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Purification and functional motifs of the recombinant ATPase of orf virus

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ABSTRACT

Our previous study showed that the recombinant ATPase encoded by the A32L gene of orf virus displayed ATP hydrolysis activity as predicted from its amino acids sequence. This viral ATPase contains four known functional motifs (motifs I–IV) and a novel AYDG motif; they are essential for ATP hydrolysis reaction by binding ATP and magnesium ions. The motifs I and II correspond with the Walker A and B motifs of the typical ATPase, respectively. To examine the biochemical roles of these five conserved motifs, recombinant ATPases of five deletion mutants derived from the Taiping strain were expressed and purified. Their ATPase functions were assayed and compared with those of two wild type strains, Taiping and Nantou isolated in Taiwan. Our results showed that deletions at motifs I–III or IV exhibited lower activity than that of the wild type. Interestingly, deletion of AYDG motif decreased the ATPase activity more significantly than those of motifs I–IV deletions. Divalent ions such as magnesium and calcium were essential for ATPase activity. Moreover, our recombinant proteins of orf virus also demonstrated GTPase activity, though weaker than the original ATPase activity.

Introduction

Orf virus belongs to the genus parapoxvirus of the family *poxviridae* and contains a linear double-stranded DNA genome with length of 137–139 kbp [1]. The morphology and size of orf virion is ovoid-shaped with a crisscross textures and approximate 260 nm in length and 160 nm in width [2]. In goats, sheep, and ruminants, contagious ecthyma caused by orf virus is characterized by papules, vesicles, and scabs around the lips, muzzle, gums, and tongue [3,4]. Despite that low mortality for adult sheep, orf is still an important contagious disease to newborn lambs which may succumb to complicated secondary infections and suffer from difficulty in milk feeding, leading to high mortality [5].

The ATPase encoded by the A32L gene which is located at the right terminal region of viral genome, and it maybe involves in viral DNA packaging. Similar to the A32 protein of vaccinia virus, IVa2 proteins of adenovirus, and gp I of small filamentous single-stranded DNA bacteriophages, four conserved motifs were found in ATPase of orf virus [6–8]. Motif I (MALVGGSGSGKT) and II (LIILDD) corresponding to the Walker boxes A and B are typical motifs of ATPases and serve as a phosphate-binding site and a magnesium-binding site for ATP–Mg complex, respectively [6,9,10]. Motif III (RHINVSLVLLCQ) which forms a beta-stranded structure ended with a polar amino acid is found in most purine NTP-binding proteins. Motif IV (GRAITHLCCCN) forms a hydrophobic beta strand leaded by an invariant histidine residue [6]. The heterogeneous diversity of residues near the C-terminal of ATPase protein of orf viruses was similar to that of ras protein (a GTPase) [8,11,12].

In the previous work, we demonstrated the A32L gene of orf virus indeed encodes a functional ATPase as expected from sequence analysis [13]. In this study, the full length of ATPase proteins from Taiping and Nantou strains which were two strains isolated in the central Taiwan and were described [8], and five mutant proteins each carrying a deletion in one of the motifs were expressed, purified and characterized. Results showed motifs I–IV and the AYDG motif are required for ATPase activity, and the Taiping strain exhibited a slightly higher ATPase activity than Nantou strain. Furthermore, a weaker GTPase activity was found in our recombinant proteins.

Materials and methods

Construction of mutant plasmids

To construct plasmids containing deletion in one of five conserved motifs, the PCR-driven overlap extension method was used [14]. All primer information was listed in Table 1. The pET32b-A32L (Taiping strain) plasmid carrying the full length A32L gene

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Table 1
List of primers used for deletion construction of each motif.

	Primer name	Sequence
1	A32L outer, primer F1	5′-GGAGATATGGATCCGGATGTCGTGCAG-3′
2	A32L outer, primer R1	5′-GGCGCTCAGCGGCCGCATTGCCGCCG-3′
3	MotifI deletion, primer R2	5′-CGAGAGCAGGTAGGCCCTGAAGGGCGCCTC-3′
4	Motif I deletion, primer F2	5′-GAGGCGCCCTTCAGGGCCTACCTGCTCTCG-3′
5	AYDG motif deletion, primer R2	5′-GTCCGGCCACACGAAGGCGTTGTAGACGGG-3′
6	AYDG motif deletion, primer F2	5′-CCCGTCTACAACGCCTTCGTGTGGCCGGAC-3′
7	Motif II deletion, primer R2	5′-CTGCGTGTCGCCCATGAACTTCTGCTTCTT-3′
8	Motif II deletion, primer F2	5′-AAGAAGCAGAAGTTCATGGGCGACACGCAG-3′
9	Motif III deletion, primer R2	5′-GATGTGCTTGTAGGTCCCGTAGTTCATGAG-3′
10	Motif III deletion, primer F2	5′-CTCATGAACTACGGGACCTACAAGCACATC-3′
11	Motif IV deletion, primer R2	5′-GTCGGAGTCGGAGACGTTGACGGGGATGTG-3′
12	Motif IV deletion, primer F2	5′-CACATCCCCGTCAACGTCTCCGACTCCGAC-3′

was used as template in the first round PCR. The scheme of PCR and cloning was illustrated in Fig. 1A.

PCR products with correct sizes and sequences were purified from agarose gel and subsequently equal quantities of each DNA fragments were used as templates for the second round PCR amplification with outer flanking primers (Fig. 1A). The conditions for the first and second PCR were 94 °C for 3 min and then 30 thermo-cycles consisting of denaturation at 94 °C for 50 s, annealing at 59 °C for 1 min, and an extension at 72 °C for 45 s with a final extension at 72 °C for 7 min. Finally, PCR products were digested with restriction enzymes *Bam*H I/*Not* I and separately cloned to prokaryotic expression vectors, pET32b. The nucleotide identity of each cloned insert was verified by automated sequencer (Mission Biotech, Taipei, Taiwan). The plasmids with deletion in motifs I–IV and motif AYDG were named as pET32b-A32L-box-1-del, pET32b-A32L-box-2-del, pET32b-A32L-box-3-del, pET32b-A32L-box-4-del, pET32b-A32L- AYDG-del, respectively, as shown in Fig. 1B.

Protein expression and purification

Plasmids of wild type and of the deletion mutants were separately transformed into the expression host, *E. coli* strain BL21 AI (Invitrogen). The transformed bacteria was induced with a final concentration of 0.8 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) and 0.2% L-arabinose (CALBIOCHEM) and then incubated at 16 °C for 24 h. After centrifugation, bacteria pellets were resuspended in binding buffer (500 mM NaCl, 50 mM Tris–HCl, and 20 mM imindazole, pH 7.4) and then subjected to freezing and thawing for three times. After addition of 0.4 mg/mL lysozyme, bacteria lysates were incubated on ice for 30 min and subsequently sonicated for 6 min. Following centrifugation, the supernatant containing the native form of recombinant proteins was recovered and purified by the chelating Sepharose Fast Flow (GE Healthcare). Briefly, the supernatant was mixed with Ni^{2+}-charged Sepharose and then incubated at 4 °C for 2–3 h for an optimal binding. After binding, the Sepharose matrix was washed 6 times with washing buffer (500 mM NaCl, 50 mM Tris–HCl, 50 mM imidazole, pH 7.4) to remove non-specifically bound protein. Finally, the bound protein was eluted in 4 mL elution buffer (500 mM NaCl, 50 mM Tris–HCl, 400 mM imidazole, pH 7.4) and then dialyzed against 1× PBS at 4 °C to remove excess imidazole.

Electrophoresis and Western blotting

Purified proteins were mixed with 2× sample buffer and boiled for 6 min. Proteins were separated by the SDS-12% PAGE, and stained with Coomassie Brilliant Blue or further transferred to a PVDF membrane (Amersham, GE Healthcare) for Western blot analysis. After blocking with TBST containing 5% dried milk (150 mM NaCl, 20 mM Tris, and 0.1% Tween 20, pH 7.6), the membrane was incubated with anti-His-tag antibody (AbD Serotec) at room temperature for 1 h. Then the membrane was washed with TBST, and a horseradish peroxidase-conjugated goat anti-mouse antibody (Jackson ImmunoResearch) was added. The signal was then developed using an enzyme-linked chemiluminescence system (ECL, Amersham, GE Healthcare) following the manufacturer's instructions.

ATPase and GTPase functional assays

Purified recombinant proteins were tested for their ATPase activity. Briefly, an assay mixture contained 10 μl of 3× reaction buffer (150 mM NaCl, 150 mM HEPES, 15 mM $MgCl_2$, pH 7.5), 2.5 μM (0.225 μCi) of radioactive [α-^{32}P] ATP, and 1 μg recombinant protein. The mixtures were incubated at 37 °C for 30 min. Reaction mixtures were spotted onto a polyethyleneimine (PEI) cellulose plate (Merck) and developed by ascending thin-layer chromatography (TLC) in a solvent system (750 mM KH_2PO_4, pH 3.5). The autoradiogram was obtained by exposing dried TLC plates to the X-ray films. Densitometric analysis for the measurement of ATPase activity on the X-ray film was performed using ImageJ software (National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html). All values were presented as mean (%) ± SD, and significant differences were analyzed by one-way ANOVA and *t* test. The conditions for GTPase activity analysis were same as those of ATPase assay except radioactive [α-^{32}P] ATP was replaced by [α-^{32}P] GTP.

Results

Expression, purification and identification of recombinant ATPases

By a multiple alignment of amino acid sequences of ATPases between vaccinia virus and five orf viruses, our previous study indicated that four conventional functional motifs (motifs I–IV) and the AYDG motif might play important roles in ATP hydrolysis [6,8]. Motifs I and II are classified as the Walker A and B motifs of ATPases, respectively. Therefore, to examine the contribution of these five motifs in the viral ATPase activity, protein expressing plasmids with deletion in motifs were constructed (Fig. 1B).

As shown in Fig. 2A and B, the expression and purification of our recombinant ATPases (Taiping strain, Nantou strain, and deletion mutants Box1-del, Box2-del, Box3-del, Box4-del or AYDG-del) were accomplished. All recombinant proteins were purified to a similar quantity, and their concentrations were determined by the Bradford method (Bio-Rad).

The recombinant ATPases contained thioredoxin and six histidine residues tagged at its N and C-terminus, respectively. For