Hormone Interactions to Leu-rich Repeats in the Gonadotropin Receptors

II. ANALYSIS OF LEU-RICH REPEAT 4 OF HUMAN LUTEINIZING HORMONE/CHORIONIC GONADOTROPIN RECEPTOR*

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The luteinizing hormone receptor (LHR) consists of an ~350-amino acid-long N-terminal extracellular exodomain and a membrane-associated endodomain of similar size. Human chorionic gonadotropin (hCG) binds to the exodomain, and then hCG/exodomain complex is thought to make a secondary contact with the endodomain and generate hormone signals. The sequence alignment of the exodomain shows imperfectly matching eight to nine Leu-rich repeats (LRRs). In the preceding article (Song, Y., Ji, I., Beauchamp, J., Isaacs, N., and Ji, T. (2001) J. Biol. Chem. 276, 3426-3435), we have shown that LRR2 and LRR4 are crucial for hormone binding. In this work, we have examined the residues of LRR4, in particular Leu103 and Ile105 in the putative \( \beta \) strand. Our data show that Leu103 and Ile105 are involved in the specific, hydrophobic interaction of the LRR4 loop, likely to form the hydrophobic core. This loop is crucial for the structural integrity of all of the LRRs. In contrast, the downstream sequence consisting of Asn107, Thr109, Gly109, and Ile110 of LRR4 is crucial for cAMP induction but not for hormone binding, folding, and surface expression. This implicates, for the first time, its involvement in the interaction with the endodomain and signal generation. The evidence for the interaction is presented in the following article.

The luteinizing hormone/chorionic gonadotropin receptor (LHR) plays crucial roles in development of the gonads in both sexes and the ovulation cycle of the females (1, 2). LHR is a member of the glycoprotein hormone receptor subfamily, which includes the follicle-stimulating hormone receptor and the thyroid-stimulating hormone receptor in the G protein-coupled receptor superfamily. It consists of an extracellular N-terminal half (exodomain) and a membrane-associated C-terminal half (endodomain) (3, 4). The ~350-amino acid-long exodomain is responsible for high affinity hormone binding (5–7) and hormone specificity (8–10). The resulting hCG-exodomain complex is thought to make a secondary contact with the endodomain, which generates hormone signal (11–13). Despite the importance of this secondary contact, it has been difficult to find any clues for the secondary contact points. These contact points and residues are likely to be the site of signal generation and play a key role in the signal generation.

The exodomain contains imperfect Leu/Ile-rich repeats (LRRs) of 22–29 amino acids (3, 14–18), which are a common structural motif found in a large family of proteins, including glycoprotein hormone receptors (19). In the crystals of ribonuclease inhibitors, individual LRRs consist of a \( \beta \) strand connected to a parallel \( \alpha \) helices despite divergent sequences. In each \( \beta \) strand, there are two conserved Leu and/or Ile residues in the alternate positions as Leu/Ile-X-Leu/Ile. Therefore, the primary homology among LRRs is the Leu/Ile-X-Leu/Ile motif in \( \beta \) strands. Furthermore, the \( \beta \) strands in ribonuclease inhibitors are involved in the interaction with ribonuclease. However, it has been unknown whether the putative LRRs of the LHR are active at all and what their nature and function are.

In the preceding article (20) we have shown that some, but not all, LRRs of the LHR and the FSH-R are crucial for hormone binding. In LHR, LRR2 and LRR4 are crucial. In this study, the residues around the Leu-Ser-Ile motif in LRR4 of LHR were examined. Our results suggest that the Leu and Ile are involved in the specific and tightly packed hydrophobic interaction in the core of LRR4 loop. In addition, our data implicate, for the first time, the involvement of LRR4 in the interaction of the hCG-exodomain complex with the endodomain. The evidence is presented in the following companion article (21).

EXPERIMENTAL PROCEDURES

Mutant human LHR cDNAs were prepared, expressed in HEK 293 cells, and assayed for hormone binding and intracellular cAMP production as described previously (20, 22). All assays were carried out in duplicate and repeated four to six times. Means and standard deviations were calculated. FLAG-LHR was prepared by inserting the FLAG epitope (23), Asp-Tyr-Lys-Asp-Asp-Asp-Lys, between the C terminus (Ser26) of the signal sequence and the N terminus (Arg27) of mature receptors. (20).

RESULTS

To investigate the Leu-Ser-Ile motif of LRR4 in LHR, we decided to examine the extended sequence encompassing the motif and the flanking residues, Arg28-Leu-Lys-Tyr-Arg-Leu-Ser-Ile-Cys-Asn-Thr-Gly-Ile-Arg-Lys (21). These residues were individually substituted with Ala, and each of the Ala substitution mutants was stably expressed in HEK 293 cells. They were assayed for \( ^{125}\text{I}-\text{hCG} \) binding on intact cells or after solubilization in Triton X-100 and for hCG-dependent cAMP induction. As shown by the binding displacement data and Scatchard...
FIG. 1. Ala scan and characterization of residues around the conserved Leu\(^{103}\)/Ile\(^{105}\) motif in LRR4 of LHR. Amino acids from Arg\(^{99}\) to Lys\(^{112}\) encompassing the conserved Leu\(^{103}\)/Ile\(^{105}\) motif in the putative β strand of LRR4 were individually substituted with Ala. The resulting mutant receptors were stably expressed on human 293 cells. Cells were assayed for \(^{125}\)I-hCG binding in the presence of increasing concentrations of nonradioactive hCG (A), and Scatchard analysis (B) was plotted against specific binding. Cells were also solubilized in Triton X-100 and assayed for \(^{125}\)I-hCG binding in the presence of unlabeled hCG (C), and the results were converted to Scatchard plots (D). In addition, intact cells were treated with increasing concentrations of unlabeled hCG and intracellular cAMP was measured (E) as described under “Experimental Procedures.” Experiments were repeated four to six times in duplicate. NS stands for not significant.
plots in Fig. 1, the cells transfected with the vector carrying the Arg$^{99}$ → Ala mutant bound $^{125}$I-hCG with a $K_d$ value of 1,410 pM as intact cells (Fig. 1, A and B) or 1,640 pM after solubilization (Fig. 1, C and D). These values were higher than the corresponding wild type values of 600 pM and 1,100 pM. In contrast, the cells that were transfected with the vector containing no receptor cDNA or were not transfected at all did not bind the hormone (data not included). These results indicate that the cells transfected with the Arg$^{99}$ → Ala plasmid expressed the mutant receptor on the cell surface as well as in the cells and that the mutant was capable of binding hCG. In addition, the cells produced cAMP in response to hCG in a dose-dependent manner with an EC$^{50}$ value of 230 pM and a maximum cAMP level of 68 fmol/1,000 cells. These results are similar to those of the Arg$^{99}$ → Ala mutant, indicating that the Arg$^{99}$ → Ala and Lys$^{101}$ → Ala mutants behaved the same and the Ala substitution for Arg$^{99}$ and Lys$^{101}$ had the similar effect. The effect of the Ala substitution for the next residue, Tyr$^{102}$, was more severe, although it did not abolish the activity of the mutant (Fig. 1). For example, the $K_d$ values for binding to intact cells and solubilized cells were 4,610 and 7,800 pM, respectively, while the EC$^{50}$ for cAMP production was 720 pM. These affinities of the mutant are only 12–14% of the wild type values, although the maximum cAMP level was 120 fmol/1,000 cells, which is nearly 80% of the wild type level (Table I).

The effects of the Ala substitution for the next three residues were similar to or more severe than the effect of the Tyr$^{102}$ → Ala substitution. For example, the Ser$^{104}$ → Ala mutant had $K_d$ values of 3,600 and 5,370 pM for hormone binding to intact cells and solubilized cells, respectively (Fig. 1, F–I). The EC$^{50}$ value for cAMP induction was 599 pM (Fig. 1J). The affinities for hormone binding to intact cells and cAMP induction were, therefore, only 15–17% of the wild type affinities (Table I). Leu$^{103}$ → Ala and Ile$^{105}$ → Ala resulted in the complete loss of hormone binding both to intact cells and solubilized cells as well as hCG-dependent cAMP induction (Fig. 1, F–J). These two residues correspond to the conserved Leu/Ile-X-Leu/Ile motif in the β strands of LRRs. All of the mutant receptors were also capable of inducing cAMP synthesis. These results indicate that the conserved Leu/Ile-X-Leu/Ile motif, Leu$^{103}$ and Ile$^{105}$, is indeed more sensitive to Ala substitution than the flanking residues except Leu$^{103}$. Interestingly, this was independent of the chemical and physical properties of the side chains of the flanking residues.

Since these results are consistent with the LRR hypothesis, we investigated the nature of the Leu$^{103}$ → Ala and Ile$^{105}$ → Ala substitutions by replacing Leu$^{103}$ and Ile$^{105}$ with a panel of amino acids containing hydrophobic, hydrophilic, neutral, anionic, and cationic side chains. First, Leu$^{103}$ was substituted with various hydrophobic amino acids. The substitutions of Val, Phe, and Ile increased the $K_d$ values up to 10-fold (Fig. 2, A–D), thus reducing the affinity to 11–44% of the wild type (Table II), but did not abrogate hormone binding and cAMP induction (Fig. 2E). It is notable that the Leu$^{103}$ → Ile mutant

| Mutant   | Hormone binding, $K_d$<sup>mut</sup> | cAMP synthesis |   |   |   |
|----------|------------------------------------|----------------|---|---|---|
|          |                                    |                | $EC_{50}$<sup>mut</sup> | Max<sub>mut</sub> | Max<sub>wt</sub>/Max<sub>mut</sub> |
| Wild type| 1.00                               |                | 1.00 | 1.00 | 1.00 |
| Arg$^{99}$ → Ala | 0.44                           | 0.39 | 0.47 | 0.88 | 1.07 |
| Leu$^{100}$ → Ala | NS                           | NS   | NS   | NS   | NS   |
| Lys$^{101}$ → Ala | 0.38                           | 0.42 | 0.44 | 1.10 | 1.16 |
| Tyr$^{102}$ → Ala | 0.14                           | 0.12 | 0.79 | 0.86 | 5.60 |
| Leu$^{103}$ → Ala | NS                           | NS   | NS   | NS   | NS   |
| Ser$^{104}$ → Ala | 0.17                           | 0.15 | 0.53 | 0.88 | 3.10 |
| Ile$^{105}$ → Ala | 0.33                           | 0.15 | 0.53 | 0.45 | 1.60 |
| Cys$^{106}$ → Ala | 0.58                           | 0.7   | 0.41 | 1.21 | 0.71 |
| Asn$^{107}$ → Ala | 0.64                           | 0.10 | 0.69 | 0.16 | 1.08 |
| Thr$^{108}$ → Ala | 0.64                           | 0.12 | 0.14 | 0.44 | 0.52 |
| Gly$^{109}$ → Ala | 0.27                           | 0.8   | 0.79 | 1.74 | 1.72 |
| Ile$^{110}$ → Ala | 0.46                           | 0.20 | 0.45 | 0.83 | 1.88 |
| Arg$^{111}$ → Ala | 0.24                           | 0.20 | 0.55 | 0.67 | 2.83 |
| Lys$^{112}$ → Ala | 0.30                           | 0.20 | 0.55 | 0.67 | 2.83 |

TABLE I
Percent $K_d$, EC$^{50}$, and maximum cAMP values of Ala substitution mutants

$K_d$<sup>mut</sup> values for Ala substitution mutants in Fig. 1 were calculated by dividing the wild type $K_d$ with the mutant $K_d$ values as $EC_{50}$ values. Maximum cAMP values were calculated by dividing maximum cAMP of each mutant with that of the wild type receptor. NS stands for “not significant.” In addition, ratios of ($EC_{50}$<sup>mut</sup>/$EC_{50}$<sup>mut</sup>)/($K_d$<sup>mut</sup>)<sup>mut</sup> and (max<sub>mut</sub>/max<sub>mut</sub>)<sup>mut</sup> are presented.
was the one with the binding affinity of only 10% of the wild type. This suggests that Ile could not replace Leu 103 without impacting the receptor function. When Leu 103 was substituted with Trp, the bulkiest hydrophobic amino acid, the mutant did not show hormone binding to intact cells and solubilized receptors. Similarly, the mutant receptor with the substitution of hydrophilic Thr, neutral Asn, anionic Asp, or cationic Lys did not bind the hormone (Fig. 2, F–H). Since these substitutions introduced an amino acid with a different side chain, it is difficult to tell whether the substitution effects were due to the missing side chain of Leu or the newly introduced side chain. To test these possibilities, Leu 103 was deleted, and the resulting mutant did not bind the hormone (Fig. 2, F–H).

Next, Ile 105 was examined after substitutions. The substitutions of Phe, Val, and Leu were tolerable, as the resulting mutants were capable of hormone binding and inducing cAMP (Fig. 3, A–E), although the affinities were reduced to 16–56% of the wild type affinity (Table II). This is similar to the Phe, Val, and Ile substitutions for Leu 103. Interestingly, the Val substitution impacted the least for both Leu 103, whereas the Phe substitution did the least for Ile 105, suggesting the distinct environment of the two residues. On the other hand, the Trp substitution resulted in the complete loss of the activity for both Ile 105 and Ile 103. The substitution for Ile 105 with non-hydrophobic Thr, Asn, Asp, or Lys completely impaired the receptor activity as was the case with Leu 103 (Fig. 3, F–H). Next, Ile 105 was examined after substitutions. The substitutions of Phe, Val, and Leu were tolerable, as the resulting mutants were capable of hormone binding and inducing cAMP (Fig. 3, A–E), although the affinities were reduced to 16–56% of the wild type affinity (Table II). This is similar to the Phe, Val, and Ile substitutions for Leu 103. Interestingly, the Val substitution impacted the least for both Leu 103, whereas the Phe substitution did the least for Ile 105, suggesting the distinct environment of the two residues. On the other hand, the Trp substitution resulted in the complete loss of the activity for both Ile 105 and Ile 103. The substitution for Ile 105 with non-hydrophobic Thr, Asn, Asp, or Lys completely impaired the receptor activity as was the case with Leu 103 (Fig. 3, F–H). To investigate the relationship between Leu 103 and Ile 105, a reciprocal mutant with Leu 103 → Ile and Ile 105 → Leu was generated. The resulting double mutant, Leu 103 → Ile/Ile 105 → Leu, poorly bound the hormone with a $K_d$ value of 3,340 nM on intact cells and with a $K_d$ value of 6,970 nM in solution (Fig. 4). These

Table II

| Mutant | Hormone binding, $K_d^{wt/mut}$ | cAMP synthesis, EC50 $^{wt/mut}$ | Maxmut/wt |
|--------|-------------------------------|-------------------------------|-----------|
| Wild type | 1.00 | 1.00 | 1.00 |
| Leu103 → Val | 0.44 | 0.22 | 0.76 |
| Leu103 → Phe | 0.17 | 0.32 | 0.69 |
| Leu103 → Ile | 0.11 | 0.17 | 0.53 |
| Ile105 → Phe | 0.56 | 0.10 | 0.37 |
| Ile105 → Val | 0.16 | 0.12 | 0.50 |
| Leu103 → Leu | 0.21 | 0.11 | 0.43 |
| Leu103 → Ile/Ile105 → Leu | 0.19 | 0.40 | 0.44 |

$K_d$, EC50, and maximum cAMP values of substitution mutants of Leu103 and Ile105

$K_d^{wt/mut}$ for Ala substitution mutants in Figs. 2 and 3 were calculated as described in Table I.
values indicate that the binding affinities are <20% of the corresponding wild type affinities, consistent with the view of the distinct environment of Leu_103 and Ile_105.

To determine whether the double mutant receptor was expressed at all, the FLAG-Leu_103 \rightarrow Ile/Ile_105 \rightarrow Leu receptor was tested. 125I-Monoclonal anti-FLAG antibody bound to the intact cells as well as solubilized cells that were transfected with the FLAG-Leu_103 \rightarrow Ile/Ile_105 \rightarrow Leu plasmid (data not included). In contrast, cells that were not transfected or transfected with the LHR with Leu_103 \rightarrow Ile/Ile_105 \rightarrow Leu plasmid did not bind the antibody. These results clearly indicate expression of FLAG-LHR with Leu_103 \rightarrow Ile/Ile_105 \rightarrow Leu on the cell surface and in cells, as well as expression of the Leu_103 \rightarrow Ile/Ile_105 \rightarrow Leu mutant lacking the FLAG epitope. When the Leu_103 \rightarrow Ile/Ile_105 \rightarrow Leu mutant was reverted to the wild type receptor, the revertant was capable of binding the hormone and inducing cAMP production. Therefore, the inability of the Leu_103 \rightarrow Ile/Ile_105 \rightarrow Leu mutant to bind the hormone and induce cAMP was due to the double substitutions, not due to unexpected random mutations during the mutagenesis and cloning.

DISCUSSION

The results observed in this work show that the Ala substitutions for Leu_103 and Ile_105 abolished the hormone binding activity of the receptor, whereas the Ala substitution for Tyr_102 and Ser_104 severely impaired the receptor. In contrast to these four crucial and tandem residues, other residues among the sequence of the 14 amino acids, Arg_99-Leu-Lys-Tyr-Leu-Ser-Ile-Cys-Asn-Thr-Gly-Ile-Arg-Lys_112, in the putative LRR4 are marginally or less severely impacted by Ala substitution except Leu_100. Therefore, the sequence of Tyr_102-Leu_103-Ser_104-Ile_105 appears to be crucial for hormone binding and may constitute the core of the putative \( \beta \) strand of LRR4. This configuration would orient Leu_103 and Ile_105 at one side and Tyr_102 and Ser_104 at the other side of the \( \beta \) strand, suggesting a hydrophobic core comprising Leu_103 and Ile_105 and a hydrophilic phase with Tyr_102 and Ser_104. The hydrophobic core may include Leu_100, as it is equally sensitive to Ala substitution. In fact, the result of multiple substitutions for Leu_103-Ile_105 indicate such a hydrophobic core, since only hydrophobic residues larger than Ala, but less bulky than Trp, are tolerable at the positions of Leu_103 and Ile_105. Substitutions with hydrophilic, neutral, cationic, and anionic residues totally impaired the receptor activity.
These results suggest that the hydrophobic core is compact and specific.

It is interesting to note that the hormone binding affinity of the reciprocal double mutant, Leu103 \( \rightarrow \) Ile and Leu, respectively, and the resulting mutant receptor was stably expressed on HEK 293 cells. Cells were assayed for \( \text{hCG} \)-dependent cAMP induction as described in the legend to Fig. 1.

### TABLE III
Comparison of the LRR4 sequence

| LHR (human) | PGAF1NLPG | LKYLICNTG | KKKPFDVTKV |
| LHR (sheep) | PG*FTN**R | *KY*S*C*** | ***I****I |
| LHR (bovine) | AG*FTN**R | *KY*S*C*** | ***I****I |
| LHR (pig) | PG*FTN**R | *KY*S*C*** | ***I****I |
| LHR (rat) | PG*FTN**R | *KY*S*C*** | ***I****I |
| LHR (canina) | PG*FTN**R | *KY*S*C*** | ***I****I |
| FSHR (human) | PE*FQN**N | *QY*L*S*** | *KHL***H*I |
| FSHR (bovine) | PD*FQN**N | *RY*L*S*** | *KHL***H*I |
| FSHR (sheep) | PD*FQN**N | *RY*L*S*** | *KHL***H*I |
| FSHR (horse) | HD*FQN**N | *QY*L*S*** | *KHL***H*I |
| FSHR (pig) | PD*FQN**N | *RY*L*S*** | *KHL***H*I |
| FSHR (rat) | PE*FQN**S | *RY*L*S*** | *KHL***H*I |
| FSHR (macaca) | PE*FQN**N | *RY*L*S*** | *KHL***H*I |
| FSHR (chick) | QD*FQH**S | *RY*L*S*** | LSF*L*V** |
| TSHR (human) | PD*LKE**L | *KF*G*F*** | LKM***L*** |
| TSHR (bovine) | SG*LKE**L | *KF*G*F*** | L*V***L**I |
| TSHR (sheep) | SG*LKE**L | *KF*G*F*** | L*V***L**I |
| TSHR (rat) | PD*LTE**L | *KF*G*F*** | L*V***L**I |
| TSHR (mouse) | PD*LTE**L | *KF*G*F*** | L*V***L**I |
| TSHR (canina) | PD*LKE**L | *KF*G*F*** | LGV******* |

These results suggest that the hydrophobic core is compact and specific.

It is interesting to note that the hormone binding affinity of the reciprocal double mutant, Leu103 \( \rightarrow \) Ile and Leu, respectively, and the resulting mutant receptor was stably expressed on HEK 293 cells. Cells were assayed for \( \text{hCG} \)-dependent cAMP induction as described in the legend to Fig. 1.
41% of the wild type value. The only exception is Gly$^{109}$ → Ala substitution, which nearly abolished cAMP induction to 14% of the wild type value. This trend is more obvious when the (maximum $cAMP_{mut/wt}$/$K_d^{wt/mut}$, where mut indicates mutant and wt indicates wild type) ratios are compared among the Ala mutants (Table I). The ratios are more than 1.00 for all except Gly$^{109}$ → Ala and Asn$^{107}$ → Ala. The ratios for the two mutants are 0.52 and 0.71, and such exceptionally low ratios are not found with any LRR2 Ala mutants (20). The sequence alignment reveals the striking conservation of the amino acids from Leu97-Pro-Gly-Leu-Ser-Ile-Cys-Asn-Thr-Gly 109 (Table III). Out of the 13 amino acids, 8 are absolutely identical among LHR, follicle-stimulating hormone receptor, and thyroid-stimulating hormone receptor of all species. Interestingly, Asn$^{107}$-Thr-Gly$^{109}$ are uniquely in tandem and their maximum $cAMP_{mut/wt}$/$K_d^{wt/mut}$ ratios are 0.71, 1.08, and 0.52, respectively. These residues are not essential for folding and surface expression, since the Ala mutants were successfully expressed on the cell surface and capable of binding the hormone. However, Ala substitution of them notably impaired cAMP induction as obvious by their significantly high EC$_{50}$ for hCG and, furthermore, is involved in the interaction of hCG-exodomain complex with the endodomain.

In conclusion, the Leu$^{103}$–Ile$^{105}$ sequence is crucial for the specific interaction to form the hydrophobic core of LRR4. In addition, the downstream sequence consisting of Asn$^{107}$, Thr$^{108}$, and Gly$^{109}$ is crucial for cAMP induction but not for hormone binding, folding, and surface expression. Therefore, they are likely involved in the interaction with the endodomain. This is the first evidence suggesting an endodomain contact point in the exodomain. Then, the inevitable question is whether LRR4 interacts with hCG at all. In the following article (21) we show evidence that LRR4 does interact with hCG and, furthermore, is involved in the interaction of hCG-exodomain complex with the endodomain.

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