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Investigation of the Multimerization Region of the Kaposi's Sarcoma-Associated Herpesvirus (Human Herpesvirus 8) Protein K-bZIP: the Proposed Leucine Zipper Region Encodes a Multimerization Domain with an Unusual Structure

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The *K8* gene of Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) shares many functional similarities with the *BZLF1* gene of Epstein-Barr virus. The protein products of *K8* and *BZLF1*, K-bZIP (RAP, K8) and Zta (BZLF1, ZEBRA, Z) have both been proposed to be members of the bZIP family of transcription factors, forming multimers via a coiled-coil motif termed a leucine zipper. Substantial evidence supporting this model for Zta is published. Here, we demonstrate that the proposed leucine zipper region of K-bZIP (amino acids 182 to 218) is required for multimer formation but that it does not fold as a coiled coil.

Kaposi's sarcoma-associated herpesvirus (KSHV) (or human herpesvirus 8 [HHV8]) is implicated in the development of three potentially fatal human diseases (reviewed in references 6, 7, 11, 20, 30, 37, 38, and 45). It is a member of the human gammaherpesvirus family, and its genome displays sequence homology with the prototypical member of the family, Epstein-Barr virus (EBV) (34). The *K8* gene of KSHV encodes K-bZIP (RAP, K8α) which is involved in viral lytic replication (5, 25, 48). K-bZIP interacts with replication structures (4, 47) and with three individual cellular proteins, p53 (31), CBP (19), and C/EBPα (51), and it regulates the expression of the cellular gene encoding $p21^{CIP1}$ (51). Thus, K-bZIP is a candidate to mediate two important events: viral replication and cell cycle arrest (41).

K8 appears to be a homologue of the EBV gene *BZLF1* (14, 26, 32). The protein encoded by *BZLF1*, Zta (BZLF1, ZEBRA, or Z), plays a key role in the EBV replicative cycle (reviewed in references 12, 29, 39, 41, and 43). Zta also shares with K-bZIP the ability to interact with its origin of lytic replication (35, 36), to promote cell cycle arrest (13), and to interact with the cellular proteins p53, CBP, and C/EBPα (1, 28, 41, 50, 52, 53). Given the functional similarities between these two proteins, it is tempting to speculate that a similar molecular mechanism is exploited by each virus to achieve the same ends.

The overall homology between K-bZIP and Zta is low but rises to 30% similarity (18% identity) within the C-terminal half. This region contains the well-characterized bZIP domain of Zta, which directs the formation of homodimers through the leucine zipper motif and interacts with DNA through the adjacent basic region (15, 16, 29, 39, 41–43). Interestingly, both K-bZIP and Zta form homo-multimers involving the C-terminal half of the protein (14, 26), suggesting that multimerization could be mediated in a similar manner for both viral proteins. In this study, we question whether K-bZIP contains a functional bZIP domain.

Similarities between Zta and K-bZIP primary and secondary structures are illustrated in Fig. 1. Alignment of the carboxy-terminal halves of both Zta and K-bZIP using ClustalW (17, 46) reveals scattered homology throughout the putative basic and leucine zipper regions. Leucine zipper multimerization domains comprise two strands of α-helix, which fold together through a hydrophobic face on each helix to form a coiled-coil structure (3, 8, 24, 44). Bioinformatics analyses using the program COILS (27) revealed that the proposed leucine zipper region of K-bZIP is predicted to fold as a coiled coil, as Zta does. This prompted us to question experimentally whether K-bZIP is able to undertake the two key functions of a bZIP protein, namely, (i) to interact with DNA or (ii) to form multimers using a coiled-coil folding motif.

In light of the scattered homology between the basic (DNA contact) region of Zta and K-bZIP, we questioned whether K-bZIP is able to interact with an AP1 site. Polyhistidine-tagged versions of both full-length proteins were generated in vitro using a reticulocyte lysate translation system and equal amounts of each protein were added to an in vitro DNA-binding assay with AP1 and a mutant version of that site (as described in reference 15). As expected, only His-Zta showed a clear interaction with the AP1 site (data not shown).

We sought to determine whether K-bZIP encodes a leucine zipper domain by undertaking domain swaps with the bZIP protein Zta. Functional swaps of the leucine zipper have been documented for this protein with leucine zippers of other members of the bZIP family (for an example, see reference 49). Using the alignment shown in Fig. 1, the first domain swap maintained the exact register of the alignment, ending Zta at amino acid Q198 and replacing the remainder of the coding sequence with amino acids A190 to the carboxyl terminus of K-bZIP (Fig. 2). His-Zta and His-K-bZIP proteins were gen-

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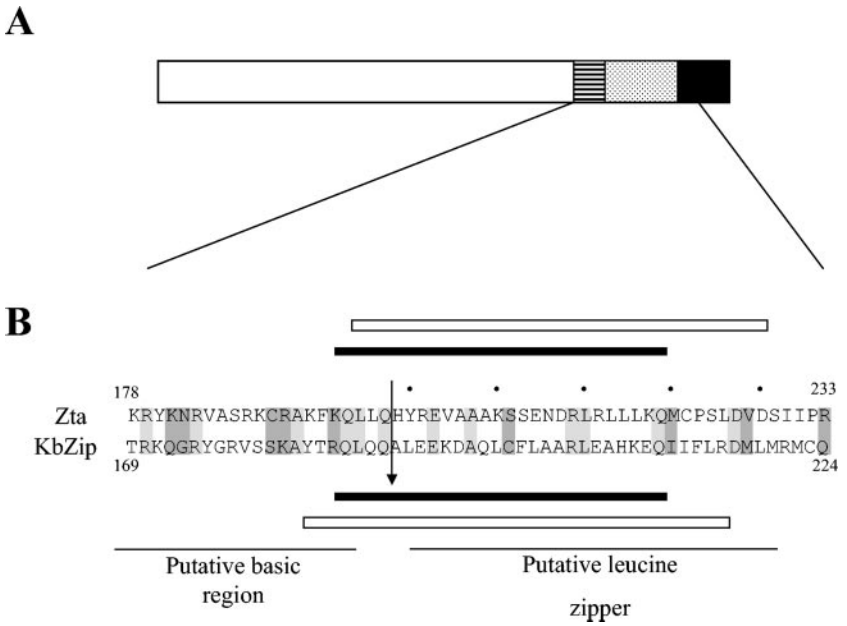

FIG. 1. Schematic diagram of Zta and K-bZIP. A. A schematic diagram of the known features of Zta is shown. The striped box represents the basic region, the stippled box the coiled coil, and the filled box the CT region. B. Analysis of the primary structure of K-bZIP (KbZip) and Zta using the bioinformatics program ClustalW revealed the indicated region of homology. Conserved amino acids are shown in on a light grey background, and conservative substitutions are shown on a dark grey background. The dots above the Zta sequence indicate those amino acids in the leucine zipper at "d" positions in the proposed coiled coil. The propensity of equivalent regions of Zta and K-bZIP to fold as coiled coils were investigated using the predictive program COILS and are shown as black bars below or above each sequence. The extent of the synthetic peptides used for biophysical analyses are indicated as boxes above or below each sequence. The position of the junction within the Z-K hybrid is indicated by an arrow.

erated in vitro, and their ability to interact with DNA was assessed using a DNA-binding assay. Surprisingly, this exchange did not result in a hybrid protein capable of interacting with AP1. The alignment of the basic and leucine zipper regions is important for the function of b-ZIP proteins (2, 23), so six further domain swaps were generated that contained between zero and four alanine residues between the basic and proposed zipper regions in order to align the proposed zipper in different conformations with respect to the basic region. However, none of the hybrids was able to interact with DNA, suggesting that a functional bZIP domain is not formed by any of the combinations tested. It is possible that this approach omitted a crucial configuration; however, the data shown lend no support to a model in which the putative zipper region of K-bZIP contains a functional leucine zipper.

In order to probe whether the predicted coiled coil within the putative bZIP region of K-bZIP is required for multimerization, a deletion mutant, lacking amino acids 182 to 218, was generated by site-directed mutagenesis. The ability of a polyhistidine-tagged version of this protein, His-K-ΔZIP, to form multimers with full-length K-bZIP (14) was assessed using an in vitro association assay with a glutathione *S*-transferase (GST)-tagged version of K-bZIP (Fig. 3). In conditions where no association between GST and His-K-bZIP or GST-K-bZIP and His-Zta was observed, His-K-bZIP showed a clear association with the full-length GST-K-bZIP. Furthermore, the deletion within His-K-ΔZIP prevented association with GST-K-bZIP, thus demonstrating a role for amino acids 182 to 218 in multimerization. We then explored the ability of the hybrid proteins to form multimers with full-length K-bZIP protein. His-K-bZIP showed a strong association with GST-K-bZIP, whereas His-Zta revealed negligible binding, setting the background level of the assay (2% of the binding of His-K-bZIP). All of the His-Z-K hybrids showed a degree of multimerization, with His-Z-K1 and His-K-6 reaching over 20% of the binding of His-K-bZIP and His-Z-K and with His-Z-K2 and His-Z-K3 clearly also forming multimers. This demonstrates that the region of K-bZIP between 190 and 237 contains a multimerization domain that can transfer its function onto another protein.

The proposed leucine zipper region of K-bZIP is predicted to have a high probability of folding as a coiled coil, according to the analysis shown in Fig. 1. We questioned whether this occurs by using two biophysical approaches. A synthetic peptide was generated that was equivalent to the region of Zta that we had previously shown to fold as a coiled coil in vitro (16). The propensity of the K-bZIP peptide to fold as an α-helix was assessed first using circular dichroism (CD) spectroscopy. Using this spectroscopic technique, α-helical peptides generate a CD spectrum with two characteristic minima, at 208 and 222 nm. The biophysical properties of the synthetic peptide were analyzed at two concentrations and two pH conditions, pH 7.0 and pH 3.7, where it displayed a greater solubility. All of the data sets generated similar results. A lack of concentration dependence is indicative of this peptide folding as a monomeric species in solution. Surprisingly, none of the data revealed α-helical minima at 222 nm and 208 nm (Fig. 4), suggesting that the peptide did not fold in an α-helical

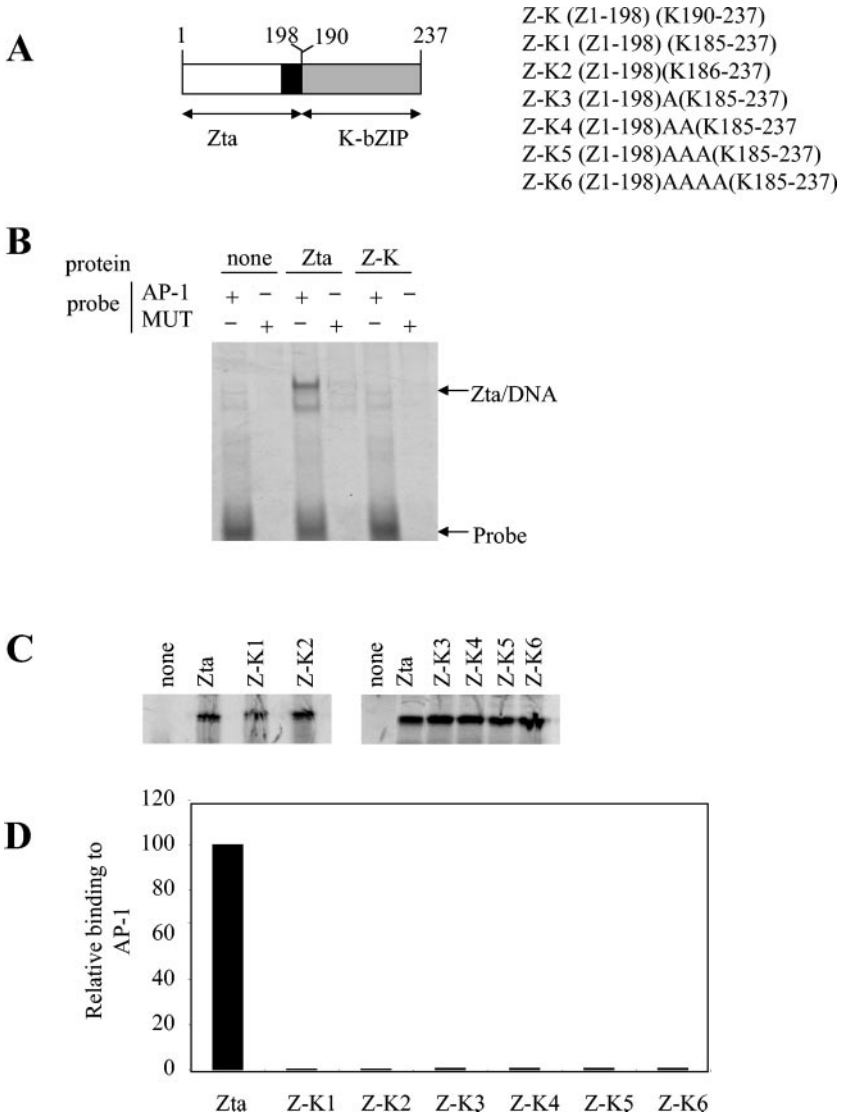

FIG. 2. K-bZIP domain swap proteins do not function as bZIPs. A. Vectors encoding hybrid proteins composed of the Zta transactivation and basic domains and the K-bZIP putative zipper domain were generated and are shown schematically here. The Zta basic region is shown in black. The region of K-bZIP from the proposed zipper to the carboxy terminus is shown as a grey box. B. Electrophoretic mobility shift assay reactions were undertaken with equal quantities of the indicated proteins and probes (as described in reference 15). Following electrophoresis, the locations of the probe and DNA complexes were visualized using phosphorimaging. C. Products from the indicated translation reactions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and quantitated by phosphorimaging. The migration of protein molecular weight markers (in kDa) is indicated on the left. D. Following the electrophoretic mobility shift assay analysis with equal amounts of protein, the specific binding of His-Zta and each hybrid protein to DNA is shown.

conformation. Indeed, a single trough was observed at 218 nm, which is the characteristic signal generated by peptides folded as β-sheets (21, 22). Further analysis of the folding of the K-bZIP peptide was undertaken using attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) (Fig. 4). This analysis revealed a peak for amide I bonds at $1{,}637 \pm 4\ cm^{-1}$ (characteristic of β-sheet structure). The second derivative of the spectrum of the amide I peaks revealed four components, which all correspond to either β-sheet or β-turn structure (10).

Given the functional homology and sequence homology between K-bZIP and Zta, it is tempting to speculate that they contain a common structural element that acts in a similar manner within each protein to achieve their common functions. The obvious candidate for this is the bZIP domain that has been so well characterized for Zta and widely proposed to exist in K-bZIP. Evidence in favor of a bZIP structure for K-bZIP is summarized below.

The initial publications describing the transcript encoding K-bZIP and the primary structure of the protein identified and highlighted the presence of a heptad repeat of leucine residues within the carboxy-terminal half of the protein and termed it a leucine zipper (14, 26, 40, 54). Furthermore, K-bZIP folds as a multimer and the carboxy-terminal half of the protein is required for multimerization (14) (26). Analysis of the primary structure reveals two regions containing clusters of basic amino

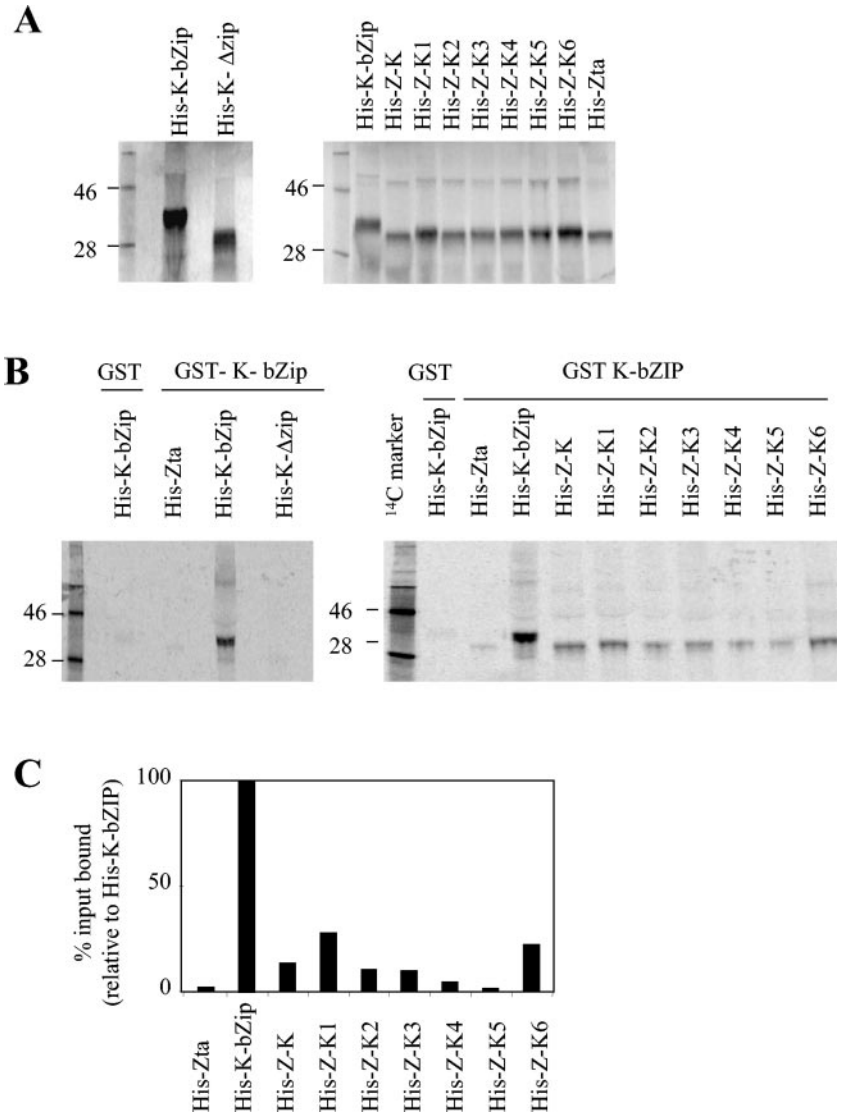

FIG. 3. The formation of multimers of K-bZIP requires amino acids within the proposed leucine zipper region. A. [^{35}S]methionine-labeled His-K-bZIP, His-K-ΔZIP, and the Z-K hybrid series were generated in reticulocyte lysate. Products from the indicated translation reactions were analyzed by SDS-PAGE and quantitated by phosphorimaging. The migration of protein molecular weight markers (in kDa) is indicated on the left of each gel. B. In vitro association assays with GST and GST-K-bZIP agarose beads were undertaken with equal quantities of the indicated proteins. C. The products from the indicated translation reactions were analyzed by SDS-PAGE and quantitated by phosphorimaging.

acid residues, but these do not have direct homology with the motif found in the basic region of bZIP proteins. Indeed, we did not observe any significant interaction between K-bZIP and an AP1 site in in vitro DNA-binding assays. However, it has recently been shown that K-bZIP expressed in vivo associates with DNA, specifically the lytic origin of replication (4, 48, 50). However, whether this occurs by a direct or indirect association and whether it involves the proposed bZIP region remain to be firmly established.

Recently, AuCoin et al. elegantly demonstrated that K-bZIP is required for replication through the lytic origin of the KSHV genome (4) and, furthermore, that this function requires contributions from both the amino- and carboxy-terminal halves of K-bZIP. During their further investigation of the contribution from the carboxy-terminal half of the protein, they generated a series of mutations in the heptad of leucine residues within the proposed leucine zipper region by replacing between one and four leucines in each mutant. Interestingly, two of the resulting mutants retained the ability to replicate through the lytic origin of replication and two lost this function. This study clearly demonstrates that K-bZIP is required for replication through the KSHV lytic origin of replication and, furthermore, it shows a contribution from some of the leucine residues within the proposed zipper to that function. However, no experiments involving multimerization or other biophysical properties were undertaken, and this set of data could be used equally well to support or to detract from a bZIP model for the K-bZIP protein.

Regarding multimerization, we demonstrate that amino acids 190 to 237 of K-bZIP contain a transferable multimeriza-

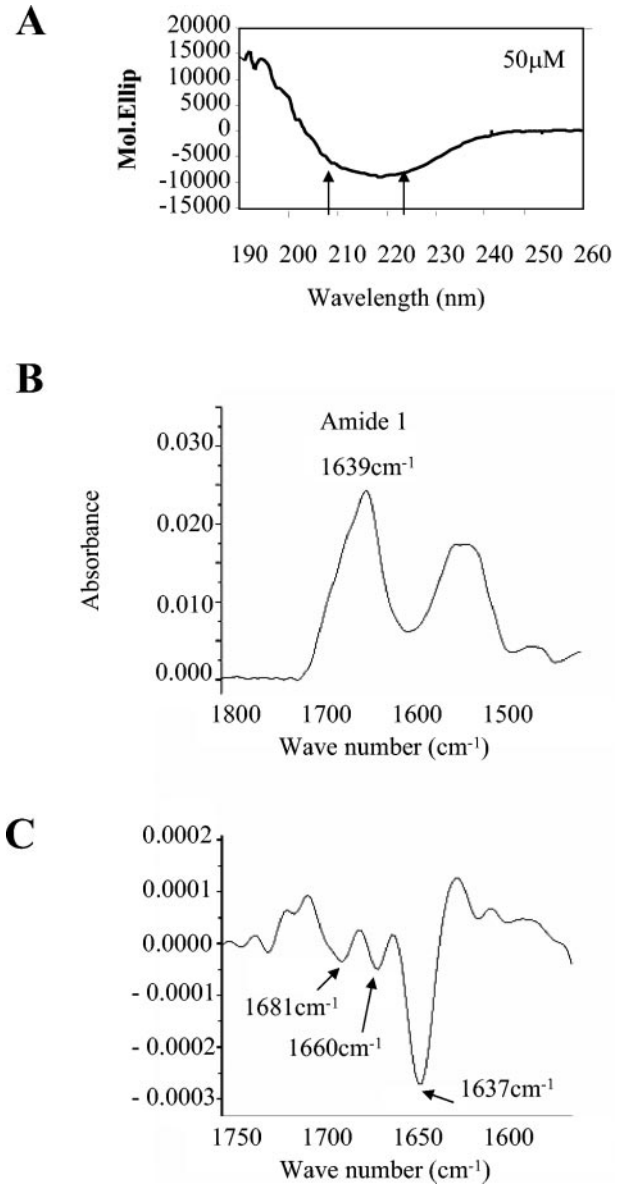

FIG. 4. The proposed leucine zipper region of K-bZIP does not fold as a coiled coil in vitro. A synthetic peptide corresponding to amino acids 182 to 218 of K-bZIP was synthesized and analyzed by circular dichroism spectroscopy as described in references 15 and 49. A. The normalized spectrum of a 50 μM peptide solution at pH 3.7 is shown. The arrows indicate the two minima, which are characteristic of α-helical secondary structure. The observed minimum, 218 nm, is characteristic of a β-sheet. B. The FTIR-ATR spectrum of peptide is shown. The position of the amide I peak is indicated. C. The second derivative spectrum of the amide I peak revealed three distinct contributions at the indicated wavelengths. The characteristic structure responsible for generating a peak at that wavelength is shown.

tion domain. Although additional contributions from amino acids out of this region cannot be excluded, these amino acids clearly contain sufficient inherent information to fold into a stable multimeric structure. K-bZIP therefore appears to conform to the bZIP model, in terms of the ability to multimerize using the proposed bZIP region; however, the similarity ends there. The proposed zipper region of K-bZIP is not able to confer DNA-binding ability onto the basic region of Zta, despite the ability of several of the hybrid proteins to fold as multimers.

Finally, our biophysical investigations not only fail to support the leucine zipper model but demonstrate that this region preferentially folds into a different secondary structure. The synthetic K-bZIP peptide corresponding to the proposed leucine zipper of K-bZIP appears to fold as a β-sheet rather than as an α-helix in two distinct in vitro assays; CD spectroscopy and FTIR-ATR (Fig. 4). It is worth noting that many sequences that were predicted to fold as α-helices have been shown, using biophysical measurements, to actually fold as β-sheets; these have been termed false zippers (18).

Although β-sheets can form multimeric interfaces in vitro (9, 33), we observed no concentration dependence on the strength of the β-sheet signal observed for the CD spectra, which suggests that the peptide contains insufficient information to fold as multimers. Therefore, despite the clear involvement of the region containing the β-sheet to the multimerization of K-bZIP, it remains to be determined whether multimerization requires a β-sheet interface or whether the β-sheet stabilizes the formation of a different multimerization interface employing amino acids that lie outside residues 182 to 218.

The balance of evidence presented here suggests that the proposed bZIP region within K-bZIP is unlikely to function as a bZIP motif, unlike that of Zta. So, although Zta and K-bZIP hold many functions in common, we conclude that it is unlikely that they exploit similar molecular mechanisms to effect those functions.

Henri Gruffat (Lyon) kindly provided a vector encoding K-bZIP, George Papadakis helped to generate the His-tagged version of K-bZIP, Matthew Hicks generated the His-tagged version of Zta, and Derek Woolfson (University of Sussex) advised on biophysical analyses.

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