FSH receptor-specific residues L⁵⁰¹ and I⁵⁰⁵ in extracellular loop 2 are essential for its function

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Abstract

The extracellular loop 2 (EL2) of FSH receptor (FSHR) plays a pivotal role in various events downstream of FSH stimulation. Because swapping the six FSHR-specific residues in EL2 (chimeric EL2M) with those from LH/choriogonadotropin receptor resulted in impaired internalization of FSH–FSHR complex and low FSH-induced cAMP production, six substitution mutants of EL2 were generated to ascertain the contribution of individual amino acids to the effects shown by chimeric EL2M. Results revealed that L⁵⁰¹F mainly and I⁵⁰⁵V to a lesser extent contribute to the diminished receptor function in chimeric EL2M. HEK293 cells stably expressing WT and chimeric EL2M FSHR were generated to track the fate of the receptors post FSH induction. The chimeric EL2M FSHR stable clone showed weak internalization and cAMP response similar to transiently transfected cells. Furthermore, reduced FSH-induced ERK phosphorylation was also observed. The interaction of activated chimeric EL2M and L⁵⁰¹F FSHR with β -arrestins was weak compared with WT FSHR, thus explaining the impaired internalization of chimeric EL2M and corroborating the indispensable role of EL2 in receptor function.

Key Words

- ▶ extracellular loop 2
- ▶ FSH receptor
- ▶ internalization
- ▶ signaling

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Introduction

The interaction of follicle-stimulating hormone (FSH) with its cognate receptor is important for folliculogenesis (Robker & Richards 1998) and spermatogenesis (Foulkes *et al.* 1993). FSH receptor (FSHR) is a class A rhodopsin-type G-protein coupled receptor (GPCR). Along with luteinizing hormone/choriogonadotropin receptor (LH/CGR) and thyroid-stimulating hormone receptor, FSHR belongs to the subfamily of glycoprotein hormone receptors (GPHRs; Kleinau & Krause 2009). It is present on the granulosa cells of the ovary in females, and on the Sertoli cells of the testis in males. Structurally, it contains a large extracellular domain (ECD) consisting of leucine-rich repeats at the

N-terminal end and a hinge region at the C-terminal that connects the ECD to the membrane spanning transmembrane domain (TMD). The TMD comprises of seven α -helices which are connected to each other by means of three extracellular loops (ELs) and three intracellular loops (ILs) and ends in a short cytoplasmic tail. Though the TMD shows high sequence homology across all GPHRs, there is variability in the sequence of the extracellular N-terminal domain thus conferring hormone-binding specificity to each receptor (Braun *et al.* 1991). The ELs and ILs also vary in length and sequence, indicating their probable role in functional specificity. However, within a given GPHR, the

loop sequences are conserved across all species, indicating a common function.

Binding of FSH to FSHR triggers multiple downstream signaling pathways, but the exact molecular mechanism remains elusive. The recently solved crystal structure of FSH in complex with the entire ECD of FSHR including the hinge region provides some explanation. FSH interacts with FSHR in a two-step process: by binding to a high-affinity site in the ECD followed by the insertion of a sulfated Tyr residue of FSHR into FSH, eventually leading to receptor activation (Jiang *et al.* 2012). This process may involve the contact of the FSH–FSHR complex with the ELs being present at the interface of the ECD and the TMD. Although the interaction of the ILs with G-proteins (Timossi *et al.* 2002), and adaptor proteins such as APPL1 and 14-3-3 τ (Nechamen *et al.* 2004, Dias *et al.* 2010) has been demonstrated, the role of the ELs is not clearly defined. However, results from various studies employing site-directed mutagenesis and of naturally occurring mutations (Touraine *et al.* 1999, Desai *et al.* 2015) indicate that mutations in the ELs of FSHR affect diverse receptor functions such as FSH binding and cAMP production (Ji & Ji 1995), cell surface trafficking (Meduri *et al.* 2003), FSH-mediated desensitization and internalization (Casas-González *et al.* 2012), and the FSH-mediated PI3K/AKT pathway (Uchida *et al.* 2013). Interestingly, alanine-scanning mutagenesis of residues in the EL3 of FSHR and LH/CGR (Ryu *et al.* 1996, Sohn *et al.* 2002) revealed different residues in FSHR and LH/CGR to be crucial for hormone-binding and cAMP response, thus indicating that EL residues play specific functional roles in each receptor.

In studies using a combination of X-ray crystallography, mutagenesis, and a computational modeling approach, EL2 has been shown to play an important role in other GPCRs with small molecular ligands. EL2 residues project into the crevice within the transmembrane bundle and form a part of the ligand-binding surface for retinal in rhodopsin (Palczewski *et al.* 2000) and in dopamine D2 receptor (Shi & Javitch 2004) and are important in ligand-binding and receptor activation in glucagon-like peptide-1 receptor (Koole *et al.* 2012), and calcitonin gene-related peptide receptor (Woolley *et al.* 2013) to name a few.

Previously our group has shown the ELs of FSHR to be surface-accessible and potential secondary hormone-binding sites (Dupakuntla & Mahale 2010). Also, the role of FSHR-specific residues (nonconserved) in the ELs in its function was shown by generating chimeras where the ELs of FSHR were substituted with the respective ELs of LH/CGR. The cell surface FSHR expression and FSH-binding affinity of chimeric EL2M, where the six

FSHR-specific residues of the EL2 were substituted with those from LH/CGR, were found to be similar to those of WT FSHR. However, determination of surface-bound and internalized hormone–receptor complex revealed that the mutant receptor showed impaired internalization. Also, the FSH-induced cAMP response of the mutant receptor was found to be low. Thus, EL2 plays an indispensable role in FSH-induced signaling and internalization of the FSH–FSHR complex (Dupakuntla *et al.* 2012).

The present study was carried out with the aim of determining the contribution of individual amino acids in EL2 to hormone-binding and signaling events and of further characterizing the effects of EL2 mutation on the fate of the activated receptor. To achieve this, single substitution mutants of EL2 of FSHR were generated where each of its specific residues was replaced with the corresponding residues of LH/CGR. Internalization of hormone–receptor complexes as well as FSH-mediated signaling events were studied and the point mutants L⁵⁰¹F and I⁵⁰⁵V were identified as being important for FSHR function. Simultaneously, stable clones of HEK293 cells expressing either WT FSHR or chimeric EL2M FSHR were generated to confirm the observations obtained using transient transfections. The clones were used to monitor FSH-induced receptor internalization by confocal microscopy and FSH-induced ERK phosphorylation. Furthermore, the interaction of activated WT FSHR, chimeric EL2M, and the point mutants L⁵⁰¹F and I⁵⁰⁵V with β -arrestins was assessed. Evaluating the effects of the single-amino-acid substitutions on hormone binding and signaling helped to identify the key residues in EL2, providing further evidence to the pivotal role of EL2 in FSH–FSHR interactions.

Materials and methods

Generation of mutant FSHR constructs

Six substitution point mutants of EL2 (S⁴⁹³N, L⁵⁰¹F, I⁵⁰⁵V, D⁵⁰⁶E, S⁵⁰⁷T, and P⁵⁰⁸T) were generated using the human FSHR (hFSHR) cloned into pSG5 vector as the template for mutagenesis (Dupakuntla *et al.* 2012) using the QuikChange Site-directed Mutagenesis Kit (Agilent Technologies, La Jolla, CA, USA). Full-length DNA sequencing was carried out to confirm the desired mutation and to ensure that there were no other deleterious mutations. Plasmid DNA extraction of mutant constructs for transfection was then carried out using a midi-prep kit (Sigma). The sequences of WT and EL2 mutant constructs are listed in Table 1.

Table 1 Sequences of WT EL2 and chimeric EL2M of FSHR. Residues shown in bold in the WT EL2 sequence are unique to FSHR. Residues shown in bold and underlined are from the corresponding position in the LH/CGR sequence

Construct	Sequence
WT EL2	⁴⁹² S S Y M K V S I C L P M D I D S P L S Q ⁵¹¹
Chimeric EL2M	⁴⁹² S <u>N Y M K V S I C F P M D V E T T L S</u> Q ⁵¹¹

EL2, extracellular loop 2; FSHR, follicle-stimulating hormone receptor; LH/CGR, luteinizing hormone/choriogonadotropin receptor; chimeric EL2M, chimeric FSHR LH/CGR EL2 mutant.

Transient transfection in HEK293 cells

HEK293 cells obtained from the Cell Repository at the National Centre for Cell Science, Pune, India, were maintained in DMEM-F12 (Gibco; Life Technologies) supplemented with 5% fetal bovine serum (Gibco) and antibiotics. Transient transfection of WT FSHR and substitution point mutants of EL2 were performed using Lipofectamine and Plus reagents (Invitrogen), and all assays were performed 48 h post transfection. Untransfected cells served as a negative control.

Stable transfection in HEK293 cells

Stable clones of WT and chimeric EL2M FSHR were generated by co-transfection of pSG5 vector containing the WT or EL2M construct (2 µg) with empty vector pcDNA 3.1+ (Life Technologies) carrying the neomycin-resistance gene (200 ng) into HEK293 cells using Lipofectamine and Plus reagents. HEK293 cells transfected with 200 ng empty pcDNA 3.1+ vector alone were also generated, which served as a negative control. The cells were maintained in DMEM-F12 medium supplemented with 750 µg/ml G418 for 14 days and the clones obtained were screened using western blotting, immunofluorescence, and radioreceptor assays for identification of positive clones. WT clone 19 (WT-19), EL2M clone J (EL2M-J), and empty vector clone a (pcDNA-a) were chosen for further studies. All assays were performed 24 h post seeding of cells.

Western blotting analysis for detection of FSHR expression and FSH-induced ERK phosphorylation

Western blotting analysis was performed to monitor total FSHR expression in transiently transfected cells and in the stable clones by the method described previously (Dupakuntla *et al.* 2012). Densitometric analysis of the FSHR and

β-actin signals of three independent experiments was carried out using ImageQuantTL Software from GE Healthcare (Little Chalfont, Buckinghamshire, UK). The band intensity for FSHR was normalized to β-actin for each construct.

To determine the FSH-induced ERK phosphorylation, HEK293 stable clones WT-19, EL2M-J, and pcDNA-a were seeded into six-well plates. Twenty-four hours post seeding, cells were serum starved for 6 h in serum-free DMEM and then incubated at 37 °C with different doses of hFSH (0, 25, 50, 100, and 200 ng) and at different times points (0, 5, 15, 30, and 60 min). The cells were then lysed with lysis buffer (150 mM NaCl; 50 mM Tris, pH 8.0; 5 mM EDTA; 1% Nonidet P-40) with 2 mM sodium orthovanadate (Sigma-Aldrich Corp.) and protease inhibitor cocktail. Equal amounts of protein were loaded onto 10% SDS-PAGE gels and proteins were transferred to nitrocellulose membrane (GE Healthcare) using a semi-dry transfer apparatus (Bio-Rad Laboratories). Detection of phospho-ERK was done using rabbit polyclonal anti-phospho-ERK1/2 antibody from Cell Signaling Technology, Inc. (Danvers, MA, USA). The same blot was stripped and reprobed with rabbit polyclonal anti-ERK2 antibody from Santa Cruz Biotech, Inc. to normalize levels of phosphorylated ERK1/2 with the total ERK2 levels in each lane.

FSH-induced ERK1/2 phosphorylation was also determined for the point mutants L⁵⁰¹F and I⁵⁰⁵V after 5 and 30 min of FSH stimulation. Densitometric analysis was performed using ImageQuantTL Software from GE Healthcare.

Immunofluorescence

Cell-surface FSHR expression was determined using a direct immunofluorescence method on transiently transfected HEK293 cells seeded on cover slips in a six-well plate. The FSHR antibody, MAb 106.105, was labeled with Alexa Fluor 488 or Alexa Fluor 568 using a MAb labeling kit (Molecular Probes, Life Technologies). The cells were fixed using 4% paraformaldehyde for 10 min at RT and processed as described in Dupakuntla *et al.* (2012). The images were collected using an oil immersion 63× objective with NA 1.4 on a LSM510-Meta confocal system (Carl Zeiss, Jena, Germany). Untransfected cells served as the negative control. Fluorescence intensity of the positive cells showing cell-surface FSHR expression was quantified using Image J Software (National Institutes of Health, Bethesda, MD, USA).

In another set of experiments, cell-surface expression of FSHR before and after FSH stimulation was monitored in HEK293 cells stably expressing WT FSHR or chimeric

EL2M FSHR. Twenty-four hours after seeding, cells were stimulated with (100 ng) or without FSH for 60 min at 37 °C and processed for immunofluorescence.

Flow cytometry

Cell-surface FSHR expression was measured using a FACS Scan Flow Cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) as described previously (Dupakuntla *et al.* 2012).

Ligand-binding assay

Assessment of binding of ^{125} I-FSH to FSHR was carried out in 24-well plates in the absence or presence of unlabeled FSH (1 µg/well) to determine non-specific binding (Dupakuntla *et al.* 2012). The radioiodination of hFSH (procured from the National Hormone and Pituitary Programme, National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), Bethesda, MD, USA) was performed by the Iodogen method (Fraker & Speck 1978).

The surface-bound and internalized counts were measured on an automatic γ -counter (Wallac 1470, WIZARD, Turku, Finland).

cAMP assay

cAMP production was estimated using a commercially available enzyme immunoassay kit (Cayman Chemical Company, Ann Arbor, MI, USA) with unstimulated cells (basal response), and after stimulation of the cells with 100 ng hFSH for 1 h at 37 °C as described previously (Dupakuntla *et al.* 2012).

Coimmunoprecipitation of activated FSHR with endogenous β -arrestins

Recruitment of endogenous β -arrestins by activated WT, chimeric EL2M, L⁵⁰¹F, and I⁵⁰⁵V FSHR was determined by the method described by Luttrell *et al.* (2001) with some modifications. HEK293 cells in 10 cm-dishes (2×10^6 cells/well) were transiently transfected with 10 µg of each receptor construct using FuGENE HD Transfection Reagent (Roche). Untransfected cells served as a negative control. Forty-eight hours post transfection, cells were serum starved for 4 h in serum-free DMEM followed by stimulation with 100 ng/ml of FSH in 4 ml of serum-free DMEM for 5 min at 37 °C. Crosslinking was done with freshly prepared sulfo-dithio-bis[succinimidyl propionate] (DSP, Pierce, Rockford, IL, USA) for 30 min at RT with gentle agitation followed by quenching by adding

Tris-HCl (pH 7.3) to a 20 mM final concentration for 15 min at RT with gentle agitation. The cells were rinsed with PBS/10 mM HEPES and lysed in freshly prepared lysis buffer containing 1% Igepal, 0.4% deoxycholate, 10 mM Tris pH 7.5, 6.6 mM EDTA, 140 mM NaCl, protease and phosphatase inhibitors. The lysate supernatant was pre-cleared, centrifuged, and collected in fresh tubes to which anti- β -arrestin antibody (beta-Arrestin 1/2 (D24H9) Rabbit MAb, Cell Signaling Technology, Inc.) was added. The beads were treated with SDS sample buffer before subjecting the samples to 7.5% SDS-PAGE followed by transfer onto PVDF membrane (Immobilon-P, Millipore, Bedford, MA, USA) using a semi-dry transfer apparatus. Detection of FSHR and β -arrestin 1/2 was done with FSHR MAb 106.105 and β -arrestin 1/2 MAb respectively.

Building mutant model structures

The modeled structure of hFSHR TMD (residues 343–625) was based on the bovine rhodopsin structure (PDB ID:1U19) and used to build the EL2M chimera as described

Figure 1

FSHR expression in WT, chimeric EL2M and EL2 substitution point mutants by western blotting. (A) The lysates from HEK293 cells transiently transfected with WT FSHR, EL2 mutants, and from untransfected cells were subjected to 7.5% SDS-PAGE followed by western blotting analysis for detection of FSHR (upper panel) and β -actin (lower panel) which served as a loading control. Three independent experiments were carried out and the image shown is a representative one. (B) The ratio of the intensity of FSHR to β -actin has been plotted. The relative intensity of FSHR was normalized to β -actin for each construct. No significant differences were observed.

previously (Dupakuntla *et al.* 2012). With the WT modeled structure of hFSHR, six point-mutant model structures of EL2 were built using Discovery Studio 3.5 (Accelrys Software, Inc., San Diego, CA, USA). These point-mutant models were minimized using Schrodinger 2013 OPLS 2005 forcefield with default parameters.

Statistical analysis

The results are expressed as mean $\pm$ s.e.m. of three independent experiments performed in duplicates. Data analysis was carried out using the unpaired Student's *t*-test of the Software 'GraphPad Prism 5.0' (GraphPad Software, Inc., San Diego, CA, USA). The values of $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ with respect to WT FSHR was considered to be statistically significant.

Results

Generation of substitution point mutants of EL2 of FSHR

In order to identify the amino acids in EL2 of FSHR that contribute to the effects observed with respect to FSH-induced signaling and internalization, individual substitution-mutant constructs were generated by replacing each of the FSHR-specific residues in EL2 with the corresponding LH/CGR residues (S⁴⁹²N, L⁵⁰¹F, I⁵⁰⁵V,

D⁵⁰⁶E, S⁵⁰⁷T, and P⁵⁰⁸T). The identity of each construct generated was confirmed by DNA sequencing.

Receptor expression following transient transfection

The six substitution point mutants of EL2 were observed to show similar FSHR expression to the chimeric EL2M receptor and also to the WT receptor (Fig. 1A) as determined by western blotting. Densitometric analysis of the FSHR signal of WT and mutant constructs normalized to β -actin revealed no significant differences (Fig. 1B). Cell-surface FSHR expression was monitored as it is essential for hormone-binding ability. EL2 point mutants showed FSHR expression similar to that observed with WT and chimeric EL2M receptors by confocal microscopy (Fig. 2A and B) and flow cytometry (Fig. 2C). Thus the receptor expression was not affected by substituting these residues.

Effect of point mutation on FSH binding and internalization

All the six point mutants generated were tested for FSH-binding ability by radioreceptor assay. Surface-bound and internalized counts of ¹²⁵I-FSH for each of the point mutants of EL2 are shown in Fig. 3A. A decreased internalization index observed in the case of the chimeric EL2M receptor was found to be mainly due to mutation of L⁵⁰¹ and to some extent by mutation of the residues S⁴⁹³ and I⁵⁰⁵ (Fig. 3B).

Figure 2

Cell-surface FSHR expression in WT, chimeric EL2M and EL2 substitution point mutants determined by immunofluorescence and flow cytometry. (A) Cell-surface FSHR expression (green) of WT and EL2 substitution point mutants of FSHR determined by a direct immunofluorescence method using FSHR antibody conjugated to Alexa 488 as a probe. DAPI (blue) was used as a nuclear stain. Untransfected cells served as a negative control. Images shown

are representative ones and show FSHR MAb fluorescence merged with DAPI (scale bar = 20 μ m). (B) Fluorescence intensity of cell-surface FSHR expression of WT FSHR and EL2 substitution mutants. No significant differences were observed. (C) Flow cytometry was carried out and the mean fluorescence intensity is plotted. No significant differences were observed. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-14-0275>.

Figure 3

Assessment of binding of FSH to WT FSHR, chimeric EL2M and EL2 substitution point mutants by radioreceptor assay. (A) Specific binding of ^{125}I -FSH to FSHR. Forty-eight hours post transfection of HEK293 cells with WT and mutant receptor constructs, cells were incubated with labeled FSH in the presence of saturating concentration of unlabeled FSH to determine surface-bound and internalized counts. Untransfected cells served as a negative control.

(B) Internalization index: the ratio of internalized counts to surface-bound counts of the ^{125}I -FSH-FSHR complex. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ with respect to WT FSHR.

FSH-induced signaling

FSH-induced signaling was monitored by estimating cAMP levels post FSH stimulation in HEK293 cells transfected with WT or mutant constructs of EL2. L⁵⁰¹F showed significantly decreased cAMP levels (** $P < 0.01$ with respect to WT FSHR) similar to the chimeric EL2M receptor. The I⁵⁰⁵V receptor also showed a slight decrease (* $P < 0.05$ with respect to WT FSHR) in the cAMP levels (Fig. 4).

Characterization of HEK293 cells stably expressing WT or chimeric EL2M FSHR

Stable cell lines expressing WT FSHR or chimeric EL2M FSHR were made in order to study the fate of the activated receptor in the events following FSH stimulation. Western blotting (Fig. 5A) showed FSHR expression in HEK293 cells stably co-transfected with either WT FSHR or EL2M FSHR

construct along with empty vector pcDNA 3.1+. The cells stably transfected with empty vector pcDNA 3.1+ alone served as a negative control. WT-19 and EL2M-J clones displayed cell-surface FSHR expression and were chosen for further studies. No FSHR expression was seen in the empty vector clone pcDNA-a. Although the hormone-binding ability of WT and chimeric EL2M FSHR was similar (Fig. 5B, surface bound counts), chimeric EL2M showed a low internalization index (Fig. 5C) indicating low internalization of the FSH-FSHR complex because of the mutation. FSH-induced signaling in terms of cAMP production was also reduced in the chimeric EL2M receptor (Fig. 5D). Thus, the results obtained with the stable clones confirm that the mutation of the FSHR-specific residues in EL2 impairs internalization and cAMP production as reported previously for transiently transfected cells (Dupakuntla *et al.* 2012).

Impairment in internalization of chimeric EL2M FSHR as monitored by confocal microscopy

Receptor density on the cell surface of unstimulated cells and post FSH stimulation was determined for stable clones as a measure of internalization of FSHR. In the case of WT FSHR (WT-19), after 60 min of FSH stimulation, almost complete disappearance of cell surface FSHR was observed corresponding to the internalization of most of the hormone-receptor complex (Fig. 6B). However, in the case of chimeric EL2M FSHR (EL2M-J), receptor density on the cell surface was much higher (Fig. 6D), supporting the observation that mutation of receptor-specific residues in EL2 impairs receptor internalization post ligand stimulation as determined using a radioreceptor assay.

Figure 4

cAMP production in response to FSH stimulation in WT FSHR, chimeric EL2M and EL2 substitution point mutants. Forty-eight hours post transfection of HEK293 cells with WT and mutant receptor constructs, cells were stimulated with 100 ng FSH for 1 h at 37 °C. The cell lysates were prepared thereafter and cAMP levels were estimated using a competitive enzyme immunoassay method. * $P < 0.05$, ** $P < 0.01$ with respect to WT FSHR.

Figure 5

Characterization of FSHR expression, FSH binding, and cAMP signaling ability of HEK293 cells stably expressing WT FSHR (WT-19) and chimeric EL2M FSHR (EL2M-J) as determined by western blotting, radioreceptor assay, and cAMP assay respectively. (A) Cell lysates from HEK293 cells transiently expressing WT FSHR (positive control), the stable clones WT-19, and EL2M-J, and cells stably transfected with empty vector clone pcDNA-a (negative control) were subjected to 7.5% SDS-PAGE followed by western blotting to detect FSHR (upper panel) and β-actin (lower panel) which

served as a loading control. (B) Specific binding of ¹²⁵I-FSH to FSHR. Surface bound and internalized counts in HEK293 cells stably expressing the different constructs were measured as described in materials and methods. The cells transfected with empty vector (pcDNA-a) served as a negative control. (C) Internalization index: ratio of internalized counts to surface bound counts. (D) cAMP production in HEK293 cells stably transfected with different constructs. The cells transfected with pcDNA-a served as a negative control. ** $P < 0.01$, *** $P < 0.001$ with respect to WT FSHR.

FSH-induced ERK phosphorylation is impaired in chimeric EL2M receptor

As EL2 mutation affects the cAMP/PKA pathway, its effect on ERK phosphorylation was studied. Total ERK and phosphorylated ERK in the stable clones in response to increasing concentrations of FSH (0–200 ng) were monitored 5 min post stimulation. The extent of ERK phosphorylation was estimated by calculating the ratio of phosphorylated ERK 1/2 to total ERK2 by densitometric analysis of the respective signals. The percentage response for each dose of FSH was calculated by comparing the value obtained for that dose with the maximum response obtained by the WT FSHR (considered as 100%).

With an increase in dose, an increase in ERK phosphorylation was observed in cells expressing WT FSHR. Maximum response was observed with the 100 ng FSH dose after which saturation was reached. In case of chimeric EL2M FSHR, the percentage response calculated was significantly lower as compared with WT FSHR (Fig. 7A).

In another set of experiments, the effect of 100 ng of FSH on ERK phosphorylation at different time points (0–60 min) was estimated. Peak ERK phosphorylation was observed in WT FSHR cells at 5 min post stimulation after which it gradually decreased with increasing time. In chimeric EL2M FSHR, a significantly weaker response was observed at all time points (Fig. 7B).

Because the FSH-induced cAMP production was low in the point mutants L⁵⁰¹ and I⁵⁰⁵, these two mutants were evaluated for their effect on FSH-induced ERK phosphorylation. Both the mutants showed reduced ERK

phosphorylation similar to that observed in the EL2M chimera as compared with WT FSHR (Fig. 8).

Effect of EL2 mutation on β-arrestin recruitment

As chimeric EL2M FSHR and L⁵⁰¹F FSHR show impairment of internalization of the FSH–FSHR complex, the ability of WT and mutant receptors to interact with endogenous β-arrestins, adaptor proteins for agonist-mediated internalization of GPCRs, was investigated by co-immunoprecipitation. As compared with WT FSHR, the interaction of chimeric EL2M and L⁵⁰¹F FSHR with β-arrestins in response to FSH stimulation was found to be very weak, whereas that of I⁵⁰⁵V FSHR was similar to that of the WT (Fig. 9). Thus, mutation of EL2 hampers β-arrestin recruitment to the activated receptors and hence their internalization.

Comparison of molecular models of FSHR mutants

In order to understand the involvement of FSHR-specific residues of the EL2 region in the interaction with neighboring residues, homology models for six of the mutants were generated and different types of interactions (such as hydrophobic, hydrogen bonding, ionic, cation–pi, aromatic–sulfur and aromatic–aromatic interactions) were studied and compared with the ones observed in the WT and chimeric EL2M FSHR. Computer models of the WT, chimeric EL2M, L⁵⁰¹F, and I⁵⁰⁵V are shown in Fig. 10. Though the loop structure was altered in the case

Figure 6

FSHR expression in HEK293 cells stably expressing WT-19 or chimeric EL2M-J constructs at 0 min (A and C) and after incubation with FSH for 60 min (B and D) as monitored by confocal microscopy. FSHR MAb conjugated to Alexa Fluor 568 (pink) was used for detection of the FSHR signal.

DAPI (blue) was used as a nuclear stain. Images shown are representative ones and show FSHR MAb fluorescence merged with DAPI (scale bar = 20 μ m). A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-14-0275>.

of chimeric EL2M as compared with WT, similar changes were not observed in the L⁵⁰¹F and I⁵⁰⁵V mutants. However, these substitutions resulted in gain of interactions with neighboring residues from EL1 and EL3

as seen in chimeric EL2M but not in WT. This indicates that these acquired interactions are important factors in the changes observed in point mutants and chimeric EL2M model structures as compared with the WT.

Figure 7

Detection of phosphorylated ERK (P-ERK) in HEK293 cells stably expressing WT-19 and chimeric EL2M-J FSHR in response to FSH stimulation. The lysates from (A) cells stimulated with different doses of FSH (0–200 ng) for 5 min and (B) cells stimulated with 100 ng of FSH at different time points (0–60 min) were subjected to 10% SDS–PAGE. The upper blots were probed with anti-P-ERK (1/2) antibody and the lower panels show the same blots that were reprobed with anti-total ERK2 antibody (from a representative

experiment). (A and B lower panels) Densitometric analysis was carried out to calculate the ratio of phosphorylated ERK (1/2) to total ERK2. The maximum response obtained with WT FSHR (WT-19) was considered to be 100%, and the percentage responses obtained at different concentrations of FSH or at different time points were determined by comparing them with the maximum response. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ with respect to WT FSHR.

Figure 8

Effect of L⁵⁰¹F and I⁵⁰⁵V mutations of EL2 on FSH-induced ERK phosphorylation. (A) Representative blot showing ERK phosphorylation in response to 100 ng FSH for 5 or 30 min was determined using HEK293 cells transiently transfected with WT, chimeric EL2M, and the point mutants L⁵⁰¹F and I⁵⁰⁵V. Basal levels of ERK phosphorylation were also determined (0 min). Untransfected cells served as negative controls. The cell lysates were subjected to western blotting and blots were probed for phospho ERK1/2. The same blot was reprobed for total ERK2 protein levels for normalization. (B) Densitometric analysis was carried out to calculate the ratio of phosphorylated ERK (1/2): total ERK2. The maximum response obtained with the WT FSHR at 5 min post stimulation with 100 ng FSH was considered to be 100% and the percentage response obtained for the mutants at 0, 5, and 30 min post FSH induction (100 ng) was determined by comparing it with the maximum response. *P < 0.05, ***P < 0.001 with respect to WT FSHR.

Discussion

We have previously reported that swapping the six FSHR-specific residues of EL2 with the corresponding residues from LH/CGR affected the receptor function.

Chimeric EL2M receptor exhibited lower cAMP production and lower internalization of the FSH–FSHR complex as compared with WT FSHR (Dupakuntla *et al.* 2012). In this study, six substitution mutants of EL2 of FSHR were generated to identify the receptor-specific residues that play a key role in internalization and ligand-induced signaling events. It was seen that the behavior of the L⁵⁰¹F substitution resembled the behavior of chimeric EL2M mutant, i.e. this substitution resulted in low internalization and low cAMP production as seen in EL2M mutant. The I⁵⁰⁵V substitution also contributed to these effects to some extent. The importance of these residues is also corroborated by the fact that these residues are conserved across FSHR of all species.

Also, HEK293 clones stably expressing either WT or chimeric EL2M FSHR were generated to overcome the limitations of low transient transfection efficiency. We observed that the amount of cell-surface-bound FSH–FSHR complex in the chimeric EL2M receptor (clone EL2M-J) was similar to that of the WT (clone WT-19) according to a radioreceptor assay. However, the amount of internalized

Figure 9

Evaluation of the ability of activated WT FSHR, chimeric EL2M FSHR, L⁵⁰¹F FSHR, and I⁵⁰⁵V FSHR to recruit endogenous β -arrestins by co-immunoprecipitation. HEK293 cells transiently expressing WT FSHR, chimeric EL2M FSHR, L⁵⁰¹F FSHR, and I⁵⁰⁵V FSHR were stimulated with FSH for 5 min at 37 °C followed by *in vivo* DSP crosslinking and immunoprecipitation with anti- β -arrestin antibody. Immunocomplexes were separated by 7.5% SDS-PAGE and western blotting was performed to reveal FSHR and β -arrestins (input lanes) and also the amount of activated WT and mutant receptors coimmunoprecipitated with β -arrestins (IP lanes). Untransfected cells served as negative controls. (A) HEK293 cells transiently expressing WT FSHR, chimeric EL2M FSHR, L⁵⁰¹F FSHR, and I⁵⁰⁵V FSHR show similar FSHR expression, whereas untransfected cells do not show a band in the input lanes (20% input). Equal amounts of β -arrestin were detected in all the input lanes. (B) Activated WT and I⁵⁰⁵V FSHR coimmunoprecipitated with β -arrestins show a prominent band, whereas chimeric EL2M FSHR and L⁵⁰¹F FSHR show a faint band. No band is seen in untransfected cells, confirming the specificity of the interaction.

Figure 10

Comparison of models of TMD and ELs of WT FSHR with chimeric EL2M, L⁵⁰¹F, and I⁵⁰⁵V. EL2 region in all the models is shown in yellow. The residues involved in interaction in all the four models are circled in cyan (Y⁴¹⁵, L⁵⁰¹/F⁵⁰¹, and M⁵⁰³). Residues showing similar interaction in chimeric EL2M and L⁵⁰¹F mutant are indicated by white square boxes (I⁴¹⁷ and I⁵⁰⁵/V⁵⁰⁵).

Residues showing similar interactions in chimeric EL2M and I⁵⁰⁵V are indicated by green square boxes (Y⁴⁹⁴ and V⁵⁸²). Hydrogen bonds and hydrophobic interactions are indicated by pink and green lines. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-14-0275>.

hormone–receptor complex was significantly reduced, as observed in transiently transfected cells, confirming our earlier observations. Next, visualization of the disappearance of cell-surface FSHR expression, post FSH stimulation, to monitor internalization of FSHR, was performed using confocal microscopy. In the case of chimeric EL2M FSHR, most of the mutant receptor was seen on the surface and only a small fraction internalized in contrast to the WT FSHR where most of the receptor internalizes, substantiating the same results obtained using a radioreceptor assay.

Also, EL2M-J showed low cAMP production post FSH stimulation. Another downstream effect of FSH stimulation is activation of the MAPK/ERK pathway by cAMP/PKA and β -arrestins (Kara *et al.* 2006). Cottom *et al.* (2003) have shown that FSH stimulation in granulosa cells results in phosphorylation of ERK by PKA, which in turn phosphorylates downstream effectors of MAPK pathway. As the cAMP production was low upon stimulation of chimeric EL2M receptor, we sought to determine the effect of EL2 mutation on PKA-mediated MAPK/ERK pathway as the PKA activity depends on cellular levels of cAMP. As one would expect, the ERK phosphorylation was affected in the

clone-expressing chimeric EL2M receptor, across different doses of FSH and over different time points as compared with WT FSHR. Also, the point mutants L⁵⁰¹F and I⁵⁰⁵V which showed low cAMP production also showed less ERK phosphorylation similar to chimeric EL2M FSHR as compared with WT FSHR. These findings indicate that the residues in EL2 are important in various FSH-mediated signaling pathways such as cAMP and MAPK.

Upon FSH stimulation, FSHR is phosphorylated on the ILs and C-tail by means of GRKs which recruit adaptor proteins called β -arrestins, which then direct the FSH–FSHR complex to clathrin-coated pits for endocytosis (Kara *et al.* 2006). Also, even though FSHR is a member of the class ‘A’ GPCRs, it has a conserved Ser/Thr cluster in its C-tail, which is a characteristic of class ‘B’ GPCRs that recruit both β -arrestins 1 and 2 for internalization (Kara *et al.* 2006). As the chimeric EL2M and L⁵⁰¹F receptors showed decreased internalization of the FSH–FSHR complex post ligand stimulation, we studied FSHR– β -arrestin interaction post FSH stimulation in HEK293 cells transiently expressing WT and EL2 mutant receptors. It was observed that the β -arrestin recruitment was affected in the case of chimeric EL2M and

L⁵⁰¹F FSHR receptor which explains their impairment in internalization. Casas-González *et al.* (2012) reported that in the case of the N⁴³¹I FSHR mutation in EL1 which resulted in impairment in receptor internalization, overexpression of β -arrestin 2 restored the internalization to levels similar to that of the WT FSHR. In this study, the authors envisaged that the EL1 mutation resulted in conformational changes in the receptor, which affected its ability to undergo phosphorylation and β -arrestin recruitment, which perhaps is the case for the EL2 mutation reported in this study too. Thus EL residues are possibly key players in hormone-mediated receptor internalization.

In our previous study, we compared the modeled structure of TMD with ELs for WT and chimeric EL2M. Significant changes in the EL2 structure were observed in the case of chimeric EL2M as compared with the WT (Dupakuntla *et al.* 2012). In addition, gain of interactions with reference to substituted amino acids (F⁵⁰¹ and V⁵⁰⁵) was observed. Experimental evidence obtained by Nishi *et al.* (2002) indicated that the ECD–EL2 interaction imposed a constraint on LHR activation which could be released only on ligand binding. On the basis of that study, Dupakuntla *et al.* (2012) speculated that substitutions in EL2 of FSHR might have retained the constraint on the receptor in spite of hormone binding and thus prevented receptor activation and downstream events to a large extent. Though the loop structure was retained, similar gain of interactions was observed even with the single-substitution mutants L⁵⁰¹F and I⁵⁰⁵V in this study. Findings from the ligand-binding assay discussed previously indicate that the decrease in internalization of the hormone–receptor complex observed in case of chimeric EL2M is mainly due to mutation of the residues L⁵⁰¹ and I⁵⁰⁵. Similarly, both these residues also had an effect on FSH-induced signaling. Findings from these experiments and the modeling studies indicate that FSHR-specific residues L⁵⁰¹ and I⁵⁰⁵ are crucial for receptor activity.

The physiological significance of mutations in EL2 of FSHR has been elucidated by *in vitro* studies on naturally occurring mutations e.g.: a Pro⁵¹⁹Thr mutation in a patient with delayed puberty and primary amenorrhea resulted in the mutant receptor being trapped intracellularly, and this caused a block in follicle maturation after the primary stage (Meduri *et al.* 2003). A M⁵¹²I mutation resulted in inactivation of the FSH-induced PI3K/AKT pathway, which is essential for the proliferation, differentiation, survival, and enhanced mRNA translation in granulosa cells (Uchida *et al.* 2013). Our group has recently reported a novel mutation Val⁵¹⁴Ala in a patient with iatrogenic ovarian hyperstimulation syndrome. The mutant receptor showed

higher cell-surface FSHR expression and higher cAMP production at lower doses of FSH (Desai *et al.* 2015). The study of the L⁵⁰¹F and I⁵⁰⁵V mutations is thus important as they affect internalization of the FSH–FSHR complex as well as cAMP signaling and ERK/MAPK pathways, which are essential in ovulation and luteinization events.

In summary, FSHR-specific residues L⁵⁰¹ and I⁵⁰⁵ in EL2 are important in internalization of the FSH–FSHR complex. Also, as reported for several other GPCRs, mutation in FSHR-specific residues of EL2 affected internalization which is most probably due to the weak β -arrestin recruitment and FSH-mediated downstream signaling pathways, thus establishing the importance of this loop in receptor function. Results of molecular modeling experiments also indicated that both L⁵⁰¹F and I⁵⁰⁵V substitutions contribute to the additional interactions observed in the EL2M chimera, which were absent in WT FSHR. Further experiments are needed to determine the mechanistic basis of these effects exerted by the EL2 and to identify the contribution of functionally significant epitopes in the ELs of FSHR to diverse FSH-mediated downstream events.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

- Braun T, Schofield PR & Sprengel R 1991 Amino-terminal leucine-rich repeats in gonadotropin receptors determine hormone selectivity. *EMBO Journal* **10** 1885–1890.
- Casas-González P, Scaglia HE, Pérez-Solís MA, Durand G, Scaglia J, Zariñán T, Dias JA, Reiter E & Ulloa-Aguirre A 2012 Normal testicular function without detectable follicle-stimulating hormone. A novel mutation in the follicle-stimulating hormone receptor gene leading to apparent

- constitutive activity and impaired agonist-induced desensitization and internalization. *Molecular and Cellular Endocrinology* **364** 71–82. (doi:10.1016/j.mce.2012.08.011)
- Cottom J, Salvador LM, Maizels ET, Reierstad S, Park Y, Carr DW, Davare MA, Hell JW, Palmer SS, Dent P *et al.* 2003 Follicle-stimulating hormone activates extracellular signal-regulated kinase but not extracellular signal-regulated kinase kinase through a 100-kDa phosphotyrosine phosphatase. *Journal of Biological Chemistry* **278** 7167–7179. (doi:10.1074/jbc.M203901200)
- Desai SS, Achrekar SK, Sahasrabudhe KA, Mehariji PK, Desai SK, Mangoli VS & Mahale SD 2015 Functional characterization of two naturally occurring mutations (Val⁵¹⁴Ala and Ala⁵⁷⁵Val) in follicle stimulating hormone receptor. *Journal of Clinical Endocrinology and Metabolism*. In Press. (doi:10.1210/jc.2014-3662)
- Dias JA, Mahale SD, Nechamen CA, Davydenko O, Thomas RM & Ulloa-Aguirre A 2010 Emerging roles for the FSH receptor adapter protein APPL1 and overlap of a putative 14-3-3 τ interaction domain with a canonical G-protein interaction site. *Molecular and Cellular Endocrinology* **329** 17–25. (doi:10.1016/j.mce.2010.05.009)
- Dupakuntla M & Mahale SD 2010 Accessibility of the extracellular loops of follicle stimulating hormone receptor and their role in hormone–receptor interaction. *Molecular and Cellular Endocrinology* **315** 131–137. (doi:10.1016/j.mce.2009.10.002)
- Dupakuntla M, Pathak B, Sur Roy B & Mahale SD 2012 Extracellular loop 2 in the FSH receptor is crucial for ligand mediated receptor activation. *Molecular and Cellular Endocrinology* **362** 60–68. (doi:10.1016/j.mce.2012.05.008)
- Foulkes NS, Schlötter F, Pévet P & Sassone-Corsi P 1993 Pituitary hormone FSH directs the CREM functional switch during spermatogenesis. *Nature* **362** 264–267. (doi:10.1038/362264a0)
- Fraker PJ & Speck JC Jr 1978 Protein and cell membrane iodinations with a sparingly soluble chloramide, 1,3,4,6-tetrachloro-3a, 6a-diphenylglycoluril. *Biochemical and Biophysical Research Communication* **80** 849–857. (doi:10.1016/0006-291X(78)91322-0)
- Ji I & Ji TH 1995 Differential roles of exolooop 1 of the human follicle-stimulating hormone receptor in hormone binding and receptor activation. *Journal of Biological Chemistry* **270** 15970–15973. (doi:10.1074/jbc.270.27.15970)
- Jiang X, Liu H, Chen X, Chen PH, Fischer D, Sriraman V, Yu HN, Arkinstall S & He X 2012 Structure of follicle-stimulating hormone in complex with the entire ectodomain of its receptor. *PNAS* **109** 12491–12496. (doi:10.1073/pnas.1206643109)
- Kara E, Crépiaux P, Gauthier C, Martinat N, Piketty V, Guillou F & Reiter E 2006 A phosphorylation cluster of five serine and threonine residues in the C-terminus of the follicle-stimulating hormone receptor is important for desensitization but not for β -arrestin-mediated ERK activation. *Molecular Endocrinology* **20** 3014–3026. (doi:10.1210/me.2006-0098)
- Kleinau G & Krause G 2009 Thyrotropin and homologous glycoprotein hormone receptors: structural and functional aspects of extracellular signaling mechanisms. *Endocrine Review* **30** 133–1351. (doi:10.1210/er.2008-0044)
- Koole C, Wootten D, Simms J, Miller LJ, Christopoulos A & Sexton PM 2012 Second extracellular loop of human glucagon-like peptide-1 receptor (GLP-1R) has a critical role in GLP-1 peptide binding and receptor activation. *Journal of Biological Chemistry* **287** 3642–3658. (doi:10.1074/jbc.M111.309328)
- Luttrell LM, Roudabush FL, Choy EW, Miller WE, Field ME, Pierce KL & Lefkowitz RJ 2001 Activation and targeting of extracellular signal-regulated kinases by β -arrestin scaffolds. *PNAS* **98** 2449–2454. (doi:10.1073/pnas.041604898)
- Meduri G, Touraine P, Beau I, Lahuna O, Desroches A, Vacher-Lavenu MC, Kuttann F & Misrahi M 2003 Delayed puberty and primary amenorrhea associated with a novel mutation of the human follicle-stimulating hormone receptor: clinical, histological, and molecular studies. *Journal of Clinical Endocrinology and Metabolism* **88** 3491–3498. (doi:10.1210/jc.2003-030217)
- Nechamen CA, Thomas RM, Cohen BD, Acevedo G, Poulidakos PI, Testa JR & Dias JA 2004 Human follicle-stimulating hormone (FSH) receptor interacts with the adaptor protein APPL1 in HEK 293 cells: potential involvement of the PI3K pathway in FSH signaling. *Biology of Reproduction* **71** 629–636. (doi:10.1095/biolreprod.103.025833)
- Nishi S, Nakabayashi K, Kobilka B & Hsueh AJ 2002 The ectodomain of the luteinizing hormone receptor interacts with exolooop 2 to constrain the transmembrane region. Studies using chimeric human and fly receptors. *Journal of Biological Chemistry* **277** 3958–3964. (doi:10.1074/jbc.M109617200)
- Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, Le Trong I, Teller DC, Okada T, Stenkamp RE *et al.* 2000 Crystal structure of rhodopsin: a G protein-coupled receptor. *Science* **289** 739–745. (doi:10.1126/science.289.5480.739)
- Robker RL & Richards JS 1998 Hormonal control of the cell cycle in ovarian cells: proliferation versus differentiation. *Biology of Reproduction* **59** 476–482. (doi:10.1095/biolreprod59.3.476)
- Ryu KS, Gilchrist RL, Ji I, Kim SJ & Ji TH 1996 Exolooop 3 of the luteinizing hormone/choriogonadotropin receptor. Lys583 is essential and irreplaceable for human choriogonadotropin (hCG)-dependent receptor activation but not for high affinity hCG binding. *Journal of Biological Chemistry* **271** 7301–7304. (doi:10.1074/jbc.271.13.7301)
- Shi L & Javitch JA 2004 The second extracellular loop of the dopamine D₂ receptor lines the binding-site crevice. *PNAS* **101** 440–445. (doi:10.1073/pnas.2237265100)
- Sohn J, Ryu K, Sievert G, Jeoung M, Ji I & Ji TH 2002 Follicle-stimulating hormone interacts with exolooop 3 of the receptor. *Journal of Biological Chemistry* **277** 50165–50175. (doi:10.1074/jbc.M207646200)
- Timossi C, Maldonado D, Vizcarro A, Lindau-Shepard B, Michael Conn P & Ulloa-Aguirre A 2002 Structural determinants in the second intracellular loop of the human follicle-stimulating hormone receptor are involved in G_s protein activation. *Molecular and Cellular Endocrinology* **189** 157–168. (doi:10.1016/S0303-7207(01)00720-1)
- Touraine P, Beau I, Gougeon A, Meduri G, Desroches A, Pichard C, Detoeuf M, Paniel B, Prieur M, Zorn JR *et al.* 1999 New natural inactivating mutations of the follicle-stimulating hormone receptor: correlations between receptor function and phenotype. *Molecular Endocrinology* **13** 1844–1854. (doi:10.1210/mend.13.11.0370)
- Uchida S, Uchida H, Maruyama T, Kajitani T, Oda H, Miyazaki K, Kagami M & Yoshimura Y 2013 Molecular analysis of a mutated FSH receptor detected in a patient with spontaneous ovarian hyperstimulation syndrome. *PLoS ONE* **8** e75478. (doi:10.1371/journal.pone.0075478)
- Woolley MJ, Watkins HA, Taddese B, Karakullukcu ZG, Barwell J, Smith KJ, Hay DL, Poyner DR, Reynolds CA & Conner AC 2013 The role of ECL2 in CGRP receptor activation: a combined modeling and experimental approach. *Journal of the Royal Society, Interface* **10** 20130589. (doi:10.1098/rsif.2013.0589)

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