Paracrine regulation of growth hormone gene expression by gonadotrophin release in grass carp pituitary cells: functional implications, molecular mechanisms and signal transduction

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Abstract

Growth hormone (GH) is known to stimulate luteinizing hormone (LH) release via paracrine interactions between somatotrophs and gonadotrophs. However, it is unclear if LH can exert a reciprocal effect to modulate somatotroph functions. Here we examined the paracrine effects of LH on GH gene expression using grass carp pituitary cells as a cell model. LH receptors were identified in grass carp somatotrophs and their activation by human chorionic gonadotropin (hCG) increased 'steady-state' GH mRNA levels. Removal of endogenous LH by immunoneutralization using LH antiserum inhibited GH release and GH mRNA expression. GH secretagogues, including gonadotrophin releasing hormone (GnRH), pituitary adenylate cyclase-activating polypeptide (PACAP) and apomorphine, were effective in elevating GH mRNA levels but these stimulatory actions were blocked by LH antiserum. In pituitary cells pretreated with actinomycin D, the half-life of GH mRNA was not affected by hCG but was enhanced by LH immunoneutralization. Treatment with LH antiserum also suppressed basal levels of mature GH mRNA and primary transcripts. hCG increased cAMP synthesis in carp pituitary cells and hCG-induced GH mRNA expression was mimicked by forskolin but suppressed by inhibiting adenylate cyclase and protein kinase A. Similarly, the stimulatory actions of hCG and forskolin on GH mRNA expression were blocked by inhibiting Janus kinase 2 (JAK2) and MAP kinase (MAPK), including P42/44^{MAPK} and P38^{MAPK}. These results suggest that LH is essential for the maintenance of GH release, GH gene expression, and somatotroph responsiveness to GH-releasing factors. The paracrine actions of LH on GH mRNA expression are mediated by a concurrent increase in GH gene transcription and GH mRNA turnover, probably through JAK2/MAPK coupled to the cAMP-dependent pathway.

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Introduction

Growth hormone (GH) is well documented to be an important pituitary hormone regulating body growth and metabolism. However, increasing evidence suggests that GH also plays a role in reproductive functions. Recently, GH has been proposed to be a 'co-gonadotrophin' in mammals (Hull & Harvey 2002). This idea is supported by the findings that correlative changes in GH secretion can be observed at various stages of the ovarian cycle, sexual maturation, pregnancy, and seasonal breeding (Hull & Harvey 2000*a,b*). Furthermore, pathological conditions with gonadal deficiency (e.g. Turner's syndrome) tend to be associated with a drop in GH secretion, which can be partially rectified by steroid replacement (Wit *et al.* 1992). Gonadal steroids also modify the pulsatile pattern of GH release (Kerrigan & Rogol 1992), probably by regulating gene expression of GH-releasing hormone (GHRH) in the arcuate nucleus (Zeitler *et al.* 1990) and somatostatin (SRIF) in

the periventricular nucleus (Argente *et al.* 1990). In addition to these central actions, gonadotrophic and somatotrophic interactions can also occur at the pituitary level. In the rat, GH binding sites have been identified in gonadotrophs (Harvey *et al.* 1993) and basal secretion of luteinizing hormone (LH) and/or follicle-stimulating hormone (FSH) *in vivo* (Schemm *et al.* 1990) and *in vitro* (Tang *et al.* 1993) can be elevated by GH treatment. The stimulatory effects of gonadotrophin (GTH)-releasing hormone (GnRH) on LH and FSH release, however, can be inhibited by GH immunoneutralization (Chandrashekar & Bartke 1998). These findings, as a whole, raise the possibility that GH stimulates GTH secretion by local interactions between somatotrophs and gonadotrophs (Schwartz 2000). In this case, GH-induced GTH release regulates reproductive functions through GTH receptors coupled to the adenylate cyclase (AC)/cAMP/protein kinase A (PKA) and phospholipase C (PLC)/inositol 1,4,5-triphosphate (IP3)/protein kinase C (PKC) cascades (Ascoli *et al.*

2002). Functional coupling of GTH receptors with mitogen-activated protein kinases (MAPK) and phosphoinositol 3-kinase (PI3K) has also been reported recently (Seger *et al.* 2001, Carvalho *et al.* 2003). Given that the direct actions of GTH on GH release and synthesis at the pituitary level have not been examined, whether GTH can also act in a reciprocal manner to modify somatotroph functions is unclear.

The bony fish, or teleosts, are unique for the lack of a hypothalamo–hypophyseal portal blood system and the median eminence is functionally integrated into the anterior pituitary (Peter *et al.* 1990). In this case, the endocrine cells in the pars distalis are directly innervated by neurons from the hypothalamus. In addition to this structural modification in the brain–pituitary axis, a clear zonation of endocrine cells is also observed in the anterior pituitary of teleosts which is different from the random pattern of distribution found in mammals (Doerr-Schott 1980). In the pituitary of teleosts, e.g. grass carp (Wong *et al.* 1998a), lactotrophs are located exclusively in the rostral pars distalis (RPD) whereas the distribution of somatotrophs and gonadotrophs are restricted to the proximal pars distalis (PPD). In some species, e.g. tuna (Kagawa *et al.* 1998), gonadotrophs can also be found in the region along the external rim of the neurointermediate lobe (NIL). In bony fish studied to date, gonadotrophs always exhibit a patchy distribution in the PPD within a matrix formed by somatotrophs. Such a conserved pattern of anatomical relationship between these two cell types may suggest that paracrine interactions between somatotrophs and gonadotrophs also exist in fish. In salmon, it is well documented that GH also plays a role in the regulation of reproductive functions, including steroidogenesis, spermatogenesis, and oocyte maturation (LeGac 1993). In Cyprinids, e.g. white sucker (Stacey *et al.* 1984), a close correlation between GH and LH (or GTH-II) release can be noted during the period of sexual recrudescence and the spawning season. Recently, a similar correlation at the transcript level has been reported for the two hormones in gilthead seabream (Meiri *et al.* 2004). In the goldfish, the preovulatory LH surge occurs with a concurrent increase in GH release *in vivo* (Peter & Yu 1997). This parallel increase in GH and LH secretion is particularly important for seasonal breeders with overlapping somatic growth and gonadal development during the reproductive cycle. This phenomenon has been largely attributed to the stimulatory effects of GnRH on GH and LH release at the pituitary level, e.g. in goldfish (Peter & Yu 1997) and common carp (Lin *et al.* 1993). Whether local interactions between somatotrophs and gonadotrophs also contribute to the coordinated release of GH and LH in fish models is still unknown.

In this study, paracrine actions of LH on GH synthesis were examined in pituitary cells prepared from one-year-old (1+) grass carps. The grass carp at this

stage undergoes a rapid growth phase during the transition from juvenile to adult stage and represents a unique model for the studies of the mechanisms regulating GH synthesis and secretion in ‘prepubertal’ teleosts. Using a static incubation approach, the effects of exogenous LH on GH mRNA expression were tested in grass carp pituitary cells. The results of these studies were also confirmed by removal of endogenous LH using immunoneutralization. To characterize the role of LH in the responsiveness of somatotrophs to GH secretagogues, the stimulatory actions of known GH-releasing factors in fish on GH mRNA expression were tested either in the presence of exogenous LH or in the absence of endogenous LH caused by LH immunoneutralization. Parallel experiments were also conducted to study the effects of LH immunoneutralization on the clearance of GH mRNA and production of GH primary transcripts. To further elucidate the post-receptor signalling mechanisms mediating LH actions at the pituitary cell level, the effects of exogenous LH on GH mRNA expression were also tested in the presence of the inhibitors for the cAMP-, Janus kinase 2 (JAK2)-, MAPK-, and PI3K-dependent pathways respectively.

Materials and methods

Animals

One-year-old (1+) Chinese grass carps (*Ctenopharyngodon idellus*) with body weights ranging from 1.5 kg to 2.0 kg were obtained from local markets and housed in well-aerated 200 litre aquaria under a 12 h light:12 h darkness photoperiod at $18 \pm 2^\circ\text{C}$. Since the carps at this stage were ‘prepubertal’ (gonadosomatic index $\leq 0.2\%$) and sexual dimorphism was not apparent, fish of mixed sexes were used for the preparation of pituitary cell cultures. During the process, the fish were killed by anaesthesia in 0.05% MS222 (Sigma) followed by spinosectomy according to the regulations of animal use at the University of Hong Kong.

Reagents and test substances

Human chorionic gonadotropin (hCG), apomorphine (APO), and MDL12330A were obtained from Sigma (St Louis, MO, USA). Salmon gonadotrophin-releasing hormone (GnRH) and ovine pituitary adenylate cyclase-activating polypeptide-38 (PACAP) were purchased from Phoenix Pharmaceuticals Inc. (Belmont, CA, USA). Forskolin, actinomycin D, H89, 3-isobutyl-1-methylxanthine (IBMX), PD98059, SB202190, AG490 and wortmannin were from Calbiochem (San Diego, CA, USA). MEM Eagle, fetal bovine serum (FBS), trypsin, and antibiotic-antimycotic stock solution were obtained from GIBCO BRL Life Technology (Rockville,

MA, USA). Forskolin, IBMX, H89, MDL12330A, PD98059, SB202190, AG490 and wortmannin were dissolved in DMSO to form 10 mM stock solutions and stored frozen in small aliquots at -80°C . Stock solutions of peptide hormones, including hCG, GnRH, and PACAP, were prepared in a similar manner except that these peptides were dissolved in double-distilled deionized water at 1 mM concentration. On the day of experiments, frozen stocks of test substances were diluted with culture medium to appropriate concentrations 30 min before adding to pituitary cells. Since APO can be easily oxidized in solution by prolonged storage, a 10 mM stock was freshly prepared in DMSO 15 min prior to drug treatment. In these experiments, the final levels of DMSO were always below 0.1% (v/v) and had no effects on GH release and GH mRNA expression.

Primary culture of grass carp pituitary cells

Grass carp pituitary cells were prepared by the controlled trypsin/DNase II digestion method (Wong *et al.* 1998a) and cultured in 24-well clustered plates (Costar, Corning Inc., New York, NY, USA) at a seeding density of $\sim 2.5 \times 10^6$ cells/ml/well ($\sim 1.25 \times 10^6$ cells/100 mm²) in carp MEM (MEM Eagle with 26 mM NaHCO₃, 25 mM HEPES, 100 units/ml penicillin, and 100 µg/ml streptomycin; pH 7.7) supplemented with 5% FBS. The average cell yield was $\sim 8.2 \times 10^6$ cells/pituitary with a mean viability of $96.5 \pm 0.5\%$ ($n=14$). Pituitary cells were routinely incubated overnight at 28°C under 5% CO₂ to allow for the recovery of membrane receptors after trypsin digestion. On the following day, culture medium was replaced with serum-free carp MEM and drug treatment was initiated for the duration as indicated in individual experiments.

Measurement of 'steady-state' GH mRNA

After drug treatment, pituitary cells were dissolved in TRIZOL (GIBCO) and total RNA was isolated according to the instructions of the manufacturer. Individual RNA samples were divided into two equal portions for measurement of GH mRNA and 18S RNA respectively. After blotting onto a positively charged nylon membrane using a Bio-Dot SF microfiltration unit (Bio-Rad, Hercules, CA, USA), GH mRNA levels in these RNA samples were quantified as described previously (Zhou *et al.* 2004) by hybridization with a digoxigenin (DIG)-labelled cDNA probe covering the region from 75 to 444 of the grass carp GH cDNA (GenBank no: M27094). In these experiments, parallel probing of a duplicated membrane using a DIG-labelled probe for grass carp 18S RNA was used as an internal control.

Measurement of cAMP production

Freshly dispersed grass carp pituitary cells were resuspended in carp MEM and evenly seeded at a density of $\sim 8 \times 10^6$ cells/2 ml/dish ($\sim 0.83 \times 10^6$ cells/100 mm²) onto poly-D-lysine coated 35 mm petri dishes. Cell attachment ($>90\%$) was completed after 3 h incubation at 28°C under 5% CO₂. After that, FBS (5%) was introduced and pituitary cells were incubated overnight to allow for the recovery of membrane receptors. On the following day, culture medium was replaced with 0.9 ml HHBSA medium (Yunker *et al.* 2000) with IBMX (0.1 mM). IBMX, the inhibitor for phosphodiesterase, was added to prevent cAMP degradation in pituitary cell cultures. After incubation at 28°C for 15 min, hCG treatment was initiated by adding 0.1 ml $10 \times$ stock solutions at appropriate concentrations. Pituitary cells were then incubated for another 20 min, culture medium was harvested for the measurement of cAMP release and cellular cAMP was extracted by adding 1 ml ice-cold absolute ethanol. These cAMP samples were freeze-dried and stored at -40°C until their cAMP contents were assayed by a Biotrak [¹²⁵I]cAMP RIA kit (Amersham). In these experiments, cAMP production was defined as the sum of cellular cAMP content and cAMP released into the culture medium.

Immunohistochemical staining of grass carp pituitary cells

Pituitaries were freshly excised from grass carps, fixed in Bouin's fixative at 4°C , and embedded in paraffin wax according to standard procedures. Pituitary sections of 5 µm in thickness were prepared and mounted onto slides precoated with 2% 3-aminopropyltriethoxy silane (Sigma). Immunohistochemical staining was performed using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions. Antisera for carp LH (or GTH-II) and prolactin (PRL) were used at 1:1000 dilution, which matched the doses of LH antiserum used in immunoneutralization. These antisera were a generous gift from Dr R E Peter (University of Alberta, Canada) and have previously been confirmed to be specific for carp LH and PRL respectively (Wong *et al.* 1998a). The LH antiserum used in immunostaining and immunoneutralization has no cross-reactivity with other pituitary hormones (Ge & Peter 1994).

Western blot for GH release and GH content

Western blot was conducted to study the effects of LH immunoneutralization on basal GH release and GH content in grass carp pituitary cells. Pituitary cells were seeded in 6-well plates at a density of $\sim 12 \times 10^6$

cells/3 ml/well ($\sim 1.25 \times 10^6$ cells/100 mm²) and incubated with increasing levels of LH antiserum for 48 h or exposed to LH antiserum (1:2500) for 2 h, 24 h and 48 h respectively. After immunoneutralization, culture medium was harvested for monitoring of GH secretion. Pituitary cells were lysed in distilled water by three cycles of freezing and thawing and the lysate obtained was cleared by centrifugation to remove cell debris. These protein samples were then size fractionated by SDS-PAGE in duplicate under denaturing conditions. One of the gels was stained with Coomassie blue to visualize protein bands whereas the other one was transblotted onto an Immobilon-P membrane (Millipore, Bedford, MA, USA) by low-current electro-transfer for 2 h at 50 V. After overnight blocking with 2% nonfat dried milk, the membrane was incubated with GH antiserum at 1:40 000 dilution for 1.5 h. The antiserum used for Western blot was previously validated to be specific for grass carp GH without cross-reactivity with GTH and PRL (Wong *et al.* 1998a). Following incubation, the membrane was washed four times to remove the unbound primary antiserum and horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) was added for signal development. Chemiluminescence signal was detected using Super-Signal West Pico (PIERCE, Rockford, IL, USA) as the substrate and quantified using an IC440 CF Kodak digital image station (Eastman Kodak). Since the antisera for LH and GH were raised in the rabbit, two 'positive' bands could be detected in the Western blot for GH release, namely a 33 kDa band for LH antiserum (used in immunoneutralization) and a 22 kDa band for GH immunoreactivity detected by GH antiserum. To differentiate the two bands, a blank control with culture medium containing LH antiserum without incubation with pituitary cells was included. In this case, only the 33 kDa band for LH antiserum was detected.

RT-PCR of LH receptor in laser capture microdissection (LCM)-isolated somatotrophs

Cytospin preparation of grass carp pituitary cells ($\sim 5 \times 10^4$ cells/slide) was prepared and subjected to immunostaining with GH antiserum (1:8000). After staining, pituitary cells were dehydrated in ethanol, cleared in xylene, and air-dried prior to laser capture microdissection (LCM). Immuno-identified somatotrophs were isolated with Capsure HS LCM Caps using a PixCell II LCM system (Arcturus Engineering Inc., Mountain View, CA, USA) with infra red (IR) laser setting at 65 mW, pulse duration at 0.8 – 1.2 ms, and beam size at 7.5 μ m in diameter. For single cell PCR, only one somatotroph was captured on individual LCM Caps and total RNA was extracted using TRIZOL. After DNase I digestion and reverse transcription, PCR was conducted using primers covering the cDNA sequence between

TMD II and III of the grass carp LH receptor (LHR), and the authenticity of PCR product (213 bp) was confirmed by Southern blot (data not shown). In these experiments, RT-PCR of β -actin was used as an internal control.

Real-time PCR measurement of mature GH mRNA and GH primary transcripts

To examine the effects of LH immunoneutralization on the expression of mature GH mRNA and GH primary transcripts, pituitary cells were cultured in 12-well clustered plates (Costar) at a seeding density of $\sim 5 \times 10^6$ cells/2 ml/well ($\sim 1.25 \times 10^6$ cells/100 mm²). After treatment with increasing levels of LH antiserum, pituitary cells were dissolved in TRIZOL and the total RNA isolated was treated with DNase I to remove genomic DNA contamination. After that, reverse transcription (RT) was performed with Superscript II (GIBCO) and the RT samples were then subjected to quantitative PCR for mature GH mRNA and GH primary transcripts (Zhou *et al.* 2004) using a RotorGene 2000 Real-Time PCR System (Corbett Research, Eight Mile Plains, NSW, Australia). The specificity of PCR reactions was confirmed by melting analysis (melting temperature = 92.2 °C for mature GH mRNA and 91.4 °C for GH primary transcript) and ethidium bromide staining of PCR products in 2% agarose gels.

Measurement of grass carp GH promoter activity

A 986 bp 5' promoter of grass carp GH gene was subcloned into pGL3.Basic (Promega) to give pGH(–986).Luc for transfection studies in α T3–1 and GH4C1 cells. α T3–1 cells were maintained in monolayer culture in high glucose DMEM with 10% FBS at a seeding density of $\sim 5 \times 10^5$ cells/0.2 ml/well in a 96-well clustered plate the day before transfection. GH4C1 cells were cultured in a similar manner except that Ham F-10 with 10% FBS was used as the culture medium and the seeding density was reduced to $\sim 5 \times 10^4$ cells/0.2 ml/well. After overnight incubation, the cells were washed in OPTIMEM (GIBCO). Transfection medium was prepared by mixing 0.3 μ l lipofectamine with 0.1 μ g plasmid DNA in 100 μ l OPTIMEM. After incubation for 30 min, the transfection medium was carefully overlaid onto the cells in individual wells. Since α T3–1 cells are a GTH cell line with endogenous expression of LH receptors but do not express Pit-1 to support GH promoter activity, co-transfection with the carp Pit-1-expressing vector pcDNA.gcPit-1 was performed. In the case of GH4C1 cells, a rat GH cell line with endogenous Pit-1 but no expression of LH receptors, co-transfection with the salmon LH receptor-expressing vector pSG5.LHR was conducted. In these studies, plasmid DNA used

for transfection (0.1 µg/well) was composed of pGH(-986).Luc, pcDNA.gcPit-1 (for αT3-1 cells) or pSG5.LHR (for GH4C1 cells) and pEGFP-N1 (Clontech) at a ratio of 8:1:1. The green fluorescence protein (GFP)-expressing pEGFP-N1 was used as an internal control to monitor transfection efficiency and to normalize the data of luciferase expression. After transfection for 6 h, transfection medium was replaced with normal culture medium and the transfected cells were allowed to recover for 16 h before the initiation of hCG treatment. The duration of drug treatment was routinely fixed at 24 h. After drug treatment, transfected cells were dissolved in 100 µl Reporter Lysis Buffer (Promega). Luciferase activity in 25 µl lysate was assayed in a Lumat LB9507 luminometer (EG & G, Gaithersburg, MD, USA) with a 2 s delay and 10 s integration in 100 µl Luciferase Assay Reagent (Promega). GFP expression in 50 µl lysate was detected by fluorescence measurement using a CytoFluor 4000 Multi-Well Plate Reader (Perspective Biosystem, Framingham, MA, USA).

Data transformation and statistics

GH mRNA levels were quantified in terms of 'arbitrary light unit' and normalized against 18S RNA of the same sample. In this study, no significant differences were observed in terms of 18S RNA expression and these normalized data were simply transformed into '%Control' for statistical analysis. In the case of real-time PCR, standard curves with a dynamic range $\geq 10^5$ and a correlation coefficient (r^2) ≥ 0.95 were used for data calibration. Levels of mature GH mRNA and GH primary transcript were expressed as 'femtomole/million cells' and 'attomole/million cells' respectively. The half-life ($T_{1/2}$) of GH transcripts was deduced based on the one phase exponential decay model using GraphPad Prism 3.02 software (GraphPad, San Diego, CA, USA). Luciferase activity was normalized against GFP expression in the same sample. Given that GFP levels were relatively stable in this study, these normalized data were also transformed into '%Control' to facilitate the pooling of data from separate experiments. Data presented were analysed using ANOVA followed by Fisher's least significance difference (LSD) test. Differences were considered significant at $P < 0.05$.

Results

Effects of LH on 'steady-state' GH mRNA expression

To test if LH can modify GH synthesis directly at the pituitary level, a static incubation approach was used to examine the effects of hCG on 'steady-state' GH mRNA expression in primary cultures of grass carp pituitary cells. hCG is a placental GTH and has been used as a

pharmacological tool to probe the biological actions mediated by LH receptors, mainly for its long half-life, higher affinity for receptors, and commercial availability (Ascoli *et al.* 2002). Recently, a lack of cross-reactivity of hCG with FSH receptors in fish has been reported (Laan *et al.* 2002), confirming that hCG can be used as a functional homologue of LH in teleosts. In this study, a 48-h incubation of pituitary cells with increasing concentrations of hCG (10–50 IU/ml) resulted in a dose-dependent increase in 'steady-state' GH mRNA levels (Fig. 1A). The minimal dose of hCG tested to trigger a rise in GH mRNA expression was noted at 20 IU/ml. The stimulatory effects of hCG reached a plateau phase at doses higher than 40 IU/ml (data not shown). To study the functional role of endogenous LH in regulating GH synthesis, immunoneutralization was performed using an antiserum raised against carp LH. Removal of endogenous LH released into the culture medium by applying increasing levels of LH antiserum inhibited basal expression of GH mRNA in a dose-related fashion (Fig. 1B). A significant drop in GH mRNA levels was noted at 1:10 000 dilution of LH antiserum. When applied at 1:2500 dilution, LH antiserum totally abolished GH mRNA expression in grass carp pituitary cells. In parallel experiments, LH immunoneutralization was not effective in altering basal expression of thyrotrophin (TSH) β and FSH β mRNA (data not shown). Apparently, the inhibitory effect on GH mRNA expression was specific to LH antiserum as normal rabbit serum (NRS) or an antiserum raised against carp PRL did not alter GH mRNA levels when applied at 1:1000 dilution (Fig. 2A). The specificity of these antisera was further confirmed by immunostaining of grass carp pituitary sections using 1:1000 dilution of LH and PRL antisera. In this case, PRL immunoreactivity was identified in lactotrophs located in the RPD. In a consecutive section, LH immunoreactivity exhibited a patchy distribution in the PPD but not in RPD or NIL (Fig. 2B). This distribution pattern of LH immunoreactivity is consistent with that reported previously for gonadotrophs in the grass carp pituitary (Wong *et al.* 1998a).

LH immunoneutralization on GH release and GH content

To test if LH also regulates GH secretion at the pituitary level, grass carp pituitary cells were treated for 48 h with increasing doses of LH antiserum (1:10 000 to 1:1000). GH released into the culture medium was analysed by Western blot using an antiserum specific for grass carp GH (Fig. 3A). In our assay system, the second antibody for signal development was raised against rabbit IgG and could recognize both the protein bands for LH antiserum (33 kDa) and GH immunoreactivity (22 kDa). In this case, treatment with increasing levels of LH

Figure 1 Effects of hCG and LH immunoneutralization on GH mRNA expression in grass carp pituitary cells. Pituitary cells were exposed to increasing doses of hCG (A, 10–50 IU/ml) and LH antiserum (B, 1:10 000–1:2500) for 48 h under static incubation. After that, total RNA samples were extracted for quantitation of steady-state GH mRNA levels. Parallel measurement of 18S rRNA was conducted to serve as an internal control. Data presented (means $\pm$ S.E.M., $n=8$) are pooled results from eight separate experiments (each with triplicate treatments). Treatment groups denoted by different letters represent a significant difference at $P < 0.05$ (ANOVA followed by Fisher's LSD test). Representative results of slot blot are also presented for these studies.

antiserum resulted in a dose-dependent increase in the 33 kDa antiserum band but a corresponding decrease in the 22 kDa GH band, suggesting that removal of endogenous LH inhibited basal GH secretion in pituitary cell cultures. Parallel analysis of protein samples prepared from pituitary cells in the same experiment also revealed that the 22 kDa GH band was increased after LH immunoneutralization. The inhibitory action of LH antiserum on GH release was also found to be time-dependent (Fig. 3B). When compared with the time-matched control, a drop in the 22 kDa GH band was noted in the culture medium collected after a 2-h incubation with LH antiserum (1:2500). Although a time-dependent increase in 22 kDa GH band was noted in the control from 2 h to 48 h, GH immunoreactivity remained at a low level in the groups treated with LH antiserum. In these experiments, equal loading of protein sample per lane and molecular weight estimation of target bands in the SDS-PAGE gel were confirmed by Coomassie blue staining. A blank control was also included with 'cell-free' culture in MEM containing 1:500 dilution of LH antiserum. Given that only a single 33 kDa band was noted in the blank control which was clearly separated from the 22 kDa GH band in the experimental control (without LH antiserum treatment), the possibility of cross-reactivity of GH antiserum with LH antiserum is highly unlikely.

Expression of LH receptors in carp somatotrophs

To test if somatotrophs were the target cells for hCG treatment at the pituitary level, RT-PCR of LH receptors was performed in LCM isolated immuno-identified carp somatotrophs (Fig. 4A). In this study, only a single somatotroph was captured on individual Capsure HS LCM Caps and potential contamination by other pituitary cell types could be avoided. Using primers specific for grass carp LH receptor, a 213 bp PCR product was detected in the RT samples of isolated somatotrophs (i.e. GH cells #1 and #2) and mixed populations of carp pituitary cells (Fig. 4B). The 213 bp PCR product for LH receptor was also detected in the gonad, which was used as a positive control for these experiments. Parallel PCR of the RT samples prepared from somatotrophs with primers for TSH β and FSH β mRNA, however, could not yield any detectable PCR products, confirming the absence of contamination by other pituitary cell types during the LCM procedures (data not shown).

Interactions of LH and GH-releasing factors on GH mRNA expression

To study the functional relevance of LH in regulating GH synthesis in grass carp, *in vitro* interactions of LH with the GH-releasing factors in fish, including PACAP,

GnRH, and dopamine, were examined. In this case, a 48-h treatment of pituitary cells with hCG (30 IU/ml), PACAP (1 μ M), GnRH (1 μ M) and the dopamine agonist, APO (1 μ M) consistently increased basal levels of 'steady-state' GH mRNA (Fig. 5A-C). When hCG (30 IU/ml) was given together with PACAP (1 μ M, Fig. 5A) or APO (1 μ M, Fig. 5B), the stimulatory effect of hCG on GH mRNA levels was enhanced in an additive manner. A similar additive effect, however, was not observed with simultaneous treatment of hCG (30 IU/ml) and GnRH (1 μ M, Fig. 5C). To confirm the role of LH on the stimulatory effects of these GH-releasing factors, LH immunoneutralization was performed to test the responsiveness of pituitary cells to these stimulators in the absence of endogenous LH. Increasing concen-

trations of LH antiserum (1:10 000 to 1:2500) dose-dependently reduced basal GH mRNA levels as well as the stimulatory effects of PACAP (1 μ M, Fig. 6A), APO (1 μ M, Fig. 6B) and GnRH (1 μ M, Fig. 6C) on GH mRNA expression. The stimulatory actions of PACAP and GnRH were abrogated by LH antiserum at 1:10 000 dilution, whereas a higher level of LH antiserum (1:5000) was required to block APO-induced GH mRNA expression. Under a high dose of LH antiserum (1:2500), GH mRNA expression was almost undetectable even in the presence of GH-releasing factors.

Molecular mechanisms for LH regulation of GH gene expression

'Steady-state' GH mRNA, a dynamic balance between GH mRNA production and degradation, was assayed in the preceding experiments to serve as an index for GH synthesis. As a logical extension of our study, we also examined the role of transcript stability and gene transcription in LH-induced GH mRNA expression in grass carp pituitary cells. To test if LH can modify the half-life ($T_{1/2}$) of GH transcripts, the clearance curves of GH mRNA were constructed after exposing pituitary cells to the transcription inhibitor, actinomycin D (8 μ M) in the presence of hCG (40 IU/ml) or LH antiserum (1:5000). When compared with the control, hCG treatment had no effect on GH mRNA clearance and did not alter the $T_{1/2}$ value of GH transcripts (19 h, 95% confidence interval: 17.7 h-22.3 h; Fig. 7A). In contrast, the GH mRNA clearance curve was shifted to the right by treatment with LH antiserum (Fig. 7B) with an increase in $T_{1/2}$ value from 12 h (95% confidence interval: 9.7 h-14.2 h) to 35 h (95% confidence interval: 32.5 h-39.9 h). These results indicate that GH transcript stability was enhanced after removal of endogenous LH by immunoneutralization.

To further examine the role of LH on GH gene transcription in grass carp pituitary cells, quantitative

Figure 2 Selectivity of LH antiserum for immunoneutralization. (A) Effects of LH antiserum, PRL antiserum, and normal rabbit serum on GH mRNA expression. Pituitary cells were incubated for 48 h with LH antiserum (LH AS; 1:1000), PRL antiserum (PRL AS; 1:1000), and normal rabbit serum (NRS; 1:1000). Data presented (means $\pm$ S.E.M., $n=8$) are pooled results from eight experiments (each with triplicate treatments). Treatment groups labelled with different letters represent a significant difference at $P < 0.05$ (ANOVA followed by Fisher's LSD test). (B) Immunohistochemical staining of gonadotrophs and lactotrophs in grass carp pituitary sections. Pituitary sections were stained with a Vectastain ABC kit using the antisera for carp LH (1:1000) and PRL (1:1000) respectively. LH immunoreactivity was noted exclusively in the proximal pars distalis (PPD), whereas PRL immunoreactivity was restricted to the rostral pars distalis (RPD). Immunoreactivity of LH or PRL could not be detected in the neurointermediate lobe (NIL).

Figure 3 Effects of LH immunoneutralization on basal GH release and cellular GH content in grass carp pituitary cells. (A) Dose dependence of LH antiserum treatment on GH release and GH content. Pituitary cells were exposed to increasing levels of LH antiserum (LH AS; 1:1000 to 1:10 000) for 48 h under static incubation. Culture medium was harvested for quantitation of basal GH release. Pituitary cells were lysed in distilled water and the protein extracts prepared were used for the measurement of cellular GH content. (B) Time course of LH immunoneutralization on GH secretion. Pituitary cells were incubated with LH antiserum (1:2500) for 2 h, 24 h and 48 h respectively, and culture medium was harvested to monitor changes in GH secretion. GH levels in culture medium or in protein extracts prepared from grass carp pituitary cells were analysed using Western blot. Coomassie blue staining of the protein gels is presented in the right panels and the corresponding Western blots for GH immunoreactivity (22 kDa) are presented in the left panels. Besides the control without antiserum treatment, a blank control with culture medium containing LH antiserum but no incubation with pituitary cells was also included to identify the immunoreactivity caused by LH antiserum (33 kDa). The experiments were repeated three times with similar results and only representative gel pictures are presented.

analysis of GH primary transcripts by real-time PCR was performed after a 48-h incubation with increasing levels of LH antiserum (1:10 000 to 1:1000). As a parallel control, mature GH mRNA levels were also measured in the same experiment. Calibration of data for mature GH mRNA (Fig. 8A) and GH primary transcripts (Fig.

8B) was conducted using linear regression of critical threshold (or CT) values of the standard curve. In this study, immunoneutralization with LH antiserum inhibited basal expression of mature GH mRNA (Fig. 8C) and GH primary transcripts (Fig. 8D) in a dose-dependent manner. A significant reduction of mature

Figure 4 Expression of LH receptors in LCM-isolated somatotrophs. (A) Isolation of single immuno-identified somatotroph from mixed populations of grass carp pituitary cells by laser capture microdissection (LCM). Somatotrophs were immunostained with GH antiserum (1:2500) using a Vectastain ABC kit and laser-captured using Capsure HS LCM cap. (B) RT-PCR of LH receptor (LHR) transcripts in LCM-isolated grass carp somatotrophs. Total RNA was prepared from individual somatotrophs or mixed populations of carp pituitary cells and reverse transcription was performed in the presence (+RT) or absence (-RT) of Superscript II. RT-PCR of LHR transcripts in the gonad was used as a positive control and PCR of β -actin was used as the internal control. PCR with distilled water substituted for RT samples was used as a negative control. GH cells #.1 and #.2 are representatives of somatotrophs captured for the present study. Since the total RNA isolated from a single cell was very minute, the -RT control was not performed in these samples.

GH mRNA levels was noted at a 1:10 000 dilution of LH antiserum, whereas a higher concentration of antiserum (1:7500) was required to induce a drop in GH primary transcripts. In these studies, PCR specificity was confirmed by melting analysis and ethidium bromide staining of PCR products (upper panels, Fig. 8C-D). A single 252 bp ($T_m=92.2^\circ\text{C}$) and 210 bp PCR products ($T_m=91.4^\circ\text{C}$) were consistently observed in samples exposed to increasing levels of LH antiserum after real-time PCR of mature GH mRNA and GH primary transcripts respectively.

To provide further evidence that LH induces GH mRNA expression via activation of GH gene transcription, transfection studies were performed in α T3-1 and GH4C1 cells. After co-transfection with the Pit-1 expression vector pcDNA.gcPit-1, basal expression of

luciferase activity in α T3-1 cells was increased in a dose-dependent manner by increasing levels of hCG (10 - 50 IU/ml, Fig. 9A). In parallel experiments with GH4C1 cells, a similar dose-dependence of hCG-induced luciferase activity was also noted after co-transfection with the salmon LH receptor expression vector pSG5.LHR (Fig. 9B). However, increasing levels of hCG were unable to further elevate basal luciferase activity in the dose-matched controls with co-transfection of the blank vector pSG5 without the LHR insert.

Post-receptor signalling mechanisms for LH-stimulated GH gene expression

To elucidate the signalling mechanisms for LH-induced GH gene expression in Chinese grass carp, the functional coupling of LH receptors with the cAMP-dependent pathway was examined at the pituitary level. In this case, grass carp pituitary cells were treated for 20 min with increasing levels of hCG (0.5 -100 IU/ml) and cAMP production was accentuated in a dose-related fashion (Fig. 10A). In parallel experiments, the stimulatory effect of hCG (40 IU/ml) on GH mRNA expression was tested with simultaneous treatment with the AC inhibitor, MDL12330A (20 μM) and the PKA inhibitor, H89 (20 μM). hCG was effective in elevating GH mRNA levels but this stimulatory effect could be abolished by treatment with MDL12330A or H89. In the same study, MDL12330A or H89 alone was also effective in reducing basal GH mRNA expression (Fig. 10B).

Given that LH is known to activate MAPK and PI3K cascades in mammals (Ascoli *et al.* 2002), the possible role of these pathways in LH-stimulated GH gene expression was also examined. hCG-induced GH mRNA expression in grass carp pituitary cells was tested in the presence of the P42/44^{MAPK} inhibitor, PD98059 (100 μM), the P38^{MAPK} inhibitor, SB202190 (20 μM), and the PI3K inhibitor, wortmannin (100 nM), and parallel treatment with the JAK2 inhibitor, AG490 (100 μM) was used as a negative control. SB202190 and AG490 suppressed basal expression of GH mRNA (Fig. 11B) whereas a similar inhibitory effect was not observed with PD98059 and wortmannin (Fig. 11A). In these studies, the inhibitors for P42/44^{MAPK}, P38^{MAPK}, PI3K and JAK2 were also effective in blocking the stimulatory action of hCG (40 IU/ml) on GH mRNA expression. To clarify if P42/44^{MAPK}, P38^{MAPK}, PI3K and JAK2 are operating downstream of the cAMP-dependent pathway after hCG stimulation, the effect of the AC activator, forskolin (10 μM) on GH mRNA expression was examined in the presence of SB202190 (20 μM), PD98059 (100 μM), AG490 (100 μM) and wortmannin (100 nM) respectively. Similar to hCG, forskolin increased GH mRNA levels in carp pituitary cells and

this stimulatory action could be blocked by SB202190 (Fig. 12A), PD98059 (Fig. 12A) and AG490 (Fig. 12B). Forskolin-induced GH mRNA expression, however, was not affected by simultaneous treatment with wortmannin (Fig. 12B).

Discussion

Gonadotrophins, including LH and FSH, are well documented to play an essential role in reproductive functions, in particular those related to the processes of gametogenesis and steroidogenesis. The molecular structure of LH receptor has been elucidated (Ascoli *et al.* 2002) and its functional coupling to steroidogenesis, e.g. in the ovary, is mediated through stimulation of steroidogenic acute regulatory (StAR) protein and cytochrome P450 expression (Wood & Strauss 2002). Recently, extragonadal expression of LH receptors has been reported, suggesting that LH may have novel functions in 'non-classical' targets (Rao 2001). In the rat, low levels of LH receptor transcripts can be detected in the anterior pituitary (Lei *et al.* 1993). This finding raises the possibility that LH may play the role of an autocrine/paracrine factor at the pituitary level. In this study, using grass carp pituitary cells as a model, we have demonstrated for the first time that LH serves as a paracrine factor regulating GH synthesis and secretion in the fish pituitary. In this case, steady-state GH mRNA levels were elevated in a dose-dependent manner by hCG treatment in grass carp pituitary cells. Basal expression of GH mRNA, on the other hand, was suppressed by immunoneutralization using LH antiserum. Besides, treatment with LH antiserum was also effective in reducing GH secretion in a time- and dose-related fashion. These results, as a whole, provide evidence that endogenous LH is essential for the maintenance of GH release and GH gene expression in fish pituitary cells. Given that (i) the inhibitory effect of LH antiserum on GH mRNA levels was not mimicked by NRS or PRL antiserum and (ii) LH antiserum at the level used for immunoneutralization could only recognize gonadotrophs but not other pituitary cell types, the inhibitory action of LH antiserum caused by a cross-reactivity with other pituitary hormones is highly unlikely. Since exogenous LH was effective in increasing

Figure 5 Interactions of hCG with GH-releasing factors in fish on GH mRNA expression in grass carp pituitary cells. Pituitary cells were incubated for 48 h with (A) ovine PACAP 38 (PACAP; 1 µM), (B) the dopamine agonist apomorphine (APO; 1 µM), and (C) salmon GnRH (GnRH; 1 µM) in the presence or absence of hCG (30 IU/ml). Data presented (means $\pm$ S.E.M., $n=8$) are pooled results from eight experiments (each with triplicate treatments). Treatment groups denoted by different letters represent a significant difference at $P<0.05$ (ANOVA followed by Fisher's LSD test).

GH mRNA levels, it is conceivable that endogenous LH produced by pituitary cells is not saturating and GH gene expression can be modulated *in situ* by LH production at the pituitary level. Using single cell PCR in LCM-isolated carp somatotrophs, the transcripts for LH receptors were also detected, implying that LH stimulation is acting directly on somatotrophs to modify GH synthesis and secretion. It is worth mentioning that there was an increase in cellular GH content in carp pituitary cells after LH immunoneutralization. This increase in GH content is suspected to be the result of suppressed GH exocytosis. In the rat, a reciprocal relationship between GH secretion and pituitary GH content has been reported after anterolateral deafferentation of the medial basal hypothalamus (Ieiri *et al.* 1988). Under static incubation, dopaminergic stimulation is known to inhibit basal GH release but increase GH content in rat pituitary cells (Wood *et al.* 1987). Apparently, intricate mechanisms operating at the level of transcription and translation may exist in somatotrophs to regulate the dynamic balance among GH gene expression, cellular GH stores and GH exocytosis.

To examine the physiological implication of the paracrine effects of LH on GH gene expression, functional interactions of hCG with documented GH-releasing factors in fish, namely GnRH, dopamine and PACAP, were also tested in carp pituitary cells. In teleosts, GnRH functions as a hypothalamic hormone-releasing factor regulating LH and GH release at the pituitary level (Chang *et al.* 2000). Its stimulatory actions on GH mRNA expression in fish pituitary cells have been reported in tilapia (Melamed *et al.* 1996) and common carp (Li *et al.* 2002). Since GnRH receptors have been identified in fish somatotrophs, e.g. in goldfish (Cook *et al.* 1991), it is commonly accepted that GnRH stimulates GH synthesis and secretion in fish by direct actions on somatotrophs. Similar to GnRH, dopamine also serves as a GH-releasing factor in fish (Wong *et al.* 1993a) via activation of pituitary D1 receptors (Wong *et al.* 1993b). In tilapia, dopaminergic stimulation can induce GH mRNA expression at the pituitary cell level (Melamed *et al.* 1996). PACAP, the latest member of the secretin/glucagon peptide family, has been proposed to be the ancestral GHRH in lower vertebrates (Montero

Figure 6 Effects of LH immunoneutralization on GH mRNA expression induced by GH-releasing factors in grass carp pituitary cells. Pituitary cells were incubated for 48 h with (A) ovine PACAP 38 (PACAP; 1 μ M), (B) the dopamine agonist apomorphine (APO; 1 μ M), and (C) salmon GnRH (GnRH; 1 μ M) in the presence or absence of increasing levels of LH antiserum (1:10 000 to 1:2500). Data presented (means $\pm$ S.E.M., $n=8$) are pooled results from eight experiments (each with triplicate treatment). Treatment groups labelled with different letters represent a significant difference at $P<0.05$ (ANOVA followed by Fisher's LSD test).

Figure 7 Effects of (A) hCG and (B) LH immunoneutralization on GH mRNA stability in grass carp pituitary cells. Pituitary cells were incubated with actinomycin D (8 μ M) in the presence or absence of either hCG (40 IU/ml) or 1:5000 LH antiserum for the duration as indicated. Total RNA samples were isolated at the respective time points for the construction of GH mRNA clearance curves. The half-life ($T_{1/2}$) of GH transcript is defined as the time required for GH mRNA levels to drop to 50% of the original value. The experiments were repeated three times (each with quadruplicate treatments) and only the representative results of a single experiment are presented ($n=4$). In these studies, the trend of $T_{1/2}$ responses to drug treatment was similar between separate experiments.

Figure 8 Quantitative real-time PCR for mature GH mRNA and GH primary transcripts after LH immunoneutralization. (A and B) Standard curves for mature GH mRNA (A) and GH primary transcript (B). A tenfold serial dilution of the PCR standard (i.e. plasmid containing GH cDNA or full-length GH gene) was used to generate the standard curve. The resulting critical threshold (CT) values were plotted as a function of the levels of PCR template. (C and D) Effects of LH immunoneutralization on the expression levels of mature GH mRNA (C) and GH primary transcripts (PT; D). Pituitary cells were incubated for 48 h with increasing concentrations of LH antiserum (1:10 000 – 1:1000). PCR was conducted using primers specific for exon II and III (for mature GH mRNA) or flanking the junction between exon II and intron II of grass carp GH gene (for GH primary transcript). Data presented (means $\pm$ S.E.M., $n=8$) are pooled results from eight experiments (each with triplicate treatments). Treatment groups denoted by different letters represent significant difference at $P < 0.05$ (ANOVA followed by Fisher's LSD test). The shaded area represents the doses of LH antiserum inducing a significant drop of mature GH mRNA or GH primary transcripts. Representative results of ethidium bromide-stained PCR products are also presented for these experiments.

et al. 2000). Its role as a novel GH-releasing factor in fish has been confirmed (Wong *et al.* 2000) and its GH-releasing effect is mediated by pituitary PAC-I receptors (Wong *et al.* 1998b). Using RT-PCR coupled to LCM, the transcripts for D1 and PAC-1 receptors have recently been detected in somatotrophs isolated from grass carp and goldfish pituitary cells respectively (W K W Ko and A O L Wong, unpublished results). These results imply that dopamine and PACAP also act directly on somatotrophs for GH regulation. In the present study, basal levels of steady-state GH mRNA in

Figure 9 Effects of hCG on grass carp GH promoter activity. Co-transfection of luciferase-expressing pGH(-986).Luc with pcDNA.gcPit-1 and pSG5.LHR was conducted in αT3-1 cells (A) and GH4C1 cells (B) respectively. After transfection, cell cultures were exposed to increasing doses of hCG (10–50 IU/ml) for 24 h. In the experiments with GH4C1 cells, transfection with the blank vector pSG5 (without LHR insert) was used as a parallel control. Data presented (means±S.E.M., $n=8$) are pooled results from eight experiments (each with triplicate treatments). Treatment groups denoted by different letters represent a significant difference at $P<0.05$ (ANOVA followed by Fisher's LSD test).

Figure 10 Functional role of the cAMP-dependent pathway in hCG-stimulated GH mRNA expression. (A) Effects of hCG on cAMP production in grass carp pituitary cells. Pituitary cells were incubated with increasing concentrations of hCG (0.5–100 IU/ml) for 20 min in the presence of IBMX (0.1 mM). After that, culture medium was harvested for measurement of cAMP release and pituitary cells were extracted with ethanol for determination of cellular cAMP content. Total cAMP production was defined as the sum of cAMP release and cellular cAMP content. (B) Effects of inhibiting adenylate cyclase (AC) and protein kinase A (PKA) on hCG-stimulated GH mRNA expression in grass carp pituitary cells. Pituitary cells were exposed to the AC inhibitor, MDL12330A (20 μM) or the PKA inhibitor, H89 (20 μM) in the presence or absence of hCG (40 IU/ml) for 48 h. In these experiments, the inhibitors were added 10 min prior to the initiation of hCG treatment. Data presented (means±S.E.M., $n=8$) are pooled results from eight experiments (each with triplicate treatments). Treatment groups denoted by different letters represent significant difference at $P<0.05$ (ANOVA followed by Fisher's LSD test).

Figure 11 Effects of inhibiting MAPK and PI3K on hCG-stimulated GH mRNA expression. Grass carp pituitary cells were incubated for 48 h with (A) the P42/44^{MAPK} inhibitor, PD98059 (100 μM) and the PI3K inhibitor, wortmannin (100 nM), or (B) the JAK2 inhibitor, AG490 (100 μM) and the P38^{MAPK} inhibitor, SB202190 (20 μM) in the presence or absence of hCG (40 IU/ml). In these studies, the inhibitors were added 10 min prior to the initiation of hCG treatment. Data presented (means±S.E.M., n=8) are pooled results from eight experiments (each with triplicate treatments). Treatment groups denoted by different letters represent a significant difference at *P*<0.05 (ANOVA followed by Fisher's LSD test).

grass carp pituitary cells were increased by GnRH, PACAP and APO, confirming that these GH secretagogues can activate GH gene expression in grass carp. The stimulatory effects of these GH secretagogues, except for

GnRH, were additive to that of hCG. Since the doses of GnRH, PACAP and APO tested have been shown to induce the maximal responses in GH mRNA expression in carp pituitary cells (Li *et al.* 2000), the additivity observed in this study may imply that the stimulatory actions of APO and PACAP are independent of hCG. This is unexpected as LH receptors (Laan *et al.* 2002), D1 receptors (Wong *et al.* 1994) and PAC-I receptors (Wong *et al.* 1998b) are coupled to the AC/cAMP/PKA pathway in fish models. Although a differential transportation of cAMP to specific subcellular organelles/compartments has been reported in fibroblast cell lines (Scott & Carr 1992), it is unclear if functional segregation of cAMP-dependent mechanisms also exists in fish by differential coupling to membrane receptors.

Unlike APO and PACAP, the stimulatory action of GnRH on GH mRNA expression was not enhanced by hCG treatment. Given that GnRH is a potent stimulator of LH release in fish models (Chang *et al.* 2000), the stimulatory action of hCG might have been masked by endogenous LH released in response to GnRH treatment. These results have prompted us to speculate that at least a part of the GnRH-induced GH gene expression is mediated through local secretion of LH in grass carp pituitary cells. In parallel experiments, the stimulatory effects of GnRH, PACAP and APO on GH mRNA expression were suppressed in a dose-dependent manner by LH antiserum. These results indicate that the sensitivity of carp somatotrophs to stimulation by these GH-releasing factors is dependent on the presence of endogenous LH. This may be related to the novel function of LH in maintaining GH synthesis in carp pituitary cells. In this case, the releasable pool of secretory vesicles for GH might have been markedly reduced in the absence of endogenous LH and therefore the cells became less responsive/non-responsive to GH-releasing factors. Alternatively, LH may play a role in regulating receptor expression for these GH-releasing factors. In the rat, hCG but not FSH suppresses the levels of GnRH receptor transcripts in the testes (Botte *et al.* 1999). In the same animal model, variations in dopamine receptor levels have been reported in the pituitary during different phases of the oestrous cycle (Zabavnik *et al.* 1993), which may be related to 17β-oestradiol production by the ovary under the influence of LH and FSH (Guivarc'h *et al.* 1998). Although PAC-I receptors have been implicated in the control of GnRH, GnRH receptor and LH β-subunit expression in the brain-pituitary axis (Choi *et al.* 2000), to our knowledge, no information is available regarding the role of LH in regulating PAC-I receptor expression. It is also worth mentioning that a higher level of LH antiserum (1:5000) was required to block APO-induced GH mRNA expression when compared with the dose (1:10 000) used in GnRH and PACAP stimulation. Since dopamine is known to inhibit LH release in

fish via pituitary D2 receptors (Chang *et al.* 2000), the lower sensitivity of APO-induced GH gene expression to LH immunoneutralization might reflect a lower level of endogenous LH in pituitary cell cultures after dopaminergic stimulation.

To elucidate the molecular mechanisms for LH-induced GH gene expression at the pituitary level, the possible involvement of GH mRNA stability and GH gene transcription were examined. Clearance analysis of GH transcripts was conducted in carp pituitary cells in the presence of the transcription inhibitor, actinomycin D. This approach has been used in mammals to estimate the $T_{1/2}$ of target mRNA and has proved to be useful in

fish to study GH mRNA stability (Melamed *et al.* 1996). In grass carp pituitary cells treated with actinomycin D, the clearance rate of GH mRNA (as indicated by the $T_{1/2}$ values) was not affected by hCG treatment. The clearance curve, however, was shifted to the right with an increase in $T_{1/2}$ by LH immunoneutralization. These results indicate that the stability of GH mRNA was enhanced following the removal of endogenous LH. Apparently, LH can act at the pituitary level to promote GH mRNA degradation. The lack of hCG effect on GH mRNA clearance may also suggest that this novel action might have been saturated by basal release of LH in carp pituitary cells. To examine the possible role of GH gene transcription in LH-stimulated GH synthesis, real-time PCR was conducted to monitor the expression of GH primary transcripts. To serve as a parallel control, the level of mature GH mRNA was also measured in these experiments. In this study, mature GH mRNA and primary transcripts were reduced in a dose-dependent manner by LH antiserum. Since primary transcripts are processed rapidly and translocated to the cytoplasm as mature mRNA, the level of primary transcripts present in the nucleus is, in general, accepted to be a faithful reflection of target gene transcription (Beyersmann 2000). This idea is also consistent with the observation that hCG was effective in stimulating grass carp GH gene promoter activity in α T3-1 cells (via mammalian LH receptors) and GH4C1 cells (via fish LH receptors). These results, as a whole, suggest that LH is essential in maintaining basal levels of GH gene transcription. Since LH removal could enhance the stability of GH transcripts, the concurrent drop in mature GH mRNA and GH primary transcripts may indicate that steady-state GH mRNA is primarily determined by GH gene transcription. In this study, two opposing effects of LH have been demonstrated, namely the stimulatory actions on (i) GH gene transcription and (ii) GH mRNA degradation. The combined actions of these two mechanisms may result in a rapid turnover of GH transcripts in grass carp pituitary cells.

At the gonadal level, LH receptors are essential for steroidogenesis and their stimulatory actions are mediated through functional coupling to Gs and

Figure 12 Effects of inhibiting MAPK and PI3K on forskolin-induced GH mRNA expression. Grass carp pituitary cells were incubated for 48 h with (A) the P42/44^{MAPK} inhibitor, PD98059 (100 μM) and the P38^{MAPK} inhibitor, SB202190 (20 μM) or (B) the JAK2 inhibitor, AG490 (100 μM) and the PI3K inhibitor, wortmannin (100 nM) in the presence or absence of forskolin (10 μM). In these experiments, the inhibitors were added 10 min prior to the initiation of hCG treatment. Data presented (means ± S.E.M., $n=8$) are pooled results from eight experiments (each with triplicate treatments). Treatment groups denoted by different letters represent a significant difference at $P<0.05$ (ANOVA followed by Fisher's LSD test).

subsequent activation of the AC/cAMP/PKA pathway (Ascoli *et al.* 2002). Recently, functional coupling of LH receptors to MAPK- and PI3K-dependent mechanisms has also been reported in granulosa cells (Salvador *et al.* 2002) and Leydig cells (Hirakawa *et al.* 2002). In this study, hCG treatment not only elevated steady-state GH mRNA levels but also increased cAMP production in carp pituitary cells. Furthermore, hCG-induced GH mRNA expression was mimicked by the AC activator, forskolin but blocked by the AC inhibitor, MDL12330A and the PKA inhibitor, H89. These results confirm that LH receptors expressed in carp pituitary cells are functionally coupled to the AC/cAMP/PKA pathway. Given that basal GH mRNA levels were markedly suppressed by AC and PKA inhibition, the cAMP-dependent cascade may represent a key component maintaining resting state GH gene expression in Chinese grass carp. This idea is consistent with the finding that the 5' promoters of GH gene, e.g. human GH gene (Shepard *et al.* 1994), are known to contain cis-acting elements responsive to cAMP stimulation (e.g. CRE). In fish, e.g. chinook salmon, cAMP induction of GH gene transcription is mediated by two CGTCA motifs in the 5' proximal promoter (Wong *et al.* 1996).

Besides the cAMP-dependent cascade, hCG-induced GH gene expression in grass carp may also involve other signalling components. In this case, the MAPK inhibitors, namely PD98059 for P42/44^{MAPK} and SB202190 for P38^{MAPK}, were effective in blocking hCG- and forskolin-stimulated GH mRNA expression, suggesting that P42/44^{MAPK} and P38^{MAPK} may be functionally coupled to the cAMP-dependent pathway to modulate GH gene expression in carp pituitary cells. In mammals, functional coupling of P42/44^{MAPK} to LH receptors through PKA phosphorylation of Raf-1/C-Raf (Dhillon *et al.* 2002) and B-Raf (Qiu *et al.* 2000) has been reported. P42/44^{MAPK} can also be activated independent of PKA by direct actions of cAMP on the Rap-1 guanine nucleotide-exchange factor Epac (Enserink *et al.* 2002). Although the coupling of P38^{MAPK} with LH receptors through cAMP-dependent mechanisms has not been reported, PKA-dependent activation/phosphorylation of P38^{MAPK} has been demonstrated in mouse cardiomyocytes (Zheng *et al.* 2000). It is worth mentioning that hCG- and forskolin-induced GH mRNA expression in carp pituitary cells were totally abolished by the JAK2 inhibitor, AG490. These results were unexpected as AG490 was used as a negative control in these experiments. The inhibitory action of AG490 may suggest that pituitary LH receptors can recruit JAK2 as a downstream effector for the AC/cAMP/PKA pathway. JAK2 is a non-receptor tyrosine kinase coupled to cytokine receptors (Kisseleva *et al.* 2002) and the present finding of its involvement in hCG-induced GH gene expression raises the possibility that LH receptors may activate P42/44^{MAPK} indirectly

via the JAK2/Grb2/SOS/Ras/Raf/MEK pathway. After ligand binding to G protein-coupled receptors, e.g. μ opioid receptors (Ai *et al.* 1999), PI3K has been shown to act as a mediator for P42/44^{MAPK} activation by cross-talk at the level of protein kinases. However, the possible existence of a similar mechanism in hCG-induced GH gene expression in grass carp pituitary cells is unlikely as the PI3K inhibitor, wortmannin, was only effective in blocking hCG- but not forskolin-induced GH mRNA expression. These results indicate that PI3K mediates hCG stimulation of GH gene expression but it is not coupled to the cAMP-dependent pathway or the downstream MAPK cascades. Apparently, multiple signalling pathways (both cAMP-dependent and cAMP-independent) are associated with pituitary LH receptors, which may be functional in fine-tuning LH-induced GH gene expression in the carp model.

In summary, using grass carp pituitary cells as a model, we have demonstrated for the first time that LH can act in a paracrine manner at the pituitary level to regulate basal GH secretion, GH gene expression, and somatotroph responsiveness to GH-releasing factors. The paracrine actions of LH on GH mRNA expression are mediated by a concurrent increase in GH gene transcription and GH transcript turnover. Furthermore, we also speculate that (i) the JAK2/MAPK cascades secondary coupled to the cAMP-dependent pathway and (ii) the PI3K cascade independent of cAMP-mediated mechanisms may be the key elements in the signal transduction for LH-induced GH gene expression. The present study not only provides new insights into the paracrine regulation of GH synthesis and secretion at the pituitary level, but also sheds light on a novel mechanism for functional interactions between the gonadotrophic and somatotrophic axes. The phenomenon described in the present study may also explain the evolution of a close anatomical relationship between gonadotrophs and somatotrophs in teleosts and accounts for the parallel changes in LH and GH secretion in fish during sexual recrudescence and the spawning season.

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