various tissues from 2-mo-old mice were ground using a mortar and pestle under liquid nitrogen. The samples were then extracted in lysis buffer containing 250 mM NaCl, 50 mM Tris-HCl (pH 7.5), 0.125% Na-deoxycholate, 0.375% Triton X-100, 0.15% NP-40, 4 mM EDTA, 10 mM aprotinin, 50 mM leupeptin, and 1 mM PMSF. After one freeze/thaw cycle, the lysates were cleared as described above. The same amount of total protein from each tissue was subsequently used for immunoprecipitation and immunoblotting analysis.

For all Western blots, following addition of the appropriate primary antibody and an alkaline phosphatase–conjugated secondary antibody, bound protein was visualized with 5-bromo-4-chloro-3-indolylphosphate toluidinium and nitro blue tetrazolium (BCIP, NBT; Promega Biotec, Madison, WI).

Cell Cycle Synchronization and Co-Immunoprecipitation

CV-1 cells were synchronized to early G1, mid-S, and mid-M phase of the cell cycle. Lovastatin was used at 40 mM for 48 h to arrest the cells in early G1. A double block of hydroxyurea, followed by a 5-h release, synchronized cells to mid-S phase. Mitotic cells were collected by shake-off following a 24-h exposure to 1.0 μg/ml nocodazole. At each time point, cells were washed twice with PBS and immediately lysed as described previously (Chen et al., 1989). Following four freeze/thaw cycles and centrifugation at 14K for 10 min at 4°C, supernatants were immunoprecipitated with mAbs to RB protein (11D7) or p84 (5E10). Protein A–Sepharose was used to pellet the immunocomplexes. After extensive washing in L250 buffer, samples were boiled for 5 min in SDS sample buffer. Complexes were separated by 8% SDS-PAGE; immunoblotting was done with 11D7 and 5E10 mAbs.

Immunocytochemistry and Image Analysis

CV1 cells were fixed either in cold methanol for 10 min at −20°C, or in 4% formaldehyde prepared in cytoskeletal buffer (CSK; 0.01 M Pipes, pH 6.7, 100 mM NaCl, 300 mM sucrose, 1.0 mM EGTA, 3 mM $MgCl_2$) for 30 min at room temperature. Formaldehyde-fixed cells were permeabilized in CSK containing 0.5% Triton X-100 for 5 min either prior to fixation, or afterwards. All three fixation protocols yielded similar staining patterns. All antibody and washing solutions were made in 0.1 M Tris-HCl, pH 7.4, 0.15 M NaCl, 0.1% Tween-20, 5% (wt/vol) dry milk, and 0.1% sodium azide. Co-localization of $p110^{RB}$ and p84 was performed using a polyclonal rabbit anti-$p110^{RB}$ antibody (0.47) mixed with the 5E10 IgG mAb to p84; the respective antigens were visualized with goat anti–mouse IgG conjugated to Texas red and goat anti–rabbit conjugated to FITC. Co-localization of p84 with an IgM mAb to the B1C8 nuclear matrix antigen (generously provided by S. Penman, Massachusetts Institute of Technology, Cambridge, MA; Wan et al., 1994), which localizes to sites involving in RNA processing (snRNP- and SC35-positive speckles; Blencowe et al., 1994), was performed by mixing both primary antibodies as above, followed by goat anti–mouse IgG (Texas red) and goat anti–mouse IgM (FITC). The Sm human autoantibody sera (kindly provided by P. Sharp, Massachusetts Institute of Technology) was detected with goat anti–human IgG (FITC). Controls included omission of the primary antibody, and in the case of p84, 5E10 mAb was pre-incubated with excess GST-N5 fusion protein (not shown). Confocal optical sections were obtained with a Zeiss 310 LSM, equipped with Ar and HeNe lasers. Digital images of FITC or Texas red fluorescence, or of Normarski differential interference contrast, were overlaid for multichannel recording. Co-localization was performed either by the LSM software, or by merging the individual channels from a single Z section with VoxelView–ULTRA 2.1 and VoxelMath 2.0 software (Vital-Images, Inc., Fairfield, IO). Intensity-based thresholding was performed with the multi-channel rendering capability of VoxelView–ULTRA 2.1. All images were kernel filtered (N = 3) by VoxelMath 2.0 using an average smoothing operation. Pseudo-color tables, contrast and opacity were optimized for each channel, before merging and thresholding, to visualize low levels of fluorescence.

Nuclear Matrix Preparation

The procedure of He et al. (1990) was followed as described, except that antipain, leupeptin, and aprotinin (1-μg/ml each), and sodium fluoride (5 mM), were added to all extraction solutions.

Results

Isolation of cDNAs Encoding $p110^{RB}$ Amino-terminal-binding Proteins

To screen for proteins able to bind the amino-terminal region of $p110^{RB}$, a modified version of the yeast two-hybrid system was utilized (Durfee et al., 1993). In this assay, co-transformants of a yeast strain, Y153, which are phenotypi-

A

N-domain   T/E1A domains

1 8   262 300   394   566   649   766   928 a.a.

Rb

Figure 1. Expression vector for Gal4-RB fusion and screening results. (*A*) Structures of RB and yeast expression vector. Bar represents the RB coding sequence with encoded protein domains and amino acid coordinates indicated. The region (encoding amino acids 1-300) fused to the Gal4 DNA-binding domain is bracketed, and the map of the resulting expression vector, pAS/N-RB, is shown. (*B*) Sum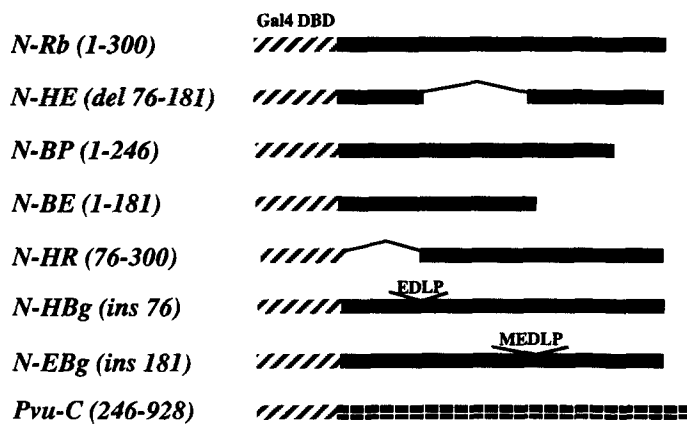mary of the screening and rescreening results. The number of total transformants was estimated by plating an aliquot of each transformant on media selecting only for the presence of the plasmids (SC-trp-leu). The approximate number of His[+] positive colonies was determined following plating of the transformation on SC-trp-leu-his + 25 mM 3-AT. The presence of β-galactosidase activity was detected using the colony filter lift method (Breeden and Nasmyth, 1985). N-RB dependency was determined by transforming Y153 with the isolated library-derived plasmid alone or together with either pAS/N-RB, pASRB2, or pAS/SNF1. Activation domain-tagged hybrids which interacted specifically with the Gal4-N-RB fusion only were counted as positives.

B

	# of transformants	# of His[+]	# of His[+], blue	# of N-Rb dependent
pAS/N-Rb + human library	1 x 10^{6}	20,000	21	6

cally His[+] and blue following the selection-screening procedure, are considered as positives, and further assessed for their reproducibility and dependence on RB protein expression. A yeast expression plasmid, pAS/N-RB, containing RB cDNA encoding the first 300 amino acids of the protein fused in-frame to sequences for the Gal4 DNA binding domain was used to transform Y153 (Fig. 1 *A*). A resulting transformant was then co-transformed with an activation domain-tagged human cDNA library created in the λACT vector (Durfee et al., 1993). Of the approximately one million transformants tested, 21 were phenotypically His[+] and blue in the initial screen, and six of those were reproducible and dependent on presence of pAS/N-RB, as shown in a rescreen using purified plasmids (Fig. 1 *B*). Sequence data eliminated four of these clones as encoding either a cytoplasmic protein or short peptides translated from cDNA in the reverse orientation. The remaining two clones, N5 and N11, encode the same gene whose protein product bound strongly to the amino-terminal domain of p110[RB]. This gene was characterized further.

A Structurally Defined Amino-terminal Domain of p110[RB] Is Required for N5 Binding

To define the sequences of the RB protein needed for binding N5, a series of amino-terminal mutations were created and subcloned into pAS1. These mutants were then used to co-transform Y153 with N5-expressing plasmid DNA, and the resulting colonies were analyzed for β-galactosidase activity (Fig. 2). Deletion mutants which affect a structurally defined amino-terminal domain (amino acids 8-262; Hensey et al., 1994), abolished N5 binding. In addition, a linker insertion mutant (pAS/N-HBg), resulting in a 5-amino acid insert at residue 76, also blocked the ability of the RB protein to interact with N5. However, another linker insertion mutant (pAS/N-EBg) at position 181 had no effect on N5 association. Thus, a largely intact domain in the NH₂ terminus is required for interaction and a small perturbation caused by an insertion at amino acid 76 can block this binding. The Pvu-C mutant, which contains RB protein sequences from 246-928 and binds T-ag in this system (data not shown), does not bind the N5 protein.

		Colony color	Units of β-galactosidase
N-Rb (1-300)	Gal4 DBD	blue	17.5 ± 0.2
N-HE (del 76-181)		white	0.7 ± 0.2
N-BP (1-246)		white	0.4 ± 0.1
N-BE (1-181)		white	0.5 ± 0.2
N-HR (76-300)		white	0.4 ± 0.1
N-HBg (ins 76)	EDLP	white	0.5 ± 0.2
N-EBg (ins 181)	MEDLP	blue	44.1 ± 23.7
Pvu-C (246-928)		white	0.4 ± 0.1

Figure 2. Detection of interactions between N5 and RB mutants in vivo. The Gal4 DNA binding domain (amino acids 1-147; *striped box*) fused to various RB mutants is shown schematically. RB amino acids present in the fusion are denoted, except in the case of linker insertions (*ins*) and an internal deletion (*del*), where the affected amino acid(s) within the N-RB fragment are indicated. Inserted amino acids are shown in single letter code. Y153 was co-transformed with the indicated panel of Gal4-RB mutants and the N5 expression plasmid.

All Gal4-RB fusions were expressed, as determined by immunoblotting (data not shown). Colony color was determined by the colony lift assay. CPRG quantitation of β-galactosidase activity was done in triplicate for each transformation.

N5-encoded Protein Binds p110^RB In Vitro

The ability of the N5-encoded protein to bind native full-length RB protein was tested using an in vitro binding assay. The cDNA fragment present in the N5 clone was fused to sequences for glutathione S-transferase (GST) (Smith and Johnson, 1988) present on an *E. coli* expression vector to create GST-N5. The fusion protein was expressed and then bound to an affinity matrix. The resulting complex was then incubated with cell extracts from a RB reconstituted WERI cell line, 3G10 (Chen et al., 1992). Complexes were washed, separated by SDS-PAGE, and immunoblotted with an anti-RB protein mAb. Fig. 3 shows that RB protein was retained in the GST-N5 column, comparable to the amount bound to the positive control column, GST-T-ag. No significant binding was seen in the GST column (Fig. 3). N5 also binds preferentially to the hypophosphorylated form of the RB protein (Fig. 3), analogous to T-ag binding (Ludlow et al., 1989). Together, these data demonstrate that the N5-encoded protein can bind full-length p110^RB and this association is specific for the hypophosphorylated p110^RB isoform.

Analysis of N5 Sequence

Northern blot analysis using the N5 cDNA as a probe shows the mRNA for this gene to be ~2.1 kb in length (data not shown). To obtain a full-length cDNA clone of this gene, a human lymphocyte cDNA library (Elledge et al., 1991) was screened, and several overlapping clones were isolated. The resulting 2,100-nucleotide (nt) sequence has a single large open reading frame (nt 15-1985) which can encode an extremely acidic (pI = 4.85) 657 amino acid protein with a predicted molecular weight of 75,633 D (Fig. 4). The predicted protein has no significant homology with any protein in the present databases. The ATG at nt 15 has a canonical purine in the −3 position (Kozak, 1989), and thus likely serves as the initiator codon; however, the lack of an upstream in-frame stop codon, together with the unknown nature of additional 5' sequences, prevents its unambiguous assignment. The 107-nt 3' untranslated region contains a

canonical poly adenylation site, AAUAAA, followed by a polyA tail.

Placing the N5 fragment isolated in the original screen within this sequence limits the region of the encoded protein necessary for p110^RB binding. Sequencing reveals that the original N5 fragment spans nucleotides 1,132–2,066 of the near full-length cDNA, and encodes amino acids 374-657
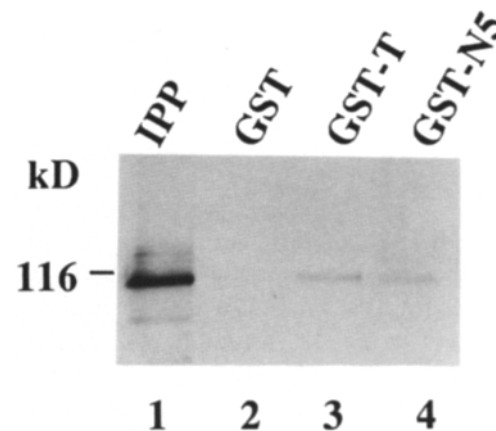

Figure 3. In vitro binding of N5 and p110^RB. Glutathione S-transferase (*GST*) and in-frame GST fusions with cDNA encoding N5 and the NH$_2$-terminal 273 amino acids of T-antigen (*GST-T*) were expressed in *E. coli*. GST and GST fusions were bound to glutathione-sepharose beads and washed extensively. Samples were quantitated by Coomassie blue staining of SDS-PAGE gels and equivalent protein amounts used in each lane. Extracts made from 3G10 cells (Chen et al., 1992) were mixed with the bound samples for 30 min at room temperature. Following extensive washing, complexes were separated by SDS-PAGE and transferred for immunoblotting. The amount of RB protein present and the extent of its phosphorylation in 3G10 cells was determined by immunoprecipitation with the 11D7 antibody (lane *1*). The blot was probed with an anti-RB protein monoclonal antibody, 11D7, and visualized by enzymatic color reaction.

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GGCCTTCGTCGAAGATGTCTCCGACGCCGCCGCTCTTCAGTTTGCCCGAAGCGCGGACGCGGTTTACGAAGTCTACCAGAGAGGCCTTGAACAACAAAAA    100
                M  S  P  T  P  P  L  F  S  L  P  E  A  R  T  R  F  T  K  S  T  R  E  A  L  N  N  K  N

CATCAAGCCATTGTTAAGTACCTTCAGCCAGGTACCTGGCAGTGAAAATGAAAAAAAATGTACCCTTGACCAAGCTTTCAGAGGTATTCTAGAAGAAGAA    200
 I  K  P  L  L  S  T  F  S  Q  V  P  G  S  E  N  E  K  K  C  T  L  D  Q  A  F  R  G  I  L  E  E  E

ATTATAAATCATTCATCATGTGAAAACGTTTTAGCTATTATTTCTCTTGCTATTGGGGGAGTAACTGAAGGTATTTGTACCGCATCTACACCTTTTGTAT    300
 I  I  N  H  S  S  C  E  N  V  L  A  I  I  S  L  A  I  G  G  V  T  E  G  I  C  T  A  S  T  P  F  V

TGTTGGGAGATGTTTTGGATTGTCTTCCTTTGGATCAGTGTGACACAATATTCACTTTTGTCGAAAAAAATGTTGCTACTTGGAAATCAAATACCTTCTA    400
 L  L  G  D  V  L  D  C  L  P  L  D  Q  C  D  T  I  F  T  F  V  E  K  N  V  A  T  W  K  S  N  T  F  Y

TGCTGCTGGGAAAAATTACTTACTACGTATGTGCAATGATCTCCTAAGAAGATTGTCTAAATCCCAGAATACAGTCTTCTGTGGACGGATTCAGCTCTTT    500
 A  A  G  K  N  Y  L  L  R  M  C  N  D  L  L  R  R  L  S  K  S  Q  N  T  V  F  C  G  R  I  Q  L  F

TTGGCCAGGCTTTTCCCTCTGTCTGAGAAATCAGGTCTTAACTTGCAGAGTCAGTTTAATCTGGAAAATGTCACTGTTTTCAATACAAATGAGCAGGAAA    600
 L  A  R  L  F  P  L  S  E  K  S  G  L  N  L  Q  S  Q  F  N  L  E  N  V  T  V  F  N  T  N  E  Q  E

GCACCCTGGGTCAGAAGCACACTGAAGATAGAGAAGAAGGAATGGATGTAGAAGAAGGCGAAATGGGAGATGAGGAAGCTCCAACAACGTGCTCTATTCC    700
 S  T  L  G  Q  K  H  T  E  D  R  E  E  G  M  D  V  E  E  G  E  M  G  D  E  E  A  P  T  T  C  S  I  P

AATTGATTACAACCTGTATCGAAAATTCTGGTCACTTCAGGATTACTTCAGGAACCCTGTGCAATGCTATGAGAAGATTTCATGGAAAACTTTTCTCAAG    800
 I  D  Y  N  L  Y  R  K  F  W  S  L  Q  D  Y  F  R  N  P  V  Q  C  Y  E  K  I  S  W  K  T  F  L  K

TATTCTGAAGAAGTTTTAGCTGTTTTTAAGAGTTATAAATTAGATGATACTCAGGCCTCAAGAAAAAAGATGGAAGAATTGAAAACAGGAGGAGAACATG    900
 Y  S  E  E  V  L  A  V  F  K  S  Y  K  L  D  D  T  Q  A  S  R  K  K  M  E  E  L  K  T  G  G  E  H

TATATTTTGCAAAATTTTTAACAAGTGAAAAGCTGATGGATTTACAACTGAGTGACAGTAACTTTCGTCGACACATCCTGTTGCAGTATCTCATTTTATT    1000
 V  Y  F  A  K  F  L  T  S  E  K  L  M  D  L  Q  L  S  D  S  N  F  R  R  H  I  L  L  Q  Y  L  I  L  F

CCAATATCTCAAGGGGCAGGTCAAATTCAAAAGTTCAAACTATGTTTTAACTGATGAGCAATCACTTTGGATTGAAGATACTACAAAATCAGTTTATCAA    1100
 Q  Y  L  K  G  Q  V  K  F  K  S  S  N  Y  V  L  T  D  E  Q  S  L  W  I  E  D  T  T  K  S  V  Y  Q

CTACTATCTGAAAACCCCCCCGATGGAGAAAGATTTTCAAAGATGGTAGAGCATATATTAAACACTGAAGAAAACTGGAACTCGTGGAAAAATGAAGGTT    1200
 L  L  S  E  N  P  P  D  G  E  R  F  S  K  M  V  E  H  I  L  N  T  E  E  N  W  N  S  W  K  N  E  G

GCCCAAGTTTTGTGAAAGAAAGAACATCAGATACCAAACCTACGAGAATAATTCGGAAGAGAACAGCACCCGAGGACTTCCTAGGGAAAGGACCCACCAA    1300
 C  P  S  F  V  K  E  R  T  S  D  T  K  P  T  R  I  I  R  K  R  T  A  P  E  D  F  L  G  K  G  P  T  K

AAAAATTCTGACGGGAAATGAGGAGTTAACAAGGCTTTGGAATCTTTGCCCTGATAATATGGAAGCCTGTAAATCAGAGACAAGGGAACACATGCCCACT    1400
  K  I  L  T  G  N  E  E  L  T  R  L  W  N  L  C  P  D  N  M  E  A  C  K  S  E  T  R  E  H  M  P  T

TTGGAGGAATTCTTTGAAGAAGCCATTGAACAGGCAGACCCTGAAAATATGGCGGGAAATGAATATAAGGCTATGAACAATTCAAATTATGGTTGGAGAG    1500
 L  E  E  F  F  E  E  A  I  E  Q  A  D  P  E  N  M  A  E  N  E  Y  K  A  M  N  N  S  N  Y  G  W  R

CCCTGAAACTATTAGCACGGAGAAGCCCTCACTTCTTCCAGCCAACCAACCAGCAGTTTAAAAGTTTACAAGAATATCTTGAAAATATGGTAATAAAGCT    1600
 A  L  K  L  L  A  R  R  S  P  H  F  F  Q  P  T  N  Q  Q  F  K  S  L  Q  E  Y  L  E  N  M  V  I  K  L

AGCCAAGGAATTACCGCCTCCTTCTGAAGAAATAAAAAACAGGTGAGGATGAAGATGAGGAAGATAATGATGCTCTACTGAAGGAAAATGAAAGTCCTGAT    1700
  A  K  E  L  P  P  P  S  E  E  I  K  T  G  E  D  E  D  E  E  D  N  D  A  L  L  K  E  N  E  S  P  D

GTTCGGCGAGACAAACCTGTAACAGGAGAACAAATAGAGGTATTTGCCAACAAGCTGGGTGAACAATGGAAGATTCTGGCTCCCTACTTGGAAATGAAAG    1800
 V  R  R  D  K  P  V  T  G  E  Q  I  E  V  F  A  N  K  L  G  E  Q  W  K  I  L  A  P  Y  L  E  M  K

ACTCAGAAATTAGGCAGATTGAGTGTGACAGTGAAGACATGAAGATGAGAGCTAAGCAGCTCCTGGTTGCCTGGCAAGATCAAGAGGGAGTTCATGCAAC    1900
 D  S  E  I  R  Q  I  E  C  D  S  E  D  M  K  M  R  A  K  Q  L  L  V  A  W  Q  D  Q  E  G  V  H  A  T

ACCTGAGAATCTGATTAATGCACTGAATAAGTCTGGATTAAGTGACCTTGCAGAAAGTCTAACTAATGACAATGAGACAAATAGTTAGCTTCTTTTTTTT    2000
 P  E  N  L  I  N  A  L  N  K  S  G  L  S  D  L  A  E  S  L  T  N  D  N  E  T  N  S

TTCTTTTTATTAAAACTGTGATAGATTTTGTTACCAAGCAGCATTTGATAAGAGGTCCACTGGTTTTGGTAAAC[AATAAA]CATTTTTATAACAAAAAAAA    2100
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B
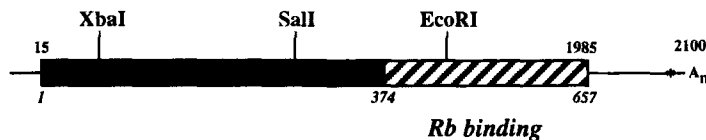
Figure 4. Nucleotide and predicted amino acid sequence of full-length N5. (*A*) Amino acids are shown in single letter code, and the first in-frame stop codon is indicated with an asterisk. The polyadenylation signal, AAUAAA, is boxed. (*B*) Schematic of the full-length N5 sequence. cDNA is depicted as a line, with the p84 coding region indicated with a black bar. The $p110^{RB}$ binding domain is overlaid on the coding region as a hatched box. Nucleotide and amino acid coordinates are shown above and below the sequence, respectively. The asterisk represents the polyadenylation signal, and the poly A tail is shown as A_n. These sequence data are available from EMBL/GenBank/DDBJ under accession number L36529.

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(Fig. 4 *B*), a highly negatively charged region of the protein. That this region binds to the p110ᴿᴮ amino-terminal domain, which is itself slightly acidic, may suggest hydrophobic interactions are involved.
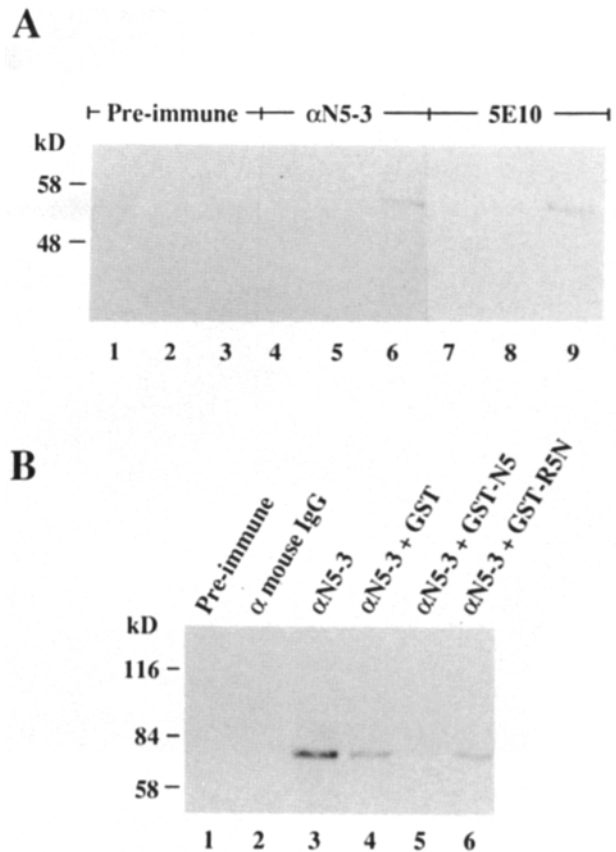
Anti-N5 Antibodies Detect an 84-kD Protein from Mammalian Cells

The GST-N5 fusion protein was used as an antigen to immunize mice and generate polyclonal antibodies. The resulting antisera specifically detected the GST-N5 antigen produced in *E. coli* (data not shown). The ability of the antisera to recognize the N5 polypeptide was further demonstrated using yeast extracts producing the Gal4 activation domain–N5 fusion. A polyclonal antibody, αN5-3, recognizes a protein of the correct molecular weight only in the N5-expressing cells (Fig. 5 *A*, lanes *4–6*). No protein was detected with preimmune serum (lanes *1–3*). In addition, a monoclonal antibody, 5E10, generated from the same antigen, produced identical results (lanes *6–9*).

To identify the authentic N5 protein in mammalian cells, CV-1 cell extracts were prepared and used for immunoprecipitation with either the αN5-3 antibody or controls. Immunoprecipitates were separated by SDS-PAGE, and immunoblotted with the 5E10 mAb (Fig. 5 *B*). The antibody recognizes an 84-kD protein from these cells which is lacking in the precipitates of both the preimmune serum and those of an irrelevant anti-mouse IgG (compare lanes *1–3*). Depletion of the αN5-3 serum by preincubation with the GST-N5 fusion protein abolishes recognition of the 84-kD protein; incubation with GST alone or GST-R5N (containing the N5 sequences in reverse orientation) has no effect (lanes *4* and *5*). Thus, the anti-N5 antibodies specifically detect an 84-kD protein from mammalian cells, in reasonable agreement with the predicted molecular weight from sequence data. Therefore, this protein will be referred to as p84 for the remainder of this report.

p84 Is a Nuclear Protein That Co-localizes with p110ᴿᴮ

The cellular localization of p84 was determined by confocal immunofluorescence microscopy. CV-1 cells were fixed by several methods (see Materials and Methods), reacted with the 5E10 antibody and subsequently visualized via a fluorescently labeled secondary antibody. All fixation methods revealed p84 to be localized within the nucleus (Fig. 6), consistent with the subcellular localization of p110ᴿᴮ (Lee et al., 1987). p84 is organized into multiple discrete foci and is not detected in the nucleolus (Fig. 6). An identical result was obtained with the αN5-3 antibody (data not shown). To assess the amount of colocalization of Rb and p84 in the nucleus, the distribution of both proteins in double labeled cells were assessed using the VoxelView–ULTRA and VoxelMath visualization software packages (VitalImages, Inc.). The opacity, contrast, and color tables were identically adjusted for each set of images to maximize visualization of low level fluorescence. When the full range (100%) of detected fluorescence was rendered individually, it was clear that Rb (Fig. 7 *a*, green) was more widespread in the nucleus than p84 (Fig. 7 *b*, red); however, many hot spots of Rb did overlap with punctate spots of p84 (Fig. 7 *c*, yellow). In an effort


A

B

Figure 5. Anti-N5 antibodies specifically detect an 84-kD protein from mammalian cells. (*A*) Characterization of anti-N5 antibodies. Yeast cell extracts were made from Y153 (lanes *1*, *4*, and *7*), Y153 transformed with pSE1107 (Durfee et al., 1993) (lanes *2*, *5*, and *8*), or Y153 transformed with N5 (lanes *3*, *6*, and *9*), separated by SDS/PAGE, and immunoblotted. Lanes *1–3* were probed with preimmune mouse serum, lanes *4–6* with a mouse anti-N5 polyclonal serum, αN5-3, and lanes *7–9* with a monoclonal antibody, 5E10, derived from αN5-3. Reacting proteins were visualized by enzymatic color reaction. (*B*) Detection of endogenous N5 protein from mammalian cells. CV-1 cells were lysed, and subjected to immunoprecipitation with antibody preparations as indicated. Preabsorption of the serum was performed as described in Materials and Methods. Precipitates were separated by SDS-PAGE, and prepared for immunoblotting. Blots were probed with 5E10, and visualized by enzymatic color reaction.

to assess, and visualize, the amount of Rb that colocalized with p84, we used thresholding (based upon intensity) to remove all but the top 10% of both channels (Fig. 7, *d–f*). The top 10% Rb signal (Fig. 7 *d*, green) and the top 10% p84 signal (Fig. 7 *e*, red) were largely confined to overlapping or adjacent regions (Fig. 7 *f*, yellow). It is interesting to note that some regions of mutually exclusive label is seen for both proteins.

## p84 Co-localizes to Nuclear Domains Involved in RNA Processing

Specific regions of the nucleus that are rich in molecules involved in RNA metabolism have been identified (for recent
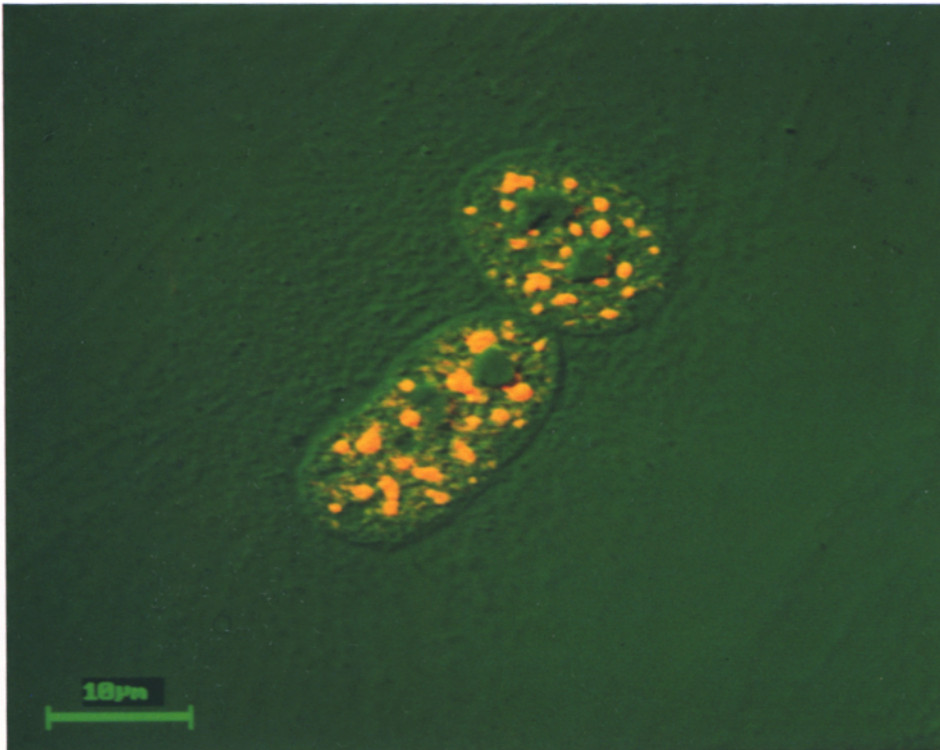
reviews see Spector, 1993; and Xing and Lawrence, 1993). To determine whether the p84 speckles are coincident with these RNA processing centers, or "transcript domains" (Carter et al., 1991; Xing and Lawrence, 1993), we used both the Sm autoimmune sera and a novel IgM mAb (B1C8) for colocalization with the 5E10 IgG mAb. The Sm autoimmune sera labels multiple bright speckles in the nucleus, and a low level of homogeneous staining as well (Spector, 1993),

which is attributed to recognition of several small nuclear ribonuclear proteins (snRNPs) involved in splicing. The B1C8 mAb identifies a 180-kD nuclear matrix protein (Wan et al., 1994) that principally colocalizes with the bright foci labeled by the anti-SM antibody and the SC35 mAb (Fu and Maniatis, 1990), and co-immunoprecipitates exon-containing RNA from in vitro splicing reactions (Blencowe et al., 1994). SC35 is an essential non-snRNP splicing factor pre-
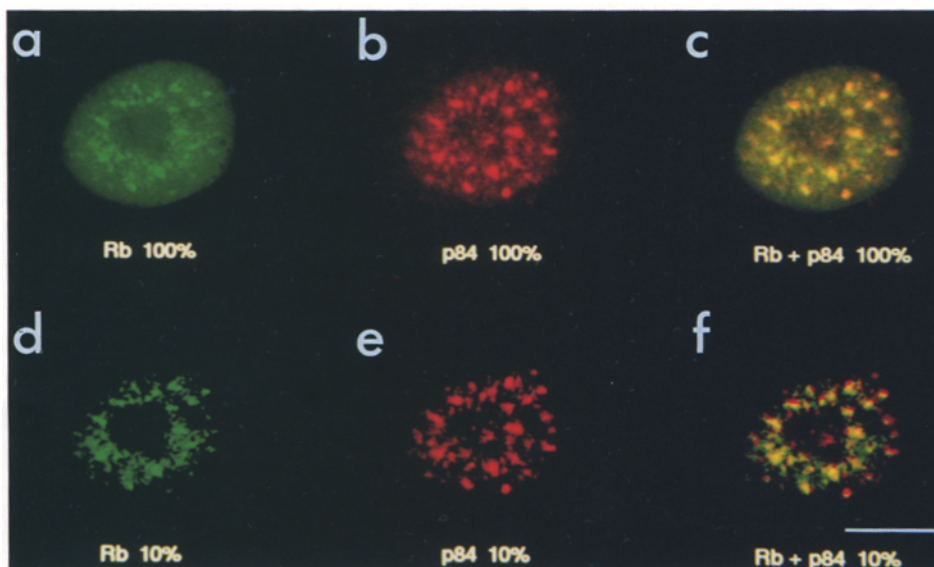
Figure 7. Regions of high p110[RB] concentration colocalize with p84. Methanol-fixed CV1 cells double labeled with p84 mAb 5E10 and rabbit anti-p110[RB] antibody 0.47. Confocal optical sections of both fluorescent channels were individually recorded. The full spectrum (*100%*) of fluorescent signals are shown in *A–C*; by thresholding out all but the top 10% of the signals in *D–F*, intranuclear regions of intense staining for both proteins is highlighted. For each set of images, the opacity, contrast and color tables were identically adjusted to maximize detection of low level fluorscence. (*A*) p110[RB] labeling (*green*) appears widely distributed within this plane of nucleus, with frequent brighter foci throughout. (*B*) p84 (*red*) appears much more punctate. (*C*) Colocalization of p110[RB] and p84 is apparent at multiple hot spots (*yellow*) in this merged image. (*D*) Distribution of the highest fluorescent intensity (concentration) of p110[RB] (*green*) is significantly more confined to specific regions. (*E*) Removing 90% of the p84 image (*red*) results in a similar pattern of intranuclear speckles as in *B*. (*F*) Areas containing high concentration of p110[RB] overlap, or are adjacent, with the focal staining of p84 (*yellow*). Bar, 5 μm.
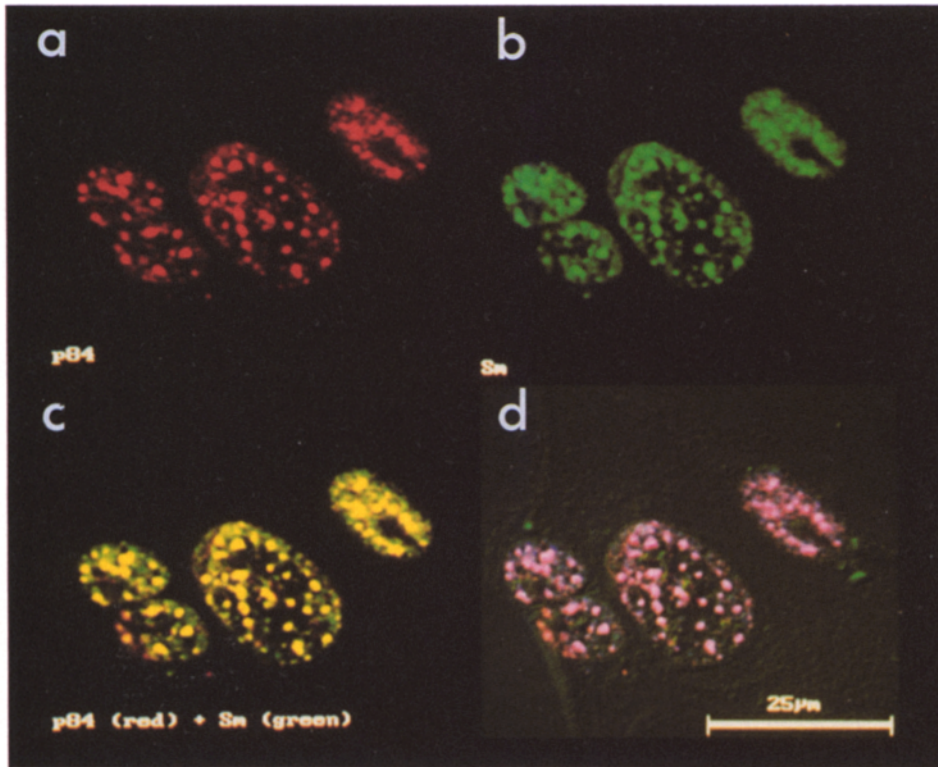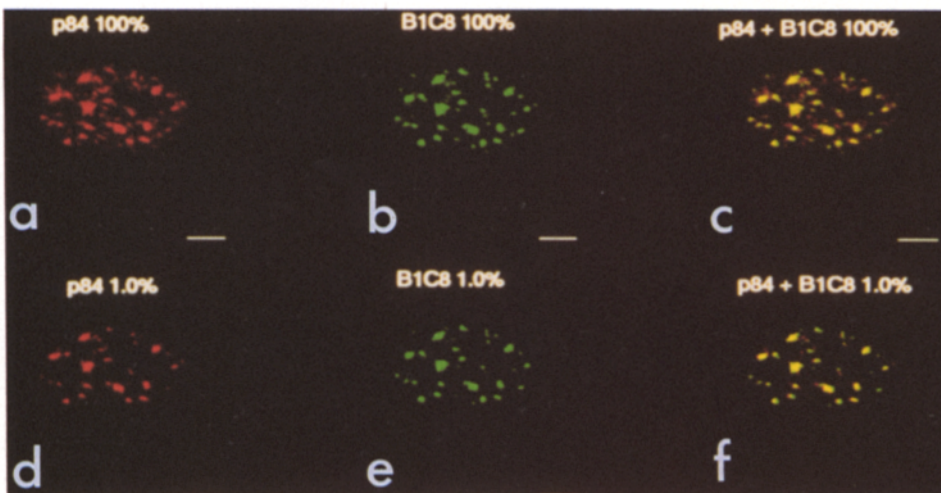
Figure 8. p84 colocalizes with the Sm antigen. A methanol-fixed CV1 cell was double labeled with mAb 5E10 and the Sm autoantibody sera. The highest concentration of both p84 (*A, red*) and the Sm antigen (*B, green*) is found in a similar intranuclear speckled pattern. The Sm antigen is somewhat more diffuse in the nucleoplasm compared with p84. When the Texas Red and FITC channels are overlaid, regions of colocalization appear as yellow (*C*). The colocalization results are superimposed with cellular morphology in *D* (in this case, B1C8 is assigned as *blue*). Bar, 25 μm.

viously shown to colocalize with snRNP antigens (Fu and Maniatis, 1990; Spector et al., 1991), poly-adenylated RNA (Carter et al., 1991), and specific gene transcripts (Huang and Spector, 1992; Carter et al., 1993). Fig. 8 shows the result of colocalization experiments using the 5E10 mAb (Fig. 8 *a, red*) and the Sm sera (Fig. 8 *b, green*). When these channels were merged, the majority of the punctate labeling pattern appears as yellow (Fig. 8 *c*), indicating a high degree of overlap within the optical section. Fig. 8 *d* shows the p84 (*red*) and Sm (in this image, shown as *blue*) fluorescent signals overlaid with a differential interference contrast image

(*green*) of the cell morphology. The resulting image shows the colocalization as purple, and indicates the relative position of these antigens against the nuclear morphology. These data strongly support the idea that p84 is abundant in centers associated with RNA processing.

As the relatively insoluble B1C8 nuclear matrix antigen is more specifically confined to regions of RNA processing as compared to the Sm antigen, i.e., highly coincident with Sc35 (Blencowe et al., 1994) we performed double-labeling experiments with 5E10 (IgG) and B1C8 (IgM) mAbs on CV1 cells briefly preextracted with detergent. When 100% of the

Figure 9. p84 colocalizes with the B1C8 nuclear matrix antigen. A CV1 cell that was briefly preextracted with 0.5% Triton X-100 was fixed and labeled with mAbs to p84 (IgG) (*A* and *D, red*); and the B1C8 (IgM) nuclear matrix/ spliced RNA-binding antigen (Wan et al., 1994) (*B* and *E, green*). *A–C* shows images in which the full range (*100%*) of collected pixels values are represented. Images in *D–F* represent only the top 1% (by intensity) of each channel. The colocalization (*yellow*) of p84 and B1C8 is shown in *C* and *F.* Note that in both the 100 and 1% panels, p84 and B1C8 are largely overlapping. Interestingly, there are areas where these antigens are mutually exclusive (*red* only or *green* only). Bar, 5 μm.
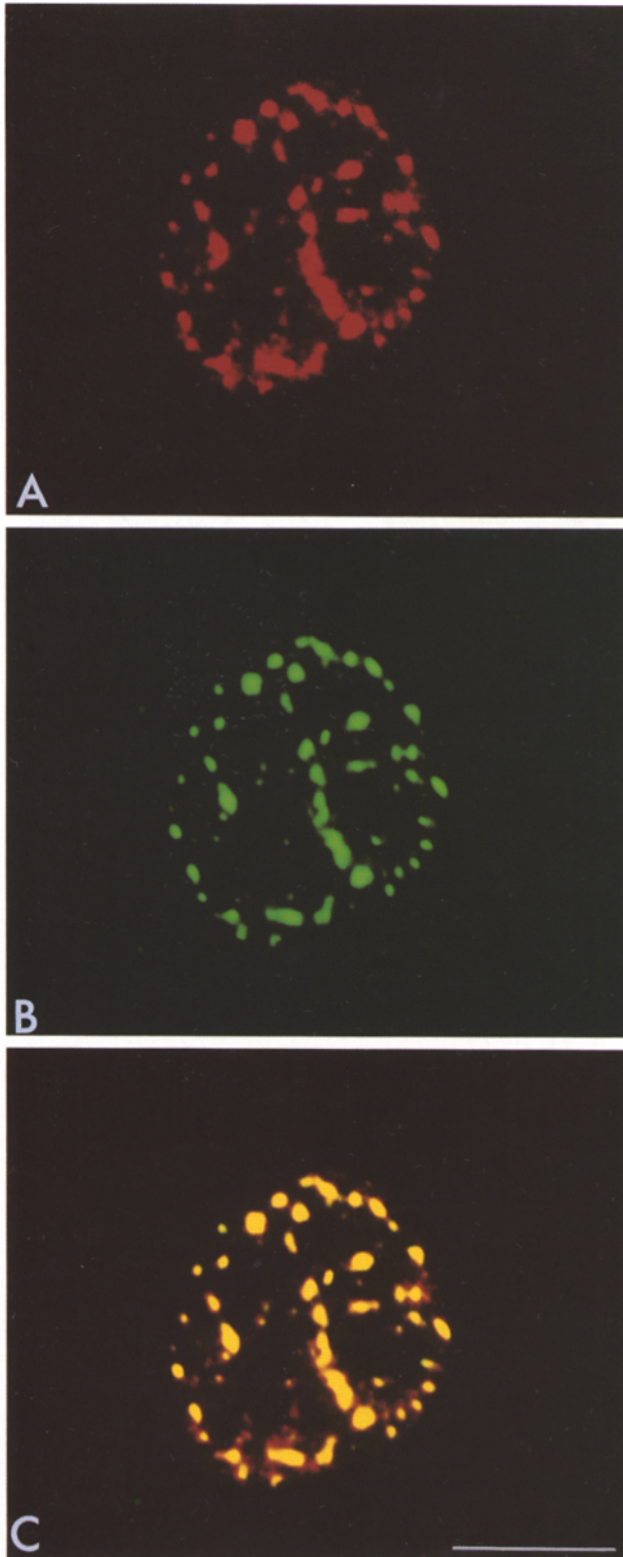
Figure 10. Localization of nuclear matrix-bound p84. (*A*) Staining pattern of p84 in the nuclear matrix prepared by sequential in situ extraction with Triton X-100, RNase-free DNaseI, 0.25 M (NH$_4$)SO$_4$, and 2 M NaCl (He et al., 1990). The strong punctate staining remains following the removal of 70% of the soluble proteins and 95% of the DNA (He et al., 1990). The B1C8 antigen (*B, green*) also remains associated with the matrix almost all regions containing p84 (*C, yellow*). Bar, 5 μm.

signal in cells double labeled with p84 (Fig. 9 *a, red*) and the B1C8 antigen (Fig. 9 *b, green*) are superimposed, a high degree of alignment is readily apparent (Fig. 9 *c, yellow*). As an indication of the degree of colocalization between p84 and B1C8 based upon fluorescent intensity, when both channels were reduced to just 1% of the highest recorded signal, the pattern of labeling of p84 (Fig. 9 *d, red*) and B1C8 (Fig. 9 *e, green*) still remained largely overlapped (Fig. 9 *f, yellow*). However, as with other matrix antigens found largely within RNA processing domains (Blencowe et al., 1994) there are instances where both proteins are mutually exclusive (*red* or *green* in Fig. 9 *d*). It remains to be determined if these smaller, p84-positive speckles represent sites of transcription, identified by Br-UTP incorporation, that are not found associated with intensely staining, SC35-rich domains (Wansink et al., 1993).

p84 Is a Component of the Nuclear Matrix

During G1, hypophosphorylated wild type p110^{RB} has been shown to resist low-salt/detergent extraction (Mittnacht and Weinberg, 1991) and associate with the nuclear matrix (Mancini et al., 1994). Further, p110^{RB} binds in vitro to the nuclear matrix proteins, lamins A and C (Mancini et al., 1994; Shan et al., 1992). Tumor-derived RB mutations, which affect the binding of the viral oncoproteins (Tag, E7, and E1A), do not associate with the matrix (Mittnacht and Weinberg, 1991; Templeton et al., 1991; Mancini et al., 1994), nor does an RB protein with a deletion from the second T/E1A domain through the carboxyl terminus bind to lamin A in vitro (Mancini et al., 1994). These data strongly suggest a functional importance to the association p110^{RB} with the nuclear matrix. In an effort to identify additional nuclear matrix proteins that bind the RB protein, we have routinely examined putative p110^{RB}-binding proteins for their affinity to the nuclear matrix. Accordingly, we tested the ability of p84 to withstand not only brief exposure to detergent (Figs. 6 and 9), but a thorough regiment of well-established extraction procedures that gently reveal the nonchromatin, insoluble system of core filaments and fibrogranular components comprising the nuclear matrix (He et al., 1990). This procedure removes greater than 85% of nuclear proteins and 95% of the DNA, leaving behind the core filament-containing nuclear matrix (He et al., 1990). Fig. 10 is a panel of images that show p84 (Fig. 10 *a, red*) and B1C8 (Fig. 10 *b, green*) remain predominantly colocalized (Fig. 10 *c, yellow*) in discrete foci following matrix isolation. A small amount of p84 is still visibly outside the B1C8 foci (Fig. 10 *c, red*).

To verify the immunostaining data on extracted cells, the 5E10 mAb and immunoblot analysis was used to examine the protein composition of sequential supernatants from the extractions, and the 2 M salt-resistant, insoluble nuclear matrix pellet. Fig. 11 shows that the extraction procedure, during both G$_1$ and S phases, does not effectively release p84, as most of the protein is found in the final, insoluble pellet. The confocal immunofluorescence images suggest that p84 localizes to many foci within the matrix (Fig. 10); however, high-resolution ultrastructural localization studies will be necessary to specifically identify the structures that contain p84. Nevertheless, the in situ localization and the biochemi-

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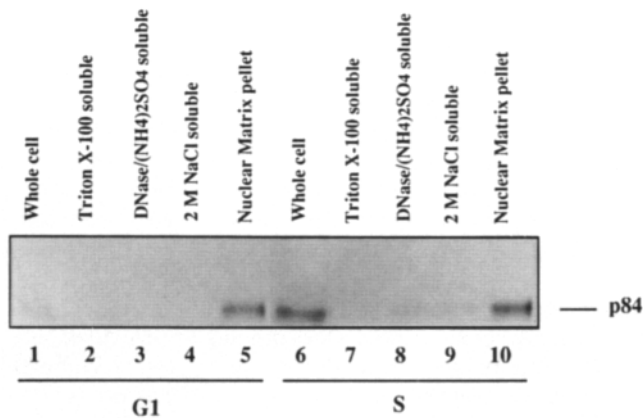
Figure 11. Immunoblot analysis of p84 in nuclear matrix fractions. CV-1 cells were cell cycle arrested in either G_1 by lovastatin (lanes *1–5*), or in mid-S by blocking twice with hydroxyurea followed by a 5-h release (lanes *6–10*), and then subjected to the extraction procedure. Whole cell lysates were compared with the extracted material from each step and the final pellet by SDS-PAGE separation, and immunoblotting with the 5E10 antibody. In both the G1 and S phase cells, most of the p84 is found in the final insoluble nuclear matrix pellet (lanes *5* and *10*). Blots were probed with 5E10, and visualized by enzymatic color reaction.

cal fractionation data support the conclusion that p84 is a component of the nuclear matrix.

Tissue Distribution of p84

To investigate the expression pattern of p84, extracts were made from various organs of an adult mouse and immunoprecipitated with the 5E10 mAb. Immunoprecipitates were then separated by SDS-PAGE and immunoblotted. As seen in Fig. 12, p84 is expressed in each tissue examined analogous to p110^{RB} (Lee et al., 1987). Similar experiments using various human cell lines confirm the ubiquitous expression of p84 in humans as well (data not shown). Thus, p84 and p110^{RB} have the same wide tissue distribution indicating their interaction may have a role in regulating a basic function(s) of either or both proteins.
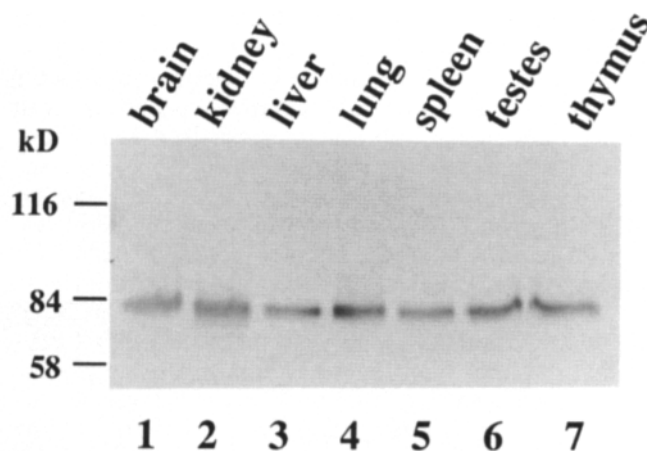
Figure 12. Tissue distribution of the p84 protein. Tissue samples were obtained from an adult mouse, and used to prepare cell extracts. 12.5 μg of total protein per sample was separated by SDS-PAGE, and immunoblotted using the anti-N5 mAb, 5E10. Bound protein was detected by enzymatic color reaction.

p84 Co-immunoprecipitates with p110^{RB} in a Cell Cycle-dependent Manner

To demonstrate that the p84/p110^{RB} association can occur at physiological concentrations, and to determine the timing of the interaction more precisely, co-immunoprecipitation experiments using CV1 cells were conducted. Cultures of CV1 cells were synchronized either in G_1, S, or M phases of the cell cycle by addition of appropriate drugs. Direct lysis with 300 mM salt, followed by several cycles of freezing and thawing, effectively solubilizes p84 and p110^{RB}. Extracts of these cultures were then immunoprecipitated by the anti-p110^{RB} mAb, 11D7 (Shan et al., 1992) or 5E10. After extensive washing, precipitates were separated by SDS-PAGE, and immunoblotted with either 11D7 or 5E10. p84 is detected in the 11D7 immunoprecipitates from G_1 cells (Fig. 13), consistent with its association with the hypophosphorylated form of p110^{RB} (Fig. 3). The very low level of hypophosphorylated p110^{RB} present in nocodozole-arrested cells (Ludlow et al., 1993), makes detection of any association during this time point unlikely. No association was observed during S phase (Fig. 13). This cell cycle dependency is not due to altered p84 expression as the 5E10 immunoprecipitates indicate the protein is present at approximately equivalent levels in G_1, S, or M phase (Fig. 13). These results demonstrate the interaction is specific for the hypophosphorylated form of p110^{RB} and confirms the GST-N5 binding
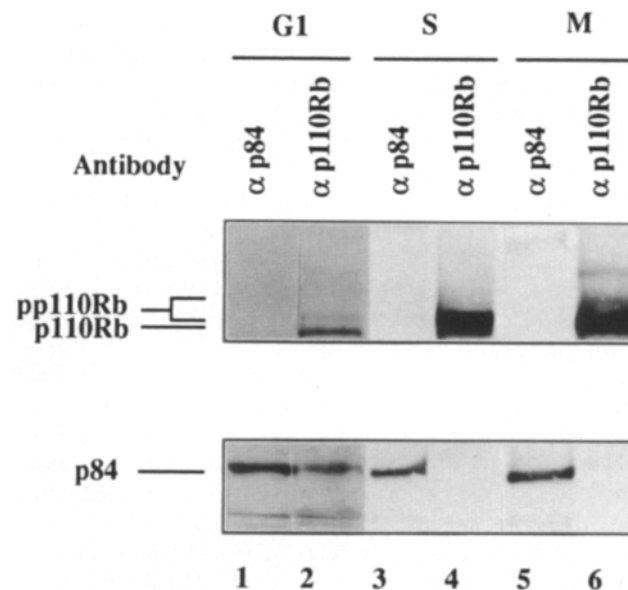
Figure 13. Cell cycle timing of the p84/p110^{RB} association. CV-1 cells were synchronized by treatment with Lovastatin (lanes *1* and *2*), a double block with hydroxyurea and a 5-h release (lanes *3* and *4*), or nocodazole (lanes *5* and *6*). Cells were then lysed, and immunoprecipitated with either the anti-RB protein monoclonal antibody, 11D7 (lanes *2, 4,* and *6*), or the anti-p84 monoclonal antibody, 5E10 (lanes *1, 3,* and *5*). Precipitates were then separated on an 8% SDS-polyacrylamide gel, and transferred to Immobilon. One portion of the blot was probed with 11D7, while the other was probed with 5E10. 11D7 mAb to p110^{RB} co-immunoprecipitates p84 only during G1. Bound protein was detected by enzymatic color reaction.

data shown in Fig. 3. The lack of p110^{RB} in the 5E10 immunoprecipitates is likely due to antibody interference with the interaction.

Discussion

We have used the yeast two-hybrid system to screen for cellular proteins that interact with the amino-terminal 300 amino acids of the RB protein. In contrast to the carboxy-terminal region of p110^{RB} which interacts with multiple cellular proteins (Kaelin et al., 1991; Lee et al., 1991), only a single clone was isolated which associates with the amino-terminal fragment. The gene encodes a novel nuclear protein of 657 amino acids with a predicted molecular weight of 75 kD; however, when separated on SDS–polyacrylamide gels, the native protein isolated from human, monkey, and mouse cells migrates more slowly with an apparent molecular weight of 84 kD. This nuclear protein, p84, is expressed in all tissues examined and throughout the cell cycle, analogous to p110^{RB} (Lee et al., 1987). Clues to the function of p84 come from our immunological and biochemical experiments which demonstrate that it is concentrated in subnuclear regions known to be involved in RNA processing. Further, similar to many critical molecules involved in the splicing/transcription machinery, p84 is a component of the nuclear matrix. As with other proteins which bind the RB gene product, p84 preferentially interacts with the hypophosphorylated isoform of p110^{RB}, and does so predominantly during G1, the time when p110^{RB} is functionally active (Goodrich et al., 1991) and associates with the nuclear matrix (Mancini et al., 1994). We cannot rule out that some interaction between p84 and Rb protein may occur during late M phase when hypophosphorylated p110^{RB} accumulates. p84, therefore, in addition to lamins A and C (Mancini et al., 1994; Shan et al., 1992), represents a potential site of interaction for p110^{RB} on the matrix.

The nuclear matrix has been implicated in most metabolic activities occurring in the nucleus, including replication, transcription, and RNA splicing and transport (Berezney and Coffey, 1975; Ciejek et al., 1983; Zeitlin et al., 1989). Many of the molecular components responsible for these activities have been identified as either constitutively or transiently associated matrix proteins. We have shown that p84 colocalizes with the intranuclear speckles labeled by the Sm autoantibody, which is often used to identify sites of RNA splicing (Spector, 1993). Further, p84 and the more punctate BlC8 nuclear matrix antigen (Wan et al., 1994) colocalize in both whole nuclei and on the matrix. BlC8 also colocalizes with essential components (snRNPs, SC35) of the RNA splicing centers (Blencowe et al., 1994). These splicing domains are also found to contain, or be adjacent to, sites of specific gene transcription (Huang and Spector, 1991; Xing et al., 1993). Taken together, the localization data suggest a role for p84 in one or more of these functions as well. Considering that most p84 foci either overlap or are adjacent with "hot spots" of p110^{RB}, p84 may also function to concentrate a portion of the RB protein to these regions. As immunogold labeling of both nuclear matrix–bound p110^{RB} (Mancini et al., 1994) and BlC8 (Wan et al., 1994) are localized to the fibrogranular masses by resinless section electron microscopy, it is highly probable that p84 will also, thus identifying the sub-matrix location where p110^{RB}/p84 inter-

actions would occur. Direct resinless section electron microscopic colocalization of p110^{RB} with p84, and other components of the splicing/transcript domains, will be required to substantiate this hypothesis.

During G1, studies indicate that low-salt/detergent extraction leaves some hypophosphorylated RB protein "tethered to the nuclear structure" (Mittnacht and Weinberg, 1991); mutant RB proteins do not display this property (Mittnacht and Weinberg, 1991; Templeton et al., 1991). A portion of wild-type hypophosphorylated p110^{RB} can also withstand the stringent conditions necessary to reveal the nuclear matrix (Mancini et al., 1994). Does binding to p84 contribute to p110^{RB} matrix association during the cell cycle? Whereas the amount of matrix-bound p84, and its subnuclear organization, appears normal in tumor cells containing RB mutation (Mancini, M. A., and W.-H. Lee, unpublished results), the fact that mutant RB protein does not tether to the matrix (Mittnacht and Weinberg, 1991; Templeton et al., 1991; Mancini et al., 1994) suggests that COOH-terminal mutations may affect binding to p84. Alternatively, the p84/p110^{RB} interaction may not be sufficient for matrix association. Experiments to address this issue are underway.

The mechanism whereby p110RB exerts a growth suppressing affect upon cells is not yet fully understood. However, several studies have clearly demonstrated that the carboxy-terminal half of the RB protein is essential, and when overexpressed, sufficient for this activity (for review see Goodrich and Lee, 1993; Hinds et al., 1992). Evidence now indicates that, in the context of the full-length protein, the amino-terminal domain is also required for growth suppression (Qian et al., 1992), and the recent isolation of an RB mutation affecting only this domain from a retinoblastoma tumor (Hogg et al., 1993), further supports this conclusion. These results indicate that the amino-terminal domain provides a function which is essential under physiological conditions, but which can be bypassed through mass action of the carboxy-terminal region alone. What is the function of the amino-terminal domain and how does p84 binding affect it? As the highest concentrations of p84 are found in regions of the nucleus associated with splicing factors (Spector, 1993) and specific gene transcription (Huang and Spector, 1991; Carter et al., 1993; Xing et al., 1993), p84 association could focus a subset of p110^{RB} to these areas while still allowing important interactions to occur through the carboxy-terminal domains. Association with several transcription factors, most notably E2F (Helin et al., 1992; Kaelin et al., 1992; Shan et al., 1992), through carboxy-terminal sequences, is thought to be essential for p110^{RB} function. The additional p84 found outside the regions labeled with SC35 may also colocalize with sites of general transcription, as studies reveal many BrUTP-positive foci associate with less-intense regions of SC35-staining (Wansink et al., 1993; Jackson et al., 1993). Localization of p110^{RB} to RNA processing centers is consistent with the relatively high RB protein concentrations found in areas of low DNA density (Szekely et al., 1991), i.e., the transcriptionally active euchromatin, and its proposed role in the regulation of several transcription factors. Thus, association with p84 would be an efficient means of concentrating the RB protein to subnuclear regions where active forms of several target-associated proteins (transcription factors) would be located.

The p110^{RB} amino-terminal domain may also exert a regulatory affect on a carboxy-terminal function(s) through either inter- or intramolecular interactions. This hypothesis is supported by two lines of evidence indicating that the tertiary structure of p110^{RB} is severely compromised by small alterations in its amino-terminal region. First, such mutations dramatically affect the ability of the protein to be hyperphosphorylated (Hamel et al., 1990; Qian et al., 1992; Hinds et al., 1992), considered to be an important indicator of the structural integrity of the RB protein. Second, amino-terminal mutants also disrupt interaction with the carboxy-terminal half of p110^{RB} (Hensey et al., 1994), an interaction that appears to be required for oligomerization (Hensey et al., 1994). Mutations with such dramatic consequences for p110^{RB} structure are likely to interfere with its function and/or regulation. As p84 interacts specifically with the active form of p110^{RB}, this association may be a mechanism for removing an inhibitory effect by the NH$_2$-terminal domain upon the active carboxy-terminal region. However, this switch would be reversible allowing p110^{RB} to be inactivated when cell division was required.

A highly complex and interactive mechanism for p110^{RB}-mediated tumor suppression is suggested by the multiple proteins it binds, and the spatially and solubility based subnuclear compartmentation of p110^{RB} itself. Does the association of p110^{RB} with both matrix-bound and soluble binding partners, particularly within regions associated with RNA processing, represent a unique mechanism whereby the RB protein exerts its tumor suppressing activity? To fully address this question, future studies must examine these interactions in the context of the full-length RB protein, and how it interfaces with architecturally based partitioning of nuclear metabolism.

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