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An artificial HIV enhancer-binding peptide is dimerized by the addition of a leucine zipper

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Abstract A 42 residue artificial peptide that binds to the HIV-1 enhancers has been described previously. The specificity of interaction of the peptide with its target DNA sequence has been demonstrated by a variety of techniques. Naturally occurring regulatory proteins frequently bind to DNA as dimers, thereby increasing the strength and specificity of the interaction, the dimer interface often being provided by a leucine zipper type coiled coil. As a suitable binding site for this kind of system is located to the 5′ end of the HIV enhancer region, it was decided to design and synthesize a fusion peptide that not only contained the DNA binding sequence of the original 42 residue peptide but also incorporated a leucine zipper based on that of the GCN4 transcriptional activator, that should, therefore, be capable of dimerizing. The resultant peptide, LZ66, has now been shown to be fully active in band shift and *in vitro* transcription assays and to exhibit about double the inhibitory activity of the parent 42 residue peptide. Preliminary CD measurements revealed that the peptide has a high α-helical content and that it adopts a stable conformation down to the low micromolar peptide concentration range. Sedimentation equilibrium studies confirmed that the principles involved in the design of the peptide are valid and that the peptide is indeed dimeric in solution.

Key words Artificially designed peptide · HIV-1 enhancer binding · Leucine zipper · Circular dichroism · Sedimentation equilibrium

Abbreviations HIV-1, human immunodeficiency virus type 1 · CD, circular dichroism · EDTA, ethylenediaminetraacetic acid

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Introduction

Sequence similarities between bacteriophage 434 operators and the HIV-1[1] enhancer region suggested that the 434 repressor-operator complex could be used as a model system for the design of a polypeptide with preferential HIV enhancer binding properties (Aggarwal et al. 1988; Anderson et al. 1987; Nabel and Baltimore 1987; Muesing et al. 1987; Hehlgans et al. 1993). A peptide, R42, that was designed on this basis contained (a) the recognition helix of the 434 repressor for the nucleotide sequence ACAAG, that occurs not only in several of the 434 operators, but also in positions –108 to –104 of the enhancer region of the HIV-1 long terminal repeat (HIV-1 LTR), and (b) an N- and C-terminal tandem repeat of the positively charged sequence that follows the recognition helix in the 434 repressor (Fig. 1a).

The specificity of HIV enhancer binding by R42 has been demonstrated by competitive band shift assays, by stepwise displacement of the p50 subunit of the NF-κB transcription factor from its two HIV enhancer binding sites and by DNAse I footprinting (Städler et al. 1995). As most proteins that interact with DNA achieve their high level of binding affinity and specificity by dimerization or tetramerization and as a potential basic region/leucine zipper-type binding site is located at the 5′ end of the HIV enhancer region (Talanian et al. 1990) (Fig. 1c), R42 was modified to include the leucine zipper moiety, GCN4-p1, of the yeast transcription activator GCN4 (Landschulz et al. 1988), with minor sequence modifications. Spatial considerations suggested that the zipper should be linked to the N-terminus rather than the C-terminus of the DNA-binding domain, contrary to the situation found in GCN4. The sequence of the resultant 66-residue polypeptide (LZ66) is shown in Fig. 1b. The proposed structure of the LZ66-HIV enhancer complex was confirmed by model building.

Here we describe the synthesis and preliminary biochemical and physiochemical examination of LZ66. One of the most important features to be determined when in-

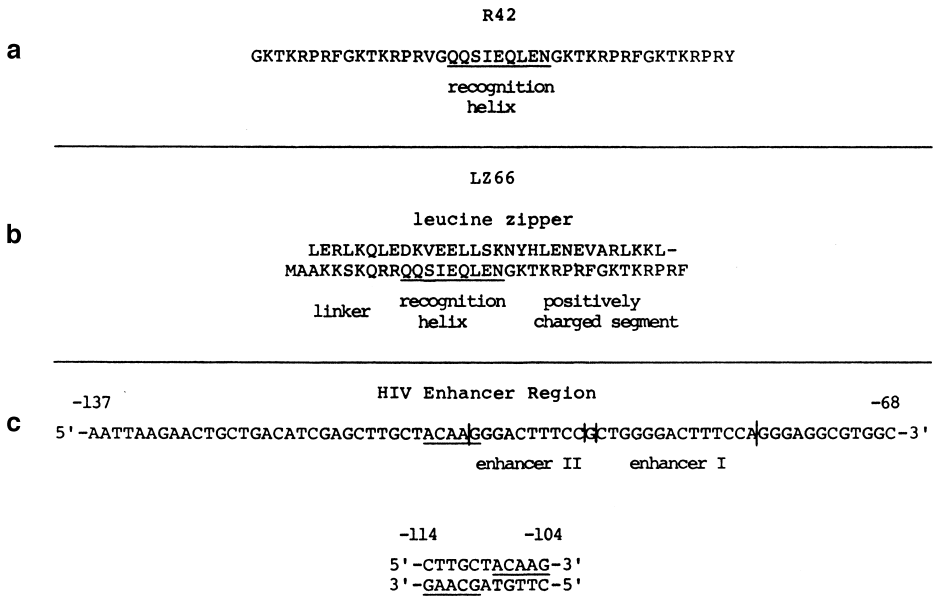

Fig. 1 a–c The sequences of polypeptides and DNA relevant to this study. **a** The original, 42 residue HIV enhancer-binding polypeptide derived from 434 repressor. **b** LZ66, a dimerizing version of the HIV enhancer-binding polypeptide shown in Fig. 1a. The components of LZ66 are leucine zipper (1–31), linker (32–41), recognition helix (42–50), and positively charged C-terminal tail (51–66). **c** 70-bp HIV DNA used as target DNA in the binding studies. *Vertical bars* indicate the locations of the two enhancer regions. The *inset* shows the proposed, almost symmetrical basic region/leucine zipper-type binding site of dimeric LZ66 at the 5′ end of the HIV enhancer region (*underlined*)

vestigating constructs of this type is the oligomerization stoichiometry and strength, and the method of choice to this end is equilibrium sedimentation in the analytical ultracentrifuge.

Experimental

Peptide synthesis and purification

LZ66 was synthesized by the solid phase method (Merrifield 1963, 1985) and was purified by standard procedures (gel filtration, ion exchange and high performance liquid chromatography). The purity of the product was demonstrated by amino acid analysis, capillary electrophoresis and ion spray mass spectrometry.

Band shift assays

^{32}P-labelled HIV-1 enhancer DNA (70 bp, 200 fmol) was incubated with LZ66 (10 pmol) in the presence of increasing concentrations of calf thymus DNA (as a competitor) in 20 µl of 20 mM Tris-HCl, 10 mM KCl, 2 mM $MgCl_2$, 0.1% nonylphenylpolyethylene glycol, 3.5% sucrose, pH 7.4, for 30 minutes at room temperature. 5–10 µl aliquots were subjected to gel electrophoresis on 8% polyacrylamide gels in Tris-borate buffer, pH 7.5. The gels were dried and analysed by phosphor imaging.

In vitro transcription

Published procedures were used for the identification and quantification of the transcripts obtained from OVEC-LTR, an HIV enhancer-containing plasmid, and from an

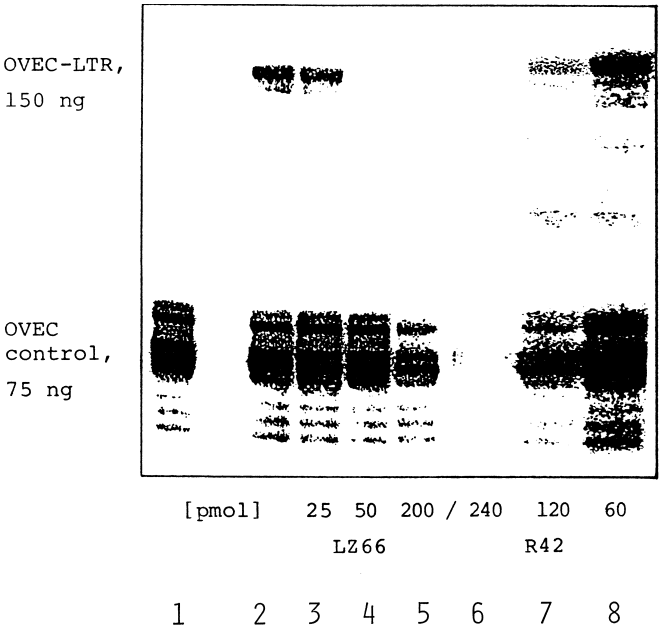

Fig. 2 Comparison of the effects of LZ66 and R42 on the *in vitro* transcription of the HIV enhancer-containing plasmid OVEC-LTR in HeLa cell nuclear extracts using polyacrylamide gel electrophoresis

OVEC control plasmid (SI nuclease mapping; Westin et al. 1987). Transcriptions (total volume 25 µl, inlcuding 2 µl HeLa cell nuclear extract) were run for 60 minutes at 30 °C. The nucleic acids were then extracted with phenol/methylene chloride (1 : 1). The DNA was digested by RNAse-free DNAse I and the plasmid-specific RNA was detected by gel electrophoresis in 7 M urea after hybridization with a complementary, ^{32}P-labelled 93 unit oligodeoxyribonucleotide and single-strand specific S1 nuclease digestion (Fig. 2).

Circular dichroism spectroscopy measurements

CD spectra were recorded on a Jasco J-715 spectropolarimeter in an approximately physiological buffer (50 mM potassium phosphate, 75 mM KCl, 10 mM NaCl, 15 mM $MgSO_4$, 1.7% glycerol, pH 7.4) at 25 °C. Cells of 0.01 and 0.1 cm pathlength were used.

Equilibrium sedimentation

Conventional equilibrium sedimentation experiments were performed with a Beckman XL-A analytical ultracentrifuge. Standard charcoal-filled epon double-sector centrepieces were used and the fluorocarbon FC43 was added to sample sectors to provide a false bottom. Typical sample loading volume was ≈120 µl. Run speeds and temperatures were varied and loading concentrations were in the range 20–180 µM. Experiments were run in either 0.02 M sodium phosphate, 0.08 M sodium chloride, pH 7.4 or in the approximately physiological buffer, 50 mM potassium phosphate, 75 mM KCl, 10 mM NaCl, 15 mM $MgSO_4$, 1.7% glycerol, pH 7.4. The partial specific volume was calculated in the usual way (Laue et al. 1992) and solvent density was either calculated or measured using a Paar DSA48 sound and density analyser. Partial specific volumes and densities were corrected, where necessary, for temperature. Equilibrium sedimentation data were analysed using software provided by the instrument manufacturer.

Results and discussion

Our original 42 residue HIV enhancer-binding polypeptide (Hehlgans et al. 1993) was derived from the DNA-binding domain of bacteriophage 434 repressor (Anderson et al. 1987; Aggarwal et al. 1988) which, as a dimer, specifically recognizes symmetrical ACAAG and ACAAT nucleotide sequences. Although the HIV enhancer binding specificity of R42 was quite remarkable (Städler et al. 1995), it needed to be increased, were the polypeptide to have appreciable HIV replication-inhibiting activity *in vivo*. One way of achieving this aim was to attempt to dimerize the peptide, assuming that a suitable, symmetrical binding site was present on the target DNA. As there was a (nearly) symmetrical, basic region/leucine zipper-type binding site at the 5′ end of the HIV enhancer region (Fig. 1c, underlined sequences), the potentially dimerizing fusion protein LZ66 (Fig. 1b) was constructed and synthesized.

Band shift assays

Band shift assay is commonly used to test the affinity and specificity of DNA binding by proteins. This assay showed that, even in the presence of a 300-fold excess of competitive calf thymus DNA, as stable complex of LZ66 and the 70 bp HIV enhancer-containing DNA (Fig. 1c) existed alongside unbound HIV DNA. Preliminary results from footprinting experiments revealed that LZ66 indeed bound to the symmetrical sequence motif adjacent to the 5′ terminus of the HIV enhancer region (Fig. 1c), indicating that the peptide might be dimeric.

In vitro transcription

The plasmids used in this study were based on pUC18. OVEC control (Westin et al. 1987) contained four Sp1 sites, the TATA box, and a truncated β-globin gene at the transcriptional start site. OVEC-LTR (G. Caderas, unpublished) contained the two enhancers and the three Sp1 sites of the HIV long terminal repeat (positions –109 to –45 of the HIV genome), the TATA box, and the β-globin gene as reporter gene.

OVEC-LTR and OVEC control were incubated with HeLa cell nuclear extracts. After incubation the mRNA was isolated, hybridized with a suitable labelled oligodeoxyribonucleotide, and treated with S1 nuclease. Gel electrophoresis of the hybridized, uncleaved mRNA fragments allowed relative quantitation of the transcription of the two plasmids. Figure 2 shows that the binding of the LZ66 dimer to OVEC-LTR was clearly more specific than its binding to OVEC control resulting in preferential inhibition of the transcription of the HIV enhancer-containing plasmid (Fig. 2, lanes 4 and 5). Figure 2 also reveals that LZ66 was about twice as active as R42 (Fig. 1a) in inhibiting the *in vitro* transcription of OVEC-LTR (Fig. 2, lanes 4 and 7).

The fact that dimerization of the original HIV enhancer-binding peptide (R42, Fig. 1a) had only a modest effect on the inhibitory activity in the *in vitro* transcription assay may have two main reasons. Firstly, the symmetry of the binding site at the 5′-end of the HIV enhancer region was not complete (Fig. 1c inset, underlined sequences); and secondly, the fit of the two DNA-binding regions of the LZ66 dimer and the target sites on the HIV long terminal repeat was probably not optimal. Work to improve the DNA binding specificity of the LZ66 construct is in progress.

Circular dichroism

The principles involved in the design of LZ66 would imply that about 50 out of the 66 residues, or 75%, should be in an α-helical conformation. Qualitatively, the major contributions to the CD spectrum are made by residues that adopt this structure and by residues in an aperiodic conformation (Fig. 3). The α-helical content is, however, lower than expected, at 60–70%. This can be explained by two distinct mechanisms. In constructs of the LZ66 type, the general assumption is that the different structural regions (in this case, the DNA-binding moiety on one hand and the zipper region, linker and recognition helix on the other) retain their structural integrity when covalently linked. This may not entirely hold, and there could be local conformational changes due to the spatial proximity of the different

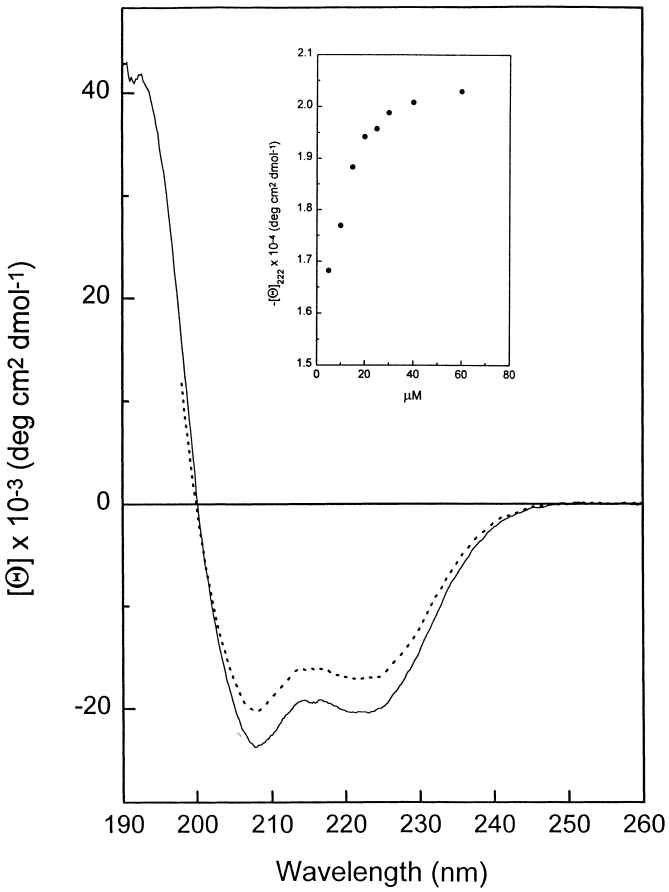

Fig. 3 The far ultraviolet CD spectrum of LZ66 in 50 mM potassium phosphate, 75 mM KCl, 10 mM NaCl, 15 mM $MgSO_4$, 1.7% glycerol, pH 7.4. Peptide concentrations were 60 μM (——) and 5 μM (·····). The *inset* shows the dependence of mean residue ellipticity on concentration

structural elements. Secondly, the dissociation of a self-associating system in which the structural content of the monomer is lower than that of the associated form will lead to a loss of intensity in a conformationally sensitive measurement, such as CD. In the leucine zipper case, the monomers of the self-associating system are unstructured, the formation of α-helix being concomitant with oligomerization. Figure 3 shows the CD spectrum of LZ66 at both 60 and 5 μM and there was a small, but measurable, reduction in intensity as the polypeptide was diluted, the inset to Fig. 3 showing this in more detail. No changes were seen above a peptide concentration of ≈30 μM, suggesting that the dissociation constant for the system is in this range.

While it is impossible to distinguish between these two possibilities, and both may, in fact, play a role, it is interesting to note that the GCN4-pl leucine zipper, on which the LZ66 coiled coil is based, also dissociated at low concentration (O'Shea et al. 1989; Thomas et al. 1995).

Equilibrium sedimentation

The apparent molar mass, $M_{w,\,app}$ of LZ66 has been determined over a range of peptide concentrations, temperatures, and operating speeds as well as in two different buffer systems. A value, $\bar{v}_{20,\,c} = 0.743$ cm^3 g^{-1}, was calculated for the partial specific volume, and the monomer molar mass obtained from the amino acid composition was 7978 Da. Whole-cell averages were calculated using standard fitting procedures, assuming that a single, ideal species was present. The results are presented in Tables 1 and 2.

Table 1 $M_{w,\,app}$ as a function of concentration, temperature and run speed in 0.02 M potassium phosphate, 0.08 M potassium chloride, pH 7.4

Concentration [μM]	Temperature [°C]	Speed [rpm]	$M_{w,\,app}$
180	20	18,000	15,750±260
		25,000	14,400±120
		32,000	12,870±115
	10	18,000	12,750±135
		25,000	12,740±110
		32,000	11,500±115
36	20	18,000	14,960±150
		25,000	14,263±200
		32,000	13,440±230
	10	18,000	13,440±130
		25,000	13,350±160
		32,000	12,710±250
18	20	18,000	14,110±160
		25,000	14,320±140
		32,000	14,200±170
	10	18,000	13,870±130
		25,000	13,920±125
		32,000	13,860±200

Table 2 $M_{w,\,app}$ as a function of concentration and run speed in 50 mM potassium phosphate, 75 mM KCl, 10 mM NaCl, 15 mM $MgSO_4$, 1.7% glycerol, pH 7.4 at 10 °C

Concentration [μM]	Temperature [°C]	Speed [rpm]	$M_{w,\,app}$
41	10	20,000	16,140±100
		26,000	16,046±130
		32,000	15,740±410
21		20,000	15,610±160
		26,000	15,810±195
		32,000	15,490±320

In general, the quality of the fits to the experimental data decreased with increasing rotor speed in a way that was typical of a self-associating system. This can be seen in the data shown in Table 1 (potassium phosphate/potassium chloride buffer), the whole-cell average mass decreasing as the rotor speed increased. There was also evidence of slight sample degradation in this solvent system, the runs at 10 °C being made on the same samples that had previously been run at 20 °C in each case. This may reflect either slow, non-specific sample aggregation or minor microbiological contamination of the samples. The data at 10 °C in approximately physiological buffer (Table 2) were

more self-consistent and there was little evidence of a dependence of $M_{w,\,app}$ on concentration in the range studied. The apparent mass measured under these conditions indicated that LZ66 existed as dimer in solution down to at least a peptide concentration of 20 μM.

These results indicate that the position of the equilibrium between monomer and dimer in LZ66 greatly favours the dimeric form in this concentration range. Comparison of the present sedimentation equilibrium data with that of the parent GCN4-p1 peptide (Thomas et al. 1995) suggests that the LZ66 dimer is the more stable of the two.

Conclusion

LZ66 has been shown to posses HIV enhancer binding specificity and the sedimentation equilibrium analysis clearly shows that the design principle of specifically creating a dimeric species has been successful. The CD data indicate that the dimer is stable down to the low micromolar concentration range and there is some, although limited, evidence that the dissociation process is detectable by analytical ultracentrifugation at the lowest concentrations studied. In contrast, under approximate physiological conditions, the peptide remains an intact dimer down to at least ≈30 μM.

The ability to engineer specific oligomerization sites without affecting the folding, and hence function, of biologically active peptides and proteins is of considerable importance. In this case the activity of LZ66 was double that of its precursor 42 residue peptide that lacked the leucine zipper region. The overall thermodynamic stability of constructs of this type is also of biological relevance. In some cases it may be advantageous that the molecule remains highly associated and tightly bound to its target, whereas in other cases, where "turnover" is required, the ability to modulate the strength of the self-association process, and hence that of the interaction with the target, by informed modifications in the peptide sequence will be of great value.

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