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Characterization of DNA-binding activity of Zα domains from poxviruses and the importance of the β-wing regions in converting B-DNA to Z-DNA

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ABSTRACT

The E3L gene is essential for pathogenesis in vaccinia virus. The E3L gene product consists of an N-terminal Zα domain and a C-terminal double-stranded RNA (dsRNA) binding domain; the left-handed Z-DNA-binding activity of the Zα domain of E3L is required for viral pathogenicity in mice. E3L is highly conserved among poxviruses, including the smallpox virus, and it is likely that the orthologous Zα domains play similar roles. To better understand the biological function of E3L proteins, we have investigated the Z-DNA-binding behavior of five representative Zα domains from poxviruses. Using surface plasmon resonance (SPR), we have demonstrated that these viral Zα domains bind Z-DNA tightly. Ability of $Zα_{E3L}$ converting B-DNA to Z-DNA was measured by circular dichroism (CD). The extents to which these Zαs can stabilize Z-DNA vary considerably. Mutational studies demonstrate that residues in the loop of the β-wing play an important role in this stabilization. Notably the Zα domain of vaccinia E3L acquires ability to convert B-DNA to Z-DNA by mutating amino acid residues in this region. Differences in the host cells of the various poxviruses may require different abilities to stabilize Z-DNA; this may be reflected in the observed differences in behavior in these Zα proteins.

INTRODUCTION

Poxviruses are the largest, most complex, double-stranded DNA viruses that have been observed to replicate in the cytoplasm of infected cells (1,2). Each poxvirus exhibits a different host range; some are extremely species specific, for example, swinepox virus, while others exhibit a broad host range (3,4). Vaccinia virus is the best-characterized member of this large family, due to its long established role in vaccination against smallpox as well as its importance as a gene transfer vehicles (1).

The E3L protein of vaccinia virus is composed of two distinct domains associated with two different nucleic acid-binding properties. The N-terminal domain (Zα) binds tightly and specifically to left-handed Z-DNA (5–8), while the C-terminal domain comprises a well-characterized double-stranded RNA (dsRNA) binding domain (9–12). The dsRNA-binding domain allows the virus to overcome host defense systems mediated by the dsRNA activated protein kinase PKR (9). Vaccinia virus lacking the dsRNA-binding domain of E3L has an increased sensitivity to IFN and restricted host range (13). The Z-DNA-binding domain is a member of the Zα family of Z-DNA-binding proteins, whose other members include the vertebrate dsRNA editing enzyme ADAR1 and the mammalian Z-DNA-binding protein ZBP1 (previously known as DLM-1).

The molecular structures of several Zα domains have been determined. Zα:Z-DNA co-crystal structures have been solved for the Zα domains of human ADAR1 (14), mouse ZBP1 (15) and yaba-like disease virus E3L (16). In each case, the protein adopts a helix-turn-helix with β-sheet (winged helix-turn-helix) fold, with the left-handed DNA backbone grasped between the recognition helix and the β-sheet by numerous hydrogen bonds. Both the precise shape of the fold and the interaction with DNA are extremely similar among these proteins. The DNA-contacting residues are highly conserved, both between species within a given protein and between different members of the Zα family (15,16), however, different

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members of the Zα family are not otherwise similar. The solution structure of free vaccinia virus $Z\alpha_{E3L}$ shows the same overall fold and supports the concept that E3L proteins share their Z-DNA-binding mode (17). There is one provocative difference between the different Zα structures: although the contacts between the β-sheet and the DNA are nearly the same, the shape and position of the β-sheet is variable, differing in each or the determined structures (16,17).

In previous studies, the Z-DNA-binding domain of E3L protein from vaccinia virus ($vZ\alpha_{E3L}$) was shown to play a key role in viral pathogenesis in mice (18,19). Furthermore, it was shown that the ability to bind Z-DNA is the essential characteristic required for the biological activity of this domain; $vZ\alpha_{E3L}$ can be replaced with the Zα domain of either ADAR1 or ZBP1 with no loss of viral pathogenicity. Mutations that decrease or abolish Z-DNA-binding activity proportionately decrease or abolish pathogenicity (19). It has been demonstrated that the Z-DNA-binding activity of $vZ\alpha_{E3L}$ is responsible for the anti-apoptotic activity of vaccinia E3L when expressed in cultured cells and can activate expression of a battery of genes (20). Therefore, it is of interest to characterize the binding activity of viral Zα domains in order to better understand poxvirus infection.

In this study, we have expressed the $Z\alpha_{E3L}$ domains from a representative group of five poxviruses: vaccinia virus ($vZ\alpha_{E3L}$), swinepox virus ($spZ\alpha_{E3L}$), yaba-like disease virus ($yabZ\alpha_{E3L}$), orf virus ($orfZ\alpha_{E3L}$) and lumpy skin disease virus ($lsZ\alpha_{E3L}$) (Figure 1).We show that these proteins bind strongly to Z-DNA and alter the equilibrium between B-DNA and Z-DNA. In addition, we have modified the β-sheets of several of these proteins using site-directed mutagenesis. These modified proteins display altered Z-DNA-binding activity, showing that changes in this region can modulate the interaction between protein and Z-DNA. These modulations are similar in magnitude to differences between the E3Ls of several poxviruses. It remains to be determined whether such changes in binding activity would alter the biology of the virus.

MATERIALS AND METHODS

Protein expression and purification

The sequences encoding viral Zα domains were either amplified from viral genomic DNAs ($orfZ\alpha_{E3L}$ and $vZ\alpha_{E3L}$) by PCR or assembled from synthesized oligonucleotides ($lsZ\alpha_{E3L}$, $spZ\alpha_{E3L}$ and $yabZ\alpha_{E3L}$) and cloned into the expression vector pET28a (Novagen), to be expressed as N-terminal-$(His)_6$-tagged fusion proteins. Expression clones were confirmed by restriction enzyme analysis and DNA sequencing. Resulting vectors were transformed into *Escherichia coli* strain BL21(DE3). Expression and purification of viral Zαs were carried out essentially as described elsewhere (16,21). Briefly, cells were grown at 37°C in LB medium supplemented with 30 μg/ml kanamycin until they reached a final concentration of OD_{600} = 0.5–0.7, at which time IPTG was added to 0.5 mM. Protein was expressed for 4 h at 37°C with the exception of $yabZ\alpha_{E3L}$, which was induced at 18°C (16). Cells were harvested by centrifugation

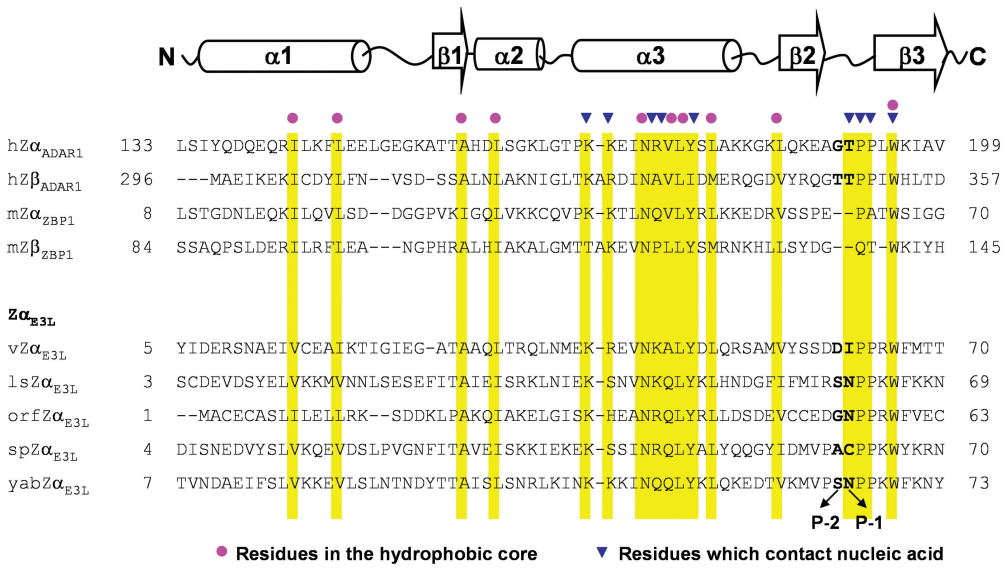

Figure 1. Sequence alignment of viral Zα domains and related Zα domains. It is shown underneath the secondary structure diagram, as revealed in the co-crystal structures of $hZ\alpha_{ADAR1}$, $mZ\alpha_{ZBP1}$ and $yabZ\alpha_{E3L}$ (14–16). Residues interacting with Z-DNA (blue triangles) and residues important for the protein fold (pink dots) are indicated. Yellow bars indicate residues that are important for the protein fold or Z-DNA recognition. Human $Z\beta_{ADAR1}$, which lacks the key tyrosine in helix α-3, does not bind to Z-DNA. In contrast, the Zβ domain from zebrafish ADAR1, which possesses this tyrosine, is capable of inducing the B–Z transition (8). The GenBank accession numbers for the various sequences are as follows: double-stranded RNA adenosine deaminase 1 (*Homo sapiens*): AAB06697 [GenBank]; Z-DNA-binding protein 1 (*Mus musculus*): NP_067369 [GenBank]; the E3L proteins: (vaccinia virus): AAA02759 [GenBank]; (orf virus): AAC08018 [GenBank]; (lumpy skin disease virus): AAK84995 [GenBank]; (swinepox): NP_570192 [GenBank]; (yaba-like disease virus): NP_073419 [GenBank]. Amino acids residues located at P-2 and P-1 positions of the β-wing regions in viral Zαs are in bold.

at 4000*g* for 10 min at 4°C. Proteins were purified a metal-chelating column (AP biotech), followed by removal of the N-terminal $(His)_6$-tag with thrombin (Boehringer Mannheim). Zα was further purified by ion exchange chromatography (GE), and dialyzed against 5 mM HEPES, pH 7.5, 10 mM NaCl, except yabZα_{E3L} and its mutants, which have limited solubility in low salt, for which 100 mM NaCl was used (16). The purified protein was adjusted to >2 mM final concentration, as determined by UV absorbance at 280 nm, using extinction coefficients deduced from amino acid sequence. The purified proteins were stored frozen at −70°C until use.

Surface plasmon resonance analysis

The binding affinities of viral Zαs for Z-DNA were determined by surface plasmon resonance (SPR) using a BIAcore 2000 as described previously (5). Briefly, ca. 300 response units (RU) of biotinylated poly (dG–dC) stabilized in the Z conformation (22) were immobilized on a SA chip (Biacore). Protein solutions at concentrations between 75 nM and 2000 nM were passed over the chip surface at 20 µl/min. All experiments were carried out at 25°C in HBS buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 0.01 mM EDTA). Regeneration was performed with a pulse of 0.05% SDS. The association and dissociation times were 180 and 200 s, respectively. For analysis, binding curves were fitted using BIA evaluation 3.0 and the 1:1 binding drifting baseline model.

Circular dichroism (CD)

Poly (dG–dC) (AP biotech) was rehydrated with 10 mM Tris–Cl, pH 7.4, 100 mM NaCl prior to use. The conversion of poly (dG–dC) from the B to the Z conformation was monitored by circular dichroism (CD). CD spectra were taken at 25°C using a Jasco J-810 CD spectrophotometer. Measurements were carried out on 150 µg/ml (225 µM base pair) DNA in CD buffer (10 mM HEPES, pH 7.4, 10 mM NaCl and 0.1 mM EDTA) in a 2 mm quartz cell for all proteins except yabZα_{E3L} and its mutants, which included 100 mM NaCl. To the DNA, 90 µM (final concentration) protein was added. The maximum volume of protein added to the sample did not exceed 5% of the total. Wavelength spectra were recorded at 1 nm interval averaged over 3 s. For kinetic measurements, CD signal changes at 255 nm were recorded at 1 s intervals for 1 h.

Mutagenesis of viral Zαs

Mutant proteins were constructed using the QuikChange® site-directed mutagenesis kit (Stratagene), according to the instructions provided by the supplier. After PCR and cloning, the sequence of each construct was verified.

RESULTS AND DISCUSSION

We chose five representative viral Zαs from several subfamilies of poxvirus for careful examination. The amino acid sequences of these Zα domains show relatively little sequence identity (between 19% and 39%); in contrast to the proteins as a whole, the residues that make contact with DNA are highly conserved (Figure 1) (14–16). Many, including the asparagine and tyrosine in the recognition helix α-3, and the first proline and tryptophan in the wing β-3 are invariant in proteins that bind Z-DNA, while the rest show mostly conservative changes. An exception is the Thr-191 of hZα_{ADAR1}; this residue makes contact with the DNA in the hZα_{ADAR1} co-crystal structure, but is not well conserved in other Zαs. Even between poxvirus proteins there is no observable conservation of residues that do not contact DNA. In order to determine the effect on DNA binding of sequence variability in the Zα domains from E3L proteins, the activity of these domains was examined, with a focus on the effect of the residues preceding the invariant proline. We will henceforth refer to the two residues preceding the conserved proline as 'P-2' and 'P-1' (Figure 1).

Characterization of the interaction between the Zα_{E3L} poxviruses and Z-DNA by SPR

The viral Zα domains shown in Figure 1 were purified from *E. coli*. Z-DNA-binding activities of these viral Zαs were determined using SPR. The equilibrium-binding constant (K_D) values were calculated from association (k_{on}) and dissociation (k_{off}) rate constants determined by fitting real-time kinetic data. As shown in Table 1, the binding constants ranging from 60 to 177 nM. This is comparable to the binding affinity of hZα_{ADAR1}, 57 nM (Table 1). Data from a typical SPR experiment is shown in supporting information (Figure S1).

Conversion of B to Z-DNA by poxviral Zα proteins

DNA with the sequence $d(CG)_n$ can be stabilized into Z-form by the binding of hZα_{ADAR1}; the B–Z transition has been observed by CD (5,6). Zα domains from other proteins have been characterized by CD for their ability to induce the B–Z transition (15,16,23,24), and variability between proteins with comparable binding constants has been seen. For example, the two Zα family domains from human ZBP1 produce a slower B to Z transition than hZα_{ADAR1} (23).

As shown in Figure 2, all the tested Zα proteins were able to alter the equilibrium between B- and Z-DNA in these experiments, with the exception of vZα_{E3L}. Although, vZα_{E3L} is not able to change the B–Z equilibrium under these conditions, its binding to

Table 1. Z-DNA-binding affinities of viral Zαs

Protein	k_{on} (1/Ms)	k_{off} (1/s)	K_D (nM)
hZα_{ADAR1}	9.6×10^4	5.47×10^{-3}	57
yabZα_{E3L}	1.27×10^5	7.56×10^{-3}	60
vZα_{E3L}	1.14×10^4	1.36×10^{-3}	120
spZα_{E3L}	4.21×10^4	7.44×10^{-3}	177
lsZα_{E3L}	4.84×10^4	7.98×10^{-3}	165
orfZα_{E3L}	7.18×10^4	12.4×10^{-3}	173

The binding affinities (K_D) of Zαs for Z-DNA were calculated from association (k_{on}) and dissociation (k_{off}) rate constants determined using surface plasmon resonance (Biacore).

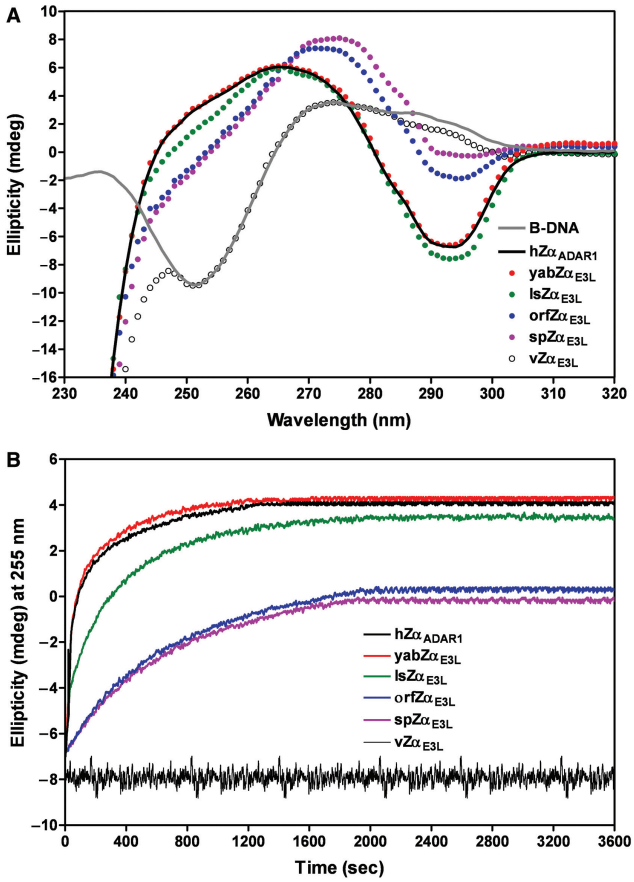

Figure 2. The conversion of poly (dG–dC) from the B to Z conformation by Zα. (**A**) Conversion of B-DNA to Z-DNA by Zα proteins measured by CD in the range of 230–320 nm. The spectra of the B-form (gray) and Z-form (black, stabilized by $hZ\alpha_{ADAR1}$) of poly (dG–dC) are shown for comparison. The spectra of $yabZ\alpha_{E3L}$ (red), $lsZ\alpha_{E3L}$ (green), $orfZ\alpha_{E3L}$ (blue), $spZ\alpha_{E3L}$ (pink) and $vZ\alpha_{E3L}$ (black) are also shown as circles. Equilibrium states are shown. The drop in ellipticity below 240 nm is due to the protein. (**B**) Kinetics of the conformation change from B-DNA to Z-DNA in the presence of Zα proteins. The change in ellipticity at 255 nm was monitored as a function of time. $hZ\alpha_{ADAR1}$ (thick black), $yabZ\alpha_{E3L}$ (red), $lsZ\alpha_{E3L}$ (green), $orfZ\alpha_{E3L}$ (blue), $spZ\alpha_{E3L}$ (pink) and $vZ\alpha_{E3L}$ (thin black) are shown.

Z-DNA has been demonstrated previously, both *in vitro* and *in vivo* (8). On the other hand, the rate of B to Z conversion by $yabZ\alpha_{E3L}$ and the equilibrium state are the same as that of $hZ\alpha_{ADAR1}$ (16). The other Zα proteins yield slower and less complete B–Z transitions than $yabZ\alpha_{E3L}$, but faster and more complete than $vZ\alpha_{E3L}$.

One way to quantify the results obtained by CD is to normalize the data with respect to $hZ\alpha_{ADAR1}$. $hZ\alpha_{ADAR1}$ is the best characterized Zα family protein and binds extremely tightly and specifically. Two comparisons can be made: (i) the extent of Z-DNA stabilization and (ii) the time required to reach equilibrium. By both of these criteria, the order of B to Z conversion was $hZ\alpha_{ADAR1} \sim yabZ\alpha_{E3L} > lsZ\alpha_{E3L} > orfZ\alpha_{E3L} \sim spZ\alpha_{E3L}$. Proteins that produce a faster, more complete conversion from the B to the Z-form tend to have a faster k_{on} as determined by SPR, however the correlation is not consistent. $orfZ\alpha_{E3L}$ has a k_{on} of 7.2×10^4, higher than

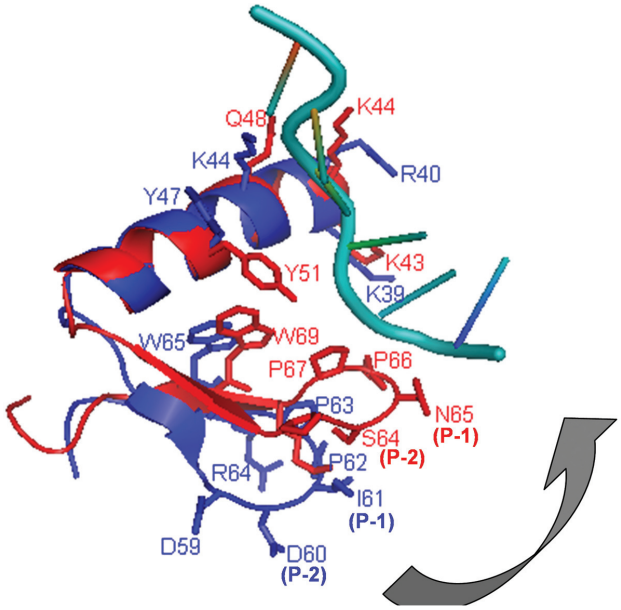

Figure 3. The interaction between Z-DNA and the β-wing region of $yabZ\alpha_{E3L}$ (red) and $vZ\alpha_{E3L}$ (blue) as determined structurally. The structure of $vZ\alpha_{E3L}$ determined by NMR (17) is superimposed on the structure of $yabZ\alpha_{E3L}$ in a complex with Z-DNA (16) using α3 as the superposition template. The β-wing is positioned parallel to the DNA backbone in $vZ\alpha_{E3L}$, leaving the P-1 and P-2 residues some distance from the DNA. In contrast, these residues are very close to the DNA backbone in $yabZ\alpha_{E3L}$. Although contacts between the P-2 residue and DNA have not been seen in structural studies of these complexes, it is possible that an interaction forms between this residue and intermediates between B-DNA and Z-DNA.

$spZ\alpha_{E3L}$or $lsZ\alpha_{E3L}$, but results in slower and less complete stabilization of Z-DNA than $lsZ\alpha_{E3L}$. It should be noted that the SPR experiments measure the binding of Zα protein to preformed Z-DNA, while the CD experiments observe a conformational change in dsDNA induced by the protein. Factors including the off rate, the specific geometry and contacts between the protein and the Z-DNA are likely to affect the conversion of B-DNA to Z-DNA in a different way than the binding to pre-stabilized Z-DNA.

Gain of B to Z-DNA conversion activity in a $vZ\alpha_{E3L}$ mutant

The Zα domain from vaccinia virus E3L appears inert in the CD experiments shown above. However, tight and specific binding to Z-DNA has been shown previously (8), and SPR shows that it binds preformed Z-DNA more tightly than $spZ\alpha_{E3L}$, $lsZ\alpha_{E3L}$ or $orfZ\alpha_{E3L}$. This apparent contradiction correlates with a low on rate, an order of magnitude less that that of $yabZ\alpha_{E3L}$ (Table 1). The solution structure of $vZ\alpha_{E3L}$ shows a considerable difference between the position and sequence of this wing and that of $yabZ\alpha_{E3L}$, which binds tightly and specifically in all assays [Figure 3 and (16,17)]. In order to assess the importance of residues in the β-wing (β-2, β-3 and the loop region in-between, Figure 1), Asp-60 (P-2) and Ile-61 (P-1) in $vZ\alpha_{E3L}$ were both changed to threonines. For comparison, $yabZ\alpha_{E3L}$ was also mutated to more closely resemble $vZ\alpha_{E3L}$ in sequence: Ser-64 (P-2) and

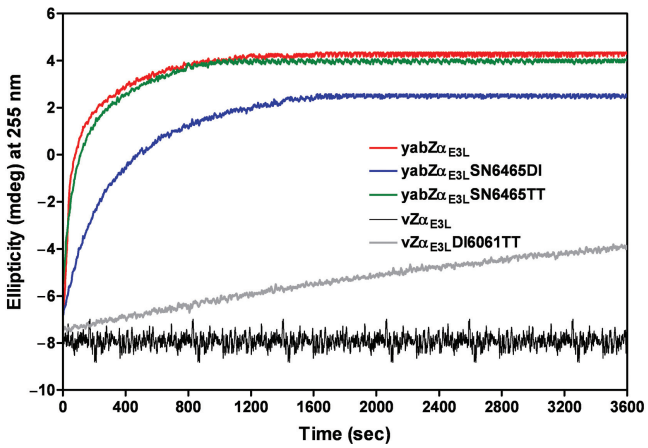

Figure 4. Effect of changes in the β-wing of yabZα_{E3L} and vZα_{E3L}. Kinetics of the change in DNA conformation in the presence of yabZα_{E3L} (red), vZα_{E3L} (black), yabZα_{E3L}SN6465TT (green), yabZα_{E3L}SN6465DI (blue) and vZα_{E3L}DI6061TT (gray) are shown. vZα_{E3L}DI6061TT, containing two threonines at P-1 and P-2, gains significant activity. The mutation yabZα_{E3L}SN6566TT does not affect activity, but yabZα_{E3L}SN6566DI has reduced activity.

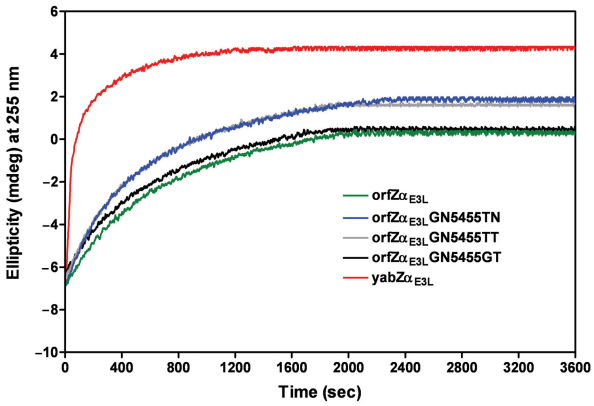

Figure 5. Effects of hydrogen bond forming mutations at P-1 and P-2 in the variable regions of orfZα_{E3L} on B to Z-DNA conversion activity. Kinetic measurements of DNA conformation change from B-DNA to Z-DNA in orfZα_{E3L} (green) and its variable region mutants—orfZα_{E3L}GN5455TN (blue), orfZα_{E3L}GN5455TT (blue) and orfZα_{E3L}GN5455GT (black)—were carried out to investigate effects of hydrogen bond forming potentials by amino acid residues in the variable region (P-1 and P-2). When both amino acid residues at P-1 and P-2 have abilities to form hydrogen bond(s), these mutants show better B to Z-DNA conversion activities than wild types or other mutants that have Gly (orfZα_{E3L}) or Ala (spZα_{E3L}) at P-2 position, respectively. This may indicate that the amino acid residue at P-2 could contribute to B to Z-DNA conversion possibly by hydrogen-bond interaction(s) with Z-DNA backbones as is found in P-1.

Asn-65 (P-1) were changed to Asp and Ile, respectively. Changing the residues at P-2 and P-1 in the β-wing can significantly affect the ability of a Zα protein to convert DNA from the B to the Z conformation, as shown in Figure 4. The mutation yabZα_{E3L}SN6465TT has no effect—rate and equilibrium of the B-DNA to Z-DNA conversion are unchanged. In contrast, yabZα_{E3L}SN6465DI produces a decreased rate and lower equilibrium.

Examination of the structure of the β-wing (Figure 3) suggests an explanation for this effect. The wing from vZα_{E3L} is positioned parallel to the DNA, aligning the two prolines nearest to the backbone, while the wing from yabZα_{E3L} extends toward the backbone, providing DNA interactions not only with the prolines but also with the Asn at P-1. It is possible that both P-1 and P-2 amino acid residues can make DNA contacts, possibly with DNA in an intermediate state between B and Z. These residues would then play a larger role in the conversion of DNA from the B to the Z-form than in binding to pre-stabilized Z-DNA. If this is true, positively charged and polar residues at positions P-1 and P-2 should effect the B to Z-DNA transition better non-polar amino acids and much better than negatively charged amino acids. The yabZα_{E3L} mutants described above both satisfy this prediction. In the case of vZα_{E3L}, the negatively charged Asp at P-2 could decrease binding to DNA, and the neutral Ile at P-1 cannot form hydrogen binds. The mutations D60T and I61T, vZα_{E3L}DI6061TT, remove one negative charge and offer the possibility of hydrogen bonds at both sites.

Effect of changes in P-1 and P-2 in other viral Zαs

To further test the hypothesis, mutations were made in other Zα domains from E3L proteins. When both P-1 and P-2 were changed to threonine in orfZα_{E3L}, a pronounced increase in the proportion of Z-DNA at equilibrium and the rate of conversion were seen (Figure 5 and Table 2).

In order to determine whether changes at both P-1 and P-2 were required for this effect, the single mutations orfZα_{E3L}GN5455TN and orfZα_{E3L}GN5455GT were tested. As shown in Figure 5, the orfZα_{E3L}GN5455TN mutation was sufficient to produce the increased binding. Changing Gly at P-2 to another amino acid acts to stiffen the β-turn; this will increase Z-DNA binding, except in the case of a negatively charged amino acid, which will destabilize binding due to electrostatic effects, as demonstrated by orfZα_{E3L}GN5455DI (Table 2).

Similar experiments with spZα_{E3L} verify that for these proteins, a change to threonine at position P-2 is sufficient to increase the stabilization of Z-DNA (Table 2). This result is unexpected because the residue at position P-1 contacts the Z-DNA in hZα_{ADAR1} and yabZα_{E3L}; therefore an effect of optimizing the residue at that position is more expected. The notable effect of sequence at P-2 supports the idea that this residue plays a role in either making a binding intermediate between protein and DNA, or, attractively, in shifting the equilibrium between B-DNA and Z-DNA by stabilizing an intermediate. This later possibility explains the discrepancy between K_D values and CD data.

When positions P-2 and P-1 are changed to aspartic acid and isoleucine, respectively, in orfZα_{E3L} or spZα_{E3L}, the effect is the same as that seen in yabZα_{E3L}SN6465DI (Table 2). The mutant proteins bind Z-DNA less well. This supports the hypothesis that these residues are not optimized for DNA binding in vZα_{E3L}.

Although a single threonine at position P-2 increases the B- to Z-DNA conversion activity of orfZα_{E3L} and spZα_{E3L} as much as the double mutant, this is not true for vZα_{E3L} (Table 2). In the case of the vaccinia protein,

Table 2. Effects on the degrees of B to Z-DNA conversion and the time to saturation for mutations in the β-wing region

Protein	% conversion	Time to saturation (s)	Mutation (s) in the β-wing
hZα$_{ADAR1}$	100	1000	Wild type
yabZα$_{E3L}$	100	1000	Wild type
SN6465TT	100	1000	P-1/P-2
SN6465DI	80	1600	P-1/P-2
vZα$_{E3L}$	0	∞[a]	Wild type
DI6061TT	35	>3600[b]	P-1/P-2
DI606DT	0	∞[a]	P-2
DI6061TI	0	∞[a]	P-1
DI6061KT	40	>3600[b]	P-1/P-2
orfZα$_{E3L}$	65	2000	Wild type
GN5455TT	80	2000	P-1/P-2
GN5455GT	65	2000	P-2
GN5455TN	80	2000	P-1
GN5455KT	80	2000	P-1/P-2
GN5455DI	50	>3600[b]	P-1/P-2
spZα$_{E3L}$	65	2000	Wild type
AC6162TT	80	2000	P-1/P-2
AC6162AT	65	2000	P-2
AC6162TC	80	2000	P-1
AC6162KT	80	2000	P-1/P-2
AC6162DI	50	>3600[b]	P-1/P-2
lsZα$_{E3L}$	90	1400	Wild type

Results are normalized to hZα$_{ADAR1}$, which is set to 100% conversion. Time to saturation is measured from $t = 0$ to the point where the curve becomes horizontal.
[a]No activity.
[b]Does not reach saturation within 1 h.

neither vZα$_{E3L}$DI6061TI nor vZα$_{E3L}$DI6061DT shows any activity in the CD assay. This suggests that the position of the β-wing of vZα$_{E3L}$ remains different for that of yabZα$_{E3L}$, even in the presence of a single mutation. It is likely that other residues also play a role in positioning the wing.

Effect of a charged amino acid in position P-2

It has been hypothesized that the presence of a positively charged amino acid at position P-2 could increase the ability of a Zα protein to alter the equilibrium between B-DNA and Z-DNA. This was tested by making mutants of vZα$_{E3L}$, orfZα$_{E3L}$ and spZα$_{E3L}$, in each of which P-2 and P-1 were changed to lysine and threonine, respectively. As predicted, the presence of the positively charged lysine improved the ability of the protein to stabilize Z-DNA (Table 2). In each case, the KT mutant performed better than the TT mutant. This supports the idea that residues P-1 and P-2 are close to or make contact with the DNA backbone, as part of a binding intermediate and/or in the final Z-DNA–protein complex. Binding to and stabilization of Z-DNA by a Zα protein can be optimized if residue P-2 is positively charged and residue P-1 is able to form a hydrogen bind to a backbone phosphate.

Biological implications of modulation of Z-DNA binding by viral Zαs

Although residues that are required for binding to Z-DNA are well conserved in Zα domains from viral E3L gene products, we have shown that there are residues that modulate DNA binding, which are conserved poorly or not at all. On one hand, yabZα$_{E3L}$ binds to Z-DNA extremely tightly, and is fully capable of stabilizing appropriate sequences in the Z conformation. On the other, vZα$_{E3L}$ cannot stabilize Z-DNA in the absence of other factors such as cobalt hexamine or negative supercoiling. Nevertheless, it is in vaccinia virus that it has been shown that the ability of E3L to bind Z-DNA is essential for viral pathogenicity (19).

In vaccinia infections, it is essential for Zα$_{E3L}$ to bind Z-DNA; mutations that decrease Z-DNA binding of vZα$_{E3L}$ decrease the pathogenicity of the virus (19). However, it is possible that viral E3L proteins do not have to stabilize Z-DNA in the absence of other factors, but rather bind to Z-DNA stabilized by factors such as negative supercoiling. Perhaps certain viral Zαs, e.g. vZα$_{E3L}$, do not need to stabilize Z-DNA on their own. Substitution of a stronger Zα domain such as hZα$_{ADAR1}$ or mZα$_{ZBP1}$ for vZα$_{E3L}$ in a chimeric E3L maintains pathogenicity but does not increase it (19).

Our study demonstrates that viral Zαs from different poxviruses have different ability for Z-DNA stabilization. The variability in the sequence of the β-wing and the modulation in Z-DNA-binding activity may reflect the lifecycle of the virus. In each of viruses, different degrees of modulation of Z-DNA-binding activity may be essential. Too weak binding will inactivate the Zα, but too much binding may result in binding to inappropriate targets or the activation of genes that will hamper viral activity.

The biological action of the different viral Zα domains can only be the object of speculation at present. When transfected into a cultured cell, Zα can prevent apoptosis, stopping one of the most powerful host defenses against viral infection. Expression of Zα also regulates the expression of many genes (20), and may act thus in viral pathogenesis. Finally, viral Zαs may compete with cellular Zαs or other, as yet undiscovered, Z-DNA-binding proteins, much as the C-terminal dsRNA-binding domain acts by competing for substrate with PKR.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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