Structures of inactive retinoblastoma protein reveal multiple mechanisms for cell cycle control

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Cyclin-dependent kinase (Cdk) phosphorylation of the Retinoblastoma protein (Rb) drives cell proliferation through inhibition of Rb complexes with E2F transcription factors and other regulatory proteins. We present the first structures of phosphorylated Rb that reveal the mechanism of its inactivation. S608 phosphorylation orders a flexible “pocket” domain loop such that it mimics and directly blocks E2F transactivation domain (E2F^{TD}) binding. T373 phosphorylation induces a global conformational change that associates the pocket and N-terminal domains (RbN). This first multidomain Rb structure demonstrates a novel role for RbN in allosterically inhibiting the E2F^{TD}–pocket association and protein binding to the pocket “LxCxE” site. Together, these structures detail the regulatory mechanism for a canonical growth-repressive complex and provide a novel example of how multisite Cdk phosphorylation induces diverse structural changes to influence cell cycle signaling.

[**Keywords:** Retinoblastoma protein; cell cycle regulation; multisite phosphorylation; cyclin-dependent kinase; X-ray crystal structure; small-angle X-ray scattering (SAXS)]

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Cyclin-dependent kinases (Cdks) control key events in the cell cycle through protein phosphorylation. Multisite phosphorylation of Cdk substrates induces complex signaling properties such as sensitivity and switch-like behavior and permits diverse outputs (Nash et al. 2001; Mimura et al. 2004; Kim and Ferrell 2007; Holt et al. 2009; Koivomagi et al. 2011). The structural effects of Cdk substrate phosphorylation are less well characterized, as studied examples have been limited to proteins in which phosphorylation creates a linear binding epitope for direct association with degradation factors (Orlicky et al. 2003; Hao et al. 2005). Thus, the structural mechanisms by which phosphorylation of a single substrate can generate multiple distinct signaling outputs are largely unknown.

Retinoblastoma protein (Rb) is inactivated by multisite Cdk phosphorylation in normal and cancerous cell cycles (Buchkovich et al. 1989; DeCaprio et al. 1989; Lees et al. 1991; Hinds et al. 1992; Weinberg 1995; Burkhart and Sage 2008). Rb regulates transcription to affect a number of processes related to cell growth and differentiation. Its best-characterized activity is control of the G1–S transition in the cell cycle. Rb binds and inhibits E2F transcrip-

tion factors, thereby preventing activation of E2F genes that stimulate S-phase progression. In addition to its association with E2F, Rb is found in complexes with a number of other proteins, such as regulators of chromatin and chromosome structure and ubiquitin ligases (Brehm et al. 1998; Nielsen et al. 2001; Ji et al. 2004; Binne et al. 2007; van den Heuvel and Dyson 2008; Manning and Dyson 2011). The association of Rb with E2F and many of these other complexes is regulated by Cdk phosphorylation (Buchkovich et al. 1989; DeCaprio et al. 1989; Lees et al. 1991; Hinds et al. 1992; Knudsen and Wang 1997; Zarkowska and Mittnacht 1997; Brown et al. 1999; Harbour et al. 1999; Rubin et al. 2005; Lents et al. 2006; Gorges et al. 2008; Burke et al. 2010); however, it is unknown how Rb phosphorylation changes its structure to inhibit these interactions.

Rb contains the N-terminal (RbN) and pocket domains and several intrinsically disordered regions: the interdomain linker between the two independently folded domains (RbIDL), the large loop within the pocket domain (RbPL), and the C-terminal domain (RbC) (Fig. 1A). Structures of isolated domains have been determined; however, interdomain interactions and their relevance for Rb function are less well characterized (Lee et al. 1998; Rubin et al. 2005; Hassler et al. 2007). The Rb–E2F complex is stabilized primarily by an association between the E2F transactivation domain (E2F^{TD}) and the Rb pocket domain

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Figure 1. Overall Rb structure and phosphorylation-induced conformational changes. (A) Domain architecture of Rb. The two structured domains, RbN and the pocket, are colored gold and blue, respectively. Disordered sequences, including RbIDL, RbPL, and RbC, are uncolored. Conserved Cdk consensus sites are indicated. (B) Phosphorylation-induced conformational changes presented here that result in Rb–E2F^{TD} complex inhibition. Phosphorylation of S608 causes RbPL to bind to the pocket domain in a manner that competitively inhibits E2F^{TD} binding. Phosphorylation of T373 induces an interdomain association that allosterically inhibits E2F^{TD} binding.

(Lee et al. 2002; Xiao et al. 2003). Tumorigenic viral proteins such as the human papillomavirus E7 protein use an “LxCxE” motif to associate with the pocket domain at a site distinct from E2F^{TD} binding (Lee et al. 1998). Other cellular proteins bind the LxCxE cleft or other sites in the pocket domain, but the precise determinants for these associations have not been found (Brehm et al. 1998; Nielsen et al. 2001; Ji et al. 2004; van den Heuvel and Dyson 2008; Manning and Dyson 2011).

Cdk phosphorylation beginning in G1 occurs at 13 consensus sites in unstructured regions of Rb, including RbIDL, RbPL, and RbC (Lees et al. 1991; Zarkowska and Mittnacht 1997). Several studies have indicated that distinct phosphorylation events modulate specific Rb associations with E2F and other proteins. For example, T821/T826 phosphorylation inhibits histone deacetylase and viral protein binding to the pocket domain (Knudsen and Wang 1996; Harbour et al. 1999; Rubin et al. 2005). The specific association between E2F^{TD} and the pocket domain is inhibited by both T356/T373 phosphorylation in RbIDL and S608 phosphorylation in RbPL (Knudsen and Wang 1997; Burke et al. 2010). Here we characterize the structural effects of these phosphorylation events using X-ray crystallography and small-angle X-ray scattering (SAXS). We found that T373 and S608 phosphorylation each produce unique structural changes that result in allosteric and direct E2F^{TD} inhibition (Fig. 1B). Our study reveals a novel

role for RbN in the mechanism of Rb inactivation and provides the first insights into the overall structure of the multidomain Rb protein. The distinct structural changes induced by particular phosphorylation events explain how multisite phosphorylation can differentially regulate Rb interactions with other proteins.

Results

Phosphorylated RbPL binds the pocket domain at the E2F^{TD} site

We first aimed to elucidate the mechanism of E2F inhibition by S608 phosphorylation in RbPL (Fig. 1B). S608 phosphorylation inhibits E2F^{TD} binding even in the context of the isolated pocket domain (Knudsen and Wang 1997; Burke et al. 2010). To observe the structural effect of phosphorylation, we solved the 2.0 Å crystal structure of a pocket domain construct with a phosphoserine-mimetic S608E and a shortened RbPL (Table 1; Fig. 2). The crystallized protein (Rb^{380-787Δ616-642/S608E/S612A/S780A}; hereafter called Rb^{PL-P}) binds E2F^{TD} with a reduced affinity that indicates that the glutamate mutation functionally mimics S608 phosphorylation (Supplemental Fig. 1). The structure was solved by molecular replacement using the

Table 1. Statistics from X-ray crystallography analysis

	Rb ^{PL-P}	Rb ^{N-P}
Data collection		
Space group	H3	P2 ₁ 2 ₁ 2 ₁
Cell dimensions		
a, b, c	249.66 Å, 249.66 Å, 35.11 Å	51.62 Å, 129.51 Å, 135.04 Å
α, β, γ	90°, 90°, 120°	90°, 90°, 90°
Resolution	36–2.0 Å	58–2.7 Å
R _{pim} ^a	4.2 (33.1)	5.9 (31.0)
I/σ ¹	14.4 (2.7)	8.2 (2.3)
Completeness	99.9% (100.0%)	92.3% (90.0%)
Redundancy	6.0 (5.9)	4.9 (5.0)
Refinement		
Resolution	36–2.0 Å	58–2.7 Å
Number of reflections	57,103	23,354
R _{work} /R _{free}	19.3/23.8	21.2/26.6
Number of atoms		
Protein	5606	4851
Water	355	57
RMS deviations		
Bond lengths	0.008 Å	0.004 Å
Bond angles	1.0°	0.9°
Average B factor	39.5 Å ²	62.1 Å ²
Ramachandran analysis		
Preferred	97.7%	95.0%
Allowed	2.3%	4.7%
Outliers	0.0%	0.3%

Values in parentheses correspond to the highest-resolution shell of data (1.98–2.03 Å for Rb^{PL-P} and 2.70–2.77 Å for Rb^{N-P}).

^aR_{pim} = $\sum_{hkl} \{1/(N-1)\}^{1/2} \sum_i |I_i(hkl) - \bar{I}(hkl)| / \sum_{hkl} \sum_i I_i(hkl)$, where *i* indexes the *i*th measurement of reflection *hkl*, and *N* indicates the total number of times a given reflection is measured.

Figure 2. Structure of the Rb pocket domain bound by RbPL (Rb^{PL-P}). (A) Electron density is shown for the bound RbPL fragment. The mesh corresponds to a 1.5σ $f_o - f_c$ map that was generated from the molecular replacement solution and before the peptide was built into the model. (B) RbPL (yellow) binds at the interface between the A and B subdomains of the pocket (blue) and partially occludes the E2F^{TD}-binding site (pink; rendered from PDB: 1N4M). (C) Detailed interactions stabilizing the RbPL-pocket interface and comparison with the E2F^{TD}-pocket interface.

unliganded pocket domain as a search model (Balog et al. 2011), and density corresponding to RbPL was readily observable (Fig. 2A). Residues 600–610 of RbPL are ordered and bound to the pocket at the E2F^{TD} site (Fig. 2B), which resides in a cleft between the A and B subdomains (Lee et al. 2002; Xiao et al. 2003). RbPL residues 602–607 form a short α helix similar to the helix found in the C-terminal half of E2F^{TD}. Two of these RbPL residues structurally align with E2F^{TD} side chains and contact the pocket in the same manner (Fig. 2C): D604 (D424 in E2F^{TD}) forms a salt bridge with R467, and Y606 (F426 in E2F^{TD}) makes van der Waals contacts with I481 and F482. E2F residues D424 and F426 are strictly conserved, and each forms interactions that are critical for tight binding with the Rb pocket (Lee et al. 2002; Xiao et al. 2003); thus, it is significant that RbPL makes analogous side chain interactions to act as an inhibitor.

Additional important RbPL contacts are not superimposable with E2F^{TD} (Figs. 2C, 3). T601 in RbPL makes a side chain hydrogen bond with E464 from the pocket. The L607 side chain is buried within a hydrophobic pocket composed of side chains from residues N472 and L476 and the aliphatic portion of K475. The phosphoserine-mimetic S608E binds the N terminus of helix α P11, stabilizing the positive helix dipole and acting as a hydrogen bond acceptor for the amide protons of residues S644 and T645 and the hydroxyl side chains of S644 and S646. Interestingly, a similar interaction at the N terminus of α P11 is made by an aspartate in the adenovirus E1A protein, which binds and dissociates Rb–E2F complexes for cellular transformation (Liu and Marmorstein 2007). When phosphorylated, S608 could form the hydrogen bond contacts observed for the glutamate mutant and also interact with the positive helix dipole (Supplemental Fig. 1). The critical role of D604, Y606, L607, and phosS608/S608E at the RbPL–pocket domain interface is consistent with previous observations of their importance in E2F^{TD} inhibition (Burke et al. 2010). In sum, the Rb^{PL-P} structure demonstrates that S608 phosphorylation results in a bound and inhibitory conformation of RbPL that directly blocks the E2F^{TD}-binding site.

Structure of the phosphorylated RbN–pocket complex

We next set out to determine the mechanism of Rb inactivation by T356/T373 phosphorylation (Fig. 1B). The inhibitory effect of these sites in RbIDL on E2F^{TD} binding requires the presence of RbN (Knudsen and Wang 1997; Burke et al. 2010). We therefore hypothesized that RbN and phosphorylated T356/T373 act on the pocket to form an inhibited structure. We generated an Rb construct suitable for structural studies that contains RbN, RbIDL, and the pocket, but lacks RbPL and an analogous disordered loop in RbN (Rb^{53–787,Δ245–267,Δ582–642}; called Rb^{ΔLoops}). Isothermal titration calorimetry experiments demonstrate that phosphorylation of Rb^{ΔLoops} modulates E2F^{TD} binding, as previously observed for the T356/T373 sites (Supplemental Fig. 2). We successfully determined the structure of phosphorylated Rb^{ΔLoops} (hereafter called Rb^{N-P}) containing two mutations (K289A and Y292A) that allow crystal packing and an S780A mutation that facilitates homogeneous phosphorylation (Table 1). These mutations do not affect E2F^{TD} binding or the overall conformation in solution (Supplemental Figs. 2, 3).

The 2.7 Å crystal structure of Rb^{N-P} reveals a closed conformation with RbN and the pocket associated across an extensive interface (Fig. 4A). The overall structures of the individual domains are similar to their structures observed in isolation (Lee et al. 1998; Hassler et al. 2007); both contain two subdomains composed primarily of helical cyclin folds. The RbN–pocket association is composed of two sets of contacts, each involving residues from a unique pair of subdomains.

The larger interface (2277 Å² buried surface area) is formed between pocket subdomain A and RbN subdomain B and is mediated by T373 phosphorylation (Fig. 4B). The phosphothreonine side chain forms an interdomain salt

Structural mechanism of Rb inactivation

Figure 3. Summary of Rb crystal structures and interdomain interactions. Rb sequence conservation and secondary structure analysis. The bottom secondary structure markings are assigned from the Rb^{PL-P} structure (Fig. 2), and the top markings are assigned from the Rb^{N-P} structure (Fig. 4). Residues that make interdomain contacts in each crystal structure are indicated. The degree of conservation is based on alignment of the human, mouse, chicken, frog, and zebrafish sequences.

bridge with K164, which is found on the long helix (αN6) that connects the two RbN subdomains (Hassler et al. 2007). The phosphate also makes an N-terminal helix-capping interaction in the first helix of the pocket domain (αP1). The phosphate oxygens serve as hydrogen bond acceptors to backbone amide protons from R376 and V375 (Fig. 4B). This capping stabilizes αP1 such that two extra turns at its N terminus are ordered relative to the unphosphorylated structure (Fig. 3; Lee et al. 1998). These two turns position V375 and M379 to pack against RbN L161 and a conserved patch of hydrophobic residues (L212, V213, and F216), which were previously suggested to constitute a protein interaction surface in RbN (Figs. 3, 4B; Hassler et al. 2007). The C-terminal half of the αP1 helix packs against the pocket domain, with residues I382 and L385 forming a hydrophobic interface with V494, T497, and Y498. In sum, T373 phosphorylation lengthens the αP1 helix and positions it to form an interface with RbN, holding both domains in the docked conformation.

The second interdomain interface is between pocket subdomain B and RbN subdomain A (Fig. 4). This smaller

interface (387 Å² buried surface area) is formed exclusively by polar contacts from highly conserved residues (Figs. 3, 4C). Q736 from the pocket makes a side chain hydrogen bond with D145; the latter reaches the interface from the N-terminal end of the RbN-bridging helix αN6. K740 makes a hydrogen bond with the backbone carbonyl T140 and a salt bridge with D139 in RbN. K740 is part of the previously identified “lysine patch,” a set of conserved lysine residues in pocket subdomain B thought to play a role in binding phosphorylated RbC (Harbour et al. 1999; Rubin et al. 2005; Singh et al. 2005).

Electron density corresponding to phosphorylated T356 was not present in the Rb^{N-P} crystal structure. Helix αN13, which immediately precedes T356 and is present in the structure of RbN alone (Hassler et al. 2007), is also not observable here (Fig. 3). One possible explanation for the disordering of αN13 in Rb^{N-P} is that T356 phosphorylation has a destabilizing effect at the electrostatically negative helix C terminus. Calorimetry and SAXS experiments confirm that T373, but not T356, is primarily responsible for the E2F^{TD} inhibition and interdomain association effects induced by RbIDL phosphorylation (Supplemental

Figure 4. Structure of the phosphorylated RbN-pocket complex. (A) Overall structure of RbN^{N-P}. RbN and the pocket domain are colored gold and blue, respectively. (B) The interface between pocket subdomain A and RbN subdomain B is mediated by T373 phosphorylation. Phosphothreonine T373 directly contacts K164 and orders the two N-terminal turns of helix αP1, which form a hydrophobic interface with RbN. (C) The interface between pocket subdomain B and RbN subdomain A is mediated by pocket residues Q736 and K740.

Figs. 2, 3). Accordingly, the function of the structural change that occurs upon T356 phosphorylation is not yet clear.

T373 phosphorylation induces RbN-pocket docking

The RbN^{N-P} structure suggests that T373 phosphorylation is essential for the RbN and pocket domain association. This observation raises the question of whether the domains are undocked in the unphosphorylated state. To explore the conformation of Rb in the unphosphorylated state and test whether RbIDL phosphorylation induces a significant conformational change in solution, we used SAXS (Putnam et al. 2007). SAXS curves for the unphosphorylated and phosphorylated Rb^{ΔLoops} are notably distinct, and the phosphorylated protein has a smaller radius of gyration ($R_g^{\text{unphos}} = 36.8 \text{ \AA}$ and $R_g^{\text{phos}} = 31.7 \text{ \AA}$) (Fig. 5A). Shapes calculated from the SAXS curves reflect this change in R_g and suggest a conformational change from an extended to a compact structure upon phosphorylation.

Analysis of the Porod-Debye region of the SAXS curves indicates that T373 phosphorylation induces structural ordering within Rb (Fig. 5B). SAXS intensities from com-

pact structures decay as q^4 in intermediate resolution regions of the curve, and in a plot of $I(q) \cdot q^4$, compact structures plateau (Rambo and Tainer 2011). In contrast, unfolded proteins decay as q^2 and do not plateau in the $I(q) \cdot q^4$ plot. Data for only the phosphorylated Rb^{ΔLoops} show the characteristic plateau (see $\sim 0.08 \text{ \AA}^{-1}$) of a compact structure. The Porod-Debye decay of the phosphorylated state is best fit by an exponent of 3.9, which is close to the value of 4 for a compact, globular protein. The curve for the unphosphorylated state decays with an exponent of 3.3, which is consistent with the presence of a significant structural disorder in the unphosphorylated state.

We further analyzed the Rb^{ΔLoops} SAXS data by comparison with theoretical curves calculated from atomic models based on the RbN^{N-P} crystal structure. A complete atomic model for Rb^{ΔLoops} was first generated in which flexible loops not visible in the electron density were built in using Modeller (Supplemental Fig. 4). A large ensemble of possible solution conformations was then generated with a molecular dynamics simulation. The conformations in the ensemble whose calculated scattering curves best fit the experimental data for phosphorylated ($\chi^2 = 1.4$) and unphosphorylated ($\chi^2 = 1.6$) are shown in Figure 5A.

Figure 5. RbIDP phosphorylation induces RbN–pocket docking. (A) SAXS data from phosphorylated (cyan) and unphosphorylated (magenta) Rb^{ΔLoops}. Envelopes calculated from the SAXS curves are shown in the *inset*. Models based on the Rb^{N-P} crystal structure that best fit the SAXS data are shown as black ribbons within the envelopes. The calculated scattering curves of the best models are shown as black lines through the SAXS data. (B) Porod-Debye region of the experimental scattering curves for phosphorylated (cyan) and unphosphorylated (magenta) Rb^{ΔLoops}. The plateau of the phosphorylated curve indicates an ordered, compact structure.

The single best-fitting model to the phosphorylated state is similar to the Modeller structure and has the same R_g and D_{max} . The best-fitting model to the unphosphorylated state has the two domains undocked, consistent with the larger R_g and the role of phosT373 in creating the RbN–pocket interface observed in the Rb^{N-P} structure.

While these best-fitting models are plausible solution conformations, equivalent fits may exist with Rb in an ensemble of states, with no single structure representing the entire population. Using a minimal ensemble approach, we found that the unphosphorylated data are best fit by an ensemble of both undocked and docked structures, while the phosphorylated data are best fit by predominantly docked structures ($\chi^2 = 1.0$ for both) (Supplemental Fig. 4). This analysis suggests an equilibrium between associated and dissociated RbN and pocket domains, in which phosphorylation of RbIDP shifts the equilibrium toward the associated conformation. The presence of a small population of associated molecules even in the unphosphorylated state is consistent with previous observations of a weak

RbN–pocket association that is phosphorylation-independent (Hassler et al. 2007).

RbN–pocket docking inhibits protein binding at the pocket LxCxE site

The RbN docking in pocket subdomain B is proximal to the LxCxE-binding cleft, which is a well-characterized binding site for cellular and viral proteins as well as phosphorylated RbC (Harbour et al. 1999; Rubin et al. 2005; Singh et al. 2005). Alignment of the Rb^{N-P} structure with the pocket structure bound by the E7 LxCxE peptide shows some steric clashing between RbN subdomain A and the LxCxE peptide (data not shown). With a quantitative binding assay, we tested whether T356/T373 phosphorylation, which drives RbN–pocket docking, inhibits the affinity of peptides known to associate at the LxCxE cleft (Table 2; Supplemental Fig. 5). Using the Rb^{ΔLoops,S780A} construct, which only contains the RbIDL sites, we found that the affinity of the LxCxE peptide from E7 for phosphorylated protein ($K_d = 0.8 \pm 0.2 \mu\text{M}$) is weaker than its affinity for unphosphorylated protein ($K_d = 0.12 \pm 0.06 \mu\text{M}$). We also found a similar weak affinity for a phosphorylated construct that contains all of the RbN, RbIDL, and pocket phosphorylation sites (Rb⁵⁵⁻⁷⁸⁷; $K_d = 1.1 \pm 0.1 \mu\text{M}$). E7 binding to the pocket domain phosphorylated on S608/S612 ($K_d = 0.14 \pm 0.01 \mu\text{M}$) is similar to that previously reported for the unphosphorylated pocket domain ($K_d = 0.11 \pm 0.03 \mu\text{M}$) (Lee et al. 1998), indicating that phosphorylation of RbPL sites does not inhibit LxCxE binding to the pocket. Finally, we found that phosphorylation of T373 is necessary for inhibition of LxCxE binding, as the affinity of E7 peptide for a phosphorylated T373A mutant (phosRb^{ΔLoops,T373A,S780A}; $K_d = 0.29 \pm 0.07 \mu\text{M}$) is more similar to its affinity for unphosphorylated Rb (Rb^{ΔLoops,S780A}; $K_d = 0.12 \pm 0.06 \mu\text{M}$). This result is consistent with the observation that T373 phosphorylation, but not T356 phosphorylation, is necessary and sufficient for RbN–pocket docking (Supplemental Figs. 2, 3).

Phosphorylation of RbC at T821/T826 induces binding to the pocket domain at a site that overlaps with the LxCxE site and potentially involves the lysine patch in pocket subdomain B (Harbour et al. 1999; Rubin et al. 2005). Considering the proximity of the docked RbN subdomain A to this site in the Rb^{N-P} structure (Fig. 4), we next tested whether RbIDP phosphorylation also inhibits phosRbC binding to the pocket (Table 2; Supplemental Fig. 5). Rb⁷⁷¹⁻⁹²⁸, which includes RbC and seven Cdk consensus sites (S780, S788, S795, S807, S811, T821, and T826), was quantitatively phosphorylated and mixed with Rb^{N-P} in the calorimetry assay. We found that the affinity of phosRb⁷⁷¹⁻⁹²⁸ was similar for unphosphorylated ($K_d = 20 \pm 4 \mu\text{M}$) and phosphorylated Rb^{N-P} ($K_d = 25 \pm 13 \mu\text{M}$). Both of these values are similar to that previously reported for a comparable phosRbC construct binding to the pocket domain (Rubin et al. 2005). The affinity of a synthetic peptide containing phosphorylated T821 and T826 to phosphorylated Rb^{N-P} ($K_d = 11 \pm 2 \mu\text{M}$) is also similar to that previously reported for an unphosphory-

Table 2. T373 phosphorylation inhibits binding of E7 but not phosRbC at the pocket LxCxE site

Rb construct	Phosphorylated Cdk sites	Binding protein	K _d
Rb ^{ΔLoops,S780A}	None	E7 LxCxE	0.12 μM ± 0.06 μM
Rb ^{ΔLoops,S780A}	T356, T373	E7 LxCxE	0.8 μM ± 0.2 μM
Rb ^{55–787}	S249, T252, T356, T373, S608, S612	E7 LxCxE	1.1 μM ± 0.1 μM
Rb ^{379–792}	None	E7 LxCxE	0.11 μM ± 0.03 μM ^a
Rb ^{380–787}	S608, S612	E7 LxCxE	0.14 ± 0.01
Rb ^{ΔLoops,T373A,S780A}	T356	E7 LxCxE	0.29 μM ± 0.07 μM
Rb ^{N-P}	None	phosRb771–928	20 μM ± 4 μM
Rb ^{N-P}	T356, T373	phosRb771–928	25 μM ± 13 μM
Rb ^{373–787}	None	phosRb786–874	8 μM ± 4 μM ^b
Rb ^{N-P}	T356, T373	phosRb818–842	11 μM ± 2 μM
Rb ^{373–787}	None	phosRb818–839	7 μM ± 1 μM ^b

Isothermal titration calorimetry (ITC)-measured binding affinities for similar experiments using E7 LxCxE peptides and phosRbC. Sample ITC curves are shown in Supplemental Figure 5.

^aMeasurements reported in Lee et al. (1998).

^bMeasurements reported in Rubin et al. (2005).

lated pocket domain (K_d = 7 ± 1 μM) (Rubin et al. 2005). These measurements indicate that RbIDL phosphorylation and the corresponding domain closure do not affect binding of phosRbC to the pocket domain. We conclude that the RbN- and phosRbC-binding sites in the Rb pocket are not exclusive and that these domains may regulate different protein–protein interactions involving pocket subdomain B.

RbN-pocket docking allosterically inhibits E2F^{TD} binding

RbN binds the pocket domain on the face opposite from E2F^{TD} (Fig. 4), suggesting that direct competition is not the mechanism by which RbIDL phosphorylation inhibits the Rb–E2F complex. Instead, comparison between this Rb^{N-P} structure and structures of the pocket with E2F^{TD} bound reveals that T373 phosphorylation inhibits E2F^{TD} binding through an allosteric mechanism. RbN docking to the pocket induces a relative rotation of the pocket A and B subdomains by 9.6° about an axis that bisects them. To better visualize how this structural change is inconsistent with tight E2F^{TD} binding, we aligned the A subdomains of Rb^{N-P} and the pocket–E2F^{TD} structure (Protein Data Bank [PDB]: 1N4M) (Fig. 6). In this alignment, contacts between E2F^{TD} and residues in pocket A of Rb^{N-P} can be maintained; however, distances to several residues in pocket B are too far for proper binding. For example, K652 is translated 2.5 Å away from its position in the pocket–E2F^{TD} structure (relative C_α position) and is too distant to make its requisite interactions (Fig. 6B; Supplemental Fig. 6).

The Rb^{N-P} structure suggests two features that are likely critical for the observed rotation of the pocket subdomains. First, RbN binding can influence the relative orientation of pockets A and B because both subdomains are contacted in forming the overall interface. Second, the relative orientation of the RbN subdomains remains fixed upon pocket docking, likely because of the rigidity of the unique RbN-bridging helix (αN6) (Figs. 4A, 6A; Hassler et al. 2007). Residues on both ends of the same αN6 helix, K164 and D145, make respective contacts at

each of the pocket subdomain A and B interfaces, providing a constraint to the pocket subdomain geometry required for the overall RbN association. To support the allosteric model for E2F^{TD} inhibition suggested by the structure, we tested E2F^{TD} binding to an Rb^{ΔLoops} construct in which Q736 and K740—two residues in pocket domain B that make critical contacts at the smaller, polar RbN interface (Fig. 4C)—are mutated to alanine. We found that phosphorylation of this mutant protein does not weaken E2F^{TD} affinity (Supplemental Fig. 6), confirming the requirement of both interfaces for the inhibitory pocket domain conformation.

Discussion

Our results demonstrate the phosphorylation-induced structural changes in Rb that result in loss of E2F^{TD} affinity. The crystal structures together specifically implicate T373 and S608 as the key phosphorylation events for E2F^{TD} inhibition. These observations are consistent with assays for E2F binding and Rb inactivation in cancer cell models that found critical phosphorylation events in RbPL and RbIDL (Knudsen and Wang 1997; Zarkowska and Mittnacht 1997; Brown et al. 1999; Lents et al. 2006; Gorges et al. 2008). In particular, the importance of T373 phosphorylation in the mechanism of Rb inactivation is supported by the observation that only T373 phosphorylation is sufficient for E2F dissociation and activation in cells (Lents et al. 2006; Gorges et al. 2008). The Rb^{N-P} structure and SAXS data also explain the critical role for RbN in Rb inactivation previously suggested by cellular assays (Knudsen and Wang 1997). RbN docking to the pocket, which is stimulated by T373 phosphorylation, induces a change in the relative pocket subdomain orientation that perturbs the E2F-binding site.

We found that T373 and S608 phosphorylation stimulate distinct and independent mechanisms for decreasing E2F^{TD} binding, explaining how multisite Rb phosphorylation cumulatively induces Rb–E2F inhibition and E2F activation (Brown et al. 1999; Burke et al. 2010). T373 phosphorylation and RbN docking disrupt the pocket

Figure 6. Structural change in the pocket domain induced by RbN binding and its effect on the E2F^{TD}-binding site. (A) Structural comparison of Rb^{N-P} (gold and blue) with the E2F^{TD}-bound pocket domain (red, PDB: 1N4M), generated by aligning the pocket subdomain A of each structure. The pocket subdomain B of Rb^{N-P} is rotated by 9.6° relative to the E2F^{TD}-pocket subdomain B. (B) Close-up of the E2F^{TD}-binding cleft in the same structural alignment as in A. The subdomain orientation induced by RbN docking is incompatible with optimal E2F^{TD} binding. For example, in this alignment, pocket A residues (E533, I536, and K537) can contact E2F^{TD}, but the position of K652 in subdomain B of Rb^{N-P} is too distant.

structure at the overlapping E2F^{TD}- and phosRbPL-binding site, yet do not abrogate the inhibitory effect of S608 phosphorylation on E2F^{TD} binding (Burke et al. 2010). We suggest that while RbN docking likely weakens the association of phosRbPL as well as E2F^{TD} to the pocket, phosRbPL is still present as a competitive inhibitor to reduce the apparent E2F^{TD} affinity further.

Together with previous studies characterizing the effects of phosphorylation in RbC (Knudsen and Wang 1997; Harbour et al. 1999; Rubin et al. 2005), our results demonstrate that specific phosphorylation events generate remarkably diverse structural changes in Rb. In several cases, phosphorylation takes place in an intrinsically disordered region of the protein and induces structure formation. T373 phosphorylation in RbIDL induces pocket–RbN docking, S608 phosphorylation induces RbPL binding to the pocket domain, and T821/T826 phosphorylation induces RbC association with the pocket domain (Rubin et al. 2005). In two other cases, there is

evidence that phosphorylation induces the surrounding sequence to undergo a structured-to-disordered transition. S788/S795 phosphorylation directly inhibits binding of part of RbC to the E2F-DP marked box domains (Rubin et al. 2005), and here we observe that T356 phosphorylation disorders a helix in RbN. Now that these independent structural changes have been characterized, it will be important to investigate how they are coordinated to generate different cellular effects.

The differences in the two inhibitory mechanisms described here offer new insights into the importance of diverse phosphorylation pathways leading to Rb inactivation. It is noteworthy that phosphorylated RbPL directly competes with E2F^{TD} for pocket binding, while phosphorylation-induced RbN docking weakens E2F^{TD} affinity through an allosteric interaction. Direct RbPL competition for binding is an efficient mechanism for inhibiting E2F complex formation, but likely not for dislodging E2F that is already tightly bound. The allosteric mechanism in which phosT373-induced RbN docking opens the E2F-binding site is better suited for dissociating preformed Rb–E2F complexes. These distinct mechanisms for E2F inhibition may be relevant and used depending on the particular cellular context. Interestingly, the observation of an allosteric interaction mediating E2F release suggests the possibility of therapeutically targeting the RbN–pocket interface to prevent Rb inactivation.

The particular Rb conformations that result from distinct phosphorylation events differ in their ability to bind other protein factors. We found here that in addition to reducing E2F affinity, T373 phosphorylation uniquely inhibits binding at the LxCxE site. We propose that an additional important role for multisite phosphorylation in E2F inhibition is that distinct phosphorylations differentially modulate other Rb complexes. This function of multisite phosphorylation in cell cycle signaling is novel compared with previous well-characterized examples in which the enzymatic mechanisms of multisite phosphorylation tune the signaling properties of a single output (Nash et al. 2001; Kim and Ferrell 2007; Koivomagi et al. 2011). Here, the structural diversity of different Rb phosphoforms supports a model in which multisite Cdk phosphorylation generates multiple signaling outputs by assembling distinct protein complexes.

Materials and methods

Protein production and binding assays

Proteins were overexpressed in *Escherichia coli* as fusions with glutathione S-transferase. Proteins were purified by glutathione affinity chromatography, followed by cation exchange chromatography. Quantitative phosphorylation of purified protein was achieved with 2% Cdk6–CycK or 10% Cdk2–CycA overnight at 4°C. Phosphate incorporation was verified by electrospray mass spectrometry. Detailed procedures for protein expression, purification, phosphorylation, and isothermal titration calorimetry (ITC) experiments were previously described (Burke et al. 2010). Reported K_d values are the average of two to three ITC experiments, and the standard deviation of the mean is reported as the error.

Crystallization, X-ray data collection, structure determination, and model refinement

Proteins were prepared for crystallization by elution from a Superdex 200 column in a buffer containing 25 mM Tris, 200 mM NaCl, and 5 mM DTT. Proteins were crystallized by sitting drop vapor diffusion at 4°C. Rb^{PL-P} crystals grew for 1 wk in a solution containing 100 mM sodium citrate, 1 M LiCl, and 18% PEG 8K (pH 5.5) and were frozen in the same solution with 30% ethylene glycol. Rb^{N-P} crystals grew for 3 wk in a solution containing 100 mM HEPES, 100 mM ammonium fluoride, and 16% PEG 4K (pH 6.5) and were frozen in the same solution with 30% ethylene glycol.

Data were collected on Beamline 7.1 at the Stanford Synchrotron Radiation Lightsource (Rb^{PL-P}) and on Beamline 23-IDB at the Advanced Photon Source, Argonne National Laboratory (Rb^{N-P}). Diffraction spots were integrated with Mosflm (Leslie 2006) and scaled with SCALE-IT (Howell and Smith 1992). Phases were solved by molecular replacement using PHASER (McCoy et al. 2007). For Rb^{PL-P}, the unliganded Rb pocket (PDB ID: 3POM) was used as a search model, and the unliganded pocket and RbN (2QDJ) were used as search models for Rb^{N-P}. The initial model was rebuilt with Coot (Emsley and Cowtan 2004) and was refined with Phenix (Adams et al. 2010). Several rounds of position refinement with simulated annealing and individual temperature factor refinement with default restraints were applied. The Rb^{PL-P} structure has two molecules in the asymmetric unit, while the Rb^{N-P} molecule has one. In one of the asymmetric unit molecules in Rb^{PL-P}, residues 579–589 extend to a crystallographic symmetry mate and contact its LxCxE-binding site. Considering that these residues are not well conserved, that the Rb pocket is a monomer in solution (size exclusion chromatography and SAXS) (data not shown), and that other pocket crystal structures show nonspecific crystallographic interactions at this site (Lee et al. 2002; Liu and Marmorstein 2007), we believe that this observed association is a crystallographic packing artifact. Water was modeled into the electron density using Phenix with default parameters. An electron density feature corresponding to two to three water molecules is visible at the smaller RbN–pocket interface in Rb^{N-P}. We had difficulty refining water at this site, perhaps due to heterogeneity in the precise water geometry throughout the crystal, and left the density unmodeled. Buried surface areas were calculated using Chimera (Pettersen et al. 2004), and the pocket subdomain rotation was calculated using the program DynDom (Hayward and Berendsen 1998). Coordinates and structure factors have been deposited in PDB for Rb^{PL-P} and Rb^{N-P} under accession codes 4ELL and 4ELJ, respectively.

SAXS and analysis

SAXS data were collected at the SIBYLS beamline (12.3.1) at the Advanced Light Source, Lawrence Berkeley National Laboratory. Scattering data are plotted as a function of $q = 4\pi [\sin(\theta/2)]/\lambda$, where θ is the scattering angle, and λ is the X-ray wavelength in angstroms. An automated pipeline was applied for collection and partial analysis as previously described (Hura et al. 2009). Three concentrations of each sample were collected with three exposure times to check for concentration dependence and radiation damage. No concentration dependence was observed. Data were merged using PRIMUS (Konarev et al. 2003), maximizing signal to noise but excluding radiation-affected data points. The radius of gyration was determined to better than an angstrom of precision by using the Guinier approximation. GNOM (Svergun 1992) was used to determine the $P(r)$ function and assign a $D_{\max}$. The output of GNOM was used as input into GASBOR (Svergun et al. 2001) for shape calculations (Fig. 5A). Ten runs of GASBOR were

averaged together using the program DAMMAVER. The suite of programs is collectively assembled in the ATSAS suite (Konarev et al. 2006) available at <http://www.embl-hamburg.de/biosaxs/software.html>.

Missing amino acids were modeled onto the crystal structure using the program Modeller (Sali and Blundell 1993). Both Rb^{PL-P} and Rb^{N-P} structures were used as inputs into Modeller. The following Rb amino acids were modeled: three residues remaining from a cleaved N-terminal protease site to 54, 84–94, 172–185, 353–376 (RbIDL), 500–509, and 772–787. The resulting structure is shown in Supplemental Figure 4A overlaid on the solved Rb^{N-P} structure. The scattering curves calculated from atomic resolution coordinates were generated by FoXS (Schneidman-Duhovny et al. 2010). The programs BilboMD and MES (Pelikan et al. 2009) were used to generate a large ensemble of conformations and define a minimal ensemble of conformations with best fit to the data (Fig. 5A; Supplemental Fig. 4). The structure from Modeller was used as a starting conformation for BilboMD. In the simulation, RbN and the pocket were treated as rigid domains. RbIDL was unrestrained such that the relative distance and orientation of the two structured domains could vary while remaining tethered. The loops and termini were also unrestrained, except their N-terminal and C-terminal positions were fixed within their respective rigid domains. In total, 7200 conformations from 36 trajectories run in BilboMD were used in the analysis.

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