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 ZαE3L 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
members of the Zα family are not otherwise similar. The solution structure of free vaccinia virus ZαE3L 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 of the determined structures (16,17).

In previous studies, the Z-DNA-binding domain of E3L protein from vaccinia virus (vZαE3L) 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; vZαE3L 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 vZαE3L 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 ZαE3L domains from a representative group of five poxviruses: vaccinia virus (vZαE3L), swinepox virus (spZαE3L), yaba-like disease virus (yabZαE3L), orf virus (orfZαE3L) and lumpy skin disease virus (lsZαE3L) (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 (orfZαE3L and vZαE3L) by PCR or assembled from synthesized oligonucleotides (lsZαE3L, spZαE3L and yabZαE3L) 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 OD600 = 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 yabZαE3L, 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 hZαADAR1, mZαZBP1 and yabZαE3L (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 ZβADAR1, 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.
Surface plasmon resonance analysis

The binding affinities of viral Zzs 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 bacterially expressed poly (dG–dC) stabilized in the Z conformation (22) were immobilized on a SA chip. 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 yabZ_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 Zzs

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 Zzs from several subfamilies of poxvirus for careful examination. The amino acid sequences of these Zz 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 hZz_ADAR1; this residue makes contact with the DNA in the hZz_ADAR1 co-crystal structure, but is not well conserved in other Zzs. 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 Zz 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 Zz_E3L poxviruses and Z-DNA by SPR

The viral Zz domains shown in Figure 1 were purified from E. coli. Z-DNA-binding activities of these viral Zzs 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 hZz_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 Zz proteins

DNA with the sequence d(CG)_n can be stabilized into Z-form by the binding of hZz_ADAR1; the B–Z transition has been observed by CD (5,6). Zz 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 Zz family domains from human ZBP1 produce a slower B to Z transition than hZz_ADAR1 (23).

As shown in Figure 2, all the tested Zz proteins were able to alter the equilibrium between B- and Z-DNA in these experiments, with the exception of vZz_E3L. Although, vZz_E3L is not able to change the B–Z equilibrium under these conditions, its binding to

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

| Protein   | k_on (1/Ms) | k_off (1/s) | K_D (nM) |
|-----------|-------------|-------------|----------|
| hZz_ADAR1 | 9.6 x 10^4  | 5.47 x 10^-3| 57       |
| yabZ_E3L  | 1.27 x 10^4 | 7.56 x 10^-3| 60       |
| vZ_E3L    | 1.14 x 10^4 | 1.36 x 10^-3| 120      |
| spZ_E3L   | 4.21 x 10^4 | 7.44 x 10^-3| 177      |
| lsZ_E3L   | 4.84 x 10^4 | 7.98 x 10^-3| 165      |
| orfZ_E3L  | 7.18 x 10^4 | 1.24 x 10^-3| 173      |

The binding affinities (K_D) of Zzs for Z-DNA were calculated from association (k_on) and dissociation (k_off) rate constants determined using surface plasmon resonance (Biacore).
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 yabZαE3L and the equilibrium state are the same as that of hZαADAR1 (16). The other Zα proteins yield slower and less complete B–Z transitions than yabZαE3L, but faster and more complete than vZαE3L.

One way to quantify the results obtained by CD is to normalize the data with respect to hZαADAR1. hZαADAR1 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 hZαADAR1 > yabZαE3L > lsZαE3L > orfZαE3L > spZαE3L. Protein 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. orfZαE3L has a \( k_{on} \) of \( 7.2 \times 10^8 \), higher than spZαE3L or lsZαE3L, but results in slower and less complete stabilization of Z-DNA than lsZαE3L. 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 vZαE3L 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 spZαE3L, lsZαE3L or orfZαE3L. This apparent contradiction correlates with a low on rate, an order of magnitude less that that of yabZαE3L (Table 1). The solution structure of vZαE3L shows a considerable difference between the position and sequence of this wing and that of yabZαE3L, 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 vZαE3L were both changed to threonines. For comparison, yabZαE3L was also mutated to more closely resemble vZαE3L in sequence: Ser-64 (P-2) and
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 yabZ3E3L,SN6465TT has no effect—rate and equilibrium of the B-DNA to Z-DNA conversion are unchanged. In contrast, yabZ3E3L,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 vZ3E3L is positioned parallel to the DNA, aligning the two prolines nearest to the backbone, while the wing from yabZ3E3L 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 than non-polar amino acids and much better than negatively charged amino acids. The yabZ3E3L mutants described above both satisfy this prediction. In the case of vZ3E3L, the negatively charged Asp at P-2 could decrease binding to DNA, and the neutral Ile at P-1 cannot form hydrogend bonds. The mutations D60T and I61T, vZ3E3L,D60I61TT, 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 orfZ3E3L, 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 orfZ3E3L,GN5455TN and orfZ3E3L,GN5455GT were tested. As shown in Figure 5, the orfZ3E3L,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 orfZ3E3L,GN5455DI (Table 2).

Similar experiments with spZ3E3L 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 hZ3ADAR1 and yabZ3E3L, 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 latter possibility explains the discrepancy between KD values and CD data.

When positions P-2 and P-1 are changed to aspartic acid and isoleucine, respectively, in orfZ3E3L or spZ3E3L, the effect is the same as that seen in yabZ3E3L,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 vZ3E3L.

Although a single threonine at position P-2 increases the B- to Z-DNA conversion activity of orfZ3E3L and spZ3E3L as much as the double mutant, this is not true for vZ3E3L (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 |
|---------------|--------------|------------------------|---------------------------|
| hZzADAR1      | 100          | 1000                   | Wild type                 |
| yabZzE3L      | 100          | 1000                   | Wild type                 |
| SN6465TT      | 100          | 1000                   | P-1/P-2                   |
| SN6465DI      | 80           | 1600                   | P-1/P-2                   |
| vZzE3L        | 0            | ∞^a                    | Wild type                 |
| DI6061TT      | 35           | >3600^b                | P-1/P-2                   |
| DI6061DT      | 0            | ∞^a                    | P-2                       |
| DI6061TI      | 0            | ∞^a                    | P-1                       |
| DI6061KT      | 40           | >3600^b                | P-1/P-2                   |
| orfZzE3L      | 65           | 2000                   | Wild type                 |
| GN5455TT      | 80           | 2000                   | 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                   |
| spZzE3L       | 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                   |
| hsZzE3L       | 90           | 1400                   | Wild type                 |

Results are normalized to hZzADAR1, which is set to 100% conversion. Time to saturation is measured from t = 0 to the point where the curve becomes horizontal.

^aNo activity.
^bDoes not reach saturation within 1 h.

neither vZzE3L DI6061TI nor vZzE3L DI6061DT shows any activity in the CD assay. This suggests that the position of the β-wing of vZzE3L remains different for that of yabZzE3L, 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 vZzE3L, orfZzE3L and spZzE3L, 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 bond to a backbone phosphate.

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

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, yabZzE3L binds to Z-DNA extremely tightly, and is fully capable of stabilizing appropriate sequences in the Z conformation. On the other, vZzE3L 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 Zzs to bind Z-DNA; mutations that decrease Z-DNA binding of vZzE3L 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 Zzs, e.g., vZzE3L, do not need to stabilize Z-DNA on their own. Substitution of a stronger Z domain such as hZzADAR1 or mZzBP1 for vZzE3L in a chimeric E3L maintains pathogenicity but does not increase it (19).

Our study demonstrates that viral Zzs 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 Zzs may compete with cellular Zzs 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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