The retinoblastoma tumor suppressor protein (pRb) is a key negative regulator of cell proliferation that is frequently disregulated in human cancer. Many viral oncoproteins (for example, HPV E7, E1A) are known to bind to the pRb pocket domain via a LXCXE binding motif. There are also some 20 cellular proteins that contain a LXCXE motif and have been reported to associate with the pocket domain of pRb. Using NMR spectroscopy and isothermal calorimetry titration, we show that LXCXE peptides of viral oncoproteins bind strongly to the pocket domain of pRb. Additionally, we show that LXCXE-like peptides of HDAC1 bind to the same site on pRb, with however a weak (micromolar) and transient association. Systematic substitution of residues other than conserved L, C and E show that the residues flanking the LXCXE are important for the binding, whereby positively charged amino acids in the XLXCXEXXX sequence significantly weaken the interaction.

The retinoblastoma protein, pRb, is a 928 amino acid protein that belongs to the family of so-called pocket proteins (other members being p107 and p130) (1, 2). The small pRb pocket, which is the major focus of tumorigenic mutations in pRb, comprises the A and B cyclin-like domains (pRb-AB, amino acids 379-578 and 641-791, respectively) (3-5). The tumor suppressor action of pRb stems from its ability to arrest cells at the G1 phase of the cell cycle by suppressing the activity of the E2F family of transcription factors (6, 7). It is now believed that the pRb together with p53 tumor-suppressor gene pathways are inactivated in most of the cancers (8).

The small pocket of pRb binds to the LXCXE-like sequence containing proteins (9) and includes also a primary binding site for E2Fs (10-15). Biochemical and structural studies showed that the E2F peptide binding site is separated by ~30 Å from the LXCXE peptide binding site (16, 17). There are other contact points on viral oncoproteins for pRb, but the major interaction is through the LXCXE motif. The crystal structure of the [pRb-AB]-[domain of SV40 large T antigen] shows that two-thirds of the total surface interaction between the two proteins is via the LXCXE motif (18). This motif interacts with the pRb-AB on the B subdomain in an extended conformation exactly like the LXCXE peptide from HPV E7 (18, 19). The large pocket region of pRb (amino acids 379-928) is known to have additional binding to E2Fs and is necessary for binding to other cellular proteins, for example, MDM2 (20). About 120 proteins have been reported to physically interact with pRb, mostly through the pRb pocket domain (21, 22).

DNA tumor virus oncoproteins, such as adenovirus E1A, SV40 large T antigen, HPV E7, (23-26) and about 20 cellular proteins, such as for example, HDAC1, HDAC2, BRG1, cyclin D1, BRAC1 and PAI, possess a LXCXE-like motif in their sequences (27-30, 31, 32, 33, 34, 35, 36). The viral oncoproteins inactivate the pocket proteins by direct association through their LXCXE sequences. As viral oncoproteins utilize the LXCXE motif, it was proposed that cellular proteins would also interact with the pRb through this sequence (27, 35-37). However, this model of interaction would not account for binding of protein like HDAC3 to pRb as it lacks any LXCXE-like motif (37, 38). There are also reports that suggest that HDAC1 and HDAC2 do not utilize their LXCXE-like sequences for interaction with the small pocket of pRb (39-41); instead, recruitment of HDACs to pRb occurs via an intermediary, LXCXE containing protein, RBP1 (31). How pRb recruits the class-1 HDACs to the repressor complex at the promoter remains a subject of controversy (41). Similar conflicting reports abound in the literature regarding the interaction of other proteins, for example BRG1, with the pRb (31, 32). Some reports suggest direct interaction between BRG1 and pRb-AB, whereas other
reports contradict this notion (38, 39, 42, 43). One report suggested that BRG1 and HDAC1 interact separately with pRb via differing modes of interaction (38).

Since the assays reported in the literature were based on inhomogenous protein preparations, we decided to use homogenously purified proteins and check for pRb-AB/protein or peptide associations using direct in vitro experiments. These experiments included NMR spectroscopy, isothermal titration calorimetry (ITC), gel filtration and mass spectrometry. This multi-method approach can unambiguously characterize the interaction between pRb and LXCXE sequences. We studied a series of LXCXE peptides or protein domains, derived from HDAC1, BRG1, PAI2 (plasminogen activator inhibitor 2) and the N-terminal pRb (Figure 1 A, B and Table 1).

The pRb-N and BRG1 LXCXE domains were also cloned into the pET30 LIC/Xa vector. Protein expression was carried out in a similar manner to that of pRb-AB except that induction was performed at 24°C for overnight. The proteins were purified over a Ni-NTA agarose column, followed by size exclusion chromatography. The identity of proteins was confirmed with the SDS-PAGE, Western blot, N-terminal sequencing and mass spectrometry. Final protein samples used for all the studies were more than 95% purified as judged by SDS-PAGE analysis (Figure 2 A, B, C).

Peptide synthesis - The peptides were synthesized using solid phase, purified with C8 reverse phase chromatography and checked by mass spectrometry. Table 1 shows the peptides used in our study.

Isothermal titration calorimetry - Binding of various peptides to pRb-AB was measured by ITC using a MicroCal Omega VP-ITC system (Micro-Cal, Amherst, MA). The peptides, at concentrations between 200 and 400 µM, were titrated into 20–50 µM pRb-AB at 20-22°C. pRb-AB was dialysed against 50 mM NaH2PO4·H2O, pH 7.8 and 10 mM βME buffer and all the peptides were dissolved in the same buffer. After subtraction of the dilution heats, calorimetric data were analyzed with the MicroCal ORIGIN V5.0 (MicroCal Software, Northampton, MA). For all of the titrations, the stoichiometry of ligand binding to pRb was close to 1.0.

Mass spectrometry - Mass spectrometry was carried out on an ESI–MS API 165 Perkin–Elmer Sciex (Langen) spectrometer coupled with
HPLC (column: Macherey-Nagel EC 125/2 Nucleosil 300–5 C4 MPN; pump system: Microgradient System 140B/C Perkin Elmer (solvent A: water, 0.05 % TFA, B: MeCN, 0.05% TFA; gradient 10–95% B); photodiode array Agilent HP1100PDA; software: Masschrom, Biomultiview). Protein and peptide were mixed at 1:3 to 5 molar ratio and complex was passed through analytical S75 Superdex (Amersham-Pharmacia) gel filtration column. The peak corresponding to the complex was collected and concentrated using Centricon (Millipore). Samples were then analysed by mass spectrometry for both the mass spectrometry for both the protein and peptides.

NMR spectroscopy - 15N uniformly labeled or specifically 15N lysines labeled samples of pRb-AB were used for binding studies. Several 1H-15N HSQC spectra were recorded to observe chemical shift behaviour of lysines resonances upon addition of peptides. NMR spectra were recorded on the AV900 MHz with Cryo-Probe or DRX 600 MHz Bruker spectrometers with standard TXI-probe heads. 128 increments for uniformly labeled and 72 increments for the 15N lysine labeled sample were recorded in an indirect dimension. The protein concentration used was ~0.5 mM concentration. Concentrations of peptides used were between 2 mM to 5 mM depending on the solubility of a particular peptide. Both protein and peptide samples were dissolved in the same buffer as pRb-AB (50 mM NaH₂PO₄·H₂O, 10 mM βME, pH 7.3). The measurement time was of 16 h per HSQC for 0.5 mM sample, and extended up to 24 h for more diluted samples.

RESULTS

The LXCXE sequence of HPV E7 and SV40 large T antigen bind tightly to the small pocket of pRb, while the IXCXE sequence of HDAC1 associates transiently - We used the following peptides in the first round of our study: the LXCXE peptides from HPV E7, SV40 large T antigen, and PAI2 proteins; and the IXCXE peptides from HDAC1 and a peptide derived from the transactivation domain of E2F-1. Also LXCXE-containing fragments of BRG1 and the N-terminal of pRb were used (Figure 1 A, B and Table 1). We first measured the binding of these various peptides to pRb-AB using ITC. In our construct of pRb-AB domains A and B comprise amino acids 379-578 and 642-791, respectively. The equilibrium dissociation constant, K_D, for the binding between the pRb pocket and IXCXE peptide of HDAC1 (17mer) was difficult to measure but could be estimated to be ca. 10 μM (Figure 3A and Table 1), which is weaker compared to the binding of LXCXE containing peptides from HPV E7 or SV 40 large T antigen (Figure 3B and 3C) and the peptide derived from the transactivation domain of E2F-1 (Table 1). Three HDAC1 peptides were tested: the HDAC1 peptide of Table 1 (17mer), a shorter version, RIACEEEFS, and a peptide extended by two N- and one C-terminal residues, SS and E, respectively (20mer). ITC showed the pRb-AB binding for all three peptides but for the two latter quantification of the data was difficult to obtain.

We also checked the strength of this binding using mass spectrometry. The pRb-AB and the IXCXE HDAC1 peptide were mixed in 1:3 molar ratio and passed through a S75 analytical Superdex column. A mass corresponding to pRb-AB was detected in the eluent. The same procedure was used for the LXCXE peptide of large T antigen and in this case the masses of both the protein and the peptide were observed (data not shown).

We next used NMR spectroscopy to check the binding. NMR measurements consisted of monitoring changes in chemical shifts and line widths of the backbone amide resonances of 15N-enriched pRb samples upon addition of unlabeled peptides (45, 46). As the assignment of the NMR spectra was not possible up to now (and not intended), NMR resonances influenced by binding to known binding partners (E7 peptide, E2F peptide, T Ag peptide) were identified (only K713 has been assigned using an 15N lysine labeled sample of the pRb-AB K713S mutant) (Supplementary Figure S1A). From the published crystal structure of the complex between the pRb small pocket and the HPV E7 peptide, and from a number of biochemical and biophysical assays, it has been shown that several lysine residues of pRb participate in the LXCXE-pRb interface (18, 19, 37-41). In particular, the crystal structure of the pRb/E7 peptide shows that there are four lysine residues, which are close to and make contact with the E7 peptide backbone, viz. K713, K720, K722 and K765; beside these lysines there are two more lysine residues near to the LXCXE binding site namely, K729 and K740 (18, 19). All these lysine residues are situated in the B domain of pRb (40, 47) (Figure 4 A, B). We have therefore prepared an 15N lysine labeled sample of pRb-AB and carried out titration with this sample (the
The positive control experiment constituted titration of the Lys-pRb-AB with the unlabeled E7 peptide. As can be seen in Figure 5A, five lysines exhibited changes in position and intensities of peaks upon complex formation with the E7 peptide. These induced shifts are proportional to the strength of the intermolecular interaction to a first approximation and therefore these lysine residues form the major E7-binding region of pRb-AB (45, 46, 48). NMR spectra showed that the E7 petide/pRb-AB complex was long-lived and was too crowded to be of practical use in these type of experiments (Supplementary Figure S1B). The 1H-15N HSQC spectrum of 15N lysine labeled pRb-AB exhibited 27 cross peaks corresponding to the total of 27 lysines present in pRb-AB (Figure 5 A, B).

The molecular basis of the LXCXE interaction with pRb-AB - We then addressed the question why the LXCXE sequence from HDAC1 binds more weakly to the pRb-AB compared to the LXCXE sequences from the E7 or T antigen. In order to determine which residues are important for binding to the LXCXE motif, we systematically designed various mutants of the basic sequences of E7 and HDAC1 (Table 1). We introduce here a nomenclature in which the cysteine residue of the LXCXE motif is denoted as the P0 site and residues on the target peptide N-terminal to this are denoted P-1, P-2 etc., and C-terminal P+1, P+2 etc (Table 1).

The first small but clear difference between LXCXE-like sequences of HDAC1 and HPV E7 (or SV40 large T antigen) is the presence of isoleucine at the P-2 position instead of conserved leucine. ITC titrations showed that the E7 peptide with Leu to Ile mutations also binds to the pRb-AB with approximately same affinity (Kd 0.32 ± 0.05, affinity lowered by only 1.68 times) (Table 1). This result shows that this change is not significant for the binding.

Structures of complexes of the [pRb-AB]-[E7 LXCXE peptide] or the [pRb-AB]-[domain of SV40 large T antigen] reveal that the side chain of residues outside the minimal LXCXE sequence - leucine in position P+4 (DLYCYEQLN) in HPV E7 and methionine (NLFCSEEMP) in SV40 large T antigen - also make strong hydrophobic interactions to pRb-AB (18, 19). There is a larger phenylalanine residue in the HDAC1 at this position (RIACEEFS). In order to check whether this substitution significantly affects binding, we used variants DLYCYEQFN from E7 [HPV E7 L(P+4)F] and RIACEEELS of HDAC1 [HDAC1 F(P+4)L], in which the E7 wild type Leu at position P+4 was changed to Phe and the HDAC1 wild type Phe at position P+4 was changed to Leu, respectively. ITC titration showed that the HPV E7 L(P+4)F peptide still binds strongly (Table 1). The HDAC1 F(P+4)L peptide did not show any binding in ITC and NMR titration showed that the HDAC1 F(P+4)L peptide binds weakly to the pRb-AB (Table 1). These results show that the change of Leu to Phe is not a determinant for the binding, although we believe that the residue at this position should be hydrophobic, based on the crystal structures.
For three major interactions of L, C, and E in the minimal LXCXE, the side chains of the conserved leucine and cysteine fit tightly to the hydrophobic groove on pRb-AB, whereas the carboxylate group of glutamic acid of the terminal LXCXE makes two hydrogen bonds with two backbone amide groups of helix-15 of pRb-AB. As mentioned earlier, a lysine patch surrounds the LXCXE binding site groove on the B half of the AB pocket (Figure 4A). Six lysine residues that surround this binding cleft are K713, K720, K722, K729, K740 and K765. We speculated that this highly positive surface on the LXCXE binding cleft would repel any positive residue in the LXCXE sequences or the flanking residues. The first confirmation of this hypothesis came from the observation that the longer peptide from HDAC1 bound better than the short peptide (Table 1). The longer peptide differs from the short one by having a number of extra negatively charged residues in the C terminal segment (Table 1). In viral oncoproteins such as HPV E7 or SV 40 large T antigen, the LXCXE motif is followed by a stretch of negatively charged residues. It has been proposed that these acidic residues may interact with the lysine patch. However, the [pRb-AB]- [domain of SV40 large T antigen] structure showed that this stretch is flexible, with relatively few interactions that were further proposed to be driven by the phosphorylation of pRb (18). The second substantiation of the hypothesis of repulsion via positive flanking residues came from the observation that the peptide from the PAI2 protein did not show any binding to pRb-AB (Table 1). The PAI2 sequence has positively charged residues at the C terminus of the peptide. We also confirmed the lack of interaction between pRb-AB and PAI2 protein or pRb-large pocket (amino acids 379-928) and PAI2 protein using gel filtration chromatography (data not shown).

We designed two single mutant peptides, RLYCYEQLN, D(P-3)R peptide (Asp at P-3 changed to Arg), from HPV E7 and DIACEEEFS, R(P-3)D peptide (Arg at P-3 changed to Asp) from HDAC1 (Table 1). The ITC results showed that the HPV D(P-3)R peptide binds to the pRb-AB with 5.5 fold reduced affinity, and the HDAC1 R(P-3)D peptide also showed better binding compared to the normal peptide. ITC and NMR suggested K[D values of ~10 µM and 8 µM, respectively, the lowest values among all HDAC1 peptides so far. However, complex formation between pRb and the peptide was still in the fast exchange regime (Table 1). These results clearly show that any positive residue in or around the LXCXE sequences reduces the binding affinity to the pRb-AB.

The crystal structure shows that the E7 peptide binds to the pRb-AB in an extended conformation. There are interactions among the side chains of the peptide residues. In the E7 peptide DLYCYEQLN, the side chain aromatic ring of Tyr at P+1 makes a stacking interaction with the side chain aromatic ring of Tyr at P-1, and also makes a long distance hydrogen bond with the side chain of Asp at P+3 position (Figure 4B)(19). Since the residues at the corresponding positions in HDAC1 differ significantly (RL4CCEEFS), we decided to design two peptides, each with two substitutions compared to wild type sequences: RLYCCEQLN [from HDAC1 with substitutions D(P-3)R and Y(P+1)E] from HPV E7 and DIACEEEFS, [from HPV E7 with substitutions R(P-3)D and E(P+1)Y], see Table 1. ITC measurements showed that the HPV E7 D(P-3)R,Y(P+1)E peptide binds to pRb-AB with a 25 fold reduced affinity (K[D = 4.79 ± 0.20 µM) compared to wild type E7 peptide and that the HDAC1 R(P-3)D,E(P+1)Y peptide also binds with relatively high affinity (K[D = 3.27 ± 0.07 µM) (Table 1 and Figure 7 A, B). We also checked these interactions using gel filtration chromatography followed by mass spectrometry, as discussed previously. In case of the pRb-AB/HPV E7 D(P-3)R,Y(P+1)E peptide interaction, a mass corresponding only to pRb-AB was detected, but for the pRb-AB/ HDAC1 R(P-3)D,E(P+1)Y peptide interaction, both protein and peptide masses were detected (data not shown). These results demonstrate clearly the importance of intramolecular interactions between the peptide amino acid side chains and pRb.

Binding of a BRG1 domain and an N-terminal pRb domain to the pRb-AB - The BRG1 protein, which was reported to interact with the pRb-AB, has a LXCXE motif in its sequence (...)EVERLTCCEEEEK(...) (31, 32, 47). There is a LXCXE like motif (...)RIIE LCKEHE...) present in the N terminal domain of pRb, however, no data in the literature could be found about binding of the N-terminal pRb to the pRb-AB small pocket (Figure 1A, B). Both these domains have positive residues in or flanking the LXCXE sequence. In gel filtration under native conditions, we detected no complex formation between the BRG1 LXCXE domain and pRb.
AB or the N-terminal pRb and pRb-AB, as all the protein eluted separately (data not shown). ITC titrations also showed no binding (Table 1). These results supplement the data presented in the previous sections, indicating that any positive charge around the LXCXE sequence are detrimental to the binding.

**Binding of the pRb-AB mutants to LXCXE sequences.** We tested the binding of two pRb-AB mutants with the E7 LXCXE peptide - It has been reported that a pRb-AB mutant C706F is defective in its ability to bind to the LXCXE sequence containing viral proteins (37, 38, 47, 50, 51). Another mutant, K713S, was also shown not to bind LXCXE sequences in one report (37), whereas other reports suggested the opposite (37, 47, 50). Our binding experiments showed that both of these mutants bind tightly to the LXCXE peptide of HPV E7 (Table 1). We have used NMR to check the structural integrity of the mutants (46, 52). The NMR showed that these mutations do not disrupt the structural integrity of the small pocket as no noticeable differences were observed compared to the wild type pRb-AB pocket in the NMR spectra (for K713S mutant see Supplementary figure S1A).

**DISCUSSION**

Most of the studies on the pRb/LXCXE interaction have relied on mutagenesis whereby the LXCXE binding site on the pRb pocket was mutated and tested for binding with possible target proteins in cell lysates coupled with immunoprecipitation and Western blot assays. Our approach is different from these approaches in that we use direct binding methods in vitro. Since our titration experiments use purified proteins, the results are less ambiguous and direct protein-protein interactions can easily be checked. In order to check for any interaction that could be weak, we have carried out our binding study also with NMR spectroscopy. Standard molecular biology methods for protein binding, for example ELISA or RIA, and Biacore fail to detect ligands with weak (i.e. millimolar) affinities. The ability of NMR to detect such ligands has made NMR increasingly important in drug discovery and structural proteomics (45, 46).

Amino acids that form the LXCXE binding site in pRb are highly conserved across pRb homologs of different species (53, 54). Many cellular pRb binding proteins contain LXCXE-like sequences. The simplest model would predict that pRb uses the LXCXE binding site to interact with target proteins, and viruses evolved a LXCXE sequence to mimic this interaction (50, 55). The model for the pRb-HDAC interaction has been tested by making the LXCXE binding site mutants of pRb (37-40). pRb alleles deficient in LXCXE binding retain at least some ability to repress transcription in all the reports, but different conclusions were reached regarding the HDAC interaction. In two reports the authors concluded that the LXCXE mutant pRb retained the ability to bind HDAC1 (40, 41), whereas other reports showed reduced or no binding (37-39). From our data we conclude that although the E7 and HDAC1 LXCXE-like sequences bind to the same site on pRb, binding between the small pocket of pRb and E7 LXCXE peptide is tight whereas the HDAC1 IXCXE peptide binding is weak and the complex formation between the two is transient.

There have also been conflicting reports regarding the interaction of BRG1 with pRb-AB. One model suggests that BRG1 interacts with pRb through the LXCXE motif (31). However, using the LXCXE binding site mutant alleles of pRb, others reported that LXCXE motif may not be required for binding of BRG1 to pRb-AB (38, 39), and moreover Zhang et. al. showed that HDAC1 and BRG1 interact with separate sites of pRb (43). Recently, Kang et al. have shown that the direct physical interaction of BRG1 with the pRb is not required for the BRG1 ability to arrest growth and control flat cell formation, but rather it controls the activity of pRb via regulation of p21CIP/WAF1/SDI, an upstream regulator of pRb (42). Our results showed no binding between pRb-AB and a LXCXE containing BRG1 domain. Moreover no binding was detected between pRb-AB and the LXCXE containing peptide from the PAI2 protein and the N-terminal domain of pRb. We would like to note that these results do not rule out other interactions among the full length or larger domains of these proteins.

We also examined a series of E7 and HDAC1 derived peptides with single or double amino acid substitutions. Whereas substitutions Ile to Leu at the position P-2 and Leu to Phe at P+4 did not influence the binding, any positively charged residue in the peptides had significant effect on binding. In agreement with this trend, the peptide from the PAI2 protein, which has a series of positive residues at the C-terminus of LXCXE, did not show any binding to pRb-AB. An Asp to Arg mutation at P-3 in HPV E7
reduced binding to pRb-AB ~6 fold, a reverse Arg to Asp change at P-3 in HDAC1 showed tighter binding (with the K_D ~8-10 µM), showing that any positive charge in the XLXCXEXXX sequence can be detrimental for the interaction. In fact, a frequently used mutation in biochemical assays Glu(107)Lys at P+1 position in SV40 large T antigen leads to its dissociation from pRb-AB (56). The double mutant, (an Asp to Arg substitution at P-3 coupled with Tyr to Glu at P+1) in HPV E7 reduced the binding ~25 fold, whereas an Arg to Asp substitution at P-3 coupled with Glu to Tyr at P+1 showed binding of 3.27 ± 0.07 µM, the tightest among all the HDAC1 derived peptides, indicating that the Tyr to Glu change is also important for this interaction. These results are in agreement with the crystal structure of pRb-AB/E7, which shows that the E7 amino acids at these positions make contact with each other, which may help keep the LXCXE sequence in a correct, extended configuration. We can thus expect that a LXCXE motif that is a part of a regular secondary structure element, such as an α-helix or β-sheet, would present a conformation that is not optimal for the interaction with pRb.

Our results also showed that single point mutations C706F and K713S in pRb are not able to disrupt the structure of pRb-AB and these mutants were still able to bind HPV E7 LXCXE peptide strongly. We concluded therefore that a single amino acid substitution may not be sufficient for inactivation of the pRb binding to LXCXE sequences.

In conclusion, our data show that the LXCXE-like sequences with only the determinant triplet Leu, Cys and Glu in are not sufficient for tight complex formation with pRb-AB. These residues might provide specificity, but high affinity binding requires other flanking residue interactions. Specifically, a sequence XLXCXEXXX, where X should not be a positively charged amino acid (e.g. Lys or Arg), and X should preferably be a hydrophobic residue, should bind tightly to pRb-AB. Positively charged amino acids do not abolish binding, but weaken it to differing extents such that fast exchange XLXCXEXXX/pRb complexes form. Moreover, it is worth noting that the Leu, Cys and Glu amino acids in the IXCXE motif of HDACs homologues across different species are not conserved, whereas the amino acid residues N-terminal to the IXCXE motif are highly conserved (Figure 8). In fact, the yeast homologue of class I HDACs, Rpd3, does not have any sequence homology in the IXCXE region of the HDAC1. It has been shown that in yeast, the HDAC dependent transcriptional repression by pRb does not require the pRb LXCXE binding cleft; rather, Msilp(RbAp48) was proposed to act as a bridging protein between pRb/Rpd3p. Similarly in some reports RBP1, a LXCXE containing protein, was shown to act as a bridging protein between pRb/HDAC (30, 41). We envisage that class I HDACs (HDAC1, HDAC2 and HDAC3) have some functions common with their deactylase activity, whereas HDAC1 and HDAC2, which have IXCXE motifs, have other functions unique to this interaction. All these observations, combined with our results, suggest that the IXCXE motif in HDAC1 and HDAC2 evolved to serve a function unique to higher organisms. Thus, for example, the HDAC1 RIACEEF/pRb interaction may not be important in some in vivo situations, but this interaction does exist and its weak feature should be taken into consideration when considering models of the pRb/HDAC interaction. It should be mentioned here that a very different outcome would be unlikely if full-length proteins were used in these studies. This is because the primary interaction sites are located in the fragments we have studied.

Multiple functions of pRb (e.g. proliferation, apoptosis, cell differentiation, cellular senescence, control of developmental processes in extra-embryonic tissues, and maintenance of tissue homeostasis - to name a few) require the interaction with many cellular partners directly or indirectly (through multiprotein complexes). These functions can be achieved not only by adding interacting partners to the system, but also by modulating the strength of the interaction by, for example, posttranslational modifications, such as phosphorylation and acetylation, or simply (without any modifications) by the primary amino acid sequence present in binding sequences.

Viral proteins associate with pRb by displacing cellular proteins, such as E2F and HDAC1, showing the greatest viral advantage presumably when the interactions are strong. Interaction between the cellular proteins and pRb is highly regulated, so that the regulatory interactions need to be reversible. For example, the phosphorylated C-terminus of pRb is suggested to interact with the positively charged lysine patch and displace HDAC1. This can only
be achieved if the interaction between HDAC1 and the pRb B domain is transient (57).

Another example of the complexity of the LXCXE/pRb interactions is provided by a recent study of the SUMO modification of pRb (58). This study showed that pRb is SUMOylated at lysine 720 and the level of SUMOylation is controlled by the interaction of pRb with LXCXE-motif proteins. The viral E1A, E7 and the cellular EID-1, E1A-like inhibitor of differentiation proteins, completely abolished SUMO modification of pRb, while HDAC1 showed reduced SUMOylation of pRb, but failed to completely block it. Our data provide an explanation for these observations: the SUMO inhibitory potential of these LXCXE proteins most likely reflects their different binding affinities for pRb.

REFERENCES


ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1. The domains of pRb and BRG1 used in this study. (A) pRb-AB and pRb-N terminal domain. (B) BRG1 LXCXE domain, compared with the full-length protein.

Figure 2. Final purity of protein preparations used in this study. (A) pRb-AB. (B) pRb-N. (C) BRG1 LXCXE containing domain. SDS-Page analysis combined with the mass spectrometry, western blot and NMR spectroscopy showed the protein to be purified close to homogeneity. First lane shows the protein molecular weight marker.

Figure 3. ITC titration curve for various LXCXE peptides with the pRb-AB. (Upper) The raw data of ITC experiment each performed at 20-22°C. (Lower) The integrated heat changes, corrected for the heat of dilution, and fitted curve based on a single site model. (A) pRb-AB titrated with HDAC1 peptide (17mer). (B) pRb-AB titrated with HPV E7 peptide. (C) pRb-AB titrated with Large T antigen peptide.

Figure 4. (A) Surface of the B domain of pRb bound to the LXCXE peptide from HPV E7 (taken from the PDB file 1GUX (32). Blue colour indicates positive charge and red colour indicates negative charge. Lysine residues in the binding site and the residues of the LXCXE E7 peptide are highlighted. (B) Intramolecular interactions among the amino acid side chains. Two tyrosine aromatic rings stack with each other (dashed circle), while the hydroxyl group of tyrosine (P+1) makes a long distance hydrogen bond with the aspartic acid side chain at position (P-3)(dashed line).

Figure 5. (A) The $^{15}$N lysine labeled pRb-AB was titrated with increasing concentrations of the E7 peptide and $^1$H-$^{15}$N HSQC spectra were recorded at each step of ligand addition. Only two steps of titrations are shown: the starting (red, untitrated) and the final step (blue, at an E7 peptide/pRb-AB molar ratio of 1.60). Upon addition of the E7 peptide, five resonances were perturbed (arrows) indicating that these lysines are involved in the binding to pRb-AB, including the resonance peak from Lys713 (at the chemical shifts for $^1$H 7.91 ppm, and for $^{15}$N 123.9 ppm; the assignment of this peak is described in the Supplementary Figure S1A). (B) A fresh sample of the $^{15}$N lysine specifically labeled pRb-AB was titrated with the IXCXE peptide from HDAC1. Only two steps of titrations are shown: red, the starting (untitrated) and blue, the final (at HDAC1 peptide/pRb-AB molar ratio of 3.76). Features of the cross peak (marked box, at 118.75 ppm, $\omega_{1-15N}$; 7.56 ppm, $\omega_{2-1H}$ chemical shift values) in (A) and (B) are described in detail in Figure 6.

Figure 6. The cross peak (at 118.75 ppm $\omega_{1-15N}$; 7.56 ppm $\omega_{2-1H}$, Figure 5) was chosen to demonstrate the difference between tight (A) (E7 peptide/pRb-AB) and weak (B) (HDAC1 peptide/pRb-AB) binding. Four steps of titrations are shown here at the specified peptide/pRb-AB molar ratio.

Figure 7. ITC titration curve for the variant LXCXE peptides and pRb-AB. (A) pRb-AB titrated with HPV E7 D(P-3)R,Y(P+1)E peptide. (B) pRb-AB titrated with HDAC1 R(P-3)D,E(P+1)Y peptide.

Figure 8. Sequence alignment of L/IXCXE –motif of various HDACs from different organisms. Amino acids in red indicate the position of I, C and E or corresponding residues. Conserved residues N-terminus to the IXCXE motif are highlighted by a box.
TABLE 1

**ITC and NMR titration results data for small pocket of pRb and various peptides/protein domains**

K_D values were determined by NMR in case of fast exchange or weak binders. First column shows the various peptides or protein domains used in this study and second column shows the LXCXE sequence in the peptides or protein. The Leu (Ile), Cys, and Glu residues in the LXCXE motif are highlighted with red color and residues in blue were mutated.

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<th>Interaction of pRb-AB with:</th>
<th>Peptide sequence</th>
<th>ITC K_D (µM)</th>
<th>NMR K_D (µM)</th>
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<td>Position P</td>
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<tr>
<td>HPV E7 peptide</td>
<td>DLNCYEQLN</td>
<td>0.19 ± 0.07 (0.11) a</td>
<td>tight binding</td>
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<td>HPV E7 L(P-2)I peptide</td>
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<td>HPV E7 L(P+4)F peptide</td>
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<td>HPV E7 D(P-3)R peptide</td>
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<td>RLYCEQEQNL</td>
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<td>T antigen peptide</td>
<td>NLFCSEEMD</td>
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<td>HDAC1 peptide (17mer)</td>
<td>DKRIACEEFFSDSEEEG</td>
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<td>40.0 c</td>
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<td>SSDKRIACEEFFSDSEEEGE</td>
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<td>HDAC1 short peptide</td>
<td>RIACEEEFS</td>
<td>20.0 ± 4.0</td>
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<td>HDAC1 R(P-3)D peptide</td>
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<td>LDYHFGLEEGRGIRDLFD</td>
<td>1.24 ± 0.20 (0.49)</td>
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<td>PAI2 peptide</td>
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<td>pRb-N domain</td>
<td>...IEVLCKHEH... d</td>
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<tr>
<td>BRG1 LXCXE domain...</td>
<td>...ERLTCEEEEK... d</td>
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<td>pRb-AB (K713A) e + E7 peptide</td>
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* The data from Lee et al., (1998) are in parentheses.

b-, not determined

c Specific values refer to the complexes in fast exchange between binding components, the estimated error is 10%.

d Protein domains having LXCXE sequences rather than peptides are used in these experiments.

e pRb-AB mutants are used in these experiments.
Figure 1

A

\[ \begin{array}{c}
pRb \\
pRb-AB \\
pRb-N \\
\end{array} \]

B

\[ \begin{array}{c}
BRG1 \\
BRG1 LXCXE containing domain \\
\end{array} \]
Figure 6

A

Molar ratio of E7 peptide/pRb-AB

0

0.16

0.64

1.60

ω₂-¹H (ppm)

7.56

7.48

B

Molar ratio of HDAC1 peptide/pRb-AB

0

0.51

1.50

3.76

ω₂-¹H (ppm)

7.56

7.48
## Figure 8

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<td>I</td>
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Supplementary information for:

**Molecular determinants for the complex formation between the retinoblastoma protein and LXCXE sequences**

Mahavir Singh, Marcin Krajewski, Aleksandra Mikolajka, and Tad A Holak

**Figure S1.** (A) Assignment of Lys713. Overlay of $^{15}$N HSQC spectra pRb-AB (red) and pRb-AB (K713S) mutant (blue), all cross peaks except one are present in both spectra. The missing resonance peak corresponds to lysine 713. This experiment also tells us that the overall integrity of the mutant protein remains intact. (B) The perdeuterated pRb-AB. Overlay of $^{15}$N HSQC spectra of pRb-AB (red) with the pRb-AB/E7 peptide (blue).
Molecular determinants for the complex formation between the retinoblastoma protein and LXCXE sequences
Mahavir Singh, Marcin Krajewski, Aleksandra Mikolajka and Tad A. Holak

J. Biol. Chem. published online August 23, 2005

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