Mutagenesis and Chimeric Genes Define Determinants in the β Subunits of Human Chorionic Gonadotropin and Lutropin for Secretion and Assembly

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Abstract. Chorionic gonadotropin (CG) and lutropin (LH) are members of a family of glycoprotein hormones that share a common α subunit but differ in their hormone-specific β subunits. The glycoprotein hormone β subunits share a high degree of amino acid homology that is most evident for the LHβ and CGβ subunits having >80% sequence similarity. However, transfection studies have shown that human CGβ and α can be secreted as monomers and can combine efficiently to form dimer, whereas secretion and assembly of human LHβ is less efficient. To determine which specific regions of the LHβ and CGβ subunits are responsible for these differences, mutant and chimeric LHβ-CGβ genes were constructed and transfected into CHO cells. Expression of these subunits showed that both the hydrophobic carboxy-terminal seven amino acids and amino acids Trp^{8}, Ile^{15}, Met^{42}, and Asp^{77} together inhibit the secretion of LHβ. The carboxy-terminal amino acids, along with Trp^{8}, Ile^{15}, Met^{42}, and Thr^{58} are implicated in the delayed assembly of LHβ. These unique features of LHβ may also play an important role in pituitary intracellular events and may be responsible for the differential glycosylation and sorting of LH and FSH in gonadotrophs.

HUMAN chorionic gonadotropin (CG),[1] lutropin (LH), follitropin (FSH), and thyrotropin (TSH) are a family of heterodimeric glycoprotein hormones that share a common α subunit but differ in their hormone-specific β subunits (8, 45, 52). Combination of the α and β subunits begins in the endoplasmic reticulum (18, 43) and for CG, dimerization is completed before the addition of the *O*-linked oligosaccharides in the Golgi (43). Although the β subunits determine biological specificity of the hormones, there is a high degree of amino acid homology between these subunits (50), which is most apparent for LHβ and CGβ. They are 85% homologous in the first 114 amino acids (51), and this relationship is responsible for the binding of CG and LH to a common gonadal receptor (8, 45, 52). However, CGβ and LHβ contain two prominent structural differences: (*a*) LHβ contains one *N*-linked oligosaccharide at position 30, whereas CGβ contains two *N*-linked units at sites 13 and 30; and (*b*) CGβ contains a 31-amino acid hydrophilic COOH-terminal extension with four *O*-linked oligosaccharides (3, 7, 21, 23) compared with a shorter, 7-amino acid, hydrophobic stretch on LHβ (24, 48, 51). Fiddes and colleagues (13, 51) have suggested that the longer CGβ COOH-terminal extension was due to a frameshift mutation at codon 114 in the ancestral LHβ/CGβ gene which resulted in a read-through of the 3′ untranslated region.

Earlier transfection studies from our laboratory (11, 35) demonstrated that human LHβ and CGβ subunits display different intracellular behavior. Whereas CGβ can be secreted as monomer and assembles rapidly, LHβ is secreted inefficiently and is slow to combine with α. Thus, the unique characteristics of the individual β subunits represent a determinant step in the expression of dimer in vivo. To elucidate the structural basis for these intracellular differences, we constructed chimeric and mutant human LHβ-CGβ genes. Transfection of these mutants and chimeric genes in the presence or absence of the α gene reveal that an interaction of the LHβ hydrophobic COOH terminus and other LHβ-specific residues play a critical role in delaying secretion and assembly of LH.

Materials and Methods

Enzymes used to prepare vectors were purchased from New England Biolabs, (Beverly, MA); or Bethesda Research Laboratories (Gaithersburg, MD). Klenow fragment was a gift of Dr. John Majors (Washington University, St. Louis, MO). The DNA vector, M13mp19 (38), was obtained from New England Biolabs. Oligonucleotides used for the site-directed mutagenesis were prepared by the Washington University Sequencing Facility (St. Louis, MO). [^{35}S]Cysteine (>1,000 Ci/mmol) was purchased from ICN Biochemicals (Irvine, CA). All other reagents are as described previously (34, 36).

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1. *Abbreviations used in this paper*: BiP, immunoglobulin heavy chain binding protein; CG, chorionic gonadotropin; ER, endoplasmic reticulum; FSH, follitropin; GalNAC, *N*-acetylgalactosamine; HA, hemagglutinin; LH, lutropin; TSH, thyrotropin.

Mutagenesis and Vector Constructions

Hind III-Bam HI fragments containing exons II and III of the CGβ gene (3,250 bp) or the LHβ gene (1,270 bp) were inserted into M13mp19 and the single-stranded viral recombinant DNA were isolated for mutagenesis. Mutant oligonucleotides (22–28mers) were synthesized for the mutagenesis. The mutagenesis and hybridization conditions were as described previously (36). The tetramethylammonium chloride wash temperature for the 22–28-mers is as described by Wood et al. (53). To construct the mutant genes for LHβΔT and CGβΔT, codons 115 were mutated to stop codons (TAA), resulting in genes coding for LHβ and CGβ, respectively, lacking the carboxy-terminal extension (Fig. 3). Alteration of the glycosylation site at asparagine 13 in CGβ via a Thr^{15} to Ala^{15} change has been described (37). All other LHβ mutations were changed as follows: LHβ2, Arg^2 to Lys^2; LHβ8, Trp^8 to Arg^8, LHβ8/10, Trp^8 and His^{10} to Arg^8 and Arg^{10}; LHβ15*, Ile^{15} to Thr^{15}; LHβ15(A), Ile^{15} to Ala^{15}; LHβ42, Met^{42} to Thr^{42}; LHβ47/51, Ala^{47} and Pro^{51} to Gly^{47} and Ala^{51}; LHβ58, Thr^{58} to Asn^{58}, LHβ77, Asp^{77} to Asn^{77}; LHβ82/83, Phe^{82} and Pro^{83} to Tyr^{82} and Ala^{83}. The asterisk in LHβ15* denotes that this change reconstitutes the Asn-X-Thr consensus sequence and allows for glycosylation of Asn^{13}. These mutants were all subcloned into vectors containing exon I to reconstitute the entire CGβ or LHβ gene containing all three exons. These subcloned genes were rechecked to ensure that the mutation was still present and that there was no wild-type contamination.

To construct chimeric genes, we took advantage of the common restriction endonuclease sites between the two genes (see Fig. 3). Thus, the LCβ41 mutant gene was constructed by transferring the Sac I-Bam HI fragment of CGβ (Fig. 3 *B, right*) into the same sites in LHβ (Fig. 3 *B, left*) to reconstruct a chimeric gene that coded for the first 41 amino acids of LHβ and the remainder CGβ. The CLβ41 mutant was constructed by doing the opposite transfer. The LCβ87ΔT and CLβ87 mutants were constructed by transferring the Pvu II-Bam HI segments of CGβΔT and LHβ into the same sites in LHβ and CGβ, respectively. Because Pvu II cuts at the codon for amino acid 87, this transfer allows for construction of chimeras with the first 87 amino acids of one β subunit and the remainder of the other. Chimeric genes that lacked the terminus of either CGβ or LHβ were made by transferring the segment from the CGβΔT or LHβΔT mutants instead of the wild-type gene. The mutants CLβ41-15ΔT and LCβ41-15* are chimeras analogous to CLβ41ΔT and LCβ41, respectively, except that amino acid 15 has been altered in each case.

All subcloned mutant and chimeric genes were contained on Bgl II-Bam HI fragments which were inserted into the eukaryotic expression vector, pM^2 (34) downstream of the Harvey murine sarcoma virus long terminal repeat (12). Genes that contained the COOH terminus and 3′ end of the CGβ gene were 3,600 bp, whereas those that contained the COOH-terminus and the shorter 3′ nontranslated region of the LHβ gene were only 1,400 bp. The following expression vector plasmids were used for these studies: pM^2-CGβ, pM^2LHβ, pM^2CGβΔT, pM^2LHβΔT, pM^2CLβ41, pM^2LCβ41, pM^2-CLβ41ΔT, pM^2LCβ87ΔT, pM^2CLβ87ΔT, pM^2CLβ41-15ΔT, pM^2LHβ15*, pM^2LHβ15*ΔT, pM^2LHβ15(A)ΔT, pM^2LCβ41-15*, pM^2LHβ2ΔT, pM^2-LHβ8, pM^2LHβ8/10, pM^2LHβ8ΔT, pM^2LHβ8/10ΔT, pM^2LHβ42ΔT, pM^2-LHβ47/51ΔT, pM^2LHβ58ΔT, pM^2LHβ77ΔT, and pM^2LHβ82/83ΔT.

Transfection, Clone Selection, and Cell Culture

The plasmids described above which contained the mutant genes were transfected alone or cotransfected with pM^2CGα (34, 36) into CHO cells as described previously (34, 36). Cells containing the expression vectors were selected by growing in culture medium containing 0.25 μg/ml of the neomycin analogue G418 (49). Expression of the mutants and chimeras was detected by immunoprecipitation of metabolically labeled cells (see below). Both monomer- and dimer-secreting cell lines were selected for these studies. All stably transfected CHO cell lines were maintained in medium I (Ham's F12 medium supplemented with penicillin [100 U/ml], streptomycin [100 μg/ml], and glutamine [2 mM]) containing 5% (vol/vol) FCS and 0.125 mg/ml G418 in a humidified 5% CO_2 incubator.

Metabolic Labeling and Protein Analysis

Cells were plated into 12-well dishes (300,000–350,000 cells/well) in 1 ml of medium I supplemented with 5% FCS 1 d before labeling. For continuous labeling experiments, cells were washed twice with cysteine-free medium II (medium I supplemented with 5% dialyzed calf serum) and labeled for 6 h in 1 ml of cysteine-free medium II containing 20 μCi/ml [^{35}S]cysteine. For pulse-chase experiments, the cells were washed twice and preincubated for 1.5 h in cysteine-free medium II, followed by a 20-min labeling in cysteine-free medium II containing 100 μCi/ml [^{35}S]cysteine. The labeled cells were then washed twice with medium II containing 1 mM unlabeled cysteine and incubated in this medium for the indicated times.

Medium and cell lysates were prepared, immunoprecipitated, and treated as described (11, 34). Polyclonal antisera against α, LHβ, and CGβ were prepared in our laboratory. Each antiserum has been tittered and was added in excess to ensure complete precipitation. The antiserum generated against CGβ cross-reacts fully with LHβ when compared with MAbs to LHβ (11) and was used for all β mutants and chimeras. All subunit and dimer immunoprecipitates were resolved on 15% $NaDodSO_4$-polyacrylamide gels by the method of Laemmli (28). Gels were soaked for 10 min in 1 M sodium salicylate, dried, and autoradiographed with preflashed film as described (11). Autoradiographs for the pulse-chase experiments were scanned with a laser densitometer (Ultrascan XL; LKB Instruments, Gaithersburg, MD). At least two autoradiographs from separate experiments were used to determine the secretion rates and amount which is recovered in the medium.

Results

N-linked Oligosaccharides and COOH Termini

The β subunits of human CG and human LH are 85% homologous in the first 114 amino acids (17 amino acid replacements; Fig. 1), suggesting that the two genes evolved from the same ancestral LHβ/CGβ gene (51). However, a deletion in this ancestral gene at codon 114 allowed a readthrough, which resulted in a CGβ subunit protein of 145 amino acids compared with the 121 amino acids of LHβ (51), and thus LHβ and CGβ subunits have different COOH-terminal sequences. The 7-amino acid terminus of LHβ is very hydrophobic, but the terminus of CGβ is hydrophilic, containing an abundance of serine residues, four of which are *O*-glycosylated. Furthermore, CGβ contains two *N*-linked oligosaccharides, whereas LHβ contains only one *N*-linked unit due to the presence of Ile at position 15, which disrupts the Asn^{13}-X-Thr^{15} consensus sequence (17, 33) found in CGβ.

Earlier studies in our laboratory (11) have shown that, unlike CGβ, LHβ produced from transfected C127 cells fails to exit the cell as monomer and is inefficient at dimerization. We had also shown that differences are not cell specific because similar effects were seen in CHO cells (35) and AtT-20 cells (11). Earlier studies (11) have also shown that absence of the LHβ in the medium is due to inefficient secretion rather than extracellular degradation, because the amount of LHβ secreted into the medium is stable for several hours. Exogenous LHβ incubated with a cell monolayer was quantitatively recovered, further emphasizing that LHβ is not degraded extracellularly and does not adhere to cells or culture dishes (Corless, C., and I. Boime, unpublished observations). In a continous labeling experiment (Fig. 2), CGβ accumulates in the medium (lane *2*) with less of the intracellular forms accumulating (lane *1*), whereas very little LHβ is secreted (lane *4*) and the intracellular LHβ accumulates (lane *3*). To analyze whether only absence of the *N*-linked oligosaccharide at Asn^{13} of LHβ is responsible for the effects seen, the ATC triplet coding for Ile^{15} was mutated to the AAC coding for the CGβ Thr^{15}. Addition of an extra glycosylation site seen in mutant LHβ15* leads to a small increase in secretion and recovery compared to LHβ but is still slow and inefficient compared with CGβ when examined by pulse-chase analysis (Table I, nos. 1, 2, and 3). These results are consistent with previous mutagenesis studies in which absence of the *N*-linked oligosaccharide at position 13 of CGβ did not significantly affect the secretion kinetics (37).

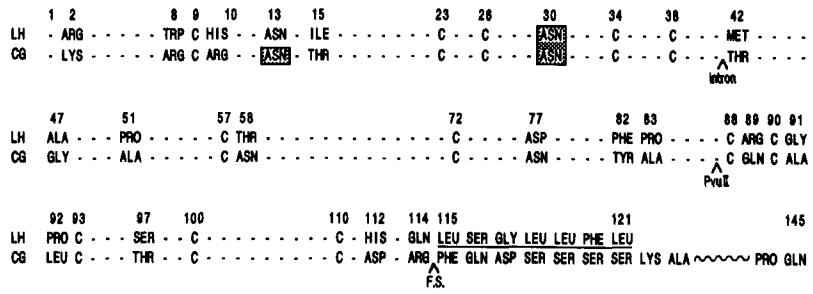

Figure 1. Differences in protein sequences between LHβ and CGβ. The amino acid differences between LHβ and CGβ are shown (51). Dashes denote identical amino acids; *C* denotes conserved cysteines that are aligned exactly in the two proteins. *Intron* between amino acids 41 and 42 designates the position where the second intron divides the coding sequences in the DNA. *PvuII*, site for shuffling fragments to construct chimeras. *F.S.*, frameshift mutation in the CGβ gene, resulting in a 31-amino acid COOH-terminal extension in the CGβ protein unlike the corresponding seven-amino acid LHβ sequence (underlined). The Asn residues that are *N*-linked glycosylated are enclosed by boxes. The CGβ sequence between 123 and 144 is indicated by a curved line.

We next examined how the different carboxy-terminal extensions affected the secretion of the LHβ and CGβ subunits. Mutants LHβΔT and CGβΔT, which terminate at amino acid 114 due to placement of a stop codon at position 115 (Fig. 3 *c*), were expressed in CHO cells. Absence of the LHβ terminus in LHβΔT (Fig. 2, lanes *5* and *6*) results in a slight increase in the amount of LHβΔT secreted (lane *6*) compared with LHβ (lane *4*). Although pulse-chase analysis shows that the rate of secretion and the amount recovered for LHβΔT is greater than LHβ, secretion of this mutant remains inefficient compared with CGβ (Table I, nos. 1, 2, and 4). Pulse-chase analysis of the CGβΔT mutant reveals that absence of the 31-amino acid hydrophilic tail has only marginal effects on secretion, indicating that this region alone does not explain the enhanced secretion of CGβ compared with LHβ (Table I, no. 5). Earlier studies (34) using an *O*-glycosylation mutant cell line (26) showed that absence of the carboxy terminus *O*-linked units on CGβ does not alter secretion or assembly. Thus, absence of the *O*-linked units and the carboxy-terminal segment have only minor effects on CGβ secretion.

Chimeric LHβ-CGβ Genes

Because the alterations at the positions described above did not have dramatic effects on the secretion of LHβ and CGβ, we reasoned that multiple changes may be responsible for their intracellular differences. To address this issue, chimeric LHβ-CGβ genes were constructed. These chimeras were designed to localize unique LHβ-CGβ sequences to a specific region and then determine if interactions between different amino acids are responsible for the intracellular effects seen. Three internal restriction enzyme sites (Hind III, Sac I, and Pvu II) are conserved and two other sites (Bgl II and Bam HI) have been constructed at unique sites in each gene (Fig.

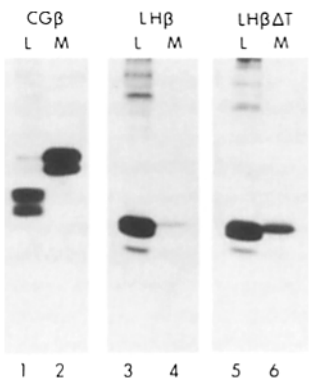

Figure 2. CHO expression of wild-type subunits and COOH terminus mutant. CHO cells expressing CGβ (lanes *1* and *2*), LHβ (lanes *3* and *4*), and LHβΔT (lanes *5* and *6*) were labeled with 20 μCi/ml [^{35}S]-cysteine for 7 h and the lysate (*L*) and medium (*M*) were immunoprecipitated with human CGβ antiserum.

Table I. Secretion and Assembly of LHβ-CGβ Chimeras and Mutants

		Subunit	Monomer $t_{½}$*	Monomer Recovery‡	Dimer $t_{½}$	Dimer Recovery
			h	%	*h*	%
A. Wild-type	1.	CGβ	1.9	>95	1.2	>95
	2.	LHβ	10	<20	5.5	42
B. *N*-linked/ COOH-terminal mutants	3.	LHβ15*	7.4	40	7.7	30
	4.	LHβΔT	7	40	6	80
	5.	CGβΔT	2.6	91	2	90
C. Chimeras	6.	LCβ41	5	85	1	>95
	7.	CLβ41	6.6	50	2.5	50§
	8.	LCβ87ΔT	10	25	5.5	32
	9.	CLβ87	5.3	85	3	45
	10.	CLβ87ΔT	2.3	95	1.6	>95
	11.	CLβ41ΔT	2.5	>95	1	94§
D. NH_2-terminal mutants	12.	LHβ15*ΔT	4.2	83	3.1	>95‖/40§
	13.	LHβ8	10	25	5.5	32
	14.	LHβ8ΔT	5.1	91	2.0	91
	15.	LHβ8/10ΔT	5.2	90	2.3	95
	16.	CLβ41-15ΔT	2.5	>95	–	–
	17.	LCβ41-15*	4.2	91	–	–
E. Mutants in region 42–87	18.	LHβ42ΔT	6.0	80	2.6	84
	19.	LHβ47/51ΔT	7.5	28	–	–
	20.	LHβ58ΔT	6.9	40	2.2	>95
	21.	LHβ77ΔT	5.7	70	–	–
	22.	LHβ82/83ΔT	10	50	–	–

* The $t_{½}$ is the average of two independent experiments. The range is ≤0.6 h. The rate of secretion in cases where the subunit is inefficiently secreted is based on disappearance from the lysate (see also reference 11).
‡ For mutants that are slowly secreted, recovery is estimated by comparing the amount secreted and the amount that has disappeared from the lysate in 10 h.
§ Monoglycosylated form recovered in the medium because α obscures proper quantitation.
‖ Recovery of diglycosylated form is estimated.

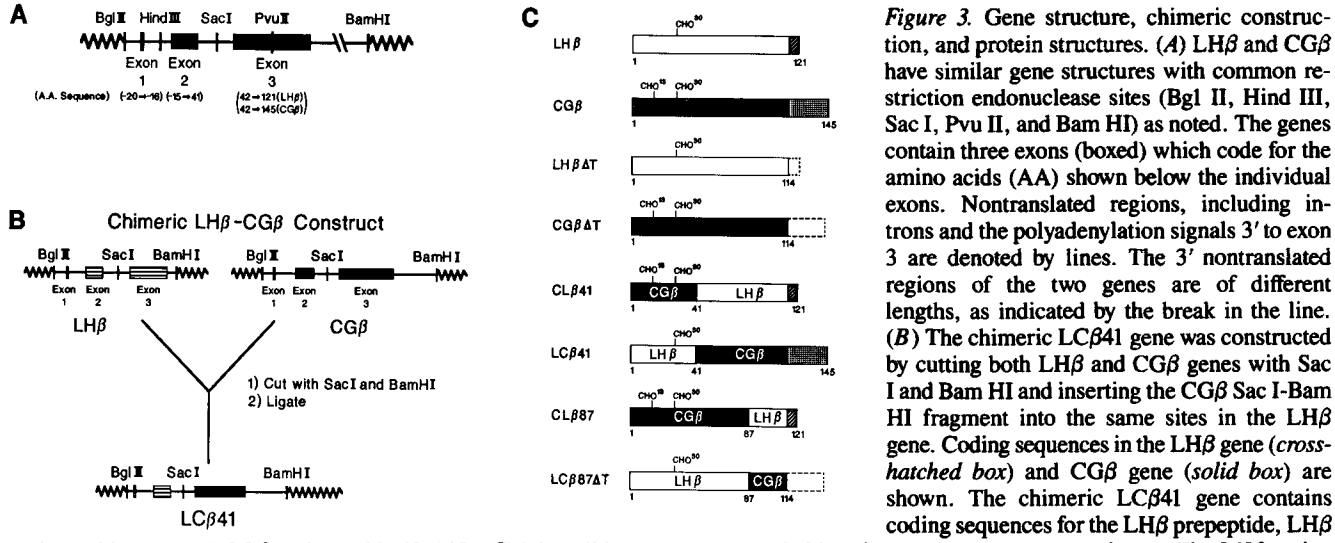

Figure 3. Gene structure, chimeric construction, and protein structures. (*A*) LHβ and CGβ have similar gene structures with common restriction endonuclease sites (Bgl II, Hind III, Sac I, Pvu II, and Bam HI) as noted. The genes contain three exons (boxed) which code for the amino acids (AA) shown below the individual exons. Nontranslated regions, including introns and the polyadenylation signals 3′ to exon 3 are denoted by lines. The 3′ nontranslated regions of the two genes are of different lengths, as indicated by the break in the line. (*B*) The chimeric LCβ41 gene was constructed by cutting both LHβ and CGβ genes with Sac I and Bam HI and inserting the CGβ Sac I-Bam HI fragment into the same sites in the LHβ gene. Coding sequences in the LHβ gene (*crosshatched box*) and CGβ gene (*solid box*) are shown. The chimeric LCβ41 gene contains coding sequences for the LHβ prepeptide, LHβ amino acids 1–41 and CGβ amino acids 42–145. (*C*) The wild-type, mutant, and chimeric protein structures are shown. The LHβ amino acid sequences (*open box*) with a seven-amino acid hydrophobic terminus (*crosshatched area*) is shown. The CGβ amino acid sequences (*solid box*) with its 31-amino acid hydrophobic extension (*dotted area*) is also shown. The presence of an *N*-linked oligosaccharide is noted by *CHO* and the absence of the COOH-terminal extensions on LHβ or CGβ is presented as a dashed line box. LHβ-CGβ chimeras and the amino acids from each subunit are shown by either open or solid boxes.

3 *a*) (11). Furthermore, the Sac I site (in intron II between codons 41 and 42) and the Pvu II site (located at codon 87) divide the LHβ and CGβ genes into three convenient regions for analysis. An LHβ-CGβ chimeric gene (Fig. 3 *b*) was made by digestion of the CGβ and LHβ genes with Sac I and Bam HI to generate the LCβ41 derivative, which codes for the first 41 amino acids of LHβ and the remainder of CGβ (Fig. 3 *c*). Similar manipulations were used to construct the CLβ41 derivative coding for the first 41 amino acids of CGβ and the remainder of LHβ. Pvu II was used to construct the derivatives LCβ87ΔT (the first 87 amino acids of LHβ and the remainder of CGβΔT) and CLβ87 (the first 87 amino acids of CGβ and the remainder of LHβ) (Fig. 3 *c*).

Pulse-chase analysis of the chimeras described above reveals that all four chimeras (LCβ41, CLβ41, LCβ87ΔT, and CLβ87) are secreted slower than CGβ and vary in the amount recovered in the medium (Fig. 4; Table I, nos. 6–9). Because these chimeras contain either the NH_2- or COOH-terminal regions of LHβ, these data imply that variations in both regions are responsible for the differences in secretion of LHβ and and CGβ. However, we can attribute the different secretion kinetics of CLβ87 ($t_{½}$ = 5.3 h) and CGβΔT ($t_{½}$ = 2.6 h) to either the six amino acid changes between residues 87 and 114 (Fig. 1) or the presence of the LHβ 7-amino acid COOH terminus in CLβ87 (Fig. 3 *c*). To examine if this COOH terminus is responsible, we constructed another chimera, CLβ87ΔT, which was identical to CLβ87, except that it lacked the LHβ seven-amino acid COOH terminus. Both CLβ87ΔT (Fig. 4 *f*) and CGβΔT are secreted with similar kinetics (Table I, nos. 5 and 10), and at a rate twice that of CLβ87, which contains the LHβ terminus. Thus, the presence of the extension rather than the amino acid differences between 87 and 114 in CLβ87ΔT and CGβΔT is partly responsible for the inefficient secretion of LHβ. However, because absence of this extension alone in the mutant LHβΔT has only a small effect on secretion (Fig. 2; Table I, no. 4) compared with the greater effect seen for CLβ87ΔT, there must be an interaction between this extension and the first 87 amino acids of LHβ that is responsible for the inhibited secretion.

To localize which of the first 87 amino acids were interacting with the LHβ COOH-terminus, we constructed CLβ41ΔT. This chimera contains the first 41 amino acids of CGβ and the remaining amino acids of LHβ and, in addition, lacks the 7-amino acid LHβ COOH terminus. Pulse-chase kinetics of CLβ41ΔT (Fig. 4 *c*; Table I, no. 11) shows that absence of the LHβ terminus markedly enhances secretion ($t_{½}$ = 2.5 h) and recovery (>95%) of this derivative compared with CGβ. Thus, both the NH_2-terminal 41 amino acids and the LHβ COOH-terminal extension play a critical role in secretion. However, because LCβ41, which contains the first 41 amino acids of LHβ, is secreted much more efficiently than LCβ87ΔT, which contains the first 87 amino acids of LHβ, the LHβ/CGβ amino acid differences between 42 and 87 also influence secretion (Fig. 4, *A* and *D*; Table I, nos. 6 and 8 [see below]). Thus, the data implicate residues in two regions of the first 87 amino acids of LHβ that interact with the carboxy terminus to impede monomer secretion.

Identification of Residues Critical for LHβ Secretion

We next analyzed the eleven amino acid differences in the NH_2-terminal 87-amino acid region of LHβ and CGβ (Fig. 1). Several mutants with changes of individual amino acids in the first 87 amino acids and absence of the LHβ COOH terminus were constructed (Fig. 5). As described above, the mutant LHβ15*, which contains two *N*-linked units, was secreted poorly similar to the secretion of LHβ. However, mutant LHβ15*ΔT (Fig. 5), which also contains the extra *N*-linked unit but in addition lacks the COOH terminus of LHβ, is secreted more efficiently (Fig. 6, lanes *1* and *2*). Its rate of secretion ($t_{½}$ = 4.2 h) and recovery (83%) is greater than either LHβ15* or LHβΔT ($t_{½}$ ≈ 7 h), which contain only the single changes (Table I, nos. 3, 4, and 12). The presence of the extra *N*-linked glycosylation site, associated with

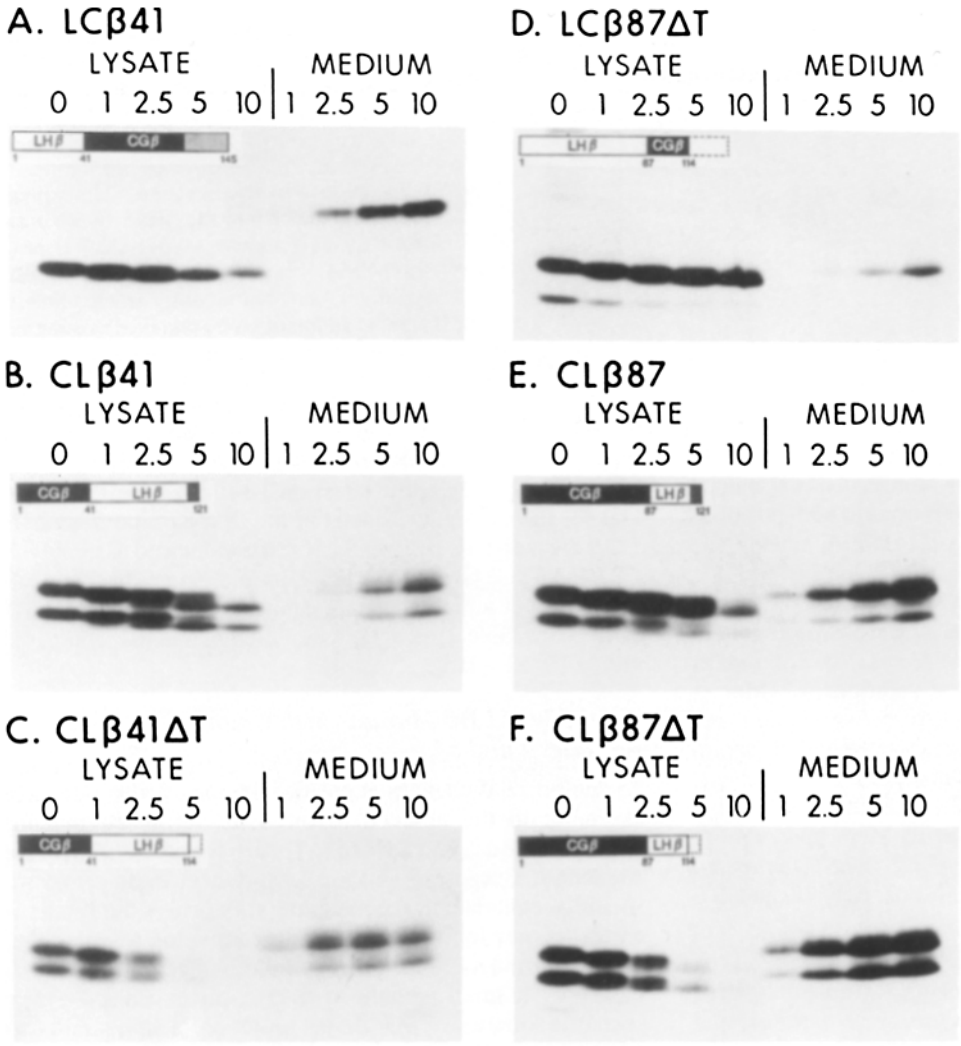

Figure 4. Kinetics of chimeric LHβ-CGβ subunit secretion from CHO cells. Cells expressing chimeras LCβ41 (*A*), CLβ41 (*B*), CLβ41ΔT (*C*), LCβ87ΔT (*D*), CLβ87 (*E*), and CLβ87ΔT (*F*) were pulse-labeled with 100 μCi/ml [^{35}S]cysteine for 20 min, chased for the indicated times (h), immunoprecipitated with human CGβ antiserum, and prepared for SDS-PAGE analysis as described in Materials and Methods.

the change of Ile^{15} to Thr^{15}, and the absence of the seven-amino acid COOH terminus of LHβ thus enhances secretion. Another mutant, LHβ15(A)ΔT (Fig. 5), which contains an Ile^{15} to Ala^{15} change, and thus lacks the Asn^{13} *N*-linked oligosaccharide, is also secreted efficiently compared with LHβ15*ΔT (data not shown). The absence of Ile^{15} alone rather than the presence of the extra oligosaccharide thus is responsible for the enhanced secretion of these two derivatives.

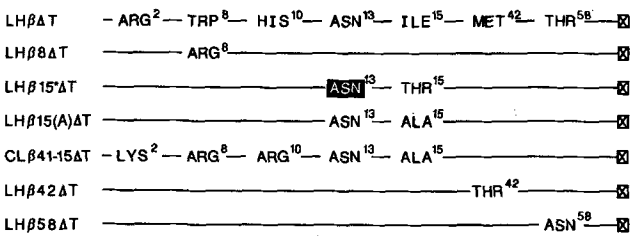

Figure 5. LHβ mutants with sequence variations between positions 2 and 58. Six of the eight different amino acids in the first 58 residues of LHβΔT compared with CGβ are shown. LHβ mutants have their amino acids changed to the corresponding residue seen in CGβ. [X] denotes absence of the seven-amino acid LHβ COOH-terminal extension in these mutants.

A change of Arg to Lys at position 2 in the absence of the LHβ seven-amino acid carboxy terminus (LHβ2ΔT) has little effect on secretion compared with LHβΔT (data not shown) as would be expected given their high degree of charge similarity and similar hydrophilic characteristics (42). We also changed the LHβ Trp^{8} to Arg^{8} (LHβ8; Fig. 5) and both the Trp^{8} and His^{10} to Arg^{8} and Arg^{10} (LHβ8/10; see Fig. 1). These residues may be expected to play an important role for the following reasons: (*a*) Trp is the most hydrophobic amino acid and thus it is less accessible to the surface compared with the Arg present in CGβ (42); and (*b*) Trp^{8} and His^{10} are adjacent to Cys^{9}, and thus their presence may alter disulfide pairing of Cys^{9} with its cognate, Cys^{90}.

Mutants LHβ8 (Fig. 6, lanes *3* and *4*) and LHβ8/10 (data not shown), containing the 7-amino acid carboxy-terminal extension of LHβ, accumulate in the lysate and are secreted inefficiently (Table I; no. 13). However, mutants LHβ8ΔT (Fig. 5, lanes *5* and *6*) and LHβ8/10ΔT (data not shown), which lack the seven-amino acid LHβ extension, are secreted faster and more efficiently (Table I, nos. 14 and 15). Because both mutants behaved similarly, His^{10} apparently plays a minor role. Because less of LHβ8ΔT ($t_{½}$ = 5.1 h) and LHβ15*ΔT ($t_{½}$ = 4.2 h) are secreted compared with CLβ41ΔT, both the Trp^{8} and Ile^{15} changes must have an ad-

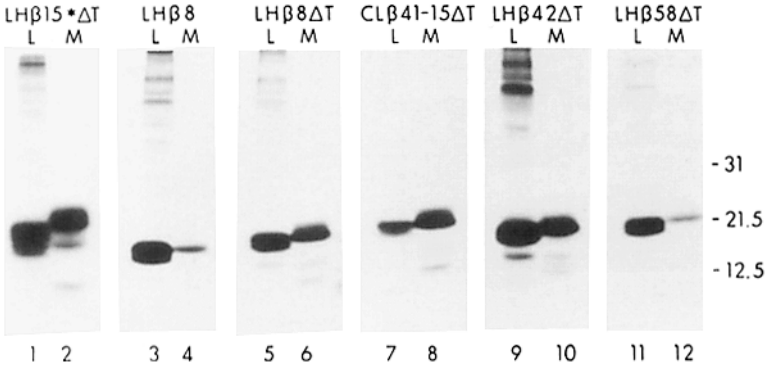

Figure 6. Secretion of mutant subunits. Cells expressing LHβ15*ΔT (lanes *1* and *2*), LHβ8 (lanes *3* and *4*), LHβ8ΔT (lanes *5* and *6*), CLβ41-15ΔT (lanes *7* and *8*), LHβ42ΔT (lanes *9* and *10*), and LHβ58ΔT (lanes *11* and *12*) were continuously labeled and the lysate (*L*) and medium (*M*) were prepared as described in Fig. 2.

ditive effect to enhance secretion in the absence of the LHβ COOH terminus. This is further confirmed by mutant CLβ41-15ΔT (Fig. 5), which has alterations at both positions 8 and 15 and is secreted twofold faster (Fig. 6, lanes *7* and *8*; Table I, no. 16).

Mutants lacking the LHβ COOH terminus and containing changes between amino acids 42 and 87 were also generated. Expression of these mutants (Table I, nos. 18–22; Fig. 6, lanes 9–12) revealed that changes at positions 42 (Met → Thr; Fig. 6, lanes *9* and *10*) or 77 (Asp → Asn; Table I, no. 21) increased the amount of β subunit secreted and the secretion rate compared with mutants at positions 47/51 (Table I, no. 19), 58 (Fig. 6, lanes *11* and *12*), or 82/83 (Table I, no. 22). Thus, the presence of amino acids Met^{42} and Asp^{77} in LCβ87ΔT impedes the secretion of LCβ87ΔT compared with the rate seen for LCβ41 (Table I, nos. 6, 8, 18, and 21).

Assembly of Mutant and Chimeric LHβ-CGβ Subunits

To analyze the structural determinants for assembly, chimeric and mutant β expression vectors were cotransfected with $pM^2CG\alpha$ (34, 36), and clones expressing both subunits were selected. To ensure that assembly of the β subunit was not limited by the amount of α present, clones expressing α in excess of β were isolated. If α is in excess and all of the β subunit can combine, the entire population of the β subunit synthesized will appear as dimer in the medium.

We have previously shown (35) that in transfected CHO cells CGβ combines efficiently and is secreted rapidly ($t_{½}$ = 1.2 h; Table I, no. 1), whereas LHβ is slower to assemble and less dimer is recovered ($t_{½}$ = 5.5 h; recovery ≈ 42%; Table I, no. 2). To identify the residues that may be responsible for the differential rates of combination, we examined the assembly of clones expressing both α and either CLβ41, LCβ41, or CLβ87. Whereas CLβ41, LCβ41, and CLβ87 are secreted slowly as free subunits as described above (Fig. 4; Table I, nos. 6, 7, and 9), LCβ41 combines more efficiently ($t_{½}$ ≈ 1 h; recovery >95%) than CLβ41 ($t_{½}$ ≈ 2.5 h; recovery ≈ 50%) and CLβ87 ($t_{½}$ ≈ 3 h; recovery ≈ 45%) (Fig. 7; Table I, nos. 6, 7, and 9). However, absence of the 7-amino acid COOH terminus in CLβ41ΔT (Fig. 7 *c*; Table I, no. 11) or CLβ87ΔT (Table I, no. 10) enhances assembly and secretion of dimer compared with CLβ41 (Fig. 7 *b*; Table I, no. 7) or CLβ87 (Table I, no. 9). Thus, the presence of the LHβ COOH-terminal seven-amino acid extension in the chimeras CLβ41 and CLβ87 is partly responsible for their less efficient and delayed assembly. However, because absence of only the LHβ COOH terminus in clones secreting α and LHβΔT has a negligible effect on assembly (Table I, nos. 2 and 4), the different residues in the first 87 amino acids of CGβ must also be responsible for the enhanced assembly of CLβ41ΔT and CLβ87ΔT. For example, some determinants in the first 41 amino acids are candidates because CLβ41ΔT (Fig. 7 *c*; Table I, no. 11) assembles efficiently compared with LHβΔT (Table I, no. 4).

Assembly of LHβ Mutants with Changes between Residues 1 and 87

To determine if changes at positions 8 and 15 also influence assembly in the absence of the LHβ terminus, mutants LHβ8, LHβ8ΔT, and LHβ15*ΔT were coexpressed with the α subunit. Because α is in excess of β in these clones, β should accumulate in the medium, with less in the lysate, if combination is efficient. Immunoprecipitation revealed that the LHβ8 mutant accumulates in the lysate (Fig. 8, lane *1*) with only a small amount exiting as dimer (lane 2). The secretion kinetics of this dimer secretion is also similar to LHβ (Table I, nos. 2 and 13). However, analysis of a clone expressing α and LHβ8ΔT shows that the α subunit causes a rapid secretion of LHβ8ΔT with very little β subunit remaining in the cell (Fig. 8, lanes *4–6*). This is confirmed by pulse-chase analysis of these clones (Table I, no. 14). A similar pattern was seen for clones expressing α and either LHβ8/10ΔT (Table I, no. 15) or LHβ8/10 (data not shown). Therefore, His^{10} plays a minor role in assembly, similar to its effect on monomer secretion. A clone expressing α and LHβ15*ΔT (containing Thr^{15} instead of Ile^{15}), devoid of the 7-amino acid terminus, assembles more efficiently and is associated with faster kinetics (Table I, no. 12; $t_{½}$ = 3.1 h) than mutants with only the Ile^{15} change (LHβ15*; Table I, no. 3) or lacking only the LHβ terminus (Fig. 6 *d*; Table I, no. 4). Thus, alteration of either Trp^{8} or Ile^{15} in the absence of the LHβ hydrophobic extension increases the rate of dimer secretion and the amount of dimer recovered in the medium.

LHβ mutants containing alterations between residues 42 and 87 and lacking the COOH terminus were coexpressed with the α subunit. Whereas mutations at positions 42 (Fig. 7, lanes *7–9*) and 58 (lanes *10–12*) enhance disappearance from the lysate (lanes *7* and *10*) with simultaneous appearance in the medium (lanes *8* and *11*), mutants with changes at positions 47/51, 77, or 82/83 did not assemble efficiently (data not shown). Pulse-chase analysis confirms that both LHβ42ΔT and LHβ58ΔT (Table I, nos. 18 and 20) have an

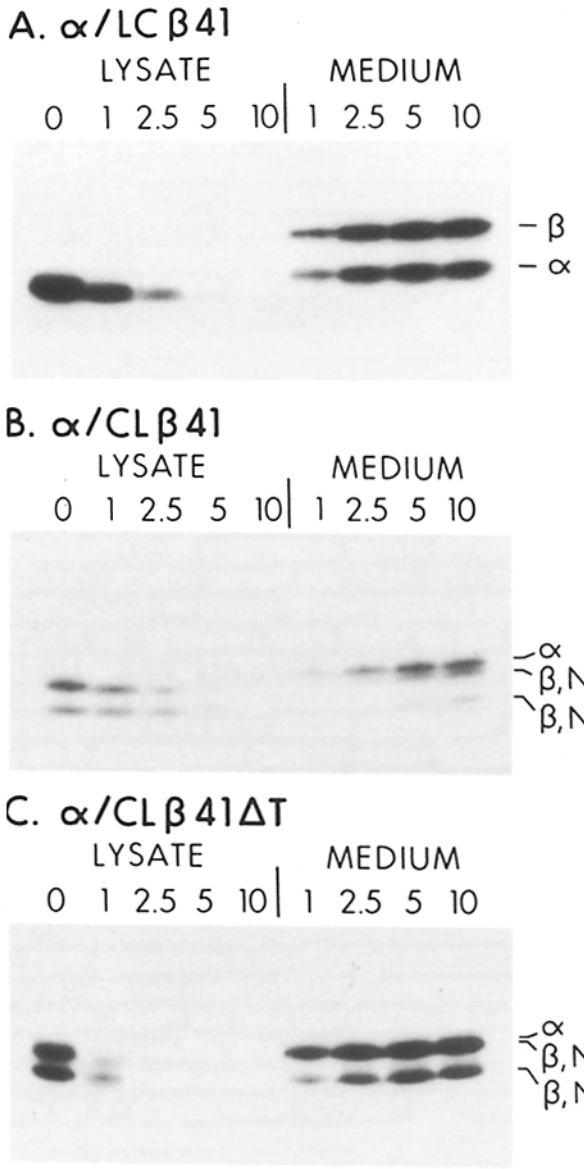

Figure 7. Kinetics of chimeric and mutant LH-CG dimer secretion. Cells expressing α and either LCβ41 (*A*), CLβ41 (*B*), and CLβ41ΔT (*C*) were pulse-labeled and prepared as described in Fig. 4. All chimeras were immunoprecipitated with CGβ antiserum. The migration of α and β subunits are shown. N-1 and N-2 denote the number of *N*-linked units present on the β forms. The α subunit appears in the medium before CLβ41 (medium, 1-, and 2.5-h time points) because there is an intracellular pool of unlabeled β subunit which combines initially with newly synthesized (labeled) α subunit as has been shown previously for LH assembly (11, 35).

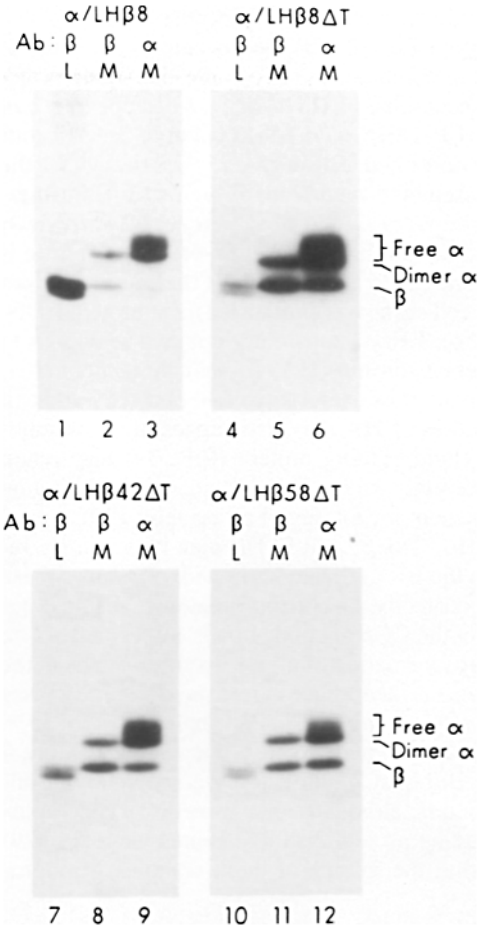

Figure 8. Assembly of mutant LHβ subunits. Cells expressing α and LHβ8 (lanes *1–3*), LHβ8ΔT (lanes *4–6*), LHβΔ42 (lanes *7–9*), and LHβΔ58 (lanes *10–12*) were labeled continuously for 7 h with 20 μCi/ml [^{35}S]cysteine. An aliquot of the lysate (*L*) from each clone was immunoprecipitated with β antiserum and equivalent aliquots of the medium (*M*) from each clone were immunoprecipitated with either α or CGβ antisera (Ab). The migration of dimer α, free α, and β subunits are indicated.

increased rate of dimer formation compared to LHβΔT and alterations of both Met^{42} and Thr^{58} have an additive effect to enhance assembly.

Discussion

Previous studies from our laboratory (11, 35) had shown that the highly homologous LHβ and CGβ subunits differed greatly in their rate of secretion and assembly from the common α subunit. The studies presented here use mutagenesis and chimeric genes to define the structural determinants responsible for these differences. The major findings are that the seven-amino acid hydrophobic COOH-terminal extension acts cooperatively with hydrophobic residues at the NH_2 terminus (Trp^8 and Ile^{15}) and residues Met^{42} and Asp^{77} to cause the LHβ to be retained inside the cell and slowly degraded. Alterations in the assembly of LH are also the result of this interaction, except that changes of Thr^{58} to Asn^{58} in the absence of the LHβ carboxy-terminal extension and not Asp^{77} to Asn^{77} enhance dimer formation. Several changes in the ancestral LHβ/CGβ gene thus have resulted in glycoprotein β subunits with very different intracellular characteristics. Even TSHβ and FSHβ differ from LHβ; although they are retained intracellularly as monomers (35, 22), they nevertheless combine efficiently to form dimer similar to CG. These studies also show that the 31-amino acid extension on CGβ plays a minor role in secretion and assembly of CG, confirming earlier studies (4, 34).

What is the mechanism whereby LHβ is retained inside the cell? LHβ that accumulates in transfected C127 cells is sensitive to endoglycosidase H (11), suggesting that it is retained

in the endoplasmic reticulum (ER). Because assembly normally occurs in the ER (18, 43), and because ≈50% of the retained LHβ can combine, the ER is the likely site of accumulation of uncombined LHβ. Also, the disappearance of unassembled LHβ, TSHβ, and FSHβ subunits could occur via the recently described "ER degradation pathway" for the disposal of unassembled membrane proteins (30). Furthermore, several groups (14, 31, 54; see reference 32 for review) have shown that the rate-limiting step in protein secretion is the transport from ER to Golgi and that the transport from the Golgi to the cell surface is similar for most proteins (≈20 min; reference 54). LHβ is apparently retained analogous to the influenza hemagglutinin (HA) protein monomers (10a, 16); HA monomers must trimerize to fold correctly and exit the ER. The studies of HA (16) also suggest that immunoglobulin heavy-chain binding protein (BiP; 39) may retain some of the incorrectly folded HA protein, and BiP binding may be a requirement for folding of all proteins (20). Incorrectly folded LHβ, TSHβ, and FSHβ may be similarly retained by BiP in the ER until assembly with α induces these subunits to fold correctly. Data from Ruddon et al. (46) suggest that not all of the CGβ disulfide bonds are formed before assembly. Perhaps formation of all the disulfide bonds in the pituitary β subunits is incomplete unless the α subunit causes rearrangement of protein domains, bringing specific cysteines into contact with one another to generate a mature β subunit. Thus, BiP may retain these incompletely disulfide-bonded subunits, although other proteins or a complex of proteins including protein disulfide isomerase (6, 15, 29) may be involved in the folding of these complex glycoproteins.

If LHβ, TSHβ, and FSHβ are retained as monomers intracellularly, why is LHβ the only subunit that is slow to assemble? The differences between LHβ and the other β subunits may reflect the ability of different folding intermediates to assemble with α. Because cysteines 9 and 90 and cysteines 26 and 110 are purported to form disulfide pairs (45, 47), this would bring the NH_2- and COOH-terminal residues in close apposition. The presence of the hydrophobic residues at amino acids 8 and 15 and in the COOH-terminal extension might prevent these contacts. Alternatively, these residues may cause disulfide mispairing, which might contribute to the slow dimer formation and the increased degradation compared with the other β subunits. Furthermore, the derivatives LCβ41 and LHβ58ΔT are secreted slowly as monomers yet assemble quickly and are efficiently secreted as dimers, reemphasizing that determinants for secretion and assembly are different.

What are the advantages of having a β subunit that is retained in the ER? Because the free α subunit is synthesized in excess of β in the pituitary, this would facilitate complete dimer formation and subsequent transport into granules. That LHβ is slow to assemble into dimer compared with CGβ suggests that there may be differences in pituitary (10) versus placenta (1, 5) secretion (i.e., regulated versus constitutive), or that CG dimer is required at high extracellular levels to maintain the corpus luteum of pregnancy. Alternatively, these differences may indicate sequences involved in other functions of LHβ. It is known that LH and FSH are synthesized in the same pituitary cell type in several species (40, 44). However, these hormones can be sorted into separate secretory vesicles and are found in separate regions of the same cell (9, 10). Because bovine LHβ exits the cell only when combined with α (19), the intracellular events seem to be a common phenomenon of all gonadotropes. The residues that delay the secretion and assembly of LH may be associated with the segregation of FSH and LH analogous to the sorting of membrane and secretory proteins in the *trans*-Golgi (41). Whereas LHβ contains a seven-amino acid hydrophobic COOH-terminal extension, FSHβ lacks such an extension and therefore the hydrophobic tail on LHβ may act like a membrane anchor and thereby direct LH to separate granules from FSH.

The structure of LHβ is associated with directing the addition of *N*-acetylgalactosamine (GalNAC) and sulfate to the NH_2 terminus of both the LHβ and α subunits, unlike the galactose and sialic acid present on the FSH subunits (2). The determinants that are recognized by the GalNAC transferase may be these hydrophobic areas (27), because the disulfide bond 26–110 would likely bring the hydrophobic terminus (115–121) in close apposition to the *N*-linked oligosaccharide at LHβ Asn^{30}, and thereby influence the posttranslational processing. Further support for this possible interaction is that the lysate forms containing two *N*-linked oligosaccharides (e.g. CLβ41, CLβ87; Fig. 4 *b* and *e*) show increased oligosaccharide processing as seen in earlier studies of CGβ (34). Furthermore, absence of the CGβ terminus in the derivative CGβΔT causes increased heterogeneity of the secreted form compared with wild-type CGβ (data not shown). This suggests that the LHβ and CGβ carboxy termini may influence one or more posttranslational steps. Thus, the role of specific LHβ amino acids in the sorting and posttranslational processing of LH compared with FSH may be more important in the evolution of LHβ than their effects on dimer assembly, and these mutants may aid in determining structural determinants critical for GalNAC addition. Another interesting finding is that whereas both LHβ and TSHβ contain hydrophobic COOH-terminal extensions and are recognized by the GalNAC transferase, FSHβ lacks a comparable extension and is not similarly modified. Determination of the LHβ structural regions that may be important in GalNAC addition and sorting into granules may help pinpoint regions in other hormones and polypeptides which are critical for similar functions.

Because generation of LHβ-CGβ chimeras does not totally perturb these proteins, it reinforces the idea that the 4 glycoprotein hormone β subunits, with 12 conserved cysteine residues, show identical disulfide pairing. Use of other mutants containing alterations at the cysteine residues will further aid in understanding how the β subunits fold and if dimerization is needed for the final disulfide bonds to form. Furthermore, the disulfide loop 38–57, which has been proposed to be important for receptor binding and signal transduction of LH and CG (25), would cause amino acids 42 and 58 to lie close together. Because these amino acids are important in CG assembly, perhaps this region may also be important in determining the conformation of the β subunit that is needed to bind to its specific receptor. Further use of these chimeras and mutants to more precisely localize unique β subunit epitopes recognized by antibodies that bind specifically to LH or CG may be critical to determining the three-dimensional structure of these hormones and for defining critical regions that determine gonadotropin-specific posttranslational modifications.

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