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Pf1, a Novel PHD Zinc Finger Protein That Links the TLE Corepressor to the mSin3A-Histone Deacetylase Complex

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The mSin3A-histone deacetylase corepressor is a multiprotein complex that is recruited by DNA binding transcriptional repressors. Sin3 has four paired amphipathic alpha helices (PAH1 to -4) that are protein-protein interaction motifs and is the scaffold upon which the complex assembles. We identified a novel mSin3A-interacting protein that has two plant homeodomain (PHD) zinc fingers we term Pf1, for PHD factor one. Pf1 associates with mSin3A in vivo and recruits the mSin3A complex to repress transcription when fused to the DNA binding domain of Gal4. Pf1 interacts with Sin3 through two independent Sin3 interaction domains (SIDs), Pf1SID1 and Pf1SID2. Pf1SID1 binds PAH2, while Pf1SID2 binds PAH1. Pf1SID1 has sequence and structural similarity to the well-characterized 13-amino-acid SID of the Mad bHLHZip repressor. Pf1SID2 does not have sequence similarity with either Mad SID or Pf1SID1 and therefore represents a novel Sin3 binding domain. Mutations in a minimal fragment of Pf1 that encompasses Pf1SID1 inhibited mSin3A binding yet only slightly impaired repression when targeted to DNA, implying that Pf1 might interact with other corepressors. We show that Pf1 interacts with a mammalian homolog of the *Drosophila* Groucho corepressor, transducin-like enhancer (TLE). Pf1 binds TLE in an mSin3A-independent manner and recruits functional TLE complexes to repress transcription. These findings suggest that Pf1 may serve to bridge two global transcription networks, mSin3A and TLE.

Over the last decade, a wealth of genetic and biochemical evidence has demonstrated that transitions between "active" and "inactive" chromatin states are catalyzed by targeting large multiprotein complexes to DNA via sequence-specific DNA binding proteins. Generally, transcriptional activators interact with histone acetyltransferase (HAT)-containing complexes to drive the formation of active, or "open," chromatin, while transcriptional repressors interact with histone deacetylase (HDAC)-containing complexes to drive the formation of inactive, or "closed," chromatin (for reviews, see references 21, 24, 29, and 38). ATP-dependent chromatin-remodeling complexes were initially thought to catalyze formation of active chromatin only, but it is now apparent that they facilitate the access of both HAT and HDAC complexes (4, 35, 60). While we have extensive knowledge concerning the composition and function of isolated HAT, HDAC, and chromatin-remodeling complexes, current evidence only hints at the regulation of these complexes, the existence of subcomplexes, and the cooperation between complexes (for examples, see references 17, 18, 27, 34, 37, 58, and 64).

Many transcriptional corepressors have been identified through mutagenic screens and through specific interactions with DNA binding repressor proteins. The Sin3 and Groucho complexes were originally isolated in lower organisms (22, 45, 54) and have been extensively characterized. The mammalian orthologs of Sin3p, mSin3A and mSin3B, were isolated as corepressors for the Mad1 family of transcriptional repressors (6, 52). Groucho was identified in *Drosophila melanogaster* as a corepressor utilized by the Hairy basic helix-loop-helix protein and hairy-related proteins (47). Similar to the Sin3 family, a family of Groucho orthologs—transducin-like enhancers (TLE) (human TLE1 [hTLE1] to -3, mTLE1 and -3, mGrg2, hAES1 and -2, and mAES1 and -2)—has been identified in mammalian cells (14, 55). While originally thought to function with specific families of transcriptional repressors, it is now known that both Sin3 and Groucho/TLE corepressors are targeted to a diverse set of promoters by an ever-increasing number of repressors (for reviews, see references 4, 14, 20, and 35).

mSin3A is part of a 1.2-MDa multiprotein complex comprised of 8 to 10 tightly associated polypeptides that are present in apparently stoichiometric amounts (23, 62, 65). Sin3 proteins themselves have four paired amphipathic alpha helix domains (PAH1 to -4) that serve as protein-protein interaction motifs (4, 35). Isolation and characterization of mSin3A-associated proteins have proved instrumental in gaining insight into mSin3A complex function. These associated proteins include HDAC1, HDAC2, SAP30, RbAP46, RbAP48, and SAP18. Experiments using deacetylase inhibitors and mutant HDACs with reduced enzymatic activity demonstrated that the majority of Sin3-dependent repression can be attributed to associated HDAC activity (23, 25, 30, 39, 65). Furthermore, targeting of Sin3 by the UME6 repressor in *Saccharomyces cerevisiae* resulted in localized deacetylation of the nucleosomal template within one or two nucleosomes of the targeting site (32, 50), implying that Sin3 may be primarily involved in short-range transcriptional repression. The function of the other mSin3A-associated proteins is less clear. SAP30 targets mSin3A to a subset of NCoR-regulated promoters (39), RbAp46 and RbAp48 likely direct mSin3A-associated HDAC activity to the amino-terminal tails of histones H3 and H4 (60), and SAP18 enhances Mad1-dependent repression by an unknown mechanism (65).

The Groucho/TLE family also appears to mediate transcrip-

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tional repression via interactions with the underlying chromatin template. For example, TLE1 can interact with the amino-terminal tail of histone H3 (46), and both genetic and biochemical interactions have been observed between *D. melanogaster* Groucho and dRPD3, a *Drosophila* HDAC1 homolog (15, 42). Furthermore, repression by promoter-targeted Groucho can be partially relieved by HDAC inhibitors (15). Groucho/TLE proteins share sequence and structural similarity to the *S. cerevisiae* Tup1p long-range repressor, which also uses associated HDACs (59, 61), suggesting that Groucho/TLE may function in long-range repression. In support of this hypothesis, Groucho can function at distances of 1 kb or more (7, 11). Like Sin3, members of the Groucho/TLE family appear to be members of multiprotein complexes (46); however, the components of Groucho/TLE complexes are only now beginning to be identified (15).

To fully understand the mechanisms of mSin3A-dependent repression, it is essential to identify its complete complement of associated proteins and investigate potential cooperation with other corepressor networks. Here we describe the cloning and characterization of a novel mSin3A-interacting protein that we have called PHD factor 1 (Pf1) because it has two plant homeodomain zinc fingers (PHD). Pf1 has two independent binding sites for mSin3A; one interacts with PAH1, while the other interacts with PAH2. Surprisingly, Pf1 also interacts with TLE proteins independently of mSin3A. Together, our data suggest that Pf1 links mSin3A and Groucho/TLE complexes, thereby providing a means for cooperation between these two global transcriptional corepressors.

MATERIALS AND METHODS

Yeast two-hybrid screen. A fusion protein containing the LexA DNA binding domain linked to PAH2 of mSin3A was used to screen a VP16 transactivation domain fusion library of mouse day 9.5 and 10.5 embryonic cDNAs as previously described (6, 8). Directed two-hybrid assays were performed using LexA fusions to PAH1 (amino acids 119 to 196), PAH2 (amino acids 297 to 385), PAH3 (amino acids 459 to 526), and PAH4 (amino acids 885 to 955).

cDNA cloning and Northern analysis. cDNAs encoding Pf1 were identified by screening a human fetal cDNA library with a ^{32}P-labeled, random-primed fragment of Pf1 identified in the two-hybrid screen. A complete cDNA containing the open reading frame of Pf1 was compiled using Sequencher software (Gene Codes). The complete clone was assembled from three overlapping cDNAs. For Northern analysis, whole-tissue and developmental Northern blots (Clontech) were probed with a 250-bp ^{32}P-labeled Pf1 fragment encompassing the first PHD zinc finger. Blots were washed in 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate at 65°C and exposed for autoradiography.

Plasmids. Full-length Pf1 was amplified by the PCR using *Pfu* polymerase (Stratagene), and the product was inserted into pcDNA3.1 (Invitrogen). Pf1 truncations were generated, FLAG epitope tagged using PCR, and cloned into pcDNA3.1. Ga14-Pf1 fusion proteins were made by using the pFA vector (Stratagene). FLAG-AES1 (56) was provided by S. Stifani (McGill University). Site-directed mutagenesis was performed using the QuikChange mutagenesis kit (Stratagene). All mutations and constructs made were verified by sequencing; and details concerning their generation are available upon request.

Transfections, immunoprecipitations, and Western blots. Polyclonal antibodies were raised against a glutathione *S*-transferase–Pf1 fusion protein containing amino acids 40 to 228 of Pf1. Specificity was determined by immunoprecipitating in vitro-transcribed and -translated Pf1 (Promega) under high- and low-stringency conditions.

HEK293 cells (American Type Culture Collection) were grown in Dulbecco's modified Eagle's medium (Gibco) with 10% defined calf serum (HyClone). For each immunoprecipitation, 8×10^5 cells were transfected with 5.0 μg of expression construct using calcium phosphate. Twenty-four hours later, the cells were washed in 1× phosphate-buffered saline (PBS), and whole-cell lysates were prepared in L buffer (1× PBS, 0.1% NP-40, 0.1 mM phenylmethylsulfonyl fluoride, 0.2% aprotinin, 2 nM leupeptin, and 1.5 nM pepstatin) by sonication. Sonication was performed using a Fisher Sonic Dismembrator using a power setting of 1.5 twice for 30 s each time. Proteins were immunoprecipitated with a 30-μl bed volume of M2-FLAG agarose (Sigma) while rocking for 1 h at 4°C. Parallel immunoprecipitations were blocked with 20 μg of FLAG peptide on ice for 30 min. Immunoprecipitates were washed four times with L buffer and analyzed by Western blotting using polyclonal antibodies recognizing mSin3A (23), SAP30 (39), HDAC1 (Affinity Bioreagents), and TLE (46) (kindly provided by S. Stefani). Generation of the FLAG-SAP30-stable HEK293 cell line is described elsewhere (39).

Western blotting was performed by wet transfer of polyacrylamide-resolved polypeptides using polyvinylidene difluoride membranes (MSI). Membranes were blocked in PBST (1× PBS, 0.025% Tween 40) with 5% nonfat dry milk and probed with polyclonal antibodies in PBST with 1% dry milk. Primary and secondary antibodies were used at 1:1,000 and 1:5,000, respectively. Blots were washed three times for 10 min each time and probed with horseradish peroxidase-conjugated secondary antibodies (Amersham). Blots were treated with ECL (Amersham) and were analyzed using a Lumiimager (Boehringer Mannheim).

Immunofluorescence. NIH 3T3 cells were transfected with FLAG-Pf1 and washed with 1× PBS 24 h later. The cells were fixed in formaldehyde (3.7% formaldehyde, 1× PBS) for 15 min at room temperature, washed three times with 1× PBS, and blocked for 30 min with PBT (1× PBS, 0.1% Triton X-100, 1% bovine serum albumin, 0.1% sodium azide). Cells were stained for 1 h at room temperature with anti-FLAG antibody (Sigma) diluted 1:1,000 in PBT and then washed twice in PBT. The cells were incubated with anti-mouse Alexa 488 (Molecular Probes) diluted 1:500 in PBT for 30 min at room temperature and stained with Hoechst 33342 (5 μg/ml). Slides were mounted with Prolong antifade (Molecular Probes) and visualized using a Zeiss microscope.

Generation of the stable Pf1-expressing cell line. FLAG epitope-tagged full-length Pf1 was amplified by PCR using *Pfu* polymerase (Stratagene) and inserted into pIRESpuro2 (Clontech). The linearized expression construct was transfected into HEK293 cells. Transfected cells were selected for Pf1 expression using 1 μg of puromycin per ml. Clones were isolated, and Pf1 expression was confirmed by Western blot analysis.

Transcription assays. For luciferase assays, 2×10^5 HEK293 cells were transfected with 100 ng of 14DG4-Luc reporter, 200 ng of Ga14 expression plasmid, and 25 ng of cytomegalovirus (CMV)-β-galactosidase (β-Gal). Twenty-four hours following transfection, cells were harvested and assayed for β-Gal and luciferase activities according to the manufacturers' guidelines (Promega and Tropix). For the HDAC inhibition experiment, cells were washed 24 h after transfection and treated with 100 ng of trichostatin A (TSA) per ml for 10 h. Data are presented as relative light units normalized to β-Gal activity to control for transfection efficiency.

HDAC assays. FLAG immunoprecipitates were resuspended in 250 μl of L buffer. Acid-extracted histones (13) from HeLa cells (50,000 dpm) were added, and the mixtures were incubated with agitation at 37°C for 1 h. Reactions were quenched with 50 μl of 0.16 M acetic acid–1 M HCl, and the aqueous phase was extracted with 600 μl of ethyl acetate. Deacetylase activity was measured as released [^{3}H]acetic acid and quantified by scintillation counting.

Nucleotide sequence accession number. The GenBank accession number for the Pf1 cDNA is AY030283.

RESULTS

Identification and cloning of Pf1. The PAH domains of mSin3A function as protein-protein interaction domains. To identify proteins that interact with PAH2 of mSin3A, PAH2 was fused to the LexA DNA binding domain and used as bait in a yeast two-hybrid screen. Of 30 interacting clones, 1 cDNA encoded a 188-amino-acid segment of a novel PHD zinc finger protein. We termed this protein Pf1 (see below). The complete open reading frame of Pf1 was cloned from a human fetal liver cDNA library and encodes a 704-amino-acid protein with a predicted molecular mass of 77 kDa. Pf1 has two PHD zinc fingers. The first finger, amino acids 59 to 102, fits the PHD C_4HC_3 consensus sequence (C^1-X_{1-2}-C^2-X_{9-21}-$C^3X_{2-4}C^4$-X_{4-5}-H^5-X_2-C^6-X_{12-46}-C^7-X_2-C^8) (1, 51), while the second finger, amino acids 274 to 318, has a histidine in place of cysteine at the last position of the consensus (Fig. 1A). The segment of

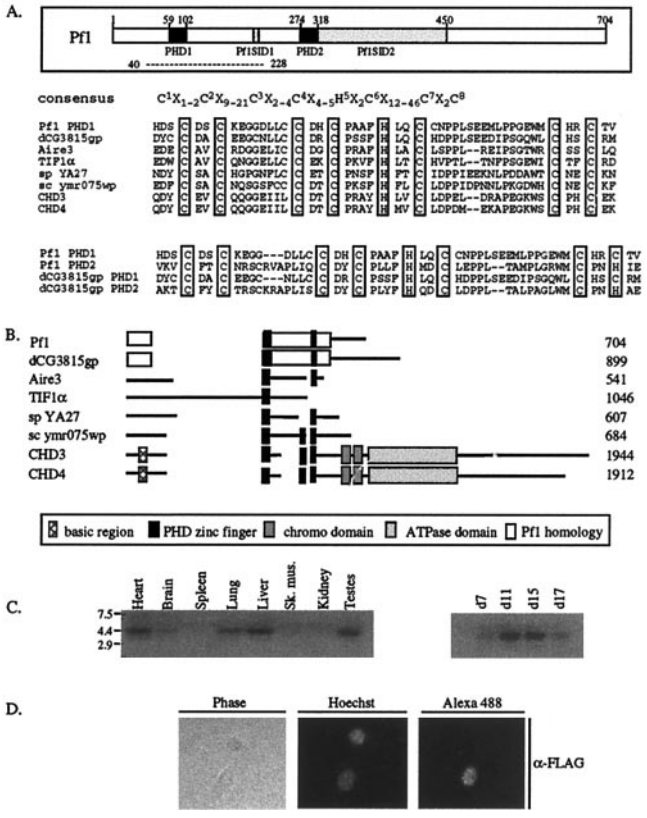

FIG. 1. Cloning and expression of a novel PHD zinc finger protein. (A) Schematic of Pf1. The dotted line indicates the region of Pf1 isolated as a VP16 fusion in the original two-hybrid screen. Pf1SID1 and Pf1SID2 are shaded (top). Alignments are shown of the first Pf1 PHD zinc finger with PHD zinc fingers found in the indicated proteins and the two PHD zinc fingers from both Pf1 and dCG3815gp (bottom). (B) Alignment of Pf1 with other PHD zinc finger proteins. Blocks denote basic regions, PHD zinc fingers, chromo domains, ATPase domains, and the region of homology between human Pf1 and dCG3815gp. Lines indicate that there is no significant similarity. Accession numbers for the proteins are as follows: dCG3815gp, AE003437; Aire3, NM000383.1; TIF1α; 015164; sp YA27; Q09698; sc ymr075wp, NC001145.1; CHD3, NM001272.1; and CHD4, NM001273.1. (C) Northern blot analysis of *Pf1* expression in adult mouse tissues (left) and in the developing mouse embryo (right). d7, d11, d15, and d17, days postcoitum that RNA was isolated. (D) Immunofluorescence of NIH 3T3 cells transfected with FLAG-Pf1 using an anti-FLAG antibody.

Pf1 isolated in the two-hybrid screen contains amino acids 40 to 228 of full-length Pf1 and the amino-terminal PHD finger. Database searches reveal that several proteins align with the PHD zinc fingers of Pf1. Of these, only the product of the *Drosophila* open reading frame dCG3815 has sequence similarity to Pf1 outside of the PHD zinc fingers, suggesting that it may be a Pf1 homolog (Fig. 1B). Pf1 lacks chromo domain and ATP-dependent helicase domains characteristic of the CHD family and is therefore most similar in overall structure to the Aire3 and TIF1α members of the PHD zinc finger family.

Northern blotting was performed to determine *Pf1* expression in adult mouse tissues and during murine embryogenesis. Pf1 is encoded by an mRNA of 4.5 kb and is expressed primarily in heart, brain, lung, liver, and testes (Fig. 1C). *Pf1* expression levels are low at day 7 of embryonic development, peak at day 11, and then decrease through day 17. Searches of the High Throughput Genome Sequence database revealed that *Pf1* is localized to chromosome 17 (accession number AC042267) and includes 12 exons (data not shown). Therefore, *Pf1* is developmentally regulated and widely, but not ubiquitously, expressed in adult mouse tissues. We were unable to detect Pf1 protein in 14 cell lines and nuclear extracts prepared from rat livers using a polyclonal antibody raised against amino acids 40 to 288 of Pf1 in a Western blot. The Pf1 antibody was able to detect 10 ng of purified recombinant Pf1, suggesting that Pf1 protein levels are low in most cell sources (data not shown).

Many PHD zinc finger proteins are localized to the nucleus and are thought to regulate transcription (1, 41). To determine the subcellular localization of Pf1, we transfected several different cell types with an expression construct encoding FLAG epitope-tagged Pf1 and determined localization by indirect immunofluorescence. Like other PHD zinc finger proteins, Pf1 localized to the nucleus when expressed in NIH 3T3, HEK293, and HeLa cells (Fig. 1D and data not shown), consistent with a potential role in transcriptional regulation.

Pf1 interacts with mSin3A in vivo. To confirm the two-hybrid interaction between Pf1 and mSin3A, we determined whether full-length Pf1 could interact with endogenous mSin3A. An expression construct encoding FLAG epitope-tagged Pf1 was transfected into HEK293 cells, and Pf1-associated proteins were collected by immunoprecipitation with FLAG antibodies. mSin3A association was detected by Western blotting. Pf1 coprecipitated mSin3A, and this interaction was blocked when the FLAG antibody was preincubated with FLAG peptide (Fig. 2A). We were concerned that the interaction between Pf1 and mSin3A might have occurred because Pf1 was expressed at a high level following transient transfection. To address this concern, we made a HEK293 cell line that stably expresses FLAG-Pf1. Pf1 levels in this cell line were much lower on a per-cell basis than levels in cells transfected with Pf1 expression vectors and much lower than endogenous mSin3A levels (Fig. 2A and B). Pf1 still coprecipitated mSin3A and HDAC1 from the stable cell line, suggesting that Pf1 specifically interacts with mSin3A in vivo and the interaction does not result from high expression of Pf1.

The majority of mSin3A is in high-molecular-weight complexes (28, 62); however, it is possible that Pf1 interacted with free mSin3A and HDAC1 rather than the complex. To show that Pf1 interacted with the mSin3A complex, we used a HEK293 cell line that stably expresses FLAG-SAP30. Using this cell line, it has been shown that SAP30 associated with all known members of the mSin3A complex (39). Untagged Pf1 was transfected into the FLAG-SAP30-expressing cells, and the mSin3A complex was isolated by FLAG immunoprecipitation. Pf1, mSin3A, and HDAC1 all coprecipitated with SAP30, suggesting that Pf1 associated with an mSin3A complex rather than free mSin3A (Fig. 2C). These data might also be explained by an mSin3A-independent interaction between Pf1 and SAP30. However, no interaction between in vitro-synthesized Pf1 and SAP30 was detected, suggesting that Pf1 and SAP30 do not interact directly (data not shown).

To identify the regions of Pf1 responsible for interaction with mSin3A, we made a series of Pf1 constructs with amino- and carboxy-terminal deletions (Fig. 3A). These truncations

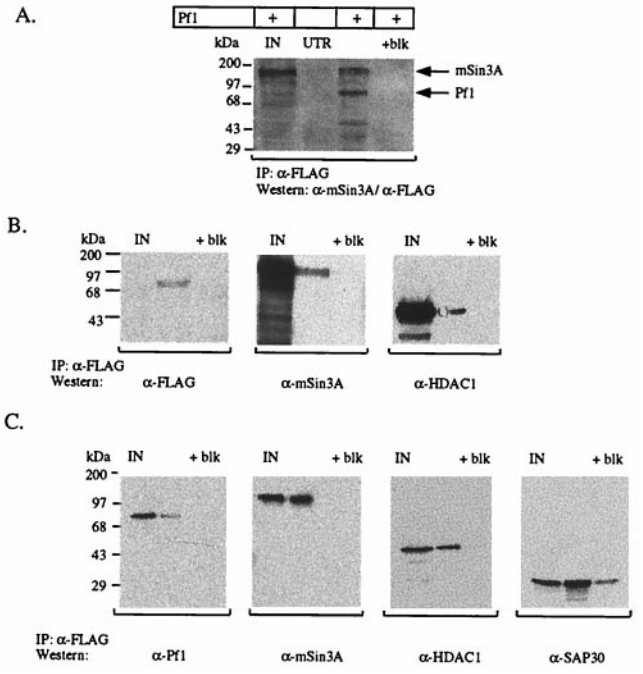

FIG. 2. Pf1 interacts with endogenous mSin3A in vivo. (A) Anti-mSin3A and anti-FLAG Western blot of FLAG immunoprecipitates (IP) of lysates prepared from HEK293 cells transfected with FLAG-Pf1. (B) Western blots of FLAG immunoprecipitates of lysates from HEK293:FLAG-Pf1, a cell line that stably expresses FLAG-Pf1. (C) HEK293:SAP30, a cell line that stably expresses FLAG-SAP30, was transfected with wild-type untagged Pf1. Cell lysates were prepared, and SAP30-associated proteins were isolated by FLAG immunoprecipitation. Associated proteins were identified by Western blotting using the indicated antibodies. IN, 1/15 input lysate used in each immunoprecipitation; UTR, FLAG immunoprecipitates from untransfected HEK293 cells; +blk, FLAG agarose incubated with FLAG peptide prior to immunoprecipitation.

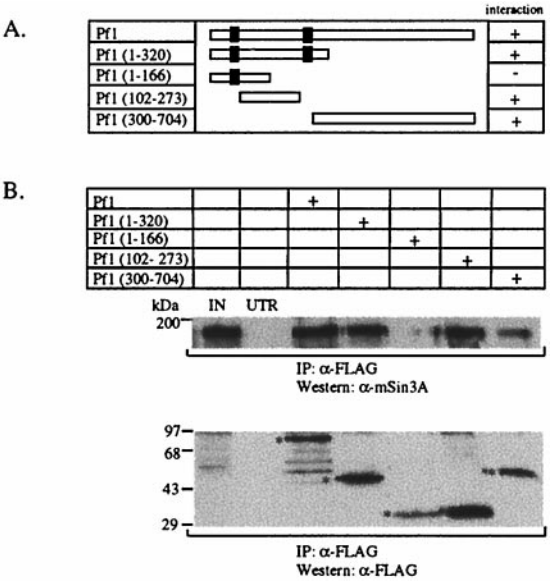

FIG. 3. Pf1 has two independent mSin3A binding sites. (A) Schematic of FLAG-tagged amino- and carboxy-terminal deletions of Pf1. Numbers correspond to the region of Pf1 expressed. Each construct was FLAG tagged at its amino-terminus. (B) Anti-mSin3A and anti-FLAG Western blots of FLAG immunoprecipitates (IP) of lysates prepared from HEK293 cells transfected with the Pf1 constructs shown in panel A. Asterisks mark the position of each FLAG-Pf1 protein. IN, 1/15 input lysate used in each immunoprecipitation; UTR, FLAG immunoprecipitates from untransfected HEK293 cells.

were tagged with the FLAG epitope at their amino termini and transfected into HEK293 cells. Association with endogenous mSin3A was determined by FLAG immunoprecipitation and Western blotting. Pf1 (1–320) and Pf1 (300–704) both interacted with mSin3A, suggesting that the mSin3A binding site lies between amino acids 300 and 320 (Fig. 3B). However, Pf1 (102–273), which lacks the potential binding site between amino acids 300 and 320, also bound mSin3A, ruling out this possibility. These data suggest that Pf1 has two independent mSin3A interaction domains (SIDs). To distinguish these binding sites from the SID of Mad1, we refer to them as Pf1SID1 and Pf1SID2 (Fig. 1A). mSin3A interacted with Pf1 (102–273) and not Pf1 (1–166), suggesting that Pf1SID1 lies between amino acids 166 and 273 (Fig. 3B). Interaction between mSin3A and Pf1 (300–704) demonstrated that Pf1SID2 is carboxy terminal of amino acid 300. Finally, PHD zinc fingers are proposed to function as protein-protein interaction domains. However, Pf1 (102–273) and Pf1 (300–704), which lack the PHD zinc fingers, both bound mSin3A, demonstrating that the PHD zinc fingers are not required for mSin3A interaction.

To determine if Pf1 could interact with HDACs independently of mSin3A, we transfected HEK293 cells with our panel of FLAG-Pf1 deletion constructs and determined which regions of Pf1 interacted with HDAC1. We also measured the HDAC activity associated with each FLAG-Pf1 protein. Each of the Pf1 proteins that interacted with mSin3A (Fig. 3), i.e., Pf1, Pf1 (102–273), and Pf1 (300–704), precipitated both HDAC protein and activity (Fig. 4). By contrast, Pf1 (1–166) did not interact with mSin3A and did not immunoprecipitate HDAC protein or activity. Together, these results suggest that Pf1 associates with enzymatically active mSin3A-HDAC complexes and that it does not interact with HDACs independently of mSin3A.

Pf1 functions as a transcriptional repressor when targeted to DNA. Sin3 does not have regions of similarity to known DNA binding domains, nor does it bind DNA nonspecifically (6, 57). To repress transcription, Sin3 must be recruited by site-specific DNA binding factors, such as Mad or UME6 (23, 31, 40). However, it has been shown that mSin3A can be artificially targeted to DNA using a heterologous DNA binding domain fused to different components of the complex (39, 65, 66). To demonstrate that Pf1 interacted with functional mSin3A complexes, it was fused to the Gal4 DNA binding domain (Gal4-Pf1) and, following transfection into HEK293 cells, the transcriptional activity of this construct was tested on a Gal4-responsive (14DG4-Luc) minimal luciferase reporter gene (5). Gal4-Pf1 repressed activity of the reporter gene fivefold relative to Gal4 alone (Fig. 5A), suggesting that DNA-bound Pf1 was capable of recruiting functional mSin3A complexes.

To determine whether the regions of Pf1 that interacted with mSin3A also mediated repression, the Pf1 truncations diagrammed in Fig. 3A were fused to Gal4 and tested for their ability to repress transcription. A construct that did not inter-

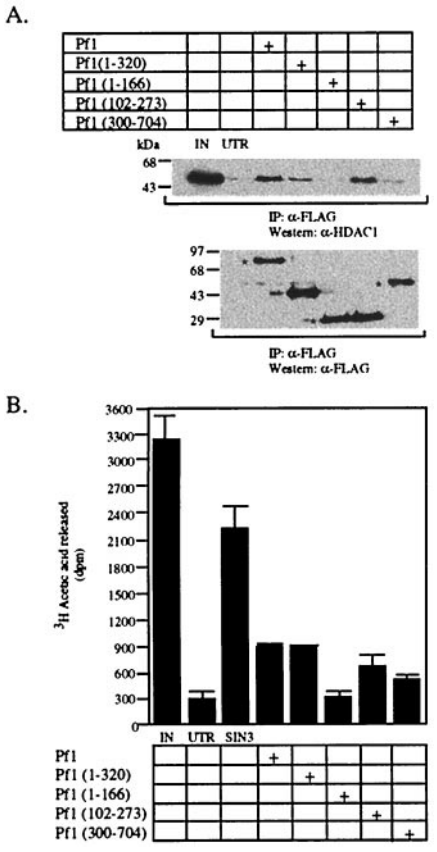

FIG. 4. Pf1 associates with HDAC1 and HDAC activity. (A) Anti-HDAC1 and Anti-FLAG Western blots of FLAG immunoprecipitates (IP) of lysates prepared from HEK293 cells transfected with the FLAG-tagged Pf1 deletion series; (B) HDAC activity of FLAG immunoprecipitates from HEK293 cells transfected with the FLAG-Pf1 deletion series. IN, 1/15 input lysate used in each immunoprecipitation; UTR, FLAG immunoprecipitates from untransfected HEK293 cells; SIN3, mSin3A immunoprecipitates from untransfected HEK293 cells.

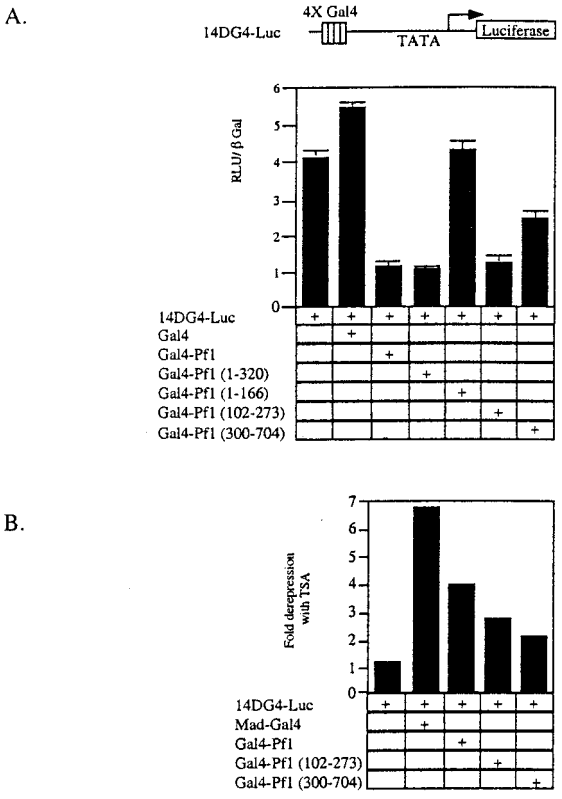

FIG. 5. Gal4-Pf1 interacts with functional mSin3A complexes. (A) HEK293 cells were cotransfected with plasmids encoding a minimal Gal4-responsive luciferase reporter (14DG4-Luc), CMV β-Gal, and the indicated Gal4-Pf1 fusions. Luciferase activity was determined 24 h after transfection. At the top is a schematic of the reporter construct. RLU, relative light units. (B) HEK293 cells were cotransfected with the indicated plasmids. Samples were left untreated (not shown) or treated with 100 ng of TSA per ml for 10 h prior to determination of luciferase activity. Fold derepression with TSA relative to untreated controls is shown.

act with mSin3A, Gal4-Pf1 (1–166), did not repress, while a construct containing Pf1SID1, Gal4-Pf1 (102–273), repressed transcription as effectively as Gal4-Pf1 (Fig. 5A). Pf1 (300–704) bound mSin3A less well than full-length Pf1 did (Fig. 3B), and consistent with this finding it repressed less effectively than Gal4-Pf1. The Gal4-Pf1 fusions were expressed equally, suggesting that the different levels of repression reflect differences in mSin3A recruitment rather than differences in expression (data not shown). Pf1 therefore recruited functional mSin3A complexes to DNA when targeted using Gal4. Furthermore, constructs encoding Pf1SID1 and Pf1SID2, each independently recruited functional mSin3A.

To determine whether Pf1 recruits functional HDACs to repress transcription, we tested if Pf1-mediated repression was sensitive to the HDAC inhibitor TSA. TSA relieved repression of both our positive control, Mad-Gal4 (23), and Pf1, suggesting that Pf1 requires an enzymatically active HDAC(s) to repress transcription (Fig. 5B). Furthermore, the degree to which the different Pf1 constructs were derepressed by TSA paralleled the relative levels of HDAC protein and activity (compare Fig. 5B with 4A and B). These results suggest that Pf1 represses transcription at least in part through the activity of an associated HDAC(s).

Characterization of the two mSin3A interaction domains of Pf1. Our original two-hybrid screen demonstrated that Pf1SID1 interacted with PAH2. We used two approaches to determine which PAH domain interacted with Pf1SID2. Initially, a yeast two-hybrid screen using full-length Pf1 fused to the LexA DNA binding domain as bait identified interacting Sin3 clones that contained PAH2 and clones that contained PAH1. To investigate this further, full-length Pf1 or regions encompassing either Pf1SID1, amino acids 1 to 273, or Pf1SID2, amino acids 300 to 704, were tested for interaction with VP16-PAH1 or VP16-PAH2. Full-length Pf1 bound both PAH domains, while Pf1SID1 bound PAH2 and Pf1SID2 bound PAH1 (Table 1). PAH1 bound full-length Pf1 with apparent higher affinity than isolated Pf1SID2, suggesting that the fusion construct [LexA-Pf1 (300–704)] lacked determinants required for high-affinity binding. Further mapping studies demonstrated that Pf1 (300–450) interacted with PAH1 similarly to full-length Pf1, suggesting that sequences carboxy terminal of amino acid 450 interfere with Pf1SID2-PAH1 in-

TABLE 1. Pf1 has two binding sites for mSinA[a]

Protein or fusion	β-Gal activity		
	VP16	VP16-PAH2	VP16-PAH1
LexA	−	−	−
LexA-Pf1	−	+++	+++
LexA-Pf1 (1–273)	−	+++	−
LexA-Pf1 (300–704)	−	−	+
LexA-Pf1 (300–450)	−	−	+++

[a] The yeast strain L40 was transformed with different LexA-Pf1 fusions and VP16 fusions to PAH1 or PAH2. β-Gal activity was scored as follows: −, none; +, weak; +++, strong.

teraction. Therefore, PAH1 and PAH2 bound independent SIDs on Pf1.

Recently, it has been shown that the 13-amino-acid alpha-helical SID located at the amino terminus of Mad1 was sufficient for interaction with PAH2 (10, 19). Mutagenesis and nuclear magnetic resonance studies demonstrated that the conserved sequence LLEAA, which is centrally located within the SID, made key contacts with PAH2 (10, 19, 53). PAH2 bound Pf1 between amino acids 166 and 273, and we noticed a region, between amino acids 208 and 220, which is similar to the SID of Mad1. Importantly, this region has the sequence LLIAA between amino acids 211 and 217, and we thought that this sequence might contribute to Pf1SID1. To test this hypothesis, helix-destabilizing proline substitutions were engineered at leucine 212 and alanine 216 in the context of Pf1 (102–273) (Fig. 6A). As before, Pf1 (102–273) interacted with endogenous mSin3A, but the proline substitutions almost completely abolished the Pf1-mSin3A interaction (Fig. 6B). Therefore, Pf1SID1 is centered around amino acid 214 and, like the SID of Mad1, is likely to form an alpha helix that mediates interactions with PAH2 (see Discussion).

Pf1 (102–273 L212P/A216P) does not interact with mSin3A, and we predicted that it would not be able to target transcriptional repression machinery. As before, Gal4-Pf1 (102–273) repressed transcription relative to Gal4 alone (Fig. 6C). However, Gal4-Pf1 (102–273 L212P/A216P) also repressed transcription, suggesting that Pf1 can repress transcription by mSin3A-independent mechanisms.

Pf1 interacts with TLE. The Groucho/TLE proteins are members of an abundant corepressor family, and we hypothesized that Pf1 might interact with TLE family members. To explore this hypothesis, we determined whether Pf1 could coimmunoprecipitate endogenous TLE. In these experiments, endogenous TLE proteins were detected using a pan-specific monoclonal antibody that recognizes the carboxy-terminal WD repeats of TLE family proteins (46). TLE interacted specifically with FLAG-Pf1, FLAG-Pf1 (102–273), and FLAG-Pf1 (102–273 L212P/A216P), demonstrating that mSin3A and TLE bind independent sites on Pf1 (Fig. 7A and data not shown).

To test whether TLE mediates repression by Gal4-Pf1 (102–273 L212P/A216P), we utilized a TLE protein, AES1, that has only the tetramerization, or Q, domain (14, 56). Because TLE function is dependent on tetramerization (16), AES1 is predicted to act as a dominant negative. Consistent with this notion, AES1 relieved repression by Gal4-Pf1 (102–273 L212P/A216P) in a dose-dependent manner but had no effect on the activity of Gal4 alone, Gal4-Pf1 (102–273), or Gal4-Pf1 (Fig. 7B and data not shown). AES1 had no effect on Gal4-Pf1 (102–273) repression, suggesting that it could not titrate mSin3A from Pf1. Finally, AES1 coprecipitated endogenous TLE, suggesting that it relieved repression by Gal4-Pf1 (102–273 L212P/A216P) by sequestering TLE (Fig. 7C). Together, these data suggest that in the absence of interactions with mSin3A, Gal4-Pf1 (102–273 L212P/A216P)-dependent repression can be attributed to interaction with endogenous TLE.

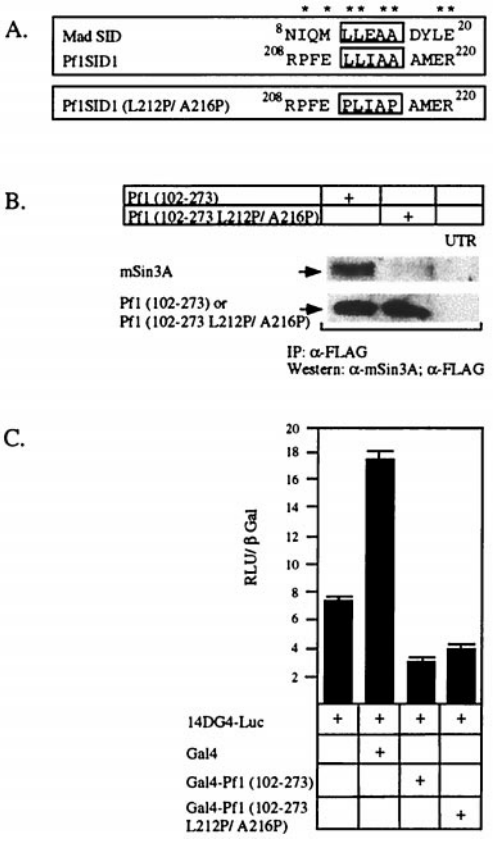

FIG. 6. Pf1 has a SID between residues 102 and 273. (A) Alignment of Pf1SID1 with the SID of Mad1. The conserved minimal core SID is boxed, and numbers are the amino acid position within each protein. Key contact residues in the SID of Mad1 required for interaction with PAH2 are marked with asterisks. Pf1SID1(L212P/A216P) has proline substitutions at conserved leucine and alanine positions 212 and 216. (B) HEK293 cells were transfected with FLAG-Pf1 (102–273) or FLAG-Pf1 (102–273 L212P/A216P) as indicated. FLAG immunoprecipitations (IP) were performed 24 h after transfection, and associated mSin3A was detected by Western blotting. UTR, FLAG immunoprecipitation from untransfected HEK293 cells. (C) Luciferase activity of cells transfected with 14DG4-Luc and the indicated Gal4-Pf1 fusion constructs. RLU, relative light units.

DISCUSSION

We have cloned and characterized a new member of the PHD zinc finger family called Pf1 that interacts with two global transcription corepressors: mSin3A and TLE. Both corepressors are abundant and broadly expressed. Furthermore, they function as corepressors for a broad spectrum of transcriptional repressors, each of which plays pivotal role in controlling different developmental pathways (for reviews, see references

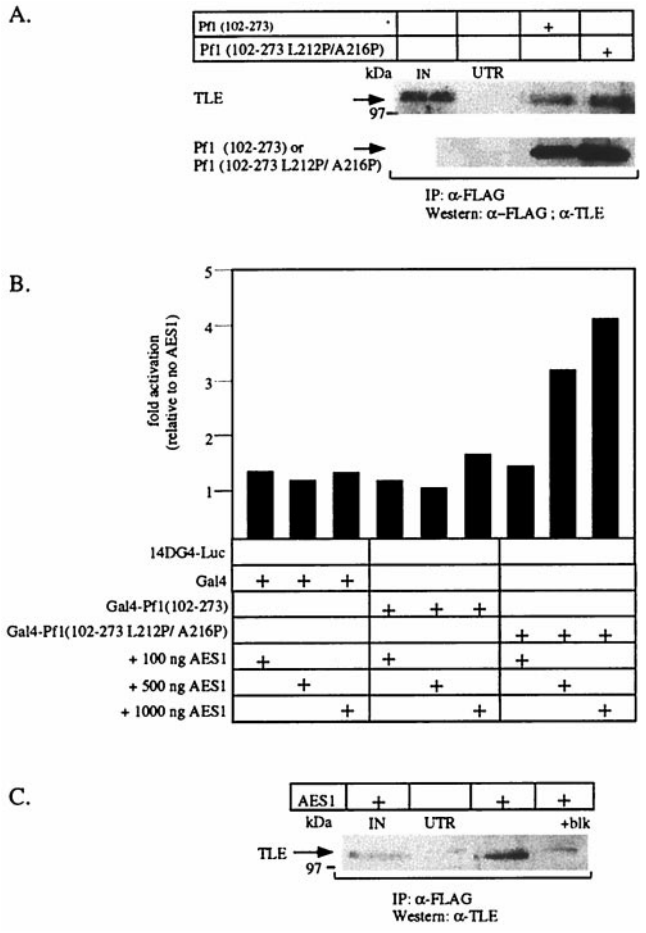

FIG. 7. Pf1 interacts with endogenous TLE. (A) Anti-TLE and anti-FLAG Western blot of FLAG immunoprecipitates (IP) of lysates from HEK293 cells transfected with FLAG-Pf1 (102–273) or FLAG-Pf1 (102–273 L212P/A216P). (B) HEK293 cells were cotransfected with plasmids encoding a minimal Gal4-responsive luciferase reporter (G414D-Luc), CMV β-Gal, and either Gal4, Gal4-Pf1 (102–273), or Gal4-Pf1 (102–273 L212P/A216P) and increasing amounts of AES1 as indicated. Luciferase activity was measured 24 h later. (C) Anti-TLE Western blot of FLAG immunoprecipitates of lysates prepared from FLAG-tagged AES1-transfected HEK293 cells. IN, 1/15 input lysate used in each immunoprecipitation; UTR, FLAG immunoprecipitates from untransfected HEK293 cells; +blk, FLAG agarose incubated with cognate peptide prior to immunoprecipitation.

2, 4, 14, 20, and 35). Our finding that mSin3A and TLE can be linked via Pf1 hints at cooperation between these two corepressors in regulating a diverse set of developmental pathways.

Several lines of experimental evidence support the physiological relevance of Pf1's association with both the mSin3A and TLE corepressors. With regard to mSin3A, Pf1 bound endogenous complex in cells stably expressing low levels of FLAG-Pf1. Pf1 is unlikely a stoichiometric component of the mSin3A complex, due to its low abundance and the absence of proteins with similar molecular weights in purified mSin3A complexes (23, 62, 65). Targeting of Pf1 to DNA with the heterologous DNA binding domain of Gal4 resulted in transcriptional repression, in a manner that correlated with interaction with the mSin3A complex. We have identified two independent binding sites for mSin3A on Pf1 (see below). A fragment containing the first of these sites, amino acids 102 to 273, repressed transcription when tethered to DNA. However, a mutant version of this construct that no longer interacted with mSin3A still repressed transcription, suggesting that additional corepressors might interact with Pf1. This observation led to the discovery that Pf1 can also target functional TLE corepressors to DNA.

Gal4-Pf1 transcriptional repression was sensitive to TSA, suggesting that targeted Pf1 requires active HDACs for it to function. By contrast, Gal4-Pf1 (102–273 L212P/A216P), which does not interact with mSin3A but retains TLE interaction, was not affected by TSA (data not shown). This finding was somewhat surprising, because repression by Groucho depends at least in part on associated HDAC activity (15). However, not all Groucho-regulated genes are derepressed in dRPD3 mutants, demonstrating that Groucho function does not depend solely on dRPD3 (42). Our data suggest that the lack of effect of TSA on Gal4-Pf1 (102–273 L212P/A216P) is due to HDAC-independent mechanisms of repression by TLE.

Pf1 has two independent mSin3A binding sites, or SIDs. Pf1SID1 interacts with PAH2, and Pf1SID2 interacts with PAH1. The SID of Mad1 adopts an amphipathic alpha-helix, and its apolar face binds to a deep hydrophobic pocket formed by PAH2 (10, 53). Given the sequence similarity between Mad1 SID and Pf1SID1, it seems likely that Pf1SID1 would interact similarly with PAH2. Consistent with this hypothesis, Pf1SID1 can be modeled as an amphipathic alpha helix, bind PAH2 directly with nanomolar affinity, and induce structural changes in PAH2 very similar to those observed with Mad1 (K. Brubaker, I. Radhakrishnan, G. S. Yochum, and D. E. Ayer, unpublished data). The interaction between Pf1SID2 and PAH1 appears to be more complex. The PAH domains are predicted to adopt similar structures and therefore interact with related domains (10). However, Pf1SID2 and other PAH-binding proteins, including NRSF/REST (44, 49), MNF-β (63), SAP30 (39, 66), and NCoR (3, 26), do not have homology to the SID of Mad1 or Pf1SID1. Therefore, PAH domains likely adopt different structural conformations to bind a variety of protein surfaces.

Both mSin3A and TLE complexes are recruited by sequence-specific DNA binding repressors. Because Pf1 does not have a recognizable DNA binding domain, we propose that it may function to tether the mSin3A and/or TLE complexes to DNA via a sequence-specific DNA binding protein(s). Alternatively, Pf1 may function as a bridging molecule to bring TLE to mSin3A-dependent repressors and vice versa. We cannot exclude the possibility that Pf1 has a novel DNA binding domain and interacts with DNA directly. However, current evidence suggests that most, if not all, PHD zinc finger proteins are targeted to DNA indirectly (33, 36, 43).

The role of the PHD zinc fingers in Pf1 is unknown. It has been proposed that PHD zinc fingers may function as DNA binding motifs. We found that in vitro-translated Pf1 does not interact with naked DNA or nucleosomal templates, which suggests alternative roles for PHD zinc fingers in Pf1 (data not shown). A second hypothesis is that PHD zinc fingers serve as protein-protein interaction domains. Our findings demonstrate that Pf1 does not require its PHD zinc fingers to bind mSin3A or TLE. It is still possible, however, that the PHD zinc fingers may stabilize a Pf1 complex of proteins through either intra- or interprotein interactions. This notion is supported by the fact

that PHD zinc fingers are structurally similar to RING finger domains (12, 48) and that RING finger domains are postulated to function in the assembly of multiprotein complexes (9).

Whether a single molecule of Pf1 can interact simultaneously with a single TLE and two mSin3A complexes is unknown. Alternatively, it is possible that Pf1 interacts with a single mSin3A complex via concurrent interactions with PAH1 and PAH2. Mutations in Pf1SID1 that abolished interaction between Pf1 and mSin3A had no effect on Pf1-TLE1 interaction, demonstrating independent binding sites for TLE and mSin3A on Pf1. Furthermore, repression by Gal4-Pf1 (102–273) was not affected by AES1, due to probable mSin3A binding. Therefore, simultaneous interactions are possible. By targeting multiple corepressors to DNA, Pf1 may play a specialized role in establishing and/or maintaining silenced chromatin structure. For example, the localized corepressor function of the mSin3A complex may allow the establishment of a repressed chromatin state, while the long-range repressor function of the TLE complex may facilitate the spreading of the silenced chromatin domain from the site of targeting. Alternatively, TLE proteins may function in maintenance by binding the deacetylated amino-terminal tails of the core histone, generated by the targeted mSin3 complex, and thereby block access to the tails by other chromatin-modifying activities.

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