FOR THE RECORD

The role of helix stabilizing residues in GCN4 basic region folding and DNA binding

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Abstract

Basic region leucine zipper (bZip) proteins contain a bipartite DNA-binding motif consisting of a coiled-coil leucine zipper dimerization domain and a highly charged basic region that directly contacts DNA. The basic region is largely unfolded in the absence of DNA, but adopts a helical conformation upon DNA binding. Although a coil $\rightarrow$ helix transition is entropically unfavorable, this conformational change positions the DNA-binding residues appropriately for sequence-specific interactions with DNA. The N-terminal residues of the GCN4 DNA-binding domain, DPAAL, make no DNA contacts and are not part of the conserved basic region, but are nonetheless important for DNA binding. Asp and Pro are often found at the N-termini of α -helices, and such N-capping motifs can stabilize α -helical structure. In the present study, we investigate whether these two residues serve to stabilize a helical conformation in the GCN4 basic region, lowering the energetic cost for DNA binding. Our results suggest that the presence of these residues contributes significantly to helical structure and to the DNA-binding ability of the basic region in the absence of the leucine zipper. Similar helix-capping motifs are found in approximately half of all bZip domains, and the implications of these findings for in vivo protein function are discussed.

Keywords: Basic region; leucine zipper; N-cap; DNA binding; conformational change

Basic region leucine zipper (bZip) proteins contain a bipartite DNA-binding motif consisting of a coiled-coil leucine zipper dimerization domain and a highly charged basic region that directly contacts DNA (for reviews, see Hu and Saver 1992; Hurst 1995). In the presence of DNA, the bZip domain forms a dimer of uninterrupted α -helices (Ellenberger et al. 1992; König and Richmond 1993; Keller et al.

1995). The leucine zipper region is both necessary and sufficient for dimerization (Gentz et al. 1989; Landschulz et al. 1989; Ransone et al. 1990), forming a stable, two-stranded, parallel coiled coil (O'Shea et al. 1989a, 1989b, 1991). The basic region, on the other hand, is largely unstructured in the absence of DNA, but adopts a helical structure upon DNA binding (O'Neil et al. 1990; Patel et al. 1990; Talanian et al. 1990; Weiss 1990; Weiss et al. 1990; O'Neil et al. 1991; Saudek et al. 1991; Talanian et al. 1992; Cuenoud and Schepartz 1993; Richards et al. 1996).

Although the coil $\rightarrow$ helix transition observed in bZip proteins upon DNA binding is entropically unfavorable, this conformational change positions the DNA-binding residues appropriately for sequence-specific interactions with DNA. However, the requirement for a structural change can lead to a reduction in specificity if there are nonspecific modes of binding that do not require the same folding transition (Johnson et al. 1994). Indeed, Record et al. have observed that extended, 17-residue tetracationic peptides bind non-

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Abbreviations: bZip, basic region leucine zipper; b-HLH-ZIP, basic/helix-loop-helix/leucine zipper; CD, circular dichroism; $[\theta]$, molar ellipticity; $[\theta]_{222}$, molar ellipticity at 222 nm; CRE, cAMP-responsive element; bp, base pairs; K_d , apparent monomeric equilibrium dissociation constant; HPLC, high-performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; PBS, phosphate-buffered saline; EDTA, ethylenediaminetetraacetic acid; DTT, DL-dithiothreitol; BSA, bovine serum albumin.

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specifically to DNA with lower affinity than do compact, five-residue peptides with the same overall charge. This result suggests an energetic penalty for helix formation or other conformational changes required for the longer peptides to bind to DNA (Padmanabhan et al. 1997). Nonetheless, a number of proteins in a variety of structural classes undergo folding transitions upon protein-DNA complexation (Spolar and Record 1994).

Recent studies of bZip variants suggest that a coil $\rightarrow$ helix conformational change is not necessary for high-affinity, specific binding (Takemoto and Fisher 1995; Lajmi et al. 2000). Shin and coworkers have shown that an Ala-rich variant of the GCN4 basic region binds to DNA with only a sevenfold decrease in affinity relative to wild-type (Lajmi et al. 2000). Importantly, high-affinity specific DNA binding is maintained despite the loss of favorable Coulombic interactions. As expected, this variant is substantially more helical than wild-type in the absence of DNA. Similarly, multiple alanine substitutions in the basic domain of b-HLH-ZIP proteins enhance DNA binding affinity (Takemoto et al. 1995). Taken together, these results suggest that the energetic penalty associated with DNA binding by a flexible domain can be avoided, at least in part, by stabilizing nascent or partial helix formation in the absence of DNA.

The C-terminal 56 residues of the yeast transcriptional activator GCN4 (residues 226–281) are both necessary and sufficient for DNA binding (Hope and Struhl 1986; Oakley and Dervan 1990). This domain contains both the leucine zipper dimerization domain and the basic region. The N-terminal residues, DPAAL, make no DNA contacts and are not part of the conserved basic region, but are nonetheless important for DNA binding (Oakley and Dewan 1990). Asp and Pro are often found at the N-termini of α -helices, and they are followed by the excellent helix-forming residues Ala and Leu (Richardson and Richardson 1988). We therefore considered the possibility that residues 226–230 of GCN4 play a structural role, facilitating helix formation and reducing the entropic cost of the coil $\rightarrow$ helix transition that takes place upon DNA binding.

Statistical analysis of peptides and proteins has shown that there is a strong preference for certain amino acid residues at the N- and C-termini of α -helices (Richardson and Richardson 1988). Because the first four amide groups and the last four carbonyl groups necessarily lack the intrahelical $i \rightarrow i + 4$ hydrogen bonds that are characteristic of α -helices, there is a preference at these positions for residues that can accommodate the unique hydrogen bonding requirements of the backbone amide and carbonyl groups. Amide hydrogens at the helix N-terminus often pair with side-chain H-bond acceptors, and carbonyl oxygens at the C-terminus are satisfied primarily by hydrogen bonds with backbone amide groups from the adjacent loop or turn (Presta and Rose 1988).

Helix-capping motifs have been shown to stabilize α -helices in peptide models (Bruch et al. 1991; Chakrabartty et al. 1993; Forood et al. 1993; Harper and Rose 1993; Lyu et al. 1993; Yumoto et al. 1993; Seale et al. 1994; Zhou et al. 1994; Petukhov et al. 1996; Esposito et al. 1997; Reymond et al. 1997) and in intact proteins (Serrano and Fersht 1989; Bell et al. 1992; Thapar et al. 1996). In the present study, we investigate whether the Asp and Pro residues at the N-terminus of the GCN4 bZip domain serve to stabilize a helical conformation for the GCN4 basic region, lowering the energetic cost for DNA binding. In addition, we investigate the role of these residues on the affinity and specificity of GCN4-DNA interactions. The implications of our findings for in vivo protein function are discussed.

Results

To investigate the importance of putative helix-capping residues for the structure and function of the GCN4 basic region, the bZip peptides GCN4–56 and GCN4–54 were overexpressed and purified from *Escherichia coli*. GCN4–56 contains the 56 C-terminal residues (226–281) of GCN4, including both the leucine zipper dimerization domain and the basic region (Fig. 1a). GCN4–56 also contains a Met-Lys dipeptide at the N-terminus for efficient translation initiation of the recombinant protein fragment in *E. coli* (Looman et al. 1987). The resulting 58-residue peptide is the same GCN4 construct that was used for crystallization of the peptide complex with AP-1 DNA (Ellenberger et al. 1992) and for NMR studies of basic region dynamics in the absence of DNA (Bracken et al. 1999), with the exception of a single Met-to-Val substitution to avoid problems with Met oxidation. GCN4–54 differs only in the absence of two amino acid residues, Asp226 and Pro227, at the N-terminus of the bZip domain (Fig. 1a).

The effect of helix-capping residues on basic region structure

We used CD spectroscopy to determine whether the presence of the Asp-Pro dipeptide affects the degree of helicity in the GCN4 bZip domain in the absence of DNA. At 25°C, GCN4–56 is ~74% α -helical based on a mean residue ellipticity of $-28,100 \text{ deg cm}^2 \text{ dmol}^{-1}$ at 222 nm (Fig. 2a). This observation is consistent with a helical leucine zipper domain and basic region that is not well structured, as others have observed (O'Neil et al. 1990; Talanian et al. 1990; Weiss, 1990; Weiss et al. 1990; Saudek et al. 1991; Cuenoud and Schepartz 1993). GCN4–54 is slightly less helical (~69%) under the same conditions. This small difference in θ_{222} is reproducible. However, the CD signal of both proteins is dominated by the helical leucine zipper domain, making differences in the structure of the basic region dif-

BR29^{ox} at the same temperature (~41% helical; data not shown). Thus, our data suggest that the entropic penalty for helix formation in the presence or the absence of DNA is reduced by the putative helix-capping residues, Asp and Pro.

The CD difference spectra shown in Figure 2b were obtained by subtracting the CD spectrum of the CRE DNA solution from the CD spectrum of each peptide-DNA complex. The CD spectrum of the DNA duplex (data not shown) is consistent with B-form DNA, with a minimum at ~247 nm and a maximum at ~221 nm. We note that upon complexation, there is a small change in the difference spectra at ~247 nm, where the protein contributes little, if any, to the observed signal. It therefore appears that a small conformational change in the DNA accompanies protein binding, consistent with the observations of previous workers (Cuenoud et al. 1993; Podust et al. 2001). This DNA conformational change is not surprising, as the DNA fragment in the crystal structure of the GCN4-CRE complex is distorted from B-form by a 20° bend toward the leucine zipper (König and Richmond 1993).

The effect of helix-capping residues on DNA binding

To determine if the presence of helix-capping residues contributes to the DNA-binding affinity and specificity of the GCN4 bZip domain, we probed the DNA binding properties of both BR31^{ox} and BR29^{ox} using an electrophoretic mobility-shift assay. We monitored peptide-DNA complex formation in the absence (Fig. 3a) and presence (Fig. 3b)

Fig. 3. Equilibrium binding of BR29 and BR31 to CRE DNA in PBS buffer in the (A) absence and (B) presence of calf thymus DNA (100 μ M in base pairs). Peptide concentrations are 6 μ M (lanes 1 and 6), 1 μ M (lanes 2 and 7), 600 nM (lanes 3 and 8), and 100 nM (lanes 4 and 9). Lanes 5 and 10 contained 32 P-end-labeled DNA only.

of excess nonspecific DNA (calf thymus, 100 μ M in bp). Although the pseudosymmetric AP-1 sequence, 5'-TGA(C/G)TCA-3', is the optimal recognition site for GCN4 (Hill et al. 1986; Oliphant et al. 1989; Mavrothalassitis et al. 1990), high-affinity binding to the perfectly symmetric CRE site, 5'-ATGACGTCAT-3' has also been observed (Sellers et al. 1990). Interestingly, we were not able to detect DNA binding in the presence of AP-1 DNA with either BR31^{ox} or BR29^{ox} (data not shown). This observation is consistent with those of Talanian et al. (1992).

Both BR31^{ox} and BR29^{ox} form a stable complex with CRE DNA in the absence of excess nonspecific DNA. However, BR29^{ox} appears to bind to DNA with somewhat lower affinity than does BR31^{ox} (cf. lanes 4 and 9 of Fig. 3a). Unfortunately, the degree of DNA binding site saturation is low for both peptides, precluding precise measurements of apparent dissociation constants. It appears, however, that the binding site saturation is lower for BR29^{ox} than for BR31^{ox}, perhaps due to faster dissociation rates for the less stable protein-DNA complex.

Upon the addition of nonspecific competitor DNA (calf thymus, 100 μ M in bp) to the binding reactions, no complex formation is observed with BR29^{ox}, even at the highest peptide concentration (6 μ M) (Fig. 3b). Significantly, although BR31^{ox} appears to have slightly lower affinity for CRE DNA in the presence of competitor DNA than in its absence, the helix-cap-containing peptide still forms a detectable protein-DNA complex at a protein concentration of 100 nM. Thus, removal of the Asp-Pro dipeptide from the N-terminus of the basic region appears to reduce both the affinity and specificity of DNA binding.

Because helix-capping residues have a clear impact on DNA binding by disulfide-linked basic region peptides, we examined the effect of these residues in the context of the intact bZip domain. We measured the apparent K_d of each GCN4 peptide for AP-1 DNA in the presence of increasing amounts of calf thymus DNA. Calculated values for the apparent monomeric equilibrium dissociation constants are shown in Table 1. In the absence of nonspecific competitor DNA, GCN4-56 binds AP-1 DNA with an apparent dissociation constant of $(4 \pm 2) \times 10^{-8}$ M, in agreement with previously published values (Hope and Smith 1985; Weiss et al. 1990; Metallo and Schepartz 1994; Berger et al. 1996; Hollenbeck and Oakley 2000). GCN4-54 has comparable affinity for the AP-1 site ($K_{d(\text{apparent})} = (3 \pm 1) \times 10^{-8}$ M). Importantly, at concentrations of calf thymus DNA between 0 and 300 μ M bp, there was no detectable difference in the affinity of either protein for AP-1 DNA, suggesting that the helix cap does not affect DNA-binding affinity or specificity in the context of the dimeric bZip domain.

Discussion

In addition to defining the beginnings and ends of α -helices, helix-capping motifs play a role in stabilizing protein struc-

Table 1. Binding constants for GCN4-56 and GCN4-54 to AP-1 DNA in the presence of increasing amounts of competitor DNA as measured by an electrophoretic mobility-shift assay

[CT DNA] (μ M bp)	K_d (M) ^a	
	GCN4-56	GCN4-54
0	$4 \pm 2 \times 10^{-8}$	$3 \pm 1 \times 10^{-8}$
100	$1.1 \pm 0.2 \times 10^{-7}$	$1.2 \pm 0.3 \times 10^{-7}$
300	$2.8 \pm 0.7 \times 10^{-7}$	$1.8 \pm 0.4 \times 10^{-7}$

^a Values reported are the mean values $\pm$ the standard deviation in the values measured from three individual data sets.

ture. The importance of N-terminal capping has been demonstrated using synthetic model peptides (Chakrabartty et al. 1993; Harper and Rose 1993; Yumoto et al. 1993; Seale et al. 1994; Petukhov et al. 1996) and site-directed mutagenesis of helix termini in native proteins (Serrano and Fersht 1989; Bell et al. 1992; Thapar et al. 1996). Indeed, N-capping motifs can stabilize monomeric helices by up to 2 kcal mol⁻¹ relative to the unfolded random coil state (Lyu et al. 1992; Doig and Baldwin 1995; Petukhov et al. 1996).

In a statistical survey of α -helices in globular protein structures, Richardson and Richardson observed a preference for Asn, Asp, Ser, Thr, and Gly in the N-cap position and a strong preference for Pro in the N-cap + 1 (N1) position (Richardson and Richardson 1988). As an N-cap, Asp can interact favorably with the helix dipole (Wada 1976; Hol 1985) as well as form a hydrogen bond to one of the exposed backbone NH groups in the first turn of helix. In the N + 1 position, Pro is ideal because it lacks an exposed backbone NH group that would need to be satisfied by a side-chain H-bond acceptor (Presta and Rose 1988; Richardson and Richardson 1988). In the crystal structure of the GCN4-AP-1 DNA complex, the side chain of Asp226 in one monomer is indeed folded in a conformation suitable for H-bond formation to the exposed backbone amide of Ala228. However, the distance between the side chain carbonyl oxygen and the nitrogen of Ala228 is 4.31 Å, outside of H-bonding distance. In the crystal structure of the GCN4-CRE DNA complex, no electron density is visible for Asp226 suggesting multiple conformations for its side chain. Thus, it is not clear whether Asp226 forms an energetically significant H-bond with an unpaired amide nitrogen at the N-terminal end of the basic region helix.

Nonetheless, we have found that the residues Asp and Pro at the N-terminus of the GCN4 basic region do indeed increase the extent of helix formation in the absence of DNA (Fig. 2b). Importantly, upon DNA binding, there is little difference in the helical content of either peptide, suggesting that the two DNA-bound complexes are similar in structure. Thus, the presence of helix-capping residues at the N-terminus reduces the amount of free energy required to complete the coil $\rightarrow$ helix transition observed upon binding DNA.

Because these residues are important for GCN4 basic region structure, we searched for helix-capping motifs in other naturally occurring bZip proteins (see Materials and Methods). A search of the Swiss-Prot database identified 104 unique bZip sequences. Of these, 50 (~50%) have putative helix-capping residues near the N-terminus of the bZip domain. One representative sequence of each of the 13 families of cloned bZip proteins identified by Hurst (1995) is shown in Figure 4. Importantly, the presence or absence of helix-capping residues is strongly conserved within each family. Taken together, these data suggest that helix-stabilizing residues occur commonly at the N-terminus of the basic region and that these sequences are functionally significant in the bZip classes in which they are found.

Intriguingly, although the Asp-Pro dipeptide has a clear impact on DNA binding by disulfide-linked basic region peptides, the presence of these residues at the N-terminus of the basic region does not appear to affect DNA-binding affinity or specificity in the context of the full bZip domain (Table 1). One explanation for this observation is that a helical leucine zipper domain itself stabilizes nascent or partial helix formation in the basic region. Both GCN4-56 and GCN4-54 are considerably more helical in the absence of DNA than would be expected if only the leucine zipper region were folded. Because approximately 30 residues within the coiled coil are folded (O'Shea et al. 1991; Ellenberger et al. 1992), we would expect GCN4-56 to be 52% helical in the absence of DNA. The observed higher degree of helicity (~74%, Fig. 2a) can be explained by partial helix formation in the basic region. Indeed, NMR studies have demonstrated that the GCN4 basic region adopts fluctuating helical structure in the absence of DNA (Saudek et al. 1991; Bracken et al. 1999). Similarly, the GCN4 leucine zipper region appears to stabilize DNA-dependent α -helix formation in a hybrid protein in which the GCN4 basic region is grafted into a small helical domain from the cytoskeletal protein villin (Mori et al. 2002). If the leucine zipper stabilizes helix formation more effectively than does the Asp-Pro dipeptide, or if the effects of the N-cap and the leucine

Fos	SP EEEEKRRIRRRERNKMAAAKCRNRRRELDTL
Jun	ESQERIKAEKRMRNRIAAASKCRKRKLERIARL
C/EBP- α	VDKNS NE YRVRRERNNIIVRKSQDKAKQNRVET
HLF	DDLKDDKYWARRRKNMMAAKRSRDARRLKENQI
CREB	AEEAARKREVRMLKNREAARECRKKKEYVKCL
ATF2	NE DP DEKRRKFLERNRAAASRCRQKRKVWVQSL
ATF4	K GE KLDKKLKKMEQNKTAATRYRQKKRAEQEAL
NF-E2	AQLALIRDIRRRGKNKVAAQNCRRKRKLENIVEL
c-Maf	EEVIRLKQKRRTLKNRGYAQSCRFRVQQRHVL
YAP1	QDL DP ETKQKRTAQNRAAQRAFRERKERKMKEL
TGA1	TSKPVEKVLRRILAQNRQAARKSRLRKKAYVQQL
GCN4	VPES SD PAALKRARNTAARRSRARKLQRMKQL
XBP1	HL SP EKKALRRKLKNRVAAQTARDKKARMSEL

Fig. 4. Amino acid sequences of naturally occurring bZip basic regions. Putative helix-capping residues are shown in bold and underlined.

zipper are not additive, little or no change in the DNA binding properties of the dimeric bZip domain would be expected.

Significantly, the relatively high (μM) dimerization constants of bZip proteins (O'Shea et al. 1989a; O'Neil et al. 1991; Kohler et al. 1999) and the short lifetime of the GCN4 dimer (Weiss et al. 1990) suggest that GCN4 exists in vivo primarily as unfolded monomers. Indeed, several groups have observed that bZip peptides can bind to DNA sequentially as monomers that subsequently dimerize on the DNA (Metallo and Schepartz 1997; Berger et al. 1998; Kohler et al. 1999). In this case, the coiled-coil domain will not affect helix formation in the basic region as GCN4 leucine zipper monomers are primarily unfolded in solution (Zitzewitz et al. 1995). The impact of helix-stabilizing residues at the N-terminus of the basic region is therefore expected to be maximal in the case of monomer-DNA binding. Schepartz et al. have demonstrated that excess nonspecific DNA slows the dimer binding pathway, but not the sequential monomer binding pathway, suggesting that the monomer pathway may allow bZip proteins to find their target sites more quickly (Kohler et al. 1999). Thus, in vivo, in the presence of a large excess of nonspecific DNA, the basic region helix-capping motif is likely to contribute significantly to DNA-binding affinity and specificity.

Materials and methods

Peptide production and purification

Construction of the plasmid, pGCN4-56, encoding the basic region and leucine zipper of GCN4 (residues 226–281), was described previously (Hollenbeck and Oakley 2000). pGCN4-54, encoding residues 228–281 of GCN4, was constructed by mutagenesis using QuikChange Site-Directed Mutagenesis (Stratagene). The primary sequence of each construct was confirmed by DNA sequencing (Sanger and Coulson 1977). GCN4-54 and GCN4-56 were overexpressed in *E. coli* strain BL21(DE3) pLysS using the T7 expression system (Studier et al. 1990) and purified from cell lysates as described previously (Hollenbeck and Oakley 2000).

The peptides BR29 and BR31 were synthesized by solid-phase methods and purified by reverse-phase HPLC with a Vydac C₁₈ column and a linear water/acetonitrile gradient containing 0.1% (v/v) trifluoroacetic acid as described previously (McClain et al. 2001). All peptides were acetylated at the N-terminus and amidated at the C-terminus. Peptide concentrations were determined by UV absorbance in 6 M guanidinium hydrochloride and 0.02 M phosphate (pH 6.5) assuming an extinction coefficient of 5690 M⁻¹ cm⁻¹ for Trp at 280 nm (Edelholz 1967). The molecular mass of each purified peptide was confirmed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry. Covalently constrained dimers were produced through air oxidation (phosphate buffer, pH 8–9), and purified by reverse-phase HPLC as described previously (O'Shea et al. 1989a).

Electrophoretic mobility shift assays

Oligonucleotides (Genosys) containing the DNA binding sites AP-1 and CRE (Fig. 1c) were purified by preparative denaturing

(19% polyacrylamide, 19:1 acrylamide:bisacrylamide) gel electrophoresis and 5'-end-labeled by standard procedures (Sambrook et al. 1989). Radiolabeled oligonucleotides were annealed to their complement by heating an equimolar mixture of the two single-strand DNAs to 90°C for 2 min and then cooling slowly to room temperature.

Protein-DNA complex formation was monitored in PBS binding buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM sodium phosphate, 1.4 mM potassium phosphate, 1 mM EDTA, 1 mM DTT, 0.1% Igepal CA-630, and 0.4 mg/mL BSA [pH 7.4]) (Metallo and Schepartz 1994; Hollenbeck and Oakley 2000) supplemented with calf thymus DNA (Hollenbeck and Oakley 2000). Apparent equilibrium dissociation constants were determined for the GCN4-56-AP-1 DNA and GCN4-54-AP-1 DNA complexes as described previously (Hollenbeck and Oakley 2000).

CD spectroscopy

CD spectra were acquired with a Jasco J-715 spectropolarimeter. Samples were prepared in 50 mM KCl and 12.5 mM potassium phosphate (pH 7.0). The wavelength dependence of $[\theta]$ was monitored at 25°C by five scans acquired in 1-nm increments with a sampling time of 4 sec. The helix content was determined by the method of Chen et al. (1974). We find from repeated measurements that the error in $[\theta]_{222}$ for protein samples prepared from the same stock solution is approximately 1%, and we estimate that the error in the concentration determination of the protein stock solutions is approximately 5%. The CD spectrum shown for each peptide-DNA complex was obtained by subtracting the CD spectrum of the DNA solution from the CD spectrum of the complex. The spectrum of the CRE DNA duplex is consistent with B-form DNA, with a minimum at ca. 247 nm and a maximum at ca. 221 nm.

Statistical survey of bZip protein sequences

A search of the Swiss-Prot database using the identifier "bZip" yielded 176 sequences. These sequences were aligned with the GCN4 basic region using ClustalW (European Bioinformatics Institute, <http://www.ebi.ac.uk/clustalw/>). Three sequence fragments were eliminated as were 18 sequences bearing little or no homology to the consensus sequence NXX(A)(A)XX(C/S)R (Hurst 1995). The unique sequences (104/155) were surveyed for the presence of helix-capping residues at the N-terminus of the basic region.

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