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Identification of a Cyclin-cdk2 Recognition Motif Present in Substrates and p21-Like Cyclin-Dependent Kinase Inhibitors

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Understanding how cyclin-cdk complexes recognize their substrates is a central problem in cell cycle biology. We identified an E2F1-derived eight-residue peptide which blocked the binding of cyclin A and E-cdk2 complexes to E2F1 and p21. Short peptides spanning similar sequences in p107, p130, and p21-like cdk inhibitors likewise bound to cyclin A-cdk2 and cyclin E-cdk2. In addition, these peptides promoted formation of stable cyclin A-cdk2 complexes in vitro but inhibited the phosphorylation of the retinoblastoma protein by cyclin A- but not cyclin B-associated kinases. Mutation of the cyclin-cdk2 binding motifs in p107 and E2F1 likewise prevented their phosphorylation by cyclin A-associated kinases in vitro. The cdk inhibitor p21 was found to contain two functional copies of this recognition motif, as determined by in vitro kinase binding/inhibition assays and in vivo growth suppression assays. Thus, these studies have identified a cyclin A- and E-cdk2 substrate recognition motif. Furthermore, these data suggest that p21-like cdk inhibitors function, at least in part, by blocking the interaction of substrates with cyclin-cdk2 complexes.

Progression through the mammalian cell cycle is driven by the orderly activation of cyclin-dependent kinases (cdks) (30, 31, 56). cdk activity, in turn, is regulated through posttranslational modifications, such as phosphorylation, and by interactions with regulatory proteins such as cyclins and cdk inhibitors (CDKIs) (38, 51, 72). Each cyclin binds to a preferred subset of cdks, and the resulting cyclin-cdk complexes typically display peak kinase activity during a defined period in the cell cycle. For example, cyclin E-cdk2 is active at the G_1/S transition whereas cyclin B-cdc2 functions at the G_2/M transition (31). Presumably, different cyclin-cdk complexes phosphorylate and regulate different downstream effector proteins, thus giving rise to the different biochemical events characteristic of each stage of the cell cycle. Consistent with this view, different cyclin-cdk complexes display differences in substrate specificity in vitro (11, 12, 14, 35, 37, 49, 61, 88), although the sequence S/T-P-X-Z (where Z is typically a basic residue) appears to be a preferred cdk phosphorylation site (55, 76, 77, 90). How different cyclin-cdk complexes recognize their respective substrates is largely unknown.

Cyclin D-cdk4, cyclin A-cdk2, and cyclin E-cdk2 complexes have all been shown to be involved in control of progression from late G_1 to S phase (1, 2, 20, 34, 53, 57–59, 65–69, 85, 94). Each of these complexes can phosphorylate the retinoblastoma protein (pRB) in vitro and, when overproduced, lead to hyperphosphorylation of pRB in vivo (14, 28, 35, 49, 50, 67, 68). Moreover, overproduction of cyclin A, E, or D can overcome a pRB-induced G_1/S block, suggesting that pRB is a physiological target of cdk4 and cdk2 action (11, 14, 28). Indeed, it has been proposed that pRB is the only important physiological substrate of cyclin D-cdk4 (3, 36, 44–46, 52). In contrast, numerous reports suggest that important cdk2 substrates other than pRB exist. For example, dominant negative cdk2 mutants (81), neutralizing anti-cyclin E antibodies (58), and the CDKIs p21 and p27 (25, 62, 79, 86) arrest cells lacking functional pRB at the G_1/S boundary. Furthermore, comparison of the effects of induced overproduction of cyclins E and D on pRB phosphorylation and the kinetics of entry into S phase suggests that the effects of the cyclin E-dependent kinase are not dependent on pRB phosphorylation (68). Putative in vivo substrates of cdk2, other than pRB, include E2F1 (16, 37, 60), p107 (13, 17, 39, 61), and p130 (9, 41).

A number of cell cycle regulatory proteins can form stable complexes with cyclin A and E-cdk2 complexes. For example, the cell cycle regulatory transcription factor E2F1 binds to cyclin A-cdk2 (12, 37, 88). This complex is required for the timely phosphorylation of the E2F1 heterodimeric partner DP1, which, in turn, leads to a loss of E2F1-DP1 DNA-binding capability in mid to late S phase. p107 and p130, two members of the pRB family, also form stable complexes with cyclin A-cdk2 and cyclin E-cdk2 (9, 13, 17, 39, 41, 73). In addition, p107 appears to bind to the c-myc oncoprotein. Recent data suggest that p107-bound cyclin A-cdk2 phosphorylates c-myc and consequently inhibits its ability to serve as a transactivator (4, 22, 29). Finally, each of the cloned p21-like CDKIs can bind to, and inhibit, cyclin A- and E-cdk2 complexes (23, 25, 62, 79, 86, 87).

In this study, we have identified an eight-residue E2F1-derived sequence which is necessary and sufficient for binding to cyclin A- and E-cdk2 complexes. This sequence enabled us to identify similar cyclin A- and E-cdk2 binding sites in the putative substrates, p107 and p130, and in the p21-like CDKIs, p21, p27, and p57. Synthetic peptides derived from these sequences inhibited cyclin A-associated kinase activity toward pRB despite promoting stable complex formation between cyclin A and cdk2. Mutations affecting the cyclin A- and E-cdk2 binding sites in p107 and E2F1 prevented their phosphorylation by cyclin A-dependent kinases in vitro. We found that p21 contained two cyclin A- and E-cdk2 recognition motifs, one located in the N terminus and, unexpectedly in light of previously published data, one located in the C terminus (7, 18, 21, 43, 47, 54, 78). Both binding sites, in the context of full-length p21, were found to be functional as determined by in vitro

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kinase binding and inhibition assays and in vivo growth suppression assays. Thus, multiple cell cycle regulatory proteins contain a modular cyclin A- and E-cdk2 recognition motif. This motif is necessary for targeting of substrates to cyclin A- and E-cdk2 complexes. The p21-like CDKIs appear to function, at least in part, by blocking the interaction of substrates with cyclin A- and E-cdk2 complexes.

MATERIALS AND METHODS

Cell culture and transfections. U20S 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. Cells were transfected by the calcium phosphate method (5).

Plasmids. The following have been described previously: pGEX2TK-E2F1 (32), pGEX2TK-E2F1Δ24 (37), pGEX2TK-p107(252-816) (15), pGEX2TKcs-p21 (25), pRcCMVp21 (86), pCMV-CD19 (64), pGEX2TK-RB(379-928) (32), and pGEX2T-RB(792-928) (63). pBS-cyclin A was a gift of Ed Harlow, and pRcCMV-cyclin E was a gift of R. Weinberg. The proliferating cell nuclear antigen (PCNA) cDNA from pUC19-PCNA (a gift of R. Bravo) was excised by digestion with *Bam*HI and subcloned into pcDNA3 (InVitrogen) to make pcDNA3-PCNA. Site-directed mutagenesis of pRcCMVp21 was used to generate the mutants pRcCMVp21ΔN, pRcCMVp21ΔC, and pRcCMVp21ΔN+C. Mutagenesis was carried out with a mutagene kit as instructed by the manufacturer (Bio-Rad). The synthetic oligonucleotides GTCCACTGGGCCGAATGC GGCCGCGGCGGCCTTGCTGCCGCA and CCTCTTGGAGAAGATTGCG GCCGCGGCGGAGTGGTAGAAATC were used to introduce the ΔN (CRRL to AAAA [amino acids 18 to 21]) and ΔC (KRRL to AAAA [amino acids 154 to 157]) mutations, respectively. The double mutant was made by inclusion of both oligonucleotides in a single mutagenesis reaction. Each mutant was confirmed by DNA sequence analysis. The pGEX2TKp21ΔN, -ΔC, and -ΔN+C mutants were generated by PCR of the corresponding p21 mutant cDNAs in pRcCMV, using *Pfu* polymerase, the 5′ oligonucleotide GCGCGGATCCGAATTCGCACTCA GAGGAGG, and the 3′ oligonucleotide GCGCCAATTGTCTAGAACTAGT GGATCACC. The PCR products were restricted with *Bam*HI and *Mun*I, gel purified, and cloned into pGEX2TK linearized with *Bam*HI and *Eco*RI.

Site-directed mutagenesis of pSG5-TETr-RB (379-792; p107Spacer) (71) was used to create plasmids encoding TETr-RB (379-792; p107Spacer) mutants in which the KRRL sequence within the p107 spacer was replaced with AAAA [TETr-RB (379-792; p107Spacer {Δ657A4})] or in which the sequence AGSA KRRLFGE was replaced with NAAIRS [TETr-RB (379-792; p107Spacer {Δ653NAAIRS})]. The former was made using the synthetic oligonucleotide ACCGCAGGGAGTGCTGCCGCGGCTGCTTTTGGAGAGGACCCC, and the latter was made with TACAGTTCTCCTACGAATGCCGCCATTCGTTC TGACCCCCCAAAGGAA. The corresponding p107 cDNAs were excised as *Hin*dIII-*Sal*I fragments and cloned into pGEX2TKp107A/B-RBSpacer (13, 15) that had been restricted with *Hin*dIII and *Sal*I.

Peptide synthesis. Peptides were synthesized by solid-phase synthesis on an Applied Biosystems 431A synthesizer (E2F1, p27, and p57) or were purchased from Biosynthesis, Inc. (Lewisville, Tex.) (p21, p107, and p130) and were dissolved in phosphate-buffered saline (PBS) prior to use. The p57 peptide has a histidine-to-leucine substitution at the position corresponding to residue 40 of the full-length protein due to an error during its synthesis.

Antibodies. Anti-p21 monoclonal antibody sc-817 was purchased from Santa Cruz, Inc. Anti-CD19 monoclonal antibody B9 was a gift of John Gribben. The anti-cyclin A antibody, C160, and the anti-simian virus 40 (SV40) T antibody, 419, were gifts of Ed Harlow. The anti-cyclin B antibody, CB169, was a gift of Jim DeCaprio.

GST-fusion protein purification and protein binding assays. Recovery and purification of glutathione *S*-transferase (GST) fusion proteins on glutathione-Sepharose for use in binding assays were done as described previously (33). GST fusion protein binding assays to radiolabeled in vitro translation products were performed essentially as described previously (37) in approximately 500 μl of NETN (20 mM Tris [pH 8], 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40) containing 1 μl of cyclin A (binding to GST-E2F1 and GST-cdk2), 4 μl of cyclin A and 4 μl of cyclin E (binding to GST-p21), or 4 μl of PCNA (binding to GST-p21) in vitro translation product. Approximately 1 μg of GST fusion protein was used in each binding reaction. Peptides were added prior to the addition of translation products as indicated in the figure legends. For analysis of kinase activity bound to GST–p107(252-816) and mutants thereof, the fusion proteins were immobilized on glutathione beads, incubated with extracts from U2OS cells, washed, and subjected to an in vitro kinase assay essentially as described below for immunoprecipitations and in vitro kinase assays.

For use as a substrate in kinase assays, GST–pRB(379-928) and GST–pRB(792-928) were solubilized in buffer containing Sarkosyl according to the method of Frangioni and Neel (19). Briefly, bacterial cultures expressing the protein of interest were resuspended and sonicated in NETS (20 mM Tris [pH 8], 100 mM NaCl, 1 mM EDTA, 1.5% Sarkosyl). The supernatant was cleared by centrifugation at 10,000 × *g* for 10 min at 4°C and rocked with 1/4 volume of NET-TX-100 (20 mM Tris [pH 8], 100 mM NaCl, 1 mM EDTA, 20% Triton X-100) for 10 min at 4°C. The solubilized proteins were then immobilized on glutathione-Sepharose as previously described (33), washed three times in NETN, and eluted in GST elution buffer (100 mM Tris [pH 8], 120 mM NaCl, 20 mM reduced glutathione). The eluted proteins were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and assessed by Coomassie blue staining for purity and concentration. The eluted proteins were frozen in elution buffer in a dry ice-ethanol bath and stored at −80°C.

In vitro kinase assays. In vitro kinase assays were carried out essentially as described previously (37). CB169 and C160 immunoprecipitations were performed on EBC (50 mM Tris [pH 8], 120 mM NaCl, 0.5% Nonidet P-40, 100 mM NaF, 0.2 mM sodium orthovanadate, 50 μg of phenylmethylsulfonyl fluoride per ml, 10 μg of aprotinin per ml, 5 μg of leupeptin per ml) lysates derived from asynchronously growing U2OS cells. Immune complexes were collected on protein A-Sepharose beads that had been previously coupled to a rabbit anti-mouse secondary antibody (Cappel). The Sepharose was washed five times in NETN and then three times in IP-kinase buffer (50 mM Tris [pH 7.5], 10 mM $MgCl_2$, 1 mM dithiothreitol [DTT]). Kinase reactions were performed for 20 min at 30°C in a volume of 32 μl (20 μl of IP-kinase buffer, 10 μl of GST elution buffer containing 5 to 500 ng of GST fusion protein as the substrate as indicated in the figure legends, 1 μl of [γ-^{32}P]ATP [10 mCi/ml; 6,000 Ci/mmol], and 1 μl of peptide in PBS buffer or PBS alone as indicated in the figure legends). Reactions were stopped by addition of 30 μl of 3× Laemmli sample buffer (6% SDS, 30% glycerol, 300 mM DTT, 0.03% bromophenol blue), boiled, and fractionated by SDS-polyacrylamide gel electrophoresis.

Reimmunoprecipitation of in vitro-phosphorylated proteins was performed essentially as described previously (80). Briefly, after the in vitro kinase reactions, SDS and DTT were added to final concentrations of 1% and 1 mM, respectively. The samples were boiled, diluted 30-fold in NETN, and immunoprecipitated with the indicated antibodies as described above.

Western blotting (immunoblotting). Western blot analysis was performed essentially as described previously (63). Briefly, cells were harvested by trypsinization and lysed by boiling in 1× Laemmli sample buffer (62 mM Tris [pH 6.8], 10% glycerol, 2% SDS, 100 mM DTT, 0.01% bromophenol blue). One hundred micrograms of protein, as determined by the Bradford method, was resolved by electrophoresis in an SDS–12% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. The membrane was probed with an anti-p21 antibody (sc-187) followed by goat anti-mouse conjugated to alkaline phosphatase.

Fluorescence-activated cell sorting (FACS). Determination of the cell cycle distribution of CD19-positive cells was performed as described elsewhere (64).

RESULTS

An eight-residue E2F1 sequence is sufficient for binding to cyclin A-cdk2 complexes. In an attempt to understand the structural basis of stable binding to cyclin-cdk complexes, we examined the ability of cyclin A-cdk2 to bind to E2F1. A 42-residue peptide corresponding to the previously identified cyclin A-cdk2 binding domain in E2F1 (residues 67 to 108) (37) efficiently blocked the binding of radiolabeled cyclin A, translated in vitro, to recombinant GST-E2F1 (data not shown). Note that the ability of cyclin A to bind to E2F1 in these assays likely depends on the presence of cdk2 in rabbit reticulocyte lysate (37, 88). A series of smaller peptides derived from this region was similarly assayed (Fig. 1A and B). An eight-residue peptide (PVKRRLDL) was the smallest peptide which retained the ability to efficiently prevent complex formation between cyclin A and E2F1 (Fig. 1A and B). Peptides lacking the first or last residue in PVKRRLDL, while not inert, were at least 10-fold less potent in this regard, and the peptide VKRRLD was seemingly inactive (Fig. 1A). As expected, cyclin A did not bind to a GST-E2F1 mutant [GST-E2F1(Δ24)] in which this region of E2F1 was deleted (Fig. 1A, lane 1) (37).

In the next set of experiments, alanine scanning was used to assess the importance of the individual residues in the PVKRRLDL peptide. Alanine substitution at residue 1 or 8 diminished the ability of the corresponding peptide to compete with GST-E2F1 for binding to radiolabeled cyclin A (Fig. 1C and D), thus corroborating the results obtained with the deletion mutants (Fig. 1B). Alanine mutations at positions 2 and 7 were tolerated, although the former substitution (valine to alanine) was only a conservative change (see also Fig. 2). Ala-

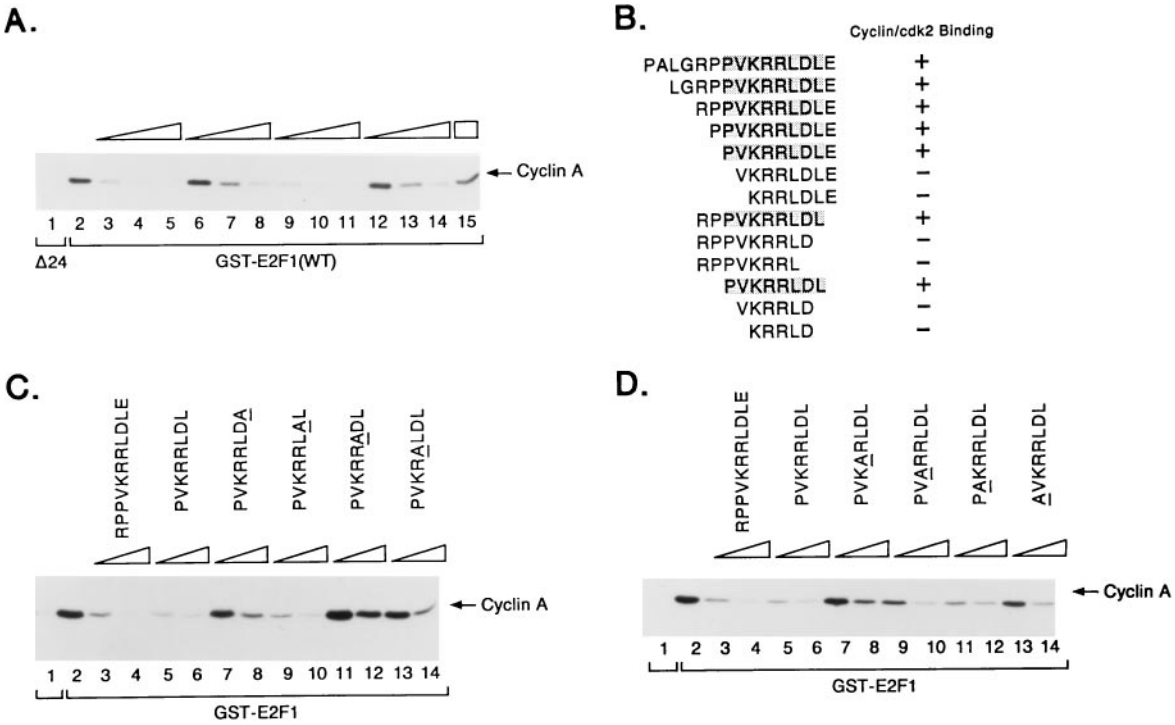

FIG. 1. Short peptides from E2F1 block the binding of cyclin A-cdk2 complexes to full-length E2F1. (A) Autoradiogram of ^{35}S-labeled cyclin A bound to glutathione-Sepharose preloaded with GST-E2F1(Δ24) (lane 1) or wild-type GST-E2F1 [GST-E2F1(WT); lanes 2 to 15]. The following E2F1-derived peptides were added to the reaction mixtures prior to the addition of cyclin A: none (lanes 1 and 2); 1, 10, and 50 μg of RPPVKRRLDLE (lanes 3, 4, and 5, respectively); 1, 10, and 50 μg of VKRRLDLE (lanes 6, 7, and 8, respectively); 1, 10, and 50 μg of PVKRRLDL (lanes 9, 10, and 11, respectively); 1, 10, and 50 μg of RPPVKRRLD (lanes 12, 13, and 14, respectively); and 50 μg of VKRRLD (lane 15). (B) Summary of E2F1 peptide data. +, greater than 50% competition of cyclin A binding by 10 μg of peptide (~10 μM); −, less than 50% competition by 10 μg of peptide (~10 μM). (C and D) Alanine scanning mutagenesis of E2F1 peptides. Shown is an autoradiogram of ^{35}S-labeled cyclin A bound to glutathione-Sepharose preloaded with GST-E2F1(Δ24) (lane 1) or wild-type GST-E2F1 (lanes 2 to 14). Five micrograms (odd-numbered lanes) or 50 μg (even-numbered lanes) of the indicated peptides was added prior to the addition of ^{35}S-labeled cyclin A.

nine mutations at positions 4, 5, and 6 were the least tolerated changes (see also Fig. 2).

Multiple cell cycle regulatory proteins contain similar sequences which are necessary and sufficient for cyclin-cdk2 binding. We noted that multiple cell cycle regulatory, cyclin-binding proteins contain a sequence with homology to the cyclin A-cdk2 binding sequence of E2F1 (Fig. 2). Among these are two additional members of the E2F family, E2F2 and E2F3, the p21-like CDKIs p21, p27, and p57, and the putative cdk2 substrates p107 and p130. There are two such motifs

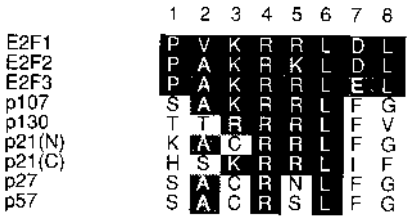

FIG. 2. Sequence alignment of putative cyclin-cdk2 binding regions from multiple cell cycle regulators. Shown is sequence alignment of the putative cyclin-cdk recognition motifs of E2F1, E2F2, E2F3, p107, p130, p21^{cip1}, p27^{kip1}, and p57^{kip2}. Note that p21^{cip1} has two such motifs, one at the N terminus [p21(N)] and one at the C terminus, [p21(C)]. Residues highlighted in black are identical to the E2F1 sequence, and those highlighted in grey are conserved. The numbers to the left and right of each sequence are the residue numbers of the N- and C-terminal amino acids of the sequence, respectively. The numbering of residues 1 to 8 above the alignment corresponds to the numbering of residues of the E2F1 8-mer in the text.

within p21, one at the N terminus and one, unexpectedly in light of previously published data, at the C terminus (7, 18, 21, 43, 47, 54, 78). Of note, given the alanine scanning results presented above, is the relatively high conservation of residues 3, 4, 5, and 6 in this alignment, which can be represented as Z-arginine-X-leucine (ZRXL), where Z is basic or cysteine and X, in seven of nine cases, is basic. Fourteen-residue synthetic peptides spanning these sequences in p107, p130, and either the N or C terminus of p21 likewise inhibited the binding of cyclin A to E2F1, whereas scrambled versions of these peptides did not (Fig. 3A and data not shown). Conversely, synthetic peptides spanning the cyclin binding motifs in E2F1, p21, p27, p57, p107, and p130 blocked the binding of cyclin A and cyclin E to GST-p21 (Fig. 3B to D). To date there is no evidence that E2F1 forms a stable complex with cyclin E in vivo (12, 37, 88). Thus, the ability of the E2F1 peptide to block the interaction of cyclin E with p21 might, among several possibilities, suggest that cyclin binding specificity is influenced by the context of this motif. Thus, residues outside this motif may participate directly (through physical interaction) or indirectly (through conformational effects) in determining cyclin binding specificity. None of the peptides tested here, including those derived from p21, blocked the binding of cyclin B to p21 (data not shown). Thus, this motif does not appear to be sufficient for binding to cyclin B under these assay conditions (see also Fig. 5A).

The cyclin binding motifs in p107 and p130 are located in their spacer elements. The spacer is, in turn, contained within a larger region of the protein, the pocket (residues 252 to 816

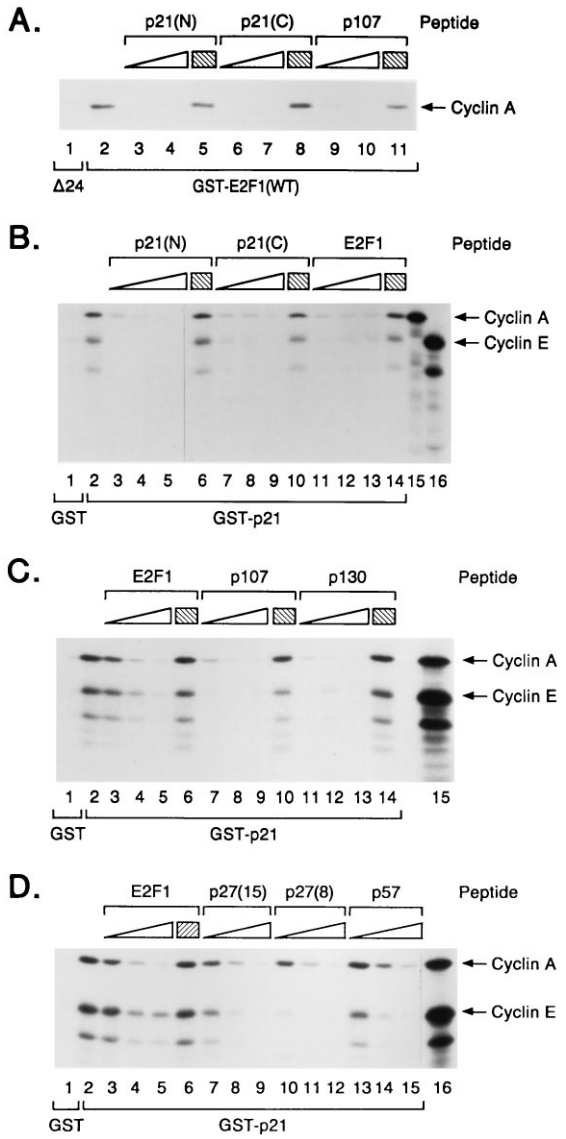

FIG. 3. Cross-competition of cyclin-cdk2-binding peptides. (A) Autoradiogram of ^{35}S-labeled cyclin A bound to GST-E2F1(Δ24) (Δ24; lane 1) or wild-type GST-E2F1 [GST-E2F1(WT); lanes 2 to 11]. The following peptides were added to the reaction mixtures prior to the addition of cyclin A: none (lanes 1 and 2); 5 and 50 μg of CGSKACRRLFGPVD (p21N, wild type [WT]; lanes 3 and 4, respectively); 50 μg of CDARCPGKGRFSLV (p21N, scrambled; lane 5); 5 and 50 μg of DFYHSKRRLIFSKR (p21C, WT; lanes 6 and 7, respectively); 50 μg of RIKFRYRHKDFLSS (p21C, scrambled; lane 8); 5 and 50 μg of TAGSA KRRLFGEDP (p107, WT; lane 9 and 10, respectively); and 50 μg of ADFTRG GLRSKAEP (p107, scrambled; lane 11). (B) Autoradiogram of ^{35}S-labeled cyclins A and E bound to GST (lane 1) or GST-p21 (lanes 2 to 14). The following peptide additions were made: none (lane 1 and 2); 1, 10, and 100 μg of CGSKA CRRLFGPVD (p21N, WT; lanes 3, 4, and 5, respectively); 100 μg of CDARC PGKGRFSLV (p21N, scrambled; lane 6); 1, 10, and 100 μg of DFYHSKRRLIFSKR (p21C, WT; lane 7, 8, and 9, respectively); 100 μg of RIKFRYRHKDFLSS (p21C, scrambled; lane 10); 1, 10, and 100 μg of RPPVKRRLDLE (E2F1, WT; lanes 11, 12, and 13, respectively); and 100 μg of ETDHQYLAESSGPA (E2F1 control, residues 95 to 108; lane 14). One-fourth of the input cyclins A and E translation product was loaded into lanes 15 and 16, respectively. (C) Autoradiogram of ^{35}S-labeled cyclins A and E bound to GST (lane 1) or GST-p21 (lanes 2 to 14). The following peptide additions were made: none (lanes 1 and 2); 1, 10, and 50 μg of RPPVKRRLDLE (E2F1, WT; lanes 3, 4, and 5, respectively); 50 μg of ETDHQYLAESSGPA (E2F1 control, residues 95 to 108; lane 6); 1, 10, and 50 μg of TAGSAKRRLFGEDP (p107, WT; lanes 7, 8, and 9, respectively); 50 μg of ADFTRGGLRSKAEP (p107, scrambled; lane 10); 1, 10, and 50 μg of PASTTRRRLFVEND (p130, WT; lanes 11, 12, and 13, respectively); and 50 μg of SRENDRTLFATRPV (p130, scrambled; lane 14). One-fourth of the input cyclins A and E was loaded into lane 15. (D) Autoradiogram of ^{35}S-labeled cyclins A and E bound to GST (lane 1) and GST-p21 (lanes 2 to 15). The following peptide additions were made: none (lanes 1 and 2); 1, 10, and 50 μg of RPPVKRRLDLE (E2F1, WT; lanes 3, 4, and 5, respectively); 50 μg of ETDHQYLAESSGPA (E2F1 control, residues 95 to 108; lane 6); 1, 10, and 50 μg of PSACRNLFGPVDHEE (p27, WT; lane 7, 8, and 9, respectively); 1, 10, and 50 μg of SACRNLFG (p27, WT; lanes 10, 11, and 12, respectively); and 1, 10, and 50 μg of SSACRSLFGPVDLEE (p57, WT; lanes 13, 14, and 15, respectively). One-fourth of the input cyclins A and E was loaded into lane 15.

in p107), that is sufficient for binding to the viral oncoproteins E1A and SV40 large T antigen (15). The p107 spacer is sufficient for binding to cyclins A and E in vitro (13). p107 deletion mutants which eliminate the cyclin binding motif identified here no longer bind to cyclins (75, 91, 92).

Using site-directed mutagenesis, we created p107 mutants containing residues 252 to 816 in which the ZRXL motif was replaced with either four alanines or a "plastic" sequence (NAAIRS) (48, 84), p107(252-816, Δ657A4) and p107(252-816, Δ653NAAIRS), respectively. These mutants were produced in bacteria as GST fusion proteins and purified by glutathione-Sepharose affinity chromatography. The levels of recovery of all GST fusion proteins were comparable, as determined by SDS-polyacrylamide gel electrophoresis and Coomassie blue staining (data not shown). GST-p107(252-816) wild type, but not the GST-p107(252-816) mutants, bound to in vitro-translated cyclin A (data not shown). The purified GST-p107(252-816) fusion proteins, immobilized on glutathione-Sepharose, were next incubated with mammalian extracts prepared from asynchronous U2OS cells. The Sepharose was washed, and in vitro kinase assays were performed. Using this approach, we detected kinase activity bound to wild-type p107 but not to the two ZRXL mutants (Fig. 4A). Each of these p107 mutants did, however, bind to E1A with seemingly wild-type efficiency (data not shown). These results, taken together, underscore the importance of this motif for cyclin-cdk2 binding by p107. They do not exclude, however, that residues outside this region modulate p107 cyclin binding in vivo, as has been suggested by others (91).

Similarly, site-directed mutagenesis was used to convert the N-terminal or C-terminal, or both, ZRXL sequences present in p21 to four alanines (Fig. 4B). All of the corresponding p21 mutants gave rise to stable GST fusion proteins of comparable solubility, as determined by Coomassie blue staining (data not shown). The single-site mutants each retained the ability to bind to in vitro-translated cyclins A and E with seemingly wild-type efficiency, whereas the double mutant did not (Fig. 4C). Likewise, both single-site mutants, but not the double-site mutant, bound to cyclin D1 (data not shown). Each p21 mutant retained the ability to bind to radiolabeled PCNA in vitro translate (Fig. 4D), in keeping with an earlier report which placed the PCNA binding site in p21 outside the two cyclin A- and E-cdk2 binding motifs identified here (82). The N-terminal CRRL motif of p21 and the CRNL motif of p27 defined here fall within regions of p21 and p27 that have been shown by others to be functionally significant (7, 18, 43, 47, 54, 62, 78). Likewise, in a previous study, two E2F1 mutants carrying deletions (Δ7 and Δ24) which, in retrospect, violate the PVKRRLDL sequence, were found to no longer interact with cyclin A-cdk2 in vitro and in vivo (37). These results, taken together, suggest that the cyclin-cdk2 binding sequences which we have identified are functional in the context of the corresponding native proteins. Furthermore, it appears that in p21, either copy of the ZRXL motif is sufficient, and at least one is necessary, for binding to cyclin A- and E-cdk2 in vitro.

The cyclin-cdk2 binding motif, as a synthetic peptide, inhibits cyclin A-associated kinase activity. The presence of similar

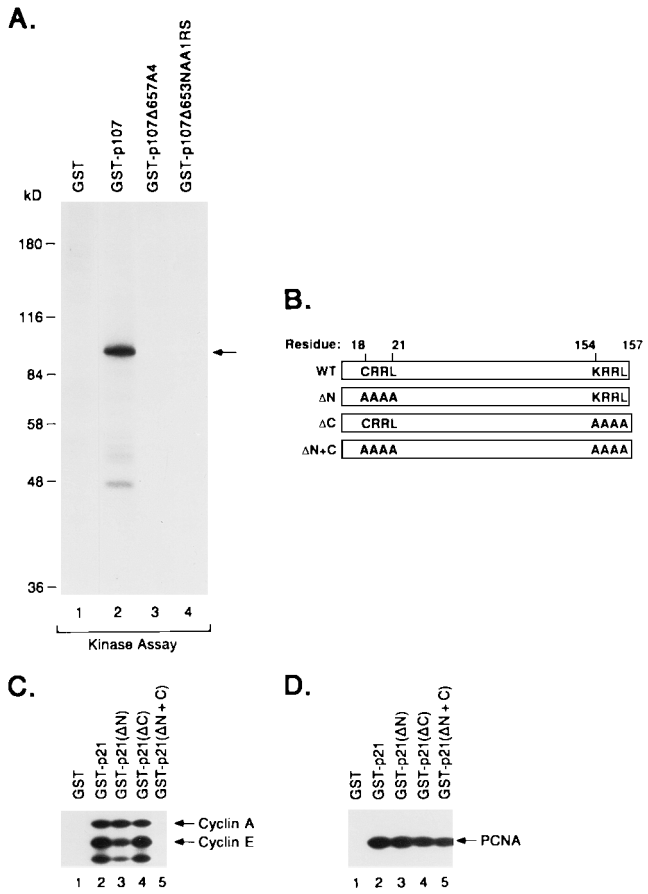

FIG. 4. Binding of cyclins to p107 and p21 depends on the ZRXL motif. (A) Glutathione-Sepharose was preloaded with GST (lane 1), GST-p107(252-816, wild type [WT]) (lane 2), GST-p107(252-816, Δ657A4) (lane 3), or GST-p107(252-816, Δ653NAAIRS) (lane 4) and incubated with extracts from U2OS cells. The Sepharose was then washed and subjected to in vitro kinase analysis. Phosphorylated proteins were resolved by SDS-polyacrylamide gel electrophoresis and detected by autoradiography. The arrow marks the position of the ^{32}P-labeled GST-p107(252-816). (B) Schematic illustration of p21 mutants. Residues 18 to 21 were converted to alanines in ΔN and ΔN+C, and residues 154 to 157 were converted to alanines in ΔC and ΔN+C. (C and D) GST (lane 1), GST-p21(WT) (lane 2), GST-p21(ΔN) (lane 3), GST-p21(ΔC) (lane 4), and GST-p21(ΔN+C) were incubated with radiolabeled in vitro translated cyclins A and E (C) or PCNA (D). Bound proteins were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography.

cyclin A- and E-cdk2 binding sequences in multiple cell cycle regulatory proteins, including known CDKIs, led us to examine whether this motif, as a synthetic peptide, might affect cyclin A-associated kinase activity. To this end, immunoprecipitates were prepared from asynchronous U2OS cells by using anti-cyclin A or anti-cyclin B antibodies and subjected to in vitro kinase analysis using recombinant GST-pRB fusion proteins as substrates. The addition of an 11-residue E2F1 peptide containing the PVKRRLDL sequence inhibited cyclin A-associated, but not cyclin B-associated, kinase activity (Fig. 5A), whereas a control peptide, corresponding to E2F1 residues 95 to 108, did not inhibit GST-pRB phosphorylation. Similarly, the wild-type peptides corresponding to the two cyclin A- and E-cdk2 binding motifs present in p21, as well as the p27-, p57-, p107-, and p130-derived peptides, inhibited cyclin A-associated kinase activity, whereas the corresponding scrambled peptides did not (Fig. 5B and data not shown).

These experiments were performed with a region of pRB, large pocket, that is capable of stably interacting with E2F1 (26, 27, 63). Thus, it was possible that E2F1, coprecipitating with cyclin A-cdk2, mediated an association between cyclin A-cdk2 and pRB in these assays. If so, the observed inhibition of kinase activity might be due to displacement of E2F1 from cyclin A-cdk2 (Fig. 1). To address this possibility, these experiments were repeated with GST-pRB(792-928) as a substrate. This molecule contains many of the pRB cyclin-cdk phosphorylation sites which have been mapped to date (40, 42) but lacks the pRB pocket and can no longer bind to E2F (63). Phosphorylation of pRB(792-928) was also inhibited by the E2F1 peptide (Fig. 5C). Thus, the observed phosphorylation is not dependent on the E2F and E1A binding region of pRB and the observed inhibition of phosphorylation is not a consequence of disrupting an E2F1-cyclin A interaction.

Following in vitro kinase analysis of cyclin A immunoprecipitates, an approximately 20-kDa ^{32}P-labeled protein was observed by autoradiography (Fig. 5B, band indicated by the asterisk). The presence of this protein was not dependent on addition of exogenous substrate, indicating that it coprecipitated from the cell extracts with cyclin A. This ^{32}P-labeled protein was reimmunoprecipitated with an anti-p21 monoclonal antibody, but not a control antibody, suggesting that it was p21 or a related protein (data not shown). Phosphorylation of this protein, like that of pRB, was inhibited by the cyclin-cdk binding peptides (Fig. 5A and B).

The cyclin-cdk2 binding motif promotes formation of stable cyclin A-cdk2 complexes. The results described above still left open the possibility that the cyclin A and E-cdk2 binding peptides disrupted cyclin A and E-cdk2 complexes, thereby preventing them from binding to proteins such as E2F1 (Fig. 1 and 3) or from acting as kinases (Fig. 5A to C). To address this possibility, GST-cdk2, immobilized on glutathione-Sepharose, was incubated with radiolabeled cyclin A in the presence or absence of the wild-type peptides derived from E2F1 and p21 (Fig. 5D). Bound cyclin A was resolved by SDS-polyacrylamide gel electrophoresis and detected by autoradiography. As expected, binding of cyclin A to GST-cdk2 was detectable in the absence of peptide (Fig. 5D; compare lanes 7 and 12 with lane 1). Each of the wild-type, but not scrambled, peptides promoted, rather than prevented, the stable association of cyclin A with cdk2 under conditions in which cyclin A binding to p21 was inhibited (Fig. 5D; compare lanes 2 to 6 with lanes 7 to 16). Thus, the observed inhibition of cdk2 kinase activity by the peptides is not a consequence of disruption of cyclin-cdk complexes.

Deletion of the cyclin-cdk2 binding motifs in substrates specifically inhibits their phosphorylation by cyclin A-cdk2. At least two possible mechanisms could be envisioned to account for the inhibition of kinase activity by the ZRXL peptides. One possibility was that they induced an allosteric change in cyclin-cdk2 complexes such that they were no longer active as kinases. This seemed unlikely, however, as cyclin A- and E-cdk2 complexes are active as kinases when bound to these sequences in the context of full-length p107 and E2F1 (12, 29, 37, 61, 88). A second possibility was that these ZRXL sequences bound to a region of cyclin A- and E-cdk2 responsible for substrate recognition. According to this second model, the ZRXL peptides were blocking access of substrates to cyclin A- and E-cdk2 complexes.

To begin to address this question, immunoprecipitates were prepared from asynchronous cells by using an anti-cyclin A antibody and subjected to in vitro kinase analysis using recombinant GST-E2F1 and GST-E2F1(Δ24) fusion proteins as substrates (Fig. 6A). The former, but not the latter, was efficiently phosphorylated under these conditions. Likewise, GST-p107,

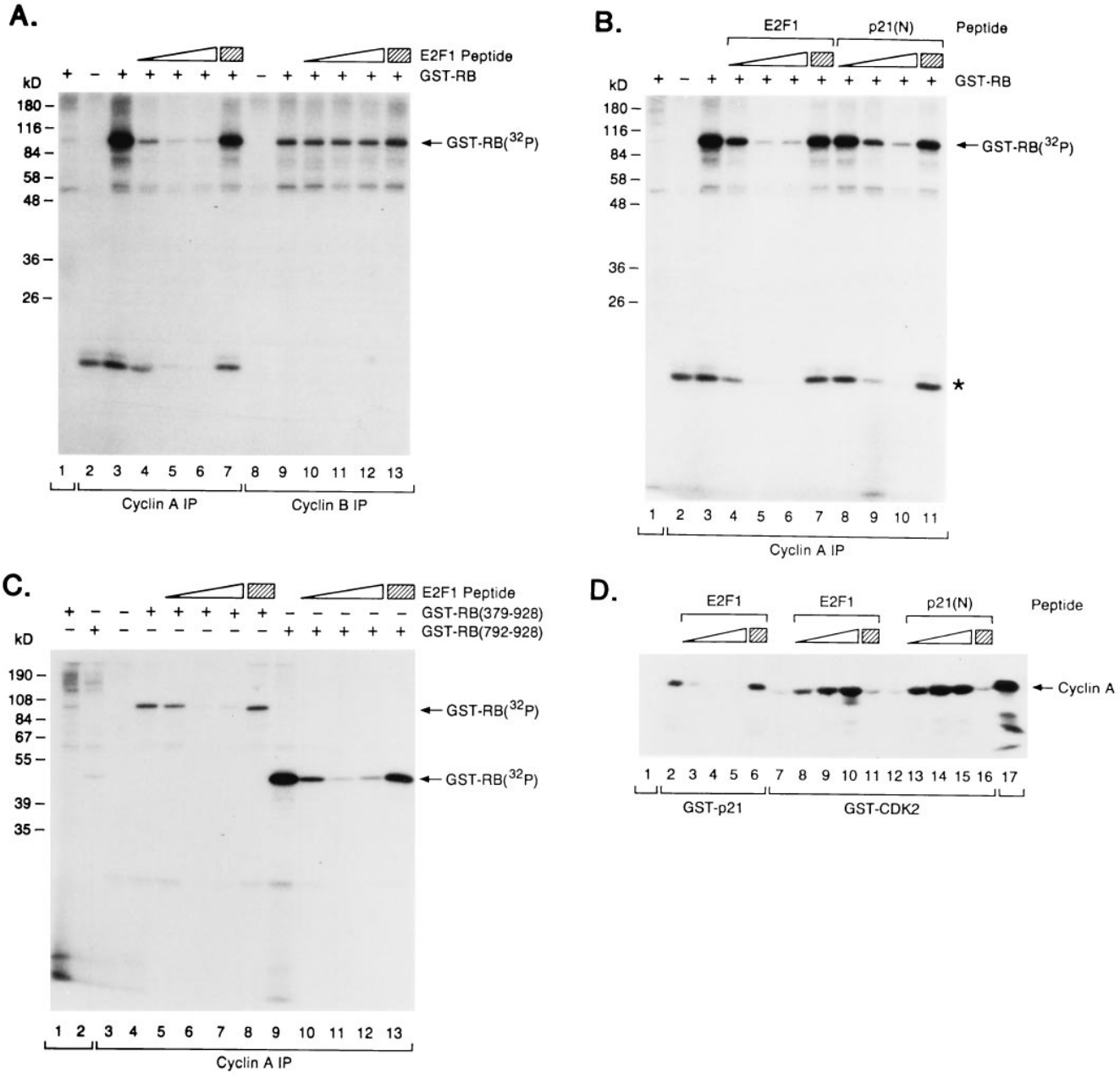

FIG. 5. Inhibition of cyclin A-associated kinase activity by cyclin-cdk2 binding peptides. Immunoprecipitates prepared from U2OS cells were washed and subjected to in vitro kinase analysis (A to C). Phosphorylated proteins were fractionated by SDS-polyacrylamide gel electrophoresis and detected by autoradiography. (A) Immunoprecipitation with anti-SV40 T (lane 1), anti-cyclin A (lanes 2 to 7), and anti-cyclin B (lanes 8 to 13) monoclonal antibodies. Shown are results of in vitro kinase analysis in the presence (lanes 1, 3 to 7, and 9 to 13) or absence (lanes 2 and 8) of ~200 ng of GST-pRb(379-928). Peptides added prior to addition of substrate: none (lanes 1 to 3, 8, and 9); 50, 500, and 5,000 ng of RPPVKRRLDLE (E2F1, wild type [WT]; lanes 4, 5, and 6, respectively, and 10, 11, and 12, respectively); 5,000 ng of ADFTRGGLRSKAEP (p107, scrambled; lanes 7 and 13). (B) Immunoprecipitation with anti-SV40 T (lane 1) and anti-cyclin A (lanes 2 to 11) monoclonal antibodies. Shown are results of in vitro kinase analysis in the presence (lanes 1 and 3 to 11) or absence (lane 2) of ~200 ng of GST-pRB(379-928). Peptides added prior to addition of substrate: none (lanes 1 to 3); 50, 500, and 5,000 ng of RPPVKRRLDLE (E2F1, WT; lanes 4, 5, and 6, respectively); 5,000 ng of ADFTRGG LRSKAEP (p107, scrambled; lane 7); 50, 500, and 5,000 ng of CGSKACRRLFGPVD (p21N, WT; lanes 8, 9, and 10, respectively); and 5,000 ng of CDARCPGKG RFSLV (p21N, scrambled; lane 11). * indicates a ^{32}P-labeled protein that can be reimmunoprecipitated with an anti-p21 antibody. (C) Immunoprecipitation with anti-SV40 T (lanes 1 and 2) and anti-cyclin A (lanes 3-13) monoclonal antibodies. Shown are results of in vitro kinase analysis in the absence (lane 3) or presence of ~200 ng of GST-pRB(379-928) (lanes 1 and 4 to 8) or ~100 ng of GST-pRB(792-928) (lanes 2 and 9 to 13). Peptides added prior to the addition of substrate: none (lanes 1 to 4 and 9); 50, 500, and 5,000 ng of RPPVKRRLDLE (E2F1, WT; lanes 5, 6, and 7, respectively, and 10, 11, and 12, respectively); and 5,000 ng of SRENDRTLFATRPV (p130, scrambled; lanes 8 and 13). (D) Autoradiogram of ^{35}S-labeled cyclin A bound to glutathione-Sepharose preloaded with GST (lane 1), GST-p21 (lanes 2 to 6), or GST-cdk2 (lanes 7 to 16). The following peptides were added prior to the addition of ^{35}S-labeled cyclin A: none (lanes 1, 2, 7, and 12); 1, 10, and 100 μg of RPPVKRRLDLE (E2F1, WT; lanes 3, 4, and 5, respectively, and lanes 8, 9, and 10, respectively); 100 μg of ETDHQYLAESSGPA (E2F1 control, residues 95 to 108; lanes 6 and 11); 1, 10, and 100 μg of CGSKACRRLFGPVD (p21N, WT; lanes 13, 14, and 15, respectively); and 100 μg of CDARCPGKGRFSLV (p21N, scrambled; lane 16). A volume of cyclin A translate equal to one-fourth of the input of lanes 2 to 6 and equal to the input of lanes 1 and 7 to 16 was loaded in lane 17.

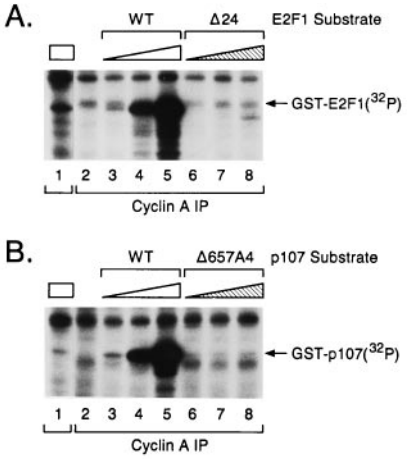

FIG. 6. Phosphorylation of p107 and E2F1 by cyclin A-associated kinase depends on the ZRXL motif. (A and B) Anti-cyclin A (lanes 2 to 8) or anti-SV40 T (lane 1) immunoprecipitates (IP) were prepared from U2OS cells and subjected to in vitro kinase analysis using 5, 50, or 500 ng of GST-E2F1 (A, lanes 1 and 3 to 5), GST-E2F1(Δ24) (A, lanes 6 to 8), GST-p107 (B, lanes 1 and 3 to 5), or GST-p107(Δ657A4) (B, lanes 6 to 8) as the substrate. Amounts of exogenous substrate added are indicated by the triangles (increasing substrate from left to right), and the squares indicate 500 ng of substrate (A and B, lane 1). No exogenous substrate was added in lane 2. Phosphorylated proteins were resolved by SDS-polyacrylamide gel electrophoresis and detected by autoradiography. WT, wild type.

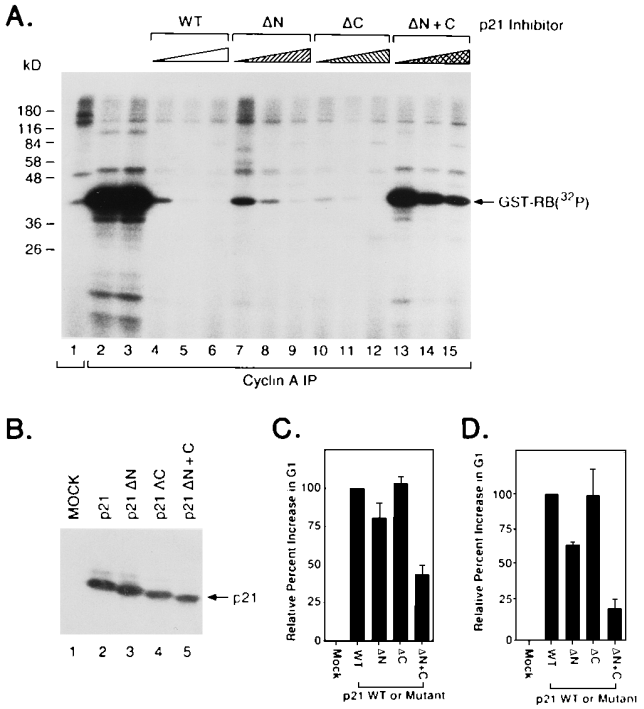

FIG. 7. Both ZRXL motifs in p21 are functional in kinase inhibition and G_1/S growth arrest. (A) Anti-cyclin A (lanes 2 to 15) or anti-SV40 T (lane 1) immunoprecipitates derived from U2OS cells were subjected to in vitro kinase analysis using ~100 ng of GST-pRB(792-928) as the substrate in the absence (lanes 1 and 2) or presence of 1 μg of GST (lane 3) or 0.04, 0.2, and 1 μg of GST–wild-type p21 (p21 WT) (lanes 4, 5, and 6, respectively), 0.04, 0.2, and 1 μg of GST-p21(ΔN) (lanes 7, 8, and 9, respectively), 0.04, 0.2, and 1 μg of GST-p21(ΔC) (lanes 10, 11, and 12, respectively), and 0.04, 0.2, and 1 μg GST-p21(ΔN+C) (lanes 13, 14, and 15, respectively). Phosphorylated proteins were resolved by SDS-polyacrylamide gel electrophoresis and detected by autoradiography. (B and C) U2OS cells were transiently transfected with mammalian expression plasmids (2 μg) encoding the indicated p21 proteins (or with the backbone expression plasmid) together with 1 μg of CD19 expression plasmid; 48 h later, cells were harvested by trypsinization. (B) An aliquot was lysed in Laemmli sample buffer, resolved by SDS-polyacrylamide gel electrophoresis, and analyzed by Western blotting with an anti-p21 antibody. Samples analyzed: mock (vector alone) (lane 1), p21 WT (lane 2), p21(ΔN) (lane 3), p21(ΔC) (lane 4), p21(ΔN+C) (lane 5). (C) The remainder of the cells were stained with propidium iodide, and the cell cycle distribution of the CD19-positive cells was determined by FACS. The results are expressed as the relative percent increase in the G_1 population. The relative percent increase in G_1 in each case was determined as follows: $[(C - B)/(A - B)] \times 100$, where A is the percent G_1 in cells transfected with p21 WT, B is the percent G_1 in mock-transfected cells, and C is the percent G_1 in cells transfected with the plasmid of interest. The results are the means of four separate experiments (three of which were performed in duplicate) with standard deviation from the mean. The mean percents in G_1 were as follows: mock, 28.6; and p21 WT, 75.9. (D) SAOS-2 cells were transiently transfected as for panels B and C. The cell cycle distribution of the CD19-positive cells was determined and expressed as in panels B and C. Results are the means of three separate experiments performed in duplicate. The mean percents in G_1 was were as follows: mock, 55.5; and p21 WT, 73.5.

but not GST-p107(Δ657A4), was phosphorylated by cyclin A-associated kinase(s) (Fig. 6B). Thus, synthetic peptides containing the ZRXL motif inhibited cyclin A-associated kinase activity and yet the ZRXL motif was necessary for phosphorylation of p107 and E2F1 by cyclin A-associated kinase. Taken together, these observations suggest that the ZRXL motif serves to target substrates to a discrete site on cyclin A-cdk2 and that the peptides inhibit kinase activity by blocking this interaction.

The ZRXL sequences found in proteins which stably associate with cyclin-cdk2 complexes, such as E2F1, p107, p130, and p21, may reflect binding sites which favor slow complex dissociation. In contrast, substrates such as pRB and histone H1 may contain suboptimal binding sites which, although necessary for recognition by cyclin-cdk2 complexes, favor rapid complex dissociation typical of most enzyme-substrate interactions. A prediction of this model is that pRB(792-928) might contain one or more sequences which, at least at the level of secondary structure, loosely resemble the ZRXL motif identified here. The observation that cyclin A kinase activity can be copurified with pRB under certain experimental conditions is consistent with this view (24, 83). Indeed, pRB(792-928) contains a number of primary sequence elements which loosely resemble the sequence ZRXL. Conversion of one of these, pRB residues 873 to 875 (KKL) to three alanine residues, reproducibly led to an approximately fivefold reduction in its phosphorylation by cyclin A-associated kinase relative to wild-type pRB (data not shown).

p21 contains two functional copies of the cyclin-cdk2 binding motif. To begin to address the significance of the two cyclin A- and E-cdk2 binding sites in p21, two additional sets of experiments were performed. In the first set of experiments, the GST-p21 fusion proteins described above were tested for the ability to inhibit the phosphorylation of exogenous pRB protein by anti-cyclin A immunoprecipitates (Fig. 7A). Inhibition by the double-site mutant, but not the two single-site mutants, was significantly impaired. Next, wild-type p21, as well as the corresponding single-site and double-site mutants, were introduced into asynchronous U2OS ($RB^{+/+}$) and SAOS2 ($RB^{-/-}$) cells by transient transfection along with a plasmid encoding the cell surface marker CD19. Each mutant gave rise to a stable protein as determined by steady-state Western blot analysis using an anti-p21 monoclonal antibody (Fig. 7B). Following cotransfection, the cell cycle distribution of CD19-positive cells, as determined by FACS, indicated that wild-type p21, as well as each of the two single-site mutants, efficiently induced a G_1/S block, although the N-terminal mutant was reproducibly slightly less effective than wild-type p21

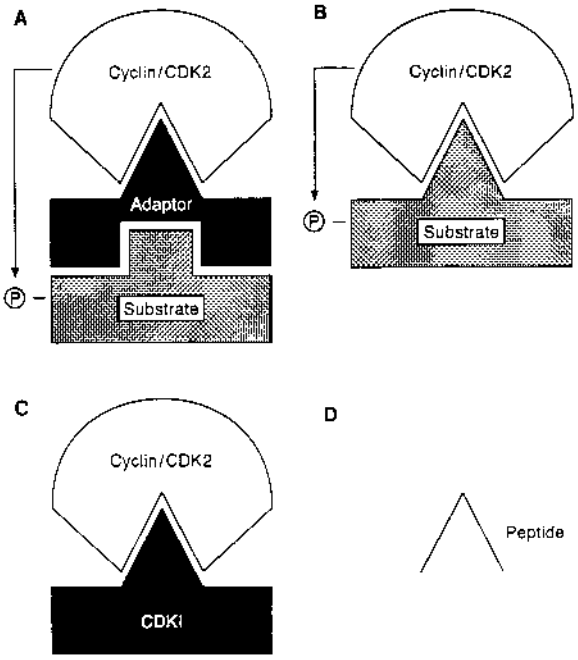

FIG. 8. Model for the role of the ZRXL motif in targeting CDKIs, substrates, and adapters for substrates to cyclin-cdk2 complexes. (A) A ZRXL-containing adapter protein, such as E2F1, binds to the cyclin-cdk2 complex and allows phosphorylation, in *trans*, of a substrate such as DP1. (B) A ZRXL-containing substrate, such as E2F1 or p107, binds to the cyclin-cdk2 complex and is phosphorylated. (C) A ZRXL-containing CDKI, such as p21, p27, or p57, binds to the cyclin-cdk2 complex and prevents binding to substrates. (D) Peptides encompassing the ZRXL motif will bind to the cyclin-cdk2 complex and block binding of substrates and adapters.

or the C-terminal mutant. In contrast, the double-site mutant, although not inert, was clearly defective in these assays (Fig. 7C and D). The residual activity of this mutant might, among several possibilities, reflect its ability to bind to PCNA (Fig. 4D).

DISCUSSION

We have identified a short, colinear, cyclin-cdk recognition motif present in multiple cell cycle regulatory proteins. This motif has, at its core, a consensus sequence of Z-arginine-X-leucine, where Z is basic or cysteine and X is basic in most cases. Short peptides containing this motif blocked the binding of substrates to cyclin A- and E-cdk2 complexes. Moreover, despite the fact that these peptides promoted the formation of stable cyclin A-cdk2 complexes, they inhibited cyclin A-associated, but not cyclin B-associated, kinase activity. In addition, mutation of this motif in two putative in vivo substrates, p107 (13, 17, 39, 61, 73) and E2F1 (16, 37, 60), prevented their phosphorylation by cyclin A-associated kinase in vitro. This latter observation, coupled with the knowledge that cyclin A- and E-cdk2 complexes are clearly active as kinases when bound to p107 and E2F1 (12, 29, 37, 61, 88), strongly suggests that this motif targets both substrates and p21-like CDKIs to cyclin A- and E-cdk2 complexes (Fig. 8). Implicit in this proposal is the idea that the p21-like CDKIs function, at least in part, by blocking the interaction of substrates with cyclin A- and E-cdk2 complexes. The model depicted in Fig. 8 is also in keeping with recent studies which showed that p107 and p21 bind to cyclin-cdk2 in a mutually exclusive manner (10, 91, 92) and that p21 can likewise disrupt the interaction of cyclin-cdk2 complexes with p130 (74).

Our results further demonstrate that the presence of a consensus S/T-P motif is not sufficient for efficient phosphorylation of a polypeptide substrate by cyclin A- and E-cdk2 complexes and suggest a model whereby cyclin A and E-cdk2 complexes first bind to a substrate recognition sequence prior to acting as catalysts. Conceivably, the cyclin-cdk2 substrate recognition and catalytic domains are separate, allowing certain S/T residues to be brought into the catalytic site once the enzyme is tethered to the substrate recognition motif. Indeed, according to this model, the cyclin-cdk recognition motif and the phosphorylatable serine or threonine could be on separate, but physically associated, proteins. This appears to be the case for the E2F1-DP1 complex, in which E2F1 serves as an adapter for DP1 phosphorylation (12, 37, 88). Likewise, p107 may serve as an adapter that allows cyclin A-cdk2 to phosphorylate the c-myc transactivation domain (29).

For E2F1, it has been previously shown that this motif is within a region that is required for binding of E2F1 to cyclin A-cdk2 in vivo (37, 88). However, deletion of the E2F1 cyclin A-cdk2 binding motif had no effect on its ability to be phosphorylated, in vivo, as determined by orthophosphate labeling followed by one-dimensional SDS-polyacrylamide gel electrophoresis (37). To our knowledge, however, it has not been shown that the phosphorylation of this E2F1 mutant, in vivo, is mediated by cyclin A-cdk2.

Our data, and the model depicted in Fig. 8, are entirely consistent with the recently solved crystal structure of cyclin A-cdk2-p27 (70). In particular, the p27 8-mer peptide (Fig. 2) which we showed blocked the interaction of cyclin A-cdk2 complexes with p21 and E2F1 forms a rigid coil structure toward the N terminus of p27 and coincides perfectly with the region of p27 which makes the major contacts with cyclin A. The ZRXL motif is at the center of this coil and fits into a peptide binding groove in cyclin A. Of particular note, the crystal structure indicates that the R and L of the ZRXL motif participate in three hydrogen bonds with cyclin A, and the L also makes multiple van der Waals contacts. We noted that these two amino acids are 100% identical between all of the substrates and inhibitors which we analyzed (Fig. 2) and that in E2F1 they are particularly important for the cyclin-cdk interaction (as demonstrated by the alanine scanning mutagenesis experiments [Fig. 1C and D].

Our results strongly suggest that p21 contains two cyclin-cdk binding motifs and that both are functional in the context of the full-length protein, as measured by the ability of single-site, but not double-site, p21 mutants to inhibit cyclin A-associated kinase activity in vitro and to efficiently induce a G_1/S block in transformed cells. The presence of two cyclin-cdk2 binding motifs in p21 was unexpected given prior studies which had assigned the cdk binding/inhibition function exclusively to the N-terminal half of the molecule (7, 18, 43, 47, 54, 78). One possible explanation is that the C-terminal cdk2 binding motif may be denatured and/or inaccessible in the context of the mutants used in these earlier studies. Our results are, however, entirely in keeping with a recent study by Chen et al. (8).

In one study (6), a synthetic peptide spanning the N-terminal cyclin-cdk2 recognition motif of p21 (corresponding to residues 15 to 40) was found to bind to cyclin-cdk2, but not to inhibit cyclin-cdk2 in vitro kinase activity, in apparent disagreement with our own results. There are technical differences between our study and that of Chen et al. (6), however, which might account for this discrepancy. The interpretation of their results is further complicated by the observation that p21 residues 15 to 40 bound to full-length p21 and, perhaps as a result,

antagonized p21 activity. Nonetheless, it is possible that p21 residues outside the core motifs identified here contribute to the stable binding of p21 to cyclin-cdk2 complexes under certain conditions, as first suggested by others (6, 8, 18, 21, 54).

Our results do not exclude the possibility that p21-like CDKIs, in addition to blocking enzyme-substrate interactions, perform additional functions when bound to cyclin-cdk2 complexes. In this regard, p27 has been shown to prevent the phosphorylation of cdk2 by CAK (62, 79). Furthermore, the cyclin A-cdk2-p27 crystal structure suggests that p27 can, in addition to preventing the binding of substrates to the ZRXL binding groove of cyclin A, alter the conformation of the cdk2 amino-terminal lobe and prevent the binding of ATP to the cdk2 catalytic cleft (70).

That the cyclin-cdk2 recognition motif peptides promoted the formation of stable cyclin A-cdk2 complexes is in keeping with earlier studies using full-length p107 and p21 (61, 86). The data presented here suggest a generalization whereby any protein that binds to cyclin A- and E-cdk2 through a ZRXL motif might promote complex formation. If this is so, differences in substrate availability during the cell cycle might influence the formation of the appropriate cyclin-cdk complexes necessary to promote cell cycle progression. The cyclin A-cdk2-p27 crystal structure, as well as binding studies performed with highly purified components, suggests that the ZRXL motif contacts exclusively cyclin A (8, 70). One possibility, among several, is that binding of the ZRXL motif to cyclin A induces an allosteric change which promotes stable complex formation with cdk2. In this regard, it is perhaps important that the cyclin A-cdk2 structures solved to date have used a C-terminal fragment of cyclin A (70). Thus, potential effects of the cyclin A N terminus on the accessibility of the cyclin A ZRXL-binding groove could not be detected.

It is possible that cyclin-cdk complexes other than cyclin A-cdk2 and cyclin E-cdk2 are likewise targeted to their substrates via distinct short, colinear motifs. Thus, the model depicted in Fig. 8 may provide a general paradigm for substrate recognition by different cyclin-cdk complexes. Members of the p21 family appear capable of inhibiting all known cyclin-cdk complexes, at least in vitro. Preliminary evidence indicates that the cyclin-cdk binding motifs that we have identified in p21 also play a role in binding to D-type cyclins (data not shown). In contrast, this motif does not appear to be sufficient, and may not be necessary, for binding to cyclin B-cdc2 complexes by p21. Whether p21 contains a separate, dedicated, cyclin B-cdc2 binding motif remains to be determined.

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