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Cell surface transglutaminase required for nodavirus entry into freshwater prawn hemocytes

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ABSTRACT

To identify molecules involved in *Macrobrachium rosenbergii* nodavirus (*Mr*NV) entry into hemocytes of the giant freshwater prawn *M. rosenbergii*, biotinylated prawn hemocyte membrane proteins were prepared, purified and separated by SDS-PAGE. The proteins were blotted on the nitrocellulose membrane before incubation with the *Mr*NV capsid protein (*Mr*NV-CP) by a VOPBA technique. Subsequent mass spectrometry and analysis of immune-reactive bands represent putative binding partners including transglutaminase (TG), actin, α2-macroglobulin, α1-tubulin, F_1-ATP synthase β-subunit and a currently uncharacterized protein. The sequence of TG has been characterized and found 5 amino acids differences to a previously reported *Mr*TG (ADX99580), mainly at its N-terminal part and thus, we named it *Mr*TGII (KM008611). Recombinant *Mr*TGII was prepared to produce a polyclonal antibody against it, which was successfully revealed the presence of *Mr*TGII (100 kDa) in prawn hemocyte lysates. Using the pentylamine-biotin incorporation assay, an acyl transfer reaction was observed when hemocyte lysates were added to solutions containing *Mr*NV-CP, suggesting that hemocyte *Mr*TG could use *Mr*NV-CP as the substrate. The expression levels of *Mr*TGII were changed during the course of *Mr*NV infection. By using immunostaining technique, location of *Mr*TGII on the hemocyte surface was confirmed. Specific interaction between *Mr*TGII with *Mr*NV-CP in a dose-dependent manner was confirmed by *in vitro* ELISA assay. The highest binding activity of *Mr*NV-CP was found with the N-terminal portion of the protein. *In vitro* neutralization using *anti-Mr*TGII antibody resulted in inhibition of *Mr*NV attachment to the hemocyte surface, accompanied by a dramatic reduction in viral replication. This is the first time that crustacean TG has been shown to be involved in viral entry, in addition to its roles in blood clotting and haematopoiesis.

1. Introduction

Successful crustacean aquaculture is limited by viral disease outbreaks. White tail disease (WTD) in the giant freshwater prawn *Macrobrachium rosenbergii* is caused by *M. rosenbergii* nodavirus (*Mr*NV), usually in association with extra small virus (XSV) [1]. Affected prawns exhibit white, opaque muscles in the abdominal segments, commonly accompanied by progressive reduction in feeding and swimming. Mortalities may reach between 50 and 70% within 2 weeks after transfer of post-larvae (PL) to grow-out ponds, causing high economic losses [2,3]. Until now, there is no strategy other than prevention to control *Mr*NV outbreaks in prawn culture.

Transglutaminase (TG) (EC 2.3.2.13) comprises a family of Ca^{2+}-dependent enzymes responsible for post-translational modification/cross-linking of proteins [4]. In mammals, TGs catalyze reactions for several protein substrates and are involved in intra- and extracellular physiological functions, including cell death, cell adhesion and migration, cell-matrix interactions, and stabilization and maintenance tissue integrity [5]. In crustaceans, TG is a crucial molecule in the blood (hemolymph) coagulation reaction [6] which is part of the innate immune defense mechanism. It was first reported as a key coagulation factor in the system of the freshwater crayfish *Pacifastacus leniusculus*

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[7]. Later, crayfish TG was also shown to be involved in hemocyte homeostasis since loss of TG activity in hematopoietic tissue (hpt) caused the stem cells to differentiate and move into the peripheral circulation [8]. In the black tiger shrimp *Penaeus (Penaeus) monodon*, two types of TGs called *Pm*TGI [9] and *Pm*TGII [10] have been reported. *Pm*TGII and its heterologously expressed form (r*Pm*TGII) could catalyze stable clot formation [10] but *Pm*TGI (first reported from a hemocyte cDNA library) could not [9]. The TG of Pacific white shrimp, *P. (Litopenaeus) vannamei* could also be divided into two types based on sequence comparison and phylogenetic analysis [11]. The sequence of TG from *M. rosenbergii* (*Mr*TG) was found to be most closely related to that of shrimp *Pm*TGII [12]. *Mr*TG-mRNAs were found in various tissues but mainly in hemocytes. After injection with bacterial cells of *Lactococcus garvieae*, *Mr*TG expression and activity was depressed and this resulted in a delay in hemolymph clotting time [12]. The mRNA relative expression level of *Mr*TG was also significantly changed after challenges with *Vibrio harveyi* or with white spot syndrome virus (WSSV) [13]. In *P. monodon*, infection with Taura syndrome virus (TSV) was accompanied by poor hemolymph coagulation as a result of decreased TG activity [14]. In Chinese shrimp, *P. (Fenneropenaeus) chinensis*, WSSV was reported to affect TG expression in shrimp hemocytes [15].

In this study, we found that TG in *M. rosenbergii* not only involved in blood coagulation but also required for *Mr*NV entry into hemocytes. *Mr*TGII has been shown to bind to the capsid protein of *Mr*NV (*Mr*NV-CP) by VOPBA (Virus Overlay Protein Binding Assay) analysis. *Mr*TGII-cDNA sequence has been characterized and cloned into the bacterial expression vector. The recombinant *Mr*TGII and the antibodies raised against each region of *Mr*TGII were synthesized to confirm its function involved in viral entry. By using immunofluorescence microscopy, it was found that prawn *Mr*TGII was localized on the surface of adult prawn hemocytes and that pre-incubation of the hemocytes with *anti-Mr*TGII antibody inhibited viral replication. Specific binding between *Mr*TGII and *Mr*NV-CP was also shown using an *in vitro* binding assay. Finally, using the pentylamine-biotin incorporation assay, *Mr*NV-CP might be a substrate for *Mr*TGII. Thus, all the results indicated that *Mr*TGII has a novel functional role in viral binding and entry into giant freshwater prawn hemocytes.

2. Materials and methods

2.1. Animals

Adult prawns (*M. rosenbergii*) were purchased from the Farmers' Marketing Organization, Bangkok, Thailand. Upon arrival at the laboratory, prawns were screened to be *Mr*NV-free by nested RT-PCR before they were used for the preparation of hemocyte surface proteins and primary hemocyte cultures. Healthy post-larvae (PL 10) free of white tail disease (WTD) were obtained from a hatchery at Amphoe Ban Laem, Phetchaburi, Thailand and were used for viral challenge tests. The prawns were maintained in aerated tap water at 28 °C.

2.2. Preparation of biotinylated cell surface

Healthy *M. rosenbergii* were surface cleaned with 70% ethanol. Hemolymph was withdrawn using a 1-ml sterile syringe containing modified AC-1 anticoagulant (0.1 M NaCl, 0.1 M glucose, 30 mM sodium citrate, 26 mM citric acid, 15 mM EDTA, pH 4.6 with a final osmolarity of 417–420 mOsm/kg) in 1:1 ratio [16]. Hemocytes were washed twice with AC-1, pH 8.0 and biotin labeled for 30 min on ice at 4 °C with 10 mM working biotinylation reagent (EZ-link NHS-SS biotin, Invitrogen, USA) at a final cell concentration of 10^6 cells/ml. The biotinylated hemocytes were lysed in 1% Triton X-100 in streptavidin binding buffer (20 mM sodium phosphate, 0.15 M NaCl, pH 7.5, 1 mM PMSF and 1X proteinase inhibitor cocktail) before being subjected to freeze-thawing. The supernatant portion was collected by centrifugation at 3000 × *g* for 10 min at 4 °C and soluble biotinylated hemocyte surface proteins were purified by streptavidin affinity beads (GE healthcare, USA) according the manufacturer's protocol. The eluted fraction was collected and called cell surface proteins (CSPs). Acetone precipitation was used to concentrate the CSPs.

2.3. Virus overlay protein binding assay (VOPBA)

To identify hemocyte cell surface proteins that interact with r*Mr*NV-CP, VOPBA was carried out using CSPs (30 μg) that had been previously separated on 10% SDS-PAGE and transferred to nitrocellulose membranes (GE Healthcare, USA). The blotted membranes were treated with 5% skim milk in TBS buffer (50 mM Tris-HCl, 150 mM NaCl, 0.05% tween-20, pH 7.4) at room temperature for 1 h with constant agitation. After washing twice, the blot was incubated with 0.5 mg of purified r*Mr*NV-CP in TBS dilution buffer (TBS buffer containing 0.02% BSA) at 4 °C, overnight. To detect the r*Mr*NV-CP binding protein(s), the membrane blot were thoroughly washed with TBS buffer before being incubated with 1:500 dilution of *anti-Mr*NV-CP antibody at 4 °C, overnight. After washing, the blots were incubated with alkaline phosphatase-conjugated goat anti-mouse IgG antibody (Zymed, USA) at a dilution of 1:1,000 in TBS dilution buffer at 4 °C for 4 h. Positive signals were visualized by staining the blot with a commercial solution of the alkaline phosphatase substrate NBT/BCIP (Invitrogen, USA). The NBT/BCIP reaction was stopped by extensive washing with dH_2O.

2.4. Mass spectrometry analysis

Candidate cell surface binding proteins to r*Mr*NV-CP were excised from gels stained with coomassie blue. In-gel digestion was performed overnight with trypsin at 37 °C before carrying out Liquid chromatography-electrospray ionization tandem mass spectrometry (LC-MS/MS). Results from LC-MS/MS were analysed by the MASCOT program (http://www.matrixscience.html). Sequence homology searches were performed using the BLAST program with the NCBI database (http://blast.ncbi.nlm.nih.gov.html).

2.5. The pentylamine-biotin incorporation assay

This assay measures the catalytic activity of transglutaminase (TG) by cross-linking 5' (biotinamido)-pentylamine ($BPNH_2$) to substrate residues. It was carried out in 96-well plates coated with r*Mr*NV-CP substrate (10 μg/well) at 4 °C overnight. The wells were blocked with 0.5% skim milk in 0.1M Tris-HCl, pH 8.5 for 1 h at room temperature. The activity of *Mr*TG in hemocyte lysate preparation was evaluated by its ability to covalently cross-link $BPNH_2$ (Pierce, USA) to the r*Mr*NV-CP coated in the well. The reaction was carried out by adding the following reagents: 0.5 mM $BPNH_2$, various dilutions of hemocyte lysate in a total volume 200 μl of reaction buffer (0.1M Tris-HCl, pH 8.5, 10 mM $CaCl_2$, 5 mM DTT). The reactions were incubated for 30 min at 37 °C, stopped with 200 μl of EDTA (200 mM) and washed twice with 200 μl of 0.1 M Tris-HCl, pH 8.5. Activity was detected using added streptavidin-HRP conjugates (dilution 1:200) incubated at room temperature for 1 h, followed by extensive washing with 0.1 M Tris-HCl, pH 8.5 and then addition of HRP substrate (Sigma, USA) as described previously.

2.6. Isolation, characterization and analysis of MrTGII cDNA sequence

Total RNA was extracted from hemocytes and cDNA was prepared as template according to the protocol of the Superscript III RTase kit (Invitrogen, USA). To validate the full length *Mr*TGII cDNA sequence, *Mr*TG primers were designed based on the GenBank record JF309296 (Table 1) and focused on major domains of *Mr*TG genes found in the NCBI database (http://www.ncbi.nlm.nih.gov) identified by the LC-MS/MS analysis described above. PCR products were analyzed by agarose gel electrophoresis and bands of expected sizes were excised

Table 1
Primers used for amplification of *Mr*TGII regions and their nucleotide sequences.

Prime name	Sequence (5′→3′)
*Mr*TG-N-F	CATATGACCAACGAGATCGCCAAC
*Mr*TG-N-R	GGATCCTTA(ATGGTG)$_3$TCCAATAGCTCTGCAAACAGT
*Mr*TG-catalytic core-F	CATATGAAGTGGACAGGCTCCATTGC
*Mr*TG-catalytic core-R	GGATCCTTA(ATGGTG)$_3$AGCAGTGTACAGCGTGAGCCT
*Mr*TG-C-F	CATATGATGGCCAACAGGACAGTGACAATG
*Mr*TG-C-R	GGATCCTTA(ATGGTG)$_3$TTCCGTGCTCAAGATGGTGAC

Underlines indicate restriction enzyme sequences.

and purified using a gel/PCR DNA Fragment Extraction kit (Geneaid, Taiwan). The purified DNA fragments were cloned into pGEM-T Easy vector (Promega, USA) and sequenced by using the vector primers. Amplification of three major domains of the *Mr*TGII gene was achieved by PCR using Pfu DNA polymerase (NEB, UK) before separate cloning and sequencing of the three domains. Deduced amino acids translated from the cDNA were analyzed with Translate tool (http://www.Expasy.org). Protein domains and identity of deduced amino acid sequences were determined using clustalW2 multiple alignment software (http://www.ebi.ac.uk/Tools/clustalw2) and SMART analysis tools (http://smart.embl-heidelberg.de/).

2.7. Recombinant protein production and expression

A recombinant pGEX-6P1 *Mr*NV-CP plasmid in *Escherichia coli* BL21 and mouse monoclonal *anti-Mr*NV-CP antibody were kindly provided by Prof. P. Sithigorngul, Srinakarinwirote University, Bangkok, Thailand. The preparations of the recombinant *Mr*NV-CP protein was followed the protocol described in Wangman et al., 2012 [17]. The *Mr*TGII mRNA sequence was used to design forward primers containing a recognition site for restriction enzyme *NdeI* and reverse primers containing a *BamHI* (NEB, UK) site and a 6X-His tag. The cDNA preparation from hemocytes was used as a template. The PCR protocol consisted of 30 cycles of 98 °C for 30 s, 57 °C for 30 s and 72 °C for 1 min. Amplicons of the three major domains of the *Mr*TGII gene were cloned into pET17-b vector (Novagen, USA) and sequenced. All of these recombinant plasmids were transformed into competent *E. coli* strain BL21 (Novagen, USA). Protein expression was induced by 1 mM isopropyl-b-D-thiogalactopy-166 ranoside (IPTG) and purification was achieved by using affinity glutathione sepharose 4B beads (GE healthcare) or affinity Ni-NTA beads (Qiagen, USA) following the manufacturers' protocols. Total purified recombinant protein concentrations were determined using Bradford's reagent (Bio-Rad, USA). Purified r*Mr*NV-CP was subjected to western blot analysis using 1:500 dilution of *anti-Mr*NV-CP antibody provided for its detection while the three purified, His-tagged *Mr*TGII subunit proteins (r*Mr*TGII-N terminal, r*Mr*TGII-catalytic core and r*Mr*TGII-C terminal) were detected using goat anti-histidine antibody at 1:3000 dilution (GE healthcare). For specific detection of the three *Mr*TGII subunit proteins, polyclonal antibodies were commercially raised in rabbits at the Biomedical Technology Research Center, Chiang Mai University, Thailand.

2.8. MrTGII expression in response to MrNV challenge

Crude viral stock was prepared in TN buffer (0.02 M Tris–HCl, 0.4mNaCl, pH 7.4) from *Mr*NV-infected PL with clinical signs of WTD and confirmed by the nested RT-PCR for *Mr*NV infection. Briefly, a 1 g lot of *Mr*NV infected post-larvae was homogenized with glass tissue homogenizer in 1 ml sterile ice cold TN buffer supplemented with 1x proteinase inhibitor cocktail. After homogenization, the suspension was centrifuged at 4000 × g for 20 min at 4 °C. The collected supernatant was defined as 10% w/v viral stock and stored in − 80 °C.

*In vivo Mr*NV challenges in post-larvae were carried out by immersion. Prawn were divided into two groups: the *Mr*NV challenge group and the PBS mock challenge group. The viral inoculum was added to the rearing water at a final volume equal to 1% (v/v) of the total rearing medium (approximately 2×10^8 viral copies). Three post-larvae (PLs) from each group were collected at 0, 6, 12, 24, 48 and 72 h post exposure. The post-larvae were washed 3 times with sterile water and subjected to analysis for *Mr*TGII mRNA and protein levels. For total RNA extraction, PLs were collected in Trizol reagent (Invitrogen, USA) and the extraction method was followed the manufacturer's protocol. The SuperScript™ III One-Step RT-PCR System (Invitrogen, USA) was used to prepare cDNA and PCR amplification. The mRNA expression levels were determined with the primers; *Mr*TGII-Forward 5′-AGGTGGGACGGAAACTATGAA-3′and *Mr*TGII-Reverse 5′-GAGTTTCTTGAAGCCGTCTGG-3´. The internal control was EF1- α (Accession no. GH624925) with *Mr*EF1-α-Forward; 5′-CTCTGGCAAGTCAACCACCA and *Mr*EF1-α-Reverse CACGTTCACGTTCAGCCTTG-3´. The PCR conditions were 50 °C for 30 min and 94 °C for 2 min followed by 25 cycles of 94 °C for 30 s, 57 °C for 30 s and 68 °C for 30 s with a final extension step at 68 °C for 10 min. The PCR amplicons were detected by 1.2% agarose gel electrophoresis. The intensity of gel bands was measured using Quantity One (Bio-Rad, USA).

Total proteins from homogenized PL in the experiment were subjected to *Mr*TGII protein level analysis by ELISA. Total protein (2.5 μg) extracted from experimental PL at various times after challenge were coated on 96 well-ELISA plates. *Mr*TGII protein was detected using polyclonal *anti-Mr*TGII-N antibody at a dilution 1:16000 overnight at 4 °C. After extensive washing with PBS-T (PBS, pH7.4 buffer containing 0.5% Tween), bound *anti-Mr*TGII antibody was detected using HRP labeled goat anti-rabbit antibody (Zymed, USA) at a dilution of 1:2000 followed by visualization using the HRP substrate tetramethylbenzidine (Sigma, USA). To stop the reaction, 50 μl of 2N H_2SO_4 was added followed by immediate reading of absorbance at 450 nm using an ELISA reader (VersaMax, UK).

2.9. Specific binding by ELISA

To evaluate the specificity of *Mr*TGII subunit binding to r*Mr*NV-CP, 96-well ELISA plates (Corning) were coated with purified r*Mr*NV-CP (1 μg/well) or BSA (negative control) at 4 °C overnight. After removal of the unbound proteins with PBS-T, various dilutions of r*Mr*TGII subunits (0.1–1 μg/well) were added and incubated for 1 h at 37 °C followed by extensive washing. Specific binding of r*Mr*TGII to *Mr*NV-CP was measured by monoclonal anti-histidine antibody (dilution 1:2000) or polyclonal antibody raised against specific r*Mr*TGII subunits (dilution 1:16000) at room temperature for 1 h. After extensive washing, HRP conjugated goat anti-mouse antibody was added and incubated at room temperature for 1 h. Visualization was achieved as described above.

2.10. Primary hemocyte cultures for immunofluorescence assays

Hemocytes were collected in modified AC-1 anticoagulant and washed twice in culture medium that consisted of Leibovitz-15 (L-15) (Gibco, USA) growth medium supplemented with 15% fetal bovine serum, 0.1% glucose, 0.5% NaCl and an antibiotic cocktail (100 U/ml penicillin, 100 U/ml streptomycin) with a final osmolarity of 417–420 mOsm/kg. The cells were washed twice with culture medium by centrifugation at 800 × g for 5 min at 4 °C. They were seeded gently at 1–2 × 10^5 cells/ml in each well of 24-well plates (Corning, Germany) and incubated at 28 °C with medium refreshed at 2-day intervals. Viability of the cultured cells was assessed with 10% trypan blue staining.

2.11. Immunofluorescence microscopy for location of hemocyte binding

Aliquots a 50 μl of prawn hemocytes (2–1 × 10^5 cells/ml) treated as described above were transferred to coverslips in a moist chamber and allowed to adhere overnight in an incubator at 28 °C. The adhered cells were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 15 min at room temperature. Cells were treated with PBS containing 0.1% triton-X for 15 min and blocked (PBS containing 10% FBS) for 1 h at room temperature. After washing twice, the cells were treated overnight with *anti-Mr*TGII-N antibody at a dilution of 1:1000 in blocking solution at 4 °C. After extensive washing, the cells were incubated with fluorochromeAlexa Fluor® 546-conjugated goat anti-rabbit IgG antibodies (Molecular probe, USA) at a dilution of 1:1000 in blocking solution for 1 h at room temperature in a dark, humid chamber. TO-PRO-3 iodide (Molecular probe) was used for nucleic acid counter staining at a dilution of 1:1000. The treated coverslips were mounted with antifade reagent (Molecular probe) before being observed under a confocal laser microscope (FV1000, Olympus). To evaluate the location of antigens, in house antibodies raised against β-subunit of penaeid Na + /K + -ATPase, kindly provided by Dr. S. Asuvapongpatana, Mahidol University, Bangkok, Thailand, was used as marker for membrane compartment [18].

2.12. In vitro antibody neutralization

One day-old primary hemocyte cultures were prepared to adhere to 96-well plates. After medium removal, the cells were treated with various dilutions of *anti-Mr*TGII-N antibody for 1 h before gentle washing once with L-15 culture medium. Then, purified r*Mr*NV-CP (10 μg/well) was added followed by incubation for 30 min at 28 °C. The cell treated with BSA was performed as negative control of binding on cell surface. After gentle washing twice with PBS to remove unbound r*Mr*NV-CP protein, the cells were then fixed immediately with 4% paraformaldehyde for 10 min at 4 °C. Binding of r*Mr*NV-CP to hemocytes was evaluated by *anti-Mr*NV-CP antibody (1:100) and positive signals were detected using a 1:2,000 dilution of HRP-conjugated goat anti-mouse antibody as previously described.

3. Results

3.1. VOPBA identification of proteins binding to MrNV-capsid protein

Results of the VOPBA to identify candidate hemocyte surface proteins from *M. rosenbergii* that could bind to the capsid protein of *Mr*NV are shown in Fig. 1. Two distinct bands with molecular masses of 112 and 50 kDa were revealed. When the gel pieces (C1 and C2) were cut from the gel and subjected to mass spectrometry analysis using the MASCOT program (Table 2), the C1 piece yielded peptide fragments with similarity to transglutaminase from *M. rosenbergii* (*Mr*TG, GenBank accession no. ADX99580), to actin from the tigertop shell *Oxystele tigrina* (GenBank accession no. AAZ28876) and to α2-macroglobulin from *M. rosenbergii* (GenBank accession no. ABK60046). The C2 piece yielded peptide fragments with similarity to alpha-1-tubulin of the blackback land-crab *Gecarcinus lateralis* (GenBank accession no. AAC47522), to an uncharacterized protein LOC767806 from the zebrafish *Danio rerio* (GenBank accession no. NP_001070241) and to F_1-ATP synthase beta subunit of the signal crayfish *Pacifastacus leniusculus* (GenBank accession no. ABI34071). From all of these hits, *Mr*TG sequence was chosen for further investigation into it possible role in viral pathogenesis. Using the primers designed from the sequence of *Mr*TG (GenBank accession no. ADX99580) with cDNA template from our adult *M. rosenbergii* hemocytes, we obtained, cloned and sequenced an *Mr*TG that differed from ADX99580 at 5 different amino acid positions (206, 207, 244, 263 and 362) (Fig. 2). It was thus named *Mr*TGII (GenBank accession no. KM008611). *Mr*TGII consisted of 766 deduced amino acids with a calculated mass of 86.39 kDa and a predicted pI of 5.48. The six of possible N-linked glycosylation sites were found together with two integrin-binding motifs (RGD) at the N-terminus region.

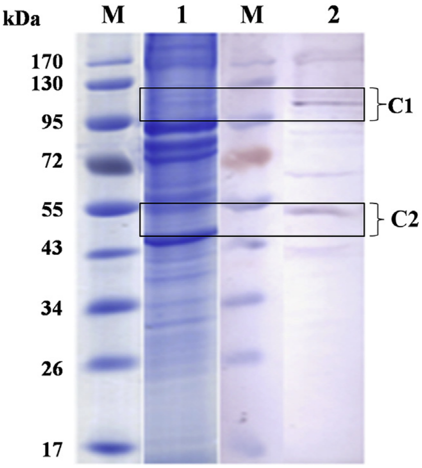

Fig. 1. Analysis of *Mr*NV-CP binding proteins by the VOPBA technique. Purified r*Mr*NV-CP was overlayed on the nitrocellulose membrane of separated, biotinylated hemocyte surface proteins. Protein-protein interactions were detected using *anti-Mr*NV-CP antibody. M, prestained protein marker; Lane 1, SDS-PAGE of affinity-purified biotinylated hemocyte surface proteins;
Lane 2, r*Mr*NV-CP-interacting bands detected with *anti-Mr*NV-CP antibody (1:500) and with C1 and C2 bands indicated.

3.2. Bacterial expression of rMrTGII fragments

Using a bacterial expression system, recombinant *Mr*TGII fragments comprising the N-terminal region (r*Mr*TGII-N), the catalytic core (r*Mr*TGII-Core) and the C-terminal domain (r*Mr*TGII-C) (Fig. 3A) were produced with apparent molecular masses of 36 kDa, 26 kDa and 23 kDa, respectively, including their 6X-His tags. In western blots, antibodies raised against these three *Mr*TGII fragments were found to be specific for their corresponding expressed proteins (Fig. 3B–D). By using *anti*-r*Mr*TGII-N antibody, full *Mr*TGII protein was found in the hemocyte lysate with an approximate mass of 100 kDa (Fig. 3E), corresponding to the protein mass of protein C1 revealed by VOPBA (Fig. 1). The molecular mass of *Mr*TGII detected by using its antibody is higher than those calculated from its deduced amino acid sequences suggests that *Mr*TGII has post-translational modification such as glycosylation as the glycosylation sites are found in its sequence.

3.3. The N-terminal region of MrTGII binds to MrNV-CP

When ELISA with anti-histidine antibody was used to characterize the specific binding of *Mr*TG subunits to purified r*Mr*NV-CP coated on 96-well plates at concentrations of 0–1 μg (Fig. 4A–C), it was found that the highest binding activity was for r*Mr*TGII-N (Fig. 4A), when compared to the lower binding activities of r*Mr*TGII-Core (Fig. 4B) and r*Mr*TGII-C (Fig. 4C). The binding of r*Mr*TGII-N to *Mr*NV-CP was dose-dependent (Fig. 4D) and no binding was observed with the BSA negative control. These results revealed that the *Mr*TGII N-terminal domain bound specifically with *Mr*NV. In hemocyte cell cultures pre-incubated with *anti-Mr*TGII-N antibody, binding to r*Mr*NV-CP increased as the amount of *anti-Mr*TGII-N antibody was diluted (Fig. 4E) while the binding of BSA did not show any binding activity. Previous results revealed any background of antibody neutralizing of pre-immune serum (data not shown).

3.4. Incorporation of 5-(biotinamido)-pentylamine to MrNV-CP

TG activity in hemocyte lysates (measured by its ability to catalyze incorporation of $BPNH_2$ into coated r*Mr*NV-CP) was revealed to be dose-dependent (Fig. 5). Activity was enhanced by the addition of $CaCl_2$ (10 mM) to the reaction buffer. In contrast, addition of EDTA (20 mM) resulted in reduced incorporation (Table 3). The result indicated that

Table 2
Mass spectrometry analysis of hemocyte cell surface-r*Mr*NV-CP interacting proteins.

Gel piece	Accession no.	Similar protein (protein name/organism)	Nominal Mass (Da)	MASCOT score (sequence coverage %)
C1	ADX99580	Transglutaminase [*M. rosenbergii*]	88,133	410 [12]
	AAZ28876	Actin [*Oxystele tigrina*]	24,943	120 [15]
	ABK60046	Alpha-2-macroglobulin [*M. rosenbergii*]	164,611	107 [2]
C2	AAC47522	Alpha-1-tubulin [*Gecarcinus lateralis*]	50,832	360 [17]
	NP_001070241	Uncharacterized protein LOC767806 [*Danio rerio*]	50,354	288 [12]
	ABI34071	F_1-ATP synthase beta subunit [*Pacifastacus leniusculus*]	55,914	287 [16]

the prawn hemocyte lysate contains TG activity and could use rMrNV-CP as a substrate for the acyl transfer reaction.

3.5. *Time course of MrTGII expression during MrNV infection*

Analysis of changes in *Mr*TGII-mRNA protein expression during the course of *Mr*NV infection (Fig. 6) revealed that the level of *Mr*TGII mRNA was significantly reduced during *Mr*NV replication ($P < 0.05$) at 24 h post infection (Fig. 6A), while the level of *Mr*TGII protein was gradually increased from 24 h to 72 h post challenge (Fig. 6B) with significant difference of protein changed ($P < 0.05$) at 72 h post infection). Although the inverse correlation of transcriptional and translational level of *Mr*TGII were found during *Mr*NV infection, the results suggest that *Mr*TGII expression was affected by MrNV infection.

3.6. *MrTGII is localized on hemocyte surfaces*

Using a confocal microscope for immunofluorescence assays revealed that *Mr*TGII was located on the prawn hemocyte extracellular membrane. The primary hemocyte culture was processed according to

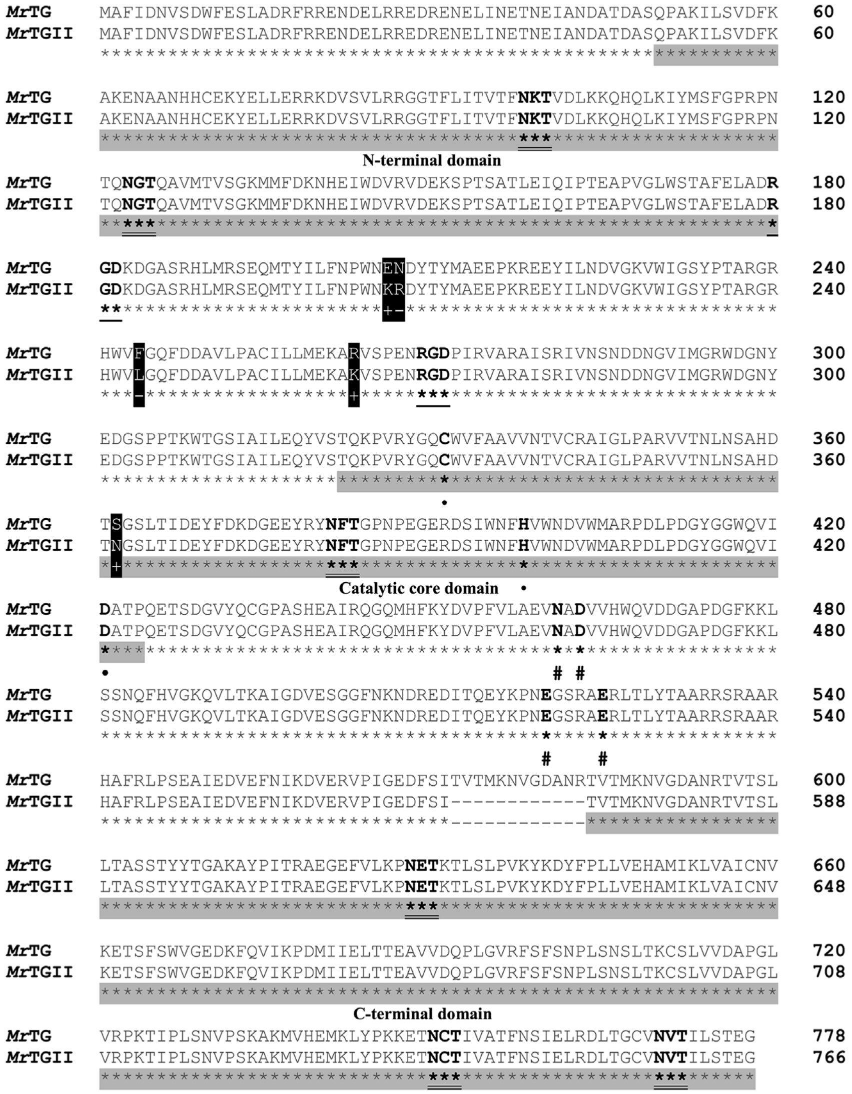

Fig. 2. Comparison of deduced amino acid sequences of *Mr*TG (accession no. ADX995850) and *Mr*TGII (accession no. KM008611). Identical amino acids are indicated by asterisks. Non-identical amino acids are outlined in black and conserved domains of the transglutaminase family are indicated by gray bars under the sequence. The amino acids shown in black typeface and with bold, black bullets (•) or with bold hatch signs (#) indicate the catalytic triad and calcium binding sites, respectively. Two RGD motifs (single underline) and possible glycosylation sites (double underline) are also indicated.

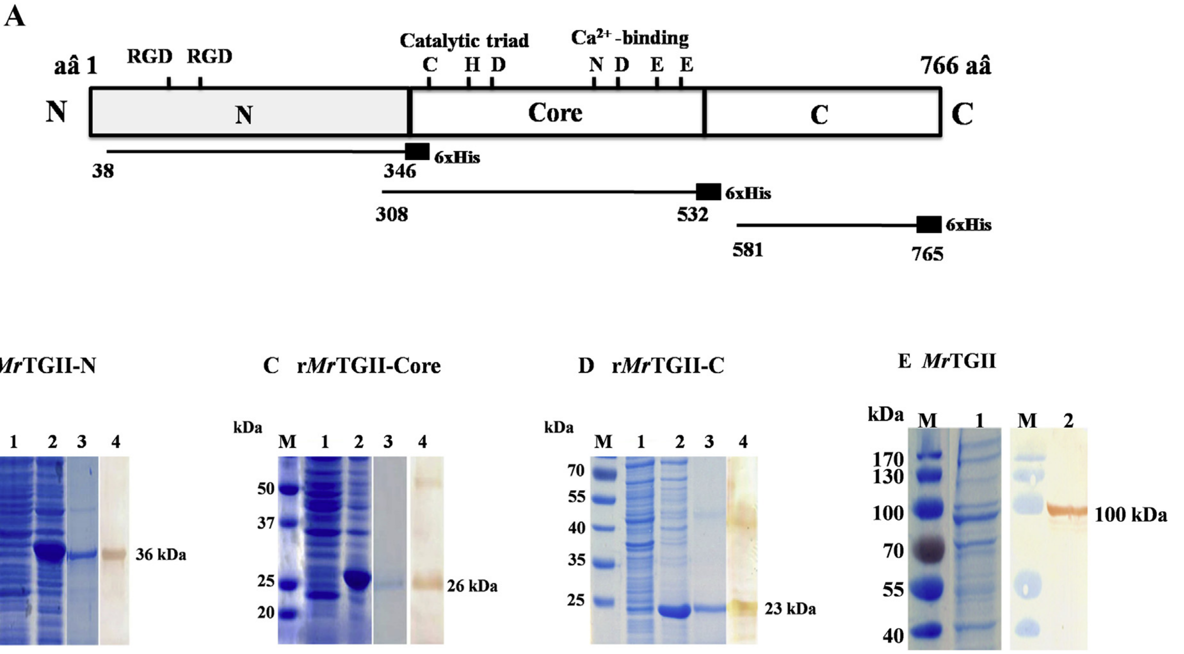

Fig. 3. Bacterial expression of recombinant *Mr*TGII and production of antibodies to three *Mr*TGII domains. (A) Schematic presentation of *Mr*TGII expression domains. All recombinant proteins were tagged with 6-Histidine at the C-terminal region. The two RGD motifs, the catalytic triad and the Ca^{2+} binding sites are indicated. (B–D) SDS-PAGE profiles of recombinant *Mr*TGII expression with apparent molecular masses (including 6X-His tags). (B) The r*Mr*TGII-N domain of 36 kDa. (C) The r*Mr*TGII-Core of 26 kDa. (D) The C-terminus r*Mr*TGII-C of 23 kDa. M, prestained protein marker; Lane 1, non-induced bacterial cell lysate; Lane 2, induced bacterial cell lysate; Lane 3, protein purified with Ni-NTA beads; Lane 4, purified r*Mr*TGII detected with rabbit polyclonal antibodies. (E) Detection of *Mr*TGII in the cell surface protein fraction. Lane 1, coomassie blue stained cell surface proteins; Lane 2, *Mr*TGII-reactive band detected with *anti-Mr*TGII-N antibody. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

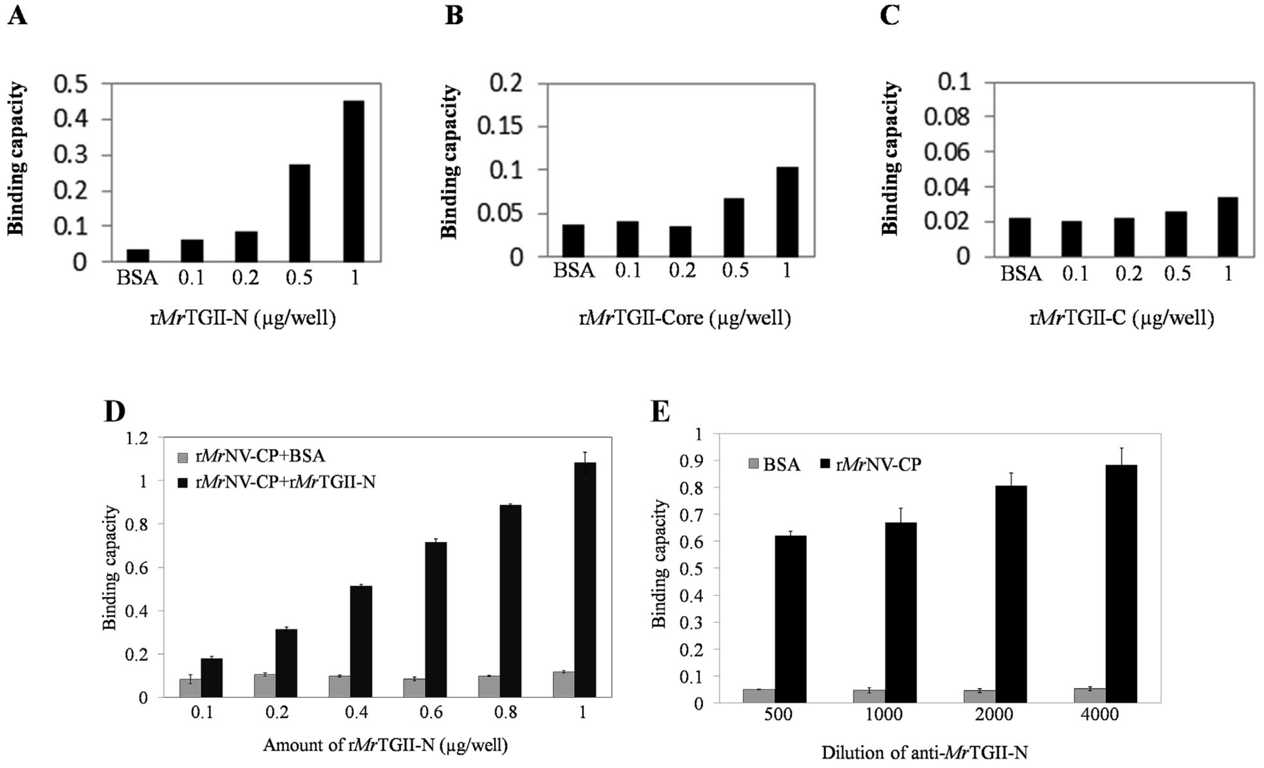

Fig. 4. Binding of three *Mr*TGII domains to MrNV-CP. Purified r*Mr*NV-CP (1 μg) coated onto 96-well plates was incubated with different concentrations of r*Mr*TGIIs (0–1 μg) and interactions were detected using anti-histidine antibody. (A) High binding of *Mr*TGII-N to r*Mr*NV-CP. (B) Low binding of *Mr*TGII-Core to rMrNV-CP. (C) Low binding of *Mr*TGII-C to r*Mr*NV-CP. There was no binding to BSA coated plates (negative control). (D) Confirmation the specific binding of r*Mr*TGII-N to r*Mr*NV-CP using *anti*-r*Mr*TGII-N antibody detected by HRP-conjugated secondary antibody. (E) Cell-surface blocking by *anti-Mr*TGII-N antibody reduced r*Mr*NV-CP binding as detected using *anti-Mr*NV-CP antibody and secondary antibody conjugated with HRP. Bars indicate means plus SD of 3 replicates.

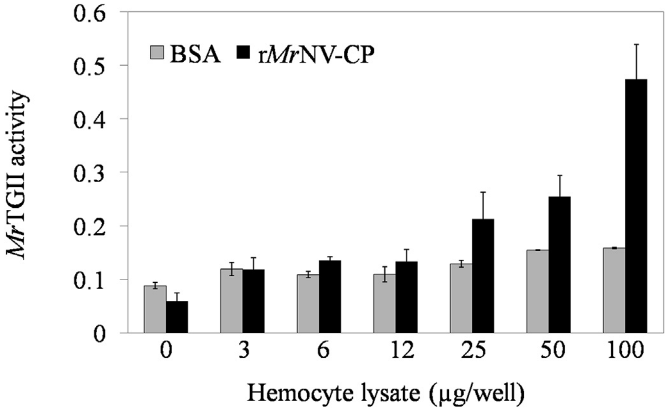

Fig. 5. *Mr*TG catalyzed labelling of r*Mr*NV-CP. Purified r*Mr*NV-CP coated on 96 well-plates (10 μg/well) was incubated with 5′ (biotinamido)-pentylamine ($BPNH_2$) and whole hemocyte lysate containing *Mr*TG. Negative controls consisted of plates coated with BSA. Covalent binding of $BPNH_2$ to *Mr*NV-CP was measured by absorbance at 450 nm. Bars indicate mean absorbance with standard deviations for 3 replicates.

Table 3
Effect of $CaCl_2$ and EDTA on the acyl transfer activity.

HLS (μg/well)	Mean proportion of *Mr*TG activity ± SD		
	r*Mr*NV-CP + $CaCl_2$	r*Mr*NV-CP + EDTA	BSA
0	0.059 ± 0.016	0.034 ± 0.028	0.088 ± 0.006
3	0.119 ± 0.022	0.067 ± 0.021	0.120 ± 0.013
6	0.136 ± 0.007	0.056 ± 0.011	0.109 ± 0.006
12	0.133 ± 0.023	0.065 ± 0.029	0.110 ± 0.014
25	0.213 ± 0.050	0.072 ± 0.034	0.129 ± 0.006
50	0.256 ± 0.039	0.057 ± 0.038	0.155 ± 0.001
100	0.475 ± 0.064	0.066 ± 0.031	0.159 ± 0.002

the protocol described for immunofluorescence microscopy examination. Two types of antibodies were used to stain the hemocyte including *anti-Mr*TGII antibody, anti-Na^+K^+-ATPase antibody (as a membrane marker). Using the non-permeabilized cells, it was found that anti-Na^+K^+-ATPase antibody (green fluorescence, Fig. 7A) and *anti-Mr*TGII-N antibody (red fluorescence, Fig. 7B) both stained the hemocyte surface indicating that *Mr*TGII was in the cell-surface compartment, as well as the membrane-bound Na^+K^+-ATPase protein (Fig. 7C).

3.7. Binding of MrNV to MrTGII occurs on the hemocyte surface

Using cultured hemocytes (10^5 cells/ml) incubated with various dilutions of purified r*Mr*NV-CP prior to exposure to anti *Mr*NV-CP revealed that binding to *Mr*TGII occurred on the hemocyte surface a in dose-dependent manner (Fig. 8A). In addition, use of RT-PCR to measure *Mr*NV replication after cell-surface blocking of *Mr*TGII with *anti-Mr*TGII-N antibody revealed that blocking could inhibit *Mr*NV replication. As the concentration of the *anti-Mr*TGII-N antibody was reduced by dilution, viral replication levels increased (Fig. 8B). The positive control (without blocking) gave the highest *Mr*NV replication whereas no *Mr*NV-RNA was detected in the negative control. All the evidence indicated that cell surface TG was required for the entry of *Mr*NV into prawn hemocytes.

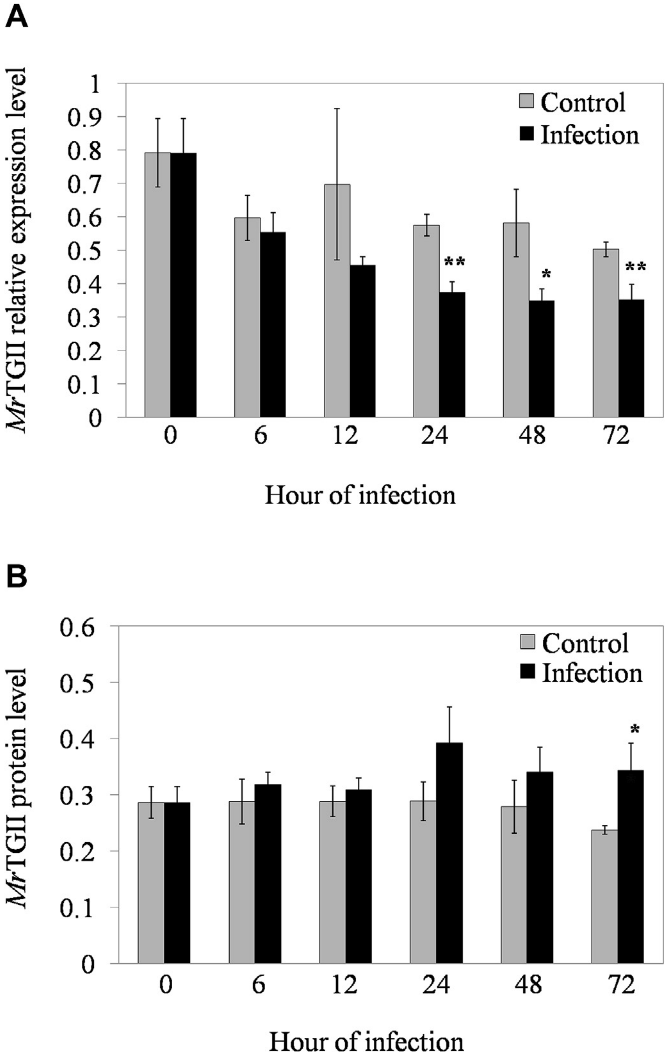

Fig. 6. Transcription and translation of *Mr*TGII after *Mr*NV challenge. (A) *Mr*TGII expression was significantly lower in the challenged than the control group after infection after post infection at 24 h ($p < 0.05$). (B) *Mr*TGII protein levels were significantly different in challenged and control shrimp ($p < 0.05$) at 72 h after infection. Bars indicated means plus standard deviation of triplicate samples.

4. Discussion

There are many reports demonstrating that TG is involved in viral pathogenesis in vertebrates [19]. For example, human immunodeficiency virus (HIV) requires HIV-type1 aspartyl protease (HIV-1PR) for replication and maturation and the activity of HIV-1PR is enhanced via a post-translational process catalysed by TG [20]. TG has been reported to act as a cellular interfering factor. Tissue TG (tTG) regulates the RNA binding activity of hepatitis C virus (HCV) core protein [21]. TG2 can interrupt polyamines retinoblastoma (Rb) binding ability of E7 oncoprotein of human papillomavirus type 18 (HPV E7) [22].

Research on crustacean TGs has so far revealed roles in the clotting process and in blood cell production (haematopoiesis) [6,8]. This report is the first to demonstrate that a crustacean TG present on the hemocyte surface involved in the entry of a nodavirus. *Mr*TGII has been isolated from prawn hemocyte membrane fraction and could use the capsid protein of *Mr*NV (*Mr*NV-CP) as their substrate by mediating the cross-linking of *Mr*NV-CP with the primary amine group of other cell surface proteins. *Mr*TGII contains two RGD motifs, which is known as integrin-targeting peptide sequence and thus, it might interact with cell surface integrin through direct non-covalent interaction. In vertebrate, cell surface tissue transglutaminase (tTG) interacts with a 42 kDa-fibronectin (Fn) and integrin (β1 and β3 subfamilies) to promote cell adhesion and spreading [23]. We hypothesize that *Mr*TGII via its N-terminus can bind to *Mr*NV-CP and to cell surface integrin through its

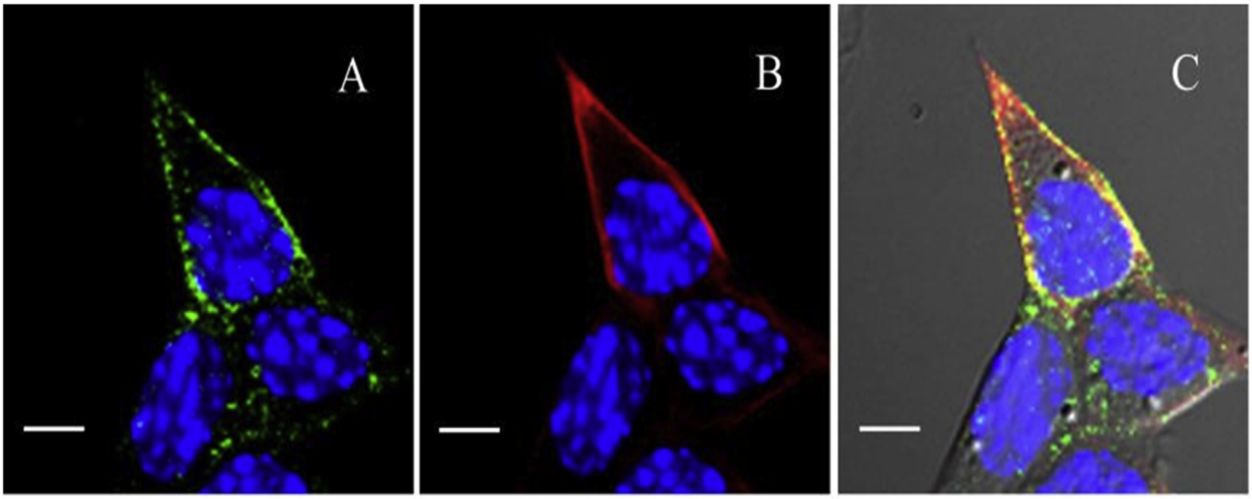

Fig. 7. Localization of *Mr*TGII in hemocytes. The non-permeabilized cells immunostained with (A) the membrane marker anti-Na$^+$K$^+$ATPase (green fluorescence) and (B) *anti-Mr*TGII-N (red fluorescence) show co-localization in the cell membrane. The merged images are shown in (C). Nuclei are stained with ToPro-3 (blue fluorescence). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

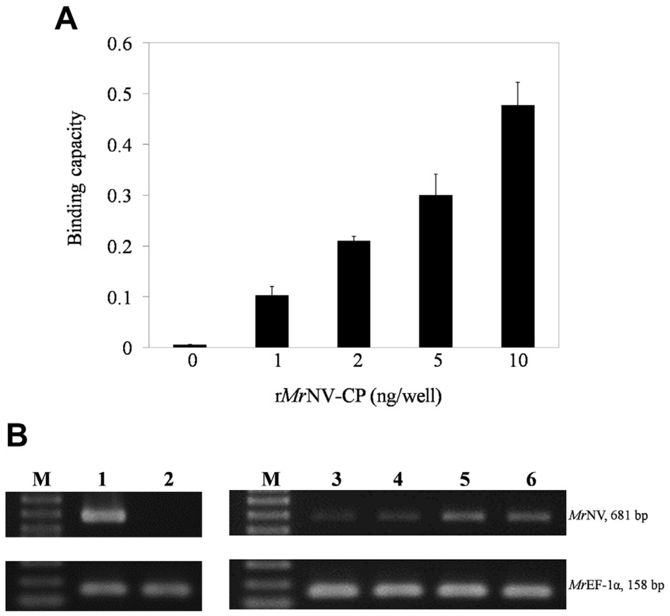

Fig. 8. Effect of blocking *Mr*TGII on the hemocyte surface.(A) Cultured hemocyte were incubated with various dilutions of purified r*Mr*NV-CP for 1 h before detection of binding using *anti-Mr*NV-CP antibody and secondary antibody conjugated with HRP. Binding increased with concentration of r*Mr*NV-CP used. Bars indicate means plus standard deviations for 3 replicates. (B) *Mr*TGII on the hemocyte cell surface was incubated with various concentrations of *anti-Mr*TGII-N antibody before challenge with *Mr*NV. Then RT-PCR was used measure viral replication. Lane 1, Positive control with no antibody; Lane 2, Negative control with PBS alone; Lanes 3–6, pre-treatment with *anti-Mr*TGII-N antibody at dilutions of 50, 100, 200 and 500, respectively.

integrin binding motif resulting in viral entry which is an essential step for viral penetration into the cell. The fact that blocking of cell surface *Mr*TGII resulted in significant reduction in *Mr*NV replication indicated that *Mr*TGII might be a facilitating factor for successful *Mr*NV infection. Integrin has been reported to involve in the infection process of another shrimp virus, WSSV [24]. However, molecular study of *Mr*TGII-integrin should be further investigated.

In vertebrates, TG was originally found in the cytoplasmic compartment [25], but more recently, it has been suggested that TG2 is also expressed on the surface of many cells, including fibroblasts, macrophages, hepatocytes and endothelial cells [26–28]. Since vertebrate TGs possess no secretory signal sequence, an unconventional secretion pathway was suggested for its release on the cell surface [29]. Extracellular binding partners of TG2 such as heparin sulphate proteoglycans (HSPGs) [30] and platelet derived growth factor receptor (PDGFR) [31] have been proposed. By immunofluorescence microscopy in non-permeabilized cells, positive signals for *anti-Mr*TGII antibody were seen at the cell surface indicating that *Mr*TGII binding activity was present on the cell surface. However, the possibility that the *anti-Mr*TGII antibody might stain more than one type of TG must also be considered. For example, Arockiaraj et al. [13] showed that *Mr*TG transcriptional regulation occurred after prawns were injected with WSSV. This contrasts with the results from our study where there was a dramatic reduction in *Mr*TGII transcription during the course of *Mr*NV infection, even though the *Mr*TGII protein level increased. This discrepancy might be explained if the polyclonal *anti-Mr*TGII-N antibody can detect more than one type of TG in prawn hemocytes. Blocking of cell surface *Mr*TGII with its specific antibody resulted in the significant reduction of *Mr*NV replication. The capsid protein can serve as a substrate for catalytic incorporation of 5´(biotinamido)-pentylamine in the presence of hemocyte lysate containing *Mr*TGs. TG has also been reported to catalyze modification of HIV envelope proteins, gp120 and gp41, that are suggested to be involved in post-binding events and in virus entry to the host cell [32,33]. Our preliminary results also demonstrated that *Mr*NV infection had an effect on clotting activity in prawns. The increasing of clotting time was also investigated after *Mr*NV infection (data not shown) suggests that *Mr*NV might interfere the clotting reaction to be able to infect the target cells.

5. Conclusions

In conclusion, we have found evidence that prawn *Mr*TGII has a novel, cell surface function in facilitating binding and entry of *Mr*NV into the hemocytes. This adds to the meagre but growing information available on the mechanism of viral entry into shrimp and prawn cells. It is hoped that further research in this area may lead to the development of a new strategies for control of crustacean viral diseases.

Declarations of interest

None.

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