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Constitutive Activating and Inactivating Mutants of Eel Luteinizing Hormone Receptor

Munkhzaya Byambaragchaa[1], Dong-An Kim[1], Dae-Jung Kim[2], Sun-Mee Hong[3],

Myung-Hwa Kang[4] and Kwan-Sik Min[1,*]

[1]Animal Biotechnology, Graduate School of Future Convergence Technology, School of Animal Life Convergence Science, Institute of Genetic Engineering, Hankyong National University, Ansung, Republic of Korea; monkhzaya_b@yahoo.com (M.B); wrm032@daum.net (D-A, K)

[2]Jeju Fisheries Research Institute, National Institute of Fisheries Science (NIFS), Jeju, Republic of Korea; djkim4128@korea.kr (D-J, Kim)

[3]Department of Technology Development, Marine Industry Research Institute for Eastrim (MIRE), Ulgin, Republic of Korea; hongsunmee@mire.re.kr (S-M, Hong)

[4]Department of Food Science and Nutrition, Hoseo University, Asan, Republic of Korea; mhkang@hoseo.ac.kr

* Correspondence: ksmin@hknu.ac.kr; Tel.: +82-31-670-5421; Fax: +82-31-670-5417

Abstract

We analyzed signal transduction of three constitutively activating mutants (M410T, L469R, and D590Y) and two inactivating mutants (D417N and Y558F) of the eel luteinizing hormone receptor (eel LHR), known to occur in human LHR. The objective of this study was to assess the functional effects of these mutations in signal transduction and cell surface loss of receptor. Mutant receptors were transiently expressed in Chinese hamster ovary (CHO-K1) cells. Eel LH-stimulated accumulation of cyclic adenosine monophosphate (cAMP) was measured by homogeneous time-resolved fluorescence (HTRF) assays. The loss of receptors from the cells surface was measured using an enzyme-linked immunosorbent assay (ELISA) in human embryonic kidney (HEK) 293 cells. The cAMP response in cells expressing the wild type eel LHR was increased in a dose-dependent manner using eel LH ligand stimulation. Compared with the wild type, cells expressing the activating mutants (M410T, L469R, and D590Y), exhibited a 4.0-, 19.1-, and 7.8-fold increase in basal cAMP response without agonist stimulation, respectively. Their maximal responses to agonist stimulation were approximately 65%, 52%, and 98%, respectively, of those of the wild type. The inactivating mutants (D417N and Y558F) did not completely impair signal transduction,

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and their maximal responses were only 33% and25 % of those of wild type. These data clearly showed that the eel *LHR*-L469R and D590Y, activating mutants enhanced the rate of the loss of cell surface receptors following treatment with eel LH. Thus, the loss of cell surface receptors in cells expressing mutant eel LHRs was consistent with the eel LH agonist-induced production of cAMP. Our results suggested that the activation of the eel LHR requires appropriate loss of LHR-ligand complexes from the cell surface.

Keywords: eel luteinizing hormone receptor, constitutively activating mutation, inactivating mutation, cyclic adenosine monophosphate response, cell surface loss of receptor

1. Introduction

The luteinizing hormone receptor (LHR) is a member of the family of the 7 transmembrane G protein-coupled receptors (GPCRs), one of the largest gene families [1]. In particular, the LHR and follicle-stimulating hormone receptors (FSHRs) form a subgroup of the glycoprotein hormone receptors [2, 3]. The LH receptor (*LHR*) gene has been associated with an abundance of naturally occurring mutations related to reproductive failures in mammals [4]. Data from cDNA and genomic sequences of the human LHR (*hLHR*) have made it possible to determine genetic mutations in *hLHR* that could be connected to particular reproductive failure disorders [5-8]. Accordingly, mutations in a specific site of the *hLHR* gene have been demonstrated in familial male-limited precocious puberty (FMPP) [9, 10]. Boys with this condition show increased concentrations of testosterone, but prepubertal levels of gonadotropin-releasing hormone (GnRH) and LH [6, 11], suggest that the LHR-signaling can be activated even without ligand stimulation. These *hLHR*-activating mutations have also been shown to result in significantly increased levels of cAMP in the absence of hormonal stimulation, constituting the main cause of FMPP [12, 13].

Cells expressing the *hLHR*-D578Y mutant (equivalent to D590Y in eel *LHR*) have been reported to display a prominently increased cAMP response in the absence of ligand stimulation [6]. Such constitutive activity of the *hLHR* gene has been associated with causing LH-releasing hormone-independent premature puberty in boys with FMPP [8]. The Asp578 residue of the *hLHR* has been shown to serve as an appropriately placed acceptor of hydrogen bonds to help conserve the inactive condition of the receptor [14]. In a study analyzing the genomic DNA from 32

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unrelated patients with FMPP, 3 activating mutation sites (Asp578Gly, Met571Ile, and Thr577Ile) and 4 other mutation sites (Ile542Leu, Asp564Gly, Asp578Tyr, and Cys581Arg) were identified in *hLHR* [14, 15]. The 1624–1741 nucleotides of the *hLHR* are known to be a hotspot for heterogeneous specific mutations, suggesting that mutations in this region constitutively activate *hLHR* [13]. Germline *hLHR* mutations that lead to the continuous activation of the signal transduction of Gs have also been detected in cases of Leydig cell hyperplasia [11].

Another activating LHR mutation, M398T (equivalent to M410T in eel *LHR*), located in the second transmembrane helix, has been described in a patient with precocious puberty, as well as in the mother and brother of the patient [16, 17]. This mutation is of special interest, as one member of the family that carries the mutation has not exhibited any evidence of precocious puberty [18]. Another study reported that the M398T mutant caused constitutively high basal levels of cAMP, relevant to the activation of Leydig cells and premature adolescence in the patient [7]. The production of cAMP in the absence of ligand was shown to be elevated up to 25-fold in the mutant as compared with the basal level of the wild type receptor [5]. In contrast, the double mutant receptors (D578G and M410T) were demonstrated to lead to lesser production of cAMP, with the basal cAMP response being increased up to 10- to 15-fold compared with that of the wild type receptor. The *hLHR*-L457R mutant (equivalent to L469R in eel *LHR*) was first identified on the basis that cells expressing this mutant displayed remarkably higher basal levels of cAMP (7- to 14-fold) compared with those of the wild type *LHR* [19]. The levels of wild type and these mutant cell surface receptors were similar, but the L457R mutation were reported to cause a marked increase in the basal cAMP response [20]. However, cells expressing the L457R mutant were unresponsive to further hormonal stimulation [9, 21], indicating that the L457R mutant was not routed to the lysosomes, with most of it being recycled to the cell surface [22]. A potential activating mutation (*hLHR*-D578H) has also been reported in boys with testicular adenomas [23, 24]. The ratLHR (*rLHR*)-D556H mutation (*rLHR*-D556H; equivalent to the *hLHR*-D578H mutation) has also been shown to result in a marked increase in the basal cAMP response [4]. The majority of constitutively activating mutations in *hLHR* have been identified in boys with intermittent or more common forms of FMPP [25, 26].

Although the inactivating *rLHR*-D383N (equivalent to D417N in eel *LHR*) and -R442H mutations have been reported to not affect the binding of the human chorionic gonadotropin (hCG), they do impair signal transduction [27]. In addition, the cAMP responsiveness of the *rLHR*-D383N

and -R442H mutant receptors was characterized by an 18- and 7-fold increase in EC_{50}, respectively, compared with the EC_{50} of an equivalent density of wild type *rLHR* [27]. We have also previously reported that the *rLHR*-D383N and Y524F (equivalent to Y558F in eel *LHR*) mutations were found to be signal impairing mutations, showing low-to-normal levels of cAMP under basal conditions. Thus, 2 signal-impairing mutants are known to decrease the rate of the internalization of hCG [28]. Cells expressing the *rLHR*-D383N mutant have been shown to exhibit significantly lower affinity and impaired cAMP response, indicating the requirement for the structure and function of receptors, conserved in all glycoprotein hormone receptors [29]. All inactivating mutations are known to result in an underlying hCG stimulated cAMP response in mutant receptor cells, suggesting a clear correlation between the intensity of the frequency of the clinical phenotype and the signal efficiency of receptors, which concern both the quantities of cell-surface expression and coupling efficiency [15].

The GPCR signal transduction including glycoprotein hormone receptors has been studied in detail with respect to loss of cell surface receptors, internalization, and recycling [30-32]. Recent research studies from our laboratory have elucidated several characterizations of signal transduction of the eel LHR [33] and eel FSHR [34-36] on deglycosylated ligands. Although the activation effects of these mutants have been relatively well demonstrated in *hLHR*, very less is known about the signal transduction leading to the activation and inactivation in fish *LHR*.

Thus, this study aimed to determine these mechanisms by analyzing 3 constitutively activating (M410T, L469R, and D590Y), and 2 inactivating (D417N and Y558F) mutations in highly conserved regions of the LHR. We also aimed to delineate the means by which activating/inactivating mutations might affect the signal pathway in the eel LHR-LH complex. Mutant receptors were transiently expressed in CHO-K1 cells, the production of cAMP and loss of cell surface receptors were measured in HEK 293 transfected cells. Here, we report that the basal cAMP response was constitutively involved in activating eel LHRs, and the mutant receptors exhibited a decrease in cell surface loss similar to the wild type receptor, but the inactive mutants impaired the signal transduction and cell surface loss.

2. Results

2.1. Construction of wild type and mutant eel luteinizing hormone receptors

As previously reported, the eel *LHR* is known to consist of 2115 nucleotides encoding 705

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amino acids [33]. In order to generate substitute mutations at target amino acids, we used an overlap extension PCR strategy with primers designed to change target nucleotides. To investigate the effects on the interaction of hormones to the receptor in the eel LHR activation system, we generated 3 constitutively activating mutations in the II, III, and VI transmembrane helices of LHR. These mutant receptors were designated M410T (equivalent to M398T in *hLHR*), L469R (equivalent to L457R in *hLHR*), and D590Y (equivalent to D578Y in *hLHR*). We also constructed 2 inactivating mutations of eel *LHR*, designated as D417N and Y546F in the II and V transmembrane helices, respectively (**Figure 1**).

2.2. Cyclic adenosine monophosphate responsiveness induced by agonist in activating mutants

The effects of activating mutations on the basal and eel LH-stimulated cAMP responsiveness are summarized in **Figure 2A and Table 1.** The basal and Rmax cAMP responses in wild type receptor were demonstrated to be 1.2 and 87.5 nM/ 10^4 cells, respectively. The production of cAMP was exhibited an increased in a dose-dependent manner. The EC_{50} value of the eel LH-stimulated cAMP response was shown to be approximately 18.9 ng/mL.

In cells expressing the constitutively activating mutants (M410T, L469R, and D590Y) the induced basal cAMP responsiveness was increased by 4.8 ± 0.3, 22.9 ± 1.5, and 9.3 ± 0.8 ng/10^4 cells, respectively **(Figure 2B**). In contrast to CHO-K1 cells harboring the wild type receptor, cells expressing the M410T mutant exhibited a 4.0-fold increase in basal production of cAMP, indicating that the receptor was constitutively active. In addition, cells expressing the L469R and D590Y mutants exhibited a 19.1- and 7.8-fold increase in the amounts of basal cAMP, respectively, as compared to those of cells expressing the wild type eel *LHR*, as shown in **Table 1**. We noted that eel LH produced a concentration-dependent increase in the production of cAMP in cells expressing the activating mutants (M410T, L469R, and D590Y), with an EC_{50} (50% effective concentration) of 8.7 ng/mL, 3.8 ng/mL, and 77.3 ng/mL, respectively.

The maximum cAMP response induced by eel LH in the activating mutants (M410T, L469R, and D590Y) with respect to the maximal response of the wild type was found to be approximately 0.65-, 0.52-, and 0.98-fold, respectively, as shown in **Table 1**. Cells expressing the M410T and L469R mutants were shown to not respond to further stimulation by higher concentrations of agonist. However, the D590Y mutant was observed to reach approximately 98% of the maximal response of the wild type. The concentration-response curve of the accumulation of cAMP in eel

LHR-D590Y was characterized by a 4-fold increase in the EC_{50}, but no change in the maximal response when compared with cells expressing wild type receptor. Thus, compared with the wild type eel *LHR*, the 3 activating mutations were demonstrated to produce a higher basal cAMP response in CHO-K1 cells, consistent with constitutive activation of the receptor. The mutant exhibiting the highest level of basal production of cAMP (L469R) was found to not react to eel LH with a further increase in maximal cAMP responsiveness. The basal production of cAMP in the L469R mutant represented 49% of the maximal stimulation produced by eel LH **(Table 1).** Thus, the high basal production of cAMP in the L469R mutant prevented a further increase in the cAMP responsiveness despite a higher agonist stimulation.

2.3. Cyclic adenosine monophosphate responsiveness induced by agonist in inactivating mutants

To directly assess the functional effects of the 2 inactivating mutations, we transiently expressed these mutant receptors in CHO-K1 cells. The D417N and Y558F mutants were evaluated by quantifying the cAMP stimulation in cells incubated with increasing concentrations of eel LH (**Figure 3A and Table 2**). As predicted, the cAMP signaling was impaired in cells expressing both mutant receptors compared with those expressing the wild type receptor. The basal cAMP response was shown to not be affected by the inactivating mutations, and showed little increase under high concentration of eel LH. The EC_{50} value exhibited an approximately 2.6-fold decrease in D417N mutant compared with that of the wild type eel *LHR*. However, the maximal response of this mutant was demonstrated to only be 33% of the response of the wild type eel *LHR*. In the Y558F mutant, the EC_{50} value was also shown to exhibit a 2.2-fold decrease of the value of the wild type eel *LHR*; however, the maximal response was only 25% of that of the wild type. As shown in **Table 2**, the signal responsiveness of the receptors was severely affected by the inactivating mutations. The maximal response of these cells was observed to be 67-75% lower than the maximal response of cells expressing the wild type eel *LHR* **(Figure 2B).**

2.4. Loss of cell surface receptors induced by treatment with eel luteinizing hormone agonist

To more accurately quantitate the rate of loss of cell surface receptors, we performed experiments in which the loss of receptors from the cell surface was measured in a time-dependent manner under the continuous presence of eel LH. The results of the time-dependent loss of eel

LHRs for 2 activating and 2 inactivating mutations are shown in **Figure 4 and Figure 5.** Loss of the expression of cell surface receptors was measured for 30 min as shown **in Figure 4**. Results were then expressed as a percentage of the loss of the expression of surface receptor measured in control cells pre-incubated in the absence of the eel LH agonist (considered as 0% of loss of surface receptors).

Cells expressing the wild type eel LHR treated with eel LH agonist (500 ng/mL) for 30 min were observed to exhibit an increased loss (>45 %) of cell surface receptors. In cells harboring the L469R activating mutation, the loss of cell surface receptors was considerably similar (44%) to that observed in the wild type. We also found that cells expressing the D590Y mutant exhibited slower rates (36%) of expression as compared with those harboring the corresponding wild type receptor. In cells expressing the inactivating D417N and Y557F mutants, the loss of cell surface receptors induced upon stimulation with eel LH agonist was demonstrated to be only 19% and 21%, respectively.

Next, we determined, in more detail, the time-dependent loss of cell surface receptors as a result of the expression of wild type or mutant eel LHRs (**Figure 5**). The expression of surface receptors in cells harboring the wild type eel LHR was shown to be gradually decreasing until it reached 55% of the pretreatment value. Similarly, the expression of cell surface receptors in cells expressing the activating mutants (L469R and D590Y) was shown to be decreased up to approximately 70-80% in the first 5 min and then a little further decreased in the following 15 min. Finally, the loss of receptors was noted to remain to levels between 60% and 64% for 60 min. The rates of loss of cell surface agonist-receptor complexes in both the wild type and activating mutant receptors were observed to be very rapid (2.6~ 6.2 min), as shown in **Table 3**. However, the D417N and Y558F inactivating mutants, were observed to almost not result in the loss of cell surface receptors.

3. Discussion

The present study was designed to determine the possibility that the activation/inactivation of eel LHRs might be necessary for the signal transduction and loss of cell surface receptors induced by ligand agonist binding to hLHRs. To pursue this goal, we constructed eel LHR mutants containing single point mutations in 5 distinct amino acid residues known to be highly conserved among glycoprotein hormone receptors, including LHR, FSHR, and TSHR. The Met-410, Leu-469, Asp-590, Asp-417, and Tyr-558 residues in eel LHR have been reported to be conserved

among all LHRs [27, 28]. However, studies on the effect of these residues in the signal transduction of eel LHR have not been previously described. The present study described mutations inducing constitutive activation or impairing signal transduction in the eel LHR, consistent with previously reported mutations in mammalian LHRs causing FMPP and elevated levels of cAMP in the absence of agonist [12, 27-29, 37].

Many studies have suggested that similar dynamic modifications of mammalian LHRs were closely involved in the activity of G proteins [5, 12, 28]. In humans, mutations that constitutively activate *hLHR* and cause FMPP have been previously described [13, 19]. Differences observed in the phenotypic appearance of FMPP might be attributed to the distinctions in the basal activity of *hLHR* [13]. In a previously studied case from Scotland, a patient exhibited signs of pubertal development at 1 y of age [38]. In this case the patient was found to harbor the D578Y mutation (equivalent to D590Y in the eel *LHR*), a mutation that was reported to induce higher basal production of cAMP compared with that of wild type LHR.

In the present study, our results showed that the 3 activating mutations, eel *LHR*-M410T, -L469R, and -D590Y, resulted in a distinctly increased cAMP response under basal conditions, suggesting that these mutations might cause the constitutive activation of the eel LHR. Compared with wild type, the eel *LHR*-M410T, -L469R, and -D590Y mutants were shown to result in the production of a 4-, 19.1-, and 7.8-fold increase in the basal cAMP response, respectively, indicating that these 3 mutants were constitutively active, as previously reported in mammalian LHRs [17, 19, 28].

Cells expressing the *hLHR*-M398T mutant (equivalent to M410T in the eel LHR) exhibited high basal levels of cAMP [7]. This mutation is of special interest, as one member of this family that carrier the mutation has not exhibited any evidence of precocious puberty [18]. The basal levels of the production of cAMP were observed to be 15- to 25-fold higher in the M398T mutant compared with those of the wild type receptor [5]. Our results also described the M410T constitutively activating mutation in the eel LHR, which was shown to be located in the same second transmembrane region. However, the maximal cAMP response was observed to reach only 65 % of the activity detected in cells expressing the wild type receptor. Apparently, the amino acid substitution led to changes in the basal cAMP responsiveness, but the maximal response was not changed under treatment with high concentration of agonist employed for hormone-induced conformational shift and posttranslational modification.

The *hLHR*-L457R mutation (equivalent to L469R in eel LHR) was the first activating mutation identified in hLHR and cells expressing this mutant receptor were noted to exhibit markedly higher basal levels of cAMP (7- to 14-fold) relative to those harboring the wild type receptor [19]. Previous results from our colleagues have also shown that basal cAMP responses in cells expressing the *rLHR*-L435R (equivalent to L469R in eel LHR) displayed a 47-fold increase in the absence of agonist, without leading a further increase in the cAMP response following stimulation by hCG [28]. The complex of *hLHR*-L457R and hCG has been reported to not migrate to lysosomes, but rather get recycled to the cell surface, with hormone degradation being hardly detectible [22]. These results were consistent with our current data, showing a remarkable increase (19.1-fold) in the basal cAMP response of cells expressing the eel *LHR*-L469R mutant. However, the maximal cAMP response to agonist was shown to be approximately 52% of the wild type receptor, as previously described in hLHR [28]. Compared with the basal cAMP production, the maximal cAMP response in cells expressing the L469R mutant was found to be only 2-fold greater. Hence, we suggested that the L469R mutant did not increase its maximal cAMP responsiveness despite treatment with a high concentration of agonist. Previous data on *rLHR*-L457R revealed that the low hormonal responsiveness induced by treatment with high concentration of agonist was not due to an impairment in the binding activity of the ligand to the receptor, because the mutant was shown to bind hCG with the same affinity as it did with the wild type hLHR [39]. In the present study, our data suggested that the L469R constitutively active mutant was easily distinguishable from agonist-activated eel LHRs. Thus, the activating eel *LHR*-L457R mutant could serve as a specific model in figuring out the basic molecular mechanisms of the activation of eel LHR induced by treatment with high concentration of agonist.

The *hLHR*-D578Y and -D578G mutants (equivalent to D590Y in the eel LHR) were first reported to be inherited in an autosomal dominant manner and associated with signs of puberty by 4 y of age [6]. The D578G mutant was shown to result in a 4.5-fold increase in the cAMP response under basal conditions, with an EC_{50} similar to that of the wild type receptor. Agonist-independent stimulation of the production of cAMP by this mutant receptor represented 42% of the maximal stimulation [6]. However, substitution with D578Y (8.5-fold) and D578F (7.5-fold) was noted to have a greater activating effect in the basal levels of cAMP. These results were consistent with our results, showing that the eel *LHR*-D590Y mutation resulted in the constitutive activity of the receptor (7.8-fold); however, the maximal response in our results reached approximately 98% of

the wild type receptor. Thus, our results suggested that the D590Y mutant has a specific characteristic compared with the M410T and L469R mutants, which were markedly decreased in their maximal cAMP activity. The aspartic acid residue at position 590 is known to be conserved in all LH receptors, including the eel LHR, but is not found in any other GPCRs. Another study suggested that the Asp^{578} side chain in hLHR has the most appropriate position to act as an acceptor of hydrogen bonds and was therefore significant for stabilizing the impaired state of the LHR [12]. Thus, substitutions at position 590, which lead to the constitutive activation of the cAMP response, might not always be consistent with the high basal cAMP responsiveness, and mutations might be involved in maintaining the active state after the formation of receptor-ligand complexes. Previous results from our studies have also reported that *rLHR*-D556Y (equivalent to D590Y in eel LHR) resulted in a high increase in the concentration of basal cAMP responses without stimulation by hormones with the maximal cAMP response to hCG being about 75% of that of the wild type receptor [28]. These results on the *hLHR*-D578Y and *rLHR*-D556Y were consistent with our results, indicating that eel *LHR*-D590Y displayed a high basal cAMP response, and exhibited a maximal cAMP response similar to that of the wild type receptor. This suggested that activating mutations, including eel *LHR*-M410T, -L469R, and -D590Y might not be unique for the species, and that the basal cAMP response would be markedly increased irrespective of whether these mutants are expressed in mammals (human and rat) or fish.

By analyzing the genomic DNA from 32 unrelated cases with FMPP, other constitutively activating mutations (Ile542Leu, Asp564Gly, Met571Ile, and Cys581Arg) have also been identified [13]. These sites were shown to be preserved among glycoprotein hormone receptors, suggesting an important function in the receptor signaling pathway. These data suggested that the 1624–1741 specific nucleotide region in *hLHR* is an important site for the occurrence of heterogeneous mutations that activate the receptor and cause FMPP. Based on the above summarized results, we expected that mutation in eel LHR that induce continuous activation of the receptor would result in specific changes to the receptor-ligand complex. The D590Y mutant was noted to induce an elevated basal level of cAMP corresponding to approximately 10% of the maximal cAMP response. However, the L469R mutant was found to induce a highly elevated basal level of cAMP corresponding to approximately 49% of the maximal cAMP response. These results suggested that the distinct configurations of these mutants induced different signal transduction pathways, resulting in different maximal cAMP responses to LH.

In the case of the inactivating mutants, we mutated the highly conserved amino acids present in the second (eel *LHR*-D417) and fifth (eel *LHR*-Y558) transmembrane helices to asparagine and phenylalanine, respectively. As predicted from results obtained with other GPCRs [27-29, 37], these mutations (eel *LHR*-D417N and eel *LHR*-Y558F) were expected to impair signal transduction. Previous studies reported that cells expressing *rLHR*-D383N displayed a rightward shift in the EC_{50} for cAMP stimulation, but normal maximal levels [27]. The EC_{50} levels in *rLHR*-Y524F was increased 27-fold compared with that of the wild type receptor, but its maximal response was shown to be only 14% lower [28]. These results were consistent with our data, showing that eel *LHR*-D417N and -Y546F were signaling-impairing mutations. However, the maximal response was found to be a little different from our results, displaying only 25 – 33% of the maximal response of wild type eel LHR. In the present study, the eel LH-induced increase of the production of cAMP in cells harboring the eel *LHR*-D417N and -Y558F mutations did not completely impair signal transduction. The maximal response to eel LH was shown to be markedly lower than the cAMP response detected in the wild type receptor. These inactivating mutants were assumed to impair the signal transduction through conformational changes following the formation of the receptor-ligand complexes despite the prolonged treatment with the agonist.

Based on the activation model, we would expect the rate of the loss of the L469R and D590Y mutated cell surface receptors to be the same as the rate of the loss of receptors from the cell surface in cells expressing the wild type receptor. The rate of loss of the eel *LHR*-L469R cell surface receptor was the same as that of the wild type. Although the basal cAMP response was associated with a marked increase in the D590Y mutant, the rate of its loss from cell surface was demonstrated to be slightly slower than that of the agonist-occupied wild type receptor. The results presented here revealed that the rate of loss of the constitutively active mutants (L469R and D590Y) from the cell surface following a 5 min preincubation with agonist was decreased by approximately 30%, whereas the loss of the D417N and Y558F inactivating mutants was shown to be dramatically slower than that of the wild type receptor. These results were also in agreement with the finding regarding the cAMP response following treatment with the agonist, which was shown to markedly increase in activating mutants, whereas it was impaired in inactivating mutants. Our results were consistent with previous data reported by our colleagues studying equivalent mutations in rLHR, in which they demonstrated that 2 signaling-impairing mutations, *rLHR*-D383N and *rLHR*-Y524F, decreased the rate of internalization of hCG [27], whereas *rLHR*-L435R and *rLHR*-D556Y

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activating mutants enhanced its internalization rate [28]. The results presented here showed for the first time that 3 constitutively activating mutants of eel LHR were able to conclusively effect the basal cAMP response and loss of these receptors from the cell surface, but did not enhance the maximal cAMP response. Our study further highlighted the importance of the crucial transmembrane regions of eel LHR in exhibiting the agonist-induced loss of eel LHRs from the cell surface. Characterization of these constitutively activating mutants during their internalization, degradation, and recycling, would be the next step.

4. Materials and Methods

4.1. Materials

The pcDNA3 mammalian expression vector, CHO-S suspension cells, MAX transfection reagent, and Lipofectamine-3000 were obtained from Invitrogen (Carlsbad, CA, USA). The pGEM-T easy cloning vector was purchased from Promega (Madison, WI, USA). Chinese hamster ovary cells were obtained from the Japanese Cancer Research Resources Bank (JCRB, Tokyo, Japan). A homogeneous time-resolved fluorescence (HTRF) cAMP assay kit was purchased from Cisbio (Codolet, France). Monoclonal antibodies (5A11, 11A8, and 14F5) and rec-eel LH from CHO-K1 cells were produced in our lab, as previously reported [34]. The horseradish peroxidase (HRP) labeling of the 8A11 monoclonal antibody was generously performed by Medexx Inc. (Seongnam, Korea). Eel LHR cDNA was cloned from eel ovaries and testes, as previously reported [33]. QIAprep-Spin plasmid kits were purchased from Qiagen Inc. (Hilden, Germany). Polymerase chain reaction (PCR) reagents and endonucleases were purchased from Takara (Osaka, Japan). Oligonucleotides were synthesized by Genotech (Dajeon, Korea). Disposable spinner flasks were purchased from Corning Inc. (Corning, NY, USA). Centrifugal Filter Devices were purchased from Amicon Bio (Billerica, MA, USA). All other reagents used were obtained from Sigma-Aldrich (St. Louis, MO, USA) or Wako Pure Chemicals (Osaka, Japan). The procedures and protocols used in this study were ethically reviewed and approved in accordance with the guidelines of the Hankyong National University committee (Number: 2018-03-01).

4.2. Site-directed mutagenesis of active and inactive sites

An overlap extension PCR strategy was used to create the activating and inactivating mutants in eel LHR cDNA, as previously described [33]. Two different sets of polymerase chain reactions (PCRs) were conducted. In step 1, the first set of fragments were amplified using forward and reverse primers (mutation primer). The second set of fragments were then amplified using forward (mutation primer) and reverse primers. In step 2, the amplified fragments (first and second set of fragments) from step 1 were used as templates to amplify the completely mutated fragments. The primer sequences used in these experiments are shown in **Table 4**. The full-length PCR products synthesized in step 2 were cloned into a pGEM-T easy vector. Plasmids were extracted and sequenced to confirm the presence of the mutations. A schematic representation of the naturally occurring mutation sites for the 3 activating (M410T, L469R, and D590Y) and 2 inactivating (D417N and Y558F) mutants in eel LHR is shown in **Figure 1**.

4.3. Vector construction

cDNAs encoding wild type (WT) and mutant eel LHR were digested with the Eco *RI* and Xho *I* restriction enzymes. The resulting fragments were then ligated into the pcDNA3 and pCORON1000 SP VSV-G expression vector, as previously described [33]. Plasmids were then purified, and the presence of the correct insert was confirmed through analysis with restriction enzymes. Finally, we constructed a total of 6 receptor genes, including wild type, M410T, L469R, D590Y, D417N, and Y558F eel *LHR*.

4.4. Transient transfection and production of recombinant eel luteinizing hormone protein

Chinese hamster ovary cells were cultured in growth medium (Ham's F-12 medium containing 2 mM glutamine, 50 U/mL penicillin, 50 μg/mL streptomycin, and 10% fetal bovine serum). Cells were grown to 80 – 90% confluence in 6-well plates followed by transfection with mutant plasmids. Chinese hamster ovary growth medium containing 20% fetal bovine serum (FBS) was added to each well 5 h after transfection. Cells were used for cAMP analysis at 48 h after transfection.

For ligand production, the rec-eel LH expression vector was transfected into CHO-suspension (CHO-S) cells using the FreeStyle™ MAX reagent transfection method according to the manufacturer's instructions, and as was previously reported in our lab [33]. On the day of

transfection, cell density was approximately 1.2–1.5 × 10^6 cells/mL. The FreeStyle™ MAX Reagent and eel LH plasmid were diluted, and gently mixed by inverting the tube. The DNA-FreeStyle™ MAX reagent was incubated for 10 min at 25 °C to allow the formation of complexes. The complexes were added to 200 mL of cell-containing medium. Culture media were collected on day 7 after transfection; supernatants were collected and frozen at -80 °C. The concentration of rec-eel LH was analyzed using an enzyme-linked immunosorbent assay (ELISA), previously developed in our laboratory [34].

4.5. ELISA analysis of recombinant eel luteinizing hormone protein

Rec-eel LH was quantified using a double-sandwich ELISA performed in plates coated with the 5A11 monoclonal antibody, as previously described [34]. A volume of 100 µL of the rec-eel LH sample was added to the wells and then incubated for 1 h at 25 °C. After washing 3 times with PBS-T, HRP-conjugated anti-eel11A8 antibody in PBS was added to the plates and they were incubated for 1 h at 25 °C. After washing, wells were incubated with 100 µL of substrate solution for 20 min at 25 °C. The reaction was stopped by adding stop solution (50 µL of 1 M H_2SO_4). Absorbance at 450 nm was measured in each well using a microplate reader Cytation 3 (BioTek, Winooski, VT, USA).

4.6. Analysis of cyclic adenosine monophosphate by homogeneous time-resolved fluorescence (HTRF)

The accumulation of cAMP in CHO-K1 cells expressing WT and mutant eel LHRs was measured using cAMP Dynamic 2 assay kits (Cisbio Bioassays, Codolet, France), as previously described [33]. Briefly, cells transfected with WT and mutant eel LHRs were added at 10 000 cells per well into a 384-well plate 48 h after transfection. Cells were stimulated by incubation with the agonist for 30 min at 25 °C. cAMP level was detected by measuring the decrease in HTRF energy transfer (665 nm/620 nm) using an Artemis K-101 HTRF microplate reader (Kyoritsu Radio, Tokyo, Japan). The specific signal-Delta F (energy transfer) is inversely proportional to the concentration of cAMP in the standard or sample. Results were calculated based on the 665 nm/620 nm ratio and expressed as Delta F% (cAMP inhibition), according to the following equation: [Delta F% = (standard or sample ratio-sample negative) × 100/ratio negative]. The cAMP concentrations were calculated from the Delta F% values using the Prism software (GraphPad, Inc., La Jolla, CA,

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USA).

4.7. Agonist-induced loss of cell surface receptors

Loss of eel LHR from the cell surface was assessed by ELISA as previously described [32, 40]. We excluded the M410T mutant, which displayed the lowest basal cAMP response among the activating mutants. Accordingly, we characterized 2 activating (L469R and D590Y) and 2 inactivating (D417N and Y558F) mutants for the loss of receptors from the cell surface. Cells were plated at a density of 6 x 10^5 cells per 60 mm dish, and then split into 96-well dishes (1 × 10^4 cells) coated with poly-D-lysine 24 h post-transfection. For the experiment of loss of cell surface receptors, cells were preincubated with 500 ng/mL rec-eel LH for the time-dependent tests (5, 15, 30, and 60 min). Briefly, cells were fixed using 4 % paraformaldehyde in Dulbecco's PBS for 5 min at 25 °C. After washing 3 times with DPBS, all wells were incubated with blocking solution (TBS with 1% BSA) for 30 min. The primary antibody reaction was performed using a rabbit anti-VSVG antibody (1/1000; Abcam, USA), followed by incubation with horseradish peroxidase-conjugated anti-rabbit secondary antibody (1500; Abcam, USA). After washing 4 times with blocking solution, 80 µL PBS and 10 µL SuperSignal ELISA Femto Maximum substrate (Thermo Fisher Scientific, USA) was added to each well for detection. Luminescence was measured using a Cytation 3 plate reader (BioTek, Winooski, VT, USA). The expression level of the wild type receptor was set as 100%. The loss of wild type and mutant eel FSHRs from the cell surface was calculated by comparing the levels in the presence of rec-eel LH to the levels in the absence of stimulation with the agonist (taken as 0% of loss of cell surface receptors).

4.8. Data analysis

The MultAlin interface-multiple sequence alignment software was used for sequencing results. The GraphPad Prism 6.0 was used for the analysis of the production of cAMP and GraFit 5.0 (Erithacus Software Limited, Surrey, UK) was used for the cAMP EC_{50} values and analyses of stimulation curves. Curves fitted in a single experiment were normalized to the background signal measured for mock-transfected cells in Figure 6. Each curve was drawn using data from three independent experiments. One-way ANOVA and Turkey's Multiple Comparison tests were used to compare the results between samples, using GraphPad Prism 6.0. Differences were indicated as significant between the groups ($P<0.05$).

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5. Conclusions

In summary, we showed that constitutively activating mutations in eel *LHR* (M410T, L469R, and D590Y) resulted in a significant increase in the basal production of cAMP, but did not respond to eel LH stimulation with a concentration-dependent increase in the production of cAMP, as has been reported for mutations of these highly conserved amino acids in mammalian LHRs. In contrast, the inactivating mutations (D417N and Y558F) demonstrated to not completely impair the signal transduction associated with cAMP responsiveness. The rate of loss of the D590Y mutant from the cell surface was found to be slightly slower, whereas that of the L469R mutant was shown to be similar to that of the wild type receptor. However, the loss of the D417N and Y558F inactivating mutants from the cell surface was considerably slower than that of the agonist-occupied wild type receptor. Thus, we suggested that the activation process might involve an agonist-induced conformational change in the receptor. The fundamental mechanisms through which the constitutively activating mutants resulted in a significant increase in the basal cAMP response, whereas the inactivation mutants that impaired signal transduction require further investigation. Future studies using these glycoprotein hormone receptors could provide very valuable information regarding the structure-function relationship of LHR-LH complexes in signal transduction.

Authors' contributions

Munkhzaya Byambaragchaa and Dong-An Kim were responsible for data and collection. Dae-Jung Kim and Sun-Mee Hong interpreted the results and critically revised the manuscript for important intellectual content. Myung-Hwa Kang and Kwan-Sik Min designed the experiments and contributed to manuscript preparation. All authors reviewed and approved the final manuscript.

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Conflict of Interest

The authors declare no conflict of interest.

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Figures Legends

Figure 1. Schematic representation of the eel LHR structure. The location of the 3 constitutively activating mutations (M410T, L469R, and D590Y) and the 2 inactivating mutations (D417N and Y558F) are indicated. Amino acid sequences at the mutated sites in the transmembrane domains of the eel LHR are shown. Alignment of the eel LHR sequence was performed using homologous mammalian LH/CGR sequences obtained from the NCBI database. The activating and inactivating sites were determined by comparison with the corresponding sites in the eel LHR. Red circles indicate constitutively activating mutations, whereas blue circles indicate inactivating mutations. EC, extracellular domain; TM, transmembrane domain; IC, intracellular domain.

Figure 2. Total levels of cAMP stimulated by eel LH in CHO-K1 cells transfected with constitutively activating eel LHR mutants. (A) CHO-K1 cells transiently transfected with wild type and activating mutations (M410T, L469R, and D590Y) of the eel LHR were stimulated with eel LH (0–1000 ng/mL) for 30 min. The production of cAMP was detected using a homogenous time-resolved fluorescence (HTRF) assay. The accumulation of cAMP was calculated as Delta F%. The concentration of cAMP was recalculated using the GraphPad Prism software. The mock-transfected results were subtracted from each data set. (B) Basal cAMP response without agonist and maximal cAMP response were presented using bar graphs. *Statistically significant differences ($P < 0.05$) when compared with the wild type receptor. A representative data is shown as the mean of three independent experiments.

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Figure 3. Production of cAMP stimulated by treatment with eel LH in CHO-K1 cells transfected with inactivating eel LHR mutants. (A) CHO-K1 cells transiently transfected with wild type and inactivating mutant (D417N and Y558F) eel LHRs were stimulated with eel LH (0–1000 ng/mL) for 30 min. Total levels of cAMP were analyzed using a homogenous time-resolved fluorescence (HTRF) assay. Empty circles denote wild-type eel LHR, whereas black circles denote mutants. Data was subtracted from the results of the mock-transfected cells. (B) The maximal cAMP response is presented by a bar graph. *Statistically significant differences ($P < 0.05$) when compared with the wild type receptor. A representative data set performed as the mean of three independent experiments.

Figure 4. Loss of wild type and activating/inactivating mutant eel LHRs from the cell surface. Each mutant plasmid was transiently transfected into HEK-293 cells. Cells were incubated without or with 500 ng/mL eel LH for 30min. At the end of this incubation, cells were used to determine the expression of receptors on the cell surface. Results were expressed as percentage of the loss of receptors from the cell surface. The loss of wild type and mutant eel LHRs from the cell surface was calculated by comparing the levels in the presence of eel LH to the levels in the absence of treatment with the agonist (taken as 0% of loss of cell surface). A representative data set performed as the mean of three independent experiments. *Statistically significant differences ($P < 0.05$) when compared with the wild type receptor.

Figure 5. Time-dependent loss of wild type and activating/inactivating mutant eel LHRs from the cell surface. HEK-293 cells transiently expressing wild type or activating/inactivating eel LHRs were incubated with 500 ng/mL for up to 60 min. The expression of receptors on the cell surface in nonpretreated groups was taken as 100% (see Materials and Methods for details). The loss of each receptor is shown using the GraphPad Prism software. A representative data set performed as the mean of three independent experiments. In this figure, mean data were fitted to the one phase exponential decay equation.

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Tables

Table 1. Bioactivity of eel LH receptors in cells expressing activating receptor mutants

eel LH receptors	cAMP responses		
	Basal[a] (nM / 10^4 cells)	EC_{50} (ng/mL)	Rmax[b] (nM / 10^4 cells)
eel LHR-wt	1.2 ± 0.5 (1-fold)	18.9 (15.3 to 24.9)[c]	87.5 ± 2.2 (1-fold)
eel LHR-M410T	4.8 ± 0.3 (4.0-fold)	8.7 (5.5 to 20.1)	57.3 ± 2.6 (0.65-fold)
eel LHR-L469R	22.9 ± 1.5 (19.1-fold)	3.8 (2.4 to 8.1)	46.2 ± 1.1 (0.52-fold)
eel LHR-D590Y	9.3 ± 0.8 (7.8-fold)	77.3 (52.5 to 147)	85.9 ± 4.1 (0.98-fold)

Values are the means ± SEM of three independent experiments. EC_{50} values were determined from the concentration-response curves obtained in vitro bioassays.
[a] Basal average level of cAMP without treatment with agonist.
[b] Rmax average level of cAMP/10^4 cells.
[c] Geometric mean (95% confidence limit).

Table 2. Bioactivity of eel LH receptors in cells expressing inactivating receptor mutants

eel LH receptors	cAMP responses		
	Basal[a] (nM / 10^4 cells)	EC_{50} (ng/mL)	Rmax[b] (nM / 10^4 cells)
eel LHR-wt	2.6 ± 0.1	31.7 (26.1 to 40.6)[c]	69.5 ± 1.5 (1-fold)
eel LHR-D417N	1.2 ± 0.1	11.9 (9.4 to 16.5)	22.7 ± 0.6 (0.33-fold)
eel LHR-Y558F	0.7 ± 0.1	13.8 (12.2 to 15.7)	17.3 ± 0.2 (0.25-fold)

Values are the means ± SEM of three independent experiments. EC_{50} values were determined from the concentration-response curves obtained in vitro bioassays.
[a] Basal average level of cAMP without treatment with agonist.
[b] Rmax average level of cAMP/10^4 cells.
[c] Geometric mean (95% confidence limit).

Table 3. Rates of loss of receptors from the cell surface in transiently transfected cell lines expressing the wild type and mutant eel LHRs

eel LHR cell lines	$t_{1/2}$ (*min*)	plateau (*% of control*)
eel LHR-wild type	2.6 ± 0.3	56.9 ± 4.5
eel LHR-L469R	2.7 ± 0.5	60.3 ± 5.8
eel LHR-D590Y	6.2 ± 0.8	64.4 ± 5.2
eel LHR-D417N	-[a]	81.9 ± 6.9
eel LHR-Y558F	-	80.2 ± 7.1

Data were fitted to one phase exponential decay curves to obtain values of $t1/2$ and plateau (i.e. maximum reduction). Data from individual experiments performed as mean of three independent experiments.
[a]Nondetectable.

Table 4. List of primers used to construct eel LHR mutants

	Primer name	Primer sequence
1	eelLHR-wt forward	5'-AT**GAATTC**ATGTCCAATCTGCTCTTGTGGACGATG-3' EcoRI site
2	eelLHR-wt reverse	5'- C**CTCGAG**TTATTTAGGACCTCTGTTGAGAAT-3' XhoI site
3	M410T forward	5'- TCTCCCGCTTCCTCACGTGCAACCTGGCCTT-3'
4	M410T reverse	5'- AAGGCCAGGTTGCACGTGAGGAAGCGGGAGA-3'
5	L469R forward	5'- TGTCCGTCTACACCCGGACCGTCATCACCCT -3'
6	L469R reverse	5'- AGGGTGATGACGGTCCGGGTGTAGACGGACA-3'
7	D590Y forward	5'- TGCTCATATTCACCTACTTCCTGTGCATGGC-3'
8	D590Y reverse	5'- GCCATGCACAGGAAGTAGGTGAATATGAGCA -3'
9	D417N forward	5'- CTGGCCTTCGCCAACCTCTGCATGGGC -3'
10	D417N reverse	5'- GCCCATGCAGAGGTTGGCGAAGGCCAG-3'
11	Y558F forward	5'- GTCTGCGTCTGCTTCGGCCGCATCTAC-3'
12	Y558F reverse	5'- GTAGATGCGGCCGAAGCAGACGCAGAC-3'

*Underlined nucleotides are sites of mutagenesis, while bold nucleotides shown the Eco*RI* and Xho*I* restriction sites for cloning into the expression vector.

Figures

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Fig. 1.

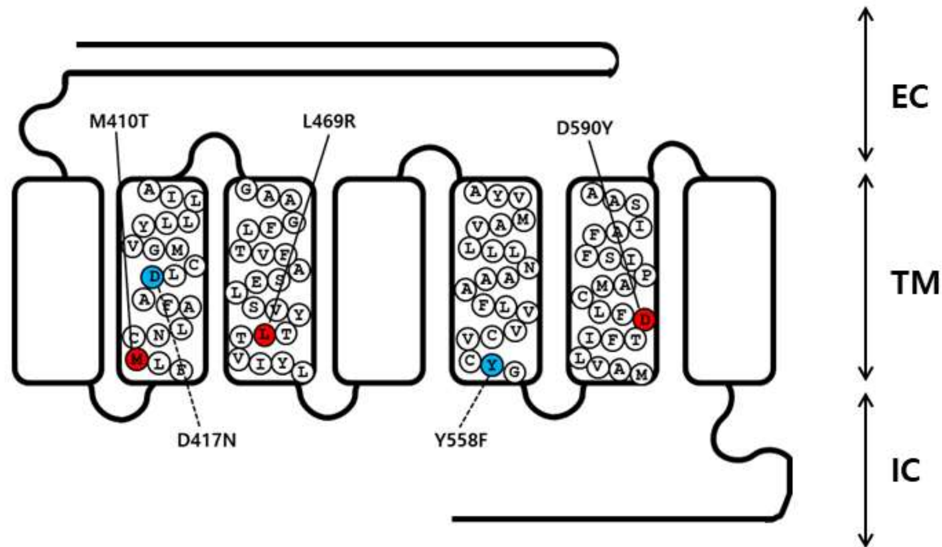

Fig. 2.

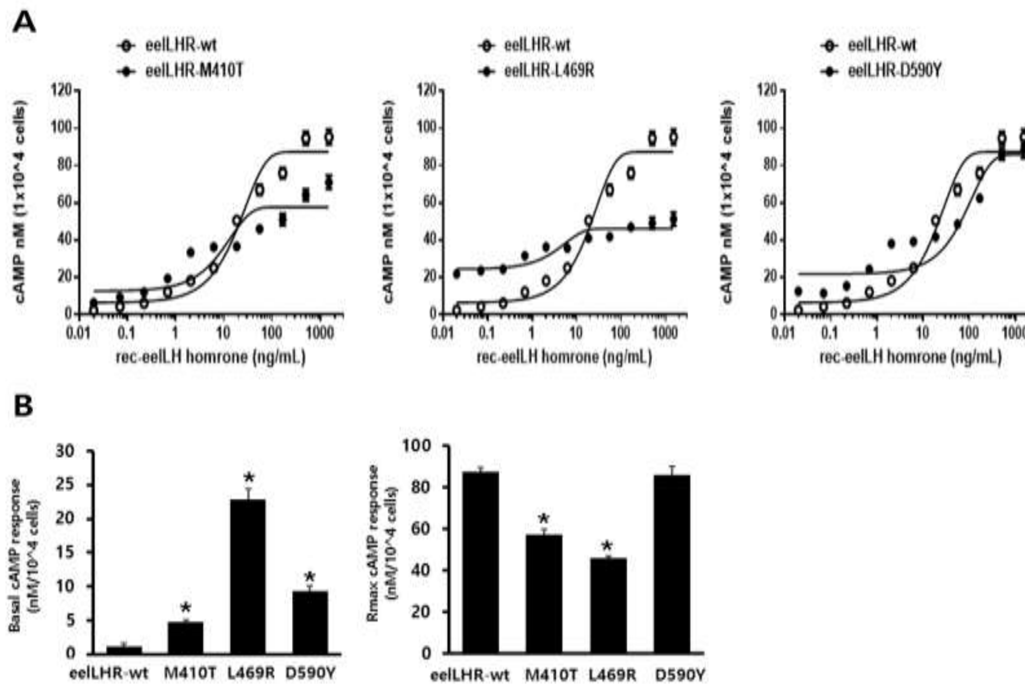

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Fig. 3.

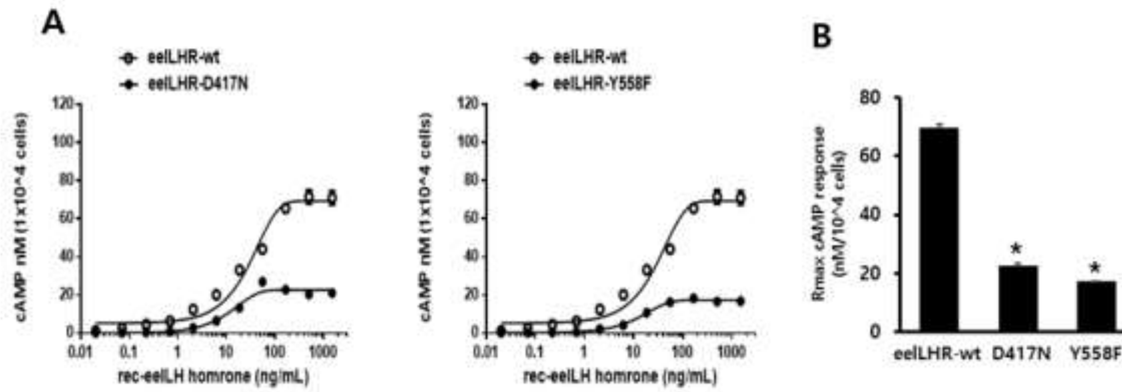
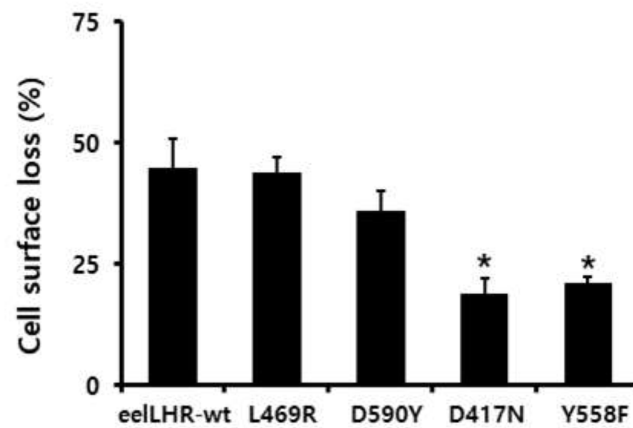


Fig. 4.



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