NTP binding and phosphohydrolase activity associated with purified bluetongue virus non-structural protein NS2

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The bluetongue virus ssRNA-binding protein, NS2, is a phosphoprotein that forms viral inclusion bodies in infected cells. Recombinant NS2 was expressed in the baculovirus expression system and purified to homogeneity from insect cells. Purified NS2 bound nucleosides. Further investigation revealed that the protein bound ATP and GTP and could hydrolyse both nucleosides to their corresponding NMPs, with a higher efficiency for the hydrolysis of ATP. The increased efficiency of hydrolysis of ATP correlated with a higher binding affinity of NS2 for ATP than GTP. Ca²⁺, Mg²⁺ and Mn²⁺ were able to function as the required divalent cations in the reactions. The phosphohydrolase activity was not sensitive to ouabain, an inhibitor of cellular ATPases, suggesting that this activity was not the result of a cellular contaminant.

Bluetongue virus (BTV), the aetiological agent of the arthropod-borne disease bluetongue, is a member of the genus Orbivirus in the family Reoviridae. Its genome consists of 10 dsRNA segments. Each segment, except for the smallest (segment 10), encodes a single protein (Mertens et al., 1984; French et al., 1989). The genome is encapsidated within a core of VP3 and VP7 along with the minor core proteins VP1, VP4 and VP6 (Verwoerd & Huismans, 1972; Huismans et al., 1987). An outer shell of VP2 and VP5 covers the VP7 layer of the core. In addition to these seven structural proteins, four non-structural proteins (NS1, NS2, NS3 and NS3a) are found in virus-infected cells (Roy et al., 1990). Although the functions of the non-structural proteins are not clear, it is believed that they may be involved in virus replication and assembly.

NS2, the 41 kDa product of the S8 gene, binds ssRNA but does not bind dsRNA (Thomas et al., 1990). It is the only phosphorylated BTV protein. The effect of phosphorylation on its RNA-binding capability has been investigated: Thomas et al. (1990) reported that there was no reduction in RNA binding when baculovirus-expressed NS2 was dephosphorylated, while Theron et al. (1994) used E. coli-expressed NS2 and observed a reduction, but not complete elimination, of RNA binding. NS2 forms viral inclusion bodies (VIBs) in both BTV-infected mammalian cells (Brookes et al., 1993) and insect cells when expressed from a recombinant baculovirus (Thomas et al., 1990), suggesting that neither other viral proteins nor a full complement of viral RNAs is necessary for VIB development. Neither the amino nor carboxy terminus of NS2 is required for VIB formation (Zhao et al., 1994). VIBs are believed to be the location at which virus assembly occurs.

In order to characterize NS2 biochemically, we obtained highly purified protein from recombinant baculovirus-infected insect cells. Spodoptera frugiperda cells, grown in suspension in TC100 medium supplemented with 5% foetal calf serum, were infected at an m.o.i. of 5 with a recombinant baculovirus (AcBTV10-NS2; Thomas et al., 1990) that expresses NS2 protein. Three days after infection, the cells were harvested by centrifugation at 2400 g for 10 min. The pellet was washed once with PBS and resuspended in NS2 lysis buffer [0.1 M NaH₂PO₄–Na₂HPO₄ buffer (NaPh) pH 8.0, 0.1 M NaCl, 1 mM EDTA, 1 mM DTT, 0.5% Triton X-100, 1 mM Pefabloc SC (Pentapharm AG), 1 mM 4-amidinophenylmethanesulphonyl fluoride, 10 µM E-64], using 5 ml per 100 ml culture fluid. Cells were lysed by ten strokes of a Dounce homogenizer and cell debris was pelleted at 60000 r.p.m. for 45 min in a Ti70 rotor at 10 °C.

The supernatant was passed over a Q-Sepharose column (Pharmacia) equilibrated with 0.1 M NaCl, 1 mM EDTA (pH 8.0) and the protein was eluted by a gradient of 0.1–0.5 M NaCl plus 1 mM EDTA (pH 8.0). Fractions above 0.25 M NaCl were analysed by SDS−PAGE (Fig. 1A). The peaks were pooled, diluted 1:4 with 0.1 M NaPi pH 8.0, 1 mM EDTA and passed over a heparin column (Pharmacia) equilibrated with 25 mM NaPi pH 8.0, 0.1 M NaCl, 1 mM EDTA. The column was washed with 25 mM NaPi pH 8.0, 0.1 M NaCl, 1 mM...
1 mM EDTA. A step gradient of 0–4 M NaCl was washed with 25 mM NaPi pH 8 with 25 mM NaPi pH 8 [prepared in-house from Sigma poly(U) agarose] equilibrated with 25 mM NaPi pH 8.0, 0·1 M NaCl, 1 mM EDTA. The column was washed with 25 mM NaPi pH 8·0, 0·1 M NaCl, 1 mM EDTA. A step gradient of 0·4, 0·5 and 1·0 M NaCl in 25 mM NaPi pH 8·0, 1 mM EDTA was applied and fractions were collected. Fractions were analysed by SDS–PAGE (Fig. 1 C) and concentrated by using Centricon-10 (Ambion) centrifuge concentrators with a molecular mass cut-off of 10 kDa. Faint bands of approx. 26 kDa observed in the final preparation (Fig. 1 C, lanes 5–7) were identified by Western blot as NS2 degradation products (data not shown). Degradation products observed in Fig. 1(B) lanes 5 and 6 were also identified by Western blot as NS2. Aliquots of NS2 were stored at −70 °C. Typical yields were 1 mg NS2 per 100 ml culture medium.

During earlier studies of NS2 RNA binding, an ATP-binding activity was observed (Horscroft, 1998). NTP binding and phosphohydrolase activity are key features of various enzymes such as ATPases, helicases and guanylyltransferases. ATPase activity is required for the splicing of RNA (Schwer & Guthrie, 1992a, b), in transcription (Lowery & Richardson, 1977a, b) and in helicases (Bisaillon et al., 1997; Staeuber et al., 1997). The capping of viral RNA transcripts is performed by a guanylyltransferase that binds and hydrolyses GTP (Liu et al., 1992; Mertens et al., 1992; Pizarro et al., 1991). Additionally, NTP hydrolysis serves as a crucial source of energy to enable movement or conformational change in proteins and nucleic acids (Palacios et al., 1996; Rodnina et al., 1997). In BTV, phosphohydrolase activity is found in two core proteins, the guanylyltransferase, VP4, and the RNA helicase, VP6 (Mertens et al., 1992; Staeuber et al., 1997).

Both the helicase and guanylyltransferase activities of BTV have been localized to specific proteins and therefore the significance of ATP binding by NS2 was not immediately apparent. Phosphohydrolase activity relies on the ability of a protein to bind NTPs. To test for phosphohydrolase activity associated with NS2, a TLC assay was used (Martinez-Costas et al., 1998). Purified NS2 (1 µg) was incubated at 37 °C for 30 min with 1 µCi [α-32P]ATP in buffers and salts as indicated in the legend of Fig. 2(A). The reaction was stopped by the addition of an equal volume (10 µl) of 10% TCA. The products were separated by TLC by spotting 1·5 µl of the reaction onto a PEI–cellulose plate (Merck) along with known standards and the plate was developed in a TLC tank with 0·5 M KH2PO4 as the ascending phase. The results indicated that purified NS2 can hydrolyse ATP to ADP and AMP (Fig. 2A, lanes 3 and 4). As observed for all other NTPases, the reaction requires a divalent cation, in this case Mg2+. The reducing agent DTT did not appear to influence the reaction (Fig. 2A, lanes 1 and 2).

These experiments were repeated with [α-32P]GTP to investigate the specificity of the NTP hydrolysis. Lanes 3 and 4 of Fig. 2(B) show that NS2 can also hydrolyse GTP, but only as far as GDP. Overexposure of the X-ray film allowed a small amount of GMP to be detected (data not shown).

To characterize further the binding of ATP and GTP by NS2, aliquots of the NTPase reactions from the previous experiments were resolved by SDS–PAGE under denaturing conditions. A 10 µl aliquot of each reaction was mixed with 2 µl 5 × SDS–PAGE sample buffer, heated to 100 °C for 5 min
Purified BTV NS2 binds and hydrolyses NTPs

Fig. 2. NTPase activity and NTP binding by NS2. (A) Purified NS2 was incubated with 1 µCi [α-32P]ATP and Tris buffer in the presence or absence of 10 mM MgCl₂ and/or 1 mM DTT. Reactions were incubated at 37 °C for 30 min and stopped by the addition of 1 vol 10% TCA. The products were resolved by PEI–cellulose TLC. The positions of cold AMP, ADP and ATP, visualized by fluorescence, are indicated to the right. (B) GTPase activity of NS2. Purified NS2 was incubated with 1 µCi [α-32P]GTP as described in (A). (C)–(D) Aliquots of the ATPase reactions described in (A) and aliquots of the stopped GTPase reactions described in (B) were separated by 10% SDS–PAGE and blotted onto PVDF Immobilon membranes and labelled proteins were detected by autoradiography.

and run on a 10% SDS–PAGE gel. The gels were transferred to Immobilon membranes before exposure to X-ray film to reduce the excessive background radiation observed in preliminary experiments. The bound label migrated as a band identical in size to NS2. The results (Fig. 2 C) suggest that NS2, and not a contaminating protein, binds ATP in the presence of Mg²⁺ (lanes 3 and 4). Without Mg²⁺, NS2 was unable to bind either nucleoside (Fig. 2 C, D; lanes 2), suggesting that the lack of hydrolysis in the TLC assay (Fig. 2 A, B; lanes 2) was due to the inability of NS2 to bind ATP or GTP. These experiments indicate that NS2 binds and hydrolyses both ATP and GTP but that there appears to be a difference in the hydrolysis of the two nucleosides. The binding of UTP by NS2 was also investigated in other experiments, but the level of binding was very low compared with binding of ATP and GTP, even after long exposures to X-ray film (data not shown).

A TLC assay was used to determine any preference of NS2 for the divalent cation in the phosphohydrolase reaction. Assays were set up that differed only in their divalent cation content, with either [α-32P]ATP (Fig. 3 A) or [α-32P]GTP (Fig. 3 B) as the phosphate donor. The reactions contained either MgCl₂, MnCl₂ or CaCl₂ at a concentration of 10 mM (Fig. 3 A, B; lanes 2–4) or no divalent cation (Fig. 3 A, B; lane 1). For the ATPase activity, Mn²⁺ ions gave the greatest hydrolysis of ATP (Fig. 3 A, lane 3). In terms of hydrolysis to ADP, Ca²⁺ and Mg²⁺ appeared to give similar results. However, there was less hydrolysis to AMP in the presence of Ca²⁺ than of Mg²⁺ (Fig. 3 B; lanes 2 and 4). With GTP, the presence of both Mn²⁺ and Ca²⁺ gave better results than Mg²⁺, with little difference in the low levels of GMP produced (Fig. 3 B).

While the purified NS2 used in these experiments appeared pure by SDS–PAGE, a co-purified cellular NTPase could result
in the observed hydrolysis of nucleosides. To determine whether the phosphohydrolase activity observed was the result of a contaminant, we performed a phosphohydrolase reaction in the presence of ouabain (Sigma), an inhibitor of cellular ATPases (Borsa et al., 1970). Ouabain had no effect on the reactions with either ATP or GTP at concentrations of up to 3-5 mM (data not shown). Additionally, assays performed with BTV VP7 purified from baculovirus-infected insect cells did not result in hydrolysis of ATP or GTP (data not shown). These two lines of evidence, along with the data indicating that the protein labelled by the binding of radioactive nucleosides is identical in size to NS2 (Fig. 2C, D), suggest strongly that NTP-binding hydrolysis is a function performed by NS2 and not by a contaminating cellular protein.

These experiments identify enzymatic activity associated with purified NS2. NTP binding and hydrolysis by NS2 represent novel findings. The results indicate that NS2 can bind and hydrolyse ATP and GTP, with a higher affinity for the former.

NTP binding and hydrolysis are indicative of a number of enzymatic activities including the mRNA capping and dsRNA helicase activities already identified in BTV. NTP hydrolysis also plays a crucial role in the function of molecular motors, proteins or protein complexes that use chemical energy from phosphate hydrolysis to generate mechanical force. In this regard, the role of the bacteriophage φ6 P4 protein and its associated NTPase activity in packaging and condensation of the positive-sense RNA genome of φ6 is of interest (Gottlieb et al., 1992; Paatero et al., 1995). φ6 is a bacteriophage with a segmented dsRNA genome. This genome consists of three segments that are packaged precisely within assembled procapsids in a specific order. Energy is required for this packaging, and the NTPase activity of P4, a component of the polymerase complex, is thought to provide this energy (Paatero et al., 1995).

NS2 has a possible functional homology with the rotavirus non-structural protein NSP2. NS2 forms 7S multimers (Uiteweerde et al., 1995), while NSP2 forms 10S multimers, and both proteins bind RNA (Kattoura et al., 1992, 1994). Recently, NSP2 was shown to be phosphorylated and to have an NTPase activity (Taraporewala et al., 1999). NSP2 catalyses the hydrolysis of each of the four NTPs to NDPs. Although NSP2 expressed by itself can form VIBs, NSP2 assembles into viroplasms only in infected cells. Another rotavirus non-structural protein, NSP5, localizes to viroplasms in infected cells but not when transfected alone, a feature that it shares with NSP2 (Kattoura et al., 1994; Poncet et al., 1997). Both proteins are required for viroplasm assembly. NSP2 and NSP5 interact with each other and the detection of multiply phosphorylated forms of NSP5 has been shown to correlate with localization to viroplasms (Poncet et al., 1997). Also, although NSP5 alone can bind poly(U) and ssRNA, binding is enhanced by the presence of NSP2 (Matton et al., 1994). Additionally, NSP2 is a component of the rotavirus replicase complex (Aponte et al., 1996).

Taken together, it appears that BTV NS2 combines most of the features of rotavirus NSP2 and NSP5. Although there is no apparent sequence similarity between the three proteins (Horscroft, 1998), the functions performed by these proteins are clearly analogous. The NTPase activity described for both NSP2 and NS2 may play a role in providing energy for the assortment, movement or packaging of the ssRNA that they each bind, therefore making them critical in virus replication and assembly.

References


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Received 24 February 2000; Accepted 26 April 2000