FUSION PROTEINS ENGINEERED TO CHARACTERIZE GLYCOPROTEIN HORMONE-RECEPTOR INTERACTIONS

By

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(Under the direction of Dr. J. David Puett)

ABSTRACT

The family of glycoprotein hormones consists of four heterodimeric hormones: human chorionic gonadotropin, luteinizing hormone, thyroid stimulating hormone, and follicle stimulating hormone. Each hormone consists of a common α subunit and a hormone-specific β subunit, which together bind to the N-terminal extracellular domain of their G protein-coupled receptors, stimulating production of cAMP. Proper activation of these receptors is critical for control of normal reproduction and metabolism. Both hCG and LH bind the LH receptor, whereas TSH and FSH bind their unique receptors, TSHR and FSHR, respectively. Structure-function studies of homologous sequences within this family are hampered by the excessive amounts of hormone necessary to study non-cognate or low affinity interactions. By fusing a hormone or part of a hormone to a receptor, weak binding events can be evaluated that would otherwise be undetectable. These methods revealed that hCG can activate the non-cognate receptors, TSHR and FSHR, in addition to its cognate receptor, LHR. The minimum number of residues of hCGβ required to exhibit binding and activation activity can also be determined in this system. Low molecular weight hCG hormones, or mini-gonadotropins, retaining just one-third of the β subunit were sufficient to cause weak activation of LHR in the absence of full-length hCG. These studies provide valuable information concerning regions of specificity in hCG and the glycoprotein hormone receptors that are responsible for hormone binding and receptor activation, which will aid in the development of clinically relevant low molecular weight agonists and antagonists for these receptors.

INDEX WORDS: Follicle stimulating hormone, Follicle stimulating hormone receptor, Glycoprotein hormones, Glycoprotein hormone receptors, G protein-coupled receptors, Human chorionic gonadotropin, Luteinizing hormone, Luteinizing hormone receptor, Mini-gonadotropins, Thyroid stimulating hormone, Thyroid stimulating hormone receptor

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DEDICATION

I dedicate this to my father, who always believed in me, inspired me, and gave me the strength to continue when I felt like I couldn't go any further. Thank you, Dad. I love you.

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I would like to recognize my family members, who have been a constant source of support throughout my life. They didn't always understand what I was doing, but always offered encouragement, and I pulled from them strength, courage, and motivation.

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LIST OF ABBREVIATIONS

BSA ..... bovine serum albumin

CPM ..... counts per minute

CTP ..... C-terminal peptide of hCGβ

DMEM ..... Dulbecco's modification of Eagle's medium

ECD ..... extracellular domain

ED_{50} ..... dose for 50% maximal receptor activation

FSH ..... follicle stimulating hormone

FSHR ..... follicle stimulating hormone receptor

hCG ..... human chorionic gonadotropin

HEK 293 cells ..... human embryonic kidney 293 cells

^{125}I ..... iodine 125 labeled

IBMX ..... isobutylmethylxamthine

IC_{50} ..... concentration for 50% maximal hormone binding

LH ..... luteinizing hormone

LHR ..... luteinizing hormone receptor

LRR ..... leucine-rich repeat

PBS ..... phosphate buffered saline

RIA ..... radioimmunoassay

TSH ..... thyroid stimulating hormone

TSHR ..... thyroid stimulatng hormone receptor

Y ..... yoked (fused)

YCG/R ..... yoked hCG-receptor complex

YMG ..... yoked mini-gonadotropin

YMG/R ..... yoked mini-gonadotropin receptor complex

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Human chorionic gonadotropin (hCG), luteinizing hormone (LH), thyroid stimulating hormone (TSH), and follicle stimulating hormone (FSH) constitute the heterodimeric family of glycoprotein hormones (1, 2). The four members share a common α subunit and possess a distinct β subunit that confers receptor specificity to their respective receptors. LH, FSH, and TSH are products of the anterior pituitary, while hCG is derived from the placenta (1). Under physiological conditions, hCG and LH bind a common receptor, the LH receptor (LHR), while TSH and FSH each uniquely bind the TSH receptor (TSHR) and FSH receptor (FSHR), respectively (1, 2). The glycoprotein hormone receptors are members of the superfamily of G protein-coupled receptors (GPCRs), which are characterized by a large N-terminal extracellular domain, seven transmembrane helices, three intracellular and three extracellular loops, and a short C-terminal cytoplasmic tail (3). The extended N-terminal extracellular domain distinguishes the glycoprotein hormone receptors as a unique subset of GPCRs and imparts high affinity hormone binding (3). Accurate interactions between hormone and receptor ensure proper regulation of the physiological processes under their control.

Physiology of the glycoprotein hormones and their G protein-coupled receptors

The glycoprotein hormones control many aspects of reproduction and metabolism. LH, TSH, and FSH are released by the anterior pituitary in response to peptide hormonal signals from the hypothalamus (4). Although constitutively produced in small amounts by the anterior pituitary, hCG is usually thought of as a hormone of pregnancy and is secreted in large amounts by the trophoblastic cells of the placenta (5). The gonadotropins, LH and FSH, occupy a central position in gamete maturation and sexual functioning.

In the human ovary, LH and FSH mediate synthesis and secretion of sex steroids and release of a mature ovum every 28-30 days (4, 6). LH receptors are found in two types of ovarian cells, the theca and granulosa cells, while FSH receptors are found only in granulosa cells. Activated LHR stimulates the production of androgens, which are converted to estrogens in granulosa cells by aromatase, and of progesterone in luteal cells (4, 6). FSHR activation regulates growth and differentiation of follicles and increases production of mRNAs encoding aromatase and other granulosa cell proteins, including LHR (6). Positive and negative feedback loops perpetuate the cycle of follicle maturation and atresia.

In the testes, LH and FSH function together to maintain spermatogenesis and testicular function (4). LH receptors are found on the surface of the interstitial Leydig cells and receptor activation triggers the secretion of testosterone. FSH receptors reside on the surface of Sertoli cells and their activation is important in normal spermatogenesis

(4). Their activation also stimulates secretion of androgen binding protein and inhibin. Inhibin circulates to the pituitary, causing negative feedback of gonadotropin release (4).

Thyrotropin releasing hormone from the hypothalamus acts on the anterior pituitary to release TSH, which in turn stimulates TSH receptors located in the thyroid. TSHR controls production of thyroxine (T_4) and triiodothyroxine (T_3), potent hormones that regulate many physiological events in the body such as growth, development, and metabolism (4). Activated TSHR prompts general stimulation of metabolism via the thyroid hormones with effects manifested throughout the organism.

Because the glycoprotein hormones and their receptors govern such important processes, and malfunction of these, either from aberrant structure or regulation, results in many different diseases, the molecular basis of hormone binding and receptor activation needs to be fully understood. Constitutive activation of the glycoprotein hormone receptors results in increased activation of the cAMP pathway and is commonly caused by heterozygous mutations (7-9). In contrast, inactivating mutations of these receptors severely affect either hormone binding or signal transduction, and can be caused by homozygous point mutations or deletions (8, 9).

Constitutively activating mutations of LHR leading to familial and sporadic male-limited precocious puberty have been identified and mapped to many locations within the receptor (7, 9). In contrast, male patients with LHR inactivating mutations have been diagnosed with Leydig cell hypoplasia, male pseudohermaphroditism, undifferentiated sex organs, undervirilization and micropenis (9). Primary amenorrhea results when LHR inactivating mutations occur in females (9).

Currently, only one constitutively activating mutation has been recognized for FSHR (10). This mutation was discovered in a hypophysectomized male who continued to exhibit normal spermatogenesis. Apparently, the effects of constitutively activated FSHR are masked in men with a normal hypothalamus.

Activating and inactivating mutations of TSHR produce hyperthyroidism and hypothyroidism, respectively (11). Because TSHR activation results in the production of thyroid hormones as well as cell proliferation, constitutive activation can also lead to the presence of hyperfunctioning thyroid adenomas (9-11). In these cases, the adenoma results in hyperproduction of the thyroid hormones, which in turn causes negative feedback of TSH. This negative feedback results in quiescence of normal thyroid tissue (1, 9, 11).

Elucidating the glycoprotein hormones and their G protein-coupled receptors: A history

Gonadotropic hormones were first detected in 1926 by two independent research groups (6, 12, 13). Zondek and Aschheim observed that urine from pregnant and postmenopausal women had opposing effects on follicular development in rhesus macaques (6). Extracts from postmenopausal women induced follicular development, while extracts from the urine of pregnant women induced ovulation and development of the corpus luteum. These results led to the identification of hCG in 1927 (6, 13). The following year, it was shown that the pituitary gland plays a role in ovarian function when it was observed that pituitary extracts administered to rhesus macaques caused sexual

maturation (6). In 1931, two active hormone fractions were found in pituitary extracts (12). One fraction stimulated follicular growth in the ovary and was designated follicle stimulating hormone; the second caused rupture of the follicles and their transformation into corpora lutea, or luteinization, and was named luteinizing hormone (12). The difficulty of separating and purifying LH and FSH to homogeneity led some investigators to believe that FSH and LH were not separate hormones, but that both activities were performed by one molecule (14). It was not until 1942 that Greep and co-workers were able to obtain complete separation of FSH and LH (6).

Experimental studies with thyroid stimulating hormone began in 1922 with the work of Smith and Smith (15). They demonstrated that atrophied thyroids of hypophysectomized tadpoles could be repaired and reactivated by an injection of bovine anterior pituitary extracts in saline solution. Isolation of TSH began in 1931 with the work of Janssen and Loeser (15); however, TSH preparations were not produced until 1940.

In 1964, Li and Starman first suggested that the glycoprotein hormones are not a single polypeptide chain as previously believed, but rather are composed of two subunits (16). Upon re-examination of ovine LH sedimentation behavior, they noted that the molecular weight of the hormone seemed to change with the pH of the buffer. The molecular weight of LH was calculated to be 28,000-30,000 Daltons at neutral pH, however, when the pH was reduced to 1.3 the molecular weight fell to 16,000 Daltons, suggesting the presence of two subunits. In 1965, it was demonstrated that these components were not associating monomers as previously believed, but were in fact non-identical subunits (17). In 1967, counter-current distribution was utilized to obtain intact

subunits from two chemically and electrophoretically different fractions of LH (17-20). Each fraction was shown to be biologically inactive, or nearly inactive; however, when the fractions were mixed and incubated, a significant amount of activity returned. This was the first successful reversible separation of LH into its non-identical subunits.

In 1970, researchers separated, purified, and determined the amino acid sequence of the TSH subunits (21, 22). While investigating the carbohydrate moieties of TSH, it was discovered that three distinct tryptic peptides could be isolated (22). Two of the three TSH peptides that were isolated were identical to two peptides previously reported (22). Examination of bovine LH and TSH glycoproteins showed that these hormones shared an identical subunit (22).

According to Papkoff, "all essential elements of the subunit nature of glycoprotein hormones had been demonstrated in a matter of four years after the separation and isolation of the ovine LH subunits in 1967" (17). In 1970, FSH was separated into its non-identical subunits (23) and the determination of the amino acid sequences of the FSH subunits followed in 1971 (24). The amino acid sequences of the α and β subunits of hCG were determined in 1973 (25, 26). Initial studies indicated hCG was composed of two identical chains, but as with the other glycoprotein hormones, further experiments revealed that the subunits were distinct (25).

Cloning of the glycoprotein hormones and their receptors

The common human α and hCGβ subunits were cloned in 1979 and 1980, respectively, by Fiddes and Goodman (27, 28). The α subunit clone was synthesized by

reverse transcriptase from first trimester polyadenylated mRNA (27), followed by hCGβ one year later (28). The cloning of murine TSHβ cDNA from a mouse thryotropic tumor and the determination of its nucleotide sequence were accomplished in 1983 (29). The cDNA encoding the rat LHβ subunit was cloned from a rat liver DNA library and subsequently characterized in 1984 (30), whereas the cloning and sequencing of the bovine FSHβ subunit from a bovine pituitary cDNA library was determined in 1986 (31). Complementary DNA clones were now available for all of the glycoprotein hormones in at least one species, and the cloning of these subunits in different species has since been accomplished. There is a high degree of sequence homology within the β subunits. hCGβ, however, is the largest of the glycoprotein hormone β subunits due to a 30 amino acid extension on the C-terminus known as the C-terminal peptide (CTP) (5). An alignment of each of the subunits of the human hormones is shown in Fig. 1.1.

Cloning and sequencing of the glycoprotein hormone receptors soon followed. The sequences of rat LHR, generated from mRNA obtained from rat ovaries (32), and porcine LHR obtained from mRNAs isolated from porcine testes (33), were published in the same issue of *Science* in 1989. The cloning and sequencing of canine TSHR cDNA from a dog thyroid cDNA library (34) followed later in 1989, and the cDNA of the last of the glycoprotein hormone receptors, rat FSHR, was cloned and sequenced in 1990 from RNA isolated from rat testicular Sertoli cells (35). With the cloning of the LHR cDNAs in 1989 came the first suggestion that the glycoprotein hormone receptors are members of the G-protein coupled receptor superfamily (32). These receptors were shown to have a large N- terminal extracellular domain (ECD), seven transmembrane helices, three

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hα      1APDVQDCPECTLQENPFFSQPGAPILQCMGCCFSRAYPTPLRSKKTMLVQKNVTSESTCCVAKSYNRVTVMGGFK75
        76VENHTACHCSTCYYHKS92

hCGβ    1SKEPLRPRCRPINATLAVEKEGCPVCITVNTTICAGYCPTMTRVLQGVLP..ALPQVVCNYRDVRFESIRLPGCP73
hLHβ    1-R-----W-H--------------------------------------..P------T--------------73
hFSHβ         1NS-ELT-I-I-I---E-RF--SI---W-----Y-RDL-YKDPAR..PKI-KT-TFKELVY-TV-V---A67
hTSHβ          1F-I-TEY-MHI-RRE-AY-L-I---------M-RDINGKLF--KY--S-D--T---FIYRTVEI----68

        74RGVNPVVSYAVALSCQCALCRRSTTDCGGPKDHPLTCDDPRFQDSSSSKAPPPSLPSPSRLPGPSDTPILPQ145
        74---D----FP-----R-GP-----S-------------H-QLSGLLFL121
        68HHADSLYT-P--TQ-H-GK-DSDS---TVRGLG-SY-SFGEMKE111
        69LH-A-YF--P-----K-GK-NTDYS--IHEAIKTNY-TK-QKSY112
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Fig. 1.1: The amino acid sequences of the common human α subunit and each of the four human glycoprotein hormone β subunits. Using hCGβ as the reference sequence in the β subunit alignment, the dashes (-) represent identical residues and the dots (.) represent gaps in the sequence. Alignments were made using web-based programs utilizing CLUSTAL W (36).

intracellular and three extracellular loops, and a short C-terminal cytoplasmic tail (32-35). An alignment of the extracellular domains of the three glycoprotein hormone receptors is depicted in Fig. 1.2.

Until the cloning of the glycoprotein hormone receptors, little was known about their structures. Biochemical studies resulted in conflicting reports; some experiments indicated these receptors were single glycoproteins while others suggested the congregation of multiple subunits (32). The cloning of these receptors provided the information needed to make a definitive determination of the structures of this family of receptors.

The extracellular domain is the site for high affinity hormone binding (3, 37). It is believed that after high affinity binding occurs in this location, lower affinity binding occurs in the third extracellular loop. After the binding event, the conformation of the transmembrane portion of the receptor changes to allow interaction of the intracellular loops and cytoplasmic tail with G_s, which then leads to activation of adenylate cyclase and the subsequent production of cAMP as a second messenger (3). Activating mutations in the glycoprotein hormone receptors causes a change in the conformation of the receptor, resulting in an arrangement of the transmembrane helices that allows association of the receptor with G_s in the absence of ligand (9).

Fig. 1.2. Amino acid sequence alignment of the ECDs of the glycoprotein hormone receptors. Using hLHR as the reference sequence, the dashes (-) indicate identical residues and the dots (.) indicate gaps in the sequence. Alignments were made using web-based programs that utilized CLUSTAL W (36).

The primary structures of the glycoprotein hormone receptors illuminate their evolutionary history. Determination of the genomic structure of the LHR gene led to the suggestion that all of the glycoprotein hormone receptors evolved from a common ancestral gene (38, 39). The LHR gene contains eleven exons. Most of the ECD of LHR is encoded by the first 10 exons; the eleventh exon encodes a small portion of the ECD as well as the remainder of the receptor (38, 39). It is likely that the glycoprotein hormone receptors evolved from an ancestral heptahelical receptor gene that was created by the fusion and subsequent duplication of a leucine-rich repeat protein (LRR) (exons 1-10) and an intronless GPCR gene (exon 11) (38). The ECDs of the glycoprotein hormone receptors contain LRR pseudorepeats, the exact number of which has been debated (40). A model of the ECD of LHR (Fig. 1.3) using nine imperfect LRRs was previously developed, and was based on the structure of porcine ribonuclease inhibitor, a LRR protein (40).

Fig. 1.3: Ribbon diagram of a model of the ECD of LHR (amino acid residues 27-235) based on nine leucine-rich repeats (40).

Fusion proteins of the glycoprotein hormones and receptors

The cloning of the glycoprotein hormones and their receptors enabled researchers to manipulate and utilize these proteins in ways that were not previously allowed. Many laboratories soon began to apply different techniques to conduct structure-function studies of the glycoprotein hormones and their receptors, including the use of single-chain proteins (38). The use of single-chain proteins has proven to be beneficial for these studies because the hormone and receptor are in close proximity to one another, allowing interactions to occur (38).

In 1995, two research groups independently constructed the first single-chain gonadotropins (41, 42). These single-chain proteins were constructed to determine if an active hormone could be synthesized when the α and β subunits were linked in a single chain and to facilitate structure-function studies (42). Two yoked hCG (YhCG) constructs were created by the Puett laboratory (41) in the same year that Boime and co-workers constructed the tethered hCGβ–α mutant (42).

The single chain hCGβ-α complexes were designed so that the N-terminal end of the α subunit was fused to the C-terminal end of the hCGβ subunit (Fig. 1.4). A biologically active single-chain hCG molecule was produced when this chimeric gene was expressed in baculovirus-infected insect cells (42) and CHO cells (41), indicating that a single-chain gonadotropin adopts an active conformation.

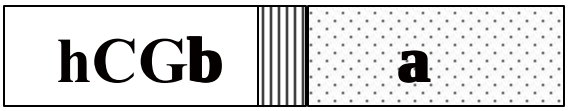

Fig. 1.4: Schematic of YhCG1. The common α subunit is linked to hCGβ using the endogenous CTP of hCG, indicated by vertical stripes, as a flexible linker.

The two YhCG constructs created by the Puett laboratory, designated YhCG1 and YhCG2, differed only in the length of the CTP linker, YhCG2 having a shorter flexible linker region than YhCG1 (42). The YhCG constructs were expressed in baculovirus-infected insect cells and proven to have similar binding affinities to LHR (42). The single-chain hormones elicited increases in intracellular cAMP comparable to wild-type hCG. Because of the success of the YhCG constructs, these studies were expanded to include the construction of a yoked hormone receptor (YHR) complex (43). YHR is made up of YhCG1 fused to the N-terminus of the LHR, using an additional CTP as a flexible linker (Fig. 1.5). Expression of YHR in COS-7 and HEK 293 cells resulted in a high basal level of intracellular cAMP, i.e. constitutive activation, resulting from the stable and functional interaction between LHR and the covalently linked YhCG1. Yoked hormone-receptor complexes, such as YHR, are very useful for the evaluation of ligand-receptor interactions (38, 43). One advantage of using the yoked hormone-receptor system is that it allows the evaluation of hormone-receptor interactions, even when the occurrence of these interactions is low. This enables the characterization of a variety of

ligand-receptor interactions, such as lower expression mutants, single subunits, non-cognate associations, as well as hormone truncations.

Fig. 1.5. Schematic of YHR. YhCG1 was fused to the N-terminus of rat LHR using an additional CTP as a flexible linker region. The β subunit is shown by the open box, the α subunit is depicted by light stippling, rat LHR in gray, and the CTP regions are depicted by vertical lines.

Studies involving fusion protein techniques have been expanded to include single subunits fused to rat LHR in the YαR and YβR constructs designed and characterized by Narayan, Gray and Puett. In these fusion proteins, each of the individual subunits was fused to the N-terminus of rat LHR in order to determine if one subunit alone was sufficient to elicit receptor activation. It was shown that both subunits are required for hormone binding and subsequent receptor activation (Narayan, Gray and Puett, unpublished data).

In addition, Boime's research group has extended their single-chain hormone studies to include single chain TSH (44) and FSH (45), as well as multi-functional hormones. The first of these multi-functional proteins was developed in 1999 to generate a protein with both hCG and FSH function (46). Using one α subunit and the hCGβ and FSHβ subunits in a single polypeptide, both LH and FSH receptors were able to be

stimulated (46). These studies were then expanded to construct a tri-functional hormone in which one α subunit was linked with all of the glycoprotein hormone β subunits in tandem; imparting the ability to stimulate LHR, TSHR, or FSHR (47).

The use of fusion proteins permits the evaluation of even low-affinity hormone-receptor interactions such as non-cognate ligand-receptor interactions, or trials in drug design. In my research projects, I used fusion protein studies to test the hypothesis that hCG can bind to and activate each of the human glycoprotein hormone receptors (Chapter 2). Additionally, I used fusion protein and co-transfection techniques in order to test the effectiveness of two mini-gonadotropins as LHR agonists (Chapter 3). There is still a wealth of opportunities in this exciting and challenging area of research. These studies were designed not only to investigate the specificity of hormone-receptor interactions within these important families of hormones and receptors, but also to provide useful insights into the functional regions that dictate these interactions, which can be used in the development of low molecular weight agonists and, ideally, antagonists for the glycoprotein hormone receptors. Because of the critical function of these receptors in the human body, low molecular weight agonists and antagonists would prove to be valuable additions in this area of medical treatment.

References

1. **Pierce JG, Parsons TF** 1981 Glycoprotein hormones: structure and function. Annu Rev Biochem 50:465-495
2. **Hearn MTW, Gomme PT** 2000 Molecular architecture and biorecognition process of the cysteine knot protein superfamily: part I. The glycoprotein hormones. J Mol Recognition 13:223-278
3. **Ascoli M, Fanelli F, Segaloff DL** 2002 The lutropin/choriogonadotropin receptor, a 2002 perspective. Endocr Rev 23:141-174
4. **Ganong WF** 1989 Review of Medical Physiology 14 ed. Prentice-Hall, Englewood Cliffs, NJ
5. **Puett D** 1986 Human Choriogonadotropin. BioEssays 4:70-75
6. **Hopkin K** 1997 Evolutions: Mammalian Oogenesis and Ovulation. J NIH Res 9:58-64
7. **Scheer A, Cotecchia S** 1997 Constitutively active G protein-coupled receptors: potential mechanisms of receptor activation. J Recept Signal Transduct Res 17:57-73
8. **Spiegel AM** 1997 Inborn errors of signal transduction: mutations in G proteins and G protein-coupled receptors as a cause of disease. J Inherit Metab Dis 20:113-121
9. **Gromoll J, Brocker M, Derwahl M, Hoppner W** 2000 Detection of mutations in glycoprotein hormone receptors. Methods 21:83-97

10. **Simoni M, Gromoll J, Nieschlag E** 1997 The follicle-stimulating hormone receptor: biochemistry, molecular biology, physiology, and pathophysiology. Endocr Rev 18:739-773

11. **Paschke R, Ludgate M** 1997 The thyrotropin receptor in thyroid diseases. N Engl J Med 337:1675-1681

12. **Gemzell C, Roos P** 1966 The Physiology and Chemistry of Follicle-Stimulating Hormone. In: Harris GW, Donovan BT (eds) The Pituitary. University of California Press, Berkeley and Los Angeles, vol 1:492-517

13. **Zondek B, Aschheim S** 1927 Das Hormon des Hypophysenvorder-Lappens. Klin Wochschr 6:248-252

14. **Segaloff A** 1966 The Physiology of Luteinizing Hormone. In: Harris GW, Donovan BT (eds) The Pituitary. University of California Press, Berkeley and Los Angeles, vol 1:518-526

15. **Bate RW, Condliffe PG** 1966 The Physiology and Chemistry of Thyroid Stimulating Hormone. In: Harris GW, Donovan BT (eds) The Pituitary. University of California Press, Berkeley and Los Angeles, vol 1:374-210

16. **Li CH, Starman B** 1964 Molecular Weight of Sheep Pituitary Interstitial Cell-stimulating Hormone. Nature 202:291-292

17. **Papkoff H** 1991 Glycoprotein hormones were always composed of subunits--we just had to find out the hard way. Endocrinology 129:579-581

18. **Papkoff H, Samy TSA** 1967 Isolation and partial characterization of the polypeptide chains of ovine Interstitial cell-stimulating hormone. Biochem Biophys Acta 147:175-177

19. **Sairam MR, Papkoff H, Li CH** 1972 The primary structure of ovine interstitial cell-stimulating hormone. I. The α -subunit. Arch Biochem Biophys 153:554-571

20. **Sairam MR, Samy TS, Papkoff H, Li CH** 1972 The primary structure of ovine interstitial cell-stimulating hormone. II. The β-subunit. 572-586

21. **Liao TH, Pierce JG** 1970 The Presence of a Common Type of Subunit in Bovine Thyroid-Stimulating and Luteinizing Hormones. J Biol Chem 245:3275-3281

22. **Pierce JG** 1971 Eli Lilly Lecture: The Subunits of Pituitary Thyrotropin-Their Relationship to other Glycoprotein Hormones. Endocrinology 89:1331-1334

23. **Papkoff H, Edblad M** 1970 Ovine Follicle Stimulating Hormone: Preparation and Characterization of its Subunits. Biochem Biophys Res Commun 40:614-621

24. **Saxena BB, Rathnam P** 1971 Dissociation Phenomenon and Subunit Nature of Follicle-stimulating Hormone from Human Pituitary Glands. J Biol Chem 246:3549-3554

25. **Bellisario R, Carlsen RB, Bahl OP** 1973 Human chorionic gonadotropin. Linear amino acid sequence of the alpha subunit. J Biol Chem 248:6796-6809

26. **Carlsen RB, Bahl OP, Swaminathan N** 1973 Human chorionic gonadotropin. Linear amino acid sequence of the beta subunit. J Biol Chem 248:6810-6827

27. **Fiddes JC, Goodman HM** 1979 Isolation, cloning and sequence analysis of the cDNA for the alpha-subunit of human chorionic gonadotropin. Nature 281:351-356

28. **Fiddes J, Goodman, HM** 1980 The cDNA for the β-subunit of human chorionic gonadotropin suggests evolution of a gene by readthrough into the 3'-untranslated region. Nature 286:684-687

29. **Gurr JA, Catterall JF, Kourides IA** 1983 Cloning of cDNA encoding the pre-beta subunit of mouse thyrotropin. Proc Natl Acad Sci U S A 80:2122-2126

30. **Jameson L, Chin WW, Hollenberg AN, Chang AS, Habe ner JF** 1984 The gene encoding the beta-subunit of rat luteinizing hormone. Analysis of gene structure and evolution of nucleotide sequence. J Biol Chem 259:15474-15480

31. **Maurer RA, Beck A** 1986 Isolation and nucleotide sequence analysis of a cloned cDNA encoding the beta-subunit of bovine follicle-stimulating hormone. DNA 5:363-369

32. **McFarland KC, Sprengel R, Phillips HS, Kohler M, Rosemblit N, Nikolics K, Segaloff DL, Seeburg PH** 1989 Lutropin-choriogonadotropin receptor: an unusual member of the G protein-coupled receptor family. Science 245:494-499

33. **Loosfelt H, Misrahi M, Atger M, Salesse R, Vu Hai-Luu Thi MT, Jolivet A, Guiochon-Mantel A, Sar S, Jallal B, Garnier J, Milgrom E** 1989 Cloning and sequencing of porcine LH-hCG receptor cDNA: variants lacking transmembrane domain. Science 245:525-528

34. **Parmentier M, Libert F, Maenhaut C, Lefort A, Gerard C, Perret J, Van Sande J, Dumont JE, Vassart G** 1989 Molecular cloning of the thyrotropin receptor. Science 246:1620-1622

35. **Sprengel R, Braun T, Nikolics K, Segaloff DL, Seeburg PH** 1990 The testicular receptor for follicle stimulating hormone: structure and functional expression of cloned cDNA. Mol Endocrinol 4:525-530

36. **Thompson JD, Higgins DG, Gibson TJ** 1994 CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence

weighting, position-specific gap penalities and weight matrix choice. Nuc Acids Res 22:4673-4680

37. **Xie YB, Wang H, Segaloff DL** 1990 Extracellular domain of lutropin/choriogonadotropin receptor expressed in transfected cells binds choriogonadotropin with high affinity. J Biol Chem 265:21411-21414

38. **Puett D, Wu C, Narayan P** 1998 The tie that binds: design of biologically active single-chain human chorionic gonadotropins and a gonadotropin-receptor complex using protein engineering. Biol Reprod 58:1337-1342

39. **Segaloff DL, Ascoli M** 1993 The lutropin/choriogonadotropin receptor ... 4 years later. Endocr Rev 14:324-347

40. **Bhowmick N, Huang, J, Puett, D, Isaacs, NW, and Lapthorn, AJ** 1996 Determination of residues important in hormone binding to the extracellular domain of the luteinizing hormone/chorionic gonadotropin receptor by site-directed mutagenesis and modeling. Mol Endocrinol 10:1147-1159

41. **Sugahara T, Pixley MR, Minami S, Perlas E, Ben-Menahem D, Hsueh AJ, Boime I** 1995 Biosynthesis of a biologically active single peptide chain containing the human common alpha and chorionic gonadotropin beta subunits in tandem. Proc Natl Acad Sci U S A 92:2041-2045

42. **Narayan P, Wu C, Puett D** 1995 Functional expression of yoked human chorionic gonadotropin in baculovirus-infected insect cells. Mol Endocrinol 9:1720-1726

43. **Wu C, Narayan P, Puett D** 1996 Protein engineering of a novel constitutively active hormone-receptor complex. J Biol Chem 271:31638-31642

44. **Fares FA, Yamabe S, Ben-Menahem D, Pixley M, Hsueh AJ, Boime I** 1998 Conversion of thyrotropin heterodimer to a biologically active single- chain. Endocrinology 139:2459-2464

45. **Sugahara T, Sato A, Kudo M, Ben-Menahem D, Pixley MR, Hsueh AJ, Boime I** 1996 Expression of biologically active fusion genes encoding the common alpha subunit and the follicle-stimulating hormone beta subunit. Role of a linker sequence. J Biol Chem 271:10445-10448

46. **Kanda M, Jablonka-Shariff A, Sato A, Pixley M, Bos E, Hiro'oka T, Ben-Menahem D, Boime I** 1999 Genetic fusion of an α-subunit gene to the Follicle-Stimulating Hormone and Chorionic Gonadotropin-β subunit genes: Production of a bifunctional protein. Mol Endocrinol 13:1873-1881

47. **Garcia-Campayo V, Boime I** 2001 Covalent union of three members of the glycoprotein hormone family: A model of multi-functional relationships. Program and Abstracts of The Endocrine Society's 83rd Annual Meeting 506, abstract P3-269

CHAPTER 2

SPECIFICITY OF COGNATE LIGAND RECEPTOR INTERACTIONS: FUSION PROTEINS OF HCG AND THE HEPTAHELICAL RECEPTORS FOR HUMAN LH, TSH, and FSH[1]

[1]Schubert, R.L., Narayan, P., and Puett, D. To be submitted to *Endocrinology*.

Abstract

The family of glycoprotein hormones, consisting of a common α subunit and a hormone-specific β subunit, and their homologous G protein-coupled receptors, characterized by high affinity ligand binding to the ectodomain, represent an excellent system for comparative structure-function studies. We have used protein engineering to study single chain molecules of hCG fused to its cognate receptor, LHR, and to the non-cognate receptors, TSHR and FSHR. Each of the human glycoprotein hormone receptors was fused to hCG in the manner N-β-α-receptor-C in order to create the yoked (Y) hormone-receptor complexes YCG/LHR, YCG/TSHR, and YCG/FSHR. Expression and bioactivity of these fusion proteins were examined after transient transfection of each cDNA into HEK 293 cells via hormone and antibody binding assays, as well as cAMP measurements. Western blot analysis indicated that each of the YCG/R proteins was expressed. In the case of YCG/LHR, no binding of exogenous hormone was observed due to the occupation of receptor by the fused ligand. The presence of hCG in the non-cognate hormone-receptor fusion proteins, however, did not prevent binding of exogenous cognate ligand. When compared to the results for cells expressing LHR, the fusion of hCG to LHR in the single chain YCG/LHR complex results in constitutive activation of LHR that is approximately 20-fold higher than the basal cAMP production of cells expressing wild-type LHR. An increase in basal cAMP production was observed when hCG was fused to TSHR and FSHR, e.g. 13-fold and 4-fold increases, respectively. Whereas the specificity of hCG for its cognate receptor, LHR, is extraordinarily high, the hormone is capable of binding to and activating both TSHR and FSHR under these conditions that mimic high ligand concentrations. While the functional interaction of

hCG and TSHR has been recognized clinically, there are no reports linking hCG to FSHR activation, although this study suggests that it is capable of occurring.

Introduction

There are four hormones that comprise the family of glycoprotein hormones, human chorionic gonadotropin (hCG), luteinizing hormone (LH), thyroid stimulating hormone (TSH), and follicle stimulating hormone (FSH) (1, 2). These hormones are heterodimers, consisting of a common α subunit and a receptor-specific β subunit, and confer activity by binding to their respective G protein-coupled receptors (GPCRs) (2). The glycoprotein hormone receptors are a special class of GPCRs, characterized by their relatively large ectodomain, which is responsible for high affinity hormone binding (3). Because of the high sequence similarity in their β subunits, both hCG and LH bind to the same receptor, LHR, while TSH and FSH bind unique receptors, TSHR and FSHR, respectively (3). There is a high degree of sequence homology among the glycoprotein hormone β subunits (Fig. 2.1A) and between the glycoprotein hormone receptor ectodomains (Fig. 2.1B).

In 1995, the crystal structure of aglyco-hCG was determined independently by two groups (4, 5), which was later followed by that of partially glycosylated hFSH (6). This information has provided a molecular framework for structure-function studies designed to elucidate the regions of these gonadotropins that impart receptor binding and activation.

We, and others, have used fusion protein techniques to further delve into structure-function studies within the families of glycoprotein hormones and their GPCRs (7, 8). Single-chain, or yoked (Y) hormones have been designed in the manner N-hCGβ-α-C (7-9) and N-α-hCGβ-C (10-12). Our group (7) and that of Boime's (8) first designed YhCG1 with hCGβ at the N-terminus, we then created YhCG3 in which hCGβ is located at the C-terminus (10). Both conformations were effective in activating LHR, however, YhCG1 was able to bind LHR with a higher affinity than that found with YhCG3. This fusion protein technique was later expanded to include yoked hormone-receptor complexes in which YhCG1 and YhCG3 were fused to the N terminus of rat LHR in the manner, N-hCGβ-α-CTP-LHR-C and N-α-CTP-hCGβ-LHR-C, respectively (10, 13). This approach of using a fused ligand-receptor complex permits the study of receptor binding and activation by individual subunits, subunit mutants that fail to efficiently form holoproteins, and ligand mutants that bind with low affinity to their cognate receptor.

Since the glycoprotein hormones are comprised of a common α subunit and homologous β subunits, and their receptors are homologous as well, we have prepared and characterized fusion proteins of single chain hCG with the three human receptors, using the configuration N-β-α-CTP-R, to yield YCG/LHR, YCG/TSHR, and YCG/FSHR (Fig. 2.2) in order to investigate the anticipated weak binding of hCG to and the activation of its non-cognate receptors, TSHR and FSHR. While hCG stimulation of TSHR results in pathophysiological conditions such as gestational hyperthyroidism (14), there are no known clinical cases involving hCG activation of FSHR. This non-cognate

hormone-receptor interaction involving hCG and TSHR documents that cross-reactivity occurs within these families.

The goal of the current study is to comparatively evaluate the ability of hCG to activate each of the human glycoprotein hormone receptors, hLHR, hTSHR, and hFSHR. This information is important in elucidating basic structure-function relationships of these families of hormones and receptors; furthermore, the information gained should aid in the design of agonists and antagonists for the glycoprotein hormone receptors.

Materials and Methods

PCR was used to add a portion of the CTP to the 5' end of each of the human glycoprotein hormone receptors, as well as an Xho I restriction site to the 3' end of each receptor using the following primer designs:

5' primer: 5'-(Afl III/CTP/Factor Xa/human receptor sequence)-3'

3' primer: 5'-(human receptor sequence/Xho I)-3'

Each of the human glycoprotein hormone receptors was digested with EcoRI and XhoI and then ligated to both YhCG1 that had been digested with BamHI and EcoRI and pcDNA3 that had been digested with Bam HI and XhoI to create vectors containing YCG/LHR, YCG/TSHR, and YCG/FSHR.

Cell culture and transient transfections

HEK 293 cells were grown in monolayer culture in growth media consisting of Dulbecco's Modification of Eagle's Medium (DMEM) fortified with 10% (v/v) newborn

calf serum, 10 mM HEPES buffer, pH 7.4, 50 U/ml penicillin, 50 µg/ml streptomycin, 50 µg/ml Fungizone, and 0.125 µg/ml Amphotericin B. Cells were sustained at 37C in humidified air containing 5% CO_2. The cDNAs were transiently transfected using Lipofectamine 2000 as recommended by Gibco BRL into 75 cm^2 tissue culture flasks containing HEK 293 cells (5 µg cDNA for each fusion protein).

Western blot analysis

Solubilized membrane fractions were electrophoresed on 10% SDS-PAGE and transferred to PVDF membranes. The membrane was probed with a 1:1000 dilution of an anti-CTP polyclonal antibody and visualized by chemiluminescence via a secondary HRP-labeled anti-rabbit antibody.

Hormone binding

Cells were resuspended in 36 ml of growth media approximately 16-18 h after transfection, and 1 ml of medium was added to each well of a 12-well tissue culture plate coated with 0.1% gelatin in PBS. The cells were assayed for ^{125}I-hormone binding approximately 24 h later. In order to perform competitive binding assays, increasing concentrations of unlabeled hCG, bTSH, (both hCG and bTSH were kindly provided by Dr. Albert Parlow and the NIDDK), or hFSH (Sigma Chem. Co., St. Louis, MO) in Waymouth's media with 0.1% BSA (w/v) and 50 pM ^{125}I-hCG, 50 pM ^{125}I-bTSH or 100 pM ^{125}I-hFSH was added to each well and incubated at 37C for 6 h. Nonspecific binding was determined by addition of 1 µg/ml of unlabeled hormone for hCG and bTSH, and 3 µg/ml of unlabeled hFSH. The incubation medium was aspirated, the cells washed with

PBS then lysed by addition of 0.5 ml of 1 N NaOH. All binding assays were performed in duplicate. Mock transfected cells treated in the same manner were used as negative controls.

Cell surface antibody binding assay

Approximately 16-18 h after transfection, the cells were processed as described above and the cells assayed for binding of a receptor-specific primary antibody and then detected by secondary ^{125}I-anti-rabbit antibody. For hLHR, a 1:4000 dilution of an anti-rat LHR antibody, raised against a synthetic peptide to residues 15-38 (kindly provided by Dr. Patrick Roche, Mayo Medical School, Rochester, MN), was used. Dr. Mariusz Szkudlinski (Trophogen, Inc., Rockville, MD) kindly provided an anti-hTSHR antibody raised against residues 352-366, and Dr. Mario Ascoli (University of Iowa, Iowa City, IA) generously provided an anti-rat FSHR antibody raised against residues 19-29. Dilutions of 1:1000 and 1:500 were made of the TSHR and FSHR antibodies, respectively. The cells were incubated in the appropriate dilution of the primary antibody, as indicated, in Waymouth's media with 0.3% (w/v) BSA for the LHR antibody and 0.1% (w/v) BSA for the TSHR and FSHR antibodies for 4 h at room temperature while shaking. After washing twice with Waymouth's media, containing 0.3% (w/v) BSA for the LHR antibody and 0.1% (w/v) BSA for the TSHR and FSHR antibodies, the cells were incubated with the ^{125}I-anti-rabbit secondary antibody (400,000 CPM/well) in Waymouth's media containing 0.3% or 0.1% BSA for 1 h at room temperature with shaking. The medium was aspirated and the cells washed with PBS after the second

incubation period. Lysates were prepared as described above and the samples counted in a gamma counter. Each binding experiment was performed in duplicate.

cAMP assay

The cells were replated and washed as described above some 16-18 h after transfection, then incubated in Waymouth's media with 0.1% (w/v) BSA and 0.8 mM isobutylmethylxanthine (IBMX) for 15 min at 37C. The cells were incubated with increasing concentrations of hCG, bTSH, or hFSH for 30 min at 37C in Waymouth's media containing 0.1% (w/v) BSA and 0.8 mM IBMX immediately following the first incubation. The incubation medium was removed and the cells lysed in 100% ethanol at -20C overnight. The extract was collected, dried under vacuum, and cAMP concentrations were determined by RIA as recommended (DuPont NEN). Each data point was performed in duplicate, and mock transfected cells were used as negative controls.

Data analysis

Competitive binding and cAMP results were analyzed by non-linear regression using the Prism software program (Graph Pad Software, San Diego, CA). The results are given as mean ± SEM, based on 3-5 independent transfections, and significance was determined from Student's t-test. The figures showing competitive binding and hormone-mediated cAMP production are representative experiments. The results in Table 2.1 refer to the average of each individual parameter from multiple transfections.

Results

Cell surface expression of the yoked hormone receptor complexes

After transient transfection into HEK 293 cells, membrane preparations of cells expressing the YCG/R complexes were prepared. Western blot analysis using a 1:1000 dilution of an anti-CTP antibody showed that all YCG/R proteins were expressed in the molecular weight range of 120-150 kDa (Fig. 2.3A).

In order to compare expression of the YCG/R complexes with that of their corresponding receptors, the transfected cells were incubated in a solution containing a primary antibody against the extracellular portion of the receptors, whose binding was detected with an ^{125}I-anti-rabbit secondary antibody. As shown in Fig. 2.3B, there is no significant difference in expression of the YCG/R complexes with their corresponding wild type receptors, signifying that any differences in the functional assays are not due to differences in expression levels.

Specific binding of each of the YCG/R complexes and corresponding wild-type receptors are depicted in Fig. 2.3C. There is no apparent binding of ^{125}I-hCG to cells expressing YCG/LHR, likely due to the presence of the fused ligand. Cells expressing TSHR and YCG/TSHR were able to bind ^{125}I-bTSH at approximately the same level, while those expressing YCG/FSHR displayed reduced binding of ^{125}I-hFSH as compared to cells expressing FSHR.

Yoked hCG-receptor complexes: Characterization of single-chain ligand receptor complexes

hLHR and YCG/LHR: Cells expressing YCG/LHR showed essentially no binding of 50 pM ^{125}I-hCG due to the occupancy of binding sites by the fused hormone (Fig. 2.3C), as expected (10, 13). These cells, however, exhibited a high constitutive level of cAMP production that was not elevated upon addition of exogenous hormone (Fig. 2.4). Comparisons of the binding and signaling parameters of cells expressing hLHR and YCG/LHR cells are shown in Table 2.1.

hTSHR and YCG/TSHR: Competitive binding studies with ^{125}I-bTSH and bTSH were conducted with cells expressing hTSHR and YCG/TSHR (Fig. 2.5A), and no differences were found in the IC_{50} values (Table 2.1). An approximate 13–fold increase in basal cAMP in cells expressing YCG/TSHR compared to that seen in cells expressing hTSHR was found. Interestingly, these cells were further stimulated upon addition of exogenous bTSH, and the same maximal cAMP production was observed as that obtained in cells expressing hTSHR (Fig. 2.5B). Moreover, there is no difference between the ED_{50} values for cells expressing hTSHR and YCG/TSHR (Table 2.1).

hFSHR and YCG/FSHR: For competitive binding of cells expressing hFSHR and YCG/FSHR, 100 pM ^{125}I-hFSH was used with increasing concentrations of unlabeled hFSH (Fig. 2.6A). Again, there was no difference in the IC_{50} values for hFSHR and YCG/FSHR (Table 2.1). Cells expressing YCG/FSHR exhibited an approximate 4-fold increase in basal cAMP production compared to that of hFSHR; moreover, as with cells expressing YCG/TSHR, those cells were further stimulated following addition of exogenous hFSH, reaching a maximum cAMP level comparable to that of cells

expressing hFSHR (Fig. 2.6B). No differences in the ED_{50} values were observed (Table 2.1).

Discussion

The results reported herein demonstrate that there is indeed recognition of hCG by the three glycoprotein hormone receptors. In the yoked hormone-receptor system, hCG binds to and activates not only its cognate receptor, LHR, but also the non-cognate receptors, TSHR and FSHR. Both YCG/TSHR and YCG/FSHR can be further stimulated by addition of exogenous, cognate ligand, indicating neither of the interactions of hCG, even when fused with a non-cognate receptor, were strong enough to prevent cognate hormone binding, as in the homologous system, i.e. ^{125}I-hCG binding to YCG/LHR. However, since all of the glycoprotein hormones share a common α subunit and, as shown in Fig. 2.1, there is a high degree of sequence homology in both the glycoprotein hormone β subunits and glycoprotein hormone receptor ectodomains that, evidently, allows for some cross-reactivity within the families. An understanding of the differences and similarities between the abilities of these hormones to bind to and activate cognate and non-cognate receptors will provide important information on sequence determinants responsible for specificity, but also on common binding and receptor activation elements, e.g. α and conserved β contact sites to identical receptor domains. Such results will help guide the development of small molecule agonists and antagonists for these GPCRs.

Fig. 2.1 is arranged to depict the three glycoprotein hormone receptors in the context of the model proposed by Bhowmick et al. (15) for the LHR extracellular domain. In their homology model based on the leucine-rich repeat (LRR) structure of pancreatic ribonuclease (16), residues 27-235 of rat LHR were proposed to form 9 LRRs as shown for the human receptors. The N-terminal and C-terminal regions of the extracellular domain do not exhibit a LRR structure, but it was suggested that the hinge region may adopt a chemokine-like fold (15). Identical amino acid residues in the extracellular domains of the three glycoprotein hormone receptors are shown in italiced, bold font and conserved residues, e.g. hydrophobic, ionizable or identical charge (K/R and D/E), and hydroxylated (S/T), are indicated by open boxes. We know from several studies that high affinity hCG binding to LHR occurs primarily in the region encoded by exons 1-8 (3, 17, 18). While there is no assurance that the binding of TSH and FSH to their cognate receptor extracellular domains will be comparable to that of hCG and the LHR extracellular domain, we will assume for the sake of argument that similar binding domains exist in the three receptors.

A careful examination of Fig. 2.1 shows that the regions encoded by exons 1, 10, and a portion of 11 are the most divergent in structure. A highly conserved region in the three extracellular domains is encoded by exon 9, L(T/S)YPSHCCAFXN, as is an identical region in exon 11, FNPCEDIMGY. Both of these domains have been shown to influence hCG-mediated LHR activation, but not ligand binding (19-22). Within the region of the LHR extracellular domain believed to be responsible for high affinity ligand binding, the N-terminal sequence and that of LRR1 are the most variable, and LRRs 2-8 exhibit about 52 ± 8% amino acid sequence identity/homology in the three receptors. It

is tempting to assign binding specificity to the β subunits making contacts with the N-terminal region, LRR1, and portions of LRRs 2-8, while the α subunit is more likely to have contact sites in the identical/conserved regions of LRRs 2-8. Those are but suggestions, but they provide a useful framework for data interpretation and to guide further experiments.

We know that in cases of gestational hyperthyroidism, hCG is able to bind to and activate TSHR (14), consistent with our YCG/TSHR findings. In addition, Rodien et al. showed that a mutation in TSHR, in which Lys-183 is replaced with Arg, increases the receptor's affinity for hCG significantly above that of wild-type hTSHR (23). Again, this finding supports our studies and exemplifies the importance of the slight differences in primary structure within theses families in order to direct hormone binding and allow for receptor activation. To the best of our knowledge, there are no reported clinical cases in which hypersecretion of hCG activates FSHR, but our results suggest that this can occur.

Yoked hormones and yoked hormone-receptor systems have been used to study interactions within the families of glycoprotein hormones and the glycoprotein hormone receptors. We and others have created YhCG complexes in which the fusion protein has been found to be active in either orientation, N-hCGβ-α-C (7-9) and N-α-hCGβ-C (10, 11). In addition to single-chain TSH (24) and FSH (25) hormones, both of which are as active as the heterodimers, Boime's group has also created multifunctional fusion proteins (26, 27). In one such protein, the common α subunit was fused to FSHβ as well as hCGβ, creating a hybrid that could react with FSHR as well as LHR (26). These studies were then expanded to include a fusion protein that included all three glycoprotein hormone β subunits and a single α subunit to create a trifunctional protein.

This protein was found to bind with high affinity to each of the glycoprotein hormone receptors and result in cAMP production (27).

Although we cannot discount the possibility that the presence of hCG in the YCG/R complexes forces a constraint on receptor conformation that promotes an increase in basal cAMP levels, we do not believe that this happens based on the documentation of hCG-TSHR interactions (14), as well as the absence of receptor activation when the a subunit, as a monomer or homodimer, was fused to hTSHR (28). In addition, previous studies from our group showed that the α subunit, CGβ, or prolactin, an unrelated protein, when fused to LHR, produced no increase in basal cAMP production (Narayan, Gray and Puett, unpublished data). Given these data, we believe that the increases in basal cAMP levels that we observe are caused by specific interactions of hCG with each of the glycoprotein hormone receptors. In summary, the findings from this study have indicated an ability of hCG to bind to and activate its non-cognate receptors, hTSHR and hFSHR.

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TABLE 2.1. Summary of binding and signaling parameters of YCG/Rs and human receptors.

	IC_{50} (nM) (n)	*ED_{50} (nM) (n)*	*Basal cAMP (pmol/ml) (n)*	*Maximum cAMP (pmol/ml) (n)*	*R_{max} (pmol/ml) (n)*
hLHR	0.6 ± 0.2[a] (5)	0.4 ± 0.1 (3)	4.5 ± 0.4 (3)	241.5 ± 25.7 (3)	237.0 ± 39.9 (3)
YCG/LHR	N/A	N/A	95.1 ± 15.9[b] (3)	110.6 ± 17.8[b] (3)	15.6 ± 3.0[b] (3)
hTSHR	2.4 ± 0.6 (5)	0.3 ± 0.1 (3)	5.0 ± 0.5 (3)	251.6 ± 29.7 (3)	246.5 ± 46.0 (3)
YCG/TSHR	2.9 ± 0.1 (5)	0.4 ± 0.2 (3)	69.2 ± 8.0[b] (3)	196.1 ± 16.9 (3)	127.0 ± 13.2 (3)
hFSHR	1.5 ± 0.5 (3)	0.8[c] (1)	3.5 ± 0.4 (3)	87.5 ± 13.5 (3)	84.0 ± 20.5 (3)
YCG/FSHR	2.3 ± 1.0 (3)	1.2[c] (1)	14.8 ± 2.3[b] (3)	78.9 ± 18.9 (3)	61.7 ± 21.6 (3)

HEK 293 cells were transiently tranfected with 5 μg of cDNA of pcDNA3 for mock-transfected cells, receptor, or YCG/R. Dose-reponse curves were performed using hCG, bTSH, or hFSH in order to compete with a constant level of the corresponding ^{125}I-hormone. Maximum cAMP levels refer to the maximum production of cAMP when stimulated with 250 ng/ml of hCG (LHR) or bTSH (TSHR), or 1000 ng/ml of hFSH (FSHR) with no correction for basal cAMP production. R_{max} values refer to the difference between maximum and basal cAMP levels, showing the amount of cognate ligand-mediated stimulation of the receptors.

[a] Data obtained from previous study (Schubert, R.L. and Puett, D., unpublished data).

[b] Significantly different from the corresponding human receptor ($p<0.05$).

[c] Replicate transfections were executed, but only one gave a well defined plateau with two points.

References

1. **Pierce JG, Parsons TF** 1981 Glycoprotein hormones: structure and function. Annu Rev Biochem 50:465-495
2. **Hearn MTW, Gomme PT** 2000 Molecular architecture and biorecognition process of the cysteine knot protein superfamily: part I. The glycoprotein hormones. J Mol Recognition 13:223-278
3. **Ascoli M, Fanelli F, Segaloff DL** 2002 The lutropin/choriogonadotropin receptor, a 2002 perspective. Endocr Rev 23:141-174
4. **Lapthorn AJ, Harris DC, Littlejohn A, Lustbader JW, Canfield RE, Machin KJ, Morgan FJ, Isaacs NW** 1994 Crystal structure of human chorionic gonadotropin. Nature 369:455-461
5. **Wu H, Lustbader JW, Liu Y, Canfield RE, Hendrickson WA** 1994 Structure of human chorionic gonadotropin at 2.6 A resolution from MAD analysis of the selenomethionyl protein. Structure 2:545-558
6. **Fox KM, Dias JA, Van Roey P** 2001 Three-dimensional structure of human follicle-stimulating hormone. Mol Endocrinol 15:378-389
7. **Narayan P, Wu C, Puett D** 1995 Functional expression of yoked human chorionic gonadotropin in baculovirus-infected insect cells. Mol Endocrinol 9:1720-1726
8. **Sugahara T, Pixley MR, Minami S, Perlas E, Ben-Menahem D, Hsueh AJ, Boime I** 1995 Biosynthesis of a biologically active single peptide chain

containing the human common alpha and chorionic gonadotropin beta subunits in tandem. Proc Natl Acad Sci U S A 92:2041-2045

9. **Heikoop JC, Van Beuningen-De VMM, Van Den Boogaart P, Grootenhusi PDJ** 1997 Evaluation of subunit truncation and the nature of the spacer for single chain human gonadotropins. Eur J Biochem 245:656-662

10. **Narayan P, Gray J, Puett D** 2000 A biologically active single chain human chorionic gonadotropin analog with altered receptor binding properties. Endocrinology 141:67-71

11. **Sen Gupta C, Dighe RR** 2000 Biological activity of single chain choriogonadotropin, hCGαβ is decreased upon deletion of five carboxyl terminal amino acids of the α subunit without affecting its receptor binding. J Mol Endocrinol 24:157-164

12. **Ben-Menahem D, Jablonka-Shariff A, Hyde RK, Pixley MR, Srivastava S, Berger P, Boime I** 2001 The position of the α and β subunits in a single chain variant of human chorionic gondotropin affects the heterodimeric interaction of the subunits and receptor-binding epitopes. J Biol Chem 276:29871-29879

13. **Wu C, Narayan P, Puett D** 1996 Protein engineering of a novel constitutively active hormone-receptor complex. J Biol Chem 271:31638-31642

14. **Burrow G** 1993 Thyroid function and hyperfunction during gestation. Endocr Rev 14:194-201

15. **Bhowmick N, Huang, J, Puett, D, Isaacs, NW, and Lapthorn, AJ** 1996 Determination of residues important in hormone binding to the extracellular

domain of the luteinizing hormone/chorionic gonadotropin receptor by site-directed mutagenesis and modeling. Mol Endocrinol 10:1147-1159

16. **Kobe B, Deisenhofer J** 1993 Crystal structure of porcine ribonuclease inhibitor, a protein with leucine-rich repeats. Nature 366:751-756

17. **Thomas D, Rozell TG, Segaloff DL** 1996 Mutational analyses of the extracellular domain of the full-length lutropin/choriogonadotropin receptor suggest leucine-rich repeats 1-6 are involved in hormone binding. Mol Endocrinol 10:760-768

18. **Hong S, Phang T, Ji I, Ji TH** 1998 The amino-terminal region of the luteinizing hormone/choriogonadotropin receptor contacts both subunits of human choriogonadotropin. I. Mutational analysis. J Biol Chem 273:13835-13840

19. **Alvarez CA, Narayan P, Huang J, Puett D** 1999 Characterization of a region of the lutropin receptor extracellular domain near transmembrane helix 1 that is important in ligand-mediated signaling. Endocrinology 140:1775-1782

20. **Nakabayashi K, Kudo M, Kobilka B, Hsueh AJW** 2000 Activation of the luteinizing hormone receptor following substitution of Ser-277 with selective hydrophobic residues in the ectodomain hinge region. J Biol Chem 275:30264-30271

21. **Huang J, Puett D** 1995 Identification of two amino acid residues on the extracellular domain of the lutripin/choriogonadotropin receptor important in signaling. J Biol Chem 270:30023-30028

22. **Zeng H, Phang T, Song YS, Ji I, Ji TH** 2001 The role of the hinge region of the luteinizing hormone receptor in hormone interaction and signal generation. J Biol Chem 276:3451-3458

23. **Rodien P, Bremont C, Sanson ML, Parma J, Van Sande J, Costagliola S, Luton JP, Vassart G, Duprez L** 1998 Familial gestational hyperthyroidism caused by a mutant thyrotropin receptor hypersensitive to human chorionic gonadotropin. N Engl J Med 339:1823-1826

24. **Fares FA, Yamabe S, Ben-Menahem D, Pixley M, Hsueh AJ, Boime I** 1998 Conversion of thyrotropin heterodimer to a biologically active single- chain. Endocrinology 139:2459-2464

25. **Sugahara T, Sato A, Kudo M, Ben-Menahem D, Pixley MR, Hsueh AJ, Boime I** 1996 Expression of biologically active fusion genes encoding the common alpha subunit and the follicle-stimulating hormone beta subunit. Role of a linker sequence. J Biol Chem 271:10445-10448

26. **Kanda M, Jablonka-Shariff A, Sato A, Pixley M, Bos E, Hiro'oka T, Ben-Menahem D, Boime I** 1999 Genetic fusion of an α-subunit gene to the follicle-stimulating hormone and chorionic gonadotropin-β subunit genes: Production of a bifunctional protein. Mol Endocrinol 13:1873-1881

27. **Garcia-Campayo V, Boime I** 2001 Covalent union of three members of the glycoprotein hormone family: A model of multi-functional relationships. Program of the 83rd Annual Meeting of The Endocrine Society, Denver, CO 506

28. **Angelova K, Jain R, Puett D, Szkudlinski MW, Narayan P** 2001 Fusion of the commong glycoprotein hormone α-subunit to the TSH receptor results in partial

antagonism of TSH activation. Program of the 83rd Anuual Meeting of The Endocrine Society, Denver, CO 265

29. **Thompson JD, Higgins DG, Gibson TJ** 1994 CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalities and weight matrix choice. Nuc Acids Res 22:4673-4680

Figure Legends

Fig. 2.1. Alignments of the amino acid sequences of the glycoprotein hormone β subunits (A) and glycoprotein hormone receptor ectodomains (B). The dashes (-) indicate identical sequence and the dots (.) indicate gaps in the sequence as analyzed by CLUSTAL alignment (29). The alignment of the receptor ectodomains is separated to show the N-terminal region (ca. 30 amino acid residues), the 9 leucine-rich repeats (LRR) predicted by Bhowmick, et al. (15) for rat LHR, and the Cys-rich region prior to transmembrane helix 1. Bold, italicized lettering indicates identical residues in the three receptors, and open boxes denote conservative replacements, e.g. hydrophobic, charge residues, and T/S.

Fig. 2.2. Schematics of the YCG/R fusion protein constructs. YhCG is fused to the N-terminus of each of the glycoprotein hormone receptors using the hCGβ CTP, designated with vertical stripes, as a flexible linker region to give YCG/LHR, YCG/TSHR, and YCG/FSHR.

Fig. 2.3. Expression of the YCG/R proteins. A. Western blot analysis using a 1:1000 dilution of CTP antibody and a 1:5000 of secondary anti-rabbit HRP antibody shows that all three YCG/R proteins are expressed. B. Cell surface expression is documented by receptor-specific primary antibody detected with ^{125}I-anti-rabbit secondary antibody. There is no difference in cell surface expression when comparing the expression of each YCG/R protein with the corresponding wild type human glycoprotein hormone receptor. The data are corrected for binding of antibody to mock transfected cells (n=3). C.

Specific binding of ^{125}I-hCG, bTSH, and hFSH to their cognate receptors and yoked constructs. Cells expressing YCG/LHR show no apparent binding of ^{125}I-hCG due to the occupation of binding sites by the fused hormone. There was no difference in ^{125}I-bTSH binding to cells expressing either protein, while cells expressing YCG/FSHR showed reduced binding of ^{125}I-hFSH as compared to those expressing hFSHR.

Fig. 2.4. Characterization of hLHR and YCG/LHR proteins. cAMP production in cells expressing hLHR and YCG/LHR in response to increasing concentrations of hCG. ED_{50} values for cells expressing hLHR are given in Table 2.1. (Values could not be determined for cells expressing YCG/LHR because of the inablility of exogenous hCG to bind YCG/LHR.)

Fig. 2.5. Characterization of hTSHR and YCG/TSHR proteins. A. Competitive binding of cells expressing hTSHR and YCG/TSHR. 50 pM ^{125}I-bTSH with increasing concentrations of unlabeled bTSH was used in order to determine the affinity of exogenous bTSH to cells expressing hTSHR or YCG/TSHR. B. cAMP production of cells expressing hTSHR and YCG/TSHR upon stimulation with increasing amounts of bTSH; mock tranfected cells were used as a negative control. IC_{50} and ED_{50} values are given in Table 2.1.

Fig. 2.6. Characterization of hFSHR and YCG/FSHR proteins. A. Competitive binding of cells expressing hFSHR and YCG/FSHR. 200 pM ^{125}I-hFSH with increasing concentrations of unlabeled hFSH was used to determine the affinity of hFSH for each of

the expressed proteins. B. Cells expressing hFSHR and YCG/FSHR were assayed for production of cAMP after stimulation with increasing concentrations of hFSH, with mock transfected cells serving as a negative control. IC_{50} and ED_{50} values are listed in Table 2.1.

Fig. 2.1A

```
hCGb   [1]SKEPLRPRCRPINATLAVEKEGCPVCITVNTTICAGYCPTMTRVLQGVLP..ALPQVVCNYRDVRFESIRLPGCP
hLHb   [1]-R----W-H----I-----------------------M----A----..P-----T----------------
hTSHb  ........[1]F-I-TEY-MHI-RRE-AY-L-I---------M-RDINGKLF--KY--S-D--T---FIYRTVEI----
hFSHb  ........[1]NSCELT-I-I-I---E-RF--SI---W-----Y-RDL-YKDPAR..PKIQKT-TFKELVY-TV-V---A

       RGVNPVVSYAVALSCQCALCRRSTTDCGGPKDHPLTCDDPRFQDSSSSKAPPPSLPSPSRLPGPSDTPILPQ[145]
       ---D----FP-----R-GP-----S----------H-QLSG.....[111]-.....-.....F-[121]
       LH-A-YF--P-----K-GK-NTDYS--IHEAIKTNY-TKPQKSY[111]
       HHADSLYTYP--TQ-H-GK-DSDS---TVRGLG-SY-SFGEMKE[110]
```

Fig. 2.1B

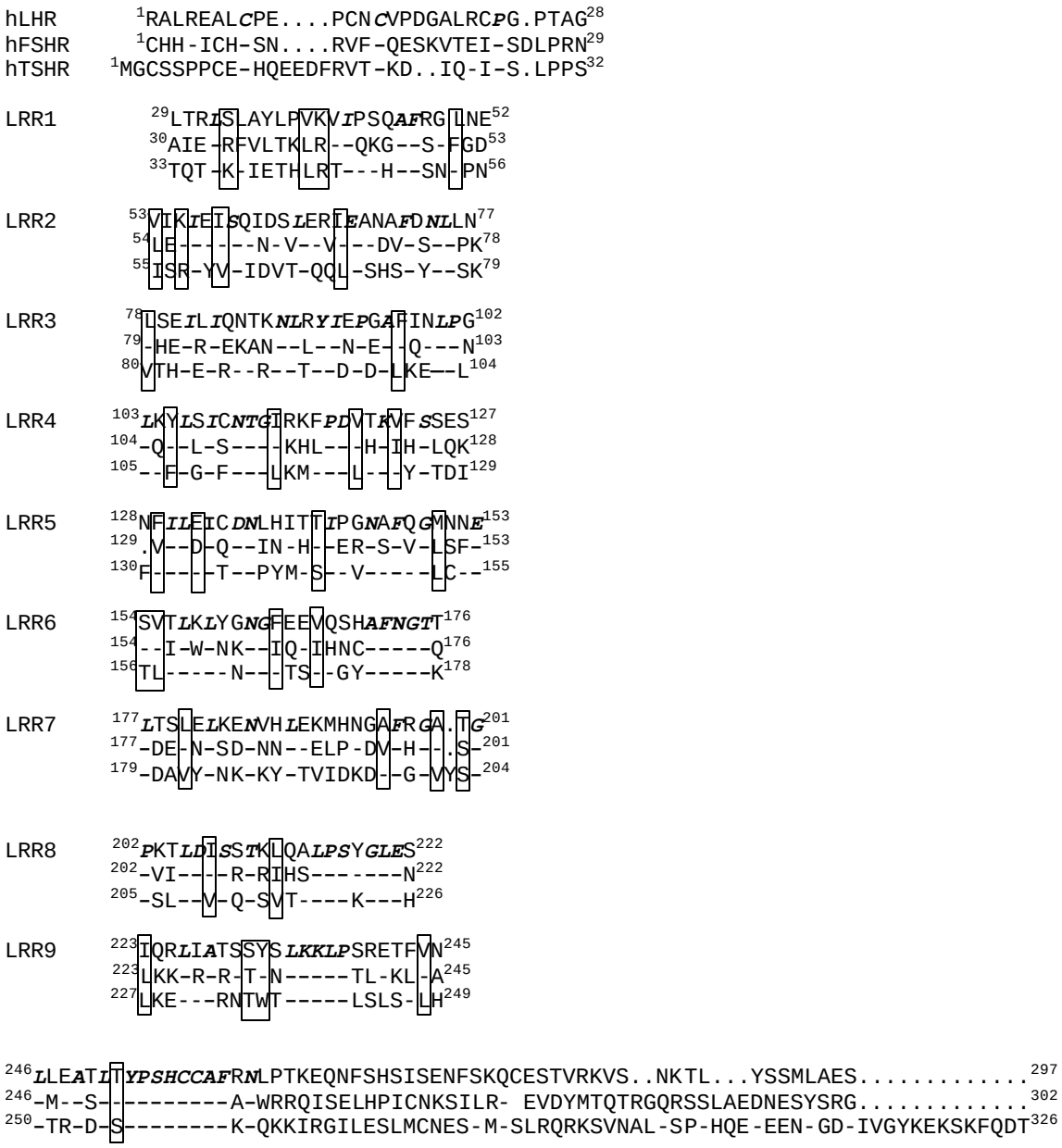
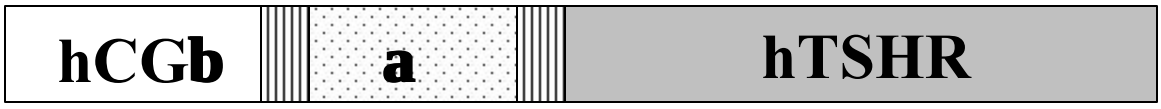


Fig. 2.2

YCG/LHR

YCG/TSHR



YCG/FSHR

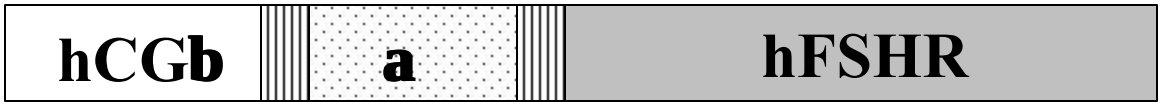
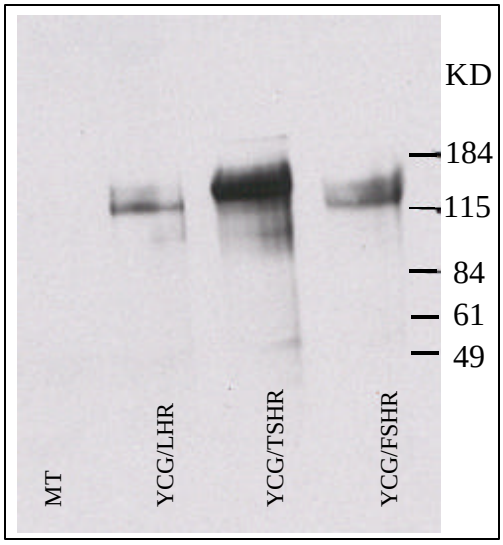
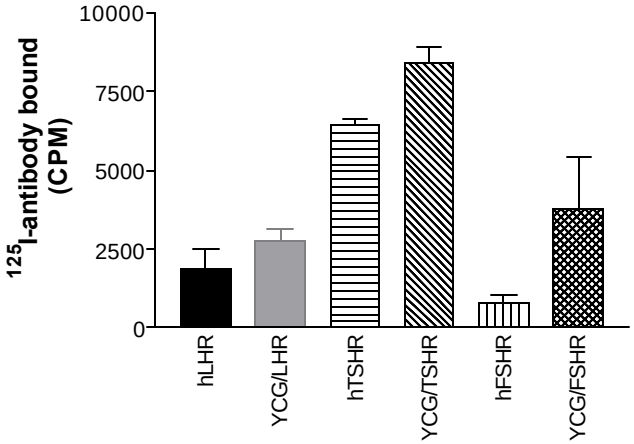


Fig. 2.3

A.


KD
184
115
84
61
49
MT
YCG/LHR
YCG/TSHR
YCG/FSHR

B.


^{125}I-antibody bound (CPM)
10000
7500
5000
2500
0
hLHR
YCG/LHR
hTSHR
YCG/TSHR
hFSHR
YCG/FSHR

C.

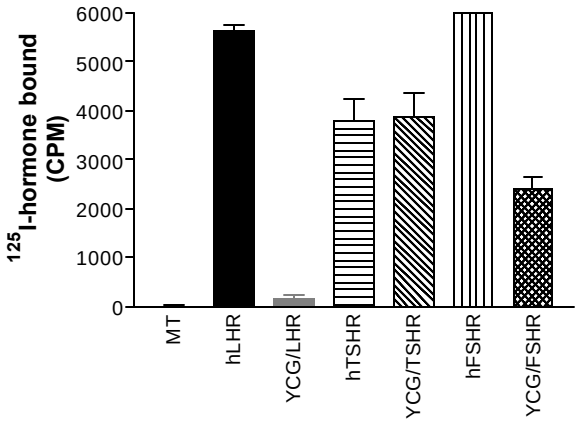

^{125}I-hormone bound (CPM)
6000
5000
4000
3000
2000
1000
0
MT
hLHR
YCG/LHR
hTSHR
YCG/TSHR
hFSHR
YCG/FSHR

Fig. 2.4

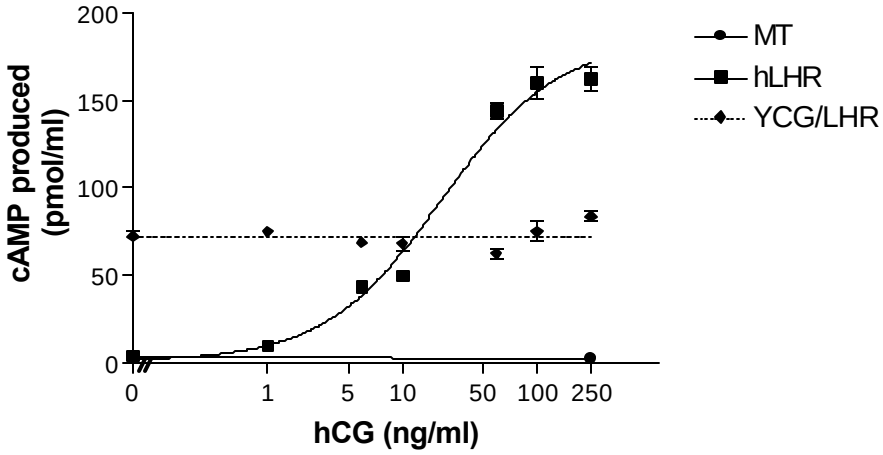
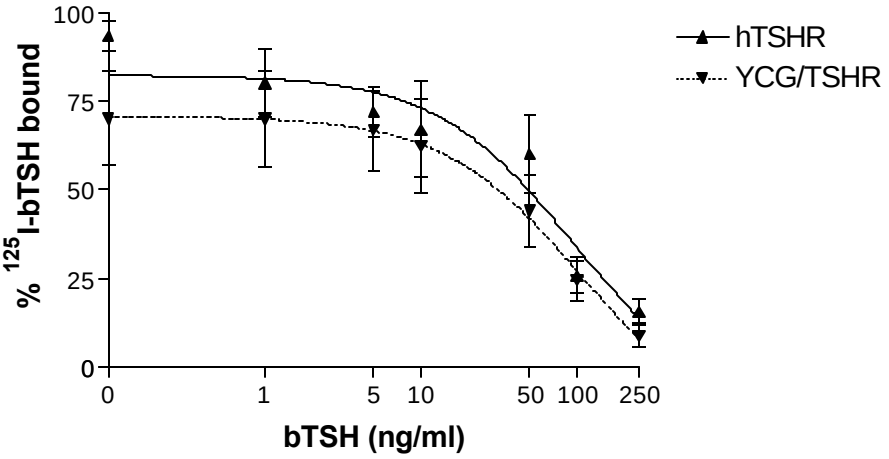

cAMP produced (pmol/ml)
200
150
100
50
0
0
1
5
10
50
100
250
hCG (ng/ml)
MT
hLHR
YCG/LHR

Fig. 2.5

A.


100
75
50
25
0
% 125I-bTSH bound
0
1
5
10
50
100
250
bTSH (ng/ml)
hTSHR
YCG/TSHR

B.

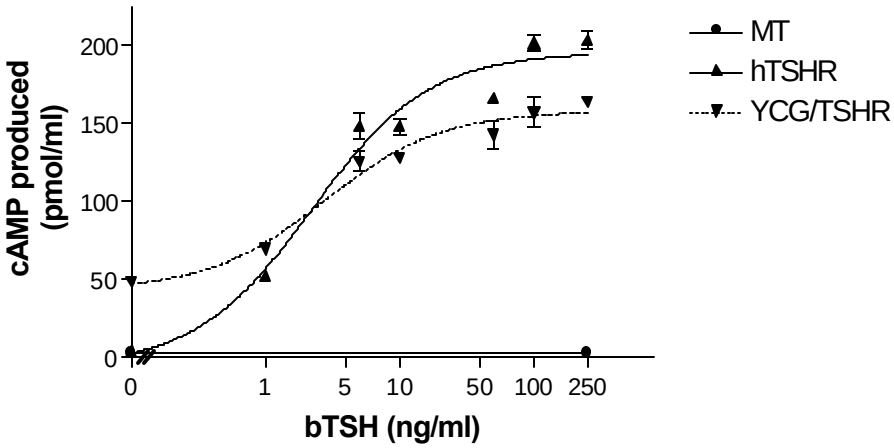
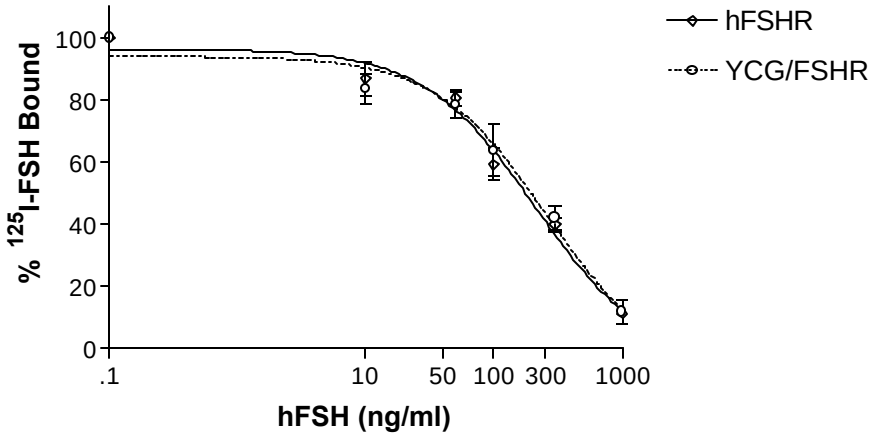
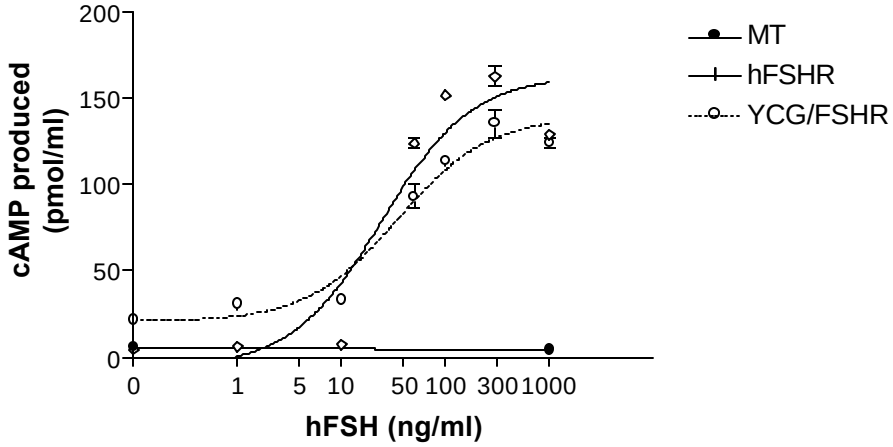

200
150
100
50
0
cAMP produced (pmol/ml)
0
1
5
10
50
100
250
bTSH (ng/ml)
MT
hTSHR
YCG/TSHR

Fig. 2.6

A.


hFSHR
YCG/FSHR
% 125I-FSH Bound
100
80
60
40
20
0
.1
10
50
100
300
1000
hFSH (ng/ml)

B.


MT
hFSHR
YCG/FSHR
cAMP produced (pmol/ml)
200
150
100
50
0
0
1
5
10
50
100
300
1000
hFSH (ng/ml)

CHAPTER 3

BIOLOGICALLY ACTIVE SINGLE-CHAIN HCGs WITH A MINIATURIZED β SUBUNIT*

*[1]Schubert, R.L. and Puett, D. Submitted to Molecular and Cellular Endocrinology.

Abstract

hCG binds the large ectodomain of human luteinizing hormone receptor (hLHR), leading to increased intracellular production of cAMP. Using protein engineering, two miniaturized hCGβ subunits have been separately fused to the N-terminus of the α-subunit to give N-*des*[1-91]hCGβ-α-C and N-*des*[1-91,110-114]hCGβ-α-C. Bioactivity was assessed in two systems following transient transfections into HEK 293 cells and subsequent cAMP measurements. In one, each mini-β-α cDNA was fused to that of hLHR, using the carboxyl-terminal peptide of hCGβ as a linker, and transfected into cells to create yoked miniaturized hCG-receptor complexes; in the other, the cDNA of each mini-β-α was co-transfected with that of hLHR to produce non-covalent miniaturized hCG-receptor complexes. Ligand and antibody binding assays confirmed expression, and each mini-hCG was found to lead to LHR activation approximately 1.5-2.0-fold over basal in the yoked and non-yoked complexes, respectively, indicating an intrinsic activity of the mini-β-α fusion protein.

Introduction

The four human (h) glycoprotein hormones are heterodimers that share a common α-subunit, comprised of 92 amino acid residues, and a distinct β-subunit that confers hormonal specificity (Hearn and Gomme, 2000). hCGβ contains 145 amino acid residues, including a 30 residue extension on its C-terminus, referred to as the C-terminal peptide (CTP). The four hormones act via three G protein-coupled receptors, with the β-subunits of hCG and LH being sufficiently similar that the two gonadotropins both bind to and activate the human LH receptor (hLHR) (Dufau, 1998, Ascoli et al., 2002).

hCG is the most comprehensively studied of the human glycoprotein hormones, and therefore there is an abundance of information available concerning the regions of the two subunits believed to be important for bioactivity. Using various techniques such as site-directed mutagenesis (Bielinski and Boime, 1992, Chen et al., 1992, Yoo et al., 1993, Liu et al., 1993, Puett et al., 1994), protein chimeras (Campbell et al., 1991, Dias et al., 1994), and synthetic peptides (Salesse et al., 1990, Keutmann, 1992), many of the regions of hCG important for heterodimer formation, receptor binding, and receptor activation have been determined. The combined findings from these studies concerning ligand-receptor interactions have indicated that the central portion of the α-subunit and the determinant loop of hCGβ (residues 93-100) are required for hLHR binding and activation, although whether the determinant loop directly contacts the receptor remains controversial. The crystal structure of aglyco-hCG (Lapthorn et al., 1994, Wu et al., 1994), followed by that of hFSH (Fox et al., 2001), demonstrated that these regions of α and β, delineated as putative receptor contact sites, are in relatively close proximity to one another.

We and others have shown that biologically active fusion proteins of hCG could be constructed in the manner, N-hCGβ-α-C (Narayan et al., 1995, Sugahara et al., 1995, Heikoop et al., 1997) and N-α-CTP-hCGβ-C (Narayan et al., 2000, Sen Gupta and Dighe, 2000). These studies were expanded to include yoked hCG-rat LHR complexes (YHR) of the form, N-hCGβ-α-CTP-LHR-C (Wu et al., 1996) and N-α-hCGβ-LHR-C (Narayan et al., 2000), resulting in ligand-mediated constitutive receptor activation. The goal of the current investigation is the design and characterization of miniature hCG

hormones, a continuation of earlier work focusing on N-terminal and C-terminal deletion mutants of hCGβ that retained the ability to form heterodimers and activate LHR (El-Deiry et al., 1989, Chen and Puett, 1991, Huang et al., 1993). We have created single-chain mini-gonadotropins in which a large N-terminal portion of hCGβ has been removed (residues 1-91) and the remainder of the subunit, containing the determinant loop/seat belt region and the CTP, was fused to the N-terminus of the full-length α-subunit to create YMG1. YMG2 incorporates a further deletion of hCGβ, residues 110-114, eliminating a portion of the C-terminal region of the seat belt that includes Cys-110, which pairs with Cys-26 in the native structure (Lapthorn et al., 1994, Wu et al., 1994). These deletions were chosen in order to retain in the seat belt with the important determinant loop region, and the CTP as a linker. Low affinity interaction of these hCG analogs with LHR was anticipated, and difficulty experienced in expressing sufficient quantities of the mini-gondotropins in mammalian cells for dose response curves thus, we have used the yoked hormone-receptor system, as well as co-transfection studies, to evaluate the activity of the two miniaturized yoked hCG hormones and found that each activates hLHR to a small degree.

Materials and Methods

Construction of yoked mini-gonadotropins and yoked mini-gonadotropin-receptor complexes

MG1 and MG2 were created using PCR to generate the hCGβ fragments in the following manner:

MG1: 5'-BamHI restriction site/hCGβ signal sequence/flag tag/hCGβ residues 92-145/EcoRI-3', and

MG2: 5'-BamHI restriction site/hCGβ signal sequence/flag tag/hCGβ residues 92-109, 114-145/EcoRI-3'.

In order to create the miniaturized hCG fusion proteins, the N-terminal deletion cDNAs of hCGβ were ligated in place of full length hCGβ in N-hCGβ-α-C, which has already been prepared and characterized by our laboratory (Schubert, Narayan and Puett, unpublished data), to give N-MG1-α-C (YMG1) and N-MG2-α-C (YMG2).

A BamHI/EcoRI double digestion was used to excise the miniaturized hCGβ subunits from YMG1 and YMG2. The full length hCGβ was then removed from the existing N-hCGβ-α-hLHR-C construct by BamHI/EcoRI digestion. The miniaturized hCGβ subunits were then ligated in place of full length hCGβ to produce Y(MG1/LHR) and Y(MG2/LHR).

Cell culture and transient transfections

HEK 293 cells were grown in monolayer culture in Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 10% (v/v) newborn calf serum, 10 mM HEPES buffer, 50 U/ml penicillin, 50 μg/ml streptomycin, 50 μg/ml Fungizone, and 0.125 μg/ml Amphotericin B. Cells were maintained at 37C in humidified air containing 5% CO_2. The cDNAs were transfected into 75 cm^2 tissue culture flasks containing HEK 293 cells using lipofectamine as the transfection agent as recommended by Gibco BRL (5

μg cDNA for each fusion protein; 5 μg of each cDNA per co-transfection for a total of 10 μg).

Hormone binding

Approximately 16-18 h after transfection, the cells were resuspended in 36 ml of growth media, and 1 ml of medium was added to each well of a 12-well tissue culture plate coated with 0.1% gelatin in phosphate buffered saline (PBS). Approximately 24 h later the cells were assayed for ^{125}I-hCG binding. For competitive binding, increasing concentrations of unlabeled hCG (kindly provided by Dr. Albert Parlow and the NIDDK) in Waymouth's media with 0.1% BSA (w/v) and 50 pM ^{125}I-hCG was added to each well and incubated at 37C for 6 h. Nonspecific binding was determined by addition of 1 μg/ml of unlabeled hormone. After the 6 h incubation period, the medium was aspirated and the cells were washed with PBS. Cells were lysed by adding 0.5 ml of 1 N NaOH to each well, followed by incubation at room temperature for approximately 5 min. The cell lysate was transferred to test tubes, and the wells were rinsed with 0.5 ml PBS. The samples were pooled appropriately, and counted in a gamma counter. All assays were performed in duplicate and mock transfected cells were used as a control.

Anti-LHR antibody binding assay

Approximately 16-18 h after transfection, the cells were processed as described above and the cells assayed for binding of an anti-rat LHR polyclonal antibody (raised against a synthetic peptide to residues 15-38 and kindly provided by Dr. Patrick Roche, Mayo Medical School, Rochester, MN) that was detected by secondary ^{125}I-anti-rabbit

antibody. The cells were incubated in a 1:4000 dilution of the primary antibody in Waymouth's media and 0.3% (w/v) BSA for 4 h with shaking. After washing twice with Waymouth's media and 0.3% (w/v) BSA, the cells were incubated with the ^{125}I-anti-rabbit secondary antibody (400,000 CPM/well) in Waymouth's media and 0.3% BSA for 1 h with shaking. After the second incubation period, the medium was aspirated and the cells were washed with PBS. The cells were removed from the plates as described above and the radioactivity counted. Each experiment was performed in duplicate and all steps were performed at room temperature.

cAMP assay

About 16-18 h after transfection, the cells were replated and washed as described above, then incubated in Waymouth's media with 0.1% (w/v) BSA and 0.8 mM isobutylmethylxanthine (IBMX) for 15 min at 37C. Following removal of the medium, the cells were incubated with increasing concentrations of hCG for 30 min at 37C in Waymouth's media containing 0.1% (w/v) BSA and 0.8 mM IBMX. Incubation medium was again removed and the cells were lysed in 100% ethanol at -20C overnight. The extract was collected, dried under vacuum, and resuspended in the sodium acetate buffer provided in the ^{125}I-cAMP assay kit. cAMP concentrations were determined by RIA as recommended (DuPont NEN). The results are presented in two units for basal cAMP: pmol/ml and pmol/fmol of bound ^{125}I-hCG to correct for receptor expression differences. (The correction for receptor expression is based on B_o, i.e. the specific binding of 50 pM ^{125}I-hCG. However, since the K_d values of the constructs are the same as that of LHR,

this serves as a relative correction for receptor densities.) Each experiment was performed in duplicate.

Data analysis

Competitive binding and cAMP data were analyzed by non-linear regression using the Prism software program (Graph Pad Software, San Diego, CA). The results are given as mean ± SEM, based on 3-7 independent transfections, and significance was determined from Student's t-test. The figures showing competitive binding and hCG-mediated cAMP production are based on overall averages from replicate transfections, and the IC_{50}s and ED_{50}s in Table 3.1 refer to the average of each individual parameter from each transfection. The IC_{50} and ED_{50} values are given in Table 3.1 in units of nM; by convention, the dose-response curves are presented in units of ng/ml. To convert the latter to nM, simply multiply by 0.027.

Results

Structures of the yoked mini-hCGs and yoked mini-hCG-hLHR complexes

Schematics of the two yoked mini-gonadotropins, YMG1 and YMG2, are shown in Fig. 3.1A and compared to YhCG. YMG1 lacks the N-terminal 91 amino acid residues of hCGβ, and thus both sites of N-glycosylation and the Cys-knot cluster, but retains the remainder of the β-subunit, including the determinant loop/seat belt and the CTP with its four sites of O-glycosylation, that is fused to α. YMG2 is similar but has an

additional five amino acid residue sequence deleted, 110-114, hence removing Cys-110 that pairs with Cys-26 in hCGβ. The two yoked mini-gonadotropin-hLHR structures are given schematically in Fig. 3.1B and compared to that of yoked hCG-hLHR, or Y(CG/LHR). The crystal structure of aglyco-hCG (Lapthorn et al., 1994, Wu et al., 1994) is shown in Fig. 3.2 with the region of hCGβ preserved in the mini-gonadotropins highlighted.

Anticipating that any discernable binding of the miniaturized hCGβs, fused to α, would be of low affinity, experimental conditions were chosen to maximize association with hLHR. One paradigm, based on protein engineering to produce fusion proteins, was developed by our laboratory to give constitutive ligand-mediated receptor activation and involved yoking a single-chain hCG to the full length receptor (Narayan et al., 2000, Wu et al., 1996) and to the receptor ectodomain (Fralish et al., 2001). The former approach was used in the present study with full length hLHR in order to permit an evaluation of receptor activation in transfected HEK 293 cells. Another paradigm involved co-transfection of the hLHR cDNA and each of the cDNAs of the yoked mini-gonadotropins to evaluate non-covalent association at the relatively high intracellular concentrations of each component. In each experimental paradigm, a negative control consisted of just hLHR expression (or mock transfection), while positive controls involved Y(CG/LHR) expression and co-expression of YhCG and hLHR.

Yoked mini-hCG-hLHR complexes: expression of a single-chain ligand-receptor complex

Addition of 50 pM ^{125}I-hCG to cells expressing Y(MG1/LHR) and Y(MG2/LHR) led to specific binding similar to that of hLHR (Fig. 3.3A). In contrast, cells expressing Y(CG/LHR) bound negligible amounts of ^{125}I-hCG as expected (Wu et al., 1996), attributable to the nearly constant high-affinity association of single-chain hCG fused to hLHR. No difference in binding of exogenous hCG was observed due to the low affinity of YMG1 and YMG2 to hLHR. Transfected cells were incubated with an anti-LHR antibody and receptor densities were then estimated by binding an ^{125}I-labeled secondary antibody. As shown in Fig. 3.3B, there is no significant difference in expression levels of hLHR, whether free or fused to YMG1, YMG2, or YhCG.

Competition binding curves using ^{125}I-hCG and hCG are presented in Fig. 3.4A for cells expressing Y(MG1/LHR), Y(MG2/LHR), or hLHR. The IC_{50}s obtained from the three competition curves are essentially identical (Table 3.1). cAMP dose-response curves were determined at various concentrations of hCG, and the ED_{50}s for Y(MG1/LHR), Y(MG2/LHR), and hLHR are also similar (Fig. 3.4B, Table 3.1). As expected from earlier studies (Wu et al., 1996), expression of Y(CG/LHR) results in a high level of basal cAMP that is not further increased at high concentrations of exogenous hormone, and untransfected HEK 293 cells do not express hLHR (Fig. 3.4B). Interestingly, the basal cAMP values of Y(MG1/LHR) and Y(MG2/LHR) are slightly elevated with reference to that of hLHR when corrected for expression levels, although only that for Y(MG2/LHR) is significantly different from hLHR if corrections are not made for receptor expression levels (Fig. 3.5A, Table 3.1).

Yoked mini-hCGs and hLHR: co-expression of ligand and receptor

Binding measurements with ^{125}I-hCG suggested comparable receptor densities in cells expressing hLHR only and hLHR with each of the mini-hCGs (Fig. 3.6). Competition binding and cAMP activation curves show that IC_{50}s and EC_{50}s are similar in the hLHR control and when co-expressed with the YMGs. Co-expression of YhCG and hLHR results in a high basal level of cAMP that is not increased upon addition of hCG, analogous to that found with Y(CG/LHR). These results are summarized in Fig. 3.7 and Table 3.1. Compared to hLHR and corrected for expression levels, the fold-increases in basal cAMP values are slightly greater in cells co-expressing hLHR and each of the mini-gonadotropins (Fig. 3.5B). In this paradigm, however, only YMG2 co-expressed with hLHR differs from hLHR whether the data are corrected for expression levels or not (Table 3.1).

Discussion

Recent studies from our laboratory have shown that when yoked to the N-terminus of rat LHR, neither the α-subunit, hCGβ, prolactin, nor CTP, results in increased basal cAMP over that of LHR (P. Narayan and D. Puett, unpublished results). Thus the small increase in basal cAMP found with YMG1 and YMG2, particularly when fused to hLHR, appears to be specific and, coupled with earlier studies showing important functional roles of α and hCGβ in receptor binding (Bielinski and Boime, 1992, Chen et al., 1992, Yoo et al., 1993, Liu et al., 1993, Puett et al., 1994), suggests that portions of both subunits are required for LHR activation.

Previous studies with heterodimeric hCG have shown that C-terminal fragments of hCGβ, including *des*[122-145]β (El-Deiry et al., 1989), *des*[115-145]β (Matzuk et al., 1990, Chen and Bahl, 1991), and *des*[111-145]β (Huang et al., 1993), associate well with α and the resulting heterodimers are equipotent to wild type hCG in *in vitro* measurements. *des*[101-145]β also bound to α, but the heterodimer exhibited reduced potency; *des*[93-145]β, on the other hand, failed to associate with α (Chen and Puett, 1991). Two N-terminal deletion mutants of hCGβ have been described, *des*[1-7]β (Huang et al., 1993) and *des*[2-8]β (Slaughter et al., 1995). Although association with α is impaired in the latter, both deletion fragments bound α, and the resulting heterodimers were equipotent to wild type hCG. We also reported that a combined N-terminal and C-terminal deletion fragment of hCGβ, *des*[1-7,111-145]β, bound to α, resulting in a heterodimer equipotent to wild type hCG, while *des*[1-7,101-145]β associated poorly with α, but some activity was present in the heterodimer (Huang et al., 1993). A single-chain hCG in the same configuration described here, i.e. N-β-α-C, but with a shortened CTP, *des*[124-145]β was shown to be active (Wu et al., 1996), as were fusion proteins of the form, *des*[103-145]β-$(Ser\text{-}Gly)_x$-α and *des*[112-145]β-$(Ser\text{-}Gly)_x$-α, where X=4, 5, or 6 (Heikoop et al., 1997).

These studies, based on heterodimeric and single-chain hCG derivatives, establish that the CTP does not contribute significantly to α association or receptor binding. It was, however, retained in our characterization of the mini-hCGs to serve as a spacer between the β seat belt and the α subunit; the CTP was also used as a spacer between the α-subunit and hLHR in the yoked mini-hCG/hLHR complexes. Prior to the present

study, the shortest fragment of hCGβ that yielded some activity *in vitro*, in combination with α, was *des*[1-7,101-145]β (Huang et al., 1993). The retention of the determinant loop was based on previous studies using synthetic peptides (Keutmann et al., 1989, Keutmann, 1992), site-directed mutagenesis (Chen and Bahl, 1991, Chen and Puett, 1991, Huang et al., 1993), and protein chimeras (Campbell et al., 1991, Dias et al., 1994) suggesting this region of hCGβ to be important in receptor binding/activation, either directly or indirectly. YMG1 contains the full seat belt region of the β-subunit, while YMG2 has a truncated portion of the seat belt region. The latter may be somewhat more potent than the former, perhaps reflecting a deleterious effect of the free sulfhydryl at Cys-110 in YMG1.

Using synthetic peptides corresponding to amino acid residues 93-101 of hCGβ, with an additional Tyr at the the C-terminus for iodination, Keutmann et al. (Keutmann et al., 1989) showed that the wild type sequence inhibited ^{125}I-hCG binding to rat LHR (using rat ovarian membranes) with an IC_{50} of 0.2 mM, compared to that of 0.1 nM for hCG. In contrast, a linear peptide obtained by substituting the two Cys residues with Ala failed to inhibit hCG binding to rat LHR at 0.5 mM, the highest concentration used. Thus, the potency of the linear peptide is much less than that of the loop peptide, presumably reflecting conformational constraints imposed by the Cys-93-Cys-100 disulfide. Although effective in inhibiting hCG binding to LHR, the β-93-100 peptide did not stimulate testosterone production in dispersed Leydig cells (Keutmann et al., 1989).

On the other hand, in a single-chain hCG of the form, N-hCGβ-α-C, Ben Menahem et al. (Ben-Menahem et al., 1997) showed that engineered mutations replacing each of Cys-93 and Cys-100 with Ala led to an analog that was only slightly less effective than the wild type single-chain gonadoptropin in LHR binding and activation, e.g. the IC_{50} and ED_{50} values increased by about 1.5-fold and 3-fold, respectively. In this case, however, the determinant loop may be constrained in a native-like conformation by the remainder of the fusion protein.

While we have retained the full determinant loop as part of the β seat belt, we of course have no assurance that the disulfide bond forms properly between Cys-93 and Cys-100, as in the native structure (Lapthorn et al., 1994, Wu et al., 1994). Other regions of hCGβ have been mapped to contain LHR contact sites (Puett et al., 1994, Salesse et al., 1990, Keutmann, 1992), and it is likely that potent engineered LHR agonists and antagonists will require two or more segments of the β-subunit, in conjuction with the α-subunit or derived fragments.

Much less has been done with deletions of the 92-amino acid residue α-subunit. The C-terminal Ser can be removed without greatly impairing association with hCGβ and subsequent LHR binding and activation; however, progressive deletion of residues 91-92, 90-92, and 89-92 results in reduced signaling, while not eliminating receptor binding (Yoo et al., 1993). The N-terminal fragment, *des*[88-92]α, associates with hCGβ but has reduced LHR binding and minimal signaling (Chen et al., 1992, Yoo et al., 1993). In contrast to these observations, the fusion protein, N-hCGβ-*des*[88-92]α-C, was found to bind to LHR with the same affinity as the full-length control, but signaling was diminished (Sen Gupta and Dighe, 2000).

The finding of activity, albeit low, in the fusion proteins, N-*des*[1-91]hCGβ-α-C and N-*des*[1-91,110-114]hCGβ-α-C, offers promise for the design of linked α and β fragments that would serve as hLHR agonists, particularly since modest increases in cAMP can, in many target cells, result in a robust reponse of steroidogenesis. From this, the design of antagonists may be feasible. In any case, the results reported herein contribute to our understanding of hCG structure-function relationships and demonstrate the power of using fusion proteins to probe weak biological interactions.

Acknowledgments

This work was supported by NIH (DK33973). We wish to thank John D. Calhoun for his assistance with the preparation of the cDNAs for the miniaturized hCGβ subunits used to create the yoked mini-hCGs and Mike Ford for his help in preparing Fig. 3.2. We gratefully acknowledge all members of the laboratory, particularly Dr. Krassimira Angelova and Dr. Lisa Kelly, for their helpful suggestions and support.

TABLE 3.1 Summary of binding and signaling parameters of YMGs and hLHR

	IC_{50} (nM) (n)	*ED_{50} (nM) (n)*	*Basal cAMP*[a] *(pmol/fmol) (n)*	*Basal cAMP*[a] *(pmol/ml) (n)*	*Maximal cAMP (pmol/ml) (n)*
Fusion proteins of YMGs and hLHR:					
Y(MG1/LHR)	0.46 ± 0.11 (7)	0.36 ± 0.09 (7)	7.54 ± 0.93[b] (7)	4.81 ± 0.80 (7)	198 ± 24 (7)
Y(MG2/LHR)	1.02 ± 0.55 (7)	0.33 ± 0.11 (6)	7.41 ± 0.87[b] (7)	5.03 ± 0.55[b] (7)	229 ± 32 (6)
hLHR	0.56 ± 0.15 (5)	0.26 ± 0.04 (7)	4.9 ± 0.71 (7)	3.20 ± 0.42 (7)	201 ± 28 (7)
Y(CG/LHR)	_c	_c	_c	107 ± 9.17[b] (7)	110 ± 12.5[b] (6)
Co-expression of YMGs and hLHR:					
YMG1 & hLHR	0.88 ± 0.48 (3)	0.19 ± 0.006 (3)	11.57 ± 3.57 (3)	5.8 ± 1.27 (3)	338 ± 91 (3)
YMG2 & hLHR	2.24 ± 1.50 (4)	0.23 ± 0.06 (4)	10.80 ± 1.53[d] (4)	5.95 ± 0.68[d] (4)	307 ± 40 (4)
pcDNA3 & hLHR	0.75 ± 0.21 (4)	0.21 ± 0.03 (4)	7.17 ± 0.67 (4)	3.98 ± 0.44 (4)	265 ± 70 (4)
YhCG & hLHR	_c	_c	_c	114 ± 21[b] (4)	173 ± 63 (4)

HEK 293 cells were transiently transfected with cDNAs to YMG1 and LHR, YMG2 and LHR, Y(MG1/LHR), and Y(MG2/LHR), with LHR serving as negative control and Y(CG/LHR) and (YhCG & LHR) serving as positive controls. Dose-response curves were conducted with hCG, and competitive binding also included 50 pM ^{125}I-hCG. The maximal cAMP levels refer to maximum production at 250 ng/ml hCG with no correction for basal cAMP levels.

[a]Basal cAMP values are presented in two units: pmol/fmol of bound ^{125}I-hCG, to correct for minor differences in the expression levels, and in pmol/ml. Values showing the fold-activation over the LHR control are given in Fig. 3.5.

[b]Significantly different from LHR ($p<0.05$).

[c]Expression of yoked hCG-hLHR and co-expression of yoked hCG and hLHR results in minimal binding of ^{125}I-hCG and in elevated levels of basal cAMP, compared to LHR, that are not further increased upon addition of exogenous hCG.

[d]$p=0.0506$ when compared to LHR.

References

Ascoli, M., Fanelli, F. and Segaloff, D. L. (2002) *Endocr Rev,* **23,** 141-174.

Ben-Menahem, D., Pixley, M., Sato, A., Suganuma, N., Perlas, E. and Hsueh, A. J. W. (1997) *J Biol Chem,* **272**.

Bielinski, M. and Boime, I. (1992) *Mol Endocrinol,* **6,** 267-271.

Campbell, R. K., Dean-Emig, D. M. and Moyle, W. R. (1991) *Proc Natl Acad Sci U S A,* **88,** 760-764.

Chen, F. and Puett, D. (1991) *J Biol Chem,* **266,** 6904-6908.

Chen, F., Wang, H. and Puett, D. (1992) *Mol Endocrinol,* **6,** 914-919.

Chen, W. and Bahl, O. P. (1991) *J Biol Chem,* **266,** 6246-6251.

Dias, J. A., Zhang, Y. and Liu, X. (1994) *J Biol Chem,* **269,** 25289-25294.

Dufau, M. L. (1998) *Annu Rev Biochem,* **60,** 461-496.

El-Deiry, S., Kaetzel, D., Kennedy, G., Nilson, J. and Puett, D. (1989) *Mol Endocrinol,* **3,** 1523-1528.

Fox, K. M., Dias, J. A. and Van Roey, P. (2001) *Mol Endocrinol,* **15,** 378-389.

Fralish, G. B., Narayan, P. and Puett, D. (2001) *Endocrinology,* **142,** 1517-1524.

Hearn, M. T. W. and Gomme, P. T. (2000) *J Mol Recognition,* **13,** 223-278.

Heikoop, J. C., Van Beuningen-De, V. M. M., Van Den Boogaart, P. and Grootenhusi, P. D. J. (1997) *Eur J Biochem,* **245,** 656-662.

Huang, J., Chen, F. and Puett, D. (1993) *J Biol Chem,* **268,** 9311-9315.

Keutmann, H. T. (1992) *Mol Cell Endocrinol,* **86,** C1-C6.

Keutmann, H. T., Mason, K. A., Kitzmann, K. and Ryan, R. J. (1989) *Mol Endocrinol,* **3,** 526-531.

Lapthorn, A. J., Harris, D. C., Littlejohn, A., Lustbader, J. W., Canfield, R. E., Machin, K. J., Morgan, F. J. and Isaacs, N. W. (1994) *Nature,* **369,** 455-461.

Liu, C., Roth, K. E., Shepard, B. A., Shaffer, J. B. and Dias, J. A. (1993) *J Biol Chem,* **268,** 21613-21617.

Matzuk, M. M., Hsueh, A. J. W., Lapolt, P., Tsafriri, A., Keene, J. L. and Boime, I. (1990) *Endocrinology,* **126,** 376-383.

Narayan, P., Gray, J. and Puett, D. (2000) *Endocrinology,* **141,** 67-71.

Narayan, P., Wu, C. and Puett, D. (1995) *Mol Endocrinol,* **9,** 1720-1726.

Puett, D., Huang, J. and Xia, H. (1994) In *Glycoprotein Hormones*(Eds, Lustbader, J. W., Puett, D. and Ruddon, R. W.) Springer-Verlag, New York, pp. 122-134.

Salesse, R., Bidart, J.-M., Troalen, F. and Garnier, J. (1990) *Mol Cell Endocrinol,* **68,** 113-119.

Sen Gupta, C. and Dighe, R. R. (2000) *J Mol Endocrinol,* **24,** 157-164.

Slaughter, S., Wang, Y., Myers, R. V. and Moyle, W. R. (1995) *Mol Cell Endocrinol,* **112,** 21-25.

Sugahara, T., Pixley, M. R., Minami, S., Perlas, E., Ben-Menahem, D., Hsueh, A. J. and Boime, I. (1995) *Proc Natl Acad Sci U S A,* **92,** 2041-2045.

Wu, C., Narayan, P. and Puett, D. (1996) *J Biol Chem,* **271,** 31638-31642.

Wu, H., Lustbader, J. W., Liu, Y., Canfield, R. E. and Hendrickson, W. A. (1994) *Structure,* **2,** 545-558.

Yoo, J., Zeng, H., Ji, I., Murdoch, W. J. and Ji, T. H. (1993) *J Biol Chem,* **268,** 13034-13042.

Figure Legends

Fig. 3.1. Schematics of yoked mini-gonadotropins and yoked hCG-hLHR complexes. A. YMG1 and YMG2 compared to YhCG with the CTP represented by vertical stripes. YMG1 contains amino acid residues 92-145 of hCGβ, i.e. residues 92-114 plus the CTP (residues 115-145, indicated by vertical stripes), fused to the full length α-subunit (92 amino acid residues). YMG2 contains a further deletion of hCGβ residues 110-114, which removes Cys-110 that is present in YMG1. B. YMG1/LHR and YMG2/LHR compared to YCG/LHR. The CTP is again denoted by vertical stripes. The two sites of N-glycosylation on each of the intact subunits and the four sites of O-glycosylation on the CTP are not shown.

Fig. 3.2. Crystal structure of deglycosylated hCG. The α-subunit from residues 5-89 is shown in orange and the β-subunit from residues 2-111 is shown in blue. The other amino acid residues did not appear in the electron density maps (Lapthorn et al., 1994, Wu et al., 1994), presumably due to their flexible nature. The region of the miniaturized β-subunit from residues 92-111 is highlighted in dark blue.

Fig. 3.3. Cell surface expression of hLHR and the yoked ligand-receptor complexes, Y(CG/LHR), Y(MG1/LHR), and Y(MG2/LHR). A. Specific binding following addition of 50 pM ^{125}I-hCG to HEK 293 cells expressing hLHR and each of the three yoked ligand-receptor complexes. The levels of exogenous ligand binding to hLHR, Y(MG1/LHR), and Y(MG2/LHR) are similar, while Y(CG/LHR), as expected (Wu et al.,

1996), binds negligible amounts of ^{125}I-hCG. B. Expression of hLHR, free and in yoked complexes, as monitored by a rabbit anti-rat LHR antibody, followed by binding of ^{125}I-anti rabbit secondary antibody. The data are corrected for binding to mock-transfected cells, and there is no significant difference between the receptor levels.

Fig. 3.4. Competitive binding and cAMP dose-response curves with HEK 293 cells expressing hLHR and fusion proteins. A. Competitive binding with 50 pM ^{125}I-hCG and various concentrations of unlabeled hCG for hLHR and the two yoked mini-gonadotropin-receptor complexes. B. cAMP production of mock-transfected (MT) cells and cells expressing hLHR and each of the three yoked ligand-receptor complexes. The IC_{50}s and EC_{50}s are given in Table 3.1.

Fig. 3.5. Basal cAMP production of HEK 293 cells expressing Y(MG1/LHR) and Y(MG2/LHR) and co-expressing YMG1 & hLHR and YMG2 & hLHR. A. Results from cells expressing Y(MG1/LHR) and Y(MG2/LHR). B. Results from cells expressing YMG1 & hLHR and YMG2 & hLHR. The results are an average of each individual experiment, shown as fold-increase over that of hLHR production. Averages were made from values expressed in pmol of cAMP produced/fmol of bound ^{125}I-hCG, which corrects for protein expression levels. Values are presented in Table 3.1, where * denotes a significant difference from LHR ($p<0.05$) and + denotes $p= 0.0506$.

Fig. 3.6. Cell surface expression of hLHR when co-expressed with yoked ligand receptor complexes. Specific binding after addition of 50 pM ^{125}I-hCG to HEK 293 cells expressing hLHR or co-expressing hLHR and YMG1, YMG2, or YhCG.

Fig. 3.7. Competitive binding and cAMP dose-response curves with HEK 293 expressing hLHR only or co-expressing hLHR with the yoked ligand-receptor complexes. A. Competitive binding with 50 pM ^{125}I-hCG and various concentrations of unlabeled hCG for hLHR alone and hLHR co-expressed with YMG1 or YMG2. B. cAMP production in mock-transfected (MT) cells, in cells expressing hLHR only (hLHR and pcDNA3), and in cells co-expressing hLHR with YCG, YMG1, or YMG2. The IC_{50}s and EC_{50}s are listed in Table 3.1.

Fig. 3.1

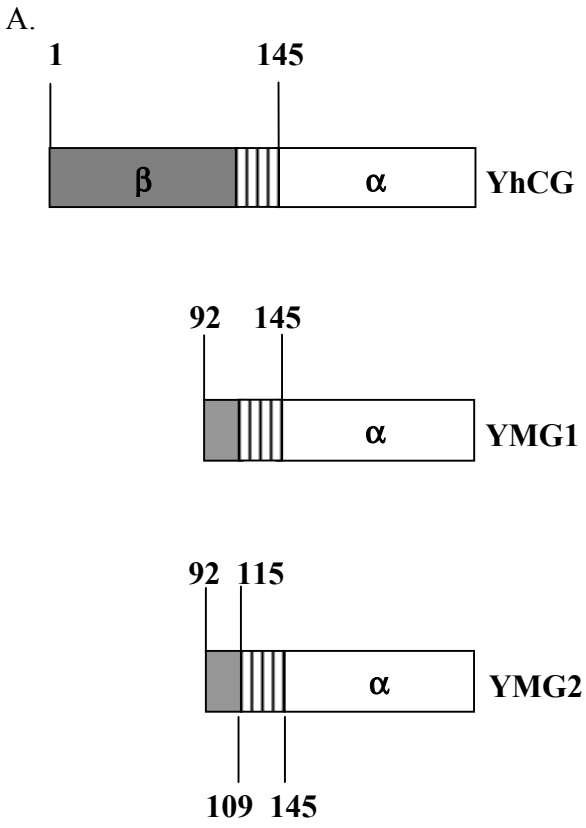

A.
1
145
β
α
YhCG
92
145
α
YMG1
92
115
α
YMG2
109
145
B.
YCG/LHR
β
α
hLHR
YMG1/LHR
α
hLHR
YMG2/LHR
α
hLHR

Fig. 3.2

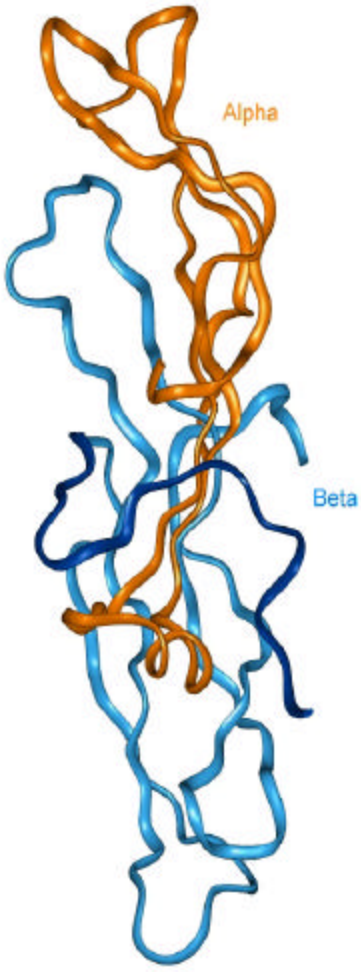
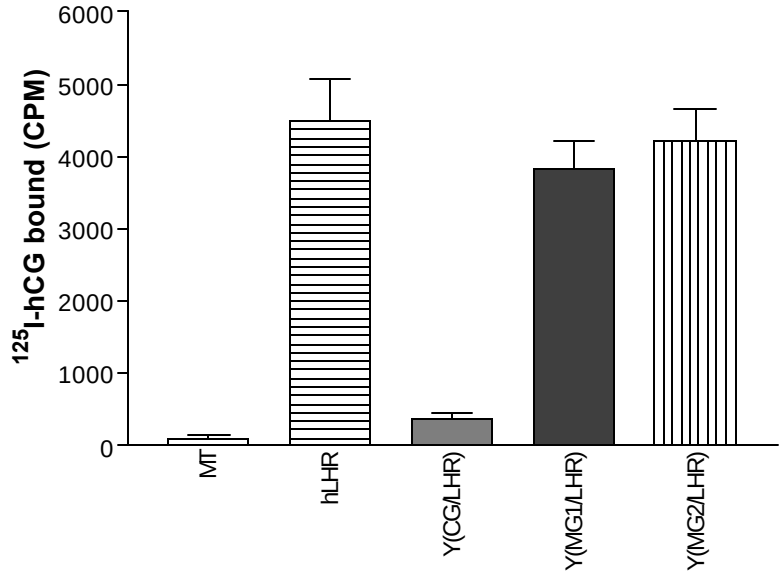


Fig. 3.3

A.


^{125}I-hCG bound (CPM)
6000
5000
4000
3000
2000
1000
0
MT
hLHR
Y(CG/LHR)
Y(MG1/LHR)
Y(MG2/LHR)

B.

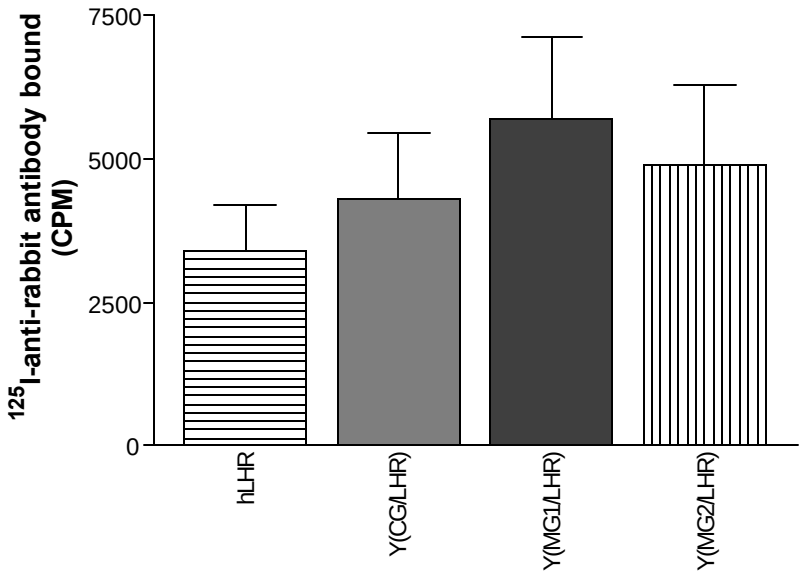
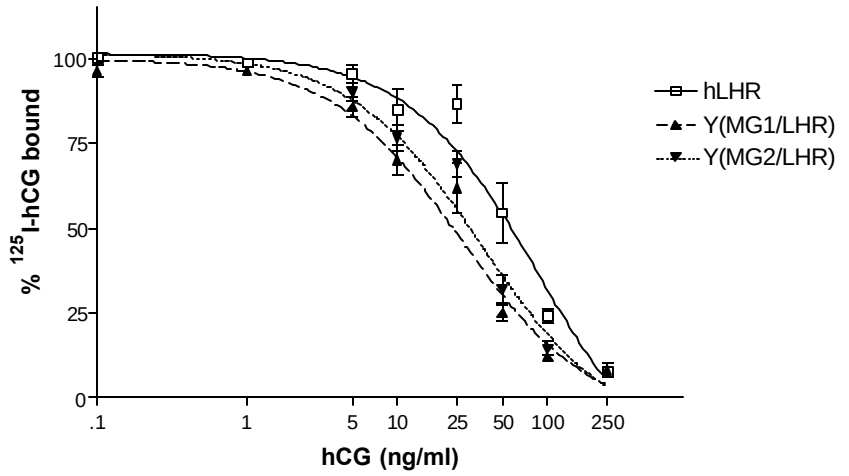

^{125}I-anti-rabbit antibody bound (CPM)
7500
5000
2500
0
hLHR
Y(CG/LHR)
Y(MG1/LHR)
Y(MG2/LHR)

Fig. 3.4

A.


100
75
50
25
0
% 125I-hCG bound
.1
1
5
10
25
50
100
250
hCG (ng/ml)
hLHR
Y(MG1/LHR)
Y(MG2/LHR)

B.

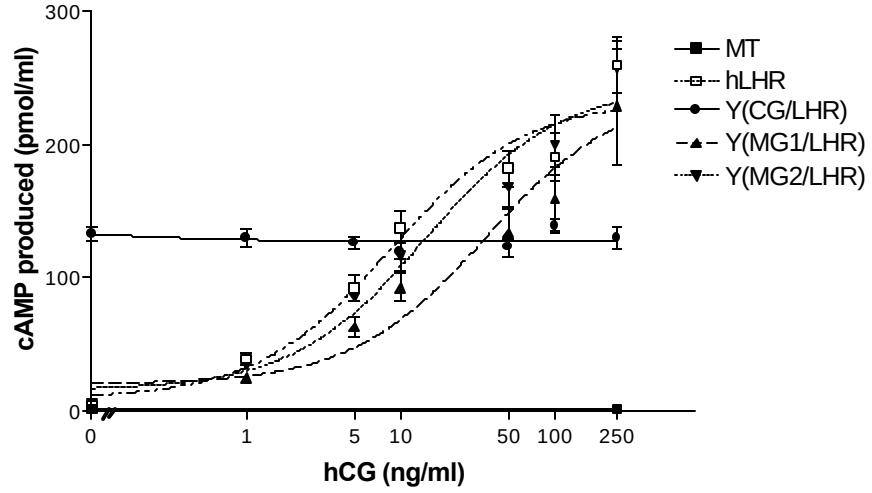
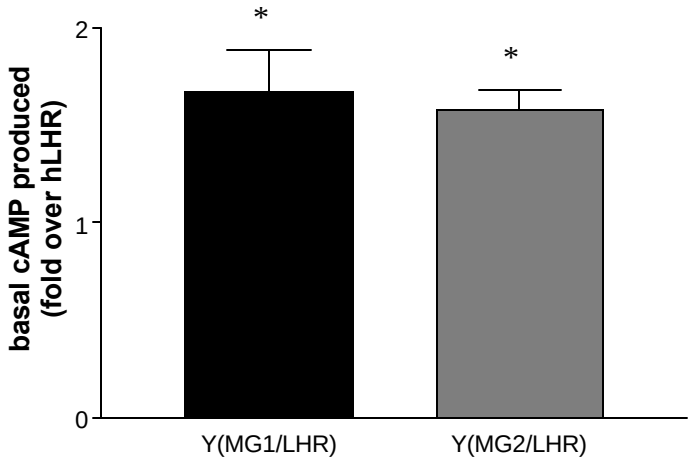

300
200
100
0
cAMP produced (pmol/ml)
0
1
5
10
50
100
250
hCG (ng/ml)
MT
hLHR
Y(CG/LHR)
Y(MG1/LHR)
Y(MG2/LHR)

Fig. 3.5

A.


basal cAMP produced
(fold over hLHR)
2
1
0
*
*
Y(MG1/LHR)
Y(MG2/LHR)

B.

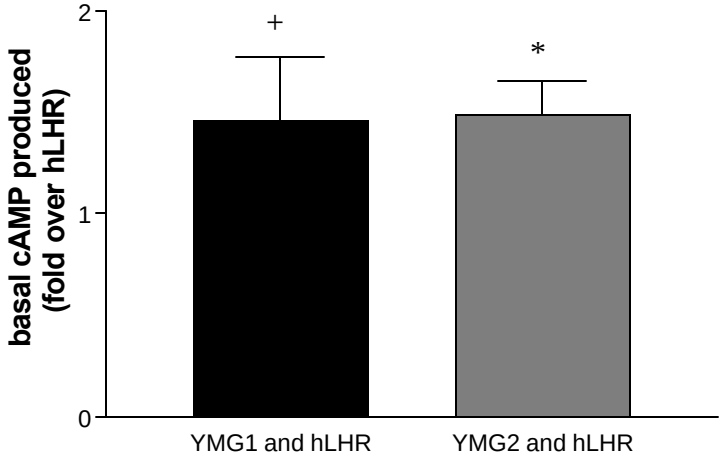

basal cAMP produced
(fold over hLHR)
2
1
0
+
*
YMG1 and hLHR
YMG2 and hLHR

Fig. 3.6

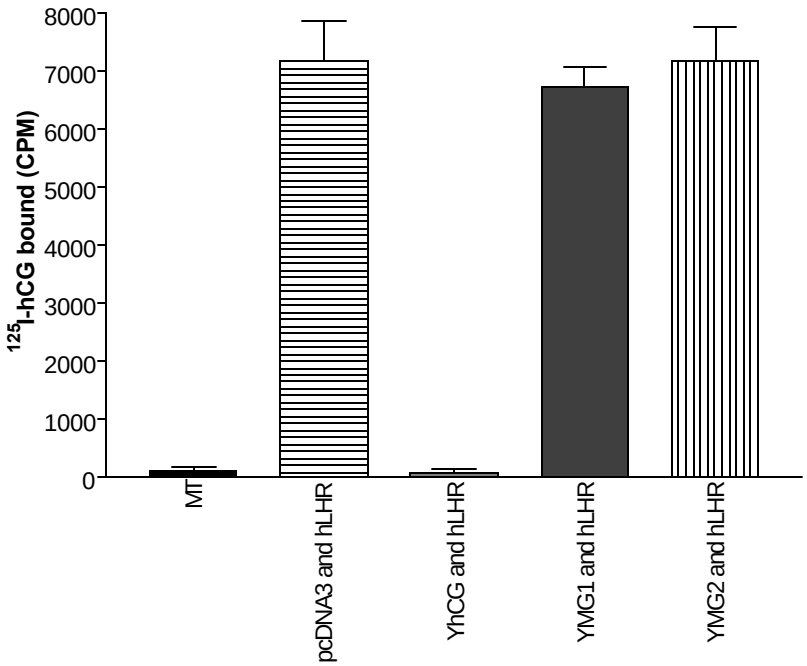
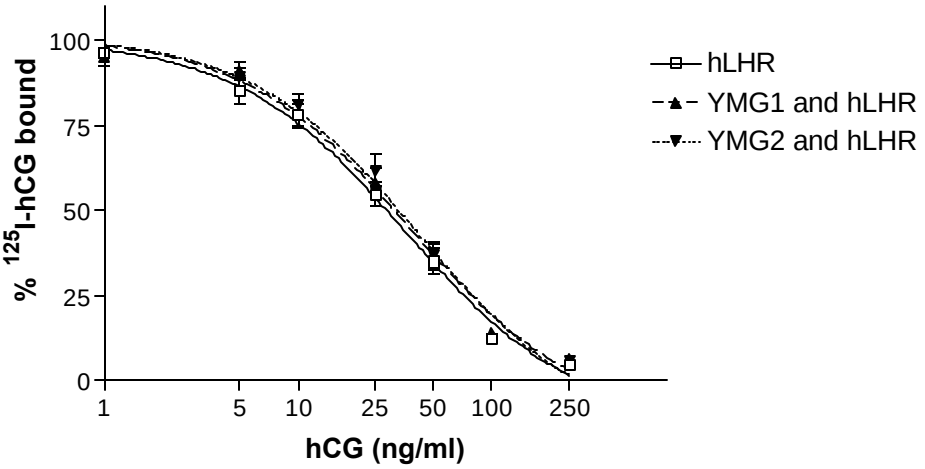
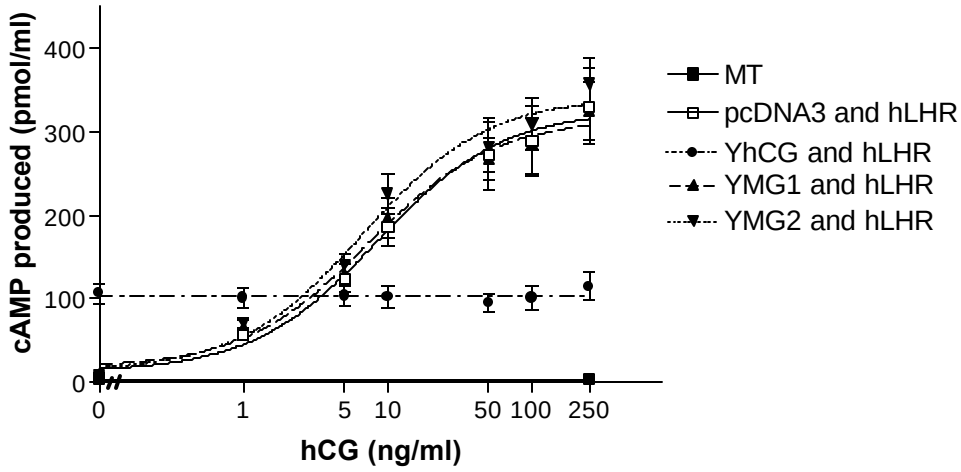

^{125}I-hCG bound (CPM)
8000
7000
6000
5000
4000
3000
2000
1000
0
MT
pcDNA3 and hLHR
YhCG and hLHR
YMG1 and hLHR
YMG2 and hLHR

Fig. 3.7

A.


% 125I-hCG bound
100
75
50
25
0
1
5
10
25
50
100
250
hCG (ng/ml)
hLHR
YMG1 and hLHR
YMG2 and hLHR

B.


cAMP produced (pmol/ml)
400
300
200
100
0
0
1
5
10
50
100
250
hCG (ng/ml)
MT
pcDNA3 and hLHR
YhCG and hLHR
YMG1 and hLHR
YMG2 and hLHR

CHAPTER 4

CONCLUSIONS

The glycoprotein hormones and their three G-protein coupled receptors are vital for normal growth, development, metabolism and reproduction. Knowledge elucidating the ability of hormones to bind and activate these receptors will provide information that can aid fertility control and treatment, as well as enhance the treatment of thyroid disorders.

The YCG/R studies discussed in Chapter 2 were designed to investigate the ability of hCG to activate its non-cognate receptors, TSHR and FSHR. The purpose of this study was not only to evaluate cross-reactivity between the hCG and different members of the glycoprotein hormone receptor family, at least one of which is known to occur in certain pathophysiological states, but also to use the findings from these studies to learn more about the regions in the hormones and receptors that are important and specific for hormone binding and receptor activation. The YCG/R system has helped illuminate the similarities and differences in binding and activation abilities among the hormones and receptors, which possess a high degree of sequence homology. Fusing the same hormone to different members of the glycoprotein hormone receptor family demonstrated the ability of hCG to bind these other receptors and revealed information about the relevant sequence diversity among the receptors. Constructing and evaluating YTSH/R and YFSH/R proteins would provide a comprehensive evaluation of cross-reactivity within this family and give a complete comparison of the differences in amino

acid sequences, revealing the various regions of each receptor critical for hormone binding and subsequent receptor activation. By comparing the differences in receptor primary structures and the ability of each hormone to bind these receptors, one can make conclusions about the various regions of the receptors that are necessary for binding of each hormone and subsequent receptor activation. The sequences of the N-terminal regions of the receptor ECDs are quite different, suggesting that this region may be important for conferring specificity to the β subunits. However, many conserved sequences are found in the LRR regions of the receptor ECDs, suggesting possible contact sites for the α subunit or conserved regions within the β subunits.

This information complements the studies carried out in Chapter 3, where yoked hormone-receptor studies were coupled with co-transfection studies in order to evaluate the effectiveness of the two mini-hCG hormones, YMG1 and YMG2. These miniature gonadotropins represent the smallest truncations of hCG reported that retain hormonal activity. In addition, these mini-hCGs possess a low affinitiy for hLHR and are capable of activating the receptor in absence of full-length hCGβ.

The results obtained from the YCG/R studies can aid in future mini-hormone studies. By using the knowledge forthcoming from the yoked hormone receptor studies, coupled with new information that is constantly becoming available in the literature and the eventual YTSH/R and YFSH/R studies, one could make better decisions about the design of possible agonists for the glycoprotein hormone receptors and, ideally, expedite the rational design of antagonists. Low molecular weight LHR agonists could be used for fertility treatment, while antagonists could be used as contraceptives and abortifacients. TSHR agonists could be used to treat hypothyroidism, and antagonists could be used to

treat hyperthyroid conditions, of which there are several. There are currently no low molecular weight agonists or antagonists for any of these receptors. The advantage to the creation of such molecules is that, when used as therapeutic agents, the delivery of these miniaturized hormones might be facilitated due to their reduced size. Future work in this area could include the truncation of the α-subunit in the YMG1 and YMG2 constructs, as well as the generation of other miniaturized glycoprotein hormones.

In summary, the findings from this study concerning cross-reactivity between the glycoprotein hormones and their G-protein coupled receptors provides valuable insight into specific regions of the hormones and receptors that are important for hormone binding to and activation of cognate and non-cognate receptors, which in turn can help advance the construction and characterization of viable receptor agonists and antagonists. Moreover, the retention of some bioactivity in a small hCGβ fragment fused to the α-subunit offers encouraging results that receptor agonists/antagonists may be constructed from miniaturized heterodimeric glycoprotein hormones.