DISSERTATION

LOCALIZATION OF LUTEINIZING HORMONE RECEPTORS IN SMALL MEMBRANE COMPARTMENTS DURING SIGNAL TRANSDUCTION

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY JINGJING LIU ENTITLED LOCALIZATION OF LUTEINIZING HORMONE RECEPTORS IN SMALL MEMBRANE COMPARTMENTS DURING SIGNAL TRANSDUCTION BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

LOCALIZATION OF LUTEINIZING HORMONE RECEPTORS IN SMALL MEMBRANE COMPARTMENTS DURING SIGNAL TRANSDUCTION

Luteinizing hormone receptors (LHR) are G protein-coupled membrane proteins with important functions in reproduction. These receptors, upon binding of hCG, translocate into plasma membrane compartments of low buoyant density. To further explore ligand-receptor structures necessary for localization of LHR in small membrane compartments, we have used single particle tracking methods to monitor the lateral diffusion of individual LH receptors and the size of plasma membrane compartments accessed by the receptor on viable cells. Following treatment of CHO cells expressing FLAG-tagged rat LHR with 100nM hCG, LHR become confined in compartments with an average diameter of 86 ± 36 nm. These compartments are significantly smaller than the 230 ± 79 nm diameter regions occupied by the untreated receptor. Following treatment of cells with 0.01nM hCG, LHR are distributed in two groups. Approximately 65% of the receptors are confined in small compartments with a diameter of 69 ± 38 nm while the remaining receptors exhibit unconfined lateral diffusion in large compartments typical of untreated LHR. Palmitoylation of the LHR C-terminus appears to be important for raft localization and for confinement in small membrane compartments. LHR mutants lacking potential palmitoylation sites at position 621 and 622 in the C-terminus do not translocate into membrane rafts. These receptors remain in large compartments and their rapid diffusion coefficients are not affected by hCG treatment. The

cytoskeleton also appears to play a role in restricting hormone-treated receptors within small compartments: LHR on cells treated with cytochalasin D, a microfilament disruptor, exhibit fast lateral diffusion within large compartments both before and after exposure to 100 nM hCG. Possible membrane models to explain this behavior include compartmentalization by cytoskeletally-anchored proteins that serve as protein fences. However, any such model must also explain why only hormone-occupied palmitoylated receptors are laterally restricted.

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CHAPTER 1

BACKGROUND

The plasma membrane is a dynamic structure that maintains cell integrity and regulates the internal environment of the cell. Many membrane functions are carried out by proteins that, in addition to signal transduction, undergo movement and reorganization in the plane of the membrane. We are interested in the ability of membrane proteins to move between and within specialized compartments that exist in the plasma membrane. Here we used single particle tracking methods to study luteinizing hormone receptors involved in cell signaling. Specifically, we have evaluated effects of receptor structure on the localization of the receptor in small compartments and examined membrane features such as cholesterol-enriched microdomains and the actin cytoskeleton that might limit the motions of these receptors.

Plasma membrane structure and organization

Singer and Nicholson proposed the fluid mosaic model of the cell membrane in the early 1970s. In their model (Figure 1), the two-dimensional phospholipid bilayer contains proteins (1), most of which are embedded in the bilayer. There is a diverse mixture of various membrane lipids that make up the bilayer, most of which consist of a polar head group and acyl chains. Interestingly, lipids undergo nonrandom segregation within the bilayer and from

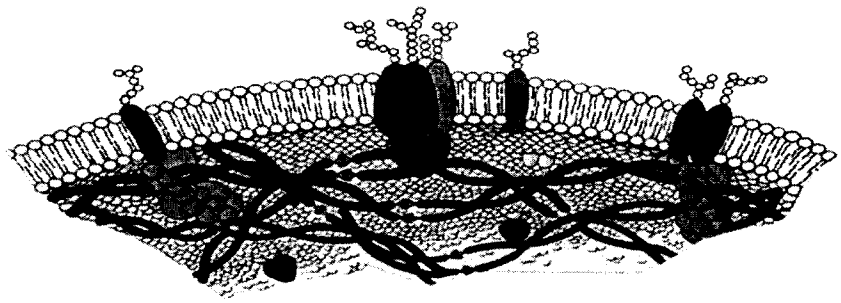

Figure 1: Membrane structure, modified from Stryer, Biochemistry, 4th edition (W. H. Freeman & Company, 1995).

lipid microdomains (2).

The Singer and Nicolson model suggests that proteins can move within the plane of the membrane. Since the introduction of this model, instrumental and microscopic methods for evaluating such motions have been developed. In 1974, the lateral diffusion of rhodopsin within the frog visual membranes was measured by Poo and Cone (3). In 1976, Axelrod and coworkers (4) developed fluorescence photobleaching recovery (FPR), an optical approach to measure the lateral diffusion coefficient of membrane proteins. FPR experiments use an attenuated laser beam focused onto a fluorochrome-labeled cell membrane. After recording of initial fluorescence intensity for several seconds, a brief pulse of laser light photo bleaches the fluorophores in the beam path. The attenuated beam is then used to monitor diffusion of unbleached fluorophores into the interrogated spot. Fluorescence recovery after photobleaching reveals the diffusion coefficient and fractional mobility of the cell surface protein being observed.

In addition to lateral diffusion over long distances, membrane proteins may also move within and between membrane compartments (5, 6). Baird and coworkers (7) have demonstrated that native, monomeric type I Fcϵ receptors (FcϵRI) occupied by IgE are localized mainly within the "bulk" plasma membrane regions. Upon binding of antigen by IgE, about 50% of the total receptor population is found in membrane fragments enriched in cholesterol and sphingomyelin called "rafts" that float into lower density sucrose within sucrose concentration gradients. These structures can also contain certain members of the heterotrimeric G protein family, namely G_s and G_i (8), as well as glycosylphosphotidylinisitol-(GPI)-anchored membrane proteins (9). Horejsi and coworkers

have isolated GPI-anchored proteins from human lymphocytes using cold Triton X-100 (10). These proteins were part of detergent-insoluble complexes that included src family tyrosine family kinases. This suggests that these cholesterol-enriched microdomains may function as signaling platforms (11).

Luteinizing hormone receptors

Rafts may transiently contain luteinizing hormone receptors (LHR) and serve as signaling platforms for signal transduction by LHR (12). Luteinizing hormone receptors are seven transmembrane domain-containing membrane receptors involved in reproduction. LHR has a single polypeptide chain containing 674 amino acid residues, 340 of which make up the large, N-terminal extracellular domain which is extensively glycosylated. The seven transmembrane domains consist of 24 amino acids each and the intracellular C-terminal tail is 70 amino acids long (13, 14). Because LHR is a single peptide chain, there are three extracellular and intracellular loops connecting the seven transmembrane domains, as shown in Figure 2. Rat LHRs have extensive glycosylation and, as a result, the cell surface receptor demonstrates a native molecular weight of approximately 85-95 kDa (13).

Human luteinizing hormone receptors bind either luteinizing hormone (LH) or human chorionic gonadotropin (hCG). These hormones are members of the glycoprotein hormone family that includes LH, hCG, follicle stimulating hormone (FSH) and thyroid stimulating hormone (TSH). Both LH and hCG exist as heterodimers consisting of one α and one β subunit and are assembled by non-covalent interactions (15). Both subunits are required for

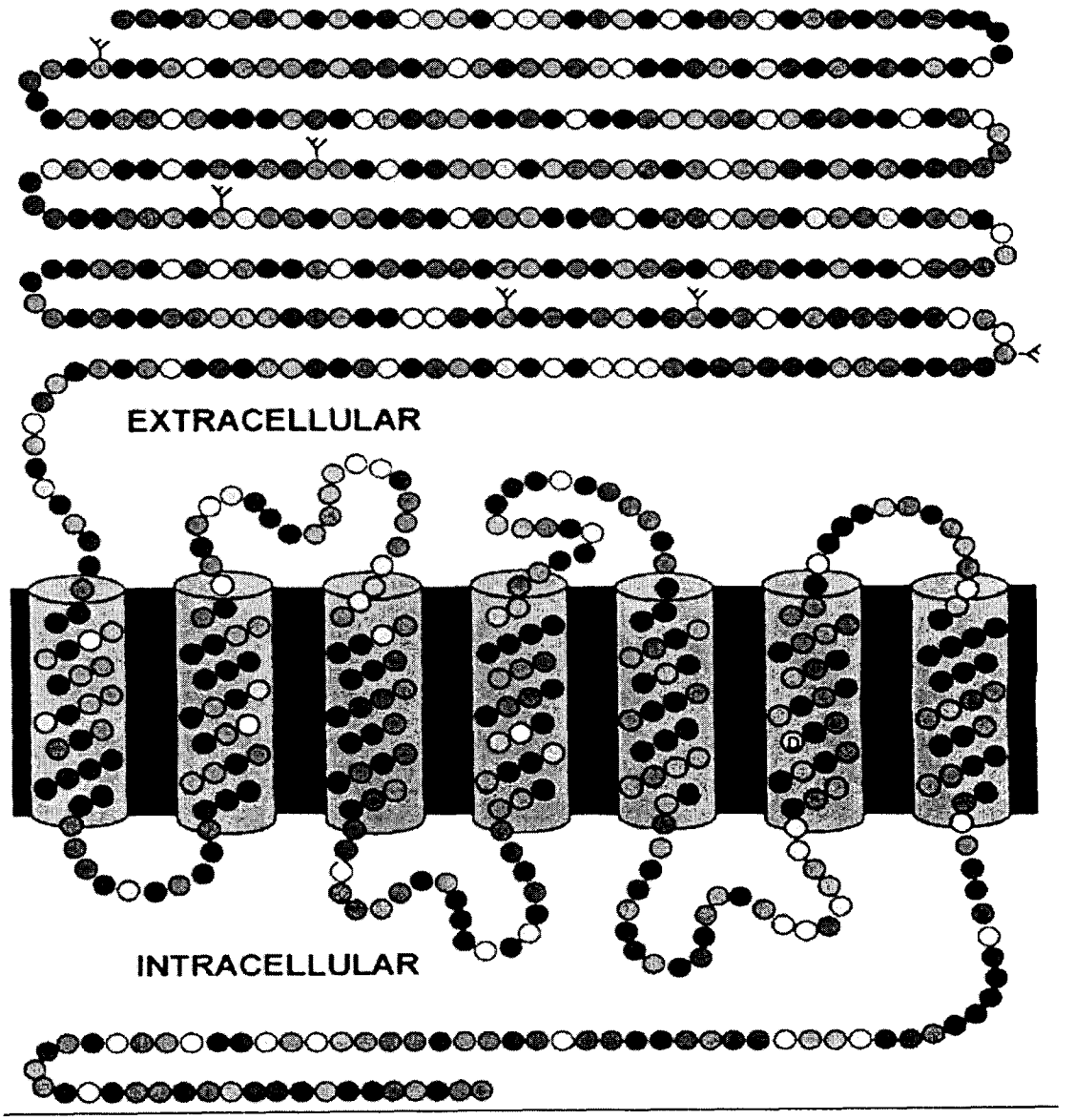

Figure 2: LH receptor organization from McFarland et al (22).

high affinity binding to LHR. The α subunit, a 92-96 amino acid peptide, is highly conserved for LH and hCG as well as the other glycoprotein hormones. The β subunit is specific to each hormone and more variability. The β subunit confers receptor specificity. The β subunit of LH contains 117 amino acids while the β subunit for hCG has 142 amino acids (16).

LHRs must function properly in both males and females for normal reproduction. In females, LHR serves two functions, both essential for successful reproduction. At approximately day 14 in the menstrual cycle, an LH surge from the anterior pituitary results in binding of LH to receptors on theca interna and follicular cells. LH binding leads, within approximately thirty-six hours, to ovulation. Then in early pregnancy, a placentally-derived structure, the chorion begins to secrete hCG in increasingly quantities. hCG binds to LHRs on the corpus luteum. Corpus luteum synthesis and release of progesterone is necessary for maintenance of the uterine lining in early pregnancy. Production of hCG reaches maximum levels by the tenth week of pregnancy. hCG release, however, maintain at a low but measurable level for the duration of the pregnancy.

In males, LH is responsible for many important functions, including embryonic sexual differentiation, development of the testes and normal functioning of the Leydig cells, and testosterone production. During fetal development, LHR on Leydig cells binds LH. These cells then secrete testosterone which drives growth and development of the male reproductive tract structures, including penis, prostate, and epididymis. After puberty, Leydig cells produce more testosterone in response to LH and this leads to the development of the secondary sex characteristics such as increased muscle mass, pubic and facial hair,

voice deepening and sperm production by the testes.

Signal transduction by LHR

LHR-mediated responses occur as a result of signal transduction following binding of either LH or hCG. The initial step in signal transduction involves interaction between the receptor and G proteins. There are three subunits that make up the G proteins, which are coded for by three distinct genes. The α-subunit binds guanine nucleotides with high affinity and specificity and has intrinsic GTPase activity. The β and γ polypeptides are non-covalently associated in a functional dimer subunit. Activation of the LHR causes the heterotrimeric Gs to dissociate into α, β and γ subunits, with the α subunit activating adenylyl cyclase. Adenylyl cyclase is a membrane protein that enzymatically converts adenosine monophosphate into cyclic AMP. Mediators of second messenger metabolism such as adenylyl cyclase, as well as phospholipase C and a variety of ion channels thus alter intracellular concentrations of important second messengers and ions. The results can be rapid effects on hormone secretion, muscle contraction and a variety of other physiologic functions. This process is summarized in Figure 3.

Signaling by the LHR is, as discussed earlier, carefully regulated. However, in Familial Male-limited Precocious Puberty (FMPP), a dominant mutation of LHR results in constitutive activation of the receptor (17) and overproduction of cAMP. cAMP accumulation causes Leydig cell hyperfunction and, in some cases, tumor formation (18). The naturally-occurring mutations affect thirteen different residues in LHR, with the majority

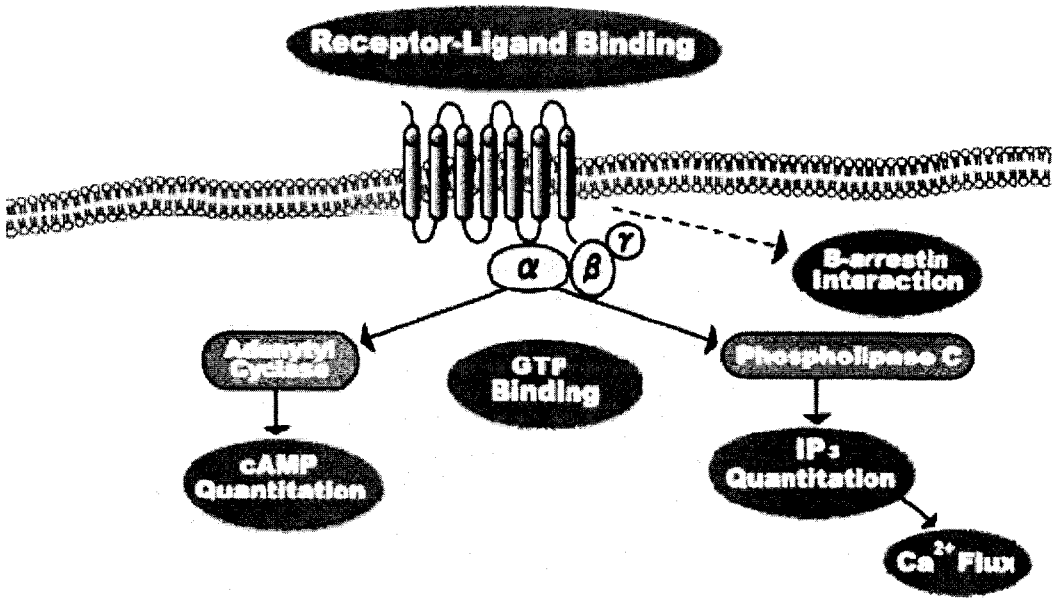

Figure 3: G protein-coupled receptor signaling. Modified from Perkin-Elmer product literature, 2006.

clustered in the cytoplasmic half of the 6th transmembrane helix. Signaling by constitutively active LHR appears to depend on the rearrangement of key electrostatic, hydrogen-bond, and hydrophobic interactions that normally stabilize the inactive LHR conformation (17).

Compartmentalization of membrane proteins

LHRs, in addition to signaling, become localized in membrane microdomains (12). Single particle tracking methods have been used to study the compartmentalization of membrane proteins. Single particle tracking (SPT) techniques allow observation of the movements of individual membrane proteins or lipids at the cell surface with nanometer spatial resolution. A gold nanoparticle is specifically attached to the molecule of interest. The displacement of the particle over time is recorded using video microscopy. Gold colloid is an electron-dense negatively charged hydrophobic particle suspension stabilized by electrostatic repulsions. Gold particles are sized from 1 to 150nm. Large gold particles (30-40nm) can be used as probes for labeling the surface of living cells.

SPT may help to answer questions about particle motions raised by fluorescence recovery after photobleaching (FRAP) measurements. SPT has several advantages over FRAP measurements: The spatial resolution is approximately two orders of magnitude higher than FRAP, the minimum detectable diffusion coefficient is lowered by approximately two orders of magnitude and it can measure individual trajectories. Another major advantage of SPT is its ability to resolve modes of motion of individual molecules (12).

Colloidal gold particles of 40 nm diameter microinjected into living cells are invisible

to the eye in the light microscope. Individual particles, can, however, easily be discerned using transmitted light at high numerical aperture by use of video contrast enhancement. In a light microscope, they appear as light dots with orange-red coating on a darker background. In epipolarization microscopy, they appear as brightly staining dots on a background consisting of the interference reflection picture of the cells. Because colloidal gold is a strong light scatterer that acts as a light sink rather than light source, in high resolution darkfield microscopy, gold particles are easily recognized.

Most preparations of colloidal gold consist of particles varying in diameter from about 5nm to around 150nm. The diameter D of the gold particle is much less than the wavelength of light, so the particle is a Rayleigh scatterer. The minimum detectable diameter is 15nm and the typical diameter used is 30-40nm. Gold particle are much stronger scatterers than cell organelles, so the organelles are almost invisible in bright field microscopy.

Nanometer-scale SPT is possible because the center of a small particle can be located with a precision well below the wavelength of light. The particle is much smaller than the wavelength of light, so its image is an Airy disk. Two nearby particles give partially overlapping Airy disks and, if the particles are too close, the pair cannot be resolved. For a wavelength of 546nm and a numerical aperture of 1.4, the radius of the Airy disk is 238nm. The limiting spatial accuracy in an SPT measurement is set by the mechanical stability of the apparatus and is obtained from trajectories of stationary particles. The scatter in position is 1-30nm, yielding a minimum observable D of $5x10^{-14}$- $1x10^{-13}$ cm^2s^{-1}. For mobile particles, the spatial resolution is decreased by the motion of the particle during the acquisition time of the image and is, therefore, a function of D. The acquisition time depends on the label. For

gold labels, images are usually obtained at the standard video rate.

Careful analysis of the trajectories is required to distinguish between the possible different modes of motion and can reveal submicroscopic or larger membrane structures. The goal of SPT data analysis is to sort trajectories into various modes of motion and to find the distribution of quantities characterizing the motion, such as the diffusion coefficient, velocity, directed diffusion exponent, corral size and escape probability. The analysis of the trajectories relies on the calculation for each of the trajectories of the mean square displacement (MSD).

There are several methods that are used to classify trajectories. Cherry and colleagues use the shape of the <r(t)> curve to classify trajectories (19). They calculate the experimental MSD and determine which analytical expression yields the best fit. Kusumi and colleagues characterize the shape of the <r(t)> curve in terms of the relative deviation (RD). They used their RD parameter to classify trajectories, reporting histograms of RD for their experimental values and Monte Carlo values for a pure random walk (20). Several modes of motion have been observed including: immobile, directed, confined, tethered, normal diffusion, and anomalous diffusion.

Confined motion may result from corrals formed by cytoskeletal proteins including actin filaments near the membrane. It might be caused by tethering to immobile species, or by restrictions to motion within lipid domains. In SPT measurements, several works have reported a significant fraction of confined motion with corral sizes in range of 250-1500 nm, and average residence times in the range of 3-35s.

Our goal in this project was to evaluate compartimentalization of murine and human LH

receptors using single particle tracking methods. In Chapter 2, raft localization of LHR is explored in more detail with additional characterization of LHR retention in small membrane compartments. Chapter 3 focuses on studies describing the structural relationship between the receptor and motions including those of constitutively activated LHR. In Chapter 4, we take several approaches to examining effects of LH receptor structure on receptor distribution in membranes. These studies used chimeric GnRH-LHR in which the LH receptor C terminus is truncated and or modified to prevent palmitoylation.

CHAPTER 2[1]

Luteinizing Hormone Receptors Translocate to Plasma Membrane Microdomains Following Binding of Human Chorionic Gonadotropin[1]

ABSTRACT

Receptor-mediated signal transduction by G protein-coupled receptors can involve redistribution of plasma membrane receptors into membrane structures that are characterized by insolubility in Triton X-100 and low buoyant density in sucrose gradients. Here we describe the translocation of wild type rat luteinizing hormone receptors from the bulk membrane into membrane microdomains (rafts) following the binding of hCG. In sucrose gradient ultracentrifugation of plasma membranes from cells stably expressing FLAG-tagged LHR-wt, receptors were located in high density membrane fractions before binding of hormone and in low density fractions following hCG treatment. Receptor translocation to low density sucrose fractions did not occur when cells were pretreated with 1% MβCD which reduces membrane cholesterol and disrupts rafts. Single-particle tracking of individual FLAG-LHR-wt receptors showed that hCG-treated receptors become confined in small compartments with a diameter of 86 ± 36 nm, which is significantly smaller than the

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This chapter was adapted from Steven M. L. Smith, Yin Lei, Jingjing Liu, George Barisas and Deborah A. Roess (2006) Luteinizing hormone receptors translocate to plasma membrane microdomains following binding of human chorionic gonadotropin, *Endocrinology*, 147, 1789-1795.

regions accessed by the untreated receptor (230 ± 79 nm). After disruption of rafts by MβCD, receptors were no longer confined in these small compartments which also decreased levels of cAMP production in response to hCG. Finally, translocation of LH receptors into rafts required a functional hormone-receptor complex, but did not occur after extensive receptor crosslinking which elevated cAMP levels. Thus, retention of LHR in rafts or small membrane compartments is a characteristic of functional, hormone-occupied LHR-wt. Although raft translocation was not essential for cAMP production, it may be necessary for optimizing hormone-mediated signaling.

INTRODUCTION

Several lines of evidence suggest that functional LHRs are clustered within relatively large structures following the binding of hormone. Electron micrographs of LHR on rat granulosa cells show large clusters of receptors that form only after binding of hormone (21) Immunofluorescent labeling of rat LH receptors shows similar responses in granulosa cells (22). Wild type rat LH receptors tagged with green fluorescent protein (LHR-GFP) aggregated within minutes following receptor binding of either LH or hCG on viable cells (23). This aggregation was accompanied by close interactions between individual receptors, as described previously by other strategies for evaluating fluorescence resonance energy transfer between LH receptors (24), and indicated by comparatively high values for fluorescence energy transfer efficiency between receptors tagged with variants of green fluorescent protein (25). The presence of receptors in relatively large structures has also been

suggested by lateral diffusion studies of the LH receptor in luteal cells from sheep (26) and rat (27) in which most LH receptors were laterally immobile.

Clustering of the rat LH receptor involves the movement of diffusely distributed receptors into discrete membrane sites (25). One question raised by such observations is whether the receptors cluster at arbitrary sites on the plasma membrane or become confined in membrane microdomains. Membrane microdomains include so-called rafts which, because of their high cholesterol and sphingolipid content, "float" in sucrose gradients. Rafts are enriched, not only with sphingolipids and cholesterol (2), but with GPI-anchored proteins (28). The lateral diffusion of specific membrane proteins within these microdomains is also reduced (29). Membrane domains, which on some cells may comprise a substantial fraction of the plasma membrane (30, 31), can contain membrane proteins necessary for cell signaling such as G proteins (32) and adenylyl cyclase (33). There is also evidence for transient protein targeting to membrane rafts including proteins involved in the regulation of G protein signaling (34). Together, these observations have led to suggestions that rafts might serve as signaling platforms for G protein-coupled receptors. We believe LHR signaling may be accomplished by targeting the receptors to environments that favor receptor-receptor interactions and G protein-mediated signaling.

An additional question raised by these observations is whether LHR function is dependent on receptor translocation into rafts. There are physical differences between functional hormone-receptor complexes, i.e., hCG- and LH-occupied wild type LH receptors, and those complexes that do not activate adenylyl cyclase. As examples, binding of the hCG antagonist deglycosylated hCG (35) to rat LHR does not produce receptor self-association

(36) or slow receptor rotational diffusion to the same extent observed for hCG-occupied LHR (37). These results indicate that larger receptor-containing structures do not form after receptor binding of deglycosylated hCG. Similarly, there is no self-association of rat LH receptors containing a substitution of arginine for lysine at amino acid 583 (LHR-K583R) which either partially (38) or fully (39) eliminates the cAMP response to hCG. Thus, if rafts serve as signaling platforms for rat LH receptors, non-functional receptors may be excluded in some manner from these structures or, alternatively, lack some critical receptor feature needed to direct the hormone-occupied receptor to rafts.

To address these questions, we have isolated membrane fragments from hCG- or deglycosylated hCG-treated CHO cells expressing wild type rat LH receptors and from hCG-treated LHR-K583R which have been all been labeled at their N-terminus with the FLAG sequence. To further demonstrate the localization of functional LH receptors within rafts, we have treated cells with MβCD which can efficiently remove cholesterol from the plasma membranes of live cells (40, 41) and thus disrupt raft structure. To independently examine the effects of hCG on LH receptor motions within the plasma membrane, we have used single-particle tracking methods to evaluate the size of membrane compartments accessed by individual receptors as visualized microscopically on viable cells (42). Finally, we examined whether disruption of membrane rafts is accompanied by altered signaling in CHO cells in response to hCG (36)

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose was purchased from VWR (West Chester, PA). Geneticin was purchased from Invitrogen (Carlsbad, CA). MβCD, HEPES and non-essential amino acids were purchased from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum (FBS) was purchased from Gemini Bio Products (Woodland, CA). hCG was purchased from Research Diagnostics Inc. (Flanders, NJ). Cellular cAMP was measured using a "Direct Cyclic AMP Correlate-EIA" kit (Assay Designs, Ann Arbor, MI) as per the Manufacturers instructions. Colloidal gold (40 nm) was purchased from Ted Pella, Inc. (Redding, CA). Deglycosylated hCG was a kind gift from Dr. Henry Keutman. Cytochalasin D (20 μg/ml).

Cell lines

To test whether the rat wild type LH receptor becomes associated with membrane rafts following binding of ligand, we generated a stable cell line expressing the FLAG-tagged receptor. Dr. K.J. Menon from the University of Michigan kindly provided us with N-terminal FLAG- tagged LHR subcloned into the pFLAG vector (Sigma, St. Louis, MO). Using the FLAG-LHR vector we made a mutation of lysine 583 to arginine (FLAG-LHR-K583R). CHO cells were stably transfected with 5 μg of the FLAG-LHR or K583R-FLAG-LHR vector using Lipofectamine-Plus (Gibco-BRL, Gaithersburg, MD) as per Manufacturers instructions. Selection of stable clones expressing the FLAG-tagged receptors was based on

the acquisition of geneticin (G418) resistance. CHO cell lines were maintained in Dulbecco's modified Eagle media supplemented with 4.5 g/mL glucose and containing 10% FBS, 100U/mL penicillin, 100 μg/mL streptomycin and 1x MEM non-essential amino acids (Sigma Chemical Co., St.Louis, MO) that was supplemented with 400μg/mL G418.

Isolation of plasma membrane rafts

Cells were incubated with either 100nM hCG or 100 nM deglycosylated hCG (35), or PBS for 1 hour at 37°C prior to cell lysis. To isolate membrane rafts from LHR-wt and LHR-K583R cells, 5 x 10^7 cells were washed two times with phosphate-buffered saline, pH 7.2 (PBS) and lysed for 5-10 minutes on ice in 1ml of a buffer containing 25mM MES, 150mM NaCl, 2mM EDTA, 20% glycerol, 0.25% Triton-X100, and protease inhibitors including aprotinin, leupeptin, EDTA, and PMSF (Roche). A low speed 300 x g spin was used to remove cell nuclei and large cell debris. For raft studies, 1 mL of this supernatant containing plasma membrane fragments was then combined with 1mL of 80% sucrose containing 0.25% Triton-X100 and protease inhibitors to produce a 40% sucrose solution. A discontinuous sucrose gradient from 10-80% was created with the sample in 40% sucrose layered within this gradient. The gradient was loaded into a Beckman SW-41 swinging bucket rotor and spun at 175,000x g for 20 hours at 4°C. After the spin, eighteen 650μL fractions were carefully collected from the top of the gradient downward. A 50 μL aliquot from each fraction was diluted 1:1 with 95% SDS and 5% β-mercaptoethanol. After separation of proteins from each fraction using SDS-PAGE and transfer of proteins to

nitrocellulose, the LH receptor was identified using 30μg of an anti-FLAG M2 monoclonal antibody (Sigma, St. Louis, MO). The amount of receptor in each fraction was measured using a Bio-Rad GS-800 calibrated densitometer. The sucrose concentration in each fraction was determined using a Bausch and Lomb refractometer. In some experiments, cells were pretreated for 1 hr at 37°C with 1% methyl-β-cyclodextrin (MβCD) in serum-free Dulbecco's modified Eagle medium containing high glucose prior to incubation with hCG or PBS or with 100 nM anti-FLAG antibody for 45 min at room temperature followed by a second 45 min incubation with 1 μM anti-mouse IgG (Sigma). MβCD neither binds nor inserts in the plasma membrane of cells but rather extracts cholesterol by including it in a central non-polar cavity of cyclic oligomers of glucopryanoside in α-1,4-glycosidic linkages (28). At this concentration, it was non-toxic to cells and did not compromise cell integrity (data not shown) (43).

Single-particle tracking of FLAG-LHR-wt receptors on individual cells.

Lateral dynamics and the size of domains accessed by individual FLAG-LHR-wt were evaluated using single-particle tracking methods as described by Kusumi et al. (42). Our probe was prepared using 40 nm nanogold particles conjugated with the lowest possible concentration of anti-FLAG monoclonal antibody (mAb) needed to stabilize the gold solution. This gold-labeled antibody was then incubated with CHO cells expressing FLAG-LHR-wt receptors. The anti-FLAG-gold concentration, typically 15 μg/mL, was then further reduced by addition of 1% BSA in PBS until there were approximately 1-4 gold particles per

cell. Probe binding was specific for FLAG-tagged receptors; when cells were preincubated with a 10-fold excess of anti-FLAG antibody, no anti-FLAG-gold particles were detected on cells. In some experiments, cells were treated with 100 nM hCG for 1 hr after labeling receptors with anti-FLAG mAb or cells were pre-treated with 1% MβCD for 1 hr prior to labeling with anti-FLAG antibody. Some cells were pretreated for 1 h at 37 C with cytochalasin D (20 μg/ml), a microfilament disruptor.

Individual nanoparticles were imaged using differential interference contrast with a 1.4 N.A. 63x objective in a Zeiss Axiovert 135 microscope. Images were acquired using a Dage IFG-300 camera and were recorded for 2 min (3600 frames) at approximately 30 nm/pixel under the control of Metamorph software from Universal Imaging (Downingtown, PA). The trajectories for individual gold particles were segmented into domains by calculation of statistical variance in particle position over times using procedures developed previously(44-46). The variance of a particle's position was calculated within windows of varying duration. These windows were translated along the particle trajectory, producing a variance plot that exhibits peaks that indicate inter-domain boundaries. These results were analyzed to yield the domain size and residence time for each particle. Effective macroscopic diffusion constants were calculated as the square of the domain diagonal divided by four times the residence time in the domain as previously described (46).

RESULTS

Functional, but not non-functional, LH receptors appear in membrane rafts following hCG

binding.

After isopycnic centrifugation of plasma membrane fractions from CHO cells expressing FLAG-LHR-wt, unoccupied LH receptors were found in sucrose fractions of relatively high densities (Figure 4). Immunoblots of FLAG-LHR-wt receptors identified by anti-FLAG antibody were developed as previously shown (12) and the relative amount FLAG-LHR-wt in each sucrose fraction was quantified using densitometry. Over 90% of FLAG-LHR-wt receptors were localized in sucrose fractions 10-15 where sucrose concentrations ranged from approximately 36-56%. Following treatment of cells with 100 nM hCG, there was marked change in the distribution of receptors to the lower density sucrose fractions. Over 80% of hCG-treated FLAG-LHR-wt receptors consistently appeared in fractions 3-7 which contained 14-26% sucrose. In addition, over 90% of the hormone-bound receptors appeared in these less dense sucrose fractions than did the untreated receptors.

The translocation of LH receptors from high to low density membrane fractions required functional hormone-receptor complexes; LHR from CHO cells expressing FLAG-LHR-K583R treated with hCG or FLAG-LHR-wt cells treated with deglycosylated hCG remained associated with higher density sucrose fractions (Figure 5) despite the presence of bound ligand. Crosslinking of FLAG-LHR-wt receptors using an anti-FLAG antibody either alone or together with an excess of a secondary polyclonal anti-mouse antibody (Figure 5) had no significant effect on the distribution of FLAG-LHR-wt within the sucrose gradient.

Depleting membrane cholesterol disrupts membrane rafts and decreases cAMP signals in

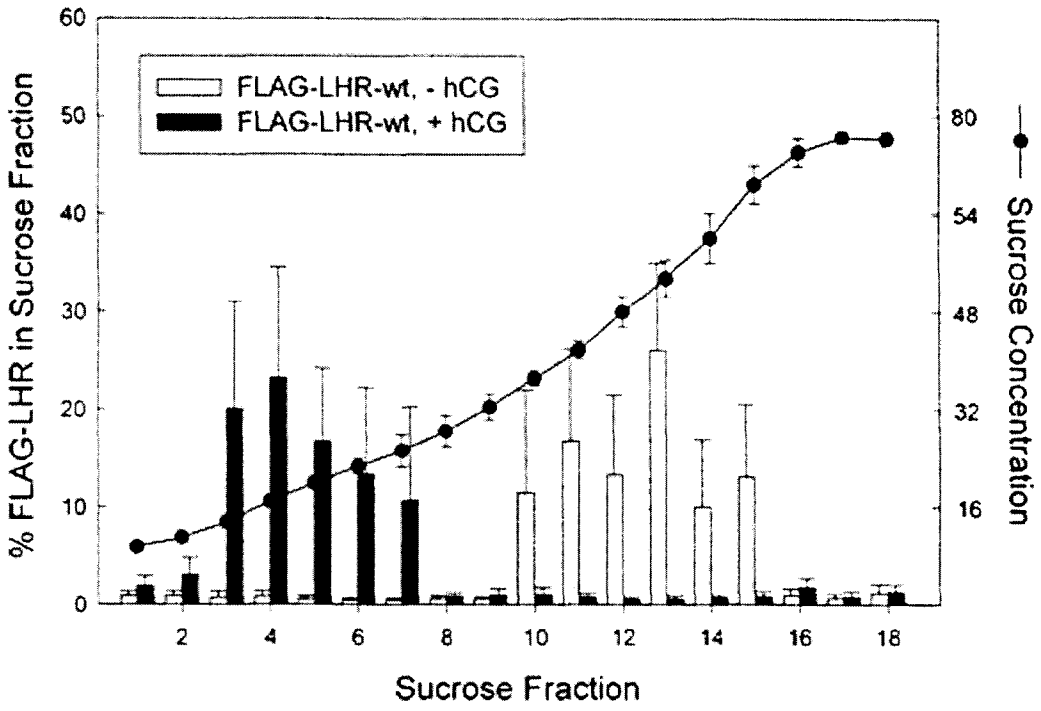

Figure 4. Before exposure to hCG, LHR-wt appear in high-density fractions of the sucrose gradient. After treatment of CHO cells with 100 nM hCG, receptors appear in lower-density fractions. Membrane fractions were separated as described in Materials and Methods, and densitometry was used to estimate the relative amount of LHR contained in each fraction. Results shown are the mean and SEM for at least five individual experiments. The sucrose concentration in each fraction was evaluated in five separate experiments using a Bausch and Lomb refractometer together with a standard curve. Because, for any given fraction, the sucrose concentration did not vary appreciably from experiment to experiment, the average sucrose concentration for five representative experiments is shown.

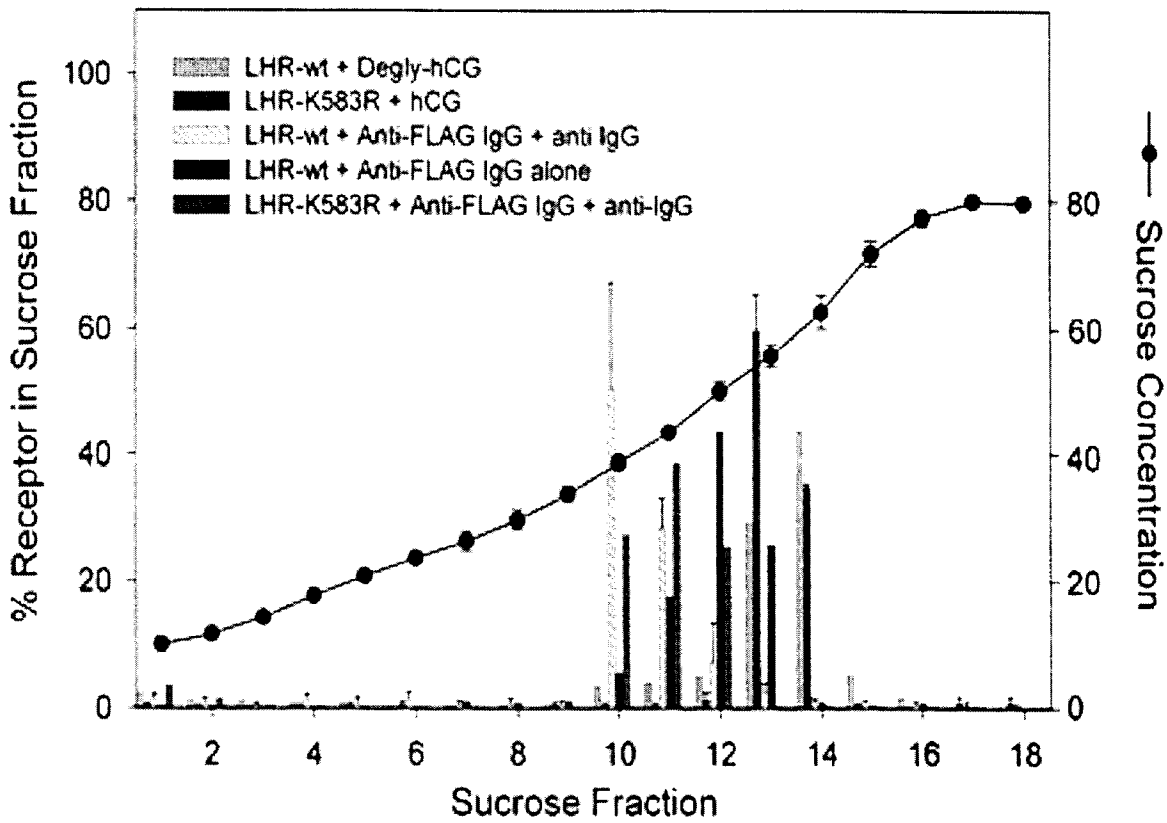

Figure 5. Translocation of LHR into buoyant membrane fractions required functional LHR. LHR-wt treated with 100 nM deglycosylated hCG (Degly-hCG), LHR-K583R treated with hCG or antibody treated (anti-FLAG antibody alone), or extensively cross-linked LHR-wt (anti-FLAG antibody followed by excess antimouse IgG) were not found in plasma membrane rafts.

response to hCG treatment.

Preincubation of cells for 30 minutes with 1% MβCD disrupted membrane rafts containing the LH receptor. Over 93% of hCG-treated FLAG-LHR-wt were found in fractions 10-12 following exposure of cells to 1% MβMD (Figure 6) which was essentially unchanged from the distribution of unoccupied FLAG-LHR-wt receptors on CHO cells treated with MβCD (data not shown) and to the distribution of unoccupied receptors shown in Figure 4. Interestingly, disruption of LHR-containing rafts with MβCD did not affect caveolae. Western blots prepared in tandem with those used to identify FLAG-LHR-wt were probed with an anti-caveolin antibody from Santa Cruz Biotechnology. Caveolin remained broadly distributed in lower density sucrose fractions under all experimental conditions (Figure 6) suggesting that LH receptors are associated with membrane microdomains that are distinct from caveolin-containing membrane regions.

Disruption of membrane rafts also reduced cell signaling in response to hormone. It has been suggested that plasma membrane rafts may serve as signaling platforms, effectively concentrating receptors and other plasma membrane molecules necessary for initiating down-stream signaling events. If this were correct, disruption of membrane rafts should reduce or eliminate selective signaling events dependent on this membrane micro-environment. We examined accumulation of cAMP in response to hCG in cells pretreated with 1% MβCD. Although, in our hands, changes in cAMP levels in CHO cells were modest, typically 2 to 7-fold over basal levels, pretreatment of cells with MβCD, nonetheless reduced hCG-mediated increases in intracellular cAMP to basal levels (Figure 7).

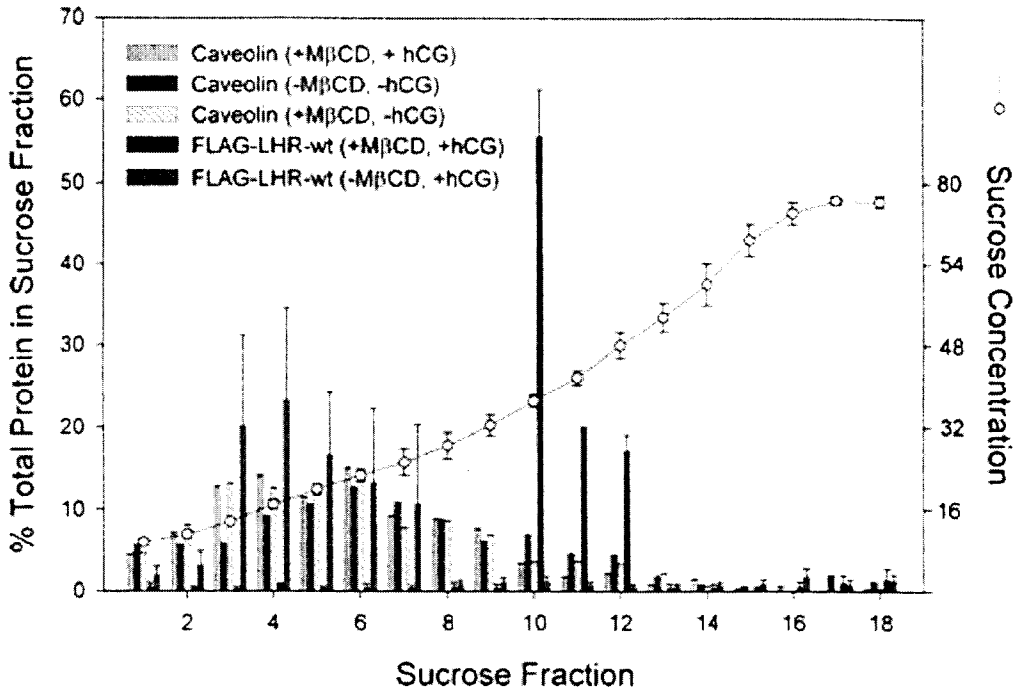

Figure 6. Disruption of plasma membrane rafts by extraction of cholesterol from the plasma membrane eliminated translocation of FLAG-LHR-wt into low-density sucrose fractions. Results shown are the mean and SEM for two separate experiments. In addition, anti-caveolin-1 antibody was used to identify caveolin in sucrose fractions. The distribution of caveolin in fractions 1–10 remained relatively constant in untreated cell samples and in samples exposed to hCG or MßCD.

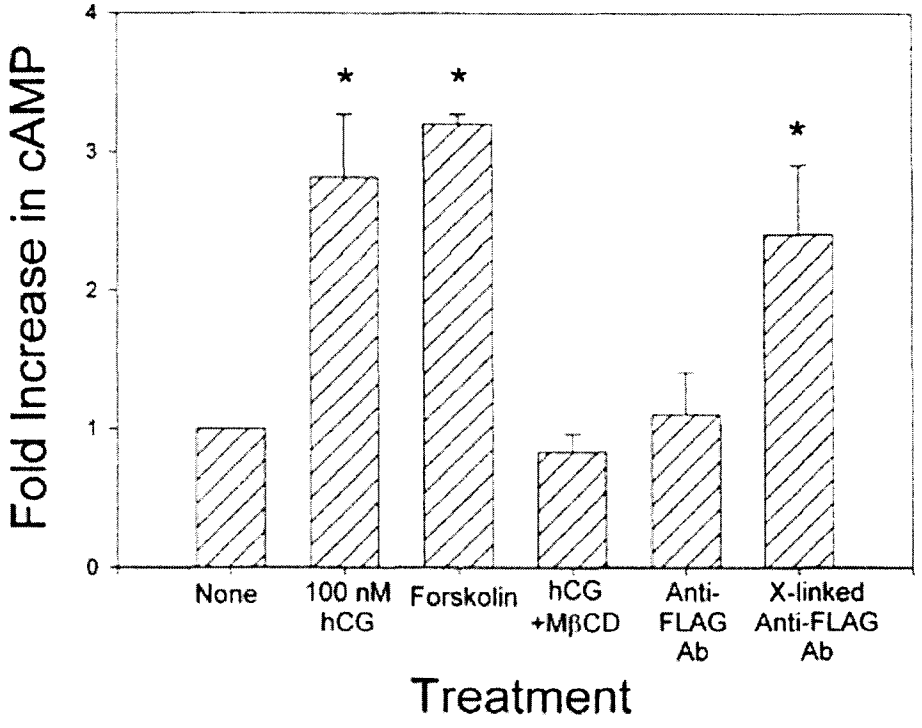

Figure 7. cAMP levels were assayed in CHO cells using a colorimetric cAMP kit from Assay Designs. There was an approximately 3-fold increase in cAMP in response to cell treatment with either 100 nM hCG or forskolin, and 1% MßCD reduced cAMP levels in hCG-treated samples to basal levels. Although exposure of FLAG-LHR-wt to monoclonal anti-FLAG antibody (Ab) had no effect on cAMP levels, cross-linking of the receptor with an excess of antimouse IgG elevated cAMP levels to values comparable to those of hCG-treated cells. Results are the mean and SEM for at least five experiments performed in triplicate.

Interestingly, extensive crosslinking of LHR-wt caused a significant increase in cAMP production to levels comparable to those of hCG-treated CHO cells or cells treated with forskolin even though this antibody-induced form of receptor self association was not sufficient to drive receptor translocation from the bulk membrane to rafts (Figure 5).

Single-particle tracking of hCG-occupied FLAG-LHR-wt receptors demonstrates "trapping" of receptors in small membrane compartments.

Figure 8 shows a representative particle track for an untreated LH receptor and for an hCG-treated receptor. The single-particle tracking results demonstrate that a gold particle bound to FLAG-LHR-wt exhibits distinctive motions in the presence and absence of hCG. hCG treatment reduces the size of compartments containing FLAG-LHR-wt from 230 ± 79 to 86 ± 36 nm (Table 1). Although the average residence time for receptors within microdomains and the number of microdomains, 6 ± 2 and 5 ± 2, accessed by the receptor in two minutes did not differ significantly for untreated and hCG-treated cells, respectively, an individual receptor's rate of diffusion (D) within each domain was reduced by a factor of ten following hCG treatment. Figure 9 illustrates these results for untreated and hCG-treated cells and shows that as receptors access progressively larger membrane microdomains, their rate of diffusion within those microdomains increases. We then treated CHO cells with hormone concentrations ranging from 0.1nm to 100nm. After treatment with 1nm and 100nm hCG, 62% and 91% of the receptors were confined in small compartments with a diameter of less than 100nm. The remaining receptors exhibit unconfined lateral diffusion in large

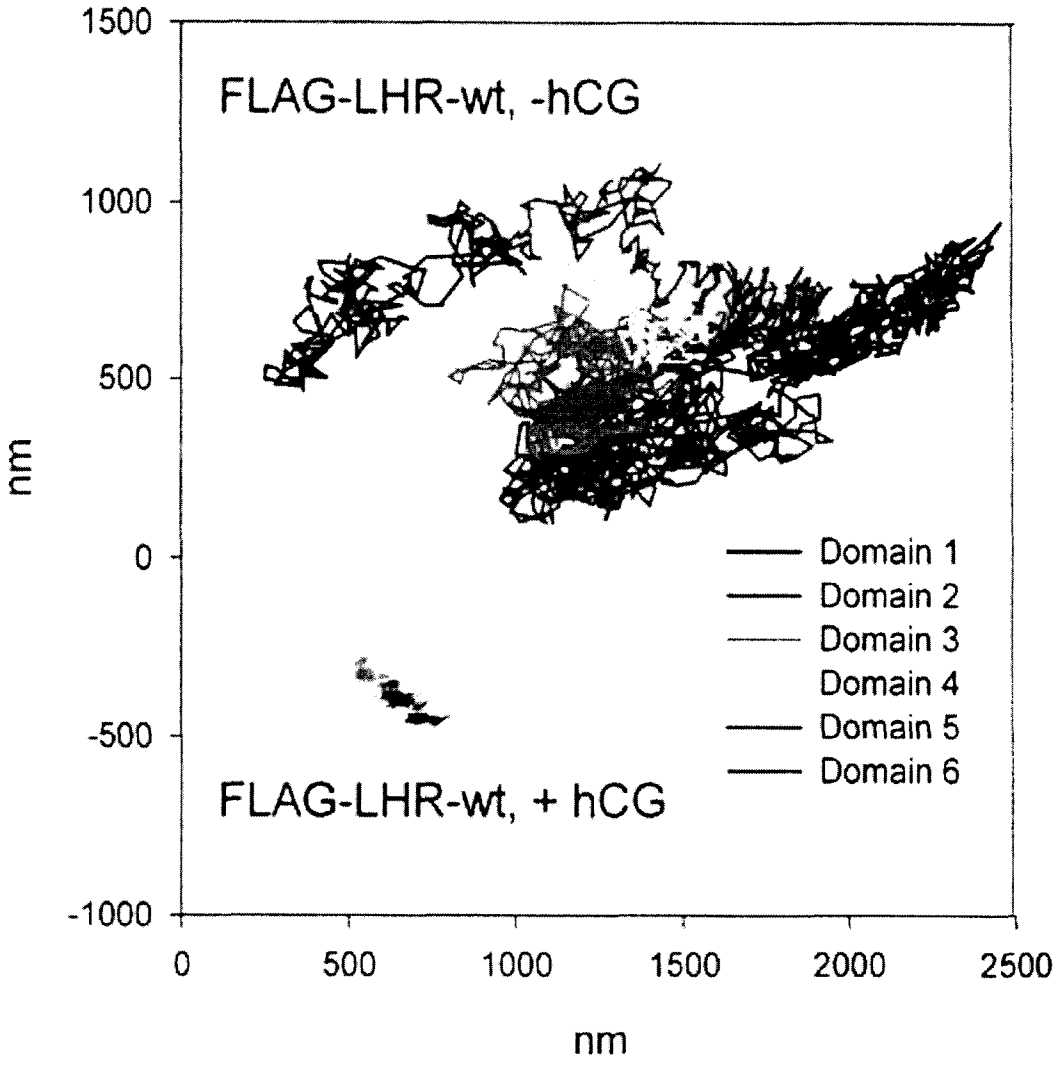

Figure 8. Representative trajectories for a single FLAG-LHR-wt receptor on an untreated cell or a cell treated with hCG. Each track represents data obtained during a single experiment of approximately 2 min. The representative track for untreated FLAG-LHR-wt cells shows receptor confinement within five compartments, whereas the representative track for hCG-treated cells shows confinement within three compartments. Individual compartments within a given particle trajectory were identified as described in Materials and Methods.

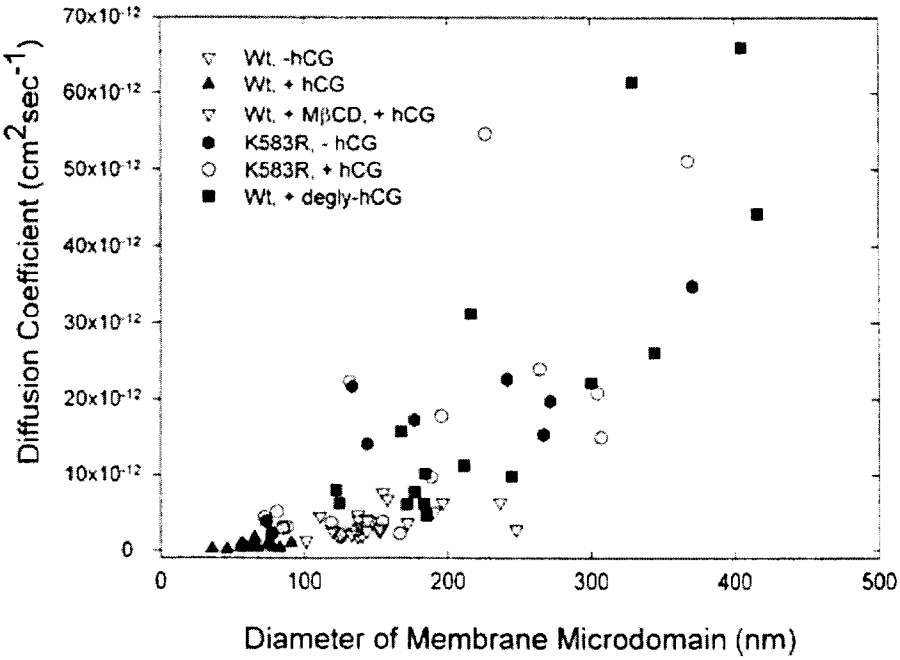

Figure 9. Single-particle tracking of individual FLAG-LHR-wt receptors labeled with gold-conjugated anti-FLAG antibody. The compartment size and diffusion coefficient for the LHR within that compartment were calculated as described in Materials and Methods. Results shown are from individual cells that were untreated (), treated with hCG after labeling of receptors with gold-conjugated anti-FLAG antibody (), or pretreated with 1% MßCD before labeling with anti-FLAG antibody and treatment with hCG (). Data presented in this figure for each condition are from cells examined in separate experiments on three different days.

Table 1: Tracking of individual anti-FLAG-gold particles bound to FLAG-LHR-wt on CHO cells.

Cell Line	Pretreatment	Hormone	Number of particles analyzed	Number of domains/ 2 min trajectory	$D_{0\text{-}1}$ [a] ($10^{-11}cm^2sec^{-1}$)	$D = L_r^2/4t$ [b] ($10^{-11}cm^2sec^{-1}$)	Time[d] (sec)	Domain Diameter (L_r)[c]
wt	none	none	20	6 ± 2	6.8 ± 3.8	1.2 ± 0.8	16 ± 13	230 ± 79
wt	none	hCG	20	5 ± 2	2.9 ± 1.1	0.1 ± 1.8	20 ± 11	86 ± 36
wt	MβCD	hCG	10	5 ± 1	4.8 ± 2.8	0.3 ± 0.8	19 ± 5	150 ± 12
wt	none	degly-hCG	10	2 ± 1	3.1 ± 1.9	3.4 ± 3.9	17 ± 14	256 ± 103
K583R	none	none	10	2 ± 1	3.3 ± 1.3	2.3 ± 2.2	27 ± 11	200 ± 92
K583R	none	hCG	10	2 ± 1	2.5 ± 4.0	1.8 ± 2.0	29 ± 11	164 ± 94

[a] $D_{0\text{-}1}$: Diffusion coefficient of the first 2 points.
[b] D presents the diffusion coefficient within a domain.
[c] The average diameter of an individual domain.
[d] Average time for residence within a domain

compartments typical of untreated cells (Figure 10).

Because MβCD pre-treatment largely reversed the effects of hCG on compartment size and receptor lateral diffusion, it seems plausible that compartments retaining hCG-treated receptors are rafts. As shown in Table 1 and illustrated in Figure 9, MβCD pre-treatment results in a significant increase in the size of membrane compartments containing LHR-wt. The size of these compartments does not differ significantly from those accessed by untreated FLAG-LHR-wt receptors and, together with results from sucrose density gradient centrifugation experiments, suggests that these unbound receptors are residing in the bulk membrane rather than rafts.

Then we evaluated single-particle tracks for non-functional hormone-receptor complexes. As shown in Figure 9, FLAG-LHR-wt receptors treated with deglycosylated hCG as well as FLAG-LHR-K583R receptors treated with hCG remained in large membrane compartments where they exhibited comparatively fast lateral motions. These results were consistent with previous studies of LH receptor rotational dynamics which suggest that receptors within non-functional hormone-receptor complexes exhibit comparatively short rotational correlation times suggesting that they are located within smaller complexes (37).

Finally, to determine whether the cytoskeleton played a role in restricting movement of individual LH receptors, we evaluated receptor motions in cells retreated with cytochalasin D, a microfilament disruption. As shown in Table 2, this treatment resulted in receptor motions that resembled those of unoccupied receptors. Particles diffused over large areas of the membrane and did not appear constrained by any membrane abstacles.

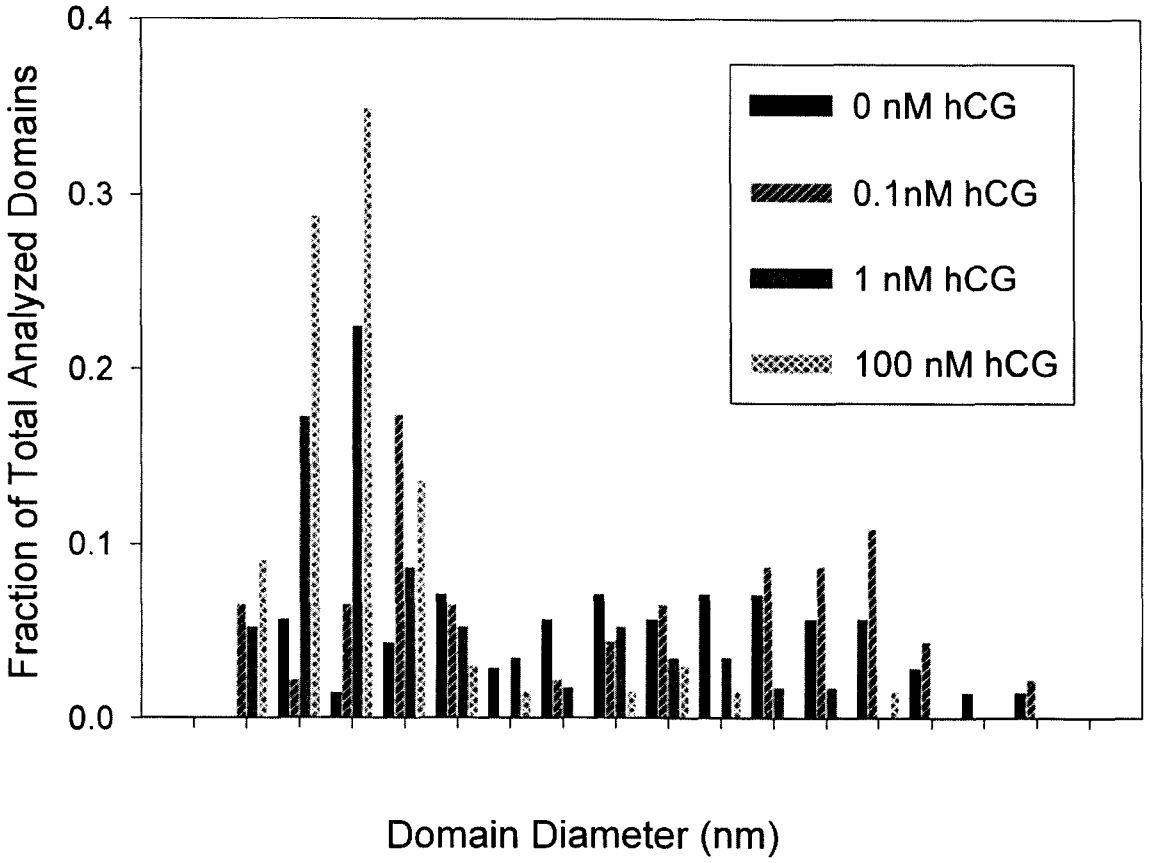

Figure 10: Dose-dependent LH movement into small compartments. We treated CHO cells with hormone concentrations ranging from 0.1nm to 100nm. After treatment with 1nm and 100nm hCG, 62% and 91% of the receptors were confined in small compartments with a diameter of less than 100nm. The remaining receptors exhibit unconfined lateral diffusion in large compartments typical of untreated cells.

Table 2. Effect of Cytochalasin D on SPT

Cell Line	Pretreatment	Hormone	Number of particles analyzed	Number of domains/ 2 min trajectory	D_{0-1}[a] ($10^{-11}cm^2sec^{-1}$)	$D = L_r^2/4t$[b] ($10^{-11}cm^2sec^{-1}$)	Time[c] (sec)	Domain Diameter (L_r)[d]
Human	Cytochalasin	none	20	2 ± 1	3.6 ± 3.9		2.8 ± 2.4 25 ± 18	221 ± 80
Human	Cytochalasin	hCG(100nM)	20	2 ± 1	2.8 ± 1.8		2.2 ± 1.7 29 ± 14	204 ± 66
Human	none	none	20	6 ± 2	6.8 ± 3.8		1.2 ± 0.8 16 ± 13	230 ± 79
Human	none	hCG(100nM)	20	5 ± 2	2.9 ± 1.1		0.1 ± 1.8 20 ± 11	86 ± 36

[a] D_{0-1}: Diffusion coefficient of the first 2 points

[b] D presents the diffusion coefficient within a domain as described by Saxton.

[c] The average diameter of an individual domain was calculated as described by Daumas et al. and Murase et al..

[d] Average time for residence within a domain

DISCUSSION

Our results demonstrate that wild type LHRs, if occupied by a functional ligand, are capable of moving from the bulk membrane into small nm-diameter plasma membrane compartments with characteristics of lipid rafts. The translocation of LHR to low density membrane regions appears to be characteristic of the functional hormone-receptor complex and is not observed when either the LHR is non-functional or when a hormone antagonist has bound to the wild type receptor. Moreover, the regions within which hormone-treated LHR-wt receptors are confined are small. Translocation of receptors into rafts is also implicated in receptor-mediated signaling as demonstrated by the decrease in hCG-mediated cellular levels of cAMP when rafts were disrupted. The appearance of receptors within low density, detergent-insoluble membrane fractions occurs upon binding of hormone agonist to LHR. This is similar to results of Bramley and Ryan (47) who separated membrane fragments from homogenated ovaries of superovulated ewes on continuous sucrose gradients. They isolated two distinct membrane fractions that displayed hCG binding and, as a result, suggested that there might be two populations of LH receptors present on granulosa cells. They also characterized selected proteins in "heavy" and "light" membrane fractions containing LHR including adenylyl cyclase which, interestingly, associated with the "heavy" receptor population.

The mechanism involved in targeting of LHR to rafts is not clear. Proteins found in plasma membrane rafts include transmembrane proteins with attached lipid groups including, most commonly, GPI or palmitate (48). There is evidence for palmitoylation of the LH

receptor at two cysteines located in the receptor's intracellular carboxy terminal (49). Although mutations to the palmitoylated cysteines did not affect cAMP production, the mutated receptors were internalized more quickly than wild type receptors, apparently through an arrestin-mediated pathway (50). Point mutations to palmitoylation sites on the LH receptor C terminus also eliminates LHR-wt translocation into rafts (51). However, it is not known whether there is reversible palmitoylation of the wild type LHR or whether the binding of ligand alters equilibrium between palmitolylated and non-palmitoylated LH receptors. This appears to be the case for β-adrenergic receptors, G protein-coupled receptors structurally related to LHR, which undergo palmitoylation/depalmitoylation cycling with binding of agonist favoring receptor depalmitoylation (52). If palmitoylation were necessary for targeting of a ligand-activated G protein-coupled receptor to rafts, depalmitoylation would be predicted to reduce the likelihood of finding, for example, β-adrenergic receptors in rafts. However, the role for palmitoylation in receptor-mediated signaling (53) and raft localization is generally unclear and may prove ultimately to be receptor-specific.

Receptor aggregation, either together with receptor palmitoylation or independently, may increase the affinity of the LH receptor for rafts. Signaling by multi-chain receptors such as T-cell and B-cell antigen receptors are present in the bulk membrane prior to crosslinking by multivalent antigens (54, 55). Crosslinking may shift the equilibrium distribution for membrane proteins within the lipid bilayer and favor interactions between proteins and more ordered lipid microdomains such as rafts as has been demonstrated for lipid-anchored proteins within artificial lipid monolayers (9). In our hands, LH receptor self-association

(36) as well as raft localization are characteristic of functional hormone-receptor complexes. One could hypothesize that agonist-induced associations between LH receptors causes translocation of receptors to rafts. Interestingly however, presumably random interactions between LHR receptors following antibody crosslinking, are not sufficient to drive receptor translocation to rafts but are sufficient for activation of adenylyl cyclase in the absence of hCG. Although targeting of proteins to rafts can be initiated by antibody crosslinking of, for example, T-cell and B-cell antigen receptors (54, 55) and the Type I Fcϵ receptor on rat basophilic leukemia cells (56), this was not the case for antibody-crosslinked LH receptors, suggesting that LH receptors required binding of a hormone agonist and potentially specific receptor conformations for targeting of activated receptors to rafts.

It appears that membrane rafts may serve as a signaling platforms for the LH receptor although the specific signaling event(s) are uncertain. Spatial coordination of key signaling proteins in lipid rafts may provide a rapid, efficient and specific mechanism for promoting signal transduction from extracellular to intracellular mechanisms while also preventing cross-talk between pathways (8, 57). As an example, Type I Fcϵ receptors form, at a minimum, receptor dimers that are tyrosine phosphorylated by Lyn and then colocalize with Lyn in membrane rafts (56). Other downstream signaling molecules also appear within rafts. Oh and Schnitzer have shown that G_i and G_s are targeted to, and concentrated in, lipid rafts (8) and some isoforms of adenylyl cyclase are associated with these membrane structures (58-60). If rafts serve to concentrate these proteins, cholesterol depletion and raft disruption may disperse raft-associated proteins and reduce the likelihood of protein-protein interactions necessary for cell signaling. Indeed, MβCD treatment reduced FRET between GPI-anchored

proteins (61) and signal transduction by these proteins (62). Other studies have shown that cholesterol is important for lipid raft formation and that its depletion decreases signal transduction efficiency (43, 63).

Single-particle tracking studies provide some insight into the nature of LH receptor-containing structures. Upon binding ligand, the LHR-wt becomes largely confined within small compartments with an average diameter of 86 nm. For the most part, the receptor remains within these regions for comparatively long times and appear to diffuse pseudo-randomly before being captured within another compartment of similar size. Similar behavior has been described and analyzed by Kusumi and coworkers (44) for selected phospholipids and for transferrin receptor (64) and by Daumas et al. (45) for the μ opioid receptor, a G protein-coupled receptor involved in pain responses. Daumas argues that μ opioid receptor motions reflect its diffusion within the bulk membrane followed by confinement within a domain that itself diffuses slowly and suggests that this confinement is due to interactions with the confining molecules. Alternatively, Ritchie et al. (65) suggest that these interactions may be with proteins forming a continuous barrier (fences) or discontinuous protein barrier (pickets). Fences or pickets can confine and limit receptor diffusion within small membrane regions while still permitting intermittent escape from a compartment zone followed by faster diffusion in the bulk membrane. Results from experiments using cytochalasin D pretreatment of CHO cells support this notion. After disruption of actin microfilaments on CHO cells expressing FLAG-LHR, hormone-treated receptors remained in large compartments suggesting that small membrane compartments containing LHR maybe defined by protein pickets or fences.

Finally, disruption of the membrane rafts reduced, but did not completely eliminate, LH signaling. This result is reasonable if hormone-mediated signaling is most efficient within rafts where higher concentrations of downstream signaling molecules exist. Nonetheless, some signaling proteins remain available within the bulk membrane, albeit at reduced concentrations, where they are capable of relaying a productive signal. The questions that remain are what role receptor aggregation plays in signaling and raft localization, whether small membrane compartments accessed by hCG-occupied receptors are the same structures isolated in low density sucrose fractions, and whether rafts are essential for LH receptor function. These questions seem likely to be resolved only if it can be demonstrated that forcing an LH receptor, either with or without ligand, into the raft environment, can produce a downstream signal and if some, or all, of the components involved in LH-receptor mediated signaling can be localized with LH receptors in the same membrane compartments. Alternatively, raft localization may be a convenient, but not essential method, for concentrating membrane proteins involved in signal transduction.

CHAPTER 3[2]

Constitutively-Active Human LH Receptors are Self-Associated and Located in Rafts

Summary

Several naturally-occurring mutations in human luteinizing hormone receptors (LHR) at position 578 are associated with constitutive activation of the receptor. To determine whether human LHRs signal in the absence of ligand are self-associated, fluorescence resonance energy transfer (FRET) between receptors was evaluated. Values for FRET between wild type LHR in the absence of ligand were less than 1% and increased significantly to over 11% after exposure to hCG. Constitutively active receptors exhibited 11-15% FRET efficiency in the absence of hormone and these values did not change with hCG treatment. A large fraction of constitutively active LHR-D578H receptors were also associated with plasma membrane rafts and with small membrane compartments evaluated using single particle tracking. Disruption of these membrane microdomains reduced FRET efficiency but did not affect signaling through cAMP. Thus, in the absence of ligand, constitutively-active receptors are self-associated and located in high buoyancy membrane fractions, both

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This chapter has been adapted from Ying Lei, Guy M. Hagen, Steven M. L. Smith, Jinging Liu, George Barisas and Deborah A. Roess (2007), Constitutively-Active Human LH Receptors are Self-Associated and Located in Rafts, Molecular and Cellular Endocrinology, 260-262: 65-72.

characteristics of the hormone-treated wild type receptor.

Introduction

Functional luteinizing hormone (LH) receptors are critical to fertility in both males and females. In females, the LH receptor is found on granulosa and thecal cells in the follicle and on luteal cells. In males, the receptor is found on Leydig cells. Binding of LH from the anterior pituitary results in signaling cascades leading to follicle maturation, steroidogenesis or spermatogenesis. Mutations in the human luteinizing hormone (hLH) receptor aspartic acid residue at position 578 are associated with constitutive activation of Gs by the receptor (66) as well as with naturally-occurring pathologies, such as Familial Male-limited Precocious Puberty (FMPP) and Leydig cell adenomas (18).

Although details of G protein-coupled receptor signaling through intracellular mediators are increasingly well-characterized, membrane events involved in signaling are less understood including receptor interactions such as receptor dimer or oligomerization or interactions with other membrane proteins. Several lines of evidence suggest that functional LH receptors, i.e., receptors that have bound hormone and are actively transducing signal, are associated within large molecular weight structures following the binding of hormone. Electron micrographs of LHR on rat granulosa cells show large clusters of receptors that form only after binding of hormone (67) as does immunofluorescent labeling of rat receptors in granulosa cells (68). Large clusters of wild type rat LH receptors tagged with green fluorescent protein (LHR-GFP) also form within minutes following binding of either LH or

hCG to receptors on viable cells (23). These clusters may reflect aggregation of receptors cis-activated by ligand or aggregated receptors in which ligand-occupied receptors have trans-activated nearby unliganded neighbors (69). The presence of receptors in physically large structures is also suggested by lateral diffusion studies of hormone-treated LH receptors on luteal cells from sheep and rat in which most LH receptors were laterally immobile (reviewed in (12)).

The LH receptor within these membrane clusters appears to be self-associated. Rat LH receptors become self-associated upon binding of either LH or hCG (12) and when desensitized in plasma membrane preparations from porcine granulosa cells (70). Tao et al. (71) have used immunoprecipitation methods to show that some human LH receptors stably expressed in 293 cells exist as receptor dimers or oligomers and that the relative amounts of these receptor structures increases upon binding of hCG.

Receptor self-association may also be accompanied by a redistribution of the LH receptor within the plasma membrane. Upon binding of ligand, rat LH receptors partition into high buoyancy membrane fractions that can be isolated via density gradient centrifugation (12). Because of their high lipid content, these specialized membrane microdomains or rafts are found in membrane fractions with low density and "float" in sucrose gradients. The outer leaflet of the raft membrane is enriched with sphingolipids and cholesterol as well as glycosylphosphatidyl-inositol (GPI)-anchored proteins and can limit the lateral diffusion of specific membrane proteins (72). In addition to sequestering receptors, such domains may serve "signaling platforms" for a diverse group of signaling molecules (11) as well as G protein-coupled receptors such as the rat LH receptor (73) and

the gonadotropin releasing hormone (GnRH) receptor (74). The presence of membrane microdomains with higher affinity for activated, signaling receptors than the bulk membrane could also explain, at least in part, why receptor clustering occurs within minutes and, upon microscopic inspection, involves the movement of diffusely distributed LH receptors into discrete membrane locations (23). In single particle tracking experiments of rat LH receptors on viable cells, the size of compartments accessed by hCG-treated receptors is reduced by over 60% (73). Thus, the question raised by these various observations is whether constitutively-active hLHRs, but not wild type receptors, are self-associated in the absence of hormone, are restricted in their motions within the plane of the membrane and localized in membrane rafts involved in receptor-mediated signaling.

Materials and Methods

Materials. Dulbecco's modified Eagle medium containing high glucose was purchased from Irvine Scientific, Santa Ana, CA. Geneticin was purchased from GIBCO, Grand Island, NY. Non-essential amino acids were purchased from Sigma-Aldrich, St. Louis, MO. Fetal bovine serum (FBS) was purchased from Invitrogen (Carlsbad, CA). hCG was purchased from Research Diagnostics Inc. (Flanders, NJ). Methyl-β-cyclodextrin (MβCD) and the FLAG vector were purchased from Sigma-Aldrich (St. Louis, MO). CFP and YFP vectors were purchased from Clontech. Vectors containing human LHR (LHR-wt) receptors or receptors with mutations D578G, D578H or D578Y were gifts from Dr. Andrew Shenker. Intracellular cAMP was measured using a TiterFluor cAMP EIA kit obtained from

Assay Designs, Ann Arbor, MI.

Preparation and maintenance of CHO cells expressing visible fluorescent proteins (VFP) or epitope tags. Stable CHO cell lines expressing wild type human LHR receptors or receptors with mutations, D578G, D578H or D578Y, associated with constitutive activation, were coupled to one of the visible fluorescent proteins, either enhanced cyan fluorescent protein (CFP) or enhanced yellow fluorescent protein (YFP), at their C terminus using N-terminal Protein Fusion Vectors cECFP-Ca (6900-1) and pEYFP-C1 (6006-1), respectively. To prepare these cell lines, vectors were constructed for LHR-wt-CFP and -YFP, LHR-D578G-CFP and -YFP, LHR-D578H-CFP and -YFP, and LHR-D578Y-CFP and -YFP as previously described for transfection of rat LHR coupled to GFP (23). CHO cells in 60 mm dishes at 40-80% confluence were transfected with 5μg DNA in 20μL lipofectamine. DNA was added as either a single vector or, to accomplish co-transfection of two vectors, at a 1:3 ratio of CFP:YFP. After 3-4 weeks, clones expressing both CFP and YFP were selected using fluorescence microscopy. To determine whether LHRs partitioned into membrane rafts, stable CHO cell lines were prepared expressing either LHR-wt or LHR-D578H coupled to the FLAG epitope at the receptor N terminus and were maintained in CHO cell medium (12).

Imaging analysis of FRET using fluorescence dequenching. To evaluate the effects of hCG treatment on energy transfer efficiency, flasks containing 3-4 x 10^6 cells were selected. The medium was discarded and cells were removed from the flask using PBS containing 5mM EDTA, washed with 12mls of PBS, and spun down. The cell pellet was resuspended in 500 μL of PBS alone or in PBS containing 100nM hCG. The cells were then incubated

at 37°C for 1 hour, washed once and resuspended in PBS for FRET measurements. Because aggregation of rat LH receptors is visible within minutes (23)and receptors clusters do not dissociate for several hours (25), it is likely that the extent of LH receptor self-association is relatively stable on the time-scale of these experiments. FRET between hLHR-CFP and hLHR-YFP was evaluated on individual cells by measuring the intensity of the plasma membrane localized fluorescence donor CFP in the presence and absence of a fluorescence acceptor YFP (75). More intense signals from CFP, the fluorescence donor, after photobleaching of YFP, the fluorescence acceptor, were indicative of energy transfer from fluorescence donor to acceptor. For this donor-acceptor pair, the Förster r_0 is calculated to be 56Å (76)and energy transfer occurs to a measurable extent only when the donor and acceptor are separated by distances less than about 100Å. FRET measurements were made using a Zeiss Axiovert 135 microscope or a Zeiss Axiovert 200m microscope, Omega Optical filter sets for imaging of CFP and YFP and Metamorph software from Universal Imaging. Before photobleaching YFP, CFP and YFP were imaged separately using a Princeton Instruments1300YHS ICCD camera. Imaging the cell involved careful focusing through the entire cell and selection of an image plane that demonstrated "ring-like" fluorescence at the cell's outermost plasma membrane. FRET measurements were made only on fluorescence emitted from the anular image region containing the plasma membrane. After photobleaching YFP for 5 minutes using a mercury arc lamp source and an Omega Optical XF1074 filter, YFP and CFP were imaged again. Five minute exposure to 525nm light was sufficient to bleach essentially all YFP signal. The intensity of CFP signals before and after YFP photobleaching was then compared. After subtracting the

background signal from each image and correcting for the small extent of donor bleaching during the bleaching of acceptor, energy transfer efficiency was calculated using %E= (donor fluorescence after photobleaching - donor fluorescence before photobleaching / donor fluorescence after photobleaching) x 100.

Isolation of plasma membrane rafts. To evaluate the localization of LH receptors in membrane fractions with high buoyancy, stable cell lines were prepared that expressed either LHR-wt or LHR-D578H coupled to the FLAG epitope on their N-terminus as has been described for rat LHR-wt (12). Cells were incubated with either 100nM hCG or PBS for 1 hour at 37°C prior to cell lysis. As previously described (12), 1 mL cell lysate was combined with 1ml of 80% sucrose and the sample was layered at 40% sucrose within a discontinuous sucrose gradient from 10-80%. After centrifugation at 175,000x g for 20 hours at 4°C, fractions were collected from the top of the gradient downward and an aliquots from each fraction were diluted 1:1 in Laemmli SDS buffer. After separation of proteins using SDS-PAGE and transfer to nitrocellulose, the LH receptor was identified using anti-FLAG M2 monoclonal antibody (Sigma-Aldrich, St. Louis, MO). In some experiments, cells were pretreated for 1 hr at 37°C with 1% MβCD in serum free DMEM medium prior to incubation with hCG or PBS.

Single particle tracking of FLAG-LHR-wt receptors on individual cells. Lateral dynamics and the size of domains accessed by individual FLAG-LHR-wt were evaluated using single-particle tracking methods as described by Kusumi and coworkers (42) and Smith et al. (73). 40 nm nanogold particles were conjugated with the lowest possible concentration of anti-FLAG monoclonal antibody (mAb) and then incubated with CHO

cells expressing FLAG-LHR-wt receptors at concentrations typically less 15 μg/mL to produce 1-4 gold particles per cell. The trajectories for individual gold particles were segmented into domains by calculation of statistical variance in particle position over times using a procedure similar to that developed by a number of investigators (44-46). Results were analyzed to yield the domain size, the residence time for each particle and the effective macroscopic diffusion constants as described by Saxton (46).

Results

hLHR-wt become self-associated and translocate into membrane rafts following treatment with hormone. We used fluorescence dequenching of the fluorescence donor and imaging methods to examine conditions in which wild type human LHR were self-associated. This FRET method has a number of advantages, the most important being that all measurements of fluorescence emission from the fluorescence donor are accomplished on the same cell. To perform these experiments, CHO cells were stably cotransfected with both CFP- and YFP-coupled LH receptors. Stable transfection of LH receptors is reported to decrease immature or misfolded hLHR and increase cell membrane expression (71). We imaged CFP and YFP fluorescence separately using fluorescence filter sets for these visible fluorescent proteins that minimized the fluorescence contribution from CFP when imaging YFP (75) and that, in subsequent steps, permitted photobleaching of YFP only. Following photobleaching, each cell was reimaged using the same filter sets. As shown in Figure 11, after photobleaching of YPP, an increase in CFP dequenching and thus a brighter CFP

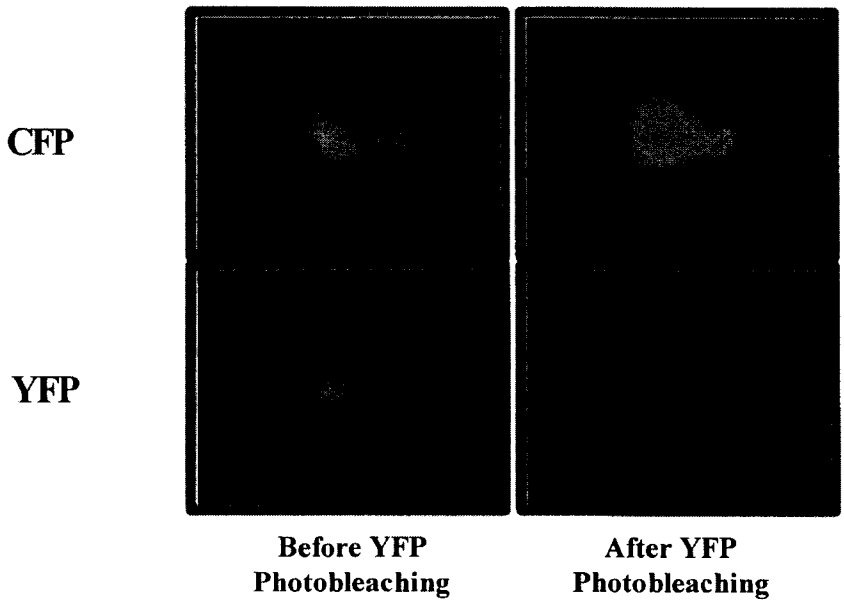

Figure 11. After photobleaching of YPP, an increase in CFP dequenching and thus a brighter CFP image occurred when there was energy transfer between the visible fluorescence proteins. In this case, the energy transfer efficiency is 24%.

image was obtained when there was energy transfer between the visible fluorescence proteins.

We used two methods to analyze data from these studies (Figure 12). In both methods, background fluorescence was subtracted from the four images obtained in each individual experiment. This involved selection of a cell-free site on the complete image and subtraction of the average intensity of that site from the complete image. This typically produced images of cells on a dark background. The first method used for analysis of fluorescence donor intensity averaged fluorescence intensity from the entire cell before and after photobleaching to calculate energy transfer efficiency. Figure 12 shows donor fluorescence from a representative cell before and after photobleaching of the fluorescence acceptor. We compared this method with one in which only fluorescence from the periphery of the cell was used. In the example shown in Figure 12, the average intensity before photobleaching from the entire cell as compared to "plasma membrane only" was reduced from 1530 cps to 487 cps. After photobleaching, values increased to 1753 cps and 560 cps. Energy transfer efficiency (%E) was not statistically different (12.7% and 12.9%, respectively) using these two methods for analysis of FRET. More generally, examining only the plasma membrane of each of the cells lines used for FRET analysis produced a decrease in the mean and S.D. of 2.3 ± 1.4% for %E (data not shown) which was not significant while reducing the overall number of photon counts used to evaluate FRET.

A summary of results using the whole cell imaging method is shown in Table 3. In the absence of ligand, values for energy transfer efficiency between LHR-wt receptors were, on average, less than 1%. Energy transfer efficiency increased significantly to 11.5 ± 1.2 %

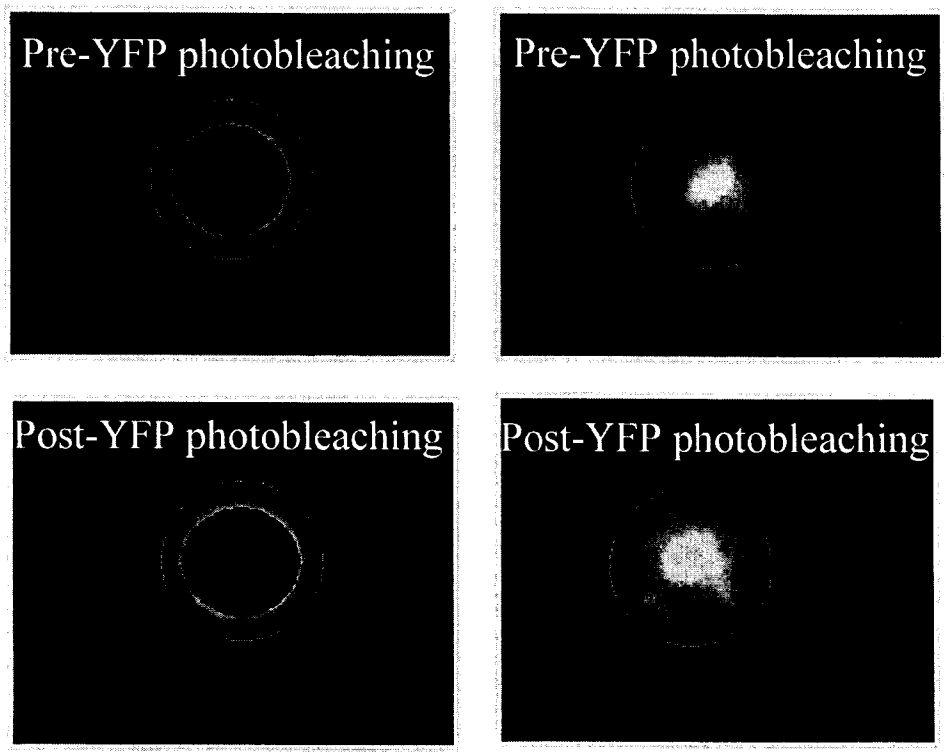

Figure 12. Different analysis methods of imaging FRET. We used two methods to analyze data from these studies. In both methods, background fluorescence was subtracted from the four images obtained in each individual experiment. Figure shows donor fluorescence from a representative cell before and after photobleaching of the fluorescence acceptor.

following exposure of LHR-wt to 100 nM hCG. Since energy transfer occurs to a measurable extent only when the CFP (donor) and YFP (acceptor) are separated by distances less than about 100 Å(76), FRET results indicate that wild type human LHRs, if occupied by a functional ligand, undergo receptor self-association.

LHR-wt receptors also translocated from the bulk plasma membrane into high buoyancy membrane fractions (rafts) following ligand treatment (Figure 13and upper panel of Figure 14). Following isopycnic centrifugation of plasma membrane fractions from CHO cells, over 95% of unoccupied receptors were found fractions containing 39-56% sucrose. About 75% of hCG-treated FLAG-hLHR-wt consistently appeared in sucrose fractions containing 24-34% sucrose and thus had "floated" to lower sucrose densities during centrifugation. The remaining receptors were found in fractions containing higher sucrose concentrations. To further demonstrate the presence of hormone-treated LH receptors within rafts, we treated cells with 1% MβCD, a cholesterol-sequestering reagent that is efficient in removing cholesterol from the plasma membranes of live cells and that disrupts raft structure (Figure 13 and the upper panel of Figure 14). At low concentrations MβCD extracts membrane cholesterol by placing it in a central non-polar cavity of cyclic oligomers of glucopyranoside in 1,4 glycosidic linkages (28) without affecting cell viability (data not shown). Pretreatment of CHO cells with MβCD followed by exposure to 100 nM hCG reduced the relative number of receptors in fractions containing less than 34% sucrose from 75% to 13%, presumably by disrupting membrane microdomains.

Constitutively-active human LHR are self-associated in the absence of hormone. In contrast to wild type human LH receptors, constitutively active receptors in the absence of

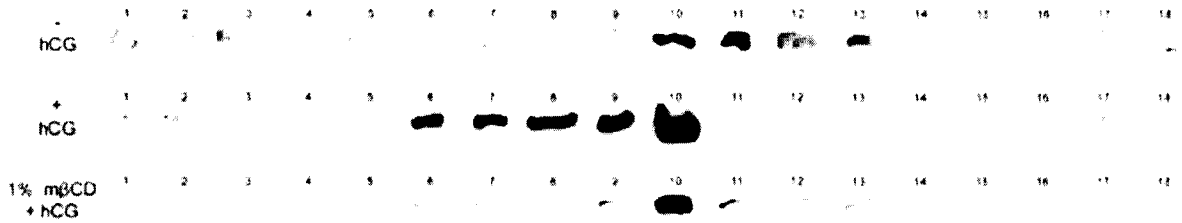

Figure 13. Distribution of wild type human LH receptors in sucrose fractions.

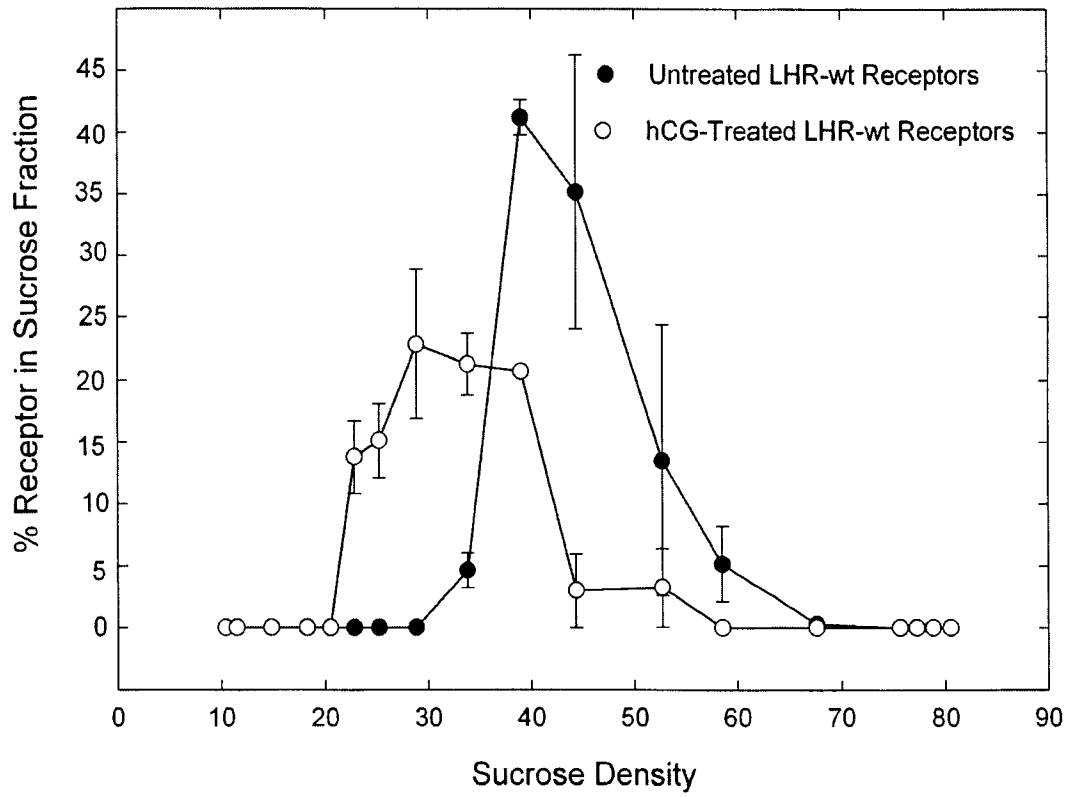

Figure 14. Relative fractions of wild type human LH receptors +/- hCG appearing at each sucrose concentration. Following isopycnic centrifugation of plasma membrane fractions from CHO cells, over 95% of unoccupied receptors were found fractions with low buoyancy.

hormone exhibited relatively high values for fluorescence energy transfer efficiency that ranged from about 11-15% (Table 3). Hormone treatment had no statistically significant effect on FRET values. Following treatment of cells with 100 nM hCG, energy transfer between LHR-D578Y increased from 11.7 ± 2.2 % to 14.1 ± 4.2% while energy transfer efficiency for cells expressing LHR-D578H decreased slightly. The absence of any statistically significant effect suggests that there was little, if any, overall change in the extent of receptor association when constitutively active receptors were occupied by ligand.

MβCD treatment disrupts plasma membrane rafts and reduces the extent of energy transfer between FLAG-LHR-D578H receptors but does not affect cAMP signaling. To examine localization of LHR-D578H in rafts, the receptor was epitope-tagged on the N terminus using FLAG. Western blots of sucrose gradient fractions obtained from isopycnic ultracentrifugation showed approximately 20-25% of FLAG-LHR-D578H associated with sucrose fractions containing 24-34% sucrose both before and after binding of hCG (Figure 14). As expected, MβCD treatment shifted the constitutively active receptor to lower buoyancy membrane fractions which was consistent with disruption of membrane rafts (Figures 13 and 14). Because it has been suggested that plasma membrane rafts may concentrate receptors and other plasma membrane molecules necessary for the transduction of a productive ligand-mediated signal, we examined whether disruption of membrane rafts reduced or eliminated cAMP signaling by FLAG-LHR-D578H cells. Interestingly, there was no decrease in cell signaling via cAMP following MβCD treatment (Figure 15) suggesting that localization of LHRs to rafts may not be required for receptor signaling. There was, however, a significant decrease in the extent of receptor self association from

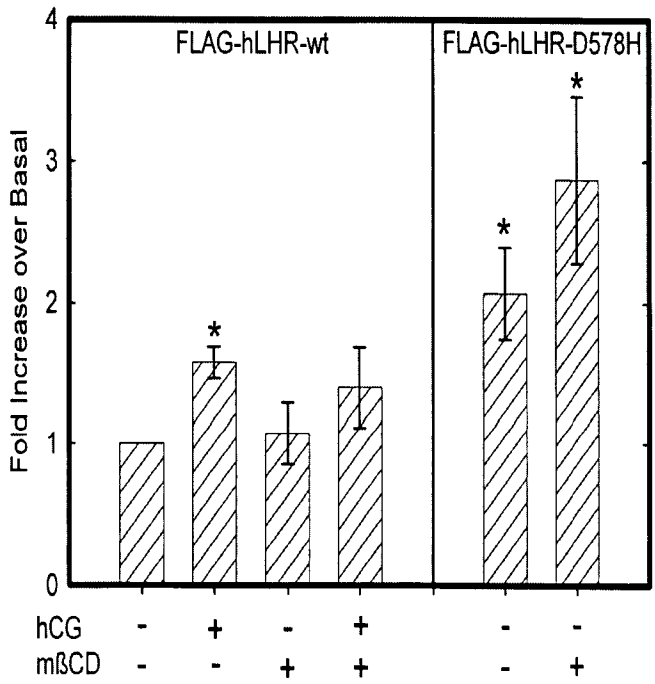

Figure 15 cAMP levels for hLHR-wt and hLHR-DH cells. There was no decrease in cell signaling via cAMP following MβCD treatment suggesting that localization of LHRs to rafts may not be required for receptor signaling.

Table 3. Efficiency of Fluorescence Energy Transfer between Wild Type LH receptors and Constitutively Active Receptors. In the absence of ligand, values for energy transfer efficiency between LHR-wt receptors were, on average, less than 1%. Energy transfer efficiency increased significantly to 11.5 ± 1.2 % following exposure of LHR-wt to 100 nM hCG. Since energy transfer occurs to a measurable extent only when the CFP (donor) and YFP (acceptor) are separated by distances less than about 100 Å(80), FRET results indicate that wild type human LHRs, if occupied by a functional ligand, undergo receptor self-association.

Cell Line	Ligand	Treatment	%Efficiency	n
hLHR-wt	none	none	0.8 ± 1.3	17
	100 nM hCG	none	11.5 ± 1.2	24
hLHR-DG	none	none	15.3 ± 1.9	11
	100 nM hCG	none	11.1 ± 2.5	14
hLHR-DH	none	none	13.8 ± 1.6	39
	100 nM hCG	none	13.0 ± 1.3	23
	none	1% mβCD, 45 min	8.0 ± 1.4	15
hLHR-DY	none	none	11.7 ± 2.2	14
	100 nM hCG	none	14.1 ± 4.3	11

an average of 14% to 8% in MβCD-untreated cells (Table 3).

A more detailed analysis of this decrease in FRET efficiency is presented in Figure 16 which shows individual FRET values for both untreated and MβCD-treated cells. The efficiency of energy transfer for untreated and MβCD-treated cells varied on a cell-to-cell basis over a fairly large range. When LHR-D578H cells were treated with MβCD, higher values for FRET efficiency were absent as reflected in the left shift in accumulated FRET values. One explanation for this result is that there are two populations of self-associated, constitutively-active receptors on LHR-D578H cells with more extensive receptor self-association occurring in membrane rafts. The persistence of self-associated receptors in the bulk membrane following cell treatment with MβCD may be sufficient to maintain adenylate cyclase activation and elevated levels of cAMP in cells.

Single particle tracking of hCG-occupied FLAG-LHR-wt receptors demonstrates "trapping" of receptors in small membrane compartments. To independently assess the localization of hCG-treated FLAG-LHR-wt in membrane compartments, single particle tracking methods were used. This technique identifies individual LH receptors on the surface of viable cells and tracks their motions over approximately two minutes. The image of a 40 nm gold particle attached to an individual receptor can be identified visually on video obtained from each experiment and the motions of its centroid can be quantitatively described. hCG treatment reduced the average diameter of compartments containing human FLAG-LHR-wt approximately 2-fold from 309 ± 131 nm to 146 ± 119 nm as well as the diffusion coefficient for receptors within compartments (Figure 17 and Table 4). For both unoccupied and hormone-treated FLAG-LHR-D578H, the compartments

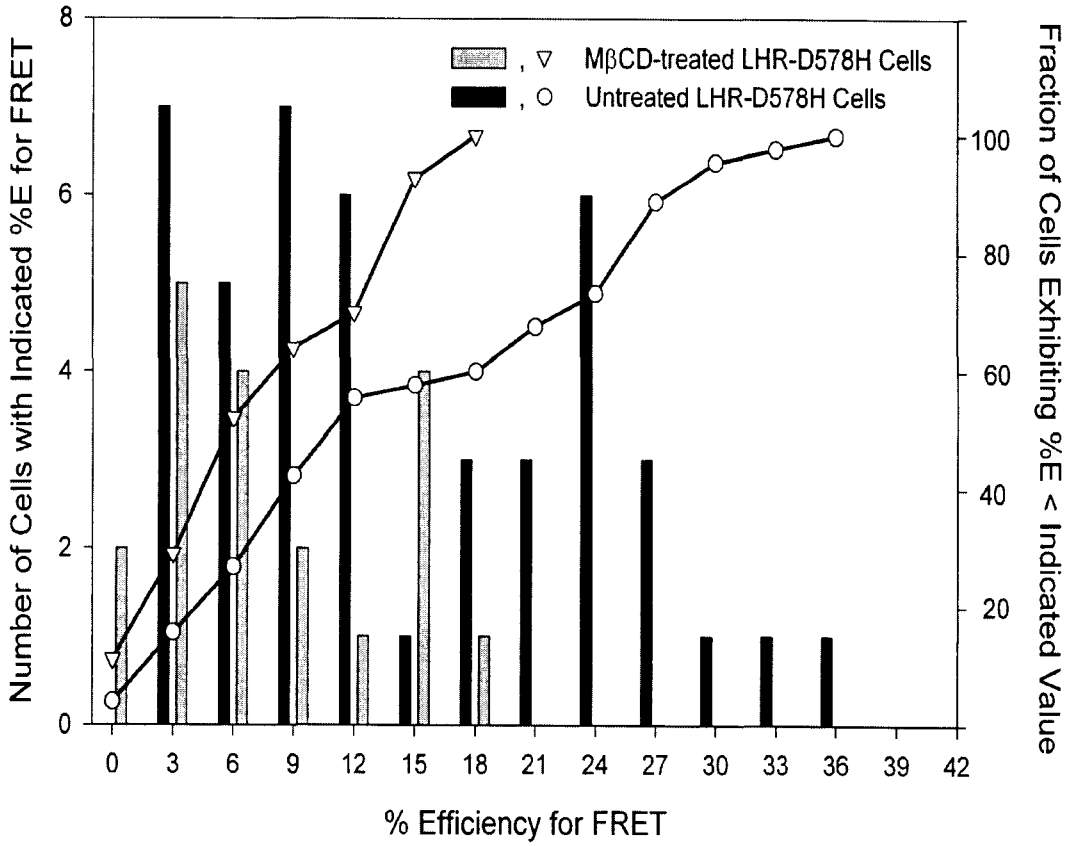

Figure 16. Individual FRET Measurements for LHR-D578H cells +/- CD.

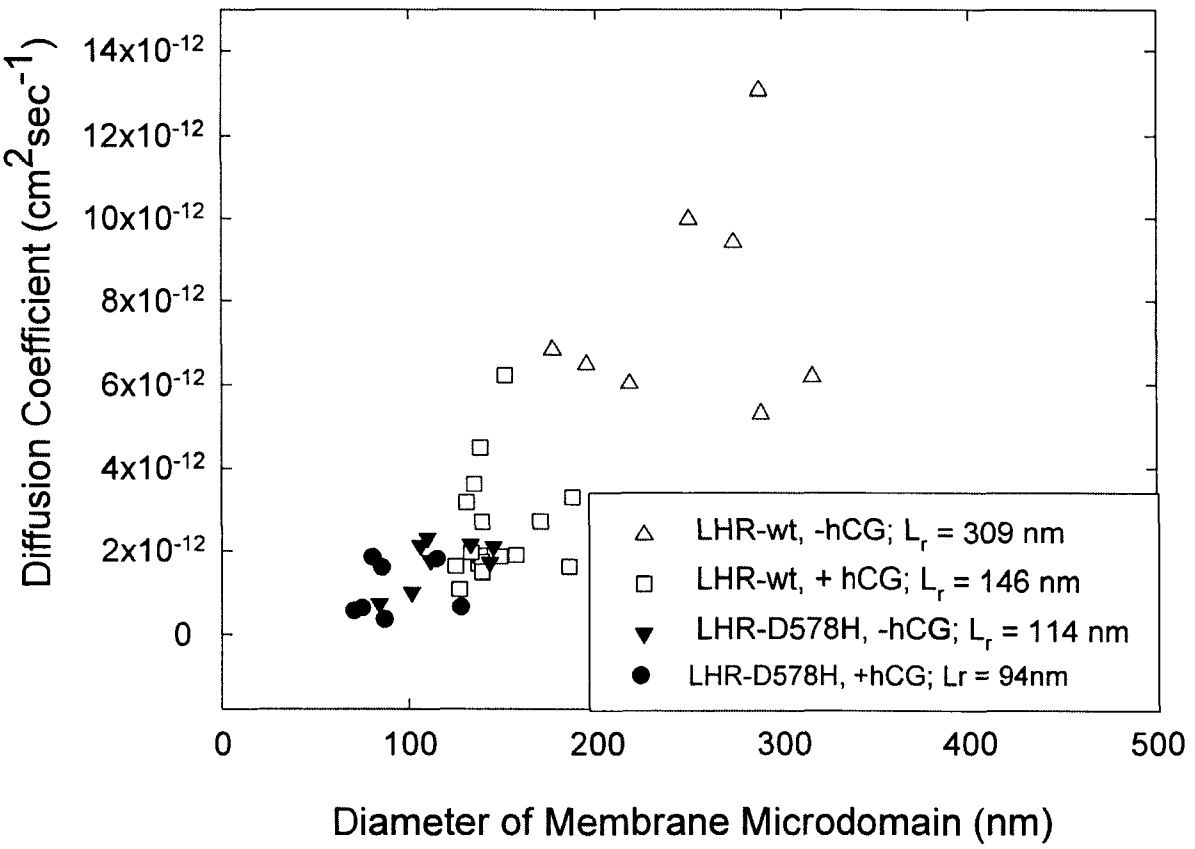

Figure17. For both unoccupied and hormone-treated FLAG-LHR-D578H, the compartments containing receptors were small with domain diameters, L_r, of 114 ± 22 nm and 94 ± 33 nm, respectively. Diffusion coefficients within these small compartments (0.2 x $10^{-11}cm^2sec^{-1}$ and 0.1 x $10^{-11}cm^2sec^{-1}$, respectively) were comparable to those obtained for hCG-treated FLAG-LHR-wt (0.3 x $10^{-11}cm^2sec^{-1}$).

Table4. For both unoccupied and hormone-treated FLAG-LHR-D578H, the compartments containing receptors were small with domain diameters, L_r, of 114 ± 22 nm and 94 ± 33 nm, respectively. Diffusion coefficients within these small compartments (0.2 x $10^{-11}cm^2sec^{-1}$ and 0.1 x $10^{-11}cm^2sec^{-1}$, respectively) were comparable to those obtained for hCG-treated FLAG-LHR-wt (0.3 x $10^{-11}cm^2sec^{-1}$).

Cell Line	Pretreatment	Number of of particles analyzed	Number of domains/ 2 min trajectory	D_{0-1}[a] ($10^{-11}cm^2sec^{-1}$)	$D = L_r^2/4t$[b] ($10^{-11}cm^2sec^{-1}$)	Time[c] (sec)	Domain Diameter (L_r)[d]
FLAG-LHR-wt	none	8	4 ± 0	5.1 ± 2.1	1.28 ± 1.06	27 ± 9	309 ± 131
FLAG-LHR-wt	hCG(100nM)	8	4 ± 1	1.7 ± 0.8	0.25 ± 0.13	25 ± 11	146 ± 19
FLAG-LHR-D578H	none	8	4 ± 1	0.7 ± 0.3	0.17 ± 0.06	23 ± 11	114 ± 22
FLAG-LHR-D578H	hCG(100nM)	8	4 ± 1	0.7 ± 0.0	0.12 ± 0.08	19 ± 8	94 ± 33

[a] D_{0-1}: Diffusion coefficient of the first 2 points

[b] D presents the diffusion coefficient within a domain as described by Saxton.

[c] The average diameter of an individual domain was calculated as described by Daumas et al. and Murase et al..

[d] Average time for residence within a domain

containing receptors were small with domain diameters, L_r, of 114 ± 22 nm and 94 ± 33 nm, respectively. Diffusion coefficients within these small compartments (0.2 x $10^{-11}cm^2sec^{-1}$ and 0.1 x $10^{-11}cm^2sec^{-1}$, respectively) were comparable to those obtained for hCG-treated FLAG-LHR-wt (0.3 x $10^{-11}cm^2sec^{-1}$).

Discussion

In addition to exhibiting unregulated signaling, constitutively-active LH receptors have characteristics of hormone-treated, actively signaling wild type human receptors and rat LH receptors (73). These receptors are self-associated, present in small membrane compartments on viable cells and isolated within plasma membrane rafts. The extent of receptor self-association is characteristic of wild type rat (25) and human LH receptors treated with a high concentration of hCG with values for energy transfer efficiency ranging from 11-15%. The LHR-D578H receptor also appears to be distributed into sucrose fractions with densities comparable to those in which hormone-treated rat (73) or human LHR-wt receptors are found.

We find no evidence for extensive self-association of the LHR-wt receptor on the viable cells used in these studies prior to binding of ligand. This is in contrast to reports by Tao et al. (71) and Urizar et al. (77). Whether this is because of differences in the experimental approaches used by these investigators or other factors such as receptor density is difficult to determine. However, in our hands, results with human LH receptors are consistent with those seen for rat LH receptors (25) using a related fluorescence method to evaluate FRET

on intact, viable cells. Moreover, the evaluation of single particle tracking of individual LH receptors also suggests that extensive aggregation of the receptor has not occurred prior to hormone binding. Although lateral diffusion measurements are only weakly correlated with the size of the diffusing structure, it appears that prior to hormone treatment, wild-type LH receptors are diffusing freely in the membrane over large distances while hormone-treated receptors are significantly restricted in their ability to move laterally in the plane of the membrane. The restriction of LH receptor motions may be due to extensive receptor self association, aggregation of the receptor into large structures containing other proteins, receptor anchoring by, for example, cytoskeletal components, or confinement of the receptor by membrane architectural features such as raft as discussed in Chapter 2.

Unlike constitutively active LHR-D578H receptors, human wild type receptors undergo translocation into rafts upon binding of ligand. In addition to rat and human LH receptors, there are a number of examples of plasma membrane receptors that are transiently associated with rafts during signaling. As examples, the β-adrenergic receptor becomes transiently associated with lipid rafts together with nitric oxide synthase and adenylate cyclase (78)and requires the raft environment for adenylate cyclase activity by Gs. The Type I Fcϵ receptor is also transiently associated with lipid rafts and exhibits increased signaling capability in that environment. Baird and coworkers suggest that, although the Type I Fcϵ receptor appears to be associated with Lyn in the bulk membrane, Lyn has low activity under these conditions (56). Rather, it is translocation of Type I Fcϵ receptors together with Lyn into the raft environment together as well as aggregation of the Fcϵ receptor that promotes the coupling of Type I Fcϵ receptors with Lyn phosphorylation.

There are also examples of constitutive association of proteins with rafts including gonadotropin releasing hormone receptor, a G protein-coupled receptor (74).

Factors driving translocation of either the wild type receptor into membrane rafts or residence of constitutively-active receptors within these structures are not known. Although the possibility remains that factors regulating LH receptor association with rafts differ for wild type and LHR-D578H, these studies indicate that association of LH receptors with rafts does *not* involve direct interactions between glycosylated hCG and raft components. While binding of hCG to the wild type receptor is necessary for translocation of the receptor into raft domains, the increase in association of the LHR-D578H with raft domains following addition of hormone is small, approximately 6% of the total receptor number. Moreover, studies of an rat LH receptor with a point mutations in the sixth transmembrane domain (73) suggest that these receptors, which exhibit little if any capacity to signal, remain localized in bulk membrane fractions despite binding of hCG.

On the other hand, conformational differences between the wild type and constitutively active receptor may be sufficient to promote constitutive interactions between the receptor and raft components. An appropriate conformation for the constitutively active receptor and acquisition of a similar conformation by the hormone-occupied wild type receptor may increase receptor affinity for raft-associated proteins. It is also possible that a critical receptor conformation is needed for association of ligand-activated receptors with other non-receptor raft components and it is these interactions that result in retention of the receptor in rafts.

Alternatively, structural modification of receptors such as lipidation could cause

preferential localization of the receptor in the cholesterol and spingomyelin-rich outer leaflet that defines the raft environment. There is evidence of palmitoylation of the wild type rat LH receptor upon binding of hormone (79) and both the constitutively active human LH receptor and the human wild type receptor contain the palmitoylation motif. Menon and coworkers (50, 80) have suggested that rat LH receptor depalmitoylation may be involved in internalization of the receptors: wild type receptors, which are more extensively palmitoylated, are also more rapidly internalized than a less palmitoylated, constitutively-active mutant. Whether palmitoylation is a dynamic process, occurring in response to, for example, binding of ligand, is not known although there are examples of other G-protein coupled receptors for which this process is dynamic (53). Moreover, palmitoylation of other membrane proteins such as Thy-1 (42) and linker for activation (LAT) of T cells (81) is necessary for retention in low density membrane fractions.

In these studies, a minimum of two events appear to be necessary for signaling by the LH receptor. First, productive signaling by a hormone occupied receptor appears to involve receptor-receptor interactions that produce comparatively high positive values for energy transfer efficiency. Second, migration to the raft environment or retention in small membrane compartments may also play a role in signaling. Although we anticipated that disruption of lipid rafts would impair signaling by constitutively active LH receptors, as it did for rat LHR-wt receptors (73), this was not the case. Rather, receptor-mediated cAMP accumulation by the constitutively-active receptor was not affected by cholesterol depletion although it did reduce the extent of receptor-receptor interaction. Thus, it remains possible that the key event in signaling by the constitutively-active receptor, in contrast to the wild

type receptor, is establishing and maintaining receptor interactions rather than their membrane microenvironment.

CHAPTER 4

Chimeric GnRH-LH Receptors and LH Receptors Lacking C-terminus Palmitoylation Sites Do Not Localize to Small Membrane Compartments[3]

Abstract

Luteinizing hormone and gonadotropin releasing hormone receptors (LHR and GnRHR, respectively) are G protein-coupled receptors with important functions in reproduction. We have used chimeric GnRHR-LHR that contain the full GnRHR coupled to various forms of the LH receptor C-terminus to explore the role of the LH receptor C-terminus in membrane compartmentalization. We (unpublished results) and others have previously shown that addition of the full-length LHR C-terminus to GnRHR resulted in localization of the resting chimeric receptor in the bulk membrane rather than plasma membrane rafts as has been reported for the wild-type GnRHR (74). With truncation of the LHR C-terminus, approximately 3% of chimeric receptors appeared in low density membrane fractions . We

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[3] This chapter was adapted from a full length manuscript entitled "Modulation of LH Receptor Motions by Hormone Concentration, Mutation of C-Terminus Palmitoylation Sites and Disruption of the Actin Cytoskeleton" by Jinjing Liu, Ying Lei, Steven M. Smith, George Barisas and Deborah A. Roess, in preparation. An abstract summarizing these results was presented at the 2006 Annual Meeting of the Society for the Study of Reproduction by Jingjing Liu entitled "Chimeric GnRH-LH Receptors and LH Receptors Lacking C-terminus Palmitoylation Sites Do Not Localize to Small Membrane Compartments".

used single particle tracking method to analyze the dynamics of the receptors. Palmitoylation of sites on the LHR C-terminus appears important for membrane compartmentalization and raft localization. Mutations to C-terminus palmitoylation sites eliminated translocation of LH receptors from the bulk membrane to rafts upon binding of hCG. Particle tracks for these receptors suggest that these receptors are able to access large membrane areas. Because these mutant receptors retained the ability to signal via cAMP, it is not clear whether receptor compartmentalization is required for productive signaling by these chimeric receptors.

Introduction

LH receptors are single-strand receptors with seven transmembrane (TM) domains and a C-terminal intracellular tail which has about 70 amino acids (13, 14). The LH receptor C-terminus has been implicated in modulation of receptor function and turnover. Progressive truncation of the C-terminus suggests that a region(s) between residues 616 and 631 of the rat LHR is required for proper insertion and/or targeting of the receptor to the plasma membrane. Truncation of the LH receptor at position 631 in the C terminus slows receptor internalization following binding of hormone (82). Palmitoylation at cysteine residues 621 and 622, in addition to providing anchoring sites for the cytoplasmic tail in the plasma membrane, may also affect receptor turnover: mutations in these palmitoylation sites enhance the rate of ligand-induced receptor internalization although other studies have reported no effect on the intracellular trafficking of the receptor (53, 79).

Here, we explore whether the LH receptor C-terminus, when coupled to the GnRHR terminal C-terminus amino acid, alters the distribution of these chimeric receptors within the membrane and whether this distribution is affected by mutations to LHR C-terminus cysteine residues at positions 621 and 622 or truncation of the C-terminus at position 631. We also compare the membrane distribution of these chimeric receptors with that of a full-length LHR with mutations to cysteines at positions 621 and 622 that eliminated receptor palmitoylation.

Methods

Geneticin and Dulbecco's modified Eagle medium containing high glucose was purchased from Mediatech, Inc (Herndon, VA). Non-essential amino acids and penicillin-streptomycin solution were purchased from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum (FBS) was purchased from Hyclone Laboratories (Logan, UT). D-Ala-GnRH, FLAG vector and anti-FLAG M2 monoclonal antibody were purchased from Sigma (St. Louis, MO). hCG was purchased from Research Diagnostics, Inc. (Flanders, NJ). QuikChange site-directed mutagenesis kit was purchased from Stratagene (La Jolla, CA) and Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA).

To construct cell lines used in these studies, we insert mutated cysteines in the rat LH receptor at positions 621 and 622 to serines using the QuikChange site-directed mutagenesis kit from Stratagene according to Manufacturer's instructions. Using PCR, GnRH receptors were coupled to the full length rat LH receptor C-terminus tail (amino acids

604-674; GnRHR-LHR-C_{full}), the rat LH receptor truncated C-terminus tail (amino acids 604-631; GnRHR-LHR-C_{t631}), or the rat LH receptor C-terminus with C621S and C622S mutations (GnRHR-LHR-$C_{C621,622S}$). Stable CHO cell lines were constructed expressing mutated rat LH receptors or chimeric GnRHR-LHR coupled to FLAG epitope on their N-terminus. To prepare these cell lines, vectors were constructed for FLAG-GnRHR-LHR-C_{full}, FLAG-GnRHR-LHR-C_{t631}, or FLAG-GnRHR-LHR-$C_{C621,622S}$. Then, 8μg DNA and 20 μL of Lipofectamine 2000 were added to CHO cells that were 90-95% confluent in 60 mm polystyrene plates. After 3-4 weeks, clones expressing FLAG were selected based on membrane fluorescence following cell labeling with Cy3 conjugated anti-FLAG antibody. Single particle tracking of FLAG-tagged chimeric receptors or mutant LHRs were performed as diccused in Chapter 2 for FLAG-rLHR.

Results and Discussion

LHR-C621,622S receptors do not translocate into small compartments upon binding hCG.

Protein palmitoylation, in addition to anchoring proteins in the plasma membrane (83), may affect the localization of these proteins in membrane microdomains. We have previously shown that LH receptors, upon binding of ligand, translocate to membrane microdomains (12). In addition, individual rat LHR identified with 40nm gold particles remain confined to these small membrane regions for comparatively long times and exhibit slow lateral diffusion. Treatment of cells with methyl-β-cyclodextrin disrupts membrane

microdomains containing hormone-occupied LHRs and increases the average rate of receptor lateral diffusion (Chapter 2).

To determine whether palmitoylation of the receptor affected receptor localization in membrane microdomains, we constructed receptors, originally described by Menon and coworkers, that contained mutations to both of the two possible palmitoylation sites on the LHR C-terminus (49, 84). We confirmed that receptors with these mutations retained the ability to signal via cAMP, as previously reported (49, 84), and, in fact, produced levels of cAMP that were comparably to wild type receptors (data not shown). Nevertheless, FLAG-tagged LHR-C621,622S did not translocate into small compartments following binding of hormone as shown in Table 5.

The LH receptor C-terminus has also been implicated in receptor function. Progressive truncation of the C-terminus affects targeting of the receptor to the membrane and, if the receptor is expressed in the membrane, the rate of receptor desensitization (82). As discussed earlier, palmitoylation of the C-terminus is implicated in the rate of receptor internalization (53, 79).

To determine whether the C-terminus of the LH receptor also played a role in receptor compartmentalization, we constructed the rat LH receptors with truncated C-terminus tail (amino acids 604-631; LHR-C_{t631}). Single particle tracking data also shows that receptors with truncated C-terminus tail LHR-C_{t631}, with or without binding of functional ligand binding, remain in large compartments in Table5.

Together these studies demonstrate a role for the LH receptor C-terminus in regulating the localization of the receptor within the membrane. It appears that

Table 5. FLAG-tagged LHR-C621,622S did not translocate into small compartments following binding of hormone .

Cell Line	Pretreatment	Number of particles analyzed	Number of domains/ 2 min trajectory	$D_{0\text{-}1}$[a] ($10^{-11}cm^2sec^{-1}$)	$D = L_t^2/4t$ [b] ($10^{-11}cm^2sec^{-1}$)	Time[c] (sec)	Domain Diameter (L_t)[d]
FLAG-LHR-wt	none	10	3 ± 1	1.3 ± 0.3	0.2 ± 0.2	26 ± 10	100 ± 9
FLAG-LHR-wt	hCG(100nM)	10	3 ± 1	1.0 ± 0.4	0.1 ± 0.03	23 ± 7	81 ± 10
FLAG-LHR-C621,622S	none	10	3 ± 1	1.1 ± 0.3	0.4 ± 0.6	29 ± 12	204 ± 74
FLAG-LHR-C621,622S	hCG(100nM)	10	3 ± 1	1.0 ± 1.0	0.1 ± 0.2	32 ± 11	195 ± 47

[a] $D_{0\text{-}1}$: Diffusion coefficient of the first 2 points

[b] D presents the diffusion coefficient within a domain as described by Saxton.

[c] The average diameter of an individual domain was calculated as described by Daumas et al. and Murase et al..

[d] Average time for residence within a domain

palmitoylation is important in driving receptor translocation into small compartments but whether compartmentalization occurs as a consequence of the affinity of palmitate for lipids wether these compartments or whether this is due to a structural change in the receptor itself, is not clear. Addition of the large C-terminus to GnRH receptors also appears to actively exclude the GnRH receptor from small compartments.

One lingering question is whether these membrane compartments are important in signaling by the LH receptor. Although Navratil *et al.* (74) have demonstrated that constitutive localization of the GnRH receptor in low density membrane microdomains is necessary for GnRH signaling to ERK, LHR with point mutations in the receptor's C-terminus that prevented palmitoylation, retained the ability to signal via cAMP following hCG treatment but did not translocate to small compartments or rafts (85). This is consistent with other results suggesting that disruption of rafts by methyl-β-cyclodextrin reduces, but does not completely eliminate, either receptor self-association or signaling. Localization of small compartments, while enhancing signaling efficiency, may not be essential to the production of cAMP in response to binding of hormone by luteinizing hormone receptors

CHAPTER 5

Discussion and Future Directions

LH receptor function is critically important for male and female reproductive success. These LH receptors signal through G-proteins and participate in ovulation, regulation of sex steroid synthesis, and maternal recognition of pregnancy in humans. We have investigated LH receptor organization in membrane receptor-mediated intracellular signaling using single particle tracking methods. Our results suggest that, in general, receptors capable of inducing productive signaling, are localized in small membrane compartments. These structures can be disrupted either by MβCD treatment which extracts cholesterol from the membrane or by cytochalasin D treatment to disrupt the cytoskeleton. It appears that the cytoskeleton may serve as a fence to restrict motion of the LH receptor following binding of ligand (Figure 18).

Together these studies raise several questions, some of which can be addressed using contemporary biophysical techniques.

First, are small compartments seen in single particle tracking experiments the same structures isolated using biochemical methods (rafts)? Fluorescence resonance transfer studies may address this question. If we assume that GM1 is a marker for membrane rafts, it may be possible to label FLAG-LHR with, for example, a large fluorescent tag that permits single particle tracking of the receptor and can be used as an energy transfer donor to fluorescent GM1 in FRET experiments. Sequentially obtaining images of confined

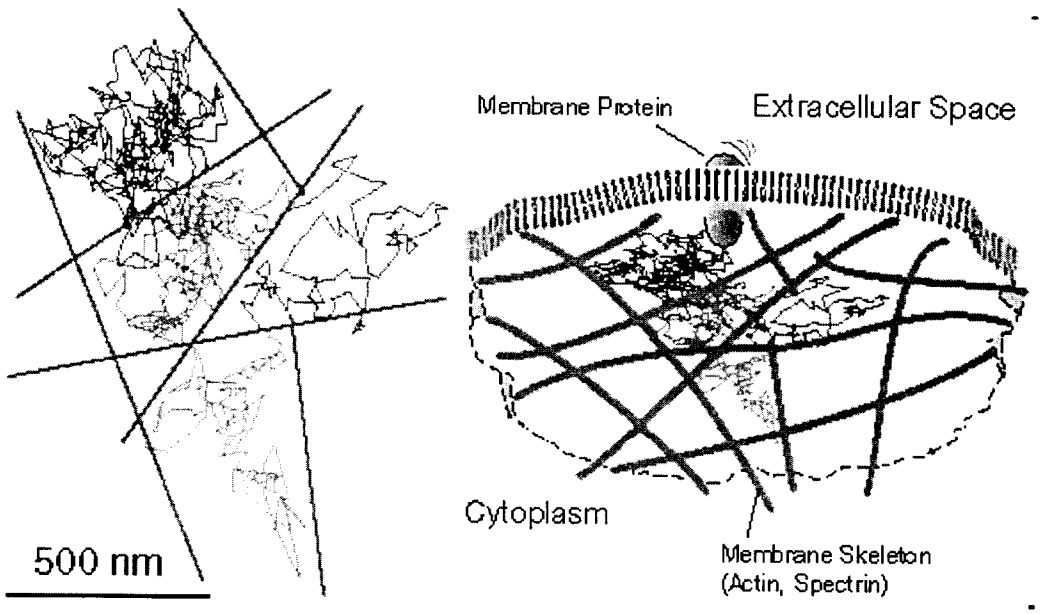

Figure 18. Trapping of individual particles within actin fences. This figure is adapted from Kusumi and coworkers (42).

fluorescent particles and energy transfer to GM1 might resolve such a question at the single molecule level.

Second, it remains unclear whether plasma membrane rafts/small compartments represent specialized membrane areas in which signaling is enhanced. An approach to this questions would again require imaging of two events. In this case, confinement of a particle within a compartment might be effectively imaged together with, for example, activation of a membrane-localized cAMP reporter molecule. Such a reporter molecule has recently been developed (86). This reporter uses energy transfer methods to examine changes in cAMP levels. Combining such a reporter with, for example, FLAG-LHR activated using hCG conjugated to 40nm gold particles would reveal whether cAMP levels were locally affected by hCG binding to its receptor.

Finally, our working model for LHR motions in the membrane includes cytoskeletal fences that define small membrane compartments. Keith Lidke and coworkers at the University of New Mexico have recently described an imaging method in which the cytoskeleton is labeled with GFP-actin and the membrane receptor is identified by a quantum dot. The challenge in single particle tracking of a quantum dot is that these dots "blink" on and off. Thus, a track may be interrupted by the dot turning off briefly. Lidke has solved this problem by designing software that uses a particle track established prior to when the dot turns off to identify the dot once it turns on again. Imaging of the particle track of the quantum dot-tagged LH receptor and actin filaments expressed with GFP, permits an evaluation of the compartment size both from the perspective of the receptor and as defined by actin. Interestingly, Lidke has shown the small breaks in actin fences permit

receptors to "escape" from confined membrane regions and diffuse in the membrane until captured again, an approach that we could employ with LHR receptors as well.

In conclusion, it is interesting to speculate that receptor compartmentalization may ultimately be as important for productive signaling as ligand binding. The receptor environment may be important for receptor conformation, may provide high concentrations of molecules needed for downstream signaling such as G proteins, and may also provide a mechanism for turning off signaling (receptor desensitization). Biophysical and imaging strategies for evaluating the role of the receptor environment are improving and may ultimately provide insight into signaling mechanisms functioning at the membrane level.

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LIST OF ABBREVIATIONS

AC: Adenylyl cyclase

ATP: Adenosine triphosphate

BSS: Hank's balanced salt solution

cAMP: Cyclic adenosine monophosphate

CFP: Cyan fluorescent protein

CHO: Chinese hamster ovary

D: Diffusion coefficient

Da: Dalton (molecular weight)

DAG: Diacylglycerol

DMEM: Dulbecco's modified minimum essential medium

EGFR: Epidermal growth factor receptor

Fab: Fraction antigen binding

FBS: Fetal bovine serum

FITC: Fluorescein isothiocyanate

FMPP: Familial male-limited precocious puberty

FPR: Fluorescence photobleaching recovery

FRET: Fluorescence resonance energy transfer

G418: Geneticin

GDP: Guanosine diphosphate

GFP: Green fluorescent protein

G_i: Inhibitory G protein

GM1: Membrane ganglioside

GnRH: Gonadotropin releasing hormone

GPCR: G protein-coupled receptor

GPI: Glycosylphosphatidylinositol

G_s: Stimulatory G protein

GTP: Guanosine triphosphate

hCG: Human chorionic gonadotropin

hLHR: Human luteinizing hormone receptor

IP_3: Inositol 1,4,5-triphosphate

LH: Luteinizing hormone

mAb: Monoclonal antibody (intact)

MβCD: Methyl-β-cyclodextrin

MEM: Minimum essential medium

PAGE: Polyacrylamide gel electrophoresis

PBS: Phosphate buffered saline

PIP_3: Phosphatidylinositol triphosphate

PKA: Protein kinase A

PKC: Protein kinase C

PLCγ: Phospholipase C gamma

RCT: Rotational correlation time

rLHR: Rat luteinizing hormone receptor

SDS: Sodium dodecylsulfate

TNFR: Tumor necrosis factor receptor

TPA: Time-resolved phosphorescence anisotropy

TrITC: Tetramethyl rhodamine isothiocyanate

VFP: Visible fluorescent protein

YFP: Yellow fluorescent protein