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 (E2FTD) 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 E2FTD–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 transcription 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 (E2FTD) and the Rb pocket domain.
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 E2FTD site

We first aimed to elucidate the mechanism of E2F inhibition by S608 phosphorylation in RbPL (Fig. 1B). S608 phosphorylation inhibits E2FTD 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 crystalized protein (Rb380–787

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We found that T373 and S608 phosphorylation each produce unique structural changes that result in allosteric and direct E2FTD inhibition (Fig. 1B). Our study reveals a novel structural mechanism of Rb inactivation.

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–E2FTD complex inhibition. Phosphorylation of S608 causes RbPL to bind to the pocket domain in a manner that competitively inhibits E2FTD binding. Phosphorylation of T373 induces an interdomain association that allosterically inhibits E2FTD binding.

(1996). 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 E2FTD 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 (Lee 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 E2FTD 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 E2FTD inhibition (Fig. 1B). Our study reveals a novel
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 E2FTD 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 E2F2 TD] forms a salt bridge with R467, and Y606 [F482 in E2F2 TD] makes van der Waals contacts with I481 and F482. E2F residues D424 and F482 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 αP11, 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 αP11 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 RbPL–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, D245–267, D582–642; called Rb DLoops). Isothermal titration calorimetry experiments demonstrate that phosphorylation of Rb DLoops modulates E2F TD binding, as previously observed for the T356/T373 sites (Supplemental Fig. 2). We successfully determined the structure of phosphorylated Rb DLoops (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桥.
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 RbN–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 RbN–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 RbDL phosphorylation [Supplemental
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 Rb\textsuperscript{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 Rb\textsubscript{IDL} phosphorylation induces a significant conformational change in solution, we used SAXS (Putnam et al. 2007). SAXS curves for the unphosphorylated and phosphorylated Rb\textsubscript{D Loops} are notably distinct, and the phosphorylated protein has a smaller radius of gyration ($R_g^{unphos} = 36.8$ Å and $R_g^{phos} = 31.7$ Å) (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 Poro-Deby region of the SAXS curves indicates that T373 phosphorylation induces structural ordering within Rb (Fig. 5B). SAXS intensities from compact structures decay as $q^4$ in intermediate resolution regions of the curve, and in a plot of $I(q)q^4$, compact structures plateau (Rambo and Tainer 2011). In contrast, unfolded proteins decay as $q^2$ and do not plateau in the $I(q)q^4$ plot. Data for only the phosphorylated Rb\textsubscript{D Loops} show the characteristic plateau (see ~0.08 Å$^{-1}$) of a compact structure. The Poro-Deby 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\textsuperscript{D Loops} SAXS data by comparison with theoretical curves calculated from atomic models based on the Rb\textsuperscript{N–P} crystal structure. A complete atomic model for Rb\textsuperscript{D Loops} 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.
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 phosphoT373 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 predominately 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 RbIDL 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 RbN-P structure with the pocket structure bound by the E7 LxCxE peptide shows some steric clashes 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 (Fig. 2; Table 2; Supplemental Fig. 5). Using the RbN--pocket phosphorylated 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–pocket interface observed in the RbN-P structure.

RbN–pocket docking inhibits protein binding at the pocket LxCxE site. Structural analysis suggests a balance between RbN–pocket docking and Rb N–P in the calorimetry assay. We found that the affinity of phosphorylated RbC at T821/T826 is similar to that previously reported for an unphosphorylated pocket peptide (K_d = 0.11 ± 0.02 μM) (Lee et al. 1998), indicating that phosphorylation of RbC at pocket 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 (pRb IDL loops,S780A;K_d = 0.29 ± 0.07 μM) is more similar to its affinity for unphosphorylated Rb (K_d = 0.12 ± 0.06 μM). 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 RbN-P structure (Fig. 4), we next tested whether RbIDL phosphorylation also inhibits LxCxE binding to the pocket (Table 2; Supplemental Fig. 5). Rb_771–928, which includes RbC and seven Cdk consensus sites (S780, S788, S795, S807, S811, T832, and T826), was quantitatively phosphorylated and mixed with RbN-P in the calorimetry assay. We found that the affinity of pRb IDL loops,S780A,T821A,T826A;K_d = 20 ± 4 μM) and phosphorylated RbN-P (K_d = 25 ± 13 μM). 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 T832 to phosphorylated RbN-P (K_d = 11 ± 2 μM) 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

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<th>Rb construct</th>
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<td>Rb&lt;sup&gt;Δloops,S780A&lt;/sup&gt;</td>
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<td>E7 LxCxE</td>
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Phosphorylation and RbN docking disrupt the pocket conformationally. Measurements reported in Rubin et al. (2005).^<sup>a</sup> Measurements reported in Lee et al. (1998).^<sup>b</sup> Measurements reported in Rubin et al. (2005).

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.

Discussion

Our results demonstrate the phosphorylation-induced structural changes in Rb that result in loss of E2F<sup>TD</sup> affinity. The crystal structures together specifically implicate T373 and S608 as the key phosphorylation events for E2F<sup>TD</sup> 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 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<sup>N–p</sup> 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<sup>TD</sup> 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.
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<sup>TD</sup> for pocket binding, while phosphorylation-induced RbN docking weakens E2F<sup>TD</sup> 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 phophoforms 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<sub>d</sub> 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. RbPL−P crystals grew for 1 wk in a solution containing 100 mM sodium citrate, 1 M LiCl, and 18% ethylene glycol. RbN−P crystals grew for 3 wk in a solution containing 100 mM HEPES, 100 mM ammonium fluoride, and 16% ethylene glycol. Data were collected on Beamline 7.1 at the Stanford Synchrotron Radiation Lightsource (RbPL−P) and on Beamline 23-IDB at the Advanced Photon Source, Argonne National Laboratory (RbN−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 RbPL−P, the unliganded Rb pocket [PDB ID: 3POM] was used as a search model, and the unliganded pocket and RhN (2QDJ) were used as search models for RbN−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 RbPL−P structure has two molecules in the asymmetric unit, while the RbN−P molecule has one. In one of the asymmetric unit molecules in RbPL−P, residues 579–589 extend to a crystallographic symmetry mate and contact its LxCEX-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 crystallographic interactions at this site (Lee et al. 2002; Liu and Marmorstein 2007), we believe that this observed association is.

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 $q = 4\pi \sin(\theta)/\lambda$, where $\theta$ is the scattering angle, and $\lambda$ 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 Dmax. 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.embbl-hamburg.de/biosaxs/software.html.

Missing amino acids were modeled onto the crystal structure using the program Modeller [Sali and Blundell 1993]. Both RbPL−P and RbN−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 (RhD1), 500–509, and 772–787. The resulting structure is shown in Supplemental Figure 4A overlaid on the solved RbN−P structure. The scattering curves calculated from atomic resolution coordinates were generated by FoXS [Schneider-Duhoyn 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, RhN and the pocket were treated as rigid domains. RhD1 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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