The Testicular Receptor for Follicle Stimulating Hormone: Structure and Functional Expression of Cloned cDNA

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Cloned cDNA encoding the rat Sertoli cell receptor for FSH was isolated from a cognate library and functionally expressed in cultured mammalian cells. The FSH receptor (FSH-R), as predicted from the cDNA, is a single 75K polypeptide with a 348 residue extracellular domain which contains three N-linked glycosylation sites. This domain is connected to a structure containing seven putative transmembrane segments which displays sequence similarity to G protein-coupled receptors. Thus, the FSH-R is identical in its structural design to the LH/CG receptor (LH/CG-R). Furthermore, both receptors display 50% sequence similarity in their large extracellular domains and 80% identity across the seven transmembrane segments. Expression of the cloned cDNA in mammalian cells conferred FSH-dependent cAMP accumulation. The selectivity for FSH is attested by the fact that the related human glycoprotein hormones human CG and human TSH do not stimulate adenylyl cyclase in FSH-R expressing cells even when these hormones are present at high concentrations. (Molecular Endocrinology 4: 525–530, 1990)

INTRODUCTION

A receptor specific for FSH and present on testicular Sertoli cells and ovarian granulosa cells (1, 2) mediates the folliculogenic and spermatogenic actions of this pituitary-produced gonadotropin. FSH shares sequence similarity with LH, CG, and TSH. These four hormones are 28–38K molecular weight heterodimeric glycoproteins composed of a common α-subunit non-covalently bound to distinct β-subunits that confer receptor binding specificity (3, 4). The similarity of the glycoprotein hormones and the observation that they act on their respective receptors to increase adenylyl cyclase activity (2, 5) suggest that the structural design of these receptors reflects a common mechanism of hormone-induced activation as well as the selective recognition of the individual members of this hormone family.

Several groups have reported on the biochemical characterization and purification of the FSH receptor (FSH-R) from ovarian and testicular sources (2, 6–10). However, these groups have differed on both the size and possible subunit structure of this receptor. Recently, we have reported the structure of the LH/CG receptor (LH/CG-R) as deduced by cDNA analysis (11). This receptor is a single glycoprotein containing an unusually large extracellular domain in excess of 300 residues composed of internal repeats which is joined to a structure characterized by seven putative transmembrane segments, TMI–TMVII. This latter structure displays sequence homology to G protein-coupled receptors. The unusual design of the LH/CG-R suggested a new mechanism for gonadotropin-evoked receptor activation (11). To elucidate further structural requirements of the molecular events decisive for reproductive competence we have isolated cloned cDNAs encoding the FSH-R. In this report we present the primary structure and functional expression of this receptor and discuss its mechanism of activation based on a detailed structural comparison of the two gonadotropin receptors.

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RESULTS

Molecular Cloning of the FSH-R

A cDNA library constructed from RNA isolated from rat testicular Sertoli cells was screened using molecular probes derived from selected coding regions of the LH/CG-R cDNA (11). While no LH/CG-R clone was found, several clones were isolated which encoded a sequence bearing similarity to the LH/CG-R. Upon functional expression this sequence was seen to encode the FSH-R (see below). The nucleotide and predicted amino acid sequences of this receptor are shown in Fig. 1.

The translation initiation codon at position 1 defines the start of a 2076 nucleotide open reading frame specifying an N-terminal 17 residue signal sequence followed by a largely hydrophilic domain of 348 residues of putatively extracellular location. This domain contains three N-linked glycosylation sites. It is followed by a structure of 264 residues which comprises seven transmembrane segments. These segments are the hallmark of G protein-coupled receptors. Similar to other such receptors, the 63 residue C-terminus of the FSH-R is proposed to be located intracellularly and contains several amino acids (Ser, Thr, Tyr) whose phosphorylation may regulate receptor activity (12, 13). However, these residues are not part of consensus phosphorylation sites as in other receptors. The mature FSH-R is predicted to comprise 675 amino acids (75K mol wt) and to constitute an integral membrane glycoprotein.

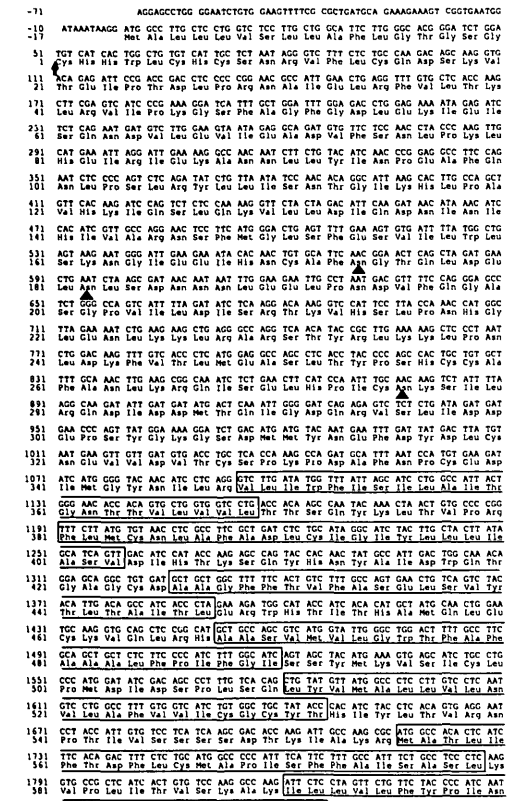

Fig. 1. The cDNA and Predicted Amino Acid Sequence of Rat Testicular FSH-R

Amino acid numbering begins at the N-terminal sequence for the predicted mature receptor protein, with negative numbers denoting the signal sequence. The predicted signal cleavage site (37) is indicated by an *arrow*. Consensus N-linked glycosylation sites are marked by *triangles* and the proposed seven transmembrane segments TMI-TMVII are *boxed*.

Comparison between FSH-R and LH/CG-R

It is illuminating regarding the proposed similarities in function to compare the two gonadotropin receptors (Fig. 2). Both molecules are of similar size and display the same structural design. On the level of primary structure, the extracellular domains share approximately 50% sequence similarity while the domains defined by the seven transmembrane segments display 80% sequence identity. The areas of highest sequence divergence comprise the N-terminus, a 40 residue region preceding the first transmembrane segment and the 30 residues encompassing the C-terminus.

As noted for the extracellular domain of the LH/CG-R, the homologous domain in the FSH-R can be viewed as being composed of 14 imperfectly replicated units of approximately 20 residues each (Fig. 3). The motif underlying this repeat is also found in other proteins and is known as leucine-rich repeat (14). A characteristic feature of members of the leucine-rich repeat family is a purported tendency to interact with both hydrophilic and hydrophobic protein surfaces (15). This property may be important regarding the function of the extracellular domain of gonadotropin receptors (see below).

The alignment of the extracellular domains of both receptors (Fig. 2) shows that repeats with the highest sequence divergence (repeats 12 and 13) are also the least conserved relative to the underlying motif. Notably, this region is of different length between the gonadotropin receptors and the recently characterized TSH receptor (TSH-R) (16–18) and is variable in sequence between glycoprotein hormone receptors from different species (17), indicating that it is unlikely to be involved in hormone recognition. The alignment further reveals eight conserved cysteine residues, two of which are in adjacent positions. Interestingly, several of these residues are found in well conserved regions comprising 13 and 15 consecutive amino acids. Since these cysteines are also conserved in the TSH-R (16–18), the formation of disulfide bonds seems to be crucial for the conformational integrity of the large extracellular domain of glycoprotein hormone receptors.

A differential pattern of sequence conservation is also observed for the seven transmembrane segments. While TMIII and TMVII are highly conserved, TMIV and TMV contain many substitutions. Although the overall sequence similarity to other G protein-coupled receptors is low (11), the aspartic acid residue within TMII and the asparagine within TMVII, two conserved resi-

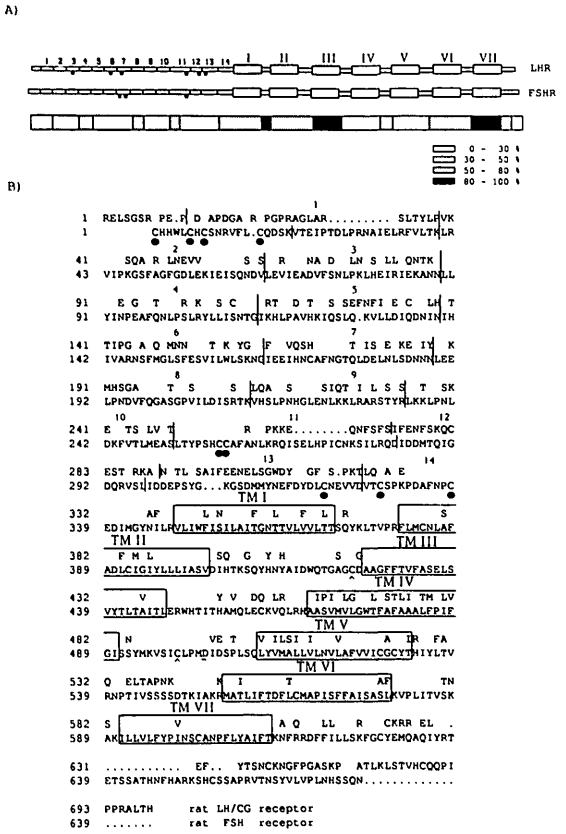

Fig. 2. Structural Comparison between the Gonadotropin Receptors

A, Sequence similarities of receptor domains. The N-terminal half representing the extracellular domain is subdivided into 14 imperfectly duplicated units of approximately 20 residues each and the C-terminal half shows the seven transmembrane segments. Potential glycosylation sites are indicated by *filled squares*. *Different shadings of gray* indicate the degrees of sequence conservation for different receptor areas. B, Sequence comparison of receptors in the one letter code. The FSH-R sequence is shown as the lower sequence and differences as well as substitutions in the LH/CG-R are presented above. *Dots* denote insertions introduced for optimal alignment. The extracellular repeats are numbered and demarked by *vertical lines*. Conserved cysteine residues in the extracellular domain are denoted by *filled ovals*. Transmembrane regions TMI-TMVII) are *boxed*. *Small arrows* indicate conserved cysteine residues in the second and third extracellular loops of the receptor.

dues in G protein-coupled receptors (11, 19), are also present in the two gonadotropin receptors. So are proline residues in TMIV, TMVI, and TMVII and the two cysteine residues that are thought to form a disulfide bridge between the second and third extracellular loops in many G protein-coupled receptors (20).

In the gonadotropin receptors, the third intracellular loop flanked by TMV and TMVI is short and quite divergent. The low degree of sequence similarity in this region is as seen for subtypes of other G protein-coupled receptors (21). In some of these receptors the region bordered by, and comprising part of, TMV and

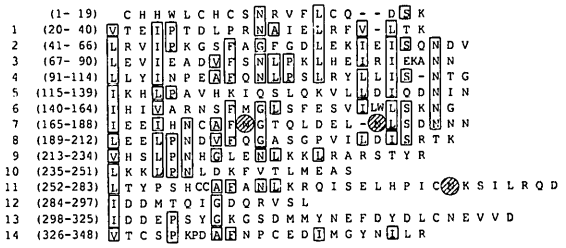

Fig. 3. Alignment of Repeated Motifs in the Extracellular Domain of FSH-R Illustrating the Differential Extent of Sequence Conservation between the Repeats

N-Linked glycosylation sites are indicated by *hatched circles*. The alignment and numbering is according to that of the LH/CG-R (11).

VI appears to be involved in the coupling to G protein (22, 23). A sequence of eight amino acids at the C-terminal end of this intracellular loop is well conserved between both gonadotropin receptors (21). A similar sequence implicated in coupling to G_s can be found in the β-adrenergic receptor (23). The interaction with the G protein may occur via an amphiphilic α-helical structure (24) formed by this peptide sequence. A helical wheel analysis performed on the conserved eight residue sequence in the FSH-R and LH/CG-R reveals that the charged side chains are located on one side and the hydrophobic ones on the opposite face of the helix, as proposed for the homologous region of α_2 and β_2 adrenergic receptors (24).

Functional Expression of the FSH-R

To test the hormone-mediated activation of the FSH-R we constructed the expression vector pCFSH-R in which the cloned cDNA containing the complete coding sequence is under the transcriptional control of the human cytomegalovirus promoter (25). Cultured human embryonic kidney 293 cells were transfected with pCFSH-R and tested for their ability to respond to ovine FSH with an increase in cAMP levels. As shown in Fig. 4 and in Table 1, cells expressing the cloned receptor displayed an FSH-dependent and saturable increase in intracellular cAMP. Untransfected and mock-transfected cells did not show this response.

The concentration of FSH required to elicit half-maximal stimulation of this response (2–3 ng/ml, 80 pM) is comparable to that seen for human CG (hCG) and its receptor (11) and is well within the range of values reported for the FSH-R (26). In contrast, hCG, even at concentrations up to 25 nM did not evoke a cAMP response in FSH-R expressing cells (Table 1). In the absence of data obtained with recombinantly produced FSH and LH, we conclude that the receptor recognition of different gonadotropins is selective.

DISCUSSION

The FSH-R displays structural similarities with the LH/CG-R, a finding we predicted based upon the known

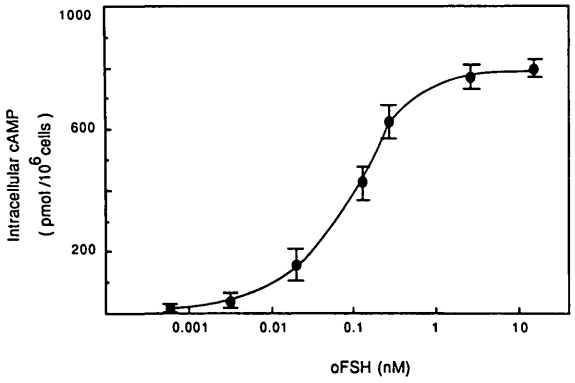

Fig. 4. Functional Expression of the FSH-R

FSH-Stimulated cAMP accumulation in 293 cells transiently transfected with expression plasmid pCFSH-R. Intracellular cAMP was measured as a function of hormone concentration. Each data *point* represents the mean + range of duplicate determinations.

Table 1. Adenylyl Cyclase Stimulation in FSH-R Expressing Cells

Hormone (25 nM)	cAMP (pmol/10^6 cells)
none	9.0
oFSH	700.0
hCG	7.6
hTSH	12.0

293 cells were transiently transfected using the pCFSH-R expression construct and incubated in the presence of 26 nM several glycoprotein hormones. The cAMP accumulated during 30 min of hormonal stimulation reflects the specificity of the FSH-R to its natural ligand.

homology of the gonadotropin hormones. Although some studies by others have suggested that the LH/CG-R and the FSH-R are composed of multiple subunits (2, 6–10, reviewed in Ref. 28), biochemical studies on the LH/CG-R have shown that it is composed of a single polypeptide with a mol wt of 92K when analyzed on sodium dodecyl sulfate gels in the presence or absence of disulfide reducing agents (27). As the LH/CG-R has been shown to be readily proteolyzed into smaller sized fragments (see Ref. 28 for review), it is reasonable to postulate that the FSH-R may be similarly susceptible to proteolysis, and that this may account for the discrepant reports on its structure. Clearly, the molecular cloning and functional expression of the cDNAs for the rat ovarian LH/CG-R (11) and now the rat testicular FSH-R demonstrate conclusively that the gonadotropin receptors are indeed single polypeptides. Furthermore, a comparison to our FSH-R sequence reveals that a recently described cloned human DNA fragment (16) generated by the polymerase chain reaction and related to the LH/CG-R and TSH-R coding sequences can now be defined to encode part of the human FSH-R.

Reflecting a unique mechanism of receptor activation, both the FSH-R and the LH/CG-R are characterized by the presence of a large, glycosylated domain of putative extracellular location which is grafted onto a structure containing seven transmembrane segments and displaying homology to G protein-coupled receptors. The same structural design also characterizes the TSH-R (16–18), another member of the glycoprotein hormone family. In comparison to other G protein-coupled receptors, this unique design suggests that the extracellular domain is responsible for the recognition and binding of the dimeric hormones. In support of this notion, the extracellular domain of the LH/CG-R binds hCG with high affinity when singly expressed in cultured cells (Braun, T. B., and Sprengel, R., in preparation).

The three-dimensional structure of this extracellular receptor domain is likely to be stabilized by disulfide bridges involving cysteine residues conserved in the glycoprotein hormone receptors. Interestingly, the positions of these conserved cysteines do not correlate with the internal repeat structure of the extracellular domain. Some of these cysteines could be the target of dithiol-disulfide interchanges and oxidation-reduction reactions catalyzed by gonadotropins. Such reactions have been envisioned to contribute to receptor hormone interaction and, possibly, receptor activation (29, 30).

The functional significance of the internal repeat structure of the extracellular domain of glycoprotein hormone receptors can only be the subject of conjecture. It is likely that the amphiphilic nature of the repeats confers the dual property of interacting with hormone and transmembrane domains. Such an interaction seems crucial for receptor activation which, for most other G protein-coupled receptors, is effected by the binding of a small ligand to a spatially defined site within the seven transmembrane segments. Considering the evolutionarily conserved basic mechanism of receptor activation, it is entirely possible that selected amino acid residue side chains of the gonadotropins substitute for the customary small ligands. In this model, the activating residues are correctly positioned by the binding of the hormone to the extracellular domain. In a variation of this model, residues of the extracellular domain itself, upon binding hormone, may contact essential sites in the transmembrane segments.

One important factor to bear in mind when considering the possible mechanisms by which the binding of glycoprotein hormones to their respective receptors causes the activation of the G_s protein is the role of the hormone carbohydrate moieties in this activation process (31, 32). Thus, although deglycosylated glycoprotein hormones bind with high affinity to their receptors, they elicit little or no activation of cAMP production (32). In fact, the concept of antihormones has been proposed to describe the FSH antagonistic effects of naturally occurring glycosylation variants of FSH (33). It is intriguing to question whether the hormone-attached carbohydrate moieties themselves interact with the transmembrane helices of the receptor or whether they position selected amino acid side chains in a conformation that permits receptor activation.

Further elucidation of the molecular events governing glycoprotein hormone-evoked receptor activation will necessitate the construction and analyses of selected receptor mutants and chimaeras.

MATERIALS AND METHODS

Hormones

Highly purified ovine FSH (NIDDK-oFSH-17; 20 U/mg), hCG (CR-123; 12,780 IU/mg), and human TSH (NIDDK-hTSH-I-6; 17 IU/mg) were generous gifts from the National Hormone and Pituitary Program of the NIDDK (NIH).

Isolation of FSH-R cDNA

Polyadenylated RNA isolated from rat testicular Sertoli cells was used as a template for reverse transcriptase. The resulting cDNA served for the construction of a library in λgt10. An aliquot (1×10^6 clones) was screened for clones with sequence similarity to two probes derived from the LH/CG-R cDNA (nucleotides 1–483 and 1499–2604). Several positive clones were isolated and cloned cDNAs sequenced (34) after subcloning into M13 vectors (35).

Functional Expression of FSH-R cDNA

The expression vector pCFSH-R was constructed by introducing the entire coding region of the cloned cDNA contained on an *Eco*RI-*Bam*HI fragment (nucleotides -77 to 2095) into the pCIS vector (25).

Exponentially growing human embryonic kidney cells 293 (ATCC CRL 1573) in 34-mm dishes were transfected with this expression vector (36). After 42 h intact cells were assayed for hormone-stimulated cAMP production. Each dish was washed once with 3 ml warm Dulbecco's modified Eagle's medium containing 10% fetal calf serum and placed in 1 ml serum-free Dulbecco's modified Eagle's medium buffered with 25 mM HEPES, pH 7.4, containing 0.1 mM 3-isobutyl-1-methylxanthine. After a 15-min incubation period at 37 C, highly purified glycoprotein hormone (oFSH, hTSH, or hCG) was added and the incubation was continued for 30 min at 37 C. The assay was stopped by rapid freezing and thawing of the cells in liquid nitrogen and then 1.2 ml cold ethanol was added to each dish. The cell debris and precipitated protein were removed by centrifugation (10 min; 13,000 × *g*) and 5 μl or 50 μl supernatant wereassayed for cAMP using an Amersham kit.

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