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Biological Functions of N- and O-linked Oligosaccharides of Equine Chorionic Gonadotropin and Lutropin/Chorionic Gonadotropin Receptor

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ABSTRACT

Members of the glycoprotein family, which includes CG, LH, FSH and TSH, comprise two noncovalently linked α- and β-subunits. Equine chorionic gonadotropin (eCG), known as PMSG, has a number of interesting and unique characteristics since it appears to be a single molecule that possesses both LH- and FSH-like activities in other species than the horse. This dual activity of eCG in heterologous species is of fundamental interest to the study of the structure-function relationships of gonadotropins and their receptors. CG and LH β genes are different in primates. In horse, however, a single gene encodes both eCG and eLH β-subunits. The subunit mRNA levels seem to be independently regulated and their imbalance may account for differences in the quantities of α- and β-subunits in the placenta and pituitary. The dual activities of eCG could be separated by removal of the N-linked oligosaccharide on the α-subunit Asn 56 or CTP-associated O-linked oligosaccharides. The tethered-eCG was efficiently secreted and showed similar LH-like activity to the dimeric eCG. Interestingly, the FSH-like activity of the tethered-eCG was increased markedly in comparison with the native and wild type eCG. These results also suggest that this molecular can implay particular models of FSH-like activity not LH-like activity in the eCG/indicate that the constructs of tethered molecule will be useful in the study of mutants that affect subunit association and/or secretion. A single-chain analog can also be constructed to include additional hormone-specific bioactive generating potentially efficacious compounds that have only FSH-like activity.

The LH/CG receptor (LH/CGR), a membrane glycoprotein that is present on testicular Leydig cells and ovarian theca, granulosa, luteal, and interstitial cells, plays a pivotal role in the regulation of gonadal development and function in males as well as in nonpregnant and pregnant females. The LH/CGR is a member of the family of G protein-coupled receptors and its structure is predicted to consist of a large extracellular domain connected to a bundle of seven membrane-spanning a-helices. The LH/CGR phosphorylation can be induced with a phorbol ester, but not with a calcium ionophore. The truncated form of LHR also was down-regulated normally in response to hCG stimulation. In contrast, the cell lines expressing LHR-t631 or LHR-628, the two phosphorylation-negative receptor mutant, showed a delay in the early phase of hCG-induced desensitization, a complete loss of PMA-induced desensitization, and an increase in the rate of hCG-induced receptor down-regulation. These results clearly show that residues 632~653 in the C-terminal tail of the LHR are involved in PMA-induced desensitization, hCG-induced desensitization, and hCG-induced down-regulation. Recently, constitutively activating mutations of the receptor have been identified that are associated with familial male-precocious

puberty. Cells expressing LHR-D556Y bind hCG with normal affinity, exhibit a 25-fold increase in basal cAMP and respond to hCG with a normal increase in cAMP accumulation. This mutation enhances the internalization of the free and agonist-occupied receptors ~2- and ~17-fold, respectively. We conclude that the state of activation of the LHR can modulate its basal and/or agonist-stimulated internalization. Since the internalization of hCG is involved in the termination of hCG actions, we suggest that the lack of responsiveness detected in cells expressing LHR-L435R is due to the fast rate of internalization of the bound hCG. This statement is supported by the finding that hCG responsiveness is restored when the cells are lysed and signal transduction is measured in a subcellular fraction (membranes) that cannot internalize the bound hormone.

I. INTRODUCTION

Equine chorionic gonadotropin (eCG), known as pregnancy mare serum gonadotropin (PMSG), has a number of interesting and unique characteristics since it appears to be a single molecule that possesses both luteinizing hormone (LH)- and follicle-stimulating hormone (FSH)-like activities in other species than the horse (Min et al., 1994, 1996a, 1997). This dual activity of eCG in heterologous species is of fundamental interest to the study of the structure-function relationships of gonadotropins and their receptors. A second unusual property of eCG is its remarkable carbohydrate content (over 40% w/w) (Min et al., 1996a) which is the highest in the glycoprotein hormones, and extents its persistence *in vivo*. eCG is of commercial value because it is readily collected and a potent agent for induction of folliculogenesis in domestic and laboratory mammals. Members of the glycoprotein family, which includes CG, LH, FSH and thyroid stimulating hormone (TSH), comprise two noncovalently linked α- and β-subunits. The α-subunits are common to these glycoproteins and the β-subunits differ among them. CG and LH β genes are different in primates (Murphy & Martinuk, 1991). In horse, however, a single gene encodes both eCG and eLH β subunits (Min et al., 1994). The difference between eCG and eLH lies in the structure of their glycoresidues, which are both sialylated and sulfated in LH and sialylated in CG. We previously studied the regulation of rat and equine placental function at different stages of pregnancy and identified pregnancy-stage specific placental functions which include secretion of growth modulators called placental lactogens (Hirosawa et al., 1994; Shiota et al., 1997), equine choriogonadotropin (eCG), equine relaxin (Min et al., 1994, 1996a, 1996b), rat CG (rCG) (Shinozaki et al., 1997), and leukemia inhibitory factor receptor (Aikawa et al., 1997).

The lutropin/choriogonadotropin receptor (LH/CGR), a membrane glycoprotein that is present on testicular Leydig cells and ovarian theca, granulosa, luteal, and interstitial cells, plays a pivotal role in the regulation of gonadal development and function in males as well as in nonpregnant and pregnant females (Wang et al., 1993). The LH/CGR is a member of the family of G protein-coupled receptors (GPCRs) and its structure is predicted to consist of a large extracellular domain connected to a bundle of seven membrane-spanning a-helices. Recently, constitutively activating mutations of the receptor have been identified that are associated with familial male-precocious puberty (FMPP) (Shenker et al., 1993; Yano et al., 1996; Min et al., 1998). A FMPP is a form of isosexual precocious puberty in boys in which testosterone levels are elevated independent of changes in luteinizing

hormone-releasing hormone and serum luteinizing hormone levels (Kraaij et al., 1995). Although many activating GPCR mutations have now been described, the molecular basis of the activating effects has only been explored in a few cases.

The present work is an attempt to summarize recent literature. Throughout this review the data obtained from our laboratory have been used to illustrate some findings that we believe are critical in the understanding of the structure and function of the CG. To address these further functions, we used site-directed mutagenesis. The mutant receptor genes were expressed in human embryonic kidney 293 cells, and hCG binding, cAMP response were measured in wild type and activating mutant receptors transfected cells.

Ⅱ. BIOLOGICAL FUNCTIONS OF EQUINE CHORIONIC GONADOTROPIN

1. Primary Structure of Subunits

Within a given species, the α-subunit is identical, except for differences in the oligosaccharides, whereas the β-subunit is hormone-specific and contains the determinant domains for receptor specificity. Both subunits are required for these glycoprotein hormones and combination of the subunits is essential for the expression of their biological activities. A molecular weight of eCG has been estiamted to be about 45～65 kDa, 17 kDa for the α-subunit and 44 kDa for the β-subunit. The amino-acid sequences of both eCG subunits have been determined. They are exactly identical to those of eLH subunits, containing an α-subunit of 96 amino acids and β-subunit of 149 amino acids.

Using the cDNA prepared from mRNA extracted from equine placental and pituitary tissues, we cloned the eCG α- and β-subunits cDNA and eFSH β-subunit cDNA. The cDNA fragment with

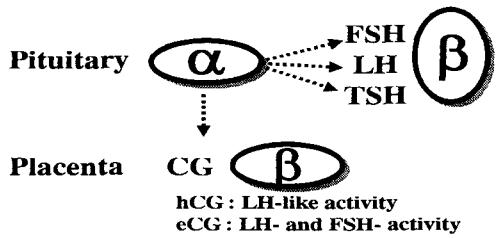

Fig. 1. Combination of subunits in the glycoprotein hormones.

387 bp predicted for the eCG α-subunit was amplified. A cDNA band of 524 bp for the eCG β-subunit was also amplified using a set of eCG β primers (Min et al., 1994). eCG α-subunit is very similar to that of the other species (74～84% at the nucleotide and 69～80% at the amino acid level). The β-subunits of the glycoprotein hormones also appear to be highly conserved with the porcine, bovine, and human LH β-subunits, with an overall homology of 80%. The nucleotide sequence of the marmoset exhibits lower homology with the eCG β-subunit (73.7%). The analysis between the first 110 amino acids of eCG β and hCG β demonstrates a 66% homology. But, homology between the primary structures of the C-terminal tails was much lower. We also cloned the cDNA of eFSH β-subunit from equine pituitary using the mixed primers designed from the nucleotide sequence of human, rat, and bovine FSH β-subunits. Sequence data analysis showed that cDNA encoded the eFSH β-subunit including the signal peptide region consists of 18 amino acids and mature protein of 111 amino acids (Saneyoshi et al., manuscript in preparation).

2. Glycosylation

There have two N-glycosylation sites on the α-subunits of all these glycoproteins, whereas the numbers of such sites on their β-subunits differ depending on the hormone: LH β- and FSH β

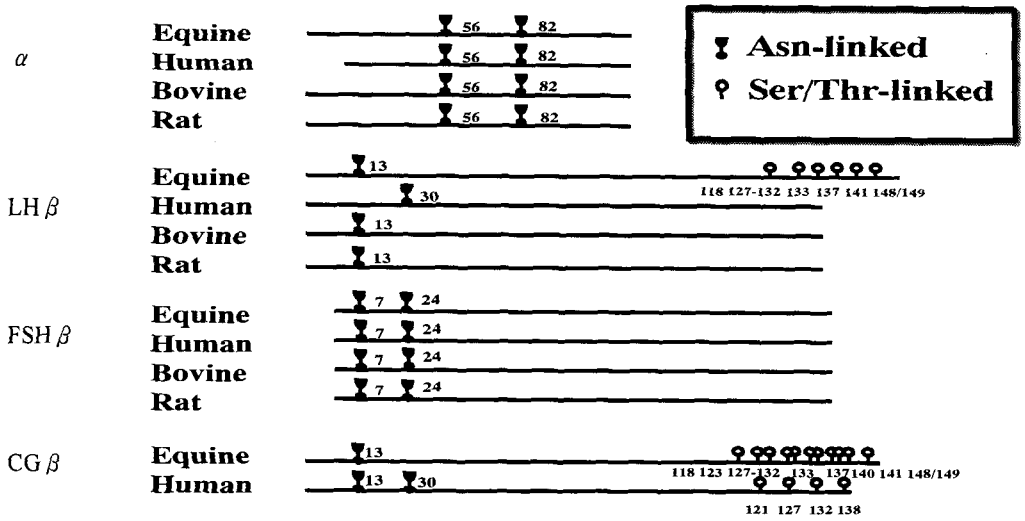

Fig. 2 . Glycosylation sites on the α- and β-subunits of glycoprotein hormone.
The α- and β-subunits of equine, human, bovine and rat glycoprotein hormones are shwon, and their homologies with respect to the Asn glycosylation site location and numbers of amino acids are indicated. The α-subunits of all these hormones from four species have two Asn glycosylation sites located at homologous positions (Asn^{56} and Asn^{82} *in equine* α). LHβ has only a single Asn glycosylation site and two analogous sites are present on FSHβ from all four species. Unlike LHβ and FSHβ, CGβ has a 25~29 amino acid residue extension peptide at its carboxyl terminus, and eCGβ possesses several O-glycosylation sites in its C-terminal extension, at least 11 of which are glycosylated.

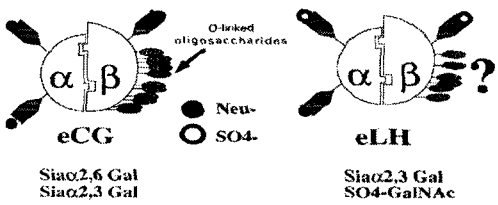

Fig. 3. Oligosaccharide structure of equine chorionic gonadotropin.

-subunits bear one and two sites, respectively, there are two on hCG, whereas eCG has only one. In horse, a single gene encodes both eCG and eLH β subunits (Min et al., 1994). The difference between eCG and eLH lies in the structure of their glycoresidues, which are both sialylated and sulfated in LH and sialylated in CG. The role of oligosaccharides in the function of eCG is distinct from that in the case of hLH, hFSH and hCG. Removal of the N-linked oligosaccharides from gonadotropins by enzymatic or chemical methods has been shown to reduce their adenylate cyclase stimulating activities, suggesting that, in general, these oligosaccharides are required for efficient signal transduction (Min et al., 1996a, 1997). Recombinant eCGs including the mutants which lack oligosaccharides will be useful tools for analyzing the structure-function relationships of gonadotropins in the horse as well as other species.

3. Pattern of α and CG β mRNA Expression

Northern blot analysis of mRNA prepared from equine placenta revealed a transcript of the predicted size. An intense mRNA ratio of α to β

at first trimester placenta was about 1: 5 (Min et al., 1994; Min, 2000a). Interestingly, Couture et al. (1993) showed that the free eCG β-subunit exists in serum and urine, whereas the free eCG α-subunit was undetectable. In our results, the expression of α-subunit in the equine placenta was lower than that of the eCG β-subunit, suggesting that the biosynthesis of eCG β is dominant and secretion of free eCG α-subunit is reduced in the equine placenta at day 70 of gestation. We also analysed mRNA expressions of α-, LH β- and FSH β-subunits from equine pituitary. The intensity of the α-subunit RNA was greater than that of the β-subunit, suggesting that the expression of α-subunit was dominant in the equine anterior pituitary (Min et al., 1994; Min, 2000).

Boothby et al. (1983) reported the presence of twice as much hCG α mRNA as hCG β mRNA. In contrast, the amount of eCG β mRNA was higher than that of eCG α mRNA in the equine placenta. Thus, the subunit mRNA levels seem to be independently regulated and their imbalance may account for differences in the quantities of α- and β-subunits in the placenta. Animals, including horses, have a single copy of the cAMP response element (CRE)-like element in the 5'-flanking region of the α-subunit gene (Fenstermaker et al, 1990), while the human α-subunit gene has the repeated copies of the CRE. Inspection of the genomic DNA sequence in the 5'-flanking region that contains the α-gene' CRE revealed by Steger et al, (1991).

4. Differential Role of eCG Oligosaccharides

To determine the biological role of the N-linked oligosaccharide at Asn 56 of the α-subunit and O-linked oligosaccharides at the carboxyl-terminal peptite(CTP) of the β-subunit, Two mutant eCGs were produced by site-directed mutagenesis and transfected into Chinese hamster ovary (CHO-K1) cells. LH- and FSH-like activities were assayed in terms of testosterone production and aromatase activity in primary cultured rat Leydig cells and granulosa cells, respectively. The wild type eCG showed similar LH-and FSH-like activities to native eCG in the *in vitro* bioassay. The LH-like activity of eCG α56/β was greatly reduced, whereas that of eCG α/β-CTP was unaffected, demonstrating that the oligosaccharide at Asn 56 of the eCG α-subunit plays an indispensable role in LH-like activity. Interesting, the FSH-like activity of eCG α 56/β was increased markedly in comparison with the wild type, and that of eCG α/β-CTP was also considerably increased (Min et al., 1996a, 1997). These data indicate that the dual activities of eCG, LH- and FSH-like activities, could be separated by removal of the N-linked oligosaccharide on the α-subunit Asn 56 or CTP-associated O-linked oligosaccharides.

5. Increasing of FSH-like Activity in a Single Chain eCG

To determine whether α- and β- subunits can be synthesized as a single polypeptide chain (tethered-eCG) and also display biological activity, the tethered-eCG molecule by fusing the carboxyl terminus of the eCG β-subunit to the amino terminus of the α-subunit was constructed and transfectdd into CHO-K1 cells. The tethered-eCG was efficiently secreted and showed similar LH-like activity to the dimeric eCG expressed in the CHO-K1 cells. Interestingly, the FSH-like activity of the tethered-eCG was increased markedly in comparison with the native and wild type eCG (Min et al., manuscript in preparation). These results also suggest that this molecular can implay particular models of FSH-like activity not LH-like activity in the eCG/indicate that the constructs of tethered molecule will be useful in the study of mutants that affect subunit association and/or

secretion. A single-chain analog can also be constructed to include additional hormone-specific bioactive generating potentially efficacious compounds that have only FSH-like activity.

Ⅲ. BIOLOGICAL FUNCTIONS OF LUTROPIN/RECEPTOR

1. Phosphorylation of the Gonadotropin Receptors

When target cells are exposed to a hormone, their responsiveness wanes with time, in spite of the continuous presence of the hormone. This phenomenon, referred to as desensitization, is due to regulatory steps that occur at the level of the hormone receptor as well as at post-receptor steps.

Using the immunoprecipitation techniques and a clonal cell line stably transfected with the LH/CG receptor, we show that exposure of cells to hCG induces phosphorylation of its cognate receptor. The hCG-induced increase in receptor phosphorylation requires receptor activation because it cannot be elicited with a hCG antagonist and is mediated at least in part by the cAMP second messenger system. And we show that LH/CG receptor phosphorylation can be induced with a phorbol ester, but not with a calcium ionophore. Cells expressing LHR-wt or LHR-t653 responded to hCG, or phorbol 12-myristate-13-acetate (PMA) stimulation with a similar increase in rLHR phosphorylation. Neither of these two stimuli increased LHR phosphorylation in cells expressing LHR-t631 or LHR-t628, however. The cell line expressing LHR-t653, the phosphorylation-positive receptor mutant, desensitized normally in response to PMA or hCG stimulation. This truncated form of LHR also was down-regulated normally in response to hCG stimulation. In contrast, the cell lines expressing LHR-t631 or LHR-628, the two phosphorylation-negative receptor mutant, showed a delay in the early phase of hCG-induced desensitization, a complete loss of PMA-induced desensitization, and an increase in the rate of hCG-induced receptor down-regulation. These results clearly show that residues 632-653 in the C-terminal tail of the LHR are involved in PMA-induced desensitization, hCG-induced desensitization, and hCG-induced down-regulation.

2. Constitutive Activation Mutations that Induce Signal Transduction of Lutropin/Choriogonadotropin Receptor

We have analyzed two naturally occurring, constitutively active mutants of the human LHR. These mutations were introduced into the LHR and are designated L435R and D556Y. Cells expressing LHR-D556Y bind hCG with normal affinity, exhibit a 25-fold increase in basal cAMP and respond to hCG with a normal increase in cAMP accumulation. This mutation does not affect the internalization of the free receptor, but it enhances the internalization of the agonist-occupied receptors ~3-fold. Cells expressing LHR-L435R also bind hCG with nomal affinity, exhibit a 47-fold increase in basal cAMP, and do not respond to hCG with a further increase in cAMP accumulation. This mutation enhances the internalization of the free and agonist-occupied receptors ~2- and ~17-fold, respectively. We conclude that the state of activation of the LHR can modulate its basal and/or agonist-stimulated internalization. Since the internalization of hCG is involved in the termination of hCG actions, we suggest that the lack of responsiveness detected in cells expressing LHR-L435R is due to the fast rate of internalization of the bound hCG (Min et al., 1998). This statement is supported by the finding that hCG responsiveness is restored when the cells are lysed and signal transduction is measured in a subcellular fraction (membranes) that cannot internalize the bound

hormone.

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