Deformed epidermal autoregulatory factor-1 (DEAF-1) is a DNA-binding protein required for embryonic development and linked to clinical depression and suicidal behavior in humans. Although primarily nuclear, cytoplasmic localization of DEAF-1 has been observed, and this suggests the presence of a nuclear export signal (NES). Using a series of fluorescent fusion proteins, an NES with a novel spacing of leucines (LXL2LXL2LXL-L) was identified near the COOH-terminal MYND domain at amino acids 454–476. The NES was leptomycin B-sensitive and mutation of the leucine residues decreased or eliminated nuclear export activity. In vitro pull downs and an in vivo fluorescent protein interaction assay identified a DEAF-1/DEAF-1 protein interaction domain within the NES region. DNA binding had been previously mapped to a positively charged surface patch in the novel DNA binding fold called the “SAND” domain. A second protein-protein interaction domain was identified at amino acids 243–306 that contains the DNA-binding SAND domain and an adjacent zinc binding motif and a monopartite nuclear localization signal (NLS). Deletion of these adjacent sequences or mutation of the conserved cysteines or histidine in the zinc binding motif not only inhibits protein interaction but also eliminates DNA binding, demonstrating that DEAF-1 protein-protein interaction is required for DNA recognition. The identification of an NES and NLS provides a basis for the control of DEAF-1 subcellular localization and function, whereas the requirement of protein-protein interaction by the SAND domain appears to be unique among this class of transcription factors.

Identification of a Nuclear Export Signal and Protein Interaction Domains in Deformed Epidermal Autoregulatory Factor-1 (DEAF-1)*

Deformed epidermal autoregulatory factor-1 (DEAF-1) was first identified in Drosophila as a DNA-binding protein and potential regulator of the homeotic gene Deformed (1). The human, rat, and monkey homologs of Drosophila DEAF-1 (dDEAF-1) were previously called “nuclear DEAF-1 related” (NUDR) because of the limited protein similarity (46%) to dDEAF-1 (2). Genomic sequencing projects have confirmed a single gene in metazoan genomes with the overall structure of dDEAF-1, therefore we have adopted the DEAF-1 designation for all orthologs of the gene. DEAF-1 proteins are structurally defined as having both a DNA binding SAND domain and an adjacent cysteines or histidine in the zinc binding motif not only inhibits protein interaction but also eliminates DNA binding, demonstrating that DEAF-1 protein-protein interaction is required for DNA recognition. The identification of an NES and NLS provides a basis for the control of DEAF-1 subcellular localization and function, whereas the requirement of protein-protein interaction by the SAND domain appears to be unique among this class of transcription factors.

* This work was supported by National Institutes of Health Grant CA89436 (to J. I. H. and M. W. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: DEAF-1, deformed epidermal autoregulatory factor-1; SAND, Sp100, AIRE-1, NucP41/75, DEAF-1; MYND, myeloid translocation protein 8, Nervy, DEAF-1; GMEB, glucocorticoid modulatory element-binding; NLS, nuclear localization signal; NES, nuclear export signal; GST, glutathione S-transferase; GFF, green fluorescent protein; SUMO-1, small ubiquitin-related modifier; HA, hemagglutinin; WT, wild type; LMB, leptomycin B; TRITC, tetramethylrhodamine isothiocyanate.
Because nuclear and cytoplasmic trafficking may play an important role in the regulation of DEAF-1 function, we have sought to further define the nuclear localization and nuclear export signals in DEAF-1. We have found that the nuclear localization signal (NLS) and newly identified NES of DEAF-1 can participate in DEAF-1/DEAF-1 protein interaction. In addition, the NLS, and the zinc binding motif adjacent to the SAND domains were shown to be required for DEAF-1 binding to DNA. Thus, these protein regions appear to provide unique functions to DEAF-1 relative to other SAND domain-containing factors.

MATERIALS AND METHODS

Plasmid Construction—All DEAF-1 constructs were derived from human nuclear DEAF-1-related cDNA, accession number AF049459, unless otherwise noted. COOH-terminal hemagglutinin (HA)-tagged or FLAG-tagged DEAF-1 constructs in pcDNA3 were generated using PCR with the reverse primer 5′-GGGATCCCTCAGCATATGTCGACCTTCTCCATCAC-3′ for the HA tag or the reverse primer 5′-GGGATCCCTACTGGTGTGTGCTGTCCTGTTAGTCCAGGTCACCTCTTCACC-3′ for the FLAG tag. Both primers eliminate the DEAF-1 stop codon and provide an in-frame HA or FLAG sequence followed by a stop codon and a BamHI site. Plasmid constructs of the K304T mutation in the DEAF-1 (K304T) were previously described (2). A region that encompassed the K304T mutation was excised with restriction enzymes and used to replace the wild type region in DEAF-1-HA to create the HA-tagged K304T construct. The DNA encoding K315A/K316A, K304R, and K304T/L465A/L466A mutations was generated by ligating a HindIII/AatII fragment of K304T or H275S and an AatII/BamHI fragment of K315A/K316A into HindIII/BamHI cut pcDNA3. The 2xGFP construct was previously described methods (28). The DEAF-1 construct with the K304T mutation was excised with restriction enzymes and used to construct the K304T mutation in DEAF-1 and the GFP-DEAF-1-FLAG sequence followed by a stop codon and a BamHI site. Plasmid constructs were confirmed by DNA sequencing at the Southern Illinois University sequencing facility.

Functional Domains of DEAF-1

Localization of Fusion Proteins by Intrinsic Fluorescence and Immunofluorescence—CV-1 cells were grown on slides and plasmid DNA constructs were transfected for 3 h using Superfect (Qiagen). Fourteen hours after transfection, cells were fixed, permeabilized, and incubated with mouse anti-HA (Covance), mouse anti-FLAG (Sigma), rabbit anti-SUMO-1 (American Biosciences) or rabbit anti-HA antibody (Santa Cruz) and visualized with secondary anti-rabbit antibody conjugated to horseradish peroxidase followed by ECL treatment (Pierce).

Protein Purification—GST and GST fusion proteins were constructed in pGEX-2T vector (Amersham Biosciences) and expressed in CAG748 Echerichia coli cells (New England Biolabs) by 90 min induction with 0.2 mM isopropyl-1-thio-β-D-galactopyranoside at 37 °C. Cells were pelleted and lysed by sonication in 8 ml of lysis buffer (200 mM NaCl, 50 mM Tris (pH 8.0), 5 mM β-mercaptoethanol, and 1 protease inhibitor mixture tablet (Roche)). After sonication 800 μl of 10% Triton X-100 and 800 μl of 10% Tween 20 were added and the lysate was left on ice for 10 min. Cell debris was removed by centrifugation and the lysate was incubated with glutathione-Sepharose beads overnight at 4 °C. The beads were washed 4 times with lysis buffer and proteins were left bound to the beads for pull down experiments. Protein concentrations of the beads were determined by both SDS-PAGE comparisons to bovine serum albumin standards and by BCA assay (Bio-Rad). His-tagged fusion proteins were made from a modified pET-24d vector (Novagen Inc.) and were expressed in BL21-CodonPlus-RIL E. coli (Stratagene) by 2 h induction with 0.4 mM isopropyl-1-thio-β-D-galactopyranoside at 37 °C. Cells were pelleted and lysed by sonication in 8 ml of lysis buffer (200 mM NaCl, 40 mM Tris (pH 8.0), 5 mM β-mercaptoethanol, 0.2 mM isopropyl-1-thio-β-D-galactopyranoside, and the protease inhibitor mixture tablet (Roche)). After sonication 800 μl of 10% Triton X-100 and 800 μl of 10% Tween 20 were added and the lysate was left on ice for 15 min. Cell debris was removed by centrifugation and the lysate was incubated with nickel-nitrilotriacetic-agarose beads (Qiagen) overnight at 4 °C. The beads were then washed once with lysis buffer, twice with lysis buffer plus then twice with 30 mM imidazole and the beads were resuspended in 100 μl of 1× NaCl. Proteins were eluted with three aliquots of 750 μl of lysis buffer containing 300 mM imidazole. Eluted proteins were dialyzed against lysis buffer with 3 buffer exchanges. Proteins were checked for purity by SDS-PAGE and quantified by BCA assay.

GST Pull Down—[35S]Met- and [35S]Cys-labeled proteins were produced by in vitro translation using a TNT coupled reticulocyte lysate system (Promega). Two μg of GST fusion proteins coupled to GST beads were incubated with 10 μl of [35S]-labeled proteins for 16 h at 4 °C in interaction buffer (50 mM NaCl, 20 mM Tris (pH 7.9), 0.1% Nonidet P-40, 10% glycerol, 1 mM dithiothreitol, and 0.25% bovine serum albumin). Proteins coupled to the beads were washed 3 times with interaction buffer plus bovine serum albumin and then twice with interaction buffer without bovine serum albumin. Proteins were eluted from the beads with 30 μl of Laemmli sample buffer and separated by SDS-PAGE. [35S]-Labeled proteins were detected by PhosphorImager (Amersham Biosciences 44551).

Electrophoretic Mobility Shift Assays—His-tagged fusion proteins were purified from E. coli as described above. [35S]-Labeled DNA probes were synthesized using Klenow fill-in reactions as previously described (24) and incubated with 1 μg of the indicated proteins for 30 min at room temperature in 10 mM Tris (pH 7.5), 40 mM KCl, 6% glycerol, 1 mM dithiothreitol, 0.05% Nonidet P-40, 2 mM EDTA, and 1 μg of dA dT. After electrophoresis on nondenaturing gels, migration of the DNA probes were visualized by PhosphorImager.

RESULTS

Defining the Nuclear Localization Signal of DEAF-1—A potential bipartite NLS in human DEAF-1 was initially attributed to two clusters of basic amino acids between amino acids 299 and 316 (Fig. 1A) and had originally been proposed based upon similarity to the NLS motifs in nucleoplasmin and glucocorticoid receptor (2). Mutation of lysine 304 to threonine in the DEAF-1 protein (K304T) changes localization from strong nuclear expression to strong cytoplasmic expression, whereas mutation of the two lysines at positions 315 and 316 to alanines (K315A/K316A) does not affect nuclear localization and is similar to wild type DEAF-1 (Fig. 1B). These results indicate that the basic amino acids between 301 and 305 comprise a mono-
partite NLS rather than the bipartite NLS previously proposed (2). Analysis of the DEAF-1 protein sequence indicates that there is a potential SUMO-1 binding site, KKE-(304–306), which overlaps with the NLS in an arrangement similar to that found in cAMP-response element-binding protein. The KKE site in the cAMP-response element-binding protein was recently shown to be sumoylated and mutation of the first lysine resulted in loss of cAMP-response element-binding protein nuclear localization (30). To examine if potential sumoylation at a similar site in DEAF-1 affects localization, lysine 304 was mutated to an arginine (K304R) to maintain the basic charge of an NLS but disrupt the potential sumoylation site. The K304R mutant protein was localized to the nucleus like wild type DEAF-1, indicating that this site is unlikely to be sumoylated, or that sumoylation has no effect on DEAF-1 nuclear import or export in transfected cells (Fig. 1B). No change in DEAF-1 localization was observed by co-expression with SUMO-1 (Fig. 1B). Transfection of wild type or dominant-negative forms of Ub9 and HA-tagged SUMO-1 also did not alter DEAF-1 localization or produce anti-HA immunoreactivity in DEAF-1 immunoprecipitates (data not shown), again suggesting a lack of DEAF-1 sumoylation.

Identification of a Nuclear Export Signal in DEAF-1—To identify a region of DEAF-1 that may contain a nuclear export signal, specific regions of DEAF-1 were amplified by PCR and inserted into a 2xGFP construct to produce fusion proteins with GFP. Regions of 70 to 72 amino acids that were rich in hydrophobic residues were examined as potential NES sites, including amino acids 161–233, 284–357, and 411–484. As an NES-negative control, a 2xGFP construct was transfected into cells, and for a positive control, the 2xGFP with an insert encoding 20 amino acids that encompass the characterized NES of p73 (p73NES) (29) was also examined. The 2xGFP was distributed throughout the cell, whereas the p73NES showed strong cytoplasmic distribution, indicating that GFP localization was dependent on the inserted sequence (Fig. 2). An NH2-terminal DEAF-1 region (161–233) showed diffuse distribution throughout the cell (similar to 2xGFP) and indicated a lack of NES or NLS function in this region. Amino acids 284–357 of DEAF-1, which includes the DEAF-1 NLS, localized the GFP fusion protein to the nucleus. The same region with the K304T mutation showed both nuclear and cytoplasmic localization demonstrating that although this mutation disrupts the NLS, it is not sufficient to produce the complete cytoplasmic distribution observed with proteins containing more COOH-terminal regions. A region COOH-terminal of the NLS (411–484) showed cytoplasmic localization indicating a possible NES sequence (Fig. 2). Based on the documented NES motif defined by the consensus sequence, LX1−3LX2−3LXLIV (31–33), a potential NES does exist in this region (42). The 411–484 region was divided into three smaller overlapping regions to...
further distinguish the NES sequence. Both the 411–448 region, which includes the NES consensus mentioned above, and the 429–467 region localized GFP throughout the cell indicating a lack of NES activity (summary in Fig. 2). The 449–484 region fused to GFP showed strong cytoplasmic expression, indicating NES activity. This region contains a number of leucine residues but it does not contain a well defined NES sequence. To determine the minimal sequence of the putative NES, 4–7 amino acids from either the COOH- or NH₂-terminal ends were deleted from the 449–484 sequence in the 2xGFP construct. Upon deletion of four amino acids from the NH₂-terminal end, 453–484 showed NES activity, however, when an additional 6 amino acids were removed, 459–484, NES activity was lost. COOH-terminal deletions showed that region 449–476 retained NES activity, but further deletion to 449–471 lacked NES activity. Based on this mapping, a minimal NES is contained within the sequence 453WLYLEEMVSLNNTAQQLKTLFEQ 476 and was confirmed in both CV-1 and PC3 cells (Fig. 2). This amino acid sequence is highly conserved among human, monkey, rat, and mouse DEAF-1 orthologs.

Characterizing the Nuclear Export Signal of DEAF-1—Because the 453–476 region behaves as an NES and is leucine-rich yet does not conform to a consensus NES motif, we sought to determine which leucines were critical for nuclear export. Six mutations were designed in the 453–476 region and inserted into the 2xGFP construct (Fig. 3A); namely, three pairs of leucines and three neighboring but non-leucine amino acids were changed to alanines. Ninety-two percent of cells transfected with WT 453–476 showed strong cytoplasmic expression of GFP (export positive). Mutation of leucines to alanines at positions 454 and 456 (NESm1) decreased the percentage of export positive cells to 76%. Mutation of leucines 463 and 464 to alanines (NESm3) and leucines 470 and 473 to alanines (NESm5) strongly diminished export positive cells to 3 and 8%, respectively. The mutation of non-leucine amino acids in this region (NESm2, NESm4, and NESm6) appeared to have no effect on nuclear export. We conclude that the NES of DEAF-1 is located between amino acids 453 and 473 with the critical leucines in positions LₓLₓLₓLₓLₓL.

To determine whether export of the DEAF-1 NES was dependent on the export factor chromosome region maintenance-1, we examined the effects of the chromosome region maintenance-1 inhibitor, leptomycin B (LMB). The p73NES has been shown to
be LMB sensitive and was used as a positive control (29). Cells were transfected with p73NES or 453–476 of DEAF-1, which was transfected with full-length DEAF-1 containing a nuclear localization mutation (K304T-GFP) alone or in combination with a HA-tagged full-length DEAF-1 (wt-HA) or with various HA-tagged deletions or mutations as indicated. Intracellular localization of the HA-tagged proteins and (K304T)-GFP was observed by fluorescent microscopy as in Figs. 1 and 2, and relocation of (K304T)-GFP from the cytoplasm to the nucleus was scored as protein-protein interaction.

The effects of an NES mutation alone (L463A/L464A) or in combination with an NLS mutation were examined in the full-length DEAF-1 protein (Fig. 3C). Mutations in the NES (L463A/L464A) showed strong nuclear localization of the protein similar to WT-DEAF-1, whereas the K304T mutation in the NLS showed strong cytoplasmic localization. The combination of both NES and NLS mutations, L463A/L464A and K304T, resulted in the mutant protein being equally distributed between the nucleus and cytoplasm, indicating that the strong cytoplasmic localization of the NLS mutation (K304T) is because of a functional NES.

The NES Region Mediates DEAF-1 Protein-Protein Interaction in Vivo—Potential protein-protein interactions of DEAF-1 with itself were investigated in CV-1 cells using the DEAF-1(K304T)-GFP fusion protein (abbreviated (K304T)-GFP) and various HA-tagged DEAF-1 proteins (Fig. 4A). Intrinsic fluorescence of GFP allowed for visualization of the (K304T)-GFP mutant and an antibody to HA allowed visualization of the HA-tagged proteins. The (K304T)-GFP fusion protein showed similar cytoplasmic localization (Fig. 4) as the HA-tagged versions of the same mutant protein (Figs. 1B and 3C). When the NLS mutant (K304T)-GFP was coexpressed with WT-DEAF-1, the mutant protein was relocalized to the nucleus indicating an interaction between the two proteins. To map the location of this interaction, a number of NH2-, COOH-terminal, and internal deletions in DEAF-1 were generated and tested in a similar manner. Removal of the first 292 amino acids of DEAF-1 (1–292) produced a protein that was still able to relocate (K304T)-GFP to the nucleus, indicating that the interaction was in the COOH-terminal half of the protein. Deletion of the last 49 amino acids (1–341) also allowed relocation of (K304T)-GFP to the nucleus, eliminating the MYND domain as a region of protein interaction. However, when an additional 30 amino acids at the COOH terminus were removed (1–310), the (K304T)-GFP mutant remained in the cytoplasm.
in vivo and in vitro. A, HA-tagged full-length DEAF-1, NH2-, or COOH-terminal deletion expression constructs were transfected into CV-1 cells with DEAF-1-FLAG (odd lanes) or HA-tagged DEAF-1 (control even lanes). Cell lysates were prepared and immunoprecipitated with anti-FLAG-agarose, followed by Western blot analysis using an anti-HA antibody. Confirmation of expression of all constructs was confirmed by Western blot of inputs with either anti-FLAG or anti-HA antibodies. B, in vitro translated, 35S-labeled DEAF-1 deletion or mutation proteins were incubated with full-length DEAF-1 GST fusion protein or GST followed by pull downs with glutathione beads. Eluted proteins were separated by SDS-PAGE and proteins were visualized by autoradiography.

not show interaction with either 260–565 or 292–565. These results indicate that the region between 243 and 306 is a DEAF-1/DEAF-1 interaction domain and includes the monopartite NLS between amino acids 301 and 306 (Fig. 1).

In addition to the DNA binding SAND domain and NLS, within the 243–306 region of DEAF-1 is a zinc binding motif (Fig. 5C) (27). Mutations of histidine and several cysteines would coordinate zinc were generated to ascertain if this motif is involved in DEAF-1 self-interactions. A GST-(173–375) fusion protein was incubated with [35S]methionine-labeled DEAF-1-(1–326) or mutated forms of DEAF-1-(1–326, H275S), -(1–326, C279S/C281S), and -(1–326, C284S/C285S) and assayed for interaction by pull downs. Wild type DEAF-1-(1–326) showed interaction with GST-(173–375), but mutations of the
histidine and cysteines in the zinc finger motif eliminated this interaction (Fig. 5D). This demonstrates that the zinc binding motif adjacent to the SAND domain is required for protein-protein interaction of this DEAF-1 region.

The Zinc Binding Motif and NLS Are Necessary for DNA Binding of the DEAF-1 SAND Domain—Because the zinc binding motif resides close to the DNA binding domain, we sought to determine the effects of mutating the zinc binding motif and COOH-terminal deletions on DNA binding. A fusion protein between GST and amino acids 167–370 of DEAF-1 (peptide J, GST-167–370) was previously shown to bind a DNA ligand that contained two TTGC elements spaced apart by 9 base pairs (N52–69 probe), and mutation of the CG residue(s) in a single TTGC element (Mut 1) or in both TTGC elements (Mut 2) eliminated DNA binding (24). In addition, the shorter SAND domain (187–324) had previously bound the N52–69 probe as both a low and a high mobility species, and was also able to bind the Mut 1 probe but not the Mut 2 probe (24). In the current set of experiments, a His-tagged fusion protein of DEAF-1 peptide J (His-167–370) and various derivatives of this protein with mutations in the zinc binding motif or COOH-terminal deletions were examined for their ability to bind DNA (Fig. 6). The His-(167–370) fusion protein showed a single, shifted band with the N52–69 probe as previously described for GST-(167–370). Although the GST-(167–370) fusion protein did not bind the Mut 1 probe in the previous study (24), the His-(167–370) fusion protein does bind the Mut 1 probe albeit at a reduced level (Fig. 6). This suggests that the small His tag moiety probably has reduced steric hindrance relative to the GST moiety and allows region 167–370 to bind a single TTGC site. Mutations in the zinc binding motif (H275S or C279S/C281S) eliminated DNA binding to both the N52–69 and Mut 1. Deletions from the COOH-terminal end of the protein, 167–326 and 167–306, bound the N52–69 and Mut 1 probes, but further deletions that removed the NLS sequence (167–300) eliminated recognition of both DNA ligands. None of the Histagged fusion proteins showed binding to the Mut 2 probe, demonstrating specificity and the requirement of at least one CG dinucleotide. These results indicate that the zinc binding motif and NLS are required for DNA binding of the SAND domain, and indicate that DEAF-1 protein-protein interaction is a prerequisite for DNA binding.

The SAND and NES Regions Mediate Independent Self-Interaction in Vivo and in Vitro—The results of an in vivo relocalization assay (Fig. 4) defined a DEAF-1/DEAF-1 interaction region and NES at amino acids 447–475, whereas an in vitro pull down assay defined a second interaction region containing the SAND domain at amino acids 243–306 (Fig. 5). These regions were further evaluated for their influence on DEAF-1 protein-protein interaction both in vivo and in vitro. Co-immunoprecipitation experiments were performed to confirm the interactions in vivo. Cells were transfected with expression vectors for DEAF-1-FLAG and/or with HA-tagged DEAF-1 peptides. Transfected cell lysates were then immunoprecipitated with anti-FLAG (Fig. 7A). DEAF-1-FLAG showed interaction with DEAF-1-HA, (1–326)-HA, and (292–565)-HA indicating that protein interaction occurs with both the SAND domain and NES regions in vivo. We note that while there is a slight overlap in the regions used for this assay, smaller NES-containing regions fused to GFP, GST, and epitope tags can fail in relocalization, immunoprecipitation, or pull down assays probably because of improper folding or steric constraints in the fusion proteins. GST pull downs were then designed to address how both interaction regions of DEAF-1 might influence interaction with full-length DEAF-1. GST-DEAF-1 was incubated with various 35S-labeled in vitro translated DEAF-1 mutant proteins (Fig. 7B). As previously observed, GST-DEAF-1 interacted with WT-DEAF-1. Interaction was also observed with the DEAF-1-(1–306) and (292–565) peptides. Each of these regions encompasses one of the interaction sites, indicating that the sites can act independently of each other. When one of the interaction sites was mutated in the full-length protein (H275S or Δ447–475), interaction with GST-DEAF-1 was still observed, again supporting the concept that both sites can act independently. However, when both the H275S mutation and the NES deletion (Δ447–475) were introduced into the full-length protein, interaction with GST-DEAF-1 was eliminated, indicating that at least one of the sites is necessary for in vitro interactions. GST-DEAF-1 also interacted in pull downs with the NLS mutants K304T and K304T/Δ447–475, suggesting that a functional NLS is not required for the in vitro interaction and implying that loss of protein-protein interaction is not responsible for the cytoplasmic localization of the K304T mutation in vivo.

DISCUSSION

We have identified a leucine-rich NES motif between amino acids 453 and 476 of human DEAF-1. This NES was shown to confer cytoplasmic localization to the heterologous protein GFP and was sensitive to leptomycin B. However, the spacing of leucines that was found critical for export function, LXXLXXLXXL, differs from previously described sequences (32) and thus represents a novel chromosome region maintenance-1 interaction sequence. Sequence analyses of known DEAF-1 orthologs show that the mammalian proteins contain this NES sequence but there is little or no amino acid homology to the invertebrate orthologs of Drosophila and Anopheles, suggesting that the nuclear export function of the NES in mammalian DEAF-1 proteins may not be conserved in the invertebrate proteins. However, computer analysis indicates a high probability that the region between 450 and 500 in both Drosophila and mammalian DEAF-1 proteins will form a coiled-coil structural motif (8).

Coiled-coil motifs are formed from two to five amphipathic α-helices that may form higher order structures and have been shown to mediate versatile protein folding and oligomerization (34). Amino acids 456–476 in human DEAF-1 are predicted to form amphipathic α helices with seven residue periodicity (leucines at 456, 463, and 470) (35). This structure has been identified in other NES motifs (36) and has also been shown important in protein-protein interaction. Both in vitro (Fig. 7B) and in vivo assays (Fig. 4 and 7A) confirmed that the NES region of DEAF-1 functions in protein-protein interaction. An assay that examines the subcellular relocalization of fluorescent proteins was useful in demonstrating that the NES interaction domain included amino acids 453–476 (Fig. 4B). However, some discrepancies were observed for the assay and indicated that subcellular relocalization may be a more stringent criteria in assaying protein interactions relative to in vitro pull downs or in vivo coprecipitations. For example, the (Δ447–475)-HA protein that lacks the NES region but contains the SAND interaction domain was unable to relocalize the NLS mutant to the nucleus (Fig. 4A). This suggests the while the SAND domain functions as a protein interaction domain in pull downs, this is not sufficient to produce subcellular relocalization of a partner. Conversely, the (1–476)-HA peptide contains both the SAND and NES interaction domains but was unable to relocalize the NLS mutant to the nucleus (Fig. 4A). A possible explanation for this is that the peptide is at the very limit of the defined NES protein interaction domain and possibly does not achieve proper folding relative to the 2xGFP-(453–476) region that does relocalize to the nucleus (Fig. 4B).
GST pull downs demonstrated that amino acids 243–306 participate in protein-protein interactions and this region includes the DNA binding SAND domain, an adjacent zinc finger motif, and the NLS. Mutation of the histidine and cysteines in the zinc binding motif eliminated these interactions (Fig. 5D), suggesting that a potential function of the zinc finger is to promote protein-protein interaction in the SAND domain. Amino acids 243–306 include the monopartite nuclear localization signal of DEAF-1 and deletion of the NLS (30KRKK305) also eliminates protein-protein interaction (Fig. 5B). Mutations in the zinc finger or deletion of the NLS also eliminated DNA binding (Fig. 6), indicating that protein-protein interactions facilitated by these regions are required for DNA binding. A similar zinc binding motif was initially characterized in the GMEB SAND domain (27). Although DEAF-1 and GMEB show considerable homology in their SAND domains, the zinc binding motif in GMEB was shown not to be required for DNA binding and not directly involved in dimerization, but was suggested to serve an auxiliary role in stabilization of the overall structure of GMEB or with protein partners (27, 37). GMEB was found not to interact with DEAF-1 (38), further suggesting that the protein interaction domain of DEAF-1 will demonstrate partner specificity.

The potential importance of DEAF-1 in human depression and suicide, embryonic development, and cancer necessitates a comprehension of its interactions and trafficking in cells. The identification of an NES indicates that DEAF-1 may be regulated by nuclear/cytoplasmic shuttling while the presence of two protein-protein interaction domains suggests the possibility of cooperative binding and/or multimerization at target genes. Thus, the present study should provide a structural basis for understanding DEAF-1 regulation and function under normal and aberrant conditions.

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J. Biol. Chem. 2004, 279:32692-32699.
doi: 10.1074/jbc.M400946200 originally published online May 25, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M400946200

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