Protein kinase-like ER kinase (PERK) regulates autophagy of hemocytes in antiviral immunity of Pacific oyster *Crassostrea gigas*

Shujing Liu\(^a\),\(^c\),\(^d\), Weilin Wang\(^a\),\(^c\),\(^d\), Yu Liu\(^a\),\(^c\),\(^d\), Wanqing Cao\(^a\),\(^c\),\(^d\), Pei Yuan\(^a\),\(^c\),\(^d\), Jiaxin Li\(^a\),\(^c\),\(^d\), Xiaorui Song\(^a\),\(^c\),\(^d\), Lingling Wang\(^a\),\(^b\),\(^c\),\(^d\), Linsheng Song\(^a\),\(^b\),\(^c\),\(^d\),\(^*\)

\(^a\) Liaoning Key Laboratory of Marine Animal Immunology, Dalian Ocean University, Dalian 116023, China
\(^b\) Laboratory of Marine Fisheries Science and Food Production Process, Qingdao National Laboratory for Marine Science and Technology, Qingdao 266235, China
\(^c\) Liaoning Key Laboratory of Marine Animal Immunology and Disease Control, Dalian Ocean University, Dalian 116023, China
\(^d\) Dalian Key Laboratory of Aquatic Animal Disease Prevention and Control, Dalian Ocean University, Dalian 116023, China

**A R T I C L E   I N F O**

**Keywords:**
*Crassostrea gigas*
Protein kinase-like endoplasmic reticulum kinase
poly(I:C)
Autophagy
Unfolded protein response

**A B S T R A C T**

The maintenance of cellular homeostasis is an important process for successful immune defense against pathogenic invading, in which unfolded protein response (UPR) pathway regulates endoplasmic reticulum (ER) homeostasis upon exposure to environmental changes. Protein kinase-like ER kinase (PERK) is an important ER stress sensor to be activated during the UPR to regulate cells homeostasis. In the present study, one PERK homologue was identified from Pacific oyster *Crassostrea gigas* (designated as CgPERK). The cdNA of CgPERK was of 4307 bp with a 3174 bp open reading frame (ORF) encoding a polypeptide of 1058 amino acids. There were two conserved protein kinases domains and two conserved autophosphorylation sites at Lys618 and Thr980 in CgPERK. The mRNA transcript of CgPERK was constitutively expressed in all the tested tissues including mantle, adductor muscle, hepatopancreas, gill, gonad and labial palp with the highest expression level in hemocytes (31.15-fold compared to mantle). The CgPERK protein was found to be located mainly in the cytoplasm of hemocytes. The mRNA expression level of CgPERK in hemocytes was significantly up-regulated and reached the highest level (5.25-fold compared to seawater group, \(p < 0.01\)) at 48 h after the oysters were stimulated with poly(I:C). Meanwhile, a significant increase of fluorescence autophagosome spots in hemocytes was also observed at 36 h post stimulation. After the mRNA expression of CgPERK was knocked down (0.49-fold compared to dsGFP group, \(p < 0.01\)) by injection of CgPERK dsRNA, the mRNA expression of autophagy related 12 (ATG12) in hemocytes was significantly decreased at 12 h post poly(I:C) stimulation, which was 0.53-fold (\(p < 0.01\)) compared to dsGFP injected oysters. When the CgPERK was inhibited by its inhibitor GSK26656157 stimulation, the autophagosomes rate of hemocytes decreased significantly at 12 h post poly(I:C) stimulation, which was 0.34-fold (\(p < 0.01\)) of that of DMSO group. Collectively, these results suggested that CgPERK, as an UPR initiator, was involved in autophagosomes formation upon poly(I:C) stimulation by regulating the expression of ATG12, and ER stress stimulated the autophagosome formation on an ATG protein-dependent manner in oysters.

1. Introduction

In eukaryotic cells, the folding of secreted and transmembrane proteins occurs in endoplasmic reticulum (ER). This process is perturbed when cells are exposed to detrimental environments. The accumulation of incorrectly folded proteins triggers an ER-stress and induces an unfolded protein response (UPR) to orchestrate the recovery of ER function [1]. In this process, inositol-requiring protein 1 (IRE1) and activating transcription factor 6 (ATF6) in UPR pathway firstly induce the expression of ER resident proteins to promote the folding of newly synthesized peptides in ER lumen. The protein kinase-like ER kinase (PERK) controls the second step of a profound and rapid repression of most protein synthesis [2]. These three proteins (IRE1, ATF6 and PERK) play key roles in UPR and ER hemostasis.

As an important type I transmembrane protein, PERK is structurally conserved in all metazoans. The N-terminal region of PERK lies inside the ER lumen, while the C-terminal region contains the kinase domains and autophosphorylation sites in cytosolic [3]. The inactive PERK is bound by Glucose-regulated protein of 78 kDa (GRP78). It has been proposed that the misfolded proteins within ER lumen compete with PERK for GRP78 binding [3]. Accumulation of misfolded proteins causes the dissociation of GRP78 from PERK and induces the autophosphorylation of PERK at several residues in its C-terminal domain [4]. The activated PERK phosphorylates the serine 51 residue of eukaryotic initiation fac-

---

\(^*\) Corresponding authors.

E-mail addresses: wangweilin@dlou.edu.cn (W. Wang), lshsong@dlou.edu.cn (L. Song).

https://doi.org/10.1016/j.fsiirep.2020.100002

Received 4 October 2020; Received in revised form 18 November 2020; Accepted 19 November 2020

Available online 28 November 2020

2667-0119/© 2020 Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/)
Phosphorylation of eIF2α inhibits the GDP-GTP exchange activity of eIF2α and decreases the concentration of translation preinitiation complex. The specific mRNAs that harbor upstream open reading frames (uORFs) in their 5’ UTRs can be upregulated translationally by eIF2α phosphorylation, such as ATF4 and Gadd34 [6, 7]. As a transcription factor, ATF4 can migrate into the nucleus and induce the expression of autophagy genes, such as Beclin 1 (BECN1), autophagy related 5 (ATG5) and autophagy related 12 (ATG12) to regulate stress response [8, 9].

Recently, the regulation roles of PERK in embryonic development and stress response have been documented in invertebrates, such as nematodes, arthropods, and mollusks. PERK from nematode Caenorhabditis elegans was reported to be involved in the conserved PERK-eIF2α pathway for larval development and pathogenesis of disease [10]. The PERK transcripts were highly expressed in the embryos of Drosophila melanogaster, which were concentrated in gut and gonads during the later stages of embryogenesis [11]. The conserved eIF2α-ATF4-CHOP pathway was reported to participate in anoxia tolerance and freezing survival in Littorina littorea [12]. However, there are few reports about the involvement of PERK in anti-virus immune response of invertebrates.

The Pacific oyster Crassostrea gigas is a kind of worldwide economic mollusk, which inhabits in the intertidal zone and has to cope with harsh and dynamically changing environments. Recently, the oyster aquaculture industry has suffered serious summer mortality, and ostreid herpes virus 1 is suspected to be one of the main pathogens [13, 14]. Analyzing and analyzing of genomics data reveal that oyster has developed a sophisticated response and adaption system, among which UPR is an important mechanism to maintain homeostasis [15]. The knowledge about the immune regulatory functions of PERK pathway would provide new clues to develop the strategies for the disease prevention and control in oyster aquaculture. The major objectives of the present study were to (1) identify and characterize PERK in the oyster C. gigas, (2) investigate the spatiotemporal expression patterns in hemocytes, and (3) determine its impact on autophagosomes formation of oysters after poly(I: C) stimulation. The results would provide knowledge about the regulation of PERK on the autophagy under viral stimulation.

2. Materials and methods

2.1 Animals

Adult oysters, two-years-old with average length of 12.0 cm, were collected from a local farm in Dalian, China. Before the experiment, the oysters were cultured in tanks with aerated seawater for seven days. The water was changed and the oysters were fed with powdered spirulina every day.

Six-week-old female mice, employed for preparation of polyclonal antibodies, were purchased from the Dalian Institute of Drug Control in Liaoning Province, China, and raised in laboratory for one week prior to use. The experiments with mice were approved by our ethic committee.

2.2 The analysis of CgPERK sequence and structure

By screening the genome of oyster C. gigas, a gene encoding PERK was identified (CgPERK, NCBI accession NO. XM011452385.2). The protein domain of CgPERK was predicted with the simple modular architecture research tool (SMART) (http://www.smart.embl-heidelberg.de/) and Expert Protein Analysis System (http://www.expasy.org/). Multiple alignment was analyzed with the ClustalW multiple alignment programs (http://www.ebi.ac.uk/clustalw/). A neighbor-joining (NJ) tree of various PERK proteins from different organisms was established using MEGA 6.0 software package with 1000 pseudo-replicates of bootstrap resampling to test the reliability of the clade. The number at the nodes indicated the bootstrap value. The scale bar corresponds to 0.1 estimated amino-acid substitutions per site.

2.3. Poly(I: C) stimulation and sample collection

After temporarily cultured, 108 oysters were employed and divided into two group. The oysters in control group received an injection of 100 µl sterilized seawater, while the oysters in stimulation group received an injection of 100 µl poly(I: C) (1 mg mL⁻¹ in seawater) (Sigma, USA, synthetic double stranded RNA). All the oysters were returned to culture system, and nine oysters from each group were randomly sampled at 0,3,6,12,24,48 h after injection. The hemolymphs were withdrew and centrifuged at 600 g, 4 °C for 10 min to harvest hemocytes. The tissues including hemolymph, gills, mantle, hepatopancreas, labial palp, gonad and adductor muscle were sampled from non-stimulated oysters and stored in 1 mL TRIzol™ reagent (Invitrogen, California, USA) at -80 °C to detect the tissue distribution of C. gigas mRNA.

To detect the autophagy activity in hemocytes, 27 oysters were divided into three groups. To inhibit autophagosome degradation, oysters were firstly treated with NH₄Cl at a final concentration of 1mM (10 L) for 36 h according to previous report [8]. Then oysters were treated by an injection with 100 µL poly(I: C) (1 mg mL⁻¹ in seawater) and cultured in aquariums for 36 h to induce autophagy.

2.4. RNA extraction, cDNA synthesis and real-time quantitative PCR

The total RNA was extracted from hemocytes or tissues using Trizol (Invitrogen, USA) according to the protocol. The first strand cDNA was synthesized using PrimeScript™ RT reagent Kit (Takara) according to the manufacture’s instruction, with oligo (dt) (Table 1) as primer and DNasel-treated total RNA as template. The mixture was incubated in 37 °C for 15 min, 85 °C for 5 s. The cDNA was diluted to 1:40 and stored at 4 °C for subsequent gene cloning and real-time PCR (qRT-PCR). The qRT-PCR experiment was conducted according to previous description [18]. The qPCR reaction mixture (10 µL) consisted of SYBR Green PCR Master mix, ROX Reference Dye, 0.4 mM each of the forward and reverse primers, and 2 µL of cDNA template. The program of qPCR was operated as follow: 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s and 60 °C for 34 s. Dissociation curve analysis was conducted to confirm that the only one product was amplified at the end of each PCR. Specific primers CgPERK-RT-F and CgPERK-RT-R, CgGRP78-RT-F and CgGRP78-RT-R (Table 1) were designed to amplify the fragments of CgPERK and CgGRP78, respectively. Primers CgEF-F and CgEF-R were designed to amplify the endogenous control gene elongation factor (CgEF) according to the previous report [16]. In dsRNA-injected oysters, the mRNA expressions of autophagy related 12 (ATG12) and beclin 1 (BECN1) in C. gigas hemocytes were detected by qRT-PCR with the primers in Table 1 at 12 h after poly(I: C) injection. The relative expression level of CgPERK mRNA was calculated by the comparative CT (2⁻ΔΔCT) method.

2.5. Recombination, purification, antibody preparation of CgPERK and western blot analysis

A pair of specific primers CgPERK-F and CgPERK-R (Table 1) was designed to amplify the full length ORF of CgPERK. The cDNA fragment encoding the cytoplasmic terminus of CgPERK was amplified using the primers rCgPERK-F and rCgPERK-R (Table 1) with restriction enzyme sites BamHI and XhoI. The PCR products were cloned into PET-30a expression vector. The recombinant plasmid (pET-30a-CgPERK) was transformed into Escherichia coli (E. coli) BL21 (DE3) (TransGen, China). The recombinant protein of CgPERK (rCgPERK) was purified by Ni-NTA Sepharose column and used to prepare mouse polyclonal antibody referred to the previous report [17]. Briefly, rCgPERK (0.5 mg mL⁻¹) were emulsified with isometric Freund’s complete adjuvant (Sigma, USA) and then used to immunize each female mouse. The second and third immunizations were executed on the 14th and 21th day with incomplete adjuvant (Sigma, USA). The fourth inoculation was executed on the 28th day using purified proteins, and finally the serum was collected on the
The anti-rCgPERK serum was stored at -80 °C for subsequent experiment.

For western blot analysis, samples (purified rCgPERK) were separated by SDS-PAGE and electrophoretically transferred onto nitrocellulose membrane. The membrane was blocked by 5% skim milk powder solution in TBS-T (Tris 2.42 g L⁻¹; NaCl 8.80 g L⁻¹; 0.1% Tween-20, v/v; pH 7.4) at 37 °C for 2 h. After washing in TBS-T for three times (5 min each time), the membrane was incubated with the rCgPERK antibody solution (diluted with 1:1000 in 5% skim milk, v/v) at 4 °C overnight. Pre-immune serum was used as negative control. After thoroughly washing with TBS-T for three times, the membrane was incubated with horse radish peroxidase (HRP)-conjugated anti-mouse IgG (diluted with 1:2000 in 5% skim milk, v/v) for 1 h. After a final three times of washing with TBS-T, the membrane was incubated with enhanced chemiluminescence (ECL) detection reagents (Thermo scientific, USA) and the image was captured by Amershams Imager 600 system (GE Healthcare, USA).

2.6. The immunofluorescence assay

Hemocytes from oyster were collected and washed two times using modified L15 medium (M-L15, supplemented with KCl 0.54 g L⁻¹; CaCl₂ 0.6 g L⁻¹; MgSO₄ 1.0 g L⁻¹; MgCl₂ 3.9 g L⁻¹; NaCl 20.2 g L⁻¹) [18]. The cells were fixed by 4% paraformaldehyde for 20 min. After blocking with 20 μL of 3% BSA for 1 h, the sample was incubated with anti-rCgPERK antibody (1:1000) as first antibody and Alexa Fluor 488-conjugated anti-mouse IgG (1:2000) as secondary antibody at 37 °C for 1 h. The serum from pre-immunization mice was used as negative control. The nucleus was stained blue by 4′,6-Diamidino-2-Phenylindole (DAPI). After the last three times washing, the hemocytes were observed and captured by using Laser Scan Confocal Microscope (Carl Zeiss, Germany).

2.7. Inhibitor treatment and in vivo RNA interference (RNAi) of oysters

The oysters in control group received an injection of 100 μL 1% DMSO, while the oysters in stimulation group received an injection of 100 μL GSK2656157 (2 mg mL⁻¹ in 1% DMSO). To enhance the effect of Inhibitor treatment, a second injection was performed at 12 h after the first injection. The hemocytes from oysters in each group were collected for autophagy detection at 24 h after injection.

To knock down the expression of CgPERK, dsRNAs of CgPERK gene and GFP gene were synthesized using Transcription T7 Kit (Takara, Japan). The cDNA fragment of CgPERK was amplified by the primers CgPERK-F and CgPERK-R (Table 1) and this fragment was used as template to synthesize dsRNA (designated as dsCgPERK). Primers GFP-F and GFP-R (Table 1) were designed to amplify DNA fragment of GFP and this fragment was used as template to synthesize dsRNA (designated as dsGFP). Oysters were divided into two groups with nine individuals in each group and received an injection (100 μL, 1 μg mL⁻¹) of dsCgPERK (designated as dsCgPERK group) or dsGFP (designated as dsGFP group). To enhance the effect of RNAi, a second injection was performed at 12 h after the first injection. The untreated oysters were used as blank group. The hemocytes were collected from oysters in each group at 24 h after dsRNA injection to detect the RNAi efficacy by primers CgPERK-F and CgPERK-R (Table 1).

2.8. Assay of autophagy activity

The percentages of hemocytes with autophagy activity were quantified using the commercial Cytoid® autophagy detection kit (ENZO Life Science, ENZ-51, 031-0050) following the manufacturer’s instruction. Hemolymph was collected with modified Alsever’s solution (MAS, glucose 20.8 g L⁻¹; EDTA 3.36 g L⁻¹; NaCl 22.5 g L⁻¹; sodium citrate 8.0 g L⁻¹; pH 7.4) at the ratio of 1:1, and then centrifuged immediately at 600 g, 4 °C for 10 min. After washing with modified L15 medium twice, the pellet containing hemocytes was suspended in 100 μL CytoID® green detection reagent (diluted with 1:2000 in modified L15 medium, v/v), and incubated in the dark at room temperature for 90 min. After thoroughly washing with modified L15 medium for two times, hemocytes were incubated with Hoechst 33342 Nuclear Stain (1mM diluted 1:1000 in modified L15 medium) at room temperature for 20 min. After the last two times of washing, the hemocytes were re-suspended in 100 μL modified L15 medium, and observed and cap-
tured by using Laser Scan Confocal Microscope (Carl Zeiss, Germany). The autophagosome rate of hemocytes in each group was analyzed by FACS Aria II flow cytometry (BD Biosciences, USA). The percentage of hemocytes with autophagosomes was calculated, and the ratio of percentage between tested group and control group was used to determine the autophagy activity.

2.9. Statistical analysis

All data were given as means ± S.D. The two-samples Student’s test was performed for the comparisons between groups. Multiple group comparisons were executed by one-way ANOVA and followed by a Turkey multiple group comparison test using Statistical Package for Social Science (SPSS) 17.0 software. The results were graphed by Origin 8.1. The differences between treatments for each assay were considered significant at $p < 0.05$ and extremely significant at $p < 0.01$.

3. Results

3.1. The sequence characteristics and phylogeny relationship of CgPERK

A 4307 bp DNA fragment of CgPERK was identified after the genome annotation of Pacific oyster C. gigas, which contained an ORF of 3174 bp encoding a putative polypeptide of 1058 amino acids. ExPASy analysis showed that CgPERK could be divided into ER luminal terminus and cytoplasm terminus on the basis of the transmembrane region (368-392aa). SMART program analysis revealed a protein kinase domain (537-618 aa) and a Tyrosine protein kinases domain (825-1024 aa) in C-luminal cytosolic position (Fig. 1A). The conserved Lys618 and Thr980 phosphorylation sites were identified at the C-terminal cytoplasmatic kinase domain. The deduced amino acid sequence of CgPERK was conservative with that of those PERKs from other animals (Fig. 1B), and shared relatively high similarity with PERKs from Mizuhopecten yessoensis (OWF40340.1, 56.1%), Biomphalaria glabrata (XP_013090857.1, 44.5%) and Octopus bimaculoides (XP_014772435.1, 43.7%), and relatively low similarity with PERKs from Xenopus tropicalis (XP_012811205.1, 32.4%) and Drosophila melanogaster (NP_649538.1, 35.2%) (Table 2). An NJ polygenetic tree of CgPERK and PERKs from other species was constructed, and all the members were distinctly divided into invertebrate clade and vertebrate clade. CgPERK was closely clustered with PERK from Mizuhopecten yessoensis (OWF40340.1) and then gathered with PERKs from Octopus bimaculoides (XP_014772435.1), Biomphalaria glabrata (XP_013090857.1) and Limulus polyphemus (XP_014772435.1) to form invertebrate clade (Fig. 1C). The other PERKs from Homo sapiens, Mus musculus, Parus major, Pogona vitticeps, Dario rerto and Pogona vitticeps, were clustered into vertebrate clade, with the outer clade of Drosophila melanogaster and Strongylocentrotus purpuratus.

3.2. Recombinant protein and polyclonal antibody of CgPERK

After IPTG induction, a distinct band with molecular mass of 65 kDa was revealed (Fig. 2A). The purified rCgPERK protein was used to prepare polyclonal antibody. The antibody specificity was examined by western blotting assay and a highly specific reaction band was revealed (Fig. 2B). No band was detected with mouse pre-immune serum in negative group (data not shown).

3.3. The distribution of CgPERK mRNA transcripts and protein in tissues

Quantitative real-time PCR was employed to detect the expression level of CgPERK mRNA in different tissues. CgPERK mRNA was constitutively expressed in all the tested tissues including hemocytes, mantle, adductor muscle, hepatopancreas, gill, gonad, and labial palp. The highest expression level CgPERK mRNA was detected in hemocytes, which
was 31.15-fold ($p < 0.01$) higher than that in mantle. The higher expression level CgPERK mRNA was also detected in labial palp, which was 4.93-fold ($p < 0.05$) higher than that in mantle. There was no significant difference of CgPERK mRNA transcripts among the other tissues, including adductor muscle (3.69-fold compared to mantle, $p > 0.05$), hepatopancreas (3.96-fold compared to mantle, $p > 0.05$), gill (4.06-fold compared to mantle, $p > 0.05$), and gonad (4.13-fold compared to mantle, $p > 0.05$) (Fig. 3A).

The subcellular localization of CgPERK protein in hemocytes was observed using polyclonal-antibody against CgPERK protein. In the bright field, the hemocytes of oyster were observed in irregular cellular shapes. The nuclei were dyed in blue by DAPI. In the FITC channel, the positive signals of CgPERK were observed in green. According to the merged images, the green signals were distributed mainly in cytoplasm of untreated oyster hemocytes (Fig. 3B).

### 3.4. The expression patterns of CgPERK after Poly(I:C) stimulation

The temporal mRNA expression changes of CgPERK in oyster hemocytes after poly(I:C) stimulation were examined by qPCR. After the stimulation with poly(I:C), the expression level of CgGRP78 mRNA in oyster hemocytes increased significantly at 24 h (4.09-fold compared to seawater group, $p < 0.01$) and 48 h (3.22-fold compared to seawater group, $p < 0.01$) (Fig. 4A). Meanwhile, the expression of CgPERK mRNA in hemocytes was also significantly up-regulated at 48 h after poly(I:C) stimulation, which was 5.25-fold compared to seawater group ($p < 0.01$) (Fig. 4B).

### 3.5. Autophagy in oyster hemocytes after poly(I:C) stimulation

Fluorescence microscopy was employed to examine the formation of autophagosome. The NH$_4$Cl was used to inhibit the degradation of autophagosomes [8]. In the control group, the green fluorescence intensity of autophagosome spots was low, while it increased at 36 h after poly(I:C) treatment in the stimulation group. The increased fluorescence intensity was even stronger in poly(I:C) + NH$_4$Cl group compared with that in poly(I:C) group when the degradation of induced autophagosome was inhibited by NH$_4$Cl pre-treatment (Fig. 5).

### 3.6. The expression changes of autophagy related genes in hemocytes of C. gigas after CgPERK knock-down

The expression changes of ER chaperones and anti-apoptosis related genes in oyster hemocytes were determined after CgPERK was in vivo knocked-down to explore the possible immune function of CgPERK. After injection of dsCgPERK, the expression of CgPERK in oyster hemocytes was knocked down, which was 0.49-fold ($p < 0.01$) compared to dsGFP group (Fig. 6A). Meanwhile, the mRNA expression of CgBcl2 and CgATG12 were detected in dsCgPERK group at 36 h after poly(I:C) stimulation. No significant change of CgBcl2 expression was observed (Fig. 6B), while the mRNA expression of CgATG12 decreased significantly at 36 h after poly(I:C) stimulation, which was 0.51-fold ($p < 0.01$) compared to dsGFP oysters (Fig. 6C).

### 3.7. The effects of CgPERK inhibition on the formation of autophagosome

The formation of autophagosome was observed after the oysters were treated with Inhibitor GSK2656157. The percentage of hemocytes with autophagosomes was calculated, and the ratio of percentage between tested group and control group was used to determine the autophagy activity. In blank group, 36 ± 1.7% of the total hemocytes were stained positive (Fig. 7A). The percentage of positive hemocytes in poly(I:C) + DMSO group was 47 ± 1.5%, which was significantly higher ($p < 0.05$) than that in the blank group and DMSO group (38.2 ± 1.3%) (Fig. 7B). However, a significant decrease of positive hemocytes was observed in poly(I:C) + GSK2656157 inhibitor treatment, which was 0.74-fold ($p < 0.05$) compared to poly(I:C) + DMSO group.

### 4. Discussion

Eukaryotic cells are able to respond to various types of stress caused by changes in the extracellular environment. For example, pathogen infections are reported to impair cellular immunity and reduce cellular homeostasis [21]. Autophagy can be induced after infection and involves in clearing diverse intracellular pathogens to maintain homeostasis [22, 23]. PERK pathway, as one of the most important mechanism in
Fig. 3. The spatial expression patterns and subcellular localization of CgPERK. (A) Relative mRNA expression level of CgPERK in different tissues (gonad, gill, hepatopancreas, labial, muscle) and hemocytes were compared to that in mantle. Vertical bars show mean ± S.E. (N = 3). (B) Subcellular localization of CgPERK in hemocytes of C. gigas. After incubation of polyclonal antibody of CgPERK, Alexa Fluor 488-conjugated anti-mouse IgG was used to detect CgPERK. Hemocytes morphology is shown in bright field. Nucleus was stained blue by DAPI. Positive signals of CgPERK were shown in green. Scale bar = 20 μm.

Fig. 4. Temporal expression patterns of CgPERK and CgGRP78 in hemocytes after poly(I:C) stimulation. The mRNA of hemocytes was extracted at a series of time points (0, 3, 6, 12, 24 and 48 h) after poly(I:C) stimulation. The expression levels of CgGRP78 (A) and CgPERK (B) were detected by real-time PCR. The significant differences among the seawater group and treated groups were labeled with different letters (p < 0.05). Vertical bars show as mean ± S.E. (N = 3).

Fig. 5. The changes of autophagy level of hemocytes in oyster post stimulation with poly(I:C). The autophagy level of hemocytes was reflected by the intensity of green fluorescence and determined by fluorescence microscopy after treatment with poly(I:C) or poly(I:C) + NH₄Cl.

UPR, has been identified to play vital roles in the process of autophagy in model organisms [24]. However, the information about PERK path-
to mammalian PERK. eIF2α phosphorylation was reported to upregulate the translation of ATF4 which subsequently migrated to nucleus to induce the expression of autophagy relative genes [29]. In the phylogenetic tree, CgPERK was firstly clustered with mollusc PERKs from M. yessoensis and then gathered with O. bimaculoides, B. glabrata and L. polyphemus, and fell in the invertebrate clade. In view of the great similar structural characteristics and phylogeny relationship, CgPERK was confirmed to be a conserved homologue of vertebrates PERK, which might be involved in regulating autophagy relative genes.

To evaluate the possible function of CgPERK in immune response, its expression pattern and distribution were examined. The mRNA transcript of CgPERK was ubiquitously detected in all the tested tissues with the highest expression level in hemocyte of C. gigas. The subcellular localization of PERK was previously reported to be distributed in the endoplasmic reticulum [12]. In the present study, CgPERK was found to be located mainly in the cytoplasm of oyster hemocytes. GRP78 has been characterized as an ER resident protein and its synthesis can be promoted by a variety of environmental and physiological stresses that perturb ER function and homeostasis [30]. The expression of CgGRP78 in oyster hemocytes was significantly up-regulated at 24 h after poly(I: C) stimulation, indicating that ER function and homeostasis were perturbed by poly(I: C) stimulation. PERK is responsible for sensing ER stress resulted from various stimuli, such as chemicals, hypoxia, and virus [31]. The expression of CgPERK in oyster hemocytes was signifi-
cantly up-regulated at 48 h after poly(I: C) stimulation, indicating that CgPERK was sensitive to the exogenous stress and could be activated quickly after poly(I: C) stimulation. The significant up-regulation of CgPERK mRNA appeared later compared to that of CgGRP78, implying that CgPERK might participate in the response against ER stress.

Previous reports have demonstrated that autophagy is an essential strategy of Drosophila immunity against vesicular stomatitis virus [32]. In Pacific oyster, autophagy pathway was reported to function in mantle to protect animals from OsHV-1 infection [33], and the accumulation of autophagosomes was also observed in the hemocytes of C. gigas [34]. In the present study, the increased green fluorescence spots of autophagosomes were observed in the hemocytes from oysters exposed to poly(I: C) compared with that in blank group, implying autophagy was induced by poly(I: C) stimulation. The autophagosome formation is an intermediate stage in the whole dynamic autophagy process. The accumulation of autophagosomes represents either an increased generation of autophagosomes or the blocked conversion to autolysosomes. NH2Cl is well known to inhibit the conversion to autolysosomes and degradation of autophagosomes. In the present study, when the conversion and degradation of autophagosomes was inhibited by NH2Cl pretreatment, the increased fluorescence intensity was even stronger in poly(I: C) + NH2Cl group compared with that in poly(I: C) group, indicating an increased generation of autophagosomes. These results suggested that poly(I: C) stimulation induced the formation of continuous autophagy flux in hemocytes of C. gigas.

It has been reported that PERK pathway plays an important role in autophagy by regulating the transcription of autophagy relative genes. In mammals, the phosphorylation of eIF2α by PERK promotes the translation of ATF4 [35], and the phosphorylation of Beclin 1 is required for full autophagic induction [36]. ATG12, a member of the autophagy protein family involving in protein conjugation systems, is required for the elongation stage of autophagy [37]. Beclin 1 and ATG12 have been reported to be regulated by PERK pathway [38,39]. However, the involvement of PERK in the regulation of Beclin 1 and ATG12 expression to induce autophagy is still not clear in invertebrates. In the present study, the expressions of CgATG12 and CgBeclin 1 in hemocytes of oysters receiving an injection of dsCgPERK were detected in order to explore the effects of CgPERK activation on the expression of downstream genes. The transcripts of CgATG12 in hemocytes from dsCgPERK group were significantly down-regulated at 36 h after poly(I: C) stimulation compared to that in dsGFP group. However, the mRNA expression of CgBeclin 1 in dsCgPERK group after poly(I: C) stimulation did not change significantly compared with that in dsGFP oysters. These results indicated that CgPERK could positively regulate the expression of CgATG12 under immune response, which might further regulate autophagy. Mammal ATG12 protein is a key regulator to induce cell autophagy. When PERK pathway was blocked, the expression of ATG12 mRNA was significantly decreased concomitant with the decreased autophagy level of C2C5 cells [8]. The expression change of CgATG12 in present study was consistent with that in mammals, indicating a conserved role of CgPERK in promoting the expression of CgATG12. Increasing evidences have documented the vital roles of this PERK pathway in innate and adaptive immunity [40,41]. In the present study, the autophagosomes ratio of hemocytes was found to decrease significantly in the oysters from poly(I: C) + GSK2656157 group compared with that in poly(I: C) + DMSO group, indicating that PERK regulated the formation of autophagosome in immune response. All these results indicated that CgPERK might positively regulate the expression of CgATG12 and promote the autophagy of hemocytes in the immune response of oyster.

In conclusion, a conserved CgPERK with Tyrosine protein kinases domain and conserved phosphorylation sites at Lys618 and Thr980 was identified from oyster C. gigas. The CgPERK mRNA was expressed constitutively in all the tested tissues with the highest level in hemocytes. Its expression in hemocytes was significantly up-regulated after poly(I: C) stimulation. When CgPERK was knocked down by dsCgPERK-injection, the transcripts of CgATG12 and autophagosome formation decreased significantly after poly(I: C) stimulation. All the results indicated that the conserved CgPERK positively regulated the expression of CgATG12 and autophagy formation of hemocytes to maintain cell homeostasis against the invasion of virus.

Declaration of Competing Interest
The authors declared that they have no conflicts of interest to this work.

Acknowledgements
We are grateful to all the laboratory members for their technical advice and helpful discussions. This research was supported by National Key R&D Program (2018YFD0900304), grants (Nos. 41961124009, U1706204) from National Science Foundation of China, earmarked fund (CARS-49) from Modern Agro-industry Technology Research System, the Outstanding Talents and Innovative teams of Agricultural Scientific Research in Ministry of Agriculture, Key R&D Program of Liaoning Province (2017203001 to L. W.), Dalian High Level Talent Innovation Support Program (2015R020), Aoshan Talents Cultivation Program supported by Qingdao National Laboratory for Marine Science and Technology (No. 2017ASTCP-OS13), Program for Innovative Talents in Higher Education of Liaoning Province (LR2016036), the Research Foundation for Distinguished Professor in Liaoning (XLYC19R012) and Talented Scholars in Dalian Ocean University.

References
[1] Hampton, Y. Randolph, ER stress response: getting the UPR hand on misfolded proteins, Curr. Biol. 10 (14) (2000) 851A–852A.
[2] David Ron, Translational control in the endoplasmic reticulum stress response, J. Clin. Invest. 110 (10) (2002) 1383–1388.
[3] J. Lee, U. Ozcan, Unfolded protein response signaling and metabolic diseases, J. Biol. Chem. 289 (5) (2014) 1203–1211.
[4] N. Donnelly, A.M. Gorman, S. Gupta, et al., The eIF2α kinases: their structures and functions, Cell Mol. Life Sci. 70 (19) (2013) 3493–3511.
[5] C. Koumenis, C. Nazaki, M. Kozlitinsky, et al., Regulation of protein synthesis by hydrogen peroxide via activation of the endoplasmic reticulum kinase PERK and phosphorylation of the translation initiation factor eIF2α, Mol. Cell. Biol. 22 (21) (2002) 7405–7416.
[6] H.P. Harding, I. Novoa, Y. Zhang, et al., Regulation translation initiation controls stress-induced gene expression in mammalian cells, Mol. Cell 6 (5) (2000) 1099–1108.
[7] Y.Y. Lee, R.C. Cevallos, E. Jan, An upstream open reading frame regulates translation of GADD34 during cellular stresses that induce eIF2α phosphorylation, J. Biol. Chem. 284 (11) (2009) 6661–6673.
[8] Y. Kosorok, E. Fujita, I. Tanida, et al., ER stress (PERK/eIF2α phosphorylation) mediates the polyglutamine-induced LC3 conversion, an essential step for autophagy formation, Cell Death Differentiation 14 (2) (2007) 230–239.
[9] K.M.A. Koushop, T. Van Den Beucken, L. Dubois, et al., The unfolded protein response protects human tumor cells during hypoxia through regulation of the autophagy genes MAP1LC3B and ATG5, J. Clin. Invest. 120 (1) (2010) 127–141.
[10] N. Pomar, J.J. Berlanga, S. Campuzano, et al., Functional characterization of Drosophila melanogaster PERK eukaryotic initiation factor 2α (eIF2α) kinase, Eur. J. Biochem. 270 (2) (2003) 293–306.
[11] K.B. Storey, B. Lant, O.O. Anozie, et al., Metabolic mechanisms for anoxia tolerance and freezing survival in the intertidal gastropod, Littorina littorea, Comp. Biochem. Physiol. A Mol. Integr. Physiol. 154 (4) (2012) 448–459.
[12] H.P. Harding, Y. Zhang, A. Bertolotti, et al., PERK is essential for translational regulation and cell survival during the unfolded protein response, Mol. Cell 5 (5) (2000) 897–904.
[13] T. Renault, P. Moreau, N. Faury, et al., Analysis of clinical osteoid herpervirus 1 (Malacoherperviridae) specimens by sequencing amplified fragments from three virus genome areas, J. Virol. 86 (10) (2012) 5942–5947.
[14] A. Segarra, J.F. Pépin, I. Arzul, et al., Detection and description of a particular Ostreid herpesvirus 1 genotype associated with massive mortality outbreaks of Pacific oysters, Crassostrea gigas, in France in 2008, Virus Res. 153 (1) (2010) 92–99.
[15] G. Zhang, X. Fang, X. Guo, et al., The oyster genome reveals stress adaptation and complexity of shell formation, Nature 490 (7416) (2012) 49–54.
[16] T. Renault, N. Faury, V. Barbosa-Solomieu, et al., Suppression subtractive hybridization (SSH) and real time PCR reveal differential gene expression in the Pacific cupped oyster, Crassostrea gigas, challenged with Ostreid herpesvirus 1, Develop. Comparative Immunol. 35 (7) (2011) 725–735.
[17] Z. Lv, L. Qiu, M. Wang, et al., Comparative study of three C1q domain containing proteins from pacific oyster Crassostrea gigas, Develop. Comparative Immunol. 78 (2018) 48–51.
[18] W. Wang, M. Li, L. Wang, et al., The granulocytes are the main immunocompetent hemocytes in Crassostrea gigas, Develop. Comparative Immunol. 67 (2017) 221–228.

[19] J.M. Axten, S.P. Romeril, A. Shu, et al., Discovery of GSK2656157: an optimized PERK inhibitor selected for preclinical development, ACS Med. Chem. Lett. 4 (10) (2013) 964–968.

[20] J. Krishnamoorthy, K. Rajesh, F. Mirzajani, et al., Evidence for eIF2α phosphorylation-independent effects of GSK2656157, a novel catalytic inhibitor of PERK with clinical implications, Cell Cycle 13 (5) (2014) 801–806.

[21] G. Pasqual, D.J. Burri, A. Pasquato, et al., Role of the host cell’s unfolded protein response in arenavirus infection, J. Virol. 85 (4) (2011) 1662–1670.

[22] V. Deretic, Autophagy as an immune defense mechanism, Curr. Opin. Immunol. 18 (4) (2006) 375–382.

[23] A. Orvedahl, B. Levine, Autophagy in mammalian antiviral immunity, in: Autophagy in Infection and Immunity, Springer, Berlin, Heidelberg, 2009, pp. 267–285.

[24] S. Lv, E.C. Sun, Q.Y. Xu, et al., Endoplasmic reticulum stress-mediated autophagy contributes to bluetongue virus infection via the PERK-eIF2α pathway, Biochem. Biophys. Res. Commun. 466 (3) (2015) 406–412.

[25] J. Dong, H. Qiu, M. Garcia-Barrio, et al., Uncharged tRNA activates GCN2 by displacing the protein kinase moiety from a bipartite tRNA-binding domain, Mol. Cell 6 (2) (2000) 269–279.

[26] S. Nanduri, F. Rahaman, B.R.G. Williams, et al., A dynamically tuned double-stranded RNA binding mechanism for the activation of antiviral kinase PKR, EMBO J. 19 (20) (2000) 5567–5574.

[27] S.J. Marciniak, L. Garcia-Bonilla, J. Hu, et al., Activation-dependent substrate recruitment by the eukaryotic translation initiation factor 2 kinase PERK, J. Cell Biol. 172 (2) (2006) 201–209.

[28] S.J. Sequeira, A.C. Ranganathan, A.P. Adam, et al., Inhibition of proliferation by PERK regulates mammary acinar morphogenesis and tumor formation, PLoS One 2 (7) (2007) e615.

[29] G. Zhang, X. Ling, L. Liu, et al., The eIF2α/ATF4 pathway links endoplasmic reticulum stress to autophagy following the production of reactive oxygen species in mouse spermatocyte-derived cells exposed to dibutyl phthalate, Free Radic. Res. 50 (7) (2016) 698–707.

[30] L.G. Haas, B.P. GORP78, an essential hsp70 resident protein in the endoplasmic reticulum, Experientia 50 (11-12) (1994) 1012–1020.

[31] L. Feng, Y. Shen, J. Li, Endoplasmic-reticulum stress and inflammatory response, Chin. Pharm. Bull. 29 (6) (2013) 756–760.

[32] S. Shelly, N. Lukinova, S. Bambina, et al., Autophagy is an essential component of Drosophila immunity against vesicular stomatitis virus, immunity 30 (4) (2009) 588–598.

[33] P. Moreau, K. Moreau, A. Segarra, et al., Autophagy plays an important role in protecting Pacific oysters from OsHV-1 and Vibrio aestuarianicus infections, Autophagy 11 (3) (2015) 516–526.

[34] S. Picot, B. Marga, N. Faury, et al., A study of autophagy in hemocytes of the Pacific oyster, Crassostrea gigas, Autophagy 15 (10) (2019) 1801–1809.

[35] M. Kozak, Pushing the limits of the scanning mechanism for initiation of translation, Gene 299 (1-2) (2002) 1–34.

[36] R.C. Russell, Y. Tian, H. Yuan, et al., ULK1 induces autophagy by phosphorylating Beclin-1 and activating VPS34 lipid kinase, Nat. Cell Biol. 15 (7) (2013) 741–750.

[37] D.J. Klionsky, Autophagy revisited: a conversation with Christian de Duve, Autophagy 4 (6) (2008) 740–743.

[38] J. Wang, R. Kang, H. Huang, et al., Hepatitis C virus core protein activates autophagy through EIF2AK3 and ATF6 UPR pathway-mediated MAP1LC3B and ATG12 expression, Autophagy 10 (5) (2014) 766–784.

[39] E. Zalickv, H. Berini, L. Mizrahy, et al., DAP-kinase-mediated phosphorylation on the BE3 domain of beclin 1 promotes dissociation of beclin 1 from Bcl-XL and induction of autophagy, EMBO Rep. 10 (3) (2009) 285–292.

[40] B. Levine, V. Deretic, Unveiling the roles of autophagy in innate and adaptive immunity, Nat. Rev. Immunol. 7 (10) (2007) 767–777.

[41] D. Schmid, C. Münz, Innate and adaptive immunity through autophagy, Immunity 27 (1) (2007) 11–21.