Characterization of constitutive activation and inactivation mutants in conserved regions of the eel luteinizing hormone receptor

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Abstract

Background: We analyzed signal transduction of three constitutively activating mutants (designated M410T, L469R, and D590Y) and two inactivating mutants (D383N and Y546F) of the eel luteinizing hormone receptor (LHR), known to be naturally occurring in humans as LHR. The objective of the present study is to assess the functional effects of these mutations in signal transduction.

Methods: 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.

Results: The cAMP response in cells expressing the wild-type eel LHR (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 the basal cAMP response, respectively. However, their maximal responses to agonist were approximately 73, 53, and 92%, respectively, of the maximal response of the LHR-WT. The L469R mutant exhibited a particularly marked increase in cAMP concentration in the absence of ligand. The inactivating mutations did not completely impair signal transduction. The maximal responses of the inactivating mutants, D383N and Y546F, were 32% and 24% of the LHR-WT, respectively.

Conclusion: We report here the first characterization of activating and inactivating mutations in eelLHR. These results provide important data on the signal transduction of constitutively active and inactive eelLHR mutants.

Keywords: eel LHR, constitutively activating/inactivating mutation

Introduction

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

Cells expressing the hLHR D578Y constitutively activating mutant displayed a prominently increased cAMP response in the absence of ligand stimulation [6] . Such constitutive activity of the hLHR gene causes LH-releasing hormone-independent premature puberty in boys and FMPP [8].

The Asp578 residue of the hLHR serves as an appropriately placed hydrogen bond acceptor to help conserve the inactive condition of the receptor [14]. An M398T hLHR mutant has been shown to cause constitutively high basal cAMP levels, relevant to Leydig cell activation and premature adolescent in the patient [7]. In a study of genomic DNA from 32 unrelated FMPP patients, three activating mutation sites (Asp578Gly, Met571Ile, and Thr577Ile) and four other mutation sites (Ile542Leu, Asp564Gly, Asp578Tyr, and Cys581Arg) were identified in hLHR [14, 15]. The nucleotides 1624–1741 of the hLHR are a hotspot for heterogeneous specific mutations, suggesting that mutations in this region constitutively activate hLHR [13]. Germline hLHR mutations that display continuous activation of Gs signal transduction have also been detected in cases of Leydig cell hyperplasia [11].

Another activating LHR mutation, M398T, in the second transmembrane helix, has been described in a precocious puberty patient and in the patient's mother and brother [16, 17]. This mutation is of special interest, as one member of the family has the mutation, with no evidence of precocious puberty [18]. Latronico et al. [19] first identified the hLHR activating mutation, L457R, and showed that cells expressing this mutant display remarkably higher basal levels of cAMP (7-to 14-fold) compared to the LHR wild-type (LHR-WT). A potential activating mutation (D578H) has also been reported in boys with testicular adenomas [20, 21]. The D556H mutation in rat LHR (rD556H; equivalent to the hD578H mutation) also results in an increase in the basal levels of cAMP [4]. The majority of constitutively activating mutations in hLHR have been identified in boys with intermittent or more common forms of FMPP [22, 23].

The inactivating LHR mutations, D383N and R442H, do not affect human chorionic gonadotropin (hCG) binding, however, they do impair signal transduction [24]. However, the cAMP responsiveness of the D383N and R442H mutant receptors was characterized by an 18- and 7-fold increase in

EC_{50}, respectively, compared to the EC_{50} of an equivalent density of rLHR-WT [24]. We have also previously reported that the L435R and D556Y mutations induce constitutive receptor activation and result in a 25- and 47-fold increase in basal cAMP responsiveness, respectively [25]. The D383N and Y524F mutations are signal impairing mutations and show low-to-normal levels of cAMP under basal conditions. Thus, two signal-impairing mutants are known that decrease the rate of hCG internalization [25]. All inactivating mutations result in an underlying hCG-stimulated cAMP response in mutant receptor cells, suggesting a clear correlation between the intensity of the clinical phenotype frequency and receptor signal efficiency, which concern both quantities of cell-surface expression and coupling efficiency [15].

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

Results

Construction of eelLHR mutants

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 the eelLHR, designated as D383N and Y546F.

Analysis of eelLHR-wild type and constitutively activating mutations

Cells transfected with eelLHR-WT exhibited an increased levels of cAMP in response to a high concentration of rec-eelLH, when 10^4cells were analyzed. The EC_{50} value 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 identify the functional effects of the three receptor mutations directly, eelLHR-WT and constitutively active mutant receptors were transiently transfected in CHO-K1 cells. Cells expressing eelLHR-WT DNA exhibited very low basal cAMP production. And then the cAMP levels were further stimulated by rec-eelLH, in a dose-dependent manner

In contrast, cells expressing the constitutive active mutants (M410T, L469R, and D590Y) had highly increased amounts of basal cAMP that were 4.8 ± 0.3, 22.9 ± 1.5, and 9.3 ± 0.8 ng/10^4 cells, respectively. Cells expressing eelLHR-L469R and eelLHR-D590Y exhibited a 19.1- and 7.8-fold increase in amounts of basal cAMP, respectively, compared to cells expressing the 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 the eelLHR-WT. The maximum level of cAMP production of all the constitutively active mutants in response to stimulation with rec-eelLH was lower than that of the eelLHR-WT. However, compared with the eelLHR-WT, all eelLHRs with activating mutations produced higher basal levels of cAMP in CHO-K1 cells

than the 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.

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 mutant was only 32% of the response of the eelLHR-WT. In the Y546F mutant, the EC_{50} value was 67.2% of the value for the eelLHR-WT; however, the maximal response was only 24% lower than that of the eel-LHR-WT. Basal cAMP levels in cells expressing D383N and Y546F mutants were 1.2 nM and 0.7 nM, respectively, which were slightly lower than the basal cAMP levels in cells expressing the eelLHR-WT. As shown in Table 2, the rec-eelLH responsiveness of the 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.

Discussion

The present study describes mutations that induce constitutive activation or impaired signal transduction in the eelLHR consistent with previously reported mammalian LHR mutations that cause FMPP and elevated cAMP levels in the absence of agonist. Thus, we constructed eelLHR mutants

containing single point mutations in five distinct amino acid residues that were highly conserved among GPCRs. These mutations have been shown to stimulate basal cAMP responsiveness or attenuate agonist-induced activation in a dose-dependent [12, 24-27].

Many studies have suggested that the similar dynamic modification of mammalian LHRs is closely involved in the activity of G proteins [5, 12, 25]. In humans, mutations have previously been described that constitutively activate hLHR and cause FMPP [13, 19]. The differences observed in the phenotypic appearance of FMPP may be accounted for by distinctions in basal hLHR activity [13]. In a previously studied case from Scotland, a patient exhibited signs of pubertal development at 1 year of age [28]. This case was found to have the D578Y mutation (equivalent to D590Y in the eelLHR), which is the mutant that induces higher basal cAMP production than that of the eelLHR-WT. The residues Met-410, Leu-469, Asp-590, Asp-383, and Tyr-546 in eelLHR are conserved among LHRs, suggesting that these residues are important for normal receptor function [24, 25]. Receptor mutations in these residues in the eelLHR have not previously been described.

In the present study, we showed that the three activating mutations in eelLHR resulted in a distinctly increased cAMP response under basal conditions, suggesting that these mutations cause constitutive activation of the eelLHR, as also seen in the FMPP-causing mutations in hLHR. Compared to the eelLHR-WT, the eelLHR-M410T, -L469R, and -D590Y mutants produced a 4-, 19.1-, and 7.8-fold increase, respectively, in the basal cAMP response in CHO-K1 cells, indicating that these three mutants were continuously active, as previously reported in mammalian LHRs [17, 19, 25]. Cells expressing the hLHR-M398T mutant exhibited high basal cAMP levels [7]. 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, one member of this family has the mutation, but with no evidence

of precocious puberty [18]. The basal levels of cAMP production were 15- to 25-fold higher in the M398T mutant receptor compared to the WT receptor [5]. In this paper, we also described a constitutively activating mutation in the 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 markedly higher basal cAMP levels (7-to 14-fold) than that of the WT receptor [19]. We have also reported that basal cAMP responses in cells expressing rLHR-L435R (equivalent to L469R in eelLHR) display a 47-fold increase in the absence of agonist and do not react to hCG with a further stimulation of the cAMP response [25]. In the hLHR, the complex of hLHR-L457R and hCG does not migrate to the lysosomes, but most of it is returned to the cell surface and hormone degradation is hardly detectible [29]. These results are consistent with our current data, showing that the L469R mutant of the eelLHR remarkably increased (19.1-fold) the basal cAMP response in the absence of agonist. However, the maximal cAMP response to agonist was approximately 53% lower than that of the WT receptor, as previously described in hLHR [25]. 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 the eelLHR, but is not found in any other GPCRs. The D578Y mutant (equivalent to D590Y in the 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 in the cAMP response under basal conditions, 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 most appropriate position to act as a hydrogen bond acceptor and is significant for stabilizing the impaired state of the LHR. Other constitutively activating mutations (Ile542Leu, Asp564Gly, Met571Ile, and Cys581Arg) have been identified by analyzing genomic DNA from 32 unrelated FMPP [13]. These sites are preserved among glycoprotein hormone receptors, suggesting an important function in the receptor signaling pathway. These data suggest that the specific nucleotide regions, 1624–1741 in hLHR, are an important point for heterogeneous occurrence mutations that activate the receptor and cause FMPP. The basal cAMP production of activating rat LHR mutant, rLHR-D556Y, also exhibits a 25-fold increase in the absence of agonist, but responds to agonist with a normal increase in cAMP stimulation [25]. Based on the activation and the results summarized above, we expected that eelLHR mutations that induce continuous 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 induced different signal transduction pathways, resulting in different maximal cAMP responses to LH.

In the inactive mutants, the 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 [24-27], 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 normal maximal levels [24]. The Y524F mutant in rLHR was

also a signaling-impairing mutation. Cells expressing this mutant exhibit normal cAMP levels in the absence of agonist, however, their maximal response to agonist is only 14% compared to the WT receptor [25]. These results are consistent with our data, showing that D383N and Y546F are signaling-impairing mutations in the eelLHR. The maximal response of these mutant receptors to agonist was only 24–32% of the maximal response of eelLHR-WT. The internalization of the inactivation mutants, rLHR-D383N and rLHR-Y524F, was much slower than the rLHR-WT [24]. In the present study, D383N and Y546F mutations were predicted to induce the inactivation of the eelLHR. However, these mutations did not completely impair signal transduction in the eelLHR. Thus, we suggest that these mutations are system-dependent or species-specific and therefore, may not have the same effects in fish systems.

Conclusion

In this study, we showed that the constitutive activation mutations (M410T, L469R, and D590Y) of the eelLHR resulted in a significant increase in basal cAMP production, but responded to rec-eelLH stimulation with a concentration-dependent increase in cAMP production, 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. These mutations were not totally impaired in the signal transduction of cAMP responsiveness. Thus, we suggest that the activation process involves an agonist-induced conformational change in the receptor. The fundamental mechanisms whereby the constitutively active mutants resulted in a significant increase in the basal cAMP response and inactivation mutants impaired signal transduction require further investigation. Future studies using these glycoprotein hormone receptors could provide very valuable information regarding the structure-function relationship of GPCRs in signal

transduction.

Methods

Materials

The pcDNA3 mammalian expression vector, CHO-S suspension cells, MAX transfection reagent, and Lipofectamine-3000 were obtained from Invitrogen (Carlsbad, CA, USA). The pGEMTeasy cloning vector was purchased from Promega (Madison, WI, 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 a 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]. QIAprep-Spin plasmid kits were purchased from Qiagen Inc. (Hilden, Germany). Polymerase chain reaction (PCR) and endonucleases reagents 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).

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 conducted. In step 1, the first fragments were amplified with forward and reverse primers (mutation primer). The second fragments were then amplified with forward (mutation primer) and reverse primers. In step 2, the amplified fragments (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 cloned into a pGEMTeasy vector. Plasmids were extracted and sequenced to confirm the presence of the mutations. A schematic representation of the mutations is shown in **Figure 3.** We selected naturally occurring mutation sites for three activating (M410T, L469R, and D590Y) and two inactivating (D383N and Y546F) mutations in eelLHR.

Vector construction

cDNAs encoding WT and mutant eelLHR were digested with the EcoRI and XhoI restriction enzymes. The resulting fragments were then ligated into the pcDNA3 expression vector, 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).

Transient transfection and rec-eelLH protein production

CHO-K1 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. CHO growth medium containing 20% fetal bovine serum (FBS) was added to each well 5h after transfection. The cells were used for cAMP analysis at 48h after transfection.

For ligand production, 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, and as previously reported in our lab [31]. On the day of transfection, the cell density was approximately 1.2–1.5 × 10^6 cells/mL. FreeStyle™ MAX Reagent and eelLH WT plasmid was diluted and mixed gently by inverting the tube. The DNA-FreeStyle™ MAX mix was incubated for 10 min at RT to allow complexes to form. The complexes were added to 200 mL of medium containing cells. Culture media were collected on day 7 after transfection; supernatants were collected and frozen at -80°C. The concentration of rec-eelLH was analyzed using an enzyme-linked immunosorbent assay (ELISA), previously developed in our laboratory [30].

ELISA analysis of rec-eelLH protein

Rec-eelLH was quantified using a sandwich ELISA performed in plates coated with the monoclonal antibody, 5A11, as described previously [30]. A volume of 100µL of rec-eelLH sample was added to the wells and then incubated for 1h at room temperature (RT). After washing three times with PBS-T, HRP-conjugated anti-eel11A8 antibody in PBS was added and plates were incubated for 1h at RT. After washing, the wells were incubated with 100µL of substrate solution for 20 min at RT. The reaction was stopped by adding stop solution (50µL of 1M H_2SO_4). Absorbance at 450nm was measured in each well using a microplate reader (Cytation 3).

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 assay kits (Cisbio Bioassays, Codolet, France), as described previously [30]. Briefly cells transfected with eelLHR-WT and eelLHR mutants were added at 10,000 cells per well into a 384-well plate 48h after transfection. Cells were stimulated by incubation with the agonist for 30 min at room temperature. cAMP was detected by measuring the decrease in HTRF energy transfer (665nm/620nm) 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 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) ×100/ratio negative]. The cAMP concentrations for Delta F% values were calculated using Prism software (GraphPad, Inc., La Jolla, CA, USA).

Data analysis

The MultAlin interface-multiple sequence alignment software was used for sequence results. 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.

Acknowledgments

The authors thank Dr. HW Seong (Institute of animal Science) for his helpful discussions.

Authors' contributions

KSM and MHK designed the study. MB and DAK were responsible for data and collection. DJK and SMH were interpreted the results and revised it critically for important intellectual content. All authors reviewed and approved the final manuscript.

Funding

This work was supported by a grant from the Korean Research Foundation Program of Korea (2018R1A2B6007794).

Availability of data and materials

The data used and analyzed in the current study are available from the corresponding author on reasonable request.

Ethics approval

The study protocol was approved by the Ethics Committee of the Hankyong National University.

Consent for publication

Not applicable

Competing interests

Not applicable

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Figures Legends

Fig. 1 Total cAMP levels stimulated by rec-eelLH inCHO-K1 cells transfected with constitutively active eelLHRmutants. CHO-K1 cells transiently transfected with the eelLHR-WT and mutant eelLHRs (M410T,L469R, and D590Y) were stimulated with rec-eelLH (0–1,000 ng/mL) for 30 min. cAMP production was detected using a homogenous time-resolved fluorescence (HTRF) assay. cAMP levels stimulated by rec-eelLH are shown as Delta F%. cAMP concentration was calculated using GraphPadPrism software.

Fig. 2 Rec-eelLH-stimulated cAMP productionin CHO-K1 cells transfected with the 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 30min. Total cAMP levels were 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.

Fig. 3 Schematic representation of the eelLHR structure. The location of thethree constitutively activating mutations (M410T, L469R, and D590Y) and the two inactivating mutations (D383N and Y546F) are indicated. Amino acid sequences at the mutated sites in the transmembrane domains of the eelLHR are shown. The 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 the eelLHR. Red circles indicate constitutively activating mutations and the blue circles indicate inactivating

614 mutations.EC, extracellular domain; TM, transmembrane domain; IC,
615 intracellular domain.

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