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Crystal Structure of a Poxvirus-Like Zalpha Domain from Cyprinid Herpesvirus 3

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Zalpha domains are a subfamily of the winged helix-turn-helix domains sharing the unique ability to recognize CpG repeats in the left-handed Z-DNA conformation. In vertebrates, domains of this family are found exclusively in proteins that detect foreign nucleic acids and activate components of the antiviral interferon response. Moreover, poxviruses encode the Zalpha domain-containing protein E3L, a well-studied and potent inhibitor of interferon response. Here we describe a herpesvirus Zalpha-domain-containing protein (ORF112) from cyprinid herpesvirus 3. We demonstrate that ORF112 also binds CpG repeats in the left-handed conformation, and moreover, its structure at 1.75 Å reveals the Zalpha fold found in ADAR1, DAI, PKZ, and E3L. Unlike other Zalpha domains, however, ORF112 forms a dimer through a unique domain-swapping mechanism. Thus, ORF112 may be considered a new member of the Z-domain family having DNA binding properties similar to those of the poxvirus E3L inhibitor of interferon response.

Zalpha domains represent a subfamily of winged helix-turn-helix (wHTH) domains with the unique property of specific binding to purine/pyrimidine repeats in the left-handed helical conformation known as Z-DNA (1). Typically, Zalpha domains are 66 amino acids long (Pfam 02295) adopting a fold consisting of 3 alpha helices and 3 beta strands arranged in α1β1α2α3β2β3 topology. Helix 3 and the loop connecting the β2 and β3 strands, called the wing, have been found to interact with DNA in the cocrystal structure of the ADAR1 Zalpha domain with a short $(CG)_3$ oligonucleotide (2). The surprising feature of the Zalpha-DNA complex is that the DNA adopts the left-handed helical conformation, rather than the naturally occurring right-handed B form (3). It is still an open question whether Zalpha actively induces the conformational transition or selectively binds the left-handed form, which is in equilibrium with the right-handed conformation.

Interestingly, a very similar behavior was found to occur with $(CG)_3$ oligonucleotides in double-strand RNA (dsRNA) (4), allowing Zalpha to bind both dsDNA and dsRNA of the same sequence. The ADAR1 Zalpha DNA cocrystal structure and several structures of Zalpha domains from other proteins (5–7) have established key features of the domain necessary for binding DNA/RNA: a tyrosine, an asparagine, and a tryptophan (Tyr177, Asn173, and Trp195 in ADAR1 Zalpha) form a unique and conserved structural motif in all known structures and sequences of Zalpha domains (1). A mutation of any of these three residues results in the loss of specific DNA binding, and this triplet, along with hydrophobic residues involved in the folding of the protein, defines a characteristic signature of the Zalpha domain that distinguishes it from the rest of the wHTH DNA binding domains (8).

Significantly, members of this domain subfamily have been found in the vertebrate proteins ADAR1, DAI, and PKZ (3, 5, 9), all of which are involved in the antiviral interferon response pathway. Functionally, DAI and Z-DNA-dependent protein kinase (PKZ) are components of the machinery responsible for the recognition of foreign nucleic acids in the cytoplasm and have been implicated in the initiation of a cascade that blocks cellular translation and leads to the production of type I interferons. Similarly, a Zalpha domain is also found in a virally encoded protein (E3L) that acts as an inhibitor of the interferon response (10). E3L is a key protein in the pathogenesis of poxviruses. Thus, strains with nonfunctional E3L dramatically lose their proliferation potential. The E3L protein consists of two domains, a C-terminal dsRNA-binding domain (dsRBD) and an N-terminal Zalpha domain, and both domains have been shown to be required to fully block host responses (11). Surprisingly, however, no other virus family has been found to encode a Zalpha-containing protein.

The most recently discovered Zalpha-containing protein, PKZ, is uniquely found in bony-fish species of the orders Cypriniformes and Salmoniformes, including the laboratory model zebrafish (Danio rerio) and common species, such as Atlantic salmon (Salmo salar) and goldfish (Carassius auratus). This protein bears a kinase domain strongly related to protein kinase R (PKR) and two Zalpha domains (9). The key difference between the two proteins is that PKZ contains two Z-DNA binding domains instead of the dsRNA binding domains (dsRBDs) found in PKR. The latter dsRBDs are responsible for sensing viral dsRNA in the cytoplasm, leading to the self-activation of PKR and the phosphorylation of eIF2α and eventually resulting in the shutdown of cellular translation. This and additional functions attributed to PKR render this protein the central node of innate responses in vertebrates. PKZ blocks cellular translation in a similar manner but in response to DNA CG repeats (12). Thus, in these fish species, a different and unstudied nucleic acid sequence/structure appears to be active as a pathogen-associated molecular pattern (PAMP).

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Herpesviruses of the family *Alloherpesviridae* are important pathogens of fish and amphibians species (13). Despite their similar morphology, members of the family *Alloherpesviridae* have gene content distinct from that of members of the *Herpesviridae*, which infect mammals, birds, and reptiles. Significantly, *Alloherpesviridae* genomes contain at least three genes encoding proteins that are characteristic of poxviruses (14). Horizontal gene transfer or a common ancestor has been proposed as a source of the gene overlap between poxviruses and *Alloherpesviridae*. Cyprinid herpesvirus 3 (CyHV3) is a typical member of *Alloherpesviridae* and a major pathogen of common carp and koi (15). Infection by CyHV3 has become a significant threat to aquaculture as well as the wild population of carp, as its mortality rate can be as high as 95%, threatening fish farms worldwide. Recently the genome of CyHV3 was sequenced, providing key information for understanding the biology of the infection and particularly host-pathogen interactions.

Here we show that cyprinid herpesvirus 3 open reading frame 112 encodes a protein that, in vitro, has a structural and functional Zalpha domain, similar to both the poxviral interferon response inhibitor E3L and the PKZ protein of the fish species that CyHV3 infects. Our findings suggest that *Alloherpesviridae* and poxviruses may use a similar mechanism to subvert the interferon response, and this allows us to assign a potentially crucial role in virus pathogenesis to *Alloherpesviridae* ORF112 function.

MATERIALS AND METHODS

Cloning and expression. Sequences encompassing M221-A278, S219-A278, and N216-A278 of ORF112 (BAF48926.1) were amplified from CyHV-3 genomic DNA (kindly provided by the Friedrich-Loeffler Institute) and cloned in a pET28a vector as a fusion with a 6His tag at the NheI-XhoI restriction sites. Protein expression was induced in *Escherichia coli* BL21(DE3) cells with the addition of 0.4 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 3 h. Cell pellets from 1-liter cultures were lysed with Bugbuster (Novagen) in the presence of 1 mM phenylmethylsulfonyl fluoride (PMSF) and Benzonase (Novagen) for 2 h at 4°C. The proteins were loaded on a HiTrap nickel-nitrilotriacetic acid (Ni-NTA) column on an Äkta purifier system and eluted using a gradient between 20 mM and 300 mM imidazole. The first construct did not result in any detectable protein production. The eluted protein from the other two constructs was dialyzed against 0.5× thrombin buffer (10 mM Tris [pH 8.4], 75 mM NaCl, 1.25 mM $CaCl_2$) and incubated with thrombin overnight at 4°C. The cleaved proteins were directly applied to a MonoS column and then eluted with a 75-to-600 mM NaCl gradient. Selected fractions were concentrated and buffer exchanged to a final concentration of 7.5 mg/ml in 20 mM Tris (pH 7.5), 50 mM KCl or 10 mM HEPES, 50 mM NaCl (pH 7.4) for use in crystallization and biochemical characterization.

Protein crystallization. The purified protein was used for crystallization screens (Crystal Screen I-II from Hampton Research and the Joint Center for Structural Genomics from Molecular Dimensions) in complex with $T(CG)_3$ duplex oligonucleotides, and crystals were obtained under several conditions. However, in all cases the crystals were also reproducible in controls in the absence of nucleic acids, suggesting that only the protein component had been crystallized. Highest-quality rod-shaped crystals (~0.2 by 0.05 by 0.05 μm) were obtained using a reservoir containing 0.2 M NaCl, 0.1 M cacodylate (pH 6.5), and 2 M ammonium sulfate. Well-shaped crystals were subsequently frozen at 100 K in the presence of Paratone N or 20% glycerol as a cryoprotectant.

Data collection and structure determination. Flash-frozen crystals at 100 K were exposed to X rays at the ID23-1 beam line of ESRF/Grenoble using X rays of 0.97 Å in wavelength. The best crystal diffracted up to 1.76 Å. Data processing was performed using the XDS software package (16). The ORF112 Zalpha crystals belong to the P212121 space group with the following parameters: $a = 51.7$ Å, $b = 54.6$ Å, $c = 86.6$ Å, and $\alpha = \beta = \gamma = 90.00°$. Phasing performed using molecular replacement with ADAR1 Zalpha (PDB code 1QBJ) as the starting model was successful when a C-terminally truncated model only was used, leading to a solution with four monomers per asymmetric unit. The structure was refined from the starting model using Phenix (17) and iterative rebuilding using Coot (18). The resulting electron density clearly showed the C terminus of the protein leading to a neighboring monomer, suggesting domain swapping of the last beta-strand and resulting in an arrangement of two dimers per asymmetric unit. The final refined model has an R/R_{free} ratio of 20.1/24.7 and comprises 248 protein residues and 213 solvent molecules, among which are 9 sulfate ions (see Table 1 for statistics). The backbone of P217-A278, S215-P277, H212-A278, and S219-A278 from chains A, B, C, and D, respectively, was visible in the electron density map. Some side chains of solvent-exposed amino acids (S215B, N216B, H212C, M213C, R266C, R266D, and Q270D) had no visible electron density, while the side chain of H188C shows two alternative conformations which were both modeled. The nine sulfate ions could be located in the structure in similar positions in each the four monomers.

TABLE 1 Data collection and refinement statistics

Parameter	Result
Data set	Cr2_19w1
Beam line	ID23-1
Resolution	46.18–1.75 Å
Space group	$P2_12_12_1$
Cell parameters	$a = 51.7$ Å, $b = 54.6$ Å, $c = 86.6$ Å, $\alpha = \beta = \gamma = 90.0000°$
Wavelength	0.97 Å
R_{merge} (%)	3.6
$I/\sigma I$	13.1
Completeness (%)	94.8
Unique reflections	23721
Multiplicity	4.2
Wilson B	31.1
Refinement	
Resolution	46.18–1.76 Å
Reflections	21,681
R/R_{free} (%)	20.1/24.7
R/R_{free} (%), outer cell	32.1/41.8
RMS bond	0.003
RMS angles	0.677

DNA binding assays. The ability of the purified protein to bind DNA was evaluated by gel mobility shift analysis using a $T(CG)_3$ duplex oligonucleotide (Integrated DNA Technologies). Mixtures of protein with 50 μM DNA at ratios of 1/1 to 4/1 were incubated at 37°C for 20 min and then subjected to electrophoresis on nondenaturing polyacrylamide gels and stained with SYBR followed by Coomassie blue. A distinct band corresponding to the protein-DNA complex was clearly visible. Complete conversion to Z-DNA could be observed at protein/DNA ratios of 2:1. The conformation of the oligonucleotides in the complex was evaluated using circular dichroism (CD) spectroscopy on a Jasco J-815 CD system in a 0.1-mm cuvette. A clear inversion of the trace, characteristic of the Z-DNA helix, was observed at a wavelength of 255 nm (see Fig. 2C).

Size exclusion chromatography characterization. We used size exclusion chromatography to characterize the oligomerization state of the protein in solution. An S75 prepacked column (GE Healthcare) was equilibrated with 50 mM Tris (pH 7.5) and 100 mM KCl and calibrated using protein standards (molecular mass, 6,500 to 66,000 Da; Sigma-Aldrich). ORF112 Zalpha protein (1 mg) in a volume of 200 μl was loaded on the column, and the elution profile was compared with that of the standards showing a single peak corresponding to a molecular mass of 7.7 kDa, in

FIG 1 Identification of ORF112 of cyprinid herpesvirus 3 as a Zalpha domain-containing protein. (A) The annotated predicted protein is 278 amino acids (aa) long, with a highly repetitive N terminus (BAF48926.1). An alternative, more likely product starts at position 186 and corresponds to slightly more than the Zalpha domain (shading). The starting residues of the described Zalpha constructs are highlighted (dark shading). (B) Alignment of Zalpha ORF112 sequences (CyHV3) with PKZ Zalpha1, E3L, and ADAR1 Zalpha domains. SS, *Salmo salar*; CA, *Carassius auratus*; CI, *Ctenopharyngodon idella*; DR, *Danio rerio*; GR, *Gobiocypris rarus*; VV, vaccinia virus. The vertical bars over the sequence indicate the degree of conservation. The three key DNA-interacting residues in ADAR1 Zalpha are indicated with arrows, along with the residue numbering of ORF112 (corresponding to Asn174, Tyr177, and Trp195 in hADAR1).

good agreement with the predicted molecular mass for the monomer. A similar procedure using the buffer used for crystallization revealed a novel peak at the expected molecular mass for the dimer. The transition from monomer to dimer was found to be induced by ammonium sulfate at concentrations of 50 and 200 mM.

Protein structure accession numbers. The Zalpha domain crystal structure determined here has been deposited in the RCSB database (RCSB code RCSB075715; PDB code 4HOB).

RESULTS

A putative Z-DNA binding domain containing protein from cyprinid herpesviruses. Using sequence homology searches for new members of the Z-domain family, we found a gene (*orf112*) encoding a Z-domain-containing hypothetical protein (BAF48926.1) in the cyprinid herpesvirus 3 genome sequence (15). The gene, as annotated, encodes a 278-amino-acid protein with the Z-domain signature located at its C terminus (Fig. 1A), and its expression as cDNA has been confirmed (19). No recognizable homology could be detected for the N-terminal part of the protein. Thus, unlike the poxviral E3L protein, ORF112 does not contain an obvious dsRNA binding domain. A shorter version of a 92-amino-acid protein, encompassing mostly the Zalpha domain and starting from an internal methionine (M187) residue, is possible.

The Zalpha domain of ORF112 shows highest identity (Fig. 1B) with the Zalpha domain of PKZ from *Gobiocypris rarus* (20) and *Danio rerio* (9), suggesting a functional and/or evolutionary relationship between PKZ and ORF112. ORF112 Zalpha conserves all residues known to be critical for interaction with DNA, as defined by the prototypic ADAR1 Zalpha. Thus, residues Tyr257, Asn253, and Trp274 correspond to Tyr177, Asn173, and Trp195 of ADAR1 and are highly conserved among known Zalpha domains. Similarly, the pattern of hydrophobic core residues important for folding of the wHTH domain is also conserved in ORF112. The fact that no viral Zalpha domains have previously been described outside the poxvirus family combined with the relevance of Zalpha domains in viral pathogenicity prompted us to clone and express the protein for further biochemical and structural characterization.

Cloning, protein expression and biochemical characterization. Expression of the full-length ORF112, as annotated, in a bacterial expression system was unsuccessful, resulting in a largely insoluble protein (data not shown). However, we successfully expressed two ORF112 Zalpha constructs differing in length and covering the regions from Ser219 to Ala278 and from Asn216 to Ala278 as fusions with a cleavable His tag. Both constructs were expressed as soluble proteins, but the shorter version was a temperature-sensitive protein which, upon short incubation at 37°C, formed protein aggregates and precipitated (data not shown). The longer-form S216-A278 ORF112 Zalpha protein was purified to homogeneity using affinity and ion-exchange chromatography and was used for further biochemical characterization. Gel-mobility shift assays with the purified protein against a $T(CG)_3$ duplex oligonucleotide showed a robust band shift (Fig. 2A) similar to that observed with Zalpha ADAR1, confirming the ability of ORF112 Zalpha to interact with CpG repeats. In contrast, no specific complex was observed when ORF112 Zalpha1 was incubated with sequences with a low propensity for forming Z-DNA (Fig. 2B). We obtained similar results using a protein starting at position M187 (not shown) and likely representing the full-length product of the gene. We concluded that the bound DNA is in the left-handed conformation, as circular dichroism revealed the characteristic Z-DNA inversion at 255 nm (Fig. 2C).

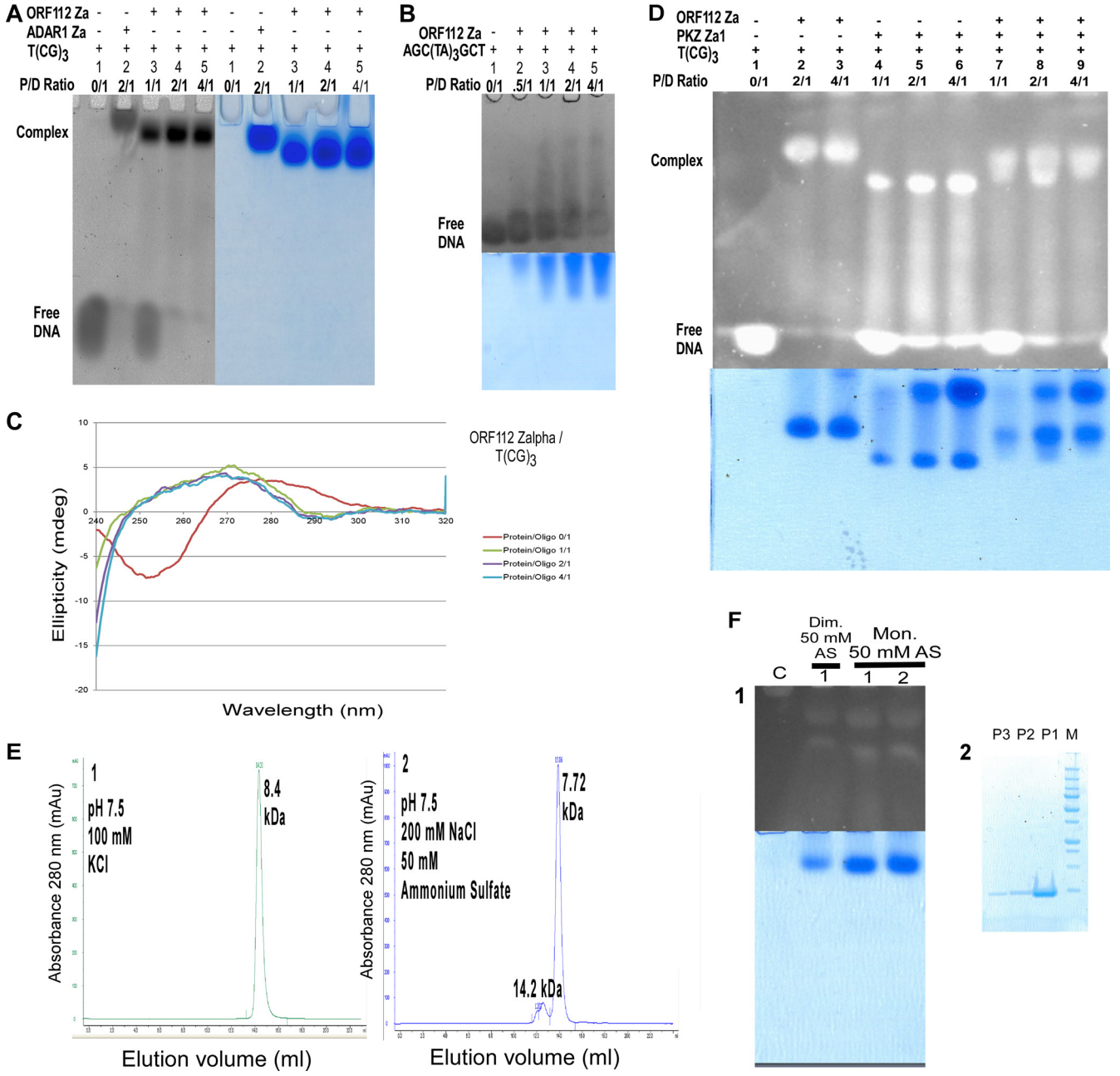

FIG 2 Zalpha ORF112 binds CpG repeats. (A) Zalpha ORF112 forms a complex with a $T(CG)_3$ oligonucleotide, as demonstrated by electrophoresis on a native polyacrylamide gel stained for nucleic acids with SYBR (left) and for proteins with Coomassie blue (right). The molar ratio of protein to DNA is indicated above the lanes. A gel mobility shift of the same oligonucleotide with ADAR1 Zalpha at a protein/DNA ratio 2/1 is also shown (lane 2). (B) No specific bands are formed between Zalpha ORF112 and a control duplex oligonucleotide $AGC(TA)_3GCT$, as seen once again with SYBR staining (top) and Coomassie blue stain (bottom) at molar ratios similar to those use for panel A. (C) Circular dichroism spectra of mixtures of ORF112 Zalpha with $T(CG)_3$ DNA at ratios of 1/1, 2/1, and 4/1. A characteristic inversion is observed at 255 nm compared to the B-DNA control (red line) indicating Z-DNA formation. (D) Gel retardation of a $T(CG)_3$ oligonucleotide in the presence of ORF112 Zalpha (lanes 2 and 3) and PKZ Zalpha1 domains (lanes 4, 5, and 6) and in the presence of both domains (1:1 protein ratios) (lanes 7, 8, and 9); lane 1 is the DNA-only control. Protein/DNA ratios in lanes 7, 8, and 9 reflect the total protein mixture-to-DNA ratio. Protein/DNA ratios are given above the lanes. As in panels A and B, the same gel was stained with SYBR stain (top) and Coomassie stain (bottom) to visualize DNA and protein, respectively. (E, panel 1) Size exclusion chromatography on a Superdex 75 column in the absence of sulfate ions reveals a single peak corresponding to the monomeric protein. (E, panel 2) Size exclusion chromatography in the presence of 50 mM ammonium sulfate (AS) revealed peaks corresponding to both monomer and dimer oligomeric species. (F, panel 1) Gel retardation experiments with the $T(CG)_3$ duplex oligonucleotide and the gel filtration fractions representing the dimer (ratio 1/1) (Dim. lane 1) and monomer (protein/DNA ratios, 1/1 and 2/1) (Mon. lanes 1 and 2) states of ORF112 Zalpha. Lane C, no-DNA control. AS, ammonium sulfate. (F, panel 2) Denaturing SDS-PAGE of gel filtration peak fractions P1 (monomer), P2 (dimer), and P3 (unknown) reveals that all peaks have similar molecular weights.

To investigate a potential role for ORF112 as an inhibitor of PKZ, we studied the ability of the ORF112 domain to compete for DNA binding with the Zalpha domains of the zebrafish PKZ (drPKZ). The two Zalpha domains (Za1 and Za2) of drPKZ were expressed and purified to homogeneity, and Za1 was shown to be a strong binder of CpG DNA (9, 21; our unpublished results). In competition experiments comparing the binding of equimolar amounts of ORF112 Zalpha and PKZ Zalpha1 to the $T(CG)_3$ oligonucleotide, the complex of ORF112 Zalpha/DNA was preferentially formed (Fig. 2D). Thus, ORF112 Zalpha is an efficient competitive inhibitor with PKZ Zalpha for DNA binding under the conditions we tested.

Having confirmed the ability of ORF112 to interact in vitro with nucleic acids in the left-handed Z-DNA conformation, we proceeded to its structural analysis by protein crystallography.

The crystal structure of the ORF112 Zalpha domain. Although crystals of the protein alone were readily obtained, cocrystallization of ORF112 Zalpha with DNA oligonucleotides failed to produce crystals of the protein-DNA complex. The structure of Zalpha ORF112 was determined at a resolution of 1.75 Å by molecular replacement using a truncated Zalpha ADAR1 (PDB code 1QBJ) as starting model. The asymmetric unit of the crystals is composed of four Zalpha ORF112 monomers organized in two nonidentical dimers (Fig. 3A). The two dimers differ in their solvent structure and the angle formed between the two monomers. Each monomer adopts a fold characteristic of the wHTH domains and shows a high degree of structural similarity with the prototypic ADAR1 Zalpha domain (Fig. 3B). Superposition of each of the four monomers up to residue Lys185 with ADAR1 Zalpha (PDB code 1QBJA) results in alignments with root mean square difference (RMSDs) of 0.511, 0.570, 0.491 and 0.693 for monomers A, B, C, and D, respectively. Despite the clear similarity in folding between the monomers of ORF112 Zalpha and Zalpha ADAR1, dimer formation between Zalpha domains has never been observed before. Interestingly, the formation of dimers is mediated by a C-terminal sequence exchange ("domain swapping"). Thus, the two monomers reciprocally exchange their last 10 amino acids (a sequence which normally forms the wing) and β3, the last β-strand of the domain (Fig. 3A). In contrast, the crystal structures of ADAR1, DAI, and E3L Zalpha domains reveal an entirely monomeric structure, despite the fact that two monomers are typically bound to a single binding site, one on each DNA strand. In ORF112 Zalpha, the C-terminal exchange creates a hinge region that flexibly connects the two monomers. The hinge is formed by the residues of the wing where single or double proline residues are a conserved feature of Z domains, raising the possibility that similar domain swapping might be a widespread strategy for dimer formation but has escaped detection until now. Significantly, the exchanged residues include Trp274 (Trp195 in ADAR1), a key residue for the interaction of the domain with DNA, and thus the dimer creates a conserved DNA interaction surface that is formed with the contribution of both monomers.

A possible role for phosphate groups in the induction of domain swapping. The electron density of the Zalpha ORF112 crystals allowed us to clearly locate a number of sulfate ions bound to the surface of the protein. That was not unexpected given the high positive charge of the domain's surface and the high concentration of sulfate ions present in the crystallization conditions (2 M ammonium sulfate). Surprisingly, however, the superposition of the ORF112 Zalpha domains on ADAR1 Zalpha revealed a precise positioning of sulfate ions at locations occupied by the backbone DNA phosphates in the protein-DNA complex and in contact with residues involved in domain swapping (Fig. 3B). This observation suggested that sulfate ions may have a role in inducing the domain swapping phenomenon. To shed light on the mechanism of the domain swapping, we used size exclusion chromatography to characterize the oligomeric state of the protein under different conditions. Interestingly, under our standard conditions (50 mM Tris [pH 7.5], 100 mM KCl), the protein eluted as a single clear peak corresponding to 7.67 kDa, close to the molecular mass expected for the monomeric form of the protein (Fig. 2E, panel 1). A smaller second peak, roughly corresponding to the expected molecular mass of the dimer, appeared only when 50 mM ammonium sulfate was introduced into the buffer, clearly confirming a role of the sulfate ions in dimer formation (Fig. 2E, panel 2). A third minor peak was also observed, probably corresponding to a higher-order oligomerization of the protein. All peaks were shown to contain a protein of identical molecular weight in SDS-PAGE, suggesting that indeed they represent different oligomeric states of the same protein (Fig. 2F, panel 2).

Domain swapping and DNA binding. We were puzzled by the results of the crystallization using a mixture of protein and DNA, which consistently resulted in protein-only crystals, despite the strong interaction of ORF112 Zalpha with DNA, as revealed by gel shift experiments. Modeling of the DNA-bound form of ORF112 Zalpha, based on the structure of the complex of Zalpha ADAR1 with Z-DNA, showed that steric hindrance between the two monomers comprising the dimer, now located at a fixed distance from the binding site, might interfere with DNA binding (Fig. 3B). Nevertheless, we cannot exclude the possibility that the flexible nature of the hinge region may allow a repositioning of the second monomer in a way that permits the interaction of one of the monomers with DNA. Whatever the explanation, our modeling clearly suggests that the dimer of ORF112 Zalpha cannot recapitulate the way two monomers of ADAR1 Zalpha interact with a single $(CG)_3$ binding site. If the dimeric form of ORF112 Zalpha does not bind DNA, we hypothesized that the equilibrium between a monomeric form and a dimeric form might allow the transition between a monomeric DNA binding-competent form and an inactive ORF112 Zalpha dimer. Therefore, we performed band shift experiments using the isolated oligomers from size exclusion chromatography experiments. With the caveat that there was some overlap between monomer and dimer peaks, significantly reduced binding of DNA was observed for the dimer fraction compared to the monomer (Fig. 2F, panel 1).

DISCUSSION

Here we describe a new member of the Zalpha domain family found in a protein (ORF112) of cyprinid herpesvirus 3, a member of the family *Alloherpesviridae*. This is an interesting finding as, to date, no Zalpha domains have been found in any member of the *Herpesviridae*, which infect mammals, birds, and reptiles. On the other hand, Zalpha domains are a feature of the poxviral protein E3L, which is present in all known poxviruses and which is crucial for the subversion of the host interferon response. The E3L protein acts through the inhibition of PKR (11) and has also been suggested to inhibit the action of DAI, a cytoplasmic sensor of nucleic acids with two Zalpha domains. Finding a similar virus evasion protein in *Alloherpesviridae* is consistent with previous work which identified similarities between genes encoding thymi-

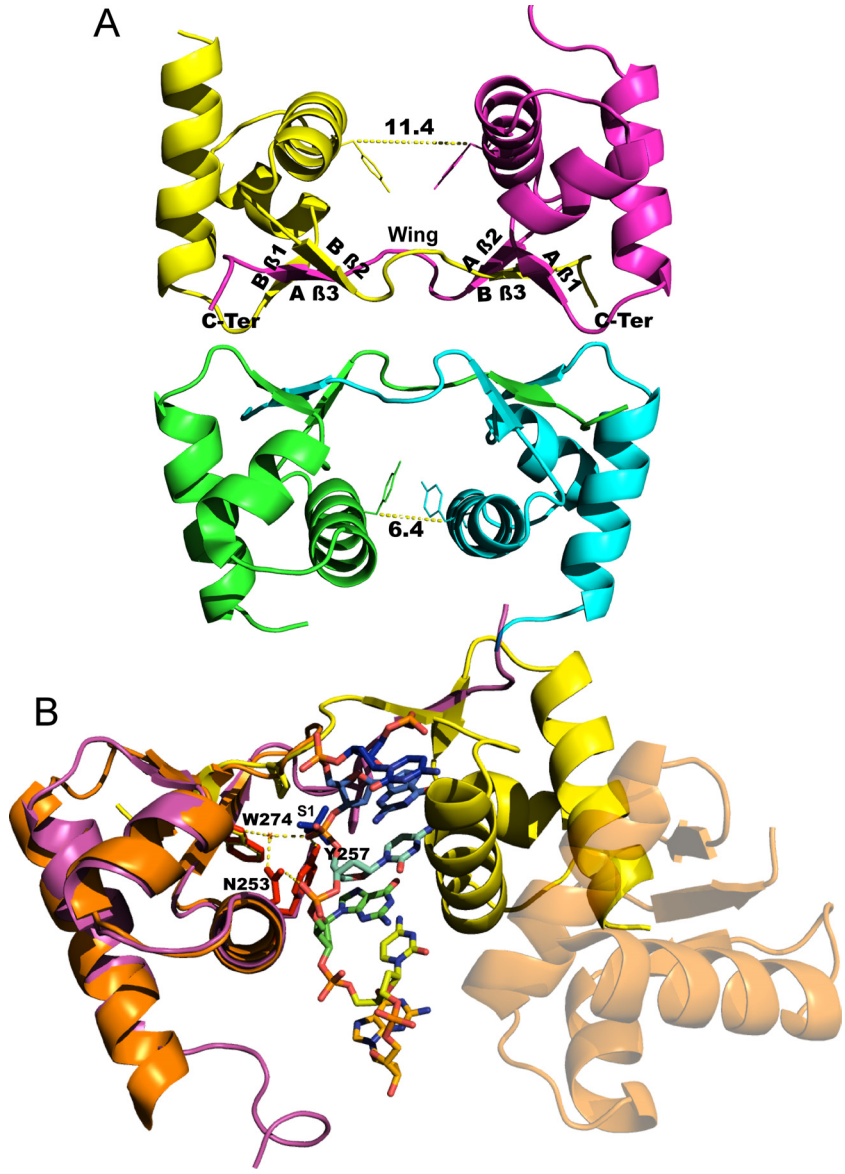

FIG 3 Overview of the structure of the ORF112 Zalpha domain. (A) Four monomers of ORF112 are present in the asymmetric unit. The monomers form dimers through domain swapping involving the last β-strand of each domain and residues P269 to A278. The swapped β-strand intercalates in the β-sheet between strands β1 and β2. The two dimers have different angles between monomers at the hinge region, resulting in a different distance between monomers. The distance between Cβ of Tyr257 residues of each dimer is shown as a reference. (B) Monomer A of ORF112 Zalpha (magenta) was superimposed on a Zalpha ADAR1 monomer (orange) from the crystal structure of the Zalpha-DNA complex (PDB, 1QBJ). Also shown are the ORF112 monomer B (yellow) and the second monomer from the ADAR1 Zalpha structure (translucent orange). For clarity, only one DNA strand along with key DNA-interacting residues Tyr177/Tyr257, Asn173/Asn253, and Trp 195/Trp274 (ADAR1/ORF112) are shown in stick representation. The location of a sulfate ion (S1) occupying the same location as a critical Z-DNA recognition backbone phosphate and contacting the domain-swapping hinge region is indicated in blue.

dylate monophosphate kinase (TmpK), ribonucleotide reductase and thymidine kinase in poxviruses and the family *Alloherpesviridae*, suggesting that *Alloherpesviridae* may be evolutionarily related to poxviruses (14).

Fish of the cyprinid family, including zebrafish, were recently shown to have, in addition to PKR, a protein called PKZ, with two Z-DNA binding Zalpha domains replacing the dsRNA binding domain. The PKZ protein inhibits cellular protein translation, as does PKR, but in this case in response to DNA repeats which can adopt a Z-DNA conformation (22). We show that the Zalpha domain of ORF112 is homologous to PKZ Zalpha domains and it can compete with Zalpha1 of PKZ in DNA binding in vitro. These results suggest that the function of ORF112 may be to block the activation of PKZ and the subsequent antiviral host responses. The mechanism of such inhibition would be competition between ORF112 and cellular receptors for nucleic acids, as has been shown for E3L. Our structure also suggests potential alternative models that involve inactive heterodimer formation between host Zalpha and ORF112. Further work could evaluate the formation of domain-swapped heterodimers with other members of the Zalpha family. Based on the above results, and because CyHV3 is emerging as a source

of devastating disease for carp and koi worldwide, ORF112 may represent an important pharmacological target.

Despite a relatively low sequence identity, structurally the Zalpha domain of ORF112 shares the fold of Zalpha domains from ADAR1, DAI, and E3L. Moreover, key DNA-interacting residues are conserved, as well as the major features of its hydrophobic core, and consistent with this, ORF112 firmly binds oligonucleotides bearing CpG repeats in vitro. At the same time, the crystallographic structure of ORF112 Zalpha forms dimers through domain swapping of the C-terminal strand, a unique feature not previously observed for Zalpha domains. The possible functional significance of this observation is currently unknown. It is possible that the dimer formation may have been induced by the crystallization conditions, as we did not detect these structures under physiological conditions. Intriguingly, however, in the crystal structure we found sulfate ions occupying positions that accurately reflect the positions of DNA phosphates in the complex as well as at key hinge positions of the swapped loops (Fig. 3B). Indeed we show that sulfate binding is necessary for the induction of domain swapping (Fig. 2E). This finding raises the possibility that DNA binding may induce similar reorganization and oligomerization of the protein, although it is unclear how the conformational changes could occur without disrupting the complex. Indeed, oligomerization of Zalpha domain-containing proteins on DNA has been previously suggested to play important role in their function (23). Further work using the entire PKZ kinase would be needed to clarify the interaction between ORF112 and PKZ.

In conclusion, we have identified a new member of the Z domain family, a potential inhibitor of the interferon response in cyprinid herpesvirus 3. This protein exhibits the novel characteristic of domain swapping as a mechanism for its dimerization and oligomerization.

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