The Role of the Hinge Region of the Luteinizing Hormone Receptor in Hormone Interaction and Signal Generation*

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Luteinizing hormone receptor, a G protein-coupled receptor, consists of two halves, the N-terminal extracellular hormone binding domain (exodomain) and the C-terminal membrane-associated, signal-generating domain (endodomain). The exodomain has seven to nine Leu-rich repeats, which are generally thought to form a 1/3 donut-like structure and interact with human choriodionadotropin (hCG). The resulting hCG-exodomain complex adjusts the structure and its association with the endodomain, which results in signal generation in the endodomain. It is unclear whether the rigid 1/3 donut structure could provide the agility and versatility of this dynamic action. In addition, there is no clue as to where the endodomain contact point (the signal modulator) in the exodomain is. To address these issues, the exodomain was examined by Ala scan and multiple substitutions, while receptor peptides were used for photoaffinity labeling and affinity cross-linking. Our results show that the C-flanking sequence (hinge region), Thr250–Gln268, of the Leu-rich repeats (LRRs) specifically interacts with hCG, preferentially hCGα. This interaction is inhibited by exoloop 2 of the endodomain but not by exoloops 1 and 3, suggesting an intimate relationship between Thr250–Gln268, exoloop 2, and hCG. Taken together, our observations in this article suggest a new paradigm that the LRRs contact the front of hCG, while both flanking regions of the LRRs interact with the sides of hCG. This would trap hCG in the 1/3 donut structure of the LRRs and enhance the binding affinity. In addition, mutations of conserved Ser255 in the sequence can constitutively activate the receptor. This provides a clue for the signal modulator in the exodomain. In contrast, a phenyl or phenolic group is necessary at conserved Tyr256 for targeting the receptor to the surface.

The glycoprotein hormone receptors comprise two equal halves; an extracellular N-terminal half (exodomain) and a membrane-associated C-terminal half (endodomain). The exodomain is ~350 amino acids long, which alone is capable of high affinity hormone binding (1–3) without hormone action (3, 4). The endodomain consists of seven transmembrane domains, three exoloops, three cytoloops, a C-terminal tail, and a short extracellular extension connected to transmembrane domain 1.

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Receptor activation occurs in the endodomain (3, 5, 6), which is structurally equivalent to the entire molecule of many other G protein-coupled receptors (7). Existing data suggest that these two domains are associated together before and after hormone binding (8, 9). A hormone initially binds to the exodomain, and the resulting hormone-exodomain complex adjusts its conformation (10) and then interacts with the endodomain (7). This change in the interaction in the ternary complex, hCG-exodomain-endodomain, is thought to generate a signal (6, 7, 11).

There is ample evidence that the exodomain of the luteinizing hormone/choriogonadotropin receptor (LH/CG-R)† alone is capable of binding hCG with high affinity. The truncated exodomain lacking the endodomain binds hCG with a high affinity similar to or slightly better than the wild type affinity (1–3). Exodomain fusion proteins are capable of binding hCG with a high affinity (12, 13). A number of sites in the exodomain are important for hormone binding (14–16) and hormone specificity (17–19). LHR peptides corresponding to three regions in the exodomain are capable of inhibiting hCG binding to the receptor (20).

A major feature in the structure of the LHR exodomain is the Leu-rich repeat (LRR) motif (21, 22). LRRs are found in a large number of proteins (23). The existing crystal structures of a few LRR proteins show up to 15 LRRs, which form a rigid 2/3 donut structure (24, 25). Based on these LRR crystal structures, the glycoprotein hormone receptors have been modeled (26–28). They show a 1/3 donut structure consisting of 7–9 LRRs and suggest that the inner lining interacts (16, 29, 30) with the concave, front side of the hormones (31, 32). Recently, it has been shown that the putative LRRs in the gonadotropin receptors are indeed active and that part of them interact with hCG (33–35).

However, it is unclear whether the rigid 1/3 donut structure of the LRRs alone interacts with hCG, adjusts its conformation, contacts with the endodomain, and activates the endodomain or if other regions of the exodomain are involved in the process. This agility and versatility may come from regions other than the LRRs. For example, we have shown that the N-terminal flanking region of the LRRs makes strong contact with both subunits of hCG (36, 37). However, it is unknown whether the N-terminal flanking region of the LRRs is the only exodomain region outside of the LRRs to interact with hCG. In addition to the insufficient knowledge on the exodomain/hCG interaction, little is known about the contact sites between the exodomain and endodomain. There is evidence that exoloops 2 and 3 of the

† The abbreviations used are: LH, luteinizing hormone; CG, choriogonadotropin; CG-R, CG receptor; h, human; LHR, LH receptor; PBS, phosphate-buffered saline; NHS-AB, the N-hydroxysuccinimide of 4-azidobenzoic acid; SES, ethylene glycolbis(sulfosuccinimidyl succinate); LRR, Leu-rich repeat; FSH, follicle-stimulating hormone; FSHR, FSH receptor; TSH, thyroid-stimulating hormone.
endodomain are involved in the interaction with unliganded exodomain and constrain the hormone binding at the exodomain (8, 9). It has just been reported that LRR4 (34, 35) and the C-terminal flanking (hinge) region of the LRRs (38) modulates signal generation in the exodomain. However, there is no clue to where the endodomain contact point is in the exodomain. In this article, the first evidence is presented that the hinge region interacts with hCG and that the hCG-exodomain complex makes contacts with exoloop 2 of the endodomain.

EXPERIMENTAL PROCEDURES

Materials—The N-hydroxysuccinimide of 4-azidoenobac acid (NHS-A
B) was synthesized as described previously (39). The N-hydroxysulfo-
succinimide esters of ethylene glycolbis(sulfosuccinimidylsuccinate) (SES) were purchased from Pierce. The hCG CR 127 and hCG subunits were supplied by the National Hormone and Pituitary Program. Denatura
ted hCG was prepared by boiling hCG in 8 M urea for 30 min. Receptor peptides were synthesized and N-acetylated and C-amidated by Biosynthesis (Lewisville, TX). They were purified on a Vydac C$_{18}$ high performance liquid chromatography column using a solvent gradi
dient from 100% of 0.1% trifluoroacetic acid in water to 20% of 0.1% trifluoroacetic acid in water and 80% 1-propanol. Mutagenesis and Functional Expression of LHR—Mutant LHR cDNAs were prepared in a pSELECT vector using the Altered Sites

Mutagenesis System (Promega), sequenced, subcloned into pcDNA3 (Invitrogen) as described previously (40), and sequenced again to verify mutation sequences. This procedure does not involve polymerase chain reaction, and therefore, there are no polymerase chain reaction-associated unintended mutations. To produce truncated receptors, a codon was substituted with a stop codon. Mutant and truncated LHR con
taxtions were transfected into human embryonic kidney 293 cells by the calcium phosphate method. Stable cell lines were established in minimum essential medium containing 10% horse serum and 500 μg/ml of G-418. They were used for hormone binding and cAMP production. All assays were carried out in duplicate and repeated four to six times. Mea

125$I$-hCG Binding and Intracellular cAMP Assay—Stable cells were assayed for 125$I$-hCG binding in the presence of a constant amount of 125$I$-hCG (39) and increasing concentrations of unlabeld hCG. The K$_d$ values were determined by Scatchard plots. For intracellular cAMP assay, cells were washed twice with Dulbecco’s modified Eagle’s me

RESULTS

Truncation of the C′ Terminus—LHR is encoded by 11 exons (22, 43), and the exodomain is primarily comprised of exons 1–10, whereas the exodomain is mainly encoded in exon 11. When exons 1–10 were expressed in mammalian cells after truncating exon 11, the exodomain fragment was trapped within the cells and could not be detected on the intact cell surface or in the culture medium (2, 3). However, when the cells were solubilized in nonionic detergent, the exodomain fragment was capable of binding hCG with a high affinity similar to the hormone binding affinity of the wild type recep

dence of the following solutions were sequen
duced from 100% of 0.1% trifluoroacetic acid in water to 20% of 0.1% trifluoroacetic acid in water and 80% 1-propanol. Mutagenesis and Functional Expression of LHR—Mutant LHR cDNAs were prepared in a pSELECT vector using the Altered Sites

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The resulting Tyr253 deletion mutant was not expressed on the surface, supporting the hypothesis. One may argue that cells expressing trapped receptors might be unable to bind hormone because they are in the range of 22,000–57,000/cell, except for Thr252 to Cys258 were expressed on the surface. This result suggests that the first half, but not the second half, of the sequence may be important for receptor targeting to the cell surface.

Substitutions for Tyr253 Impair Surface Expression—To further investigate the impact on surface expression, Tyr253 was substituted with an array of amino acids (Table II). The substitution of Phe did not impact the surface expression or the receptor concentration. In contrast, the His substitution significantly impaired the surface expression, and other substitutions blocked it. These results show that Tyr253 to Phe and a phenyl group at the Tyr253 position are permissible for receptor targeting and hormone binding. Therefore, the hydroxyl moiety of the Tyr's phenolic group does not appear to influence the targeting and hormone binding. The partial blockage of surface expression by the substitution of His suggests that an imidazole at the Tyr253 position is tolerable but not desirable. Substitutions of nonaromatic hydrophobic, hydrophilic, neutral, and acidic amino acids for Tyr253 prevented surface expression of the receptor, although the concentration of the mutant receptors trapped in cells was significant and sometimes considerably higher than the total concentration of the wild type receptor. These results suggest that a phenolic or phenyl group is necessary at the Tyr253 position for efficient receptor transport to the surface membrane. To test this possibility and the essential role of Tyr253, the residue was deleted. The resulting Tyr253 deletion mutant was not expressed on the surface, supporting the hypothesis.

One may argue that cells expressing trapped receptors might have developed a defective mechanism for receptor transport, perhaps when stable cell lines were being established. To test this unlikely possibility, the cells stably expressing the Tyr253→Ala mutant were transfected again with the wild type LH/CGR or the human FSH receptor plasmid. The transfected cells were capable of binding hCG or FSH, respectively (Table III), indicating that the receptor transport mechanism was functional. To exclude the possibility that the second transfection rescued the receptor transport mechanism, additional experiments were performed. Cells stably transfected with the FSH receptor or wild type LH receptor were transfected again with the Tyr253→Ala mutant plasmid. These doubly transfected intact cells were capable of binding FSH or hCG, respectively, indicating that the receptor-targeting mechanism was not affected by transfection of the Tyr253→Ala mutant plasmid and expression of the Tyr253→Ala mutant.

Constitutive Activation of cAMP Induction by Substitution for Ser255—The examination of cAMP induction discloses that the mutations impaired the EC50 value, maximum level, or both for cAMP induction (Fig. 2, A and B). The only exception is Ser255→Ala. It showed a similar EC50 and higher maximum cAMP as compared with the wild type values, suggesting an improved efficacy of cAMP induction. To follow up this interesting observation, Ser255 was replaced with several amino acids (Table IV). The resulting mutants were transiently surface-expressed in the 20,000–40,000/cell range. Some of mutations, Val and Ile, resulted in strikingly high basal cAMP levels, despite lower receptor concentrations on the surface. This is unusual because all activating mutations uncovered to date are found in the endodomain (11, 46). A simple explanation is that the region may be involved in signal generation in the endodomain. If so, it may interact with hCG, the endodomain, or both. However, it is also possible that the mutations might have impacted protein processing including folding and thus, the global structure, which in turn resulted in the observed high basal cAMP level. One way to resolve this difficult issue is to determine its interaction, which can be done by affinity labeling.

Affinity Labeling of hCG with Receptor Peptide LHR—We synthesized a 24-mer peptide, Ac-Leu246–Asn269-NH2 (LHR246–269), derivatized it with NHS-AB, and radioiodinated it to produce AB-125I-LHR246–269. NHS-AB couples to the Lys residues, and Lys265 and Lys266 are present in the peptide. To determine
whether AB-125I-LHR246–269 would bind and label hCG, and if so, which subunit is labeled, the peptide derivative was incubated with hCG and irradiated with UV for increasing time periods (Fig. 3A). The samples were solubilized in SDS under reducing conditions and electrophoresed as described under “Experimental Procedures.” The autoradiographic phosphoimage of the gel shows that AB-125I-LHR246–269 labeled conspicuously both the α and β subunits in hCG as well as faintly the αβ dimer. The positions of hCGα, hCGβ, and the hCGαβ dimer were determined by comparing the respective positions of 125I-hCGα, 125I-hCGβ, and the cross-linked 125I-hCG αβ dimer on the autoradiograph (35, 37). The labeling was dependent on UV irradiation, reaching the maximum level at 60-s exposure. The labeling of the αβ dimer implies that there were two AB groups

![Image](https://example.com/image.png)

**Fig. 2. Ala scan of Thr250–Gln268.** Residues in the Thr250–Gln268 sequence were individually substituted with Ala, except the Ala259 → Gly substitution. The resulting mutants were stably expressed in 293 cells and assayed for hCG binding to intact cells and cAMP induction as described under “Experimental Procedures.” Nonbinding cells were solubilized in Nonidet P-40 and assayed for hCG binding. Each experiment was repeated four to six times in duplicate and means ± S.D. were calculated. NS stands for not significant.

### Table: Binding on Cell

| Kd (pM) | Receptor/Cell |
|---------|---------------|
| 450 ± 70 | 40,000 ± 4,800 |
| 630 ± 110 | 22,000 ± 3,000 |
| NS       | NS            |
| 1,600 ± 150 | 4,400 ± 500  |
| NS       | 40,000 ± 5,400 |
| 830 ± 140 | 42,700 ± 3,600 |
| NS       | NS            |
| NS       | NS            |
| 1,160 ± 170 | 27,500 ± 3,800 |
| NS       | NS            |
| NS       | 750 ± 120     |
| 1,010 ± 170 | 33,200 ± 4,300 |

### Table: Binding in Solution

| Kd (pM) | Receptor/Cell |
|---------|---------------|
| 810 ± 180 | 45,000 ± 6,800 |
| 120 ± 30  | 41,700 ± 5,700 |
| 550 ± 60  | 40,800 ± 8,500 |
| 640 ± 100 | 45,000 ± 6,700 |
| 680 ± 90  | 40,000 ± 5,800 |
| 750 ± 130 | 40,000 ± 5,800 |
| 600 ± 90  | 30,000 ± 4,600 |
| 680 ± 120 | 36,000 ± 4,600 |
| 1,000 ± 210 | 36,000 ± 4,100 |
| 930 ± 170 | 49,000 ± 6,600 |

### Table: cAMP Induction

| EC50 (pM) | Max (fmol) |
|-----------|------------|
| Wild Type | 93 ± 11    | 140 ± 15  |
| Thr250Ala | 71 ± 14    | 123 ± 14  |
| Ph250Ala  | 449 ± 67   | 16 ± 4    |
| Ar250Ala  | 112 ± 10   | 111 ± 15  |
| As250Ala  | 100 ± 8    | 127 ± 21  |
| Le250Ala  | 100 ± 14   | 113 ± 17  |
| Pn260Ala  | 134 ± 17   | 108 ± 13  |
| Ly260Ala  | 87 ± 16    | 140 ± 23  |
| Ly260Ala  | 134 ± 19   | 130 ± 12  |
| Gl260Ala  | 108 ± 7    | 121 ± 15  |
| Gl260Ala  | 114 ± 11   | 131 ± 14  |
TABLE II
Multiple substitutions for Tyr<sup>253</sup>

| Receptor plasmid       | 125<sup>1</sup>-bCG  | 125<sup>1</sup>-FSH  | EC<sub>50</sub> | Max  |
|------------------------|----------------------|----------------------|----------------|------|
| Wild type              | 52,000 ± 4,000        | 83,000 ± 9,000       | 30,000 ± 2,000 | 30,000 ± 2,000 |
| Tyr<sup>253</sup> → Ala | 39,000 ± 3,000        | NS                   | 34,000 ± 3,000 | 37,000 ± 4,000 |
| LHR<sup>253</sup> → Ala/LHR<sup>WT</sup> | 27,000 ± 2,000        | 49,000 ± 5,000       | 30,000 ± 2,000 | 30,000 ± 2,000 |
| FSHR<sup>WT</sup>      | 34,000 ± 3,000        | NS                   | 32,000 ± 6,000 | 38,000 ± 5,000 |
| FSHR<sup>WT</sup>/LHR<sup>253</sup> → Ala | 41,000 ± 3,000        | NS                   | 88,000 ± 9,000 | NS   |

TABLE III
Activities of cotransfected receptors

Cells that were stably expressing the Tyr<sup>253</sup> → Ala mutant receptor in cells were transiently transfected with the wild type LHR or wild type FSH receptor plasmid. The cells were assayed for hormone (hCG or FSH) binding as described under “Experimental Procedures.” Conversely, cells that were stably expressing the FSH receptor on the cell surface were transiently transfected with the Tyr<sup>253</sup> → Ala mutant receptor plasmid and assayed for hormone binding. NS stands for not significant; WT indicates wild type.

| Receptor plasmid       | 125<sup>1</sup>-bCG  | 125<sup>1</sup>-FSH  | 129<sup>1</sup>-bCG | 129<sup>1</sup>-FSH  |
|------------------------|----------------------|----------------------|---------------------|---------------------|
| LHR<sup>253</sup> → Ala | 52,000 ± 4,000        | 83,000 ± 9,000       | 50,000 ± 4,000      | 35,000 ± 3,000      |
| LHR<sup>253</sup> → Ala/LHR<sup>WT</sup> | 39,000 ± 3,000        | 34,000 ± 3,000       | 49,000 ± 5,000      | 37,000 ± 4,000      |
| LHR<sup>253</sup> → Ala/FSHR<sup>WT</sup> | 27,000 ± 2,000        | 32,000 ± 6,000       | 35,000 ± 4,000      | 38,000 ± 5,000      |
| FSHR<sup>WT</sup>      | 34,000 ± 3,000        | 32,000 ± 6,000       | 35,000 ± 4,000      | 38,000 ± 5,000      |
| FSHR<sup>WT</sup>/LHR<sup>253</sup> → Ala | 41,000 ± 3,000        | 41,000 ± 3,000       | 88,000 ± 9,000      | NS                 |

TABLE IV
Multiple substitutions for Ser<sup>255</sup>

Ser<sup>255</sup> was substituted with several amino acids. The mutant receptors were expressed and assayed for hCG binding to intact cells and in solution as well as cAMP induction as described under “Experimental Procedures.”

| Receptor plasmid       | 125<sup>1</sup>-bCG  | 125<sup>1</sup>-FSH  | 129<sup>1</sup>-bCG | 129<sup>1</sup>-FSH  |
|------------------------|----------------------|----------------------|---------------------|---------------------|
| Wild type              | 40,000 ± 4,800        | 42,700 ± 3,600       | 42,700 ± 3,600      | 42,700 ± 3,600      |
| Ser<sup>255</sup> → Ala | 40,000 ± 4,800        | 42,700 ± 3,600       | 42,700 ± 3,600      | 42,700 ± 3,600      |
| Ser<sup>255</sup> → Thr | 32,800 ± 5,900        | 28,700 ± 3,400       | 28,700 ± 3,400      | 28,700 ± 3,400      |
| Ser<sup>255</sup> → Tyr | 33,000 ± 2,500        | 33,000 ± 2,500       | 33,000 ± 2,500      | 33,000 ± 2,500      |
| Ser<sup>255</sup> → Val | 18,000 ± 3,000        | 18,000 ± 3,000       | 18,000 ± 3,000      | 18,000 ± 3,000      |
| Ser<sup>255</sup> → Ile | 24,000 ± 2,600        | 24,000 ± 2,600       | 24,000 ± 2,600      | 24,000 ± 2,600      |

attached to the peptide and that one was apparently closer to hCGα and labeled it, whereas the other was closer to and labeled hCGβ. This is in contrast to an LHR peptide derivatized with an AB that is capable of labeling only one of the subunits but not both (35, 37). It suggests the specificity of photoaffinity labeling.

Next, increasing concentrations of hCG were incubated with a constant amount of AB-125<sup>1</sup>-LHR<sup>246–269</sup> and irradiated with UV for 60 s (Fig. 3B). The results show that the hCG labeling was also dependent on the hCG concentration, reaching a maximum labeling at and above 200 ng. In the following experiment, 200 ng of hCG was incubated with increasing concentrations of AB-125<sup>1</sup>-LHR<sup>246–269</sup> and UV-photolyzed for 60 s (Fig. 4A). The result shows the dependence of the labeling on the AB-125<sup>1</sup>-LHR<sup>246–269</sup> concentration with the maximum labeling at and above 200 ng of AB-125<sup>1</sup>-LHR<sup>246–269</sup>. This experiment, taken together with the UV- and hCG-dependent experiments, indicate that the labeling of hCGα, hCGβ, and hCGαβ is saturable and requires the peptide derivative, hCG, and UV photolysis. In addition, the results indicate that hCG was covalently labeled by AB-125<sup>1</sup>-LHR<sup>246–269</sup> and that this labeling was specific. To test whether the derivatization of AB impacted the activity of the peptide, AB-125<sup>1</sup>-LHR<sup>246–269</sup> was incubated with hCG in the presence of increasing concentrations of nonlabeled peptide and photolyzed (Fig. 4B). Nonlabeled peptide competitively inhibited the labeling. To further test the labeling specificity, AB-125<sup>1</sup>-LHR<sup>246–269</sup> was incubated with increasing concentrations of denatured hCG and photolyzed. No labeled bands appeared (data not included), indicating that AB-125<sup>1</sup>-LHR<sup>246–269</sup> labeled active, but not denatured, hCG.
hCGα and 10% for hCGβ, indicating that the labeling is saturable and specific. In addition, hCGα was preferentially labeled compared with hCG, which is consistent with the preferential photoaffinity labeling of the hCGα subunit. This affinity cross-linking is more effective than photoaffinity labeling. A possible explanation is that SES can reach further than AB can, because of the maximum cross-linkable distance, ~13 Å for SES and ~7 Å for AB. The SES cross-linking results also show that either or both amino groups of Lys265 and Lys266 of 125I-LHR246–269, the only amino groups in the peptide, were cross-linked to one of seven hCGα amines, five hCGβ amines, or those in both subunits. The SES-dependent cross-linking of 125I-LHR246–269 to the hCG subunits was competitively inhibited by nonlabeled peptide, suggesting a specificity. Furthermore, SES failed to cross-link 125I-LHR246–269 to denatured hCG (data not included).

**Differential Effects of Exoloop Peptides on Photoaffinity Labeling of hCG—**Our mutational analysis suggests that the Thr250–Gln268 region may be involved in signal generation in the endodomain, perhaps interacting with hCG, the endodomain, or both. Such interactions, if true, might impact the specific affinity labeling of hCG with LHR246–269. To this end, hCG was incubated with AB-125I-LHR246–269 in the presence of an excess amount of exoloop peptides and irradiated with UV (Fig. 7A). The labeling was inhibited most conspicuously by the exoloop 2 peptide, whereas the inhibition by the exoloop 3 peptide or exoloop 1 peptide was significantly less or barely noticeable, respectively (Fig. 7, B and C).

**DISCUSSION**

The results of our mutagenesis and affinity labeling studies indicate that the Thr250–Gln268 sequence of the LHR exodomain interacts with both subunits of hCG, particularly with hCGα. In previous reports, we have shown that the LRRs function in the LHR exodomain and interact with hCG (33–35) and that the N-flanking region of the LRRs also interacts with hCG (36, 37). Receptor peptide mimics were useful in these studies, although synthetic peptide mimics have limitations. They do not necessarily mimic the function of the corresponding sequences in proteins, probably due to structural differences. This is underscored by the fact that an extension of either terminus or substitution of a single amino acid in peptides mimics deprives their activity, specificity, or both (20, 35, 37). Therefore, we have carefully chosen the sequence and size of the hinge peptide based on our mutational analysis and others’ studies (20, 36). Furthermore, short synthetic peptides can be structured for a stable tertiary structure (47) and assume a potent biological activity (48–50).

Taken together, our observations show that the LRRs and their flanking regions interact with hCG. This suggests an intriguing possibility that the LRRs face hCG, probably at the front of hCG with the β seat belt and α C terminus (31, 32), whereas the flanking regions of the LRRs face the sides of hCG and may reach the back of hCG. This would trap hCG in the 1/3 donut structure of the LRRs, slowing the dissociation rate of hCG and LHR (51) and enhancing the affinity. This is consistent with the fact that high affinities are generally ascribed to slow dissociation rates rather than fast association rates (52). For example, kinetic studies on interactions between hormones and receptors, antibodies and antigens, and enzymes and substrates show that association rate constants, $k_{on}$ are in the
narrow range of $10^5$ to $10^7$ M$^{-1}$ s$^{-1}$, whereas dissociation rate constants, $k_{off}$, vary widely from 1 s$^{-1}$ for low affinity complexes to a half-life of more than 3 months (10$^{-7}$ s$^{-1}$) (52).

An hCG contact point in the Thr$^{250}$-Gln$^{268}$ sequence is near or not too far from the two tandem Lys residues at 265 and 266 in the second half of the sequence. This is consistent with the fact that most Ala substitutions for the second half residues reduced the binding affinity by up to 2.3-fold but did not noticeably impact the surface expression and maximum cAMP induction level (Fig. 3, A and B). In contrast to this primary role of the second half in hCG binding, the first half of the sequence seems to be involved in the signal generation. For example, some substitutions for Ser$^{255}$ resulted in substantially high basal cAMP levels. In particular, the cAMP level was dramatically high for Val and Ile substitutions, indicating constitutive activation of adenyl cyclase (38). Taken together with the results that the interaction of the Thr$^{250}$-Gln$^{268}$ sequence with hCG is inhibited by the exoloop 2 peptide, our observations implicate the involvement of the Thr$^{250}$-Gln$^{268}$ sequence in the interaction of hCG-exodomain complex with the endodomain and the subsequent signal generation. This is the first clue to the identification of the endodomain contact points in the exodomain. The exoloop 2 peptide may directly compete with LHR$^{246-269}$ for the binding site or allosterically interfere with the interaction between LHR$^{246-269}$ and hCG. On the other hand, the exoloop 3 peptide marginally inhibited the labeling of hCG by AB-125I-LHR$^{246-269}$, and the inhibition by the exoloop 1 peptide was barely detectable. This suggests several possibilities that these peptides bind hCG, but the affinities are relatively low, do not share the binding site of LHR$^{246-269}$, or do not bind hCG at all. Since exoloop 3 impacts hCG binding to the exodomain, it is possible that exoloop 3 is capable of binding the exodomain or hCG-exodomain complex, but the affinity is low.

In addition, exoloop 3 and the LHR$^{246-269}$ sequence may not share a significant portion of their binding sites in hCG and the hCG-exodomain complex.

A phenyl or phenolic group at Tyr$^{253}$ is crucial for the recognition by the surface-targeting machinery, since both Tyr and Phe are permissible. It is striking to see that substitutions of other amino acids, including other hydrophobic amino acids, completely blocked the surface expression, suggesting a high specificity. The attenuation of surface expression by Ala substitution for Leu$^{251}$, Thr$^{252}$, Tyr$^{253}$ and His$^{256}$-Cys$^{257}$-Cys$^{258}$ is specific, and therefore, these residues may assume a particular structure. The two groups consist of 3 contiguous amino acids, and a secondary structure analysis suggests interesting possibilities. The Leu$^{251}$, Thr$^{252}$, Tyr$^{253}$ sequence is predicted to be part of a $\beta$ sheet (53), thus orienting Leu$^{251}$ and Tyr$^{253}$ on one side of the $\beta$ sheet and Thr$^{252}$ on the other. In such a structure, Leu$^{251}$ and Tyr$^{253}$ face one side, whereas Thr$^{252}$ would face the opposite side. This may explain why Leu$^{251}$ and Tyr$^{253}$ were more important for receptor transport than Thr$^{252}$. Furthermore, it has been suggested that Leu and Tyr are part of a transport determinant of membrane proteins (54). Interestingly, the $\beta$ sheet is followed by a turn at Pro$^{254}$ and Ser$^{255}$ (53, 55). Therefore, the two groups, Leu$^{251}$-Thr$^{252}$-Tyr$^{253}$ and His$^{256}$-Cys$^{257}$-Cys$^{258}$, are likely to be in close proximity, which could allow them to form a contiguous determinant. This is also consistent with the observation that Pro$^{254}$ and Ser$^{255}$ play a role distinct from the flanking residues, in particular the unique role in modulating signal generation (38).

In conclusion, the results presented in this study show that the C-terminal flanking (hinge) region (38), Thr$^{250}$-Gln$^{268}$, of the LRRs in the LHR exodomain interacts with hCG, contacting primarily hCGa. This interaction is specifically inhibited by exoloop 2 but not by exoloops 1 and 3 of the endodomain, suggesting an intimate relationship in the ternary complex of Thr$^{250}$-Gln$^{268}$, exoloop 2 and hCG. Taken together, our observations in this and previous articles (33–35) suggest a new paradigm that the LRRs face the front of hCG, while both of the flanking regions of the LRRs interact with the sides of hCG and possibly reach the back of hCG as well. This would trap hCG in the 1/3 donut structure of the LRRs, increasing the binding affinity. In addition, mutations of Ser$^{255}$ in the exodomain sequence can constitutively activate the receptor. This result, along with the ternary complex, provides a new clue for the identity of the enigmatic signal modulator in the exodomain.

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The Role of the Hinge Region of the Luteinizing Hormone Receptor in Hormone Interaction and Signal Generation

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