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The evolution of tachykinin/tachykinin receptor (*TAC*/*TACR*) in vertebrates and molecular identification of the TAC3/TACR3 system in zebrafish (*Danio rerio*)

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ABSTRACT

Tachykinins are a family of peptides that are conserved from invertebrates to mammals. However, little is known about the evolutionary history of tachykinin (*TAC*) and tachykinin receptor (*TACR*) genes in vertebrates, especially in the teleost group. In the present study, five *TACs* and six *TACRs* genes were identified in the zebrafish genome. Genomic synteny analysis and phylogenetic tree analysis indicate that the increased numbers of *TAC* and *TACR* genes in vertebrates are the result of both genome duplications and local individual gene duplication. The full-length cDNA sequences encoding multiple *TAC3*s (*TAC3a* and *TAC3b*) and *TACR3*s (*TACR3a1*, *TACR3a2* and *TACR3b*) were subsequently cloned from zebrafish brain samples. Sequence analysis suggested that four putative neurokinin B (NKB)-like peptides (NKBa-13, NKBa-10, NKBb-13 and NKBb-11) might be generated by the processing of two zebrafish TAC3 precursors. Tissue distribution studies in zebrafish revealed that *TAC3* and *TACR3* are mainly expressed in the brain regions. The biological activities of four zebrafish NKB peptides and three TACR3s were further examined using transcription reporter assays in cultured eukaryotic cells. All the synthetic NKB peptides were able to evoke the downstream signaling events of TACR3s with the exception of NKBb-11. These results indicated that the multiple *TAC*/*TACR* genes identified in vertebrates evolved from gene duplication events and that the TAC3/TACR3 systems also operate in the teleost group.

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1. Introduction

Tachykinins are a family of phylogenetically conserved peptides present from invertebrates to mammals (for review, see Van Loy et al., 2010). The members of tachykinin family include SP, NKA, NKB, HK-1 and EKs, which are characterized by a consensus C-terminal sequence of Phe-X-Gly-Leu-Met-NH_2 (for review, see Pennefather et al., 2004). The mammalian tachykinins are encoded by three distinct genes (*TAC1*, *TAC3* and *TAC4*) (for review, see Page, 2005). SP and NKA are products of the TAC1 precursor (Carter and Krause, 1990; Nawa et al., 1984); NKB is derived from the TAC3 precursor (Kotani et al., 1986; Page et al., 2000); and HK-1 and EKs are generated from the TAC4 precursor (Kurtz et al., 2002; Page et al., 2003; Zhang et al., 2000). The biological actions of tachykinins are mediated via their endogenous receptors. Three tachykinins receptors have been identified in mammals and are named NK_1, NK_2 and NK_3, which are encoded by the *TACR1*, *TACR2* and *TACR3* genes, respectively (Buell et al., 1992; Gerard et al., 1990, 1991; Takahashi et al., 1992; Takeda et al., 1991). The tachykinin receptors belong to the G protein-coupled receptor (GPCR) family and exhibit differential ligand selectivity to endogenous tachykinins (Gerard et al., 1993; Krause et al., 1994; Maggi, 1995; Nakanishi, 1991). NK1 is SP-preferring, NK2 is NKA-preferring, and NK3 is NKB-preferring (Lecci and Maggi, 2003; Maggi, 2000; Mussap et al., 1993; Regoli et al., 1994). Tachykinins and NKRs are widely expressed in the central and peripheral nervous

Abbreviations: NKB, neurokinin B; GnRH, gonadotropin releasing hormone; CRE, cAMP response element; SRE, serum response element; SP, substance P; NKA, neurokinin A; HK-1, hemokinin-1; EK, endokinin; GPCRs, G protein-coupled receptors; NK_1, tachykinin-1 receptor; NK_2, tachykinin-2 receptor; NK_3, tachykinin-3 receptor.

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system and participate in a broad range of physiological processes, such as pain transmission, neurogenic inflammation, gastrointestinal control, smooth muscle contraction and immune system activation (Grandordy et al., 1988; Greco et al., 2004; Patacchini and Maggi, 1995; Pernow, 1985).

Although NKB is one of the best known members of the tachykinin family, its physiological functions are still largely undetermined. In 2009, the significance of NKB signaling on reproduction was uncovered. Loss-of-function mutations in either *TAC3* or *TACR3* could lead to hypogonadotrophic hypogonadism in human, indicating that the NKB/NK_3 system is indispensable for the human reproductive axis (Topaloglu et al., 2009). Neuroanatomical studies have revealed that NKB neurons are mainly located in the arcuate nucleus, sending their projections to the GnRH terminals in the median eminence where *TACR3* is expressed (Abel et al., 1999; Duarte et al., 2006; Foradori et al., 2006; Goodman et al., 2007; Navarro et al., 2009; Pillon et al., 2003; Ramaswamy et al., 2010; Sandoval-Guzmán and Rance, 2004; Wakabayashi et al., 2010). These data suggest that the TAC3/TACR3 system controls reproduction by modulating GnRH secretion.

Tachykinin-like peptides have already been isolated from several fish species to date (for summary, see Mi et al., 2010). However, the evolutionary relationships among these fish peptides are ambiguous. Moreover, to the best of our knowledge, there are no any reports so far on a piscine TAC3/TACR3 system. Given that fish are the largest and most evolutionarily divergent assemblage of vertebrates, studies in these species will expand our understanding on the structural and functional evolution of the *TAC*/*TACR* gene family. Our goals of this study are to (1) identify the *TAC* and *TACR* genes in the zebrafish genome using a homology search, (2) clarify the evolutionary history of the *TAC* and *TACR* genes using chromosomal synteny analysis and phylogenetic analysis and (3) functionally characterize the TAC3/TACR3 system in zebrafish using molecular techniques.

2. Materials and methods

2.1. Animals and chemicals

Zebrafish were obtained from a local fish market in Guangzhou, China. All animal experiments were conducted in accordance with the guidelines and approval of the Animal Research and Ethics Committees of the Sun Yat-Sen University.

Peptides corresponding to zebrafish NKB peptides (NKBa-13, NKBa-10, NKBb-13, and NKBb-11) were synthesized by GL Biochem, Shanghai, China. The purity was >95% as determined by analytical HPLC.

2.2. Data mining, genomic synteny analysis and phylogenetic analysis

To identify the *TAC* and *TACR* genes in teleost fish, tblastn was used to search the genome database of zebrafish (*Danio rerio*) and the EST database from NCBI (http://www.ncbi.nlm.nih.gov) using the mammalian tachykinin peptides as the query sequences. Genomic synteny analysis was performed using the NCBI genome databases. The *TAC* and *TACR* gene sequences obtained were then searched against the genomes of zebrafish and human to determine the chromosomal locations of the *TAC* and *TACR* genes. Genes proximal to the human *TAC* and *TACR* genes were used to search for orthologs in the zebrafish genome database.

TAC and *TACR* amino acid sequences were aligned with Clustal X 1.81 (Thompson et al., 1994). A phylogenetic tree was constructed with MEGA 3.1 using neighbor-joining (NJ) with default settings (Kumar et al., 2004). One thousand bootstrap replications were conducted.

2.3. Cloning of zebrafish TAC3 and TAC3R cDNAs and sequence analysis

On the basis of the predicted open reading frames (ORFs) of the zebrafish *TAC3a*, *TAC3b*, *TAC3Ra1*, *TAC3Ra2* and *TAC3Rb* sequences, gene-specific primers were designed. All primers used in this study are listed in Table 1.

Total RNA from zebrafish hypothalamus samples was isolated using Trizol reagent (Invitrogen, USA). One microgram of isolated RNA was used to synthesize the first-strand cDNA using the ReverTra Ace-α First-strand cDNA Synthesis Kit (TOYOBO, Japan). The PCR reactions were performed with an initial denaturation at 94 °C for 5 min, followed by 40 cycles of 94 °C for 25 s, 53–58 °C for 20 s and 72 °C for 35–70 s. The reactions were completed with a final extension of 5 min at 72 °C. The amplification products were separated by 1.5% agarose gel electrophoresis, and the band of the desired size was excised and purified using the E.Z.N.A. Gel Extraction Kit (Omega BioTek, USA). This excised and purified product was then ligated into the pTZ57R/T vector (Fermentas, USA). The positive clones containing the expected size inserts were identified by the same PCR reactions described above. Five different individual positive clones were selected to sequence in order to confirm the sequence information (Applied Biosystems, USA).

The signal peptide and the neuropeptide prohormone cleavage sites of *TAC3s* were predicted using Signal P 3.0 (Bendtsen et al., 2004) and Neuropred software (Southey et al., 2006), respectively. Putative seven-transmembrane domains were predicted using TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/). The *TAC* and *TACR* amino acid sequences were aligned with Clustal X 1.81 (Thompson et al., 1994).

2.4. Tissue distribution of TAC3 and TACR3 in zebrafish

The expression patterns of zebrafish *TAC3a*, *TAC3b*, *TACR3a1*, *TACR3a2* and *TACR3b* mRNA in various tissues were analyzed by real-time PCR. Total RNA was isolated from 12 tissues, including telencephalon, optic tectum-thalamus, cerebellum, hypothalamus, pituitary, heart, liver, kidney, muscle, intestine, gill and ovary ($n = 3$). One microgram of total RNA from each tissue type was digested with DNase I (Invitrogen) and reverse transcribed into cDNA, as de-

Table 1
Nucleotide sequences of the primers used for full-length and real-time PCR.

Primers	Sequence (from 5′ to 3′)
Primers for full-length PCR	
zfTAC3a-full-F	GTCTGATAGATAGTCCATCAC
zfTAC3a-full-R	TCGTGACAGGGTGGTGCAGAC
zfTAC3b-full-F	CAGCTCATACGAGTACTTGG
zfTAC3b-full-R	GAAAGCTTCATACACCACAG
zfTACR3a1-full-F	ATGGCACAGTCACAGAACGG
zfTACR3a1-full-R	ACCGGTACGCGTAGAATCG
zfTACR3a2-full-F	ATGGCTGGTCCTCAGAGC
zfTACR3a2-full-R	TCAGCTGAGCTGCTCTGTT
zfTACR3b-full-F	ATGTCCTCTCCAAGAAACTC
zfTACR3b-full-R	TCAGCTCAGAGTTTCGGGA
Primers for real-time PCR	
zfTAC3a-real-F	GATCAGTTTCAAGCGAGAGTC
zfTAC3a-real-R	GACGTCCGGATTCAGGTTCAG
zfTAC3b-real-F	CTTCACTGACAACAGCGAC
zfTAC3b-real-R	CTTTCTTTTCTCATAGACGAC
zfTAC3Ra1-real-F	AGTGGTGACCTTTGCCTTCT
zfTAC3Ra1-real-R	CTACGGTGGTCTCCATTCG
zfTAC3Ra2-real-F	TTGTCTGTATTTGGGCACTG
zfTAC3Ra2-real-R	CACCACCTTCCTCTTAGCAC
zfTAC3Rb-real-F	GCCAAGAGGAAGGTTGTGAAG
zfTAC3Rb-real-R	CGACATCTGAATGAAGGGACA
ef1a	
ef1a F	AAGACAACCCCAAGGCTCTCA
ef1a R	CCTTTGGAACGGTGTGATTGA

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