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Histone Deacetylase Activity Is Required for Full Transcriptional Repression by mSin3A

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Summary

Members of the Mad family of bHLH-Zip proteins heterodimerize with Max to repress transcription in a sequence-specific manner. Transcriptional repression by Mad:Max heterodimers is mediated by ternary complex formation with either of the corepressors mSin3A or mSin3B. We report here that mSin3A is an in vivo component of large, heterogeneous multiprotein complexes and is tightly and specifically associated with at least seven polypeptides. Two of the mSin3A-associated proteins, p50 and p55, are highly related to the histone deacetylase HDAC1. The mSin3A immunocomplexes possess histone deacetylase activity that is sensitive to the specific deacetylase inhibitor trapoxin. mSin3A-targeted repression of a reporter gene is reduced by trapoxin treatment, suggesting that histone deacetylation mediates transcriptional repression through Mad-Max-mSin3A multimeric complexes.

Introduction

The Mad family of basic region-helix-loop-helix-leucine zipper (bHLH-Zip) proteins plays an important role in controlling cell proliferation and differentiation (Amati and Land, 1994; Bernards, 1995). Four identified Mad family members—Mad1, Mxi1, Mad3, and Mad4 (Ayer et al., 1993; Zervos et al., 1993; Hurlin et al., 1995)—form heterodimers with another bHLH-Zip protein, Max, to repress transcription (Ayer et al., 1993; Hurlin et al., 1995) and are thought to play a negative role in the control of cell proliferation.

Two mammalian homologs of the Saccharomyces cerevisiae transcriptional corepressor Sin3p, mSin3A and mSin3B, have recently been identified as Mad-interacting proteins and are required for Mad-mediated transcriptional repression (Ayer et al., 1995; Schreiber-Agus et al., 1995). The most conserved regions of these proteins correspond to four putative paired amphipathic helix (PAH) motifs, which have been proposed to constitute protein–protein interaction surfaces (Wang et al., 1990). The second PAH motif in mSin3A, mSin3B, and Sin3p interacts with the mSin3 interaction domain, or SID, in the amino terminus of the four Mad family members (Ayer et al., 1995; Hurlin et al., 1995; Schreiber-Agus et al., 1995; Kasten et al., 1996). Mad1, Max, and mSin3A form ternary complexes capable of binding DNA (Ayer et al., 1995). Point mutations in the SID domain of Mad1 disrupt its ability to bind mSin3A, negate its function as a transcriptional repressor (Ayer et al., 1995), and eliminate Mad1 function in several biological assays (Koskinen et al., 1995; Roussel et al., 1996). These findings suggest that Mad:Max heterocomplexes repress transcription by tethering either mSin3A or mSin3B to DNA. A chimeric protein fusing the SID of Mad1 to the GAL4 DNA-binding domain results in repression of simple and complex promoters in a manner that is dependent on mSin3 binding, suggesting that targeting mSin3 to DNA is necessary for repression (Ayer et al., 1996). Nevertheless, the molecular mechanism(s) for mSin3A-mediated repression remain unknown.

Recently, a mammalian histone deacetylase has been identified, and cDNAs encoding the protein—histone deacetylase 1 or HDAC1 (formerly HD1, renamed HDAC1 according to GDB Genetic Nomenclature Guide)—have been cloned (Taunton et al., 1996b). HDAC1 is approximately 60% identical to the S. cerevisiae Rpd3p protein, which is a component of a yeast histone deacetylase complex (Rundlett et al., 1996). Single mutations in either *RPD3* or *SIN3* give the same phenotypes as *RPD3/SIN3* double mutants, suggesting that they function in the same pathway (Stillman et al., 1994). Because Mad family proteins use mSin3A as a corepressor and Mad1 can repress transcription in wild-type yeast but not yeast having a null mutation in *SIN3* (Kasten et al., 1996) or *RPD3* (D. J. Stillman, personal communication), it is likely that the mechanism of transcriptional repression by Mad proteins may be conserved between yeast and higher eukaryotes. Consistent with this hypothesis, the DNA-binding transcription factor YY1 interacts with a mammalian Rpd3p homolog (mRpd3) to repress transcription of a heterologous reporter gene (Yang et al., 1996a). These results demonstrate that mRpd3-like activity functions in transcriptional regulation.

Several lines of evidence suggest that the acetylation status of conserved lysines in the amino-terminal domains of histones H3 and H4 play a role in the regulation of transcription. In general, histone hyperacetylation correlates with transcriptionally active or poised genes; conversely, hypoacetylation correlates with transcriptionally repressed heterochromatin (for reviews, see Turner, 1993; Loidl, 1994; Wolffe, 1996). While little is known about the targeting and regulation of histone acetyltransferases and deacetylases, it has been recently shown that several transcriptional coactivators possess inherent acetyltransferase activity (Brownell et al., 1996; Ogryzko et al., 1996) or associate with acetyltransferases (Yang et al., 1996b). We report that mSin3A and HDAC1 associate in vivo and that the histone deacetylase inhibitor trapoxin interferes with mSin3A-mediated transcriptional repression.

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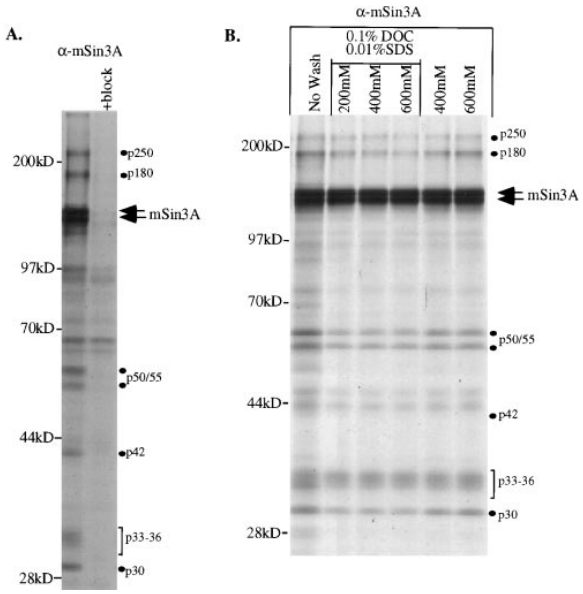

Figure 1. mSin3A Is Present in Cells as a Large Stable Multiprotein Complex

Nuclear lysates were prepared from U937 cells metabolically labeled with ^{35}S-methionine and low stringency immunoprecipitations performed with antiserum specific for mSin3A. ''+block'' shows proteins immunoprecipitated when the anti-mSin3A was preincubated with purified GST-PAH2 (A). In (B), low stringency mSin3A immunoprecipitates were washed for an additional 60 min using the salt and detergent conditions indicated. In (A) and (B), the immunoprecipitates were analyzed by SDS–PAGE and autoradiography. Apparent molecular mass of the coprecipitating proteins and the sizes of the molecular mass markers are given in kilodaltons.

Results

mSin3A Is Present in Cells as a Large Stable Multiprotein Complex

To study the in vivo function of mSin3A we generated polyclonal antiserum specific for the PAH2 domain of mSin3A. We tested this antiserum by immunoprecipitation using nuclear lysates made from the myeloid leukemia cell line U937 that had been metabolically labeled with ^{35}S-methionine. Analysis of immunoprecipitates showed an intensely labeled doublet with an apparent molecular mass of 150 kDa that was present in the anti-mSin3A immunoprecipitates (Figure 1A). This doublet comigrated with in vitro–translated mSin3A, shared identical V8 protease digestion profiles with in vitro–translated mSin3A, and was absent from immunoprecipitations using preimmune serum or immune serum preincubated with the cognate immunogen (data not shown).

Fractionation of U937 nuclear extracts by size exclusion chromatography indicated that mSin3A is present in large molecular weight complex(es) (D. E. A., unpublished). To address this possibility, we performed immunoprecipitations from metabolically labeled U937 cells under conditions that should preserve protein–protein interactions. In addition to mSin3A, the low stringency mSin3A immunoprecipitates contained several labeled polypeptides of apparent molecular masses of 250 kDa, 180 kDa, 55 kDa, 50 kDa, 42 kDa, 33–36 kDa, and 30 kDa (Figure 1A). These proteins were not detected in immunoprecipitates using mSin3A antiserum blocked with the cognate immunogen, suggesting that the proteins detected are specifically associated with mSin3A. Furthermore, none of these proteins were detected using high stringency immunoprecipitation or by Western blotting of whole-cell lysates using anti-mSin3A, suggesting that they do not share epitopes with mSin3A and are not proteolytic breakdown products of mSin3A (data not shown). All of the associated proteins appear to be present in substoichiometric amounts to mSin3A, suggesting that mSin3A complexes are heterogeneous.

To test the stability of the mSin3A complex, we subjected low stringency mSin3A immunoprecipitates to different salt concentrations and ionic detergent conditions. The proteins that remained bound to mSin3A in the immunocomplex were analyzed by SDS–PAGE. Under the most stringent conditions, we observed only a slight loss of mSin3A-associated proteins in the immunocomplex (Figure 1B). One exception to this finding was the apparently quantitative loss of p42 under slightly elevated salt concentrations. These findings demonstrate that the mSin3A complex is stable in vivo and suggest that some or all of the mSin3A-associated proteins may facilitate mSin3A function as a transcriptional corepressor.

HDAC1 and RbAp48 Are Components of the mSin3A Complex

Because Sin3p and Rpd3p appear to function in the same pathway in yeast and two components of the mSin3A complex, p50 and p55, are similar in apparent molecular weight to HDAC1, we hypothesized that HDAC1 or related proteins might be components of the mSin3A repressor complex. To test this hypothesis, the proteins bound to mSin3A immunocomplexes were eluted with ionic detergents and reprecipitated with affinity-purified antibodies specific for an internal peptide of HDAC1. Only two of the proteins eluted from the mSin3A complex were reprecipitated by HDAC1 antiserum (Figure 2A). These polypeptides comigrated with p50 and p55 from the low stringency mSin3A immunoprecipitation, suggesting that proteins highly related to HDAC1 are complexed to mSin3A in vivo. The p55 polypeptide comigrates with in vitro–translated HDAC1 and is recognized by an antibody specific for a carboxy-terminal epitope unique to HDAC1. Another cDNA encoding an HDAC1 homolog, HDAC2, has recently been identified (Yang et al., 1996a). It is likely that p50 represents HDAC2 (data not shown and accompanying paper, Laherty et al., 1997 [this issue of Cell]).

In a reciprocal experiment, we performed low stringency immunoprecipitations using antiserum specific for an epitope at the carboxyl terminus of HDAC1. HDAC1 immunoprecipitates contain several proteins that were specifically competed with the immunizing peptide (Figure 2B). A polypeptide doublet that comigrated with mSin3A was detected in the HDAC1 immunocomplexes (Figures 2B and 2C). To confirm that the doublet coprecipitating with HDAC1 is mSin3A, the HDAC1 immunocomplex was eluted and reprecipitated with antiserum specific for mSin3A (Figure 2C). The two proteins in this precipitate comigrated with mSin3A,

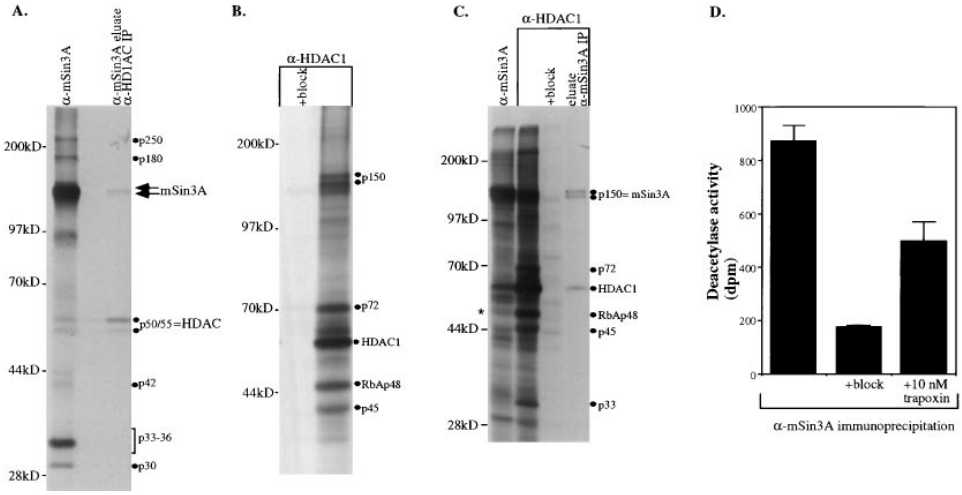

Figure 2. mSin3A and HDAC1 Associate In Vivo

Immunoprecipitations were performed using nuclear extracts from ^{35}S-methionine-labeled U937 cells. (A) The left lane shows proteins from an anti-mSin3A immunoprecipitate. The right lane shows proteins eluted from an anti-mSin3A immunoprecipitate and reprecipitated with antiserum specific for HDAC1. In (B) and (C), low stringency immunoprecipitations were performed using antiserum specific for the carboxyl terminus of HDAC1. ''+block'' indicates that the HDAC1 antiserum was preincubated with the immunizing peptide. In (C), proteins immunoprecipitated with anti-mSin3A are shown for reference, proteins eluted from a low stringency anti-HDAC1 immunoprecipitate and reprecipitated with anti-mSin3A are shown in the far right lane. In (A), (B), and (C), autoradiographs of SDS-PAGE gels are shown. Apparent molecular mass of the coprecipitating proteins and the sizes of the molecular mass markers are given in kilodaltons. In (D), in vitro histone deacetylase activity in anti-mSin3A immunoprecipitates is shown. Human Jurkat cell extracts (12 mg) were immunoprecipitated using anti-mSin3A polyclonal antibodies. ''+block'' indicates that the anti-mSin3A antibody was preincubated with GST-PAH2; ''+10 nM trapoxin'' indicates that the immunoprecipitated proteins were pretreated with 10 nM trapoxin for 30 min at 4°C prior to being assayed for histone deacetylase activity.

confirming that mSin3A and HDAC1 are associated in vivo.

To determine whether HDAC1 associated with mSin3A in vivo is enzymatically active, we assayed low stringency immunoprecipitates for histone deacetylase activity. We used a synthetic peptide corresponding to the first 24 amino acids of histone H4 as a substrate for our deacetylase assay (Taunton et al., 1996b). Low stringency anti-mSin3A immunoprecipitates contained deacetylase activity; however, only background levels of deacetylase activity were detected in the immunoprecipitates if the mSin3A anti-serum was blocked with cognate immunogen (Figure 2D). To confirm the authenticity of the mSin3A-associated activity, we treated the immunoprecipitates with synthetic trapoxin, a specific inhibitor of histone deacetylase activity (Taunton et al., 1996a). Treatment of mSin3A complexes in vitro with 10 nM trapoxin reduced deacetylation by approximately 50% (Figure 2D), suggesting that the precipitated deacetylase activity can be attributed to trapoxin-sensitive histone deacetylases bound to mSin3A.

We detected an interaction between HDAC1 and RbAp48 in vivo (Figures 2B and 2C; also Taunton et al., 1996b). The low stringency mSin3A immunoprecipitation shown in Figure 2C also contained a protein that comigrated with RbAp48 (marked with an asterisk) that was not readily visible on the shorter exposures of low stringency mSin3A immunoprecipitations (Figure 2A). We have identified RbAp48 in mSin3A immunoprecipitates from cell extracts of nontransfected cells by Western blotting, further demonstrating that mSin3A and RbAp48 associate in vivo (Figure 3A).

To address further the association between HDAC1 and RbAp48 with mSin3A, we expressed the mammalian proteins in insect cells using recombinant baculoviruses. To this end, we expressed recombinant FLAG-epitope-tagged HDAC1 (HDAC1-F), which could be immunopurified by anti-FLAG antibodies, and histidine-tagged mSin3A (mSin3A-H), which could be purified by nickel affinity (data not shown). HDAC1-F was immunoprecipitated from infected Sf9 cell extracts by anti-FLAG antibodies in the presence or absence of mSin3A-H. HDAC1-F was also precipitated by Ni^{2+}-NTA agarose in a manner that was dependent on coexpression of mSin3A-H (Figure 3B), demonstrating that a complex between HDAC1 and mSin3A is formed in insect cells using exogenously expressed human proteins.

Consistent with our finding that RbAp48 is associated with mSin3A and HDAC1 in vivo, we show that baculovirus-expressed Flu-epitope-tagged RbAp48 (p48-HA) is specifically precipitated from infected Sf9 cell extracts using anti-FLAG antibody only when HDAC1-F is coexpressed. Furthermore, p48-HA is specifically retained by Ni^{2+}-NTA in the presence of mSin3A-H (Figure 3C). Coexpression of p48-HA did not appear to affect the association between HDAC1-F and mSin3A-H, suggesting that the regions of interaction are distinct and that all three proteins can associate simultaneously. These data suggest a direct interaction between mSin3A, HDAC1, and RbAp48 in vivo.

Transcriptional Repression by mSin3A Requires Histone Deacetylase Activity

To investigate whether histone deacetylation plays a role in mSin3A-mediated transcriptional repression in vivo, we examined mSin3A-specific repression in the

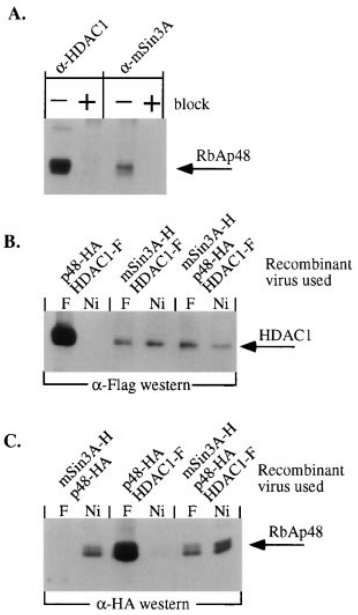

Figure 3. RbAp48 Is Associated with mSin3A In Vivo and Recombinant HDAC1, RbAp48, and mSin3A Copurify from Insect Cell Extracts

(A) TAg Jurkat cell lysates were immunoprecipitated using antibodies specific to the C-terminal portion of HDAC1 (left) or antibodies specific to PAH2 of mSin3A (right). Parallel immunoprecipitations were blocked as described in Figure 2. Immunopurified proteins were analyzed by SDS–PAGE and immunoblotted with α-RbAp48 monoclonal antibody 12B1.

(B and C) Equal amounts of baculovirus-coinfected Sf9 cell extracts were affinity purified using Ni^{2+}-NTA-agarose (Ni) or α-FLAG-M2-agarose (F). Purified recombinant proteins were analyzed by SDS–PAGE, transferred to Immobilon-P (Millipore) and immunoblotted with α-FLAG to detect HDAC1-F (B) or α-Flu (12CA5) to detect p48-HA (C). We observe a reduction in expression of HDAC1-F and p48-HA when coexpressed with mSin3A.

presence and absence of the histone deacetylase inhibitor trapoxin. Human 293 cells were transfected with a luciferase reporter gene construct containing a minimal promoter consisting of only a TATA box and initiation site derived from the myelomonocytic growth factor gene (Figure 4A). This reporter has four consensus binding sites for the DNA binding domain of the S. cerevisiae transcriptional activator GAL4 and therefore is responsive to chimeric proteins containing the GAL4 DNA binding domain (GALDBD) (Sterneck et al., 1992). We have used this reporter construct previously to demonstrate that fusion of the SID repressor region of Mad1 to the GALDBD is necessary for mSin3A-dependent transcriptional repression. Furthermore, we have shown that fusion of SID to the potent transcriptional activator GALVP16, MadN35GALVP16, can cancel the activation function of VP16 in an mSin3A-dependent manner. Consistent with our previous results (Ayer et al., 1996), MadN35GALVP16 activated transcription from the reporter gene approximately 100-fold less well than GALVP16 (data not shown). As a negative control, we engineered two proline substitutions into the SID of Mad1; this Mad(Pro) protein cannot bind mSin3A in vitro (Ayer et al., 1995). Consistent with an inability to interact with mSin3A, Mad(Pro)N35GALVP16 is a much less potent repressor (Figure 4B). In control experiments, we have shown that the observed effects require the presence of GAL4 sites in the promoter and that both MadN35GALVP16 and Mad(Pro)N35GALVP16 are expressed to equivalent levels in these cells and bind GAL4 sites with similar affinities (data not shown). To test the role of histone deacetylation on the repression observed in our transfection assays, we first examined the effect of trapoxin on histone deacetylase activity in 293 cells. As expected, in vivo treatment with 10 nM trapoxin for 8 hr reduced deacetylase activity of both crude 293 extracts and anti-HDAC1 immunopurified complexes by approximately 46% and 58%, respectively (Figure 4C).

To test the effect of a histone deacetylase inhibitor on MadN35GALVP16- and Mad(Pro)N35GALVP16-mediated repression, we treated a duplicate set of transfections with 10 nM trapoxin for 8 hr prior to harvest. In the representative experiment shown, 10 nM trapoxin treatment derepressed the activity of MadN35GALVP16 9-fold while it had little effect on the activity of Mad(Pro)N35GALVP16, suggesting that histone deacetylation plays a direct role in mSin3A transcriptional repression (Figure 4B). In addition, there was typically less than a 2-fold effect of trapoxin on the activity of the reporter gene in cells transfected with the expression vector alone or in cells transfected with GALVP16 (data not shown). Following trapoxin treatment, the repression observed for MadN35GALVP16 was still seven times greater than that of Mad(Pro)N35GALVP16, suggesting that the residual deacetylase activity following trapoxin treatment (Figure 4B) continues to drive mSin3A-mediated repression; however, we can not rule out that mSin3A is capable of repression by mechanisms independent of histone deacetylation.

Discussion

Earlier studies implicated mSin3 as the primary candidate responsible for the negative transcriptional function of the DNA-binding transcription factor Mad (Ayer et al., 1995; Schreiber-Agus et al., 1995; Ayer et al., 1996). We present evidence that the mSin3A corepressor is part of a high molecular weight, multicomponent complex(es) that contains active histone deacetylase, thereby implicating histone deacetylation as a potential mechanism for mSin3A-mediated repression. Furthermore, we observe a pronounced increase in the transcriptional activity of an mSin3A-silenced reporter gene upon treatment in vivo with the specific histone deacetylase inhibitor trapoxin, suggesting that full transcriptional repression by mSin3A requires histone deacetylation. These results suggest a mechanism of gene regulation through the targeting of an enzyme that alters chromatin structure.

These observations are consistent with genetic experiments in yeast, suggesting that the yeast orthologs of mSin3A and HDAC1, Sin3p and Rpd3p, respectively, are epistatic transcriptional regulators (Stillman et al., 1994). Furthermore, recent biochemical evidence demonstrates that Rpd3p is a component of a large molecular weight histone deacetylase complex in yeast (Rundlett

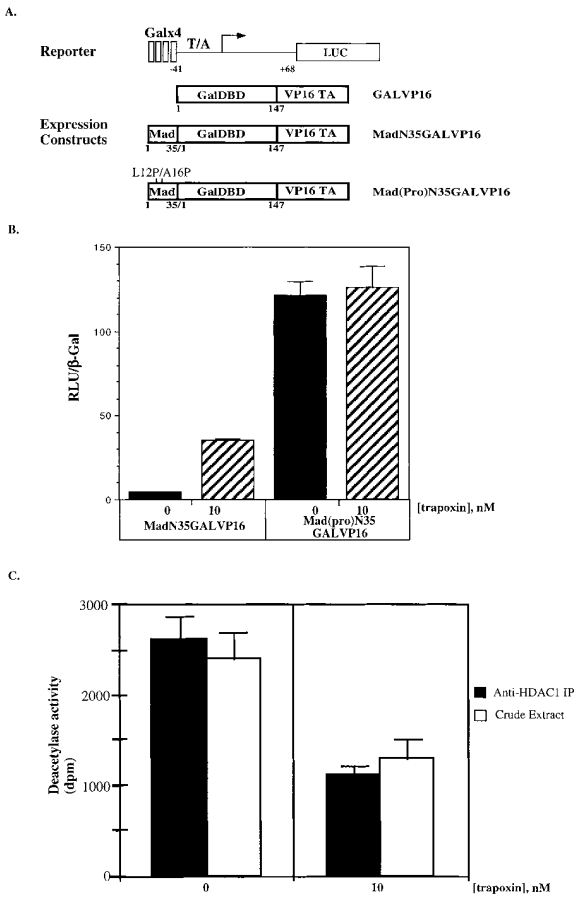

Figure 4. Trapoxin Reverses Transcriptional Repression by mSin3A

(A) The structure of the minimal reporter gene derived from the myelomonocytic growth factor gene and the expression vectors. Mad(Pro)N35GALVP16 has leucine at position 12 and alanine at position 16 mutated to proline as indicated. These point mutations prevent association between mSin3A and Mad (Ayer et al., 1995).

(B) The transcriptional activity of MadN35GALVP16 and Mad(Pro)N-35GALVP16 was determined by measuring luciferase activity (relative light units, RLU) of transfected 293 cells following an 8 hr treatment with 0 (closed bars) or 10 nM trapoxin (striped bars). To control for differences in transfection efficiency, the RLU values were normalized to the β-galactosidase activity produced by a cotransfected CMV-βGAL construct. Shown is data from a representative experiment, and the error (SEM). This experiment has been done a minimum of five times in triplicate with similar results. An 8 hr treatment of 293 cells with 10 nM trapoxin is within the linear range of the response of the reporter gene. Furthermore, trapoxin treatment did not prevent association between mSin3A and HDAC1 (data not shown).

(C) Trapoxin inhibits histone deacetylase activity of human 293 cells in vivo. Cells (2×10^8) were cultured for 8 hr in the absence (0) or in the presence of (10) 10 nM trapoxin. Cells were harvested and crude extracts from approximately 1×10^7 cells (closed bars) or anti-HDAC1 immunoprecipitations of extracts from approximately 4×10^7 cells (open bars) were assayed for histone deacetylase activity in vitro.

et al., 1996). Together with our results, these findings predict a conservation of the mSin3/HDAC1 functional association in yeast.

We have used chimeric transcriptional regulators to discern the effects of trapoxin on the activity of our reporter genes. The MadN35GALVP16 chimera functioned as a repressor by a mechanism that was dependent on the binding of mSin3A and that was sensitive to trapoxin. The same mutations that inactivate MadN3-5GALVP16 as a transcriptional repressor (i.e., Mad-(Pro)N35GALVP16), also block interaction between Mad1 and mSin3A in vitro and Mad1 function in vivo. Therefore, it is likely that Mad:Max heterocomplexes repress transcription in a manner dependent on an mSin3A-associated histone deacetylase.

By coimmunoprecipitation, we have demonstrated that mSin3A and HDAC1 associate in vivo. Consistent with these data, we observed nuclear colocalization of mSin3A and HDAC1 by immunofluorescence microscopy (data not shown). Finally, overexpression in insect cells facilitates copurification of mSin3A and HDAC1 (Figures 3B and 3C), suggesting that the interaction between mSin3A and HDAC1 is either direct or requires a conserved cofactor. The finding that mSin3A has different associated histone deacetylases (HDAC1 and HDAC2) suggests that the mSin3A complex(es) may have multiple substrate or target specificities. The heterogeneous nature of the mSin3A complex potentially reflects a diverse array of repressors, histone deacetylases, and different targeting molecules that facilitate mSin3A-dependent alterations in gene expression.

At least five additional polypeptides, whose functions are currently unknown, are stably associated with mSin3A (Figures 1 and 2), but tight association with mSin3A in both U937 cells and Jurkat T cells (data not shown) suggests that they in some way mediate mSin3A function. Furthermore, we have identified an association between mSin3A and RbAp48 in vivo, suggesting that this protein may play a role in regulating mSin3A-targeted deacetylation. RbAp48 was originally identified as a retinoblastoma-binding protein that contains WD repeats and shares homology with the β subunit of G proteins (Qian et al., 1993). Subsequently, it has been shown that RbAp48 or its orthologs are involved in targeting different histone modifying enzymes to chromatin (Parthun et al., 1996; Taunton et al., 1996b; Tyler et al., 1996; Verreault et al., 1996). The mSin3A/RbAp48 complex isolated from U937 cells (Figure 2) is likely to represent only a small fraction of the mSin3A complexes, but its detection implies that mSin3A may play a role in the control of different aspects of chromatin physiology as well as transcriptional repression.

It is unclear how different chromatin states facilitate transcriptional repression and activation or how their distinct biochemical states arise; however, there is ample cytological, genetic, and biochemical evidence supporting the model that hyperacetylated chromatin is transcriptionally more active than hypoacetylated chromatin. Acetylation levels in β-heterochromatin of Drosophila melanogaster polytene chromosomes are significantly reduced at lysine positions 5, 8, and 16 of histone H4, while the transcriptionally hyperactive X chromosome of male flies is uniquely hyperacetylated at position 16 (Turner et al., 1992). In yeast, mutation of acetyl-accepting lysines in histone H4 reduces the activity of the *GAL1*, *PHO5*, and *CUP1* promoters in vivo (Durrin et al., 1991). The transcriptionally silent regions in yeast, HML and HMR, are hypoacetylated, and their activation

is correlated with acetylation of histone H4 (Braunstein et al., 1993). Additionally, biochemical studies showed that certain transcription factors have higher affinity for their binding sites when those sites are embedded in chromatin assembled from hyperacetylated histones (Lee et al., 1993; Vettese-Dadey et al., 1996). Finally, evidence suggesting that acetylation is required for activation comes from the recent demonstration that several coactivators are acetyltransferases or are associated with acetyltransferases. Thus, our data support this general model for the control of gene expression by histone acetylation status and provide a biochemical mechanism for deacetylation-mediated repression.

The acetylation status of a particular chromatin region represents a balance between competing acetylation and deacetylation reactions. We propose that MadN35-GALVP16 recruits mSin3A–HDAC complexes to specific sites on DNA and shifts this equilibrium toward deacetylation and subsequent transcription repression by creating a high effective molarity of the histone deacetylase. In yeast, the activation domain of VP16 has been shown to use the acetyltransferase Gcn5p as a coactivator (Marcus et al., 1994; Brownell et al., 1996), suggesting that in mammalian cells VP16 will also use an acetyltransferase as a cofactor. Thus, trapoxin treatment could shift the equilibrium from deacetylation to acetylation and thereby drive activation.

Whether histone deacetylation will always have a negative effect on gene expression is unclear. Mutants in *SIN3* and *RPD3* can have both positive and negative effects on gene expression (Vidal and Gaber, 1991; Yoshimoto et al., 1992); however, for *SIN3* there is evidence that positive effects may be indirect (Wang et al., 1994). In addition, mutations or deletions in *RPD3* have recently been shown to enhance telomeric silencing both in yeast and in fruit fly (Sussel et al., 1995; De Rubertis et al., 1996; Rundlett et al., 1996). In mammalian cells, deacetylase inhibitors can inhibit MyoD- (Johnston et al., 1992) and steroid receptor–activated transcription (McKnight et al., 1980; Bresnick et al., 1990). While it remains to be shown that the effects of *RPD3* on silencing are direct, this evidence suggests that histone deacetylation can elicit both positive and negative effects on gene expression. Determining the factors that govern the functional outcome of histone deacetylation will provide fertile ground for further experimentation.

Experimental Procedures

Antibodies, Cell Culture, and Immunoprecipitations

To generate antiserum specific for mSin3A, a GST fusion protein encoding amino acids 251–405 of mSin3A was used to immunize a New Zealand White rabbit. The crude serum was passed over a GST column to remove the anti-GST antibodies. U937 cells were grown in RPMI supplemented with 10% calf serum (Hyclone), glutamine, and penicillin-streptomycin. Low and high stringency immunoprecipitations were performed essentially as described (Ayer and Eisenman, 1993). To elute proteins from low stringency immunoprecipitates, they were incubated for 60 min at room temperature in antibody buffer and reprecipitated under high stringency conditions.

Luciferase Assays

293 cells were seeded in triplicate onto 60 mm dishes at 3×10^5 cells in 4 ml DME with 10% calf serum (Hyclone). Six hours after seeding, cells were transfected with 50 ng luciferase reporter, 50 ng CMV-β-gal, 50 ng expression construct, and 2.85 μg carrier DNA using the BBS/$CaPO_4$ method. Trapoxin was added to 10 nM 8 hr prior to the luciferase assays. Cell lysates were prepared 20–24 hr following transfections, and luciferase and β-galactosidase activities were assayed according to manufacturers' directions (Promega, Tropix). Luciferase values (relative light units) were normalized for transfection efficiency by dividing by β-gal activity.

Histone Deacetylase Assays

In vitro histone deacetylase activity was assayed essentially as described with either 50 μl of crude cell extract (approximately 5×10^6 cells) or immunopurified cell extracts (approximately 2×10^7 cells) for 2.5 hr at 37°C (Taunton et al., 1996b). Pretreatment of crude or immunopurified extract with synthetic trapoxin was performed for 30 min at 4°C prior to addition of peptide substrate. TAg Jurkat and 293 cell extracts for histone deacetylase assays were prepared as in Taunton et al., 1996b. Anti-HDAC1 and anti-mSin3A immunoprecipitations were performed as described above and in Figure 2. The Protein A–conjugated immunoprecipitates were washed three times in J buffer plus 1 mM EDTA and resuspended in J buffer without Triton X-100, and histone deacetylase activity was measured as described.

Baculoviruses

cDNAs encoding FLAG-tagged HDAC1, HA-tagged RbAp48, and His-tagged mSin3A were cloned into the transfer vector pVL1392 (specific details on the construction of these vectors are available upon request). Recombinant virus was generated using Baculogold DNA according to the manufacturer's instructions (Pharminigen). Sf9 or High 5 cells were infected at high multiplicity, extracts prepared 48 hr postinfection, and immunoprecipitations performed as described above. Ni^{2+}-NTA agarose and anti-FLAG antibody were purchased from Qiagen and Kodak-IBI, respectively.

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