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Penaeus monodon Tudor staphylococcal nuclease preferentially interacts with N-terminal domain of Argonaute-1

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RNA interference (RNAi) plays a crucial role as an antiviral defense in several organisms including plants and invertebrates. An understanding of RNAi machineries especially protein components of the RNA-induced silencing complex (RISC) is essential for prior to applying RNAi as a tool for viral protective immunity in shrimp. Tudor staphylococcal nuclease (TSN) is an evolutionarily conserved protein and is one of the RISC components. In previous study, suppression of *Penaeus monodon* TSN (*PmTSN*) by double-stranded RNA (dsRNA) resulted in decreasing dsRNA-mediated gene silencing activity. To elucidate the functional significance of PmTSN in shrimp RNAi pathway, interactions between PmTSN and three Argonaute proteins (PmAgo) were characterized by yeast two-hybrid and *in vitro* pull-down assays. The results demonstrated that PmTSN interacted with PmAgo1, but not with PmAgo2 or PmAgo3. The interaction between PmAgo and PmTSN was mediated through the N-terminal domain of PmAgo1 and the SN1-2 domains of PmTSN. Analysis of the nuclease activity of the recombinant PmTSN indicated that PmTSN possessed calcium-dependent nuclease activity specific to single-stranded RNA (ssRNA), but not dsRNA and DNA. Knockdown of *PmAgo1* and *PmTSN* diminished the ability of dsRNA-Rab7 to knockdown *PmRab7* expression, indicating the involvement of PmAgo1 and PmTSN in shrimp RNAi pathway. Taken together, the results imply that PmTSN is one of the components of PmAgo1-RISC, thus providing new insights in the RNAi-based mechanism in shrimp.

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1. Introduction

Invertebrates employ RNAi as a major host-defense mechanism against viruses [1]. In *Drosophila melanogaster*, the dsRNA-replicative intermediate of viruses is recognized and cleaved by Dicer, generating viral-derived small RNA which is subsequently incorporated into RISC, and mediated the degradation of viral RNA [1–3]. In addition, mutation of the core RNAi machineries such as Dicer-2, Ago2, and R2D2 substantially increases susceptibility of the mutant flies to viruses, indicating the crucial role of RNAi as an antiviral immunity in invertebrates [4–7]. A number of evidences emphasized that dsRNA triggered an antiviral response in shrimp in both sequence-independent [8] and sequence-specific manners [9–12]. Even though, there are several evidences revealing the existence of RNAi, the mechanism by which the RNAi operates in shrimp cells is not yet completely understood. An understanding of the RNAi machineries especially the components of RISC will provide insights into an innate antiviral immunity and the host–viral interaction in this economically important species.

Tudor staphylococcal nuclease (TSN, also known as SND1 or p100) is an evolutionarily conserved protein which is composed of four tandem repeats of staphylococcal nuclease-like domains (SN), followed by Tudor and C-terminal SN domains. It is a multifunctional protein involved in a variety of cellular processes. For example, TSN can interact with several transcription factors to modulate their activities such as a transcriptional co-activator of Epstein–Barr virus nuclear antigen 2 (EBNA2), STAT5, STAT6, c-Myb, and Pim-1 [13–16]. In RNA splicing, TSN regulates small nuclear ribonucleoprotein (snRNP) assembly by interacting with SmB/B′ and SmD1/D3, the core proteins of snRNP complex [17]. In addition, cleavage of TSN by caspase-3 is important for the execution of apoptosis in plant and human [18]. Despite its roles in several biological processes as described above, Caudy et al., 2003 revealed that TSN also involved in RNAi pathway in *Caenorhabditis elegans*, *D. melanogaster*, and mammals. Biochemical fractionation of RISC from these organisms revealed that TSN was co-purified with other proteins of RISC including Ago, VIG, and FMRP, and also resided in miRNA-ribonucleoprotein complex (miRNP) [19]. In addition,

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suppression of *TSN* expression impaired the RNAi activity indicating the importance of this protein in the RNAi mechanism [19–21].

In previous study, we identified and characterized a TSN gene from the black tiger shrimp, *Penaeus monodon*. The *P. monodon* TSN gene (*PmTSN*) encoded a polypeptide of 889 amino acids with an estimated molecular weight of 99.7 kDa. It contained the four tandem repeats of SN domains followed by a Tudor and a partially truncated C-terminal SN domain. Silencing of *PmTSN* expression by dsRNA resulted in diminishing of the dsRNA-mediated gene silencing in shrimp [22]. In this study, to elucidate the functional insights of PmTSN as one of the components of RISC, we characterized the interaction between PmTSN and the catalytic engine of RNAi, PmAgo by using yeast two-hybrid (Y2H) and *in vitro* pull-down assays. In addition, the nuclease activity of the recombinant PmTSN was also investigated.

2. Materials and methods

2.1. Shrimp

The black tiger shrimps, *P. monodon* (~10 g body weight) were purchased from the commercial shrimp farms in Thailand. They were reared in the laboratory tanks with continuous aerated artificial sea water (10 ppt) for 2–3 days before processing to allow acclimatization, and were fed *ad libitum* with commercial shrimp feed. Apparently healthy shrimp free of yellow head virus (YHV) and white spot syndrome virus (WSSV) were selected and used in all experiments.

2.2. Plasmid construction

The plasmids pGEM-T containing coding regions of PmTSN (GenBank acc. no. JF429696), PmAgo1 (GenBank acc. no. DQ663629), PmAgo2 (kindly provided by Dr. Apinunt Udomkit, Mahidol University, Thailand), and PmAgo3 (GenBank acc. no. JX845575) were used as templates for PCR. VENT® DNA polymerase (New England Biolabs) was used for all PCR amplifications. For Y2H assay, the DNA fragment of PmTSN (amino acid residues 1–889) was amplified and cloned into *Nde*I/*Sal*I sites of pGBKT7 to create plasmid expressing GAL4-DNA-binding domain fusion protein (BD-TSN). To generate plasmids expressing GAL4-activation domain fusion protein of PmAgo (AD-Ago), the DNA fragments of PmAgo1 (amino acids residues 1–939) and PmAgo2 (amino acid residues 1–810) were amplified and cloned into *Nde*I/*Xho*I sites, and PmAgo3 (amino acid residues 1–825) was amplified and cloned into *Eco*RI/*Xho*I sites of pGADT7, respectively.

For *in vitro* pull-down assay, the DNA fragments of TSN (amino acid residues 1–889), SND (amino acid residues 1–660) and SN34 (amino acid residues 320–889) were amplified and cloned into *Xma*I/*Xho*I sites of pGEX-5X-1 to generate N-terminal GST-fusion constructs of PmTSN and its deletion mutants. For N-terminal His_6-tagged constructs, the DNA fragments of PmAgo1 (amino acid residues 1–939), NTD-Ago1 (amino acid residues 1–291), NPAZ-Ago1 (amino acid residues 1–409), PAZ-Ago1 (amino acid residues 274–409), PIWI-Ago1 (amino acid residues 402–939), PmAgo2 (amino acid residues 1–810), and PmAgo3 (amino acid residues 1–825) were amplified and cloned into *Eco*RI/*Xho*I sites of pET-28a(+). All primers used for cloning are listed in Table 1. All constructs were subsequently sequenced to confirm gene sequences and correct reading frames.

2.3. Yeast two-hybrid assay

Matchmaker GAL4 Two-hybrid System 3 (Clontech) was used for yeast two-hybrid studies, according to the manufacturer's protocol. *Saccharomyces cerevisiae* strains Y187 and AH109 were kindly provided by Dr. Saengchan Senapin (Biotec, Thailand). The yeast strain Y187 harboring pGBKT7-TSN was used for mating with the yeast strain AH109 harboring pGADT7-Ago1, -Ago2, or -Ago3. The mated cultures were selected on synthetic double dropout medium (SD/-L/-W). Protein interaction was further indicated on synthetic quadruple dropout medium (SD/-L/-W/-H/-A) containing X-α-Gal (40 μg/ml). Yeast harboring pGBKT7-TSN and pGADT7-Laminin receptor (AD-Lamr) served as a positive control whereas yeast harboring pGBKT7-TSN and empty pGADT7 served as a negative control. Preparation of yeast protein extracts was performed according to Yeast Protocol Handbook (Clontech). Expression of BD-TSN and AD-Ago in yeasts were confirmed before performing two-hybrid experiments using anti-c-Myc antibody and anti-HA antibody (US Biological), respectively.

2.4. Protein expression and purification

All GST-fusion and His_6-tagged constructs were expressed in *Escherichia coli* Rossetta (DE3) (Novagen). The recombinant GST-TSN, GST-SND, and GST-SN34 were expressed by using auto-induction approach [23]. Briefly, overnight cultures in non-inducing media ZYP-505 (1% N-Z-amine, 0.5% yeast extract, 50 mM Na_2HPO_4, 50 mM KH_2PO_4, 25 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.5% glycerol, and 0.05% glucose) were inoculated in auto-inducing media ZYZ-5052 (1% N-Z-amine, 0.5% yeast extract, 50 mM Na_2HPO_4, 50 mM KH_2PO_4, 25 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.5% glycerol, 0.05% glucose, and 0.2% lactose) containing ampicillin (100 μg/ml) and chloramphenicol (34 μg/ml) and incubated at 37 °C with shaking until A_{600} reached ~1.0. The cultures were then incubated at 18 °C for 20 h with shaking. For GST-fusion protein purification, cells were collected by centrifugation, resuspended in lysis buffer (PBS, pH 7.4, 0.2% Triton X-100, 1 mM DTT, 1 mM PMSF, and 100 μg/ml lysozyme) and lysed by sonication. Following centrifugation, the clear cell lysates containing GST-fusion proteins were filtrated and applied to the GSTrap FF column (GE Healthcare) for affinity purification. GST-TSN and GST-SN34 were further purified by anion-exchange chromatography using HiTrap Q column (GE Healthcare). On the other hand, GST-SND was further purified by cation-exchange chromatography using HiTrap SP column (GE Healthcare). The purified proteins were dialyzed against binding buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM DTT, 0.2% Triton X-100, 10% glycerol, 1 mM PMSF), concentrated by Vivaspin 6 (GE Healthcare), and determined the concentration by Bradford's assay.

Expression of His_6-tagged fusion proteins were induced by the addition of Isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.2 mM at A_{600} ~ 0.6 and proceeded for 6 h for His-Ago1 and its deletion mutants, or 20 h for His-Ago2 and His-Ago3 at 18 °C with shaking. Following centrifugation, the cells were resuspended in binding buffer at the ratio of 100 OD per ml, lysed by sonication, and centrifuged at 48,000× *g* for 30 min at 4 °C to separate inclusion bodies and cell debris. The clear cell lysates containing His_6-tagged proteins were then divided into small aliquots, flash frozen with liquid nitrogen, and stored at −80 °C until used.

2.5. In vitro pull-down assay

In vitro pull-down assay was performed according to Adachi et al. with some modifications [24]. Ten micrograms of the purified GST-fusion proteins or GST alone were immobilized on 30 μl of 50% (v/v) glutathione-agarose resin (Sigma) for 2 h at 4 °C with rotating. The resins were then washed three times with washing buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM DTT, 1% Triton X-100, 10% glycerol, 0.2 mM PMSF). Subsequently, the resins were incubated with the lysates of *E. coli* expressing His_6-tagged proteins for 4 h at 4 °C

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