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Dimerization of CtIP may stabilize *in vivo* interactions with the Retinoblastoma-pocket domain

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Abstract

CtIP is a tumor suppressor that interacts with Retinoblastoma protein (Rb) to regulate the G1/S-phase transition of the cell cycle. Despite its large size (897 residues) CtIP has few known structured regions. Rather it contains several linear motifs that interact with known binding partners, including an LXCXE motif that binds the pocket domain of Rb-family proteins. This LXCXE motif lies at the C-terminus of the only known structured domain, an N-terminal coiled-coil dimerization domain (DD; residues 45–160). Yeast two-hybrid (Y2H) and GST-pulldown analyses showed that CtIP requires the LXCXE motif to bind the Rb-pocket. Although isothermal titration calorimetry data indicates that the LXCXE motif is the sole determinant of binding affinity for the Rb-pocket domain ($K_A \sim 10^6\ M^{-1}$), Y2H data indicates that the DD is required to stabilize the interaction *in vivo*. Thus dimerization may increase the apparent stability of the proteins and/or the lifetime of the complexes.

Keywords: Protein dimerization; CtIP; Retinoblastoma; Protein–protein interactions; Stabilization; Auto-activation

CtIP (CtBP interacting protein) is a widely expressed tumor suppressor protein [1] that interacts with the co-repressor CtBP (C-terminal binding protein) [2] and the tumor suppressors BRCA1 (breast cancer associated protein 1) [3] and Rb (retinoblastoma protein, a crucial regulator of the G1/S-phase transition of the cell cycle, reviewed in [4]) [5]. CtIP appears to have roles in transcriptional regulation, DNA damage response, and cell cycle control [1,6–8]. In particular, the interaction of CtIP with Rb activates the transcription of S-phase genes [8], whereas depletion of CtIP results in an Rb-dependent arrest in the G1 phase of the cell cycle [1].

CtIP is 897-residue nuclear protein that contains a coiled-coil dimerization domain near the N-terminus (CtIP-DD, residues 45–160) [9], the biological role of which has not yet been determined. CtIP contains few other obvious structural features, but possesses several short peptide regions (linear motifs) that mediate interactions with several of its known partner proteins: a PLDLS motif for interaction with CtBP (residues 490–494) [2]; a phosphoserine motif centered on S327 for interaction with BRCA1 [10]; and an LXCXE motif for interaction with the Rb family, Rb, p130 and p107 (LECEE, residues 153–157) [5].

LXCXE motifs are found on numerous Rb-binding cellular and viral proteins and bind to a conserved cleft in the so-called "pocket-domains" of Rb-family proteins [11,12]. The viral oncoproteins that exploit this motif disrupt normal Rb function within cells, particularly by disrupting interactions with the E2F family of transcription factors [13].

In CtIP, the LXCXE motif lies at the C-terminal end of the defined CtIP-DD. Dimerization is a feature common to many proteins, which may confer numerous possible

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advantages on proteins (reviewed in [14]), including stabilization and the ability to up- or down-regulate activity by generating or obscuring an active site upon dimer formation. Thus, it seemed feasible that dimerization of CtIP could affect binding to the Rb-pocket domain. When both the DD and LXCXE motif of CtIP were deleted, or when the pocket domain of p130 was disrupted, the interaction between CtIP and p130 was abrogated [6]. In contrast, when the pocket domain of Rb was disrupted by mutation, it could still form an interaction with CtIP [15], possibly through a PENF motif near the C-terminus of CtIP and the C-terminal C domain of Rb [16], which is not common to all pocket proteins. Thus, in order to specifically determine whether dimerization of CtIP has the potential to modulate binding of the LXCXE motif to a target protein, a study was carried out to investigate the mode of binding between CtIP and the isolated Rb-pocket domain.

A combination of *in vivo* and *in vitro* techniques was used to confirm that CtIP binds the Rb-pocket domain through an LXCXE motif, with a lower binding affinity than many viral partners of Rb, but a higher affinity than other known cellular partners. The CtIP-LXCXE motif appears to be the sole determinant of binding to the Rb-pocket, but dimerization of CtIP apparently stabilizes the interaction *in vivo*, probably by enhancing the lifetime of complex formation.

Materials and methods

Generation of constructs. Rb and CtIP constructs were generated through PCR amplification of cDNA clones of CtIP (J. Visvader) and Rb (R. Reddel) and ligated into pGEX-2T, pGAD10, pRSET or pGBT9 plasmids using *Bam*HI and *Eco*RI restriction sites. RbAB contains residues 375–767, whereas RbA′B′ contains residues 375–577 and 644–767 fused by a flexible linker (GSIEGRG). An internal *Eco*RI site in CtIP was removed through a silent mutation.

Yeast two-hybrid and auto-activation assays. Yeast (AH109; Clontech) were transformed with Rb-pGBT9 and CtIP-pGAD10 plasmids according to standard procedures (Yeast Protocol Handbook, Clontech) and maintained in media deficient in leucine and tryptophan. Yeast cultures were normalized for cell density by dilution in sterile Milli-Q™ water to an OD_{600} of 0.2. Serial dilutions were made ($2 \times 1{:}10$), and solutions were spotted onto selection plates and incubated at 30 °C for ~72 h. Three levels of selection were used: low (histidine deficient media); medium (histidine deficient with the addition of 1 mM 3-amino-1,2,4-triazole; 3-AT, Sigma); and high (histidine and adenine deficient media). All selection media included 40 μg/ml X-α-gal (5-bromo-4-chloro-3-indolyl-αD-galactopyranoside; Astral), and interactions were evaluated by yeast growth and blue color development. Auto-activation assays used CtIP-pGAD10 plasmids only and the media contained leucine.

GST-pulldown assays. RbA′B′ was produced as a GST fusion and immobilized on GSH beads (Amersham Biosciences). ^{35}S-Met-labelled CtIP was produced by *in vitro* transcription/translation, using the TnT®T7 Coupled Reticulocyte Lysate System (Promega), from CtIP-pRSET constructs. Immobilized GST-Rb (~10 μg) was incubated with 5 μl ^{35}S-Met-labelled CtIP in 20 mM phosphate, 150 mM NaCl, 0.5% IGEPAL, 2.5 mg/ml bovine serum albumin (BSA), 0.1% β-mercaptoethanol pH 7.4, at 4 °C for 1 h, and washed with the above buffer containing 10% glycerol in the absence of BSA. Levels of bound CtIP protein were evaluated using SDS–PAGE and phosphorimage analysis using ImageQuant™ Version 4.2 (Molecular Dynamics).

Protein expression and purification. The CtIP dimerization domain (CtIP-DD; CtIP residues 45–160) was purified as described previously [9]. His_6-Rb proteins were expressed from Rb-pRSET constructs in *Escherichia coli* BL21(DE3), and purified by Ni–NTA chromatography (Qiagen) and size-exclusion chromatography. Where appropriate the His_6-tag was removed by treatment with thrombin.

Peptide synthesis. Starting materials and reagents were purchased from Auspep (Victoria, Australia). CtIP peptide (residues 148–162) was manually synthesized by solid-phase methods using standard Fmoc chemistry [17]. Following cleavage from the resin with trifluoroacetic acid, the peptide was purified by reverse-phase high-pressure liquid chromatography and its molecular weight was confirmed by electrospray mass spectrometry.

Gel filtration/MALLS analysis. Purified proteins were applied to a Superose12™ column (Amersham Biosciences) equilibrated with 20 mM Tris, 150 mM NaCl, 1 mM dithiothreitol (DTT) pH 7.5 at a flow rate of 0.6 ml/min. MALLS was performed using a mini-DAWN instrument inline with an Optilab DSP interferometric refractometer (Wyatt Technology Corp), with a laser wavelength of 690 nm. BSA monomer was used for normalizing the signals of the detectors relative to the 90° detector's signal. Data were analyzed with Astra Version 4.90.07 (Wyatt Technology), using a dn/dc value of 0.19 ml/g.

Isothermal titration calorimetry. Isothermal titration calorimetry (ITC) experiments were carried out on a Microcal VP-ITC at 4 °C in 20 mM Tris pH 7.4, 150 mM NaCl, 0.5 mM Tris(2-Carboxyethyl) phosphine Hydrochloride. De-gassed samples were titrated as an initial injection of 1 μL followed by 20–25 sequential injections of 10 μL. Normalized and baseline corrected data was fitted by a single-site model using Origin (Microcal Software, Northampton, MA).

Results

CtIP and the Rb-pocket domain interact in yeast two-hybrid assays

The Rb-pocket consists of two conserved 'boxes' (A and B) with a less conserved spacer region lying in between. Structural studies of the Rb-pocket domain showed that the boxes fold together to form a compact structure [11,18], whereas the region between is unstructured [12]. Our experiments used two constructs of Rb: the native-pocket domain, containing the A and B boxes and the intervening spacer region (RbAB); and, a truncated version where the spacer region was replaced with a short linker (RbA′B′; Fig. 1A).

An interaction between CtIP and both Rb-pocket constructs (Fig. 1B) was observed using yeast two-hybrid (Y2H) analysis, confirming that the structured region of the Rb-pocket was responsible for mediating an interaction with full-length CtIP. Subsequent experiments to determine the key binding regions of the proteins were carried out using the truncated form of the pocket.

As expected, the removal of the LECEE motif completely abolished the interaction between the two proteins (Fig. 1B). An interaction was observed between CtIP-DD and the Rb-pocket, but with an apparently lower binding affinity than the full-length CtIP construct, and removal of most of the dimerization domain from CtIP while retaining the LECEE motif weakened the interaction. Thus, the dimerization domain could interact directly with Rb, or may indirectly stabilize the interaction; however, there might also be a region C-terminal to the DD that also contributes to the interaction with the Rb-pocket.

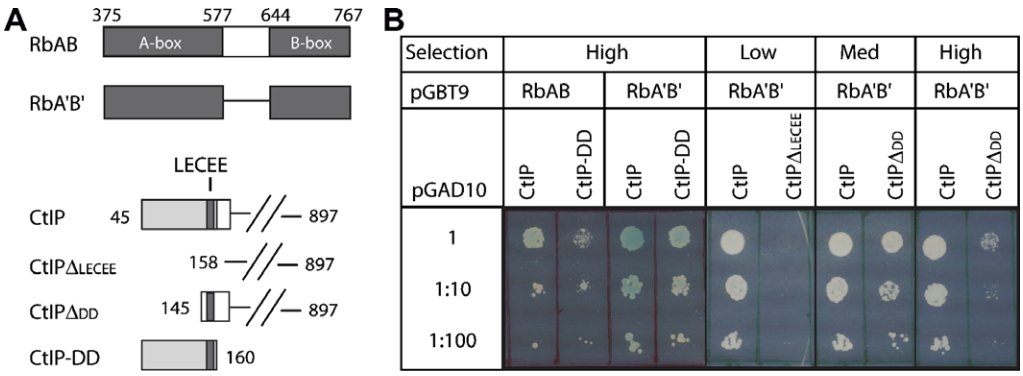

Fig. 1. CtIP interacts with Rb *in vivo*. (A) Schematic of Rb and CtIP constructs used in Y2H assays. (B)Y2H assays showing combinations of constructs evaluated. Only tests for interaction (as three different dilutions) are shown; control experiments on −LW plates all showed yeast growth. Stringency of selection conditions were: low: −LWH; medium: −LWH + 1 mM 3AT; high: −LWHA.

CtIP displays a strong transcriptional activation activity in Y2H assays

To look for an additional pocket-binding domain, C-terminal truncations of CtIP (45–897) were then tested for their ability to interact with the Rb-pocket domain in Y2H assays (Fig. 2; Supplementary data1). There were differences in the apparent strength of binding between the various constructs, but no consistent trend was observed. We had previously observed that when fused to the GAL4 DNA-binding domain (GAL4DB) full-length CtIP displayed a strong auto-activation activity in yeast that might artefactually boost the level of transcription (and hence yeast growth) in these experiments. To explore this possibility, all CtIP constructs were singly transformed as GAL4DB fusions and tested for auto-activation. By using a range of selection stringencies, differences in auto-activation were seen (Fig. 2; Supplementary data1) that matched the patterns of binding seen in the Rb-binding Y2H assays. Thus, the apparent differences in binding from different C-terminal truncations of CtIP likely originate from auto-activation of CtIP in this system, roughly localized to residues 161–897. This is consistent with observations that CtIP can interact with one or more proteins that have transcriptional activities, including the basal transcription factor TFIIB [19].

C-terminal regions of CtIP are not involved in Rb binding

To provide an alternative test of this theory, the interaction between CtIP and Rb was also examined *in vitro* using GST-pulldown experiments. Two constructs of CtIP that displayed differences in apparent Rb-binding in the Y2H assays were used: CtIP(45–897) and CtIP(45–371). Quantification of phosphorimaged bands in Fig. 3A indicated that Rb bound to 35% and 36% of the full-length species of each CtIP input. Although this is not a true quantitative technique, these data suggest that there is no significant difference in levels of binding between these C-terminal truncations of CtIP, and that the differences seen in the Y2H assays stemmed from the auto-activation properties of those constructs. Overall, these data imply that no region of CtIP C-terminal to the LECEE motif contributes significantly to its interaction with the Rb-pocket.

CtIP interacts with the Rb-pocket with a moderate affinity

To examine the strength and stoichiometry of the interaction between the Rb-pocket domain and CtIP-DD, these proteins were produced in milligram quantities. The purified proteins were first subjected to gel filtration in conjunction with multi-angle laser light scattering (MALLS) analysis to determine the solution oligomeric

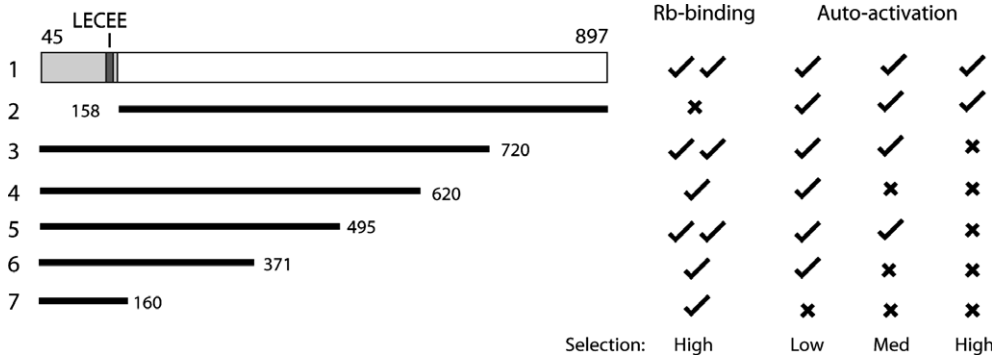

Fig. 2. Apparent increases in binding affinity stem from autoactivation of CtIP in Y2H. Summary of results and schematic of CtIP constructs used in Y2H assays. Interaction assays are shown in Supplementary data1. Stringency of selection conditions as in Fig. 1.

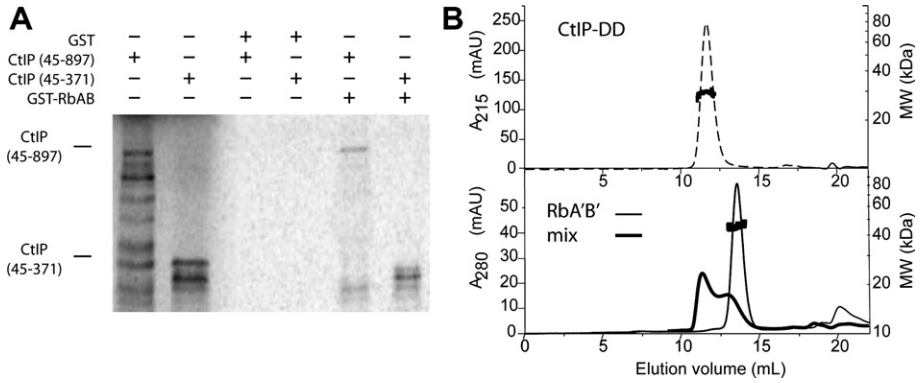

Fig. 3. Formation of CtIP/Rb-pocket complexes. (A) Pulldown of ^{35}S-Met *in vitro* transcribed/translated CtIP with GST-RbAB. Samples of CtIP proteins are 25% of input. Equal loadings of GST proteins were used (Supplementary data2). (B) Gel filtration and MALLS analysis of CtIP-DD (top; ------) and Rb-pocket domain (bottom; —), showing distribution of molecular mass across the peak, and gel filtration analysis of a 1:1 mix of CtIP and Rb (bottom; ▬).

states of the individual proteins. CtIP-DD has no significant UV signal at 280 nm, but its elution was monitored by absorbance at 215 nm, and it eluted as a single peak at ~12 min (Fig. 3B). The weight average molecular weight of this peak determined by MALLS was 29.4 ± 0.6, compared with a theoretical monomer value of 28.4 kDa, indicating that CtIP-DD formed a dimer in solution which agrees with data obtained from analytical ultracentrifugation experiments [9]. The Rb-pocket does have a significant absorbance at 280 nm, and eluted as a single species at ~13.5 min (Fig. 3B), with a weight average molecular weight of 43.3 ± 0.5, indicating that the protein is monomeric (theoretical monomer mass 40.4 kDa). Note that CtIP-DD has an elongated coiled-coil structure [9], which is the likely cause of its relatively low elution volume.

In order to test for protein complex formation a 1:1 mixture of Rb and CtIP-DD (based on monomer concentrations) was subjected to gel filtration and monitored at 280 nm to specifically detect the elution of the Rb-pocket. The appearance of a second peak at ~11.5 min indicated the formation of a complex between the two proteins (Fig. 3B). However, a significant proportion of the Rb-pocket domain remained in the uncomplexed form. The overlap between the elution times of the three different species meant that MALLS could not be used to determine the molecular weight (and thus the stoichiometry) of the complex. Similar results were obtained with CtIP-DD and the native Rb-pocket domain (data not shown).

Isothermal titration calorimetry (ITC) analysis was then used to determine the affinity and stoichiometry of the interaction. At the concentrations used in this experiment CtIP-DD is largely dimeric [9]. When fitted by a model describing 1:1 binding, these data showed that the CtIP-DD bound to both Rb-pocket constructs with a similar affinity (K_A of $0.7–1.4 \times 10^6$ M^{-1}; Fig. 4A and B), with a stoichiometry of 1:1 using the monomer concentrations of both proteins, i.e., two Rb molecules bind one CtIP dimer.

CtIP dimerization has no affect on Rb-binding in vitro

In Y2H assays, the removal of the dimerization domain appeared to weaken the interaction (Fig. 1B). To further assess this, ITC analysis was also carried out between the Rb-pocket and a 14-residue synthetic peptide derived from the CtIP sequence that spanned the LECEE motif (residues 148–162). This short peptide probably exists as a monomer in solution, but given the small size and spectral properties of the peptide, this could not be confirmed. The ITC data revealed much lower enthalpy changes than did the same experiment with CtIP-DD, meaning that the confidence levels on the fitted data are low, but they indicate a similar affinity for Rb as CtIP-DD (Fig. 4C). Unfortunately it was not possible to repeat the ITC experiments at higher protein concentrations to improve the signal-to-noise for this interaction. However, these data indicate that the LECEE motif is likely to be the sole determinant of direct binding between CtIP and Rb.

Discussion

The coiled-coil dimerization domain of CtIP is one of the few domain structures evident in the sequence of CtIP, but the biological significance of this domain has not been established. CtIP-DD comprises residues 45–160, which encompasses the LXCXE motif (residues 153–157). Far-UV circular dichroism showed that this domain was highly helical [9], which implies that the LXCXE motif might also have helical structure. As structures of the pocket domain of Rb bound by LXCXE motifs indicate that those motifs bind in an extended conformation [11,12], it is possible that dimerization could stabilize the helicity of the LXCXE motif and disfavour Rb-pocket binding. However, the ITC data for CtIP-DD and the LXCXE-containing peptide showed that the binding affinities were identical, suggesting that no helix-to-coil transition is required. CtIP-DD was designed from a combination of limited proteolysis data and coiled-coil predictions from MULTICOIL, including

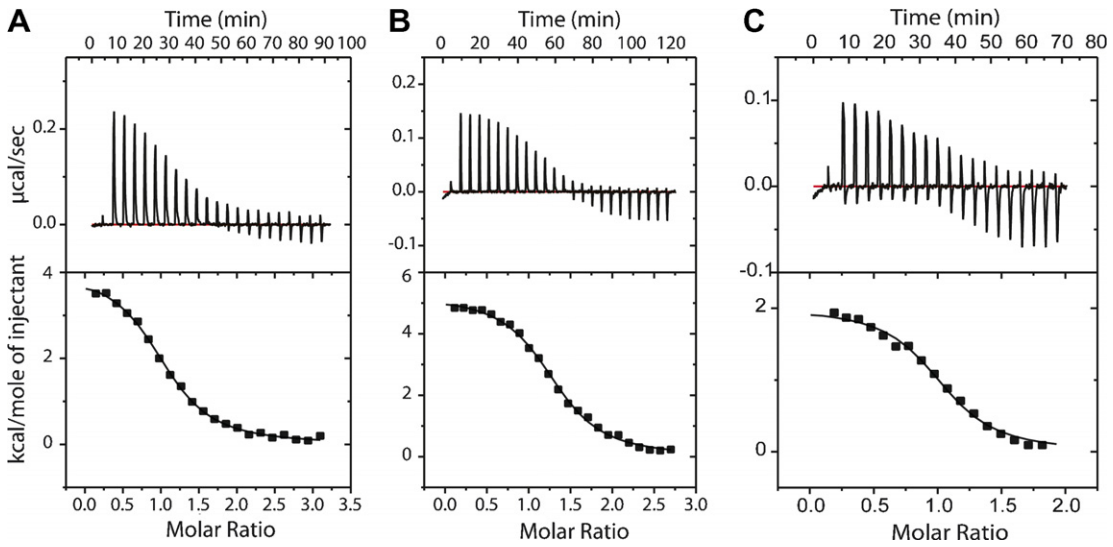

Fig. 4. ITC analysis of CtIP/Rb binding. (A) RbAB (183 μM) titrated into CtIP-DD (18 μM). $N = 1.07 \pm 0.01$, $K_A = [0.70 \pm 0.05] \times 10^6\ M^{-1}$, $\Delta H = 4930 \pm 50$ cal/mol (B) RbA′B′ (123 μM) into CtIP-DD (9.8 μM). $N = 1.30 \pm 0.01$, $K_A = [1.39 \pm 0.09] \times 10^6\ M^{-1}$, $\Delta H = 5240 \pm 60$ cal/mol (C) CtIP-LECEE (100 μM) peptide (20 μM) into RbAB. $N = 1.02 \pm 0.02$, $K_A = [1.02 \pm 0.15] \times 10^6\ M^{-1}$, $\Delta H = 2000 \pm 40$ cal/mol.

some lower probability residues at the end of the domains to ensure that the full coiled-coil domain was included in the sequence [9]. It is reasonable to assume that the ends of the domain, including the LECEE motif, are not structured.

We had previously proposed that dimerization of CtIP might facilitate the formation of multiprotein complexes and/or exchange of complex components [9]; however, results from this present study suggest an additional role. The Y2H data indicated that dimerization of CtIP may stabilize the interaction with the Rb-pocket *in vivo*, whereas ITC data indicated that dimerization of CtIP has no effect on the binding affinity. Rather, the LECEE motif of CtIP appears to be the sole determinant of the binding affinity for the Rb-pocket. This apparent contradiction can be reconciled by considering that both the thermodynamic stability (as measured by ITC) and the lifetime of the complex *in vivo* will contribute to the extent of reporter gene activation within the Y2H assay. In the case of the Rb/CtIP interaction, the kinetics of complex assembly and disassembly may be controlling the level of transcriptional output observed. The kinetics could be affected without affecting the apparent binding affinities of individual interactions such that a complex containing multiple components (i.e., a CtIP dimer and two Rb monomers) should effectively dissociate more slowly than a simpler two-component system. To date, it has not been established if the oligomerization of other oligomeric LXCXE-containing proteins [20–22], might also have an effect on LXCXE-mediated interaction with Rb.

Over 20 known binding partners of Rb contain LXCXE motifs and must compete for binding the Rb-pocket. CtIP binds the Rb-pocket with a higher affinity than does the cellular protein HDAC1 ($K_D = 10$ μM) [23] but a lower affinity than known viral proteins (E7: $K_D = 0.11$–0.19 μM; TAg: 0.44 μM [12,23]). The CtIP/Rb-pocket interaction would be blocked upon infection by those viruses. Notably, differences in the Rb affinity displayed by variants of the HPV E7 protein correlate with the corresponding viral strains association with anogenital cancer; high risk strains have a higher affinity for Rb [24], which highlights the importance of interactions with Rb during tumorigenesis triggered by viral infection.

Our data add to evidence that the relative Rb affinities for different LXCXE motifs could help determine the binding patterns of Rb and other pocket proteins in any given cellular context. It will be interesting to determine how the inclusion of a putative additional binding interface, possibly between the Rb-C domain and the C-terminus of CtIP might additionally contribute to the binding patterns and activity of these two tumor suppressors.

In conclusion, we have confirmed the presence of an interaction between CtIP and the Rb-pocket and have established that CtIP utilizes its LECEE motif to bind to the pocket domain with a lower affinity compared with viral Rb-pocket partners. The dimerization of CtIP does not appear to affect the binding affinity *in vitro*, but may stabilize the interaction *in vivo*.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.12.178.

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