Molecular cloning and expression analysis of the ASC gene from mandarin fish and its regulation of NF-κB activation

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Abstract
Apoptosis-associated speck-like protein containing a CARD (ASC) is an adaptor protein that has a bipartite domain structure, an N-terminal PYRIN domain and a C-terminal caspase-recruitment domain (CARD). In this study, we cloned the mandarin fish ASC cDNA (mfASC), which consisted of 899 bp with a 115 bp 5′-UTR and a 181 bp 3′-UTR. The open reading frame encoded 201 amino acids. The mfASC shows 37% identity to an ASC orthologue from zebrafish. The mfASC has two protein–protein interaction domains, an N-terminal PYRIN domain and a C-terminal CARD domain. The mfASC gene structure was determined and had a length of 3954 bp with four exons separated by three introns. Northern blot analysis showed that mfASC mRNA is constitutively expressed in the head kidney, gill, hind kidney, spleen and intestine. *In vitro* studies, mfASC fused with green fluorescent protein appeared as a speck in the transfected 293T cells. When transiently overexpressed in 293T cells, mfASC inhibited NF-κB activity with or without tumor necrosis factor (TNFα) or lipopolysaccharide (LPS) stimulation.
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1. Introduction

Proteins containing the death domain fold (DDF), originally identified to be involved in apoptosis [1], have gradually also been found to participate in inflammatory responses [2]. These proteins exist ubiquitously in vertebrates and invertebrates and have been detected in the viruses.

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Presently, the DDF superfamily has four subfamilies: death domains (DDs), death effector domains (DEDs), caspase-recruitment domains (CARD) and the PYRIN domain. Although the sequences of these four motifs are very diverse, they have a similar structure fold with a six-helical bundle as revealed by nuclear magnetic resonance [2–4]. Interestingly, proteins with the same domain can take part in protein–protein interactions by self-association or with other proteins containing the same domain. Through protein–protein interaction, members of the DDF super-family regulate the delicate balance between cell survival and death via activation of the main effectors of NF-κB and caspase. As a consequence, more discoveries indicated that mutations or expression changes in the proteins containing DDFs could lead to serious diseases [5,6].

Apoptosis-associated speck-like protein containing a CARD (ASC) is an adaptor protein that has a bipartite domain structure, an N-terminal PYRIN domain and a C-terminal CARD domain [7,8]. From its structural features, it can be anticipated that ASC will play a key role in PYRIN and CARD-dependent pathways. First, ASC can mediate caspase-1 activation [9–13] and induce apoptosis [7,14–18] by DDs interaction. Second, as an intracellular adaptor protein, ASC plays a central role in the inflammasome, a complex of proteins that have distinct roles in innate defense systems [19–22]. With its PYRIN and CARD domains, ASC acts as a direct bridge between the sensor NACHT-, LRR- and pyrin domain containing proteins (NALPs) and the effector caspase-1 for the secretion of IL-1β [9,23]. Third, ASC works as a dual regulator that can either enhance or suppress NF-κB activity depending upon stimulation by tumor necrosis factor α (TNFα) or lipopolysacharide (LPS) [24]. Fourth, ASC was also named Target of Methylation-Induced Silencing-1 (TMS1). In many reports, methylation of the TMS1 CpG island was found to be a cancer-specific event in primary tumors, with corresponding normal tissues lacking TMS1 methylation [25–30]. Furthermore, during the host–pathogen interaction, ASC was reported to interact with poxvirus-encoded PYRIN domain protein and thus inhibit host inflammatory and apoptotic responses to infection [31].

ASC is well characterized in mammals, while in fishes there was only one report about ASC in zebrafish [32]. A PYRIN-containing protein, Caspy, from the zebrafish could interact with the zebrafish orthologue of ASC to induce specific proteolytic activation of Caspy and enhance Caspy-dependent apoptosis.

The mandarin fish, *Siniperca chuatsi*, is an important farmed fish species in China. In the last decade, spread of infectious spleen and kidney necrosis virus (ISKNV) has resulted in high mortalities of mandarin fish and caused significant economic losses in China [33–35]. The ISKNV-infected cells were characterized by hypertrophy in the spleen, kidney, cranial connective tissue, and endocardium and necrosis in hematopoietic tissues diffused in the spleen and kidney. To understand the virus–host interaction, forward and reverse suppression subtractive hybridization cDNA libraries were constructed in our lab from spleens of mandarin fish infected with ISKNV and an ASC homolog was identified [36]. In the present report, we cloned the ASC gene from mandarin fish and characterized its distribution in immune organs. We showed that transient overexpression of mandarin fish ASC cDNA (mfASC) in 293T cells inhibited NF-κB activity with or without TNFα or LPS stimulation.

2. Materials and methods

2.1. Fish and reagents

Mandarin fish were obtained from fish farms in Nanhai, Guangdong Province, China, and kept in an aquarium with fresh water at 28 °C for more than 1 week before the experiment. Experimental fish were randomly selected from these fish and kept individually, one 40 L aquaria uniform for one fish, at 28 °C. Tank water was passed through a sand-filter with a carbon layer and aerated before use.

2.2. Rapid amplification of cDNA ends (RACE)

An EST homologous to ASC was found using forward and reverse suppression subtractive hybridization cDNA libraries constructed from the spleens of mandarin fish infected with ISKNV [36]. Total RNA was extracted from spleen using the SV Total RNA Isolation System (Promega, USA) according to the manufacturer's instructions. RNA quality was assessed by electrophoresis on 1% agarose. Using high-quality total RNA as starting template, the 5′ and 3′ ends of the mfASC mRNA were amplified with a SMART RACE cDNA amplification kit (Clon-tech, USA) following the manufacturer's protocol. The PCR reaction was performed with gene-specific primers (5′ ASC RACE primer: 5′-GGTTCCACTAGCTTTCAGGCAACCAGAG-3′, 5′ ASC nest RACE primer: 5′-TGCCATGATATTTACACCAGTTGCTC-CA-3′, 3′ ASC RACE primer: 5′-GGGTCAGACGCAGCAGGGTGGA-GAG-3′ and 3′ ASC nest RACE primer: 5′-GGCTCTTCAGG-TGGCTGTGGAGTTATTG-3′) designed based on the known EST sequence and the RACE primers supplied in the kit. PCR products were gel purified and ligated into the T/A cloning vector pGEM-T Easy (Promega, USA) at 4 °C overnight and transformed into *Escherichia coli* DH5α competent cells. Positive clones were examined by PCR and sequenced.

2.3. Analysis of nucleotide and amino acid sequences

The nucleotide and deduced amino acid sequences of mfASC were analyzed in the Expasy search program. DNA and protein sequence comparisons were conducted with BLAST at the National Centre for Biotechnology Information Website (http://www.ncbi.nlm.nih.gov/BLAST/) and Scan-prosite programs (http://au.expasy.org/tools/scanprosite/). Multiple alignments were generated with the Clustal W program (http://www.ebi.ac.uk/clustalw/).

2.4. Genomic DNA

Genomic DNA was isolated from mandarin fish spleen using the Tissue DNA kit (Omega, USA). MfASC genomic DNA was amplified with primers F1 (5′-GCA GTT TGG GAT CTC TTG CTG AG-3′) and R1 (5′-GTT GTT CAG GTA GAG TGT ATG TTT GGT CT-3′), which were designed in the 5′- and 3′-UTR, respectively. PCR was performed with 30 cycles of 95 °C for 30 s, of 58 °C for 30 s, of 72 °C for 4 min, followed by a final

extension at 72 °C for 10 min. The PCR products were purified, cloned and sequenced as described above.

2.5. Northern blot analysis

For the tissue expression analysis, total RNA was isolated from the gill, liver, spleen, head kidney, hind kidney and intestine of mandarin fish using Trizol Reagent (Invitrogen, USA) as recommended in the manufacturer's instructions. Each sample of total RNA (15 ug) was loaded onto a 1% glyoxal-based agarose gel (Ambion, USA) and subjected to electrophoresis. Northern blotting was performed by vacuum transfer onto a positively charged nylon membrane using NorthernMax Transfer Buffer (Ambion, USA) and RNA was immobilized by UV cross-linking. MfASC-specific primers, F2 (5′-AAG AAT TCG AGA AAT GCC CCC CCA A-3′) and R2 (5′-GGC TCG AGA TTT ATT CTT GAG GTC AGC A-3′) for the full-length mfASC were used to obtain the transcription template for probe synthesis. PCR products were subcloned into pGEM-T vector (Promega, USA) with positive orientation, and the plasmid was linearized by digestion with the restriction enzyme *Eco*R I. The cRNA probe was transcribed *in vitro* with SP6 RNA polymerase following the manufacturer's protocol (Roche, USA). Probe was labeled with digoxigenin (DIG) according to the protocol supplied with the RNA DIG Probe Synthesis Kit (Roche, USA). Hybridization was performed overnight at 68 °C in ULTRAHyb hybridization buffer (Ambion, USA). The membrane was washed once in $2 \times$ SSC containing 0.1% SDS for 10 min at room temperature and twice in $0.1 \times$ SSC, 0.1% SDS at 68 °C for 15 min. Hybridized bands were detected with the DIG Detection Kit (Roche, USA) and CDP Star Luminescent (Roche, USA).

2.6. Expression plasmids

The entire open reading frame of mfASC was amplified by primers F2 and R2, inserted into pCDNA3.1-V5-His (Invitrogen, USA) at *Eco*R I and *Xho* I sites, and by primers F3 (5′-AAC TCG AGA TGC CCC CCC AA-3′) and R3 (5′-CCG GAT CCT TTA TTC TTG AGG-3′), inserted into pEGFP-C3 (Clontech, USA) at *Xho* I and *BamH* I sites. Both constructs were confirmed by sequencing. DNA constructs were transfected into cells using Lipofectamine 2000 reagent (Invitrogen, USA) according to the manufacturer's instruction.

2.7. Cell culture and subcellular localization analysis

HEK293T cells were cultured in DMEM supplemented with 10% heat-inactivated FBS (GIBCO, USA) at 37 °C in a 5% CO_2-humidified chamber. HEK293T cells were transiently transfected with pEGFP-C3-mfASC in a 12-well chamber slide. After 24 h, cells were fixed with ice-cold methanol and 50 ug ml^{-1} propidium iodide (PI, Sigma, USA) nuclear dye was added and then detected by confocal laser-scanning microscopy. Transfected 293T cells were lysed in SDS-loading buffer, subjected to 10% SDS-polyacrylamide electrophoresis and detected by Western blotting using anti-V5 and anti-GFP antibody. Anti-V5 antibody and anti-GFP antibody were purchased from Invitrogen (USA) and Beyotime (China), respectively.

2.8. NF-κB assay

About 2×10^4 HEK293T cells cultured in DMEM containing 10% (v/v) serum in 96-well plates were co-transfected with a total of 0.2 ug pcDNA3.1-V5-His empty vector or pcDNA3.1-V5-His-mfASC, including 1 ng of pRL-TK vector (Promega, USA) and 50 ng of pNF-κB-Luc or pTAL-Luc vector (Clontech, USA), using Lipofectamine 2000 reagent (Invitrogen, USA). At 24 h after transfection, cells were treated with 10 ng ml^{-1} human TNFα (Promega, USA) or 600 ng ml^{-1} LPS (*E. coli* O55:B5, Sigma, USA) for 8 h. Lysates were analyzed using the Dual Luciferase kit (Promega, USA). Significance of differences was analyzed by one-way ANOVA followed by Bonferroni's post hoc adjustment. All statistics were performed using SPSS program.

3. Results

3.1. cDNA cloning and sequence analysis of ASC

The full-length mfASC cDNA sequence consisted of 899 bp with a 115 bp 5′-UTR and a 181 bp 3′-UTR. There was one polyadenylation signal (AATAAA), found in 23 nucleotides upstream of the poly(A) tail. The open reading frame of mfASC cDNA encoded 201 amino acids with a predicted isoelectric point of 5.16 and a mass of 22.9 kDa (Fig. 1A). Analysis of the deduced protein sequence by the Scanprosite programs (http://au.expasy.org/tools/scanprosite/) showed that the mfASC contains a 90-residue N-terminal PYRIN and a 90-residue C-terminal CARD domain, the typical domain structure of ASC proteins (Fig. 1B).

3.2. Homology analysis of mfASC

The deduced amino acid sequence of mfASC was aligned with five reported proteins of zebrafish, human, mouse, rat and cattle (Fig. 2). The deduced amino acid sequence of mfASC exhibited 37% identity with zebrafish (203aa), 33% with human (195aa), 33% with mouse (193aa), 32% with rat (193aa) and 30% with cattle (195aa). The typical N-terminal PYRIN and C-terminal CARD domain in mfASC have been identified by SCANPROSITE. Residues with low identity mainly existed beyond these two domains. Secondary structure prediction (sec. str. pred.) indicated that these two domains have six α-helices which are common in these proteins interaction DDF superfamily [37,38]. There are 11 conserved hydrophobic residues: LEU10, LEU14, LEU17, PHE22, PHE25, LEU29, VAL38, ILE51, LEU55, LEU72, LEU84 in the PYRIN domain [39] and 15 conserved hydrophobic residues: VAL9, LEU16, VAL20, ILE23, ILE26, LEU27, LEU30, VAL35, ILE36, ILE44, LEU57, PHE72, LEU76, LEU83, LEU87 in the CARD domain (numbered based on the mandarin fish sequence).

3.3. Genomic organization

The mfASC gene has a length of 3954 bp with four exons separated by three introns. The gene structure of mfASC was found to be different from that of the *D. rerio* ASC, with five exons and four introns, and *H. sapiens* ASC, with three exons

A

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  1  tctttttccgggtgagaggggggacacatcactactggcgccttcttcggtcggactaacc
 62  tcgaagaaagactttggacaaccaggacggcagtttgggatctcttgctgagaaatgccc
                                                                  M  P
122  ccccaaaccataagaaaggctttagcagacatgctggaggacctgtcacagctggacttt
       P  Q  T  I  R  K  A  L  A  D  M  L  E  D  L  S  Q  L  D  F
182  gagaagttctgccgccagcttctggaccgcagagaagagcagcgggtcagacgcagcagg
       E  K  F  C  R  Q  L  L  D  R  R  E  E  Q  R  V  R  R  S  R
242  gtggagagtaaaagcttcttggatatcgcagacgtcctggtttcaacttttggcgagcct
       V  E  S  K  S  F  L  D  I  A  D  V  L  V  S  T  F  G  E  P
302  aatgctcttcaggtggctgtggaggtattgagacagattgactgcaacttggatgcagac
       N  A  L  Q  V  A  V  E  V  L  R  Q  I  D  C  N  L  D  A  D
362  agactcgttgaagagacatctgcactactctcaaaatctggttccagtgacactgcaaga
       R  L  V  E  E  T  S  A  L  L  S  K  S  G  S  S  D  T  A  R
422  ccctcagctggagcaactggtgtaaatatcatggcagatgacatacactttgtggataaa
       P  S  A  G  A  T  G  V  N  I  M  A  D  D  I  H  F  V  D  K
482  catagaacggcgctgatcaatagagtgagcaacattgcacccattctggatgagctcgtc
       H  R  T  A  L  I  N  R  V  S  N  I  A  P  I  L  D  E  L  V
542  gacaaaaatgtcatccaacaagagacctacgatagaatcaggattctgcctaccactcag
       D  K  N  V  I  Q  Q  E  T  Y  D  R  I  R  I  L  P  T  T  Q
602  gaaaggatgagggagctctactctggttgcctgaaagctagtggaacctgcaaagatatc
       E  R  M  R  E  L  Y  S  G  C  L  K  A  S  G  T  C  K  D  I
662  ttctacaaaatccttgaggaaaatgagaaatatctcattgctgacctcaagaataaataa
       F  Y  K  I  L  E  E  N  E  K  Y  L  I  A  D  L  K  N  K  *
722  atgtttcatgtaatttccatttttagaccaaacatacactctacctgaacaacaaaaat
782  aattatacattaataacggggatatattaatgctatgtctctgtggatttttcttaaaaa**a**
842  **ataaa**taaaaaacacttttttccactgtaaaaaaaaaaaaaaaaaaaaaaaaaaaaa 899
```

B

Fig. 1 Structure of mfASC. (A) cDNA (GenBank accession no AY909488) and deduced amino acid sequences of ASC in *Siniperca chuatsi*. The nucleotide residues are numbered on the left. (B) Schematic structure of mfASC. mfASC has two domains that are homologous to the N-terminal PYRIN domain (black box) and a C-terminal CARD domain (dark gray box).

and two introns (Fig. 3). It is 2509 bp larger than the human ASC gene, but 2426 bp smaller than the zebrafish ASC gene. When comparing mfASC with zebrafish ASC, the first exon of mfASC was obviously shorter by 18 bp than the first exon of zebrafish. Further analysis demonstrated that the second exon of mfASC was equivalent to a combination of these 18 bp with the second exon of zebrafish. The third exon of mfASC was corresponding to the third and fourth exons of zebrafish ASC. In the mfASC gene, the PYRIN domain was interrupted by the first intron, whereas in the zebrafish and human ASC gene, the PYRIN domain was completely encoded by the first exon. Typical GT/AG intron splice motifs were also identified flanking each intron.

3.4. Expression of the mfASC mRNA

Northern blot analysis was used to characterize the tissue specificity of mfASC mRNA using a DIG-labeled RNA probe. A single specific transcript of approximately 1 kb was found

to be constitutively expressed in various mandarin fish normal tissues (Fig. 4). The expression of mfASC transcript was predominantly detectable in the head kidney, and to a lesser degree in the gill, hind kidney, spleen and intestine. However, a very weak hybridization signal was detected in skin and liver.

3.5. Transient expression of mfASC

The characteristics of mfASC were ascertained by a transient expression experiment in 293T cells. The sub-cellular localization of mfASC was performed by fusing a GFP reporter to the N-terminus of mfASC (Fig. 5A). GFP-mfASC appeared as perinuclear speck-like aggregates in the transfected 293T cells. Western blots revealed a 26 kDa band in 293T cells transfected with pcDNA3.1-V5-His-mfASC and a 49 kDa band in 293T cells transfected with pEGFP-C3-mfASC (Fig. 5B), suggesting that recombinant mfASC and GFP-mfASC fusion proteins were expressed in 293T cells.
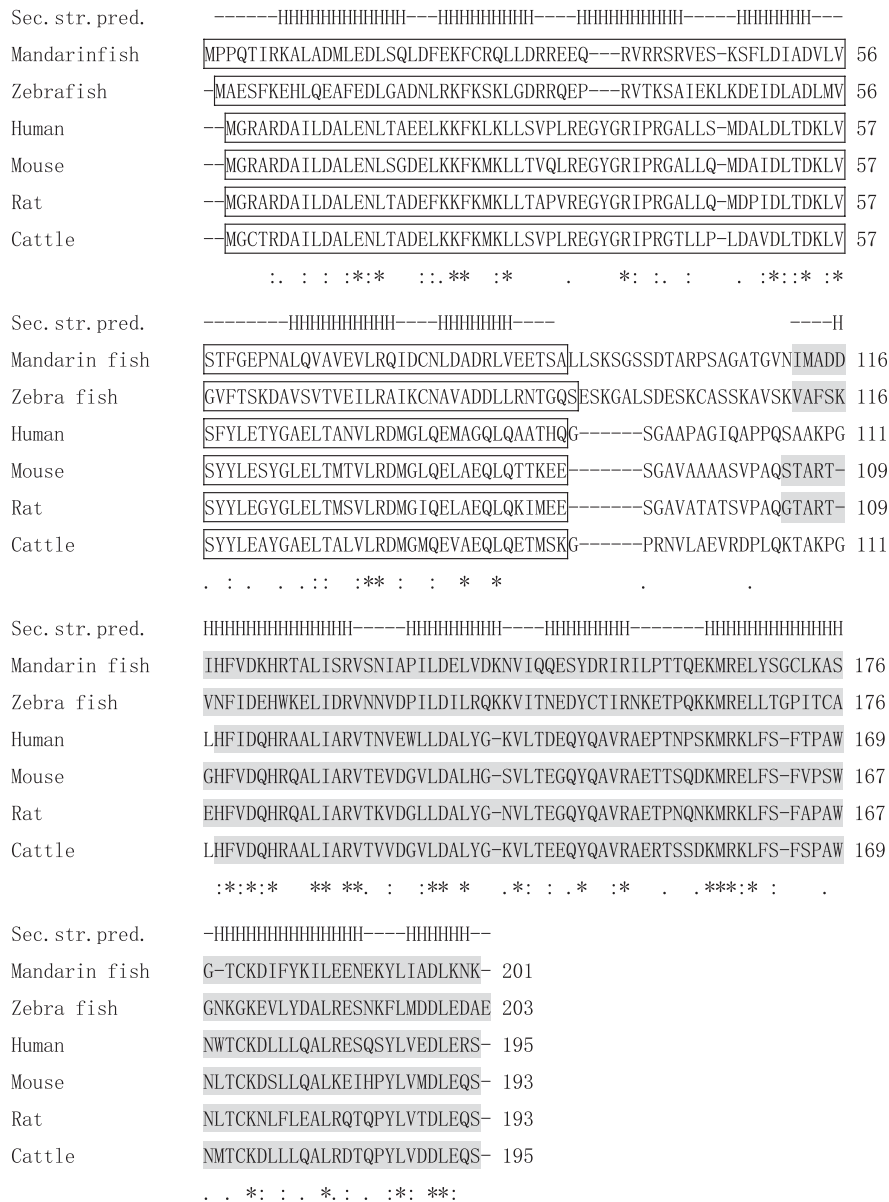
```
Sec. str. pred.      ------HHHHHHHHHHH---HHHHHHHH----HHHHHHHHHH-----HHHHHHH---
Mandarinfish    MPPQTIRKALADMLEDLSQLDFEKFCRQLLDRREEQ---RVRRSRVES-KSFLDIADVLV 56
Zebrafish      -MAESFKEHLQEAFEDLGADNLRKFKSKLGDRRQEP---RVTKSAIEKLKDEIDLADLMV 56
Human          --MGRARDAILDALENLTAEELKKFKLKLLSVPLREGYGRIPRGALLS-MDALDLTDKLV 57
Mouse          --MGRARDAILDALENLSGDELKKFKMKLLTVQLREGYGRIPRGALLQ-MDAIDLTDKLV 57
Rat            --MGRARDAILDALENLTADEFKKFKMKLLTAPVREGYGRIPRGALLQ-MDPIDLTDKLV 57
Cattle         --MGCTRDAILDALENLTADELKKFKMKLLSVPLREGYGRIPRGTLLP-LDAVDLTDKLV 57
                       :. : : :*:*  ::.**  :*     .    *: :. :   . :*::* :*

Sec. str. pred.      --------HHHHHHHHHH----HHHHHHH----                    ----H
Mandarin fish   STFGEPNALQVAVEVLRQIDCNLDADRLVEETSALLSKSGSSDTARPSAGATGVNIMADD 116
Zebra fish      GVFTSKDAVSVTVEILRAIKCNAVADDLLRNTGQSESKGALSDESKCASSKAVSKVAFSK 116
Human          SFYLETYGAELTANVLRDMGLQEMAGQLQAATHQG------SGAAPAGIQAPPQSAAKPG 111
Mouse          SYYLESYGLELTMTVLRDMGLQELAEQLQTTKEE-------SGAVAAAASVPAQSTART- 109
Rat            SYYLEGYGLELTMSVLRDMGIQELAEQLQKIMEE-------SGAVATATSVPAQGTART- 109
Cattle         SYYLEAYGAELTALVLRDMGMQEVAEQLQETMSKG------PRNVLAEVRDPLQKTAKPG 111
                . :. . ..:: :**: : * *          .          .

Sec. str. pred.      HHHHHHHHHHHHHH----HHHHHHHHHH----HHHHHHHH-------HHHHHHHHHHHHH
Mandarin fish   IHFVDKHRTALISRVSNIAPILDELVDKNVIQQESYDRIRILPTTQEKMRELYSGCLKAS 176
Zebra fish      VNFIDEHWKELIDRVNNVDPILDILRQKKVITNEDYCTIRNKETPQKKMRELLTGPITCA 176
Human          LHFIDQHRAALIARVTNVEWLLDALYG-KVLTDEQYQAVRAEPTNPSKMRKLFS-FTPAW 169
Mouse          GHFVDQHRQALIARVTEVDGVLDALHG-SVLTEGQYQAVRAETTSQDKMRELFS-FVPSW 167
Rat            EHFVDQHRQALIARVTKVDGLLDALYG-NVLTEGQYQAVRAETPNQNKMRKLFS-FAPAW 167
Cattle         LHFVDQHRAALIARVTVVDGVLDALYG-KVLTEEQYQAVRAERTSSDKMRKLFS-FSPAW 169
                       :*:*:*   ** **.. :  :** *   .*: : .* :*   .  .***:* :    .

Sec. str. pred.      -HHHHHHHHHHHHHHH----HHHHHH--
Mandarin fish   G-TCKDIFYKILEENEKYLIADLKNK- 201
Zebra fish      GNKGKEVLYDALRESNKFLMDDLEDAE 203
Human          NWTCKDLLLQALRESQSYLVEDLERS- 195
Mouse          NLTCKDSLLQALKEIHPYLVMDLEQS- 193
Rat            NLTCKNLFLEALRQTQPYLVTDLEQS- 193
Cattle         NMTCKDLLLQALRDTQPYLVDDLEQS- 195
                . . *: : . *.: . :*: **:
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Fig. 2 Multiple sequence alignment of ASC proteins. Multiple sequence alignment of ASC proteins from mandarin fish, zebrafish, human, mouse, rat and cattle. The alignments were conducted using the Clustal W program. (*) Fully conserved residues, (:) conservation of strong groups and (.) conservation of weak groups. Amino acid numbering is indicated on the right. Secondary structure prediction (sec. str.pred.): H, α-helix, the deduced PYRIN and CARD domains were highlighted in box and shading. Genebank accession numbers are: ASC: mandarin fish (AY909488); zebrafish (Q9I9N6); human (NP_037390); mouse (NP_075747); rat (NP_758825) and cattle (NP_777155).

3.6. mfASC inhibits NF-κB activity in response to TNFα and LPS stimulation

To study the regulation of NF-κB activation by mfASC, we overexpressed the mfASC in 293T cells by transiently transfecting the pcDNA3.1-V5-His-mfASC. We noticed that TNFα-induced NF-κB activity increased almost 3-fold compared to unstimulated in 293T cells, but LPS had no effect on NF-κB activity (Fig. 6). The expression of mfASC reduced ~30% NF-κB activity in TNFα and LPS stimulated cells, and also decreased NF-κB activity in the unstimulated cells. Reduction of NF-κB activity in cells that express recombinant mfASC was significant ($P < 0.05$) compared to control cells that did not express mfASC.

4. Discussion

In recent findings, proteins containing a CARD and PYRIN domain have played a key role in innate immune response [20,40–42]. ASC is one of the only two genes in the human genome encoding proteins that contain both PYRIN and CARD domains. ASC was first identified in 1999 as a speck that appeared during apoptosis in HL-60 cells [7]. In 2001,
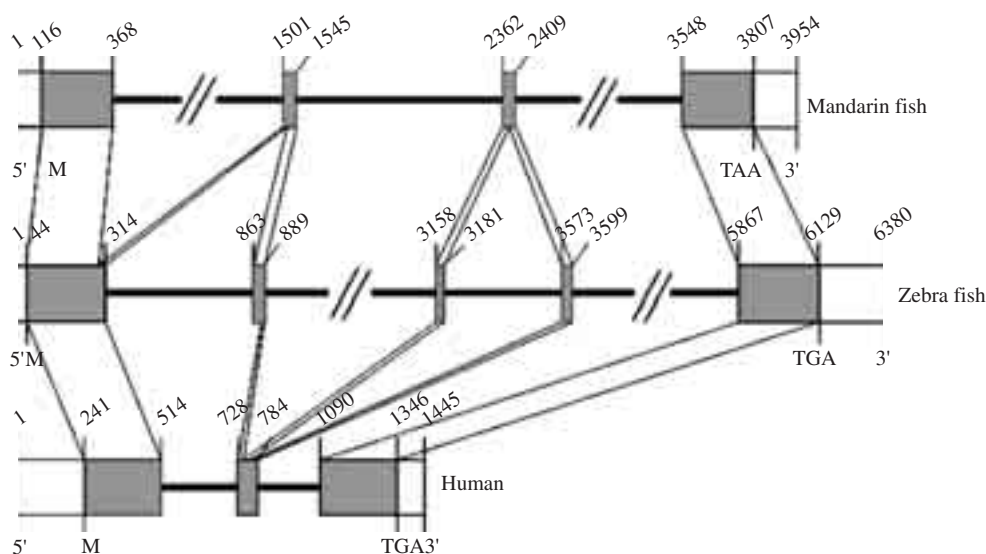
Fig. 3 Genomic structure of the mandarin fish ASC gene compared with zebrafish and human ASC gene. Black boxes represent exons while solid lines between them indicate introns (larger than 1 kb are interrupted by two lines), the white boxes represent untranslated regions (UTRs). The homological regions of exon are indicated by lines between the different species. Genebank accession numbers are: mandarin fish (EF564193); zebrafish (NC_007127) and human (NC_000016).
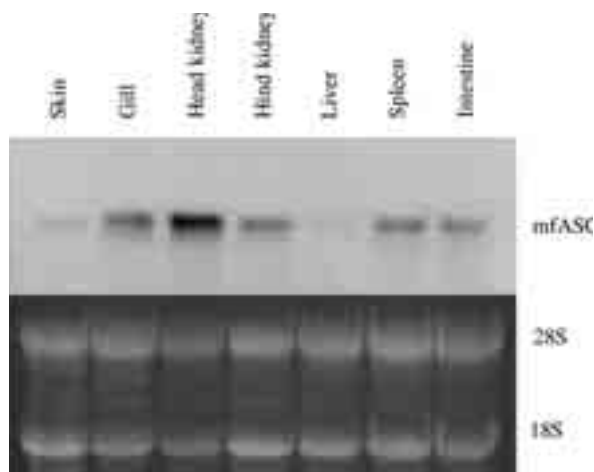

Fig. 4 Northern blot analysis of mfASC mRNA in mandarin fish normal tissues. 15 ug of total RNA was run on a 1% glyoxal-based agarose gel (Ambion) and blotted onto nylon membranes. The membrane was hybridized with DIG-labeled mfASC RNA probe and detected using chemiluminescence. The lower panel shows ethidium bromide stained ribosomal RNA.

the murine orthologue of ASC was cloned and it appeared as a speck due to self-association [8]. Since then, more and more researches have focused on the role of this protein in apoptosis and the inflammatory response. In fish, ASC has been sequenced and reported only in zebrafish [32]. Therefore, more information on genomic structure and regulation of protein expression is required to better understand the immune defense mechanism of ASC in fish.

The genomic organization of the ASC gene is different among the species (Fig. 3). More introns were found in the ASC genes of zebrafish and mandarin fish than in mammals. It is generally known that the exon–intron structure of

orthologous genes is highly conserved through vertebrate species. It was also found that introns were lost during evolution [43,44].

The expression pattern of mfASC was different from that of human and mouse ASC gene. In humans, ASC mRNA had a high level of expression in the spleen and was expressed normally in liver [7]. In mouse, there was a high level of expression in the small intestine, but very low expression in the kidney and liver [8]. The expression pattern of zebrafish ASC was only detected by *in situ* hybridization in the embryonic development. At the embryonic development stages of 48 and 72 h post-fertilization, zebrafish ASC mRNA was detected primarily in the epidermis, mouth and pharyngeal arches [32]. Noticeably, mfASC was found to be constitutively expressed in the head kidney, spleen, hind kidney, gill and intestine, suggesting it may play an important role in the mandarin fish immune system.

To investigate *in vitro* functions of mfASC, we expressed mfASC in the human 293T cells that do not express ASC [24]. Bright perinuclear speck-like signals were detected in living 293T cells after transient transfection with plasmids containing mfASC fused to GFP. Expressions of recombinant V5-His-tagged mfASC (26 kDa with 23 kDa of mfASC and 3 kDa of V5-His tag) and mfASC-GFP fusion protein (49 kDa with 23 kDa of mfASC and 26 kDa GFP) were confirmed by Western blotting. These results indicate that mfASC, like human and mouse ASC proteins, has the ability to aggregate via the PYRIN and CARD domains [7,8]. This aggregation is very important for the function of ASC as an adaptor protein in the inflammasome [10,13]. It was reported that some proteins containing CARD or PYRIN domains could modulate ASC in caspase-1 activation by interrupting this speck aggregating ability [10,45].

The role of ASC in the activation of the NF-κB pathway remains somewhat controversial. ASC could inhibit NF-κB activation by various proinflammatory stimuli, including
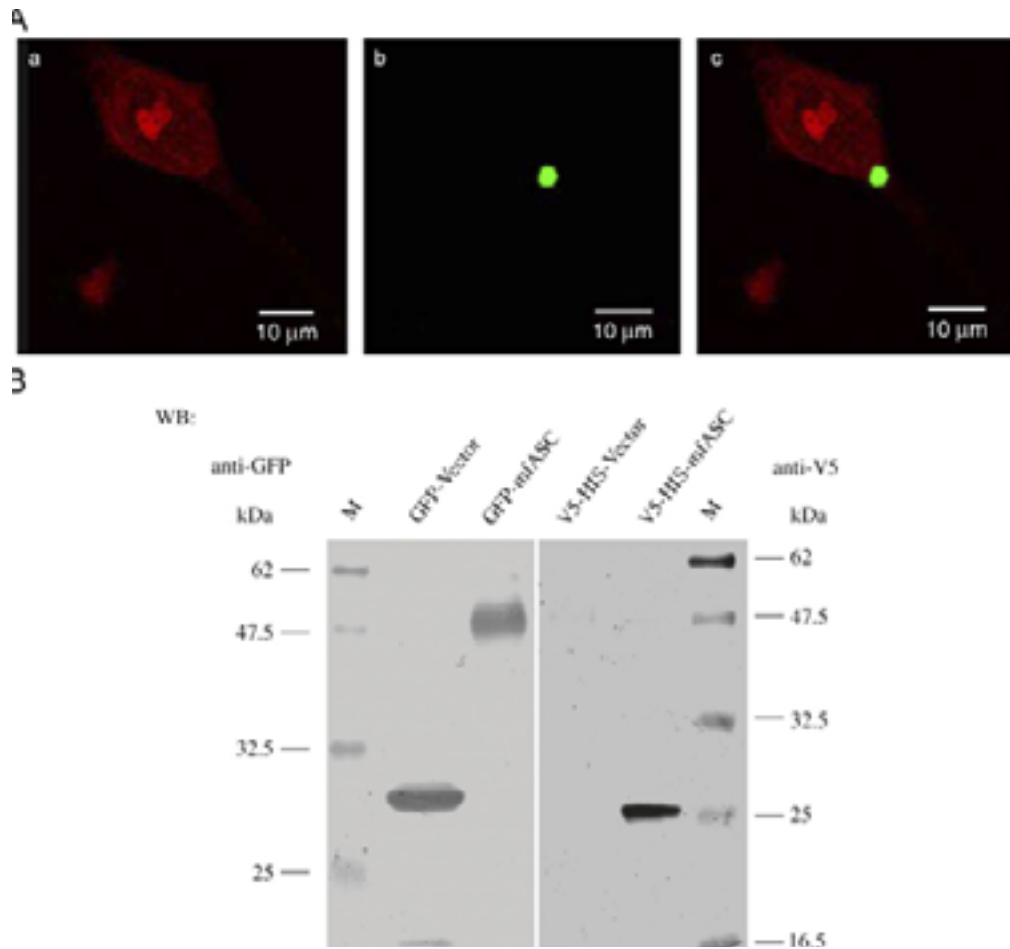
Fig. 5 Transient expression of mfASC. (A) Subcellular localization of GFP-fused mfASC protein. 293T cells were transiently transfected with pEGFP-C3-mfASC. After 24 h, cells were fixed in ice-cold methanol and detected by confocal laser-scanning microscopy. (a) Red nuclei were stained with PI dye. (b) A green speck-like signal was seen in the same field. (c) Overlay of the two channels in the same field. (B) 1×10^6 293T cells were transiently transfected with pcDNA3.1-V5-His vector, pcDNA3.1-V5-His-mfASC, pEGFP-C3 and pEGFP-C3-mfASC. After 24 h, cells were lysed in SDS sample buffer and analyzed by Western blotting using an anti-GFP mAb (left) or anti-V5mAb (right). M: marker. Sizes of protein standards are shown in kilodaltons.

TNFα, IL-1β and LPS [24]. Moreover, the PYRIN domain of ASC bound only the same domain in pyrin or Nalp3 proteins, allowing these proteins to collaborate in induction of NF-κB activity when coexpressed in 293T cells [14,24,46]. However, in macrophages from ASC$^{-/-}$ mice, the activation of NF-κB was normal after TNFα and LPS stimulation [23]. In our research, we studied the effects of mfASC on NF-κB induction by overexpression of the mfASC in 293T cells after transient transfection. It was obvious that the expression of mfASC reduced NF-κB activity not only in response to TNFα and LPS stimulation, but also in the cells without TNFα and LPS stimulation.

Recent identification of ASC involved in innate immune responses to microorganisms has gained more attention. In ASC-null macrophages, activation of caspase-1 and secretion of IL-1β in response to an intracellular Gram-negative bacterial pathogen (*Salmonella typhimarium*) was abrogated severely [23]. Similarly, the other highly infectious gram-negative coccobacillus of *Francisella tularensisis* led to markedly increased bacterial burdens and mortality in ASC-deficient mice compared with wild-type mice [47]. Secretion of IL-1β and processing of pro-caspase-1 induced

by bacterial RNA was also abrogated in the ASC-deficient macrophage, but was independent in TLR7- and MyD88-deficient macrophages [48]. During innate immune recognition of viral infection, myxoma virus encodes a PYRIN-containing protein, M13L, that interacts with ASC to inhibit caspase-1 activation and apoptotic responses to infection [31]. Although the exact mechanism is not known, it provides new insights to better understand how pathogens manipulate or suppress the innate immune responses. We examined the mfASC mRNA expression in the spleen and kidney after infection with ISKNV, and showed that mfASC transcription did not change significantly after ISKNV infection (data not shown).

In conclusion, our current study has clearly identified and characterized an ASC adaptor protein in mandarin fish. We found that the predicted PYRIN and CARD domains are similar to those of zebrafish, human and mouse. However, the gene structure and expression pattern of mfASC are different from those of zebrafish, human and mouse. Interestingly, the inhibitory effect of mfASC on NF-κB activity by transiently transfected mfASC is not related to stimulation. Our future work will focus on the exact
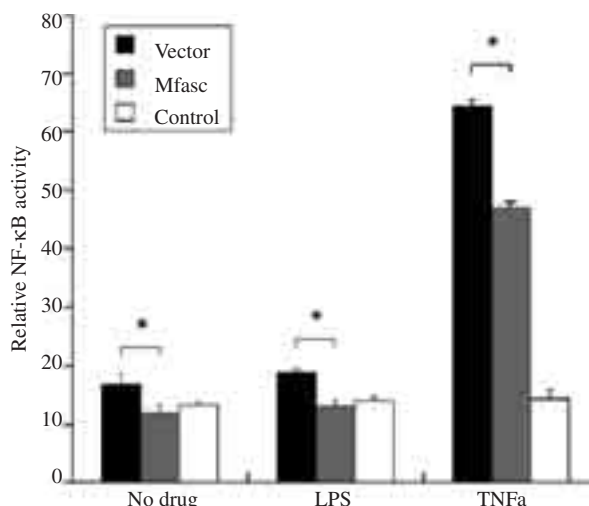
Fig. 6 MfASC inhibits NF-κB activity in response to different stimuli. 293T cells were transfected with 50 ng of pNF-κB-Luc or pTAL-Luc (negative control, white bars), 1 ng of pRL-TK reporter vectors and either 150 ng of pcDNA3.1-V5-His empty vector (black bars) or the same amount of pcDNA3.1-V5-His-mfASC (gray bars). At 24 h after transfection, cells were left untreated (left) or stimulated for 8 h with LPS (middle) or TNFα (right). Cells were then harvested and analyzed using the Dual Luciferase kit. Data indicate the relative induction of luciferase activity. The values are the mean $\pm$ SD ($n = 3$) of three independent biological samples (three cell cultures) with three replicates each. Asterisks indicate significant difference at $p < 0.05$ compared to empty vector (black bars) in each group.

mechanism of how ASC regulates NF-κB activation and its role in innate immunity.

Acknowledgments

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