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Use of Protein Knobs to Characterize the Position of Conserved α-Subunit Regions in Lutropin Receptor Complexes*

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Efforts to identify the manner in which human choriogonadotropin (hCG) contacts lutropin receptors (LHR) have been stymied by the complex structure of the hormone and the likelihood that it contacts the receptor at multiple sites. During studies of hCG assembly in mammalian cells, we found that addition of a cysteine to the long disordered β-subunit COOH terminus (βCT) enabled it to become cross-linked by a disulfide to cysteines that are substituted for residues in loop α2 or in the α-subunit COOH terminus (αCT). This created a "knob" on the α-subunit at the location of the cysteine. Knobs of various sizes and charges were useful for probing surfaces of the α-subunit thought previously to contact the LHR. Attachment of the βCT to residues in loop α2 facing loops β1 and β3 reduced hormone activity only a few fold revealing that this surface does not participate in essential high affinity receptor contacts, a finding inconsistent with our earlier view of the hCG-LHR complex. In contrast, this approach showed that the opposite surface of loop α2 appeared to be nearer the receptor interface. Although attachment of knobs to portions of the αCT reduced hormone activity substantially, this finding was difficult to interpret. As discussed, this procedure should be adapted readily to other proteins and may facilitate the introduction of fluorophores, enzymes, or other reagents at specific sites on protein surfaces. It may also permit one to cross-link proteins or to obscure specific protein surfaces during the development of "Trojan Horse" therapeutics.

Glycoprotein hormones control reproduction and development by their abilities to bind receptors on gonadal and thyroid tissues. Gonadotropins are used widely to induce ovulation and to stimulate the production of oocytes prior to assisted reproduction therapies such as *in vitro* fertilization. Whereas the potencies of these hormones can be tailored by altering their circulating half-lives (1) or receptor binding specificities (2–4), efforts to produce hormone antagonists have been limited largely to modifying their oligosaccharides (5–7). The design of useful hormone analogs has been hindered by the lack of information as to how these hormones interact with their receptors. This appears due in part to the complex structures of these hormones and to the manner in which they appear to interact with their receptors.

Glycoprotein hormones are heterodimers that have a highly unusual architecture (8–10). Unlike heterodimers that are stabilized by hydrophobic contacts or intersubunit disulfide bonds, hormones such as hCG,[1] human follicle-stimulating hormone, and presumably human leutininzing hormone and human thyrotropin stimulating hormone are stabilized by a strand of their hormone-specific β-subunits that surrounds α-subunit loop 2 like a "seatbelt" (8, 9). The seatbelt reduces motions of loop α2 that would disrupt hydrogen bonds with backbone atoms of residues in the β-subunit cysteine knot and a small loop in the seatbelt that has a crucial role in hCG assembly (33). The seatbelt is responsible for much of the influence of the β-subunit on hormone function (2, 3, 11), but it remains to be seen if this is because of contacts between the seatbelt and the receptor, the influence of the seatbelt on hormone conformation, or both. Indeed, it is nearly impossible to distinguish seatbelt mutations that disrupt a receptor contact site from those that cause subtle alterations in the conformation of the ligand.

Glycoprotein hormone receptors contain a leucine-rich repeat domain and a rhodopsin-like transmembrane domain needed for G-protein coupled signal transduction (12). Whereas high affinity interactions between the hormone and the leucine-rich repeat domain have been known for years (13), other portions of the extracellular domain have also been shown to interact with mammalian lutropins such as bovine LH (14). This led to the proposal that glycoprotein hormones contact their receptors at two or more distant sites, which would explain how small changes in the conformation of the hormone can have profound influences on receptor binding (15). Unfortunately, these changes in conformation are not readily apparent and are difficult to detect.

We have attempted to circumvent the influence of conformational changes on data interpretation by monitoring portions of the ligand that can be detected in the hormone-receptor complex. Surfaces of glycoprotein hormones that can be recognized when they are bound to receptors provide an unequivocal glimpse of the ligand in the complex regardless of changes to other portions of the molecule (16, 17). Once these surfaces have been identified, the remaining potential contact sites can be identified by reference to a crystal structure of the ligand. Application of this approach to hCG revealed that the convex surfaces of both hormone subunits are exposed in the LHR complex (18–20). This suggested that the "opposite" surface of the ligand, which includes loop α2, the most highly conserved portion of all glycoprotein hormones, might have key roles in receptor interaction (19). Unfortunately, it has been difficult to obtain probes that can be used to detect this portion of the

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[1] The abbreviations used are: hCG, human choriogonadotropin; LHR, lutropin receptor; α1, α2, α2, hCG α-subunit loops 1, 2, or 3; β1, β2, β3, hCG β-subunit loops 1, 2, or 3; αCT, α-subunit carboxyl-terminal residues 88–92; βCT, β-subunit carboxyl-terminal residues 111–145.

protein even in the free hormone. Our finding that the seatbelt can be "latched" to several residues in loop α2 without abolishing the activity of hCG (21) suggested that this portion of the hormone did not have the role in hormone-receptor interaction we had envisioned originally (19). This caused us to search for a better method for testing the proximity of this portion of the hormone to the receptor.

Another portion of the α-subunit that has long been presumed to be essential for its biological activity is its COOH terminus; removal of the αCT diminished hormone activity by more than 100-fold (22, 23). Unfortunately, this also altered the conformation of the heterodimer, a phenomenon that changed its ability to be recognized by monoclonal antibodies (19). As in the case of loop α2, the αCT is a highly conserved portion of the α-subunit. Along with its small size, this has made it difficult to use antibody probes to determine its contribution to hormone-receptor interaction.

During studies of hCG assembly in the endoplasmic reticulum, we found that the seatbelt of hCG scans the surface of the β-subunit until it finds its "latch" site (34). We reasoned that the long disordered βCT might also scan much of the surface of the heterodimer and enable a cysteine that had been introduced near its end to form a disulfide with a cysteine that had been introduced into α-subunit loop 2 or into the αCT. In principle, this would enable us to create "knobs" of different sizes and charges at nearly any desired site in these portions of the α-subunit. As described here, this approach permitted the addition of different types of probes to loop α2 and to the αCT. The high activities of these analogs revealed that many residues in loop α2, particularly those that face loops β1 and β3, do not participate directly in essential LHR interactions. Indeed, it was possible to design active analogs in which a part of the βCT passed directly through the groove between loops α2 and β1/β3. The results of these findings suggest that loop α2 is near the receptor in the hCG-LHR complex but that much of it does not participate in essential contacts with the receptor. The finding that knobs were more inhibitory when they are attached to portions of the αCT suggests that this portion of the hormone may be near the receptor interface, although we cannot exclude the possibility that attachment to this site alters the conformation of the heterodimer.

MATERIALS AND METHODS

The sources of hCG and antibodies used in these studies have been described (14, 17, 19). Vectors used to express α-subunit cysteine substitutions have been described (21). All constructs are named by their mutations. Thus, α-L48C refers to an analog of the α-subunit in which αLeu-48 has been converted to cysteine. A construct capable of expressing hCGβ-S138C was prepared by cassette mutagenesis between the natural ApaI site in the hCG cDNA and a BamHI site that had been engineered downstream of the termination codon as described (2). Constructs that encode short and long linkers were derived from hCGβ-S138C by elimination of hCG residues 116–135 and 121–135. We define the linker region as the sequence between the last residue in the core of the hCG β-subunit, which is βCys-110, and the cysteine used for cross-linking, which corresponds to βCys-138 in hCGβ-S138C. The full-length linker used in these studies has the same amino acid sequence as the hCG β-subunit between residues 111 and 137, namely: DDPRFQDSSSSKAPPPSLPSPSRLPGP-C. The "short" and "long" linker constructs were prepared by removing hCG β-subunit residues 116–135 and 121–135 to give: DDPRFGP-C and DDPRFQDSSSGP-C, respectively. Constructs that contain β-lactamase have two linkers. The first is the full-length linker found in hCGβ-S138C between residues 110 and 138 and the second linker connects the cross-linking cysteine (*i.e.* S138C) to β-lactamase. We used two different sequences for the second linker. The amino acid sequence of the longer of these two is: DTPILPQ. The shorter one consists of a single aspartic acid residue. We fused the NH_2-terminal codon of β-lactamase to these second linkers to create the following sequences: GPC-DTPILPQHPFTLVKVKD-(remainder of β-lactamase) and GPC-DHPFTLVKVKD-(remainder of β-lactamase). The codons for the dipeptides proline-arginine and glycine-proline in these sequences contain SstII and ApaI restriction sites, which can be used to attach these tails to other proteins that terminate in these restriction sites.

TABLE I
Production of cysteine containing α-subunit heterodimeric analogs by transfected COS-7 cells

COS-7 cells were co-transfected with plasmids encoding the indicated α-subunit analog and the native β-subunit. Heterodimers secreted into the culture media were quantified using a sandwich immunoassay employing anti-α-subunit antibody A113 and radioiodinated anti-β-subunit antibody B110. Values shown are mean ± S.E. for triplicate transfections.

Transfection	Amount of dimer in the medium (ng/50 μl) mean ± S.E.
αY37C + β	Not detected
αP38C + β	1.710 ± 0.026
αP40C + β	0.588 ± 0.093
αL41C + β	7.617 ± 0.438
αR42C + β	0.962 ± 0.135
αS43C + β	3.571 ± 0.156
αK44C + β	2.788 ± 0.358
αK44A + β	5.677 ± 0.046
αK45C + β	1.090 ± 0.093
αK44E,K45Q + β	1.989 ± 0.403
αT46C + β	7.044 ± 0.229
αM47C + β	9.491 ± 0.524
αL48C + β	1.497 ± 0.170
αV49C + β	2.579 ± 0.297
αQ50C + β	1.985 ± 0.153
αN52C + β	0.615 ± 0.081
αV53C + β	6.108 ± 0.356
αM71C + β	6.153 ± 0.332
αG73C + β	Not detected
αT86C + β	4.973 ± 0.027
αY88C + β	7.930 ± 0.290
αY89C + β	Not detected
αH90C + β	0.971 ± 0.170
αK91C + β	1.049 ± 0.055
αS92C + β	2.595 ± 0.106

Constructs encoding the human α-subunit or the cysteine containing α-subunit analogs were co-expressed with the hCG β-subunit or hCGβ-S138C in COS-7 cells using methods that have been described (2). Materials secreted into the culture media were quantified by sandwich immunoassays (16) employing α-subunit antibody A113 for capture and radioiodinated β-subunit antibody B110 for detection. They were treated at acid pH to promote the dissociation of heterodimers that lack an intersubunit disulfide cross-link, also as described (21). Chinese hamster cells cells that overexpress the rat LHR were used to monitor the influence of the analogs on the ability of ^{125}I-hCG to bind LHR and to elicit cyclic AMP accumulation as described (3, 14, 17). Binding and signaling assays were performed in 100 and 60 μl, respectively.

RESULTS

COS-7 cells that were co-transfected with vectors encoding most α-subunit containing cysteine analogs and either the native β-subunit or hCGβ-S138C were capable of assembling the heterodimer and secreting it into the culture medium (Tables I and II). Heterodimers secreted poorly or not at all included those containing the native hCG β-subunit and α-subunit analogs in which a cysteine had been substituted for residues αTyr-37, αPro-40, αAsn-52, αGly-73, and βTyr-89 (Table I). The poor secretion of αN52C/hCGβ may reflect the absence of the *N*-linked glycosylation signal normally found at this position of hCG that is required for efficient heterodimer secretion (24).

Cross-linking the α-subunit analogs to hCGβ-S138C improved the secretion of heterodimers containing αY37C, αP40C, or αN52C, but not those containing αG73C or αY89C (Table II and Fig. 1). Many of these heterodimers appeared to contain an intersubunit cross-link as shown by the finding that unlike hCG they remained intact following a brief treatment at low pH (Table II). Heterodimers containing hCGβ-S138C and the native α-subunit, αG22C, and αV53C were destroyed by low pH treatment, suggesting that they lacked an intersubunit

TABLE II
Production of heterodimers by COS-7 cells transfected with the indicated cysteine-containing α-subunit constructs and hCGβ-S138C

α-Subunit analog	Heterodimer	Cross-linked[a]	Seatbelt latch[b] (B111/B110)
	ng/50 μl	*%*	
Native α	26.2 ± 0.8	Undetectable	Not done
αQ5C	14.3 ± 1.3	17.5 ± 9.9	0.98
αG22C	9.1 ± 0.4	Undetectable	Not done
αQ27C	3.3 ± 0.0	21.1 ± 4.2	1.03
αR35C	6.0 ± 0.0	98.4 ± 4.9	0.97
αY37C	5.4 ± 1.2	80.2 ± 0.5	0.18
αP38C	0.3 ± 0.04	Not tested	Not done
αP40C	2.1 ± 0.04	52.8 ± 4.3	0.64
αL41C	3.2 ± 0.04	37.2 ± 0.6	0.49
αR42C	22.1 ± 5.3	67.7 ± 5.3	0.48
αS43C	29.7 ± 1.4	81.3 ± 1.9	0.60
αK44C	4.0 ± 0.1	55.4 ± 1.3	0.84
αK45C	5.8 ± 0.1	82.9 ± 6.2	0.85
αT46C	21.4 ± 0.4	76.8 ± 0.6	1.14
αM47C	4.9 ± 0.8	72.0 ± 2.0	0.82
αL48C	14.3 ± 0.9	88.5 ± 2.1	0.86
αV49C	6.0 ± 0.5	70.5 ± 6.1	0.76
αQ50C	0.8 ± 0.1	66.7 ± 7.4	0.82
αK51C	4.1 ± 1.2	27.5 ± 1.4	0.86
αN52C	6.3 ± 0.2	70.6 ± 3.9	0.88
αV53C	6.1 ± 0.4	4.3 ± 2.9	Not done
αS64C	4.4 ± 0.3	82.5 ± 8.9	0.85
αM71C	6.2 ± 0.3	14.1 ± 3.2	Not done
αG73C	Undetectable	Not done	Not done
αV76C	4.3 ± 0.0	41.9 ± 4.9	1.10
αY88C	7.4 ± 0.3	83.0 ± 6.2	0.87
αY89C	Undetectable	Not tested	Not done
αH90C	7.2 ± 1.3	79.2 ± 1.0	0.86
αK91C	9.9 ± 5.7	69.4 ± 3.3	0.91
αS92C	15.1 ± 0.8	140.7 ± 14.4	0.98

[a] This value was calculated as the percentage of material that remained in the sample following treatment at acid pH as described in the text.

[b] This value was calculated as the ratio of activity determined in sandwich assays employing A113 and ^{125}I-B111 relative to that observed in A113 and ^{125}I-B110 after low pH treatment. Detection of any B111 binding indicates that the seatbelt is latched. The low values observed in some cases may reflect a steric influence of the β-subunit carboxyl terminus on the ability of B111 to interact with the cross-linked heterodimer.

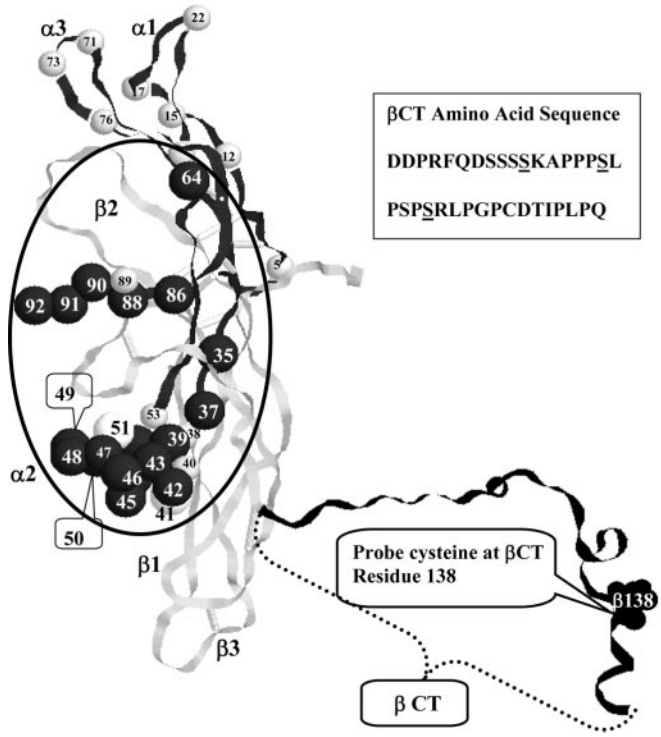

FIG. 1. **Locations of α subunit residues that became coupled to the βCT.** This figure depicts a visual summary of data in Table II in conjunction with the structure of hCG. The α- and β-subunit backbones are shown as *dark* and *light gray ribbons*, respectively. The βCT is shown as a *black ribbon*. The locations of the Cα carbons of cysteine substitutions that were cross-linked efficiently to the βCT are shown as *dark spheres*. Those that gave lesser amounts of cross-linking are depicted in a *lighter shade of gray*. The *small pale spheres* refer to α-subunit locations that did not become cross-linked to the βCT. Note, α-subunit residues 90, 91, and 92 cannot be seen in the crystal structure of hCG and are shown here in arbitrary positions to reflect their approximate locations relative to the α-subunit and the βCT. The *inset* illustrates the amino acid sequence of the βCT with the putative *o*-linked glycosylation sites *underlined* and a *box* drawn around Cys-138.

disulfide. Only a fraction of the heterodimers that contained αL41C and αK51C appeared to be stabilized by intersubunit disulfides, however, suggesting that they became cross-linked inefficiently (Table II). The cysteines in the α-subunit that formed little or no cross-linked heterodimers are at the subunit interface or are distant from the βCT (Fig. 1), a phenomenon suggesting that most intersubunit disulfide cross-links formed after the subunits had assembled into a heterodimer similar in structure to hCG. Most other cysteine-modified α-subunit analogs became cross-linked readily to the cysteine in the βCT. These included analogs that contained a cysteine in the αCT such as α-T86C, α-Y88C, α-H90C, αK91C, and αS92C (Table II, Fig. 1).

We did not attempt to prove that the disulfide introduced into these analogs had formed between the cysteine substituted into the α-subunit and that at β-subunit residue 138 because of the difficulty in identifying disulfides in glycoprotein hormones. The complexity of these analogs suggests this would probably require determining the crystal structures of each analog, as was the case for hCG (8, 9). Nonetheless, it seems highly unlikely that any other disulfide could have been formed because each of the cross-linked heterodimers was recognized by conformation-sensitive antibodies that depend on formation of the cystine knots in each subunit. We have found that βCys-110, the cysteine that latches the carboxyl-terminal end of the seatbelt to the β-subunit core can become cross-linked to the cysteine that had been introduced into many of these α-subunit analogs, but only if it is prevented from forming a disulfide with Cys-26 in β1 (21). This phenomenon is detected readily because it disrupts the ability of the heterodimer to be detected by monoclonal antibody B111, an antibody that recognizes the hCG β-subunit near its seatbelt latch site. Although the seatbelt does not need to be latched for this antibody to bind hCG, it will not bind hLH or analogs of hCG in which the seatbelt is latched to the α-subunit (21). Each of the cross-linked heterodimers in these studies was recognized by antibody B111 (Table II), an observation that showed its seatbelt was latched to βCys-26 as it is in hCG. This finding revealed that formation of the disulfide between βCys-138 and the cysteine added to the α-subunit occurred after subunits had combined with one another, a process that takes place after the seatbelt is latched to βCys-26 (35).

A few analogs were recognized less well by antibody B111 than B110, however, and while we cannot exclude the possibility that βCys-110 is latched to the α-subunit in a fraction of these analogs, this seems highly unlikely. All of the β-subunits used in these studies contain a cysteine at residue 26. We have found that elimination of this cysteine is required to cause the seatbelt to become cross-linked to a cysteine introduced into any site of the α-subunit (21). A more likely explanation for the reduced abilities of some analogs to be recognized by B111 relative to B110 is that the cross-link may have stabilized a position of the β-subunit carboxyl terminus of these analogs in a conformation that interfered with the access of B111 to the heterodimer. This is supported by the observation that the

TABLE III

Influence of the mutations in α-subunit loop 2 and carboxyl terminus on heterodimer lutropin activity

The concentration of each analog was determined by sandwich immunoassay. These values were calculated from the IC_{50} and ED_{50} values from experiments summarized in Figs. 2–10. Several analogs were not tested in these assays due to the fact that only small amounts were produced by transfected COS-7 cells as indicated in Fig. 1. As also shown in Table I, we did not obtain any stable heterodimer following acid treatment of α/hCGβ-S138C. This was consistent with the notion that a free cysteine in the α-subunit was required for formation of a disulfide cross-link.

	α-Subunit analog	Native β-subunit containing analogs (% hCG potency, 95% CL)		Acid-stable hCGβ-S138C analogs (% hCG potency, 95% CL)	
		Receptor binding[a]	Signaling[a]	Receptor binding[a]	Signaling[a]
Loop α2	α	100	100	Not done	Not done
	αR42C	113 (97–132)	103 (85–124)	327 (305–351)	332 (287–385)
	αR42E	Not done	80 (66–98)	Not prepared	Not prepared
	αS43C	35 (31–39)	74 (50–110)	17 (15–20)	43 (37–49)
	αK44C	66 (60–72)	97 (75–124)	250 (228–274)	191 (148–247)
	αK44A	120 (101–143)	101 (70–147)	Not prepared	Not prepared
	αK44E,K45Q	58 (54–61)	42 (32–55)	Not prepared	Not prepared
	αK45C	93 (87–100)	91 (71–117)	223 (212–257)	168 (129–217)
	αT46C	38 (35–42)	56 (43–73)	50 (44–57)	52 (43–64)
	αM47C	8.7 (7.4–10)	23 (16–32)	4.1 (2.9–5.9)	2.4 (2.1–2.7)
	αM47A	83 (71–97)	50 (36–68)	Not prepared	Not prepared
	αM47E	74(61–89)	53 (37–76)	Not prepared	Not prepared
	αM47K	Not done	23 (18–28)	Not prepared	Not prepared
	αL48C	60 (54–68)	147 (96–226)	66 (60–72)	99 (81–120)
	αV49C	70 (64–77)	133 (102–174)	71 (65–76)	99 (84–119)
	αQ50C	81 (76–85)	216 (163–285)	79 (62–100)	308 (241–395)
	αK51C	Undetectable	Undetectable	Not done	Not done
	αN52C	Not done	Not done	61 (57–65)	118 (73–192)
	αV53C	149 (137–162)	Not done	Not done	Not done
αCT	αS64C	Not done	Not done	89 (78–101)	17 (15–21)
	αY88C	141 (128–156)	74 (64–84)	0.05 (indeterminate)	0.12 (0.09–0.16)
	αY89C	Not done	Not done	Not done	Not done
	αH90C	30 (26–34)	65 (48–89)	3.1 (1.8–5.4)	1.0 (0.96–1.1)
	αK91C	41 (38–45)	56 (46–68)	2.2 (1.1–4.4)	Undetectable
	αK91E	4.3 (3.4–5.5)	1.1 (0.1–2.2)	Not prepared	Not prepared
	αK91M	68 (36–144)	66 (45–140)	Not prepared	Not prepared
	αS92C	100 (91–110)	38 (31–47)	28 (23–34)	13 (12–14)
	δαCT	0.30 (0.15–0.57)	0.47 (0.40–0.56)	Not prepared	Not prepared

[a] Values are averages of triplicates. Those in parentheses refer to the 95% confidence limit.

location of the α-subunit cysteine in the cross-linked analogs that were recognized least by B111 (*e.g.* αL41C and αR42C) is nearest the B111 binding site or partially obscured by the seatbelt (*e.g.* αY37C).

Except for residues αMet-47 and αLys-51, substitution of a cysteine for most α-subunit residues in loop 2 had relatively little influence on the receptor-binding and signal-transduction activities of hCG (Table III and Fig. 2). Replacing α-subunit residue αMet-47 with cysteine reduced the activity of the heterodimer more than 10-fold; replacing αLys-51 with cysteine was shown earlier to nearly eliminate ligand activity (25). Whereas an analog in which αLys-51 had been replaced by alanine also had considerably lower activity than hCG (25), hCG-αM47A, an analog in which αMet-47 had been replaced by alanine was nearly as active as hCG in both assays (Table III). This suggested that the presence of a methionine at α-subunit residue 47 was not essential for hCG activity. Replacing αMet-47 with glutamate disrupted hormone-receptor interaction, however (Table III). This suggested that whereas the side chain of loop α2 residue αMet-47 is not crucial for hormone activity, this residue may have an important role in receptor interaction or in the conformation of the heterodimer. The role of αLys-51 in receptor interaction also remains to be determined. Based on the finding that a heterodimer in which α-subunit residue 51 is cross-linked to β-subunit residue 99 by a disulfide was more active than those in which αLys-51 is replaced by cysteine or alanine (25), it appears likely that replacing the αLys-51 side chain may alter the conformation of the heterodimer. This residue may participate in hydrogen bonds with backbone atoms in the small seatbelt loop (8, 26) and its removal may distort the heterodimer.

Changing loop α2 residue αLys-44 to alanine has been reported to reduce hCG activity 100-fold or more (27). This contrasts with the finding that replacing αLys-44 with cysteine had relatively little influence on LHR signaling or binding (Table III) and led us to study this portion of the hormone further. We prepared hCG-αK44A and found that it had high activity in both assays (Table III). We also tested the requirement for a positively charged residue in this portion of the α-subunit using hCG-αK44E,K45Q, a negatively charged analog. This analog also had high activity in signaling and binding assays (Table III and Fig. 3), indicating that neither of the positively charged amino acids at these positions in loop α2 is essential for hCG activity *in vitro* despite its highly conserved sequence (28). Unlike the earlier report (27), the results of these studies were consistent with what we had observed by replacing either αLys-44 or αLys-45 with cysteine (Table III and Fig. 2).

Many of the acid-stable analogs in which the carboxyl terminus of the β-subunit was cross-linked to residues in α2 had considerable activities in receptor-binding and signal-transduction assays (Table III and Fig. 4). These include those in which it was attached to α2 residues 42, 44, 45, 48, 49, 50, and 52 (Table III). The activities of analogs in which α-subunit residues 48, 49, and 50 were cross-linked to the cysteine substituted in the βCT (Table III) were considerably higher than those in which they were cross-linked to seatbelt residue βCys-110 (21). This suggested that the low activity of the latter was because of the constraints of the cross-link on the conformation of the heterodimer, not contacts between these residues and the LHR. The side chains of α2 residues in nearly all the most active analogs project toward β1/β3. This suggests that the surface of α2 that faces β1/β3 does not contact the LHR.

Attachment of the βCT to residue 47 nearly abolished receptor binding activity; coupling it to residues 43 and 46 reduced the activities of the heterodimer by half (Table III and Fig. 4).

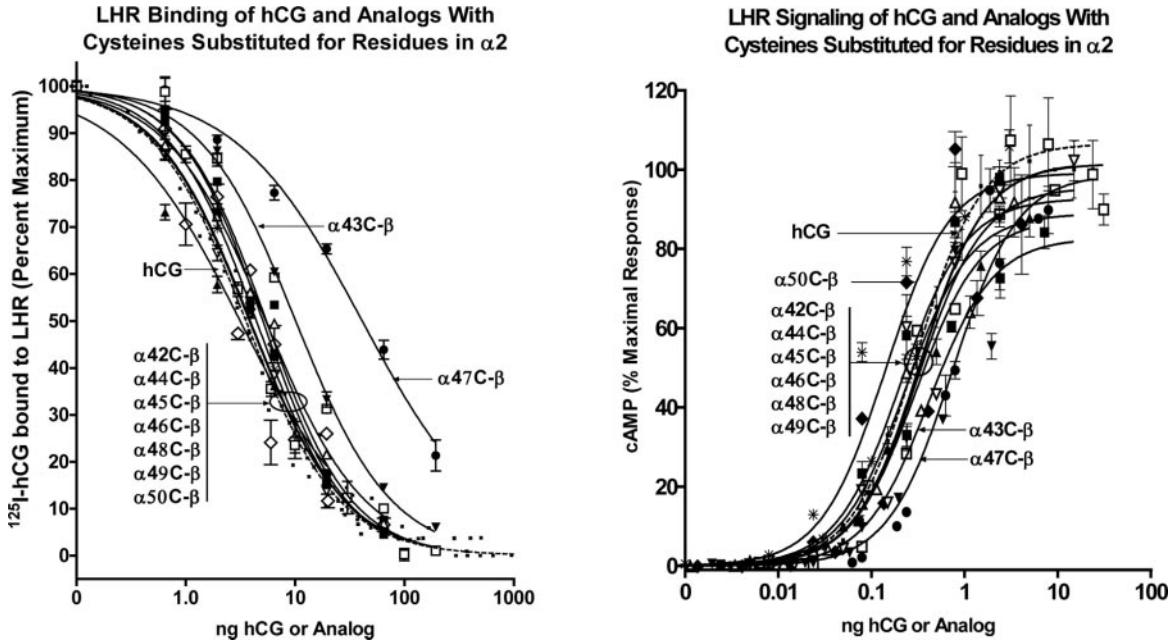

FIG. 2. **Results of LHR binding (*left panel*) and signaling assays (*right panel*) in response to hCG and analogs encoding the indicated α2 cysteine substitutions.** Constructs encoding the indicated α2 cysteine were co-expressed in COS-7 cells with the construct encoding the hCG β-subunit. Heterodimers secreted into the culture media were concentrated by ultrafiltration, quantified in A113-^{125}I-B110 sandwich immunoassays, and assayed in LHR binding and signaling assays in triplicate. Binding was assessed by comparing the abilities of hCG and the analogs to inhibit the binding of ^{125}I-hCG to Chinese hamster ovary cells that express rat LHR. Signaling was assessed by measuring the abilities of hCG and the analogs to elicit cyclic AMP accumulation from the LHR expressing Chinese hamster ovary cells. *Vertical bars* extend to the limits of the S.E. The volumes of the binding and signaling assays were 100 and 60 μl.

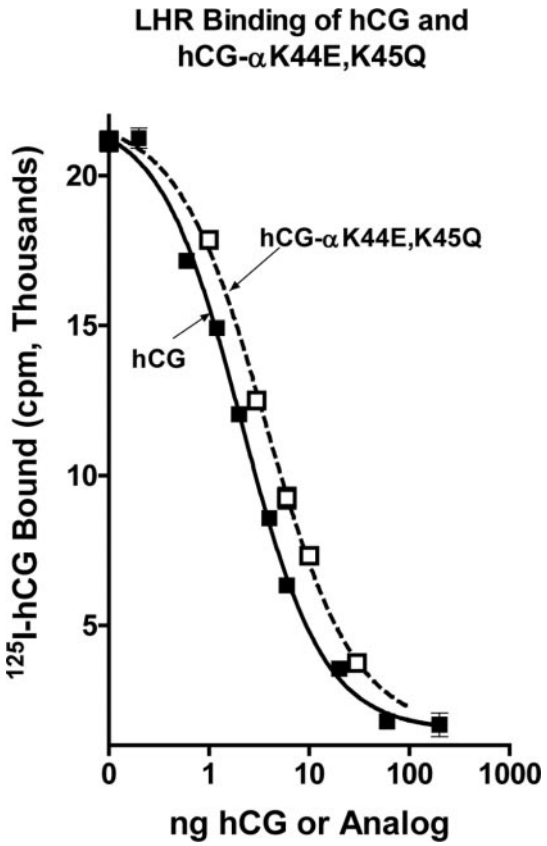

FIG. 3. **LHR binding in response to hCG and hCG-αK44E,K45Q.** Constructs encoding the αK44E,K45Q subunits were co-expressed in COS-7 cells with a construct encoding the hCG β-subunit. Heterodimer secreted into the culture media were concentrated by ultrafiltration, quantified in A113-^{125}I-B110 sandwich immunoassays, and assayed in LHR binding in triplicate as noted in the legend to Fig. 2. *Vertical bars* extend to the limits of the S.E. and are within the sizes of the symbols in most cases.

The side chains of α-subunit residues 43 and 46 project toward the seatbelt and that of residue 47 faces the small seatbelt loop formed by the disulfide between Cys-93 and Cys-100. Although the loss in activity caused by attaching the βCT probe to α-subunit residues 43, 46, or 47 could be because of several factors such as disrupting an interaction between these residues and the receptor or altering the conformation of the heterodimer, additional studies will be needed to distinguish these possibilities.

The length of the β-subunit carboxyl terminus suggests that residue 138 might be capable of being latched to cysteines in loop α2 without passing through the groove between loop α2 and loops β1/β3 (Fig. 1). To test the possibility that this groove formed a key element of the receptor binding site, we compared the activities of hCG and cross-linked analogs lacking β-subunit residues 116–135 and 121–135. In these analogs, the β-subunit carboxyl terminus is too short to be cross-linked to many α-subunit residues unless it passes directly through the groove between α2 and β1/β3. These analogs had substantial activities in LHR binding assays (Fig. 5), providing additional support for the concept that this hormone groove does not participate in essential high affinity LHR contacts.

The knob created by cross-linking hCGβ-S138C to α-subunit loop 2 is only of moderate size and charge. To learn how changes in the size and charge of the knob affected ligand binding and signaling, we designed knobs that contained 4 aspartic acid residues or arginine residues near the attachment site. We also prepared knobs in which β-lactamase was attached to the α-subunit at varying distances from residues in loop α2. The latter enabled us to probe the possibility that loop α2 is near the receptor interface even though it appears not to be required for high affinity contacts with it.

Knobs that had 4 arginine residues surrounding the disulfide tether reduced the activity of hCG by only 2–3-fold (Fig. 6, *left*). Thus, hCG analogs with these additional residues near α-subunit residues 42, 44, 45, 46, and 48 had nearly the same activity as hCG. Analogs that contained 4 aspartic acids attached to

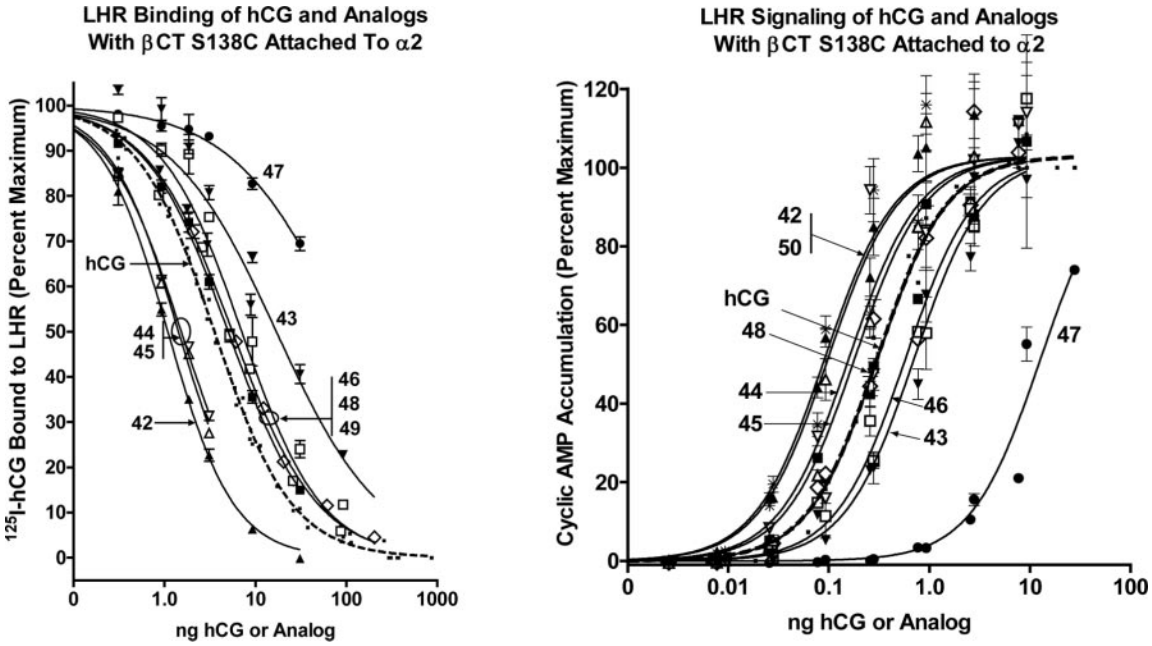

FIG. 4. **LHR binding (*left panel*) and signaling (*right panel*) responses to analogs in which the βCT was coupled to α2.** Constructs encoding the indicated α2 cysteine were co-expressed in COS-7 cells with the construct encoding hCG-βS138C. Acid-stable heterodimers secreted into the culture media were concentrated by ultrafiltration, quantified in A113-^{125}I-B110 sandwich immunoassays, and assayed in LHR binding and signaling assays in triplicate as noted in the legend to Fig. 2. *Vertical bars* extend to the limits of the S.E.

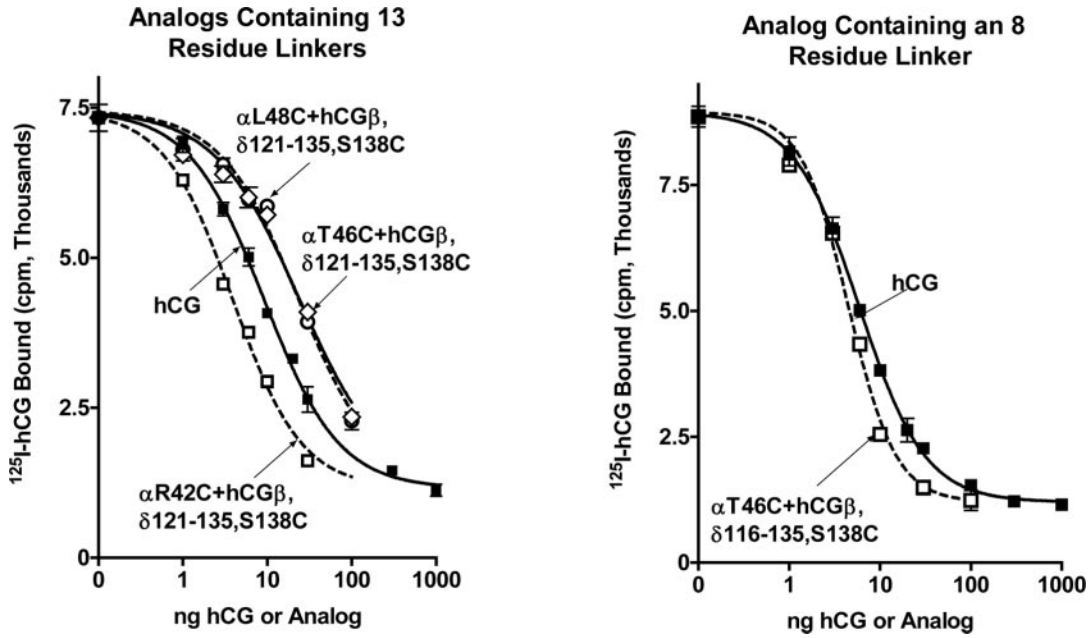

FIG. 5. **LHR binding of hCG and analogs in which the βCT was coupled to α2 by 13-residue (*left panel*) and 8-residue (*right panel*) linkers.** The indicated analogs were prepared in COS-7 cells, quantified, and monitored in triplicate for their abilities to block the binding of ^{125}I-hCG to Chinese hamster ovary cells that express rat LHR. *Vertical bars* extend to the limits of the S.E. and are within the sizes of the symbols in most cases. The assay volume was 100 μl.

residues 42, 44, 48, and 49 were 5-fold less active than hCG (Fig. 6, *right*). The findings that neither positively charged nor negatively charged knobs at the tip of loop α2 disrupted hCG activity indicated that this portion of the subunit did not fit tightly into a receptor pocket although it might be close to the receptor interface.

To learn if loop α2 might be close to the receptor interface, we studied the activities of analogs that had β-lactamase knobs, a protein similar in size to hCG. The β-lactamase knobs are attached to the α-subunit by two linkers, an upstream linker needed to hold the knob near the α-subunit until formation of the intersubunit disulfide, and either of two shorter downstream linkers between the site of the cross-link in the α-subunit and the β-lactamase knob. The influence of each cross-linker must be considered during data analysis. Although the influence of the upstream linker was low for most β-lactamase knobbed hCG analogs, it affected the signal transduction activities of those in which the cross-link is attached to α-subunit residue 64. Thus, the signal transduction activity of hCG was reduced 6-fold (*i.e.* to 17%) by the upstream linker before β-lactamase was added (Table III, αS64C, fourth column). The presence of a β-lactamase knob reduced this by only a small amount, even when tethered by the shortest linker (Fig. 8, *right*).

As a rule, addition of the β-lactamase knob reduced the

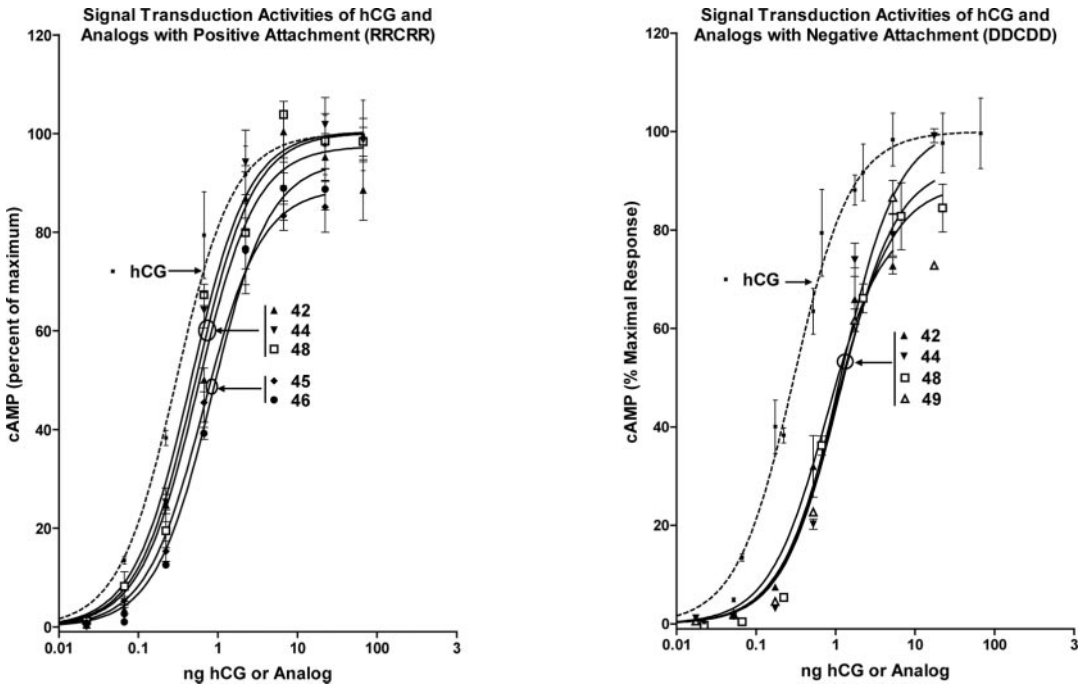

FIG. 6. **Signal transduction assays employing constructs in which positively (*left panel*) and negatively (*right panel*) charged probes were attached to selected residues in loop α2.** Preparation, quantification, and analysis of these hCG analogs employed methods described in the legend to Fig. 2. The assay volume was 60 μl.

binding (Fig. 7) and signaling (Fig. 8) activities of hCG, particularly when the enzyme was tethered to the α-subunit by a short linker (Figs. 7, *right*, and 8, *right*). For example, attaching β-lactamase to loop α2 residues 46 and 48 with a 7-residue linker reduced hormone binding 10-fold (Fig. 7, *left*). Attaching it to these residues with a single residue linker nearly abolished hormone binding (Fig. 7, *right*). Although the reduction in binding might have been caused in part by the increased size of the protein, which would slow its diffusion rate, this would not explain the influence of linker length. Furthermore, some antibody-hormone complexes that have similar sizes bind to the LHR with high affinity, provided that the antibody is complexed with a portion of the hormone that does not contact the receptor (29). Taken together, these findings suggested that much of loop α2 is near the receptor interface. Clearly, it does not participate in key receptor contacts in the manner that we had anticipated earlier (19).

To learn how the β-lactamase probes affected signal transduction we monitored their influence in cyclic AMP accumulation assays. Knobs having the short linker were usually much less active than those with the longer linker (Fig. 8), a phenomenon that is also consistent with the notion that loop α2 is near the receptor interface. Attachment of β-lactamase to residues 44, 49, and 50 with an 8-residue linker reduced hormone activity by only few fold; attaching it to residues 46 and 48 reduced activity further but not as much as attaching it to residue 92 (Fig. 8, *left*). All analogs except that in which β-lactamase was attached to residue 50 by a short linker had very low activities (Fig. 8, *right*). Considered together, these data suggest that loop α2 residue 50 is furthest from the receptor interface and that residues in the vicinity of the tip of loop α2 are nearest the receptor.

The αCT is a portion of the glycoprotein hormone that is often thought to make essential receptor contacts (22). As had been reported by others (22, 23), we found that truncation of the αCT disrupted hormone activity (Table III, analog δαCT). We have also shown that this alters the conformation of the heterodimer (19), making it difficult to know if this portion of the hormone makes essential receptor contacts. Most of the residues in the αCT could be replaced by cysteine without disrupting the ability of hCG to bind to LHR or to stimulate cyclic AMP accumulation more than a few fold (Table III and Fig. 9). This suggested that none of these residues participated in essential receptor contacts. We were surprised by the finding that substitution of a cysteine for α-subunit residue Lys-91 did not have a more dramatic influence on signal transduction because of the report that this residue is needed for hormone efficacy (30). Therefore, we tested the activities of analogs in which this lysine was replaced by methionine and glutamate, substitutions that had been shown to eliminate signal transduction. In our hands, both analogs had the same efficacy as hCG, although glutamate substitution reduced the potency of the heterodimer 25–100-fold (Table III).

Analogs in which the cysteine added to αCT had been cross-linked to β-subunit residue 138 were much less active. Except for the analog in which the cross-link was to α-subunit residue 92, which had ~10% the activity of hCG, the remainder were nearly devoid of activity (Fig. 10). This suggested that this region of the hormone was near the receptor or that cross-linking the αCT to the βCT had distorted the heterodimer, a phenomenon that would also be expected to render it inactive. Attachment of a β-lactamase knob to residue 92 reduced its activity substantially (Figs. 7 and 8), regardless of the length of the linker.

DISCUSSION

The strategy outlined here for introducing cross-linked probes into hCG should be applicable to most proteins that have disordered NH_2-terminal or COOH-terminal ends or to proteins that can be modified by addition of this type of terminus. hCG is a complex glycoprotein heterodimer in which part of one subunit is wrapped around another. The finding that we could introduce disulfide cross-links into nearly any desired portion of the molecule suggests that this approach should be readily adapted to other less complex proteins. One of the most obvious uses for this technique is to prepare cross-linked pro-

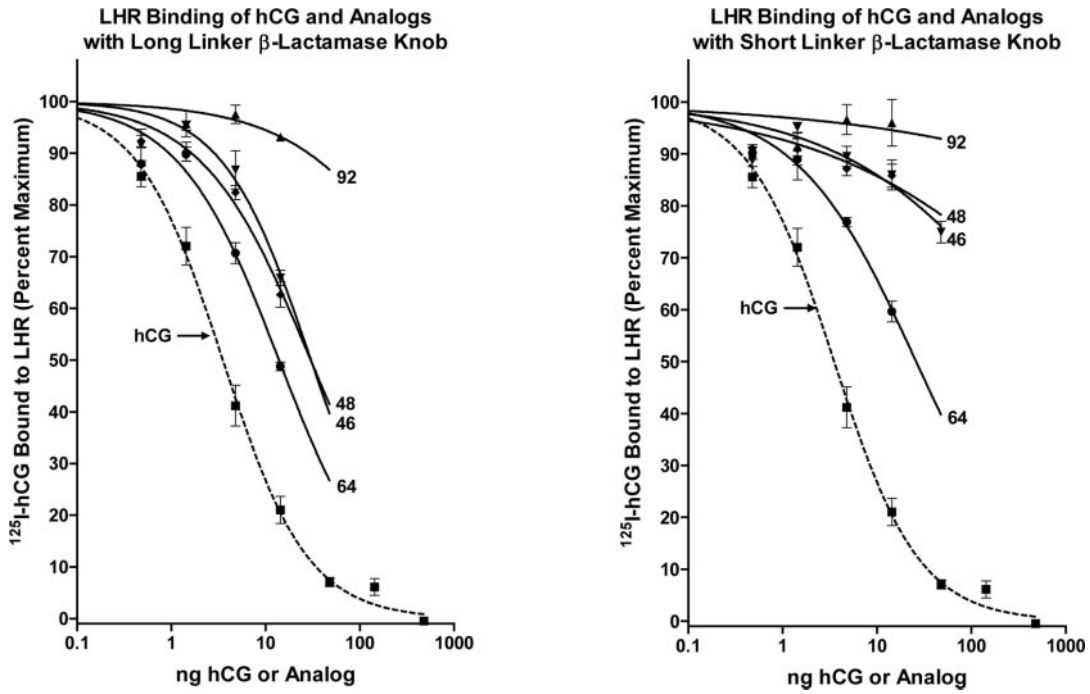

FIG. 7. **Binding characteristics of hCG analogs that contained a β-lactamase knob attached by a 7-residue linker (*left panel*) and a single residue linker (*right panel*).** Constructs encoding the indicated α-subunit analogs were co-expressed with those encoding hCG β-subunit analogs upstream of a 7-residue linker or a single residue linker and β-lactamase. Material secreted into the culture media was concentrated, quantified in sandwich immunoassays, and characterized in receptor binding assays. Note that these analogs contain two linkers, a long linker between the core of hCG and the cysteine that attaches the knob to the α-subunit and either a long or short linker that attaches β-lactamase to the cysteine cross-linker. As discussed in the text, this will account for much of the reduction in activity observed when the β-lactamase probe is attached to α-subunit residue 64. Values are means of triplicates and the *vertical bars* extend to the limits of the S.E. The assay volume was 100 μl.

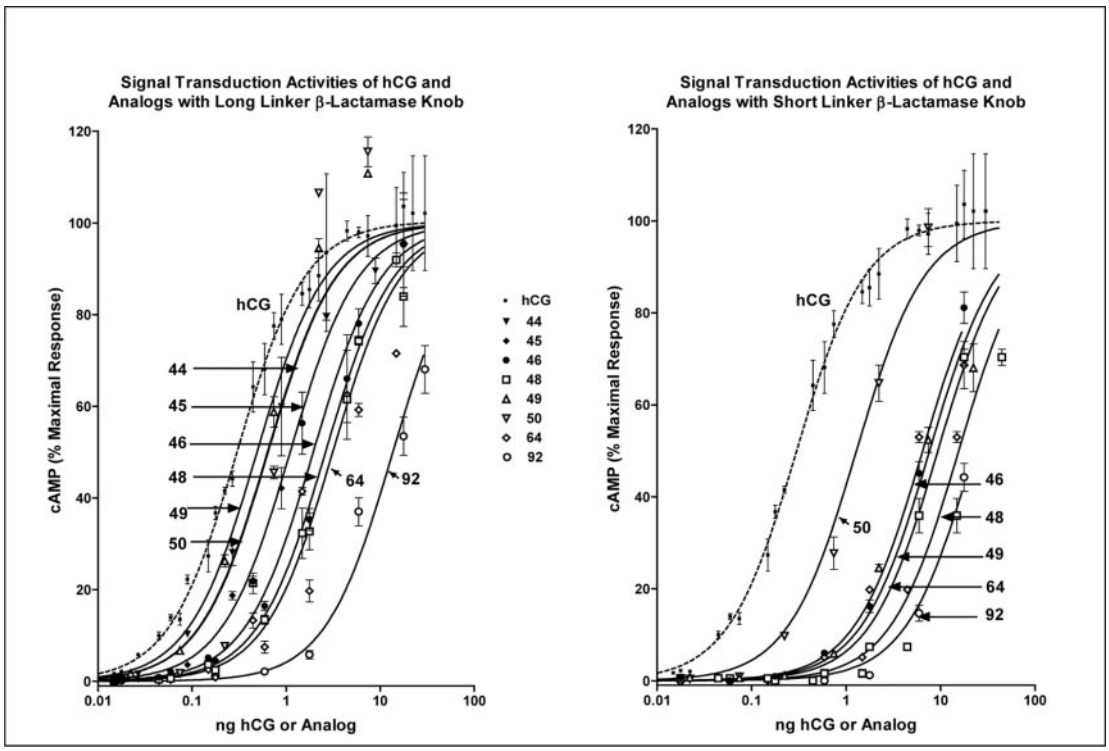

FIG. 8. **Signaling characteristics of hCG analogs that contained a β-lactamase knob attached by an 8-residue linker (*left panel*) and a single residue linker (*right panel*).** Constructs encoding the indicated α-subunit analogs were prepared as described in the legend to Fig. 7 and analyzed in cyclic AMP accumulation assays. Values are means of triplicates and the *vertical bars* extend to the limits of the S.E. The assay volume was 60 μl.

teins. We have found that it is possible to introduce cross-links into hCG without regard to its crystal structure. The resulting heterodimers are acid stable and many have high biological activity. Indeed, their activities depend on the location of the disulfide cross-links.

This technique takes advantage of the natural abilities of

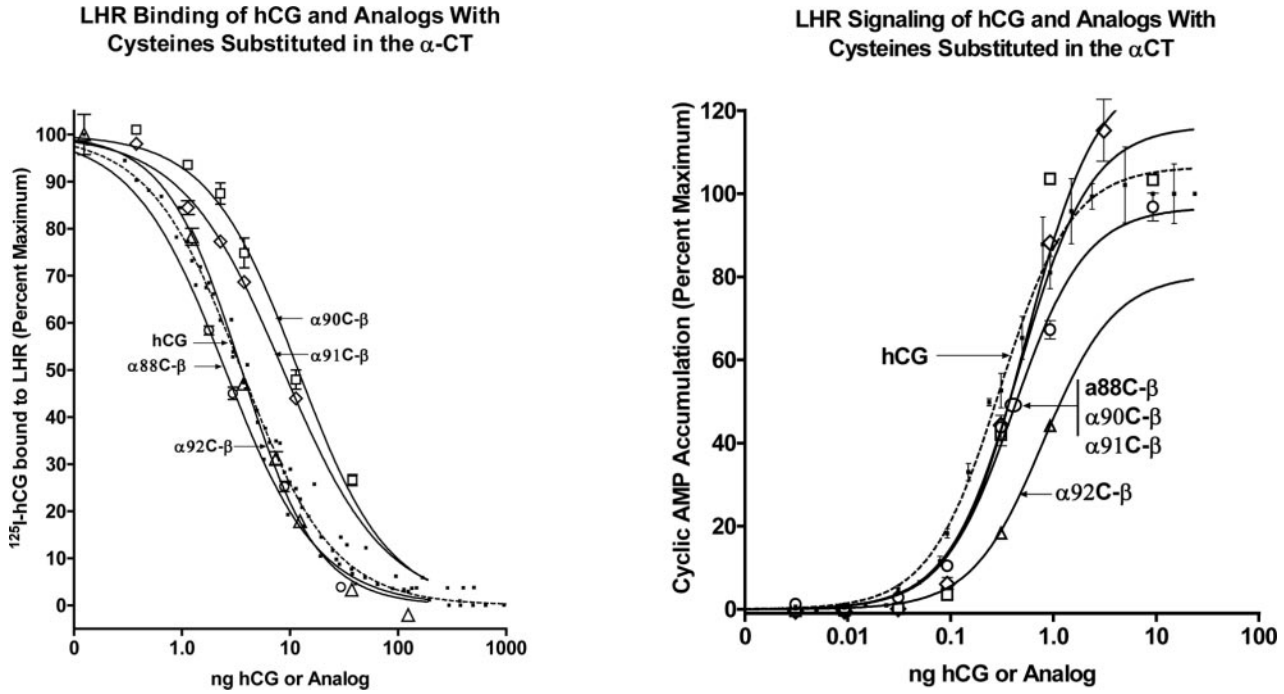

FIG. 9. **Influence of cysteine substitutions in the *α*CT on the activity of hCG in LHR binding assays (*left panel*) and signaling assays (*right panel*).** Heterodimers were prepared by co-expressing the indicated *α*-subunit construct and the native hCG *β*-subunit. Material secreted into the medium was concentrated, analyzed by sandwich immunoassays, assayed in LHR binding and signaling assays. Values are means of triplicates and the *vertical bars* extend to the limits of the S.E. The volumes of the assays were 100 *μ*l (binding) and 60 *μ*l (signaling).

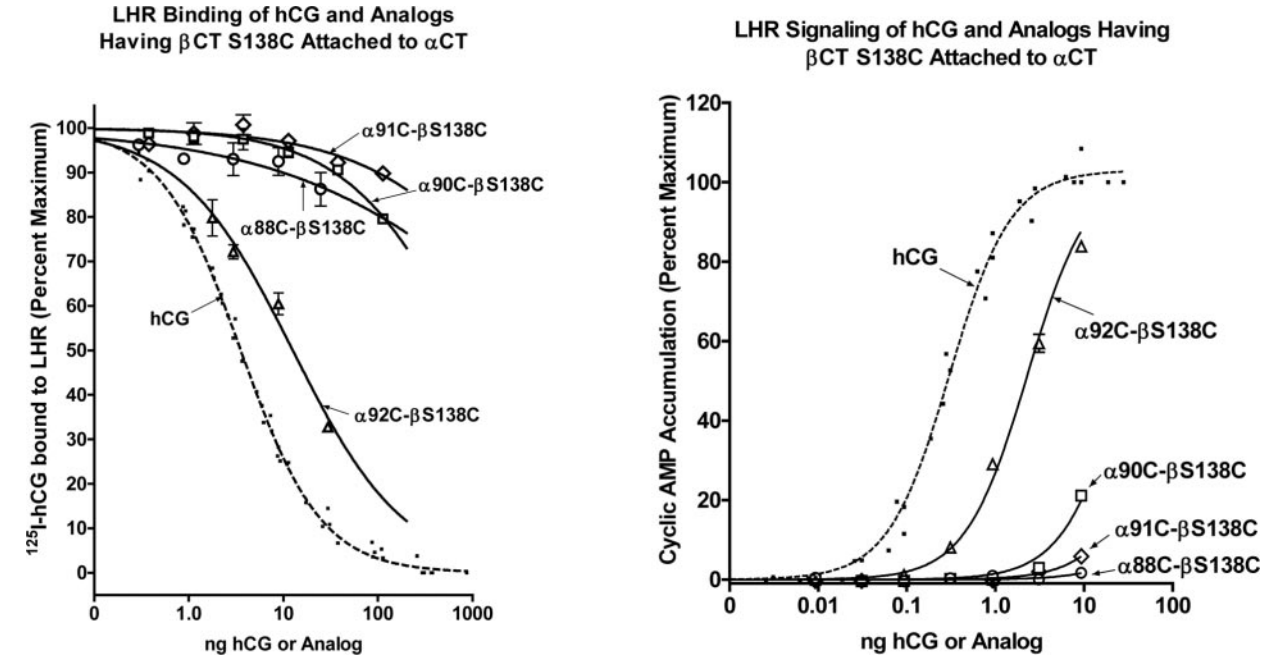

FIG. 10. **LHR binding activity (*left panel*) and signaling activity (*right panel*) of hCG analogs in which the *β*CT was cross-linked to various residues in the *α*CT.** Constructs encoding the indicated *α*CT cysteine were co-expressed in COS-7 cells with the construct encoding hCG-*β*S138C. Acid-stable heterodimers secreted into the culture media were concentrated by ultrafiltration, quantified in A113-^{125}I-B110 sandwich immunoassays, and assayed in LHR binding and signaling assays in triplicate as noted in the legend to Fig. 2. *Vertical bars* extend to the limits of the S.E.

cells to fold proteins as part of the secretory process. All of our attempts to prepare cross-linked proteins were successful provided that the linker used was sufficiently long. This suggests that disordered terminal portions of proteins, such as the *β*CT, "scan" the protein surface during the secretory process and will form a disulfide bond if and when two cysteines become adjacent. Furthermore, as exemplified by our abilities to attach *β*-lactamase to various sites on hCG, by fusing two proteins to a disordered linker it is possible to attach knobs to various sites on protein surfaces. Indeed, we have also used this approach to attach green fluorescent protein to specific sites on hCG.[2]

There are several obvious modifications that should enhance the utility of the knob technique. Inclusion of a protease cleav-

[2] Y. Xing, D. Cao, R. V. Myers, W. Lin, M. P. Bernard, and W. R. Moyle, unpublished results.

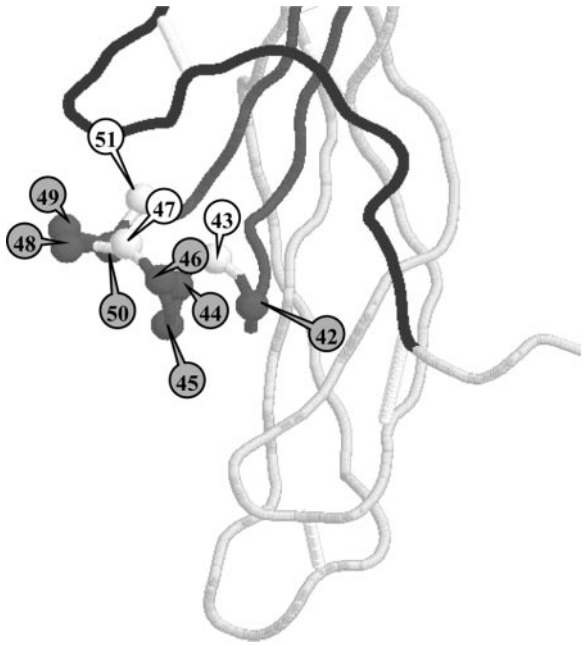

FIG. 11. **Summary of residues in loop α2 that are nearest the receptor interface.** Shown here is a *tube diagram* of loop α2 (*gray*) and a portion of β-subunit loops β1 and β3 (*white*) and seatbelt (*black*). Residues of α2 that appear to be furthest from the LHR interface are colored *gray* and are labeled with *dark colored residue number flags*. Those that appear to be nearest the LHR are colored *white* and are labeled with the *white residue number flags*. The directions of the flags indicate the positions of the side chains. Note that the original model (19) suggested that the residues that are colored *gray* were presumed to form the key receptor contacts along with residues near the tips of loops β1 and β3. This is now known to be incorrect.

age site in the linker should enable it to be clipped after formation of the stabilizing disulfide. This should enable one to create new NH_2-terminal or COOH-terminal ends at any site on the protein surface, a technique that would permit the site-specific introduction of epitope tags in proteins at positions other than their ends. We envision that the procedure will also be useful for blocking active sites of an enzyme such as a protease substrate binding site or for preventing the interactions of protein ligands with their receptors as with the case described here. Addition of enzyme cleavage sites to the linkers of these proteins would permit the targeted activation of the enzyme or restoration of the binding site. Finally, this procedure may be useful for cross-linking proteins, even when their structures are unknown. Unlike earlier studies in which intersubunit disulfides were introduced into hCG based on its crystal structure (25, 31), the cysteines in the disulfides of these cross-linked hCG analogs were not located at the intersubunit interface.

The application of this technique to hCG enabled us to study a surface of the protein that is highly conserved and partially concealed. The observations described here virtually exclude the idea that the surface loop α2 facing β-subunit loops 1 and 3 forms a key receptor binding surface, a tenet of our original model of the hormone-receptor complex (19). They show that parts of α-subunit loop 2 are likely to be near the receptor interface, however. These include residues having side chains that face the seatbelt. As a result the activities of analogs in which the βCT was cross-linked to residues 43, 46, and 47 were substantially lower than those whose side chains face β-subunit loops 1 and 3 (Fig. 11). The abilities of β-lactamase probes to alter receptor interactions is also consistent with the notion that α-subunit loop 2 is near the receptor interface, even though many of the side chains of residues in this portion of the hormone do not participate in essential receptor contacts. We have used this information to build a refined model of the hormone-receptor complex in which this portion of the α-subunit is near the leucine-rich repeat domain of the receptor and in which the hormone has an orientation 180° opposite to that proposed earlier (32).

Our studies did not enable us to determine how the αCT functions in receptor binding. The data described here are consistent with the notion that the αCT has a role in ligand-receptor interactions, but the finding that nearly all its residues can be replaced by cysteines without altering hormone activity more than a few fold (Table III) or without disrupting hormone efficacy (Fig. 9, *right*) makes it hard to understand how it functions in receptor interactions. The observation that the βCT can be cross-linked to α-subunit residue 92 without destroying the activity of the molecule shows that the αCT does not project into a receptor crevice. Unfortunately, we cannot exclude the possibility that attachment of the βCT cross-link to residues in the αCT altered the conformation of the heterodimer, a phenomenon that also would have reduced its activity. Residues of hCG that can be probed without disrupting its activity in LHR binding and signaling assays significantly are not likely to participate in LHR contacts. The converse of this may not be true, however. Probes that reduce the activity of the hormone may do so by disrupting a receptor contact, by altering hormone conformation, or by other unforeseen mechanisms that cannot be readily distinguished. Thus, whereas our observations are consistent with the notion that parts of the hormone near the αCT contact the receptor, they certainly do not prove this possibility.

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Use of Protein Knobs to Characterize the Position of Conserved α-Subunit Regions in Lutropin Receptor Complexes

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