A Cyclin D1/Cyclin-dependent Kinase 4 Binding Site within the C Domain of the Retinoblastoma Protein

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ABSTRACT

Phosphorylation of the retinoblastoma protein (Rb) by the cyclin D1/cyclin-dependent kinase (cdk) 4 complex (cdk4/D1) is a key regulatory step for maintaining the orderly progression of the cell cycle. The B domain of Rb contains a site that recognizes and binds the LXCXE motif found in D-type cyclins. This interaction is important for phosphorylation of Rb by cdk4/D1, although in vitro the Rb C domain alone is efficiently phosphorylated by cdk4/D1. A mutation in the C domain of Rb, L901Q, has been identified that completely abolishes cdk4/D1 phosphorylation of the isolated C domain. By contrast, the L901Q mutation has no effect on phosphorylation by either cyclin E/cdk2 or cyclin B/cdk1, suggesting that the interaction between L901Q and cdk4/D1 is specific. Introduction of the L901Q mutation into Rb containing the A, B, and C domains results in phosphorylation becoming predominantly dependent on the LXCXE binding region. However, when the LXCXE binding region of Rb is mutated, phosphorylation becomes dependent on the L901 site within the C domain. The L901 binding site can supplement the LXCXE binding site for the cdk4/D1-dependent phosphorylation of S780 and S795 but not S807/S811. Despite the limited homology between C domains of Rb, p107, and p130, the L901 site is conserved and introduction of the L925Q mutation into the isolated C domain of p107 also inhibits phosphorylation by cdk4/D1. These data support a model for cdk4/D1 recognizing two independent binding sites in Rb and suggests a conservation of this C domain binding motif for cyclin D1/cdk4 kinase among the Rb family of proteins.

INTRODUCTION

Rb is a key regulator of cell cycle progression. The activity of Rb is modulated by its phosphorylation states that in turn are controlled by the action of different cdks and phosphatases (for recent reviews, see Refs. 1–5). Cyclin D1/cdk4 phosphorylates Rb late in G1 prior to the cell traversing the G1 checkpoint and entering S phase. As a key initiator of cell cycle progression, cdk4/D1 possesses restricted substrate specificity, acting on a limited number of proteins in the cell including Rb and its “pocket protein” family members p107 and p130 (2, 3, 6, 7, 8). This substrate specificity is also reflected in vitro where cdk4/D1 acts only on Rb family proteins but not on more general substrates, such as histones, which are phosphorylated by other cdks (8–12). In addition to the substrate specificity for Rb and its family members, not all potential sites within Rb are phosphorylated equally (13–18). The significance of these findings is not currently understood but suggests an important role for sequence-specific phosphorylation in governing the interaction of Rb with various proteins within the cell.

Substrate-specific phosphorylation implies the existence of unique contacts between the cdk cyclin complex and its substrate. Located within the B domain of Rb is a site critical for binding the LXCXE motif found in the D-type cyclins (1, 3, 19–23) and also various oncoproteins including SV40 T antigen (24), adenovirus E1A protein (21, 25–27), and the human papillomavirus E7 protein (19, 20, 23). The importance of this LXCXE binding site has been confirmed by mutational inactivation of the LXCXE sequence in cyclin D resulting in the loss of Rb-cdk4/D1 complexes in vivo (11, 28, 29). Nevertheless, in vitro work has demonstrated that the C domain of Rb can be efficiently phosphorylated by cdk4/D1 in the complete absence of the B domain (17, 18). For cdk4/D1, the K_m for the Rb C domain is nearly identical to the K_m for Rb containing the A, B, and C domains (17). This raises the question of how the C domain can be efficiently phosphorylated in the absence of the LXCXE binding domain.

Recent evidence has indicated that there are additional modes for docking substrate Rb with the cyclinE-cdk2 and the cyclin A-cdk2 complexes that do not rely on recognition by the LXCXE binding motif. This binding motif, ZRXL (where Z and X are usually basic residues), has been identified in the C domain of Rb as well as a number of other proteins that all bind cdk2 complexes (30–37). These include the Rb family proteins p107 and p130; the human papillomavirus replication protein E1; transcription factor E2F1, and the cdk inhibitors p21, p27, and p57. Mutation of the ZRXL motif at position 870 in Rb resulted in a loss of phosphorylation of the isolated C domain of Rb by both cdk2/A and cdk2/E (35). A small decrease in phosphorylation of this substrate was also observed with cdk4/D1, implying this motif could also contribute to efficient phosphorylation of Rb by cdk4/D1 (35).

In this report, we demonstrate that there is at least one additional site in the C domain of Rb at position L901 that plays a critical role in phosphorylation by cdk4/D1. The evidence suggests that this residue is part of a specific interaction site for cdk4/D1 independent of the LXCXE binding site located in the B domain of Rb. The existence of two separate binding sites for cdk4/D1 on Rb could have important consequences on the specificity of phosphorylation in vivo.

MATERIALS AND METHODS

Plasmid Construction. Site-directed mutagenesis of the GST-Rb fusion proteins was performed as described previously (17, 18).

Expression and Purification of GST-Rb Fusion Proteins. Purification of the various GST-Rb fusion proteins was performed as described previously (17, 18).

In Vitro Kinase Reactions. Cyclin/cdk reactions containing either cdk4/D1, cdk2/E, or cdk1/B were performed as described previously (17, 18, 38). Briefly, reactions (300 μl) containing 50 mM Tris-HCl (pH 7.6), 10 mM MgCl_2, 10% DMSO, 1 mM DTT, 50 μM ATP, 6 μCi of [γ-32P]ATP, and 180 μg of GST-Rb fusion protein were initiated by the addition of 10–25 units of purified cyclin/cdk. After incubation at room temperature, reactions were terminated at the times indicated in Figs. 1–4 by extraction of 25–50 μl aliquots into 50 μl of cold PBS containing 100 mM Na2EDTA, 10 mM ATP, 200 μg/ml BSA, and 0.2% NP-40. Aliquots (50 μl) were transferred to glutathione-Sepharose beads (Pharmacia, Piscataway, NJ) and processed as described previously (38).

Alternatively, reactions (50 μl) were stopped after 15 min by the addition of 2% SDS and 600 mM mercaptoethanol. After heat denaturation, the reaction products were separated by SDS-PAGE on 4–15% polyacrylamide gels (Bio-Rad, Hercules, CA) and stained with Coomassie Blue. The 32P-labeled Rb was visualized by autoradiography and the bands were quantitated using the Phos...
phorImager (Molecular Dynamics, Sunnyvale, CA). The LXCXE-containing peptide, acetyl-TDLYCYEQLN-amide (28), was prepared by Research Genetics (Huntville, AL).

**Dissociation Constant (K_d) for the Binding of cdk4/D1 to Rb.** cdk4/D1 (64 units, 128 ng) was mixed with either 60 kDa GST-Rb (30 μg) or 15 kDa GST-Rb (130 μg) in 100 μl of 50 mM sodium-HEPES (pH 7.6), 5 mM MgCl₂, 1 mM DTT, 0.5 mM EDTA, 100 μg/ml BSA, and 10% glycerol on ice for 2 h. The reaction mixture was transferred to a microcentrifuge tube containing 100 μl (packed volume) of GSH-Sepharose beads equilibrated in the same buffer and incubated on ice for an additional 30 min. The free cdk4/D1 was separated from the GST-Rb-bound cdk4/D1 by centrifugation. The amount of free cdk4/D1 in the supernatant was determined using the in vitro kinase reaction. The dissociation constant was calculated using the equation, \( K_d = \frac{[\text{free cdk4/D1}]}{[\text{Rb}]/[\text{bound cdk4/D1}]} \). Controls were performed using Sepharose 4B in place of the GSH-Sepharose.

**Western Blot Analysis.** Western blot analysis was performed using phosphospecific polyclonal antibodies as described previously (39). Briefly, GST-Rb fusion proteins were separated by SDS-PAGE on 4–20% polyacrylamide gels (NOVEX, San Diego, CA) and electrotransferred to an Immobilon-P membrane (Millipore, Bedford, MA). After blocking with BSA, samples were probed with rabbit polyclonal antibodies that specifically recognize the phosphorylated forms of either Rb S780, Rb S795, or Rb S807/S811 (New England Biolabs, Beverly, MA). Immune complexes were detected using horseradish peroxidase-conjugated donkey antirabbit antibodies and the enhanced chemiluminescence detection system (Amersham).

**GST-Rb Binding Assay.** cdk4/D1 (32 units, 64 ng) was incubated with 15 μg of either 60 kDa GST-Rb or 15 kDa GST-Rb on ice for 1 h in 50 mM Tris-HCl (pH 7.6), 10 mM MgCl₂, 1 mM DTT, and 50 μM ATP (50 μl). Protein complexes were captured on GSH-Sepharose and washed 5 times with 200-μl aliquots of cold reaction buffer. The beads were heated at 70°C for 30 min in 50 mM Tris-HCl (pH 7.6), 2% SDS, 600 mM mercaptoethanol, and 5% glycerol. Proteins were separated by SDS-PAGE and Western blot analysis was performed as described above. Blots were probed with rabbit polyclonal antibodies against cdk4 (Santa Cruz Biotechnology, Santa Cruz, CA).

**RESULTS**

A Mutation in the C Domain of Rb Affects Phosphorylation. During the course of characterizing mutations in the 15 kDa GST-Rb C domain, a spontaneous mutation, L901Q, was fortuitously identified that was refractory to phosphorylation by cdk4/D1. DNA sequencing of the mutant demonstrated that L901Q was the only mutation within the 15 kDa Rb. A comparison of the initial rates of phosphorylation between the wild-type 15 kDa GST-Rb and the L901Q mutant using purified cdk4/D1 is shown in Fig. 1A. The 15 kDa GST-Rb in which the five potential cdk phosphorylation sites have been changed to alanine, KO₅ (17, 18), was included as a control. Under conditions of substrate excess, the wild-type protein is phosphorylated linearly for 30 min. By contrast, the L901Q mutant protein is phosphorylated at less than one-tenth the rate of the wild-type Rb protein. If the L901Q mutation simply disrupted the folding of the 15 kDa GST-Rb, then the 15 kDa Rb. A comparison between the initial rates of phosphorylation by cdk4/D1 for the wild-type p107 C domain and mutant L925Q, corresponding to Rb L901Q, is shown in Fig. 2A. The rate of phosphorylation of the L901Q mutant C domain, a spontaneous mutation, L901Q, was fortuitously identified that was refractory to phosphorylation by cdk4/D1. DNA sequencing of the mutant demonstrated that L901Q was the only mutation within the 15 kDa Rb. A comparison of the initial rates of phosphorylation between the wild-type 15 kDa GST-Rb and the L901Q mutant using purified cdk4/D1 is shown in Fig. 1A. The 15 kDa GST-Rb in which the five potential cdk phosphorylation sites have been changed to alanine, KO₅ (17, 18), was included as a control. Under conditions of substrate excess, the wild-type protein is phosphorylated linearly for 30 min. By contrast, the L901Q mutant protein is phosphorylated at less than one-tenth the rate of the wild-type Rb protein. If the L901Q mutation simply disrupted the folding of the 15 kDa GST-Rb, then the 15 kDa Rb. A comparison between the initial rates of phosphorylation by cdk4/D1 for the wild-type p107 C domain and mutant L925Q, corresponding to Rb L901Q, is shown in Fig. 2A. Although the phosphorylation of the wild-type p107 by cdk4/D1 is linear for 20 min, introduction of the L925Q mutation prevents phosphorylation by cdk4/D1. Again, phosphorylation of p107 L925Q by cdk2/E was only slightly affected (Fig. 2B). Similar results were obtained with cdk1/B (data not shown), indicating that the interaction between L901 and cdk4/D1 is specific and is conserved among the Rb “pocket” proteins.
in vitro kinase assay as described in “Materials and Methods.” In mutant KO 6, potential cdk phosphorylation sites were mutated to alanine. A significant impact on the initial rate of phosphorylation by cdk4/D1 M851A, K873A, E884A, and R908A. None of these mutations had a substantial effect on the distribution of sites phosphorylated by cdk4/D1 could have been altered in the L901Q-containing mutant. This possibility will be examined in more detail below.

Two Separate Binding Sites for cdk4/D1 Exist in Rb. The phenotypic silencing of the L901Q mutation in the context of the A and B domains is consistent with two separate modes of interaction between cdk4/D1 and Rb. The stronger one driven by the LXCXE binding motif in the B domain and the weaker one driven by the L901 site in the C domain. The validity of this model was examined in three different ways. First, the effect of an LXCXE peptide on cdk4/D1 phosphorylation of the 60 kDa GST-Rb was tested. In this experiment, either 60 kDa GST-Rb or 60 kDa GST-Rb L901Q were mixed with varying concentrations of the LXCXE peptide and the inhibition of the cdk4/D1 phosphorylation was determined. Equimolar concentrations of 15 kDa GST-Rb were included in each reaction as a control since its phosphorylation by cdk4/D1 is independent of the LXCXE peptide. The products of the reaction were then separated by gel electrophoresis, visualized by autoradiography, and quantitated. In each reaction, the total amount of phosphorylation (the sum of the 60 kDa and 15 kDa Rb substrates in each lane) remains relatively constant. However, the level of 60 kDa GST-Rb phosphorylation decreases in the presence of increasing LXCXE peptide to a minimum of 60% of total phosphorylation at 40 μM peptide concentration used (Fig. 5, A and C). By contrast, when the 60 kDa GST-Rb L901Q construct is used, phosphorylation in the presence of the LXCXE peptide decreases to <10% at 40 μM (Fig. 5, B and C). These results are consistent with a model in which the LXCXE peptide blocks the D cyclin-specific binding site within the B domain of Rb (23), but cdk4/D1 can still use the site surrounding L901. With the 60 kDa GST-Rb fusion protein containing the A, B, and C domains. The rate of phosphorylation of the L901Q mutant 60 kDa GST-Rb fusion protein (□) was compared to the wild-type 60 kDa GST-Rb protein (○) using the in vitro kinase assay as described in “Materials and Methods.” In mutant KO3 (×), all of the potential cdk phosphorylation sites were mutated to alanine. (data not shown). Together these data demonstrate that L901 represents a critical residue within the C domain of Rb that has important consequences on phosphorylation by cdk4/D1. These results are also consistent with our earlier data showing that small COOH-terminal deletions of Rb profoundly influence the level of phosphorylation by cdk4/D1 (17).

Effect of L901Q Mutation on Phosphorylation of 60 kDa Rb. The above data demonstrate that the L901Q mutation had a pronounced effect on phosphorylation of the isolated C domain. To test the influence of this mutation on cdk4/D1 phosphorylation in the context of the A, B, and C domains, the L901Q mutation was then introduced into an otherwise wild-type background of 60 kDa GST-Rb. Surprisingly, the rate of phosphorylation is identical to that observed with the wild-type 60 kDa Rb protein (Fig. 4). Although the initial rate of phosphorylation is the same, the possibility exists that the distribution of sites phosphorylated by cdk4/D1 could have been altered in the L901Q-containing mutant. This possibility will be examined in more detail below.

To determine whether the effect on phosphorylation was specific for the amino acid substitution at L901, four additional amino acid substitutions were made at this position in Rb. Only the conservative substitution to isoleucine resulted in partial phosphorylation by cdk4/D1, whereas alanine, serine, or asparagine resulted in complete loss of phosphorylation (Fig. 3). Six other residues within the C domain of Rb that are identical in p107 and p130 (41) were mutated to alanine to test the effect on cdk4/D1 phosphorylation: I848A, N849A, M851A, K873A, E884A, and R908A. None of these mutations had a significant impact on the initial rate of phosphorylation by cdk4/D1
reactions were separated by SDS-PAGE and visualized by autoradiography. LXCXE were quantitated using the PhosphorImager (E, C, 2.5 m), 1.25 mm. The wild-type 60 kDa GST-Rb is preferentially phosphorylated in the L901 site and that binding is not the rate-limiting step in the reaction (see “Discussion”). Next, a 60 kDa GST-Rb mutant protein was included as a control. The products of in vitro kinase reactions were separated by SDS-PAGE and visualized by autoradiography. LXCXE peptide: 0 μM (Lane 1), 40 μM (Lane 2), 20 μM (Lane 3), 10 μM (Lane 4), 5 μM (Lane 5), 2.5 μM (Lane 6), 1.25 μM (Lane 7), 0.63 μM (Lane 8), and 0.31 μM (Lane 9). The gels were quantitated using the PhosphorImager (C, ○, wild-type and □, L901Q).

Fig. 5. An LXCXE-containing peptide differentially inhibits the cdk4/D1 phosphorylation of 60 kDa GST-Rb fusion protein containing the L901Q mutation. The phosphorylation of the wild-type (A) or L901Q mutant (B) 60 kDa GST-Rb fusion protein by cdk4/D1 was determined in the presence of 0–40 μM LXCXE peptide. The wild-type 15 kDa GST-Rb fusion protein was included as a control. The products of in vitro kinase reactions were separated by SDS-PAGE and visualized by autoradiography. LXCXE peptide: 0 μM (Lane 1), 40 μM (Lane 2), 20 μM (Lane 3), 10 μM (Lane 4), 5 μM (Lane 5), 2.5 μM (Lane 6), 1.25 μM (Lane 7), 0.63 μM (Lane 8), and 0.31 μM (Lane 9). The gels were quantitated using the PhosphorImager (C, ○, wild-type and □, L901Q).

CDK4/D1 binds to the C domain of Rb

GST-Rb containing the L901Q mutation, the L901 site is no longer available and, thus, the LXCXE peptide completely inhibits Rb phosphorylation by cdk4/D1.

This model was tested in a second, complimentary experiment by introducing a series of mutations into the 60 kDa GST-Rb at both the LXCXE binding site in the B domain (23) and the L901 site in the C domain of Rb. The phosphorylation of these mutant proteins by cdk4/D1 was examined in the presence or absence of an equimolar concentration of wild-type 15 kDa GST-Rb added as a competitor. The wild-type 60 kDa GST-Rb is preferentially phosphorylated in the presence of 15 kDa GST-Rb by nearly 6-fold (Fig. 6, Lanes 1 and 2). We have previously shown that the $K_m$ values for the individual 60 kDa GST-Rb and 15 kDa substrates are nearly identical (17). These results indicate that cdk4/D1 binds tighter to the LXCXE binding site than the L901 site and that binding is not the rate-limiting step in the reaction (see “Discussion”). Next, a 60 kDa GST-Rb mutant protein was used in which the LXCXE binding site had been disrupted by alanine substitutions at both Y709 and K713 (23). This mutant Rb protein can still be efficiently phosphorylated by cdk4/D1 (Fig. 6, Lane 3), but the phosphorylation of the wild-type 15 kDa GST-Rb has increased 4-fold in the presence of this mutant protein lacking the LXCXE binding site (Fig. 6, compare Lanes 2 and 4). This is analogous to the result obtained using the LXCXE-containing peptide (Fig. 5, A and B). As expected, the phosphorylation of the 60 kDa GST-Rb containing the L901Q mutation by cdk4/D1 (Fig. 6, Lane 5) is efficient (see Fig. 3A). However, there is nearly a 3-fold increase in the level of 15 kDa GST-Rb phosphorylation in the presence of 60 kDa GST-Rb containing the L901Q (Fig. 6, Lane 6) over that seen in the presence of wild-type 60 kDa Rb (Fig. 6, Lane 2). These data suggest that the interaction of cdk4/D1 with the LXCXE binding site is attenuated in the presence of the L901Q mutation, implying the possibility of an interaction between these two cdk4/D1 binding sites. Combining the mutations of the LXCXE binding site and the L901 site (Y709A, K713A, and L901Q) into the 60 kDa GST-Rb nearly eliminates the ability of cdk4/D1 to phosphorylate this substrate (Fig. 6, Lane 7). The small amount of phosphorylation is easily competed away in the presence of the 15 kDa GST-Rb (Fig. 6, Lane 8). Together, these results indicate that cdk4/D1 binds preferentially to the LXCXE binding site but can also bind to the 901 site when access to the LXCXE binding site is restricted.

Finally, the ability of cdk4/D1 to bind the Rb LXCXE binding site mutant was compared to the Rb L901 mutant. cdk4/D1 was mixed with a 200-fold molar excess of the various 60 kDa GST-Rb and 15 kDa GST-Rb substrates. Complexes were captured on GSH-Sepharose, separated by SDS-PAGE, and analyzed by Western blot with anti-cdk4 antibodies. As expected (21, 23, 29), mutation of the LXCXE binding site of Rb diminishes the binding to cdk4/D1 when compared to wild-type 60 kDa GST-Rb (Fig. 7, Lanes 1 and 2). The L901Q mutation in 60 kDa GST-Rb also reduces the binding to cdk4/D1 but to a lesser extent (Fig. 7, Lane 3), whereas the combination of both mutations nearly eliminates cdk4/D1 binding (Fig. 7, Lane 4).
Fig. 8. Mutation of the LXCXE binding site of Rb prevents cdk4/D1 phosphorylation of S807/S811. cdk4/D1 phosphorylation of equal amounts of the various mutant forms of 60 kDa GST-Rb was compared to the wild-type 60 kDa GST-Rb protein using phosphospecific antibodies that recognize specific phosphorylation sites in the Rb C domain. Western blot analysis was performed as described in “Materials and Methods.” Wild-type 60 kDa GST-Rb (Lane 1); 60 kDa GST-Rb LXCXE binding site mutants, Y709A and K713A (Lane 2); 60 kDa GST-Rb L901Q mutant (Lane 3; the 60 kDa GST-Rb double mutant containing both the LXCXE binding site mutations and the L901Q mutation (Lane 4); 60 kDa GST-Rb with all 10 potential cdk sites mutated to alanine (Lane 5), A, antiphosphorylated S780; B, antiphosphorylated S795; C, antiphosphorylated S807/S811; D, the blot from A was stripped and reprobed with antiphosphorylated S807/S811; E, the blot from C was stripped and reprobed with antiphosphorylated S795.

Comparison between the L901 Site and the LXCXE Binding Site on Site-specific Phosphorylation. Since the above data are consistent with Rb having two binding sites for cdk4/D1, the access to these sites could regulate differential phosphorylation of the sites within the C domain of Rb. For example, the negative effect of L901Q could be confined to S795. To address this possibility, Western blot analysis was performed using antibodies specific for phosphorylated S780, S795, and S807/S811 (39). The results demonstrate that there is no effect on the cdk4/D1 phosphorylation of either S795 or S780 using the 60 kDa GST-Rb wild-type, the LXCXE binding site mutant, or the L901Q mutant as substrates (Fig. 8, A and B, Lanes 1–3, respectively). As anticipated, cdk4/D1 phosphorylation of S795 and S780 is substantially reduced when the 60 kDa GST-Rb containing mutations in both binding sites is the substrate (Fig. 8, A and B, Lane 4). Unexpectedly, cdk4/D1 phosphorylation of S807/S811 is completely dependent on the presence of the Rb LXCXE binding site (Fig. 8C, Lane 2). These results were confirmed by stripping the blots (Fig. 8, A–C) and reprobing either with antiphosphorylated S807/S811 (Fig. 8, D and E) or with antiphosphorylated S795 (Fig. 8, F), respectively. In similar experiments with cdk2/E, there was no effect on site-specific phosphorylation by mutation in either the Rb LXCXE binding site, the L901 binding site, or both (data not shown). Thus, in the larger context of the Rb A, B, and C domains, when the LXCXE binding site is not available, the L901 binding site can facilitate cdk4/D1 phosphorylation of the S780 and S795 sites but not the phosphorylation of S807/S811.

DISCUSSION

We have fortuitously identified a mutation in the C domain of Rb, L901Q, that defines a second interaction site for cdk4/D1 that is distinct from the LXCXE binding site in the B domain. Interestingly, this is one of the few identical amino acids shared among the otherwise divergent C domains of the other pocket proteins, p107 and p130 (41). The loss of cdk4/D1 activity when this mutation was inserted into the C domain of p107 along with the lack of effect on either cdk2/E or cdk1/B support the involvement of this site in cdk4/D1-specific phosphorylation. When the L901Q mutation was transferred to the context of the larger Rb protein encompassing the A, B, and C domains, the effect of the L901Q mutation on the overall phosphorylation of Rb by cdk4/D1 was abrogated. This result suggested a subordinate role for the L901Q binding site in the context of the LXCXE binding motif in the B domain. This hypothesis was confirmed by following the cdk4/D1 phosphorylation using a combination of mutant Rb protein substrates as well as the inhibition of cdk4/D1 by an LXCXE-containing peptide.

Recently, a series of articles has described the substrate specificity of cdk2/E and cdk2/A (33–37). Although these cell cycle-dependent kinases have more promiscuous substrate specificity than does cdk4/D1, they too interact with Rb and the other substrates in a multifaceted manner. The interactions are directed by a ZRXL motif that has been identified in p107 and p130, E2F1, the human papillomavirus E1 protein, p21, p27, p57, and in the C domain of Rb (30–37). These sites are different from those described here for cdk4/D1. One site, K873, is identical in Rb, p107, and p130. We had independently mutated this conserved site, as well as five other conserved sites, to test the effect on cdk4/D1. Our results indicated that K873 is not essential for cdk4/D1 activity whereas it is for cdk2/A and cdk2/E (35). A model has been proposed in which these binding sites increase the local concentration of the substrate and correctly orient it relative to the enzyme active site (33). Our data agree with this interpretation for cdk4/D1 in general but differ in a significant way. Namely, since two independent cdk4/D1 binding sites are present in one Rb substrate, our results suggest that if one site is occupied the other site can act as an alternative binding site for efficient phosphorylation.

At least two different explanations can be invoked to account for the complete loss of cdk4/D1 phosphorylation of the isolated Rb C domain containing the L901Q mutation. First, the L901Q mutation could result in a global unfolding of the isolated C domain preventing efficient phosphorylation by cdk4/D1. This would be consistent with previous results indicating that peptides or COOH-terminal deletions of the C domain are inefficient substrates (17). However, since both cdk2/E and cdk1/B phosphorylate the 15 kDa GST-Rb containing the L901Q mutation as efficiently as wild type, global unfolding represents a less likely possibility for the loss of cdk4/D1 activity. A more likely explanation is that L901 defines an interaction site for cdk4/D1 and amino acid substitution at this site blocks the interaction and, hence, the phosphorylation. This model agrees with the recent reports cited above that show the Rb C domain contains short sequence motifs that are necessary for interaction and phosphorylation by cdk2/E and cdk2/A (33–37). Both of these findings suggest that docking via specific interactions is an important step for efficient phosphorylation by cdks. This is particularly true for cdk4/D1 since short peptides lacking the docking site or phosphorylation sites transplanted to protein scaffolds missing the docking site are such poor substrates3 (17). Thus far, only the position at L901 in Rb has been identified as part of the cdk4/D1-docking site in the isolated C domain. However, although there is very little homology between the C domains of the

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Rb family of proteins, the position at L901 can be aligned with position L925 in p107 (41). The observation that the identical mutation in the C domain of p107 illicits the same phenotype suggests that these sequences are functionally equivalent and validates this alignment. Given that L901 probably represents part of a discontinuous epitope, it will be more difficult to identify the other members of the epitope without appropriate structural information.

The loss of phenotype when the L901Q mutation was transferred back in the context of the Rb A, B, and C domains led us to the model that Rb has two distinct binding sites for cdk4/D1 and that the availability of either site can lead to phosphorylation. Previously, we have shown that the K_c for phosphorylation of the isolated C domain is roughly equivalent to the K_c for Rb containing the A, B, and C domains (17). However, when these two substrates are present in the same reaction mixture, the 60 kDa GST-Rb is clearly the preferred substrate in vitro over the 15 kDa GST-Rb (Figs. 5 and 6) while cdk2/E phosphorylates both substrates equally (data not shown). These results agree with earlier reports on the importance of the LXCXE binding motif in the B domain of Rb (1, 3, 11, 21, 28, 29). Nevertheless, binding to the B domain of Rb is not the slow step in the enzymatic reaction since this would have been reflected in the kinetic analysis. Whereas cyclin binding to the LXCXE binding motif in the B domain of Rb could be viewed as an obligatory step in order for phosphorylation to occur, this is probably an oversimplification of the process. Our results clearly show that the LXCXE binding motif is necessary for the cdk4/D1 phosphorylation of S807/S811. However, when the LXCXE binding site is unavailable, the L901 binding site is an efficient substitute for the cdk4/D1 phosphorylation of the S795 and S780 sites. These results and the multitude of diverse protein partners that interact with the various domains of Rb suggest the potential for a multifaceted mechanism for Rb phosphorylation by cdk4/D1 in vivo.

It has been suggested that nearly all tumors have mutations that impact the Rb pathway (42). The C domain of Rb has been shown to interact with E2F (21), Mdm2 (43), and c-Abl (44). Recently, a 58-amino acid deletion from the COOH terminus of Rb was shown to have a low penetrance phenotype for developing retinoblastoma (45). A truncated form of Rb was detected, suggesting that this deleted domain is essential for suppression of retinoblastoma. This COOH-terminal truncation had partially defective nuclear localization and E2F repression but had completely lost its ability to interact with Mdm2. The effect on Rb phosphorylation has not yet been reported but it should be significant since the cdk4/D1, as well as the cdk2/A and cdk2/E, binding sites have been deleted. Similarly, the biological consequences of the L901Q mutation will be part of future experiments to examine in vivo Rb phosphorylation. A thorough understanding of this key step in the phosphorylation of Rb by cdk4/D1 should enhance our knowledge of these molecular mechanisms of transformation and suggest crucial points for therapeutic intervention.

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REFERENCES


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