THESIS

INVESTIGATING LUTEINIZING HORMONE RECEPTOR SIGNAL TRANSDUCTION USING THE CYCLIC AMP REPORTER ICUE1

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WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERVISION BY XIN HUANG ENTITLED INVESTIGATING LUTEINIZING HORMONE RECEPTOR SIGNAL TRANSDUCTION USING THE CYCLIC AMP REPORTER ICUE1 BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

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ABSTRACT OF THESIS

INVESTIGATING LUTEINIZING HORMONE RECEPTOR SIGNAL TRANSDUCTION USING THE CYCLIC AMP REPORTER ICUE1

Mechanisms involved in initiation of signaling by LH receptors have been under active investigation because they play important roles in human fertility and development of gonadal tumors. Assay of cAMP levels in response to hormone treatment is usually used to demonstrate LH receptor activation and has historically relied on biochemical methods. ICUE1 is an Epac-based cAMP reporter which undergoes conformational changes after binding cAMP. Unlike traditional biochemical assays, ICUE1 combined with FRET techniques is capable of real-time monitoring of cAMP levels in individual cells.

In this project, Epac reporters have been used to evaluate LH receptor activity in cells expressing constitutively-active LH receptors or cells where transactivation of LH receptors is reported to occur. For the investigation of constitutively active LH receptors, DNA of ICUE1 and yoked LH receptor were co-transfected into CHO cells and expressed on the cell membrane. For the study of LH receptor trans-activation, CHO cells were developed using two plasmids encoding LH receptors with defects either in ligand binding or coupling of LH receptor to Gs. Our results show ICUE1 offers an useful tool for evaluating cAMP levels in real-time using single cell imaging methods. Further application of this technique to studies evaluating cAMP level in cells

where ligand binding to receptor can be visualized using, for example, quantum dots or nano-gold particles linked to individual molecules of hCG, will be of interest as will studies of cAMP levels during LH receptor desensitization and resensitization in response to brief pulses of hCG.

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CHAPTER I

INTRODUCTION

A. LUTEINIZING HORMONE RECEPTOR STRUCTURE

Luteinizing hormone (LH) and human chorionic gonadotropin (hCG) are members of the glycoprotein hormone family (1). Both LH and hCG can bind luteinizing hormone receptor (LHR), a 93 kDa glycoprotein that is a member of the G protein-coupled receptor superfamily. LH receptors are single polypeptide chains with seven hydrophobic α-helices that comprise the transmembrane regions of the receptor. The receptor endodomain consists of seven membrane spanning segments linked by three intracellular and three extracellular loops and intracellular C-terminus. The large N-terminal LH receptor exodomain binds LH or hCG with high affinity and is joined to the receptor endodomain by a linker region (2)(Figure 1).

Mechanisms involved in initiation of signaling by LH receptors are of interest because of the receptor's importance in fertility. 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 (3). Binding of LH from the anterior pituitary results in signaling cascades leading to follicle maturation, steroidogenesis or spermatogenesis.

B. LUTEINIZING HORMONE AND HUMAN CHORIONIC GONADOTROPIN STRUCTURE

Human chorionic gonadotropin (hCG) plays a central role in human reproduction, serving as a critical hormone involved in maternal recognition of pregnancy. It is synthesized by the syncytiotrophoblast cells in the placenta and is needed for corpus luteum rescue and the stimulation of progesterone production during early pregnancy. hCG is a member of the glycoprotein hormone family which also includes pituitary-derived luteinizing hormone, follicle-stimulating hormone, and thyroid-stimulating hormone. These hormones are structurally related; each has two subunits, α and β. The α subunit is common to all members of the glycoprotein family, whereas each hormone has a unique β subunit that determines the receptor specificity (4, 5). The non-covalent association of the two subunits into a heterodimer is required for hormonal activity (4). A distinct feature of hCGβ is the presence of a C-terminal extension of about 30 amino acid residues (CTP) that is not essential for hormone activity (6, 7) but may reduce the rate of hormone degradation in the plasma.

C. LUTEINIZING HORMONE RECEPTOR ACTIVATION AND SIGNAL TRANSDUCTION

The human luteinizing hormone receptor (LH receptor) has 674 amino acids which are organized into an extracellular N-terminus (exodomain) and an endodomain made up of seven transmembrane domains with connecting intracellular and extracellular loops and an intracellular C-terminus. Binding of either LH or hCG to the LH receptor's exodomain results in interactions between the hormone-occupied exodomain and the receptor's transmembrane regions and extracellular loops. The receptor endodomain becomes activated and capable of interacting with Gs. This process is called cis-activation (Figure 2).

Initially the G-protein α subunit is bound to GDP and the α, β and γ subunits are complexed together. $G_{\beta\gamma}$, the complex of β and γ subunits, inhibits G_α activity. Hormone binding to the extracellular domain of the LH receptor causes a conformational change in the receptor that is transmitted to the G-protein on the cytosolic side of the membrane. The nucleotide-binding site on G_α becomes more accessible to the cytoplasm where the concentration of GTP is usually higher the GDP concentration. G_α releases GDP and binds GTP, a process refers to as GDP-GTP exchange. Substitution of GTP for GDP causes another conformational change in G_α. G_α-GTP dissociates from the inhibitory βγ subunit complex and can now bind to and activate adenylate cyclase which is a membrane-associated enzyme. Adenylate cyclase, activated by the stimulatory G_α-GTP, catalyzes synthesis of cAMP (Figure 3). Protein Kinase A (cAMP-Dependent Protein Kinase) catalyzes transfer of phosphate from ATP to serine or threonine residues of various cellular proteins, altering their activity.

Structural features of the LH receptor are necessary for hormone-mediated receptor activation. Ryu et al. have shown that a lysine residue at position 583 (Lys583) in exoloop 3 is crucial and irreplaceable for receptor activation of cAMP synthesis. Exoloop 3 is comprised of 11 amino acids and flanked by two Lys residues, Lys573 and Lys583, that are located at the boundaries with the transmembrane sequences 6 and 7, respectively. All substitutions including arginine for Lys583 did not affect high affinity hCG binding to the receptor but did result in the complete loss of cAMP synthesis induced by hormone (8).

Activation of LH receptor by hormone is also accompanied by receptor-receptor aggregation. Large clusters of wild type LH receptors tagged with green fluorescent protein (LHR-GFP) form within minutes following binding of either LH or hCG to receptors on viable cells (9). It is not known whether these clusters reflect aggregation of receptors cis-activated by

ligand or aggregated receptors in which ligand-occupied receptors have trans-activated nearby unliganded neighbors (10). The presence of receptors in physically large structures is also suggested by lateral diffusion studies of hormone-treated LH receptors on luteal cell from sheep and rat in which most LH receptors were laterally immobile (11).

D. LUTEINIZING HORMONE RECEPTOR TRANS-ACTIVATION

Recently, a novel mechanism of intermolecular G protein-coupled receptor (GPCR) activation, referred to as trans-activation, has been described by Ji et al.(10) (Figure 4). Trans-activation of GPCR refers to binding of hormone to one receptor that leads to activation of an adjacent receptor (10).

To investigate rescue of cAMP production via LH receptor trans-activation, Ji et al. constructed mutant LH receptors either defective in hormone binding (LHR-K583R and LHR-I105A) but capable of cAMP production, or capable of hormone binding but defective in cAMP production (LHR-I55A and LHR-I80A). Their results showed that when 293 cells were cotransfected with the LHR-K583R and LHR-I55A plasmids, hCG bound to available receptors with the same high affinity it bound wild type receptors (10). The cells also produced cAMP in response to hCG in a dose-dependent manner. The maximum cAMP level of LHR-K583R and LHR-I55A was approximately 43-fold higher than basal cAMP levels and 33% of the wild type maximum cAMP level. A higher hCG concentration was needed to induce maximum cAMP response for LHR-K583R and LHR-I55A as compared to the wild-type LHR (10).

To test whether the rescue of cAMP production was caused by changes in the G protein or adenylate cyclase activity, cells were treated with cholera toxin, an activator of Gsα that leads

to an increase in adenylate cyclase activity and cAMP production (10). All of the cells expressing the wild-type LH receptor, LHR-K583R, LHR-I55A, LHR-I80A, LHR-I105A, LHR-K583R and LHR-I55A, LHR-K583R and LHR-I80A, or LHR-K583R and LHR-I105A produced similar amounts of cAMP. These results show that functional hormone-occupied LH receptor exo-domain was capable of trans-activating the endo-domain of an unliganded LH receptor (10) .

Ji et al. also investigated whether an LH receptor exo-domain capable of binding hCG but separated from the LH receptor endodomain could activate an LH receptor incapable of binding ligand. In these experiments Ji et al. constructed a hybrid receptor composed of the LH receptor exo-domain attached to the single membrane spanning transmembrane domain of CD 8 (ExoCD). ExoCD was expressed on the cell surface and bound hCG with high affinity, but was incapable of inducing cAMP formation. Coexpressing ExoCD with LHR-I55A or LHR-I80A and introducing hCG did, however, successfully induce cAMP production. Coexpressed of ExoCD with LHR-I105A, LHR-K583R together with LHR-I55A or LHR-K583R together with LHR-I80A did not result in cAMP production when hCG was introduced. Thus the exo-domain did not have to be attached to the LH receptor endodomain for hCG binding and activation of available, functional LH receptor endodomains (10).

E. CONSTITUTIVELY ACTIVE LUTEINIZING HORMONE RECEPTORS

Naturally-occurring mutant LH receptors have been identified including constitutively active LH receptors that are continuously active in the absence of exogenous hormone. In vitro, there are two ways to obtain constitutively active LH receptors. Several mutations in LH receptor

at position 578 are associated with constitutive activation of the receptor (3). Asp578Gly was the first human mutant LH receptor (12) to be identified in males with precocious puberty. It remains the most frequently encountered LH receptor mutant (13). The presence of this mutant receptor results in constitutive activation of Gs by the receptor (14) in the absence of LH or hCG. Furthermore, other mutations in the human luteinizing hormone (hLH) receptor aspartic acid residue at position 578 are associated with constitutive receptor activation and naturally-occurring pathologies such as familial male-limited precocious puberty (12) and Leydig cell adenomas (15).

Lee et al. investigated how the LHR-D578G mutant of LH receptor constitutively stimulates cAMP production and whether this activating mutant induced activation of adenylate cyclase using the same mechanism as the hCG-activated wild-type receptor (16). Lee et al. hypothesized that the LHR-D578G mutant interacts with Gαs and activates it using the same mechanism used by liganded wild-type receptor (16). Normally, hCG binds to the wild type LH receptor and the receptor activates Gαs which, in turn, activates adenylate cyclase to convert ATP to cAMP (17, 18). To test whether G-protein-coupled receptors interact with the C-terminal region of Gα subunits, corresponding C-terminal minigenes were used (19). Lee et al. utilized the C-terminal region of Gα subunits to compare induction of cAMP by wild-type LH receptor and LHR-D578G receptors. Their results indicated that different mechanisms were involved in elevated basal cAMP induced by the activating mutations of LH receptor and increases in cAMP levels induced by the wild-type receptor activated by hCG binding (16).

To determine whether constitutively-active LH receptors were also constitutively associated with one another in the absence of ligand, FRET methods were used (3). Lei et al. used wild type LH receptor and LH receptor with mutations at position 578 and constructed

vectors attaching these receptors to either cyan fluorescent protein (CFP) or yellow fluorescent protein (YFP). Using fluorescent acceptor bleaching and imaging methods, she showed that stably-expressed constitutively active receptors exhibited 11-15% FRET efficiency while FRET efficiency for wild type LH receptor was less than 1%. This suggested that that constitutively active LHRs, unlike wild type LH receptors, were self-associated in the absence of ligand (3). Interestingly, values for FRET efficiency between constitutively active receptors did not change with the addition of hCG (3). Moreover, the constitutively active LHRs were generally located in high buoyancy membrane fractions, so-called plasma membrane rafts (3).

Another constitutively active form of LH receptor is the yoked LH receptor (YHR) developed by Chengbin Wu and Prema Narayan (20). The yoked receptor is composed of a single chain hCG molecule that has been covalently coupled to the LH receptor. To construct the complex, Wu et al. generated the entire coding sequence of yoked hCG followed by the first half of the C-terminal peptide (CTP) sequence, and ligated it with an LH receptor sequence containing the second half of the CTP sequence, upstream of the coding sequence for the mature receptor. The ligated product was subcloned into the BamHI site of the eukaryotic expression vector pcDNA3 (20). Wu et al. generated both COS-7 and HEK 293 cell lines transfected with wild type LH receptor or yoked LH receptor to investigate receptor expression in transfected cells. Western blot analysis with an antibody against CTP showed that the yoked LH receptor complex was expressed in transfected cells (20).

Cells transfected with yoked LH receptor were unable to bind significant amounts of exogenous hormone (20) but, nonetheless, had elevated basal levels of cAMP suggesting that yoked LH receptor was constitutively active. To investigate further, ^{125}I-hCG was added to COS-

7 cells transfected with either wild-type LH receptor or yoked LH receptor cDNA (20). No significant amounts of exogenous hCG bound yoked LH receptor expressed in COS-7 cells.

The yoked LH receptor is also functional *in vivo*. Coonce et al. investigated the effects of yoked LH receptor expressed in transgenic mice. They detected elevated testosterone levels in male mice expressing yoked LH receptor at 3 and 5 weeks of age (21). There was also a decrease in testicular weight and serum levels of LH and follicle stimulating hormone (21). Coonce et al. also reported that the mRNA levels for insulin-like growth factor binding protein 3 were up-regulated in 3- and 5-week-old yoked LH receptor mice, and the mRNA levels for several germ cell-specific proteins were up-regulated at 5 weeks of age in both wild type and yoked LH receptor mice (21).

F. FLUORESCENT RESONANCE ENERGY TRANSFER (FRET)

It remains unclear whether constitutively-active LH receptors become self-associated when transducing hormone. This can be examined on viable cells using fluorescence resonance energy transfer between well matched fluorescent donor and acceptor pairs which indicates that donor and acceptor molecules are at distances less than 100Å apart. Hetero-FRET is FRET between two different fluorescent protein while homo-FRET refers to FRET between two identical fluorescent proteins. In hetero-FRET, excitation of a fluorescence donor leads to energy transfer to fluorescence acceptor and emission by that acceptor when a donor-acceptor pair are in close proximity (Figure 5).

Imaging FRET is a sequential procedure in which images of CFP (fluorescence donor D) and YFP (fluorescence acceptor A) are obtained separately, the fluorescent acceptor is irreversibly photobleached, and images of CFP and YFP are obtained again. When there is FRET between donor and acceptor, the fluorescence signal from donor increases after acceptor photobleaching. Energy transfer efficiency is calculated using:

$$\%E = [(D_{after} - D_{before}) / D_{after}] \times 100$$

G. ICUE1, an Epac-based cAMP reporter

Evaluation of cAMP levels in cells has historically relied on biochemical assays. As fluorescence techniques have improved, one active area of research has been the development of fluorescent sensors, including a cAMP sensor, for real-time monitoring of cell events in living cells. Cyclic AMP is a second messenger that regulates many cellular functions through its effectors including protein kinase A (PKA) and Epac, exchange proteins directly activated by cAMP (22). Epac is a family of molecules that are guanine nucleotide exchange factors (GEFs) for Rap1 and Rap2 (23). Rap GTPases cycle between an inactive GDP-bound state and an active GTP-bound state, with GEFs mediating the exchange of GDP for GTP. Rap proteins are involved in many biological processes, most notably the regulation of cell adhesion through integrins and cadherins (24) (Figure 6).

The GEF Epac1 has a C-terminal catalytic domain characteristic of exchange factors for Ras family GTPases and an N-terminal regulatory domain. The N-terminal regulatory domain contains a cAMP-binding site similar to those of protein kinase A (PKA). In addition it has a

DEP domain that mediates membrane attachment (23, 25). Zhang et al. constructed fluorescent indicators that report intracellular cAMP levels and Epac activation by sandwiching the full-length Epac 1 between CFP and YFP (22) (Figure 8). Elevations in cAMP decrease FRET between CFP and YFP and increase the ratio of cyan-to yellow emissions by 10-30% in living mammalian cells (Figure 9). This response can be reversed by removing cAMP-elevating agents or abolished by mutating the critical residue responsible for cAMP binding (22). Targeting of the reporter to the plasma membrane, where cAMP is produced in response to the activation of β-adrenergic receptor, has been used to demonstrate a faster cAMP response at the membrane than in the cytoplasm and mitochondria (22).

In this project, the Epac reporters has been used to evaluate LH receptor activity in cells expressing constitutively active LH receptors or when receptor activation occurs via trans-activation of LH receptor pairs.

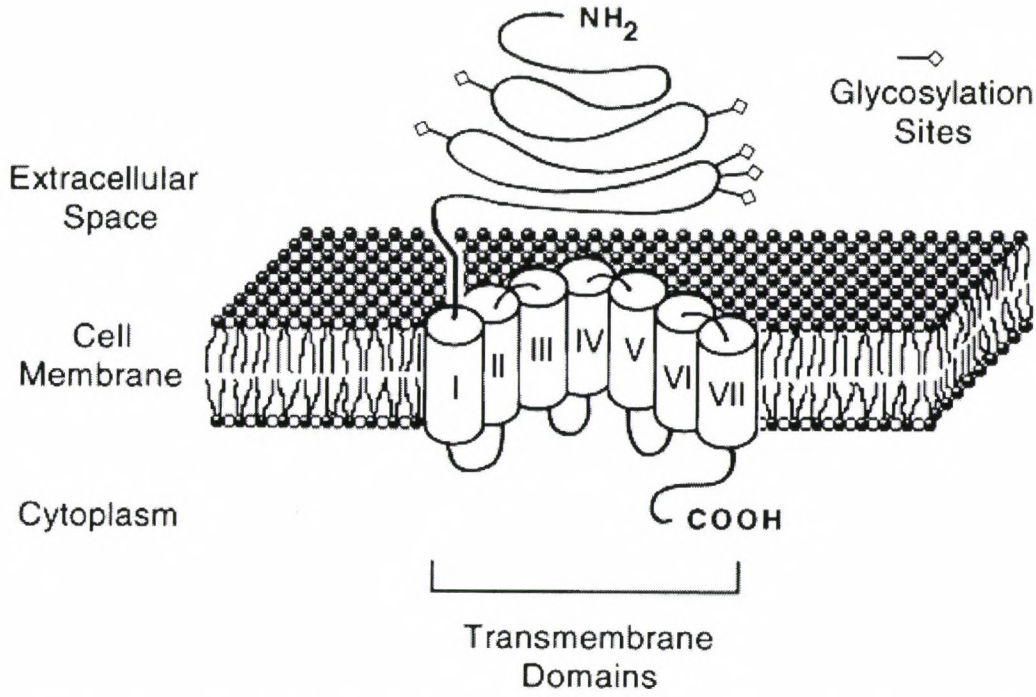

Figure 1. Luteinizing hormone receptor structure (modified from Segaloff DL, Sprengel R, Nikolics K, Ascoli M: The structure of the lutropin/choriogonadotropin receptor. Recent Prog Hormone Res 46:261–303, 1990.)

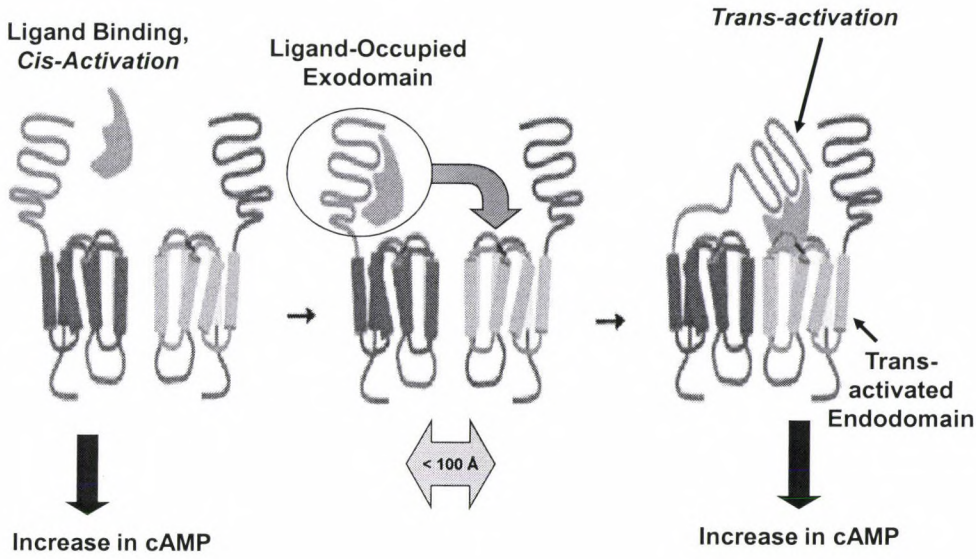

Figure 2. Cis-activation of the Luteinizing hormone receptor (adapted from Ji et al. 2002) (10). Hormone binding to the LH receptor exodomain results in activation of the receptor's endodomain which consists of the seven transmembrane sequences, the extracellular and intracellular loops and the long cytoplasmic tail.

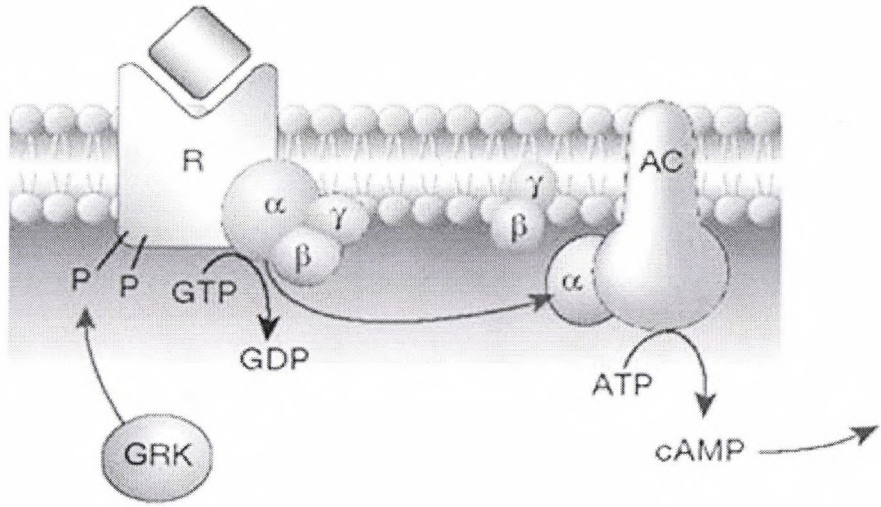

Figure 3. LHR activation of G_s and adenylate cyclase (AC) (Adapted from Peter H. Raven, George B. Johnson, Jonathan B. Losos, and Susan R. Singer, Biology (7th edition), McGraw-Hill Co. NY, Chapter 7).

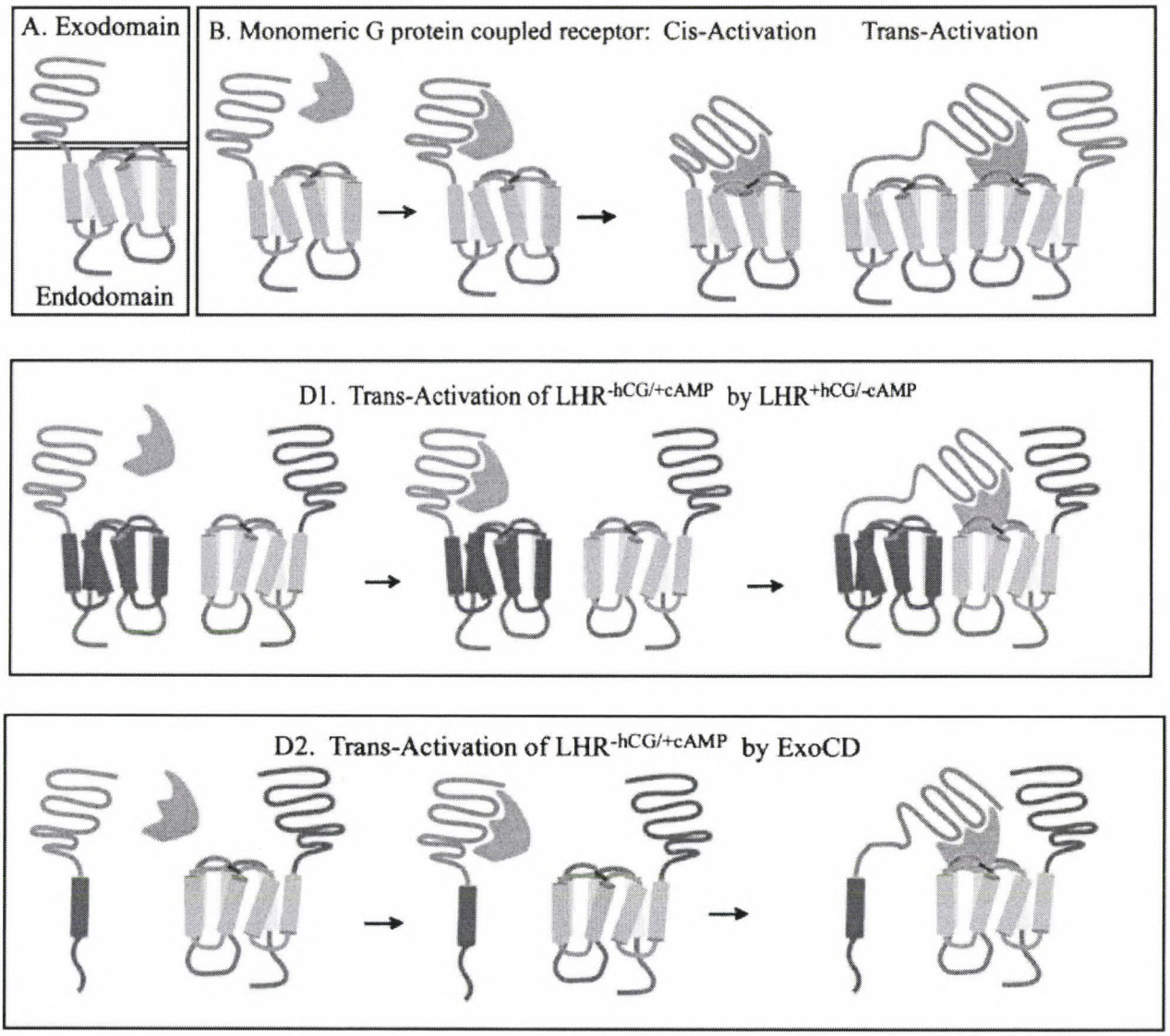

Figure 4. A schematic representation of LHR cis- and trans-activation (adapted from Ji et al., 2002) (10).

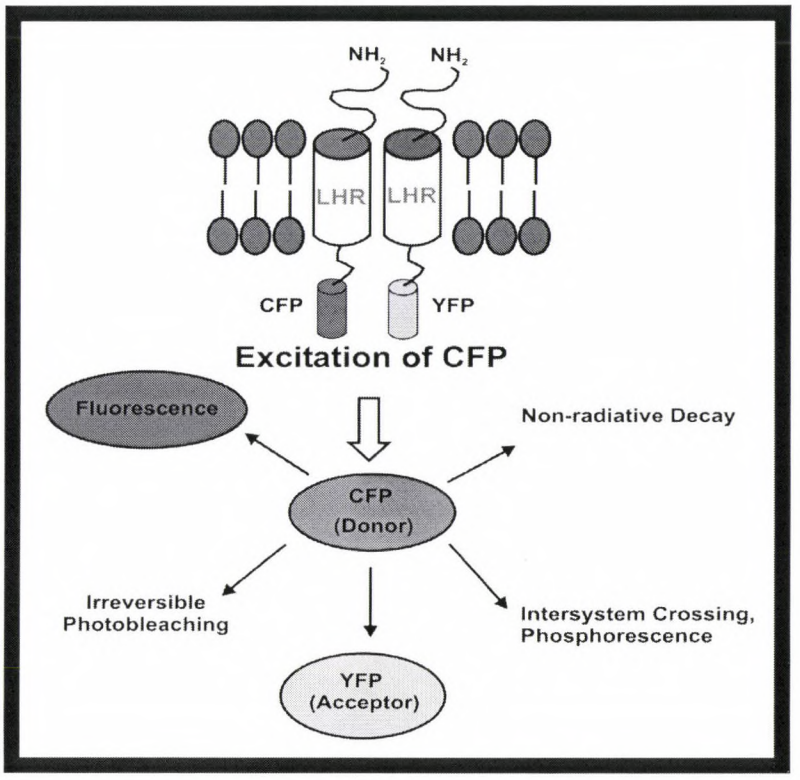

Figure 5. Hetero-FRET between CFP-LHR and YFP-LHR. Activation of the fluorescence donor CFP results in energy transfer to the YFP acceptor when these molecules are less than 100Å apart. When energy transfer is occurring between CFP and YFP, photobleaching of the YFP acceptor using a mercury arc lamp or other light source, results in an increase in fluorescence from the CFP donor.

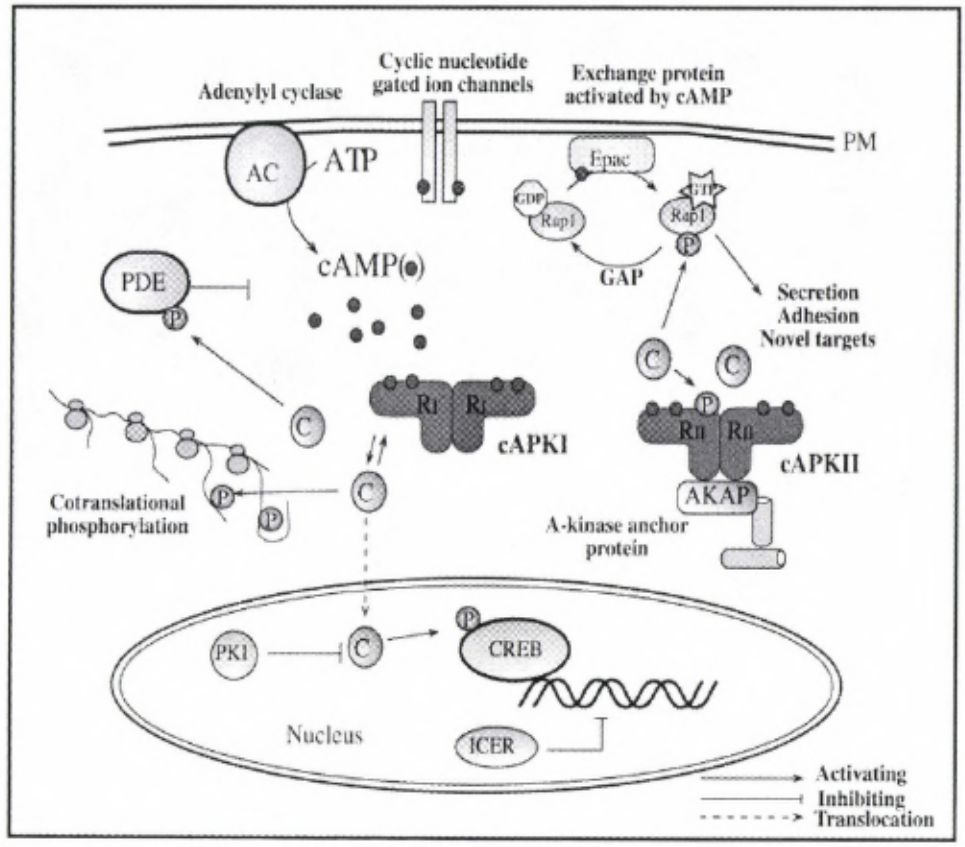

Figure 6. Epac is an exchange protein activated by cAMP. This molecule can be targeted to the plasma membrane using a membrane-targeting amino acid sequence and modified to include CFP and YFP. In the absence of cAMP, these fluorophores are close to one another and energy transfer from CFP to YFP occurs. When cAMP binds to Epac, a conformational change in Epac results in physical separation of CFP and YFP and a reduction in energy transfer efficiency (from Kopprud et al. 2003) (26).

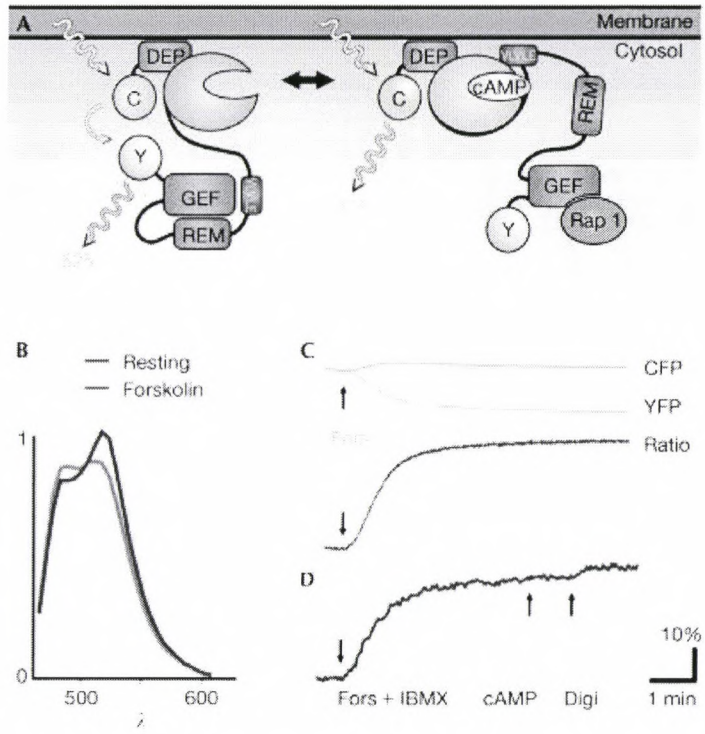

Figure 7. An Epac-based cAMP reporter has been engineered to undergo a decrease in FRET in the presence of cAMP. This decrease in FRET occurs between enhanced cyan (ECFP) and citrine fluorescent proteins due to the conformational change of Epac when binding cAMP(adapted from Bos et al. 2003) (24).

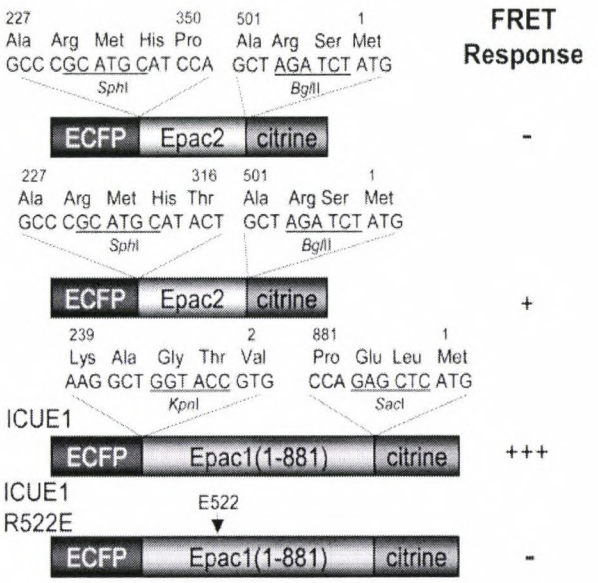

Figure 8. Domain structure and comparison of FRET responses for Epac-based cAMP reporters. Sandwiched between ECFP and citrine are truncated forms of Epac2, full-length Epac1 with or without R522E mutation, with R522 corresponding to R279 in Epac1. The construct that produced the highest FRET response in the absence of cAMP was designated as ICUE1 (adapted from Jin Zhang et al., 2004) (22).

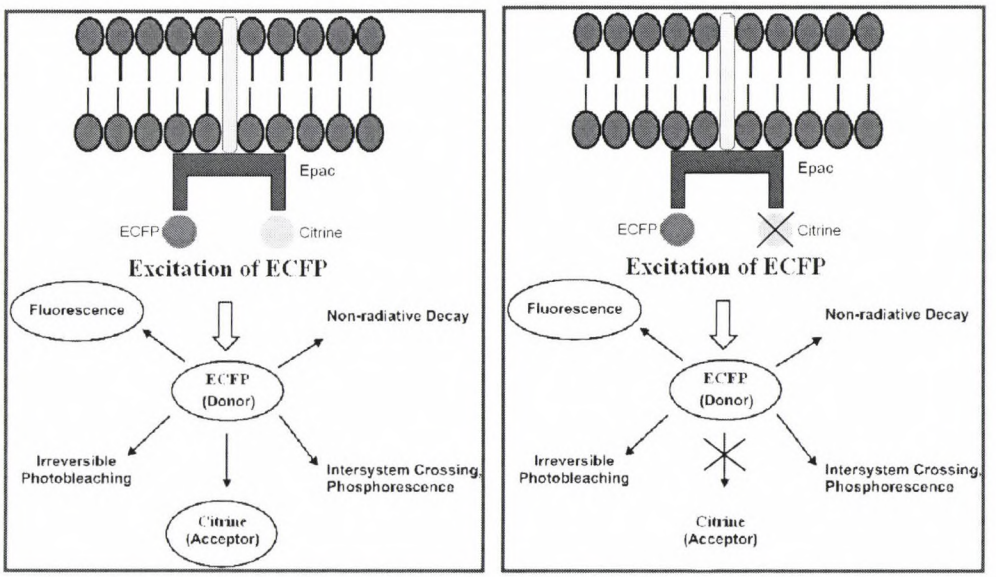

Figure 9. FRET between ECFP and Citrine on Epac. In the absence of cAMP, ECFP and citrine are close together and values for energy transfer efficiency are high. The extent of energy transfer is evaluated by comparing ECFP fluorescence before and after irreversible photobleaching of citrine on Epac. When these molecules are close, photobleaching of citrine results in an increase in fluorescence emission from ECYP. (adapted from Zhang et al., 2004) (22).

CHAPTER II

USE OF AN EPAC-BASED cAMP REPORTER TO INVESTIGATE SIGNAL TRANSDUCTION OF CONSTITUTIVELY ACTIVE LUTEINIZING HORMONE RECEPTORS

INTRODUCTION

Levels of cAMP during LH receptor signaling have been investigated using several methods including colorimetric cAMP assays, ELISA techniques, radiolabeled cAMP, etc. Recently, a novel Epac-based cAMP reporter associated with fluorescent resonance energy transfer method has been introduced to study cell signaling.

The goal of this project was to create cell lines that expressed different forms of LH receptor including constitutively active receptors and evaluate cAMP levels using a FRET-based cAMP reporter. ICUE1 was used as a Epac-based cAMP reporter. This molecule undergoes a conformational change upon binding cAMP that reduces FRET between the fluorescent donor and acceptor that are integral components of this reporter molecule.

Hetero-FRET between ECFP and citrine (EYFP) on ICUE1 was performed using an imaging FRET method to detect a conformational change in ICUE1 upon binding cAMP. CHO cells were used and cell lines were prepared that transiently expressed either ICUE1 alone, LHR-D578G and ICUE1 or yoked LH receptor and ICUE1. This is the first time that an Epac-based cAMP reporter has been used to investigate cAMP production by a constitutively active luteinizing hormone receptor.

MATERIALS AND METHODS

The DNA for ICUE1 cloned in pcDNA 3 was a gift from Lisa DiPilato at The Johns Hopkins University School of Medicine. The DNA for the yoked LH receptor cloned in pcDNA 3 was a gift from Dr. Prema Narayan at Southern Illinois University. The CHO-D578G cell line was prepared by Dr.Ying Lei (3).

For amplification, DNA was transformed into *E.Coli*, DH5α, which readily takes up DNA when heat shocked at 42°C. Cells were then incubated on ice for 35 minutes. The mixture of *E.Coli* and DNA was plated on L-broth agar plates with ampicillin and grow overnight at 37°C. Mono-clones were picked up and inoculated in 3ml L-broth medium with ampicillin, then grown at 37°C on a shaker for 15 hours. The DNA was extracted from *E.Coli* according to the Qiagen mini-prep protocol. The DNA samples were detected by running 0.8% agarose gel stained with ethidium bromide to determine whether the extracted DNA was the correct size. DNA concentration was determined by using Smart Spec 3000 (Bio-Rad).

Untransfected Chinese hamster ovary (CHO) cell medium was made using 450ml Dulbecco's modified Eagle medium containing high glucose from Cellgro (Manassas, VA), 50ml fetal bovine serum from Clontech (Palo Alto, CA), 5ml non-essential amino acids solution from Sigma (St. Louis, MO), 5ml L-glutamine (Gemini Bio-Products), and 5ml penicillin-streptomycin solution from Sigma (St. Louis, MO). Transfected CHO cells medium was made with all of the ingredients used for untransfected CHO cells medium with the addition of 0.2mg geneticin from Gibco (Grand Island, NY).

Chinese hamster ovary (CHO) cells were cultured in 2mL untransfected CHO cell medium and CHO-D578G cells were cultured in 2mL transfected CHO cell medium in 35x10 mm dishes at 37°C. When cells were at about 90% confluency, CHO cells were transfected with 2.5 μg ICUE1 DNA in 6.25 μl Lipofectamine 2000 according to the Invitrogen Lipofectamine 2000 protocol. The remained dishes of CHO cells were co-transfected with a total of 2.5 μg of ICUE1 and yoked hCG LHR DNA. CHO-D578G cells were transfected with 2.5 μg ICUE1 DNA using the Lipofectamine 2000 protocol. Transiently transfected cells were then incubated at 37°C in culture medium for 24 hours.

To prepare stable cell lines, cells diluted 1:10 dilution in medium were placed into larger dishes using PBS containing 5mM EDTA and cultured with transfected CHO cells medium. When cells confluency was greater than 90%, they were placed in glass-bottom dishes for FRET studies. In some experiments, 100 nM forskolin was added to CHO cells transfected with ICUE1. All were then fixed using 2% paraformaldehyde and 1M glycine, washed twice using PBS and maintained for the duration of FRET measurements in 2 mL PBS.

For hetero-FRET studies of ICUE1, cells were imaged using a Zeiss Axiovert 200M microscope and Omega Optical filter sets for imaging of CFP and YFP. Metamorph software from Universal Imaging was used to acquire and analyze images. Before photobleaching YFP, CFP and YFP were imaged separately using exposure times of 200 – 1000ms. After photobleaching YFP for 5 minutes using the Zeiss mercury arc lamp source and the appropriate Omega Optical filter, YFP and CFP were imaged again. Five minute exposure to 525 nm light was sufficient to bleach essentially all YFP signal. The intensity of CFP signals before and after YFP photobleaching was then compared. Image backgrounds were corrected by subtracting

background fluorescence without cells from the emission intensities of fluorescent cells expressing reporters. Energy transfer efficiency was calculated as

$$\%E = [(D_{after} - D_{before}) / D_{after}] \times 100$$

RESULTS AND DISCUSSION

Different values of E% was obtained from CHO cells expressing ICUE1 alone, CHO cells expressing ICUE1 and treated with 100 nM forskolin, CHO-D578G transiently expressing ICUE1 and for CHO cells transiently expressing both yoked LH receptor and ICUE1. A representative experiment is shown in Figure 10. Untreated CHO cells expressing ICUE1 cells had an average FRET efficiency of 26.2 ± 13.7% which suggested that basal levels of cAMP were low in these cells and that there was no significant conformational change in ICUE1 due to cAMP.

When CHO cells expressing ICUE1 were treated with 100 nM forskolin, energy transfer efficiency was marked reduced to 5.9 ± 1.93%. This is consistent with forskolin effects on adenylate cyclase activity and resulting increases in cellular levels of cAMP. A representative experiment is shown in Figure 11.

CHO cells that stably express LHR-D578G are constitutively active as shown by Lei et al. (3). These cells exhibit higher basal levels of cAMP than do untransfected CHO cells or CHO cells expressing wild type LH receptor. In addition, these receptors become constitutively self-associated and localized in high buoyancy membrane fractions that may be involved in signal transduction. In these studies, CHO cells expressing LHR-D578G had lower values for FRET efficiency than did CHO cells transfected with ICUE1 alone suggesting that the basal level of

cAMP in the presence of the LHR-D578G was higher. A representative experiment is shown in Figure 12.

Finally, we evaluated the activity of yoked hCG-LHR (YHR) in CHO cells. This receptor has been reported to be constitutively active in the absence of exogenous hCG. In cells expressing both yoked hCG-LHR and ICUE1, reduced FRET efficiency suggests that yoked LH receptor is constitutively active in CHO cells and the basal levels of cAMP are higher than in normal cells.

Together these results suggest that the ICUE1 reporter is a useful probe for evaluating cAMP levels in viable cells. Results from these various cell lines were consistent with reported changes in cAMP evaluated using other, traditional biochemical assays such as colorimetric assays or assays requiring radiolabeled cAMP. The major advantage of ICUE1, however, is that this probe makes it possible to evaluate cAMP levels in real-time as cell conditions change and to evaluate these changes in cAMP within a single cell using single cell imaging methods.

Applications of this probe to studies of LH receptor function including an application outlined in Chapter 3 below are numerous. In particular it would be of interest to know whether locally high levels of cAMP can be evaluated in cell membranes where ligand binding to receptor can be visualized using, for example, quantum dots or nano-gold particles linked to individual molecules of hCG. It would also be of interest to follow cAMP levels during LH receptor desensitization and resensitization in response to brief pulses of hCG.

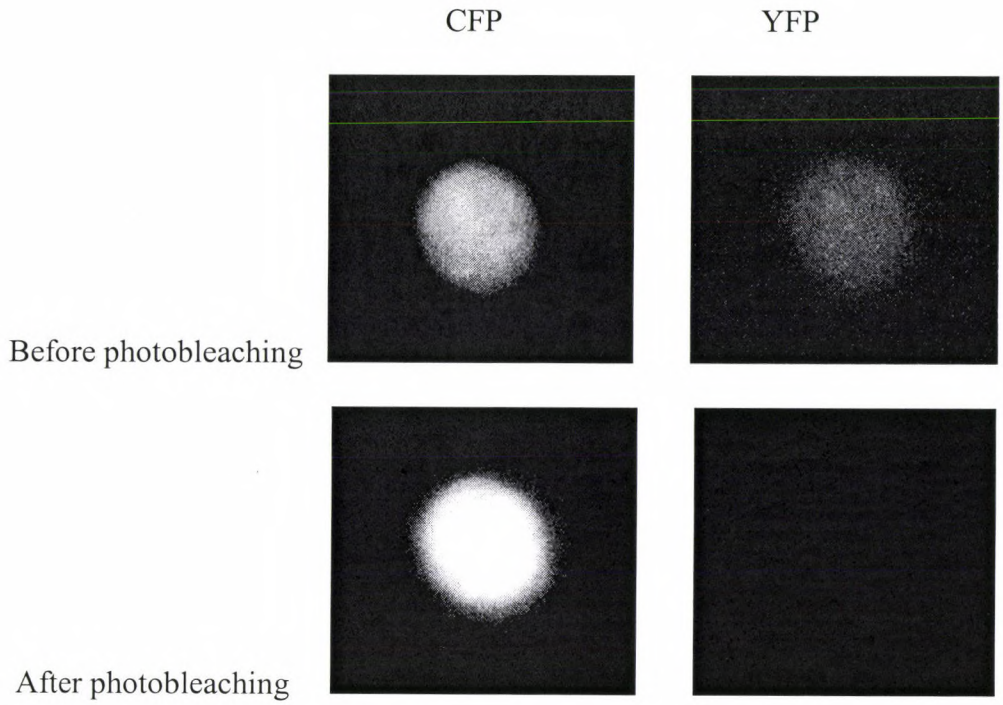

Figure 10. Hetero-FRET of a CHO-ICUE cell showed CFP fluorescence increased in 41.8% after photobleaching YFP.

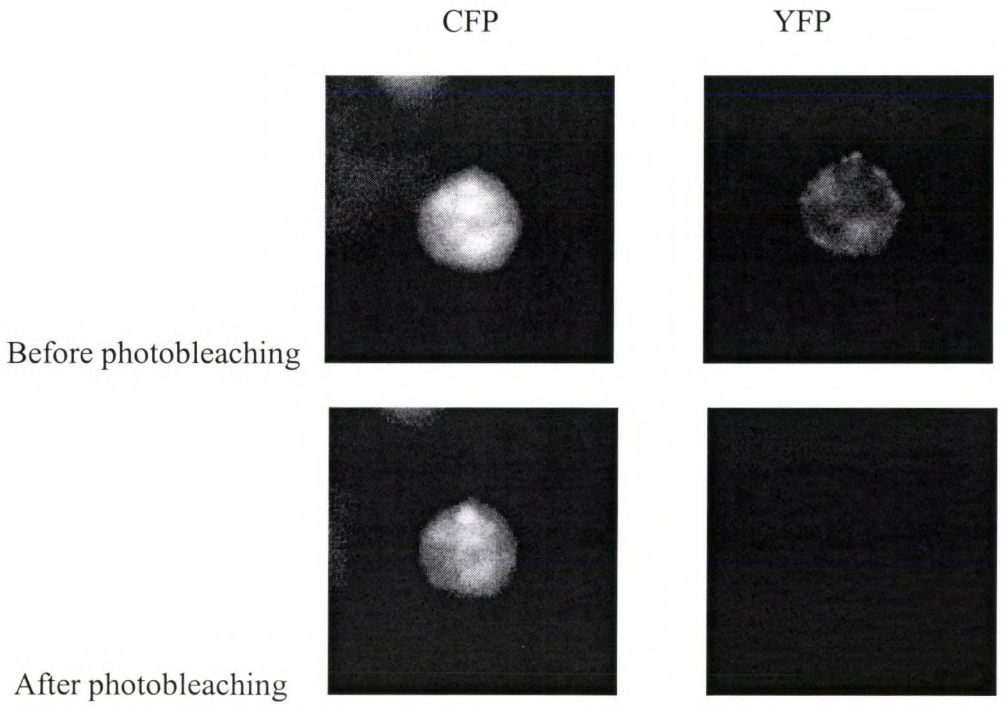

Figure 11. Hetero-FRET of a CHO-ICUE1 cell treated with 100nM forskolin for 5 minutes showed that the increase of CFP fluorescence was only 7.6% after photobleaching YFP.

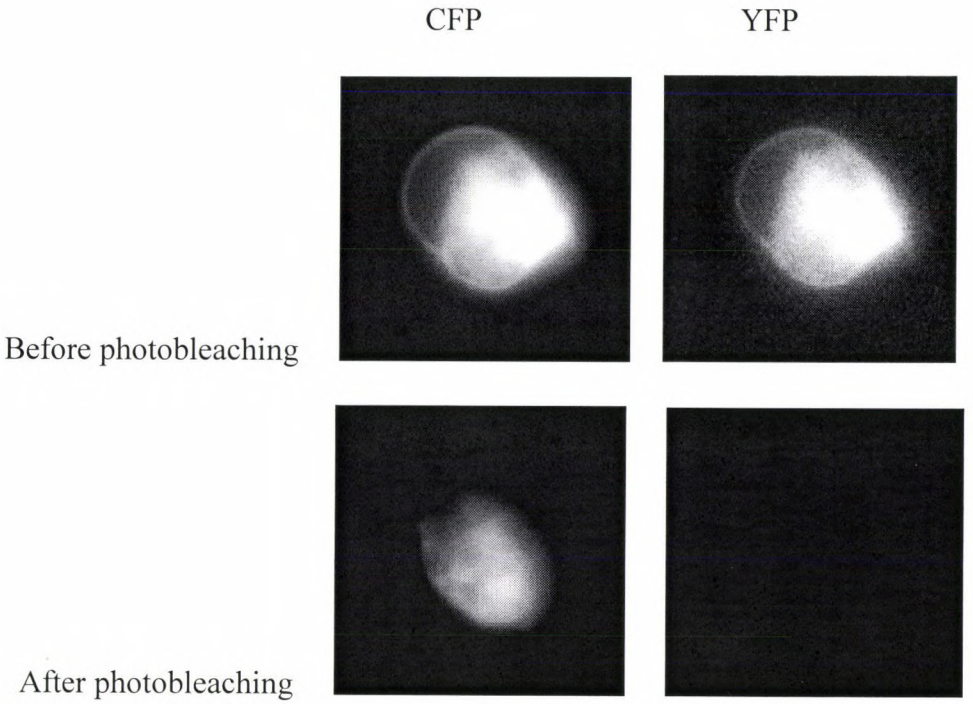

Figure 12. Hetero-FRET of a CHO-D578G-ICUE1 cell showed that CFP fluorescence increased 4.1% after photobleaching YFP.

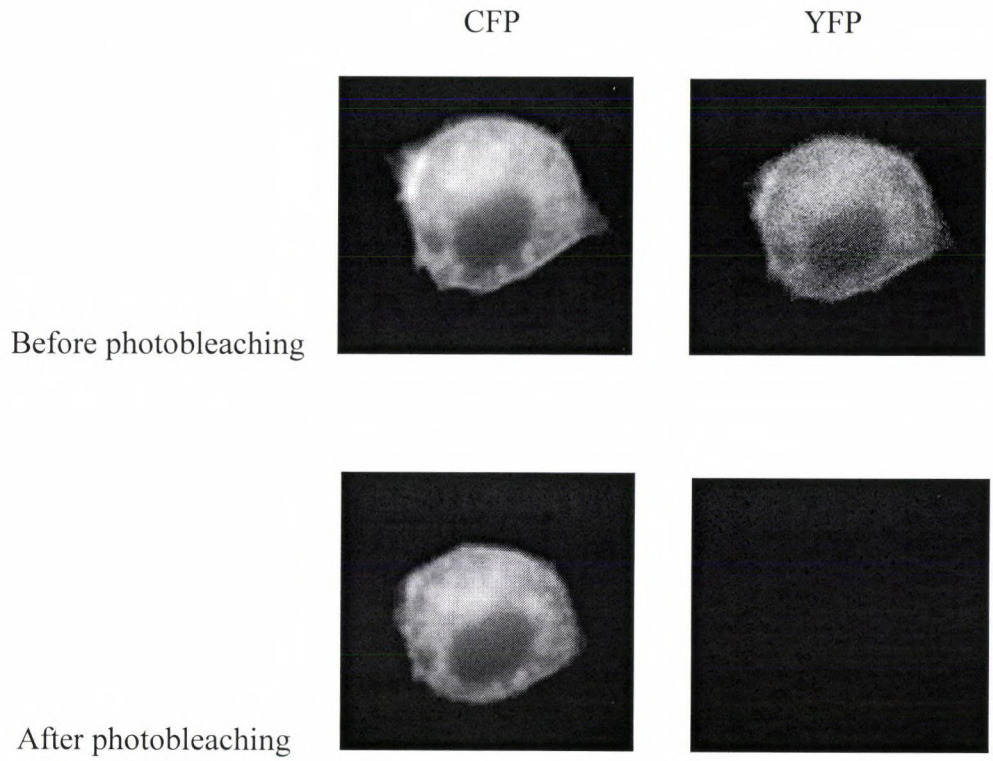

Figure 13. Hetero-FRET of a CHO-YHR-ICUE1 cell showed that the increase of CFP fluorescence was 9.7% after photobleaching YFP.

Table I Summary of ICUE1 results

Sample	% Energy Transferred Efficiency	% Standard Deviation	n
CHO-ICUE1	26.2 %	13.7%	3
CHO-ICUE1 treated with 100 nM forskolin	5.9%	1.93%	3
CHO-D578G-ICUE1	6.2%	2.90%	2
CHO-YHR-ICUE1	10.6%	1.27%	2

CHAPTER THREE

INVESTIGATING LUTEINIZING HORMONE RECEPTOR TRANS-ACTIVATION USING ICUE1 AND CONCLUSIONS

INTRODUCTION

G protein-coupled receptors are the largest class of plasma membrane receptors and important targets for drug discovery (27) including receptor antagonists and, for constitutively-active G protein-coupled receptors, reverse agonists. Although mechanisms involved in signal transduction by these receptors have received considerable attention, several fundamental steps in ligand-mediated receptor activation are far from settled. Thus, it is not resolved whether G protein-coupled glycoprotein hormone receptors such as LH receptor undergo *only* cis-activation following binding of ligand or can also trans-activate other receptors via interactions between their hormone-occupied exodomains and the endodomains of nearby receptors (see Figure 2). To address this question, we have proposed to examine whether signal amplification occurs via coupling of receptor cis-activation with trans-activation using the Epac1 cAMP reporter. An outline for these studies is described below.

METHODS

The studies proposed to use intact rat LH receptor and rat LH receptor exodomains. The vectors needed to construct these cell lines have been generously provided by Dr. Tae Ji at the University of Kentucky. Although it would be convenient to use transiently transfected cells for these studies, it is difficult to control the total number of receptors expressed per cell and thus to interpret many of the studies we propose. In addition, transiently expressed receptors are more likely to be retained intracellularly due to problems with folding (28). Therefore, our goal was to prepare stable lines as previously described (3). These cell lines would ideally have approximately 8,000-15,000 of each receptor involved in trans-activations and 20,000-30,000 total receptors, numbers reported for trans-activating LH and FSH receptors by Ji and coworkers (10, 29, 30). For receptors with functional exodomains, fluorescent derivatives of hCG would be used to evaluate the number of ligand-binding receptors via flow cytometry. Receptors that cannot bind ligand would be epitope tagged and evaluated using a fluorescently-conjugated antibody specific for the epitope tag. As controls for cell lines expressing two receptor constructs, we also planned to develop stable cell lines that express only one or the other receptor at levels similar to that receptor's level of expression in co-transfected cells or at a higher level that was comparable to the total receptor number in co-transfected cells.

PROJECT DESCRIPTION

We hypothesized that LH receptor trans-activation amplified the hormone signal that resulted from cis-activated nearby receptors. Although Ji and coworkers have shown

biochemically that signaling occurs in hCG-treated cells expressing functional rat LH receptor exodomains and functional receptor endodomains (29), the mechanism involved in LH receptor trans-activation is not known. We planned to examine whether LH receptor trans-activation was accompanied by interactions between receptor pairs where one receptor can bind ligand and a second receptor is capable of coupling that hormone signal to G_s (see Figure 2).

To trans-activate LHR-I55A , which is incapable of binding ligand but which has a fully functional receptor endodomain, we proposed to construct molecules which bound hormone and potentially interactd with nearby receptors that had functional endodomains (29).

The following cell lines were to be constructed.

a. Exo^{wt}-CD trans-activating LHR-I55A. The transmembrane and cytoplasmic tail of CD8 would be coupled to the wild type rat LHR exodomain at CD8's extracellular N-terminus.

b. LHR-K583R trans-activating LHR-I55A. LHR-K583R (9) has a functional exodomain that binds hCG with high affinity (8, 31). Ligand binding, however, results in little or no cAMP production, indicating that signaling by the receptor's endodomain is impaired. LHR-I55A would be coupled to the FLAG epitope.

c. Exo^{wt}-GPI trans-activating LHR-I55A. We proposed to use c-Myc- and FLAG epitope tags coupled in both cases to the N terminus of the LHR exodomain. Fab fragments of antibodies specific for these epitope tags (available from Santa Cruz Biotechnology and Sigma, respectively) would be prepared using a Pierce ImmunoPure Fab kit. The use of Fab fragments reduces crosslinking of target proteins which, in our experience, can occur with monoclonal as well as polyclonal antibodies (32). To quantitate the number of receptors expressed per cell, Fab

fragments would be derivatized with Cy3 or Cy5 (Amersham Biosciences). In addition we planned to co-express wild type LH receptor with FLAG-LHR-I55A/K583R.

FRET measurements of transiently transfected Epac1 would be used to evaluate whether binding of ligand (100 nM hCG) to Exo^{wt}-CD, LHR-K583R or FLAG-Exo^{wt}-GPI is accompanied by increased cAMP in CHO cells. Increased fluorescence intensity from the fluorescence donor, after photobleaching of the fluorescence acceptor is indicative of energy transfer from fluorescence donor to acceptor (Figure 4). Cells would be grown on coverslips and examined using an Olympus 63x 1.0 NA water immersion objective. Initially, an image of the CFP would be obtained using an Olympus FV5-LD440-2 laser. YFP protein would then be imaged. A selected membrane region would then be bleached with full power from the 488nm laser for YFP and the YFP fluorescence re-imaged to verify at least 80% bleaching of the acceptor. CFP fluorescence would then be re-imaged. Fluorescence intensities in the acceptor-bleached (I_D) and unbleached portions of the sample (I_{DA}) would be calculated after background subtraction and corrections for donor photobleaching during acceptor bleaching and for laser drift. Olympus software allows numerical average intensities to be calculated for regions of interest which is particularly useful when evaluating FRET efficiencies from the plasma membrane. Energy transfer efficiency %E is calculated as $(1-I_{DA}/I_D) \times 100\%$.

We anticipate that receptor pairs capable of trans-activation in biochemical studies would interact with one another. We would then treat cells with increasing concentrations of hCG (0.1-100 nM hCG) to determine whether the extent of LH receptor trans-activation, as indicated by FRET efficiencies and cAMP levels, is dose-dependent.

Although previous results suggest that it is unlikely, we would also examine whether there are interactions between hormone-occupied exodomains, either LHR-K583R, Exo^{wt}-CD or FLAG-Exo^{wt}-GPI, used to trans-activate other receptors. Our previous FRET studies of LHR-K583R suggest that they do not interact despite binding ligand with high affinity (1). However, receptors with a single membrane spanning domain are sometimes capable of signal transduction following dimerization or oligomerization (2). Thus it is important to rule out possible aggregation of Exo^{wt}-CD-YFP, particularly if there is an evidence of elevated cAMP levels in hormone-treated cells expressing this LH receptor exodomain.

This would be done by evaluating homo-transfer FRET between YFP molecules using polarization-FRET methods. Hetero-transfer FRET (hetero-FRET), used in the experiments described above, arises when the emission spectrum of an excited donor chromophore overlaps the absorption spectrum of a spectroscopically distinct acceptor chromophore. Thus, excitation of a fluorescence donor leads to energy transfer to a fluorescence acceptor and emission by that acceptor when a donor-acceptor pair are in close proximity. FRET, however, can also occur between like molecules. For most fluorophores, the absorption spectrum overlaps substantially with the emission spectrum and permits like molecules to readily interchange excitation energy. Thus FRET between VFP moieties of the same type can be as efficient as FRET between different VFPs. For example, yellow fluorescent protein (YFP) exhibits a Förster distance (R_0) for homo-transfer FRET (homo-FRET) (33) that is comparable to the values for hetero-FRET between common donor-acceptor pairs such as CFP and YFP. Homo-FRET is manifest in reduced fluorescence polarization of the single fluorescent species (34). Using an Olympus FV300 confocal microscope fitted with a polarizing beam splitter in the detector dichroic slider, sequential YFP images are recorded using 488nm excitation and acquisition until YFP intensities

have fallen to ~10% of their initial values. The YFP image sequence is then unpacked into sequences of images of fluorescence polarized parallel (v_i) and perpendicular (h_i) to the exciting light. The intensity and anisotropy in image sequences, s_i and r_i respectively, are calculated as $s_i=v_i+2gh_i$ and $r_i=[v_i-gh_i]/s_i$, respectively. In each image, mean anisotropy is calculated for the same plasma membrane region of interest and this value is plotted versus illumination time. Homo-FRET is indicated by an initial anisotropy substantially below the 0.37 observed for free YFP and by increasing anisotropy as the sample is bleached.

RESULTS

Construction of plasmids for preparation of cells expressing rLHR-K583R-ECFP and rLHR-I55A-EYFP. The overall plan for this project was first to insert rLHR DNA into ECFP and EYFP vectors to construct LHR-ECFP and LHR-EYFP and then to use gene mutation to obtain LHR-K583R-ECFP and LHR-I55A-EYFP vectors. Several protocols were tried of which the final method involved use of rLHR DNA in pcDNA3 and of ECFP and EYFP vectors. rLHR DNA in pcDNA3 was obtained from Dr. Prema Narayan and ECFP and EYFP vectors were obtained from Dr. James Bamburg. Transformation in *E.Coli* was used to amplify the plasmids which were then examined using restriction enzyme digestion. Since there were no appropriate digestion sites in the rLHR plasmid, EcoRI and BamHI sites in the rLHR plasmid were created by PCR using appropriately designed primers.

At this point, it became apparent that sequence information for rLHR was not correct. Despite repeated efforts, the digestion products did not have the correct molecular weight suggesting that putative restriction sites were not available. Should digestion products of these vectors have exhibited the correct molecular weights, rLHR DNA would have been sepatated from PCR products and purified from a gel. We would have then digested rLHR, ECFP and EYFP using EcoRI and BamHI, purified rLHR, ECFP and EYFP from digestion products and ligated rLHR with ECFP or with EYFP before beginning steps leading to receptor mutation.

DISCUSSION AND FURTHER STUDIES

If trans-activation of the LHR-I55A occurs following binding of hCG to Exo-CD or LHR-K583R, we anticipate seeing a decrease in the efficiency of energy transfer, indicative of an increase in cAMP, that is dependent on hormone concentration. Of interest is whether FRET values obtained using ICUE1 are comparable to those observed for wild-type rat LH receptors treated with 100 nM hCG and whether there are dose-dependent increases in cellular levels of cAMP.

The nature of interactions between LH receptors involved in trans-activation will require further study using various FRET methods for evaluating receptor-receptor interactions. We expect, based on Ji's results (10), that cAMP will be elevated in hCG-treated cell lines expressing receptor pairs capable of trans-activation. If cAMP levels increase in response to hCG but there is no evidence of hetero-FRET between trans-activating receptor pairs, we will

conclude that receptors are interacting with one another transiently. Together with Epac1 evaluation of cAMP levels, these studies will clarify the mechanism used by receptors to transactivate signal in response to hormone binding.

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