Infectious Bursal Disease Virus VP3 Upregulates VP1-Mediated RNA-Dependent RNA Replication

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Genome replication is a critical step in virus life cycles. Here, we analyzed the role of the infectious bursal disease virus (IBDV) VP3, a major component of IBDV ribonucleoprotein complexes, on the regulation of VP1, the virus-encoded RNA-dependent RNA polymerase (RdRp). Data show that VP3, as well as a peptide mimicking its C-terminal domain, efficiently stimulates the ability of VP1 to replicate synthetic single-stranded RNA templates containing the 3' untranslated regions (UTRs) from the IBDV genome segments.

Infectious bursal disease virus (IBDV) is an immunosuppressive avian pathogen belonging to the *Birnaviridae*, a family of viruses harboring bisegmented double-stranded RNA (dsRNA) genomes enclosed within naked T=13 icosahedral capsids (1). Genome segment A (3.2 kb) harbors two partially overlapped open reading frames (ORF). ORF A1 encodes VP5, a small (17-kDa) polypeptide associated with virus dissemination (2, 3). ORF A2 encodes a large (107-kDa) polypeptide that undergoes a rapid self-proteolytic processing, yielding three polypeptides: the precursor of the capsid protein (pVP2; 54 kDa), the protease (VP4; 25 kDa), and VP3 (28 kDa). VP3 is a multifunctional protein involved in (i) virus morphogenesis, acting as a scaffolding element during the assembly of the virus particle (4–6) and recruiting the virus encoded RNA-dependent RNA polymerase (RdRp) to the virus particle (5, 6); (ii) RNA binding, shielding the viral genome in stable ribonucleoprotein complexes (RNPs) (6–8) and controlling the host's innate antiviral responses triggered by dsRNA molecules (9–12), and (iii) regulation of the RdRp activity (13). The crystal structure of the central region of IBDV VP3 (residues 90 to 220) showed that the polypeptide is arranged as a dimer, with each monomer folded in two α -helical domains connected by a long and flexible hinge (13).

Genome segment B (2.8 kb) contains a single ORF encoding VP1, the viral RdRp, which is present within the virion in two molecular forms, i.e., as a soluble protein and as VPg, covalently linked to the 5' ends of both genome segments in the RNPs (14). VP1 belongs to a special group of unconventional polymerases exhibiting a permutation of the conserved sequence motifs found in the palm subdomain that contains the catalytic site (15).

In a previous work, we mapped the VP1-binding domain within the 16 C-terminal residues of the VP3 polypeptide (16). Thereafter, we solved the X-ray structures of the IBDV RdRp VP1 both in its apo form and bound to a peptide harboring the RdRp-binding domain of VP3 (pepVP3; sequence GRLGRWIRTVSDE DLE). Although pepVP3 was disordered in the VP1-pepVP3 complex, comparisons of the RdRp catalytic cavities showed an ~ 8 -Å movement of a loop at the N terminus of motif B (called the B loop) from a completely closed conformation in the unbound state, in which the B loop protrudes toward the catalytic cavity, to an open catalytically competent conformation in the pepVP3-bound form (17). Important movements of the B loop have also been observed in RdRp structures from other RNA virus families

(18). In addition, another IBDV VP1 structure obtained under different crystallization conditions showed that the B loop can adopt an open conformation even in the absence of VP3 (19). Furthermore, it was recently reported that the conformational flexibility of this B loop is required for proper enzyme performance (20). The conformational rearrangements observed when the unbound and pepVP3-bound VP1 structures were compared prompted us to hypothesize a possible role of VP3 in regulating VP1 activity (17). This hypothesis was preliminarily confirmed by the characterization of the effect of both pepVP3 and the full-length VP3 on VP1 replicative activity assays on a single-stranded RNA (ssRNA) template containing the 5' and 3' untranslated regions (UTR) from IBDV genome segment B. The results of that study showed that preincubation of VP1 with either pepVP3 or full-length VP3 significantly increased the VP1 RdRp activity (17).

Recently, the structure of the RdRp encoded by another member of the *Birnaviridae* family, i.e., the infectious pancreatic necrosis virus (IPNV), was solved in complex with a 12-amino-acid peptide derived from the IPNV VP3 C terminus (21). Interestingly, the IPNV VP3 peptide binds to an extended groove at the surface of the VP1 fingers located ~ 25 Å away from the B loop (21). In contrast to data gathered with the IBDV RNA polymerase, preincubation of the IPNV RdRp with the VP3 peptide does not stimulate the replicative activity of the enzyme, as determined using a replication assay based on the use of a non-IPNV-related ssRNA template.

These apparently conflicting observations prompted us to undertake an in-depth analysis of the effect of the VP3 polypeptide

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FIG 1 Stimulation of the RNA polymerization activity of IBDV VP1 by pepVP3 and VP3. (A) Representative autoradiograms of *in vitro* RdRp activity of VP1 analyzed in 7% acrylamide–TBE gels on the segment A-derived template showing the polymerization products obtained at different incubation times. Reactions were carried out in the absence (left) or presence (right) of pepVP3. (B) Kinetics profile of the RdRp activity under conditions shown in panel A. Relative activity values were calculated by considering the maximum activity obtained in samples incubated in the absence of pepVP3 as 100%. Each point is the average from three independent experiments. (C and D) *In vitro* RdRp activity of VP1 on templates derived from the 3′-terminal regions of the IBDV genome segments A and B. A_m corresponds to the A template with a 20-nt deletion at the 3′ end. Assays were performed in the absence (–) or presence (+) of the full-length VP3 protein.

on the replication activity of the IBDV RdRp and the role of the template RNA in such regulation. For this, a series of polymerization assays comparing the activity of the IBDV VP1 in absence or presence of either pepVP3 or the full-length VP3 protein were performed. The ssRNA templates used in these assays were prepared by T7 polymerase-based (Promega) *in vitro* transcription using purified plasmids harboring cDNAs flanked by the bacteriophage T7 promoter and the hepatitis δ ribozyme sequence. After transcription, reaction mixtures were subjected to digestion with DNase I (Roche), extracted with phenol-chloroform, and precipitated with ethanol. Purified ssRNAs were resuspended in diethyl pyrocarbonate (DEPC)-treated H_2O and used as templates for IBDV VP1 replication assays.

Replication assays were carried out according to a previously described protocol (17). Briefly, reaction mixtures contained 1 μ g of freshly purified hVP1 and hVP3 in a molar hVP1/hVP3 ratio of 1:15 (hVP1 and hVP3 are recombinant versions of the IBDV proteins VP1 and VP3, both harboring an N-terminal polyhistidine tag) or synthetic pepVP3 (molar hVP1/pepVP3 ratio of 1:2). In order to compensate for the observed tendency of the hVP3 recombinant polypeptide to undergo a proteolytic trimming of its C-terminal domain (4), a higher molar hVP3 ratio was used in reactions performed with the full-length protein. Reaction mixtures were prepared in 40 μ l of transcription buffer (100 mM Tris-HCl [pH 8.5], 125 mM NaCl, 4 mM $MgCl_2$, 0.01 mM EGTA, 20 units of RNasin, 1 mM ATP, CTP, and GTP, and 0.02 mM UTP). Samples were incubated at 25°C for 30 min, and then supplemented with 5 μ l of the corresponding ssRNA template (0.2 mg/ml) diluted in transcription buffer containing 10 μ Ci of [α - ^{32}P]UTP. After incubation at 40°C for 2 h, or the time indicated for each experiment, reactions were halted by heating at 100°C for

3 min, and the products were digested with proteinase K (100 μ g/ml) for 1 h at 37°C, heated at 65°C for 5 min, and then subjected to nondenaturing electrophoresis on 7% acrylamide–TBE (90 mM Tris, 64.6 mM boric acid, and 2.5 mM EDTA [pH 8.3]) gels. Radioactive signals were detected with a Storm gel imaging system (Molecular Dynamics), and results were analyzed and quantified using tools from the ImageJ software package (<http://imagej.nih.gov/ij/>).

We first characterized the VP1 polymerization activity using a 639-nucleotide (nt) RNA template corresponding to the 3′-terminal region, including the UTR, of IBDV segment A. Reactions were carried out in the absence or presence of pepVP3, and samples were collected at different postincubation times. As shown in Fig. 1, a radioactive band was detected in both cases, thus demonstrating the activity of the hVP1 enzyme. In agreement with our previously reported data (17), a conspicuous difference was observed in the kinetics of accumulation of the specific RNA reaction product, thus confirming that the addition of pepVP3 results in a major boost of VP1 enzymatic activity. It is noteworthy that although the polymerization reaction leads to formation of dsRNA molecules, ssRNA molecules generated by *in vitro* transcription of selected DNA templates were used as indicative molecular size markers.

To further assess the boosting effect caused by pepVP3, a quantitative analysis was performed using data from three independent experiments. The results of this analysis, shown in Fig. 1B, demonstrate that in the presence of pepVP3, accumulation of the specific polymerization product follows a typical sigmoidal kinetics, reaching a plateau as early as at 30 min postincubation, most likely reflecting the complete consumption of the radioactive UTP used to monitor the reaction. In contrast, in the absence of the pep-

FIG 2 Effect of the template on IBDV VP1 activity. (A) Effect of pepVP3 on VP1 replication activity. Polymerization reactions were performed in the absence (–) or presence (+) of pepVP3 using RNA templates derived from IBDV segments A and B and from an unrelated virus (U). (B) Increase in VP1 activity promoted by the presence of pepVP3 in the replication. Activity enhancement values were calculated by considering data obtained in the absence of VP3 as 100%. Each value is the average from three independent experiments. (C) Electrophoretic mobility shifts for the ssRNA templates derived from viral segment A (A) and the unrelated RNA sequence (U), when incubated for 10 min at room temperature with different amounts of purified VP1 protein (1.4, 1.0, 0.36, and 0.12 μ g). Protein-RNA complexes were resolved in 1.5% agarose-TBE gels, stained with SYBR Safe Gold (Life Technologies), and photographed under UV light. RNA controls (–), loaded in the absence of VP1, are shown in the first lane of each gel.

tide, the reaction follows a linear kinetics, reaching its maximum at 2 h postincubation. Finally, as shown in Fig. 1C and D, the stimulatory effect on VP1 activity is even higher when the purified full-length VP3 protein is used instead of the pepVP3. The increased enhancement effect observed with the full-length VP3 polypeptide might reflect the higher VP3 molar ratio used in these assays.

In order to evaluate whether the VP1 replication activity is dependent upon the specificity of the RNA template, parallel assays were carried out using three ssRNA templates: (i) the 639-nt template (template A) corresponding to the IBDV segment A 3'-terminal region described above; (ii) a 539-nt RNA template (template B) corresponding to the IBDV segment B; and (iii) an unrelated 631-nt RNA template (template U), generated by *in vitro* transcription of the pVOTE.2 expression plasmid digested with EcoRI (22). Polymerization reactions were performed either in the presence or absence of pepVP3 for 1 h. As shown in Fig. 2A, addition of pepVP3 to reaction mixtures resulted in a significant enhancement on the accumulation of reaction products corresponding to IBDV-derived RNA templates. Quantification of samples from three independent experiments showed that addition of pepVP3 to reaction mixtures results in average increases in the accumulation of specific reaction products of over 300 and 220% for templates derived from segments A and B, respectively (Fig. 2B). As expected from previous experiments, very faint bands were detected in reaction samples corresponding to the unrelated RNA template. The weakness of the radioactive signal prevented a reliable quantification of the effect that pepVP3 may exert on the replication of this IBDV-unrelated template. It is noteworthy that similar results were obtained with other IBDV-unrelated templates.

Results described above show that the ability of VP1 to replicate unrelated RNA templates is dramatically lower than that of IBDV-specific RNAs. It has been shown that the 3' UTRs of both

IBDV genome segments contain predicted stem-loop structures that are essential for efficient virus replication (23). To assess whether this predicted secondary structure might affect VP1 polymerization, a series of assays were carried out using IBDV segment A- and B-derived templates lacking nucleotide sequences thought to be involved in stem-loop formation (i.e., 20-nt and 30-nt deletions at the 3' end of segment A and B, respectively). The results of this analysis did not reveal significant differences in either the ability of VP1 to replicate IBDV-derived templates or the pepVP3 boosting activity (Fig. 1D).

Indeed, the enhanced replication of specific RNA templates might reflect an increased ability of the VP1 polypeptide for the binding of IBDV-specific RNA templates. Therefore, we next analyzed the binding of VP1 to both specific and unspecific RNA templates. This analysis was performed using electrophoretic mobility shift assays following a previously described protocol (7), with templates A and U described above. The results of this analysis showed that VP1 binds both templates with similar efficiency (Fig. 2B). Although information about the molecular mechanism(s) underlying the low efficiency on the ability of VP1 to replicate unspecific templates is pending, our results highlight the critical importance of using specific templates for a proper analysis of VP1 replication activity.

Data described in this report unambiguously show that the IBDV VP3 protein enhances the enzymatic activity of the RNA polymerase VP1. Such enhancement, however, seems to be subject to the use of viral genome sequences as templates for the polymerase. Remarkably, removal of the 3'-most sequences of both genomic segments, which contain a predicted stem-loop putatively involved in viral replication (23), did not prevent the VP3 boosting effect, suggesting the presence of other elements in the IBDV genome able to modulate the VP1 polymerase activity.

At this point, we cannot rule out the existence of differences in the regulation of the RdRp activity between different birnavirus

genera. The VP3 binding site identified in the X-ray structure of IPNV VP1-VP3 peptide complex is located far from the B loop, consistent with its proposed role as a mediator of VP1 recruitment into birnavirus capsids during morphogenesis (21). It remains to be determined whether the regulation of VP1 activity shown here requires VP3 binding to a different site of the polymerase or whether VP3 acts as an allosteric modulator, not simply turning on VP1 activity but offering selective control of the substrate that will be used for replication. A similar example of allosteric regulation of RdRp activity by other viral factors has been reported for HCV NS5B, showing that the HCV replication reaction *in vitro* is enhanced by the protein NS5A in a template-specific manner (24).

Birnaviruses exhibit a number of structural and functional features that differentiate them from prototypical icosahedral dsRNA viruses. A prominent difference is the lack of a transcriptional core, a structure ubiquitously present in all other icosahedral dsRNA viruses, that shelters the virus genome throughout the replication cycle, preventing the exposure of dsRNA to specialized cellular sentinel devices (25, 26). This vital structural element is replaced by RNPs in members of the *Birnaviridae* family (8). We recently showed that that RNPs act as capsid-independent functional units during the IBDV replication process (27). In this context, the ability of the VP3 polypeptide to activate VP1-mediated RNA replication might play a key role in preventing the formation of unspecific RNA duplexes capable of triggering harmful antiviral responses by cellular dsRNA sensors.

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