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Molecular Cloning of Pituitary Glycoprotein α -Subunit and Follicle Stimulating Hormone and Chorionic Gonadotropin β -Subunits from New World Squirrel Monkey and Owl Monkey

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Abstract

The goal of this study was to characterize the gonadotropins expressed in pituitary glands of the New World squirrel monkey (*Saimiri* sp.) and owl monkey (*Aotus* sp.). The various subunits were amplified from total RNA from squirrel monkey and owl monkey pituitary glands by reverse transcription-polymerase chain reaction and the deduced amino acid sequences compared to those of other species. Mature squirrel monkey and owl monkey glycoprotein hormone α -polypeptides (96 amino acids in length) were determined to be 80% homologous to the human sequence. The sequences of mature β subunits of follicle stimulating hormone (FSH β) from squirrel monkey and owl monkey (111 amino acids in length) are 92% homologous to human FSH β . New World primate glycoprotein hormone α -polypeptides and FSH β subunits showed conservation of all cysteine residues and consensus N-linked glycosylation sites. Attempts to amplify the β -subunit of luteinizing hormone from squirrel monkey and owl monkey pituitary glands were unsuccessful. Rather, the β -subunit of chorionic gonadotropin (CG) was amplified from pituitaries of both New World primates. Squirrel monkey and owl monkey CG β are 143 and 144 amino acids in length and 77% homologous with human CG β . The greatest divergence is in the C terminus, where all four sites for O-linked glycosylation in human CG β , responsible for delayed metabolic clearance, are predicted to be absent in New World primate CG β s. It is likely that CG secreted from pituitary of New World primates exhibits a relatively short half-life compared to human CG.

Keywords

reverse transcription polymerase chain reaction; O-linked glycosylation; N-linked glycosylation; phylogenetic analysis

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INTRODUCTION

Follicle stimulating hormone (FSH), luteinizing hormone (LH), and chorionic gonadotropin (CG) are a family of hormones that regulate gonadal function (Themmen and Huhtaniemi, 2000; Garcia-Campayo and Boime, 2001; Dias et al., 2002; Ben-Menahem, 2004). LH and FSH are synthesized and secreted by cells in the anterior pituitary gland, whereas CG is made predominantly by placental trophoblasts during early pregnancy. Each of these hormones, as well as thyroid stimulating hormone (TSH) that regulates thyroid function, is made up of a heterodimer of a common α -subunit non-covalently bound to a β -subunit. The α -subunit is encoded by a single gene, whereas the unique β -subunits arise from separate genes and confer the biological specificity of each of the hormones. Each of the chains is extensively glycosylated, and the nature of the oligosaccharide chain plays an important role in the processing and biological activities of the respective hormones (Baenziger, 1996; Ulloa-Aguirre et al., 2001).

The coordinated secretion and activities of FSH and LH are essential for control of ovarian and testicular function. In females, FSH is important for follicular development and maturation. In males, FSH regulates gonadal maturation and spermatogenesis. In females, LH is essential for follicular rupture, release of the oocyte, and maintenance of progesterone secretion from the corpus luteum. In males, LH stimulates steroidogenesis and androgen production by Leydig cells of the testis. CG, expressed in the placenta of higher primates (Maston and Ruvalo, 2002), is indispensable for successful progression of pregnancy. Acting through the LH/CG receptor, CG is responsible for preventing regression of the corpus luteum and stimulates continued progesterone output that maintains the uterine lining in a state receptive for implantation.

The goal of the present study was to obtain the primary amino acid sequences of pituitary gonadotropins in two New World primate species, squirrel monkey and owl monkey, and compare them to sequences in Old World primates and man. The study was conducted for several reasons. Squirrel monkeys and owl monkeys are among the most commonly used New World primates in biomedical research (Abee, 2000). As the availability of Old World primates, such as rhesus monkeys, for research in a number of arenas is becoming limiting, there is support for expanding the use of New World primates (Abbott et al., 2003). An area of interest is assisted reproductive technologies, which offers opportunities for *in vitro* fertilization, embryo transfer, and embryo and gamete cryopreservation. It is critical in these studies to achieve ovarian hyperstimulation with exogenous FSH. However, New World primates are quite refractory to the effects of recombinant human FSH (Marshall et al., 2003; Schuler et al., 2006). These results suggest that human FSH may exhibit low binding to the New World primate FSH receptor. The β -subunits of two New World marmoset species were cloned and sequenced in 2001 (Wolfgang et al., 2001), but the sequences of squirrel monkey and owl monkey FSH had yet to be determined and compared to Old World primate FSH sequences.

The need to obtain the sequences of other pituitary gonadotropins in the squirrel monkey and owl monkey is perhaps more pressing. Recent studies have suggested that in common marmosets the β -subunit of LH is not present in the pituitary gland. Rather, CG is the only gonadotropin with luteinizing function expressed in the pituitary gland of the common marmoset (Gromoll et al., 2003; Muller et al., 2004). This intriguing finding, which is accompanied by the expression in common marmosets of a mutant form of the LH receptor that is activated by CG but not LH (Muller et al., 2003), warrants confirmation in other New World primates. We attempted to amplify LH and CG β -subunit cDNAs from squirrel monkey and owl monkey pituitary glands. The CG β -subunit cDNAs were successfully amplified, and the primary sequences and predicted glycosylation patterns compared to those of common marmoset and human CG and human LH.

METHODS AND MATERIALS

Total RNA was isolated with RNA Stat-60 (Tel-Test “B”, Friendswood, TX) from pituitary glands from female Bolivian squirrel monkeys (*Saimiri boliviensis boliviensis*, animal numbers 90802, 1331, and 90795) and from a male (85277) and a female (87001) owl monkey (*Aotus nancymaae*). Tissues were obtained at necropsy from the Tissue and Biological Fluids Resource of the Center for Neotropical Primate Research and Resources (CNPRR) at the University of South Alabama, supported by grants RR-01254 and RR-20052 (to C.R. Abee) from the National Institutes of Health. Squirrel monkey 1331 was colony born and 19 years of age, whereas squirrel monkeys 90802 and 90795 were wild-caught, but had been at the CNPRR for more than 16 years. Owl monkeys were wild-caught and had been at the CNPRR since at least 2003. Owl monkey 85277 was at least 16 years of age, whereas monkey 87001 was at least five-years-old. Prior to necropsy, squirrel monkeys were housed indoors in laboratory breeding groups and exposed to a natural light/dark cycle that tracked local sunrise and sunset. Owl monkeys were also housed indoors in breeding pairs, but were exposed to an offset light/dark cycle (light 3 a.m. to 3 p.m.; dark 3 p.m. to 3 a.m.).

cDNAs were obtained from pituitary gland RNA by reverse transcription-polymerase chain reaction (RT-PCR) using the QIAGEN OneStep RT-PCR Kit (QIAGEN, Valencia, CA). Amino acid sequences were deduced from nucleotide sequences of the PCR products. Full-length squirrel monkey and owl monkey glycoprotein hormone α -subunits were amplified using primers: 5'-AAG CCC AGA GAA AGG AGC GCC AT-3' and 5'-GTC ATC AAG ACA GCA CTT GG-3', positions 81–103 and 480–461 in human glycoprotein hormone α -polypeptide cDNA (GenBank accession number NM_000735), respectively. Full-length squirrel monkey and owl monkey FSH β -subunits were amplified using the upstream primer 5'-TAA GGT CGA CCG AGG ATG-3', based on the primer used to amplify rhesus monkey FSH β -subunit (Wolfgang et al., 2001), and downstream primer 5'-GTA TGT GGC CTG AAA TGT CCA CTG-3' that represents positions 487–464 in the human FSH β -subunit cDNA (GenBank accession number NM_000510), respectively. Full-length squirrel monkey and owl monkey CG β -subunits were amplified using primers: 5'-GAC GCA CCA AGG ATG GAG-3' and 5'-GCG GAT TGA GAA GCC TTT A-3', positions 354–371 and 879–861 of the human CG β -subunit cDNA (GenBank accession number NM_000737), respectively. Sequences were obtained across both strands and were confirmed in DNA fragments amplified from RNA isolated independently from multiple pituitary glands.

For phylogenetic analysis, the alignment of the amino acid sequences used ClustalX (1.83) (Thompson et al., 1994). The alignment parameters were those suggested by Hall (2004). Values used for pairwise alignments were gap opening penalty 35 and gap extension penalty 0.75. Values for multiple alignment were gap opening penalty 15, gap extension penalty 0.3, and delay divergent sequences 25%. Protein weight matrix chosen from the multiple alignment parameters menu was Gonnet series. Phylogenetic analyses were conducted with MEGA version 3.1 software (Kumar et al., 2004). Maximum parsimony, minimum evolution, and neighbor-joining methods with Poisson-correction for multiple amino acid substitutions produced very similar topologies. The tree inferred from the neighbor-joining method is shown.

RESULTS AND DISCUSSION

Squirrel monkey and owl monkey glycoprotein hormone α -polypeptides

PCR products of approximately 400 bp were amplified by RT-PCR of total RNA isolated from squirrel monkey or owl monkey pituitary glands using primers corresponding to sequences in the 5'- and 3'-untranslated regions of human glycoprotein hormone α -subunit cDNA. cDNA fragments were sequenced across both strands, and nucleotide sequences deposited in GenBank with accession numbers DQ143872 (squirrel monkey α -subunit) and DQ200806 (owl monkey

α -subunit). Nucleotide sequences of squirrel monkey and owl monkey α -subunits are 92% identical to each other and 83% and 84% identical to that of human α -subunit cDNA (NM_000735), respectively. Squirrel monkey and owl monkey α -subunit cDNAs share 93% identity with that of another New World primate, the common marmoset (U04446).

The amino acid sequences of squirrel monkey and owl monkey glycoprotein hormone α -polypeptides were deduced from the cDNA sequences and compared to those of other species (Figure 1A). The cDNAs of squirrel monkey and owl monkey α -subunits each encode proteins of 120 amino acids, including putative signal peptides of 24 amino acids and mature proteins of 96 amino acids (predicted by SignalP 3.0 server on-line program (Bendtsen et al., 2004), <http://www.cbs.dtu.dk/services/SignalP/>). The position of the signal peptide cleavage site is similar to that in glycoprotein hormone α -subunits of other species (Dias et al., 2002). The amino acid sequences of mature squirrel monkey and owl monkey α -subunits are 86% identical (93% similar) to each other and 69% identical (79–80% similar) to that of mature human α -subunit. Mature squirrel monkey and owl monkey α -subunits are 89% and 87% identical (96% similar) to the mature common marmoset α -subunit. Only human α -subunit has a gap of four amino acids at position three of the mature protein, most likely as a result of a mutation at the exon/intron splice site (Chin et al., 1983). Using the ExPASy ProtParam tool (<http://us.expasy.org/cgi-bin/protparam>), the M_r s of mature squirrel monkey and owl monkey α -subunits were calculated to be 10884.5 and 10681.2, and the theoretical isoelectric points of the polypeptides were determined to be 8.34 and 8.54, respectively. Mature human α -subunit has a M_r of 10205.7 and an isoelectric point of 8.38. Two potential N-linked glycosylation sites, at positions 56 and 82 of the mature protein, were identified in squirrel monkey and owl monkey glycoprotein hormone α -subunits using the NetNGlyc 1.0 Server on-line program (<http://www.cbs.dtu.dk/services/NetNGlyc/>). The N-linked glycosylation sites as well as ten cysteine residues involved in disulfide bridge formation (Lapthorn et al., 1994) are conserved across all species examined. Also relatively well conserved is the sequence PLRSKK (positions 40–45 of human α -glycopeptide) that serves as a recognition marker for addition of N-acetylgalactosamine sulfate to oligosaccharides (Mengeling et al., 1995). The sequence PF/V/LRSQK of New World primate α -glycopeptide (Figure 1A) is predicted to support this process efficiently. Glycoprotein hormone α -subunit is not thought to undergo O-linked glycosylation (Bousfield et al., 1996).

Squirrel monkey and owl monkey follicle stimulating hormone β -subunits

Complimentary DNA fragments of the β -subunits of squirrel monkey and owl monkey FSH were also amplified from pituitary glands using RT-PCR. Fragments (approximately 400 bp in length) were sequenced across strands and deposited in GenBank with accession numbers DQ143873 (squirrel monkey FSH β -subunit) and DQ200807 (owl monkey FSH β -subunit). Nucleotide sequences of squirrel monkey and owl FSH β -subunits are 97% identical to each other and 92% identical to that of human FSH β -subunit cDNA (NM_000510). Deduced amino acid sequences were compared to FSH β -subunits of other primate species (Figure 1B) and analyzed by the SignalP 3.0 program (Bendtsen et al., 2004). cDNAs of squirrel monkey and owl monkey FSH β -subunits each encode a protein of 129 amino acids that includes a signal peptide of 18 amino acids, similar to the size of the signal peptide of FSH β of other species (Ulloa-Aguirre and Timossi, 1998). Mature FSH β -subunits demonstrate high homology across species (Wolfgang et al., 2001; Dias et al., 2002), and squirrel monkey and owl monkey FSH β -subunits are 97% identical (98% similar) to each other and 90–91% identical (92% similar) to human FSH β -subunit. The M_r s of squirrel monkey and owl monkey FSH β -subunits were calculated (ExPASy ProtParam tool) to be 12371.8 and 12451.9, respectively, and the theoretical isoelectric point of each protein was determined to be 4.63. Mature human FSH β -subunit has a M_r of 12485.1 and an isoelectric point of 5.91. The lower isoelectric point of the peptide backbone of New World primate FSH β likely reflects the substitution of neutral amino

acids in New World primate FSH β for basic amino acid residues in human FSH β . We do not know the influence of oligosaccharide composition on the overall charge of New World primate FSH, but putative N-linked glycosylation sites at positions 7 and 24 (NetNGlyc 1.0 Server) and twelve cysteines are conserved among species. FSH β is not thought to undergo O-linked glycosylation (Bousfield et al., 1996).

Recent studies have suggested that New World marmosets and squirrel monkeys are relatively refractory to ovarian stimulation with recombinant human FSH (Marshall et al., 2003; Schuler et al., 2006). There is no structural information on FSH receptors from any New World primates. However, the X-ray structure of human FSH in complex with the FSH receptor was recently reported (Fan and Hendrickson, 2005). It is recognized that even single nucleotide polymorphisms leading to changes in FSH receptor structure can influence FSH responsiveness (Gromoll and Simoni, 2005). Structural differences between New World primate and human FSH receptors may be responsible for conferring low responsiveness of New World primate FSH receptor to human FSH.

Squirrel monkey and owl monkey chorionic gonadotropin β -subunits

We initially attempted to amplify LH β -subunit cDNA from squirrel monkey and owl monkey pituitary glands using primers representing 5'- and 3'-untranslated sequences of human LH cDNA (NM_000894). A fragment of approximately 450 bp similar to human LH β -subunit was amplified from the squirrel monkey pituitary gland, but no product was amplified from owl monkey pituitary. The fragment amplified from squirrel monkey pituitary gland was sequenced. But, comparison to human LH β cDNA showed that it contained a frame shift mutation resulting from a single base deletion in the 3'-end leading to a read-through into the 3'-untranslated region (gacta-cgcca in squirrel monkey cDNA fragment; gaccaccccca in human LH β cDNA, nucleotides 400–410, NM_000894). However, Gromoll and colleagues (Gromoll et al., 2003; Muller et al., 2004) recently demonstrated that in another New World primate, the common marmoset, LH β -subunit is not expressed in the pituitary gland. Rather, CG β -subunit is expressed in marmoset pituitary. We attempted therefore to amplify CG β -subunit cDNA from squirrel monkey and owl monkey pituitary gland RNA using primers corresponding to sequences in the 5'- and 3'-untranslated regions of the human CG β -subunit cDNA. We generated amplicons of approximately 500 bp that were sequenced across both strands and found to be similar to CG β -subunits of other species. The nucleotide sequences of squirrel monkey and owl monkey CG β -subunits are 91% identical to each other. Squirrel monkey CG β -subunit cDNA is 91% and 78% identical, whereas owl monkey CG β -subunit cDNA is 92% and 80% identical, to the nucleotide sequences of common marmoset and human CG β -subunits, respectively. When compared to the human LH β -subunit, the nucleotide sequences of squirrel monkey and owl monkey CG β -subunits are 79% and 81% identical, respectively. The squirrel monkey CG β -subunit nucleotide sequence was deposited in GenBank with accession number DQ174773, whereas the owl monkey CG β -subunit sequence was deposited as DQ200808.

Deduced amino acid sequences of squirrel monkey and owl monkey CG β -subunits were compared to full or partial sequences of CG β -subunits from other primates as well as the β -subunit of human LH (Figure 2A). Predicted sizes of the signal peptide are the same in each of the CG β -subunits as well as the LH β -subunit, in agreement with direct determinations by others (Simula et al., 1995). Mature squirrel monkey CG β -subunit is 143 amino acids in length, compared to human CG β that is 145 amino acids in length. Squirrel monkey CG β -subunit lacks valine and serine residues at positions 48 and 118, respectively, of human CG β -subunit. Owl monkey CG β is 144 amino acids in length and, like CG β of New World marmoset and dusky titi monkey, lacks serine at position 118. The M_r s of mature squirrel monkey and owl monkey CG β -subunits were calculated to be 15601.0 and 15721.1, respectively, and the

theoretical isoelectric points of the mature polypeptides were determined to be 8.28. Mature human CG β -subunit has an M_r of 15531.9 and a pI of 8.65. The amino acid sequences of mature squirrel monkey and owl monkey CG β -subunits are 82% identical (88% similar) to each other, and respectively 84% and 86% identical (88% and 92% similar) to that of the common marmoset. New World primate CG β -subunits are at most 66% identical (77% similar) to the human CG β sequence. The greatest divergence is in the C-terminal part of the molecule. Comparisons were also made to human LH β -subunit that lacks the carboxy-terminal 24 amino acid peptide of CG β (Figure 2A). Human CG β and LH β are 81% identical, whereas squirrel monkey and owl monkey CG β are 62% and 64% identical to human LH β , respectively.

Predicted glycosylation patterns are different in New World primate and human CG β -subunits. Squirrel monkey, owl monkey, and common marmoset CG β -subunits lack one of the two consensus sequences for N-linked glycosylation in human CG β (at position 13), but have gained a unique recognition sequence for N-linked glycosylation at position 125 (of squirrel monkey CG β) (Figure 2B). The corresponding residue in human CG β (position 127) is serine and is a known site of O-linked glycosylation, as are serine residues at positions 121, 132, 138 (Birken and Canfield, 1977; Keutmann and Williams, 1977; Kessler et al., 1979). However, none of the corresponding residues in squirrel monkey CG β are predicted to undergo O-linked glycosylation (NetOGlyc 3.1 Server on-line program, <http://www.cbs.dtu.dk/services/NetOGlyc/>). The absence of serine in New World primate CG β at position 118 of human CG β did not significantly affect predicted O-linked glycosylation in the C terminus. As expected, all four sites in human CG β received high predictive scores for O-linked glycosylation (Julenius et al., 2005). CG β in New World dusky titi monkey shares with other New World primates the differences from human CG β in glycosylation in the C terminus, but it appears that N-linked glycosylation at position 13 is retained.

Differences in glycosylation in the C terminus of the β -subunit of CG may have important functional consequences. The four O-linked glycosylation sites in human CG β are thought to confer a long circulating half-life (Matzuk et al., 1990; Klein et al., 2002). The different glycosylation pattern of New World primate CG β likely results in a shorter half-life. As CG is the only gonadotropin with ovulation and luteinizing function synthesized in the pituitary gland of New World primates, a shorter half-life would be appropriate for a pituitary hormone released in a pulsatile manner for this purpose. Furthermore, expression of CG, not LH, as the relevant pituitary hormone plays an essential physiological role in New World primates. Alterations in the LH/CG receptor gene of New World primates leads to failure of the splicing machinery and expression of a receptor that lacks exon 10 at the mRNA level (Gromoll et al., 2003). LH/CG receptor mRNA in humans is transcribed from 11 exons (Ascoli et al., 2002). New World primate LH/CG receptors, termed type II LH receptors, are refractory to stimulation by LH, but respond normally to CG (Muller et al., 2003). Thus, New World primates express a form of the LH/CG receptor that for normal reproductive function necessitates the pituitary expression and secretion of a shorter-lived form of CG β .

Phylogenetic analysis of CG β , FSH β , and α -glycoprotein hormone subunit families

To further characterize the relationships of the primate glycoprotein hormone subunit families, we created a neighbor-joining phylogenetic tree from multiple alignment of the amino acid sequences shown in Figures 1 and 2. As in a previous evolutionary analysis of α - and β -subunits of the glycoprotein hormone gene family (Li and Ford, 1998), we used human transforming growth factor β (TGF- β) as a distantly related outgroup to root the tree. TGF- β and the glycoprotein hormones are members of the cysteine-knot superfamily that share a common overall topology (Sun and Davies, 1995). Figure 3 shows the inferred neighbor-joining tree. Within each of the subunit families, the New World primate sequences formed separate

monophyletic groups supported by bootstrap values of 98%, 82% and 87%, respectively, for CG β , FSH β , and α -glycoprotein families. Old World monkey and human sequences grouped together in the CG β family (bootstrap value 99%); however, this grouping was not statistically supported for the FSH β and α -glycoprotein families where separate groups for Old World monkeys were strongly supported. This difference is likely related to the relatively recent origin within primates of the CG β subunit family (Maston and Ruvolo, 2002). Unfortunately, this determination provides little insight into the phylogenetic relationship between owl monkey, squirrel monkey and common marmoset, a grouping that remains uncertain (Steiper and Ruvolo, 2003; Opazo et al., 2006).

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A

		-24					+1							
SqM	α -glyc	MDSYRKYAAI	ILVTL	SVFLH	ILHSL	PEGEF	TTEEC	PECKL	KENKY	FSKLG	TPIYQ	CTGCC	36	
OM	α -glyc	-----V	-----L	----	S---	V-D-D-	-AQ	-----	-----	-----	A-V---	A---	36	
Mar	α -glyc	--Y-----	--I-----	-----	-----	D---	-A	-----	-----	R--	S----	M---	36	
Hum	α -glyc	--Y-----	F-----	-----	V---	A-D***	*VQD	---T-	Q--	PF--	QP-	A--L--	M---	32
RhM	α -glyc	--Y-----V	-----	-----	---	F-D---	-MQD	---P	R---	F---	P-	A----	M---	36
CM	α -glyc	--Y-----V	-----	-----	---	F-D---	-MQD	---P	R---	F---	P-	A----	M---	36
Rat	α -glyc	--C--R--V	--M--M--	-----	---	D-D-	IIQG	-----P	-----	-----	A----	M---	36	
Consensus		MD YR AA	L L L		LHS P		CPEC		N F		P QC GCC			
SqM	α -glyc	FSRAYPTPFR	SQKTM	LVKPN	VTSESS	CCVA	KAYTK	ATVMG	NVKVEN	HTEC	HCSTC	YHKF	96	
OM	α -glyc	-----V-	-----S	----	-----	----	-T	-----	-I	-----	-----	H---	96	
Mar	α -glyc	-----L-	-----	-----	-----	T---	-----	-----	-IR	-----	-----	H---	96	
Hum	α -glyc	-----L-	-K----	Q--	-----	T---	-S-NRV	----	GF	-----	A-	-----	S	92
RhM	α -glyc	-----V-	-K----	Q--	-----	T---	-SL-RVM	----	S-R	-----	-----	-----	96	
CM	α -glyc	-----L-	-K----	Q--	-----	T---	-SL-RVM	----	-R	-----	Q-	-----	96	
Rat	α -glyc	-----A-	-K----	Q--	-----	T---	-F	-----	-AR	-----	-----	S	96	
Consensus		FSRAYPTP R	S KTM V KN		TSE CCVA		K	MG		ENHT C		CSTCY HK		

B

		-18				+1													
SqM	FSHβ	MKT	VQFCFLF	CCWKA	ICCN	S	CEL	TNITIAI	ENEECH	FCIS	INTT	WCAGYC	YTRDL	VFKDP	42				
OM	FSHβ	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	Y---	42				
Mar	FSHβ	--S-----	-----	-----	-----	-----	-----	-----	-K---	R---	-----	-----	-----	Y---	42				
Pyg	FSHβ	--S-----	-----	-----	-----	-----	-----	-----	-K---	R---	-----	-----	-----	Y---	42				
Hum	FSHβ	---L--F---	-----	-----	-----	-----	-----	-----	-K---	R---	-----	-----	-----	Y---	42				
RhM	FSHβ	-----	-----	-----	-----	-----	-----	-----	-K---	R---	-----	-----	-----	Y---	42				
CM	FSHβ	-----	-----	-----	-----	-----	-----	-----	-K---	R---	-----	-----	-----	Y---	42				
Rat	FSHβ	M--SI-L-I-L	W-LR-V--H-	-----	-----	SV	-K---	R---	-----	E---	-----	-----	-----	Y---	43				
Consensus		MK	Q	L	C	A	CC	S	C	LTNITI	E	EEC	FCIS	INTTWC	GYC	YTRDLV DP			
SqM	FSHβ	ATPNIQT	TCT	FKELV	YETVR	VP	GCA	HADS	LYTYP	VATQC	HCGK	CDS	DST	DCTTQ	GLGPD	102			
OM	FSHβ	-----	-----	-----	-----	-----	F	-----	-----	-----	-----	-----	-----	---	M-----	102			
Mar	FSHβ	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	E---	-----	---	M-----	102			
Pyg	FSHβ	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	E---	-----	---	M-----	102			
Hum	FSHβ	-R-K--K---	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	---	VR----	S	102		
RhM	FSHβ	-R----K---	---	V--D---	-----	-----	-----	-----	-----	-----	-----	-----	-----	---	VR----	S	102		
CM	FSHβ	-R----K---	---	V--D---	-----	-----	-----	-----	-----	-----	-----	-----	-----	---	VR----	S	102		
Rat	FSHβ	-R--T-KV--	-----	I-	L----	R-S--	-----	E-	-----	-----	-----	-----	-----	---	VR----	S	103		
Consensus		A	P	Q	CT	FKE	VY	T	PGCA	H	DS	Y	TYPVAT	C	HC	KC	DST	DCT	GLGP
SqM	FSHβ	YCSF	SEMKE																111
OM	FSHβ	-----	-----																111
Mar	FSHβ	---	IR----																111
Pyg	FSHβ	---	IR----																111
Hum	FSHβ	---	G----																111
RhM	FSHβ	-----	-----																111
CM	FSHβ	-----	-----																111
Rat	FSHβ	---	G----																112
Consensus		YCS	E																

Figure 1.

(A) Alignment of the predicted amino acid sequences of squirrel monkey (SqM α -glyc) and owl monkey (OM α -glyc) glycoprotein hormone α -subunits with those of common marmoset (Mar, U04446), human (Hum, NM_000735), rhesus monkey (RhM, (Golos et al., 1991)), cynomolgus monkey (CM, AJ781394), and rat (P11962) glycoprotein hormone α -subunits. Four amino acids deleted in the human glycoprotein hormone α -subunit sequence are indicated by asterisks. (B) Alignment of the predicted amino acid sequences of squirrel monkey (SqM FSH β) and owl monkey (OM FSH β) FSH β -subunits with those of common marmoset (Mar, (Wolfgang et al., 2001)), pygmy marmoset (PygM, (Wolfgang et al., 2001)), human (Hum, NM_000510), cynomolgus monkey (CM, AJ781395), rhesus monkey (RhM, AY083550 and

(Wolfgang et al., 2001)), and rat (NM_001007597) FSH β -subunits. Dashes indicate amino acid residues that are identical to the squirrel monkey sequences. Numbering indicates residues in the mature subunit sequences. Consensus sequence (in bold) was obtained from completely conserved residues among mammalian species identified by Dias et al. (Dias et al., 2002) and squirrel monkey and owl monkey subunits. Conserved N-linked glycosylation sites are underlined in the consensus sequence.

[illegible]

B

(A) Alignment of the predicted amino acid sequences of the squirrel monkey (SqM CG β) and owl monkey (*Aotus nancymae*) (OMna CG β) CG β -subunits with those of owl monkey *Aotus trivirgatus* (OMtr CG β , AF397604, partial sequence), common marmoset (Mar CG β , U04447), dusky titi monkey (Titi CG β , AF397603, partial sequence), human (Hum CG β , NM_000737), baboon (Bab CG β , M14966), and rhesus monkey (RhM CG β , AF397601 and AY011015) CG β -subunits, and human LH (Hum LH β , NM_000894) β -subunit. Dashes indicate amino acid residues that are identical to the squirrel monkey CG β -subunit sequence. Deleted amino acids are indicated with an asterisk. Numbering indicates residues in the mature CG β -subunits. Consensus sequence in CG β among all primate species described here is indicated in bold. N-

linked glycosylation sites are underlined. (B) Schematic showing predicted glycosylation sites in New World squirrel monkey, owl monkey, common marmoset, and dusky titi monkey CG (SqM, OM, Mar, and Titi CG β , respectively). Human CG β possesses N-linked oligosaccharide chains at positions 13 and 30 and O-linked oligosaccharide chains at positions 121, 127, 132, and 138. No sites for O-linked glycosylation are predicted to exist in New World monkey CG β . Rather, New World primate CG β s have gained a unique recognition sequence for N-linked glycosylation in the C terminus.

Figure 3.

Phylogenetic tree of the β sub-units of chorionic gonadotropin (CG-beta), luteinizing hormone (LH-beta), follicle stimulating hormone (FSH-beta), and of α -glycopeptide (alpha-glyc) sequences from selected primates and rat. The phylogenetic tree was constructed with Mega version 3.1 software (Kumar et al., 2004). Minimum evolution, neighbor-joining and maximum parsimony methods were used with Poisson correction for multiple amino acid substitution and with 1000 random bootstrap replications. The figure shows the neighbor-joining tree (the three methods produced very similar topologies). Brackets to the right indicate the monophyletic groups encompassing New World monkey (NWM) subunits. Bootstrap values $\geq 50\%$ are shown. The human transforming growth factor β (TGF- β) is used as an outgroup to root the tree (Li and Ford, 1998). The scale at the bottom left is in units of amino acid substitutions per site.