Mutations in conserved regions of eel luteinizing hormone receptor result in constitutive activation or inactivation

Munkhzaya Byambaragchaa¹, Dong-An Kim¹, Dae-Jung Kim², Sun-Mee Hong³, Myung-Hwa Kang⁴ and Kwan-Sik Min¹,*

¹Animal Biotechnology, Graduate School of Future Convergence Technology, Department of Animal Life Science, Institute of Genetic Engineering, Hankyong National University, Ansung 17579, Korea

E-Mail: monkhzaya_b@yahoo.com
E-Mail: wrm032@daum.net

²Jeju Fisheries Research Institute, National Institute of Fisheries Science (NIFS), Jeju 63610, Korea

E-Mail: djkim4128@korea.kr

³Dept. of Research and Development, Institute of Gyeongbuk Marine Bioindustry, Ulgin 36315, Korea

E-Mail: hongsunmee@gimb.or.kr

⁴Department of Food Science and Nutrition, Hoseo University, Asan 31499, Korea

E-Mail: mhkang@hoseo.ac.kr

* Author to whom correspondence should be addressed; E-mail address: ksmin@hknu.ac.kr,

Tel.: +82-31-670-5421; Fax: +82-670-5417
**Abstract:** Luteinizing hormone receptor (LHR) is a member of the seven-transmembrane (TM) receptor family. Several mutations in LHR have been identified in many mammalian species, leading to either constitutive activation or inactivation of the receptor. Mutations in highly conserved regions of the TM domain have been reported. In this study, we analyzed signal transduction by three constitutively active mutants (designated M410T, L469R, and D590Y) and two inactivating mutants (D383N and Y546F) of eelLHR known as naturally occurring in human LHR. To directly assess the functional effects of these mutations, site-directed mutant receptors were transiently expressed in CHO-K1 cells and cAMP accumulation stimulated by recombinant eelLH (rec-eelLH) was measured by homogeneous time-resolved fluorescence (HTRF) assays. The cAMP response in cells expressing eelLHR wild-type (eLHR-WT) increased in a dose-dependent manner with rec-eelLH ligand stimulation. Cells expressing the activating eelLHR mutants, M410T, L469R, and D590Y, exhibited a 4.0-, 19.1-, and 7.8-fold increase in basal cAMP response, respectively. However, their maximal responses were approximately 73, 53, and 92%, respectively, of the maximal response of LHR-WT. The L469R mutant exhibited a particularly marked increase in cAMP production in the absence of agonist. The maximal responses of the inactivating mutants, D383N and Y546F, were 32 and 24% of LHR-WT, respectively. However, the inactivating mutations did not completely impair signal transduction. Thus, we report here the first characterization of activating and inactivating mutations in eelLHR and we show that these mutations have similar effects as those reported for mammalian LHRs. Moreover, eelLHR with activating mutations showed constitutive cAMP responses. These results provide important data on the signal transduction of constitutively active and inactive LHR mutants. Further studies should aim to identify the mechanism responsible for the significant increase in basal cAMP response in the constitutively activated eelLHR mutants.

**Key words:** eel luteinizing hormone receptor, constitutively activating mutation, inactivating mutation, cAMP responses.
1. Introduction

The seven-transmembrane G protein-coupled receptors (GPCR) represent one of the largest gene families in eukaryotic organisms [1]. Receptors for luteinizing hormone (LH) and follicle-stimulating hormone (FSH) form a subgroup of glycoprotein hormone receptors within the GPCR family [2,3].

There is a great abundance of naturally occurring mutations in the LH receptor (LHR) gene that are related with reproductive failures in mammals [4]. The elucidation of the cDNA and genomic sequences of the human LH receptor (hLHR) have made it possible to identify hLHR mutations that can be directly connected to particular reproductive disorders [5-8]. Several dominant gain-of-function mutations in the hLHR gene have been identified in males with sporadic or familial male-limited gonadotropin-independent pseudo-precocious puberty, also known as “testotoxicosis” [9,10]. In the presence of a heterozygous activating mutation, boys with this condition display elevated levels of testosterone, but prepubertal levels of gonadotropin-releasing hormone (GnRH) and LH [6,11], suggesting that the LHR-signaling pathway in their Leydig cells is activated, even in the absence of hormonal stimulation. These activating hLHR mutations also result in markedly increased cAMP production in the absence of agonist and this is the most common cause of male-limited precocious puberty [12,13].

Cells expressing the hLHR mutant, D578Y, exhibit markedly increased cAMP production in the absence of agonist [6]. Such constitutive activity of the hLHR gene causes LH-releasing hormone-independent precocious puberty in boys and the autosomal dominant disorder, familial male-limited precocious puberty (FMPP) [8]. The Asp578 residue of hLHR serves as an appropriately positioned hydrogen bond acceptor to help stabilize the inactive state of the receptor [12]. An M398T hLHR mutant has been shown to cause constitutively high basal cAMP levels, consistent with Leydig cell activation and precocious puberty in the patient [7]. In a study of genomic DNA from 32 unrelated FMPP patients, 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 region spanning nucleotides 1624-1741 of exon 11 is a hotspot for heterogeneous point mutations that constitutively activate hLHR [13]. Germline hLHR mutations that display constitutive activation of the Gs pathway have also been found in cases of Leydig cell hyperplasia [11].

3
Another LH receptor mutation, M398T, in the second transmembrane helix, has been reported in a precocious puberty patient and in the patient's mother and brother [16,17]. This mutation is of special interest, since one member of the family has the mutation, with no evidence of precocious puberty [18]. Latronico et al. [9] first identified the hLHR activating mutation, L457R, and showed that cells expressing this mutant exhibited significantly higher basal levels of cAMP (7- to 14-fold) than cells expressing the LHR wild-type (LHR-WT). A potent activating somatic mutation (D578H) has also been identified in boys with testicular adenomas [19,20]. The D556H mutation in rat LHR (rD556H; equivalent to the hD578H mutation) also results in an increase in the basal levels of cAMP [4]. A large number of constitutively activating mutations in LHR have been found in boys with sporadic or more common forms of FMPP [21,22].

The inactivating LHR mutations, D383N and R442H, do not affect human chorionic gonadotropin (hCG) binding, but they do impair signal transduction [23]. However, the cAMP responsiveness of the D383N and R442H mutant receptor was characterized by an 18- and 7-fold increase in EC50, respectively, compared to the EC50 of an equivalent density of rLHR-WT [23]. We have also previously reported that L435R and D556Y mutations induce constitutive receptor activation and result in a 25- and 47-fold increase in basal cAMP responsiveness, respectively [24]. These mutations also enhance the internalization of free and agonist-occupied receptors by ~2- and ~17-fold, respectively. The D383N and Y524F mutations are signal impairing mutations and show low-to-normal levels of cAMP under basal conditions. Thus, there are two known signal-impairing mutants that decrease the rate of hCG internalization [24]. All inactivating mutations result in impaired hCG-stimulated cAMP production in cells expressing the mutant receptors, suggesting a clear correlation between the severity of the clinical phenotype and overall receptor signal capacity, which reflects both cell-surface expression levels and coupling efficiency [15].

Although the hormone-binding and activation properties of these mutants are generally well characterized in hLHR, little is known about signal transduction in fish LHR. In the present study, we examined the effects of several constitutively activating (M410T, L469R, and D576Y) and inactivating mutations (D383N and Y546F) in highly conserved residues of eelLHR. We report here that the basal cAMP response of constitutively active eelLHR mutants and the maximal cAMP levels of the inactive mutants differed from those of eelLHR-WT.
2. Results

2.1. Construction of eelLHR mutants and Constitutively active mutant receptors

eelLHR consists of 2,115 nucleotides encoding 705 amino acids. In order to generate substitution mutations at target amino acids, an overlap extension PCR strategy was used with primers designed to change target nucleotides (Table 1). We generated three constitutively activating mutations in transmembrane helices II, III, and VI to investigate how they affect the hormone-receptor interaction and receptor activation system. These mutant receptors were designated M410T (equivalent to M398T in hLHR), L469R, and D590Y. We also constructed two constitutively inactive mutants of eelLHR, designated as D383N and Y546F. Cells transfected with eelLHR-WT exhibited an increased production of cAMP in response to a high concentration of rec-eelLH, when 10^4 cells were analyzed. The EC_{50} of the rec-eelLH-stimulated cAMP response was approximately 24 ng/mL. The basal and Rmax cAMP responses were 1.2 and 96 nM/10^4 cells, respectively.

To assess the functional effects of the three receptor mutations directly, eelLHR-WT and constitutively active mutant receptors were transiently expressed in CHO-K1 cells. Cells transfected with eelLHR-WT DNA had very low basal cAMP production. In contrast, cells expressing the constitutive active mutants (M410T, L469R, D590Y) had highly increased basal cAMP accumulation that was further respond by rec-eelLH in a dose-dependent manner. It was 4.8 ± 0.3, 22.9 ± 1.5, and 9.3 ± 0.8 ng/10^4 cells, respectively. Cells expressing eelLHR-L469R and -D590Y exhibited a 19.1- and 7.8-fold increase in basal cAMP accumulation respectively, compared to cells expressing eelLHR-WT. The EC_{50} values of these mutants for rec-eelLH-stimulated cAMP production were 15 and 827% of LHR-WT values, respectively and their maximum cAMP production was lower than that of the eelLHR-WT. However, the M410T mutant showed only a 4-fold increase in basal cAMP production. The EC_{50} level and Rmax values for this mutant were only 20% and 73%, respectively, of the values of eelLHR-WT. The maximum level of cAMP production of all the constitutively active mutants was lower than that of eelLHR-WT. Compared with eelLHR-WT, all eelLHRs with activating mutations produced higher basal levels of cAMP in CHO-K1 cells than eelLHR-WT, which is consistent with constitutive activation (Figure 1 and Table 2). The mutant with the highest level of basal cAMP production (L457R) did not react to rec-eelLH with a further increase in cAMP responsiveness.
The maximum levels of cAMP production were the lowest in eelLHRs with activating mutations. Thus, high basal cAMP production was not consistent with cAMP responsiveness to agonist stimulation.

### 2.2 Characterization of inactivating mutations

The activity of eelLHRs with the inactivating mutations, D383N and Y546F, was measured by quantifying cAMP stimulation in cells incubated with increasing concentrations of rec-eelLH (Figure 2 and Table 2). As predicted, signaling was impaired in both mutant receptors. The EC$_{50}$ for the rec-eelLH-induced increase in cAMP levels was 2.1-fold higher in the D383N mutant than in eel-LHR-WT. However, the maximal response of this manner was only 32% of the response of eelLHR-WT. In the Y546F mutant, the EC$_{50}$ value was 67.2% of the value for eelLHR-WT, but the maximal response was only 24% lower than that of eel-LHR-WT. Basal cAMP levels in cells expressing D383N and Y546F mutants were 1.2 and 0.7 nM respectively, which were slightly lower than the basal cAMP levels in cells expressing eelLHR-WT. As shown in Table 2, the rec-eelLH responsiveness of receptor was severely affected by the inactivating mutations. The maximal response of these cells was 68~76% lower than the maximal response of cells expressing eelLHR-WT.

### 3. Discussion

The present study describes mutations that induce constitutive activation or impaired signal transduction in eelLHR consistent with previously reported mammalian LHR mutations that cause FMPP and elevated cAMP levels in the absence of agonist. Thus, we prepared mutant eelLHRs containing single point mutations in five distinct amino acid residues that are highly conserved among GPCRs. These mutations have been shown to stimulate basal cAMP responsiveness or attenuate agonist-induced activation of other GPCRs.

Many studies have suggested that the similar active conformation of mammalian LHRs is involved in the stimulus of G proteins [5,12,24]. In humans, mutations have previously been described that constitutively activate hLHR and cause mutation of FMPP [9,13]. The differences observed in the phenotypic appearance of FMPP may be accounted by distinctions in basal hLHR activity [13]. In a previously reported case from Scotland, a patient presented with signs
of pubertal development at 1 year of age [25]. This case was found to have the D578Y mutation (equivalent to D590Y in eelLHR), which is the mutant that reduces the highest basal level of cAMP production in transfected CHO-K1 cells. The residues Met-410, Leu-469, Asp-590, Asp-383, and Tyr-546 in eelLHR are conserved among LHRs, suggesting that these are important for normal receptor function. Receptor mutations correlating to these residues in eelLHR have not previously been described.

In the present study, we show that the three activating mutations in eelLHR resulted in markedly increased cAMP stimulation under basal condition, suggesting that these mutation cause constitutive activation of eelLHR, as also seen in the FMPP-causing mutations in hLHR. Compared to eelLHR-WT, the eelLHR-M410T, -L469R, and -D590Y mutants produced a 4-, 19.1-, and 7.8-fold increase, respectively, in basal cAMP production in CHO-K1 cells, indicating that these mutants were constitutively active, as previously reported in mammalian LHRs [9,17,24,26,27]. Cells expressing the hLHR-M398T mutant exhibited high basal cAMP levels (Yano et al., 1996). The same mutation has been detected in an FMPP patient and in the patient's mother and brother [17]. This mutation is of special interest, since one member of this family has the mutation, with no evidence of precocious puberty [18]. The basal levels of cAMP production increased approximately 15- to 25-fold in the M398T mutant receptor compared to WT receptor [5]. In this paper, we also describe a constitutively activating mutation in eelLHR, M410T, which is located in the same second transmembrane region. The L457R mutation (equivalent to L469R in eelLHR) was the first activating mutation identified in hLHR and cells expressing this mutant receptor exhibit significantly higher basal cAMP accumulation (7-to 14-fold) than cells expressing the WT receptor [9]. We have also reported that cells expressing rLHR-L435R (equivalent to L469R in eelLHR) display a 47-fold increase in basal cAMP responses and do not react to hCG with a further stimulation in cAMP accumulation [24]. In the hLHR, the complex form between hLHR-L457R and hCG is not migrated to the lysosomes, but most of it is returned to the cell surface and hormone degradation is hardly detectable [26]. These results are consistent with our current data, showing that the L469R mutant of eelLHR remarkably increases (19.1-fold) the basal cAMP response in the absence of agonist. But the maximal cAMP response was approximately 53% lower than that of the WT receptor as previously described in hLHR [24].
Thus, our data suggested that the constitutively active mutant, L469R, was easily distinguishable from agonist-activated eelLHRs analyzed in this study.

The aspartic acid residue at position 590 is conserved in all LH receptors, including eelLHR, but is not found in any other GPCR. The D578Y mutant (equivalent to D590Y in eelLHR) was first reported to be inherited in an autosomal dominant and is associated with signs of puberty by 4 years of age. This mutant hLHR results in a 4.5-fold increase cAMP production under basal condition, with an EC$_{50}$ similar to that of the WT receptor. Agonist-independent stimulation of cAMP production by this mutant receptor represented 42% of the maximal stimulation [6]. This is consistent with our results showing that the eelLHR-D590Y mutation, at a conserved in glycoprotein hormone receptor site, resulted in constitutive receptor activity.

Kosugi et al. [12] suggested that the Asp$^{578}$ side chain in hLHR has the appropriate position to recognize as a hydrogen bond acceptor and is important for vitalizing the inactive state of LHR. Other constitutively activating mutations (Ile$^{542}$Leu, Asp$^{564}$Gly, Met$^{571}$Ile, and Cys$^{581}$Arg) have been identified by analyzing genomic DNA from 32 unrelated FMPP [13]. These sites are conserved among glycoprotein hormone receptors, suggesting an important role in receptor function. These data suggest that the region spanning nucleotides 1624–1741 of exon 11 is a hotspot for heterogeneous point mutations that activate hLHR and cause FMPP. The rat LHR-D556Y mutant also exhibits a 25-fold increase in basal cAMP production, but responds to hCG with a normal increase in cAMP accumulation [24]. Based on the model of activation and the observations summarized above, we expected that eelLHR mutations that induce constitutive activation would result in specific changes to the receptor-ligand complex. The D590Y mutant induced an elevated basal cAMP level corresponding to approximately 10% of the maximal cAMP response. However the L469R mutant induced a highly elevated basal cAMP level corresponding to approximately 44% of the maximal cAMP response. These results suggested that the configurations of these three mutants are different in the signal transduction of maximal cAMP responses by LH.

In the inactive mutants, highly conserved amino acids present in the second TM helix (codon 383) and in the fifth TM helix (codon 546) were mutated to asparagine and phenylalanine, respectively. As predicted from results obtained with other GPCRs [23,24,28,29], these mutations (eelLHR-D383N and eelLHR-Y546F) were expected to impair signal transduction.
Cells expressing rLHR-D383N display a rightward shift in the EC$_{50}$ for cAMP stimulation, but a normal maximal response [23]. The Y524F mutant in rLHR was also a signaling-impairing mutation. Cells expressing this mutant respond a normal level of cAMP in the absence of agonist, but their maximal response is only 14% lower than that of the WT receptor [24]. These results are consistent with our data, showing that D383N and Y546F are signaling-impairing mutations in eelLHR. The maximal response by these mutant receptors was only 24–32% of the maximal response of eelLHR-WT. The internalization in inactivation mutants, rLHR-D383N and rLHR-Y524F, was much slower than in rLHR-WT [23]. In the present study, D383N and Y546F mutations were predicted to induce the inactivation of eelLHR. However, these mutations did not completely impair signal transduction in eelLHR. Thus, we suggest that these mutations are system-dependent or species-specific and therefore, may not have the same effects in fish systems.

4. Experimental Section

4.1. Materials

The pGEMTeasy cloning vector was purchased from Promega (Madison, WI, USA). The mammalian expression vector, pcDNA3, FreeStyle MAX transfection reagent, FreeStyle CHO-S suspension cells, and Lipofectamine-2000 were obtained from Invitrogen (Carlsbad, CA, USA). Ham’s F-12 medium, CHO-S-SFMII medium, and fetal bovine serum, OptiMEM medium were bought from Gibco BRL (Grand Island, NY, USA). CHO-K1 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-eelLH from CHO-K1 cells were produced in our lab as previously reported [30]. Rec-eelLH produced using baculovirus expression system was kindly donated by Dr. Sun-Me Hong (Institute of Gyeongbuk Marine Bio-Industry). The horseradish peroxidase (HRP) labeling of 8A11 monoclonal antibody was generously performed by Medexx Inc. (Seongnam, Korea). EelLHR cDNA was cloned from eel ovaries and testes as previously reported [31]. Polymerase chain reaction (PCR) and endonucleases reagents were from Takara (Osaka, Japan). QIAprep-Spin plasmid kits were bought from Qiagen Inc.
Oligonucleotides were synthesized by Genotech (Dajeon, Korea). Disposable spinner flasks were purchased from Corning Inc. (Corning, NY, USA). Fetal bovine serum was bought from HyClone Laboratories (Logan, UT, USA). Centrifugal Filter Devices were from Amicon Bio (Billerica, MA, USA). All other reagents used were from Sigma-Aldrich (St. Louis, MO, USA) or Wako Pure Chemicals (Osaka, Japan).

4.2. Site-directed mutagenesis of activation and inactivation sites

An overlap extension PCR strategy was used to create activating and inactivating mutants in eelLHR cDNA, as previously described [32]. Two different sets of PCR were performed. In step 1, forward and reverse primers (mutation primer) were used to amplify the first fragment. The second fragments were then amplified with forward (mutation primer) and reverse primers. In step 2, the amplified products (first and second 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 1. The full-length PCR product synthesized in step 2 was eluted and cloned into a pGEMTeasy vector. This clone was used to transform DH5α competent cells. Plasmids were extracted and sequenced to confirm the presence of the mutations. A schematic representation of the mutations is shown in Fig. 1. We selected naturally occurring mutation sites for three activating (M410T, L469R, and D590Y) and two inactivating (D383N and Y546F) mutations in eelLHR.

4.3. Vector construction

cDNAs encoding WT and mutants eelLHR were digested with EcoRI and XhoI enzymes. The resulting fragments were then ligated into the eukaryotic expression vector, pcDNA3, as previously described [31,33]. Plasmids were then purified and the presence of the correct insert was confirmed by digestion with EcoRI and XhoI restriction enzymes. Finally, we constructed a total of six receptor genes, including eelLHR-WT (designated as pcDNA3-eelLHR-WT, pcDNA3-M410T, pcDNA3-L469R, pcDNA3-D590Y, pcDNA3-D383N, and pcDNA3-Y546F).

4.4. Transient transfection
CHO cells were transfected using the liposome transfection method, as previously described [32]. CHO cells were cultured in growth medium (Ham’s F-12 medium containing 50 U/mL penicillin, 50 μg/mL streptomycin, 2 mM glutamine, and 10% fetal bovine serum). CHO cells were grown to 80–90% confluence in 6-well plates and plasmid DNAs were transfected using Lipofectamine reagent. After combining the diluted DNA with Lipofectamine samples were incubated for 20 min. CHO cells were washed with Opti-MEM and the DNA-Lipofectamine complex was added to each well. After 5 h, CHO growth medium containing 20% fetal bovine serum was added to each well. The cells were used for cAMP analysis at 48 h after transfection.

For ligand production, a rec-eelLH expression vector was transfected into CHO-suspension (CHO-S) cells using the FreeStyle MAX reagent transfection method, according to the manufacturer’s instructions. One day prior to transfection, CHO-S cells were passaged at 5 × 10^5 cells/mL. Flasks were placed on an orbital shaking platform, rotating at 120–135 rpm at 37°C in a humidified atmosphere of 8% CO_2 in air. On the day of transfection, the cell density was approximately 1.2–1.5 × 10^6 cells/mL. Next, 260 μg of plasmid DNA was diluted into OptiPRO™ serum-free medium (SFM) to a total volume of 4 mL. In a separate tube, 260 μL of FreeStyle™ MAX Reagent was diluted in Opti-PRO™ SFM to a total volume of 4 mL and mixed gently by inverting the tube. After the solutions were mixed, the DNA-FreeStyle™ MAX mix was incubated for 10 min at RT to allow complexes to form. DNA-FreeStyle™ MAX Reagent complexes were slowly added to 200 mL of medium containing cells. Transfected cell cultures were incubated at 37°C in a humidified atmosphere of 8% CO_2 on an orbital shaking platform, rotating at 135 rpm. Finally, culture media were collected on day 7 after transfection and centrifuged at 100,000 × g for 10 min at 4°C to remove cell debris. Supernatants were collected and frozen at -80°C. The samples were concentrated using a Centricon filter or by freeze-drying and mixed with PBS. The concentration of rec-eelLH was analyzed by an enzyme-linked immunosorbent assay (ELISA), previously developed in our laboratory (Kim et al., 2016).

**4.5. ELISA analysis of rec-eelLH protein**

Rec-eelLH was quantified using a double-sandwich ELISA performed in plates coated with the monoclonal antibody, 5A11, which is directed against the α-subunit of eelLH as described previously [30]. The standard of rec-eelLH was produced from *E. coli* and purified by
performing Ni-NTA Sepharose column chromatography with 250 mM imidazole. The wells were blocked by incubation with 1% skim milk in PBS for 1 h at 37°C. After removal of the blocking reagent, the wells were washed with filtered PBS containing 0.05% Tween 20 (PBS-T). Next, 100 μL of rec-eelLH sample was added to the wells and incubated for 1–2 h at 37°C. After washing three times with PBS-T, a 400-fold diluted, HRP-conjugated anti-eel 11A8 antibody in PBS was added and plates were incubated for 1 h at room temperature. The wells were washed 5 times and incubated with 100 μL of substrate solution (tetramethylbenzidine) for 20 min at room temperature. The reaction was stopped by adding 50 μL of 1 M H₂SO₄. Absorbance at 450 nm was measured in each well using a microplate reader (Cytation 3; Biotek, Winooski, VT, USA).

4.6. cAMP analysis by homogeneous time-resolved fluorescence (HTRF)

cAMP accumulation in CHO-K1 cells expressing eelLHR-WT and eelLHR mutants was measured using cAMP Dynamic 2 competitive immunoassay kits (Cisbio Bioassays, Codolet, France), as described previously [31]. In briefly cells transfected with eelLHR-WT and eelLHR mutants were added at 10,000 cells per well into a 384-well plate. Cells were stimulated by incubation with the agonist for 30 min at room temperature. The assay was terminated by incubation with the Cisbio detection reagents, cAMP-d2 and anti cAMP-cryptate (diluted 5-fold in lysis buffer and used at 5μL/well), for 1 h at room temperature. cAMP 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). This method is a competitive immunoassay between native cAMP produced by cells and cAMP labeled with the dye, d2. Tracer binding is visualized using a monoclonal anti-cAMP antibody labeled with Eu3+Cryptate. The specific signal-Delta F (energy transfer) is inversely proportional to the concentration of cAMP in the standard or sample. Results were calculated from 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) x100/ratio negative]. The cAMP concentrations for Delta F% values were calculated using Prism software (GraphPad, Inc., La Jolla, CA, USA).

4.7. Data analysis
The Multalin interface-multiple sequence alignment tool was used for sequence analysis. GraphPad Prism 6.0 was used for the analysis of cAMP production and Grafit 5.0 (Erithacus Software Limited, Surrey, UK) was used for cAMP EC$_{50}$ value and stimulation curve analyses. Curves fitted in a single experiment were normalized to the background signal measured for mock-transfected cells. Each curve was drawn using data from at least three independent experiments.

5. Conclusions

In summary, our mutagenesis data suggest that the constitutive activation mutations (M410T, L469R, and D590Y) of eelLHR result in a significant increase in basal cAMP production, but respond to rec-eelLH with a concentration dependent increase in cAMP accumulation, as reported for mutations of these highly conserved amino acids in mammalian LHRs. However, the inactivation mutants (D383N and Y546F) were almost completely abolished in hormone-induced receptor activation. However, these mutations did not totally impair in signal transduction of cAMP responsiveness. Thus, we suggest that the activation process involves an agonist-induced conformational change in the receptor. The mechanism whereby the constitutively active mutants result in a significant increase in the basal cAMP response and inactivation mutants impair signal transduction require further investigation. Future studies using these glycoprotein hormone receptors could provide important information regarding the structure-function relationship of GPCRs in signal transduction.

Acknowledgements

This work was supported by the Korean Research Foundation Program (2018R1A2B6007794), Republic of Korea.

Author contributions

M.B performed the experiment. D-A.K was financially support. D-J.K, S-M.H, M-H.K and K-S.M conceived the study, designed the experiment and drafted the manuscript. All authors read and approved the final manuscript.
Conflicts of Interest

The authors have no financial or personal relationship that could inappropriately influence this research.

References

10. Narayan, P. Genetic models for the study of luteinizing hormone receptor function. *Front Endocrinol.* 2015, 6, 152


**Figures Legends**

**Figure 1. Total cAMP levels stimulated by rec-eelLH in CHO-K1 cells transfected with constitutively active eelLHR mutants.** CHO-K1 cells transiently transfected with eelLHR-WT and mutant eelLHRs (M410T, L469R, and D590Y) were stimulated with rec-eelLH (0–1,000 ng/mL) for 30 min. Finally, the cAMP production was detected using homogenous time-resolved fluorescence (HTRF) assay. The cAMP accumulation stimulated by rec-eelLH is shown as Delta F%. cAMP concentration was calculated using GraphPad Prism software.
Figure 2. Rec-eelLH-stimulated cAMP production in CHO-K1 cells transfected with inactivating eelLHR mutants. CHO-K1 cells transiently transfected with eelLHR-WT and mutant eelLHRs (D383N and Y546F) were stimulated with rec-eelLH (0–1,000 ng/mL) for 30 min. Total cAMP accumulation was analyzed using a homogenous time-resolved fluorescence (HTRF) assay. The empty circles denote wild-type eelLHR and black circles denote the mutants. Each point is the mean of duplicate experiments and one result from three similar experiments is presented.

Figure 3. Schematic representation of the eelLHR structure. The location of the three constitutively activating mutations (M410T, L469R, and D590Y) and the two inactivating mutations (D383N and Y546F) are indicated in the structure of eelLHR. Amino acid sequences at the mutated sites in the transmembrane domains of eelLHR are shown. EelLHR sequence alignment was performed with homologous mammalian LH/CGR sequences obtained from the NCBI database. The activating and inactivating sites were determined by comparison with the corresponding sites in eelLHR. Red circles indicate constitutively activating mutations and the blue circle indicate inactivating mutations. EC, extracellular domain; TM, transmembrane domain; IC, intracellular domain.

**Table 1.** List of primers used to construct eelLHR mutants

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 eelLHR-wt forward</td>
<td>5’-ATGAATTTCATGTCCAAATCTGCTCTTGTGGACGATG-3’ EcoRI site</td>
</tr>
<tr>
<td>2 eelLHR-wt reverse</td>
<td>5’-CCTCGAGTTATTTAGAGAACCCTGTTTGAATT-3’ Nhol site</td>
</tr>
<tr>
<td>3 M410T forward</td>
<td>5’-TCTCCCGCTCTCCTCACTGCAACCTGCTCTTT-3’</td>
</tr>
<tr>
<td>4 M410T reverse</td>
<td>5’-AACGCTTCAAGGCACCTGCAAGGAACCGGGAGA-3’</td>
</tr>
<tr>
<td>5 L469R forward</td>
<td>5’-TTCGGCGTCTACACCGGACCCTACACCT-3’</td>
</tr>
<tr>
<td>6 L469R reverse</td>
<td>5’-AGGTGTAGCAGGCAGGCAGTACACAC-3’</td>
</tr>
<tr>
<td>7 D590Y forward</td>
<td>5’-GCTCATATCTACACTTCTCTTGTGCATGGC-3’</td>
</tr>
<tr>
<td>8 D590Y reverse</td>
<td>5’-GCCATGCAAGGAAGTGATGAAAATGAGGA-3’</td>
</tr>
<tr>
<td>9 D383N forward</td>
<td>5’-CTGGCCTTCGCCCAACCTCTGCATGGG-3’</td>
</tr>
<tr>
<td>10 D383N reverse</td>
<td>5’-GCCATGCAAGAGGTGGCGCAAGGCGCAG-3’</td>
</tr>
<tr>
<td>11 Y546F forward</td>
<td>5’-GTTCGGCATGCTGGCCGCACTCTA-3’</td>
</tr>
<tr>
<td>12 Y546F reverse</td>
<td>5’-TGTTAGATCGGCCGCAAGCAGCAGCAAGC-3’</td>
</tr>
</tbody>
</table>

*Underlined nucleotides are the site of mutagenesis.
**Table 2. Bioactivity of eelLH receptors in cells expressing activating and inactivating receptor mutants**

<table>
<thead>
<tr>
<th>eelLH receptors</th>
<th>Basal (nM / 10^4 cells)</th>
<th>EC_{50} (ng)</th>
<th>R_{max} (nM / 10^4 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>eelLHR-WT</td>
<td>1.2 ± 0.5 (1-fold)</td>
<td>24.0 ± 4.0 (100%)</td>
<td>96.0 ± 6.2 (100%)</td>
</tr>
<tr>
<td>eelLHR-D590Y</td>
<td>9.3 ± 0.8 (7.8-fold)</td>
<td>152.1 ± 5.2 (15%)</td>
<td>89.1 ± 4.5 (92%)</td>
</tr>
<tr>
<td>eelLHR-L469R</td>
<td>22.9 ± 1.5 (19.1-fold)</td>
<td>2.9 ± 0.1 (827%)</td>
<td>50.2 ± 5.0 (53%)</td>
</tr>
<tr>
<td>eelLHR-M410T</td>
<td>4.8 ± 0.3 (4.0-fold)</td>
<td>118.0 ± 5.2 (20%)</td>
<td>70.1 ± 4.3 (73%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>eelLH receptors</th>
<th>Basal (nM / 10^6 cells)</th>
<th>EC_{50} (ng/mL)</th>
<th>R_{max} (nM / 10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>eelLHR-WT</td>
<td>1.6 ± 0.0 (1-fold)</td>
<td>28.5 ± 4.0 (100%)</td>
<td>72.1 ± 5.6 (100%)</td>
</tr>
<tr>
<td>eelLHR-D383N</td>
<td>1.2 ± 0.1 (2.1-fold)</td>
<td>13.5 ± 5.3 (211%)</td>
<td>23.2 ± 2.1 (32%)</td>
</tr>
<tr>
<td>eelLHR-Y546F</td>
<td>0.7 ± 0.1 (67%)</td>
<td>42.4 ± 3.5 (67%)</td>
<td>17.7 ± 0.7 (24%)</td>
</tr>
</tbody>
</table>

Values are the means ± SEM of triplicate experiments. EC_{50} values were determined from the concentration-response curves from *in vitro* bioassays.
**Fig. 1.**

![Graphs showing cAMP levels for different hormone concentrations.](image1)

**Fig. 2.**

![Graphs showing cAMP levels for different hormone concentrations.](image2)