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A novel tilapia prolactin receptor is functionally distinct from its paralog

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SUMMARY

A novel tilapia prolactin (PRL) receptor (*Om*PRLR2) was identified based on its induction during hyperosmotic stress. *Om*PRLR2 protein shows 28% identity to tilapia *Om*PRLR1 and 26% identity to human PRLR. Comparison of *Om*PRLR1 and *Om*PRLR2 revealed conserved features of cytokine class I receptors (CKR1): a WS domain and transmembrane domain, two pairs of cysteines and *N*-glycosylation motifs in the extracellular region, CKR1 boxes I and II, and three tyrosines in the intracellular region. However, *Om*PRLR2 lacked the ubiquitin ligase and 14-3-3 binding motifs. *Om*PRLR2 mRNA was present in all tissues analyzed, with highest expression in gills, intestine, kidney and muscle, similar to *Om*PRLR1. Transfer of fish from fresh water to sea water transiently increased gill *Om*PRLR2 mRNA levels within 4 h but decreased its protein abundance in the long term. *Om*PRLR2 is expressed in part as a truncated splice variant of 35 kDa in addition to the 55 kDa full-length protein. Cloning of the mRNA encoding the 35 kDa variant revealed that it lacks the extracellular region. It is expressed at significantly higher levels in males than in females. In stably transfected HEK293 cells over-expressing tetracycline-inducible *Om*PRLR1 and *Om*PRLR2, activation of these receptors by tilapia PRL_{177} and PRL_{188} triggered different downstream signaling pathways. Moreover, *Om*PRLR2 significantly increased HEK293 salinity tolerance. Our data reveal that tilapia has two PRLR genes whose protein products respond uniquely to PRL and activate different downstream pathways. Expression of a short PRLR2 variant may serve to inhibit PRL binding during osmotic stress and in male tissues.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/212/13/2007/DC1

Key words: prolactin, prolactin receptor, tilapia, osmotic stress, salinity adaptation, osmosensory signal transduction.

INTRODUCTION

Prolactin (PRL) regulates several important physiological processes in fish, including salinity adaptation (Manzon, 2002; Sakamoto and McCormick, 2006), modulation of immune functions (Harris and Bird, 2000), pigment dispersion (Kitta et al., 1993), reproduction (Cavaco et al., 2003) and development (Nguyen et al., 2008). In euryhaline species, such as tilapia, PRL is known to promote freshwater adaptation. Relevance of PRL in freshwater adaptation has been shown since Pickford and Phillips demonstrated that hypophysectomized killifish required PRL to survive in fresh water (Pickford and Phillips, 1959). Recent studies also support a critical function of this hormone for teleost osmoregulation (Hirano, 1986; Sakamoto and McCormick, 2006). Two PRL variants, PRL_{177} and PRL_{188}, are expressed in tilapia (Specker et al., 1985; Yamaguchi et al., 1988). However, based primarily on binding assays it was argued that only a single PRL receptor (PRLR) is present in fish (Auperin et al., 1994a). This argument has been consistently supported since the first PRLR was cloned from *Oreochromis niloticus* (Auperin et al., 1995; Sandra et al., 1995). At the same time the existence of only a single PRLR has been debated because of the different actions exerted by the two kinds of PRL (Auperin et al., 1994b; Specker et al., 1989). Shedding light on this controversy, recently it was reported that two PRLR genes exist in seabream, which conclusively demonstrated the occurrence of PRLR1 and PRLR2 in teleosts (Huang et al., 2007).

In a previous study aimed at the identification of genes up-regulated rapidly during osmotic stress in the euryhaline tilapia (*Oreochromis mossambicus*) we isolated an unidentified clone (SSH#828) transiently induced upon osmotic stress (Fiol et al., 2006a). In this work, we were able to identify clone SSH#828 as *Om*PRLR2 after sequencing the full-length mRNA. A partial sequence of a PRLR1 gene with high similarity to the *O. niloticus* PRLR1 had been cloned before in *O. mossambicus* (Prunet et al., 2000). In the present study we report the cloning of full-length cDNAs for *O. mossambicus* PRLR1 (*Om*PRLR1) and the novel *O. mossambicus* PRLR2 (*Om*PRLR2). Furthermore, we characterized the effect of environmental salinity, tissue type and sex on *Om*PRLR1 and *Om*PRLR2 expression at mRNA and protein levels in tilapia. In addition, we performed functional analyses of *Om*PRLR1 and *Om*PRLR2 using a heterologous expression system, observing a differential capability of activating downstream signaling pathways and responsiveness to tilapia PRL variants. Our studies revealed the presence of a truncated *Om*PRLR2 protein variant that is regulated in response to salinity stress and more highly expressed in males than in females. The implications of these findings for tilapia osmoregulation, in particular during the process of transitioning between plasma hypo-osmotic and plasma hyperosmotic milieus, are discussed.

MATERIALS AND METHODS

Animals and experimental protocol

Tilapia (*Oreochromis mossambicus*, Peters 1852) were maintained in large (4 ft, ~1.22 m, diameter) tanks supplied with flow-through heated (26°C) Davis well water (fresh water, FW, Na^{+}=28 mg l^{-1},

K^+<5 mg l^{-1}, Ca^{2+}=33 mg l^{-1}, Mg^{2+}=36 mg l^{-1}, pH 8.0) at the Center for Aquatic Biology and Aquaculture (CABA) of the University of California, Davis. Five days prior to treatments, fish were transferred to 20 gallon (~76 l) recirculation aquaria containing FW at 25–27°C. Fish were transferred from FW to sea water (SW) by making successive changes of a fraction of the water (about 20–30% of the volume) in order to minimize handling stress during experiments. Full-strength SW was reached over a period of *ca.* 90 min; then fish were kept with recirculation of SW. Control fish transferred from FW to FW were exposed to the same water changes but without altering the salinity. For long-term acclimation to SW fish were kept for at least 4 weeks in 29 gallon (~110 l) recirculation SW aquaria. Osmotic stress was applied by making successive changes of a fraction of the water to reach the desired condition over a period of *ca.* 90 min. SW was collected at Bodega Bay, California (1000 mosmol kg^{-1}, ~32 p.p.t.) or prepared with Instant Ocean sea salt to the same concentration. Animals were processed and sorted into batches based on sex and size to exclude these factors from compounding the analysis. Three all-male and three all-female batches were created and at least six animals from each batch were sampled at each point. Therefore, only same-sex animals were compared with PRLR expression values normalized to the respective FW controls within each batch. Relative abundance values for each batch were comparable regarding the effects of salinity acclimation and combined (averaged). Fish were sampled at the times indicated, gills were perfused and gill epithelium was collected by scraping it off the cartilage of individual gill arches as described in previous work (Kültz and Somero, 1995). In addition, muscle (posterior to the dorsal fin), and the whole intestine, kidneys, heart, brain, liver and testes or ovaries were collected. All different tissues were kept at –80°C and subsequently utilized for RNA or protein isolation.

Cloning of full-length *OmPRLR1* and *OmPRLR*

Full-length sequences for each gene were cloned using a SMART RACE cDNA amplification kit (Clontech, Mountain View, CA, USA). *OmPRLR1* fragment AAC31825 was used as a starting point and extended with 3′ RACE until completion of the full-length cDNA. *OmPRLR2* full-length cDNA was obtained from the previously unknown sequence SSH#828 (GenBank DQ465388) using 5′ RACE. Novel full-length cDNA sequences were submitted to GenBank (EU999783, EU999784, EU999785).

Quantitative real-time PCR

Total RNA from mIMCD3 cells or tilapia tissues was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) as specified by the manufacturer. RNA was treated with DNase (Turbo DNA free, Ambion, Austin, TX, USA) and purity was confirmed and quantity determined by measuring absorbance of the samples at 260 and 280 nm with a Nanodrop spectrophotometer. For cDNA synthesis, RNA (1 μg) was reverse-transcribed using Superscript III first-strand synthesis reagents (Invitrogen) with a random hexamer:oligo(dT) mix (1:1) as primers. Abundance of all transcripts was quantified with a PRISM 7500 real-time thermal cycler (Applied Biosystems, Foster City, CA, USA). Reactions were performed in duplicate in a 20 μl reaction volume using SYBR Green PCR Master Mix (Applied Biosystems) and 30 pmol of each primer. PCR conditions were 50°C/2 min and 95°C/10 min, followed by 40 cycles of 95°C/15 s, 60°C/1 min. Data were collected at 60°C. The ΔCt method was used to determine the relative amounts of different genes, normalized to the abundance of either β-actin (tilapia tissues) or ribosomal protein L32 (HEK293 cells), and expressed as normalized RNA level in arbitrary units. Normalization genes were selected based on constant levels of expression during all experimental conditions as determined previously (Fiol et al., 2006b). Efficiencies of individual PCR reactions were analyzed using LinRegPCR and were always ~2. Gene-specific primer sequences were designed with Primer Express software (Applied Biosystems). The absence of unwanted by-products was confirmed by automated melting curve analysis and agarose gel electrophoresis of PCR products. Primers utilized in this study are shown in Table 1.

Protein extraction and western blot analysis

For protein extraction, cells were lysed in a buffer that contained 50 mmol l^{-1} Tris HCl, pH 7.4, 1% nonidet P-40, 0.25% sodium deoxycholate, 150 mmol l^{-1} NaCl, 1 mmol l^{-1} EDTA, 1 tablet of minicomplete protease inhibitor mixture (Roche, Indianapolis, IN, USA) per 10 ml, 1 mmol l^{-1} activated Na_3VO_4 and 1 mmol l^{-1} NaF. Protein concentrations were determined by BCA protein assay according to the manufacturer's instructions (Pierce Chemical, Rockford, IL, USA). Proteins were separated by SDS-PAGE. Equal amounts of protein (25 μg) were loaded in each lane of 10% Tris-glycine SDS-PAGE gels. Samples were electrophoresed at 120 V, the gels briefly rinsed in transfer buffer (25 mmol l^{-1} Tris, 200 mmol l^{-1} glycine, 20% methanol), and proteins blotted onto PDVF membranes (Millipore, Bedford, MA, USA) at 1 mA cm^{-2} for 90 min using a TransBlot SD semidry transfer cell (Bio-Rad, Hercules, CA, USA). Membranes were blocked for 1 h at room temperature in a solution containing 137 mmol l^{-1} NaCl, 20 mmol l^{-1} Tris, pH 7.6 (HCl), and 5% (w/v) non-fat dry milk.

Table 1. Primers employed for full-length cloning and real-time PCR

Gene	Forward (5′ to 3′)	Reverse (5′ to 3′)	Product (bp)
	Cloning primers		
OmPRLR1	ATGATGACGAAAGTCGGAGAAGT	CTACTAGGTGTGCAGGGAGAA	
OmPRLR2	ATGGTTTGTGCCAGGATGGTG	CTTTAGATTACAGCTGGTGGGG	
	Real-time primers		
Om β-actin	ACAGAGCGTGGCTACTCCTT	CCCATCTCCTGCTCGAAGTC	103
OmPRLR1	CCAGTCCCAGGTCCTAAAATCA	CGACCATCAGTTCCTGCTGTT	177
OmPRLR2	TACCTGATCGTGTCTGATAAAGAAACC (E9)	CGACGACTTGGTCTTCTCTGAA (E9)	182
h *L32*	AACGTCAAGGAGCTGGAAGTG (E2)	CACGATGGCTTTGCGGTTCTT (E3)	95
h *c-Fos*	GGGCAAGGTGGAACAGTTATC (E2/3)	CCGCTTGGAGTGTATCAGTCA (E3)	125
h *Spi2.1*	GGAACCTATGATCTGAAGAGCG (E3)	TCCCTTTCTCGTCGATGGTCA (E4)	151
h *c-Myc*	AGGCGAACACACAACGTCTT (E3)	TTGGACGGACAGGATGTATGC (E3)	156

When known, the exon number (E) where primers are located is indicated in parentheses.
PRLR, prolactin receptor; *Om*, *Oreochromis mossambicus*; h, human.

They were then incubated for 2h in blocking buffer containing anti-PRLR2 antibody at 1:500 dilution (custom-made, raised in rabbits using a KLH-conjugated peptide SESSSEESSEKTKSSQ; Sigma-Genosys, Woodlands, TX, USA). Goat anti-rabbit secondary antibody conjugated to horseradish peroxidase was used at 1:2000 dilution (Pierce catalogue no. 31460). Blots were developed with SuperSignal Femto (Pierce) and imaged with a ChemiImager (Alpha Innotech, San Leandro, CA, USA).

Details of antibody production are available from D.K. on request.

Overexpression of OmPRLR1 and OmPRLR2 in HEK293 T-Rex cells

PRLR1 and PRLR2 open reading frames (ORFs) were amplified with the respective ATG forward and END reverse primers (Table 1). PCRs were performed using the Advantage HF-2 PCR kit (Clontech) in a MasterCycler (Eppendorf, Hamburg, Germany) using the following cycling parameters: 94°C/1min, 32 cycles of 94°C/30s, 60°C/30s, 72°C/1.5min, and then 72°C/5min. PCR products were extracted from agarose gels using the GeneClean system (Q-BIO gene, Carlsbad, CA, USA) and then double-pass sequenced on an ABI 3730 automated DNA sequencer (Foster City, CA, USA). pcDNA5/FRT/PRLR1 and pcDNA5/FRT/PRLR2 constructs were created by cloning the respective PCR products into pcDNA5/FRT/TO TOPO TA expression vector (Invitrogen). The constructs were then propagated in *Escherichia coli* strain DH5α (Invitrogen). Endotoxin-free plasmid mega-preps were performed using a kit as described by the manufacturer (Qiagen GmbH, Hilden, Germany). Stable cell lines were established by transfecting HEK293 T-Rex cells (Invitrogen) with 2μg of a 1:9 mix of pcDNA/FRT/PRLR1 or pcDNA5/FRT/PRLR2 plasmid DNA:pOG44 plasmid DNA and 4μl of LipofectAMINE 2000 reagent (Invitrogen). Twenty-four hours after transfection cells were exposed to selection in medium containing 0.15 $\mu g\,ml^{-1}$ hygromycin (Invitrogen). After 2 weeks individual colonies were picked, expanded, and tested for expression of *OmPRLR1* or *OmPRLR2* by RT-PCR and quantitative real-time PCR analysis.

Cell survival assay

Cells were cultured in 96-well plates and exposed to hypersaline media. Cell survival was analyzed with ViaLight cytotoxicity assay (Lonza, Walkersville, MD, USA) following the manufacturer's indications. The method incorporates bioluminescence detection of cellular ATP as a measure of viability; alive cells, which contain higher amounts of intracellular ATP than dead or dying cells, will generate after cell lysis a higher amount of luminescence. Thus, a higher value in relative luminescence units correlates directly with a higher number of alive cells. Similar results were obtained when viable cells were counted in Neubauer hemocytometer chambers using 0.2% Methylene Blue as an indicator of dead cells.

Bioinformatics and statistical analysis

Multiple sequence alignments were performed using AlignX, a multiple sequence alignment application of Vector NTI Advance 9.0 (InforMax Software, Invitrogen), which uses a modified ClustalW algorithm.

Differences between pairs of data were analyzed by *F*-test and Student's unpaired *t*-test. Differences in time series data sets were statistically evaluated using ANOVA. Significance threshold was set at $P<0.05$ and data are presented as means ± s.e.m.

RESULTS

Cloning of *OmPRLR1* and *OmPRLR2*

We identified *OmPRLR2* from a sequence previously classified as unknown in a study aimed at identifying genes that are rapidly upregulated during osmotic stress in tilapia (Fiol et al., 2006a). To enable characterization of prolactin receptors in tilapia, we performed full-length cloning of both *OmPRLR1*, which had previously been partially cloned, and *OmPRLR2*. *OmPRLR1* and *OmPRLR2* full-length cDNA sequences were obtained by RACE-PCR using GenBank IDs AAC31825 and DQ465388 as starting sequences. The nucleotide and deduced amino acid sequences of *OmPRLR1* and *OmPRLR2* are shown in supplementary material Fig.S1. *Om*PRLR1 and *Om*PRLR2 share only 28.3% identity of sequence overall. When separately comparing sequences in the transmembrane region, the extracellular domain (ECD) of the amino-terminus and the intracellular domain (ICD) of the carboxy-terminus, we noticed that the degree of sequence conservation was much higher in the ICD than in the ECD (Table 2). A comparison of tilapia PRLRs and the human PRLR long variant showed higher similarity between *Om*PRLR1 and hPRLR than between the two tilapia PRLRs (Table 2). We were able to identify conserved features of the cytokine class I receptor (CKR1) family, including two pairs of cysteine residues, *N*-glycosylation sites and the functional WS, and Box 1 and Box 2 domains in both tilapia PRLRs. However, ubiquitination and 14-3-3 binding domains were only present in hPRLR and *Om*PRLR1 but not in *Om*PRLR2. The location of these domains in human and tilapia PRLRs is depicted and summarized in Fig. 1 and Table 3. Furthermore, three tyrosine phosphorylation sites were identified in *Om*PRLR1 and four in *Om*PRLR2. Analysis of the amino acids surrounding the tyrosine residues revealed that three of them (I, II and IV) are conserved and present in both receptors while site III is only present in *Om*PRLR2 (Table 2; Fig. 1). An alignment of selected PRLR1 and all reported PRLR2 sequences is shown in supplementary material Fig.S2.

Expression and tissue distribution of PRLRs in tilapia

Levels of expression of *Om*PRLR1 and *Om*PRLR2 mRNAs were analyzed in different organs of FW-acclimated tilapia. Both *Om*PRLR1 and *Om*PRLR2 showed highest expression levels in gills, kidney, intestine and muscle, although expression was also detected in brain, liver and heart for both receptors (Fig. 2).

Using specific *Om*PRLR2 antibodies, we analyzed the protein levels in the different organs of fish acclimated to FW. Two different isoforms were detected: the expected full-length protein of ~55kDa and a smaller peptide of about 35kDa (Fig. 3). *Om*PRLR2 was expressed widely but with organ-specific expression profiles.

We investigated the response of *Om*PRLR1 and *Om*PRLR2 mRNAs after acute hyperosmotic and hyposmotic stress in gills and confirmed that *Om*PRLR2 transcript increased in gills 6h after transfer of fish from FW to SW compared with the handling control (FW to FW transfer; Fig. 4). Comparison of *Om*PRLR1 and *Om*PRLR2 transcript levels in fish that were long-term acclimated to FW and SW showed that steady-state levels of *Om*PRLR1 and

Table 2. Comparison of PRLR protein sequences

	Total	Extracellular	Intracellular
*Om*PRLR1–*Om*PRLR2	28.3 (40.0)	45.6 (57.7)	17.3 (28.4)
*Om*PRLR1–hPRLR	35.8 (48.0)	47.7 (62.2)	28.1 (38.0)
*Om*PRLR2–hPRLR	25.9 (35.8)	39.7 (50.8)	16.7 (25.5)

Percentages of identity and similarity (in parentheses) are indicated.

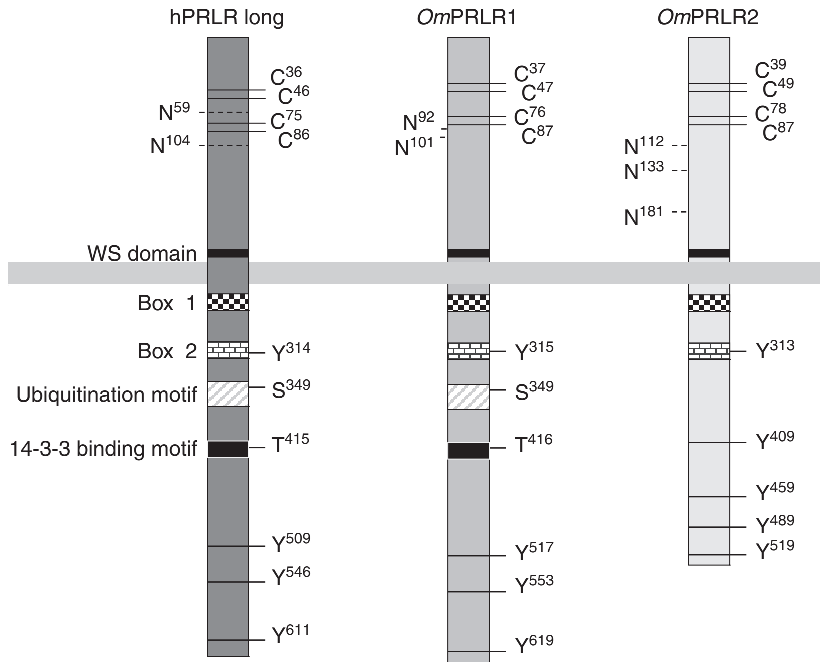

Fig. 1. Representation of human prolactin receptor (PRLR) long isoform, *Om*PRLR1 *and Om*PRLR2. Features that are characteristic for the cytokine class I receptor protein family, including conserved domains and key residues are indicated.

*Om*PRLR2 were higher in FW gills than in SW gills (FW–FW *versus* SW–SW in Fig. 4). We also compared *Om*PRLR2 protein abundance in fish that were long-term acclimated to FW *versus* SW. While no differences were detected in any organ for the 55 kDa isoform, expression of the 35 kDa truncated protein was significantly higher in fish acclimated to FW in gills, kidney, muscle, heart and brain (Fig. 5). These results suggest that *Om*PRLR2 regulation during salinity stress is complex and consists of multiple phases at transcript and protein levels.

Interestingly, expression of the 35 kDa truncated variant of *Om*PRLR2 was significantly higher in gills of males than females in FW-acclimated fish (Fig. 6).

Identification of an *Om*PRLR2 splice variant

To confirm the presence of a truncated variant of *Om*PRLR2 at the mRNA level we investigated the presence of splice variants. Using RT-PCR and primers including the initiation codon and stop codons, we were able to detect two transcripts. One of them comprised a 1590 bp ORF and the other a 1050 bp ORF (Fig. 7A). The shorter transcript was found to be expressed ubiquitously as is illustrated in Fig. 7A for heart, brain and eye. Isolation and sequencing of the shorter (1050 bp) band verified that it indeed represented a mRNA coding for a *Om*PRLR2 variant. A graphical representation of both *Om*PRLR2 variants, indicating key residues and domains, is depicted in Fig. 7B. Synteny analysis comparing all available genomic data for the fish PRLR2 locus (*Danio rerio*, *Fugu rubripes*, *Gasterosteus aculeatus*, *Oryzias latipes* and *Tetraodon nigroviridis*) permitted identification of a conserved exon structure and high nucleotide conservation at splice sites. At least nine main exons are present in PRLR2 genes; exon I corresponding to the 3′ UTR and the coding region being composed of exons II–IX. The presence of additional exons spanning the 5′ UTR is expected but cannot be predicted because the 5′ UTR was not included in the known or predicted orthologous sequences. Nevertheless, we were able to predict the exon composition of *Om*PRLR2 variants in the coding region. Full-size *Om*PRLR2 showed a typical exon composition with exons II–IX comprising the coding region and a high degree of conservation of exon sizes. On the other hand, the short isoform lacked exons IV, V, VI and part of exon VII (Fig. 7C). Thus, the short *Om*PRLR2 variant seems to result from alternative splicing between exon III and exon VII.

Heterologous overexpression of *Om*PRLR1 and *Om*PRLR2

Stably transfected cell lines expressing *Om*PRLR1 and *Om*PRLR2 were established in HEK293 T-Rex cells to functionally characterize tilapia PRLRs. Quantification of changes in the expression of PRLR target genes was used to monitor responsiveness to hormone treatment in cell lines expressing either *Om*PRLR1 or *Om*PRLR2.

Table 3. Conserved domains in the intracellular region of human and tilapia PRLRs

Motif	hPRLR long		*Om*PRLR1		*Om*PRLR2	
	Sequence	Key residue	Sequence	Key residue	Sequence	Key residue
Box 1	PPVPGPKIK		PPVPGPKIK		PPVPAPKIR	
Box 2	DLLVE**Y**LEV	Y314	DLLVE**Y**LEV	Y315	DQNED**Y**LIV	Y313
UL	D**S**GRGSCDS	S349	D**S**GRGSCDS	S349	Absent	
14-3-3	KCS**T**WPL	T415	KVK**T**WPS	T416	Absent	
Y-I	LD**Y**VE	Y509	TE**Y**VE	Y517	SG**Y**VD	Y409
Y-II	KE**Y**AK	Y546	DD**Y**SK	Y553	DD**Y**SR	Y459
Y-III	Absent		Absent		KG**Y**ID	Y489
Y-IV	LDYLD	Y611	SG**Y**VD	Y619	CG**Y**VD	Y519

Key residues are shown in bold. UL, ubiquitination motif; Y, tyrosine phosphorylation site.

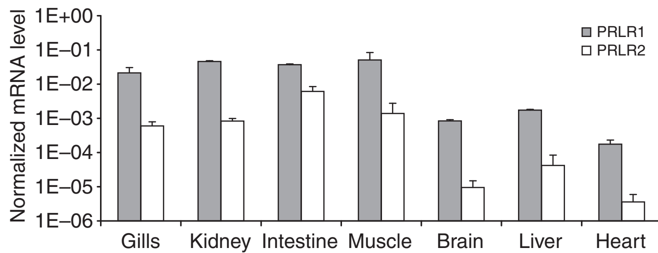

Fig. 2. Expression analysis of *Om*PRLR1 and *Om*PRLR2 transcripts. mRNA levels were analyzed by quantitative RT-PCR in different organs of fresh water (FW)-acclimated tilapia. Data are presented as means ± s.e.m. (*N*=6).

Multiple conserved genes whose expression was previously shown to be modulated by prolactin were analyzed; *c-Fos*, *p53*, *p21*, *Bcl-xl*, *Socs2*, *c-Myc* and *Spi2.1* expression levels were quantified by quantitative real-time PCR after 4 h treatment with either tilapia PRL_{177} or tilapia PRL_{188} at $20\,ng\,ml^{-1}$.

Based on their expression profiles in response to prolactin treatment, PRLR target genes could be separated into three different groups. First, *c-Myc* expression was induced for both kinds of receptor, when cells were treated with PRL_{177} but not with PRL_{188} (Fig. 8A). The second group comprises most of the genes analyzed, including *c-Fos*, *p53*, *p21*, *Bcl-xl* and *Socs2*, which were induced only in cells expressing *Om*PRLR2 when treated with PRL_{177} but not PRL_{188} (Fig. 8B shows *c-Fos* as a representative example of this group). The third group includes *Spi2.1*, which was induced only in cells expressing *Om*PRLR1 when treated with either PRL_{177} or PRL_{188} (Fig. 8C).

When cell lines expressing *Om*PRLR1 and *Om*PRLR2 were challenged with hyperosmotic media, a significant increase in osmotic stress tolerance was observed for *Om*PRLR2-expressing cells compared with control cells transfected with the empty vector (Fig. 9). On the other hand, *Om*PRLR1-expressing cells showed no difference in osmotic stress tolerance. When exposed to water of $550\,mosmol\,kg^{-1}$ for 24 h, *Om*PRLR2-expressing cells showed >80% survival, while *Om*PRLR1-expressing and empty vector controls showed <20% survival (Fig. 9). When cells were exposed to similar osmotic stress conditions but with heat-inactivated fetal bovine serum (FBS) instead of regular FBS in the medium, no increase in osmotic stress tolerance was observed (data not shown). This result suggests that a PRLR2-activating substance such as prolactin or a related hormone is present in FBS and is necessary to stimulate PRLR2 downstream signaling pathways.

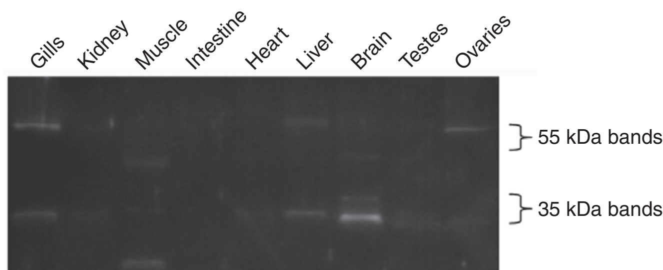

Fig. 3. Tissue-specific expression of two *Om*PRLR2 protein variants: 55 kDa and 35 kDa variants of *Om*PRLR2 were consistently detected by western blot in different organs of FW-acclimated tilapia. Note that the abundance of the short and long variant is about equal in gill epithelium.

DISCUSSION

Teleosts have two prolactin receptor genes

The CKR1 family includes growth hormone receptor, prolactin receptor, leptin receptor, erythropoietin receptor, and receptors for several leukins (Bazan, 1989). Several common features have been described in these proteins, such as conserved domains and key residues (Bole-Feysot et al., 1998). A single-pass transmembrane region divides the amino-terminal ECD from the carboxy-terminal ICD. The ECD contains two pairs of conserved disulfide-linked cysteines and a conserved penta-peptide named the WS domain, which are present in all members of the family and were demonstrated to be involved in ligand binding (Bole-Feysot et al., 1998). A number of *N*-glycosylation sites are commonly present in the ECD and are involved in positioning the receptor on the cell surface (Buteau et al., 1998). Several conserved domains have also been characterized in the ICD, including Box 1 (Edery et al., 1994; Kelly et al., 1991), Box 2 (Murakami et al., 1991) and multiple tyrosine phosphorylation sites (Endo et al., 2003). Additionally, some less ubiquitous motifs have been described, including the ubiquitination domain (Li et al., 2004) and the 14-3-3 binding domain (Olayioye et al., 2003). Both tilapia PRLRs contain all the above-mentioned ECD features and the single-pass transmembrane region. Furthermore, Box 1 and Box 2 could be also identified in the ICD of both tilapia PRLRs. But several important differences between *Om*PRLR1 and *Om*PRLR2 were observed in the ICD: while three tyrosine phosphorylation residues were present in *Om*PRLR1, four were identified in *Om*PRLR2. Moreover, the ubiquitination and 14-3-3 binding domains were only present in *Om*PRLR1. The lack of these regulatory motifs in *Om*PRLR2 suggests considerable differences in the post-translational regulation of both tilapia receptors. These differences between tilapia prolactin receptors were found to be ubiquitous for all published teleost sequences.

Two variants of tilapia PRLR2 are expressed

We were able to immunologically detect the presence of a shorter 35 kDa *Om*PRLR2 variant, in addition to the expected full-length 55 kDa peptide. The corresponding transcript of the shorter variant was cloned and sequenced and shown to represent a splice variant (short *Om*PRLR2). It is possible that additional splice variants of *Om*PRLR2 are expressed that were not detected in this study because they lack the antigenic region detected by our *Om*PRLR2 antibody. The presence of shorter PRLR variants is a common feature in mammals but was only recently described in fish when alternatively spliced PRLR1 forms having a truncated ICD were detected in carp

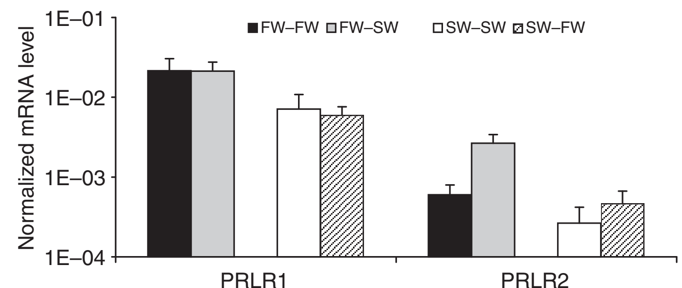

Fig. 4. Responses of *Om*PRLR1 and *Om*PRLR2 transcripts to osmotic stress. Expression levels were quantified by quantitative real-time PCR in gill epithelium of tilapia transferred for 8 h from FW to SW, from SW to FW, and the corresponding handling controls (FW to FW and SW to SW). Prior to all transfers fish were acclimated to FW and SW for 4 weeks. Data shown are means ± s.e.m. (*N*=6).

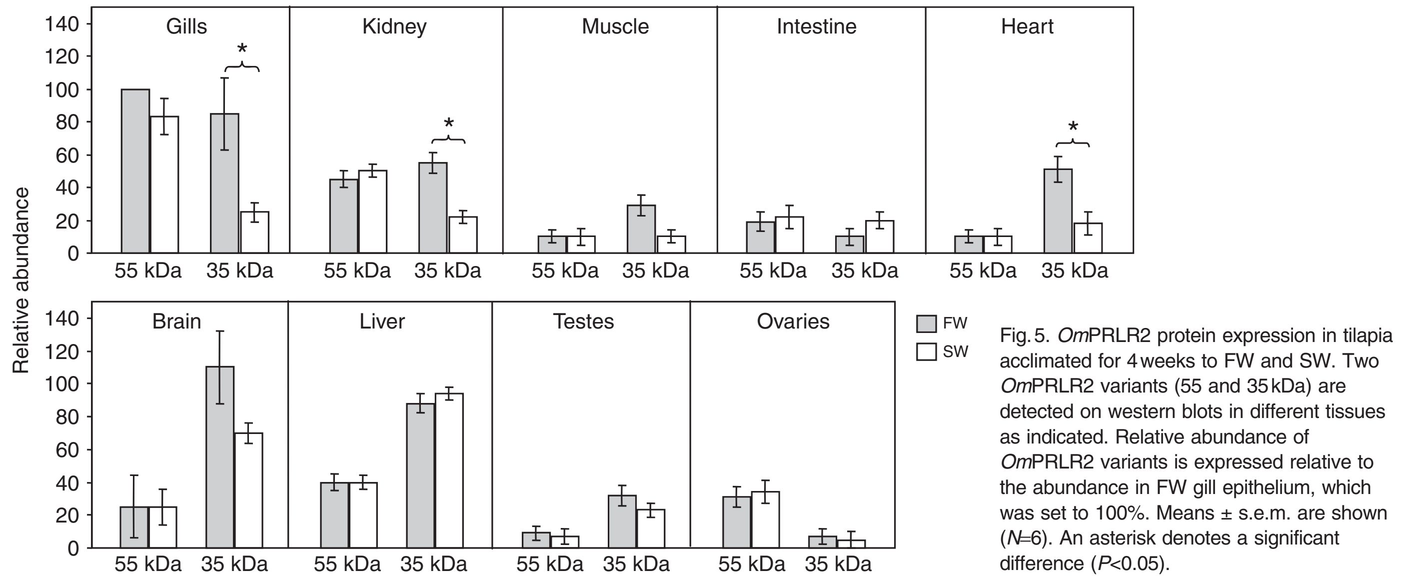

Fig. 5. *Om*PRLR2 protein expression in tilapia acclimated for 4 weeks to FW and SW. Two *Om*PRLR2 variants (55 and 35 kDa) are detected on western blots in different tissues as indicated. Relative abundance of *Om*PRLR2 variants is expressed relative to the abundance in FW gill epithelium, which was set to 100%. Means ± s.e.m. are shown (N=6). An asterisk denotes a significant difference (P<0.05).

(San Martin et al., 2007). Carp variations resemble human PRLR splice variants but are different from the short *Om*PRLR2 variant found in this study, where the ICD remains constant compared with the full-length variant.

The exon composition of both *Om*PRLR2 variants was deduced based on synteny analysis of the PRLR2 loci of five fish with fully sequenced genomes (*Danio rerio*, *Fugu rubripes*, *Gasterosteus aculeatus*, *Oryzias latipes* and *Tetraodon nigroviridis*). The exon composition of the coding region of the long *Om*PRLR2 variant showed the conserved pattern of exon number and size. In contrast, the short variant represents a truncated protein that lacks a region flanked by exons III and VII, which is likely to be a result of alternative splicing.

Given the required dimerization step of PRLR during receptor activation, the presence of distinct PRLR2 variants may allow the formation of hybrid receptors with altered PRL binding and downstream pathway activation capabilities. A schematic model illustrating signaling consequences of *Om*PRLR1 and *Om*PRLR2 activation as well as a possible hybrid between long and short *Om*PRLR2 is presented in Fig. 10. According to this model, transient up-regulation of the short *Om*PRLR2 variant observed during salinity stress may constitute a regulatory event that sequesters long *Om*PRLR2 variants and inhibits PRL binding. Of interest, higher expression of the short *Om*PRLR2 variant in tilapia males than in females may indicated that prolactin signaling is gender specific and inhibited at the receptor level in males. Recently, gender-specific differences were also found in the expression of a variety of other tilapia receptors, including growth hormone receptor and several estrogen receptors (Davis et al., 2008).

Both tilapia prolactin receptors are widely expressed and regulated by salinity

Both PRLRs showed ubiquitous expression in all analyzed tissues, consistent with a multifunctional role of PRL. High expression levels were especially detected in gills, kidney, muscle and intestine. Earlier studies in tilapia, *Sparus aurata* and *Takifugu rubripes* reported comparable expression profiles for prolactin receptors (Lee et al., 2006; Pierce et al., 2007; Santos et al., 2001). The two tilapia PRLRs are both more highly expressed in gills of long-term FW-acclimated tilapia than in SW-acclimated fish. However, upon acute transfer from FW to SW mRNA levels of *Om*PRLR2 increase significantly although transiently for up to 12 h, confirming our previous data (Fiol et al., 2006a). The reason for such complex regulation of *Om*PRLR2 abundance at transcript and protein levels is currently not understood.

Tilapia prolactin receptors differ in their responses to ligands and mediate distinct signaling events

Functional analysis of *Om*PRLR1 and *Om*PRLR2 in HEK293 cells allowed us to characterize the activation of major target genes that are involved in downstream signaling pathways in response to ligand binding. The two tilapia PRLRs responded differently to treatment with the two tilapia prolactins (PRL_{177} or PRL_{188}). Based on the responses of a number of genes previously shown to be activated by prolactin, we were able to distinguish targets that are activated only *via Om*PRLR1 by both PRL_{177} and PRL_{188} (*Spi2.1*), targets that are activated *via* both receptors by PRL_{177} only (*c-Myc*) and targets that

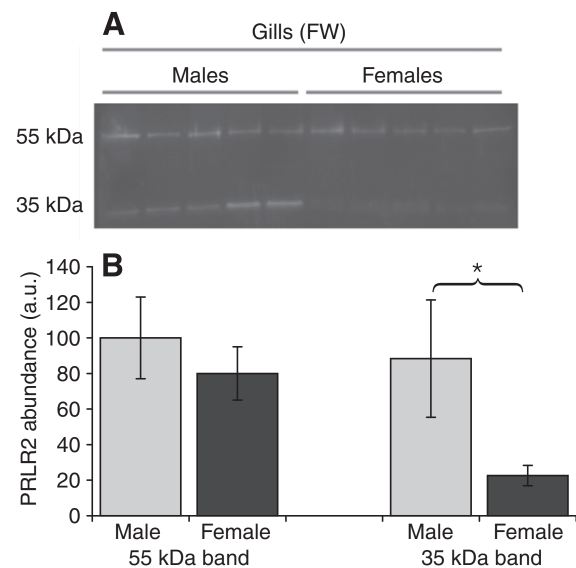

Fig. 6. Expression of the *Om*PRLR2 35 kDa variant in gills is higher in males. Western blot (A) and quantitative densitometry data (B) of *Om*PRLR2 protein abundance in gills of FW-acclimated tilapia. Data shown in B (a.u., arbitrary units) are means ± s.e.m. (N=6). An asterisk denotes a significant difference (P<0.05).

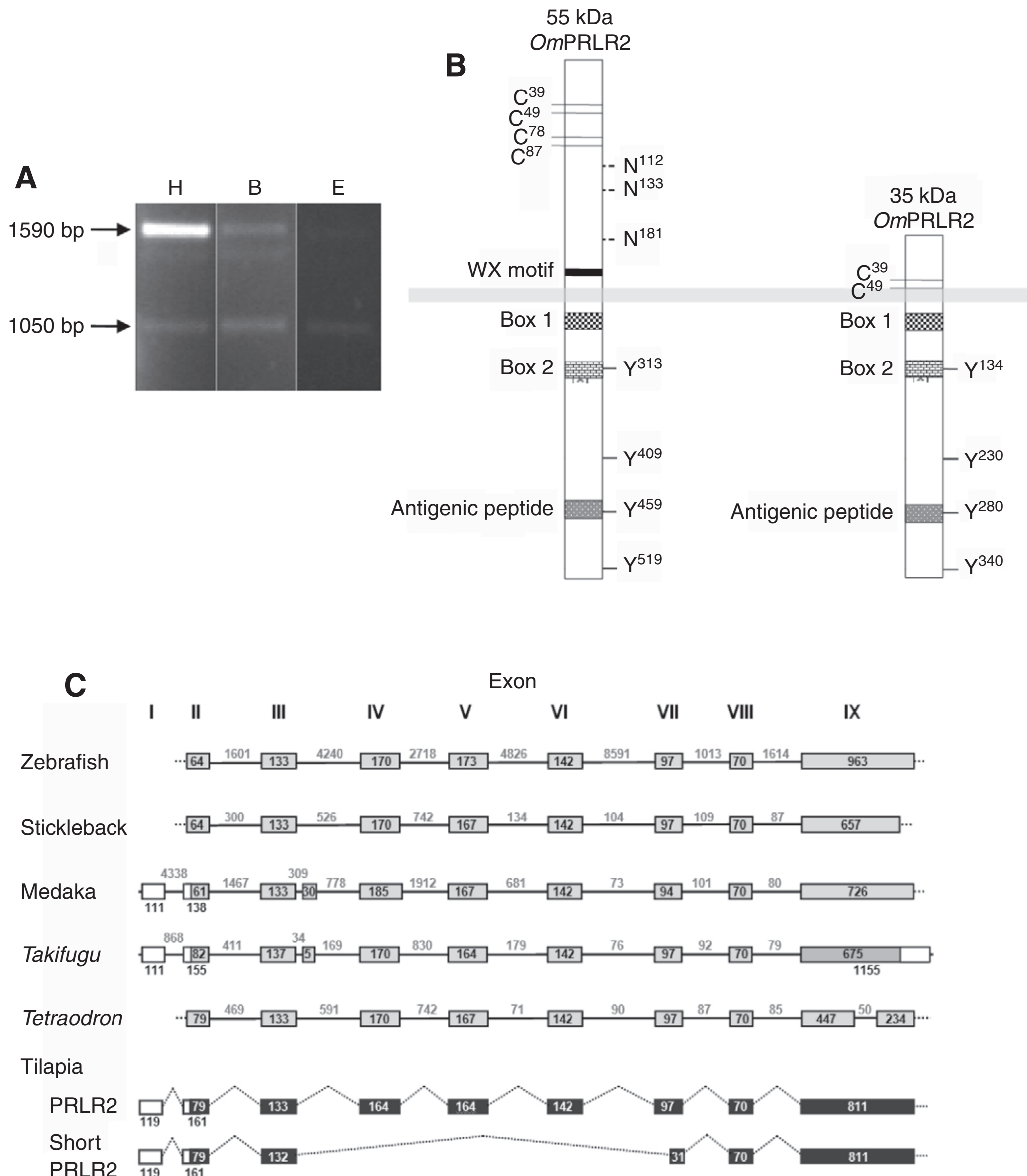

Fig. 7. Identification of alternatively spliced *Om*PRLR2 transcripts. (A) RT-PCR products obtained with *OmPRLR2* cloning primers using heart (H), brain (B) and eye (E) as templates. Asterisk indicates a non-specific PCR product. (B) Schematic representation of full-length *Om*PRLR2 and the product of the 1050 bp band. (C) Deduced exon composition of both *Om*PRLR2 splice variants based on synteny analysis of fully sequenced fish genomes: *Danio rerio*: transcript ENSDART00000039549 (Q6UA22_DANRE), located on chromosome 21 (4,397,350-4,423,764); *Gasterosteus aculeatus*: transcript ENSGACT00000018219, located on group XIII (17,294,549-17,298,05); *Oryzias latipes*: transcript ENSORLT00000000408 located on chromosome 9 (586,788-598,322); *Takifugu rubripes*: ENSTRUT00000043279 (NP_001072093.1) located on scaffold 4 (3,270,178-3,275,428); *Tetraodron nigroviridis*: transcript ENSTNIT00000004287 located on chromosome 12 (5,140,564-5,144,291). Gray rectangles represent coding exons, white rectangles represent non-coding regions. Lengths of exons and introns are indicated in base pairs.

are only activated *via Om*PRLR2 by PRL_{177} only (*c-Fos*, *p53*, *p21*, *Bcl-xl* and *Socs2*). Similar results were obtained for *Spi2.1* activation by seabream PRLR1 and PRLR2 when treated with salmon prolactin (Huang et al., 2007). However, these authors found that a *c-Fos* promoter reporter gene was induced by activation of both prolactin receptors. The discrepancy with our results may be attributed to the different expression systems employed (transient *versus* stably inducible in our study), the hormone concentrations used (300 $ng\,ml^{-1}$ *versus* 20 $ng\,ml^{-1}$ in our study), and the use of heterologous receptor–hormone combinations *versus* utilization of all receptors and hormones from the same species (*O. mossambicus*) in our study. Differential effects of PRL variants were observed previously for transiently expressed *O. niloticus* PRLR1. In this case, a higher affinity for PRL_{188} was observed and a correspondingly lower concentration of hormone was required to activate the Jak2/Stat5 pathway (Sohm et al., 1998). In addition, different potencies were reported for the two prolactins with regard to their effect on pigment dispersion in cultured xanthophores and erythrophores (Oshima et al., 1996). Pigment dispersion was affected by nanomolar concentrations of tilapia PRL_{177} but only by micromolar concentrations of tilapia PRL_{188}. Specific activation of c-Fos, p21, p53, Bcl-xl, and other key components of cellular stress response networks and cell survival signaling by *Om*PRLR2 binding of PRL_{177} can explain the increased osmotolerance of HEK293 cells over-expressing *Om*PRLR2.

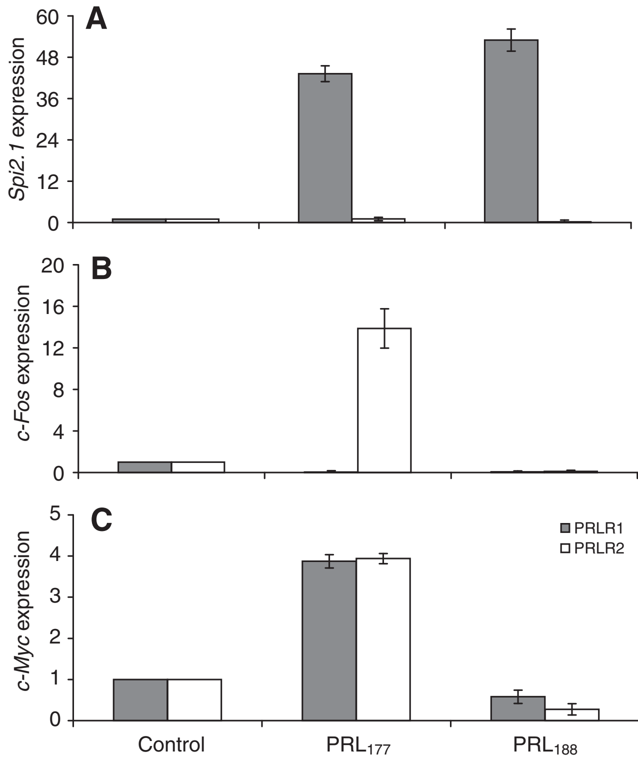

Fig. 8. *Om*PRLR1 and *Om*PRLR2 have differential responsiveness to tilapia PRL variants and distinct downstream targets. HEK293 cells expressing *OmPRLR1* or *OmPRLR2* from a tetracycline-inducible promoter were treated with 20 ng ml^{-1} tilapia PRL_{177} or PRL_{188}. *c-Myc* (A), *c-Fos* (B) and *Spi2.1* (C) gene expression was quantified by quantitative real-time PCR. Data represent means ± s.e.m. (*N*=6).

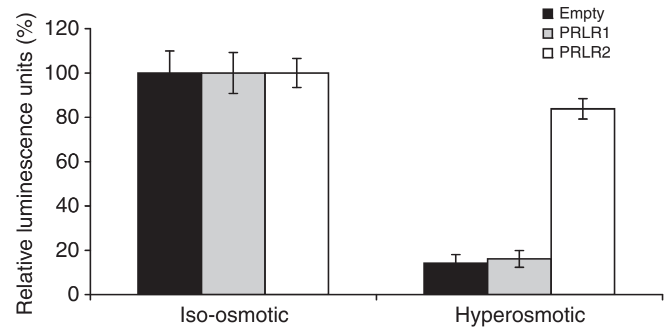

Fig. 9. *Om*PRLR2 confers increased tolerance to hyperosmotic stress in HEK293 cells. Cells expressing *Om*PRLR1 or *Om*PRLR2 and corresponding empty vector controls were exposed for 24 h to hyperosmotic medium (550 mosmol kg^{-1}). Survival was assessed as described in Materials and methods. Results represent means ± s.e.m. for three independent experiments.

However, we cannot exclude the possibility that *Om*PRLR2 activates additional survival pathways that are not activated *via* *Om*PRLR1 and lead to increased osmotolerance. Nonetheless, a transient increase in osmotolerance, whether mediated *via c-Fos* and the other *Om*PRL2 target genes identified in this study or by as yet unidentified PRLR2 targets, may be beneficial during the transient 'crisis period' encountered by tilapia upon transfer to SW. During this period, which lasts about 1–3 days, major restructuring of the gill epithelium and changes in the direction and activity of ion transport take place. The transient increase in PRLR2 expression may serve to support cell survival during this time.

In summary, we have identified a novel prolactin receptor in tilapia (*Om*PRLR2) and compared its expression at mRNA and protein levels to that of *Om*PRLR1. We identified a short *Om*PRLR2 splice variant, which is differentially expressed by males and females and regulated by environmental salinity. Functional characterization of both *Om*PRLRs revealed differences in their responsiveness to tilapia prolactins (177 or 188) and several shared but also unique downstream targets. In particular, activation of *c-Fos via* transient induction of *Om*PRLR2 and/or inhibition of prolactin signaling *via*

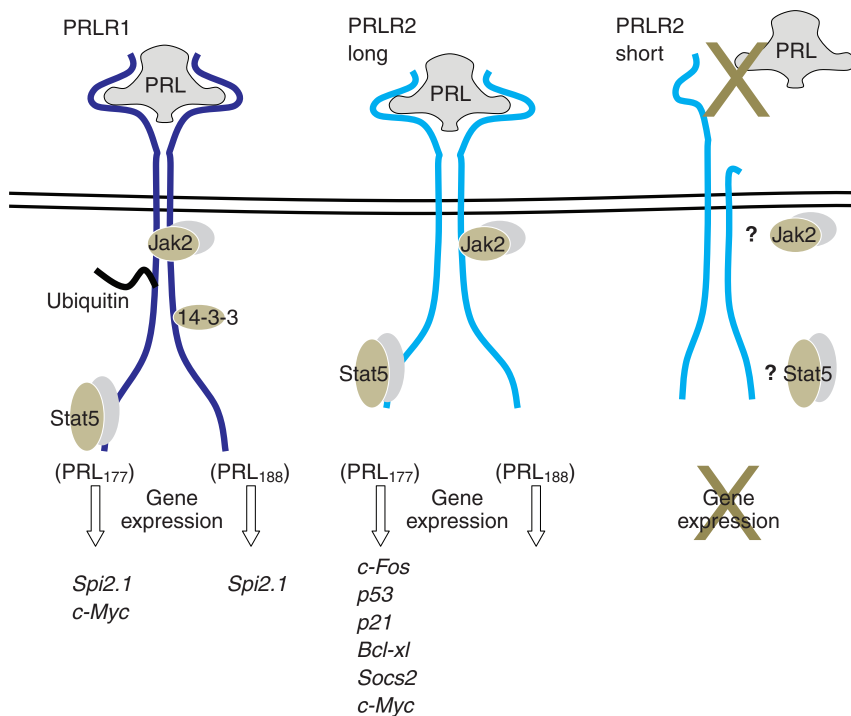

Fig. 10. Model illustrating the structure and potential mechanisms of action of *Om*PRLR1 and the two *Om*PRLR2 splice variants. Known interactions, including Jak2 binding, Stat5 binding, 14-3-3 binding and ubiquitination, are depicted. Specific induction of survival genes *via* PRL_{177} activation of *Om*PRLR2 and/or inhibition of prolactin binding to hybrid receptors consisting of long and short *Om*PRLR2 splice variants represent potential mechanisms through which transient induction of *Om*PRLR2 during salinity stress could trigger pro-survival signaling and increase osmotic stress tolerance (see text).

formation of non-functional receptor hybrids may contribute to osmoprotection of gill cells during the period of active restructuring of gill epithelium in response to salinity stress. Further dissection of prolactin signaling pathways is necessary to discern how each variant of this hormone (PRL_{177} and PRL_{188}) contributes to physiological osmoregulation during steady-state conditions and during periods of acute osmotic stress.

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