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Retinoblastoma Protein Contains a C-terminal Motif That Targets It for Phosphorylation by Cyclin-cdk Complexes

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Stable association of certain proteins, such as E2F1 and p21, with cyclin-cdk2 complexes is dependent upon a conserved cyclin-cdk2 binding motif that contains the core sequence ZRXL, where Z and X are usually basic. In vitro phosphorylation of the retinoblastoma tumor suppressor protein, pRB, by cyclin A-cdk2 and cyclin E-cdk2 was inhibited by a short peptide spanning the cyclin-cdk2 binding motif present in E2F1. Examination of the pRB C terminus revealed that it contained sequence elements related to ZRXL. Site-directed mutagenesis of one of these sequences, beginning at residue 870, impaired the phosphorylation of pRB in vitro. A synthetic peptide spanning this sequence also inhibited the phosphorylation of pRB in vitro. pRB C-terminal truncation mutants lacking this sequence were hypophosphorylated in vitro and in vivo despite the presence of intact cyclin-cdk phosphoacceptor sites. Phosphorylation of such mutants was restored by fusion to the ZRXL-like motif derived from pRB or to the ZRXL motifs from E2F1 or p21. Phospho-site-specific antibodies revealed that certain phosphoacceptor sites strictly required a C-terminal ZRXL motif whereas at least one site did not. Furthermore, this residual phosphorylation was sufficient to inactivate pRB in vivo, implying that there are additional mechanisms for directing cyclin-cdk complexes to pRB. Thus, the C terminus of pRB contains a cyclin-cdk interaction motif of the type found in E2F1 and p21 that enables it to be recognized and phosphorylated by cyclin-cdk complexes.

Orderly progression through the mammalian cell cycle requires that different cyclin-cdk complexes phosphorylate specific substrates in a temporally controlled fashion (38, 39, 71). For example, the retinoblastoma protein, pRB, is phosphorylated by cyclin D-cdk4 and cyclin E-cdk2 complexes as cells exit G_1 and enter S phase (66). Cyclin A-cdk2 complexes probably contribute to the maintenance of pRB phosphorylation during S phase (82, 90). Phosphorylation of pRB results in the release of E2F family members and hence in transcription of the E2F-responsive genes that are required for entry into S phase (1, 69). In contrast, entry into M phase is driven by the phosphorylation of various substrates by cyclin B-cdc2 and cyclin A-cdc2 complexes (65, 67).

Several lines of evidence suggest that pRB is a critical downstream target of cyclin D-cdk4. For example, inhibition of cyclin D-cdk4 activity induces a G_1/S block in a pRB-dependent manner (3, 25, 50, 61, 62, 68). In contrast, neutralization of cdk2 induces a G_1/S block in cells lacking pRB (30, 73, 77, 88, 89, 94). Furthermore, there is mounting evidence that cyclin E-cdk2 acts both upstream and downstream of pRB. In particular, the cyclin E promoter is under the control of pRB (4, 23, 32, 40, 72, 84) and the induction of S-phase entry by overproduced cyclin E does not appear to require phosphorylation of pRB (55, 60). Thus, there must be physiologically important cdk2 substrates in addition to pRB.

How cyclin-cdk complexes recognize substrates in general, and pRB in particular, is largely unknown apart from their requirement for a (serine/threonine)-proline (S/T-P) phosphoacceptor site and a preference for a basic residue at position +3 (where the S/T position is position 0) (36, 70, 83, 85, 98). It is clear, however, that the cyclin moiety contributes to substrate specificity. For example, cyclin A-cdc2 but not cyclin B-cdc2 phosphorylates the pRB-related protein, p107, in vitro (76). Furthermore, certain substrates can bind stably to cyclin-cdk complexes, suggesting that physical association may play a role in establishing substrate specificity in at least some cases.

Cyclin-cdk2 complexes bind stably to cell cycle-regulatory proteins including the transcription factor E2F1 (15, 51, 95), the pRB family members p107 and p130 (8, 17, 21, 53, 57), and all of the p21-like cyclin-dependent kinase inhibitors (CDKIs) (24, 30, 77, 88, 93, 94). E2F1 and p107 are putative cyclin-cdk2 substrates (17, 20, 21, 51, 53, 75, 76). We and others recently identified an octamer cyclin-cdk2 binding motif in E2F1, p107, and p21 that was required for the efficient phosphorylation of E2F1 and p107 by cyclin-cdk2 and for the action of p21 as a cyclin-cdk2 inhibitor (2, 7, 100). This motif has, at its core, the sequence ZRXL, where Z and X are typically basic (2, 80). Thus, these studies identified a potential cyclin-cdk2 substrate recognition motif and provided evidence that p21-like CDKIs act, at least in part, by competing with substrates for binding to cyclin-cdk2 complexes. This model is supported by analysis of the cyclin A-cdk2-p27 crystal structure (79).

We previously showed that a peptide replica of the above-mentioned motif, derived from E2F1, blocked the phosphorylation of pRB by cyclin A-associated kinase activity (2). pRB, unlike E2F1 and p107, does not bind stably to cyclin-cdk2 complexes. Nonetheless, the observation that phosphorylation of pRB by cyclin A-cdk2 was inhibited by such a peptide suggested that pRB contained an analogous sequence. According to this model, this pRB sequence would mediate a transient

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interaction with cyclin-cdk2 complexes that was necessary for a productive enzyme-substrate interaction.

Examination of the primary sequence of the pRB C terminus showed that this region contained several candidate cyclin-cdk2 binding motifs between residues 829 and 928. Site-directed mutagenesis identified one such motif, beginning at residue 870, that was necessary for efficient phosphorylation of pRB. A synthetic peptide spanning this motif inhibited the phosphorylation of pRB by cyclin-cdk2 complexes. A pRB mutant with C-terminal truncation, pRB(1–829), was not phosphorylated by cyclin-cdk2 complexes despite retention of all of the potential cyclin-cdk phosphoacceptor sites of pRB. Fusion to 10 amino acids spanning the ZRXL-like motif of pRB or 12 amino acids spanning the cyclin-cdk2 binding motifs present in either E2F1 or p21 restored its ability to be phosphorylated by these complexes. Thus, pRB contains a dedicated cyclin-cdk2 substrate recognition motif. Furthermore, these findings suggest that the paradigm for substrate recognition, based upon the stable interaction of cyclin-cdk2 complexes with proteins such as E2F1 and p107, can be extended to include substrates that do not form such stable complexes.

MATERIALS AND METHODS

Cell culture and transfections. U2OS and SAOS2 osteosarcoma cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal clone I (HyClone), penicillin, and streptomycin and maintained at 37°C in a humidified 10% CO_2-containing atmosphere. The cells were transfected by the calcium phosphate method (6).

Plasmids. pRcCMV cyclin A and pRcCMV cyclin E were gifts from Jonathan Pines. pGEX2TRB(792–928) has been described previously (78). pGEX2TRB (794–910), pGEX2TRB(794–896), pGEX2TRB(794–884), pGEX2TRB(794–876), pGEX2TRB(794–864), pGEX2TRB(794–857), pGEX2TRB(794–844), and pGEX2TRB(794–829) were generated by PCR amplification of a human RB cDNA with *Pfu* polymerase and the 3′ primers GCGCGAATTCCTCGAGTC ATCGTGTTCGAGTAGAAGTCAT, GCGCGAATTCCTCGAGTCATTTGG ACTCTCCTGGGAGATG, GCGCGAATTCCTCGAGTCATTCATCTGATC CTTCAATATC, GCGCGAATTCCTCGAGTCAGCGTAGTTTTTTCAGTG GTTT, GCGCGAATTCCTCGAGTCATTCAGCACTTCTTTTGAGCAC, GC GCGAATTCCTCGAGTCAACGGTCGCTGTTACATACCAT, GCGCGAAT TCCTCGAGTCACTTCTCAGAAGTCCCGAATGA, and GCGCGAATTCT CACTCGAGGTCTGCTGCTGCTGCTACGGTACCTCATCATGATCTTGG AGTCATTTTTG respectively. The 5′ primer was GCGCGGATCCTCACCCT TACGGATTCCTG in each case. The PCR products were digested with *Bam*HI and *Eco*RI and ligated into pGEX2T (Pharmacia PL). Plasmids were verified by restriction analysis and visualization of the corresponding glutathione *S*-transferase (GST) fusion proteins, expressed in *Escherichia coli*, by Coomassie blue staining following sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. pGEX2TRB(794–829)E2F1CyWT and pGEX2TRB(794–829) E2F1CyMut were generated by PCR amplification of a human RB cDNA with *Pfu* polymerase and the 3′ primers GCGCGAATTCTCACTCGAGGTCGAG ACGACGTTTTACTGGTGGTCGACCTGATCTTGGAGTCATTTTTG and GCGCGAATTCTCACTCGAGGTCTGCTGCCGCGGCTACTGGTGGTCG TCCTGATCTTGGAGTCATTTTTG, respectively. The 5′ primer was GCGC GGATCCTCACCCTTACGGATTCCTG in each case. The PCR products were digested with *Bam*HI and *Eco*RI and ligated into pGEX2T. Plasmids were verified by restriction analysis and visualization of the corresponding GST fusion proteins by Coomassie blue staining.

pGEX2TRB(794–928)Δ870 and pGEX2TRB(794–928)Δ889 were generated by site-directed mutagenesis of pSG5LHARB(1–928) (81) with the Bio-Rad Muta-Gene kit as specified by the manufacturer and the oligonucleotides GGA AGCAACCCTCCTAATGCCGCCATTCGATCGCGCTTTGATATTGAA and GAAGCAGATGGAAGTGCCGCGGCACCAGGAGAGTCCAAA to produce pSG5LHARB(1–928)Δ870 and pSG5LHARB(1–928)Δ889 respectively. The former results in the replacement of the amino acid sequence KPLKKL by a flexible linker (NAAIRS) (2, 64, 92), and the latter results in the replacement of the sequence KHL by AAA. pSG5LHARB(1–928)Δ870 and pSG5LHARB(1–928)Δ889 were used as templates in PCRs with *Pfu* polymerase, the 5′ primer GCGCGGATCCTCACCCTTACGGATTCCTG, and the 3′ primer GCGCGA ATTCAGATCTTCATTTCTCTTCCTTGTTTGAG. The PCR products were digested with *Bam*HI and *Eco*RI and ligated into pGEX2T. pGEX2TRB(794–928)Δ870,889 was generated in the same way except that both oligonucleotides were included in the initial mutagenesis reaction. The constructs were verified by restriction analysis, DNA sequencing, and visualization of the corresponding GST fusion proteins by Coomassie blue staining.

To make GST-RB fusion proteins beginning at pRB residue 773 or 792, the oligonucleotides GCGCGGATCCTCCACCAGGCCCCCTACCTTG and GCG CGGATCCTTTCCTAGTTCACCCTTACGG, respectively, were used in the above reactions.

pGEX2TRB(794–829)RBCy and pGEX2TRB(794–829)p21Cy were generated by PCR from pSG5LHARB with *Pfu* polymerase, the 5′ primer GCGCG GATCCTCACCCTTACGGATTCCTG, and the 3′ primers GCGCGAATTCT CAATCAAAGCGTAGTTTTTTCAGTGGTTTAGGTGATCTTGGAGTCAT TTTTGT and GCGCGAATTCCTCGAGTCATGGTCCGAATAGTCGTCGG CATGCTTTTGATCCGCAAGATCTTGGAGTCATTTTTG, respectively. The PCR products were digested with *Bam*HI and *Eco*RI and ligated into pGEX2T. The products were confirmed by restriction analysis and visualisation of the corresponding GST fusion proteins by Coomassie blue staining.

pSG5LHARB(1–896), pSG5LHARB(1–864) and pSG5LHARB(1–829) were generated by PCR from pSG5LHARB with *Pfu* polymerase, the 5′ primer TGCAGAGACACAAGCAACCTC, and the same 3′ primer used to generate the corresponding pGEX2T-RB mutants. For pSG5LHARB(1–896) and pSG5LHARB(1–864), the PCR products were digested with *Nhe*I and *Xho*I and ligated into pSG5LHARB that had been digested with these enzymes. For pSG5LHARB(1–829), the PCR product was digested with *Nhe*I and *Eco*RI and ligated into pSG5LHARB digested with *Eco*RI-*Bam*HI and *Bam*HI-*Nhe*I (three-way ligation). The constructs were verified by restriction analysis. pSG5LHARB (1–829)E2F1CyWT and pSG5LHARB(1–829)E2F1CyMut were generated by PCR from pSG5LHARB with *Pfu* polymerase, the 5′ primer TGCAGAGACA CAAGCAACCTC, and the same 3′ primer used to generate the corresponding pGEX2T-RB mutants. The PCR products were digested with *Nhe*I and *Eco*RI and ligated into pSG5LHARB digested with *Eco*RI-*Bam*HI and *Bam*HI-*Nhe*I (three-way ligation). The constructs were verified by restriction analysis.

pSG5LHARB(1–864)E2F1CyWT, pSG5LHARB(1–864)E2F1CyMut, and pSG5LHARB(1–864)E2F1CySCR were generated by PCR from pSG5LHARB with *Pfu* polymerase, the 5′ primer TGCAGAGACACAAGCAACCTC, and the 3′ primers GCGCGAATTCCTCGAGTCATTCGAGGTCGAGACGACGTTT TACTGGTGGCCGGCCTTCAGCACTTCTTTTGAGCAC, GCGCGAATTC CTCGAGTCATTCGAGGTCTGCTGCCGCGGCTACTGGTGGCCGGCCT TCAGCACTTCTTTTGAGCAC, and GCGCGAATTCCTCGAGTCAACGA CGGAGGTCTACTGGCCGGCCTTTGAGTGGTTCTTCAGCACTTCTTTT GAGCAC, respectively. The PCR products were digested with *Nhe*I and *Xho*I and ligated into pSG5LHARB digested with *Nhe*I and *Xho*I. The constructs were verified by restriction analysis.

Peptides. Peptides were purchased from Biosynthesis, Inc. (Lewisville, Tex.) and were dissolved in phosphate-buffered saline prior to use.

Antibodies. The anti-cyclin A antibody, C160, and the anti-simian virus 40 (SV40) T antibody, pAB419, were gifts of Ed Harlow. The anti-cdk2 antibody, M2, and the anti-cyclin E antibody, HE111, were purchased from Santa Cruz, Inc. The anti-HA antibody, 12CA5, was purchased from Boehringer Mannheim. The Anti-E2F4 antibody, LLF4, was a gift of Jackie Lees. The monoclonal antibody against phospho-Ser780 and the polyclonal antibodies against phospho-Thr821, phospho-Ser795, and phospho-Ser612 were made essentially as described by Kitagawa et al. (46). The generation and validation of these reagents will be described elsewhere (86).

Purification of recombinant cyclin A-cdk2 and cyclin D1-cdk4. The production and purification of cyclin A-cdk2 and cyclin D1-cdk4 by baculoviral infection of insect cells was performed as described previously (9).

In vitro kinase assays and purification of GST fusion proteins. In vitro kinase assays were performed as described previously (2). Purification of GST fusion proteins for use as substrates in kinase assays was performed by the method of Frangioni and Neel (22).

Immunoprecipitation and Western blot analysis. Immunoprecipitations for Western blot analysis were performed essentially as described previously for immunoprecipitation kinase assays, except that after being washed five times in NETN (20 mM Tris [pH 8.0], 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40) the protein A-Sepharose was boiled in Laemmli sample buffer (62 mM Tris [pH 6.8], 2% SDS, 10% glycerol, 100 mM dithiothreitol, 0.01% bromophenol blue), fractionated by SDS-polyacrylamide gel electrophoresis, and transferred to a polyvinylidene difluoride membrane (2). Western blot analysis of total soluble protein was performed as follows. At 36 h after transfection, U2OS or SAOS2 cells were washed once in cold PBS and scraped into 0.2 ml of EBC lysis buffer (50 mM Tris [pH 8.0], 120 mM NaCl, 0.5% Nonidet P-40). The 150 μg of soluble protein (as determined by the Bradford assay) was fractionated by SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The membranes were probed with either the 12CA5 or 419 antibody, as indicated, followed by a goat anti-mouse antibody conjugated to alkaline phosphatase.

RESULTS

The pRB C-terminus contains a cluster of seven candidate in vivo cdk phosphorylation sites (see Fig. 2A) (9, 46, 54, 58, 96). GST-RB(792–928) contains five of these sites (residues 795, 807, 811, 821, and 826) and is phosphorylated in vitro by cyclin A, cyclin E, and cyclin D-associated kinases (Fig. 1 and data not shown). We and others previously showed that phosphor-

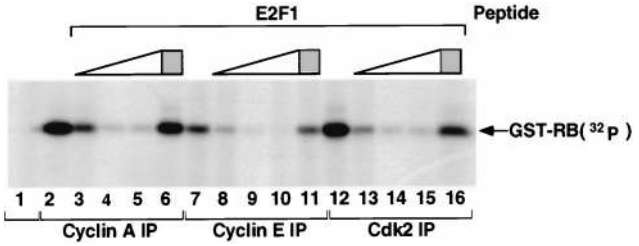

FIG. 1. Inhibition of cyclin-cdk2 by an E2F1-derived peptide. U2OS osteosarcoma cells were lysed and immunoprecipitated (IP) with anti-cyclin A, anti-cyclin E, anti-cdk2, or control (lane 1, anti-SV40 T-antigen) antibodies as indicated. In vitro kinase assays were performed with GST-RB(792–928) in the presence of no competitor peptide (lanes 1, 2, 7, and 12), 1.4, 14, or 140 μM wild-type E2F1-derived cyclin-cdk2 binding peptide (open triangles), or 140 μM scrambled version of the cyclin-cdk2 binding peptide (shaded squares) (lanes 6, 11, and 16). Phosphorylated proteins were resolved by SDS-polyacrylamide gel electrophoresis and detected by autoradiography.

ylation of GST-RB(792–928) by cyclin A-associated kinase activity was inhibited by synthetic peptides spanning the cyclin-cdk2 binding sequences present in either E2F1 or the cdk inhibitor p21 (2, 7). The same E2F1-derived peptide also inhibited the phosphorylation of GST-RB(792–928) by cdk2- and cyclin E-associated kinase activities immunoprecipitated from asynchronously growing cells (Fig. 1). The observation that phosphorylation of pRB by cyclin-cdk2 complexes was inhibited by the above-mentioned peptides suggested that pRB might contain a sequence, or sequences, capable of forming a structure that interacted, at least transiently, with cyclin-cdk2 in an analagous fashion.

Examination of the primary amino acid sequence of the pRB C terminus revealed five potential cyclin-cdk2 binding motifs, related to the ZRXL motif defined previously (2), between residue 829 and the C terminus (Fig. 2A). To test whether these motifs were required for the phosphorylation of pRB, we generated a series of GST-RB C-terminal truncation mutants for use as substrates in in vitro kinase assays. The GST moiety was fused to fragments of pRB starting at residue 794 and ending at residue 910, 896, 884, 876, 864, 857, 844, or 829 as indicated (Fig. 2B). GST-RB(794–884), like GST-RB(792–928), was efficiently phosphorylated by cyclin A-associated kinase activity, whereas GST-RB(794–864) was not. Thus, residues C-terminal to residue 884, including the KXL beginning at residue 889 (Fig. 2A), were not necessary, and the RXL sequences beginning at residues 830 and 857 (Fig. 2A) were not sufficient, for cyclin A-associated kinase(s) to efficiently phosphorylate pRB under these assay conditions. Notably, sequences between residues 864 and 884, which includes the sequence KXLKXL (KPLKKL) beginning at residue 870, were necessary for the efficient phosphorylation of the S/T-P motifs located between residues 794 and 829.

We next tested whether these sequences were necessary for phosphorylation of pRB in vivo. It has been previously shown that pRB is not phosphorylated in $RB^{-/-}$ SAOS2 osteosarcoma cells in the absence of ectopic production of an appropriate cyclin (12, 18, 34, 43). Plasmids directing the expression of HA-tagged versions of wild-type pRB [pRB(1–928)] or C-terminally truncated versions thereof were transfected into SAOS2 cells. All of the pRB species were produced at comparable levels as determined by anti-HA immunoblot analysis and migrated as single bands in the absence of an ectopically produced cyclin (Fig. 2C and D). pRB(1–928) and pRB(1–896), but not pRB(1–864) and pRB(1–829), displayed a characteristic electrophoretic mobility shift, previously shown to be due to phosphorylation (34), when coproduced with either cyclin A or cyclin E. All of the pRB species bound to SV40 T antigen when produced in COS cells, suggesting that they were not grossly denatured (Fig. 2E). Thus, consistent with the in vitro results, sequences between residues 864 and 896 were required for the efficient phosphorylation of pRB by cyclin A- and E-associated kinases in vivo.

The experiments described above suggested that a sequence between residues 864 and 884 played a key role in targetting pRB for phosphorylation by cyclin-cdk2 complexes. To test whether the KXLKXL beginning at reside 870 could play such a role, site-directed mutagenesis was used to replace this sequence with the sequence NAAIRS (the NAAIRS sequence has been found in both α-helical and β-sheet regions of protein secondary structure and therefore is thought to be a highly flexible linker that should only minimally disrupt the confirmation of proteins) (Fig. 3A) (64, 92). Phosphorylation of the NAAIRS substitution mutant by cyclin A-, cyclin E-, and cdk2-associated kinase activity was grossly impaired relative to that of GST-RB(792–928) (Fig. 3B). The mutant was, however, more efficiently phosphorylated than was GST-RB(794–829) (see Discussion). Consistent with the analysis of the C-terminal truncation mutants, replacement of the KXL sequence beginning at residue 889 with three alanine residues had no effect (Fig. 3).

We next asked whether a synthetic peptide spanning the KXLKXL sequence could, like the E2F1-, and p21-derived peptides, inhibit cyclin-cdk2 kinase activity. A 15-mer peptide corresponding to pRB residues 864 to 880, but not a sequence-scrambled version thereof, inhibited the phosphorylation of GST-RB(792–928) by cyclin A-associated kinase (Fig. 4). The pRB-derived peptide appeared to be less potent than the corresponding E2F1-derived peptide, perhaps reflecting the fact that E2F1 forms stable complexes with cyclin-cdk2 complexes whereas pRB does not. Thus, the relative stabilities of the complexes and the relative potencies of the peptides might both reflect, for example, differences in the off-rates for cyclin A bound to E2F1 versus pRB.

If the inability to phosphorylate pRB species truncated at residue 829 was due to the loss of a specific cyclin-cdk2 interaction motif rather than to nonspecific conformational changes, then providing a homologous or heterologous cyclin-cdk2 recognition motif in *cis* should restore their ability to be phosphorylated by cyclin-cdk2 complexes. To test this, a chimera, GST-RB(794–829)RBCy, in which residues 869 to 878 of pRB (PKPLKKLRFD) were fused to the C terminus of GST-RB(794–829), was made (Fig. 5A). GST-RB(794–829)RBCy was phosphorylated as efficiently as GST-RB(792–928) by both cyclin A- and cyclin E-associated kinases (Fig. 5B). In the next set of experiments, 12 amino acids spanning the cyclin-cdk2 binding site of E2F1 (GRPPVKRRLDLE) or p21 (CGSKAC RRLFGP) were fused to the C terminus of GST-RB(794–829) to generate GST-RB(794–829)E2F1CyWT and GST-RB(794–829)p21Cy, respectively (Fig. 5A). As a control, GST-RB(794–829)E2F1CyMut, in which the KRRL present in the E2F1 sequence was replaced with four alanine residues (GRPPVAAAADLE), was also generated. The analogous mutation in the cyclin-cdk2 binding sequence of p107 has previously been shown to inactivate cyclin-cdk2 binding (2). Fusion to either the wild-type E2F1 or p21 cyclin-cdk2 binding sequences restored the ability of GST-RB(794–829) to serve as a substrate for both cyclin A- and E-associated kinases whereas fusion to the mutated E2F1 sequence did not (Fig. 5C and D). These results strongly suggest that GST-RB(794–829) is not phosphorylated by cyclin-cdk2 because it lacks a substrate recognition motif, rather than as a result of improper folding and

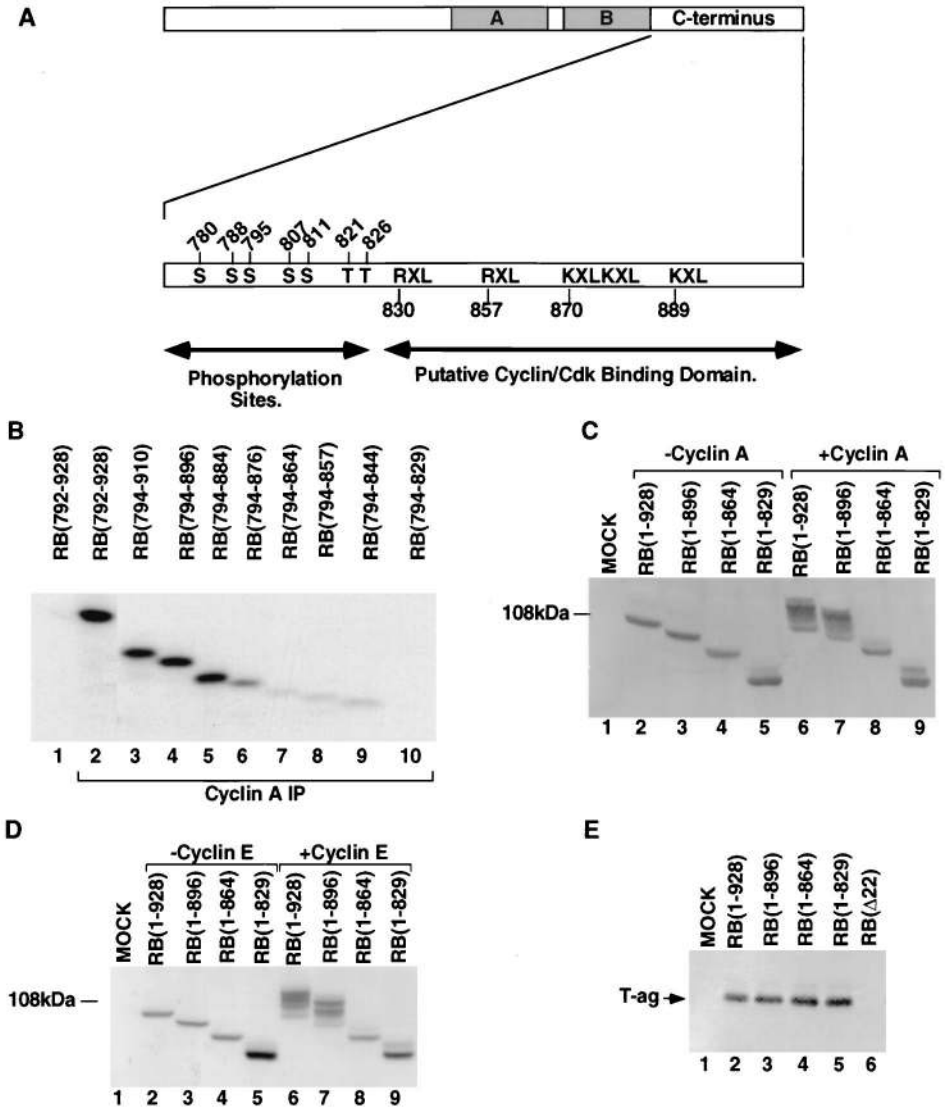

FIG. 2. Residues 864 to 884 are required for phosphorylation of pRB in vitro and in vivo. (A) Schematic of pRB protein and its C terminus. The top line shows the full-length pRB protein. Shaded boxes correspond to the A and B subdomains of the pRB viral oncoprotein binding domain (also referred to as the pRB pocket). The bottom line shows an expansion of the pRB C terminus. Indicated are the locations of the candidate S/T-P cyclin-cdk in vivo phosphorylation sites and sequences related to the previously defined cyclin binding sequence. The actual sequences in each case are as follows: at 830, RIL; at 857, RVL; at 870, KPLKKL; at 889, KHL. There are no RXL or KXL motifs between residues 780 and 829 and no S/T-P motifs downstream of residue 829. (B) U2OS osteosarcoma cells were lysed and immunoprecipitated with anti-cyclin A or control (lane 1, anti-SV40 T-antigen) antibodies as indicated. In vitro kinase assays were performed in the presence of 1 μg of the indicated GST-RB fusion proteins. Phosphorylated proteins were resolved by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and detected by autoradiography. (C and D) SAOS2 $RB^{-/-}$ osteosarcoma cells were transiently transfected with plasmids encoding HA-tagged versions of the indicated pRB mutants in the presence or absence of plasmids encoding cyclin A (C) or cyclin E (D). Cell lysates were fractionated by SDS-polyacrylamide gel electrophoresis (6% polyacrylamide) and Western blotted with an anti-HA antibody. (E) Cells stably producing SV40 T antigen (COS cells) were transiently transfected with plasmids encoding HA-tagged versions of the indicated pRB mutants, lysed, and immunoprecipitated with an anti-HA antibody. Coimmunoprecipitation of SV40 T antigen was detected by anti-SV40 T-antigen Western blot analysis.

inaccessible phosphoacceptor sites. Furthermore, these results underscore the finding that the cyclin-cdk2 binding motifs derived from pRB, E2F1, and p21 are functionally analogous.

We next tested whether the E2F1 cyclin-cdk2 binding motif could restore the ability of cyclin A-associated kinases to phosphorylate a C-terminally truncated pRB species in vivo. To this end, plasmids encoding HA-tagged wild-type pRB [pRB(1–928)], pRB(1–829), or pRB(1–829) fused at its C terminus to either the wild-type or mutant E2F1 cyclin-cdk2 binding sequence [pRB(1–829)E2F1CyWT and pRB(1–829)E2F1CyMut, respectively] were transfected into SAOS2 cells in the presence or absence of a plasmid encoding cyclin A. All of the proteins were produced at comparable steady-state levels as determined by anti-HA immunoblot analysis (Fig. 6A). Phosphorylation was detected by the appearance of pRB species with retarded mobility, as described above. Consistent with the in vitro experiments, fusion of the wild-type but not mutant E2F1-derived cyclin-cdk2 binding sequence to the C terminus of pRB(1–829) enabled it to be phosphorylated by cyclin A-associated kinases (Fig. 6A). Similarly, the wild-type but not mutant cyclin binding site from E2F1 restored phosphorylation by cyclin E-associated kinase in this assay (data not shown).

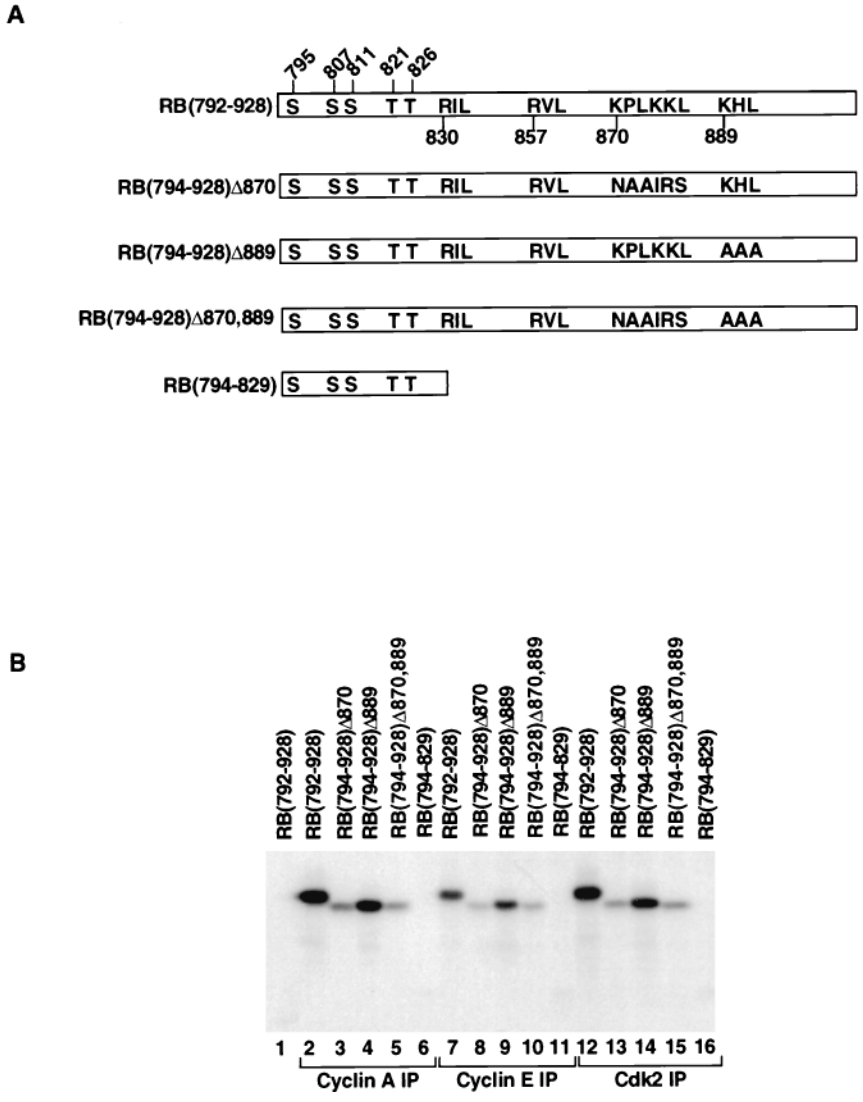

FIG. 3. A consensus cyclin-cdk binding motif between residues 870 and 875 mediates efficient phosphorylation of pRB. (A) Schematic illustrating the mutants used in the experiment in this figure. (B) U2OS osteosarcoma cells were lysed and immunoprecipitated with anti-cyclin A, anti-cyclin E, anti-cdk2, or control (lane 1, anti-SV40 T-antigen) antibodies as indicated. In vitro kinase assays were performed in the presence of 1 μg of the indicated GST-RB fusion proteins. Phosphorylated proteins were resolved by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and detected by autoradiography. Comparable levels of the GST-RB proteins were confirmed by anti-GST Western blot analysis (data not shown).

In contrast to SAOS2 cells, U2OS osteosarcoma cells are capable of phosphorylating ectopically produced wild-type pRB in the absence of an ectopically produced cyclin (78). As additional confirmation of the ability of the E2F1 cyclin-cdk2 binding sequence to direct the phosphorylation of pRB (1–829), the same pRB expression plasmids were transfected into U2OS cells. The cells were metabolically labeled with [^{32}P]orthophosphate, lysed, and immunoprecipitated with an anti-HA antibody. Bound proteins were resolved by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and detected by anti-HA immunoblot analysis (Fig. 6B, bottom) or autoradiography (Fig. 6B, top). In this experiment, electrophoretic conditions were chosen so that the pRB species would migrate as single bands. Anti-HA immunoblot analysis showed that all of the pRB species were produced at comparable steady-state levels. The phosphorylation of pRB (1–829) was reproducibly diminished by approximately 50% relative to wild-type pRB as determined by quantitative phosphorimager analysis (Fig. 6C). Consistent with the results obtained with SAOS2 cells, pRB(1–829)E2F1CyWT but not pRB(1–829)E2F1CyMut, incorporated ^{32}P to the same extent as did wild-type pRB. Thus, in keeping with the results obtained in vitro, pRB residues 829 to 928 contain one or more cyclin-cdk2 recognition motifs, and this function can be provided by a heterologous cyclin-cdk2 recognition motif.

Results similar to those depicted in Fig. 6 were also obtained when heterologous cyclin-cdk2 binding sites were fused to pRB(1–864) rather than to pRB(1–829) (Fig. 7A). The residual phosphorylation of pRB(1–829) and pRB(1–864) following reintroduction into U2OS cells left open several possibilities. For example, phosphorylation of all of the cdk phosphoacceptor sites in pRB might be reduced by similar amounts in these mutants relative to wild-type pRB. Alternatively, different cdk phosphoacceptor sites might exhibit differential requirements for the C-terminal cyclin-cdk2 binding site studied here. Finally, the residual phosphorylation of these mutants might

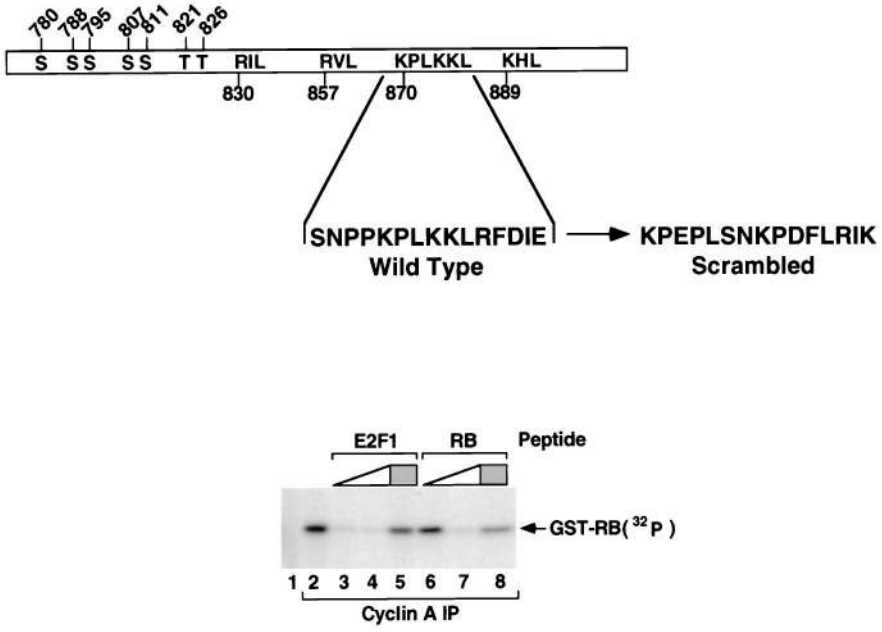

FIG. 4. A synthetic peptide spanning the KPLKKL residues of pRB inhibits phosphorylation of pRB by cyclin A-cdk2. The schematic shows the amino acid sequences of the wild type and scrambled versions of the pRB-derived peptide (residues 866 to 880) tested as inhibitors of cyclin A-associated kinase activity. U2OS osteosarcoma cells were lysed and immunoprecipitated with anti-cyclin A or control (lane 1, anti-SV40 T-antigen) antibodies as indicated. In vitro kinase assays were performed with GST-RB(792–928) in the presence of no competitor peptide (lanes 1 and 2), 1.4 or 140 μM wild-type E2F1-derived cyclin-cdk2 binding peptide (open triangle) (lanes 3 and 4), 1.4 or 140 μM wild-type pRB peptide (residues 866 to 880) (open triangle) (lanes 6 and 7), or 140 μM scrambled versions of the E2F1 and pRB peptides (shaded squares) (lanes 5 and 8).

reflect phosphorylation by kinases other than cdks (see also below).

To this end, U2OS cells were transfected to produce HA-tagged pRB, pRB(1–896), pRB(1–864), or pRB(1–864) fused to either wild-type or mutant E2F1 cyclin-cdk2 binding sequences. Cell extracts were prepared and immunoblotted with anti-HA antibody or antibodies against specific pRB-derived phosphopeptides corresponding to known cdk phosphorylation sites (Fig. 7B). Interestingly, phosphorylation of T821, S780, and S795 was undetectable in pRB(1–864) and was fully restored in the chimera containing an intact E2F1-derived cyclin-cdk2 binding site. In contrast, residual phosphorylation of S612 was detectable in pRB(1–864) as well as in pRB(1–864) species fused to mutated or scrambled E2F1-derived cyclin-cdk2 binding sites (Fig. 7B, lanes 4, 6, and 7, respectively). These data suggest that some cdk phosphorylation sites, such as T821, are strictly dependent upon an intact RXL-like motif C-terminal to pRB residue 864 whereas at least one (S612) is not. Furthermore, they leave open the possibility that kinases other than cdk2 contribute to the phosphorylation of pRB(1–829) and pRB(1–864) in vivo. pRB(1–864) did not induce a cell cycle arrest when introduced into these cells (data not shown), perhaps reflecting the residual phosphorylation of this mutant. It was previously shown that phosphorylation of S612 inhibits the binding of pRB to E2F (49).

In pilot experiments, we confirmed that pRB(1–864) is sufficient to bind to E2F, in keeping with earlier reports (33, 78). To ask whether phosphorylation of pRB under the control of a heterologous KRXL motif was functionally significant, asynchronously growing SAOS2 cells were transfected with plasmids encoding HA-tagged pRB(1–864) fused to 12 amino acids (GRPPVKRRLDLE) spanning the wild-type cyclin-cdk2 binding sequence of E2F1 [pRB(1–864)E2F1CyWT] or HA-tagged pRB(1–928) in the presence or absence of plasmids encoding cyclin A and cdk2. As negative controls, similar plasmids in which the cyclin-cdk2 sequence was alanine substituted (GRPPVAAAADLE) [pRB(1–864)E2F1CyMut] or scrambled (EPLKGRPVDLRR) [pRB(1–864)E2F1CySCR] were tested in parallel. Subsequently, the cells were lysed and immunoprecipitated with an anti-E2F4 antibody, and bound pRB was detected by anti-HA immunoblot analysis. In the presence of ectopically produced cyclin A and cdk2, pRB(1–928) and pRB(1–864)E2F1CyWT, but not pRB(1–864)E2F1CyMut and pRB(1–864)E2F1CySCR, were detectably phosphorylated, as judged by their relative mobilities following SDS-polyacrylamide gel electrophoresis (Fig. 8, bottom) and in keeping with the data presented in Fig. 6. In the absence of ectopic cyclin A and cdk2, E2F4 associated with wild-type pRB and all three of the pRB mutants (Fig. 8, top). In the presence of ectopically expressed cyclin A and cdk2, however, E2F4 no longer bound to wild-type pRB and pRB(1–864)E2F1CyWT but remained bound to pRB(1–864)E2F1CyMut and pRB(1–864)E2F1CySCR. Thus, the phosphorylation of pRB(1–864) by cyclin A-cdk2 under the direction of the wild-type cyclin-cdk2 recognition motif was associated with the same outcome as phosphorylation of wild-type pRB by cyclin-cdk2, namely, disruption of E2F binding. Furthermore, the failure of cyclin A-cdk2 to disrupt the binding of E2F4 to pRB(1–864)E2F1CyMut and pRB(1–864)E2F1CySCR strongly suggests that the displacement of E2F4 from wild-type pRB and pRB(1–864)E2F1WT is a direct consequence of phosphorylation of pRB by cyclin A-cdk2.

pRB is a critical target of cyclin D-cdk4 complexes. Previous studies suggested that short RXL-containing peptides were unlikely to stably interact with cyclin D-cdk4 complexes (2, 7). Nonetheless, these studies left open the possibility that such sequences, in the appropriate molecular context, also contribute to substrate recognition by cyclin D-cdk4. To begin to

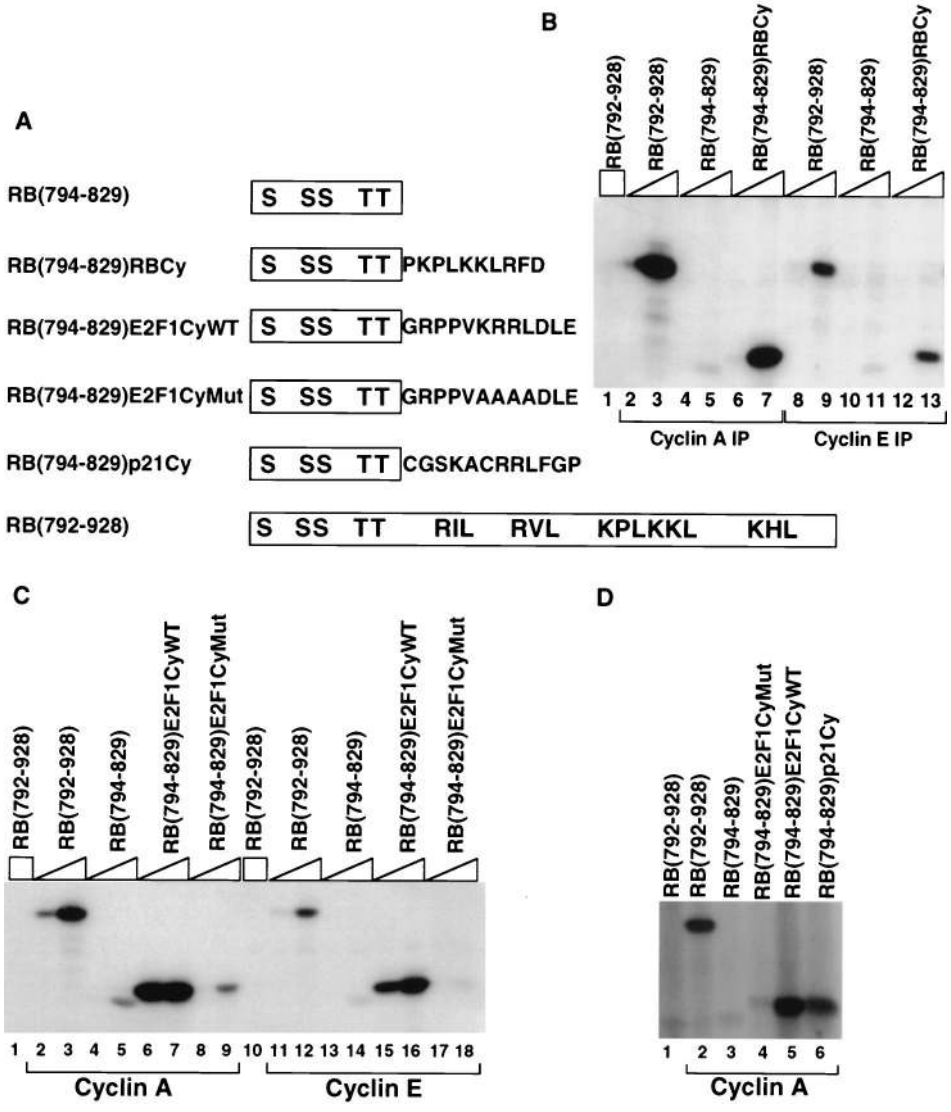

FIG. 5. A minimal cyclin-cdk2 targeting sequence derived from pRB, E2F1, or p21 will promote pRB phosphorylation in vitro. (A) Schematic of the pRB mutants used in the experiment in this figure. (B to D) U2OS osteosarcoma cells were lysed and immunoprecipitated with anti-cyclin A, anti-cyclin E, or control (lane 1, anti-SV40 T-antigen) antibodies as indicated. In vitro kinase assays were performed in the presence of 1 μg of GST–RB(792–928) (open squares) or 0.1 or 1 μg of the indicated GST-RB fusion proteins (open triangles). Phosphorylated proteins were resolved by SDS-polyacrylamide gel electrophoresis and detected by autoradiography.

address this, GST-pRB(792–928) and C-terminally truncated version thereof were tested as substrates in cyclin D1-cdk4 in vitro kinase assays (Fig. 9). Due to the difficulty in recovering high levels of cdk4 kinase activity from mammalian cell extracts, recombinant cyclin D1-cdk4 was used. The amount of recombinant cyclin A-cdk2 and cyclin D1-cdk4 necessary to achieve comparable phosphorylation of GST-pRB(792–928) was determined in pilot experiments and was used subsequently. In contrast to cyclin A-cdk2, deletion of the C-terminal 32 amino acids from pRB led to a dramatic decrease in its ability to undergo phosphorylation by cyclin D1-cdk4 (Fig. 9A), in keeping with a recent report (74). Similar results were obtained with respect to phosphorylation of GST-pRB(773–928) and derivatives thereof by cyclin D1-cdk4 (data not shown). Thus, sequences outside of the KXLKXL motif beginning at residue 870 contribute to the recognition of pRB by cyclin D1-cdk4. Nonetheless, as observed for cyclin-cdk2 (Fig. 3), site-directed mutagenesis of the KXLKXL motif led to a demonstrable decrease in pRB phosphorylation by cyclin D1-cdk4 (Fig. 9B, compare lanes 2 and 4 to lane 1). Finally, fusion of the E2F1-derived cyclin-cdk2 binding site to pRB(792–829) restored its ability to be phosphorylated by cyclin D1-cdk4 (Fig. 9B, compare lane 6 to lanes 5 and 7). Thus, in certain contexts, the KRXL cyclin-cdk2 binding motif can also serve as a recognition site for cyclin D1-cdk4 complexes.

DISCUSSION

Using cyclin-cdk2 as a model, we have attempted to understand how cyclin-cdk complexes recognize their substrates. Our results suggest that cyclin-cdk2 complexes must physically interact with a substrate recognition motif prior to phosphorylation of the S/T-P phosphoacceptor sites and suggest that this model holds true not only for substrates that stably interact with cyclin-cdk2 complexes, such as E2F1 and p107, but also for substrates that interact only transiently, such as pRB. Ac-

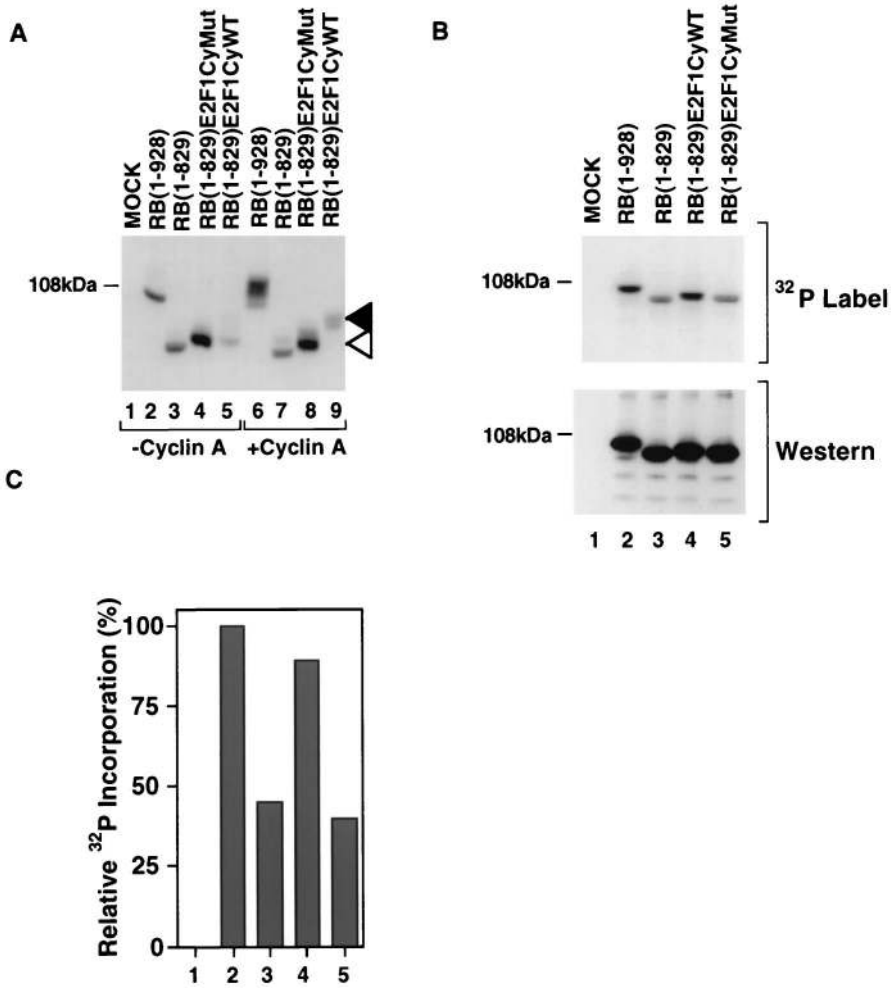

FIG. 6. A minimal cyclin-cdk targeting sequence derived from pRB, E2F1, or p21 will promote pRB Phosphorylation in vivo. (A) SAOS2 $RB^{-/-}$ osteosarcoma cells were transiently transfected with plasmids encoding HA-tagged versions of the indicated pRB mutants in the presence or absence of a plasmid encoding cyclin A as indicated. The cells were lysed, and soluble proteins were fractionated by SDS-polyacrylamide gel electrophoresis (6% polyacrylamide) and Western blotted with an anti-HA antibody. The open arrowhead marks the position of unphosphorylated RB(1–829)CyE2F1Mut and RB(1–829)CyE2F1WT. The solid arrowhead marks the position of phosphorylated RB(1–829)CyE2F1WT. (B) U2OS $RB^{+/+}$ osteosarcoma cells were transiently transfected with plasmids encoding HA-tagged versions of the indicated pRB mutants, metabolically labelled with [^{32}P]orthophosphate, lysed, and immunoprecipitated with an anti-HA antibody. Bound proteins were resolved by SDS-polyacrylamide gel electrophoresis (10% polyacrylamide) and detected by autoradiography (top) and anti-HA Western blot analysis (bottom). Note that the characteristic pRB mobility shift following phosphorylation is not apparent under these electrophoretic conditions, in contrast to the conditions used in panel A. (C) ^{32}P incorporation relative to wild-type pRB was determined by phosphorimager analysis of panel B.

cording to this model, the cyclin-cdk2 targeting function may be provided in *cis*, as part of the same molecule as the S/T-P phosphoacceptor site, or in *trans* by an adapter molecule that contacts both the substrate and the cyclin-cdk2 complex simultaneously. For example, E2F1 functions as an adapter for phosphorylation of its heterodimeric partner, DP1, by cyclin A-cdk2 (15, 51, 95) and p107 has been suggested to likewise act as an adapter for the phosphorylation of c-myc by cyclin A-cdk2 (35). This model is consistent with two recent observations. First, the N terminus of E2F1, containing its cyclin-cdk2 binding motif, can direct the phosphorylation of E2F4 (which lacks such a motif) by cyclin-cdk2 as an E2F1-E2F4 chimera (16). Second, cyclin A mutants that cannot bind to the RXL motif but retain the ability to bind to cdk2 are unable to phosphorylate substrates such as pRB and are unable to promote S-phase entry (80).

With this general model in mind, a novel function can now be ascribed to the pRB C terminus. That is, the C-terminal 100 amino acid residues of pRB serve to target pRB to cyclin A- and E-cdk2 complexes. In this regard, the pRB C terminus might be viewed as performing a function analogous to that performed by the spacer elements of the pRB family members p107 and p130 (17, 21). Furthermore, the physical separation of the cdk phosphorylation sites (upstream of residue 829) and the cyclin-cdk2 binding region (downstream of residue 829) allows the pRB C terminus to be viewed as two separate subdomains. The N-terminal half contains a number of candidate in vivo phosphorylation sites, residues 780, 788, 795, 807, 811, 821, and 826, whereas the C-terminal half contains a number of candidate cyclin-cdk2 targeting motifs (9, 46, 48, 49, 54, 58, 96). At least one of these, the KPLKKL sequence beginning at residue 870, appears to be functional in directing pRB phosphorylation.

Most of the proteins that stably interact with cyclin-cdk2 contain the sequence RXL (2, 7, 100). Lisztwan et al. recently showed that p45/Skp2 uses the sequence KXL, rather than

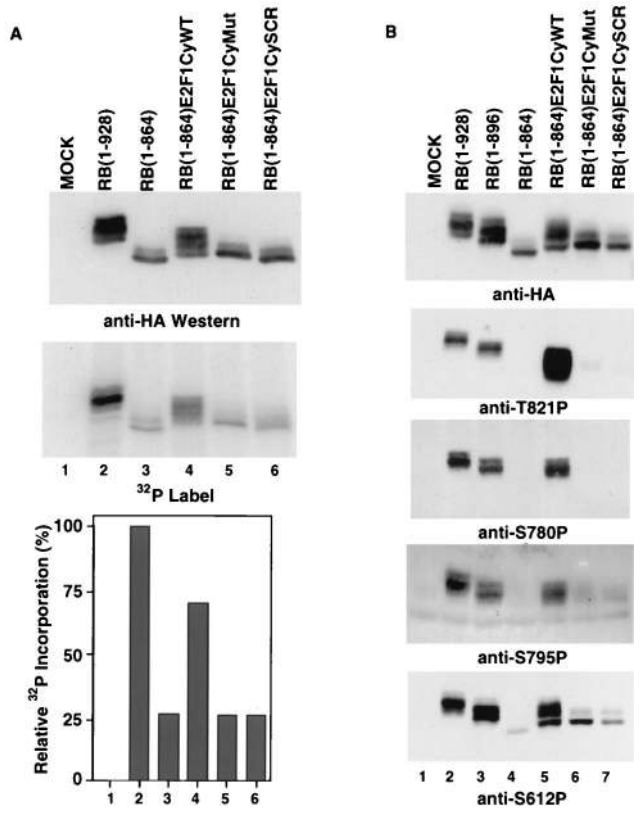

FIG. 7. Identification of pRB phosphosites that require a C-terminal cyclin-cdk2 binding site. (A). U2OS $RB^{+/+}$ osteosarcoma cells were transiently transfected with plasmids encoding HA-tagged versions of the indicated pRB mutants, metabolically labelled with [^{32}P]orthophosphate, lysed, and immunoprecipitated with an anti-HA antibody. Bound proteins were resolved by SDS-polyacrylamide gel electrophoresis (6% polyacrylamide) and detected by anti-HA Western blot analysis (top) and autoradiography (middle). ^{32}P incorporation relative to wild-type pRB was determined by phosphorimager analysis (bottom). (B) U2OS $RB^{+/+}$ osteosarcoma cells were transiently transfected with plasmids encoding HA-tagged versions of the indicated pRB mutants. Cell extracts were prepared and immunoblotted with the indicated antibodies.

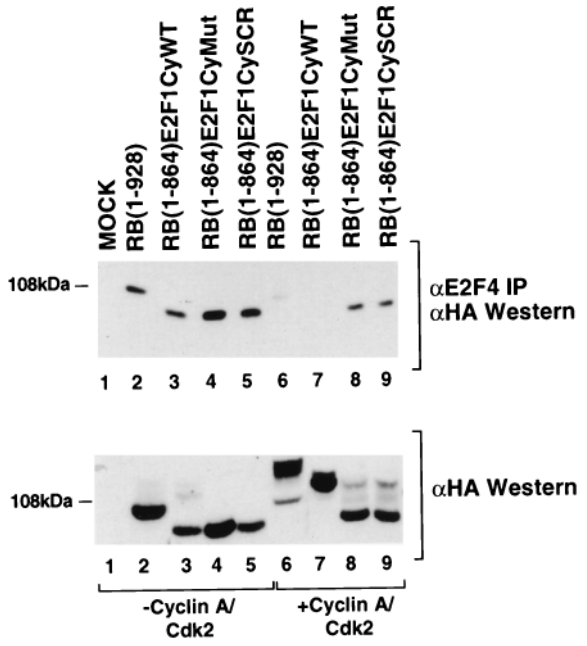

FIG. 8. Phosphorylation of pRB mediated by the heterologous cyclin-cdk2 binding sequence of E2F1 results in disruption of pRB-E2F4 complexes. Asynchronously growing SAOS2 cells were transfected with plasmids encoding the HA-tagged versions of the indicated pRB mutants in the presence or absence of cyclin A and cdk2, as indicated. The cells were lysed, and an aliquot of the soluble proteins (150 μg) was fractionated by SDS-polyacrylamide gel electrophoresis (6% polyacrylamide) and Western blotted with an anti-HA antibody (bottom). The remainder of the lysate was immunoprecipitated with an anti-E2F4 monoclonal antibody. The immunoprecipitates were fractionated by SDS-polyacrylamide gel electrophoresis (6% polyacrylamide) and Western blotted with the anti-HA antibody (top).

RXL, to bind to cyclin A-cdk2 (59). It is possible that the use of KXL instead of RXL, in addition to contextual effects, allows pRB to bind to cyclin-cdk2 complexes with a high off-rate typical of most substrate-enzyme interactions. Nonetheless, our findings are consistent with earlier studies that showed that cyclin A can physically interact with pRB (18, 91).

We cannot presently exclude the possibility that pRB contains an additional (R/K)XL-like sequence that contributes to its phosphorylation by cyclin-cdk2 complexes. In this regard, we have not yet evaluated pRB mutants in which the RXL motifs beginning at either residue 830 or residue 857 were specifically altered. The presence of a second cyclin-cdk2 recognition motif in the pRB C terminus might account for the residual phosphorylation of GST-RB(794–928)Δ870 and GST-pRB(794–844) relative to GST-RB(794–829) and would raise the interesting possibility that different cyclin-cdk2 substrate recognition motifs within pRB lead to phosphorylation of different S/T-P sites. Our data thus far suggest that phosphorylation of at least some pRB phosphoacceptor sites, such as T821 and S795, is under the control of the KXLKXL motif beginning at pRB residue 870.

Conceivably, this general model holds true for other cyclin-cdk complexes as well. In this regard, pRB can form complexes with cyclin D (12, 18, 43). Notably, the region of cyclin A that binds to the RXL motif is fairly well conserved among different cyclins (79, 80). Furthermore, mutation of the KXLKXL beginning at residue 870 impaired the phosphorylation of pRB by cyclin D1-cdk4 in vitro. In addition, the E2F1-derived cyclin-cdk2 binding motif restored the phosphorylation of GST-pRB(792–829) by cyclin D1-cdk4. Thus, the (R/K)XL might be viewed as a general cyclin-cdk docking site. Several lines of evidence suggest that flanking residues influence whether this sequence will form a productive complex with a given cyclin-cdk complex. First, short RXL-containing peptides inhibit the activity of cyclin-cdk2 complexes at much lower concentrations than are required to inhibit cyclin D-cdk4 complexes (2, 7). Second, pRB mutants that lack residues 910 to 928 but retain the phosphoacceptor and (R/K)XL sites are inefficiently phosphorylated by cyclin D-cdk4 (reference 74 and results described above). Finally, E2F1 binds to cyclin A-cdk2 but not to cyclin E-cdk2 whereas the E2F1-derived cyclin-cdk2 binding peptide interacts with both (references 2 and 51 and results described above).

The pRB pocket, corresponding to pRB(379–792), binds to proteins containing the consensus sequence LXCXE. Intriguingly, the D-type cyclins and cyclin E contain LXCXE sequences, which might serve to target them to pRB (12, 45). In addition, pRB pocket mutants are hypophosphorylated in vivo (28, 29, 44, 87). Nonetheless, pRB(792–928) is efficiently phosphorylated by cyclin-cdk2 and cyclin-cdk4 complexes in vitro whereas phosphorylation of pRB(1–829), which contains an intact pocket, is clearly impaired. Furthermore, cyclin D1 LXCXE mutants can phosphorylate pRB (9, 37). Our data provide a potential reconciliation of these findings. Specifically, it

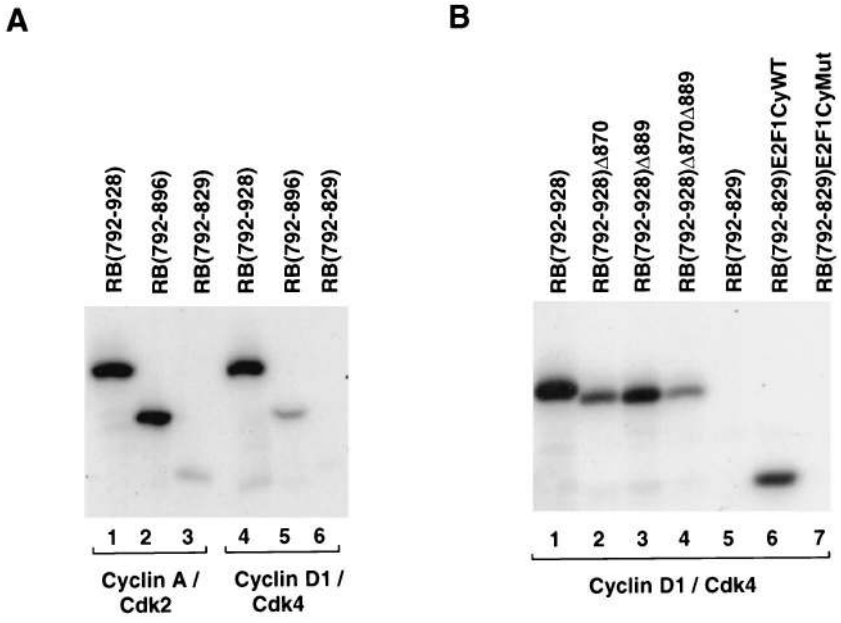

FIG. 9. A cyclin-cdk2 binding motif can participate in substrate recognition by cyclin D1-cdk4. In vitro kinase reactions were performed with recombinant cyclin A-cdk2 or cyclin D1-cdk4 and the indicated GST-RB fusion proteins. Comparable amounts of each GST-RB fusion protein were added in each reaction, as determined by Bradford assay and confirmed by Coomassie blue staining. Phosphorylated proteins were resolved by SDS-polyacrylamide gel electrophoresis and detected by autoradiography.

is possible that cyclin-cdk complexes can interact with pRB via the pocket or via the (R/K)XL motif(s). Mutation of the pocket might affect the phosphorylation of pRB in vivo in at least two ways. First, it is possible that certain phosphoacceptor sites strictly depend upon docking of cyclin-cdk complexes to the pRB pocket in an LXCXE-dependent manner. Second, mutations in the pRB pocket might lead to conformational changes that affect the accessibility of the (R/KXL) motifs.

Short phosphoacceptor peptides that can serve as substrates for cyclin A-cdk2 or cyclin D-cdk4 complexes have been selected or identified (9, 46, 74, 83). Pan et al. found that a pRB-derived peptide containing a putative cyclin D-cdk4 phosphoacceptor site (Ser 795) was phosphorylated 1,000-fold less efficiently than was RB(792–928) by cyclin D1-cdk4 (74). This result is entirely consistent with our data implicating the need for a cyclin-cdk-docking site in conjunction with a phosphoacceptor site for efficient phosphorylation by cyclin-cdk complexes.

There are precedents for cellular kinases being targeted to their substrates in this fashion. For example, JNK kinase interacts with a docking site on its substrate, c-jun, that is separate from the phosphoacceptor sites (26, 42). Indeed, there is evidence to suggest that the relative specificity of the JNK family kinases for the Jun family members, c-jun, junD, and junB, is in part determined by their relative affinities for the docking site. Other examples include mitogen-activated protein kinase and its substrate $p90^{rsk}$ (99) and receptor tyrosine kinases and substrates such as phospholipase Cγ (5).

The finding that a p21-derived cyclin-cdk2 could direct the phosphorylation of a heterologous substrate [pRB(1–829)] suggests that the binding of cyclin-cdk2 to this sequence, per se, does not prevent it from acting as a kinase. This is in keeping with earlier observations that suggested that p21, at least when present in low concentrations, could be found in complexes that contained catalytically active cyclin-cdk2 (97). This has led to the speculation that p21, under certain situations, might act as a substrate or adapter (2, 52, 100). The RXL motif, based on the crystal structure of cyclin A-cdk2 bound to the p21 family member p27, is likely to interact with a potential substrate-docking site or cleft formed by cyclin A (79). Inhibition of cyclin-cdk2 by full-length p27 and, by extension, by p21, probably also involves the induction of conformational changes in the cyclin-cdk2 complex and steric effects on the ATP binding site (79).

It has been suggested that Ser780 (46, 96) and Ser795 (9, 74) are preferentially phosphorylated by cyclin D-cdk4 complexes rather than cyclin-cdk2 complexes. Nonetheless, these sites were phosphorylated in pRB(1–896) but not in pRB(1–864) in vivo. This is in apparent disagreement with our in vitro studies, as well as those of Pan et al. (74), suggesting a requirement for the C-terminal 18 residues of pRB for recognition by cyclin D-cdk4 complexes. One possibility is that these sites can also be phosphorylated by cyclin-cdk2 complexes in vivo under certain conditions. A second possibility stems from the fact that U2OS cells lack the cdk4 inhibitor p16INK4A. It is possible that the residual interaction of cyclin D-cdk4 with the KXLKXL motif beginning at residue 870 can lead to the phosphorylation of these sites in this setting.

pRB phosphorylation occurs in an orchestrated fashion as cells exit G_0, traverse G_1, and enter S (10, 66). The initial phosphorylation of pRB coincides with activation of the D-type cyclins, whereas hyperphosphorylation of pRB in late G_1 coincides with activation of cyclin E (66). Recent studies suggest that D-type cyclins and cyclin E cooperate to neutralize the ability of pRB to suppress cell growth (19, 31, 63). Furthermore, recent studies suggest a requirement for continued phosphorylation of pRB by cyclin A during S phase (47). On the other hand, it is clear that cyclin E-cdk2 can also act downstream of, or perhaps in parallel with, pRB (4, 11, 13, 14, 23, 32, 40, 55, 56, 60, 72). Our studies have not addressed the relative contributions of cdk4 and cdk2 to the control of pRB function. In particular, the ability of cyclin A to promote the

dissolution of pRB-E2F complexes in SAOS2 cells when overproduced cannot be taken as evidence that it performs this function under physiological conditions. The creation of pRB mutants that are not recognized by specific cyclin-cdk complexes should facilitate studies aimed at addressing these issues.

Emerging data suggest that different pRB phosphoacceptor sites regulate distinct biochemical functions such as binding to LXCXE proteins or E2F (48, 49, 96). It is possible that combinatorial regulation of pRB phosphorylation, and hence its biochemical activities, is achieved through the use of different cyclin-cdk complexes, different cyclin-cdk docking sites, and different phosphoacceptor sites.

The majority of human cancers harbor RB gene mutations or mutations which lead to the untimely phosphorylation and hence functional inactivation of pRB by cyclin-cdk complexes (27, 41). Understanding the structural requirements for pRB phosphorylation by cyclin-cdk complexes and the identification of peptides which specifically block pRB phosphorylation may facilitate the development of small molecules which modulate pRB phosphorylation in cancer cells.

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