Zinc-Finger Motifs

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Specific DNA Recognition by a Synthetic, Monomeric Cys_2His_2 Zinc-Finger Peptide Conjugated to a Minor-Groove Binder**

*Olalla Vázquez, M. Eugenio Vázquez, Juan B. Blanco, Luis Castedo, and José L. Mascareñas**

Dedicated to Professor Paul A. Wender on the occasion of his 60th birthday

The control of eukaryotic gene transcription is dependent on the interaction of certain proteins called transcription factors (TFs) with specific DNA sequences.[1] It is known that aberrant recognition properties in some of these proteins are at the origin of numerous diseases[2] and, therefore, there is growing interest in understanding the molecular basis of their supramolecular behavior as well as in developing non-natural DNA-binding mimetics. In general, high affinity and specific DNA recognition by such TFs involves the cooperative action of several domains, whereas monomeric modules are usually unable to form stable DNA complexes.[1,3] This requirement makes it quite challenging to develop minimized synthetic versions of natural TFs that show DNA-binding properties that are comparable with those of their natural counterparts.[4] Indeed, successful approaches in this area have been scarce and mostly restricted to the leucine-zipper (bZIP) family of TFs, a type of proteins that interacts with DNA by means of relatively simple dimeric coil motifs.[5] In this context, we recently demonstrated that it is possible to promote the DNA binding of a relatively small bZIP basic region (BR) upon appropriate cross-linking to a distamycin-like tripyrrole that binds in the minor groove adjacent to the BR target site.[6]

It would be highly desirable to design minimized versions of other types of TFs, and in this respect a particularly relevant target involves the well-known zinc finger proteins (ZFP), the largest group of eukaryotic DNA-binding proteins.[7] It is well established that the interaction of classical Cys_2His_2 ZFP with DNA requires the presence of several tandem zinc-finger repeats, with each finger unit binding to a three base-pair subsite on the DNA.[7,8] Although there have been numerous advances in the preparation of recombinant zinc-finger proteins with designed DNA sequence selectivities,[9] DNA binding by non-natural systems containing a single Cys_2His_2 zinc-finger unit has not yet been established. Herein, we demonstrate that appropriate tethering to a minor-groove-binding tripyrrole unit transforms a non-DNA-binding monomeric zinc-finger peptide into a high affinity and sequence-specific DNA binder.

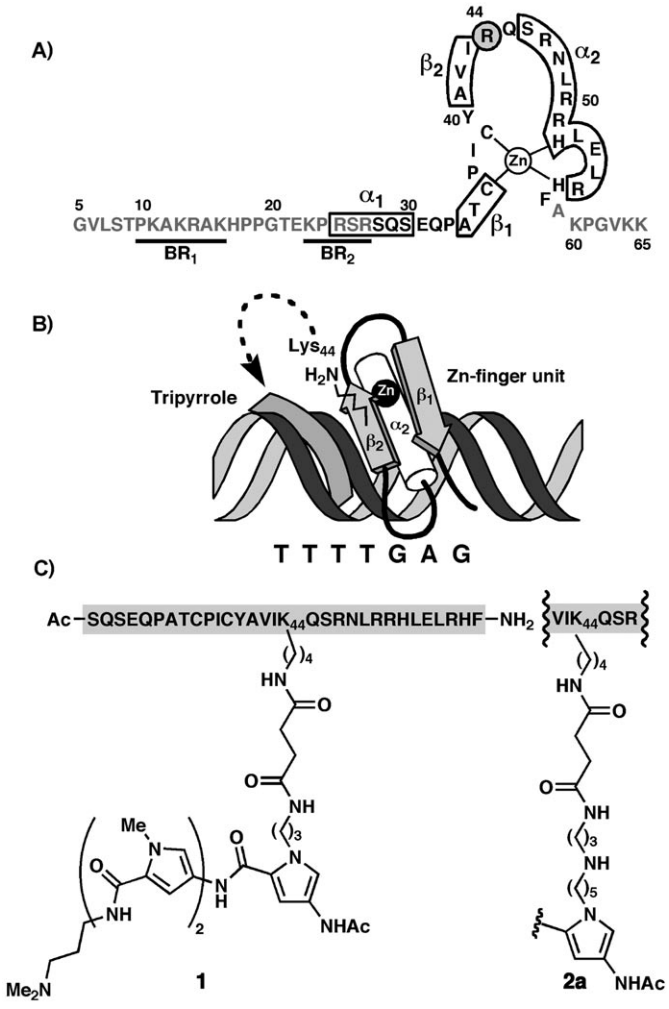

Figure 1. A) Sequence of the GAGA DBD showing the location of the two basic regions (BR1 and BR2) as well as the secondary-structure elements. Residue numbering corresponds to the intact GAGA-DBD structure as in reference [10]. Arg44 is highlighted in a gray circle. B) Outline of the hypothetical bipartite major–minor-groove recognition. C) Hybrids **1** and **2a** used in this study.

[*] O. Vázquez, Dr. M. E. Vázquez, Dr. J. B. Blanco, Prof. Dr. L. Castedo, Prof. Dr. J. L. Mascareñas
Dept. de Química Orgánica y Unidad Asociada al CSIC
Universidade de Santiago de Compostela
Facultade de Química, 15782 Santiago de Compostela (Spain)
Fax: (+34) 981-595-012
E-mail: qojoselm@usc.es

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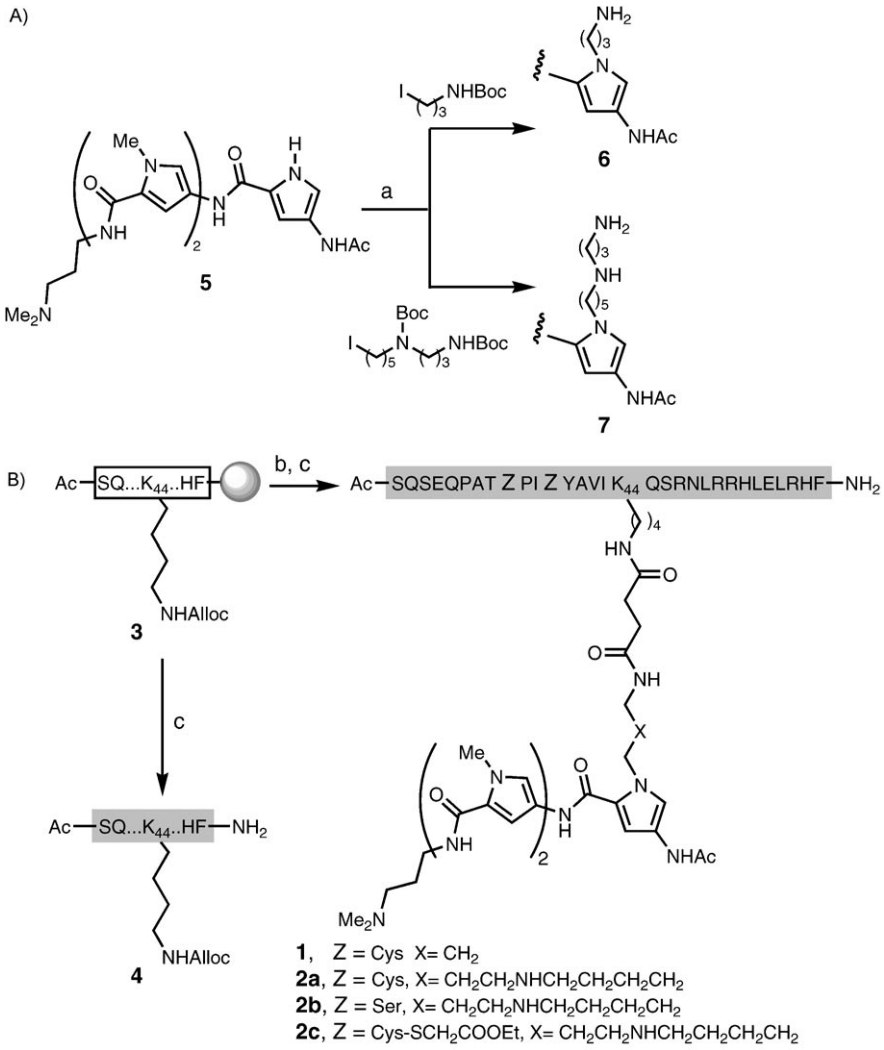
As the reference protein for design purposes, we chose the minimal DNA-binding domain (DBD) of the GAGA factor of *Drosophila melanogaster*, an atypical DNA-binding ZFP that binds its consensus DNA sequence (GAGAG) by means of a trivalent interaction involving a classical zinc-finger module and two basic regions, BR_1 and BR_2 (Figure 1 A).[10] Although DNA binding by this domain of 65 amino acids is mediated by a single zinc-finger module, it has been well established that both basic regions are strictly required for high-affinity DNA recognition as truncated versions of this GAGA DBD lacking either of the basic regions fail to bind DNA.[11]

On the basis of the reported solution structure of the DNA complexes of the GAGA DBD[10] and distamycin A,[12] we built a hypothetical model for the simultaneous interaction on adjacent DNA sites of a distamycin-like tripyrrole and a truncated zinc-finger module of the GAGA DBD that lacked both basic regions (amino acids 28–58, Figure 1 A). Qualitative modeling suggested that Arg44, which is exposed to the solvent face of the β hairpin unit of the ββα fold, could be an appropriate position for connection to the tripyrrole unit without perturbing any of the key DNA interactions of the zinc-finger module. We therefore decided to synthesize an Arg44→Lys44 mutant to obtain an appropriate handle for conjugation to the tripyrrole minor-groove-binding unit. To explore the viability of the approach, we synthesized two hybrids with different linkers; one with a relatively short linker but still long enough to span the distance between the binding units (**1**) and the other with a longer linker containing an amine group in the tethering chain (hybrid **2a**). Previous studies with bZIP conjugates suggest that the presence of such amine units in the tether might increase the DNA affinity of the system.[6b]



Scheme 1. Synthesis of GAGA-DBD–tripyrrole hybrids. A) Synthesis of the aminotripyrrole derivatives **6** and **7**: a) K_2CO_3, acetone, reflux 4 h, then TFA/CH_2Cl_2; B) Synthesis of peptide derivatives **1**, **2a**–**c**, and **4**: b) 1. $[Pd(PPh_3)_4]$ (1 equiv), morpholine (190 equiv), 2% H_2O/CH_2Cl_2 overnight;[13] 2. Succinic anhydride, DIEA, DMF; 3. HATU, DIEA, DMF, and the corresponding tripyrrole (**6** or **7**), 2 h. c) 95% TFA, ethanedithiol, H_2O, triisopropylsilane, 1.5 h, RT. Alloc = allyloxycarbonyl, Boc = *tert*-butoxycarbonyl, DIEA = *N*,*N*-diisopropylethylamine, DMF = *N*,*N*-dimethylformamide, HATU = *o*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, TFA = trifluoroacetic acid.

Hybrids **1** and **2a** were synthesized as outlined in Scheme 1. The key synthetic steps involved the selective derivatization of the resin-bound peptide **3** at Lys44 with succinic anhydride followed by coupling to the amino tripyrrole units **6** or **7**. As controls, we also prepared hybrids **2b** and **2c**, in which the coordinating Cys residues are either substituted by Ser or capped by reaction of **2a** with ethyl bromoacetate.

CD experiments showed that upon addition of $ZnCl_2$, hybrids **1** and **2a** display spectral changes in the 200–250-nm region that are consistent with the expected increase in secondary structure and similar to the changes observed with the truncated GAGA-DBD fragment **4** (see Figure 2 and the Supporting Information).[8f] These results indicate that the ability to form the expected Cys_2His_2–Zn^{2+} complex is retained after conjugation of the peptide to the tripyrrole unit. Addition of a double-stranded (ds) oligonucleotide containing the hybrid target sequence (*T*/GAGAG; *T* = tripyrrole DNA target sequence) to the complex of the Zn^{2+} ion and **2a** did not lead to significant changes in the far-UV region of the CD spectrum, but induced a positive ellipticity band at 330 nm, which is indicative of a DNA interaction of the tripyrrole unit.[14] Additionally, UV absorption measurements

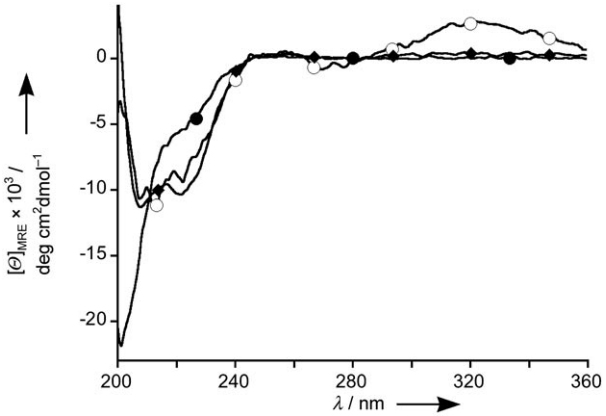


Figure 2. CD spectra of GAGA-DBD–tripyrrole hybrid **2a** (●), **2a** in the presence of $ZnCl_2$ (◆), and **2a** in the presence of $ZnCl_2$ and the target DNA sequence *T*/GAGAG (dsDNA contribution substracted; ○). Spectra were recorded in 18 mM Tris-HCl buffer solution (pH 7.0), 50 mM KCl, 0.5 mM EDTA, 0.55 mM DTT, and 1.8 mM $MgCl_2$ at 20 °C. Hybrid **1** underwent similar changes in the CD spectra (see the Supporting Information). *T*/GAGAG = 5′-d(GAGGA*TTTT*GAGAG TACGCT)-3′. DTT = 1,4-dithiothreitol, EDTA = ethylendiaminetetraacetate, Tris = tris(hydroxymethyl)aminomethane. MRE = mean residue ellipticity.

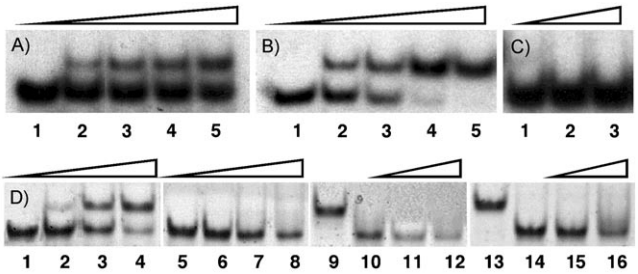


Figure 3. EMSA results showing the binding of peptides **1**, **2a**–**c**, and **4** to dsDNA *T*/GAGAG. Experiments (A–C) were carried out with 45 pM ^{32}P dsDNA and analyzed by autoradiography, whereas experiments shown in (D) were carried out with 50 nM dsDNA and analyzed by SyBr-gold staining. All experiments were carried out in the following buffer solution: 18 mM Tris-HCl (pH 7.0), 50 mM KCl, 0.5 mM EDTA, 0.55 mM DTT, 1.8 mM $MgCl_2$, 9% glycerol, 0.11 mg mL^{-1} BSA, and 4.2% NP-40. All experiments were performed in the presence 10 equivalents of $ZnCl_2$ except when indicated. A) Hybrid **1** in the presence of dsDNA, [**1**] = 0, 20, 400, 600, 1000 nM. B) Hybrid **2a** in the presence of dsDNA, [**2a**] = 0, 2, 5, 10, 15 nM. C) Control peptide **4** in the presence of dsDNA, [**4**] = 200, 600, 1000 nM. D) Binding of peptides: **2a**, **2b**, and **2c** to the dsDNA; lanes 21–4: hybrid **2a**, [**2a**] = 0, 50, 100, 200 nM; lanes 5–8: hybrid **2a** in the absence of Zn^{2+}, [**2a**] = 50, 200, 300 nM; lanes 9 and 13: controls with hybrid **2a**, [**2a**] = 400 nM; lanes 10–12: hybrid **2b**, [**2b**] = 100, 300, 500 nM; lanes 14–16: hybrid **2c**, [**2c**] = 200, 500, 1000 nM. BSA = bovine serum albumin, NP-40 = Nonidet P40 (octylphenolpoly(ethyleneglycol ether)).

of peptide **4**, by using previously described Co^{2+}-ion-displacement techniques, are consistent with the formation of tetrahedral zinc complexes[15] and further support tight Zn^{2+} ion binding (see the Supporting Information).

The DNA binding properties of the synthetic hybrid peptides were studied by gel electrophoresis mobility shift assays (EMSA) at 20 °C. Incubation of hybrids **1** and **2a** with the ^{32}P-dsDNA *T*/GAGAG promoted the appearance of new, slower-migrating bands in the gels. These bands must arise from the formation of complexes between the peptides and the DNA. Hybrid **1** binds to the target DNA with a modest affinity (Figure 3A), whereas hybrid **2a** showed very good DNA affinity with almost complete formation of the complex at concentrations over 15 nM (Figure 3B). As expected, the truncated GAGA–ZF module **4** was incapable of eliciting stable DNA complexes, even at peptide concentrations of 1 μM (Figure 3C). The higher DNA affinity of **2a** relative to **1** is consistent with the effect of the amino group present in the linker, but might also be related to the higher flexibility provided by the longer tether.[16] On the basis of these observations, we decided to focus our efforts on further characterizing the DNA binding properties of hybrid **2a**.

An EMSA titration of *T*/GAGAG in the presence of increasing amounts of hybrid **2a** allowed the calculation of a K_d value of approximately 2.5 nM at 20 °C (see the Supporting Information), which represents one of the best DNA affinities achieved to date with synthetic DNA binders.[4] Subsequent EMSA experiments were analyzed by fluorescence staining with SyBr gold, which required the use of larger amounts of dsDNA ($\approx$ 50 nM). On using these conditions, we were able to confirm that the DNA binding of hybrid **2a** is dependent on the presence of Zn^{2+} ions (Figure 3D); hence, in the presence of Zn^{2+} ions, half-maximal binding is observed with peptide concentrations near 100 nM, whereas complexation bands were not observed in its absence (Figure 3D, lanes 5–8). Control experiments with peptides **2b** and **2c**, which can not fold into the required ββα structure, confirmed that these peptides are unable to elicit the formation of stable DNA complexes (Figure 3D, lanes 10–12 and 14–16). These results further confirm the requirement of specific Zn^{2+} ion complexation for high-affinity DNA recognition.[17]

Finally, we studied the sequence selectivity of hybrid **2a** by running a series of gel-shift experiments in the presence of different oligonucleotides. As expected, mutation of the tripyrrole-binding DNA site (*T*^{m}/GAGAG) completely abolished complexation (Figure 4A and B). This result highlights the requirement for the accessory interactions provided by the minor-groove binder for the formation of stable DNA complexes and supports our proposed binding model. Selective DNA recognition by the ZF fragment of the hybrid was assessed by using dsDNA molecules containing specifically designed mutations in the GAGAG sequence. As shown in Figure 4C and D, incubation of **2a** with dsDNA molecules *T*/GAGct and *T*/cAGAG led to the formation of sharp complexation bands that are comparable with those observed with the initial target DNA. However, further mutations that remove one of the remaining G units promote a critical decrease in the DNA binding affinity (*T*/cAGct and *T*/cAtAG, Figure 4E and F). The slower migration of the band observed in the case of dsDNA *T*/cAGct is consistent with a relatively weak and nonspecific recognition mode in which the Cys_2His_2 peptide fragment does not interact specifically with the base edges in the DNA major groove.[18]

These results are consistent with the reported structure of the GAGA-DBD/DNA complex in which the Cys_2His_2 module makes specific contacts with a GAG triad, whereas

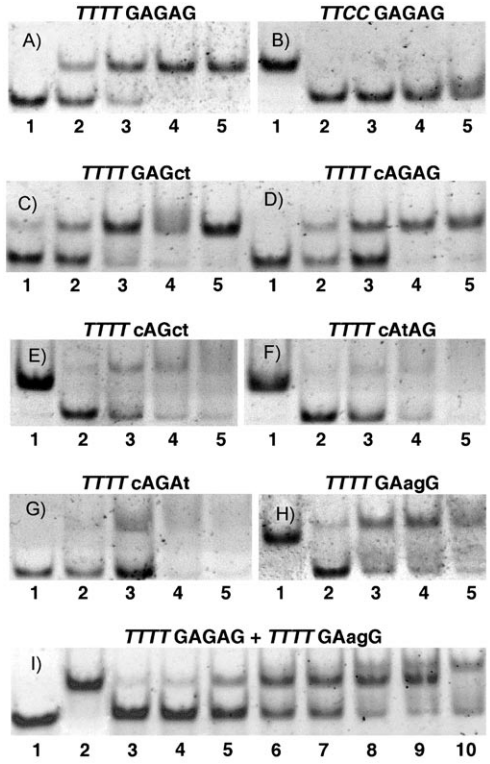


Figure 4. Binding of hybrid **2a** to different dsDNA molecules. The experiments were performed with 50 nM of the dsDNA molecules in the presence 10 equivalents of $ZnCl_2$. Lanes 2–5 in experiments (A–H) contain [**2a**] = 100, 200, 400, 1000 nM. Lane 1 in experiments B, E, F, and H are controls containing [**2a**] = 400 nM and dsDNA *T*/GAGAG. I) Competition experiment with *T*/GAGAG and *T*/GAagG in the presence of **2a**, lane 1: *T*/GAGAG and *T*/GAagG, [**2a**] = 0; lane 2 (control): *T*/GAGAG, [**2a**] = 400 nM; lanes 3–9, [**2a**] = 50, 100, 200, 300, 400, 600, 1000 nM; lane 10 (control): *T*/GAagG, [**2a**] = 400 nM. A) *T*/GAGAG = d(GAGGA *TTTT*GAGACTACGCT). B) T^m/GAGAG = d(GAGGC *TTCC*GAGAGTACGCT). C) *T*/GAGct = d(GAGGA *TTTT*GAGctTACGCT). D) *T*/cAGAG = d(GAGGA *TTTT*cAGAGTACGCT). E) *T*/cAGct = d(GAGGA *TTTT*cAGctTACGCT). F) *T*/cAtAG = d(GAGGA *TTTT*cAtAGTACGCT). G) *T*/cAGAt = d(GAGGA *TTTT*cAGAtTACGCT). H) *T*/GAagG = d(GAGGA *TTTT* GAagG TACGCT). Only one of the strands of the oligonucleotides is indicated; mutated residues, with respect to the reference sequence *T*/GAGAG, are shown in lower-case letters, the peptide DNA target site is underlined, and the tripyrrole DNA target sequence is in italics.

both basic regions BR_1 and BR_2 specifically bind to the other A and G units of the GAGAG string. High-affinity specific DNA binding of hybrid **2a** therefore requires the presence of a GAG sequence at an appropriate distance from the AT-rich site. In agreement with this hypothesis, high-affinity binding does not occur when such spacing is increased to 4 bp (see the Supporting Information) or with other dsDNA molecules that feature a broken GAG sequence (Figure 4G and H). Although the EMSA results in Figure 4H might suggest a relatively good affinity of **2a** for the dsDNA *T*/GAagG, a competition experiment with the target *T*/GAGAG confirmed that this latter dsDNA is strongly preferred (Figure 4I).

In summary, we have shown that linking a tripyrrole to a non-DNA-binding Cys_2His_2 fragment provides sequence-specific and high-affinity recognition of GAG sites near to 5′ A-rich sequences. The binding mode must involve bipartite major-groove and minor-groove interactions. To the best of our knowledge, hybrid **2a** represents the first example of a synthetic, monomeric Cys_2His_2 zinc-finger peptide capable of specific and tight DNA binding. Given the well-known possibilities for the design of custom zinc-finger modules to target desired DNA triplets,[7,8] a rational and specific targeting of a wide range of DNA sequences should be within reach. On the other hand, the findings described herein validate the general utility of our minor-groove-binder tethering strategy to recover the DNA-binding capabilities of truncated TF fragments and to discover new DNA-binding entities.

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[17] Hybrid **2a** interacts with the target DNA *T*/GAGAG only in the presence of the Zn^{2+} ion or the coordinatively similar Co^{2+} ion, but not in presence of other metals (see the Supporting Information).

[18] The disappearance of bands at higher peptide concentrations is probably due either to the formation of multiple species in low concentration or to the precipitation of the DNA. See, for instance: E. K. Liebler, U. Diederichsen, *Org. Lett.* **2004**, *6*, 2893 – 2896.

