Detection of the expression of a *Bombyx mori* Atypical Protein Kinase C in *BmPLV*-Infected Larval Midgut

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Protein kinase C (PKC) is involved in many cellular signaling pathways, it participates in many physiological processes, such as cell cycle, growth, proliferation, differentiation and apoptosis. To investigate the effect of PKC on the silkworm midgut tissue infection of *Bombyx mori* parvo-like virus (*BmPLV*), a *B. mori* atypical protein kinase C (*BmaPKC*) gene was cloned from larval midgut tissue, expressed in *E. coli* and purified. Additionally, the *BmPLV* susceptible silkworm strain and resistant silkworm strain were used to test the effect of the *B. mori* infection on *BmPLV*. The result showed that *BmaPKC* encodes a predicted 586 amino acid protein, which contains a C-terminal kinase domain and an N-terminal regulatory domain. The maximum expression amount of the soluble (His)6-tagged fusion protein was detected after 0.8 mmol/L IPTG was added and cultured at 21°C. The (His) 6-tagged fusion protein revealed about 73 kDa molecular weight which confirmed by western blot and mass spectrography. Furthermore *BmaPKC* protein were detected at 0–72 h post-infection in *BmPLV*-infected larval midgut tissue, western blot showed that as time went on, the expression of *BmaPKC* increased gradually in susceptible strain, the expression quantity on 72 h is 5 times of 0 h. However, in resistant strain, the expression quantity is slightly lower than susceptible strain. But no significant change in resistant strain was observed as time went on. The available data suggest that *BmaPKC* may involve in the regulation of *BmPLV* proliferation.

Key words: *Bombyx mori* Protein kinase C, Expression, *Bombyx mori* parvo-like virus

Introduction

Nishizuka *et al.* discovered protein kinase C (PKC) isoenzymes when they study the extracts obtained from rat brains in 1977 (Uno *et al.*, 2006). PKC participates in many physiological processes in regulating cell cycle, growth, proliferation, differentiation and apoptosis. The PKC family contains 10 members encoded by 9 genes in mammals. Based on primary structure and biochemical characteristics, the enzymes can be divided into three sub-families: classical or conventional PKCs (α , β I, β II and γ ; cPKCs), novel PKCs (δ , θ , ϵ and η ; nPKCs), and atypical PKCs (ζ and λ (mouse)/ ι (human); aPKCs). The conventional and novel PKCs have two types of lipid binding domains, a tandem repeat of C1 domain and a C2 domain in the regulatory region. The C1 domain is a cysteine-rich domain involved in the binding of sn-1,2-diacylglycerol (DAG) and phorbol ester. In the cPKCs, the C2 domain follows the C1 domains, making these isoenzymes Ca^{2+} -responsive and diacylglycerol-sensitive; in the case of nPKCs, the Ca^{2+} independent C2 domain in the N-terminal regulatory region that is followed by the C1 domain (Stahelin *et al.*, 2004, 2005; Melowic *et al.*, 2007). Furthermore, these C2 domains of nPKCs do not respond to Ca^{2+} , making these enzymes diacylglycerol-sensitive but Ca^{2+} independent; The aPKCs lack binding motifs for these cofactors (Ca^{2+} or diacylglycerol), The enzyme activity of aPKCs is not dependent on diacylglycerol, Ca^{2+} , or phospholipid. Instead, they contain a Phox/Bem 1 (PB1) domain located in the N-terminus (Moscato *et al.*, 2006). This domain binds to other PB1 domain-containing proteins through the PB1–PB1 domain interaction. Although processes of atypical protein kinase C have been investigated in fly (Kim *et al.*, 2009) mouse (Kovac *et al.*,

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2007) and human (Wang *et al.*, 2009), but only a few studies have been performed in *Bombyx mori* (Uno *et al.*, 2006; Dedos *et al.*, 2008). In *B. mori*, the characters of protein kinase C alpha and iota were examined, and the protein kinases C operates a negative feedback mechanism at the level of extracellular Ca^{2+} entry through capacitative Ca^{2+} entry channels. But *Bombyx mori* atypical protein kinase C remains to be investigated.

As the domesticated silkworm, *B. mori* is a model insect; in a certain level it can reflect the biological characteristics of higher eukaryotes. *B. mori* parvo-like virus is a novel virus. It infects the midgut tissue of *B. mori*, causes flacherie diseases of silkworm. It is reported that nonstructural proteins 1 (NS1) of Parvovirus minute virus of mice (MVM) is able to initiate viral DNA replication and becomes covalently attached to replicated DNA (Dettwiler *et al.*, 1999). Atypical PKC as the active component can regulate the NS1 DNA unwinding and replicative functions (Lachmann *et al.*, 2003). Nevertheless, no evidence indicates that BmaPKC affect the replicate of virus when the silkworm *B. mori* was infected by virus. Therefore, the study of *B. mori* atypical protein kinase C will help to elucidate the functional roles of aPKCs orthologues in higher eukaryotes and further explore the relationship between protein kinase C and virus.

In this work, the open reading frame (ORF) of aPKC was cloned and the recombinant enzyme was expressed in *Escherichia coli*. The amino acid sequence of recombinant aPKC was verified by mass spectrometry analysis. After expression and purification of recombinant protein, we prepared the polyclonal antibody of aPKC. We also examined the expression profiles of aPKC gene in the midgut tissue of BmPLV-infected highly susceptible silkworm strain and resistant silkworm strain.

Materials and Methods

Materials

The expression vector pET-30a (+) and *E. coli* strains BL21 (DE3) were obtained from Novagen (CA, USA). All primers, RNase-free DNaseI, LA Taq polymerase, restriction enzymes, T4 DNA ligase and DNA the subcloning vector pMD18-T were purchased from TaKaRa (Dalian, China). Chemicals are all from Sigma (MO, USA) or a domestic provider in China if not state otherwise. *B. mori* susceptible strain Jingsong, resistant strain Qiufeng was reared in our lab.

RNA extraction and cDNA synthesis

The fifth larvae midgut tissue of *B. mori* were dissected, and washed with cold PBS (137 mM NaCl, 2.7 mM KCl,

4.3 mM Na_2HPO_4 and 1.4 mM KH_2PO_4). Then the tissue sample was frozen immediately in liquid nitrogen and stored at -80°C until extraction of RNA. Total RNA was extracted from frozen sample with RNeasy Mini Kit (Qiagen, USA). DNA was digested with RNase-free DNaseI at 37°C for 20 min. The RNA was further extracted with phenol-chloroform and precipitated with ethanol. The RNAs dissolved in DEPC-treated ddH₂O were used to make cDNAs with the M-MLV RTase (Promega, USA) and an oligo-dT primer following the manufacturer's instructions.

Construction of expression vector containing BmaPKC gene

The *BmaPKC* specific primers: forward primer (5'-CGCGGATCCATGATGCCGACACAACCTTG-3') with a *Bam* HI site (underlined), and reverse primer (5'-CCGCTCGAGT-CACACGCAGTCCTCCAG-3') with an *Xho* I site (underlined) were designed to amplify the ORF of the putative *BmaPKC* gene (GenBank accession no. NM_001043458). The PCR reaction was carried out with 35 amplification cycles (94°C for 30 s, 55°C for 45 s, and 72°C for 2 min) in a Gene Amp 2400 System thermocycler. The PCR product was ligated into pMD18-T vector using T4 DNA ligase and then transformed into *E. coli* TG1. Recombinant plasmid pMD18-*BmaPKC* was verified by restriction enzyme digestion (*Bam* HI and *Xho* I) and sequenced. The result showed that the *BmaPKC* ORF was correctly. A fragment between *Bam* HI and *Xho* I containing the insert was excised from the recombinant plasmid. The purified fragment was subcloned into the pET-30a (+) expression vector and frame with 6×His tag at the N terminus. The recombinant plasmid, pET-30a-*BmaPKC*, was verified by restriction analysis.

Expression of BmaPKC recombinant protein

The pET-30a-*BmaPKC* was then transformed into *E. coli* BL21 (DE3). In order to express recombinant protein, a freshly transformed colony was cultured in LB medium supplement with kanamycin (50 $\mu\text{g}/\text{ml}$) at 37°C overnight. This overnight culture was inoculated into fresh LB medium and cultured at 37°C with vigorous shaking. When OD_{600} reach 0.4, the expression of BmaPKC was induced by 0.2 mmol/L, 0.4 mmol/L, 0.8 mmol/L, 1.0 mmol/L concentrations of IPTG at 37°C for approximate 4 h, the target protein expression was highest with the add 0.8 mmol/L IPTG. Cells were harvest by centrifugation ($4500\times g$, 4°C , 15 min) and SDS-PAGE analysis on 12% gel was performed to estimate the expression level of *BmaPKC*. Western blot analysis using anti-6×His tag antibody showed that the fusion protein was successfully expressed.

Mass spectrometry

The specific bands corresponding to BmaPKC were

excised manually from the gel with a sterile scalpel and digested with trypsin according to Li's method (Li *et al.*, 2006). The digested samples were analyzed by an ultraflex II MALDI-TOF-TOF (BRUKER, GERMANY). Peptide mass fingerprinting (PMF) was performed by comparing the masses of peptides to NCBI protein database using the MASCOT search engine (<http://www.matrixscience.com>).

Purification of recombinant protein

A freshly transformed colony of pET-30a-*BmaPKC* was culture at 37°C overnight, then inoculated into fresh LB medium and cultured at 37°C with vigorous shaking. When OD₆₀₀ reach 0.4 the expression of *BmaPKC* was induced with 0.8 mmol/L IPTG and further cultured at 16°C, 21°C, 25°C, 27°C for another 12 h. Cells were harvest by centrifugation (4500× g, 4°C, 15 min) and SDS-PAGE analysis on 12% gel was performed to estimate the expression level of *BmaPKC*. At 21°C the target protein expression was highest. Then mass culture the transformed colony of pET-30a-*BmaPKC* in the same condition (21°C, 0.8 mmol/L IPTG). Also, cells were harvest by centrifugation (4500× g, 4°C, 15 min). The cell pellet was resuspended in buffer A (50 mM sodium phosphate, 300 mM NaCl, 1 mM EDTA, 0.5 mM PMSF, pH 8.0), then the suspension was lysed by sonication. The lysate was clarified by centrifugation (16,000× g, 4°C, 25 min). The supernatant was loaded onto a Ni-NTA affinity column (Qiagen). Purification conditions were standardized by optimizing pH, the concentration of salt and imidazole. After washing the captured column with 20 mM and 40 mM imidazole, the fusion protein was eluted with 250 mM imidazole. The eluted protein was dialyzed against buffer B (50 mM sodium phosphate, 150 mM NaCl, pH 7.5) at 4°C. The concentration of protein was determined by the Bradford method using bovine serum albumin as the standard (Bradford, 1976).

Polyclonal antibody preparation and immunoblot analysis

The 6×His-tagged recombinant *BmaPKC* protein was purified on a Ni²⁺-NTA column (Novagen) and used to raise polyclonal antibodies in rabbits. The antibody was prepared by standard techniques. Briefly, purified *BmaPKC* protein (about 2 mg) was injected subcutaneously to immunize New Zealand white rabbits in complete Freund's adjuvant, followed by two booster injections in incomplete Freund's adjuvant within a gap of 2 weeks before exsanguinations.

The Polyclonal antibody immunogenicity was detected by western blot. Using recombinant protein *BmaPKC* as antigen, the steps as mentioned above, the Rabbit anti-*BmaPKC* polyclonal antibodies (1:1,000 dilution) and horseradish per-

oxidase (HRP)-conjugated goat anti-rabbit IgG antibodies (1:2,000 dilution, Jackson) were used, and signals were detected by diaminobenzidine (DAB) (Sigma, USA).

Detection of the expression of *BmaPKC* in *BmPLV*-infected larval midgut

The 5th instar larvae of susceptible strain Jingsong and resistant strain Qiufeng were infected through oral with 5 µl of *BmPLV* and the midgut were harvested at 0, 12, 24, 36, 48, 60 and 72 h post-infection (h p.i), the midgut of 5th instar larvae which not infected with *BmPLV* were also harvested as the contrast. Protein samples of the midgut were lysed in SDS-PAGE loading buffer by boiling for 10 min. Then, the proteins were separated on 12% SDS-PAGE and subjected to western blot assay.

Western blot was performed as described previously (Towbin *et al.*, 1979). After SDS-PAGE electrophoresis, proteins were transferred onto a PVDF membrane in cold Towbin buffer (0.025 M Tris, 0.19 M glycine, 20% methanol). The membranes were blocked in 5% skimmed milk powder in PBST for 3 h followed by incubation with the anti-*BmaPKC* polyclonal antiserum for 1.5 h at room temperature. Subsequently, the membrane was incubated with a goat anti-rabbit IgG conjugated to HRP for 2 h at room temperature. The signal was detected with a ECL substrate.

Results

Cloning of *BmaPKC* gene from in *BmPLV*-infected larval midgut

The cDNA fragment of *BmaPKC* ORF was first amplified

Fig. 1. Agarose gel electrophoresis of PCR products. Lane 1, DNA marker; lane 2, PCR products after amplification with *BmaPKC* specific primers.

Fig. 2. Agarose gel electrophoresis of recombinant plasmids digested with *Bam* HI and *Xho* I. Lane 1, DNA marker; lane 2, pMD18-T-BmaPKC.

from *B. mori* fifth larval midgut tissue by a pair of specific primer (Fig. 1). The PCR products were cloned into pMD18-T vector. The recombinant plasmid was verified by restriction enzyme digestion (*Bam* HI and *Xho* I) (Fig. 2) and sequenced. The sequences corresponded with the expected size which is 1761 bp. This fragment encoding 586 amino acid residues, which contains a C-terminal kinase domain and an N-terminal regulatory domain and its catalytic activity is not dependent on diacylglycerol, Ca^{+2} , or phospholipid, lack binding motifs for these cofactors (Uno *et al.*, 2006).

Expression, western blot and mass spectrometry of *BmaPKC* gene in *E. coli*

Although BmaPKC was expressed in eukaryotes cells, in order to detect the expression of BmaPKC in *BmPLV*-infected larval midgut, we adopted a highly efficient prokaryotic expression system (The 1761 bp fragment including *BmaPKC* was excised from pMD18-T by *Bam* HI and *Xho* I and ligated into pET-30a (+) expression vector), in frame with 6×His tag at the N-terminus to generate pET-30a-BmaPKC (Fig. 3).

The (His)₆-tagged fusion protein His-BmaPKC was expressed in *E. coli* BL21 harboring the expressing plasmid pET-30a-BmaPKC. The expression of fusion protein could be induced by 0.2 mmol/L, 0.4 mmol/L, 0.8 mmol/L, 1.0 mmol/L concentrations of IPTG at 37°C for approximate 4 h respectively. SDS-PAGE analysis showed that The recombinant (His)₆-tagged protein His-BmaPKC of approximately 73 kDa was expressed in *E. coli*. And the amount of insoluble BmaPKC proteins is a maximum when 0.8 mmol/L of IPTG was added (Fig. 4A). We induced the expression of BmaPKC at 16°C, 21°C, 25°C, 27°C, with 0.8 mmol/L IPTG. Generally, lower tempera-

Fig. 3. Agarose gel electrophoresis of recombinant plasmids digested with *Bam* HI and *Xho* I. Lane1, DNA maker; lane 2, pET-30a- BmaPKC.

Fig. 4. The SDS-PAGE results stained with Coomassie blue and western blotting analysis of fusion protein His-BmaPKC. (A) M, protein molecular weight marker; lane 1, *E. coli* BL21 (DE3) lysate; lane 2, lysate of *E. coli* BL21 (DE3) transformed with pET-30a (+) expression vector; lanes 3-6, lysate of *E. coli* BL21 (DE3) harboring pET-30a(+)-BmaPKC induced with 0.2, 0.4, 0.8, and 1.0 mM IPTG. (B) M, marker: Size markers in kDa are indicated on the left side of the panel; lane 1, lysate of *E. coli* BL21 (DE3) with expression of BmaPKC; lanes 2-3, recombinant BmaPKC purified by Ni-NTA resin column; lane 4, western blotting analysis of recombinant BmaPKC with anti-6×His antibody.

1 MMPTQLVNQD NHVNDVRVKT VYNGDVMITY INHNITFEET THEMVATCRF
 51 APDQVFTIKW VDEEGDPCTI STQLELDESL RLYELNRDSE LTVHVFPNVP
 101 AAPGMPCAGE DRSIYRRGAR RWRKLYRVNG HIPQAKRFNR RAFCAFCQDR
 151 IWGLGRQGFK CIQCKLLVHK KCHKLVQKPC SNEHVDPIEV KDDANGEATL
 201 GRASSVRSRA EEQPPLPETP PAPAPPARPE ELEPGSQROY SLDDFELIRV
 251 IGRGSYAKVL MVELKRTKRI YAMKVIKKAL VTDDDEDIWW QTEKHVFETA
 301 SNHPFLVDLH SCFQTPSRLE FVIEFVRGGD LIFHIQRQRR LPEKHAFYA
 351 AEISLALHFL HERGVIIYRDL KLDNVLLDHE GRIKLTIDYGM CKEGVVRPGDT
 401 TSTFCGTPNY IAPILRLEE YGFSVDWWAL GVLTYEMLAG RSPFDIAQAA
 451 DNPQNTEDY LQQVILEKTI RIPRSLSVKA ASVLKGFINT NPVERLGCGE
 501 NGFLDIVNHP FPKSIEWEML EQQVYPPFK PRLEGERDLA NFPPEFTDEP
 551 VHLTPDNESV IADIDQSEFE GFVEYNPLLM SLEDCV

Fig. 5. Identification of BmaPKC protein by MALDI-TOF analysis. Amino acid sequences of BmaPKC protein. Matched peptide sequences are underlined.

tures for culturing and expressing are beneficial to expression of soluble protein. At 21°C, both soluble and insoluble BmaPKC proteins were produced and the expression of soluble proteins was highest.

The target protein was separated in 12% SDS-PAGE and confirmed by western blot analysis using the anti-6×His tag antibody. A band about 73 kDa was clearly visible in the PVDF membrane. Moreover, the target protein was excised to perform MS analysis (Fig. 5). By comparing the masses of identified peptides to the hypothetical tryptic peptides for proteins in NCBI database using the MASCOT search engine, BmaPKC was obviously identified with a high score of 110, with 11 peptides matched and the sequence coverage was 14%, which demonstrated the credibility of our result. The results showed that the fusion protein was successfully expressed.

Purification of recombinant protein and polyclonal antibody preparation

The soluble BmaPKC was purified by a Ni-NTA column (Fig. 4B). All procedures were carried out at 4°C. Under optimal conditions that wash the captured column with 20 mM and 40 mM imidazole, the fusion protein was eluted with 250 mM imidazole. The soluble BmaPKC was purified to 80% pure by a Ni-NTA column. Immunoblotting using an antibody against the His-tag peptide indicated that His-BmaPKC was detected. The purified fusion protein was used to prepare the specific antiserum against BmaPKC. Then, the polyclonal rabbit antibody against 6×His-BmaPKC was obtained and used for immunoassay. The specificity of the purified polyclonal antibodies was examined by western blotting analysis; the result showed that the antibody prepared could serve as a good tool to detect BmaPKC protein.

Fig. 6. Western blot analysis of BmaPKC protein in the midguts of *BmPLV*-infected and non infected Jingsong and Qiufeng strains. The midguts of *BmPLV*-infected Jingsong and Qiufeng strains were collected at 0, 12, 24, 36, 48, 72 h p.i., and non infected Jingsong and Qiufeng strains were collected at 0, 12, 24, 36, 48, 72 h, and then processed for western blot using anti-BmaPKC antiserum followed by incubated with a goat anti-rabbit IgG conjugated to HRP. The signal was detected with ECL substrate.

Detection of the expression of BmaPKC in *BmPLV*-infected larval midgut

As far as we know, the *BmPKC* only had been cloned from the fat body of *B. mori*. To study the expression of BmaPKC in silkworm midgut tissue with *BmPLV*-infected, we first obtained and expressed the BmPKC from *B. mori* midgut tissue. A time course of expression of BmaPKC in *BmPLV*-infected 5th instar larvae, the midgut susceptible strain and resistant strain was analyzed by western blot using anti-BmaPKC antiserum. The signal was detected with ECL substrate, a specific immunoreactive band of approximately 73 kDa was observed at 0 h, 12 h, 24 h, 36 h, 48 h, 60 h and 72 h in the midgut of susceptible and resistant strain, which is in agreement with BmaPKC fusion protein molecular weight. It indicated that as time went on, the expression of BmaPKC increased in susceptible strain Jingsong gradually, but has no significant change in resistant strain (Fig. 6). Also, there was no significant change in both strain which not infected by *BmPLV* (Fig. 6).

Discussion

Protein kinase C (PKC) is a member of a family of Ser/Thr phosphotransferases that is involved in many cellular signaling pathways. As a subfamilies of protein kinase C, atypical PKCs has been studied in *Bos taurus*, *Mus musculus*, *Rattus norvegicus*, *Homo sapiens*, *Drosophila melanogaster* and so on, which suggest that these genes are evolutionarily conserved. Moreover, differ from other subfamilies, atypical PKCs play critical roles in the signaling pathways that control cell growth, differentiation,

and survival through interact with other proteins. At present, the biologic significance of heterogeneity as well as the function of aPKC largely remains unknown. In contrast to the many reports concerning mammal such as mice, little is known about atypical PKCs in insect, especially silkworm. The *B. mori* serves as an ideal system to investigate the primary role of the aPKCs. It may have a guiding role of higher eukaryotes. So the study of *B. mori* atypical protein kinase C will help to elucidate the functional roles of aPKCs orthologues in higher eukaryotes and further explore the relationship between protein kinase C and virus infection.

In this study, we have successfully cloned, optimized the expression and purified BmaPKC of *B. mori* in the *E. coli* BL21. The gene which includes an ORF of 1761 bp encoding 586 amino acids was clone from silkworm midgut, and with no difference between the gene that from the fat body. It suggests that it is conserved in different tissues.

Appropriate temperature for the growth of silkworm is about 20-28°C, so we get the maximum amount of BmaPKC soluble protein at 21°C. Western blot analysis showed that the translational product of the BmaPKC was about 73 kDa, consistent with the predicted protein size. We observed the midgut tissue which BmPLV-infected of susceptible silkworm strain and resistant silkworm strain from 0 to 72 h. The result showed that as time went on, the expression of BmaPKC increases in susceptible strain gradually, but has no significant change in resistant strain. A recent study demonstrated that nonstructural proteins 1 (NS1) of parvovirus minute virus of mice (MVM) is able to initiate viral DNA replication and becomes covalently attached to replicated DNA (Dettwiler *et al.*, 1999). Moreover, it has been reported that protein kinase C phosphorylates residues in vitro and in vivo, leading directly to an activation of NS1 helicase function, and as the active component participate in the rolling circle replication of NS1 (Lachmann *et al.*, 2003). In particular, the unwinding function might be modulated by atypical PKCs. Thereby, we speculated that atypical PKCs may promote parvovirus replication, but the mechanism by PKC may regulate virus entry is currently a matter of speculation. So explore the causes of this mechanism has certain directive significance.

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