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The adenovirus-2 E1B-55K protein interacts with a mSin3A/histone deacetylase 1 complex

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Abstract The adenovirus E1B-55K protein is a multifunctional phosphoprotein that regulates nuclear to cytoplasmic export of host cell and viral mRNAs during lytic viral growth. E1B-55K also blocks apoptosis by binding and functionally inactivating the human tumor suppressor protein p53. Here, we show that E1B-55K interacts with histone deacetylase 1 (HDAC1) and the transcriptional corepressor protein mSin3A, both in the adenovirus-transformed 293 cell line and during a lytic adenovirus infection. Furthermore, we show that the central amino acids 156–261 in E1B-55K are necessary for efficient HDAC1 interaction. Importantly, the E1B-55K/mSin3A/HDAC1 complex is also enzymatically active, catalyzing deacetylation of a histone substrate peptide. Collectively, our results suggest that E1B-55K interaction with mSin3A/HDAC1 containing complexes may be significant for one or several of the multiple activities ascribed to this protein.

Key words: Adenovirus; E1B-55K; Histone deacetylase 1; mSin3A

1. Introduction

The adenovirus (Ad) E1B-55K protein is a multifunctional phosphoprotein that serves several functions during virus growth. For example, the E1B-55K protein interacts and functionally inactivates the cellular tumor suppressor protein p53 (reviewed in [1]). This interaction is specific since Ad5 E1B-55K does not interact with the structural and functional p53 homologous proteins, p51 and p73 [2]. E1B-55K interacts with the amino-terminal transactivation domain of p53 [3]. This interaction blocks p53 as a transcriptional activator of several promoters, such as mdm2, cyclin G, bax and p21WAF1 [4]. Furthermore, tethering E1B-55K to a promoter represses basal and activated transcription from that promoter [5,6]. Available data suggest that the silencer activity of E1B-55K requires an unknown cellular corepressor protein that copurifies with RNA polymerase II [5].

Additionally, a protein complex consisting of the adenoviral E1B-55K and the E4-ORF6 proteins mediates the selective transport of viral mRNAs late during lytic virus growth (reviewed in [7]). The observation that the Ad5 E1B-55K protein interferes with mRNA export in *Saccharomyces cerevisiae* indicates that this protein is the primary adenovirus protein blocking host cell mRNA export [8]. Interestingly, E1B-55K interaction with the host cell protein, E1B-AP5 (E1B-55K-associated protein 5) appears to be critical for the E1B-55K-dependent inhibition of host cell mRNA export [9]. Recent data indicate that the E1B-55K protein shuttles between the cytoplasm and the nucleus, also in the absence of E4-ORF6 [10].

Reversible acetylation of core histones is an important regulatory mechanism controlling gene expression. In general, histone acetylation is associated with activated transcription, and deacetylation with transcriptional repression. The acetylation and deacetylation reactions are catalyzed by a number of evolutionary conserved histone acetyltransferases (HATs) and histone deacetyltransferases (HDACs), respectively (reviewed in [11]). In mammalian cells, HDAC1 and HDAC2 are found in a multiprotein complex with mSin3A, the mouse homologue of the yeast global transcription repressor protein SIN3 [12]. The mSin3A/HDAC complex has been implicated as a corepressor complex utilized by a diverse collection of transcriptional repressor proteins, including SMRT, N-CoR, Mad, MeCP2, Ski (reviewed in [13]).

Here we report that the adenovirus-2 E1B-55K protein makes a specific complex with HDAC1 in vitro and in transiently transfected cells. In vivo, E1B-55K appears to exist in a complex with mSin3A and HDAC1. Importantly, the E1B-55K/mSin3A/HDAC1 complex is enzymatically active, catalyzing deacetylation of a histone substrate peptide. Collectively, our results suggest that E1B-55K binding to mSin3A/HDAC1 complexes may be significant for one or several of the multiple functions associated with this protein.

2. Materials and methods

2.1. Plasmids

Plasmid maps, sequences are available on request, or at http://www.bmc.uu.se/IMIM/res/GA.html. Cloning and amplification of E1B-55K expressing plasmids were done in *Escherichia coli* SURE cells (Stratagene). Plasmid pBSE1B-55K (kindly provided by E. Bridge) encodes the adenovirus type 2 E1B-55K protein, and was the starting material for reconstruction of all E1B-55K expressing plasmids. Plasmid pHis-E1B-55K was generated by recloning E1B-55K into the pQE30 expression vector (Qiagen). For eukaryotic expression, E1B-55K was recloned into pcDNA3.1 (Invitrogen). pGSTHDAC1 was generated by recloning the HDAC1 cDNA from pING14AHDAC1 [14] into pGEX2T (Pharmacia). Plasmids pING14AHDAC1, pCMV5′3THDAC1(FL) and pGEXp53 were generously provided by Tony Kouzarides and Klas Wiman, respectively.

2.2. Purification of proteins expressed in bacteria

His-E1B-55K expression was induced in 1 l cultures at 37°C to an optical density of 0.4. IPTG was added to a final concentration of 0.4 mM and the incubation continued, at room temperature, for an additional 16 h. Cells were collected and His-E1B-55K purified on Ni-NTA as described by the manufacturer (Qiagen). Expression and

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purification of GST-HDAC1, GST-p53 and GST were done as previously described [15].

2.3. In vitro transcription/translation

^{35}S-Labeled wild-type E1B-55K and deletion mutant proteins were synthesized by coupled in vitro transcription/translation using the reticulocyte TnT system (Promega). The template DNA was generated by polymerase chain reaction (PCR) amplification using a forward primer containing a T7 RNA polymerase promoter and a Kozak sequence positioned upstream of the translation initiation codon, and a reverse primer complementary to the 3′ end of the gene. Carboxy-terminal E1B-55K deletion mutants were generated by restriction endonuclease cleavage of the PCR template DNA; *Pvu*II (mutant 1–155), *Hin*dIII (mutant 1–261), *Bgl*II (mutant 1–435). E1B-55K mutant 261–421 was generated by PCR and recloned into pcDNA3.1(+). HDAC1 was transcribed from plasmid pING14AHDAC1.

2.4. In vitro binding assays

Approximately 400 ng of His-E1B-55K or His-p32 [16] was incubated in 400 μl of NET(0.2) buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, plus 0.2% NP40) with ^{35}S-labeled HDAC1 for 30 min at 4°C. Anti-His (Clontech) or anti-FLAG (Kodak) antibodies were added to the reaction mixture and incubation continued for 1 h. Precipitates were collected on protein A Sepharose (Amersham Pharmacia Biotech) and washed four times with NET(0.2) buffer. GST pull-down experiments were performed with 1 μg of GST, GST-HDAC1 or GST-p53 proteins incubated with in vitro translated E1B-55K proteins in NET (0.5% of NP40) buffer. Proteins were separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and visualized by autoradiography.

2.5. In vivo co-immunoprecipitation

Subconfluent monolayers of COS-7 cells were transfected with 2 μg of HA-HDAC1 plus or minus 2 μg of pcDNAE1B-55K plasmids. Transient transfections were done by using FuGene6 (Roche) according to the manufacturer's protocol. Cells were lysed with NET(0.2) containing COMPLETE Protease Inhibitor Mix (Roche), and the cell suspension passed five times through a 21 G needle. The soluble extract was precleared with 20 μl of 50% protein A Sepharose slurry. The precleared extracts were immunoprecipitated with 1 μg of anti-HA (clone 12CA5), anti-His antibodies or with 20 μl of anti-E1B-55K antibody (clone 2A6 [17]) for 1 h. 20 μl of a 50% protein A Sepharose slurry was added and incubation continued for another 2 h. The precipitates were washed four times in NET(0.2) buffer, separated on SDS–PAGE, transferred to a nitrocellulose membrane, and probed with the appropriate antibodies. Co-immunoprecipitations from 293 cells and late adenovirus-infected HeLa cells were done in a similar way. For 293 cells, a total of 3 mg of protein was incubated with 1 μg of anti-mSin3A, anti-HDAC1 or anti-Gal4 antibodies (all antibodies from Santa Cruz). Protein A Sepharose bound immunocomplexes were recovered after overnight incubation and washed in NET(0.2) buffer. For HeLa cells, nuclear extracts were prepared from uninfected and late adenovirus-infected cells as previously described [18]. Approximately 200 μg of precleared nuclear extracts was used in the assay and immunoprecipitated with the same antibodies as described for 293 cells. Proteins were visualized on nitrocellulose filter by Western blot analysis using the enhanced chemiluminescence system (Amersham Pharmacia Biotech).

2.6. Histone deacetylation activity assay

As substrate, a peptide corresponding to the first 24 amino acids of bovine H4 was synthesized and chemically acetylated with 0.1 mCi of [^{3}H]sodium acetate (DuPont) in the presence of BOP and triethylamine [19]. Immunoprecipitations were done using anti-mSin3A, E1B-55K or three different p53 antibodies (Ab-3, Calbiochem; DO-1, Santa Cruz; and 421, generously provided by Toivo Maimets) per 1 mg of total 293 cell extract in NET(0.2) buffer. Protein A Sepharose beads were washed four times with NET(0.2) buffer and once with HDAC(10) buffer (10 mM Tris pH 7.5, 10 mM NaCl, 10% glycerol). Beads were finally resuspended in 100 μl of HDAC(10) buffer, mixed with 0.5 μg of acetylated peptide (50 000 cpm) and incubated at 37°C for 20 min. Reactions were terminated by addition of 1 M HCl/0.16 M acetic acid and extracted with ethyl acetate. The ethyl acetate fraction was counted in a liquid scintillation counter. Deacetylation was blocked by incubating the immunoprecipitates with 300 nM of trichostatin A (TSA) for 15 min on ice before addition of the peptide substrate. Samples were assayed in duplicate in three different experiments.

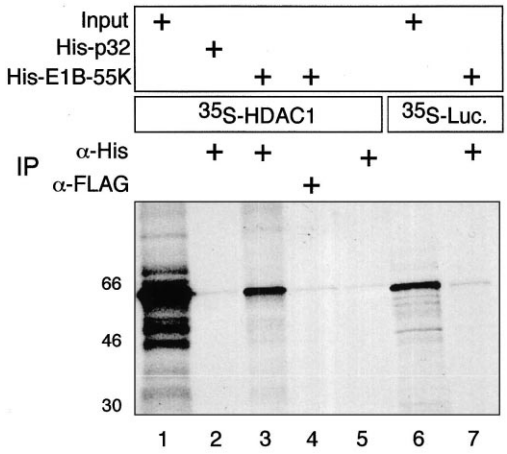

Fig. 1. The adenovirus-2 E1B-55K protein interacts with HDAC1. In vitro translated ^{35}S-labeled HDAC1 and ^{35}S-labeled luciferase were incubated together with His-p32 or His-E1B-55K and immunoprecipitated with an anti-His antibody (lanes 2, 3, 5 and 7) or a control anti-FLAG antibody (lane 4). Lanes 1 and 6 contain 1/5 and 1/10 of the input protein used in binding reactions. The positions of protein size markers (in kDa) are indicated on the left.

3. Results

3.1. E1B-55K interacts with HDAC1 in vitro

To investigate whether the Ad2 E1B-55K protein can interact with HDAC1, we performed in vitro co-immunoprecipitation experiments. In these experiments, bacterially expressed His-E1B-55K was incubated with ^{35}S-labeled HDAC1. Reaction mixtures were immunoprecipitated with an anti-His antibody. As shown in Fig. 1, mixing HDAC1 and His-E1B-55K results in a co-precipitation of HDAC1 (lane 3), in a reaction that requires His-E1B-55K (lane 5). Importantly, under the same experimental conditions, HDAC1 did not bind to an unrelated His-tagged protein, His-p32 (lane 2). Furthermore, HDAC1 was not precipitated by a control, anti-FLAG antibody (lane 4). Also, E1B-55K did not show a significant binding to in vitro translated luciferase protein (lane 7), further emphasizing that the E1B-55K/HDAC1 interaction was specific. Collectively, our results show that E1B-55K interacts specifically with HDAC1 under in vitro co-immunoprecipitation assay conditions.

To delineate the amino acid sequences in E1B-55K required for HDAC1 interaction, we performed the reverse assay using bacterially produced GST-HDAC1 in a pull-down assay with in vitro translated ^{35}S-labeled E1B-55K deletion mutant proteins (Fig. 2). GST-p53 was used as a positive control in this assay. As shown in Fig. 2, GST-HDAC1 and GST-p53 interact with the full-length E1B-55K protein (1–495), mutants E1B-55K (1–435) and E1B-55K (1–261), but not with E1B-55K (1–155). Using the amino-terminal deletion mutant E1B-55K (261–421) shows that GST-p53, and particularly GST-HDAC1, interacted weakly with a carboxy-terminal fragment of E1B-55K.

Based on these results, we conclude that HDAC1 interacts primarily with the central part of E1B-55K (amino acids 156 and 261) and weakly with amino acids 261–421. We further

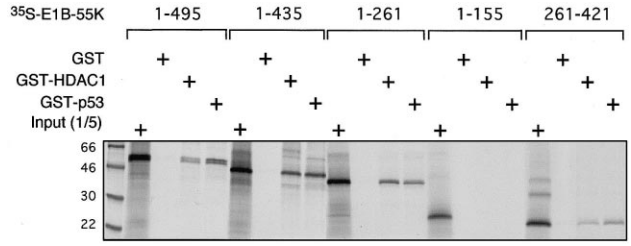

Fig. 2. Mapping of the primary amino acid sequences in E1B-55K required for HDAC1 and p53 interaction. GST, GST-HDAC1 and GST-p53 were incubated with equal amounts of ^{35}S-labeled wild-type and mutant E1B-55K proteins. Proteins were separated on 12% SDS–PAGE. The position of protein size markers (in kDa) is indicated on the left.

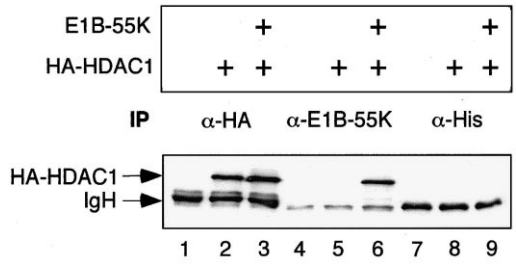

Fig. 3. E1B-55K interacts with HDAC1 in vivo. COS-7 cells were transfected with pCMV5′3THDAC1(FL) plus or minus pcDNAE1B-55K. Cell extracts were split into equal fractions and immunoprecipitated with an anti-E1B-55K antibody (lanes 4–6) or a non-specific control anti-His antibody (lanes 7–9). Lanes 1–3 show the input, corresponding to 1/10 of the cell extract used for immunoprecipitation with the anti-E1B-55K or the anti-His antibodies (lanes 4–9). Proteins were transferred onto a nitrocellulose membrane and HDAC1 visualized by probing with an anti-HA antibody.

conclude that the binding specificity of GST-HDAC1 and GST-p53 to E1B-55K was identical, whereas the binding affinity varied slightly (Fig. 2).

3.2. E1B-55K interacts with HDAC1 in vivo

To analyze whether E1B-55K and HDAC1 also interact in vivo, we performed co-immunoprecipitation experiments in COS-7 cells, transiently transfected with plasmids encoding E1B-55K and HA-HDAC1. As shown in Fig. 3, immunoprecipitation with anti-E1B-55K antibody brought down HA-HDAC1 (lane 6). This effect was E1B-55K specific since the same antibody did not immunoprecipitate HA-HDAC1 in the absence of E1B-55K (lane 5). Furthermore, immunoprecipitation with an irrelevant anti-His antibody did not precipitate either E1B-55K (data not shown) or HA-HDAC1 (lanes 8 and 9). Importantly, HA-HDAC1 was expressed at the same level irrespective of E1B-55K cotransfection (lanes 2 and 3).

Based on these results, we conclude that E1B-55K binds specifically to HA-HDAC1 in vivo, in transiently transfected COS-7 cells.

3.3. E1B-55K interacts with HDAC1 and the mSin3A corepressor protein in vivo

To further demonstrate the relevance of the E1B-55K/HDAC1 interaction, we determined whether an interaction could be detected in 293 cells, an adenovirus-transformed cell line expressing E1B-55K [20]. Since previous studies have shown that HDAC1 exists in a multiprotein complex, which includes mSin3A [12], we also assayed for a possible interaction between E1B-55K and mSin3A. As shown in Fig. 4A, immunoprecipitation with anti-mSin3A brought down high levels of E1B-55K and HDAC1 (lane 2). This interaction was specific since we could not detect E1B-55K, mSin3A or HDAC1 in immunoprecipitates using an unrelated anti-Gal4 antibody (lane 4). We also detected a weak precipitation of E1B-55K with the anti-HDAC1 antibody (lane 3). Despite the fact that anti-mSin3A and anti-HDAC1 antibodies precipitated similar amounts of HDAC1, the anti-mSin3A antibody precipitated significantly more E1B-55K (lanes 2 and 3). This result indicates that E1B-55K interaction with mSin3A does not require HDAC1. Potentially, E1B-55K may interact directly with mSin3A, or alternatively with other members of mSin3A complex. A strong interaction between E1B-55K and mSin3A was also detected in nuclear extracts prepared from late adenovirus-infected HeLa cells (Fig. 4B), suggesting a

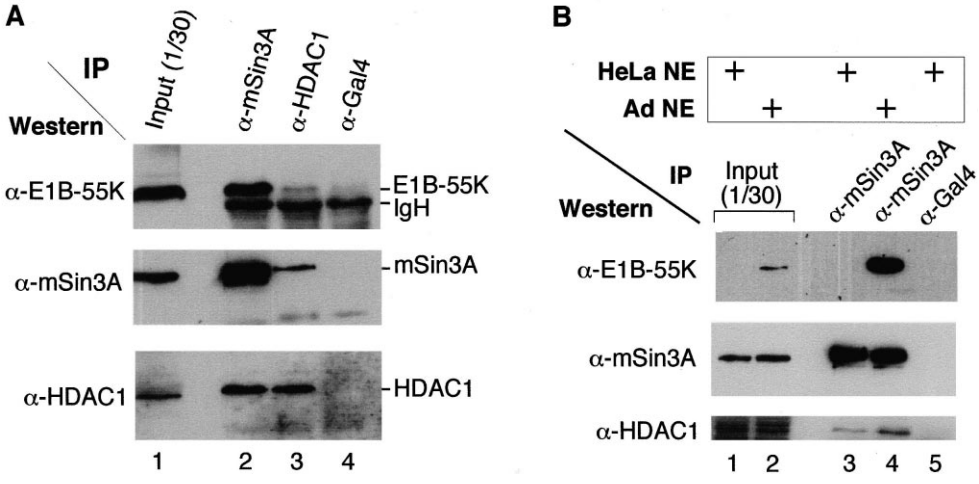

Fig. 4. E1B-55K interacts with HDAC1 and mSin3A expressed in vivo. A: Cell extracts prepared from 293 cells were immunoprecipitated with anti-mSin3A (lane 2), anti-HDAC1 (lane 3) or with anti-Gal4 antibodies (lane 4). B: Nuclear extracts prepared from uninfected or adenovirus-infected HeLa cells were immunoprecipitated with anti-mSin3A (lanes 3 and 4) or with anti-Gal4 antibodies (lane 5). In both panels, precipitated proteins were sequentially detected on the same Western blot filter by probing with anti-E1B-55K, anti-mSin3A or anti-HDAC1 antibodies. Input (lane 1) represents 1/30 of the extracts used for immunoprecipitations.

potential significance of these interactions for lytic virus growth.

Collectively, our results show that E1B-55K interacts with mSin3A and HDAC1 in vivo. The data are compatible with, but do not prove, the hypothesis that E1B-55K forms a multimeric complex with mSin3A and HDAC1. Potentially, E1B-55K interacts independently with HDAC1 and mSin3A.

3.4. The E1B-55K and mSin3A/HDAC complex is enzymatically active

The establishment of an interaction between E1B-55K and mSin3A/HDAC1 is interesting, and potentially very important. However, a key question is whether this interaction generates a complex that is enzymatically active. To test this, we immunoprecipitated the endogenously expressed mSin3A and E1B-55K proteins in 293 cell extracts and incubated the precipitate with a ^{3}H-acetylated peptide corresponding to the 24 N-terminal amino acids of histone H4. Deacetylation was measured as a release of [^{3}H]acetate, which was quantified by scintillation counting. As shown in Fig. 5, anti-mSin3A immunoprecipitation recruited a high deacetylation activity (lane 1), an activity that was reduced to background levels by treatment with TSA (lane 2), a potent HDAC inhibitor [21]. Interestingly, immunoprecipitation of E1B-55K also precipitated a substantial amount of deacetylase activity, only 5-fold less compared to mSin3A (compare lanes 1 and 3). This result is important, because it demonstrates that E1B-55K interaction with mSin3A/HDAC (Fig. 4) generates an enzymatically active complex. Treatment of E1B-55K immunoprecipitates with TSA reduced deacetylation back to the background levels (compare lanes 3 and 4). Similarly, immunoprecipitation of E1B-55K from adenovirus-infected nuclear extracts resulted in a recruitment of deacetylase activity (data not shown).

We also tested whether the enzymatic activity recruited by E1B-55K was indirect, mediated by p53. Recent studies have

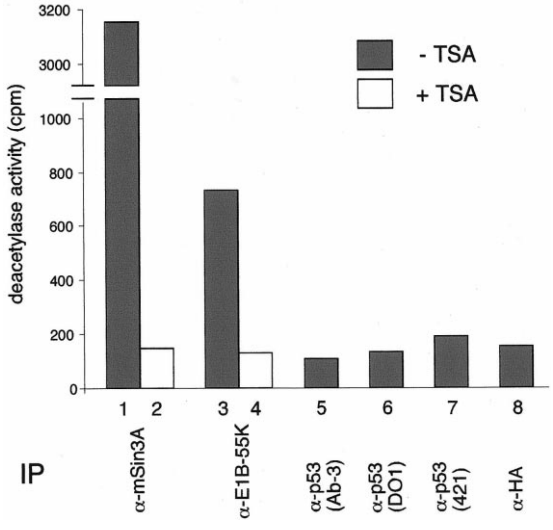

Fig. 5. E1B-55K interaction with mSin3A/HDAC generates an enzymatically active complex. Extracts from 293 cells were immunoprecipitated with anti-mSin3A, anti-E1B-55K, anti-p53 or anti-HA antibodies. Deacetylase activity was detected as radioactivity released from a ^{3}H-labeled acetylated histone H4 peptide [19]. Pretreatment with 300 nM TSA was done for 15 min before addition of the substrate peptide. The results represent the average of three different experiments, assayed in duplicate.

suggested that p53 binds to the mSin3A/HDAC1 complex in murine and human cells [22]. Therefore, it became important to determine whether the E1B-55K recruitment of HDAC activity was indirect, mediated through an interaction between p53 and HDAC1. However, as shown in Fig. 5 (lanes 5–7), we could not detect a recruitment of a deacetylation activity in 293 cells by immunoprecipitation of p53 with three different p53 antibodies. Thus, we conclude that p53 immunoprecipitated from 293 cells does not bring down a HDAC activity.

Collectively, these results demonstrate that the E1B-55K and mSin3A/HDAC1 complex is enzymatically functional, and furthermore suggest that recruitment of this enzymatic activity is not mediated by p53.

4. Discussion

Here we demonstrate that the Ad2 E1B-55K protein interacts with HDAC1, and mSin3A containing complexes in a way that generates an enzymatically active complex.

Two distinct classes of HDACs have been identified in higher eukaryotes, based on their homology with yeast HDACs. Class I HDACs (HDAC1–3) show a homology to the yeast Rpd3 protein, and class II HDACs (HDAC4–6) are homologous with the yeast Hda1 protein [23]. Since yeast Rpd3 and Hda1 interact specifically with different accessory proteins, it appears likely that the class I and II HDACs expressed in higher eukaryotes will also make functionally distinct complexes [13]. Interestingly, Ad5 E1B-55K does not interact with HDAC4, a member of class II HDACs [24]. Thus, E1B-55K may show specificity towards the class I HDACs in vivo. Importantly, the interaction between E1B-55K and mSin3A/HDAC1 generates a complex that has a deacetylation activity (Fig. 5).

What is the biological significance of the E1B-55K interaction with mSin3A/HDAC1? The most obvious hypothesis is that E1B-55K repression of p53 as a transcriptional activator protein requires recruitment of this complex. Recruitment of HDAC containing complexes has been coupled to transcriptional repression by several repressor proteins [13]. Although an attractive model, we have not been able to demonstrate that cotransfection of a plasmid expressing HDAC1 significantly affects the repressive effect of E1B-55K on p53-induced transcription (data not shown). Similarly, Gal4-E1B-55K repression of basal transcription from a herpes simplex thymidine kinase promoter, containing Gal4 DNA binding sites, was not sensitive to TSA treatment (data not shown). Collectively, these experiments argue against a function of the E1B-55K/mSin3A/HDAC1 complex in transcriptional regulation. Similar results have previously been reported by others [24,25]. However, the function of the E1B-55K/mSin3A/HDAC complex may be promoter specific. Precedence for such a promoter-dependent mechanism comes from studies of pRB repression of transcription. Here it has been shown that pRB inhibits the adenovirus major late promoter by HDAC recruitment, but not the SV40 enhancer or the herpes simplex thymidine kinase promoters [26]. It should be noted that in all our experiments, we have used artificial promoter constructs. Thus, analyzing the effect of E1B-55K/mSin3A/HDAC1 on native p53 regulated promoters may give a different result.

Alternatively, it is possible that transcriptional repression by E1B55K is not mediated by the deacetylation activity of

HDAC1, but by the activity of other subunits in the mSin3A complex. For example, mSin3A itself can mediate transcription repression in the absence of deacetylation. In fact, mSin3A binds the general transcription factor TFIIB, suggesting that mSin3A may interfere with formation of a functional preinitiation complex [27].

Although much of the current work has focused on transcriptional regulation through acetylation/deacetylation of core histones, it is important to note that recent studies have shown that a number non-histone proteins are substrates for acetylation. For example, p53, GATA1, E2F1, Importin-α all have been shown to be acetylated by the same HATs acetylating core histones [28]. Acetylation of these proteins regulates many functions in the cell, including DNA binding, protein–protein interactions and protein stability. Very little is known about the deacetylation of non-histone proteins. However, the E2F1 transcription factor has been shown to be deacetylated by HDAC1 [29]. It appears likely that future studies will further increase the number of HDAC targets, and furthermore show that reversible acetylation is an important mechanism regulating a diverse set of cellular functions.

Of particular interest is the recent finding that Importin-α is acetylated by p300 [30]. Importin-α is a nuclear import factor, initiating the nuclear entry of target proteins [31]. This finding may provide a link between the E1B-55K nucleo-cytoplasmic shuttling activity [10] and E1B-55K interaction with HDAC1 (Fig. 1). Although attractive, this hypothesis remains to be experimentally tested.

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