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Microfibrillar-associated protein 4 (MFAP4) genes in catfish play a novel role in innate immune responses

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ABSTRACT

The lectin pathway of the complement system is characterized by two groups of soluble pattern recognition molecules, mannose-binding lectins (MBLs) and ficolins. These molecules recognize and bind carbohydrates in pathogens and activate complement leading to opsonization, leukocyte activation, and direct pathogen killing. While MBLs have been reported in many fish species, ficolins do not appear to be present in the teleost lineage, despite their importance in invertebrate and higher vertebrate innate immunity. A protein with a similar fibrinogen-like domain, microfibrillar-associated protein 4, MFAP4, is present in fish, albeit with no described immune function. We examined whether MFAP4 genes in fish may potentially act as pathogen receptors in the absence of ficolin. We isolated and characterized five MFAP4 genes from channel catfish. Linkage mapping and phylogenetic analysis indicated that at least three of the catfish MFAP4 genes are tightly clustered on a single chromosome, suggesting that they may have arisen through tandem duplication. Divergent, duplicated families of MFAP4 genes are also present in other teleost species. Expression analysis of the catfish MFAP4 transcripts revealed unique patterns of homeostatic expression among the genes in gill, spleen, skin, liver, and muscle. Expression of the five MFAP4 transcripts showed significant changes in expression as soon as 4 h after infection with either *Edwardsiella ictaluri* or *Flavobacterium columnare* with modulation of expression continuing up to 7 d following pathogen exposure. Several different tissues and gene-specific patterns were captured and transcript expression changes of >30-fold were observed over the course of the bacterial challenges. Our results suggest a novel role for MFAP4 in teleost immune responses.

1. Introduction

The ability of lectins to act against pathogens by aggregating and opsonizing them has been well-established in a host of vertebrate and invertebrate species, including teleost fish (Endo et al., 2006). Further potency is afforded a subset of lectins through their association with the complement system. In the lectin pathway of the complement system, mannose-binding lectins (MBLs) and ficolins serve as pathogen recognition molecules, activating the complement cascade that is at the heart of many innate immune strategies. While a diverse group of lectins, including MBLs, have been described and their pathogen responses well-characterized in teleost fish and invertebrates (Vasta et al., 1999; Nakao et al., 2006), relatively little research has been conducted on ficolins or other fibrinogen-related proteins (FREPs, also FREDs) in these groups.

FREPs are a family of glycoproteins that encode a fibrinogen-like (FBG) domain in the C-terminal end but differ in the N-terminal region (Romero et al., 2010). FREP members include fibrinogen/fibrin, ficolins, angiopoietin, tenascins, tachylectins, fibroleukin, FIBCD1, and microfibrillar-associated protein 4 (Thomsen et al., 2010). Ficolins, with their proven abilities to detect pathogens, enhance phagocytosis, and activate complement, are the best characterized molecules of the family. In spite of the importance of ficolins in mammals, these molecules do not appear to be present in teleost fish (Garred et al., 2010). Interestingly, ficolins have been reported from amphibians, birds (Kakinuma et al., 2003; Garred et al., 2010) and several invertebrate species (Kenjo et al., 2001), although phylogenetic relationships remain unclear. The apparent absence of ficolin genes in some species groups has caused some to examine other FBG-containing members for their ability to mediate similar functions. The tachylectins, a unique

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Table 1
Primers used for the study of the five catfish MFAP4 genes.

Gene	Amplicon sizes (bp)	Primer	Forward primer (5′–3′)	Reverse primer (5′–3′)
MFAP4-1	157	For qRT-PCR	TTGTATGTGCGGCTCCTGCCAC	TCACTGTCCATCCACGGGCTGA
	729	For Southern blot	GCATCTGCTCCCCATCACTT	TGGCCCACACGTTACCAATA
	160	For linkage map	GTTTTTTCCTGCCACATT	TTATTCCCAAAGCCATTC
MFAP4-2	143	For qRT-PCR	GTTGTGTGTGCTCCCCCTGCTG	CCTGGACAGGTGTGTCTGCTGT
	675	For Southern blot	GGCACCTTAGGACATGGT	CCGATGGCGTAATAAGTG
	210	For linkage map	TTCCTTTCTGTGATTGTGAC	TAATATCGCTGTTGTTGATG
MFAP4-3	145	For qRT-PCR	ATGCTGCTGCTGTTCGTAGCACTG	CAGACCCAGCAGGGAAGATGGTGT
	879	For Southern blot	GACGATAATACAGACGAAACAGT	AATGCTTTGTGGTTATACAGAGT
	168	For linkage map	GATTTTAACTGGACTTTGATGT	GCATTATTATTATTGTTTCAGC
MFAP4-4	185	For qRT-PCR	CTGGGACTTGAGACAATTCATC	GCACCACCATCTTCAAAATCAG
	750	For Southern blot	TATGACTCTGAAATCCCTGGTT	TAAAGCCTTTCCAAGTTCTCC
	176	For linkage map	CAAATTCAGGCACAGAGAC	GAAAGACAACATAGAGGCA
MFAP4-5	163	For qRT-PCR	AACACGTCCCTGCCGATGGACT	CATGGCCTCCGGTTTCCAAGCA
	995	For Southern blot	ACACGAGAATTAGCACAGAAAGA	ACAGAGAGGATATACCACAGGAA
	151	For linkage map	AGTGCGTGTCACTTAGCC	GATCTTCCAGACCCATCC

lectin group from horseshoe crab (*Tachypleus trimentatus*) with a C-terminal fibrinogen domain, were shown to bind and agglutinate a variety of bacterial types (Gokudan et al., 1999). Recent studies in a range of invertebrate species have revealed large clusters of FREPs with tremendous coding diversity and immune responsiveness after pathogen challenge (Middha and Wang, 2008; Romero et al., 2010). A similar examination of alternative FREPs has not been carried out in fish species.

In previous research from our group on the innate immune responses of channel catfish (*Ictalurus punctatus*) and blue catfish (*Ictalurus furcatus*) to a Gram-negative bacterium (Peatman et al., 2007, 2008), we observed strong upregulation of FREP family member microfibrillar-associated protein 4 (MFAP4) by microarray analysis. Little is known about MFAP4 function. After its original discovery in porcine aorta (Kobayashi et al., 1989), it was found to be one of several genes deleted in connection with Smith-Magenis Syndrome (Zhao et al., 1995). MFAP4 was purified from bovine lung washings and found to bind the collagen domain of surfactant protein D (SP-D) as well as mannan (Lausen et al., 1999), while human recombinant MFAP4 was reported to bind the collagen domain of surfactant protein A (SP-A), suggesting that MFAP4 may play a role in inflammatory processes of the mammalian lung (Schlosser et al., 2006). Limited reports of additional functions of MFAP4 in aortic structure and function suggest that it is likely a multifunctional protein with different roles in several organ systems (Toyoshima et al., 2005). Structurally, MFAP4 possesses a characteristic FBG domain at the C-terminal end. Additionally, MFAP4 has an integrin-binding motif with a single cysteine residue and an Arg-Gly-Asp (RGD) sequence located at the N-terminal region in mammals (Schlosser et al., 2006). The RGD sequence motif is often associated with cell adhesion activity and is known to be a ligand motif for cell surface integrins (Ruoslahti, 1996).

Given our previous observation of MFAP4 upregulation during bacterial infection and the potential of FREPs to serve as pattern recognition molecules in early innate immune responses, here we set out to characterize MFAP4 in catfish and to determine immune responsiveness to pathogen challenge. We isolated and characterized five unique MFAP4 cDNAs from channel catfish. Expression of the five MFAP4 genes showed significant changes in transcript expression as soon as 4 h after infection with either *Edwardsiella ictaluri* or *Flavobacterium columnare* with modulation of expression continuing up to 7 d following pathogen exposure. Several different tissue and gene-specific patterns were captured and transcript expression changes of >30-fold were observed over the course of the bacterial challenges. Our results represent the first functional characterization of MFAP4 genes in fish and suggest a novel role for MFAP4 in teleost immune responses.

2. Materials and methods

2.1. Identification and sequencing of MFAP4 cDNAs

Zebrafish MFAP4 (NP_998054) was used as a query to search cDNAs encoding MFAP4 from channel catfish expressed sequence tags (ESTs) using *tblastn*. Contigs were assembled using Vector NTI 10.0 (Invitrogen, Carlsbad, CA) to identify clones that potentially contain full open reading frames (ORFs). These clones were resequenced using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) following manufacturer's protocols with modifications (Xu et al., 2006) on an ABI 3130XL automated DNA sequencer (Applied Biosystems). Assembly and comparison of existing transcripts with *de novo* sequences allowed high certainty about transcript identity and sequence accuracy.

2.2. Sequence analysis

The MFAP4 amino acid sequences were either identified by simple key-word searches; or with *blastp* searches using zebrafish MFAP4 amino acid sequences to query the NCBI non-redundant (nr) amino acid sequence database (http://www.ncbi.nlm.nih.gov/BLAST). Protein sequences retrieved from the public database were used for ORF and domain searches; alignment; and phylogenetic reconstruction.

ORFs were predicted using Open Reading Frame Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html), and signal peptides and fibrinogen-like domain (FBG) were identified by the NCBI conserved domain feature of *blastp* (http://www.ncbi.nlm.nih.gov/BLAST) and by the Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de).

2.3. Phylogenetic analysis

Sequences of MFAP4: microfibrillar-associated protein 4, FCN: ficolin, FB: fibrinogen, TNR: tenascin R, FIBCD1: fibrinogen c domain containing 1, ANGPT: angiopoietin of human, mouse, frog, zebrafish and ascidian (*Ciona intestinalis*) retrieved from databases were aligned using the ClustalW2 program (http://www.ebi.ac.uk/Tools/clustalw2/). The complete list of species, gene names and accession numbers are listed in Supplementary Table 1. Phylogenetic trees were constructed using the neighbour-joining (NJ) method based on the deduced full-length amino acid sequences with 10,000 bootstrapping replications within the Molecular Evolutionary Genetics Analysis