Zoological Studies **49**(1): 19-27 (2010)

Zoological Studies

Endocytosis and NF-κB Are Involved in the Signal Pathway of the Degranulation and Expression of Prophenoloxidase Messenger RNA of Prawn Hemocytes Triggered by CpG Oligodeoxynucleotides

Hung-Hung Sung*, Pang-Hung Chen, Che-Pei Chuo, Yu-Lun Cheng, and Po-Tzung Chang

Department of Microbiology, Soochow University, Taipei 111, Taiwan

(Accepted April 21, 2009)

Hung-Hung Sung, Pang-Hung Chen, Che-Pei Chuo, Yu-Lun Cheng, and Po-Tzung Chang (2010) Endocytosis and NF-κB are involved in the signal pathway of the degranulation and expression of prophenoloxidase messenger RNA of prawn hemocytes triggered by CpG oligodeoxynucleotide. *Zoological Studies* **49**(1): 19-27. Microscopic observations showed that granulocytes were the primary CpG-oligodeoxynucleotide (ODN)-stimulated hemocyte population of the prawn *Macrobrachium rosenbergii*. To clarify whether endocytosis and the NF-κB and ERK signal pathways are required to initiate and activate degranulation in prawn ODN-triggered hemocytes, the total extracellular phenoloxidase (PO) activity (PO_T) was examined. When hemocytes were separately treated *in vitro* with ODN2006 and inhibitors of specific signaling components, there was a decrease in the PO_T of hemocytes treated with either chloroquine (1 and 2 mM), an inhibitor of endosomal acidification, or pyrrolidine dithiocarbamate (PDTC; 5-30 μM), which inhibits the NF-κB pathway. After ERK-inhibiting PD98059 treatment, a reduction in the PO_T was detected in 1/2 of all hemocyte samples tested in this experiment; however, the PO_T of the remaining samples did not significantly differ from that of the ODN2006-stimulated sample. In addition, expression of the proPO gene was monitored after prawns were injected with ODN2006 (5 μg/prawn) and PDTC (1.5 mM/prawn) by a semiquantitative reverse-transcription polymerase chain reaction analysis. The results showed that a decreased level of prophenoloxidase (proPO) messenger RNA was detected at 1 and 3 h post-treatment. All data suggest that in addition to the protein kinase C-activating signaling pathway demonstrated in our previous study, both the endocytic and NF-κB pathways are involved in activation of the proPO system of CpG ODN-induced prawns.
http://zoolstud.sinica.edu.tw/Journals/49.1/19.pdf

Key words: Phenoloxidase, CpG oligodeoxynucleotide, Endocytic signaling pathway, NF-κB pathway.

In crustaceans, hemocytes play a critical role in immune reactions and are capable of phagocytosis, encapsulation of foreign material, nodule formation, mediation of cytotoxicity, and cell agglutination and melanization by activation of the prophenoloxidase (proPO)-activating system (Söderhäll and Cerenius 1992). Several proteins associated with the proPO system of granulocytes play important roles in non-self recognition and host defense in eliminating foreign particles from the body cavity of crayfish and other crustaceans (Ratcliffe et al. 1985, Smith and Söderhäll 1991, Söderhäll 1992, Söderhäll et al. 1994). Therefore, the proPO system can be used as a defense indicator in crustaceans, including cultured prawns (Rodriguez and Moullac 2000). In addition to the effect of several environmental and biological factors, the proPO system can also be activated by several biotic immunostimulants, such as β-1,3-glucan and zymosan from fungal cell walls (Unestam and Söderhäll 1977, Söderhäll and Unestam 1979, Perazzolo and Barracco 1997, Sung et al. 1998), lipopolysaccharides (LPSs) from gram-negative bacteria (Söderhäll and Hall 1984,

*To whom correspondence and reprint requests should be addressed. E-mail:hhsung@scu.edu.tw

Söderhäll et al. 1990, Perazzolo and Barracco 1997, Sung et al. 1998), peptidoglycan from gram-positive bacteria (Ashida et al. 1983), laminarin from algae (Hernandez et al. 1996), and bacterial DNA (Chuo et al. 2005).

In *Drosophila* and mammals, Toll and Toll-like receptor (TLR) homologues recognize broad structural motifs, called pathogen-associated molecular patterns (PAMPs) (Medzhitov et al. 1997, Medzhitov and Janeway 2000). All of the biotic immunostimulants described above are also PAMPs. In *Drosophila*, defense mechanisms are activated by some PAMPs via 2 distinct signal pathways, the Toll pathway and the immune-deficiency (Imd) pathway (De Gregorio et al. 2000, Hoffmann and Reichhart 2002, Tzou et al. 2002). Some nucleic acid motifs, such as unmethylated CpG DNA from bacteria (Krieg et al. 1995), a type of PAMP, can be recognized by the TLR9 of mammals (Hemmi et al. 2000, Takeshita et al. 2001). Typically, signals activated through TLR9 cause transcriptional activation and the synthesis and secretion of cytokines, which effectively bridge innate and acquired immunity (Wagner 2000). However, to the present, there are no reports on the role of CpG DNA in the innate immunity of *Drosophila*.

In mammals, much evidence suggests that CpG DNA actively signals in intracellular compartments, that endocytosis, including internalization and endosomal maturation, is required for stimulation (Häcker et al. 1998, Yi et al. 1998, Hemmi et al. 2000, Ahmad-Nejad et al. 2002, Latz et al. 2004), and that CpG DNA activates cells through the TLR pathway via myeloid differentiation factor 88 (MyD88) and TRAF6 (Hoffmann and Reichhart 2002), which can indirectly activate protein kinases and transcription factors such as mitogen-activated protein kinases (MAPKs) (Shirakabe et al. 1997) and nuclear factor (NF)-κB (DiDonato et al. 1997). A number of studies indicated that induction of innate immunity in both mammals and flies leads to the activation of similar effector mechanisms (Hoffman et al. 1999). The most significant common feature of innate immunity throughout the animal kingdom is the central role of the NF-κB/Relish family of transcriptional activators (Karin and Ben-Neriah 2000). It was found that the mechanisms involved in the antimicrobial immunity of insects are similar to those required for the activation of NF-κB in mammals (Silverman and Maniatis 2001).

Our previous study found that activation of the proPO system of prawns can be enhanced by different sequence motifs of CpG oligodeoxynucleotides (ODNs) via a protein kinase C (PKC)-activating signaling pathway (Chuo et al. 2005). Preliminary tests showed that CpG ODN2006 can bind to hemocytes. In this study, we further determined whether endocytosis is required in the ODN-inducing signal pathway and which transcriptional factors participate in activating the proPO system of ODN-stimulated hemocytes, including hemocytic degranulation and proPO gene expression. An understanding of the CpG ODN-stimulated signaling pathway will be of benefit in developing powerful, inexpensive, and stable immunostimulants for use in prawn farming.

MATERIALS AND METHODS

Experimental prawns

A batch of apparently healthy giant freshwater prawn *Macrobrachium rosenbergii* ranging 25-35 g in weight were purchased from a commercial shrimp farm and were acclimated in aerated fresh water in 120 L plastic containers held at 25°C for at least 7 d prior to each experiment. Stocking densities were generally maintained at 4 or 5 prawns/m^2, and artificial feed was given twice a day.

Reagents

All products of unmethylated phosphorothioate ODN used in this study were synthesized by and purchased from MDBio (Taipei, Taiwan), including ODN2006 (Hartmann et al. 2000) and 5'-end-labelled carboxyfluorescein-5-succimidyl ester (FAM)-ODN2006. The inhibitors of specific signaling components used in this study were chloroquine (an inhibitor of the endosomal acidification of endocytosis; Fluka, 25745; Milwaukee, Wisconsin, USA), pyrrolidine dithiocarbamate (PDTC, an NF-κB inhibitor; Sigma, P8765; St. Louis, MO, USA), and PD98059 (a specific inhibitor of extracellular signal-regulated kinase (ERK) belonging to the MAPK family; Sigma, P-215). In order to measure the PO activity, cacodylate (CAC), trypsin, and L-dihydroxyphenylalanine (L-dopa) were also purchased from Sigma-Aldrich (St. Louis, MO, USA). M-199 medium without phenol red (M-199), used for culturing hemocytes, was purchased from GIBCO (Grand I., NY, USA).

Hemocyte preparation

Hemocyte samples were prepared according to procedures described by Sung et al. (2004). A hemolymph sample (0.6 ml) was drawn from the 1st abdominal segment of each prawn with a 26 gauge hypodermic needle containing 0.3 ml of anticoagulant (0.114 M trisodium citrate and 0.1 M sodium chloride; pH 7.45) with an osmolarity of 420 ± 20 mOs/kg. The hemolymph was centrifuged at 300 ×*g* for 5 min at 4°C, and the resultant hemocyte pellet was suspended in 0.6 ml of M-199. Hemocyte concentrations were adjusted to 5×10^6 cells/ml. In this study, only cell suspensions with a viability of ≥ 85%, as determined by trypan blue exclusion (0.4% in 0.01 M PBS), were used to determine the PO activity.

Hemocytes labeled by FAM-ODN2006

Hemocyte suspensions (100 μl for each sample) were centrifuged and then resuspended in 100 μl M-199 containing 50 μg/ml of FAM-ODN2006. The stimulating reaction was performed at room temperature for 1 and 10 min. After washing with M-199, hemocyte samples were fixed using 10% formaldehyde in 0.01 M phosphate buffer (PBS; pH 7.4) for 10 min. Fixed hemocytes were observed with an epifluorescence microscope (Nikon Eclipse E-800, Tokyo, Japan) and a confocal microscope (Zeiss LSM 5 Pascal, Oberkochen, Germany).

Hemocytes treated *in vitro* with ODN2006 and inhibitors

Cell pellets collected from 200 μl of the hemocyte suspension were separately treated with an equal volume (200 μl) of (1) M-199 as a negative control, and M-199 containing (2) 50 μg/ml of ODN2006 as a positive control, as well as (3) various inhibitors, and (4) ODN2006 plus an inhibitor, as indicated in the figure legends. For example, in order to examine whether endocytosis is involved in activation of the proPO system triggered by ODN2006, hemocytes were simultaneously treated with ODN2006 (50 μg/ml) and the inhibitor, chloroquine (1 or 2 mM). Following incubation at 28°C for 30 min, hemocyte sample viability was counted by trypan blue exclusion and centrifuged. Hemocyte samples with a viability of ≥ 75% were used to determine the PO activity. According to a concept described by Chuo et al. (2005), supernatants were collected, and the total extracellular PO activity (PO_T) was determined, which is an indicator of hemocytic degranulation. The protein concentration of the resultant supernatants was determined with a Bio-Rad Protein Assay Kit II (Hercules, California, USA), using bovine serum albumin as a standard.

Prawns treated *in vivo* with ODN2006 or inhibitors

In order to determine whether the NF-κB pathway is involved in activation of the proPO system triggered by ODN2006 in prawns, proPO gene expression was detected after the prawns were injected in the 1st abdominal segment with ODN2006 or inhibitors. Prawns (with an average body weight of 32 g) were divided into 4 groups and separately injected with 0.1 ml of (1) 0.01 M PBS with an osmolarity of 420 ± 20 mOs/kg, (2) 50 μg/ml of ODN2006 in PBS, (3) 15 mM of PDTC (an NF-κB inhibitor) in PBS, or (4) ODN2006 plus an inhibitor in PBS. The expression of the proPO gene was determined before the injection and at 1 and 3 h after the injection.

Assay of PO_T

The PO activity in the supernatant was determined according to procedures described by Chuo et al. (2005). Briefly, 50 μl of the supernatant sample, which was collected from the extracellular solution of the treated hemocyte sample, was treated with 50 μl of trypsin (1 mg/ml of 0.01 M CAC buffer, pH 7.0). After incubation for 10 min at room temperature, 100 μl of 0.01 M L-dopa in CAC buffer was added and reacted for 1 min. Subsequently, the optical absorbance at 490 nm was measured. One unit of enzyme activity was defined as an increase in the absorbance of 0.001/min/mg protein (Unestam and Söderhäll 1977). Values given in this study are the ratio of PO_T activity from each individual. The value of the ratio was calculated using the formula: PO activity of a treated sample/ PO activity of the negative control sample.

Semiquantitative reverse-transcription polymerase chain reaction (RT-PCR) of proPO messenger (m)RNA

In order to quantify the proPO mRNA of hemocytes, a semiquantitative RT-PCR was performed as described by Lu et al. (2006). A

2098 bp fragment of proPO cDNA (GenBank accession no. AY947400) (Lu et al. 2006) was specifically amplified using the primer pair, SP9F (5'-GATACTGCCACTTGCTCTCT-3') and SP8R (5'-CTTGCAGGGTTCAAGAAG-3'). In addition, the gene expression of β-actin was used as a meaningful internal control for the semiquantitative experiment using a pair of specific primers, actin-F (5'-CCCAGAGCAAGAGAGGTA-3') and actin-R3 (5'-GCGTATCCTTCGTAGATGGG-3'), which yielded a 309 bp fragment (GenBank accession no. AY947402) (Lu et al. 2006). This internal control was also amplified by its own specific primers at the same time for each sample.

One RNA sample was isolated from hemocytes of 3 prawns treated with ODN2006 or an NF-κB inhibitor using the TriPure Reagent (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's protocols. mRNA of hemocytes was purified using a GeneStrips™ Kit (RNAture, Irvine, California, USA). After purification, the mRNA bound to the tube was washed 3 times with 100 μl of wash buffer (RNAture), and then it was added to 50 μl of the RT mixed solution (10 μl of 5x RT buffer, 5 μl of 100 mM DTT, 5 μl of 10 mM dNTP mixtures, 0.5 μl of a ribonuclease inhibitor, and 2.5 μl of RT). The 1st-strand cDNA was synthesized at 42°C for 1 h. After carefully washing the cDNA-containing tube with 100 μl of Tris-HCl (10 mM, pH 7.5) 3 times, 50 μl of the PCR mixture solution, which contained 1 μl of the RT product, 5 μl of 10x PCR buffer (Finnzymes, Massachusetts, USA), 1 μl of 10 mM dNTP mixtures, 1 μl of 20 μM of the forward primer, 1 μl of 20 μM of the reverse primer, and 0.5 μl of *Taq* DNA polymerase (5 U/μl; Dynazyme™, Finnzymes), was added to the tube. The PCR was performed at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min for 35 cycles. All PCR products were analyzed on 1% agarose gels and quantified using ImageMaster™ (TotalLab Software vers. 2.00, Amersham, Uppsala, Sweden). The band intensity was expressed in relative absorbance units. The semiquantitative determinations were expressed as a ratio of the absorbance units of the proPO band to the absorbance units of the actin band in the same sample.

Statistical analysis

Since outbred prawns were used as samples in this study, the physiological status significantly varied among individuals. With the exception of data from the gene expression experiment that were statistically analyzed using analysis of variance (ANOVA) and Duncan's multiple-range tests with a specified significance level of $p < 0.05$, for all results, data from 4-8 individuals are listed and were analyzed for the effects of CpG ODN and inhibitors on the POT using a paired *t*-test with a specified significance level of $p < 0.05$.

RESULTS

Based on cellular granules, prawn hemocytes are grouped into agranulocytes (hyaline cells) and granulocytes. To observe whether ODN2006 can bind to hemocytes, viable hemocytes were directly incubated with FAM-ODN2006 for 1 or 10 min. The use of an epifluorescence microscope revealed that ODN2006-bound hemocytes were almost all granulocytes in the hemocyte sample treated for 10 min (Figs. 1A, B). Observations by confocal microscopy revealed that many FAM-ODN-bound granules were located within hemocytes treated for 1 min (Fig. 1C), and that an increase in the fluorescent intensity of hemocytes was observed after stimulation with FAM-ODN2006 for 10 min (Fig. 1D).

In order to clarify whether endocytosis is required to initiate activation of the proPO system triggered by CpG ODN, the effect of chloroquine, an inhibitor of endosomal acidification, on the PO_T was examined. When hemocytes were treated with different concentrations of chloroquine, a reduction in PO_T was detected at concentrations of 2, 3, and 4 mM after treatment for 5-30 min (Fig. 2A). After ODN2006-stimulated hemocytes were separately treated with 1 and 2 mM chloroquine for 30 min, the PO_T was reduced and did not differ from that of the control group (Fig. 2B).

In order to determine whether the NF-κB and ERK signaling pathways are involved in activation of the proPO system of CpG ODN-stimulated hemocytes, the effects of inhibitors of specific signaling components of the PO_T were measured. When ODN-stimulated hemocytes were separately treated with different concentrations of the NF-κB inhibitor, PDTC, the PO_T from 8 individuals was reduced compared to either the ODN alone-stimulated group or the control group (Fig. 3). After different concentrations of the ERK inhibitor, PD98059, were used to treat ODN-stimulated hemocytes, the PO_T from 4 individuals was shown to have been reduced, and the greatest reduction was detected at a concentration of 5 μM PD98059 (Fig. 4A); however, PO activities of 4 other

individuals did not significantly differ from those of the ODN-stimulated group (Fig. 4B). Thereafter, in order to further clarify that the NF-κB pathway is truly involved in intrahemocytic signaling triggered by ODN2006, the proPO mRNA level was detected by a semiquantitative RT-PCR. As shown in figure 5, elevation of proPO gene expression was observed at 1 h after an injection with ODN2006 (5 μg/prawn), but it was not detected in the control group or PBS-injected prawns. The mRNA level of

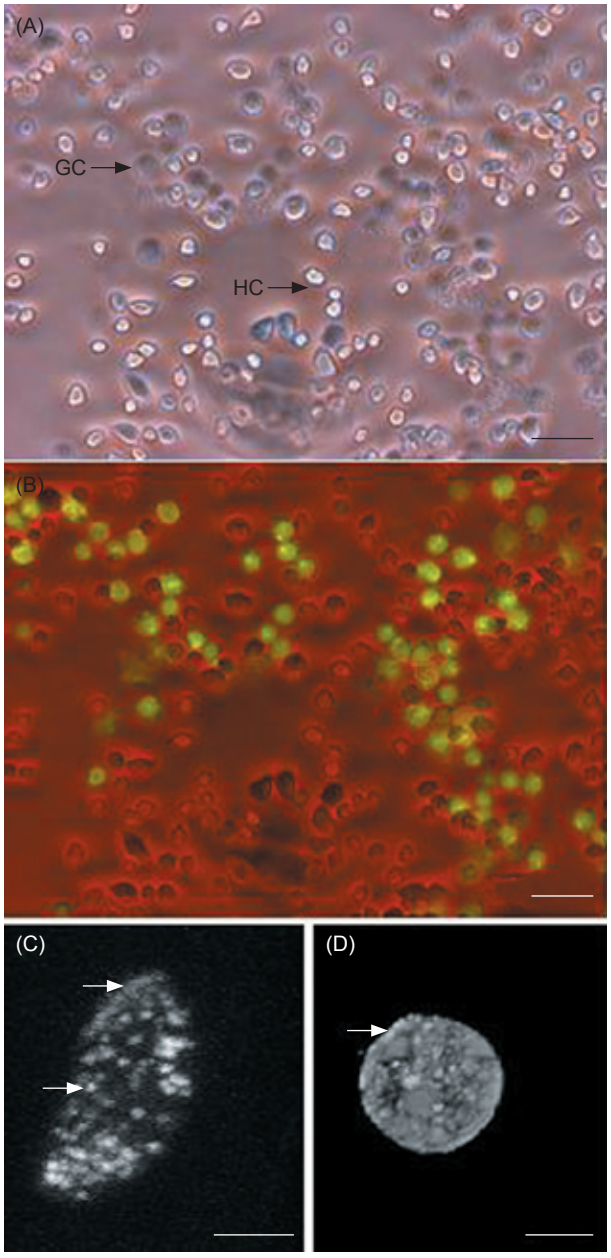

Fig. 1. Microscopic observations of FAM-oligodeoxynucleotide (ODN)2006-stimulated hemocytes from *Macrobrachium rosenbergii*. (A) Light-view and (B) fluorescence-view photographs showing hemocytes treated with FAM-ODN2006 for 10 min as observed with an epifluorescence microscope. GC, granular cell (black letter and arrow); HC, hyaline cell (blue letter and arrow). Hemocytes shown in the lower 2 photographs were separately treated with FAM-ODN2006 for 1 (C) and 10 min (D) and observed with a confocal microscope. Arrow (in C and D), ODN2006-labeled portion of hemocytes. Scale bars: A, B = 25 μm; C, D = 5 μm.

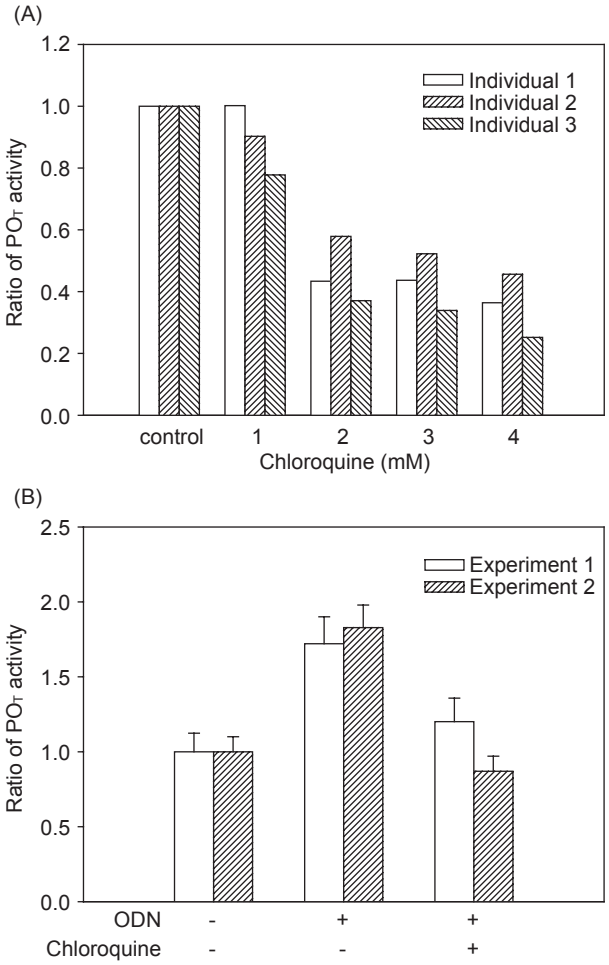

Fig. 2. Total extracellular phenoloxidase activity (PO_T) of *in vitro* ODN2006-stimulated hemocytes inhibited by the endosomal-acidification inhibitor, chloroquine. (A) Hemocytes from 1 individual were separated into 5 samples and treated with different concentrations of chloroquine (0, 1, 2, 3, and 4 mM). The PO_T was measured in supernatant prepared from hemocytes treated for 30 min. (B) Hemocytes from 1 individual were separated into 3 samples and treated with neither ODN nor chloroquine which served as the negative control, with ODN (50 μg/ml) which served as the positive control, and simultaneously treated with ODN2006 and chloroquine as the experimental sample. There were 2 experiments. The experimental samples in experiments 1 and 2 were treated with 1 and 2 mM of chloroquine, respectively. Data from the means of the average relative value of each experiment from 5 individuals were determined.

proPO of PDTC-injected prawns was less than that of PBS-injected prawns at 1 h after the injection. Comparing this with the ODN-stimulated prawns, the mRNA level of proPO was reduced at 1 h after ODN-stimulated prawns were injected with PDTC (1.5 mM/prawn), but it did not significantly differ from that of PBS-injected prawns (Fig. 5).

DISCUSSION

Most studies of bacterial DNA and certain CpG ODNs focused on mammals. Many studies indicated that bacterial DNA and biologically active CpG ODNs are powerful activators of cells of the innate immune system of mammals, especially of mice and humans (Tokunaga et al. 1999, Wagner 1999). Much evidence indicates that receptor-mediated endocytosis of CpG-DNA, endosomal acidification (maturation), and CpG-DNA recognition of endosomal/lysosomal vesicles by TLR9 are essential steps in cellular activation (Häcker et al. 1998, Yi et al., 1998, Ahmad-Nejad et al. 2002). Conventional CpG DNAs exert their activity through TLR9 signaling, which depends on the cytoplasmic adaptor, MyD88 (Hemmi et al. 2003), CpG-DNA colocalizes with recruited MyD88, and CpG-DNA interfaces with TLR9 at the lysosomal compartment where signaling is initiated (Ahmad-Nejad et al. 2002, Latz et al. 2004).

Our previous study was the first report on the effect of CpG ODN in activating the proPO system of crustaceans and the first demonstration of the signal transduction of the proPO system of prawn hemocytes activated by CpG ODN (Chuo et al. 2005). We concluded that the CpG ODN induces activation of the proPO system of prawns, including degranulation and the PO activity of hemocytes, through activation of G-protein-coupled receptors, which use cAMP as a secondary messenger to stimulate the PKC signaling pathway. In this study, many fluorescent vesicles appeared within granulocytes after treatment with FAM-ODN for 1 min; furthermore, the fluorescent intensity clearly increased when the duration of treatment with ODN was prolonged (Fig. 1). Our results are similar to those of Takeshita et al. (2001), who found that after HEK 293 cells were treated with Cy3-labelled CpG ODN for 10 min, the complex of CpG ODN-TLR was internalized, and the size and number of CpG DNA-containing vesicles increased after treatment for 2 h.

Microscopic observations of this study suggest that receptor-mediated endocytosis of CpG-DNA may also be an essential step in cellular activation by the prawn, such as activation of the proPO system. This hypothesis was supported by the results of treating hemocytes with chloroquine, an inhibitor of endosomal acidification, which reduced the CpG ODN-induced PO_T of hemocytes (Fig. 2).

TLRs are germ-line-encoded non-clonal receptors that recognize conserved microbial products. Dimerization of TLRs on cells such as antigen-presenting cells triggers activation of MAPKs and NF-κB transcription factors and the production of inflammatory cytokines (DiDonato et al. 1997, Shirakabe et al. 1997). The evolutionary conservation of NF-κB transcription factors, from *Drosophila* to humans, underscores their pivotal role in the immune response. In *Drosophila melanogaster*, NF-κB homologues (Dorsal, Dif, and Relish) are responsible for regulating several biological roles, including humoral immunity and development (Wasserman 2000, Hoffmann 2003). *Limulus* is the most ancient arthropod and has evolved a formidable host defense system (Iwanaga 2002). Wang et al. (2006) identified NF-κB and IκB homologues in a species of *Limulus* and concluded that the NF-κB/IκB signaling

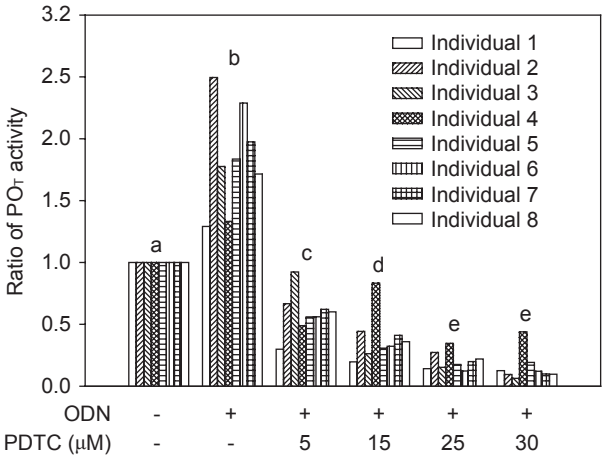

Fig. 3. Total extracellular phenoloxidase activity (PO_T) of in vitro oligodeoxynucleotide (ODN)2006-stimulated hemocytes inhibited *in vitro* by different concentrations of the NF-κB inhibitor, pyrrolidine dithiocarbamate (PDTC). The PO_T was measured in supernatant prepared from hemocytes treated with ODN2006 (50 μg/ml) or simultaneously treated with PDTC and ODN2006 for 30 min. The M-199-treated sample with neither ODN nor PDTC served as the control. Data from 8 individuals (separately represented by 8 bars) are listed and were analyzed by paired *t*-test. Values with different lowercase letters above the bars significantly differed ($p < 0.05$). The letter, a, corresponds to the control.

pathway was evolutionarily entrenched and has coevolved and remained well conserved from the horseshoe crab to humans, playing an archaic but crucial and fundamental role in the innate immune response by regulating the expression of critical immune defense molecules.

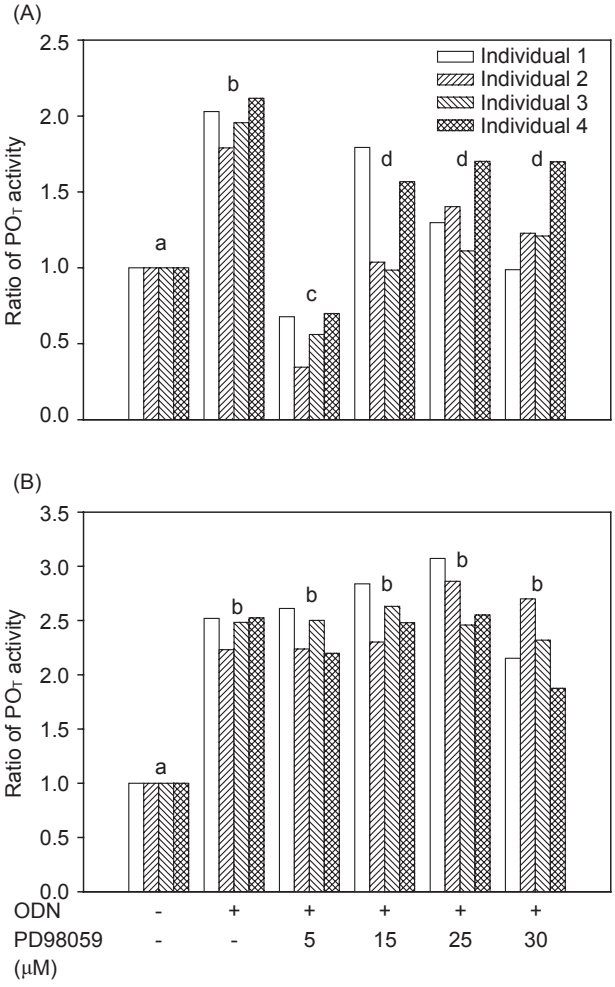

Fig. 4. Total extracellular phenoloxidase activity (PO_T) of in vitro oligodeoxynucleotide (ODN)2006-stimulated hemocytes inhibited *in vitro* by different concentrations of the extracellular-regulated kinase (ERK) inhibitor, PD98059. The PO_T measured in supernatant prepared from hemocytes treated with ODN2006 (50 μg/ml) served as the positive control, or hemocytes were simultaneously treated with PD98059 and ODN2006 for 30 min. The M-199-treated sample with neither ODN nor PD98059 served as the negative control. (A) The ODN2006-induced effect on PO_T was inhibited by PD98059 (5 μM) in 4 individuals; (B) the ODN2006-induced effect on PO_T was not affected by PD98059 in another 4 individuals. In (A) and (B), data from 4 individuals (separately represented by 4 bars) are listed and were analyzed by a paired *t*-test. Values with different lowercase letters above the bars significantly differed ($p < 0.05$). The letter, a, corresponds to the control.

In this study, we further clarified whether the intracellular signaling pathways, including NF-κB and ERK signaling, follow the PKC or endocytic pathway to induce activation of the proPO system in prawn. The results from hemocytes separately treated with the NF-κB inhibitor and ERK inhibitor showed that the CpG ODN-induced PO_T was reduced by the NF-κB inhibitor (Fig. 3), but precise conclusions about the reducing effect in those treated with the ERK inhibitor (Fig. 4) cannot be deduced at the moment. Furthermore, the expression of the hemocytic proPO gene of prawns was detected by a semiquantitative RT-PCR analysis. The CpG ODN-induced level of proPO mRNA was clearly reduced after prawns were treated *in vivo* with an NF-κB inhibitor (Fig. 5). These data reveal that the transcription factor, NF-κB, is required to activate the proPO gene of CpG ODN-stimulated prawn.

In conclusion, in addition to the PKC-activating signaling pathway (Chuo et al. 2005),

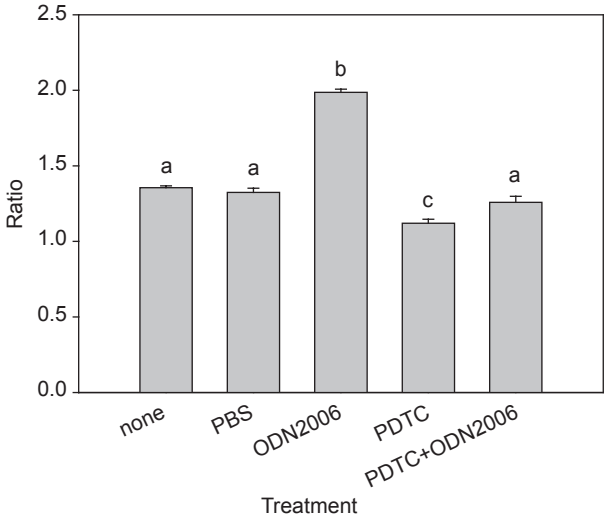

Fig. 5. Semiquantitative analysis of prophenoloxidase (proPO) gene expression at the RNA level. Bar 1, pre-injection; bar 2, 1 h post-injection with 0.1 ml of phosphate buffered saline (PBS) as the control; bars 3, 4, and 5, oligodeoxynucleotide (ODN)2006 (5 μg/prawn) which served as the positive control, pyrrolidine dithiocarbamate (PDTC) (1.5 mM/prawn), and ODN2006 and PDTC, respectively. The semiquantitative determination of proPO mRNA and the internal control was carried out using ImageMaster™ (TotalLab Software vers. 2.00, Amersham) and was expressed as a ratio between the density of the proPO band and that of the actin band. The PBS-treated sample served as the control. Data were statistically analyzed using ANOVA and Duncan's multiple-range tests. Values with different lowercase letters above the bars significantly differed ($p < 0.05$). The letter, a, corresponds to the control.

endocytosis is one of the essential pathways for initiating signal transduction of the degranulation of CpG ODN-triggered hemocytes in prawn, and the NF-κB signaling pathway plays a role in regulating proPO gene expression. Furthermore, our earlier studies demonstrated that the activity of respiratory bursts and the expression of several immune-related genes of prawn hemocytes can also be enhanced by CpG ODN (Lu et al. 2007, Sung et al. 2008). Therefore, in the future, CpG ODNs may function as powerful and stable immunostimulants for activating the innate immunity of prawns.

Acknowledgments: This research was supported by the National Science Council, Taiwan (NSC94-2313-B-031-001 and NSC95-2313-B-031-004-MY3).

REFERENCES

Ahmad-Nejad P, H Häcker, M Rutz, S Bauer, RM Vabulas, H Wagner. 2002. Bacterial CpG-DNA and lipopolysaccharides activate Toll-like receptors at distinct cellular compartments. Eur. J. Immunol. **32:** 1958-1968.

Ashida M, Y Ishizaki, H Iwahana. 1983. Activation of prophenoloxidase by bacterial cell walls or β-1,3-glucan in plasma of the silkworm, *Bombyx mori*. Biochem. Biophys. Res. Commun. **113:** 562-568.

Chuo CP, SM Liang, HH Sung. 2005. Signal transduction of the prophenoloxidase activating system of prawn haemocytes triggered by CpG oligodeoxynucleotides. Fish Shellfish Immunol. **18:** 149-162.

De Gregorio ES, J Han, WJ Lee, ML Baek, T Osaki, SI Kawabata, BL Lee, S Iwanaga, B Lemaitre, PT Brey. 2002. An immune-responsive serpin regulates the melanization cascade in *Drosophila*. Develop. Cell **3:** 581-592.

DiDonato JA, M Hayakawa, DM Rothwarf, E Zandi, M Karin. 1997. A cytokine-responsive IkappaB kinase that activates the transcription factor NF-kappaB. Nature **388:** 548-554.

Häcker H, H Mischak, T Miethke, S Liptay, R Schmid. 1998. CpG-DNA-specific activation of antigen-presenting cells requires stress kinase activity and is preceded by non-specific endocytosis and endosomal maturation. EMBO J. **17:** 6230-6240.

Hartmann G, RD Weeratna, ZK Ballas, P Payette, S Blackwell, I Suparto. 2000. Delineation of a CpG phosphorothioate oligodeoxynucleotide for activating primate immune responses *in vitro* and *in vivo*. J. Immunol. **164:** 1617-1624.

Hemmi H, T Kaisho, K Takeda, S Akira. 2003. The roles of toll-Like receptor 9, MyD88, and DNA-dependent protein kinase catalytic subunit in the effects of two distinct CpG DNAs on dendritic cell subsets. J. Immunol. **170:** 3059-3064.

Hemmi H, O Takeuchi, T Kawai, S Sato, H Sanjo, M Matsumoto, K Hoshino, H Wagner, K Takeda, S Akira. 2000. A Toll-like receptor recognizes bacterial DNA. Nature **408:** 740-745.

Hernandez LJ, GT Gollas, AF Vargas. 1996. Activation of the prophenoloxidase system of the brown shrimp (*Penaeus californiensis* Holmes). Comp. Biochem. Physiol. **113C:** 61-66.

Hoffmann JA. 2003. The immune response of *Drosophila*. Nature **426:** 33-38.

Hoffman JA, FC Kafatos, CA Janeway, RA Ezekowitz. 1999. Phylogenetic perspectives in innate immunity. Science **284:** 1313-1318.

Hoffmann JA, J Reichhart. 2002. *Drosophila* innate immunity: an evolutionary perspective. Nat. Immunol. **3:** 121-126.

Iwanaga S. 2002. The molecular basis of innate immunity in the horseshoe crab. Curr. Opin. Immunol. **14:** 87-95.

Karin M, Y Ben-Neriah. 2000. Phosphorylation meets ubiquitination: the control of NF-[κ]B activity. Annu. Rev. Immunol. **18:** 621-663.

Krieg AM, A Yi, S Matsin, TJ Waldschmidt, GA Bishop, R Teasdale et al. 1995. CpG motifs in bacterial DNA trigger direct B-cell activation. Nature **374:** 546-549.

Latz E, A Schoenemeyer, A Visintin, K Fitzgerald, BG Monks. 2004. TLR9 signals after translocating from the ER to CpG DNA in the lysosome. Nat. Immunol. **5:** 190-198.

Lu KY, YT Huang, HH Lee, HH Sung. 2006. Cloning the prophenoloxidase cDNA and monitoring the expression of proPO mRNA in prawns (*Macrobrachium rosenbergii*) stimulated *in vivo* by CpG oligodeoxynucleotides. Fish Shellfish Immunol. **20:** 274-284.

Lu KY, HJ Sung, HH Sung. 2007. Genes expression in hemocytes of the giant freshwater prawn (*Macrobrachium rosenbergii*) elicited by CpG oligodeoxynucleotide by suppression subtractive hybridization. J. Fish. Soc. Taiwan **34:** 275-290.

Medzhitov R, CA Janeway Jr. 2000. Innate immunity. N. Engl. J. Med. **343:** 338-344.

Medzhitov R, P Preston-Hurlburt, CA Janeway. 1997. A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. Nature **388:** 394-397.

Perazzolo LM, MA Barracco. 1997. The prophenoloxidase activity system of the shrimp *Penaeus paulensis* and associated factors. Develop. Comp. Immunol. **21:** 385-395.

Ratcliffe NA, AF Rowley, SW Fitzgerald, CP Rhodes. 1985. Invertebrate immunity: basic concepts and recent advances. Int. Rev. Cytol. **97:** 183-350.

Rodriguez J, GL Moullac. 2000. State of the art of immunological tools and health control of penaeid shrimp. Aquaculture **191:** 109-119.

Shirakabe K, K Yamaguchi, H Shibuya, K Irie, S Matsuda, T Moriguchi. 1997. TAK1 mediates the ceramide signaling to stress-activated protein kinase/c-Jun N-terminal kinase. J. Biol. Chem. **272:** 8141-8144.

Silverman N, T Maniatis. 2001. NF-κB signaling pathways in mammalian and insect innate immunity. Genes Develop. **15:** 2321-2342.

Smith VJ, K Söderhäll. 1991. A comparison of phenoloxidase activity in the blood of marine invertebrates. Develop. Comp. Immunol. **15:** 251-261.

Söderhäll K. 1992. Biochemical and molecular aspects of cellular communication in arthropods. Bull. Zool. **59:** 141-151.

Söderhäll K, A Aspàn, B Duvic. 1990. The proPO system and associated proteins: role in cellular communication in arthropods. Res. Immunol. **141:** 896-907.

Söderhäll K, L Cerenius. 1992. Crustacean immunity. Annu. Rev. Dis. **2:** 3-23.

Söderhäll K, L Cerenius, MW Johansson. 1994. The prophenoloxidase activating system and its role in invertebrate defence. Annu. NY Acad. Sci. **712:** 155-161.

Söderhäll K, L Hall. 1984. Lipopolysaccharide-induced activation of prophenoloxidase activating system in crayfish haemocyte lysate. Biochem. Biophys. Acta **797:** 99-104.

Söderhäll K, T Unestam. 1979. Activation of serum prophenoloxidase in arthropod immunity. The specificity of cell wall glucan activation and activation by purified fungal glycoproteins of crayfish phenoloxidase. Can. J. Microbiol. **25:** 406-414.

Sung HH, HJ Chang, CH Her, JC Chang, YL Song. 1998. Phenoloxidase activity of hemocytes derived from *Penaeus monodon* and *Macrobrachium rosenbergii*. J. Invertebr Pathol. **71:** 26-33.

Sung HH, PH Chen, CL Liu. 2008. Initiation of signal transduction of the respiratory burst of prawn haemocytes triggered by CpG oligodeoxynucleotides. Fish Shellfish Immunol. **24:** 693-700.

Sung HH, YT Huang, LT Hsiao. 2004. Phenoloxidase activity of *Macrobrachium rosenbergii* after challenge with two pathogens: *Aeromonas veronii* and *Lactococcus garvieae*. Fish Pathol. **39:** 1-8.

Takeshita F, CA Leifer, I Gursel, KJ Ishii, S Takeshita, M Gursel et al. 2001. Role of Toll-like receptor 9 in CpG DNA-induced activation of human cells. J. Immunol. **167:** 3555-3558.

Tokunaga T, T Yamamoto, S Yamamoto. 1999. How GCG led to the discovery of immunostimulatory DNA. Jpn J. Infect. Dis. **52:** 1-11.

Tzou P, DG Ennio, L Bruno. 2002. How *Drosophila* combats microbial infection: a model to study innate immunity and host-pathogen interactions. Curr. Opin. Microbiol. **5:** 102-110.

Unestam T, K Söderhäll. 1977. Soluble fragments from fungal cell walls elicit defence reactions in crayfish. Nature **267:** 45-46.

Wagner H. 1999. Bacterial CpG DNA activates immune cells to signal infectious danger. Adv. Immunol. **73:** 329-368.

Wagner H. 2000. Interactions between bacterial CpG-DNA and TLR9 bridge innate and adaptive immunity. Curr. Opin. Microbiol. **5:** 102-110.

Wang XW, NS Tan, B Ho, JL Ding. 2006. Evidence for the ancient origin of the NF-κB/IκB cascade: its archaic role in pathogen infection and immunity. Proc. Natl. Acad. Sci. **103:** 4204-4209.

Wasserman SA. 2000. Toll signaling: the enigma variations. Curr. Opin. Genet. Dev. **10:** 497-502.

Yi AK, T Tuetken, T Redford, M Waldschmidt, J Kirsch, AM Krieg. 1998. CpG motifs in bacterial DNA activate leukocytes through the pH-dependent generation of reactive oxygen species. J. Immunol. **160:** 4755-4761.