The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit

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The retinoblastoma protein (p110RB) interacts with many cellular proteins in complexes potentially important for its growth-suppressing function. We have developed and used an improved version of the yeast two-hybrid system to isolate human cDNAs encoding proteins able to bind p110RB. One clone encodes a novel type 1 protein phosphatase catalytic subunit (PP-1c2), which differs from the originally defined PP-1c1 by an amino-terminal 11-amino-acid insert. In vitro-binding assays demonstrated that PP-1c1 isoforms preferentially bind the hypophosphorylated form of p110RB. Moreover, similar p110RB sequences are required for binding PP-1c2 and SV40 large T antigen. Cell cycle synchrony experiments revealed that this association occurs from mitosis to early G1. The implications of these findings on the regulation of both proteins are discussed.

[Key Words: Two-hybrid system; protein–protein interaction; retinoblastoma; protein phosphatase 1; cell-cycle regulation]

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The retinoblastoma susceptibility gene (Rb) was the first tumor suppressor gene to be cloned and characterized [Friend et al. 1986; Lee et al. 1987a]. Inactivation of both alleles of this gene has been found in all retinoblastomas examined, and additional studies have implicated Rb mutations in the development of a variety of tumors (for review, see Bookstein and Lee 1991). Introduction of a wild-type copy of this gene into Rb– tumor cells suppresses their tumorigenicity in nude mice [Huang et al. 1988; Bookstein et al. 1990; Takahashi et al. 1991], providing further evidence that the cloned gene has properties consistent with those predicted for a tumor suppressor.

The Rb protein [p110RB] is a nuclear protein of 110 kD [Lee et al. 1987b], and is phosphorylated on both serine and threonine residues [Buchkovich et al. 1989; Ludlow et al. 1989; Shew et al. 1989]. These modifications are thought to be important regulators of p110RB and have been shown to occur in a cell cycle-dependent manner [Buchkovich et al. 1989; Chen et al. 1989; DeCaprio et al. 1989]. Cells in early G1 phase of the cycle contain exclusively unphosphorylated or underphosphorylated p110RB. At an as yet undefined point later in G1, the protein is hyperphosphorylated [p110RB] and exists in this state until M phase, although the level of phosphorylation does fluctuate slightly. Several studies have implicated the cyclin-dependent kinase (cdk) family of kinases as enzymes responsible for this phosphorylation [Lees et al. 1991; Lin et al. 1991]. Dephosphorylation of p110RB occurs in mitosis, possibly initiated during anaphase [Ludlow et al. 1993]. Recent studies have shown that both type 1 and 2A protein phosphatases can dephosphorylate p110RB in vitro, and strongly suggest that type 1 protein phosphatase is critical for this event in vivo [Alberts et al. 1993; Ludlow et al. 1993].

The mechanism by which p110RB suppresses tumorigenicity remains unknown. However, a possible explanation emerged with the discovery that p110RB physically associates with oncoproteins of many DNA tumor viruses, namely, the adenovirus EIA protein [Whyte et al. 1988], the SV40 T antigen [DeCaprio et al. 1988], and the E7 protein of human papillomavirus 16 [HPV16] [Dyson et al. 1989]. p110RB sequences required for these interactions were mapped to two large domains in the carboxy-terminal portion of the protein [Hu et al. 1990; Huang et al. 1990]. These domains are affected in all known naturally occurring mutant Rb proteins, suggesting an important role for this region of the protein in tumor suppression (for review, see Bookstein and Lee 1991). In addition, T antigen specifically binds the unphosphorylated form of the Rb protein, implying that this isoform is important in cell growth control [Ludlow et al. 1989]. One possibility, by analogy to the viral pro-
teins, is that this region of p110RB associates with cellular factors and, through these complexes, asserts its tumor-suppressing function. It has been shown that the carboxy-terminal half of p110RB is capable of complexing with a variety of cellular proteins using Rb protein affinity columns (Kaelin et al. 1991; Lee et al. 1991). Subsequently, using a protein-screening method, a number of potential p110RB-binding proteins have been identified; these include RBP-1 and RBP-2 (Defeo-Jones et al. 1991), a 46-kD protein (Huang et al. 1991), the E2F transcription factor (Helin et al. 1992, Kaelin et al. 1992, Shan et al. 1992), and several other proteins (Shan et al. 1992). The c-myc and N-myc proteins (Rustgi et al. 1991), the Cdc2 kinase (Hu et al. 1992), and the activating transcription factor 2 (ATF-2) (Kim et al. 1992) were also identified as likely p110RB-associating proteins by the investigation of logical candidates in vitro. However, it is likely that many unknown targets of Rb interaction remain, and a thorough understanding of Rb function requires their identification.

To further our understanding of the role of Rb in tumor suppression, an in vivo strategy was employed to identify human proteins capable of physically associating with p110RB. The yeast two-hybrid system devised by Fields and Song (1989) was chosen to provide a physiological environment in which to detect potential interactions involving the Rb protein. Recently, this system has been used to screen cDNA libraries for clones encoding proteins capable of binding to a protein of interest (Chien et al. 1991; Dalton and Treisman 1992; Yang et al. 1992). Here, we describe several improvements to this approach and the application of this modified technique to isolate cDNA clones encoding proteins that interact with the carboxy-terminal portion of p110RB. One clone isolated in this screen encodes a novel protein phosphatase type 1 catalytic subunit (PP-1ct2). The type 1 class of serine/threonine phosphatases is important for many cellular processes, including cell cycle regulation (for review, see Cohen 1989; Cyert and Thorner 1989). The cloning and characterization of this phosphatase is described, as well as its association with p110RB and the possible regulatory implications of this interaction.

Results

Strategy

To screen for cDNAs encoding proteins able to interact with p110RB, the general scheme outlined in Figure 1A was employed. First, two sets of fusion proteins are constructed; one generates a hybrid between sequences for the DNA-binding domain of the yeast transcription factor Gal4 (amino acids 1–147; Keegan et al. 1986) and a portion of the Rb protein. A second expression plasmid contains sequences for the Gal4 activation domain II (amino acids 768–881; Ma and Ptashne 1987a), fused to a cDNA library generated from human lymphocytes. As first demonstrated with Gal4–Gal80 interactions (Ma and Ptashne 1988) and later generalized by Fields and Song (1989), if the two proteins expressed in yeast are able to interact, the resulting complex will regain the ability to activate transcription from promoters containing the yeast transcription factor Gal4 (amino acids 1–147; Keegan et al. 1986) and a portion of the Rb protein. A second expression plasmid contains sequences for the Gal4 activation domain II (amino acids 768–881; Ma and Ptashne 1987a), fused to a cDNA library generated from human lymphocytes. As first demonstrated with Gal4–Gal80 interactions (Ma and Ptashne 1988) and later generalized by Fields and Song (1989), if the two proteins expressed in yeast are able to interact, the resulting complex will regain the ability to activate transcription from promoters containing Gal4-binding sites, upstream activating sequence from GAL1 (UASG).

A new yeast strain, Y153, has been constructed that provides a dual selection system to efficiently screen cDNA expression libraries for clones interacting with a...
protein of interest. The strain carries two chromosomally located reporter genes whose expression is regulated by Gal4. First, the *Escherichia coli* *lacZ* gene is under the control of the GAL1 promoter, and its usefulness in this system has been described (Fields and Song 1989). A second reporter, the selectable *HIS3* gene, was chosen because very low levels of the enzyme [imidazole glycerol phosphate (IGP) dehydratase] are required for prototrophy. To provide Gal4 control, the *HIS3* regulatory sequences have been replaced by the *GAL1* UAS <sub>G</sub>. Because Y153 is deleted for *gal4* (and its negative regulator *gal80*), expression of both reporters should be off in the absence of exogenous Gal4. However, the *GAL1—HIS3* fusion has residual *HIS3* expression sufficient to allow growth without exogenous histidine, even in the absence of Gal4. This can be overcome by growing cells in the presence of 25 mM 3-aminotriazole [3-AT], a chemical inhibitor of IGP dehydratase, which restores histidine auxotrophy (Kishore and Shah 1988). The low requirement for His<sup>+</sup> protein makes this selection very sensitive such that proteins that only weakly interact can be selected. To screen for associated proteins, Y153 cells expressing a protein of interest fused to the Gal4 DNA-binding domain are transformed with an activation domain-tagged cDNA library. Interacting hybrids are isolated by selecting for His<sup>+</sup> prototrophs and subsequently screened for the *HIS3* gene of the organism from which the library is derived. Colonies that are His<sup>+</sup> and blue are considered positives and are isolated for further analysis.

A second advantage of the *HIS3* selection/*lacZ* screen combination involves the elimination of false positives. We and others (P. Bartel and S. Fields, pers. comm.) have observed that a class of false positives appear in these library screens that depend on both plasmids but that will activate other nonspecific fusions bound to a DNA-binding domain. These positives are often transcription factors that are thought to access the promoter DNA adjacent to the target protein when overproduced. Because the two different reporter promoters, *HIS3* and *GAL1*, share only a small region of DNA sequences in common [150 bp that should mostly be protected by the binding of target protein fusions], this class of false positives will be largely diminished.

The efficacy of the His selection in identifying interacting proteins was tested using fusions to *SNF1* and *SNF4*, two proteins known to physically associate in vivo and whose interaction can be detected using the two-hybrid system (Fields and Song 1989). Simultaneous expression of both hybrid proteins is required to generate His prototrophy under selective conditions (Fig. 1B). Surprisingly, the *HIS3* transcription produced by the *SNF1—SNF4* interaction provided more resistance to 3-AT than the wild-type *HIS3* gene itself, indicating the potential for interacting hybrids to increase expression above wild-type levels, thus increasing the sensitivity of the selection. This selection was also found to work efficiently directly following cotransformation of the two test plasmids.

The Rb protein can specifically interact with SV40 large T in yeast

To determine whether the two-hybrid system was applicable for isolating genes encoding Rb-associated proteins, the ability of a known p110<sup>RB</sup>-binding protein, SV40 large T antigen (DeCaprio et al. 1988) to complex with a Gal4—Rb fusion in yeast was examined. A cDNA encoding amino acids 301–928 of the Rb protein, containing all the sequences required for T binding, was fused to the Gal4 DNA-binding domain sequences in a new expression vector, pAS1 [described below], to create pASRb2. On a second expression plasmid, YIpPTG10, the cDNA encoding the first 273 amino acids of T antigen was joined to those for the Gal4 activation domain II [amino acids 768–881; Ma and Ptashne 1987b]. Y153 cotransformants were then assayed for their ability to activate transcription of the *lacZ* gene. When both wild-type Rb and T fusions were present, cells were able to produce β-gal [Fig. 2], demonstrating that Rb and T antigen can interact in this system.

The specificity of the Rb—T antigen association was shown using two pAS1 constructs containing mutations in the Rb sequence. RbM6 contains a small deletion of the first T/E1A domain (amino acids 567–621), whereas H209 contains a naturally occurring point mutation changing the cysteine residue at position 706 to phenylalanine, and both fail to bind T in vitro (Bignon et al. 1990; Huang et al. 1990). As expected, when cotransformed with the T—Gal4 hybrid, these mutants fail to bind and activate transcription [Fig. 2]. This demonstrates that physical association detected in this system reflects the known in vitro-binding properties of both Rb and T antigen.

**Generation of activation domain-tagged cDNA libraries**

The use of a genetic selection to detect interacting proteins allows much larger cDNA libraries to be analyzed for associating clones. To facilitate construction of these cDNA libraries, a λ phage vector, λ ACT [activation domain], was created that fuses sequences for the Gal4 activation domain to cDNAs [Fig. 3A]. λ ACT is similar in principle to λ YES, yeast—E. coli shuttle [Elledge et al. 1991], and has several advantages over conventional methods for the production of large plasmid libraries. It has the ability to generate large cDNA libraries [10<sup>9</sup> recombinants/µg of cDNA] with a high percentage of inserts (>95%), has a large insert capacity (8.5 kb), can replicate and express fusion proteins in yeast, and can be simply converted from a phage to a plasmid using cre-lox mediated site-specific recombination. The ability to convert phage to plasmids with high efficiency facilitates the recovery of inserts and introduction of libraries into yeast. pACT, the plasmid excised from λ ACT [Fig. 3A], contains the ColE1 origin of replication and bla gene for replication and selection in E. coli, and LEU2, 2 µ origin, and the *ADCl* [Adh1] promoter sequences for selection, replication, and expression in *Saccharomyces*
**Figure 2.** Rb and SV40 T antigen interaction in yeast. (A) Fusion constructs used to test Rb and T association in yeast. The Rb2 fusion protein contains the Gal4 DNA-binding domain (amino acids 1-147, black hatched box) joined to a carboxy-terminal fragment of Rb. The T/E1A-binding domains of Rb are shown as stippled boxes. RbM6 is a deletion affecting the first T/E1A domain; RbH209 is a point mutation affecting the second domain. Neither mutant is able to bind T in vitro. The Gal4 trans-activation domain (amino acids 768-881, white hatched box) was fused to the amino terminus of T antigen (solid box). (B) Determination of β-galactosidase activity in transformed yeast cells. Y153 was transformed with various plasmids as indicated, and β-gal activity was determined by the colony lift method and quantitated using an ONPG assay. Three independent colonies per transformation were used for each ONPG determination.

*cerevisiae.* The ADC promoter drives expression of a hybrid protein consisting of the SV40 large T antigen nuclear localization signal and sequences encoding the activation domain II of Gal4. Fused to GAL4 at amino acid 881 is a polylinker containing an XhoI site into which cDNAs are inserted.

A human cDNA library was constructed in λ ACT using mRNA prepared from Epstein-Barr virus (EBV)-transformed human peripheral lymphocytes. The library contained $1.1 \times 10^6$ total recombinants with >95% inserts. It was amplified and converted to plasmid form, and plasmid DNA was prepared in bulk for yeast transformation.

A companion plasmid, pAS1, was constructed to facilitate creation of target protein fusions with the Gal4 DNA-binding domain (for details of construction, see Fig. 3B and Materials and methods). This plasmid contains TRP1, 2 μ origin, and the ADC1 promoter driving expression of the Gal4 DNA-binding domain (amino acids 1-147; Keegan et al. 1986) fused to a polylinker. The Gal4 derivative is tagged with the hemagglutinin (HA) epitope recognized by a commercially available monoclonal antibody (mAb 12CA5) (Babco, Richmond, CA). The polylinker contains several useful cloning sites, including Ncol and Ndel, that each have an in-frame ATG codon in their recognition sequences.

**Screening for cellular proteins that interact with the Rb protein**

Tumor suppression by the Rb protein likely requires association with cellular factors through sequences in the carboxy-terminal half of the protein. To screen for human proteins able to interact with this portion of p110RB, Y153 containing pASRb2 was transformed with the λ ACT human lymphocyte cDNA library and transformants were subjected to the screening procedure described above (Fig. 1). A total of ~2 million transformants were placed under selection (Fig. 4B). Of the transformants spread on synthetic complete (SC)—His, Leu, Trp plus 25 mM 3-AT plates, ~1% grew into colonies within 3–5 days. Many colonies were very small but were nevertheless included in the count. It is likely that the use of higher 3-AT levels would further reduce the background; however, we chose to use a lower level so that weak interactions would also be detected. At 25 mM 3-AT, the selection behaves more like a 100-fold enrichment.

After selection, transformants were screened for their ability to produce β-galactosidase using a filter lift assay (Breeden and Nasmyth 1985). One hundred thirty-nine His+ colonies were also blue in this assay (Fig. 4B). In general, larger His+ colonies were much more likely to be blue than the small colonies. These His+ blue colonies were considered positives in the initial screen and were used for additional studies.

**Identification of the positive clones dependent on Rb hybrid expression**

To test further whether the phenotype observed in the original screen was reproducible and dependent on the Rb hybrid, library-derived plasmids were selectively recovered by virtue of the yeast LEU2 gene carried on those plasmids to complement a leu8 mutation present in the *E. coli* strain, JA226 (Tschumper and Carbon 1980). Plasmids isolated were then used to transform Y153 either alone or with three test fusions in pAS1. The test fusions were pASRb2, pAS/N-Rb (containing cDNA for amino acids 1–300 of p110RB), and pAS/SNF1 (pSE1112), which contains the yeast SNF1 gene. Transformants were assayed for β-gal activity, and those showing activity only in the presence of pASRb2 were considered positive (Fig. 4A). Of the 139 original isolates, 28 of the recovered plasmids induced the expression of lacZ and did so only in the presence of pASRb2 (Fig. 4B). This
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Figure 3. Expression vectors for target protein and cDNA library construction. (A) λ ACT allows construction of cDNA libraries fused to sequences for the Gal4 transcriptional activation domain with subsequent conversion to plasmid form using cre–lox site-specific recombination as with λ YES [Elledge et al. 1991]. (B) pAS1 contains the sequences encoding Gal4 DNA-binding domain followed by a polylinker to facilitate construction of hybrids. Expression of the fusions in yeast is driven from the ADC1 promoter. Sequences for replication and selection in yeast and E. coli are indicated. The sequence of the polylinker region in each vector is shown as in-frame triplets to facilitate the creation of in-frame fusion proteins.

is likely to be an underestimate of the true number of associating clones isolated in the initial screen owing to the possibility of multiple library-derived plasmids present within the same yeast cell, only one of which would be likely to encode an associating protein. Because only a single E. coli transformant per positive was used to prepare plasmid DNA for retesting in yeast, several Rb-associating fusions could have been missed. No plasmids were recovered that produced β-gal in the absence of pAS1 derivatives.

One clone encodes a novel type 1 protein phosphatase

Sequence analysis from one of the positive clones, C27,
revealed that the cDNA insert encoded a protein with a predicted molecular mass of 38.6 kD and was identical to the PP-1α catalytic subunit [Barker et al. 1990] with two exceptions. First, an 11-amino-acid insert was found in the amino terminus starting at amino acid 18 (Fig. 5). This insertion is located at some distance from a highly conserved region [amino acids 60–130] that likely serves as the active site for the phosphatase [Bollen and Stalmans 1992], and thus should not interfere with the enzymatic function of the protein. Second, the 5’-untranslated region was different from the published sequence. Although the authenticity of the differences seen at the 5’ end need to be verified further by analysis of multiple isolates, the insert seen in the coding region is likely real, as both flanking sequences correspond to PP-1α. These findings suggest the potential for alternative splicing in the generation of the phosphatase messages. On the basis of the identity between the two human clones, we have named the C27 encoded isoyme PP-1α2.

Both PP-1α isoforms bind unphosphorylated p110Rb in vitro

To confirm and extend the binding data obtained in yeast, we then expressed PP-1α2 as a glutathione S-transferase (GST) fusion protein in E. coli [Smith and Johnson 1988]. To test the ability of this protein to bind p110Rb in vitro, a GST–PP-1α2-containing affinity matrix was incubated with human cell extracts from 2E3 cells, a WERI (Rb+) reconstituted cell line [Chen et al. 1992]. Following extensive washing, proteins were eluted and run on a SDS-PAGE gel and Western blotted, and the blot was probed with an anti-Rb antibody, 11D7 [Shan et al. 1992]. GST–PP-1α2 was able to complex with p110Rb, as was the GST–T antigen [GST–T] control, whereas a matrix of GST alone was not (Fig. 6, lanes 2–4). This result establishes that the PP-1α2 protein can interact with the native, full-length Rb protein.

The sequence identity of these isoforms outside of the amino-terminal insert suggested that PP-1α should also bind p110Rb. To test this possibility, a common carboxy-terminal region [amino acids 181–342 of PP-1α2] was fused to GST and the resultant protein was used for the in vitro-binding assay. As expected, this truncated protein was still able to bind the Rb protein [Fig. 6, lane 5] albeit with lower affinity than the full-length protein. Nonetheless, this confirms the idea that both PP-1α isoforms can bind p110Rb.

Both PP-1α isoforms interact preferentially with the unphosphorylated form of p110Rb [Fig. 6] analogous to the binding characteristics of T antigen [Ludlow et al. 1989]. This was confirmed using completely unphosphorylated Rb protein produced in E. coli, which was also able to bind GST–PP-1α2 in vitro [data not shown]. Although the ability of PP-1α to bind unphosphorylated Rb protein is clear, the possibility that the enzyme binds to the phosphorylated form of p110Rb and catalyzes the removal of phosphate from the protein cannot be ruled out. In support of this hypothesis, it has been shown recently that PPI is capable of dephosphorylating pp110Rb in vitro [Alberts et al. 1992; Ludlow et al. 1993].

Similar regions of Rb protein are required for binding PP-1α2 and T antigen

The p110Rb sequences required for binding PP-1α2 were examined to determine whether they coincided with the T/E1A-binding domains defined previously [Hu et al. 1990; Huang et al. 1990]. To accomplish this, a deletion set that had originally been used to delineate the T/E1A domains was employed [Huang et al. 1990]. Several carboxy-terminal deletion mutants, as well as the H209 point mutant, were subcloned into pAS1 [Fig. 7A] and
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Figure 5. Sequence of the PP-la isozyme isolated in this screen, PP-la2, is shown on the top line and compared with the previously reported sequence (Barker et al. 1990). Broken lines in the PP-la sequence indicate identity with the PP-la2 sequence. The 33-nucleotide insert found in the PP-la2 sequence and the resulting amino acid sequence are shown in italics. Two stretches of the pentapeptide LXSXE are underlined, and the polyadenylation signal AATAAA is boxed.

used to cotransform Y153 with the Gal4 activation domain–PP-la fusion-expressing plasmid [C27] and YIpPTG10. Western blot analysis showed that each of

Figure 6. In vitro binding of PP-la2 and p110RB. GST and in-frame GST fusions with cDNA encoding full-length PP-la2 [GST–la2], the carboxy-terminal 161 amino acids of PP-la2 [GST–ledc2], and the amino-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-polyacrylamide gels, and equivalent protein amounts were used in each lane. Extracts made from 2E3 cells (Chen et al. 1992) were mixed with the bound samples for 30 min at room temperature. Following extensive washing, complexes were separated by SDSPAGE and transferred for immunoblotting. The amount of Rb protein present and the extent of its phosphorylation in 2E3 cells was determined by immunoprecipitation with mAb 11D7 antibody (lane 1). The blot was probed with anti-Rb mAb 11D7 and visualized by fluorography.

the Rb fusions was expressed at a level that did not vary more than two- to threefold (data not shown). The resulting transformants were then assayed for β-gal activity. As seen in Figure 7B, binding of the PP-la2 fusion protein to Gal4–Rb was diminished by many of the same mutations, including the H209 point mutant, which eliminated T antigen binding with two exceptions. First, PP-la2 was able to bind the XS mutant, which deletes part of the second T domain (amino acids 634–774; Huang et al. 1990), although with greatly reduced affinity. Second, PP-la2 was unable to bind the Ssp mutant, which lacks the carboxy-terminal 160 amino acids of the Rb protein, whereas T can bind. However, previous studies with full-length T showed that this Rb mutant could not interact (Huang et al. 1990). Whether this difference is the result of the sensitivity of the assay or the portion of T used is unclear. The M1 deletion (amino acids 612–632), which affects the spacer region between the T/E1A domains, was the only mutation able to bind both PP-la2 and T. Clearly, a similar, although not identical, region of the Rb protein is required for the binding of both T and PP-la2.

PP-la can be coimmunoprecipitated with p110RB in a cell cycle-dependent manner

To further characterize the association between PP-la and p110RB, we examined whether these two proteins could be coimmunoprecipitated from human cells and whether the interaction was specific for any phase of the cell cycle. CV-1 cells were first synchronized in the Go/G1 phase of the cycle by density arrest, released, and
Figure 7. PP-Iα2 and T antigen bind to similar regions of the Rb protein. (A) Schematic of Gal4-Rb fusions used to determine binding domains. The Gal4 DNA-binding domain (amino acids 1–147; hatched box) is fused to various Rb mutants. The T/E1A-binding domains A and B, as defined by in vitro-binding experiments, are shown as stippled boxes. Domains affected by mutation are depicted as spotted boxes. (B) Detection of interactions between PP-Iα2 and Rb mutants in vivo. Y153 was cotransformed with the indicated panel of Gal4–Rb mutants and either the Gal4–PP-Iα2-expressing clone (C27) or YIpPTG10. All Gal4–Rb fusions were expressed at approximately the same level 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.

Figure 8. PP-Iα and p110RB associate in a cell cycle-dependent manner. (A) Analysis of p110RB immunoprecipitates. CV-1 cells were density arrested and released, and samples were taken at various time points. Extracts from each sample were immunoprecipitated with anti-Rb mAb 0.47 (Huang et al. 1990). The immunoprecipitates were then resolved by SDS-PAGE, and the gel was immunoblotted and probed with an antibody specific for the catalytic domain of PP1 (Heng-chun Li, pers. comm.). A band corresponding to PP1 was seen in G0/G1 and mid-G1 cells and was then lost as cells progressed through S and G2 before reappearing in M-phase cells (Fig. 8). The low intensity of the coimmunoprecipitated PP1 band in G0 and G1 is not unexpected, as immunostaining experiments have demonstrated that the enzyme is predominantly cytoplasmic at this time (Fernandez et al. 1992). This places the time of interaction between these proteins from M phase to approximately mid-G1 of the following cell cycle.

Discussion

An improved version of the yeast two-hybrid method has been developed and employed to isolate potential Rb-associated proteins. The newly developed ACT vector provides a simple means of generating large activation domain-tagged cDNA libraries that can then be converted to plasmid form for transformation of yeast. Introduction of a genetic selection, together with a convenient screening method, allows these large cDNA expression libraries to be efficiently searched for interacting proteins. This combination may also reduce the number of false positives by limiting common promoter sequences. The ability to modulate the stringency of the
His selection, by varying the amount of 3-AT used, potentially provides flexibility in the number and strength of the interactions that will be detected. A more relaxed selection was employed here to ensure that even weak interactions would be detected. Furthermore, it is not yet clear that all protein fusions to the activation domain of Gal4 are equally competent at transcriptional activation owing to potential geometrical constraints. Thus, even strong interactions may give weak transcriptional activation under certain circumstances.

Using this method, cellular proteins capable of interacting with the carboxy-terminal two-thirds [amino acids 301–928] of the Rb protein were identified. This portion of the protein was chosen for two reasons. First, it contains all sequences required for interacting with viral oncoproteins whose binding to p110RB is hypothesized to contribute to cellular transformation, at least in part, by competing with important cellular factors for p110RB binding. It has been shown that several cellular proteins bind to this region of the Rb protein and that T antigen can compete with them for binding [Kaelin et al. 1991; Lee et al. 1991]. Thus, it is not surprising that 28 potential associated proteins were isolated using this sensitive assay. Second, genetic evidence from human tumors containing mutant Rb proteins strongly implicates this region of the protein as being important for normal p110RB function [for review, see Bookstein and Lee 1991]. It is likely that a subset of clones isolated here interact with the Rb protein through this region and in vivo may play important roles in cellular growth control.

The ability of the two-hybrid system to detect potential enzyme–substrate interactions has been demonstrated recently through the identification of a likely substrate for the yeast SNF1 kinase using this method [Yang et al. 1992]. One clone isolated in this screen encodes the catalytic subunit of a type I protein phosphatase, PP-1α2, which may dephosphorylate p110RB in vivo. The protein encoded by this gene is identical to PP-1α except for an 11-amino-acid insert following residue 18, and both isoforms were shown to bind to the Rb protein. Examination of the PP1α amino acid sequence showed that it contains two stretches the pentapeptide, LXSXE, occurring at positions 51–55 and 191–195 of p110RB [Fig. 4]. This sequence is closely related to the LXXE stretch found in the viral Rb-associated proteins [Figge et al. 1988; Dyson et al. 1989], as well as RBP-1 and RBP-2 [Defeo-Jones et al. 1991], and known to be important for p110RB-binding by those proteins. Mutation of the cysteine residue to serine was found not to affect the ability of HPV E7 to bind p110RB [Phelps et al. 1992], although changing the same residue to glycine abolished binding [Barbosa et al. 1990]. While not all p110RB-binding proteins require this motif [Hclin 1992], Rb deletion analysis mapped the binding of PP-1α to the T/EIA-binding domains, suggesting a possible similarity between the cognate Rb-binding sequences. The role that these sequences play in the association between PP-1α and p110RB remains to be determined.

PP1 proteins are the major serine/threonine phosphatases in the nucleus and are among the most highly conserved enzymes known [Cohen et al. 1990]. They are important regulators of, among other processes, the cell cycle [for review, see Cyert and Thorner 1989]. As first shown in Aspergillus nidulans, mutation of the PP-1 homolog, bimC, led to an inability to progress through mitosis and a defect in normal polar growth [Doonan and Morris 1989] that could be fully complemented by the rabbit muscle PP-1α gene but not the PP2A gene [Doonan et al. 1991]. Two PP1 proteins, dis2+ [defective in sister chromatid disjoining] and sds21+, have also been shown to play an important role in the cell cycle of the fission yeast Schizosaccharomyces pombe [Ohkura et al. 1989]. dis2+ was originally defined as a mutant capable of entering mitosis normally but failing to exit properly and was later shown to encode a protein of striking homology to the rabbit PP-1 catalytic subunit [82% identity] [Ohkura et al. 1989]. sds21+ was isolated as a high copy suppressor of the cold-sensitive dis2-11 mutation and also shown to be highly homologous to both dis2+ [79% identical] and the rabbit PP-1 [74% identity] [Ohkura et al. 1989]. The proteins appear to have redundant functions in that only double mutations are inviable, with the exception of the dis2-11 mutation [Ohkura et al. 1989]. In addition, mutation of one of the PP1 isoforms in Drosophila also leads to a variety of mitotic defects [Axton et al. 1990].

What is the significance of the p110RB–PP1 interaction? Presumably the Rb protein is acting in a growth control pathway that is influenced by extracellular signals. These signals may be integrated via phosphorylation of p110RB. In this model negatively acting regulatory factors such as Cdk/cyclin complexes might promote cell cycle progression by phosphorylating and inactivating p110RB. PP1 would act as a positive activator of Rb function, acting in direct opposition to the cdk/cyclin functions. Progression through G1 would be determined by the balance of these opposing activities in much the same way that the wee1 and mik1 protein kinases and the cdc25 protein phosphatase antagonistically regulate the activity of the Cdc2/cyclin B complex for entry into mitosis [Russell and Nurse 1987]. Consistent with this model, the Rb protein–PP1 association is first observed when p110RB is thought to be dephosphorylated, namely in M phase. Although recent evidence suggests that dephosphorylation of p110RB may begin during anaphase [Ludlow et al. 1993], the precise timing that these events first occur is unclear. Subsequent binding to the unphosphorylated or hypophosphorylated p110RB isoforms by PP1 would likely enhance the ability of the enzyme to maintain the Rb protein in this underphosphorylated state. That such associations exist is supported by two pieces of evidence. First, PP1 can bind unphosphorylated Rb protein as shown by the in vitro-binding experiment [Fig. 6]. Second, the duration of the complex appears to extend well into the G1 phase of the next cycle, a time when p110RB is thought to be predominantly unphosphorylated [Buchkovich et al. 1989; Chen et al. 1989, DeCaprio et al. 1989]. Furthermore, it has recently been shown that p110RB can serve as a substrate for PP1 in vitro [Alberts et al. 1992; Ludlow et al. 1993].
Currently, it is hypothesized that the unphosphorylated form of the Rb protein is active in growth suppression. This notion is supported by several lines of evidence. Biochemically, T antigen and cellular factors such as E2F bind preferentially to the unphosphorylated form of the Rb protein [Ludlow et al. 1989; Chellappan et al. 1991], and it is this form of the protein that is found tethered to the “nuclear structure,” whereas phosphorylated and mutant forms of the protein are not [Mittnach and Weinberg 1991; Templeton 1992]. Functionally, microinjection of unphosphorylated Rb protein early in G1 is capable of inhibiting progression of cells through the cell division cycle [Goodrich et al. 1991]. Thus, temporarily the PPI–p110RB association also correlates with the presence of an active form of the Rb protein in the cell and may be required for the dephosphorylation event at the end of the cell cycle that regenerates this isoform. These observations suggest that this complex is important for the p110RB function in vivo.

A second, though not mutually exclusive, explanation is that p110RB regulates PPI. PPI activity and subcellular localization is known to be modulated by its association with other cellular factors. For example, mammalian PPI is found complexed with a 161-kD glycogen-binding protein that directs and stimulates PPI activity to proteins involved in glycogenolysis and glycogen synthesis [Cohen 1989]. Also, the enzyme is negatively regulated by protein inhibitors 1 and 2 [Cohen 1989], as well as newly defined nuclear inhibitors [Beullens et al. 1992]. In S. pombe, dis2+ immunocomplexes have been reported to contain additional proteins potentially involved in its regulation [Kinoshita et al. 1990]. One candidate for such a regulator, the sds22+ gene product, isolated as a suppressor of the dis2-11 mutation, has been shown to positively modulate PPI activity possibly through direct interaction with one of the PPI proteins [Ohkura and Yanagida 1991]. Whether the Rb protein can serve as a regulator and direct PPI activity to specific substrates during the mid-M to early G1 period is an intriguing possibility. In this connection the tethering of unphosphorylated Rb protein to the nuclear structure [Mittnach and Weinberg 1991; Templeton 1992] may be important for the subcellular localization of PPI, which is also found in a largely insoluble form in nuclear preparations [Beullens et al. 1992].

The phosphorylation of cellular proteins is recognized as an important regulatory mechanism governing a variety of cellular processes (for review, see Hunter 1987). Protein phosphatases are now emerging as critical enzymes in a wide range of cellular events as well. The inhibition of PPI activity (as well as PP2A) by the potent tumor-promoting factor, okadaic acid, has prompted some to hypothesize that PPI activity may play a role in maintaining normal restraints on cell growth [Barker et al. 1990]. Consistent with that, microinjection of PPI into cells in G1 phase blocks progression into S phase, similar to the Rb protein [Alberts et al. 1992]. The PPIα association with p110RB may provide an important link in understanding these phenomena. Conversely, cells with PP-1α mutations that specifically disrupt its interaction with p110RB might be expected to have a transformed phenotype because of inappropriate Rb protein phosphorylation or similar effects on other proteins requiring an active phosphatase–p110RB complex. The chromosomal location of the PPIα gene, 11q13, is associated with the etiology of certain cancers [Barker et al. 1990], although the involvement [if any] by the enzyme in these neoplasias is unknown. A thorough understanding of the influences imparted by this interaction should yield important information on how these proteins regulate cell growth.

Materials and methods

Bacterial and yeast strains

E. coli [M107 {endA1, gyrA96, thi, hsdR17, supE44, relA1, Δ[lac-proAB], [F', traD36, proAB+, lacF' lacZAM15}] [Yannisch-Perron et al. 1985], and DH5 [F-, recA1, endA1, hsdR17, supF44, thi, gyrA, relA1] were the transformation recipient for all plasmid constructions. \( \text{JM}^2 \text{B} \text{L}2 \text{I}-\text{LysS} \) [Studier et al. 1990] was used for the expression of GST fusion proteins. Y153, \( \text{MAT}^a \text{leu}2-3,112, \text{ura}3-52, \text{trp}1-901, \text{his}3-\Delta 200, \text{ade}2-101, \text{gal}4^+ \text{gal}80^+ \text{URA3}^{33} \text{GAL}^{101} \text{LACZ} \) [Lehter and Johnston 1992] was used to recover expression plasmids from yeast. The E. coli B strain BL21–LysS [Studier et al. 1990] was used for the expression of GST fusion proteins. Y153, \( \text{MAT}^a \text{leu}2-3,112, \text{ura}3-52, \text{trp}1-901, \text{his}3-\Delta 200, \text{ade}2-101, \text{gal}4^+ \text{gal}80^+ \text{URA3}^{33} \text{GAL}^{101} \text{LACZ} \) [Lehter and Johnston 1992] to create Y152. YOJO-Z contains a GAL–lacZ reporter integrated at an unknown chromosomal site. The integration of pB10499 was targeted adjacent to the LYS2 locus by cleavage with Stul, which cleaves in plasmid-borne LYS2 sequences before transformation and was selected by Ura prototrophy. The second recombination event was selected by growth on plates supplemented with 5-fluoro-orotic acid as described [Flick and Johnston 1990] and screening for the His+ phenotype. A second GAL–lacZ fusion gene was introduced into Y153 by two-step gene replacement by integration of pB10499 (URA3 GAL–HIS3) [Flick and Johnston 1990] into YJO–Z (\( \text{MAT}^a \text{leu}2-3,112, \text{ura}3-52, \text{trp}1-901, \text{his}3-\Delta 200, \text{ade}2-101, \text{gal}4^+ \text{gal}80^+ \text{GAL}^{101} \text{LACZ} \) [Lehter and Johnston 1992] to create Y152. YOJO-Z contains a GAL–lacZ reporter integrated at an unknown chromosomal site. The integration of pB10499 is targeted adjacent to the LYS2 locus by cleavage with Stul, which cleaves in plasmid-borne LYS2 sequences before transformation and was selected by Ura prototrophy. The second recombination event was selected by growth on plates supplemented with 5-fluoro-orotic acid as described [Flick and Johnston 1990] and screening for the His+ phenotype. A second GAL–lacZ fusion gene was introduced into this background to increase the sensitivity to X-gal staining. The GAL–lacZ reporter contained on the Yip plasmid, pRY17 [URA3 GAL–lacZ] [Yocum et al. 1984] was introduced by selection for Ura prototrophy to create Y153.

Media, enzymes, assays, and genetic methods

For drug selections, Luria broth [LB] plates were supplemented with ampicillin [50 \( \mu \text{g/ml} \)]. Minimal media plates for E. coli, lacking leucine and containing ampicillin were used. Yeast YEPD and SC media were prepared as described [Rose et al. 1990]. Restriction endonucleases, E. coli DNA polymerase I large fragment, T4 polynucleotide kinase, T4 DNA polymerase, and T4 DNA ligase were purchased from New England Biolabs. Deoxyribonucleotides and ATP were purchased from P-L Biochemicals. Drugs were purchased from Sigma.

Construction of a ACT

pSE1107 was constructed in several steps. First the Hpol–SalI LEU-containing fragment from YEP13 was inserted into YEP24 cleaved with Stul and SalI, replacing the URA3 gene, to create pSE1101. The large SalI–PvuII fragment of pSE1101 was ligated in a three-way ligation to an 844-bp Sphi–BanHI fragment from pBTM146 [F. Bartel and S. Fields, unpubl.], in which Sphi was made blunt by T4 polymerase, and a 435-bp BanHI–
Sall fragment of pBTM116, to create pSE1106. The 844-bp fragment contains the ADC1 promoter driving a fusion protein consisting of the nuclear localization sequence from SV40 T antigen fused to the activation domain of GAL4 starting at amino acid 768. The 435-bp fragment contains the sequence starting at amino acid 222 of 100 mM spermine, incubated on ice for 30 min, pelleted for 15 min in an Eppendorf centrifuge, washed three times for 30 min each with 1 ml of spermine wash buffer [70% EtOH, 10 mM MgAc\textsubscript{2}], 0.3 M NaAc at pH 7 and once with 1 ml of 70% EtOH (Hoopes and McClure 1981). cDNA was resuspended in 50 μl of TE buffer and made flush by treatment with T4 DNA polymerase under the conditions suggested by the supplier, followed by the addition of 5 μl of 0.5 M EDTA and phenol/chloroform extractions, and ethanol precipitation. cDNA, approximately 4 μg, was resuspended in 7 μl of TE buffer and was ligated to 2 μg of an adapter in a total volume of 10 μl at 4°C overnight. The sequence of the kinased adaptors was 5'-GGGCTTTAGTGCCC-3' (top strand), and 5'-GGAGGCCCGAGGCCC-3' (bottom strand). After ligation, 170 μl of TE, 20 μl of 1 M KCl, and 10 μl of 100 mM spermine were added, incubated on ice for 30 min and precipitated and washed as described above. Adapted cDNA was resuspended in 20 μl of TE buffer and run on a 1% low melting point agarose gel. cDNA of 600 bp and longer was gel purified for ligation into λ ACT arms. cDNA (0.1 μg) was ligated to 2 μg of T-filled λ ACT plasmid DNA, prepared as described previously (Elledge et al. 1991) in a volume of 4 μl at 4°C overnight and packaged using one Gigapack Gold packaging extract (Stratagene, La Jolla, CA). Total recombinants (1.1 x 10\textsuperscript{9}) were obtained. Phage libraries were amplified on LE392.

Automatic subcloning conversion of the human cDNA library in λ ACT into plasmid was accomplished by incubation of 10° phage with 2 ml of a fresh overnight culture of BNN132 in 10 mM MgCl\textsubscript{2} for 30 min at 30°C without shaking. Two milliliters of LB was added, and cells were incubated with shaking for 1 hr at 30°C. Cells were then plated on ten 150-mm LB plates with 50 μg/ml of ampicillin and incubated at 37°C overnight. Ap\textsuperscript{r} cells were scraped from these plates, added to 3 liters of terrific broth (Maniatis et al. 1982) with 100 mM spermine and grown to stationary phase. Plasmid DNA was prepared and purified using CsCl density gradients by standard methods (Maniatis et al. 1982).

Construction of pAS1

The ADC – GAL4[1-147] – ADC terminator fragment from pMA424 [Ma and Ptashline 1987a] was cloned into plc20H [Marsh et al. 1984] by a three-way ligation between gel-purified fragments including the EcoRV-Xhol ADC1 promoter and Xhol-SphI GAL4 – ADC terminator fragments from pMA424 and SphI – EcoRV-cleaved plc20H to create pHI. An EcoRI – BamHI double-stranded oligonucleotide encoding an HA epitope tag and several cloning sites was fused to the GAL4 DNA-binding domain of EcoRI – BamHI-cut pHI. The sequence of this oligonucleotide is GAA TTA ACT TAC CCA TAC GAT GTT CCA GAT TAC GTT TTG GGT GGT CAT ATG GCC ATG GAG GCC CCG GGG ATC C and has the following restriction sites that are unique in pAS1: EcoRI, Ndel, NotI, SalI, Smal, BamHI. The sequence is presented as in-frame triplets. The NcoI and Ndel sites contain in-frame ATG codons that are particularly useful for fusing full-length cDNAs generated by polymerase chain reaction (PCR) to the DNA-binding domain encoding sequences of GAL4.

Rb and T expression plasmids

pASRb2 was created as follows: The HindIII site in p44-1 [Huang et al. 1992] was extended by polymerase chain reaction (PCR) to the DNA-binding domain II and the ADH1 transcriptional terminator, was subcloned into BKS[+]. This vector was cleaved with HindIII and the 823-bp HindIII fragment from Y62-25-2 encoding T antigen amino acids 1–273 fused in-frame with the GAL4 sequence resulting in pT225. A SacI – EcoRI fragment containing the ADC1 promoter from YC-DE2 [Beier and Young 1982] was then inserted into pT225 upstream of the T – GAL4 fusion. This plasmid, pPTG7, was cleaved with XhoI and BamHI, and the insert was subcloned into Yep13, resulting in YlpPTG10. The plasmid was linearized at the unique SacI site in the LEU2 gene preceding yeast transformation to increase the integration frequency.

Library screening

Y153 was transformed to Trp prototrophy with pASRb2 by the method of Schiestl and Geitz (1989). A single colony was grown in SC – Trp medium and transformed with library DNA using total yeast RNA as carrier. Aliquots were taken from each transformation mix before plating and used to determine the transformation efficiency by plating on SC media lacking tryptophan and leucine. The transformation mix was then plated on 15-cm petri dishes containing SC media lacking tryptophan, leucine, and histidine but including 25 mM 3-AT (Sigma), and incubated at 30°C for 3–5 days. His\textsuperscript{+} colonies were then screened for B-gal activity using a filter lift assay [Breeden and Nasmyth 1985]. Colonies were transferred onto nitrocellulose filters, permeabilized by freezing in liquid nitrogen (1 min), and thawed at room temperature. Filters were then overlaid on Whatman 3MM paper saturated with a LacZ-X-gal solution [Breeden and Nasmyth 1985].
Hoffman and Winston (1987) and used to transform JA226 via electroporation using a Bio-Rad GenePulser according to the manufacturer's specifications. Transformations were plated on minimal media lacking leucine and containing ampicillin.

Recovery of plasmids from yeast

Total DNA from yeast was prepared according to the method of Dur|ee et al. (1985; 8 ml/15-cm dish) and incubated at 30°C. 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 analyzed further.

Sequence analysis

C27 and related subclones were sequenced using dyeoxy NTPs and Sequenase 2.0 according to the manufacturer's specifications (U.S. Biochemical). Sequence analysis and homology searches were performed using DNASTAR software (DNASTAR, Inc., Madison, WI).

Quantitation of β-gal activity in yeast

 Cultures of 2.5 ml were grown in the appropriate selecting media to OD600 of 1.0-1.2. Cells were then prepared and permeabilized as described (Guarente 1983). For quantitation using α-nitrophenyl-β-d-galactoside (ONPG), standard conditions were used (Guarente 1983). When using chlorophenyl-red-β-d-galactopyranoside (CPRG; Boehringer Mannheim), the same procedure was followed, except cell pellets were resuspended in 900 μl of H buffer (100 mM HEPES, 150 mM NaCl, 2 mM MgCl₂, 1% BSA at pH 7.0), 100 μl of 50 mM CPRG was added following permeabilization, and the amount of liberated CPR was determined by OD574.

Construction of GST fusions, protein preparation, and in vitro binding

To construct GST–PP-Ia2, the plasmid C27 was partially digested with BglII and the 1.4-kb insert fragment was subcloned into the BamHII site of pGEX-3X. GST–PP-Ia was created by subcloning the 850-bp fragment following complete digestion of C27 with BglII into pGEX-3X. GST–T was made by cutting pY62-25-2 with HindIII, blunt-ending with Klenow, and subcloning the 823-bp fragment into pGEX-3X cut with Smal. Expression of GST fusion proteins was induced with 0.2 mM IPTG. Cultures were centrifuged at 10K for 5 min, and the resultant pellet resuspended in buffer A [150 mM NaCl, 5 mM DTT, 2 mM EDTA, 50 mM Tris (pH 8.0), 1 mM phenylmethylsulfonyl fluoride (PMSF), 8 μg of leupeptin, and 8 μg of antipain]. Four milligrams of lysozyme was added, and the cells were held at 4°C for 30 min. Four hundred microliters of 10% NP-40 was added, and the cells were lysed by sonication. Cell debris was removed by centrifugation (10K for 30 min), and the supernatant was added to glutathione-coated beads.

The in vitro-binding assay was performed as follows. Extracts made from 2 × 10⁶ 2E3 cells (Chen et al. 1992) were incubated with beads containing 2–3 μg of GST or GST fusion proteins in lysis buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.1% NP-40, 50 mM NaF, 1 mM PMSF, 1 μg of leupeptin/ml, 1 μg of antipain/ml] for 30 min at room temperature. Complexes were washed extensively with lysis buffer, boiled in loading buffer, and run on 7.5% SDS-polyacrylamide gels. Gels were transferred to immobilon membranes and immunoblotted with anti-Rb mAb 11D7. Following the addition of an alkaline phosphatase-conjugated secondary antibody, bound Rb protein was visualized with 5-bromo-4-chloro-3-indolylphosphate toluidinium and nitro blue tetrazolium (BCIP/NBT; Promega, Madison, WI).

Cell cycle synchrony and immunoprecipitation

CV-1 cells were grown to confluence and maintained in 10% fetal calf serum (FCS) for 2 weeks. Cells were then split 1:2 and grown in 10% FCS. Aliquots were lysed in 500 μl of lysis 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 of leupeptin/ml, 1 μg of antipain/ml] and cleared by centrifugation [14K for 2 min at room temperature]. The supernatant was diluted in 400 μl of lysis buffer. The same as lysis 250, except lacking NaCl, 850 μl was immunoprecipitated with anti-Rb polyclonal antibody 0.47 (Huang et al. 1990), and 50 μl was directly assayed for PP-Ia content by Western blotting with an antibody raised against the catalytic subunit of PP-Ia prepared from bovine heart (kindly provided by Heng-chun Li, Mt. Sinai Medical School, NY). Immunoprecipitates were separated by SDS-PAGE and immunoblotted using the same anti-PP-Ia antibody. Proteins were visualized using BCIP/NBT. Using an independently prepared anti-PP1 antibody (Brautigan et al. 1985), identical results to those in Figure 8 were obtained (data not shown).

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