Role of melanocortin receptor accessory proteins in the function of zebrafish melanocortin receptor type 2

Maria Josep Agulleiro [a,1], Simon Roy [b,1], Elisa Sánchez [a], Sara Puchol [a], Nicole Gallo-Payet [b,2], José Miguel Cerdá-Reverter [a,*,2]

[a] Department of Fish Physiology and Biotechnology, Instituto de Acuicultura de Torre de la Sal, Consejo Superior de Investigaciones Científicas, Ribera de Cabanes, Castellón, Spain
[b] Service of Endocrinology, Department of Medicine, University of Sherbrooke, Sherbrooke, Québec, Canada

ABSTRACT

In this paper, we identify three different MRAPs in zebrafish, zfMRAP1, zfMRAP2a and zfMRAP2b, and demonstrate that zfMC2R is not functional in the absence of MRAP expression. ZfMRAP1 expression was restricted to adipose tissue and the anterior kidney whereas MRAP2a and MRAP2b were expressed in all the tissues tested. Quantification of surface receptor and immunofluorescence studies indicated that the receptor is unable to translocate to membrane in the absence of MRAP isoforms. MRAP1 and MRAP2b are localized in the plasma membrane in the absence of zfMC2R expression but MRAP2b is retained in perinuclear position. MRAP1 and MRAP2a displayed an equivalent translocation capacity to the membrane of zfMC2R but only zfMRAP1 expression led to intracellular cAMP increases after ACTH stimulation. ZfMRAP2b had no effect on zfMC2R activity but both zfMRAP2 isoforms enhanced the zfMRAP1-assisted cAMP intracellular increase, suggesting an interaction between zfMRAP1 and zfMRAP2s when regulating zfMC2R activity.

1. Introduction

Melanocortin 2 receptor (MC2R) is the smallest member of a G protein-coupled receptor subfamily, which includes five receptors (MC1R–MC5R). These receptors are involved in the regulation of diverse physiological functions, including skin pigmentation, stress response, metabolism, energy balance and exocrine secretion (Cone, 2006). MC2R is adrenocorticotropic hormone (ACTH)-specific, whereas the other four MCRs distinctively recognize MSHs (Schiöth et al., 2005). ACTH is posttranscriptionally processed from proopiomelanocortin (POMC) after tissue-specific cleavage in the corticotrophs of the anterior pituitary (Castro and Morrison, 1997), and secreted in response to hypothalamic corticotropin-releasing factor (CRF). ACTH binding to MC2R increases intracellular cAMP, thus stimulating steroidogenesis and protein synthesis in the adrenal gland (Sewer and Waterman, 2003). The expression of the MC2R mRNA is mainly restricted to the adrenal cortex and adipose tissue (Mountjoy et al., 1992; Boston and Cone, 1996) and

is a key point in the peripheral response to stress (Dallman et al., 2004).

Unlike in the case of other melanocortin receptors, the study of MC2R activation has been constrained because of the lack of a readily transfectable heterologous expression system. Suitable expression data have been obtained only in adrenocortical-derived cell lines (Y6 or OS3), in which endogenous MCR expression is absent (Schimmer et al., 1995). The expression experiments of MC2R in a range of non-adrenal cells suggested a common explanation for failed functional expression in that the receptor cannot reach plasma membrane and is retarded in the endoplasmic reticulum. This explanation involved the presence of an accessory factor that works as an MC2R-specific transport system in the adrenocortical cells (Noon et al., 2002). One gene candidate that encodes for a small single-transmembrane domain protein with high expression in the adrenal cortex was identified in humans, showing familial glucocorticoid deficiency (FGD) and no MC2R mutations. This syndrome is characterized by resistance to ACTH, i.e. high plasma ACTH levels but lacking adrenal glucocorticoids. The protein is now known as melanocortin 2 receptor accessory protein (MRAP), even though alternative splicing of the last two exons gives rise to two isoforms that differ in the C-terminal region (MRAPα and MRAPβ) (Metherell et al., 2005). Knockdown of endogenous mouse MRAP in Y1 adrenocortical cells, which express a functional endogenous MC2R, leads to insensitivity to ACTH, demonstrating that MRAP is essential for producing an ACTH responsive MC2R (Cooray et al., 2008). MRAP interacts with the MC2R to facilitate correct folding,

* Corresponding author at: Department of Fish Reproductive Physiology, Instituto de Acuicultura de Torre de la Sal, 12595 Torre de la Sal, Ribera de Cabanes, Castellón, Spain. Tel.: +34 964319500; fax: +34 964319509.
  E-mail addresses: cerdarev@iats.csic.es, cerdarev@iats.csic.es (J.M. Cerdá-Reverter).
  [1] Both authors have contributed equally to this study.
  [2] Co-senior authors.

and subsequent glycosylation and receptor cell surface expression (Metherell et al., 2005) but they are also essential for ACTH binding and ACTH-induced cAMP production (Sebag and Hinkle, 2009a; Roy et al., 2007). Recent experiments have identified a second form of MRAP in the human genome called MRAP2. This protein shares the structural characteristics of MRAP but is expressed only in the human brain and adrenal gland. Similar to MRAP1, MRAP2 assists MC2R cell surface expression and allows the ACTH-induced cAMP production (Sebag and Hinkle, 2009a; Chan et al., 2009).

Our preliminary experiments expressing sea bass MC2R in HEK-293 cells failed to generate an ACTH-stimulated cAMP increase. However, when human MRAPs were co-transfected, the sea bass MC2R was able to mediate ACTH-induced cAMP production (Agulleiro MJ, Sánchez E, Puchol S, and Cerdá-Reverter, unpublished results), suggesting the presence of a conserved MC2R trafficking system in non-mammalian vertebrates. Taking advantage of the genomic facilities available for zebrafish, we report here the characterization of three different zebrafish MRAPs (zfM-RAP), which are essential for the translocation of zfMC2R to the plasma membrane and the formation of a functional ACTH responsive receptor.

2. Material and methods

2.1. Animals and reagents

Wild-type zebrafish were purchased from local suppliers and maintained at the facilities of the Instituto de Acuicultura de Torre la Sal under standard conditions. Animals were anesthetized in 2-phenoxy-ethanol (0.1%) for 4 min prior to sacrifice by rapid decapitation. All experiments were carried out in accordance with the principles published in the European animal directive (86/609/CEE) concerning the protection of experimental animals and were approved by the internal committee. Unless otherwise indicated, all reagents were purchased from Sigma.

2.2. Identification of MC2R and MRAP genes

Related MRAP sequences in zebrafish were identified from the databases found at http://www.ncbi.nlm.nih.gov. Tblastn searches on the databases using the fragment (11–58) of the human MRAP1a provided three different hypothetical proteins (XP_001345808.1, for zfMRAP1, XP_001342923.2 for zfMRAP2a and XP_001344880.1 for zfMRAP2b) with low identity to human MRAP1a. These sequences were used to identify potential orthologue genes in the genomes of different species using tblastn searches on available databases at http://www.ensembl.org/index.html

and http://www.ncbi.nlm.nih.gov/Genbank/. The following proteins were identified and retrieved form databases: DW031694 and ENSGACP00000012524 for three-spined stickleback (*Gasterousteus aculeatus*) MRAP1 and MRAP2, respectively, ENSTRUP00000046738 and FUGU4:scaffold_19:1:2116366:1 for pufferfish (*Takifugu rubripes*) MRAP2 and MRAP1, respectively, AM953338 for gilthead seabream (*Sparus aurata*) MRAP1, DY628664 for African cichlid fish (*Haplochromis burtoni*) MRAP1, GR635328 for Nile tilatia (*Oreochromis niloticus*) MRAP1, ENS-MUSP00000108774 for mouse (*Mus musculus*) MRAP2, ENSRNOP00000014006 for rat (*Rattus norvergicus*) MRAP2, ENSFCAP00000006314 for human (*Homo sapiens*) MRAP2, ENSCAFP00000004413 for dog (*Canis familiaris*) MRAP2, ENS-GALP00000025492 for chicken (*Gallus gallus*) MRAP2, ENSXETP00000039367 for pipid frog (*Xenpus tropicalis*) MRAP2, ENSORLP00000016093 for medaka (*Orizyas latipes*) MRAP2, ENSRNOP00000030039 for rat MRAP1, ENSP00000382684 for human MRAP1a, ENSP00000343661 for human MRAP1b, ENSMUSP00000043890 for mouse MRAP1, ENSCAFP00000013046 for dog MRAP1. Chicken MRAP1 amino acid sequence was retrieved from genbank database (XP_416703.1). Zebrafish MC2R (zfMC2R) sequence was obtained from (Logan et al., 2003), accession number AY161848.

2.3. Molecular cloning of zfMC2R and zfMRAPs and tissue expression studies

The zfMC2R and zfMRAPs were amplified by RT-PCR. Total RNA was extracted form the whole animal using Trizol (Invitrogen) and treated with RQ1-DNAse (Promega). Superscritp II reverse transcriptase (Invitrogen) was used for cDNA synthesis by priming with random hexaprimers (Invitrogen). The cDNA was subsequently used as template for PCR reactions with Taq DNA polymerase (Invitrogen) and specific primers (see Table 1 for sequences). PCR products were isolated from agarose gels and ligated into pGEM-T easy vector (Promega). One clone that contained an insert of expected size was sequenced on both strands.

For tissue expression experiments, cDNA was synthesized as above from RNA purified from fresh tissues (muscle, intestine, liver, gill, brain, testis, ovary, eye, posterior kidney, heart, spleen, skin, fat, anterior kidney and whole fish) or HEK-293/FRT cells and PCR fragments for each gene separated onto 1.2% agarose gel (see Table 1 for primer sequences). As internal control of the reverse transcription step, PCR for β-actin mRNA was carried out.

2.4. DNA constructs

The full coding regions of the zebrafish genes were released from pGEM-T easy vector and subcloned directionally into Hind III/Xho I restricted pcDNA3 (Invitrogen). Different N- or C-terminal epitope tagged proteins (MRAPs and MC2R) were made by PCR using Phusion® DNA Polymerase (Finnzymes) and pcDNA3/zfMRAP1, pcDNA3/zfMRAP2a, pcDNA3/zfMRAP2b, pcDNA3/zfMC2R constructs as templates. Proteins were N- or C-terminally tagged with Flag (DYKDDDDKC) or c-Myc (EQK-LISEEDL) epitopes. The expected size products were cloned directionally into Hind III and Xho I restricted pcDNA5/FRT (*Flp* recombinase target) vector, and sequenced (see Table 1 for primer sequences).

Table 1
Primers used for RT-PCR cloning of zfMC2R and zfMRAPs.

| Primer	Sequence (5′–3′)
HindIII_zfMC2R_fw	TATAAGCTTATGAACCCCAGCGCTGAATC
XhoI_zfMC2R_rev	TTACTCGAGTCAAGCACACCGCTTGCAGA
HindIII_zfMRAP2a_fw	TATAAGCTTATGCCGAGGTTCCAGCTTTC
XhoI_zfMRAP2a_rev	TTACTCGAGATCAATGATATGATGAGTACAGC
HindIII_zfMRAP2b_fw	TATAAGCTTATGAGTGAATATTCAAACCGC
XhoI_zfMRAP2b_rev	TTACTCGAGTCAATGTCTGATGATATCATAG
HindIII_zfMRAP1_fw	TATAAGCTTATGAAAAACTCCTCAGAATACG
XhoI_zfMRAP1_rev	TTACTCGAGTTACTGAGATGATGCATAACC
zfβAct_fw	CTCTTCCAGCCTTCCTTCCT
zfβAct_rv	CTTCTGCATACGGTCAGCAA
hMRAP1_fw	GGAAAAGTGCCACAGACATG
hMRAP1_rev Reverse	AGGCGGACCAGGACATGTAG
hMRAP2_fw	AGCAATCGGCATCTAATTCTGAT
hMRAP2_rev Reverse	GTTGGACGTCACTGTCAAGG
Hind/Myc-zfMC2R S	TAACTTAAGCTTACCATGGAACAAAAACTCATCTCAGAAGAGGATCTGATGAACCCCAGCGCTGAATC
zfMC2R/SalI	TAAGCAAGACACATGTCGAC TCAAGCACACCGCTTGCAG
h/k/Flag/zfMRAP2a S	TGTCAACTAAGCTTACCATGGATTACAAGGATGATGATGATAAGTGTATGCCGAGGTTCCAGC
zfMRAP2a/s AS	TTGTCTATGTCGACTCAATCAATGATATGATGAGTAC
h/k/Flag/zfMRAP2b S	TGTCAACTAAGCTTACCATGGATTACAAGGATGATGATGATAAGTGTATGAGTGAATATTCAAAC
zfMRAP2b/s AS	TTGTCTATGTCGACTCAATGTCTGATGATATCATAG
h/k/Flag-zf1 S	TGTCAACTAAGCTTACCATGGATTACAAGGATGATGATGATAAGATGAAAAACTCCTCAG
zf1/s AS	TGTCTATGTCGACTTACTGAGATGATGCATAAC
h/k/zf1 S	TGTCAACTAAGCTTACCATGAAAAACTCCTCAGAATACGTGTGGGGCTATG
zf1-Flag/s AS	TGTCTATGTCGACTTACTTATCATCATCATCCTTGTAATCCTGAGATGATGCATAAC
h/K/MRAP2b S	TTGTCAACTAAGCTTACCATGAGTGAATATTCAAACC
zfMRAP2b/Flag/s AS	TTGTCTATGTCGACTCAGCACTTATCATCATCATCCTTGTAATCATGTCTGATGATATCATAG
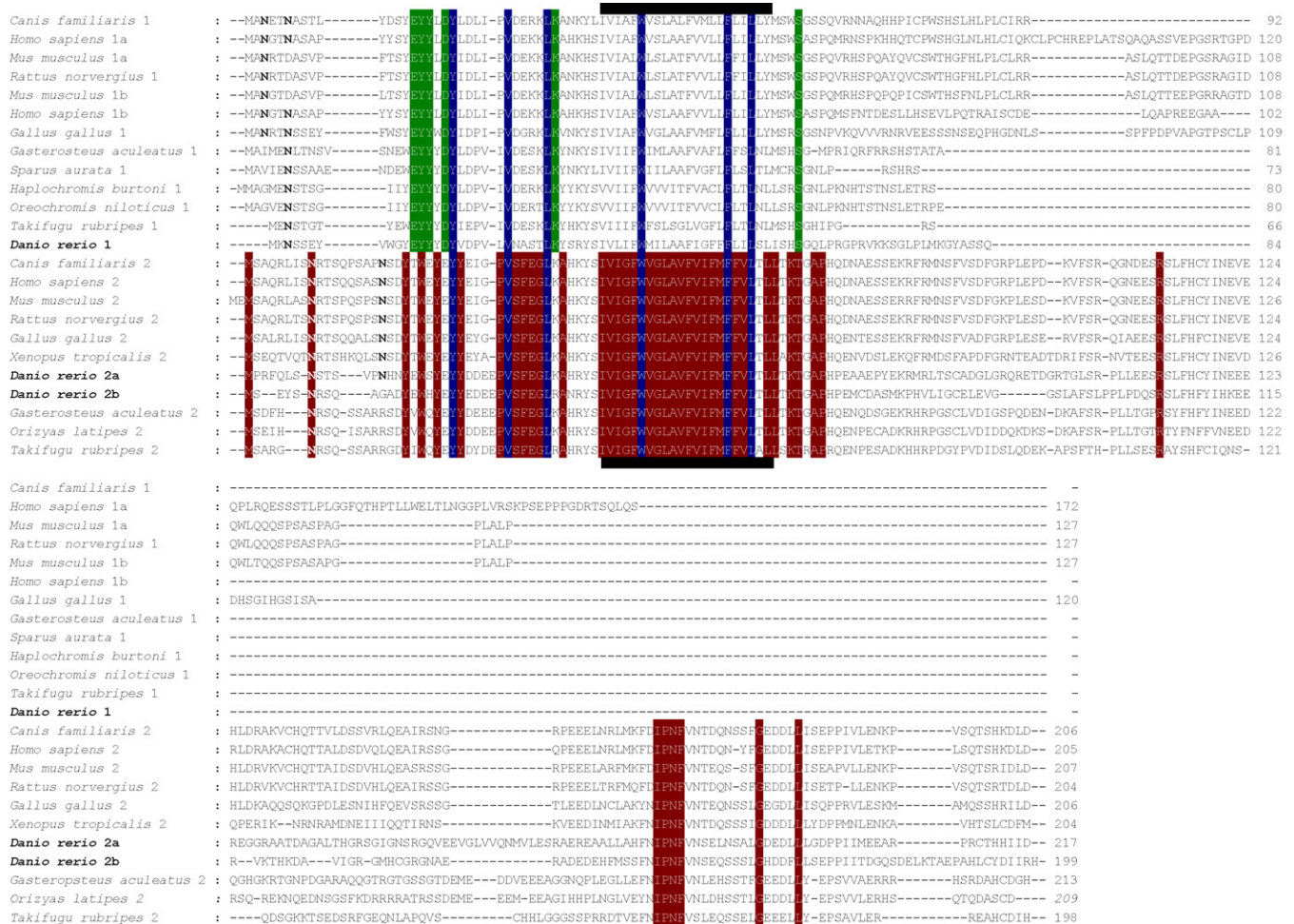
Fig. 1. Alignment of MRAP amino acid sequences. Amino acid numbers are indicated on the right. Dashes were introduced to improve alignment. The black bar shows putative transmembrane domains. Bold amino acids indicate consensus sequence for N-linked glycosylation sites. Residues in blue are identical in all sequences, while residues in green or red are identical in all MRAP1 or MRAP2 sequences, respectively.

2.5. Cell culture, transfection and stable cell lines

Flp recombinase-mediated homologous recombination system (Flp-InTM) was used to produce cells lines stably expressing N-terminus c-Myc tagged zfMC2R (Roy et al., 2007). The development of isogenic cell lines was carried out according to the manufacturer recommendations. The HEK-293/FRT cell line (HEK293 cell line with single genome-integrated FRT) was maintained in DMEM supplemented with 7% fetal bovine serum (Invitrogen) and 2 mM GlutaMAX (Invitrogen) at 37 °C, and 5% CO$_2$. Cells were transfected with 0.5 μg of the DNA specific construct using lipofectamine and PLUS reagent (Invitrogen). Cells were subsequently selected with 100 μg/ml zeocine and negatively selected to confirm the insertion into the genomic FRT site only with hygromicin at 100 μg/ml. *Flp* system ensures the insertion of only one copy of recombinant pcDNA5/FRT into the FRT genomic site of native HEK-293/FRT cells to generate cell lines that show isogenic expression levels. Cell lines developed for this study were named as 293/FRT/Myc-zfMC2R. Functionality and epitope tag detection of each construction was confirmed by immunofluorescence and cAMP production (see below).

2.6. Cell surface ELISA

To measure cell surface receptor expression, 293/FRT/Myc-zfMC2R cells were seeded in poly-L-lysine-coated 24-well plate (1×10^5 per well) and transfected independently with pcDNA5/FRT/zfMRAP1, pcDNA3/zfMRAP2a or pcDNA3/zfMRAP2b with 0.125 μg DNA/well. Twenty-four hours after transfection, cells were washed with phosphate saline buffer (PBS), fixed on ice for 15 min with 1.85% formaldehyde to evaluate the presence of the receptor in the plasma membrane, or for 5 min with methanol for total receptor measurements. Cells were then processed for ELISA as previously described (Roy et al., 2007). Non-specific OD$_{492}$ values were determined by transfecting the untagged versions of each construct when possible or with EGFP. Positive controls included human Myc-MC2R or human MRAPβ-Flag as required. Experiments were repeated three independent times in triplicate.

2.7. Immunofluorescence microscopy

293/FRT/Myc-zfMC2R cells grown onto poly-L-lysine-coated coverslips were transiently transfected with 0.5 μg/well of zfMRAP1-Flag, Flag-MRAP1, Flag-MRAP2a, MRAP2b-Flag and Flag-MRAP2b constructs. Twenty-four hours later, cells were fixed and permeabilized by incubation in methanol for 5 min and subsequently in acetone for 1 min (Roy et al., 2007). Cells were rehydrated, washed in PBS, blocked and incubated with mouse monoclonal anti-c-Myc and/or rabbit polyclonal anti-Flag antibodies. Primary antibodies were detected with goat anti-mouse or anti-rabbit secondary antibodies coupled to Alexa-Fluor488 or Alexa-Fluo647 (Invitrogen) as required. DAPI (2 μM) was used to stain nuclei. Coverslips were mounted in VectaShield mounting medium for fluorescence (Vector Laboratories). Cells were also examined with a laser-scanning confocal microscope and a 60× oil objective (Olympus URFL-T, MAG Biosystems). All images were acquired in order to occupy the widest dynamic range of grey tones as possible (0–4095 tones) and images were analyzed as previously described (Battista et al., 2009).

2.8. cAMP measurements

Intracellular cAMP production was determined in HEK-293/FRT native or 293/FRT/Myc-zfMC2R cells independently transfected with 0.5 μg of pcDNA5/FRT/zfMRAP1, pcDNA3/zfMRAP2a or pcDNA3/zfMRAP2b constructs after stimulation with human ACTH (1–24) or NDP-MSH. Experiments were always made 24 h post-transfection. Conversion of [^{3}H]ATP to [^{3}H]cAMP was measured by separation of [^{3}H]cAMP from [^{3}H]ATP by chromatography on Dowex and alumina columns. cAMP formation was calculated as % conversion = [$100 \times$ [^{3}H]cAMP/([^{3}H]cAMP + [^{3}H]ATP)]. Positive controls included hMC2R or MRAPβ-tranfected cells as required and EGFP transfection was used as negatives. Results are the mean ± SEM of three independent experiments performed in duplicate.

2.9. Data analysis and statistics

Sequence comparisons and alignments were performed using public domain ClustalX. A phylogenetic tree was derived using CulstalX (Larkin et al., 2007), which uses the Neighbor-Joining method on a matrix of distances. Receptor activation data were fitted using GraphPad Prism 5.0. Statistical analysis was conducted by one-way analysis of variance, followed by the Holm–Sidak test.

3. Results

3.1. Identification of zebrafish MC2R and MRAPs

Zebrafish MC2R was cloned by RT-PCR using specific primers designed according to the receptor sequence previously reported (Logan et al., 2003). The amino acid alignment of the cloned zfMC2R exhibited eight substitutions (S88L, G110S, K169T, L225K, I226K, P250Q, Y277N and M291Q) when compared against the published sequence (accession number NM_180971). An intensive search in the Genbank database revealed that the clone DKEY-181C1 in linkage group 16 of the zebrafish genome (accession number CR848742) includes a different version of the published zfMC2R. The last protein exhibit only one substitution V99I with the sequence here reported (data not shown).

BLASTP searches using the (11–58) fragment of the human MRAP1a provided three different hypothetical proteins with low identity to tetrapod MRAPs. The phylogenetical analysis constructed on the basis of the amino acid sequence alignments (Fig. 1) showed that XP_001345808.1 (zfMRAP1) clustered with tetrapod MRAP1 sequences whereas XP_001342923.2 (zfMRAP2a) and XP_001344880.1 (zfMRAP2b) proteins clustered together with the MRAP2 sequences (Fig. 2). We also found MRAP2 orthologue genes in the genome of dog, chicken, pipid frog, medaka, pufferfish and three-spined stickleback. However, no MRAP1 orthologue genes were found in fish other than zebrafish or non-mammalian species other than chicken. The zfMRAP1 is more similar to MRAP1 (17–33%) than MRAP2 sequences (10–12%). Also, zfMRAP2s shares more identity with MRAP2 (37–47%) than MRAP1 sequences (12–16%). Most of the sequence divergence was located within the C-terminal region of the peptide. When the region following the transmembrane domain was removed from the analysis, the identity of zfMRAP1 to the tetrapod MRAPs ranged from 38 to 55% whereas zfMRAP2s identity to remaining MRAPs ranges from 62 to 76%. However, it is noticeable that five aminoacids within the C-terminal region (^{178}IPNFV^{182}, zfMRAP2a numbering) are fully conserved in all MRAP2 sequences.

Similar to tetrapod MRAPs, all three zebrafish sequences display putative glycosylation sites within the N-terminal region, a tyrosine-rich N-terminal region that includes the conserved motif YEYY (Fig. 1) and a putative 23-amino acid hydrophobic transmembrane domain (residues 34–56 zfMRAP1 numbering) which is fully conserved for MRAP2s. A consensus pattern for glycosaminoglycan attachment site is also present in zfMRAP2a (^{141}SGIG^{144}).

3.2. Tissue expression patterns

RT-PCR with specific primers resulted in bands of the expected size (Fig. 3). The zfMC2R was expressed in the testis, ovary, spleen, fat and anterior kidney, with minor levels in the skin. The expression of zfMRAP1 was restricted to fat tissue and anterior kidney, whereas the expression of zfMRAP2 s was detected in all tested tissues. Inverse transcriptions and cDNA quality were corroborated by PCR amplification of β-actin. The cDNA made from total RNA from the whole fish was used as positive control. Water or total RNA after cDNA synthesis without reverse transcriptase, were used as negative controls. In addition, RT-PCR experiments demonstrated that HEK293/FRT cell line endogenously express hMRAP2 mRNA but no MRAP1 mRNA (data not shown).
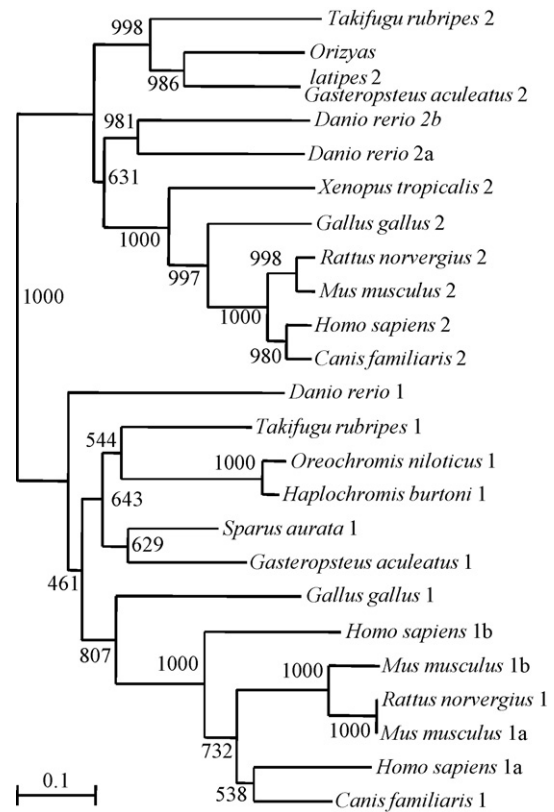

Fig. 2. Phylogenetic tree of MRAPs amino acid sequences built using CulstalX, which uses the Neighbor-Joining method on a matrix of distances. Numbers at branch nodes represent the confidence level of 1000 bootstrap replications. The accession numbers of the sequences are described in Section 2.

3.3. Influence of zfMRAPs on the plasma membrane expression of zfMC2R

The levels of zfMC2R in the cell surface of 293/FRT/Myc-zfMC2R cells were similar to that observed in native 293/FRT cells (Fig. 4A). The N-terminal c-Myc epitope was detected in the surface of 293/FRT/Myc-zfMC2R cells only when zfMRAP1 or zfM-RAP2a were transfected (Fig. 4A). However, MRAP2b transfection did not increase the levels of cell surface receptor. Immunofluorescence assays performed on 293/FRT/Myc-zfMC2R transiently
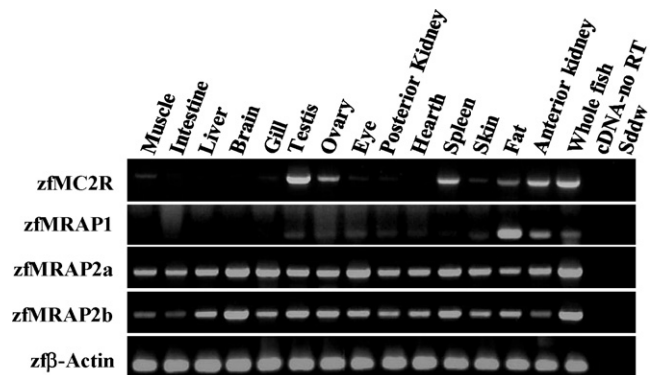

Fig. 3. Distribution of zfMC2R and zfMRAPs mRNA expression in different tissues, as revealed by RT-PCR. Ethidium bromide-stained agarose gels showing zebrafish receptor and accessory proteins. Amplifications of β-actin mRNA were used as internal control of the reverse transcription.
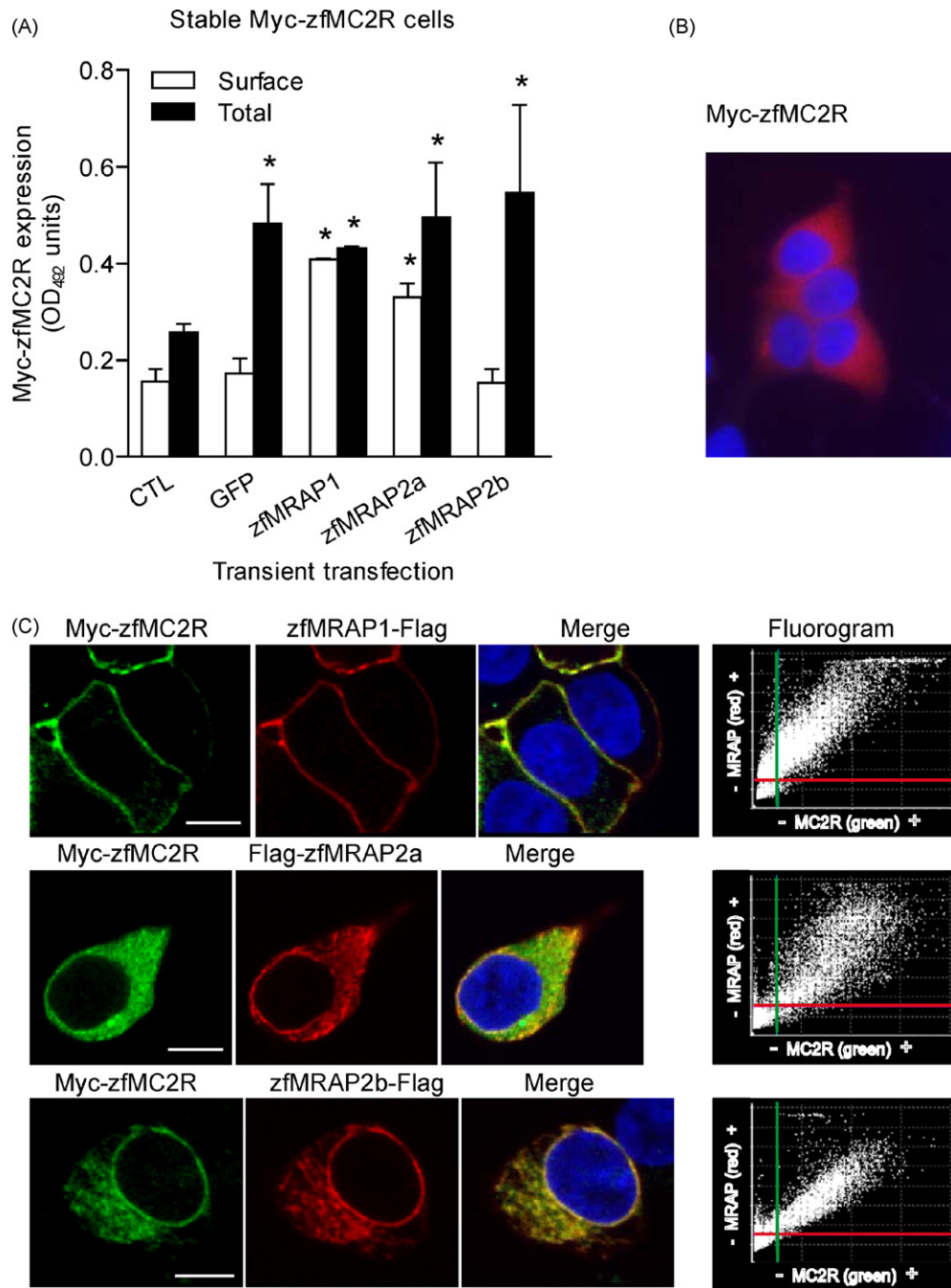
(A)

Stable Myc-zfMC2R cells

(B)

Myc-zfMC2R

(C)

Fig. 4. (A) Total and cell surface detection of Myc-zfMC2R using anti-c-Myc antibodies. Control corresponds to non-transfected HEK-293/FRT cells. Stable 293/FRT/Myc-zfMC2R cells were transiently transfected with pEGFP (zfMC2R), pcDNA5/FRT/zfMRAP1, pcDNA3/zfMRAP2a, and pcDNA3/zfMRAP2b and assayed for total and extracellular c-Myc detection by whole-cell ELISA. The results represent the mean ± SEM of three independent experiments, each performed in triplicate. Asterisk shows significant differences after one-way ANOVA and Holm–Sidak method ($P < 0.05$). (B) Immunofluorescence assays in live cells showing the cytoplasmatic expression of zfMC2R in 293/FRT/Myc-zfMC2R cells in absence of MRAPs expression. (C) Immunofluorescence assays in live cells showing the expression of MRAPs (green pseudo-color) and/or zfMC2R (red pseudo-color) in 293/FRT/Myc-zfMC2R cells. Nuclei are counterstained in blue. C- or N-terminally Flag tagged MRAPs were transiently expressed in 293/FRT/Myc-zfMC2R cells. Photomicrographs are taken with a 60× objective and are from a single optical section obtained within an acquisition of z-stacks (0.44 μm/slice) to enable 3D reconstructions of the cells (not shown). Colocalization fluorograms based on 3D reconstructions are shown in the right column. The x-axis of fluorograms is representative of green-labeling and the y-axis is representative of red staining. Colocalization between green-labeling (zfMC2R) and red-labeling (zfMRAPs) voxels are shown as being proportional to each other in fluorograms and correspond to the yellow color in merged slices.

transfected with N- or C-terminal tagged zfMRAPs with Flag, corroborated data obtained by ELISA (Fig. 4B and C). Experiments showed that zfMC2R was exclusively located in the cytoplasm of non-transfected 293/FRT/Myc-zfMC2R cells (Fig. 4B). In the presence of zfMRAP1, zfMC2R was targeted at the plasma membrane, where it colocalized with zfMRAP1 (Fig. 4C). ZfMRAP2a was also able to target zfMC2R at the plasma membrane; however, a large

amount of the receptor remained in the cytoplasm, suggesting that cell surface expression of zfMC2R (Fig. 4C) depends on the interaction with zfMRAP1. On the other hand, co-transfection of MRAP2b with zfMC2R induced intracellular colocalization (including the perinuclear region), but, in these conditions, zfMC2R was not expressed at the plasma membrane (Fig. 4C). As shown in the fluorograms in Fig. 4C, a linear relationship of colocalized voxels
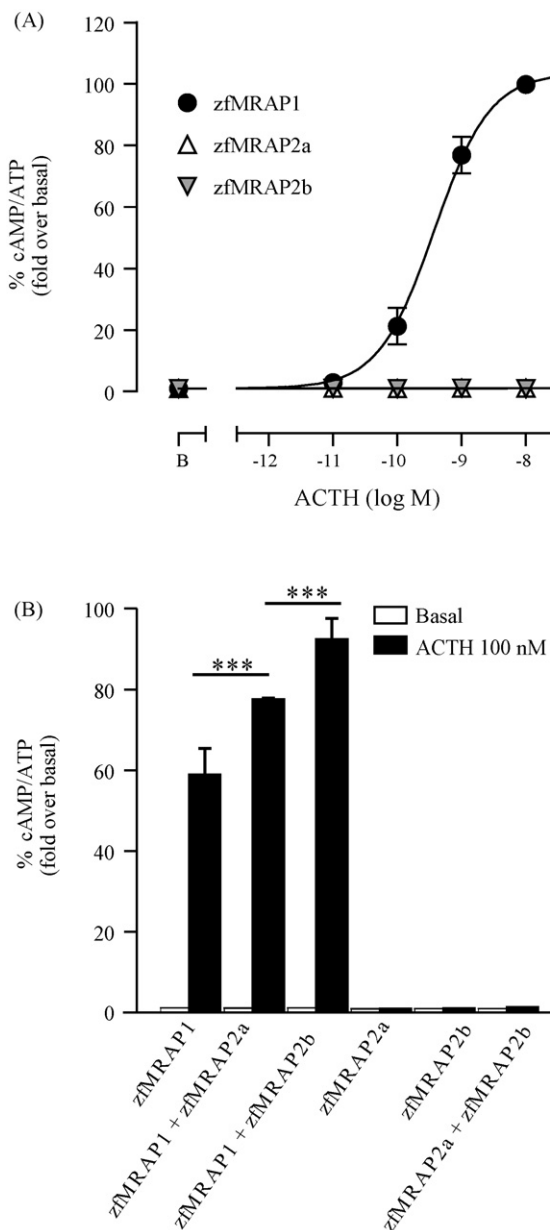
Fig. 5. Cyclic AMP production in 293/FRT/Myc-zfMC2R cells transiently transfected with different zfMRAPs. (A) Cells were stimulated with ACTH ranging from 10 pM to 100 nM for 15 min in the presence of 1 mM IBMX. Results represent the mean ± SEM of three independent experiments, each performed in duplicate. (B) Cyclic AMP production in 293/FRT/Myc-zfMC2R cells transiently transfected with different combinations of zfMRAPs. Cells were stimulated with 100 nM ACTH for 15 min in the presence of 1 mM IBMX. Results represent the mean ± SE of two independent experiments, each performed in duplicate. Statistical analysis was conducted by one-way analysis of the variance followed by Holm–Sidak test (***$P < 0.001$).

was observed in the upper and the lower panels, while a more dispersed distribution of the voxels was observed in the central panel. In addition, N-terminal or C-terminal tagging of MRAPs did not modify receptor or MRAP trafficking to plasma membrane (data not shown).

3.4. Receptor signaling

cAMP levels in HEK-293/FRT cells stably transfected with Myc-zfMC2R showed no differences from cAMP values recorded in native HEK-293/FRT after stimulation with 100 nM ACTH. Similar results were obtained after transient transfection of

MRAPs and subsequent stimulation with 1 μM NDP-MSH (data not shown). Only 293/FRT/Myc-zfMC2R cells transiently transfected with zfMRAP1 (Fig. 5A) displayed a dose–response increase in cAMP levels after stimulating with ACTH (EC$_{50}$ = 0.373 nM). Transfection of both zfMRAP2a and zfMRAP2b did not enable ACTH-induced cAMP production. In order to further investigate zfMRAP2a and zfMRAP2b function, different zfMRAPs combinations were transiently co-transfected in 293/FRT/Myc-zfMC2R and stimulated with 100 nM ACTH (Fig. 5B). Both zfMRAP2a and zfMRAP2b significantly increased ACTH-stimulated cAMP production in 293/FRT/Myc-zfMC2R cells transiently expressing zfMRAP1. The effect of zfMRAP2b on zfMRAP1-stimulated cAMP production was significantly higher than observed with zfMRAP2a.

4. Discussion

MRAP is a small single-transmembrane domain protein, which is required for the functional expression of MC2R. MRAP interacts with MC2R, promoting trafficking to the cell surface. In previous experiments with sbMC2R, we observed a lack of intracellular cAMP increase after ACTH stimulation when the receptor was expressed in HEK-293 cells. However, sbMC2R was completely functional when human MRAP1s where coexpressed in the same cellular system. Therefore, we hypothesized the evolutionary conservation of the system MC2R/MRAP in fish. In this paper, we report the molecular characterization of three different zfMRAPs, i.e. zfMRAP1, zfMRAP2a and zfMRAP2b, and demonstrate that zfMRAP1 is essential for trafficking to the cell membrane and signaling of zfMC2R. In addition, while zfMRAP2a is able to mediate zfMC2R trafficking to the plasma membrane, it did not enable ACTH responsiveness. Furthermore, even if both zfMRAP2a and 2b had no effect on zfMC2R function by themselves, their presence enhanced cAMP production when coexpressed with zfMRAP1.

Tblastn searches in fish databases identified three different proteins in the zebrafish showing similarity to mammalian MRAPs. After phylogenetic analysis, one protein clustered with tetrapod MRAP1, whereas the remaining two grouped with MRAP2. We also found MRAP2 orthologues in all the fish genomes screened but only zebrafish exhibited MRAP2 paralogues. An additional genome duplication process in teleost fish supports the presence of extra genes in this lineage (Amores et al., 1998). We also found MRAP1 orthologues in several fish species. Zebrafish proteins displayed similar structural features to mammalian MRAPs—no putative signal peptide, a single predicted hydrophobic transmembrane domain, a tyrosine-rich region within the N-terminal domain and putative glycosylation sites in the N-terminal region (Hinkle and Sebag, 2009). The transmembrane domain is entirely conserved in all MRAP2, except one amino acid in pufferfish, however the identity is much lower (39%) in MRAP1 sequences. Experiments in humans have demonstrated that the transmembrane domain of MRAP1 is sufficient for interaction with MC2R but it is not able to support receptor trafficking to the cell membrane. The tyrosine-rich region between residues 9 and 24 of human MRAP1 seems to be a key region controlling trafficking since constructs carrying a deletion of the above region are unable to promote MC2R cell surface expression (Webb et al., 2009). This region contains the segment EYY, which is fully conserved in all MRAP sequences except including zebrafish. The above region also contains the segment LDYI, which seems to be critical for ACTH binding to mouse MC2R. Transfer of this segment to mouse MRAP2 does not modify the MC2R cell surface expression but sharply increases the ACTH-induced cAMP production via MC2R activation (Sebag and Hinkle, 2009a). Non-mammalian MRAP1s exhibit complete conservation of DY residues. Interestingly, there is a fully conserved aspartic residue (D) just downstream of this segment, except for pufferfish MRAP1,

which suggests that the MRAP1 segment allowing ACTH binding to MC2R is shortened (to DY) or that the responsible segment has the sequence DYXD, where X is a highly hydrophobic residue. Experiments in mouse have demonstrated that MRAP1 forms antiparallel homodimers and is found upon both Nexo/Ccyt and Ncyt/Cexo topologies in similar quantities (Sebag and Hinkle, 2007). The segment LKANKHS of the mouse MRAP1 seems to be necessary and sufficient to infer this dual topology, since deletion of this area containing three positive charges induces N_{exo}/C_{cyt} topology exclusively and avoids the formation of stable dimers. This mutated protein failed to facilitate MC2R trafficking to the plasma membrane, suggesting that dual topology is required for MC2R function. In addition, the larger segment KKLKANKHS is sufficient to transfer dual topology to other transmembrane proteins (12). This region is not well conserved in zebrafish MRAP1 since only three residues (XXLKXXXXS) were coincident. ZfMRAP1 exhibited only two positively charged amino acids and two negatively charged arginines (R), whereas mouse MRAP1 displays five but no negatively charged amino acids. This would suggest that zebrafish protein does not adopt a dual topology but more experiments are required to corroborate this hypothesis. The C-terminal domain is highly divergent in all MRAP, and zebrafish sequences are no exception. Entire deletion of the C-terminal region in human MRAP1 does not impair MC2R trafficking or signaling but does modulate the amount of receptor reaching the cell surface (Webb et al., 2009). Accordingly, while the C-terminal region of zfMRAPs is not conserved, the presence of a fully conserved small segment in the MRAP2 sequences (IPNFV) suggests a conserved role in MRAP2 activity.

In mammals, MC2R is predominantly expressed in the adrenal cortex where it regulates adrenal steroid synthesis and secretion. In teleosts, the steroidogenic cells, together with closely intermingled chromaffin cells, are embedded in the head kidney, forming the interrenal organ, the homolog of the mammalian adrenal gland (Wendelaar Bonga, 1997; To et al., 2007). We show that zfMC2R is mainly expressed within the anterior kidney, spleen, fat, ovary and testis. Similar distribution was reported in trout, carp and zebrafish (Alsop et al., 2009; Aluru and Vijayan, 2008; Metz et al., 2005). MRAP1 was mainly expressed in the adipose tissue and anterior kidney, where zfMC2R is profusely expressed, but low levels were also detected in gonadal tissue, eye and skin. On the contrary zfMRAP2s expressions were detected in all tested tissues. In humans, MRAP1 isoforms are expressed at different levels in all tissues tested (Metherell et al., 2005), whereas MRAP2 expression is limited to adrenal gland and brain (Chan et al., 2009). The restricted expression patterns of zfMRAP1, as well as, its coincidence with the receptor expression, suggests that its function is primary linked to zfMC2R activity, whereas the wide expression pattern of zfMRAP2s, including in tissues where zfMC2R is not produced, indicates putative additional functions. Suggestively, several experiments have demonstrated that human MRAPs are involved in the negative regulation of the remaining MCRs (Chan et al., 2009; Sebag and Hinkle, 2009b). It is therefore possible that zfMRAPs may also modulate the activity of other zfMCRs but more experiments are required to test this idea.

Our *in vitro* experiments demonstrated that zfMC2R is nonfunctional when expressed in HEK293/FRT cells and only zfMC2R coexpression with zfMRAP1, but not with zfMRAP2s, promoted an intracellular cAMP increase after ACTH stimulation. Mammalian receptors also require the presence of MRAP1 to induce cAMP increases (Roy et al., 2007; Sebag and Hinkle, 2007) and, similarly to zebrafish, human MRAP2 promotes a marginal intracellular cAMP increase when it is overexpressed and no response at endogenous levels in HEK293/FRT (Sebag and Hinkle, 2009a). Both zfMRAP2s enhanced zfMRAP1-assisted intracellular cAMP increases in cells stably expressing zfMC2R after ACTH stimulation. This indicates that the combination of MRAPs may modulate

the response to ACTH, increasing the hormonal response in MC2R-expressing tissues with low levels of MRAP1 transcripts. In addition, the results suggest that both MRAP1 and MRAP2 could form stable heterodimers. The formation of MRAP1/MRAP2 heterodimers has previously been suggested in humans (Chan et al., 2009), although this may be a particular feature of the MRAP system in zebrafish since human MRAP2 has no effect on MRAP1-assisted cAMP signaling (Chan et al., 2009).

In mammals, MRAP is essential for MC2R localization at the plasma membrane of CHO cells. Protein interaction has been demonstrated by the formation of stable immunoprecipitable complexes (Metherell et al., 2005; Cooray et al., 2008; Sebag and Hinkle, 2007). In the absence of mouse MRAP1/2, mouse MC2R is trapped in the endoplasmic reticulum (ER), and is rapidly degraded. However when coexpressed with MRAPs, the receptor is localized at the plasma membrane, where it binds ACTH and positively couples to adenylyl cyclase (Metherell et al., 2005; Chan et al., 2009; Sebag and Hinkle, 2007). Conversely, previous studies reported that human MC2R does not require MRAP coexpression to reach the plasma membrane in HEK293/FRT cells but is required for receptor signaling (Roy et al., 2007). Using RT-PCR, we detected that HEK293/FRT cells express human MRAP2 endogenously (data not shown). Therefore, it is reasonable that endogenous human MRAP2 can mediate the membrane receptor expression but is insufficient for receptor signaling. Accordingly, it has been reported that mouse MRAP2-assisted cAMP production is much lower than that promoted by mouse MRAP1 (Sebag and Hinkle, 2009a). Our results demonstrate that endogenous hMRAP2 in HEK293/FRT cells cannot promote zfMC2R plasma membrane expression, suggesting high sequence specificity for the protein interaction. However, quantification of cell surface receptor by ELISA demonstrated that either zfMRAP1 or zfMRAP2a, but not zfMRAP2b, are sufficient for zfMC2R trafficking to the plasma membrane in HEK293/FRT cells. Immunofluorescence assays corroborated the ELISA data showing that MC2R is not present in the plasma membrane in the absence of zfMRAP expression. However, both proteins were colocalized at the plasma membrane when coexpressed in HEK293/FRT cells. When zfMRAPs were expressed independently, both zfMRAP1 and MRAP2a were present at the cell membrane, indicating that they do not require zfMC2R to reach this position. In contrast, MRAP2b was localized in a perinuclear region and in the cytoplasm, colocalized with zfMC2R but was unable to promote ACTH-induced cAMP; thus the exact functions remain to be determined.

In summary, we demonstrate the evolutionary conservation of the MC2R trafficking and signaling system, adding a new regulatory step in the hypothalamic–pituitary–interrenal (HPI) control axis of fish. ZfMC2R plasma membrane expression and signaling after ACTH stimulation were fully dependent on the presence of MRAP1, whereas MRAP2a only poorly promotes receptor trafficking to the plasma membrane but not receptor signaling. MRAP2b had no effect on zfMC2R functionality but both MRAP2s were able to enhance MRAP1-assisted MC2R signaling. Developmental studies have demonstrated that zebrafish can be used as a model to study adrenal organogenesis (To et al., 2007). Our studies further extend the use of the zebrafish as a model to study regulation of the pituitary adrenal axis. Future experiments will focus the zfMRAP interactions and topology, as well as the MRAP effect on the remaining zfMCRs trafficking and signaling.

References

Alsop, D., Ings, J.S., Vijayan, M.M., 2009. Adrenocorticotropic hormone suppresses gonadotropin-stimulated estradiol release from zebrafish ovarian follicles. PLoS ONE 4 (7), e6463.

Aluru, N., Vijayan, M.M., 2008. Molecular characterization, tissue-specific expression, and regulation of melanocortin 2 receptor in rainbow trout. Endocrinology 149, 4577–4588.

Amores, A., Force, A., Yan, Y.L., Joly, L., Amemiya, C., Fritz, A., Ho, R.K., Langeland, J., Prince, V., Wang, Y.L., Westerfield, M., Ekker, M., Postlethwait, J.H., 1998. Zebrafish hox clusters and vertebrate genome evolution. Science 282, 1711–1714.

Battista, M.C., Roberge, C., Martinez, A., Gallo-Payet, N., 2009. 24-Dehydrocholesterol reductase/seladin-1: a key protein differentially involved in adrenocorticotropin effects observed in human and rat adrenal cortex. Endocrinology 150, 4180–4190.

Boston, B.A., Cone, R.D., 1996. Characterization of melanocortin receptor subtype expression in murine adipose tissues and in the 3T3-L1 cell line. Endocrinology 137, 2043–2050.

Castro, M.G., Morrison, E., 1997. Post-translational processing of proopiomelanocortin in the pituitary and the brain. Crit. Rev. Neurobiol. 11, 35–57.

Chan, L.F., Webb, T.R., Chung, T.T., Meimaridou, E., Cooray, S.N., Guasti, L., Chapple, J.P., Egertová, M., Elphick, M.R., Cheetham, M.E., Metherell, L.A., Clark, A.J., 2009. MRAP and MRAP2 are bidirectional regulators of the melanocortin receptor family. Proc. Natl. Acad. Sci. 106, 6146–6151.

Cone, R.D., 2006. Studies on the physiological functions of the melanocortin system. Endocr. Rev. 27, 736–749.

Cooray, S.N., Almiro Do Vale, I., Leung, K.Y., Webb, T.R., Chapple, J.P., Egertová, M., Cheetham, M.E., Elphick, M.R., Clark, A.J., 2008. The melanocortin 2 receptor accessory protein exists as a homodimer and is essential for the function of the melanocortin 2 receptor in the mouse y1 cell line. Endocrinology 149, 1935–1941.

Dallman, M.F., Akana, S.F., Strack, A.M., Scribner, K.S., Pecoraro, N., La Fleur, S.E., Houshyar, H., Gomez, F., 2004. Chronic stress-induced effects of corticosterone on brain: direct and indirect. Ann. N. Y. Acad. Sci. 1018, 141–150.

Hinkle, P.M., Sebag, J.A., 2009. Structure and function of the melanocortin 2 receptor accessory protein (MRAP). Mol. Cell. Endocrinol. 300, 25–31.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., Higgins, D.G., 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23, 2947–2948.

Logan, D.W., Bryson-Richardson, R.J., Pagán, K.E., Taylor, M.S., Currie, P.D., Jackson, I.J., 2003. The structure and evolution of the melanocortin and MCH receptors in fish and mammals. Genomics 81, 184–191.

Metherell, L.A., Chapple, J.P., Cooray, S., David, A., Becker, C., Rüschendorf, F., Naville, D., Begeot, M., Khoo, B., Nürnberg, P., Huebner, A., Cheetham, M.E., Clark, A.J., 2005. Mutations in MRAP, encoding a new interacting partner of the ACTH receptor, cause familial glucocorticoid deficiency type 2. Nat. Genet. 37, 166–170.

Metz, J.R., Geven, E.J., van den Burg, E.H., Flik, G., 2005. ACTH, alpha-MSH, and control of cortisol release: cloning, sequencing, and functional expression of the melanocortin-2 and melanocortin-5 receptor in *Cyprinus carpio*. Am. J. Physiol. 289, R814–R826.

Mountjoy, K.G., Robbins, L.S., Mortrud, M.T., Cone, R.D., 1992. The cloning of a family of genes that encode the melanocortin receptors. Science 257, 1248–1251.

Noon, L.A., Franklin, J.M., King, P.J., Goulding, N.J., Hunyady, L., Clark, A.J., 2002. Failed export of the adrenocorticotrophin receptor from the endoplasmic reticulum in non-adrenal cells: evidence in support of a requirement for a specific adrenal accessory factor. J. Endocrinol. 174, 17–25.

Roy, S., Rached, M., Gallo-Payet, N., 2007. Differential regulation of the human adrenocorticotropin receptor [melanocortin-2 receptor (MC2R)] by human MC2R accessory protein isoforms alpha and beta in isogenic human embryonic kidney 293 cells. Mol. Endocrinol. 21, 1656–1669.

Schimmer, B.P., Kwan, W.K., Tsao, J., Qiu, R., 1995. Adrenocorticotropin-resistant mutants of the Y1 adrenal cell line fail to express the adrenocorticotropin receptor. J. Cell. Physiol. 163, 164–171.

Schiöth, H.B., Haitina, T., Ling, M.K., Ringholm, A., Fredriksson, R., Cerdá-Reverter, J.M., Klovins, J., 2005. Evolutionary conservation of the structural, pharmacological, and genomic characteristics of the melanocortin receptor subtypes. Peptides 26, 1886–1900.

Sebag, J.A., Hinkle, P.M., 2007. Melanocortin-2 receptor accessory protein MRAP forms antiparallel homodimers. Proc. Natl. Acad. Sci. U.S.A. 104, 20244–20249.

Sebag, J.A., Hinkle, P.M., 2009a. Regions of melanocortin 2 (MC2) receptor accessory protein necessary for dual topology and MC2 receptor trafficking and signalling. J. Biol. Chem. 284, 610–618.

Sebag, J.A., Hinkle, P.M., 2009b. Opposite effects of the melanocortin-2 receptor accessory protein MRAP on MC2 and MC5 receptor dimerization and trafficking. J. Biol. Chem. 284, 22641–22648.

Sewer, M.B., Waterman, M.R., 2003. ACTH modulation of transcription factors responsible for steroid hydroxylase gene expression in the adrenal cortex. Microsc. Res. Technol. 61, 300–307.

To, T.T., Hahner, S., Nica, G., Rohr, K.B., Hammerschmidt, M., Winkler, C., Allolio, B., 2007. Pituitary-interrenal interaction in zebrafish interrenal organ development. Mol. Endocrinol. 21, 472–485.

Webb, T.R., Chan, L., Cooray, S.N., Cheetham, M.E., Chapple, J.P., Clark, A.J., 2009. Distinct melanocortin 2 receptor accessory protein domains are required for melanocortin 2 receptor interaction and promotion of receptor trafficking. Endocrinology 150, 720–726.

Wendelaar Bonga, S.E., 1997. The stress response in fish. Physiol. Rev. 77, 591–625.