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Alternatively Folded Choriogonadotropin Analogs

IMPLICATIONS FOR HORMONE FOLDING AND BIOLOGICAL ACTIVITY*

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Most heterodimeric proteins are stabilized by intersubunit contacts or disulfide bonds. In contrast, human chorionic gonadotropin (hCG) and other glycoprotein hormones are secured by a strand of their β-subunits that is wrapped around α-subunit loop 2 "like a seatbelt." During studies of hCG synthesis in COS-7 cells, we found that, when the seatbelt was prevented from forming the disulfide that normally "latches" it to the β-subunit, its carboxyl-terminal end can "scan" the surface of the heterodimer and become latched by a disulfide to cysteines substituted for residues in the α-subunit. Analogs in which the seatbelt was latched to residues 35, 37, 41–43, and 56 of α-subunit loop 2 had similar lutropin activities to those of hCG; that in which it was latched to residue 92 at the carboxyl terminus had 10–20% the activity of hCG. Attachment of the seatbelt to α-subunit residues 45–51, 86, 88, 90, and 91 reduced lutropin activity substantially. These findings show that the heterodimer can form before the β-subunit has folded completely and support the notions that the carboxyl-terminal end of the seatbelt, portions of α-subunit loop 2, and the end of the α-subunit carboxyl terminus do not participate in lutropin receptor interactions. They suggest also that several different architectures could have been sampled without disrupting hormone activity as the glycoprotein hormones diverged from other cysteine knot proteins.

The heterodimeric placental glycoprotein hormone hCG[1] binds LHR and stimulates ovarian steroid synthesis during early pregnancy (1). The structure of hCG is known (2, 3), but the structures of the LHR and the hormone receptor complex remain to be elucidated. Each hCG subunit is divided into three elongated loops by cystine knots (2, 3), and the heterodimer is stabilized by a part of the β-subunit termed the "seatbelt" (2) that is wrapped around α-subunit loop 2. The composition of the seatbelt determines the receptor binding specificity of hCG (4–6), but it is not known if this portion of the hormone contacts the receptor. The LHR is a G protein-coupled receptor that contains a large extracellular domain that binds hCG with high affinity and specificity (7). Based on its leucine-rich repeat motif (7), the LHR extracellular domain is usually presumed to be horseshoe-shaped, similar to ribonuclease inhibitor (8).

The surfaces of hCG most likely to contact the LHR remain debated. The carboxyl-terminal end of the α-subunit, which is adjacent to a portion of the seatbelt in the heterodimer (2, 3), has been found to influence the affinity of all the glycoprotein hormones for their receptors (1, 9) and was proposed to be a receptor contact more than 25 years ago (1). Based partially on this observation, Jiang *et al.* (10) suggested that the long axis of hCG docks with the concave surface of a horseshoe-shaped extracellular domain. In their view, the hormone is perpendicular to the extracellular domain of the receptor such that the α-subunit carboxyl-terminal end, parts of β-subunit loop 2, and the seatbelt form key contacts with the concave surface of the receptor extracellular domain. Remy *et al.* (11) suggest that similar portions of the hormone contact a region nearer the rim of the extracellular domain. In this model the position of the hormone is tilted relative to that of Jiang *et al.* (10) such that the ends of α-subunit loops 1 and 3 contact residues in the receptor transmembrane domain to initiate signal transduction. This explained the finding that an antibody to the α-subunit bound hCG when the hormone was complexed with the receptor extracellular domain but not with the intact receptor (12). A third view of the hormone-receptor complex (13) suggested that the long axis of hCG is oriented parallel to the plane of the extracellular domain such that the hormone contacts its rim at two widely separated sites. Contacts with the amino-terminal half of the extracellular domain contribute to the high affinity of the hormone for the receptor (14) and those near the carboxyl-terminal end influence receptor binding specificity (15).

In principle, these models should be readily distinguished using site-directed mutagenesis to identify portions of the hormone and receptor that contact one another. Unfortunately, mutations of the α-subunit carboxyl terminus and β-subunit seatbelt alter the positions of the subunits within the heterodimer (13, 16) and have made it difficult to interpret the influence of these key parts of the hormone on its function. Efforts to test the role of the α-subunit in hormone-receptor interaction led us to design a scanning mutagenesis strategy useful for identifying residues that do not contact the LHR. We prepared hCG analogs in which the seatbelt is cross-linked by a disulfide to specific residues in α-subunit loop 2 and carboxyl terminus. In these analogs, β-subunit residues at the end of the seatbelt and at the beginning of the carboxyl terminus are located adjacent to specific parts of the α-subunit where they would be in a position to disrupt contacts between the α-subunit and the receptor. Cross-linked α-subunit residues of analogs that had high activities in LHR binding assays would be expected not to participate in key receptor interactions. Each model of the hormone-receptor complex makes specific predictions about contacts between the α-subunit and the LHR. Thus, a finding that one or more of these predictions is likely to be

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[1] The abbreviations used are: hCG, human choriogonadotropin; LHR, lutropin receptor; CHO, Chinese hamster ovary.

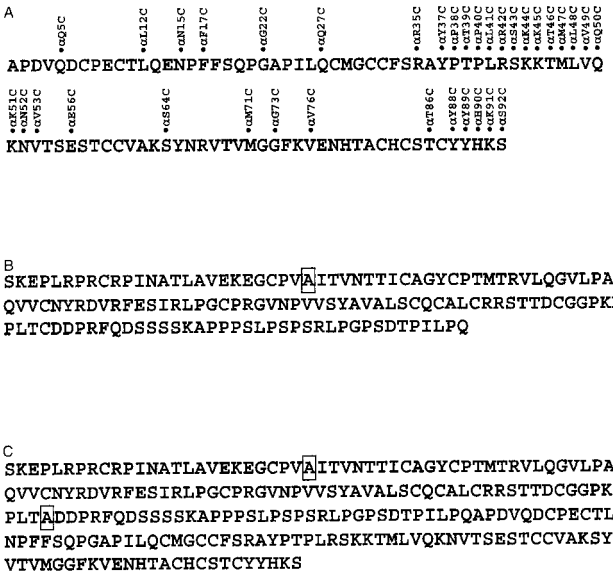


FIG. 1. **Amino acid sequences of analogs used in these studies.** Constructs encoding these sequences were prepared by standard methods and verified prior to transfection in COS-7 cells. The name of the α-subunit analog is shown *above* the amino acid that was modified.

erroneous would suggest the model is wrong. Analogs that had little or no LHR activity are much less informative, because it is not possible to determine if the cross-link reduced hormone activity by disrupting a key receptor contact, by changing the conformation of the heterodimer, or both.

Our rationale for attempting to prepare analogs having an architecture in which the seatbelt is attached to the α-subunit was based partly on the proposal made by Ruddon *et al.* (17) that the seatbelt latch disulfide forms after the α-subunit has docked with the hCG β-subunit during hCG synthesis in cells. Following docking, seatbelt residue Cys-110 is thought to scan the surface of the heterodimer until it encounters β-subunit residue Cys-26 with which it forms the disulfide that latches the seatbelt to β-subunit loop 1 and that stabilizes the heterodimer. Conversion of β-subunit Cys-26 to alanine is known to prevent heterodimer formation, presumably because it blocks formation of the seatbelt latch disulfide (18). We anticipated that Cys-110 of this β-subunit analog might form disulfides with cysteines that had been substituted for various residues in the α-subunit, resulting in an intersubunit disulfide cross-link. As shown here, many α-subunit analogs that contained an additional free cysteine combined with a β-subunit analog in which Cys-26 was converted to alanine. These were much more stable than hCG at low pH and, remarkably, some were as active as hCG in receptor binding and signal transduction assays.

EXPERIMENTAL PROCEDURES

hCG was purified from a urinary extract as described previously (19). The sources of all monoclonal antibodies used in these studies have been identified (13, 16, 20). hCG and antibodies were radioiodinated to a specific activity of ~50 μCi/μg in tubes coated with 1.5 μg of IODOGEN (Pierce, Rockville, IL). Procedures for Western blotting have also been described previously (20). The rabbit antisera used in cAMP measurements were obtained from Strategic BioSolutions (Newark, DE). cAMP was radiolabeled as described before (21).

The amino acid sequences of the analogs used in these studies are illustrated in Fig. 1. DNA sequences encoding these analogs were prepared by standard methods, including cassette and PCR mutagenesis similar to those that have been described (4, 13). The proteins were produced by transient expression in COS-7 cells in serum-free media as noted (4) and quantified by sandwich immunoassays (22) employing α-subunit antibody A113 for capture and radioiodinated β-subunit antibody B110 for detection. The biological activities of the analogs were determined in receptor binding and signal transduction assays employing CHO cells that had been transfected with the rat LHR (23). These assays were performed on samples that had been concentrated ~100-fold by ultrafiltration (Centriprep YM-10, Millipore, Bedford, MA) followed by dialysis against phosphate-buffered saline or by pouring the culture media into a dialysis bag and placing the bag on a bed of Aquacide II (Calbiochem, La Jolla, CA) until the samples were dry. The latter samples were rehydrated by dialyzing them in phosphate-buffered saline, and the concentration of the analog was determined using the A113/^{125}I-B110 sandwich immunoassay. Statistical analyses were performed with Prism (GraphPad Software, Inc., San Diego, CA). To learn if the analogs had become cross-linked by a disulfide, we added concentrated HCl to reduce the pH below 2 for 30 min at 37 °C, a condition known to promote the dissociation of the hCG heterodimer (1). The pH was neutralized by the addition of 0.017 volume of 1 M Tris (pH 7.5) plus an amount of NaOH equivalent to the HCl used for acidification, and the concentration of heterodimer was determined using the A113/^{125}I-B110 sandwich immunoassay.

RESULTS

The Seatbelt Can Be "Latched" to the α-Subunit at Several Sites—We did not detect any heterodimer in the media of COS-7 cells that had been co-transfected with vectors encoding the native α-subunit and hCG-βC26A, the β-subunit analog in which Cys-26 had been converted to alanine (Table I). This observation confirms the result obtained by Suganuma *et al.* (18) and is consistent with the idea that the stability of the native hCG heterodimer depends on the seatbelt being "latched" to Cys-26 of β-subunit loop 1. The amounts of heterodimers secreted into the medium by COS-7 cells that had been co-transfected with vectors encoding hCG-βC26A and several α-subunit constructs were comparable to that of hCG (Table I). These included α-subunit analogs having an additional cysteine at or near the α-subunit carboxyl terminus (*i.e.* αT86C, αY88C, αS92C) or in parts of α-subunit loop 2 (*i.e.* αR35C, αY37C, αL41C, αR42C, αS43C, αT46C, αL48C). The use of α-subunit analogs containing a cysteine in place of residues 5, 45, 47, 49, 51, 56, 64, 90, or 91 (*i.e.* αQ5C, αK45C, αM47C, αV49C, αK51C, αE56C, αS64C, αH90C, αK91C) led to the accumulation of less heterodimer in the medium. We failed to detect reproducible secretion of heterodimer when hCG-βC26A was expressed with the native α-subunit or analogs αL12C, αN15C, αF17C, αG22C, αP38C, αP40C, αK44C, αN52C, αV53C, αM71C, αG73C, and αY89C (Table I).

To reduce the possibility that the differences we observed in heterodimer formation were caused by limitations of our assay procedures, we compared the abilities of the α-subunit analogs to be expressed with the native hCG β-subunit using similar assay protocols. The α-subunit antibodies used to capture these analogs in sandwich assays, A113 and A116, recognize epitopes located primarily on opposite surfaces of α-subunit loop 1 (13, 24) and bind the free α-subunit as well as that combined with the hCG and hCG-βC26A β-subunits. Except for αY89C, each of the α-subunit analogs was captured by one or both of these antibodies when expressed with the native β-subunit (not shown). The β-subunit antibodies used to monitor these analogs recognize sites on loops 1 and/or 3, a portion of the native hCG β-subunit detected readily before and after it has combined with the α-subunit. The B111 epitope includes the seatbelt latch (20, 25). Although this antibody bound each of the α-subunit analogs when they were combined with the native hCG β-subunit (not shown), it was unable to recognize hCG-βC26A (Fig. 2) or any of the cross-linked heterodimers (Table I). B110 is unable to bind hCG at the same time as B111, but its epitope does not involve residues in the seatbelt and it recognizes analogs such as hCG-βC26A in which the seatbelt is not latched or heterodimers such as αL41C/hCG-βC26A in which the seatbelt is latched to the α-subunit (Fig. 2). The B112 epitope includes residue Asn-77 on a surface of β-subunit loop

TABLE I
Properties of heterodimers released into the media

Analog	Amount in medium	Remaining after pH 2	^{125}I-B111/ ^{125}I-B110[c]
	ng/50 μl[a]	*%*[b]	
hCG(α-β)	7.01 ± 0.78	0 ± 1.5	1.29 ± 0.02
α-βC26A	Not detectable	Not done	Not done
αQ5C-βC26A	1.15 ± 0.46	Not done	Not done
αL12C-βC26A	Not detectable	Not done	Not done
αN15C-βC26A	Not detectable	Not done	Not done
αF17C-βC26A	Not detectable	Not done	Not done
αG22C-βC26A	Not detectable	Not done	Not done
αQ27C-βC26A	0.39 ± 0.08	Not done	Not done
αR35C-βC26A	4.32 ± 0.28	99.2 ± 11.5	Not detectable
αY37C-βC26A	6.34 ± 0.48	98.0 ± 3.0	Not detectable
αP38C-βC26A	0.014 ± 0.007	Not done	Not done
αT39C-βC26A	2.98 ± 0.24	97.1 ± 6.3	Not detectable
αP40C-βC26A	0.36 ± 0.011	Not done	Not done
αL41C-βC26A	9.13 ± 0.63	92.2 ± 0.2	Not detectable
αR42C-βC26A	4.62 ± 0.40	97.6 ± 1.6	Not detectable
αS43C-βC26A	8.44 ± 0.37	98.3 ± 4.2	Not detectable
αK44C-βC26A	0.22 ± 0.06	Not done	Not detectable
αK45C-βC26A	1.28 ± 0.14	Not done	Not detectable
αT46C-βC26A	5.03 ± 0.12	95.3 ± 5.5	Not detectable
αM47C-βC26A	2.14 ± .010	96.5 ± 6.9	Not detectable
αL48C-βC26A	3.51 ± 0.19	87.6 ± 6.2	Not detectable
αV49C-βC26A	1.28 ± 0.04	96.1 ± 1.3	Not detectable
αQ50C-βC26A	2.11 ± 0.05	100.3 ± 4.3	Not detectable
αK51C-βC26A	1.10 ± 0.01	108.0 ± 8.1	Not detectable
αN52C-βC26A	Not detectable	Not done	Not done
αV53A-βC26A	Not detectable	Not done	Not done
αE56A-βC26A	2.15 ± 0.10	95.4 ± 1.3	Not done
αS64C-βC26A	1.46 ± 0.19	98.1 ± 4.0	Not detectable
αV76C-βC26A	0.38 ± 0.20	Not done	Not done
αT86C-βC26A	15.8 ± 0.23	96.7 ± 0.9	Not detectable
αY88C-βC26A	4.44 ± 0.31	93.2 ± 1.5	Not detectable
αY89C-βC26A	Not detectable	Not done	Not done
αH90C-βC26A	1.76 ± 0.17	99.2 ± 0.8	Not detectable
αK91C-βC26A	1.98 ± 0.09	100.6 ± 3.6	Not detectable
αS92C-βC26A	4.72 ± 0.55	102.8 ± 8.3	Not detectable
hCGscβC26A,C110A-α	Not done	Not done	0.67 ± 0.03

[a] Values are means of triplicates ± S.E. determined in A113/^{125}I-B110 sandwich immunoassays on separate transfections prior to concentration of the media.

[b] Values are triplicates ± S.E. determined in A113/^{125}I-B110 sandwich immunoassay following transient incubation at low pH for 30 min at 37 °C.

[c] The amounts of radiolabeled ^{125}I-B111 and ^{125}I-B110 bound in A113/^{125}I-B110 and A113/^{125}I-B111 sandwich assays were determined at each of several concentrations of hCG and βC26A,C110A-α. The amount of ^{125}I-B111 bound to both analogs varied linearly with that of ^{125}I-B110. Values illustrated are the slopes and the S.E. of each regression line. We were not able to detect any binding of ^{125}I-B111 to any of the disulfide cross-linked analogs, regardless of the amount used.

3 (20, 25). This surface of the β-subunit is distant from the seatbelt latch disulfide, an observation that explains why B111 and B112 bind hCG at the same time. Unlike B111, B112 binds the free hCG-βC26A subunit and heterodimers containing hCG-βC26A (Fig. 2). B112 and B110 recognized each of the cross-linked heterodimers that had been captured by A113 in an equivalent fashion (Table II). Based on this observation and the fact that we have much greater access to B110 than B112, we routinely used B110 to quantify the analogs described here even though its affinity for hCG is lower than that of B112.

We subjected the media to a brief acid treatment to learn if the analogs produced by the COS-7 cells had an intersubunit cross-link that made them more stable than hCG. As expected from the known acid instability of the glycoprotein hormones (26), transient treatment of hCG at low pH caused its subunits to dissociate, making it no longer detectable in A113/^{125}I-B110 sandwich assays (Table I). Unlike hCG, each of the heterodimeric analogs tested was readily measured in the A113/^{125}I-B110 sandwich assay following acid treatment, showing that its subunits were tethered by an intersubunit disulfide.

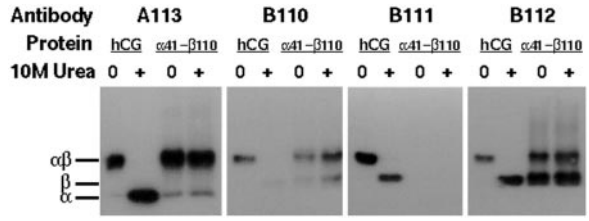


FIG. 2. **Western blots illustrating the ability of antibodies A113, B110, B111, and B112 to detect the αL41C/hCG-βC26A heterodimer before and after treatment with 10 M urea.** Equal amounts of highly purified hCG or unpurified αL41C/hCG-βC26A (200 ng) in concentrated culture medium were subjected to electrophoresis on 12% polyacrylamide gels in the presence of 1% sodium dodecyl sulfate and in the presence or absence of 10 M urea. *Each panel* illustrates a separate blot. Proteins transferred to nitrocellulose were detected with the radioiodinated antibody indicated at the *top of each panel*. Note that B111 is unable to recognize the hCG-βC26A free subunit or heterodimers in which the seatbelt is latched to the α-subunit. Note also that we were unable to detect homodimers of αL41C or hCG-βC26A, even after overexposure of the blots as shown here. The former would have been detected as a band between that of the heterodimer and the free α-subunit in the *panel at the left* (27). The latter would have been detected as a band *above* that of the heterodimer in *panels 2* and *4* but *below* that of the unknown high molecular weight material detected as a *feint band* in *panel 4* (27).

TABLE II
Detection of analogs in A113/^{125}I-B112 and A113/^{125}I-B110 sandwich immunoassays

The same samples were analyzed in each assay relative to hCG as a standard. As can be seen here, both assays gave essentially the same result.

Analog	Ratio B112/B110
α41C-βC26A	1.06
α42C-βC26A	0.99
α43C-βC26A	1.02
α48C-βC26A	1.09
α50C-βC26A	0.63
α90C-βC26A	1.08
α91C-βC26A	1.23
α86C-βC26A	1.40
α92C-βC26A	0.94
Average	1.05 ± 0.07

We also tested the stability of the analog containing a cross-link to α-subunit residue 41 to 10 M urea denaturation. Western blots (Fig. 2) revealed that urea treatment caused the complete dissociation of hCG but had no influence on the analog detected using A113, B110, or B112.

Due to the presence of a free thiol in each free subunit, it was conceivable that we might observe the formation of stable disulfide cross-linked αα and ββ homodimeric analogs. Unlike hCG β-subunit analogs that were engineered to form homodimers by replacing loop 2 with its α-subunit counterpart (27), hCG-βC26A did not appear to form significant amounts of homodimers (Fig. 2). We have also described methods for preparing α-subunit homodimers in mammalian cells (27). Although we did not test the ability of each of the α-subunit analogs to form homodimers, we were unable to detect the formation of αL41C homodimers in Western blots (Fig. 2).

Production of the Cross-linked Heterodimers Was Correlated with the Accessibility of the Carboxyl-terminal End of the Seatbelt to the Cysteine That Had Been Introduced into the α-Subunit—With the exception of αY89C, we observed that each α-subunit analog combined with the native hCG β-subunit and was secreted into the medium (not shown), albeit not always at the same level as hCG. This showed that most cysteine substitutions did not disrupt the folding of the α-subunit analog, prevent docking of the α-subunit with the hCG β-subunit, or block the passage of heterodimer through the secretory pathway. Comparisons of the amounts of cross-linked heterodimers

TABLE III
Distance between Cα carbons of residues in hCG

Values shown were measured from the crystal structure of hCG except for that of αS92C, which is indeterminate. The distance between the Cα carbons of βC26 and βC110 is typical for that of a disulfide bond. Distances between the Cα carbons of βC26-βA91 and βC26-βG101 reflect the minimum length of a strand needed to connect these residues in hCG. Some values could not be determined due to the fact that parts of the α-subunit carboxyl terminus are not visible in the crystal structure of hCG.

hCG or Analog	β-Subunit amino acid residue		
	βC110	βA91	βG101
		Å	
βC26 (hCG)	6	33	26
αQ5C	32	16	25
αQ27C	42	21	24
αG22C	57	36	41
αR35C	19	11	11
αY37C	14	15	12
αP38C	14	16	13
αT39C	13	18	12
αP40C	10	22	12
αL41C	10	25	17
αR42C	10	26	16
αS43C	13	23	12
αK44C	15	23	14
αK45C	15	27	17
αT46C	17	25	14
αM47C	19	23	14
αL48C	22	25	16
αV49C	23	25	15
αQ50C	20	21	15
αK51C	19	17	12
αN52C	18	17	14
αV53C	17	14	11
αE56C	19	13	6
αS64C	40	26	23
αM71C	57	33	37
αG73C	57	33	36
αV76C	48	26	28
αT86C	26	16	9
αY88C	28	14	7
αY89C	31	18	10
αH90C	Unknown	Unknown	Unknown
αK91C	Unknown	Unknown	Unknown
αS92C	Unknown	Unknown	Unknown

produced (Table I) with the positions of the α-subunit cysteines and their distances from parts of the seatbelt in hCG (Table III) suggested that secretion of these analogs was limited by their abilities to form a disulfide with hCG-βC26A. Residues of α-subunit analogs that combined well with hCG-βC26A were located in a circumscribed surface on the α-subunit centered near the midpoint of the seatbelt (Fig. 3). This suggested that movements of the carboxyl-terminal half of the seatbelt (*i.e.* between β-subunit residues 101 and 110) were required for formation of the intersubunit disulfide cross-links.

Not every analog containing a cysteine within the circumscribed area of Fig. 3 was incorporated into cross-linked heterodimers with equal efficiency. Those that formed heterodimers well contained cysteines located on a surface of the α-subunit that is unobstructed in the heterodimer (2, 3). Those that formed heterodimers less well contained cysteines that would appear to be partially blocked in the heterodimer (*i.e.* 44, 52) or that might alter the ability of α-subunit loop 2 to attain a conformation needed for docking with the β-subunit (*i.e.* substitution of cysteines for αPro38 and αPro40). Because the conformation of α-subunit loop 2 in the free α-subunit is known to be highly disordered (28), this observation suggested that docking of the subunits may stabilize the conformation of α-subunit loop 2 to one that is roughly similar to the conformation seen in the native heterodimer.

The Position of the Seatbelt in These Cross-linked Heterodimers Differs from That of hCG and Single-chain Analogs Lacking the Seatbelt Latch Disulfide—Attachment of the seatbelt to the α-subunit was expected to change the position of the seatbelt markedly. As noted earlier, none of the altered seatbelt analogs tested was detected in a sandwich assay employing A113/^{125}I-B111 (Table I). This result confirmed that the carboxyl-terminal end of the seatbelt in all the mutant analogs occupies a position different from that in hCG. Others have shown that the disulfide between β-subunit residues 26 and 110 that latches the β-subunit is not required for hormone folding or for receptor binding activities of single-chain analogs (29, 30). To learn if the carboxyl-terminal end of the seatbelt has a similar location in a single-chain hCG analog lacking the seatbelt latch disulfide as it has in hCG, we compared hCGscβC26A, C110A-α (Fig. 1*C*) and hCG in A113/^{125}I-B110 and A113/^{125}I-B111 sandwich assays. Each was readily detected by both of these antibodies (Table I), although the relative ability of the single-chain analog to be recognized by B111 was only half that of hCG. The finding that hCGscβC26A, C110A-α can be recognized by B111 at all, however, suggests that linkage of the carboxyl-terminal end of the β-subunit to the amino-terminal end of the α-subunit partially offsets the function of the βC26A-βC110 disulfide in stabilizing the conformation of this epitope. It also showed that the failure of B111 to recognize analogs containing hCG-αC26A was not due merely to the substitution of Cys-26 by alanine.

The Subunit Cores of These Analogs Appear to Be Oriented Similarly to Those of the Subunits in hCG—In the hCG heterodimer, α-subunit loops 1 and 3 contact portions of β-subunit loop 2; β-subunit loops 1 and 3 are near α-subunit loop 2. To learn if these "core" portions of the subunits had similar positions in the cross-linked heterodimers as they do in hCG, we compared the abilities of several analogs to be recognized by antibodies to epitopes that are obscured by the subunit interface (Table IV). All the analogs were detected readily by A113 and B110, antibodies that recognize epitopes on the free α- and

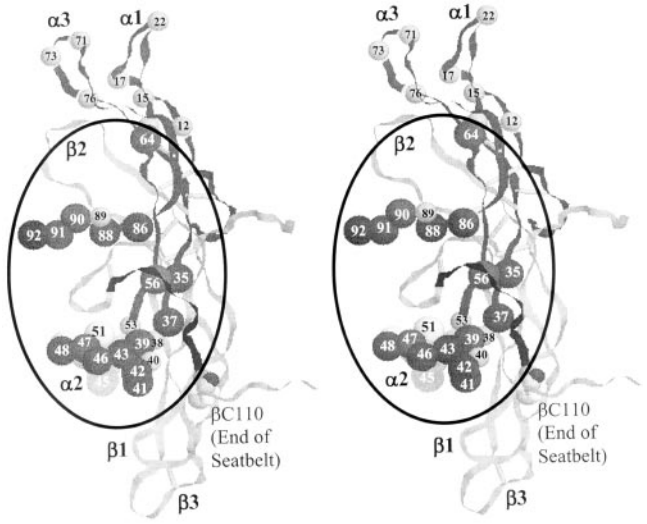


FIG. 3. **Relaxed stereo representation of the surface of hCG that appears to be scanned by the seatbelt during assembly of the heterodimer.** The α- and β-subunit backbones are shown in *dark* and *light gray ribbons*, respectively. The carboxyl-terminal half of the seatbelt is shown as a *black ribbon*. The locations of the C_α carbons of cysteine substitutions that enabled modest or efficient combination of α-subunit analogs with hCG-βC26A are shown as *dark spheres*. The *lighter gray spheres* refer to residues that gave lesser amounts of heterodimer. The *small pale spheres* refer to cysteine substitutions that did not lead to heterodimer formation. Note that α-subunit residues 90, 91, and 92 are too disordered to be seen in the crystal structure of hCG, and the arbitrary positions of these residues shown here are intended only to emphasize their apparent abilities to be latched to the seatbelt.

TABLE IV
Binding of selected analogs in alternate sandwich assays

Analogs were captured with A113, an antibody to the α-subunit, and detected with radiolabeled antibodies to the β-subunit. B110 binds an epitope on β-subunit loops 1 and 3 distant from the α-subunit. B101 binds an epitope on β-subunit loop 2 that overlaps that with A113 in the native heterodimer. B123, a free β-subunit-specific antibody, binds β-subunit loops 1 and 3 at an opposite surface that faces α-subunit loop 2 and that is on the opposite surface of these loops from that recognized by B110. B123 does not recognize the hCG heterodimer.

hCG analog	Antibody pairs			
	A113/^{125}I-B110	A113/^{125}I-B101	A113/^{125}I-B123	B123/^{125}I-B110
	cpm ± S.E.			
hCG (5 ng)	35,030 ± 1509	655 ± 724	593 ± 273	Not tested
αR42C + βC26A	32,525 ± 223	211 ± 93	−199 ± 11.5	Not tested
αS43C + βC26A	36,263 ± 546	−396 ± 3.0	1051 ± 244	Not tested
αM47C + βC26A	39,005 ± 332	−244 ± 108	−206 ± 238	Not tested
αL48C + βC26A	21,075 ± 221	156 ± 865	884 ± 1257	Not tested
αY88C + βC26A	31,343 ± 407	−999 ± 395	−372 ± 278	Not tested
αS92C + βC26A	33,200 ± 810	−83.0 ± 269	201 ± 263	Not tested
hCG β-subunit (0.5 ng)	Not tested	Not tested	Not tested	15,118 ± 172

β- subunits that are distant from one another in the heterodimer (Tables I and IV). None of the analogs tested were recognized simultaneously by antibody pairs A113 and B101 or A113 and B123, however. B101 recognizes β-subunit loop 2 in both the heterodimer and the free β-subunit. In the heterodimer, the A113 and B101 epitopes are adjacent, and, as a consequence, the two antibodies are unable to bind hCG at the same time (Table IV). The finding that A113 and B101 were unable to bind to the analogs simultaneously suggested that loop α1 is adjacent to loop β2, similar to its location in hCG. B123 binds a site on β-subunit loops 1 and 3 that is obscured in the heterodimer by α-subunit loop 2. The finding that A113 and B123 were unable to bind to the analogs at the same time suggested that the position of the B123 epitope is obscured by α2 similarly as it is in hCG. These observations are most readily explained by the view that relocating the end of the seatbelt to the α-subunit did not alter the relative positions of the subunit cores, at least grossly. This also suggests that the seatbelt is not the only factor that contributes to the stability of the heterodimer.

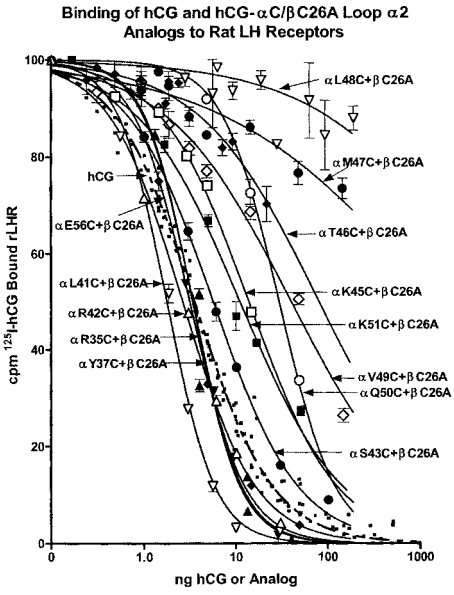
Many Cross-linked Heterodimers Had High Receptor-binding and Signal Transduction Activities Despite Their Altered Architectures—We tested the biological activities of analogs that had been produced in sufficient quantities in receptor binding and signal transduction assays. Analogs in which the seatbelt was cross-linked to α-subunit loop 2 residues 35, 37, 41, 42, 43, or 56 had high affinities for the LHR and at least 30% the potency of hCG in signal transduction assays (Figs. 4 and 5; Table V). The remaining analogs in which the seatbelt was attached to α-subunit loop 2 were less potent than this, and those with cross-links to α47, α49, and α51 also had reduced abilities to simulate cAMP accumulation (Figs. 4 and 5; Table V). The analog in which the seatbelt appeared to be latched to α-subunit carboxyl-terminal residue 92 (*i.e.* αS92C/hCG-βC26A) had 10 and 20% the activity of hCG in signal transduction and LHR binding assays (Figs. 4 and 5; Table V). Analogs in which the seatbelt was attached to residues 86–91 (*i.e.* αT86C/hCG-βC26A, αY88C/hCG-βC26A, αH90C/hCG-βC26A, αK91C/hCG-βC26A) near the carboxyl terminus had only 3–9% the activity of hCG in binding assays and even less in signal transduction assays (Figs. 4 and 5). We were unable to detect any signal transduction activity of αH90C/hCG-βC26A at the concentrations available. The heterodimer in which the seatbelt appeared to be latched to a residue in α-subunit loop 3 (*i.e.* αS64C/hCG-βC26A) had half the activity of hCG in receptor binding assays (Figs. 4 and 5) but less than 10% the activity of hCG in signal transduction assays.

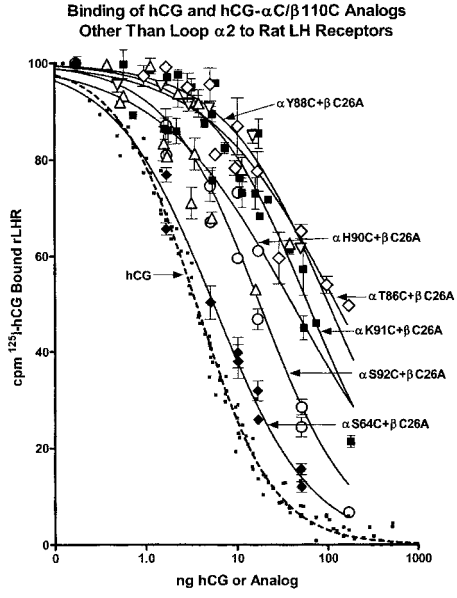
DISCUSSION

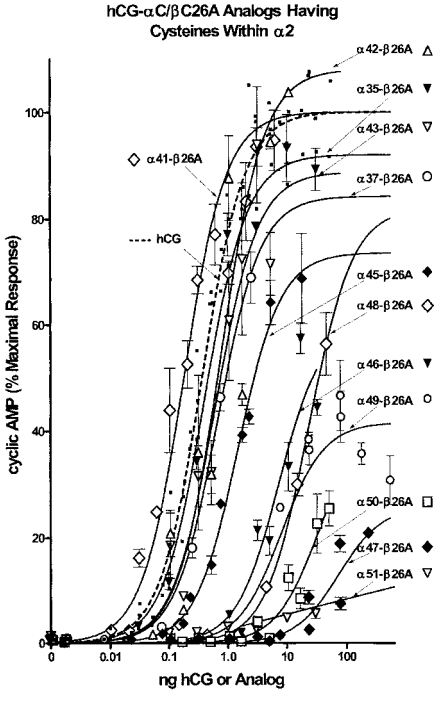
Structures of the Analogs Having Altered Seatbelts—We did not attempt to verify the acid-stable intersubunit cross-link experimentally, a difficult task beyond the scope of these studies. Several observations lead us to believe that the hCG analogs described here are stabilized by a disulfide between β-subunit Cys-110 and the cysteine that had been incorporated into the α-subunit. First, hCG-βC26A failed to combine with the native α-subunit (Table I), a phenomenon that argues against the ability of seatbelt residue βCys-110 to interrupt an existing α-subunit disulfide. Second, most of the α-subunit analogs combined efficiently with the native hCG β-subunit, but none of the resulting heterodimers were more stable than hCG at low pH (data not shown), a phenomenon that argues against the ability of the free thiol in the α-subunit to disrupt an existing β-subunit disulfide or to interfere with α-subunit folding. Third, the ability of hCG-βC26A to combine with α-subunit constructs was low when the distance of the cysteine from β-subunit residue Gly-101 was equal to or greater than that between β-subunit residues Gly-101 and Cys-26, the length of the carboxyl-terminal half of the hCG seatbelt (2, 3) or when the cysteine was on a surface of the α-subunit that would require the seatbelt to be wrapped around the α-subunit before it could form the intersubunit disulfide. The former explains the low yield of heterodimers containing αG22C, αM71C, or αG73C (Table I); the latter explains the low yield of heterodimers containing αQ5C, αL12C, αN15C, and αF17C. And fourth, hCG-βC26A combined inefficiently with α-subunit analogs containing a cysteine located at sites predicted to be near the subunit interface in hCG (2, 3). These include α-subunit analogs αQ5C, αQ27C, αN52C, αV53C, and αV76C. Coupled with the finding that the heterodimers were not recognized simultaneously by A113/B101 and A113/B123 (Table IV), these observations show that similar surfaces of the subunits face one another more or less as they do in hCG, even though the position of the seatbelt differs significantly from that in hCG as seen by the inability of the heterodimers to be recognized by antibody B111 (Table I). Considered together, these findings also suggest that the intersubunit disulfide forms only after the subunits have docked.

Implications of These Observations for hCG Assembly in the Endoplasmic Reticulum—The ability of the α-subunit analogs to combine with hCG-βC26A showed that the seatbelt does not need to be latched to its natural site before the α-subunit docks with the β-subunit. This result is consistent with the model of hCG assembly proposed by Ruddon *et al.* (17) and shows that the conformation of the seatbelt is not restricted to the position it occupies in hCG (2, 3). These data also support a prediction

FIG. 4. **Abilities of selected analogs to prevent the binding of ^{125}I-hCG to CHO cells that express the rat LHR.** Analogs or hCG were mixed with ^{125}I-hCG (~1.5 ng), and the mixture was added to the receptor bearing cells for 1 h at 37 °C as described previously (15). Binding was terminated by diluting the cell mixture to 2 ml with ice-cold 0.9% NaCl solution containing 1 mg of bovine serum albumin per milliliter. Values shown represent the amount of radiolabel bound to the cells normalized to the maximal value observed in the absence of hCG (100%) and to the minimal value observed in the presence of 1 μg of hCG (0%). These data were pooled from at least two independent studies for each analog, and in most cases are derived from three to five experiments using at least two different analog preparations. The *bars* shown for all analogs extend to the limits of the S.E. The data for hCG are shown as the *broken line*. The *small symbols* represent individual points for the hCG curves and are illustrated to reflect assay variability.







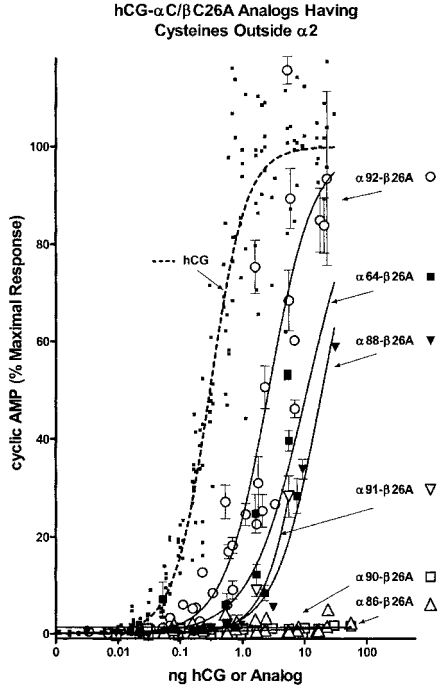


FIG. 5. **Abilities of selected analogs to stimulate the accumulation of cAMP in CHO cells that express the rat LHR.** Analogs or hCG were added to the CHO cells for 20 min at 37 °C. The reaction was stopped by immersing the incubation tubes in a 70 °C water bath, and the cAMP released into the medium was measured by radioimmunoassay (21). Values shown have been pooled from at least two independent studies with each analog, and in most cases the data have been combined from three to five assays. The minimum (0%) was determined in the absence of hCG, and the maximal response (100%) was determined in the presence of 10–30 ng of hCG. For reasons that remain unknown, factors present in the culture medium inhibit this assay. This prevented us from determining the efficacies of analogs that were produced inefficiently or that had low receptor binding activity. The data illustrated for analogs α47-β26 and α49-β26 are for three of five studies in which these analogs elicited a significant response. Two studies in which we failed to observe a response to these analogs were excluded from the estimates of potency shown in Table V. The curve for αE56C-βC26A, which is between those for hCG and αL41C-βC26A is not shown.

implicit in the Ruddon model, namely that contacts between the subunits can stabilize the heterodimer until the seatbelt latch disulfide is formed. Although these observations show that hCG assembly can occur prior to formation of the seatbelt latch disulfide as suggested by Ruddon *et al.* (17), it remains to be determined if this is more than a salvage pathway that can rescue hCG assembly. We have found that the subunits can combine readily *in vitro* in oxidizing conditions while the seatbelt disulfide remains latched (31), and it is conceivable that most subunit assembly occurs after the seatbelt has latched unless the latch disulfide has been prevented from forming as described here.

The position of the amino-terminal portion of the seatbelt in these analogs remains unknown. We did not observe the formation of heterodimers in which the seatbelt was latched to α-subunit residues beyond the distance capable of being scanned by the carboxyl-terminal half of the seatbelt (Tables I and III). This suggests that the amino-terminal half of the seatbelt is not as mobile as the carboxyl-terminal half or that the amino-terminal half of the seatbelt contributes to interactions between the subunits needed for docking when the seatbelt is not latched. This observation is also consistent with the proposal that the small seatbelt loop is formed prior to docking of the subunits (17).

Implications of These Observations for Hormone Activity and Models of Receptor Binding—The finding that the hCG seatbelt latch disulfide can be relocated from its natural position on β-subunit loop 1 to several sites on the α-subunit has important implications for the role of the seatbelt in hCG-lutropin receptor interactions. To our knowledge, this is the first report suggesting that the seatbelt can be latched to the α-subunit. The high lutropin activities of several analogs suggest strongly that the region of hCG near the seatbelt latch does not participate in key receptor contacts. Previous studies with single-

TABLE V
Potencies of the analogs relative to hCG

All values are based on the concentration of analog determined by sandwich immunoassay using hCG as the standard. The same hCG standard was used in sandwich assays, receptor binding assays, and signal transduction assays.

Analog[a]	Receptor binding	Signal transduction
	% (95% CL)	
hCG	100	100
αR35C-βC26A	102 (91–113)	75 (60–93)
αY37C-βC26A	96 (107–119)	38 (32–45)
αL41C-βC26A	203 (191–216)	190 (151–268)
αR42C-βC26A	135 (122–149)	58 (39–88)
αS43C-βC26A	62 (58–67)	48 (36–64)
αK45C-βC26A	25 (22–29)	10 (7.5–14)
αT46C-βC26A	4.4 Indeterminate[b]	1.9 (1.6–2.2)
αM47C-βC26A	0.25 Indeterminate[b]	0.3 Indeterminate[b,c]
αL48C-βC26A	0.05 Indeterminate[b]	1.1 (1.0–1.3)
αV49C-βC26A	12 (8.1–19)	0.14 Indeterminate[b,c]
αQ50C-βC26A	12 (9.7–14.3)	4.8 (2.1–8.9)
αK51C-βC26A	24 (31–40)	0.05 Indeterminate[b]
αE56C-βC26A	112 (103–121)	30 (10–45)
αS64C-βC26A	57 (49–66)	6.9 (5.6–8.4)
αT86C-βC26A	3.1 (2.3–4.1)	Indeterminate[b]
αY88C-βC26A	3.5 (2.7–4.6)	1.6 (1.4–1.8)
αH90C-βC26A	8.8 (5.3–15)	Indeterminate[b]
αK91C-βC26A	5.9 (5.1–6.9)	2.9 (2.5–3.4)
αS92C-βC26A	20 (18–24)	11.5 (9.6–13.7)

[a] Many of the analogs that were made in small amounts (Table I) were not tested in either assay due to limited amount of material available.

[b] "Indeterminate" indicates that the analog gave only a marginal response at the highest amount tested or that, due to the low activity of the material, values of potency shown were determined after extrapolation. The latter should be considered only as rough estimates.

[c] These values were estimated from the three studies in which these analogs gave a response, assuming that sufficient amounts of analog would have elicited the same maximum response as hCG.

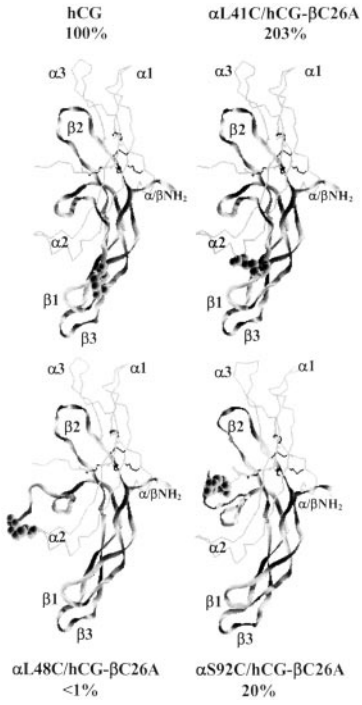


FIG. 6. **Models illustrating the location of the seatbelt in hCG and some of the analogs described here.** These models were made using Sybyl (Tripos, St. Louis, MO) and illustrate the smallest changes in the seatbelt that would enable β-subunit Cys-110 to form a disulfide with the α-subunit cysteine depicted. *Gray scale*: *light gray line*, α-subunit; *dark gray ribbon*, β-subunit; *black thin lines*, disulfide bonds; *dark spheres*, cysteine residues in the disulfide that stabilizes the heterodimer. Note that the carboxyl-terminal residues of the β-subunit are disordered in the crystal structure and are not drawn here. These would be quite bulky and extend from the seatbelt in the vicinity of the *dark spheres*.

chain gonadotropin analogs have also led to the suggestion that the region of the seatbelt near the latch disulfide is not needed for lutropin activity (29, 30, 32). Although this conclusion is consistent with the data described here, the finding that a single-chain hCG analog lacking this disulfide was recognized well by B111 (hCGscβC26A, C110A-α, Table I) suggests that fusing the subunits together offsets the role of the seatbelt latch disulfide in stabilizing this region of the protein. Thus, single-chain analogs may be less useful for studying the structure and function of this region of the glycoprotein hormones.

The activities of single-chain hCG analogs led Jackson *et al.* (33) to argue that the activities of the glycoprotein hormones are not dependent on the "complete native quaternary interactions" between their subunits. The studies described here show clearly that the position of at least a part of the seatbelt can be altered without disrupting lutropin receptor interactions (Table V), a finding that could be taken as support for this concept. Linking the seatbelt to the α-subunit did not appear to cause a gross change in the alignment of the subunits compared with that in hCG, however, even in analogs that had only trace amounts of lutropin activity (Table IV). Thus, with the exception of the seatbelt, all the analogs described here appeared to have similar quaternary configurations, despite the fact that they lack constraints imposed on the α-subunit by the arrangement of the seatbelt in the native hormones.

Modeling suggested that the carboxyl terminus of the seatbelt in αL41C/hCG-βC26A, one of the most active analogs (Table V), is likely to be located within 4–5 Å of the site that it occupies in hCG (Fig. 6). Thus, despite its inability to be recognized by antibody B111, it is conceivable that much of the seatbelt of this analog adopts a conformation very similar to that of hCG (Fig. 6, *top right panel*). In contrast, the carboxyl-terminal half of the seatbelt would not be expected to have the same conformations in hCG and αY37C/hCG-βC26A, αR35C/hCG-βC26A, or αE56C/hCG-βC26A, analogs that also have high activity, because the carboxyl-terminal end of these seatbelts are likely to be located 14–19Å away from its natural site (Table III). The finding that the seatbelt can be attached to α-subunit residues 35, 37, 41, 42, or 56 without reducing hCG receptor binding suggests the carboxyl-terminal half of the hCG seatbelt does not participate in essential LHR contacts. It is also consistent with the findings that this portion of the seatbelt can be replaced by its human follitropin counterpart with little influence on LHR interactions (5, 34). This portion of the seatbelt has been shown to control the positions of the hCG subunits (16), and its ability to influence the receptor interactions of lutropins and thyrotropins (5, 6, 35) through its contacts with α-subunit loop 2 may reflect this property.

The high activities of analogs containing αS43C and αK45C (Table V) suggest that the small helix in α-subunit loop 2 also does not participate in LHR contacts. The activities of the analog containing αK51C also suggest that this portion of α-subunit loop 2 does not contact the receptor, in agreement with conclusions reached earlier (36).

The activities of analogs in which the seatbelt is attached to other parts of α-subunit loop 2 have important implications for models in which residues in this loop are thought to contact the receptor (13). Unlike analogs in which the seatbelt was cross-linked to α-subunit loop 2 residues just discussed, those in which it was cross-linked to α-subunit loop 2 residues 46–51 had much lower receptor binding and signal transduction activities. The least active analogs were those in which the seatbelt was attached to α-subunit residues 47 or 48, a portion of the hormone predicted to contact the receptor (13). Nonetheless, the finding that all analogs in which the seatbelt is latched to a residue in α-subunit loop 2 retained detectable activity

makes it difficult for us to retain our notion that several residues of α-subunit loop 2 participate in essential receptor contacts (13). Although we continue to think that α-subunit loop 2 is near the hormone-receptor interface, the results of these studies suggest that residues in this portion of the hormone make fewer essential hormone-receptor contacts than we originally envisioned.

The carboxyl-terminal end of the α-subunit has been long thought to participate in receptor contacts (1). The activities of analogs in which the seatbelt is latched to residues at or near the carboxyl terminus, albeit low, offer additional insights into the role of this hormone region in receptor interactions. The very end of the α-subunit carboxyl terminus appears to be exposed in the lutropin receptor complex as shown by the finding that a few residues can be added to this site without disrupting hormone activity (37). This portion of the hormone may be near the receptor interface, however, as shown by the finding that the presence of hCG β-subunit residues 118–145, a region that contains several glycosylated serine residues in hCG, reduced the activity of hCG ~50-fold (38). The receptor-binding and signal-transduction activities of αS92C/hCG-βC26A, an analog in which the seatbelt is likely to be cross-linked to the α-subunit carboxyl terminus, was between these extremes (Table V). This finding surprised us considerably and led us to test and compare the activities of several preparations of this analog (Figs. 4 and 5). The residual activities of αS92C/hCG-βC26A and analogs in which the seatbelt was attached to residues near the α-subunit carboxyl terminus, which are higher than has been reported following truncation of the α-subunit (1, 9), are more readily explained by models of the hCG-receptor complex in which the hormone is thought to bind to the rim of the receptor extracellular domain (11, 13) than to other sites (10). Because the large carboxyl-terminal end of the β-subunit in these analogs is likely to be located near the carboxyl-terminal end of the α-subunit, it is difficult to imagine how the α-subunit carboxyl terminus could contact the transmembrane domain as proposed (39). Furthermore, it is hard to envision how the seatbelt could reach these α-subunit residues without passing between the receptor and other portions of the hormone thought to contact the receptor in models other than those in which the hormone is oriented parallel to the plane of the receptor extracellular domain (13).

Implications of These Observations for the Evolution of the Glycoprotein Hormones—The finding that several structural analogs of hCG have high biological activities suggests that different types of hormone intermediates could have been produced during the evolution of lutropins. The glycoprotein hormones have essential roles in the reproduction and development of all vertebrates and would be subjected to extremely high selection pressures. With the exception of some piscine hormones in which the seatbelt appears to be latched to a cysteine near the amino-terminal end of the β-subunit (40), the amino acid sequences of the vertebrate glycoprotein hormones suggest each member of this family folds similarly to hCG. As is apparent from the instability of heterodimers missing the seatbelt latch disulfide (18), the hCG subunit cores do not have high affinity for one another, at least once they have left the endoplasmic reticulum. Unless these proteins originated from dimers of subunits that had high affinities for one another, it seems likely that they could have evolved from intermediates lacking seatbelts only if their subunits were expressed in tandem (30) or if they were produced downstream of dimerization domains (23, 27). The latter mechanism appears to be responsible for the assembly of inhibins and activins, other cystine knot proteins with key roles in reproduction and development (41). The observations described here suggest a new mechanism for the evolution of the heterodimer. The attractiveness of this mechanism depends on the finding that it does not require a specific seatbelt latch disulfide for high hormone activity, a phenomenon that would greatly enhance the probability that a given mutant would lead to reproductive success. Additional selection pressure would have led to the migration of the latch disulfide to its current position on the β-subunit.

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REFERENCES

1. Pierce, J. G., and Parsons, T. F. (1981) *Annu. Rev. Biochem.* **50,** 465–495
2. Lapthorn, A. J., Harris, D. C., Littlejohn, A., Lustbader, J. W., Canfield, R. E., Machin, K. J., Morgan, F. J., and Isaacs, N. W. (1994) *Nature* **369,** 455–461
3. Wu, H., Lustbader, J. W., Liu, Y., Canfield, R. E., and Hendrickson, W. A. (1994) *Structure* **2,** 545–558
4. Campbell, R. K., Dean Emig, D. M., and Moyle, W. R. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 760–764
5. Moyle, W. R., Campbell, R. K., Myers, R. V., Bernard, M. P., Han, Y., and Wang, X. (1994) *Nature* **368,** 251–255
6. Grossmann, M., Szkudlinski, M. W., Wong, R., Dias, J. A., Ji, T. H., and Weintraub, B. D. (1997) *J. Biol. Chem.* **272,** 15532–15540
7. Segaloff, D. L., and Ascoli, M. (1993) *Endocr. Rev.* **14,** 324–347
8. Kobe, B., and Deisenhofer, J. (1993) *Nature* **366,** 751–756
9. Chen, F., Wang, Y., and Puett, D. (1992) *Mol. Endocrinol.* **6,** 914–919
10. Jiang, X., Dreano, M., Buckler, D. R., Cheng, S., Ythier, A., Wu, H., Hendrickson, W. A., Tayar, N. E., and el Tayar, N. (1995) *Structure* **3,** 1341–1353
11. Remy, J.-J., Couture, L., Pantel, J., Haertle, T., Rabesona, H., Bozon, V., Pajot-Augy, E., Robert, P., Troalen, F., Salesse, R., and Bidart, J.-M. (1996) *Mol. Cell. Endocrinol.* **125,** 79–91
12. Pantel, J., Remy, J.-J., Salesse, R., Jolivet, A., and Bidart, J.-M. (1993) *Biochem. Biophys. Res. Commun.* **195,** 588–593
13. Moyle, W. R., Campbell, R. K., Rao, S. N. V., Ayad, N. G., Bernard, M. P., Han, Y., and Wang, Y. (1995) *J. Biol. Chem.* **270,** 20020–20031
14. Braun, T., Schofield, P. R., and Sprengel, R. (1991) *EMBO J.* **10,** 1885–1890
15. Bernard, M. P., Myers, R. V., and Moyle, W. R. (1998) *Biochem. J.* **335,** 611–617
16. Wang, Y. H., Bernard, M. P., and Moyle, W. R. (2000) *Mol. Cell. Endocrinol.* **170,** 67–77
17. Ruddon, R. W., Sherman, S. A., and Bedows, E. (1996) *Prot. Sci.* **8,** 1443–1452
18. Suganuma, N., Matzuk, M. M., and Boime, I. (1989) *J. Biol. Chem.* **264,** 19302–19307
19. Bahl, O. P. (1969) *J. Biol. Chem.* **244,** 567–574
20. Moyle, W. R., Matzuk, M. M., Campbell, R. K., Cogliani, E., Dean Emig, D. M., Krichevsky, A., Barnett, R. W., and Boime, I. (1990) *J. Biol. Chem.* **265,** 8511–8518
21. Brooker, J., Harper, J. F., Terasaki, W. L., and Moylan, R. D. (1979) *Adv. Cyclic Nucleotide Res.* **10,** 1–33
22. Moyle, W. R., Ehrlich, P. H., and Canfield, R. E. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79,** 2245–2249
23. Lin, W., Ransom, M. X., Myers, R. V., Bernard, M. P., and Moyle, W. R. (1999) *Mol. Cell. Endocrinol.* **152,** 91–98
24. Myers, R. V., Wang, Y., and Moyle, W. R. (2000) *Biochim. Biophys. Acta* **1475,** 390–394
25. Cosowsky, L., Rao, S. N. V., Macdonald, G. J., Papkoff, H., Campbell, R. K., and Moyle, W. R. (1995) *J. Biol. Chem.* **270,** 20011–20019
26. Li, C. H., and Starman, B. (1964) *Nature* **202,** 291–292
27. Moyle, W. R., Myers, R. V., Wang, Y., Han, Y., Lin, W., Kelley, G. L., Ehrlich, P. H., Rao, S. N. V., and Bernard, M. P. (1998) *Chem. Biol.* (*Lond.*) **5,** 241–254
28. De Beer, T., Van Zuylen, C. W., Leeflang, B. R., Hard, K., Boelens, R., Kaptein, R., Kamerling, J. P., and Vliegenthart, J. F. (1996) *Eur. J. Biochem.* **241,** 229–242
29. Heikoop, J. C., van Beuningen-de, V. M., van, d. B., and Grootenhuis, P. D. (1997) *Eur. J. Biochem.* **245,** 656–662
30. Ben-Menahem, D., Kudo, M., Pixley, M. R., Sato, A., Suganuma, N., Perlas, E., Hsueh, A. J., and Boime, I. (1997) *J. Biol. Chem.* **272,** 6827–6830
31. Xing, Y., Williams, C., Campbell, R. K., Cook, S., Knoppers, M., Addona, T., Altarocca, V., and Moyle, W. R. (2001) *Prot. Sci.* **10,** 226–235
32. Galet, C., Chopineau, M., Martinat, N., Combarnous, Y., and Guillou, F. (2000) *J. Endocrinol.* **167,** 117–124
33. Jackson, A. M., Berger, P., Pixley, M., Klein, C., Hsueh, A. J., and Boime, I. (1999) *Mol. Endocrinol.* **13,** 2175–2188
34. Han, Y., Bernard, M. P., and Moyle, W. R. (1996) *Mol. Cell. Endocrinol.* **124,** 151–161
35. Campbell, R. K., Bergert, E. R., Wang, Y., Morris, J. C., and Moyle, W. R. (1997) *Nat. Biotech.* **15,** 439–443
36. Einstein, M., Lin, W., Macdonald, G. J., and Moyle, W. R. (2001) *Exp. Biol. Med.* **226,** 581–590
37. Marichatou, H., Martinat, N., Guillou, F., and Combarnous, Y. (2000) *Eur. J. Endocrinol.* **142,** 402–405
38. Furuhashi, M., Shikone, T., Fares, F. A., Sugahara, T., Hsueh, A. J., and Boime, I. (1995) *Mol. Endocrinol.* **9,** 54–63
39. Ji, I., and Ji, T. H. (1993) *J. Biol. Chem.* **268,** 20851–20854
40. Li, M. D., and Ford, J. J. (1998) *J. Endocrinol.* **156,** 529–542
41. Woodruff, T. K., and Mather, J. P. (1995) *Annu. Rev. Physiol* **57,** 219–244

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