Basic region-leucine zipper (B-ZIP) proteins homo- or heterodimerize to bind sequence-specific double-stranded DNA. We present circular dichroism (CD) thermal denaturation data on vitellogenin promoter-binding protein (VBP), a member of the PAR subfamily of B-ZIP proteins that also includes thyroid embryonic factor, hepatocyte leukemia factor, and albumin site D-binding protein. VBP does not heterodimerize with B-ZIP domains from C/EBPα, JUN, or FOS. We describe a dominant negative protein, A-VBP, that contains the VBP leucine zipper and an acidic amphipathic protein sequence that replaces the basic region critical for DNA binding. The acidic extension forms a coiled-coil structure with the VBP basic region in the VBP-A-VBP heterodimer. This new α-helical structure extends the leucine zipper N-terminally, stabilizing the complex by 2.0 kcal/mol. A-VBP abolishes DNA binding of VBP in an equimolar competition assay, but does not affect DNA binding even at 100-fold excess of CREB, C/EBPα, or FOS/JUND. Likewise, proteins containing the acidic extension appended to seven other leucine zippers do not inhibit VBP DNA binding. We show that conserved g → e' or i, i' + 5 salt bridges are sufficient to confer specificity to VBP by mutating the C/EBPα leucine zipper to contain the g → e' salt bridges that characterize VBP. A-VBP heterodimerizes with this mutant C/EBP, preventing it from binding to DNA. These conserved g → e' electrostatic interactions define the specificity of the PAR subfamily of B-ZIP proteins and preclude interaction with other B-ZIP subfamilies.

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To whom correspondence should be addressed: Bldg. 37, Rm. 4D06, Laboratory of Metabolism, NCI, National Institutes of Health, Bethesda, MD 20892. Tel.: 301-496-8753; Fax: 301-496-8419; E-mail: vinsone@dc37a.nci.nih.gov.

The abbreviations used are: B-ZIP, basic region-leucine zipper; A-ZIP, acidic extension-leucine zipper; DTT, dithiothreitol; deg, degree(s); PAR, proline- and acidic-rich region; VBP, vitellogenin promoter-binding protein; TEF, thyroid embryonic factor; HLF, hepatocyte leukemia factor; DBP, albumin site D-binding protein.

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B-ZIP proteins described in this paper contain the \( \phi 10 \) leader and 37 amino acids N-terminal of the first d leucine position of the leucine zipper (Fig. 1). All A-ZIP proteins, except for FOS and ATF2, which are truncated, contain the leucine zipper region starting at the first leucine position (Fig. 1) and extend until the natural C terminus. All A-ZIPS, except for A-CREB, contain the acidic extension LEQRAEELA (12). A-CREB contains a leucine instead of an asparagine (in bold) in the acidic extension (14). The LE in addition to a 5-mm rectangular CD cell. (a) Circular dichroism (CD) studies were performed using a Jasco J-720 spectropolarimeter. All protein stock solutions were in 12.5 mM potassium phosphate (pH 7.4), 150 mM KCl, and 0.25 mM EDTA. For the assay, 1 mM DTT and 4 \( \mu \)M protein sample in 1.5 ml of stock buffer (described above) was heated to 65 °C for 20 min to reduce the cysteines, cooled to room temperature for 5 min, and added to 0.25 mM EDTA, 2.5 mM DTT, 2 mg/ml bovine serum albumin, 2% \( \beta \)-d-galactopyranoside-inducible system (22). B-ZIP domains were purified over a heparin column and subsequently purified on a Rainin high performance liquid chromatography system using a C18 column chromatographed from 0% to 100% acetonitrile in 0.1% trifluoroacetic acid (12). The A-ZIP and O-VBP (the VBP leucine zipper region and a 43-amino acid leucine zipper. The B-ZIP domain is main is 80 amino acids long, containing a 37-amino acid basic region and a 43-amino acid leucine zipper. The B-ZIP domain is sufficient for sequence-specific DNA binding (16, 23). Native VBP, including the transactivation domain, is 313 amino acids long. The VBP leucine zipper homodimer is schematically presented in Fig. 1. The lower panel of Fig. 1 presents a sequence alignment showing the conserved amino acids in the PAR family of leucine zipper proteins. Four pairs of attractive g ↔ e’ interhelical salt bridges in VBP are highlighted by bold arrows. The first pair is R ↔ E, and the next three are all E ↔ R pairs. Biochemical studies indicate that PAR proteins can readily form heterodimers within the family but not with C/EBP (16, 19, 24). However, the ability of VBP to heterodimerize with a range of leucine zippers has never been tested. We mixed VBP with C/EBPs (Fig. 2) and saw no increase in thermal stability of the mixtures by CD spectroscopy. Similar results were ob-
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TABLE I

CD thermal stability of VBP, O-VBP, and A-VBP

The table presents the thermal stability, measured by circular dichroism at 222 nm, for VBP, O-VBP, and A-VBP and mixtures of VBP with either O-VBP or A-VBP. Ellipticity at 6 °C (θ, mdeg), melting temperature (T_m, °C), dimerization free energy extrapolated to T = 37 °C (ΔG in kcal/mol), ΔH (kcal/mol), and K_d at 37 °C (in μM) are presented. If the mixture has a higher melting temperature than either homodimer alone, we infer that the mixture sample is composed of heterodimers.

| Protein  | Homodimer (θ, T_m, ΔG, ΔH, K_d (37 °C)) | Heterodimer with VBP (θ, T_m, ΔG, ΔH, K_d (37 °C)) |
|----------|------------------------------------------|--------------------------------------------------|
| VBP      | −42, 50; −14.4, −928.6 × −9              | −81, 56; −11.9, −733.8 × −9                      |
| O-VBP    | −35, 56; −12.5, −851.4 × −9              | −120, 60; −13.4, −833.3 × −10                    |
| A-VBP    | −48, 53; −12.1, −882.7 × −9              | −120, 60; −13.4, −833.3 × −10                    |

Fig. 3. A, circular dichroism denaturations of VBP, O-VBP (a protein with the DNA-binding basic region deleted), and a mixture of VBP and O-VBP. B, circular dichroism thermal denaturations of VBP, A-VBP (a protein with an acidic extension replacing the basic region), and a mixture of VBP and A-VBP. Note the increased stability of the VBP-A-VBP mixture as compared with the VBP/O-VBP mixture. C, gel shifts of VBP bound to its cognate site with increasing concentrations of A-VBP and O-VBP. 1, 3, 10, 30, or 100 molar eq of these two proteins were mixed with VBP before adding DNA. D, schematic of the A-VBP dominant-negative. The calculated dimerization dissociation constant is shown: left, the VBP homodimer with the basic region unstructured; center, mixture of VBP and O-VBP; right, the mixture of VBP and A-VBP. This construct shows an increase in ellipticity and thermal stability. The right panel shows that the addition of the acidic extension stabilizes the heterodimer complex 30-fold compared with the VBP homodimer and 11-fold with respect to O-VBP.

tained for mixtures of VBP with CREB, JUND, or FOS B-ZIP domains (data not shown). This indicates that VBP does not preferentially heterodimerize with B-ZIPs from other subfamilies. From these data, it is clear that homodimers are thermodynamically significantly favored over heterodimers, but does not reveal whether heterodimers are thermodynamically unfavored relative to monomers.

A-VBP Interacts with VBP and Can Prevent VBP DNA Bind-
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To inhibit VBP DNA binding, we generated a dominant negative version of VBP termed A-VBP. The A-VBP dominant-negative contains the VBP leucine zipper, but the basic region is replaced with an amphipathic acidic protein sequence that forms a heterodimeric coiled coil with the basic region. We used A-VBP in two assays: circular dichroism at 222 nm to measure thermal stability of A-ZIP and B-ZIP protein mixtures, and a gel shift competition assay to examine if A-VBP can inhibit VBP DNA binding.

Thermal denaturations, monitored at 222 nm by CD spectroscopy in 12.5 mM phosphate (pH 7.4) and 150 mM KCl are shown for mixtures of the VBP B-ZIP domain (VBP) with either the VBP leucine zipper (O-VBP) (Table I; Fig. 3A) or A-VBP (Table I; Fig. 3B). The CD trace for the VBP B-ZIP domain thermal denaturation has an inclined low temperature base line that probably represents the non-cooperative denaturation of the basic region. Deleting the basic region from VBP to produce O-VBP reduced the ellipticity (θ) from −42 mdeg to −35 mdeg at 6 °C and increased the Tm, suggesting that the basic regions are destabilizing by 1.1 kcal/mol/dimer. The low temperature base line of O-VBP is nearly level, suggesting that the VBP leucine zipper domains denatures more cooperatively than the VBP B-ZIP domain. A-VBP is 0.4 kcal/mol less stable than O-VBP, indicating that the acidic extension is also slightly repulsive in the A-VBP homodimer. Unlike the basic region that unfolds non-cooperatively at low temperatures, the acidic extension displays a cooperative unfolding at around 20 °C that is independent of the VBP leucine zipper cooperative unfolding at 50 °C.

The mixture of A-VBP and VBP is 2.0 kcal/mol more stable than the VBP homodimer. The 43% increase in ellipticity at low temperatures implies that the basic and acidic extension form an α-helical coiled coil extension of the leucine zipper. The absence of a low temperature transition for the B-VBP-A-VBP heterodimer suggests that the basic and acidic regions form an extended coiled-coil that denatures cooperatively with the VBP zipper in the heterodimer. Analytical ultracentrifugation of the B-VBP-A-VBP mixture indicates the sample is a dimer (data not shown).

Fig. 3C shows the results of a gel shift assay after adding varying concentrations of either O-VBP or A-VBP to a DNA binding reaction containing 20-nm VBP and its cognate DNA site (ATTACGTAA) in a 28-base pair double-stranded oligonucleotide. O-VBP inhibits VBP DNA binding at between 10 and 30 molar eq. A-VBP, in contrast, almost completely abolishes DNA binding at equimolar concentrations. Fig. 3D presents a schematic of the heterodimer between A-VBP and the VBP B-ZIP domain.

A-VBP Does Not Heterodimerize with Other B-ZIP Domains—CD thermal denaturation experiments of the mixture of A-VBP and the C/EBPα B-ZIP domain show increased ellipticity at low temperatures but no increase in thermal stability (Fig. 4). Analytical ultracentrifugation of the mixture at 6 °C indicates a greater than dimer molecular weight (data not shown). We interpret this to be a A-VBP homodimer and a C/EBPα homodimer whose acidic and basic regions are interacting, resulting in both an increase in ellipticity and molecular weight. The 2.4 kcal/mol interaction between the acidic extension and the basic region of C/EBPα (12) did not overcome the repulsion between their leucine zipper regions. Similarly, A-VBP did not heterodimerize with CREB, FOS, or JUND (data not shown).

We also used gel-shift assays to measure inhibition of DNA binding by A-VBP. Fig. 5C shows that A-VBP did not inhibit the DNA binding of C/EBP, CREB, or JUND/FOS bound to their cognate sites, even at 100 molar excess.

In a corollary experiment (Fig. 5B), we examined the ability of seven A-ZIP leucine zippers to inhibit the DNA binding of VBP. The leucine zipper protein sequences used in these experiments are shown in Fig. 5A. The number of putative attractive or repulsive g ↔ e' interactions in homodimer or heterodimer with VBP is also calculated. The control A-VBP inhibited binding at equimolar concentration. However, none of the other zippers inhibited VBP DNA binding, even at 100-fold molar excess. All of these A-ZIPs did inhibit DNA binding of their normal dimerization partners.

A C/EBPα g ↔ e' Mutant Leucine Zipper Heterodimerizes with VBP—We have designed a C/EBPα leucine zipper mutant protein, called C/EBPα-L, that has the same g ↔ e' salt bridges that are found in VBP (25). This required changing four amino acids in the g and e position of C/EBPα leucine zipper (Fig. 6). We examined the interaction of C/EBPα-L with A-VBP to investigate the importance of these g ↔ e' salt bridges to VBP dimerization specificity. The thermal stabilities of C/EBPα and C/EBPα-L homodimers are shown in Table II.

Fig. 7 (A and B) presents the CD thermal denaturation curves for C/EBPα-L alone or mixed with either O-VBP or A-VBP. O-VBP did not preferentially heterodimerize with C/EBPα-L (Fig. 7A). However, A-VBP did heterodimerize with C/EBPα-L as shown by a 10 °C increase in the melt line relative to the sum line at −50 °C (Fig. 7B). Previous work has shown that the acidic extension heterodimerizes with the C/EBP basic region contributing 2.4 kcal/mol to stability (12). Thus, heterodimerization between C/EBPα-L and A-VBP implies that repulsion between the two zippers is less than 2.4 kcal/mol. In contrast, heterodimerization is not observed between A-VBP and another mutant C/EBPα protein called C/EBPα-J. C/EBPα-J has the g and e residues in the third heptad reversed (compared with C/EBPα-L) so that there are six attractive and two repulsive g ↔ e' interactions with the VBP leucine zipper (Fig. 6B). The lack of interaction between C/EBPα-J and A-VBP indicates that there are repulsive interactions between the zippers that destabilize the heterodimer by more than 2.4 kcal/mol (Fig. 7, C and D).

Fig. 8 is a gel shift that shows that the DNA binding of C/EBPα-L is abolished by A-VBP of between 1 and 10 molar eq. This is consistent with the CD results. In contrast the DNA binding of C/EBPα-J is not inhibited by 100 molar eq of A-VBP. These data indicate that the attractive g ↔ e' salt bridges play an important role in regulating the dimerization specificity of the VBP leucine zipper.
We describe A-VBP, a dominant negative to the VBP B-ZIP protein. A-VBP contains the VBP leucine zipper and an appended acidic amphipathic protein sequence that replaces the basic region critical for DNA binding. The acidic extension forms a coiled coil structure with the VBP basic region in the VBP-A-VBP heterodimer that stabilizes the complex 2.0 kcal/mol as shown by CD thermal data. A-VBP abolishes VBP.

**DISCUSSION**

We describe A-VBP, a dominant negative to the VBP B-ZIP protein. A-VBP contains the VBP leucine zipper and an appended acidic amphipathic protein sequence that replaces the basic region critical for DNA binding. The acidic extension forms a coiled coil structure with the VBP basic region in the VBP-A-VBP heterodimer that stabilizes the complex 2.0 kcal/mol as shown by CD thermal data. A-VBP abolishes VBP.

**FIG. 5.** A. Sequence alignment of leucine zippers used in experiments with VBP. Except for FOS and ATF2 that are truncated as denoted by the double dashes, all protein sequences for the B-ZIP leucine zippers start in the basic region at an invariant arginine, continue through the leucine zipper and terminate at the natural C terminus of these proteins. The proline in the C terminus of the leucine zipper is likely to be the helix-breaking residue at the natural C-terminal boundary of the α-helical leucine zipper. Below the protein sequences is a consensus sequence for the B-ZIP motif, where ψ represents any hydrophobic amino acid. The leucine zipper sequence is broken into heptads (g, a, b, c, d, e, f). Above the sequences is a bar connecting amino acids in the g position with those in the following e position, indicating amino acids that may interact interhelically in either homodimers or heterodimers. Only four heptads are shown because of the presence of the proline and lack of charged residues at the C terminus of these molecules. The number of attractive and repulsive g→e salt bridges formed in a hypothetical heterodimer between VBP and other B-ZIP proteins are presented.

B. VBP DNA binding is inhibited by A-VBP but not other A-ZIPs. 30 nM VBP was bound to its cis element and competed with 1, 10, or 100 molar eq of the indicated A-ZIP. Lane 1 contains the free probe. Lane 2 contains VBP bound to its consensus DNA sequence. 1, 10, and 100 molar eq of each A-ZIP are competed against the VBP-DNA interaction as shown. At the 100 molar eq, some of the A-ZIPs show minimal VBP binding, notably A-C/EBP and A-CREB. C. A-VBP does not inhibit DNA binding of other B-ZIP proteins. VBP (30 nM), C/EBP (30 nM), CREB (20 nM), and JUND/FOS (30 nM) were bound to their respective probes and challenged with 1, 10, or 100 molar eq of A-VBP. As previously observed, A-VBP selectively inhibits VBP DNA binding at 1 molar eq.
binding to its cognate site at equimolar concentrations in
EMSA, but does not interact with other B-ZIPs, even at 100-
fold excess. Using eight A-ZIP dominant negatives, we show by
EMSA and CD thermal melts that the PAR B-ZIPs do not
interact with the other four known subfamilies of mammalian
B-ZIP proteins: FOS, C/EBP, JUN, and CREB. These data
strongly suggest that A-VBP may be a potent and specific
reagent for inhibiting both the activation and repressive prop-
erties of the VBP family of B-ZIP proteins in biological systems.
To facilitate further design of A-ZIP dominant negatives, we
need to have a detailed understanding of the interactions that
govern dimerization specificity.

We have used A-VBP to investigate the structural determin-
ants regulating the preferential dimerization within the PAR
subfamily. We find that conserved attractive interhelical $g \leftrightarrow e'$
(i, i' + 5) salt bridges play a major role. C/EBP$\alpha$-L protein,
containing the VBP $g \leftrightarrow e'$ salt bridges in a C/EBP$\alpha$ background, interacts with A-VBP while C/EBP$\alpha$ does not. This
suggests that these $g \leftrightarrow e'$ salt bridges are critical for regulat-
ing homodimerization of the PAR subfamily. Their importance
is reinforced by the observation that a reversal of a single $E \leftrightarrow
R$ salt bridge to $R \leftrightarrow E$ in the mutant C/EBP$\alpha$-J prevented
heterodimerization with A-VBP.

The expected difference in stability between the A-VBP-C/E
BP$\alpha$-L and A-VBP-C/E$\beta$P$\alpha$-J heterodimers is 2.9 kcal/mol
(Fig. 9) (10, 23). Of this, 2.5 kcal/mol is due to coupling energy.
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The table presents the thermal stability measured by circular dichroism at 222 nm for three proteins, including ellipticity at 6 °C (θ, mdeg), melting temperature (T_m, °C), dimerization free energy extrapolated to T = 37 °C (ΔG in kcal/mol), ΔH (kcal/mol), and K_d (37 °C).

| Protein | Four g ↔ e pairs | θ | T_m (°C) | ΔG | ΔH | K_d (37 °C) |
|---------|------------------|---|----------|----|----|-----------|
| C/EBP_a | K ↔ T, D ↔ R, R ↔ S, E ↔ R | -39 | 49 | -9.9 | -58 | 9.9 × 10^{-8} |
| C/EBP_a-J | K ↔ E, E ↔ R, R ↔ E, E ↔ R | -41 | 71 | -15.7 | -81 | 7.8 × 10^{-12} |
| C/EBP_a-L | K ↔ E, E ↔ R, E ↔ R, E ↔ R | -44 | 74 | -16.9 | -87 | 1.1 × 10^{-12} |

**FIG. 9.** The calculated energetic contribution of g ↔ e' amino acids in the third heptad to stability (ΔG) and coupling energy (ΔΔG) of heterodimers between C/EBP_a-L and A-VBP or C/EBP_a-J and A-VBP.

40% of the coupling energy is from attractive g ↔ e' interactions (-1.0 kcal/mol), and 60% is from repulsive interactions (+1.5 kcal/mol). The C/EBP_a-L-A-VBP heterodimer would be stabilized by -1.3 kcal/mol for each of the two E ↔ R salt bridges relative to A ↔ A in the third heptad, resulting in a stabilization of -2.6 kcal/mol and a coupling energy of -1.0 kcal/mol. In contrast, the C/EBP_a-J-A-VBP heterodimer would be destabilized by 0.3 kcal/mol because repulsive E ↔ E and the R ↔ R interactions result in a destabilizing coupling energy of +1.5 kcal/mol. These attractive g ↔ e' interactions are not observed in the GCN4 leucine zipper (26).

A general strategy to measure repulsion between protein surfaces is to entropically constrain them. The strategy we present in this report is more limited, the heterodimer between the basic region and the acidic extension must denature cooperatively with the leucine zippers. Additionally, to measure the repulsion between the leucine zippers of interest, it must be less than the basic region and acidic region interaction.

PAR proteins are expressed in the brain (27), liver, and thyroid and have a circadian rhythm in their expression pattern (28–30). Recent work has demonstrated that a Drosophila B-ZIP protein with homology to PAR B-ZIP domains is required for a functional circadian clock (21). A chimeric protein between the B-ZIP domain of HLFI the transactivation domain E2A mediates a lethal form of childhood leukemia (24). Previous work using a TEF dominant negative containing mutations in the basic region had dominant negative properties (31). Expression of this dominant negative in UOCC-B1 cells, a human leukemia cell line that contains a chimeric protein containing the TEF B-ZIP domain, lead to apoptosis. The VBP/TEF dominant negative described in this manuscript should be more effective because the acidic extension confers higher affinity to the target B-ZIP domain. When gene therapy procedures are in place, A-VBP could be an effective therapy for this particular leukemia.

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