Identification of CDK4 Sequences Involved in Cyclin D1 and p16 Binding*

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Activation of CDK4 is regulated, in part, by its association with a D-type cyclin. Conversely, CDK4 activity is inhibited when it is bound to the cyclin-dependent kinase inhibitor, p16INK4A. To investigate the molecular basis of the interactions between CDK4 and cyclin D1 or p16INK4A we performed site-directed mutagenesis of CDK4. The interaction was examined using in vitro translated wild type and mutant CDK4 proteins and bacterially expressed cyclin D1 and p16 fusion proteins. As mutational analysis of CDC2 suggests that its cyclin binding domain is primarily located near its amino terminus, the majority of the mutations constructed in CDK4 were located near its amino terminus. In addition, CDK4 residues homologous to CDC2 sites involved in Suc1 binding were also mutated. Our analysis indicates that cyclin D1 and p16 binding sites are overlapping and located primarily near the amino terminus. All CDK4 mutations that resulted in decreased p16 binding capability also diminished cyclin D1 binding. In contrast, amino-terminal residues were identified, including the PSTAIRE region, that are important for cyclin D1 binding but are not involved in p16 binding.

The eukaryotic cell cycle is controlled by a series of regulatory "checkpoints," two of which occur at the G1/S and G2/M phase transitions. The cyclin-dependent kinases (CDKs)1 are key regulators of these checkpoints (1–4). The CDKs are serine/threonine protein kinases that are composed of a catalytic CDK subunit and a regulatory cyclin subunit. Compelling evidence suggests that D-type cyclins and their major catalytic partners, CDK4 and CDK6, are key regulators of the G1/S phase checkpoint. Cyclin D-CDK holoenzymes accumulate when resting cells are stimulated to proliferate by addition of growth factors. Their associated kinase activities are first detected in mid G1 phase and increase as cells approach the G1/S transition. Cyclin D-CDK holoenzymes accumulate in wild-type cells during G1 and decrease as cells move through the cell cycle (5). Furthermore, microinjection of cyclin D1 antibodies into cells during G1 prevents entry into S phase whereas injections at or after S phase have no effect (6, 7). Thus, cyclin D-CDK complexes are essential for the G1 to S phase transition.

Activation of individual CDKs is dictated in part by the temporal expression of their cognate cyclins and by stage-specific phosphorylation and dephosphorylation events (8). An additional layer of CDK regulation has emerged with the discovery of the CDK inhibitor proteins (CKIs) p15 (11, 12), p16 (13), p18 (14), p19 (14), p21 (15), p27 (16), and p57 (17). Binding of these inhibitory proteins to specific CDKs or specific CDK-cyclin complexes inhibits kinase activity and thus blocks cell cycle progression. The human p16INK4A/MTS1/CDKN21 (hereafter referred to as p16) gene encodes a 16-kDa nuclear protein that binds to and inhibits the catalytic activity of CDK4 and CDK6 (13). Loss of functional p16 is associated with deregulation of cell proliferation and tumorigenesis (18). Whereas this finding serves to emphasize the critical role p16 plays in regulating cell growth, the molecular mechanism by which it inhibits CDK4 or CDK6 kinase activity is unknown. Thus, to identify the regions of CDK4 required for p16 and cyclin D1 binding, we performed site-directed mutagenesis of CDK4. The data from our protein interaction studies indicate that the p16 and cyclin D1 binding sites of CDK4 are overlapping but not identical and support the hypothesis that p16 competes with cyclin D1 for binding to CDK4.

EXPERIMENTAL PROCEDURES

Protein Expression—Cyclin D1 cDNA (kindly provided by E. Harlow) was cloned as a BamHI fragment in the pET29a vector (Novagen) to allow production of an S-tag-cyclin D1 fusion protein. BL21 cells containing pET29-cyclin D1 were grown to an A695 of 0.4, and protein expression was induced for 4 h with 1 mM isopropyl-1-thio-β-D-galactopyranoside. Cells were harvested by centrifugation, resuspended in a lysis buffer (20 mM Tris, pH 7.4, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM EGTA, 100 μg/ml Pefabloc, and lysed by sonication. The insoluble protein fraction, which contains the S-tag-cyclin D1 fusion protein, was isolated by centrifugation at 30,000 rpm for 30 min in a Beckman Type 45 rotor. The pellet was resuspended in lysis buffer containing 0.5% Nonidet P-40 followed by centrifugation. The pellet was resuspended in 50 ml of DNsase buffer (40 mM Tris, pH 8.0, 10 mM NaCl, 6 mM MgCl2, 100 μg/ml Pefabloc, 10 μg/ml DNase I, 10 μg/ml RNase A) and incubated at room temperature for 30 min. The insoluble protein was precipitated again by centrifugation, resuspended in 10 ml of Tris buffer (20 mM Tris, pH 7.4, 50 mM NaCl, 1 mM DTT, 0.1 mM EGTA) containing 5 μM urea, and spun at 30,000 rpm for 30 min in a Beckman Type 45 rotor. The protein concentration of the supernatant was determined using the BCA protein assay (Pierce), and solubilized protein was diluted dropwise to a final concentration of 150 μg/ml in 2 μl urea. The protein was dialyzed for 24 h against 50 volumes of storage buffer (50 mM Tris, pH 7.4, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM MgCl2, 25% glycerol). The dialysis buffer was changed, and dialysis continued for an additional 24 h. The

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¶ The abbreviations used are: CDK, cyclin-dependent kinase; CKI, CDK inhibitor; DTT, dithiothreitol; GST, glutathione S-transferase; S-tag, S-tagged; RB, retinoblastoma.
CDK4 Mutational Analysis

protein was sized on a 10% SDS-polyacrylamide gel, stained with Coomassie Blue, and shown to be >90% pure.

CDK4 cDNA (kindly provided by E. Harlow) was cloned as a BamHI fragment into pBMS1 (19) to allow production of GST-CDK4 fusion protein in Sf9 cells. A 500-ml Sf9 culture (1 x 10^7 cells/ml) in S-900 II SF 900 II medium was infected at a multiplicity of infection of 3 and harvested 48 h after infection by centrifugation at 1500 rpm in a Beckman J6-M2 centrifuge. The cells were resuspended in 100 ml of GST buffer (150 mM NaCl, 16 mM NaHPO4, 4 mM Na2HPO4) containing 0.1% Tween 20, 0.1 mM NaF, 0.1 mM Na2VO4, 10 mM β-glycerophosphate, and 0.1 mg/ml Pefabloc. Cells were lysed by sonication, and the insoluble material that was removed by centrifugation at 30,000 rpm for 30 min in a Beckman Type 45 rotor. The Sf9 cellular lysate containing the GST-CDK4 protein was added to 10 ml of glutathione-Sepharose beads. Unbound material was removed by washing with GST buffer. Bound protein was eluted from the column with a buffer containing 50 mM Tris, pH 8.0, and 10 mM glutathione. Fractions containing the GST-CDK4 protein were pooled. The pooled fractions were dialyzed against 100 volumes of storage buffer (50 mM HEPES, pH 8.0, 50 mM NaCl, 1 mM EGTA, 1 mM DTT, 1 mM MgCl2, 25% glycerol).

p16 cDNA was cloned as a BamHI fragment in the pGEX-2T vector (Pharmacia Biotech Inc.) to allow production of a GST fusion protein in E. coli DH5α cells. To isolate the GST-p16 fusion protein, a 500-ml culture of DH5α cells containing pGEX-p16 was grown to an A660 of 0.4, and protein expression was induced with 1 mM isopropyl-1-thio-galactoside. The expressed protein was purified according to the manufacturer’s instructions and subsequently cleaved with thrombin to produce untagged protein.

The carboxyl-terminal end of the retinoblastoma open reading frame (base pairs 2564–2922) was cloned as a BamHI fragment in the pGEX-4T vector (Pharmacia) to allow production of GST-RB fusion protein in E. coli DH5α cells. The GST-RB fusion protein was purified as described above.

[35S]Methionine-labeled CDK4 proteins were translated in vitro in rabbit reticulocyte lysates using the Promega Tnt transcription and translation kit according to the manufacturer’s instructions.

Site-directed Mutagenesis—The human CDK4 cDNA was cloned into pALTER-1 (Promega). Oligonucleotide-directed mutagenesis was performed using the Altered Sites II in vitro Mutagenesis Systems kit (Promega) according to the manufacturer’s instructions. Clones containing the appropriate mutations were confirmed by DNA sequence analysis.

Protein Binding Assays—Wild type and mutant CDK4 proteins were produced by translation in reticulocyte lysates in the presence of [35S]methionine. Identical amounts of wild type or mutant CDK4 proteins were added to 700 μl of TTBS buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) containing 3% bovine serum albumin and 5 μg of either Sα2-cyclin D1 or GST-p16 fusion protein. The reactions were incubated at 4 °C for 1 h with gentle rocking. 20 μl of protein-agarose (Novagen) or glutathione-Sepharose beads (pre-equilibrated with TTBS buffer) were added to the cyclin D1 and p16 binding assays, respectively. The reactions were incubated with gentle rocking at 4 °C for an additional hour. Bound proteins were precipitated by centrifugation and washed 3 times with TTBS buffer at 4 °C. CDK4 protein was eluted by boiling the beads in 40 μl of 3 x loading buffer. Proteins were sized on a 12.5% SDS-polyacrylamide gel, quantitated using the Fuji BAS1000 PhosphorImager, and visualized by autoradiography.

Kinase Assays—The kinase reactions contained 300 ng of GST-CDK4, 300 ng of Sα2-cyclin D1, and 500 ng of GST-RB in 25 μl of kinase buffer (50 mM HEPES, pH 8.0, 10 mM MgCl2, 2 mM DTT, 5 mM EGTA, 1 μCi of [γ-32P]ATP, 25 μM ATP). Reactions were incubated at 30 °C for 1 h. 20 μl of glutathione-Sepharose was then added, and the reactions were incubated 20 min on ice. The beads were isolated by centrifugation for an additional hour. The S-protein-agarose bead complexes were precipitated with 40 μl of S-protein-agarose beads, washed 3 times with kinase buffer, and then loaded onto a 12.5% SDS-polyacrylamide gel, quantitated using the Fuji BAS1000 PhosphorImager, and visualized by autoradiography.

To identify the region(s) of CDK4 involved in the interaction with cyclin D1 and p16 we generated a collection of mutants by site-directed mutagenesis. Alanine residues were substituted for charged amino acids, a strategy that allows the evaluation of the functional significance of charged residues in a given protein. Charged-to-alanine mutagenesis has been used successfully for the identification of protein-protein interaction domains (20–22), including the regions of CDC2 that are important for cyclin and Suc1 binding (23–25). By this method, charged residues (Asp (D), Arg (R), Glu (E), His (H), and Lys (K)) were changed either singularly or in clusters to Ala (A). In addition, as phosphorylation is known to regulate CDK4 activity (26, 27), Tyr-17 and Thr-172 were also mutated because these residues have been identified as phosphoacceptor sites. Finally, all CDK family members contain a glycine residue (i.e. CDK2 Gly-43) immediately preceding the PSTAIRE motif. Crystallographic analysis has shown that this glycine residue is critical for cyclin A binding in that it allows proper folding of the PSTAIRE motif (28). Compared with other CDKs, CDK4 is unique in that it contains 7 glycines (residues 42–48) at this position and is the only CDK family member that is devoid of charged residues in this region. To assess the functional significance of this region in CDK4, 3 of the 7 glycine residues were deleted.

The high degree of sequence similarity shared between CDK family members suggests that CDK proteins have similar structures. In support of this hypothesis, crystallographic analysis has demonstrated that the cAPK catalytic subunit and CDK2 share remarkable structural similarity even though the two proteins are only distantly related at the amino acid level (29). The backbone structures of both proteins can be divided into two lobes, a smaller amino-terminal lobe and a larger carboxy-terminal lobe. Thus, it is reasonable to assume that CDK4, like CDK2, is bilobal. All 22 charged residues in the putative amino-terminal lobe of CDK4 (amino acids I–86) were mutated. In addition, 10 charged residues in the putative carboxy-terminal lobe were targeted; residues Lys-88, Glu-94, and His-95 were targeted due to their close proximity to the putative amino-terminal lobe, and 7 additional charged residues were targeted because they are homologous to CDC2 sequences involved in Suc1 binding. As our CDK4 mutants were made prior to the publication of the crystal structure of the closely related CDK2-cyclin A complex (28), the choice of sites to be mutagenized was not influenced by this study. The name of each mutant and the amino acids substituted are listed in Table I.

To examine interactions between the mutant CDK4 proteins and cyclin D1, radiolabeled CDK4 protein was expressed in vitro using a rabbit reticulocyte lysate system. Identical amounts of wild type or mutant CDK4 proteins were incubated with bacterially expressed Sα2-cyclin D1 fusion protein. Protein complexes were precipitated using S-protein-agarose beads and analyzed by SDS-polyacrylamide gel electrophoresis. The in vitro translated protein bound to cyclin D1 was quantitated using a Fuji BAS1000 PhosphorImager. The level of interaction for each mutant CDK4 protein was measured relative to the quantity of wild type CDK4 protein precipitated. Fig. 1A shows an example of a CDK4-cyclin D1 interaction assay. In these assays, approximately 30% of the wild type CDK4 protein was precipitated by cyclin D1 whereas no CDK4 protein was precipitated when Sα2-cyclin D1 fusion protein was omitted from the assay (data not shown). Each binding assay was performed five to seven times, and the average binding capacity for each mutant is summarized in Table I. In the putative
amino-terminal lobe, CDK4-MUT3, -MUT6, -MUT7, -MUT8, -MUT9, -MUT10, and -MUT13 significantly decreased cyclin binding to less than 50% of wild type. The most dramatic effect on cyclin D1 binding was observed for CDK4-MUT3 (residues 22–25), which decreased cyclin D1 binding to 10% relative to wild type. This region includes the site of a CDK4 mutation (R24C) present in a human melanoma (30). CDK4-R24C contains a cysteine residue in place of the arginine residue at position 24 and was identified as a tumor-specific antigen recognized by cytolytic T lymphocytes. The CDK4-R24C mutant, however, was reported to bind cyclin D1 as well as wild type CDK4. To investigate the involvement of the individual amino acids of the MUT3 region in cyclin D1 binding, single amino acid substitutions were made. CDK4-MUT3A (K22A) bound cyclin D1 as poorly as the triple mutant (15%), CDK4-MUT3B (R24A) bound slightly more cyclin D1 (30%), and CDK4-MUT3C (D25A) bound cyclin D1 almost as well as wild type (80%). Thus, Lys-22 appears to play a greater role in cyclin D1 binding.

The sequence of the CDK4-MUT3 region (Lys-Ala-Arg-Asp) is identical for CDK4 and CDK6, the major catalytic partners of the D-type cyclins. In contrast, the corresponding regions in CDK2 (Lys-Ala-Arg-Asn), CDK3 (Lys-Ala-Lys-Asn), and CDK5 (Lys-Ala-Lys-Asn) all contain an asparagine residue in place of the aspartic acid present at residue 25 of CDK4. To determine if substitution of Asn for Asp at position 25 affected the affinity of CDK4 for cyclin D1, CDK4-MUT3E (D25N) was constructed. As a control, Asp-25 was also mutated to glutamic acid (CDK4-MUT3D). Like the D25A mutation, D25N and D25E bound cyclin D1 almost as efficiently as wild type CDK4. Thus, Asp-25 does not appear to be involved in cyclin D1 binding.

The PSTAIRE regions of CDK1 and CDK2 play a critical role in cyclin binding (23, 28). Mutation of this region in CDK4 (CDK4-MUT7) decreases cyclin D1 binding by 85% relative to wild type. In addition, residues that are proximal to the amino and carboxyl terminus of the PSTAIRE motif also contribute to cyclin D1 binding. CDK4 Lys-35, which is located 15 amino acids proximal to the PSTAIRE regions in CDK2 (Lys-Ala-Arg-Asn), CDK3 (Lys-Ala-Lys-Asn), and CDK5 (Lys-Ala-Lys-Asn) all contain an asparagine residue in place of the aspartic acid present at residue 25 of CDK4. To determine if substitution of Asn for Asp at position 25 affected the affinity of CDK4 for cyclin D1, CDK4-MUT3E (D25N) was constructed. As a control, Asp-25 was also mutated to glutamic acid (CDK4-MUT3D). Like the D25A mutation, D25N and D25E bound cyclin D1 almost as efficiently as wild type CDK4. Thus, Asp-25 does not appear to be involved in cyclin D1 binding.

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| Mutant name | Mutation | % Binding | Cyclin D1 | p16 |
|-------------|----------|-----------|----------|-----|
| CDK4-MUT1   | R5A,E7A  | 66 ± 5    | 140 ± 25 | 165 ± 23 |
| CDK4-MUT2   | E11A     | 66 ± 14   | 41 ± 11  | 48 ± 11 |
| CDK4-MUT3   | K22A     | 12 ± 1    | 2 ± 3    | 2 ± 3  |
| CDK4-MUT3A  | R24A     | 15 ± 4    | 4 ± 3    | 4 ± 3  |
| CDK4-MUT3B  | R24A     | 30 ± 2    | 6 ± 4    | 6 ± 4  |
| CDK4-MUT3C  | D25A     | 78 ± 7    | 206 ± 36 | 206 ± 36 |
| CDK4-MUT3D  | D25E     | 64 ± 15   | 94 ± 12  | 94 ± 12 |
| CDK4-MUT3E  | D25N     | 93 ± 18   | 110 ± 40 | 110 ± 40 |
| CDK4-MUT3F  | H27A     | 91 ± 17   | 110 ± 20 | 110 ± 20 |
| CDK4-MUT5   | H30A     | 74 ± 19   | 50 ± 10  | 50 ± 10 |
| CDK4-MUT6   | K35A     | 44 ± 11   | 32 ± 6   | 32 ± 6  |
| CDK4-MUT7   | R55A,E56A| 17 ± 2    | 130 ± 40 | 130 ± 40 |
| CDK4-MUT8   | R61A,R62A,E64A| 26 ± 4 | 18 ± 4   | 18 ± 4  |
| CDK4-MUT9   | E67A,H68A| 32 ± 6    | 160 ± 25 | 160 ± 25 |
| CDK4-MUT10  | R73A,D76A| 42 ± 10   | 140 ± 24 | 140 ± 24 |
| CDK4-MUT11A | R82A,D84A| 140 ± 23  | 190 ± 52 | 190 ± 52 |
| CDK4-MUT11B | R85A,E66A| 50 ± 9    | 100 ± 24 | 100 ± 24 |
| CDK4-MUT12  | K88A     | 150 ± 15  | 411 ± 64 | 411 ± 64 |
| CDK4-MUT13  | E94A,H95A| 28 ± 6    | 95 ± 19  | 95 ± 19  |
| CDK4-MUT14  | R181A,E184A| 9 ± 2  | 8 ± 2    | 8 ± 2  |
| CDK4-MUT15  | D196A    | 58 ± 10   | 91 ± 12  | 91 ± 12  |
| CDK4-MUT16  | R225A,D228A| 110 ± 27  | 120 ± 31 | 120 ± 31 |
| CDK4-MUT17  | H281A,K282A,R283A| 8 ± 4  | 5 ± 10   | 5 ± 10  |
| Additional mutants | | | | |
| CDK4-Y17F   | Y17F     | 75 ± 18   | 129 ± 24 | 129 ± 24 |
| CDK4-3G     | Deletion Gly-46, Gly-47,Gly48 | 93 ± 21 | 84 ± 25 | 84 ± 25 |
| CDK4-T172A  | T172A    | 110 ± 35  | 268 ± 73 | 268 ± 73 |

* The numbers of mutants is as in Fig. 1.
* Normalized to a value of 100% binding in the wild type (see "Experimental Procedures"). Averages of five to seven experiments for each mutant.
* As the double mutation K35A,R38A possessed the same phenotype as K35A alone, only the K35A data are shown.

**Table I**

**Cyclin D1**

**p16**

**Fig. 1.** Binding of wild type and mutant CDK4 proteins to cyclin D1 and p16. A, mutant CDK4 mRNA was translated in the presence of [35S]methionine in vitro, incubated with S<sub>c</sub>-cyclin D1, and precipitated with S-protein-agarose. The beads were washed, boiled in SDS sample buffer, and loaded on a SDS-polyacrylamide gel. B, wild type and mutant CDK4 mRNA were translated in vitro, incubated with GST-p16, and precipitated with glutathione-Sepharose. Reactions were analyzed as described above.
mutant shows similar cyclin D1 binding relative to CDK4-MUT6 (K35A; data not shown). Three of the five amino-terminal cluster mutations located proximal to the carboxyl terminus of the PSTAIRE motif (CDK4-MUT8, -MUT9, and -MUT10) decreased cyclin D1 binding by greater than 50%. Of the three regions, CDK4-MUT8 displayed the greatest reduction in cyclin D1 binding (25%).

Seven regions were mutated in the putative carboxyl-terminal lobe of CDK4. Mutation of the activating Thr at position 172 (CDK4-T172A) had no detectable effect on cyclin D1 binding. However, two mutants, CDK4-MUT14 and CDK4-MUT17, displayed significant reductions in cyclin D1 binding (10% of wild type). CDK4-MUT14 contains a mutation of the consensus triplet Ala-Pro-Glu (residues 182–184), a conserved feature of protein kinase catalytic domains (31). Mutation of this region eliminates virtually all cyclin D1 binding. The region mutated in CDK4-MUT17 (residues 281–283) is located 20 amino acids from the carboxyl-terminal end of CDK4; mutations in the homologous region of CDC2 dramatically decrease Suc1 binding activity (24) but not cyclin A binding (23). In CDK4, however, the CDK4-MUT14 mutant displayed a dramatic decrease in cyclin D1 binding.

p16 was initially identified as a CDK4-binding protein and found to be a specific inhibitor of CDK4 and CDK6 (13). The regions of CDK4 involved in p16 binding were determined using our set of mutant CDK4 proteins. The CDK4-p16 binding assays were performed as described for the cyclin D1 binding assays except that p16 was expressed in bacteria as a GST-p16 fusion protein; therefore, protein complexes were precipitated using glutathione-linked Sepharose beads. Fe. 1B shows an example of a CDK4-p16 interaction assay. In these assays, approximately 60% of the wild type CDK4 protein was precipitated by GST-p16 whereas no binding was detected when GST was used in the assay (data not shown). Each binding assay was performed five to seven times, and the average binding capacity for each mutant is summarized in Table I. Mutations in six regions of CDK4 decreased p16 binding by greater than 50%; four of these regions (CDK4-MUT2, -MUT3, -MUT6, and -MUT8) are located in the putative amino-terminal lobe, and two regions (CDK4-MUT14 and -MUT17) are located near the carboxyl terminus. Interestingly, mutations within the PSTAIRE homology region (CDK4-MUT7) did not affect p16 binding. Of the amino-terminal mutants, CDK4-MUT3 displayed the most dramatic reduction in p16 binding (2% of wild type). Within this region, residues Lys-22 (CDK4-MUT3A) and Arg-24 (CDK4-MUT3B) play key roles in p16 binding whereas Asp-25 appears to contribute little to the binding. The two carboxyl-terminal mutants, CDK4-MUT14 and -MUT17, displayed dramatic reductions in p16 binding (90 and 95% relative to wild type, respectively). Mutations in three regions (CDK4-MUT12, -Y17F, and T172A) resulted in a 2-fold increase in p16 binding.

To validate the protein interaction assays, we examined the RB kinase activity of a subset of the CDK4 mutants. Wild type and mutant CDK4 proteins were expressed as GST-fusion proteins using a baculovirus expression system and were affinity purified. Each CDK4 protein was incubated with cyclin D1, and the kinase activity of the complexes was measured using the retinoblastoma protein as substrate (Fig. 2A). CDK4-MUT2, -MUTY17F, and -MUT5, which efficiently bind cyclin D1, possess RB kinase activities that are comparable to wild type. In contrast, mutants that displayed a greater than 80% reduction in cyclin D1 binding activity (CDK4-MUT3, -MUT7, and -MUT14) lacked appreciable RB kinase activity. Three mutants, CDK4-MUT3B, -MUT8, and -MUT10, which displayed 25–40% of cyclin binding activity, retained at least 20% of wild type RB kinase activity (Fig. 2B). As expected, CDK4-MUT6, in which the catalytic lysine at position 35 is replaced with alanine, lacks detectable RB kinase activity even though it retains 45% of cyclin D1 binding activity. Likewise, CDK4-T172A, in which the activating threonine residue at position 172 is replaced with alanine, binds cyclin D1 efficiently but does not phosphorylate RB.

The mutant CDK4-MUT3B (R24A) is particularly interesting in that a similar CDK4 mutation, R24C, has been identified in a primary human melanoma (30). The melanoma-derived CDK4-R24C mutant binds cyclin D1 and phosphorylates RB. However, CDK4-R24C does not bind p16, and consequently, its RB kinase activity is not inhibited by p16. As CDK4-MUT3B binds cyclin D1 but not p16, we examined the ability of p16 to inhibit its kinase activity (Fig. 3). The kinase activity of both wild type and CDK4-MUT5 (retains 50% of wild type p16 binding) proteins were inhibited by p16 protein. Both proteins were inhibited with an IC50 value of approximately 225 nM. In contrast, the kinase activity of CDK4-MUT3B protein was not significantly inhibited by the highest concentration of p16. Thus, the phenotype of the R24A mutation is consistent with that reported for R24C.

DISCUSSION

An important step toward understanding the mechanisms by which CDK4 is regulated is to understand the molecular basis of its interaction with cyclin D1 and p16. In this study, we generated a family of charged-to-alanine mutants of CDK4 and characterized how these mutants affect cyclin D1 and p16 binding. Alanine substitutions were chosen to minimize the risk of altering the overall structure of the mutant proteins. We
determined. A titration of increasing concentrations of p16 protein was performed for each kinase, and the percent inhibition was calculated for each concentration of p16. The percent inhibition was determined.

These data demonstrate that, in addition to the PSTAIRE homology region, Lys-22 and Arg-24 play a critical role in cyclin D1 binding. The Arg-24 residue is particularly interesting in that it has recently been shown to be mutated to a cysteine residue in a human melanoma (30). The R24C mutant retained cyclin D1 binding and RB kinase activity but was unable to bind p16. Although the R24A mutation reduced cyclin D1 binding by 70%, it retained 50% of its kinase activity. Thus, the phenotype of R24A appears to be similar to that reported for R24C. Our analysis of p16 binding activity of R24A will be discussed below.

Three cluster mutations (CDK4-MUT13, -MUT14, and -MUT17), which are located within the carboxyl-terminal lobe of CDK4, interfered with cyclin D1 binding. CDK4-MUT14 and -MUT17 are carboxyl-terminal to the T-loop region (the T-loop contains the activating threonine residue) and abolished cyclin binding by more than 90%. As observed with CDK4-MUT3, cluster mutations in the corresponding regions of CDC2 affect Suc1 binding without altering cyclin binding (23, 24). Thus, we have identified three regions of CDK4 involved in cyclin binding that are homologous to Suc1 binding regions of CDC2. Because the effect of Suc1 binding to CDC2 is unknown, the functional significance of this finding is not clear. However, the data do suggest that homologous regions of the different CDKs are involved in protein-protein interactions. It is noteworthy that residues within the CDK4-MUT14 (Glu-184) and -MUT17 (Arg-283) clusters are conserved in all protein kinases (31). The replacement of these invariant residues may perturb the conformation of CDK4 and inhibit cyclin binding.

The crystal structure of the CDK2-ATP-cyclin A complex demonstrated that residues within this region of CDK2 interact with cyclin A (28).

A primary difference among the cyclin D1-CDK4 interaction sites is that of the PSTAIRE homology region (CDK4-MUT7) decreased cyclin binding by 85%. Similar mutations in the PSTAIRE sequence of CDC2 also decreased cyclin binding significantly (23, 25). Individual amino acid changes within the PSTAIRE sequence of CDC2 showed that the arginine at position 50 is required for efficient cyclin binding. In addition, the crystal structure of the human cyclin A-CDK2-ATP complex demonstrated that the highest concentration of side chain contacts between cyclin A and CDK2 occurs around the PSTAIRE motif (28). Thus, consistent with the CDC2 and CDK2 studies, the PSTAIRE homology region of CDK4 plays a critical role in cyclin binding.

The three cluster mutations that are located proximal to the carboxyl terminus of the PSTAIRE homology region (CDK4-MUT8, -MUT9, and -MUT10) all interfered with cyclin D1 binding. Although mutations in the corresponding region of CDC2 did not dramatically interfere with cyclin binding, structural analysis of the CDK2-ATP-cyclin A complex revealed that residues within this region of CDK2 interact with cyclin A (28).
mutants contained appreciable kinase activity. All of the mutants that displayed greater than 50% cyclin binding relative to wild type CDK4 (CDK4-MUT2, -MUTY17F, and -MUT5) phosphorylated RB as well as wild type. In contrast, mutations that decreased cyclin binding by greater than 80% (CDK4-MUT3, -MUT7, and -MUT14) decreased kinase activity by greater than 90%. The three mutants that retained intermediate levels of cyclin D1 binding (CDK4-MUT3B, -MUT8, and -MUT10) retained intermediate levels of kinase activity. CDK4-MUT3B retained 30% of its cyclin binding activity and 50% of its kinase activity whereas CDK4-MUT8 retained 26% of its cyclin binding and 24% of its kinase activity. These data suggest that the overall CDK4 structure is not perturbed in these mutants. The CDK4-MUT10 mutation did not show a tight correlation between the two activities in that it retained 40% of its cyclin binding but only 20% of its kinase activity. This suggests that the R73A,D76A mutation may result in additional loss of structural integrity. In general, the level of cyclin binding of the various mutants correlates well with kinase activity.

The CDK4 mutants were also characterized for p16 binding. In general, the sequences involved in p16 binding are a subset of those involved in cyclin D1 binding. The most notable difference between the two binding sites was the PESTAIRE homology region. Whereas we demonstrated that the PESTAIRE homology region of CDK4 plays a critical role in cyclin binding, mutations within this region (CDK4-MUT7) had no effect on p16 binding. Since p16 binds specifically to CDK4 and CDK6 but not to the other CDK proteins, it is not surprising that the highly conserved PESTAIRE homology region is not involved in its binding. Four amino-terminal mutations (CDK4-MUT2, -MUT3, -MUT6, and -MUT8) and two carboxyl-terminal mutations (CDK4-MUT14 and -MUT17) decreased p16 binding by greater than 50%. With the exception of the single-site mutant, CDK4-MUT2 (E11A), these mutations also decreased cyclin D1 binding by greater than 50%. The residues mutated in the CDK4-MUT3 cluster mutation (Lys-22 to Asp-25) play a critical role in p16 binding. Analysis of single-site mutations within this cluster demonstrated that both the K22A and R24A mutations dramatically decreased p16 binding, whereas the D25A mutation displayed enhanced p16 binding (206% of wild type CDK4). Thus, while the loss of binding in the cluster can be attributed to Lys-22 and Arg-24, the enhanced binding observed for the D25A mutation suggests that this residue may also interact with p16. As previously discussed, Arg-24 is the site of a CDK4 mutation (R24C) that has been found in a human melanoma. Like R24A, the R24C mutation interferes with p16 binding while maintaining cyclin D1 binding and kinase activity. This phenotype suggests that the R24C and R24A mutations might be oncogenic.

In addition to CDK4-MUT3C, three other mutants, CDK4-Y17F, -MUT12, and -T172A, showed a significant enhancement (2-fold increase relative to wild type) of p16 binding. This enhanced binding suggests that these sites are also involved in p16 binding. Our analysis did not indicate that these regions are involved in cyclin binding with the possible exception of CDK4-MUT12, which shows a slight increase (50%) in cyclin D1 binding.

The mutational analysis described here provides a molecular framework for understanding the regulation of CDK4 kinase activity by cyclin D1 and p16. Our analysis shows that cyclin D1 and p16 interact with the same sites on CDK4, supporting the hypothesis that p16 inhibits CDK4 kinase activity by competing with cyclin D1 for binding to CDK4.

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