RBP1 Family Proteins Exhibit SUMOylation-Dependent Transcriptional Repression and Induce Cell Growth Inhibition Reminiscent of Senescence†

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The retinoblastoma binding protein 1 (RBP1) appears to be an important factor in the repression of E2F-dependent transcription by the retinoblastoma protein (pRB) family. The recent identification of the breast carcinoma associated antigen (BCAA) as an RBP1-like protein led us to investigate its biological properties and compare them to RBP1. Like RBP1, BCAA contains a carboxy-terminal R2 domain that elicits histone deacetylase (HDAC)-dependent transcriptional repression via interactions with the SAP30 subunit of the Sin3/HDAC complex. Each RBP1 family member also contains two HDAC-independent repression activities within a region termed R1, which can be subdivided into a SUMOylated moiety (R1 σ) and a predicted α -helical region (R1 α). R1 α is embedded within the ARID region and represses basal transcription only, whereas R1 σ represses both basal and activated transcription and depends on SUMOylation. Overexpression of either RBP1 or BCAA, but not the truncated BCAA^{MCF-7} isoform that is overexpressed in breast cancer cells, caused a profound inhibition of cell proliferation and induced expression of a senescence marker. In each case the presence of both R1 and R2 was necessary for suppression of cell growth, suggesting that both R1 and R2 transcriptional repression activities play a role in RBP1 family protein-mediated regulation of cellular proliferation.

The retinoblastoma gene (*Rb*) was the first tumor suppressor gene to be identified (12, 16), and its product pRB plays a major role in the regulation of the cell cycle. pRB appears to function principally as a transcriptional repressor that blocks E2F-dependent transcription, thus regulating entry into the S phase of the cell cycle. This cellular proliferation pathway is deregulated in a vast majority of cancers, if not all. pRB functions primarily via interactions with key components of the cell cycle machinery, many of which are mediated through a region of pRB termed the pocket. This region of pRB is the target of interaction for several viral oncoproteins and a range of cellular polypeptides involved in cell cycle progression, including E2Fs, involved in cell cycle progression, and its coding sequence is frequently mutated in cancer cells. Interaction with viral oncoproteins such as human papillomavirus E7 and adenovirus E1A proteins, as well as pocket mutations, are known to disrupt the interaction between pRB and E2Fs (5, 13) and lead to uncontrolled cell cycle progression. pRB and another pocket protein family member, p130, have recently been shown to be involved in the establishment and maintenance of senescence (30), which is a fail-safe mechanism that prevents indefinite cell division and therefore represents a barrier that must be overcome during tumorigenesis to achieve cellular immortality.

RBP1, which was originally identified as a cellular protein that interacts with the pocket of pRB (14), was shown by our laboratory to be implicated in the repression of E2F-dependent transcription by pRB and family members p107 and p130 and to be responsible for at least 50% of the histone deacetylase (HDAC) activity associated with pRB (26, 27). A model, illustrated in Fig. 1A, was derived from these studies. In G₁, RBP1 would function as a bridging protein to recruit the mSin3A/HDAC histone-modifying complex (25) to E2F-dependent promoters through a direct interaction between the R2 region of RBP1 and the Sin3-associated peptide (30-kDa) SAP30. In addition, RBP1 also represses in an HDAC-independent manner through its R1 region, by a hitherto-unknown mechanism, a finding that correlates with previous reports suggesting that pRB represses transcription via both HDAC-dependent and -independent mechanisms (28, 35, 36). The R1 region of RBP1 is composed of a region spanning residues 241 to 452 that overlaps with an A/T-rich interacting domain (ARID; residues 314 to 409, see Fig. 1B), a sequence that in some instances has been found to mediate protein-DNA interactions (32).

In recent studies, other groups have also reported the presence of RBP1 in additional histone-modifying complexes. RBP1 was found to interact directly with the breast metastasis suppressor 1 (BRMS1) in mSin3/HDAC-containing complexes (29). RBP1 was also identified in other mSin3/HDAC complexes also containing BRMS1, along with BRMS1-homologue p40 and the putative tumor suppressor p33^{ING1b} (31), as well as with three novel mSin3 core complex proteins: SAP180, SAP130, and mSDS3 (15). Although we have shown that RBP1, through its interaction with pRB family members, pre-

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FIG. 1. (A) Model of E2F-dependent transcription repression regulated by RBP1. The RBP1 corepressor recruits the mSin3/HDAC histone deacetylase complex to E2F-dependent promoters through a direct interaction between the R2 repression domain and the Sin3-associated peptide SAP30. A second domain (R1) within RBP1 represses by an HDAC-independent mechanism. (B) Comparison of RBP1 and BCAA functional structure. The relevant features and amino acid residues are identified in the figure. (C) Migration properties on SDS-PAGE of RBP1, BCAA, and BCAA^{MCF-7} expressed in H1299 transfected cells.

sumably targets an mSin3/HDAC complex to E2F-dependent promoters, it is still unclear what role RBP1 plays in these additional complexes.

An RBP1-like protein, termed BCAA (also reported as RBP1-like 1, ARID4B, and SAP180), was recently identified as an overexpressed cytoplasmic epitope in some breast cancer patients (3, 4, 6) and as a nuclear protein that is a component of the mSin3A/HDAC core complex (15). BCAA shares ex-

tensive similarity with RBP1 both in amino acid composition (Fig. 1B) and, as shown here, in function. BCAA is devoid of an LXCXE pRB pocket-binding motif, suggesting that it might have a role unrelated to pocket proteins, and it could possibly even act in a dominant-negative fashion toward pRB-related RBP1 functions. As depicted in Fig. 1B, BCAA and RBP1 are 34% identical in overall amino acid composition, and their putative Tudor, ARID, and Chromo domains are 80, 83, and 75% identical, respectively. BCAA and RBP1 also share similarity within the R1 (58% identity) and R2 (44%) regions previously characterized in RBP1 as repression domains. In the present study, we characterized two different isoforms of BCAA. Both are full-length cDNAs of identical size, but one encodes a 200-kDa protein (see Fig. 1C, lane 3), whereas the

other one, isolated from the breast carcinoma cell line MCF-7, encodes an aberrantly truncated peptide of 100 kDa (see Fig. 1C, lane 4) also detected endogenously as a 100-kDa cytoplasmic peptide (6), which lacks the carboxy-terminal half of the protein and therefore the R2 region as well (see Results).

Posttranslational modification by the small ubiquitin-like modifier SUMO has recently been shown to be linked to negative regulation of a growing number of transcription factors. Conjugation of SUMO to its target occurs on a lysine residue found generally within the consensus sequence Ψ -Lys-X-Glu (Ψ KXE) (where " Ψ " is a large hydrophobic amino acid and "X" is any residue). In addition to modulating transcriptional activity, SUMOylation is involved in functionally altering protein-protein interactions, cellular localization and enzymatic activity (see references 18 and 19 for reviews).

The extensive homology of BCAA with RBP1, as well as its lack of an LXCXE pRB-binding motif, led us to compare its biological functions with those of RBP1. In the present study, we examine the ability of wild-type and mutant forms of BCAA and RBP1 to induce growth arrest and expression of a senescence-associated marker and, in addition, we characterized the mechanism of action of the R1 and R2 repression domains of both proteins.

MATERIALS AND METHODS

Cell lines and transfections. CHO-K1 chinese hamster ovary (ATCC CCL-61) cells were maintained in alpha minimum essential medium (Invitrogen) supplemented with 10% fetal bovine serum (Cansera), 100 U of penicillin G/ml, 100 μ g of streptomycin/ml, and 2 mM glutamine (Invitrogen). C33-A human carcinoma (ATCC HTB-31) and H1299 non-small cell lung cancer (ATCC CRL-5803) cells were maintained in Dulbecco modified Eagle medium (Invitrogen) supplemented with 10% fetal bovine serum (Cansera), 100 U of penicillin G/ml, 100 μ g of streptomycin/ml, and 2 mM glutamine (Invitrogen). DNA transfections were conducted by using Lipofectamine (Invitrogen) or DMR1E-C (Invitrogen) according to the manufacturers' recommendations.

Plasmids. Full-length RBP1 cDNA was previously described (27). Full-length BCAA cDNA expressing truncated forms of the protein were obtained by reverse transcription, using SuperScriptII (Invitrogen), of total RNA isolated from MCF-7 breast cancer cell line (ATCC HTB-22) with TRIzol (Invitrogen), followed by PCR amplification of BCAA transcripts. The cDNA expressing the full-length BCAA protein was obtained from Donald E. Ayer (15). The truncations used in transcriptional repression assays were constructed by subcloning the various regions into pSG424 vector using the appropriate restriction endonucleases. pGL3 simian virus 40 (SV40) luc (Promega) expressing the firefly luciferase reporter gene under the control of the SV40 promoter was modified by insertion of five Gal4 DNA binding consensus sites (G5) from pG5luc (Promega) to generate pGL3 G5 SV40 luc. pG5 TK luc (herpes simplex virus minimal thymidine kinase [HSV TK] promoter) has been described previously (27). pG5 MLP luc (adenovirus major late promoter) was obtained from Promega (pG5luc). The reporter pUAS^TTATAAluc has been described previously (39). The pEBB mammalian vector driving the expression of the SUMO protease SuPr-1 and the catalytic mutant SuPr-1 (C466S) were described elsewhere (1). pcDNA3 HA-SUMO-1, -2 and -3 were provided by Ronald T. Hay (9, 10, 37). pcDNA3 HA-SUMO-4 was constructed from pCMV myc-SUMO-4, which was kindly provided by David Owerbach (2). SAP30 was cloned by reverse transcription-PCR from mouse total RNA and inserted in frame with the amino-terminal His₆ tag of pET33b(+) (Novagen). SAP30 was also inserted in frame with the 3 $\times$ Flag tag of pCMV-3Tag-1A (Stratagene).

Luciferase assays. Transcriptional repression assays were conducted by cotransfection of plasmids expressing firefly luciferase, *Renilla* luciferase to allow normalization of transfection efficiency, and either RBP1- or BCAA-expressing vectors. The cells were rinsed once with phosphate-buffered saline (PBS) then lysed with 500 μ L 1 $\times$ passive lysis buffer (Promega). Then, 20 μ L of lysate was used for measurement of luciferase activity by dual-luciferase assay (Promega) on a Lumat LB 9507 (Berthold Technologies) luminometer. The activity was normalized for transfection efficiency against the *Renilla* luciferase activity. To

assess HDAC-dependent repression activity, the cells were treated with 330 nM trichostatin A for 24 h prior to the luciferase assay measurements.

Protein purification. BL21(DE3) *Escherichia coli* (Stratagene) were transformed with plasmid expressing GST alone or GST-BCAA/RBP1 R1/R2. Cell pellets were resuspended in GST lysis buffer (HEPES-KOH [pH 7.4], 200 mM KCl) and subjected to sonication. The lysates were cleared by centrifugation and incubated with glutathione-Sepharose 4B (Pharmacia) overnight and then washed extensively in glutathione *S*-transferase (GST) lysis buffer. The GST-tagged recombinant peptides were then eluted thrice with reduced glutathione and dialyzed using an Amicon Ultra-4 10,000 MWCO (Millipore) against PBS supplemented with protease inhibitors.

His-tagged SAP30 was purified by metal ion affinity chromatography. BL21(DE3) cells were transformed with pET33b(+) His₆-SAP30 plasmids, and the expression was induced with 0.2 mM IPTG (isopropyl- β -D-thiogalactopyranoside). The cells were collected by centrifugation and lysed using Bug Buster (Novagen). The cleared lysates were passed through Ni²⁺ columns (900 cartridges [Novagen]) for purification of His₆-tagged recombinant proteins. Eluates were dialyzed using Amicon Ultra-4 10,000 MWCO (Millipore) against PBS supplemented with protease inhibitors. The purification procedure was monitored by denaturing polyacrylamide gel electrophoresis (SDS-PAGE), and the recombinant proteins were visualized by Coomassie staining or by anti-His (Pharmacia) immunoblotting.

Immunoprecipitation. CHO-K1 cells were seeded at a density of 1.5×10^6 cells per 60-mm plate. One hour prior to transfection, the cells were infected with vaccinia virus expressing T7 RNA polymerase. The cells were then transfected with 1.5 μ g of pcDNA3.1 Gal4-R1 and 1.5 μ g of pcDNA3 HA-SUMO-1, -2, -3 or -4 plasmid DNA by using DMR1E-C (Invitrogen). After 24 h, the adherent and floating cells were collected and lysed in radioimmunoprecipitation assay lysis buffer supplemented with protease inhibitors (1 mM aprotinin, 1 mM leupeptin, 1 mM pepstatin A, 1 mM phenylmethylsulfonyl fluoride, and 25 mM *N*-ethylmaleimide). The protein concentration was determined by the Bradford assay. Whole-cell protein extracts (150 μ g) were incubated with 0.5 μ g of RK5C1 antibody for 30 to 45 min at 4°C with constant mixing. A total of 20 μ L of a 1:1 Fast-Flow protein A-agarose (Upstate) slurry was added, and the immunoprecipitates were further incubated overnight at 4°C. The samples were washed 4 to 6 times with 1 ml of radioimmunoprecipitation assay buffer. The samples were separated by SDS-PAGE (10% polyacrylamide), and the proteins were transferred to polyvinylidene difluoride (PVDF) membrane. The membranes were probed by Western blotting with mouse monoclonal HA.11 antibody (1:1,000; Sigma). The membranes were then stripped with NaOH (0.2 M) and reprobed with anti-Gal4 RK5C1 antibody (1:500; Santa Cruz). The Flag immunoprecipitations were conducted similarly. Briefly, H1299 were seeded at a cell density of 5×10^5 cells per 60-mm plate the day preceding the transfection. The cells were transfected with 1.5 μ g of pCMV-Flag-SAP30 and 2.5 μ g of pcDNA3.1 HA-BCAA or HA-RBP1 expression plasmids. The cells were harvested at 48 h posttransfection and lysed in nuclear lysis buffer (27) supplemented with protease inhibitors. Whole-cell protein extracts (250 μ g) were immunoprecipitated using Flag M2 agarose (Sigma) in lysis buffer (100 mM KCl) and washed four times in 1 ml of lysis buffer (100 mM KCl), and the samples were resolved by SDS-PAGE (6% polyacrylamide), transferred to a PVDF membrane, and immunoblotted with antihemagglutinin (anti-HA) antibody.

Growth assay. C33-A cells were transfected with the appropriate plasmids. At 24 h posttransfection the cells were subjected to selection by using either 200 μ g of hygromycin B (Invitrogen)/ml or 1,000 μ g of G418 (Invitrogen)/ml for a period of 2 weeks. Finally, the cells were either treated with trypsin and counted or stained with crystal violet (0.5% in methanol).

SA- β -Gal assay. Senescence-associated β -galactosidase (SA- β -Gal) assays were performed essentially as initially described previously(11).

RESULTS

RBP1 and BCAA are potent inhibitors of cellular proliferation. Although the growth-inhibitory properties of RBP1 have been previously explored, the role of the LXCXE pRB-binding motif in RBP1 and the effects of BCAA on cell growth are not known. To address these questions, we used colony formation assays in which DNA of an empty vector (negative control) or of plasmids expressing pRB, RBP1, or BCAA was transfected into C33-A cells. Transfected cells were then seeded onto plates and colonies were allowed to form over a period of 2

FIG. 2. (A) Effect of overexpression of BCAA and RBP1 on cellular proliferation. (A) Colony formation assay with pRB and RBP1. Plasmid DNAs expressing the neomycin resistance gene and the HA tag only or the cDNA of pRB, HA-RBP1, or HA-RBP1 $\Delta LxCxE$ mutant were transfected in C33-A cells. Colonies were allowed to form for 2 weeks in the presence of G418 and then were stained with crystal violet. (B) Expression levels of HA-tagged RBP1 proteins in C33-A by Western blotting with anti-HA antibody (representative of cells in panel A). (C) Cell growth with BCAA and RBP1. Plasmid DNAs expressing the hygromycin B resistance gene and the HA tag only or the cDNA of BCAA, RBP1, or $\Delta LxCxE$ mutant were transfected in C33-A cells. Cells were selected by using hygromycin B for 2 weeks and collected by trypsinization and counted. (D) Expression levels of HA-tagged RBP1 and BCAA proteins by Western blotting with anti-HA antibody (representative of cells in Fig. 2C). These experiments were conducted at least three times in duplicate, and the error bars represent the standard deviations.

weeks in the presence G418. Cells were then either stained with crystal violet (Fig. 2A) or collected by trypsinization and counted to examine the effects of overexpression of BCAA and RBP1 on cell growth (Fig. 2C). Figure 2B shows that RBP1 and RBP1 $\Delta LxCxE$ (deletion mutant that lacks the LXCXE pocket-binding motif) were expressed at similar levels in this experiment. Overexpression of RBP1 and the LXCXE RBP1 mutant appeared to reduce colony formation as efficiently as overexpression of pRB (Fig. 2A). Figure 2C demonstrated this effect more quantitatively. The number of cells present using the empty vector negative control was arbitrarily set at 100%. Both RBP1 wild-type and the RBP1 $\Delta LxCxE$ mutant proteins had potent growth-inhibitory activity (64 and 60% inhibition,

respectively). Interestingly, overexpression of BCAA had a similar growth inhibitory effect (a 76% reduction in cell count). Figure 2D shows that equivalent expression levels of these proteins occurred in this experiment. Thus, both BCAA and RBP1 proteins appear to play a role in cellular growth inhibition, and this phenotype is independent of the presence of the LXCXE pocket-binding motif (see more in Discussion).

Since BCAA was first identified as an overexpressed epitope in breast cancers, it was postulated that it could have transforming properties. BCAA was therefore tested in transformation assays in NIH 3T3 mouse embryonic fibroblasts. NIH 3T3 cells were transduced with retroviruses expressing adenovirus E1A 12S (243-residue) protein, oncogenic Ras (V12), RBP1,

BCAA, BCAA^{MCF-7}, or empty vector. Two weeks after initial infection, the culture plates were examined and none of the cells, with the exception of Ras (V12) and E1A 12S to a lesser extent, exhibited a growth pattern characterized by multilayered cell growth or the formation of colonies as a result of transformation and loss of contact inhibition (data not shown). The growth of these cells was also tested for growth factor requirement. As expected, only Ras (V12)-expressing cells were able to grow in medium supplemented with only 0.5% serum instead of 10% (data not shown). Thus, BCAA^{MCF-7}, although isolated from a breast carcinoma cell line, does not appear to be actively involved in cellular transformation.

Growth-inhibitory functions of RBP1 and BCAA are related to induced senescence. Recent work has demonstrated a role for pRB and p130 in the establishment and maintenance of oncogenic Ras stress-induced senescence (30). We therefore postulated that RBP1 through its interaction with the pocket proteins, as well as with the Sin3/HDAC complex, might play a role in the senescence function attributed to pRB and p130. It was also possible that BCAA^{MCF-7}, while not inducing transformation, could be involved in immortalization. We used H1299 cells (p53^{-/-}) for studies on the senescence phenotype since these cells had been shown to enter into senescence in response to overexpression of tumor suppressors (40). Overexpression of the p53-like protein p73- α had previously been shown to induce a senescence phenotype (22) and was used as a positive control in this experiment. Overexpression of the p14^{ARF} protein, which prevents p53 degradation by binding to MDM2, had also been shown to induce a senescence phenotype and was used as a negative control since H1299 are deficient for p53. Figure 3A shows that, in this experimental context, BCAA and RBP1 were both capable of inducing a significant increase (~10-fold) in activity of the senescence specific biomarker SA- β -Gal (11) compared to cells transfected with empty vector or a p14^{ARF} expression vector. Moreover, BCAA^{MCF-7} was unable to induce SA- β -Gal activity, although it was expressed at level similar to that of BCAA and RBP1 (Fig. 3B). This truncated peptide of BCAA, which migrates as a protein of about 100 kDa instead of 200 kDa (see Fig. 3B), lacks the carboxy-terminal half of the protein and therefore is devoid of the potential R2 HDAC-dependent repression domain identified previously in RBP1 (see below). These results suggested that the overexpression of BCAA isoforms in cancer cells might help to bypass senescence, thus promoting immortalization. Further work is needed to resolve this hypothesis.

RBP1 and BCAA have comparable transcription repression properties. To assess the transcriptional repression properties of BCAA and RBP1, we used the well-characterized Gal4 system. Expression of the firefly luciferase gene reporter was under the control of a viral promoter (HSV TK) with five upstream regulatory elements for specific recognition by the DNA-binding domain (DBD) of Gal4, which was fused to the protein of interest. Figure 4A shows, as previously demonstrated (27), that RBP1 has little effect on luciferase reporter expression when fused only to an HA tag, whereas it significantly repressed luciferase expression when fused to the Gal4 DBD. BCAA also displayed similar effects, indicating that, like RBP1, it possesses transcriptional repression activity. Figure 4B shows that in both cases, such activity depended on target-

FIG. 3. Overexpression of BCAA or RBP1 induces a senescence-associated β -galactosidase activity phenotype. (A) H1299 cells were transfected with empty plasmid, or plasmid DNAs expressing p14^{ARF}, p73- α , RBP1, BCAA, or BCAA isoform isolated from the MCF-7 cell line. The transfected cells were grown for 10 days in the presence of G418, stained for SA- β -Gal, and positively stained cells counted. (B) Immunoblotting with anti-HA antibodies showing the representative protein levels of HA-RBP1, HA-BCAA, and HA-BCAA^{MCF-7}.

ing to the promoter by the Gal4 DBD, since repression did not occur at a promoter that lacked the Gal4 DNA consensus sites.

According to the alignment of protein sequences of BCAA and RBP1, represented in Fig. 1B, considerable homology was detected in the regions of BCAA corresponding to the R1 and R2 repression domains of RBP1 (26). To determine whether these regions are also responsible for the repression activity of BCAA, a series of constructs that express the R1 and R2 regions of each of these proteins was prepared. Figure 5 shows that the adenovirus E1B-55K protein, a known transcriptional repressor, considerably reduced luciferase expression, as found previously (38, 41). As a negative control, a form of RBP1

FIG. 4. Transcriptional repression by BCAA and RBP1. (A) Repression assay with a reporter containing Gal4 binding sites. CHO-K1 cells were seeded at a density of 2×10^5 per 35-mm plate and transfected the next day with 0.25 μ g of pG5 HSV TK luc, 0.0025 μ g of phRL Rous sarcoma virus, and 0.25 μ g of pcDNA3 cytomegalovirus Gal4 or HA using 2.5 μ l of Lipofectamine (Invitrogen). After 30 h, the cells were lysed and luciferase activity measured as described in Materials and Methods. (B) Repression assay with a reporter lacking Gal4 binding sites. Studies were performed as in Fig. 4A except that the reporters used were pGL3 SV40 luc (no Gal4 DNA binding consensus site [G0]) and pGL3 G5 SV40 luc (5 Gal4 DNA binding consensus sites [G5]) instead of pG5 HSV TK luc. These experiments were conducted three times in duplicate, and the error bars represent the standard deviations. The diagrams show the reporter constructs.

lacking both the R1 and the R2 repression domains was used, and it exhibited only very modest repression activity. Full-length RBP1 and the individual R1 and R2 regions of RBP1 exhibited significant repression activity (43, 40, and 15% lucif-

FIG. 5. Characterization of the repression domains in BCAA and RBP1. BCAA and RBP1 have two major repression domains with comparable activity. Experiments were essentially conducted as in Fig. 4A with the pG5 HSV TK luc reporter. Plasmids expressing Gal4DBD alone; Gal4-E1B-55K; full-length Gal4-RBP1; Gal4-BCAA, Gal4-dl R1/R2, Gal4-R1, or Gal4-R2 from BCAA and RBP1; or Gal4-BCAA^{MCF-7} were used.

erase activity, respectively). BCAA and its corresponding R1 and R2 regions also displayed repression activity at levels similar to those seen with RBP1 (35, 47, and 16% luciferase activity, respectively). The BCAA^{MCF-7} isoform displayed levels of repression activity (47%) comparable to the R1 region alone. Thus, BCAA appears to have transcriptional repression properties comparable to RBP1.

The R2 region of BCAA represses in an HDAC-dependent manner and physically interacts with the amino-terminal half of Sin3-associated peptide 30-kDa SAP30. Previous studies had shown that, whereas the RBP1 R2 repression activity is dependent on the association with the mSin3A/HDAC complex, the activity of R1 is independent of HDACs (25, 26). To examine this issue with BCAA, the repression activities of the corresponding R1 and R2 domains of BCAA and RBP1 were examined in the presence or absence of trichostatin A (TSA), a known inhibitor of HDAC activity. Figure 6A shows that, whereas repression of the adenovirus MLP by the R2 regions of both BCAA and RBP1 was considerably relieved by TSA, no effect was observed on R1 repression activity. Furthermore, the repression activity of BCAA^{MCF-7} remained unchanged by the addition of TSA, strengthening the idea that it lacks a functional R2 domain. These results indicated that BCAA and RBP1 are very similar, each containing both HDAC-dependent (R2) and HDAC-independent (R1) repression functions.

Although we have previously shown that RBP1 R2 HDAC-dependent transcriptional repression activity is mediated via an association with the Sin3A/HDAC complex through a direct interaction with SAP30, it is uncertain how BCAA does so. We therefore purified His-tagged SAP30 by metal ion affinity chromatography and GST-tagged R2 regions of BCAA and RBP1

FIG. 6. (A) Studies with the HDAC inhibitor TSA. Experiments were conducted as in Fig. 4A except that the pG5 major late promoter (MLP) luc reporter was used, and the cells were treated with 330 nM TSA in ethanol or ethanol only for 24 h. In addition to the reporter, plasmids expressing Gal4-tagged R1 and R2 from BCAA or RBP1, BCAA^{MCF-7}, or Gal4 alone were transfected. The data have been presented as a fold relief of repression in the presence of TSA. These experiments were conducted at least five times in duplicate, and the error bars represent the standard deviations. The insert shows the reporter construct used. (B) R2 interacts directly with SAP30. A total of 5 μ g of purified GST- and His-tagged recombinant proteins were incubated for 2 h in binding buffer (PBS containing 0.1% NP-40 supplemented with protease inhibitors) and then washed six times in binding buffer. The proteins bound to the GST-tagged proteins were resolved by SDS-PAGE, transferred to PVDF membranes, and analyzed by anti-His immunoblotting. (C) BCAA^{MCF-7} does not associate with SAP30. H1299 cells were cotransfected with empty pCMV-Flag plasmid or pCMV-Flag-SAP30 and pcDNA3.1 HA-BCAA, HA-BCAA^{MCF-7}, or HA-RBP1. Whole-cell protein extracts (250 μ g) were immunoprecipitated with Flag M2 antibody, and the immunoprecipitates were analyzed by immunoblotting with the HA.11 antibody. Lanes 1, 4, and 7 were loaded with 25 μ g of whole-cell protein extracts (1/10 input).

using glutathione-Sepharose. GST alone and GST-R1 were used as negative controls, and GST-HDAC1 was used as a positive control (25, 42). In GST-pulldown experiments (Fig. 6B), the R1 regions of BCAA and RBP1 were not able to associate with SAP30, whereas the R2 region of BCAA and RBP1 physically interacted with SAP30. Furthermore, the amino-terminal half (amino acids 1 to 120) of SAP30 was necessary and sufficient for this interaction; however, the carboxy-

A)

B)

FIG. 7. Identification of regions within R1 of RBP1 and BCAA that repress activated and/or basal transcription. (A) Repression activities of the amino- and carboxy-terminal portions of R1 of BCAA and RBP1. Repression assays were conducted as in Fig. 4A in H1299 cells expressing full-length R1, the amino-terminal part of R1 containing a predicted α -helical region (R1dSigma), and the carboxy-terminal region containing putative SUMOylation sites (R1 Sigma), using a reporter construct pUAS⁵TATAAluc containing only a TATAA box (basal transcription [■]) or the pG5TKluc (activated transcription [□]). (B) Fine mapping of the R1 repression activities. A series of amino- and carboxy-terminal deletions in BCAA Gal4-R1 was generated and tested in repression assays as conducted previously except that the TATAA box-only reporter pUAS⁵TATAAluc (illustrated in the inset) was used. The nature of these BCAA regions, including the restriction enzymes sites used to generate them and the amino acids involved, are illustrated in the figure, and the residual luciferase activity (%) associated with each is indicated. Similar studies yielding comparable results have also been done with RBP1 (data not shown). (C) Western blotting analysis with anti-HA antibodies showing the representative protein levels of the truncation mutants used in Fig. 7B. The experiments in panels A and B were conducted at least five times in duplicate, and the error bars represent the standard deviations.

terminal half (amino acids 121 to 220) did not appear to associate with R2. These results confirmed that, as with RBP1, BCAA associates directly with SAP30 in vitro.

To confirm the binding of SAP30 to both BCAA and RBP1 and, in addition, to determine whether BCAA^{MCF-7}, as predicted, is incapable of such an interaction, we immunoprecipitated Flag-tagged SAP30 in the presence of HA-tagged BCAA, RBP1, or BCAA^{MCF-7}. H1299 cells were cotransfected with plasmids expressing either Flag alone or Flag-SAP30 with BCAA, RBP1, or BCAA^{MCF-7}. Figure 6C shows that Flag alone was unable to coimmunoprecipitate either BCAA or RBP1. In contrast, Flag-SAP30 coimmunoprecipitated with both BCAA and RBP1; however, BCAA^{MCF-7} was undetectable in SAP30 immunoprecipitates. These results confirmed that the R2 region associates with SAP30 and that BCAA^{MCF-7}, lacking the R2 region, is unable to form such a protein complex.

The R1 domain represses both basal and activated transcription. Although previous studies characterized the mechanism of action of the R2 HDAC-dependent repression activity of RBP1, little is known about R1 and its function. We noted that the R1 region of both RBP1 and BCAA was composed of a predicted α -helical region within the ARID sequence and a carboxy-terminal sequence that contained two (BCAA) or three (RBP1) predicted SUMOylation sites (see Fig. 8A). We therefore assessed the transcriptional repression activities of the intact R1 regions, as well as of deletion mutants of R1 that expressed only the predicted α -helical region (R1 α) or the region containing the predicted SUMOylation sites (R1 σ) fused to Gal4. All polypeptides were expressed at

similar high levels (data not shown). Transcriptional repression activity was measured either using pG5TKluc to observe effects on activated transcription or using a reporter construct in which the expression of the luciferase gene was solely under the control of a TATAA box (pUAS⁵TATAAluc) to measure repression of basal transcription. Figure 7A shows that, whereas the removal of the carboxy-terminal R1 σ portion of R1 in both RBP1 and BCAA did not affect repression of basal transcription (R1 versus R1d σ), repression of activated transcription was significantly reduced. Furthermore, when constructs expressing only the carboxy-terminal R1 σ portion were tested, this region alone was found to repress both activated and basal transcription. These results suggested that the R1 transcriptional repression domain was actually composed of two activities: one associated with R1 α within the ARID that repressed only basal transcription and a second associated with R1 σ within the carboxy-terminal region that repressed both basal and activated transcription.

The R1 α activity was further dissected by generating a series of truncation mutants. Figure 7C shows that all constructs were expressed at equal levels. Figure 7B shows that after removal of the carboxy-terminal region of BCAA R1 domain, the remaining portion of R1 (Fig. 7B SpeI/Scal construct) was capable of repressing basal transcription (21% luciferase activity) driven solely by a TATAA box. Analysis of the series of constructs delineated a minimal region (residues 316 to 355 in BCAA; R1 α) with significant repression activity (33% luciferase activity). Based on its amino acid sequence, this region was predicted to contain a cyclin recognition motif embedded in a helix-turn-helix secondary structure. Similar repression

FIG. 8. Analysis of the role of SUMOylation within the R1 region of BCAA and RBP1. (A) Alignment of the R1 regions of RBP1 and BCAA. The amino acid sequences of the R1 regions of RBP1 and BCAA are presented, and the locations of the putative SUMOylation sites (Ψ KXE) are indicated in boldface and underlined. The RBP1 K433 putative SUMOylation site (strikethrough) was found experimentally not to be modified (data not shown). (B) Repression by RBP1 and BCAA R1 domains bearing alterations at the putative SUMOylation consensus sites. Mutants of R1 were prepared by site-directed mutagenesis that converted K418 and K444 of RBP1 and K429 and K440 of BCAA to arginines. The repression assays were conducted as in Fig. 4A. This experiment was conducted at least five times in duplicate, and the error bars represent the standard deviations. (C) Representative expression levels of Gal4-RBP1 and Gal4-BCAA proteins by Western blotting with anti-Gal4 antibodies. (D) Effect of the SUMO protease SuPr-1 on repression activity. The repression assays were conducted as in Fig. 4A using cells cotransfected with R1 or R2 of RBP1 or BCAA in the presence or absence of the SUMO protease SuPr-1. This experiment was conducted at least five times in duplicate, and the error bars represent the standard deviations. (E) Representative expression levels of Gal4-RBP1 and Gal4-BCAA proteins in the absence or presence of SuPr-1 by Western blotting with anti-Gal4. (F) Analysis of in vivo SUMOylation of RBP1 and BCAA R1 regions by SDS-PAGE and Western blotting analysis of immunoprecipitates. CHO-K1 cells were cotransfected with plasmids expressing Gal4-R1 and HA-SUMO-1, -2, -3, or -4. Cell extracts were immunoprecipitated with anti-Gal4 antibodies and precipitates were resolved by SDS-PAGE and analyzed by Western blotting with anti-HA or anti-Gal4 antibodies, as indicated in the figure. Lane 1, Gal4; lane 2, Gal4-RBP1 R1 wild type; lane 3, Gal4-RBP1 R1 K418R; lane 4, Gal4-RBP1 R1 K444R; lane 5, Gal4-RBP1 R1 K418R/K444R double mutant; lane 6, Gal4-BCAA R1 wild type; lane 7, Gal4-BCAA R1 K429R; lane 8, Gal4-BCAA R1 K440R; lane 9, Gal4-BCAA R1 K429R/K440R dou-

E)

F)

ble mutant. Results obtained with HA-SUMO-2, -3, and -4 are shown in Fig. S1A to C in the supplemental material, respectively. "*" represents the singly SUMOylated forms of R1, "**" indicates the doubly SUMOylated forms, and "#" indicates an unidentified SUMOylated peptide associated with the BCAA R1 region. The "a" denotes the doubly SUMOylated form of RBP1 R1, "b" denotes the SUMOylated doublet discussed in the Results section, "c" indicates the faster-migrating species, and "d" indicates the slower-migrating species of the doublet "b." Species "g" and "h" are the non-SUMOylated forms of RBP1 R1. Species "e" is the doubly SUMOylated form of BCAA R1, and "f" is the singly SUMOylated form of BCAA R1.

activity was detected with the RBP1 ARID region (data not shown). In summary, and as suggested by results shown in Fig. 7A, the R1 regions of both BCAA and RBP1 were found to contain two separable repression functions: one capable of

repressing activated transcription (R1 σ) and the other active against the basal transcription only (R1 α).

The R1 domain harbors SUMO-dependent repression activity. As mentioned above, close inspection of the amino acid sequence of R1 revealed the presence of three putative SUMO modification sites in RBP1 and two in BCAA within the carboxy-terminal region (amino acids 396 to 448 for BCAA and 399 to 452 for RBP1) (Fig. 8A, boldface and underlined). Figure 8B shows the results of Gal4 repression assays with BCAA and RBP1 R1 mutants in which the lysine residues within the Ψ KxE SUMOylation consensus sites (K418 and K444 in RBP1 and K429 and K440 in BCAA) were converted to arginine (see Fig. 8C for expression levels of the mutants versus wild-type R1). Conversion of individual lysine residues within each consensus site resulted in a partial relief of repression, whereas alteration of both prevented repression (Fig. 8B). It should be noted that the third putative SUMOylation site in RBP1 R1 (K433, Fig. 8A) was also mutated, and no effect on repression activity was observed (data not shown, indicated by strikethrough in Fig. 8A). To obtain further evidence that these lysine residues are SUMOylated, Gal4 repression assays were carried out in the presence of overexpressed SUMO-specific protease SuPr-1, which deconjugates SUMO from its substrate. Figure 8D shows that coexpression of SuPr-1 significantly relieved the repression associated with R1 of both BCAA and RBP1, whereas there was no effect on the HDAC-dependent repression activity of the R2 domains. In contrast, coexpression of a nonfunctional SuPr-1 catalytic mutant (C466S) had no effect on the repression activity of R1 or R2 (data not shown). Figure 8E shows that coexpression of SuPr-1 did not affect the expression level of R1. These results suggested that these lysine residues may indeed be SUMOylated and that at least some of the transcriptional repression activity resident in R1 is positively regulated by this modification.

To confirm that these residues were in fact SUMOylated, CHO-K1 cells were cotransfected with plasmids expressing either wild-type or mutant forms of Gal4-tagged R1 regions of BCAA or RBP1, as well as those encoding HA-tagged SUMO-1, -2, -3, or -4. After immunoprecipitation with a Gal4-specific antibody and separation by SDS-PAGE, Gal4-R1 species were detected by Western blotting with anti-Gal4 antibodies and the presence of SUMOylated species was confirmed by Western blotting with anti-HA antibodies. Figure 8F shows the results obtained with extracts from cells overexpressing HA-SUMO-1. The major Gal4-R1 species of both BCAA and RBP1 detected by anti-Gal4 antibodies were non-SUMOylated forms, whereas the slower-migrating SUMOylated forms (Fig. 8F, bottom panel [α -Gal4], species marked by an asterisk) represented only a small proportion of the population of both BCAA and RBP1. Although non-SUMOylated BCAA Gal-R1 predominantly migrated as a single species, two RBP1 Gal4-R1 species were evident (Fig. 8F, bottom panel [α -Gal4], species marked "g" and "h"), suggesting that two major variants existed, possibly due to some other posttranslational modification or to alternative splicing. In the case of the SUMOylated forms, singly or doubly modified forms have been indicated in Fig. 8F as single and double asterisks, respectively. With RBP1, conversion of individual acceptor lysine residues K418 and K444 to arginines resulted in the disappearance of

the slowest-migrating SUMOylated form of R1 (Fig. 8F, species "a" in lane 2), whereas simultaneous conversion of both sites eradicated the singly and the doubly SUMOylated species (Fig. 8F, species "a" and "b" in lane 2). The singly SUMOylated form of wild-type RBP1 R1 appeared as two major closely migrating species (indicated by a "b" in Fig. 8F lane 2, panel α -HA) and a minor faster-migrating doublet. We noted that the singly modified form present with the K444R mutant (Fig. 8F, lane 4, species "d") represented the slowest-migrating species in this doublet, whereas that produced by K418R (Fig. 8F, "c" in lane 3) corresponded to the fastest-migrating form. Mutation of residues K429 and K440 within BCAA resulted in a similar loss of the SUMOylated forms (Fig. 8F, "e" and "f"). Interestingly, a SUMOylated peptide appeared to associate specifically with BCAA R1 (indicated by a number sign in Fig. 8F) but not with RBP1 R1. The identity of this species and its importance are not known. Similar results were observed with the other SUMO family members SUMO-2, -3, and -4 (see Fig. S1 and additional results in the supplemental material), although the SUMOylation process seemed somewhat less efficient than with SUMO-1.

Taken together, the results in this series of experiments have delineated a region in R1 of both BCAA and RBP1 that exhibits strong repression activity that is highly dependent on SUMOylation.

The R1 and R2 regions of RBP1 and BCAA are both involved in growth inhibition and induction of a senescence marker. To demonstrate the functional roles of the R1 and R2 transcriptional repression domains of RBP1 and BCAA in the induction of growth arrest and a senescence marker, studies were carried out in which mutants that lacked R1, R2, or both R1 and R2 or in which SUMOylation sites had been altered were overexpressed. Figure 9A and B show that after overexpression of wild-type forms of RBP1 and BCAA, the growth of C33-A cells was significantly decreased, as was the case in Fig. 2. With both RBP1 and BCAA, removal of either R1 or R2 significantly reduced this growth arrest. In addition, the BCAA^{MCF-7} isoform isolated from breast cancer cells, which lacks the R2 region, was also partially defective in inducing growth arrest (Fig. 9B). Deletion of both R1 and R2 of RBP1 eliminated virtually all of its growth-inhibitory properties (Fig. 9A); however, with a similar mutant in BCAA, although growth arrest was considerably reduced, it was not completely abolished (Fig. 9B). These results may suggest that BCAA harbors additional growth arrest activity. Nonetheless, these results imply that both R1 and R2 are required for regulation of cell proliferation. Interestingly, alteration of the SUMOylation sites within R1 of RBP1 (Fig. 9A) and BCAA (data not shown) also reduced growth inhibition, suggesting that SUMOylation of the R1 σ repression function was important in R1-dependent growth arrest.

Experiments were also conducted with these RBP1 and BCAA mutants to determine their ability to induce the senescence specific biomarker SA- β -Gal. Figure 9C shows that with both RBP1 and BCAA, deletion of either R1 or R2 significantly reduced SA- β -Gal expression, as was the case with the BCAA^{MCF-7} form that lacks the R2 domain relative to full-length BCAA. Figure 9D shows the levels of expression of the various constructs used in the senescence induction assay. These results indicated that both R1 and R2 are considerably

important in the induction of both growth arrest and the senescence phenotype.

DISCUSSION

In this report, we compared the biological functions of BCAA to the known properties of RBP1 and further characterized the repression activity of the R1 region of each of these proteins, thus establishing RBP1 and BCAA as members of a family of transcriptional repressors. Since pRB is a known negative regulator of cell growth and RBP1 is involved in some transcriptional events mediated by pRB, we were interested in determining the effect of overexpression of BCAA and RBP1 on cell proliferation. Overexpression of an RBP1 mutant lacking the LXCXE pRB-binding motif, or of BCAA, which is naturally devoid of an LXCXE motif, was shown to inhibit growth to the same extent as RBP1 and pRB. These results suggested either that BCAA and RBP1 can act independently of the pRB family members or that they are able to interact with the pRB family in an LXCXE-independent fashion. We are currently investigating these possibilities.

Recently, pRB and p130 were shown to be involved in the establishment and maintenance of senescence. Furthermore, recent studies have reported that HPV18 E7 oncoprotein blocks pRB from functioning normally in the senescence pathway (8, 21, 34). E7 is an LXCXE-containing pRB-binding protein that is able to dislodge RBP1 from its association with pRB (7). Thus, it is possible that RBP1 might have a role to play in the establishment of senescence by pRB. After overexpression of BCAA or RBP1, a 10-fold increase in senescent cell-like cells was observed, as measured by the activity of the biomarker SA- β -Gal. A similar increase was observed when p73- α was overexpressed, but none occurred with DNA from either empty vector or p14^{ARF} plasmid controls. Interestingly, the BCAA^{MCF-7} isoform was unable to induce senescence. These results suggested that wild-type BCAA and RBP1, but not cancer-related isoforms, might be involved in the establishment and maintenance of senescence, possibly through interaction with pRB and/or p130. BCAA and RBP1 associate with mSin3/HDAC complexes via the R2 repression domain, and their histone-modifying activity might be required in the formation of the heterochromatic foci induced by pRB and p130 in senescent cells. The BCAA^{MCF-7} isoform encompasses the amino-terminal half of BCAA and lacks the R2 region. Thus, it is interesting to speculate that BCAA^{MCF-7} failure to induce the senescence biomarker SA- β -Gal is related to its inability to associate with HDAC complexes.

The apparent inability to induce senescence, as well as the lack of the R2 region in BCAA^{MCF-7}, led us to characterize the transcriptional repression properties of BCAA and RBP1 further. We demonstrated that both BCAA and RBP1 are effective transcriptional repressors and that in addition to their HDAC-dependent R2 repression domains, each contains two potent repression activities within their R1 regions.

The region within the ARID of both BCAA and RBP1 that exhibited basal transcription repression activity is predicted to be a cyclin recognition motif and to form a helix-turn-helix secondary structure, suggesting that this activity may originate from interactions with either general transcription factors and/or pRB. Cyclins have an evolutionarily conserved structure

FIG. 9. Role of the R1 and R2 repression regions in growth arrest and induction of a senescence marker. Experiments similar to those described in Fig. 2C and 3A were conducted in which cells were transfected with cDNAs expressing full-length RBP1 or BCAA, mutant forms lacking either the R1 or R2 regions or both, RBP1 containing alterations on the SUMOylation sites within R1, or the BCAA^{MCF-7} form that lacks the carboxy-terminal R2 domain. (A) Effect on cell growth of overexpression of RBP1 in C33-A cells; (B) effect on cell growth of overexpression of BCAA in C33-A cells; (C) effect on expression of senescence marker SA-β-Gal. These experiments were conducted at least five times (A) or twice (B and C) times in duplicate, and the error bars represent the standard deviations. (D) Representative expression levels of the various constructs used in Fig. 9C by Western blotting with anti-HA antibodies.

D)

FIG. 9—Continued.

known as the cyclin fold, which is also found in the general transcription factor TFIIB and in pRB (17, 23), suggesting that the ARID region might represent a bridging point for pRB and/or TFIIB association. Transcription of RNA polymerase II promoters occurs through an orderly assembly of the preinitiation complex. The TFIID (TBP) subunit recognizes the TATA box and TFIIB stabilizes TFIID at the promoter by contacting TFIID and DNA sequences adjacent to the TATA box. These sequences are recognized and bound by a helix-turn-helix motif at the carboxy-terminal of TFIIB. Subsequently, TFIIB recruits the RNA pol II-TFIIF subunit to the promoter, and then TFIIE and TFIIH are recruited before transcription initiation occurs. We postulate that the repression activity associated with the ARID region could occur from one or more of the following possibilities. First, the cyclin fold structure of TFIIB could interact with the ARID region, thus preventing subsequent recruitment of the RNA pol II subunit by TFIIB, and therefore aborting transcription initiation. Also, as a nonspecific DNA interaction module (32), the ARID region could, through its helix-turn-helix secondary structure, bind to the TFIIB recognition element DNA sequence in the vicinity of the TATA box, thus preventing TFIIB from binding to the promoter and stabilizing the TFIID subunit and consequently proper assembly of the preinitiation complex. Further studies are required to distinguish between these possibilities.

The R1 repression activity appeared to be controlled by posttranslational modification by the SUMO family of proteins. This observation was confirmed by mutation of the SUMO acceptor lysine residues within the R1 region, as well as by overexpression of the SUMO protease SuPr-1 that removes covalently bound SUMO from the target protein. The carboxy-terminal region of R1 appeared to be SUMOylated by all known members of the SUMO family. The functional relevance of this R1 modification by different SUMO proteins is still unknown. SUMO-2, -3, and -4, which contain the ΨKXE consensus sequence, could themselves be SUMOylated, resulting in the formation of a polymerized SUMO chain reminiscent of the polyubiquitin chains that target proteins for degradation; however, the outcome of poly-SUMO chains in transcriptional regulation is still unknown. In addition, the R1

region of RBP1, unlike BCAA, appears as two species subsequent to SDS-PAGE analysis. Mutation of lysine 418 in RBP1 resulted in migration of a species that corresponded to the fastest-migrating form of R1, and mutation of lysine 444 yielded the slowest-migrating form of R1. Moreover, a similar phenomenon has recently been reported through the dual SUMOylation of the basic Kruppel-like factor BKLF (33), as well as the zinc finger DNA-binding transcription factor Ikaros (20). Although the differential migration was not discussed, Fig. 2A from Pablo Gomez-del Arco et al. clearly shows that the K240R Ikaros mutant migrates faster than the K58R mutant in a manner similar to the K418R and K444R mutants of RBP1. Our results may suggest the existence of another type of modification (such as acetylation or phosphorylation), whose presence appears to correlate with SUMOylation at the K418 site. If such is the case, it will be important to identify this modification since it may play a role along with SUMOylation in the regulation of R1 transcriptional activity in RBP1.

Although we have identified two separate regions within R1, R1 α and R1 σ , that exhibit transcriptional repression activity when expressed alone, the R1 region within the full-length RBP1 family members should perhaps be considered as a whole. Both R1 α and R1 σ were found to be able to repress basal transcription; however, SUMOylation of the R1 σ region may, as is the case for most SUMOylated repression domains characterized thus far, enhance the activity of R1 α . Our results provide the first example of transcriptional repression activity being mediated by an ARID and the regulation of this activity by SUMOylation.

The present study indicates that in terms of transcriptional repression, BCAA and RBP1 exhibit quite similar activities, as was predicted from their high degree of sequence homology and structural organization. We confirmed that, like RBP1, the R2 region of BCAA physically interacts with the SAP30 subunit of the Sin3/HDAC complex. This interaction in conjunction with the results obtained using the HDAC inhibitor TSA, suggests that BCAA recruits the Sin3/HDAC complex via SAP30. Furthermore, this interaction occurs with the amino-terminal half of SAP30 in a similar fashion to the association with the N-COR corepressor (24).

The final experiments with RBP1 and BCAA mutants lacking the R1 and R2 regions indicated quite clearly that both of these transcriptional repression functions play an important role in growth arrest and induction of a senescence marker. Interestingly, alteration of the K418 and K444 SUMOylation sites in RBP1 had an almost identical effect as deletion of the whole R1 region, suggesting that repression of activated transcription through R1 σ as regulated by SUMOylation may represent the major contribution of the R1 region. Nonetheless, the reduced biological activity of mutants and isoforms lacking R2 clearly indicated an involvement of mSin3A/HDAC in the mediation of growth inhibition. The almost total loss of function with mutants lacking both R1 and R2, especially in the case of RBP1, indicated that these two classes of transcriptional repression activities may account for most if not all of the RBP1 family biological activity.

In conclusion, BCAA and RBP1 appear to represent a family of proteins with highly similar biological and biochemical properties. It will be interesting to determine their particular

roles in the control of cell proliferation and the establishment or progression of cancer.

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