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Only a Portion of the Small Seatbelt Loop in Human Choriogonadotropin Appears Capable of Contacting the Lutropin Receptor*

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Twenty residues of the human choriogonadotropin (hCG) β-subunit that are wrapped around α-subunit loop 2 like a "seatbelt" stabilize the heterodimer and enable the hormone to distinguish lutropin (LHR), follitropin, and thyrotropin receptors. The N-terminal portion of the seatbelt contains a small disulfide-stabilized loop needed for heterodimer assembly and is thought to mediate hCG-LHR interactions. To test the latter notion, we compared the LHR binding and signal transduction activities of hCG analogs in which the α-subunit C terminus (αCT) was cross-linked to residues in the small seatbelt loop. Analogs having an intersubunit disulfide between a cysteine in place of αCT residue αSer-92 and cysteines substituted for loop residues βArg-94, βArg-95, or βSer-96 had high activities in LHR binding and signaling assays despite the fact that both portions of the hormone are thought to be essential for hCG activity. Use of a larger probe blocked hormone activity when the αCT was cross-linked to cysteines in place of residues βArg-95 and βAsp-99, but not to cysteines in place of residues βArg-94, βSer-96, or βThr-97. This suggested that the side chains of residues βArg-95 and βAsp-99, which face in the same outward direction from the heterodimer, are nearer than the others to the LHR interface. The finding that residue 95 can be cross-linked to small αCT probes without eliminating hormone activity indicates its side chain does not participate in essential LHR contacts. We suggest that contacts between the small seatbelt loop and the LHR, if any, involve its backbone atoms and possibly the side chain of residue βAsp-99.

The crystal structure of hCG[1] revealed that 20 residues of its β-subunit surround loop α2 like a "seatbelt" (1, 2). The seatbelt has a key role in the stability of the heterodimer; elimination of the disulfide that "latches" its C-terminal end to βCys-26 in the subunit core disrupts heterodimer formation (3). Thus, glycoprotein hormones differ from most other heterodimeric proteins, which are stabilized by hydrophobic contacts or intersubunit disulfides. Whereas the evolutionary advantages of this unusual structural arrangement remain unknown, it may have facilitated the co-evolution of ligand-receptor pairs by permitting point mutations to alter the conformation of the heterodimer and thereby modulate its biological activity (4–6).

The seatbelt is divided into two regions that differ in their influence on the activities of lutropins, follitropins, and thyrotropins. Its N-terminal half contains a small disulfide-stabilized loop that has an important role in heterodimer assembly (7–9). Because a portion of this loop participates in hydrogen bonds with α-subunit loop 2 (1, 2, 10), mutations that affected its conformation or the stability of these hydrogen bonds would be expected to alter the positions of the subunits in the heterodimer. The seatbelt loop contains positively charged residues in mammalian lutropins and negatively charged residues in mammalian follitropins and thyrotropins. This led to the speculation, which was made several years before it could be tested experimentally, that the charge of this loop "determines" receptor binding specificity (11). Replacing the positively charged residues in the hCG seatbelt loop with their hFSH counterparts reduced LHR binding 10–15-fold but did not convert hCG to a follitropin (4). Changing the specificity of hCG required replacing the C-terminal half of its seatbelt, which we term the strap, with its hFSH counterpart (4, 5). Indeed, the influence of the small seatbelt loop on lutropin activity is determined largely by the composition of the strap (5). Analogs of hCG in which the strap region of the seatbelt is derived from hFSH interact with both LH and FSH receptors. Modifying the charge of the small seatbelt loop in the N-terminal half of the seatbelts of these bifunctional analogs altered binding to LHR more than 100-fold even though it had little influence on binding to FSHR (12). These findings indicate that the small seatbelt loop might affect ligand binding by interacting with the receptor, altering the conformation of the ligand, or by both. Studies described here were initiated to learn if the small seatbelt loop is located near the LHR interface and to determine how it might contact the receptor. The results of these studies indicate that only a limited surface of the seatbelt loop is in a position to contact the LHR.

MATERIALS AND METHODS

The sources of hCG and antibodies used in these studies have been described (13–15). We radioiodinated hCG and antibody B110 using an IODO-GEN procedure (16). The amino acid sequences of the hCG α- and β-subunit analogs used in these studies are shown in Fig. 1. Constructs encoding all hormone analogs were prepared by PCR or cassette mutagenesis using standard methods and expressed transiently in COS-7 cells (4). Materials secreted into the culture media were assayed by sandwich immunoassays (17) employing α-subunit antibody A113 for capture and radioiodinated β-subunit antibody B110 for detection. They were treated at acid pH to cause the dissociation of heterodimers that lack an intersubunit disulfide cross-link (18). Chinese hamster cells that overexpress the rat LHR were used to monitor the influence of the

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[1] The abbreviations used are: hCG, human choriogonadotropin; LHR, luteinizing hormone receptor; αCT, α-subunit C terminus; βCT, hCG β-subunit C terminus; strap, C-terminal half of the seatbelt; hFSH, human follicle-stimulating hormone.

α-Subunit

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Native: αAla1…Cys87-y-y-h-k-s
α92C:   αAla1…Cys87-y-y-h-k-C
αEC:    αAla1…Cys87-y-y-h-e-C
αGGC:   αAla1…Cys87-y-y-h-k-s-g-g-C
αCT100: αAla1…Cys87-y-y-h-k-s-d-d-p-r-f-g-p-C-d-t-p-i-l-p-q
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β-Subunit

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Native: βSer1…Cys90-a-l-c-r-r-s-t-t-d-c-Gly101…Gln145
β91C:   βSer1…Cys90-C-l-c-r-r-s-t-t-d-c-Gly101…Gln145
β92C:   βSer1…Cys90-a-C-c-r-r-s-t-t-d-c-Gly101…Gln145
β94C:   βSer1…Cys90-a-l-c-C-r-s-t-t-d-c-Gly101…Gln145
β95C:   βSer1…Cys90-a-l-c-r-C-s-t-t-d-c-Gly101…Gln145
β96C:   βSer1…Cys90-a-l-c-r-r-C-t-t-d-c-Gly101…Gln145
β97C:   βSer1…Cys90-a-l-c-r-r-s-C-t-d-c-Gly101…Gln145
β98C:   βSer1…Cys90-a-l-c-r-r-s-t-C-d-c-Gly101…Gln145
β99C:   βSer1…Cys90-a-l-c-r-r-s-t-t-C-c-Gly101…Gln145
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FIG. 1. **Analogs used in these studies.** The amino acid sequences of the analogs used in these studies are shown here. Residues in the native human α-subunit between αAla-1 and αCys-87 are not illustrated. The amino acids in the native hCG β-subunit between residues βSer-1 and βCys-90 and between βGly-101 and βGln-145 are also not shown. Mutated residues are shown in *uppercase*.

analogs on the ability of ^{125}I-hCG to bind LHR and to elicit cyclic AMP accumulation as reported previously (5, 13, 15). Binding and signaling assays were performed in 100 and 60 μl, respectively.

RESULTS

We have observed repeatedly that disulfide cross-linked hCG analogs are stable at pH 2, 37 °C, for 30 min or longer; hCG and heterodimers that lack a disulfide cross-link under these conditions dissociate and become undetectable in heterodimer-specific assays (9, 18). Therefore, before using cross-linked samples in binding or signal transduction assays, we routinely treated them at acid pH to destroy any heterodimers that lack a cross-link. In the few cases where we measured the amounts of total and cross-linked heterodimer in the medium before concentrating it and subjecting the concentrate to low pH treatment, we observed that a high percentage of the heterodimers having the ability to form a cross-link between the αCT and the small seatbelt loop were acid stable (Table I). This showed that these cross-links had formed efficiently. We found that sufficient acid-stable material was produced for most receptor binding and signaling assays in these studies by transient transfection of three 10-cm plates of 60% confluent COS-7 cells.

The αCT has long been known to be essential for the activities of most glycoprotein hormones (19). Fusion of peptides to this portion of the α-subunit has been shown to have varying effects on hormone activity (20, 21). Therefore, when we began these studies we were concerned that the presence of a cross-link between the αCT and the hCG β-subunit might destroy heterodimer activity by a mechanism that was unrelated to the ability of this portion of the hormone to contact the LHR. Remarkably, we found that the αCT could be attached directly to several residues in the small seatbelt loop of hCG without disrupting its ability to stimulate signal transduction. Cross-linked acid-stable heterodimers containing a cysteine in place of αSer-92 and a cysteine in place of βLeu-92 (*i.e.* hCG-α92/β92), βArg-94 (*i.e.* hCG-α92/β94), βArg-95 (*i.e.* hCG-α92/β95), or βSer-96 (*i.e.* hCG-α92/β96) stimulated cyclic AMP accumulation to the same maximum extent as hCG with at least 15–20% the potency of highly purified hCG (Fig. 2). We did not try to produce heterodimers containing a comparable cross-link to hCGβ-T97C, hCGβ-T98C, or hCGβ-D99C because of the fact that the distances between these residues might cause the cross-link to distort the heterodimer. Indeed, the addition of a three-residue linker at the end of the α-subunit (*i.e.* Gly-Gly-Cys) overcame the loss in activity caused by cross-linking the end of the α-subunit to hCG β-subunit residue 96 as can be seen by comparing the activities of hCG, hCG-α92/β96, and hCG-αGGC/β96 (Fig. 3). This finding supported the notion that the reduced activity of hCG-α92/β96 relative to hCG was because of the ability of the cross-link to alter the conformation of the heterodimer.

To probe the distance between the receptor and region of the heterodimer that contains the αCT and the small seatbelt loop, we used a longer cross-linker and created a "knob" at the site of the cross-link. In these studies we used an analog of the α-subunit (αCT100) created by fusing hCG β-subunit residues 111–116 and 136–145 to the end of the α-subunit and converting Ser-138 to cysteine (Fig. 1). We chose this sequence based on its utility for probing the roles of residues in α-subunit loop 2 (26). Cross-linking this larger probe to loop residues 95 and 99 eliminated signal transduction activity (Fig. 4, overlapping lines "β95C" and "β99C"). We did not study the ability of the knob to inhibit binding when it was attached to residue 99.

TABLE I

Acid stability of analogs containing a cross-link between the αCT and β-subunit residues 92, 94, and 96

Constructs encoding α92C and β92C, β94C, or β96C were co-transfected into COS-7 cells in triplicate 10-cm plates. Three days later the media were analyzed for the presence of heterodimer in a sandwich immunoassay using α-subunit monoclonal antibody A113 for capture and radioiodinated β-subunit antibody B110 for detection. Values shown in the second column represent the concentration of material measured relative to an hCG standard before acid treatment. Values shown in the third column represent the fraction that remained after treatment at pH 2, 37 ° C, for 30 min. These conditions dissociate all heterodimers that do not contain an intersubunit cross-link.

Analog	Total heterodimer	Acid-stable heterodimer
	ng/50 μl ± S.E.	*% total ± S.E.*
hCG-α92/β92	5.3 ± 0.3	97.2 ± 3.9
hCG-α92/β94	13.0 ± 0.4	98.3 ± 2.9
hCG-α92/β96	11.2 ± 0.5	88.7 ± 2.7

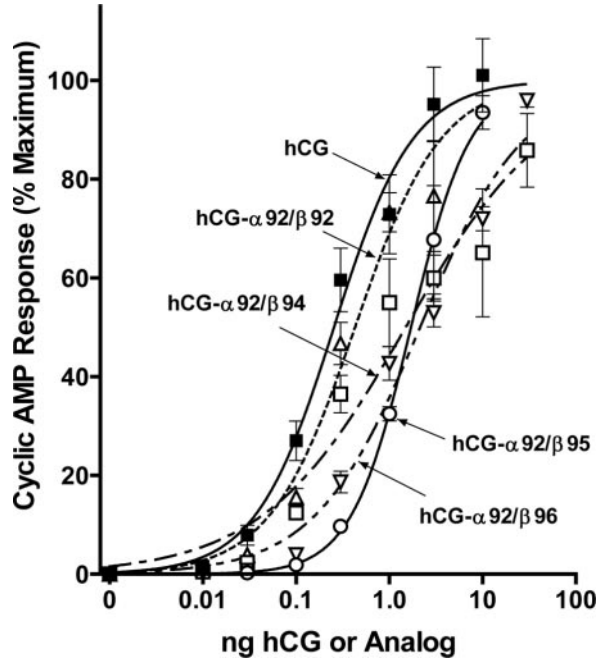

FIG. 2. **Signal transduction activities of heterodimers containing α92C to the small seatbelt loop.** Heterodimers secreted into the medium by cultured COS-7 cells that had been co-transfected with constructs encoding α92C and the indicated β-subunit analogs were concentrated, treated at pH 2, 37 °C, for 30 min to remove any non-cross-linked material and tested for their abilities to stimulate cyclic AMP accumulation in Chinese hamster ovary cells that express the rat LHR in an assay volume of 60 μl. Values were normalized to 100% of the response for hCG to facilitate comparisons of all signaling data and are means of triplicates. *Vertical bars* extend to the limits of the S.E. The maximum response was always 50–100-fold over the blank.

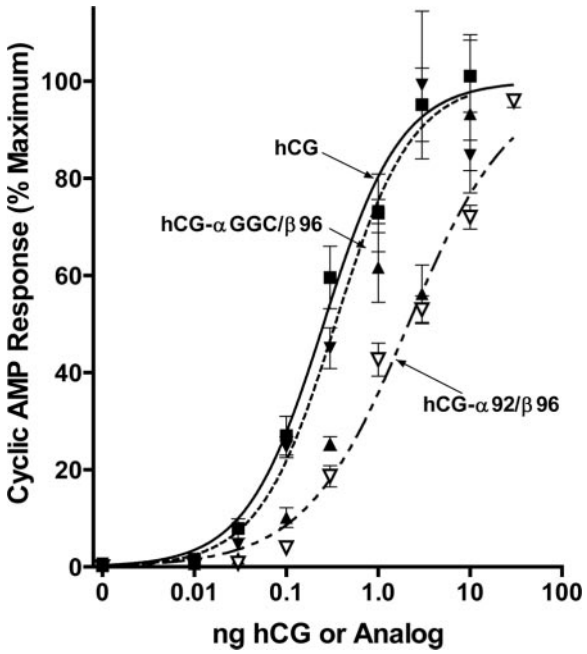

FIG. 3. **Influence of linker length on activities of heterodimers containing α92C cross-linked to β96.** Acid-stable cross-linked heterodimers containing α92C/β96C and αGGC/β96C were tested for their abilities to elicit cyclic AMP accumulation in rat LHR expressing Chinese hamster ovary cells. See the legend to Fig. 2 for other details.

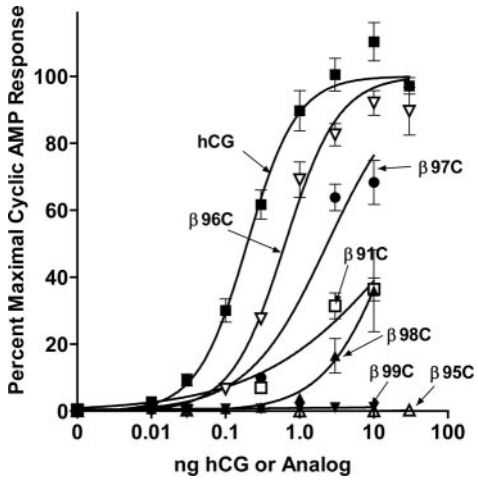

FIG. 4. **Influence of a cross-link between the αCT100 and the cysteine added to the small seatbelt loop.** Acid-stable cross-linked heterodimers containing αCT100 and the indicated β-subunit analog were tested for their abilities to elicit cyclic AMP accumulation in rat LHR expressing Chinese hamster ovary cells. See the legend to Fig. 2 for other details. Note, all the constructs contain αCT100 and the indicated β-subunit analog. Only a part of the label is included on the figure to enhance its clarity.

However, the presence of a knob at residue 95 appeared to abolish interactions with the LH receptor (Fig. 5), which would explain the inactivity of this analog in cyclic AMP accumulation assays (Fig. 4). The small reduction in signal transduction activity caused by the presence of the knob at residues 96 and 97 (Fig. 4) was not associated with a loss in receptor binding (Fig. 5). This suggested that the presence of knobs at these sites may have altered the conformation of the heterodimer slightly, making it less capable of eliciting signal transduction. The reduction in signal transduction activity because of the presence of the knob at β-subunit residue 98 (Fig. 4) appeared due largely, but not completely because of its ability to disrupt binding (Fig. 5). Thus, the presence of the knob at this site

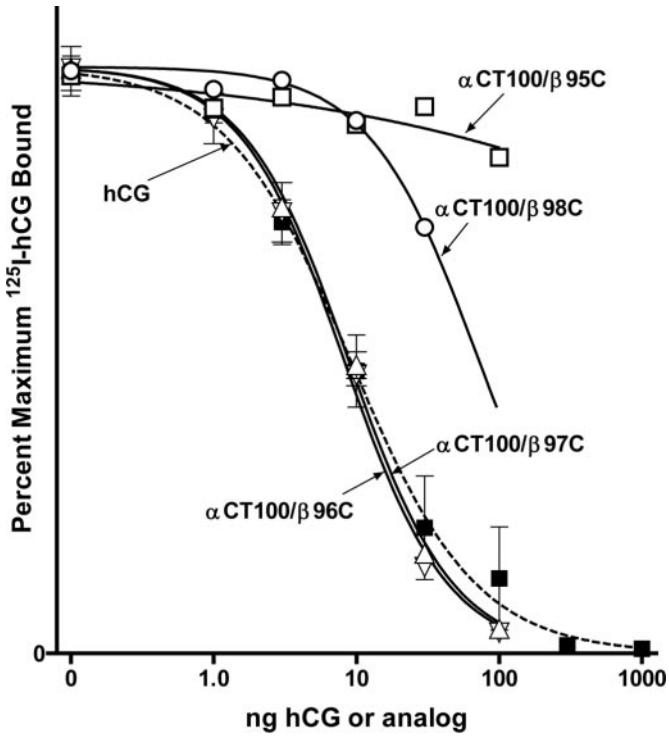

FIG. 5. **Binding of heterodimers containing αCT100 cross-linked to the small seatbelt loop.** The indicated acid-stable heterodimers were incubated with Chinese hamster ovary cells that express the rat LHR and with ^{125}I-hCG for 60 min, 37 °C, in an assay volume of 100 μl. Following the incubation, the bound and free radiolabel was separated by centrifugation and radioactivity in the cell pellets was analyzed in a γ-counter. Values are means of triplicate incubations and the *vertical lines* extend to the limits of the S.E.

appeared to reduce signaling roughly 10-fold more than binding.

DISCUSSION

These observations restrict models as to how the αCT and the small seatbelt loop participate in ligand receptor interactions. Of the eight residues in the small seatbelt loops of mammalian hormones with lutropin activity, the second, third, and fourth, *i.e.* those that correspond to hCG β-subunit residues 94, 95, and 96, vary the most in composition (19). The first and eighth residues are always cysteine, the fifth and sixth residues are usually serine or threonine, and the seventh residue is always aspartic acid. Of the three variable residues that corresponding to hCG βArg-95 appears to have the greatest influence on LHR interactions, we have found that the leucine at this position in bovine LH accounts for much of its inability to interact with the human LHR (22). Furthermore, replacing βArg-95 with a negatively charged residue has the largest impact on the lutropin activity of bifunctional chimera analogs (12). The 5–7-fold reduction in activity caused by cross-linking the αCT to residue 95 shows that this residue may be near a key contact site even though it appears not to be essential for hormone-receptor interaction. This conclusion is strengthened by the observation that a knob at this site destroyed the ability of the hormone to bind and activate the LHR (Figs. 4 and 5). The other variable residues in the small seatbelt loop do not appear to make key contacts with the receptor. Thus, we were able to cross-link the αCT to either residue 94 or 96 without disrupting the activity of the heterodimer (Figs. 2 and 3). Whereas we did not test the influence of a knob at residue 94, addition of a knob at residue 96 barely reduced heterodimer activity in signaling assays (Fig. 4) and did not reduce its activity in binding assays (Fig. 5).

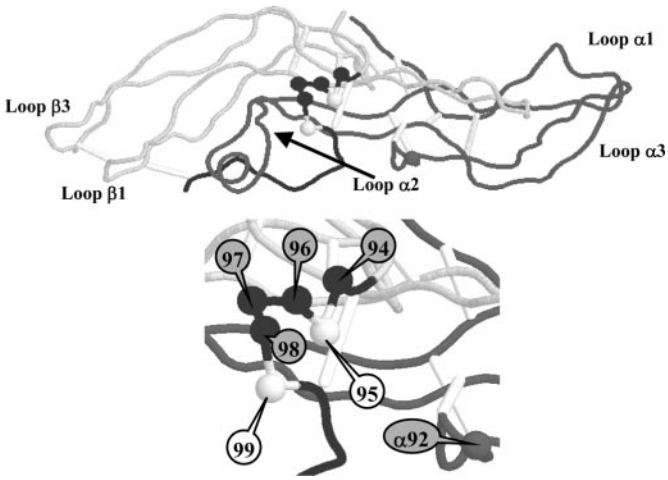

FIG. 6. **Diagram illustrating the relative positions of residues in the αCT and the small β-subunit seatbelt loop.** The *upper portion* of this figure was prepared from the crystal structure of hCG and illustrates its α- and β-subunit backbones in *dark* and *light gray*, respectively. The Cα carbon atoms of residues in the small seatbelt loop are shown as *spheres* that are colored to indicate their apparent proximity to the receptor. Cross-links that involve the residues having the *dark gray* Cα atoms had the least influence on hormone activity. Those that involve residues having *white* Cα atoms had the greatest influence on activity. The figure shown *below* illustrates the numbers of each residue. The callouts are positioned to illustrate the approximate position of the side chain. Note that the side chains of βArg-95 and βAsp-99 project away from the twisted plane of the heterodimer in the same direction. With the possible exception of the side chain of residue 98, residues 95 and 99 appear to be the only ones that are likely to make significant contacts with the receptor. The adjacent surface of loop α2 denoted by the *arrow* is the surface of this portion of the protein that also appears to be nearest the LHR interface (26).

The presence of a knob at residue 99 blocked hormone activity. Although this is one of the most conserved residues in all glycoprotein hormones, it can be replaced by some amino acids without destroying hormone activity. Indeed, in our hands replacing it with cysteine (23) or lysine (not shown) reduced its activity in assays employing Chinese hamster ovary cells engineered to overexpress the rat LHR by ~10-fold. An arginine substitution has been reported to be much more inhibitory in assays employing a murine Leydig cell line (24), but we have not tested this possibility.

The small seatbelt loop of hCG is stabilized by a disulfide between cysteine residues 93 and 100 and has the appearance of a partially twisted ring (Fig. 6). The side chains of residues βArg-94, βThr-97, and βThr-98 are on a surface of the ring that faces α-subunit loop 2 and β-subunit loop 1. The side chains of residues βArg-95 and βAsp-99 are on the opposite surface of the ring, which faces away from the heterodimer interface. The side chain of residue βSer-96 projects from the plane of the ring as do the side chains of βCys-93 and βCys-100 that form the disulfide that closes the ring. The abilities of knobs to block hormone activity when connected to residues 95 and 99 suggest that the surface of this loop that faces away from the subunit interface may be nearest the receptor interface (Fig. 6). The influence of other residues in this portion of the β-subunit on receptor binding activity is more likely to involve a change in hormone conformation, possibly by altering the position of the αCT, a portion of the hormone that is known to affect hCG-LHR interactions.

Considered together, the findings that the αCT can be cross-linked to most of the small seatbelt loop limits the types of contacts that this portion of the hCG is likely to make with the LHR. We anticipate that the surface of this planar loop that faces away from the heterodimer interface is the only portion of the loop likely to contact the LHR (Fig. 6). This argues against models in which this loop has a more important role in receptor contacts, such as those in which it contacts the central region of the concave surface of the leucine-rich repeat domain of the glycoprotein hormone receptors (25). We anticipate that the reduction in signal transduction activity caused by the presence of the knob on residues other than 95 and 99 in the small seatbelt loop is indirect, possibly because it alters the conformation of the heterodimer. Studies described in detail elsewhere (27) suggest that glycoprotein hormones need to contact two portions of the extracellular domains of their receptors to elicit signal transduction, a phenomenon that appears to be extremely sensitive to heterodimer conformation.

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