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A 13-Amino Acid Amphipathic α-Helix Is Required for the Functional Interaction between the Transcriptional Repressor Mad1 and mSin3A*

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Members of the Mad family of bHLHZip proteins heterodimerize with Max and function to repress the transcriptional and transforming activities of the Myc proto-oncogene. Mad:Max heterodimers repress transcription by recruiting a large multi-protein complex containing the histone deacetylases, HDAC1 and HDAC2, to DNA. The interaction between Mad proteins and HDAC1/2 is mediated by the corepressor mSin3A and requires sequences at the amino terminus of the Mad proteins, termed the SID, for Sin3 interaction domain, and the second of four paired amphipathic α-helices (PAH2) in mSin3A. To better understand the requirements for the interaction between the SID and PAH2, we have performed mutagenesis and structural studies on the SID. These studies show that amino acids 8–20 of Mad1 are sufficient for SID:PAH2 interaction. Further, this minimal 13-residue SID peptide forms an amphipathic α-helix in solution, and residues on the hydrophobic face of the SID helix are required for interaction with PAH2. Finally, the minimal SID can function as an autonomous and portable repression domain, demonstrating that it is sufficient to target a functional mSin3A/HDAC corepressor complex.

Transcriptional regulation depends on the assembly of large multiprotein complexes. For example, the preinitiation complex (1), chromatin remodeling complexes (2, 3), and histone deacetylase-containing corepressor complexes (4, 5) have been shown to be in the 1–2 × 10^6 dalton size range. Molecular connections between proteins in these molecular machines, and the structural basis of their assembly, are not well understood. Initially, transcription repression domains were defined by structure/function analysis, which revealed that, like activation domains, they are more likely to contain particular amino acids rather than have easily identifiable protein-protein interaction domains. This finding led to the hypothesis that activation and repression domains share similar molecular targets and that the structure of the activation or repression domain in itself was not required for function. Transcriptional repressors function by at least three distinct mechanisms: by direct contact with components of the basal transcriptional machinery, *e.g.* even-skipped (6), Dr1 (7), and MOT1 (8); by tethering histone deacetylase-containing corepressor complexes to the promoter, *e.g.* the Mad family (9, 10), Rb (11–13), and MeCP2 (14, 15); or by tethering corepressors that lack deacetylase activity to the promoter, *e.g.* hairy (16) and MATα2-MCM1 (17). In each of these cases, little or no structural data are available for the repression domain. In contrast, one theme that has emerged recently from the study of activation domains is that relatively short stretches of amino acids can adopt amphipathic α-helical structures and mediate stable functional interactions between transcriptional activators and coactivators (18–20).

Reversible acetylation of the amino-terminal tails of core histones plays an important role in the regulation of gene expression. In general, regions of chromatin that are hyperacetylated are transcriptionally active, while hypoacetylated regions are silenced (21). The recent discovery that several transcriptional co-activators are histone acetyltransferases and that co-repressor complexes contain histone deacetylases as active components has provided a mechanistic basis for this correlation (22–26). mSin3A and mSin3B were identified as corepressors required for the transcriptional and biological activities of the Mad proteins (27, 28). mSin3A has recently been shown to be a component of a large multi-protein complex(s) that also contains the histone deacetylases HDAC1[1] and HDAC2 in apparently stoichiometric amounts. The enzymatic activities of the mSin3A-bound HDACs are required for full transcriptional repression by the Mad family proteins (9, 10, 29). Subsequently, the mSin3A·HDAC complex has been implicated as a corepressor utilized by a diverse and rapidly expanding collection of transcriptional repressors, including RXR, MeCP2, estrogen receptor, RPX, and Pit1 (14, 15, 30–32).

mSin3A and mSin3B and their *Saccharomyces cerevisiae* orthologue SIN3 each contain four similar domains each suggested to form two amphipathic α-helices separated by a flexible linker (27, 33). These regions, termed PAH domains for paired amphipathic α-helix, were originally proposed to function as protein-protein interaction domains (33). Recent experiments have demonstrated this to be the case. For example, Mad proteins interact with PAH2 (27, 28), a repression domain of the nuclear hormone corepressor N-CoR interacts with PAH1 (31, 34) and the mSin3 interacting protein SAP30 binds to PAH3 (30). The four PAH domains of the different Sin3 proteins are highly conserved. For example, PAH2 is 90% similar between mSin3A and mSin3B and it is approximately 70%

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[1] The abbreviations used are: HDAC1, histone deacetylase 1; HDAC2, histone deacetylase 2; bHLHZip, basic region-helix-loop-helix-zipper; SID, mSin3 interaction domain; PAH, paired amphipathic helix; CD, circular dichroism; TFE, trifluoroethanol; PBS, phosphate-buffered saline; GST, glutathione *S*-transferase; PVDF, polyvinylidene fluoride; GALDBD, GAL4 DNA binding domain; KID, kinase-inducible domain; deg, degree(s); PAGE, polyacrylamide gel electrophoresis.

similar to the PAH2 domain of *S. cerevisiae* SIN3 (27) and recently identified SIN3 homologues from *Schizosaccharomyces pombe*, *Caenorhabditis elegans*, *Drosophila melanogaster,* and *Arabidopsis thaliana* (data not shown). Within a given protein, the four PAH domains are roughly 45% similar with the hydrophobic positions of the putative amphipathic α-helices being most highly conserved, suggesting that PAH domains may share structural features (27). With the exception of the Mad family, the domains required for SIN3 binding of the other SIN3 interacting proteins, SAP30, SAP18, N-CoR, UME6, HDAC1, and HDAC2, etc., share no obvious sequence similarity (data not shown).

The Mad family of basic region-helix-loop-helix-leucine zipper (bHLHZip) proteins functions as transcriptional repressors and antagonize the transcriptional and transforming activity of the Myc proto-oncogenes (35–39). Currently, four Mad family members have been identified: Mad1, Mxi1, Mad3, and Mad4 (35, 38, 40). These proteins share extensive sequence homology throughout their entire open reading frames, with the highest degree of conservation within the bHLHZip and for mSin3 interaction domains (SID) (38). The bHLHZip domain is required for dimerization with the bHLHZip protein Max and DNA binding, while the SID is required for interaction with mSin3A or mSin3B (28, 35, 38). This SID sequence from Mad1 has been modeled as an amphipathic α-helix (27). Recently, another bHLHZip protein termed Mnt, which shares homology to the Mad family within these two regions, has been identified. Mnt also interacts with Max and can repress transcription in a mSin3-dependent manner and therefore appears to be functionally equivalent to the Mad family proteins (41).

Several lines of experimental evidence suggest that interaction between the Mad proteins and Mnt and mSin3A or mSin3B is critical for their function as transcriptional repressors. Mad1 proteins with point mutations in the SID no longer repress transcription, block Myc+Ras cotransformation, or arrest cells in the G_1 phase of the cell cycle (27, 36, 42). Similarly, deletions of amino-terminal regions that contain the SID in Mad3, Mad4, and Mnt severely affect their biological function (38, 41). Finally, Mxi1 is encoded by two alternatively spliced mRNAs, only one of which encodes a Mxi1 protein with a SID. This protein, Mxi1-SR is much more potent at blocking Myc+Ras cotransformation than is an Mxi1 isoform which lacks a SID (28). In order to better understand the interaction between Mad family members and their corepressor mSin3A we have delineated the minimal functional SID, determined key contact residues required for interaction with PAH2 and demonstrated that the minimal SID domain is helical in solution.

EXPERIMENTAL PROCEDURES

Cloning and Interaction Assays—Fusions to the LexA DNA binding domain were made either by polymerase chain reaction amplifying SID 1–57 and SID 1–27 using pSPMad1 or pSPMad1(L12P/A16P) (27) as template or by inserting a double-stranded oligonucleotide cassette encoding the various SID constructs between the *Eco*RI and *Bam*HI sites of pBTM116 (43). Each construct was verified by sequencing. The different LexA fusion constructs and VP16PAH2 (27) were introduced into the *S. cerevisiae* strain L40 by lithium acetate transformation (44). Quantitative β-galactosidase assays were performed from three independent colonies in triplicate from liquid cultures (44). For each measurement the standard deviation was less than 10%.

Transcription Assays—293 cells were grown in Dulbecco's modified Eagle's medium with 10% defined calf serum (HyClone). 2×10^5 cells were plated on 60-mm dishes and transfected with 200 ng of the GAL4–14D luciferase reporter (45) and 1 μg of the indicated expression vector. Cells were harvested 24 h after transfection and luciferase and β-galactosidase activity measured according to the manufacturers' protocols (Promega and Tropix). Each transfection was performed at least twice in triplicate. Error shown is the standard of the mean.

Peptide Synthesis—The four SID-containing peptides were synthesized and purified by the Huntsman Cancer Institute DNA/Peptide Resource Core Facility. Each peptide includes residues 7–20 of human Mad1. The sequence of the wild type SID peptide is GGGMNIQMLLEAADYLE. The sequence of the double mutant L12P/A16P SID is GGGMNIQMPLEAPDYLE. The sequences of the two single mutant peptides A15D SID and L19D SID are GGGMNIQMLLEDADYLE and GGGMNIQMLLEAADYDE, respectively. Peptide concentrations were determined by measuring the absorbance of the peptide at 280 nm and using the extinction coefficient for a single tyrosine of 1.49×10^3 M^{-1} cm^{-1}.

Circular Dichroism (CD) Spectroscopy—CD samples contained 50 μM of peptide, 1× phosphate-buffered saline, and the percentage of trifluoroethanol (TFE, Sigma) indicated in Fig. 4. CD spectra were collected on an Aviv 62DS spectrophotometer from 280 to 195 nm at 25 °C using a cell with a 0.1-cm pathlength. The reported spectra are the average of 15 consecutive runs. The observed ellipticity was converted to mean residue molar ellipticity $[\theta]$ (deg cm^2 dmol^{-1}) using the relationship $[\theta] = \theta/(C_r\, l)$ where θ is the observed ellipticity, l is the pathlength, and C_r is the mean residue molar concentration. Fractional helicities were calculated as described using values for $[\theta]^0_{222}$ and $[\theta]^{100}_{222}$, corresponding to 0% and 100% helical content at 222 nm, of −2000 and −28,400 deg cm^2 dmol^{-1}, respectively (46).

Far Western Blotting—Mad1His and Mad1(L12P/A16P)His (27), which have a polyhistidine tag fused to their carboxyl termini, were translated *in vitro* in 50-μl reactions using the TNT coupled reticulocyte lysate system (Promega) and ^{35}[S]methionine (NEN Life Science Products) and were purified under native or denaturing conditions. Ni^{2+}-NTA agarose (Qiagen) was blocked with rabbit reticulocyte lysate (diluted 1:3 in PBS) for 30 min at 4 °C and then incubated with the *in vitro* synthesized Mad1His and Mad1(L12P/A16P)His for 30 min at 4 °C in PBS (native) or 6 M guanidine hydrochloride, 0.1 M NaH_2PO_4, and 0.01 M Tris, pH 8.0 (denaturing conditions) followed by extensive washing with the same buffers. The bound proteins were eluted in PBS containing 0.5 M imidazole and then dialyzed overnight against PBS to remove the imidazole and allow for renaturation. Recombinant GST and GST-PAH2 were expressed in bacteria and purified on glutathione-Sepharose 4B (Amersham Pharmacia Biotech). The blots were prepared by resolving 1 μg of GST and 1 μg of GST-PAH2 on 15% SDS-PAGE, followed by transfer to PVDF membrane. The blots were blocked in far Western buffer (PBS containing 0.1% Nonidet P-40, 1 mM EDTA, and 1 mM dithiothreitol) containing 5% nonfat dry milk for 1 h at 4 °C. Purified probes were added to blots in 5 ml of far Western buffer containing 1% nonfat dry milk and incubated together at 4 °C overnight with rocking. Following washing with far Western buffer, the blots were air-dried and exposed 48 h for autoradiography.

RESULTS

Mad1 and mSin3A Interact Directly—We wished to determine the structural requirements for the interaction between mSin3A and Mad1. However, it has not been conclusively demonstrated that the interaction between the two proteins is direct. For example, the interaction between Mad1 and mSin3A has been detected using the two-hybrid assay, *in vitro* translated proteins, and co-immunoprecipitation from cell extracts containing epitope-tagged Mad1 (10, 27, 28). While these experiments suggest that the interaction between Mad1 and mSin3A is direct, they do not rule out the possibility that a bridging factor could mediate the interaction between Mad1 and mSin3A.

To determine whether the interaction between Mad1 and mSin3A is direct, we used far Western blot assays. In these experiments GST-PAH2, a GST fusion to the PAH2 domain of mSin3A, and GST alone were resolved by SDS-PAGE followed by transfer to a PVDF membrane. Duplicate blots were probed with ^{35}S-labeled *in vitro* transcribed and translated Mad1His and mutant Mad1 protein, Mad1(L12P/A16P)His, that does not interact with mSin3A. Equal efficiency of transcription and translation of these proteins was confirmed by SDS-PAGE followed by autoradiography to detect the proteins (data not shown). These protein probes were purified on Ni^{2+}-NTA agarose under native or denaturing conditions. When purified under native conditions, Mad1His but not Mad1(L12P/A16P)His was able to interact with GST-PAH2. Neither

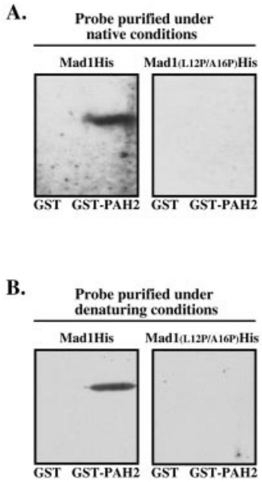

FIG. 1. **The interaction between the SID and PAH2 is direct.** PVDF blots with immobilized GST and GST-PAH2 were probed with ^{35}S-labeled IVT Mad1His (*left panel*) and Mad1(L12P/A16P)His (*right panel*) that had been purified either under native (*A*) or denaturing (*B*) conditions. The blots were dried and then exposed to detect bound ^{35}S-labeled proteins. Equivalent amounts of GST and GST-PAH2 were immobilized in each membrane.

A.

```
                        •    •    •   20                27
Mad1 (murine)     7   MNIQL|LLEAA|DYLE......RRERE...A|EHGYAS|ML|P| 35
Mad1 (human)      7   MNIQM|LLEAA|DYLE......RRERE...A|EHGYAS|ML|P| 35
Mad4 (murine)     4  .NSLLL|LLEAA|EYLE......RRDRE...A|EHGYAS|ML|P| 32
Mad4 (human)      4  .NSLLI|LLEAA|EYLE......RRDRE...A|EHGYAS|VL|P| 32
Mad3 (murine)     6  .SNIQV|LLQAA|EFLE......RRERE...A|EHGYAS|LC|P| 34
Mxi1 (human)      7  .INVQR|LLEAA|EFLE......RRERE...C|EHGYAS|SF|P| 35
Mxi1 (murine)     7  .INVQR|LLEAA|EFLE......RRERE...C|EHGYAS|SF|P| 35
Mxi1 (fish)       23  KNVQV|LLEAA|SYIE......SAERKDGKC|EHGYAS|TF|P| 53
Mnt  (human)      2  .MSIET|LLEAA|RFLEWQAQQQRARE 25
Mnt  (murine)     2  .MSIET|LLEAA|RFLEWQAQQQRARE 25
Mad  (nematode)   6  . NLGH|LLTAA|RLLD......IGALD 23.
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B.

```
SID deletion mutants:                                % control β-gal activity

SID 1-57     MAAAVRMNIQMLLEAADYLERREREAE....     100%
SID 1-57     MAAAVRMNIQMPLEAPDYLERREREAE....    0.25%
(L12P/A16P)
SID 1-27     MAAAVRMNIQMLLEAADYLERREREAE         188%
SID 1-27     MAAAVRMNIQMPLEAPDYLERREREAE         0.5%
(L12P/A16P)
SID 7-20           MNIQMLLEAADYLE                154%
SID 8-20            NIQMLLEAADYLE                 92%
SID 9-20             IQMLLEAADYLE                6.7%
SID 10-20             QMLLEAADYLE                0.9%
SID 8-19            NIQMLLEAADYL                  13%
SID 8-18            NIQMLLEAADY                  1.2%
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FIG. 2. **Determination of the minimal SID.** The amino termini of the Mad family members and Mnt were aligned using the GCG pileup algorithm (*A*). Regions of highest conservation are boxed. Amino acid positions 10, 14, and 17, numbering relative to Mad1, of the SID which are somewhat divergent among these proteins are marked with a filled circle. Interactions between the SID and the PAH2 domain were measured by a directed two-hybrid assay (*B*). Amino acids 251–404 of mSin3A encoding the PAH2 domain were fused to the VP16 activation domain and the SID and various mutants were fused to the LexA DNA binding domain. The sequences of the different SID amino- or carboxyl-terminal deletion mutants are shown along with the relative β-galactosidase activity of each SID in combination with VP16PAH2 in the yeast strain L40. For SID 1–57, only amino acids 1–27 are shown. The β-galactosidase activity of each mutant was normalized to that measured for LexA fused to amino acids 1–57 of Mad1 in combination with VP16PAH2.

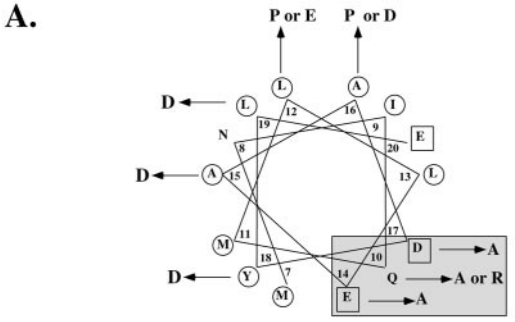
Mad1His nor Mad1(L12P/A16P)His interacted with GST alone (Fig. 1*A*). Together, these results confirm that Mad1 interacts specifically with PAH2 of mSin3A and that the interaction is sensitive to mutations in the SID. However, because there is abundant mSin3A, and presumably interacting cofactors, in reticulocyte lysate (data not shown), the possibility exists that a bridging factor may have copurified with Mad1 under native conditions and that it mediated the interaction between Mad1 and PAH2. Mad1His purified under denaturing conditions and subsequently renatured also interacted with GST-PAH2 but not with GST alone (Fig. 1*B*, *left panel*). Furthermore, Mad1(L12P/A16P)His purified under denaturing conditions did not interact with either GST or GST-PAH2 (Fig. 1*B*, *right panel*). It is very likely that any interaction between Mad1 and a putative bridging factor would have been disrupted under the denaturing conditions used for purification. Therefore, these results indicate that the interaction between Mad1 and PAH2 of mSin3A is direct and does not require a bridging factor.

The SID Is an Amphipathic α-Helix—Alignment of the Mad family proteins and Mnt from different species reveals that amino acids 7–35, numbering relative to Mad1, are highly conserved (Fig. 2*A*). Within this block of residues, the sequence LLEAA is nearly identical between the aligned molecules, suggesting that it may form the core of the interaction domain. This block of conservation is followed by a stretch of charged amino acids and the sequence EHGYAS. These downstream sequence elements are highly conserved within the mammalian Mad proteins but are absent from the Mnt proteins and an invertebrate Mad homologue. Previous mutagenesis studies in which the first 35 amino acids of Mad1 were deleted have demonstrated that this conserved amino-terminal region is necessary for interaction between Mad proteins and mSin3A (27). Another Mad1 truncation in which the first 20 amino acids of Mad1 are deleted but leaves the EHGYAS region intact was also tested. This deletion was also unable to interact with mSin3A, indicating that the conserved EHGYAS region is not sufficient for the Mad1:mSin3A interaction (27). Further experiments have shown that amino acids 1–35 of Mad1 mediate histone deacetylase-dependent repression (9, 10, 27, 38, 41). However, the minimal domain required for the interaction between Mad1 and mSin3A and the role, if any, of the conserved EHGYAS region in this interaction have not been determined.

We have used a directed two-hybrid assay to measure the relative affinity of the SID and various SID mutants for PAH2. Briefly, SID molecules based on the sequence of human Mad1 were fused to the DNA binding domain of bacterial LexA, and the PAH2 domain of mSin3A was fused to the transcriptional activation domain of VP16. Following introduction into the *S. cerevisiae* strain L40, relative affinity was measured by quantitative analysis of the β-galactosidase activity generated from an integrated LexA-dependent LacZ reporter gene.

To define the minimal sequence required for interaction with PAH2, we constructed a series of amino- and carboxyl-terminal truncations of the SID. Consistent with previous findings (27), the region from the initiating methionine to the beginning of the basic region, amino acids 1–57, was sufficient for interaction and two point mutations within the putative α-helical region of the SID, L12P/A16P, completely abolished interaction (Fig. 2*B*). A carboxyl-terminal deletion of 30 amino acids, SID 1–27, which removes the conserved EHGYAS, bound PAH2 almost 2-fold better than the longer amino-terminal construct, suggesting that the EHGYAS sequence has a slight negative effect on binding (Fig. 2*B*). Again, in the context of this protein, the L12P/A16P double mutation completely abolished interaction. Further deletion analysis demonstrated that amino acids 8–20 are necessary and sufficient for interaction. These findings suggest that the highly conserved region between amino acids 20–35 found in the vertebrate Mad proteins is completely dispensable for interaction and that the sequences that are conserved between Mad proteins across species and Mnt constitute a minimal SID.

To determine which residues of the minimal SID are re-



B.

SID point mutants	8 20	% control β-gal activity
SID 8-20(WT)	NIQMLLEAADYLE	100%
SID 8-20(Q10R)	NI**R**MLLEAADYLE	25%
SID 8-20(Q10A)	NI**A**MLLEAADYLE	235%
SID 8-20(E14A)	NIQMLL**A**AADYLE	215%
SID 8-20(D17A)	NIQMLLEAA**A**YLE	284%
SID 8-20(Q10A,E14A,D17A)	NI**A**MLL**A**AA**A**YLE	235%
SID 8-20(L13E,A16D)	NIQM**E**LEA**D**DYLE	3.8%
SID 8-20(A15D)	NIQMLLE**D**ADYLE	6.8%
SID 8-20(Y18D)	NIQMLLEAAD**D**LE	5.5%
SID 8-20(L19D)	NIQMLLEAADY**D**E	4.4%

FIG. 3. **Determination of residues required for interaction between the SID and PAH2.** Amino acids 7–20 of Mad 1 are modeled as an amphipathic α-helix (*A*). Hydrophobic and charged residues are circled and boxed, respectively. Amino acids 10, 14, and 17 are potential non-contact residues and are enclosed in a *shaded box*. The amino acid residues that were mutated are also indicated. The sequences of the different SID point mutants are shown along with the relative β-galactosidase activity of each SID in combination with VP16PAH2 in the yeast strain L40 (*B*). The β-galactosidase activity of each mutant was normalized to that measured for LexA fused to amino acids 8–20 of wild type Mad1 in combination with VP16PAH2.

quired for interaction with PAH2, we first displayed residues 7–20 on a helical wheel (Fig. 3*A*). This conceptual α-helix is amphipathic. The three residues that are less conserved within the Mad family and Mnt, positions Gln-10, Glu-14 and Asp-17, all lie on the hydrophilic face of the α-helix. Given the charged nature of the hydrophilic face and the lower conservation of Gln-10, Glu-14, and Asp-17, this surface is predicted not to be involved in the SID:PAH2 interaction. In contrast, the highly conserved hydrophobic face of this putative α-helix is predicted to mediate protein-protein interaction. To determine which face of the SID is required for interaction with PAH2, we mutated several amino acids in the context of the minimal 13-amino acid SID (Fig. 3*B*). As predicted, mutation of the presumptive noncontact face had little effect on interaction. A SID peptide containing a Q10R mutation reduced binding 4-fold, while three single mutations to alanine, Q10A, E14A, and D17A, could bind to PAH2 with approximately 2-fold higher affinity. A protein containing all three alanine mutations (Q10A/E14A/D17A) bound PAH2 with affinity similar to each of the single alanine mutants, further supporting the hypothesis that these residues are not involved in the interaction. In addition, because alanine substitutions are thought to be compatible with helical structure, this finding is consistent with the predicted helicity of the SID.

Mutation of any of the presumptive contact hydrophobic residues, Ala-15, Tyr-18, or Leu-19, to aspartic acid severely impaired binding. Our original double mutant, L12P/A16P (27), failed to interact with PAH2, suggesting that these residues may be involved in direct contact between the SID and PAH2. Alternatively, it is possible that the double proline mutations disrupt the helical nature of the SID and the mutant fails to interact for this reason. To further test whether these residues are involved directly in the interaction, amino acids 12

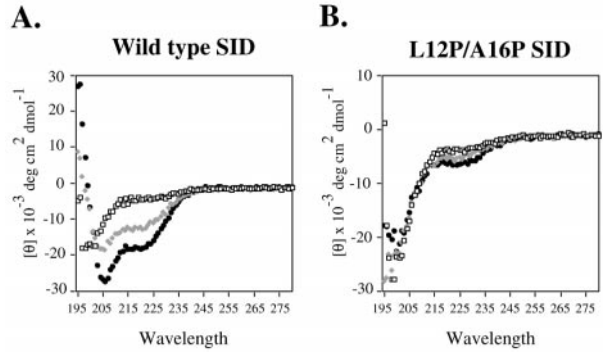


FIG. 4. **The wild type SID peptide has an inherent helical propensity.** The CD spectra of wild type SID and L12P/A16P SID peptides were measured in 1, 20, and 50% TFE. The CD spectra of the wild type SID peptide (*A*) and the L12P/A16P SID peptide (*B*) are shown. Percentages of TFE are denoted as follows: *open squares*, 1% TFE; *shaded triangles*, 20% TFE; *filled circles*, 50% TFE.

and 16 were mutated in tandem to glutamic and aspartic acid, respectively. We predicted that these alterations would not disrupt the helical nature of the SID, but would no longer make hydrophobic interactions. Like the single mutants at the presumptive contact interface, this double mutant was incapable of high affinity interaction with PAH2. This mutational analysis is consistent with the hypothesis that the SID forms an amphipathic α-helix with the hydrophobic face serving as the contact interface with PAH2.

To test directly if the SID could adopt an α-helical structure, we measured the helical content of wild type SID and mutant SID peptides using circular dichroism (CD) spectroscopy. Short peptides do not generally form secondary structures in aqueous solutions because the solvent competes for structure-stabilizing intramolecular hydrogen bonds. Therefore, spectra for the wild type and mutant SID peptides were measured in the solvent TFE, which is commonly used to stabilize α-helical conformation in peptides that have an inherent helical propensity (47, 48). In an aqueous solution containing 1% TFE, the wild type SID peptide lacks secondary structure (Fig. 4*A*). At increasing TFE concentrations, the wild type peptide adopts an α-helical structure as indicated by the strong negative peaks at 208 and 222 nm. In 20% TFE the SID is approximately 40% α-helical. This percentage increases to approximately 60% in 50% TFE. Thus, as predicted the wild type SID has helical propensity and is able to adopt an α-helical conformation.

Recently, it was demonstrated that TFE destabilizes the unfolded state of a peptide that indirectly enhances the folding of the helix (49). Therefore, we were concerned that any peptide, regardless of its inherent helical content, might be forced into a helical structure at high TFE concentrations. Unlike the wild type SID, however, the spectra of a SID peptide with two putative α-helix-destabilizing proline substitutions, L12P/A16P, remained relatively unchanged with increasing concentrations of TFE, demonstrating that it does not undergo a transition from random coil to α-helix (Fig. 4*B*). We infer that the α-helical structure observed with the wild type SID peptide in TFE is a reflection of its helical propensity. These results, along with those from the directed two-hybrid assay, suggest that the SID must adopt an α-helical conformation to allow interaction with PAH2.

The SID mutant L12E/A16D is unable to interact with PAH2 (Fig. 3*B*). We hypothesized that, unlike the helix-destabilizing proline substitutions, this mutant peptide's loss of binding may have resulted from disruption of hydrophobic interactions required for contact with PAH2 rather than disruption of helical structure. However, the CD spectrum of L12E/A16D SID indicates that in 50% TFE this peptide is not as helical as the wild

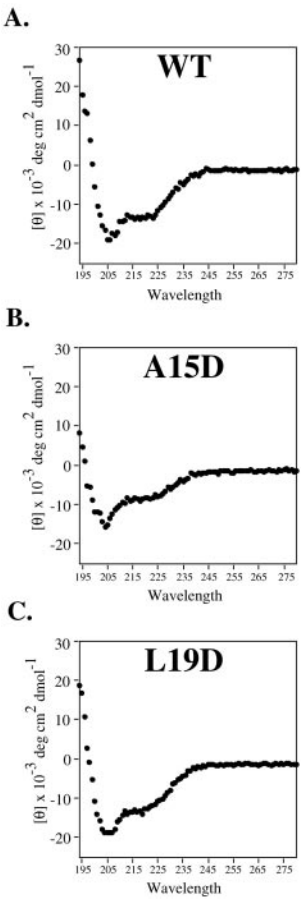


FIG. 5. **Mutant SIDs that are unable to interact with PAH2 have an inherent helical propensity.** The CD spectra of wild type SID peptide (*A*), A15D SID peptide (*B*), and L19D SID peptide (*C*) measured in 50% TFE are shown.

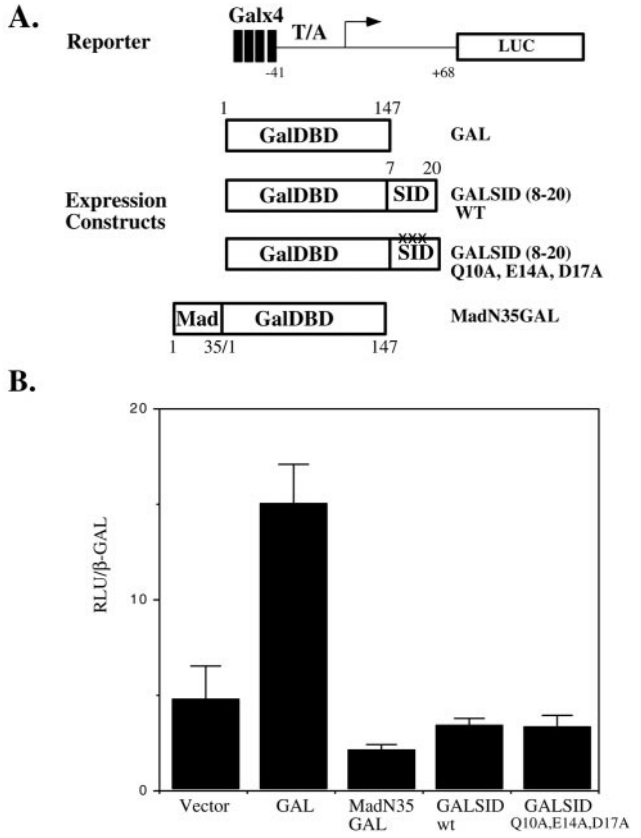


FIG. 6. **SID 8–20 functions as a portable repression domain.** The reporter plasmid and expression vectors used in this experiment are shown (*A*). Transcriptional activity of the GAL4 DNA binding domain-responsive reporter in the presence of the expression vectors indicated at the *bottom* of the figure. *LUC*, luciferase; *RLU*, relative light units (*B*).

type peptide and shows only slightly more helical nature than L12P/A16P SID (data not shown). Thus, these mutations appear to affect the structure of the SID, making it impossible to discern whether the inability of this mutant to interact with PAH2 in the two-hybrid is due to disruption of hydrophobic interactions or the disruption of secondary structure. In an attempt to clarify the role of the hydrophobic residues of the SID, we collected the CD spectra for the two single mutant peptides, A15D SID and L19D SID. The spectrum for L19D SID was nearly identical to that collected for the wild type SID, while the spectrum for A15D showed that it was slightly less helical than the wild type SID, indicating that both mutant peptides are primarily helical in 50% TFE (Fig. 5, *A–C*). Calculation of percentage helicity for the peptides indicated that the mutations A15D and L19D reduced the helicity, of the SID relative to wild type, by approximately 40% and 5%, respectively. Therefore, A15D SID and L19D SID retain helical structure but are unable to interact with PAH2 in the two-hybrid. This suggests that the hydrophobic face of the SID α-helix, which is disrupted in these mutants, is important for interaction with PAH2.

Amino Acids 8–20 of Mad1 Functions as a Portable Repression Domain—Our mutational analysis suggests that a 13-amino acid amphipathic α-helix mediates the interaction between the SID and PAH2. To test whether this minimal 13-residue interaction domain is sufficient to target a functional mSin3·HDAC corepressor complex to DNA, we fused amino acids 8–20 from Mad1 to the DNA binding domain of the yeast transcriptional activator GAL4 (GALDBD). The transcriptional activity of GALSID(8–20) WT was tested on a reporter containing four GAL4 binding sites cloned upstream of a minimal promoter (Fig. 6*A*). Consistent with our previous findings (45), the GALDBD alone activates this reporter approximately 3-fold, and a fusion between the first 35 amino acids of Mad1 and the GALDBD repressed this level of reporter activity approximately 7-fold. GALSID(8–20) WT repressed transcription to approximately the same level when fused to the GAL4 DNA binding domain (Fig. 6*B*), suggesting that this minimal SID is sufficient to target functional mSin3·HDAC complexes to DNA. Amino acids 10, 14, and 17 of the SID can be mutated to alanine without adversely affecting the interaction with PAH2 (Fig. 3). However, it is possible that these residues constitute a surface that interacts with other components of the mSin3·HDAC complex and/or components of the general transcriptional machinery. To test whether mutation of these residues may impair the ability of the SID to recruit a functional corepressor complex, we constructed a minimal SID containing the mutations Q10A, E14A, and D17A in the context of a GALDBD fusion (Fig. 6*A*). This mutant SID (GALSID Q10A, E14A, D17A) repressed transcription to the same extent as the wild type minimal SID (Fig. 6*B*), suggesting that the surface comprised of residues 10, 14, and 17 is unlikely to make functionally important contacts with other components of the mSin3A·HDAC complex.

DISCUSSION

To understand the structural basis for the direct interaction between the transcriptional repressor Mad1 and its corepressors mSin3A and mSin3B, we defined the minimal sequence of Mad1 required for interaction with PAH2, showed that this minimal interaction domain can adopt an amphipathic α-heli-

cal structure in solution, and determined that the hydrophobic face of this helix makes key contacts with mSin3A. We had previously shown that the amino-terminal 35 residues of Mad1 functions as a portable repression domain and is required to target functional mSin3A·HDAC complexes (45). Here we show that residues 8–20 of Mad1 constitute a minimal functional portable repression domain. Further, our experiments showed that residues 10, 14, and 17 are not required for interaction with PAH2 and that the surface created by these residues does not make important contacts with other components of the mSin3A·HDAC complex.

We have used two classes of mutations to determine the structural requirements for the interaction between the SID and PAH2. The first class (L12P/A16P SID and L12E/A16D SID) cannot adopt an α-helical structure and fails to interact with PAH2, suggesting a requirement for this structure in binding to PAH2. The second class of mutations (A15E and L19D) retains helical structure in TFE but fails to interact with PAH2. These mutations are in the hydrophobic face of the SID amphipathic α-helix demonstrating that the hydrophobic face of the SID makes key contacts required for high-affinity interaction with PAH2. We propose that the α-helix correctly positions the hydrophobic residues of the SID and optimizes the hydrophobic interactions required for the SID to bind PAH2.

We believe that the helical nature of the 14-amino acid SID peptide observed in our CD experiments is likely to reflect its structure in the context of full-length Mad1. The double proline mutant SID peptide is incapable of α-helix formation even at high concentrations of TFE. The same proline substituted SID in the context of full-length Mad1 disables both the transcriptional repressor and the biological functions of Mad1. Furthermore, the SID has been fused to the DNA binding domain of GAL4, LEXA, and c-Myc and in these contexts can impart transcriptional repression functions to each of these proteins (27, 36, 42, 45, 50). In each of these cases, proline substitution of positions 12 and 16 of the SID result in the loss of repression function of the fusion proteins. Therefore, there is a strict correlation between the inability of the L12P/A16P SID peptide to adopt a helical conformation in TFE and the inability of the SID to function as an autonomous transcription repression domain. The simplest interpretation of these results is that the proline-substituted SID, in the context of the different fusion proteins or full-length Mad1, cannot adopt the helical conformation that is required for a functional interaction between the SID and the mSin3A·HDAC corepressor complex.

The dependence of the interaction between the repression domain of Mad1 and mSin3A on an amphipathic helical structure is reminiscent of the interaction between several activation domains and their target proteins. The p53 activation domain and the KID domain of CREB bury the hydrophobic faces of their helical activation domains into hydrophobic pockets in MDM2 and the KIX domain of CBP, respectively (18, 19). Also, the acidic activation domain of VP16 forms an amphipathic α-helix when it contacts $hTAF_{II}31$ (20). In each of these cases, mutation of hydrophobic residues around the binding interface inhibits both interaction and transcriptional activation (20, 51, 52). These findings have led to the conclusion that the charged residues in these activation domains are generally unimportant for stable interaction and that interaction is primarily driven by Van der Waals contacts. Our mutagenesis studies on the SID suggest that similar rules will govern the interaction between transcription repressors and their co-repressors.

Another important feature emerging from structural studies on activation domains is that they tend to be unstructured in the absence of their target and adopt their helical structure upon binding (19, 20, 53). Each of our mutant SIDs containing alanine substitutions at non-contact residues interact with PAH2 approximately 2-fold better that the wild type. Because substitutions to alanine at these positions may promote helicity, we speculate that the SID may be unstructured in the absence of PAH2 and that alanine substitution at noncontact residues lowers the activation energy required for the SID to undergo the transition from random coil to helix.

It is not clear what structural features of PAH2 will be required for interaction with the SID. The PAH domains were originally suggested to consist of two amphipathic helices, helix A and B, separated by a flexible linker (33). Proline insertions into helix A of PAH2 and deletion of either the helix A or B of PAH2 eliminate binding to Mad1 or Mxi1, demonstrating the importance of these putative structures for interaction (28, 45). It may be that the PAH2 domain is most structurally similar to the KIX domain of CBP. The KIX domain consists of three α-helices, α1, α2, and α3. α1 and α3 pack approximately parallel to one another and are linked by α2, defining the hydrophobic groove that receives the hydrophobic face of the CREB KID α-helix (19). Because the linker between the helix A and helix B of PAH2 can be modeled as an α-helix (data not shown), it is possible that helix A and B form a hydrophobic cleft, analogous to that found in the KIX domain, which would receive the hydrophobic face of the SID.

The minimal SID contains 13 amino acids and is rich in hydrophobic amino acids. One mutant SID, Q10A/E14A/D17A, has hydrophobic amino acids at 10 of its 13 positions and binds 2-fold better than wild type. Therefore, the SID may be characterized as a hydrophobic region of amino acids. However, given that regions of hydrophobic amino acids are relatively common in proteins, it seems unlikely that other mSin3A/B interacting proteins will be identified through simple searches of protein data bases. Several other proteins have been identified that interact with the PAH domains of mSin3A and/or B. The 91 carboxyl-terminal residues of SAP30 interact with PAH3 (30). Two regions of N-CoR interact with mSin3A: residues 1–312 interact with PAH3, and residues 1829–1940 interact with PAH1 (31, 34). SAP30 and the amino-terminal portion of N-CoR lack regions with obvious sequence similarity to the Mad1 SID; however, deletion of an alanine-rich putative α-helix between amino acids 1833 and 1845 of N-CoR interrupts interaction with PAH1 (34). These findings suggest that protein domains that interact with PAH1 and PAH2 may be similar structurally and distinct from those that interact with PAH3. Current evidence shows that the interaction between Sin3-binding proteins and PAH domains is highly specific. For example, no interaction is detected between Mad1 and PAH1, PAH3 or PAH4 domains of mSin3A using GST pull-down experiments or directed two-hybrid experiments (27).[2] Therefore, while the PAH domains may be structurally related, each must have different requirements for specific protein-protein interaction.

The 15-amino acid region following the SID, residues 20–35, is highly conserved but it is only found in the vertebrate Mad family proteins. This conserved region is apparently not required for interaction with PAH2, and may have a destabilizing effect on the SID PAH2 interaction. Further, the minimal 13-amino acid SID functions similarly to the longer 35-amino acid SID in transcription repression experiments, suggesting that residues 20–35 are relatively unimportant for transcription repression. It is possible that this conserved domain plays an ancillary role in binding to PAH2 and repression, but this function is not revealed by the assays employed here. Because

[2] A. N. Billin and D. E. Ayer, unpublished results.

the Mad family and Mnt have overlapping, if not identical, DNA binding specificities (35, 38, 40, 41), it is possible that the residues 20–35 may themselves function as a protein-protein interaction domain that will distinguish activity of the Mad family from Mnt or other transcription repressor families that depend on SID-like domains for function.

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