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Involvement of caveolin-1 in the Jak–Stat signaling pathway and infectious spleen and kidney necrosis virus infection in mandarin fish (*Siniperca chuatsi*)

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ABSTRACT

Caveolae, the major source of caveolin-1 protein, are specialized invaginated microdomains of the plasma membrane that act as organizing centers for signaling molecules in the immune system. In the present study, we report the cloning and characterization of caveolin-1 (mCav-1) from mandarin fish (*Siniperca chuatsi*) and study on the roles of mCav-1 in the fish Jak–Stat signaling pathway and in virus infection. The cDNA sequence of mCav-1 was 707 bp in size, encoding a protein of 181 amino acids, which was different from the mammalian protein (178 amino acids). The deduced amino acid sequence of mCav-1 shared similar architecture with vertebrate caveolin-1 proteins, but mCav-1 lacked a phosphorylation site (y14). The major subcellular location of mCav-1 was in the caveolae, where the protein appeared to have major functions. Real-time PCR revealed that the expression of the mandarin fish *Mx*, *IRF-1*, *SOCS1*, and *SOCS3* genes involved in the poly(I:C)-induced Jak–Stat signaling pathway was impaired by the mCav-1 scaffolding domain peptide (mSDP). In mandarin fish fry (MFF-1) cells, the protein levels of mCav-1 were markedly up-regulated at 12 and 24 h post-infection with ISKNV (infectious spleen and kidney necrosis virus). In addition, ISKNV entry into MFF-1 cells was significantly inhibited by mSDP, and the inhibition was dose-dependent. Thus, ISKNV infection was apparently associated with mCav-1 protein and may utilize the caveolae-related endocytosis pathway. The findings reported here further our understanding of the function of caveolin-1 in the complex signal transduction network in fish immune systems and in the cellular entry mechanism of iridoviruses.

1. Introduction

Caveolae are specialized flask-shaped invaginations 50–100 nm in diameter within the plasma membrane (Palade, 1953). They are found in most mammalian cells, but are particularly abundant in terminally differentiated cells, such as adipocytes, endothelial cells, smooth muscle cells, and fibroblasts (Rothberg et al., 1992). Caveolae are highly enriched in cholesterol and glycosphingolipids and are characterized by the presence of caveolin proteins (Thomas and Smart, 2008).

The caveolins are a family of 21–24 kDa integral membrane proteins that bind cholesterol and fatty acids and that also maintain the structure of caveolae (Ikonen and Parton, 2000). Three members of the caveolin family have been identified to date: caveolin-1 (Cav-1, previously named VIP-21), caveolin-2 (Cav-2), and caveolin-3 (Cav-3, previously named M-caveolin) (Williams and Lisanti, 2004; Way and Parton, 1996). Caveolin-1 was first identified as a principal protein component of caveolae membranes (Kurzchalia et al., 1992). Subsequently, caveolin-1 was found highly expressed in cells with abundant caveolae and caveolin-1 expression was also found necessary or sufficient to generate caveolae membranes (Kim et al., 2006). Caveolin-1 directly binds to cholesterol, employing the "cholesterol recognition amino acid consensus" sequence composed of residues 95–101 in the caveolin scaffolding domain (CSD). The cholesterol-binding capacity of caveolin-1 plays a key role in maintaining a stable balance of intracellular cholesterol, allowing caveolin-1 to act as a transporter that directs cholesterol efflux, selective uptake, and the delivery of newly synthesized cholesterol to the plasma membrane (Fielding, 2006). The membrane binding

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and lipid raft interactions of synthetic peptides derived from the CSD of the caveolin-1 protein (caveolin-1 scaffolding domain peptide, SDP) have been investigated (Horton et al., 2006; Benferhat et al., 2008).

Caveolin-1 clearly plays important roles in cholesterol transport, endocytosis, and signal transduction (Frank et al., 2008). It is thought to function as a scaffolding protein that organizes and concentrates cholesterol, glycosphingolipids, and caveolae-associated signaling molecules, such as endothelial nitric oxide synthase, H-ras, Src-like kinase, G proteins, Janus kinase (Jak), and signal transducer and activator of transcription (Stat) proteins, among others (Couet et al., 1997; Ju et al., 1997; Harris et al., 2002; Schutzer et al., 2005; Baran et al., 2007; Elsasser et al., 2007; Rath et al., 2009). Caveolae are known to act as organizing centers for immune-related signal transduction pathways, such as the Jak–Stat signaling pathway or the NFκB–IκB signaling pathway, etc. (Harris et al., 2002; Garrean et al., 2006). In mammals, the Jak–Stat signaling pathway functions in host defense against viral and bacterial infections (Lad et al., 2005; Haller and Weber, 2009). The Jak–Stat signal transduction pathway is activated by a large number of cytokines and growth factors, including interferon (IFN)-α/β, IFN-γ, interleukin-2 (IL-2), IL-4, IL-5, IL-6, IL-10, and growth hormone (GH), among others (Imada and Leonard, 2000; Levy and Darnell, 2002; Gao, 2005; Chelbi-Alix and Wietzerbin, 2007; Sarasin-Filipowicz et al., 2009). In mammalian cells, caveolin-1 protein has been reported to inhibit the Jak–Stat signaling pathway through direct interaction with Stat3 protein (Shah et al., 2002).

The mandarin fish, *Siniperca chuatsi* (Basilewsky), is widely cultured and has a relatively high market value in China (Liu et al., 1998, 2000). However, outbreaks of disease caused by pathogenic parasites, bacteria, and viruses, especially the infectious spleen and kidney necrosis virus (ISKNV), now greatly threaten the aquaculture industry (He et al., 2000; Zhang et al., 2003; Sun and Nie, 2004). For this reason, research is being conducted to understand the immune system of the mandarin fish. The primary structure and functional roles of caveolin-1 protein have been described in other vertebrates, such as humans, mice, sheep, rabbits, and frogs, etc. (Glenney and Soppet, 1992; Sargiacomo et al., 1993; Tang et al., 1994; Lavialle et al., 2000; Chen et al., 2001). However, the biological roles of caveolin-1 with respect to immune responses in fish are still not well understood.

In the current study, the molecular cloning, tissue-specific expression, and subcellular distribution of mandarin fish caveolin-1 were reported. A functional role for this protein in the poly(I:C)-induced Jak–Stat signal transduction pathway and in virus infection was also postulated.

2. Materials and methods

2.1. Experimental animal, cell and virus

Healthy mandarin fish (weight ~250 g) were obtained from a fish farm in Nan-Hai (Guangdong Province, China) and maintained for at least 2 weeks in aquaria at 28 °C. Freshly dissected tissues, including brain, fat tissue, heart, hemocytes, gills, intestines, liver, muscle, kidney, and spleen, were washed thoroughly with phosphate buffered saline (PBS) and immediately homogenized with thiourea lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS) containing protease inhibitor cocktail set III (Calbiochem, USA). Protein concentrations were quantified using the Bradford reagent (Bio-Rad, USA) and 100 μg of protein was analyzed by Western blotting.

Mandarin fish fry (MFF-1) cells were cultured in Dulbecco's modified Engle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 27 °C under a humidified atmosphere containing 5% CO_2 (Dong et al., 2008). The ISKNV used in this study was originally isolated from diseased mandarin fish and maintained by our laboratory. For infection, MFF-1 cells were cultured in 25 cm^2 flasks at 5×10^6 cells overnight before further treatment. Each flask was inoculated with virus suspension (MOI = 10) and cells were harvested at various times (1, 2, 4, 8, 12, and 24 h), while the uninfected flask served as a negative control.

2.2. Molecular cloning and sequence analysis of mCav-1

Total RNA was extracted from MFF-1 cells using Trizol reagent (Invitrogen, USA) and reverse transcribed to cDNA as previously described (Guo et al., 2009). A cDNA fragment of mCav-1 was obtained by PCR amplification with degenerate primers mCav-1-F and mCav-1-R (Table 1), which were derived from conserved regions of known caveolin-1 sequences. The PCR fragment was subcloned into the pGEM-T Easy vector (Promega, USA) and selected clones were sequenced. Based on the obtained partial sequence of mCav-1, gene-specific primers were designed (Table 1) and 3′/5′ RACE was performed using a GeneRacer Kit (Invitrogen, USA). The PCR products were gel-purified and subcloned into the pGEM-T Easy vector for DNA sequencing.

The cDNA sequence and deduced amino acid sequence of mCav-1 were analyzed using the BLAST program from NCBI and the Simple Modular Architecture Research Tool (SMART) program. Sequence alignments were performed using the ClustalX v1.83 program and edited with the GeneDoc v2.6 software (Thompson et al., 1997).

2.3. Prokaryotic expression of mCav-1 and preparation of its polyclonal antibody

Based on the complete open reading frame sequence of mCav-1, a pair of primers (MBP-mCav-1-F and -R, Table 1) was designed. The DNA fragment was amplified by PCR, digested with EcoR I and Xba I, and then inserted into the expression vector pMAL-C2X (New England Biolabs, UK), which expresses a maltose binding protein (MBP). The resultant plasmid, designated as pMAL-mCav-1, was transformed into the competent *E. coli BL21* strain. MBP and the mCav-1 fusion protein (named MBP-mCav-1) were expressed after induction with 0.1 mM IPTG for 4 h at 37 °C, and the supernatants of sonicated bacterial cells were analyzed by SDS-PAGE.

Table 1
Primers used for cloning mCav-1.

Primer name	Sequence (5′–3′)	Use
mCav-1-F	AAGRTDGAYTTTGARGAYGTGATCGC	mCav-1 partial
mCav-1-R	CASTGGATCTCGATYAGGTARCTC	mCav-1 partial
3′-mCav-1-F1	TCTTCTTCGCCATCCTGTCC	mCav-1 3′ RACE
3′-mCav-1-F2	CGTGCGTCAAGAGCTACCTAATCGAGATC	mCav-1 3′ RACE
5′-mCav-1-R1	TCAGCAGCCGGTAGCACC	mCav-1 5′ RACE
5′-mCav-1-R2	CCCTGCAGGCTCGGCGATCAC	mCav-1 5′ RACE
MBP-mCav-1-F	CGGAATTCACAGGAGGACTGAAGGACGATG	Cloned into expression vector
MBP-mCav-1-R	GCTCTAGACTACACCTCCTTGGTCGTGCG	Cloned into expression vector

R = A/G; Y = C/T; S = C/G; D = A/G/T.