韓國家畜繁殖學會誌 23(4) : 347~352(1999)
Korean J. Animal Reprod.

Functional Expression of Lutropin/Choriogonadotropin and Follitropin Receptor cDNAs in 293 Cells

Min, K. S.

Breeding and Reproduction Division, National Livestock Research Institute

ABSTRACT

This cDNAs were cloned with the aid of the polymerase chain reaction (PCR) by sequences based on cloned rat LH/CG and FSH receptor cDNAs. A cDNAs of LHR and FSHR were transfected into the 293 cells. Several clonal cell lines were obtained expressing different numbers of cell surface receptors. One cell lines for each LHR and FSHR were chosen, and a corresponding cell lines expressing the wild type LHR and FSHR were selected based on the number of cell surface receptor for the particular LHR and FSHR. The abilities of the LHR and FSHR to transduce the hCG and FSH signals were measured by quantitating cAMP accumulation in cells incubated with increasing concentrations of hCG and FSH. The cAMP accumulation effects for these receptors were increased by the increasing concentrations of hCG and FSH. Thus, most of the receptors expressed in cells transfected with LHR and FSHR could be detected by measuring hormone binding and cAMP response, and can utilize to study the structrue function and signal transduction of the choriogonadotropins and glycoprotein hormones.

(Key words : LH/CG receptor, FSH receptor, Expression)

I. INTRODUCTION

The LH/CG receptor (LHR), a membrane glycoprotein that is present on testicular on Leydig cells and ovarian theca, granulosa, luteal, and interstitial cells, plays a pivotal role in the regulation of gonadal development and function in males as well as in nonpregnant and pregnant females (Wang et al., 1993). In both males and females, the LH/CG receptor recognizes the pituitary hormone LH. However, the same receptor also recognizes the placental hormone choriogonadotropin (CG) in the pregnant female (Min et al., 1996b). The glycoprotein hormone receptors are unusual members of the family of G proein-coupled receptors in that they have large extracellular domains (300~400 amino acids) and bind large ligands (28~38 kDa). Since most other members of the family of G proein-coupled receptors have small N-terminal extracellular domains (30~50 amino acids in length) and bind small ligands (200~300 Da) via interactions with amino acids present in the transmembrane helices (Segaloff and Ascoli, 1993).

A cDNAs for the LHR and Follicle stimulating hormone receptor (FSHR) from several species have been cloned, sequenced, and expressed, conclusively establishing that this re-

Corresponding Author : 564 Omock Cheon-Dong, Kweon Sun-Ku, Suwon City, Kyong Gi-Do, Korea 441-350. Tel: (0331)-290-1624. E-mail: ksmin@nlri.go.kr

ceptor is in indeed a single polypeptide chain (Segaloff and Ascoli, 1993; Simoni et al., 1997). The cloning of the cDNAs for the LH/CG receptor has also generated new knowledge and experimental tools that are rapidly being used to probe novel aspects of the functions and regulation of this important receptor. Like many other G protein-coupled receptors, the LH/CG and the FSH receptors become phosphorylated upon agonist stimulation (Hipkin et al., 1995; Wang et al., 1997). Phosphorylation is an important event in the agonist-induced desensitization of many G protein-coupled receptors (Freedman et al., 1997).

In the present study, we determined the full length of LH and FSH receptor cDNAs and investigated the expression to study the functions of gonadotropins in 293 cells.

Ⅱ. MATERIALS AND METHODS

1. Materials

The expression vector pcDNA3 was purchased from Invitrogene (San Diego, CA, USA). Endonucrease, LA PCR kit, Primers, Agarose were form Takara (Japan). Embryonic kidney 293 cells (CRL 1573/1573.1) were from American Type Culture Collection (MD, USA). Lipofectamine, DMEM, Wa/BSA, newborn calf serum (FBS) and Hepes were from Gibco BRL (MD, USA). Purified hCG (CR-127) and FSH were obtained from the National Hormone and Pituitary Agency of the NIDDK. [^{125}I]hCG and [^{125}I] FSH were prepared as described (Min et al., 1996b). The QIAprep-spin plasmid kit was from QIAGEN Inc. (Hilden, Germany) and all the other reagents were from Wako Pure Chemicals (Osaka, Japan).

2. RNA Extraction, PCR and Sequencing

Rat testes for FSH receptor around day 10 after birth and rat testes for LH receptor were prepared for total RNA extraction. Total RNA was extracted by the CsCl ultracentrifugation method as reported previously (Min et al., 1994). First-strand cDNA synthesis was done by AMV reverse transcriptase as reported previously (Min et al., 1996a). LHR primers [sense:5'agatatcgagctcacactcaggctggcgggc3' and antisense:5'ctctagctcgaattcactgatgtaaacag3'] and FSHR primers [sense:5'cgctcgagacgcaggagcctggggaatc3' and antisense:5'ctctagaggatccattttcacattgattt3'] were designed from the nucleotide sequences of the LHR and FSHR cDNAs.

PCR was carried out with the first-strand cDNAs synthesed for cloning of LH and FSH receptor cDNAs. PCR product was cloned into the pcDNA3. DNA sequencing was performed by the dideoxy chain-termination method using an AutoRead sequencing kit and an ALF DNA sequencer (Pharmacia LKB, Uppsala, Sweden). Sequencing analysis was performed with MacMolly Tetra computer software (Berlin, Germany) and compared with the database of GenBank/EMBL (MacMolly data).

3. Cell Culture and Expression

The LH receptor cDNA was inserted into the *Eco* RV and *Xba* I sites of pcDNA3. The FSH receptor cDNA also was inserted into the *Xho* I and *Xba* I sites of pcDNA3. The expression vectors were transfected into 293 cells by the liposome formulation (Lipofectamine) transfection method as previously described (Min et al, 1996b). The cells were maintained by incubation in growth medium (DMEM media containing 10 mM HEPES, 50 μg/ml gentamicin, and 10% newborn calf serum, pH 7.4) in a humidified atmosphere containing 5% CO_2. Selection was then started by supplementing the growth medium with 700 μg/ml G418. The surviving colonies were then picked up individually with the

aid of a pipetman. A series of clonal cell lines expressing LH and FSH receptors were screening using a radioligand binding assay.

4. Saturation Receptor Binding Assays

[^{125}I]hCG and [^{125}I]FSH were prepared as previously reported (Min et al., 1996b). The cells (2×10^5) were plated in 6 well plates coated with gelatin. The wells were placed on ice and the cells were washed twice with 2 ml aliquots of Wa/BSA and placed in 1 ml of the same medium containing a trace amount of [^{125}I]hCG (0.1 ~1,000 ng/ml) and [^{125}I] FSH (0.1~1,000 ng/ml). After an overnight incubation at 4C, the cells were scraped into a small volume of cold Wa/BSA and centrifuged at 1,500 × g for 10 min at 4C. The supernatants were aspirated and the cells were resuspended in 2 ml of cold Wa/BSA and collected by centrifugation again before counting in a gammer-counter. All determinations were performed in duplicate, the binding affinity and maximal binding capacity were calculated by analyzing the data using the computer program LIGAND and Deltagraph (Munson and Rodbard, 1980).

5. cAMP Assays

Cells were preincubated with 0.5 mM MIX for 15 min and then incubated with increasing concentrations of hCG (30 min), FSH (15 min) and cholera toxin 100 ng/ml (2 hours) at 37℃. The wells were then placed on ice, and after adding 1 ml of the cold stop solution (1 N perchloric acid with 360 μg/ml theophylline), the cells were scraped and transferred to glass tubes. The wells were washed with 0.5 ml of 0.5 N perchloric acid with 180 μg/ml theophylline, and this combined with the previous extract. One ml of the supernatants was neutralized with 0.5 ml of 0.72 M KOH/0.6 M $KHCO_3$ and centrifuged again to remove the salt precipitates. The samples were then used for cAMP measurement by RIA as previously described (Wang et al., 1997).

Ⅲ. RESULTS

1. PCR Results and Sequences

Using the cDNA prepared from rat tissues, PCR was performed to amplify the LHR and FSHR cDNAs. The cDNA fragments predicted for LHR (2.5 kb) and FSHR (2.2 kb) were amplified (Fig. 1).

The PCR-amplified DNA fragments were digested with *Eco* RV and *Xba* I endonucleases for LHR and digested with *Xho* I and *Xba* I endonucleases for FSHR. And then it was inserted into the same digested sites of pcDNA3 expression vector. Analyses of the nucleotide sequences of LHR and FSHR were identical to the previous data reproted by McFarland et al., (1989) and Sprengel et al., (1990). The mature LH/CG receptor is a 674 amino acids with a predicted molecular mass of 75 kDa. The predicted rat FSH receptor protein also is composed

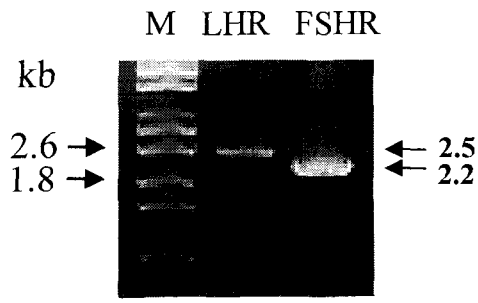

Fig. 1. PCR amplification of the LH and FSH receptors. The PCR products predicted with each primer were analyzed by 1.0% agarose electrophoresis. The major fragments obtained, which comprised 2.5 kb (LH) and 2.2 kb (FSH), were amplified. M, markers (kb).

of 692 amino acids, including 17 amino acids, which encode a hydrophobic signal peptide. Therefore, the mature protein is likely to consist of 675 amino acids.

2. Binding and cAMP Assay

Embryonic kidney 293 cells were stably transfected with the cDNAs encoding for the LHR and FSHR, and hormone binding was measured. Although several stable transfectants were isolated, only one cell line expressing each of the receptor was used for further analysis. The result of a representative experiment designed to measure parameters of hCG binding to LHR expressing cells is shown in Fig. 2. The result presented shows that cells expressing the LHR bind hCG with high affinity. The binding of FSH was bounded with high affinity (not shown).

We next tested the ability of the receptors to translate the binding of hCG into an increase in cAMP accumulation. A representative experiment is presented in Fig. 3. Since the maximal response elicited by an agonist is dependent on the binding affinity and the number of receptors,

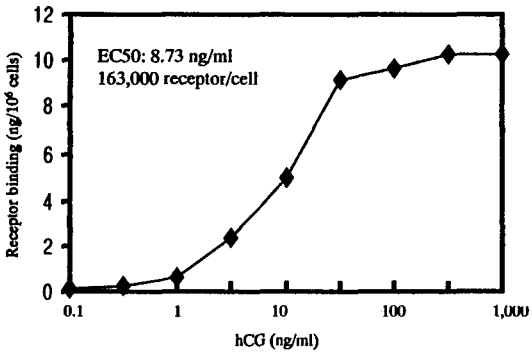

Fig. 2. Binding of ^{125}I-hCG to cell surface wild type LH/CG receptor. 293 cells were transfected with wild type LH/CG receptor and subjected to the ^{125}I-hCG binding assay described under "Materials and Methods".

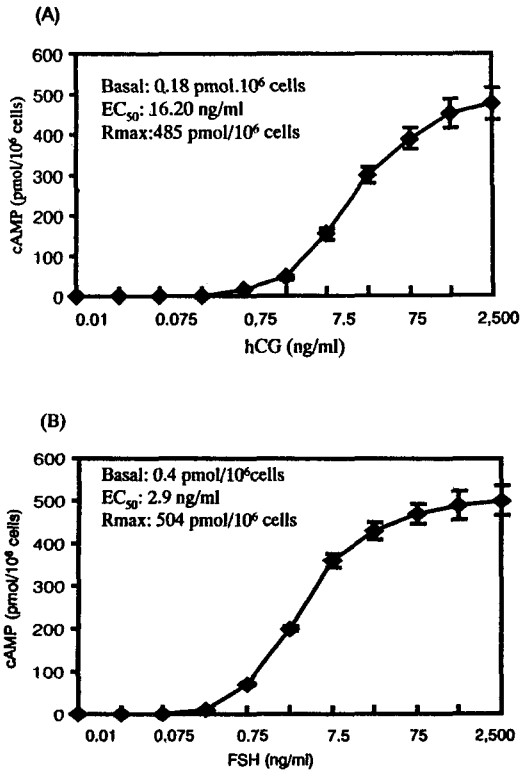

Fig. 3. Effects of increasing concentrations of hCG or FSH on cAMP accumulation in sable transfectants of 293 cells expressing LHR-wt (A) and FSHR-wt (B). Cells were incubated with the indicated concentrations of hCG and FSH in medium containing 0.5 mM 3-isobutyl 1-methyl xanthine for 30 min at 37 C before total cAMP was assayed (see Materials and Methods for details). Each point represents the average ± SEM of three independent experiments. Duplicate dishes were used for each experiment.

it is important to make these cells that express similar numbers of receptor. The basal cAMP levels were 0.18 pmol /10^6 cells for the LHR and maximal cAMP responses were 485 pmol /10^6 cells (Fig. 3A). The basal cAMP levels for the FSHR were 0.4 pmol /10^6 cells and maximal

cAMP responses were 504 pmol/10^6 cells (Fig. 3B). The maximal cAMP response seems directly proportional to the number of receptors.

Ⅳ. DISCUSSION

The present studies were designed to investigate the possibility that the clonal cell lines of LHR and FSHR are select and could be apply for the signal transduction of the choriogonadotropins containing equine CG (eCG), hCG and FSH. To pursue this goal we prepared the clones of LHR and FSHR cDNAs and transfected into the 293 cells to be express the receptors and picked up the each cells binding hCG and FSH. Theoretically, then, the hCG-induced desensitization of hCG-induced cAMP responses can be due to changes in the density of cell surface LHR (down-regulation) and/or changes in the functional properties of a constant number of cell surface LHR (uncoupling) (Wang et al., 1997). There is line of evidence presented here to indicate that the agonist-induced desensitization of the hCG-responsive adenylyl cyclase detected in transfected 293 cells under the conditions used herein is due to uncoupling rather than to down-regulation.

The increased experience of several investigators studying the LH, FSH or other G-protein coupled receptors in cell lines is now leading to a better appreciation of potentials. Future experiments of structure- function relationship with recombinant receptors should consider these aspects and, in particular, analyze whether and which cell system produces results really meaningful to clinical practice. Studies with additional receptor mutants and with cell lines that lack Gs will be research to further test this receptor functions and to better define the steps in recetor activation that are also involved in internalization and endocytosis and utilize to study the structrue function and signal transduction of the choriogonadotropins and glycoprotein hormones. Now, we are investigating to understand the functions of the activating mutant in the transmembrane Ⅵ region of the LHR.

Ⅴ. REFERENCES

1. Freedman, N., A. S. Ament, M. Oppermann, R. Stoffel, T. Exum and R. Leftkowitz. 1997. Phosphorylation and desensitization of human endothelin A and B receptors. evidence for G protein-coupled receptor kinase specificity. JBC. 272:17734-17743.
2. Hipkin, W., L. Xebo and M. Ascoli. 1995. Truncation of the C-terminal tail of the follitropin receptor does not impair the agonist-or phorbol ester-induced receptor phosphorylation and uncoupling. JBC. 270:26683-26689.
3. McFarland, K. C., R. Sprengel, H. Phillips, M. Kohler, N. Rosemblit, K. Nikolics, D. Segaloff and P. Seeburg. 1989. Lutropin-choriogonadotropin receptor: an unusual member of the G protein-coupled receptor family. Science 245:494-499.
4. Min, K. S., K. Shiota and T. Ogawa. 1996a. Molecular cloning of equine preprorelaxin cDNA. J. Reproduction and Development 42:171-178.
5. Min, K. S., M. Shinozaki, K. Miyazawa, R. Nishimura, N. Sasaki, K. Shiota and T. Ogawa. 1994. Nucleotide sequence of eCG α-subunit cDNA and its expression in the equine placenta. J. Reproduction and Development 40:301-305.
6. Min, K. S., N. Hattori, J-I. Aikawa, K. Shiota and T. Ogawa. 1996b. Site- directed mutagenesis of recombinant equine chorionic gonadotropin/luteinizing hormone: differential role of oligosaccharides in luteinizing

hormone- and follicle-stimulating hormone-like activities. Endocrine Journal 43:585-393.
7. Munson, P. J. and D. Rodbard. 1980. Ligand: a versatile computerized approach for characterization of ligand-binding systems. Anal. Biochem. 107:220-239.
8. Segaloff, D. and M. Ascoli. 1993. The lutropin/choriogonadotropin receptor. 4 years later. Endocrine Reviews 14:324-347.
9. Simoni, M., J. Gromoll and E. Nieschlag. 1997. The follicle-stimulating hormone receptor: biochemistry, molecular biology, physiology, and pathophysiology. Endocrine Reviews 18:739-773.
10. Sprengel, R., T. Braun, K. Nikolics, D. Segaloff and P. Seeburg. 1990. The testicular receptor for follicular-stimulating hormone: structure and functional expression of cloned cDNA. Mol Endocrinol 4:525-530.
11. Wang, Z., H. Wang and M. Ascoli. 1993. Mutation of a highly conserved acidic residue present in the second intracellular loop of G-protein-coupled receptors doses not impair hormone binding or signal transduction of the luteinizing hormone/chorionic gonadotropin receptor. Molecular Endocrinology 7:85-93.
12. Wang, Z., L. Xebo and M. Ascoli. 1997. Phosphorylation of the lutropin/choriogonadotropin receptor facilitates uncoupling of the receptor from adenylyl cyclase and endocytosis of the bound hormone. Molecular Endocrinology 11:183-192.

요 약

웅모성 성선자극 호르몬 및 난포 자극호르몬 수용체의 293세포에서 기능적으로 발현

민 관 식
축산기술연구소

1. 유전자 재 조합 호르몬(eCG/PMSG, hCG 및 hFSH)의 활성체크 및 세포내 signal transduction에 관한 기초연구를 위하여 rat의 웅모성 성선자극 호르몬 수용체 (LH/CGR) 및 난포 자극호르몬 수용체(FSHR)를 이미 보고되어진 염기배열에 의하여 PCR방법으로 크로닝 하여, human embryonic kidney 유래의 293 세포에 transfection하여 세포 표면에 LH/CGR와 FSHR를 발현하는 cell lines을 분리하였다.
2. hCG와 FSH의 signal을 전달하는 능력은 hCG와 FSH 또는 eCG의 농도증가에 따라 이들 수용체로 하여금 세포내의 cAMP 분비가 증가함을 알 수 있었다. 즉, transfection 되어진 이들 수용체를 발현하는 수용체의 대부분은 ligand binding 기능을 가지고, cAMP 반응에 의한 생리활성을 분석할 수 있으며, 또한 유전자 재조합당 단백질 호르몬(eCG, hCG 및 hFSH)의 signal transduction, 구조 및 기능연구에 활용할 수 있다.

(접수일자 : 1999. 10. 30./채택일자 : 1999. 11. 19.)