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Review

The early origin of melanocortin receptors, agouti-related peptide, agouti signalling peptide, and melanocortin receptor-accessory proteins, with emphasis on pufferfishes, elephant shark, lampreys, and amphioxus

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ABSTRACT

There are conflicting theories about the evolution of melanocortin MC receptors while only few studies have addressed the evolution of agouti-related peptide (AgRP) and agouti signalling peptide (ASIP), which are antagonists at the melanocortin receptors (MCRs), or the melanocortin MC_2 receptor accessory proteins (MRAP1 and MRAP2). Previously we have cloned melanocortin MC receptors (MC_a and MC_b) genes in river lamprey and here we identify orthologues to these melanocortin MC receptor sequences in the sea lamprey. We investigate the putative presence of the melanocortin MC receptor genes in lancelet (amphioxus; *Branchiostoma floridae*) but we find it unlikely that such gene exists, due to a sharp drop in sequence similarity beyond sequence clusters of known receptors. We show the presence of AgRP and ASIP in elephant shark, a cartilaginous fish belonging to the subclass of *Elasmobranchii*. However, we do not find any of these genes in lamprey or lancelet after detailed analysis of both targeted and whole proteome regular expression scans. We found MRAP2, but not MRAP1, to be present in elephant shark and sea lamprey while Fugu (*T. rubripes*) has both genes. This study shows that the most ancient presence of these melanocortin-related sequences is found in elephant shark and lampreys considering the current available sequence data.

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1. Introduction

It is well established that all the five melanocortin MC receptors subtypes are found in single copies in mammals and chicken (Boswell and Takeuchi, 2005; Metz et al., 2006; Schioth et al., 2005b). The first cloning of melanocortin MC receptors beyond these amniote type of species was in the teleost zebrafish (Ringholm et al., 2002). Teleosts include in about 30,000 species, which equals to all other vertebrate groups combined and new teleost species are being discovered each year. This first report showed that the zebrafish had at least one melanocortin MC_4 receptor subtype and two copies of the melanocortin MC_5 receptor, termed melanocortin zMC_5a, and zMC_5b receptors (Ringholm et al., 2002). These genes shared 70–71% overall amino acid identity with the respective human orthologues and over 90% in three TM regions believed to be most important for ligand binding suggesting that melanocortin MC receptor genes have high evolutionary conservation in vertebrate species. Moreover, all the three zebrafish receptors showed pharmacological properties remarkably similar to their human orthologues. The affinities and the potency order for the natural melanocyte stimulating hormones (MSH) peptides were similar when expressed and characterized in radioligand binding assay. Later, when the genome of zebrafish was sequenced it became apparent that the zebrafish also has single copies of melanocortin MC_1 receptor, melanocortin MC_2 receptor and melanocortin MC_3 receptor (Logan et al., 2003). However, another teleost, Fugu (*Takifugu rubripes*), contains single copies of four melanocortin MC receptors while the melanocortin MC_3 receptor is missing (Klovins et al., 2004a,b). This work also showed that the unique pharmacology of the melanocortin MC_2 receptor, being specific for the adrenocorticotropin hormone (ACTH) peptides is evolutionary conserved. Melanocortin MC receptors have also been cloned in other teleost species, including goldfish (melanocortin MC_4 receptor, melanocortin MC_5 receptor) (Cerda-Reverter et al., 2003), the river trout (melanocortin MC_4 receptor and melanocortin MC_5 receptor) (Haitina et al., 2004), rainbow trout (melanocortin MC_2 receptor) (Aluru and Vijayan, 2008), carp (melanocortin MC_2 receptor and melanocortin MC_5 receptor) (Metz et al., 2005), tilapia (melanocortin MC_1 receptor) (van der Salm et al., 2005), medaka (melanocortin MC_1 receptor) (Selz et al., 2007), platyfish (melanocortin MC_1 receptor) (Selz et al., 2007), sea bass (melanocortin MC_1 receptor, melanocortin MC_4 receptor, melanocortin MC_5 receptor) (Sanchez et al., 2009a,b, 2010) and barfin flounder (melanocortin MC_1 receptor, melanocortin MC_2 receptor, melanocortin MC_5 receptor) (Kobayashi et al., 2008, 2010). There are also several reports using the teleost systems for tissue expression and functional assays (Metz et al., 2006; Song and Cone, 2007).

Much less work has been performed in more ancient species. Cartilaginous fishes (*Chondrichthyes*) are represented by sharks, rays, skates and chimaeras. These are phylogenetically the oldest group of living jawed vertebrates. Sharks are one of the most enduring success stories in evolution and are today found in over 400 species. The first cloning of melanocortin MC receptors in shark showed that the spiny dogfish (*Squalus acanthias*) has highly conserved copies of melanocortin MC_4 receptor (Ringholm et al., 2003), melanocortin MC_3 and MC_5 receptors (Klovins et al., 2004b) that also pharmacologically bind MSH peptides. Interestingly, these ancient shark melanocortin MC receptors show preference to ACTH derived peptides beyond MSH peptide in contrast to the mammalian melanocortin MC receptors (Haitina et al., 2007b). The elephant shark (*Callorhinchus milii*), also known as the elephant fish and ghost shark, has the smallest genome among the known cartilaginous fish genomes. It has been proposed as a model cartilaginous fish genome for whole-genome sequencing and comparative analysis (Venkatesh et al., 2007). Recently the genome of the elephant shark has become available at 1.4× coverage providing new possibilities for evolutionary studies.

The only extant jawless vertebrates are lamprey and hagfish that form unique basal groups in the craniate lineage intermediate between amphioxius (*protochordate*) and gnathostomes (jawed vertebrates). The most ancient known melanocortin MC receptor genes are present in river lamprey and hagfish (Haitina et al., 2007a). The river lamprey receptors were designated as melanocortin MC_a and MC_b receptors and these show interesting orthology to the melanocortin MC_1 and MC_4 receptor subtypes, respectively. The sea lamprey genome is now found in 5.9× coverage and another genome that has been recently sequenced is amphioxus (*branchiostoma floridae*), also termed lancelet. This creature is a small *cephalochordate* that spends much of its time buried in the sand. It is one of the closest living relatives to vertebrates and the genome of this species has recently been sequenced, showing a high number of G protein-coupled receptors (Nordstrom et al., 2008) providing more material for investigation of the early appearance of the melanocortin system.

While the ancient melanocortin MC receptor sequences have been extensively analysed using phylogenetic analysis (Haitina et al., 2007a,b), the evolutionary history of these genes is not straightforward to delineate considering the different mechanisms

of gene evolution. Important such mechanisms involve two rounds of whole genome duplications that are proposed to have occurred early in vertebrate evolution. One of the reasons to make this issue complex is a presence of introns that seem to have been inserted in the "DRY" motif of melanocortin MC_2 and MC_5 receptors in Fugu (Schioth et al., 2005a). The authors of a recent analysis of the melanocortin MC receptor system interpreted the current data in a way that the melanocortin MC_2 and MC_5 receptors were considered highly evolutionary related (Baron et al., 2009) and forwarded a new evolutionary scenario. This scheme stands however in contrast to several other data including detailed pharmacological and sequence comparison results that indicate the melanocortin MC_4 and MC_5 receptors are more similar (Haitina et al., 2004, 2007a,b; Klovins et al., 2004a,b) and we provide further discussion of this matter here.

The AgRP and ASIP are well studied in the mammalian species (Kaelin et al., 2007). Beyond these species, it was early on shown that chickens have AgRP (Takeuchi et al., 2000) while Fugu has both AgRP and ASIP (Klovins et al., 2004a). Further annotation of AgRP/ASIP peptides were made in chicken and fish in 2005 (Klovins and Schioth, 2005) as well as in fish in 2006 (Jackson et al., 2006; Kurokawa et al., 2006). Further studies of the evolution of these peptides are highly warranted considering the recent sequencing of additional genomes.

The mammalian melanocortin MC_2 receptor accessory protein (MRAP) interacts directly with melanocortin MC_2 receptor and is essential for its trafficking from the endoplasmic reticulum to the cell surface (Webb et al., 2009). MRAP2 is a homolog of MRAP that is also able to support the cell surface expression of melanocortin MC_2 receptor, and interact with transmembrane domains of other melanocortin MC receptors. Both MRAPs and receptor activity-modifying proteins (RAMPs) have single transmembrane domains and interact with G protein-coupled receptor (Hay et al., 2006). It is known that zebrafish has both homologues of MRAPs, including isoforms of MRAP2, and that both the subtypes also exist in *T. rubripes* (Agulleiro et al., 2010). Because of high interest and importance for the function of G protein-coupled receptors, we have searched for more ancestral sequences.

In this study we investigate the origin of the melanocortin system with emphasis on melanocortin MC receptors, AgRP, ASIP and MRAP using range of bioinformatics methods. The genomes that are mostly interesting considering the early origin of the genes are the pufferfishes, elephant shark, lampreys, and lancelet. The evolution of proopiomelanocortin (POMC) has not been considered in this paper as it has been documented extensively by many groups for a long time (Metz et al., 2006; Takahashi and Kawauchi, 2006) and the presence of POMC in lamprey was already described in 1995 (Takahashi et al., 1995).

2. Methods

2.1. Melanocortin antagonists (AgRP/ASIP)

2.1.1. Finding melanocortin antagonists in elephant shark

The receptor binding domains from *T. rubripes* were used as database queries against the elephant shark genome, using TBLASTN (v. 2.2.6). Two sequences were identified that maintained the same cysteine constellation as AgRP1 and ASIP1. For these sequences, the relevant contigs were examined in the area upstream of the receptor binding domain in an effort to recover the complete sequences. In addition to manual inspection of sequence similarity, splicing profiles, and splice sites, a Genscan prediction (http://genes.mit.edu/cgi-bin/genscanw_py.cgi) was made for contig AAVX01056113.1, using default settings for vertebrates, and suboptimal exon probability cutoff 0.50. A promoter scan for vertebrates was made for the contig using a neural network tool (http://www.fruitfly.org/seq_tools/promoter.html).

2.1.2. Search for melanocortin antagonists in sea lamprey

A Perl script was used in a Linux environment to search complete contigs for partial cysteine constellations in nucleotide form. These data were presented as support vector graphics and viewed to aid manual inspection of contigs. This method was applied on contigs with TBLASTN hits (e-values<1e−07) to DDBJ ID: BR000851 (elephant shark ASIP), Fugu ASIP1, Fugu ASIP2, Fugu AgRP1, Fugu AgRP2.

2.1.3. Search for melanocortin antagonists in amphioxus

The proteome (ftp://ftp.jgi-psf.org/pub/JGI_data/Branchiostoma_floridae/v1.0/proteins.Brafl1.fasta.gz) was searched using a Perl script to identify all windows with eight or ten cysteines. The four known constellations from *T. rubripes* were included in the dataset as reference points.

2.2. Melanocortin receptors

2.2.1. Resolving the relationship between lamprey and shark melanocortin receptors

Alignments have been produced using MAFFT-EINSI (Katoh et al., 2009). Phylogenetic trees have been made using RAxML 'fast & easy' bootstrap maximum likelihood protocol (Stamatakis et al., 2005), using the WAG model, estimating proportion of invariable sites, using empirical base frequencies, and generating 100 BS trees. The standard script 'easyrax.pl' was used as interface. The bootstrap forests were edited using two tools, Summary Tree Explorer (STE), an open-source Java application for interactively exploring sets of phylogenetic trees developed by Mark Derthick, Carnegie Mellon University, Pittsburgh (http://cityscape.inf.cs.cmu.edu/phylogeny/) and "P4", a Python package for Phylogenetics developed by Peter G. Foster, The Natural History Museum, London (http://bmnh.org/~pf/p4.html). The averaged bootstrap forests were viewed as single trees in FigTree, a graphical viewer of phylogenetic trees at Edinburgh University (http://tree.bio.ed.ac.uk/software/figtree/). This tree is also used to look specifically at the branching events from lamprey melanocortin MC_b receptor.

2.2.2. Attempt to find ancestral melanocortin receptors

A search of the NCBI databases returned a machine-annotated melanocortin receptor-like sequence in *C. intestinalis* (GenBank ID: XP_002120969). The melanocortin receptors in river lamprey and hagfish (*M. glutinosa*) have previously been sequenced (GenBank ID: ABB36647.1, ABB36648.1, ABB36649.1). The vase tunicate and river lamprey sequences were blasted against the sea lamprey and lancelet genomes, respectively.

2.2.3. Melanocortin receptor-like sequence cluster in amphioxus

A search was done at the NCBI website TBLASTN, with organism limited to *B. floridae*, as of June 23, 2010. The query was river lamprey melanocortin MC_a receptor. The e-value cutoff was set to 0.001. The hits were only selected if they were annotated as "hypothetical protein, mRNA". Furthermore, the hits must have only one protein coding exon to qualify as putative G protein-coupled receptors, and have at least one "melanocortin" annotation in top 100 hits in a BLASTP version 2.2.23+ backblasting against the NR database. The selected clusters of unknown proteins were compared with shark and lamprey melanocortin receptor proteins using ClustalW.

2.2.4. Sequence similarity between human and shark melanocortin receptors

BLASTP 2.2.23+ was used to measure sequence identity between human melanocortin MC_{1-5} receptors and the corresponding receptors in elephant shark and spiny dogfish.

2.3. Melanocortin receptor accessory proteins

2.3.1. Search for MRAPs in zebrafish, tetraodon, takifugu, elephant shark

TBLASTN and contig analysis was used to search for MRAPs in tetraodon ~6× assembly, takifugu ~8.7× assembly, elephant shark 1.4× assembly. An alignment was produced in ClustalW2 2.0.12 to compare findings.

2.3.2. MRAP in sea lamprey 5.9× assembly

Fugu MRAP2 is used as TBLASTN query. The scoring matrix was set to "distant homologies". The genome is the lamprey genome at Ensembl Pre as of June 23, 2010.

2.3.3. Confirmation of absence of MRAP1 in elephant shark and absence of MRAP2 in amphioxus

MRAP1 from *T. rubripes* (Agulleiro et al., 2010) was used as a TBLASTN (v. 2.2.6) query, against the elephant shark 1.4× assembly, e-value cutoff 100, BLOSUM45 matrix (distant homology detection), no low complexity filtering, on the elephant shark website (http://blast.fugu-sg.org/). MRAP2 from sea lamprey, which was identified in this study (DDBJ ID: BR000863), is used as TBLASTN 2.2.6 query against *B. floridae* v2.0 assembly, e-value cutoff 100, BLOSUM45 matrix, no low complexity filter, on the JGI website (http://genome.jgi-psf.org/cgi-bin/runAlignment?db=Brafl1&program=tblastn&dataLib=Brafl2&email=&advanced=1&useHeader=&noHeader=0).

2.4. Comparisons between melanocortin-related proteins

2.4.1. Comparison between motifs and splice sites in ASIP and MRAP

A "SIV" motif is present in the conserved regulatory domain of ASIP (Jackson et al., 2006) in mammals and elephant shark (DDBJ ID: BR000851), close to the first splice site within the coding sequence (Klovins and Schioth, 2005). The same motif is present, close to the first splice site within the coding sequence in MRAP (EMBL ID: BN001497). The exact location of the motif in relation to the splice site was checked using NCBI's new sequence viewer (http://www.ncbi.nlm.nih.gov/nuccore/NW_003104370.1?from=16155800&to=16155900&report=graph&content=5). Splice sites were identified automatically using a neural network application (http://www.fruitfly.org/seq_tools/splice.html), using a score cutoff of 0.4, and verified manually using the 'GU–AG' rule.

2.5. Third party annotations

2.5.1. Third party annotation in GenBank/EMBL/DDBJ

Our TPA data is covered by §10, §12, §16 of INSDC TPA policy. Pre-existing entries by same submission group for melanocortin receptors include: NP_851301.1, NP_851303.1, NP_775385.1, NP_775386.1, NP_775387.1, AAQ55176.1, AAQ55177.1, AAQ55178.1, AAQ55179.1, AAO65548.1, AAO65550.1, AAO65549.1, AAO65551.1, AAO65553.1, AAO65552.1, AAS66720.1, AAS67890.1, ABB36647.1, ABB36648.1, and ABB36649.1. Pre-existing melanocortin antagonist entries by same submission group include: NP_001026628.1, NP_001129.1, CAH60801.1, CAH60803.1, and CAH60802.1. MRAP TPA data is covered by INSDC TPA policy §12, §16. In the case of sea lamprey, sequences are mapped to the NCBI trace archive using Mega BLAST (http://www.ncbi.nlm.nih.gov/blast/mmtrace.shtml).

3. Results

3.1. Melanocortin antagonists (AgRP/ASIP)

3.1.1. Finding antagonists in elephant shark

In the case of ASIP, the 5′-end of the ORF could be recovered on the same contig. In the case of AgRP, this was not possible, but it is likely that the rest of the gene exists on another contig. The Genscan prediction agreed with the manually defined gene area, and suggested a possible exon 2 (DDBJ ID: BR000863), that contained 3 lysines, and 7 asparagines. A vertebrate promoter was identified that had a probability score of 0.88, leaving a 5′ UTR with a length of 31. Depending on the exact location of the splice sites (Burge and Karlin, 1997), we arrive at the following splicing profile: {(31) 141 (317) 71 (165) 253 nts}. The new sequences have been submitted to GenBank/EMBL/DDBJ (see Table 1).

3.1.2. Examination of the elephant shark ASIP gene

The gene contains a conserved motif, "SIV", found in the regulatory domain of ASIP in other species. The N-terminal end of the protein contains a small cysteine constellation. Exon 2 in elephant shark contains fewer lysines and prolines than expected from teleosts.

3.1.3. Comparison of ASIP and AgRP receptor binding domains in elephant shark

The ASIP and AgRP cysteine constellations are identical to each other and to ASIP1 and AgRP1 in Fugu and there are segments of residues in ASIP that are AgRP vertebrate-like and AgRP residues that are ASIP vertebrate-like. There are 30 interspersed residues between the cysteines in the receptor binding domain. In ASIP, there are 6 residues that are AgRP-like, and 7 residues that are mammalian-like. In AgRP, there is 1 residue that is ASIP-like, and 11 residues that are mammalian-like. ASIP contains 4 residues that are elephant shark-specific, and AgRP 6 such residues. This convergence between the antagonists indicates that the sequences are ancestral-like.

3.1.4. Search for antagonists in sea lamprey

A partial constellation, delineated by two cysteines separated by 111 nucleotides, could be found on PMAR3 contig27900, close to the corresponding coding area in *T. rubripes* (segment 6451–6654). The smaller cysteine motifs found in the area of interest did not amount to a cysteine knot-like sequence. Furthermore, no wild-type constellation could be found anywhere else in this genome.

3.1.5. Search for melanocortin antagonists in lancelet

The most common constellation (C3C24C18C12C12C24C3C) existed in 611 copies. No further copies of the *T. rubripes* constellations were found. Constellations that were similar to the known cysteine knots were observed. These constellations were only present in 1–2 copies. They were rejected as potential cysteine knots because they were present in tandem repeats that were longer than the expected 40-mer cysteine knot unit. A good example of this type of repeat can be seen in primary sequence ABEP02028016.1 (segment 1365–1484).

Table 1
Overview of accession numbers of submitted sequences.

Accession number		Organism	Size	Description
DDBJ	BR000851	*C. milii* (Cmi)	142	ASIP exon 1, including promoter
DDBJ	BR000852	*C. milii* (Cmi)	254	ASIP exon 3
DDBJ	BR000853	*C. milii* (Cmi)	120	AgRP exon 3
DDBJ	BR000855	*C. milii* (Cmi)	852	MC1R
DDBJ	BR000856	*C. milii* (Cmi)	948	MC2R
DDBJ	BR000857	*C. milii* (Cmi)	966	MC3R
DDBJ	BR000859	*T. nigroviridis* (Tni)	483	MRAP2
DDBJ	BR000860	*T. rubripes* (Tru)	96	MRAP2
DDBJ	BR000861	*C. milii* (Cmi)	278	MRAP2
DDBJ	BR000863	*C. milii* (Cmi)	72	ASIP exon 2
DDBJ	BR000864	*P. marinus* (Pma)	189	MRAP2, exons 1 and 2
EMBL	BN001497	*C. milii* (Cmi)	168	PREDICTED: Similar to MRAP
EMBL	BN001505	*P. marinus* (Pma)	462	Melanocortin receptor A, 5′-end
EMBL	BN001506	*P. marinus* (Pma)	363	Melanocortin receptor A, 3′-end
GenBank	BK007095	*P. marinus* (Pma)	1035	Melanocortin receptor B

3.2. Melanocortin receptors

3.2.1. Resolving the relationship between shark and lamprey melanocortin receptors

The phylogenetic tree shows that melanocortin receptor 2 and melanocortin receptor 5 are separated by a common node with bootstrap support exceeding half of the bootstrap trees (56%). The phylogenetic tree shows that from the melanocortin receptor (B) receptor in lamprey, melanocortin receptor 3 have branched off first (bootstrap support 17), then melanocortin receptor 4 and melanocortin receptor 5 (bootstrap support 13).

3.2.2. Attempt to find ancestral melanocortin receptors

The melanocortin receptors were recovered from the sea lamprey genome, using our cloned sequence from river lamprey (EMBL ID: BN001505, EMBL ID: BN001506, GenBank ID: BK007095). The closest hit of the *C. intestinalis* sequence in *B. floridae* has been identified as a distant homolog of the vase tunicate sequence.

3.2.3. Melanocortin receptor-like cluster of sequences in lancelet

In the hypothetical proteins surveyed, 40 proteins had zero introns, and of these, 10 proteins had between 6 and 322 melanocortin annotations (Supplementary Fig. 4). The alignment shows that the *B. floridae* proteins are much more similar to each other than the shark or lamprey proteins. There are no proteins that constitute potential hybrids between the two clusters. The "DRY" motif, which is necessary for melanocortin receptor 4 activation (Lagerstrom et al., 2003), is not present in the hypothetical proteins. This motif is normally present in all *Rhodopsin* G protein-coupled receptors (Fredriksson et al., 2003). The cluster of conserved residues in lancelet mRNAs are centered close to "DRY" and contain "RY" (Supplementary Fig. 5).

3.2.4. Sequence similarity between human and shark melanocortin receptors

Elephant shark melanocortin MC_1 and MC_2 receptors have sequence identity of 55% (150/271) and 50% (140/279), as compared with human melanocortin MC_1 and MC_2 receptors. Elephant shark melanocortin MC_3 receptor and spiny dogfish melanocortin MC_{4-5} receptors have sequence identity 71% (221/308), 73% (244/333), and 72% (226/310), as compared with human melanocortin MC_{3-5} receptors.

3.3. Melanocortin receptor accessory proteins

3.3.1. Search for MRAPs in zebrafish, tetraodon, takifugu, elephant shark

A database search identified an MRAP sequence in zebrafish (GenBank ID: XP_001342923). A second MRAP-like sequence was identified in zebrafish on an mRNA, BC122349.1 (3–272). Isoforms of MRAP2 have been documented in zebrafish, and both lineages are documented to exist in *T. rubripes* (Agulleiro et al., 2010). Single MRAP2 sequences were reported to the NCBI databases in tetraodon (DDBJ ID: BR000859), takifugu (DDBJ ID: BR000860), elephant shark (high quality segment EMBL ID: BN001497 and exon 3 segment DDBJ

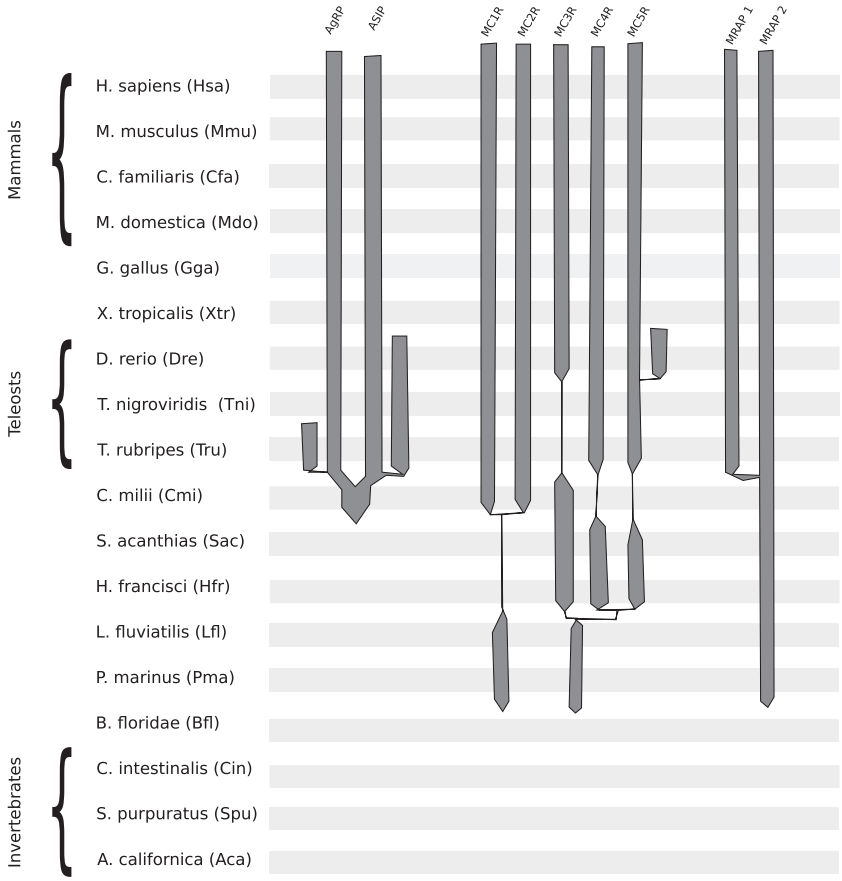

Fig. 1. Summary tree of presence/absence pattern of melanocortin sequences showing evolutionary history of antagonist, receptor, and accessory proteins. The antagonists were first found as a single pair in *T. rubripes*, and then as a double pair (Klovins and Schioth, 2005; Kurokawa et al., 2006). The antagonists die out in *C. milii*; the details of antagonists in the other shark cluster are not known. The absence of antagonist has been confirmed in lamprey, amphioxus, due to negative result of targeted and whole proteome regular expression scan. There is an extra melanocortin MC_5 receptor in zebrafish; the melanocortin MC_3 receptor is absent in pufferfishes; there are two shark clusters, melanocortin receptor 3 is common to both clusters. The melanocortin MC_3 receptor and melanocortin $MC_{4/5}$ receptor divergence emanated from melanocortin MC_b receptor. Melanocortin MC_1 receptor, melanocortin MC_2 receptor came from lamprey melanocortin MC_a receptor. The lamprey and hagfish melanocortin MC receptors are the oldest known receptors (Haitina et al., 2007a). There are isoforms of MRAP1 in human and of MRAP2 in zebrafish (Agulleiro et al., 2010). Pufferfishes constitute last point where MRAP1 is present. Only MRAP2 exists in *P. marinus*.

ID: BR000861). A splice site between exons 1 and 2 followed by the "SIV" motif exists in both mammals and elephant shark MRAP.

3.3.2. MRAP in sea lamprey

Sea lamprey MRAP has at least two exons. The splice site between exon 1 and 2 coincides with the "SIV" motif (Supplementary Fig. 6A–C). The e-values range between e−05 and e−07. 33 out of 50 residues are identical with the query in the high scoring segment (EMBL ID: BN001497). The lineage specific "FEG" motif is present.

3.3.3. Confirmation of absence of MRAP1 in elephant shark and absence of MRAP2 in amphioxus

The best MRAP1 hit in elephant shark has an e-value of 18, 26% identity, and 56% positives. The best MRAP2 hit in amphioxus has an e-value of 4.0, 24% identities, 56% positives. The MRAP2 hit in amphioxus contains a cluster of conserved residues centered around the "SIV" motif (H+S+V+GFW+). These results suggest that MRAP1 is not present in elephant shark, and MRAP2 is not present in amphioxus. The low e-values and lack of intact motifs means that the MRAP1 lineage is not found beyond the *T. rubripes*, while the MRAP2 lineage is not found beyond the sea lamprey.

3.4. Comparison between new sequences

3.4.1. Comparison between motifs and splice sites in ASIP and MRAP

In ASIP, the splice site is after SIV (SIV is on coding exon 1). In MRAP, the splice site is before SIV (SIV is on coding exon 2).

In *P. marinus*, elephant shark, and mammalian MRAP2, a colocalization is observed of a 'DRY'-like motif and a 'SIV' motif, separated by a splice site. The proto-splice site found in *T. rubripes* melanocortin $MC_{2/5}$ receptor 'DRY' (Schioth et al., 2005a) is not intact in *P. marinus*. The phasing of TMs and the DRY motif differs between *P. marinus* melanocortin receptor (A) and the single TM in MRAP2. DRY-like motifs may result in constitutive receptor activation (Lagerstrom et al., 2003).

4. Discussion

Here we present a clear hypothesis of evolution of the melanocortin MC receptors based on comprehensive mining, phylogeny and analysis of melanocortin MC receptor features (see Fig. 1) which is in good agreement with previous findings (Haitina et al., 2007a; Klovins et al., 2004a,b). This suggests that the most ancestral forms of the melanocortin MC receptors known today, named melanocortin MC_a and MC_b receptors represent two branches of the ancestral melanocortin MC receptors. The melanocortin MC_a receptor has split into the melanocortin MC_1 and MC_2 receptors perhaps during the whole genome duplications events. The melanocortin MC_b receptor has split into melanocortin MC_3 receptor and an ancestral branch that later split into melanocortin MC_4 and MC_5 receptors. These data are supported by calculations based on the latest phylogenetic methods using the conserved ancestral sequences in sharks and lamprey (see Fig. 2). We have earlier discussed the presence of the intron that is found in both melanocortin MC_2 and MC_5 receptors in Fugu (Klovins et al., 2004a,b). We suggested that these introns are inserted at a late stage independently into these melanocortin MC receptors due to the

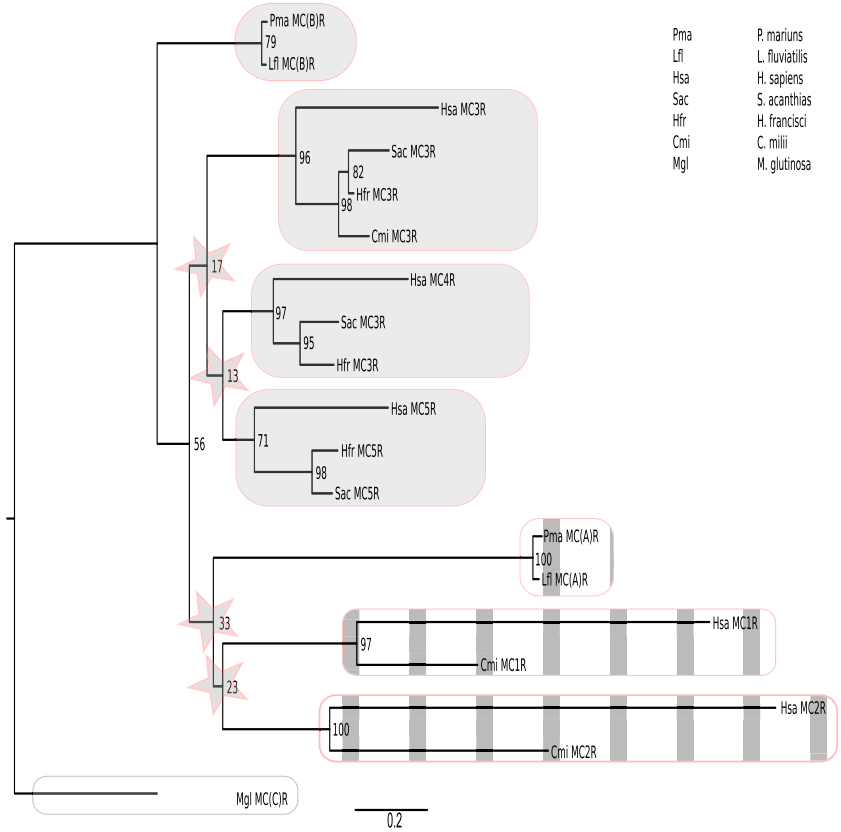

Fig. 2. Phylogenetic tree of melanocortin receptors in human and sharks to determine relationship between lamprey and shark melanocortin receptors, produced using MAFFT-EINSI, RAxML fast & easy maximum likelihood protocol generating 100 bootstrap trees and viewing the averaged tree, without any branch editing, as support vector graphics in FigTree (see Methods). The green stars indicate branches that have bootstrap values <50%. Hagfish melanocortin MC_c receptor is the outgroup. There is a clear division between grey lamprey melanocortin MC_b receptor-derived shark receptors and striped melanocortin MC_a receptor-derived ones; bootstrap support = 56%. Melanocortin MC_2 receptor is, in fact, in the melanocortin MC_a receptor-derived subtree (Baron et al., 2009). The bootstrap values are only 17 and 13%, but indicate that melanocortin MC_3 receptor split from melanocortin $MC_{4/5}$ receptors before melanocortin MC_4 receptor and melanocortin MC_5 receptor separated.

presence of a genetic code in the DRY motif that can form a protosplice site C/A, A, G, R, which is believed to be target site for insertion of spliceosomal introns by a process called reverse splicing (Dibb and Newman, 1989; Dibb, 1991, 1993). There are several examples for this among other G protein-coupled receptors (Fridmanis et al., 2007). The genomic position of melanocortin MC_2 and MC_5 receptors, showing tandem evolution (Schioth et al., 2005a) could suggest that they were formed through local duplication events. However, there is no other evidence to support this and there could be many different genomic reasons for this, which still remains as unexplained phenomena. It is unlikely that melanocortin MC_2 and MC_5 receptors were formed through a whole genome duplication event, as such duplication event would most likely leave the new genes on different chromosomal regions accompanied with synteny to other genes. It is clear that the melanocortin MC_2 and MC_5 receptors are the most divergent members in the melanocortin MC receptors and that the melanocortin MC_4 and MC_5 receptors are the most related members considering the sequences. We thus reiterate our opinion that melanocortin MC_2 and MC_5 receptors did not split directly from a common ancestor as suggested by Baron et al. (2009). The fact that the percentage identity between melanocortin MC_{1-2} receptors in elephant shark and human is lower than the percentage identity between melanocortin MC_{3-5} receptors in human and spiny dogfish is consistent with a higher evolutionary rate in melanocortin MC_{1-2} receptors.

We identified the orthologues to the melanocortin MC_a and MC_b receptors in sea lamprey using the cloned sequences from river lamprey. Lancelet (*Branchiostoma*) contains vast numbers of hypothetical mRNA sequences that appear to code for G protein-coupled receptors, many of which exist in multiple copies (Nordstrom et al., 2008). We identified a cluster of sequences that have the highest similarity to the melanocortin MC receptors. We used different selection criteria but we do not find that any of these G protein-coupled receptor-like sequences to have significant melanocortin MC receptor like properties that are clearly beyond other G protein-coupled receptors in our data sets. There appears to be a sharp drop in sequence identity beyond the cluster of known receptors, including the melanocortin MC receptors in lamprey and hagfish. The closest sequences do not have their DRY motifs intact, and the fact that they cluster more strongly with themselves than a cross-section of melanocortin MC receptors from both sharks and lampreys does not support that they can be at this stage be identified as melanocortin MC receptors. It should also be mentioned in this context that there is a sequence automatically annotated in *C. intestinalis* as a melanocortin MC receptor (GenBank ID: XP_002120969). This sequence is however not similar to the melanocortin MC receptors and should be regarded as false positive hit as melanocortin MC receptors.

Here we show that the elephant shark contains both AgRP and ASIP sequences. We have also presented convincing evidence that these melanocortin MC receptor antagonists are not present in the current versions of the lampreys or lancelet (*branchiostoma*) genomes (see Supplementary Figs. 2 and 3). These data suggest that these sequences in elephant shark are the most ancient AgRP and ASIP sequences identified so far. It is interesting to note that the elephant shark AgRP and ASIP sequences appear ancestral, because both of

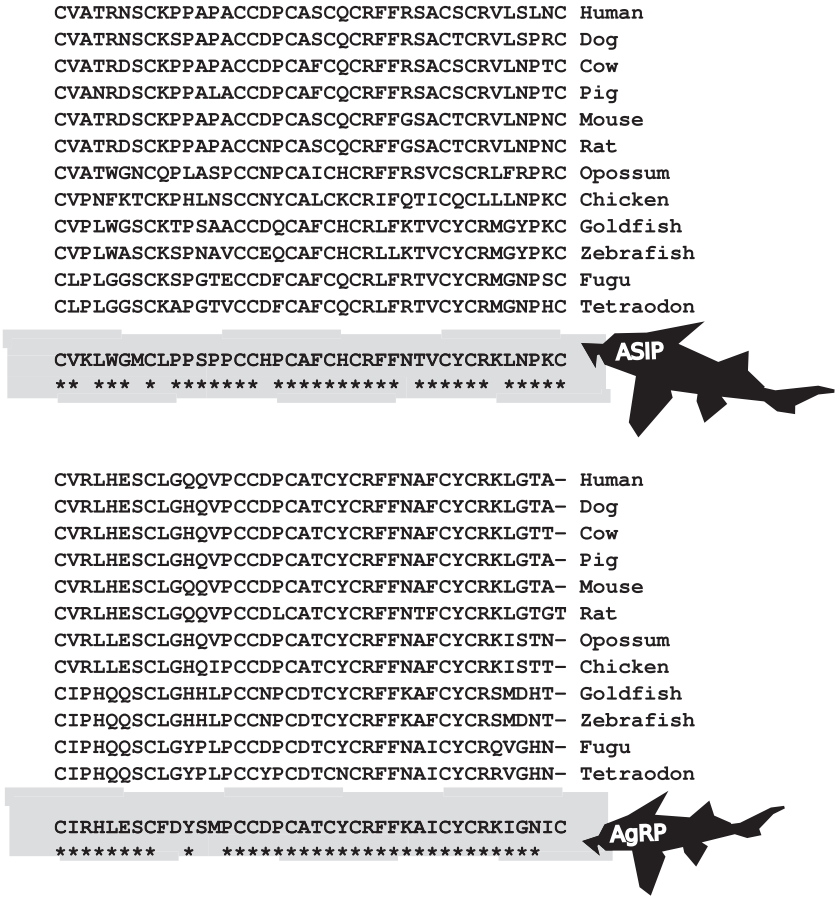

Fig. 3. Receptor binding domain of antagonist sequence: comparison with elephant shark. Shows alignment of receptor binding domain of ASIP and AgRP, including the elephant shark sequence (bottom). The top sequence is human. The following 7 sequences are mammalian in origin. The following 4 sequences are teleost/vertebrate. The alignment is based on a published alignment (Jackson et al., 2006). The receptor binding domains converge in elephant shark: AgRP contains residues that are ASIP-like and ASIP contains residues that are AgRP-like. Both ASIP and AgRP receptor binding domains have mammalian-like residue positions. There is an unexpected cysteine at the end of the AgRP receptor binding domain. The upstream portion of the ASIP alignment can be found in Supplementary Fig. 1. The stars indicate positions in the elephant sequence that are common with any of the other model organisms.

them display converging features that are mixtures of the mammalian AgRP and ASIP like features. These crossovers between ASIP and AgRP-like features in the shark genes can be seen in Fig. 3. It is also interesting that they appear more mammalian-like than their teleost counterparts, a feature also shown for the melanocortin MC receptors, highlighting the importance of sharks as an excellent model for study evolution of these genes. We developed new bioinformatics tools to search for cysteine knot-like sequences (see Supplementary Figs. 2 and 3). Interestingly we found a vast numbers of putative cysteine knot-like sequence in lancelet (see Supplementary Fig. 3). Based on this it is possible to speculate that AgRP/ASIP-like sequences could have originated from ancestors to such sequences, rather than from the wolf spider toxin sequences that are marked up as having Agouti domains in Pfam (see ACI41437).

Prior to this study, the public databases lacked manually verified, ancestral-like sequences for MRAP, except for an entry in zebrafish for one of the two subtypes. Agulleiro et al. (2010) had shown that both subtypes of MRAP exist in *T. rubripes* (Agulleiro et al., 2010). In this study, we have found MRAP2 in elephant shark and sea lamprey. We found convincing evidence for the absence of MRAP1 in elephant shark, and we also present evidence for the absence of MRAP2 in lancelet. Fig. 1 illustrates these findings that the MRAP2 is the more ancient subtype, while MRAP split from MRAP2 at a later stage. Taken together these findings also illustrate the importance of these model organisms in the emergence of the melanocortin system.

Looking through the sequence motifs in these sequences we have made some observation that may provide leads to further studies. It is notable that there exist features that could give the impression that ASIPs and MRAPs could be related. These genes share a 'SIV' motif, three coding exons, similar length, and both emergence first in the elephant shark. Any such relationship can however not been shown in the full-length alignments. Moreover, fact that the "SIV" motif is on different sides of the splice site in ASIP and MRAP would weaken the hypothesis that they are related, if we assume that splicing profiles are more evolutionary conserved than sequence similarity for orthologues (Vastermark et al., 2004). In ASIP, the SIV motif is found in the centre of the regulatory conserved domain (Jackson et al., 2006). In MRAPs, it is found on the border between the domain necessary for receptor trafficking (Webb et al., 2009) and the only transmembrane domain of MRAP. Before the splice site, in MRAP, this motif is preceeded by a DRY-like sequence. Given that both ASIP and MRAPs interact with the melanocortin receptors, it is possible that this interaction is partially exerted through the 'SIV' motif. The conservation of these sequences motif could suggest that they are essential for basic function of the proteins and it is possible that ASIP and MRAP both use this in receptor interactions. PDB models of the antagonists are limited to the receptor binding domain (1Y7J, 1Y7K, 1MR0, 1HYK). A correlation study between segments of ASIP and the receptor that are co-evolving, could be an interesting way to use ancestral sequences to infer functional relationships.

In summary we have extended the repertoire of melanocortin like sequences and these are summarized in Table 1. We find that the new sequences and our detailed analysis of the evolutionary end points for the different genes have provided additional clarification in the appearance of the melanocortin system (see Fig. 1). The studies highlight the importance of cartilaginous fishes (sharks) and jawless vertebrates (lamprey and hagfish) as important lineages for studies of the early origin of the melanocortin system.

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