Dual ATPase and GTPase activity of the replication-associated protein (Rep) of beak and feather disease virus

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Background: Psittacine beak and feather disease affects parrots resulting in an immunosuppressive disease that is often characterized by an abnormal shape and growth of the animal’s beak, feathers, and claws. Beak and feather disease virus (BFDV) is a single-stranded circular DNA virus and is classified as a member of the Circoviridae family. Two major open reading frames (ORFs) are known to encode the replication-associated (Rep) protein and the capsid-associated (Cap) protein.

Methods: The Rep and Cap genes of BFDV were fused with tags and then expressed and purified, respectively. Both the ATPase and GTPase activities of the recombinant Rep protein are measured. The substrate and ion preference, the optimal conditions, the effects of ATPase and GTPase inhibitors and the presence of Cap protein on the ATPase and GTPase activity of the Rep protein are examined. Finally, the effects of the Walker A motif, the Walker B motif, and a novel GYDG motif of the Rep protein on the ATPase and GTPase activities are studied by various mutants.

Results: The recombinant Rep protein could display ATPase activity and GTPase activity. The Rep protein was able to hydrolyze both deoxyribonucleotides and ribonucleotides. Among nucleoside triphosphates and deoxynucleoside triphosphates, the substrate preference orders were found to be ATP > GTP > CTP > UTP and dATP > dCTP > dGTP > dTTP, respectively. Both the ATPase and GTPase activity of the BFDV Rep protein required magnesium ions and the presence of calcium ions significantly inhibited the ATPase and GTPase activity of the Rep protein. The optimal temperatures for ATPase activity and GTPase activity were both 56 °C, while their optimal pH values were both pH 7.5. Both the ATPase activity and GTPase activity of the BFDV Rep protein were significantly down-regulated by polynucleotides and the dsDNA of 36 bp (located in origin of replication) of BFDV genome. The ATPase activity of the BFDV Rep protein was found to be more sensitive to sodium azide than sodium orthovanadate and N-ethylmaleimide. Linoleic acid more strongly inhibited the GTPase activity of the Rep protein than dinasore. This suggested the Rep protein of BFDV should be classified as an F-type ATPase and polynsaturated fatty acid-sensitive GTPase. In the presence of 10 ng of the Cap protein, the ATPase activity and GTPase activity of the BFDV Rep protein were significantly increased. Furthermore, the BFDV Rep protein contains the Walker A motif, the Walker B motif and a novel GYDG motif. Both the ATPase activity and the GTPase activity of various deletion and site-directed mutants of the Rep protein affecting these motifs were significantly reduced, suggesting all the three motifs contribute to the ATPase and GTPase activities; specifically. In addition, the ATPase activity and GTPase activity of the deletion mutants of the Rep protein were reversed in the presence of the Cap protein. This is the first example of dual ATPase and GTPase activity being reported in the Rep protein of BFDV.

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1. Introduction

The genome of beak and feather disease virus (BFDV) consists of a single-stranded circular DNA (ss cDNA) and the virus belongs to the Circoviridae family (Rahaus and Wolff, 2003). The virus consists of a non-enveloped capsid with a diameter of between 14 and 16 nm (Bassani et al., 1998). Interestingly, both the virion and genome size of BFDV makes it the most compact and smallest viral pathogen that affects parrots. The virus leads to successive malformation, fracture, and necrosis of the bird’s feathers, beak, and claws. In addition to the cracked outer layers of the beaks and claws, the birds are prone to secondary infections because they suffer...
from immunosuppression that is caused by lymphocytic depletion of the thymus and bursa of Fabricius. Since its first report in a psittacine species from Australia in 1970, this disease has been found in both captive and wild populations of psittacine around the world, including Southern Australia, Europe, Africa, the Americas, and Asia (Varsani et al., 2011). An epidemiology study has revealed that this disease was transmitted from Old World psittacine species to New World psittacine species via international bird movement and trade (Bert et al., 2005).

With a genome size that ranges from 1992 nucleotides to 2018 nucleotides, the virus encodes three major open reading frames (ORFs), namely V1, V2, and V3 on the sense strand together with four further ORFs, C1, C2, C3, and C4, which are found on the complementary antisense strand (Raue et al., 2004). Among them, the replication-associated (Rep) protein and the capsid-associated (Cap) protein are encoded by ORF V1 and ORF C1, respectively (Hsu et al., 2006). In Taiwan, the positive rate for BFDV among asymptomatic birds is 24% and the major source of BFDV in Taiwan has been linked to the import of exotic birds (Su et al., 2007). In such circumstances, both surveillance for the presence of BFDV and the study of BFDV have become important issues.

The Rep protein of several ss-C-DNA animal viruses, such as the Circoviridae, Geminiviridae and Nanoviridae, are involved in the initiation of rolling-circle replication (Steinfeldt et al., 2007). The Rep protein of porcine circovirus (PCV) has been shown to have both ATP binding and ATP hydrolysis activity through a conserved GKS motif. If this GKS motif is mutated, the replication of PCV is inhibited due to there being lower ATPase activity (Warren et al., 1993). The down-regulation or abolishment of the ATPase activity of the Rep protein of tomato yellow leaf curl virus (TYLCV) and of hepatitis C virus (HCV) have been shown to inhibit the replication of TYLCV and HCV (Desbiez et al., 1995; Tate et al., 1989). The ATPase activity has also been found to be associated with the NS4B protein of classical swine fever virus (CSFV), the VP4 protein of bluetongue virus (BTV), the A32L protein of orbivirus, and the α protein of avian reovirus (ARV) (Glade et al., 2011; Ramadevi and Roy, 1998; Su et al., 2007). Most of those proteins include at least two typical ATPase motifs, one of which is the Walker A motif (G/AXXXGK/ST) that is involved in the ATP binding, while the other is the Walker B motif (ihhhDD/E), which affects the chelation of the enzyme’s cofactor, magnesium ions. Both of these motifs are present in the Rep protein of BFDV (Walker et al., 1982). This contrasts with the A32L protein of orbivirus, where there are a motif III and a motif IV, which allow the formation of a beta-stranded structure with a polar amino acid at the C-terminus and a hydrophobic beta-stranded structure at the N-terminus, respectively; these structural features have been shown to be associated with the ATPase activity. No such motifs are present in the NS4B protein of CSFV or the Rep protein of BFDV. Moreover, in a number of studies GTPase activity has been found to be associated with proteins exhibiting the ATPase activity, but with varying levels of activity. For example, the A32L protein of orb virus, the α protein of ARV, and the NS4B protein of CSFV have ATPase activity levels that are much higher than their GTPase activity levels (Glade et al., 2011; Lin et al., 2011; Su et al., 2007). However, the GTPase activity of the VP4 protein of BTV has been shown to be higher than its ATPase activity (Ramadevi and Roy, 1998). In fact, cell signalling, viral replication, and membrane fusion have all been shown to be related to the hydrolysis of ATP and GTP for the NS4B protein of HCV and the 2C protein of poliovirus (Einav et al., 2004; Pfister and Wimmer, 1999). In addition to ATP and GTP being the preferred substrates of the VP4 protein of BTV and the α protein of ARV, the presence of nucleoside triphosphate hydrolase activity has also been demonstrated for UTP and CTP in these viruses but at lower activity levels (Ramadevi and Roy, 1998; Su et al., 2007).

The aim of this study is to examine the ATPase and GTPase activity of the Rep protein of BFDV and to characterize its biochemical features and enzymatic kinetics. The effects of deletions and mutations on the BFDV Rep protein in terms of ATPase activity and GTPase activity were explored. The results indicated that the BFDV Rep protein has comparable levels of ATPase and GTPase activity and that the Walker A motif, the Walker B motif, and the GYDG motif are all necessary for both ATPase activity and GTPase activity to be present.

### 2. Materials and methods

#### 2.1. Plasmid construction and verification

The Rep and Cap genes of BFDV (GenBank accession No. KC510146) were cloned into pET32b. The Rep gene was cloned into pGEX. To create mutants, the Rep gene was used as a template for a PCR-driven strategy (Heckman and Pease, 2007). All primers for the construction of the clones are listed in Table 1. To clone the Rep or Cap gene, the amplified products from the first round PCR were digested following by cloning. First round PCR products were used as templates for the second round PCR using outer flanking primers. The first round of PCR involved 94 °C for 30 min followed by 32 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s. The second round of PCR involved 94 °C for 30 min followed by 36 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min 30 s. Both SalI and XhoI were used to digest the PCR products, which were cloned into pET32b or pGEX. After sequence verification, plasmids containing the Cap gene, the Rep gene, the Rep gene with the Walker A motif deleted, the Rep gene with the Walker B motif deleted, and the Rep gene with the GYDG motif deleted were respectively named as Cap, Rep, Rep-GST, Rep-box-1-del, Rep-box-2-del, and Rep-box-3-del.
2.2. Site-directed mutagenesis

Site-directed mutagenesis of the Walker A motif (K171A) involved using the mutated primers Rep 1–2F primer: 5′-CCACCGGGGTGTGCGCAAGTAGATGGGCCAA-3′ and Rep 1–2R primer: 5′-TTGCCCATCTACTGCGCCACACCCCGTTG-3′ for PCR; the modified nucleotides are identified in Table 1 by underlining. The PCR program followed the protocol of the QuickChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, USA). Briefly, PCR involved 95 °C for 2 min followed by 18 cycles consisting of 95 °C for 20 s, 60 °C for 10 s, and 68 °C for 3 min and 24 s with a final extension of 68 °C for 5 min. After digestion with Dpn I, the products were cloned into pET32b and their sequence confirmed. This clone was named Rep-box-1-mut.

2.3. Expression of the recombinant proteins and their purification

The Escherichia coli BL21 strain (Invitrogen) or Top10 strain (ThermoFisher) transformed with the expression vectors was induced at 20 °C for 8 h or 30 °C for 4 h with 1.0 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG). The pelleted bacteria were resuspended in binding buffer (250 mM NaCl, 20 mM Tris–HCl, and 10 mM imidazole, pH 7.5) plus 1 M phenylmethylsulfonyl fluoride (PMSF). Samples were digested with 0.5 mg/mL lysozyme and then sonicated. Each supernatant was purified using Ni Sepharose 6 Fast Flow (GE Healthcare). The nickel-charged sepharose beads were incubated with the supernatant at 25 °C for 10 min and then washed with washing buffer (500 mM NaCl, 50 mM Tris–HCl, and 40 mM imidazole, pH 7.5). Bound protein was eluted by elution buffer (500 mM NaCl, 50 mM Tris–HCl, and 400 mM imidazole, pH 7.5) and dialyzed. The GST-fusion proteins were purified by incubation with glutathione-coupled sepharose beads (GE Healthcare) at 4 °C for 2 h. The GST-fusion proteins were immobilized on the beads and stored in PBS buffer (1× phosphate-buffered saline with 2 mM EDTA, 0.1% β-mercaptoethanol, 0.2 mM PMSF, and 5 mM benzamidine) at 4 °C prior to use.

2.4. Immunoprecipitation, GST-pull down assay, SDS-polyacrylamide gel electrophoresis, and Western blotting

The liver and spleen samples from BFDV-positive grey parrots were solubilized in PBS by sonication. Ten µg of the supernatant were diluted in RIPA lysis buffer (50 mM Tris, 150 mM NaCl, 10 mM EDTA, 0.1% SDS, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, pH 7.5). The lysates were incubated with anti-BFDV Cap or anti-BFDV Rep monoclonal antibodies provided by the Molecular Biology Lab of National Chung Hsing University, Taiwan and then with 75 µl of 50% agarose-coupled goat anti-mouse IgG (Sigma, USA). DF-1 cells were used as negative control. The pelleted beads were washed with RIPA lysis buffer. To assess the interaction of the Cap and Rep proteins, tissue lysates were incubated with anti-BFDV Cap monoclonal antibody and then immunoprecipitated with agarose-coupled anti-mouse IgG. The purified or precipitated proteins were separated by the 10% SDS-PAGE and then stained with Coomassie Brilliant Blue. The purified proteins were dissolved in 5% formic acid and subjected into the electrospray ionization tandem mass spectrometric analysis. The identity of the BFDV Cap and Rep proteins were confirmed by the Mascot program. For the GST-pulldown assay, 4 µg of Cap or thioredoxin (Trx) proteins were incubated with 5 µg of immobilized GST or GST-Rep proteins in Nonidet P-40 (NP-40) buffer (1% NP-40, 20 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM Na2VO4, 5 mM EDTA pH 8.0, 10% glycerol, 10 mg/ml leupeptin, 10 mg/ml aprotinin, 1 mM PMSF). After an overnight incubation, the bound proteins were washed and separated by 10% SDS-PAGE. For Western blotting, proteins were transferred, blocked with TBST (150 mM NaCl, 20 mM Tris, and 0.1% Tween 20, pH 7.5) containing 7.5% skimmed milk, and incubated with anti-His (Genetex, USA), anti-BFDV Cap or anti-BFDV Rep monoclonal antibodies or the transferred proteins on the membrane were stained with Ponceau S. After washing, the membrane was probed with a horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (Jackson ImmunoResearch, USA). An enzyme-linked chemiluminescence system (ECL Pro, Perkin Elmer, USA) was used for development.

2.5. Measurement of ATPase activity and GTPase activity

The release of free γ-phosphate (Pi) from ATP or GTP by the Rep protein was quantified using a colorimetric ATPase assay kit (Innova Biosciences, United Kingdom) followed the manufacturer’s instructions. Two hundred nmoles of protein were dissolved in 100 µL Tris–HCl (pH 7.5) and incubated with 0.5 mM ATP at 25 °C for 30 min. After termination, the absorbance at OD550, which was proportional to the amount of phosphomolybdate malachite complex present, was measured using an enzyme-linked immunosorbent assay. The value of a blank assay that assessed the non-enzymatic release of Pi contamination in the absence of proteins and substrates was then subtracted from the enzymatic activity. The ATPase and GTPase activities of Trx were measured as negative controls. A set of inorganic acids (50, 45, 40, 35, 30, 25, 20, 15, 10, 5, and 2.5 µM) was run in parallel to provide a standard curve, which was then used to calculate the total released phosphate (µM) and the release rate of phosphate (pmol/min/µg) representing the levels of ATPase or GTPase activity. To obtain a time course, the reaction was incubated for 30, 60, 90, and 120 min. For the optimal pH, the reaction was incubated in buffers at pH...
Fig. 2. The pET32b plasmids carrying Rep and Cap genes express the Rep and Cap proteins, respectively. Expression and purification of the Trx, Rep (Rep, Rep-box-1-del, Rep-box-2-del, Rep-box3-del, and Rep-box-1-mut), and Cap proteins were carried out and the proteins were separated by SDS-PAGE. Both crude bacterial lysates without (−) or with (+) IPTG-induction are shown (A). The purified Trx, Rep, Rep-box-1-del, Rep-box-2-del, Rep-box3-del, Rep-box-1-mut, and Cap proteins were resolved by SDS-PAGE (B). The purified proteins were examined using anti-His, anti-Rep protein, and anti-Cap protein monoclonal antibodies by Western blotting (C). The reactivity of anti-Rep and anti-Cap monoclonal antibodies against native Rep and Cap proteins was confirmed by the immunoprecipitation and Western blotting (D). The ATPase and GTPase activities of the Rep protein of beak and feather disease virus and the Trx protein (E). The free phosphate (µM) released from ATP and GTP by using 50 nM, 100 nM, and 200 nM proteins in assay. The means ± SD from three independent experiments are presented.
5.5, pH 6.5, pH 7.5, and pH 8.5. The effects of ATPase inhibitors, namely sodium azide (NaN₃), sodium orthovanadate (NaVO₄), and N-ethylmaleimide (NEM), and GTPase inhibitors, namely dynasore and linoleic acid, were assessed; all inhibitors were purchased from Sigma–Aldrich, USA. Specifically, the Rep protein was pre-incubated with inhibitors for 15 min and 30 min before the addition of the substrate. To assess the effect of divalent ions, ATPase and GTPase activity levels were measured in the presence of 2.5 mM Mg²⁺, Mn²⁺, Zn²⁺, and Ca²⁺. To calculate the $V_{\text{max}}$ and the Michaelis constant ($K_M$) values, the velocity of the enzymatic reactions was measured in the presence of 25, 50, 100, 200, 400, 800, 1200, and 1600 µM ATP or GTP. A Lineweaver–Burk plot using reciprocal values for the substrate concentrations and velocities was obtained and the $K_M$ and $V_{\text{max}}$ values were calculated by the least square estimates method. To assess substrate specificity, 0.5 mM ATP, GTP, CTP, UTP, dATP, dGTP, dCTP, and dTTP were used. To measure dose-dependent effects, ATPase or GTPase activity was determined when the Rep protein was present at 50, 100, and 200 nmoles. In order
to assess the effect of the presence of the Cap protein, the Cap protein (10, 25, 50, and 100 ng) was pre-incubated with the Rep protein for 30 min prior to the ATPase and GTPase activity measurements; this was repeated using 100 ng Cap protein in the presence of mutant Rep proteins. The effect of polynucleotides was determined by pre-incubating the Rep protein for 30 min with 0.25 mM poly(dA), 0.25 mM poly(dG), 0.25 mM poly(dC), 0.25 mM poly(dT), 0.25 mM poly(dA) plus poly(dT), 0.25 mM poly(dC) plus poly(dG), 0.25 mM P10 (5′-TAGTATTACC-3′) single-stranded DNA, 0.25 mM P12 (5′-TAGTATTACCCG-3′) single-stranded DNA, or 0.25 mM P36 (5′-CCGCGCTGGGCACCGGGGCACGTGACGCATTGG-3′) double-stranded DNA. The P10, P12, and P36 DNA sequences are all located in origin of replication of BFDV.

2.6. Enzyme-linked immunosorbent assays (ELISA)

The Cap protein (250 ng) in TBS buffer (25 mM Tris–HCl, 150 mM NaCl, pH 7.5) was used to coat ELISA plates. After washing with TBST buffer and blocking with 5% bovine serum albumin, the plates were washed three times with TBST and then different amounts (10 ng, 12.5 ng, 25 ng, 50 ng, 100 ng, and 150 ng) of Rep protein were added in the presence of micrococcal nuclease (40 units/ml), which was followed by incubation for 30 min. After washing three times, monoclonal antibody against the BFDV Rep protein was added and the mixture incubated for 60 min. For the negative control, the Trx protein (250 ng) was coated onto the ELISA plates and then challenged with the Rep proteins. After washing three times, horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (Jackson ImmunoResearch, USA) was added as the secondary antibody. After final washing, the chromogenic substrate was added and the absorbance values were quantified at 450 nm by spectrophotometer.

2.7. Statistical analysis

The ATPase and GTPase activity levels were determined as the mean (± standard deviation (SD)) value measured as pmol/min/μg from triplicated experiments. The relative activity or absorbance value at 450 nm was determined using the mean. Finally, one-way ANOVA and the Student’s t-test were used for sta-
3. Results

3.1. Expression and purification of the Cap and Rep proteins of BFDV and the dose-dependent ATPase and GTPase activities of the Rep protein

The Walker A motif, the Walker B motif, and the GYGD motif have been characterized as motifs associated with ATPase activity and magnesium binding; all three motifs are found in the Rep protein of BFDV. Accordingly, the following proteins, Trx, Rep, Rep-box-1-del, Rep-box-2-del, Rep-box-3-del, Rep-box-1-mut, and Cap were expressed and purified (Fig. 1). The expected sizes of these proteins in soluble form were found to be 20 kDa, 53 kDa, 53 kDa, 53 kDa, 53 kDa, 53 kDa, 53 kDa, and 49 kDa, respectively, and these were confirmed by SDS-PAGE (Fig. 2A and B). Since Trx and a 6-His tag were separately added to the N-term and C-term of these proteins, respectively, the purified proteins were also checked using anti-His antibody and Western blotting (data not shown). The mass spectrometry confirmed that the identity of recombinant proteins with 49 kDa and 53 kDa was Rep and Cap proteins of BFDV, respectively. The authenticity of the recombinant proteins was also verified by Western blotting using monoclonal antibodies against the Rep and Cap proteins of BFDV (Fig. 2C). Besides, the reactivity of anti-Rep and anti-Cap monoclonal antibodies to native Rep and Cap proteins was confirmed by the immunoprecipitation using proteins from the BFDV-infected bird. The molecular weights of native Rep and Cap proteins were 33 kDa and 29 kDa, respectively (Fig. 2D).

Free phosphate (μM) release from the substrate ATP increased to 30.41 ± 4.85, 47.34 ± 1.52, and 75.95 ± 1.79, respectively, when 50, 100, and 200 nM of the Rep protein were present. However, no change in the release of free phosphate (μM) was detected in the presence of the Trx, with release remaining at background level. The amounts of free phosphate (μM) produced in the presence of 50, 100, and 200 nM of the Rep protein were 0.12 ± 0.12, 0.72 ± 0.12, and 0.5 ± 0.17, respectively. However, the dose–dependent effect of increased amounts of the Rep protein was less obvious when the GTPase activity was measured. The free phosphate (μM) released from the substrate GTP increased to 28.25 ± 5.17, 44.57 ± 3.16, and 69.35 ± 3.26 in the presence of 50, 100, and 200 nM of the Rep protein, respectively. The background levels of GTPase activities were 0.01 ± 0.02, 1.16 ± 0.02, and 1.06 ± 0.09 μM, respectively (Fig. 2E).

3.2. The effects of divalent ions, temperature, and pH on the ATPase and GTPase activities of the Rep protein of BFDV

The relative ATPase activities of the Rep proteins in the presence of MgCl₂, MnCl₂, ZnCl₂, CaCl₂, and MgCl₂ + MnCl₂ were 100 ± 8.50%, 56.99 ± 7.47%, 48.79 ± 5.35%, 31.32 ± 5.35%, and 99.74 ± 9.59%, respectively (Fig. 3A). The relative GTPase activities of the Rep proteins in the presence of the same ions were 100 ± 4.25%, 46.77 ± 6.5%, 45.07 ± 5.35%, 19.17 ± 6.84%, and 96.32 ± 6.84%, respectively (Fig. 3B). These results suggest that the ATPase and GTPase activities of the Rep are largely depending on the presence of magnesium ions. Furthermore, ZnCl₂ is able to effectively inhibit the ATPase activity and the GTPase activity of the Rep protein. Similar to magnesium ions, calcium ions are able to activate the ATPase activity of the Rep protein. However, 2.5 mM CaCl₂ would seem to significantly suppress the GTPase activity of the Rep protein.

The relative ATPase activities of the Rep protein at 25°C, 37°C, 56°C, and 80°C were 100 ± 5.63%, 137.32 ± 9.27%, 145.07 ± 6.49%, and 23.12 ± 5.35%, respectively (Fig. 3C). Furthermore, the relative GTPase activities were 100 ± 6.5%, 119.39 ± 7.47%, 152.49 ± 8.86%, and 50.04 ± 5.35% at the same temperatures, respectively (Fig. 3D). The ATPase activity and GTPase activity of the Rep were almost abolished at 80°C.

The relative ATPase activities of the Rep protein were 100 ± 8.6%, 131.77 ± 5.35%, 134.81 ± 7.47%, and 132.78 ± 6.84% at pH 5.5, pH 6.5, pH 7.5, and pH 8.5, respectively (Fig. 3E), thus the optimal pH was 7.5. Similarly, the relative GTPase activities of the Rep protein at the same pH values were 100 ± 8.86%, 131.21 ± 4.25%, 136.8 ± 6.5%, and 135.54 ± 6.38%, respectively (Fig. 3F), which means the optimal pH for GTPase activity was pH 7.5. Thus, the GTPase activity of the Rep is the same under neutral conditions with ATPase activity of the Rep.

3.3. The time course and enzymatic kinetics of the ATPase and GTPase activities of the Rep protein of BFDV

Next we explored the time-dependence of free phosphates (μM) release from ATP in the presence of 200 nM Rep protein; this increased giving values of 31.85 ± 1.74, 65.86 ± 2.09, 83.73 ± 0.53, and 86.01 ± 2.16 after 30, 60, 90, and 120 min. There were no changes in the release of free phosphate (μM) as the various concentrations of Trx with the activity remaining at background level. The free phosphates (μM) produced by 200 nM of Trx after 30, 60, 90, and 120 min were 1.16 ± 0.06, 1.17 ± 0.03, 1.16 ± 0.04, and 1.09 ± 0.04, respectively.

The time-dependent effect was less obvious when the GTPase activity of the Rep protein was explored. Using the same time periods, the free phosphate (μM) released from the substrate GTP by 200 nM of the Rep protein were increased to 31.21 ± 0.57, 63.83 ± 1.08, 83.68 ± 1.5, and 88.13 ± 2.21, respectively, while the free phosphate (μM) released by 200 nM of Trx were 1.39 ± 0.05, 1.81 ± 0.1, 1.86 ± 0.15, and 1.8, respectively (Fig. 4A). These findings indicate that both the ATPase activity and the GTPase activity of the Rep protein approached a plateau phase after 90 min when 200 nM of the Rep protein is used. To ensure that ATP and GTP hydrolysis does followed a linear relationship between speed and substrate concentration, 50 nmoles of the Rep protein were used. A linear relationship was found to exist within a range of 25 μM and 1200 μM of ATP/GTP and the initial velocity (nmole/min/mg) between 25 μM and 400 μM for the ATPase activity of the Rep was higher than for the GTPase activity of the Rep. This is demonstrated in the inset of Fig. 3B. Least square estimation was used to determine the V_max and the K_m and straight lines were obtained using a Lineweaver–Burk plot. The K_m and V_max values for the ATPase activity and for the GTPase activity were 79.25 ± 2.51 nmole/min/mg and 2.33 ± 0.01 μM and 65.37 ± 0.92 nmole/min/mg and 2.36 ± 0.02 μM, respectively (Fig. 4B). The binding affinity of the Rep protein for GTP compared that for ATP decreased only by 1.01-fold, which suggests that, in terms of binding affinity, the ATPase activity of the Rep protein of BFDV is very similar to that of the protein’s GTPase activity.

3.4. The nucleotide substrate specificity of the Rep proteins of BFDV and the effect of polynucleotides on the ATPase and GTPase activities of the Rep protein of BFDV

The substrate specificity of the Rep protein of BFDV was determined using various nucleotides. The relative activities of the Rep protein when hydrolysing ATP, UTP, GTP, CTP, dATP, dGTP, dCTP, and dTTP were 100 ± 8.05%, 23.59 ± 5.35%, 71.27 ± 4.43%, 26.64 ± 4.43%, 145.28 ± 4.25%, 43.18 ± 3.25%, 60.38 ± 2.13%, and 20.76 ± 2.46%, respectively (Fig. 5A). Significant differences were observed with respect to all ATPase activities of the Rep protein (p < 0.05). The efficiency of hydrolysis of the ribonucleotide ATP was
higher than that of GTP, which is similar to the difference observed when the same deoxyribonucleotides (dATP and dGTP) are used. However, the efficiency of hydrolysis for different ribonucleotides and their deoxynucleotide counterparts varied. This suggested that the Rep protein of BFDV should be regarded as preferentially being an ATPase and a GTPase. The effects of single-stranded and double-stranded deoxyribonucleotides on the ATPase activity and GTPase activity were tested. The relative levels of ATPase activities of the Rep protein in the presence of single-stranded homopolymers poly(dA), poly(dT), poly(dG), and poly(dC), single-stranded P10 sequence, single-stranded P12 sequence, double-stranded poly(dA-dT), poly(dG-dC), and double-stranded P36 sequence were 100 ± 8.85%, 64.78 ± 5.81%, 87.51 ± 6.84%, 77.97 ± 8.05%, 78.83 ± 7.67%, 99.65 ± 7.67%, 98.09 ± 6.38%, 75.19 ± 7.37%, 73.28 ± 6.84%, and 68.08 ± 6.49%, respectively (Fig. 5B). A significant difference (p < 0.05) was observed for the single-stranded homopolymers poly(dA), homopolymers poly(dT), homopolymers poly(dG), homopolymers poly(dC), the double-stranded poly (dA-dT), the double-stranded poly (dG-dC), and the double-stranded P36 sequence. The relative levels of the GTPase activities of the Rep protein in the presence of the same substrates were 99.99 ± 2.03%, 72.27 ± 3.5%, 73.91 ± 2.12%, 47.33 ± 1.96%, 65.83 ± 4.32%, 82.22 ± 4.55%, 74.47 ± 1.78%, 77.68 ± 2.83%, 41.79 ± 1.58%, and 60.92 ± 3.92%, respectively (Fig. 5C). Thus except the double-stranded P10 and P12 sequences, both the ATPase and GTPase activities were found to be lower when the enzyme acted on deoxyribonucleotides.

3.5. The effect of inhibitors on the ATPase and GTPase activities of the Rep protein of BFDV

The relative ATPase activities of the Rep protein alone and after treatment with 0.1 mM, 1 mM and 10 mM NaNO3 for 10 min were 100 ± 4.42%, 49.65 ± 5.35%, 45.89 ± 6.14%, and 42.5 ± 8.86%, respectively (Fig. 6A). The relative levels of the ATPase activities alone and after the treatment with 0.1 mM, 1 mM, and 10 mM NaNO3 for 30 min were 100 ± 5.63%, 46.18 ± 5.35%, 42.16 ± 9.27%, and 39.52 ± 7.47%, respectively (Fig. 6B). Thus significant differences in the ATPase in the presence of NaNO3 (p < 0.05) were observed at 0.1 mM, 1 mM, and 10 mM at both 10 min and 30 min. The relative ATPase activities of the Rep protein were 100 ± 7.7%, 73.55 ± 5.63%, 66.93 ± 5.63%, and 68.4 ± 3.24% alone and after treatment with 0.01 mM, 0.1 mM, and 1 mM NaNO3 for 10 min, respectively (Fig. 6A). The relative ATPase activities of the Rep protein alone and treated with the same concentrations of NaNO3 for 30 min were 100 ± 5.35%, 72.82 ± 5.35%, 65.86 ± 5.35%, and 67.01 ± 7.47%, respectively (Fig. 6B). Thus significant differences (p < 0.05) were seen at 0.01 mM, 0.1 mM, and 1 mM for both 10 min and 30 min. The relative ATPase activities of the Rep protein alone and treated with 0.1 mM, 1 mM, and 10 mM NEM for 10 min were 100 ± 4.91%, 89.93 ± 6.5%, 88.06 ± 7.47%, and 78.41 ± 8.86%, respectively (Fig. 6A). The ATPase activities of the Rep protein alone and after treatment with the same concentrations of NEM for 30 min were 100 ± 8.05%, 86.55 ± 6.84%, 70.74 ± 9.27%, and 59.32 ± 8.05%, respectively (Fig. 6B). Thus significant differences (p < 0.05) were found at 0.1 mM, 1 mM and 10 mM for 10 min treatment and 0.1 mM, 1 mM, and 10 mM for 30 min treatment. The relative GTPase activities alone and after treatment with 5 mM, 50 mM, and 500 mM dynasore for 10 min were 100 ± 6.49%, 88.64 ± 3.25%, 91 ± 4.43%, and 89.35 ± 6.84%, respectively (Fig. 6C). The relative levels of GTPase activities of the Rep protein alone and after treatment with 5 mM, 50 mM, and 500 mM dynasore after 30 min treatment were 100 ± 6.14%, 84.65 ± 5.35%, 75.02 ± 5.35%, and 64.67 ± 8.05%, respectively (Fig. 6D). Significant differences were observed at 5 mM, 50 mM, and 500 mM for 10 min and 5 mM, 50 mM, and 500 mM for 30 min (all p < 0.05). This suggests that a prolonged incubation can increase the effectiveness of dynasore on the Rep protein. The relative GTPase activities of the Rep protein alone and when treated with 10 μM, 100 μM, and 1000 μM linoleic acid were 100 ± 8.86%, 85.1 ± 6.14%, 70.08 ± 6.38%, and 70.59 ± 7.47%, respectively, after 10 min treatment (Fig. 6C). The relative GTPase activities of the Rep protein alone and when treated with 10 μM, 100 μM, and 1000 μM linoleic acid for 30 min were 100 ± 7.67%, 49.47 ± 5.35%, 29.16 ± 6.84%, and 30.11 ± 8.05%, respectively (Fig. 6D). Thus significant differences were observed after 10 min at 10 μM, 100 μM, and 1000 μM after 10 min treatment and at 10 μM, 100 μM and 1000 μM after 30 min treatment.
Thus, the inhibitory effect of linoleic acid was greater than dynasore.

3.6. Interaction between the Cap protein and the Rep protein of BFDV

The interaction between the native Cap and Rep proteins from the tissue of BFDV-infected birds was verified by the co-immunoprecipitation. The native Rep protein, which has a molecular weight of 33 kDa, was detected by Western blot and this confirmed the interaction between the Cap and Rep proteins (Supplemental Fig. 1). Immobilized Cap protein was challenged with different amounts of the Rep proteins and binding of the Rep protein was found to increase in a dose-dependent manner. The mean absorbance values were 0.15, 0.16, 0.17, 0.27, 0.49, and 0.49 for 10 ng, 12.5 ng, 25 ng, 50 ng, 100 ng, and 150 ng of the Rep proteins, respectively. For the negative control, the mean absorbance values remained 0.08, 0.08, 0.1, 0.1, 0.12, and 0.14 for 10 ng, 12.5 ng, 25 ng, 50 ng, 100 ng, and 150 ng of the Trx proteins, respectively (Fig. 7A). For the GST-pull down assay, the Cap protein was precipitated with the GST-Rep protein rather than the GST protein. The precipitated Cap protein was detected by both anti-His and anti-Cap monoclonal antibodies. The Trx protein was not precipitated by the GST and the GST-Rep proteins (Fig. 7B). This excluded the possibility of the association of the Rep and Cap protein via the fusion tags and indicated the existence of an in vitro association between the Rep and Cap proteins.

3.7. The effect of the Cap protein of BFDV on the ATPase and GTPase activities of the Rep protein of BFDV

The relative ATPase activities of 100 ng Cap protein and 100 ng Rep protein were 6.61 ± 1.09% and 98.89 ± 1.04%, respectively. The relative ATPase activities of Rep protein in the presence of 10 ng, 25 ng, 50 ng, and 100 ng Cap proteins were 107.85 ± 1.8%, 106.74 ± 2.28%, 121.07 ± 1.09%, and 125.48 ± 1.95%, respectively. This increase suggests that, while there is no ATPase activity associated with the Cap protein and that the ATPase activity of the Rep protein is up-regulated by the presence of Cap protein in a dose-dependent manner and that significant differences could be observed in the presence of only 10 ng of Cap protein (all p < 0.05; Fig. 7C).

The relative GTPase activities of 100 ng Cap protein and 100 ng Rep protein were 2.42 ± 2.78% and 96.06 ± 3.41%, respectively. The relative GTPase activities of 100 ng Rep protein in the presence of 10 ng, 25 ng, 50 ng, and 100 ng of Cap protein were 109.24 ± 1.31%, 115 ± 8.71%, 124.24 ± 2.15%, and 133.79 ± 5.01%, respectively. Thus the GTPase activity of the Rep protein are also up-regulated by the Cap protein in a dose-dependent manner and a significant difference was observed in the presence of only 10 ng of Cap protein (all p < 0.05; Fig. 7D). Interestingly, the up-regulation of GTPase activity was more profound than that of ATPase activity when the Cap protein is present and this is supported by the relative ATPase activity levels of the Rep protein compared to GTPase activity levels of the Rep protein in the presence of 100 ng Cap protein (125.48 ± 1.95% vs. 133.79 ± 5.01%).
3.8. The ATPase and GTPase activity of the mutant Rep proteins and the effect of the Cap protein on the ATPase and GTPase activity of the mutant Rep proteins

The relative ATPase activities of Rep, Rep-box-1-del, Rep-box-2-del, Rep-box-3-del, and Rep-box-1-mut were 105.39 ± 5.56%, 53.99 ± 0.85%, 58.09 ± 3.19%, 51.72 ± 2.33%, and 49.97 ± 1.58%, respectively. The Walker A-del and Walker B-del mutants both lead to significant reductions in the ATPase activity. Furthermore, the ATPase activity of the AYDG-del mutants of Rep protein lost about 42% of the wild-type enzyme’s activity, which suggests that magnesium ions are necessary for the ATPase activity of the Rep protein. The relative ATPase activities of Rep-box-1-del, Rep-box-2-del, Rep-box-3-del, and Rep-box-1-mut were reversed to 112.69 ± 2.71%, 105.39 ± 8.42%, 105.62 ± 4.45%, and 104.58 ± 5.49%, respectively, in the presence of the Cap protein (Fig. 8A). The relative ATPase activities of mutant Rep proteins are able to be reverted to the levels of the Rep protein alone. As expected, all relative activities of the mutant Rep proteins were significantly lower than that of the Rep protein in the presence of the Cap protein.

Similarly, the relative GTPase activities of the Rep, Rep-box-1-del, Rep-box-2-del, Rep-box-3-del, and Rep-box-1-mut were 102.47 ± 2.46%, 60.27 ± 2.52%, 57.34 ± 1.84%, 53.64 ± 1.25%, and 55.45 ± 1.27%, respectively (Fig. 8B), which suggests that GTPase activity is also affected when magnesium ion cannot bind. Finally, the lysine (K) residue of the Walker A motif (G/XXXXGK/GST) was found to be important to both the ATPase activity and the GTPase activity of the BFDV Rep protein. The relative ATPase activities of Rep-box-1-del, Rep-box-2-del, Rep-box-3-del, and Rep-box-1-mut were respectively reversed to 119.59 ± 3.14%, 116.92 ± 3.2%, 116.97 ± 3.48%, and 120.59 ± 4.6%, respectively, in the presence of the Cap protein. The relative GTPase activities of mutant Rep proteins can reverse. Noteworthy, the relative GTPase activities of Rep-box-1-del and Rep-box-1-mut returned to a similar level to that of the Rep protein in the presence of the Cap protein (Fig. 8B).

4. Discussion

In order to obtain a high yield of the target proteins, better protein purification, good solubility of the expressed proteins, and correct post-translation modification of the expressed pro-
proteins, the proteins of interest were expressed as tagged proteins in bacteria (Patterson et al., 2013; Sarker et al., 2015). No glycosylation site was found on the Cap protein based on the prediction from the http://www.cbs.dtu.dk/services/NetNGlyc website. The N-terminal thioredoxin tag and a lower induction temperature were used in this study to preserve adequate biological activity. Such a system helps to purify the capsid proteins of circovirus, which are notoriously difficult to purify in soluble forms. In addition to the Rep protein of BFDV, this tagging system has also been used to analyse the ATPase activity of the A32L protein of orb virus (Lin et al., 2010).

If we examine the Rep protein of PCV, this protein’s ATPase activity is dependent on the presence of Mg\(^{2+}\) ions as a co-factor and is not affected by the presence of either single-stranded or double-stranded DNA (Steinfeldt et al., 2007). The hydrolysis of ATP is necessary to allow the replication of PCV because the separation and unwinding of the origin of replication of DNA would need the energy. These characteristics are very similar to those of the Rep protein of BFDV. However, an inhibition of the ATPase and GTPase activity of the Rep protein of BFDV were observed when either double-stranded DNA or single-stranded DNA was present. Several mechanisms allowing such DNA-independent ATPase and GTPase activity have been proposed. Firstly, this may be an intrinsic characteristic of the Rep protein. Secondly, because large amounts of Mg\(^{2+}\) ions are sequestered by single-stranded and double-stranded DNA, this will result in there being less cofactor available when these molecules are present, which in turn will inhibit the ATPase and GTPase activity levels. Thirdly, other host proteins are likely to be important when the above activities are involved in binding DNA to the Rep protein (Bideshi and Federici, 2000). Since the ATPase activity and GTPase activity of the Rep protein of BFDV are inhibited by nucleic acids, the enzyme is not likely to be a helicase (Staubler et al., 1997).

Similar to geminiviruses, the activation of the ATPase activity and GTPase activity of the Rep protein of BFDV occurs through the binding of ATP and GTP to their appropriate domains. This binding serves as a conformation switch and is involved in a signal transduction cascade initiated by heterotrimeric G proteins and Ha-ras p21-like GTPases (Lin et al., 1992). In such a system, the ATPase activity and GTPase activity, which are controlled by the Walker A and Walker B motifs, respectively, are able to determine the immune response of the host and the virulence of the virus. Therefore, mutations of the Walker A motif are known to result in the production of non-infectious progeny of CSFV (Gladue et al., 2011).

The GXXXXGK, DXXG, and NXD domains are known to be associated with GTP-binding proteins and are usually separated by 40 amino acids and then by another 40 amino acids. However, the GXXXXGK and GXXG domains of the Rep protein of BFDV are only separated by 32 amino acids. Similar to α/β tubulin, a GTP-binding protein, it exhibits an atypical spacing rule of less than 40 amino acids (Dever et al., 1987). When the A32L protein of orb virus and the NS4B protein of CSFV were examined, the ATPase consensus sequence with mismatches can be used as the common phosphate-binding sequence for ATP and GTP (Gladue et al., 2011; Lin et al., 2011). However, the dissociation constant (K\(_d\)) value of such a sequence within a GTP-binding protein is higher than that of a ATP-binding protein suggesting that the affinity for ATP is higher than that of GTP. Interestingly, the Rep protein of BFDV is the only one that exhibits similar levels of ATPase and GTPase activity. It is possible that, unlike larger viruses, the Rep protein of BFDV has to be multifunctional (Thompson et al., 2009). Since the GXXXXGK sequence belongs to the consensus domains of both ATPase and GTPase, it is reasonable that there is both the ATPase activity and GTPase activity of BFDV Rep protein are reduced in the Rep-box-1-del mutant protein. Similar to the μA of ARV, the lysine (K) residue of the Walker A motif (GXXXXGK) is involved in the binding of the β-phosphates and γ-phosphates of NTPs. If it was mutated into an alanine (A) residue, both the ATPase and GTPase activities are known to be abrogated (Su et al., 2007). The GTPase activity of Rep-box-1-del protein is close to that of the Rep protein with the Walker A motif mutation implying that the hydrophobic residue at the 2nd position near the glycine residue (G) plays a significant role in the GTPase activity. A negatively charge amino acid, aspartic acid (D), located at the 1st or/and 3rd positions of the DYDG of the Rep protein of BFDV is able to bind magnesium ions. This is similar to for the situation with the AYDG motif of the A32L protein of orb virus (Lin et al., 2011). Since the GTPase activity of the Rep protein with motif I, II, and III deletions are profoundly reduced, all of the identified motifs would seem to participate in ATPase and GTPase activity.

The optimal pH values for the ATPase and GTPase activities of BFDV are both 7.5, which implies that the two activities are simultaneously active at the same pH value. A significant inhibition of the ATPase activity of BFDV was observed at 80 °C suggesting the Rep protein is a thermo-sensitive protein. The optimal activity of the ATPase of the Rep protein and of the μA protein of ARV occur in the presence of Mg\(^{2+}\) ions and the presence of Zn\(^{2+}\) ions significantly inhibits both the ATPase and GTPase activities (Su et al., 2007). While the ATPase activity of the μA protein of ARV is not affected by Mn\(^{2+}\) ions, the ATPase and GTPase activity of the Rep protein of BFDV are inhibited by Mn\(^{2+}\) ions suggesting that the
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Appendix A. Supplementary data

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