Conserved ETS Domain Arginines Mediate DNA Binding, Nuclear Localization, and a Novel Mode of bZIP Interaction**

Received for publication, August 19, 2005, and in revised form, October 6, 2005. Published, JBC Papers in Press, October 13, 2005. DOI 10.1074/jbc.M509143200

James A. Listman, Nawarat Wara-aswapati, JoAnne E. Race, Lisa W. Blystone, Nancy Walker-Kopp, Zhiyong Yang, and Philip E. Auron

From the SUNY Upstate Medical University, Syracuse, New York 13210, the Khon Kaen University, Khon Kaen 40002, Thailand, the University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261, and the Department of Medicine, Harvard Medical School, Boston, Massachusetts 02115

The DNA-binding ETS transcription factor Spi-1/PU.1 is of central importance in determining the myeloid-erythroid developmental switch and is required for monocyte and osteoclast differentiation. Many monocyte genes are dependent upon this factor, including the gene that codes for interleukin-1β. It has long been known that the conserved ETS DNA-binding domain of Spi-1/PU.1 functionally cooperates via direct association with a diverse collection of DNA-binding proteins, including members of the basic leucine zipper domain (bZIP) family. However, the molecular basis for this interaction has long been elusive. Using a combination of approaches, we have mapped a single residue on the surface of the ETS domain critical for protein tethering by the C/EBPβ carboxy-terminal bZIP domain. This residue is also important for nuclear localization and DNA binding. In addition, dependence upon the leucine zipper suggests a novel mode for both protein-DNA interaction and functional cooperativity.

The ETS family of transcription factors serves a variety of roles in development and differentiation (1). A highly conserved DNA-binding domain (DBD)** defines the family. Spi-1/PU.1 (Spi-1), one of the most diverse members, is critical for myeloid and B cell differentiation and function (2–6). It also regulates the expression of many effector genes, including IL-1β, coding for the IL-1β protein (7). The Spi-1 molecule possesses a multipartite transactivation domain (TAD) consisting of the amino half of the molecule that binds TBP, RB (8), and CBP/p300 (9) (Fig. 1a). The carboxyl end of the TAD contains a phosphorylatable PEST sequence that aids in the formation of a protein-protein interaction with transcription factors IRF-4 and -8 (10, 11). Located at the COOH-terminal end of the protein is the ETS DBD. Besides DNA binding, this domain is also a tethering site for multiple transactivators, some of which occur at composite DNA-binding sites (reviewed in Ref. 12), and contains the previously coarsely mapped nuclear localization signal (NLS) (13). The structure of the Spi-1 DBD, like other ETS factors, consists of 85 conserved residues folded into a winged-helix-turn-helix (wHTH) conformation (14) (Fig. 1b). Two arginines, which are solvent-exposed in helix α3, are conserved in all ETS family members and form direct and indirect hydrogen bonds to a core DNA sequence of A/GGAA in Spi-1 (1, 7, 15). The Spi-1 DBD is 98% conserved at the amino acid level between mouse and human, suggesting stringent structural and functional requirements.

Spi-1 plays a necessary role in the regulation of the IL1B promoter by cooperating functionally with C/EBPβ, a basic leucine zipper (bZIP) family member (16, 17). This cooperativity may be derived, in part, by a physical interaction between the DBD of both molecules and the two composite DNA-binding sites in the IL1B promoter (Fig. 2a) (18). The downstream TATA-proximal site binds relatively well to Spi-1 but weakly to C/EBPβ (7). C/EBPβ DNA binding at this site appears to be supported by a protein interaction with the adjacent DNA-binding domain of Spi-1 (18). Relative binding of these two factors at the upstream site is reversed relative to the downstream site and may play an especially critical role in serving to tether Spi-1 to C/EBPβ bound at a site (located between −2768 and −2761) in the IL1B upstream induction sequence (UIS) enhancer (19), based upon a greater loss of general enhancer function when the upstream site is deleted from the promoter (7).

Analogous phenomena have been described between ETS and other transcription factors (reviewed in (20)). For example, the cooperative function observed for Spi-1 and IRF-4 on the Igl λ enhancer is explained, in part, by the enhanced DNA binding of IRF4 mediated by sidechain contacts with Arg222 and Lys225 on the adjacent bound Spi-1 (10). C-Myb, another HTH protein, was shown to interact with the bZIP domain of C/EBPβ while each was bound to a remote DNA site, demonstrating a model of tethering between an enhancer and promoter (21). In contrast, Spi-1 interactions with several proteins including C/EBPβ and human cytomegalovirus (HCMV) IE2 reveal clear cooperative function but no obvious DNA binding cooperativity (18, 22, 23). This has led to a hypothesis of protein-tethered transactivation, whereby a direct DNA-binding protein tethers another protein, resulting in either activation or inhibition of function (18, 23). Therefore, understanding the molecular basis for these kinds of interactions continues to be intensely studied.

While investigating the nature of the Spi-1/C/EBPβ interaction, we uncovered new functions for the highly conserved arginines of helix 3 (Arg252 and Arg255) within the DNA recognition site. These functions expand upon the classic role these residues play in DNA binding and suggest new mechanisms of functional cooperativity and protein-DNA interaction for transcription factors.

MATERIALS AND METHODS

Plasmids—The pGL3B HT reporter plasmid was constructed as described previously (18). The 3MEHT and 3MEHT.A16 expression plasmids contain the IL1B promoter sequence −131 to +12, and the...
Multiple Functions for Conserved ETS Domain Arginines

**FIGURE 1.** Spi-1 structural and functional correlates. a, Schematic of Spi-1 functional domains as described in the text. The ETS domain is the location of the DNA-binding domain and tetras to several transcription factors. The secondary structures identified by crystallography are shown. b, Tertiary structure of Spi-1 ETS domain (Protein Data Bank ID 1PUE) rendered by DS Viewer Pro (Accelrys, Inc., San Diego, CA). The overall complex.

**IIIB** promoter sequence –131 to +12 with deletion of the C/EBPβ-binding site at –95 to –80, respectively, ligated to pA10CAT3ME (24). The HCMV expression vector pEQ326 (IE2), gift from Adam Geballe (Fred Hutchinson Cancer Research Center, Seattle, WA), contains the genomic HCMV IE2 DNA inserted into pGEM1 vector. The C/EBPβ pCDNA3.1 expression vector contains the full-length cDNA for C/EBPβ (19). The bZIP portion of C/EBPβ from amino acids 269–345 was previously cloned into pCDNA3.1 (Invitrogen) and pGEX 2T (Amersham Biosciences) by PCR (18). The Spi-1 pRC/CMV expression vector contains the murine Spi-1 cDNA as reported previously (25). Spi mutants were constructed using PCR mutagenesis as described previously (23) or by QuikChange (Stratagene, La Jolla, CA) site-directed mutagenesis according to the manufacturer’s instructions. Spi-1/ETS DBD constructs were either mutated within parent wild type (WT) plasmids or cloned by PCR amplification into either pRC/CMV or use in transfection assays, or pEX 2T (Amersham Biosciences) for use in GST pull-down assays as described previously (23). All constructs were verified by DNA sequencing. The pCMVSPORT-β-Gal vector was used in luciferase assays for normalization purposes.

**Purification of Fusion Proteins and GST Pull-down Assays—**GST fusion proteins were harvested from *Escherichia coli* BL21(DE)pLysS (Promega, Madison, WI) using previously described methods (23). Briefly, cultures were induced with 0.5 mM isopropyl β-D-thiogalactopyranoside for 3–4 h, pellets were suspended in NETN buffer (20 mM Tris, 100 mM NaCl, 1 mM EDTA, 0.05% Nonidet P-40) with 1 mM dithiotreitol, PefaBloc (Roche Applied Science), and one Complete™ protease inhibitor mixture (Roche Applied Science) tablet/50 ml. Suspensions were sonicated on ice and supernatants collected. Glutathione-Sepharose beads were washed in NETN buffer and incubated with fusion proteins at 4 °C rotating overnight. Beads were washed three times with NETN buffer and incubated with the in vitro translated protein probe of interest for 45 min rotating at 4 °C. After three washes with ice-cold NETN, beads were boiled and separated by SDS-PAGE. Gels were stained with SimplyBlue Safestain (Invitrogen) for protein determination and analyzed for binding using autoradiography. Radiolabeled protein probes were synthesized in vitro (TNT T7 quick coupled reticulocyte lysate system, Promega) and labeled with [35S]methionine (Amersham Biosciences) according to the manufacturer’s instructions.

**Transfection and Luciferase Assay—**Luciferase assays, HeLa S3 cells were transfected by Effectene reagent according to manufacturer’s instructions (Qiagen, Valencia, CA). Briefly, 4 × 10^4 HeLa S3 cells were plated in 24 well plates 24 h before transfection. Immediately before transfections, cells were washed with phosphate-buffered saline and placed in DMEM containing 10% fetal bovine serum and penicillin-streptomycin. Plasmid DNA (1.5 μg) was mixed in 60 μl of Qiagen EC buffer, 8.5 μl of enhancer, and 12.5 μl of Effectene reagent. After 5–10 min incubation at room temperature, DMEM was added to the transfection mixture, and an equal volume was transferred to each of three wells for triplicate samples. After 24 h, cells were washed with DMEM. Cells were lysed 48 h after transfection with luciferase assay reagent (Promega), and extracts were analyzed by luminometry (Luminoskan, Thermo Labsystems, Ramsey, MN). Results represent an average luciferase value after normalization to β-galactosidase activity as measured at the time of harvest with β-galactosidase enzyme assay system (Promega). For all transfections the total DNA amounts were kept constant using empty parental vectors. The CaPO₄ transfection and CAT assays were carried out as described previously (23).

**Western Blot—**Nuclear and cytoplasmic fractions were extracted from cells using a kit from Pierce Biotechnology following manufacturer’s instructions. After extraction, samples were separated on 16% SDS-PAGE gels (Invitrogen) and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were blotted with anti-Spi-1 antibody PU.1 and horseradish peroxidase-conjugated antibody secondary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Reactivity was visualized by enhanced chemiluminescence (National Diagnostics, Atlanta, GA).

**Electrophoretic Mobility Shift Assay—**Double-stranded oligonucleotide homologous to IIIB promoter region –131 to –58 was synthesized and labeled using DNA polymerase Klenow fragment in the presence of [α-32P]dGTP. Proteins were synthesized in vitro (TNT T7 coupled wheat germ extract system, Promega) and labeled with [35S]methionine. Relative protein amounts were estimated based on densitometry of autoradiographs of in vitro translation reactions, and volumes were adjusted to ensure equal amounts (and volumes) were added to DNA-protein binding reactions. Protein and labeled DNA probes were incubated under binding conditions of 10 mM Tris, pH 7.5, 50 mM NaCl, 3.3 mM MgCl₂, 0.06 μM 2-mercaptoethanol, 1 mM EDTA, and 5% glycerol with 1 μg poly(dI-dC) in a final volume of 15 μl. The binding reactions were incubated at room temperature for 20 min and then subjected to electrophoresis on a 4% nondenaturing polyacrylamide gel using 0.5×...
RESULTS

Conserved Arginines of Spi-1 DBD Confer Binding to C/EBP-IE2—Dose-response experiments revealed vigorous transactivation of the IL1B promoter by Spi-1 and C/EBPβ (Fig. 2, b and c, respectively). IE2, a viral transactivator derived from HCMV, could improve this activity by 4–10-fold in the absence of the UIS upstream enhancer (Fig. 2d). A collection of alanine substitutions of the Spi-1 DBD were generated for use as probes with GST fusions of the C/EBPβ bZIP domain and IE2. Substitution at Arg232 resulted in 80 and 50% reductions in C/EBPβ and IE2 binding, respectively, while substitution at Arg235 had a smaller effect on binding to C/EBPβ and little or no effect on binding with IE2 (Fig. 3). Combining the substitutions resulted in a 90% reduction in C/EBPβ binding. Substitutions in homologous residues previously shown to interfere with interaction between Jun/Fos, a bZIP heterodimer, and Erg, an ETS factor with homology to Spi-1, were also made (26). These substitutions, at Asn236, Tyr237, and Lys239 in Spi-1 had no effect on bZIP binding (data not shown). Substitutions at Arg222, Lys223, and Lys224 (containing residues that mediate Spi-1 DBD interaction with IFR4 (10)) also did not abrogate interaction with bZIP or IE2 (data not shown).

Nuclear Localization of Spi-1 DBD Also Depends on Conserved Arginines—Western blotting was performed on cytoplasmic and nuclear extracts to evaluate the expression of various mutated Spi-1 or DBD expression vectors and the results quantified by densitometry and compared with the ability of the same proteins to bind DNA by EMSA. There was a dramatic reduction in Spi-1 localized to the nucleus from 13% using WT to <1% using either alanine or aspartate substitutions at Arg232 (lane 1 versus lanes 6 and 7, respectively, in Fig. 4a and the indicated columns in b). As expected, we did not observe DNA binding for these proteins (Fig. 4c). However, the R235A substitution had no effect on nuclear localization, while reversing the charge with aspartate resulted in reduction to about 1% in nuclear localization (Fig. 4a, lanes 1 versus lanes 8 and 9, respectively, and the indicated columns in b). Although no detectable binding was observed for the charge-reversal substitution R235D, there was detectable, albeit reduced, DNA binding for the R235A substitution (Fig. 4c). Combined alanine substitutions at
Arg232 and Arg235 resulted in further reduction to <0.1% of the total protein in the nucleus and no detectable DNA binding (lane 1 versus lane 10, respectively, in Fig. 4a and the indicated lanes in b and c). When compared with the control, R222A, K223E, and K224E all had equivalent or slightly greater nuclear localization, but there was reduced DNA binding observed for R222A and K223E and normal DNA binding detected for K224E (Fig. 4a, lane 1 versus lanes 3–5, respectively, and the indicated lanes in b and c). Thus, not every substitution that impaired DNA binding resulted in reduced nuclear localization. The I191E-substituted protein was not detectable in the nuclear compartment and barely observable in the cytoplasm following transient transfection (Fig. 4a, lane 2). When expressed in vitro, I191E did not detectably bind DNA (Fig. 4c). These results are consistent with a loss of function for this mutant (Fig. 4d) resulting from compromised integrity to the structural hydrophobic core of the protein.

The observation that only a fraction of total Spi-1 localized to the nucleus corresponded with fluorescent microscopic observations made using green fluorescent protein fusion proteins, revealing both cytoplasmic and nuclear localization for the full-length molecule but predominantly nuclear localization for the isolated DBD (data not shown). These
observations suggest that some upstream portion of the Spi-1 molecule interferes with or regulates Spi-1 nuclear localization. To better quantify these observations, we also tested the intracellular distribution of the Spi-1 DBD by subcellular fractionation and Western blot analysis (Fig. 4a, lane 1 versus lane 11). The results showed a dramatic increase in localization to the nuclear compartment for the DBD to ~2/3 of the total protein (Fig. 4b).

Functional Analysis of Spi-1 DBD Residues—Expression vectors for selected Spi-1 constructs described in text. a, schematic of C/EBPβ showing functional and structural regions described in text. b, deletion of last 15 residues of the bZIP domain results in loss of Spi-1 binding in GST pull-down analysis. Radiolabeled WT bZIP or bZIP Δ330–345 was incubated with the indicated GST fusion proteins, washed, and the resulting complexes resolved by SDS-PAGE and radiography as described. c, excess Spi-1 expression can compensate for loss of DNA binding by C/EBPβ. Transient transfections were carried out in HeLa S3 cells as described. Briefly, CAT gene reporters containing either WT IL18 promoter sequence ~131 to ~12 (3MEHT or 3MEHTΔ6) were co-transfected with Spi-1 in increasing amount and reporter activity measured by CAT assay. Results are reported as amount of Spi-1 vector versus relative CAT activity ± S.E. d, C/EBPβ bZIP can support cooperative activity on IL18 promoter. Transient transfections were carried out in HeLa S3 cells as described in the legend to Fig. 2 using optimized amounts of Spi-1 and C/EBPβ and the HT-luc reporter. Cells were co-transfected with WT bZIP or bZIP Δ330–345 in increasing amounts. The resulting luminescence results are reported normalized to Spi-1 + C/EBPβ alone ± S.E. These are pooled data from three independent experiments. e, bZIP Δ330–345 retains ability to bind DNA. In vitro translated protein from the indicated expression vectors were incubated with radiolabeled probe for the HD region of the IL18 promoter (see Fig. 2a) and resulting complexes resolved by EMSA as described.

Residues 330–345 at the COOH-terminal End of C/EBPβ Mediate Binding to the Spi-1 ETS Domain and Support Spi-1 Function on the IL18 Promoter—The C/EBPβ molecule contains a transactivation domain at its N terminus and a basic leucine zipper structure at its COOH terminus (Fig. 5a). C/EBPβ, like other bZIP proteins dimerizes via the leucine zipper, which positions the basic region of each monomer at two opposing locations along the DNA major groove, thus recognizing a palindromic DNA motif. The coil-coil structure of the zipper protrudes away from the DNA helix at right angles, pointing into the solvent (21, 27–29). The bZIP region of C/EBPβ can also interact with numerous transcription factors, including other ETS family members and c-Myb (18, 30–32). However, the precise region of the bZIP molecule making these contacts was uncertain until the recent demonstration that 11 residues of the COOH-terminal end of the C/EBPβ bZIP molecule are required for interaction with c-Myb while bound to a remote DNA site (21). We hypothesized that a similar kind of complex interaction could occur between C/EBPβ and Spi-1. To test this hypothesis, we generated a bZIP fragment lacking residues 330–345 at the COOH-terminal end (bZIP Δ330–345). Using GST fusion protein pull-down analysis, we found that bZIP Δ330–345 had essentially complete loss of binding to a GST-ETS fusion protein compared with wild type bZIP, thus recapitulating the interaction between the C/EBP bZIP and c-Myb (Fig. 5b).

We previously reported that a minimal promoter containing only the TATA proximal composite site (DT in Fig. 2a) retained C/EBPβ-dependent cooperativity even when the C/EBP-binding site was rendered non-functional by substitutions that prevent C/EBPβ binding (18).
Multiple Functions for Conserved ETS Domain Arginines

Using a similar approach, we investigated the role of tethering at the upstream composite site. Because of the observed multiple functions of Arg in the Spi-1 DBD, we needed an alternative method to investigate the functional role of tethering. First, we found that the activity of the reporter containing an upstream C/EBP site deletion can be completely restored by increasing the amount of co-transfected Spi-1 expression vector (Fig. 5c). This result led to a hypothesis that the COOH terminus of the bZIP domain could support Spi-1 function. This was tested by determining the responsiveness of the IL1B reporter to transfection with either bZIP or bZIP Δ330–345 when co-transfected with Spi-1 and C/EBPβ (Fig. 5d). The results showed that cooperative transactivation of the IL1B promoter was enhanced by the TAD-deficient bZIP domain but not when missing the last 15 COOH-terminal residues. The loss of function by bZIP Δ330–345 could not be explained by the loss of DNA binding by this truncated product (Fig. 5e).

**DISCUSSION**

In our quest to map protein-tethering sites on the Spi-1 DBD, we have uncovered what may be novel functions for conserved arginines found in the DBD of all ETS members. Although these residues are well recognized for their role in DNA binding, our data support other functions relating to nuclear localization and protein tethering. The NLS for ETS factors was previously localized to the DBD (13). However, consensus searching did not reveal a classical NLS signal for this family of proteins. Our results reveal that nuclear localization is mediated by a non-classical NLS seated primarily over helix 3, which has Arg and Arg protruding into the solvent. The fact that deletion of the Spi-1 TAD leads to a significantly greater proportion of protein in the nucleus suggests that a region of the NH₂ terminus physically blocks access to helix 3. This may serve to either interfere with or regulate nuclear localization. While many DNA-binding proteins have NLS activity localized to their DBD (33), we are aware of only a few that have had NLS function and DNA binding co-localized to the same residues. These include the bZIP family members CREB, C/EBP, and Jun (34–36).

An interesting finding was that alanine substitution at Arg and Arg dramatically reduced binding to C/EBPβ with a lesser effect on binding to IE2. At first glance, this did not conform to our notions regarding the location of the interaction between these tethered partners, which was to assume interaction at a surface other than that contacting DNA. We previously reported that Spi-1 could tether C/EBPβ to the promoter because the downstream TATA proximal composite site binds strongly to Spi-1 but not C/EBPβ and that activity required the presence of the C/EBPβ TAD (18). Using a similar approach here, we investigated the role of tethering at the upstream composite site. This idea was plausible because we had previously reported that the downstream high avidity Spi-1 site and the low avidity upstream site were each required for maximal activity. However, deletion of the upstream C/EBPβ site demonstrated that it was important but less crucial (7). We now show that the activity of the reporter containing the upstream C/EBPβ site deletion could be completely restored by increasing the amount of co-transfected Spi-1 expression vector.

These data suggest that the function of this site does not require the specificity of the C/EBPβ TAD but rather a non-TAD-dependent cooperativity such as a protein-protein interaction. The observed cooperative effect of a TAD-deficient C/EBPβ bZIP on Spi-1 and C/EBPβ-dependent transactivation of the IL1B promoter upstream composite site is compelling evidence to support the hypothesis that the bZIP domain facilitates Spi-1 binding to this low avidity site. This result is in contrast to the expected dominant negative function typically found for C/EBP fragments missing the TAD in other systems. Loss of bZIP cooperativity by deletion of 15 COOH-terminal residues points to a region that mediates the tethering function with Spi-1 DBD. The loss of cooperative function by the deletion construct cannot be due to loss of dimerization or DNA binding as we observed equal, if not better, DNA binding by bZIP Δ330–345.

C/EBPβ and IE2 interact at a similar site on Spi-1, but with some distinctions that remain undefined given the difference in the degree of lost binding to C/EBPβ and IE2 when Arg and Arg are substituted. More biochemical and/or direct structural studies will be needed to precisely define the topography.

Our results raise interesting questions regarding the role of protein tethering at a locus critical for DNA binding. Analysis of the Spi-1 DBD crystal structure (14) shows that helix 3 sits in the major groove with Arg residing at the outside edge of the helix exposed to solvent (Fig. 6a). However, it does not seem likely that this residue would be accessible for interaction with the COOH terminus of C/EBPβ bound to an immediately adjacent DNA binding site. However, C/EBPβ in solution or complexed with a remote DNA site could gain access to this site.

This model is somewhat similar to that shown for tethering of a remote C/EBPβ enhancer site to c-Myb on the mim-1 promoter, although the molecular interactions differ (21). The C/EBPβ leucine zipper primarily interacts with a surface of c-Myb that does not directly contact DNA, while tangentially interacting with the DNA backbone outside of the major groove (21). In contrast, the location of Spi-1 Arg within the major groove suggests a novel penetration of the groove by the COOH terminus of the leucine zipper (Fig. 6a). This is plausible given the extensive basic electrostatic field surrounding the C/EBPβ leucine zipper as compared with that of Jun/Fos (Fig. 6b–e). This field would further potentiate a mass-action model given the acidic nature of DNA at sites of transcription, because electrostatic fields are stronger over greater distances than hydrogen bonding and van der Waals forces (37). These forces could serve to dock the bZIP leucine zipper within the major groove and position the COOH terminus for interaction with at least one of the two Nγ amino groups of Spi-1 Arg located at the COOH terminus of helix α3, thus stabilizing the interaction (Fig. 6a). Alternatively, Arg and helix α3 could interact with tethering partners of DNA.

This raises the possibility of two discrete functions that would depend on specific kinetic and thermodynamic properties of the protein and DNA interactions. First, the protein interaction may serve to compartmentalize Spi-1 with partner proteins at composite DNA-binding sites (Fig. 7). Such interactions would display cooperative binding on the promoter. This model would be particularly valuable for composite promoter sites where the for C/EBPβ binding to DNA is low and Spi-1 is high, as is the case for the upstream IL1B composite site (Fig. 7a). The converse is true for the downstream TATA-proximal composite site (Fig. 7b). Therefore, C/EBPβ and Spi-1 might either pre-associate prior to targeting the IL1B DNA via a C/EBPβ-Spi-1 interaction or C/EBPβ could first bind its cognate site and serve as a protein-protein-mediated recruiter for Spi-1. In either case, the interaction of one factor with its cognate DNA target would increase the local concentration of the other factor in the vicinity of its weak DNA-binding site, thus driving the interaction via mass-action following protein-protein dissociation. This thermo-kinetic scenario could occur for other promoters containing composite Spi-1 sites that exhibit DNA binding cooperativity with partner transcription factors, including the Spi-1-IRF interaction where Spi-1 possesses a greater affinity for its site than does IRF (38). Another scenario, which is not exclusive, incorporates a tether between the protein-interacting COOH terminus of C/EBPβ bound to the upstream composite site and Spi-1 bound at the proximal composite site (Fig. 7c).
FIGURE 6. Model of Spi-1 DBD and C/EBPβ bZIP interaction. a, surface representation of DNA bound Spi-1 DBD highlighting the location and basic nature of Arg232 (blue) and the acidic nature of DNA phosphate backbone (red). Adjacent and aligned parallel to the major groove is a surface representation of one monomer of the C/EBPβ bZIP dimer (shown for clarity is the backbone structure only for the second monomer), which is poised to interact with Arg232 and the phosphate backbone of DNA. This interaction mimics the interaction with DNA of the bZIP basic region at the opposite end of the molecule and may be supported by the highly basic nature of the leucine zipper of this bZIP domain. Isopotential electrostatic contours at ±1 kT/e for the Fos-Jun dimerized bZIP domain (b) and the C/EBPβ bZIP dimer (c). Connolly solvent surface using 1.4-angstrom (water) sphere radius reveals that the Fos-Jun dimer is positively charged (blue) only over its DNA-binding region and negatively charged (red) over its leucine zipper (d), while the C/EBPβ dimer is positively charged over both the basic and leucine zipper regions (e). Electrostatic calculations were generated using the Delphi Poisson-Boltzmann calculator and displayed with the GRASP visualization program (39). Structure coordinates were derived from Protein Data Bank ID 1FOS for Jun/Fos and 1HJB for C/EBPβ and 1PUE for Spi-1.
Multiple Functions for Conserved ETS Domain Arginines

![Image of cooperative interactions between DBD and bZIP]

**FIGURE 7. Models of cooperative interactions between DBD and bZIP.** (a) Spi-1 is attracted to its weaker upstream composite site by interaction between a region surrounding Arg^{232} of helix a3 of the Spi-1 DBD with the COOH terminus of bZIP (protein-protein interaction regions shown in solid gray). b, C/EBPβ is attracted to its weaker downstream composite site by interaction with Spi-1. Observations presented in a and b support a thermokinetic model of protein and DNA interaction between Arg^{232} of DBD and the C terminus of bZIP that serves to create a “sink” for interacting proteins. c, the COOH terminus of bZIP (bound at its stronger upstream composite site) interacts with Spi-1 (bound at its stronger downstream composite site) serving to tether remote DNA sites.

Similarly, tethering between C/EBPβ bound at the IL6B UIS enhancer (which contains a functional C/EBP site) and Spi-1 bound at a downstream composite site could be the structural basis for enhancer-promoter functional cooperativity.

In conclusion, we have found evidence that the conserved ETS domain arginines have evolved multiple functions in addition to DNA binding. These include nuclear localization and protein binding with other transactivators. The ability of this region to serve both functions expands upon the evolving and complex story regarding interfaces on other transactivators. The ability of this region to serve both functions may function not only in classic bZIP homotypic dimerization but also as a cationic DNA interaction module providing an anchor for mediating long range DNA looping.

Acknowledgments—We acknowledge the assistance of Pu Zhang, Gerhard Behre, and Daniel G. Tenen for providing numerous discussions and technical help during the early stages of this work. Deborah L. Galson provided valuable technical guidance and discussion throughout our investigations. Finally, we recognize Thomas R. Welch for his critical review of the manuscript.

REFERENCES

1. Sementchenko, V. I., and Watson, D. K. (2000) *Oncogene* 19, 6533–6548
2. Friedman, A. D. (2002) *Oncogene* 21, 3377–3390
3. Shin, M. K., and Kosshland, M. E. (1993) *Genes Dev.* 7, 2006–2015
4. Oikawa, T., Yamada, T., Kihara-Negishi, F., Yamamoto, H., Kondoh, N., Hitomi, Y., and Hashimoto, Y. (1999) *Cell Death Differ.* 6, 599–608
5. Fisher, R. C., and Scott, E. W. (1998) *Stem Cells* 16, 25–37
6. Auro, P. E. (2005) in *Measuring Immunity-Basic Science and Clinical Practice* (Lotzer, T. M., and Thomson, A. W., eds) pp. 91–109, Academic Press, New York
7. Kominato, Y., Galson, D., Waterman, W. R., Webb, A. C., and Auro, P. E. (1995) *Mol. Cell. Biol.* 15, 59–68
8. Hagemeier, C., Bannister, A. J., Cook, A., and Kouzardides, T. (1993) *Proc. Natl. Acad. Sci. U. S. A.* 90, 1580–1584
9. Yamamoto, H., Kihara-Negishi, F., Yamada, T., Hashimoto, Y., and Oikawa, T. (1999) *Oncogene* 18, 1495–1501
10. Escalante, C. R., Brass, A. L., Pongubala, J. M., Shatova, E., Shen, L., Singh, H., and Aggarwal, A. K. (2002) *Mol Cell* 10, 1107–1115
11. Marecki, S., Riendeau, C. J., Liang, M. D., and Fenton, M. J. (2001) *J. Immunol.* 166, 6829–6838
12. Li, R., Pei, H., and Watson, D. K. (2000) *Oncogene* 19, 6514–6523
13. Bouloukis, K. E., Pognonec, P., Rabault, B., Begue, A., and Ghydesd %, J. (1989) *Mol. Cell. Biol.* 9, 5718–5721
14. Kodandapani, R., Pio, F., Ni, C. Z., Picciulli, G., Klemmsz, M., McBercher, S., Maki, R. A., and Ely, K. R. (1996) *Nature* 380, 456–460
15. Pio, F., Kodandapani, R., Ni, C. Z., Shepard, W., Klemmsz, M., McBercher, S. R., Maki, R. A., and Ely, K. R. (1996) *J. Biol. Chem.* 271, 23329–23337
16. Akira, S., Ishihik, H., Sugita, T., Tanabe, O., Kinoshita, S., Nishio, Y., Nakajima, T., Hirano, T., and Kishimoto, T. (1990) *EMBO J.* 9, 1897–1906
17. Williams, S. C., Cantwell, C. A., and Johnson, P. F. (1991) *Genes Dev.* 5, 1553–1567
18. Yang, Z., Wara-Aswapati, N., Chen, C., Tsukada, J., and Auro, P. E. (2000) *J. Biol. Chem.* 275, 21272–21277
19. Tsukada, J., Saito, K., Waterman, W. R., Webb, A. C., and Auro, P. E. (1994) *Mol. Cell. Biol.* 14, 7285–7297
20. Verger, A., and Duterque-Couillaud, M. (2002) *BioEssays* 24, 362–370
21. Tahirov, T. H., Sato, K., Ichikawa-Iwata, E., Sasaki, M., Inoue-Bungo, T., Shiina, M., Kimura, K., Takata, S., Fujikawa, A., Morii, H., Kusamasaka, T., Yamamoto, M., Ishii, S., and Ogata, K. (2002) *Cell* 108, 57–70
22. Behre, G., Whitmarsh, A. J., Coghlan, M. P., Hoang, T., Carpenter, C. L., Zhang, D.-E., Davis, R. J., and Tenen, D. G. (1999) *J. Biol. Chem.* 274, 4939–4946
23. Wara-Aswapati, N., Yang, Z., Waterman, W. R., Koyama, Y., Tetrasdi, S., Choy, B. K., Webb, A. C., and Auro, P. E. (1999) *Mol. Cell. Biol.* 19, 6803–6814
24. Shirakawa, F., Saito, K., Bonagura, C. A., Galson, D. L., Fenton, M. J., Webb, A. C., and Auro, P. E. (1993) *Mol Cell Biol.* 13, 1332–1344
25. Galson, D. L., Hensold, J. O., Bishop, T. R., Schalling, M., D’Andrea, A. D., Jones, C., Auro, P. E., and Housman, D. E. (1993) *Mol. Cell. Biol.* 13, 2929–2941
26. Verger, A., Buisine, E., Carrere, S., Wintiens, R., Fleurons, A., Coll, J., Stehelin, D., and Duterque-Couillaud, M. (2001) *J. Biol. Chem.* 276, 17181–17189
27. Keller, W., Konig, P., and Richmond, T. J. (1995) *J Mol. Biol.* 254, 657–667
28. Glover, J. N., and Harrison, S. C. (1995) *Nature* 373, 257–261
29. Jamieson, A. C., Miller, J. C., and Fabbri, C. D. (2003) *Nat. Rev. Drug Disc.* 2, 361–368
30. Stein, B., Cogswell, P. C., and Baldwin, A. S., Jr. (1993) *Mol. Cell. Biol.* 13, 3964–3974
31. Xie, Y., Chen, C., Stevenson, M. A., Auro, P. E., and Calderwood, S. K. (2002) *J. Biol. Chem.* 277, 11802–11809
32. Tsukada, J., Misago, M., Serino, Y., Ogawa, R., Murakami, S., Nakahishi, M., Onai, S., Kominato, Y., Morimoto, I., Auro, P. E., and Eto, S. (1997) *Blood* 90, 3142–3153
33. LaCasse, E. C., and Lefebvre, J. A. (1995) *Nucleic Acids Res.* 23, 1647–1656
34. Forewood, J. K., Larm, M. H. C., and Jans, D. A. (2001) *Biochemistry* 40, 5208–5217
35. Waecher, G., and Habener, J. F. (1991) *Mol. Endocrinol.* 5, 1431–1438
36. Williams, S. C., Angerer, N. D., and Johnson, P. F. (1997) *Gene Expr.* 6, 371–385
37. Schulz, G. E., and Schirmer, R. H. (1984) *Principals of Protein Structure*, pp. 27–36, Springer-Verlag, New York
38. Brass, A. L., Zhu, A. Q., and Singh, H. (1999) *EMBO J.* 18, 977–991
39. Honig, B., and Nicholls, A. (1995) *Science* 268, 1144–1149