Isolation of the Rb-related p130 through its interaction with CDK2 and cyclins

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A two-hybrid protein interaction screen was used to isolate cDNAs encoding human proteins that can interact with human CDK2 in yeast. A new member of the retinoblastoma susceptibility gene family, Rbr-2 (Rb-related), was obtained. The sequence of the Rbr-2 protein shares ~50% identity with p107 and homology to Rb within the pocket domain. Several lines of evidence indicate that Rbr-2 is the adenovirus E1A-associated p130. Like Rb and p107, p130^{Rbr-2} can bind to viral oncoproteins, SV40 large T antigen, and adenovirus E1A through its pocket domain. Although p130^{Rbr-2} does not bind to CDK2 *in vitro*, it can interact with cyclins, with a clear preference for D-type cyclins. Because both CDK2 and p130^{Rbr-2} show affinity for cyclins, we suggest that p130^{Rbr-2} and CDK2 interacted through a yeast-derived cyclin bridge in the two-hybrid screen. The gene encoding p130^{Rbr-2} mapped to 16q13, a region of frequent genomic alteration in human tumors.

[*Key Words:* p130^{Rbr-2}; CDK2; cyclins; E1A; Rb; two-hybrid screen]

Received August 5, 1993; revised version accepted September 29, 1993.

Passage of a mammalian cell through the cell cycle is regulated at a number of key control points. Among these are the points of entry into and exit from quiescence (G₀), the restriction point, the G₁/S transition, and the G₂/M transition (for review, see Draetta 1990; Sherr 1993). For a cell to pass through a control point and enter the next phase of the cell cycle, it must complete all of the events of the preceding cell cycle phase and, in addition, satisfy a number of checkpoint controls. Such controls act, for example, to ensure that DNA replication has been successfully completed before the onset of mitosis. Ultimately, information from these checkpoint controls is integrated through the regulated activity of a group of related kinases, the cyclin-dependent kinases (CDKs). Once a phase of the cell cycle has been successfully completed, phosphorylation of critical substrates by activated CDKs allows passage of a cell cycle transition point and execution of the next cell cycle phase.

To date, five members of the CDK family have been identified in human cells; these are cdc2 (CDK1), CDK2, CDK3, CDK4, and CDK5 (for review, see Draetta 1990; Sherr 1993). These kinases are active only if complexed with a member of a family of positive regulatory subunits known as cyclins. D-type cyclins and cyclin E have been implicated in the regulation of the cell cycle during G₁ (Baldin et al. 1993; Ohtsubo and Roberts 1993). Cell

cycle kinase complexes that control passage through G₁ are of particular interest because their regulation may link cell cycle control to the control of proliferation and differentiation. In this regard, the product of the retinoblastoma susceptibility gene (*Rb*) acts as a negative regulator of cell proliferation and may be involved in coupling proliferative signals to cell cycle control. Although the level of Rb protein remains constant throughout the cell cycle, Rb undergoes cyclic variations in its phosphorylation state. In quiescent cells or cells in early G₁, Rb is present in a hypophosphorylated state. As cells progress toward the G₁/S boundary, the overall level of Rb phosphorylation increases, and Rb is maintained in a hyperphosphorylated state through the following S, G₂, and M phases (for review, see Goodrich and Lee 1993; Muller et al. 1993). Circumstantial evidence that Rb phosphorylation is necessary for transit through the cell cycle comes from studies in which Rb was overexpressed in SAOS-2 cells that contain no endogenous Rb. These cells appeared to arrest in G₁ and contained hypophosphorylated Rb (for review, see Muller et al. 1993). Ectopic expression of cyclins A or E, or D-type cyclins, could abrogate the growth suppressive effect of Rb, and lack of growth suppression was often accompanied by the induction of Rb hyperphosphorylation (for review, see Muller et al. 1993). In addition, Rb binds D-type cyclins *in vitro* and *in vivo* (Dowdy et al. 1993; Kato et al. 1993) and can be phosphorylated by cyclin D/CDK4 kinases at sites of Rb phosphorylation *in vivo* (Kato et al. 1993). The data from these investigations support a model in

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which the ability of Rb to inhibit cell cycle progression is regulated by its phosphorylation by CDKs.

Studies of a large number of tumors and transformed cell lines suggest that loss of Rb function leads to a deregulation of growth and plays a substantial role in the expression of the neoplastic phenotype (for review, see Weinberg 1992). In this regard, Rb is targeted by the transforming proteins of a number of DNA tumor viruses. SV40 T antigen, adenovirus E1A, and HPV-16 E7 protein each bind to and possibly sequester hypophosphorylated forms of Rb (for review, see Muller et al. 1993). The ability to bind to viral oncoproteins is a defining characteristic of the Rb family of "pocket" proteins. Three additional proteins, p107, p130, and p300, have been tentatively grouped with Rb in this family. Each of these proteins was found in association with adenovirus E1A, and certain monoclonal antibodies against Rb can recognize other potential family members (Harlow et al. 1986; Hu et al. 1991). A cDNA encoding p107 has been isolated, and this protein shows significant homology to Rb within the Rb pocket domain (Ewen et al. 1991; Zhu et al. 1993). p107 also shares with Rb the ability to bind to cyclins. p107 complexes with cyclins A and E *in vivo* and can bind cyclins D2 and D3 *in vitro* (Ewen et al. 1992, 1993; Faha et al. 1992; Lees et al. 1992).

It is becoming evident that progress through the cell cycle is governed by the association of multiple proteins into stable and metastable complexes. The identification and characterization of proteins that can interact with CDKs promise to increase our understanding of the spe-

cific roles of CDKs in cell cycle regulation and the mechanisms by which the kinases themselves are regulated. One approach toward identifying proteins that can interact with CDKs is a technique known as the two-hybrid protein interaction screen (Fields and Song 1989). Here, we have used this screen to search for genes whose protein products can interact with CDK2. We have isolated one known gene, *cks1* (a human *suc1* homolog), and two previously unidentified genes. One of these genes encodes a new member of the Rb family of pocket proteins. Several lines of evidence indicate that this Rb relative is the E1A-associated p130.

Results

Isolation of CDK2-binding proteins

To identify previously uncharacterized proteins with roles in the regulation of transit through the G₁ and S phases of the cell cycle, we sought to isolate cDNAs whose protein products could interact with the cell cycle regulatory kinase CDK2. Toward this goal, we employed a two-hybrid protein interaction screening procedure similar to that originally described by Fields and Song (1989; for review, see Bartel et al. 1993; see Fig. 1A). As a target for the two-hybrid screen, we fused the entire open reading frame of human CDK2 (Tsai et al. 1991) to the Gal4 DNA-binding domain in a vector that directs a low level of fusion protein expression in yeast. The tester strain for the screen, YPB2 (Bartel et al. 1993), contained two Gal4-inducible markers, *HIS3* and *lacZ* (see Fig. 1A).

Figure 1. Two-hybrid screen with human CDK2. (A) A schematic representation of the interaction between the Gal4 DNA-binding domain (BD)-CDK2 fusion protein and a Gal4 activation domain (AD) fusion with a cDNA-encoded protein. This interaction is shown to target the activation domain to two different Gal4-dependent promoters with consequent activation of the transcription of the *HIS3* and *lacZ* marker genes. (B) Yeast strain YPB2 containing plasmids expressing the Gal4 activation domain-p130^{Rbr-2} fusion in combination with plasmids expressing a Gal4 DNA-binding domain fused to either human CDK2, human *cdc2*, human CDK5, or *S. cerevisiae* SNF1. (+ histidine) The plate lacks only leucine and tryptophan and thus selects only for the presence of the activation domain and DNA-binding domain plasmids. (- histidine) The plate lacks leucine, tryptophan, and histidine and contains 30 mM 3-AT (see Materials and methods), thus selecting not only for the presence of the two plasmids but also for the interaction of the plasmid-encoded fusion proteins.

This tester strain was cotransformed with the CDK2 interaction target and a HeLa cell cDNA library constructed in pGAD-GH, a vector that directs the synthesis of fusions between cDNA-encoded polypeptides and the Gal4 transcriptional activation domain. In cases where an activation domain fusion protein interacted with the CDK2 target, a functional Gal4 activity was reconstituted and expression of the two marker genes was activated.

Primary positives were selected by identifying colonies that could grow in the absence of histidine. Whereas transformation with the target alone gave no His⁺ colonies, cotransformation of the target and the library produced a small number of colonies that were phenotypically His⁺. The majority of His⁺ colonies were found to express β -galactosidase activity, and library plasmids were recovered from each of these positive strains. Library plasmids were used to retransform the tester strain in combination with either the original target or control plasmids. In the majority of cases, expression of the *HIS3* and *lacZ* markers was seen only in the presence of the CDK2 target and not in the presence of plasmids encoding a fusion between the Gal4 DNA-binding domain and the *Saccharomyces cerevisiae* SNF1 protein kinase.

To date, three classes of specific, positive clones have been analyzed. One class was composed of a number of related library plasmids, all of which encoded fusions between the Gal4 activation domain and *cks1* (Richardson et al. 1990), a human homolog of the *Schizosaccharomyces pombe* *suc1* protein. In fission yeast, *suc1* interacts tightly with the *cdc2* protein kinase and is a subunit of active *cdc2* kinase complexes (Brizuela et al. 1987). A second class of positive clones consisted of multiple isolates of plasmids encoding fusions between the Gal4 activation domain and a previously unknown protein that we have termed KAP (CDK-associated phosphatase). KAP was found to be a protein phosphatase that shows dual specificity for phosphotyrosine and phosphoserine residues (G. Hannon, D. Casso, and D. Beach, in prep.). The third class of positive clones was represented by a single isolate, which we termed Rbr-2 (see below). This library plasmid contained a cDNA insert of ~4.8 kb that, upon retransformation into YPB2, activated *HIS3* and *lacZ* gene expression only in the presence of the Gal4 DNA-binding domain-CDK2 fusion and not in the presence of *cdc2* or CDK5 target fusions (Fig. 1B).

Rbr-2 is the E1A-associated p130, a new member of the Rb gene family

Sequence analysis of the clone termed Rbr-2 revealed an open reading frame of ~3.3 kb fused to the Gal4 transcriptional activation domain. The predicted amino acid sequence derived from this open reading frame was used to search available data bases using the BLAST program

on the NCBI blast network server (Altschul et al. 1990). Rbr-2 was found to have clear homology to two related proteins: p107 and Rb. It was on the basis of this homology that we chose the name Rbr-2 (Rb-related). Homology is greatest to the p107 protein sequence; Rbr-2 and p107 share ~50% amino acid identity. Homology between Rbr-2 and Rb is mainly confined to regions of Rb that are conserved between Rb and p107 (see Fig. 3, below; Ewen et al. 1991).

Homology to Rb and p107 clearly placed Rbr-2 within the Rb gene family and suggested the possibility that our Rbr-2 cDNA might be a partial clone of p130 or p300, the two proteins that have also been tentatively placed in the Rb family on the basis of their physical properties (see above). Northern analysis indicated that the message corresponding to the Rbr-2 cDNA was ~5 kb in length. Because this seemed insufficient to encode p300, we further investigated the possible relationship between Rbr-2 and p130.

We raised a polyclonal antiserum to a 71-amino-acid peptide that is unique to the Rbr-2 sequence (amino acids 757–828 of Rbr-2; see Figs. 2 and 3). This antiserum specifically recognized Rbr-2 and did not cross-react with Rb or p107. In immunoprecipitations from extracts of ³⁵S-labeled human cells, the Rbr-2 antiserum recognized polypeptides that migrated as a diffuse band with roughly the same mobility as the E1A-associated p130 (data not shown). In addition, when proteins that coimmunoprecipitate with E1A were released from the precipitate and reimmunoprecipitated with the Rbr-2 antiserum, p130 was specifically recovered (Fig. 4). Considered together, these results suggested that we had isolated a partial cDNA encoding the E1A-associated p130. This was further confirmed by comparison of the Rbr-2 sequence with the sequence of the p130 cDNA independently isolated by Li et al. (this issue). The remainder of the p130 cDNA was amplified by PCR from the HeLa cell cDNA library using nested primers derived from the Gal4 activation domain and the Rbr-2 sequence present in the initial clone (see Materials and methods). The complete coding sequence of p130 and the predicted amino acid sequence of the p130 protein are shown in Figure 2.

Comparison of the amino acid sequences of p107 and Rb, in addition to a number of functional studies, has provided an outline of the shared domain structure of this protein family (Hu et al. 1990; Huang et al. 1990; Kaelin et al. 1990; Ewen et al. 1991). Binding of Rb and p107 to the viral oncoproteins T antigen and E1A is mediated through the pocket domains of Rb and p107 (so named because this domain serves as an internal receptor for these viral proteins; Kaelin et al. 1990, 1991; Ewen et al. 1991). The pocket is composed of two subdomains, A and B, which are necessary and sufficient for viral protein binding. The A and B subdomains are separated by a

Figure 2. Sequence of p130^{Rbr-2}. The DNA sequence of the coding region of the p130^{Rbr-2} cDNA, as well as the predicted amino acid sequence of the p130^{Rbr-2} protein, is shown.

Figure 2 (See facing page for legend.)

spacer that is not conserved either in length or in primary sequence. Spacer sequences are not required for viral oncoprotein binding, because their replacement by heterologous sequences does not affect such binding (Hu et al. 1990; Ewen et al. 1991).

p130^{Rbr-2} shares substantial homology to the A and B subdomains of Rb and p107 (Fig. 3, boxes A and B), suggesting that p130^{Rbr-2} may also be capable of association with a number of proteins that are known to bind Rb and p107 through this domain (see below). Whereas no homology exists between the Rb and p130^{Rbr-2} spacer sequences, p107 and p130^{Rbr-2} are homologous in this region. Conservation of these spacer sequences may be functionally significant because the p107 spacer can direct complex formation between p107 and cyclin A (Ewen et al. 1992; Faha et al. 1992). Finally, Rb, p130^{Rbr-2}, and p107 share two additional regions of homology (Fig. 3, regions 1 and 2). Region 1 was identified previously in a comparison of Rb and p107 sequences and was termed the amino-terminal homology region (Ewen et al. 1991). Although the function of these sequences is unknown, their conservation in all three family members suggests that these sequences may have some biological significance.

p130^{Rbr-2} binds to SV40 large T antigen and adenovirus E1A through the pocket domain

The amino acid sequence of p130^{Rbr-2} predicted that it contained a domain similar to that which is necessary for the binding of Rb and p107 to E1A (termed the pocket). This domain also mediates binding to SV40 T antigen. To determine whether the p130^{Rbr-2} pocket domain could mediate such interactions, we tested the ability of p130^{Rbr-2} to bind T antigen and E1A in vitro. p130^{Rbr-2} (amino acids 58–1139; Fig. 2) was produced by in vitro translation and incubated with extracts prepared from human 293 cells or VA13 cells. 293 cells express E1A; whereas VA13 cells express large T antigen. We then asked whether antibodies directed against T antigen or E1A could coimmunoprecipitate ³⁵S-labeled p130^{Rbr-2}. As shown in Figure 5, p130^{Rbr-2} was specifically coimmunoprecipitated by an E1A antibody only when mixed with lysates from 293 cells; whereas antibodies directed against T-antigen coimmunoprecipitated p130^{Rbr-2} only after incubation of p130^{Rbr-2} with extracts from VA13 cells. These experiments suggested that p130^{Rbr-2} specifically bound to these viral proteins in vitro.

To confirm that this binding required a functional pocket domain, we took two approaches. A point mutation in the B subdomain of the Rb pocket changes cys-

teine-706 to phenylalanine. This naturally occurring mutation, originally identified in the analysis of a small-cell lung carcinoma cell line, abrogates many of the growth suppressive effects of Rb and also abolishes the binding of Rb to viral oncoproteins (Kaye et al. 1990). Introduction of the same alteration at an analogous point in the p107 sequence (Cys-713) prevents association between p107 and T antigen or E1A (in addition to a number of cellular proteins; Ewen et al. 1992). Cys-894 of p130^{Rbr-2} occupies a position within the p130^{Rbr-2} pocket subdomain B similar to that of Cys-706 in Rb. Consequently, we changed this cysteine residue to phenylalanine by site-directed mutagenesis and assayed the effect of this alteration on complex formation between p130^{Rbr-2} and T antigen or E1A. Neither viral protein bound detectably to the p130^{Rbr-2} pocket mutant, suggesting that binding did require an intact pocket domain (Fig. 5). To determine whether sequences in addition to the p130^{Rbr-2} pocket domain were required for viral oncoprotein binding, we constructed a fusion protein between glutathione S-transferase (GST) and a portion of p130^{Rbr-2} consisting of only the A and B subdomains separated by the p130^{Rbr-2} spacer. Following production in bacteria, this fusion protein specifically bound to in vitro-translated T antigen (data not shown). These results suggest that the p130^{Rbr-2} pocket domain is both necessary and sufficient for viral oncoprotein binding.

p130^{Rbr-2} binds to cyclins but not CDK2 in vitro

Because the p130^{Rbr-2} gene was isolated in a protein interaction screen in which CDK2 was the interaction target, it seemed possible that p130^{Rbr-2} and CDK2 might interact physically. Therefore, we tested the ability of Rbr-2 to bind to CDK2 both in vitro and upon coexpression in insect cells; however, we failed to detect any interaction between these two proteins. Although these experiments do not exclude the possibility that p130^{Rbr-2} and CDK2 might be capable of physical interaction in vivo, they suggested that during the interaction screen, p130^{Rbr-2} and CDK2 might have associated through their mutual binding to a third protein. CDK2 associates in vivo with several cyclins (A, E, D-type), and Rb and p107 can also bind to cyclins (see above). Therefore, members of this class of proteins seemed to be good candidates for forming a bridge between CDK2 and p130^{Rbr-2}, and we tested the ability of p130^{Rbr-2} to interact with various cyclins in vitro.

We first tested the binding of p130^{Rbr-2} to cyclin proteins produced in bacteria. The complete open reading frames of cyclins A, D1, D2, D3, and E were fused to GST, and the resulting chimeric proteins were purified

Figure 3. Comparison of p130^{Rbr-2} with Rb and p107. A comparison of the amino acid sequence of p130^{Rbr-2} with p107 and Rb protein is shown. Regions of significant homology were identified using the BLAST program (Altschul et al. 1990). Vertical lines indicate identical amino acids. Regions corresponding to the A and B subdomains of the pocket domain are boxed and indicated as A and B. In Rb, the A and B subdomains have been shown to extend from amino acids 393/4 to 571/2 and 646/9 to 772/3, respectively (Hu et al. 1990; Huang et al. 1990; Kaelin et al. 1990). The area denoted 1 was identified previously as a region of homology between Rb and p107 and was termed the amino-terminal homology region. Area 2 was not noted in previous comparisons of Rb and p107.

p130^{Rbr-2}, a new member of the Rb gene family

RBr-2:	52		LC SRLNMDEAARPEAWDSYRSM	73
p107 :	24		LCQELNLDEGSAAEALDDFTAI	45
RBr-2:	74	SESYTLEGNDLHLWALACALYVACRKSVP-PTVSKGTVEGNYVSLTRILKCSEQSLIEFFNKKMKWEDITNLPHPFRERTERLERNFTVSA		160
p107 :	46	RGNYSLEGEVTHWLACSLYVACRKSIIPTVGKGIMEGNCVSLTRILRSALSLIQFFSKMKKWMDSNLPQEFREIERLERNFEVST		133
RB :		194 WITFLAKGEVLQMEDDLVISFQMLCULD 224		
RBr-2:	161	VIFKKYEPFQDIFKYQEEQPRQQRGRKRRQPCVSEIFHFCWVLIYAKGNFPMISDDLVNSYHLLLCALDLVYGNALQCSNRKE		248
p107 :	134	VIFKKYEPFILDIFQNPYEEPPKLPRSRKQRRIPCSVKDLNFNCWTLFVYTKGNFRMIGDDLVNSYHLLLCCLDLIFANAIMCPNRQD		221
RB :			316 LSK	319
RBr-2:	249	LVNPNFKGLSEDFHAKDSKPSDDPPCIEKLCSLHDGLVLEAKGIKEHFWKPYIRKLYEKKLLKGKEENLTGFLEPGNFGESFKAINK		336
p107 :	222	LLNPSFKGLPSDFHTADFTASEEPPCIIAVLCBLHDGLLVEAKGIKEHYFKPYISKLFDRKILKG--ECL---LDLSSTDNKSAVNK		304
RB :	320	RYEEIYLKNDLDARFLDHD 340	373 TPVRTVMN	381
RBr-2:	337	AYEYVLSVGNLDERIFLGEDAEIEIGTSLRCLNAGSGTETAERVQMKNILQHFQDKSKALRISTPLTGVRIKENSPPCVTPVSTATH		424
p107 :	305	EYEEYVLTGDFDERIFLGADAEIEIGTPRKFTTRDTPLGKLTAAQANVEYNIQHFQDKRSFAPSTPLTGRYLRKEAVITPVASATQ		392
RB :	382	TIQQLMMILNSASDQPSENLISYFNNCTVNPKESILKRVKDIGYIFKEKF-AKAVGQGCY--EIGSQRYKLGVLRYRVMESMLKSEE		465
RBr-2:	425	SLSRLHMTLGLRNAPSEKLEQILRTCSRDPQTAIANRLKEMFEIYSQHFPQDDE-FSNCAKEIASKHFRFAEMLYKVLSEVIEQEQ		511
p107 :	393	SVSRLQSIIVAGLKNAPSDQLINIFESCVRNPNVENIMKILKGIGETFCQHYTQSTDEQPGSHIDFAVNRLKLAELIYKILETVMVQET		480
RB :	466	ERLSIQNFSKLLNDNIFHMSLLACALEVVMATY.14.SFPWILNVLNLKAFDYKVIKESFIKAEGNLTREMIKHLECEHRIMESLAW		563
RBr-2:	512	KRLGMDMDLSGILEQDAFHRSLLACCLEVVTF SYKPPGNFPFITEIFDVPLYHFYKVIKESFIRAEDGLCREVVKHLNQIEEQILDLHLAW		599
p107 :	481	RRLHGMDMSVLLLEQDIFHRSLLMACCLEIVLFAYSSPRTFPWIEVLNLQPFYFYKVIKESFIRAEDGLCREVVKHLNQIEEQILDLHLAW		568
RB :	564	LSDSPFLDLIKQSKDR 579		
RBr-2:	600	KPESPLWEKIRDNENRVPCTCEEVMPQNLERADEICIAG-----SPLTPRRVTEVRADTGGGLGRSI-T-SPTTLVDYRYSPPASTT		678
p107 :	569	SHDSALWEALQVSANKVPTCEEVIFPNFETGNGGNVQGHLPMPMSPLMHPRVKVERTDSSGLRRDMQPLSPISVHERYSPTTAGSA		656
RBr-2:	679	RRRLFVENDSPSDGGTPGRMPQPPLVNAVVPQNVSGETVSVTPVPGQTLVTMATATVTANNGQTVTIPVQGIANENGGITFFPVQVNV		766
p107 :	657	KRRLFGEPPKEMLMDKRIITEGTLKLIAPSSSITAE---NVSILPGQTLTMTATAPVTGTTGKHVTIPLHGVANDAGEITLIPLSMNT		741
RB :			637 TQKPLKSTSLSLFYKKVYRLAYLRNLTL	665
RBr-2:	767	GGQAQAVTSGSIQPLSAQALAGSLSSQVQTGTLTQVPGQVAIQQISPGGQQKQGSVTSNNRPRKTSLSLFFRKVYHLAAVRLDL		854
p107 :	742	NQESKVKSPVSLTAHSLIGASPKQNTLTKAQEVH-----STGINRPKRTGSLALFYRKVYHLASVRLDL		806
RB :	666	CERLLSEHPELEHIIWTLFQHTLQNEYELMRDRHLQIMMCSMYGICKVKNIIDLFKIIIVTAYKDLPHAVQETFKRVLK		745
RBr-2:	855	CALD-LSDELRRKIWTCEFEFSIIQCPLEMMDRHLQDLCAIYVMAKVTKEKSFQIMRCYRTQPOARSQVYRSLVKGKRRKRRNS		941
p107 :	807	CLKLD-VSNELRRKIWTCEFEFTLVHCPDLMKDRHLQDLCAFYIMAKVTKEERTFQIMKSYRNQPOANSHVYRSLVLSIPREVVA		893
RB :			746 EEEYDSIIVFYNSVFMQRLKTNILQYA	772
RBr-2:	942	GSSDSRSHQNSPTELKNDRTSRDSSPVMRSSTLPVQPSSAPPTPTRLTGANSDEEEERGDLIQFYNNIYIKIKTFAMKYSQAN-		1028
p107 :	895	YNKNINDDFEMIDCDLEDATKTPDCSSGPVK-----EERSDLIKFYNTIYVGRVKSFALKYDLANQ		954
RBr-2:	1029	---MDAPPLSPYPFVRTGSPRRIQLSQNHPVYISPHKNETMSPREKIFYFNSNPSKRLREINSMIRTGETPTKKRGILLEDGSESP		1113
p107 :	955	DHMDAPPLSPFPPIKQPGSPRRISQHSIYISPHKNGSGLTPRSALLYKFGNSPSKSLKDINNMIQGEQRTKKRVIAIDSDAES		1042
RBr-2:	1114	AKRICPENHSALLRRLQDVANDRGSH		1139
p107 :	1043	AKRVQENDDVLLKRLQDVVERANH		1068

Figure 3. (See facing page for legend.)

Figure 4. Rbr-2 is the adenovirus E1A-associated p130. E1A and associated proteins were recovered from lysates of metabolically labeled 293 cells using a monoclonal E1A antibody (M73, lane α -E1A). Bound proteins were released and reimmunoprecipitated using a polyclonal antiserum specific for Rbr-2 (lane α -E1A then α -Rbr-2). The positions of pRb, p107, and p130 are indicated. For reference, the positions of labeled protein markers, electrophoresed in parallel, are shown.

from overexpressing bacterial strains by affinity chromatography on glutathione-Sepharose. An equal amount (0.5 μ g) of each cyclin fusion protein was mixed with in vitro-translated p130^{Rbr-2} (carboxy-terminal 1082 amino acids); and following incubation, GST-cyclins were recovered by affinity chromatography on glutathione-Sepharose. Despite stringent wash conditions (0.5 M NaCl, 0.5% NP-40), a significant proportion of the p130^{Rbr-2} was recovered in association with each GST-cyclin fusion (Fig. 6A). In the case of GST-cyclin D2, >10% of the p130^{Rbr-2} remained bound to the glutathione-Sepharose beads.

In parallel, we tested the ability of p130^{Rbr-2} to bind to CDK2 and cyclins in insect cells. We constructed a recombinant baculovirus that directed the expression of a fragment of p130^{Rbr-2} comprising its carboxy-terminal 1082 amino acids. This polypeptide contained, in addition, three tandem copies of the hemagglutinin (HA) epitope at its amino terminus (Field et al. 1988). Upon infection of insect cells (Sf9), this virus directed high-level expression of the HA-tagged p130^{Rbr-2} (see Fig. 6B, lane -). Insect cells were then coinfecting with viruses encoding p130^{Rbr-2} and either CDK2 or cyclin A, B1, D1, D2, D3 (murine), or E under conditions where CDK or cyclin expression exceeded p130^{Rbr-2} expression. At 40 hr postinfection, infected cells were pulse-labeled for 4 hr with [³⁵S]methionine. Cells were then lysed, and p130^{Rbr-2} and associated proteins were recovered by immunoprecipitation with a monoclonal antibody directed

against the HA epitope. The results of these experiments were essentially similar to those described above. Again, p130^{Rbr-2} bound several of the cyclins tested (see Fig. 6B). The best binding was to D cyclins; each of the D-type cyclins formed high stoichiometry complexes with p130^{Rbr-2} (the ratio in each case was calculated to be ~1:1 based on quantitation of ³⁵S-labeled proteins but not taking into account potential differences in turnover rates). Cyclins A and E also bound p130^{Rbr-2} to a significant extent; when compared with D cyclins, approximately three- to five-fold less association was seen between p130^{Rbr-2} and cyclin A or E. Cyclin B1 bound p130^{Rbr-2} at only a very low level.

Chromosomal location of the p130^{Rbr-2} gene

The relationship of p130^{Rbr-2} to a known tumor suppressor prompted us to determine the chromosomal location of the gene encoding p130^{Rbr-2}. A fragment of the p130^{Rbr-2} genomic locus was isolated from a human placental genomic library and used as a probe for fluorescence in situ hybridization to human metaphase chromosomes (Fig. 7). The majority of the metaphases that we examined showed specific labeling of at least one pair of sister chromatids. In many metaphases, the sister chromatids of both homologous chromosomes were labeled. In all cases, signal was confined to a small, group E chromosome. DA-DAPI banding identified this as

Figure 5. p130^{Rbr-2} binds adenovirus E1A and SV40 T-antigen. Lysates from either 293 cells (expressing E1A, as indicated) or VA13 cells (expressing T antigen, as indicated) were mixed with 1 μ l of an in vitro translation reaction that had been programmed with either wild-type p130^{Rbr-2} or a plasmid encoding a p130^{Rbr-2} protein with a single amino acid substitution in the B subdomain of the pocket (Cys-894→Phe, C $\gg$ F, as indicated). Following immunoprecipitation with antibodies directed against either T antigen or E1A [as indicated], bound proteins were released by boiling in SDS sample buffer and analyzed by SDS-PAGE. Lanes marked Rbr-2 input and Rbr-2 C C $\gg$ F input show 10 μ l (1/50) of the initial binding reactions that were removed before immunoprecipitation. For reference, the positions of ¹⁴C-labeled protein markers (electrophoresed in parallel) are shown.

Figure 6. p130^{Rbr-2} can associate with cyclins in vitro. (A) Bacterially produced fusions between GST and human cyclins A, D1, D2, D3, and E or GST alone (as indicated) were mixed with 1 μ l of an in vitro translation reaction programmed with a plasmid encoding p130^{Rbr-2} (see Materials and methods). Following incubation, GST–cyclin fusion proteins were recovered on glutathione-Sepharose. Bound proteins were released by boiling in SDS sample buffer and analyzed by electrophoresis. The lane marked Rbr-2 input shows 5 μ l (1/40) of the binding reaction that was removed before the addition of Sepharose beads. (B) Insect (Sf9) cells were infected with a baculovirus directing the expression of an epitope-tagged fragment of the p130^{Rbr-2} protein (see text) or this virus in combination with viruses encoding cyclin A, B1, D1, D2, D3, or E (as indicated). At 40 hr postinfection, cells were pulse-labeled with [³⁵S]methionine. p130^{Rbr-2} and associated proteins were recovered from cell lysates by immunoprecipitation (see Materials and methods) with a monoclonal antibody directed against the epitope tag. Bound proteins were analyzed by SDS-PAGE. The positions of p130^{Rbr-2} and of each cyclin are indicated. For reference, the locations of ¹⁴C-labeled protein markers (electrophoresed in parallel) are shown for each panel.

chromosome 16, and analysis of high-resolution chromosome spreads placed the map position at 16q13 (Fig. 7). Two other independent genomic clones of p130^{Rbr-2} also mapped to this position (data not shown).

Localization of p130^{Rbr-2} to chromosome 16 was confirmed independently using a series of hamster–human somatic cell hybrids. Each cell line harbors a defined subset of human chromosomes (see Materials and meth-

Figure 7. (A) Dual chromatid Cy3 fluorescence (arrowheads) on chromosome 16 corresponding to specific hybridization of the digoxigenin-labeled genomic p130^{Rbr-2} probe. (B) A higher resolution preparation of chromosome 16, demonstrating the localization of the p130^{Rbr-2} gene to the q13 region (white dots). Chromosomes are counterstained with DAPI/actinomycin. Kodachrome images were digitized and converted to gray scale. Red Cy3 fluorescence was pseudocolored to white.

ods). PCR analysis of genomic DNA from 25 such cell lines gave a pattern of amplification consistent only with the $p130^{Rbr-2}$ gene residing on chromosome 16 (data not shown).

Discussion

We have used a two-hybrid protein interaction screen to identify cDNAs that encode proteins capable of physically associating with the cell cycle regulatory kinase CDK2. Three such proteins were identified: *cks1*, a previously isolated human homolog of fission yeast *suc1*; KAP, a dual specificity protein phosphatase; and $p130^{Rbr-2}$, a new member of the Rb family. $p130^{Rbr-2}$ shares ~50% amino acid identity with another Rb-related protein, p107, over the entire length of p107. In addition, Rb and $p130^{Rbr-2}$ share considerable homology in their pocket domains. $p130^{Rbr-2}$, p107, and Rb all share two additional regions of significant identity near their amino termini (Fig. 3, 1 and 2); and although the function of these sequences remains unknown, their potential importance is suggested by their conservation in all three family members.

$p130^{Rbr-2}$, Rb, and p107 not only share primary sequence homology but also functional similarity. Each of these proteins can bind specifically to the viral oncoproteins, SV40 T antigen and adenovirus E1A. The alteration of a conserved cysteine residue within the pocket domain of any of these three proteins destroys its ability to bind to either T antigen or E1A. A number of cellular proteins associate with Rb and p107 through their pocket domains (Kaelin et al. 1991; Ewen et al. 1992), and it will be important to determine whether $p130^{Rbr-2}$ binds a similar set of proteins in this manner.

The structural and functional similarities between Rb and $p130^{Rbr-2}$ raise the question of whether $p130^{Rbr-2}$ might act as an inhibitor of cellular proliferation or as a tumor suppressor gene. To gain insight into this question, we have mapped the chromosomal location of the $p130^{Rbr-2}$ gene. $p130^{Rbr-2}$ resides on the short arm of chromosome 16 at the locus 16q13. Interestingly, several studies have identified a putative Wilms' tumor gene (WT3) localized to 16q; one group proposed that up to 20% of Wilms' tumor patients have a deletion in the area of 16q13–16q22 (Maw et al. 1992; Slater and Mannens 1992). Karyotypic analyses have also identified deletions of 16q as a significant abnormality in Wilms' tumors (Kaneko et al. 1991). In addition, loss of heterozygosity (LOH) at 16q has been detected in a variety of other solid tumors. Several studies of breast carcinomas have found alterations of 16q (Mitchell and Santibanez-Koref 1990; Sato et al. 1990, 1991). Up to 45% of primary breast carcinomas show LOH of 16q markers. LOH of 16q and 10q markers has also been found in up to 30% of prostatic adenocarcinomas (Carter et al. 1990; Isaacs and Carter 1991). Finally, hepatocellular carcinomas (Zhang et al. 1990; Yokata et al. 1991) and primitive neuroectodermal tumors (Thomas and Raffel 1991) show LOH of the 16q region. On the basis of these studies, it seems likely that a tumor suppressor gene is localized to 16q

(although in several cases its location has been proposed to be 16q22–16q24, distal to that of the $p130^{Rbr-2}$ gene), and the intriguing possibility that $p130^{Rbr-2}$ may act as a tumor suppressor remains to be explored.

$p130^{Rbr-2}$ was isolated in a protein interaction screen in which CDK2 was the interaction target, but $p130^{Rbr-2}$ failed to bind directly to CDK2 in vitro under a variety of conditions. Instead, $p130^{Rbr-2}$ bound to a number of different cyclins. That CDK2 is also known to interact with cyclins raises the possibility that $p130^{Rbr-2}$ and CDK2 interacted in a yeast cell through their mutual binding to an *S. cerevisiae* cyclin (Fig. 8). On the basis of this model, we suggest that two proteins may not need to interact directly for their association to be detected in a two-hybrid screen. This possibility is interesting in light of the fact that $p130^{Rbr-2}$ interacted in yeast with CDK2 but not with *cdc2*. As in *S. pombe*, control of the G_1/S and G_2/M transitions in *S. cerevisiae* is accomplished by the action of a single CDK, p34^{CDK28} (for review, see Draetta 1990). Conditional CDC28 mutants can be fully complemented by either human *cdc2* or human CDK2 (Wittenberg and Reed 1989; Elledge and Spottswood 1991), implying that both of these human proteins can bind all of the yeast cyclins that are essential for cell cycle progression. If $p130^{Rbr-2}$ and CDK2 interact in yeast via an *S. cerevisiae* cyclin (cyclin X; Fig. 8), our data suggest that this cyclin binds to CDK2 but not to *cdc2*. Candidates for such a cyclin can be found among the *S. cerevisiae* G_1 cyclins (CLN1, CLN2, and CLN3) or the recently isolated CLB cyclins (Richardson et al. 1989, 1992; Lew et al. 1991; Fitch et al. 1992). None of these proteins is individually essential for viability, and this allows for the possibility that one of them might preferentially interact with human CDK2. An alternate possibility is that elements present both in CDK2 and in a yeast cyclin contributed to the $p130^{Rbr-2}$ –CDK2 interaction.

We have found that $p130^{Rbr-2}$ can interact with several different cyclins in vitro. Similar binding specificities are seen using cyclins produced as fusion proteins in bacteria and cyclins produced in baculovirus-infected insect cells. $p130$ has been shown to interact with cyclins A and E in vivo (Faha et al. 1993; see Li et al., this issue), and we have found that similar interactions occur in vitro (see Fig. 6). It was initially surprising that such interactions were relatively inefficient; however, Peepers

Figure 8. Model for the interaction of the Gal4 DNA-binding domain–CDK2 fusion with the Gal4 activation domain– $p130^{Rbr-2}$ fusion during the two-hybrid screen. A schematic representation of the interaction of $p130^{Rbr-2}$ and CDK2 through mutual binding to a *S. cerevisiae* cyclin (cyclin X) is shown.

et al. (1993) have recently demonstrated that cyclin A binds to p107 only when complexed with CDK2. Cyclins A and E are likely to bind p130^{Rbr-2} in a similar manner based on the homology between the p130^{Rbr-2} and p107 spacer elements. Therefore, binding of cyclins A and E was probably limited in our *in vitro* experiments by the availability of CDK2 in either reticulocyte lysates (Fig. 6A) or baculovirus-infected insect cells (Fig. 6B). p130^{Rbr-2} also bound strongly to all three of the D-type cyclins (Fig. 6) and could serve as a substrate for cyclin D/CDK4 kinase complexes formed in baculovirus-infected insect cells (data not shown). In this regard, the properties of p130^{Rbr-2} are similar to those described for Rb and p107. Rb has been reported to interact with cyclins D1 and D3 (Dowdy et al. 1993) or D2 and D3 (Ewen et al. 1993), and p107 has been shown to bind D2 and D3. In addition, both p107 and Rb can be phosphorylated by insect cell lysates containing cyclin D/CDK4 kinase complexes. These findings suggested that one function of cyclin D/CDK4 complexes might be to phosphorylate Rb and abrogate its growth-suppressive effects. In a number of cases, the expression of D-type cyclins is altered upon growth factor stimulation (Matsushime et al. 1991; Cocks et al. 1992; Inaba et al. 1992; Won et al. 1992); therefore, the ability of cyclin D kinase complexes to phosphorylate Rb might provide a link between growth control and cell cycle control. p130^{Rbr-2} can also interact, at least *in vitro*, with D-type cyclins, suggesting that p130^{Rbr-2} functions might be subject to similar regulation.

Both Rb and p107 are capable of forming complexes with a transcription factor, E2F, that has been implicated in the control of a number of genes with expression critical for cell growth. These include *c-fos*, *c-myc*, and DHFR (for review, see Wagner and Green 1991). Complexes between E2F and Rb can actively silence transcription when bound to E2F sites (Weintraub et al. 1992); however, it is thought that upon phosphorylation of Rb, Rb-E2F complexes dissociate and E2F can then act to stimulate transcription (Chellapan et al. 1991; e.g., see Hiebert et al. 1992). Complexes between E2F and p107 have been less well characterized. For example, it is not known whether these complexes normally recognize a subset of E2F sites that overlaps those recognized by Rb-E2F complexes *in vivo*. During G₁, E2F and p107 are present in a multicomponent complex that also contains cyclin E and CDK2. During S phase, cyclin A takes the place of cyclin E in a similar complex (Mudryj et al. 1991; Devoto et al. 1992; Lees et al. 1992). p130^{Rbr-2} also associates with an E2F-related protein (Cobrinik et al., this issue). In this regard, it is possible that each member of the growing family of pocket proteins might regulate different sets of genes through its interaction with individual members of a family of E2F-like transcription factors. Alternatively, each member of the Rb family of proteins may regulate a similar or overlapping set of genes either in response to different cellular signals or at different phases of the cell cycle.

The differences between the roles of Rb and p107 and the putative role of p130^{Rbr-2} in linking cell cycle control

to various aspects of growth control remain to be elucidated. However, an intriguing possibility is provided by the recent demonstration that Rb can play a role in controlling the differentiation of muscle cells in culture (Gu et al. 1993). In this system, Rb influences the developmental decisions made by these cells through its interaction with *myoD*. MyoD is a transcription factor that regulates the expression of a number of muscle specific genes and with its inappropriate expression can induce the myogenic pathway in a variety of cell lineages (e.g., see Weintraub et al. 1991). The interaction between Rb and a regulator of differentiation suggests a mechanism by which the Rb family of proteins may serve to couple developmental decisions to cell cycle control. For example, each member of the Rb family might interact with a different subset of transcription factors that regulate the expression of the differentiated phenotype in specific cell lineages.

The isolation of p130^{Rbr-2} in a screen designed to reveal proteins that can interact with CDK2 underscores the intimate relationship between the Rb family of proteins and the regulation of the cell cycle. An elucidation of the specific role of each member of the Rb family in cell cycle regulation is likely to shed light on the mechanisms by which cell cycle control is linked to proliferative or developmental decisions.

Materials and methods

Construction of cDNA libraries

Fifteen micrograms of poly(A)⁺ RNA isolated from HeLa S3 suspension cells was used as a substrate for cDNA synthesis using the Unizap XR cloning kit (Stratagene) according to the manufacturer's instructions. Resulting double-stranded cDNA with *EcoRI* and *XhoI* ends had an average size of ~1.5 kb. This cDNA (375 ng) was ligated to 600 ng of *EcoRI/XhoI*-digested pGAD-GH [see below] for 2 days at 16°C. Following ligation, the library was phenol/chloroform extracted, precipitated with ethanol, and electrotransformed into *Escherichia coli* DH10B (BioRad). The library contained ~7 × 10⁶ primary recombinants. Library DNA was obtained by plating primary transformants on one hundred 150-mm LB-AMP plates. Colonies were scraped into LB media, and DNA was prepared by standard methods. pGAD-GH is a derivative of pGAD10 (generously supplied by P. Bartel and S. Fields, SUNY, Stony Brook, NY). It differs from pGAD10 essentially in that it contains a complete ADH promoter and a portion of the pBluescript (Stratagene) polylinker.

Two-hybrid screens

For two-hybrid screening, the complete open reading frame of human CDK2 was inserted into the vector pGBT9 (P. Bartel and S. Fields, unpubl.). This vector directs the expression of a fusion between the DNA-binding domain (amino acids 1–147) of Gal4 and the entire CDK2 protein from a crippled ADH promoter. This plasmid, which also contains a TRP1 marker, was cotransformed with the HeLa cell cDNA library (*LEU2* marker) into the yeast strain YPB2 [a, *ura3-52*, *his3-200*, *ade2-101*, *lys2-801*, *trp1-901*, *leu2-3*, *CAN^r*, *gal4-542*, *gal80-538*, *LYS2::gal1^{uas}-leu2^{tata}-HIS3*, *URA3::gal4_{17-mers3x}-cyc1^{tata}-lacZ*] (Bartel et al. 1993). Transformants were plated on yeast drop-out media lacking leucine, tryptophan, and histidine and containing 30 mM

3-amino-1,2,4-triazole [3-AT]. Our best estimates indicate that $\sim 1 \times 10^6$ transformants were analyzed; precise determination of the number of primary transformants is complicated by the fact that plating efficiency is much lower on plates containing 3-AT than on plates that select only for the presence of the target and library plasmids. After 10–14 days of growth, HIS⁺ colonies were scored for β -galactosidase activity as follows: Colonies (or patches derived from the original positive colonies) were lifted from plates directly onto Whatman 50 filters, quick frozen in liquid nitrogen (~ 10 sec), and immediately overlaid onto Whatman 3MM filters that had been soaked in β -galactosidase assay buffer (60 mM Na₂HPO₄·7H₂O, 60 mM NaH₂PO₄·7H₂O, 10 mM KCl, 1 mM MgSO₄·7H₂O, 50 mM β -mercaptoethanol, 0.01% X-gal). Filter sandwiches were placed in petri dishes and incubated at 30°C. Positive colonies showed blue color in from 30 min to several hours. These colonies were picked directly from the assay filters and restructured on selective media. Plasmid DNA was recovered from positive colonies and introduced by electroporation into *E. coli* MH4 (see Bartel et al. 1993). MH4 is *leuB*[−], and this defect can be complemented by the *LEU2* gene on the library plasmid. Thus, on minimal media only transformants carrying the library plasmid, and not those carrying the target plasmid, will grow.

Primary positives were tested for target specificity by retransformation into the test strain (YPB2) in conjunction with a number of different Gal4 DNA-binding domain–target fusions (fusions with CDK2, *cdc2*, CDK5, SNF1, *pim1*; the SNF1 fusion was a gift from S. Fields, the CDK5 fusion was kindly provided by H. Zhang (this laboratory), and the *pim1* fusion was a gift from H. Feilotter, this laboratory). Only library plasmids that did not activate marker expression in the presence of SNF1 or *pim1* were analyzed further. The library plasmid encoding the human *suc1* homolog was isolated using a different tester strain, YGH1 [*a*, *ura3-52*, *his3-200*, *ade2-101*, *lys2-801*, *trp1-901*, *leu2-3*, *Can*^r, *gal4-542*, *gal80-538*, *LYS2::gal1_{uas}-gal1_{tata}-HIS3*, *URA3::gal1-lacZ*]. This screen was performed identically to that described above except that 3-AT was not included in the selective media.

Analysis of cDNAs encoding p130^{Rbr-2}

For sequence analysis, cDNAs were excised from pGAD-GH and inserted into pBluescript SK(−) and pBluescript KS(−) (Stratagene). Unidirectional deletions were prepared from both of these plasmids using the double-stranded nested deletion kit (Pharmacia). The complete sequence of both strands of the p130^{Rbr-2} cDNA was determined by sequencing overlapping deletions on an ABI 373A automated sequencer. The full-length p130 cDNA was obtained by PCR from the HeLa cell cDNA library. Three successive rounds of amplification were performed using nested primers. In each case, one primer was derived from the Gal4 activation domain sequence and one primer was derived from the Rbr-2 sequence present in our original clone. After three rounds of amplification, the largest products were isolated, subcloned, and sequenced. To avoid error, fragments derived from three independent PCR reactions were analyzed.

Production of p130 antibodies and reimmunoprecipitation experiments

A plasmid encoding a fusion between GST and amino acids 757–828 of p130^{Rbr-2} was introduced into *E. coli* BL21. Fusion proteins were expressed and purified as described below. Polyclonal antisera to this fusion protein were generated by Pocono Rabbit Farm and Laboratory.

For reimmunoprecipitation experiments, human 293 cells were metabolically labeled with [³⁵S]methionine, and lysates were prepared as described (Xiong et al. 1993). E1A and associated proteins were recovered by immunoprecipitation with a monoclonal E1A antibody, M73. Proteins were released from the immunoprecipitate by boiling in 0.25× Laemmli sample buffer. Protein A–Sepharose was removed by centrifugation, and the supernatant (40 μ l) was diluted to 1 ml in 50 mM Tris (pH 8.0), 150 mM NaCl, and 0.5% NP-40. Reimmunoprecipitation of p130 was carried out using 1 μ l of the α -Rbr-2 polyclonal antisera.

In vitro binding experiments

For use in in vitro binding experiments the p130^{Rbr-2}-coding region was inserted into the translational enhancing vector pCITE (Novogen). The p130^{Rbr-2} C₈₉₄F mutation was created by in vitro mutagenesis of the p130^{Rbr-2} cDNA in pBluescript SK(−) as described (Hannon et al. 1990). An 835-bp *SacI*–*XhoI* fragment from the mutant gene was then used to replace the identical fragment in pCITE–p130^{Rbr-2}. For all experiments, in vitro-translated proteins were synthesized using a coupled transcription–translation system (TNT lysate, Promega).

For T antigen- and E1A-binding experiments, 1 μ l of an in vitro translation reaction was mixed with 125 μ g of lysate from either 293 or VA13 cells. The preparation of the cellular extracts and assay for binding was performed exactly as described (Kaelin et al. 1990).

For use in cyclin-binding experiments, the entire coding sequence of cyclins A, D1, D2, D3, and E were inserted into the expression vector pGEX-KG (Guan and Dixon 1991), which directs the synthesis of GST fusion proteins (pGEX–cyclins A, D1, D2, and D3 were gifts from Y. Xiong, this laboratory). For the preparation of fusion proteins, GST–cyclin expression vectors were transformed into *E. coli* BL21. Transformants were grown to an OD₆₀₀ of 0.5 and were then induced to express fusion protein by the addition of IPTG to 0.4 mM. Induction was allowed to proceed for 10–12 hr at 23–25°C, after which time the cells were washed once in PBS and then resuspended in 10 ml of GST column buffer [GCB: 50 mM Tris at pH 8.0, 200 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM DTT, 1× protease inhibitors (2 μ g/ml of leupeptin, 2 μ g/ml of aprotinin, 0.3 μ g/ml of benzamide, 10 μ g/ml of soybean trypsin inhibitor, 100 μ g/ml of TPCK, 50 μ g/ml of TLCK)]. Cells were lysed by five sequential 30-sec of sonications on ice, and lysates were cleared by centrifugation (10 min at 9600g). Fusion proteins were recovered by affinity chromatography on glutathione–Sepharose (Pharmacia). Chromatography was essentially as described by the manufacturer except that it was carried out in GCB and a 0.7-M NaCl wash was performed before elution.

For in vitro cyclin binding, 1 μ l of an p130^{Rbr-2} in vitro translation reaction was mixed with 0.5 μ g of each GST–cyclin fusion protein or 3 μ g of GST alone in 200 μ l of GST binding buffer [GBB: 50 mM Tris at pH 8.0, 120 mM NaCl, 1 mM DTT, 0.5% NP-40, 1× protease inhibitors] and incubated for 1 hr at 4°C. 15 μ l of glutathione–Sepharose (prewashed with GBB) was then added, and the binding reactions were then rotated for an additional 30 min at 4°C. The glutathione–Sepharose was then washed five times in GBB containing 0.5 M NaCl. Bound proteins were released by boiling in SDS sample buffer and electrophoresed on a 10% SDS-PAGE gel. Quantitation of the binding reactions was carried out on a FUJI BAS2000 bio-imager.

Interaction of p130^{Rbr-2} and cyclins in baculovirus-infected insect cells

A baculovirus that directs the expression of a fusion between

the HA epitope (Field et al. 1988) and the carboxy-terminal 1082 amino acids of p130^{Rbr-2} was constructed using the Pharmingen Baculo-gold kit. This was used to infect insect (Sf9) cells either alone or in combination with viruses directing the expression of CDK2 or human cyclins A, E, D1 or D2, or murine cyclin D3. Viruses encoding cyclins D1 and D2 were supplied by Dr. Stephen Gruenwald (Pharmingen, Palo Alto, CA) and viruses encoding CDK2, cyclin A, cyclin E, and murine cyclin D3 were a gift of Dr. Charles Sherr (St. Jude, Memphis, TN). For each experiment 4 × 10⁵ Sf9 cells were seeded in each well of a 12-well tissue culture plate. p130^{Rbr-2} virus was used at an m.o.i. of ~5; CDK2 and cyclin viruses were used at an m.o.i. of ~15. At 40 hr postinfection, cells were pulse-labeled with 0.1 mCi of [³⁵S]methionine (Express label, NEN). After 4 hr of labeling, cells were lysed in 500 μl of a buffer containing 50 mM Tris (pH 7.5), 250 mM NaCl, 0.5% NP-40, 1 mM DTT, 1 mM NaVO₄, 10 mM NaF, and 1 × protease inhibitors (see above). p130^{Rbr-2} was recovered by immunoprecipitation with a monoclonal antibody directed against the HA epitope (12CA5, Babco), and precipitated proteins were analyzed by SDS-PAGE. In each case, quantitation of proteins present in unfractionated lysates confirmed that cyclins or CDK2 were present in excess to p130^{Rbr-2}. Assay of cyclin D/CDK4 kinase activity toward p130^{Rbr-2} was performed exactly as described (Ewen et al. 1993).

Fluorescence in situ hybridization

Plasmid preparations containing genomic subclones were labeled with digoxigenin and hybridized to synchronized normal lymphocyte preparations in a manner similar to that published previously (Demetrick and Beach 1993). Briefly, plasmid mid-preps were purified on ion exchange columns (Quiagen). One microgram of plasmid containing the p130 genomic insert was labeled with digoxigenin using a BRL nick-translation kit. Labeled probe was centrifuged over a 1-ml G-50 (Pharmacia) spin column. Fifty nanograms of probe in 50% formamide, 10% dextran sulfate, 2 × SSC, and 1 mg/ml of sonicated human DNA in a total volume of 10 μl was denatured by heating for 5 min at 85°C, and suppressed for 15–30 min at 37°C before use. Metaphase chromosome spreads were prepared from normal lymphocytes stimulated with phytohemagglutinin and hybridized to probe essentially as described (Demetrick and Beach 1993).

Following hybridization, the slides were washed for 5 min in 50% formamide/2 × SSC (3 ×) and 2 × SSC (3 ×) at 42°C. The slides were then stained with 15 μg/ml of sheep antidigoxigenin (Boehringer-Mannheim) and washed for 5 min each in three changes of 2 × SSC/0.005 % CHAPS (SC buffer), then incubated with a 1/40 dilution of Cy3-conjugated donkey anti-sheep antibody (Jackson Immunoresearch). After the slides were washed, they were counterstained with DAPI and actinomycin D for DA-DAPI banding (Schweizer 1976). The slides were visualized utilizing a Nikon FXA microscope and a triple bandpass (FITC/Texas red/DAPI, Chromo Technology, Inc.) filter. Hamster-human somatic cell hybrid DNA (BIOS Laboratories, New Haven, CT) was amplified by PCR using oligonucleotides directed against the human p130^{Rbr-2} cDNA sequence (ATAGGCCT-TATTGATGGCT, GCTCCTTAAGGGAAAAGA). These primers give an amplified fragment of ~0.8 kb when used in PCR reactions containing human genomic DNA. Although these oligonucleotides gave no specific amplified fragment when used in PCR reactions containing hamster DNA, an 0.8-kb amplified band was observed in PCR reactions that included DNA prepared from somatic cell hybrid lines 967 and 1079. Both of these lines, but no others tested, contain human chromosome 16.

Acknowledgments

We are indebted to Paul Bartel and Dr. Stan Fields (SUNY, Stony Brook) for providing plasmids and strains before publication and advice on the use of their two-hybrid system. Excellent technical assistance was provided by C. Gawel and S. Matsumoto. We thank Drs. Konstantin Galaktionov, Hui Zhang, Yue Xiong, and Tim Nilsen for helpful discussions and critical reading of the manuscript. Finally, we thank Dr. Stephen Gruenwald (Pharmingen) and Dr. Charles Sherr (St. Jude) for providing baculoviruses. G.J.H. is a postdoctoral fellow of the Damon Runyon-Walter Winchell Cancer Research Fund (DRG-1159). D.D. is supported by the Medical Research Council of Canada and The Alberta Heritage Fund for Medical Research. D.B. is an investigator of the Howard Hughes Medical Institute. This work was supported in part by the National Institutes of Health.

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Note added in proof

The sequence data described in this paper have been submitted to the EMBL/GenBank data libraries.

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Genes Dev. 1993, **7**:

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