Short communication

Cloning and expression of a transcription factor activator protein-1 (AP-1) member identified from manila clam Venerupis philippinarum

Luning Wu a,b,1, Lei Zhang b,1, Jianmin Zhao b,*, Xuanxuan Ning c, Changkao Mu a, Chunlin Wang a,**

a School of Marine Science of Ningbo University, Zhejiang, Ningbo 315211, PR China
b Key Laboratory of Coastal Zone Environmental Processes and Ecological Remediation, Yantai Institute of Coastal Zone Research, Chinese Academy of Sciences, Yantai 264003, PR China
Yantai Oceanic Environmental Monitoring Central Station, State Oceanic Administration, Yantai 264006, PR China

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A B S T R A C T
The transcription factor activator protein-1 (AP-1) proteins are implicated to play a major role in the regulation of numerous genes involved in the function and development of the immune system, cell differentiation, proliferation, apoptosis, etc. It can bind to promoter of its target genes in a sequence-specific manner to transactivate or repress them. In this study, the full-length cDNA of an AP-1 was identified from Venerupis philippinarum (denoted as VpAP-1) by EST analysis and RACE approaches. Phylogenetic analysis indicated that VpAP-1 had higher evolutionary conservation to invertebrate than vertebrate counterparts and should be a new member of the AP-1 protein family. Spatial expression analysis found that VpAP-1 transcript was most abundantly expressed in the hemocytes and hepatopancreas, weakly expressed in the tissues of the gills, mantle and muscle. After Vibrio anguillarum challenge, the expression of VpAP-1 transcript in overall hemocyte population was up-regulated in the first 6 h, and then decreased to 1.5-fold of the control group at 12 h. At time progressed, a second peak of VpAP-1 expression was detected at 24 h post-infection, which was 5-fold compared with that of the control group \( P < 0.01 \). After that, the expression level was sharply decreased and dropped to 0.5-fold of the control at 96 h. The above results indicated that VpAP-1 was perhaps involved in the immune responses against microbe infection and might be contributed to the clearance of bacterial pathogens.

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1. Introduction

The regulation of transcriptional activation or control is an essential and critical point in gene expression, which is mainly governed by a group of regulatory proteins, transcription factors (Moye-Rowley et al., 1988; Sun and Oberley, 1996). They bind to specific DNA sequence in the promoter region of the downstream target genes to regulate gene expression at both transcriptional and post-translational levels (Baeuerle, 1991). As one of the most important transcription factors identified from mammals, activator protein (AP-1) consists of Jun (c-Jun, JunB, JunD), Fos (c-Fos, Fra-1, Fra-2 and Fos B) and Atf (activating transcription factor) sub-families (Karin and Shaulian, 2001). In addition, AP-1 is composed of homo- or heterodimers of Jun-Jun, Jun-Fos, or Jun-Atf and categorized into the class of basic leucine zipper (bZIP) transcription factors (Vesely et al., 2009; Shaulian, 2010; Halazonetis et al., 1988).

AP-1 has been demonstrated to be involved in the regulation of host immune responses and plays a major role in the activation of numerous genes involved in the immune responses, cell differentiation, proliferation, apoptosis, etc. (Karin and Shaulian, 2001; Vesely et al., 2009; Angel and Karin, 1991). Moreover, AP-1 can regulate gene expression in response to a variety of stimuli, including cytokines, growth factors, stresses, and bacterial or viral infections. To our knowledge, most studies on AP-1 are focused on mammals and the model organisms, while the molecular features and expression pattern of AP-1 remain poorly investigated in mollusk. Molluscan AP-1 was identified from abalone Haliotis discus discus (De Zoysa et al., 2010) and Hong Kong oyster Crassostrea hongkongensis (Xiang et al., 2014).

Manila clam, Venerupis philippinarum, is an important marine bivalve for commercial fisheries, accounting for about 80% of mudflat fishery production in China. However, with the rapid development of intensive culture and environmental deterioration, clams are continuously confronted with infectious microorganisms, which have resulted in enormous economic losses to the clam aquaculture (Munroe and McKinley, 2007). Lacking the adaptive immune system, clams are supposed to depend solely on the innate immune system to defend invading microorganisms. AP-1 functions as a central transcription factor and link with various immune functional genes. The characterization of such immune regulatory components is helpful to better understand the
innate immune defense mechanisms of mollusk and to give new insights to health management and disease control of mollusk aquaculture. In the present study, the full-length cDNA of AP-1 was cloned from manila clam V. philippinarum, and the spatial and temporal expression patterns post Vibrio anguillarum challenge were investigated in order to provide clues on the immune regulation functions of VpAP-1.

2. Materials and methods

2.1. Clams and bacterial challenge

The clams V. philippinarum (shell length: 3.0–4.0 cm) were purchased from a local farm, Yantai of Shandong Province and maintained in aerated tanks at 20–22 °C for ten days before processing. The seawater (salinity 30‰) was changed 100% daily and the clams were fed with Isochrysis galbana and Platymonas helgolandica during the experiment.

V. anguillarum was inoculated in marine broth 2216E at 28 °C to OD600 = 0.6, and then centrifuged at 3000 g for 10 min to harvest the bacteria. The pellet was re-suspended in filtered seawater and adjusted to 10^7 CFU ml\(^{-1}\), which did not cause mortalities immediately, but induced significant immune responses in clam V. philippinarum. For the bacterial challenge experiment, 300 clams were employed and kept in six aerated tanks (50 individuals in each tank). One tank with untreated clams served as control, the other five tanks were exposed to V. anguillarum with final concentration of 10^7 CFU ml\(^{-1}\). Five individuals were randomly sampled at 6, 12, 24, 48, 72 and 96 h, respectively. 0.5 ml of hemolymphs from each individual (control and infected groups) was collected using a syringe and centrifuged at 4 °C for 10 min to harvest the hemocytes. The hemocyte pellet from each sample was immediately subjected to RNA extraction separately.

2.2. RNA isolation and cDNA synthesis

Total RNA was extracted using Trizol reagent according to the manufacturer’s protocol (Invitrogen). The cDNA first-strand synthesis was carried out based on Promega M-MLV RT Usage information (Promega) using total RNA treated with DNase I as template. Single-strand cDNA was synthesized from 1 μg of total RNA in a final volume of 20 μl containing 50 pmol of random hexamers, 50 mmol/l Tris–HCl, pH 8.3, 75 mmol/l KCl, 3 mmol/l MgCl2, 50 mmol/l DTT, 0.75 U of RNasin, 0.2 mmol/l each of dATP, dCTP, dGTP, and dTTP, and 100 U of MMLV reverse transcriptase (Promega). Reactions were incubated at 37 °C for 1 h, terminated by heating the mixture at 95 °C for 5 min, and subsequently stored at −80 °C. cDNA mix was diluted to 1:50 for subsequent experiment.

2.3. Cloning the full length cDNA of VpAP-1 gene

A cDNA library was constructed from the hemocytes of manila clam V. philippinarum. For the full-length cDNA cloning of AP-1 gene from V. philippinarum, using the ZAP-cDNA synthesis kit (Stratagene). Random sequences revealed that an EST of 498 bp was highly similar to the known AP-1 sequences. This sequence was then selected for further cloning of full-length cDNA of AP-1 gene from V. philippinarum (designated VpAP-1).

Based on the above EST sequence, the 3′ and 5′ ends of VpAP-1 cDNA were obtained by RACE approaches. Four specific primers, sense primers P1 and P2, and reverse primers P3 and P4 (Table 1) were designed based on the EST sequence, the nested PCR strategy was applied to clone the 5′ end of VpAP-1 using sense primers P1 and P2 and reverse primer P7, while sense primer oligo (dg)-adaptor and reverse primers

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5′–3′)</th>
<th>Sequence information</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>ACTAAACCCAGGCACTATA</td>
<td>3′-RACE primer</td>
</tr>
<tr>
<td>P2</td>
<td>TCACTCCCCCTGATTCACA</td>
<td>3′-RACE primer</td>
</tr>
<tr>
<td>T7</td>
<td>GTAATAC CACCTATACAGGCG</td>
<td>3′-RACE primer</td>
</tr>
<tr>
<td>P3</td>
<td>CTCCTGATTCCTGACCAA</td>
<td>5′-RACE primer</td>
</tr>
<tr>
<td>P4</td>
<td>TTCCTGATTCCTGACCAA</td>
<td>5′-RACE primer</td>
</tr>
<tr>
<td>Oligo(dG)-adaptor</td>
<td>GCGACGCGGTCGACTACG10</td>
<td>Real time VpAP-1 primer</td>
</tr>
<tr>
<td>P5</td>
<td>AATGTCAATGAAACTGACGAC</td>
<td>Real time VpAP-1 primer</td>
</tr>
<tr>
<td>P6</td>
<td>TGTGCAATGCAGCAGAGTCA</td>
<td>Real time actin primer</td>
</tr>
<tr>
<td>P7</td>
<td>TCTCCCTTGAGAAAGCTGAGTAC</td>
<td>Real time actin primer</td>
</tr>
<tr>
<td>P8</td>
<td>GATACCCAGACCTATCCATAC</td>
<td>Real time actin primer</td>
</tr>
<tr>
<td>M13-47</td>
<td>CCGAGGGGATTCCTGACGAC</td>
<td>Sequencing primer</td>
</tr>
<tr>
<td>RV-M</td>
<td>CAGCGGGAATACCTTACGAGC</td>
<td>Sequencing primer</td>
</tr>
</tbody>
</table>

P3 and P4 were used to get the 5′ end of VpAP-1. The PCR temperature profile was 94°C for 5 min followed by 32 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 1 min, and the final extension step at 72°C for 10 min.

Fig. 1. The nucleotide sequence (below) and deduced amino acid sequence (above) of VpAP-1. The nucleotide and amino acid positions were numbered on the left. The start and stop codons were in bold and the canonical polyadenylation signal sequence (AATAAA) was double-underlined. The shaded amino acids indicated the predicted Jun transcription factor domain. The basic leucine zipper (bZIP) domain was underlined.
10 min. The PCR products were cloned into the pMD18-T simple vector (TaKaRa) and sequenced bi-directionally with primers M13-47 and RV-M (Table 1). The sequencing results were verified and subjected to cluster analysis.

2.4. Sequence analysis of VpAP-1

The VpAP-1 cDNA sequence was analyzed by the BLAST algorithm at the National Center for Biotechnology Information (http://www.ncbi.

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Fig. 2. Multiple alignments of the VpAP-1 with other registered counterparts from Holoccephalus discus discus (ACJ65889), Harpegnathos saltator (EFN76477), Rattus norvegicus (NP_068607), Mus musculus (NP_053471), Bos taurus (NP_001071295), Callithrix jacchus (XP_002750926), Homo sapiens (CAC46525), Gallus gallus (NP_001026460), Solano salmo (ACN11435), Oncorhyncus mykiss (NP_001117883), Danio rerio (NP_956281), Saccoglossus kowalevskii (NP_001161502), Camponotus floridanus (EFNN6194) and Geis scapolopus (XP_002404571). The black shaded regions indicated identical amino acids among the different sequences, while the gray shaded regions represented conservative replacements. The bZIP domain was indicated by asterisks (*). Gaps were indicated by dashes to improve the alignment.
PCR. The \( \Delta \) values were determined for each sample. Difference in the RT-PCR. Two difference in the amount of template and the efficiency of the RT-PCR; the difference was called \( \Delta \Delta \)Ct. The expression level of VpAP-1 was normalized to that of muscle. As concerned to the internal control, called \( \Delta \)Ct. The value stood for an n-fold difference in the hemocytes from the clams in control group were used as the reference sample.

2.5. Tissue distribution and temporal expression profile of VpAP-1 transcript post Vibrio challenge

Total RNA was extracted from various tissues of five randomly healthy clams, including the muscle, gill, hemocytes, hepatopancreas and mantle. RNA isolation and cDNA synthesis were carried out as described above. cDNA mix was diluted to 1:100 and stored at \(-80^\circ\text{C}\) for subsequent SYBR Green assay.

The tissue distribution and temporal expression of VpAP-1 transcript in the hemocytes of clams challenged with V. anguilinarum were determined by quantitative real-time PCR (Q-PCR) as previously described (Zhang et al., 2011; Zhao et al., 2007). Two VpAP-1 gene-specific primers P5 and P6 (Table 1) were used to amplify a product of 96 bp from cDNA, and the PCR product was sequenced to verify the specificity of RT-PCR. Two \( \beta \)-actin primers, P7 and P8 (Table 1) were used to amplify a 121 bp fragment as internal control. The comparative Ct method was used to analyze the relative expression level of VpAP-1. The \( C_t \) for the target amplified products of VpAP-1 and internal control \( \beta \)-actin were determined for each sample. Difference in the \( C_t \) for the target and the internal control, called \( \Delta C_t \), was calculated to normalize the differences in the amount of template and the efficiency of the RT-PCR. The \( \Delta C_t \) for each sample was subtracted from the \( \Delta C_t \) of the calibrator; the difference was called \( \Delta \Delta C_t \). The expression level of VpAP-1 was calculated by \( 2^{-\Delta \Delta C_t} \), and the value stood for an n-fold difference relative to the calibrator (Livak and Schmittgen, 2001). For tissue distribution, VpAP-1 transcript levels in the hemocyte, mantle, gill and hepatopancreas were normalized to that of muscle. As concerned to temporal expression of VpAP-1, the hemocytes from the clams in control group were used as the reference sample.

Real-time PCR amplification was carried out in an Applied Biosystem 7500 fast real-time PCR system. Dissociation curve analysis of amplification was performed at the end of each PCR reaction to confirm that only one PCR product was amplified and detected. After the PCR program, data were analyzed using SDS software V2.01 (Applied Biosystems). To maintain consistency, the baseline was set automatically by the software. All data were given in terms of relative mRNA expressed as mean ± S.E. (N = 5). One-way analysis of variance (one-way ANOVA) was performed on all data using SPSS 13.0 statistical software, and P values less than 0.05 were considered statistically significant.

3. Results and discussion

3.1. cDNA cloning and structural analysis of VpAP-1

The full sequence of VpAP-1 cDNA consisted of 1692 bp (GenBank accession no. HQ918289), encoding a polypeptide of 274 amino acids with the predicted molecular mass of 30.12 kDa (Fig. 1). No signal peptide was identified from the deduced amino acid of VpAP-1, which indicated that VpAP-1 was a non-secreted protein as the other \( \alpha \)-APs. Jun proteins (components of AP-1) were found to contain an N-terminal transcriptional activation domain, a basic domain for DNA binding, and a C-terminal leucine zipper domain for dimerization (Sun and Oberley, 1996). SMART analysis revealed that the characteristic Jun transcription factor domain (1–185 aa) and the basic leucine zipper (bZIP) region (194–258 aa) existed in the VpAP-1 amino acid sequence. In addition, the triple alpha family or small cytokine CXC (SCY) motif (170–217 aa), interleukin 4 and 13 domains (123–244 aa) and helix–loop–helix (HLH) domain (205–258 aa) were also identified in the deduced amino acid sequence of VpAP-1. All these conserved characteristics indicated that VpAP-1 should be a new member of AP-1 family.

ClustalW analysis revealed that the deduced amino acid sequence of VpAP-1 shared high identity with previously identified AP-1s (Fig. 2). For example, VpAP-1 shared 75% identity to H. discus discus AP-1 (ACJ65689), 63% identity to Salmo salar AP-1 (ACN11435), 62% identity to Homo sapiens AP-1 (CAG46525), 45% identity to Saccoglossus kowalevskii AP-1 (NP_001161502) and 43% identity to Oncorhynchus mykiss AP-1 (NP_001161502). Multiple alignments clearly showed that the C-terminus of VpAP-1 was highly conserved, while the N-terminal domain was considerably variable (Fig. 2). Although the Jun domain was not well conserved at the N-terminus, the bZIP domain still exhibited relatively high similarity among all the sequences.

![Fig. 3. A phylogenetic tree constructed by the neighbor-joining method. The common names and the GenBank accession numbers were the same as follows: Crassostrea hongkongensis (AHF51977), Haliotis discus discus (ADQ43242), Zootermopsis nevadensis (KDR21486), Crassostrea gigas (EKC41210), Homo sapiens (NP_002219), Charadrius vociferous (KGL92897), Danio rerio (NP_956281), Xenopus (Silurana) tropicalis (XP_002931634) and P. sinensis (XP_006133565). Numbers next to the branches indicated bootstrap value of each internal branch in the phylogenetic tree nodes from 1000 replicates.](image-url)
3.2. Phylogenetic analysis of VpAP-1

To evaluate the molecular evolutionary relationships of VpAP-1 against other AP-1s, a phylogenetic tree was constructed using the neighbor-joining method. According to the phylogenetic tree (Fig. 3), the AP-1 members were mainly clustered into two groups by their invertebrate and vertebrate origin. VpAP-1 was firstly clustered with AP-1 from abalone, then formed a sister group with those from invertebrates and further grouped with those from vertebrates. The sequence alignment, structure comparison and phylogenetic analysis together favored that this protein was a member of AP-1 family.

3.3. Tissues distribution of VpAP-1

To examine the spatial expression pattern of VpAP-1, total RNA was isolated from the hepatopancreas, gill, muscle, hemocytes and mantle. The expression of VpAP-1 transcript was predominantly detectable in the tissues of hemocytes and hepatopancreas, and to a less degree in the tissues of gills, mantle and muscle (Fig. 4). The highest expression of VpAP-1 in hemocytes suggested the potential immune functions of this protein, since hemocytes were crucial for immunity defense not only by direct sequestration and killing of foreign invaders, but also by synthesis and exocytosis of bioactive molecules (Hoffmann et al., 1999; Tincu and Taylor, 2004; Roch, 1999). Furthermore, the hepatopancreas is also one of the important immune centers and detoxification organs in most species (Zhang et al., 2011); the enrichment of VpAP-1 mRNA in the hepatopancreas indicated that VpAP-1 might be involved in immunity defense. However, our results are inconsistent with previous research by De Zoysa et al. (2010), where the highest and lowest expression of abalone AP-1 transcript was found in the mantle (10.8-fold) and hepatopancreas (0.3-fold) respectively. The reasons for the inconsistency still required further investigation.

3.4. The temporal expression pattern of VpAP-1 in response to bacterial challenge

The expression level of VpAP-1 transcript in the hemocytes of clams post bacterial challenge was quantified by real-time RT-PCR with β-actin as internal control (Fig. 5). During the first 6 h, the expression of VpAP-1 mRNA was obviously up-regulated and reached 4-fold of the control group (P < 0.05). As time progressed, the expression level of VpAP-1 transcript decreased markedly to 1.5-fold of the control group at 12 h. A second peak of VpAP-1 expression was detected at 24 h post-infection, which was significantly up-regulated (5-fold, P < 0.01) compared with that in the control group. After that, the expression level was sharply decreased and dropped to only 0.5-fold of the original level at 96 h. One-way ANOVA analysis with control and challenged groups showed statistically significant difference in VpAP-1 gene expression at 24 h (P < 0.05), 48 h (P < 0.01) and 96 h (P < 0.05) post-infection. However, no significant difference was observed at other time points.

Several studies have illustrated that AP-1 could mediate the up-regulation or down-regulation of various regulators of apoptosis or cell survival, in order to protect organism and activate the downstream immune response (Foletta et al., 1998). Experimental evidence implicated AP-1 in different processes such as proliferation, differentiation, apoptosis, migration and wound healing, malignant transformation, and angiogenesis (Shaulian, 2006). Activation of AP-1 is essential for the initiation of immune responses to eliminate pathogens, while its activity must be tightly regulated because too strong or prolonged activation of AP-1 was seriously detrimental to the host (Foletta et al., 1998). In the present study, a very clear noticeable observation is that the higher expression of AP-1 transcripts is at 0–6 h phase and the lower number of transcripts is at 6–12 h phase. The increase of VpAP-1 mRNA in the early stage of infection is perhaps related to its roles in regulating the immune responses against Vibrio stimulation. The increase of VpAP-1 mRNA may result from the proliferation of hemocytes, which need to investigate the total hemocyte count (THC) after Vibrio challenge. From 6 h to 12 h, the significant decrease of VpAP-1 transcript was probably due to the neutralization in localized infection, which resulted in decrease of granular hemocytes infiltrated at the site of infection (Zhao et al., 2010). After this phase, the number of VpAP-1-producing hemocytes was recruited to supplement the infiltration hemocytes and protect the other location from potential hazard, leading the expression level of VpAP-1 to up-regulate and reach the peak at 24 h post-infection. Similar phenomena were also observed for abalone AP-1 transcripts post bacteria and virus stimulation (De Zoysa et al., 2010; Xiang et al., 2014). These results collectively suggested that AP-1 was perhaps involved in the immune responses of mollusks.

Acknowledgments

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References


