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Follice-Stimulating Hormone Receptor Forms Oligomers and Shows Evidence of Carboxyl-Terminal Proteolytic Processing

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Abstract

FSH receptor (FSHR), a member of the G protein-coupled receptor superfamily, is present in the plasma membrane of ovarian granulosa cells and testicular Sertoli cells. FSH regulates normal ovarian follicle development and spermatogenesis through FSHR. The extracellular domain of FSHR is a weakly associated homodimer in the recently solved crystal structure of FSH in complex with the extracellular domain of FSHR. However, there is currently no biochemical data that demonstrate that FSHR exists as a dimer or higher-order oligomer in cell membranes. A fluorescence resonance energy transfer assay was used to determine whether full-length native FSHR is an oligomer. FSHR-specific monoclonal antibody or Fab fragments, labeled with two different fluorophores, allowed the study of nontagged receptor *in situ*. Unoccupied FSHR exhibited strong fluorescence resonance energy transfer profiles *in situ*. Complementary coimmunoprecipitation experiments of myc- or FLAG-tagged FSHR indicated that FSHR forms oligomers early in receptor biosynthesis. No effect of FSH treatment was observed. Thus, immature forms of FSHR, not yet fully processed, were observed to coimmuno-precipitate. An unexpected observation was made that the C-terminal epitope tags are removed from FSHR before arrival at the cell surface. These results provide the first evidence for oligomers of full-length FSHR *in situ* and for C-terminal proteolytic processing of FSHR and that both events take place during biosynthesis. This may explain how heterozygous mutations in the FSHR gene that affect receptor trafficking may be ameliorated by oligomer formation.

FSH IS REQUIRED for follicle development in the ovary and normal spermatogenesis in the testis (for review, see Ref. 1). The glycoprotein hormone receptors, which include FSH receptor (FSHR), LH receptor (LHR), and TSH (TSHR), are composed of a large extracellular domain (ECD) and a heptahelical transmembrane (TM) domain. Structural organization of FSHR at the cell membrane is not fully understood. Recently, a crystal structure of FSH in complex with the ECD of FSHR (FSHR_{ECD}) shows that FSHR_{ECD} forms weakly associating dimers with each molecule being occupied by one molecule of FSH (2). That observation is of interest because homo- or hetero-oligomerization of receptors has been shown to influence trafficking, ligand binding, and signaling (for review, see Refs. 3 and 4).

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In fact, oligomerization has been demonstrated among some of the glycoprotein hormone receptors but not FSHR, the only type 1A G protein-coupled receptor (GPCR) for which there is a crystal structure available. Binding of human chorionic gonadotropin (hCG) enhances the constitutive self association of LHR seen by coimmunoprecipitation (5). Clustering of LHR, which appears to require functional LHR (6), is induced by the binding of LH or hCG (7). In addition, LHR appears to move into lipid rafts (8, 9) and to form large complexes upon desensitization (10).

It should be noted that although the term cluster is often used in fluorescence resonance energy transfer (FRET) studies and the term oligomer is used in biochemical experiments, in this paper, the terms are used interchangeably to designate closely associated FSHR.

TSHR appears to form constitutive self-associated complexes. In contrast to what is observed with LHR, binding of TSH causes a dose-dependent decrease in oligomeric forms, as measured by FRET (11).

In the case of FSHR, the evidence is suggestive of oligomerization. Osuga *et al.* (12) constructed a chimera of the FSHR_{ECD} fused to the TM portion of LHR and an LHR mutant truncated at TM segment V. Neither construct alone induced cAMP in response to hCG, but coexpression restored partial signaling. That study suggested either that the TM I domain is the site of interaction in LHR oligomers or that they associated via the ECD as in the FSHR_{ECD} crystal structure. Recent biochemical gain-of-function experiments further substantiate that self-association can occur, and through a process coined transactivation, a binding-defective receptor can be activated by a binding-competent, signaling-defective receptor (13).

Alternate splicing of FSHR, in which six of the seven naturally occurring TM domains are removed, produces an FSHR species that is anchored in the membrane and binds FSH but does not signal through adenylate cyclase (14). Co-expression of the splice variant with wild-type (wt) FSHR prevents a cAMP response to FSH, implying oligomerization between wt and the splice variant (15). A study in which binding- and signaling-defective mutants of FSHR were co-expressed demonstrated the partial restoration of signaling via either adenylate cyclase or phospholipase C, but not both (13).

In this study, using the complementary techniques of imaging FRET of native receptor and coimmunoprecipitation of epitope-tagged receptor, we report the first direct evidence of oligomerization of full-length FSHR at the cell surface. Analysis of intracellular receptors revealed that oligomerization occurs as a normal part of processing and, unlike LHR and TSHR, is not affected by hormone. Another novel finding was that the C terminus of tagged FSHR appears to undergo proteolytic processing before arrival at the cell surface.

Materials and Methods

Imaging FRET analysis

The efficiency of energy transfer (E) from donor to acceptor, which is highly dependent on the distance between the fluorophores, is often reported as FRET efficiency (E %). The most direct method by which to quantify the absolute efficiency of transfer is to measure donor emission before and after photobleaching of the acceptor. This increase in emission (dequenching) of the donor is a direct measure of the FRET efficiency and is calculated from: $E \% = [1 - (\text{donor emission before acceptor photobleach}) / (\text{donor emission after acceptor photobleach})] \times 100$. In this study, Alexa 568 was used as the donor fluorophore and Alexa 647 as the acceptor fluorophore. This fluorophore pair meets the requirement for

producing FRET in which the emission spectrum of the donor overlaps the absorption spectrum of the acceptor.

To study native FSHR, FRET was performed using the monoclonal antibody (mAb) 106.105, directed against FSHR_{ECD} (16), that had been labeled with fluorophores. The mAb 106.105 or Fab fragments of mAb 106.105 were directly labeled with fluorescent dye Alexa 568 or Alexa 647 (Molecular Probes, Eugene, OR) according to the manufacturer's instructions. Fab fragments were prepared according to the manufacturer's instructions (Pierce, Rockford, IL).

Human embryonic kidney (HEK) 293 cells or HEK 293 cells stably transfected with FSHR (HEK 293/FSHR) (17) were plated in 35-mm petri dishes with glass coverslip bottoms (MatTek Corp., Ashland, MA). The cells were labeled with the fluorescently tagged antibodies essentially as described (18). Briefly, a dish was placed on ice and the tissue culture medium was replaced with ice-cold PBS supplemented with 0.9 mM CaCl₂, 0.52 mM MgCl₂, and 0.16 mM MgSO₄ (PBS²⁺) containing 1% BSA and allowed to equilibrate at 4 C. Individual cell cultures were then incubated with each labeled antibody or Fab fragment diluted in PBS²⁺ for 30 min at 4 C. The cells were washed with ice-cold PBS (twice for 5 min each) and then fixed with 4% formaldehyde in PBS, freshly prepared from a 16% formaldehyde solution (Electron Microscopy Sciences, Hatfield, PA). After fixation, the cells were washed in PBS at room temperature (twice for 5 min each). As a control for specificity of the primary antibody and Fab fragments, HEK 293 cells were incubated with the fluorescently tagged antibodies and imaged as above.

The cells were then covered with fresh PBS and imaged on a Zeiss LSM 510 META confocal microscope system on an Axiomat 200 M inverted microscope equipped with a $\times 63$ 1.4 NA, oil-immersion differential interference contrast (DIC) lens. Alexa 568 was excited with the 543-nm line of a HeNe laser, and emission was detected with a 545 dichroic and a 565- to 615-nm band pass filter. Alexa 647 was excited with the 633-nm laser line of a HeNe laser, and emission was detected with a 545 dichroic and a 650- to 710-nm band pass filter. The pinhole was set at 1.32 Airy units and a Z resolution of approximately 2.0 μ m. Images were collected at 12-bit intensity resolution over 512 $\times$ 512 pixels at a pixel dwell time of 6.4 μ sec.

FRET was detected as an increase in donor (mAb 106.105-Alexa 568) intensity after photobleaching of acceptor molecules (mAb 106.105-Alexa 647). A region of interest (ROI) traced on an image of the cells was subjected to photobleach with the 633-nm HeNe laser line for 30–90 sec at maximum power. This irradiation resulted in greater than 95% photodestruction of the acceptor fluor. Four images were collected: 1) an image of the Alexa 568 fluorescence (in the presence of Alexa 647), 2) an image of the Alexa 647 fluorescence, 3) an image of Alexa 568 fluorescence after destruction of acceptor fluorescence by photobleaching, and 4) an image of the Alexa 647 after the Alexa 647 photobleach.

The images were analyzed using the LSM FRET Tool (version 1.5) that is integrated with the LSM 510 collection of software (Zeiss, Inc., Thornwood, NY). FRET analyses were performed on ROI drawn directly on captured confocal images. The analysis uses a cell or its subregion as its own internal standard after photobleaching (19). GraphPad PRISM version 3.0 (GraphPad Software Inc., San Diego, CA) was used for the linear regression analyses of the FRET data.

Plasmid constructions

FSHR was inserted into the mammalian expression vector pRK5 and a FLAG (DYKDDDDK) or c-myc (EQKLISEEDL) epitope tag was inserted C-terminal to the FSHR

sequence. The wt FSHR was inserted into the mammalian expression vector pShuttle-CMV (Stratagene, La Jolla, CA).

Cell culture and transfection

HEK 293 cells and HEK 293/FSHR cells were maintained in Eagle's medium containing 10% fetal bovine serum. Subconfluent 60-mm dishes of HEK 293 cells were transfected (Lipofectamine Plus; Invitrogen, Carlsbad, CA) with 1 μ g each of plasmid encoding either FLAG-tagged FSHR or myc-tagged FSHR and incubated for an additional 24–48 h. Cells were treated with 1.2 nM human pituitary FSH in serum-free Eagle's medium for the indicated times.

Radioreceptor assays (RRAs)

Human pituitary FSH was radiolabeled with I^{125} (PerkinElmer Life Sciences, Boston, MA) as described (20). Transfected cells were harvested in PBS/EDTA, pelleted, and resuspended in RRA buffer [50 mM Tris (pH 7.5), 25 mM $MgCl_2$, and 0.1% BSA] at a density of 250,000 cells/0.1 ml. [I^{125}]FSH was added at 200,000 cpm/0.1 ml RRA buffer in the presence or absence of 1 μ g unlabeled recombinant single-chain FSH essentially as described (20, 21). Bound [I^{125}]FSH was determined using an LKB/Wallac γ -counter (Gaithersburg, MD).

Immunoprecipitations

HEK 293 cells cotransfected with FSHR-FLAG and FSHR-myc were harvested in Igepal-DOC lysis buffer [10 mM Tris (pH 7.5), 1% Igepal, 0.4% deoxycholate, 140 mM NaCl, 5 mM EDTA] supplemented with protease inhibitors (10 μ g/ml pepstatin A, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 16 μ g/ml benzamide, 10 μ g/ml 1,10 phenanthroline, 1 mM phenylmethylsulfonyl fluoride). Protein concentration was determined by BCA assay (Pierce), and equal amounts of cell extract (~0.5 mg) were immunoprecipitated as described in Ref. 17.

Biotinylation of cell surface proteins

Transfected cells were transferred to ice and washed once with ice-cold PBS. Cell surface proteins were biotinylated using 0.55 mg/ml cell-impermeant, Sulfo-NHS-LC-biotin (Pierce) in PBS for 30 min on ice. Unreacted biotin was quenched and removed with two washes of ice-cold PBS supplemented with 10 mM glycine. The cells were washed once with ice-cold PBS and harvested in 1 ml ice-cold Igepal-DOC lysis buffer.

Cell lysates were immunoprecipitated with mAb 106.105, specific for FSHR, FLAG M2 mAb (Sigma Chemical Co., St. Louis, MO), or myc mAb clone 9E10 (American Type Culture Collection, Manassas, VA).

Gel electrophoresis and Western immunoblotting

Samples (1/4 of immunoprecipitate) were resolved on SDS-PAGE in a discontinuous buffer system (22). After separation, the proteins were transferred to Immobilon-P membranes according to the method of Towbin *et al.* (23). Membranes were blocked in Tris-buffered saline with Tween 20 (TBST) [10 mM Tris-HCl (pH 7.2), 150 mM NaCl, 0.5% Tween 20] with 5% nonfat dry milk overnight at 4 C. Blots were washed briefly in TBST and probed with mAb 106.105 (5 μ g) for 1 h at room temperature. All antibody incubations were done in a volume of 10 ml. Blots were washed and incubated with goat antimouse Ab (1:10,000) conjugated to horseradish peroxidase (HRP) (Biosource, Camarillo, CA) for 1 h at room temperature. In the case of myc mAb, blots were blocked and probed in TBST with 3% BSA. Blots were probed with 10 μ g biotinylated myc mAb, prepared according to the

manufacturer's instructions for EZ Link Sulfo-NHS-LC-biotin (Pierce). Blots were then washed and incubated with 10 μ g HRP-streptavidin (Pierce). Alternatively, blots were probed with HRP-conjugated FLAG M2 mAb (Sigma) (1:5000) for 1 h at room temperature. Signal was developed using enhanced chemiluminescence Western blot detection reagents (Pierce).

Results

FSHR is constitutively self-associated on the cell surface

FRET experiments were performed to determine whether FSHR is an oligomer on the surface of cells. FRET occurs over intermolecular distances between 1.0 and 10.0 nm and thus provides a means of assessing protein-protein interactions and of detecting even very small clusters of proteins in the plasma membrane of a cell (19, 18, 24).

The plasma membrane of HEK 293/FSHR cells displayed labeling after incubation with mAb 106.105 conjugated to either Alexa 568 (mAb 106.105-Alexa 568) or to Alexa 647 (mAb 106.105-Alexa 647). HEK 293 cells, which do not express FSHR, showed no specific labeling when incubated with these fluorescently labeled antibodies (data not shown).

In an initial FRET experiment (Fig. 1), HEK 293/FSHR cells were incubated with a 1:1 mixture of mAb 106.105-Alexa 568 (donor) and mAb 106.105-Alexa 647 (acceptor). Pre-bleach Alexa 568 and Alexa 647 images were collected (Fig. 1, A and C). Selected ROI on the plasma membrane were photo-bleached, and post-bleach images were collected (Fig. 1, B and D). In this experiment, acceptor photobleaching in the ROI resulted in a 23.06% increase in donor fluorescence (Fig. 1B) and a 95% decrease in acceptor fluorescence (Fig. 1D).

Acceptor photobleaching experiments were performed on selected ROI on the plasma membrane of 30 cells incubated with various donor/acceptor ratios (D:A). In the absence of cellular treatment, a robust average FRET efficiency of 21.89 ± 9.38 was observed for the FSHR, indicating that the receptors are within 10.0 nm of one another in unstimulated cells. These data provide direct evidence that the receptors are oligomerized in the plasma membrane in the absence of FSH.

It should be pointed out, however, that purely random proximity of fluorophores can lead to substantial FRET, especially when the donor and acceptor fluors are confined to the surface and are present in high concentrations. Simple models for analysis of FRET on membranes have been developed and have been used to distinguish between FRET due to a random distribution of high concentrations of donors and acceptors in the membrane and FRET that is due to clustering of donors and acceptors (18, 25–27). According to these models, if donor and acceptor fluorophores (FSHR) are clustered, then E % is independent of acceptor density, in contrast with a randomly distributed population wherein E % will increase with an increase in acceptor density. Furthermore, for a random distribution, E % will trend to zero at low acceptor density values. A mixed population of random and clustered distributions will exhibit features of both. Figure 2A shows the relationship between FRET efficiency and acceptor density expressed as acceptor fluorescence (arbitrary units) at the average D:A of approximately 1.4 (range, 1.18–1.59) and approximately 2.1 (range, 1.60–2.60). At the average D:A, approximately 1.4, E % is dependent upon acceptor density, increasing with increasing acceptor density and trending to zero at low acceptor densities. In contrast, at the average D:A, approximately 2.1, E % is independent of acceptor density. Apparently, a mixed population of random and clustered receptors was uncovered using a bivalent IgG probe.

The mAb used in this study is specific to a discrete linear peptide epitope in the ECD of human FSHR (16). It is possible that the divalent whole antibody molecule could cross-link two FSHR in the plasma membrane, although it is unlikely that such cross-linking would result in the robust FRET signal observed. At most, the results would indicate that FSHR dimers can come close enough for FRET to occur between the fluorescently labeled antibodies. However, to directly test that possibility, monovalent donor and acceptor fluorescently labeled Fab fragments of mAb 106.105 were generated. Each individual Fab probe bound to the surface of HEK 293/FSHR cells and could be blocked by preincubation with intact mAb 106.105 (data not shown).

When the labeled Fab fragments were mixed at a molar ratio of 1:1 and incubated with HEK 293/FSHR cells, both probes bound to the cell surface and elicited a robust average FRET efficiency of $11.22 \pm 6.05\%$. Although a only a single 1:1 mixture of Fab-mAb 106.105-Alexa 568 and Fab-mAb 106.105-Alexa 647 was used, the actual D:A that are obtained from the experimental data showed a normal distribution of D:A around a mean of 0.62 ± 0.19 with a range of D:A of 0.2–1.2. When the E % is plotted against acceptor density for this range of actual D:A, E % is independent of acceptor density (Fig. 2B). Therefore, when Fab fragments are used as a probe, only clustered FSHR appears to be detected by FRET.

Biogenesis of self-associated FSHR

HEK 293 cells transiently transfected with myc-tagged FSHR or FLAG-tagged FSHR bound comparable levels of [125 I]FSH with high specificity in a radioreceptor assay (data not shown), indicating that neither epitope tag interferes with the trafficking of receptor to the plasma membrane or with the ability of FSHR to bind FSH.

The myc- and FLAG-tagged FSHR were coexpressed in HEK 293 cells, and cell extracts were immunoprecipitated with FLAG mAb. These samples were then assessed for the presence of myc-tagged FSHR by probing immunoblots with myc mAb. Both FLAG- and myc-tagged FSHR immunoprecipitate with FLAG mAb (Fig. 3A), confirming that FSHR forms oligomers in HEK 293 cells. Three forms of FSHR were visible on the immunoblots: relative molecular mass (M_r) 75,000, M_r 175,000, and a high-molecular-mass form of $M_r > 175,000$ (*thick arrowhead*). These molecular masses are approximate and vary depending upon the markers used.

The M_r 75,000 band represents immature FSHR localized to the endoplasmic reticulum (ER) (17). The M_r 175,000 and $M_r > 175,000$ bands may represent myc-tagged FSHR complexed with a chaperone that is not dissociable by SDS. Alternatively, the M_r 175,000 and $M_r > 175,000$ bands may be FSHR targeted for degradation. These complexes are likely to have been oligomerized with FLAG-tagged FSHR, in an SDS-dissociable form, in order for myc-tagged FSHR to be detected in a FLAG immunoprecipitate.

Similar results were observed when immunoprecipitates were solubilized in sample buffer containing dithiothreitol rather than β -mercaptoethanol or when samples were analyzed on urea gels (data not shown). This suggests that the FSHR is either complexed with another protein or is not completely dissociated by SDS, as suggested for other GPCR, including the receptors for V2 vasopressin (28), dopamine D₂ (29), melatonin (30), and TSH-releasing hormone (31).

Oligomers of FLAG- and myc-tagged FSHR are also detectable when cell extracts are immunoprecipitated with myc mAb and then probed with FLAG mAb (Fig. 3B). It is apparent that FSHR forms oligomers in the absence of FSH and that treatment with FSH has little effect. The same three forms of FSHR are observed when extracts from cells cotransfected with FLAG- and myc-tagged FSHR are immunoprecipitated with FLAG mAb

and then probed with FLAG mAb or immunoprecipitated with myc mAb and then probed with myc mAb (Fig. 3, C and D).

To ensure that the oligomeric interactions observed between tagged receptors were specific and not due to aggregation, extracts from cells individually expressing either FLAG- or myc-tagged FSHR were mixed before immunoprecipitation. No FSHR-FLAG was observed in myc immunoprecipitates from mixed lysates (Fig. 4) and vice versa (data not shown). Accordingly, the interaction between receptors occurs within the cell rather than during sample preparation.

Epitope-tagged FSHR shows evidence of C-terminal processing

Interestingly, the mature fully glycosylated form of FSHR, M_r 80,000 (17), is not detectable when myc- or FLAG-tagged FSHR is immunoprecipitated with myc or FLAG mAb, respectively, and immunoblots are probed with myc or FLAG mAb (Fig. 3), suggesting that the myc and FLAG epitopes are present in immature, but not mature, FSHR. In contrast, mAb 106.105 (specific for the ECD) recognizes both immature and mature FSHR (Fig. 5, compare lane 3 vs. lane 4 and lane 5 vs. lane 6).

Immunoprecipitation of untagged receptor with mAb 106.105 (Fig. 5) shows that untagged FSHR exhibits three forms analogous to that seen with the tagged receptors, although the monomer form of FSHR migrates as a broad band, including the immature M_r 75,000 and the mature fully glycosylated M_r 80,000 species, rather than solely the M_r 75,000 species observed in myc or FLAG immunoprecipitates of tagged receptor. A background band (M_r 60,000), occasionally observed with mAb 106.105, is seen in lanes 1 and 2. A M_r 50,000 band is also seen in lanes 1 and 2 and represents the heavy chain of mAb 106.105. This band is not visualized when HRP-106.105 is used (Fig. 5).

The detection of only immature FSHR by the myc and FLAG mAbs is suggestive of proteolytic clipping of the C terminus of tagged FSHR because the myc and FLAG mAbs recognize a C-terminal epitope tag. Biotinylation experiments were carried out to solidify this result.

Biotinylation of FSHR oligomers

The evidence presented up to this point indicates that immature FSHR oligomerizes in the ER before final glycosylation processing. To distinguish between intracellular receptor and cell surface receptor, cell surface proteins were labeled with cell-impermeant biotin in HEK 293 cells transfected with myc- or FLAG-tagged FSHR. Cell extracts were immunoprecipitated with myc, FLAG, or 106.105 mAb, and biotin-labeled proteins were detected on immunoblots with HRP-streptavidin (Fig. 6).

The mature M_r 80,000 band (compare Fig. 6, A and B) is labeled by biotin, indicating that this is the only species of FSHR on the cell surface. The M_r 75,000, M_r 175,000 and M_r >175,000 proteins, detectable when the blot is reprobed with mAb 106.105 (compare Fig. 6, A and C), appear to be intracellular forms of FSHR because these forms are not labeled by biotin.

The immunoprecipitation of low levels of myc- and FLAG-tagged mature FSHR at the cell surface (Fig. 6A) indicates that a fraction of mature FSHR on the cell surface retains the C-terminal epitope tag, either because the clipping occurs at the cell surface so that tagged and untagged FSHR are detectable or because overexpression of the tagged receptor has overwhelmed the quality control mechanism of the cell allowing some FSHR to evade proteolytic processing.

Discussion

The evidence that constitutive oligomerization of FSHR occurs in the ER is strong. Myc mAb immunoprecipitates three forms of FLAG-tagged FSHR (M_r 75,000, M_r 175,000, and $M_r > 175,000$) in cells cotransfected with myc- and FLAG-tagged FSHR and vice versa. The detection of ER-localized immature FSHR (M_r 75,000) in oligomers suggests that FSHR forms oligomers at an early stage of biosynthesis. In addition, the three forms of FSHR involved in oligomerization are found intracellularly, as shown by the observation that only the mature M_r 80,000 form of FSHR is labeled with cell-impermeant biotin.

Constitutive oligomerization has been demonstrated in a number of other GPCR, including the receptors for TSH-releasing hormone (31, 32), GABA_B (33), melatonin (30), dopamine D2 (34, 35), α -factor (36, 37), vasopressin (38), and serotonin 5-HT_{2C} (39). Constitutive oligomers have also been observed for the δ -opioid receptor (40) and β_2 -adrenergic receptor (41, 42).

The results reported in this study differ from that seen with LHR in that LHR oligomers appear to exist on the cell surface in stable cell lines but were not demonstrable in transiently transfected cells (5). Furthermore, LHR oligomer formation increases with high concentrations of hCG. Interestingly, both LHR (5) and FSHR occur in high-molecular-mass SDS-resistant forms, M_r 166,000 and 240,000 in the case of LHR and M_r 175,000 and $> 175,000$ in the case of FSHR. These high-molecular-mass forms may represent gonadotropin receptor covalently cross-linked or otherwise irreversibly associated with a protein, either a chaperone or a cargo protein. The observation of these forms in two different receptors suggests a general mechanism may be revealed by determination of the nature of these forms, a current goal of the lab. Another difference worth noting is that Tao *et al.* (5) did not observe monomers of LHR in coimmunoprecipitation experiments in contrast to the monomers of FSHR reported here. This is likely due to differences in transfection efficiencies and/or sample processing.

In contrast to the abundant evidence that LHR self-associates in response to agonist binding (6–8, 10), the results reported here indicate that FSH has little impact on FSHR oligomerization. This is not surprising in light of the observation that FSHR forms oligomers in the ER and remains in a complex on the cell membrane. However, it points to a significant difference between these two otherwise similar receptors. Oligomerization in the ER could assist in trafficking of receptor to the cell membrane, as is seen for β_2 -adrenergic receptor (43), by shielding ER retention signals or by ensuring correct folding. On the other hand, formation of oligomers in the ER does not preclude additional oligomerization occurring in response to hormone that cannot be detected by methods reported herein.

Roess and Smith (6) found that wt LHR has a lower lateral mobility and higher self-association than does signaling-defective LHR, suggesting that self-association depends on functional receptor. In addition, desensitized LHR is organized into bigger complexes than is actively signaling LHR (10), suggesting a progression from small clusters to more complex arrays as unoccupied LHR binds hormone and becomes desensitized. These studies suggest that LHR has a greater propensity to form higher-order clusters than does FSHR.

In contrast to what is observed with LHR and FSHR, oligomerization of TSHR is significantly reduced by TSH treatment (11). Dissociation of constitutive TSHR oligomers induced by TSH may serve to release TSHR from an inactive conformation. Alternatively, post-binding proteolytic clipping of the TSHR ectodomain may result in destabilization of higher-order oligomers.

Because imaging FRET was conducted with wt FSHR, the possibility of variable expression levels of tagged FSHR was not an issue. In addition, the novel use of differentially labeled mAb or Fab fragments of the mAb specific for FSHR in FRET obviated the need for C-terminal fusion proteins.

A note of caution should be interjected here. These experiments demonstrate that the valency of the FRET probes is an important consideration. Use of the bivalent mAb probes or the monovalent Fab probes produced a robust FRET signal, but upon quantitative FRET analysis, the distributions of FSHR were different with the two different probes.

What might account for these observations? FRET occurs when acceptor and donor fluorophores are in close proximity, typically 1.0–10.0 nm. An intact IgG molecule is reported to be approximately 12 nm in length, and the hinge region connecting the Fab and Fc regions is flexible such that the maximum distance between the ends of the Fab regions in a fully open configuration is approximately 14–15 nm (44). Thus, if an IgG donor and IgG acceptor were each bound to FSHR molecules and they were stretched out in a fully open conformation on the cell surface, these probes could be long enough to give a FRET signal even though the distance between the FSHR might be more than 10 nm. These divalent probes would also show FRET with FSHR that was in fact oligomerized. In this case, a mixed population of randomly associated and clustered FSHR would be observed. In fact, this is what was observed in this study.

In contrast, the Fab fragments are approximately 6 nm in length and bind a single FSHR. Thus, if the FSHR are distributed as oligomers, FRET will occur and it will be independent of increasing acceptor concentration because, in this clustered situation, a donor molecule is already associated with an acceptor molecule. Again, this was found to be the case in this study.

Receptor self association may also assist in signaling as a way of regulating multiple signaling pathways. For instance, angiotensin II type I receptor mutants, defective in ligand binding or signaling, exert a dominant-negative effect on wt angiotensin II type I receptor with regard to $G_{\alpha q}$ activation (45). ERK activation, however, is not inhibited, suggesting that $G_{\alpha q}$ signaling, but not ERK activation, is dependent on oligomerization. Receptor dimerization provides an attractive explanation for the transactivation effects observed when binding-defective, signaling-competent receptors are coexpressed with binding-competent, signaling-defective mutants of FSHR (14).

More recently, it has been proposed that receptor dimerization enables negative cooperativity (46). Negative cooperativity in a system ensures that physiological responses occur at subsaturating levels of agonist with accommodation at higher levels of agonist as well. The evidence for negative cooperativity in FSHR suggests that a conformational change of FSH and/or FSHR occurs upon binding (46). In contrast, one would predict, based on the crystal structure of FSH in complex with FSHR_{ECD}, that the FSHR hormone-binding domain would not be conformationally flexible due to hydrogen bonding of β -sheets (2). These two pieces of data are not necessarily in contradiction. Rather, they speak to the need to crystallize the entire FSHR_{ECD}, including the last 100 amino acids, which likely play a role in regulating binding *in situ* (47, 48). Indeed, circular dichroism spectroscopy of FSH in complex with the full-length FSHR_{ECD} had demonstrated a conformational change upon hormone binding (21).

The mechanism and extent (dimer, tetramer, *etc.*) of FSHR oligomerization is not known. The minimal complex is likely to be a dimer, which would be consistent with the observations described in this report. Moreover, the crystal structure of FSHR hormone-binding domain, in complex with FSH, revealed a dimer with a weakly interacting interface

(2). This interface is not likely to be the major stabilizing force for dimerization. The area of contact is small and could easily be due to crystal contacts, thus not representing a physiologically relevant protein interaction site for dimerization.

It is likely that other forces are at play, and chimeric receptor experiments suggest association through the TM domains (12). Both mechanisms may be operative here and may affect the quality of the signal generated by offering temporal and spatial constraints upon receptor recycling and internalization (49).

The discovery of C-terminal proteolytic processing of tagged FSHR, as evidenced by the relative lack of C-terminal epitope tags in mature FSHR and in FSHR at the cell surface, was serendipitous. Tools for analyzing the C terminus of FSHR have not previously been available. Although a number of studies have been published in which an epitope tag has been inserted at the N terminus of FSHR, this is the first report in which FSHR was tagged at the C terminus. In contrast, LHR has been tagged at both the N and C termini with myc, FLAG, and green fluorescent protein epitopes with no report of proteolytic processing (5, 50–52).

Alternatively, the myc and FLAG epitopes may be inaccessible to antibody. However, for that to be the case, the C-terminal epitope must be inaccessible when the protein is in its native state (immunoprecipitations) or denatured (immunoblots) because FSHR that appeared to be lacking the C-terminal epitope tag was detected under both conditions. C-terminal processing seems to be the best explanation for these data at the present time. Protein sequencing of wt FSHR would establish whether this phenomenon is restricted to epitope-tagged FSHR.

Among candidate endoproteases for the C-terminal processing of FSHR are the proprotein convertases (for review, see Refs. 53 and 54). The proprotein convertases cleave pro-proteins to induce activation. Hormones, growth factors, and membrane receptors are among the substrates of these proteases.

Finally, it is important to consider the physiological implications of receptor oligomerization in the ER in genetic disorders. In the case of heterozygous mutations of the glycoprotein hormone receptor genes that lead to trafficking defects, oligomerization with viable receptor could lead to functional receptor present on the cell surface, as has been so elegantly shown *in vitro* (12). In addition, the proximity of the two ECD domains offers another level of potential control of hormone binding.

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Abbreviations

D:A	Donor/acceptor ratio
E	efficiency of energy transfer
ECD	extracellular domain

ER	endoplasmic reticulum
FRET	fluorescence resonance energy transfer
FSHR	FSH receptor
FSHR_{ECD}	ECD of FSHR
GPCR	G protein-coupled receptor
hCG	human chorionic gonadotropin
HEK	human embryonic kidney
HRP	horseradish peroxidase
LHR	LH receptor
mAb	monoclonal antibody
M_r	relative molecular mass
ROI	region(s) of interest
RRAs	radioreceptor assays
TBST	Tris-buffered saline with Tween 20
TM	transmembrane
TSHR	TSH receptor
wt	wild type

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Fig. 1.

Oligomerization of FSHR is demonstrated by FRET. Confocal images of a 2- μ m optical slice through an HEK 293/FSHR cell stained with mAb 106.105-Alexa 568 (donor), and 106.105-Alexa 647 (acceptor) were collected. A, Donor fluorescence pre-acceptor bleach; B, donor fluorescence post-acceptor bleach; C, acceptor fluorescence pre-bleach; D, acceptor fluorescence post-bleach. Alexa 568 fluorescence is pseudocolored *green*, and acceptor fluorescence is pseudocolored *red*. An area of interest was selected (*box* in D) and pre-bleach images were collected (A and C). Acceptor fluorescence in the area of interest was photobleached with 633-nm laser light, and post-bleach images were collected (B and D).

Fig. 2.

FSHR appears to be distributed as a mix of clustered and randomly associated receptors when FRET is performed with intact mAb, whereas only clustered FSHR is detected when FRET is done with labeled Fab fragments. A and B, Cells were labeled with 1:2, 1:1, or 2:1 ratios of intact mAb 106.105-Alexa 568 (donor, D):intact mAb 106.105-Alexa 647 (acceptor, A) (A) or with 1:1 ratio of Fab-mAb 106.105-Alexa 568 and Fab-mAb 106.105-Alexa 647 (B). Experimentally determined D:A ratios ranged from 0.2–1.2 (A) or 1.18–2.6 (B). E% was plotted as a function of acceptor fluorescence intensity (arbitrary units, AU). Curve fitting is by simple linear regression.

Fig. 3. FLAG- and myc-tagged FSHR are constitutively oligomerized. HEK 293 cells were cotransfected with FLAG- and myc-tagged FSHR or mock transfected and were then treated with 1.2 nM FSH for the indicated times. A and C, Cell lysates were immunoprecipitated with FLAG mAb; immunoblots were probed with biotinylated myc mAb and HRP-conjugated streptavidin (A) to detect myc-tagged FSHR in FLAG immunoprecipitates or probed with HRP-conjugated FLAG mAb (C). B and D, Cell lysates were immunoprecipitated with myc mAb; immunoblots were probed with HRP-conjugated FLAG mAb (B) to detect FLAG-tagged FSHR in myc immunoprecipitates or probed with biotinylated myc mAb and HRP-conjugated streptavidin (D).

Fig. 4.

High-molecular-weight FSHR-specific bands are not due to aggregation. HEK 293 cells were singly transfected with myc-tagged FSHR (lane 1) or with FLAG-tagged FSHR (lane 2) or cotransfected with FLAG- and myc-tagged FSHR (lane 3). In lane 4, extracts from cells singly transfected with myc- or FLAG-tagged FSHR were mixed together before immunoprecipitation. Cell lysates were immunoprecipitated with myc mAb, immunoprecipitates were analyzed by SDS-PAGE, and the immunoblot was probed with HRP-conjugated FLAG mAb.

Fig. 5.

Myc epitope is present only in immature FSHR. HEK 293 cells were transfected with wt FSHR or with myc- and FLAG-tagged FSHR, and cell lysates were immunoprecipitated with either mAb 106.105 (105 IP) or with myc mAb. Immunoblots were probed with biotinylated myc mAb and HRP-conjugated streptavidin or with mAb 106.105 and HRP-conjugated goat antimouse Ab as indicated. Lane 1 is a longer exposure of lane 2 to highlight the M_r 175,000 and M_r >175,000 bands.

Fig. 6.

Mature M_r 80,000 FSHR is the only form of FSHR biotinylated by cell-impermeant biotin. HEK 293 cells were transfected with myc- or FLAG-tagged FSHR or mock transfected. Surface proteins were biotinylated (A and C) or treated with vehicle (B and D). Cell lysates were immunoprecipitated with 106.105, myc, or FLAG mAb, immunoprecipitates were analyzed by SDS-PAGE, and immunoblots were probed with HRP-streptavidin (A and B). Immunoblots were re-probed with mAb 106.105 (C and D).