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Structural Conservation and E2F Binding Specificity within the Retinoblastoma Pocket Protein Family

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Abstract

The human pocket proteins Rb, p107 and p130 are critical negative regulators of the cell cycle and contribute to tumor suppression. While strong structural conservation within the pocket protein family provides for some functional redundancy, important differences have been observed and may underlie why Rb is a uniquely potent tumor suppressor. It has been proposed that different E2F transcription factor binding partners mediate distinct pocket protein activities. In humans, Rb binds E2F1-5, whereas p107 and p130 almost exclusively associate with E2F4 and E2F5. To identify the molecular determinants of this specificity, we compared the crystal structures of Rb and p107 pocket domains and identified several key residues that contribute to E2F selectivity in the pocket family. Mutation of these residues in p107 to match the analogous residue in Rb results in an increase in affinity for E2F1 and E2F2 and an increase in the ability of p107 to inhibit E2F2 transactivation. Additionally, we investigated how phosphorylation by Cyclin dependent kinase (Cdk) on distinct residues regulates p107 affinity for the E2F4 transactivation domain. We found that phosphorylation of residues S650 and S975 weakens E2F4 transactivation domain binding. Our data reveal molecular features of pocket proteins that are responsible for their similarities and differences in function and regulation.

Graphical Abstract

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Keywords

Cell cycle regulation; transcription factors; protein-protein interactions; protein phosphorylation; isothermal titration calorimetry

Introduction

The retinoblastoma tumor suppressor protein (Rb) and its paralogs p107 and p130 negatively regulate proliferation through controlling the cell cycle, differentiation, senescence, and apoptosis [1–6]. Consistent with their sequence homology, the three members of the “pocket protein” family share a number of cellular functions. They all arrest cells when expressed, primarily due to their ability to associate with E2F transcription factor family members and repress E2F-mediated gene expression. Pocket proteins regulate transcription both through their direct inhibition of E2F and by recruiting transcriptional co-regulators that modify histones and chromatin structure. Similarly, all are inactivated by Cyclin-dependent kinase (Cdk) activity and by proteins from oncogenic tumor viruses such as the E7 protein from human papilloma virus.

In contrast, a number of observations point to important differences in pocket proteins. Rb knockout is embryonic lethal in mice, whereas knockout of p107 or p130 results in a viable phenotype [7–9]. Mice lacking different combinations of pocket protein genes suggest that p107 and p130 have an overlapping role in development that is distinct from Rb [7]. Importantly, the tumor suppressor properties of the Rb gene are thought to be stronger than p107 and p130. Mutations within the p107 and p130 genes are less common in cancer compared with Rb [1, 3]. Heterozygous mutant Rb mice spontaneously develop tumors, while p107 and p130 mutant mice do not [7, 8, 9, 10]. However, there is evidence that p107 and p130 have some tumor suppression function and can compensate for Rb loss in certain contexts [11]. Mutations that lead to general pocket protein inactivation through kinase upregulation are much more common in tumors than loss of Rb, suggesting that broad pocket protein inactivation is necessary in many tissues [1, 3, 11, 12]. p130 levels are inversely correlated with tumor grade in many cases, suggesting p130 can protect against tumor progression by stabilizing cell cycle exit [6]. Finally, p107 protects against Rb loss in several mouse cancer models [13]. The potential for p107 and p130 to replace Rb and each other suggests the intriguing therapeutic strategy of manipulating pocket protein functions so

they can functionally compensate for each other where needed. To realize this potential, a better understanding of the molecular origins of their functional differences is needed.

Reflecting their genetic disparities, several distinct cellular functions for pocket proteins have been observed. Pocket proteins control distinct E2F target genes and arrest cells in different cell cycle phases [14, 15]. In a striking example, a genome-wide screen of gene repression in fibroblast cells revealed a unique role for Rb in promoting senescence [16]. Other additional activities have been attributed specifically to Rb, including its ability to stabilize the Cdk inhibitor p27, promote apoptosis, and maintain genomic stability [5]. Conversely, p107 and p130 exclusively act as Cdk inhibitors and promote quiescence through their assembly into the DREAM complex [17].

These similar and divergent pocket protein functions most likely reflect overlapping and distinct sets of protein interactions made by Rb and p107 or p130 (p107/p130). The pocket domain contains a cleft that binds an LxCxE motif originally identified in viral oncoproteins [18]. Histone deacetylases, chromatin remodeling complexes, Cdh1, and a number of other cellular proteins bind Rb in an LxCxE cleft-dependent manner [19–21], although detailed structural analysis of these associations is limited [22]. The best-characterized pocket protein interactions are with transcription factors. The pocket domain binds the E2F transactivation domain (E2F^{TD}), and a second binding interface exists between a section of the pocket protein C-terminal domain and the marked box (MB) domains of E2F and its obligate heterodimer partner DP (Fig. 1) [23–25]. p107 and p130 also bind viral and chromatin factors through their LxCxE cleft, and they use this cleft to bind LIN52 in the DREAM complex [10, 26]. It has been observed in cells that p107/p130 binds E2F4 and E2F5 but not E2F1, E2F2, and E2F3, while Rb is found bound to E2F1 through E2F5 [27]. Several other protein interactions are specific for Rb or p107/p130. Most notably, Rb exclusively binds Cdh1 and PP1 [19, 28], while p107/p130 bind Cdk with higher affinity, the LIN52 component of the MuvB complex to form DREAM, and protein phosphatase 2A [10, 17, 26, 29].

Cdk phosphorylation inhibits the ability of pocket proteins to negatively regulate the cell cycle, to bind E2Fs and other factors, and to repress E2F-activated transcription [30–37]. Analysis of the effects of Cdk phosphorylation on Rb structure has revealed that specific phosphorylation events mediate distinct conformational changes in Rb that disrupt interactions with E2F and potentially LxCxE -binding partners [37]. Like Rb, p107/p130 contains a large number of Cdk phosphorylation sites that regulate their activity and protein interactions [30, 33–35], however the structural effects of p107/p130 phosphorylation have not been explored.

We have investigated here the structural conservation of p107/p130, its interactions with E2F, and how these interactions are inhibited by specific phosphorylation events. The goal is a comparative analysis of pocket protein structure, biochemical interactions, and regulation such that genetic and functional differences can be understood in the context of their structural origins. We use the recently determined crystal structure of the p107 pocket domain to identify the molecular determinants of E2F specificity among pocket proteins and to demonstrate that binding specificity can be tuned both *in vitro* and in cells.

Results

Structural conservation of the pocket domain

Our understanding of pocket protein structure primarily comes from detailed analysis of Rb. Rb contains a structured N-terminal domain (RbN), the pocket domain, and an intrinsically disordered C-terminal domain (RbC) (Fig. 1a) [5]. There are several other unstructured sequences, including an interdomain linker between RbN and the pocket (RbIDL) and a ~60 amino acid loop in the pocket domain (RbPL). p107 and p130 share 54% sequence identity with each other, share 30% identity with Rb, and appear to have similar domain structure as Rb. For example, p107 has a pocket domain [26], a predicted structured N-terminal domain (p107N) and disordered C-terminus (p107C), an interdomain linker (p107IDL), and two large internal loops within the pocket domain (p107PL and p107PL2). Notably, while Cdk sites are distributed throughout the pocket proteins, their localization to sequences within the disordered IDL, PL, and C regions is similar (Fig. 1a).

The crystal structure of the p107 pocket domain was recently determined in complexes with peptides that bind the LxCxE site [26]. We performed a detailed comparison of the p107 and Rb structures in order to understand differences that may account for separate functions, particularly differences in E2F binding specificity. The overall similarity between the p107 and Rb pocket domains is high with an RMSD in C_α position of 1.1 Å (PDB codes 1GUX and 4YOZ). Moreover, most of the secondary structural elements compare with the exception of two extra small helices in p107 (α4 and α10).

To identify the most highly conserved pocket structural features, we generated an alignment of 51 pocket domain sequences from 31 metazoan organisms (Fig. S1). The alignment produces 30 amino acids that are identical in 90% of the sequences and can be divided into four clusters (Fig. 1b). Two highly conserved clusters form the structural core of the A subdomain (in human p107: E507, F519, P520, F533, I537, L547, H554, L555) and B subdomain (Y796, L806, L810, W821, R838, D841, Q842, I864, Y868). The third group contains residues critical for the AB interface (K535, E538, E559, W568, K794, R802, H839), and the fourth group forms directly or structurally supports the LxCxE binding site (F861, F933, Y934, N935). The conservation of N935 is particularly notable, as it plays no obvious role supporting pocket domain structure. N935 faces outward and contacts proteins that bind at the cleft. In the crystal structures of p107 bound to either the LIN52 or the E7 peptide, the N935 amide sidechain makes bidentate hydrogen bonds with the LxSxE or LxCxE backbone [26], and the same interaction is observed in the Rb-E7 complex [18]. The structural conservation is consistent with the similar affinity of the E7 peptide for both Rb and p107 pocket domains [18, 26].

We further compared the p107 pocket domain with structures of the Rb-E2F^{TD} complex to examine conservation of the E2F^{TD} binding site and identify residues that may explain differences in the binding of human pocket proteins to different E2Fs. E2F^{TD} binds the A-B pocket interface by making two sets of contacts [23, 25]. At its N-terminal end, E2F^{TD} binds in an extended conformation and makes interactions with α8 and α9 in the A-subdomain and α11 in the B-subdomain. The C-terminal portion binds as a short helix to α4, α5, α6 and α8 in the A-subdomain. With the exception of E479 and K535, there is much less

conservation within the E2F binding site of pocket protein orthologs (Fig. 1). A few other conserved sidechains (E538, E559, and K794) contact E2F but also clearly play a role in stabilizing the A–B interface. By aligning the structures of human p107 and Rb pockets, we identified ten amino acids near the pocket-E2F interface that differ between the two human paralogs (Fig. 1), and we examine the effect of these residues on binding specificity below.

Structural basis for E2F^{TD}-pocket specificity determinants

We applied sequence comparison of E2F family members and the structures of the Rb and p107 pocket domains to understand the structural origins of binding specificity among E2F and pocket protein family members. The E2F transcription factor family is commonly divided into activating (E2F1-E2F3) and repressive (E2F4-E2F8) subgroups based on their respective roles in regulating transcription. While E2F1-E2F5 all contain pocket-binding domains, based on co-immunoprecipitation experiments from cell extracts, it has been understood that Rb binds all E2Fs whereas p107/p130 have specificity for the repressive E2Fs (E2F4 and E2F5) [27].

We tested whether the observed specificity in cells correlates with differences in pocket protein-E2F transactivation domain binding affinities. We purified recombinant Rb and p107 pocket domains that lack their large internal loops (Rb^{PL}: residues 380–787 with 581–642 deleted, p107^{PL}: residues 391–972 with 601–779 and 888–923 deleted). We measured affinity for the E2F1, E2F2, E2F3, and E2F4 transactivation domains using isothermal titration calorimetry (Fig. 2). Consistent with previous measurements [23, 38], we found that Rb^{PL} has higher affinity for E2F1^{TD} and E2F2^{TD} than for E2F3^{TD} and E2F4^{TD}. In contrast, we found here that p107^{PL} binds E2F4^{TD} with higher affinity than E2F1^{TD}, E2F2^{TD}, and E2F3^{TD}.

We first sought an understanding of the higher affinity of Rb for E2F1 and E2F2 compared to the other E2Fs. We looked at an E2F sequence alignment for residues that are conserved in E2F1 and E2F2 but distinct in the other E2Fs (Fig. 3a). The glycine at position 414 in E2F1 (415 in E2F2) fits this criterion. In both the Rb-E2F1^{TD} and Rb-E2F2^{TD} structures, this glycine packs against helix α 11 of the pocket B subdomain such that K652 in Rb hydrogen bonds to the glycine carbonyl oxygen (Fig. 3b). K652 also makes a sidechain hydrogen bond to E416/E417 in E2F1/E2F2, which is not conserved as a glutamate in E2F3. The close proximity of G414/G415 to α 11 suggests that the bulkier sidechains present in the other E2Fs may clash with the helix and disrupt the ideal position of K652.

We mutated G415 in E2F2 to resemble E2F4, and we found that the G415N mutant E2F2^{TD} binds Rb^{PL} with 6-fold weaker affinity (Fig. 3c). We also found that an N395G mutation in E2F4 results in a five-fold increase in affinity of E2F4^{TD} for Rb^{PL} such that its affinity is similar to E2F1^{TD} and E2F2^{TD}. These data point to G414/G415 as a key residue for mediating the high affinity interaction of Rb for E2F1 and E2F2. The importance of this glycine for E2F^{TD} binding to p107^{PL} is less evident, although we do observe a weaker affinity of E2F2^{TD} G415N compared to wild-type (Fig. 3c). p107 has R793 at the analogous position to K652 in Rb and may also clash with N395 in E2F4. We propose that other interactions between E2F4 and p107 must stabilize E2F4 binding to p107 relative to E2F2 as observed in Fig. 2.

We performed a similar analysis to attempt to understand why p107 binds E2F4^{TD} with higher affinity than the activating E2Fs. Examination of the E2F^{TD} sequences identifies E2F4 V402 and C403 as candidate specificity-determining residues that are similar in E2F4 and E2F5 but different in E2F1-E2F3. We found that a double I422V and S423C mutation in E2F2^{TD} has little effect on affinity for p107^{PL}. However, unexpectedly, a double V402I and C403S mutation increases E2F4^{TD} affinity slightly.

Structural differences explain activating E2F preference for Rb

We next compared and manipulated the pocket domain structures to determine the basis for the higher affinity of the activating E2Fs for Rb compared to p107. There are ten residues that line the E2F binding cleft in the pocket and are distinct between p107 and Rb (Fig. 1). We predicted that six mutations in p107 (T480R, V490K, G786T, N556E, E560H, H797R) would influence E2F binding based on conservation and their observed interactions with the transactivation domain in the Rb-E2F^{TD} structures (Fig. 4a). We mutated these six residues in p107^{PL} to create a construct (p107^{6x}) that has an Rb-like E2F binding cleft. We then tested by ITC and found that p107^{6x} bound E2F1^{TD} and E2F2^{TD} with three fold and twenty-four fold higher affinity than p107^{PL} respectively but bound E2F4^{TD} with similar affinity (Fig. 4b). Notably, the affinity of p107^{6x} for E2F2^{TD} is nearly similar to the affinity of Rb for the activator E2Fs.

We then mutated these six residues individually in p107^{PL} and tested binding to E2F2^{TD} (Fig. 4c). We found that E560H, H797R, and V490K each bound with approximately 3-fold increases in affinity, while the effects of the other three mutations were more modest. The structure of the Rb pocket domain bound to E2F2^{TD} provides rationale for why E560H, H797R, and V490K increase affinity of p107 for E2F2^{TD} (Fig. 4d). E560H likely creates a favorable electrostatic interaction with nearby D411 and D410. While D410 is further from the H555/E560 position in the Rb-E2F2^{TD} structure, it is notable that E2F1 and E2F4 have leucine and histidine respectively at the analogous 410 position (Fig. 3A), which possibly explains why the increase in affinity of the 6x-mutation is most prominent for E2F2. To support this idea, we mutated the E2F2 aspartates to histidines in order to create favorable electrostatic interactions with the E560 in wild-type p107^{PL}. We found that a D410H mutation increases the affinity of E2F2^{TD} for p107^{PL} about 3-fold (Fig 4c). In contrast, making the histidine mutation at D411, which is conserved across all E2Fs, does not influence the affinity. The H797R mutation creates a favorable hydrophobic interaction with L413, as the imidazole group is replaced with a methylene in the arginine sidechain. The V490K mutation creates a favorable electrostatic interaction with the sidechain of D427, which is conserved in all E2F^{TD}s.

We tested whether the mutations that increase the affinity of the p107 pocket domain for E2F2^{TD} lead p107 to display activity in cells that resembles a gain in E2F2 regulation. We engineered the six E2F-binding mutations into full-length p107 (p107^{6x-FL}) and transfected the construct into C33A cells. Consistent with our calorimetry data using the isolated domains, we find that p107^{6x-FL} co-precipitates more transfected E2F2 than wild-type p107 (Fig. 5a). We used a luciferase reporter assay to measure E2F2 driven gene expression and tested the effects of p107^{6x-FL} in repressing E2F2. This experiment reveals that p107^{6x-FL}

more efficiently represses transcription by transfected E2F2 than p107^{WT} (Fig. 5b). These data support the conclusion that structural differences between pocket protein-E2F^{TD} interactions can alter E2F activity in cells.

Conservation of phosphorylation events that inhibit E2F^{TD} binding to the pocket domain

The effects of Cdk phosphorylation on Rb structure and function have been studied *in vitro* and in cell culture [37]. In three different events, phosphorylation drives an interdomain association in Rb that weakens E2F^{TD} association: T373 phosphorylation induces RbN-pocket binding to allosterically disrupt the E2F^{TD} binding site, and S608/S612 and S788/S795 phosphorylation induces binding of RbPL and RbC respectively to the pocket to inhibit E2F^{TD} competitively. Sequence alignment of pocket proteins reveal that many of these sites are conserved in p107/p130 (Fig. 6a), and here we tested using ITC whether analogous phosphorylation events in p107 also lower its affinity for E2F.

In order to observe the effects of phosphorylation at S650 in p107 (analogous to S608 in Rb, see Fig. 6a), we examined binding of E2F4^{TD} to a p107 pocket domain construct that does not have p107PL entirely deleted (residues 391–972 with 669–779 and 888–923 deleted). We found that even in the unphosphorylated state, the affinity is weaker for the p107 pocket construct with this region of p107PL intact (Fig. 6b, compare p107^{PL} to p107). The presence of RbPL does not affect E2F^{TD} binding in similar measurements made with Rb [18]. In Rb, however, phosphorylation of S608 induces binding of RbPL to the pocket domain in a manner that competitively inhibits E2F^{TD}. Considering sequence homology, we hypothesized that even in the unphosphorylated state, the p107 loop could compete to some extent for E2F4^{TD} binding. To test this idea, we mutated Y648 in the p107 pocket construct to alanine, because the corresponding Y606 in Rb is critical for the RbPL-pocket association and phosphorylation-induced E2F binding inhibition in Rb. We found that the p107^{Y648A} mutant binds E2F4^{TD} with higher affinity than wild-type (Fig. 6b), in support of the notion that the wild-type p107PL sequence adopts a bound conformation that partially inhibits E2F binding even when not phosphorylated.

We attempted to phosphorylate the p107 pocket domain with Cdk2-CycA, but we found that phosphorylation of S650 was poor (Fig. S2a and Fig. S2b), even though the analogous pocket loop site in Rb (S608) is readily phosphorylated [39]. The poor Cdk phosphorylation is again consistent with the idea that the unphosphorylated loop is to some extent already in the bound conformation, and therefore, the Cdk site is more occluded. We were able to use Cdk to phosphorylate the p107^{Y648A} pocket and found quantitative phosphorylation on three sites (Fig. S2c), which matches the number of consensus Cdk sites in p107 pocket loop (S615, S640, and S650). Using the ITC assay, we found that phosphorylation of p107^{Y648A} pocket decreases affinity of E2F4^{TD} (Fig. 6b). We suggest that, as in Rb [40], phosphorylation changes the equilibrium to favor a conformation in which p107PL competitively inhibits the E2F^{TD} binding site. Addition of an S650A mutation to the p107^{Y648A} construct (called p107^{S650A}) results in a p107 pocket domain that binds E2F4^{TD} similar to p107^{Y648A} but does not show the same reduction in affinity upon phosphorylation of its remaining two sites (Fig. 6b and Fig. S2d). This result indicates S650 is the primary phosphorylation event that stimulates pocket loop inhibition of E2F^{TD}.

To determine the effect of S964 and S975 phosphorylation in p107, we measured affinity of E2F4^{TD} for a p107 construct that includes the pocket domain, lacks both pocket loops, and includes the p107 C-terminus to residue 982 (p107^{PL982}; residues 391–982 with 602–779 and 888–923 deleted). Phosphorylation of this construct, which includes only the S515, S975, and S964 sites, reduces affinity of p107 for E2F4^{TD} 3-fold (Fig. 6c). Our electrospray ionization mass spectrometry analysis indicates that the p107^{PL982} construct is only phosphorylated quantitatively by Cdk *in vitro* at one site, and we find no incorporation of phosphates into a construct (p107^{PL972}) that ends at residue 972 (Fig. S2e and S2f). The latter observation that phosphate is not readily incorporated into S515 and S964 is consistent with their sidechains being buried in the structure and the previous observation that the analogous S780 site in Rb is not readily phosphorylated [40]. We conclude that S975 phosphorylation is responsible for the E2F4^{TD} inhibition observed in the p107 C-terminus.

We attempted to measure the specific effects of T385 phosphorylation in p107 using a construct that extends from the beginning of the p107 N-terminal domain through the pocket domain and lacks the p107 pocket loop. While we could express this construct in *E. coli* and purify it, we could not phosphorylate it homogeneously with the expected number of phosphates using recombinant Cdk as observed by radiolabeled ³²P incorporation (data not shown). We note that phosphorylation of sites in the p107 and p130 interdomain linker has been found to be important for Cdk-dependent relief of p107/p130 inhibition of E2F and the cell cycle [26, 33]. The resistance of p107 to quantitative phosphorylation at sites on the pocket loop and interdomain linker observed here is an interesting contrast to Rb. There have been several reported observations of complexes in S phase that contain p107 and E2F4, and it is thought that p107 may play an important role in regulating E2F activity in S phase [41–44]. The presence of these complexes in S phase while Cdk2-CycA activity is high has been puzzling in light of the known effects of Cdk on dissociating Rb-E2F complexes. The resistance of p107 to Cdk2-CycA phosphorylation observed here *in vitro* may explain how these complexes persist in the cell.

Discussion

Two broad questions about the pocket protein family remain unaddressed: what are the different cellular functions of Rb, p107, and p130 that account for their different tumor suppressor properties and what are the distinguishing molecular features that account for these differences? We have explored here the idea that functional differences may arise from different interactions with E2F family members and demonstrate that the pocket protein-E2F pairings observed in cells result from sequence differences that impart different binding affinities. We have found that Rb possesses higher affinity for the transactivation domains of E2F1 and E2F2, whereas p107 has preference for E2F4. We have accounted for these preferences by examining sequence and structural features that differ among pocket protein and E2F family members, and importantly, we have shown that affinities and preferences can be altered. For example, we have observed that the p107^{6x-FL} mutant is capable of co-precipitating E2F2 and represses more efficiently E2F2-dependent transcription in cells. We propose using this reagent to examine whether the capacity to bind activating E2Fs confers other Rb-specific functions to p107 that may compensate for Rb deficiency in cells.

We examined pocket protein orthologous sequences from thirty metazoan species to identify the most conserved features. The highest conserved residues support the pocket domain structure, either by stabilizing the A and B subdomain folds or by stabilizing the interface between the two subdomains. Residues in the B subdomain at the LxCxE cleft are also highly conserved, suggesting that the best conserved and perhaps most ancient function of pocket proteins is binding protein targets at the LxCxE site. In contrast to pocket structure and LxCxE-mediated protein interactions, the detailed mechanism of E2F-binding is less conserved. The expansion of the Rb family that occurs in early vertebrates is paralleled by an expansion in the E2F family. Subtle sequence changes that subsequently occurred have lead to specificity in the interactions between family members. The inhibition of E2F binding by Cdk phosphorylation is also relatively less conserved. We do observe here phosphorylation events in human p107 that weaken E2F binding similar to previous observations for Rb (Fig. 5). However, these sites (S650 and S975 in p107 and S608 and S795 in Rb) are only conserved in vertebrate pocket proteins, suggesting that E2F regulation has evolved more recently. In general, we have found here that the inhibitory effects of p107 phosphorylation on E2F affinity are more modest than the effects of Rb phosphorylation [38, 39], and Cdk cannot quantitatively phosphorylate p107 at a key inhibitory site S650 *in vitro*. These observations are consistent with the prevalence of p107-E2F4 complexes in S phase and may be critical for how p107 and Rb differentially regulate E2F in the cell cycle [41–44].

Several of our binding measurements suggest that details of p107-E2F4^{TD} binding may be different than anticipated from the Rb-E2F1^{TD} and Rb-E2F2^{TD} structures. For example, we have not been able to understand why p107 has higher affinity for E2F4^{TD} compared to the activating E2F^{TD}s (Fig. 3). It is also interesting that the 6x-mutation, which changes p107 to mimic Rb, increases p107 affinity for E2F2^{TD} but does not weaken affinity for E2F4^{TD} in the ITC experiment. The structure of the p107 pocket domain has not yet been determined bound to an E2F^{TD}, and we expect that it may provide some unexpected details that explain the unique preference of p107 and p130 for the repressive E2Fs.

Materials and Methods

Protein expression, purification, and phosphorylation

All Rb, p107, and E2F constructs were expressed in *Escherichia coli*, using PET-derived vectors, as fusion proteins containing an N-terminal glutathione S-transferase (GST) tag. Transformed BL21(DE3) cells were grown to an OD₆₀₀ of 0.6-0.8 and induced with 1 mM IPTG. Protein expression took place overnight at 22 °C. Cells were resuspended in lysis buffer containing 25 mM Tris-HCl, 250 mM NaCl, 5 mM DTT, 5% glycerol, and 1 mM PMSF (pH 8). Cells were lysed by passing them three times through a cell homogenizer, and following centrifugation the resulting soluble fraction was purified using glutathione sepharose affinity chromatography. The subsequent eluate was further purified by anion-exchange chromatography and cleaved with GST-TEV overnight at 4 °C. To remove cleaved GST, proteins were again passed over glutathione sepharose resin, and finally subjected to size exclusion chromatography to achieve a pure sample. Proteins were phosphorylated by incubation overnight at 4 °C with 10% Cdk2-CyclinA, 10 mM MgCl₂, 1 mM ATP, 200 mM

NaCl and 25 mM Tris-HCl (pH 8). Quantitative substrate phosphorylation was determined using electrospray ionization mass spectrometry (Fig. S2).

Isothermal Titration Calorimetry

Proteins were prepared for ITC by dialyzing overnight at 4°C in a buffer containing 100 mM NaCl, 20 mM Tris-HCl, and 1mM beta-mercaptoethanol (pH 8.0). Using a Micro-Cal VP-ITC calorimeter, typical binding experiments involved injecting 0.5–1 mM peptide into a 20–40 μM solution of p107 or Rb at 25 °C. Binding constants were generated by fitting the data to a one-site binding model using Origin software. The error associated with the reported dissociation constants reflect the standard deviation calculated from 2–4 separate binding experiments.

Immunoprecipitation and Western Blotting

Immunoprecipitation was carried out as previously described [45]. To generate extracts C33A cells were plated at 6x10⁶ cells per 15 cm plate and transfected with 40μg of either CMV-FLAG-p107^{WT} or CMV-FLAG-p107^{6x-FL}, 20μg of either CMV-HA-E2F2 or CMV-HA-E2F4 and 20μg CMV-HA-DP1. Extracts were then normalized for transfection efficiency and immunoprecipitated using anti-FLAG M2 (Sigma). Immunoblotting was carried out using anti-FLAG M2 (Sigma) and anti-HA 3F10 (Roche).

Luciferase Reporter Assays

SAOS2 cells were plated at 7.5x10⁵ cells per well in a six well plate and transfected 24h later. Cells were transfected in triplicate with Fugene HD according to manufacturer s instructions (Fugene HD + 100μl DMEM). All transfections included the following: 100ng of pE2F4B-Luc reporter plasmid, 200ng CMV-βGal, 15 ng of CMV-HA-E2F2, and 15ng of CMV-HA-DP1. E2F-repression assays also included either 0, 50 ng, 100 ng, 150 ng, or 200 ng of CMV-FLAG-p107^{WT} or CMV-FLAG-p107^{6x-FL}. CMV-CD20 was added to normalize p107 and CD20 plasmids to 200ng. Luciferase and βGal assays were performed as previously described [46], and luciferase activity was normalized to βGal from the same transfected extract.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Pocket proteins (Rb, p107, and p130) and E2F transcription factors regulate cell-cycle dependent gene expression.
- Specific interactions between these proteins are due to affinity differences between the E2F transactivation domain and pocket domain.
- Mutating key interacting residues in the pocket domain of p107 results in a protein that binds E2F2 like Rb and regulates its transcription activity in cells.
- Although phosphorylation of p107 is inefficient *in vitro* compared to Rb, analogous modifications inhibit E2F binding.

Fig. 1. Structural conservation among pocket domains

(a) Overall architecture of Rb and p107. Each protein contains an N-terminal domain (RbN and p107N), a pocket domain, and disordered sequences including the C-terminus (RbC and p107C), interdomain linker (RbIDL and p107IDL), and the pocket loops (RbPL, p107PL, and p107PL2). Domains with known structure are colored, although in the case of p107N, the coloring and boundaries are based on structure prediction. Consensus Cdk phosphorylation sites (SP or TP) are indicated, and those in parentheses are not even conserved among vertebrates. (b) Pocket domain sequence alignment. Sequences are shown for human Rb, p107, and p130. Relative conservation among 51 sequences from 31 organisms is represented by the bars (full alignment shown in Supplementary Fig. 1). Residues that are conserved among >90% of the sequences are highlighted in colors and divided into clusters by location in their structure. These residues are either in the structural cores of subdomain A (red) and subdomain B (magenta), at the A-B interface (blue), or at the E2F (pink) and LxCxE (green) binding sites. Residues that line the E2F^{TD} binding site and differ between Rb and p107 are noted with a pink asterisks as is the conserved Cdk phosphorylation site in the pocket loop (blue dashed box). (c) Structure of the human p107

pocket domain (PDB Code: 4YOZ). E2F2^{TD} is modeled in a bound conformation by aligning the p107 structure with the Rb-E2F2^{TD} structure (PDB Code: 1N4M). Residues with >90% conservation are colored as in (b).

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Fig. 2. Specificity in pocket-E2F^{TD} affinities
(a) Isothermal titration calorimetry data for the indicated binding reactions and (b) table of ITC measured affinities of the Rb and p107 pocket domains for each E2F^{TD}.

Fig. 3. Preference of Rb for E2F1/E2F2

(a) Sequence alignment of E2F transactivation domains. Conserved residues are highlighted yellow, G414/G415 are highlighted pink, and residues that are only similar in E2F4/E2F5 are highlighted purple. (b) Structure of Rb^{PL}-E2F2^{TD} complex shows proximity of G415 to K652 in helix α 11. The structure predicts that glycine substitution would lead to potential steric clash. (c) ITC measurements of Rb^{PL} and p107^{PL} for the indicated E2F construct. VC is an I422V and S423C mutation in E2F2^{TD}, and IS is a V402I and C403S mutation in E2F4^{TD}.

Fig. 4. Preference of activating E2Fs for Rb over p107

(a) Overlay of Rb^{PL} and p107^{PL} structures. Sidechains are shown for six residues that are near the E2F binding site and differ between the two pocket domains. (b) ITC measurements E2F peptides binding to wild-type p107^{PL} and p107^{PL} containing six mutations that are changes of p107 to the Rb sequence (p107^{6x}). (c) ITC measurements of individual mutations as indicated. (d) Comparison of the Rb^{PL} and p107^{PL} structures explains the preference of Rb for E2F2^{TD}.

Fig. 5. Mutation of the E2F-binding cleft in p107 enhances affinity for and regulation of E2F2
 (a) C33A cells were co-transfected with Flag-tagged p107^{WT} or p107^{6x-FL}, HA-tagged E2F2 or E2F4, and DP1 plasmids. Cell extracts were immunoprecipitated with anti-Flag antibodies and western blots of extracts and precipitated proteins were probed with the indicated antibodies. (b) Saos2 cells were transfected with an HA-E2F2 expression plasmid, DP1, and a p107 mutant or wild-type construct. Transfections also included an E2F responsive luciferase reporter plasmid and transcriptional activity was determined by luciferase activity and normalized to a non-p107 transfected control. Error bars indicate one standard deviation from the mean (n=9). Means were compared by a t-test and significant differences are indicated by an asterisk ($P<0.05$).

Fig. 6. Conservation of Cdk inhibitory mechanisms in pocket proteins

(a) Sequence comparison of human Rb, p107, and p130 shows conservation of Cdk phosphorylation sites that are known to inhibit E2F^{TD} binding to Rb. (b) and (c) ITC measurements of unphosphorylated and phosphorylated p107 constructs for E2F4^{TD}.