

# Human follicle stimulating hormone receptor variants lacking transmembrane domains display altered post-translational conformations

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## Abstract

Variant splicing of gonadotropin receptor mRNA commonly occurs, however expression of receptor protein variants and their trafficking has yet to be studied in detail. To determine receptor variant trafficking and intracellular processing in mammalian cells, the intracellular fate of intentionally truncated variants of human follicle stimulating hormone receptor (hFSH-R) expressed in CHO cells was examined. Monoclonal antibodies (mAbs) were made against the hFSH-R's extracellular domain (ECD) expressed in insect cells. Four mAbs 106.156, 106.290, 106.318, and 106.263 were chosen as probes. Epitope mapping using synthetic peptides, and truncated hFSH-R variants revealed that mAb 106.156 bound to ECD residues 183–220, while mAbs 106.318, 106.290, 106.263 bound ECD residues 300–331. Immunofluorescence microscopy showed that mAbs 106.318 and 106.156 stained the surface of fixed, intact CHO cells expressing wild type hFSH-R. However, following cell permeabilization all four antibodies stained hFSH-R in Golgi and endoplasmic reticulum. Permeabilized cells expressing truncated variants ECD213 and ECD254 showed staining accumulated in the endoplasmic reticulum/nuclear envelope continuum. ECD335/His was found to accumulate in extended endoplasmic reticulum (ER). The ER location of ECD335/His was confirmed by double labeling experiments with concanavalin A and ECD mAb. Glycosidase digestion followed by Western blot analysis show ECD213 and ECD335/His to be glycosylated, but not ECD254. Both glycosylated truncated hFSH-R variants were sensitive to peptide-*N*-glycanase F and endoglycosidase H but insensitive to neuraminidase indicating that these variants possess high mannose type oligosaccharides. Thus truncated hFSH-R variants do not reach the medial or *trans* Golgi where high mannose oligosaccharides are trimmed and sialic acid is added. These data suggest that the conformation of the ECD of the wild type receptor is different from the ECD alone expressed in the endoplasmic reticulum. This information suggests that the ECD serves two distinct roles; the first is to bind FSH and the other is likely to contact the endodomain of the receptor, which presumably leads to activation of the endodomain for signal transduction. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

**Keywords:** Variant splicing; Receptor protein; hFSH-R; Immunofluorescence microscopy

## 1. Introduction

The biological activity of the human pituitary glycoprotein follicle stimulating hormone (hFSH) is essential for fertility. The hormone's biochemical action is mediated through its interaction with its receptor (R) which is an integral membrane protein localized exclusively on the surface of granulosa and sertoli cells (see reviews;

Simoni et al., 1997; Huhtaniemi and Aittomäki, 1998; Ulloa-Aguirre and Timossi, 1998). The hFSH-R is composed of a single polypeptide which possesses an amine terminal extracellular domain (ECD), seven membrane spanning segments, and a cytoplasmic carboxyl terminal tail (Minegishi et al., 1991). Unlike most G-protein-coupled receptors, the gonadotropin receptors, such as the hFSH-R have an exceptionally long amine-terminal extracellular domain (ECD) which represents approximately 50% of the mature receptor protein. The ECD is necessary and sufficient for ligand specificity and affinity.

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The hFSH-R is synthesized in the endoplasmic reticulum as a precursor protein which undergoes post-translational processing in the secretory pathway to produce a mature form of 85–92 kDa (see reviews; Simoni et al., 1997; Ulloa-Aguirre and Timossi, 1998). Post-translational processing includes cleavage of the hFSH-R signal peptide, the addition of N-linked high mannose oligosaccharides to four potential N-linked glycosylation sites, Asn<sup>174</sup>, Asn<sup>182</sup>, Asn<sup>277</sup> and Asn<sup>303</sup> (Davis et al., 1995) the trimming and processing of mannose oligosaccharides and palmitoylation at residues cys<sup>629</sup> and cys<sup>627</sup> (Bouvier et al., 1995) in the molecule's cytoplasmic tail. N-linked glycosylation allows hFSH-R to interact with the endoplasmic reticulum (ER) resident chaperone, calnexin (Rozell et al., 1998), which promotes folding of the molecule to its proper native configuration (Hammond et al., 1994; Helenius et al., 1997; Trombetta and Helenius, 1998). The molecule then passes through the medial and *trans* Golgi where the oligosaccharides are trimmed and capped with sialic acid and the mature molecule is then trafficked to the cell surface (Misrahi et al., 1996).

Several alternately spliced FSHR mRNA transcripts coding for different truncated variants of the FSH-R have been described (Gromoll et al., 1992; Khan et al., 1993; Hipkin et al., 1995; Rajapaksha et al., 1996; Sairam et al., 1996; Yarney et al., 1997; Sairam et al., 1997; Khan et al., 1997; Kraaij et al., 1998; Yaron et al., 1999), but the intracellular fate of these protein variants has yet to be studied in detail. When the hFSH-R is misfolded because of mutations (Rozell et al., 1995) or inhibition of glycosylation (Davis et al., 1995), the receptor fails to reach the cell surface, and is thought to be trapped within the cell, however, the precise subcellular location is unknown. Truncated forms of FSH-R could have physiological significance if processed and inserted in the plasma membrane where they might act as dominant negative forms of the hFSH-R. The aim of this study was to define the subcellular location of truncated hFSH-R variants and to examine whether the intracellular processing and trafficking of truncated receptor variants differs from that of the wild type hFSH-R. The data demonstrate that it is unlikely that severely truncated forms of FSH-R can act in a dominant negative manner, and are unlikely to be physiologically relevant.

## 2. Materials and methods

### 2.1. Molecular cloning and expression of recombinant wild type and truncated hFSHR variants

Eukaryotic expression vectors containing full length hFSH-R cDNA and truncated forms of the extracellular domain of hFSH-R cDNA were constructed as follows:

#### 2.1.1. Construction of pBL-hFSH-R

Full length hFSH-R cDNA was isolated from pHSRX (Ares Advanced Technologies; Randolph, MA) using endonucleases PstI and EcoRI. The 5'-end PstI site was blunt ended using T4 DNA polymerase and ligated to an EcoRI linker. An EcoRI site was introduced at the 3'-end of the CMV early promoter-enhancer of expression vector pBL-proA and the modified hFSH-R cDNA was ligated into the EcoRI site of vector pBL-proA. Sense orientation of the hFSH-R cDNA was confirmed by restriction mapping.

#### 2.1.2. Construction of pBL-ECD335/His (ECD335/His)

To express (hFSH) ECD335/His we constructed pBL-ECD335/His. Plasmid pBL-hFSH-R was digested with NsiI located at nucleotide +1035 (Ala335) and KpnI located at the 3'-end of the cDNA, thus removing the hFSH-R sequences coding for the transmembrane domain (TMD) and the carboxyl terminal region. The fragment was isolated by agarose gel electrophoresis. One sense and one antisense oligonucleotide, were designed such that when annealed they formed a linker having an NsiI overhang at the 5'-end and a KpnI overhang at the 3'-end containing codons for a histidine tag and a stop codon (TAA). This allowed for direction cloning into the pBL-hFSH-R vector at the NsiI and KpnI sites. In frame ligation of the linker was confirmed by DNA sequence analysis.

#### 2.1.3. Construction of pBL-ECD213 (ECD213) and pal-ECD254 (ECD254)

To express hFSH-ECD213 the expression vector pBL-ECD213 was constructed by introducing a stop codon at nucleotide +688 (Ile 213). This was accomplished through site directed mutagenesis, using the method of Deng and Nickoloff (Jarvis et al., 1990). A mutagenesis oligonucleotide was designed to change <sup>688</sup>ATC<sup>690</sup> to <sup>688</sup>TAA<sup>690</sup>. Similarly, plasmid pBL-ECD254 was constructed by introducing a stop codon at nucleotide +811 (Tyr 254) using site directed mutagenesis of <sup>811</sup>TAT<sup>813</sup> to <sup>811</sup>TAA<sup>813</sup> and this plasmid was used to express hFSH-R ECD254. Introduction of stop codons into these cDNAs was confirmed by DNA sequencing analysis.

### 2.2. Stable transfection of CHO cells expressing wild type and truncated hFSH-R variants

CHO cells were plated at a density of  $3 \times 10^6$  cells/100 mm — dia culture dish in MEM/F12 medium supplemented with 10% fetal bovine serum. After a 24 h incubation at 37°C the cells were transfected by liposome mediated DNA transfer, using the lipofectin reagent and Opt-MEM I reduced serum medium according to the manufacturer's protocol (GIBCO, BRL).

Expression vector (15 µg), pBL-ECD335/His, pBL-ECD213, and pBL-ECD254, was co-transfected with 5 µg of pSV2-neo, providing a dominant selectable marker. Approximately 48 h post-transfection, the cells were trypsinized and replated at a 1:5 dilution. The antibiotic G418 (GIBCO, BRL) was added (800 µg/ml) the following day. G418-resistant colonies were isolated approximately 2 weeks after transfection in cloning cylinders. Selected clones were grown to mass culture for further analysis. The stable integration of vector DNA was tested by alternatively growing these colony derived cell strains on selective and non-selective media. hFSH-R positive clones were determined by Western blot analysis using anti-FSH-R polyclonal anti-peptide antibody W970 (Liu et al., 1994).

### 2.3. Cell culture and maintenance

CHO cells were grown in Dulbecco's MEM (GIBCO, BRL) containing 10% fetal bovine serum and 600 µg G-418 (gentamycin, GIBCO, BRL) and maintained at 37°C in CO<sub>2</sub> in 75 cm<sup>2</sup> cell culture flasks. Cells were removed from culture flasks by first washing and then incubating cells with warm (37°C) PBS containing 0.5 mM EDTA.

### 2.4. Expression of ECD335/His in Hi5 insect cells

#### 2.4.1. Construction of insect cell transfer vectors

Full length hFSH-R cDNA was isolated from pSHRX by digestion with PstI and EcoRI and ligated into pVL-1392. Truncated hFSH-R cDNAs were isolated from pBL-ECD335/His using EcoRI and KpnI, and ligated into pVL-1393. Recombinant baculovirus was produced by cotransfecting Sf9 cells with 0.25 µg BaculoGold viral DNA (Pharmlingen, San Diego, CA) and 2 µg recombinant transfer vector using a calcium phosphate precipitation protocol (Keller and Simons, 1997). Recombinant virus was amplified in Sf9 cells maintained in TMN-FH medium. Recombinant virus was plaque purified and titered according to the protocol of King and Possee (1992). Hi5 cells (Invitrogen) were used for protein production as previously described. At 24 h after seeding, cells were infected with recombinant virus for 5 days. Cells were resuspended in PBS, counted and prepared for ECD335/His purification.

### 2.5. Purification of ECD335/His

ECD335/His expressed in Hi5 cells was purified by immobilized metal ion affinity chromatography on Talon Metal Affinity Resin (Clontech) according to the manufacturer's protocol. Briefly, 11 roller bottles of Hi5 cells, were infected with ECD335/His virus.

Two days after infection, cells were harvested by centrifugation, and washed with PBS. The washed pellet was frozen, and later resuspended in 100 ml of 6 M GuHCl, 0.25 M Tris, 0.1 M NaCl, pH8.0 and homogenized at maximum speed using a Omni mixer. The homogenate was spun at 10 000 rpm for 30 min in a Sorval GSA rotor. The supernatant was passed through cheese cloth and homogenized a second time. This homogenate was added to 15 ml of packed Talon resin and incubated at RT with shaking. After an hour, the resin was washed several times with GuHCl buffer, then several times with 0.025 M Tris, 0.1 M NaCl, pH 8.0. Finally, the adsorbed protein was eluted using 0.025 M Tris, 0.1 M NaCl, pH 8.0 made 50 mM with imidazole. The eluted protein was concentrated using an Amicon concentrator.

### 2.6. Immunization of Balb/c mice and preparation of hybridomas

Balb/c mice were immunized subcutaneously with injections of purified ECD335/His (50 µg/injection) with adjuvant. After 4 weeks and again after 8 weeks, animals were boosted with 50 µg of purified protein. Three days after the last boost, the mice were sacrificed and the spleen was removed. The spleen was minced and the cells washed in Dulbecco's MEM containing gentamycin (Stemcell Technologies, Inc. Vancouver, B.C.). Splenocytes ( $6 \times 10^7$ ) were mixed with  $2 \times 10^7$  freshly activated P3x63-AG8.653 (x63) cells. The cell mixture was pelleted by centrifugation at 1800 rpm for 7 min at RT. The pellet was resuspended by adding in drop-wise fashion 0.8 ml polyethylene glycol (PEG) (50% w/v) Hybrimax (Sigma, St. Louis, MO.) over a 1 min period with continuous stirring. The cell mixture was then incubated for 2 min at 37°C, and then received drop-wise 1 ml of DMEM, containing gentamycin over 1 min with continuous stirring, then 20 ml was added over 5 min at 37°C. The cell mixture was pelleted by centrifugation at 1800 rpm for 7 min at RT and then decanted. The pellet was resuspended in 40 ml of DMEM, containing preselected serum and nutrients, with gentamycin (Stemcell technologies, Inc, Vancouver, B.C.) at 37°C, and transferred to a 275 ml tissue culture flask. Cells were incubated in a humid chamber at 37°C with 5% CO<sub>2</sub>. After 16 h the cells were harvested by centrifugation at 1800 rpm for 7 min at RT. The cell pellet was resuspended into 40 ml DMEM, preselected serum, methyl cellulose in alpha MEM, HAT, and gentamycin (manufacturer's formulation; Stemcell Technologies, Inc., Vancouver, B.C.) and were mixed to evenly distribute cells, 10 ml of cell suspension was plated on each of four 10 mm dia petri dishes, tilting plates to level and spread mixture. After 7–10 days individual colonies were picked and placed in wells of 96-well plate with each well receiving 200 µl of

DMEM containing preselected serum and nutrients, HT, and genetamycin. Following 7 days of culture, tissue culture media from individual clones was removed to identify those with antibodies against ECD335/His.

## 2.7. Biospecific interaction analysis screen for monoclonal antibodies

To identify those hybridoma clones secreting hFSHR ECD reactive antibodies, conditioned media from individual clones was screened by Biospecific interaction analysis (BIAcore, Inc., Piscataway, NJ). A CM5 sensorchip was equilibrated with running buffer (10 mM Hepes, pH 7.4 containing 150 mM NaCl, 3.4 mM EDTA and 0.005% Tween-20). The ECD335/His (12.5 µg/220 µl of 10 mM sodium acetate buffer, pH4.5) was subjected to a mixture of equal parts of *N*-hydroxysuccinimide and *N*-ethyl-*N*-(dimethylaminopropyl)-carbimide (Amine Coupling Kit, Biacore, Inc.) allowing coupling of peptide's amine groups to the carboxymethyl dextran surface of the chip. To determine whether hybridoma fluids contained IgG, affinity-purified rabbit anti-mouse Fc gamma (Biacore, Inc.) was immobilized in similar fashion. Sensorchip surfaces were regenerated after each ligand/analyte interaction with 100 mM HCl-glycine, pH 2.4. Culture supernatants from 96-well micro-titer plates were harvested, diluted 1:3 with Hepes, pH7.4 running buffer and tested for IgG against anti-Fc gamma coated chips, and for hFSH-R reactivity against ECD335/His coated chips. IgG isotype character was determined by capturing each monoclonal antibody on the anti-Fc gamma chip surface followed by consecutive injections of isotype-specific antibodies to IgG3, 2b, 2a, and 1 (Isotyping Kit, Biacore, Inc.)

## 2.8. Polyacrylamide gel electrophoresis and Western blot analysis

To further map mAb epitopes, whole cell lysates were prepared from stably transfected CHO-cells expressing ECD213, ECD254, ECD335/His along with CHO-cells. The samples were boiled, and reduced in Lammeli's sample buffer, and resolved on 12% SDS-acrylamide gels ( $2 \times 10^5$  cells/lane). Following electrophoresis, gel resolved peptides were transferred onto immobilon membrane using a Trans-blot semi-dry electrophoresis transfer apparatus (Bio-Rad, Melville, NY) and 48 mM Tris, 39 mM glycine, 20% methanol, 1.3 mM SDS, pH 9.2 transfer buffer. Blots were blocked in 10 mM Tris, 145 mM NaCl, pH 7.2 (TBS) containing 5% non-fat milk (M) for 1 h at RT. After blocking, blots were incubated with either purified hFSH-R monoclonal antibody (5 µg/ml) or hybridoma conditioned tissue culture media (1:50 dil.) diluted in blocking solu-

tion for 1 h at RT. Blots were washed ( $4 \times 10$  min) in TBS with 0.05% Tween-20 (T), and were then incubated with peroxidase-coupled goat anti-mouse IgG antibody (Bioscience, Camarillo, CA) (1/50 000 dil) in TBSM. Blots were washed as above, and antigenic bands were detected by ECL (Amersham, Boston, MA).

## 2.9. Synthesis of synthetic ECD peptides

Eleven overlapping peptides spanning the entire ECD of FSH-R (I) 1–15, rat, human; (II) 15–44, rat; (III) 45–100, human; (IV) 72–100, human; (V) 101–125, human; (VI) 126–150, human; (VII) 150–183, human; (VIII) 183–220, human; (IX) 221–252, rat; (X) 265–296, human; and (XI) 300–331, human were synthesized on an Applied Biosystems 431A peptide synthesizer (Foster City, CA) by the Wadsworth Center Peptide Synthesis Core Facility. For a negative control, peptide (XII) representative of TMD6, composed of FSH-R residues 580–594, and representative of hFSH-R TMD6 was also tested. The molecular mass of purified peptides was determined by mass spectrometry at the Wadsworth Center Biological mass spectrometry core facility. Synthetic peptides were made 1 mg/ml in distilled water. ECD peptides (III–VIII, XI) were not completely water-soluble.

## 2.10. Enzyme-linked immunosorbent assay (ELISA) for epitope mapping using synthetic ECD peptides

Wells of micro-titer plates (Immulon 2, Dynatech, Alexandria, VA) were coated with synthetic ECD peptide (1 µg/100 µl) in 0.05 mM Tris base, pH 9.5, and incubated overnight at 4°C. Wells were rinsed with 0.01 M phosphate buffered saline-0.05% Tween-20 (PBST), pH 7.4 to remove unabsorbed peptide and then blocked with 10% normal goat serum (in PBST) for 2 h at RT. Once peptides were blocked the following reagents were added in the following order, with washing between each reagent: 100 µl of hybridoma conditioned media or 20 ng purified mAb for 2 h at RT, 100 µl of 1:1000 diluted alkaline phosphatase-labeled goat anti-mouse IgG (Biosource International, Camarillo, CA) for 1 h at RT and 100 µl of development substrate (*p*-nitrophenyl phosphate in diethanolamine buffer) (BioRad Labs, Hercules, CA). The reaction was covered with foil. After 30 min, the absorbance was read at 410 nm using a Dynatech MR700 plate reader and the BioLinx Version 2p20d analysis software (Dynatech Laboratories). To determine the specificity of mAbs, hybridoma conditioned media (1 ml) or purified mAb (2 µg) was pre-incubated with purified ECD335/His (10 and 20 µg per ml, respectively) before application to the micro-titer wells. Samples were tested in triplicate.

### 2.11. Indirect immunofluorescence microscopy

Cells were rinsed with Small's cytoskeleton stabilizing buffer (csb) (Small, 1981) warmed to (37°C), and then immediately fixed with 3.7% formaldehyde for 5 min at RT. For some studies, cells were permeabilized with 0.5% Triton X-100 (csb, pH7.2) for 20 min at RT. Cells were washed with csb and then incubated with 50 mM NH<sub>4</sub>Cl in csb for 30 min at RT to reduce free aldehyde groups produced by formaldehyde fixation. After cells were blocked for 1 h at RT by incubation in csb containing 1% BSA (Albumin fraction V, Boehringer Mannheim), the cells were stained with purified ECD 335/His mAb (5 µg/ml) for 1 h at RT in a humid chamber. Cells were washed with csb containing 1% BSA, and then incubated with fluorescein-conjugated goat anti-mouse IgG antibody (1:1000 dil) for 1 h at RT. The cells were washed as above and mounted in csb containing 1 mg/ml *p*-phenylenediamine (anti-fade agent) and then viewed and photographed with a Nikon-Opti-fluorescence microscope.

For co-localization studies, cells were fixed and permeabilized and incubated with 106.156 or isotype IgG2A control as above, and stained with an Alexa 568-conjugated goat anti-mouse IgG antibody (Molecular Probes, Eugene, Oregon) and counter-stained with fluorescein-conjugated wheat germ agglutinin (WGA) or fluorescein-conjugated concanavalin A (ConA) (Vector Laboratories, Inc, Burlingame, CA) (20  $\mu$ g/ml csb containing 1 mM calcium chloride, manganese chloride). Stained cells were viewed with a custom double filter cube with dual excitation (488–568 nm) and emission (515–540 nm, fluorescein; 590–610 nm, Alexa 568) (Omega) using a Nikon Opti-microscope

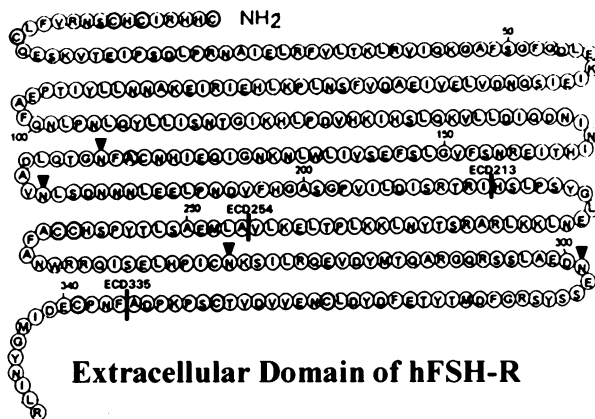


Fig. 1. Diagram displaying mature hFSH-R ECD (residues 1–349), truncated variants and sites of post-translational modification. ECD of mature human follicle stimulating hormone receptor (Minegishi et al., 1991; Ulloa-Aguirre and Timossi, 1998) showing structural characteristics, including putative glycosylation sites (▼), and the sites of truncation to yield ECD213, ECD254, and ECD335/His (┘). ECD335/His was genetically altered to possess a 5-His tag at its carboxyl-terminal end.

with a Quad-four-Epi-Flourescence attachment and a  $60 \times 1.4$  Na objective lens. Digital images were captured with a digital Spot camera (Diagnostic Imaging) and montaged using Photoshop 5.0 software.

### 2.12. Flow cytometry analysis

CHO-cells expressing full length hFSH-R were allowed to grow to confluency. Cells were removed from T-flasks by a 15 min incubation with PBS-EDTA. Cells were harvested by centrifugation at 3000 rpm, and later resuspended in PBSA (PBS with 0.02% sodium azide) at  $1 \times 10^6$  CHO-FSH-R cells (200  $\mu$ l). To block nonspecific binding, cells were incubated on ice for 1 h in 25% goat serum in PBSA. Normal mouse sera or 50  $\mu$ l unconditioned culture fluid (which acted as negative controls), or 50  $\mu$ l of hybridoma conditioned culture media was then added to cells. After 1 h incubation on ice, the cells were washed with PBS, pelleted and resuspended in PBS containing goat anti-mouse fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG and incubated for 30 min on ice. Cells were then pelleted, washed and resuspended in PBSA (0.5 ml). Cell surface immunofluorescence was measured using a flow cytometer (Becton Dickson, FACScan).

### 2.13. Glycosidase digestion of hFSH-R

For digestion with PNGase F, cells ( $1 \times 10^7$  cells) expressing truncated variants and wild type hFSH-R were solubilized in Laemmli electrophoresis sample buffer (1 ml) containing 1%  $\beta$ -mercaptoethanol, sonicated for 30 s and then denatured at 100°C for 10 min. Igepal C630 (Sigma) and PNGase F were added at final concentrations of 1% and 4 units/ $\mu$ l, respectively, and the mixture incubated overnight at 37°C. For digestion with Endo H or neuraminidase, cell lysates were solubilized in Laemmli sample buffer containing 1%  $\beta$ -mercaptoethanol, and 4 mM  $\text{CaCl}_2$ . Following denaturation, Endo H or neuraminidase was added to give a final concentration of 4 units/ $\mu$ l or 2 milliunits/ $\mu$ l, respectively, and the samples were incubated overnight at 37°C. At the end of each incubation samples were heated to 100°C for 5 min. Samples were reheated prior to Western blot analysis.

### 3. Results

### 3.1. Production of hFSH-R-specific mAbs

To make large quantities of hFSH-R antigen, ECD335/His was expressed in Hi5 insect cells. This produced (ECD335/His) a hFSH-R variant truncated at amino acid residue 335 which possessed a 5-His tag at its carboxyl-terminal end (Fig. 1). To purify the

protein, Hi5 insect cells expressing ECD335/His were solubilized, clarified by centrifugation and the supernatant was passed over a metal affinity column, which retained the truncated hFSH-R variant by its 5-His carboxyl terminal tail. ECD335/His was purified to homogeneity, and used to immunize Balb/c mice to produce mAbs.

The 576 resulting hybridomas were screened by BIA-core and ELISA using insect cell purified hFSHR-ECD335/His as antigen; 46 clones reacted with this antigen. The antibodies produced by these clones were further characterized for their reactivity to wild type hFSH-R expressed in mammalian (CHO) cells using Western blots and immunofluorescence microscopy. Based upon these analyses, mAbs 106.156, 106.263, 106.290, and 106.318 were chosen as reagents to follow the expression of truncated hFSH-R variants in CHO cells.

### 3.2. Mapping mAb epitopes using synthetic peptides

To define the epitopes of mAbs, the reactivity of mAbs to 11 overlapping synthetic peptides spanning the entire ECD of the FSH-R was examined. The length of synthetic peptides was long, averaging 30 amino acids. As shown in Table 1, mAb 106.318, 106.290, and 106.263 all reacted very strongly with peptide XI (residues 330–331), displaying absorbance values of 2.138, 1.717, 0.665, respectively, at 410 nm. No mAbs reacted to a second synthetic peptide. MAb 106.156 was found to react strongly with a peptide closer to the amine-terminal of hFSH-R, peptide VIII (residue 183–220) producing an absorbance value of 0.863 at 410 nm. To confirm the specificity of ECD-peptide mAb interactions, mAbs were absorbed with purified ECD335/His before their incubation with synthetic peptides. As shown in Table 1, pre-incubation of mAbs with purified ECD335/His inhibited their reactivity to their corresponding synthetic peptide to varying degrees. Synthetic peptide-mAb interaction for mAbs 106.156, 106.318, 106.290 was inhibited 82% or greater, while mAb 106.263 was inhibited 70% when mAbs were pre-incubated with insect cell ECD335/His. Peptide XII composed of amino acids residues 580–594, representing TMD VI, was intended to act as a negative control, and no mAbs were found to react with this peptide.

### 3.3. Mapping mAb epitopes using truncated isoforms of hFSH-R

To further characterize mAb epitopes, their reactivity with truncated hFSH-R variants was examined by Western blot analysis. Mab 106.156 reacted with truncated hFSH-R variants ECD213, ECD254, and ECD335/His, staining peptides of approximately 27.3, 34.8, 47.5 kDa, respectively (Fig. 2). MAb 106.318,

106.263, 106.290 reacted specifically only to truncated hFSH-R variant ECD335/His in agreement with synthetic peptide mapping (Table 1).

### 3.4. Indirect immunofluorescence microscopy of ECD213, ECD254, ECD335/His, and full length FSHR expressed in CHO cells

CHO cells stably expressing wild type hFSH-R were examined by indirect immunofluorescence microscopy. CHO cells were examined fixed-intact or fixed-then detergent permeabilized. This allowed selective staining of cell surface hFSH-R or intracellular hFSH-R molecules, respectively. The staining pattern produced by our four different mAbs reacting with intact and permeabilized CHO cells expressing wild type hFSH-R is shown in Fig. 3.

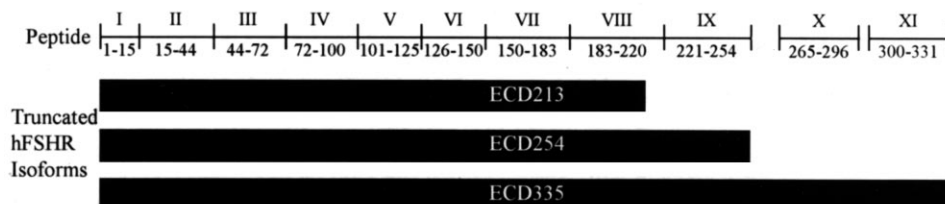
Examination of formaldehyde-fixed intact CHO cells expressing wild type hFSH-R revealed that only mAbs 106.318 and 106.156 stained cell surface hFSH-R. However, after detergent- permeabilization of CHO-hFSHR cells, all four mAbs were found to stain intracellular hFSH-R as it is transported and processed through the secretory system to the cell surface. These data suggest that mAbs 106.263 and 106.290 preferentially bind immature forms rather than mature form of hFSH-R after formaldehyde-fixation, or the epitope recognized by mAbs 106.263 and 106.290 was buried or masked by components of the plasma membrane of intact cells.

Because mAb 106.156 produced the brightest, and most concise staining of hFSH-R at both the cell surface and in the secretory system, this mAb was used to examine the intracellular trafficking of ECD213, ECD254, and ECD335/His in CHO cells. As shown in Fig. 4, staining of intact cells with mAb 106.156 produced minimal (background equivalent) surface staining of CHO cells expressing ECD213 and ECD254. Mab 106.156 stained the plasma membrane of intact CHO cells expressing wild type hFSH-R producing a punctate pattern evenly distributed over the entire cell surface. Staining of fixed intact cells expressing ECD335/His was also detected. In contrast to the staining pattern of wild type hFSH-R, the distribution of ECD335/His was perinuclear. Examination of detergent-permeabilized cells revealed that ECD213, ECD254 are trapped in the endoplasmic reticulum/nuclear envelope continuum, while both wild type hFSH-R and ECD335/His appeared to be distributed throughout the cytoplasm in organelles of the secretory system. As judged by staining intensity the intracellular expression level of ECD335/His appears to be higher than the intracellular expression level of wild type hFSH-R (Fig. 4). These data indicate that ECD213 and ECD254 are not transported to the cell surface, while ECD335/His appears as a aggregate of molecules in a perinuclear position of close proximity to the cell sur-

Table 1  
Mapping of monoclonal antibody epitopes using hFSH-R ECD synthetic peptides<sup>a</sup>

		hFSHR-ECD (+/-)							
hFSHR residues	mAb	-	-	-	-	+	+	+	+
	106.156	106.263	106.290	106.318	106.156	106.263	106.290	106.318	
	1-15 (I)	.169 .029	.091 .002	.103 .004	.106 .006	.105 .006	.096 .004	.102 .003	.101 .006
	15-44 (II)	.167 .023	.092 .003	.142 .057	.117 .005	.117 .008	.097 .007	.106 .006	.119 .014
	45-72 (III)	.117 .011	.089 .001	.098 .002	.101 .007	.091 .003	.095 .007	.096 .002	.098 .008
	72-100 (IV)	.124 .009	.090 .002	.099 .001	.102 .003	.101 .004	.092 .003	.103 .008	.106 .011
	101-125 (V)	.240 .022	.116 .003	.130 .014	.153 .013	.153 .015	.132 .018	.156 .043	.170 .013
	126-150 (VI)	.169 .019	.097 .001	.110 .009	.133 .014	.119 .003	.097 .003	.114 .010	.142 .015
	150-183 (VII)	.112 .011	.092 .002	.097 .003	.095 .005	.104 .003	.091 .002	.098 .003	.119 .033
	183-220 (VIII)	.863 .132	.093 .004	.100 .004	.101 .002	.127 .014	.093 .002	.097 .001	.103 .005
	221-254 (IX)	.112 .010	.091 .010	.094 .002	.098 .003	.097 .003	.090 .003	.094 .002	.099 .002
	265-296 (X)	.148 .014	.097 .002	.100 .002	.109 .006	.113 .001	.101 .001	.106 .004	.121 .007
	300-331 (XI)	.135 .062	.665 .056	1.717 .074	2.138 .119	.099 .006	.209 .017	.094 .004	.385 .031
	580-594 (XII)	.135 .022	.094 .001	.096 .003	.107 .008	.115 .005	.093 .003	.096 .005	.105 .004

Tissue culture media- standard deviation in lower right corner



<sup>a</sup> Wells of micro-titer plates were coated with synthetic ECD peptides, blocked and then challenged with culture fluid from hybridoma 106.318, 106.156, 106.290, and 106.263. After 1 h incubation, plates were washed, and rechallenged with alkaline phosphatase- conjugated goat anti-mouse IgG, washed, given indicator substrate and read at 410 nm using a Dyntech7000 platereader. Samples were tested in triplicate, and read 30 min after addition of indicator. The 410 nm absorbance mean value is in the upper left corner, and the standard deviation is in the lower right corner. The top row denotes the mAb tested, with the – or + at the top of the column indicating whether the mAb was pre-incubated with purified ECD335/His prior to testing. The left column denotes the synthetic ECD peptide (I-XII) tested and its amino acid residues. The line diagram below the array aligns the relative position of synthetic peptides to relative position in amino sequence of hFSH-R variants ECD213, ECD254, and ECD335/His. ECD335/His is a hFSH-R variant truncated at Ala<sup>335</sup> which possessed a 5-His tag at its carboxyl-terminal end.

face. It should be noted that no FSH binding is detected in radio-receptor binding assays examining intact CHO cells expressing ECD213, ECD254 or ECD335/His (manuscript in preparation). However, in detergent-soluble radio-receptor assays ECD335/His binds FSH with the same affinity ( $K_d$   $5.3 \times 10^{-10}$  M) as full length

hFSH-R ( $K_d$   $1.8 \times 10^{-10}$  M). Both ECD213 and ECD254 fail to bind FSH in detergent-soluble radio-receptor assays (manuscript in preparation). Experiments were not performed to determine whether intracellular sequestered truncated hFSH-R ECD variants could interfere with the transport of normal receptor forms

should both variant and wild type be expressed in the same cell. As far as non-specifically interfering with transport, this seems unlikely given the tremendous capacity of the cell to make supra-physiological levels of protein, and still function normally. One interesting possibility remains, that if the truncated form of the receptor oligomerizes with wild type full length receptor, the chimera may not traffick appropriately. That receptor oligomerization of the gonadotropin receptor extracellular domains occurs has yet to be experimentally demonstrated.

To determine whether truncated hFSH-R variants were secreted by cells expressing hFSH-R variants conditioned media was concentrated ten-fold and subjected to Western blot analysis with mAb 106.156. No truncated hFSH-R variant forms were detected (data not shown).

### 3.5. Reactivity of mAbs to native hFSH-R expressed on the cell surface

Flow cytometry was used to determine if mAbs recognized hFSH-R in its native form on the cell surface. From the mean flow data peaks (Fig. 5), none of the four mAbs tested with native hFSH-R on the cell surface. Normal mouse sera acted as our negative control, while sera from hFSHR ECD-immunized mouse acted as our positive control in flow cytometry experiments. The immunogenic character of the four hFSH-R-ECD mAbs is summarized in Table 2.

### 3.6. Post-translational processing studies

Truncated hFSH-R variants ECD213, ECD254, ECD335/His which do not appear to reach the cell surface, likely do not undergo post-translational processing to the same extent as wild type hFSH-R. In

order to test this hypothesis, the glycosylation pattern of truncated hFSHR variants was investigated. The ECD of hFSH-R contains four potential sites: Asn<sup>174</sup>, Asn<sup>182</sup>, Asn<sup>275</sup>, and Asn<sup>301</sup>, for N-linked glycosylation. ECD213 and ECD254 variants each contain two potential sites for N-linked glycosylation, while ECD335/His variant contains four potential N-linked glycosylation sites.

It is known that the synthesis of N-linked oligosaccharide chains begins with the addition of core mannose rich chains at translation. These mannose rich chains are then trimmed and new sugars are added as the protein is processed through the Golgi (Kornfeld and Kornfeld, 1985). To determine whether truncated isoforms of hFSH-R were glycosylated, stably transfected CHO cells expressing ECD213, 254, and 335/His variants were solubilized in Laemmli Sample buffer containing 1%  $\beta$ -mercaptoethanol and then incubated in the presence of PNGase F and examined by Western blot analysis. As shown in Fig. 6, the undigested truncated hFSH-R variants ECD213, ECD254, ECD335/His (lane 1) migrated as 27.3, 34.8, 47.5 kDa polypeptide bands, respectively. Treatment of truncated hFSH-R variants ECD213, and ECD335/His with PNGase F which removes N-linked glycans produced a common 3 kDa shift in their relative electrophoretic position (lane 2) as compared to undigested controls (lane 1). However, PNGase F digestion of ECD254 produced no shift (lane 2), its electrophoretic position is identical to the undigested control (lane 1).

Since truncated hFSHR variants ECD213, and ECD335/His seemed to contain N-linked oligosaccharide complexes, further endoglycosidase digestions were performed to characterize the carbohydrate chains. This was accomplished by treating cell lysates with glycosidase H. Glycoproteins that have been processed beyond the *cis* Golgi are not expected to be glycosidase

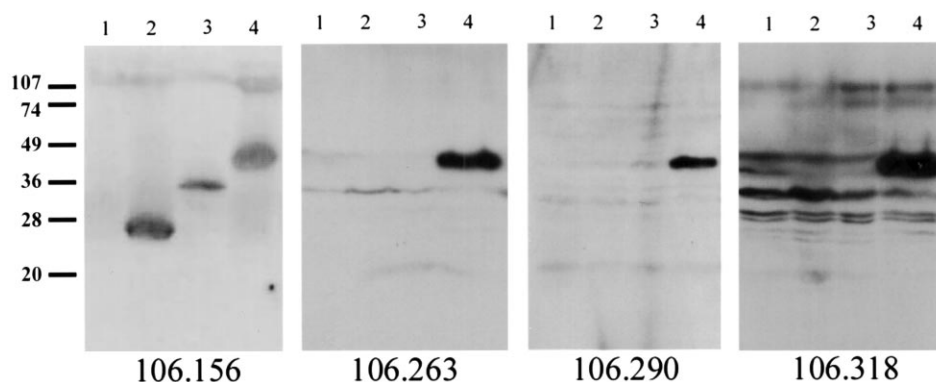


Fig. 2. Epitope mapping of mAbs by Western blot analysis of truncated hFSH-R variants. Whole cell lysates were prepared of CHO cells expressing hFSH-R variants (ECD213, ECD254, ECD335/His). Lysates were subjected to electrophoresis in 12% SDS-polyacrylamide gels, and the gel resolved proteins were then transferred to immobilon membrane. Protein blots were probed with mAbs, followed by horseradish peroxidase-conjugated goat anti-mouse IgG, and immunoreactive peptides detected by chemiluminescence. Panel 1; 106.318; panel 2; 106.290; panel 3; 106.263; panel 4; 106.156. Each panel displays mAb reactivity to lane 1, CHO; lane 2, ECD213; lane 3, ECD254; lane 4, ECD335/His. The migration position of molecular weight markers are at the left of panels.



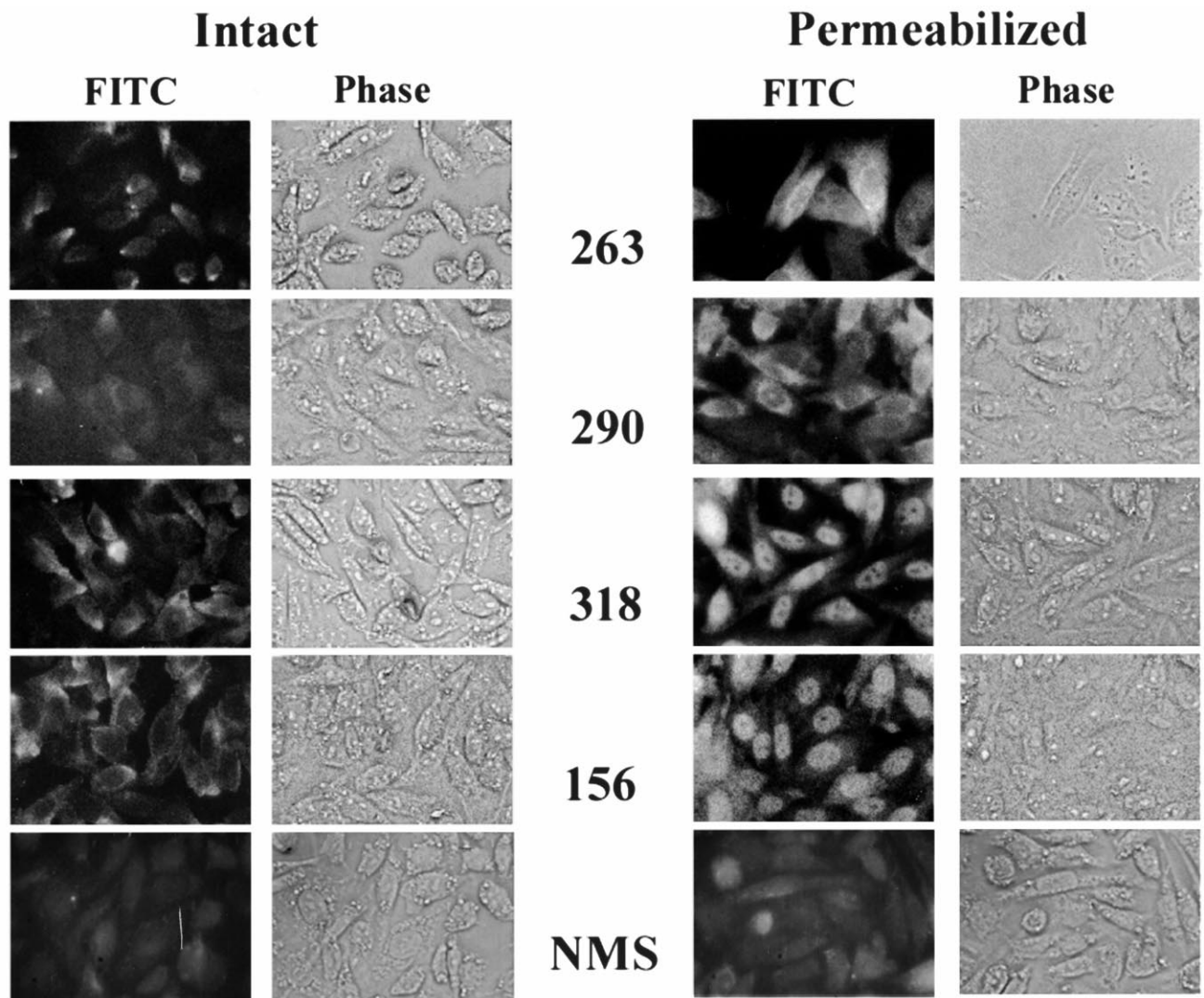


Fig. 3. mAb staining pattern of CHO cells expressing wild type hFSH-R. CHO cells expressing wild type hFSH-R and CHO cells (control) were grown on glass cover slips, washed, fixed (Intact cells) or detergent treated (Permeabilized cells). Both cell sets were blocked, stained with protein A purified mAbs (5 µg/ml), followed by FITC-conjugated goat anti-mouse IgG (1:1000 dil). The mAb used to produce the immunofluorescence micrograph is noted at the right of panel. Micrographs at the left are FITC/immunofluorescence images, corresponding phase micrographs are shown at the right.

H sensitive. The electrophoretic mobilities of truncated hFSH-R variants ECD213, and ECD335/His changed following digestion. The susceptibility to Endo H digestion suggests that truncated hFSH-R variants ECD213 and ECD335/His are not processed beyond the *cis* Golgi. The *cis* Golgi is the last point in the secretory pathway that complex mannose oligosaccharides may be found before they are trimmed by Golgi  $\alpha$  mannosidase I and  $\alpha$  mannosidase II (Kornfeld and Kornfeld, 1985).

To investigate this point further hFSH-R preparations were subjected to digestion with neuraminidase. In mammalian cells, sialic acid residues are added to the terminal ends of oligosaccharide chains of glycoproteins in the *trans* Golgi (Kornfeld and Kornfeld, 1985). Since wild type hFSH-R is sialated (Davis et al., 1995), one may expect that truncated hFSHR variants

would also possess sialic acid if fully processed and traffick past the *trans* Golgi. When neuraminidase-digested cell extracts (Fig. 6, lane 4) were compared to the undigested controls (Fig. 6, lane 1) on 12% SDS-PAGE, no detectable change in their electrophoretic position was observed. To verify enzyme activity, extracts prepared from cells expressing wild type hFSH-R were subjected to neuraminidase-treatment. Neuraminidase-treatment produced a smaller form of wild type hFSH-R compared to hFSH-R displayed by untreated lysate (Data not shown). These data indicate that post-translational processing of truncated hFSH-R variants ECD213, and ECD335/His is interrupted, by halting the molecules passage through the secretory system at some point somewhere between the endoplasmic reticulum and the *cis* Golgi.

### 3.7. Colocalization of truncated hFSH-R variant ECD335/His and endoplasmic reticulum

To further confirm the subcellular localization of hFSH-R variant ECD335/His, CHO cells expressing ECD335/His were double-labeled with 106.156 and wheat germ agglutinin (WGA) or concanavalin A (ConA). The lectin ConA recognizes  $\alpha$ -linked mannose or core oligosaccharides commonly found in the endoplasmic reticulum while WGA recognizes *N*-acetylglucosamine residues which are structures commonly added to membrane glycoproteins in the Golgi (Virtanen et al., 1980; Guasch et al., 1995). Co-localization of ECD335/His (red) and endoplasmic reticulum (green) in the digital pictures is represented in yellow (Fig. 7), the truncated hFSH-R variant (red) co-localized with ConA staining ER (green) to produce yellow signal. Non-transfected control cells stained with ConA and 106.156 and WGA show organelle staining, and the specificity of the 106.156 mAb, respectively. Truncated hFSH-R variant ECD335/His, did not colocalize with WGA staining Golgi and did not produce a yellow signal (data not shown). These results combined with our results from previous glycosylation studies, indicate that the truncated variant ECD335/His

is not processed beyond the ER, even though the variant binds FSH with equal affinity as wild type FSH-R in soluble binding assays (manuscript in preparation).

## 4. Discussion

The aim of this study was to define the subcellular location of truncated hFSH-R variants and to examine whether the intracellular processing and trafficking of truncated receptor variants differs from that of the wild type hFSH-R. To do this we progressively truncated the C-terminal end of the hFSH-R at amino acid residues 335, 254, and 214 and stably expressed the hFSH-R variants in CHO cells. Using mAbs against hFSH-R ECD, we were able to follow the intracellular trafficking and processing of truncated hFSH-R variants in CHO cells. We present evidence that all three truncated ECD variants, ECD213, ECD254, and ECD335/His were found to be trapped in the endoplasmic reticulum. ECD213 and ECD335/His display a glycosylated form containing immature, high mannose carbohydrate, and no sialic acid, while ECD254 appeared not to be glycosylated.

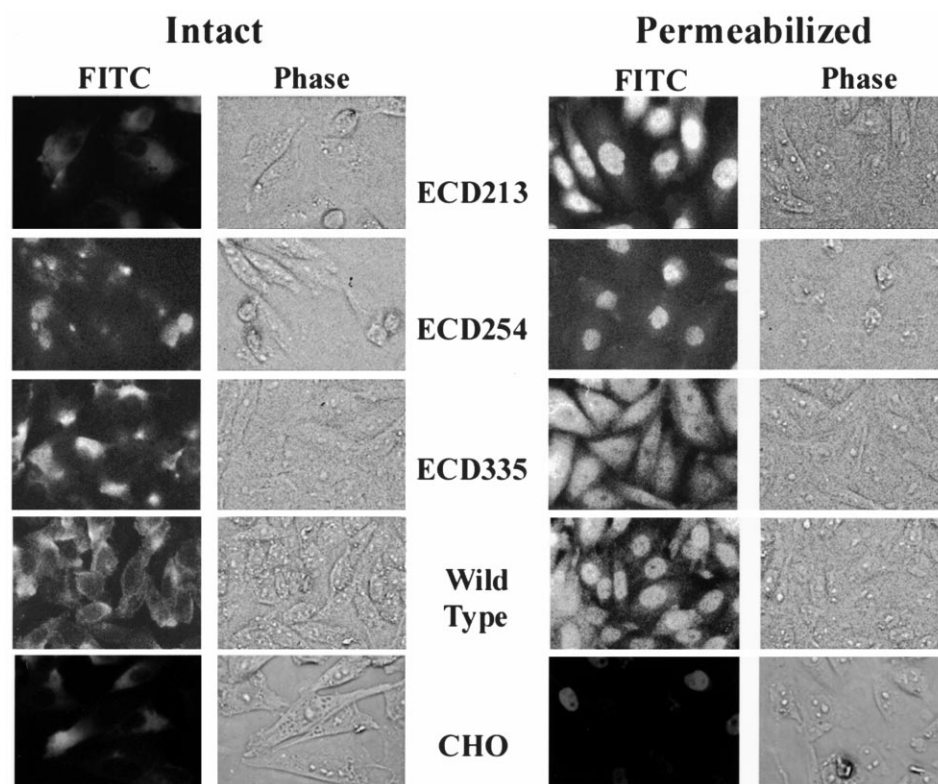


Fig. 4. Mab 106.156 staining pattern of CHO cells expressing hFSH-R variants. CHO cells expressing wild type and truncated hFSH-R variants, along with CHO cells (nontransfected) were grown on glass cover slips, washed, fixed (Intact cells) or detergent treated (Permeabilized cells). Cells were blocked, stained with protein A purified mAb 106.156 (5  $\mu$ g/ml), followed by FITC-conjugated goat anti-mouse IgG (1:1000 dil). Micrographs at the left are FITC/ immunofluorescence images, corresponding phase micrographs are shown at the right.

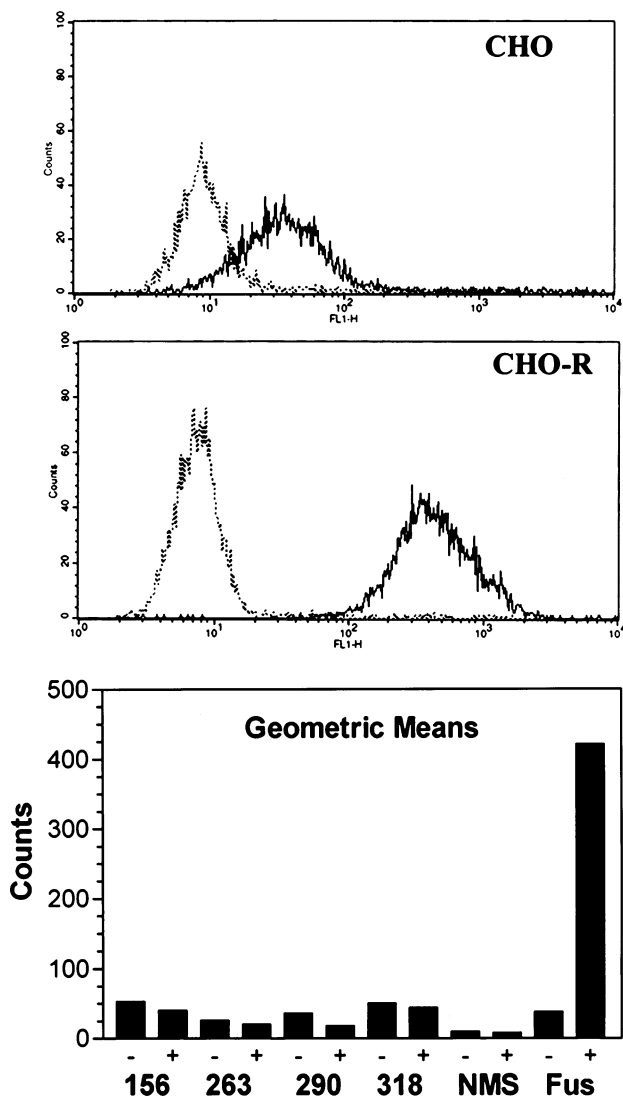


Fig. 5. Reactivity of mAbs to native hFSH-R expressed on the cell surface. Conditioned hybridoma media from clones 106.156, 106.263, 106.290, and 106.318 was tested for reactivity to native cell surface expressed hFSH-R by flow cytometry according to Section 2. Normal mouse sera (NMS) acted as the negative control (---), while sera from ECD-immunized mouse (FUS) which was used for hybridoma fusion acted as the positive control (—). Graphs display the cell surface immunofluorescence intensity with NMS and FUS against CHO cells (top), and against CHO-hFSH-R cells (middle). The geometric means were determined from Becton Dickinson FACScan flow cytometer data using cell quest software. The cell surface immunofluorescence intensity for NMS, FUS, and mAbs 106.156, 106.263, 106.290, and 106.318 against CHO (—) and CHO-hFSH-R (+) are displayed as geometric means in lower graph.

Several alternately spliced FSH-R mRNA transcripts coding for different truncated variants of the FSH-R have been described (Gromoll et al., 1992; Khan et al., 1993; Hipkin et al., 1995; Rajapaksha et al., 1996; Sairam et al., 1996, 1997; Yarney et al., 1997; Khan et al., 1997; Kraaij et al., 1998; Yaron et al., 1999; Tena-

Sempere et al., 1999). Investigators have postulated that alternative spliced forms of the FSH-R should alter hormone binding to mature wild type receptor on the cell surface and act as a regulatory mechanism to modify FSH binding affinity. This hypothesis is feasible only if truncated receptor variants are trafficked to the cell surface.

Using mAbs made against insect cell expressed ECD335/His, it was possible to detect intracellular trafficking and post-translational processing of three truncated hFSH-R variants in CHO cells. ECD213 and ECD254 are not transported to the cell surface, while ECD335/His appeared distributed perinuclear and in close proximity to the cell surface. It was also possible to study full length hFSH-R because mAbs 106.318 and 106.156 stained the cell surface of fixed, intact cells expressing wild type hFSH-R. The staining pattern of mature wild type hFSH-R was punctuated evenly over the entire cell surface (Figs. 3 and 4) similar results have been reported by us, using an anti-peptide antibody as a probe (Liu et al., 1994). In contrast to the staining pattern of wild type cell surface hFSH-R, the distribution of ECD335/His in intact cells appeared as a single stained patch (Fig. 4). We reason that if ECD335/His is expressed on the cell surface its distribution should reflect the cell surface distribution of wild type hFSH-R. When intact cells expressing truncated hFSH-R variants are used in radio-receptor binding assays no FSH binding is detected. However, after detergent treatment cells expressing ECD335/His bind FSH equally as well as detergent treated cells expressing wild type hFSH-R (manuscript in preparation). Immunofluorescence microscopy examination of detergent-permeabilized cells revealed that ECD213, and ECD254 are trapped in the endoplasmic reticulum/nuclear continuum (Fig. 4), while ECD335/His appeared in extended endoplasmic reticulum (Figs. 4 and 6). Wild type hFSH-R was found distributed in organelles of the secretory system throughout the cell cytoplasm (Fig. 4). Based upon mAb 106.156 staining intensity, cells expressing ECD335/His appear to have a higher intracellular level of receptor than cells expressing wild type hFSH-R (Fig. 4). These data taken collectively indicate that no truncated hFSH-R variants reach the plasma membrane but are trapped within the ER of the cell. It is plausible that the staining pattern observed on the cell surface of intact cells CHO expressing ECD335/His by mAb 106.156 may be due to comprised cell integrity, since Fig. 6 (ConA/106.156) displaying permeabilized CHO cells expressing ECD335/His shows perinuclear staining which co-localizes to the ER.

As expected from our microscopy studies, in contrast to wild type hFSH-R, truncated hFSH-R variants undergoes minimal post-translational processing. Digestion with PNGase F, which removes most N-linked

oligosaccharides from denatured glycoproteins indicated that ECD213, and ECD335/His were both glycosylated, while ECD254 was not. Endoglycosidase H cleaves only mannose-rich N-linked oligosaccharide chains from proteins that have yet to be processed in the *trans* Golgi. Both ECD213 and ECD335/His were sensitive to Endo H displaying a 3000 Da reduction in molecular size after enzyme digestion. The insensitivity

of truncated hFSH-R variants to the action of neuraminidase which cleaves sialic acid from N-linked oligosaccharide chains provides additional evidence that these proteins are not trafficked beyond the endoplasmic reticulum.

Our results for ECD335/His are similar to those previously reported by Davis et al. (1995) who analyzed a rFSH-R variant truncated at amino residue 346. Both

Table 2  
Characteristics of hFSH-R mAbs

Mab designation	IgG subclass <sup>a</sup>	hFSHR reactivity <sup>b</sup>	Reactive ECD peptide <sup>c</sup>	Flow reactivity <sup>d</sup>	Fixed cell reactivity <sup>e</sup>
106.156	IgG2 <sub>A</sub>	2.511	183–220	Negative	Cyto/surface
106.263	IgG <sub>1</sub>	3.990	300–331	Negative	Cyto
106.290	IgG <sub>1</sub>	3.940	300–331	Negative	Cyto
106.318	IgG <sub>1</sub>	3.596	300–331	Negative	Cyto/surface

<sup>a</sup> The isotype subclass of mAbs was determined by BIAcore analysis.

<sup>b</sup> hFSH-R reactivity is the 410 nm absorbance value produced in ELISA in which wells coated with 100 ng of purified insect cell expressed ECD335/His received 200 µl of conditioned hybridoma media (dil 1:3). ECD335/His is a hFSH-R variant truncated at Ala<sup>335</sup> which possessed a 5-His tag at its carboxyl-terminal end. After an overnight incubation at 4°C, the wells were washed, and challenged with alkaline phosphatase conjugated goat anti-mouse IgG for 1 h at RT. The wells were washed again and 100 µl of development substrate added, after 30 min the absorbance was read at 410 nm using a Dynatech MR700 plate reader.

<sup>c</sup> Reactive ECD peptide is that synthetic ECD peptide to which mAb reacted (see Table 1).

<sup>d</sup> Flow reactivity (flow cytometry) examined whether mAbs recognize native wild type hFSH-R on the cell surface (see Fig. 5).

<sup>e</sup> Fixed cell reactivity is based on the immunofluorescence pattern displayed by intact and permeabilized cells expressing wild type hFSH-R after mAb staining (see Fig. 3).

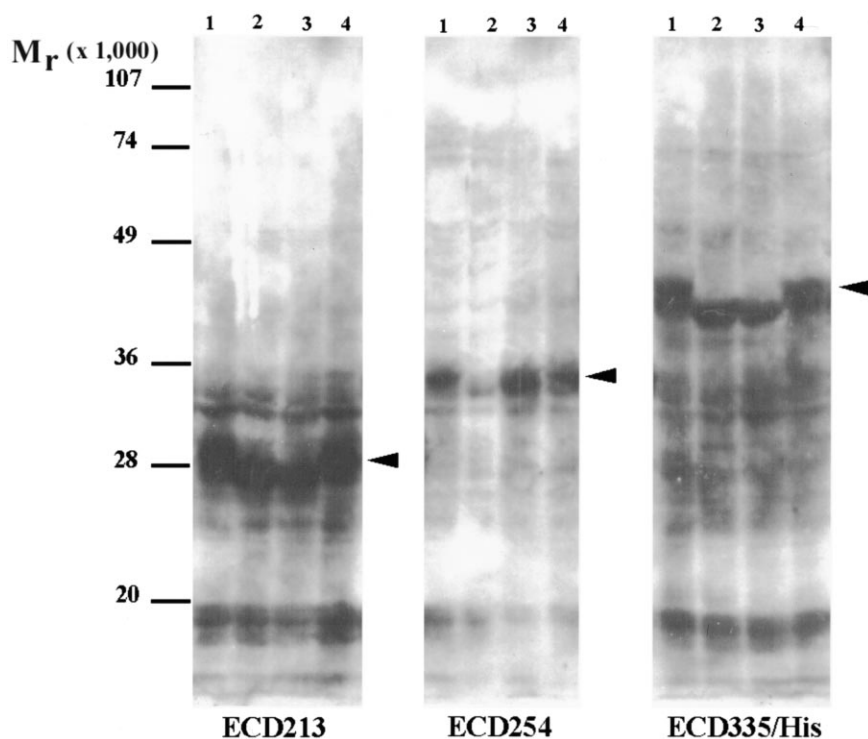


Fig. 6. Glycosidase digestion pattern of hFSH-R variants. CHO cells expressing hFSH-R truncated variants were solubilized, and then digested with either Endo H, PNGase F, or neuraminidase according to Section 2. Panel 1, ECD213; panel 2, ECD254; panel 3, ECD335/His. Lane 1, untreated cell lysate; lane 2, Endo H treated cell lysate; lane 3, PNGase F treated cell lysate; lane 4, neuraminidase treated cell lysate. Endoglycosidase treated and untreated lysates (prepared from  $2.5 \times 10^6$  cells) were analysed in a 12% SDS-polyacrylamide gel, and subjected to Western blot analysis. Membrane blots were probed with mAb 106.156 (1:10 dil), followed by horseradish peroxidase conjugated goat anti-mouse IgG (1:5000), and processed for ECL. The migration position of molecular weight markers are at the left of panels.

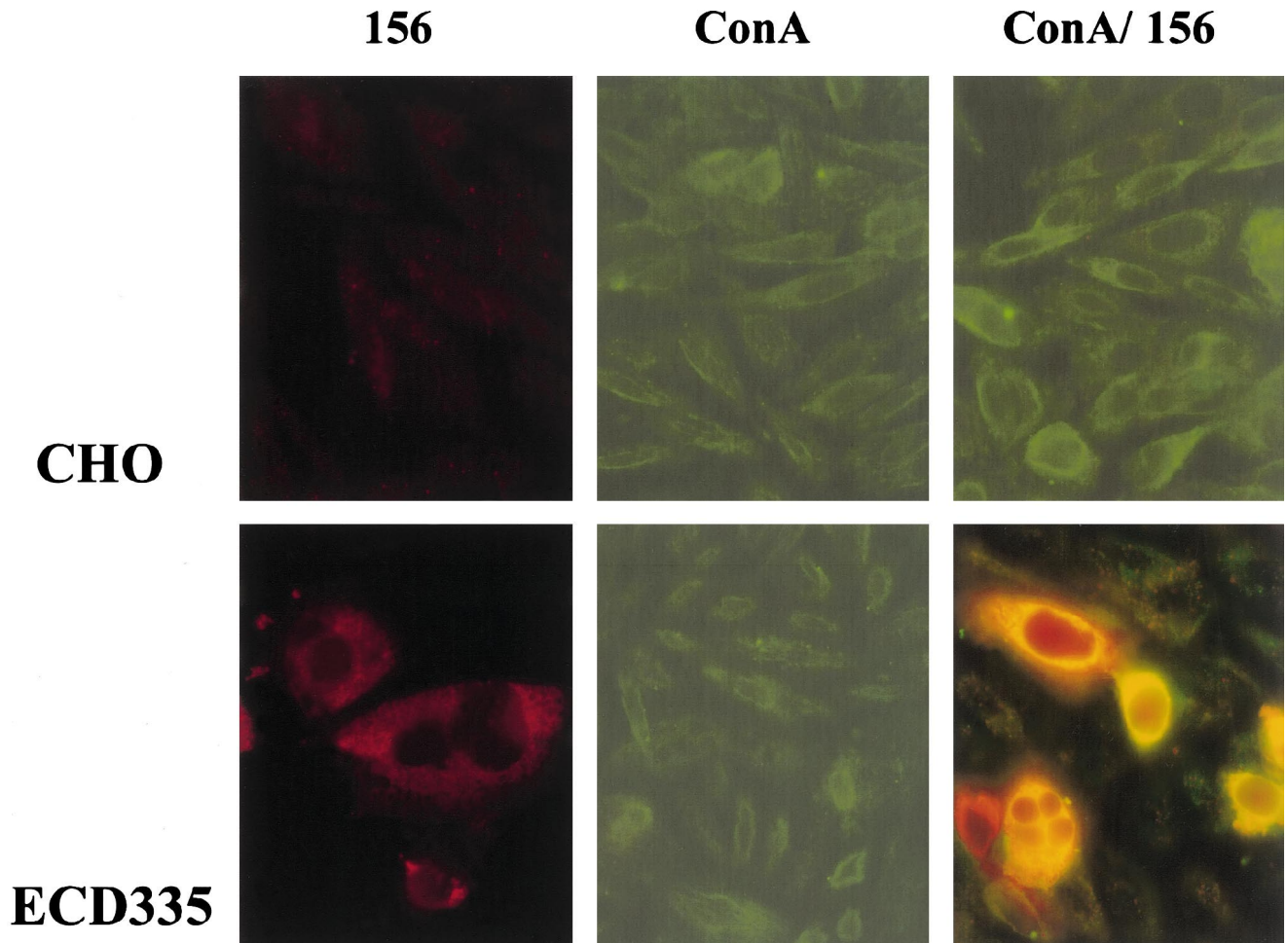


Fig. 7. Co-localization of ECD335/His and endoplasmic reticulum. Intracellular distribution and co-localization of hFSH-R ECD335/His was followed with mAb106.156, and Alexa568 conjugated goat anti-mouse IgG antibody. The endoplasmic reticulum was labeled with fluorescein-conjugated ConA. Images were collected using a double filter pack and digital camera. Co-localization of truncated hFSH-R (red) and organelles (green) in digital pictures is represented in yellow. CHO cells expressing ECD335/His stained with: ConA, mAb106.156, ConA/mAb106.156 (Top); while CHO cells stained with: ConA, mAb 106.156, ConA/106.156 (Bot).

truncated FSH-R variants display hFSH binding activity in detergent- solubilized cell extracts suggesting that these receptor variants are trapped within the cell. By Western blot analysis, rFSH-R(t346), a 55 kDa polypeptide, and ECD335/His, a 47.5 KDa polypeptide, both display an approximately 3000 Da reduction in their molecular size after incubation with endoglycosidase H. Neuraminidase digestion of ECD335/His or rFSH-R (Davis et al., 1995) has no effect on their electrophoretic mobility. These results suggest that both variants contain high mannose oligosaccharide chains. Treatment with PNGase F which cleaves N-linked oligosaccharides resulted in the molecular size change of 3000 Da as observed with endoglycosidase H. These results illustrate that N-linked oligosaccharides of variants are not as fully trimmed or processed as mature wild type FSH-R (Davis et al., 1995), yet both variants ECD335/His and rFSH-R variant (Davis et al., 1995) bind FSH as efficiently as wild type.

While variant splicing of hFSH-R mRNA transcripts commonly occurs there has been only three cases where FSH-R variants have been found expressed on the cell surface (Hipkin et al., 1995; Sairam et al., 1996; Yarney et al., 1997). One of these variants exhibits a selective alteration in its carboxyl-terminus domain and behaves as a dominant negative receptor capable of FSH binding but lacks signal transduction properties of wild type FSH-R (Sairam et al., 1996). The second variant lacked the conventional seven transmembrane domain and was anchored in the plasma membrane by a particular carboxyl-terminal of 36 amino acid residues containing a putative transmembrane segment (Yarney et al., 1997). The third variant, rFSH-R(t637), was expressed on the cell surface lacked the last 38 amino acids of the carboxyl terminus (Hipkin et al., 1995). From these studies it appears that two criteria are necessary for expression of FSH-R on the cell surface, N-linked glycosylation of Asn<sup>174</sup> for proper folding of the FSH-

R and a putative transmembrane segment at the carboxyl terminal.

The differences in intracellular trafficking of wild type and truncated hFSH-R variants may be explained by N-linked glycosylation of the receptor and the quality control mechanisms of the secretory pathway. These mechanisms regulate the biological activity of many membrane proteins by controlling the level of protein localized at the plasma membrane by a process termed quality control or architectural editing (Hurley and Helenius, 1997; Klausner, 1989). This editing operates by the following manner (see review (Hammond and Helenius, 1995)): Glucose residues are trimmed from N-linked oligosaccharides of newly synthesized proteins by glucosidase I and II, which allows proteins to bind to calnexin and calreticulin, lectins which recognize monoglucosylated oligosaccharides. Glucosidase II removes glucose residues and allows the protein to dissociate from the lectin. Partially folded proteins are reglucosylated by UDP-glucose:glycoprotein glucosyltransferase (UDP-Glc transferase) which allows the re-binding to calnexin/calreticulin and retention of the glycoprotein in the ER. Once a protein is folded correctly it is no longer recognized by the glucosyltransferase and is released from the cycle by glucosidase II.

Studies examining recombinant and wild type proteins in a variety of cell systems suggest that architectural editing tightly regulates the expression of cell surface proteins. Relatively minor defects can lead to intracellular confinement, halt intracellular processing and cause protein degradation. Sometimes the aggregation of misfolded proteins into large covalently or noncovalently cross-linked aggregates may confine misfolded products to the ER (Hurley and Helenius, 1997). The persistence of chaperone binding may result in protein retention (Gething and Sambrook, 1992; Yarney et al., 1997). Free sulfhydryl groups may result in a protein's permanent ER confinement (Sitia et al., 1990; Guenzi et al., 1994). Interestingly, 0.5–1% of the translated vertebrate genome represents genes involved in glycosylation (Dennis et al., 1999). The magnitude of this genomic commitment to glycoconjugate biosynthesis is on a par to that of protein phosphorylation and may play a role of equal importance in cell biology through its role in architectural editing.

hFSH-R plays a central role in reproductive physiology regulating antral follicle development in females and initiation and regulation of spermatogenesis in males. The ECD is encoded by parts of ten exons (Minegishi et al., 1991), consequently, architectural editing plays a critical role in assuring that only hFSH-R molecules that display proper tertiary conformation reach the cell surface. Obviously, FSH binding does not imply proper tertiary conformation of the ECD, since quality control prevents ECD335/His from reaching the cell surface. This in vivo mechanism prevents secretion

of the ECD335/His variant which could bind serum FSH, and thereby reduce physiological levels of the hormone locally, and interfere with fertility.

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