Exoloop 3 of the Luteinizing Hormone/Chorionic gonadotropin Receptor

LYS583 IS ESSENTIAL AND IRREPLACEABLE FOR HUMAN CHORIOGONADOTROPIN (hCG)-DEPENDENT RECEPTOR ACTIVATION BUT NOT FOR HIGH AFFINITY hCG BINDING*

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Ki-Sung Ryu, Roger L. Gilchrist, Inhae Ji, Seung-Jo Kim, and Tae H. Ji‡

From the Department of Molecular Biology, University of Wyoming, Laramie, Wyoming 82071-3944

The luteinizing hormone/chorionic gonadotropin (CG) receptor belongs to a subfamily of glycoprotein hormone receptors within the seven-transmembrane receptor family. It is comprised of an extracellular N-terminal half of 341 amino acids and a membrane-associated C-terminal half of 303 amino acids. The N-terminal half is capable of high affinity hormone binding whereas the C-terminal half is capable of low affinity hormone binding and receptor activation. However, the precise location of the receptor activation site is currently unknown. We present evidence for the first time that Lys583 of exoloop 3 is crucial and irreplaceable for receptor activation that results in hormone binding.

Hormone binding to receptors on the cell surface activates the receptors and generates intracellular signals (1). These events are difficult to study due to a lack of appropriate assays and, perhaps, due to their possible transient existence. The LH/C1 receptor offers a unique model to study receptor activation and signal generation, independent of high affinity hormone binding.

The LH/C1 receptor belongs to a subfamily of glycoprotein hormone receptors within the seven-transmembrane receptor family. It is comprised of an extracellular N-terminal half of 341 amino acids and a membrane-associated C-terminal half of 303 amino acids (2, 3). Both halves have hormone contact points according to studies using photoaffinity labeling (4, 5) and receptor peptides (6). The N-terminal half alone is capable of high affinity hormone binding (7–9) with no hormone action (9, 10). In contrast, the C-terminal half is capable of low affinity hormone contact with cAMP induction (9–11). These results suggest the relationship of the low affinity hormone contact with receptor activation. Such low affinity hormone contact and receptor activation are likely to occur at the extracellular domains of the C-terminal half of the receptor. The C-terminal half has three exoloops, which connect transmembrane columns. As a first step, we have examined exoloop 3. It is the shortest with 11 amino acids, from Lys573 to Lys583 (Fig. 1), whereas the other two exoloops are twice as long. In this communication, we report a novel observation that Lys583 of exoloop 3 is crucial and irreplaceable for receptor activation of the LH/C1 receptor. Lys583 is located at the boundary between exoloop 3 and the transmembrane column 7. No other amino acid of exoloop 3 including Lys573 demonstrates this crucial role.

MATERIALS AND METHODS

Mutagenesis and Functional Expression of LH/C1 Receptors—Mutant LH/C1 receptor cDNAs were prepared in pcDNA3 (Invitrogen) as described (12). Mutant LH/C1 receptor constructs were transfected into human embryonic kidney 293 cells by the calcium phosphate method. Stable cell lines were established in the presence of 500 μg/ml G418 and used for hormone binding and cAMP assays. All assays were carried out in duplicate and repeated 4–6 times. The mean and standard deviation for repeats of each mutant was calculated and analyzed by Student’s t test. In addition, values for different mutants were compared with the corresponding values of the wild type receptor using analysis of variance with 95% confidence. Untransfected cells do not express the LH/C1 receptor and, therefore, have been used as a control.

125I-hCG Binding and Intracellular cAMP Assay—Stable cells were assayed for 125I-hCG binding in the presence of 150,000 cpm of 125I-hCG and increasing concentrations of un-iodinated hCG. The Kd values were determined by Scatchard plots. hCG, batch CR 127, was supplied by the National Hormone and Pituitary Program. For intracellular cAMP assay, cells were washed twice with Dulbecco’s modified Eagle’s medium and incubated in the medium containing isobutylmethylxanthine (0.1 mM) for 15 min. Increasing concentrations of hCG were then added, and the incubation was continued for 45 min at 37 °C. After removing the medium, the cells were rinsed once with fresh medium without isobutylmethylxanthine, lysed in 70% ethanol, freeze-thawed in liquid nitrogen, and scraped. After pelleting cell debris at 16,000 g for 10 min at 4 °C, the supernatant was collected, dried under vacuum, and resuspended in 10 μl of the cAMP assay buffer, which was provided by the manufacturer (Amersham Corp.). cAMP concentrations were determined with a 125I-cAMP assay kit (Amersham Corp.) following the manufacturer’s instruction validated for use in our laboratory.

125I-hCG Binding to LH/C1 Receptor in Solution—Transfected cells were washed twice on ice with ice-cold 150 mM NaCl, 20 mM HEPES, pH 7.4 (buffer A). Cells were scraped on ice in the buffer containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 5 mM N-ethylmaleimide, and 10 mM EDTA) and pelleted by centrifugation at 13,000 × g for 10 min. Cells collected from a 10-cm plate were resuspended in 0.6 ml of the buffer containing 1% Nonidet P-40, 20%
The 11 amino acids of exoloop 3, Lys573, Val574, Pro575, Leu576, Ile577, Thr578, Val579, Thr580, Asn581, Ser582, and Lys583, were individually substituted with Ala to produce 11 substitution mutants. They are LH/CG-R-Lys573-Ala, LH/CG-R-Pro575-Ala, LH/CG-R-Leu576-Ala, LH/CG-R-Thr578-Ala, LH/CG-R-Val579-Ala, LH/CG-R-Thr578-Ala, and LH/CG-R-Thr578-Ala, respectively. These results indicate that the Ala substitution of the amino acid residues did not significantly reduce the hormone binding affinity. The number of receptors detected on intact cells was more than 120,000 for LH/CG-R-Lys573-Ala, LH/CG-R-Pro575-Ala, LH/CG-R-Leu576-Ala, LH/CG-R-Val579-Ala, and LH/CG-R-Thr578-Ala, and LH/CG-R-Thr578-Ala, respectively. On the other hand, LH/CG-R-Lys573-Ala, LH/CG-R-Pro575-Ala, LH/CG-R-Leu576-Ala, LH/CG-R-Val579-Ala, and LH/CG-R-Thr578-Ala, showed less than 100,000 receptors/cell. To determine whether any of the latter mutant receptors were trapped inside the cells, cells were solubilized in Nonidet P-40 and assayed for hormone binding. The numbers of these receptors in Nonidet P-40 solution were not significantly different from their numbers detected on intact cells, except for LH/CG-R-Pro575-Ala. Therefore, the poor surface expression of LH/CG-R-Lys573-Ala, LH/CG-R-Leu576-Ala, and LH/CG-R-Asn581-Ala appears to be caused by low level production of the receptors, which are

**RESULTS**

The 11 amino acids of exoloop 3, Lys573, Val574, Pro575, Leu576, Ile577, Thr578, Val579, Thr580, Asn581, Ser582, and Lys583, were individually substituted with Ala to produce 11 substitution mutants. They are LH/CG-R-Lys573-Ala, LH/CG-R-Pro575-Ala, LH/CG-R-Leu576-Ala, LH/CG-R-Thr578-Ala, LH/CG-R-Thr578-Ala, LH/CG-R-Val579-Ala, LH/CG-R-Thr578-Ala, and LH/CG-R-Thr578-Ala, respectively. These results indicate that the Ala substitution of the amino acid residues did not significantly reduce the hormone binding affinity. The number of receptors detected on intact cells was more than 120,000 for LH/CG-R-Lys573-Ala, LH/CG-R-Pro575-Ala, LH/CG-R-Leu576-Ala, LH/CG-R-Val579-Ala, and LH/CG-R-Thr578-Ala, and LH/CG-R-Thr578-Ala, respectively. On the other hand, LH/CG-R-Lys573-Ala, LH/CG-R-Pro575-Ala, LH/CG-R-Leu576-Ala, LH/CG-R-Val579-Ala, and LH/CG-R-Thr578-Ala, showed less than 100,000 receptors/cell. To determine whether any of the latter mutant receptors were trapped inside the cells, cells were solubilized in Nonidet P-40 and assayed for hormone binding. The numbers of these receptors in Nonidet P-40 solution were not significantly different from their numbers detected on intact cells, except for LH/CG-R-Pro575-Ala. Therefore, the poor surface expression of LH/CG-R-Lys573-Ala, LH/CG-R-Leu576-Ala, and LH/CG-R-Asn581-Ala appears to be caused by low level production of the receptors, which are

![Figure 1](image1.png)

**Fig. 1. The arrangement of the LH/CG receptor and comparison of exoloop 3 sequences of glycoprotein hormone receptors.** The LH/CG receptor was arranged according to the putative seven-transmembrane columns. The sequence of exoloop 3 is shown. The amino acids in gray circles are more important for cAMP induction than those in blank circles. The sequences of cloned LH/CG receptors and FSH receptors of different species show complete homology whereas the sequences of cloned TSH receptors show some divergence among species (b, human; d, dog; r, rat).

![Figure 2](image2.png)

**Fig. 2. Ala scan of exoloop 3.** The 11 amino acids of exoloop 3 were individually substituted with Ala, and the resulting mutant receptors were stably expressed on human 293 cells. The cells were assayed for hormone binding and hCG-dependent cAMP induction. For hormone binding, cells were incubated with a constant amount of $^{125}$I-hCG and increasing concentrations of un-iodinated hCG as described. For cAMP assay, cells were incubated with increasing concentrations of cold hCG, and intracellular cAMP was determined. Counts of empty tubes (background) were ~50 cpm, and nonspecific bindings were ~100 cpm including the background. Nonspecific bindings were normally less than 2% (~100 cpm) of specific binding. In contrast, specific bindings of samples with >70,000 receptors/cell were >10,000 cpm. Therefore, the noise/signal ratios were less than 2%. Usually, 10–30% of the total input $^{125}$I-hCG bound as suggested in the Scatchard plots. However, several mutants with <5,000 receptors/cell bound 1–10% of the total input $^{125}$I-hCG with the noise/signal ratios of 2–6%. Each experiment was repeated 4–6 times in duplicate. The mean and standard deviations of repeats for individual mutant data are presented. Their statistical significance was analyzed to determine $p$ values by Student's $t$ test. They are shown in the table section of the figure. NS, not significant. It was used when bindings were less than 2-fold of the noise level, and, therefore, $K_{d}$ values and numbers of receptors/cell could not be determined. In addition, values for different mutants were compared with the corresponding values of the wild type receptor using analysis of variance with 95% confidence. The resulting $p$ values were discussed in the text, and a sample with $p < 0.05$ was considered to have a significant difference from the wild type.
capable of hCG binding. In the case of LH/CG-RPro-575-Ala, receptors in Nonidet P-40 solution were approximately 9 times higher than that found on intact cells (data not shown). This result suggests that nearly 90% of LH/CG-RPro-575-Ala were trapped in the cells as are LH/CG-RAsn-573-Ala, LH/CG-RIle-577-Ala and LH/CG-RA sn-581-Ala, probably due to defective cell surface expression.

EC_{50} values for the cAMP induction by some of the mutant receptors are significantly diverse (p < 0.05), suggesting important roles for some of exoloop 3 amino acids. Particularly, LH/CG-RLys-583-Ala was not capable of inducing cAMP, indicating the importance of Lys^{583} in cAMP induction. Other mutant receptors showed considerably reduced affinities for cAMP induction in the order of LH/CG-RPro-575-Ala (p ≤ 0.002) > LH/CG-RAsn-573-Ala (p = 0.026) > LH/CG-RVal-579-Ala (p ≤ 0.015) > LH/CG-RIle-577-Ala (p ≤ 0.003) > LH/CG-RA sn-581-Ala (p ≤ 0.006). Since the substituted residues are hydrophobic except for neutral Asn^{581}, the results underscore the importance of strongly hydrophobic residues at those positions.

To learn more about the importance of Lys^{583}, it was substituted with a panel of amino acids consisting of basic (Arg), acidic (Asp and Glu), neutral (Glu), hydrophilic (Tyr), and hydrophobic (Leu and Ala) residues (Fig. 3). In addition, Lys^{583} was deleted to generate a deletion mutant receptor. A deletion mutant is useful to complement substitution mutations and to understand the effect of the original amino acid without introducing the effect of a new amino acid. None of these mutant receptors were capable of inducing cAMP, consistent with the essential and irreplaceable role of Lys^{583} in cAMP induction.

Our data indicate that exoloop 3 of the LH/CG receptor is important for the induction of cAMP synthesis and for the surface expression of the receptor but is not crucial for the high affinity hormone binding. Exoloop 3 consists of 11 amino acids, with two terminal Lys residues. The sequence, Lys^{573}-Val^{574}, Pro^{575}-Leu^{576}-Ile^{577}-Thr^{578}-Val^{579}-Thr^{580}-Asn^{581}-Ser^{582}. Lys^{583} is highly conserved among various species (21), suggesting its importance.

**DISCUSSION**

Exoloop 3 is the shortest among three exoloops, consisting of 11 amino acids (Fig. 1), and is involved in hormone binding. Our data indicate that exoloop 3 of the LH/CG receptor is important for the induction of cAMP synthesis and for the surface expression of the receptor but is not crucial for the high affinity hormone binding. Exoloop 3 consists of 11 amino acids, with two terminal Lys residues. The sequence, Lys^{573}-Val^{574}, Pro^{575}-Leu^{576}-Ile^{577}-Thr^{578}-Val^{579}-Thr^{580}-Asn^{581}-Ser^{582}. Lys^{583} is highly conserved among various species (21), suggesting its importance.

**Receptor Activation to Induce cAMP Synthesis—**The impacts of Ala substitutions were more pronounced on Val^{574}, Pro^{575}, Ile^{577}, Val^{579}, Asn^{581}, and Lys^{583} than on Lys^{573}, Leu^{576}, Thr^{578}, Thr^{580}, and Ser^{582} (Figs. 1 and 2). A careful examination of these two groups reveals that the first group consists of primarily hydrophilic amino acids, in particular the four upstream residues. In contrast, the second group is comprised of primarily hydrophilic residues, including a unique group of
three amino acids (Thr578-Thr580-Ser582), which possess a CH$_2$OH group. Furthermore, these two groups of amino acids are arranged in an alternate sequence except for Val574-Pro575. A simple explanation of this arrangement is that these residues may form a $\beta$-like structure (Fig. 1). The amino acids on one side of the structure may play a more important role in cAMP induction than do the residues on the other side of the structure. The group of four residues, Pro$_{575}$-Ile$_{577}$-Val$_{579}$-Asn$_{581}$, shows an interesting trend for cAMP induction. The EC$_{50}$ values for cAMP induction by the corresponding Ala substitution mutants are in decreasing order (Fig. 2). This result indicates that in this group of four amino acids the first residue, Pro$_{575}$, is the most important for cAMP induction and the following residues play gradually less important roles. Overall, Lys$_{583}$ is the most crucial and irreplaceable for cAMP induction. Therefore, the alternate sequence of the two groups of amino acids and their orderly importance for cAMP induction suggest a specific secondary structure of exoloop 3. In addition, they implicate the interaction of exoloop 3 with hCG and/or other domains of the LH/CG receptor for receptor activation to induce cAMP synthesis.

The substitution of Arg, Asp, Glu, Gln, Tyr, Leu, or Ala for Lys$_{583}$ resulted in the loss of the cAMP inducibility. This complete irreplaceability indicates the strict structural requirement for Lys$_{583}$ and appears to be specific. Also, our results indicate that the changes in the affinity (EC$_{50}$ value) and efficacy (the maximum level of synthesis) for cAMP induction by the mutant receptors are not necessarily in parallel. This further suggests the existence of distinct mechanisms to dictate the affinity and efficacy for cAMP induction.

The sequence of exoloop 3 of LH/CG receptors is conserved among species as is the exoloop 3 sequence of FSH receptors (Fig. 1). However, the sequences of the LH/CG receptor and the FSH receptor are not identical near the C-terminal end except for Lys$_{583}$. Furthermore, the exoloop 3 sequences of TSH receptors are diverse among species and divergent from those of the LH/CG receptor and the FSH receptor. Specifically, the C-terminal amino acid of exoloop 3 of TSH receptors that corresponds to Lys$_{583}$ of the LH/CG receptor varies among species. Therefore, the importance of Lys at the exoloop 3 C-terminal of the TSH receptor is open to question. Although exoloop 3 of the TSH receptor appears to be important for cAMP induction (22), the role of exoloop 3 in cAMP induction may not be identical among the three glycoprotein hormone receptors. This conclusion is consistent with the recent observation that the three C-terminal amino residues of the $\alpha$-subunits of the glycoprotein hormones play important roles in receptor activation. The roles are different in each hormone (13, 15, 16).

**Surface Expression—**LH/CG-R$\text{Lys}^{583}\rightarrow\text{Gln}$, LH/CG-R$\text{Lys}^{583}\rightarrow\text{Glu}$, LH/CG-R$\text{Lys}^{583}\rightarrow\text{Ala}$, and LH/CG-R$\text{Lys}^{583}\rightarrow\text{Val}$ were not detected at all on intact cells whereas they showed the full hormone binding activity when cells were solubilized in Nonidet P-40. This result suggests that the mutant receptors were trapped in the cells and the mechanism for surface expression was blocked. It is not clear whether this futile surface expression occurred at the endoplasmic reticulum during the membrane insertion of nascent receptors or during their transport to the plasma membrane. In addition to the three substituent receptors, the substitution of Arg and Asp for Lys$_{583}$ resulted in partial surface expression, leaving the majority of the mutant receptors trapped within the cells. The same result was seen with LH/CG-R$\text{Lys}^{583}\rightarrow\text{Glu}$ and LH/CG-R$\text{Lys}^{583}\rightarrow\text{Asp}$ (data not shown). These results indicate that Lys$_{573}$, Pro$_{575}$, Ile$_{577}$, and Asn$_{581}$ are also important for successful surface expression of the receptor.

In conclusion, our data clearly demonstrate for the first time the essential and irreplaceable role of Lys$_{583}$ of the LH/CG receptor in cAMP induction without the loss of the hormone binding affinity. Lys$_{583}$ is also important for the receptor’s surface expression as are Lys$_{573}$, Pro$_{575}$, Ile$_{577}$, and Asn$_{581}$ of exoloop 3.

**Note Added in Proof—**While this manuscript was under review, Fernandez and Puet (Fernandez, L. M., and Puet, D. (1996) J. Biol. Chem. 271, 925–930) reported the importance of Lys$_{583}$.

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