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The Lutropin/Choriogonadotropin Receptor, A 2002 Perspective

MARIO ASCOLI, FRANCESCA FANELLI, AND DEBORAH L. SEGALOFF

Departments of Pharmacology (M.A.) and Physiology and Biophysics (D.L.S.), The University of Iowa, Iowa City, Iowa 52242-1109; and Dipartimento di Chimica (F.F.), Universitá di Modena e Reggio Emilia, Via Campi 183 41100 Modena, Italy

Reproduction cannot take place without the proper functioning of the lutropin/choriogonadotropin receptor (LHR). When the LHR does not work properly, ovulation does not occur in females and Leydig cells do not develop normally in the male. Also, because the LHR is essential for sustaining the elevated levels of progesterone needed to maintain pregnancy during the first trimester, disruptions in the functions of the LHR during pregnancy have catastrophic consequences. As such, a full understanding of the biology of the LHR is essential to the survival of our species. In this review we summarize our current knowledge of the structure, functions, and regulation of this important receptor. (*Endocrine Reviews* 23: 141–174, 2002)

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I. Introduction

THIS IS THE third time that we have written a review on the lutropin/choriogonadotropin receptor (LHR) in this journal. In the first review (1), which was published in February of 1989, we contrasted and compared the conflicting data that existed on the structure of the mammalian LH/CG receptor (LHR). The controversy on the structure of the LHR that was obvious in that review was laid to rest later in 1989 with the simultaneous publication of two papers reporting the cloning, sequencing, and expression of cDNAs for the rat [r (2)] and porcine [p (3)] LHR. These two papers conclusively established that the LHR is a single polypeptide chain with an overall structure and topology that made it a member of the rhodopsin/β_2-adrenergic receptor subfamily (4, 5) of G protein-coupled receptors (GPCRs). The cloning of the rLHR and pLHR cDNAs was quickly followed by the cloning of cDNAs for the human (h) LHR (6, 7). The novel experimental tools so generated were also rapidly used to try to understand the molecular basis of the functions and regulation of the LHR, and our second review on this subject (8), which was published in June of 1993, summarized the rather large amount of data that was generated shortly after the cloning of the first two cDNAs for the LHR.

Research on the LHR has proceeded at a fast pace since our last review was published, and a few additional reviews on different aspects of the biology of the LHR have been published by other investigators (9–12). In this review we summarize the large body of literature now available on the LHR and we use these data to generate models for the structure, functions, and regulation of this important receptor.

II. Structure and Biogenesis of the LHR

A. The LHR protein

A search of the National Center for Biotechnology Information nucleotide database revealed 63 entries for "LH receptor." Most of these represent partial or complete sequences for LHR cDNAs for a variety of domestic animals (*i.e.*, cow, pig, sheep, chicken, and turkey), animals that are widely used as experimental models in modern biological research (*i.e.*, rat, mouse, and *Xenopus*), and other interesting animals such as the catfish, mink, marmoset, and black bear. Because most of the experiments to date have been performed with the rLHR and hLHR, an amino acid sequence alignment of these two receptors is presented in Fig. 1.

In agreement with the orientation and overall topology of other GPCRs (reviewed in Refs. 4, 5, and 12), one can recognize three distinct domains in the LHR, a large N-terminal domain that contains about 340 residues and is predicted to be extracellular, a serpentine region containing seven transmembrane (TM) segments connected by three extracellular loops (ELs) and three intracellular loops (ILs) and a C-terminal tail that is predicted to be located intracellularly.

Abbreviations: Arf6, ADP ribosylation factor 6; ARNO, Arf-nucleotide binding site opener; CHO, Chinese hamster ovary; EL, extracellular loop; EndoH, endoglycosidase H; EREhs, estrogen receptor response element half-site; FSHR, FSH receptor; GPCR, G protein-coupled receptor; GRK, GPCR kinase; h, human; IL, intracellular loop; LHR, lutropin/choriogonadotropin receptor; LGR, LRR-containing GPCR; LRR, leucine-rich repeat; nt, nucleotide; o, ovine; p, porcine; r, rat; SAS, Sp1c adjacent site; SNP, single nucleotide polymorphism; TM, transmembrane; TSHR, TSH receptor; wt, wild type.

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```text
Signal Peptide                                            Ascoli et.al., Figure 1

rLHR -25 MGRRVALRQLLVLAVLLLKPSQLQS -1
hLHR 1   -KQ-FSAL---K-LL--QP-LPRA 24


Mature Protein

<- N-terminal cysteine-rich region ->  <- (red) ...

                                          1|2                      2|3
rLHR 1   RELSGSRCPEPCDCAPDGALRCPGPRAGLARLS|LTYLPVKVIPSQAFRGLNEVVKI|EISQSDSLERIEANAFDNLLNLSE80
hLHR 25    -REAL-----N-V----------T---T---|-A------------------I--|----I-------------------102

           3|4                       4|5                        5|6                         6|7
rLHR 81  LL|IQNTKNLLYIEPGAFTNLPRLKYL|SICNTGIRTLPDVTKISSSEFNFIL|EICDNLHITTIPGNAFQGMNNESVTL|KLY160
hLHR 103 I-|-------R-------I--------|--------KF-----VF---S----|--------------------------|---182

                                 Leucine-rich motif region
                           7|                         8|9
rLHR 161 GNGFEEVQSHAFNGTTLISL|ELKENIYLEKMHSGAFQGATGPSIL|DISSTKLQALPSHGLESIQTLIALSSYSLKTLPSK240
hLHR 183 -----------------T--|-----VH-----N---R-----KT-|------------Y------R---T------K---R262

(red) ->  <- Hinge region
                                    9|10                          10|11
rLHR 241 EKFTSLLVATLTYPSHCCAFRNLPKKE|QNFSFSIFENFSKQCESTVRKADNETL|YSAIFEENELSGWDYDYGFCSPKTLQ320
hLHR 263 -T-VN--E----------------T--|----H--S-------------VN-K--|--SMLA-S-------E----L---PR342

(green) ->      TM-1                          IL-1             TM-2
rLHR 321 CAPEPDAFNPCEDIMGYAFLRVLIWLINILAIFGNLTVLFVLLTSRYKLTVPRFLMCNLSFADFCMGLYLLLIASVDSQT400
hLHR 343 -----------------D--------------M--M--------------------------------------------422

         EL-1             TM-3                     IL-2             TM-4
rLHR 401 KGQYYNHAIDWQTGSGCGAAGFFTVFASELSVYTLTVITLERWHTITYAVQLDQKLRLRHAIPIMLGGWLFSTLIATMPL480
hLHR 423 ------------------ST------------------------------IH------------L-----------S---ML--502

                     EL-2                TM-5                   IL-3           TM-6
rLHR 481 VGISNYMKVSICLPMDVESTLSQVYILSILILNVVAFVVICACYIRIYFAVQNPELTAPNKDTKIAKKMAILIFTDFTCM560
hLHR 503 --V--------F-----T--------T----------FI-----K-----R----M-T---------------------582

                        EL-3            TM-7
rLHR 561 APISFFAISAAFKVPLITVTNSKILLVLFYPVNSCANPFLYAIFTKAFQRDFLLLLSRFGCCKRRAELYRRKEFSAYTSN640
hLHR 583 -----------------------V--------I-------------T-----F----K--------------D--------662

rLHR 641 CKNGFPGASKPSQATLKLSTVHCQQPIPPRALTH
hLHR 663 -----T-SN----S-------L---GTALLDKTRYTEC
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Fig. 1. Amino acid sequence alignment of the rLHR and hLHR. Sequences for the rLHR (accession no. P16235) and the hLHR (accession no. P22888) were obtained from the SWISS-PROT databank. The *dashes* indicate identical residues. The boundaries of the three distinct regions of the extracellular domain discussed in the text (the N-terminal cysteine-rich region, leucine-rich motif region, and hinge region) are marked by the *green*, *red*, and *green arrows*, respectively, and the cysteine residues present in the N-terminal cysteine rich and hinge regions are *highlighted in green*. The sequences coded for by the different exons are delineated by the vertical bars and are labeled 1–11. The seven TM regions are delineated by *black boxes* and labeled TM-1–TM-7. The three ELs and four ILs that connect the TM regions are labeled as EL-1–EL-3 and IL-1–IL-4, respectively. The *blue boxes* delineate predicted helical segments based on the rhodopsin homology model discussed in *Section VI*. The consensus sequences for N-linked glycosylation are highlighted by the *gray boxes*. Polar residues that are highly conserved

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The predicted extracellular location of the N-terminal region of the rLHR and the intracellular location of the C-terminal region have indeed been confirmed in rat luteal cells using site-specific antibodies to synthetic peptides derived from the N- or C-terminal regions of the rLHR (13) and in human kidney 293 cells expressing the recombinant rLHR using antibodies directed against the FLAG epitope added to the N terminus of the rLHR (14). The amino acid sequence identity between the hLHR and the rLHR is approximately 88% in the extracellular domain, approximately 92% in the serpentine region, and approximately 69% in the C-terminal cytoplasmic tail.

By convention, residue 1 of the rLHR is taken as the N-terminal residue (R^1) of the mature rLHR. The identity of this residue was determined by sequencing the receptor protein purified from the rat ovary (2, 15). The cloning of the cDNA for the rLHR (2) revealed that the precursor form of the rLHR contains a 26-residue signal peptide, and computer algorithms that predict the most likely site of cleavage of signal peptides (http://www.cbs.dtu.dk/services/SignalP/) correctly predict the above-mentioned arginine residue to be the N terminus of the mature rLHR. In contrast, the identity of the N terminus of the mature hLHR is not known with certainty because the hLHR has never been purified and subjected to protein sequencing. Thus, by convention, the amino acid residues of the hLHR have been numbered from the initiator methionine of the hLHR precursor sequence obtained by virtual translation of the open reading frame of the cognate cDNA (6, 7). Algorithms that predict the most likely site of cleavage of signal peptides predict the N terminus of the mature hLHR to be L^{25}. For optimal alignment, L^{25} of the hLHR corresponds to L^3 of the rLHR (Fig. 1). Because of these shifts, one must move the numbering of amino acid residues up or down by 22 positions to find equivalent hLHR and rLHR residues (for example, L^{457} in the hLHR is equivalent to L^{435} in the rLHR).

The polypeptide chains of the mature rLHR and hLHR are predicted to be composed of 674 and 675 residues, respectively. Although the predicted molecular mass of these polypeptide chains is approximately 75 kDa, the molecular mass of the mature hLHR and rLHR is expected to be higher because the LHR is a glycoprotein. Mammalian cells transfected with the cDNAs for the rLHR or the hLHR display three distinct LHR species with molecular masses (estimated from SDS gels) of 65–75 kDa, 85–95 kDa, and 165–200 kDa (Fig. 2 and Refs. 16 and 17–25). The 85- to 95-kDa band is the mature LHR present at the cell surface. This form of the LHR can be readily labeled by surface biotinylation of intact cells (19) and it can be degraded by proteases under conditions that proteolyze only cell-surface proteins (16, 21). The 85- to 95-kDa band of the LHR is also susceptible to digestion with neuraminidase and peptide-N-glycosidase-F, two glycosidases that are known to act on the mature-type of carbohydrate side chains generally associated with cell-surface glycoproteins. In contrast, the 85- to 95-kDa band is not susceptible to endoglycosidase-H (EndoH), a glycosidase that removes the high-mannose type of carbohydrate side chains associated with immature glycoproteins (16, 17, 24, 25). Biosynthetic labeling of transfected cells with radioactive amino acids revealed that the 65- to 75-kDa form of the LHR is a precursor of the cell-surface receptor (16, 25). This form of the LHR is located intracellularly because it is insensitive to surface proteolysis, it cannot be detected by surface biotinylation of intact cells and is insensitive to neuraminidase digestion (16, 19, 21, 24, 25). The finding that the 65- to 75-kDa band is readily susceptible to EndoH digestion (16, 17, 24) suggests that this is an immature glycoprotein localized in the endoplasmic reticulum. The 165- to 200-kDa LHR species has been identified as an oligomer/aggregate of the 65- to 75-kDa LHR. This identification is based on three findings. First, biosynthetic labeling experiments reveal that the time course of labeling of the 65- to 75-kDa and 165- to 200-kDa forms of the rLHR are basically identical (16, 26). Second, like the 65- to 75-kDa LHR, the 165- to 200-kDa LHR is susceptible to degradation by EndoH, is resistant to degradation by proteolysis of intact cells, and is undetectable by biotinylation of intact cells (16, 19, 21, 26). Third, the 165- to 200-kDa LHR is still detectable in cells transfected with mutants of the LHR that prevent the maturation of the 65- to 75-kDa precursor into the 85- to 95-kDa cell-surface receptor (26). It should be stressed, however, that whereas studies on the properties of the 65- to 75-kDa and 85- to 95-kDa bands of the

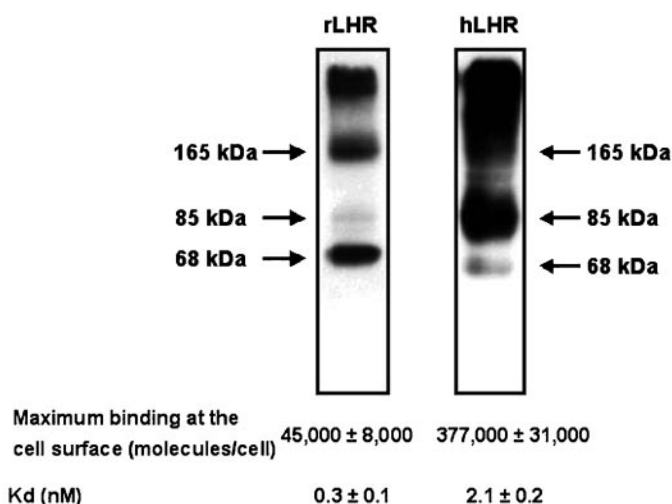

FIG. 2. Expression of the rLHR and hLHR in transfected 293 cells. The 293 cells were transiently transfected with epitope(*myc*)-tagged forms of the rLHR (21) or hLHR (19). The expression of the receptors was documented using Western blots of whole-cell lysates developed with a monoclonal antibody (9E10) to the *myc* epitope or by saturation binding analysis of ^{125}I-hCG to the intact, transfected cells.

among the rhodopsin/β_2-adrenergic receptor subfamily of GPCRs and may be involved in receptor activation are *highlighted in pink* and correspond to the *pink residues* shown in Fig. 7. Other residues that are highly conserved among the rhodopsin/β_2-adrenergic receptor subfamily are shown in *purple*. The serine residues that are phosphorylated in response to agonist stimulation are *highlighted in blue*. The residues *highlighted in yellow* are those that determine the slow rate of internalization of the rLHR and the fast rate of internalization of the hLHR. The *underlined* (GTALL) sequence present in the C-terminal tail of the hLHR highlights a short linear sequence that, when grafted into the rLHR, reroutes the internalized hCG-rLHR complex from a lysosomal degradation pathway to a recycling pathway.

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LHR have been accomplished using cells that are transiently or stably transfected with the rLHR or hLHR, the identification of the 165- to 200-kDa LHR as an oligomer/aggregate of the 65- to 75-kDa immature LHR has been accomplished only using cells expressing the recombinant rLHR. Thus, until the 165- to 200-kDa form is more fully characterized in cells expressing the hLHR, one must consider the possibility that this form of the hLHR is not necessarily an oligomer/aggregate of the 65- to 75-kDa immature receptor. This possibility is in fact likely, given the finding that the 65- to 75-kDa immature receptor is less prevalent in cells transfected with the hLHR than in cells transfected with the rLHR (Fig. 2).

It is interesting to note that the smaller species of LHR detected in cells expressing the rLHR (65–75 kDa) is clearly a glycoprotein (16, 17, 24, 25), yet its apparent size is similar to the mass of the polypeptide chain of the LHR. Moreover, cells transfected with the rLHR-wild type (wt) and treated with tunicamycin, or cells transfected with a form of the rLHR in which all glycosylation sites were mutated, yield a single receptor band with an apparent mass of approximately 51 kDa (24), a size that is clearly smaller than that expected for the polypeptide chain only. This apparent discrepancy is likely to be caused by the methods (*i.e.*, SDS gels) used to estimate the molecular weights of the receptors expressed in the transfected cells rather than by proteolysis of the polypeptide chain of the translated receptor. This statement is supported by the finding that the smallest species of rLHR detected in transfected cells (*i.e.*, the 65- to 75-kDa precursor) can be readily visualized with antibodies directed against synthetic peptides derived from the N- or C-terminal ends of the rLHR (16).

In summary then, the mature form of the LHR present at the cell surface is a glycoprotein with an apparent molecular mass of 85–95 kDa that arises from the maturation and transport of a 68- to 75-kDa precursor glycoprotein that is localized in the endoplasmic reticulum. At steady state, the relative abundance of the mature 85- to 95-kDa LHR is much lower than that of the two other forms (*i.e.*, 65–75 kDa and 165–200 kDa) in cells transfected with the rLHR, but the mature form of the LHR is more abundant than the two other forms in cells transfected with the hLHR (Fig. 2). This observation is consistent with the results of ^{125}I-hCG binding experiments performed in intact 293 cells transfected with identical amounts of the same expression vector encoding for the rLHR or hLHR (Fig. 2). It is important to note that the 68- to 75-kDa precursor of the rLHR can bind hCG with the same affinity as the mature 85- to 95-kDa rLHR (21), but the binding affinity of ovine (o) LH for the mature rLHR is higher than its binding affinity for the rLHR precursor (23). Clearly then, although the precursor of the rLHR has attained a conformation that permits hormone binding, this conformation is not the same as that of the mature form of the rLHR.

The rate of maturation of the rLHR and the turnover of the mature and immature forms of the rLHR have been studied in some detail in transfected 293 cells (16, 26–28). These studies have revealed that the conversion of the immature to the mature form of the rLHR is a slow and inefficient process (half-time of ~120 min) that seems to require the association of the immature receptor with at least one chaperone protein (calnexin, see Ref. 29). The immature rLHR is degraded with a half-life of approximately 60 min, and a large proportion of it is never converted to the mature receptor. The 85- to 95-kDa cell-surface rLHR is a fairly stable molecule that is degraded with a half-life of approximately 400 min. The degradation of the immature rLHR (but not that of the mature rLHR) was recently shown to be enhanced by a protein called p38^{JAB1} (28). The maturation and turnover of the hLHR have not been studied in detail, but the half-life of degradation of the cell-surface hLHR appears to be much slower than that of the rLHR (19). Lastly, it is important to note that the 68- to 75-kDa LHR precursor is not an artifact of overexpression in transfected cells because this form of the LHR has also been detected in Western blots of porcine testes (17, 30) and rat ovaries (16).

The extracellular domains of the rLHR and hLHR each have six consensus sites for N-linked glycosylation. These are fully conserved between the two receptors (as shown by the *shaded regions* in Fig. 1). Although it is known that the hLHR contains N-linked carbohydrates, studies determining whether all six potential sites for carbohydrate attachment are used have not been performed on this receptor species. In contrast, this question has been addressed for both the rLHR and pLHR. Using the pLHR (which also contains the same six conserved consensus sequences for N-linked carbohydrates shown in Fig. 1) purified from stably transfected L cells, Milgrom and colleagues (31) showed, by mass spectrometric analyses, that five of the six sites on the mature pLHR contain carbohydrate. Of these, three are monoantennary and three are multiantennary (31). The pLHR site lacking carbohydrate was found to be N^{299} (which corresponds to N^{277} and N^{299} in the rLHR and hLHR, respectively). Studies by one of our groups (24) using site-directed mutagenesis and endoglycosidase digestion of the rLHR expressed in 293 cells showed that all six sites of the rLHR are glycosylated. However, a study by another group (32) examining the recombinant rLHR expressed in insect cells reported that one of the six sites (corresponding to N^{77}) was not glycosylated. Although it is possible that insect and mammalian cells do not utilize the same sites for N-linked glycosylation, it is also possible that the different results obtained arise from differences in the methodology used to detect glycosylation. In the study of Zhang *et al.* (32), the presence/absence of carbohydrate on a given site was ascertained by differences in the overall molecular weight of the wt-rLHR and mutants of the rLHR in which the putative glycosylation sites were individually mutated. Because the contribution of a single oligosaccharide chain to the overall molecular weight of a large glycoprotein such as the rLHR is rather small (~2 kb), and insect cells do not glycosylate the rLHR to the same extent as mammalian cells (32), it would be difficult to detect the molecular weight contribution of a given oligosaccharide chain by the methods used. Indeed, the detection of glycosylation at each of the rLHR sites in mammalian cells was only readily detected after treatment of the cells with N-chlorosuccinimide to release a fragment of the extracellular domain containing the glycosylated sites (24). This increased the ratio of carbohydrate to protein and made small changes in molecular weight due to carbohydrate content more readily discernable. The presence of carbohydrate at N^{77} of the rLHR expressed in 293 cells could also be detected by

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comparing the molecular weight of a mutant form of the rLHR in which all potential sites for glycosylation other than N^{77} were mutated with that of a mutant in which all potential glycosylation sites were mutated (24). Moreover, the molecular weight of a mutant of the rLHR in which all potential sites for glycosylation other than N^{77} were mutated was still sensitive to endoglycosidase treatment (24).

With regards to the potential functional role of LHR glycosylation, studies with the hLHR have shown that the individual mutation of any one of the six predicted sites of N-linked carbohydrate attachment have no effect on hCG binding affinity or hCG-stimulated cAMP production in 293 cells expressing the mutants (33). Similar results were observed with the rLHR (24). Treatment of membranes prepared from human corpus luteum with peptide-N-glycosidase-F (an enzyme that removes all forms of N-linked carbohydrates) does not alter the binding affinity, specificity, or signal transduction properties of the membranes (33), suggesting that, once the hLHR has folded correctly and transported to the plasma membrane, the carbohydrates are dispensable for receptor function. Using the rLHR expressed in mammalian 293 cells, Davis *et al.* (24) examined the potential contributions of glycosylation on receptor folding and expression, in addition to hormone binding and signal transduction. It was observed that detergent solubilized extracts of cells expressing mutants of the rLHR in which all six glycosylation sites were simultaneously mutated were devoid of any hCG binding activity. However, the lack of binding activity was most likely due to the cumulative deleterious effects of the multiple mutations on the structure of the polypeptide backbone of the receptor rather than to the lack of carbohydrates. Indeed, cells treated with tunicamycin under conditions where only nonglycosylated rLHR is synthesized were found to bind hCG with high affinity (24). Notably, the tunicamycin-treated cells also responded to hCG with a stimulation of cAMP comparable to that of cells expressing a similar density of cell-surface wt rLHR. Therefore, the N-linked carbohydrates of the rLHR do not appear to be essential for the folding of the receptor or for its transport to the plasma. These results further suggest that, similar to the hLHR, once at the plasma membrane, the rLHR does not require N-linked carbohydrates for hormone binding or for hormone-stimulated second messenger production. In contrast to these results, Ji *et al.* (34) reported that tunicamycin treatment of mouse Leydig tumor cells resulted in a loss of cell-surface receptors. The cells used in the studies by this group of investigators (34) express much lower levels of LHR than those used by Davis *et al.* (24). Because culturing cells with tunicamycin not only prevents glycosylation but also results in a reduction in receptor expression (because of a general inhibition of protein synthesis), it is possible that the tunicamycin treatment of the cells in the study of Ji *et al.* decreased cell-surface expression of the rLHR to levels below detection. It should also be noted that Zhang *et al.* (32) reported that the extracellular domain of the rLHR expressed in insect cells treated with tunicamycin could not bind hCG. These observations cannot be attributed to the use of the extracellular domain (as opposed to the full-length rLHR) because Davis *et al.* (24) showed that, like the full-length rLHR, detergent extracts of 293 cells expressing the extracellular domain of the rLHR and treated with tunicamycin bound hCG with high affinity. Rather, the apparent discrepancies between the two reports can most likely be attributed to the different assays used to determine hCG binding activity of the receptor. Zhang *et al.* (32) examined hormone binding by ligand blots, which required the receptor to be refolded after electrophoresis was done under denaturing conditions. This renaturation step may require the presence of carbohydrates. When considered together, these results indicate that the LHR does not have a single carbohydrate chain that is essential for binding hormone or for signal transduction. These results stand in contrast to those obtained with hCG, where a single N-linked carbohydrate on the α-subunit is essential for the ability of hCG to stimulate Gs coupling by the LHR (35, 36). Taken altogether, therefore, it is reasonable to conclude that the nonglycosylated rLHR can be properly folded and expressed at the cell surface (albeit at reduced levels) and that the cell-surface nonglycosylated receptor can bind hormone and transduce signals. It should be noted that whereas these observations rule out an essential role for N-linked carbohydrates for the folding and trafficking of the rLHR, they do not rule out the possibility that the carbohydrates facilitate these processes. Indeed, the reduced expression of the rLHR at the cell surface observed in tunicamycin-treated cells could not only be due to the inhibition of protein synthesis but may also be due to less efficient folding and/or trafficking of the nonglycosylated rLHR. Other studies have since shown that calnexin, a chaperone protein that resides in the endoplasmic reticulum, associates with immature forms of the rLHR (29) and hLHR (D. L. Segaloff, unpublished observations). Because the effects of calnexin on the folding and trafficking of glycoproteins is thought to be mediated (at least in part) by the interaction of calnexin with glucose residues of N-linked carbohydrates (37), it is reasonable to speculate that the N-linked carbohydrates of the rLHR may play a facilitative role in these processes.

Perhaps the most salient feature of the extracellular domain of the LHR is the presence of several repeats of a structural motif of about 25 residues in length that is rich in hydrophobic amino acids and is called the leucine-rich repeat (LRR). Although it was initially thought that the extracellular domain of the rLHR had 14 or 15 of these repeats (2, 38), more recent knowledge suggests the presence of only 8 or 9 repeats (39–41). It is now generally agreed that the extracellular domain of the mature LHR can be divided into three regions: an N-terminal cysteine-rich region (labeled as such in Fig. 1), followed by 8 or 9 LRRs and a C-terminal cysteine-rich region (labeled as such in Fig. 1) that is also known as the hinge region. The most commonly accepted alignment for the LRRs of the extracellular domain of the rLHR and hLHR is shown in Fig. 3.

Tandem arrays of LRRs have been found in many proteins including hormone receptors and other hormone binding proteins, cell adhesion and extracellular matrix proteins, enzymes, and enzyme inhibitors (42, 43). The notion that LRRs are involved in protein-protein interactions is consistent with the finding that the extracellular domain of the LHR is mostly (if not entirely) responsible for the recognition and high affinity binding of its ligands (see *Section V*). The crystal

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                          β-sheet              turn                  α-helix
                    2        5     7        10       13       16        20 21      24

rLHR  LRR-1   29    L A R L S L T Y L P . V K V I P S Q A F R G L N E        52
hLHR          51    - T - - - - A - - - . - - - - - - - - - - - - - -        74

rLHR  LRR-2   53    V V K I E I S Q S D S L E R I E A N A F D N L L N        77
hLHR          75    - I - - - - - - I - - - - - - - - - - - - - - - -        99

rLHR  LRR-3   78    L S E L L I Q N T K N L L Y I E P G A F T N L P R       102
hLHR         100    - - - I - - - - - - - - R - - - - - - - I - - - G       124

                                *
rLHR  LRR-4  103    L K Y L S I C N T G . I R T L P D V T K I S S S E F     127
hLHR         125    - - - - - - - - - - . - - K F - - - - - V F - - - S     149

                                *
rLHR  LRR-5  128    N F I L E I C D N L H I T T I P G N A F Q G M N N E     153
hLHR         150    - - - - - - - - - - - - - - - - - - - - - - - - - -     175

rLHR  LRR-6  154    S V T L K L Y G N G . F E E V Q S H A F N G T T         176
hLHR         176    - - - - - - - - - - . - - - - - - - - - - - - -         198

rLHR  LRR-7  177    L I S L E L K E N I Y L E K M H S G A F Q G A T G       201
hLHR         199    - T - - - - - - - V H - - - - - N - - - R - - - -       223

rLHR  LRR-8  202    P S I L D I S S T K . L Q A L P S H G L E S             222
hLHR         224    - K T - - - - - - - . - - - - - - Y - - - -             244

rLHR  LRR-9  223    I Q T L I A L S S Y S L K T L P S K E K F T S           245
hLHR         245    - - R - - - T - - - - - - K - - - R - T - V N           267
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FIG. 3. Alignment of the LRRs in the extracellular domain of the rLHR and hLHR. Amino acid sequence alignment of the LRRs of the extracellular domains of the rLHR and hLHR. *Dashes* indicate identical residues and *dots* indicate gaps introduced for optimal alignment. The *gray boxes* highlight the location of consensus sequences for N-linked glycosylation. The model depicting nine LRRs is presented (40, 41, 53). The alignment for the eight-LRR model is basically the same as that shown here, except for the absence of LRR-9 (39). The *boxes at the top* labeled β-sheet, turn, and α-helix correspond to the proposed three-dimensional structure of the individual LRRs and extracellular domain shown in Fig. 4. The *residues enclosed in boxes* represent the conserved hydrophobic residues present in LRRs, and the *numbers at the top* correspond to the numbers associated with these residues in the structure shown on *panel b* of Fig. 4A. Also note that there are two cysteine residues present in this region (marked with an *asterisk* in LRR-4 and LRR-5). Residues in *green* have no effect on binding when mutated. Residues in *pink* abolish or decrease binding without affecting expression. Residues in *orange* abolish binding, but their effect on receptor expression was not tested (39, 40, 186, 187, 290).

structure of the porcine ribonuclease inhibitor (44), a LRR-containing protein, shows that these LRRs form repetitive, hairpin-like units comprised of a β-strand and an α-helical segment. These units in turn form a superhelix where all the β-strands and α-helical segments are oriented in a parallel fashion leading to the formation of a nonglobular, horseshoe-shaped molecule. In this molecule, all the β-strands line the inside or convex surface, whereas all the α-helical segments line the outside or concave surface (44). Although the LRRs of the extracellular domain of the LHR are somewhat shorter than those found in the porcine ribonuclease inhibitor, the crystal structure of this protein has provided the basis for several attempts to model the individual LRRs of the LHR and the overall three-dimensional structure of its extracellular domain (38, 40, 45, 46). Commonly accepted models for these two structures are shown in Fig. 4. As discussed below, these models, together with the known three-dimensional structure of hCG (47), provide a useful start point for theoretical and experimental approaches designed to understand how LH and CG interact with the LHR. Recent advances in the expression of large quantities of soluble forms of the extracellular domain of the pLHR (48) and rLHR (49) suggest that this region of the LHR will be crystallized soon, and current models are likely to be replaced by the real three-dimensional structure of the extracellular domain of the LHR in the not-too-distant future.

The homologous nature of the four glycoprotein hormones (LH, CG, FSH, and TSH) forecasted the homology of their receptors, and the cloning of the cDNAs for the two gonadotropin receptors and the TSH receptors clearly fulfilled this prediction (reviewed in Refs. 50–52). Together with the TSH receptor (TSHR) and the FSH receptor (FSHR), the LHR started a subfamily of GPCRs that is characterized by the presence of a large N-terminal extracellular domain containing several LRRs (see above). This glycoprotein hormone receptor family, which has been renamed the LRR-containing GPCR (LGR) family, has now been expanded to include four additional human LGRs (designated LGR4–7, see Refs. 41, 53, and 54), one snail LGR (55), one nematode LGR (56), one LGR from sea anemones (57), and two *Drosophila* LGRs, known as fly LGR1 and fly LGR2 (53, 58). The predicted extracellular domains of some of the new LGRs are larger than those of the gonadotropin and TSH receptors and they may have as many as 17 LRRs (41). A dendogram depicting the phylogenetic relatedness of this expanding LGR family is shown in Fig. 5. Because these new members of the LGR

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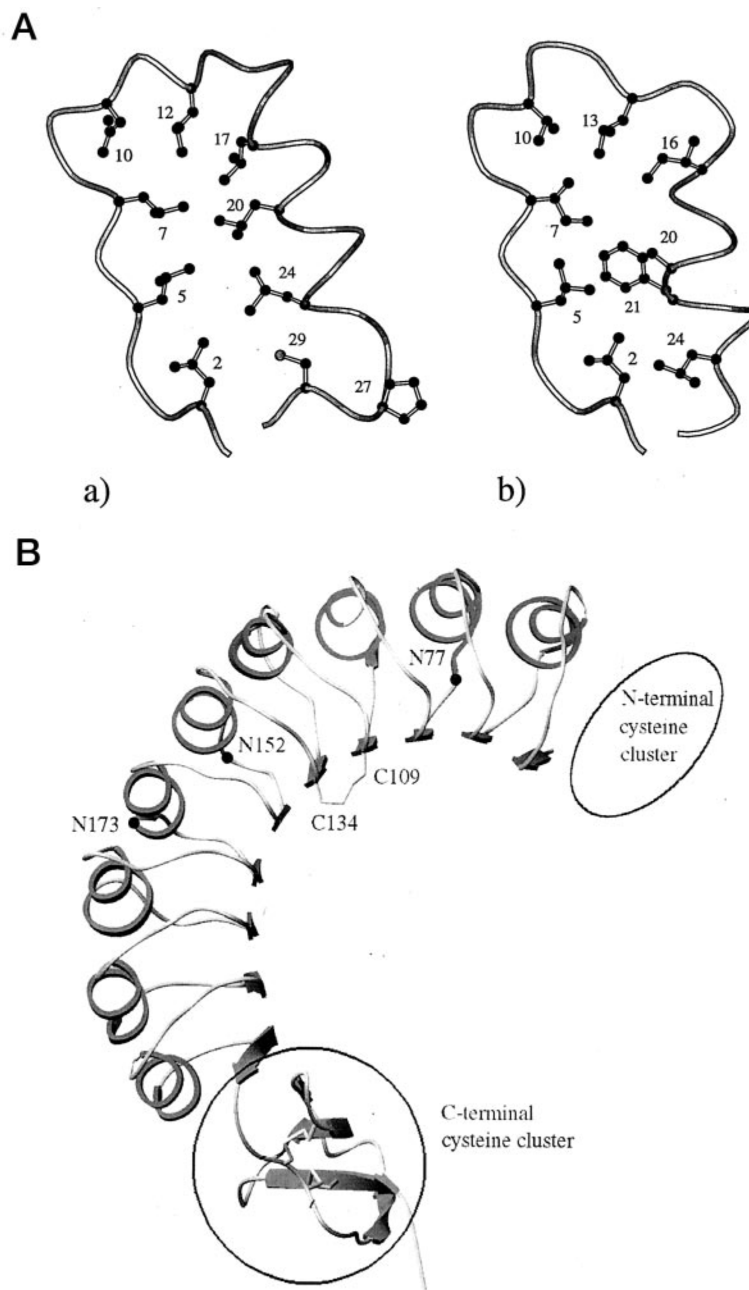

FIG. 4. Structural representation of a single LRR (A) and the leucine-rich motif region (B) of the LHR. A, The structural representation of the LRR depicted on the *left* (a) is that derived from the three-dimensional structure of the ribonuclease inhibitor (44). The structure of the LRR depicted on the *right* (b) is a proposal for the structure of the LRRs of the extracellular domain of the LHR and is based on the structure of the LRR depicted in *panel a*. The conserved hydrophobic residues are numbered based on the convention of Kobe and Deisenhofer (44), and these *numbers* also correspond to those shown at the *top* of the conserved residues on the amino acid sequence alignment shown in Fig. 3. B, A ribbon diagram of the LRR region of the extracellular domain of the LHR is shown. Note that the β-sheets (*arrows*) and the α-helices (*spirals*) align on opposite faces of the horseshoe-shaped structure. The location of the three consensus sequences for N-linked glycosylation and the two cysteine residues present in this region are also shown. [Reproduced with permission from N. Bhowmick *et al.*: *Mol Endocrinol* 10:1147–1159, 1996 (40). © The Endocrine Society.]

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have been discovered only recently, little is known about their ligands, signal-transducing properties, or physiological roles (41, 53).

The serpentine domain of the LHR is characterized by the canonical GPCR structure containing seven TM segments joined by three alternating ILs and ELs (see Fig. 1). The GPCR superfamily can now be divided into at least three major subfamilies (reviewed in Ref. 4), and the LHR is a member of subfamily A, the rodhopsin/β_2-adrenergic receptor-like subfamily of GPCRs. The serpentine domain of this family is characterized by a series of highly conserved residues. The corresponding rLHR and hLHR residues are shown in *purple* and in *pink* in Fig. 1. Several molecular models for the TM region of the hLHR have been proposed. The two initial models (59, 60) were built *ab initio* by using structural information derived from the low-resolution structure of rhodopsin (61) and from sequence comparisons performed among many GPCRs (62). The very recent availability of the crystal structure of rhodopsin in its inactive state (63) has allowed for the building of a new model of the hLHR achieved by comparative modeling with the known crystal structure of rhodopsin (Ref. 64 and F. Fanelli, submitted manuscript). As discussed below (see *Section VI* and Fig. 7), the molecular models of the hLHR have been very useful in attempting to understand the molecular basis of the mutation-induced activation of this receptor.

Our thinking of the overall structure of GPCRs will have to be modified based on the crystal structure of rhodopsin,

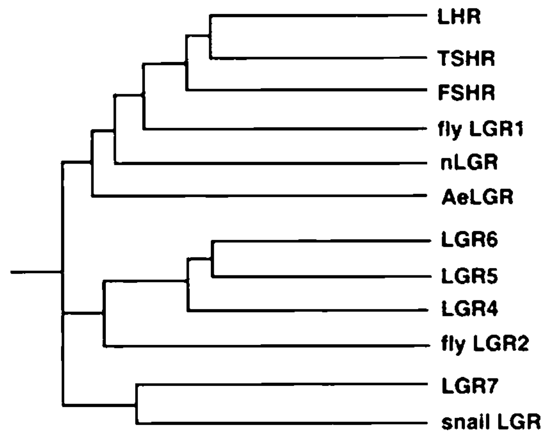

FIG. 5. Phylogenetic relatedness of LGRs from different species. This dendogram was constructed based on full-length amino sequence comparisons of the different LGRs shown. The LGRs can be basically divided into three distinct families. One family is composed of the mammalian LHR, TSHR, FSHR, the fly LGR1, the nematode LGR (nLGR), and the sea anemone LGR (AeLGR). The second family is composed of mammalian LGR4–6 and fly LGR2. The third family is composed of mammalian LGR7 and snail LGR. [Reproduced with permission S. Nishi *et al.*: *Endocrinology* 141:4081–4090, 2000 (53). © The Endocrine Society.]

which clearly shows the presence of eight α-helical segments (63). The first seven of these α-helical segments encompass what has been traditionally viewed as the seven TM segments of the GPCR superfamily. The eighth helix is entirely cytoplasmic and lies between the cytoplasmic end of TM7 and the conserved intracellular cysteine residue that is palmitoylated (see below and Fig. 1). The topology of the eight α-helical segments predicted for the hLHR is also shown in Fig. 1 for reference, but throughout this review, for ease of comparison with published data, we will still refer to the serpentine domain as being composed of seven transmembrane segments (TM1-7) with three ILs (IL1-3) and three ELs (EL1-3).

No serious attempts have been made to model the three-dimensional structure of the C-terminal tail of the LHR. This region of the LHR is the most divergent between the rLHR and the hLHR (see Fig. 1) and is the site of two known posttranslational modifications, palmitoylation and phosphorylation. An intracellular cysteine residue present in the juxtamembrane region of the C-terminal tail of the rodhopsin/β_2-adrenergic receptor-like subfamily of GPCRs is among the most highly conserved residues of this subfamily of GPCRs, and all members of this subfamily examined to date have been shown to be palmitoylated at this site. The palmitate present at this highly conserved position is thought to be imbedded in the membrane, and thus, the amino acid residues present between the cytoplasmic end of TM helix 7 and these conserved cysteines are thought to form a fourth IL for this subfamily of GPCRs (reviewed in Ref. 4). The LHR is unusual in having two adjacent cysteines in this position (Fig. 1). Although the palmitoylation of the hLHR has not been studied, the rLHR expressed in 293 cells has been shown to be palmitoylated at both of these residues (22, 26). The finding that palmitoylation can be detected only on the mature 85- to 95-kDa form of the rLHR (22, 25, 26) suggests that this posttranslational modification occurs during the maturation and transport of the immature receptor to the plasma membrane or once the LHR has reached the plasma membrane. Mutation of the palmitoylation sites of the rLHR had no effect on hCG binding or hCG-stimulated signal transduction (22, 26), but it was reported to enhance the rate of internalization of hCG (22).

Like many other GPCRs studied to date (reviewed in Refs. 65–67), the rLHR (68) and the hLHR (19) have been shown to be phosphorylated when expressed in transfected cells. Attempts to detect phosphorylation of the LHR in porcine follicular membranes, however, have failed (69). In transfected cells, basal phosphorylation of the mature, cell-surface (85–95 kDa) rLHR or hLHR is readily detectable, but phosphorylation of the immature (68–75 kDa) receptor is not detectable (19, 68). The phosphorylation of the cell-surface rLHR or hLHR can be enhanced by stimulation of the cells with hCG or phorbol esters (19, 68). Stimulation of transfected cells with cAMP analogs also induces a small and rather variable increase in phosphorylation of the rLHR (68). These analogs have not been tested on cells expressing the hLHR. The identity of the kinase(s) that phosphorylate the LHR in LH/CG-stimulated cells are not known, but they are presumed to be members of the GPCR kinase (GRK) family (67). Overexpression of one of the members of this family [GPCR kinase 2 (GRK2)] has been shown to enhance the LH/CG-induced phosphorylation of the hLHR (19). The LH/CG-induced phosphorylation of the rLHR occurs only on serine residues (70), and the residues of the rLHR that become phosphorylated in LH/CG-stimulated cells have been identified as Ser^{635}, Ser^{639}, Ser^{649}, and Ser^{652} (see *blue* residues in Fig. 1 and Refs. 70–73). The phosphorylation sites of the hLHR have not been studied in as much detail, but the mutation of the five serines that are equivalent to those phosphorylated in the rLHR (see *blue* residues in Fig. 1) causes a drastic reduction in basal and LH/CG-stimulated phosphorylation of the hLHR (19). The functional consequences of phosphorylation are discussed in *Section VII.B*.

B. The LHR gene and the LHR mRNA

The human LHR is encoded by a single gene located in the short arm of chromosome 2 (2p21) (74). In the map of the human genome the ID for the LHR is ENSG00000095001, and its detailed location can be viewed in sequence AC073082 on chromosome 2.

The general organization of the hLHR (75) and rLHR genes (76–78) are very similar. These genes are about 80 kb in size and each consists of 10 introns and 11 exons (75–78). The entire serpentine and C-terminal domains of the LHR are encoded in exon 11. This exon also codes for the C-terminal end of the hinge region of the extracellular domain. The N-terminal cysteine-rich region, all of the LRRs, and the N-terminal end of the hinge region of the extracellular domain arise from the splicing of exons 1–10 (Fig. 1).

Unlike the coding sequences, the sequences of the 5′-flanking regions of the genes for the rLHR and the hLHR are only approximately 60% homologous (75, 77, 79). The 5′-flanking region of the rLHR resembles those seen in housekeeping genes in that it lacks TATA and CAAT box consensus se-

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quences, displays multiple transcriptional start sites, and has a promoter region rich in G/C nucleotides (nt) and containing several Sp1 sites. The 5′-flanking region of the hLHR gene isolated from a human lymphocyte library by Milgrom and colleagues (75) was shown to be CG rich and to contain two putative TATA boxes and a CAAT box and one site of transcription initiation when analyzed using mRNA from testicular cells. Subsequent studies by Dufau and colleagues (80) on the 5′-flanking region of the hLHR gene that they had cloned from a human placental library suggested the presence of multiple transcriptional start sites when using mRNA from human choriocarcinoma JAR cells, human ovary, or human testes. Taken altogether, these data suggest that the 5′-flanking region of the hLHR gene, like the rLHR gene, is most likely also similar to housekeeping genes.

Transcription of the rLHR and pLHR genes gives rise to multiple mRNA species in gonadal tissues, and the relative abundance of each of the six transcripts differs between ovarian and testicular tissues (2, 81). These different transcripts are thought to occur as a result of the use of different transcriptional start sites, alternate splicing of the gene, and/or differences in polyadenylation (2, 3, 17, 79, 82–85). Interestingly, variants of the rLHR and pLHR were identified that encode for only the extracellular domain (17, 79). In the pig, 40% of the mRNA transcripts correspond to such variants (17). In the rat ovaries and testis, a 1.2-kb species encoding the extracellular domain is present but at low abundance. However, in MA-10 Leydig tumor cells, this 1.2-kb species is the predominant mRNA (79). Results obtained using heterologous cells transfected with cDNAs encoding for these truncated receptors results have varied between observations reporting total intracellular retention of the cognate protein (86), secretion into the media (87), or both secretion and intracellular retention (17). Three alternatively spliced variants of the hLHR have also been reported in gonadal cells, but these have not been fully characterized (88, 89).

Although the presence of transcripts encoding alternately spliced variants of the LHR gene, especially ones encoding only the extracellular domain, raise intriguing questions regarding their possible physiological roles, it is important to point out that at this writing there are no data yet that demonstrate the presence of proteins arising from such transcripts.

The marmoset monkey provides what may be the only documented example of a splice variant of the LHR that is translated into a stable protein. Like the genes for the human and rat LHR, the LHR gene of the marmoset monkey is made up of 11 exons, but exon 10 is spliced out of the mature mRNA (90). Despite this deletion, the mature marmoset LHR can bind hCG with a high affinity, comparable to that of the hLHR, and the binding of hCG can be translated into increases in cAMP and inositol phosphate production (90). When transfected into COS cells, however, the expression of the marmoset LHR at the cell surface is much lower than that of the hLHR (90), and the deletion of exon 10 from the hLHR results in a decrease in the cell-surface expression of the hLHR (91). Thus, although the deletion of exon 10 seems to impair the trafficking of the receptor to the cell surface, it does not affect the ability of the receptor to bind agonist or to promote cAMP accumulation. Thus, the finding that a naturally occurring homozygous deletion of exon 10 of the hLHR gene is associated with male hypogonadism is likely to be explained by the corresponding reduction in the density of cell-surface receptors rather than by changes in hormone binding and/or signal transduction (92). It should be stressed, however, that the mutant of the hLHR that lacks exon 10 is due to a mutation in the hLHR gene and is not a splice variant of the wt gene.

Several polymorphisms of the hLHR gene have been reported, and those that result in a change in amino acid sequence are summarized in Fig. 6. A 6-nt in-frame insertion/deletion between codons 18 and 19 of exon 1 results in the expression of two hLHR variants that differ by the presence (LQ) or absence (αLQ) of a Leu-Gln pair near the C terminus of the signal peptide (75, 80, 93). This polymorphism was originally thought to represent an additional gene for the hLHR (80), but the sequence of the human genome has conclusively excluded this possibility. The genotype frequency of this polymorphism in a Caucasian population is 0.088 (LQ/LQ), 0.558 (αLQ/αLQ), and 0.354 (LQ/αLQ) (93). However, the LQ allele has, thus far, not been found in a population of Japanese subjects (93). The presence/absence of the LQ allele does not appear to affect the expression or functional properties of the hLHR as assessed by hCG binding and cAMP responses of cells expressing either variant (93). This is an interesting finding because a longer (11-amino acid) in-frame insertion in the same position results in the intracellular retention of the hLHR and leads to Leydig cell hypoplasia (Fig. 6 and Ref. 94).

Two additional single nucleotide polymorphisms (SNPs) in exon 10 of the hLHR gene code for either Asp or Ser in codons 291 and 312 in the hinge region of the extracellular domain of the hLHR. The genotype frequency of this polymorphism is not known, but the presence of Asp or Ser at either of these positions does not appear to change the expression or functions of the hLHR (95). Several other SNPs of the coding region that are silent, or SNPs of intronic sequences, have also been identified. These were recently tabulated in Ref. 52. A search of the National Center for Biotechnology Information SNP database with the open reading frame of the hLHR (http://www.ncbi.nlm.nih.gov/SNP/), conducted during the writing of this review, failed to retrieve any of the SNPs previously reported but revealed a novel silent SNP (reference SNP 1042551) in codon 504 (GG**T**/GG**G**), which is translated into Gly^{504} near the cytoplasmic end of TM4.

A number of naturally occurring mutations of the hLHR gene associated with human reproductive disorders have also been reported. They will be briefly summarized here to illustrate what these mutants tell us about the processing of the hLHR (this section), as well as the mechanisms of hormone binding (*Section V*) and receptor activation (*Section VI*). A detailed discussion on the functional properties of these mutants and their role in the pathophysiology of the pituitary-gonadal axis can be found in two recent reviews (52, 96).

A tabulation of the location of the naturally occurring loss-of-function mutations of the hLHR reported to date shows that these mutations are not restricted to a particular area of the hLHR (92, 94, 97–105). They are located through-

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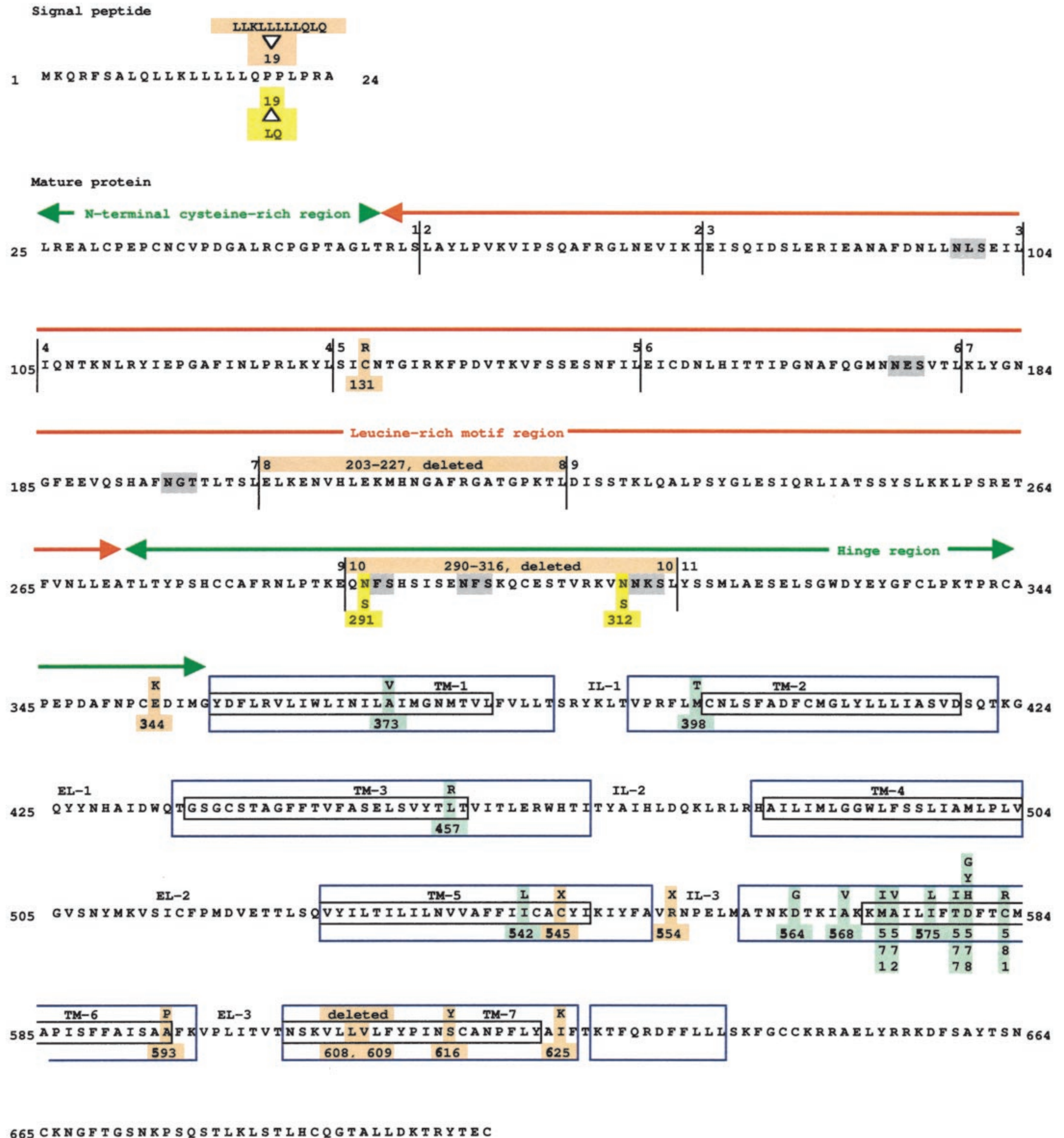

FIG. 6. Location of the naturally occurring polymorphisms and loss-of-function and gain-of-function mutations of the hLHR. The location and identity of naturally occurring mutations of the hLHR are shown. Polymorphisms are depicted in *yellow*, loss-of-function mutations are depicted in *orange*, and gain-of-function mutations are depicted in *green*. Other annotations are described in the legend to Fig. 1.

out the polypeptide chain, as shown in Fig. 6. Although some of these mutations prevent hCG binding and/or hCG-induced signal transduction, a salient feature that is common to all of them is that they impair the maturation and/or transport of the hLHR precursor so that the expression of the hLHR at the cell surface is always reduced. A comparison of the cell-surface expression of some of these loss-of-function mutants (101) reveals that this parameter can vary from

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about 50% of the hLHR-wt (*i.e.*, the I625K mutant) to less than 1% of the hLHR-wt (*i.e.*, the A593P mutant).

In contrast to the heterogeneous location of the naturally occurring loss-of-function mutations of the hLHR (Fig. 6), all naturally occurring gain-of-function mutations reported to date (106–121) are localized to exon 11 in the serpentine region of the hLHR (Fig. 6C). These mutants display different degrees of constitutive activity and, together with models of the three-dimensional structure of the serpentine region of the hLHR, have provided important information about the mechanisms involved in the activation of the hLHR (see *Section VI*). Although the restricted location of these mutations is in agreement with the perceived importance of the serpentine region of the hLHR in signal transduction (see *Section VI*), their restricted location may also be a function of the methods used to search for mutations. Because earlier efforts to find activating mutations of the hLHR were restricted to this region of the gene, it is possible that further studies will reveal the presence of activating mutations of the hLHR elsewhere. Such a finding can indeed be forecasted by the recent demonstration that certain laboratory-designed mutations of the hinge region of the hLHR can also induce constitutive activation (122, 123). In addition, naturally occurring activating mutations have been recently identified in the hinge region of the extracellular domain of the structurally related TSHR (124–126).

III. Expression of the LHR

For many years the LHR was thought to be localized strictly to gonadal cells. In the testes, the LHR is thought to be restricted to Leydig cells. In the ovary, expression of the LHR occurs in theca cells, interstitial cells, differentiated granulosa cells, and luteal cells. Certainly, the main physiological roles of the LHR can be attributed to its actions in the ovaries and the testes. Thus, women who are homozygous for loss-of-function mutations of the LHR are infertile. 46,XY individuals homozygous for severe inactivating mutations of the LHR are also infertile and present as pseudohermaphrodites, whereas less severe inactivating mutations result in micropenis and/or hypospadias. These phenotypes can be explained by the inability of the fetal Leydig cells to respond to maternal hCG with increased testosterone production, a steroid that is required for the differentiation of external genitalia to the male phenotype (52, 96). Transgenic mice in which the LHR gene has been knocked out are also infertile (127, 128). The primary difference observed between the LHR loss-of-function phenotype between mice and humans is the lack of pseudohermaphroditism in the male LHR-knockout mice.

There is increasing evidence that the LHR may be present in extragonadal tissues as well, both in the reproductive tract and elsewhere. The suggestion of LHR expression has been based in many cases upon the detection of LHR mRNA. In a few cases, the expression of the LHR protein has also been examined using antibodies or by radiolabeled hCG binding assays. Within the female reproductive tract, the LHR has been reported to be present in the bovine, porcine, rat, mouse, rabbit, and human uterus by a number of different laboratories (129–137). Whereas the LHR in human uterus has been observed in the endometrial endothelium (134), in mice the LHR has been observed in uterine stroma and subepithelial cells (129). Whether these differences reflect differences among species or result from different experimental techniques remains to be determined. It should also be noted that controversy surrounds the issue of LHR expression in the human uterus. Whereas Rao and colleagues (134) have reported the detection of LHR in human uterus, Stewart *et al.* (138) were not able to detect the LHR in the human uterine samples they examined. Two different groups have also reported the presence of the LHR in other human or mouse female reproductive tract tissues (129, 139). Rao and colleagues (139) reported the presence of the LHR in the mucosa of the human fallopian tube. In the mouse, the LHR was not detected in the oviductal mucosa, but rather, was found in the serosa and in subepithelial cells of the oviduct (129). It should also be pointed out that Rao and colleagues (reviewed in Refs. 128, 140, and 141) have reported that the LHR is present in a variety of other tissues including human sperm, human seminal vesicles, rat and human prostate, human prostate carcinomas, human skin, human breast cell lines, lactating rat mammary gland, human adrenals, neural retina, neuroendocrine cells, and rat brain. The presence of LHR mRNA and protein has also been documented in breast cancer cell lines, individual human breast cancer biopsies, and benign breast lesions by other investigators (142). In addition, a study by Frazier *et al.* (143) suggested the presence of the LHR mRNA in the human thyroid.

Certainly, the purported presence of the LHR in these various tissues raises intriguing questions about the physiological role(s) of the gonadotropins and the LHR. It is interesting to note, though, that there have been no reported clinical observations of abnormalities in these different systems in individuals with either loss-of-function or gain-of-function mutations of the LHR gene (52, 96). This finding suggests that if the LHR does indeed play a physiological role in these systems, it may be rather subtle. Determining the potential physiological roles of the LHR in nongonadal tissues in LHR-knockout mice or in humans harboring loss-of-function mutations of the LHR has been problematic because the affected individuals are infertile and they suffer from a secondary deficiency in gonadal hormones that are normally stimulated by LH. The potential development of mice with tissue-specific knockouts of the LHR gene may provide further insights into this problem, however.

The clearest evidence for the extragonadal expression of a functional LHR comes from studies done on a postmenopausal woman with Cushing's syndrome and ACTH-independent macronodular adrenal hyperplasia (144). Her clinical history suggested that she also developed Cushing's syndrome during each of four full-term pregnancies, but chronic hypercortisolism became obvious only several years after menopause. These findings led to the hypothesis that the ectopic expression of the LHR in her adrenal cortex, coupled with the elevated levels of hCG (during pregnancy) or LH (during menopause), were responsible for the hypercortisolism and adrenal hyperplasia (144). Although the expression of the LHR in the adrenal cortex of this patient was

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not directly demonstrated, the following functional evidence clearly supports the hypothesis proposed. The administration of GnRH, hCG, or recombinant hLH, but not hFSH, resulted in an increase in serum testosterone, estradiol, and cortisol levels. Administration of a long-acting GnRH analog (leuprolide) initially increased LH, FSH, and cortisol levels but eventually suppressed the levels of LH and FSH and normalized cortisol. Lastly, GnRH did not stimulate cortisol secretion when LHR levels were suppressed by chronic administration of leuprolide, thus ruling out a direct effect of GnRH on the adrenal cortex. These data clearly show that cortisol production was controlled by LH/CG and strongly suggest the presence of a functional LHR in the adrenal cortex of this individual. In fact, long-term treatment of this patient with leuprolide controlled the hypercortisolism and obviated the need for a bilateral adrenalectomy (144). A number of other cases in which clinical findings may be explained by the inappropriate expression of the LHR in the adrenal cortex or in adrenocortical tumors have been recently reviewed (145). Lastly, ectopic expression of functional LHR in the adrenal cortex also appears to occur in transgenic or knockout mouse models with elevated levels of gonadotropins (146–149).

IV. Signaling Pathways Activated by the LHR

Although most investigators agree that the LHR-mediated effects on the differentiated function of Leydig and granulosa cells are mediated mostly (if not entirely) by the activation of the Gs/adenylyl cyclase/cAMP/PKA pathway, it is abundantly clear now that this is not the only pathway activated by the LHR and that additional pathways may be involved in other LHR-dependent events such as the proliferation and/or differentiation of target cells. This issue is specially meaningful at a time when there is a growing body of evidence suggesting that GPCRs can affect the proliferation and differentiation of endocrine cells and that they may do so by using signaling mechanisms that are much more complex than previously recognized. For example, it is now generally accepted that a given GPCR can independently activate more than one subfamily of heterotrimeric G proteins (reviewed in Ref. 150), and that heterotrimeric G proteins may in fact not be the only mediators of GPCR signaling (reviewed in Refs. 151 and 152).

The LHR was one of the first GPCRs shown to independently activate two G protein-dependent signaling pathways, adenylyl cyclase and PLC. The first conclusive demonstration of this phenomenon was documented by Gudermann *et al.* using L cells (153) or *Xenopus* oocytes (154) expressing the recombinant mouse LHR. This observation has now been extensively reproduced by a number of investigators using a variety of cell lines transfected with either mLHR (153, 155), rLHR (70, 72, 156), or the hLHR (64, 115, 157–159). Although all investigators agree that the LHR can activate Gs (160–162), and thus induce the activation of adenylyl cyclase, the identity of the other G protein(s) that are activated by the LHR and which of these mediate the activation of the inositol phosphate pathway is still somewhat controversial. Using a well-established photoaffinity labeling/immunoprecipitation technique, Gudermann's group (160) reported that the mouse LHR expressed in L cells or the endogenous LHR present in bovine luteal membranes activated Gs and Gi_2 but not Gq/11, G12, or G13. Using the same methodology, Hunzicker-Dunn and colleagues (161, 162) reported that the endogenous LHR present in porcine follicular membranes activates Gs, Gi, G13, and Gq/11. The time course of the LHR-mediated activation of Gq/11, however, was exceptionally slow compared with that of Gs and Gi and to that of other receptors that activated Gq/11. Also, the subtype of Gi activated by the LHR was not examined in these experiments.

The identity of the G protein and G protein subunits that mediate the effects of the LHR on PLC have been carefully examined in only two cell types expressing the recombinant mLHR, L cells (153, 160) and Sf9 cells (155). Although the LHR-mediated activation of PLC was initially reported to be pertussis toxin insensitive in transfected L cells (153) and in *Xenopus* oocytes expressing the recombinant mLHR (154), this finding was later corrected (160). The LHR-mediated accumulation of inositol phosphates and Ca^{2+} mobilization in L cells transfected with the mLHR were later shown to be inhibited by pertussis toxin (160). The mLHR-mediated activation of PLC in transfected mouse L cells was also shown to be inhibited by scavengers of $G\beta/\gamma$ (159), and L cells were shown to express two of the different isoforms of PLC (β2 and β3) that can be activated by $G\beta/\gamma$ subunits. Lastly, overexpression of PLC-β2 was shown to potentiate the LHR-mediated increase in inositol phosphate accumulation (160). These findings led to the proposal that the LHR-mediated activation of PLC is mediated by the $G\beta/\gamma$ subunits liberated from the LHR-induced activation of Gs and Gi (160). Additional experiments performed in Sf9 cells coinfected with the LHR and different G protein subunits revealed that coexpression of $G\alpha i_2$, but not $G\alpha s$, $G\alpha$ 11, $G\alpha i1$, $G\alpha i3$, and $G\alpha q$, resulted in the potentiation of the LHR-stimulated PLC activity (155). Curiously, however, coexpression of two different combinations of $G\beta/\gamma$ subunits did not further increase the ability of $G\alpha i_2$ to enhance the LHR-stimulated PLC activity (155). This latter finding is not quite consistent with the proposal that the LHR-induced activation of PLC is mediated by $G\beta/\gamma$ (159). Thus, as of now, it seems possible that the LHR-induced activation of PLC may be mediated by the β/γ subunits liberated by the activation of Gi_2 (and possibly Gs) or by αq liberated during the activation of Gq.

It is also important to note that although the LHR-mediated activation of adenylyl cyclase is detectable in all cell types examined, the LHR-mediated activation of PLC is not always detectable. For example, two different groups (163, 164) failed to detect an effect of hCG on inositol phosphate accumulation in mouse Leydig tumor cells (MA-10) expressing the endogenous LHR even at high concentrations of hCG. It is not known whether this response is due to the lack of expression of the appropriate G proteins or PLC. These cells do express an isoform of PLC that can be activated by αq, however, as documented by the finding that their inositol phosphate response can be readily activated by the activation of an endogenous GPCR that couples to Gq [*i.e.*, one of the arginine vasopressin type 1 receptors (163)]. It is also possible that the inability of the LHR to activate PLC in MA-10 cells

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is due to their low density of endogenous LHR (165) because in transfected cells an LHR-induced inositol phosphate response is highly dependent on the density of LHR at the cell surface and is detectable only when cells expressing a high receptor density are exposed to high concentrations of hCG (70–72, 166). In fact, very recent studies from one of our labs (167) have shown that MA-10 cells expressing high densities of the recombinant hLHR-wt can indeed respond to hCG with an increase in inositol phosphate accumulation. Another problem with the interpretation of results regarding activation of the inositol phosphate *vs.* the cAMP response relates to the sensitivity of these two assays. Measurements of cAMP levels are usually done using rather sensitive techniques such as a RIA or enzyme-linked immunoassays, whereas measurements of inositol phosphates, which depend on radiolabeling the inositol-containing cellular lipids with [^{3}H]inositol, are much less sensitive. Lastly, in contrast to the well-documented role of cAMP as a mediator of the actions of LH/CG on steroidogenesis, there is little information available about actions of LH/CG that may be mediated by the activation of PLC. Because the activation of PLC requires high concentrations of LH/CG, it has been proposed that this signaling pathway is activated only in females during the preovulatory LH surge or during pregnancy (153). Because maternal hCG is also important in the development of the normal male phenotype, it is possible that exposure of the male fetus to the high levels of maternal hCG may also result in the stimulation of the PLC cascade in fetal Leydig cells.

The classification of naturally occurring mutants of the LHR as gain- or loss-of-function mutants (see above) and the impact of laboratory-designed mutations on the activation of the LHR have been traditionally based on measurements of cAMP accumulation as an index of receptor activation. Only a few experiments have compared the effects of a given LHR mutation (naturally occurring or laboratory-designed) on the cAMP and inositol phosphate responses, and the results obtained indicate that some mutations have a similar effect on the hCG-induced activation of these two pathways, whereas others have divergent effects. For example, a truncation of the C-terminal tail of the rLHR at residue 653 has little or no effect on the hCG-induced cAMP response, but it severely blunts the hCG-induced inositol phosphate response, whereas a truncation at residue 628 enhances both the hCG-induced cAMP and inositol phosphate response (71). Similarly, some single point mutations of residues present in the EL3 of the rLHR (156), IL3 of the hLHR (168), or TM3 of the hLHR (64) have been reported to have differential effects on the hCG-induced cAMP and inositol phosphate responses. These findings raise the possibility that structural features of the LHR that mediate the cAMP response are different from those that mediate the inositol phosphate response. This issue is particularly important in view of a recent proposal (115) stating that constitutive activity toward the inositol phosphate pathway may be restricted only to naturally occurring somatic gain-of-function mutations that are associated with Leydig cell adenomas. This proposal arose from the finding that a gain-of-function mutation of the hLHR associated with Leydig cell adenomas (D578H) displayed constitutive activity toward the cAMP and inositol phosphate responses, whereas a similar gain-of-function mutation of the hLHR associated with Leydig cell hyperplasia (D578Y) displayed constitutive activity only toward the cAMP response (115). It is important to note, however, that other publications from the same group of investigators (157, 159) have reported a small degree of constitutive activation of the D578Y mutant on the inositol phosphate response. In addition, a recent study utilizing another gain-of-function mutation of the hLHR associated with Leydig cell hyperplasia (L457R) has shown that constitutive activation of the inositol phosphate response could not be detected when measuring inositol phosphate accumulation by the traditional assays involving the metabolic labeling of cells with [^{3}H]inositol, but could be detected using a more sensitive reporter gene assay indicative of PKC activation (64), whereas recent studies using MA-10 cells transfected with the L457R, D578Y, and D578H mutants have also shown that they all display constitutive activation of the inositol phosphate response (167). Lastly, the only other naturally occurring gain-of-function mutation of the hLHR that has been examined for constitutive activation of the inositol phosphate response (*i.e.*, the D564G mutant) has been reported to have no constitutive activity toward this pathway by two different groups of investigators (159, 168).

Clearly, more experiments need to be done to further understand the molecular basis of the LHR-mediated activation of PLC and the physiological consequences of this activation. Also, as the number of pathways activated by GPCRs in a G protein-dependent and -independent fashion grows (reviewed in Refs. 151, 152, and 169–172), the effects of the LHR on these pathways will need to be evaluated. So far, the only other signaling pathway that has been shown to be activated by the LHR in cells expressing the endogenous or transfected hLHR is the MAPK cascade (167, 173). In fact, when expressed in MA-10 cells, two of the naturally occurring hLHR mutants associated with Leydig cell hyperplasia (D578Y and L457R) and the naturally occurring hLHR mutant associated with Leydig cell adenomas (D578H) were also shown to display constitutive activation of this pathway (167). The molecular basis of the activation of this pathway and the consequences of such activation have not, however, been explored.

V. Binding of LH/CG to the LHR

The amino acid sequence and predicted topology of the LHR deduced from the cloning efforts quickly led to the hypothesis that the large extracellular domain of this receptor was responsible for the recognition and high affinity binding of LH and CG. This hypothesis was initially tested in one of our laboratories (86) by measuring ^{125}I-hCG binding in cells transfected with a construct (designated rLHR-t338) that encoded for only residues 1–338 (*i.e.*, the extracellular domain) of the rLHR. Because the construct used was devoid of coding sequences of the serpentine and C-terminal tail, but contained the coding sequence for the signal peptide, we expected that the truncated receptor would be secreted into the culture medium. This, however, was not found to be the case. Instead, the extracellular domain of the rLHR was

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found to be retained inside of the cells. Thus, binding assays performed on the conditioned medium and intact cells failed to detect any binding. The only way to detect ^{125}I-hCG binding to cells transfected with rLHR-t338 was to solubilize the cells with a nonionic detergent and glycerol (86). Binding assays performed on solubilized cells transfected with either rLHR-wt or rLHR-t338 revealed that rLHR-t338 binds hCG with the same affinity as the full-length rLHR (K_d = 0.2–0.5 nM). The ability of the extracellular domain of the rLHR, pLHR, and hLHR to bind hCG with very similar (or identical) affinities to those of their full-length counterparts has now been confirmed by many investigators using a variety of constructs encoding for the extracellular domain (17, 78, 87), chimeras containing the extracellular domain of the LHR with the serpentine and C-terminal tails of the FSHR (174) or the β_2-adrenergic receptor (175), and fusion proteins containing the extracellular domain of the LHR with the single TM domain of CD8 (176). As expected, the extracellular domain of the LHR is also responsible for dictating hormonal specificity. Thus, whereas the extracellular domain of the rLHR binds LH and CG with high affinity, it does not bind FSH (177). Likewise, hormone binding and hormone-stimulated cAMP accumulation in cells transfected with LHR/FSHR chimeras is dictated by the identity of the extracellular domain of the chimeras (174, 178). Lastly, a soluble form of the extracellular domain of the hLHR can inhibit the binding of hCG to cells expressing the full-length hLHR, but it cannot inhibit the binding of hFSH to cells expressing the full-length hFSHR (176). Conversely, a soluble form of the extracellular domain of the hFSHR can inhibit the binding of hFSH to cells expressing the full-length hFSHR, but it cannot inhibit the binding of hCG to cells expressing the full-length hLHR (176).

The functional analysis of mutants of the LHR (and other glycoprotein hormone receptors) has often been confounded by the finding that many mutations impair the maturation and/or transport of the receptor to the cell surface, and thus, the mutant receptors are often trapped in the endoplasmic reticulum as the immature 68-kDa precursor (see above and Refs. 8, 23, 25, 26, and 179). Thus, a decrease in hCG binding in intact cells transfected with a given receptor mutant may or may not be due to an effect of that mutation on hCG binding. Because the binding of hCG can be readily measured in extracts of cells prepared with a nonionic detergent and glycerol (180), and because the 68-kDa precursor of the wt rLHR can bind hCG with the same affinity as the 85-kDa cell-surface receptor (21), the most accurate way to determine whether a given mutation affects hCG binding is to measure this parameter in extracts of transfected cells prepared with a nonionic detergent and glycerol rather than on the intact transfected cells (reviewed in Ref. 8). This complexity in data interpretation can be easily illustrated using studies on two naturally occurring loss-of-function mutations of the hLHR. The deletion of exon 10 of the hLHR is associated with Leydig cell hypoplasia (92), and heterologous cells transfected with a mutant of the hLHR that lacks exon 10 display a reduction in maximal hCG binding when measured in intact cells, but not when measured in cell extracts (91). Measurements of the binding affinity of hCG to extracts of cells transfected with the mutant hLHR lacking exon 10 also show that these cells bind hCG with the same affinity as those transfected with the full-length hLHR-wt (91). The deletion of exon 8 of the hLHR is also associated with Leydig cell hypoplasia (100), and heterologous cells transfected with a mutant of the hLHR that lacks exon 8 display a marked reduction in hCG binding in intact cells and in detergent extracts that precludes measurements of binding affinities (100). Because the expression of the mutant lacking exon 8 was not independently confirmed, we cannot make any conclusions about the involvement of this exon on hCG binding. We can, however, readily conclude that exon 10 is not necessary for hCG binding.

Truncations of the extracellular domain of the LHR as well as deletions and chimeras of the full-length LHR have contributed to a better definition of the regions of the extracellular domain of the LHR that participate in the binding of LH/CG (Table 1). Ji and co-workers (123, 181) examined several C-terminal truncations of the extracellular domain of the rLHR that systematically deleted each of the 10 exons that encode for this domain. They reported that the binding affinity of these constructs for hCG decreased in a stepwise fashion. Constructs containing exons 1–7, 1–8, and 1–9 (resulting in truncations of the extracellular domain at residues 267, 205, or 180) lowered hCG binding affinity 10- to 20-fold. Constructs containing exons 1–6 and 1–5 (resulting in truncations of the extracellular domain at residues 157 and 131, respectively) lowered hCG binding affinity 100- to 200-fold. Constructs containing exons 1–4, 1–3, and 1–2 (resulting in truncations of the extracellular domain at residues 106, 81, and 56, respectively) lowered hCG binding affinity 300- to 600-fold, whereas a construct consisting of only exon 1 (residues 1–32) did not bind hCG at all. A similar type of analysis was conducted by Thomas *et al.* (182), who systematically deleted portions of the extracellular domain based on the initial assignment of 14 LRRs in this region (see *Section II.A*). Their experiments were done using the full-length receptor, and their results are also summarized in Table 1 but, for consistency, the regions deleted are tabulated based on the current assignment of nine LRRs (see *Section II.A*). Other groups of investigators have also used progressive deletions of the extracellular domain of the rLHR (174), chimeras of the rFSHR and rLHR (174, 183), and deletions of the full-length hLHR (91, 123) to help define the LH/CG binding site, and their data are also summarized in Table 1.

There are three regions of the extracellular domain of the LHR that have been examined by several groups of investigators. One is the N-terminal end of the extracellular domain. N-terminal deletions of the full-length rLHR have shown that the deletion of residues 1–11, 12–38, 39–63, 64–88, 89–12, 113–118, and 139–164 completely abolish hCG binding (182), and chimeras of the rLHR/rFSHR have shown that the substitution of residues 1–146 of the rLHR for the corresponding residues of the rFSHR also prevents hCG binding (174). These results are in agreement with those obtained using C-terminal truncations of the extracellular domain of the rLHR. Thus, a construct encoding for residues 1–157 of the rLHR bind hCG with an affinity that is about 100 times lower than that of the rLHR-wt (181). When considered together, these data indicate that exons 1–7, which encode for the N-terminal cysteine-rich region and LRR1-LRR7, are essential for the high affinity binding of hCG to the LHR.

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TABLE 1. Effect of selected mutants of the LHR on hCG binding

Receptor construct	Exons deleted in construct	Regions deleted from construct	K_d for hCG binding (nM)[a]	References
rLHR-wt	None	None	0.3–0.5[b,c]	78, 174, 177, 181, 182
C-terminal truncations containing only the extracellular domain of the rLHR[d]				
rLHR(1–338)	11	See footnote[d]	0.3–0.8[b]	78, 123, 174, 177, 181, 182
rLHR-(1–294)	11	HR*[e]	0.3	78
rLHR(1–273)	10–11	HR*	0.7	174
rLHR(1–267)	10–11	HR*	5–6[b]	123, 181
rLHR(1–206)	9–11	LRR8*[f] + LRR9 + HR	0.7	174
rLHR(1–205)	8* + 9–11	LRR8* + LRR9 + HR	3	181
rLHR(1–180)	8–11	LRR7* + LRR8–9 + HR	4	181
rLHR(1–157)	7–11	LRR6* + LRR7–9 + HR	50	181
rLHR(1–131)	6–11	LRR5* + LRR6–9 + HR	60	181
rLHR(1–106)	5–11	LRR4* + LRR5–9 + HR	100	181
rLHR(1–81)	4–11	LRR3* + LRR4–9 + HR	150	181
rLHR(1–56)	3–11	LRR2* + LRR3–9 + HR	170	181
rLHR(1–32)	2–11	LRR1* + LRR2–9 + HR	ND[g]	181
Deletion mutants of the full-length rLHR				
rLHR(Δ1–11)	1*	*NCR[h]	ND[i]	182
rLHR(Δ12–38)	1* + 2*	NCR* + LRR1*	ND[i]	182
rLHR(Δ39–63)	2* + 3*	LRR1* + LRR2*	ND[i]	182
rLHR(Δ64–88)	3* + 4*	LRR2* + LRR3*	ND[i]	182
rLHR(Δ89–112)	4* + 5*	LRR3* + LRR4*	ND[i]	182
rLHR(Δ113–138)	5* + 6*	LRR4* + LRR5*	ND[i]	182
rLHR(Δ139–164)	6* + 7*	LRR5* + LRR6*	ND[i]	182
rLHR(Δ165–187)	7* + 8*	LRR6* + LRR7*	5	182
rLHR(Δ188–211)	8* + 9*	LRR7* + LRR8	0.9	182
rLHR(Δ214–341)	9* + 10 + 11*	LRR8* + LRR9 + HR	0.5	182
Chimeras of the rLHR and rFSHR[j]				
rLHR(1–146)	6* + 7–11	LRR5* + LRR6-LRR9 + HR	0.6	174
rLHR(146–674)	1–5 + 6*	LRR1-LRR4 + LRR5*	ND[k]	174
rLHR(1–201)	8* + 9–11	LRR8-LRR9 + HR	0.3	183
rLHR(58–201)	1–2 + 8* + 9–11	NCR + LRR1 + LRR2 + LR R8-LRR9 + HR	0.5	183
Deletion mutant of the full-length hLHR				
hLHR-wt	None	None	0.1[c]	91
hLHR(Δ290–316)	10	HR*	0.1	91

[a] All binding assays were done in detergent soluble extracts of transfected cells.
[b] Low and high values reported in the Refs. cited.
[c] Note that according to these data, there is little or no difference in the K_d for hCG binding to the hLHR and the rLHR. This is likely due to differences in the conditions used to measure binding. When assayed under the same experimental conditions the K_d for hCG binding to the hLHR and rLHR differ by about one order of magnitude (see Fig. 2).
[d] All of these constructs lack the serpentine domain and the C-terminal tail.
[e] HR, Hinge region as defined in Fig. 1. The *asterisks* indicate that only a portion of a given region was removed.
[f] LRR as defined by the nine-repeat model shown in Fig. 3. The *asterisks* indicate that only a portion of a given repeat was removed.
[g] Because the expression of this construct was not independently verified, it is possible that the lack of detectable binding is due to a lack of expression of the protein (181).
[h] NCR, N-terminal cysteine-rich region as defined in Fig. 1. The *asterisks* indicate that only a portion of a given region was removed.
[i] The lack of detectable binding cannot be attributed to a lack of expression because the expression these constructs was verified by Western blotting (182).
[j] These are full-length chimeras in which the rLHR regions deleted were substituted with the corresponding regions of the rFSHR.
[k] Because the expression of this construct was not independently verified, it is possible that the lack of detectable binding is due to a lack of expression of the protein (174).

An additional region that is roughly flanked by residues 160–190 of the rLHR and encoded mostly by exon 8 has also been examined by two different groups of investigators using C-terminal truncations of the extracellular domain (181) or by deleting it from the full-length receptor (182). Both groups concluded that the removal of this region decreases the hCG binding affinity by about one order of magnitude (Table 1).

The remaining portion of the extracellular domain of the rLHR is encoded for by exons 9 and 10. It encompasses a portion of LRR8, all of LRR9, and all of the hinge region (see Fig. 1). Although Ji's group (123, 181) reported that the deletion of exons 9 or 10 reduced the binding affinity of the extracellular domain of the rLHR by about one order of magnitude, Braun *et al.* (174) reported that the removal of this region had little or no effect on hCG binding affinity (see Table 1). Thomas *et al.* (182) also reported that the deletion of exons 9 and 10 from the full-length rLHR had no effect on

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hCG binding affinity, and a chimera of the rLHR and rFSHR that encompasses only exons 1–8 of the rLHR was reported to bind hCG with an affinity that is similar to that of the rLHR-wt (183). Lastly, the deletion of exon 10 from the hLHR was also reported to have no effect on hCG binding affinity (91). Thus, the bulk of the evidence indicates that the region encoded by exons 9 and 10 (*i.e.*, most of LRR8 all of LRR9 and the hinge region) is not necessary for the high affinity binding of hCG to the LHR.

All the binding data summarized in Table 1 were obtained using detergent/glycerol extracts of the transfected cells and their interpretation is, therefore, not confounded by the possibility that these mutants may not be expressed at the cell surface (see above). Also, because binding affinities were measured in all cases, these data are also not affected by potential differences in the levels of expression of the construct analyzed. The only instance in which conclusions can be ambiguous is when there is no detectable binding in extracts of transfected cells. Therefore, we cannot conclude that rLHR (1–32) or an rLHR/rFSHR chimera containing only residues 146–674 of the rLHR are unable to bind hCG (see Table 1) because the expression of these constructs was not independently verified (174, 181). In contrast, the lack of detectable hCG binding to many of the deletion mutants of the full-length rLHR (see Table 1) is not due to lack of expression of these mutants because their expression was independently verified using Western blots (182).

As already summarized above, the involvement of the N-terminal cysteine-rich region in hCG binding can be deduced from the finding that deletion of residues 1–11 of the rLHR abolishes hCG binding (Table 1 and Ref. 182). This conclusion is also supported by the finding that a synthetic peptide corresponding to residues 21–38 inhibits hCG binding to rat luteal membranes (184) and the finding that a synthetic peptide corresponding to residues 18–36 of the rLHR can be cross-linked to hCG (185). With the exception of mutation of individual cysteine residues (186), the effects of other point mutations of this region on hCG binding have not been examined. The individual mutation of each of the four cysteines present in this region of the rLHR were found to abolish hCG binding in detergent/glycerol extracts of transfected cells (186). Because the expression of the mutants was not independently confirmed, it is not known whether these residues participate in hCG binding or whether receptor expression was abolished (186).

The effects of discrete point mutations of the LRR region on hCG binding affinity have been examined by several investigators (39, 40, 186, 187), and a summary of their data is presented in Fig. 3. Individual mutation of the residues shown in *green* has some (2- to 4-fold reduction) or no effect on hCG binding affinity. Those shown in *red* abolish binding with little or no effect on receptor expression. Those shown in *orange* also abolish binding, but because the expression of the mutants was not independently confirmed it is not known whether the mutated residues participate in hCG binding or whether receptor expression was abolished. The two most striking features of these results are 1) mutation of the two cysteines present in this region (in LRR-3 and LRR-4) have no effect on hCG binding (186); and 2) most mutations that abolish hCG binding are located in the region of the individual LRRs that form the β-strand (39, 40, 186, 187). The location of these residues is interesting because the β-strands form the inner lining of the putative horseshoe-like structure formed by the extracellular domain of the LHR (these are represented by the *green arrows* in Fig. 4B).

Mutations of a number of residues present in the hinge region of the hLHR or rLHR, including the six cysteine residues (*cf.* Fig. 1), have been found to provoke a small reduction (*i.e.*, no more than 2-fold) or to have no adverse effect on hCG binding affinity (122, 123, 186). Because the hinge region is encoded for by the 3′ end of exon 9, all of exon 10 and the 5′ end of exon 11 (*cf.* Fig. 1), these results are in agreement with the finding that removal of exons 9–11 has no effect on hCG binding (Table 1 and Refs. 91, 174, and 182).

In summary, all investigators agree that the N-terminal extracellular domain of the LHR (*i.e.*, exons 1–10) represents the main binding site for hCG, and most investigators agree that exons 9 and 10 are not necessary for binding. This reduces the hormone binding site to roughly the N-terminal 210 residues (LRRs 1–8) of the extracellular domain (*cf.* Figs. 1 and 3). As mentioned above, the extracellular domain of the LHR is thought to form a horseshoe-like structure with the β-strands and the α-helices of the individual LRRs forming the inner and outer linings of the horseshoe, respectively (see *arrows* and *helices* in Fig. 4B). Modeling of the extracellular domain of the LHR with the known threedimensional structure of hCG (47) has led to the proposal that the binding of hCG to the LHR occurs at the inner lining of the horseshoe and the primary binding site involves intermolecular interactions between hCG and the residues present in the β-strands of the individual LRRs (38, 40, 45, 46). The results of mutations of individual LRR residues (summarized in Fig. 3) clearly support this model.

Although it is clear that the N-terminal extracellular domain of the LHR represents the primary hormone binding site, there is some evidence that suggests that other portions of the receptor participate in, or otherwise influence, hormone binding to the extracellular domain. One of the first reports to raise this possibility came from Ji's group (188), which reported that intact cells transfected with rLHR constructs in which residues 6–297 or 6–336 were deleted could bind hCG with very low affinity ($\sim$1 μM). These data were interpreted to mean that the serpentine domain of the rLHR contains a secondary low affinity binding site (188), but the data are not conclusive for two reasons. First, there was no independent assessment of either the expression or the surface localization of the transfected constructs. Second, these constructs also encoded for 5–32 residues of the N-terminal extracellular domain, and it is thus possible that these residues (as opposed to those present in the serpentine domain) were responsible for the low affinity binding reported. Other experiments from Ji's group (189) have suggested that detergent/glycerol extracts of cells expressing a truncated form of the rLHR encoding for only the extracellular domain of the rLHR (residues 1–338) bind hCG with a somewhat higher affinity ($\geq$2-fold) than detergent/glycerol extracts of cells expressing the full-length rLHR. This finding led to the suggestion that the serpentine domain has a slight inhibitory effect on the binding of hCG to the extracellular domain of the LHR. Alanine scanning mutagenesis of the second EL of

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the rLHR did in fact show that, in the context of the full-length receptor, the individual mutation of many of the residues present in this loop either enhances or decreases the binding affinity of hCG. The magnitude of these effects was rather small (≤2-fold), however. In another study involving alanine scanning mutagenesis of the second EL of the rLHR and the hLHR, one of our laboratories (20) examined the binding affinity of hCG to constructs of the hLHR in which four selected residues of this loop were individually mutated to alanine. One of the four mutants induced a 3- to 4-fold reduction in hCG binding affinity. Other groups (176), including one of our own (177), have failed to detect differences in the binding affinity of hCG or hLH to the full-length rLHR or its extracellular domain expressed alone (177) or as a fusion protein with the TM domain of CD8 (176). One of our groups has also concluded that the deletion of portions of the three ELs have no effect on the binding affinity of the rLHR for hCG (23). In a study from one of our laboratories (177), we did notice, however, that the binding of oLH to detergent/glycerol extracts of cells transfected with the extracellular domain of the rLHR (*i.e.*, rLHR-t338, see Table 1) is different from the binding of oLH to the full-length rLHR. Thus, whereas the full-length receptor-bound oLH with two apparent affinities, rLHR-t338 bound oLH with a single affinity, which was intermediate between the high and low affinities of the full-length rLHR. These data were originally interpreted as being consistent with the notion that the serpentine region of the rLHR contributes to a second low affinity hormone binding site (177). More recent studies, however, show that the lack of high affinity binding of oLH to rLHR-t338 is not unique to this mutant but is common to several other mutants that, like rLHR-t338, are retained intracellularly and are not processed into the mature cell-surface receptor (23). Because there is little or no difference in the binding affinities of hCG for the cell surface and the intracellular rLHR precursors (see *Section II.A* and Ref. 21), the intracellular retention of the extracellular domain of the rLHR could not account for the reported differences in the binding affinity of hCG for this construct and the full-length rLHR. Lastly, there are two studies (190, 191) that have examined the effects of mutating a highly conserved aspartate present in the TM2 of the rLHR (D^{383}, *shaded pink* in Fig. 1) on hCG binding affinity. Ji's group (190) reported that the mutation of this residue (to asparagine) resulted in a 200-fold decrease in the binding affinity of the rLHR for hCG, whereas one of our groups (191) reported that the same mutation had no effect on hCG binding affinity. We did report, however, that a previously documented (192) Na^+-induced increase in the binding affinity of oLH for the rLHR was completely abolished by the D383N mutation (191).

In summary then, although there is some evidence that suggests that either the ELs and/or the TM helices of the LHR affect the binding of LH or hCG to its extracellular domain, there is no agreement on how this effect may be brought about and on which regions of the receptor are involved.

VI. Activation of the LHR

The revised ternary model for GPCR activation postulates that a given GPCR exists in the plasma membrane in an unliganded inactive state (R_o) that is in equilibrium with an active (R*) state (reviewed in Ref. 4). The inactive (R_o) state is stabilized by ligands that are now referred to as inverse agonist and the active (R*) state is stabilized by ligands that have always been referred to as agonists (4). The multistate revision of this model further postulates that receptor activation involves several different active states that comprise multiple different conformations of the receptor (4). In the absence of ligand, most receptors are in the unliganded inactive (R_o) state, but some can spontaneously assume the activated (R*) state. This aspect of the model accounts for the increased basal activity of an effector system detected in cells expressing a given receptor but incubated without agonist, and the observation that this basal activity increases with increasing receptor density (193). This model also accommodates the observations that GPCRs may be activated not only by agonists, but also by certain mutations that induce receptor activation in the absence of agonist stimulation. Thus, it is now thought that agonist-induced and mutation-induced receptor activation stabilize the active (R*) state of GPCRs. Although certain key structural features of GPCRs would be postulated to assume similar conformations in the agonist-activated R* states and constitutively active R* states (as well as among R* states stabilized by different agonists and/or among constitutively active R* states stabilized by different mutations), the exact conformations of the multiple R* states that are thought to exist will not necessarily be identical.

As with other GPCRs, the regions of the LHR that are thought to interact with and activate G proteins are presumably located in the four ILs (see Fig. 1) and the juxtacytoplasmic regions of the TM helices. Although the precise areas of the LHR that activate Gs (or other G proteins, see *Section IV*) have not been mapped, there are data to suggest that the carboxyl-terminal region of IL3 (see Fig. 1) is involved in G protein activation. Thus, it has been shown that some (but not all) synthetic peptides derived from the TM helices and/or ILs of the LHR can directly activate Gs in isolated membranes (194). For example, synthetic peptides corresponding to TM1 or TM3 cannot activate Gs, but synthetic peptides corresponding to the juxtacytoplasmic regions of TM4, TM5, TM6, and TM7 were active in this assay. More importantly, a peptide corresponding to the carboxyl portion of IL3 plus the juxtacytoplasmic portion of TM6 is more effective in activating Gs than a peptide corresponding to only the juxtacytoplasmic region of TM6 (194). The importance of IL3, as well as TM5/TM6 interactions in LHR-mediated G protein activation, has also been documented by other laboratories using site-directed mutagenesis of these regions, the context of the full-length LHR (168), or in the context of chimeras of the LHR and the FSHR (195). All of these findings are in general agreement with the view that IL3 is involved in the activation of G proteins by other GPCRs (reviewed in Refs. 4, 5, and 196). For the β_2-adrenergic receptor, it had been suggested that the Gs-activating properties of the carboxyl-terminal region of IL3 may be dependent upon its ability to form an amphiphilic helix (197–200). This does not appear to be the case for the LHR, however, because mutations that would abolish the ability of this portion of the rLHR to assume an amphiphilic structure do not diminish its signal-

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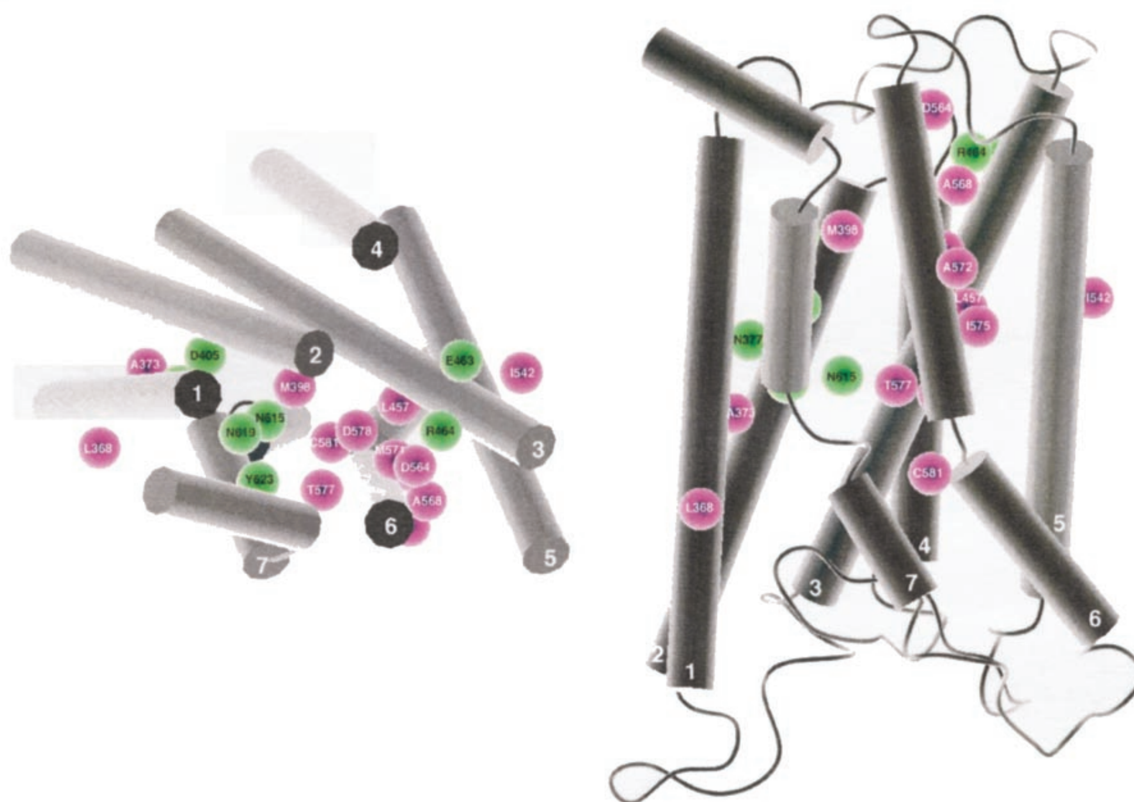

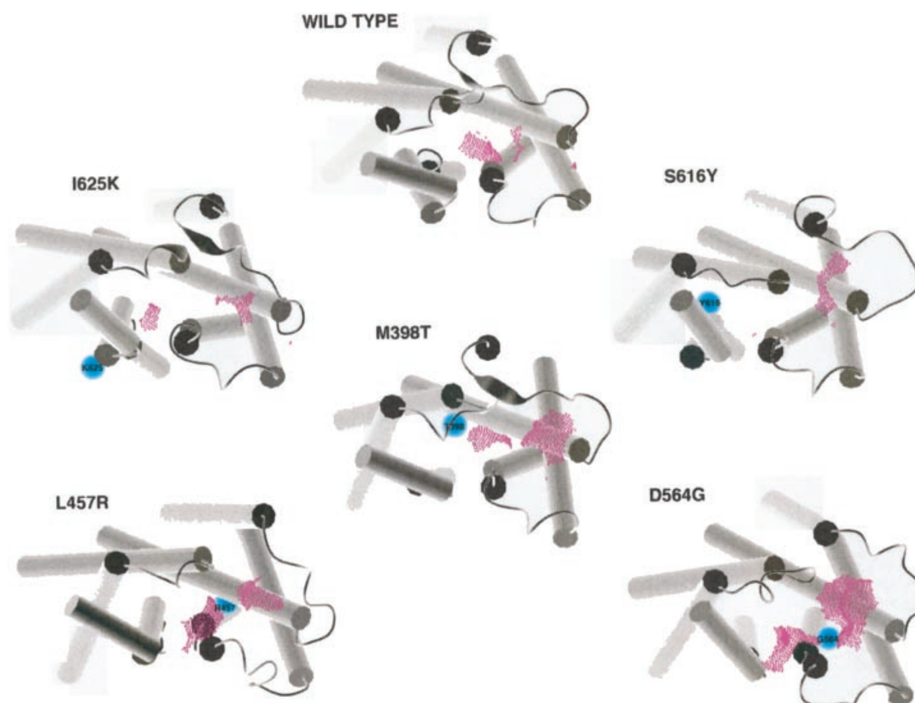

FIG. 7. Rhodopsin-based homology model of the TM region of the hLHR-wt and mutants thereof. A, The TM regions of the hLHR as viewed from the cytosol (*left*) or the plane of the membrane (*right*) are shown. The figure on the *right* is presented with the cytoplasm at the *top* and extracellular space at the *bottom*, similar to structural representations of rhodopsin. The helical segments shown by the cylinders and labeled

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ing properties (201). Lastly, it should also be stressed that IL3 is not necessarily the only region of GPCRs that is involved in G protein activation. For example, rhodopsin peptides corresponding to IL2 (the IL that connects TM3 with TM4) and IL3 (the IL that connects TM5 with TM6), but not IL1 (the IL that connects TM1 with TM2) can bind and/or activate transducin (202). Likewise, docking simulations between constitutively active mutants of the α_{1b}-adrenergic receptor and several G proteins suggest that IL2 and IL3, but not IL1, play a role in G protein recognition (203).

Our understanding of the mechanisms involved in the activation of the LHR has been greatly facilitated by the discovery of mutations that cause constitutive activation of this receptor (see Fig. 6) and by recent advances in molecular modeling. Until recently, no high-resolution structure of a GPCR was available, and thus, *ab initio* approaches were employed for building static (59) and dynamic (60) models of the hLHR TM helices and interconnecting loops. In a study done by one of our groups (60), comparisons were made between the unliganded inactive hLHR, two naturally occurring inactivating hLHR mutations, and several naturally occurring constitutively active hLHR mutants. These comparisons allowed for the determination of common structural rearrangements that accompanied the constitutive activation of the hLHR by different mutations (60). Since then, Palczewski *et al.* (63) have published the crystal structure of visual rhodopsin determined at 2.8-Å resolution. Taking advantage of this major advance in the field, another model for the hLHR (henceforth referred to as the rhodopsin-based hLHR model) has been recently built using rhodopsin as a template (Ref. 64 and F. Fanelli, submitted manuscript). The average arrangements of the seven TM helices of the unliganded inactive (R_0) state of the hLHR, as predicted by the rhodopsin-based hLHR model, are shown in Fig. 7A. The figure depicts the predicted overall structure as observed from the intracellular side (*left panel*) as well as that observed in the plane of the membrane (*right panel*). In the *right panel*, the cytoplasmic face of the membrane is shown at the *top* to keep the orientation constant relative to that of rhodopsin.

Differences between the rhodopsin-based model and the earlier *ab initio* model of the hLHR (60) are essentially restricted to the environment of the receptor sites susceptible to activating mutations, as well as the environment of a highly conserved arginine residue present in the IL2 of many GPCRs (R^{464}in the hLHR, *highlighted in pink* in Figs. 1 and 7A) and other polar conserved amino acids (also *highlighted in pink* in Figs. 1 and 7A). Despite these differences, both models support the hypothesis that the hLHR sites that are susceptible to activating mutations (*green circles* in Fig. 7A) are in interhelical positions close to amino acids that are highly conserved among the rhodopsin/β_2-adrenergic receptor subfamily and appear to be involved in signaling (*pink circles* in Fig. 7A and *pink highlighting* in Fig. 1). The importance of some of these highly conserved polar residues on LHR-mediated signaling has already been documented by mutagenesis. For example, mutation of hLHR residue D^{405} (or the corresponding rLHR residue D^{383}) in TM2 to Asn has been shown to impair signaling (19, 191, 204). Mutation of rLHR E^{441} (equivalent to hLHR E^{463}) to Gln reduced cell-surface expression, and its impact on signaling remains difficult to interpret because this property could not be examined in cells expressing an equivalent density of rLHR-wt (205). Mutation of rLHR R^{442} (equivalent to hLHR R^{464}) to His also reduced cell-surface expression and almost completely abolished signaling when compared with cells expressing an equivalent density of rLHR-wt (204), and mutation of Y^{601} to Ala in TM7 of the rLHR (equivalent to Y^{623} in the hLHR) has also been shown to impair signaling (206).

Like the *ab initio* model, the homology-based model also suggests that interactions between TM3 and TM6, TM3 and TM7, and TM6 and TM7 play an essential role in stabilizing the structure of the inactive hLHR because the majority of the residues that are susceptible to activating mutations are in or close to these interfaces (*green circles* in Fig. 7A). The importance of these interhelical interactions in stabilizing the structure of the inactive LHR has also been suggested by mutagenesis studies of selected residues present in TM6 and TM7 of the rLHR (206, 207) or the hLHR (195).

Figure 7B shows the average minimized structures (viewed from the cytoplasmic side) of several constitutively active mutants of the hLHR (D564G, M398T, L457R, and D578G), one inactivating mutation (I625K), and the unliganded wt hLHR. A comparison of these predicted structures suggests that activating mutations are able to induce structural modifications at the interfaces between the cytosolic extensions of TM3 and TM6 (60, 64). One of the structural modifications that occurs with constitutive activation is a change in the interaction patterns involving R^{464}, the highly conserved residue present in the E/D-R-Y/W motif present in the TM3/IL2 interface (*highlighted in pink* in Figs. 1 and 7A). In the inactive structures, R^{464} is predicted to be involved in a double salt bridge with both an adjacent E^{463} as well as with D^{564} in the cytosolic extension of TM6, a residue that results in constitutive activation when mutated (107, 118, 159, 168). A similar interaction pattern is found in the high resolution crystal structure of rhodopsin (63) between R^{135} (homologous to R^{464} in the hLHR) and both the adjacent E^{134} (homologous to E^{463} in the hLHR) and E^{247} (homologous to D^{564} in the hLHR). In the constitutively active hLHR mutants, R^{464} is predicted to lose its interactions with one or both of these anionic amino acids, thus becoming more exposed to the solvent. Thus, the homology model suggests that the R^{464}-D^{564} salt bridge contributes to the stabilization of the inactive state of the hLHR, linking the cytosolic extension of

1–7 correspond to the helices delineated by the *blue boxes* in Fig. 1. The *pink residues* are polar amino acids that are highly conserved in the rhodopsin/β_2-adrenergic receptor subfamily of GPCRs and correspond to the *pink residues* shown in Fig. 1. The *green residues* are those that result in constitutive activation when mutated and correspond to the *green residues* shown in Fig. 6. B, The TM regions of the hLHR as viewed from the cytosol. The helical segments shown by the cylinders and labeled 1–7 correspond to the helices delineated by the *blue boxes* in Fig. 1. In the models for the loss-of-function (I625K) and gain-of-function mutants (M398T, L457R, D564G, and D578G), the position of the mutated residue is indicated by a *blue sphere*. The *pink shading* depicts the solvent-accessible surface computed over amino acids R464, T467, and I468 in TM3 and K563 in TM6, as discussed in the text and in Refs. 60 and 64.

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TM3 and TM6. This hypothesis is consistent with the finding that substitution of D^{564} with Gly, Ala, Val, Leu, Phe, Lys, orAsn, mutations that would break or weaken the interaction with R^{464}, result in constitutive activation, whereas substitution of D^{564} with Glu, which is not predicted to weaken this interaction, does not lead to constitutive activation (159, 168).

The destabilization or breakage of the interactions involving R^{464} that accompanies activation of the hLHR is predicted to result in an increase in the solvent accessibility of the cytosolic extensions of TM3 and TM6 (indicated by the *pink dots* in Fig. 7B). This can be best demonstrated by determining a composite solvent-accessible surface value, computed over selected residues in the cytosolic extensions of TM6 and TM3 facing the core of the helix bundle (60, 64). Results of such computations reveal that the solvent-accessible surface value for constitutively active mutants is higher than the corresponding value for the hLHR-wt or for inactivating mutations (*pink dots* in Fig. 7B, and Refs. 60 and 64). Thus, when considered together, these results show that structural perturbations caused by different constitutively active mutants of the hLHR (resulting from the loss and/or gain of interactions between the mutated amino acid and its environment) induce a common structural change, including a weakening of the interactions of R^{464} with other residues (see above) and an increase in the solvent accessibility of the cytosolic extensions of TM3 and TM6, as shown by the *pink dots* in Fig. 7B. These results suggest that a structural modification at the interface between TM3 and TM6 is important in receptor activation and/or G protein recognition. This hypothesis is consistent with a number of studies demonstrating that a rearrangement in the relative position of TM3 and TM6 is a fundamental step in GPCR activation (4, 208, 209) and agrees well with the data (see above) implicating the cytosolic ends of TM5 and TM6, as well as IL3, as being involved in G protein activation by the LHR (168, 194, 195, 210).

It is important to note that the first report of a mutation causing constitutive activation of a GPCR showed that substitution of an alanine residue present in the third IL of the α_{1b}-adrenergic receptor with any of the other 19 amino acids resulted in constitutive activation (211). However, many subsequent studies performed with several other GPCRs (212–215), including the LHR (64, 157, 159), have failed to show such lack of selectivity in residues that can confer constitutive activity. For example, substitution of D^{564} (in IL3 hLHR) with Gly, Ala, Val, Leu, Phe, Lys, or Asn results in constitutive activation, whereas substitution with Glu does not (159, 168). Likewise, substitution of D^{578} (in TM6 of the hLHR) with Glu, Ser, Leu, Tyr, or Phe results in constitutive activation, whereas substitution with Asn does not (157). Lastly, recent studies from one of our groups show that a positively charged residue is required for the substitution of L^{457} (in TM3) for the hLHR to be constitutively active (64). Consistent with the experimental data, computer simulations suggest that a salt bridge formed between the cationic amino acid in position 457 in TM3, and the anionic D^{578} in TM6 is primarily responsible for stabilizing the active states of hLHR mutants in which L^{457} has been mutated to Lys, Arg, or His (64). These observations suggest that constitutive activity of the hLHR in particular (and GPCRs in general) can arise not only by the disruption of interhelical bonds stabilizing the inactive state but also by the formation of new bonds that can stabilize an active state of the receptor.

It should be clear from the foregoing discussion that the study of constitutively active mutants of the LHR has provided us with a fertile ground for the identification of regions of the LHR that may be involved in G protein activation. Although the conclusions made so far must still be considered tentative, they will continue to be tested and will eventually be excluded or shown to be correct. An important aspect of LHR activation that is still poorly understood, however, is how the binding of LH/CG to the extracellular domain of the LHR leads to the presumed rearrangement of the serpentine domain that culminates in G protein activation. Recent mutagenesis studies from several laboratories have implicated specific residues of the N- and C-terminal ends of the hinge region of the extracellular domain of the LHR (122, 123, 216) or in the third EL (217, 218) as being dispensable for hCG binding but critical for receptor activation. Studies done with the rLHR showed that individual mutations of a Pro, Glu, or Asp flanking the last Cys residue present in the C-terminal end of the hinge region (*cf.* Fig. 1) impair hCG-induced activation without affecting receptor expression or hCG binding (216), whereas studies done with the hLHR show that mutation of a Ser located two residues upstream of the first Cys residue present at the N-terminal end of the hinge region (*cf.* Fig. 1) can result in constitutive activation of this receptor without affecting receptor expression or hCG binding (122, 123). These results are particularly interesting in view of the fact that these residues are fully conserved among the LHR from different species and among the three glycoprotein hormone receptors (122, 123, 216). Studies done by two different laboratories involving mutagenesis of selected residues present in the three ELs of the rLHR have also implicated a Lys residue present in the EL3/TM6 interface (*cf.* Fig. 1) as being involved in hCG-induced activation without affecting receptor expression or hCG binding (217, 218). Alanine scanning mutagenesis of EL2 in the rLHR (189) and the hLHR (20) have also implicated several residues present in this region as being important for signal transduction. Lastly, a Glu present in the TM2/EL1 interface (*cf.* Fig. 1) was reported to be involved in receptor activation by Ji's group (219). This, however, could not be reproduced by one of our laboratories (179).

Based on these studies and those summarized in *Section V*, one may consider at least three models for the agonist-induced activation of the LHR.

Model 1. LH/CG binds to the extracellular domain of the LHR, and the bound hormone interacts with and activates the serpentine region. This model is appealing in that it takes into consideration the high degree of homology of the three glycoprotein hormone receptors and the four glycoprotein hormones. One can envision a scenario in which the specificity of binding is dictated by the amino acid sequence of the extracellular domain of a given glycoprotein hormone receptor and the amino acid sequence of the β-subunit of the hormone, whereas the activation step is common to all glycoprotein hormone receptors and mediated by specific interactions of conserved amino acids present in the common

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α-subunit of the four glycoprotein hormones and conserved amino acids present in the serpentine region of the three glycoprotein hormone receptors (45, 46, 174, 220). This model is supported by the finding that certain mutations of the hormones (LH or CG) can impair receptor activation without affecting hormone binding (36, 220–222). In agreement with the view that receptor activation may be mediated by the α-subunit of LH/CG, most of the hormone mutations that prevent activation without affecting binding are located in the α-subunit (36, 220–222). This model is also supported by the finding that intact cells transfected with constructs of the rLHR in which residues 6–297 or 6–336 were deleted could respond to extremely high concentrations of hCG with an increase in cAMP accumulation (188). As already mentioned, however (see *Section V*), it is not known whether these constructs were expressed or properly localized at the cell surface. Moreover, because these constructs also encoded for 5–32 residues of the N-terminal extracellular domain, it is possible that these extracellular residues (as opposed to those present in the serpentine domain) were responsible for the small increases in cAMP accumulation detected at high hormone concentrations.

Model 2. LH/CG binds to the extracellular domain of the LHR, thus allowing the extracellular domain of the LHR to activate the serpentine region. This model is similar to Model 1 except that the extracellular domain of the LHR, instead of the bound hormone, is the activator of the serpentine region. Model 2 is supported by the finding (see above) that discrete mutations of the extracellular domain of the LHR can impair signal transduction without impairing hormone binding (216). This finding implies that some residues of the extracellular domain of the LHR can interact with and activate the serpentine domain. It would also suggest that, in the inactive state, the extracellular domain of the LHR is not as tightly associated with the serpentine region as in the activated state. This hypothesis is supported by the observation that the hLHR-wt loses binding activity more rapidly and to a greater extent than constitutively active mutants when intact cells expressing these constructs are treated with protease (Ref. 27 and D. L. Segaloff, unpublished observations).

Model 3. The extracellular domain of the LHR may hold the serpentine domain in an inactive state, and the binding of LH/CG to the extracellular domain activates the serpentine domain by relaxing this interaction (38, 122, 223). Model 3 is supported by the finding (see above) that discrete mutations of the extracellular domain of the LHR can induce constitutive activation (122, 123). A prediction from this model is that expression of the serpentine and C-terminal regions of the LHR would result in constitutive signaling. Such a result has not been observed by two different groups of investigators that expressed constructs containing the entire C-terminal and serpentine domains plus a few residues of the extracellular domain of the rLHR (188), or a construct encoding for only the serpentine and C-terminal domains of the hLHR (224). As already mentioned above, a caveat with the interpretation of these experiments is that there was no independent assessment of either the expression or the surface localization of the transfected constructs. In considering this model, it is worth noting that the expression of a construct of the hTSHR lacking the extracellular domain results in a level of constitutive signaling that is much higher than that detected in cells expressing the full-length hTSHR (225). In contrast to the results discussed above with the lack of constitutive activity of truncated forms of the LHR, the data obtained with the TSHR are readily interpretable because the cell-surface expression of the two constructs in question was independently confirmed (225). The data obtained with the TSHR may not be extrapolated to the LHR, however, because there appear to be a number of differences in the mode of activation of the LHR when compared with the TSHR or the FSHR (122, 168, 226).

VII. Regulation of the LHR

A. Transcriptional regulation

The exposure of ovarian or testicular cells expressing the endogenous LHR to a high concentration of hCG or LH (or to a cell-permeable cAMP analog) down-regulates the levels of cell-surface receptor. Concomitant with the down-regulation of cell-surface LHR, a decrease in the abundance of all LHR mRNA transcripts is observed (227–232). In MA-10 mouse Leydig tumor cells, the hCG-induced down-regulation of LHR mRNA was shown to be mediated by cAMP and due to a decrease in the transcription of the LHR gene (79, 233, 234). However, as discussed below (see *Section VII.B*), the hCG-induced decrease in the transcription of the LHR gene was found to be insignificant to the down-regulation of the LHR (235). In rat ovaries, the preovulatory LH surge causes a marked down-regulation of cell-surface LHR and its cognate mRNA, but both of these recover upon luteinization (227, 228, 230). The down-regulation of LHR mRNA that occurs under these conditions is not to due to decreased transcription of the rLHR gene, but rather to increased degradation of rLHR mRNA (236). Menon and colleagues (237, 238) have identified a protein, designated LH receptor binding protein 1, that is present in pseudopregnant rat ovaries and binds to a region of the open reading frame of the rLHR mRNA. It has been postulated, though not yet directly shown, that LH receptor binding protein 1 regulates rLHR mRNA stability.

Transcriptional regulation of the LHR is thought to be crucial to the appropriate cell-specific expression of the LHR. The *cis*- and *trans*-acting elements governing the basal transcription of the rLHR gene have been examined by Dufau and colleagues (82, 239, 240) in murine Leydig tumor MLTC cells and nonexpressing Chinese hamster ovary (CHO) cells and by one of our groups in primary cultures of rat granulosa cells (241). Both groups have reported the promoter activity of the rLHR gene to be within the first 155–176 nt of the 5′-flanking region of the gene (where the numbering of the 5′-flanking region is relative to the start of translation due to the presence of multiple transcriptional start sites). Interestingly, there are regions upstream of the promoter that repress basal transcription of the rLHR gene. In rat granulosa cells, a repressor region was broadly mapped to within the large area between nt −186 and −1375 (241). In MLTC cells, repressor regions were mapped to between nt −173 and −626

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and −626 and −990 (82). An additional repressor region located between nt −2056 and −1237 was found in nonexpressing CHO cells but not in the MLTC cells (82). Within the promoter region of the rLHR gene, there are three Sp1 consensus sequences (designated Sp1a, -b, and -c) for Sp1 proteins, two consensus sequences for steroidogenic factor 1, binding and one for AP-2. EMSAs using extracts from rat granulosa cells show that complexes consisting of Sp1 and Sp3 transcription factors are formed at each of the three Sp1 sites (241). Mutations of any of the three Sp1 sites causes a marked reduction in reporter gene activity in the rat granulosa cells, and the combined mutagenesis of all three sites reduced basal transcription of the reporter gene to less than 10% of control (241). These results suggest that the concerted actions of the three Sp1 sites play a critical role in the basal transcription of the rLHR gene in rat granulosa cells. Although Sp1 sites were also shown to play a key role in the basal transcription of the rLHR gene in MLTC cells (239, 240), some differences between the rat granulosa cells and MLTC cells were observed. Thus, in the MLTC cells, a functional role for the Sp1b site was not observed. Furthermore, in MLTC cells it was found that Sp1 bound not only to the canonical Sp1 binding site at nt −143 to −128, but also to the upstream sequence at nt −151 to −143, and that mutation of this upstream sequence rather than the canonical Sp1 sequence reduced basal promoter activity of a reporter gene. Studies in MLTC and CHO cells further suggested that tissue-specific basal rLHR promoter activity is further regulated by three additional domains, which have been called the R domain (nt −1266 to −1377), C-box (nt −42 to −73), and the M1 domain (nt −24 to −42) (11, 82, 239, 240).

The basal transcriptional activity of the hLHR gene has been examined by Dufau and colleagues in JAR cells and simian virus 40-transformed placental cells (80, 242, 243). Similar to the rLHR, the promoter region of the hLHR appears to be within the first 176 bp of the 5′-flanking region (80). This region of the hLHR gene contains consensus sequences for two Sp1 sites, three AP-2 sites, and one estrogen receptor response element half-site (EREhs). Similar to the rLHR gene, the Sp1 sites of the hLHR gene are also involved in regulating the basal transcription of the gene, and EMSAs suggest the binding of both Sp1 and Sp3 to each of the two sites (243). Reporter gene constructs in which one or both sites were disrupted show that they each contribute to basal hLHR gene transcription (243). Upstream of these two Sp1 sites is the EREhs at nt −171. Using a yeast one-hybrid screen, Zhang and Dufau (242) identified three nuclear orphan receptors (EAR2, EAR3/COUP-TF1, and TR4) that bind to this EREhs. EMSAs were used to show that endogenous EAR2 and EAR3/COUP-TF1 from JAR cells and from human testis and TR4 from human testis bind to the hLHR EREhs (242). Functional analyses suggest that the binding of EAR2 and EAR3/COUP-TF1 to the hLHR EREhs repress, whereas the binding of TR4 to this site stimulates, hLHR gene transcription (242, 244). These studies suggest that the relative abundance of these three nuclear orphan receptors may be significant in determining the basal transcription of the hLHR gene in different cell types.

Ultimately, the identity of promoter regions of the LHR gene required for appropriate gender and tissue distribution must be confirmed *in vivo* by the expression of heterologous genes in transgenic mice. Using this approach, Huhtaniemi and colleagues (245) have constructed three different β-galactosidase fusion genes containing 7.4 kb, 1.2 kb, or 173 bp of the 5′-flanking sequence of the mLHR gene and studied their expression *in vivo* in transgenic mice and *in vitro* in MLTC Leydig tumor cells and KK-1 granulosa tumor cells (245). Interestingly, in the transgenic mice, all three constructs directed β-galactosidase expression to adult Leydig cells, but not to fetal Leydig cells or to ovarian cells. The expression in adult Leydig cells decreased with increasing lengths of the promoter. Similarly, the constructs were robustly expressed in MLTC cells (where the highest expression was with the shortest 173 bp of the 5′-flanking sequence) but minimally expressed in KK-1 cells. These data suggest that basal transcriptional activity of the mLHR promoter lies within the first 173 nt upstream of the coding sequence of the mLHR, a result that is consistent with analysis of the mLHR gene in transfected mouse Leydig tumor cells (246) and the rLHR and hLHR genes in several cell types (see above). The results observed in the transgenic mice, though, further suggest that regulatory regions outside of the 7.4-kb 5′-flanking region of the mLHR are required for directing mLHR expression to fetal Leydig cells and to the ovary.

In addition to directing the basal expression of the LHR gene, transcriptional regulation of the LHR gene is involved in the FSH-dependent induction of LHR in differentiating granulosa cells. During the growth and differentiation of granulosa cells in the developing follicles, there is an estrogen- and FSH-dependent acquisition of LHR. These actions of FSH can be mimicked, at least in part, by agents that increase intracellular levels of cAMP. The increase in LHR binding activity during this process is accompanied by an increase in LHR mRNA (227, 247). Using granulosa cells cultured from estrogen-primed immature rats, Shi and Segaloff (247) demonstrated by nuclear run-on assays that the FSH or cAMP treatment of these cells causes an approximately 10-fold increase in transcription of the endogenous LHR gene. Although these observations do not rule out a potential role for LHR induction also being mediated by increased LHR mRNA stability, they demonstrate that a cAMP-mediated induction of the LHR gene is clearly important in this process. The increased transcription of the LHR gene in response to FSH or cAMP is not observed until 24 or more hours after treatment, and increases in LHR mRNA and hCG binding activity display a similar lag time (247). Because primary cultures of rat granulosa cells transfected with a reporter gene construct containing 2.1 kb of 5′-flanking sequence of the rLHR gene respond to cAMP treatment, this system is amenable for use in identifying *cis*- and *trans*-acting elements mediating the cAMP-dependent induction of the rLHR gene. Cells thusly transfected show incremental increases in the cAMP-mediated fold induction of reporter gene activity as the length of the 5′-flanking region is extended from −40 bp to −2056 bp, suggesting the presence of multiple cAMP-responsive *cis* elements (241). Similarly, EMSAs using probes corresponding to overlapping portions of the 2.1-kb 5′-flanking sequence and extracts from control *vs.* cAMP-treated granulosa cells show the presence of multiple complexes whose intensities are increased

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upon cAMP treatment (241). Some of the elements in the rLHR gene mediating cAMP responsiveness have been identified. These include the three Sp1 sites mentioned above (Sp1a at nt −83, Sp1b at nt −103, and Sp1c at nt −174). These three sites have also been shown to bind both Sp1 and Sp3 and to be involved in the basal transcription of the rLHR gene in primary cultures of rat granulosa cells (241). Individual mutation of any one of the Sp1 sites causes a marked reduction in the 8-Br-cAMP-provoked induction of reporter gene activity, and the simultaneous mutation of all three sites reduces the induction in an additive fashion (241). Two other novel cAMP responsive elements identified thus far are located at nt −142 to −146 and at nt −924 to −933. The site in the proximal promoter region partially overlaps with the Sp1c site and, therefore, was termed an Sp1c adjacent site [SAS (248)]. Although it partially overlaps with the Sp1c site, neither Sp1 nor Sp3 are required for the cAMP-inducible complex that forms at the SAS site. Mutations that disrupt the nuclear protein/DNA complex formation at the SAS site without disturbing those at the Sp1c site reduce the cAMP-inducible reporter gene activity approximately 2-fold. The cAMP-inducible element at the distal site was found to have a somewhat different consensus sequence than the SAS site, but to bind the same nuclear protein(s). Therefore, it was termed an SAS-like element (248). The importance of this site in mediating cAMP responsiveness is underscored by the finding that its mutation abolishes cAMP responsiveness when a 2-kb fragment of 5′-flanking region of the rLHR is used to drive the expression of a reporter gene (248). The identity of the factor(s) binding to the SAS and SAS-like sites has not yet been determined. However, preliminary evidence suggests that it may be related to a family of G-string-related transcription factors (248).

In summary, although considerable progress has been made in our understanding of the mechanisms underlying both basal and hormonal regulation of the transcription of the LHR gene in recent years, much more needs to be done to piece together the role of the many elements and factors that govern the cell-specific expression and hormonal regulation of the LHR mRNA.

B. Posttranscriptional regulation

Desensitization is a word that is used to describe the ability of target cells to turn off an agonist response in the face of continuous agonist exposure. Desensitization is an important component of the regulation of hormone actions and it can occur at multiple levels. For example, experiments done 20–25 yr ago demonstrated the existence of two steps in the steroidogenic pathway that contribute to the desensitization of steroidogenic responses observed in male rats injected with LH/CG or in freshly isolated rat or mouse Leydig cells or cultured Leydig tumor cells exposed to LH/CG. These include a reduction in the activity and/or levels of 17α-hydroxylase/17,20-lyase and a reduction in the amount of cholesterol available for steroidogenesis (reviewed in Refs. 249–251). Additional studies performed using a variety of gonadal cells in culture or subcellular fractions thereof have defined two additional regulatory events that occur at the level of the LHR that may contribute to desensitization. One of them, henceforth referred to as uncoupling, is relatively fast and is caused by the impairment in the ability of the LHR to interact with and activate its cognate G proteins. The other, henceforth referred to as down-regulation, is relatively slow and is due to a reduction in the density of cell-surface receptors. As already discussed above (*Section VII.A*), changes in the levels of cell-surface receptors can be brought about by changes in transcription, but as discussed below, posttranscriptional regulation of the density of cell-surface receptors also occurs. It now appears that the LH/CG-induced uncoupling and posttranscriptional down-regulation of the LHR are mediated by a family of GPCR-binding proteins known as the nonvisual or β-arrestins. There are two members of this family, referred as β-arrestin-1 (also known as arrestin-2) and β-arrestin-2 (also known as arrestin-3).

The large number of studies conducted with the adrenergic receptors and other GPCRs have delineated the following pathway leading to the formation a GPCR/β-arrestin complex (reviewed in Refs 65–67, 172, 252, and 253). Shortly after G protein activation, GPCRs are phosphorylated in serine and/or threonine residues that are usually, but not always, located in the C-terminal tail. This phosphorylation event, which is catalyzed by a family of serine/threonine kinases known as GRKs, enhances the affinity of the GPCRs for the β-arrestins, thus leading to the recruitment of the β-arrestins from the cytosol to the plasma membrane and the formation of a stoichiometric GPCR/β-arrestin complex. The GPCR/β-arrestin complex is a common molecular intermediate in at least three events that modulate GPCR functions. First, it uncouples the GPCRs from their cognate G proteins by preventing G protein binding (254). Second, it may serve as a scaffold for the attraction and activation of other signaling components [such as Src and members of the MAPK cascade (255, 256)]. Third, it targets the GPCRs to clathrin-coated pits for subsequent internalization (172, 253). Two of these events, the β-arrestin-dependent uncoupling and internalization, seem to be involved in the posttranscriptional regulation of the LHR.

Although all investigators agree that the LH/CG-induced uncoupling of the LHR is mediated by the β-arrestins, there is a fair amount of controversy on whether the LHR undergoes phosphorylation in response to agonist stimulation and on the involvement of LHR phosphorylation in β-arrestin binding and uncoupling. In the model proposed by Hunzicker-Dunn and colleagues (257, 258), the LH/CG-induced uncoupling of the endogenous LHR present in porcine follicular cells is GTP- and β-arrestin-dependent, but is independent of the phosphorylation of the LHR. A lack of involvement of LHR phosphorylation and a need for GTP is supported by their inability to detect LH/CG-induced phosphorylation of the endogenous LHR in porcine follicular membranes (69), the lack of effect of protein kinase inhibitors on LH/CG-induced uncoupling (259), the failure of a nonhydrolyzable ATP analog (AMP-PNP) to block LH/CG-induced uncoupling (69, 260, 261), and the need for GTP analogs to induce optimal desensitization (260–262). In more recent experiments, they have been able to show that the LH/CG-induced uncoupling of the porcine LHR can be enhanced by addition of purified β-arrestins or inhibited by addition of an antibody to the β-arrestins (263). They were

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also able to show that addition of a fragment of the LHR that can bind the β-arrestins (*i.e.*, the third IL) to porcine follicular membranes inhibits LH/CG-induced uncoupling (264). Further studies from Hunzicker-Dunn's laboratory (257, 258, 265) that examined the molecular basis of the GTP dependence of the LH/CG-induced uncoupling implicated ADP ribosylation factor 6 (Arf6, a small GTPase) and Arf-nucleotide binding site opener (ARNO, its guanine nucleotide exchange factor) in LH/CG-induced uncoupling. In their most current model, the LHR is proposed to directly activate ARNO, which in turn activates Arf6 by promoting GTP/GDP exchange. The activated Arf6 then promotes the release of β-arrestin from a membrane docking site, thus allowing it to bind to the unphosphorylated LHR and to promote uncoupling (257, 258, 265). In a recent publication, however, Lefkowitz and colleagues (266) reported that ARNO can be found in a complex with β-arrestin, thus suggesting the presence of an alternative pathway whereby ARNO binds to the LHR using the LHR-bound β-arrestin as an adaptor.

Studies from one of our laboratories have shown that, when expressed in 293 cells, the rLHR (68) and the hLHR (19) become quickly phosphorylated upon addition of hCG. The phosphorylation sites have been mapped to the four (rLHR) or five (hLHR) serine residues present in the C-terminal tail (these residues are depicted in *blue* in Fig. 1). The importance of the phosphorylation of these residues in the agonist-induced uncoupling of the rLHR expressed in 293 cells is supported by a number of mutagenesis studies. Thus, the removal of the four phosphorylated serines (by truncation of the C-terminal tail, see Refs. 70, 71, and 267), their simultaneous mutation to alanine residues (72), or the individual mutation of two of them (S^{635} or S^{639}, see Ref. 73) retards the time course of LH/CG-induced uncoupling but does not affect the magnitude of this effect detected at long time points. A role for rLHR phosphorylation in the process of uncoupling is also supported by the finding that ATP (not GTP) is required for optimal uncoupling in membranes prepared from 293 cells transfected with the rLHR (268).

Because both groups of investigators agree that the uncoupling of the LHR is mediated by the formation of a β-arrestin/LHR complex, the conflicting data summarized above can be basically reduced to the question of whether phosphorylation of the LHR is necessary for β-arrestin binding. Unfortunately, this issue has not been directly examined with either of the two species of LHR used in the studies summarized above (*i.e.*, the rLHR or the pLHR). With the rLHR, it is known, however, that another β-arrestin-dependent event (*i.e.*, internalization) is also impaired by the simultaneous mutation of all four phosphorylation sites (72) or by the individual mutation of three of them (S^{635}, S^{639}, or S^{649}, see Ref. 73). With the pLHR, it is known that β-arrestin can bind to an unphosphorylated synthetic peptide corresponding to the third IL (264), but this finding does not preclude the likely possibility that other receptor regions could also participate in β-arrestin binding. Although the low levels of expression of the rLHR in transfected cells (*cf.* Fig. 2) make it difficult to examine its interaction with the β-arrestins, the high levels of expression of the hLHR in transfected cells (*cf.* Fig. 2) facilitate measurements of this interaction (269). The recent analysis of a number of hLHR mutants that impair hLHR activation, phosphorylation, or both have in fact shown that the formation of a complex between the hLHR and β-arrestin-2 is dependent mostly on receptor activation rather than on receptor phosphorylation (269). Although it would be tempting to extrapolate this finding to conclude that phosphorylation of the rLHR or the pLHR is not needed for their interaction with the β-arrestins, this extrapolation does not seem warranted in view of recent evidence showing that some of the functional properties of the LHR show substantial interspecies variation. For example, the K_d for hCG binding is about one order of magnitude lower for the rLHR than for the hLHR (*cf.* Fig. 2), the half-time of internalization of hCG mediated by the rLHR is approximately 10 times longer than that mediated by the hLHR (Fig. 8 and Ref. 270); and the internalized hCG-hLHR complex is routed to a recycling pathway, whereas the internalized hCG-rLHR complex is routed to a degradation pathway (Fig. 9 and Ref. 271). Because the two models presented above have been derived from studies conducted using the LHR from two different species (*i.e.*, porcine or rat), it is entirely possible that they are both correct. Thus, the binding of β-arrestins to the pLHR (and subsequent desensitization) may be fairly independent of receptor phosphorylation, whereas the binding of β-arrestins to the rLHR (and subsequent desensitization) may be more dependent on receptor phosphorylation. It is also possible that the differences observed are due to the use of isolated membranes by Hunzicker-Dunn and colleagues (257, 258, 265) and intact cells by one of our groups (19, 68). These issues will not be resolved until the requirements for β-arrestin binding to the rLHR and pLHR are fully understood and/or all experiments are done using the same experimental paradigm. Lastly, studies on the involvement of ARNO and Arf6 in the desensitization of the LHR have been done only using porcine follicular membranes, and the generality of this model needs to be tested in other systems. As already mentioned above, however, a recent paper (266) suggests that ARNO and the β-arrestins form a complex, thus

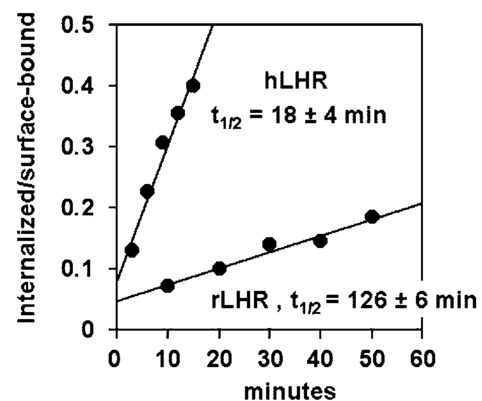

FIG. 8. Rates of internalization of hCG mediated by the rLHR and the hLHR in transiently transfected 293 cells. The 293 cells transiently transfected with the rLHR or hLHR were incubated with ^{125}I-hCG at 37 C for the times indicated. The amount of surface-bound and internalized hormone were measured, and the ratio of internalized/surface ligand *vs.* time was plotted. The *straight lines* shown were obtained using a linear least-square fit of the data points shown. The half-times of internalization were calculated from the slopes of these lines as described elsewhere (277, 291–293).

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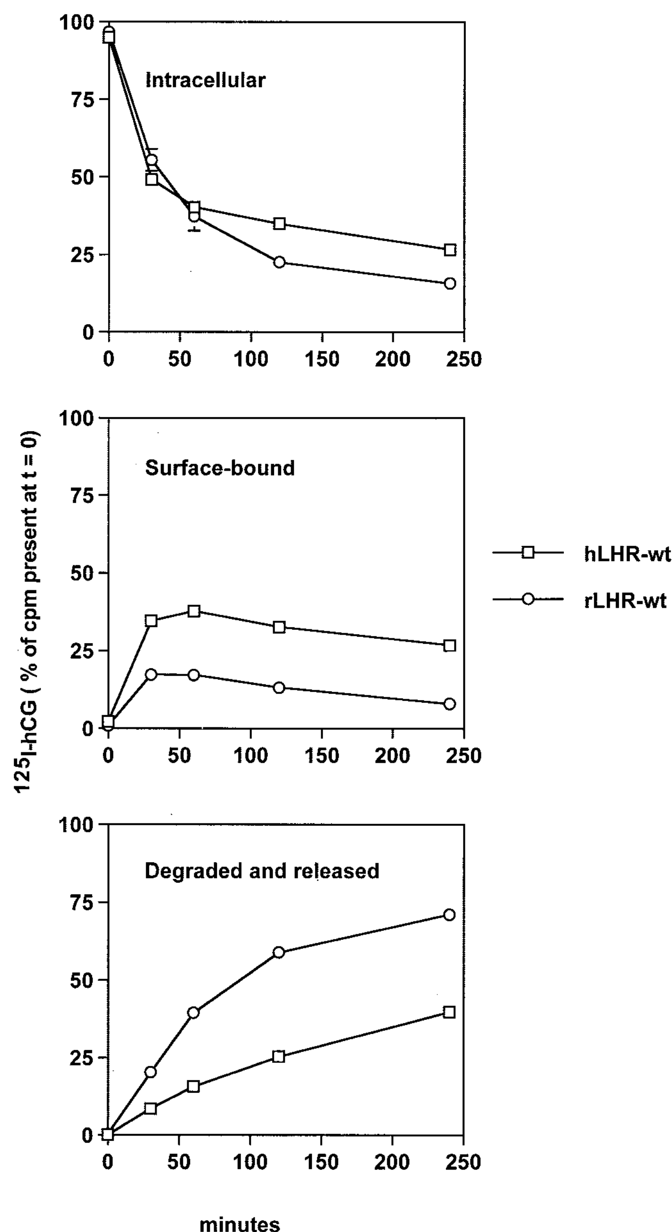

FIG. 9. Fate of the internalized hCG-rLHR and hCG-hLHR complexes. The 293 cells transiently transfected with the rLHR or hLHR were incubated with ^{125}I-hCG for 2 h at 37 C. After washing to remove the free hormone, the surface-bound hormone was released by a brief exposure of the cells to an isotonic pH 3 buffer and the cells were placed in hormone-free medium at 37 C (t = 0 in the figure). At the times indicated, the medium was removed and used to determine the amount of degraded and undegraded hormone released. The cells were washed with cold medium and they were briefly exposed again to the isotonic pH 3 buffer, thus releasing any internalized hormone that had recycled back to the surface. The acid-stripped cells were solubilized with NaOH to determine the amount of radioactivity that remained cell associated. The radioactivity that remained associated with the cells after the acid elution (*i.e.*, internalized hormone) is shown in the *top panel*, and the radioactivity released by the acid treatment (*i.e.*, recycled hormone) is shown in the *middle panel*. The *lower panel* shows the degraded and released hormone. Undegraded hormone released is not shown because it represents less than 5% of the initial radioactivity associated with the cells. [Reproduced with permission from M. Kishi *et al.*: *Mol Endocrinol* 15:1624–1635, 2001 (271). © The Endocrine Society.]

providing the molecular basis by which all GPCRs can interact with and activate ARNO.

Previous biochemical and morphological studies utilizing the porcine (272, 273), mouse (274), rat (275), and human LHR (19, 20, 270, 276) have delineated the internalization pathway followed by the agonist-LHR complex. In the absence of agonist, the endogenous LHR present in porcine Leydig cells or the pLHR expressed in transfected cells is randomly distributed on the cell surface, but upon activation by agonist, the agonist-LHR complex clusters in coated pits and is internalized into endosomes (272, 273). The activation of the LHR is essential to internalization as documented by the following findings. First, the internalization of the agonist-bound rLHR, mLHR, or hLHR is faster than that of their free counterparts (19, 27, 277). Second, the internalization of the complex formed between the mLHR and a weak partial agonist is much slower than that of the agonist-mLHR complex (278). Third, inactivating mutations of the rLHR or the hLHR impair the endocytosis of the agonist-receptor complex (19, 20, 27, 204). Last, activating mutations of the rLHR and hLHR enhance basal and/or agonist-stimulated endocytosis (19, 27, 279). These initial steps of internalization require the participation of dynamin as well as that of the β-arrestins, as documented by the ability of dominant negative mutants of these proteins to inhibit the internalization of hCG mediated by either the rLHR or the hLHR (19, 20, 73, 270, 276, 279). The potential involvement of LHR phosphorylation in the process of agonist-induced internalization is not that clear, however. In the case of the rLHR, the simultaneous mutation of the four phosphorylated serines present in the C-terminal tail (*cf.* Fig. 1) or the individual mutation of three of them (S^{635}, S^{639}, or S^{649}) slows down internalization (72, 73), whereas in the case of the hLHR, the simultaneous mutation of the equivalent residues (*cf.* Fig. 1) has little or no effect on internalization (19). This apparent difference in the importance of phosphorylation may very well be due to differences in the intrinsic rates of internalization of the rLHR and the hLHR, which in turn may be a reflection of the binding affinities of the hLHR and the rLHR for the endogenous β-arrestins. Thus, when expressed in the same cell type (*i.e.*, 293 cells), the rLHR internalizes hCG with a half-time of approximately 120 min, whereas the hLHR internalizes hCG with a half-time of approximately 20 min (Fig. 8 and Ref. 270). One can speculate then that if the hLHR already binds the endogenous β-arrestins with high affinity, the increase in affinity of the hLHR for the β-arrestins that is expected to occur as a consequence of receptor phosphorylation (see above) is likely to be minor and the mutation of the phosphorylation sites would have only a small effect on β-arrestin binding and subsequent internalization. Conversely, if the rLHR binds the endogenous β-arrestins with low affinity, the increase in affinity of the rLHR for the β-arrestins that is expected to occur as a consequence of receptor phosphorylation (see above) is likely to be more pronounced and the mutation of the phosphorylation sites would have a more substantial effect on β-arrestin binding and subsequent internalization. We have, in fact, argued that differences in the rate of internalization of the rLHR and the hLHR, together with the high degree of amino acid sequence identity between the rLHR and the hLHR, can be used to identify the

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amino acid residues of the LHR that participate in the binding of the β-arrestins (270). The analysis of several rLHR/hLHR chimeras and exchange mutants has resulted in the identification of seven residues present in distinct topological domains (see residues *highlighted in yellow* in IL2, IL3, and IL4 in Fig. 1) that are responsible for the difference in the rate of internalization, and we have speculated that these residues participate in the binding of the β-arrestins (270). Interestingly, only one of these residues (a Thr present in slightly different positions in IL3 of either the r or the hLHR, see Fig. 1) is a phosphate acceptor. In the rLHR, this residue is unlikely to be phosphorylated because all the phosphorylation sites present in the rLHR are located in its C-terminal tail and they are all serine residues (70). We cannot make the same conclusion about the hLHR, however, because the location and identity of the phosphorylation sites of the hLHR have not been fully elucidated (19).

In contrast to many other internalized agonist-receptor complexes that dissociate in the acidic environment (pH 5-6) that prevails in endosomes (280), the agonist-LHR complex is rather insensitive to dissociation in this environment (274), and the complex formed by hCG and the mouse, rat, or porcine LHR that is internalized into endosomes is delivered to the lysosomes in the intact form (*i.e.*, without dissociation of the agonist and receptor, see Refs. 272–275). The more acidic environment that prevails in lysosomes, as well as proteolysis, promote the dissociation of the agonist-receptor complex, and both subunits of the agonist are eventually degraded to single amino acids (281). Although the lysosomal degradation of the internalized LHR has not been formally documented, the net result of this pathway is to target the cell-surface LHR to a compartment where it can be degraded (*i.e.*, the lysosomes), and as such, the endocytosis of the agonist-LHR complex is involved in down-regulation of the cell-surface LHR that occurs when gonadal cells are exposed to agonist (249). The relative importance of internalization to the process of down-regulation has been particularly well studied in MA-10 cells (235). Although it is clear that hCG, acting through cAMP as a second messenger, decreases the transcription of the endogenous LHR gene and the levels of LHR mRNA in MA-10 cells (see *Section VII.A* and Refs. 233–235), this effect is quantitatively unimportant to the hCG-induced down-regulation of the LHR detected in this cell type (235).

The importance of LHR internalization to the process of down-regulation was underscored recently by a series of studies comparing these two processes in mouse (MA-10) or rat (R2C) Leydig tumor cells that express the endogenous LHR and in 293 cells expressing the recombinant rLHR (282). Using standardized assay conditions, one of our groups showed that the extent of agonist-induced down-regulation of the LHR is less in 293 cells expressing the recombinant rLHR-wt than in rat or Leydig tumor cells expressing the endogenous LHR. The difference in the extent of agonist-induced down-regulation of the LHR observed in these different cell lines was shown to be due mostly to differences in the rate at which they internalize the agonist-receptor complex (282). Thus, there is a positive correlation between the rate of internalization of the agonist-LHR complex and the extent of down-regulation in three different cell lines (MA-10, R2C, and transfected 293 cells) that express the mouse or rat LHR-wt. Second, all mutations of the rLHR that enhance the rate of internalization of the agonist-rLHR complex in transfected 293 cells also enhance the extent of agonist-induced down-regulation of the rLHR in these cells. Third, three manipulations that enhance the rate of internalization of the agonist-rLHR complex in transfected 293 cells (*i.e.*, cotransfections with GRK2 or β-arrestin-1 or -2) also enhance the extent of down-regulation of the rLHR-wt in these cells. Lastly, cotransfection with a related construct that has little or no effect on internalization (visual arrestin) does not enhance down-regulation. If the rate of internalization is indeed important to the extent of down-regulation, one would predict that manipulations that slow down internalization should also impair down-regulation. This has been found to be the case, however, in only some instances. For example, the slow rate of internalization of a weak partial agonist in MA-10 cells (278) is accompanied by a reduction in the extent of down-regulation (235). In contrast, when using transfected 293 cells, where the rate of internalization of the agonist-receptor complex is already slow compared with that of target cells, mutations of the rLHR that decrease the rate of internalization by less than approximately 2-fold are not accompanied by a reduction in the extent of down-regulation. However, one mutation that decreased the rate of internalization by 3- to 4-fold did abolish down-regulation.

The fate of the internalized LHR is another important determinant of the extent of hCG-induced down-regulation. As already mentioned above, the complex formed by hCG and the rat, mouse, or porcine LHR accumulates in the lysosomes, which is ultimately responsible not only for the degradation of hCG (272, 273, 275, 281, 282) but also for the net loss of cell-surface LHR that ensues after exposure of rodent or porcine target cells or cells expressing the recombinant rodent or porcine LHR to agonists (235, 277, 282). The targeting of these hCG-receptor complexes to the lysosomes is rather unique because, once internalized, most GPCRs are recycled back to the plasma membrane rather than being routed to the lysosomes (65, 172, 283, 284). Only a few other GPCRs (such as the thrombin receptor and the endothelin type A receptor) are routed to the lysosomes and targeted for degradation (285–289). Surprisingly, recent studies from one of our laboratories have shown that the fate of the highly related hLHR is different from that of the rLHR in that a substantial portion of the hCG-hLHR complex is routed to a recycling pathway rather than to a degradation pathway (Fig. 9 and Ref. 271). Although more work needs to be done to fully understand the cell and molecular basis of the differential fate of the internalized hCG-hLHR complex and the hCG-rodent/porcine LHR complex, it is already known that, when grafted onto the C-terminal tail of the rLHR, a short linear sequence present in the C-terminal tail of the hLHR (GTALL, *underlined* in Fig. 1) can reroute the internalized hCG-rLHR complex from the lysosomal degradation pathway to a recycling pathway (271). The removal of the GTALL sequence from the hLHR failed to affect its routing, however (271).

The last decade has led to the deduction of the amino acid sequences of many proteins, and the data obtained clearly support the maxim that structure dictates function. Because

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the LHR is a member of the large family of GPCRs, it is not surprising that some of the posttranscriptional events that regulate the LHR are common to other GPCRs. As summarized above, however, the LHR has some unique properties that set it apart from other GPCRs and justify the study of its posttranscriptional regulation. Two unique features of the LHR that should be considered in this respect are 1) the size and complexity of its two cognate ligands (LH and CG), and 2) the high affinity (*cf.* Fig. 2) of ligand binding to the LHR. Thus, whereas agonist dissociation to other GPCRs is an integral part of the process of desensitization, the high affinity binding of LH and CG to the LHR results in what can be considered to be an irreversible activation of this receptor during the short time frame needed for phosphorylation, β-arrestin binding, desensitization, phosphorylation, and internalization. As such, it is likely that the mechanisms involved in the posttranslational regulation of the LHR have evolved to take this difference into account. Lastly, the remarkable differences in the rates of internalization (Fig. 8 and Ref. 270) and the fates of the internalized rLHR and hLHR (Fig. 9 and Ref. 271) should serve as a warning in extrapolating results obtained with studies done using the pLHR, mLHR, or rLHR to the hLHR.

VIII. Summary and Conclusions

After finishing the writing of this review, we went back and read the previous two reviews on the LHR that we have published in this journal (1, 8). This exercise made us realize how much has been learned about the structure, functions, and regulation of the LHR in the past decade or so. Although many of the results discussed here are conclusive, others remain controversial and their resolution awaits further experimentation. Although current experimental approaches will undoubtedly continue to provide important and useful information about the molecular basis of ligand recognition and receptor activation, the next "quantum leap" in these two areas will take place when the three-dimensional structure of the LHR is revealed. A full characterization of the different signaling pathways activated by the LHR and the molecular basis of the regulation (transcriptional and posttranscriptional) of the LHR will also be needed to better understand normal reproductive physiology as well as the pathophysiology of reproductive disorders. Lastly, much work needs to be done to fully understand the functional significance of the extragonadal expression of the LHR.

Acknowledgments

Address all correspondence and requests for reprints to: Dr. Mario Ascoli, Department of Pharmacology, The University of Iowa, 2-319B BSB, 51 Newton Road, Iowa City, Iowa 52242-1109. E-mail: mario-ascoli@uiowa.edu

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