Functional Analysis of the Mad1-mSin3A Repressor-Corepressor Interaction Reveals Determinants of Specificity, Affinity, and Transcriptional Response

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Received 12 August 2003/Returned for modification 27 October 2003/Accepted 22 December 2003

The recruitment of corepressors by DNA-bound repressors is likely to be a critical rate-limiting step in the transcriptional regulation of many genes. An excellent paradigm for such an interaction is the association of the basic helix-loop-helix zipper protein Mad1 with the corepressor mSin3A. When bound together, the Sin3 interaction domain (SID) of Mad1 forms extensive hydrophobic contacts with the four-helix bundle formed by the paired amphipathic helix 2 (PAH2) domain of mSin3A. Using the costructure to predict the principle residues required for binding, we have carried out an extensive mutational analysis to examine the Mad1 SID-mSin3A PAH2 interaction in vitro and in vivo. Bulky hydrophobic residues in the α1 (I308 and V311) and α2 (L329 and L332) helices of the PAH2 domain are necessary to accommodate the precise arrangement of bulky (L12) and short (A15 and A16) hydrophobic residues in the amphipathic Mad1 SID. We have also used phage display to derive an optimal SID, which shows an essentially identical arrangement of key residues. By manipulating these key residues, we have generated altered-specificity Mad1 SID mutants that bind only to a PAH2 domain with a reciprocal mutation, permitting us to demonstrate for the first time that these domains interact directly in vivo. We have also found that the integrity of the PAH1 domain affects the Mad1 SID-PAH2 interaction. It is conceivable that cross talk between different PAH domains and their binding partners helps to determine the subunit composition and order of assembly of mSin3A complexes.

An important mechanistic link between transcriptional regulation and chromatin structure is the association of sequence-specific DNA-binding transcription factors with coactivators and corepressors (3, 11, 46, 57). Coactivator and corepressor recruitment is generally considered to underlie the gene-specific alterations in chromatin structure that accompany changes in gene expression during differentiation, development, and oncogenesis. An emerging theme is that many different transcription factors recruit the same set of coactivators and corepressors. For example, transcriptional activation mediated by p53, c-Myb, CREB, MyoD, MEF2, and different nuclear hormone receptors, among others, involves recruitment of the coactivator p300 or CBP, each of which possesses and binds histone acetyltransferases (for a review, see reference 54). Similarly, corepressor complexes such as mSin3 and NURD have been found to interact with many DNA-binding repressors as well as with histone deacetylases (HDACs) and other factors involved in the modification of chromatin to a repressive state (for reviews, see references 4 and 28). Given this multiplicity of associations, both the nature and the specificity of the interactions between transcription factors and their coactivators and corepressors take on considerable importance.

We have been studying the interactions between Mad proteins and the mSin3A corepressor complex. Mad family proteins (comprising Mad1, Mxi1, Mad3, and Mad4) belong to the basic helix-loop-helix zipper (bHLHZ) class and heterodimerize with the small bHLHZ protein Max to bind DNA (6, 24, 42, 72). Mad-Max heterodimers recognize the E-box sequence CA CGTG and repress transcription, in an E-box-dependent manner, from synthetic reporter genes as well as from a number of endogenous cellular target genes. Both overexpression and targeted deletion studies of Mad family proteins have demonstrated that they function to mediate the arrest of cell growth and proliferation, often in conjunction with terminal differentiation of many cell types (15). In this sense, Mad proteins appear to antagonize, at least in part, the functions of the transcriptional activator and oncoprotein Myc, which also dimerizes with Max, binds the same CACGTG consensus sequence, and has a significant number of target genes in common with Mad-Max (for reviews, see references 17, 38, 39, 53, and 75).

In addition to the bHLHZ domain, required for association with Max and DNA binding, Mad proteins contain a highly conserved 32-amino-acid N-terminal region which is necessary for Mad transcriptional repression and is capable of conferring repression when fused to heterologous DNA-binding domains (7, 8, 52). This segment is required for the ability of Mad proteins to inhibit cell proliferation, thereby underscoring the biological relevance of Mad-induced repression (30, 34, 49, 51). Importantly, this amino-terminal Mad repression domain
(termed the Sin3 interaction domain [SID]) was found to associate with the mammalian corepressor paralogs mSin3A and mSin3B (8, 52). Sin3p, the Saccharomyces cerevisiae ortholog of mammalian Sin3 proteins, had long been known to associate with transcriptional repression (58, 60, 61). The regions most highly conserved between yeast and vertebrate Sin3 proteins are four imperfect repeats, known as paired amphipathic helix (PAH) domains, and an ~400-amino-acid region located between PAH 3 (PAH3) and PAH4 (8, 52, 60). Mad family proteins interact directly with the PAH2 region of mSin3A and mSin3B. While no Mad family orthologs exist in yeast, mammalian Mad1 is also able bind to the PAH2 domain of S. cerevisiae Sin3p (27). In addition, Drosophila Sin3 associates with the SID region of Dmmt, a fly ortholog of Mad, to mediate transcriptional repression (L. W. M. Loo et al., unpublished data). Thus, the interactions between Mad proteins and Sin3 corepressors are evolutionarily conserved.

While the mammalian forms of Sin3 were first identified through their association with the Mad family proteins, subsequent work from many laboratories has shown them to be involved in multiple protein-protein interactions (4, 28). In general, three classes of Sin3-associated proteins have been defined. The first class consists of enzymes that perform post-translational modifications. These include HDAC 1 and 2 (2, 20, 26, 33), Swi/Snf chromatin remodeling complex subunits (55), O-linked N-acetylgalactosamine transferase (69), and histone methyltransferases (43, 66). The second group comprises a large number of DNA-binding transcription factors that have been reported to bind mSin3. These include the Mad family repressors p53 (40) and MNF/Neu, the neural gene repressor P53 (56). The structural studies have indicated the existence of multiple types of the Sin3 complex, the relative amounts and compositions of which remain to be determined.

To elucidate the sequence and structural requirements for transcription factor binding to mSin3, the solution structures for Mad1 SID and the PAH2 domain of mSin3A and mSin3B have been determined (9, 56). The structural studies have demonstrated that the Mad1 SID peptide exists as a random coil and the free mSin3A PAH2 region appears to be only partially folded, apparently adopting two distinct conformations in the unbound state. Upon complex formation, both Mad1 SID and PAH2 undergo mutual folding transitions, with Mad1 SID folding into an amphipathic α helix while the PAH2 domain folds into a left-handed four-helix bundle. All four helices within the bundle contribute side chains to a deep hydrophobic cleft defined principally by α1 and α2 helices. It is into this groove that the hydrophobic face of the Mad1 SID α helix is wedged (Fig. 1A). A limited site-directed mutational analysis of the interaction with full-length mSin3A confirmed the importance of hydrophobic residues on both the floor and the rim of the PAH2 hydrophobic cleft for binding to Mad1 SID in vitro. In contrast, mutation of noninteracting solvent-exposed residues had no detectable effect on binding (9).

The interaction of a DNA-bound repressor with its corepressor is likely to be the critical rate-limiting step in the regulation of many genes. In this respect, we believe that the Mad1-mSin3A interaction is an excellent paradigm, prompting us to investigate the Mad1-mSin3A interaction in greater detail. We have extended our mutational analysis and examined the binding of mutant PAH2 and Mad1 SID more extensively both in vitro and in vivo. This analysis has permitted us to predict and design altered-specificity mutants for these domains. We have also used phage display to isolate an optimal PAH2-binding peptide. Finally, we have obtained initial evidence that Mad1 SID binding to mSin3A PAH2 is influenced by PAH1, suggesting that the PAH repeats in Sin3 function cooperatively to facilitate specific interactions.

MATERIALS AND METHODS

Plasmids. Mutations were introduced into the PAH2 domain of mSin3A by recombinant PCR with Pfu polymerase (Stratagene). PCR fragments containing the desired mutations were cloned into the unique PstI and Stul sites of full-length mSin3A cDNA in pCS2.MT. PCR fragments containing truncated mSin3A molecules (amino acids [aa] 1 to 388 and aa 291 to 899) were also generated using specific oligonucleotides and inserted into the EcoRI site of pSG5. Glutathione S-transferase (GST)-PAH2 (aa 291 to 899) and mutant derivatives were generated by PCR with pCS2.MT.mSin3A plasmids as templates and inserted into the BamHI site of pGEX2TK. Plasmids expressing PAH2-VP16 fusions were generated in the same manner and then cloned into the EcoRI site of pSG.VP16 (10). The GST.Mad1-SID construct (aa 1 to 27) was generated by annealing, phosphorylating 3′ phosphates, and then cloning complementary oligonucleotides into the BamHI site of pGEX2TK. Mutant Gal4.Mad1-SID and GST.Mad1-SID constructs were generated by using the same primers in conjunction with a QuikChange mutagenesis kit (Stratagene) according to the manufacturer’s instructions. Proline mutations in each of the PAH domains (PAH1 to PAH4) were also generated by using specific oligonucleotides in conjunction with the QuikChange mutagenesis kit. All constructs were verified by sequencing.

GST pulldown assays. Recombinant cDNAs for mSin3A and mutant derivatives in vector pCS2.Myctag were transcribed and translated in vitro by using a TNT reticulocyte lysate kit (Promega) in the presence of [35S]methionine according to the manufacturer’s instructions. The GST.Mad1-SID construct and mutant derivatives were expressed in Escherichia coli DH5α and purified using glutathione-Sepharose (Pharmacia). mSin3A proteins labeled with [35S]methionine were incubated with the GST.Mad1-SID construct bound to Sepharose beads in a buffer containing 20 mM Tris (pH 8.0), 1 mM EDTA, and 0.5% Nonidet P-40 for 3 h and then washed three times in the same buffer. Bound proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then visualized with a Storm PhosphorImager.

Cell culture and transfection experiments. 293 cells were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum, penicillin-streptomycin, and glutamine. Cells were transiently transfected with plasmid constructs by using Fugene (Roche) according to the manufacturer’s instructions. For transient repression assays, cells were plated on gelatinized 24-well tissue culture plates and then transfected with Gal4.LexA.TATA.Luc (500 ng), β-actin.LacZ control plasmid (150 ng), pSG.Mad1-SID (100 ng), and Lex.AVP16 (100 ng). For mammalian two-hybrid assays, the same reporter constructs were used in addition to pSG.Mad1-SID (100 ng) and pSG.VP16-PAH2 (100 ng) constructs. A constant amount of DNA was maintained in each sample by the addition of empty vector pSG5. At 48 h after transfection, cells were harvested and assayed for luciferase and β-galactosidase activities by using standard protocols (3a).
Fluorescence polarization assay. Purified GST-PAH2 (aa 291 to 388) was added in increasing amounts (2 to 500 nM) to a constant amount (2 nM) of fluorescein-tagged Mad1 SID peptide (aa 8 to 20) in phosphate-buffered saline-0.01% bovine serum albumin and incubated for 30 min at room temperature in the dark. The polarization of each sample was measured in duplicate by using a fluorescence polarization system (Beacon), and the changes were recorded.

Differential peptide phage display selection. The L8 phage display library expresses completely degenerate 8-aa peptides in a linear sequence and was described in detail previously (16, 59). PFU containing 2,000 times the library complexity (3.6 × 10^11 PFU for round 1 and 5 × 10^10 PFU for round 2) were applied to GST-PAH2 in a total volume of 200 μl of phosphate-buffered saline-0.1% Tween 20. Each round of phage library screening consisted of three cycles of affinity purification with GST-PAH2 (100 μg for cycle 1 and 50 μg for cycles 2 and 3) on glutathione-agarose. Phage specific for GST were removed by immunodepletion with 100 μg of agarose-bound GST after cycles 1 and 2 of affinity purification (differential phage display). The sequences of isolated clones were determined by initially using PCR to amplify the region containing the peptide open reading frame and then directly sequencing the PCR product (59).

RESULTS

Importance of shape complementarity at the interface in Mad1 SID-mSin3A PAH2 interactions. Various investigators had shown previously that replacing hydrophobic residues with charged amino acids at the mSin3A PAH2-Mad1 SID interface perturbed the interaction (9, 12). In order to better understand...
the nature of the hydrophobic contacts made between PAH2 and Mad1 SID, we engineered mutations aimed at altering the length and shape, rather than the charge, of specific residues and then monitored their ability to interact. The principal residues in PAH2 that mediate the interaction with Mad1 SID are hydrophobic amino acids with long side chains—I308 and V311 in the α1 helix and L329 and L332 in the α2 helix—as determined from the PAH2-Mad1 SID holostructure (Fig. 1A) (9). To determine the necessity of these particular residues, we began by replacing them with alanines in the context of full-length mSin3A and measuring their interaction with Mad1 SID in a GST pulldown assay. Substituting alanine for either I308 or V311 in the α1 helix reduces binding to Mad1 SID only marginally, if at all, compared with wild-type binding (Fig. 1B). However, replacing both residues with alanines (I308A/V311A) results in a fourfold reduction in binding. Single alanine substitutions at the leucine residues in the α2 helix had a much more marked effect than their α1-helix counterparts. Alanine point mutations of L329 or L332 reduce the binding of mSin3A by almost fourfold compared to wild-type binding (Fig. 1B, lower panel), whereas an L329A/L332A double mutant abolishes mSin3A binding almost completely (only 4% relative binding compared to the input). Changing the α2-helix leucines to valines (L329V, L332V, and L329V/L332V) also results in submaximal binding to Mad1 SID, emphasizing the requirement for leucines at these positions. Consistent with previous results (9), mutating hydrophobic residues in the α1 helix to aspartic acid (I308D, V311D, and I308D/V311D) completely abolishes the interaction with Mad1 SID, further emphasizing the necessity for hydrophobic residues at the Mad1 SID-PAH2 interface (Fig. 1B). However, mutation of a solvent-exposed residue to aspartic acid (N309D) does not affect binding.

To quantitate the interactions of PAH2 mutants with Mad1 SID more rigorously, we used a fluorescence polarization assay (37). A Mad1 SID peptide (aa 9 to 21 of human Mad1) was synthesized and tagged with fluorescein. Purified PAH2, either wild type or mutant, was added in increasing amounts to a constant amount of Mad1 SID peptide (2 nM), and the change in polarization was measured. The wild-type PAH2 domain bound to the Mad1 SID peptide and produced a classical binding curve with a $K_d$ of $\sim60$ nM (Fig. 2A). Mutation of hydrophobic residues I308, V311, and L329 to Ala reduced the relative binding of the PAH2 domain to the Mad1 SID peptide dramatically. However, we were unable to add PAH2 mutants at sufficient concentrations to produce saturated binding and therefore were unable to measure their affinities for Mad1 SID. As a control, we also mutated N309, the solvent-exposed residue that is not involved in the interaction with Mad1, to Asp; in agreement with the GST-binding assay results, binding was not adversely affected (Fig. 1B, upper panel, and 2A). Mutation of α1-helix residues I308A and V311A caused a drastic reduction in binding in the fluorescence polarization assay, in contrast to the modest effects seen in the GST pulldown assay (Fig. 1B, upper panel). We believe that this result was due to the nature of the pulldown assay, which represents only a single point on the binding isotherm.

The Mad1 SID-PAH2 interaction can also be demonstrated in vivo by using a mammalian two-hybrid assay. The N-terminal 35 amino acids of Mad1 (including SID) were fused to Gal4 (the Gal4.Mad1-SID construct); the resulting fusion was used to recruit PAH2 linked to the VP16 activation domain (PAH2-VP16). Transfection of 293 cells with Gal4.Mad1-SID alone produced weak repression of the reporter plasmid. However, cotransfection of PAH2-VP16 with Gal4.Mad1-SID produced a 60-fold increase in transcriptional activity, indicative of a strong interaction (Fig. 2B). In contrast, Gal4.Mad1-SID showed a markedly reduced interaction with PAH2-VP16 proteins containing the hydrophobic mutations I308A, V311A, I308A/V311A, L329A, and L323A, similar to the results observed in the fluorescence polarization assay.

We conclude that for efficient binding to PAH2, Mad1 SID requires hydrophobic residues to occupy specific positions in the interacting surface, and both the size and the shape of their side chains play roles in binding.

**Correlations between mSin3A binding and transcriptional activity in a functional analysis of the Mad1 SID.** As in PAH2, the amphipathic helix that forms Mad1 SID contains hydro-
phobic residues in a specific spatial arrangement. However, Mad1 SID differs from PAH2 in that it contains hydrophobic residues with both bulky (L12, L13, and L19) and short (A15 and A16) side chains. Mad1 SID does not lie flat across the PAH2 surface; instead, the N terminus protrudes down and into the hydrophobic cleft, in what has been described as a “wedge helix” (56). A leucine at position 12 points directly into a large cleft and likely forms an anchor to stabilize Mad1 SID binding (Fig. 3A shows a schematic diagram). The C terminus of Mad1 SID contains at positions 15 and 16 a pair of alanines which contact V311 and L329 at the end of the α1 helix and the beginning of the α2 helix, respectively. The short side chains of the alanines at positions 15 and 16 accommodate the bulkier hydrophobic side chains of V311 and L329, forming the basis of the “knobs-into-holes” specificity of the Mad1-Sin3 interaction. We would therefore predict that a shorter hydrophobic residue at position 12 or a longer residue at position 15 or 16 would abolish the tight specificity of the interaction. To test this prediction, we used the GST.Mad1-SID construct and changed each of the leucines at positions 12, 13 and 19 to alanines and the alanines at positions 15 and 16 (separately or jointly) to leucines. The resulting GST fusion proteins were used in pulldown experiments to precipitate full-length mSin3A (Fig. 3B). As predicted, an L12A mutation completely abolished Mad1 SID binding to mSin3A, emphasizing its importance in contributing to the affinity of the interaction. Because the surface area formed by the folding of the four PAH2 helices is large, approximately 200 Å², we wanted to test whether PAH2 could accommodate a bulky hydrophobic residue, such as phenylalanine. An L12F mutation in Mad1 SID reduced binding to mSin3A, but not to the same extent as an alanine at the same position (Fig. 3B). Surprisingly, L13A and L19A point mutations had little effect on Mad1 SID binding to mSin3A. Both of these residues make numerous contacts with PAH2; however, they appear to be dispensable for the interaction with mSin3A. A15L and A16L mutations, individually or in combination (A15L/A16L), prevented binding to mSin3A. These particular mutations in effect replace a “hole” with a “knob,” and the “knob-knob” arrangement precludes an interaction.

The recruitment of mSin3A is essential for Mad1 to function as a transcriptional repressor (7, 8, 52). We fused the wild-type and mutant Mad1 SID constructs used in the pulldown assays to the heterologous DNA-binding domain of Gal4 and measured the ability of the fusions to repress transcription, presumably through the recruitment of mSin3A. Mutations in Mad1 SID which prevent Sin3 binding, such as L12A, A15L, and A16L, completely abolish repression (Fig. 3C). However, L13A and L19A, which have little effect on Sin3 binding, still repress transcription. Thus, alterations in specific Mad1 SID residues that influence the anchoring and knobs-into-holes interactions with mSin3A PAH2 correlate well with the ability of Mad1 SID to repress transcription.

**Screen for an optimal PAH2-binding domain.** The PAH2 domain of Sin3A is capable of binding a variety of different proteins in addition to the Mad family proteins, including Pf1, TGIF, TIEG1/2, and MNFβ. With the exception of Pf1, the Sin3-interacting regions of these proteins lack a sequence that resembles that of Mad1 SID. The large variety of PAH2-interacting sequences led us to try to identify an optimal PAH2-binding peptide. To do this, we used purified GST-PAH2 to screen a phage display library containing completely degenerate peptides of 8 aa (16, 59). After three rounds of selection, the interacting clones were isolated, tested for binding to PAH2, and then sequenced. The sequence data from 10 interacting clones are shown in Fig. 4. The purified clones show a remarkably well-conserved consensus sequence with considerable similarity to the Mad family SID sequence. The leucine at position 12 in Mad1, which we demonstrated was critical for binding to PAH2, was either a leucine or an isoleucine in all of the clones isolated. The strongest selection at any position was for two alanines at the C terminus of the peptide. These invariant alanines are in the same positions as the alanines at positions 15 and 16 in Mad1 SID (Fig. 4, lower panel). Residues in Mad1 SID which are solvent exposed, such as Glu14, show no selection for any particular amino acid. The crypto-
GST.Mad1-SID constructs to bind full-length mSin3A contain- tion. To test this hypothesis, we used wild-type and mutant instead bind a cognate domain with the complementary muta- mutant domains which cannot bind their wild-type partners but would generate two domains with altered speci- action by swapping knobs and holes. The resulting interaction of the complementary residue in PAH2 may rescue the inter- interaction, we reasoned that shortening the side chain of A16 with a bulkier leucine prevents the Mad1 SID- (holes) side chains (Fig. 1 to 3). Since replacing the short side chain of A16 with a bulkier leucine prevents the Mad1 SID-PAH2 interaction, we reasoned that shortening the side chain of either V311 or L329 by using alanine increases the binding of GST-.Mad1-SID A16L, to approximately nine- or sevenfold more than that seen with the wild type, respectively. The altered-speciﬁcity Mad1 SID-PAH2 interaction can also be demonstrated by using a mammalian two-hybrid assay. The Gal4.Mad1-SID construct interacts with wild-type PAH2-VP16 but not with PAH2-VP16 containing the V311A or L329A mutation (Fig. 5B). Conversely, Gal4.Mad1-SID A16L does not bind wild-type PAH2-VP16 but interacts strongly with PAH2-VP16 containing the V311A or L329A mutation. The interaction of Gal4.Mad1-SID A16L with PAH2-VP16 containing V311A and L329A is quite speciﬁc, as it is unable to bind PAH2-VP16 containing I308A, a mutation that is similar but that is located at a much lower position in the hydrophobic cleft and therefore distal from A16 (Fig. 3A). Gal4.Mad1-SID A15L, like its GST counterpart (Fig. 5A), does not bind PAH2-VP16 or any of the PAH2-VP16 mutants examined. Therefore, using both pulldown and two-hybrid assays, we can demonstrate binding of the altered-speciﬁcity mutants. In contrast to the results obtained with the GST pulldown assay, the association of the two mutant domains appears to be stronger than that of the wild type in the two-hybrid assay. We believe that this result may be due to the fact that our bait, Gal4.Mad1-SID, is also a repression domain which recruits endogenous mSin3A to the reporter gene. Competition between endogenous Sin3A and exogenous PAH2-VP16 likely results in a lower level of activity. Because Gal4.Mad1-SID A16L cannot bind wild-type Sin3A, it does not have to compete to bind PAH2-VP16 containing V311A (or L329A). Thus, although the mammalian two-hybrid system does not provide relative affinities for the interacting proteins, it clearly demonstrates the interaction of altered-speciﬁcity mutants. With these experiments, we have shown conclusively for the ﬁrst time that Mad1 SID and mSin3A PAH2 interact directly in vivo.

To extend the analysis of altered-speciﬁcity mutants beyond proof of the principle, we also tested the ability of full-length mSin3A containing the L329A substitution to rescue the repressive function of Gal4.Mad1-SID A16L in vivo (Fig. 5C). The introduction of mSin3A containing an L329A substitution (mSin3A L329A) produces a partial but consistent restoration of Gal4.Mad1-SID A16L function, while wild-type mSin3A has no effect on transcriptional levels. In contrast, cotransfection of

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**FIG. 4.** Isolation of an optimal PAH2-binding peptide. Purified GST-PAH2 was used in a phage display screen to isolate 8-aa-binding peptides. The isolated clones were tested for their interaction with GST-PAH2 and then sequenced. (Upper panel) Sequence data from 10 interacting clones. Lowercase indicates a weak consensus. (Lower panel) Helical-wheel representation of the Mad1 SID sequence compared to the consensus sequence derived from the screen. The approximate position of the PAH2 hydrophobic cleft is also shown.
either wild-type mSin3A or the altered-specificity mutant (mSin3A L329A) has little effect on the ability of Gal4.Mad1-SID to repress transcription in 293 cells. The inability of mSin3A L329A to restore Gal4.Mad1-SID A16L repression to wild-type levels likely is a reflection of the failure of transfected mSin3A to effectively compete with the high levels of endogenous mSin3A for the components of a higher-order repression complex. Thus, we conclude that Gal4.Mad1-SID A16L and full-length mSin3A L329A are capable of forming weak but functional complexes in cells.

Implications of PAH1-PAH2 cooperativity for complex formation. Although PAH2 is both necessary and sufficient for binding to Mad1 SID, we find that truncated versions of mSin3A that lack PAH1 bind relatively poorly compared to the wild type. Thus, full-length mSin3A and mSin3A with residues 1 to 388 (including both PAH1 and PAH2; Fig. 6A) bind Mad1 SID equally well, whereas Sin3 with residues 291 to 899 (including PAH2 through the HDAC interaction domain) exhibits ~6-fold less binding (Fig. 6B). In addition, mutations within PAH2 (V311A and L329A) that reduce the binding of Mad1 SID to PAH2 have a considerably more drastic effect on mSin3A with residues 291 to 899 than on either full-length mSin3A or mSin3A with residues 1 to 388. Preliminary data suggest that PAH1 forms a four-helix bundle structure, similar to PAH2 (S. Sahu and I. Radhakrishnan, personal communication). However, PAH1 does not appear to interact with Mad1 SID, since it does not compensate for the deletion of Mad1 SID or the introduction of acidic residues into the PAH2 hydrophobic cleft in full-length mSin3A (Fig. 1B) (18).

Introducing prolines into the PAH1 helices (L127P/L130P) in the context of mSin3A residues 1 to 388 reduced the binding, as expected, of the PAH1-binding protein SAP25 (Y. Shiio and R. N. Eisenman, unpublished data) (Fig. 6C). Surprisingly, we also observed a reduction in the binding of Mad1 SID to PAH2 in this PAH1 mutant. We favor the idea that PAH1 or a PAH1-binding protein helps stabilize the PAH2-Mad1 SID holostructure. This potential cooperativity between PAH1 and PAH2 appears to be unidirectional, as PAH2 mutants fail to affect the binding of SAP25 to PAH1 (data not shown). Structural studies showed that in the absence of Mad1 SID, PAH2 is largely unstructured (9). Therefore, it is tempting to hypothesize that during the transitional folding process, PAH1 facilitates PAH2 folding from an unstructured apostructure to the ordered holostructure. We thus extended our study to test whether other PAH domains may influence Mad1 binding to PAH2. However, the introduction of proline residues into PAH3 and PAH4 did not deleteriously affect the Mad1 SID-PAH2 interaction; only PAH1 reduced Mad1 SID binding to full-length mSin3A (Fig. 6D). It is possible that cross talk between PAH domains or their binding partners can help determine the components of heterologous mSin3A complexes.

**FIG. 5.** Generation of PAH2 and Mad1 SID altered-specificity mutants. (A) Wild-type and mutant GST.Mad1-SID fusion proteins were used to pull down full-length mSin3A, either wild type or containing the indicated PAH2 mutations. The amount of binding was quantitated using a PhosphorImager and expressed as a percentage relative to one-fifth of the starting protein used. (B) 293 cells were co-transfected with constructs expressing Mad1 SID, either wild type or with the A15L or A16L mutation, fused to Gal4 or PAH2-VP16, either wild type or containing the indicated mutations. The relative interaction was measured as the resultant increase in normalized luciferase activity. (C) 293 cells were transfected with a luciferase reporter plasmid containing both Gal4- and LexA-binding sites. The ability of the Gal4.Mad1-SID or Gal4.Mad1-SID A16L construct to repress LexA. VP16-activated transcription in the presence or absence of full-length

wild-type or mutant (L329A) mSin3A was measured as the resultant normalized luciferase activity averaged over four experiments. Error bars in panels B and C indicate standard deviations.
**DISCUSSION**

A recurring theme in transcriptional regulation is the association between transcription factors and coactivators or corepressors that in many instances involves the interaction of a short, helical region of one partner with a larger, more complex structured domain of the other. For example, the short helical LXXLL motif, present in members of the p160 nuclear receptor coactivator family, inserts into a groove formed by three helices in the ligand-binding domain of nuclear receptors (63). Similarly, a helical region of the phosphorylated kinase-inducible domain of CREB interacts with a hydrophobic groove defined by two helices of the KIX domain in CBP (48). Likewise, a single short helical region in multiple repressors is responsible for associations with mSin3A and mSin3B corepressors. Two additional repressor-corepressor complexes for which high-resolution structures have been described also follow a similar theme (19, 65). In all instances, the information required for a highly specific activator-coactivator or repressor-corepressor interaction is apparently confined to a few residues within a single helix. This scenario was elegantly demonstrated by Hu and Lazar (22), who replaced the LXXLL motifs in the GRIP-1 coactivator with the CoRNR boxes from the NCoR corepressor, creating a novel coactivator that binds nuclear receptors only in the absence of a ligand. Further elucidating the structures and specificities involved in such interactions is important in understanding a crucial step in gene regulation.

In this article, we describe a comprehensive functional analysis of the interaction between the PAH2 domain of the mSin3A corepressor and the SID, a short amino-terminal region, of the Mad1 repressor. Previous studies showed Mad1 SID to be a portable repression domain likely to account for the repressive activity of Mad1 (7). Prior to the publication of the PAH2-Mad1 SID structure, Eilers et al. showed the necessity of hydrophobic residues in a helical structure for the SID to interact with PAH2 (12). This study was followed by a limited mutational analysis of PAH2 residues aimed at testing the validity of the PAH2-Mad1 SID nuclear magnetic resonance structure (9). Using information based on the previously published solution structures for mSin3 PAH2-Mad1 SID complexes (9, 56), we have expanded on these previous studies to show not only that hydrophobic residues are required at the PAH2-Mad1 SID interface but also that the arrangement of short and bulky side chains defines the specificity of the interaction. In addition, we have demonstrated that specific hydrophobic mutations in Mad1 SID inhibit its ability to repress transcription in vivo, consistent with its interaction with mSin3 PAH2.

**Affinity and specificity determinants of the Mad1-mSin3A interaction.** Analogous to the LXXLL motif found in nuclear receptor coactivators, Brubaker et al. proposed a sequence motif for proteins that interacted specifically with mSin3 PAH2 (9). The functional analysis presented here suggests that the minimal Mad1 SID consists of just eight residues, of which only L12, A15, and A16 appear to be crucial for a high-affinity PAH2 interaction. The side chains of other residues in the minimal SID (including L13 and L19) are somewhat exposed to the surface and interact with PAH2 to a lesser degree. Substitution of L13 and L19 with alanine appears to reduce binding only slightly in pulldown assays. Of particular importance is the bulky hydrophobic side chain of L12, which inserts deeply into the large hydrophobic cleft formed by the four-helix bundle structure of PAH2. Truncating the side chain of L12 by replacing it with alanine strongly diminishes PAH2 binding. Adding substantial bulk by replacing it with phenylalanine also has a similar effect. L12 forms extensive contacts with A307, Y310, V311, L329, F376, and F379, thus forming the anchor for the Mad1 SID interaction (Fig. 1A and 3). Truncating the side chains of some of these residues in PAH2 by replacing them with alanines reduces but does not abolish binding, possibly because sufficient hydrophobicity is still retained by the substituted residues (Fig. 1). On the other hand, replacing these residues with aspartates greatly diminishes binding due to the burial of a negative charge at the hydrophobic interface. The two alanines at positions 15 and 16 in SID appear to determine the specificity of the association with PAH2 by virtue of their close proximity to the bulky side chains of V311 and L329 in the complex (Fig. 3). Indeed, the Mad1 SID interaction with PAH2 is nearly abolished when A16 is mutated to leucine. The interaction can be partially “rescued” by the reciprocal V311A and L329A mutations in PAH2 (Fig. 5). These altered-specificity mutants also support the activation of a reporter gene in a mammalian two-hybrid assay, demonstrating that the two domains interact directly and specifically in vivo (Fig. 5). That mutated PAH2 or SID fails to interact efficiently, while mutants with reciprocal mutations in both proteins clearly associate, is consistent with the knobs-into-holes model proposed earlier (9). The altered-specificity mutants thus potentially provide a useful tool for studying functional domains of mSin3 in vivo.

When we attempted to extend these findings by using full-length mSin3A in a repression assay, we found that while the altered-specificity mutants generated repression, it was significantly weaker than that observed with wild-type proteins (Fig. 5C). We surmise that the relatively weak repression by transfected mutant mSin3A is related to competition for other components of the higher-order repression complex (e.g., HDACs and SAP30) with the large pool of endogenous mSin3.

Our studies have implicated specific residues that act to determine the affinity and specificity of the Mad1 SID interaction with Sin3 PAH2. These residues are also present in the PAH2-binding regions of other Mad family proteins (Mxi1, Mad3, and Mad4 as well as the related protein Mnt and the more distantly related protein Puf1) (39, 70). However, mSin3 is a corepressor that is recruited in a PAH2-dependent manner by numerous other DNA-binding repressors (for reviews, see references 4 and 28). In addition to the Mad family, PAH2 is also able to bind MNFβ, TGIF, TIEG1/2, REST/NRSEF, and the yeast repressor Ume6 (18, 62, 64, 67, 73). The Ume6 interaction underscores the fact that Sin3 is an ancient molecule that is evolutionarily conserved, particularly in its PAH2 domain. The key residues in the α1 and α2 helices that interact with Mad1, namely, A307, I308, Y310, V311, K315, L329, and L332, are all conserved in yeast Sin3. However, since Ume6 SID is highly divergent from that of Mad1 (62), the contacts are likely to be different, although we would predict that the overall structure of yeast PAH2 would be conserved. Sequence analysis of other mammalian Sin3 PAH2-binding proteins failed to reveal good matches with the PAH2 interaction motif.
described for the Mad proteins. We carried out a phage display screen to test whether alternative PAH2 interaction motifs may exist (16). Remarkably, the majority of the peptides identified in the screen exhibit a strong similarity to the minimal Mad1 SID (Fig. 4), suggesting that, at least under the conditions examined, Mad1 SID may comprise an optimal binding sequence. This scenario is consistent with the results of binding studies performed in vitro with isolated PAH2 and SID, which suggested that for the SIDs examined, the PAH2 domain has the highest affinity for Mad1 SID (9; S. M. Cowley, R. S. Kang, I. Radhakrishnan, and R. N. Eisenman, unpublished observations). It is conceivable then that the mSin3 PAH2 domain preferentially binds Mad family proteins. However, lower-affinity binding by other repressors may involve mSin3 complexes not already engaged in binding to Mad proteins. The tight regulation of Mad expression and the short half-lives of Mad family proteins (5, 24, 47) may ensure that mSin3 complexes are available for association with other transcription factors. In addition, the association of PAH2 with lower-affinity SIDs could be facilitated by chaperonins or perhaps by

FIG. 6. Mutation of PAH1 reduces the binding of Mad1 to PAH2. (A) Schematic representation of mSin3A. The amino acid boundaries of the PAH1 to PAH4 domains (1, 2, 3, and 4) and the truncated mSin3A proteins used are shown. HID, HDAC interaction domain. (B) The GST-Mad1-SID construct was used to bind full-length or truncated mSin3A containing either wild-type (wt) or mutated PAH2. 1/5th indicates 20% of the starting protein added to each reaction. (C) GST-SAP25, which binds to PAH1, and the GST-Mad1-SID construct, which binds to PAH2, were used to pull down a truncated mSin3A molecule (positions 1 to 388) containing either wild-type or mutated PAH1. (D) Full-length mSin3A, either wild type or containing proline residues inserted into the indicated PAH domain, was used in a pulldown assay with the GST-Mad1-SID construct. The amount of binding for each of the pulldown experiments was determined by using a PhosphorImager and expressed as a percentage relative to one-fifth of the starting material used for each mutant protein.
allosteric changes within mSin3 itself. One approach to understanding the association of PAH2 with non-Mad-like SIDs is to determine the structures of these complexes. Such studies are currently under way.

With the availability of a high-affinity Sin3 PAH2 interaction motif sequence at hand, we sought to identify novel factors that may regulate transcription by recruiting the Sin3 corepressor in a PAH2-dependent manner. Toward this end, we searched the SWISS-PROT database for matches to the PROSITE pattern (FIMVL)-P-P-(LIMV)-(LIMV)-P-A-A-P-P-(LIMV)-(ED) by using PattinProt at the Network Protein Sequence Analysis web server. This procedure yielded a large number of hits. The results were "filtered" by selecting only proteins from eukaryotic organisms that were associated with a nuclear function and in which the putative SID was predicted to have a helical structure (Table 1). The factors identified included the transcriptional coregulator TRRAP, the zinc finger repressor PLZF, the leukemia- and lymphoma-related factor cLRF, the replication inhibitor Plutonium, and a component of SCF ubiquitin ligase SkpA. Of these, TRRAP and PLZF are intriguing because TRRAP is known to function as a coactivator of c-Myc, while PLZF has been shown to be recruited to Sin3, although the interaction surface has been mapped to the PAH1 domain rather than the PAH2 domain. Additional studies are required to test these predictions.

mSin3 PAH1 influences the binding activity of PAH2. Unexpectedly, our studies also revealed that full-length mSin3A and an mSin3A protein that contains both PAH1 and PAH2 (mSin3A residues 1 to 388) interact with Mad1 more strongly than do the isolated PAH2 domain and mSin3A which either lacks or has a mutated PAH1. Because deletion or point mutation of PAH2 completely abolishes the ability of Mad1 SID to bind mSin3A (Fig. 1B) (7, 8, 52), it is unlikely that PAH1 itself serves as an independent binding site for Mad1. A likely explanation for the requirement for PAH1 is that it may interact with PAH2 to cooperatively bind Mad1 SID. The idea that the PAH domains of mSin3A may participate in cross talk has implications for higher-order complex formation. Evidence suggests that mSin3A forms the central "plank" in a number of large multiprotein complexes, each with discrete components (31, 32, 55). It is unlikely that these separate complexes are formed in a purely stochastic fashion. However, if the binding of, for example, a specific transcription factor to one of the PAH domains modulates the ability of other PAH domains to recruit its binding partners, then both the constituents of the complex and the order of complex assembly may be regulated coordinately. Interestingly, mutation of either PAH3 or PAH4 had little effect on Mad1 SID binding to PAH2. Perhaps the binding of specific factors to PAH3 and PAH4 is required to modulate the conformation of other domains. In addition, the HDAC interaction domain between PAH3 and PAH4 has now been demonstrated to bind not only HDAC1 and HDAC2 but also the MeCP2 repressor (25, 44) and at least three other transcriptional regulatory proteins (14). Further structural and biochemical studies are required to understand the molecular basis for specific complex formation.

ACKNOWLEDGMENTS

We are grateful to Susan Parkhurst for the gift of reporter plasmid constructs, to Yuzuru Shio for SAP25 and communication of unpublished data, to Will Salerno for conducting the pattern searches, and to Sara Hook for critical reading of the manuscript.

This work was supported in part by NIH grant R21/R33 CA-88245 to J.V.F., by grants from the March of Dimes Birth Defects Foundation (5-FY00-605) and the NIH (GM 64715) to I.R., by a Rett Syndrome Research Foundation fellowship award to S.M.C., and by NIH/NCI grant R37CA57138 and an American Cancer Society research professorship to R.N.E.

REFERENCES


TABLE 1. Database search for putative PAH2-interacting proteins

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
<th>Accession no.</th>
<th>Putative SID</th>
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<tr>
<td>Home sapiens Mad1</td>
<td>Transcriptional repressor</td>
<td>AAA36194</td>
<td>9-JQMLEAAADYLE-20</td>
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<tr>
<td>Gallus gallus cLRF</td>
<td>Leukemia/lymphoma-related factor</td>
<td>AAC35368</td>
<td>104-VDILNAAKLLE-115</td>
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<td>DNA replication inhibitor</td>
<td>P42570</td>
<td>147-LRGMIKAAGRLD</td>
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<tr>
<td>H. sapiens PLZF</td>
<td>Promyelocytic leukemia zinc finger</td>
<td>AAD3619</td>
<td>99-LDDLYAAEILE-110</td>
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<tr>
<td>D. melanogaster SkpA</td>
<td>SCF complex protein</td>
<td>O77430</td>
<td>99-LFELILAANYLD</td>
</tr>
<tr>
<td>H. sapiens TRRAP</td>
<td>Transformation or transcription domain-associated protein</td>
<td>Q9Y6H4</td>
<td>3806-VNTLVAANSLD-3817</td>
</tr>
</tbody>
</table>

a The SWISS-PROT database was searched for matches to the PROSITE pattern (FIMVL)-P-P-(LIMV)-(LIMV)-P-A-A-P-P-(LIMV)-(ED) by using PattinProt at the Network Protein Sequence Analysis web server. This procedure yielded a large number of hits. The results were filtered by applying several criteria, including (i) restricting the source organism to eukaryotes, (ii) eliminating putative and hypothetical proteins, (iii) selecting proteins known to bind DNA, localize to the nucleus, or regulate transcription, and (iv) selecting sequences that were predicted to form a helix.

b Numbers indicate amino acid positions spanned.


