RAPID COMMUNICATION

Chromosomal localization of three somatostatin genes in zebrafish. Evidence that the [Pro²]-somatostatin-14 isoform and cortistatin are encoded by orthologous genes

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Abstract

There is now evidence for the existence of two somatostatin genes in most vertebrate species, and even three somatostatin genes in teleosts. To help clarify the evolutionary relationships between the different somatostatin isoforms currently known, we characterized the somatostatin loci in a teleost species, the zebrafish *Danio rerio*, and compared them with the corresponding regions in the human and pufferfish genomes. The occurrence of three somatostatin genes, termed SS1, SS2 and SSII, has been previously demonstrated in the zebrafish. Radiation hybrid mapping assigned these three genes to linkage groups 15, 23 and 2, respectively. Conserved synteny of the zebrafish SS2 gene and the human cortistatin gene was revealed by comparative genomic analysis, indicating that mammalian cortistatin is orthologous to the SS2 variant of non-mammalian species. In contrast, using a similar approach, it was not possible to identify the evolutionary relationships between the atypical SSII gene of zebrafish and the other teleost SSII genes.

Journal of Molecular Endocrinology (2004) **33**, R1–R8

Introduction

Somatostatin (SS1) is a cyclic tetradecapeptide that was originally identified in ovine hypothalamic extracts from its ability to inhibit growth hormone release (Brazeau *et al.* 1973). It has been subsequently shown that SS1 is widely distributed in the central nervous system and in various peripheral organs where it acts as both a neurotransmitter/neuromodulator and a hormone (Epelbaum *et al.* 1994, Barnett 2003). SS1 is synthesized as part of a larger precursor molecule which is proteolytically cleaved to generate the biologically active peptides somatostatin-14 (SS1) and the N-terminally extended form somatostatin-28 (Esch *et al.* 1980, Pradayrol *et al.*

1980). The primary structure of SS1 is identical in all vertebrate species investigated so far, from agnathans to mammals (Conlon *et al.* 1997, Tostivint *et al.* 2004).

In addition to prepro-SS1 (PSS1), a second somatostatin precursor, encoded by a distinct gene, has been characterized in several representative species, including sturgeon (Trabucchi *et al.* 2002), goldfish (also named PSSIII; Lin *et al.* 1999), zebrafish (also named PSS3; Devos *et al.* 2002), lungfish (Trabucchi *et al.* 1999), frog (Tostivint *et al.* 1996), chicken (Trabucchi *et al.* 2003), mouse (de Lecea *et al.* 1997a), rat (de Lecea *et al.* 1996) and human (de Lecea *et al.* 1997a, Fukusumi *et al.* 1997). Peptides derived from this second precursor (named SS2 in non-mammalian species and

SS1		A	G	C	K	N	F	F	W	K	T	F	T	S	C
Frog SS2		-	P	-	-	-	-	-	-	-	-	-	-	M	-
Zebrafish SS2		-	P	-	-	-	-	-	-	-	-	-	-	-	-
Rodent CST			P	-	-	-	-	-	-	-	-	-	S	-	K
Human CST		M	P	-	R	-	-	-	-	-	-	-	S	-	K
Goldfish SSII		E	-	-	-	-	Y	-	-	G	-	-	-	-	-
Eel SSII		-	-	-	-	-	Y	-	-	G	P	-	-	-	-
Tilapia SSII		-	-	-	-	-	Y	-	-	G	L	-	-	-	-
Other typical SSII		-	-	-	-	-	Y	-	-	G	-	-	-	-	-
Zebrafish atypical SSII		T	-	-	-	Y	-	-	-	S	R	T	A	-	-
Catfish atypical SSII	D	N	T	V	T	S	K	P	L	N	-	M	-	Y	-

Figure 1 Comparison of the primary structures of SS1-, SS2- and SSII-related peptides from different vertebrate species. A dash indicates residue identity. The sequence of SS1 is identical in all vertebrate species investigated so far; the SS2 sequence in zebrafish is the same as in *Fugu*, sturgeon, goldfish, lungfish and chicken.

cortistatin (CST) in mammals) exhibit a more variable structure than SS1 (Spier & de Lecea 2000, Tostivint *et al.* 2004) (Fig. 1). The occurrence of a third somatostatin precursor (PSSII) has also been established in teleost fish (Conlon *et al.* 1997). Most peptides derived from PSSII contain the [Tyr⁷,Gly¹⁰]-somatostatin-14 sequence at their C-terminal extremity. However, in some species such as the catfish (Magazin *et al.* 1982, Andrews *et al.* 1984) and zebrafish (Devos *et al.* 2002), this third precursor generates a peptide that shows only very limited sequence similarity to other teleost SSII (Fig. 1).

Several lines of evidence suggest that PSS2 and PCST are derived from orthologous genes (Tostivint *et al.* 2004): (1) their processing products, SS2 and CST, both exhibit the Gly→Pro substitution at position 2 (Fig. 1); (2) they are almost exclusively expressed in the brain but not in the pancreas or gut and (3) the mouse, rat and human genomes do not appear to encompass any sequence that would be more related to PSS2 than PCST. It should be noted however that, apart from their C-terminal region, PCST and PSS2 possess very little sequence similarity. In addition, their distributions in the brain are relatively different: while the SS2 gene is expressed in various subdivisions of the brain in all species studied so far (Tostivint *et al.* 1996, Lin *et al.* 1999, Trabucchi *et al.* 1999, 2002, 2003), the CST gene is almost exclusively expressed in the telencephalon (de Lecea *et al.* 1996, 1997a, 1997b). Although the parentage between the PSS2 gene and the PCST gene is supported by phylogenetic analysis (Trabucchi *et al.* 1999, 2003,

Devos *et al.* 2002), the orthology of the two genes has not yet been demonstrated. The question of the evolutionary significance of the genes encoding the atypical SSII in catfish and zebrafish is also currently unanswered.

The recent development of comparative genomics now provides new opportunities for identifying orthologous genes in vertebrates (O’ Brien *et al.* 1999, Postlethwait *et al.* 2000). In the present study we applied a comparative approach utilizing the zebrafish, in which three distinct somatostatin isoforms have been previously identified (Argenton *et al.* 1999, Devos *et al.* 2002), to help understanding of the evolutionary history of the somatostatin genes in vertebrates (Barbazuk *et al.* 2000, Woods *et al.* 2000). The chromosomal localization of these three genes is compared with those of the somatostatin genes in the human (Lander *et al.* 2001, Venter *et al.* 2001) and *Fugu* (Aparicio *et al.* 2002) genomes.

Materials and methods

Sequences

The sequences used for this study were obtained from GenBank. Accession numbers were as follows: AF435965 for the zebrafish PSS1 sequence; BI472739 and BI473045 for the zebrafish PSS2 (also named PSS3) sequence; AJ8017 for the zebrafish PSSII (also named PSS2) sequence; CAAB01010628 for the *Fugu* PSSII sequence.

Table 1 Sequence of the oligonucleotides used for PCR amplification

Primer	Sequence (5'→3')							
Somato II F1	CTC	CAC	CTG	ACA	GCA	ACT	CTT	CTC
Somato II B1	TCT	CTG	GTA	TCT	CTT	CAT	CCG	CAG
Somato 2 F2	TCA	CTA	CTC	TTA	CTG	ACC	TTT	CGC C
Somato 2 B5	TGC	TAT	CTC	TCC	AGC	CAG	AAC	G
Somato 1 F10	AAC	TCG	CCA	GAT	ACA	CAC	TCG	CAG
Somato 1 B3	GGG	AAA	CGC	TTT	GTC	ATA	AGG	G

Linkage analysis by radiation hybrid mapping

Radiation hybrids of the LN54 panel (zebrafish DNA in a mouse background; Hukriede *et al.* 1999, 2001) were used to map the three somatostatin genes, i.e. the SS1, SS2 and SSII genes, to a specific zebrafish linkage group by PCR. DNA (100 ng) from each of the 93 zebrafish×mouse radiation hybrids was amplified using a pair of gene-specific primers which amplify part of the 3'-untranslated region sequence of the three genes (Table 1). The reactions contained 1×PCR buffer, 1.5 mM MgCl₂, 0.25 µM each forward and reverse primer, 0.2 mM each dNTP and 1 U Taq DNA polymerase. The PCR templates for the controls were 100 ng DNA from the two parental cell lines. Following an initial denaturation at 94 °C for 4 min, PCR was performed for 32 cycles: 30 s at the appropriate annealing temperature for a given primer set, 30 s at 72 °C, 30 s at 94 °C and a final extension at 72 °C for 7 min. The entire reaction (20 µl) was fractionated by electrophoresis in 1.5% (w/v) agarose. The radiation hybrid panel was scored based on the absence (0) or presence (1) of the expected DNA fragments, or an ambiguous result (2) to generate the radiation hybrid vector.

Phylogenetic analysis

The amino acid sequences of the N-termini of all teleost somatostatin precursors were aligned using CLUSTAL X (Thompson *et al.* 1994) and optimized manually using the SEAVIEW program (Galtier *et al.* 1996). Tree construction and bootstrap analysis were carried out with the PHILO_WIN program (Galtier *et al.* 1996). Distances were calculated according to Saitou & Nei (1987).

Results and discussion

Chromosomal localization of the three zebrafish somatostatin genes

To localize the three somatostatin genes to zebrafish linkage groups (LGs), radiation hybrid mapping using the LN 54 panel of radiation hybrids (Hukriede *et al.* 1999, 2001) was performed. The SS1 mapped to LG 15 at a distance of 6.72 cR from the marker z3760. The SS2 gene (also named SS3) was assigned to LG 23 at 18.03 cR from nadl1.2. The SSII gene (also named SS2) mapped to LG 2 at 2.02 cR from fa56a06. Primary data and the radiation hybrid vector for linkage analysis is available upon request to the corresponding author.

Evolutionary relationships between the somatostatin 2 and CST genes

It has been previously reported that the human chromosomal region 3q29, where the SS1 gene is located, shares only limited conserved synteny with zebrafish LG 15 (Woods *et al.* 2000). Accordingly, we did not detect any zebrafish counterparts of human genes or other genetic markers located in 3q29 in the vicinity of the zebrafish SS1 gene locus. In contrast, the zebrafish SS2 gene appears closely linked to at least eight loci, namely PMSC12, SDR1, HKR3, FLJ20321, PGD, TARDBP, CTNNBIP and FLJ10737, each of which possesses an apparent orthologous locus in the human genome (http://fisher.wustl.edu/fish_lab/cgi-bin/human_int_map.cgi). Interestingly, in human, all of these eight loci are located at 1p36, a region where the CST gene has been mapped previously (de Lecea *et al.* 1997a). Although the order of these loci

LG23

Figure 2 Map showing chromosomal position of the SS2 gene in zebrafish and conserved synteny on zebrafish LG 23 (left) and on human chromosome 1p36 (right). The distances are expressed in cR on the zebrafish map and in Mb on the human map. CTNNBIP1, catenin β -interacting protein 1; HKR3, *Homo sapiens* GLI-Kruppel family member 3; PGD, phosphogluconate dehydrogenase; PMSCL2, polymyositis/scleroderma autoantigen 2; TARDBP, TAR DNA-binding protein; SDR1, short-chain dehydrogenase/reductase 1.

has not been totally conserved, probably due to intrachromosomal rearrangements (Postlethwait *et al.* 2000), the chromosomal environments of the SS2 gene and the CST gene appear very similar (Fig. 2). The occurrence of this conserved synteny provides strong evidence that the CST gene is the mammalian counterpart of the SS2 gene known in fish, birds and amphibians, thus corroborating the results previously obtained from phylogenetic analysis (Trabucchi *et al.* 1999, 2003, Devos *et al.* 2002). The fact that the cryptic sequences of the SS2 and CST precursors are very divergent, as compared with the cryptic sequences of the SS1 precursors, indicates that the SS2/CST gene underwent a more rapid evolution in the mammalian lineage than in other vertebrates (Trabucchi *et al.* 2003) resulting in important changes in both its structure and expression pattern

(Tostivint *et al.* 2004). The origin of the SS2/CST gene remains uncertain. The occurrence of an SS2 gene has been demonstrated in all osteichthyes (see Introduction), suggesting that it appeared before the transition from chondrichthyes to osteichthyes. The exact time of the duplication event from which it arose may have coincided with one of the whole-genome duplications that occurred during early vertebrate evolution (Furlong & Holland 2002; Fig. 3). It is interesting to note that lampreys, like all extant osteichthyes, also possess a second somatostatin gene. The lamprey [Ser¹²]-somatostatin-14 variants (Andrews *et al.* 1988, Conlon *et al.* 1995a, 1995b) may thus represent an SS2 gene product. Alternatively, these two isoforms may be the result of a gene duplication within the lamprey lineage (Escriva *et al.* 2002).

Figure 3 Schematic representation illustrating the phylogenetic relationships among the somatostatin-encoding genes in vertebrates. The possible emergence of the various somatostatin isoforms is shown relative to the established phylogeny of vertebrates. This model is based on the most parsimonious scenario of the history of the somatostatin family assuming that the lamprey [Ser¹²]-somatostatin-14 variant and the ratfish [Ser⁵]-somatostatin-14 are both orthologous to SS2. Black line, SS1 gene; dark-grey line, SS2/CST gene; dark-grey arrow, duplication generating the SS2/CST gene; light-grey line, SSII gene; light-grey arrow, duplication generating the SSII gene; broken line, non characterized genes. The atypical SSII gene is not shown.

Evolutionary significance of the atypical zebrafish somatostatin II gene

In contrast to other teleost SSII, which generally contain the [Tyr⁷,Gly¹⁰]-somatostatin-14 sequence (Conlon *et al.* 1997), zebrafish SSII exhibits a totally atypical structure (Devos *et al.* 2002; Fig. 1). In order to clarify the evolutionary history of the SSII gene, we compared its chromosomal localization in zebrafish with that of the gene encoding the classical SSII in the pufferfish *Fugu rubripes*. *In silico* screening of the pufferfish genome (Aparicio *et al.* 2002) made it possible to identify one sequence that corresponds to a PSSII-encoding gene (GenBank accession no. CAAB01010628). Although the

sequence of this precursor is truncated at its C-terminus and does not contain the SSII sequence, as a result of the lack of the second exon of the corresponding gene, its identity as a PSSII gene is attested by phylogenetic analysis. Indeed, the tree presented in Fig. 4 shows that the *Fugu* PSSII sequence is more closely related to the [Tyr⁷,Gly¹⁰]-somatostatin-14-containing-PSSII than to the atypical PSSII isoforms from zebrafish (Devos *et al.* 2002) and catfish (Magazin *et al.* 1982). The absence of the second exon in the *Fugu* SSII gene sequence can likely be ascribed to an erroneous annotation. Interestingly, in the pufferfish genome, the SS1 gene and the SSII gene are localized in tandem. This organization suggests that the SSII gene arose by duplication of the SS1 gene, and probably reflects the initial position of the two genes in the common ancestor of the teleost lineage. In contrast, in zebrafish, we found that the SS1 gene and the SSII gene are located on distinct chromosomes. Two hypotheses can be proposed to explain this observation: (1) the zebrafish atypical SSII gene and other teleost SSII genes are not orthologous or (2) the two genes are orthologous, in agreement with a previous report (Su *et al.* 1988), but, in zebrafish, the SS1 and SSII genes have been physically separated by chromosomal rearrangement. In support of the latter hypothesis, it should be noted that, up to now, no species has been shown to possess both the SSII gene and its atypical variant. However, although the species possessing an atypical SSII gene – i.e. the catfish, the zebrafish and probably the pacu (Ferraz de Lima *et al.* 1999) – are all members of a single group, the ostariophysi, surprisingly the goldfish, which belongs to the same group, possesses the classical SSII gene (Lin *et al.* 1999). Teleosts are known to possess more genes than other vertebrates. Recent studies provide evidence that these additional genes may have been produced during a complete fish-specific genome-duplication event (Amores *et al.* 1998, 2004, Vandepoele *et al.* 2004). However, the fact that, in *Fugu*, the SS1 and SSII genes are arranged in tandem indicates that the classical SSII gene has not been generated by tetraploidization. In support of this view, we have recently reported the occurrence in *Fugu* of a second isoform of the SS1 gene (Tostivint *et al.* 2004) that is probably a product of this whole-genome duplication event.

In conclusion, the present results show for the first time that SS2 and CST are encoded by

Figure 4 Phylogenetic tree of the somatostatin gene family in teleosts. The N-terminal amino acid sequences of trout PSS1 (Kittilson *et al.* 1999) and PSSII (Moore *et al.* 1995), goldfish PSS1, PSS2 (also named PSSIII) and PSSII (Lin *et al.* 1999), anglerfish PSS1 and PSSII (Hobart *et al.* 1980), medaka PSSII (GenBank accession no. AU168379), zebrafish PSS1 (accession no. AF435965), PSS2 (also named PSS3; accession nos BI472739 and BI473045) and PSSII (also named PSS2; accession no. AJ8017), catfish PSS1 (Minth *et al.* 1982) and PSSII (Magazin *et al.* 1982), *Tetraodon* PSS2 (accession no. AL296478) and *Fugu* PSSII (accession no. CAAB01010628) were aligned. The obtained alignment was used to calculate a neighbour-joining unrooted tree using the PHYLO_WIN program. The values above the branches are the results (%) of a bootstrap analysis that calculates the percentage of confidence that can be given to inferred nodes.

orthologous genes. These data demonstrate that the [Pro² Met¹³]-somatostatin-14 variant isolated from the frog brain (Vaudry *et al.* 1992) was actually the first CST-like peptide to be identified.

Acknowledgements

We thank Dr Philippe Vernier (Institut Alfred Fessard, Gif sur Yvette, France) for helpful discussions. This work was supported by grants from INSERM (U-413), the Natural Sciences and Engineering Research Council of Canada and the Conseil Régional de Haute-Normandie.

Note Added in Proof

The Genbank accession number of the [Tyr⁷, Gly¹⁰]-somatostatin-14 encoding sequence of the fugu PSSII gene is CAAB01010582. At the time this manuscript went to press, the chromosomal localization of this sequence was not yet known.

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