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Differential Specificity for Binding of Retinoblastoma Binding Protein 2 to RB, p107, and TATA-Binding Protein

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The growth suppressor activities of the RB and p107 products are believed to be mediated by the reversible binding of a heterogeneous family of cellular proteins to a conserved T/E1A pocket domain that is present within both proteins. To study the functional role of these interactions, we examined the properties of cellular retinoblastoma binding protein 2 (RBP2) binding to RB, p107, and the related TATA-binding protein (TBP) product. We observed that although RBP2 bound exclusively to the T/E1A pocket of p107, it could interact with RB through independent T/E1A and non-T/E1A domains and with TBP only through the non-T/E1A domain. Consistent with this observation, we found that a mutation within the Leu-X-Cys-X-Glu motif of RBP2 resulted in loss of ability to precipitate p107, while RB- and TBP-binding activities were retained. We located the non-T/E1A binding site of RBP2 on a 15-kDa fragment that is independent from the Leu-X-Cys-X-Glu motif and encodes binding activity for RB and TBP but does not interact with p107. Despite the presence of a non-T/E1A binding site, however, recombinant RBP2 retained the ability to preferentially precipitate active hypophosphorylated RB from whole-cell lysates. In addition, we found that cotransfection of RBP2 can reverse in vivo RB-mediated suppression of E2F activity. These findings confirm the differential binding specificities of the related RB, p107, and TBP proteins and support the presence of multifunctional domains on the nuclear RBP2 product which may allow complex interactions with the cellular transcription machinery.

An important step toward defining a mechanism underlying tumor suppressor activity of the *Rb* gene was the observation that the transforming products of adenovirus (E1A), simian virus 40 (large T), and human papillomavirus (E7) could precipitate wild-type RB protein (8, 16, 57, 59). This, in turn, led to the identification of a family of cellular proteins that can reversibly bind to a discrete domain on RB, referred to as the T/E1A pocket by using the same specificity as the viral products (10, 12, 33–35, 39, 51, 52, 54). The subsequent observation that protein binding was inhibited following RB phosphorylation in the late G_1 phase of the cell cycle suggested the hypothesis that RB, as well as the related p107 product, may regulate the functional activity of its binding partners by a cell cycle-dependent pattern of physical association (19, 48, 53).

The binding domains within the RB protein have been delineated by a series of in vitro and naturally occurring mutants as two noncontiguous regions designated domains A and B which are interrupted by a spacer sequence (29, 30, 32) (Fig. 1A). This structure generates a hypothetical pocket conformation that (i) is conserved among a family of E1A-binding proteins, including RB, p107, and p130 (19, 26, 40, 45); (ii) demonstrates binding to a series of viral and cellular proteins which can be blocked by short peptides containing a leucine-X-cysteine-X-glutamic acid motif (LXCXE, where X represents any amino acid); (iii) can bind to members of the E2F transcription family to modulate gene transcription (48, 58); and (iv) is the target for all of the naturally occurring RB mutants that have been isolated to date (42). The importance of the pocket structure was further underscored by the recent observation that the conserved C-terminal region of the TATA-binding protein, TBP, has significant homology with domain A of the RB pocket and can precipitate transcription factors in common with RB (24). To date, three different transcription factors, Pu.1 (24), c-Myc (27, 44), and E2F1 (25), have been shown to bind with both RB and TBP, and it has been proposed that RB may mimic the binding surface of TBP to block transcriptional activation (25). These findings imply a central role for the pocket-binding activity of RB and suggest that understanding the properties of the RB-binding partners is necessary to define tumor suppressor pathways.

Alternatively, mutational analysis of the viral oncoproteins has identified conserved regions within the E1A, large T, and E7 transforming products that can interact noncovalently with the RB protein (1, 16, 21, 50). In the case of E1A, two discrete domains designated regions 1 (spanning amino acids 37 to 54) and 2 (amino acids 115 to 132) have been shown to bind independently, but with differing affinities, to RB (14, 15). E1A region 2 encompasses a consensus LXCXE sequence which encodes a high-affinity RB-binding site (14). This LXCXE sequence is also present in the large T and E7 transforming proteins, as well as in cellular proteins RBP1 and RBP2 and the cyclin D1-3 family (10, 12, 18, 35), and a 14-amino-acid large T antigen synthetic peptide containing this domain can compete with the viral and cellular proteins for binding to RB (9, 34). In addition to the binding sites for RB, however, E1A has evolved a complex multifunctional structure that encodes independent protein-binding domains that are essential for transactivation or transformation activities (15, 55, 56).

We have studied the properties of the cellular RB-binding protein RBP2 and have found evidence of distinct domains that can interact with the RB, p107, and TBP products with differing specificities. While RBP2 precipitates the p107 pocket exclusively through its LXCXE motif, RBP2 can also bind to RB and TBP by a mechanism independent from the T/E1A

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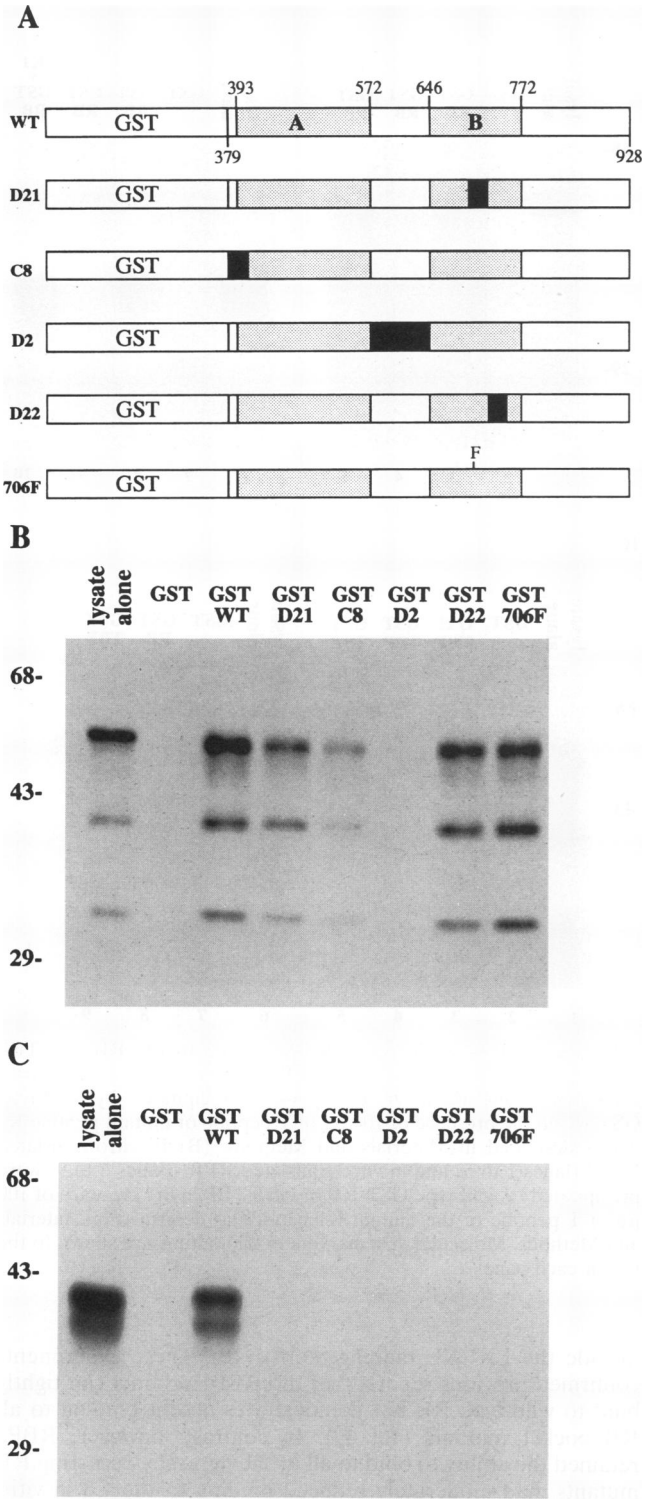

FIG. 1. RBP2 retains the ability to bind in vitro to a series of RB pocket mutants. (A) GST-RB (amino acids 379 to 928) fusion constructs representing the wild type (WT); naturally occurring mutants D21, D22, and 706F; and in vitro-generated mutants C8 and D2 (34). Noncontiguous domains A and B of the RB pocket (29, 30, 32) are depicted as stippled boxes separated by a spacer region. Deletions are shown as black boxes, and a missense cysteine-to-phenylalanine (F) substitution within exon 21 is indicated as previously described (706F) (34, 36). (B) RBP2 (amino acids 1105 to 1558) (20) was in vitro translated in the presence of [^{35}S]methionine and precipitated by the GST leader peptide or the indicated GST-RB fusion constructs, and the washed pellets were resolved by SDS-PAGE and fluorography. (C) RBP1 (isoform I containing amino acids 857 to 1204) (20, 49) was in vitro translated in the presence of [^{35}S]methionine and precipitated by the GST leader peptide or the indicated GST-RB fusion constructs, and the washed pellets were resolved by SDS-PAGE and fluorography. Molecular size markers in kilodaltons are shown to the left of panels B and C.

pocket, and we have located this non-T/E1A binding domain of RBP2 on a 15-kDa fragment that is present C terminally with respect to the LXCXE sequence. These findings demonstrate important differences between the binding properties of the related RB, p107, and TBP proteins and suggest a model in which cellular binding proteins with multifunctional domains, such as RBP2, may allow complex multimer formation with its binding partners.

MATERIALS AND METHODS

Cell lines. Human tumor cell lines were obtained from the American Type Culture Collection (Rockville, Md.) or as previously described (4) and were propagated in RPMI medium supplemented with 10% fetal calf serum and antibiotics.

Plasmids. *RBP2* cDNA encoding amino acid residues 1105 to 1558 (20) was subcloned into bacterial expression vector pGEX3X (Pharmacia) to generate a glutathione *S*-transferase (GST)-RBP2 fusion protein. GST–wild-type RB, GST pocket mutant RB plasmids, GST-p107, GST-p107 (Cys-713 to Phe), and GST-p107 A/B (RB spacer switch) have been described previously (17, 19, 34, 38). GST-p107m with a switched RB spacer sequence (GST-p107m/RBS) was constructed by substituting a 3′ *Eco*RI fragment from pGEX2TK-p107 A/B RBS (17) with the analogous *Eco*RI fragment from pGEX2TK-p107 Cys-713 to Phe. *RBP2* cDNA containing a point mutation within the LXCXE sequence (glutamic acid to lysine; E-to-K substitution) was generated by PCR amplification by using synthetic oligonucleotides carrying the GAA-to-AAA mutation, followed by subcloning into pGEM4 (Promega, Madison, Wis.) and pGEX3X. pGEM4-RBP1 (isoform I) has been previously described (49). The nucleotide sequences of the chimeric GST fusion plasmids and the in vitro transcription plasmids were confirmed by nucleotide sequencing, and diagnostic sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the fusion proteins was performed to confirm synthesis of comparable levels of protein prior to each assay. Chloramphenicol acetyltransferase (CAT) reporter vectors pTA-ATF-E2F-CAT and pTA-ATF-E2FM-CAT were supplied by S. Weintraub and D. Dean (Washington University) (58). RB expression vectors pRC-CMV-Rb and pRC-CMV-Rb (deletion exon 21) have been described previously (38). RBP2 mammalian expression vector pCMV-Neo-CMV-Bam-RBP2 was supplied by D. Defeo-Jones and A. Oliff (Merck Research Laboratories).

In vitro protein-binding assays. GST fusion plasmids were expressed and purified as previously described (34, 38), and the pGEM4-RBP1, pGEM4-RBP2, and pGEM4-mutant RBP2 plasmids were subjected to in vitro transcription and translation with a rabbit reticulocyte lysate system and [^{35}S]methionine as described by the manufacturer (Promega). Binding assays were performed by incubating the Sepharose-linked GST fusion proteins (approximately 20 ml of transformed bacterial growth for each incubation) with the [^{35}S]methionine-labeled in vitro translation products in 500 μl of NETN buffer (20 mM Tris [pH 7.5], 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride) for 1

h at 4°C. Peptide competitions were performed with 100 μg of the synthetic wild-type (T) and mutant (K1) large T peptides as previously described (9). The sequence of the T peptide is NLFCSEEMPSSDDE, and that of the K1 peptide is NLFCSKEMPSSDDE. The beads were washed six times in cold NETN buffer, resolved by SDS-PAGE, and visualized by fluorography.

Antibodies. Polyclonal α-RBP2 antisera were generated by injecting GST-RBP2 fusion protein (codons 1105 to 1558) three times over a 6-week period into New Zealand White rabbits. Antiserum was precleared twice with the GST-Sepharose leader sequence and then used at a dilution of 1:200 in phosphate-buffered saline (PBS) for immunoblotting. Monoclonal antibodies to RB (G3-245) were used as recommended by the manufacturer (PharMingen).

Immunoblotting. Subconfluent cultures (approximately 2×10^6 cells) of tumor cell lines were washed twice in PBS and then resuspended in 500 μl of lysis buffer (50 mM Tris [pH 7.5], 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride) at 4°C for 30 min. Lysates were centrifuged at 10,000 × *g* for 5 min at 4°C, and the supernatant was frozen until used. Nuclear and cytoplasmic proteins were fractionated as previously described (11). Unlabeled whole-cell lysates (400 μg of total cell protein) were rocked for 1 h at 4°C with purified GST fusion protein in a final volume of 500 μl of lysis buffer. After being washed five times in cold buffer, pellets were resuspended and subjected to SDS-PAGE followed by electroblotting to nitrocellulose. Nitrocellulose filters were incubated overnight with a 1:200 dilution of a monoclonal anti-RB (α-RB) antibody (PharMingen) in PBS containing 5% powdered milk and 1% bovine serum albumin. Conjugation with secondary antibodies and visualization were performed by using an ECL kit (Amersham) as recommended by the manufacturer.

CAT assay. Transfections were done in Rb(−/−) H2009 non-small-cell lung carcinoma cells by using Lipofectin as described by the manufacturer (Life Technologies). Ten micrograms of pTA-ATF-E2F-CAT (E2F-CAT) or pTA-ATF-E2FM-CAT (mE2F-CAT), 5 μg of CMV-RB or pRC-CMV-RB (deletion exon 21), and 5 μg of pCMV-Neo-CMV-Bam-RBP2 were transfected as indicated into 100-mm-diameter subconfluent plates. A 0.5-μg luciferase vector sample was cotransfected with each CAT construct to control for transfection efficiency. CAT activity was assayed as previously described (7).

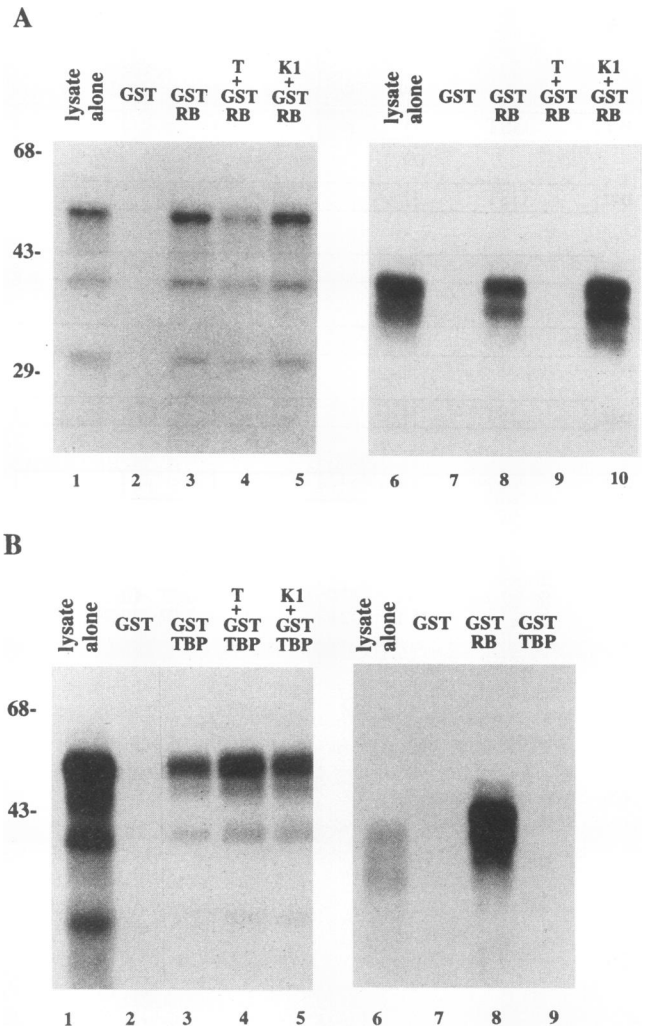

FIG. 2. RBP2 encodes non-T/E1A binding to the RB and TBP products. (A) In vitro-translated RBP2 (lanes 1 to 5) and in vitro-translated RBP1 (lanes 6 to 10) were precipitated by wild-type GST-RB in the presence of 100 μg of T peptide or mutant peptide K1 (9) as described in Materials and Methods. (B) In vitro-translated RBP2 (lanes 1 to 5) and in vitro-translated RBP1 (lanes 6 to 9) were precipitated by wild-type GST-RB or GST-TBP in the presence of 100 μg of T peptide or the mutant K1 peptide as described in Materials and Methods. Molecular size markers in kilodaltons are shown to the left of each panel.

RESULTS

RBP2 binds in vitro to a family of RB T/E1A pocket mutants. The *RBP1* and *RBP2* genes were initially isolated because of the ability of their products to bind to a 60-kDa C-terminal fragment of the RB protein (10). In the course of experiments done to study the functional roles of the RBP1 and RBP2 gene products, we compared their binding patterns with those of a series of in vitro and naturally occurring RB mutants that had been previously demonstrated to exhibit defective protein-binding activity (34). cDNA encoding wild-type RB, a deletion of exon 21 isolated from small-cell lung cancer line H1436 (D21), an in vitro deletion from domain A of the RB pocket (C8), an in vitro deletion of the RB spacer region (D2), a deletion of exon 22 from small-cell lung cancer line H69 (D22), or a point mutant with a single cysteine-to-phenylalanine substitution from small-cell lung cancer line H209 (706F) were expressed as GST fusion proteins (34) (Fig. 1A) and then used to precipitate either in vitro-translated RBP1 (isoform I) (49) or RBP2 (spanning 454 amino acids that include the LXCXE binding motif) (10). These experiments confirmed previous reports that the RBP1 product can tightly bind to wild-type RB but demonstrates absent binding to all RB pocket mutants (10, 49). In contrast, however, RBP2 retained the ability to bind to all of the naturally occurring RB mutants, had moderately reduced binding to the C8 in vitro mutant, and lost the ability to bind only to the D2 spacer deletion mutant (Fig. 1B and C).

Differential specificity of RBP2 binding for the RB and p107 pockets and the related TBP product. To address whether the binding surface mediating the interaction between RBP2 and RB overlaps with the T/E1A pocket, we repeated the precipitations with GST-RB fusion protein and in vitro-translated RBP1 and RBP2 in the presence of 100 μg of either T peptide (T) or the mutant K1 peptide (K1) (9) (Fig. 2A). The T peptide is a synthetic polypeptide that spans 14 amino acids and

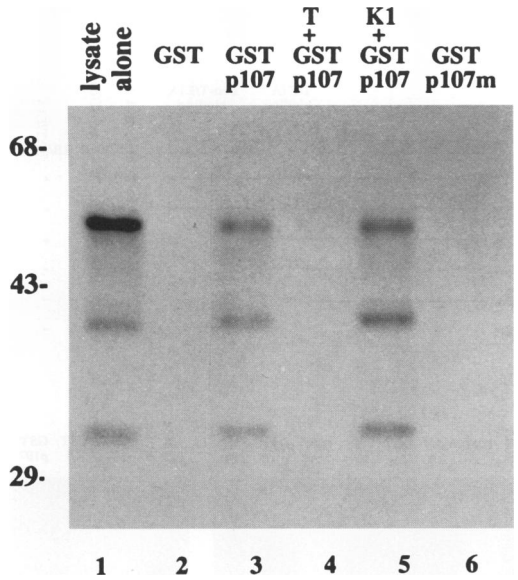

FIG. 3. RBP2 binds p107 exclusively in the T/E1A pocket. In vitro-translated RBP2 was precipitated by the GST leader peptide (lane 2), GST-p107 in the presence or absence of 100 μg of T peptide or the K1 peptide (lanes 3 to 5), or the p107$^{(713F)}$ point mutant (GST-p107m) (lane 6) and then subjected to SDS-PAGE and fluorography. Molecular size markers in kilodaltons are shown on the left.

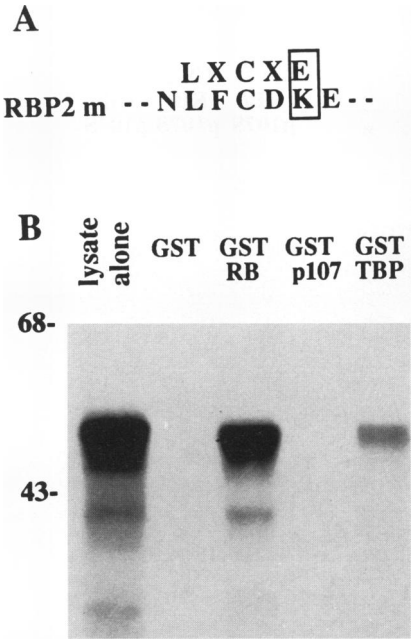

FIG. 4. An LXCXE mutation abolishes T/E1A binding to p107 but not non-T/E1A binding to RB. (A) Partial amino acid sequence of the RBP2 product showing the LXCXE binding motif at codons 1373 to 1377 (10) and the single glutamic acid (E)-to-lysine (K) substitution generated in a mutant RBP2 clone (RBP2m). (B) In vitro-translated RBP2m was precipitated by either the GST leader peptide or fusion protein GST-RB, GST-p107, or GST-TBP and then subjected to SDS-PAGE and fluorography. Molecular size markers in kilodaltons are shown to the left.

includes the LXCXE motif that is conserved in viral oncoproteins and in selected cellular RB-binding partners, while the K1 peptide contains the same sequence except for a single glutamic acid-to-lysine substitution (9). Consistent with our earlier precipitation experiments with the RB pocket mutants, we detected a reduced ability of the T peptide to compete for binding of RB to RBP2 (Fig. 2A, lane 4), while in contrast, the T peptide completely abolished RB binding to RBP1 (10, 49) (Fig. 2A, lane 9). In addition, the T peptide also failed to compete for binding of RBP2 to the D21, D22, and 706F pocket mutants of RB (data not shown). These data suggest that RBP2 can interact with RB through a non-T/E1A mechanism.

We also examined whether TBP, which has homology to RB domain A and can interact with regulatory transcription factors in common with RB (24), also encodes binding to RBP2. We performed precipitation experiments by incubating GST-TBP fusion protein with in vitro-translated RBP2 and RBP1 and demonstrated that the TBP product can bind RBP2 but not RBP1. In addition, the interaction between RBP2 and TBP could not be blocked by excess T peptide (Fig. 2B).

We next addressed whether the ability of RBP2 to exhibit non-T/E1A binding is common to other related pocket proteins, such as p107. A cDNA encoding either wild-type p107 or a point mutant containing a cysteine-to-phenylalanine substitution at codon 713 that inactivates the T/E1A pocket (p107m) and is analogous to the RB 706F mutant (17) was expressed as a GST fusion protein for precipitation experiments with in vitro-translated RBP2 in the presence of excess T or K1 peptide (Fig. 3). We found that in contrast to RB and TBP, the T peptide, but not K1, abolished p107 binding to RBP2 (Fig. 3, lanes 3 to 5). In addition, we observed that the p107m point mutant was unable to precipitate RBP2 (Fig. 3, lane 6), while the analogous RB mutant (706F) retained binding (Fig. 1).

Since an LXCXE sequence located within the conserved region 2 domain of E1A (15) and within the binding domains for large T, E7, and RBP1 (10) has been demonstrated to be essential for oncoprotein binding to the T/E1A pocket of RB and p107, we constructed an RBP2 plasmid (RBP2m) containing the single E-to-K substitution observed within the K1 mutant of simian virus 40 large T (Fig. 4A). We observed that while GST-p107 had lost the ability to precipitate RBP2m, GST-RB and GST-TBP retained efficient RBP2m-binding activity (Fig. 4B). This finding is also consistent with a non-T/E1A mechanism that is operational for RB and TBP but not for the related protein p107.

The RB spacer sequence is necessary but not sufficient for non-T/E1A binding activity. We next examined whether the RB spacer region (between domains A and B of the RB pocket) is required for non-T/E1A RBP2-binding activity. This was suggested by the observations that (i) GST-short pocket RB, encoding codons 379 to 796, which span only the domain A-spacer-domain B region, also shows non-T/E1A binding to RBP2 (data not shown); (ii) p107 and RB contain a unique amino acid sequence within the spacer region (19); (iii) the p107 spacer, but not the RB spacer, encodes non-T/E1A cyclin A-binding activity (17); and (iv) the D2 (spacer deletion) RB mutant had lost RBP2-binding activity (Fig. 1A). We repeated the precipitation experiments with a GST-RB chimeric fusion protein that had switched the p107 and RB spacer sequences, designated GST-RB/p107S. We demonstrated that although GST-RB/p107S retained the ability to precipitate RBP2, the binding could be efficiently blocked with excess T peptide, suggesting that the T/E1A pocket binding activity was unmasked by the spacer switch (Fig. 5A). In addition, we

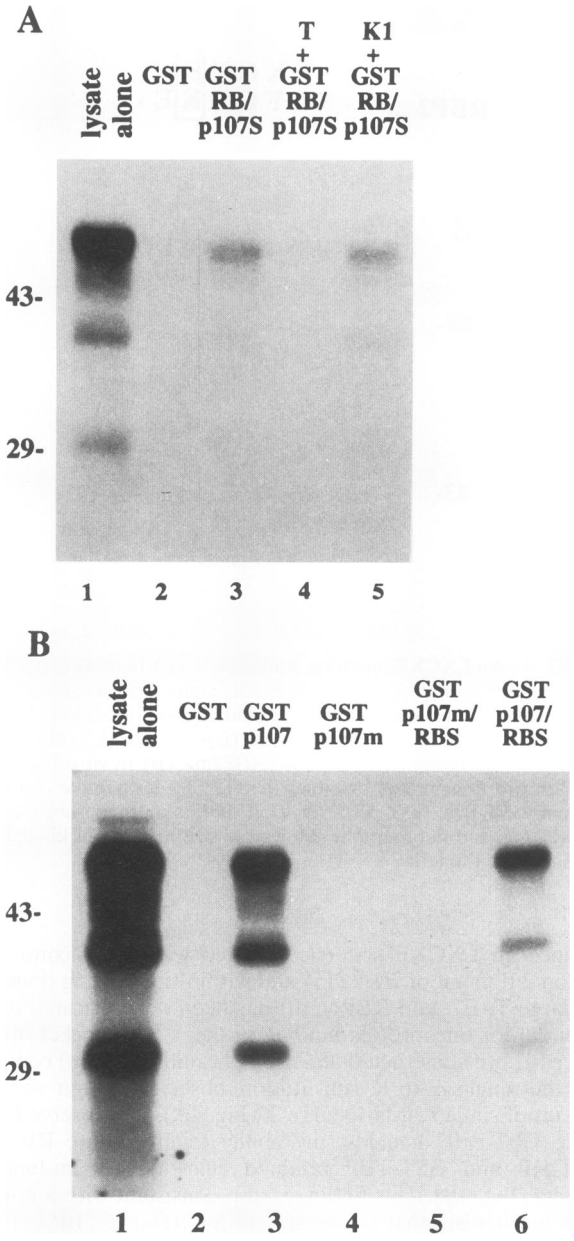

FIG. 5. The RB spacer is essential, but not sufficient, for non-T/E1A binding to RBP2. (A) In vitro-translated RBP2 was precipitated by the GST leader peptide (lane 2) or wild-type GST-RB with a switch of the p107 spacer for the RB spacer (GST-RB/p107S) in the presence of peptide T or K1 (lanes 3 to 5) and then subjected to SDS-PAGE and fluorography. (B) In vitro-translated RBP2 was precipitated by the GST leader peptide, GST-p107, point mutant GST-p107 (GST-p107m), or GST-p107m and GST-p107 with a switch of the RB spacer for the p107 spacer (GST-p107m/RBS and GSTp107/RBS) (lanes 2 to 6, respectively) and then subjected to SDS-PAGE and fluorography. Molecular size markers in kilodaltons are shown to the left.

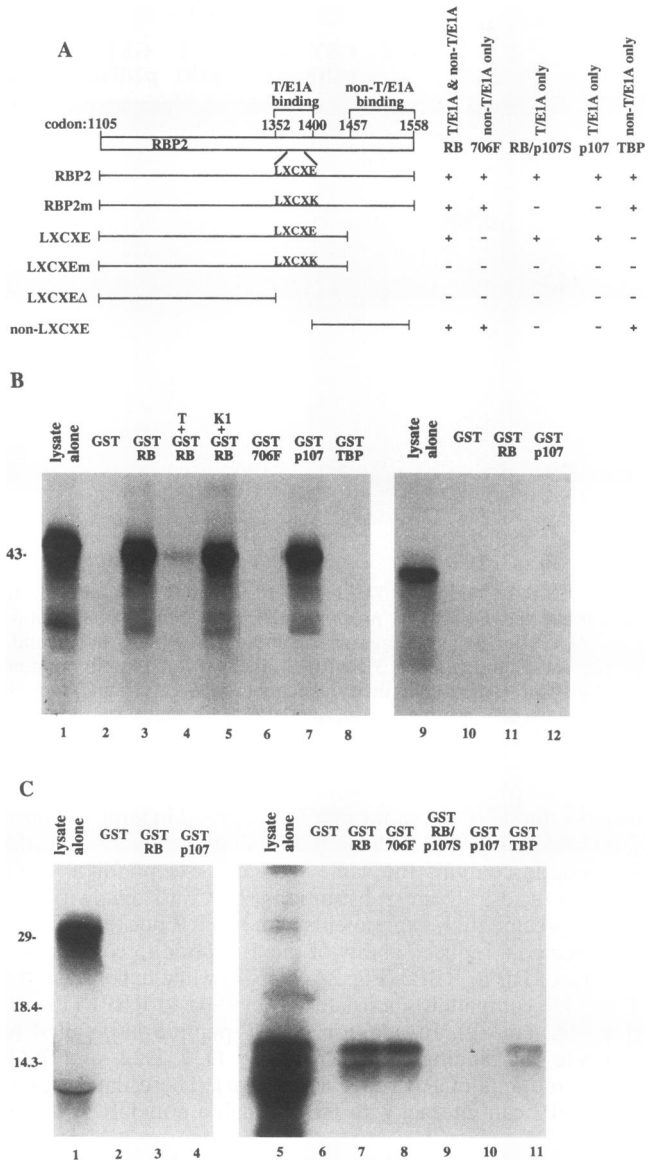

FIG. 6. A 15-kDa RBP2 fragment, distinct from the LXCXE domain, encodes non-T/E1A binding to RB and TBP but not p107. (A) A schemata of the protein-binding regions of the RBP2 product is depicted with the several RBP2 constructs tested (RBP2, RBP2m, LXCXE, LXCXEm, LXCXEΔ, and non-LXCXE). A plus or minus sign indicates the presence or absence, respectively, of ability to bind to the corresponding GST fusion protein. (B) In vitro-translated LXCXE (lanes 1 to 8) and LXCXEm (lanes 9 to 12) were precipitated by the indicated GST fusion proteins in the presence or absence of peptides T and K1. (C) In vitro-translated LXCXEΔ (lanes 1 to 4) and non-LXCXE (lanes 5 to 11) were precipitated by the indicated GST fusion proteins. Molecular size markers in kilodaltons are shown to the left of panels B and C.

observed that GST-RB/p107S had lost the ability to precipitate RBP2m containing the point mutation within the LXCXE motif (Fig. 6A). These findings show that the T/E1A and non-T/E1A binding activities of RBP2 could be isolated from each other. Because deletion mutants can result in unpredictable protein conformational changes, we addressed whether the RB spacer sequence alone can encode the non-T/E1A binding activity observed by testing a chimeric wild-type p107 plasmid and a point mutant $p107^{(713F)}$ fusion plasmid with switched RB and p107 spacers (designated GST-p107/RBS and p107m/RBS, respectively) (Fig. 5B). We observed that the RB spacer sequence did not confer RBP2-binding activity on the $p107^{(713F)}$ point mutant plasmid, demonstrating that this region alone (in the context of p107 domains A and B) does not encode non-T/E1A binding.

A 15-kDa RBP2 fragment, independent from the LXCXE motif, encodes non-T/E1A binding for RB and TBP but does not bind p107. To define the non-T/E1A binding site within RBP2 and to determine whether this region can function independently from the LXCXE domain, we generated transcription vectors that contained different portions of the RBP2 open reading frame (Fig. 6A). We observed that deletion of approximately 100 amino acids between codons 1457 and 1558 (plasmid LXCXE) resulted in loss of non-T/E1A binding to TBP and the RB pocket mutants (Fig. 6B, lanes 1 to 8). This deletion (with an intact LXCXE sequence), however, did not affect binding to the RB and p107 T/E1A pockets. This was confirmed by the finding that binding to RB could be efficiently blocked by excess T peptide (Fig. 6B, lane 4). When an E-to-K substitution was generated within this deletion plasmid (LXCXEm) or when the LXCXE domain was deleted, all protein binding was lost (Fig. 6B and C). Finally, a 15-kDa fragment, C-terminal to the LXCXE region, demonstrated non-T/E1A binding to RB, mutant RB, and TBP (Fig. 6C, lanes 5 to 11). These findings, therefore, support a model in which cellular protein RBP2 encodes independent protein-binding domains. In addition, these data suggest a similarity between the RBP2 product and the E1A transforming protein in that both encode independent T/E1A and non-T/E1A binding domains for RB. To test whether the non-LXCXE binding site of E1A (region 1) can cross-compete with RBP2, we synthesized a polypeptide sequence spanning E1A residues 35 to 59. This peptide, however, did not compete for non-T/E1A binding of RBP2, consistent with the lack of primary amino acid homology between E1A conserved region 1 and RBP2 (data not shown).

GST-RBP2 preferentially precipitates hypophosphorylated RB from whole-cell lysates. A hypothesis for the tumor suppressor activity of RB has proposed that a family of cellular binding proteins preferentially binds to the hypophosphorylated form of RB that predominates in resting cells (i) during the G_1 phase of the cell cycle, (ii) after terminal differentiation, and (iii) following senescence (3, 9, 22, 43, 46). In this model, activation of a specific cyclin-dependent kinase(s) near the G_1-S boundary results in progressive phosphorylation of RB with concomitant release of these nuclear binding proteins (53). In the E2F/DP1 heterodimeric transcription factor family (28, 31), synchronized binding of RB and E2F with specific stages of the cell cycle is believed to mediate the coordinated expression of a cassette of enzymes important for DNA synthesis (48). To address whether RBP2 also demonstrates preferential binding to hypophosphorylated forms of RB, we precipitated a whole-cell lysate from asynchronously growing RB(+/+) H630 colon carcinoma cells with fusion proteins containing either wild-type GST-RBP2 or GST-RBP2m, encoding only non-T/E1A binding activity, and performed an α-RB immunoblot (Fig. 7). We observed that both the RBP2 and RBP2m fusion proteins preferentially precipitated hypophosphorylated RB, although wild-type RBP2 demonstrated a higher affinity for RB under the conditions used in this assay.

RBP2 is a 190-kDa nuclear protein that is widely expressed and does not appear to be a target for inactivation in tumor cell lines. To characterize the RBP2 protein in vivo, we raised polyclonal antisera directed against a GST-RBP2 fusion protein. By using cell fractionation techniques and immunoblot analysis, we demonstrated that RBP2 is expressed exclusively as a nuclear protein that migrates on SDS-PAGE at approximately 190 kDa (Fig. 8A). This is consistent with a recent report that showed RBP2 to be a nuclear phosphoprotein of 195 kDa (20) and confirmed the prediction that RB-associated peptides would be localized to the nuclear compartment. We were unable, however, to show in vivo associations between RB and RBP2 by using successive immunoprecipitation-immunoblot techniques with α-RBP2 and α-RB (data not shown). Possible explanations for the difficulties in detecting these in vivo RB complexes have been proposed, including the relatively low levels of RBP2 in the cell, the inability to extract bound RB-RBP2 products in the immunoprecipitation buffers required to demonstrate their interactions (20), and competition by other cellular binding proteins. To address whether RBP2 is widely expressed in cells of different lineages and to determine whether it might serve as a target for somatic mutations in lung cancers, we performed immunoblot analyses on a series of RB(+) and RB(−) carcinoma cell lines. We observed a detectable 190-kDa RBP2 signal in all of the cells examined (Fig. 8B), suggesting that inactivation of the *RBP2* gene is not a common event in pulmonary tumorigenesis.

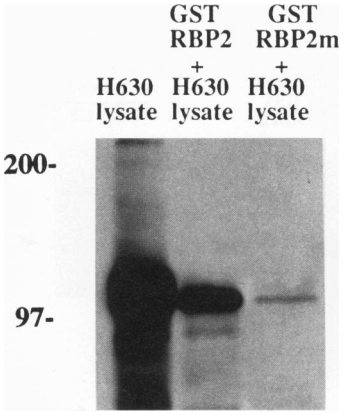

FIG. 7. Recombinant RBP2 protein preferentially precipitates hypophosphorylated RB from whole-cell protein lysates. Protein lysate from H630 colon carcinoma cells (400 μg) was subjected to immunoblot analysis with α-RB antibody following precipitation with either GST-RBP2 or GST-RBP2m. The numbers on the left are molecular sizes in kilodaltons.

Expression of RBP2 reverses RB-mediated inhibition of E2F activity. To test whether exogenous expression of RBP2 might interfere with RB function in vivo, we cotransfected RBP2 and RB either with a wild-type E2F-CAT reporter plasmid that contained tandem E2F DNA-binding sites (E2F-CAT) or with a reporter plasmid that contained tandem mutated E2F sites (mE2F-CAT) as previously described (58). We confirmed that E2F functions as an activator in RB(−/−) cells and that wild-type RB (but not a mutant RB with an exon 21 deletion) is able to suppress E2F CAT activity in these cells (Fig. 9, lanes 1 to 4 and 7). In addition, we demonstrated that cotransfection of RBP2 resulted in partial reversal of the RB-mediated inhibition of E2F CAT activity, while no effect of RBP2 was demonstrated with the mutant E2F CAT plasmid (Fig. 9, lanes 5 and 6, respectively).

DISCUSSION

Naturally occurring mutations within the *Rb* tumor suppressor gene appear to specifically target the ability of RB to achieve a pocket conformation with protein-binding activity. This observation has supported the hypothesis that RB mediates tumor suppression by modulating the activities of its cellular binding partners in response to cell cycle signals (57). The characterization of these associated proteins, therefore, is an essential step toward defining tumor suppressor pathways

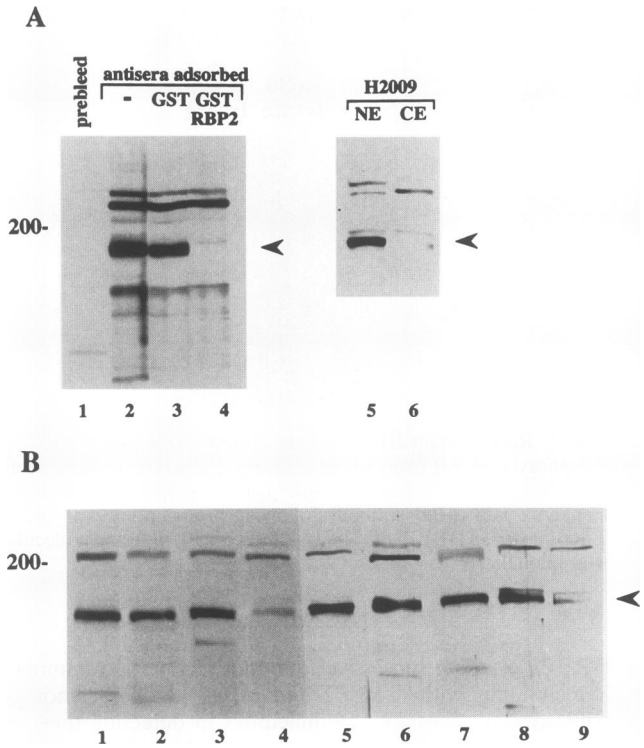

FIG. 8. RBP2 is a 190-kDa nuclear protein that is expressed in a wide range of carcinoma cell lines. (A) H630 lysates (200 μg) were subjected to immunoblot analysis with prebleed serum (lane 1), unadsorbed polyclonal antiserum directed against a GST-RBP2 fusion peptide (lane 2), GST-adsorbed antiserum (lane 3), or GST-RBP2-adsorbed antiserum (lane 4). Molecular size markers in kilodaltons are shown on the left, and the arrowhead on the right of each panel designates a 190-kDa species that is specific for the RBP2 peptide. H2009 lung carcinoma cells were fractionated as described in Materials and Methods, and the nuclear extract (NE; lane 5) and cytoplasmic extract (CE; lane 6) were subjected to immunoblot analysis with GST-preadsorbed α-RBP2 antiserum. (B) Immunoblot analysis of H2172, H2110, H378, H2369, H2342, H2009, and 2250 lung cancer cells (lanes 1 to 6 and 9, respectively), K562 leukemia cells (lane 7), and H630 colon cancer cells (lane 8) with GST-preadsorbed α-RBP2 antiserum. The number on the left is a molecular size in kilodaltons.

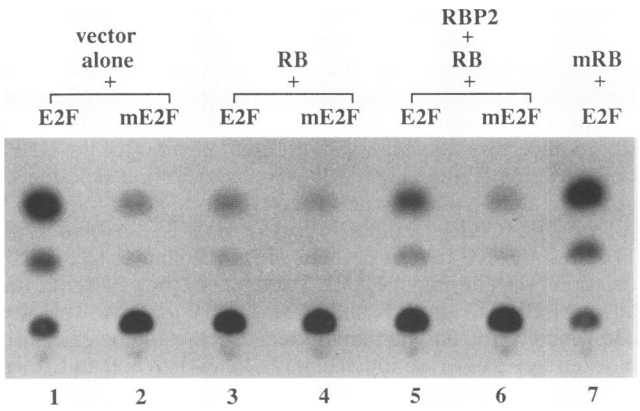

FIG. 9. RBP2 partially reverses RB-mediated inhibition of E2F CAT activity. RB(−/−) lung carcinoma cells were transfected with either an E2F or a mutant E2F (mE2F) CAT reporter gene plasmid as previously described (58). In addition, cells were cotransfected with mammalian expression vectors encoding wild-type RB, mutant RB (mRB representing a deletion of exon 21), or RBP2, as indicated. Transfection efficiency was assayed by using a luciferase reporter gene, and CAT activity was measured as described in Materials and Methods.

and may also identify additional targets for somatic mutations in human cancers with wild-type RB expression.

We have studied the functional role of the RBP2 product, which was first isolated by the screening of a cDNA expression library with recombinant RB protein (10). A full-length open reading frame for RBP2 was recently obtained and shown to contain 1,722 codons and to predict a protein with a molecular mass of 196 kDa (20). Inspection of the primary amino acid sequence demonstrated several structural features, including a potential zinc finger motif and a region of homology with a homeobox domain, that suggested a potential role as a DNA-binding protein (20). By using polyclonal antisera, we have shown that RBP2 is a 190-kDa nuclear protein that is expressed in a wide range of RB(+) and RB(−) tumor cells, which confirms recent findings that used independent polyclonal antibodies (20). In addition, we have demonstrated that the RBP2 fusion protein preferentially precipitates hypophosphorylated RB. This property is characteristic of the large T (43) and E7 (47) viral antigens, as well as the cellular RBP1 (33) and E2F (5) products, and is consistent with a physiologic role of RBP2 as a cell cycle-regulated binding protein. We were unable, however, to show an RB-RBP2 binding complex by using sequential immunoprecipitation-immunoblotting methods. By using similar methods, we were also unable to consistently detect binding with RB and RBP1, although a recent study using polyclonal antibodies demonstrated weak in vivo binding activity (20). Several hypotheses for the difficulty in detecting these interactions in cells have been suggested, including low steady-state levels of cellular RBP2, technical difficulties with the extraction of intact RBP2 complexes from the nucleus, interference with the antisera employed, and competition by other cellular binding proteins (20).

To further investigate whether RBP2 can interact with RB in vivo, we have examined whether expression of RBP2 can modulate RB-mediated inhibition of E2F transcriptional activity. By using an RB(−/−) carcinoma cell line, we have confirmed that E2F functions as a transcriptional activator that can be suppressed by wild-type, but not mutant, RB. We have also demonstrated that cotransfection of RBP2 can partially the block RB-mediated inhibition of E2F CAT activity and has no effect on expression of the mutant E2F-CAT plasmid. In addition, RBP2 in the absence of RB had no effect on either E2F-CAT or mutant E2F-CAT expression (data not shown). Although these experiments suggest that overexpression of RBP2 can compete for binding with RB to interfere with transcription suppression in vivo, they do not directly address the question of whether these interactions occur in solution under normal conditions or whether RBP2 can modulate other binding proteins as well.

To further study the functional role of the RBP2 product, we have examined its abilities to bind RB, p107, and the related TBP product (Fig. 10). Since RBP2 encodes an LXCXE motif within its protein-binding region, we expected to observe a characteristic pattern of specific binding to the T/E1A pocket of RB, as previously reported for other cellular LXCXE binding proteins, such as RBP1 (10, 33, 49) and members of the cyclin D family (12, 18, 35). We observed, however, that RBP2 could precipitate RB by using independent T/E1A (blocked by T peptide and abolished by a mutation within the LXCXE sequence) or non-T/E1A (not blocked by T peptide and encoded within a 15-kDa fragment C terminal to the

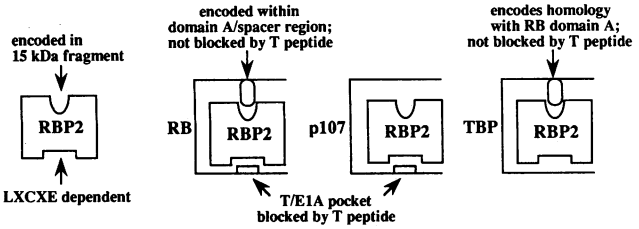

FIG. 10. Speculative model depicting differential patterns of RBP2 binding to RB, p107, and TBP.

LXCXE sequence) domains. Surprisingly, RBP2 could precipitate p107 by using only the T/E1A pocket. This supports the presence of a differential specificity for the RB and p107 binding surfaces, which has also been suggested by the observation that RB and p107 appear to associate with different molecular forms of E2F (13). In addition, we tested binding of RBP2 to TATA-associated protein TBP. We investigated potential interactions with TBP because this essential component of the RNA polymerase II transcription machinery shares significant homology with RB domain A and a short portion of the RB spacer (38% homology over 151 residues, allowing for conservative substitutions) (24, 25) and can bind in vitro as a holo-TFIID complex to E1A (2) and to several transcription factors in common with RB (24). We observed that TBP encoded the ability to precipitate RBP2 by using exclusively the non-T/E1A binding surface, while in contrast, it exhibited no binding activity toward RBP1. We have located the non-T/E1A binding region of RBP2 in a 100-amino-acid domain that encodes the ability to bind to RB and TBP independently of the rest of the RBP2 molecule. This portion of RBP2 is highly charged with predominantly basic residues (28% lysine and arginine), which predicts an α-helical structure by the empirical Chou-Fasman (6) and Robson-Garnier (23) algorithms. The finding that RB and TBP show in vitro binding to RBP2 in common with E1A and cellular transcription factors Pu.1 (24) and E2F (25) further suggests that the nuclear RBP2 product also functions as a transcription factor, but direct evidence of this is lacking. In addition, the similarity between the RBP2-binding patterns of RB and TBP and the homology within the primary sequence of RB domain A and the conserved C-terminal activation domain of TBP suggest that these two proteins have similar three-dimensional structures. Preliminary efforts to overlap the primary amino acid sequence of RB domain A with the recently published TBP three-dimensional protein coordinates (see comments in reference 37), however, have been unsuccessful, and the model depicted in Fig. 10, which suggests that RB functions as a competitor with TBP, is only speculative. These findings, therefore, reveal that RBP2 is a complex molecule with independent binding domains that are reminiscent of the distinct binding domains of E1A (15). In addition, since E1A was recently shown to bind in vitro to several different classes of transcriptional regulators (41), it will be of interest to test whether RBP2 encodes this general binding activity as well. In summary, the present data suggest that RBP2, similar to E1A, encodes multifunctional domains which may allow interactions with the cellular transcription machinery.

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