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Disruption Effect of *Microplitis bicoloratus* Polydnavirus EGF-like Protein, MbCRP, on Actin Cytoskeleton in Lepidopteran Insect Hemocytes

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Abstract *Microplitis bicoloratus* is a braconid endoparasitic wasp associated with the polydnavirus named *Microplitis bicoloratus* bracovirus (MbBV). Parasitism of *Spodoptera litura* larvae leads to an impaired cellular immune response and to the disappearance of the 42 kDa actin in host hemocytes. In this work, we investigated if the absence of actin in blood cells was related to MbBV infection. An MbBV gene similar to *egf*-like genes identified in another bracovirus was partially cloned and named *Mbcrp1*. The full-length gene, named *Mbcrp*, is transcribed throughout the course of parasitism in host hemocytes and the 30 kDa MbCRP protein was detected in hemocytes 6–7 d post-parasitization. The *Mbcrp1* gene contains the cysteine-rich trypsin inhibitor-like (TIL) domain coding sequence and the expression of recombinant MbCRP1 inhibited the expression of the 42 kDa actin in Hi5 cells. The 34.1 kDa MbCRP1-green fluorescent protein fusion protein locate specifically in the cytoplasm. These results suggest that expression of MbCRP in lepidopteran insect cells is related to the disruption of the actin cytoskeleton.

Key words actin cytoskeleton; polydnavirus; *Microplitis bicoloratus*; cellular immune response; trypsin inhibitor-like domain; MbCRP1

The Polydnaviridae family consists of segmented, double-stranded DNA viruses that are specifically associated with certain types of parasitoid wasps [1]. Polydnaviruses (PDVs) are divided into two families, bracoviruses (BVs) and ichnoviruses (IVs) that coexist with wasps from the families Braconidae and Ichneumonidae, respectively [2]. The virion morphologies of BVs and IVs differ significantly. BVs have cylindrical nucleocapsids of various lengths, with virions made of one or more nucleocapsids surrounded by a single membrane, whereas those of IVs have lenticular nucleocapsids of uniform size, individually surrounded by two membranes [2,3]. Nevertheless, for both BVs and IVs, virion assembly takes place in the nuclei of calyx cells situated between the ovarioles and lateral oviducts, and viral particles are injected in the lepidopteran along with the wasp's egg. The expression of a viral gene causes the suppression of the host's immune system that is essential for the survival of the parasitoid's progeny [1,4–6].

PDVs can infect most larval tissues, particularly hemocytes [7,8]. In parasitized host larvae, hemocytes might become apoptotic [5,9] or lose their ability to adhere to a substrate [4,9–12]. Some polydnaviral genes are reportedly involved in hemocyte function disruption. For example, Glc1.8, a protein encoded by the *Microplitis demolitor* bracovirus (MdBV), induces a loss of adhesion and phagocytosis in insect Hi5 and S2 cells [13]. Cr1, a protein encoded by the *Cotesia congregata* bracovirus, prevents cell surface exposure of lectin-binding sites and microparticle formation during immune stimulation of hemocytes. Cr1 is a secreted glycoprotein that has been implicated in depolymerization of the actin cytoskeleton of hemocytes, resulting in hemocyte inactivation [14]. Despite evidence that PDV infection is correlated with

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hemocyte cytoskeleton disruption, the mechanisms by which PDV proteins affect the cytoskeleton remain unclear.

MdBV encodes three genes, *egf1.0*, *egf1.5* and *egf0.4* (GenBank accession No. U76034, U76033 and DQ000240, respectively), which are characterized by an epidermal growth factor-like cysteine-rich motif and an amino-terminal trypsin inhibitor-like (TIL) domain [15,16]. The MdBV *egf* genes are transcribed 12–24 h post-parasitization (p.p.) and the peak of their expression coincides with physiological changes observed in host hemocytes. This suggests that the proteins encoded by *egf* genes are related to immune suppression [15].

The closely related species *Microplitis bicoloratus* is a parasitoid of *Spodoptera litura* larvae and is associated with the *Microplitis bicoloratus* bracovirus (MbBV). As for other parasitoids, parasitization of *S. litura* larvae by *M. bicoloratus* leads to suppression of the immune response and developmental arrest of the host larvae. Interestingly, we found that parasitism was correlated with an absence of 42 kDa actin in *S. litura* hemocytes.

In the present study, we isolated a sequence from MbBV using primers designed according to MdBV *egf* sequences which was named *Mbcrp1*. The recombinant MbCRP1 contains a TIL domain, and is concomitant with actin decrease in infected Hi5 cells. We also detected a 30 kDa protein, MbCRP, in hemocytes 6–7 d post-parasitization using antiserum of the recombinant MbCRP1.

Materials and Methods

Cell lines, polydnavirus and parasitoid rearing

Spodoptera frugiperda (Sf9) and *Trichoplusia ni* (Hi5) cells were maintained in Grace's medium (Gibco BRL, Gaithersburg, USA) supplemented with 10% fetal bovine serum.

The parasitoid colony was maintained on *S. litura* larvae reared in the laboratory; adults were provided honey as a dietary supplement. MbBV DNA was extracted and analyzed according to established methods [17].

Electron microscopy

Reproductive tracts of *M. bicoloratus* females were pulled out in Pringle's solution [18]. Isolated ovaries were fixed in 2.5% glutaraldehyde overnight at 4 ºC, washed three times with phosphate-buffered saline (PBS) buffer (pH 7.2) and post-fixed in 1% osmium tetroxide in PBS for 1 h at 4 ºC. After fixation, ovaries were washed three times with PBS buffer, dehydrated in graded ethanol and soaked in acetone. Samples were embedded in Spurr's resin. Ultra-thin sections were stained with uranyl acetate and lead citrate and observed under a JEM-100CXII transmission electron microscope (Jeol, Tokyo, Japan) operating at 100 kV.

For negative staining, ovaries isolated from three female wasps were placed in 50 μl ice-cold Pringle's solution and gently dissected to allow the calyx fluid to diffuse. The extract was then centrifuged at 1000 *g* for 3 min to remove cellular debris and eggs. The supernatant was adsorbed on a Formvar-coated (Nisshin EM) electron microscopy grid stained with 2% phosphotungstic acid, dried on filter paper and observed with the JEM-100CXII microscope.

egf-like gene cloning

Two primers were designed based on sequence alignment of MdBV *egf* genes. These primers, P1S, 5′-CACCTGCTTTTCCTGTTTGCATTTT-3′ and P1A, 5′-CCGTTTTGAAGAATCATTGTTGGC-3′, correspond to the N-terminal cysteine-rich conversed region of MdBV *egf* genes. To isolate an *egf*-like gene from MbBV, a polymerase chain reaction (PCR) was carried out using MbBV DNA as a template. The amplified fragment was cloned into pMD18-T (TaKaRa, Dalian, China) vector and sequenced. The sequence was named *Mbcrp1* and submitted to GenBank with accession No. DQ286649.

Sequence analysis

Nucleotide BLAST searches were carried out against the GenBank nr database at the National Centre for Biotechnology Information website (www.ncbi.nlm.nih.gov/blast). Protein analysis tools were found at the ExPASy molecular biology server (http://au.expasy.org/tools) and EMBL-EBI (http://www.ebi.ac.uk).

Plasmid construction and preparation of MbCRP1 antiserum

Two primers were designed to amplify the coding sequence of *Mbcrp1* with open reading frame using MbBV DNA as a template: P2S, 5′-GGATCCATGCCTACTAAAGAAAGTGA-3′ (*Bam*HI site underlined); and P2A, 5′-GTCGACTTAGTTGTAATAGCAGTAAA-3′ (*Sal*I site underlined). The amplified fragment was cloned into pMD18-T vector, then into the expression vector pQE30 (Qiagen, Hilden, Germany) with a 6×His-tag sequence. The resulting plasmid pQE30-MbCRP1 was checked by sequencing and transformed into *Escherichia coli* M15.

The *Mbcrp1* expression under control of bacteriophage T5 promoter in *E. coli* M15 was induced with 1 mM iso-

propyl-β-*D*-thiogalactopyranoside as recommended by the manufacturer (Qiagen). Expressed 6×His-MbCRP1 was purified from *E. coli* M15 by Ni-NTA agarose under denaturing conditions as described in the handbook (QIAexpressionist; Qiagen). Purified 6×His-MbCRP1 protein was used to raise antiserum in rabbits according to the method described by Sambrook *et al.* [19] using Freund's adjuvant (Gibco BRL).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted using the Qiagen RNA Kit (Invitrogen, Valencia, USA). For *S. litura* larvae, total RNA was isolated from 100 μl hemocytes recovered from non-parasitized and parasitized *S. litura* larvae. For Hi5 cells, total RNA of Hi5 cells was isolated from 5×10^5 mock-infected and recombinant baculovirus (see below) infected Hi5 cells at multiplicity of infection (MOI) of 5 at various time points post-infection (p.i.).

RT-PCR was carried out using an RNA-PCR-AMV Kit (Version 2.1; TaKaRa) with 3 μg of total RNA as the template. First-strand cDNA was synthesized using avian myeloblastosis virus (AMV) reverse transcriptase and random 9-mer according to the manufacturer's instructions. The cDNA mixture was amplified with MbCRP1-specific primers P2S and P3A-5′-TCTAGAGTTGTAATAGCAGTAAA-3′ (*Xba*I site underlined). The obtained PCR products were analyzed in 1.0% agarose gel.

Construction and identification of the recombinant viruses

Transfer-vector plasmids were constructed to generate recombinant AcMNPV expressing MbCRP1 protein. MbCRP1 was cloned into the *Bam*HI and *Xba*I sites of the pFastBacI vector (Invitrogen, Rockville, USA). Green fluorescent protein (GFP) gene was also cloned into the *Xba*I and *Pst*I sites of pFastBac-MbCRP1 vector as a C-terminus fusion to MbCRP1. pFastBacI vector was used as a control. The pFastBac-MbCRP1-GFP and pFastBac-GFP vectors were transformed into *E. coli* DH10Bac cells (Gibco BRL) and positive colonies were selected as described by the manufacturer. To obtain recombinant viruses, high molecular mass DNA was purified from the selected colonies as recommended by the manufacturer and transfected into Sf9 cells. The recombinant baculoviruses Ac-MbCRP1-GFP and Ac-GFP, named reAcMG and reAcG, respectively, were amplified to passage 3 in Sf9 cells. Titers of recombinant viruses were determined by tissue culture infectious dose 50 assay with infected Sf9 cells.

Collection of protein samples in hemocytes and Western blot analysis

S. litura larvae were bled from a proleg on ice. A total of 50–100 μl of hemocytes was pooled at various time points and centrifuged at 3000 *g* for 3 min. Supernatant (cell-free hemolymph) was collected and one volume of 2×sodium dodecylsulfate (SDS) loading buffer was added. The cellular pellet was resuspended in 50 μl of double distilled water and one volume of 2×SDS loading buffer was added. Equal non-heated protein samples were electrophoresed on denaturing 12% SDS-polyacrylamide gels [20]. Protein bands were either stained with Coomassie brilliant blue or transferred to a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany) as described earlier [19]. The membranes were blocked with 1% blocking solution (Roche, Mannheim, Germany) then probed with anti-MbCRP1 antiserum at a dilution of 1:200. Immunoreactive proteins were visualized using goat anti-rabbit immunoglobulin G and Ap-conjugate (Roche) following the protocol provided by the manufacturer. Actin blots were probed with a 1:5000 dilution of anti-actin (Calbiochem, San Diego, USA) and visualized with Ap-conjugated goat anti-mouse secondary antibodies at a 1:10,000 dilution (Roche).

Assessment of transcription and expression of MbCRP1 in infected Hi5 cells

Transcription and expression of MbCRP1 in infected Hi5 cells was assessed by RT-PCR and Western blot. Hi5 cells were seeded at a density of 5×10^5 cells per well in a 35 mm plate (Corning, Corning, USA). Cells were infected with recombinant virus at MOI of 5. Cells were harvested 6–72 h p.i.. RT-PCR and Western blot were carried out as described above.

Fluorescence microscopy

Hi5 cells (1×10^5) were grown on glass cover slips in 35 mm Petri dishes (Corning) and infected with reAcMG and reAcG at MOI of 5. At 12–48 h p.i., the cells were examined with a confocal-laser scanning fluorescence microscope (Leica SDK; Leica, Heidelberg, Germany).

Results

The virion morphology and DNA analysis of MbBV

The polydnavirus associated with *M. bicoloratus* is a typical bracovirus, as indicated by virion morphology and

genome characteristics. The negative staining of MbBV virions showed evident tail-like appendages [**Fig. 1(A)**, arrows] and heads [**Fig. 1(A)**, asterisks]. The particles consisted primarily of one or more nucleocapsids surrounded by a single envelope [**Fig. 1(B)**, arrows]. The MbBV genome had estimated 11 segments ranging from 8000 to 50,000 bp [**Fig. 1(C)**].

The cloning of a partial *egf*-like gene in MbBV

Due to the congeneric nature of MbBV and MdBV, we expected that the MbBV genome can also encode members of the *egf*-like family. Therefore we used primers designed in conserved regions of MdBV *egf* genes and obtained a 652 nt amplifier using MbBV DNA as a template. Sequencing confirmed that the amplified fragment was the 5′ region of an *egf*-like gene (BlastN e-value=5e–131; BlastX e-value=4e–11). The full *egf*-like gene was named *Mbcrp*, and the fragment we obtained was named *Mbcrp1*. Alignment with MdBV genomic sequences indicated that the *Mbcrp1* contains partial exon sequence of *Mbcrp* encoding the TIL domain (data not shown). This was confirmed by InterPro Scan and MotifScan analysis of MbCRP1 that MbCRP1 consists of 61 amino acids and contains a TIL domain (**Fig. 2**) encompassing a cysteine-rich repeat, located in position 35–51. And *egf* motif is absent in the *Mbcrp1*.

Fig. 2 Nucleotide and deduced amino acid sequence for the partial *Mbcrp* gene, *Mbcrp1* (GenBank accession No. DQ286649), of the *Microplitis bicoloratus* bracovirus

The putative intron 1 is in italic, based on alignment with *Microplitis demolitor* bracovirus genomic sequences. The amino acid sequence of putative exon 2 is shown. The cysteine-rich trypsin inhibitor-like domain is underlined. The open reading frame of *Mbcrp1* sequence is in shade without changing the frame of exon 2. Primers P1A, P1S, P2A and P2S are indicated in open squares.

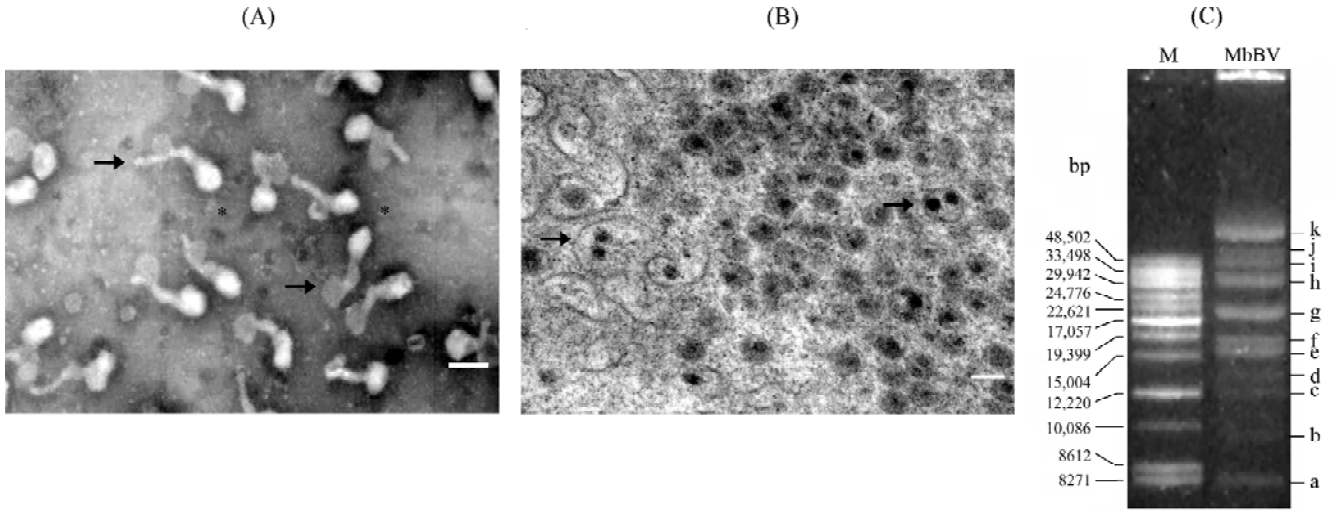

Fig. 1 Transmission electron micrographs of polydnavirus (PDV) virions from the braconid wasp *Microplitis bicoloratus* and *Microplitis bicoloratus* bracovirus (MbBV) DNA

(A) Negative staining of PDV virions of *M. bicoloratus*. The arrows indicate the viral "end structures" and the asterisks indicate the viral "head structures". Bar=100 nm. (B) MbBV particles in the nucleus of a calyx cell of the braconid wasp *M. bicoloratus*. One or more nucleocapsids (arrows) are found in a single envelope. Bar=200 nm. (C) Agarose gel (0.4%) electrophoresis of MbBV DNA (24 h, 30 V/cm). M, molecular weight marker (high weight linear DNA). The MbBV genome had estimated 11 segments ranging from 8000 to 50,000 bp (a–k).

MbCRP is expressed in hemocytes of *S. litura* larvae naturally parasitized by *M. bicoloratus*

Transcription of *Mbcrp1* in hemocytes of naturally parasitized *S. litura* larvae was examined by RT-PCR and Western blot (**Fig. 3**). A band of 195 bp was detectable 1–7 d p.p., although the bands were very faint 1–3 d p.p. [**Fig. 3(A)**]. This indicated that the *Mbcrp* gene is transcribed in *S. litura* hemocytes and transcription appears to be persistent during parasitoid larval development.

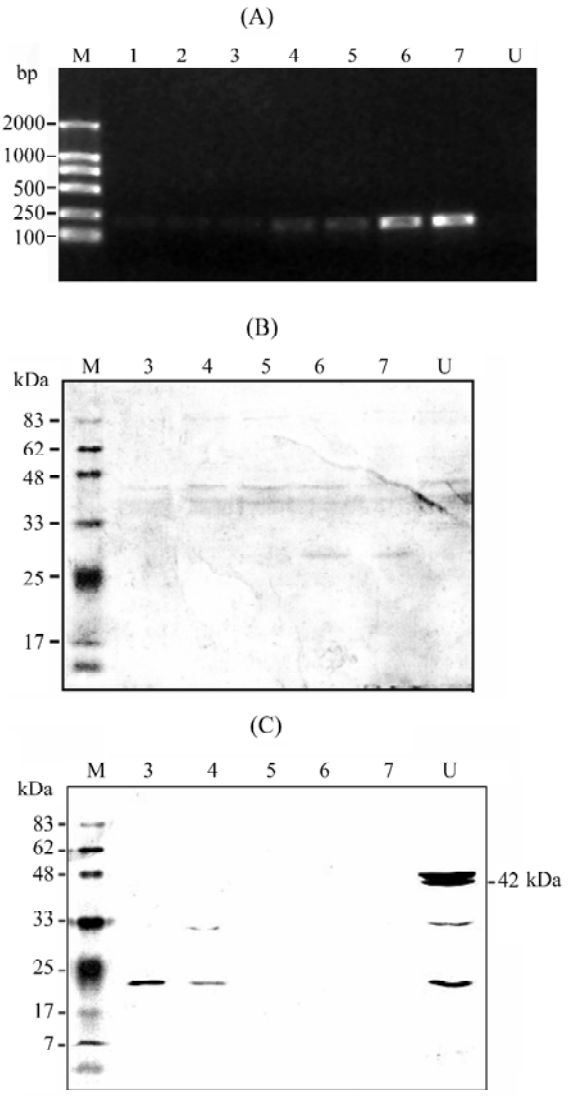

Fig. 3 Reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analysis of MbCRP and actin in hemocytes of *Spodoptera litura* larvae naturally parasitized by *Microplitis bicoloratus*

(A) RT-PCR analysis of *Mbcrp* transcripts. Total RNA was extracted from 100 μl hemocytes (1×10^6). M, DL2000 DNA marker; U, non-parasitized larvae. (B) Western blot analysis of MbCRP expression in hemocytes 3–7 d post-parasitization. M, pre-stained protein molecular weight marker; U, non-parasitized larvae. (C) Expression of 42 kDa actin in hemocytes 3–7 d post-parasitization. M, pre-stained protein molecular weight marker; U, non-parasitized larvae. 1–7, 1–7 d post-parasitization, respectively.

This result was confirmed by Western blot analysis. Indeed, in parasitized *S. litura*, the MbCRP1 antiserum recognized a specific protein of approximately 30 kDa (putative MbCRP protein) in larvae hemocyte lysate. The 30 kDa band was observed 6 and 7 d p.p. in hemocyte lysate [**Fig. 3(B)**] but was absent from cell-free hemolymph (data not shown). This indicated that the putative MbCRP protein was present only within the cell.

Effects of *M. bicoloratus* parasitism on actin in *S. litura* hemocytes

To test the effects of *M. bicoloratus* parasitization on actin in hemocytes, we analyzed expression of the 42 kDa actin in hemocyte lysate collected from parasitized *S. litura* larvae. In our experiments, the monoclonal anti-actin antibody recognized the 42 kDa actin. Western blot analysis showed that this monoclonal antibody recognized the 42 kDa band only in hemocytes from non-parasitized larvae [**Fig. 3(C)**, lane U]. Indeed, no signal was detected in hemocytes of parasitized larvae 3–7 d p.p.. This result suggests that the expression of the 42 kDa actin is inhibited by parasitism, which might lead to the disappearance of functional actin filaments in *S. litura* hemocytes.

Transcription and expression of MbCRP1 in Hi5 cells infected by reAcMG and effects of infection on actin of Hi5 cells

To further investigate if there is a link between absence of actin and expression of MbCRP in infected lepidopteran cells, we constructed a recombinant baculovirus expressing the MbCRP1 TIL domain of MbCRP and analyzed expression of actin in the baculovirus-infected Hi5 insect cell line.

After infection of the Hi5 cell line with the baculovirus reAcMG, transcription of MbCRP was analyzed by RT-PCR. The 195 bp PCR-amplified fragment corresponding to *Mbcrp1* was detected as early as 6 h p.i. and continued to be present until 72 h p.i. [**Fig. 4(A)**]. Hi5 cells were thus successfully infected by recombinant baculovirus reAcMG and MbCRP1 was persistently transcribed in infected cells. Western blot analysis using the anti-MbCRP1 antiserum confirmed the presence of the 34.1 kDa MbCRP1-GFP fusion protein in infected Hi5 cell lysate [**Fig. 4(B)**]. The 34.1 kDa band was first detected 12 h p.i., with a peak of expression 48 h p.i., then expression clearly declined 72 h p.i. [**Fig. 4(B)**].

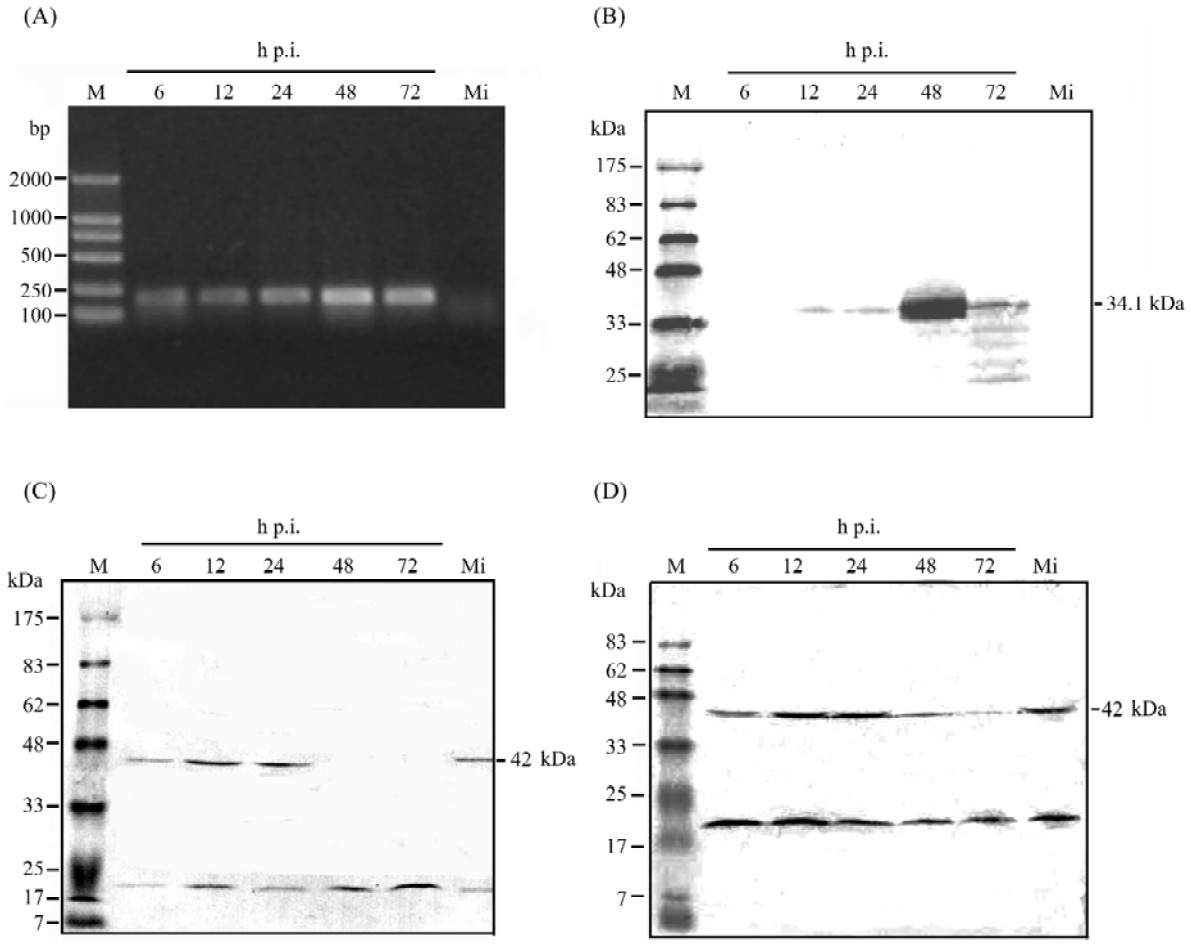

Fig. 4 Reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analysis of MbCRP1 and actin in Hi5 cells 6–72 h post-infection (p.i.) with recombinant baculoviruses

(A) RT-PCR analysis of MbCRP1 transcripts in *Trichoplusia ni* (Hi5) cells infected by recombinant baculoviruses Ac-MbCRP1-GFP (reAcMG). Total RNA extracted from 5×10^5 cells. M, DL2000 DNA marker; Mi, mock-infected Hi5 cells. (B) Western blot analysis of MbCRP1 expression in Hi5 cells infected by reAcMG. (C) Western blot analysis of 42 kDa actin proteins in Hi5 cells infected by reAcMG. (D) Western blot analysis of 42 kDa actin proteins in Hi5 cells infected by recombinant baculoviruses AcGFP (reAcG). (B–D) M, pre-stained protein molecular weight marker; Mi, mock-infected Hi5 cells. (A–D) Other lanes represent 1×10^5 cells observed 6–72 h p.i..

Expression of 42 kDa actin in Hi5 cells infected by reAcMG and reAcG was analyzed by Western blot. The 42 kDa band was detected in mock-infected Hi5 cells [**Fig. 4(C,D)**, lane Mi] and in Hi5 cells infected by reAcG recombinant virus 6–72 h p.i. [**Fig. 4(D)**]. In contrast, in Hi5 cells infected by reAcMG the 42 kDa band was detected 6–24 h p.i., but was absent 48–72 h p.i. [**Fig. 4 (C)**]. Therefore, in baculovirus-infected Hi5 cells, expression of MCRP1 seems to be correlated with an absence of 42 kDa actin expression.

At the time when MbCRP1 shows a peak of expression and 42 kDa actin is no longer expressed, approximately 48 h p.i., Hi5 cells infected with reAcMG had lost their adhesive properties and floated in the culture medium. In contrast, cells infected with reAcG remained attached to the dishes. This observation confirms the hypothesis that MbCRP1 is in some way related to disruption of the actin cytoskeleton of Hi5 cells.

The subcellular localization of the MbCRP1 protein was investigated using confocal laser scanning microscopy. Hi5 cells were infected with reAcG and reAcMG, incubated and examined for fluorescence at various time points p.i. (**Fig. 5**). GFP alone showed homogeneous fluorescence in the cytoplasm and nucleus 12–48 h p.i. The MbCRP1-GFP fusion protein was localized predominantly in the cytoplasm.

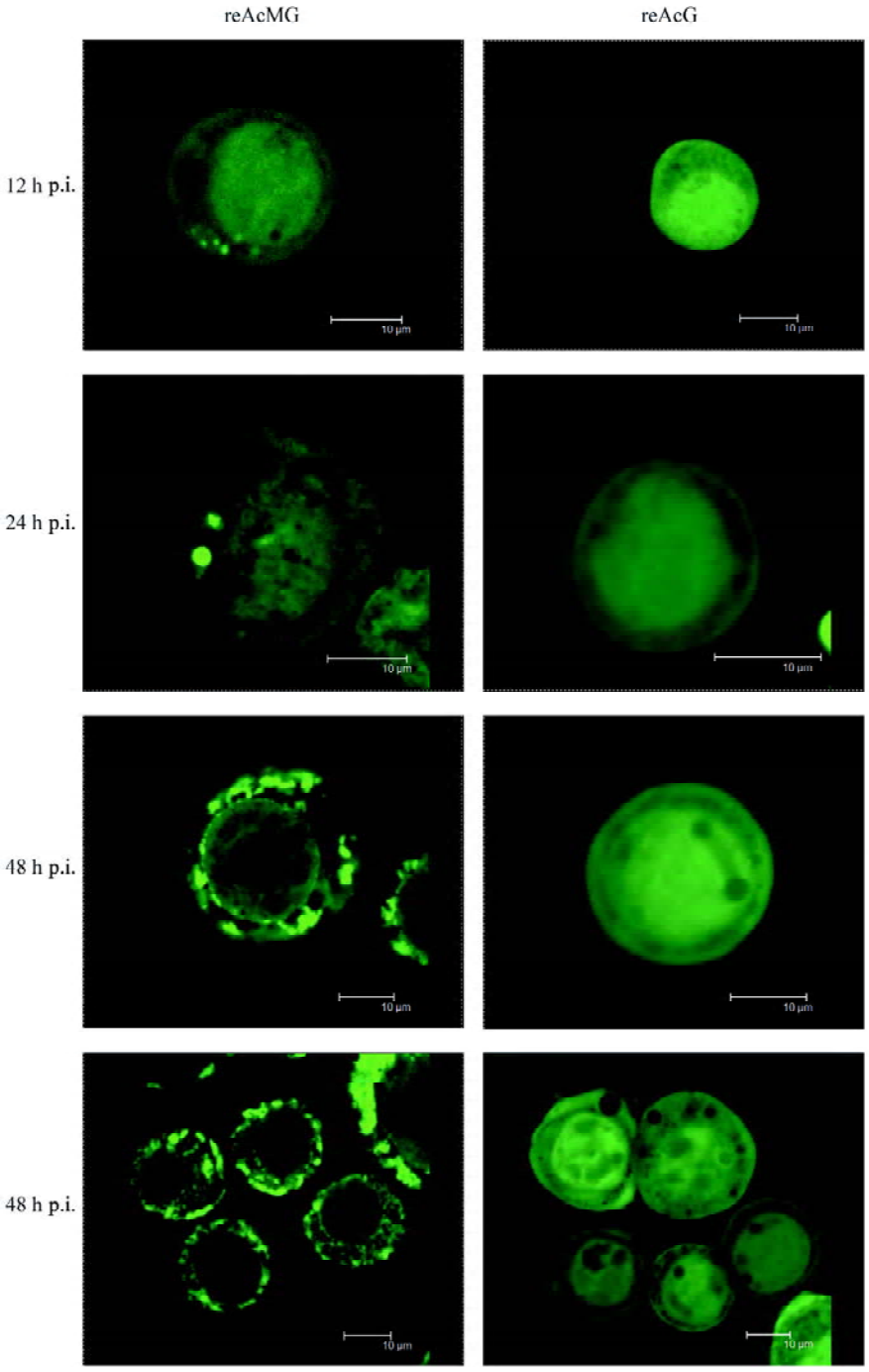

Fig. 5 Localization of the MbCRP1-GFP fusion protein in Hi5 cells infected by recombinant baculoviruses Ac-MbCRP1-GFP (reAcMG) 12–48 h post-infection (p.i.) using confocal laser scanning microscopy

MbCRP1-GFP fusion protein in *Trichoplusia ni* (Hi5) cells infected by recombinant baculoviruses AcGFP (reAcG) were used as control.

Discussion

Our study indicates that the presumed cysteine-rich EGF-like protein MbCRP of MbBV is probably related to the disruption of the actin cytoskeleton observed in the hemocytes of *S. litura* larvae parasitized by *M. bicoloratus*. Indeed, we found that infection of lepidopteran cell lines

with a baculovirus expressing the MbCRP TIL domain resulted in inhibition of expression of the 42 kDa actin. Moreover, in infected cell lines, the peak expression of MbCRP1 coincided with the complete disappearance of the 42 kDa actin [**Fig. 4(B,C)**].

In parasitized *S. litura* larvae, *Mbcrp1* is transcribed in host hemocytes throughout the course of parasitism [**Fig. 3(A)**] and the MbCRP protein is detected in the cytoplasm of polydnavirus-infected hemocytes 6–7 d p.p. using MbCRP antiserum [**Fig. 3(B)**]. By this time, the 42 kDa actin is no longer expressed in hemocytes [**Fig. 3(C)**].

Cytoskeletal proteins are found in highly organized arrays within the cytoplasm of higher eukaryotic cells. These proteins are intimately involved in functions such as cell and intracellular organelle movement, maintenance of cell shape and endocytosis [21,22]. The cytoskeleton of most eukaryotic cells includes the microfilaments composed of actin, the microtubules composed of tubulin and the intermediate filaments composed of vimentin or desmin.

The absence of functional actin filaments in MbCRP-expressed cells was probably the primary cause of cells or hemocyte inactivation. Although little is known about the molecular mechanisms leading to hemocyte activation, processes such as attachment, aggregation [23,24] and coagulation reaction [25] involve complex rearrangement of the actin cytoskeleton [26]. *In vitro*, the expression of the MdBV *glc1.8* causes the disruption of F-actin in the cytoplasm and induces the accumulation of F-actin at the periphery of cells leading to a loss of adhesion by hemocyte-like cell lines, namely, Hi5 [14]. In our study, the expression of recombinant MbCRP1 also greatly reduced the ability of these cells to adhere to a substrate, which was probably linked to the absence of the 42 kDa actin (**Fig. 4**). Some other PDV genes have been implicated in disruption of the actin cytoskeleton but their subcellular localizations were distinctly different: Glc1.8 protein localized to the surfaces of infected cells [13] and Cr1 protein was secreted [14], whereas MbCRP localized in the cytoplasm (**Fig. 5**).

The unique intracellular location of MbCRP protein suggested it is probably involved in specific roles within the actin cytoskeleton. Further studies would be necessary to investigate whether MbCRP protein is an actin-binding protein or not, through protein-protein interactions. In our experiments, we also found that the 42 kDa actin declined in Hi5 cells infected by reAcG at 48 and 72 h p.i. [**Fig. 4 (D)**]. We presume that this side-effect might cause due to Bac-to-Bac expression system. To test this hypothesis, other insect cell expression systems should be used in further research.

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