Partial Restoration Of Lutropin Activity by an Intersubunit Disulfide Bond: Implications For Structure/Function Studies

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Gonadal function is controlled by lutropins and follitropins, heterodimeric cystine knot proteins that have nearly identical α-subunits. These heterodimeric proteins are stabilized by a portion of the hormone-specific β-subunit termed the "seatbelt" that is wrapped around α-subunit loop 2 (α2). Here we show that replacing human chorionic gonadotropin (hCG) α2 residue Lys51 with cysteine or alanine nearly abolished its lutropin activity, an observation that implies that αLys51 has a key role in hormone activity. The activity of the heterodimer containing αK51C, but not that containing αK51A, was increased substantially when β-subunit seatbelt residue βAsp99 was converted to cysteine. As had been reported by others, heterodimers containing αK51C and βD99C were crosslinked by a disulfide. The finding that an intersubunit disulfide restored some of the activity lost by replacing αLys51 suggests that this residue is not crucial for receptor binding or signaling and also that hCG and related hormones may be particularly sensitive to mutations that alter interactions between their subunits. We propose the unique structures of hCG and related family members may permit some subunit movement in the heterodimer, making it difficult to deduce key residues involved in receptor contacts simply by correlating the activities of hormone analogs with their amino acid sequences. [Exp Biol Med Vol. 226(6):581–590, 2001]

Key words: LH receptor; FSH receptor; hCG; bifunctional gonadotropins; crosslinked hCG analogs

Human reproduction is controlled by the anterior pituitary gland hormones human follitropin-stimulating hormone (hFSH) and human lutropin hormone (hLH) and by the placental hormone human choriogonadotropin (hCG). These hormone heterodimers are composed of an α-subunit encoded by the same gene and a unique β-subunit encoded by one (hFSH and hLH) or multiple (hCG) genes (1–3) responsible for the abilities of these hormones to distinguish follitropin receptors (FSHR) and lutropin receptors (LHR) (4). Both subunits have similar architectures and are divided into three loops by cystine knots (5, 6). The β-subunit also contains 20 additional amino acids termed the "seatbelt" (5) that are wrapped around the second α-subunit loop (α2) to stabilize the heterodimer. The seatbelt is the primary portion of the hCG β-subunit responsible for its influence on gonadotropin receptor binding specificity (7). hCG analogs containing hFSH seatbelt residues 94 through 109 or 94 through 114 have low LH activity and high FSH activity (7, 8). Others in which seatbelt residues 101 through 109 are derived from hFSH are bifunctional and have high LH and FSH activities (9, 10).

The mechanism by which the seatbelt controls the receptor binding specificity of hCG is unknown and may involve contacts with the receptor (11), an affect on hormone conformation (12), or both. Several observations suggest the seatbelt affects receptor binding specificity through its influence on hormone conformation. First, most mammalian lutropins bind the rat LHR well, in spite of the fact that their seatbelts are not highly conserved (4). Second, most seatbelt mutations have relatively minor influences on the ability of hCG to bind LHR. Dramatic changes in amino acid size and charge within the small seatbelt loop are required to alter the lutropin activities of hCG and bifunctional hCG analogs by 100-fold (10, 13), the amount characteristically observed in other ligand receptor pairs when a single key contact residue is replaced by alanine (14, 15). And third, there is at least one example in which the seatbelt appears to influence receptor specificity by altering subunit interaction. hCG binds the human LHR with 1,000- to 10,000-fold higher affinity than most mammalian lutropins such as bovine LH (16, 17). The inability of bovine LH to bind human LHR is due primarily to interactions between its seatbelt and the α-subunit (18). Thus, while analogs of hCG containing both the bovine LH α-subunit and seatbelt had little ability to recognize the human LHR, those containing only one of these bovine LH components bind the human LHR like hCG.

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Analogs of hCG in which the C-terminal one-half of the seatbelt is derived from hFSH have receptor binding and signal transduction activities normally associated with both gonadotropins (9, 10). Except for the disulfide that latches the seatbelt to β-subunit loop 1, the C-terminal one-half of the seatbelt contacts few hormone residues other than those in loop α2. This part of the α-subunit has a unique conformation only in the heterodimer (19), where it is constrained by its location between the seatbelt and residues in the "core" of the β-subunit (5). The latter includes parts of the cystine knot, several residues in β-subunit loop 1, and a few residues in β-subunit loop 3 (6). Thus, α2 would be expected to have different conformations in hCG and in bifunctional hCG analogs that are identical in composition to hCG except for the C-terminal halves of their seatbelts (10). Indeed, it is conceivable that α2 might assume two or more conformations in bifunctional analogs, thereby allowing the hormone to interact with both receptors.

Studies described here were initiated to investigate the possibility that the positions of the subunits in bifunctional hCG analogs can move within the heterodimer to acquire conformations that enable them to interact with LH and FSH receptors. For reasons just discussed, we expected that the most likely changes in the conformations of these analogs involved movements of α2 that are controlled by the seatbelt. This idea implied that it might be possible to limit the follitropin activities of the bifunctional analogs by tethering the distance between α2 and the seatbelt to that seen in hCG. To test this idea we converted α2 residue Lys51 and seatbelt residue Asp99 of the hCG analog in which amino acid residues 101 through 114 are replaced by their hFSH counterparts (CFC) to cysteine (Cys) mutations that have been shown to form an intersubunit disulfide in hCG (20). As controls, we prepared the same mutations in hCG. Here we show that converting αLys51 to Cys nearly abolished the lutropin activities of hCG and greatly reduced the lutropin and follitropin activities of CFC. Crosslinking residues α51 and β99 by a disulfide restored some lutropin activity to hCG, but did not restore lutropin or follitropin activities to CFC. The gain in lutropin activity of the disulfide-crosslinked hCG analog suggested that αLys51 does not participate in a key receptor contact and that converting it to Cys or alanine (Ala) reduced the activity of hCG due to an affect on hormone conformation. While these studies did not enable us to understand the basis for the abilities of bifunctional analogs to interact with LH and FSH receptors, the finding that a disulfide can "rescue" some of the activity lost due to conversion of αLys51 to Cys supports the idea that the hCG subunits are much more free to move within the heterodimer than commonly assumed. This may explain the difficulty in identifying receptor contacts by monitoring the influence of point mutations on the activities of hCG and other glycoprotein hormones. The lutropin activity of the seatbelt/α2 disulfide-crosslinked analog of hCG had been noted briefly (10) and appears greater than that reported by Heikoop *et al.* (20).

Materials and Methods

Coordinates describing the structure of hCG (5) were obtained from Dr. Neil Isaacs (Glasgow University, Glasgow, Scotland). hCG and antibodies A407 and B109 were obtained from Dr. Robert Canfield (Columbia University, New York). Antibodies A113, B111, and B112 were obtained from Drs. Robert Wolfert and Glenn Armstrong (Hybritech, Inc., San Diego, CA). Purified hFSH for use in radioiodination was obtained from Zymed Laboratories (San Francisco, CA). hFSH for receptor displacement and signal transduction assays was produced in this laboratory from C127 cells stably transfected with pBMT2x vectors (21) encoding the human α-subunit and the hFSH β-subunit cDNA's downstream of the mouse metallothionein gene. pBMT2x was a gift from Dr. George Pavlakis (NCI, Frederick, MD). CFC is an hCG analog that has the ability to interact with LH and FSHR. The first 110 residues of CFC are identical to those of CF101-109, an analog that has been described (9). Unlike CF101-109, CFC also contains hFSH β-subunit residues 105 through 108 in place of their hCG counterparts (i.e., hCG β-subunit residues 111–114), as well as the entire hCG β-subunit C-terminus (i.e., residues 115–145). CFC was prepared by replacing CF101–109 β-subunit codons 102 through 114 found between its SstII-BamHI sites with β-subunit codons 102 through 145 found between the SstII-BamHI sites of CFC94-114, an analog that has also been described (8). Differences between the amino acid sequences to hCG and CFC β-subunits are diagrammed in Figure 1.

Molecular Modeling. Distances between residues in the hCG α- and β-subunits were measured using the program Sybyl (Tripos Associates, St. Louis, MO) to identify sites that would be the most appropriate locations for introduction of disulfide bonds. These included residues in which the Cα and Cβ atoms in the α- and β-subunits were separated by roughly 6.5Å and 4Å, the approximate distances between the Cα-Cα and Cβ-Cβ atoms of Cys residues in a typical disulfide bond. To constrain the positions of the seatbelt and α2, we introduced a disulfide between residues α51 and β99 by making analogs αK51C, hCGβD99C, and CFCβD99C. To learn if crosslinking the subunits would influence the activity of the hormone, we introduced a disulfide between the cystine knots of each subunit, i.e., between residues α31 and β37. Preparation of an α-subunit analog containing a free Cys at residue 31 was accomplished by converting αCys7 to serine or Ala to create αC7S or αC7A, thereby disrupting the α-subunit disulfide normally found between residues α7 and α31. Its β-subunit partner was prepared by converting Tyr37 to Cys to create hCGβY37C and CFCβY37C.

α-Subunit Constructs. Figure 1A illustrates the relative locations of restriction sites that were used to prepare the α-subunit constructs. To prepare αC7S, we replaced the fragment between the XhoI and BsmI sites of the pKBM-hCGα (7) with the polymerase chain reaction (PCR)

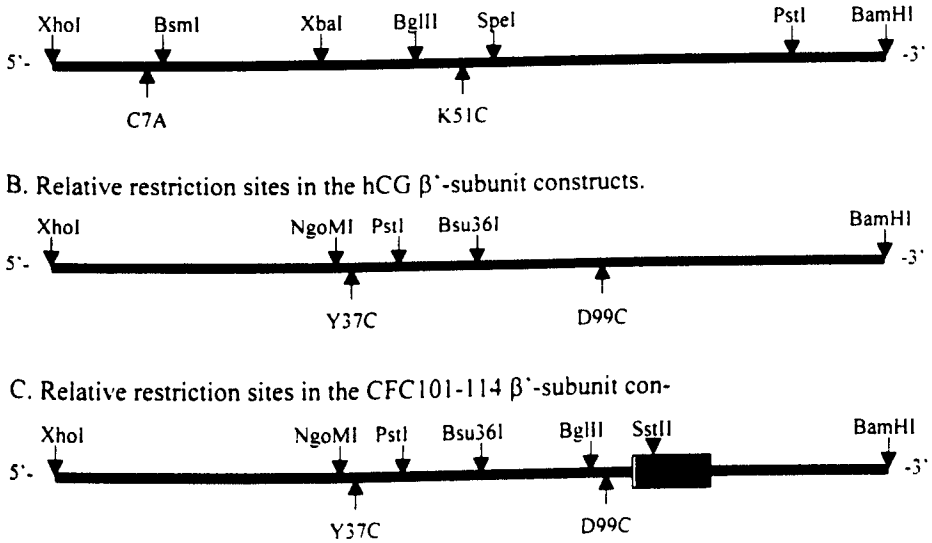

Figure 1. Description of constructs used to express the disulfide crosslinked analogs. These panels illustrate the relative locations of the restriction enzyme cleavage sites and the Cys that were introduced into the α-subunit (A), hCG β-subunit (B), and bifunctional analog (C). The cloning procedures used to prepare each analog are described in the text.

product made using pSVL-hCGα (7) as template and primers 5′-AACCGCCCTGAACACATCCTGCAAA-AAGCCCAGA-3′ and 5′-CTGTAGCGTGCATTCCG-GACTATCCTGCACATCAGGAGC-3′. (The former is complementary to a site in the pSVL vector.) These restriction sites are 5′ of the initiation codon and near the codon for Cys10 of the α-subunit cDNA (Fig. 1), respectively. The XhoI-BsmI-digested PCR product was cloned into the corresponding sites in pKBM-hCGα. Upon confirmation of the DNA sequence by dideoxy methods, the XhoI-BamHI fragment of the resulting vector was subcloned into the XhoI-BamHI sites of pSVL (Pharmacia, Piscataway, NJ) for expression in COS-7 cells (7). This fragment was also cloned into the same sites of pCI′, a derivative of pCI (Promega, Madison, WI) in which the BamHI site had been moved to the polylinker, just 5′ of the polyadenylation signal. This permitted expression in COS-7 and Chinese hamster ovary (CHO) cells. αK51C and αK51A were prepared from an existing α-subunit construct that lacked the 3′ and 5′-untranslated sequences found in the α-subunit cDNA (22) and in which the codons for amino acids Arg42-Ser43 and Thr54-Ser55 had been converted to AGATCT and ACTAGT (i.e., BglII and SpeI restriction sites, respectively). αK51C was prepared by replacing the BglII-SpeI fragment of this vector with a cassette composed of oligonucleotides 5′-GATCTAAGAAGACTATGCTTGTACAATG-TAACGTTA-3′ and 5′-CTGAGTAACGTTACATTGTA-CAAGCATAGTCTTCTTA-3′. αK51A was prepared by replacing the BglII-SpeI fragment of this vector with a cassette composed of oligonucleotides 5′-GATCTAAGAA-GACTATGCTTGTTCAAGCTAACGTTA-3′ and 5′-CTAAGTAACGTTAGCTTGAACAAGCATAGTCTTC-TTA-3′. The DNA sequences of the resulting constructs were confirmed and the constructs were subcloned into XhoI and BamHI sites of pSVL and pCI′. pCI′-αC7S.K51C was prepared by ligating the small fragment obtained by digestion of pSVL-αK51C with BsmI and the large fragment was produced by digestion of pCI′-αC7S with BsmI.

hCG β-Subunit Constructs. Figure 1B illustrates the relative locations of the restriction sites used to prepare the hCG β-subunit constructs. hCGβY37C was made by ligating a cassette containing the complementary oligonucleotides 5′-CCGGCTGTTGTCCTACCATGACACGT-GTGCTGCA-3′ and 5′-GCACACGTGTCATGGTAGGA-CAACAG-3′ into the unique NgoMI and PstI sites of pKBM-hCBβ′ (7). These restriction sites are found in the codons for amino acids Ala35-Gly36 and Leu45-Gln46, respectively. Following confirmation of the sequence of hCGβ′Y37C by dideoxy sequencing, the XhoI-BamHI fragments of this vector were cloned into pSVL and pCI for expression. hCGβD99C was created by PCR mutagenesis starting with pSVL-hCGβ′ (7) as a template and oligonucleotides 5′-TGCCGCAGATCTACTACTTGCTGCGG-GGGTCCCAAGGACCAC-3′ and 5′-CTAGCCTA-GAAGCTCTGACTGTCCTAGTTGTGGTTTGTCCA-AACTCATC-3′. The latter is complementary to a site in the pSVL vector 3′ of the BamHI site. The hCG β-subunit double mutant, hCGβY37C.D99C was produced by replacing the Bsu36I-BamHI fragment of hCGβY37C with that from hCGβD99C.

CFC101-114 β-Subunit Constructs. Figure 1C illustrates the relative locations of the restriction sites that were used to prepare the CFC101-114 β-subunit constructs. CFC101-114 is an hCG analog in which β-subunit amino acids 101 through 114 were replaced with their hFSH β-subunit counterparts (i.e., amino acids 95–108) and that interacts with both LHR and FSHR. CFCβY37C was made by replacing the XhoI-Bsu36I fragment of CFC101-114β

with that from hCGβY37C. CFCβD99C was made by inserting a cassette containing oligos 5′-GGACTGTACAACAAGTAGTA-3′ and 5′-GATCTACTACTTGTTGTACAGTCCGC-3′ between the BglII and SacII sites of CFC101-114β.

Biological Assays. Heterodimers secreted into the medium were quantified in sandwich immunoassays using anti-α-subunit monoclonal antibody A113 for capture, radioiodinated anti-β-subunit monoclonal antibody B112 for detection, and hCG as a standard. These antibodies recognize α- and β-subunit epitopes distant from the locations of the amino acid substitutions. Receptor binding activities were determined from the relative abilities of hCG, hFSH, and the analogs to inhibit binding of ^{125}I-hCG or ^{125}I-hFSH to CHO cells expressing rat LHR or human FSHR (9). Signal-transduction activities were determined from the abilities of hCG, hFSH, and the analogs to stimulate cyclic AMP accumulation in these same cell lines (9). Cyclic AMP was quantified by radioimmunoassay (23). With the exception of hCGα51-β99 and αK51A/hCGβ99C, which were purified using anti-β-subunit antibody B110 as described (24), most analogs were not purified.

Data Analysis. Dose-response curves were fitted with the four-parameter logit function in the program Prism (Graph Pad Software, San Diego, CA). Due to the low affinities of some analogs for the receptors, we did not obtain full inhibition curves at the concentrations of analogs tested. In these cases we assumed that sufficient amounts of analog would inhibit the binding of ^{125}I-hCG or ^{125}I-hFSH to cells expressing LH or FSHR to the same extent as hCG or hFSH. The "blank" was calculated as the amount of radiolabeled ^{125}I-hCG or ^{125}I-hFSH obtained in the presence of an excess of hCG or hFSH. The potencies of the analogs relative to hCG or hFSH in receptor binding assays were calculated by dividing the amount of hCG or hFSH needed to inhibit binding of ^{125}I-hCG or ^{125}I-hFSH by the amount of analog needed to inhibit binding of ^{125}I-hCG or ^{125}I-FSH and then multiplying by 100. Values illustrated in Table I are the means and SEs of these values for at least three independent experiments, except as noted.

The signal transduction activity of each analog was determined by its ability to elicit cyclic AMP accumulation relative to hCG or hFSH. Except as noted, values shown in Table I were calculated by fitting curves to the "raw" data obtained by monitoring the amount of ^{125}I-cyclic AMP bound to the anti-cyclic AMP antibody as a function of hCG, hFSH, and analog concentration. This was done to minimize errors caused by the extra step of transforming the raw data into picomoles cyclic AMP. This procedure tends to underestimate the "potencies" of analogs that have greatly reduced efficacy, however. As the case for the receptor binding data, the relative receptor binding potency of each analog was calculated by dividing the amount of hCG needed to inhibit binding of ^{125}I-hCG by the amount of analog needed to inhibit binding of ^{125}I-hCG and then multiplying by 100. Values illustrated in Table I are the means and SEs of these values for at least three independent experiments, except as noted.

Results and Discussion

Synthesis and Secretion of Analogs. These studies were based on the premise that we could introduce additional Cys residues and disulfide bonds into the subunits of hCG and hCG analogs without interfering with the ability of the proteins to fold or combine to form heterodimers. One of our concerns during the design of these studies was that the presence of additional disulfides, particularly those in which Cys had been introduced within the cystine knots (Fig. 2), would interfere with protein folding and subunit combination. COS-7 cells transfected transiently with cDNAs encoding hCG subunits secreted heterodimers into the media regardless of the Cys residues that had been introduced (Table II). These observations suggested that the mutations did not prevent formation of the

Table I. Summary of the Relative Potencies of the Analogs Described in These Studies

	hCGβ Analogs (hCG = 100%) LH receptor-expressing cells		CFCβ Analogs (CFC = 100%)			
			LH receptor-expressing cells		FSH receptor-expressing cells	
Mutation	Cyclic AMP	Binding	Cyclic AMP	Binding	Cyclic AMP	Binding
None	100%	100%	100%[a]	100%[b]	100%[c]	100%[d]
αK51A/β	0.27% ± 0.06%	0.49% ± 0.10%	0.067% ± 0.016%	1.7% ± 0.2%	Undetectable	Not tested
αK51C/β	<0.001%	<0.001%	0.30% ± 0.04%	0.039% ± 0.004%	5.4% ± 0.7%	Undetectable
α/βD99C	8.8% ± 2.6%	8.1% ± 0.9%	11.6% ± 2.6%	19.2% ± 5.6%	2.5% ± 0.6%	Undetectable
αK51A/βD99C	0.5% ± 0.1%[e]	0.43% ± 0.02%[e]	Not tested	Not tested	Not tested	Not tested
α51-β99	2.8% ± 0.7%[e]	6.0% ± 0.7%[e]	0.30% ± 0.05%	1.1% ± 0.7%	1.7% ± 1.5%	Undetectable
α31-β37	101% ± 22%	106% ± 13%	36% ± 24%	Not tested	48%[f]	Not tested
α51-β99/ α31-β37	0.64% ± 0.16%	2.6% ± 0.7%	Not tested	Not tested	Not tested	Not tested

[a] Relative to hCG, this value is 31.8% ± 3.3%.
[b] Relative to hCG, this value is 29.1% ± 7.9%.
[c] Relative to hFSH, this value is 2.16% ± 0.17%.
[d] Relative to hFSH, this value is 43.2% ± 1.9%.
[e] Determined using purified analogs.
[f] One assay only.

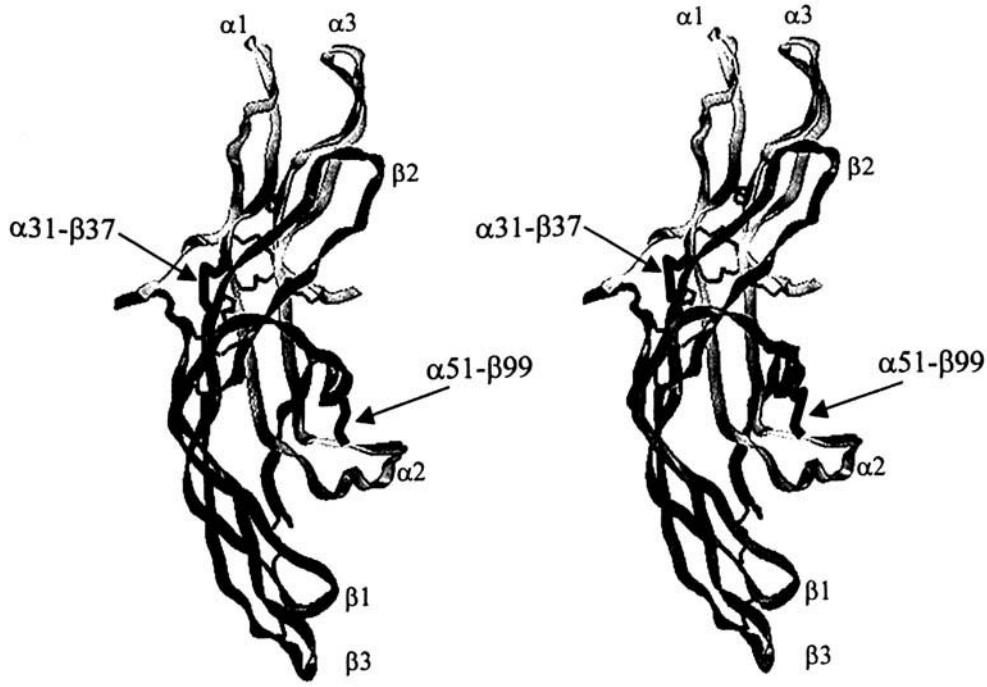

Figure 2. Stereo views illustrating the locations of the α31-β37 and α51-β99 in hCG. This figure was generated using the modeling package Sybyl and illustrates the α-subunit (light gray), β-subunit (dark gray), disulfides normally found in hCG (thin dark gray lines), and α31-β37 and α51-β99 disulfides (thick black lines) following energy minimization. The locations of the latter two disulfides are identified by arrows. The three loops of each subunit are also labeled. Seatbelt residues C-terminal to the small seatbelt loop and shown behind α2 are derived from hFSH in CFC.

Table II. Production of Hormone Analogs in COS-7 Cells Transfected Transiently

Analog	Experiment 1 (ng/ml)	Experiment 2 (ng/ml)	Experiment 3 (ng/ml)
hCG	101.4	390.0	706.6
hCGα31-β37	235.2	Not done	Not done
hCGα51-β99	Not done	97.0	Not done
hCGα31-β37, α51-β99	Not done	Not done	732.0

Note. The analogs were estimated by sandwich immunoassay as described in the text using an hCG standard.

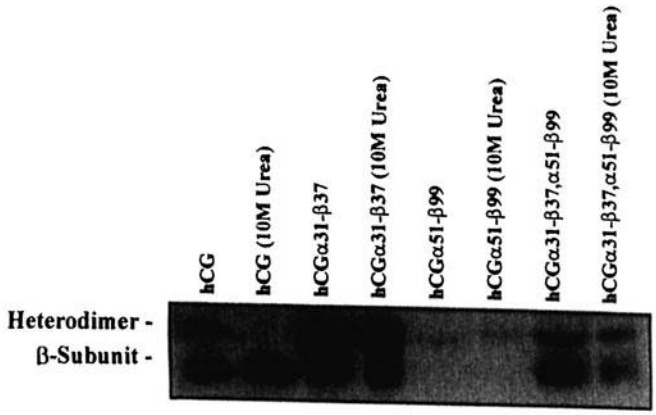

Figure 3. Western blot analysis of hCG and disulfide containing analogs. Analogs secreted into the media were incubated in the absence or presence of 10 M urea, subjected to electrophoresis on 12% polyacrylamide gels, electroblotted onto nitrocellulose, and detected with ^{125}I-B112, an anti-β-subunit antibody.

cystine knots or otherwise grossly disrupt protein folding. They also suggested that the potential to form intersubunit disulfides did not substantially inhibit or enhance combination of these subunit analogs.

Intersubunit Disulfide Bone Formation. To assess disulfide bond formation, we monitored the stabilities of the heterodimers to urea (25) and heat-induced denaturation (26). Consistent with the idea that intersubunit disulfides can stabilize proteins (27), modification of hCG to introduce a disulfide between its cystine knots or between α2 and the seatbelt prevented the subunits from dissociating in the presence of 10 M urea (Fig. 3). Furthermore, these analogs were much more stable than hCG or CFC at 85°C (Fig. 4). These observations suggested that intersubunit disulfides had formed between residues α31-β37 and α51-β99. The latter observations are in agreement with those of Heikoop *et al.* (20) who found intersubunit disulfide bonds between α5-β8 and α35-β35 increased the heat stability of hCG at 65°C. The thermal stability of analogs containing the α51-β99 disulfide appeared to be greater than those containing the intercystine knot disulfide. This may indicate that thermal denaturation begins with the escape of α2 from beneath the seatbelt, a process that is expected to be reduced greatly by the presence of a disulfide between α2 and the seatbelt.

Influence of the Intercystine Knot Disulfide (α31-β37) on the Activity of hCG. The primary goal of these studies was to determine how constraining the distance between the seatbelt and α2 with a disulfide would influence the lutropin and follitropin activities of a bifunctional hCG analog. To test the possibility that any intersubunit disulfide would alter hormone activity, we prepared a crosslinked "control analog" containing a disulfide between the cystine knots. Several factors influenced our decision to make this analog. First, computer simulations suggested that the introduction of a disulfide into this site would not alter the structure of the hormone (Fig. 2). Second, the cystine knot of each subunit is stabilized by three disulfides, making

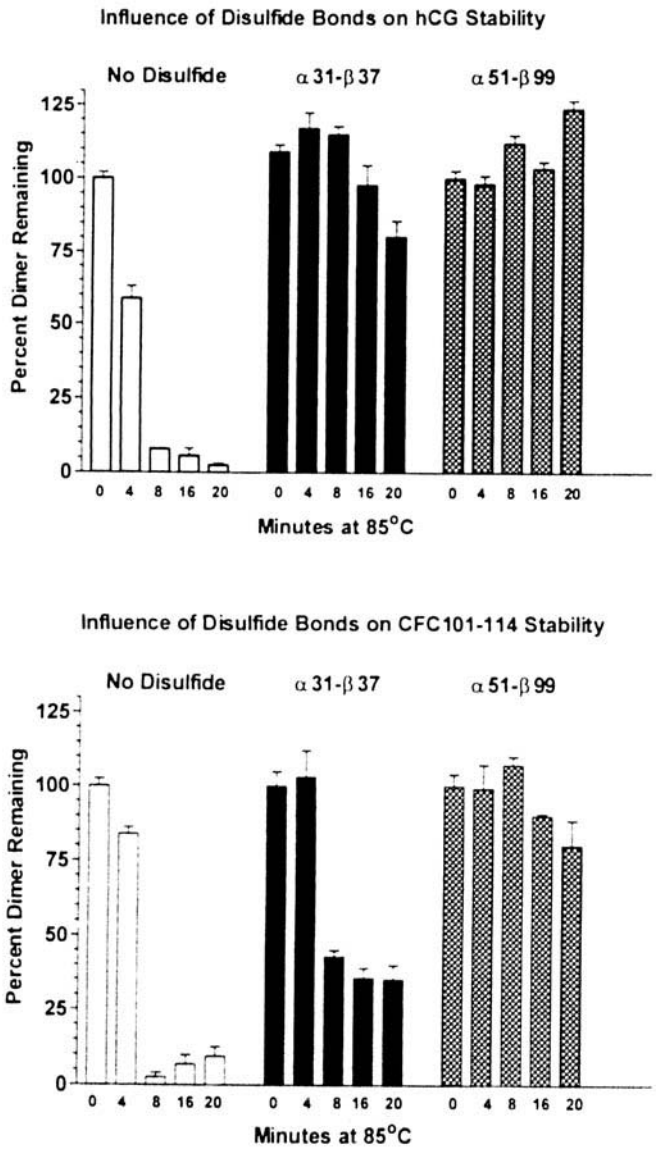

Figure 4. Temperature stability of hCG, CFC, and disulfide crosslinked analogs. The hormones and analogs were incubated at 85°C for the intervals indicated on the abscissa. The samples were rapidly cooled in an ice bath and the heterodimer was measured using a sandwich immunoassay employing antibody A115 for capture and radioiodinated antibody B105 for detection. Values are normalized relative to amount of heterodimer at the start of the assay procedure (approximately 20,000 cpm above the blank).

it one of the most rigid parts of the molecule. Therefore, the introduction of a disulfide between the cystine knots was not expected to alter the relationship between the seatbelt and α2 or to distort any subunit loop. Third, it was known that changing β-subunit residue Tyr37 to phenylalanine or leucine (28), histidine (R.K. Campbell and W.M. Moyle, unpublished data), or methionine (R.V. Myers and W.R. Moyle, unpublished data) had relatively little influence on hormone activity. Thus, the presence of a Cys residue at this site was not expected to alter hormone activity. And finally, it was known that Cys7 is not needed for the lutropin activity of hCG (29). Thus, we expected that elimination of this Cys, to disrupt the Cys7-Cys31 disulfide and create a free thiol at α-subunit residue 31, would not disrupt lutropin activity.

hCGα31-β37, the hCG analog containing an intersubunit disulfide between its cystine knots, bound LHR and stimulated signal transduction similar to hCG (Fig. 5 and Table I). This suggested that the presence of a disulfide crosslink *per se* did not alter hormone activity and it supported conclusions reached several years ago (30) that the hormone did not need to dissociate to function. These observations extend those of Heikoop *et al.* (20) who showed the presence of disulfides between residues α5-β8, α35-β35, and α37-β33 also had no influence on the lutropin activity of hCG.

Influence of the α2-Seatbelt Disulfide (α51-β99) on the Activity of hCG. hCGα51-β99, the analog containing the α51-β99 intersubunit disulfide, had only 3% to 6% the activity of hCG in signal-transduction and receptor binding assays (Fig. 6 and Table I). While the activity of the analog was much lower than that of hCG, it appeared to be greater than that observed by Heikoop *et al.* (20), and its activity in binding assays was similar to what we had observed with bovine LH (31). hCGα51-β99 stimulated cyclic AMP accumulation, but not to the same maximum as hCG,

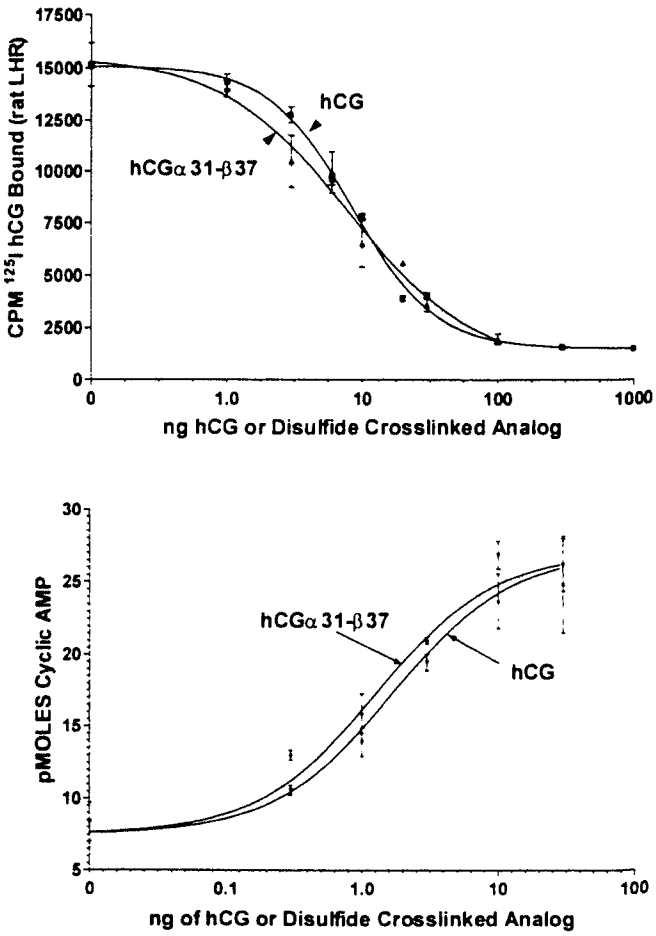

Figure 5. Influence of the α31-β37 intercystine knot disulfide on the ability of hCG to bind rat LHR (upper panel) and initiate signal transduction (lower panel).

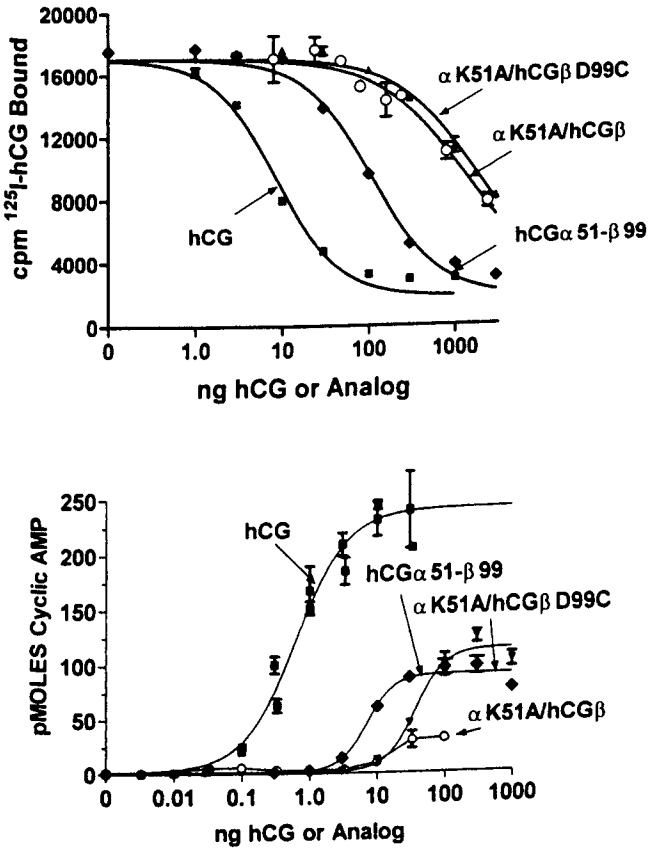

Figure 6. Effect of hCG, αK51A/hCGβD99C, αK51A/β, and hCGα51-β99 on LHR binding (upper panel) and signaling (lower panel). hCGα51-β99 and αK51A/hCGβD99C used in these studies were purified by immunoaffinity chromatography on B110 resin. The data shown for αK51A/β were taken from a separate study employing material that had not been purified.

indicating that its efficacy was approximately one-half that of hCG (Fig. 6).

To learn if the low activities of the disulfide-crosslinked analog were due to the presence of the crosslink or to the absence of either αLys51 or βAsp99, we compared the activities of hCG, αK51C/hCGβ, α/hCGβD99C, αK51A/hCGβ, and αK51A/hCGβD99 with hCGα51-β99, the only disulfide-crosslinked analog in this series (Figs. 6 and 7 and Table I). α/hCGβD99C, the analog containing Cys in place of β-subunit residue 99, had approximately 8% to 9% the activity of hCG (Table I). The activity of α/hCGβD99C was similar to that of an hCG analog in which β-subunit aspartic acid residue 99 had been replaced by asparagine (13). This suggested that by itself, the presence of a Cys at β-subunit residue 99 was not responsible for the reduced activity of α/hCGβD99C relative to hCG. The finding that α/hCGβD99C had only 1.5- to 3-fold more activity than hCGα51-β99 (Table I) also suggested that the presence of Cys at β-subunit residue 99 may have been responsible for much of the loss in hormone activity associated with formation of the disulfide in hCGα51-β99. In contrast, replacing αLys51 with Cys nearly eliminated the receptor binding and signal transduction activities of hCG. To learn if this was due to the presence of Cys at this site rather than the absence of lysine, we tested the activity of αK51A/hCGβ, an hCG analog in which αLys51 had been replaced by Ala. While the activity of αK51A/hCGβ was greater than that of αK51C/hCGβ, it was substantially lower than that of hCGα51-β99, the disulfide-crosslinked analog (Table I). If the activity of the disulfide crosslinked analog were the product of the individual activities of αK51C/hCGβ and α/hCGβD99C, it would have been lower than that of either of these analogs and barely detectable in our assays. However, the activity of the crosslinked heterodimer exceeded that of αK51C/hCGβ, αK51A/hCGβ, and αK51A/hCGβD99C (Fig. 6) and was 30% to 75% that of α/hCGβD99C (Fig. 7 and Table I). This showed that the introduction of the disulfide mitigated nearly all the deleterious effects of mutating αLys51 to Ala and suggested that αLys51 was not essential for hormone activity.

An hCG analog that had the potential to form two intersubunit disulfides (i.e., hCGα31-β37 and α51-β99) had somewhat lower activities than the analog that formed a disulfide between α2 and the seatbelt (Table I). This supports the idea that the α31-β37 disulfide has little influence on hormone activity. However, since we are unable to prove that both intersubunit disulfide bonds had formed, we did not characterize similar versions of CFC.

Influence of Intersubunit Disulfide Bonds on the Lutropin and Follitropin Activities of the Bifunctional Analog. We found previously that substituting hCG β-subunit residues 101 through 109 with their hFSH β-subunit counterparts (i.e., residues 95–103) led to a heterodimer termed CF101-109 that interacted with FSHR much better than hCG and that retained its ability to interact with LHR (9). The presence of the additional FSH residues in CFC appeared to reduce its lutropin activity relative to that of CF101-109.

The α31-β37 disulfide had little influence on the LH and FSH activities of CFC (Fig. 8 and Table I). This showed that the location of this disulfide did not disrupt hormone activity, a phenomenon we have since confirmed for hFSH (R.V. Myers *et al.*, unpublished data). In contrast, the α51-β99 disulfide reduced the lutropin and follitropin activities

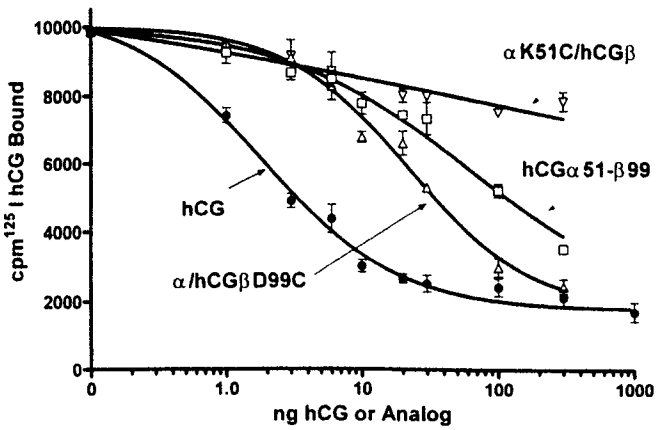

Figure 7. Effects of the hCG, hCGα51-β99, α/hCGβD99C, and αK51C/β on binding of ^{125}I-hCG to rat LHR.

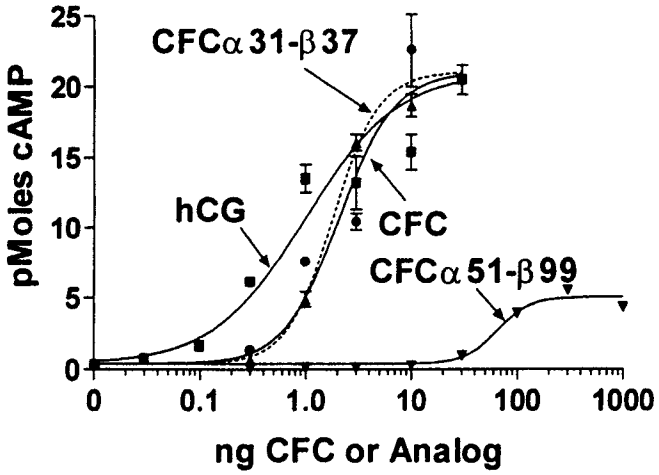

Figure 8. Effects of the α51-β99 and α31-β37 disulfide bonds on the signal transduction of CFC.

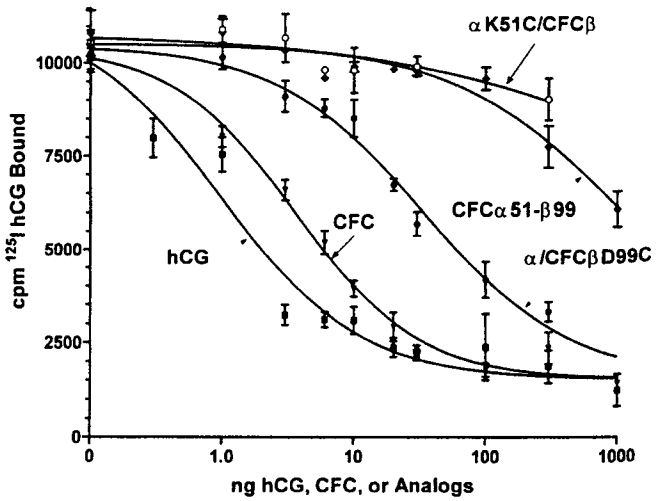

Figure 10. Effects of hCG, CFC, α/CFCβD99C, αK51C/CFCβ, and CFCα51-β99 on binding of ^{125}I-hCG to LHR.

of CFC. CFCα51-β99 had much lower LH activity than hCGα51-β99 and little, if any, FSH activity (Figs. 8 and 9 and Table I). To learn if this was due to the effect of replacing αLys51 or βAsp99 of CFC by Cys, we compared the LH and FSH activities of CFC, αK51C/CFCβ, α/CFCβD99C, and CFCα51-β99 (Figs. 9 and 10 and Table I). Replacing βAsp99 with Cys reduced the LH activities of CFC and hCG to a similar extent (Table I). However, this change effectively abolished the FSH activity of CFC (Fig. 9 and Table I), suggesting that Asp99 had a greater influence on FSH activity than on LH activity. This observation extended the earlier demonstration that residues in the C-terminal one-half of the seatbelt were much more important for FSH activity than LH activity (9). While the LH activity of αK51C/CFCβ was low, it was somewhat greater than that of αK51C/hCGβ (Table I). Unlike hCGα51-β99, which was nearly as potent as α/hCGβD99C and much more active than α51C/hCGβ (Fig. 7 and Table I), CFCα51-β99 was much less active than α/CFCβ99 and similar in potency to αK51C/CFCβ (Fig. 10 and Table I). Thus, FSH residues in CFC offset the influence of the α51-β99 disulfide, a finding consistent with the idea that α2 and the seatbelt have different conformations in hCG and CFC. Due to the extremely low FSH activity of CFC analogs caused by replacing αLys51 and βAsp99 with Cys, we were not able to evaluate the influence of the α51-β99 disulfide on the FSH activity of CFC.

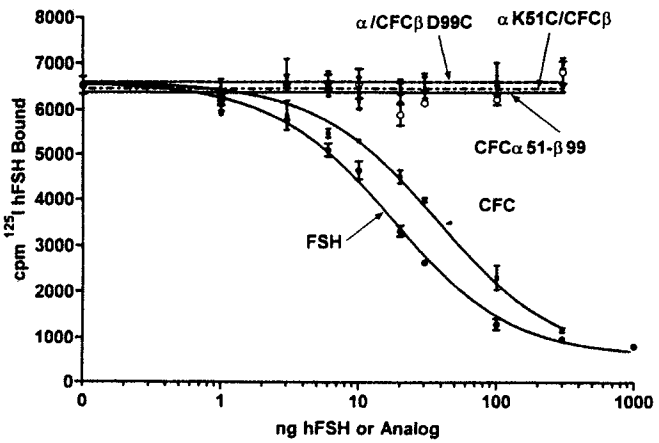

Figure 9. Effects of hFSH, CFC, α/CFCβD99C, αK51C/CFCβ, and CFCα51-β99 on binding of ^{125}I-hFSH to human FSHR.

Implications of These Observations for Receptor Interaction. The reduced potencies of analogs containing substitutions at α-subunit residue Lys51 and/or β-subunit residue Asp99 might reflect the possibility that either of these residues participates directly in receptor contacts, that these substitutions alter the conformation of the heterodimer, or both. While we cannot exclude the possibility that the 10- to 12-fold reduction in the lutropin activities caused by replacing βAsp99 in hCG and CFC is the result of disrupting a receptor contact, we favor the idea that it is due to a change in hormone conformation. The reduction in potency of hCG caused by replacing βAsp99 with Cys was similar in magnitude to the loss in activity caused by changing βAsp99 to asparagine (13). This argues against the idea that the loss in potency was due to the introduction of Cys *per se*. Conceivably, the influence of these mutations is related primarily to their influence on the charge of the hormone at this site. βAsp99 is in the center of a positively charged triangle created by the side-chains of αLys51, βArg95, and βLys104 residues found in α2, the small seatbelt loop, and the carboxyterminal one-half of the seatbelt, respectively. The negative charge of βAsp99 may offset repulsive ionic interactions between these positively charged residues and its replacement by asparagine or Cys might lead to subtle changes in the positions of α2 and parts of the seatbelt. Replacing βAsp99 with arginine led to a further reduction in activity (13), an observation consistent with this idea.

The ability of an hCG β-subunit analog containing Cys in place of Asp99 to offset much of the loss in activity caused by changing α-subunit residue Lys51 to Cys sug-

gests strongly that this α-subunit residue does not contact the receptor. If this lysine made a key essential contact with the LH receptor, we would expect that this role in hormone binding could not be replaced by a disulfide. Furthermore, conversion of βAsp99 to Cys, a mutation that by itself reduced hormone activity, might be expected to augment the reduction in hormone potency caused by mutation of the lysine. The finding that αK51A/hCGβ and αK51A/hCGβD99C had lower activities than hCGα51-β99 (Table I) suggested that the enhancement of activity by the Cys at β-subunit residue 99 depended on the formation of a disulfide with α-subunit residue 51. Thus, the gain in lutropin activity of hCG analogs lacking αLys51 appears to have been the result of constraining the distance between residues α51 and β99 to that seen in the heterodimer. Nonetheless, the finding that the α51-β99 disulfide did not restore lutropin activity to CFC suggested that the conformations of other parts of α2 and the seatbelt are important for receptor interaction. Together, the loss in activity caused by converting hCG α-subunit residue Lys51 to Cys and the restoration of activity by insertion of the α51-β99 disulfide suggest to us that the glycoprotein hormones are particularly sensitive to mutations that alter their conformations, a phenomenon that may result from their unique structures. This would explain why it has been so difficult to identify hormone residues responsible for key receptor contacts.

Finally, it is not clear why the efficacies of hCGα51-β99 and hCGαK51A-βD99C were reduced. One possibility is that α-subunit Lys51 is adjacent to a residue that is glycosylated in hCG. Glycosylation of Asn52 has been shown to influence the efficacy (32) and structure (33) of hCG. Modification of αLys51 may have a similar effect on the structure of the hormone.

1. Fiddes JC, Talmadge K. Structure, expression, and evolution of the genes for the human glycoprotein hormones. In: Greep RO, Ed. Recent Progress in Hormone Research. New York: Academic Press. **40:**43–78, 1984.
2. Bo M, Boime I. Identification of the transcriptionally active genes of the chorionic gonadotropin β gene cluster *in vivo*. J Biol Chem **267:**3179–3184, 1992.
3. Watkins PC, Eddy R, Beck AK, Vellucci V, Leverone B, Tanzi RE, Gusella JF, Shows TB. DNA sequence and regional assignment of the human follicle stimulating beta-subunit gene to the short arm of human chromosome 11. Gene **6:**205–212, 1987.
4. Pierce JG, Parsons TF. Glycoprotein hormones: structure and function. Annu Rev Biochem **50:**465–495, 1981.
5. Lapthorn AJ, Harris DC, Littlejohn A, Lustbader JW, Canfield RE, Machin KJ, Morgan FJ, Isaacs NW. Crystal structure of human chorionic gonadotropin. Nature **369:**455–461, 1994.
6. Wu H, Lustbader JW, Liu Y, Canfield RE, Hendrickson WA. Structure of human chorionic gonadotropin at 2.6Å resolution from MAD analysis of the selenomethionyl protein. Structure **2:**545–558, 1994.
7. Campbell RK, Dean Emig DM, Moyle WR. Conversion of human choriogonadotropin into a follitropin by protein engineering. Proc Natl Acad Sci U S A **88:**760–764, 1991.
8. Wang Y, Han Y, Myers RV, Macdonald GJ, Moyle WR. New recombinant hCG analogs with potent FSH activity *in vivo*. In: Filicori M, Flamigni C, Eds. Ovulation Induction: Basic Science and Clinical Advances. Amsterdam: Excerpta Medica International Congressional Series 1046. Elsevier Science B.V., pp191–196, 1994.
9. Moyle WR, Campbell RK, Myers RV, Bernard MP, Han Y, Wang X. Co-evolution of ligand-receptor pairs. Nature **368:**251–255, 1994.
10. Han Y, Bernard MP, Moyle WR. hCGβ Residues 94–96 alter LH activity without appearing to make key receptor contacts. Mol Cell Endocrinol **124:**151–161, 1996.
11. Jiang X, Dreano M, Buckler DR, Cheng S, Ythier A, Wu H, Hendrickson WA, Tayar NE, el Tayar N. Structural predictions for the ligand-binding region of glycoprotein hormone receptors and the nature of hormone-receptor interactions. Structure **3:**1341–1353, 1995.
12. Moyle WR, Campbell RK, Rao SNV, Ayad NG, Bernard MP, Han Y, Wang Y. Model of human chorionic gonadotropin (hCG) and lutropin receptor (LHR) interaction that explains signal transduction of the glycoprotein hormones. J Biol Chem **270:**20020–20031, 1995.
13. Chen F, Wang Y, Puett D. Role of the invariant aspartic acid 99 of human choriogonadotropin beta in receptor binding and biological activity. J Biol Chem **266:**19357–19361, 1991.
14. Muller YA, Li B, Christinger HW, Wells JA, Cunningham BC, De VA. Vascular endothelial growth factor: Crystal structure and functional mapping of the kinase domain receptor binding site. Proc Natl Acad Sci U S A **94:**7192–7197, 1997.
15. Clackson T, Wells JA. A hot spot of binding energy in a hormone-receptor interface. Science **267:**383–386, 1995.
16. Davies TF, Walsh PC, Hodgen GD, Dufau ML, Catt KJ. Characterization of the primate luteinizing hormone receptor in testes homogenates and Leydig cells. J Clin Endocrinol Metab **48:**680–685, 1979.
17. Jia X-C, Oikawa M, Bo M, Tanaka T, Ny T, Boime I, Hsueh AJW. Expression of human luteinizing hormone (LH) receptor: Interaction with LH and chorionic gonadotropin from human but not equine, rat, and ovine species. Mol Endocrinol **5:**759–768, 1991.
18. Cosowsky L, Lin W, Han Y, Bernard MP, Campbell RK, Moyle WR. Influence of subunit interactions on lutropin specificity: Implications for studies of glycoprotein hormone function. J Biol Chem **272:**3309–3314, 1997.
19. De Beer T, Van Zuylen CW, Leeflang BR, Hard K, Boelens R, Kaptein R, Kamerling JP, Vliegenthart JF. NMR studies of the free alpha subunit of human chorionic gonadotropin: Structural influences of N-glycosylation and the beta subunit on the conformation of the alpha subunit. Eur J Biochem **241:**229–242, 1996.
20. Heikoop JC, van den boogaart P, Mulders JWM, Grootenhuis PDJ. Structure-based design and protein engineering of intersubunit disulfide bonds in gonadotropins. Nature Biotechnol **15:**658–662, 1997.
21. Pavlakis GN, Felber BK, Wright CM, Papamatheakis J, Tse T. Applications of bovine papilloma virus and retroviral vectors. In: Miller JH, Calos MP, Eds. Gene Transfer Vectors for Mammalian Cells. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, pp29–38, 1987.
22. Fiddes JC, Goodman HM. Isolation cloning and sequence analysis of the cDNA for the α-subunit of human chorionic gonadotropin. Nature **281:**351–356, 1979.
23. Brooker J, Harper JF, Terasaki WL, Moylan RD. Radioimmunoassay of cyclic AMP and cyclic GMP. Adv Cyclic Nucl Res **10:**1–33, 1979.
24. Moyle WR, Myers RV, Wang Y, Han Y, Lin W, Kelley GL, Ehrlich PH, Rao SNV, Bernard MP. Functional homodimeric glycoprotein hormones: Implications for hormone action and evolution. Chem Biol **5:**241–254, 1998.
25. Swaminathan N, Bahl OP. Dissociation and recombination of the subunits of human chorionic gonadotropin. Biochem Biophys Res Commun **40:**422–427, 1970.
26. Forastieri H, Ingham KC. Thermal stability of human chorionic gonadotropin. J Biol Chem **257:**7976–7981, 1982.
27. Sauer RT, Hehir K, Stearman RS, Weiss MA, Jeitler-Nelson A, Suchanekk EG, Pabo CO. An engineered inter-subunit disulfide enhances the stability and DNA binding of the N-terminal domain of λ repressor. Biochemistry **25:**5992–5998, 1986.
28. Xia H, Fernandez LM, Puett D. Replacement of the invariant tyrosine

in the CAGY region of the human chorionic gonadotropin beta subunit. Mol Cell Endocrinol **92:**R1–R5, 1993.
29. Furuhashi M, Ando H, Bielinska M, Pixley MR, Shikone T, Hsueh AJW, Boime I. Mutagenesis of cysteine residues in the human gonadotropin α-subunit: Roles of individual disulfide bonds in secretion, assembly, and biologic activity. J Biol Chem **269:**25543–25548, 1994.
30. Dufau ML, Catt KJ, Tsuruhara T. Biological activity of hCG released from testis binding sites. Proc Natl Acad Sci U S A **69:**2414–2416, 1972.
31. Bernard MP, Myers RV, Moyle WR. Lutropins appear to contact two independent sites in the extracellular domain of their receptors. Biochem J **335:**611–617, 1998.
32. Matzuk MM, Keene JL, Boime I. Site specificity of the chorionic gonadotropin N-linked oligosaccharides in signal transduction. J Biol Chem **264:**2409–2414, 1989.
33. Purohit S, Shao K, Balasubramanian SV, Bahl OP. Mutants of human choriogonadotropin lacking N-glycosyl chains in the alpha-subunit. 1. Mechanism for the differential action of the N-linked carbohydrates. Biochemistry **36:**12355–12363, 1997.