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REVIEW

Role of Glycosylation in Function of Follicle-Stimulating Hormone

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The oligosaccharide structures of heterodimeric glycoprotein hormones, such as follicle-stimulating hormone (FSH), have been shown to play an important role in the biosynthesis, secretion, metabolic fate, and regulation of potency of the hormone. The oligosaccharide structures attached to each subunit of the protein seem to exhibit distinct roles in some of these functions. Glycans attached to the α-subunit are critical for dimer assembly, integrity, and secretion, as well as for signal transduction; although β-subunit glycans are also important for dimer assembly and secretion, they play a crucial role in clearance of the dimer from the circulation. Alternative glycosylation on FSH and other glycoprotein hormones not only may affect the metabolic clearance and net in vivo biopotency of the hormone, but also offers the interesting possibility that some glycosylation variants of the hormone may provoke differential or even unique effects at the target cell level. Glycosylation of FSH is regulated by hypothalamic and/or end products from the glands under the control of this hormone. In particular, estrogens regulate terminal sialylation and thus some functional properties of the gonadotropin influenced by sialic acid. Through these extrapituitary inputs, the gonadotroph may regulate not only the amount but also the intensity of the gonadotropin signal to be secreted by the pituitary in a given physiological condition.

Key Words: Glycosylation; follicle-stimulating hormone; structure; function; action.

Introduction

Follicle-stimulating hormone (FSH) is one of the master signals produced by the anterior pituitary gland. This pituitary hormone, which acts at the ovarian granulosa cells and the testicular Sertoli cells, is involved in the regulation of several essential reproductive processes such as gametogenesis, follicular growth, and ovulation. It belongs to the glycoprotein family of hormones, which is composed of FSH, luteinizing hormone (LH), thyrotropin (TSH), and chorionic gonadotropin (CG) *(1)*. As with all members of this family, FSH is a heterodimer, formed by a common α-subunit noncovalently associated with a β-subunit, which is structurally unique in its peptide sequence to each member of the family *(2)*. Nevertheless, there is a variable degree of amino acid homology among the β-subunits of all these hormones, particularly within those regions that interact with their corresponding α-subunit. Within a given animal species, the α-subunits arise from a single gene whereas the β-subunits arise from separate genes, conferring to each dimer a high degree of biochemical and biological specificity *(3–6)*. Thyrotropin is synthesized by a distinct pituitary cell type, the thyrotrophs, whereas LH and FSH share the cell type in which they are synthesized. Although CG is mainly synthesized by the placental trophoblasts, there is compelling evidence indicating that this gonadotropin is also normally produced by the anterior pituitary gland *(7)*. The subunits of all glycoprotein hormones contain various Asn-linked (N-linked) oligosaccharide structures with different degrees of sulfation and sialylation *(8,9)*. This wide spectrum in glycosylation, particularly in sialylation and sulfation, constitutes the chemical basis for isoform formation and the extensive charge heterogeneity seen with all these glycoprotein hormones *(10)*. The primary sequence of FSH α- and β-subunits each encodes two glycosylation sequons. Therefore, carbohydrate is a major structural component of FSH, comprising in excess of 30% of the mass of the hormone. In human (h) FSH, N-linked oligosaccharides are located at positions N52 and N78 of the α-subunit, whereas the β-subunit includes glycosylation sites at positions N7 and N24 *(2,11,12)*. The complexity of glycosylation of the common α-subunit is different for each of the glycoprotein hormones, illustrating that although the α-subunit primary sequence is identical, the α-subunit of each gonadotropin is in fact quite different, depending on the genetic background of the cell where it is synthesized *(13)*.

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The crystal structure of hCG *(14,15)* provided the first physical map of the three dimensional (3D) gonadotropin structure. It revealed that each gonadotropin subunit has a cystine knot motif fold. For example, the 3D structure of β-and α-subunits can be superimposed at the knot. In the α-subunit, the cystine knot is formed by three of five intrachain disulfide bonds, whereas in the β-subunit the knot is formed by three of six disulfide bonds. α- and β-subunits of all gonadotropins are likely to share this fold, and the association of the α- with the β-subunit is of high fidelity. The two gonadotropin subunits combine in an antiparallel orientation with the cystine knot motif at the center of the molecule and the two long β hairpin loops of each subunit tightly associated with the less-structured longer loop of the complementary subunit. This final configuration of the subunits results in the display of β-subunit glycans at one end of the molecule on the β hairpin loop 1 (βN7, βN24), α-subunit glycan on the α hairpin loop 3 (αN78), and a glycan moiety on the α hairpin loop 2 (αN52) (Fig. 1). Remarkably, this latter glycan moiety is proximal to a discrete set of residues in hFSH that is required for receptor binding, and is located in loops 2 and 3 of α *(16)* and a small cystine noose in β *(17)*. The significance of this is discussed, subsequently.

As in other glycoproteins from multicellular eukaryotes, oligosaccharide structures on glycoprotein hormones are highly variable (Fig. 2) and play a key role in determining several functional properties of the hormone, including metabolic clearance, regulation of potency, subunit assembly, and intracellular sorting *(8–10,18,19)*. This review focuses on the functional role of carbohydrates present in the hFSH molecule.

Role of Glycosylation on Folding, Assembly, and Secretion of hFSH

The complexity of carbohydrate at each subunit glycosylation sequon is dependent on the cell type, and this complexity is a major factor in circulatory characteristics *(20)*. Glycosylation of the subunits of glycoprotein hormones begins in the rough endoplasmic reticulum (RER) with the cotranslational transfer of dolichol-linked $Glc_3Man_9GlcNAc_2$ oligosaccharide precursor to asparagines at glycosylation consensus sites Asn-*X*-Ser/Thr *(8)*. In the glycoprotein hormones, N-linked oligosaccharides exhibit a common core consisting of two *N*-acetylglucosamine and three mannose residues *(8,9)* (Fig. 2). After dimer formation and trimming of glucose and mannose residues to a $Man_5GlcNAc_2$ in the RER and *cis*-Golgi, extensive processing of the oligosaccharides attached to the protein core of the hormone occurs in the medial and *trans*-Golgi to form the mature oligosaccharides. The numerous carbohydrate intermediates resulting from this posttranslational processing, several of which become final forms of the complex carbohydrate chains attached to the protein core, are responsible for many of the glycoforms synthesized and secreted by the pituitary and the placenta *(8,10,21)* (Fig. 2). The majority of asparagine-linked oligosaccharides in hFSH are mono-, di-, and trisialylated biantennary structures, which confer an overall negative charge to the molecule *(8,10)*. This contrasts with LH, which contains mainly terminal sulfate residues *(8,22)*. Differences in the extent of sialylation and sulfation between these two gonadotropins are of paramount importance for the in vivo bioavailability and bioactivity of the hormone, since these terminal residues determine the rate at which the hormone is cleared from the circulation (see below). In contrast to ovine and bovine FSH, in which ~30 and ~12% of their carbohydrates bear one or two terminal sulfate residues, respectively, only 5–7% of the oligosaccharides in hFSH are terminally sulfated *(8,9)*.

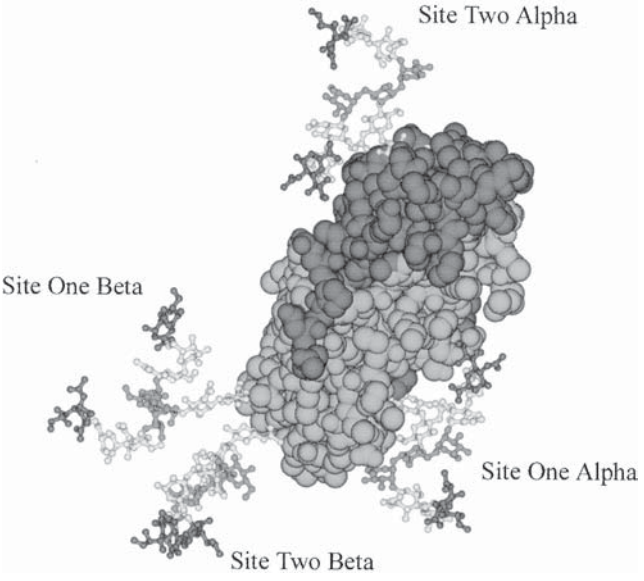

Fig. 1. How a fully glycosylated, fully sialylated hFSH molecule might appear, rendered as previously decribed with the exception that the molecular visualization program RASMOL was used *(16)*. Carbohydrate are rendered as ball and stick. Sialic acid is dark grey, N-acetylgalactosamine and N-acetyl glucosamine moieties are light grey, and mannose units are medium grey. Protein is rendered as space filling. The α-subunit is dark grey, and the β-subunit is light grey. Starting at 12 o'clock and proceeding clockwise, the carbohydrate is site-two alpha, site-one alpha, site-two beta and site-one beta.

The regulation of the remarkably different distribution of sialylated and sulfated oligosaccharides in LH and FSH is of particular interest considering that both glycoprotein hormones are synthesized in the same cell *(8,23–25)*. In fact, it has been shown that the β-subunits influence the oligosaccharide processing of bound α-subunit, particularly of the αN52-linked oligosaccharide, thus accounting in part for the differences in N-linked glycosylation among these gonadotropin molecules *(25,26)*. In this regard, it has been postulated that the presence of the β-subunit may hinder the action of some processing enzymes or, alterna-

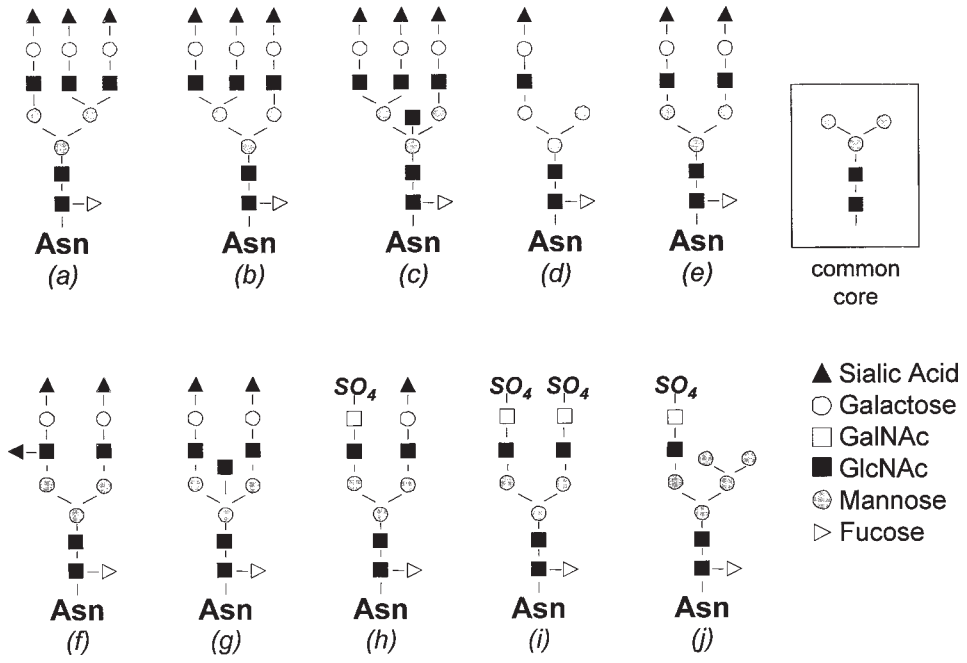

Fig. 2. Some of the N-linked oligosaccharide structures present on ovine, bovine, and human FSH. Only the complete structures are shown. Many other glycans may be found that are incompletely processed versions of these structures, mainly lacking terminal residues such as sulfate, sialic acid, and fucose.

tively, that conformational changes between free and combined α-subunit may be distinctly recognized by glycosidases and glycotransferases *(25)*. Moreover, an *N*-acetylgalactosamine transferase has been identified in pituitary membranes that recognizes a tripeptide motif (-Pro-Xaa-Arg/Lys-) found in the α-subunit sequence which forms an LHα/β dimer interface, adding a GalNAc residue to its oligosaccharides and leading to a terminally sulfated sequence *(27–29)*. This peptide sequence is present in the α-subunit as well as in βLH- and βCG-subunits, but not in βFSH. Although it has been postulated that the presence of βFSH may mask this recognition site on the αFSH-subunit, thus facilitating that N-linked oligosaccharides on FSH may terminate predominantly in a galactose-sialic acid sequence, only hFSH (in which ~7% of its oligosaccharides are sulfated) fits completely this model since nearly 30% of oligosaccharides on ovine FSH bear terminal sulfates *(8)*.

Although still unproven, the presence of distinct oligosaccharides in FSH and LH together with other specific conformational and/or β-subunit peptide-dependent structural determinants on the mature glycoprotein, may direct both gonadotropins into separated secretory granules and/or pathways *(18,30)*. Heterogeneity of naturally occurring hFSH is due solely to the carbohydrates, in which 95% of FSH is acidic owing to sialic acid *(8,9)*. Since trimming and further processing of the high-mannose precursor in the Golgi determines the final carbohydrate fingerprint, it follows that in times of increased gonadotropin production during the menstrual cycle, the complexity of blood-borne FSH can vary *(10,31–34)*. Indeed, in the perimenopausal period in which serum gonadotropins are rising, the profile of FSH isoforms in blood may have profound effects at the level of the receptor *(10)*.

For the gonadotropins to be present in the circulation, the individual subunits must be folded, assembled, and secreted. Our current knowledge on the potential role of the carbohydrate residues of FSH in subunit folding and assembly as well as on dimer secretion has been derived primarily from studies with other gonadotropin hormones, mainly hCG. Using molecular biology techniques, it has been possible to dictate the protein sequence of gonadotropins and whether or not each subunit will be glycosylated and to express each subunit individually, allowing for the study of the role of glycosylation in folding, assembly, and secretion. Despite a striking structural homology to the cystine knot motif growth factors *(35)*, there has been no evidence that naturally occurring, properly glycosylated homodimeric (α/α or β/β) gonadotropins form in vivo. Thus, glycosylation can affect formation of heterodimeric gonadotropins. In the instance of expression of α-subunit produced in early pregnancy, which is more branched than α-subunit found in the hCG heterodimer, this form of α-subunit cannot combine with βhCG *(36)*. Further, the removal of N-linked glycans from placental free α-subunit promoted conversion of the undimerizable subunits into dimerizable forms. In contrast to the α-subunit present in α/β dimers, the free α-subunit fraction produced by bovine pituitary bears an O-linked oligosaccharide attached in Thr_{43} *(37)*; although this additional O-linked oligosaccharide prevents binding of the subunit to the β-subunit *(38)*,

it does not interfere with its regulated secretion by the gonadotroph.

Early work demonstrated that there is only limited formation of native structure of gonadotropin subunit when expressed in bacteria *(39)*. Assembly of gonadotropin subunit produced in bacteria into heterodimeric gonadotropin is only possible with some denaturation and refolding, suggesting that glycosylation gives greater conformational flexibility during assembly *(40)*. Glycosylation of the subunits enhances rates of protein folding and secretion largely by facilitating the formation of correct disulfide bonds *(41,42)*. Misfolded subunits accumulate in the endoplasmic reticulum, forming complexes with chaperones and are not secreted *(41)*.

In particular, glycosylation of αhCG at N52 plays a critical role in dimer assembly and secretion *(25,42,43)*, whereas glycosylation at N78 is apparently more important for maintaining the integrity of the subunit than for subunit dimerization. Loss of α-subunit carbohydrate at N78 but not at N52 results in degradation of the mutant subunit and <20% secretion when expressed alone, a loss that can be rescued somewhat by coexpression of the β-subunit *(42)*. Glycosylation at hCGβ N13 is inconsistent, and is not likely to influence biosynthesis greatly *(44)*. In fact, it has been shown that hCGβ N30 but not hCGβ N13 is most important for folding *(45)*. Thus, the biosynthesis and secretion of hCG, and presumptively of hFSH and other pituitary glycoprotein hormones, is dependent on glycosylation. Further processing of the carbohydrate moiety affects, in more subtle ways, activity at the level of the receptor, as discussed next.

Hormonal Control of Glycosylation

Several studies indicate that glycosylation of all anterior pituitary glycoprotein hormones is regulated by hypothalamic inputs and/or end products from the target glands under the control of these tropic hormones *(46–55)*. For example, it has been shown that carbohydrate branching and sialylation of secreted hTSH vary depending on the functional status of the thyroid gland, with an increased degree of sialylation in conditions characterized by decreased pituitary exposure to thyroid hormones, e.g., in primary hypothyroidism *(50–53)*. In rodents and humans, the relative distribution of both stored and secreted LH and FSH charge isoforms undergoes major changes during the preovulatory period (a condition characterized by both increased pituitary sensitivity to gonadotropin-releasing hormone and estrogen exposure), in which an increase in shorter-lived, less acidic/sialylated isoforms may be observed *(31–34,54)*. Conversely, more sialylated forms are seen in hypoestrogenic states *(31,56)*.

The cellular mechanisms whereby these end products regulate hormone glycosylation have also been investigated. *In situ* hybridization studies have shown that the mRNA levels of several glycosyltransferases (such as α-2,6-sialyltransferase, β-1,4-galactosyltransferase, and α-mannosidase-II) are significantly increased in thyrotrophs from mice in which the synthesis of thyroid hormones was blocked by administration of propylthiouracil *(57,58)*. On the other hand, Dharmesh and Baenziger *(59)* observed that the activity of both pituitary *N*-acetylgalactosamine transferase and sulfotransferase increased severalfold after ovariectomy, rendering more LH oligosaccharides terminating with the sequence GalNAc-4-SO_4, and that estrogen administration returned the activities of these transferases to basal levels.

More recent studies have shown that the mRNA expression of another sialyltransferase, the pituitary α-2,3-sialyltransferase, changes according to the time and day of the rat estrous cycle and that the dynamics of these changes in the enzyme mRNA levels correlate with variations in serum estradiol concentrations *(60)*. Specifically, during the preovulatory days (diestrus 2 and proestrus), a progressive decrease in this enzyme mRNA may be observed in the face of increasing serum estradiol concentrations, reaching its lowest values immediately before and after maximal pituitary exposure to this sex steroid, i.e., during the morning of proestrus and estrus, respectively (Fig. 3); conversely, a significant rise in enzyme mRNA levels occurs at the time of lowest estradiol output, thus suggesting that changes in estrogen levels may influence the rate of transcription and/or stability of this glycosyltransferase mRNA. This possibility was in fact experimentally documented by studies showing that administration of estradiol benzoate to both intact and castrated female rats blocked the naturally occurring increase in α-2,3-sialyltransferase mRNA imposed by the low estrogenic milieu, whereas the opposite effect was observed when the estrogen receptor antagonist ICI 182,780 was injected to block the effects of the endogenously or exogenously provided estrogens (Fig. 3) *(60)*.

Glycosylation Effects on Extracellular Sorting and Biological Activity of the Hormone

Effects on Metabolic Clearance Rate and In Vivo Bioactivity

There is compelling evidence that the oligosaccharide structures on glycoprotein hormones influence the plasma half-life and consequently the in vivo bioactivity of the hormone *(61,62)*. As already described, oligosaccharides present in hFSH terminate predominantly in sialic acid; this sugar content, and particularly the number of exposed terminal galactose residues, play an essential role in determining the survival time of the hormone in the circulation *(61)*. As with other glycoproteins, exposure of terminal galactose residues on FSH oligosaccharides dramatically increases their rate of clearance from plasma through a mechanism that involves hepatocyte receptors for the asialo galactose-terminated complex molecules. As a consequence, heavily sialylated glycoproteins, such as FSH and CG, circulate for longer times than less-sialylated

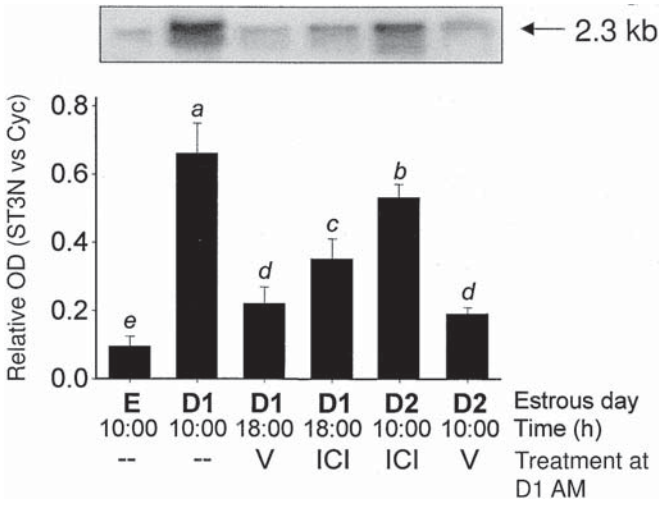

Fig. 3. Changes in pituitary α2,3-sialyltransferase (ST3N) mRNA during the days of estrus (E), diestrus 1 (D1), and diestrus 2 (D2) in rats untreated or treated with either vehicle (V) or the potent estradiol receptor antagonist ICI 182,780 (ICI) at 10:00 of D1. Data are expressed as the ST3N/cyclophilin (Cyc) mRNA relative optical density (OD) ratios. Different letters above the bars indicate the existence of significant ($p < 0.05$) differences between each animal group. The maximal ST3N mRNA expression found at 10:00 of D1 was mimicked by administration of a single dose (2 mg/kg body weight) of the estradiol antagonist. The bands on the top of the graph show a representative Northern blot of total RNA from rat anterior pituitary glands probed with radiolabeled ST3N.

molecules *(63,64)*. By contrast, the presence of terminal *N*-acetylgalactosamine-4-sulfate residues or oligosaccharides bearing terminal mannose or *N*-acetylglucosamine accelerates clearance of the molecule by specific receptors present in hepatic endothelial and Kupffer cells *(65,66)*. The half-life of FSH is therefore longer than that exhibited by LH, whose oligosaccharides terminate mainly in sulfate residues, but shorter than that of CG, which bears O-linked sialylated oligosaccharides in the COOH-terminus of its unique β-subunit *(8)*. Further, in contrast to the different, sialic acid–dependent, plasma clearance rates of rat and human FSH isoforms, ovine FSH variants, which are known to contain higher percentages of terminal sulfates than their counterparts in other animal species *(8)*, exhibit similar plasma clearance rates *(67)*. Although FSH and the other glycoprotein hormones are also degraded in the kidney *(68)*, it is unknown whether this particular mechanism requires specific structural determinants that may facilitate kidney cell uptake of the molecule.

The impact of the varying degrees in sialylation of circulating FSH on its net plasma half-life has been recently documented. Several studies have shown that during the preovulatory phase of the human menstrual cycle, an increased amount of less-sialylated FSH forms is released from the pituitary *(31–34)*; in one of these studies *(34)*, the increased release of less-sialylated isoforms correlated with a significant reduction in the plasma half-life of FSH secreted during this particular cycle phase.

Recent mutagenesis studies have confirmed the role of the oligosaccharide structures, particularly the sialic acid residues, on the circulatory survival and in vivo bioactivity of FSH. Recombinant human FSH molecules produced by mutant Chinese hamster ovary (CHO) cells deficient in either the glycosylation enzyme *N*-acetyl glucosamine transferase I (thus resulting in the synthesis of FSH molecules with oligosaccharide structures bearing only the common core shown in Fig. 2) or in sialic acid transport into the Golgi (resulting in the production of FSH molecules containing oligosaccharides devoid of sialic acid) were as active as FSH produced by wild-type CHO cells in evoking estrogen production by rat granulosa cells in vitro but not in vivo *(62)*. On the other hand, Bishop et al. *(20)* found that removal of βN24, βN7, or both amino acid residues on FSH (whose oligosaccharide structures would be located on the periphery of the molecule, as suggested by the crystal structure of CG and the primary sequence of its β-subunit [*14*]) significantly reduced the metabolic clearance rate of FSH as well as the in vivo bioactivity of the hormone as assessed by the Steelman and Pohley bioassay, which is based on the CG-augmented, FSH-stimulated increase in ovarian weight of immature female rats *(69)*. Some minor effects on clearance rate but not in in vivo bioactivity of the hormone were observed when αN78 was deleted. Further, no effect on clearance rate or bioactivity was found when αN52 was deleted, an observation that is particularly interesting considering the crucial role of its oligosaccharide structure in signal transduction in vitro (see below).

Studies employing hFSH isoforms with variable degrees of sialylation as well as different in vivo biological end points suggest that factors other than the metabolic clearance rate may also influence the net in vivo biological activity of the hormone. Although it has been observed that the in vivo increase in ovarian weight provoked by repeated administration of hFSH isoforms is closely related to the sialic acid content of each variant (with the long-lived, more-sialylated isoforms exhibiting the highest bioactivity) *(70)*, when an acute effect (such as induction of tissue-type plasminogen activator [tPA] enzyme activity [tPAea] and mRNA expression) was examined, less-sialylated, shorter-lived FSH molecules also exhibited a pronounced effect *(71)* (Fig. 4). In a more recent study, less-sialylated hFSH variants were equally or even more potent than heavily sialylated forms for inducing granulosa cell proliferation or preventing follicular atresia over a 6- to 12-h period, when administered in a single dose to hypophysectomized immature rats *(72)*. Thus, additional factors, such as receptor-binding affinity and capability of the ligand to activate its receptor and efficiently trigger intracellular signal transduction (an effect that is highly dependent on the oligosaccharide structures [*73,74*]; see below), seem to be also important in determining the overall in vivo effects of the hormone. In fact, the results of a recent

pharmacokinetics study suggest that the intrinsic differences in in vivo bioactivities between human LH glycoforms may be primarily located to the initial interaction of the hormone with the target cell rather than to differences in their metabolic clearance rate *(75)*.

Role of Carbohydrates on Receptor Binding and Signal Transduction

The initial event in the action of glycoprotein hormones is the binding to highly specific receptors located in the membrane of the target cell. The receptor for FSH belongs to the superfamily of seven transmembrane-spanning domain, G-protein-coupled receptors. These G-protein-linked cell surface receptors mediate their intracellular actions through the activation of one or more guanine-nucleotide-binding signal-transducing proteins. Receptors of this superfamily consist of a single polypeptide chain of variable length that threads back and forth across the lipid bilayer seven times, forming characteristic α-helical membrane-spanning domains, connected by alternating extracellular and intracellular loops oriented to form a ligand-binding pocket *(76,77)*. The receptors for the glycoprotein hormones FSH, LH/CG, and TSH represent a small subclass of the rhodopsin/β-adrenergic large subfamily, to which the majority of G-protein-linked receptors identified to date belong *(78,79)*. These glycoprotein hormone receptors have large extracellular domains that display 9 to 14 sequence repeats built on a motif similar to other leucine-rich glycoproteins *(80–82)*. In the FSH and LH receptors, these leucine-rich repeats are involved in high-affinity binding and binding specificity. The particular structure of this region and the conformational changes occurring upon recognition of the β-subunit of the hormone allow receptor activation. Presumably, signal transduction occurs after proper orientation and positioning of specific regions located in both subunits of the dimer into a receptor activation site defined by the exoloops and the transmembrane domains of the receptor *(83)*. The intracellular loops of the hFSH receptor are preferentially coupled to the protein G_s, which activates the enzyme adenylyl cyclase to enhance the synthesis of the second-messenger cyclic adenosine monophosphate (cAMP), which in turn activates protein kinase A *(84)*. Unlike the rat FSH, hLH/CG, and hTSH receptors, which are also coupled to the protein $G_{q/11}$ (associated to activation of phospholipase-Cβ), the hFSH receptor is weakly coupled to this G-protein-mediated signaling pathway *(85)*.

The roles of α and β oligosaccharides on receptor binding activity have been studied extensively in hCG. Oligosaccharides at site N52 in isolated α-subunit appear mobile and unrestricted whereas GlcNAc-1 at site N78 appears less accessible and more restricted *(86)*. More important, similarity in disposition of the glycan moieties in both free α-subunit and in combination with the β-subunit demonstrates that their effect on the full biological activity of hCG is not owing to sugar units beyond

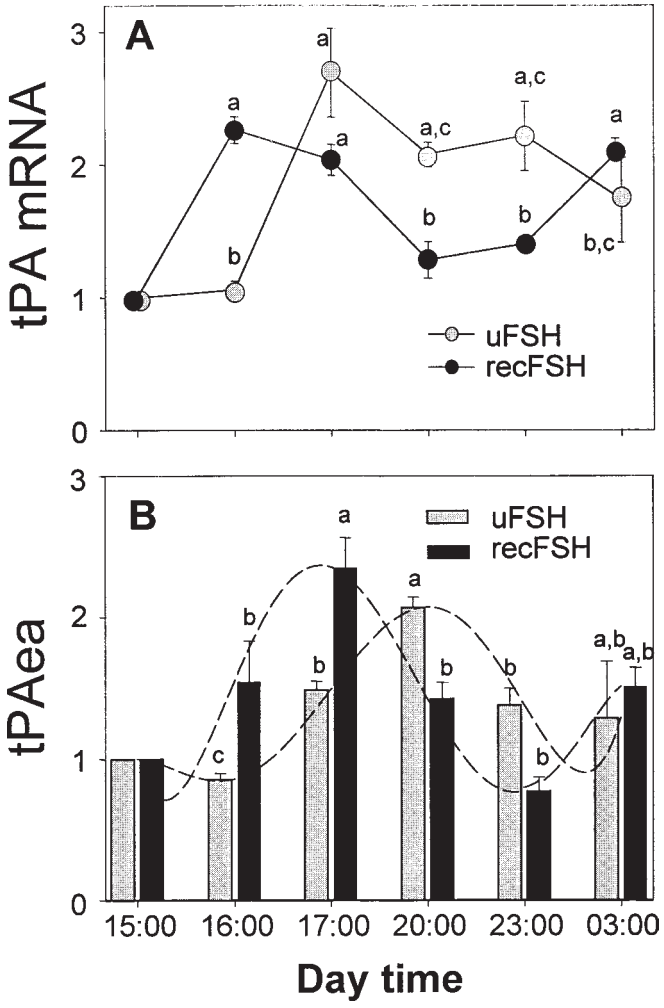

Fig. 4. Induction of ovarian tPA mRNA **(A)** and tPA enzyme activity (tPAea) **(B)** after administration of urinary human FSH (uFSH) or recombinant FSH (recFSH) (plasma clearance rates, 0.14 ± 0.01 and 0.34 ± 0.09 mL/min, respectively) to phenobarbital-blocked proestrous rats. Rats were treated with phenobarbital at 12:00 h on the day of proestrus and then with saline solution (control groups), uFSH, or recFSH at 15:00 h. Ovaries collected at each time point were examined for tPAea and mRNA expression by zymography and Northern blot hybridization analysis, respectively. Also shown are the kinetic curves for tPAea as disclosed by fifth-order linear regresssion. Results are presented as the relative ratios between treatment and control (phenobarbital/saline-treated) groups by setting the normalized tPAea and tPA mRNA of each control group at an arbitrary level of 1.0. Different letters above symbols or bars indicate the existence of significant ($p < 0.05$) differences within each treatment group (recFSH or uFSH). (Adapted from ref. *71* with permission from Oxford University Press.)

GlcNAc-1 inducing or stabilizing a conformation of the protein *(87)*. Indeed, αN78 GlcNAc-1 does appear to be tightly packed against the protein *(88)*, and an effect of glycosylation beyond αN78 GlcNAc-1 on stabilizing the structure of the free α-subunit has been noted *(89)*. It seems unlikely that the absence of oligosaccharide beyond αN78 GlcNAc-1 has any effect on the final conformation of the

α-subunit when combined with β-subunit; instead, the role of the glycans at this site may be greater for protein folding. High affinity binding of gonadotropin to receptor is observed with αN52 deglycosylated gonadotropin. The high-affinity binding is likely owing to the irreversible nature of the binding, which produces noncompetitive inhibition kinetics of fully glycosylated gonadotropin *(90)*. Thus, carbohydrate may actually act as a dampener for hormone binding, reducing the affinity of FSH for its receptor. Since sialic acid moieties of the carbohydrate appear to be accessible in the hormone-receptor complex *(91)*, it seems likely that the branched mannan units of the oligosaccharides are critical for steric hindrance in the complex. In this vein, the negatively charged terminal sialic residues present on FSH and TSH glycans may interfere with effective binding of the hormone to its receptor, explaining the enhanced receptor-binding capacity and in vitro biological activity exhibited by the less negatively charged/sialylated variants of both hormones *(92)*.

Early observations identified α-subunit glycosylation as essential for FSH- and LH- induced signal transduction *(93,94)*. At the cell level, the literature is replete with examples in which disruption of glycosylation of the α-subunit at N52 renders FSH *(73,74,95)* and hCG *(43)* marginally competent for steroidogenesis but not for receptor binding. TSH is similarly affected *(96)*. These observations suggested that binding and signal transduction were dissociable functions, which would have important implications for development of second-generation gonadotropins. There has been no elucidation of the underpinning of why glycosylation at this sequon is critical for signal transduction activity but not receptor-binding activity. One possibility is that deglycosylation may prevent hormone-induced aggregation of the receptor. In support of this idea, it has been reported that antibodies against hCG can restore the signal transduction defect caused by deglycosylation of hCG *(97)*. Another possibility is that the length of the Man(α1-6)Man oligosaccharide branch at site one of the α-subunit may provide steric hindrance, decreasing receptor-hormone affinity, and allowing conformational flexibility necessary for signal transduction *(98)*.

However, the explanation for the defect in signal transduction may in fact be quite simple: Recently it was demonstrated that the signal transduction defect caused by removing glycosylation at αN52 can be abrogated by covalently crosslinking the subunits of hCG together with engineered disulfide bridges *(99)*. This finding raises the intriguing possibility that the signal transduction defect caused by removing glycosylation at αN52 is due to instability of the heterodimeric hFSH at 37°C. This is a critical insight because it rules out any role for a lectin in gonadotropin-induced signal transduction. Also, Heikoop et al. *(99)* observations assign a role for αN52 oligosaccharide in stabilization of the heterodimer. This seems extremely likely, since mutations in the long loop of α-subunit decrease heterodimer formation *(100)*. Of interest, mutations in the long loop of the β-subunit also predispose to instability of the heterodimer *(101)*. This suggests that the gonadotropin long loops have evolved different molecular mechanisms to ensure stability of the non-covalent interactions between α- and β-subunits.

Consequences of Heterogeneity of FSH at the Systemic and Cellular Levels

A physiological significance of alternatively glycosylated forms of gonadotropin is that, in addition to their net effects on metabolic clearance rate and in vivo bioactivity of the hormone, they may function as antagonists of fully glycosylated gonadotropin in the blood. Basically charged hFSH inhibits FSH induced aromatase and tPAea but can stimulate cAMP formation (Fig. 5) *(102)*. These and other *(103)* data suggest that signaling pathways of FSH are not fully explained by cAMP accumulation and that alternatively glycosylated forms of hFSH may play striking roles at the cellular level, even in the presence of fully active glycosylated hFSH in circulation *(102)*. Deglycosylated hFSH or recFSH expressed in insect cells has been reported to be partially or strictly inhibitory when incubated with an ED_{50} or ED_{80} dose of fully glycosylated hFSH, respectively *(104)*. That finding was somewhat surprising, since *N*-glycosylation mutants of hCG produced in insect cells exhibits similar properties as when expressed in mammalian cells *(105)*. Also, Hi5 insect cell (T. ni) lines produced predominantly dimannose and linear trimannose structures devoid of α1-3-linked mannose *(106)*, and CHO cells deficient in *N*-acetylglucosamine transferase-I produced $(GlcNAc)_2(Mannose)_5$ type hFSH, which is fully biologically active *(62)*. In fact, we found that highly purified hFSH produced in this cell line does not have inhibitory properties ascribed to chemically deglycosylated hFSH (Fig. 6). The reason for this discrepancy remains unknown, but at this time, insect cell–derived hFSH appears to be fully active.

Another effect of alternatively glycosylated forms of FSH (and other glycoprotein hormones) may be the induction of differential responses at the cellular level, which may be achieved through different abilities of the glycovariants to induce and/or stabilize distinct receptor conformations that may permit preferential or different degrees of activation/inhibition of a given signal transduction pathway *(107)*. In fact, a recent study of CHO cells and COS-7 cells expressing the hTSH receptor (G_s- and $G_{q/11}$-coupled) has shown that some alternatively glycosylated TSH variants may differentially activate the two signal transduction pathways to which their cognate receptor is coupled *(108)*.

A better understanding of how carbohydrate influences signal transduction may be obtained when a 3D structure of fully glycosylated, biologically active gonadotropin is determined. If there is no difference in the structures of glycosylated vs nonglycosylated gonadotropins, the role of carbohydrate in directing a particular conformation that is required for signal transduction will be ruled out. This new information could help us understand the mechanism of receptor-G protein signal transduction prior to activation of adenylate cyclase.

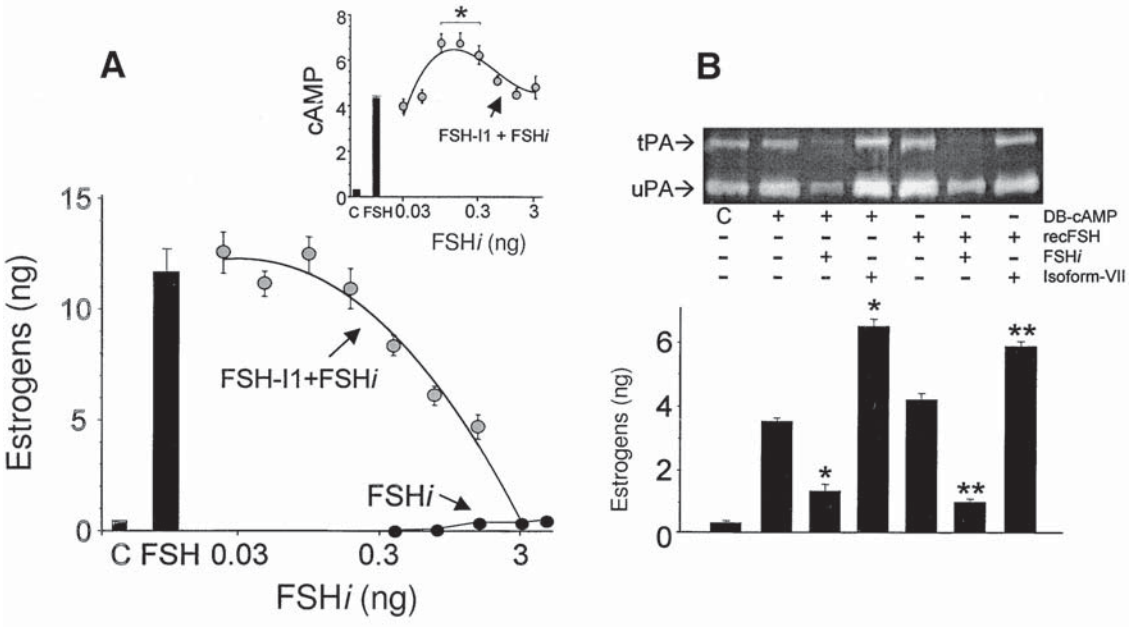

Fig. 5. Inhibitory effects of a basically charged human FSH isoform on FSH-induced androgen aromatization and tPAea in vitro. **(A)** Changes in concentrations of estrogens released into the culture medium of granulosa cells cultured during 48 h in the presence of a basically charged anterior pituitary FSH isoform (FSH*i*, solid circles), the highly purified pituitary standard FSH-I1 (National Hormone and Pituitary Program, Torrance, CA) (FSH, ED_{50} dose, black bar), and FSH plus increasing concentrations of FSH*i* (shaded circles). (Inset) cAMP production by FSH-I1 (FSH, black bar) and FSH plus FSH*i* (shaded circles). C, Control incubations in the absence of hormones; *, $p < 0.01$ vs the lower and higher FSH*i* doses and vs FSH alone. **(B)** Detection of tPAea **(top)** and total estrogens **(bottom)** in conditioned media from granulosa cells exposed for 48 h to human recFSH (ED_{50} dose) produced by CHO cells and dibutyryl cAMP (DB-cAMP) in the presence or absence of a basically charged human FSH isoform (FSH*i*) or a highly sialylated isoform (Isoform-VII). The location of tPA (M_r ~ 70,000) and high molecular weight (M_r ~ 50,000) urokinase-type plasminogen activator (uPA) activities are noted. C, Control incubations with no hormones or DB-cAMP added; *, $p < 0.01$ vs DB-cAMP; **, $p < 0.01$ vs recFSH in the abscence of FSH*i* or Isoform-VII and p N.S. recFSH plus FSH*i* vs DB-cAMP plus FSH*i* and recFSH plus Isoform-VII vs DB-cAMP plus Isoform-VII. (Adapted from ref. *102* with permission from Karger AG, Basel.)

Conclusion

The function of carbohydrate in FSH action is relegated (1.) systemically, to circulatory survival parameters; (2.) intracellularly, to proper folding and secretion; and (3.) at the target cell level, where binding and signaling are affected. These three levels of function impact the clinical use of hFSH. Thus, it has been demonstrated in principle that it may be possible to design gonadotropins with unique circulatory properties and specificities useful for superovulation for in vitro fertilization cycles *(109)*. Already, longer-acting hFSH agonists have been designed by fusing the carboxy-terminal tail of hCGβ to FSHβ *(110)*. Superagonist forms of TSH have been prepared by substituting positive charges found in domestic animal α-subunits, for the human α-subunit *(111)*. Minimized forms of hCG, roughly one-third of its native molecular weight, have been prepared *(112)*. Chimeras that mix and match residues from one gonadotropin to another provide for broader spectrum effects *(113,114)*. Thus, the design of new forms of gonadotropins is imminent.

On the other hand, further studies on FSH glycoforms are still necessary in order to assign a physiological role to alternative glycosylation; although recent studies employing novel in vitro systems have provided useful information on the role of these variants in follicular and oocyte development *(115,116)*, their regulatory actions in vivo still remain to be fully defined. The application of this knowledge and technical developments may be crucial to obtaining better control of controlled ovarian hyperstimulation, allowing for a small number of follicles to develop, ovulate, and fertilize and to avoid hyperstimulation, a potentially deadly syndrome.

Note Added in Proof

Recently the crystal structure of fully glycosylated hCG complexed to two Fv fragments has been determined. The hCG structure in the ternary complex was very close to the deglycosylated hormone structure. This suggests that neither the saccharides nor the Fv had any substantial influence on hormone structure. Alternatively, the Fv's stabilized a conformer that resembles the deglycosylated form of the hormone *(117)*.

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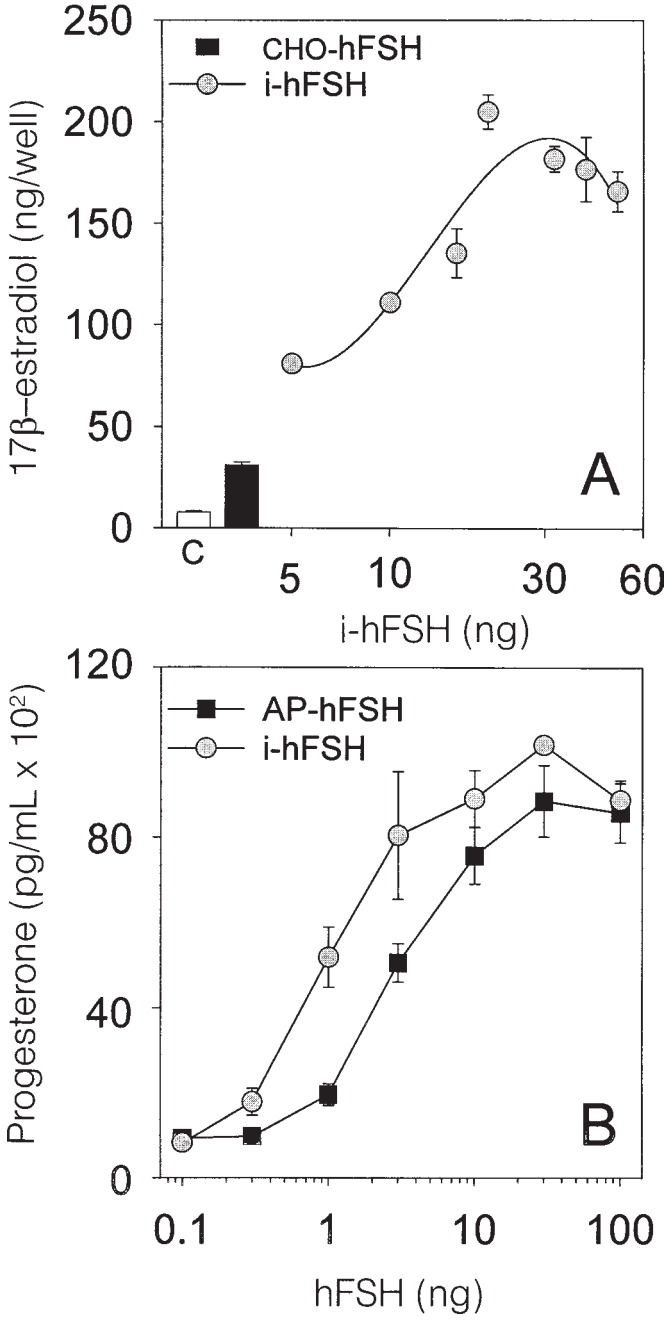

Fig. 6. The effect of insect cell–expressed recombinant hFSH (i-hFSH) on estradiol and progesterone production by cultured rat granulosa cells and Y-1 cells, respectively. **(A)** Rat granulosa cells were cultured for 48 h in the presence of recombinant hFSH (at an ED_{50} dose) produced by CHO cells (CHO-hFSH) or CHO-hFSH plus increasing concentrations of i-hFSH. C, Control incubations in the absence of hormones. **(B)** Progesterone production by Y-1 cells expressing the recombinant hFSH receptor exposed to increasing concentrations of anterior pituitary hFSH (AP-hFSH) or i-hFSH. (From Dias, J. and Ulloa-Aguirre, A., unpublished observations.)

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References

1. Pierce, J. G. and Parsons, T. F. (1981). *Annu. Rev. Biochem.* **50,** 465–495.
2. Garibh, S. D. Wierman, M. E., Shupnik, M. A., and Chin, W. W. (1990). *Endocr. Rev.* **11,** 177–199.
3. Godine, J. E., Chin, W. W., and Habener, J. F. (1982). *J. Biol. Chem.* **257,** 8368–8371.
4. Fiddes, J. C. and Talmadge, K. (1984). *Recent Prog. Horm. Res.* **40,** 43–78.
5. Gordon, D. F., Wood, W. M., and Ridgway, E. C. (1988). *DNA* **7,** 679–690.
6. Mercer, J. E. and Chin, W. W. (1995) *Hum. Reprod. Update* **1,** 363–384.
7. Patton, P. E., Hess, D. L., Cook, D. M., Loriaux, D. L., and Braunstein, G. D. (1998). *Am. J. Obstet. Gynecol.* **178,** 1138–1142.
8. Baenziger, J. U. and Green, E. D. (1988) *Biochim. Biophys. Acta* **947,** 287–306.
9. Stockell Hartree, A. and Renwick, G. C. A. (1992). *Biochem. J.* **287,** 665–679.
10. Ulloa–Aguirre, A., Midgley, A. R. Jr., Beitins, I. Z., and Padmanabhan, V. (1995). *Endocr. Rev.* **16,** 765–787.
11. Fiddes, J. C. and Goodman, H. M. (1979). *Nature* **281,** 351–356.
12. Watkins, P. C., Eddy, R., Beck, A. K., Vellucci, V., Leverone, B., Tanzi, R. E., Gusella, J. F., and Shows, T. N. (1987). *DNA* **6,** 205–212.
13. Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. (1990). *Methods Enzymol.* **185,** 60–89.
14. Lapthorn, A. P., Harris, D. C., Littlejohn, A., Lustbader, J. W., Canfield, R. E., Machilin, K. J., Morgan, F. J., and Isaacs, N. W. (1994). *Nature* **369,** 455–461.
15. Wu, H., Lustbader, J. W., Liu, Y., Canfield, R. E., and Hendrickson, W. A. (1994). *Structure* **15,** 545–558.
16. Dias, J. A., Lindau–Shepard, B., Hauer, C., and Auger, I. (1998). *Biol. Reprod.* **58,** 1331–1336.
17. Lindau–Shepard, B., Roth, K. E., and Dias, J. A. (1994). *Endocrinology* **135,** 1235–1240.
18. Green, E. D., Boime, I. and Baenziger, J. U. (1986). *Mol. Cell. Biochem.* **72,** 81–100.
19. Drickamer, K. and Taylor, M. E. (1998). *Trends Biochem. Sci.* **23,** 321–324.
20. Bishop, L. A., Nguyen, T. V., and Schofield, P. R. (1995) *Endocrinology* **136,** 2635–2640.
21. Ulloa–Aguirre, A., Mendez, J. P., Cravioto, A., Grotjan, E., Damián–Matsumura, P., and Espinoza, R. (1990). *Hum. Reprod.* **5,** 661–669.
22. Green, E. D. and Baenziger, J. U. (1988). *J. Biol. Chem.* **263,** 36–44.
23. Hebert, D. C. (1975). *Am. J. Anat.* **144,** 379–386.
24. Hebert, D. C. (1976). *Endocrinology* **98,** 1554–1559.
25. Bielinska, M., Matzuk, M. M., and Boime, I. (1989). *J. Biol. Chem.* **264,** 17,113–17,118.
26. Corless, C. L., Bielinska, M., Ramabhadran, T. V., Daniels–McQueen, S., Otani, T., Reitz, B. A., Tiemer, D. C., and Boime, I. (1987). *J. Biol. Chem.* **262,** 14,197–14,203.
27. Smith, P. L. and Baenziger, J. U. (1988). *Science* **242,** 930–932.
28. Smith, L. and Baenziger, J. U. (1990). *Proc. Natl. Acad. Sci. USA* **87,** 7275–7279.
29. Smith, L. and Baenziger, J. U. (1992). *Proc. Natl. Acad. Sci. USA* **89,** 329–333.
30. Muyan, M., Ryzmkiewicz, D. M., and Boime, I. (1994). *Mol. Endocrinol.* **8,** 1789–1797.
31. Padmanabhan, V., Lang, L. L., Sonstein, J., Kelch, R. P., and Beitins, I. (1988). *J. Clin. Endocrinol. Metab.* **67**, 465–473.
32. Wide, L. and Bakos, O. (1993). *J. Clin. Endocrinol. Metab.* **76**, 885–889.
33. Zambrano, E., Olivares, A., Méndez, J. P., Guerrero, L., Díaz–Cueto, L., Veldhuis, J. D., and Ulloa–Aguirre, A. (1995). *J. Clin. Endocrinol. Metab.* **80**, 1647–1656.

34. Anobile, C. J., Talbot, J. A., McCann, S. J., Padmanabhan, V., and Robertson, W. R. (1998). *Mol. Hum. Reprod.* **4,** 631–639.
35. Sun, P. D. and Davies, D. R. (1995). *Annu. Rev. Biophys. Biomol. Struct.* **24,** 269–291.
36. Blithe, D. L. and Iles, R. K. (1995). *Endocrinology* **136,** 903–910.
37. Parsons, T. F., Bloomfield, G. A., and Pierce, J. G. (1983) *J. Biol. Chem.* **258,**240–244.
38. Parsons, T. F. and Pierce, J. G. (1984) *J. Biol. Chem.* **259,** 2662–2666.
39. Strickland, T. W., Thomason, A. R., Nilson, J. H., and Pierce, J. G. (1985). *J. Cell Biochem.* **29,** 225–237.
40. Ren, P., Sairam, M. R., and Yarney, T. A. (1995). *Mol. Cell. Endocrinol.* **113,** 39–51.
41. Feng, W., Matzuk, M. M., Mountjoy, K., Bedows, E., Ruddon, R. W., and Boime, I. (1995). *J. Biol. Chem.* **270,** 11,851–11,859.
42. Matzuk, M. M. and Boime, I. (1988). *J. Cell Biol.* **106,** 1049–1059.
43. Matzuk, M. M., Keene, J. L., and Boime, I. (1989). *J. Biol. Chem.* **264,** 2409–2414.
44. Liu, C. L. and Bowers, L. D. (1997). *J. Mass Spectrom.* **32,** 33–42.
45. Feng, W., Huth, J. R., Norton, S. E., and Ruddon, R. W. (1995). *Endocrinology* **136,** 52–61.
46. Liu, T. and Jackson, G. L. (1978). *Endocrinology* **103,** 1253–1263.
47. Galle, P. C., Ulloa–Aguirre, A., and Chappel, S. C. (1983) *J. Endocrinol.* **99,** 31–39.
48. Weintraub, B. D., Gesundheit, N., Taylor, T., and Gyves, P. W. (1989). *Ann. NY Acad. Sci.* **553,** 205–213.
49. Weintraub, B. D., Wondisford, F. E., Farr, E. A., Steinfelder, H. J., Radovick, S., Gesundheit, N., Gyves, P. W., Taylor, T., and DeCherney, G. S. (1989). *Horm. Res.* **32,** 22–24.
50. Gyves, P. W., Gesundheit, N., Thotakura, N. R., Stannard, B. S., DeCherney, G. S., and Weintraub, B. D. (1987). *Proc. Natl. Acad. Sci USA* **87,** 3792–3796.
51. DeCherney, G. S., Gesundheit, N., Gyves, P. W., Showalter, C. R., and Weintraub, B. D. (1989). *Biochem. Biophys. Res. Commun.* **159,** 755–762.
52. Miura, Y., Perkel, V. S., Papenberg, K. A., Johnson, M. J., and Magner, J. A. (1989). *J. Clin. Endocrinol. Metab.* **69,** 985–995.
53. Papandreu, M. J., Persani, L., Asterial, C., Ronin, C., and Beck–Precoz, P. (1993). *J. Clin. Endocrinol. Metab.* **77,** 393–398.
54. Ulloa–Aguirre, A., Damian–Matsumura, P., Espinoza, R., Domínguez, R., Morales, L., and Flores, A. (1990). *J. Endocrinol.* **126,** 323–332.
55. Ulloa–Aguirre, A., Schwall, R., Cravioto, A., Zambrano, E., and Damián–Matsumura, P. (1992). *J. Endocrinol.* **134,** 97–106.
56. Wide, L. and Naessén, T. (1994). *Clin. Endocrinol. (Oxf.)* **40,** 783–789.
57. Helton, E. T. and Magner, J. A. (1994). *Endocrinology* **134,** 2347–2353.
58. Helton, E. T. and Magner, J. A. (1994). *Endocrinology* **135,** 1980–1985.
59. Dharmesh, S. M. and Baenziger, J. U. (1993). *Proc. Natl. Acad. Sci. USA* **90,** 11,127–11,131.
60. Damián–Matsumura, P., Zaga, V., Maldonado, A., Sánchez–Hernández, C., Timossi, C., and Ulloa–Aguirre A. (1999). *J. Mol. Endocrinol.* **23,** 153–165.
61. Morell, A. G., Gregoriadis, G., Scheinberg, I. H., Hickman, J., and Ashwell, G. (1971). *J. Biol. Chem.* **246,** 1461–1467.
62. Galway, A. B., Hsueh, A. J. W., Keene, J. L., Yamoto, M., Fauser, B. C. J., and Boime, I. (1990). *Endocrinology* **127,** 93–100.
63. Ulloa–Aguirre, A., Cravioto, A., Damián–Matsumura, P., Jimenez, M., Zambrano, E., and Díaz–Sánchez, V. (1992). *Hum. Reprod.* **7,** 23–30.
64. Diaz–Cueto, L., Barrios–de–Tomassi, J., Timossi, C., Méndez, J. P., and Ulloa–Aguirre, A. (1996). *Mol. Hum. Reprod.* **2,** 643–650.
65. Fiete, D., Srivastava, V., Hindsgaul, O., and Baenziger, J. U. (1991). *Cell* **67,** 1103–1110.
66. Fiete, D. and Baenziger, J. U. (1997). *J. Biol. Chem.* **272,** 14,629–14,637.
67. Robertson, D. M., Foulds, L. M., Fry, R. C., Cummings, J. T., and Clarke, I. (1991) *Endocrinoogy* **129,** 1805–1813.
68. Emmanouel, D. S., Stavropoulos, T., and Katz, A. I. (1984). *Am. J. Physiol.* **247,** E786–E792.
69. Steelman, S. L. and Pohley, F. M. (1953). *Endocrinology* **53,** 604–646.
70. Mulders, J. W. M., Derksen, M., Swolfs, A., and Maris, F. (1997). *Biologicals* **25,** 269–281.
71. Timossi, C., Damián–Matsumura, P., Domínguez–González, A., and Ulloa–Aguirre, A. (1998). *Mol. Hum. Reprod.* **4**, 1032–1038.
72. Barrios–de–Tomasi, J., Merchant, H., Quintanar, A., Avalos, J. M., and Ulloa–Aguirre, A. (1999). *32nd Annual Meeting of the Society for the Study of Reproduction,* abstract no. 571.
73. Flack, M. R., Froehlich, J., Bennet, P. A., Anasti, J., and Nisula, B. C. (1994) . *J. Biol. Chem* **269,** 14,015–14,020.
74. Bishop, L. A., Robertson, D. M., Cahir, N., and Schofield, P. R. (1994). *Mol. Endocrinol.* **8,** 722–731.
75. Burgon, P. G., Stanton, P. G., and Robertson, D. M. (1996). *Endocrinology* **137,** 4827.
76. Baldwin, J. M. (1993). *EMBO J.* **12,** 1693–1703.
77. Strader, C. D., Fong, T. M., Tota, M. R., and Underwood, D. (1994). *Annu. Rev. Biochem.* **63,** 101–132.
78. Probst, W. C., Snyder, L. A., Schuster, D. I., Brosius, J., and Seaflon, S. C. (1992). *DNA Cell. Biol.* **11,** 1–11.
79. Ulloa–Aguirre, A. and Conn, P. M. (1998). In: *Handbook of physiology–endocrinology: section 7, cellular endocrinology.* Conn, P. M. (ed.). Oxford University Press: New York.
80. Braun, T., Schofield, P. R., and Sprengel, R. (1991). *EMBO J.* **10,** 1885–1890.
81. Thomas, D., Rozell, T. G., Liu, X., and Segaloff, D. L. (1996). *Mol. Cell. Endocrinol.* **10,** 760–768.
82. Bhowmick, N., Huang, J., Puett, D., Isaacs, N. W., and Lapthorn, A. J. (1996). *Mol. Endocrinol.* **10,** 1147–1159.
83. Ji, I., Murdoch, W. J., and Ji, T. H. (1995). *Endocrine* **3,** 187–194.
84. Simoni, M., Gromoll, J., and Nieschlag, E. (1997). *Endocr. Rev.* **18,** 739–773.
85. Hirsch, B., Kudo, M., Naro, F., Conti, M., and Hsueh, A. J. W. (1996). *Mol. Endocrinol.* **10,** 1127–1137.
86. Van Zuylen, C. W., De Beer, T., Rademaker, G. J., Haverkamp, J., Thomas–Oates, J. E., Hard, K., Kamerling, J. P., and Vliegenthart, J. F. (1995). *Eur. J. Biochem.* **231,** 754–760.
87. Weller, C. T., Lustbader, J., Seshadri, K., Brown, J. M., Chadwick, C. A., Kolthoff, C. E., Ramnarian, S., Pollak, S., Canfield, R., and Homans, S. W. (1996). *Biochemistry* **35,** 8815–8823.
88. De Beer, T., Van Zuylen, C. W. E. M., Leeflang, B. R., Hard, K., Boelens, R., Kaptein, R., Kamerling, J. P., and Vliegenthart, J. F. (1996). *Eur. J. Biochem.* **241,** 229–242.
89. van Zuylen, C. W., Kamerling, J. P., and Vliegenthart, J. F. (1997). *Biochem. Biophys. Res. Commun.* **232,** 117–120.
90. Dunkel, L., Jia, X. C., Nishimori, K., Boime, I., and Hsueh, A. J. (1993). *Endocrinology* **132,** 763–769.
91. Petaja–Repo, U. E., Merz, W. E., and Rajaniemi, H. J. (1991). *Endocrinology* **128,** 1209–1217.
92. Zambrano, E., Zariñán, T., Olivares, A., Barrios–de–Tomasi, J., and Ulloa–Aguirre A. (1999). *Endocrine* **10,** 113–121.
93. Calvo, F. O., Keutmann, H. T., Bergert, E. R., and Ryan, R. J. (1986). *Biochemistry* **25,** 3938–3943,.
94. Sairam, M. R. and Bhargavi, G. N. (1985). *Science* **229,** 65–67.
95. Keene, J. L., Nishimori, K., Galway, A. B., Matzuk, M. M., Hsueh, A. J. W. and Boime, I. (1994). *Endocr. J.* 2, 175–179.
96. Papandreou, M., Sergi, I., Medri, G., Labbe–Jullie, C., Braun, J. M., Canonne, C., and Ronin, C. (1991). *Mol. Cell. Endocrinol.* **78,** 137–150.
97. Rebois, R. V. and Liss, M. T. (1987). *J. Biol Chem.* **262,** 3891–3896.

98. Butnev, V. Y., Gotschall, R. R., Butnev, V. Y., Baker, V. L., Moore, W. T., and Bousfield, G. R. (1998). *Biol. Reprod.* **58,** 458–469.
99. Heikoop, J. C., van den Boogaart, P., de Leeuw, R., Rose, U. M., Mulders, J. W., and Grootenhuis, P. D. (1998). *Eur. J. Biochem.* **253,** 354–356.
100. Liu, C. and Dias, J. A. (1996). *Arch. Biochem. Biophys.* **329,** 127–135.
101. Roth, K. E. and Dias, J. A. (1996). *Biochemistry* **35,** 7928–7935.
102. Timossi, C., Barrios–de–Tomasi, J., Zambrano, E., and Ulloa–Aguirre, A. (1998). *Neuroendocrinology* **67**, 153–163.
103. Padmanabhan, V., Sairam, M. R., Hassing, J. M., Brown, M. B., Riding, J. W., and Beitins, I. Z. (1991). *Mol. Cell. Endocrinol.* **79**, 119–128.
104. Arey, B. J., Stevis, P. E., Deecher, D. C., Shen, E. S., Frail, D. E., Negro–Vilar, A., and López, F. J. (1997). *Mol. Endocrinol.* **11,** 517–526.
105. Purohit, S., Shao, K., Balasubramanian, S. V., and Bahl, O. P. (1997). *Biochemistry* **36,** 12,355–12,363.
106. Kulakosky, P. C., Shuler, M. L., and Wood, H. A. (1998). *In Vitro Cell. Dev. Biol. Anim.* **34,** 101–108.
107. Timossi, C., Barrios–de–Tomasi, J., and Ulloa–Aguirre A. (1999). *81st Annual Meeting of the Endocrine Society,* abstract no. P2–23.
108. Schaaf, L., Leiprecht, A., Saji, M., Hübner, U., Usadel, K. H., and Khon, L. D. (1997). *Mol. Cell. Endocrinol.* **132**, 185–194.
109. Dierich, A., Sairam, M. R., Monaco, L., Fimia, G. M., Gansmuller, A., LeMeur, M., and Sassone–Corsi, P. (1998). *Proc. Natl. Acad. Sci. USA* **95,** 13,612–13,617**.**
110. Fares, F. A., Suganuma, N., Nishimori, K., LaPolt, P. S., Hsueh, A. J., and Boime, I. (1992). *Proc. Natl. Acad. Sci. USA* **89,** 4304–4308.
111. Szkudlinski, M. W., Teh, N. G., Grossman, M., Tropea, J. E., Weintraub, B. D., and Grossmann, M. (1996). *Nat. Biotechnol.* **14,** 1257–1263.
112. Heikoop, J. C., Huisman–de, W. B., and Grootenhuis, P. D. (1999). *Eur. J. Biochem.* **261,** 81–84.
113. Campbell, R. K., Dean–Emig, D. M., and Moyle, W. R. (1991). *Proc. Natl Acad. Sci. USA* **88,** 760–764.
114. Dias, J. A., Zhang, Y., and Liu, X. (1994). *J. Biol. Chem.* **269,** 25,289–25,294.
115. Vitt, U. A., Kloosterboer, H. J., Rose, U. M., Mulcers, J. W. M., Kiesel, P. S., Bete, S., and Nayudu, P. (1998). *Biol. Reprod.* **59,** 854–861.
116. Yding Andersen, C., Leonardsen, L., Ulloa–Aguirre, A., Barrios–de–Tomasi, J., Moore, L., and Byskov, A. G. (1999). *Mol. Hum. Reprod.* **5,** 726–731.
117. Tegoni, M., Spinelli, S., Verhoeyen, M., Davis, P., Cambillau, C. (1999). Crystal structure of a tenary complex between human chorionic gonadotropin (hCG) and two Fv fragments specific for the alpha and beta subunits. *J. Mol. Biol.* **289,** 1375–1385.