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Review

Deciphering B-ZIP transcription factor interactions in vitro and in vivo

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Abstract

Over the last 15 years, numerous studies have addressed the structural rules that regulate dimerization stability and dimerization specificity of the leucine zipper, a dimeric parallel coiled-coil domain that can either homodimerize or heterodimerize. Initially, these studies were performed with a limited set of B-ZIP proteins, sequence-specific DNA binding proteins that dimerize using the leucine zipper domain to bind DNA. A global analysis of B-ZIP leucine zipper dimerization properties can be rationalized using a limited number of structural rules [J.R. Newman, A.E. Keating, Comprehensive identification of human bZIP interactions with coiled-coil arrays, Science 300 (2003) 2097–2101]. Today, however, access to the genomic sequences of many different organisms has made possible the annotation of all B-ZIP proteins from several species and has generated a bank of data that can be used to refine, and potentially expand, these rules. Already, a comparative analysis of the B-ZIP proteins from *Arabidopsis thaliana* and *Homo sapiens* has revealed that the same amino acids are used in different patterns to generate diverse B-ZIP dimerization patterns [C.D. Deppmann, A. Acharya, V. Rishi, B. Wobbes, S. Smeekens, E.J. Taparowsky, C. Vinson, Dimerization specificity of all 67 B-ZIP motifs in Arabidopsis thaliana: a comparison to Homo sapiens B-ZIP motifs, Nucleic Acids Res. 32 (2004) 3435–3445]. The challenge ahead is to investigate the biological significance of different B-ZIP protein–protein interactions. Gaining insight at this level will rely on ongoing investigations to (a) define the role of target DNA on modulating B-ZIP dimerization partners, (b) characterize the B-ZIP transcriptome in various cells and tissues through mRNA microarray analysis, (c) identify the genomic localization of B-ZIP binding at a genomic level using the chromatin immunoprecipitation assay, and (d) develop more sophisticated imaging technologies to visualize dimer dynamics in single cells and whole organisms. Studies of B-ZIP family leucine zipper dimerization and the regulatory mechanisms that control their biological activities could serve as a paradigm for deciphering the biophysical and biological parameters governing other well-characterized protein–protein interaction motifs. This review will focus on the dimerization specificity of coiled-coil proteins, particularly the human B-ZIP transcription family that consists of 53 proteins that use the leucine zipper coiled-coil as a dimerization motif.
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Establishing that a protein motif can either homodimerize or heterodimerize does not indicate that all proteins possessing these motifs form physiologically relevant dimers. Two mechanisms regulate whether individual members of a protein family can dimerize in vivo. The first involves the cellular location of two proteins: do their spatial and temporal expression patterns overlap. While progress has been rapid in characterizing the transcription factor profile of individual cells and tissues from many organisms, there is limited information on the relative levels of proteins that are co-expressed in cellular samples or on the role of intracellular transport in sequestering proteins in separate cellular compartments.

The second mechanism regulating protein dimer formation is the subject of this review—namely, the structural details of a motif that impact dimerization specificity. For example, the 53 B-ZIP proteins can potentially form 53 homodimers and 52 × 52 i.e. 2,704 heterodimers, however, this represents both AB and BA heterodimers which are identical so there are 1,352 potential heterodimers for a total of 1,405 potential human B-ZIP dimers. It is essential to know the structural rules governing dimerization specificity to evaluate potential dimer formation and convert protein expression data into biologically useful information.

The coiled-coil motif were first identified in cytoskeletal proteins, including myosins, tropomyosins, and intermediate filaments [3,4]. The coiled-coil consists of a 7 amino acid motif that is termed a heptad. In the late 1980s, it was noted that a group of transcription factors, termed B-ZIP proteins, also used

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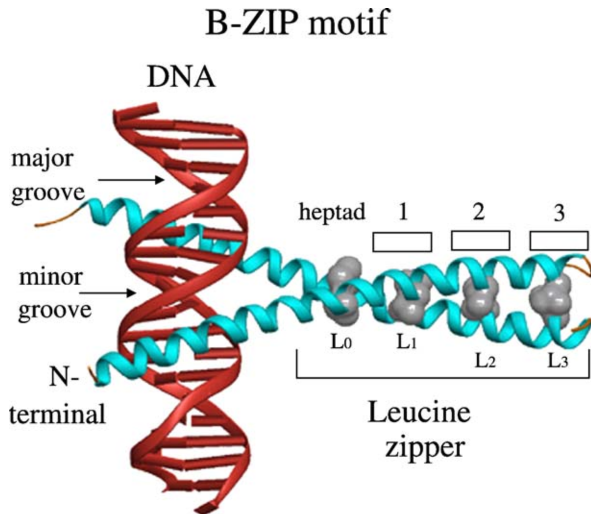

Fig. 1. X-ray structure of GCN4 B-ZIP dimer bound to double-stranded DNA [10]. The DNA is in red. The B-ZIP α-helices are in blue with the leucines in the "d" position shown in gray. The N-terminal of the protein, the basic region, and the leucine zipper are labeled. The first three heptads of the leucine zipper are numbered. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

a coiled-coil motif to mediate dimerization. This dimerization domain was termed a leucine zipper due to the presence of a leucine residue every seven amino acids [5]. The short and stable coiled-coil dimerization domains of leucine zipper transcription factors are an ideal sample for detailed biophysical analysis. Typically, a minimum of four or five heptads are required to form a productive, coiled-coil dimerization interface. Several other protein motifs are also repeated within a single protein and these tandem arrays are necessary to produce a structure with biological activity. [6,7].

The leucine zipper coiled-coil dimerization domain is found in three families of transcription factors. The basic region, leucine zipper proteins (B-ZIP) [5] and the basic region, helix–loop–helix leucine zipper proteins (B-HLH-LZ) [8] are found in both the plant and animal kingdoms. The third family, which contains a homeobox DNA binding domain adjacent to the leucine zipper motif [9], is found exclusively in plants. In all three families, the DNA binding motif is positioned immediately N-terminal to the dimerization domain.

1. Structure of the leucine zipper coiled-coil

Fig. 1 presents the X-ray structure of a B-ZIP dimer bound to DNA [10]. Each monomer is a long bipartite α-helix. The N-terminal half contains basic amino acid residues that interact with the major groove of DNA in a sequence-specific manner. The C-terminal half is a leucine zipper—an amphipathic α-helix that dimerizes to generate a parallel coiled-coil. Fig. 2 is a schematic presenting side and end views of a leucine zipper coiled-coil and the seven unique amino acid positions (a, b, c, d, e, f, and g) found in each heptad [3]. The a and d residues are typically hydrophobic and pack in a regular "knobs and holes" pattern [11] along the dimerization interface to create a hydrophobic core that contributes most of the energy needed to stabilize the leucine zipper [12]. The regular spacing of hydrophobic amino acids in a 3–4–3–4 "stutter" pattern {a–b–c–d–e–f–g–a–b–c–d} is critical for coiled-coil formation. The end-view of the coiled-coil structure (Fig. 2B) shows that the a and d positions are not structurally equivalent. The amino acid

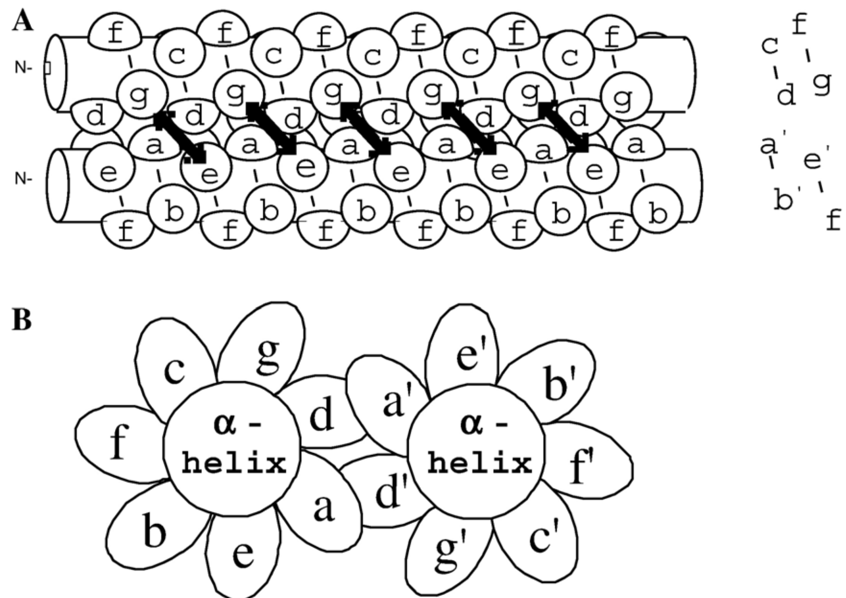

Fig. 2. Coiled-coil schematic (A) Side view of a leucine zipper with the amino acids presented in the circles. To the right, the seven unique positions (a, b, c, d, e, f, and g) of leucine zipper coiled-coil are identified. Amino acids on the second helix of the dimer are designated a′, b′, c′, d′, e′, f′, and g′. The potential electrostatic interactions between the g position of one helix and the following e′ position of the opposite helix (g↔e′) are indicated by the solid black arrow. The opposite side of a leucine zipper coiled-coil is identical in a homodimer, but different in a heterodimer. (B) End view, looking from N-terminus to C-terminus, with the amino acids in the seven unique positions of the heptad presented as ellipses oriented as the amino acid side chains would exit the α-helix.

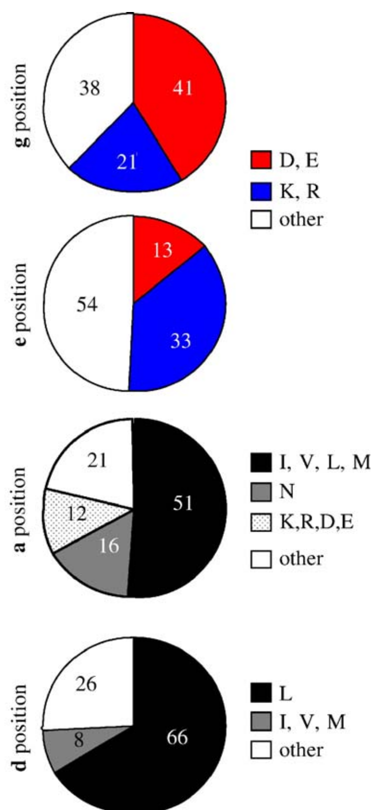

Fig. 3. Pie chart presenting the frequency of amino acids in the g, e, a, and d positions of heptads from the leucine zippers of all 53 human B-ZIP proteins.

side chain in the d position projects into the hydrophobic core, while the side chain in the a position is oriented away from the hydrophobic core and toward the aqueous surface. This helps explains why leucine is the favored hydrophobic residue for the d position [13] and isoleucine is the favored residue in the a position [14]. The g and e positions of the leucine zipper frequently contain charged amino acids [4,15]. Atomic structures of leucine zipper dimers have revealed that oppositely charged amino acids in the g position and the following e′ position lie across the hydrophobic interface and interact interhelically [10,16–21], stabilizing the structure and helping to regulate the specificity of B-ZIP protein dimerization.

Several features of the leucine zipper coiled-coil make the motif a favored model for quantitative studies that examine the energetic contribution of individual amino acids to protein dimer stability. Leucine zippers can be reversibly denatured by heat or by chaotropic chemicals, and the transition from α-helical dimer to non-helical monomer can be monitored by circular dichroism (CD) spectroscopy. The unfolding of the zipper is cooperative and well-modeled by a two-step process making is amenable to quantitative thermodynamic analysis. These biophysical properties, coupled with the simple, heptad repeat structure of the leucine zipper, have allowed for a careful examination of how individual amino acids within the dimer interface, the a, d, e, and g positions of the leucine zipper coiled-coil, impact both dimerization stability and dimerization specificity.

2. Specificity of leucine zipper dimerization: measurement of coupling energy

In contrast to the longer coiled-coils of cytoskeletal proteins, the leucine zippers of B-ZIP transcription factors contain a limited repertoire of amino acids in the a, d, e, and g positions (Fig. 3). This has been helpful in identifying amino acids capable of generating the most stable leucine zipper structure. We have examined the energetic contribution of these abundantly occurring amino acids to leucine zipper stability. We have focused on the d position [13], the g↔e′ interaction, and the a–a′ interaction. These studies have examined the 3rd and 4th heptads of the VBP leucine zipper, the portion of the leucine zipper that drives leucine zipper formation [22].

It is important to distinguish the contribution of an individual amino acid to dimerization stability versus dimerization specificity. Specificity essentially occurs when amino acids on opposite monomers energetically interact either favorably or unfavorably. One method to evaluate contributions to dimerization specificity is through a double mutant alanine thermodynamic cycle that involves analysis of four protein variants [23].

Table 1
Alanine based coupling energy for 16 g↔e′ pairs [25] and 25 a–a′ pairs [14]

Coupling energies for g↔e′ pairs relative to A↔A ($\Delta\Delta\Delta G_{int}$) (kcal/mol)

↓g\e′→	E	Q	R	K
E	+0.7	+0.2	−0.5	−0.3
Q	+0.2	−0.0	+0.3	+0.3
R	−1.1	+0.4	+0.7	+0.8
K	−0.9	+0.3	−0.6	+0.6

Coupling energies for a–a′ pairs relative to A↔A ($\Delta\Delta\Delta G_{int}$) (kcal/mol)

Protein	A-RR34(I)	A-RR34(V)	A-RR34(L)	A-RR34(N)	A-RR34(K)
	$\Delta\Delta G_{37}$ kcal/mol	$\Delta\Delta G_{37}$ kcal/mol	$\Delta\Delta G_{37}$ kcal/mol	$\Delta\Delta G_{37}$ kcal/mol	$\Delta\Delta G_{37}$ kcal/mol
B-EE34(I)	−0.9	+0.4	+0.9	+4.9	−0.4
B-EE34(V)	+0.2	−0.7	+0.2	+3.2	−0.5
B-EE34(L)	+0.5	+0.2	−0.6	+2.8	−0.1
B-EE3(N)	+4.3	+3.0	+2.6	−0.5	−0.2
B-EE34(K)	−0.7	−0.5	−0.2	−0.3	+0.3

Lets examine the case of the interhelical g↔e′ interaction between glutamic acid (E) and arginine (R) [24,25]. A protein containing an E↔R g↔e′ pair is 2.6 kcal/mol /dimer more stable that a second protein that contains alanine in both positions (A↔A). Since a B-ZIP dimer contains two g↔e′ pairs per heptad, the E↔R pair is 1.3 kcal/mol more stable than the A↔A pair. To understand specificity of molecular interaction, one needs to know how much of the 1.3 kcal/mol stabilization from the E↔R pair is from the interaction between the E and the R. To answer this question, one examines two additional proteins. One contains only E in the g position, producing an E↔A pair and the second contains only R in the following e position producing an A↔R pair. The E↔A pair is 0.1 kcal/mol more stable than A↔A while the A↔R pair is 0.7 kcal/mol more stable than A↔A. This, if the two amino acids act independently, they should contribute 0.8 kcal/mol to stability, not the measured 1.3 kcal/mol. We attribute the missing 0.5 kcal/mol (1.3–0.8) of energy from the E↔R pair to the interaction between the two amino acids, E and R which is helps regulate dimerization specificity.

A double mutant alanine thermodynamic cycle analysis has been used to calculate coupling energies and investigate the contribution of g↔e′ [25] and a↔a′ interactions [14] to leucine zipper dimerization specificity (Table 1). The general rules that result from these studies are presented in Fig. 4. g↔e′ pairs containing oppositely charged amino acids such as E↔R and E↔K have stabilizing coupling energies of −0.5 kcal/mol/pair and −0.3 kcal/mol/pair, respectively. g↔e′ pairs containing similarly charged amino acids are destabilizing, with coupling energies of +0.7, +0.6 and +0.8 kcal/mol/pair for E↔E, K↔K and R↔R, respectively.

a↔a′ pairs can also regulate dimerization specificity [14,26]. The most dramatic known example is that homotypic N↔N and I↔I interactions have stabilizing coupling energies of −0.5 and −0.9 kcal/mol/a–a′ pair respectively while the heterotypic I↔N pair has a destabilizing coupling energy of +4.3 kcal/mol/pair. As a result, an asparagine (N) residue in the a position does not interact favorably with hydrophobic amino acids and thus forms a homotypic N↔N pairs [14]. In contrast, a lysine residue in the a position does not like to form homotypic pairs but does form heterotypic pairs with aliphatic amino acids as well as asparagines thus favoring heterodimerization [14]. These results indicate that a position regulation of homodimerization is easier to predict than heterodimerization.

An enigma with longer coiled-coils is to identify what part of the structure contributes to stability as fragments of native coiled-coils rarely fold [27]. However, in cortexillin I, a 18 heptads long coiled-coil, folding is dependent on two heptads, termed the “trigger sequence”, that nucleates the folding of the

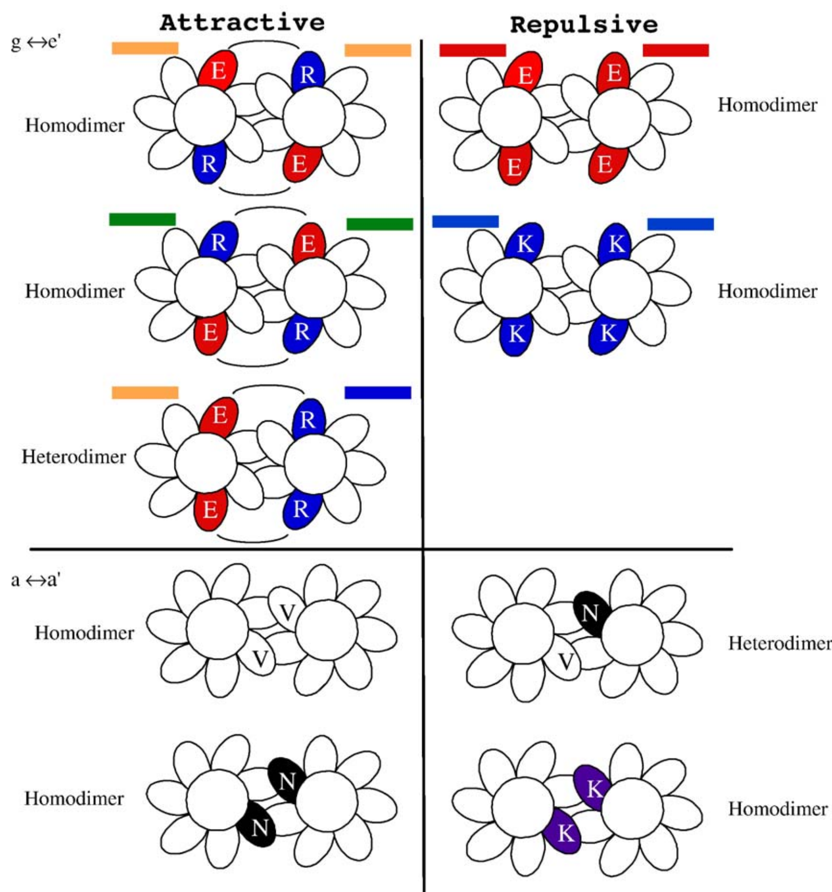

Fig. 4. Schematic of a leucine zipper depicting favorable and unfavorable g↔e′ and a↔a′ interactions. Favorable (Attractive) interactions: g↔e′ interactions containing oppositely charged amino acids (E↔R, R↔E, E↔K, and R↔E) can drive dimer formation between B-ZIP proteins (homodimers and heterodimers). For a↔a′ interactions, the homotypic V↔V and N↔N interactions are favorable. Unfavorable (Repulsive) interactions. We present two unfavorable g↔e′ interactions, (E↔E and R↔R). For a↔a′, the homotypic K↔K and the heterotypic N↔V are unfavorable.

Family	Consensus	BB BN L	1 gabcdef	2 gabcdef	3 gabcdef	4 gabcdef	5 gabcdef	6 gabcdef	7 gabcdef	8 gabcdef	9 gabcdef	10 gabcdef
CREB	CREB	AARKREVRLMKNREAARECRRKKKEYVKCLEN	RVAVLEN	QNKTLIE	ELKALKD	LYCHKSD*						
	ATF-1	PQLKREIRLMKNREAARECRRKKKEYVKCLEN	RVAVLEN	QNKTLIE	ELKTLKD	LYSNKSV*						
	CREM	ATRKRELRLMKNREAAKECRRRKKEYVKCLES	RVAVLEV	QNKKLIE	ELETLKD	ICSPKTD	Y*					
	HCREM-1	ATRKRELRLMKNREAARECRRKKKEYVKCLEN	RVAVLEN	QNKTLIE	ELKALKD	LYCHKVE*						
PAR	TEF	KDEKYWTRRKKNNVAAKRSRDARRLKENQITI	RAAFLEK	ENTALRT	EVAELRK	EVGKCKT	IVSKYET	KYGPL*				
	DBP	KDEKYWSRRYKNNEAAKRSRDARRLKENQISV	RAAFLEK	ENALLRQ	EVVAVRQ	ELSHYRA	VLSRYQA	QHGAL*				
	HLF	KDDKYWARRRKNNMAAKRSRDARRLKENQIAI	RASFLEK	ENSALRQ	EVADLRK	ELGKCKN	ILAKYEA	RHGPL*				
NFIL3	NFIL3	KDAMYWEKRRKNNEAAKRSREKRRLNDLVLEN	KLIALGE	ENATLKA	ELLSLKL	KFGLISS	TAYAQEI	QKLSNST	AVYFQDY	QTSKSNV	SSFVDEH	EPSMVSS
ATF6	ATF-6	AVLRRQQRMIKNRESACQSRKKKKEYMLGLEA	RLKAALS	ENEQLKK	ENGTLKR	QLDEVVS	ENQRLKV	VPSPKRR	VVCVMIV	LAFILIN	YGPMSML	EQDSRRM
	CREBL1	KLLKRQQRMIKNRESACQSRRKKKEYLQGLEA	RLQAVLA	DNQQLRR	ENAALRR	RLEALLA	ENSELKL	GSGNRKV	VCIMVFL	LFIAFNF	PVSISEP	PSAPISG
	XBP-1	PEEKALRRKLKNRVAAQTARDRKKARMSELEQ	QVVDLEE	ENQKLLL	ENQLLRE	KTHGLVV	ENQELRQ	RLGMDAL	VAEEEAE	AKGNEVR	PVAGSAE	SAALRLR
OASIS	OASIS	KALKRVRRKIKNKISAQESRRKKKEYVECLEK	KVETFTS	ENNELWK	KVETLEN	ANRTLLQ	QLQKLQT	LVTNKIS	RPYKMAA	TQTGTCL	MPSRSLL	FYDDGAG
	CREB-H	RVLKKIRRKIRNKQSAQESRKKKKEYIDGLET	RMSACTA	QNQELQR	KVLHLEK	QNLSLLE	QLKKLLQ	AIVVQST	SKSAQTG	TCVAVLL	LSFALII	LPSISPF
	CREB3	QILKRVRRKIRNKRSAQESRRKKKVYVGGLES	RVLKYTA	QNMELQN	KVQLLEE	QNLSLLD	QLRKLQA	MVIEISN	KTSSSST	CILVLLV	SFCLLLV	PAMYSSD
	AIBZIP	RVLKKVRRKIRNKQSAQDSRRRKKEYIDGLES	RVAACSA	QNQELQK	KVQELER	HNISLVA	QLRQLQT	LIAQTSN	KAAQTST	CVLILLF	SLALIIL	PSFSPFQ
HCF	HCF	PRKAAAAAARLNRLKKKEYVMGLESRVRGLAA	ENQELRA	ENRELGK	RVQALQE	ESRYLRA	VLANETG	LARLLSR	LSGVGLR	LTTSLFR	DSPAGDH	DYALPVG
C/EBP	C/EBPα	NSNEYRVRRERNNIAVRKSRDKAKQRNVETQQ	KVLELTS	DNDRLRK	RVEQLSR	ELDTLRG	IFRQLPE	SSLVKAM	GNCA*			
	C/EBPβ	HSDEYKIRRERNNIAVRKSRDKAKMRNLETQH	KVLELTA	ENERLQK	KVEQLSR	ELSTLRN	LFKQLPE	PLLASSG	HC *			
	C/EBPδ	GSPEYRQRRERNNIAVRKSRDKAKRRNQEMQQ	KLVELSA	ENEKLHQ	RVEQLTR	DLAGLRQ	FFKQLPS	PPFLPAA	GTADCR*			
	C/EBPε	DSLEYRLRRERNNIAVRKSRDKAKRRILETQQ	KVLEYMA	ENERLRS	RVEQLTQ	ELDTLRN	LFRQIPE	AANLIKG	VGGCS*			
	C/EBPγ	NSDEYRQRRERNNMAVKKSRLKSKQKAQDTLQ	RVNQLKE	ENERLEA	KIKLLTK	ELSVLKD	LFLEHAH	NLADNVQ	SISTENT	TADGDNA	GQ*	
	chop10	DQGRTRKRKQSGHSPARAGKQRMKEKEQENER	KVAQLAE	ENERLKQ	EIERLTR	EVEATRR	ALIDRMV	*				
	HP8	RTATSTGYGATNNIAVRKSRDKAKQRNVETQQ	KVLELTS	DNDRLRN	GVEQLSR	ELDTLRG	ALIDRMV	NLHQA*				
ATF4	ATF4	KLDKKLKKMEQNKTAATRYRQKKRAEQEALTG	ECKELEK	KNEALKE	RADSLAK	EIQYLKD	LIEELRK	ARGKKRV	P*			
	XP_093185	PYDPPGEKMVAAQVKGTRYRQKKRVEQEVLTG	ECKAVEK	KNEALQE	RADSLAE	EIQYMKD	SIEEVCK	ARGKKRV	L*			
	ATF5	RGDRKQKKRDQNKSAALRYRQRKRAEGEALEG	ECQGLEA	RNRELKE	RAESVER	EIQYVKD	LLIEVYK	ARSQRTR	SC*			
ATF2	ATF-2	PDEKRRKFLERNRAAASRCRQKRKVWVQSLEK	KAEDLSS	LNGQLQS	EVTLLRN	EVAQLKQ	LLLAHKD	CPVTAMQ	KKSGYHT	ADKDDSS	EDISVPS	SPHTEAI
	ATF7	PDERRQRFLERNRAAASRCRQKRKLWVSSLEK	KAEELTS	QNIQLSN	EVTLLRN	EVAQLKQ	LLLAHKD	CPVTALQ	KKTQGYL	ESPKESS	EPTGSPA	PVIQHSS
	CREBPA	PDERRRKFLERNRAAATRCRQKRKVWVMSLEK	KAEELTQ	TNMQLQN	EVSMLKN	EVAQLKQ	LLLTHKD	CPITAMQ	KESQGYL	SPESSPP	ASPVPAC	SQQQVIQ
JUN	JUND	ERIKAERKRLRNRIAASKCRKRKLERISRLEE	KVKTLKS	QNTELAS	TASLLRE	QVAQLKQ	KVLSHVN	SGCQLLP	QHQVPAY*			
	JUN	ERIKAERKRMRNRIAASKCRKRKLERIARLEE	KVKTLKA	QNSELAS	TANMLRE	QVAQLKQ	KVMNHVN	SGCQLML	TQQLQTF*			
	JUNB	ERIKVERKRLRNRLAATKCRKRKLERIARLED	KVKTLKA	ENAGLSS	TAGLLRE	QVAQLKQ	KVMTHVS	NGCQLLL	GVKGHAF*			
S-MAF	MAFK	TRLKQRRRTLKNRGYAASCRIKRVTQKEELER	QRVELQQ	EVEKLAR	ENSSMRL	ELDALRS	KYEALQT	FARTVAR	GPVAPSK	VATTSVI	TIVKSTE	LSSTSVP
	MAFG	VQLKQRRRTLKNRGYAASCRVKRVTQKEELEK	QKAELQQ	EVEKLAS	ENASMKL	ELDALRS	KYEALQT	FARTVAR	SPVAPAR	GPLAAGL	GPLVPGK	VAATSVI
	MAFF	TRLKQRRRTLKNRGYAASCRVKRVCQKEELQK	QKSELER	EVDKLAR	ENAAMRL	ELDALRG	KCEALQG	FARSVAA	ARGPATL	VAPASVI	TIVKSTP	GSGSGPA
FOS	FOS	EEEKRRIRRERNKMAAAKCRNRRRELTDTLQA	ETDQLED	EKSALQT	EIANLLK	EKEKLEF	ILAAHRP	ACKIPDD	LGFPLEE	MSVASLD	LTGGLPE	VATPESE
	FOSB	EEEKRRVRRERNKLAAAKCRNRRRELTDRLQA	ETDQLEE	EKAELES	EIAELQK	EKERLEF	VLVAHKP	GCKIPYE	EGPGPLA	EVRDLPG	SAPAKED	GFSWLLP
	FRA1	EEERRRVRRERNKLAAAKCRNRRKELTDFLQA	ETDKLED	EKSGLQR	EIEELQK	QKERLEL	VLEAHRP	ICKIPEG	AKEGDTG	STSGTSS	PPAPCRP	VPCISLS
		EEERRRVRPERNKLAAAKCRNWRKELTDFLQA	ETDKLED	EKSGLQR	EIEELQK	QKERLEL	VLEAH*					
	FRA2	EEEKRRIRRERNKLAAAKCRNRRRELTEKLQA	ETEELEE	EKSGLQK	EIAELQK	EKEKLEF	MLVAHGP	VCKISPE	ERRSPPA	PGLQPMR	SGGGSVG	AVVVKQE
	ATF-3	EDERKKRRRERNKIAAAKCRNKKKEKTECLQK	ESEKLES	VNAELKA	QIEELKN	EKQHLIY	MLNLHRP	TCIVRAQ	NGRTPED	ERNLFIQ	QIKEGTL	QS*
	JDP2	EEERRKRRREKNKVAAARCRNKKKERTEFLQR	ESERLEL	MNAELKT	QIEELKQ	ERQQLIL	MLNRHRP	TCIVRTD	SVKTPES	EGNPLLE	QLEKK*	
	JDP-1	DDDRKVRRREKNRVAAQRSRKKQTQKADKLHE	EYESLEQ	ENTMLRR	EIGKLTE	ELKHLTE	ALKEHEK	MCPLLLC	PMNFVPV	PPRPDPV	AGCLPR*	
	BATF	DDVRRVQRREKNRIAAQKSRQRQTQKADTLHL	ESEDLEK	QNAALRK	EIKQLTE	ELKYFTS	VLNSHEP	LCSVLAA	STPSPPE	VVYSAHA	FHQPHVS	SPRFQP*
CNC	BACH1	DCIHDIRRRSKNRIAAQRCRKRKLDCIQNLES	EIEKLQS	EKESLLK	ERDHILS	TLGETKQ	NLTGLCQ	KVCKEAA	LSQEQIQ	ILAKYSA	ADCPLSF	LISEKDK
	NRF1	SLIRDIRRRGKNKMAAQNCRKRKLDTILNLER	DVEDLQR	DKARLLR	EKVEFLR	SLRQMKQ	KVQSLYQ	EVLFGRL	RDENGRP	YSPSQYA	LQYAGDG	SVLLIPR
	NFE2	ALVRDIRRRGKNKVAAQNCRKRKLETIVQLER	ELERLTN	ERERLLR	ARGEADR	TLEVMRQ	QLTELYR	SGCQLLP	DESGNSY	SPEEYAL	QQAADGF	LVPRGTK
	BACH2	EFIHDVRRRSKNRIAAQRCRKRKLDCIQNLEC	EIRKLVC	EKEKLLS	ERNQLKA	CMGELLD	NFSCLSQ	EVCRDIR	QSPEQIQ	ALHRYCP	VLRPMDL	PTASSIN
	NRF2	ALIRDIRRRGKNKVAAQNCRKRKLENIVELEQ	DLDHLKD	EKEKLLK	EKGENDK	SLHLLKK	QLSTLYL	EVFSMLR	DEDGKLY	SPSEYSL	QQTRDGN	VFLVPKS
	NRF3	SLIRDIRRRGKNKVAAQNCRKRKLDIILNLED	DVCNLQA	KKETLKR	EQAQCNK	AINIMKQ	KLHDLYH	DIFSRLR	DDQGRPV	NPNHYAL	QCTHDGS	ILIVPKE
L-MAF	NRL	LRLKQRRRTLKNRGYAQACRSKRLQQRRGLEA	ERARLAA	QLDALRA	EVARLAR	ERDLYKA	RCDRLTS	SGPGSGD	PSHLFL*			
	MAFB	IRLKQKRRTLKNRGYAQSCRYKRVQQKHHLEN	EKTQLIQ	QVEQLKQ	EVSRLAR	ERDAYKV	KCEKLAN	SGFREAG	STSDSPS	SPEFFL*		
	C-MAF	IRLKQKRRTLKNRGYAQSCRFKRVQQRHVLES	EKNQLLQ	QVDHLKQ	EISRLVR	ERDAYKE	KYEKLVS	SGFRENG	SSSDNPS	SPEFFM*		

Fig. 5. Amino acid sequence of all the 53 *H. sapiens* B-ZIP domains. Proteins are arranged into groups with similar dimerization properties. Amino acids predicted to regulate dimerization specificity are color-coded. If the g and following e positions contain charged amino acids, the heptad is colored from g to the following e position. We use four colors to represent g↔e′ pairs. Green is for the attractive basic–acidic pairs (R↔E and K↔E); orange is for the attractive acidic–basic pairs (E↔R, E↔K, D↔R, and D↔K); red is for repulsive acidic pairs (E↔E and E↔D); blue is for repulsive basic pairs (K↔K and R↔K). If only one of the two amino acids in the g↔e′ pair is charged, we color that residue blue if it is basic and red if it is acidic. If the a or d position is polar (N, S, and T), they are colored black and if they are charged, they are colored purple. The prolines and glycines are colored red to indicate a potential break in the α-helical structure. The natural C-terminus of each leucine zipper is noted by an asterisk.

longer coiled-coil [28]. Similar sequences have been identified in other longer coiled-coils [29]. These studies suggest that the measured energetic contribution of individual amino acids to B-ZIP leucine zipper stability may be valuable information for evaluating "trigger sequence". However, the energetic contribution of these amino acids when they occur in non-trigger like sequences that cannot nucleate their own formation remains unknown. For example, mutating the 5th d position of JunD and Fos from histidine to either alanine or leucine did not change the thermal stability of the JunD|Fos heterodimer suggesting that this part of the structure was not contributing to stability (data not shown). The enigma is why this position of the leucine zipper is not contributing to stability and highlights that our understanding of the limits of the coiled-coil structure is incomplete and the energetic contribution to stability throughout the coiled-coil is incomplete.

3. Dimerization properties of human B-ZIP proteins

Access to the complete sequence of the human genome permitted the global identification of B-ZIP proteins and the subsequent prediction of potential B-ZIP dimerization partners. The dimerization rules reviewed here were used to predict dimerization specificity of B-ZIP proteins in *Homo sapiens* [30], *Drosophila melanogaster* [31] and *Arabidopsis thaliana* [2]. Recently, a landmark paper experimentally determined the dimerization specificity of 49 human B-ZIP proteins [1]. These data supported our previous predictions and, in doing so, validated the accuracy of the dimerization predictions made using these simple rules. However, the accuracy of such predictions can be improved upon using computational methods [32] and structure based analysis [33] suggesting that coupling energy analysis completed to date does not reveal all the secretes of coiled-coil dimerization specificity.

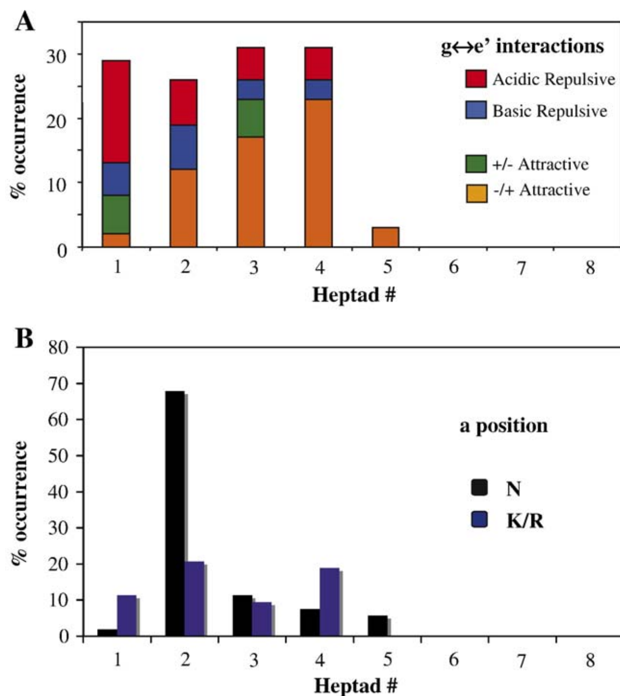

Fig. 6. (A) Histogram presenting the frequency of attractive or repulsive $g \leftrightarrow e'$ pairs per heptad of the leucine zippers for *H. sapiens* B-ZIP proteins. (B) Histogram of the frequency of asparagine and charged amino acids in the a position of the leucine zippers for *H. sapiens* B-ZIP proteins.

Fig. 5 presents an alignment of the amino acid sequences for the 53 known human B-ZIP motifs. The proteins are grouped into families based on dimerization specificity [1,30]. The N-terminal boundary of the leucine zipper is clearly defined by the basic region, while the C-terminal is identified by the presence of a proline or a pair of glycines, either of which disrupts the α-helical structure characteristic of a leucine zipper. Over half of *H. sapiens* leucine zippers contain five or six heptads, and no zippers are greater than nine heptads in length. Fig. 6A presents the frequency of attractive and repulsive $g \leftrightarrow e'$ pairs for each heptad. The total absence of these interactions beyond heptad 5 is consistent with the limited length of the leucine zippers.

Asparagine is typically found in the a position of the second heptad of the human leucine zipper proteins, but is rarely observed in the a positions of other heptads (Fig. 6B). Since asparagine produces a stable $a \leftrightarrow a'$ interaction with another asparagine and does not interact favorably with aliphatic amino acids, the presence of asparagine favors homodimer formation [14]. Charged amino acids, which are destabilizing relative to alanine [14,34], are common in the a positions of the FOS, CNC, large MAF, and small MAF families of B-ZIP proteins and drive the well-characterized heterodimerizing properties of these transcription factors (Fig. 6B).

4. B-ZIP proteins in plants

Sixty-seven B-ZIP proteins were identified in a recent annotation of the *Arabidopsis thaliana* genome [2]. No *A. thaliana* B-ZIP domains have homologs in *H. sapiens*. The kinds of amino acids found in the a, d, e, and g positions of *A. thaliana* B-ZIP domain are similar to those found in mammals suggesting that these amino acids are uniquely suited for regulating dimerization stability and specificity. Interestingly, many *A. thaliana* B-ZIP leucine zippers are eight or more heptads in length which is in sharp contrast to the four to six heptads found in *H. sapiens* leucine zippers. Furthermore, it appears that *A. thaliana* has 14 families of homodimerizing B-ZIP proteins which more than twice the number found in *H. sapiens.* This suggests that longer leucine zippers may be needed to generate more homodimerizing families, and supports the idea that the number of homodimerizing proteins in humans is severely limited by the shorter overall length of the leucine zippers.

5. DNA binding of B-ZIP proteins modulates dimerization

An important issue to examine is the role of DNA binding in affecting B-ZIP dimerization specificity. The role is significant since, in certain circumstances, a chimeric DNA binding site can catalyze the formation of a stable heterodimer between two, otherwise incompatible B-ZIP proteins [15]. In addition, a number of studies have shown that members of the Jun B-ZIP family form heterodimers with either the FOS or the ATF2 protein and that these different heterodimers differ in DNA

binding preference [35,36]. The ability of dimers to discriminate between related DNA sequences is independent of the zipper region and is specified by amino acids both in the basic region and in the “linker” region immediately N-terminal to the beginning of the leucine zipper [37,38]. Using alanine scanning mutagenesis, it was demonstrated that charged amino acids on the solvent exposed surface of CREB were critical for the observed selectivity of CREB dimers for the CRE site (consensus: TGACGTCA) over the highly related TRE, or AP-1 site (consensus: TGACTCA) [39]. Thermodynamic signatures of the yeast GCN4-B-ZIP homodimer bound to DNA revealed a role for water in discriminating between CRE and TRE sites [40]. B-ZIP complexes generate a substantially larger negative enthalpy and non-electrostatic entropy when bound to a TRE than to a CRE, implying that a TRE complex (e.g., FOS: JUN) incorporates significantly more water molecules than a CRE bound, B-ZIP dimer (e.g., ATF/CREB). Interestingly, even though the basic region of FOS binds to a TRE [41], the strong repulsion between the leucine zippers of two FOS molecules effectively prevents the FOS homodimer from binding DNA. In this case, stabilization by DNA binding is not sufficient to overcome the intrinsic repulsion between two FOS leucine zippers.

6. Kinetics of B-ZIP DNA binding

The stability of B-ZIP proteins in the absence or presence of DNA has been discussed. However, it is clear that the kinetics of assembly of B-ZIP protein-DNA complexes is important and impacts the efficiency with which these complexes carry out their biological functions. Studies on the kinetics of B-ZIP \DNA complex assembly indicate that many potential folding pathways can occur. The generally accepted mechanism involves initial B-ZIP dimer formation and the subsequent binding of pre-formed dimers to DNA. Alternatively, for some B-ZIP proteins, including FOS and JUN [42], each protein can bind DNA as monomers and then dimerize [43]. Non-B-ZIP proteins with an affinity for leucine zippers also can influence the assembly pathway [44]. For example, the X protein of the hepatitis B virus enhances the transcriptional activity of many B-ZIP transcription factors [45] by interacting with the basic and linker region, stabilizing the B-ZIP–DNA complex, and enhancing the monomer assembly pathway [46]. Green and colleagues identified a nuclear protein, B-ZIP enhancing factor (BEF) that stimulates DNA binding by recognizing the unfolded leucine zipper and promoting the folding of B-ZIP monomers to dimers; the elevated concentration of the bZIP dimer then drives the DNA binding reaction [47].

Detailed examination of the leucine zipper using calorimetry, fluorescence and CD spectroscopic studies has shown that the unfolding of the B-ZIP structure is not a simple two-state transition, but involves many folding intermediates which is the basic region unfolding independent of the leucine zipper, particularly the heptads C-terminus of the invariant asparagines in the 2nd heptad [48]. It has been suggested that the intrinsic helicity of unfolded monomers is the rate-limiting factor in the formation of a coiled-coil [22,49]. These biophysical observations are in agreement with biochemical experiments that show that ancillary proteins can drive B-ZIP binding to DNA by simply stabilizing the folding of each monomer.

7. B-ZIP dimerization in vivo

In vitro analysis of B-ZIP protein–protein interactions has produced a set of dimerization rules that are supported by biological experiments using exogenous protein expression and/or functional inactivation of various B-ZIP proteins to elicit the predicted effects on cell growth, target gene expression and on the composition of B-ZIP dimers that exist in cells [50,51]. Despite these successes, however, it remains a challenge to accurately assess if the stability and specificity predicted for particular dimers translate into actual differences in the levels of B-ZIP dimer complexes in cells. For example, if JUN, FOS and ATF2 proteins are expressed at equivalent levels in a cell, what are the relative levels of FOS–JUN, JUN–JUN, JUN–ATF2 and ATF2–ATF2 dimers in this cell? Are these levels what is predicted based on relative affinities, or do additional cellular mechanisms (such as post-translational modifications and/or intracellular transport) impinge upon the intrinsic abilities of the proteins to interact and thus bias the dimer repertoire?

Recently, two new techniques have been developed that are addressing this issue of B-ZIP dimer partner in vivo. The first method is chromatin immunoprecipitation (ChIP). This method covalently crosslinks proteins to DNA in living cells, then the protein of interest is immunoprecipitated, the covalent crosslinks are reversed, the immunoprecipitated DNA is amplified either with specific DNA primers to address a specific location in the genome or globally for use on Chip on Chip microarrays that evaluate where in the entire genome a particular transcription factor is bound. This elaborate method is starting to generate some impressive results. For example, a recent report examined where c-Jun and ATF2 are bound in the genome. Cisplatin leads to activation of the NH2-terminal Jun kinase (JNK) which in turn phosphorylates c-Jun and ATF2. ChIP and promoter microarrays under cisplatin-induced genotoxic stress identified 210 promoters bound by phospho-c-Jun and 181 promoters bound by phopho-ATF2 [52]. The majority of promoters (121) were bound by both factors suggesting that c-Jun and ATF2 form heterodimers at the promoter of these genes. Evaluation of the promoter sequence where c-Jun|ATF2 heterodimers identified CRE like sequences (5′-TGACGTCA-C-3′) in many of these bound promoters helping to identify what DNA sequence are bound in living cells by b-ZIP dimers.

The second method to evaluate what B-ZIP dimers can occur in vivo is based on the direct visualization and quantification of B-ZIP dimers in living cells. The bimolecular fluorescence complementation assay (BiFC) is a procedure in which B-ZIP proteins are expressed as fusions tagged with complementing halves of either the yellow (YFP) or cyan (CFP) fluorescent protein [53]. When co-expressed in a cell, the leucine zipper interactions will allow for the reconstitution of YFP or CPF, resulting in the emission of measurable auto-fluorescence.

Conveniently, if the C- terminal half of YFP is used on one B-ZIP protein and the N-terminal halves of YFP and CFP are used on two competing partner proteins, the emission from a reconstituted YFP can be distinguished from that of a CFP/YFP hybrid protein. This makes it possible to quantify the levels of two dimer complexes in a single cell [54]. To date, BiFC has been used primarily to visualize proteins already known to interact, yet, in doing so, these studies have uncovered novel mechanisms of B-ZIP protein regulation related to protein sequestration, signaling and accessory protein interaction that alter the dynamics of these interactions in vivo [53,55–59].

Currently, BiFC assays are hampered by the irreversibility of the matured chromophore, although work to genetically engineer less stable versions of YFP and CFP eventually may circumvent this problem. A second technique that has the advantage of being reversible is two-hybrid fluorescence cross-correlation spectroscopy (FCCS) [60]. Once again, the utility of the approach has been demonstrated recently using the FOS and JUN B-ZIP proteins. In this technique, the proteins are co-expressed in living cells as fusions with two different auto-fluorescent peptides. The correlated motion of the two distinct fluorophores is monitored by FCCS and protein dimerization which, in this case, is controlled solely by the reversible interaction of the leucine zippers, is measured over time. Quantification of the interaction, but also of the impact that secondary cellular processes (such as DNA binding) have on the stability of the interaction, can be obtained using this approach.

The further refinement of the BiFC and FCCS techniques will eventually allow for the assessment of the spatial and temporal control of various B-ZIP dimers during development using cell culture systems and whole animals. It will permit a thorough examination of the signaling events and additional cellular control mechanisms that impact the dimerization rules established through biophysical studies and lead to a more complete understanding of B-ZIP protein interaction networks in vivo. These new methods will help answer the questions: What B-ZIP dimers form in living cells, what DNA sequences do they bind, and how does this binding modulate gene expression?

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