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Receptor for Activated C Kinase-1 protein from *Penaeus monodon* (Pm-RACK1) participates in the shrimp antioxidant response

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ABSTRACT

Cellular oxidative stress responses are caused in many ways, but especially by disease and environmental stress. After the initial burst of reactive oxygen species (ROS), the effective elimination of ROS is crucial for the survival of organisms and is mediated by antioxidant defense mechanisms. In this paper, we investigate the possible antioxidant function of *Penaeus monodon* Receptor for Activated C Kinase-1 (Pm-RACK1). When Pm-RACK1 was over-expressed in *Escherichia coli* cells or *Spodoptera frugiperda* (*Sf9*) insect cells exposed to H_2O_2, it significantly protected the cells from oxidative damage induced by H_2O_2. When recombinant Pm-RACK1 protein was expressed as a histidine fusion protein in *E. coli* and purified with a Ni^{2+}-column it possessed antioxidant functions that protected DNA from metal-catalyzed oxidation. Shrimp (*Penaeus vannamei*) held at an alkaline pH had a much higher hepatopancreatic expression of *Pm-RACK1* than in those held at pH 7.4. The exposure of shrimp to alkaline pH is also known to increase ROS production. These results provide strong evidence that Pm-RACK1 can participate in the shrimp antioxidant response induced by the formation of ROS.

1. Introduction

Shrimp are the most economically important aquaculture species in Thailand. However, a rapid increase in shrimp production has led to increased infectious diseases, and especially those caused by viruses and bacteria. During infection, reactive oxygen species (ROS) are produced by the host immune system to assist in attacking and killing pathogens and for cell signaling. An infecting virus can take advantage of this kind of response as the oxidative stress can enhance viral replication and increase cell-to-cell transmission of virus (reviewed in [1–3]). For shrimp, apart from their response to viral infections, this ROS response is known to occur after exposure to environmental stress such as acidic or alkaline pH, extreme temperatures, high pressure, poor nutrition and pollution [4–8]. All organisms must maintain a reducing environment within their cells, to avoid accumulation of these reactive species such as peroxides and free radicals as they can damage essential components such lipids, proteins, carbohydrates and nucleotides [9].

Oxidative stress is linked to a reduction in survival and causes significant economic losses in the shrimp farming industry [10]. This occurs whenever the equilibrium between the rate of ROS production and ROS elimination is disrupted. Thus, an effective and rapid mechanism for eliminating ROS by antioxidant defense mechanisms is crucial for the maintenance of this equilibrium [11].

Prokaryotic and eukaryotic organisms, including shrimp, employ both enzymatic and non-enzymatic antioxidant defenses to remove oxidants and also to limit their formation and catalyze of O_2^- to H_2O_2 that is also toxic but can be readily removed by catalase and glutathione peroxidase [12,13]. Superoxide dismutase enzymes (SODs), glutathione-S-transferase, glutathione reductase, reduced glutathione and thioredoxin are known enzymatic and non enzymatic antioxidants for shrimp [5,14,15].

RACK1 (Receptor for Activated C Kinase-1) is a 36 kDa highly conserved cytosolic protein found in a diverse range of eukaryotes from plants to mammals. The importance of RACK1 is manifested by its functional roles in a variety of biological processes including signal transduction, cell development, cell growth, cell adhesion and cell survival [16]. It has also been reported to be involved in the host response to viruses such as to Epstein-Barr virus (EBV), and Adenovirus in humans, Walley dermal sarcoma virus (WDSV) in fish and White spot syndrome virus (WSSV) in shrimp [17–20]. A RACK1 ortholog, Cpc2 protein in fission yeast, is known to regulate cellular defenses against oxidative stress through the positive synthesis of stress responsive catalase, a detoxification enzyme induced by treatment with H_2O_2 [21]. When confronted with environmental stress, RACK1 functions as a mediator of the stress response. In response to certain types of stress such as hypoxia, heat shock and arsenite (type 1 stress), RACK1 assembles cytoplasmic stress gran-

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ules (SGs) that activate defense mechanisms. Type 2 stress, induced by such things as X-rays, genotoxic drugs and H_2O_2 results in RACK1 binding to stress-responsive MTK1 [22]. In addition, RACK1 overexpression protected the cardiomyocyte cell line H9c2 from H_2O_2-induced cell death [23].

We recently discovered a *Pm-RACK1* gene in an EST library of the black tiger shrimp infected with WSSV [20]. We showed that the mRNA of *Pm-RACK1* was expressed in all shrimp tissues but was up-regulated in the hepatopancreas, stomach and hemocytes during WSSV infection. Pm-RACK1 was identified as a specific cellular target protein for VP9, a nonstructural protein of WSSV. However, a role for the Pm-RACK1 in the shrimp oxidative stress defense mechanism remained to be demonstrated. Here, we have demonstrated such a role by showing protection against oxidative damage from H_2O_2 in *Escherichia coli* and *Spodoptera frugiperda* (*Sf9*) insect cells that over-express recombinant Pm-RACK1. We also demonstrate, *in vitro*, that purified recombinant Pm-RACK1 protein protects DNA from oxidative damage in a metal-catalyzed oxidation system. In addition, we show that *Pm-RACK1* expression increases in the hepatopancreas of *Penaeus vannamei* after it was exposed to an alkaline pH stress that is known to cause increased production of ROS.

2. Materials and methods

2.1. Animal collection and maintenance

Shrimp (*P. vannamei*), weighing 3–4 g were obtained from commercial shrimp farms in Thailand. The shrimp were checked for WSSV infection by using PCR technology [24]. Shrimp free from viral pathogens were reared in 50-liter tanks containing 30-liters of continuously aerated artificial saltwater at 10 ppt salinity and 20–22 °C. Prior to experimental use, they were acclimatized to laboratory conditions for 1 week and fed with commercial feed until 24 h before the experimental treatments began.

2.2. pH experiment

Experiments on pH stress were performed essentially as described by Wang et al. [7] but with the following modifications. Plastic aquaria (12) containing 10% seawater (pH 7.4) were prepared, and approximately 10 shrimp were placed in each aquarium. The pH was then raised to 9.3 in four aquaria by the gradual addition of 1 M each of Na_2CO_3 and $NaHCO_3$. It was reduced to 5.6 in another set of four aquaria by adding 1 M HCl. Measurement of the pH was accomplished using a 713 Metrohm pH-meter (Metrohm, Herisau). In each experiment, hepatopancreatic tissue was collected from the shrimp in each experimental group (pH 5.6, 7.4, and 9.3) at 0 and 24 h.

2.3. Quantitative real-time PCR

Total RNA was extracted from the hepatopancreas of shrimp after exposure to pH stress using TRIZOL reagent (Invitrogen). 1 μg of total RNA was reverse transcribed to synthesize cDNA by using the SuperScript™ First-Strand Synthesis System (Invitrogen) and random hexamers as primers following the manufacturer's recommendations. The targeted cDNAs were amplified in a reaction volume of 25 μl containing iQ™ SYBR® Green Supermix (Bio-Rad) on the MX3000P™ real-time PCR system (STRATAGENE®), 0.2 μM Pm-RACK1_F1 forward primer (5′-ATG GTC ACT TCG CCC TCT CT-3′), and 0.2 μM Pm-RACK1_R1 reverse primer (5′-CTT GAC AGC CTT GTC CCA TCC ACAT-3′) with 300 ng of DNA template. The *β-actin* gene was used as a control using the primers Actin_F1 forward primer (5′-GAC GAY ATG GAG AAG ATC TGG-3′ and Actin_R1 reverse primer (5′-AAG GCG TGG GGC AGG GCR TA-3′). The real-time PCR protocol consisted of 5 min at 95 °C followed by 40 cycles of 30 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C. All samples were run in triplicate and all the reactions were independently repeated twice to ensure reproducibility. In each 96-well plate, a standard curve was generated from a serial dilution of the cDNA of the target gene and was used to determine of the mRNA copy number of samples. For each time point, the amount of the target genes and a reference gene was determined. The level of the *Pm-RACK1* transcripts was normalized to the level of the *β-actin* gene transcripts. Statistical significance was determined via a one way ANOVA analysis (SPSS software, version 14.0). Values were considered to be significant at $p < 0.05$.

2.4. *Sf9* cell culture and transfection

The *Sf9* cells (Invitrogen) were seeded onto a 96-well plate (5×10^4 cells/well) and grown with insect express Sf9-S2 medium (PAA) overnight at 28 °C. The *Sf9* cells were transfected with 0.2 μg pcDNA4B-Pm-RACK1 and also some with an empty vector using TransFast™ Transfection reagent (Invitrogen) according to the manufacturer's instructions. Cells were disrupted with TRIZOL reagent at 24, 48 and 72 h after transfection. The total RNA was isolated, followed by RNase free DNase (Promega) treatment and an RT-PCR analysis was performed.

2.5. RT-PCR analysis

1 μg of total RNA was reverse transcribed using the SuperScript™ First-Strand Synthesis System (Invitrogen). Amplifications were obtained using the Pm-RACK1_F2 5′-GGA ATT CCA TGA ATG AGA GCT TAC AGCT-3′ and Pm-RACK1_R2 5′-CTT GAC AGC CTT GTC CCA TCC ACAT-3′ primers. PCR cycles were as follows: 1 cycle (94 °C for 5 min); 35 cycles (94 °C, 30 s; 55 °C, 30 s; 72 °C, 30 s) followed by an extension step (72 °C for 10 min). A shrimp *β-actin* gene was also amplified as a control using the primers Actin_F2 (5′-CAG ATC ATG TTY GAG ACC TTC-3′) and Actin_R2 (5′-GAT GTC CAC GTC RCA CTT CAT-3′). RT-PCR products were analyzed by electrophoresis in a 1.5% agarose gel.

2.6. H_2O_2 tolerance assay

For an *in vivo* oxidative stress bioassay, pQE-40-Pm-RACK1 and pQE-40 transformed bacteria (M15) were grown in Lauria-Bertani (LB) medium containing ampicillin and kanamycin. Cultures were then induced with 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and assayed for the expression of protein by 12.5% SDS–PAGE. An H_2O_2 survival assay was then carried out as described by Konola et al. [25] with the following modifications. Induced bacterial cells were diluted and grown with shaking to mid-exponential phase (OD_{600} of 0.4). Cultures were divided into five groups. In four groups, a thirty percent (w/w) H_2O_2 solution (Sigma) was added to each of four of the cultures to obtain 2.5, 5, 7.5 or 10 mM H_2O_2. The remaining cultures were not treated with H_2O_2. The cultures were incubated with shaking for 1 h and diluted with LB broth, plated onto LB agar plates, and the surviving cells were assessed by counting colonies after 18 h. Bacterial cells expressing DHFR were treated and plated similarly to serve as a control.

Another *in vivo* oxidative stress bioassay was carried out using *Sf9* insect cells transfected with the pcDNA4B-Pm-RACK1 plasmid or an empty vector. The transfected cells were incubated for 48 h and exposed or not exposed to 600 μM H_2O_2 for 24 h, this concentration has been detected in H9c2 cells [23]. Surviving cells were assessed using the MTT survival assay by adding a freshly prepared 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution followed by measurement of the absorbance on a microplate reader using a 630-nm filter.

2.7. Expression, solubilization and purification of Pm-RACK1

The methods for expression, solubilization and purification of Pm-RACK1 have been described by Favacho et al. [26] with the following modifications. The expression of recombinant pQE-40-Pm-RACK1 in *E. coli* M15 was induced by the addition of 0.5 mM IPTG. The cells were resuspended in lysis buffer (50 mM Tris–HCl, and 0.5 M NaCl, pH 8.0). The pellet containing inclusion bodies was solubilized in denaturing buffer B (0.01 M Tris–HCl, 0.1 M sodium phosphate buffer, and 8 M urea, pH 8.0). The His-tagged recombinant protein was purified using a Ni–NTA column and eluted by buffer B containing 100 mM EDTA and 2 mM β-mercaptoethanol. The protein was dialyzed against Tris buffer saline (TBS) and decreasing amounts of urea (8, 6, 4, 2, and 1 M). Then, two more dialysis steps were carried out against TBS and analyzed by 12.5% SDS–PAGE.

2.8. Metal-catalyzed oxidation assay

To demonstrate the ability of Pm-RACK1 protein to protect supercoiled DNA from metal-catalyzed oxidation (MCO), tests were performed essentially as described by Cheong et al. [27] with the following modifications. The pUC19 plasmid DNA was used as a substrate for detecting DNA cleavage protection. Approximately 200 ng of pUC19 plasmid DNA incubated with 4 μg purified Pm-RACK1 protein and the MCO system consisting of 3 μM $FeCl_3$ and 10 mM DTT. The reaction was carried out in a 20 μl volume for 6 h at 37 °C. Also 4 μg bovine serum albumin (Promega) was used as a control protein. The resulting reaction mixture was then analyzed by electrophoresis on a 0.8% agarose gel to examine DNA cleavage. The DNA band on agarose gel was stained with EtBr (5 μg/ml).

3. Results

3.1. Overexpression of Pm-RACK1 conferred resistance to H_2O_2 damage in E. coli

To assess if Pm-RACK1 itself can protect cells from oxidative stress induced by H_2O_2 in an *in vivo* assay, we monitored the expression of Pm-RACK1 and the DHFR (control vector) after induction with 0.5 mM IPTG as illustrated in Fig. 1A. Cells harboring a recombinant *Pm-RACK1* showed a significant increase in survival relative to those harboring a control vector when subjected to oxidative stress by exposure to increasing concentrations of H_2O_2 (Fig. 1B). The most significant improvement (90%) was with exposure to 2.5 mM H_2O_2 while improvements at 5 mM (50%) and 7.5 mM (6%) were less significant. There was no significant improvement at 10 mM H_2O_2. The survival of *E. coli* following treatment with a low dose of H_2O_2 was higher than at the higher doses of H_2O_2 because surviving cells induced RecA and RuvA proteins, critical factors that contribute to repair of the oxidative DNA damage. Exposure to the higher doses of H_2O_2 probably damaged many cellular constituents directly, including the Pm-RACK1 and decreased the efficiency of repair for oxidative DNA damage [25].

3.2. Overexpression of Pm-RACK1 conferred resistance to H_2O_2 damage in Sf9 cells

Overexpression of Pm-RACK1 in *Sf9* cells was clearly shown by RT-PCR when compared to control cells comprising untransformed cells, mock-transformed cells and cells transfected with an empty vector (Fig. 2A). The highest expression of Pm-RACK1 was found at 48 h after transfection. In the presence of a cell death-inducing amount of H_2O_2, added after 48 h of induction and left for a further

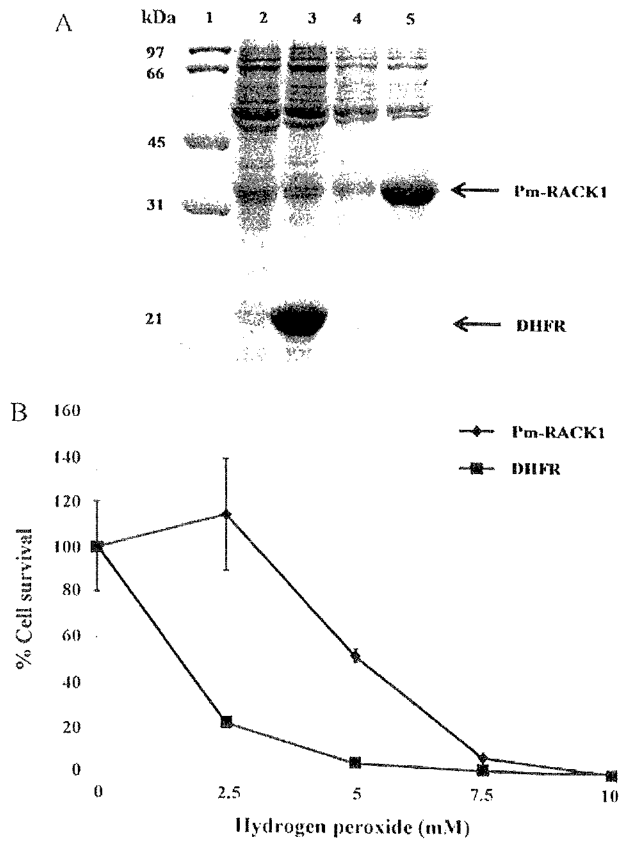

Fig. 1. Overexpression of Pm-RACK1 in *E. coli* cells that induced them to become more resistant to damage by H_2O_2. (A) The expression of the DHFR (control vector) and Pm-RACK1 protein was assessed by 12.5% SDS–PAGE: lane 1, protein molecular weight markers; lane 2 and 3, the expression of the control vector before and after induction; lane 4 and 5, the expression of Pm-RACK1 before and after induction. (B) Cells overexpressing DHFR or Pm-RACK1 were treated with H_2O_2 for a further 1 h and plated onto LB agar plates. After incubation of the plates for 18 h at 37 °C, growth of bacterial cells was evaluated by counting colonies. The percentage survival data are the mean of three experiments ± S.D.

24 h. The MTT assay revealed that the survival of cells overexpressing Pm-RACK1 increased approximately 25% relative to the survival of the control cells (Fig. 2B).

3.3. Expression and purification of Pm-RACK1

The His-Tag Pm-RACK1 fusion protein was induced in *E. coli* M15. Cells from the induced culture were suspended in lysis buffer. After cellular disruption, the recombinant Pm-RACK1 was associated with the insoluble fraction. The pellet was washed with buffer B (8 M urea) to disrupt the inclusion bodies. This treatment resulted in dissolution of the aggregated protein (Fig. 3A, lane 2), which was then applied to the Ni–NTA agarose bead for purification. The recombinant Pm-RACK1 was eluted from the column still in its denatured form (Fig. 3A, lanes 6–8). It was then allowed to refold during dialysis (Fig. 3A, lane 9).

3.4. Metal-catalyzed oxidation assay

The role of Pm-RACK1 in protecting against ROS was reinforced by an *in vitro* assay for protecting DNA from oxidative-nicking in a metal-catalyzed oxidation (MCO) system. Recombinant Pm-RACK1 protein produced in *E. coli* and purified with a Ni^{2+}-column did restrict DNA degradation, while the radicals produced in the MCO system caused complete degradation of the control DNA in the presence or absence of BSA within 6 h of incubation (Fig. 3B). These

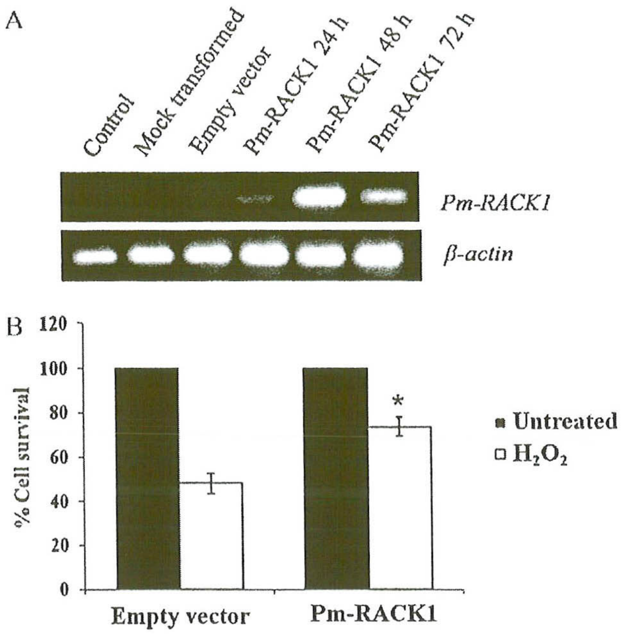

Fig. 2. Overexpression of Pm-RACK1 in *Sf9* cells. (A) The expression of *Pm-RACK1* mRNA was measured by RT-PCR in: *Sf9* cells (control), *Sf9* cells treated with a transfection reagent (mock transformed), *Sf9* cells transformed with pcDNA4B (an empty vector) and with pcDNA4B-Pm-RACK1 vector. Samples from cells with Pm-RACK1 were taken at 24, 48 and 72 h post-transfection. *β-actin* was used as an internal control for comparison of gene expression. (B) Pm-RACK overexpressing cells and empty vector cells were treated with 600 μM H_2O_2. MTT was added to the cells and absorbance was read at 630 nm. Data are presented as means with S.D. of percentage of viable cells in triplicate wells and control untreated cells with 100% viability. *$p < 0.05$ compared to H_2O_2 treated vector cells.

findings reveal that the Pm-RACK1 protein can function to inhibit MCO induced DNA degradation.

3.5. Effects of pH stress on Pm-RACK1 gene expression in shrimp

When shrimp were maintained at pH 7.4 (control) or exposed to an acidic or alkaline pH for 24 h. Real-time PCR, showed that the mRNA level of *Pm-RACK1* gene was highly expressed in the hepatopancreas of shrimp exposed to an alkaline pH but not to an acidic pH when compared to those held at pH 7.4 (Fig. 4).

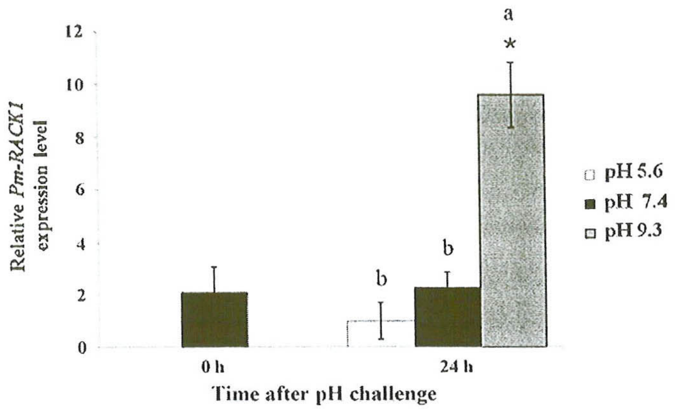

Fig. 4. Transcription of the *Pm-RACK1* gene in the hepatopancreas of *P. vannamei* exposed to pH extremes for 0 and 24 h as revealed by real-time PCR. Values shown are normalized expression levels (*Pm-RACK1* mRNA/*β-actin* mRNA). Significant differences ($p < 0.05$) in *Pm-RACK1* expression between the challenged and control groups (pH 7.4) are indicated by the letters (a, b). Significant differences ($p < 0.05$) in *Pm-RACK1* expression between challenged and untreated shrimp (0 h) are indicated by an asterisk. Each bar indicates a corresponding mean ± SD, $n = 3$.

4. Discussion

Several lines of evidence have revealed the involvement of an inducible oxidative stress response in the pathogenesis of viral infections, such as those by hepatitis B virus, hepatitis C virus, and the human immunodeficiency virus (HIV) [29]. Our previous studies have shown that *Pm-RACK1* is up-regulated in the hepatopancreas after WSSV-infection of shrimp [20].

However, the cellular function of Pm-RACK1 upon viral infection is not known. A study by Mohankumar and Ramasamy [30] demonstrated that WSSV infection induces oxidative stress via the release of ROS that are toxic to the cells. Here we have checked the possibility that the Pm-RACK1 is a cellular antioxidant required to prevent cell damage during exposure to oxidative stress. We have demonstrated that *E. coli* cells harboring Pm-RACK1 show substantial resistance to H_2O_2. This phenomenon was also observed in *Sf9* cells, when Pm-RACK1 was over-expressed in *Sf9* cells treated with H_2O_2. Previous studies have shown that enzymatic and non-enzymatic antioxidants such as ascorbic acid, catalase, alkylhydroperoxidase C, and translationally controlled tumor protein that catalyze the

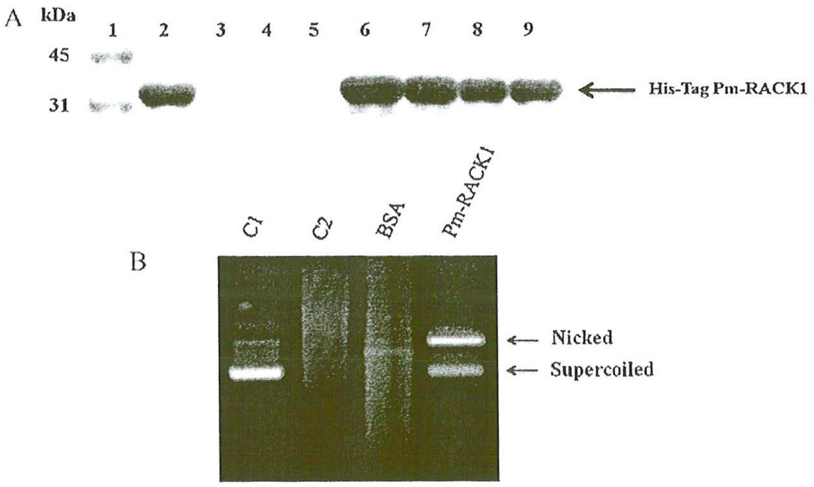

Fig. 3. Protection of DNA supercoiling. (A) 12.5% SDS–PAGE analysis of the purified Pm-RACK1 by Ni–NTA agarose after dissolution of the inclusion bodies: lane 1, protein molecular weight markers; lane 2, crude supernatant; lane 3, flow-through; lane 4–5, sequential column washings; lane 6–8, first, second and third elution of Pm-RACK1 protein from the column; lane 9, refolded-Pm-RACK1. (B) Agarose gel showing Pm-RACK1 protein, protecting supercoiled plasmid DNA against ROS degradation: (C1) 200 ng untreated pUC19 plasmid DNA, (C2) 200 ng pUC19 plasmid DNA incubated in the MCO system, (BSA) same as in C2 but with addition of 4 μg BSA and, (Pm-RACK1) same as in C2 but with addition of 4 μg purified Pm-RACK1.

conversion of H_2O_2 to H_2O and O_2, were efficient at scavenging H_2O_2 [4,12,31,32]. The results of *in vivo* studies may indicate that Pm-RACK1 is a non-enzymatic antioxidant that prevented cell death by protecting against the effects of H_2O_2 and minimizing the oxidative damage. The antioxidant role of Pm-RACK1 protein was reinforced by an *in vitro* assay for protecting DNA from an MCO system. In an MCO-based assay, Fe^{3+} ions in the MCO catalyze DTT oxidation to H_2O_2 and initiates a free radical chain and damage to plasmid DNA [32]. Pm-RACK1 protein showed a strong protective action against DNA degradation in the MCO assay system, and this is in agreement with previous observations with 2-Cys peroxiredoxin, alkylhydroperoxidase C, translationally controlled tumor protein, thioredoxin peroxidase, peroxiredoxin and a natural killer enhancing factor. These proteins are classified as natural antioxidant proteins [27,28,31–36]. Our *in vitro* results demonstrate for the first time that Pm-RACK1 protein can act directly as a natural antioxidant. A study with *P. vannamei* has revealed that the DNA of the hepatopancreas of shrimp exposed to acidic and alkaline pH stress became damaged, as a result of a respiratory burst and an increase in ROS production [7]. We therefore investigated the expression of *Pm-RACK1* in the hepatopancreas of shrimp exposed to acidic and alkaline pH stress for 24 h. The transcription level of *Pm-RACK1* mRNA was significantly increased after exposure to a high pH because this species is more sensitive to alkaline than acidic conditions [10]. A previous study in shrimp had also demonstrated that the expression of the antioxidants glutathione peroxidase and thioredoxin in the hepatopancreas was up-regulated compared to controls after 12 h exposure to a high pH stress that had resulted in increased ROS production from 3 h up to 24 h post exposure [7].

The present study provides strong evidence from both *in vivo* and *in vitro* work that Pm-RACK1 functions as a non-enzymatic antioxidant protein. This non-classical process of antioxidation activity is relatively poorly understood. The MCO-based assay indicates that the protein may operate in a sacrificial manner to inhibit the formation of free radicals [36]. The WD40-repeats found in Pm-RACK1 were reported to be involved in protein–protein interaction and signal transduction in the cell [37]. A stressed cell may activate different pathways involving many types of responsible proteins, depending on the type of stress and the cellular context.

Knockdown of RACK1 expression by short hairpin RNA inhibition (shRNAi) reduced MTK1 activation in response to cellular stress [22]. Knocking down RACK1 increased the cellular frequency of DNA breaks in Werner's syndrome, a rare autosomal disease [38]. Adenovirus E1A protein enhances the accumulation of ROS and impairs the cellular response to oxidative stress [39] by inhibiting the transcription of the Ferritin H gene. In a cell that overexpresses RACK1 can block the translocation of E1A into the nucleus and impede its function [40].

Further studies are required to investigate the physiological roles of Pm-RACK1 and maybe its binding protein in the pathway of oxidation and this contributes to the survival of organisms.

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