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ORIGINAL ARTICLE

Characterization of the Interaction Between Arginine Kinase and siRNA

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Abstract RNAi, a crucial pathway in animals to defend against virus infection, is mediated directly by RNA-induced silencing complex (RISC) in an ATP-dependent manner. The RISC comprises one strand of short interfering RNA (siRNA) and multiprotein including Argonaute protein, which can cleave target RNAs. However, the proteins interacted with siRNA are not extensively explored. In this study, an antiviral siRNA (vp28-siRNA) targeting the vp28 gene of shrimp white spot syndrome virus was characterized. Based on the biotin/streptavidin affinity screening, it was found that the shrimp arginine kinase was specifically bound with the vp28-siRNA. The co-immunoprecipitation assays revealed that the siRNA was directly interacted with arginine kinase, suggesting that arginine kinase was an essential component of RNA-induced silencing complex. Therefore, our study presented a novel finding on the RISC components, which would be helpful to reveal the molecular events in the RNAi pathway.

Keywords RNAi · siRNA · Arginine kinase

Introduction

Over the past decade, the expanding researches and applications on RNA interference (RNAi) have revealed its crucial roles in gene expression regulation. At present, there is a common agreement that RNAi is widely involved in controlling gene expression and in defending the genome integrity against foreign nucleic acids or endogenous repetitive elements, retroelements, and transposons (Lippman and Martienssen 2004). RNAi is a gene-silencing process triggered by double-stranded RNAs (dsRNAs) (Fire et al. 1998), which can cause the cleavage of homologous mRNA (Schwarz et al. 2002). In RNAi, the short interfering RNAs (siRNAs), ~21–25-nt double-stranded fragments, serve as the core mediators of RNAi and are produced from dsRNAs by a dsRNA-specific RNase III family ribonuclease called Dicer (Bernstein et al. 2001; Elbashir et al. 2001; Tijsterman et al. 2002; Xie et al. 2004). The documented data reveal that the siRNA-mediated RNAi is regarded as a conserved innate antiviral immunity, which was firstly discovered in plants and subsequently in invertebrates (Waterhouse et al. 2001; Li et al. 2002; Ding and Voinnet 2007; Xu et al. 2007). It is found that the RNAi is employed as an antiviral immune response in *Caenorhabditis elegans* (Wilkins et al. 2005). In the marine shrimp, the viruses in the virus-infected shrimp can be completely eradicated by a siRNA (Xu et al. 2007), showing a strong antiviral activity of siRNA in invertebrates. Up to this date, the evidence demonstrates that RNAi serves as an important component of invertebrates' antiviral immunity (Ding and Voinnet 2007).

The siRNAs accompanied with the argonaute family proteins (Ago proteins) and some auxiliary proteins comprise the RNA-induced silencing complex (RISC) which directly mediates target gene degradation (Hammond et al. 2000). There is evidence that Ago2 is the core protein discovered in RISC which contains two conserved domains called PAZ and PIWI domains. The PAZ domain provides a binding site for siRNAs, and the PIWI domain, which is homologous with endonuclease, has been implicated in cleavage of RNA (Carmell et al. 2002). The dsRNA binding proteins can interact with dsRNAs even as short as 11 bp and play very important roles in the assembly of RISC. As a class of dsRNA binding proteins in human cells, the TAR RNA binding protein has been reported to function in the

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recruitment of Ago2 to the siRNAs complex (Chendrimada et al. 2005). In *Drosophila*, the R2D2 protein, which contains two dsRNA binding domains, is required for the incorporation of siRNAs into RISC (Liu et al. 2003; Schwarz et al. 2003; Khvorova et al. 2003). As documented, the process of RISC assembly includes sophisticated steps, in which many proteins are involved besides argonaute proteins. However, the proteins participated in the process of RNAi have not been extensively explored.

To address this issue, in the present study, an antiviral siRNA (vp28-siRNA) targeting the vp28 gene of shrimp white spot syndrome virus (WSSV) was characterized. The results showed that the siRNA was directly interacted with arginine kinase, suggesting that arginine kinase was an essential component of RNA-induced silencing complex. Therefore, our study presented a novel finding on the RISC components.

Materials and Methods

Shrimp Culture

Marsupenaeus japonicus shrimp, approximately weighing 5–6 g and length of 6–7 cm each, were raised at a group of 20 individuals in aerated seawater at 20 °C. Shrimp were maintained temporarily for 2–3 days prior to experiments. Three shrimp of each group were randomly selected for WSSV detection with WSSV-specific primers (5′-TATTGTCTCTCCTGACGTAC-3′ and 5′-CACATTCTTCACGAGTCTAC-3′) to ensure that the shrimp were virus-free before assays.

Screening for Proteins Bound with siRNA

As reported (Xu et al. 2007), the vp28-siRNA presented a strong antiviral activity. In this study, the vp28-siRNA was used to screen for proteins bound with siRNA. The sense strand of vp28-siRNA was labeled with biotin at hydroxyl of ribose sugar of 5′ end (Invitrogen, USA). Then, the biotinylated vp28-siRNA (sense strand 5′-biotin-A ACAU CACUGGUAUGCAGAUG-3′ and antisense strand 5′-CAUCUGCAUACCA GUGAUGUU-3′) was incubated with Streptavidin agarose resins (Thermo, USA) for 30 min at 4 °C in 0.1 M sodium phosphate buffer (0.077 mM Na_2HPO_4, 0.023 mM NaH_2PO_4, pH 7.4).

The shrimp tissues except for heart and digestive organs were homogenized in lysis buffer (0.1 M phosphate buffer, 120 mM NaCl, 0.5 % NP40, pH 7.4) containing protease inhibitor and ribonuclease inhibitor. After centrifugation at 13,000×*g* for 2 min, the supernatant was incubated with the biotinylated vp28-siRNA-coupled streptavidin agarose resins for 1 h at 4 °C in 0.1 M sodium phosphate buffer (pH 7.4). Streptavidin agarose resin only was used as control. The resin was washed with 0.1 M phosphate buffer for several times. Subsequently, the proteins bound with siRNA were eluted with 0.1 M citric acid (pH 3.0), followed by immediate neutralization with 1 M Tris–Cl (pH 8.0). The protein samples of eluates were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and then stained with Coomassie brilliant blue.

Mass Spectral Analysis

The proteins specifically bound with the vp28-siRNA were identified by mass spectrometry using the ultraflex TOF/TOF mass spectrometer (Bruker Autoflex, Germany). After separation by SDS–PAGE, the protein bands were excised and dehydrated several times with acetonitrile, followed by incubation with 10 mM DTT in 100 mM ammonium bicarbonate buffer at 57 °C for 60 min and subsequently with 55 mM iodoacetamide (Sigma) in 100 mM ammonium bicarbonate buffer at room temperature for 60 min. All in-gel protein digestions were performed using sequencing grade modified porcine trypsin (Promega, USA) in 50 mM ammonium bicarbonate buffer at 37 °C for 16 h. Then, the digests were subjected to the mass spectrometer. Data mining was performed using Mascot search engine against the NCBI database. A mass deviation of 100 ppm and modifications such as carbamidomethyl, oxidation, and Pyro-glu were usually allowed in the database searches.

Recombinant Expression of Arginine Kinase in *Escherichia coli* and Antibody Preparation

Total RNAs were extracted from shrimp using a commercial kit (Ambion, USA). Then cDNAs were created from total RNAs by reverse transcription with PrimeScript 1st Strand cDNA Synthesis Kit (Takara, Japan). The arginine kinase gene was amplified from cDNAs with primers 5′-CC*GGATCC*ATGGCTGACGCTGCTGTTAT TG-3′ (BamHI, italic) and 5′-TCT*CTCGAG*TCATCTCCT TCTCCATCTTGATGAGC-3′ (XhoI, italic), followed by its cloning into the pET-28 vector (Invitrogen, USA). The recombinant arginine kinase gene was expressed in *E. coli* BL21 (DE3) as a fusion protein with 6× His tag. The purified arginine kinase was used as antigen to immunize mice to prepare antibody. The titers of antisera were determined by enzyme-linked immunosorbent assay. The immunoglobulin fraction was purified by protein A-Sepharose (Bio-Rad, America) and stored at −70 °C before use.

Western Blot

After separation in a 12 % SDS–PAGE gel, the proteins were transferred to a nitrocellulose membrane (Amersham

Biosciences, UK). The membrane was immersed in phosphate-buffered saline containing 5 % nonfat dried milk at 4 °C overnight. Then, the membrane was incubated with the primary antibody and incubated with alkaline phosphatase (AP)-conjugated secondary antibody. Two hours later, the blot was detected with NBT/BCIP reaction.

RNA Immunoprecipitation Assay

RNA immunoprecipitation assay was performed to precipitate protein–nucleic acid complexes with the specific antibody against arginine kinase. The vp28-siRNA was synthesized in vitro using Transcription T7 Kit (Takara) as described previously (Xu et al. 2007). Subsequently, the vp28-siRNA was injected into shrimp at 50 μg per shrimp. Six hours later, the shrimp hemolymph was exsanguinated and centrifuged at 600×*g* for 5 min. The precipitate was dissolved in lysis buffer (0.077 mM Na_2HPO_4, 0.23 mM NaH_2PO_4, 120 mM NaCl, 0.5 % NP40, pH 7.4) containing protease inhibitor cocktail (Roche, Basel, Switzerland) and ribonuclease inhibitor (Takara). The antibody against arginine kinase was added to the magnetic Dynabeads Protein A (Invitrogen) and incubated with rotation for 10 min at room temperature. The anti-GST antibody was used as a control. After removal of the supernatant, the antibody-coupled Dynabeads Protein A was incubated with the lysis buffer containing shrimp hemolymph for 1 h at 4 °C, followed by washes with 0.1 M phosphate buffer. The samples were eluted from the dynabeads and subject to Northern blot and SDS–PAGE analyses.

Northern Blot Analysis

The total RNAs of the shrimp hemolymph or the immunoprecipitated samples were extracted using a commercial kit (Ambion, USA). The extracted RNAs were separated by electrophoresis on a 15 % non-denatured polyacrylamide gel in 1× TBE buffer (90 mM Tris–boric acid, 2 mM EDTA, pH 8.0). Then, the RNAs were transferred to a nylon membrane (Amersham Biosciences, UK) after alkaline denaturation. The blots fixed on the nylon membrane by UV cross-linking were probed with the digoxigenin (DIG)-labeled antisense strand of vp28-siRNA. The immunological detection was performed using the AP-conjugated anti-DIG antibody and NBT/BCIP reaction (Roche, USA).

Results

Screening for Proteins Bound with siRNA

In an attempt to obtain the proteins interacted with siRNA, the biotinylated vp28-siRNA was coupled with streptavidin to make an affinity column (Fig. 1a). Subsequently, the lysate of homogenized shrimp tissues was incubated with the vp28-siRNA column. The results demonstrated that five proteins were found to bind with the affinity column of vp28-siRNA in the elute (Fig. 1b). However, three of the five proteins were also found for the control (resin only; Fig. 1b), indicating nonspecific bindings of the three proteins. In this context, the data revealed that the two proteins with molecular masses of 40 and 50 kDa were specifically bound with the vp28-siRNA.

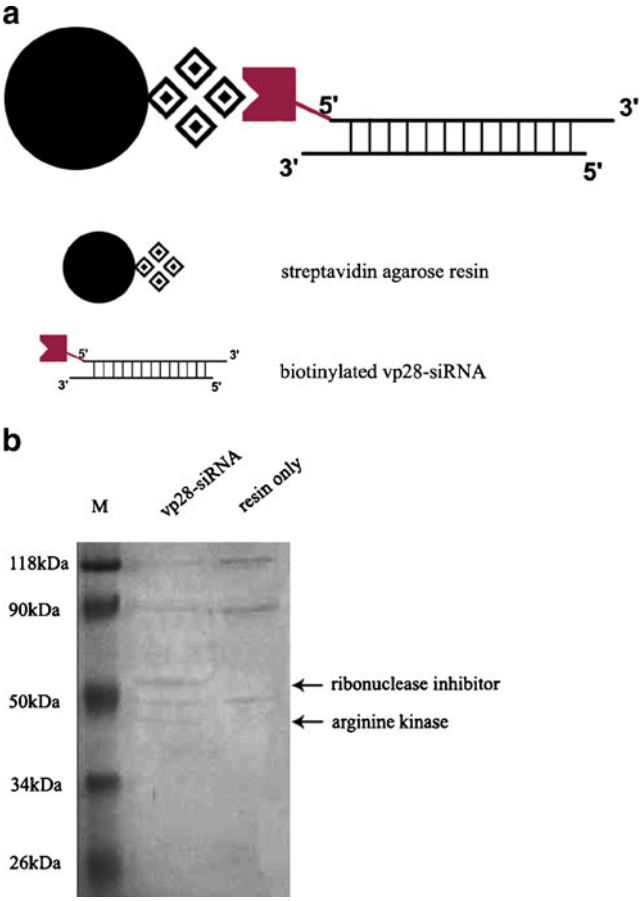

Fig. 1 Screening for proteins bound with siRNA. **a** Schematic diagram of the vp28-siRNA-coupled affinity column. **b** Proteins bound with vp28-siRNA. The lysate of homogenized shrimp tissues was incubated with the vp28-siRNA column. Then, the elutes were subjected to SDS–PAGE, followed by mass spectrometric identification of proteins. The agarose resin only was used as control. The lane headings showed the affinity columns. The proteins identified by mass spectrometry were indicated on the *right*. *M* protein marker

The mass spectrometric analysis showed the two siRNA-binding proteins were arginine kinase with 44 % peptide sequence coverage and ribonuclease inhibitor with 32 % peptide sequence coverage (Fig. 1c; Table 1), respectively. To protect the RNAs from degradation, in this study, the ribonuclease inhibitor was used. As well known, the ribonuclease inhibitor could form extremely tight complex with certain ribonuclease, and the latter could bind to RNAs. In

Table 1 Identifications of siRNA-binding proteins by mass spectrometry

Gene	Species	Molecular mass (kDa)	GenBank accession number	Mascot scores	Sequences of matched peptides (underlined)
Arginine kinase	*Marsupenaeus japonicus*	40	P51545	105	1 VDAAVLEKLQ AGFKKLEAAT DCKSLLKKYL SKDIFDKLKG QKTSLGATLL 51 DVIQSGVENL DSGVGIYAPD AEAYTLFAPL FDPIIEDYHV GFKQTDKHPN 101 KDFGDVSSFV NVDPEGQYVI STRVRCGRSM EGYPFNPCLT EAQYKEMQQK 151 VSSTLSSLEG ELKGTYFPLT GMSKEVQQKL IDDHFLFKEG DRFLQAANAC 201 RYWPAGRGIY HNDNKTFLVW VNEEDHLRII SMQMGGDLGQ VFRRLTSAVN 251 EIEKRIPFSH HDRLGFLTFC PTNLGTTVRA SVHIKLPKLA ANRDKLEEVA 301 GKYNLQVRGT RGEHTEAEGG IYDISNKRRM GLTEFQAVKE MQDGILQLIK 351 MEKEM
Ribonuclease inhibitor	*Sus scrofa*	51	P10775	74	1 MNLDIHCEQL SDARWTELLP LLQQYEVVRL DDCGLTEEHC KDIGSALRAN 51 PSLTELCLRT NELGDAGVHL VLQGLQSPTC KIQKLSLQNC SLTEAGCGVL 101 PSTLRSLPTL RELHLSDNPL GDAGLRLLCE GLLDPQCHLE KLQLEYCRLT 151 AASCEPLASV LRATRALKEL TVSNNDIGEA GARVLGQGLA DSACQLETLR 201 LENCGLTPAN CKDLCGIVAS QASLRELDLG SNGLGDAGIA ELCPGLLSPA 251 SRLKTLWLWE CDITASGCRD LCRVLQAKET LKELSLAGNK LGDEGARLLC 301 ESLLQPGCQL ESLWVKSCSL TAACCQHVSL MLTQNKHLLE LQLSSNKLGD 351 SGIQELCQAL SQPGTTLRVL CLGDCEVTNS GCSSLASLLL ANRSLRELDL 401 SNNCVGDPGV LQLLGSLEQP GCALEQLVLY DTYWTEEVED RLQALEGSKP 451 GLRVIS

this context, there might exist an interaction between arginine kinase and vp28-siRNA.

Identification of Protein Bound with siRNA

The mass spectrometric results showed that arginine kinase was directly bound with vp28-siRNA (Fig. 1). To confirm this binding, the arginine kinase gene of shrimp was cloned by RT-PCR. Then, it was expressed in *E. coli* BL21. After induction with IPTG, the results indicated that a protein band corresponding to the arginine kinase was observed in the induced recombinant bacteria, but not in the non-induced recombinant bacteria (Fig. 2a). The data presented that shrimp arginine kinase was expressed in bacteria. The recombinant arginine kinase protein was purified (Fig. 2a), which was subjected to antibody preparation. In order to evaluate the specificity of the antibody against arginine kinase, the shrimp hemolymph and the purified 6× His-arginine kinase were detected by Western blots with the anti-arginine kinase or anti-His antibodies, respectively. The results showed that the anti-arginine kinase antibody was highly specific (Fig. 2b). After separation on SDS–PAGE gel, the elute samples of the vp28-siRNA affinity chromatography were transferred to a blot and probed with the antibody against arginine kinase. The Western blots indicated that a protein corresponding to arginine kinase was probed, while no protein was detected for the control (Fig. 2c). The results showed that the arginine kinase was directly interacted with the vp28-siRNA.

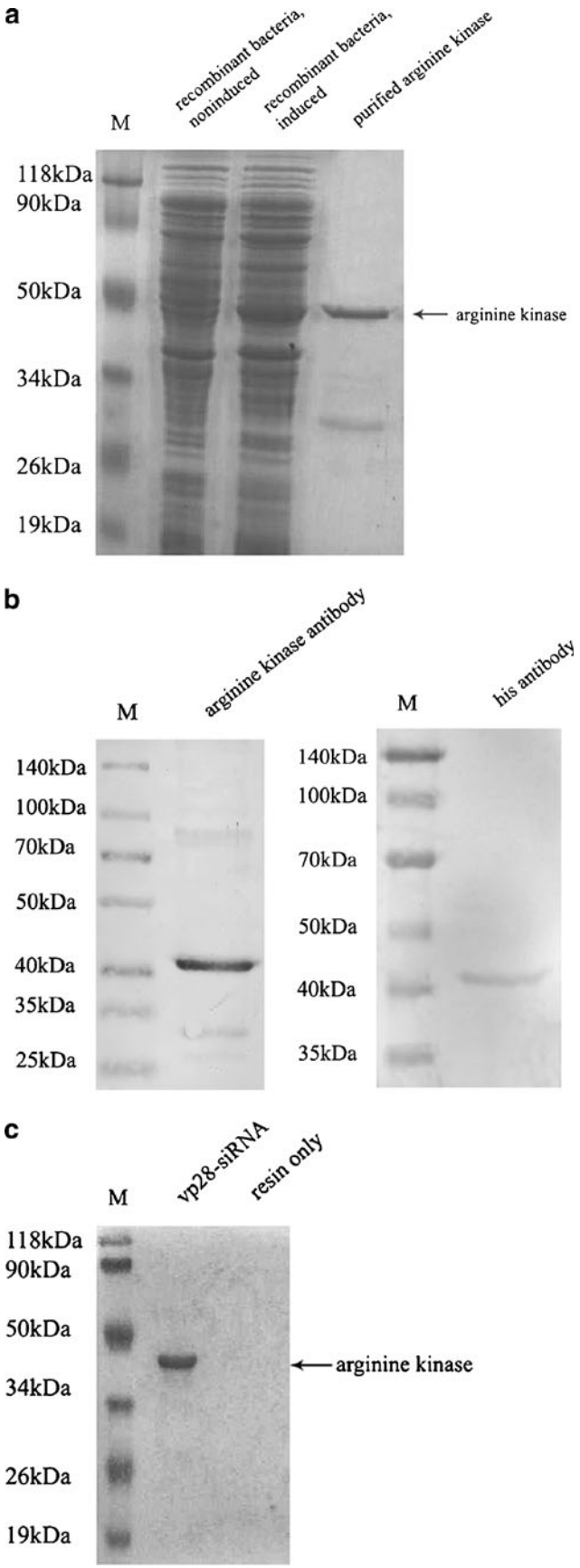

Fig. 2 The interaction between arginine kinase and vp28-siRNA. **a** SDS–PAGE of recombinant expression and purification of arginine kinase. The shrimp arginine kinase gene was expressed in *E. coli* BL21 (DE3) as a fusion protein with 6× His tag. After induction with IPTG, the recombinant protein was purified. The lane headings showed the treatments. *M* protein marker. **b** Specificity of the antibody against arginine kinase using Western blot analysis. The shrimp hemolymph was collected and subjected to Western blots using the anti-arginine kinase antibody (*left*). As control, the purified 6× His-arginine kinase was included in the detection with the anti-his tag antibody (*right*). The lane headings showed the antibodies used. **c** Western blots analysis of the elutes of affinity chromatography. The lysate of homogenized shrimp tissues was incubated with the vp28-siRNA affinity column. The resin only was used as control. Then, the elutes of affinity chromatography were probed with the antibody against arginine kinase. The headings indicated the affinity columns. *M* protein marker

Interaction Between vp28-siRNA and Arginine Kinase In Vivo

To evaluate the interaction between vp28-siRNA and arginine kinase in vivo, the vp28-siRNA was introduced into shrimp by injection, followed by RNA immunoprecipitation assay using the specific anti-arginine kinase antibody. The SDS–PAGE results showed that the arginine kinase of shrimp was precipitated by the anti-arginine kinase antibody, while no specific protein was obtained for the treatment of anti-GST antibody (Fig. 3a). When the precipitated protein–siRNA complexes by the anti-arginine kinase antibody or by the anti-GST antibody were probed with the antibody against arginine kinase, the Western blots showed that the arginine kinase was detected in the precipitated protein–siRNA complex treated with the anti-arginine kinase antibody (Fig. 3b). Based on the Northern blot analysis, it was found that the vp28-siRNA was detected in the protein–siRNA complex immune-precipitated by the anti-arginine kinase antibody, but not in the complex by the anti-GST antibody (Fig. 3c), indicating that the vp28-siRNA was directly bound with the arginine kinase in vivo.

Taking the above data together, it could be concluded that the vp28-siRNA was directly interacted with the arginine kinase in vivo, suggesting that arginine kinase contributed to the RNA-induced silencing complex (RISC).

Discussion

Since the discovery of siRNAs in the regulation of gene expression, the growing understanding of the mechanism about small RNAs has been obtained in the past decade. The siRNAs are widespread in eukaryotic kingdoms which can mediate a conserved antiviral mechanism in eukaryotes (Wang et al. 2006). It is evidenced that the mature siRNA and the Ago2 protein, comprising the RISC, serve as the core determinants of RNAi (Carmell et al. 2002). Except for the core Ago 2 protein in RISC, it is believed that there are

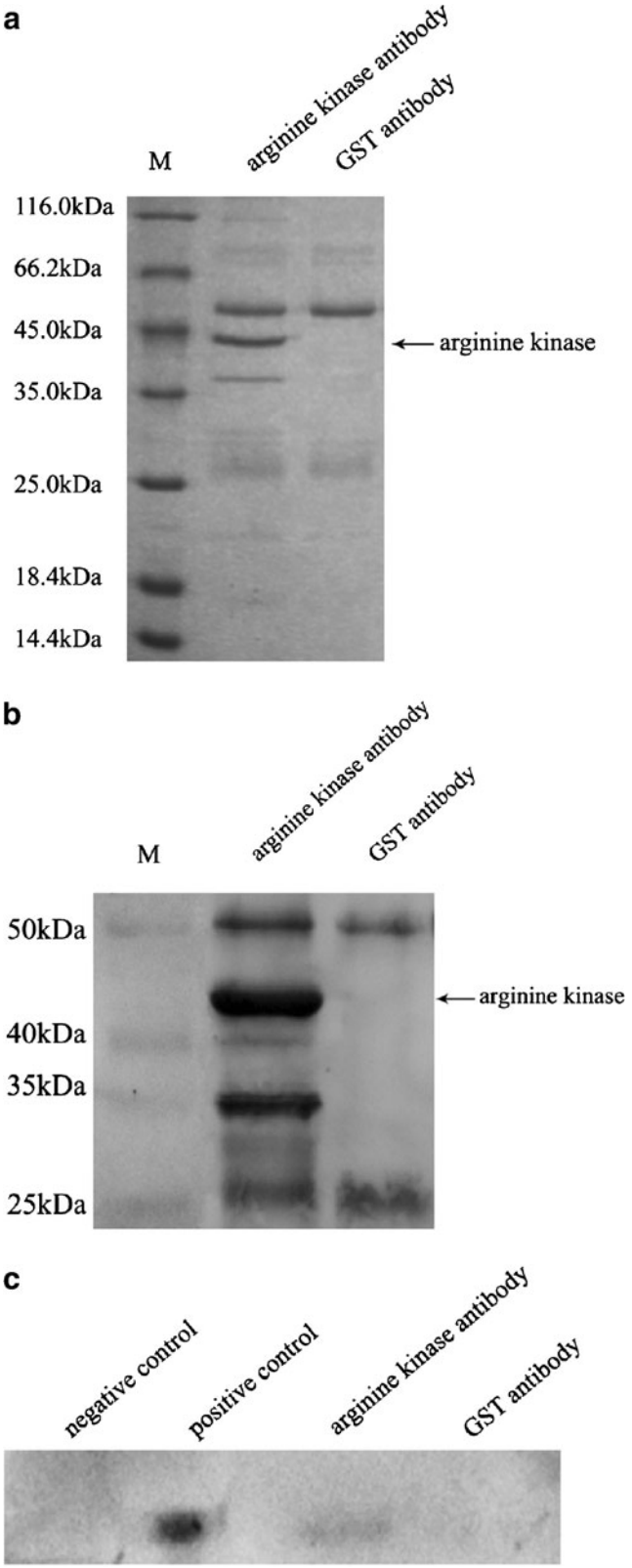

Fig. 3 The interaction between vp28-siRNA and arginine kinase identified in vivo. **a** SDS–PAGE of the protein–siRNA complexes immune precipitated by the antibodies against arginine kinase or GST as shown above every lane. The GST was used as control. *M* protein marker. **b** Detection of the protein–siRNA complexes by Western blot analysis using the antibody against arginine kinase. The lane headings indicated the antibodies used for immune precipitation. *M* protein marker. **c** Northern blot detection of the protein–siRNA complexes with the vp28-siRNA specific probe. The antibodies used for immune precipitation were shown *above* every lane. *Negative control* shrimp hemolymph without vp28-siRNA. *Positive control* shrimp hemolymph containing vp28-siRNA

many other proteins which are implicated in this complex. However, the RISC components, especially the siRNA binding proteins, are not well investigated so far. In the present study, the results showed that the siRNA was specifically bound with arginine kinase, suggesting that arginine kinase was an essential player in siRNA-mediated RNAi. Our study presented a novel component of RISC, which would be very helpful to reveal the molecular mechanisms of RNAi.

Arginine kinase, a class of transferases that catalyze the transferring of phosphorus-containing groups between arginine and arginine phosphate, plays a critical role in energy metabolism by maintaining ATP levels. This constituent arginine kinase/AP energy buffering system is widely distributed in the animal kingdom except for vertebrates. In vertebrates, this phosphagen system has evolved into the creatine kinase/phosphocreatine energy system (Ellington 2001). It is evident that the role of ATP in the RNA interference (RNAi) pathway is indispensable. The RNA interference including the incorporation of siRNAs into RISC and the subsequent unwinding of siRNA duplex is finished in an ATP-dependent manner in invertebrates (Nykanen et al. 2001). In human, although there exist arguments, it has been identified that ATP is required for siRNA duplex loaded into RISC but not necessary for the conversion of siRNA duplex into single strand (Yoda et al. 2010). In shrimp, arginine kinase is found to be involved in antiviral immunity, due to its significant upregulation in the WSSV-infected shrimp (Astrofsky et al. 2002). However, there is no comprehensive information available about the immune mechanism mediated by arginine kinase. In this context, the interaction between arginine kinase and siRNA, which was revealed in this study, implied that the arginine kinase might assist siRNA in executing its role in RNAi pathway to defend against virus infection through the unique and important phosphagen system existing in animals.

Considering the important and indispensible functions of arginine kinase in *M. japonicus* shrimp as revealed in this study, the interaction between arginine kinase and siRNA thus revealed a novel mechanism for the function of siRNA and implied the likelihood that arginine kinase might provide the energy in the process of siRNA-mediated RNAi pathway. Arginine kinase, which has evolved into creatine kinase in vertebrates (Ellington 2001), might illustrate the crucial function of this conserved protein in RNAi pathway

of vertebrates. The mechanism that the arginine kinase rendered its function in RNAi pathway still merited to be investigated in future studies.

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