A combined computational and experimental approach reveals the structure of a C/EBPβ–Spi1 interaction required for IL1B gene transcription

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From the Departments of Biological Sciences and Chemistry and Biochemistry, Duquesne University, Pittsburgh, Pennsylvania 15282, the Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts 02115, the Department of Medicine, UPMC Hillman Cancer Center, University of Pittsburgh, Pittsburgh, Pennsylvania 15213, the Department of Computational and Systems Biology, University of Pittsburgh, Pittsburgh, Pennsylvania 15260, the Department of Microbiology & Molecular Genetics, University of Pittsburgh, Pittsburgh, Pennsylvania 15219

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We previously reported that transcription of the human IL1B gene, encoding the proinflammatory cytokine interleukin 1β, depends on long-distance chromatin looping that is stabilized by a mutual interaction between the DNA-binding domains (DBDs) of two transcription factors: Spi1 proto-oncogene at the promoter and CCAAT enhancer–binding protein (C/EBP) at a far-upstream enhancer. We have also reported that the C-terminal tail sequence beyond the C/EBPβ–leucine zipper is critical for its association with Spi1 via an exposed residue (Arg-232) located within a pocket at one end of the Spi1 DNA-recognition helix. Here, combining in vitro interaction studies with computational docking and molecular dynamics of existing X-ray structures for the Spi1 and C/EBPβ DBDs, along with the C/EBPβ–C-terminal tail sequence, we found that the tail sequence is intimately associated with Arg-232 of Spi1. The Arg-232 pocket was computationally screened for small-molecule binding aimed at IL1B transcription inhibition, yielding L-arginine, a known anti-inflammatory amino acid, revealing a potential for disrupting the C/EBPβ–Spi1 interaction. As evaluated by ChIP, cultured lipopolysaccharide (LPS)-activated THP-1 cells incubated with L-arginine had significantly decreased IL1B transcription and reduced C/EBPβ’s association with Spi1 on the IL1B promoter. No significant change was observed in direct binding of either Spi1 or C/EBPβ to cognate DNA and in transcription of the C/EBPβ-dependent IL6 gene in the same cells. These results support the notion that disordered sequences extending from a leucine zipper can mediate protein–protein interactions and can serve as drugable targets for regulating gene promoter activity.

Human interleukin 1β (IL-1β), 3 a member of the IL-1 family of cytokines coded by the IL1B gene, is an important mediator of inflammation in response to microbial invasion and tissue injury. Many rapidly induced/immediate-early genes such as FOS and EGR1 (1), TNF (2), and a majority of the 6,511 protein genes in human K562 cells (3) possess prebound RNA polymerase II (Pol II) co-present with TATA-binding protein (TBP), and TFIIB transcription preinitiation factors paused 30–50 bp upstream of the transcription start site. In contrast, we previously reported that the human IL1B gene promoter is deficient in prebound Pol II and constitutively binds the Spi1/PU.1 (Spi1) transcription factor in the almost complete absence of TBP and Pol II (2). Spi1, which is required for IL1B gene expression in monocytes (2), is a myeloid cell lineage-determining factor that has a C-terminal ETS family winged helix-turn-helix (wHTH) DNA-binding domain (DBD) and an N-terminal transactivation domain (TD). We also reported that the DNA-binding domain of constitutively promoter-bound Spi1 directly interacts by long-range chromatin looping (2) with the DBD of the C/EBPβ transcription factor, which in turn binds to a far upstream superenhancer following lipopolysaccharide (LPS)/ Toll-like receptor 4 (TLR4) signaling. The critical protein–protein interaction between Spi1 and C/EBPβ in monocytes

3 The abbreviations used are: IL, interleukin; DBD, DNA-binding domain; LPS, lipopolysaccharide; TLR-4, Toll-like receptor 4; Pol II, RNA polymerase II; TBP, TATA-binding protein; wHTH, winged helix-turn-helix; TD, transactivation domain; bZIP, basic leucine zipper; HTH, helix-turn-helix; HCMV, human cytomegalovirus; GST, glutathione S-transferase; MD, molecular dynamics; aa, amino acid(s); NAMD, Nanoscale Molecular Dynamics; MOE, Molecular Operating Environment; VMD, Visual Molecular Dynamics; SASA, solvent-accessible surface area; C/EBP, CCAAT enhancer–binding protein; PDB, Protein Data Bank; qPCR, quantitative PCR; NVT, constant number of atoms, volume, and temperature; NPT, constant number of atoms, pressure, and temperature.

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This article contains Table S1 and Figs. S1–S8.
occurs along with TBP and Pol II recruitment, gene expression, and an apparent chromatin loop stabilization (2, 4, 5). We have also reported that the C-terminal tail extension of the C/EBPβ basic leucine zipper (bZIP) is critical for C/EBPβ–Spi1 association via an exposed arginine residue (Arg-232) within a pocket at one end of the DNA-recognition helix in the Spi1–DNA X-ray structure (5) previously reported by others (6).

In an attempt to investigate the molecular nature of this critical protein–protein interaction, computational docking of the existing independent X-ray structures for the C/EBPβ and Spi1 DBDs yielded a conformation reminiscent of a C/EBPβ–cMyb X-ray structure previously reported by others (7). Although the cMyb structure provided a basis for a C/EBPβ–Spi1 interaction, the absence of the C/EBPβ C-terminal tail suggested a distinct binding mode for C/EBPβ with Spi1. In vitro interaction studies between the DBDs of the two proteins argues for a larger surface footprint on Spi1 than would be expected for a cMyb-like interaction with C/EBPβ. Our docking of the isolated C/EBPβ C-terminal tails to Spi1 revealed an intimate association of the terminal cysteine carboxylate of the tail with Arg-232 of Spi1. A composite C/EBPβ–Spi1 structure was then generated and evaluated by molecular dynamics simulation, revealing a stable structure consistent with the new and previously reported data.

To test the computational model, the Arg-232 pocket in the Spi1–DNA X-ray structure (6) was used to screen known small molecules for competitive potential. L-Arginine, a known anti-inflammatory, was computationally identified to bind in this pocket, suggesting a potential for disrupting the C/EBPβ–Spi1 interaction. Subsequent ex vivo studies using LPS-activated THP-1 cells incubated with L-arginine demonstrated a significant decrease in IL1B transcription in parallel with a reduction in C/EBPβ association with Spi1 on the IL1B promoter, as evaluated by ChIP. No significant change was observed for parallel transcription of the Spi1-independent C/EBPβ-dependent IL6 gene. Importantly, direct binding of Spi1 and C/EBPβ to cognate DNA was unaffected. These results are consistent with an L-arginine-dependent decrease in IL1B gene expression being due to its ability to inhibit the protein–protein interaction between C/EBPβ and Spi1 in the absence of any effect on direct DNA binding for either factor.

Results and discussion

Computational modeling predicts interaction between the C/EBPβ leucine zipper C terminus and the Spi1 DNA-binding domain

Our previous reports described a long-range DNA loop-mediated promoter-proximal protein–protein interaction between the Spi1 wHTH DBD and the carboxyl end of the C/EBPβ leucine zipper dimer DBD that is critical for IL1B gene transcription (2, 4, 5). This interaction is similar to that we reported for human cytomegalovirus (HCMV) IE2 protein with Spi1 (8) and by others for C/EBPα with GABPα (9–11). Although the structure of HCMV IE2 protein is unknown, the structures of the Spi1 and GABPα DBDs are known and represent the two most distantly related ETS wHTH DBDs of the 12 ETS domain homology types (12). The ETS family of DNA-binding proteins all contain a common ETS DBD localized to either end or the middle of the overall protein sequence (12). The structure of several ETS domains have been solved, all of which contain three interacting α-helices, one of which serves as a DNA sequence recognition element, along with two loops closed by antiparallel β-strands that constitute a wing motif that provides additional DNA backbone interactions. An HTH structure is also found in other DNA-binding domains that possess a similar three-α-helix backbone without β-strands and minimal sequence conservation with the ETS subfamily. Three tandem copies of the non-ETS HTH domains are contained within the cMyb DNA-binding protein, which also binds the C/EBPβ leucine zipper dimer. The X-ray crystal structure of the C/EBPβ leucine zipper coiled-coil attached to its basic (i.e. positively charged) DNA-binding sequence interacting with DNA-bound cMyb (a DNA–bZIP–C/EBPβ–HTH–cMyb–DNA complex) reveals a C/EBPβ bZIP dimer interaction with a single cMyb HTH domain (7). In this structure, the short C-terminal peptide tails that extend beyond each of the leucine zipper monomers were deleted, resulting in the C-terminal glutamate of one bZIP monomer and a second glutamate within the body of the zipper generating salt bridges to one cMyb DBD (Fig. S1).

The structure of the C/EBPβ–cMyb complex provides a potential paradigm for other bZIP–HTH domain interactions. This resulted in our earlier studies aimed at exploring the involvement of the C/EBPβ leucine zipper C-terminal glutamate (5) and a detailed inspection of the contact surface between the two domains that were generated from the PDB-deposited X-ray coordinates using transformations described within the 1H89 PDB file (Fig. S1). The C/EBPβ–cMyb contact surface consists of an asymmetric interaction between the C terminus of the C/EBPβ leucine zipper dimer and a single cMyb HTH domain involving four side-chain, one backbone, and nine van der Waals contacts with the bZIP chain A, along with five backbone, two van der Waals contacts, and two DNA salt-bridge interactions with the bZIP chain B (7, 13). Strikingly, the leucine zipper dimer extra-ZIP C-terminal peptides, each consisting of nine residues beyond the terminal Glu-336, were deleted from the molecule used for crystallography, providing circumstantial evidence that complex formation did not require sequences beyond the structural terminus of the bZIP dimer. This conclusion appeared to agree with the hypothesis provided in our previous report (5) suggesting that Glu-336 might directly interact with Arg-232 of Spi1 for the C/EBPβ–Spi1 interaction, although the importance of the chain B DNA contact was never addressed. Consequently, we set out to attempt computational docking to evaluate possible requirements for the C/EBPβ–Spi1 interaction.

The initial computational approach took advantage of the ZDOCK webserver (14), which uses rigid-body protein–protein docking via a fast Fourier transform algorithm that relies on a combination of shape complementarity, electrostatics, and statistical potential. The C/EBPβ bZIP, basic leucine zipper, sequence from aa 266 to 336 was docked to the Spi1 DBD. Fig. 1 displays models for the eight top-ranking zipper dimer interactions, all of which reveal tight clustering to a local-
Figure 1. Computational docking using the ab initio ZDOCK server. Shown are six different views of a composite backbone ribbon structure presenting the general orientation of the top eight C/EBPβ leucine zipper coiled-coil dimers (long blue coils representing aa 296–336) with an invariant Spi1 DBD, represented as three α-helices (short blue coils) and four β-strands (red bands). The Spi1 α3 DNA-recognition helix in two views is labeled with a red arrow pointing to the location of Arg-232 (R232). A green arrow locates the position of the amino end of the Spi1 DBD (residue 171). The first 170 N-terminal residues of the full-length Spi1 protein TD is not contained within the solved X-ray structure. The four structures in the top row are related by incremental 90° rotations, whereas the lower row presents top and bottom views. Because of its flexible behavior in the absence of DNA, the aa 266–295 DNA-binding basic region of the bZIP is not shown in the figure.

Figure 2 presents these coded with respect to indicated regions in three of which are with the terminal C/EBP residues that are either provides a data-driven approach to docking by requiring a list of 336 residue, because C/EBP these interactions absolutely depend upon the terminal Glu-336 residue, did not yield a consistent result (Fig. S2). This docking geometry, lacking the extra-ZIP C-terminal unstructured peptides, resembles the C/EBPβ–cMyb interaction (7) and did not provide structural evidence for the involvement of protein loops. The resulting LowModeMD docking and the ZDOCK results in the absence of Glu-336 (Fig. S2) appears similar, but not identical, to that obtained with ZDOCK (Fig. 3), once again supporting a C/EBPβ–cMyb-like interaction. In particular, salt bridges between Glu-336 in C/EBPβ chains A and B with the side chains of Lys-248 and Lys-244 in Spi1, respectively, are consistent with both the cMyb tail-less mode of docking, and the ZDOCK results in the absence of Glu-336 (Fig. S2). Unfortunately, neither ZDOCK nor HADDOCK provide direct evidence for the involvement of the C-terminal tails and, specifically, Arg-232. However, the GST interaction results (Fig. 2) suggest that the Spi1 α3 helix, containing Arg-232, may be a part of an extended contact surface for the chain B C-terminal tail.

A critical salt bridge between C/EBPβ and Spi1 is stable throughout the NAMD simulation

To model the Arg-232 interaction, an independent approach was used for docking of the C/EBPβ C-terminal extra-zipper tails. This involved the use of LowModeMD (16), a method of short molecular dynamics simulations to predict the likely orientation of protein loops. The resulting LowModeMD docking of the C/EBPβ C-terminal extra-zipper tails revealed a salt bridge between the side-chain guanidinium group of Arg-232 in the Spi1 α3 helix and the C-terminal carboxylate group of Cys-345 on the chain B C/EBPβ tail (Fig. S4A), consistent with our previous report suggesting that Arg-232 on Spi1 is critical.
for the interaction between the two proteins (5). The chain A tail docked into a pseudosymmetrical location on the Spi1 α3 helix, interacting with Lys-224 (Fig. S4B). The LowModeMD docking of the C/EBPβ C-terminal extra-zipper tails to the Spi1 DBD was executed in the absence of the C/EBPβ leucine zipper–cMyb-like interaction to test whether the two contacts were independent, an hypothesis derived from the apparent independent targeting of specific substructures within the Spi1 wHTH ETS domain revealed by the in vitro GST studies (Fig. 2). Superimposition of the chain A LowModeMD C/EBPβ C-terminal tail structure revealed significant coincidence with the C/EBPβ chain A leucine zipper monomer from HADDOCK, permitting manual manipulation of the backbone to generate a peptide bond (Fig. S5A). The chain B LowModeMD Spi1 C-terminal tail structure was also reasonably close to the Glu-336 terminus of the HADDOCK C/EBPβ chain B, which was also computationally attached to the tail.

The resulting composite structure, consisting of 1) the HADDOCK-derived bZIP dimer docked to Spi1; 2) the two peptide-bonded LowMode-docked C-terminal extra-zipper tails; and 3) both fragments of dsDNA from the two original X-ray crystal structures, was then energy-minimized. This resulted in the loss of the chain A leucine zipper Glu-336 salt bridge and a rotational movement of this chain with respect to chain B. Because the in vitro data and LowModeMD docking supported an interaction between Cys-345 of C/EBPβ and Arg-232 of Spi1, the chain B tail was computationally replaced with the LowModeMD tail structure, and the Cys-345–Arg-232 contact was constrained prior to energy minimization and solvation, following which the constraints were removed, and the structure was subjected to 104 ns of NAMD simulation. Strikingly, the Glu-336 salt bridges that appeared critical for C/EBPβ–cMyb–like docking in the absence of the C-terminal tails were both destabilized in the presence of the Arg-232–docked chain C-terminal tail. The loss of the salt bridges was accompanied by a 13 Å displacement of the leucine zipper dimer from its original HADDOCK location toward the Arg-232–docked chain C-terminal tail. The stability of the chain B interaction between Arg-232 of Spi1 and Cys-345 at the C/EBPβ C terminus was maintained (Fig. 4A) versus that for the chain A tail, which rapidly destabilized during the first 20 ns of the simulation (Fig. 4B) and folded into a compact domain that directly interacted with the Spi1 DBD, providing 320 Å² of buried contact surface (Fig. S6) along with a compensatory C-terminal Cys-345 salt bridge to Lys-198 of Spi1 (Fig. 4C). The resulting dynamically stable chain B-biased model (Figs. 5 and 6) is not only compatible with our previously reported muta-
Figure 3. Computational docking using the data-driven HADDOCK server. Shown are two views of the top four docking solutions for the C/EBPβ leucine zipper coiled-coil dimer (red coils) to Spi1 (pink surface) bound to DNA (gray surface). The diagram to the right presents the interactions between Spi1 and each C/EBPβ (aa 266–336) chain.
ponent of the observed complex stability (5). Strikingly, in contrast to the extended nature of the chain B interaction that positions Cys-345 in the DNA–protein pocket at the end of the Spi1 α3 DNA-recognition helix, the NAMD simulation positions the C-terminal extension of chain A beyond Glu-336 with the backbone of the following residue folded upon itself to form a globular structure from Pro-337 through the terminal Cys-345 carboxylate that forms a salt bridge to Lys-198 of Spi1 (Fig. 6A). Globular folding of a C-terminal bZIP extension for protein–protein interaction has previously been reported for the Epstein–Barr virus ZEBRA bZIP (23).

L-Arginine reduces C/EBPβ binding to Spi1 on the IL1B gene promoter

The critical importance of Spi1 in TLR4-dependent induction of the human IL1B gene (2) suggests a unique and specific
role in promoter regulation that could be targeted for inhibition. Although specific inhibition of Spi1 binding to DNA by small molecules has been explored (24), this approach suffers from its inability to discriminate among the highly conserved Spi1–DNA binding sites, an important issue because Spi1 regulates at least 219 genes (25) almost exclusively as a functional enhancer–binding factor. However, the involvement of Spi1 as a promoter, rather than an enhancer, factor for IL1B, as well as
its unique mode of action in physically integrating C/EBPβ bound to the far-upstream C/EBPβ superenhancer into the promoter, suggests that disruption of this interaction could provide a highly specific target for disrupting Spi1 action at the IL1B promoter.

Consequently, we used the Arg-232 interaction pocket between Spi1 and the C/EBPβ chain B extra-zipper C-terminal tail to screen existing public database libraries for small compounds. It is important to note that this screening was conducted with the original 1PUE Spi1–DNA X-ray structure (6), independent of our C/EBPβ-docking models. Only those compounds capable of competitive binding to this interaction pocket, without affecting direct/cognate Spi1–DNA binding, were selected. One of the highest-scoring compounds resulting from multiple computational docking trials was L-arginine, a common semiessential amino acid. Fig. 7 presents interaction diagrams for one of the highest-scored L-arginine binding modes docked into the Spi1–C/EBPβ chain B interaction pocket. Interestingly, this mode involves contacts with Arg-232 and Asn-236, closely mimicking the Cys-345 interaction with Spi1 from NAMD (Fig. 6B).

This potential inhibitory interaction of L-arginine with Spi1 was tested by examining TLR-dependent de novo IL1B gene expression in cultured THP-1 cells in L-arginine-deficient RPMI 1640 medium. The titration of L-arginine in the presence and absence of a 2.5-h 1 μg/ml LPS treatment revealed an
inhibitory trend of IL1B gene expression between 5 and 50 mM L-arginine, beyond the 1.15 mM concentration of conventional RPMI medium. A 10 mM concentration was chosen for subsequent experiments because the lowest concentration generating a significant change was 10 mM. At the highest concentration of 50 mM, cells exhibited an altered morphology, although retaining viability (Fig. 8A). Fig. 8B demonstrates inhibition of IL1B transcription with 10 mM LPS at various concentrations of L-arginine. In contrast to IL1B, the IL6 gene does not possess a Spi1-dependent promoter but is otherwise activated via similar TLR-activated transcription factors as IL1B (26). Consequently, IL6 mRNA was assayed in parallel with IL1B, revealing no significant decrease in the presence of L-arginine (Fig. 8B). ChIP revealed an L-arginine–dependent decrease in C/EBPβ binding to Spi1 at the critical promoter-proximal Spi1-binding site (2) on the human IL1B gene promoter. As a control, C/EBPβ binding to promoter-bound Spi1 was compared with a downstream site on the IL1B gene that does not bind either C/EBPβ or Spi1. The PCR amplicon covering the Spi1-binding site on the IL1B promoter is centered at 19 nucleotides upstream of the transcription start site (−19 relative to the transcription start site), whereas the PCR amplicon control site is centered at +4858 nucleotides downstream of the transcription start site. Direct C/EBPβ binding to DNA was also evaluated by ChIP at a well-known avid C/EBPβ-binding site (27) with a PCR amplicon centered at −77 of the IL6 gene in the same cells. Incubation of THP-1 cells in the presence of 10 mM L-arginine decreased C/EBPβ association with Spi1 at the critical −19 site on the IL1B gene promoter following a 2.5-h LPS treatment (Fig. 8C). Under these conditions there was no significant effect on direct binding of C/EBPβ to its cognate DNA at position −77 of the IL6 gene promoter. Strikingly, direct binding of constitutively bound Spi1 to its DNA site at +4858 increased significantly under these conditions (Fig. 8D), suggesting the possibility of L-arginine stabilization of Spi1 DNA binding. No enrichment of either C/EBPβ or Spi1 was observed at the downstream +4858 control site on IL1B. These ChIP studies provide evidence that the L-arginine–dependent decrease in IL1B mRNA expression is due to its ability to inhibit the protein–protein interaction between C/EBPβ and Spi1.
Structural model for C/EBPβ–Spi1 complex at IL1B promoter

Figure 9. Four rotational views of the final DNA–C/EBPβ–Spi1–DNA composite model. Shown are the locations of the two bZIP chains and the Spi1 whTH DBD, along with the likely long-range enhancer–promoter DNA interaction, for four 90° rotational views in association with DNA segments representing the long-range enhancer and promoter sites of the IL1B gene.

and Spi1 on the IL1B gene promoter, without having any inhibitory effect on either direct DNA binding of C/EBPβ or another C/EBPβ-dependent gene (IL6).

Conclusion

Our prior understanding of the mechanism for transcriptional regulation of the human IL1B gene was based on the realization that its transcription depends on a cell type–specific transcription start site promoter and a far-upstream inducible superenhancer (28). This was followed by the identification of TLR rapid activation of C/EBPβ homo- and heterodimers binding to the enhancer (18) and constitutive Spi1 binding to the IL1B promoter (29) as key elements of function. However, it remained unclear how these two important gene regulatory elements, separated by almost 3 kbp of DNA sequence, might mechanistically cooperate. One hint was that the two proteins are capable of a direct interaction that depends upon the C terminus of C/EBPβ and the integrity of a single amino acid in the Spi1 DBD (5). Our report of a long-range chromatin loop that positions the enhancer in close proximity to the promoter prior to gene induction, which rapidly increases in stability after TLR signaling in human monocytes along with concomitant recruitment of TBP and Pol II, provided a reasonable mechanistic model (2). What still remained elusive was the nature of the structural interaction between C/EBPβ and Spi1 and how it contributes to TBP and Pol II recruitment. Using computational docking, in vitro interaction, and a predicted inhibitory compound, a reasonable molecular structure is now envisioned for a complex that anchors the enhancer to the promoter (Fig. 9). The interaction is distantly related to that of C/EBPβ–cMyb in that the C/EBPβ leucine zipper interacts with a DNA-distal surface of the Spi1 whTH domain by involving the same two chains that bind to cMyb but is also dependent upon the entire length of the chain B tail to provide a critical interaction with Arg-232 of Spi1. Therefore, it is interesting to speculate whether cMyb might support an interaction mode similar to that of C/EBPβ with Spi1, if it were crystallized with intact tails. This is because a salt bridge between Glu-336 of C/EBPβ chain B with Lys-248 of Spi1 in our initial ZDOCK and HADDOCK structures, which destabilized early in the NAMD simulation (Fig. 4D), is similar to the reported Glu-336 salt bridge to Arg-114 of cMyb (7). This suggests that the C/EBPβ–cMyb structure may be representative of the first stage of a two-step docking mode for C/EBPβ with Spi1 in which a less stable cMyb-like leucine zipper-only docking provides both an anchor and an increased effective concentration that supports increased collision and sampling by the chain B tail. The proximity of the chain B tail increases the probability of Arg-232 interaction with the terminal cysteine of C/EBPβ. This interaction may also be preferred because of the asymmetry of chemical interaction accessibility associated with the chain B pocket formed by the Spi1–DNA interaction (Fig. 7C). It should also be noted that the C/EBPβ interaction with cMyb, in contrast to Spi1, includes one direct and one water-mediated salt bridge with a DNA backbone phosphate, possibly abrogating the requirement for C-terminal tail interactions. The presence of the C-terminal tails and the absence of a nucleic acid salt bridge between C/EBPβ and Spi1 is consistent with the ability of this interaction to be DNA-independent during in vitro GST pulldown experiments, a situation also reported for C/EBPα with GABPα (9–11). This also reflects the conservation between the leucine zippers of C/EBPα and C/EBPβ, as well as the ETS domains of Spi1 and GABPα (Fig. S7).

It is also noteworthy that our data-supported computational model positions the final C/EBPβ-tailed structure on a surface of the 102-amino acid Spi1 DBD that is 180° removed from the attachment point of the 170-amino acid Spi1 TD (labeled K171 in Fig. 9). This is important because it places the C/EBPβ docking site in a region that is more likely to be free of permanent obstruction from the substantial mass of the Spi1 N terminus. One might speculate that the TLR4-dependent docking of C/EBPβ might maintain the Spi1 TD, which is reported to directly recruit TBP (30), in an open conformation essential for TBP and subsequent Pol II recruitment, consistent with the kinetics associated with IL1B gene induction (2).

The anti-inflammatory properties of l-arginine are well-known (31). However, the complexities of l-arginine metabolism have resulted in a vast array of possible targets (32). Our
results suggest that L-arginine can block C/EBPβ recruitment by Spi1 at the human IL1B gene promoter, resulting in decreased TLR4-dependent induction of gene transcription and suggesting one mode of action for its use as an anti-inflammatory therapeutic. This report deals with the involvement of L-arginine in a specific transcription mechanism and suggests one possible target that may serve as a model for the design of a novel and specific pro-inflammatory gene inhibitor. The central involvement of IL-1β protein in numerous pro-inflammatory acute diseases and cancer has underscored the importance of antibody therapy (33, 34). However, antibody therapy targets the huge number of IL-1β protein molecules that are derived from IL1B gene transcription in each activated cell. Blocking transcription provides an upstream target that precedes the amplified “storm” of IL-1β protein expression and can theoretically provide a much more effective method for inhibition. Of course, such inhibition requires both a reasonable efficiency for cell permeation, as well as high specificity for a target. Unfortunately, virtually all protein-coding genes use a common promoter transcription initiation mechanism and most transcription factors function as pleiotropic enhancer-binding proteins with broad gene activity that obviates therapies that depend on either direct promoter inhibition or specific enhancer DNA binding. This is certainly the case for Spi1, which predominantly functions as an enhancer-binding factor. Our observation that the human IL1B gene promoter utilizes a unique protein–DNA interaction at a core promoter that can be selectively targeted and repressed offers the potential for a correspondingly novel therapeutic mode targeting protein–protein interaction rather than protein–DNA interaction within the cell. It also begs the question of whether there are other genes that are similarly regulated either by Spi1 or by other transcription factors that function both as enhancer-dependent activators, as well as core promoter general transcription factors. Regardless, our results demonstrate that at least one gene can be selectively repressed by inhibition of protein–protein interaction at a core promoter.

**Experimental procedures**

**Cell culture, reagents, and treatment conditions**

THP-1 cell line (ATCC, TIB-202) was initially cultured in RPMI 1640 medium (Corning, 10-040-CV) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, SH30070.03), 1% penicillin/streptomycin Solution (Corning, 30-002-Cl) and 500 μL of 2-mercaptoethanol (Gibco, 21985023). RPMI 1640 medium lacking amino acid l-leucine, l-lysine, and l-arginine was purchased from Sigma (R1780). This medium was supplemented with 50 mg/liter of l-leucine (Sigma, L8912) and 40 mg/liter of l-lysine monohydrochloride (Sigma, L8662), which is same amount of these amino acids in classic RPMI 1640 medium but without l-arginine. It was also supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, SH30070.03), 1% penicillin/streptomycin solution (Corning, 30-002-Cl) and 500 μL of 2-mercaptoethanol (Gibco, 21985023). For mRNA and ChIP analyses, the THP-1 cells grown in classic RPMI 1640 medium were washed and transferred into RPMI 1640 medium lacking l-arginine. These cells were pretreated with different concentrations of L-arginine (Sigma, A8094) for 12 h prior to Escherichia coli 055:B5 LPS (Sigma, L2880) stimulation for 2.5 h.

**Small molecule docking with MOE**

Two potential binding pockets proximal to our amino acid residue of interest, Arg-232, on opposite sides of DNA-bound Spi1 from PDB code 1PUE (6, 35) (resolution, 2.1 Å; Rfree value, 0.287; Rwork value, 0.225) were identified with MOE SiteFinder (36, 37). Conformations of small molecule drugs (78–180 g/mol) from DrugBank (38) were docked to both pockets using the MOE Amber10 extended Hückel theory parameterization force field (39) and the induced fit docking protocol, which relaxes the structure as it docks. The potential binding sites were identified using dummy atoms defining the binding pockets, potential poses were determined via the Alpha Triangle match method, and initially the poses were scored with the Affinity dG method. Each pose and the pocket atoms (residues greater than 6 Å from the pocket are not included in the energy minimization or the energy evaluation; backbone atoms are held fixed) were energy-minimized using the generalized Born solvation model (40). After energy minimization, the poses are rescored with the generalized Born solvation model/weighted surface area dG method. Top ranked compounds with established links to IL-1β or inflammation in literature were selected as compounds of interest.

**Protein–protein docking**

The protein–protein docking of the Spi1–C/EBPβ DNA-bound complex used the murine Spi1 and human C/EBPβ structures found in PDB entries 1PUE (6) and 1H89 (7) (resolution, 2.8 Å; Rfree value, 0.277; Rwork value, 0.222), respectively. A series of docking runs was performed using the ZDOCK server (14), using a fast Fourier transform-based docking algorithm that takes into account pairwise shape complementarity, desolvation, electrostatics, and statistical potential. When both proteins were DNA-unbound and also when Spi1 was DNA-bound and C/EBPβ unbound with its bZIP marked as passive at residues 315 and below, ZDOCK results reliably indicated that the C/EBPβ bZIP domain associates with the Spi1 β-turn domain in an orientation notably not proximal to the residue of interest, Arg-232.

To reinforce the results from ZDOCK, additional docking was performed using the HADDOCK webserver (41, 42), which incorporates biochemical interaction data to drive docking. The Easy Interface was used with active (involved in contact) and passive (solvent-accessible neighbors) residues entered based on previous experimental data. For Spi1 (des-DNA), actives were at positions 243 and 245–254 (β3/β4 strands and loop) and passives were at positions 171–219 (α helices not in contact with DNA and β1/β2 strands and loop). On C/EBPβ, the dimer chains were combined into one, and the residues of one chain were renumbered, with active 330–336 and passive 276–293. Again, results showed the C/EBPβ bZIP domain docked to the Spi1 β turn domain in the same orientation.

Because C/EBPβ did not dock particularly close to Spi1 Arg-232, it was suggested that the disordered nine-residue C terminus end strands, which are not found in the C/EBPβ 1H89 crystal structure, could be associating with Arg-232 on one or both...
ChIP was performed using a modification of the Millipore/Upstate protocol (MCPROTO407). Following treatments, a total of $1 \times 10^7$ TPH-1 monocytes in RPMI 1640 medium lacking L-arginine were plated into 6-well plates (Thermo Fisher BioLite, 130184). Following the L-arginine and LPS treatments, the cells were pelleted, and supernatant was removed. The cell pellet was resuspended in 500 μl of TRIzol reagent (Invitrogen, 15596026). Following the addition of 170 μl of chloroform (Fisher, C606-1), the samples were vortexed well and incubated at room temperature for 15 min. Then the samples were centrifuged at 13,200 rpm for 15 min at 4 °C. The aqueous layer was transferred into a fresh tube and combined with 500 μl of isopropanol (Fisher, BP2632-4) and 1 μl of glycogen (Ambion, AM9510). The samples were incubated at room temperature for 10 min and then centrifuged at 13,200 rpm for 10 min at 4 °C. The RNA pellets were washed with 500 μl of 75% ethanol (Pharmaco-AAPER, 111ACS200) and centrifuged for 10 min in room temperature at 14,000 rpm. Then the ethanol was aspirated, and the pellets were allowed to air dry for 10–15 min. Air-dried pellets were resuspended in 25 μl of diethyl pyrocarbonate–treated water (Ambion, AM9920). The samples were incubated at 65 °C for 10 min and subjected to DNase treatments using Turbo DNA-free reagents (Ambion, AM9107) according to the manufacturer’s instructions to eliminate genomic DNA contamination. RNA concentration and 260/280 and 260/230 values were measured using NanoDrop 1000 spectrophotometer (Thermo Fisher, ND-1000). mRNA was converted into cDNA using GoScript reverse-transcription system (Promega, A5001). cDNA was analyzed using quantitative PCR (qPCR) carried out in a StepOnePlus Applied Biosystems real-time instrument (Thermo Fisher, 4376600). Relative expression levels were calculated using $\Delta \Delta C_t$ method with GAPDH (reference gene) and unstimulated TPH-1 cells as a control. Primer sequences used for mRNA analysis are indicated in Table S1.
psfgen module in VMD. A 76 × 70 × 150 Å solvation box containing 22,623 water molecules was placed around the protein–DNA complex using the Solute package of VMD; no additional molecules were added to the system.

Simulation details—The NAMD software package (49) was used to perform MD simulations on the Spi–C/EBPβ–DNA complex. The CHARMM36 force field (50) was used for all simulations to assign atomic partial charges, calculate forces, and potential energies. Constant volume, temperature, and number of atoms (NVT) were maintained during the preliminary equilibration simulations, and volume was allowed to vary while pressure was held constant (NPT) during the final equilibration run and data production simulations. All simulations were carried out at 300 K, and 1,013,25 atm was maintained during NPT simulations. The velocity Verlet algorithm was used for MD integration with a 1-fs time step. For nonbonded interactions, pair lists were maintained for pair distances less than 12 Å with an interaction cutoff distance of 10 Å, and a switching function was applied at a distance of 8 Å. All positional constraints mentioned below were held with a 0.5 kcal Å⁻¹ mol⁻¹ force constant. Full system electrostatics was calculated with the particle mesh Ewald summation (51) method. The TIP3P water model (52) was used for explicit water solvation, and cubic periodic boundary conditions were utilized. A Langevin thermostat and piston were used to maintain constant temperature and pressure, respectively.

MD simulations—Conjugate gradient minimization was performed for 5000 steps with positional constraints placed on protein–DNA heavy atoms, followed by 5000 more steps of minimization without constraints. An NVT equilibration (52) was then performed for 2 ns with the position of the protein–DNA backbone heavy atoms constrained and 2 ns with these constraints removed to enable the solvent to relax before the larger molecules. Throughout all the simulations carried out, positional constraints were maintained on the terminal residues of the DNA chains to disable the system from shifting in a manner that would disrupt the periodic boundary conditions. Otherwise, DNA chains were allowed to freely move. Following the NVT equilibration, NPT equilibration was carried out for 5 ns with no additional constraints. 104 ns of data production MD simulation was performed under the NPT ensemble, with trajectory and observable data being recorded at 2-ns intervals.

Data analysis—All data analysis was performed with VMD. Interatomic distance time traces for the various residue pairs discussed in this work were calculated each step from the DCD trajectory. Buried surface area calculations were carried out using Tcl scripts in VMD.

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