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Model of Glycoprotein Hormone Receptor Ligand Binding and Signaling*

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Studies described here were initiated to develop a model of glycoprotein hormone receptor structure and function. We found that the region that links the lutropin receptor leucine-rich repeat domain (LRD) to its transmembrane domain (TMD) has substantial roles in ligand binding and signaling, hence we term it the signaling specificity domain (SSD). Theoretical considerations indicated the short SSDs in marmoset lutropin and salmon follitropin receptors have KH domain folds. We assembled models of lutropin, follitropin, and thyrotropin receptors by aligning models of their LRD, TMD, and shortened SSD in a manner that explains how substitutions in follitropin and thyrotropin receptors distant from their apparent ligand binding sites enable them to recognize lutropins. In these models, the SSD is parallel to the concave surface of the LRD and makes extensive contacts with TMD outer loops 1 and 2. The LRD appears to contact TMD outer loop 3 and a few residues in helices 1, 5, 6, and 7. We propose that signaling results from contacts of the ligands with the SSD and LRD that alter the LRD, which then moves TMD helices 6 and 7. The positions of the LRD and SSD support the notion that the receptor can be activated by hormones that dock with these domains in either of two different orientations. This would account for the abilities of some ligands and ligand chimeras to bind multiple receptors and for some receptors to bind multiple ligands. This property of the receptor may have contributed significantly to ligand-receptor co-evolution.

Several models have been devised to account for the interactions of hCG[1] and other glycoprotein hormones with their receptors, membrane proteins that contain a ligand binding NH_2-terminal extracellular domain, a TMD consisting of seven-membrane spanning helices, and a cytosolic COOH-terminal domain (1, 2). The NH_2-terminal three-fourths of the LHR extracellular domain, which we term the LRD, contains several leucine-rich repeats that are likely to give it a curved shape similar in structure to other leucine-rich repeat proteins such as the SCF ubiquitin ligases (3). As shown here, the remaining quarter of the extracellular domain also makes important contributions to ligand binding and signaling and for this reason we refer to it as the SSD or signaling specificity domain (SSD). The amino acid sequence of the SSD is not similar to any known protein and its structure has not been modeled. The TMD appears to be similar in conformation to bovine rhodopsin (4), but it was not known how it is coupled to the LRD and TMD.

The manner in which ligands interact with these receptors has been controversial. One view suggests the α-subunit COOH terminus and the small seatbelt loop contact the concave surface of the LRD such that the ends of loops $\alpha1/\alpha3$ and $\beta1/\beta3$ are exposed (5). This model does not provide an obvious means by which ligand binding results in signal transduction. Portions of the hormone (6), including α-subunit loops 1 and/or 3 (7), have also been suggested to contact the TMD. This view implies that the extracellular domain snares the ligand and delivers it to the transmembrane domain. We had proposed that interactions of the ligand with the LRD and SSD are needed for signaling (8, 9), even though the LRD by itself is sufficient for hCG binding to the LHR (10). A key postulate of our original model, namely that the groove between hormone loops $\alpha2$ and $\beta1/\beta3$ contacts the rim of the LRD to form a high affinity binding site, is no longer tenable. We have found that portions of loop $\alpha2$ facing this groove are unlikely to participate in high affinity rat LHR contacts, even though they appear to be near the hormone-receptor interface (75). Observations described here suggest that β-subunit loops 1 and 3 of lutropins contact the SSD domain rather than the LRD.

The SSD is the least understood region of the receptor extracellular domain. This portion of the human LHR is largely responsible for its ability to distinguish hCG and bovine LH (9), indicating that it contacts the ligand. The SSD may have a role in lutropin signaling as shown by the finding that some mutations increase the basal activity of the LHR (11). To increase the probability that we could deduce a structure for the SSD, we limited our studies to the smallest natural SSDs known, namely those of the marmoset LHR (12) and the salmon FSHR (13). The SSD of the marmoset receptor lacks residues derived from exon 10, which may be responsible for its unusual ability to distinguish hCG and LH (14). Human LHRs that lack exon 10 also respond better to hCG than to LH (15). Because the SSD of the human LHR can limit its ability to bind hormones such as bLH (9), we concluded that the primate LHR might have a conformation that constrains ligand binding in a unique manner. Reasoning that a marmoset analog of the rat LHR, which interacts with lutropins from many species, would be a

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[1] The abbreviations used are: hCG, human choriogonadotropin; hLH, human lutropin; bLH, bovine lutropin; hFSH, human follitropin; hTSH, human thyrotropin; LHR, LH receptor; FSHR, follicle-stimulating hormone receptor; TSHR, thyroid-stimulating hormone receptor; LRD, leucine-rich repeat domain; SSD, signaling specificity domain; TMD, transmembrane domain; IBMX, isobutylmethylxanthine; CHO, Chinese hamster ovary.

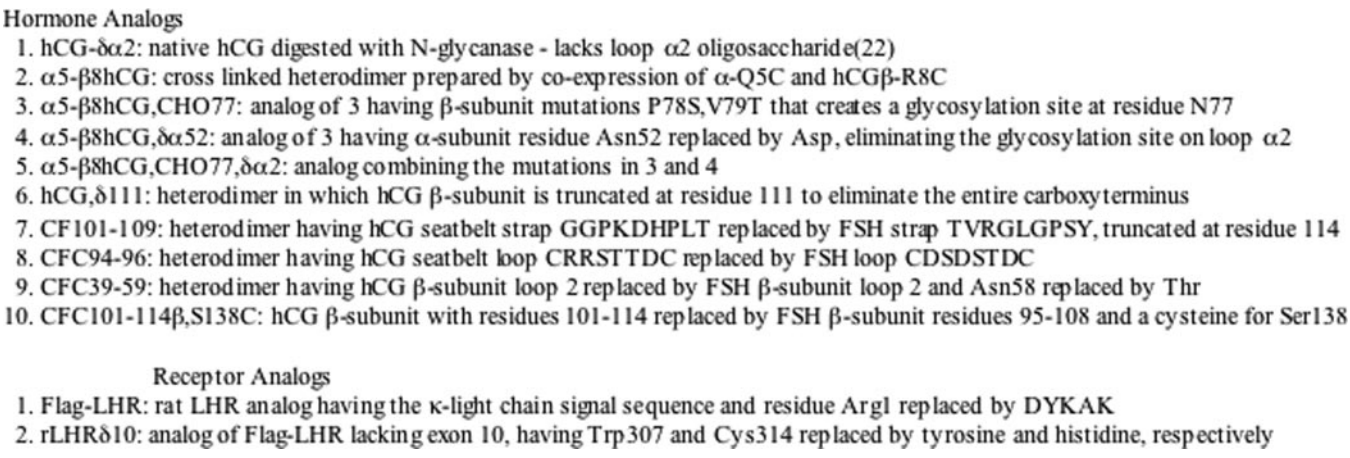


FIG. 1. **Description and nomenclature of analogs used in these studies.** The analogs were prepared as noted in the figure and their concentrations determined by sandwich immunoassays (51) employing an hCG standard and antibodies A113 and ^{125}I-B110. The receptor analogs were also prepared as noted in the figure.

more useful tool to study the role of the SSD in hormone function, we characterized a rat LHR analog that lacks residues encoded by exon 10 and that has a histidine in place of Cys-314. It also contains a tyrosine in place of Trp-307, a change that appeared to increase receptor expression, and an NH_2-terminal FLAG epitope tag. As shown here, these changes altered the ability of the rat LHR to respond to bLH, hCG partial agonists, and other hCG analogs in ways that provided new insights into receptor function. The small size of these SSD enabled us to develop models of the glycoprotein hormone receptors that are consistent with most data on ligand binding and signaling, including the finding that only two mutations of the FSHR are needed to enable it to interact with hFSH and hCG (16). Models described here will also explain many features of the TSHR, including its subunit nature (17), high basal activity (18, 19), and ability to be stimulated by anti-receptor antibodies (20).

MATERIALS AND METHODS

The sources of hCG and antibodies used in these studies have been described (8, 21). hLH was obtained from Dr. Robert Campbell (Serono Reproductive Biology Institute, Rockland, MA). hCG-αδ2, an analog of hCG lacking the α-subunit loop 2 oligosaccharide was prepared by *N*-glycanase digestion of hCG after the subunits had been dissociated and permitted to recombine (22). Other constructs encoding hormone analogs (Fig. 1) were produced by PCR. These included a disulfide cross-linked analog of hCG (α5-β8hCG) in which α-subunit residue Gln^5 and β-subunit residue Arg^8 were converted to cysteines, an analog of α5-β8hCG lacking the α-subunit glycosylation signal at loop 2 residue 52 (α5-β8hCG-αN52D), an analog of α5-β8hCG having a glycosylation signal at β-subunit loop 3 residue 77 made by converting βPro^{78} and βVal^{79} to serine and threonine (α5-β8hCG-βCHO77), and an analog of α5-β8hCG-αN52D having the β-subunit loop 3 glycosylation signal (α5-β8hCG-αN52DβCHO77). PCR mutagenesis was also used to construct rLHRδ10, an analog of the native rat LHR in which residue Glu^{267} was joined to Tyr^{295}, residue Trp^{307} was converted to tyrosine, and residue Cys^{314} was converted to histidine. (Note, the numbering system used in these studies reflects the absence of the presumed signal peptide, which we assumed to be 26, 22, 17, and 21 residues for the rat LHR, human LHR, rat FSHR, and rat TSHR, respectively.) The hormone and receptor constructs were subcloned into the polylinker of pCI (Promega, Madison, WI), a mammalian expression vector that had been modified as described (23) and expressed transiently in COS-7 cells or stably in Chinese hamster ovary cells. Radioiodinated hCG and monoclonal antibodies were prepared using IODO-GEN (Pierce) as described (24). Ligand binding was monitored by quantifying the abilities of hCG and bLH to compete with ^{125}I-hCG for binding to the LHR and LHRδ10 on intact cells in physiological buffers (9). The total volume of the assay was 100 μl. Cyclic AMP accumulation was monitored by radioimmunoassay using a rabbit cyclic AMP antibody (Strategic Biosolutions, Ramona, CA) and 2′-*O*-monosuccinyladenosine 3′5′-cyclic monophosphate tyrosine methylester (Sigma) that was radioiodinated as described (25). The total volume of this assay was 60 μl. Statistical analyses were performed using Prism (GraphPad Software, San Diego CA). Protein threading was performed with the molecular modeling packages Look (26) and Sybyl (Tripos, St. Louis, MO). Depictions in Figs. 7 and 8 were prepared using Molscript (27). Those in Fig. 12, *center* and *right*, were prepared using VMD (28) and Raster3D (29) following molecular dynamics using the Amber force field (30). Assumptions used during modeling are described under "Results."

RESULTS

Influence of the SSD on Ligand Binding—rLHRδ10 was derived from an analog of the rat LHR that contains a modified FLAG tag that does not require calcium for binding of the M1 anti-FLAG tag antibody. The presence of the epitope tag did not affect the ability of the rat LHR to bind hCG or bovine LH or to make cyclic AMP in response to hCG-δα2 (Figs. 2*A* and 3*A*). In most studies, the concentrations of hCG required to prevent binding of ^{125}I-hCG to CHO cells that expressed rLHRδ10 were similar to those that inhibited binding of ^{125}I-hCG to CHO cells that express the rat LHR (Fig. 2*B*). This suggested that both receptors had roughly equivalent affinities for hCG. Smaller amounts of hCG-δα2 were required to inhibit the binding of ^{125}I-hCG to CHO to cells expressing rLHRδ10, however (Fig. 2*B*), indicating that it appeared to have a slightly greater ability to bind an hCG analog lacking the oligosaccharide on α-subunit loop 2. In contrast, bLH competed poorly with ^{125}I-hCG for binding to rLHRδ10 (Fig. 2*B*). Thus, whereas 200–300 ng of bLH were required to inhibit the binding of ^{125}I-hCG to the rat LHR by 50% in this assay, more than 10 μg of bLH was required to halve the binding of ^{125}I-hCG to rLHRδ10 (Fig. 2). This showed that the ability of bLH to recognize hLHRδ10 was at least 30–50-fold lower than its ability to recognize the rat LHR. In this regard, rLHRδ10 behaved more like the human LHR than the rat LHR (9). These differences in the abilities of bLH to bind the rat LHR and rLHRδ10 showed that residues missing or replaced in the SSD of rLHRδ10 have roles in the binding of some lutropins. These finding are consistent with reports that regions of the rat LHR outside the LRD can influence the LHR binding of ovine LH, a lutropin similar to bLH (31).

The seatbelt is responsible for much of the influence of the β-subunit on receptor binding specificity (32–35). To learn if the seatbelt can affect interactions of the glycoprotein hormones with the SSD, we tested the abilities of rLHRδ10 to recognize hCG/hFSH chimeras. One of these has its small seatbelt loop derived from the hFSH β-subunit and is known to bind LHR roughly 8–12% as well as hCG (32). This hormone

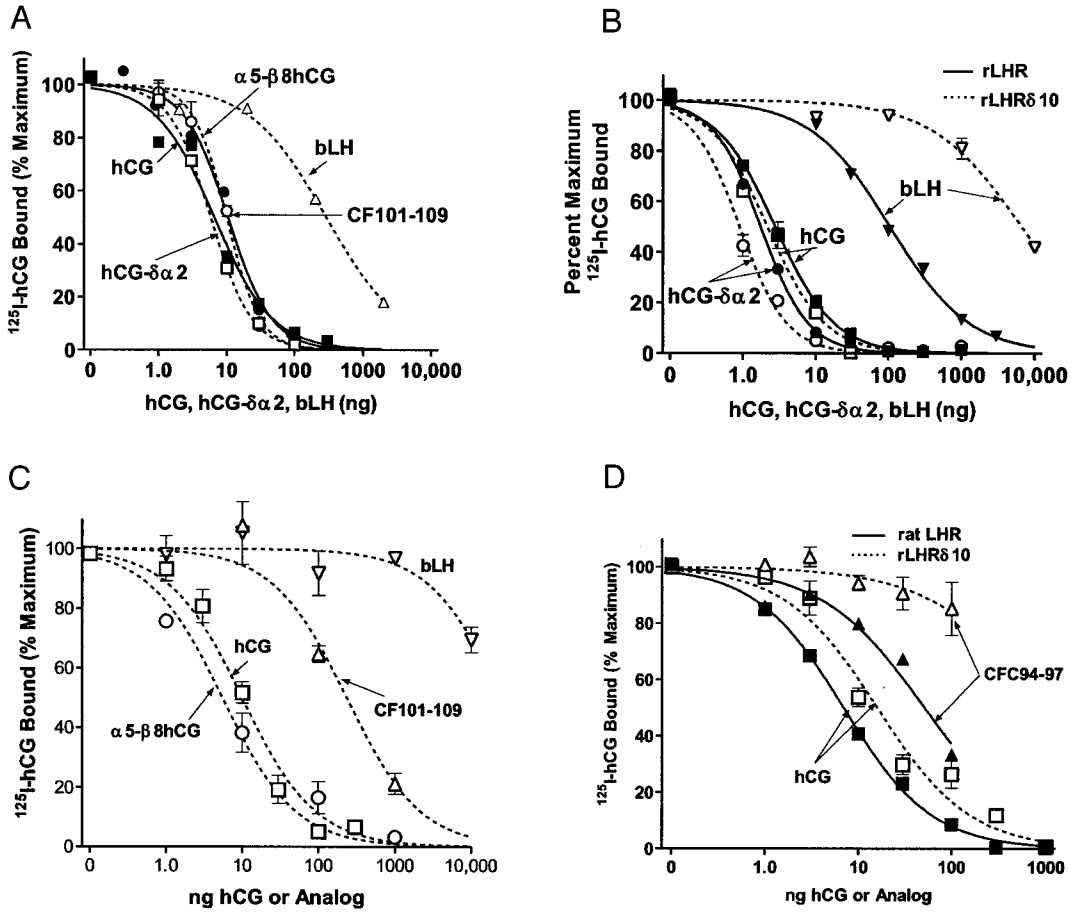


FIG. 2. **Abilities of hCG, hCG analogs, hLH, and bLH to compete with ^{125}I-hCG for binding to the rat LHR, FLAG-tagged rat LHR, and rLHRδ10.** *Panel A*, binding to FLAG-tagged rat LHR analog. This panel shows that hCG, α5-β8hCG, hCG-δα2, and CF101–109 have similar abilities to bind to the FLAG-tagged rLHR. In contrast, the ability of bLH to bind to this receptor is roughly 20–30-fold lower. These properties are similar to those observed with the native rat LHR (9, 32). *Panel B*, binding to the rat LHR and to rLHRδ10. This panel shows that hCG and hCG-δα2 have similar abilities to bind the rat LHR and rLHRδ10. The mutations introduced into the SSD greatly reduced the ability of the rat LHR to recognize bLH. *Panel C*, abilities of hCG α5-β8hCG, CF101–109, and bLH to bind to rLHRδ10. This panel shows that addition of an NH_2-terminal cross-link to hCG did not affect its ability to bind rLHRδ10. The presence of FSH residues in the strap region of the seatbelt reduced binding to the rLHR ~10-fold (compare with *panel A*). *Panel D*, interactions of hCG and an analog containing negatively charged FSH β-subunit residues in the small seatbelt loop with the rat LHR and rLHRδ10. The presence of negatively charged residues in the small seatbelt loop has been shown to reduce the affinity of hCG for the rat LHR by about 10-fold as also seen in this study. These residues reduced the ability of hCG to bind to rLHRδ10 by a greater extent. All data have been normalized to facilitate comparisons of the analogs and their abilities to bind to these receptors. Similar numbers of cells (200,000) were used in each assay except that shown in *panel A* (300,000). Values are means of triplicates ± S.E. The assay volume was 100 μl.

chimera inhibited ^{125}I-hCG binding to the rat LHR ~10% as well as hCG, but had a much lower ability to inhibit the binding of ^{125}I-hCG to the rLHRδ10 (Fig. 2*D*). This showed that the SSD domain may compensate for mutations in the small seatbelt loop that are known to reduce the ability of hCG to interact with the rat LHR. Substitution of hFSH residues for those in the hCG strap, *i.e.* the COOH-terminal half of the seatbelt, creates bifunctional chimeras that interact with the rat LHR like hCG and that bind the rat FSHR roughly one-third as well as hFSH (33). Bifunctional chimera CF101–109 bound to the FLAG-tagged rat LHR receptor similar to hCG (Fig. 2*A*), but poorly to rLHRδ10 (Fig. 2*C*). Although this finding is consistent with the notion that hCG residues in the strap interact with parts of the SSD that are altered or missing in the rLHRδ10, this seems unlikely because many hCG analogs in which the seatbelt is latched to different parts of the α-subunit (36) recognized the rat LHR much better than CF101–109 bound to rLHRδ10 (Fig. 2*C*). The hFSH residues in the straps of these chimeras alter the conformation of the heterodimer (37), indicating that the reduced abilities of CF101–109 and CFC101–114 to bind rLHRδ10 might reflect their altered conformations. hFSH has less than 0.01% the activity of hCG in rat LHR binding assays; hCG/hFSH chimeras in which both the loop and the strap regions are derived from hCG have less than 1% of the activity of hCG in rat LHR assays (32). Both hFSH and these chimeras were inactive in assays employing the rLHRδ10 receptor (not shown).

Influence of the SSD on Efficacy—To learn if the SSD contributes to hormone efficacy, we measured cyclic AMP accumulation in response to hCG and hCG-δα2, a partial agonist analog that lacks the oligosaccharide at residue Asn^{52} in loop α2. As noted earlier, the rLHRδ10 receptor has an NH_2-terminal FLAG epitope tag. To learn if this would influence its ability to respond to hCG and hCG-δα2, we tested the abilities of these analogs to stimulate cyclic AMP accumulation in assays employing the FLAG-LHR. The presence of the epitope tag was not responsible for the reduced ability of the rLHRδ10 bearing cells to respond to hCG-δα2 (Fig. 3*A*). In assays employing cells that express the FLAG-LHR, the maximum amount of cyclic AMP accumulation observed in the presence of IBMX in response to hCG-δα2 was 70% that observed in response to hCG (Fig. 3*A*), a value that is equivalent to or greater

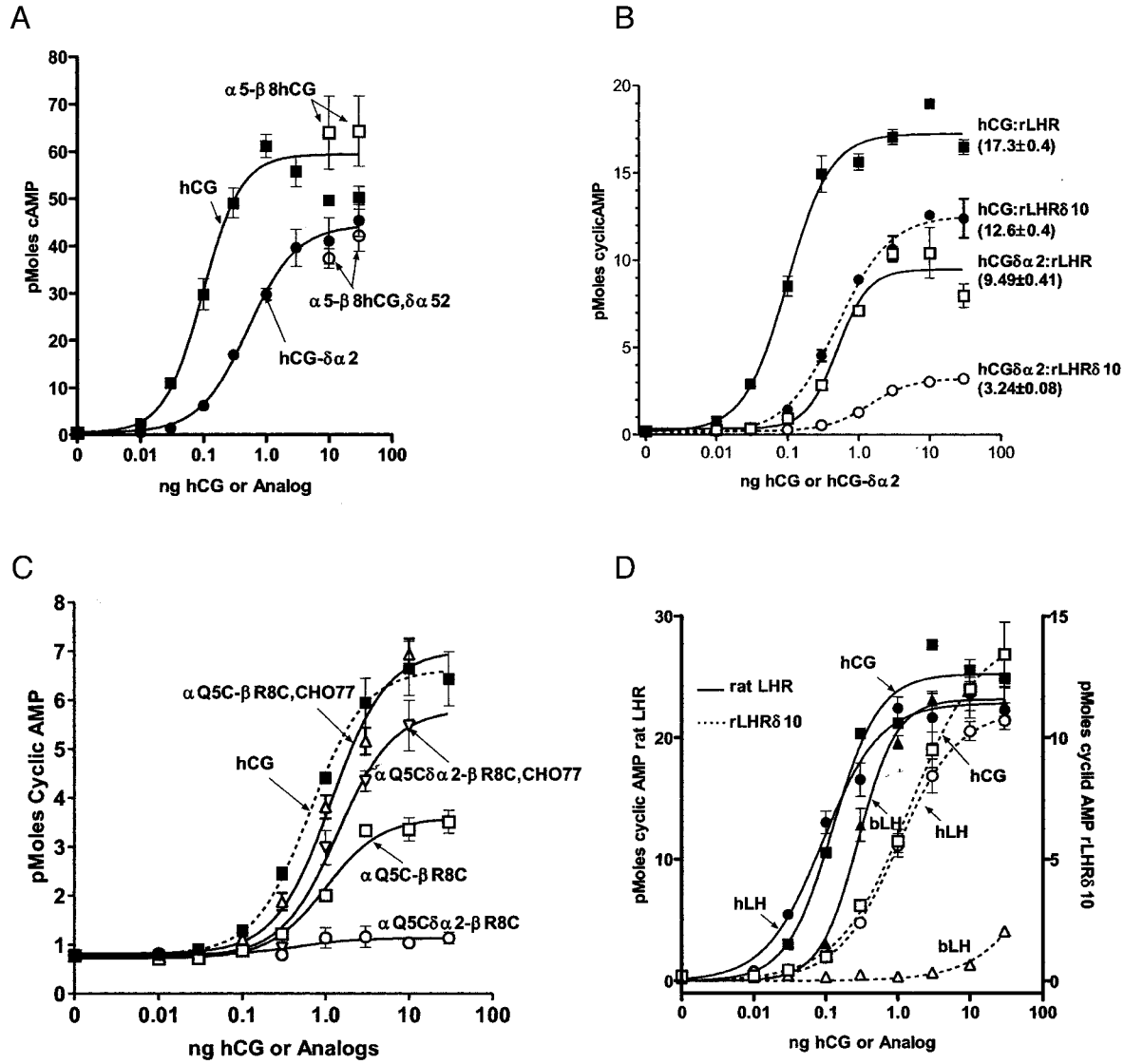


FIG. 3. **Influence of the SSD on signal transduction.** *Panel A*, hCG and an analog prepared by deglycosylation of the same highly purified hCG preparation was used to stimulate the FLAG-tagged rat LHR in the presence of 2 mM IBMX, an inhibitor of phosphodiesterase. Values illustrate the total amounts of cyclic AMP produced (*i.e.* that in the medium plus that in the cells, means of triplicate incubations). *Solid squares* and *circles* represent the responses to hCG and hCG-δα2. *Open squares* and *circles* represent the responses to 10 and 30 ng of α5-β8hCG and α5-β8hCG-N52D, analogs containing an NH_2-terminal cross-link. The efficacy of α5-β8hCG was not different from that of hCG. hCG-δα2 had ~70% the efficacy of hCG in this assay (*solid circles*) as did the NH_2-terminal cross-linked analog lacking the oligosaccharide on α-subunit loop 2 (*open circles*). *Panel B*, efficacy of hCG and hCG-δα2 in cells expressing the rat LHR and rLHRδ10. Cells expressing the rat LHR and rLHRδ10 were exposed to increasing amounts of hCG and hCG-δα2 in the presence of IBMX. hCG-δα2 had ~55% the efficacy of hCG in cells expressing the rat LHR (*solid lines*). It had only 25% the efficacy of hCG in cells expressing the rLHRδ10 receptor (*broken lines*). *Panel C*, influence of an oligosaccharide added to β-subunit residue 77 on signal transduction through the rLHRδ10 receptor. The indicated hormone analogs were produced by transient expression of COS-7 cells. The media were concentrated by ultrafiltration and the concentrations of analogs were determined by sandwich immunoassays. As shown here, the presence of the intersubunit disulfide cross-link reduced the efficacy of hCG in assays that involve the rLHRδ10 receptor. Efficacy was diminished further by elimination of the α-subunit loop 2 oligosaccharide. Addition of an oligosaccharide near the tip of β-subunit loop 3 at residue 77 led to an increase in efficacy in both the cross-linked and cross-linked deglycosylated analogs. *Panel D*, signal transduction responses to hCG, hLH, and bLH in rat LHR and rLHRδ10 receptor expressing CHO cells. Cells expressing the rat LHR and the rLHRδ10 receptor were treated with hCG, hLH, and bLH. Note that the response to hLH was equivalent to that of hCG in both cell types. bLH had a significantly lower potency than either hCG or hLH in both cell types. These assays were performed in a volume of 60 μl.

than that observed in assays employing the rat LHR (Fig. 3*B*). Furthermore, the FLAG epitope did not affect the maximal responses to α5-β8hCG, an analog that has an NH_2-terminal cross-link, or to α5-β8hCG,δα52, a cross-linked analog that is missing the oligosaccharide on α-subunit loop 2 (Fig. 3*A*).

Cells expressing the rLHRδ10 receptor recognized hCG-δα2 better than they recognized hCG (Fig. 2*B*); their abilities to accumulate cyclic AMP in response to hCG-δα2 were less than half of those of cells that express the rat LHR in media lacking the inhibitor of phosphodiesterase (not shown). This might reflect the fact that the rLHRδ10 bearing CHO cell line used in these studies expressed roughly one-third to one-half the number of receptors per cell as the CHO cell line that expressed the rat LHR. To minimize the influence of receptor expression on the determination of efficacy, we repeated these studies in the presence of IBMX and monitored the total amount of cyclic AMP produced (*i.e.* that in the cells plus that in the medium) to avoid missing cyclic AMP that was released from the cells. hCG-δα2 elicited 55% of the maximal amount of cyclic AMP seen in response to hCG treatment of cells expressing the rat

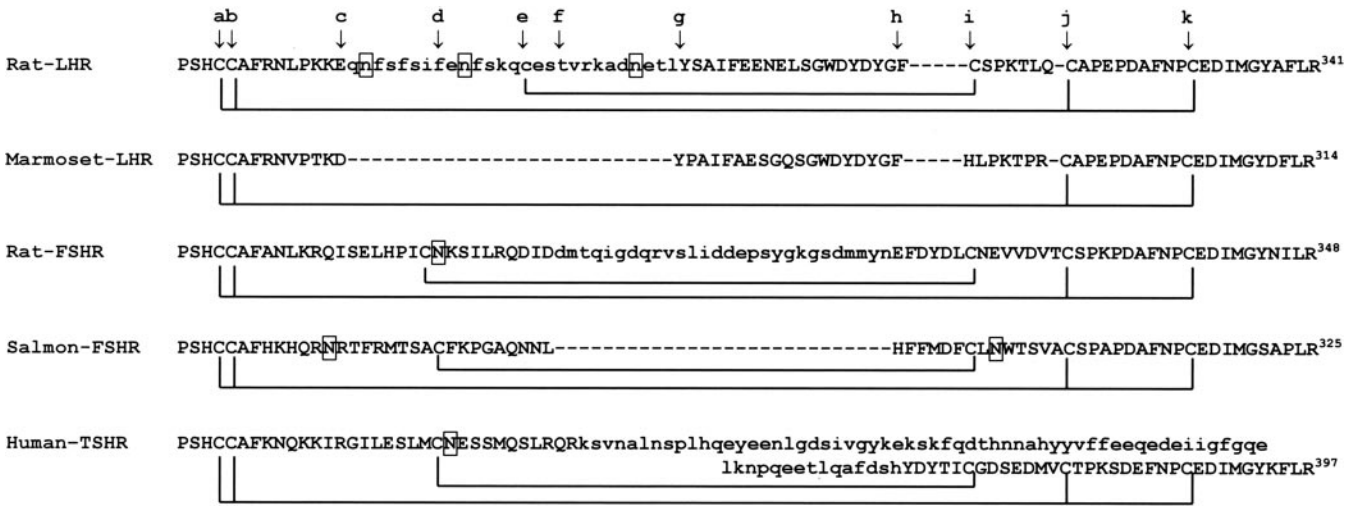


FIG. 4. **Sequences used to build minimal SSD models of the rat LHR, rat FSHR, and human TSHR.** The sequences of the SSD of the rat LHR, FSHR, and TSHR are illustrated adjacent to the smaller SSD of the marmoset LHR and the salmon FSHR. Residues shown as *broken lines* are absent from the receptor. Only those residues denoted by *uppercase letters* are included in the models of the receptor. The manner in which the folding pattern was determined is described in the text. The first cysteine shown corresponds to residues 257 (rat and marmoset LHR), 258 (rat FSHR), 259 (salmon FSHR), and 262 (rat TSHR) in our numbering system, which omits the leader sequences to facilitate alignments. The arginine at the end of these sequences is also numbered. These sequences were used to construct models of the SSD in the rLHR and rFSHR. The human sequence shown was used to construct models of the SSD in the hTSHR. *Solid lines* refer to potential disulfide bonds. Potential glycosylation sites are *boxed*. Except for the glycosylation signals in exon 10, which are not modeled, the side chains of all asparagine residues that might be glycosylated face away from the cell surface.

LHR (Fig. 3*B*), a finding consistent with the well known influence of this oligosaccharide on hormone efficacy (38). This value was significantly greater than the efficacy observed in response to hCG-δα2 in the absence of IBMX, which was typically 40% of that seen in response to hCG (not shown). In contrast, hCG-δα2 stimulated cells expressing the rLHRδ10 receptor with less than half of this efficacy (Fig. 3*B*). This showed that contacts of the hormone with the SSD have significant roles in signal transduction.

Introduction of an intersubunit disulfide bond between residues α5-β8 reduced the efficacy of hCG in assays employing cells that express rLHRδ10 (Fig. 3*C*), even though it had no influence on ligand binding (Fig. 2*C*) or the activity of hCG in cells expressing the FLAG-LHR (Fig. 3*A*). This disulfide cross-link is in a region of hCG that does not contact the receptor and that can be detected by monoclonal antibodies when hCG is bound to the LHR (37). Thus, the reduced ability of the cross-linked hCG analog to elicit a signal through the rLHRδ10 receptor showed that its conformation differs slightly from that of hCG or that it is unable to assume a conformation needed to stimulate rLHRδ10 fully.

As can be seen by comparing the abilities of rLHRδ10 bearing cells to respond to hCG analogs (Fig. 3*C*), the presence of an oligosaccharide at βAsn^{77} reversed the effect of the α5-β8 cross-link. Thus, even in the absence of a phosphodiesterase inhibitor, the amount of cyclic AMP detected following αQ5C-βR8C,CHO77 stimulation was equal to that made by rLHRδ10 in response to hCG (Fig. 3*C*). Removing α-subunit loop 2 oligosaccharide from the cross-linked analog of hCG reduced its efficacy further, which can be seen by comparing the responses to αQ5C-βR8C and αQ5Cδα2-βR8C (Fig. 3*C*). Nearly full efficacy was restored by the presence of an oligosaccharide at residue Asn^{77} in β-subunit loop 3 as can be seen by comparing the responses of rLHRδ10 bearing cells to hCG and to αQ5Cδα2-βR8C,CHO77 (Fig. 3*C*). Based on the similarities in the ED_{50} of these analogs, we anticipate that these hormone mutations did not affect the primary ligand binding site, which we presume to be in the first 200 residues of the LRD (10). In contrast, the increase in efficacy caused by the presence of the oligosaccharide near the tip of β-subunit loop 3, which is well away from the subunit interface, is likely to be mediated via its influence on the SSD. This is consistent with the notion that β-subunit loop 3 is near the SSD.

Human LH was as active as hCG and much more active than bLH when tested for its ability to elicit signal transduction through the rLHRδ10 receptor (Fig. 3*d*). The higher potency of hLH than bLH is probably related to its greater similarity to hCG. Relative to hCG, bLH had a greater potency in signal transduction assays than in receptor binding assays, a phenomenon readily apparent by comparing the bLH binding data in Fig. 2, *A* and *B*, with the signal transduction data in Fig. 3*D*. This observation may be related to differences in the kinetics of receptor binding and signaling. bLH has a lower affinity than hCG for the rat LHR, which suggests that it dissociates more rapidly from the rLHR. This would permit ^{125}I-hCG to displace bLH from the receptor during competition studies used to monitor binding. In contrast, signal transduction assays were not done in a competitive fashion. Therefore, the dissociation rate will have less influence on the potency of bLH in these assays, particularly if events needed to terminate cyclic AMP accumulation, *e.g.* the conversion of the Gsα-GTP complex to Gsα-GDP, is slower than rebinding of bLH to the receptor.

The SSD May Have a KH Domain Folding Pattern—A major goal of these studies was to develop a structural model of the SSD and to integrate this into models of the receptor. Unfortunately, the amino acid sequence of the SSD is not related in an obvious way to that of proteins of known structure. Because the SSDs in the marmoset LHR and salmon FSHR are small, we reasoned that we might find structures that could be used for model building by deducing as many distance constraints as possible from their amino acid sequences (Fig. 4). We presumed that all the cysteines in the SSD are involved in disulfide bonds, a notion supported by the finding that elimination of four of the six cysteines in the SSD of the rat LHR disrupted its expression (39). Cysteines of the rat LHR that could be eliminated without disrupting receptor expression, namely Cys^{282} and Cys^{314}, are missing in the marmoset LHR, suggesting that these form a disulfide. This would make residues Cys^{282} and Cys^{314} (*i.e.* points "e" and "i," Fig. 4) adjacent in the full-length SSD. Because the cysteine found in the FSHR and in the TSHR

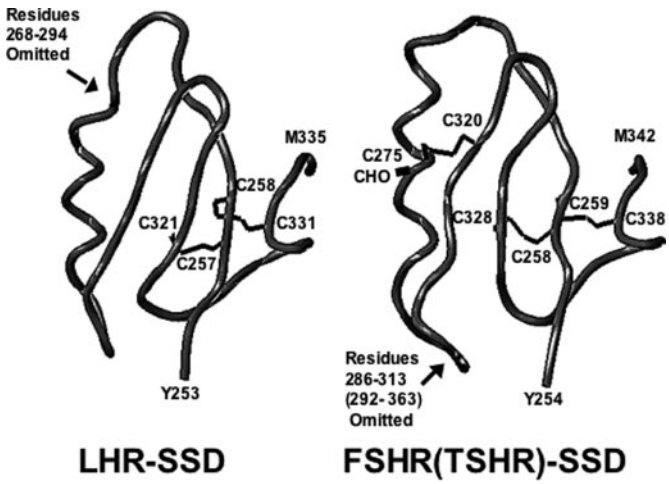


FIG. 5. **Use of the KH domain folding pattern to describe the minimal structure of the SSD domain.** The proximity of residues in minimal forms of the SSD determined as described in the text suggested that this region has a KH domain fold. Models of the minimal SSD of the LHR, FSHR, and TSHR were prepared by threading their sequences through that of Protein Data Bank code 2aw0 to give the diagrams illustrated here. Note that the positions of residues in exon 10 (LHR residues 268–294) that were omitted from models of the LHR are located in a different portion of the SSD than those that were omitted from models of the FSHR (residues 286–313) and TSHR (residues 292–363). The SSD of the minimum TSHR is similar to that of the FSHR and only that for the FSHR is shown. The cysteines of the TSHR are not shown on the diagram for reasons of clarity. These are 262, 263, 280, 363, 377, and 387. They correspond to FSHR cysteines 258, 259, 275, 320, 328, and 338, respectively. The oligosaccharides of the FSHR and TSHR are at residues 276 and 281. Note that the TSHR appears to contain proteolytic cleavages that remove residues in the vicinity of 296–347, but the exact cleavage sites are unknown. These models suggest that an even larger peptide could be removed from the SSD of the TSHR.

at point "d" would be expected to form a disulfide bond with the cysteine corresponding to LHR Cys^{314} (Fig. 4), we assumed that points "d" and i are adjacent in the SSD of these receptors. The proximity of Cys^{257} to Cys^{258} makes it unlikely that these cysteines form a disulfide with one another. Therefore, we reasoned that Cys^{321} and Cys^{331}, the remaining cysteines in the SSD, are most likely to be involved in disulfides with Cys^{257} and Cys^{258}. This indicated that point "a" is likely to be near points "j" or "k" and, because point a is near point "b," that points a, b, j, and k are likely to be near one another (Fig. 4). This location of the disulfides is also consistent with that proposed earlier for the TSHR based on the finding that this receptor contains proteolytic cleavage sites between the third and fourth cysteines in the SSD (20).

We were able to identify another set of adjacent residues by considering sequence differences between the rat and marmoset LHR and between the rat and salmon FSHR. The finding that rLHRδ10 was readily expressed at the cell surface showed that deletion of exon 10 did not disrupt the receptor or its ability to bind hCG. One explanation for this is that exon 10 forms a loop and that point "c" is usually adjacent to point "g" in both the rat and marmoset receptors (Fig. 4). Based on a similar comparison of the rat and salmon FSHR, we anticipated that points "f" and "h" are adjacent in the rat FSHR (Fig. 4).

A manual search of the SCOP data base showed that proteins having the KH fold would satisfy these distance constraints despite their lack of disulfide bonds. The KH fold is found in many proteins including those that bind metals (40) and nucleic acids (41). We considered several structures having this fold and built models of Protein Data Bank codes 1k1g (41), 1mwy (40), and 1vig (42), to name a few. Ultimately, we chose 2aw0 (43) to construct models of the minimal SSD found in the marmoset LHR and the salmon FSHR (not shown). These were then used to make models of minimal SSDs that would be present in the rat LHR, FSHR, and TSHR (Fig. 5, *A* and *B*). None of the proteins that we found in the Protein Data Bank based on searches of the DALI data base or using the VAST function of the NCBI data base had residues that corresponded to exon 10 of the LHR or to residues that are present in the rat FSHR but missing in the salmon FSHR. Therefore, none of our models contain either of these portions of the SSD.

Residues Missing from Models of the TSHR and FSHR do Not Prevent Ligand Binding or Signaling—Portions of the TSHR that are missing from the model structure (Fig. 4) do not appear to contribute to TSH binding or signaling (17, 19, 44). Thus, it seemed likely that predictions of ligand binding and signaling based on the TSHR model should have physiological relevance. To learn if the model used to build the FSHR were sufficient to explain hFSH-FSHR interactions, we built and expressed the protein having the sequence in the model (Fig. 4). We also prepared constructs encoding analogs of the rat FSHR in which the SSD was derived from the rLHR and rLHRδ10. The rat FSHR and all three analogs bound ^{125}I-hFSH and produced cyclic AMP in response to hFSH stimulation (Table I). None of them responded to a concentration of hCG that is 30-fold higher than that required to elicit maximal cyclic AMP production of cells expressing LHR. The presence of the SSD derived from the rLHR and rLHRδ10 hFSH appeared to reduce binding and signaling (Table I), indicating that the SSD of the FSHR contributes to FSH binding. We did not attempt to prove this, however.

Models of the Entire Extracellular Domain—The sequences of the LRD in the glycoprotein hormone receptors are more similar to ubiquitin ligase, *e.g.* Protein Data Bank code 1fqv (3), than ribonuclease inhibitor (45), the structure used to make our earlier model. To model the LRD of the glycoprotein hormone receptors, we aligned the leucine-rich repeat regions of each receptor with that of ubiquitin ligase (Fig. 6) and made homology models of the amino acid sequences of the rat LHR, FSHR, and TSHR using LOOK (26). These were then refined extensively with Sybyl. The COOH-terminal end of the ubiquitin ligase LRD is an α-helix and has a coil that spans its concave surface. The corresponding region of the LRD in the glycoprotein hormone receptors is thought to form a hinge and we assumed that this region of the ubiquitin ligase could be used to model the junction between the LRD and the SSD (Fig. 6*A*). Regions of the LRD that are thought to contact the ligand, SSD, and TMD are outlined on a three-dimensional view of this domain (Fig. 6*C*), which also indicates how the structure of this domain corresponds to the sequence layout in Fig. 6*A*.

To distinguish the surfaces of the LRD and SSD that are furthest from the transmembrane domain, we relied on the fact that both domains are glycosylated (46). The glycosylation signals on the LRD of all vertebrate glycoprotein hormone receptors are located on the same surface, which we assumed is the most distant from the plasma membrane. The glycosylation signals in the SSD of the LHR are located in exon 10. Because all the models of the LHR described here lack exon 10, we were unable to determine the orientation of the shortened SSD in the LHR from its amino acid sequence. In contrast, glycosylation signals are present in portions of the vertebrate FSHR and TSHR corresponding to the shortened SSD in many teleost FSHR that had been modeled on the KH domain (Fig. 5*B*) and we assumed this surface of the SSD is furthest from the transmembrane domain. Similarities in the models of the SSD from the LHR and those in the FSHR and TSHR led us to orient all three SSD in the same fashion (Figs. 5 and 7). Note that the

TABLE I

Binding of ^{125}I-hFSH and signal transduction of rat FSHR containing SSD derived from the rat LHR, the rLHRδ10 receptor, and the minimum sized SSD used to model the rat FSHR

DNA constructs encoding the indicated rat FSHR analog were transfected transiently into COS-7 cells. Two days later the cells were harvested and analyzed for their abilities to bind ^{125}I-hFSH in the presence and absence of 0.5 μg of hFSH in an incubation volume of 100 μl and for their abilities to accumulate cyclic AMP in response to the absence of hormones or 30 ng of either hCG or hFSH in an incubation volume of 60 μl. All values are means of triplicates ± S.E. All values in the column headed "No hFSH" are significantly different from the controls ($p < 0.001$). All values in the column headed "30 ng of hFSH" are significantly different from the controls ($p < 0.001$). No value in the column headed "30 ng of hCG" is significantly different from the control ($p > 0.05$).

Source of SSD	Cpm ^{125}I-FSH bound		Pmol of cyclic AMP		
	No hFSH	0.5 μg of hFSH	No hormone	30 ng of hCG	30 ng of hFSH
Rat FSHR-SSD	3156 ± 44	583 ± 7	3.89 ± 0.22	4.35 ± 0.14	13.12 ± 0.33
Model FSHR-SSD	4224 ± 91	608 ± 20	5.07 ± 0.74	4.52 ± 0.27	20.70 ± 0.40
Rat LHR-SSD	1630 ± 63	679 ± 47	3.87 ± 0.26	4.34 ± 0.57	7.72 ± .026
rLHRδ10-SSD	1236 ± 33	602 ± 70	3.85 ± 0.43	4.58 ± 0.46	8.66 ± .069

SSD of the "minimal" LHR differs from those of the FSHR and TSHR because of the location of the inserted residues. Only one model has been shown for the minimal SSD of the FSHR and TSHR because they appear to contain an insertion in the same region of the protein. The insertion in the TSHR appears to contain two cleavage sites, which causes the LRD and part of the SSD to be separated from the COOH-terminal portion of the SSD and the TMD. These would correspond to the A and B "subunits" of the TSHR, respectively (19).

The location of the NH_2-terminal end of the SSD is constrained by the fact that it is connected to the COOH-terminal end of the LRD. To determine the position of the remainder of the SSD, we relied on published data illustrating substitutions that improve the ability of the TSHR to bind hCG (47). We positioned the LRD and the SSD of the TSHR such that LRD residues Lys^{37} and Tyr^{61} are near the turn in the SSD that appears to include residues Asn^{371}, Glu^{372}, and possibly Asp^{370} (Fig. 7). This brought the corresponding residues in the LRD of the LHR, namely Ser^{33} and Glu^{57} near SSD residues Pro^{316} and Lys^{317}. In our view, substitutions in this region of the TSHR permit hCG binding indirectly by making minor alterations in the relative positions of the LRD and SSD. This contrasts with the more widely held view that the charged surface of the concave surface of the LRD contacts the ligand (5).

Interaction of the LHR with hCG—The relative positions of the SSD and LRD (Figs. 7 and 8) can account for contacts of hCG with both the LRD and SSD even though a large portion of the hormone remains exposed in the hormone-receptor complex (21, 23, 37). This model also suggests that a substantial surface of the hormone contacts the receptor, a notion that would explain the high affinity of most glycoprotein hormone-receptor interactions. This orientation of the hormone in the receptor complex accounts for the finding that much of α-subunit loop 2 appears to be near the receptor interface although few of its residues participate in essential receptor contacts (75). Finally, the position of the hormone accounts for the finding that the COOH-terminal end of the α-subunit can be cross-linked by a disulfide to most portions of the small seatbelt loop without disrupting the biological activity of hCG (76). Indeed, relatively few residues in this loop, notably Arg^{95} and Asp^{99}, appear to contact the receptor interface.

The surface of hCG in the region between α-subunit loop 2, the small seatbelt loop, and α-subunit loop 3 has a curvature similar to that of the top surface of the LRD (Fig. 8*A*). Based on the abilities of hCG to bind TSHR/LHR and FSHR/LHR chimeras (33, 47, 48), which are consistent with the hypothesis that the ligand contacts residues in leucine-rich repeats 4 and 5, we docked this surface of the hormone to the LRD such that β-subunit residue Asp^{99} is near LHR residue Lys^{104} and that the tips of β-subunit loops 1 and 3 contact the SSD (Fig. 8, *B* and *C*). This placed the highly conserved negatively charged residue in the small seatbelt loop, *i.e.* Asp^{99}, in a positively charged area of the LRD (Fig. 6*A*, *boxed* residues in upper rim of repeat 4). This would explain how replacing βAsp^{99} with less neutral or positively charged residues reduced hormone activity (49, 50), although we cannot exclude the possibility that these observations are due entirely to their abilities to alter the conformation of the heterodimer.

As shown (Fig. 8*C*), hCG makes extensive contacts with repeats 4 and 5. This would keep the nearby LHR oligosaccharides that are located in the upper rim of LHR repeats 3, 6, and 7 from interfering with binding (Fig. 6*A*). This orientation of the ligand also suggests that β-subunit loop 2 does not contact the receptor even though it is near the interface of the hormone with the LRD. This would explain the abilities of antibodies to β-subunit loop 2 to block hCG receptor interaction (51, 52) and account for the finding that this portion of hCG can be replaced with its FSH counterpart without altering ligand binding or signaling (32). It is also consistent with the binding sites of several antibodies such as B105, B110, and B111 (Fig. 8, *upper left*) and will explain why these are much less inhibitory to hCG-LHR interactions than B101 (53). Contacts between the tips of β-subunit loops 1 and 3 with the SSD would explain the change in conformation that occurs in this region of many mammalian lutropins when they bind to the rat LHR (21), a phenomenon detected readily using antibody B105. Finally, this orientation of the hormone is consistent with the abilities of an hCG antibody that we term B301 to block the binding of hCG to the LHR.[2] The binding site of this antibody has been determined by crystallography (54).

Contacts between the Hormone and the SSD May Be Essential for Signal Transduction—Antisera to the hCG β-subunit have long been known to restore the efficacy of deglycosylated hCG (55, 56). We assumed this was related to the abilities of antibodies in the antisera to increase the size of the β-subunit or to enhance its contacts with the LHR. To identify portions of the β-subunit that might contribute to the abilities of these antisera to enhance hormone efficacy, we repeated these studies using monoclonal antibodies to known hCG epitopes. Many antibodies to the β-subunit block binding of hCG to the LHR even though they can bind to the hormone-receptor complex (21). To minimize the possibility that these antibodies would block the binding of hCG and hCG-δα2 to the receptors, we preincubated cells that expressed the receptors with these ligands overnight at 4 °C, a condition that prevents signaling. Antibodies were permitted to combine with the hormone-receptor complexes during a second incubation at 4 °C. Finally, the cells were warmed to 37 °C to enable them to make cyclic AMP

[2] W. R. Moyle, M. P. Bernard, and R. V. Myers, unpublished observations.

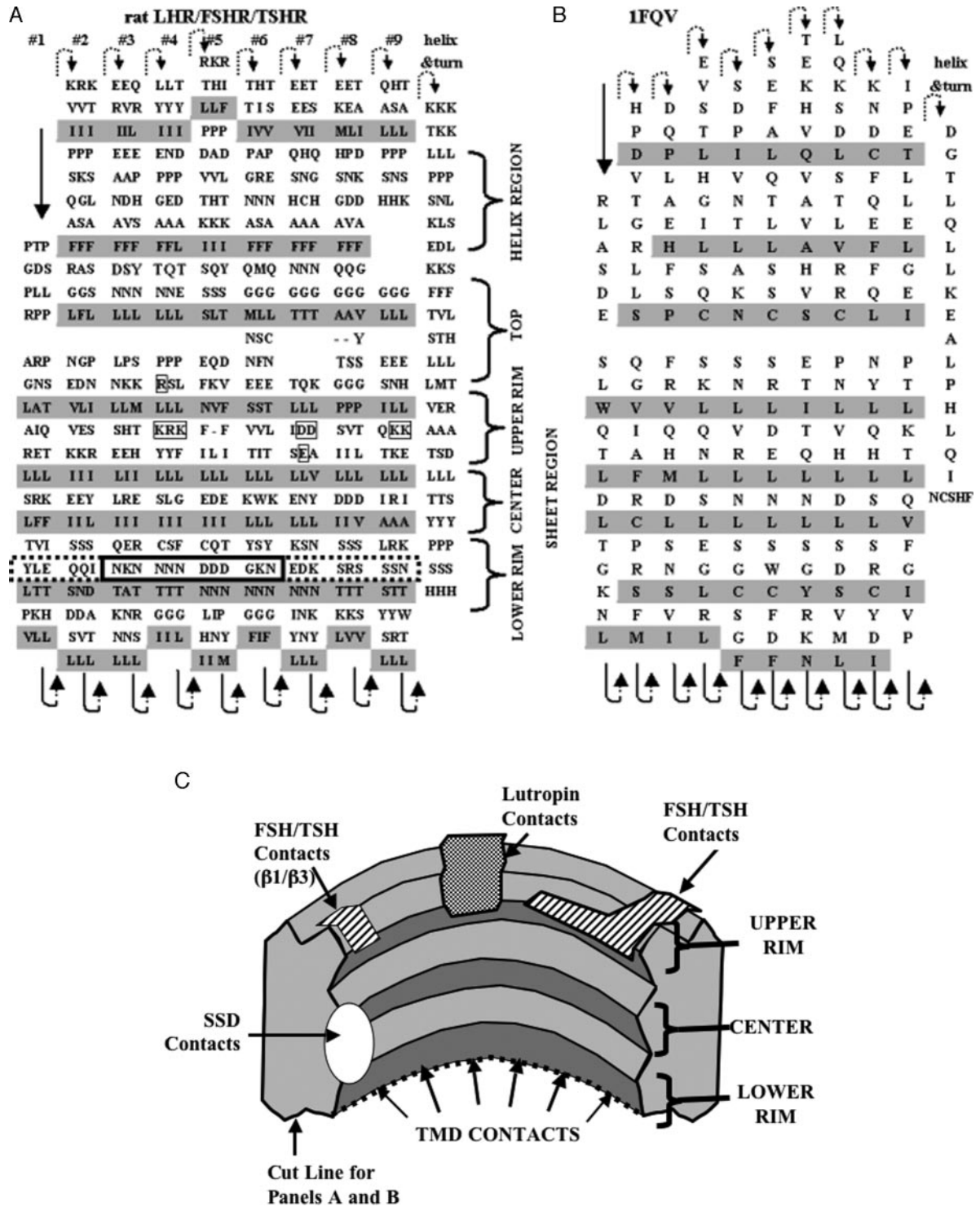
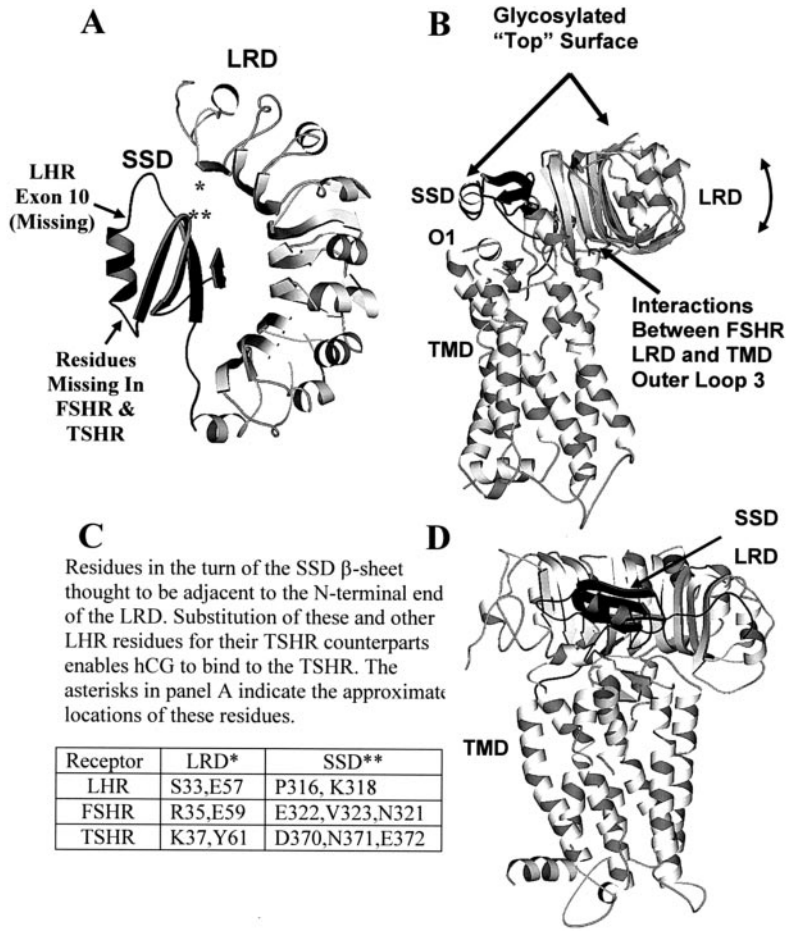


FIG. 6. **Alignments of the LRD derived from the rat LHR, FSHR, TSHR, and ubiquitin ligase used to model the LRD.** *Panels A and B*, we aligned residues that are most likely to form the hydrophobic cores of the LRD and ubiquitin ligase (highlighted residues) and arranged them in an open face format. This format was prepared by "cutting" the model along an imaginary line at the site indicated in *panel C* and folding the helical portion of the protein upwards so that adjacent residues in each leucine-rich repeat can be visualized readily. Residues that are *boxed* in the *upper rim* of repeats 4 and 9 are thought to be nearest the conserved aspartic acid residue in the small seatbelt of lutropins and follitropins/thyrotropins, respectively. Negatively charged residues in the *upper rim* of repeat 7 are thought to be near αArg^{42} in the α-subunits of hFSH and hTSH. *Heavily boxed* residues in the lower rim are thought to contact the TMD at the sites of *arrows* in *panel C*. Other boxed residues in the *lower rim* are thought to be near the TMD and to make fewer contacts with it. Each series of three residues in *panel A* illustrates the amino acid of the rat LHR, FSHR, and TSHR, respectively. The *arrows* indicate the direction of the amino acid sequence that is arranged to be read *vertically* starting at the *upper left corner*. *Panel C*, this figure shows the overall conformation of the LRD in a format designed to illustrate its contacts with the ligands, the SSD and TMD. The junction of the LRD and the SSD, which would be at the right-hand side of the drawing has been omitted.

in response to the bound ligands. Two β-subunit antibodies (B111, and to a lesser extent B110) enhanced the response to hCG-$\delta\alpha$2 (Table II). The B111 effect was readily detected in cells that expressed either receptor, but the B110 effect appeared to be greater in cells that express the rat LHR than those that express rLHRδ10. B111 binds to a region of hCG



Receptor	LRD*	SSD**
LHR	S33,E57	P316, K318
FSHR	R35,E59	E322,V323,N321
TSHR	K37,Y61	D370,N371,E372

FIG. 7. **Overview of receptor structure.** *Panel A*, view of the LRD and SSD as would be seen looking toward the cell surface. The portions of the SSD that are missing in the SSD of models of the LHR and FSHR are indicated by *arrows*. The *asterisks* refer to residues shown in the table (*panel C*). *Panels B* and *D*, views of the LRD and SSD as seen from the transmembrane domain. That in *panel D* is rotated 90° relative to that in *panel B*. *Panel C*, table describing potential contacts in the LRD and SSD of all three glycoprotein hormone receptors. The *single* and *double asterisks* refer to residues in the LRD and SSD, respectively.

TABLE II
Influence of β-subunit antibodies on the signal transduction response to hCG-δα2

Cells expressing the receptors were incubated at 4°C overnight with the indicated amount of hormone in a 50-μl volume. Next, we added 10 μl of incubation buffer containing 600 ng of the indicated antibody. After an additional incubation for 30 min at 4°C, the cells were incubated at 37°C for 20 min to permit cyclic AMP accumulation. Values are means of triplicates ± S.E. B111 increased the responses to hCG-δα2 of cells that express either receptor ($p < 0.001$). B110 increased the responses to hCG-δα2 in cells that express the rat LHR ($p < 0.001$), but not to cells that express rLHRδ10. The reduction in signaling seen in response to antibody B112 and A113 treatment of hCG-treated cells that express the rLHRδ10 may reflect their increased ability to remove the hormone that is bound to the rLHRδ10 receptor.

	Rat LHR		
	No hormone	30 ng hCG	30 ng hCG-δα2
None	0.85 ± 0.20	30.65 ± 4.44	8.67 ± 0.74
B111	1.05 ± 0.20	34.04 ± 2.42	27.52[a] ± 2.52
B101	1.12 ± 0.37	32.90 ± 1.72	6.06 ± 0.44
B112	0.50 ± 0.06	22.91 ± 5.33	8.49 ± 0.76
B110	1.43 ± 0.34	32.11 ± 2.79	22.95[a] ± 0.98
A113	1.43 ± 0.10	32.07 ± 2.40	8.10 ± 0.40
		rLHRδ10	
None	1.73 ± 0.32	24.82 ± 1.15	7.45 ± 1.61
B111	0.90 ± 0.10	27.91 ± 3.69	15.86[a] ± 0.99
B101	1.71 ± 0.63	19.99 ± 2.27	5.18 ± 0.87
B112	1.08 ± 0.07	10.81[a] ± 1.29	5.11 ± 0.48
B110	0.89 ± 0.23	20.14 ± 0.80	9.14 ± 1.55
A113	0.87 ± 0.09	13.42[a] ± 0.94	5.33 ± 0.60

[a] Refers to differences having $p < 0.001$ as determined in Dunnett's multiple comparison test by comparing the values obtained in response to the no antibody control.

near the disulfide bond that latches the seatbelt to β-subunit loop 1 (36). B110, the less effective antibody, recognizes an overlapping region of the β-subunit that includes Pro^{24}. Unlike B110, which binds to hCG analogs lacking the entire β-subunit COOH terminus, B111 binding requires residues 111–114 in addition to other portions of the β-subunit near the seatbelt latch site. All other antibodies were without effect on cyclic AMP accumulation in this assay, including A113 and B101, antibodies that do not bind the hormone-receptor complex and that had been included as controls. This supported the notion that the antibody effect involved an interaction of the hormone β-subunit with the SSD, an observation consistent with the model of the hCG-LHR complex (Fig. 8*C*).

The location of the B111 epitope near the junction of the β-subunit COOH terminus with the subunit core suggested that its influence on efficacy might be related to its proximity to the β-subunit COOH terminus. Consequently, the presence of B111 would cause this region of the β-subunit to make additional contacts with the LHR. To test this possibility, we determined the efficacy of an hCG analog that lacks the entire β-subunit COOH terminus (*i.e.* hCG-δ111) in cells that express the rat LHR and rLHRδ10. As had been found previously (57), hCG-δ111 had the same efficacy as hCG in assays employing the rat LHR. It had only half the efficacy of hCG in assays employing the rLHRδ10 receptor, however (Fig. 9). These findings are consistent with the notion that signal transduction involves contacts of the tips of β-subunit loops 1 and 3 with the SSD of the receptor. The ability of B111 to enhance the efficacy of hCG-δα2, most likely by its ability to increase contacts between the β-subunit COOH terminus and the SSD, suggests that docking of ligands to the glycoprotein hormone receptors increases the distance between the top portions of the SSD and LRD.

hFSH and hTSH Appear to Recognize a Different Region of the LRD Than hCG—Despite extensive mutagenesis of all

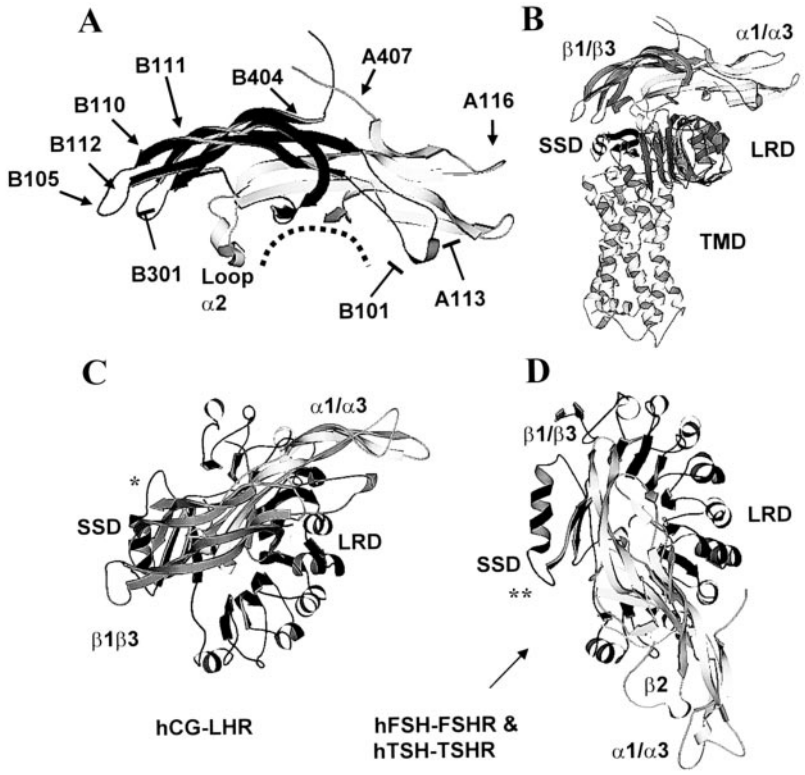


FIG. 8. **hCG and hormone receptor complexes.** *Panel A*, the structure of hCG illustrating the relative positions of antibodies that bind to the hormone-receptor complex (*pointed arrows*) and those that do not (*blunted arrows*). Although antibodies A113, B101, and B301 are potent inhibitors of hCG-LHR interaction, some of the antibodies that bind to the hormone-receptor complex such as B112 and to a lesser extent B105 and B110 also inhibit complex formation. The *broken curved line* is drawn to illustrate the top surface of the LRD. *Panel B*, this panel illustrates the docking of hCG to the extracellular domain of the receptor. *Panels C* and *D*, these panels illustrate the orientations that we anticipate are most favorable for docking of hCG and hFSH/hTSH, respectively. Note that the structure of the receptor enables either orientation of the ligand to contact the SSD.

three classes of glycoprotein hormone ligands and their receptors, specific hormone-receptor contacts have yet to be determined. We suggest this is because the most important contacts between these ligands and their receptors are not dominated by a single hydrophobic site, such as that observed in the interaction of growth hormone and its receptor (58). Identification of the contacts also appears to have been confounded by the possibility that residues in parts of the receptor that are unlikely to contact the ligand can also influence ligand binding specificity (16, 47) and that many hormones, *e.g.* equine LH (59), and hormone analogs, *e.g.* hCG/hFSH chimeras (60), can dock with multiple receptors. Indeed, some hCG/hFSH chimeras bind well to TSHR even though they have no TSH-specific residues (60). This suggests that receptor binding specificity is determined as much by subtle differences in the conformations of the hormones and their receptors as by the presence of ligand-specific and receptor-specific contact residues. Our current view of the receptor (Figs. 6 and 8) indicates that interactions of the SSD with the LRD and the TMD would be expected to augment ligand binding specificity and that this is readily changed by mutating one or more contacts between these receptor domains. Although this phenomenon would contribute to the difficulty of identifying the ligand docking site, it may have facilitated the fine tuning of ligand binding specificity during the co-evolution of these hormones and their receptors.

The similarities of the LRD with those of several other leucine-rich repeat proteins suggests strongly that it is "banana" shaped. This notion and the observation that it is composed of repeating elements raised the possibility that the LRD contains more than one ligand binding site (Figs. 6*C* and 8, *C* and *D*). Consequently, glycoprotein hormone ligands may be able to interact with two sites in the LRD, either of which would be capable of contacting the SSD and thereby eliciting a biological signal. Each of these sites contains a positively charged region that appears capable of interacting with the highly conserved aspartic acid residue in the small seatbelt loop of the hormones. These residues are "boxed" in the upper rim region of repeats 4 and 9 (Fig. 6).

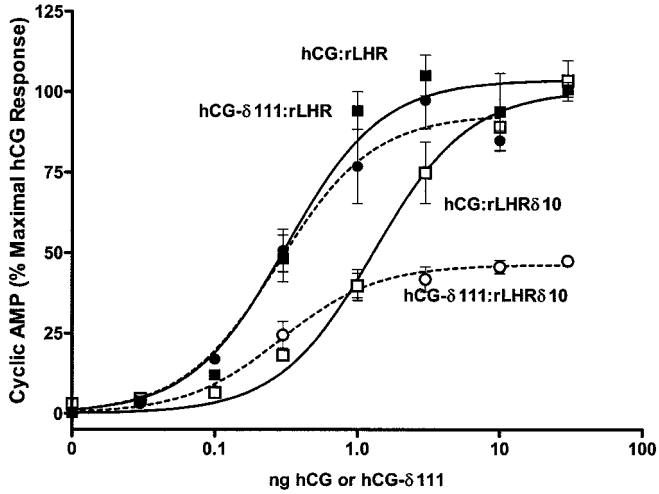


FIG. 9**. Influence of the β-subunit COOH terminus on the ability of the hormone to signal through the rat LHR and rLHRδ10.** Cells expressing the rat LHR and rLHRδ10 were tested for their abilities to respond to hCG and a β-subunit analog that is truncated at residue 111 (hCG-δ111). Both hCG and hCG-δ111 had equal efficacy in assays employing the rat LHR. In contrast hCG-δ111 had only 50% the efficacy of hCG in assays employing the rLHRδ10 receptor. The assay volume was 60 μl.

FSH and TSH appear to dock with receptors differently than hCG (Fig. 8*D*). This would explain why a portion of the α-subunit that appears to be exposed in LHR complexes is hidden in FSHR and TSHR complexes (Fig. 10). To identify the manner in which the hormones dock with their receptors, we devised a technique that permits us to identify surfaces of α-subunit loop 2 that are near or distant from the receptor (75). Briefly, we created "knobs" at different sites on loop α2 by cross-linking cysteines that are substituted for different residues to a cysteine that had been added to the carboxyl terminus of the β-subunit. In the case of hCG and a bifunctional analog that binds to both LH and FSH receptors, this cysteine replaced

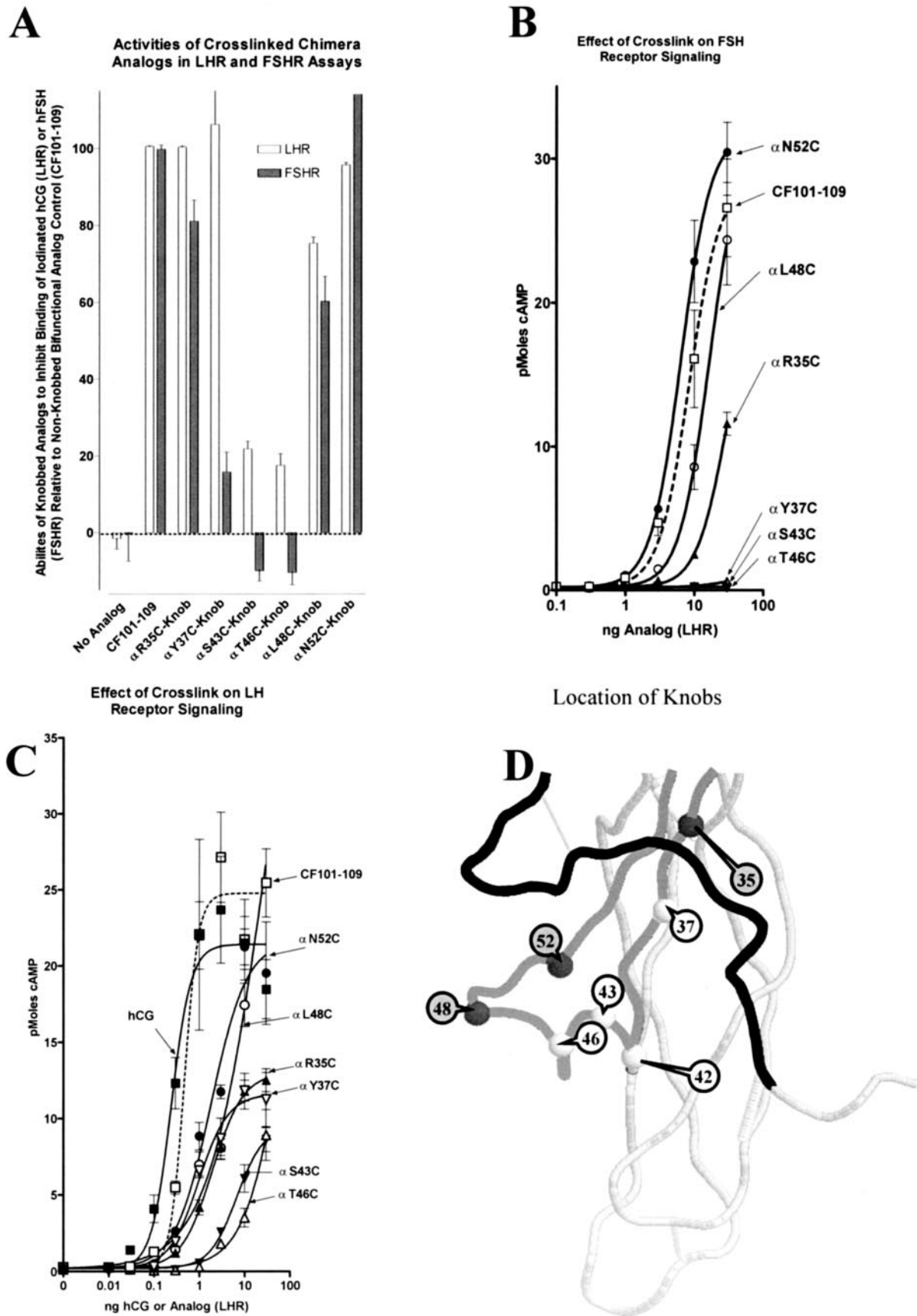


FIG. 10. **Portions of the α-subunit that are obscured in the FSHR are consistent with the notion that FSH docks with its receptor in a different orientation than hCG binds to the LHR.** A cysteine residue that had been added to the β-subunit COOH terminus of a bifunctional hCG analog (*i.e.* CFC101–114,S138C) that binds LHR and FSHR was found to become cross-linked to cysteines added to the α-subunit during heterodimer synthesis in COS-7 cells. This created a knob at the location of the cysteine in the α-subunit that was used to probe surfaces that are exposed or hidden when the heterodimer docks with LHR and FSHR. *Panel A*, results of binding studies to determine the ability of the analog to compete with ^{125}I-hCG and ^{125}I-hFSH for binding to rat LHR and FSHR. Values are expressed relative to the binding ability of a

Ser^{138}. For studies of FSH and TSH, we used β-subunit analogs containing the hCG β-subunit COOH terminus and in which hCG β-subunit residue Ser^{138} was converted to cysteine. Although cysteines in α-subunit loop 2 are normally not near a cysteine in the COOH terminus of the β-subunit, movements of the COOH terminus following heterodimer assembly enable it to "scan" the surface of the heterodimer and form an intersubunit disulfide. The cross-link that stabilizes the knob can be detected readily by its ability to prevent dissociation of the heterodimer during a 30-min incubation at pH 2, 37 °C. Under these conditions, non-cross-linked heterodimers dissociate and do not recombine.

Bifunctional hCG analogs containing α-subunit knobs in place of α-subunit residues 35, 48, and 52 had similar abilities to block the binding of ^{125}I-hFSH to FSHR and ^{125}I-hCG to LHR as CF101–109, a bifunctional control analog that lacks the β-subunit COOH terminus and that does not contain any knobs (Fig. 10*A*). Because "knobbed" ligands would be capable of competing with labeled hFSH or hCG for these receptors only if the knob did not interfere with binding, this result showed that α-subunit loop 2 residues 35, 48, and 52 are not part of the hFSH-FSHR or hCG-LHR binding sites. In contrast, bifunctional analogs containing knobs at α-subunit residues 37, 43, and 46 inhibited binding of ^{125}I-hCG to LHR much better than they inhibited binding of ^{125}I-hFSH binding to FSHR (Fig. 10*A*). This indicated that these α-subunit residues are more exposed in LH receptor complexes than in FSH receptor complexes. Similar differences were observed when we monitored the abilities of these cross-linked analogs to stimulate FSHR and LHR mediated signal transduction (Fig. 10, *B* and *C*). This indicated that bifunctional hormone analogs capable of binding to both LHR and FSHR dock with each receptor differently.

We also observed that some portions of the α-subunit that were exposed in hCG-LHR complexes were obscured in the α-subunits of hFSH-FSHR and hTSH-TSHR complexes. Thus, whereas the presence of a knob at α-subunit residue 42 reduced the potency of hCG less than 2-fold in LHR assays (75), it reduced the potency of hFSH and hTSH 100-fold or more in FSHR and TSHR assays (not shown). Other portions of α-subunit loop 2 were exposed in all three complexes. For example, the presence of a knob at α-subunit residue 48 reduced the potency of hCG, hFSH, and hTSH by only 2-, 2-, and 9-fold in LHR, FSHR, and TSHR assays, respectively (not shown). In contrast, α-subunit loop 2 residue 47 appeared to be hidden in all three receptor complexes as the presence of a knob at this site blocked the abilities of all three hormones to elicit cyclic AMP accumulation (not shown). Together, these findings suggested that surfaces of α-subunit loop 2 between αTyr^{37}–αThr^{46} are much more obscured in FSHR and TSHR complexes than they are in LHR complexes (Fig. 10*D*). The portion of loop α2 centered near αMet^{47} appears to be obscured in hCG-LHR complexes (75), as well as in FSHR and TSHR complexes. The surface of α-subunit loop 2 between residues αLeu^{48}–αGln^{50} and αAsn^{52} do not appear to be near the ligand interface in any receptor complex (Fig. 10*D*). We have also found that replacing αArg^{42} with glutamate did not alter the potency of hCG in LHR signal transduction assays. This substitution reduced the potency of hFSH in FSHR signal transduction assays ~3-fold. For example, the ED_{50} values (95% confidence limits) of hCG and hCG-αR42E in LHR cyclic AMP accumulation assays were 3.3 (2.5–4.5 ng/ml) and 3.0 ng/ml (2.7–3.5 ng/ml). Those for hFSH and hFSH-αR42E in FSHR cyclic AMP accumulation assays were 2.9 (2.1–4.0 ng/ml) and 10.1 ng/ml (6.3–16.3 ng/ml). Studies with hTSH revealed that conversion of αArg^{42} to glutamate had a similar effect (not shown), supporting the notion that hFSH and hTSH dock with their receptors in similar orientations (Fig. 8*D*). These findings were incorporated into models of the FSH-FSHR and TSH-TSHR complexes, which place αArg^{42} near the negatively charged residues that have been "boxed" in the upper rim of repeat 7 (Fig. 6*A*).

Docking of hFSH and hTSH enables the strap regions of their seatbelts to contact residues in the upper rim of several repeats (Figs. 6*C* and 8*D*). This is consistent with the findings that the straps of hFSH and hTSH have important roles in their abilities to bind FSHR and TSHR (33, 35). The strap region of hCG can be attached to several portions of the α-subunit without disrupting its ability to interact with the LHR (36), suggesting that it has a limited role in ligand binding. This finding is consistent with the lack of contact between the hCG seatbelt strap and the LHR (Figs. 6*C* and 8*C*). The model of the FSH-FSHR (Fig. 8*D*) complex also suggests that much of the small seatbelt loop is not near the receptor, a phenomenon that would explain why substitutions in this portion of the hormone have a relatively small influence on binding of hFSH to FSHR (33, 61).

Alignment of the LRD and SSD Domains with the TMD: Models of the Entire Receptor—Efforts to position the extracellular and transmembrane domains are hampered by a lack of experimental data indicating how these domains interact. We built models of the TMD based on the structure of bovine rhodopsin (4) and connected the COOH-terminal end of the SSD to the NH_2-terminal end of the TMD. This placed the extracellular loops of the TMD opposite the glycosylated surface of the extracellular domain (Fig. 6*B*). Although the SSD is connected to helix 1 of the TMD, by itself this information did not enable us to determine how the extracellular domain interacted with the TMD. By incorporating a bend in the COOH-terminal end of the SSD, we found that it was possible to make several alignments of the extracellular and transmembrane domains, including those in which either outer loop 1 or outer loop 3 was in contact with the LRD.

Two sets of data support the notion that the most likely orientation of the extracellular and transmembrane domains of the glycoprotein hormone receptors is one in which the NH_2- and COOH-terminal ends of outer loop 3 are located beneath leucine-rich repeats 6 and 3, respectively. First, this position of the TMD will explain how replacing Lys^{87} and Lys^{162} in LRD repeats 3 and 6 of the FSHR with asparagine and glycine, their respective LHR counterparts, creates a chimeric receptor that binds both hCG and hFSH (47). FSHR residues Lys^{87} and Lys^{162} become adjacent to residues Lys^{580} and Lys^{588} when leucine-rich repeats 6 and 3 are positioned adjacent to outer loop 3 (Fig. 11). Replacing FSHR residues Lys^{87} and Lys^{162} with their uncharged LHR counterparts (Fig. 6) would relieve

bifunctional analog that lacks the β-subunit COOH terminus (CF101–109) and that is not cross-linked. *Panels B* and *C*, results of signal transduction assays in which the cross-linked analogs were tested for their abilities to elicit FSHR and LHR responses. Both studies showed that the cross-linked analog functioned differently in FSHR and LHR, revealing that different portions of the α-subunit are likely to contact each receptor. The volume of each assay was 60 μl. *Panel D*, location of residues in loop α2 that are obscured by contacts of ligands with FSHR. The backbone atoms in hCG loop α2 (*light gray*), loops β1/β3 (*white*), and the seatbelt (*dark gray*) are shown here as *tubes*. The Cα carbon atoms of residues in loop α2 that are discussed in the text are illustrated as *spheres*. Cross-links to cysteines to residues denoted by the *gray spheres* did not impede the ability of ligands to interact with FSHR; those to residues depicted in *white* reduced FSH binding and signaling. The callouts are drawn to show the orientations of their side chains. When the perspective shown in this diagram is flipped about a *horizontal axis*, one obtains the perspective of the ligand shown in Fig. 8*D*.

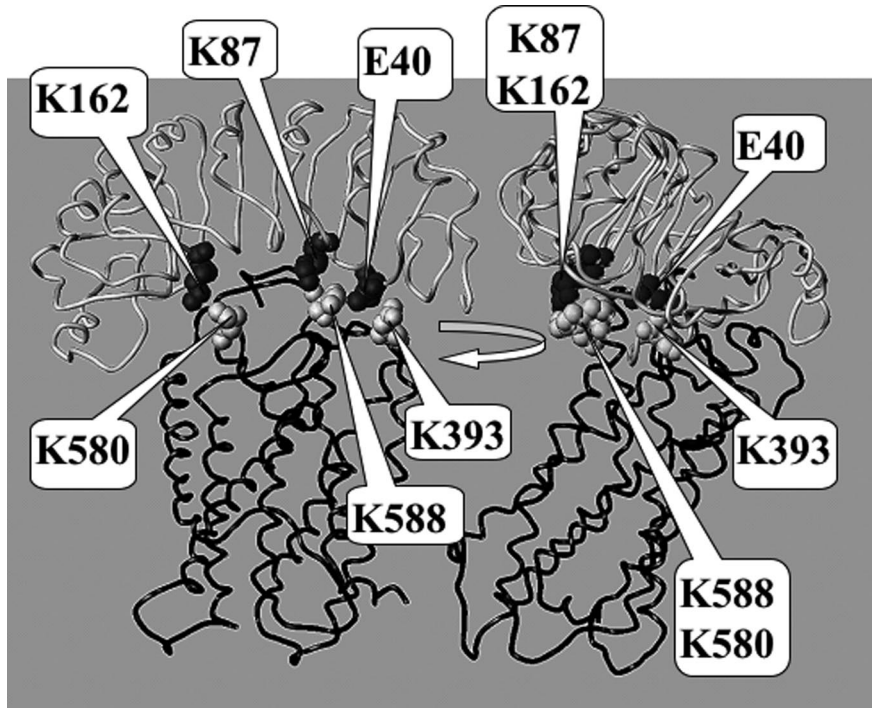


FIG. 11. **Location of key residues in the FSHR and TSHR that block binding of hCG.** When the extracellular and transmembrane domains of the glycoprotein hormone receptors are aligned such that the lower portions of leucine-rich repeats 3–6 are positioned near outer loop 3 of the transmembrane domain, several residues that are important for ligand binding specificity become adjacent. As illustrated here and discussed in the text, FSHR residues Lys^{162} and Lys^{87} in leucine-rich repeats 6 and 3 become adjacent to Lys^{580} and Lys^{588} in helices 6 and 7, respectively. Disrupting these charge-charge interactions appears sufficient to permit high affinity binding of hCG to the FSHR. TSHR residue Glu^{40} in leucine-rich repeat 1 would also be adjacent to Lys^{393}, a residue at the start of transmembrane helix 1. Disrupting this interaction also appears to have a role in enabling the TSHR to bind hCG. The illustration in the *left-hand side* of this figure shows a view of the LRD on top of the TMD. The SSD is omitted to make the figure clearer. The illustration in the *right-hand side* of the figure shows a view that is turned 90° to the left. In this perspective, the lysines in repeats 3 and 6 appear superimposed. The SSD is also shown in the *upper right-hand* area of the figure.

potential charge repulsion in this region of the receptor. We anticipate that this would permit the position of the LRD to be turned slightly downward relative to that of the SSD-TMD complex (Fig. 7*B*, *curved arrow*) and thereby abrogate the ability of the SSD to inhibit hCG binding. Second, this alignment of the extracellular and transmembrane domains is also consistent with the abilities of some TSHR analogs to bind hCG (47). The sequence of the TSHR in the region where the SSD joins TMD (*i.e.* Gly-Tyr-Lys^{393}-Phe-Leu-Arg) contains a lysine at residue 393. The corresponding region of the rat LHR has the same sequence except that Lys^{393} is replaced by an alanine. When the TMD is oriented relative to the LRD and SSD in the fashion just discussed (Fig. 11) replacing residue Glu^{40} in leucine-rich repeat 1 of the TSHR (Fig. 6*A*) with its rat LHR equivalent (*i.e.* Tyr^{36}, Fig. 6*A*) would disrupt a potential salt bridge between TSHR-Glu^{40} and TSHR-Lys^{393}. The change is likely to alter the positions of the LRD and SSD in the TSHR slightly, explaining how this mutation may enhance the binding of hCG to the TSHR (47).

In this orientation outer loop 3 is near the small space between the LRD and SSD and outer loop 1 is situated beneath the helical portion of the SSD (Figs. 7 and 8). Furthermore, the portion of outer loop 3 that is conserved in all vertebrate glycoprotein hormone receptors is adjacent to residues in leucine-rich repeats 5 and 4, *i.e.* asparagine in repeat 4 and aspartic acid in repeat 5, that are also conserved in all known vertebrate glycoprotein hormone receptors (Fig. 12*A*).

The extensive contacts between the SSD and TMD (Figs. 7 and 8), which also appear to include a salt bridge between SSD residue Glu^{332} and TMD outer loop 2 residue Lys^{488}, make it unlikely that ligand binding will promote a movement of the SSD relative to the TMD. These contacts are also likely to offset the influence of many alanine substitutions in TMD loop 2, which would account for the finding that many of these substitutions had relatively little influence on receptor function (62).

In contrast, the LRD appears to contact the SSD-TMD complex at relatively few sites. Most of these contacts involve a single residue in each leucine-rich repeat (Fig. 6, *boxed row*, *lower rim*). A few contacts appear to be made between residues in the distal end of the SSD (*e.g.* Arg^{261} and Lys^{317}) and residues in leucine-rich repeats 1 and 2. Others are made in the hinge region near the junction of the LRD and SSD. Differences in the manners in which the SSD and LRD interact with the TMD imply that ligand binding results in movements of the LRD relative to the SSD-TMD complex. We expect that these movements are a key component of signal transduction.

DISCUSSION

Role of the SSD in the LHR—The notion that the LRD is responsible for glycoprotein hormone receptor interactions was established shortly after the amino acid sequences of these receptors were determined (10), and was based primarily on the ability of hCG to interact with truncated LHR analogs. In hindsight, it appears that the very high affinity of hCG for this portion of the receptor may have obscured the role of the SSD in lutropin receptors, which becomes apparent only during studies of mammalian lutropins other than hCG (9, 31, 63). The studies described here were initiated to learn how the SSD might participate in LHR function and to use this information to develop a model of hormone induced signaling. The finding that the SSD contributes to interactions of bLH with lutropin receptors is consistent with our earlier observation that this domain is primarily responsible for the ability of the human receptor to distinguish hCG and bLH (9). Although we had assumed this was because of its ability to block binding of bLH to the human receptor, it seems clear from studies described here that the SSD may also make positive contributions to ligand binding. The finding that the SSD may be required for efficient binding of lutropins other than those produced by the higher primates raises further questions about the role of the "B-form" of the receptor in mammals, which lacks this domain (2) and that may be unable to bind lutropins at the concentrations found in most species. Both the LRD and SSD of the TSHR (48, 64) and FSHR (33) appear to be essential for ligand binding.

Models of the Glycoprotein Hormone Receptors and Ligand Binding—The models of the glycoprotein hormone receptors described here are capable of explaining most mutagenesis

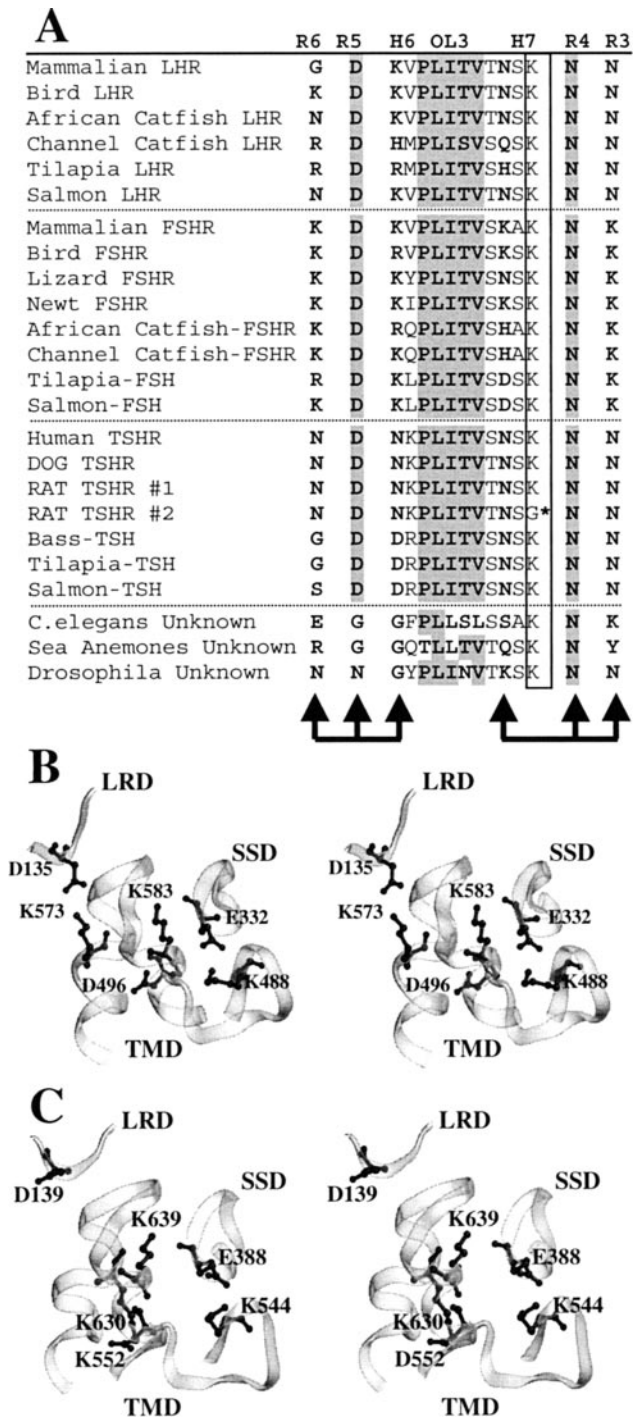


FIG. 12. **Comparison of sequences in regions of the LRD and TMD that we propose are essential for signaling.** *Panel A*, several residues that we anticipate form important interactions between LRD repeats 3–6 (R3, R4, R5, R6), TMD helices 6 and 7 (H6, H7), and TMD outer loop 3 (OL3) are highly conserved in each receptor class. This is particularly evident for asparagine and aspartic acid in R4 and R5, for the lysine in H7, and for the sequence "PLITV" that forms all of outer loop 3. Backbone atoms of outer loop 3 form most of a binding site for the side chain of the lysine in H7 (*panels B* and *C*), which is shown *boxed* and that appears to be required for signaling. The presence of a glycine that corresponds to human TSHR residue Lys^{639} in one sequence of the rat TSHR (AAA53209.1) is the only receptor that we have been able to find that has a residue other than lysine at this position. A second sequence in the data base (AAG2421.1) suggests that there may be a lysine at this site. All mammalian TSHR have a lysine near the top of helix 6 that appears to face toward the TMD and helix 7. Molecular dynamics simulations suggest the side chains of this lysine and an arginine in the piscine TSHR are near a negatively charged residue in outer loop 2 of the TMD in the TSHR (*panel C*). This would be expected to attract the top of helix 6 to the TMD. Contacts between LRD repeats 3 (R3) and 6 (R6) and residues at the top of helices 6 (H6) and 7 (H7) appear to influence ligand binding specificity and differ between the hormone classes. The *arrows* indicate the proximity of residues in these repeats relative to residues in outer loop 3. *Panel B*, relative positions of key residues in a model of the rLHR lacking exon 10 following molecular dynamics simulation. Asp^{135}, which is located in R5 of the LRD, appears to form a salt bridge with Lys^{573}, which is located at the top or H6 in the TMD. Lys^{583} at the top of H7 appears to be stabilized by hydrogen bonds with backbone atoms of residues in outer loop 3 and with the backbone oxygen of a highly conserved glutamate in the SSD. The side chain of this glutamate also appears to have a role in stabilizing Lys^{488} in outer loop 2 of the TMD, an interaction that would be expected to contribute to the stability of the SSD-TMD complex. *Panel C*, relative positions of residues in a model of the human TSHR lacking residues 292–363. Unlike rLHR-Asp^{135}, the corresponding hTSHR residue (*i.e.* Asp^{139}) does not appear to participate in contacts with H6, most likely because an asparagine is substituted for the lysine corresponding to rLHR-Lys^{573}. Several other contacts between residues in the LRD and outer loop 3 of the TSHR appear to stabilize interactions between the LRD and the TMD, however (not shown). Lys^{630} in H6 is located in a position in which it appears to form a salt bridge with Asp^{552} in outer loop 2, a phenomenon that would be expected to potentiate movements of H6 toward the TMD following TSHR binding, antibody binding, or trypsin digestion. As in the case of the LHR, the lysine in H7 of the TSHR appears to form hydrogen bonds with backbone atoms of residues in outer loop 3 and Glu^{388} of the SSD. Also, as in the case of the rLHR, the side chain of Glu^{388} appears to form a salt bridge with the side chain of Lys^{544}, a lysine in outer loop 2. This interaction would be expected to help stabilize the positions of the SSD and TMD.

data, including the puzzling observation that two residues in a region of the FSHR that is likely to be located near the plasma membrane prevent it from binding hCG (47). Although it is usually assumed that the concave surface of the LRD is the major ligand binding site for hCG and that negatively charged residues in this portion of the LHR contact positively charged residues in the small seatbelt loop (*i.e.* the "determinant loop") and the α-subunit COOH terminus, this requires that the ligand be oriented perpendicular to the LRD (5), an orientation that does not lend itself well to models of signal transduction. Furthermore, it does not explain the findings that the α-subunit COOH terminus can be cross-linked to most residues of the hCG small seatbelt loop by a disulfide without altering hormone activity (76).

In constructing our original model (8) as well as that outlined here, we relied on several assumptions, one of which is that portions of the receptor that are glycosylated are likely to be on a surface furthest from the membrane (Figs. 7 and 8). We also assumed that the model would need to explain antibody binding data that showed a single surface of the ligand contacts the SSD as well as the LRD. Antibody binding data also revealed that the conformation of the tip of β-subunit 3 is altered on receptor binding (53). In revising our initial model, we made use of unpublished data (75, 76) obtained with a technique developed to probe distances of specific residues in all three hormones and their receptors (data described in Fig. 10). We also took advantage of the recent finding that relatively few substitutions enable the FSHR and TSHR to bind hCG (47) and older data on the influence of replacing residues in LHR loops 1, 2, and 3 with alanine (62, 65, 66).

Our current model suggests that glycoprotein hormone ligands fit within a receptor pocket created by the apposition of the LRD and SSD. As a result, ligand binding is influenced by residues that modulate the position of the LRD relative to the SSD, by residues that alter the positions of the subunits in the glycoprotein hormone heterodimer, and by residues that contribute to high affinity interactions. This would explain why it has been so difficult to understand how these hormones bind their receptors. Indeed, the ability of hCG to bind to an FSHR analog containing only two LHR-specific residues and to a

TSHR analog that has only a few more LHR-specific residues (47) suggests that there are few if any key determinants of ligand binding specificity in the LRD. This may have permitted the rapid optimization of reproduction and development at a stage in vertebrate evolution prior to the time that the amino acid sequences of the hormones and their receptors became separated into three easily recognizable lines.

The model also provides a "structural" basis for the paradigm of ligand-receptor co-evolution that we proposed earlier (33). The presence of repeating elements in the LRD, its shape, and its position near the SSD suggests that glycoprotein hormone receptors have the potential to accommodate ligands in at least two orientations, both of which would be capable of eliciting a biological signal. Either of these could have been altered independently following gene duplication without destroying the activity of the receptor. This might explain why a region in leucine-rich repeat 5 that we anticipate is near a major contact site for mammalian lutropins (Fig. 6*A*) varies substantially in vertebrate receptors. Most follitropins from species other than fish contain a deletion in this site. It might also explain the insertion in leucine-rich repeat 8 of all TSHR. The ability of receptors to accommodate two orientations of the ligand would have permitted the use of a single receptor for multiple functions or for one hormone to function with multiple receptors. The latter is exemplified by the ability of some piscine follitropins to interact with FSHR and LHR (67). It would also explain the peculiar abilities of hormones from one species to interact with different receptors from another (59, 68).

The possibility that different surfaces of the LRD are responsible for binding of lutropins, follitropins, and thyrotropins also explains why it has been possible to produce FSHR and TSHR analogs that bind hCG and why reciprocal mutations do not create LHR analogs that bind hFSH and hTSH (33, 48). In the models described here, the docking site for hCG resides in a central region of the LRD rim, whereas the docking sites for hFSH and hTSH appear to be near the COOH-terminal end of the LRD rim (Figs. 6*C* and 8, *C* and *D*). Therefore, replacing the central portion of the LRD of either the FSHR or TSHR with its LHR counterpart would create a chimeric receptor having binding elements for FSH and hCG or for TSH and hCG. Conversely, replacing the central portion of the LRD of the LHR with those of FSH or TSH would eliminate both hormone binding sites. These orientations of the ligand binding sites are also consistent with the results of studies designed to probe the position of the α-subunit in the receptor complexes. As would be expected, different portions of the α-subunit appear to be hidden when bifunctional ligands bind LHR and FSHR, a finding that is consistent with the models illustrated in Fig. 8, *C* and *D*.

Relationship of Contacts with the SSD to Efficacy—The notion that hormone efficacy depends on formation of contacts between the ligand and both the LRD and SSD will readily account for the loss in efficacy caused by deglycosylation of hCG (69), a finding that has remained mysterious for 30 years. Deglycosylated hCG was less active in assays employing rLHRδ10, a receptor analog that has a smaller SSD than that of the rat LHR, making it less likely to contact the ligand. The loss in efficacy was effectively reversed by the presence of an oligosaccharide that increased the size of the tip of β-subunit loop 3. It was also reversed by an antibody (*i.e.* B111) that enlarges the tip of β-subunit loop 1 (Fig. 9). Neither the oligosaccharide nor the antibody would be expected to make specific contacts with the SSD. This indicates that the oligosaccharides function by increasing steric contacts between the hormone and SSD, which would tend to increase the distance between the SSD and the LRD. Because the LRD appears to be stabilized in part by contacts that it makes with the SSD and TMD, disrupting these contacts is likely to require a significant amount of energy. This may explain the finding that deglycosylated hCG has a significantly greater affinity for the rLHRδ10 than the rat LHR as seen by the fact that it is more potent than hCG in receptor binding assays (Fig. 2*B*).

Implications of Receptor Structure for the Constitutive Activity of the TSHR and Its Activation by Antibodies—The notion that signal transduction results from movements of the LRD relative to the SSD can explain the abilities of antibodies to stimulate the TSHR or to block TSH activity. Stimulatory antibodies are responsible for important clinical syndromes such as Graves disease and a substantial effort has been made to understand how they elicit TSHR signaling (20). We anticipate that binding of antibodies to the LRD and/or the SSD that alters the position of the LRD relative to the SSD will activate the TSHR. This would explain why antibodies that recognize the TSHR A subunit, a portion of the TSHR that contains the LRD and the NH_2-terminal half of the SSD, are very effective in eliciting a response (70). Antibodies that bind to a surface of the A subunit that is nearer the SSD might be more likely than those that bind to other portions of the LRD to increase the distance between the upper surfaces of these two receptor domains. This would also explain why they appear to bind the free A subunit better than the intact receptor (70). Antibodies that bind to the TSHR LRD and/or SSD without contacting both of these domains at the same time or that do not alter the positions of these receptor components may be in a position in which they can block TSH binding. Thus, they would be expected to inhibit TSH induced stimulation even though they cannot initiate signaling by themselves.

The structure of helix 6 in all TSHR may also account for its greater ligand independent activity, the role that the LRD has in suppressing this activity, and the ability of trypsin to activate the TSHR (18). Helix 6 of the LHR and FSHR contains a lysine that may interact with an aspartic acid in LRD repeat 5 corresponding to LHR Asp^{135} (Fig. 12*A*). This residue is replaced by asparagine in the mammalian TSHR and by an aspartic acid in the piscine TSHR. We anticipate that this reduces the interaction between these portions of the LRD and TMD. This may enable the TSHR to be much more readily activated, particularly after enzymatic treatments that would be expected to reduce interactions between the LRD and SSD.

Mechanism of Signal Transduction—Mutations in TMD helix 6 of the LHR, notably the substitution of residues for Asp^{556} (71) often enhance its constitutive activity. This suggests that helix 6 may have a central role in ligand-induced signaling. The central region of the TMD is stabilized by a hydrogen bond network that interconnects helix 6 with most other transmembrane helices (72). The region of helix 6 near the extracellular domain is stabilized primarily by its contacts with the LRD, some of which are indirect and involve contacts with helix 7 and TMD outer loop 3. Thus, the position of helix 6 could be changed by mechanisms that disrupt one or more of the hydrogen bonds that stabilize the TMD, by movements of the LRD, or both. Polar residues that form the hydrogen bond network in the TMD appear to be located too far from polar residues in the SSD, LRD, and the outer loops of the TMD to permit direct interactions between these two regions of the receptor. Therefore, we consider it unlikely that ligand binding causes a direct interaction between these regions. Instead, we anticipate that the positions of helix 6, helix 7, and outer loop 3 are regulated primarily by their contacts with the LRD.

How do ligands alter the position of the LRD? Hormone efficacy appears to depend largely on the size of the ligand. Efficacy can be enhanced substantially by increasing the size of the ligand through the addition of oligosaccharides to β-sub-

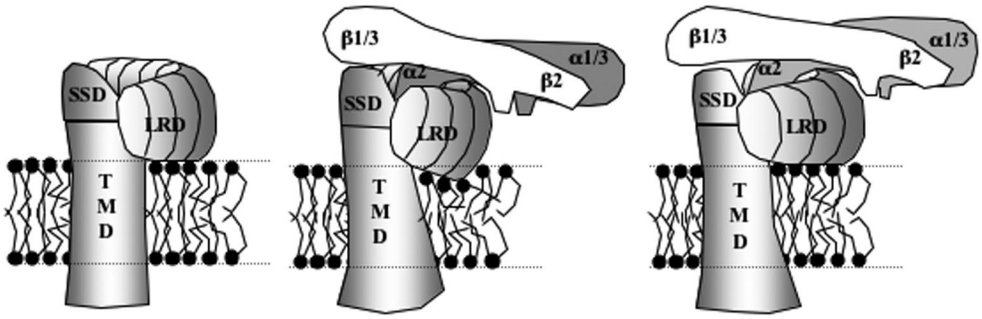


FIG. 13. **Proposed model of signal transduction.** We postulate that the SSD is stably positioned on the extracellular surface of the TMD in contact with outer loops 1 and 2. The LRD is near the SSD-TMD complex as shown such that residues in the lower rim of repeats 3–6 contact outer loop 3 of the TMD and parts of helices 6 and 7. The NH_2- and COOH-terminal portions of the LRD contact the ends of the SSD and TMD helices 1 and 5. In this model, the LRD prevents interactions of outer loop 3 and helices 6 and 7 with the TMD needed for signal transduction, possibly by preventing the formation of a binding pocket for the conserved lysine in helix 7. Mammalian lutropins and follitropins that have the salmon folding pattern dock are expected to dock with the receptor as shown here and in Fig. 8*C*. Most other vertebrate glycoprotein hormones appear to dock with their receptors in an orientation that is turned roughly 90° as shown in Fig. 8*D*. For reasons discussed in the text, binding in either orientation leads to signal transduction by increasing the distance between the top of the SSD and the top of the LRD. This leads to a rotation of the LRD (*panel B*), a gate-like movement of the LRD (*C panel*), or a combination of the two (not shown). This creates a binding pocket for the conserved lysine in helix 7 that initiates movements of outer loop 3 and helices 6 and 7 toward the core of the TMD, thereby causing a rearrangement of the TMD needed for signaling. We anticipate that ligand binding may also displace some nearby phospholipids, which may help hold the receptor in an inactive state by preventing movements of the LRD. Dissociation of the ligand would permit the lipids to reverse this position of the LRD, which would restore outer loop 3 and helices 6 and 7 to the positions they occupy in the inactive receptor. Ligand binding could also disrupt contacts between the NH_2-terminal end of the LRD and the SSD. This would also alter the positions of helices 6 and 7 that lead to a reorganization of the TMD and signal transduction (*C panel*).

unit loop 2 or by the presence of some antibodies to the β-subunit (Fig. 3 and Table I). Conversely, efficacy can be reduced by deglycosylation of the ligand, a phenomenon known for nearly 30 years (69) and restored using antisera to the β-subunit (55, 56). Efficacy can also be reduced by removing residues encoded by exon 10 (Fig. 3), which reduces the size of the SSD. TSHR antibodies that bind to the A subunit, a region largely composed of the LRD have been found to activate the TSHR (70). Based on these observations, we propose that ligand binding promotes signal transduction by increasing the distance between the top surfaces of the SSD and LRD (Fig. 13).

Contacts of the ligand with the SSD and LRD would be expected to move the LRD by either or both of two mechanisms. In one, ligand binding causes a small rotation of the LRD that enables helices 6 and 7 to move toward the TMD (Fig. 13*B*). Each end of the LRD would remain in approximately the same position because of its interactions with either end of the SSD. Alternatively, ligand binding may disrupt the interaction between the NH_2-terminal end of the LRD and the SSD, enabling it to swing outward from the TMD (Fig. 13*C*). The number of contacts that would need to be disrupted suggests that this mechanism is less likely, however. Regardless of the mechanism employed, we favor the notion that the LRD maintains contact with residues in outer loop 3, helices 6, and/or helix 7 during signal transduction. This would explain the finding that deletion of the LRD does not lead to constitutive receptor activity, even in receptor mutants that are forced to the cell surface (73).

Potential interactions between residues in the SSD, LRD, and TMD suggest that signal transduction is more likely to involve movements of helices 6 and 7 toward the TMD than away from the TMD. The LHR contains a lysine at residue 583. This lysine, which appears to be near the top of helix 7 and to be capable of making contacts with the SSD, is essential for signal transduction (66), an observation we have confirmed. Molecular dynamics simulations[3] suggest that the side chain of this lysine is recognized by a binding pocket formed by backbone atoms of residues in outer loop 3 and by the backbone oxygen of Glu^{332}, a residue in the SSD (Fig. 12, *B* and *C*). This notion is supported by the finding that substitution of serine or glycine for Val^{579} in outer loop 3 prevents LHR signaling.[4] The essential nature of this lysine suggests that its side chain must occupy this binding pocket during signal transduction. The reverse process, *i.e.* ligand binding induced disruption of the binding pocket, is much less likely to be lysine specific. Because the binding pocket appears to contain elements of the SSD, movements of the LRD that enable residues in outer loop 3 to form this binding pocket would cause helix 7 to move toward the SSD. Although we cannot exclude the possibility that the lysine side chain is in this binding pocket at all times and that helix 7 does not move during signaling, we consider this possibility unlikely.

Differences in the abilities of trypsin to activate the TSHR and analogs in which alanine has been substituted for lysine residues in helices 6 and 7 support the notion that similar movements also occur in the TSHR. TSHR residue Lys^{630} is located at the top of helix 6 in a position that suggests its side chain projects toward TMD outer loop 2 residue Asp^{552} (Fig. 12*C*). Molecular dynamics simulations suggest this lysine could form a salt bridge with this aspartic acid, a phenomenon that would be expected to facilitate movements of helix 6 toward the TMD. Conversion of this lysine to alanine reduced the ability of trypsin to activate the TSHR substantially, albeit not completely (18). In a similar fashion, conversion of TSHR residue Lys^{639} to alanine reduced the ability of trypsin to activate the TSHR (18). The side chain of this lysine, which is found at the top of helix 7 at the location corresponding to LHR residue Lys^{583} and FSHR residue Lys^{580} (Fig. 12), would be expected to participate in a similar binding pocket in all three receptors. Conversion of TSHR residue Lys^{544} to alanine also reduced the ability of trypsin treatment to activate the TSHR (18). This lysine, which occupies a position in outer loop 2 that is conserved in all three receptor classes projects toward helix 7 and may also participate in interactions with backbone atoms of residues in this helix. Conversion of other lysine and arginine

[3] Molecular dynamics simulations were preformed with the program Amber (version 8) running on the Academic Computing Services SunFire 6800 computer. Receptor models were embedded in lipid bilayers containing an outer leaflet of phosphatidylcholine and a cytosolic leaflet of a 1:1 ratio of phosphatidylserine:phosphatidylethanolamine. Both surfaces of the membrane were well hydrated in a water box. Results of these analyses, which are still ongoing, will be described elsewhere.

[4] W. R. Moyle, W. Lin, D. Cao, and M. P. Bernard, unpublished observations.

residues in the TSHR that have been studied have much less influence, if any, on the ability of trypsin to activate the receptor (18). We expect that trypsin activation of the TSHR differs significantly from that of TSH and that it permits constitutive activation of the TSHR by mechanisms that would otherwise be blocked by the position of the LRD in the unoccupied receptor.

Limitations of the Models—At best, the models illustrated here are approximations of the structure of the glycoprotein hormone receptors that have been assembled by combining structural information from several proteins including some that have no sequence similarity to the SSD. Furthermore, we have yet to develop a framework for much of the receptor, including exon 10 of the LHR, parts of the FSHR, and TSHR missing in salmon FSHR analogs, and the cytosolic COOH-terminal domains of all the receptors, a portion of the receptors that is important for their expression, turnover, and signaling (74). Indeed, the structures of bovine rhodopsin suggest that at least two conformations of TMD inner loop 3 may contribute to signaling (4).

Despite these serious limitations, the receptor models provide a conceptual framework that can explain most of the puzzling data regarding ligand binding and signaling that have been accumulated during the long history of work on these proteins. They account for the remarkable finding that substituting two LHR amino acids for their FSHR counterparts enables the FSHR to bind hCG with high affinity despite the fact that these residues are located in a part of the LRD that is likely to be near the TMD. The models explain why the oligosaccharides are important for signaling. Finally, they outline a simple mechanism that can explain how contacts with the extracellular surface of the receptor alter the structure of the receptor in a fashion that would be expected to lead to signal transduction. As such, we expect these models will facilitate studies of glycoprotein hormone and receptor function.

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Model of Glycoprotein Hormone Receptor Ligand Binding and Signaling

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