Specific Roles for the Asparagine-Linked Carbohydrate Residues of Recombinant Human Follicle Stimulating Hormone in Receptor Binding and Signal Transduction

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FSH comprises two distinct subunits, both of which contain asparagine-linked carbohydrate residues, located at positions 52 and 78 on the α-subunit and positions 7 and 24 on the β-subunit. These carbohydrate chains have been shown to regulate the biological activity of FSH, including signal transduction and receptor binding. However, the specific roles of the individual carbohydrate chains have been poorly defined. Using site-directed mutagenesis we disrupted the consensus sequences for glycosylation and expressed the mutated cDNAs in Chinese Hamster Ovary cells. Specifically deglycosylated FSH variants were secreted from all clonal cell lines expressing the mutated FSH cDNAs except for the cell line that lacked all four glycosylation sites. Analysis of the singly or doubly deglycosylated FSH mutants revealed that removal of the carbohydrate residue at position 78 on the α-subunit significantly increased the receptor binding affinity of human FSH by 72%. Removal of the other carbohydrate residues had no significant effect on receptor binding. The carbohydrate residue at position 52 on the α-subunit was found to play an essential role in signal transduction as its removal resulted in a significant decrease in potency to 26% of wild type levels. The other individual carbohydrate residues appear to play a minor role in signal transduction, although removal of each residue results in reduced maximal response. The removal of both α-subunit carbohydrates resulted in a significant decrease in biopotency, to 41% of wild type levels; whereas, the removal of both β-subunit carbohydrate chains resulted in a significant increase in biopotency, to 216% of wild type levels. These studies have allowed the identification of site-specific roles for the carbohydrate residues of human FSH. Our data suggest that the carbohydrate residues play a greater role in determining the biological activity of FSH than has been suggested in similar studies of other glycoprotein hormones. (Molecular Endocrinology 8: 722–731, 1994)

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INTRODUCTION

FSH, a member of the glycoprotein hormone family, comprises a common α- subunit and unique β-subunit. Each subunit has two asparagine-linked (Asn-linked) carbohydrate chains which, in human FSH (hFSH), are present at positions 52 and 78 on the α-subunit and on position 7 and 24 of the β-subunit (1). These residues have been shown to play a role in determining the biological activity of FSH, including receptor binding, signal transduction (2–5), and plasma half-life (6–9). However, these roles have been poorly defined. Most attempts to deglycosylate FSH have utilized either chemical cleavage or enzymatic digestion methods (for review see Ref. 10), which cannot discriminate between individual sites and are nonspecific, providing only completely deglycosylated subunits or hormone. Consequently, the study of these molecules has resulted in conclusions that are limited and conflicting and provide little insight into the role of the carbohydrate residues in the biological activity of FSH. Several groups have observed that removal of all four carbohydrate residues from FSH had no affect on receptor binding (4, 5). In contrast, other groups have observed a 6-fold increase in receptor affinity after deglycosylation of FSH (2).

Although it is clear that the carbohydrate residues are important in signal transduction of FSH, it is unclear which specific carbohydrate residue(s) are responsible for this activity. After deglycosylation of the α-subunit of FSH a significant reduction in biopotency, as determined by cAMP production and aromatase activity, has been observed (3). However, in contrast, Padmanabhan *et al.* (5) observed that removal of the α-subunit carbohydrate residues had little effect on signal transduction or biopotency. Moreover, based upon complete deglycosylation of the hormone, they have suggested that the carbohydrate residues on the β-subunit are responsible for the reduction in FSH-stimulated adenylate cyclase activity and steroidogenesis (5).

We have expressed the α- and β-subunit cDNAs in Chinese Hamster Ovary (CHO) cells and shown that the secreted recombinant hFSH (rhFSH) is biologically active *in vitro* (11) and *in vivo* (12). In order to identify and define the roles of the carbohydrate residues it is essential to specifically and completely remove each of the carbohydrate structures. Using site-directed mutagenesis we have substituted the codon for asparagine (Asn) with the codon for glutamine (Gln) and expressed the mutated cDNAs in CHO cells. Disruption of the consensus sequence for Asn-linked glycosylation (Asn-Xaa-Thr/Ser) prevents glycosylation. The rhFSH variants secreted are specifically deglycosylated, with one or more designated carbohydrate residues removed. Determination of the biological activity of these variants has allowed the identification of specific carbohydrate residues that are important in mediating receptor binding and activating signal transduction pathways.

RESULTS AND DISCUSSION

Construction of Mutant cDNAs

Disruption of the recognition signal for Asn-linked glycosylation (Asn-Xaa-Thr/Ser) prevents glycosylation. Site-directed mutagenesis was used to construct mutated α-subunit cDNAs in which the codon for Gln was substituted for the codon for Asn at either one or both of the Asn residues (52, 78, or 52 and 78) and to construct the mutated β-subunit cDNAs in which Gln was substituted for one or both of the Asn residues (7, 24, or 7 and 24). DNA sequence analysis confirmed that the codon for Gln (CAG) had been substituted for the codon for Asn (AAT/C) (data not shown) for each cDNA.

The conservative substitution of Gln for Asn has been reported not to affect protein conformation when used to produce deglycosylated variants of human CG (hCG) (13) and erythropoietin (14, 15). Therefore, the tertiary structure of FSH is least likely to be affected by this substitution. Differences observed in biological activity can thus be primarily attributed to the loss of the carbohydrate residues.

Construction and Clonal Isolation of Mutant Cell Lines

Wild type or mutant subunit expression plasmids were cotransfected into the CHO cell line. Seven mutant cell lines were constructed, four of which expressed a wild type subunit cDNA and a subunit cDNA lacking a single glycosylation site, denoted as α52/β, α78/β, α/β7, and α/β24 cell lines. Two cell lines were constructed that expressed a mutant subunit cDNA lacking both glycosylation sites and the corresponding wild type subunit cDNA, denoted as α52,78/β and α/β7,24 cell lines. A single cell line expressing both α- and β- subunit cDNAs which lacked both glycosylation sites was also constructed and denoted as α52,78/β7,24. The cell line expressing both the wild type α- and β- subunit cDNAs has been previously described (11).

Between four and six stably transfected pools of cells expressing the various wild type mutated cDNAs were selected for neomycin resistance. Clonal cell lines were isolated from G418 selected pools secreting highest levels of rhFSH. Analysis of 10 to 33 clonal cell lines allowed selection for up to 30-fold increases in secretion. For the α52,78/β7,24 cell line 16 G418-resistant pools were made and 83 clonal cell lines screened. None secreted rhFSH.

Southern blot analysis of the genomic DNA of transfected cell lines was carried out in order to confirm the integration of the desired DNA sequences. Subunit-specific primers were used to amplify the genomic DNA from each cell line, which gave a positive autoradiographic signal when hybridized with either an α- or β-subunit ^{32}P-radiolabeled cDNA, respectively (Figs. 1 and 2). These results confirmed that each selected cell line possessed an α- and a β-subunit cDNA, necessary for rhFSH production. To confirm that each cell line contained the correctly mutated subunit cDNA, the polymerase chain reaction (PCR)-amplified DNA fragments were further analyzed by hybridization with site-specific oligonucleotides. The presence or absence of the desired mutation within the α- or β-subunit cDNAs was established using the mutagenesis primers, αN52Q, αN78Q, βN7Q, and N24Q as ^{32}P-radiolabeled probes and by washing under stringent conditions (Figs. 1 and 2).

Immunoassay of Deglycosylated Forms of rhFSH

The degreee of glycosylation is known to influence the immunoreactivity of glycoprotein hormones (16). The dimer-specific monoclonal antibody used in the immunoradiometric assay (IRMA) recognizes each isoform (glycoform) of FSH equally, as isoforms are equally quantified by the IRMA or by use of a separate polyclonal RIA (our unpublished data). These data suggest that the IRMA recognizes the polypeptide portion of the hormone and quantification is not influenced by the degree of glycosylation. Nevertheless, without determination of the absolute mass of rhFSH present, the methods used for quantification of rhFSH isoforms need to be noted when examining our results.

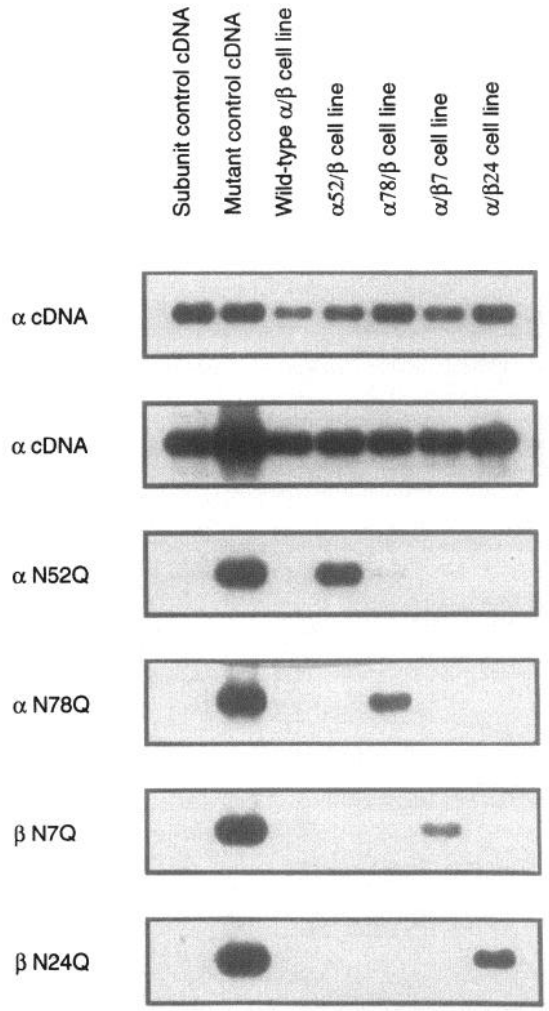

Fig. 1. Southern Blot Analysis of Integrated DNA Sequences in Cell Lines Secreting Singly Deglycosylated rhFSH

Autoradiographs of Southern blots of PCR-amplified α- or β-subunit DNAs derived from genomic DNA from each cell line are shown. Each blot was hybridized with the following probes: α-subunit cDNA, β-subunit cDNA, mutagenesis oligonucleotide αN52Q, mutagenesis oligonucleotide αN78Q, mutagenesis oligonucleotide βN7Q, and mutagenesis oligonucleotide βN24Q. Expression plasmids carrying the mutation of interest were used as positive controls for each probe, and nonmutated α- or β-subunit cDNA was used as a negative control.

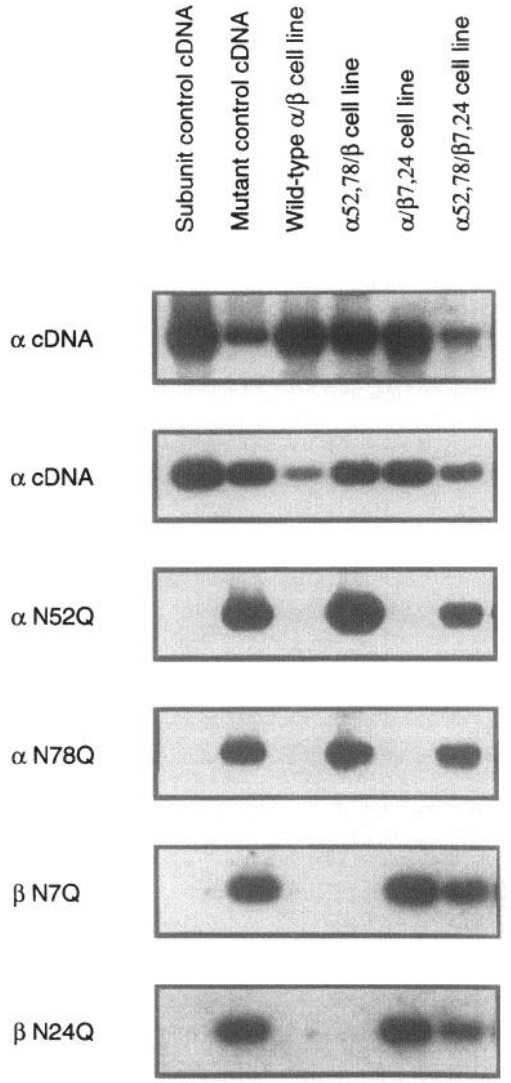

Fig. 2. Southern Blot Analysis of Integrated DNA Sequences in Cell Lines Secreting Doubly Deglycosylated rhFSH

Autoradiographs of Southern blots of PCR-amplified α- or β-subunit DNA, derived from genomic DNA from each cell line, are shown. Each blot was hybridized with the following probes: α-subunit cDNA, β-subunit cDNA, mutagenesis oligonucleotide αN52Q, mutagenesis oligonucleotide αN78Q, mutagenesis oligonucleotide βN7Q, and mutagenesis oligonucleotide βN24Q. Expression plasmids carrying the mutation of interest were used as positive controls for each probe and nonmutated α- or β-subunit cDNA was used as a negative control.

Rate of rhFSH Secretion by Mutant Cell Lines

The suggestion that the carbohydrate residues of FSH are important for secretion has been based primarily on studies with the other glycoprotein hormones (13, 17, 18). Comparison of the rates of secretion of rhFSH by the wild type and mutant cell lines shows that the carbohydrate residues have a significant role in the secretion of the hormone. No rhFSH is secreted by the cell line expressing the completely deglycosylated α- and completely deglycosylated β-subunits, the α52,78/β7,24 cell line. Moreover, removal of only a single carbohydrate residue, especially from the α-subunit, also substantially reduced the secretion rate of rhFSH. This trend suggests that the carbohydrate residues on the α-subunit are more important for secretion of rhFSH than the carbohydrate residues on the β-subunit. This is consistent with the results of Matzuk and Boime (13, 17) who studied the assembly and secretion of deglycosylated hCG variants and showed that the carbohydrate residues on the α- but not the β- subunit were essential for efficient secretion of the dimer. Loss of the carbohydrate residue from α52 position of hCG affected the combination of the subunit with the β-subunit, and

loss of the carbohydrate residue from the α78 site reduced the stability of the dimer (13).

No rhFSH was secreted from the fully deglycosylated α52,78/β7,24 cell line despite numerous separate transfections and the isolation of more than 80 clonal cell lines. Serum-containing or serum-free conditioned medium from α52,78/β7,24 G418-resistant pools was concentrated 10-fold and assayed by IRMA to determine whether the deglycosylated rhFSH mutant produced rhFSH in concentrations below the sensitivity of the immunoassay. No hFSH was detected (data not shown). The α52,78/β7,24 cells were lysed and the intracellular lysate assayed for hFSH. Trace quantities of rhFSH were observed, suggesting that some rhFSH was being synthesized but not being secreted (data not shown). The lack of intra- or extracellular rhFSH is unlikely to be due to lack of detection by the immunoassay as all other forms of deglycosylated rhFSH are recognized by the immunoassay. Moreover, forms possessing only a single carbohydrate residue (*e.g.* α52/β7,24 rhFSH) are readily detected (data not shown). These results suggest that loss of all four carbohydrate residues prevents secretion of rhFSH. This is in contrast to the studies of deglycosylated variants of hCG, in which the totally deglycosylated hormone was secreted (17). This difference may be explained by the fact that fully glycosylated hCG is much more efficiently secreted (19) than fully glycosylated rhFSH (20). Thus, loss of the carbohydrate residues from rhFSH has a greater impact on secretion than does loss of carbohydrate residues from hCG.

Determination of the Dissociation Constant (K_d) of rhFSH

Analysis of binding isotherm data (Fig. 3) allowed determination of the K_d for hFSH, which was found to be 6.4×10^{-11} M. This value is similar to the K_d reported for FSH binding to receptors purified from rat testicular homogenates ($K_d = 1.9 \times 10^{-10}$ M) (21). It is lower than that observed with the recently cloned hFSH testicular receptor ($K_d = 17 \times 10^{-10}$ M) (22). This may be due to species differences between the receptor preparations.

RRA of rhFSH Mutants

The affinity of each of the deglycosylated mutants for the FSH receptor was determined by their ability to competitively displace iodinated, glycosylated hFSH from rat testis membranes in a RRA. The α78/β rhFSH mutant demonstrated an increased affinity for the FSH receptor, as the displacement curve was notably shifted to the left (Fig. 4a). This is reflected in a significantly lower ($P < 0.05$) inhibition constant (K_i) = 138 pM compared to the wild type hormone K_i = 300 pM (Table 1). The other singly deglycosylated mutants, α52/β rhFSH, α/β7 rhFSH, and α/β24 rhFSH, all showed an equal affinity for the receptor as seen with the fully glycosylated hormone (Fig. 4, a and b and Table 1). These results strongly suggest that the carbohydrate residue at position 78 on the α-subunit plays an important role in receptor binding of FSH.

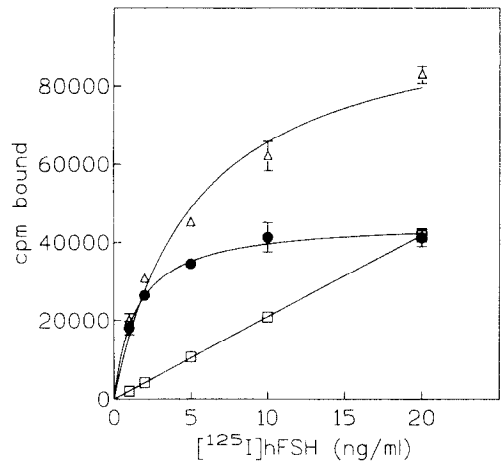

Fig. 3. Saturation Isotherm of [^{125}I]hFSH Binding to FSH Receptors

Increasing concentrations of [^{125}I]hFSH were added to rat testes membranes containing FSH receptors to determine specific binding. Nonspecific binding was determined in the presence of a 10-fold excess of unlabeled rhFSH. Calculation of specific binding of [^{125}I]hFSH was done using the program InPlot. Data from a representative experiment showing mean and SEM is shown. Total binding, *triangle*; nonspecific binding, *square*; specific binding, *solid circle*.

Surprisingly, when the carbohydrate residue from position 78 on the α- subunit was removed in combination with the carbohydrate residue from position 52 on the same subunit (α52,78/β rhFSH), no effect on receptor binding was observed. α52,78/β rhFSH (K_i = 290 pM) and wild type rhFSH (K_i = 300 pM) had similar K_i values. The shift to the left of the displacement curve for the doubly deglycosylated β-subunit (α/β7,24 rhFSH) indicates a slight decrease in receptor binding by α/β7,24 rhFSH (Fig. 4c). This is reflected in the lower, although not significant, K_i for α/β7,24 rhFSH (K_i = 208 pM) compared to that of wild type rhFSH (Table 1). This observation suggests that the β-subunit carbohydrate residues could have a minor role in FSH receptor binding.

Our results are consistent with previous studies which have demonstrated that the carbohydrate residues are involved in receptor binding. A 200–400% increase in receptor binding was observed with chemically deglycosylated FSH in rat and pig granulosa cells and rat and bull testis membranes (10). However, these earlier studies were unable to identify which specific carbohydrate residue was responsible for the increased receptor affinity observed. In contrast to our studies with hFSH, no site-specific role for the carbohydrate residues of hCG in receptor binding was observed (13, 17). All of the deglycosylated hCG variants bound to the LH/CG receptor with affinity equal to that of the fully glycosylated hormone. The difference in the role of the carbohydrate residues of hFSH and hCG may be attributed to differences in glycosylation (*i.e.* the type

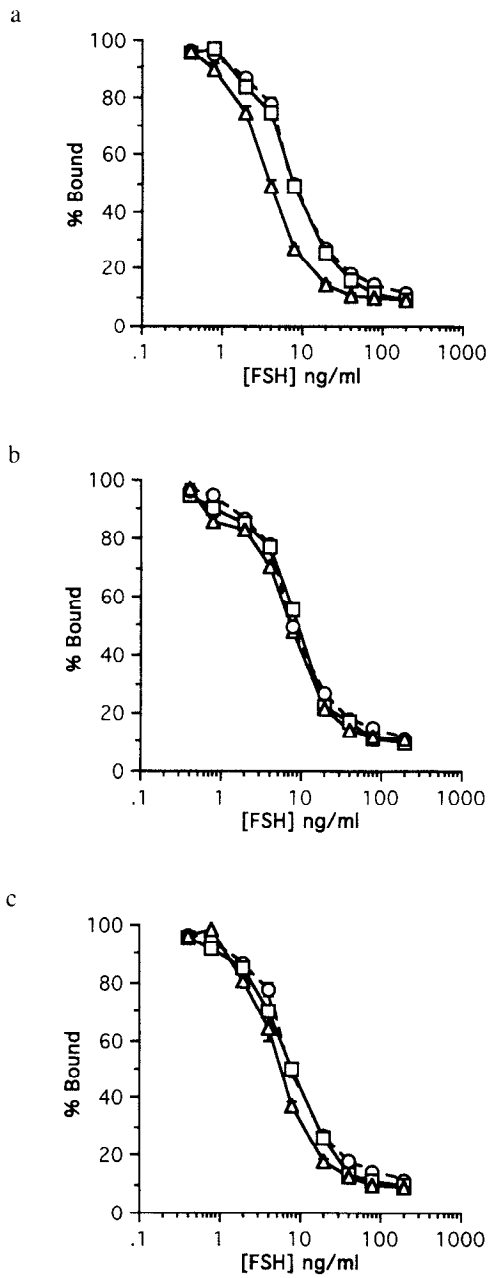

Fig. 4. Displacement of Radiolabeled hFSH by Wild Type and Deglycosylated rhFSH

Receptor binding was measured by the displacement of [^{125}I]hFSH from rat testes membranes by rhFSH and its variants. Bound FSH was separated by centrifugation, and the radioactivity remaining was determined by γ- counting. Addition of 3.7–5.8 pM of [^{125}I]FSH resulted in 32% of the total counts being bound. Results were normalized to this value. Data presented are the mean and SEM of a representative experiment. a, Wild type rhFSH, *circle*; $\alpha 52/\beta$ rhFSH, *square*; $\alpha 78/\beta$ rhFSH, *triangle*. b, Wild type rhFSH, *circle*; $\alpha/\beta 7$ rhFSH, *square*; $\alpha/\beta 24$ rhFSH, *triangle*. c, Wild type rhFSH, *circle*; $\alpha 52,78/\beta$ rhFSH, *square*; $\alpha/\beta 7,24$ rhFSH, *triangle*.

Table 1. Inhibition Constants (K_i), IC_{50} Values, and Hill Coefficients of Wild Type and Deglycosylated rhFSH

FSH	K_i (pM)	IC_{50} (ng/ml)	Hill coefficient (n)
Wild-type	300 ± 17	7.42 ± 0.47	1.72
Single mutations			
$\alpha 52/\beta$	300 ± 43	7.43 ± 1.09	1.54
$\alpha 78/\beta$	138 ± 31[a]	3.44 ± 0.78[a]	1.69
$\alpha/\beta 7$	337 ± 33	8.40 ± 0.81	1.91
$\alpha/\beta 24$	298 ± 43	7.37 ± 1.11	1.71
Double mutations			
$\alpha 52,78/\beta$	290 ± 30	7.16 ± 0.77	1.41
$\alpha/\beta 7,24$	208 ± 44	5.16 ± 1.11	1.85

The IC_{50} values (mean ± SEM) and Hill coefficients for each of the deglycosylated rhFSH mutants and wild type rhFSH were derived using regression analysis. The K_i values (mean ± SEM), derived using the Cheng-Prusoff equation, were calculated for each assay and statistical significance was determined using an analysis of variance. (*, $P < 0.05$.)
[a] $P < 0.05$.

of carbohydrate structures present) between the hormones (23–25). Such differences have been shown to influence receptor binding (26, 27).

As removal of the carbohydrate at position 78 on the α-subunit resulted in increased binding of hFSH to its receptor, it is possible that this carbohydrate residue restricts the binding of the fully glycosylated hormone. The position of the carbohydrate residue within the putative receptor binding domain (28) and the bulky nature of the oligosaccharide may explain such restricted binding. Surprisingly, increased receptor binding is not observed when the carbohydrate residues at both positions $\alpha 52$ and $\alpha 78$ are removed. This suggests that some change (conformational or otherwise) must be occurring.

In Vitro Bioactivity of rhFSH Mutants

As the deglycosylated rhFSH mutants were capable of binding to FSH receptor, their ability to stimulate aromatase activity in Sertoli cells was assessed. The dose-response curve of $\alpha 52/\beta$ rhFSH was notably shifted to the right (Fig. 5a). This is clearly reflected by the significantly lower calculated biopotency of $\alpha 52/\beta$ rhFSH, which is 3-fold lower than that of the wild type rhFSH (Table 2). Moreover, $\alpha 52/\beta$ rhFSH only reaches 50% of the maximal stimulation achieved by the wild type molecule. These results clearly implicate the carbohydrate residue at position 52 on the α-subunit as playing a major role in signal transduction of the hormone. In contrast, the dose-response curves of the other singly deglycosylated rhFSH mutants, $\alpha 78/\beta$ rhFSH, $\alpha/\beta 7$ rhFSH, and $\alpha/\beta 24$ rhFSH, more closely match that of the wild type rhFSH, indicating equivalent bioactivity (Fig. 5, a and b).

The carbohydrate residues of the α-subunit of FSH have previously been shown to be essential for signal transduction (3). Sairam and Bhargavi (3) showed that

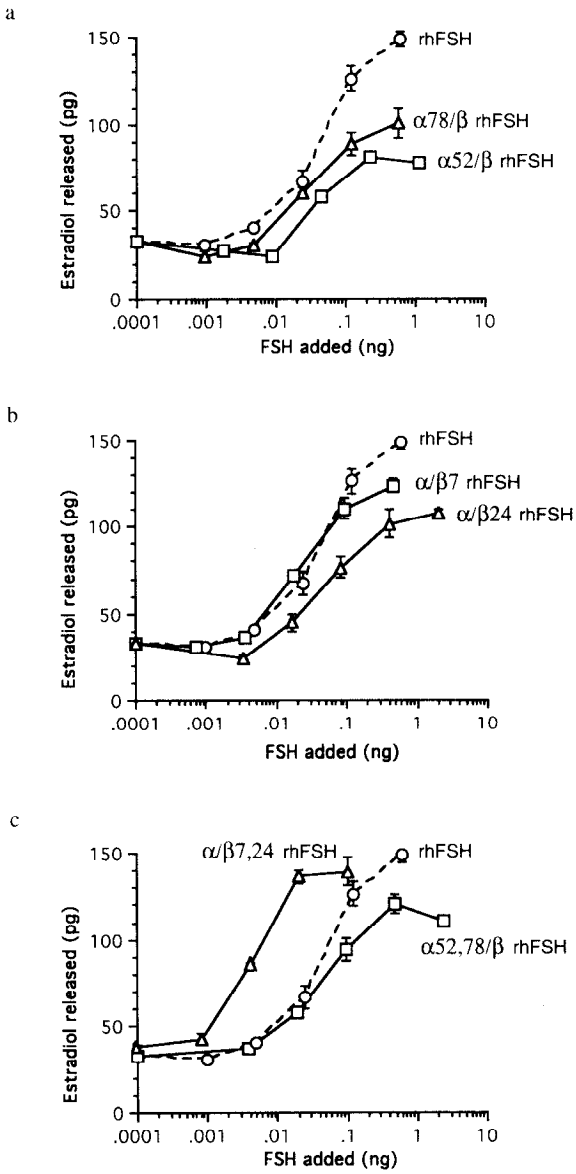

Fig. 5. Stimulation of Sertoli Cell Estradiol Production by Wild Type rhFSH and Deglycosylated rhFSH

Sertoli cells were incubated with varying doses of rhFSH and estradiol (E_2) released (picograms) measured by RIA. Data presented are the mean and SEM of a representative experiment. The control points in which no rhFSH was added are indicated on the *ordinate axis*. a, Wild type rhFSH, *circle*; $\alpha 52/\beta$ rhFSH, *square*; $\alpha 78/\beta$ rhFSH, *triangle*. b, Wild type rhFSH, *circle*; $\alpha/\beta 7$ rhFSH, *square*; $\alpha/\beta 24$ rhFSH, *triangle*. c, Wild type rhFSH, *circle*; $\alpha 52,78/\beta$ rhFSH, *square*; $\alpha/\beta 7,24$ rhFSH, *triangle*.

recombination of a deglycosylated hFSH α-subunit with a glycosylated hFSH β-subunit could not stimulate cAMP production, whereas a glycosylated hFSH α-subunit recombined with a deglycosylated hFSH β-subunit could stimulate cAMP production as effectively as the wild type hormone. Likewise, specific removal of the carbohdyrate residue at position 52 on the α-subunit of hCG, by site-directed mutagenesis, was shown

Table 2. *In Vitro* Biological Activity of Wild Type rhFSH and Deglycosylated rhFSH Mutants

rhFSH	FSH Biological Activity (IU/μg)		Maximum Estradiol Released (pg)	
Wild-type	323	(205–512)	149	(141–157)
Single mutants				
$\alpha 52/\beta$	84[a]	(58–124)	78[a]	(76–79)
$\alpha 78/\beta$	395	(269–599)	100[a]	(86–114)
$\alpha/\beta 7$	221	(148–327)	124[a]	(117–130)
$\alpha/\beta 24$	241	(156–390)	108[a]	(103–112)
Double mutants				
$\alpha 52,78/\beta$	132[a]	(88–205)	110[a]	(108–113)
$\alpha/\beta 7,24$	697[a]	(697–1098)	140	(136–146)

The biological activity of each rhFSH molecule was determined by *in vitro* Sertoli cell bioassay with the 1st International Standard, human pituitary FSH (83/575) as standard. Biological activities are normalized to equal masses of FSH as determined by IRMA. 95% confidence limits are indicated.
[a] $P < 0.05$.

Table 3. Relative Receptor Affinity and *In Vitro* Bioactivity of rhFSH and Deglycosylated rhFSH Mutants

FSH	Relative Receptor Affinity ($1/K_i$) (%)	Relative *in Vitro* Bioactivity (%)
Wild type	100	100
Single mutants		
$\alpha 52/\beta$	95	26[a]
$\alpha 78/\beta$	172[a]	122
$\alpha/\beta 7$	96	69
$\alpha/\beta 24$	98	75
Double mutants		
$\alpha 52/78/\beta$	91	41[a]
$\alpha/\beta 7,24$	141	216[a]

The relative activities of each of the deglycosylated rhFSH mutants, compared to wild type rhFSH are presented. The inverse of the K_i (affinity instead of inhibition) of the wild type is taken to be 100%.
[a] Significant differences ($P < 0.05$), as determined in Tables 1 and 2.

to significantly diminish adenylate cyclase activity (13). Removal of any one of the other hCG carbohydrate residues did not affect cAMP production. Our results are consistent with these reports. As the α-subunit is common to both hCG and hFSH it is not surprising that the same $\alpha 52$ carbohydrate residue has a significant role in both FSH- and hCG-mediated signal transduction.

The carbohydrate residues, other than that at position 52 on the α-subunit, also contribute to signal transduction. Removal of the carbohydrate residues at position 78 on the α-subunit or at either position on the β-subunit (7 or 24) of hFSH prevented the hormone from achieving maximal stimulation in the Sertoli cell bioassay, despite equipotent activity at lower doses (Table 3). Removal of the equivalent carbohydrate res-

idues from hCG did not affect the maximal stimulation of the deglycosylated hCG variants (13, 17). These results, taken together with those of receptor binding and secretion of the deglycosylated rhFSH variants, strongly suggest that the carbohydrate residues play a much greater role in determining the biological activity of hFSH than they do in the biological activity of hCG. Despite having a common α-subunit, hCG and hFSH do not necessarily share the same structure/function properties. For example, removal of the residue Lys 91 from the carboxy terminal of the α-subunit prevents hFSH from binding to its receptor, whereas the same deletion has no effect on hCG receptor binding (29).

One explanation for the inability of α78/β rhFSH, α/β7 rhFSH, and α/β24 rhFSH to reach maximal levels of stimulation is that the deglycosylated rhFSH molecules function as partial agonists rather than as full agonists. Alternatively, it is possible that the carbohydrate residues may affect signal transduction by modulating secondary messengers other than cAMP. Recently, it has been documented that hFSH induces changes in intracellular Ca^{2+} levels, potentially modulating hFSH signal transduction (30–32). It is unclear whether the FSH-stimulated Ca^{2+} influx acts as an alternate primary signal transduction mechanism or whether the fluxes act to amplify or modulate the primary cAMP response.

Loss of both carbohydrate residues from the α-subunit (α52,78/β rhFSH) resulted in significantly lower ($P < 0.05$) bioactivity (132 IU/μg) compared to wild type rhFSH (323 IU/μg) (Fig. 5c). It is likely that the loss of the carbohydrate residue at position 52 on the α-subunit is primarily responsible for this decreased activity. Removal of both carbohydrate residues from the β-subunit (α/β7,24 rhFSH) resulted in significantly ($P < 0.05$) increased bioactivity. However, maximal stimulation by α/β7,24 rhFSH was equivalent to that observed for the fully glycosylated wild type hormone. These results may be explained by the concept of 'spare receptors.' It has been shown that human LH (hLH) activation of less than 1% of the LH/CG receptor population is required for maximal induction of steroidogenesis. Occupancy of the remaining 99% of receptors (spare receptors) results in production of excess cAMP but no increase in Leydig cell testosterone (33, 34). Hence, it is likely that α/β7,24 rhFSH, which is more potent than wild type rhFSH, will activate the 1% of available receptors more rapidly than the less potent wild type rhFSH. However, once the available receptors are activated, no further increase in steroidogenesis will occur; thus maximal estradiol production by α/β7,24 rhFSH and wild type rhFSH will be equal.

The relative receptor affinity and *in vitro* bioactivity data for each of the rhFSH variants are presented in Table 3. There is no simple correlation between receptor binding and signal transduction. The high affinity of the mutant α78/β rhFSH is not reflected by an increased bioactivity of the hormone. Similarly, the mutant α52/β rhFSH has normal binding but significantly decreased bioactivity. Current understanding of receptor binding and signal transduction suggests that after FSH binds to its receptor, the combined hormone-receptor complex undergoes a conformational change in order to activate the G protein complex (2). It is likely that the carbohydrates play a direct role in the interaction of the hormone-receptor complex with the G proteins. Recent studies with proteoliposomes have suggested that the oligosaccharides on hCG are necessary for the interaction of the LH/CG receptor with the G_s proteins (35). It is therefore likely that the carbohydrates are not essential for binding to the receptor, as on no occasion does their loss result in decreased affinity for the receptor. The carbohydrates may, however, play a major role in the interaction of the hormone-receptor complex with the G proteins, by potentially altering the conformation of the hormone and/or the hormone-receptor complex.

MATERIALS AND METHODS

Hormones and Reagents

Plasmid DNA containing expression cassettes encoding the wild type hFSH α-subunit (pAxNeoRx/α) and hFSH β-subunit (pAxNeoRx/β) cDNAs under the transcriptional control of the human β-actin promoter were provided by Pacific Biotechnology Ltd. (Sydney, Australia) (11). Highly purified preparations of human pituitary FSH (80/1 and 80/2) were gifts of the hormone distribution program of the Endocrine Society of Australia with *in vitro* potencies of 4479 IU/mg and 5276 IU/mg, respectively, using the first International Reference Preparation pituitary FSH/LH for bioassay (69/104) as standard.

Construction of Mutant cDNAs

M13 site-directed mutagenesis (36) was used to construct the mutant cDNAs. The cDNA encoding wild type hFSH α-subunit [a 620-base pair (bp) *Hin*dIII fragment] and the cDNA encoding wild type FSH β-subunit (a 400-bp *Eco*RI-*Sal*I fragment) were subcloned into the bacteriophage M13 mp18. 27-mer oligonucleotide primers, αN52Q (5′-TTG GTC CAA AGG CAG GTC ACC TCA GAG-3′) and αN78Q (5′-TTC AAA GTG GAG CAG CAC ACG GCG TGC-3′), were used to substitute the codon for the amino acid Asn (N), AAC/T with that for Gln (Q), CAG at positions 52 and 78 on the α-subunit cDNA, respectively. The 27-mer oligonucleotide primers βN7Q (5′-TGT GAG CTG ACC CAG ATC ACC ATT GCA-3′) and βN24Q (5′-TGC ATA AGC ATC CAG ACC ACT TGG TGT-3′) were used for mutagenesis of the Asn at positions 7 and 24 on the β-subunit cDNA. Competent *Escherichia coli* JM109 cells were transformed with the recombinant phage and screened using the appropriate [^{32}P]γATP-labeled mutagenesis primers as probes. Tetramethylammonium chloride (TMACl, 3M) was used for stringent washing (37). The integrity of the mutant expression plasmid was verified by restriction enzyme analysis and DNA sequencing.

The expression plasmid pAxNeoRx/β7,24, in which both Asn 7 and Asn 24 were substituted with Gln, was generated using the β-subunit mutant cDNA pAxNeoRx/β7 as a template for a second round of mutagenesis. The expression plasmid pAxNeoRx/α52,78, in which both Asn 52 and Asn 78 codons are substituted with the Gln codon, was generated using a recombinant PCR method (38). The oligonucleotide primers, 5′pAx (5′-AAG GAC TCG GCG CGC CGG AA-3′) and 3′pAx (5′-AAC TCA TCA ATG TAT CTT ATC ATG 3′) flanked 5′- and 3′-regions, respectively of the α-subunit cDNA within the expression plasmid pAxNeoRx/α78. These oligonucleotide primers covered the *Hin*dIII restriction site. The primers αN78Q and 3′αN78Q (5′-GCA CGC CGT GTG CTG CTC CAC TTT

GAA-3′) spanned the Asn 78 codon to introduce the mutation from the 5′- and 3′-ends of this residue. Two overlapping primary PCR products were generated using the primers 5′pAx and 3′αN78Q and primers 3′pAx and αN78Q, respectively. In a second PCR reaction, the two overlapping primary products were denatured and allowed to reanneal, and the resulting heteroduplexes were amplified using the primers 3′pAx and 5′pAx. The amplified product was digested with *Hin*dIII and subcloned into pAxNeoRx and transformed into *E. coli* JM109. Resulting colonies were screened and plasmid DNA sequenced to confirm incorporation of the mutation.

DNA Transfection and Clone Selection

Plasmid DNA was transfected into CHO cells using a $CaCl_2$ precipitation procedure (39). The α-subunit cDNA was cotransfected with the β-subunit cDNA in a ratio of 2:1. Stable cell lines were selected on the basis of neomycin resistance, encoded on the pAxNeoRx plasmid, by addition of the analog G418 (800 μg/ml). G418-resistant cells were switched to serum-free conditions for 48 h and the conditioned medium then assayed for the secretion of rhFSH. To obtain a clonal isolate with highest secretion rate, the stable cell lines were cloned by limiting dilution. High producing cell lines were grown to confluency and switched to serum-free medium, and the rhFSH was harvested from the conditioned media every 48 h. Cells were maintained in culture for up to 2 weeks, after which production declined. The conditioned medium was concentrated and diafiltered against PBS using an Amicon diafiltration unit (Amicon Corp., Lexington, MA; mol wt cut-off 10K). The concentrated rhFSH was stored at −70 C to prevent neuraminidase activity.

PCR of Integrated Genomic DNA and Southern Blot Analysis

Cells from stably transfected cell lines were trypsinized from a 125-cm^2 tissue culture flask and washed in ice-cold PBS. The cells were resuspended in 1 ml extraction buffer, 0.1 M NaCl, 10 mM Tris-HCl, pH 8.0, 0.5% sodium dodecyl sulfate, and 0.1 mg/ml Proteinase K and incubated at 65 C for 2 h. The DNA was purified by phenol/chloroform extraction and ethanol precipitation. The α- and β-subunit cDNAs were amplified using PCR with the following primer pairs. A subunit, 5′α (5′-TCA AGC TTG GCA GTA ACC-3′), 3′α (5′-TAG AAA GCT TAA TGC TGT AT-3′); β-subunit, 5′β (5′-CGA CCA TGA AGA CAC TCC AGT-3′), 3′β (5′-TCA CCA AAG GAG CAG TAG CTG-3′). The integrity of the cDNA and introduced mutation were verified using Southern blot analysis. [^{32}P]γATP-labeled oligonucleotide primers αN52Q, αN78Q, βN7Q, and βN78Q were used as probes to check for the presence of the desired mutation. Hybridized filters were washed under stringent conditions in the presence of 3 M TMACl.

Immunoassay for Quantification of rhFSH

A commercially available IRMA (Bioclone Australia, Sydney, Australia) was used to quantify rhFSH and the deglycosylated rhFSH. One of the monoclonal antibodies used in this assay recognizes only the intact hFSH dimer, believed to be a protein only epitope. The IRMA shows no cross-reactivity with either free α- or free β-subunits of FSH nor with the other glycoprotein hormones. The intraassay coefficient of variation was 2.5%, and the interassay coefficient of variation was 4.6%. A standard curve ranging from 0.2 ng/ml to 200 ng/ml was constructed using pituitary hFSH preparation 80/1 as standard. Recombinant hFSH receptor affinities and biological activities are normalized to equal mass values of immunoreactive rhFSH.

Binding Isotherm and RRA

Crude membrane preparations were obtained from decapsulated testes of 21-day-old Wistar rats (Garvan Institute, Sydney, Australia) and stored at −20 C for use in the RRA, as previously described (40). Wild type rhFSH or deglycosylated rhFSH and labeled [^{125}I]hFSH tracer (NEN-Dupont, Wilmington, DE; 130–150 μCi/μg) were incubated with the receptor preparation at room temperature for 20 h and then centrifuged. The K_d of rhFSH for the receptor preparation was determined by analysis of binding isotherm data using the program InPlot (GraphPad, San Diego, CA). The percentage of bound rhFSH (where binding of [^{125}I]hFSH in the absence of unlabeled hormone was taken to be 100%) was determined by competitive displacement of the iodinated hFSH with increasing doses of rhFSH. The Cheng-Prusoff equation was used to calculate the K_i of rhFSH and the deglycosylated mutants. The interassay coefficient of variation was less than 15%.

FSH *in Vitro* Bioassay

FSH *in vitro* bioassay is based on the measurement of estradiol converted from 19-hydroxy-androstene-dione by FSH-induced aromatase activity in Sertoli cells from immature rats as described (41) with modifications (42). Briefly, Sertoli cells were isolated from 10-day old male Sprague-Dawley rats (Monash University, Clayton, Australia) and cultured in Dulbeco's modification of Eagle's medium with fetal calf serum for 24 h. 19-Hydroxy-androstene-dione was added and the cells were incubated for 2 h at room temperature. FSH, in the presence of a phosphodiesterase inhibitor, was added to the cells which were then incubated for 24 h at 37 C. Estradiol content of the conditioned medium was measured by RIA. The intraassay coefficient of variation was 12%.

Statistical Analysis

Differences between wild type rhFSH and deglycosylated rhFSH preparations were determined by analysis of variance with significant differences defined by $P < 0.05$.

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Erratum

The article entitled "Unique sequences in the guinea-pig glucocorticoid receptor induce constitutive transactivation and decrease steroid sensitivity" by Keightley and Fuller (*Molecular Endocrinology* **8:** 431–439, 1994) includes studies using a chimeric glucocorticoid receptor (GR) expression vector, gpGR-wt, in which the ligand-binding domain (LBD) of the human GR expression vector (pRShGRnx) was replaced by the guinea-pig GR LBD. Resequencing of this construct during the course of the construction of further human:guinea-pig GR chimeric LBDs has revealed that an adenine occurring in a run of seven adenines at position 1458 was deleted by Taq polymerase during the PCR generation of the guinea-pig LBD cassette. The new reading frame immediately encodes a stop codon; the constitutive activity seen in Fig. 3B is thus the result of this truncation mutant. What remains unexplained is the clear dexamethasone induction (Fig. 3B) which is blocked by RU486 (Fig. 5). This is not due to endogenous receptors in CV-1 cells nor to contamination of the plasmid with wild type expression vector. Analysis using the correct guinea-pig LBD in the same expression vector yields the dose-response curve shown below. This exhibits half-maximal induction at ~3 nM dexamethasone of the human GR at ~0.8 nM (Fig. 3A). The guinea-pig GR LBD in the cysteine-632 mutant (Fig. 6) contains the correct sequence which clearly explains its lack of constitutive activity. The relative importance of the tryptophan substitution is now less clear. In addition, when the correct guinea-pig LBD chimera is treated with 8-bromo-cAMP, the paradoxical response (Fig. 5) is no longer observed. In conclusion, while the guinea-pig LBD does not confer constitutive activity, it does confer resistance which must be due to one of the 24 substitutions; the elucidation of the critical residue(s) is the basis of our ongoing studies. The authors are extremely embarrassed by this and sincerely apologize for any inconvenience caused.