Differential expression of the thyrostimulin subunits, glycoprotein $\alpha 2$ and $\beta 5$ in the rat pituitary

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Abstract

Two glycoprotein hormone subunits, (glycoprotein hormone $\alpha 2$ -subunit GPA2) and (glycoprotein hormone $\beta 5$ -subunit GPB5) have been recently discovered which, when expressed *in vitro*, heterodimerize to form a new hormone called thyrostimulin. Thyrostimulin activates the thyroid-stimulating hormone receptor (TSHR) and has thyrotropic activity. Immunological studies have indicated that both subunits co-localize in pituitary cells. To explore the function of thyrostimulin in the rat, we have cloned rat GPA2 and GPB5, reconstituted the heterodimers *in vitro*, and confirmed that rat thyrostimulin activates TSHR with an affinity similar to that of TSH. *In situ* hybridization of the pituitary showed that while GPA2 is expressed in the anterior lobe, GPB5 is not detected in any of the lobes. A quantitative analysis showed that the co-localization of GPA2 and GPB5 is restricted in the rat to the eye and the testis. We found that GPB5 can be detected in the pituitary by quantitative-PCR, but at extremely low levels, 2000-fold lower than TSH β -subunit (GPBtsh). Furthermore, the levels of GPB5 remain constant during the estrus cycle, while those of GPA2 vary. Finally, we found that none of the thyrostimulin subunits was induced by TRH in pituitary cell culture. These data point at the thyrostimulin system as being functionally different to the TSH system.

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Introduction

By mining human sequence databases for similarities to the known glycoprotein hormone subunits, two new potential subunits have been recently discovered (Nakabayashi *et al.* 2002). The first one was named GPA2 in view of its homology to the common glycoprotein α -subunit (GPA1). The second one was called GPB5, as the potential fifth member of the glycoprotein hormone β -subunit family. Genomic surveys revealed that both GPA2 and GPB5 are well conserved among species; corresponding genes have been identified not only in mammals, but also fish and amphibian (Hsu *et al.* 2002). These two glycoproteins have conserved cysteine-knot and N-glycosylation motifs. It has been shown that recombinant human GPA2 and GPB5 form a heterodimeric glycoprotein hormone named thyrostimulin. Thyrostimulin binds to the thyroid-stimulating hormone receptor (TSHR) with an affinity similar to that of thyroid-stimulating hormone (TSH), and has thyroid-stimulating activity *in vivo*. GPA2 does not form heterodimers with any of the known β -subunits (GPBtsh, GPBlh (lh, luteinizing hormone), GPBfsh (fsh, follicle stimulating hormone), or GPBcg (cg, chorionic gonadotropin)) except with GPB5 and, vice versa, GPB5 does not dimerize with GPA1.

Analyses of the tissue distribution of the subunits using RT-PCR showed that GPA2 exhibits a wider distribution than GPB5, whose expression is restricted to the pituitary, thyroid, heart, and oviduct in the rat. Immunohistological studies on anterior pituitary using antihuman GPA2 and GPB5 antisera have shown that both subunits co-localize in the same cells and that these are different from any known cell pituitary types (Nakabayashi *et al.* 2002). These findings correlate with the RT-PCR studies that show that both GPA2 and GPB5 mRNAs are expressed in human pituitary. The pituitary expression of thyrostimulin has been confirmed in diverse species, and immunoreactive GPA2 and GPB5 antigens have been detected in mouse and frog pituitaries (Hsu *et al.* 2002). It was therefore proposed that thyrostimulin is a new member of the pituitary hormones and that it regulates energy metabolism through the thyroid.

In this study, we further explored the biological function of thyrostimulin in the rat. We first cloned GPA2 and GPB5 from rat pituitary RNA, expressed them in cells in culture, and determined the pharmacological characteristics of the reconstituted thyrostimulin. We then analyzed the expression profiles of GPA2 and GPB5 in rat and human tissues by quantitative-PCR (q-PCR). Finally, to identify a hypothetical regulator,

we determined whether thyrotropin-releasing hormone (TRH) can stimulate GPA2 and GPB5 mRNA levels in pituitary culture.

Materials and method

Drugs

Bovine TSH (bTSH) was purchased from Sigma, TRH was purchased from Bachem (Torrence, CA, USA), and disuccinimidyl substrate (DSS) was purchased from Pierce Biotechnology (Rockford, IL, USA).

Cloning of human and rat GPA2/B5

Oligonucleotides were synthesized from the genomic DNA sequences obtained by database search (Table 1). For the human GPA2 and GPB5, Marathon human pituitary cDNA library (Clontech) was used as a template. For rat GPA2 and GPB5, rat pituitary mRNA was prepared using Oligotex mRNA purification kit (Qiagen) and used as a template for RT-PCR, followed by nested PCR. PCR products were subcloned into pcDNA3.1/V5/His-TOPO vector (Invitrogen). The coding regions of the mature part of human and rat GPA2/B5 cDNAs were individually subcloned to pFLAG-CMV-1 expression vector (Sigma) in the multiple cloning sites in-frame with N-terminal FLAG sequence (flag-tag (short peptide DYKDDDDK)). Each insert was confirmed by DNA sequencing.

Cell culture

Plasmid DNA was mixed with LipofectAMINE transfection reagents (Life Technologies), and the mixture was diluted with opti-MEM and added to 60–70% confluent human embryonic kidney (HEK) 293T cells plated on 100-mm dishes. The transfected cells were cultured in DMEM containing 10% fetal bovine serum (FBS). In order to measure TSHR activation by calcium

mobilization assay and indirect cAMP assay in the same cell, we have established a cell line that expresses both rat TSHR cDNA and cAMP-responsive element (CRE)-luciferase reporter gene (HEK-293T/LUC/TSHR). Rat TSHR cDNA (a kind gift from Dr Toyoshi Endo, Department of Internal Medicine, Yamanashi Medical School, Yamanashi University, Japan) was subcloned to mammalian-expression vector, pcDNA3.1/zeocin (Invitrogen). And this construct was stably transfected to the cell line expressing luciferase cDNA with tandem CRE repeat at the 5'-upstream region by zeocin selection.

In vitro expression of GPA2/B5

Each of the FLAG-tagged GPA2/B5 constructs were individually or simultaneously transfected into HEK 293T cells to obtain monomeric subunits or heterodimeric complexes. Forty eight hours after transfection, regular medium was replaced with serum-free DMEM, and the conditioned medium was harvested 8 h after incubation. FLAG-tagged proteins were purified from conditioned medium using ANTI-FLAG M2 affinity gel (Sigma). The concentrations of the recombinant proteins were determined densitometrically with reference to the standard curve of the bTSH on the silver-stained SDS-PAGE gel. Some of the purified proteins were incubated with 1 mM DSS for chemical cross-linking for 30 min at room temperature before SDS-PAGE and identified by silver staining.

Cyclic AMP assay

In HEK-293T/LUC/TSHR cell line, the increased intracellular cAMP level in response to TSHR activation induces the expression of luciferase in a dose-dependent manner that enables to measure relative cAMP level by luciferase assay. HEK-293T/LUC/TSHR cells were seeded onto poly-D-lysine coated 96-well culture plates. Washed with PBS once, the cells were incubated with the samples dissolved in opti-MEM for 6 h. The cells were washed with PBS, and harvested with 100 µl of 1× cell culture lysis reagent. Ten milliliters of the cell lysate was used for luciferase assay using Luc-Screen kit (Tropix, Inc., Bedford, MA, USA).

Calcium mobilization assay

HEK293T cells stably expressing rTSHR were seeded on black-walled 96-well plates (Sigma). Cell wells were incubated for 1 h at 37°C with Ca²⁺-sensitive fluorescent dye, fura-4 (Molecular Devices, San Francisco, CA, USA) in Hanks' balanced salt solution containing 20 mM HEPES (pH 7.5). The level of [Ca²⁺]_i was monitored using a Fluorometric Imaging Plate Reader system (Molecular Devices). Test samples were loaded

Table 1 Sequences of oligonucleotides used for RT-PCR cloning GPA2 and GPB5 cDNA

	Sequence
Oligo	
Rat GPB5 F	5'-tcgactctagaagcagctccagcggaacctac
Rat GPB5 R	5'-accgaggatcctcagatggctcacactcagttg
Human GPA2 F	5'-gccgcgaattccaggaggcagtcacccaggctgccac
Human GPA2 R	5'-ggatcctctagactagtagcgagagaggcgaca
Human GPB5 F	5'-ctccggaattccagcatgaagctggcattcc
Human GPB5 R	5'-ggaagtctagatgcatgtgtctgcacacagg

into the cell wells, and the fluorescent change (arbitrary units) was monitored for 3 min.

Animals

Eight-week-old Sprague–Dawley adult rats were obtained from Charles River Laboratories (Wilmington, MA, USA). Rats were housed individually and maintained on a 12 h light: 12 h darkness cycle (0600–1800 h light) with free access to tap water and rat chow. Rats were handled and habituated for 7 days, and sacrificed in a CO₂ chamber and each tissue was dissected immediately. This study design was approved by the Institutional Animal Care and Use Committee, University of California, Irvine.

In situ hybridization

[³⁵S] UTP-labeled cRNA probes were prepared for rGPA2, and rGPB5 from cDNA subclones in transcription vectors. Both of the cDNAs include the entire coding sequences. Non-radioactive cRNA probes were prepared using fluorescein: 11-UTP for detection of mRNAs of rPOMC (rat proopiomelanocortin), rGPBtsh, rGPBlh, rGH and rProlactin from cDNA subclones in transcription vectors. Gonadotrophs were examined using the rGPBlh (rat glycoprotein β subunit of luteinizing hormone) probe. The rGH and rProlactin probes were provided by Dr Fukushi Kambe (Research Institute of Environmental Medicine, Nagoya University, Japan).

Quantitative-PCR

Quantitative-RT-PCR was performed using gene-specific primers to human GPA2/GPB5, rat GPA2/GPB5, rat GPBtsh, rat/human β -actin (Table 2). All the primer

Table 2 Sequences of oligonucleotides used for real-time PCR studies

	Sequence
Oligo	
Rat actin-F	5'-ttcaacaccccagccatgt
Rat actin-R	5'-gtggtacgaccagaggcatac
hGPA2-F	5'-cgccttcctctcggctact
hGPA2-R	5'-tgagagacgaggtgatgtgtg
hGPB5-F	5'-cctggagaaacccattctg
hGPB5-R	5'-gtcacctgtttggtcgtgttag
hGPA1-F	5'-tgtcgggtgtttctgcattgtc
hGPA1-R	5'-gcgtgcattctgggcaa
hGPBtsh-F	5'-ccaccatctgtctgtgattg
hGPBtsh-R	5'-tgggacagagcatattggga
rGPA1-F	5'-cacgtgctgtgtggccaa
rGPA1-R	5'-cagtgccagtcctgtgtgtg
rGPBtsh-F	5'-catctgcctgaccatcaaca
rGPBtsh-R	5'-cctgagagagtgctgacttg
rGPB5-F	5'-tgacgggtgaagctgcctaact
rGPB5-R	5'-ggacagccatagggttaggtgtaga
rGPA2-F2	5'-aggcagccgtcccaatc
rGPA2-R2	5'-tcacttcgactgtcacgttaa

sets were designed to span exon–intron boundaries by Primer Express software (Applied Biosystems, Foster City, CA, USA). Each primer set was confirmed for not producing non-specific amplifications resulting from self-dimerization or contamination of genomic DNA. Expression profiles were analyzed using Premium human total RNAs (Clontech) or rat mRNA from various tissues prepared with Oligotex mRNA kit (Qiagen). For nuclease-rich tissue including pancreas, spleen, and thymus, guanidium–isothiocyanate extraction method was used to lyse the tissues. With each of the RNA sample in the tissue panel, the 18S and 28S bands were identified to check the stability of RNA. PCRs were performed using SYBR Green reagents (Applied Biosystem), and analyzed by ABI 7000 sequence detection system (Applied Biosystems). For each sample, β -actin was used as an internal control. The copy number for each target gene and internal control were determined in duplicate assay from each of the standard curve within the exponential range. The relative value to β -actin was calculated from the copy numbers.

Pituitary organotypic culture

Adult male rats were killed by decapitation after anesthesia with halothane, and whole pituitaries were harvested. Neurointermediate and anterior lobes were removed and immediately soaked for ~2 min in ice-cold, oxygenated preparation buffer containing (in mM): 124 NaCl, 26 NaHCO₃, 10 glucose, 3 KCl, 1.25 NaH₂PO₄, 2 CaCl₂, and 2 MgCl₂. Pituitary slices (400 μ m thick) were prepared by tissue chopper and placed on the interface between air and medium, supported by 30 mm sterile, 0.4 μ m porous membrane (Millicell-CM, Millipore, Bedford, MA, USA). The culture medium was a 2:1 mixture of Basal Medium Eagle (Sigma; B9638) and Earle Balanced Salts Solution (Sigma; E7510), supplemented with (in mM): NaCl (20), NaHCO₃ (5), CaCl₂ (0.2), MgSO₄ (1.7), glucose (48), HEPES (26.7), 5% horse serum (GIBCO; 26050) and 10 ml/l penicillin–streptomycin (Gibco; 10378) were added, and the pH was adjusted to 7.2. Slices were maintained for 10–14 days in culture medium, which was replaced weekly, until cells formed a thin layer. At this time, they were washed once with PBS and treated in medium with or without 10^{−7} M TRH. The cells were incubated before being washed with PBS and total RNA was extracted using TRIzol reagent (Invitrogen).

Results

Cloning of rat GPA2 and rat GPB5

The rat GPA2 and GPB5 cDNAs were cloned from pituitary RNA. The cDNA sequence of GPA2 was found to be identical to that cloned from rat pancreas

Figure 2 Pharmacology of recombinant thyrostimulin. (A) FLAG-tagged recombinant thyrostimulin subunits on silver-stained SDS-PAGE gel. Lane 1, FLAG-rGPA2; lane 2, FLAG-rGPB5; lane 3, rGPA2/rGPB5 without DSS; lane 4, rGPA2/rGPB5 with 1 mM DSS; lane 5, mock transfected. Intracellular cAMP stimulation by indirect cAMP assay (B) and intracellular Ca^{2+} mobilization (C) in HEK-293T/LUC/TSHR cells in response to recombinant human rat thyrostimulin, and bovine TSH (bTSH) are shown. Circle, bTSH; square, rat FLAG-thyrostimulin; diamond, human FLAG-thyrostimulin; triangle, FLAG-GPA2; inverted triangle, FLAG-GPB5.

with the mice and human homologs respectively. The cysteine residues in the presumed mature polypeptide are all conserved, including the cysteine-knot motif and the putative N-glycosylation site. When compared

with other glycoprotein β -subunits, the rat GPB5 is truncated at its C-terminus, resulting in the losses of the third disulfide bridge and of the seatbelt structure.

Pharmacology of rat GPA2 and GPB5

FLAG-tagged GPA2 or GPB5 were expressed in HEK293T cells, affinity-purified from the conditioned medium and analyzed by SDS-PAGE (Fig. 2A). When expressed individually, the resulting FLAG-rGPA2 and FLAG-rGPB5 proteins migrate at 19.5 and 16.6 kDa respectively. (The band at 21 kDa is non-specific protein that is co-purified by FLAG-affinity column.) These MW are larger than the ones expected from the sequence of mature peptides (rat FLAG-GPA2, 134 a.a. and rat FLAG-GPB5, 133 a.a. have an expected MW of about 14.7 kDa), suggesting the addition of N-linked glycosylation chains. When GPA2 and GPB5 cDNA were co-expressed and purified, a band appeared at 36.6 kDa after chemical cross-link reaction in addition to 19.5 and 16.6 kDa, considered as the heterodimer of FLAG-rGPA2 and FLAG-rGPB5. The same cross-linking reaction did not cause homo-dimerization with either of the subunits (data not shown). The concentrations of purified proteins were determined densitometrically, and their activity for rTSHR activation was measured by both cAMP and intracellular Ca^{2+} mobilization (Fig. 2B, C). In the double-stable HEK293T cells expressing the rat TSHR cDNA and a CRE-luciferase reporter construct, TSH stimulation promoted luciferase expression due to the CRE element upstream of the luciferase cDNA. Luciferase value is known to correlate with cAMP levels (Chen *et al.* 1999). In the same cell line, we have also monitored $[\text{Ca}^{2+}]_i$ in response to TSH or recombinant proteins. The EC_{50} values of bTSH were 0.3 nM for cAMP accumulation and 3.83 nM for Ca^{2+} mobilization (Table 3) that are compatible with a previous report (Nagayama *et al.* 1989, Parmentier *et al.* 1989). FLAG-tagged rat and human thyrostimulin exhibited EC_{50} s similar to that of bTSH but the magnitudes of their responses were ~70% that of bTSH. Each of the

Table 3 EC_{50} values of recombinant GPA2 and GPB5 on TSHR

	$[\text{cAMP}]_i$ (nM)	$[\text{Ca}^{2+}]_i$ (nM)
Bovine TSH	0.30 ± 0.15	3.83 ± 0.54
Rat thyrostimulin	0.488 ± 0.34	3.62 ± 0.47
Human thyrostimulin	0.417 ± 0.18	5.67 ± 0.74
Rat GPA2	> 1000	> 1000
Rat GPB5	> 1000	> 1000
Human GPA2	> 1000	> 1000
Human GPB5	> 1000	> 1000

EC_{50} values for both cAMP accumulation and $[\text{Ca}^{2+}]_i$ in the HEK-293T/LUC/TSHR cell are shown. Data expressed as mean $\pm$ S.E.M. of triplicate assays.

Figure 3 Distribution of the GPA2 and GPB5 in rat and human tissues. The level of expression of the GPA2 and GPB5 mRNAs was measured using q-PCR in rat (A, B) and human (C, D) tissues using rat or human-specific primer-sets and normalized to that of β -actin (mean value of triplicate assay $\pm$ S.E.M.).

monomeric subunits failed to activate the rat TSHR when added individually. Heteromers generated by the combination of individually purified GPA2 and GPB5 also exhibited affinities equal to that of TSH (data not shown).

Expression profile of GPA2/B5 in human and rat tissues

We then analyzed the sites of GPA2 and GPB5 expression in the organism and compared their levels of expression in rat and human tissues by using q-PCR (Fig. 3). In rat, GPA2 mRNA was found to be relatively high in the eye (including the retina), the pituitary, and the testis. GPB5 was found to be expressed in the eye and other reproductive organs including testis, uterus, and placenta. Notably, GPB5 was virtually absent in rat pituitary and thyroid. It should be emphasized, however, that the GPB5 mRNA levels are 100–200 times lower than those of GPA2 in both rat and human. We also detected dissimilarities between the human and rat expression profiles. GPA2 mRNA is significantly expressed in the human pancreas, while it is detected at very low level in the rat. GPB5 is detectable in the human but not in the rat pituitary.

In situ hybridization of GPA2 and GPB5 in rat pituitary

As q-PCR analyses may not detect a mRNA if it is expressed in a few selective cells, we performed *in situ* hybridization (ISH) studies on adult male rat pituitary. We found GPA2 mRNA diffusely expressed in the anterior lobe, but not in the intermediate or in the neural lobe (Fig. 4A), and were unable to detect GPB5 mRNA in any parts of the pituitary. To locate the cell types expressing GPA2, ³⁵S-labeled GPA2 riboprobes were co-hybridized with fluorescein-labeled riboprobes derived from POMC, prolactin, GPB1h, and GPBtsh mRNAs, thus covering all the known cell types of the anterior pituitary (Fig. 4B, C). We could not detect co-localization of GPA2 mRNA with POMC, prolactin, LH, GH, and TSH mRNAs.

GPA2 and GPB5 mRNA levels in the rat pituitary

The fact that we were not able to detect GPB5 mRNA in the rat pituitary, while GPB5 immunoreactivity has been reported to exist (Nakabayashi *et al.* 2002), suggested to us that GPB5 may be tightly regulated in this tissue and led us to carry out a more detailed study. We analyzed GPA2 and GPB5 expression by q-PCR in the anterior and neurointermediate lobes from male and female rats, and compared with GPA1

and GPBtsh of TSH (Fig. 5A). The majority of GPA2 was localized to the anterior lobe; on the other hand, GPB5 was detectable at very low levels (100-fold lower than GPA2) in both lobes (about 30% more in anterior lobe than in neurointermediate lobe). We did not see any sexual differences in the levels of GPA2/GPB5 expressions (data not shown). These findings also document the low levels of thyrostimulin compared to those of TSH in the rat pituitary. The ratio between GPA1/GPBtsh/GPA2/GPB5 was approximately 4/1/0.05/0.0005 and explains why we did not detect GPB5 signal in ISH study in the rat pituitary (Fig. 5B). In an effort to discriminate changes in expression during the estrus cycle, we have examined the female rat pituitaries at each stage of estrus cycle (Fig. 5C). GPA2 levels were found to vary being highest at metestrus and lowest at proestrus phases. The GPA2 level at the metestrus was 3 times higher than at the proestrus phase. There were however, no significant changes in GPB5 levels (Fig. 5C), which indicates that GPB5 is not regulated by the estrus cycle.

Modulation of GPA2 and GPB5 expression by TRH in rat pituitary organotypic cultures

We carried out an attempt at finding a factor that may upregulate the thyrostimulin subunits in pituitary organotypic cultures. As thyrostimulin acts on the TSHR, we tested the effect of TRH on the mRNA levels of the thyrostimulin and TSH subunits using q-PCR. As previously reported, TRH (10 nM) induced GPBtsh mRNA by a factor of 2.5 (Fig. 6). GPA2 levels in the primary cultures were 20 times higher than those found *in vivo* in the anterior pituitary, reaching almost the same levels as GPBtsh. However, TRH did not affect GPA2 expression. The GPB5 levels were as low as those found in anterior pituitary and were not affected by TRH.

Discussion

To understand the biological function of thyrostimulin in the rat, we have cloned GPA2 and GPB5 cDNAs from pituitary RNA. The cDNA sequence of the rat GPA2 was found to be identical to the one previously reported (accession no NM 133619), while the newly cloned rat GPB5 cDNA exhibits 86.0 and 93.6% sequence similarities to its human and mouse homologs respectively. The rat GPA2 and GPB5 amino acid sequences share all structural features expected of glycoprotein hormone subunits. As a member of the cysteine knot growth factors, rGPB5 has a central cysteine knot and three loops, two β -hairpin loops on one side of a cysteine knot and a long loop on the other.

Figure 4 *In situ* hybridization histochemistry of GPA2 and GPB5 mRNAs in rat pituitary. (A) *In situ* hybridization histochemistry of GPA2 and GPB5 in sagittal slice. SEN, sense probe; αS, antisense probe. Bar, 1 mm. (B) Representative examples of fluorescein-labeled riboprobes of each anterior pituitary hormone. (C) Representative examples of dual labeling *in situ* hybridization histochemistry of GPA2 in individual anterior pituitary cell populations. The labeled GPA2 mRNA is represented by silver grains, which are revealed as white dots. The labeled hormone probe is represented by fluorescein-conjugated immunohistochemical precipitation, shown by dark staining. Bars, 20 μm.

Figure 5 q-PCR of TSH and thyrostimulin mRNA levels in rat pituitary. (A) GPA1, GPBtsh, GPA2, GPB5 mRNA levels in anterior lobe (AL; closed bar) or neurointermediate lobe (NIL, open bar) of adult male rat pituitary were determined by q-PCR. Data from three individual pituitaries, relative value to β -actin mRNA are shown (mean value, error bar represents S.E.M.). * $P < 0.05$, ** $P < 0.01$: ANOVA/Bonferoni multiple comparison test for the anterior lobe vs neurointermediate lobe. (B) The TSH and thyrostimulin mRNA levels in anterior pituitary from male rat are expressed in logarithmic scale. Each of the four glycoprotein composing TSH or thyrostimulin mRNA levels in adult male rat are illustrated for comparison. (C) GPA2 or GPB5 mRNA levels in anterior pituitary from female rats were determined. Pituitary was dissected at each stage of estrus cycle, determined by the observation of vaginal smear for more than three cycles. Relative value to β -actin mRNA are shown (mean value of six rats. Error bar represents S.E.M.). * $P < 0.05$: ANOVA/Bonferoni multiple comparison test for the proestrus versus metestrus.

In order to study the pharmacological properties, FLAG-tagged recombinant rGPA2/B5 were obtained from conditioned medium of transfected cell lines. FLAG-rGPA2 and FLAG-rGPB5 are capable of producing heterodimers. Both recombinant proteins need

each other to stimulate rTSHR followed by activation of second messenger pathways involving cAMP, and Ca^{2+} mobilization at higher concentration. The EC_{50} value of recombinant rat thyrostimulin was found to be identical to that of human thyrostimulin and purified bTSH.

Figure 6 TRH effect on GPA2 and GPB5 expression. Pituitary slices were incubated with 10 nM TRH. GPA2/GPB5 mRNA levels were measured using q-PCR. Slices from one pituitary were cultured in single well and total RNA was extracted. Each data point represents mean value of six pituitaries, relative to β -actin (mean $\pm$ S.E.M.). * $P < 0.05$: ANOVA/Bonferroni multiple comparison test for 10 nM TRH versus control. n.s., not significant.

This result is in agreement with the previous report showing that human recombinant thyrostimulin binds to TSHR at the same range as bTSH (Nakabayashi *et al.* 2002).

We have shown by *in situ* hybridization histology that GPA2 mRNA is diffusely expressed in anterior lobe of rat pituitary. As we did not find co-localization of GPA2 mRNA with any of the anterior pituitary hormone mRNAs, including TSH, LH, POMC, GH, and prolactin, we conclude that GPA2 mRNA might be expressed in an unidentified cell type. In contrast, GPB5 mRNA was not detected in the rat pituitary by *in situ* hybridization. This result is in contradiction with the data reporting GPB5 immunoreactivity in male rat anterior pituitary (Nakabayashi *et al.* 2002, Li *et al.* 2004). We therefore quantified the levels of GPA2 and GPB5 mRNAs in the rat anterior pituitaries and found that the ratio of GPA1/GPBtsh/GPA2/GPB5 was 4/1/0.05/0.0005, indicating that GPB5 is practically non-existent. This discrepancy with the immunocytochemical data suggests the following possibilities: (1) the GPB5 protein is stored in the pituitary but its mRNA expression is practically undetectable in the normal conditions, or (2) the antisera used in the previous studies cross-reacts with other antigens. Noteworthy, the specificity of the polyclonal GPB5 antisera was confirmed against the recombinant protein (Nakabayashi *et al.* 2002) or by preabsorption studies (Li *et al.* 2004), techniques which do not address the possible cross-reactivity issues.

We further analyzed the levels of GPA2 and GPB5 mRNAs in the rat pituitary during the estrus cycle. We found that, while GPA2 mRNA is regulated, GPB5 mRNA is not subject to change. This is in contrast to what has been found for other glycoprotein hormones. LH and FSH β -subunit levels are regulated during the estrus cycle, while the levels of their α -subunit

remain mostly constant (Zmeili *et al.* 1986). From the heterodimeric nature of thyrostimulin, the lack of change in GPB5 levels suggests that it may not be involved in the reproductive system regulation, but this might also indicate that GPA2 binds a partner other than GPB5, and thus far unknown, in the pituitary.

In the hypothalamic–pituitary–thyroid (HPT) axis, TRH positively regulates GPBtsh transcription (Shupnik *et al.* 1996), and thyroxine (T4) negatively regulates TRH and TSH (Shupnik *et al.* 1985, Koller *et al.* 1987). Nakabayashi *et al.* (2002) have shown that i.v. administrations of recombinant thyrostimulin increase serum T4 level in T3-treated rat when both TRH and TSH are suppressed by excess amounts of orally administered thyroid hormone. Although this illustrates that thyrostimulin is a potent thyroid stimulator, it does not necessarily mean that this novel hormone is positively regulated by hypothalamic TRH in the same manner, as does TSH. In an attempt to clarify this issue, we have analyzed whether TRH regulates thyrostimulin expression in primary culture of rat pituitary. While confirming that TRH increases GPBtsh expression, we found that the levels of GPA2 and GPB5 expression in slice cultures were refractory to TRH. This opens the possibility that thyrostimulin would control thyroid function through a mechanism independent of the classical HPT axis. If that were the case, one would expect a hyperthyroid state caused by thyrostimulin in the patients showing normal to high thyroxine level with the absence of TSH. This ‘hyperthyroidism with suppressed TSH’ is known with patients suffering Graves’ disease, hyperemesis gravidarum, and trophoblastic diseases. The pathogenic agents causing these diseases have been identified: thyroid-stimulating antibodies in Graves’ disease, excess levels of human chorionic gonadotropin (hCG) and in hyperemesis gravidarum (Lao *et al.* 1988, Rodien *et al.* 1998) and in trophoblastic diseases (Nisula & Ketelslegers 1974, Nisula & Taliadouros 1980, Hoermann *et al.* 1994). Recently, two cases of goitrous euthyroid patients presenting low TSH have been reported (Ikekubo *et al.* 2005). Upon administration of TRH, the TSH levels stay undetectable, while those of tri-iodo thyronine remain in the normal range. The authors suggest the existence of a putative thyroid-stimulating factor to explain the etiology. Thyrostimulin may be this factor, since we show that it is not positively regulated by TRH and thus quantification of thyrostimulin in these patients would be of interest. However, in view of the differential expression of GPA2 and GPB5 in the adult rat anterior pituitary, one may predict that: (1) GPA2 acts as a monomeric protein, or (2) GPA2 heteromerize with a partner that is different to GPB5, and (3) GPB5 appears only transiently in the life of the pituitary, for example, during embryogenesis, as has been found for GPBtsh (Lin *et al.* 1994).

Although we have focused on the pituitary as a primary site of thyrostimulin synthesis, some other tissues such as the eye, testis, and pancreas express more GPA2 and GPB5 than the pituitary. Thyrostimulin might therefore be produced in extrapituitary tissues as has been shown for TSH, which is expressed in lymphocytes (Harbour *et al.* 1989, Peele *et al.* 1993), thymus, and various tissues in chick embryo (Murphy & Harvey 2001). Indeed, a cross-talk between the immune and neuroendocrine systems (hypothalamic–lymphoid–thyroid regulatory axis) has been postulated, since TRH increase GPB5 expression in lymphocyte (Wang *et al.* 1997). Furthermore, binding sites for TSH, as also for thyrostimulin, have been described in a variety of extrathyroidal tissues, including lymphocytes, adipocytes, and testicular and adrenal tissues (Pekonen & Weintraub 1978, Davies *et al.* 1987). One of the tissues of interest is the testis, where both ligand and receptor (Feng *et al.* 1993, Montagne *et al.* 1999, Kumar *et al.* 2000) co-localize. However, the presence of thyrostimulin in the testis is a species-specific event, as it does not occur in human reproductive tissues.

In summary, we have analyzed the expression of the two glycoprotein subunits, GPA2 and GPB5, composing the novel thyroid-stimulating agent, thyrostimulin, in rat and human tissues. We have focused our analysis on the rat pituitary and conclude that the low levels of GPB5 expression in the rat pituitary suggest that the pituitary is not the primary source of thyrostimulin. This led us to propose that thyrostimulin is not part of the typical HPT axis. Further studies will be necessary to elucidate the roles of thyrostimulin.

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