5-1-2011

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**Recommended Citation**  
Integrative and Comparative Biology, volume 50, number 1, pp. 110–123

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SYMPOSIUM

Functional Divergence of Glycoprotein Hormone Receptors

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Synopsis

Two lamprey glycoprotein hormone receptors (lGpH-R I and II) highly similar with gnathostome GpH-Rs were cloned from sea lamprey testes and thyroid, respectively. Vertebrate glycoprotein protein receptors have a large extracellular domain (ED) containing a leu rich domain (LRD) linked to a rhodopsin-like transmembrane domain (TMD) through a highly divergent linker region (signal specificity domain, SSD or ‘hinge’ region) and a third major segment, the intracellular domain. To determine the potential roles of the different domains in the activation of the receptor following ligand-receptor binding, functional assays were performed on lGpH-R I/rat luteinizing hormone (LH)-R domain swapped chimeric receptors. These results show that the functional roles of the lamprey glycoprotein-receptor I (lGpH-R I) domains are conserved compared with its Gnathostome homologs. The ability of different glycoprotein hormones to activate chimeric lamprey/rat receptors suggests that the selectivity of the GpH-Rs in respect to their ligands is not controlled exclusively by a single domain but is the result of specific interactions between domains. We hypothesize that these interactions were refined during millions of years of co-evolution of the receptors with their cognate ligands under particular intramolecular, intermolecular and physiological constraints.

Introduction

The thyroid and gonadal steroid hormone secretions as well as development of the thyroid and gonads in vertebrates are controlled by three closely related glycoprotein hormone (GpH)/glycoprotein hormone receptor (GpH-R) tandems. Thyrotropin hormone (TSH) is released from anterior pituitary thyrotrhops and binds to the thyrotrpin hormone receptor (TSH-R) in the thyroid modulating the gene expression in this tissue (Szkudlinski et al. 2002). Similarly, the pituitary follicle stimulating hormone (FSH) binds to the cognate receptor (FSH-R) expressed on the membrane of the testicular Sertoli or ovarian granulosa cells (FSH-R) (Simoni et al. 1997). The expression of the receptor for the pituitary luteinizing hormone (LH-R) was detected predominantly in the Leydig cells of the testes and in theca, interstitial, granulosa, and luteal cells of the ovary (Dufau 1998). Glycoprotein hormones have a similar molecular organization, being dimeric proteins with one subunit (alpha) present in all GpHs, while the beta subunit is specific to each hormone. Correspondingly, their receptors are highly similar, having a large extracellular domain (ED) containing a leu rich domain (LRD) linked to a rhodopsin-like transmembrane domain (TMD) through a highly divergent linker region (signal specificity domain, SSD or ‘hinge’ region). The third major segment is the intracellular domain (ID), which shows a lower similarity score.

The physiology of the hypothalamic–pituitary–thyroid (HPT) and hypothalamic–pituitary–gonadal (HPG) axes and in this context the interactions of the glycoprotein hormones with their receptors have been extensively studied in vertebrates, particularly in mammals. However, little is known about the evolutionary origin of this endocrine control system. It is widely accepted that all glycoprotein hormones, as well as their receptors, are descendants of common molecular ancestors and that their duplication and divergent sub-functionalization started before the divergence of the vertebrates. In recent years, a number of hormones and receptors from this group have
been identified and described in some early-evolved vertebrate lineages, particularly in fish (Freematt et al. 2006; Freematt and Sower 2008a; reviewed by Freematt and Sower 2008b; MacKenzie et al. 2009; Levavi-Sivan et al. 2010). This opened a perspective on the remarkable degree of conservation of these hormone/receptor systems across the different branches of vertebrates. The search for the patterns of endocrine control similar to HPT/G axes in the closest relatives of vertebrates, the Protochordates, has provided important clues, but no definitive answer, to the question of how a high physiological specificity has emerged from interaction of molecular species which show otherwise close functional and structural characteristics. Nine forms of gonadotropin-releasing hormone (GnRH) were identified in different species of tunicates (Di Fiore et al. 2000; Adams et al. 2003). It was reported that mammalian GnRH is able to induce steroid synthesis in isolated Ciona intestinalis gonads (Di Fiore et al. 2000) and synthetic forms of urochordate GnRH induced release of gametes when injected into adult C. intestinalis (Adams et al. 2003). Although protochordates lack thyroid follicles, the presence of thyroid-like hormones has been described in blood, endostyle, and tunic of adult urochordates as well as in their larvae (Sherwood et al. 2005). To date, no evidence has been found for the presence of gonadotropin or thyrotropin-like hormones in urochordates (Sherwood et al. 2006). The closest relative of the glycoprotein hormone receptors annotated or detectable by Blast searches with GpH-R sequence queries in the C. intestinalis genome (http://genome.jgi-psf.org/Cioin2/Cioin2.home.html) was a homolog of the relaxin receptor LGR7 (data not shown).

In cephalochordates, mammalian GnRH (type 1) peptide sequence was identified using mass spectrometry in European amphioxus (Chambery et al. 2009), although genomic searches to date have not yielded a GnRH coding sequence (Tello and Sherwood, 2009). Tello and Sherwood (2009) have identified and performed functional studies on four GnRH receptor sequences identified from the amphioxus genome; two of the four GnRH receptors responded to type 1 and 2 GnRHS suggesting that these receptors are prototypes for vertebrate GnRH receptors. In earlier studies, immunoreactive (ir)-LH had been shown in these protochordates (Nozaki and Gorbman 1992; Fang et al. 2001). More recently, the beta subunit of the glycoprotein hormone, AmpGPB5, was cloned in amphioxus (Tando and Kubowawa 2009). However, there has been no evidence for the presence of a glycoprotein hormone receptor in these organisms. These data suggest a possible direct relationship between neuroendocrine factors (GnRH, TRH) and secretion of thyroid-like or gonadal-steroid-like hormones from peripheral glandular tissues and/or activation of gametogenesis or thyroid-like functions in these primitive organisms (Gorbman and Sower, 2003; Sower et al. 2009; Tando and Kuobowa 2009). Thus, the available evidence offers little support for the existence of a pituitary-like gland acting as a relay between the brain and peripheral hormone systems prior to the emergence of vertebrates. A projection of the brain contacting the Hatschek’s pit in amphioxus was described as a possible predecessor of the hypothalamic-pituitary system in vertebrates (Gorbman et al. 1999; however, genome analyses indicate that amphioxus probably lacks homologs of several key pituitary hormones (Holland et al. 2008). From this perspective, the appearance of the hypothalamic-pituitary/thyroid/gonadal axes in vertebrates coincides with the interpolation of a new level (Hartenstein 2006; Sower et al. 2009) in the respective neural-peripheral hierarchy of gland control: the glycoprotein hormone/glycoprotein hormone receptor, concomitantly with the development of specialized glandular tissues (the pituitary).

In mammals, the GpH/GpH-R system exhibits two characteristics tightly related to their proper function under normal physiological conditions: the specificity of their temporal and tissue expression profiles and selectivity in their interaction with the ligands (Simoni et al. 1997; Ascoli 2005; Costagliola et al. 2005; Farid and Szkudlinski 2004). These characteristics have evolved during divergent evolution of the ancestral duplicated genes that were inherently neither specific in their expression nor selective in their ligand affinities.

The sea lamprey and hagfish are the only extant representatives of the jawless organisms (Agnatha). It was estimated, based on DNA data (Donoghue and Purnell 2005), that the cyclostomes (lamprey and hagfishes) diverged from the ancestral agnathans during the early Paleozoic era, 535–462 million years ago. Moreover, the fossil records suggest that the lamprey lineage diverged directly from the ancestral extant agnathans (Gess et al. 2006; Janvier 2006). This makes this species an excellent object of study for understanding the endocrinology of the ancestors of present-day vertebrates.

Two novel glycoprotein hormone receptors were identified and described in lamprey (Freematt et al. 2006; Freematt and Sower 2008a) as well as the beta chain of a novel glycoprotein hormone (Sower et al. 2006). This is the only GpH detected so far in lamprey using either molecular biological methods or...
extensive screening of the lamprey genome sequencing data. Molecular phylogenetic relationships between vertebrate GpH beta chains shows the lamprey sequence far removed from the beta TSH, FSH, and LH clade, acting as an outgroup for all vertebrate glycoprotein hormone beta subunits (Sower et al. 2006). The receptor sequences on the other hand are clustered as a sister group of the thyrotropin receptors when the molecular phylogenetic relationships are derived using the whole coding sequence data. A different picture was observed when individual functional domains were used in phylogenetic analysis (Freamat and Sower 2008a, 2008b). The ED tree exhibits the same topology as the full coding sequence. The TMD suggests a phylogeny where the lGpH-R I acts as an outgroup to vertebrate GpH-Rs while the lGpH-R II keeps its monophyletic position with the TSH-R sequences (Freamat and Sower 2008a, 2008b). We hypothesize that lamprey GpH-R I and II are homologs of later-evolved vertebrate LH, FSH, and TSH receptors, descending from a common ancestor which was TSH-R-like (Freamat et al. 2006; Freamat and Sower 2008a). The difference in the phylogenetic signals for different domains is likely due to the functional constraints acting differentially at the level of different segments of the receptor. The ED tree topology reflects a similar pattern of substitution due to the interaction with a single ligand while the TMD is more significant in respect to the actual evolutionary relationships between members of the GpH-R subfamily (Castresana 2000). In this article, functional assays were performed on lGpH-R I/rat LH-R domain swapped chimeric receptors testing various ligands to determine the potential roles of the different domains.

Materials and methods

**Glycoprotein hormone receptors**

LH-R receptor was a gift from Dr William Moyle. Lamprey glycoprotein hormone receptor I was obtained through molecular cloning from lamprey testes tissue (Freamat et al. 2006).

**Hormones**

Ovine LH and ovine TSH were obtained from National Hormone & Peptide Program (NHPP). Human FSH, human CG, and the pregnant mare serum gonadotropin (PMSG) were purchased from Sigma (Sigma-Aldrich Corporation, St. Louis, MO).

**Preparation of the chimeric lamprey/rat glycoprotein hormone receptor chimeric constructs**

LH-R receptor was subcloned in pcDNA3.1/V5-His TOPO (Invitrogen, Carlsbad, CA). The lamprey GpH-R I and rat LH-R DNA fragments were combined in chimeric functional receptors using the general SOE-PCR (splicing by overlap extension) methodology (Fig. 1). The method or variants of this method have been described by different authors and applied to a wide range of molecules (Horton et al. 1990; Yang and Zwieb 2001; Hobert 2002; Heckman and Pease 2007). The two up- and down-stream receptor fragments intended to be spliced together were first amplified with gene-specific primers and universal primers (T7 and BGH, respectively). The 3' reverse primer for the upstream fragment was usually chosen as a mutagenic primer, containing at its 5' end a region complementary to the 5' end of the downstream fragment. The amplifications were done using the Phusion(r) high fidelity polymerase from Finnzymes (Finnzymes/NEB, Ipswich, MA).

Typical amplification conditions at this step included an initial denaturation at 98°C for 3 min followed by cycling with denaturation step at 98°C for 30 s, annealing 68°C 10 s and extension 30 s at 72°C, repeated 40 times; the final extension was performed for 10 min at 72°C. The amplification products were then purified by gel electrophoresis and their approximate amount estimated by densitometry. The PCR splicing step was conducted in two phases. In the first phase, the receptor fragments were added to the Phusion(r) master mix in amounts adjusted to ensure an approximate 1:1 molar ratio between them, and then run through 10 cycles amplification with denaturation step 30 s at 98°C, annealing 10 s at 60°C and extension 30 s at 72°C. In the second phase, primers designed against the 5' end of the upstream fragment and 3' end of the downstream fragment were added to the reaction to allow subcloning of the full-length hybrid receptor. The 5' primers also included the Kozak consensus for expression in eukaryotic cells.

After amplification, the PCR product was checked on agarose gel (not shown). Usually the spliced product was easy to recognize and isolate, based on its size compared with the expected total size of the construct. After purification from the gel fragments,
the chimeric receptors were digested with Kpn I/Age I restriction enzymes (51U/25 μl reaction, o/n) and ligated into the Kpn I/Age I linearized pcDNA3.1/V5-His TOPO eukaryotic expression vector and transfected into the TOP10 (Invitrogen, Carlsbad, CA) cells. The plasmids were prepared for transfection by miniprep (Wizard DNA Preparation System, Promega Corp., Madison, WI), resuspended in endotoxin free TE buffer and verified by Kpn I/Age I restriction digest. The amount and quality of plasmid DNA was estimated by measurements of optical density at 260 and 280 nm.

Concentrations of plasmids in the stock solutions used for transfection were adjusted at the same value based on their OD concentrations and on agarose gel densitometric measurements of restriction digest products. The pCF3CF2 and pCF1CF4, containing all the lamprey/rat inter-domain junctions were verified by sequencing (SeqWright, Houston, TX) in order to validate the experimental methodology for construction of chimeric receptors, using lamprey GpH-R I gene specific and universal (T7 and BGH) primers.

The procedure for maintenance and transfection of COS-7 cell cultures

We followed the same protocols as described previously (Freamat et al. 2006; Freamat and Sower 2008a).

The cAMP-dependent signal-transduction assay

The experimental protocol for the functional assays of cAMP-dependent signal-transduction activation by membrane receptors using the Secreted Alkaline Phosphatase (SEAP) reporter system was described in Freamat and Sower (2008b). Briefly, the day before transfection the COS-7 cultures were grown in T75 flasks in CO₂ (5%) humidified incubator in DMEM with 10% FBS and were cut with 2 ml trypsin, resuspended in the same medium at 4 x 10⁵ cells/ml and plated on 6 or 3.5 cm petri dishes or in 12-well plates, depending on the design of the experiment. The next day, 3 h before transfection, the medium was replaced with fresh DMEM 10% FBS then co-transfected with receptor construct/reporter plasmid mixtures at a constant receptor:reporter mass ratio of 1:7 using the Lipofectamine 2000 transfection reagent following the manufacturers’ (Invitrogen) instructions. The next day, the cells were detached with Trypsin, resuspended at 5 x 10⁵ cells/ml count, plated on 96-well plates at 100 μl/well and returned in the CO₂ incubator over night. Different layouts for the placement of the constructs on the plate were used in order to minimize the systematic errors associated with the position on the plate. In the next step, the culture medium was removed, cells washed with serum-free DMEM, overlayed with 100 μl serum-free DMEM and incubated over night. Different concentrations of hormones (stimulation media) were prepared in

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**Fig. 1** Preparation of chimeric receptors by splicing by overlap extension (SOE-PCR). The figure shows an example of application of the procedure at the level of the rat LRD/lamprey SSD junction.
a base buffer containing phenol-red-free DMEM, 1 mg/ml BSA and 0.2 mM IBMX for all samples. The serum-free DMEM was carefully aspirated using a multichannel pipette and immediately replaced with 100 μl of the stimulation medium and returned in the CO2 incubator for overnight incubation. The assay for SEAP activity in the culture medium was started with centrifugation of the culture plates and transfer of the medium to 96 new well plates which were incubated subsequently at 65°C for 20 min to inhibit the intrinsic alkaline phosphatase activity. The culture medium was then mixed with 2× SEAP reaction buffer (2 M diethanolamine/HCl DEA, 1 mM MgCl2, pH = 9.8) containing 18 mM paranitrophenylphosphate (pNPP) chromogenic alkaline phosphatase substrate and immediately transferred to the plate reader (BioTek Inc., Winooski, VT) set at 405 nm for measuring the initial point in the progress curve. The optical density measurements were then taken at various intervals of time, increasing from a higher frequency of around 10 min in the first hour of incubation to 6 h over a period of 24 h. The absorbance data was immediately saved into a text file and the automatically inserted timestamps were used to construct the SEAP reaction progression curves.

**Experimental data analysis**

The alkaline phosphatase activity (initial rate) is a measure of the relative impact of different transfected recombinant proteins and/or treatments on the cAMP-dependent signal-transduction pathway. Initial reaction rates were derived from the raw OD versus time progress curves in two steps. In the first step the parameters of an empirical function describing a saturation kinetics [Equation (1), $A_{\text{max}}$, $K$, empirical parameters, $t$ is time] were estimated by non-linear regression. The initial estimates of the parameters were obtained by linear regression on the first points of the curve (usually corresponding to a reaction time of $<2$ h). The initial rates ($S$) were then calculated using the analytical expression for the first derivative of the saturation function using the parameters derived from regression [Equation (2)].

The initial rate for each experimental sample is the sum of the contributions of three processes [Equation (3)]: (i) the basal accumulation of cAMP due to the inhibition of the phosphodiesterase by IBMX ($S_{\text{basal}}$), (ii) the accumulation of cAMP due to the constitutive activity of the receptor construct ($S_{\text{constit}}$), and (iii) the accumulation of cAMP due to the activation of the receptor construct in response to the treatment to which the culture was exposed (hormone or forskolin) $S_{\text{treat}}$. The value for SEAP activity determined in the culture media for the concentration of ligand equal to zero ($S_0$) represents the sum between the basal value ($S_{\text{basal}}$) and the effect of the constitutive activity of the receptor constructs ($S_{\text{constit}}$) [Equation (5)]. The values of $S_{\text{basal}}$ were estimated experimentally as the enzyme activity in the medium samples obtained from cultures transfected with the blank vector (pcDNA3.1/V5-His TOPO) [Equation (6)].

\[
f(x) = A_{\text{max}} \times (1 - \exp(-K \times t)), \quad (1)
\]

\[
S = f'(0) = A_{\text{max}} \times K, \quad (2)
\]

\[
S_{\text{total}} = S_{\text{basal}} + S_{\text{constit}} + S_{\text{treat}}, \quad (3)
\]

\[
S_{\text{totalfSk}} = S_{\text{basal}} + S_{\text{constit}} + S_{\text{fSk}}(\text{forskolin}), \quad (4)
\]

\[
S_0 = S_{\text{basal}} + S_{\text{constit}}(\text{dose} = 0), \quad (5)
\]

\[
S_{\text{basal}} \approx S_{\text{blank}}(\text{pcDNA3.1}), \quad (6)
\]

\[
\%S_{\text{constit}} = 100 \times \frac{(S_0 - S_{\text{blank}})}{(S_{\text{totalfSk}} - S_0)}, \quad (7)
\]

\[
\%S = 100 \times \frac{(S_{\text{total}} - S_0)}{(S_{\text{totalfSk}} - S_0)}. \quad (8)
\]

Differences in means on the dose/response curves were tested using one-way ANOVA ($R$/oneway.test) and pairwise comparison of means was carried out using Welch two sample $t$-test ($R$/t.test). All numerical values are reported as means ± SEM calculated from experimental outcomes of at least two different transfections with duplicate treatment samples (dose/response data) or at least five different transfections in duplicate (constitutive activity data).

Calculations and data plots were done in the R (http://www.r-project.org/; Ihaka and Gentleman 1996) environment; the graphic files were subsequently edited in the inkscape SVG editor (http://www.inkscape.org/) in order to include the supplementary information about the constructs and ligands used.

**Results**

The lamprey lGpH-R I/rat LH-R chimeric constructs

Figure 2 shows a diagrammatic representation of the receptor constructs prepared and used in this study. They are grouped in six series, series A contains domain-swapped constructs downstream of a rat signal peptide; series B contains the same constructs.
Fig. 2 Diagram of the chimeric rat LH-R/lamprey GpH-R I chimeric receptors. The systematic interchange between rat and lamprey GpH-R homologous segments followed an experimental scheme comprised of three steps. In the first step (panels A and B) the target of recombination were the major structural domains of the two receptors. Group A contains the chimeras built downstream the rat signal peptide while the Group B have the lamprey signal peptide as common characteristic. In the next step (C and D), the chimeras of the group A and the native rat LH-R were modified by insertion of a FLAG epitope downstream their rat signal peptide. The third step in panels E and F involved changes of the signal specificity domain (SSD) of the receptors of the groups C and D, respectively. The chimeras of the group E and F differ by the type of their LRD (lamprey GpH-R I in group E, rat LH-R in group F).

Fig. 3 Effect of oLH and oTSH on cAMP signal-transduction activation by rat/lamprey chimeric receptors series A and B (Fig. 2) in COS-7 cells.
with rat signal peptide replaced by lamprey signal peptide. These first two chimeric receptor sets lack the FLAG tag and were used for cAMP signal transduction assays using two concentrations of ovine LH and ovine TSH as stimuli. The purpose of the experiments involving these constructs was to test for the possible effect of the signal peptide on the capability of cells transfected with these constructs to respond to hormone stimulation and to obtain a preliminary estimate of the effect of gonadotropins versus thyrotropin. Series C and D were obtained using the constructs of the series A as starting material; therefore all of them have the rat signal peptide upstream to the chimeric receptors. This prevents differential expression levels of chimeras due to differences in the processing of lamprey versus rat signal peptide containing precursors. The FLAG epitope (DYKDDDDK) sequence (corresponding to the GACTACAAGGAGCAGACGATAAG oligo) was inserted downstream from the rat signal peptide, at the N-terminal end of the mature peptide. The presence of the FLAG tag allows usage of commercial anti-FLAG antibodies for detection of the protein either on the cell surface by a cell-based ELISA type of assay or intracellularly by Western blotting. However, the tag was not used in this study at this stage. Constructs of the series C and D differ by the presence of the lamprey (series C) or rat (series D) ED (including SSD) upstream of all four possible combinations of the transmembrane and IDs.

The constructs of C and D series were used as templates for synthesis of the chimeric receptors classified under the series E and F which were prepared in order to test for the effect of the reciprocal exchange of the full or partial SDs of lamprey GpH-R I and rat LH-R on the ability of the constructs to interact with and be activated by mammalian glycoprotein hormones.

The SSD or 'hinge' segment is present in all glycoprotein hormone receptors in between the leu rich repeat domain (LRD) and the N-terminal end of the TMD. This is a region of poorly defined structural organization considered initially to be a mere flexible arm linking the LRD and TMD. However, new experimental evidence (Moyle et al. 2004, 2005; Lin et al. 2007) suggests that it contains functionally important residues, critically involved in the selective interaction with the ligand and in signal transduction. The SSD contains the exon9/exon10 junction in all GpH-Rs, as well as the exon10/exon11 junction in luteinizing hormone receptors.

A detailed diagram of the rat and lamprey SSD is presented in Fig. 4. Fragments of the chimeric receptors from series C and D (domain swapped) were spliced at the level of the LRD/SSD boundary (more precisely eight residues upstream of the N-terminal end of the SSD), of the exon9/exon10 junctions (rat, lamprey) and exon10/exon11 (rat) junction. This resulted in six different combinations of the SSD fragments inserted in the original eight domain-swapped chimeras (Fig. 2E and F).

**Estimation of the response of the GpH-R chimeras to oLH and oTSH**

The effects of ovine LH and ovine TSH were tested using the constructs of the series A and B (Fig. 3).
The results were calculated as fold increase over the corresponding values obtained when concentration of stimulus was zero. This implies that the values obtained here do not account correctly for the relative contribution of the constitutive activity over the effect of exposure to the hormone. This can be noticed for example in the case of the pRF8LF9 construct (rat LHR with lamprey ID) where the effect of the oLH is underestimated due to the high constitutive activity of this chimera (see next section). However, the results in this experiment indicate a high response for the constructs containing the rat ED and suggest that the presence of the lGpH-R I signal peptide does not impair the activation of the signal transduction by oLH treatment. The response to the TSH treatment on the other hand was low for
all constructs so this ligand was not used in the next steps.

**Analysis of the constitutive activity**

The constructs of the series C to D (FLAG containing chimeras), D and F (SSD modified chimeras) were used for assessment of the constitutive activity in COS-7 cells. Figure 5 shows the SEAP activities in the culture media at concentration of ligand equal to zero, normalized in respect to the response to forskolin 5 μM treatment in the same experiment. Three constructs showed a significantly (P<0.05) higher constitutive activity compared with both constitutive activity of lGpH-R I (pRF2LF3 construct) and rLH-R (pRF1 construct): pCF40CF20 (rat LH-R with lamprey SSD and ID), pCF40LF13 (lamprey GpH-R I with rat ED) and pRF8LF9_FLAG (rat LH-R with lamprey ID).

**Response to treatment with ovine LH, human FSH, human CG, and PMSG**

Figures 6 and 7 show the dose/response curves obtained by treatment of each of the chimeric constructs from series C and D with four glycoprotein hormones (ovine luteinizing hormone, oLH; human chorionic gonadotropin, hCG; human follicle stimulating hormone, hFSH; and pregnant mare serum gonadotropin, PMSG).

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**Fig. 6** cAMP response of domain-swapped rat LH-R/lamprey GpH-R I chimeras (series C, lamprey ED) to oLH, hFSH, hCG, and PMSG in COS-7 cells.
Analysis of series C (with lamprey ED) chimeras did not result in a significant response for any of the hormones tested with one exception: the response of the pCF3CF2_FLAG construct (lamprey GpH-R I with rat TMD) to luteinizing hormone oLH stimulation. Receptors of the series D (rat ED) resulted in a high signal detected in response to stimulation with any of oLH, hCG, and PMSG hormones. The follicle stimulating hormone hFSH was inactive with the exception of one construct, pRF8LF9_FLAG (rat LH-R with lamprey ID) for which a signal almost as high as for hCG and PMSG was detected.

**Effect of SSD modification on the response to ovine LH treatment**

Twenty-three lamprey/rat chimeras were prepared by reciprocal exchanges of the fragments of the SSD segment of both receptors applied to each of the constructs in series C and D. These constructs were then used for screening the change in their response upon exposure of the transfected cells to mammalian (ovine) LH. Only the most significant results are reported here. Insertion of the rat SSD in the wild-type IgPGr-R I resulted in increased response to hormone treatment (Fig. 8, left panel, pCF36CF23). This
increase was lost when the N-terminal fragment of lamprey SSD (see Fig. 4) replaced the corresponding region of the rat SSD (pCF37CF29). The responsiveness of the construct to oLH was restored after removal of the rat fragment corresponding to exon 10 (pCF38CF32), this chimera inducing an even higher stimulation of SEAP secretion than the rat SSD only receptor at higher concentrations of ligand.

A similar pattern was observed when the same manipulations of the SSD were applied to the lamprey ED/rat TMD hybrid (Fig. 8, right panel): insertion of the rat SSD upstream of the rat TMD (pCF36CF24) resulted in increased response of the chimera to treatment with oLH. Replacement of N-terminal fragment with lamprey sequence (pCF37CF30) resulted in an unresponsive construct. Further elimination of the 10th exon sequence of rat (pCF38CF33) restored the activation of the construct at even higher levels.

**Discussion**

Construction of chimeric proteins is an experimental tool that is widely used in the study of functional properties of glycoprotein hormone receptors. This approach was particularly effective in identification and localization of the structural determinants of the ligand binding selectivity among thyrotropin, lutropin and follitropin members of this subfamily of receptors (Dufau 1998; Ascoli et al. 2002; Costagliola et al. 2005; Moyle et al. 2005). Depending on the specifics of the experimental designs applied, smaller or larger fragments of one receptor are inserted into a second one and the properties of the new construct are evaluated by different experimental methods in respect to the properties of the original proteins as a reference. Application of this methodology to investigate the functional divergence between a lamprey GpH-R (lGpH-R I) and a mammalian (rat) GpH-R (rLH-R) resulted in identification of specific combination of domains exhibiting functional properties which are distinct from their parent receptors (lamprey and rat) in respect to both ligand selectivity and constitutive activity. This suggests that evolutionary change at the level of individual domains did not alter fundamentally their original functions, but receptor selectivities have been refined by subtle correlated changes in multiple domains under the specific constraint of binding of increasingly divergent ligands.

Although not always explicitly stated, this experimental approach is based on an assumption of a non-random effect of the transfer of a particular protein fragment from one molecule to another, i.e., on an assumption of functional homology between the protein segments under investigation. This relies ultimately on the concept of the proteins as strings of domains, each domain being a functional unit, exposed to specific evolutionary constraints. In the case of the GpH-R subfamily of receptors, the main structural/functional units usually characterized are the ED, the TMD, and the ID. Their functions are, respectively, the binding of the ligand, the transfer of signal to the intracellular medium, and the regulation of silencing and internalization of the receptor.

Experimental evidence collected especially in the past decade suggested a finer functional specialization
within these broader structural units, leading to identification of the roles of conserved residues within the leu rich repeat domain (LRD) (Smits et al. 2003) and the cys rich boxes 1, 2, and 3 (Bonomi et al. 2006). Moreover, the formerly neglected, poorly conserved, low complexity or intrinsically disordered regions of proteins have received new attention after the collection of experimental evidence indicating that these regions may play an important role in the protein function both in general (Chen et al. 2006) or in the particular case of the LRD/TMD linker of GpH-Rs (Bernard et al. 1998; Moyle et al. 2004). Lamprey GpH-R is the most distant member of the vertebrate group of glycoprotein hormone receptors identified to date (Freemat and Sower 2008b). A glycoprotein hormone beta chain was also described in lamprey and its properties are being investigated but no data are available at this point on the binding to IgP-H-R I or on activation of signal transduction (Sower et al. 2006). The IgP-H-R I domains were assigned based on a multiple alignment with the vertebrate FSH-Rs, LH-Rs, and TSH-Rs, but the only support for their putative roles is the similarity scores with homologous regions of vertebrate GpH-Rs (Freemat et al. 2006). The identity scores between lamprey and rat domains are as follows: 40% for LRD [comparable with the identity scores calculated between members of the vertebrate GpH-Rs which varies between 39 and 46% (135)], 25% for SSD (low identity score but one of the highest found between IgP-H-R I and all other vertebrate GpH-Rs), 67% for TMD (again, comparable with vertebrate scores 68–72%), and 20% for ID (the most divergent segment) (Freemat and Sower 2008b).

Given the lack of mechanistic data for IgP-H-R activity and the large evolutionary distance separating them, it is difficult to draw an uncontroversial parallel between the functions of the similar sequence segments of these two receptors. Moreover, the possibility exists that the chimeric receptors exhibit new emergent properties, not directly related to the properties of the parent receptors, properties resulting from novel chains of interactions between residues and domains put in close proximity for the first time (Campbell et al. 1997). Taking into account these factors, we attempt to interpret the effects of glycoprotein hormones on the chimeric lamprey/rat receptors in three respects: (i) as indicative of the functional roles of the lamprey GpH-R I segments formally identified, based on their similarity scores in the MSA, (ii) as providing further mechanistic information in respect to the roles of the rat LH-R domains, and (iii) as emergent properties, uniquely characteristic to the respective lamprey/rat combination of domains.

The traditional concept on the mechanism of ligand binding and signal transduction (Model A) assigns distinct roles to the extracellular and TMDs of the GpH-Rs. The ligand contacts both the ED at the level of the Leu rich repeat (LRD) and the TMD at the level of extracellular loops 2 and 3. The specificity of interaction is encoded in a small number of residues located on the internal face of the horseshoe-shaped LRD reviewed by Dufau (1998) and Fan and Hendrickson (2005).

Identification of naturally occurring mutations in the TMD of human FSH-R resulting in an increased sensitivity of the receptor to hCG challenged the common understanding of the role of the serpentine region in the mechanisms determining the specificity of interaction of glycoprotein hormone receptors with the cognate ligands. The signal transduction activity of the receptor (TSH-R then extended to all GpH-Rs) is inhibited by the ED in the absence of the ligand (Model B) (Vlaeminck-Guilleum et al. 2002). The specific binding of the ligand induces a conformational change in the LRD which, in turn, results in activation of the signal transduction via interaction with the extracellular loops of the TMD. The specificity in this model is also encoded in the ED. However, this model does not require direct interaction between the ligand and the TMD (Costagliola et al. 2005). A third model of the specificity of activation of GpH-Rs by their ligands (Model C) (Bernard et al. 1998; Moyle et al. 2004, 2005) also indicates the Leu rich repeat domain as the primary site for binding of hormone but instead of restraining the specificity determinants to the LRD (as in Model A) or delegating the specificity of activation to a ‘locked’ state of the TMD (as in Model B), it describes the LRD, ‘hinge’ and TMD as an integrated functional unit, the selectivity of the receptor being the result of complex interactions between these three structural units and the ligand during binding and activation of the receptor. The ED/TMD linker segment is identified in this case as a SSD.

**Response of chimeric receptors to gonadotropin and thyrotropin**

In this experiment a preliminary cAMP signal-transduction assay was performed in order to screen for gonadotropin/thyrotropin response of domain-swapped constructs. Half of the constructs have the lamprey signal peptide at the N-terminal end. Constitutive activity profile of chimeric receptors suggests a role of the LRD + SSD unit in modulating the intrinsic constitutively activated state of the TMD. The constitutive (basal) activation of cAMP
pathways by the ligand-free receptor is the result of the TMD being locked in an activated state in the absence of the ligand. The basal activity of wild-type constructs pRF1 (rLH-R) and pRF2LF3 (lGpH-R I) show low levels, if any. The results in this experiment can be summarized as follows: (i) I1D induces increased constitutive activity in rLH-R; (ii) the concomitant presence of the ID and SSD segments of the lamprey in rat receptor seems to act synergistically to induce a very high level of basal activity; and (iii) this effect seems to be dependent primarily on the presence of the rat ED.

Analysis of domain-swapped mutant response to gonadotropins indicates a role of the TMD in ligand selectivity mechanisms. All chimeras were modified to include the rat signal peptide at the end of the N-terminal to ensure the homogeneity of protein expression on the surface of the cell. Four gonadotropin hormones were used in the cAMP assay in order to test for the changes in selectivity of binding and activation by gonadotropins of domain-swapped receptors. Insertion of the rat TMD into the wild-type lamprey receptor resulted in increased sensitivity to LH only. Presence of the IID downstream of the rat LRD + SSD + TMD combination altered the selectivity of the construct in respect to follitropin.

The rat SSD is capable of transferring the sensitivity to lutropin in the lamprey receptor. The presence of the rat exon 10 inhibits the activating effect of the lamprey N-terminal SSD + rat C-terminal SSD. In the last series of experiments, the effect of different reciprocal exchanges at the level SSD (‘hinge’) fragments of lGpH-R I and rLH-R on the response to mammalian lutropin was measured in an attempt to determine the possible role of this region in receptor lGpH-R I selectivity. Exon 10 is dispensable for the binding of hCG by hLH-R (Ascoli et al. 2002).

In summary, the results indicate that although the lamprey GpH-R I shows a low capability to be activated by a tetrapod gonadotropin (oLH), replacement of the C-terminal half of its SSD with its rat counterpart drastically changes its responsiveness to oLH treatment. This indicates that the functionality of the glycoprotein hormone receptors was remarkably well conserved in spite of the hundreds of millions of years of divergent evolution.

Acknowledgments

We thank Dr Bill Moyle from the University of Medicine and Pharmacy of New Jersey for his much appreciated help with materials and for suggestions; and to all the members of the Sower Laboratory at the University of New Hampshire. We thank Erica Spater for her technical assistance with preparation of the manuscript.

Funding

This research was supported by National Science Foundation grants IBN-0421923 and IOS-0849569 to SAS. This is scientific contribution 2422 from the New Hampshire Agriculture Experiment Station. National Science Foundation IOS-0938240 provided funds to support the symposium speaker.

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