Activation of gene transcription by tilapia prolactin variants tiPRL188 and tiPRL177

Frédéric Sohm[1,a,b], Alain Pezet[a,b], Olivier Sandra[c], Patrick Prunet[c], Amaury de Luze[a,b], Marc Edery[b,*]

[a] *Laboratoire de Physiologie Générale et Comparée, Muséum National d'Histoire Naturelle, Unité de Recherche Associée 90, Centre National de la Recherche Scientifique, 75231 Paris Cedex 05, France*
[b] *Unité 344, Endocrinologie Moléculaire, Institut National de la Santé et de la Recherche Médicale, Faculté de Médecine Necker Enfants Malades, 156, rue de Vaugirard, 75730 Paris Cedex 15, France*
[c] *Laboratoire de Physiologie des Poissons, Institut National de la Recherche Agronomique, 35042 Rennes Cedex, France*

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Abstract In the tilapia species *Oreochromis niloticus*, the pituitary releases two forms of prolactins ($tiPRL_{188}$ and $tiPRL_{177}$). The binding parameters and the activation of tiPRL-induced JAK2/Stat5 signalling pathway were analysed using a mammalian cell line transiently transfected with the tiPRL receptor (tiPRLR). Our data indicate that the tiPRLR is able to mediate transcriptional activation of the PRL responsive element. At nanomolar concentrations, $tiPRL_{188}$ activates gene transcription whereas at micromolar concentrations it inhibits luciferase transcription from the lactogenic responsive element. This is consistent with a model of receptor dimerisation. In contrast, the activation by $tiPRL_{177}$ was only reached at high (μM) concentrations. The transcriptional activities induced by $tiPRL_{177}$ and $tiPRL_{188}$ are discussed in the context of the physiology of these hormones.

Key words: Prolactin; Signal transduction; Receptor dimerization; Fish

1. Introduction

Growth hormone (GH), prolactin (PRL) and somatolactin belong to a family of vertebrate pituitary hormones. It has been postulated that these hormones evolved from a common ancestral gene through duplication and subsequent divergence [1]. PRL is the most versatile of the pituitary hormones but its primary action in fish is its osmoregulatory role during freshwater adaptation [2].

Two forms of PRL have been identified in the tilapia species *Oreochromis niloticus* and *O. mossambiscus*, one, a 177 aa form ($tiPRL_{177}$) and the other, a 188 aa form ($tiPRL_{188}$) which most closely resembles other fish PRLs [3–5]. In both species, the two tiPRLs clearly play a role in freshwater osmoregulation. Studies aimed at identifying specific actions of each highly purified tiPRL in osmoregulation have not shown differences in hypophysectomised tilapia [3,6]. However, more recent studies suggest that recombinant $tiPRL_{188}$ possesses greater sodium-retaining and calciotropic activity than does recombinant $tiPRL_{177}$ [7–9]. The actions of $tiPRL_{177}$ may be dependent on physiological status or may even be distinct, this hormone possibly having acquired new functional activities [10–12]. The recent cloning of a tilapia prolactin receptor (tiPRLR) has furnished a new tool [13], allowing investigations on the intracellular dependent pathways induced by the tiPRLs.

Activation of the mammalian PRL receptor (PRLR) occurs following ligand-induced receptor dimerisation [14,15]. A mathematical model has been developed for quantitative evaluation of biological responses associated with sequential dimerisation [16]. PRL binding to PRLR activates intracellular signalling factors such as Jak2, a non-receptor tyrosine kinase that associates with the cytoplasmic domain of the PRLR [17–19]. The signalling molecules recruited and activated by the PRLR/Jak2 complex include the signal transducer and activator of transcription 5 (Stat5) [20].

In this report, we focus our attention on the effects of the two tiPRLs on the induction of the tiPRLR signal transduction. In the absence of a characterised tilapia cell line, we transiently transfected the tiPRLR together with a lactogenic hormone responsive element (LHRE) fused to a luciferase reporter gene in the human HEK 293 cell line. We found that the binding parameters and transcriptional activities dependent on $tiPRL_{177}$ and $tiPRL_{188}$ parallel their biological potencies on osmoregulation.

2. Materials and methods

2.1. Cultures and cells

Culture media and sera were purchased from Life Technologies, Inc. The HEK 293 cell line was derived from human embryonic kidney fibroblasts transformed by human adenovirus type 5 (ATTC CRL-1573).

2.2. Hormones

Recombinant $tiPRL_{177}$ and $tiPRL_{188}$ hormones were a gift from F. Rentier-Delrue [5]. Recombinant tiGH was a gift from J. Smal (Pharos, Sart Tilman, Liège, Belgium). Labelled [^{125}I]$tiPRL_{177}$ and [^{125}I]$tiPRL_{188}$ were obtained according to the chloramine T method [7] and purified on Sephadex G75 columns by elution with phosphate buffer (0.05 M, 0.1% BSA, pH 7.5). Fractions were aliquoted and kept at −20°C until used. The specific activities of labelled hormones were 35–60 μCi/μg (1.3–2.2 MBq/μg).

2.3. Plasmids and construction of a mutant tiPRLR

cDNAs encoding the full length tiPRLR [13] and truncated mutant tiPRLR263 were cloned into pcDNA3 vector (Invitrogen) under the control of a cytomegalovirus promoter. The luciferase (*Photinus pyralis*) reporter gene was cloned into pUC19 vector under the control of thymidine kinase minimal promoter with or without (control: pUC19-tk-Luc) a six times repeat of the LHRE, a Stat5 responsive element, linked to the coding region of the luciferase reporter gene [21]. Transfection was normalised with a β-galactosidase coding sequence under

*Corresponding author. Fax: (33) (1) 43 06 04 43.
E-mail: marc.edery@necker.fr

[1]Present address: University of Southampton, Biomedical Sciences Building, Basset Crescent East, Southampton SO16 7PX, UK.

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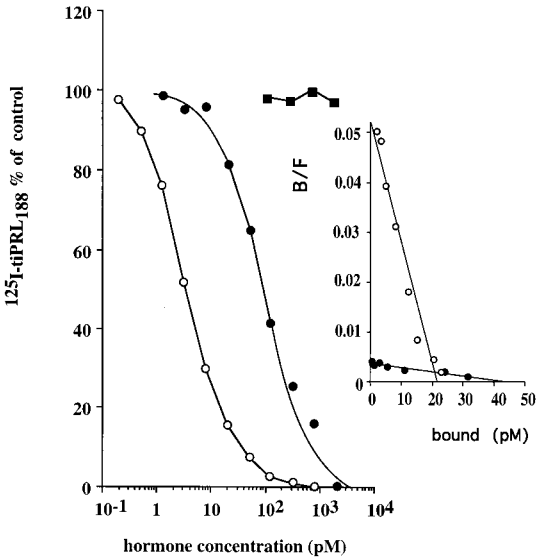

Fig. 1. Homologous competition binding and Scatchard plot of ^{125}I-labelled tiPRL$_{177}$ (●), tiPRL$_{188}$ (○) and competition binding of [^{125}I]tiPRL$_{188}$ by tiGH (■) to membrane from HEK 293 cells transfected with tiPRLR cDNA. Membranes were incubated with various concentrations of unlabelled hormones. Results are presented as percentage of maximal specific binding observed without the competitor. Each point represents the mean of three experiments. Representative Scatchard plots of each hormone, normalised per μg protein, are presented in the inset.

control of a CMV promoter (CMV-β-gal). The truncated mutant tiPRLR263 was constructed by PCR using tiPRLR cloned into pcDNA3 vector and the T7 primer (5′-ATACA-GACTCACTATAG-GGAGAC-3′) of pcDNA3 as sense primers and an antisense primer located after a proximal region of the transmembrane domain conserved among cytokine receptors and called box1 (5′-CACCTCTCT-AGAATCGCCACTCTTGAG-3′). A stop codon was inserted at position Lys^{264} with a restriction site for *Xba*I. The PCR product contains the 5′-untranslated sequence, extracellular and transmembrane domains and stop codon downstream of the box1 coding region of intracellular domain of the receptor. The tiPRLR263 thus retains only 29 amino acids in its cytoplasmic domain instead of 372 in the wild type tiPRLR [13]. The fragment was inserted into the *Xba*I and *Hin*dIII sites of pcDNA3 vector and attested by sequencing [22].

2.4. Transient transfection in HEK 293 cells and dominant negative effects assays

Cells were cultured at 37°C in 5% CO_2 with DMEM/F12 medium (Gibco) containing 10% calf foetal serum, 2 mM glutamine (Sigma) and antibiotics (Sigma). Before transfection the cells were plated into six well plates at 50% confluence (500 000 cells per well) into 2 ml of rich medium (culture medium supplemented with 1.5 g glucose/l). Cells were transfected by the phosphate calcium method [23]. In each well, cells were co-transfected with 10 ng of the receptor encoding vector, 100 ng of the vector expressing the LHRE luciferase reporter gene and 500 ng of the β-galactosidase encoding vector. Within 24 h after transfection, cells were shifted to serum-free medium containing the hormone to be tested. Hormones were assayed at concentrations ranging from 25 pM to 1.2 μM. After 16 h stimulation, cells were lysed and luciferase and β-galactosidase activities measured (Lumat LB 9501, Berthold, Nashua, NH). The relative light units were normalised by β-galactosidase activity for each sample. The data are presented as fold induction, defined as the activity of stimulated samples (with hormone) versus activity of non-stimulated samples (without hormone). For the dominant negative assay, increasing quantities of vector expressing the mutant tiPRLR263 (0, 20 and 50 ng) were co-transfected with the full length tiPRLR expression vector (10 ng), the LHRE-luciferase reporter vector (100 ng) and the β-galactosidase control vector (500 ng) and completed to a final concentration of 660 ng DNA with empty pcDNA3 vector [24]. After transfection the cells were stimulated with 24 nM of tiPRL$_{188}$.

2.5. Receptor binding assays

HEK 293 cells were transfected, in 100 mm dishes, with 4 μg of full length tiPRLR or truncated mutant/dish. After 48 h cells were scraped in 2 ml cold 1×PBS and centrifuged for 30 min at 5000×*g*. Cells were resuspended in 0.5 ml of cold assay buffer (25 mM Tris-HCl, pH 7.5/10 mM $MgCl_2$) lysed by three freeze-thawed cycles and centrifuged 20 min at 15 000×*g*. The pellet was resuspended in assay buffer and protein concentration determined by the Bradford method [25]. Membrane preparations (5–10 μg) were incubated in assay buffer with 0.5% BSA for 15 h at 20°C, in the presence of 21 000 cpm of ^{125}I-labelled hormone and various concentrations of unlabelled hormones (final volume: 0.4 ml). Reactions were stopped by the addition of 2 ml chilled assay buffer. Bound hormone was separated from unbound hormone by centrifugation at 3000×*g* for 30 min, and the supernatant removed. Pellets were counted in a LKB γ-spectrometer. Binding parameters were determined using the LIGAND program [26].

2.6. Alignment of the PRLs

In order to compare differences in functional domains involved in binding affinity between tiPRL$_{177}$ and tiPRL$_{188}$, amino acid sequence alignments of these two hormones were performed using Clustal V program of BISANCE [27]. The positions of helix and loop like the first and second sites associated with binding are shown in Fig. 5, as determined from the sequence alignment of human PRL [28].

3. Results

3.1. Competition and Scatchard analysis

The tiPRLR was transiently expressed in HEK 293 cells and used for binding experiments. The association constant (K_a) of the expressed receptor for [^{125}I]tiPRL$_{188}$ was $2.4 \pm 0.2 \times 10^9$ M^{-1} (Fig. 1). The association constant of [^{125}I]tiPRL$_{177}$ to tiPRLR transfected in HEK 293 cells was $6.0 \pm 2 \times 10^7$ M^{-1}. The K_as of the two hormones are significantly different (n=3, $P < 0.01$, Student's t-test). Next we per-

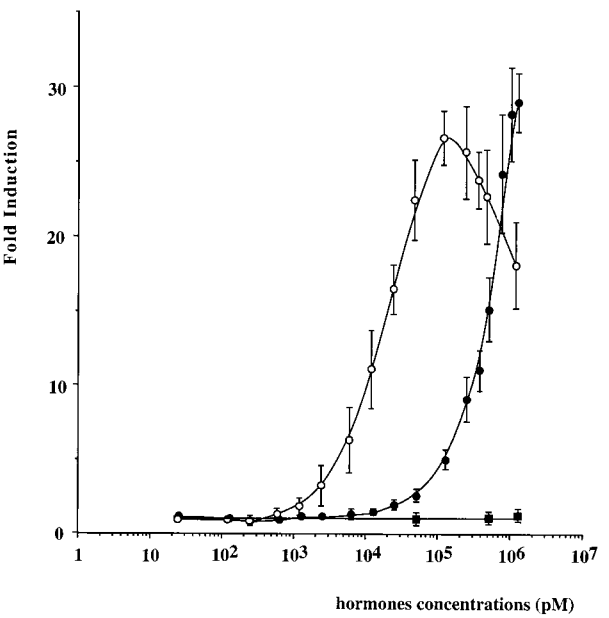

Fig. 2. Induction of the LHRE-luciferase reporter gene by tiPRLR stimulated with tiPRL$_{188}$ (○), tiPRL$_{177}$ (●) or tiGH (■). The vector expressing tiPRLR (10 ng) was co-transfected with vectors sharing the inducible LHRE-tk-luciferase reporter gene (100 ng) and the constitutive CMV-β-galactosidase reporter gene (500 ng). Cells were stimulated and the fold induction of the LHRE-tk-luciferase promoter calculated. Fold induction is defined as the activity of the promoter in presence of hormone on the activity without hormone. Each point is the mean of three independent duplicates. Vertical bars indicate the S.E.M.

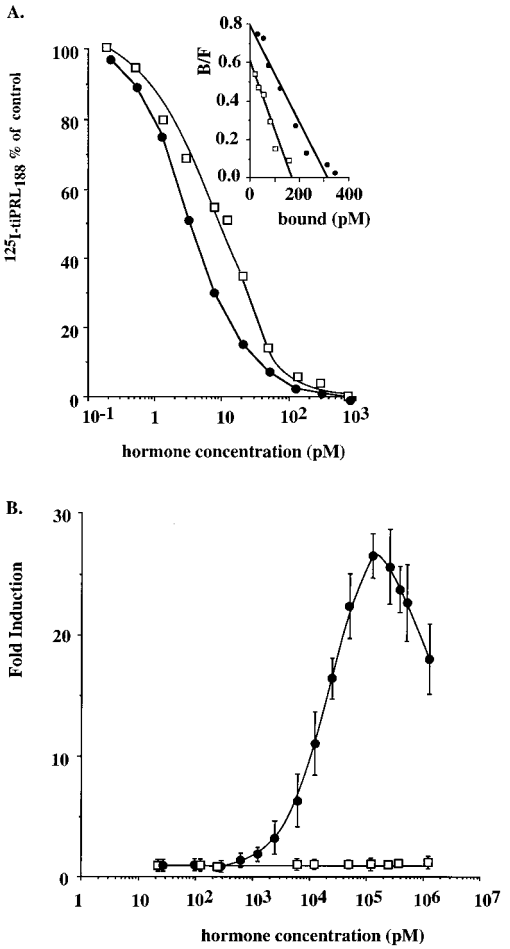

Fig. 3. A: Competition binding and Scatchard plots of [^{125}I]tiPRL$_{188}$ on wild type tiPRLR (●) and mutant tiPRLR263 (□) expressed in HEK 293 cell line. Membranes were incubated in the presence of ligand and various concentrations of unlabeled tiPRL$_{188}$. The results are expressed as percentage of the maximal specific binding observed in the absence of competitor. Wild type tiPRLR: $K_a = 2.4 \pm 0.2 \times 10^9$ M^{-1}; tiPRLR263 $K_a = 2.7 \pm 0.5 \times 10^9$ M^{-1} ($n = 3$). B: Induction of LHRE-tk-luciferase transcription in the presence of wild type tiRPRL (●) or mutant tiPRLR263 (□). Experimental conditions as in Fig. 2. Each point is the mean of three independent duplicates. Vertical bars indicate the S.E.M.

formed competition experiments of [^{125}I]tiPRL$_{188}$ by tiPRL$_{177}$ and tiGH (Fig. 1). As expected, tiGH was unable to compete with [^{125}I]tiPRL$_{188}$ in binding to the receptor. In agreement with its binding affinity, the tiPRL$_{177}$ was seen to be a less efficient competitor for displacement than tiPRL$_{188}$ and displays the same displacement curve when using [^{125}I]tiPRL$_{177}$ (data not shown).

3.2. tiPRL-induced transactivation of transcription

Both tiPRLs can activate transcription of the chimeric LHRE reporter gene but each hormone induced a specific response. Interestingly, the tiPRL$_{188}$ form activates transcription at low concentrations (600 pM) with an EC$_{50}$ of 15 nM and maximal induction (~25×) between 25 and 50 nM. Up to 120 nM, tiPRL$_{188}$ showed a bell-shaped induction curve, characteristic of self inhibition. The estimated self IC$_{50}$ was 1.4–1.6 μM. The other form of prolactin, tiPRL$_{177}$, is able to activate LHRE transcription, but at concentrations much higher than those for tiPRL$_{188}$, with maximal induction (27×) at 1.27 μM. The EC$_{50}$ was 510 nM. Thus, the tiPRL$_{188}$ which showed 35 times greater affinity than tiPRL$_{177}$ for the receptor activated it at concentrations 33 times less. Due to the limited quantity of tiPRL$_{177}$ available, we were unable to observe any bell-shaped curves with tiPRL$_{177}$ at the concentrations tested (two different batches of purified hormone were used with identical results). In agreement with the absence of tiGH binding to the tiPRLR, this hormone was unable to activate the transcription of luciferase gene up to a concentration of 1.25 μM (Fig. 2). As expected, the minimal promoter tk-luciferase did not induce any activation alone (data not shown).

3.3. Effects of a cytoplasmic deletion in the tiPRLR

The mutant receptor, tiPRLR263, was constructed by directed mutation resulting in loss of the majority of the cytoplasmic domain of tiPRLR but retaining the box1 region. The affinity of tiPRL$_{188}$ for this truncated receptor tiPRLR263 was determined by Scatchard analysis of competition experiments and found to be $2.6 \pm 0.5 \times 10^9$ M^{-1}, similar to that of the wild type receptor ($n = 3$, Fig. 3A). However, the truncated tiPRLR263 was unable to transduce a signal (Fig. 3B). The co-expression of the wild type tiPRLR and the truncated tiPRLR263 inhibited transcription induced by the wild type receptor in a dose-dependent manner (Fig. 4).

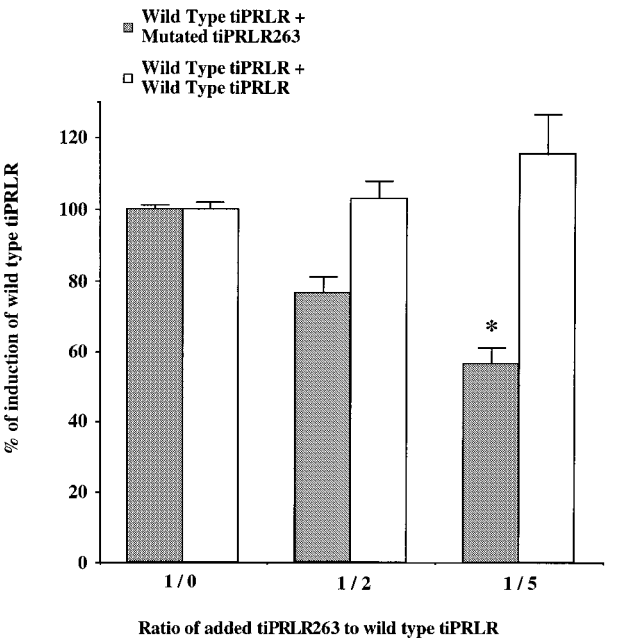

Fig. 4. Dominant negative effect of tiPRLR263 on wild type tiPRLR activity. The wild type tiPRLR (10 ng of vector) was co-transfected with increasing quantities (0, 20 and 50 ng of vector) of mutant or wild type receptor in HEK 293 cells (see Section 2). For all studies, the overall quantity of expression vector was equalised to 60 ng with empty pcDNA3 vector. The activity of tiPRLR is measured as the capacity to induce transcription of LHRE-tk-luciferase. The results are presented as percentage of activity in absence of mutant receptor (10 ng of wild type tiPRLR+50 ng of pcDNA3). The asterisk indicates significant inhibition of transcription compared to both control without mutant receptor and tiPRLR transfected under the same conditions ($n = 3$, $P < 0.005$, Duncan's multiple range test) performed in triplicate. The mutant tiPRLR263 expressed without wild type receptor is unable to induce transcription at all concentrations of vectors (10, 30 and 60 ng) (data not shown).

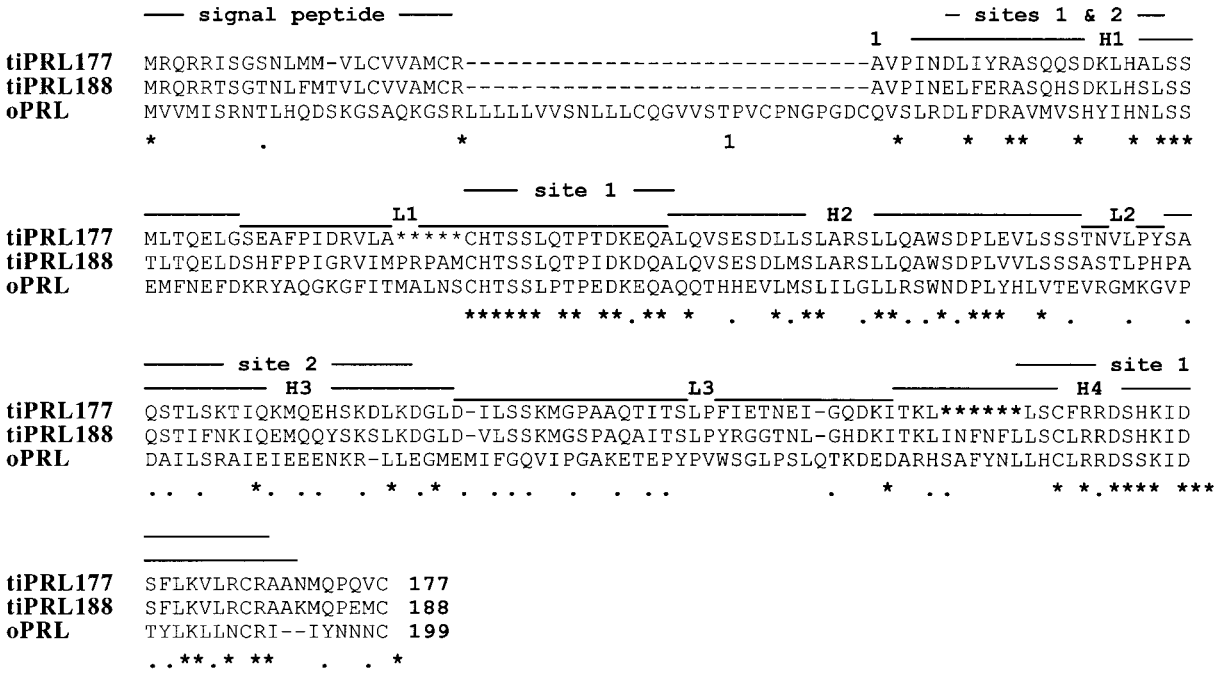

Fig. 5. Alignment of the two prolactins of tilapia (*O. niloticus*) and ovine prolactin. H1, H2, H3 and H4 are the four α helices; L1, L2 and L3 the three interhelical loops. The positions of loops and helices, like the emplacement of residues forming the first and second binding sites (respectively site 1 and 2), are reported according to sequence alignment with human prolactin (not shown). Asterisks indicate amino acids fully conserved between the three PRLs, the dots similar amino acids, and hyphens the deleted amino acids.

4. Discussion

Both tiPRLs are able to bind the tiPRLR expressed in HEK 293 cells and induce a dose-dependent transcriptional activities of the LHRE-tk-luciferase construct. The tiPRLR shares the same overall structure as the long form of the mammalian PRLR [13]. However, amino acid conservation in the cytoplasmic domain, implicated in the signal transduction, is low, corresponding to only 26–30% sequence identity [13]. Nevertheless the strong conservation of restricted regions along this domain, like the proline-rich region (box1 = PPVPGP), appears sufficient to ensure a significant activation of the LHRE-tk-luciferase reporter gene, a Stat5 responsive gene in mammalian cell lines. Moreover, although no difference in binding affinity was observed between the wild type receptor and the tiPRLR263, the latter was unable to activate the Stat5-dependent transcription when expressed alone. Furthermore, co-transfection of the tiPRLR263 inhibited the activation induced by the wild type tiPRLR in a dose-dependent manner, possibly indicating an effect due to increasing quantities of inactive wild type/mutated heterodimers. Thus, although no short form PRLR has so far been observed in tilapia, the tiPRLR263 mutant appears to behave like the mammalian short form receptor [24]. Taken together, these results indicate a strong conservation of the structure functionality required for the PRLR signalling pathways from fishes to mammals, as limited common features are required for Jak/Stat pathway activation.

Another feature associated with PRL or GH effects in mammals resides in the bell-shaped dose-response curves displayed during promoter activation [16,29,30]. Briefly, the activation of the receptor is a two-step process directed by the hormone. During the first step, the hormone recruits, via a first interaction site, one receptor molecule. This complex (1:1 hormone/receptor) is unable to transduce the signal. In a second step, the complex binds another receptor molecule to form the active receptor homodimer complex (1:2 hormone/receptors). The first and second hormone binding sites are different. Because the second site remains masked until the first site has bound a receptor molecule, the process of dimerisation is sequential. As a consequence, if the affinity of the first binding site for the receptor is greater than that of the second site, formation of inactive (1:1) hormone/receptor complexes are favoured at high hormone concentrations, and self antagonism occurs displaying a bell-shaped activation curve. The profile of luciferase expression using $tiPRL_{188}$ in our studies agrees with such homodimerisation model. These data clearly indicate that the $tiPRL_{188}$-induced homodimerisation of tiPRLR is the preliminary and necessary event for its biological effect.

Unfortunately, the quantities of $tiPRL_{177}$ available were not sufficient to obtain a bell-shaped curve with this hormone. This does not mean that the dimerisation is not necessary to the activation of the receptor by the $tiPRL_{177}$. In fact, the perfect proportionality observed comparing the binding (K_a) and activation (EC_{50}) data for both hormones argues rather for a similar mechanism. If a dimerisation-dependent activation of the receptor by the $tiPRL_{177}$ is postulated, the $tiPRL_{177}$ peak shifting towards high concentrations might indicate that the $tiPRL_{177}$ displays a lower ability to bind the first receptor molecule than does the $tiPRL_{188}$. Similarly, the maximal induction achieved by $tiPRL_{177}$, equivalent to that obtained with $tiPRL_{188}$, might indicate that the second site of both hormones is equipotent. The comparison of the sequences of the tiPRLs shows that all the essential charged amino acid residues (hydrophobic or hydrophilic) in the first site

forming region (helix 1/helix 4/loop 1) are either unmodified or substituted by homologous residues in both tilapia PRLs, the main difference being the five residues deleted in both loop 1 and helix 4 of the $tiPRL_{177}$ (Fig. 5). These deletions might create a steric hindrance in the first binding site, reducing its potency to bind the first molecule of receptor and then the ability of the $tiPRL_{177}$ to activate the receptor. Similar results have been recently obtained using 22 kDa hGH and its 20 kDa isoform on hGH receptor [31].

These data are in good agreement with the observations performed in short-term acclimatised tilapia after transfer to brackish water. Only one single class of tiPRL receptor was detected, with $tiPRL_{188}$ being the most potent ligand compared to $tiPRL_{177}$ [7,32,33]. Furthermore, the biological effectiveness of $tiPRL_{188}$ was also significantly higher on plasma osmolality, thus corroborating binding parameters [7–9,34]. In *O. niloticus* species, a converse situation implicating these two tiPRL hormones has been described recently [10,11]. The effects of the tiPRLs on pigment dispersion in cultured xanthophores and erythrophores showed inverted potencies. These comprised dose-related effects in nM range for $tiPRL_{177}$ versus effectiveness for $tiPRL_{188}$ only at μM concentrations. The mechanisms underlying the reverse responsiveness to each of the two tiPRLs in these two models in vivo are yet unknown and may implicate as well hormone/cell type-specific signalling pathways as the existence of different receptors displaying inverted affinities for the $tiPRL_{188}$ and the $tiPRL_{177}$.

In our study based on the tiPRL/tiPRLR-induced Jak2/Stat5 transduction pathway, the biological effectiveness of $tiPRL_{188}$ and $tiPRL_{177}$ requires respectively low (nM) and high hormone concentrations close to μM. Thus, the parallel responsiveness of the two tiPRLs in our model and osmoregulatory organs strongly suggests that their effect on water, sodium and chloride movements in fish might be dependent on the Stat5 signalling pathway.

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