Identification and functional analysis of VP3, the guanylyltransferase of Banna virus (genus Seadornavirus, family Reoviridae)

Fauziah Mohd Jaafar,1 Houssam Attoui,1 Peter P. C. Mertens,2 Philippe de Micco1 and Xavier de Lamballerie1,3

Correspondence Houssam Attoui h-attoui-ets-ap@gulliver.fr

1Unité des Virus Emergents: EFS Alpes-Méditerranée and Faculté de Médecine de Marseille, Université de la Méditerranée, 27 Boulevard Jean Moulin, 13005 Marseille cedex 5, France
2Institute for Animal Health, Pirbright Laboratory, Ash Road, Pirbright, Woking, Surrey GU24 0NF, UK
3Maladies Virales Émergentes et Systèmes d’Information UR034, Institut de Recherche pour le Développement, Marseille, France

Received 2 September 2004
Accepted 13 December 2004

Banna virus (BAV) particles contain seven structural proteins: VP4 and VP9 form an outer-capsid layer, whilst the virus core contains three major proteins (VP2, VP8 and VP10) and two minor proteins (VP1 and VP3). Sequence analysis showed that VP3 contains motifs \[Kx(I/V/L)S\] and (Hx\(n\)H) that have previously been identified in the guanylyltransferases of other reoviruses. Incubation of purified BAV-Ch core particles with \[^{32}\text{P}GTP\] resulted in exclusive covalent labelling of VP3, demonstrating autoguanylation activity (which is considered indicative of guanylyltransferase activity). Recombinant VP3 prepared in a cell-free expression system was also guanylated under similar reaction conditions, and products were synthesized (in the presence of non-radiolabelled GDP) that co-migrated with GMP, GDP and GpppG during TLC. This reaction, which required magnesium ions for optimum activity, demonstrates that VP3 possesses nucleoside triphosphatase (GTPase) activity and is the BAV guanylyltransferase (RNA ‘capping’ enzyme).

The family Reoviridae is divided into 12 distinct genera (Mertens et al., 2004), four of which contain viruses that infect mammals, including humans (Coltivirus, Orbivirus, Rotavirus and Seadornavirus) (Mertens et al., 2000, 2004). Banna virus (BAV) is the type species of the genus Seadornavirus (Attoui et al., 2000; Mertens et al., 2004) and was first isolated from sera and cerebrospinal fluids of humans suffering from encephalitis in China (Xu et al., 1990; Li, 1992; Chen & Tao, 1996). Purified BAV particles are icosahedral with an external diameter of 72–75 nm, which is typical of ‘non-turreted’ reoviruses (Mohd Jafaar et al., 2005). The intact BAV virion is composed of an outer-capsid layer (containing proteins VP4 and VP9), surrounding the virus core composed of three major structural proteins [VP2 (subcore shell), VP8 (core-surface layer) and VP10] and two minor proteins (VP1 and VP3).

The reoviruses (a term used here to indicate all members of the family Reoviridae) share a common overall replication strategy that is dictated by their multisegmented, double-stranded RNA (dsRNA) genomes, which involves the synthesis of full-length mRNA copies from each of the genome segments (Estes, 2001; Nibert & Schiff, 2001; Mertens & Diprose, 2004). These individual mRNAs not only serve as templates for translation into viral proteins within infected cells, but are also reassembled as templates for minus-strand synthesis (during morphogenesis of virus particles), which reforms the dsRNA segments of the progeny virus genome (Chen et al., 1999; Mertens, 2004). Reoviruses provide their own transcriptase and capping enzymes to initiate replication.

With the possible exception of the cypoviruses, the reoviruses manage to avoid direct contact between the virus genome and the cell cytoplasm (which could activate dsRNA-dependent defence mechanisms; Beattie et al., 1995; Yue & Shatkin, 1997) by using transcription and capping enzymes, which are structural components, to synthesize the viral mRNAs within the virus core (Mertens, 2004; Taraporewala & Patton, 2004). In particular, the capping of nascent viral mRNA strands is mediated by a guanylyltransferase that uses GTP (or in some cases GDP) to form a covalently bound enzyme–substrate intermediate (autoguanylation) before the GMP residue is transferred to an NDP (\(N=\text{A or G}\) at the 5’ end of the nascent mRNA (Mertens et al., 1992; Ramadevi & Roy, 1998; Ramadevi et al., 1998; Chen et al., 1999; Luongo, 2002; Qiu & Luongo, 2003).
Based on sequence analyses and identification of conserved motifs, VP1 is believed to be the BAV polymerase (Attoui et al., 2000), suggesting that VP3 may also provide one or more of the other enzyme functions that are usually associated with the reovirus core, e.g. transmethylation, guanylyltransferase [capping enzyme (CaP)], helicase or nucleoside triphosphatase (NTPase).

The RNA-dependent RNA polymerase of the orbiviruses, orthoreoviruses and rotaviruses is encapsidated as 12 monomers, situated at the fivefold axes within the innermost (T = 2) layer of the core particle (Stuart et al., 1998; Mertens et al., 2000, 2004; Taraporewala & Patton, 2004). In contrast, the guanylyltransferases of the 'turreted' reoviruses form pentameric projections ('turrets' or 'spikes') located at each of the 12 fivefold axes on the surface of the innermost shell of the core, with a copy number of 60 protein molecules per particle (Nibert & Schiff, 2001). Although the guanylyltransferase of the 'non-turreted' reoviruses is also situated at the 12 fivefold axes, it is associated with the transcriptase complexes on the inside of the T = 2 layer. There is evidence that the capping enzyme, VP4 (CaP), of Bluetongue virus (BTV) is a functional dimer (Ramadevi et al., 1998) and it is packaged as 24 copies per particle in both the orbiviruses and rotaviruses (Lawton et al., 1997; Stuart et al., 1998; Mertens et al., 2004). Information concerning the RNAs and proteins of dsRNA viruses can be found at http://www.iah.bbsrc.ac.uk/dsRNA_virus_proteins/.

The guanylyltransferases of the orthoreoviruses (j2), aquareoviruses (VP1), orbiviruses (VP4) and rotaviruses (VP3) were initially identified by autoguanylation assays using core particles or recombinant proteins (Mertens et al., 1992; Martinez-Costas et al., 1998; Chen et al., 1999; Luongo et al., 2000; Luongo, 2002). Functional analyses have shown that the rotavirus capping enzyme is non-specific, as it was able to cap both viral and non-viral RNAs (Patton & Chen, 1999). The aim of this study was to identify and characterize the BAV guanylyltransferase.

A Chinese strain of BAV (BAV-Ch), originally isolated from a patient with encephalitis, was propagated in C6/36 cells as described elsewhere (Attoui et al., 2000). BAV core particles were purified on discontinuous cesium chloride gradients (Mohd Jaafar et al., 2005). The virus band was recovered and dialysed overnight at 25 °C against 100 mM Tris/HCl (pH 8–0), 10 mM MgCl2. BAV VP3 was also expressed as a recombinant protein in a cell-free coupled transcription/translation system (Roche), as described below.

The sequence of the VP3 of three seadornaviruses [BAV-Ch, BAV-In6423 and Kadipiro virus (KDV) Ja7075; GenBank accession nos AY549307, AF134515 and AF134510, respectively] were investigated for the presence of the sequence motif Kx(I/V/L)S, previously identified in the guanylyltransferases of Mammalian orthoreovirus (MRV), BTV and Rotavirus A (RvA) (Luongo, 2002). KSIS, KTIS and KAIS motifs were identified at amino acid positions 109–112 of VP3 from BAV-Ch, BAV-In6423 and KDV-Ja7075, respectively (Fig. 1a).

Fig. 1. Partial sequence comparison of seadornavirus VP3 proteins with the guanylyltransferases of other reoviruses. (a) Sequence comparison of seadornavirus VP3 proteins and the guanylyltransferases of other reoviruses in the vicinity of the putative active motif Kx(V/I)S (the motif is shown in bold). Identical and similar residues are shaded. BAV, Banna virus; KDV, Kadipiro virus (Seadornavirus); RvA, Rotavirus A (Rotavirus); MRV3, Mammalian orthoreovirus serotype 3 (Orthoreovirus); BTV, Bluetongue virus; AHSV, African horse sickness virus; CHUV, Chuan virus (Orbivirus). (b) Sequence comparison of seadornavirus VP3 proteins and the guanylyltransferases of other reoviruses in the vicinity of the two histidine residues (shown in bold) that are involved in the guanylyltransferase activities of aquareoviruses (GCRV, grass carp reovirus positions 229 and 238) and orthoreoviruses (MRV3 positions 223 and 232; ARV, avian reovirus positions 221 and 230). Identical or similar residues are shaded.
Two histidine residues, separated by 9 aa (HxH motif), that are also involved in the guanylyltransferase activity of MRV \(p2\) (positions 223 and 232) and aquareovirus VP1 (positions 229 and 238) have been previously identified by site-directed mutagenesis (Qiu & Luongo, 2003). Two histidine residues separated by 13 aa (positions 229 and 238) have been previously identified for the guanylyltransferase activity of both aquareoviruses and orthoreoviruses (Fig. 1b).

VP3 of BAV also shows amino acid identity of 28 % (aa 467–540) to the RvA guanylyl- and methyltransferase VP3 (CaP) between aa 449 and 710. The VP3 of KDV (a species of seadornavirus that is distinct from BAV) has only 21 % amino acid identity, but over a longer sequence (aa 224–654) than the simian SA11 RvA VP3 (CaP), between aa 252 and 632. Overall, these data suggest that VP3 is the guanylyltransferase of both seadornaviruses.

Purified BAV cores (5 \(\mu\)g) were analysed in vitro for guanylyltransferase activity by incubation with 10 \(\mu\)Ci \([\alpha-32P]GTP\) in 50 \(\mu\)l 100 mM Tris/HCl (pH 8-0) containing 10 mM MgCl2, for 30 min at 30 °C (Mertens et al., 1992). The cores were solubilized immediately by boiling in denaturation buffer [160 mM Tris/HCl (pH 6-8), 4 mM EDTA, 3-6 % SDS, 60 mM dithiothreitol, 0-2 % \(\beta\)-mercaptoethanol, 0-8 % methionine] and then analysed by 10 % SDS-PAGE. The dried gel was autoradiographed, showing strong and exclusive labelling of VP3 (Fig. 2a). Boiling with SDS prior to PAGE did not release the label, indicating covalent bonding to the VP3 molecule and identifying it as a putative guanylyltransferase.

The open reading frame (nt 11–2170) of BAV-Ch segment 3 was transcribed into cDNA and amplified by PCR using the primers BAVchpivexS and BAVchpivexR, shown below. These contain virus-specific sequences (underlined) and NotI and XhoI restriction-enzyme sites (bold) to facilitate cloning into the pIVEX 2.3 MCS vector (Roche), allowing the expression of recombinant VP3 tagged with 6 × histidine at the carboxyl terminus (VP3–6 × His): [BAVchpivexS: 5′-GCACATATGAGCCGCGCATGGAATTTATTTAGTGAAGAGAAG-3′ (BAV segment 3 positions 11–36); BAVchpivexR: 5′-ACTGAGCTCGCTGAAGATCAATCTGATTATACATTCA-3′ (BAV segment 3 positions 2170–2145)]. The vector and PCR products were digested with NotI and XhoI, gel-purified, ligated by using T4 DNA ligase into pIVEX 2.3 MCS and the plasmids were transformed into competent XL-Blue MRF\(^+\) bacteria. Clones were screened by PCR with primers pivex5 (5′-TAATACGTACTCATAAGGG-3′, positions 620–636) and pivex3 (5′-GCTAGTTATGGTCAGCG-3′, positions 881–863). One positive clone was selected, grown in LB medium and the recombinant pIVEX-VP3 plasmid was purified by using a QIAprep Miniprep kit (Qiagen).

VP3–6 × His was expressed by incubating 15 \(\mu\)g plasmid in 1 ml translation reaction (bacterial lysate, energy source, amino acids, recombinant pIVEX vector) for 20 h at 30 °C, with stirring at 120 r.p.m., in a coupled transcription/translation bacterial cell-free system (RTS500 circular-template kit; Roche). The recombinant VP3–6 × His was purified by nickel-affinity chromatography using Ni-NTA Sepharose matrix (Qiagen) and eluted in 200 mM imidazole, Tris/HCl (pH 8-0). The protein was dialysed overnight at 15 °C against 100 mM Tris/HCl (pH 8-0) containing 8 % methionine, Tris/HCl (pH 8-0), 4 mM EDTA, 3-6 % SDS, 60 mM dithiothreitol, 0-2 % \(\beta\)-mercaptoethanol, 0-8 % methionine and then analysed by 10 % SDS-PAGE.

The dried gel was autoradiographed, showing strong and exclusive labelling of VP3 (Fig. 2a). Boiling with SDS prior to PAGE did not release the label, indicating covalent bonding to the VP3 molecule and identifying it as a putative guanylyltransferase.

open reading frame (nt 11–2170) of BAV-Ch segment 3 was transcribed into cDNA and amplified by PCR using the primers BAVchpivexS and BAVchpivexR, shown below. These contain virus-specific sequences (underlined) and NotI and XhoI restriction-enzyme sites (bold) to facilitate cloning into the pIVEX 2.3 MCS vector (Roche), allowing the expression of recombinant VP3 tagged with 6 × histidine at the carboxyl terminus (VP3–6 × His): [BAVchpivexS: 5′-GCACATATGAGCCGCGCATGGAATTTATTTAGTGAAGAGAAG-3′ (BAV segment 3 positions 11–36); BAVchpivexR: 5′-ACTGAGCTCGCTGAAGATCAATCTGATTATACATTCA-3′ (BAV segment 3 positions 2170–2145)]. The vector and PCR products were digested with NotI and XhoI, gel-purified, ligated by using T4 DNA ligase into pIVEX 2.3 MCS and the plasmids were transformed into competent XL-Blue MRF\(^+\) bacteria. Clones were screened by PCR with primers pivex5 (5′-TAATACGTACTCATAAGGG-3′, positions 620–636) and pivex3 (5′-GCTAGTTATGGTCAGCG-3′, positions 881–863). One positive clone was selected, grown in LB medium and the recombinant pIVEX-VP3 plasmid was purified by using a QIAprep Miniprep kit (Qiagen).

VP3–6 × His was expressed by incubating 15 \(\mu\)g plasmid in 1 ml translation reaction (bacterial lysate, energy source, amino acids, recombinant pIVEX vector) for 20 h at 30 °C, with stirring at 120 r.p.m., in a coupled transcription/translation bacterial cell-free system (RTS500 circular-template kit; Roche). The recombinant VP3–6 × His was purified by nickel-affinity chromatography using Ni-NTA Sepharose matrix (Qiagen) and eluted in 200 mM imidazole, Tris/HCl (pH 8-0). The protein was dialysed overnight at 15 °C against 100 mM Tris/HCl (pH 8-0) containing 8 % methionine, Tris/HCl (pH 8-0), 4 mM EDTA, 3-6 % SDS, 60 mM dithiothreitol, 0-2 % \(\beta\)-mercaptoethanol, 0-8 % methionine and then analysed by 10 % SDS-PAGE.

The dried gel was autoradiographed, showing strong and exclusive labelling of VP3 (Fig. 2a). Boiling with SDS prior to PAGE did not release the label, indicating covalent bonding to the VP3 molecule and identifying it as a putative guanylyltransferase.

**Fig. 2.** Autoradiography of radiolabelled VP3 of BAV-Ch. (a) Autoguanylation of VP3 from native BAV-Ch cores. Lanes: 1, core particles of BAV-Ch were incubated with \([\alpha-32P]GTP\) in the presence of 10 mM MgCl2 and analysed by SDS-PAGE and Coomassie blue staining; 2, autoradiography of the core shown in lane 1; M, size markers. The position of VP3 is indicated by an arrow. (b) Autoguanylation of VP3 from recombinant-expressed protein. Lanes: M, size markers labelled in kDa; 1, purified recombinant VP3 of BAV-Ch stained with Coomassie blue; 2, autoradiography of recombinant VP3, autoguanylated in the presence of \([\alpha-32P]GTP\) and 10 mM MgCl2. (c) Magnesium-ion dependence of autoguanylation by recombinant-expressed VP3. Lanes: 1, autoradiography of recombinant VP3, autoguanylated in the presence of \([\alpha-32P]GTP\) and 10 mM MgCl2; 2, BSA incubated with \([\alpha-32P]GTP\) and 10 mM MgCl2 (negative control); 3, autoradiography of recombinant VP3, autoguanylated in the presence of \([\alpha-32P]GTP\) but in the absence of 10 mM MgCl2 (the weak autoguanylation signal is indicated by an arrow).
10 mM MgCl₂ and quantified by UV spectrophotometry. The expressed and purified VP3–6 × His was analysed by 10% SDS-PAGE (Fig. 2b) and stained with Coomassie brilliant blue. Western immunoblots using anti-His tag antibodies (Qiagen) revealed a single band of protein at ~84 kDa [the expected size of the expressed protein (data not shown)].

Expressed VP3–6 × His (5 µg) was reacted with 10 µCi [α-³²P]GTP in 50 µl autoguanylation assays, containing 100 mM Tris/HCl (pH 8·0) and 10 mM MgCl₂, as described previously (Luongo et al., 2000). The VP3–6 × His became covalently labelled with a similar efficiency to that of the native protein from virus particles (Fig. 2b, 2c lane 1), confirming that none of the other virus structural proteins are required for this activity (autoguanylation) of BAV VP3. No labelling was detected in a negative control containing only BSA (Fig. 2c lane 2).

Studies of autoguanylation by reovirus guanylyltransferases (β2 of MRV, VP4 of BTV and VP3 of rotavirus) have shown that the activity of these enzymes is dependent on divalent cations (magnesium and manganese) (Yamakawa et al., 1982; Le Blois et al., 1992; Mertens et al., 1992; Martinez-Costas et al., 1995; Cho et al., 1997; Ho et al., 1998; Luongo et al., 1998; Ramadevi & Roy, 1998; Ramadevi et al., 1998; Wen et al., 1998; Luongo, 2002; Qiu & Luongo, 2003). The dependence of BAV VP3 on magnesium ions during autoguanylation was demonstrated by carrying out the assays in the absence of magnesium chloride, resulting in only weak labelling of the VP3 band (Fig. 2c lane 3). This suggests that, like the guanylyltransferases of other eukaryotes and other reoviruses (Yamakawa et al., 1982; Martinez-Costas et al., 1995; Ho et al., 1998; Qiu & Luongo, 2003), the rate of cap-structure synthesis by VP3 is also dependent on magnesium ions.

Reactions (20 µl) containing recombinant VP3–6 × His protein and magnesium ions (10 mM MgCl₂) (with the addition of 0·5 mM final concentration of non-radioactive GDP; Luongo et al., 2000) were incubated and then spotted directly onto polyethyleneimine cellulose (PEI-C) TLC plates containing a fluorescent indicator (Sigma). The plate was placed vertically into a sealed tank, with 0·5 M NH₄HCO₃ as the mobile phase, and left to run for 2 h. The plates were air-dried for 1 h, covered with Saran wrap and the reaction products were detected by autoradiography. Radioactive products were detected (Fig. 3a lane 4) that co-migrated with unlabelled GMP, GDP, GTP and GpppG used as markers (Fig. 3a lane 5), as shown previously for β2 of MRV (Martinez-Costas et al., 1998; Luongo et al., 2000; Luongo, 2002), confirming directly that BAV VP3 possesses both GTPase and guanylyltransferase activities. However, GpppG was not detected in reactions that did not contain the unlabelled GDP (Fig. 3a lane 3). Chromatographic markers for radiolabelled GDP, GMP and inorganic phosphate (Pi) were generated by treating [α-³²P]GTP with 5 U calf intestinal phosphatase (CIP; Roche) as described previously (Chen et al., 1999) at 30 °C for 5 min (Fig. 3a lane 1).

Some non-viral guanylyltransferases (including the mRNA capping enzyme of Saccharomyces cerevisiae; Itoh et al., 1984) do not transfer GMP onto GDP, but rather onto longer oligonucleotides (minimum of 3 nt). However, BAV-Ch VP3 can synthesize GpppG by the transfer of GMP onto GDP, suggesting that capping occurs at an early stage of RNA synthesis.

Guanylyltransferases of members of the family Reoviridae, such as the VP4 of BTV, also possess an inorganic pyrophosphatase activity (Martinez-Costas et al., 1995, 1998). This activity degrades inorganic pyrophosphate (PPi) that is generated as a by-product of GMP transfer onto the

---

**Fig. 3.** TLC analysis of guanylyltransferase reaction products. (a) TLC analysis of the guanylyltransferase reaction products generated by recombinant VP3. The reaction was carried out in the absence (lane 3) or presence (lane 4) of non-radioactive GDP. Lanes: 1, [α-³²P]GTP treated with CIP (5 min at 30 °C), showing GTP and the products GDP, GMP and Pi; 2, [α-³²P]GTP; 5, positions of authentic but non-radiolabelled GDP, GMP and GpppG (observed under UV light at 254 nm) that were used as markers. (b) Pyrophosphatase activity assay of VP3. Lanes: 1, the products when 5 µCi PPi was incubated with the reaction buffer alone [100 mM Tris/HCl (pH 8·0), 10 mM MgCl₂]; 2, products when the same amount of PPi was incubated with reaction buffer containing recombinant BAV-Ch VP3.
enzyme. To determine whether VP3 possesses an inorganic pyrophosphatase activity, 5 μg purified expressed protein was incubated in a 50 μl reaction volume containing 5 μCi [32P]-labelled PPi (Perkin Elmer) in 100 mM Tris/HCl (pH 8-0), 10 mM MgCl2, for 30 min at 30 °C. An aliquot of the reaction was then spotted onto a PEI-C plate and developed with 0.5 M NH4HCO3 as the mobile phase. Fig. 3b shows that VP3 has a pyrophosphatase activity that can cleave PPi, to yield Pi.

VP3 is the least abundant of the BAV core structural proteins, with an estimated seven copies per particle (Mohd Jaafar et al., 2005). However, structural studies of other reoviruses suggest that the transcriptase complexes are situated at each of the fivefold axes of the core particle, possibly as one complex per genome segment (Grimes et al., 1998; Stuart et al., 1998; Gouet et al., 1999; Diprose et al., 2001). It is therefore predicted that VP3 should be present as at least 12 copies per core particle (one for each transcription site), to allow capping of all nascent RNAs.

Cores of several other reoviruses also possess transmethylase activities that are required for synthesis of complete, methylated Cap1 structures at the 5’ termini of their RNAs. VP4 of BTV cores is the transmethylase as well as the guanylyltransferase (Ramadevi et al., 1998). As BAV particles do not contain other minor proteins (apart from VP1 and VP3) that are candidate enzymes, it is considered likely (but as yet unconfirmed) that VP3 is also a transmethylase.

Acknowledgements

This study was supported by EU grant ‘Reo ID’ number QLK2-2000-00143. The ‘Unité des Virus Emergents’ is an associated research unit of the Institut de Recherche pour le Développement (IRD). This study was supported in part by the IRD and EFS Alpes-Méditerranée.

References


