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Models of Glycoprotein Hormone Receptor Interaction

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The glycoprotein hormones regulate reproduction and development through their interactions with receptors in ovarian, testicular, and thyroid tissues. Efforts to design hormone agonists and antagonists useful for treating infertility and hyperthyroidism would benefit from a molecular understanding of hormone–receptor interaction. The structure of a complex containing FSH bound to a fragment of its receptor has been determined at 2.9 Å resolution, but this does not explain several observations made with cell-surface G protein receptors and may reflect the manner in which FSH binds a short alternate spliced receptor form. We discuss observations that must be explained by any model of the cell-surface G protein–coupled glycoprotein hormone receptors and suggest structures for these receptors that satisfy these requirements. Glycoprotein hormones appear to contact two distinct sites in the extracellular domains of their receptors, not just the leucine-rich repeat domain. These dual contacts contribute to ligand binding specificity and appear to be essential for signal transduction. As outlined in this minireview, differences in the manners in which these ligands contact their receptors explain why some ligands and ligand analogs interact with more than one class of receptor and why some receptors and receptor analogs bind more than one ligand. The unique manner in which these ligands appear to interact with their receptors may have facilitated hormone and receptor co-evolution during early vertebrate speciation.

Key Words: Glycoprotein hormones; glycoprotein hormone receptors; FSH; LH; TSH; hCG.

Overview of Glycoprotein Hormone Receptor Structure

Glycoprotein hormone receptors (Fig. 1) contain large extracellular domains having multiple leucine-rich repeat motifs, a transmembrane domain (TMD) comprised of seven helical membrane-spanning segments, and a cytosolic domain (reviewed in ref. *1*). Binding of glycoprotein hormone ligands to these receptors leads to G protein–coupled signal transduction. Several alternate spliced forms of these receptors are produced, including some that do not encode a TMD *(2)*. The ligand binding capabilities of truncated, alternate spliced, and chimeric receptor analogs *(3–6)* showed that the extracellular portion of the receptor contains the ligand docking site. Most truncated receptor analogs are retained within the cell, however, unless they are attached to anchors *(7)* or unless they are co-expressed with their ligands *(8)* or with the TMD *(9)*. While alternate spliced receptors may be capable of signaling *(10,11)*, they do not appear to do so through G proteins.

The extracellular domains of the glycoprotein hormone receptors contain several leucine-rich repeats and this portion of the LHR was found to bind hCG shortly after the LHR was cloned *(3)*. The leucine-rich repeat domain (LRD) forms the first 250 residues of the extracellular domain in most receptors and is separated from the TMD by a "linker." The linker varies in length from approx 65 to 150 residues, depending on the type of receptor and the species from which it is derived (Fig. 1). It is usually much longer in the TSHR than in the LHR or FSHR. As had been anticipated *(12–14)*, the folding pattern of the LRD in crystals of hFSH complexed with a fragment of the human FSHR extracellular domain *(15)* was found to be related to that of other leucine-rich repeat proteins (Fig. 2).

The amino acid sequences of the linker region are not similar to proteins of known structure, and this region of the receptor is usually not modeled. The linker is often thought to function as a flexible "hinge" that enables the LRD to convey the ligand to the TMD (Fig. 3), a region of the receptor for which it has little or no affinity. Models based on the crystal structure of hFSH bound to a fragment of its receptor *(15)* suggest that the linker tethers the extracellular domain to the TMD in a manner that permits the tips of α-subunit loops 1 and 3 to contact its outer loops (Fig. 4).

A folding pattern for the linker has been proposed *(16)* in only one model of the glycoprotein hormone receptors (Figs. 5 and 6). It was deduced for the shortest linkers, which were identified by comparing the sequences of several vertebrate glycoprotein hormone receptors. An extensive manual search of the SCOP database revealed that the KH domain,

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Fig. 1. Overview of the glycoprotein hormone receptors. Glycoprotein hormone receptors contain an extracellular domain composed of a leucine-rich repeat domain (LRD) and a linker. Both regions contribute to binding of most, if not all ligands, although this is not often appreciated. The LRD is formed from multiple leucine-rich repeats. The linker has usually been considered to function as a hinge that merely enables the ligand to interact with the TMD. This view does not explain the contributions of the linker to ligand binding and signaling, however. An alternate view of the linker *(16)* suggests that it should be termed the signaling-specificity domain (SSD) to reflect its role in ligand binding and signaling. We have proposed that interactions of the LRD and SSD have a key role in the mechanism of signal transduction *(16)*. The transmembrane domain (TMD) and cytoplasmic domain convey the signal that is initiated by ligand binding. The latter domain makes major contributions to receptor regulation and turnover, but these are not considered in this minireview.

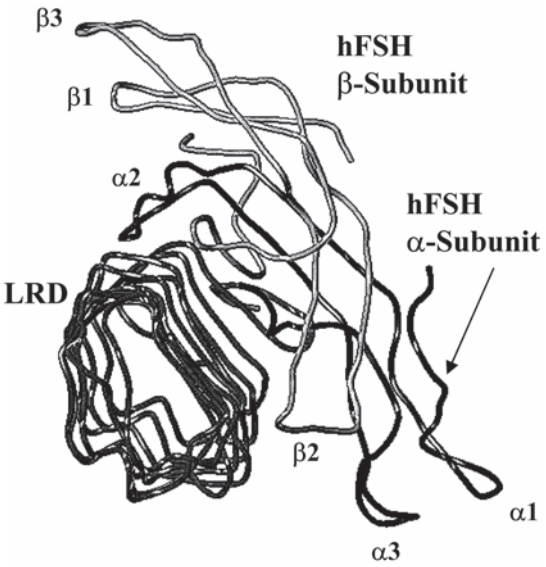

Fig. 2. Crystal structure of hFSH bound to a fragment of the human FSHR. The fragment of the receptor in the crystals contains most of the LRD and has a conformation related to that of other leucine-rich repeat proteins *(15)*. hFSH docks with the concave portion of the LRD such that residues in both subunits contact the concave surface of the LRD. As had been predicted earlier *(13)*, receptor-binding specificity is thought to involve β-subunit residues in loop 2, the small seatbelt loop, and parts of the seatbelt near its latch site *(15)*. Abbreviations: α1, α2, and α3, α-subunit loops 1, 2, and 3, respectively; β1, β2, and β3, β-subunit loops 1, 2, and 3, respectively; LRD, leucine-rich repeat domain.

a common folding motif in nucleic acid and metal-binding proteins *(17,18)*, was a good paradigm for these short linkers *(16)*. Remarkably, KH domain proteins normally lack disulfide bonds and their sequences are not related to those of the linkers in any glycoprotein hormone receptor. Nonetheless, when modeled on the KH domain, these short linker sequences formed structures that had appropriate disulfides and could be aligned readily with the LRD and the TMD to explain the roles of the linker in LHR, FSHR, and TSHR

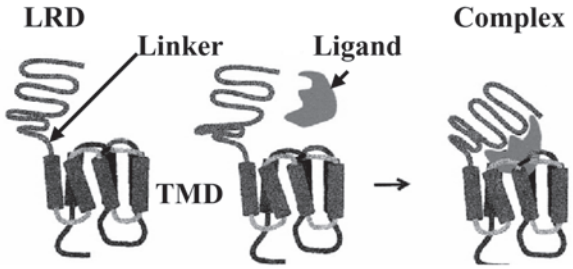

Fig. 3. View of the receptor in which the linker serves as a hinge. In some models of the glycoprotein hormone receptors, the linker functions as a flexible hinge that permits the LRD to capture the ligand so that it can then bind to the transmembrane domain. This suggests that two surfaces of the ligand be obscured in the hormone–receptor complex. In contrast to this prediction, most of the ligand is exposed in the hormone–receptor complex and only one surface appears to be blocked by the receptor. This figure has been modified from Ji et al. *(30)*.

ligand binding and signaling *(16)*. The linker does not function as a hinge in any of these receptor models and is termed the signaling-specificity domain (SSD) to reflect its key roles in ligand binding, ligand-binding specificity, and signal transduction *(16)*. These functions of the SSD are readily apparent from its position in the models (Figs. 5 and 6).

The TMD appears to be composed of seven-membrane-spanning α-helices and is usually modeled on the structure of bovine rhodopsin, the only G protein for which a crystal structure has been reported *(19)*. The positions of the TMD helices are stabilized by a hydrogen bond "cage" (Fig. 7). Mutations of residues that constrain the cage often lead to constitutive hormone activity *(20)* and can influence ligand binding specificity *(21,22)*.

Contrasting Views of Hormone–Receptor Interactions

The manner in which glycoprotein hormone ligands interact with the extracellular domains of their receptors remains controversial. Jiang et al. *(13)* developed the view of the hormone receptor complex that has dominated this field for 10 yr. This model was constructed by docking the crystal structures of hCG *(23,24)* to the concave surface of ribonuclease inhibitor *(25)*, a horseshoe-shaped protein composed entirely of leucine-rich repeats, each of which contains more residues than those in the LRD of the glycoprotein hormone receptors. The Jiang model was built on the notions that charge–charge interactions control ligand–receptor binding specificity and that all three hormones contact a similar region of the concave surface of the LRD. Several predictions of the model *(13)*, namely, that parts of the α-subunit C-terminus (αCT), β-subunit loop 2, and most of the small seatbelt loop contact the surface of the LRD, approximate the manner in which hFSH docks with the LRD fragment in the crystal structure (Fig. 2).

A major shortcoming of the Jiang model was that it did not provide an explanation of ligand-induced signaling. In

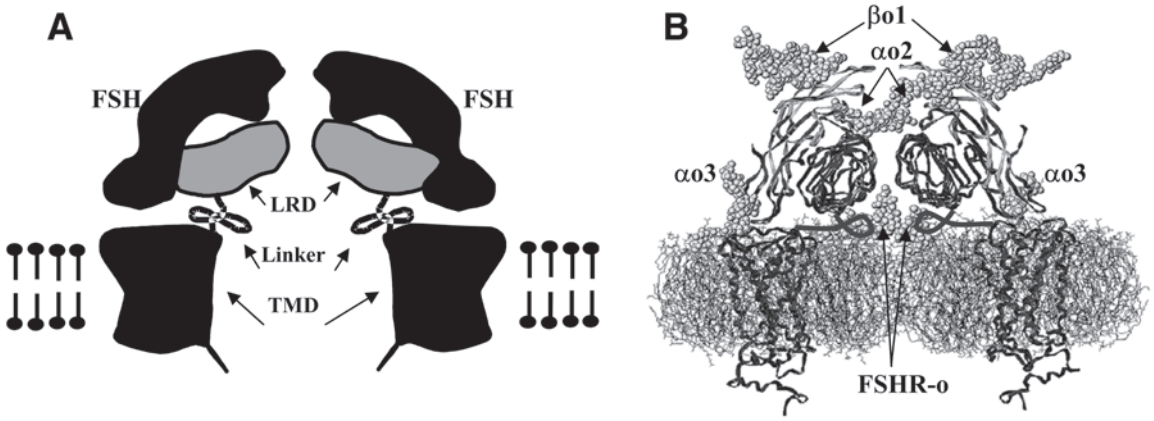

Fig. 4. Signal transduction for receptor models in which ligands contact the concave surface of the LRD. Panel **A** (Left): Cartoon model redrawn from Fan and Hendrickson *(15)*. In this model the receptor is thought to function as a homodimer containing two molecules of ligand and two molecules of receptor. Panel **B** (Right): Figure assembled from components found in the crystals, model oligosaccharides, and a model of the transmembrane domain in a lipid bilayer to show the sizes and positions of the oligosaccharides relative to components of the receptor and the plasma membrane. The TMD and plasma membrane were subjected to extensive molecular dynamics before being incorporated into this figure. Signal transduction is thought to be caused by dimerization of the TMD, although the mechanism of signaling remains to be determined because it is unlikely that dimerization of the receptor would permit interactions between each TMD. The oligosaccharides (gray spheres) on α-subunit loop 3 (αo3) and the receptor (FSHR-o) would appear to contact the TMD and lipid bilayer. The oligosaccharides on α-subunit loop 2 (αo2) have an important role in hormone efficacy, but their role in signaling is difficult to visualize since they project away from the receptor. The receptor components are labeled on panel A and correspond to those parts of hFSH identified in Fig. 2. Remaining abbreviation: βo1, the two oligosaccharides on β-subunit loop 1.

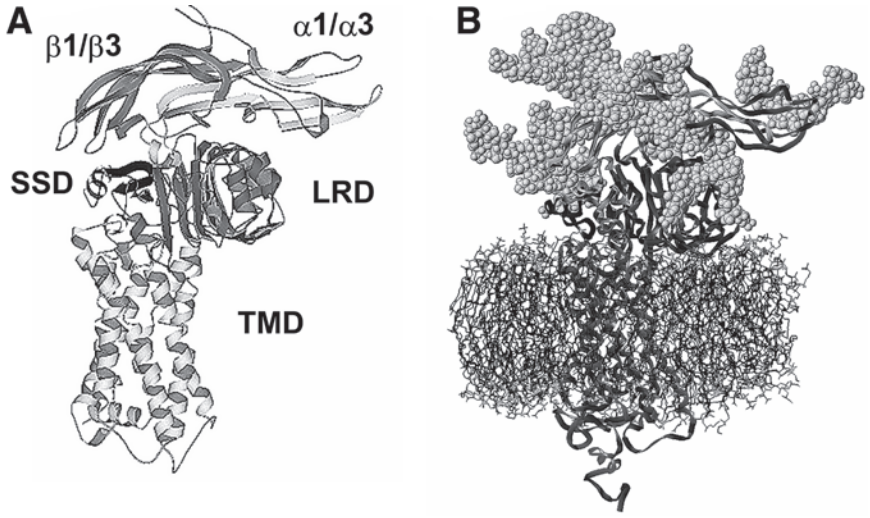

Fig. 5. Interaction of lutropins with a model of the LHR in which the LRD, SSD, and TMD are interconnected and in which ligands interact with both the SSD and LHRD. Panel **A** (left) Molscript drawing of the complex labeled to show its components. In this model of the receptor the ligand contacts surfaces of the LRD and the SSD distant from the TMD. Panel **B** (right) Figure showing the structure of a glycosylated LHR in a lipid bilayer following extensive molecular dynamics. (Note that the oligosaccharides on β-subunit loop 1 were added to the figure after the dynamics simulations.) In this model, the ligand contacts a different region of the LRD than in hinge models (Figs. 3 and 4). As can be seen, β-subunit loops 1 and 3 make significant contacts with the SSD, which has a key role in ligand binding and signal transduction. Loops α1 and α3 do not contact the receptor. Part of loop α2 (not labeled) contacts a groove between the SSD and LRD. Both the LRD and SSD contact the TMD, but none of the oligosaccharides on the hormone and the receptor project toward the lipid bilayer even though the LHR is more heavily glycosylated than the FSHR shown in Fig. 4. The oligosaccharides on the hormone and receptor in panel **B** are shown as clusters of gray spheres. The figure in panel A was taken from Moyle et al. *(16)*.

models in which the linker functions as a "hinge" (Fig. 3), this problem is solved by the ability of the linker to constrain the LRD-bound ligand near the TMD. Theoretically, this would permit a second surface of the ligand to interact with the TMD, a portion of the receptor for which it has little affinity. Movements of the LRD have been proposed to permit the conserved lysine in the αCT to interact with negatively charged residues in the outer loops of the TMD *(26–28)*. The notion that the hormone contacts both the LRD and the TMD also predicts that two or more surfaces of the

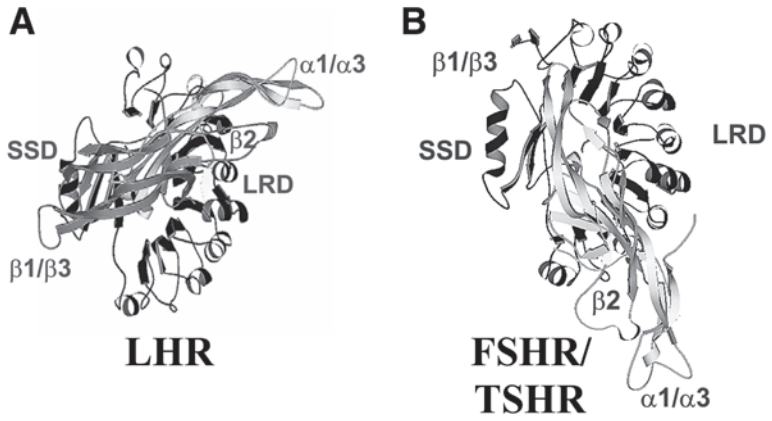

Fig. 6. Ligand recognition by LHR, FSHR, and TSHR. Panel **A** (left). Top view of hCG bound to the LHR. Panel **B** (right). Top view of FSH or TSH bound to the FSHR or TSHR. Receptor models in which the LRD, SSD, and TMD form an integrated unit (Fig. 5) suggest that ligand binding can occur in two (or more) fashions. Mammalian lutropins and many piscine follitropins are thought to bind to their receptors as shown in Panel **A**. Mammalian follitropins, thyrotropins, and most other vertebrate glycoprotein hormones are thought to dock with their receptors as shown in Panel **B**. A portion of α-subunit loop 2, the most highly conserved portion of all vertebrate glycoprotein hormones, is thought to contact the groove between the LRD and SSD. Owing to the organization of the receptors, α-subunit loop 2 is located in similar portions of the groove between the SSD and LRD regardless of the orientation in which the ligands bind. This enables some ligands to bind and activate receptors of each type. It also enables some receptors to bind multiple ligands. This mode of ligand binding will also account for the co-evolution of these hormones and their receptors since mutations in one part of the receptor can occur independently of those in another part of the receptor *(5)*. These figures are modified from Moyle et al. *(16)*.

hormone would be obscured by the receptor (Fig. 3). As discussed later, neither of these predictions appears to be true. Nonetheless, the notion that the LRD can "flip" from one portion of the receptor to another would explain hormone-induced signaling observed when receptor analogs that contain complementary defects were co-expressed in mammalian cells *(29,30)*. This finding may indicate that the receptors function as homodimers, but this has yet to be determined by direct measurements.

The observation that an α-subunit monoclonal antibody was capable of binding to truncated receptors, but not to intact receptors *(31)*, led Remy et al. *(32)* to suggest that docking of the hormone to the LRD enabled the tips of α-subunit loops 1 and 3 to contact the transmembrane domain. In this model (Fig. 4), signaling results from interactions of the convex surface of α-subunit loops 1 and 3 with the outer surface of the transmembrane domain *(32)*. Fan and Hendrickson *(15)* also proposed that the tips of α-subunit loops 1 and 3 contact the transmembrane domain and noted that formation of this type of complex would require a long linker (Fig. 4). Based on the observations that the unit cell contained two molecules of FSH bound to the FSHR fragment and that these can be crosslinked using appropriate chemical reagents, Fan and Hendrickson *(15)* suggested that signal transduction may involve receptor dimerization. Nonetheless, the TMD of each receptor component would remain widely separated in the receptor dimer (Fig. 4) and the manner in which dimerization of the receptor would lead to G protein activation remains unexplained.

An alternate view of the hormone–receptor complex proposed that the hormone docks with the rim of the receptor extracellular domain at two separate sites *(12)*, an upstream site in its N-terminal two-thirds and a downstream site near its C-terminal end. Signal transduction required the ligand to alter the conformation of the extracellular domain, which in turn altered the conformation of the TMD. These notions were refined recently to incorporate a structure for the C-terminal portion of the extracellular domain—now termed the SSD—and new information on the manner in which the ligands dock with LHR, FSHR, and TSHR (Figs. 5 and 6). In the current version of this model *(16)*, the orientation of the ligand is reversed such that β-subunit loops 1 and 3 contact the SSD, not the LRD. The overall mechanism of signaling remains the same, however, namely that interactions of the ligand with the LRD and the SSD are required for signal transduction *(12,16)*. In the revised model, the LRD, SSD, and TMD are thought to contact one another and to function as an "interacting" unit. Docking of the ligand to the SSD and LRD moves the position of a few residues in the switch region of the SSD toward the LRD (Moyle and Kerrigan, unpublished molecular dynamics simulations), thereby initiating the change in the TMD needed for signaling. The notion that the ligand does not need to contact the TMD for signaling is consistent with the abilities of TSHR antibodies to the extracellular domain of the TSHR to stimulate thyroid function *(33)*. Few, if any, of these antibodies would be expected to interact with the TMD in the fashion that was proposed based on the crystal structure of hFSH bound to a fragment of the FSHR *(15)*.

In models of the hormone receptor complex that depend on a hinge for signal transduction, the linker functions only to tether the LRD to the TMD (Figs. 3 and 4). In the model

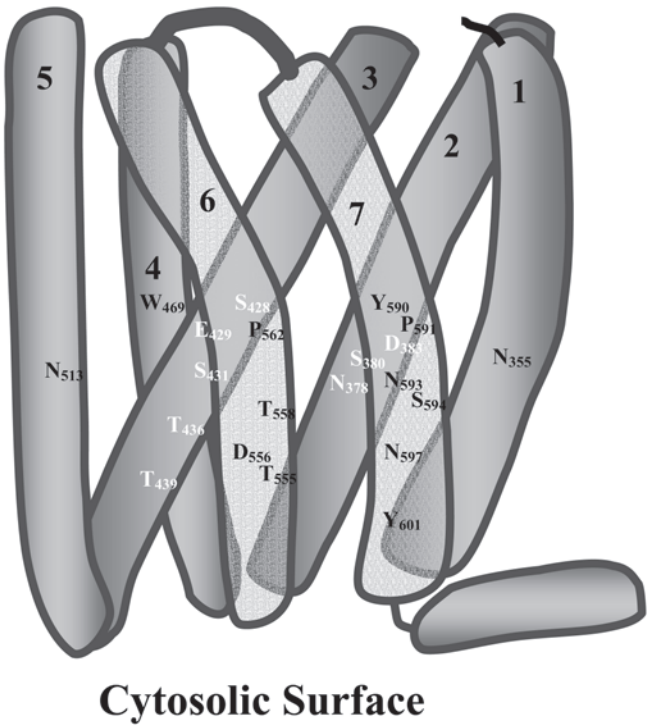

Fig. 7. Cartoon describing the relative positions of residues that form the TMD cage. The approximate positions of each TMD helix are represented as "sausage-shapes" that are numbered to reflect their relative positions in the membrane. In this perspective, helix 4 is behind helix 3; helices 6 and 7 are nearest the reader and in front of helices 1, 2, and 3; helix 1 is in front of helix 2. Residues shown in black are in helices 1, 4, 5, 6, and 7. Those shown in white are in helices 2 and 3. Hydrogen bonds between residues that are labeled on the helices stabilize the transmembrane domain in an arrangement similar to that represented here. The SSD rests on top of helices 1–5 and the LRD forms a "banana-shaped" structure that is located above the TMD. In this orientation the convex surface of the LRD faces the viewer and obscures the SSD. A few residues at the bottom rim of the LRD are thought to contact residues in the outer loops of the TMD near the tops of helices 5, 6, 7, and 1.

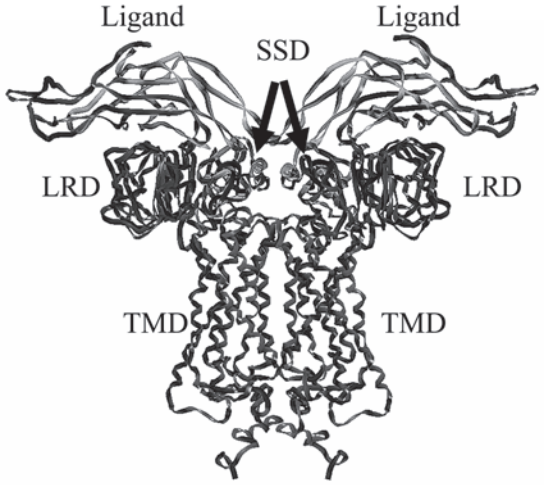

Fig. 8. Potential homodimer formation by contacts between two receptors in models in which the LRD, SSD, and TMD form an integrated unit. This model describes a mechanism for the formation of receptor homodimers based on the model of the hCG–LHR complex depicted in Fig. 5. The homodimer is this illustration was formed by aligning the models such that the SSD and TMD of one receptor contact those of another receptor. This type of homodimer differs from that described by Fan and Hendrickson *(15)* in which the TMD of each receptor are unable to contact one another (Fig. 4). There is no evidence that this type of homodimer forms on the cell surface, however. Using total internal reflection fluorescence microscopy, we have found that if this type of homodimer does form, it binds only one molecule of ligand and would differ from that shown here, which has two molecules of bound ligand.

in which the linker is folded into an SSD *(16)*, it is located adjacent to the concave surface of the LRD and contacts both ends of the LRD and a portion of the TMD (Figs. 5 and 6). The space between the SSD and the LRD forms an elongated arc-shaped groove (Fig. 6) that will accommodate a portion of the highly conserved helix at the end of α-subunit loop 2. Docking of ligands with the outermost surface of the receptor (Fig. 5) would enable this helix to contact the groove between the SSD and LRD even though other parts of lutropins, follitropins, and thyrotropins appear to bind to different sites in the LRD of their receptors (Fig. 6).

Unlike models in which ligands are recognized only by similar concave surfaces of the LRD *(15)*, those in which the LRD and SSD contribute substantially to ligand binding and signaling *(16)* permit lutropins, follitropins, and thyrotropins to interact with their receptors in different fashions (Fig. 6). This will explain the abilities of some natural hormones—e.g., equine CG or PMSG *(34)* and salmon LH *(37)*—to interact with mammalian and salmon FSHR, respectively, even though their sequences are unlike those of follitropins. This is most remarkable in the case of the salmon follitropins and lutropins since their folding patterns, not just their sequences, differ from one another. It will also explain the abilities of analogs of hCG *(5,35,36)* and hFSH *(38)* to interact with both lutropin and follitropin receptors. The notion that lutropins interact with different portions of the LRD than follitropins and thyrotropins also explains the abilities of mammalian receptor chimeras to bind and respond to multiple ligands *(5,6,39)*.

The positions of the LRD and SSD relative to the TMD in models in which all three components form an interactive complex suggest that this type of receptor can form homodimers in which the TMD of both receptors contact one another (Fig. 8). While there is no evidence that this occurs on the cell membrane normally, this type of complex could also account for the abilities of some defective receptor analogs to complement one another *(29,30)*.

Observations That Describe How Glycoprotein Hormones Recognize Their Receptors

The glycoprotein hormones have long been known to be αβ heterodimers containing a common α-subunit and a hormone-specific β-subunit *(40)*. The structures of hCG *(23,24)*

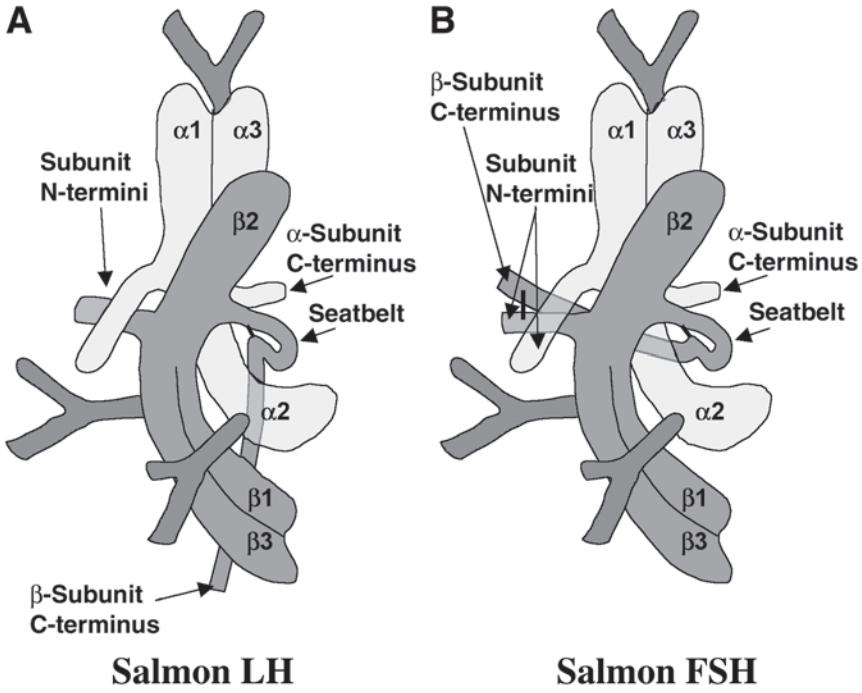

Fig. 9. Cartoon comparing the structures of the two folding patterns found in vertebrate glycoprotein hormones. All vertebrate lutropins and thyrotropins have the folding pattern seen in the left panel, as do most follitropins. Some teleost fish follitropins have the folding pattern seen in the right panel. The salmon FSH receptor binds salmon LH and salmon FSH, indicating that it must be capable of recognizing heterodimers that have altered folding patterns. Since the overall structure of the piscine receptors is similar to that of all other vertebrates, it seems unlikely that the manner in which the piscine LH and FSH receptors recognize these ligands is unique.

and hFSH *(41)* revealed that both subunits are divided into three large loops by cystine knots and that the subunits contact one another such that α-subunit loops 1 and 3 are near β-subunit loop 2 and that β-subunit loops 1 and 3 are near α-subunit loop 2 (Fig. 9). Loop 2 of the α-subunit is surrounded by 20 β-subunit residues known as the "seatbelt" *(23)*. The seatbelt is "latched" by a disulfide to a cysteine in β-subunit loop 1 in most glycoprotein hormones (Fig. 9, left panel). The seatbelts of some teleost fish follitropins are latched to a cysteine in the N-terminal end of the β-subunit (Fig. 9, right panel). This results in a marked difference in the positions of the hormone residues between the eleventh and twelfth β-subunit cysteines. Models of the salmon LH receptor must be able to account for their abilities to interact with salmon LH and FSH *(37)*, proteins in which the seatbelt is latched differently (Fig. 9).

In addition to its role in heterodimer stability, the seatbelt is responsible for much of the influence of the β-subunit on receptor binding specificity *(5,35,36,38)*, a phenomenon that has implications for the manner in which these ligands dock with their receptors. Models of glycoprotein hormone action must explain how the seatbelt influences receptor binding specificity and why other portions of the β-subunit that differ substantially in all three classes of hormones—e.g., β-subunit loop 2—do not appear to contribute to the abilities of these ligands to distinguish their receptors. The models must also explain how some ligands—e.g., equine CG—and hormone analogs can interact with multiple receptors *(5,34)*. Finally, they should also account for the co-evolution of these ligands and their receptors *(5,42)*.

Unlike most dimeric proteins, the glycoprotein hormones are not stabilized by hydrophobic intersubunit contacts. Instead, they contain a hydrogen bond stabilized β-sheet that forms when the stem region of α-subunit loop 2 becomes located adjacent to residues in the β-subunit cystine knot and part of the small seatbelt loop *(43)*. Formation of this sheet appears to drive heterodimer assembly *(43)*. One surface of this β-sheet lies under the seatbelt, which restricts movements of α-subunit loop 2 that would disrupt the heterodimer. This structural arrangement may be responsible for small differences in the conformations of hCG and hFSH and for the influence of the seatbelt on receptor binding specificity *(5,35,36,38)*. It may also make the conformation of the heterodimer easily modified during studies designed to identify receptor contacts, a phenomenon that would confound analysis of ligand receptor interactions.

Much of the information used to devise early models of the hormone–receptor complex was obtained before the structures of hCG and hFSH were determined *(13)*. Carboxypeptidase removal of the αCT reduced hormone binding and signaling activities dramatically *(40)*. This observation is usually interpreted to mean that the αCT might be a key re-

ceptor contact. Differences in the abilities of carboxypeptidase to remove residues from the αCT in the heterodimer and the free subunit suggested that the αCT was near the subunit interface, a prediction confirmed by the crystal structures of hCG *(23,24)* and hFSH *(41)*. The apparent location of the αCT near the subunit interface led Pierce and Parsons to caution against interpreting the finding that the loss in hormone activity caused by removal of the αCT *proves* that it is a receptor contact site *(40)*. They noted that its removal might reduce hormone activity by altering the conformation of the heterodimer. We found that removal of the αCT from hCG increased its ability to be recognized by monoclonal antibodies that have higher affinity for the free α-subunit than the heterodimer *(12)*. Because this showed that the αCT can influence heterodimer conformation, conclusions as to its role in receptor contacts based solely on its ability to influence hormone activity appeared to be unwarranted and, for reasons discussed next, this led us to rely on alternate methods to identify regions of the hormones that dock with their receptors.

The heterodimer has long been known to be much more active than either isolated subunit in receptor mediated assays *(40)*. Thus, both subunits might participate in receptor interactions and/or a conformation of one subunit unique to the heterodimer may be required for receptor interaction. Consequently, small changes in subunit interaction could have an undue influence on receptor binding. To avoid the inevitable difficulty in distinguishing the influence of hormone mutations that disrupt specific receptor contacts from those that alter binding by changing the conformation of the heterodimer, we identified portions of the ligand that do not contact the receptor. These can be determined unambiguously using monoclonal antibodies to detect hormone epitopes that remain exposed in the hormone–receptor complex *(44–46)*. Hormone residues that participate in the binding sites of these antibodies have been identified by determining the abilities of the antibodies to bind hormone chimeras and other analogs. By mapping the binding sites of antibodies that recognize hCG and hFSH receptor complexes on the surfaces of hCG and hFSH, we determined the maximum surface of the ligand that could interact with the receptor *(16)*. This large surface, which is found exclusively on one face of the hormone (Fig. 10), includes portions of both subunits. Ligand residues in some areas of the hormone that cannot be detected with antibodies are not likely to participate in key receptor contacts and have been excluded using other methods. For example, β-subunit loop 2 appears to be one of the most variable regions in the glycoprotein hormones and differs substantially in lutropins, follitropins, and thyrotropins (Table 1). This suggests it would alter ligand binding if it contributes contacts that reflect contributions of the β-subunit to receptor binding. The finding that β-subunit loop 2 can be swapped among the hormones without disrupting receptor binding or enabling the hormone to bind a different

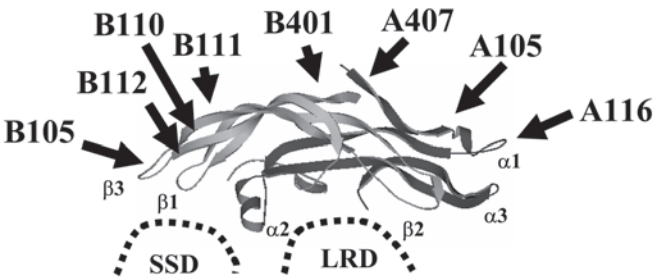

Fig. 10. Surface of the ligand exposed in the hormone receptor complex. A large surface of hCG and hFSH can be detected with monoclonal antibodies after these ligands bind LHR and FSHR. The numbers refer to the identities of monoclonal antibodies that recognize hCG and/or hLH–LHR complexes. Some of these anti-α-subunit antibodies, i.e., A105 and A116, also recognize FSH–FSHR complexes. Many β-subunit antibodies, e.g., B105, B110, and B112, recognize hCG–hFSH chimeras while they are bound to FSHR complexes. Binding of hCG to the rat LHR increases its affinity for B110 *(55)*. B105 has almost no affinity for bovine LH or most other mammalian LH molecules until after they become bound to rat LHR *(55)*. These observations indicate that the conformations of loops β1 and β3 become altered during LHR binding, a phenomenon that suggests they are near the receptor interface. Loop β2 can be replaced by its hFSH counterpart without disrupting binding to FSHR *(35)*, indicating that this portion of the molecule is unlikely to participate in key receptor contacts. The relative positions of the LRD and SSD are indicated below the hormone.

receptor shows that it is not a key receptor contact *(5,35, 36,38)*.

Most models of the hormone–receptor complex (13;15; 16) suggest that the αCT contacts the receptor, albeit in different ways. The notion that the lysine in the αCT projects into a binding pocket created by the outer loops of the TMD *(26–28)* is not consistent with the observation that the end of the αCT can be crosslinked to several residues in the small seatbelt loop of hCG without disrupting its lutropin activity *(47)*. The latter finding also makes it unlikely that the αCT and the small seatbelt loop contact cell-surface G protein–coupled receptors in the same fashion that FSH contacts the FSHR fragment in crystals. The only model that is consistent with the finding that the αCT can be crosslinked to several residues in the small seatbelt loop without destroying hormone activity *(16)* is that in which the α-CT contacts the receptor but is not buried between the ligand and the receptor (Fig. 5).

Sequence comparisons led to the "determinant loop" hypothesis *(48)*, which suggested that charged residues in the small seatbelt loops of mammalian lutropin, follitropin, and thyrotropin β-subunits (i.e., residues 94–96, 88–90, and 89–91, respectively) are receptor contacts that confer binding specificity. This widely accepted notion was used to dock hormones with their receptor in the Jiang models *(13)*. As noted earlier, the αCT can be crosslinked to the small

Table 1
Sequences in Loop 2 and the Seatbelt of Selected β-Subunits[a]

β-Subunit	Receptor activity	β-Subunit Loop 2
hCG	LHR	ptmtrvl**Q**g**--VLPP**lpqvv
hLH	LHR	ptmmrvl**Q**a**--VLPP**lpqvv
Equine LH	LHR,FSHR,TSHR	psmvrvm**P**a**--ALPA**ipqpv
hFSH	FSHR	ytrdlvy**K**d**--PARP**kiqkt
hTSH	TSRH	mtrding**K**lfl**PKYA**lsqdv
CF(94–117)	LHR,FSHR,TSHR	ptmtrvl**Q**g**--VLPA**lpqvv
CF(101–109)	LHR and FSHR	ptmtrvl**Q**g**--VLPA**lpqvv
CFC(39–58)	LHR	ytrdlvy**K**d**--PARP**kiqkt
		Seatbelt Region <-Loop->< — Strap— > βCT->
hCG	LHR	alcr**RS**tt**DCGGPKD**hpl**T**c**D**dpr
hLH	LHR	gpcr**RS**ts**DCGGPKD**hpl**T**c**D**hpq
Equine LH	LHR,FSHR,TSH	gpcq**IK**tt**DCGVFRD**qpl**A**c**A**pqa
hFSH	FSHR	gkcd**SD**st**DCTVRGL**gps**Y**c**S**fge
hTSH	TSRH	gkcn**TD**ys**DCIHEAI**ktn**Y**c**T**kpq
CF(94–117)	LHR,FSHR,TSHR	alcd**SD**st**DCTVRGL**gps**Y**c**S**fge
CF(101–109)	LHR and FSHR	alcr**RS**tt**DCTVRGL**gps**Y**c**D**dpr
CFC(39–58)	LHR	alcr**RS**tt**DCGGPKD**hpl**T**c**D**dpr

[a]Upper case bold letters correspond to β-subunit residues found to contact the LRD in the crystal structure of hFSH bound to the human FSHR *(15)*. The small seatbelt loop (Loop) contains residues between the tenth and eleventh cysteines. The "strap" region of the seatbelt is its C-terminal half and contains residues between the eleventh and twelfth cysteines. The βCT contains residues downstream of seatbelt. Note that equine LH and equine CG (PMSG) have the same amino acid sequences but the latter is more highly glycosylated. We have found that equine LH has substantial activity in TSHR assays employing CHO cells that express the human TSHR. The data for the activities of the hCG/hFSH chimeras [CF(94–117), CF(101–109), and CFC(39–58)], in which the indicated hCG β-subunit residues are replaced with their hFSH counterparts have been described *(7,10,54)*.

seatbelt loops of hCG *(47)* and to hCG analogs that bind FSHR (Bernard, Cao, Lin, and Moyle, unpublished observations) without disrupting their abilities to elicit LHR and FSHR cyclic AMP accumulation. In the case of hCG–LHR interactions, we studied the activities of analogs containing disulfide crosslinks between the αCT and each residue in the small seatbelt loop (i.e., residues 94, 95, 96, 97, 98, and 99) to learn which of these did not block the action of hCG *(47)*. Disulfide crosslinks between the αCT and residues 95 and 99, but not those between other residues in this region prevented interactions of hCG with the LH receptor. Remarkably, the crosslink at residue 95 blocked binding only when a linker was introduced between the end of the αCT and the β-subunit *(47)*. These findings showed that most residues in the small seatbelt loop do not participate in critical LH-receptor contacts, a phenomenon consistent with a model *(16)* in which this portion of the hormone contacts the receptor but is not buried in the ligand-receptor interface (Fig. 5).

hCG analogs in which the small seatbelt loop is derived entirely from hFSH have approx 10–12-fold lower affinity than hCG for rat LH receptors and do not bind to FSH receptors *(35)*. The abilities of charged residues in the small seatbelt loop to influence LH-receptor binding is increased by an order of magnitude when the remainder of the seatbelt—i.e., residues between the eleventh and twelfth β-subunit cysteines—is derived from hFSH despite the fact that this region of the seatbelt does not influence binding of hCG to the full-length rat LHR *(5,49)*. This suggests that changes in receptor binding caused by many substitutions in the small seatbelt loop may reflect their abilities to alter the conformation of the heterodimer. Finally, an essential role for the small seatbelt loop in LH-receptor contacts is not consistent with the observation that a large number of lutropins interact with most mammalian LH receptors even though the composition of residues 94–96 is not highly conserved.

The notion that charged residues in the small seatbelt loop participate in essential FSH receptor contacts is contradicted by the observation that introduction of positively charged residues into hFSH did not prevent it from interacting with FSHR receptors even though it increased its

ability to bind LH receptors *(38)*. The role of the small seatbelt loop in binding to the full-length cell-surface FSHR differs from predictions of ligand-binding specificity based on the crystal structure of hFSH bound to the truncated FSH receptor fragment *(15)*. The crystal structure suggested that βSer89 and βAsp90 in the small seatbelt loop of hFSH—i.e., residues that are arginine and serine in hCG—make key receptor contacts. This notion is not consistent with the finding that the entire FSH small seatbelt loop can be replaced with its hCG equivalent without preventing FSH from binding to its receptor *(38)*. This substitution converted Ser89 and Asp90, residues proposed to make key contacts with the receptor *(15)*, to arginine and serine. The postulate that the small seatbelt loop makes essential FSH-receptor contacts is also not consistent with the finding that this loop can be modified extensively in bifunctional hCG analogs—i.e., those that bind LH and FSH receptors—without reducing their affinities for FSH receptors *(49)*. Thus, the small seatbelt loop appears to make different contributions to LHR and FSHR interactions, a notion that is readily explained by a model in which lutropins and follitropins contact their receptors in different orientations *(16)*.

The idea that β-subunit loop 2 makes a key receptor contact has been given undue weight in models of hormone–receptor interaction. Experimental support for the notion that this loop is a receptor contact is based primarily on reports that very high concentrations of synthetic peptides having the sequences of β-subunit loop 2 in hCG and hFSH may interact with lutropin and follitropin receptors *(50,51)* and that monoclonal antibodies to this loop block the binding of ligands to the receptors *(44)*. Additional support for this notion comes from the finding that this region of hFSH contacts the receptor in crystals of FSH bound to the FSH receptor fragment *(15)*. Several observations suggest that this loop has little, if any role in ligand interactions with cell-surface G protein–coupled receptors, however. Loop 2 is one of the least conserved portions of the β-subunit and differs markedly in lutropins, follitropins, and thyrotropins (Table 1). Therefore, it would be expected to influence receptor binding specificity if it made key contacts with these receptors. In contrast to this prediction, swapping β-subunit loop 2 from one hormone to another had little influence on receptor binding. For example, hCG analogs containing hFSH β-subunit loop 2 in place of their hCG counterparts bound LH receptors like hCG and did not bind FSH receptors *(35)*. Furthermore, substitution of hFSH β-subunit loop 2 into hCG analogs that bound to FSH receptors did not improve their affinities for the FSH receptor *(35)*. Loop 2 of the TSH β-subunit can also be replaced without altering its ability to bind and activate TSH receptors *(36,52)*. Nonetheless, amino acid substitutions in β-subunit loop 2 that alter the natural sequences found in this region of the hormone can reduce binding to receptors *(53,54)*. This may be a consequence of the fact that this loop makes important contacts with α-subunit loops 1 and 3 and that mutations that alter these contacts are likely to alter the conformation of the heterodimer *(23,24)*.

The biological activities of natural hormones and hormone analogs are difficult to explain based on the key ligand–receptor contacts observed in crystals of hFSH complexed with the LRD fragment of its receptor. hFSH β-subunit residues thought to form important contacts in the crystal structure *(15)* include some from loop 2 (i.e., K-PARP), the small seatbelt loop (SD), and the C-terminal half of the seatbelt (Y-S). These residues are highlighted in Table 1 and many of them are not found in hormones that have high activities in FSHR binding and signaling assays. For example, the contacts seen in the crystal structure do not explain how hCG analogs containing only those FSH β-subunit seatbelt residues between the eleventh and twelfth cysteines bind FSHR with at least 30% the affinity of hFSH *(5)*. Furthermore, the crystal structure does not explain how equine CG binds FSH receptors *(34)*. There are only 3 β-subunit residues that are identical in hFSH and equine CG out of the 16 that were found to contact the receptor in the crystals of hFSH bound to the FSHR fragment *(15)*. Two of these three are found in nearly all β-subunits. These observations suggest that the binding of hFSH to a fragment of its receptor in the crystal structure may differ considerably from the manner in which it binds cell-surface G protein–coupled receptors.

Surfaces of the Hormones That Remain Exposed in the Receptor Complex

As noted earlier, the abilities of antibodies to bind hCG receptor complexes revealed that most of the convex surfaces of α-subunit loop 1 and β-subunit loops 1 and 3 do not contact the LH receptor (Fig. 10). Some of these regions in the β-subunit appear to be near the receptor interface; however, even though this would not be expected based on the crystal structure of hFSH bound to a fragment of its receptor *(15)*. Antibodies that recognize the seatbelt latch site and nearby residues of β-subunit loop 1—i.e., B111 (Fig. 10)—restore efficacy to hCG analogs that lack the oligosaccharide on α-subunit loop 2 *(16)*. Furthermore, a monoclonal antibody to an overlapping region of hCG that has almost no ability to bind bovine LH in solution—i.e., B105 (Fig. 10)—had high affinity for bovine LH and several other mammalian lutropins when they were complexed with rat LHR *(46,55)*. B105 has high affinity for an hCG β-subunit epitope that includes loop 3 near residue Arg74 *(46)*. Arg74 is replaced by proline in most mammalian lutropins, a change that is primarily responsible for the inability of B105 to bind most mammalian lutropins *(46)*. The presence of glycine and proline residues in the tip of β-subunit loop 3 of lutropins suggests this region may be deformed easily, which would account for the increase in the affinity of B105 for this site following LHR binding *(55)*. Although it is con-

ceivable that the change in affinity is due to the α-subunit, this appears unlikely because B105 binds hCG and its free β-subunit equally well. Thus, we interpret the data obtained with antibodies B105, B110, and B111 as support for the idea that the tips of lutropin β-subunit loops 1 and 3 are near the receptor interface. This notion is readily explained by the model of the ligand–receptor complex in which the LRD, SSD, and TMD contact one another (Fig. 5), but not by other models, including those based on the crystal structure of FSH complexed with a fragment of its receptor (Figs. 2 and 4). Indeed, it is difficult to explain the activities of these antibodies in the latter model *(15)* because the tips of β-subunit loops 1 and 3 are distant from the receptor interface.

Antibodies are large molecules and even their Fv fragments are too big for them to detect ligand residues that are near the receptor interface, but that do not contact the receptor. To circumvent this problem and to facilitate studies of more localized regions of the hormone–receptor interface, we developed a technique that employs "protein knobs" to probe contacts between protein ligands and their receptors *(47,56)*. This procedure involves the introduction of disulfide stabilized peptides or proteins at predetermined sites on the glycoprotein hormone surface (Fig. 11). It was based on our finding that disordered parts of hCG appear to "scan" the surrounding surfaces of the subunits as they fold, a process that continues after the heterodimer is assembled. A knob can usually be created at a desired site by replacing the residue to be "knobbed" with a cysteine and by incorporating a cysteine in the disordered region that is to be used as a knob. This region is usually at the N- or C-terminal end of the protein to be knobbed or at either end of its binding partner. For example, it is possible to use the disordered region of the hCG β-subunit as a linker when knobs are to be added to the glycoprotein hormone α-subunit *(56)*. If the cysteine in the disordered region encounters the cysteine at the residue to be knobbed as the protein folds in the endoplasmic reticulum, it can form a stable disulfide at that site.

In principle, this approach can be used to tether any protein to a specific residue on the surface of another protein (Fig. 11). Because a knob can be varied in size from a single residue to large proteins—i.e., the cysteine used to form the crosslink to β-lactamase or GFP—it is often possible to approximate the relative distances between ligands and their receptors by monitoring how different sized knobs alter ligand–receptor interactions. Furthermore, the distance between specific ligand residues and the receptor can also be probed using linkers and connectors of different lengths (Fig. 11). Finally, by introducing a proteolytic cleavage site into the linker that is clipped during passage of the protein through the Golgi, it is possible to minimize the influence of the linker on estimates of ligand–receptor interactions. An attractive aspect of this approach is that attachment of the knob occurs during protein folding in the cell.

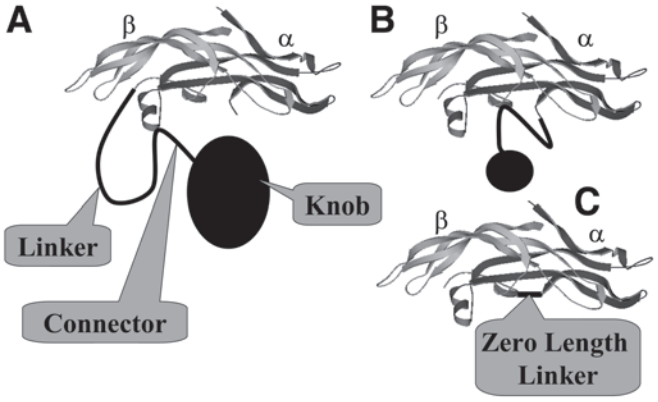

Fig. 11. Basis of the protein knob technique. Protein knobs are produced by forming a disulfide crosslink between a cysteine substituted for a desired test site on a protein and cysteines in a disordered linker. The illustration in panel **A** depicts the protein that formed when a construct containing a cysteine in place of a residue in α-subunit loop 2 was expressed with an hCG β-subunit β-lactamase (βLA) fusion protein. The fusion protein contained a cysteine substituted for a residue in the disordered β-subunit C-terminus (βCT) that links the β-subunit and the enzyme. The resulting analog contained a disulfide crosslink that tethered the βLA to the α-subunit *(56)*. By varying the lengths of the linker and the connector, the sizes of the knob, and the position of the crosslink, one can probe the distance between various hormone residues in α2 and the receptor. Panels **B** and **C** show that this technique can also be used to probe the positions of residues in the small seatbelt loop using the α-subunit C-terminus (αCT) as a linker and replacing a residue to be knobbed in the β-subunit loop with cysteine. Note that formation of a knob results in a disulfide crosslink between the subunits. This renders the heterodimer stable to low pH treatment, which promotes dissociation of heterodimers lacking knobs. Using this approach we monitored the roles of α2 residues in ligand–receptor interaction and found that the position of αArg42 differs in LHR and hFSHR/hTSHR complexes *(16)*. We also used this technique to monitor the positions of the αCT and the roles of small seatbelt residues in hormone signaling. These studies revealed that the αCT is likely to be near the small seatbelt loop on the surface of the hormone receptor complex *(47)*. As noted in the text, we also found that only one surface of the small seatbelt loop contacts the receptor.

Sites that can be "knobbed" without blocking the binding of the glycoprotein hormones to their receptors are clearly not involved in essential receptor contacts. The converse is not necessarily true, however. Thus, it may not be possible to interpret data obtained from this procedure if small knobs block hormone–receptor interactions because the crosslink and/or the knob might alter the conformation of the heterodimer. Nonetheless, by using different sized linkers and by comparing the abilities of small and large knobs to interfere with hormone activity, one can often determine the relative distances of hormone residues from the receptor interface *(56)*.

The knob approach revealed that much of the small seatbelt loop of hCG and a few surrounding residues appear to be exposed in the hormone–receptor complex. This includes residues 91, 92, 94, 96, 97, and 98 *(47)*. Only the smallest

knob was tolerated at residue 95, however. Similar studies of α-subunit loop 2 indicated that residue αArg42 appears to be further from the hormone-receptor interface in hCG–LHR complexes than it is in hFSH–FSHR and hTSH–TSHR complexes. This finding is consistent with the structures of these hormone–receptor complexes as proposed in Fig. 5 *(16)*. Residue αArg42 is thought to be highly exposed in the crystal structure of hFSH bound to the LRD fragment of the FSHR *(15)* and in models of all glycoprotein hormone receptors in which the ligands contact the concave surface of the LRD *(13)*. This prediction is not consistent with the observation that residue αArg42 has different roles in hCG–LHR and hFSH–FSHR and hTSH–TSHR complexes. The inability of models based on the crystal structure to explain the role of αArg42 adds further support to the notion that they do not describe the interactions of these ligands with cell-surface G protein–coupled receptors.

Observations That Describe How Glycoprotein Hormone Receptors Contact Their Ligands

Cloning of the rat and porcine lutropin receptor cDNAs was a major milestone in efforts to unravel the actions of the glycoprotein hormones *(1)*. LHR, FSHR, and TSHR from all species contain a series of leucine-rich repeats, most of which are encoded by single exons. The prediction that the LRD of these receptors would fold similarly to those of other leucine-rich proteins that have fewer *(57)* or more *(25)* residues in each repeat has now been confirmed *(15)*. The LRD is connected to a TMD composed of seven hydrophobic helical segments by a linker that has an influence on ligand binding and signaling *(16,58)*. Mutations in the linker and the TMD are known to influence signal transduction. Many of these lead to constitutive hormone activity and altered ligand binding *(20,59)*. Therefore, models of the receptor must explain how interactions among the LRD, the linker, and the TMD influence binding and signaling. Finally, models of the receptor need to explain how ligand binding leads to G-protein activation.

The abilities of truncated LHR to bind hCG led to the conclusion that the LRD is sufficient for ligand binding *(3)*, a notion that is consistent with the finding that alternate spliced forms of the LH receptor are able to bind hCG with high affinity *(1)*. Nonetheless, the notion that the LRD is the *only* portion of the LH receptor that is responsible for ligand binding is clearly incorrect. The human LHR does not bind most mammalian lutropins, including bovine LH *(58,60)*. Differences in the abilities of the rat and human LHR to bind bovine LH were found to be caused primarily by residues in the linker—i.e., the SSD *(58)*. This showed clearly that the linker participates in ligand-binding specificity and indicated that it is likely to make significant contacts with the ligand *(58)*. Indeed, some of the confusion in this area stems from the assumption that data obtained by monitoring the interactions of hCG with the LHR can be extrapolated to the abilities of all other lutropins to bind the LHR and to the abilities of FSH and TSH to interact with FSHR and TSHR. hCG and hLH appear to have much better abilities to interact with most mammalian LHR than the native ligands. This is due in part to the presence of β-subunit Arg95 in hLH and hCG *(61)*, a residue that is either leucine or isoleucine in the lutropins of most mammals.

Other observations also suggested that the SSD has an important role in ligand binding. Some chimeras prepared from parts of the TSH and LH receptors or from parts of the FSH and LH receptors interacted with TSH and hCG or FSH and hCG, respectively *(5,6)*. While their abilities to bind hCG depended only on the presence of residues from the central region of the LRD, their abilities to bind TSH and FSH required the presence of residues from the TSH and FSH receptors in the N-terminal and C-terminal parts of the LRD and in the SSD. Indeed, replacing only a few residues in either the TSH and FSH receptors with their LH-receptor counterparts was sufficient to enable the chimeras to bind TSH and hCG or FSH and hCG *(20,39)*. However, it has not been possible to prepare functional chimeras having the reciprocal mutations—i.e., LH receptors having a few residues of the FSH and TSH residues. This is largely because the source of the SSD has a much greater influence on FSH and TSH binding than on hCG binding to most receptor analogs *(5,6,58)*.

The LH receptor from the marmoset monkey lacks residues derived from exon 10 *(62)*. This receptor binds choriogonadotropins much better than some lutropins *(63)*, as does a rat LH receptor analog that lacks residues derived from exon 10 *(16)*. hCG analogs lacking the α-subunit loop 2 oligosaccharide have lower efficacy in assays employing this receptor analog than they have in assays employing the full-length rat LH *(16)*. This showed that the SSD has an important role in signaling in addition to its role in ligand binding.

Models of the Hormone–Receptor Complex in Which the Ligand Contacts the LRD and SSD Explain Binding and Signaling Better Than Those in Which it Contacts the Concave Surface of the LRD

The concept that hormone ligands bind the concave surface of the LRD is difficult to reconcile with several observations of glycoprotein hormone binding or signaling. As noted earlier, models based on this orientation of the hormone usually posit that the region of the receptor that connects the LRD to the TMD functions as a hinge or as an elongated linker that enables a portion of the ligand that is bound to the LRD to interact with the TMD *(15)*. The notion that the ligand contacts the TMD in this fashion suggests that regions of the glycoprotein hormones that interact with the TMD would be conserved in all vertebrates. The most

Table 2
Sequences of Hormone and Receptor Residues Postulated to Initiate Signaling in Models of Receptors in Which the Linker Acts as Hinge

	Residues in hormone α-subunit loops 1 and 3[a]	
Species	α-subunit loop 1	α-subunit loop 3
Human	TLQENPFFSQPGAPILQ	VAKSYNRVTVMGGFKVENHTA
Mammal	K K KY K D Y	AFTKA NVR E
Bird	K K R K Y	AFTKI LKDNV L E
Frog	R K LR NMGIGRIYG	TQY DNV I
Fish-1	K K NI K VY	EVK L ND-V LV D
Fish-2	KP TI——— PN I	EGE TKD P T E

	TMD residues in outer loops 1 and 2[b]	
Species	Outer Loop 1	Outer Loop 2
hLHR	DSQTKGQYYNHAIDWQTGSG	GVSNYMKVSICFPMDVETTLSQ
hFSHR	IH S H Y A	I S L IDSP
hTSHR	LY HSE P	I S A L T P AL
sLHR	LH R H SE A	S SR M L K P A
sFSHR	VR R L S A	S S L SLP
sTSHR	LH ME E P	S Q L TKSTMA

[a]Underlined residues represent those at the tips of these loops. Note that this region of the α-subunit is not highly conserved in most vertebrates and differs substantially in the subunits of fish that contain two α-subunits. Underlined regions are the subunit tips, which have been postulated to contact the TMD *(15)*.

[b]The sequences in TMD outer loops 1 and 2 often differ as much between the receptors in one species as they do between species as distant as humans and salmon. Shown here are the sequences of outer loops 1 and 2, the largest TMD loops.

conserved regions of the hormones are α-subunit loop 2 and the αCT, both of which have similar sequences in all vertebrate glycoprotein hormones *(40,64)*. In the hinge models, these portions of the α-subunit occupy positions near the LRD *(13,15)*, which would prevent them from contacting other parts of the receptor. As was also discussed earlier, the notion that the αCT is buried within a TMD pocket is inconsistent with the finding that it can be crosslinked to much of the small seatbelt loop in the β-subunit of lutropins without disrupting ligand binding or signaling *(47)*. The remainder of the α-subunit, namely α-subunit loops 1 and 3, is much less conserved. The notion that the tips of α-subunit loops 1 and 3 contact the outer loops of the TMD is not consistent with the observations that these regions differ in hormones that are known to interact with receptors from different species (Table 2). Indeed, some species of teleost fish have two α-subunits that differ markedly in loops 1 and 3 even though they are incorporated into heterodimers that interact with all three piscine receptors. One of these α-subunits lacks all four residues that form the tip of loop 1 of the other α-subunit (Table 2), which argues against the notion that this region of the α-subunit makes essential receptor contacts. While these observations are a problem for models of the hormone receptor complex in which the hormone docks with the concave surface of the LRD (Figs. 2–4), they are readily explained by a model in which the ligands dock with the surfaces of the LRD and SSD that are distant from the TMD. In the latter models, the highly conserved portions of the α-subunit face the LRD and SSD; portions of the α-subunit that are not conserved face away from the hormone–receptor interface (Figs. 5 and 6).

Models of the receptor in which the ligand is bound to the concave surface of the LRD and the LRD is tethered to the TMD by a highly mobile hinge suggest that the LRD is free to move relative to the remainder of the receptor (Figs. 3 and 4). Therefore, it would be expected that truncation of the receptor at nearly any site in the linker would cause secretion of the LRD. In contrast to this prediction, the LRD remains intracellular when the linker is truncated *(1)*, indicating that in the intact receptor it may be associated with the TMD and/or that it interacts with the lipids in the membrane. The model of the receptor in which the LRD, SSD, and TMD form a tightly interacting complex (Figs. 5 and 6) is compatible with the idea that truncation of the receptor would disrupt its structure *(16)*. Separation of the receptor components is likely to cause the receptor to be recognized as a misfolded protein, which would account for its retention in the ER and/or Golgi *(1)*.

The hinge models predict that two or more surfaces of the hormone contact the receptor (Figs. 3 and 4). This contrasts with the finding that most of the hormone surface including the convex regions of loops 1 and 3 in both sub-

units remains exposed after hCG and hFSH dock with their receptors (Fig. 10). Some regions of hFSH that have been postulated to dock with the TMD in hinge models are also known to be recognized by monoclonal antibodies while the hormone is bound to the receptor *(12,46)*. For example, antibody A116, which recognizes the tip of α-subunit loop 1 (Fig. 10), binds both hCG–LHR and hFSH–FSHR receptor complexes *(65)*. Thus, it is highly unlikely that α-subunit loops 1 and 3 contact the TMD in the manner proposed *(15)*. The model in which the hormones dock with surfaces of the LRD and SSD distant from the TMD *(16)* is consistent with all experimental data describing the exposed regions of the ligand in the hormone–receptor complexes (Figs. 5, 6, and 10).

The manner in which the ligand contacts the LRD in hinge models of the receptor is also difficult to reconcile with the locations of the receptor oligosaccharides, all of which are located on the same surface of the LRD in LHR, FSHR, and TSHR. Consequently, in the receptor model proposed by Fan and Hendrickson *(15)*, these oligosaccharides would contact the plasma membrane (Fig. 4B), a very unusual phenomenon for a membrane protein. The receptor and ligand oligosaccharides are distant from the cell surface in the model of the hormone–receptor complex in which the ligand contacts both the LRD and SSD (Fig. 5B).

Most hinge models suggest that the linker has little or no role in ligand binding and signaling other than to prevent the LRD from dissociating from the TMD. This assumption is based largely on the ability of truncated LHR fragments to bind hCG *(3)*. It does not explain the influence of the linker on the binding of other lutropins to the LHR or its role in signal transduction *(16,58)*. Because the LRD and TMD are separated in these models, they also fail to explain how mutations to regions of the TMD near the inner membrane leaflet alter ligand binding specificity *(21,22)*. In contrast, the model in which the LRD, SSD, and TMD interact tightly *(16)* accounts readily for the influence of the SSD on ligand binding since this portion of the receptor is a key part of the ligand-binding site. Furthermore, it explains the ability of the TMD to influence ligand binding since all three regions of the receptor interact with one another. Thus, contacts of the ligand with the LRD and SSD promote a change in the conformation of the TMD to initiate signaling; vice versa, changes in the TMD promote changes in the positions of the SSD and LRD that would affect ligand binding (Figs. 5 and 6).

Unlike models in which the LRD is separated from the TMD by an elongated SSD linker (Figs. 2 and 4), the model in which the LRD, the SSD, and the TMD form a well-integrated structure (Fig. 5) is capable of explaining how subtle changes in the interactions of these domains alter ligand-binding specificity. Ligands are known to contact the LRD and the SSD *(16,58)*. Therefore, binding specificity can be controlled by the relative positions of the LRD and SSD as well as by contacts of the ligand with specific residues in each of these domains. Mutations that affect contacts between specific residues of the ligand and the LRD or SSD would be expected to alter binding specificity as would mutations that could alter the position of the LRD *(20)*. The latter can also include those that alter the interaction of the SSD with the TMD *(20)*. Thus, the model described in Fig. 5 will account for the ability of mutations to the LRD that enable TSH and FSH receptors to bind hCG *(21,22)*.

The model in which the LRD, SSD, and TMD form a functional unit will also explain how equine LH, equine CG *(34)* and hCG/hFSH chimeras *(5)* can initiate signaling in LHR, FSHR, and TSHR. Indeed, some hCG/hFSH chimeras can initiate signaling at TSHR as well as LHR and FSHR even though they lack all residues unique to TSH *(52)*. The shape of the LRD and the SSD creates an elongated curved groove in the surface of the extracellular domain of the receptor that can accommodate a portion of the helical end of α-subunit loop 2 at different sites along its length (Fig. 5). Contacts between the stem of α-subunit loop 2 and nearby residues in the β-subunit—including parts of the seatbelt—with several leucine-rich repeats in the LRD will permit the helical end of α-subunit loop 2 to contact the groove between the LRD and SSD. This will also cause the ends of β-subunit loops 1 and 3 to contact the SSD differently in lutropins (Fig. 6A) than in follitropins and thyrotropins (Fig. 6B). Contacts of the ligands with two distinct regions of their receptors require that several residues in both ligand subunits interact with the receptor, which would explain why it has been so difficult to identify individual contacts and why small changes in hormone conformation have a marked influence on ligand binding and signaling.

The seatbelt contributes more than any other region of the β-subunit to its role in ligand-binding specificity *(5,35, 36,38)*. Mammalian lutropins appear to make important contacts with the central region of the LRD in an orientation in which residues in the small seatbelt loop—i.e., residues between the tenth and eleventh β-subunit cysteines—make much more important contributions to binding than those in the C-terminal half of the seatbelt—i.e., residues between the eleventh and twelfth β-subunit cysteines (Fig. 6A). As a consequence, changes to the small seatbelt loop usually have a greater influence on the binding of lutropins to LHR than mutations to the C-terminal half of the seatbelt *(5)*. In contrast, mammalian follitropins and thyrotropins contact the C-terminal end of the LRD in an orientation in which residues between the eleventh and twelfth β-subunit cysteines make more important contributions to binding than those in the small seatbelt loop (Fig. 6B). This would explain why the charges of the residues in the N-terminal half of the small seatbelt loop had little influence on the binding of analogs to FSHR *(38,49)*. These differences in the receptor binding positions of lutropins, follitropins, and thyrotropins will also explain why human α-subunit residue Arg42 is more exposed in lutropins than in follitropins and thyrotropins *(16)*.

The notion that docking to the central region of the LRD is more important for binding of mammalian lutropins and that docking to the N- and C- terminal ends of the LRD is more important for binding of mammalian follitropins and thyrotropins will explain the abilities of TSH/LH receptor chimeras and FSH/LH receptor chimeras to recognize TSH and hCG or FSH and hCG, respectively *(5,6)*. It will also explain the reversal in ligand–receptor binding specificity observed when hormones from some species were assayed with receptors from another *(66)*.

Finally, models of receptors in which the LRD and SSD contribute to ligand binding and in which ligand binding can occur in multiple orientations would readily explain the co-evolution of ligand and receptor-binding specificity during vertebrate evolution. The LRD is composed of many leucine-rich repeat domains that are encoded by single exons. Therefore, unequal crossing over and other methods of gene duplication would be expected to add, remove, or modify repeats without disrupting the abilities of the receptors to interact with ligands needed for reproduction or development. This will account for the fact that tilapia follitropin receptors contain an additional leucine-rich repeat in their LRD *(67)*. Since mutations in one region of the LRD can accumulate independently from those in another region of the LRD without disrupting signaling, this architecture would have readily permitted the co-evolution of the ligands and their receptors. It would also have permitted the seatbelt latch site of some fish follitropins to migrate from its location in β-subunit loop 1 to the N-terminal end of the β-subunit, a change that occurred during the evolution of ancient fish such as sharks and sturgeons to salmon (Fig. 9).

The fact that the SSD of the LH receptors contains an additional exon permits alternate splicing to regulate ligand-binding affinity, which may have a key role in regulating the response to LH. Thus, receptors in which this exon is spliced out would be expected to have a lower affinity for lutropins. During evolution, this may have led to the loss of this exon from the marmoset and the replacement of its LH β-subunit gene by that for choriogonadotropin *(68)*.

Models of Signal Transduction

Models of the receptor must contain a mechanism for signal transduction and explain the roles of the hormone oligosaccharides in this process. The hormone oligosaccharides are not needed for ligand–receptor interactions, although the presence of large quantities of negatively charged sugars can impede receptor binding as seen by the ability of neuraminidase to increase in the ability of equine CG—e.g., pregnant mares serum gonadotropin (PMSG)—to bind LH and FSH receptors *(69)*. The oligosaccharides contribute substantially to hormone efficacy *(70)*, an observation that has been confirmed often. Those on the α-subunit were found to be most important for efficacy *(71)*, a phenomenon refined by site-directed mutagenesis *(72)*. Removal of the oligosaccharide from α-subunit loop 2, the only oligosaccharide near the subunit interface (Fig. 9), had the greatest influence on the efficacy of hCG *(72)*.

The notion that the oligosaccharides are essential for full efficacy was challenged by the report of Heikoop et al. *(73)*, who claimed that crosslinking the deglycosylated heterodimer prevented it from losing its efficacy and who speculated that the deglycosylated heterodimer was unstable in the assay conditions. We have not been able to confirm this finding. In fact, we observed that removing the α-subunit loop 2 oligosaccharide accelerates hCG subunit combination at the pH used in most receptor binding and signaling assays *(74)*. The resulting heterodimer is stable during SDS-polyacrylamide gel electrophoresis and has only 40% the efficacy of hCG in cyclic AMP assays performed in the absence of a phosphodiesterase inhibitor and 70% the efficacy of hCG in the presence of a phosphodiesterase inhibitor *(16)*. Addition of disulfide crosslinks did not increase the efficacy of deglycosylated hCG *(16,75)*. Indeed, some disulfide crosslinks reduced its efficacy in assays in which cyclic AMP accumulation was measured directly *(75)*.

The ability of the α-subunit loop 2 oligosaccharide to influence the efficacy of hCG more than any other oligosaccharide may be due to its location near the subunit interface. This is consistent with its position in models of the receptor in which the LRD, SSD, and TMD interact with one another (Fig. 12). In this model, the oligosaccharide on α-subunit loop 2 would influence the interaction of loop 2 with the groove between the LRD and SSD, a phenomenon that appears required for signaling *(16)*. The position of the α-subunit loop 2 oligosaccharide is distant from the receptor in the model in which the tips of a-subunit loops 1 and 3 are thought to interact with the TMD (Figs. 2 and 4). Therefore, it is difficult to visualize how the oligosaccharides would contribute to efficacy in this model *(15)*.

The abilities of the oligosaccharides to influence efficacy do not appear to require them to make specific contacts with the glycoprotein hormone receptors. Fab fragments prepared from antisera to the hCG β-subunit restored the efficacy of deglycosylated hCG to that seen in hCG *(76)*. Monoclonal antibodies that recognize a region of β-subunit loop 1 near its junction with the seatbelt restored the efficacy of hCG lacking its α-subunit loop 2 oligosaccharide *(16)*; those that recognize other regions of the β-subunit were without effect. This suggested that the region of the hCG β-subunit near the junction of the seatbelt with β-subunit loop 1 may have an important role in signal transduction (Fig. 12). Furthermore, addition of an oligosaccharide to hCG β-subunit residue 77, a residue that appears to be near the receptor interface in the model described in Figs. 5 and 12, increased the efficacy of the deglycosylated heterodimer analogs lacking the α-subunit loop 2 oligosaccharide *(16)*. These findings also indicate that the influence of the oligosaccharides on hormone efficacy may occur by altering the conformation of the heterodimer or its position in the receptor complex.

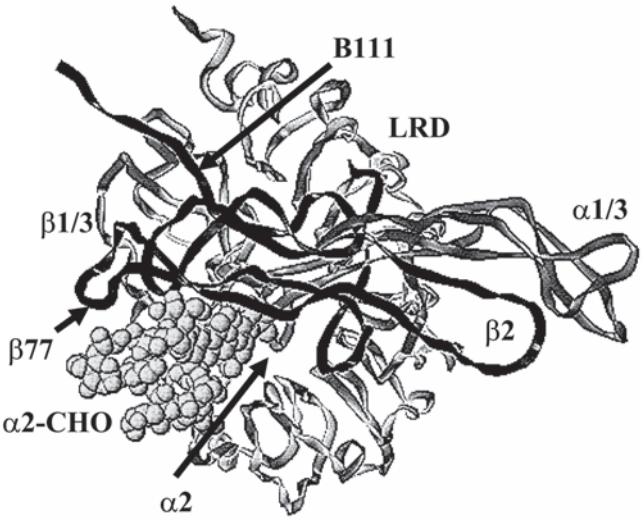

Fig. 12. Regions of the hormone that influence efficacy. Shown here is a view of the hCG–LHR complex as seen from above the cell surface. The α-subunit loop 2 (α2) is located above the groove between the LRD and SSD (not labeled). The α2 oligosaccharide (α2-CHO) is in a position that would influence the ability of α2 to alter the positions of residues in the SSD relative to those in the LRD. Removal of the α2-CHO reduces the efficacy of hCG by approx 60%. This loss in efficacy can be reversed by addition of an oligosaccharide at β-subunit residue 77 (β77) or by the addition of a monoclonal antibody (B111) that recognizes residues in the β-subunit near the seatbelt latch site. Molecular dynamics simulations suggest that efficacy is caused by a small movement of a loop within the SSD (Moyle, W. R. and Kerrigan, J. E., unpublished observations).

The abilities of some receptor fragments to restore signal transduction activity to receptor analogs that are unable to bind ligand suggest that signaling may be the result of receptor dimerization. The notion that the receptors can aggregate is supported by studies with fluorescent-labeled hCG *(77)* and by the finding that it is possible to crosslink the FSH–FSH receptor fragment *(15)*. Nonetheless, we have been unable to detect the presence of two molecules of hCG in cell-surface hCG–LHR complexes using total internal reflection fluorescence microscopy (Xing, et al., presented at the Biophysical Society Meeting, Baltimore, MD, February 2004). This technique can be used to distinguish cell-surface receptors from those that are localized within the cell. Our methods would not have detected receptor dimers that bind only a single molecule of ligand, however, and the role of dimerization in receptor activation and/or turnover remains unresolved. The model based on the crystal structure of FSH complexed to the FSH receptor fragment (Figs. 2 and 4) suggests that contacts between residues in the convex surface of the LRD are responsible for receptor dimerization *(15)*. It is not clear how the hormone would promote dimerization of the LRD, particularly at low hormone concentrations where it would be unlikely that adjacent receptors would be occupied by ligand. Furthermore, dimerization of the receptor in this model would not cause the TMD to interact with one another (Fig. 4), making it difficult to visualize how this would alter the ability of the receptor to interact with G proteins. In contrast, the homodimers derived from receptor models in which the LRD, SSD, and TMD contact one another are likely to contain contacts between the TMD (Fig. 8), a phenomenon that would be expected to alter G protein coupling.

Summary and Conclusions

Models in which the LRD, SSD, and TMD are tightly integrated into a single unit (Fig. 5) are consistent with most, if not all, experimental data on glycoprotein hormone binding and signaling *(16)*. Initially, these were based on the minimal sizes of the SSD found in the marmoset LH receptor, the salmon FSH receptor, and analogs of the TSH receptor that are produced naturally by proteolysis *(33)*. Rat LHR and FSHR analogs of the modeled receptors are expressed on the cell surface and bind and respond to hCG and hFSH with high affinity and specificity *(16)*. These models *(16)* were designed to explain all the reproducible data describing the binding and function of all three glycoprotein hormones that were known prior to publication of the crystal structure of FSH complexed with a fragment of its receptor *(15)*. Undoubtedly, they will require modification as more information becomes available on the structure of the SSD and the portion of the transmembrane domain that interacts with G proteins. Recently, we have prepared models of the LHR that incorporate a folding pattern for exon 10 (Moyle, unpublished observations). Contacts between this and the ligands will explain the ability of this SSD region to influence the binding of lutropins to LHR. These receptor models have been incorporated into simulated lipid bilayers and subjected to extensive molecular dynamics simulations in an aqueous environment (Kerrigan, J. E. and Moyle, W. R., unpublished observations). The simulations show that ligand binding leads to a small movement of a few SSD residues toward the LRD, which may be sufficient to alter the TMD and thereby initiate signaling.

Models of the glycoprotein hormone–receptor complex in which the LRD, SSD, and TMD are separated have received the most attention, but these do not explain the G protein–coupled activities of these receptors. As typified by the model based on the crystal structure of hFSH with a fragment of its receptor, models in which the LRD is separated from the TMD by a linker or hinge fail to explain the role of the SSD on ligand binding and signaling. They predict unusual interactions between oligosaccharides on the hormone and the receptor with the receptor TMD and the plasma membrane. They suggest that a portion of the hormone that can be recognized by monoclonal antibodies in the hormone receptor complex makes key contacts with the receptor that would be needed for signaling. Finally, they do not explain ligand binding specificity or the abilities of ligand analogs to bind multiple receptors and vice versa, the abilities of receptor analogs to bind multiple ligands. The

portion of the FSH receptor contained in the crystal structure is similar to that in an alternatively spliced portion of the receptor *(10,11)*, Therefore, the crystal structure *(15)* is more likely to indicate how FSH interacts with this receptor form than the G-protein coupled receptor.

Until crystal structures of the entire free receptors and ligand-bound hormone receptors have been determined, there will continue to be some uncertainty in the manner in which these complex signal transduction machines function. In spite of this reservation, we suggest that it is time to abandon models of glycoprotein hormone G protein–coupled signaling in which the LRD, SSD, and TMD are separated in favor of those in which these three components are united into a functional unit *(16)*.

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