Variant Forms of the Pig Lutropin/Choriogonadotropin Receptor†

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Received February 19, 1992; Revised Manuscript Received May 29, 1992

ABSTRACT: The cloning and sequencing of porcine lutropin/choriogonadotropin (LH/hCG) receptor messenger RNAs have shown the presence of a full-length receptor (pLHR-A) and of shorter variants lacking either the transmembrane and the intracellular domains (pLHR-B and pLHR-C) or only the transmembrane domain (pLHR-D). Moreover, immunoblotting of testicular membrane extracts has detected 85-, 68-, and 45–48-kDa proteins reacting with antireceptor antibodies. Transfection experiments were performed to assign the protein species to the various messenger RNAs and to study the function of the various receptor species. COS-7 and L-cells transfected with an expression vector encoding full-length receptor pLHR-A yielded a protein of apparent molecular mass of 105 kDa. This corresponded to the complete receptor which had undergone a different glycosylation pattern to that found in testis, since after digestion with peptide *N*-glycosidase F both the 105-kDa COS-7 protein and the 85-kDa testicular glycoprotein yielded a holoprotein of ~63 kDa. Transfection with pLHR-A also yielded a high proportion of the 68-kDa glycoprotein which was shown by digestion with endoglycosidase H to be a high-mannose precursor of the full-length receptor. The existence of a large pool of precursor species in both transfected cells and Leydig cells evokes possible physiological regulations at the level of receptor maturation. Cells transfected with expression vectors encoding pLHR-B, pLHR-C, or pLHR-D yielded 45–48-kDa proteins which bound hormone with an affinity similar to that of the full-length receptor, confirming that there is no apparent contribution of the transmembrane or of the intracellular domains to hormone binding. These variant receptors were in part secreted from the cells and in part present in the cytosol. When they were cotransfected with the full-length receptor, a limited increase (144.4 ± 7.6%; mean ± SEM five determinations) of the stimulation of adenylate cyclase by hormone was observed. Although this suggested the existence of interactions between receptor monomers, no oligomers could be observed in the solubilized testicular receptor.

The structure of the LH/hCG receptor has been the subject of extensive studies and various controversies [see review in Ascoli and Segaloff (1989)] until the cloning of its messenger RNA in the pig and in the rat (Loosfelt et al., 1989; McFarland et al., 1989).

In the pig, the majority of the transcripts (pLHR-A) encoded a protein of 699 amino acids (plus 27 amino acids corresponding to the signal peptide) having a calculated molecular mass of 75 kDa. This protein contained a putative extracellular domain of 333 amino acids preceding a 266 amino acid long transmembrane region (with 7 transmembrane spans) and a 70 amino acid putative intracellular domain. Forty percent of the messenger RNA species corresponded to variants lacking the transmembrane domain. Two (pLHR-B and pLHR-C) were very similar and encoded proteins of 329 and 331 amino acids, lacking both the transmembrane and the intracellular domains. A third variant (pLHR-D) encoded a protein of 384 amino acids lacking only the transmembrane domain but containing the extracellular and the intracellular domains. All these variants arose by alternative splicing (Loosfelt et al., 1989; Tsai-Morris et al., 1991; Koo et al., 1991). In the rat, the initial cloning only detected the full-length receptor (McFarland et al., 1989) but further studies also identified a variant lacking the transmembrane domain (Segaloff et al., 1990a; Tsai-Morris et al., 1990; Bernard et al., 1990).

An alternative approach to the study of the structure and variety of molecular species of the LH receptor has been the use of monoclonal antibodies in immunoblot experiments. In testicular membrane extracts or in immunoaffinity-purified receptors, three bands were observed: one at 85 kDa, another at 68 kDa, and a third wide band probably due to multiple proteins at 45–48 kDa (VuHai-LuuThi et al., 1989). No report has been published of immunoblot studies in other species due perhaps to the lack of adequate antibodies.

The present study was designed to match the different messenger RNA species with the various protein species. Moreover, studies were also performed to try to understand the functional significance of the variant forms of the LH receptor.

MATERIALS AND METHODS

Materials. Pure hCG was obtained from Dr. R. Canfield (NIH, Bethesda) (activity, 13 400 units/mg); ^{125}I-hCG was from Du Pont New England Nuclear (specific radioactivity, 70–90 μCi/μg; biological activity, 50–60%); peptide *N*-glycosidase F (PNGase F; EC 3.2.2.18) and endo-β-*N*-acetylglucosaminidase H (endoglycosidase H or endo H; EC 3.2.1.96) were obtained from Boehringer Mannheim (Mannheim, Germany). Immersible CX-30 was from Millipore (Bedford, MA). G418 (geneticin), Dulbecco's-modified Eagle's medium (DMEM), and fetal bovine serum were from Gibco (Paisley, Great Britain). All other chemicals were of the highest purity commercially available.

† This work was supported by the Insitut National de la Santé et de la Recherche Médicale, the Centre National de la Recherche Scientifique, the Faculté de Medecine Kremlin-Bicêtre, the Association pour la Recherche sur le Cancer, and the Fondation pour la Recherche Médicale.

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0006-2960/92/0431-8377$03.00/0

Construction of an Expression Vector Encoding the Full-Length and the Variant Forms of the LH/hCG Receptor. A clone containing the entire coding region of the LH/hCG receptor and part of the 5'-noncoding region was constructed by ligating an *Eco*RI–*Bgl*II fragment (positions −64 to +428, position +1 being assigned to the initiation codon ATG) to a *Bgl*II–*Bal*I fragment (ending at position +2213, 60 nucleotides after the TAA stop codon). These fragments were isolated from two λgt10 clones (Loosfelt et al., 1989). This insert was subcloned into the Blue Script (Stratagene, La Jolla, CA) vector previously digested by *Eco*RI and *Sma*I.

An expression vector (pCMV-LHR-A) encoding the full-length receptor was obtained by inserting the *Eco*RI–*Bam*HI fragment from the Blue Script vector into the *Hind*III site of $pcDNA_1$ (In Vitrogen, San Diego, CA) downstream from the cytomegalovirus (CMV) promoter. All the restriction sites were blunted before the insertion.

Expression vectors encoding the variant forms of the LH/hCG receptor were obtained by replacing the *Bgl*II–*Eco*RI fragment of the pCMV-LHR-A vector (the *Eco*RI site originating from the $pcDNA_1$ polylinker) by the corresponding shorter fragments of each variant cDNA clone. The inserts were deleted of 253, 732, and 930 bp in the variants encoding pLHR-B, pLHR-C, and pLHR-D, respectively.

Transfection of COS-7 Cells. COS-7 cells were grown in Dulbecco's-modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and were transfected using the calcium phosphate precipitation method (Graham & Van Der Eb, 1973). When only one expression vector was used, the cells were exposed to a precipitate of calcium phosphate formed in the presence of 10 μg of vector DNA and of 10 μg of salmon sperm carrier DNA (Sigma) (1 mL of precipitate per 10^6 cells in each 10-cm dish). In some experiments, the expression vector encoding pLHR-A (2 μg of DNA) was cotransfected with the expression vectors encoding pLHR-C or pLHR-D (8 μg of DNA) (0.5 mL of precipitate was then used per 5×10^5 cells in each 6-cm dish). Controls of the latter experiments involved the replacement either of the expression vector encoding pLHR-A or of that of vectors encoding pLHR-C or pLHR-D by equivalent amounts of carrier DNA (2 and 8 μg, respectively).

L-Cells Expressing Permanently the LH Receptor. The pCMV-LHR-A expression vector (18 μg of DNA) was cotransfected with the pSV-neo, neomycin resistance vector (Southern & Berg, 1982) (2 μg of DNA) (1 mL of precipitate per 10^6 cells in each 10-cm dish) as previously described. Neomycin-resistant cells were selected in DMEM supplemented with 10% fetal bovine serum and G418 (geneticin) (1 mg/mL). The resistant clones were then screened for LH receptor expression by an immunocytochemical test using the antireceptor antibody LHR 38 as described (Meduri et al., 1992). The transfected cells were maintained in the medium containing G418 (200 μg/mL) for further study.

Receptor Distribution between the Culture Medium and the Cellular Cytosol and Membranes. Forty-four hours after transfection of COS-7 cells, the cell culture medium from four 10-cm dishes was collected and concentrated to a volume of 2 mL with immersible CX-30.

The cells were scraped into 2 mL of Hepes [*N*-(2-hydroxyethyl)piperazine-*N'*-[2-ethanesulfonic acid], 25 mM, pH 7.4, buffer containing 100 mM NaCl, 5 mM EDTA, 1.5 mM $MgCl_2$, 5 mM *N*-ethylmaleimide (NEM), 10% glycerol, and protease inhibitors (phenylmethanesulfonyl fluoride, 1 mM; bacitracin, 100 μg/mL; aprotinin, 38 μg/mL; leupeptin, 5 mM; pepstatin, 1 μg/mL). The cells were homogenized with a Dounce apparatus and centrifuged at 14 000 rpm for 30 min at 4° C in an SS 34 Sorvall rotor. The supernatant (cytosol) was saved.

The pellet was extracted in 2 mL of the same buffer containing Triton X-100 (1.2%), and after centrifugation at 105000*g* for 65 min at 4° C, the supernatant (membrane extract) was saved. In some cases, the cells were directly collected into the buffer containing Triton X-100 (1.2%) (500 μL of buffer per 10-cm dish), and a total cell extract was obtained by centrifugation as above.

Immunopurification of the LH/hCG Receptor. The antireceptor monoclonal antibody LHR 38 was coupled to the Affi-Gel 10 as described (VuHai-LuuThi et al., 1990). Solubilized membrane receptors obtained from testes or from COS-7 cells (or L-cells) transfected with the expression vectors encoding pLHR-A, pLHR-C, pLHR-D were chromatographed through LHR 38–Affi-Gel 10 immunomatrix, and after extensive washes [see VuHai-LuuThi et al. (1990)], the receptor was eluted by a 50 mM glycine hydrochloride, pH 2.8, buffer. The eluate was then neutralized with 1 M Tris-HCl, pH 10, buffer and frozen at −20 °C for further studies.

Immunoblotting of Purified LH/hCG Receptors. The purified receptors were electrophoresed in sodium dodecyl sulfate/7.5% polyacrylamide gels under reducing conditions. The proteins were electrotransferred onto nitrocellulose sheets and immunoblotted using antireceptor monoclonal antibody LHR 775 as described (VuHai-LuuThi et al., 1990).

Treatment of Purified LH/hCG Receptors with Peptide N-Glycosidase F (PNGase F) and Endoglycosidase H (endo H). Purified receptor (3 pmol/30 μL) was incubated with PNGase F (final concentration 4 units/mL) in 50 mM Tris–glycine, pH 7.5, buffer containing 50 mM EDTA or with endo H (final concentration 0.1 unit/mL) in 50 mM Tris–glycine, pH 5.5, buffer in the presence of 1 mM phenylmethanesulfonyl fluoride at 35 °C overnight. The enzymes were omitted from control incubations. The reaction products were detected by immunoblotting.

Hormone Binding Assays. Different subcellular fractions (cytosol and membrane extract) and the cell culture medium were incubated with 5 nM ^{125}I-hCG in the presence or absence of 1 μM unlabeled hCG. ^{125}I-hCG–receptor complexes were immunoprecipitated with antireceptor antibody LHR 38 and antimouse immunoglobulin antibody as described (VuHai-LuuThi et al., 1990). The Scatchard analysis described in Figure 3 was performed by incubating 50 μL of total cellular extract with ^{125}I-hCG in the presence of serial dilutions of unlabeled hCG. The incubations were carried out at 4 °C overnight; nonspecific binding was determined as above. Hormone–receptor complexes were precipitated with 15% poly(ethylene glycol) 6000.

Measurement of the Synthesis of cAMP. Forty-four hours after transfection, the COS-7 cells were washed twice with warm DMEM medium containing 25 mM Hepes, pH 7.4, and gelatin (500 μg/mL) (1.5 mL of medium per 6-cm dish). Each dish was then incubated for 15 min at 37 °C with 1.5 mL of the same medium containing 3-isobutyl-1-methylxanthine (0.5 mM) and for 10 min at 37 °C with various concentrations of hCG in 1.5 mL of the same medium. The cell culture medium was removed, and 1 mL of cold perchloric acid (1 M) was added to the cells. The cell debris were collected by centrifugation, and the supernatants were neutralized with 0.72 M KOH/0.6 M $KHCO_3$ buffer. cAMP was measured by radioimmunoassay using the Amersham kit (RPA 509). The cell debris were collected by centrifugation, and the supernatants were neutralized with 0.72 M KOH/0.6

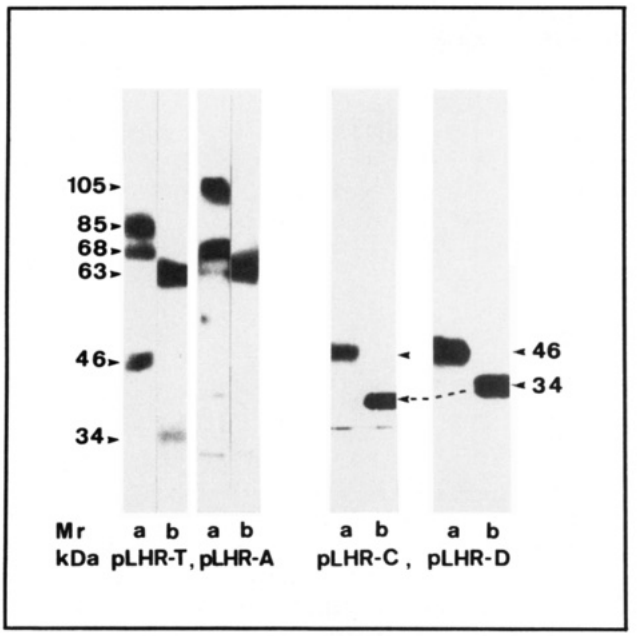

FIGURE 1: LH receptor species obtained by transfection of expression vectors encoding various forms of receptor messenger RNA. Effect of peptide *N*-glycosidase F. The purified receptors were detected by immunoblot (see Materials and Methods). pLHR-T, testicular receptors; pLHR-A, COS-7 cells transfected with an expression vector encoding pLHR-A (the full-length receptor); pLHR-C, COS-7 cells transfected with an expression vector encoding pLHR-C (receptor devoid of the transmembrane and intracellular domains); pLHR-D, COS-7 cells transfected with an expression vector encoding pLHR-D (receptor devoid of the transmembrane domain); lane a, untreated receptors; lane b, receptors treated with the peptide *N*-glycosidase F.

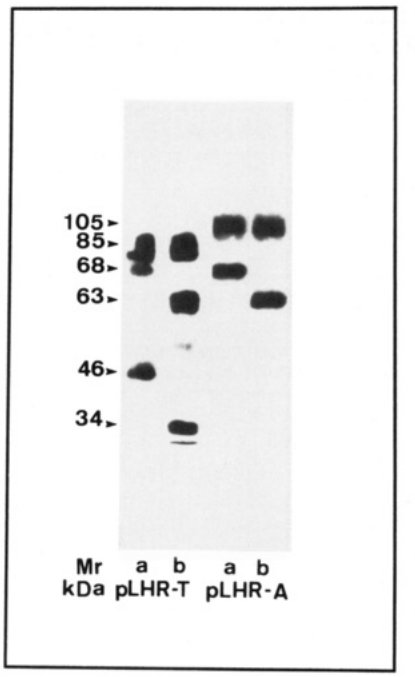

FIGURE 2: Effect of endoglycosidase H on testicular LH receptors and on receptors obtained by transfection of COS-7 cells. The purified receptors were detected by immunoblot (see Materials and Methods). pLHR-T, testicular receptors; pLHR-A, COS-7 cells transfected with an expression vector encoding the full-length receptor pLHR-A; lane a, untreated receptors; lane b, receptors treated with endoglycosidase H.

M $KHCO_3$ buffer. cAMP was measured by radioimmunoassay using the Amersham kit (RPA 509).

Sucrose Gradients. Testicular membranes were incubated with ^{125}I-hCG and extracted with Triton X-100 as described (VuHai-LuuThi et al., 1990). Aliquots (23 000 cpm of ^{125}I-hCG–receptor complexes/150 μL) were incubated overnight at 4 °C with 150 μg/50 μL of purified monoclonal antibodies. The sample (200 μL) was then centrifuged through a 10–40% sucrose gradient at 48 000 rpm in an SW 50 rotor for 17 h at 4 °C.

Immunoenzymatic Assay of LH Receptors. Monoclonal antibodies were coated onto Nunc plates (10 μg/mL) in sodium phosphate buffer, 10 mM, pH 8 (80 μL/well). The wells were saturated with sodium phosphate buffered saline containing Tween-20 (0.1%) and bovine serum albumin (1 g/L) (PBS–Tween–BSA). Purified receptor (50 fmol of receptor/100 μL of PBS–Tween–BSA) was incubated overnight at 4 °C. After five washes, biotinylated antibodies were added, and their binding was measured as described (VuHai-LuuThi et al., 1990).

RESULTS

LH Receptor Species Derived from the Full-Length Messenger RNA. COS-7 cells were transfected with the expression vector encoding full-length receptor. Immunopurification was used to enrich for LH receptor, and the resulting preparation was analyzed by immunoblot with LHR 775 antireceptor antibody. Two bands were observed (Figure 1, pLHR-A-a). The largest receptor species was ~105 kDa (Figure 1, pLHR-A-a), whereas the largest receptor species in testicular extracts was ~85 kDa (Figure 1, pLHR-, T-a). The smallest band was ~68 kDa, identical to that observed in testicular extracts. After digestion with peptide *N*-glycosidase F, which excises all carbohydrate side chains, both bands in transfected COS-7 cells were transformed into a single species of apparent molecular mass 63 kDa (Figure 1, pLHR-A-b). Similarly, in the testicular extracts, the 85- and 68-kDa species became a 63-kDa band (Figure 1, pLHR-T-b). Endoglycosidase H was then used which digests only the precursor high-mannose moieties of glycoproteins (Hubbart & Ivatt, 1981). It did not modify the molecular weight of the 85-kDa species in the testis (Figure 2, pLHR-T-b) or of the 105-kDa species in COS-7 cells (Figure 2, pLHR-A-b), but the 68-kDa species in both cell types was transformed into a 63-kDa protein. These experiments thus showed that the translation of the full-length LH receptor mRNA yielded two receptor species which differed by their glycosylation pattern.

The first species, which has an apparent molecular mass of 85 kDa (in the testis) or of 105 kDa (in COS-7 cells), contains complex oligosaccharides. The second one of 68 kDa is a precursor form containing carbohydrate of the "high-mannose" type.

L-Cells transfected with the vector encoding full-length receptor also yielded 105- and 68-kDa species of similar characteristics (data not shown).

LH Receptor Species Derived from Variant Forms of Messenger RNA. Transfection of COS-7 cells with expression vectors encoding either receptor devoid of the transmembrane domain (Figure 1, pLHR-D-a) or both the transmembrane and the intracellular domains (Figure 1, pLHR-C-a) yielded immunolabeled species of ~45 kDa (similar to those observed in the testis) which upon deglycosylation with both peptide *N*-glycosidase F and endoglycosidase H gave a protein of ~34 kDa (see Figures 1 and 2).

Distribution of Various Forms of LH Receptor between the Culture Medium, the Cellular Cytosol, and Cellular Membrane Extracts. The COS-7 cells transfected with

Table I: Distribution of LH Receptors in Culture Medium, Cellular Cytosol, and Cellular Membranes after Transfection of COS-7 Cells with Various Forms of the Receptor[a]

	pLHR-A	pLHR-B	pLHR-C	pLHR-D
cell culture medium (%)	8.5	43	58	33
cellular cytosol (%)	26	55	40	65
cellular membrane extract (%)	65.5	2	2	2

[a] COS-7 cells were transfected with vectors encoding full-length receptor (pLHR-A) or receptors devoid of the transmembrane and intracellular domains (pLHR-B and pLHR-C) or only devoid of the transmembrane domain (pLHR-D). Results are expressed as the percentage of the total ^{125}I-hCG binding sites present in the different fractions for a given transfection (see Materials and Methods).

expression vectors encoding the full-length or variant forms of LH receptor were used to study the subcellular distribution of receptors. Three fractions were isolated: the culture medium, the soluble fraction obtained after homogenization of the cells, and the Triton X-100 extract of the membranes. After incubation with ^{125}I-hCG, hormone–receptor complexes were measured by incubation with antireceptor monoclonal antibody LHR 38 and precipitation with an antimouse immunoglobulin antibody. As shown in Table I, the majority of the full-length receptor molecules were present in the membrane extract. The hCG binding activity in the cytosol and in the cell culture medium could be due to receptor proteolysis (West & Cooke, 1991) or to the presence of very small membrane fragments.

The two variants lacking both the transmembrane and the intracellular domains (pLHR-B and pLHR-C) were about equally divided between the culture medium and the soluble intracellular fractions. The variant lacking only the transmembrane domain (pLHR-D) was partitioned between the culture medium (one-third) and the soluble intracellular fraction (two-thirds).

Hormone Binding by the Variant LH Receptor Species. COS-7 cells were transfected with expression vectors encoding either the full-length receptor (pLHR-A) or variant forms lacking either only the transmembrane domain (pLHR-D) or both the transmembrane and intracellular domains (pLHR-C). Binding of ^{125}I-hCG to Triton X-100 cellular extracts was then determined. As shown in Figure 3, all receptor species had similar affinities for the labeled hormone (K_d, 0.7–0.8 nM). The concentration of the binding sites was variable, but this may have been related to varying efficiencies of transfection or to differences in the biosynthesis or turnover rates of the proteins.

The full-length messenger RNA produced two receptor species, and the method followed above did not allow us to distinguish binding of hormone to each of them individually. Moreover, previous experiments have shown that in testicular extracts the 68-kDa receptor does not bind hormone (or binds it with markedly lower affinity than the 85-kDa species) (Vu-Hai-LuuThi et al., 1990). To examine this problem in membrane extracts of COS-7 cells transfected with the full-length receptor mRNA, we incubated the extract with hCG and chromatographed it on an immunomatrix containing the anti-hCG antibody D_1E_8 [this antibody has been shown to bind to hCG receptor complexes (Bidart et al., 1987)]. Both the 105- and the 68-kDa species were retained on the immunomatrix, demonstrating that they have bound the hormone (data not shown).

Stimulation of Adenylate Cyclase Activity by Various Species of LH Receptor. Effect of Combined Transfections. As expected, when COS-7 cells were transfected with

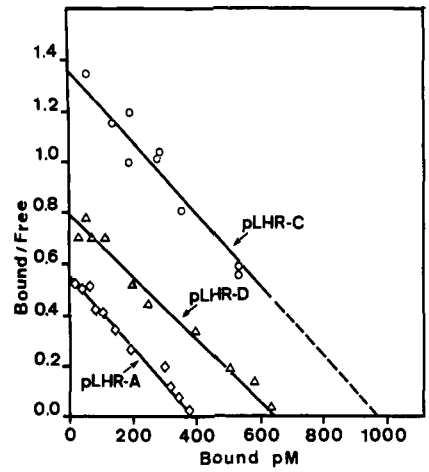

FIGURE 3: Scatchard plot for specific binding of hCG to the LH receptor species produced by transfection with expression vectors encoding full-length or variant messenger RNAs. COS-7 cells were transfected with expression vectors encoding either full-length receptor (pLHR-A) or variant pLHR-C (lacking both the transmembrane and intracellular domains) or variant pLHR-D (lacking the transmembrane domain). The total cellular extracts were incubated with a constant amount of ^{125}I-hCG and increasing amounts of unlabeled hCG (see Materials and Methods). Each point presented is the average of duplicate determinations.

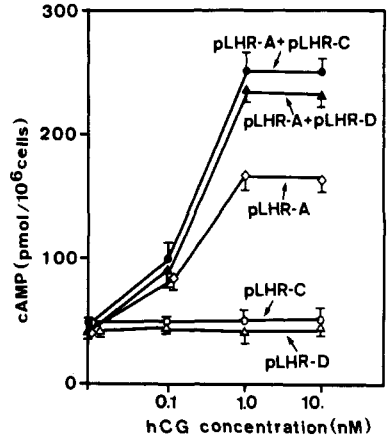

FIGURE 4: Stimulation of adenylate cyclase by various species of LH receptor. Effect of combined transfections. COS-7 cells were transfected with expression vectors encoding the full-length receptor (pLHR-A) or the receptor species devoid of the transmembrane and intracellular domains (pLHR-C) or devoid of the transmembrane domain (pLHR-D); cotransfections were also performed with either pLHR-A and pLHR-C or pLHR-A and pLHR-D. Forty-four hours after transfection, the cells were incubated for 10 min at 37 °C with various concentrations of hCG. The production of cAMP was measured by radioimmunoassay (see Materials and Methods). Each experiment was performed in quadruplicate: mean ± SD are shown.

expression vectors encoding various forms of LH receptor and incubated with hCG, only the full-length receptor provoked increased adenylate cyclase activity (Figure 4). Lower amounts of the DNA encoding full-length receptor were then used in combination with a 4-fold excess of the DNA encoding either pLHR-C or pLHR-D. The cells were thereafter incubated with various concentrations of hCG, and the production of cAMP was measured. In all three experiments, combinations of full-length LH receptor with pLHR-C or pLHR-D gave higher stimulations of adenylate cyclase than full-length receptor alone. However, the extent of enhancement was variable (144.4 ± 7.6%, mean ± SEM, five determinations) at maximal hormonal stimulation. No significant difference could be observed between the effect of pLHR-C and pLHR-D. There was always enhancement of

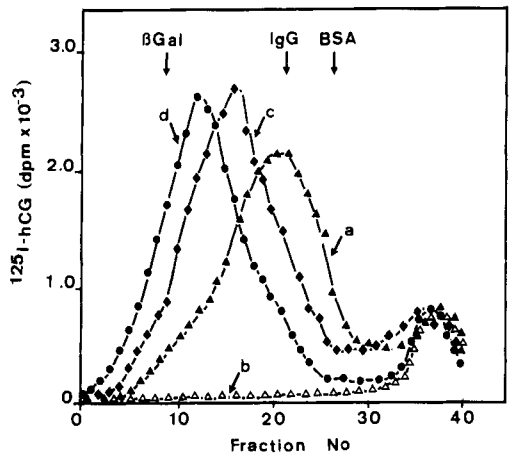

FIGURE 5: Density gradient analysis of the number of molecules of monoclonal antireceptor antibodies reacting with ^{125}I-hCG–receptor complexes. The following incubation mixtures were centrifuged on sucrose gradients (see Materials and Methods). (a) ^{125}I-hCG–receptor complexes were extracted from membranes and incubated in the absence of antibody [a similar pattern was observed in the presence of the non-receptor-related antibody Li 533 (VuHai et al., 1989)]. (b) Membranes were incubated with ^{125}I-hCG and a 100-fold excess of unlabeled hCG and processed as in (a). (c) ^{125}I-hCG–receptor complexes were incubated with LHR 442 antireceptor antibody (a similar pattern was observed with antireceptor antibodies LHR 38 and LHR 436). (d) ^{125}I-hCG–receptor complexes were incubated with both LHR 436 and LHR 442 antireceptor antibodies. (βGal, β-galactosidase; IgG, immunoglobulin; BSA, bovin serum albumin).

the maximal level of cAMP formation, whereas the concentration of hormone necessary to elicit the half-maximal stimulation was not changed. The stimulatory effect of pLHR-C and pLHR-D on the biological activity of the full-length receptor was probably even greater than suggested by these figures. The concentration of ^{125}I-hCG binding sites in the membranes of cells transfected with pLHR-A was compared to that of cells cotransfected with pLHR-A and pLHR-C or pLHR-D. This concentration mainly reflects that of the full-length receptor (see Table I). In cotransfected cells, this concentration was decreased to 70% of the concentration in control (pLHR-A-transfected) cells. Thus, a decreased number of full-length receptor molecules were exhibiting enhanced biological activity. This decrease in the membrane expression of pLHR-A might have been due to nonspecific (saturation of the cell synthetic capacity) or specific (interaction between the full-length and the truncated protein species during the synthesis and the cellular traffic of the receptors) mechanisms.

Monomeric Structure of the Solubilized LH Receptor. The cotransfection experiments suggested the existence of interactions between receptor subunits. We thus tested the hypothesis that the receptor existed as a dimer or as a higher order oligomer. Two types of experiments were performed to answer this question.

The first approach used density gradient sedimentation. Solubilized hormone–receptor complexes were incubated with antireceptor monoclonal antibodies and sedimented on a sucrose gradient (Figure 5). In the absence of antibodies (or in the presence of nonrelated antibodies), the hormone–receptor complexes displayed a sedimentation coefficient of 7.8 S [confirming previous reports (Rajaniemi et al., 1981)]. In the presence of antireceptor monoclonal antibody, their sedimentation was shifted to 10.5 S, suggesting the binding of one IgG molecule. [Monoclonal antihormone antibody D_1E_8 provoked the same shift of hormone–receptor complexes, showing the existence of the same number of antigenic determinants for hormone and for receptor in the active hormone–receptor complex (not shown)]. This was confirmed by incubating the hCG–receptor complexes in the presence of two different antireceptor antibodies recognizing two different antigenic determinants: a displacement to 13.5 S was then observed.

The second approach analyzed the additive binding of the antibodies to the receptor. The antibodies were coated onto ELISA plates; the purified receptor was added, followed by biotinylated antireceptor antibodies (Table II). None of the biotinylated antibodies could bind to the receptor when the same antibody had been coated onto the plate, showing again that the receptor behaved as a monomer.

DISCUSSION

Two types of information have previously been obtained on the structure of the different species of LH receptor: first, the results of the cloning and sequencing of the messenger RNAs; second, the observations provided by immunoblot and immunoaffinity purification experiments. In order to match the protein species which have been observed with the various messenger RNAs which have been cloned, we expressed the latter in COS-7 and L-cells. The full-length messenger RNA yielded, in about equal amounts, proteins of 105 and 68 kDa. The former was a mature receptor species which was more extensively glycosylated than the testicular or ovarian (VuHai-LuuThi et al., 1990) receptors which have an apparent molecular mass of ~85 kDa. Upon deglycosylation with peptide *N*-glycosidase F, the COS-7 and testicular cell receptors yielded a similar holoprotein of 63 kDa. Cell-specific variabilities in protein glycosylation have previously been described (Hughes, 1990). This difference in glycosylation does not impart any obvious biological difference between the COS-7 or L-cell receptor on the one hand and the testicular receptor on the other hand in terms of hormone binding and adenylate cyclase activation.

Transfection with vectors encoding full-length receptors also yielded a 68-kDa receptor species similar to the one present in the testis. (However, when both preparations were run on the same gel, the ~68-kDa band of receptor present in COS-7 cells seemed to be very slightly larger than the one present in the testis. Digestion with endoglycosidase H showed that this glycoprotein contains high-mannose carbohydrate moieties and is thus probably a precursor of the mature receptor (pulse–chase experiments will, however, be necessary to establish this point). The accumulation in high concentration of such a precursor is unusual for glycoproteins. In the case of transfection experiments, it could be argued that the cellular machinery which processes the glycoproteins was saturated by the extraphysiological concentrations of receptor being synthetised; however, this explanation is not valid for the testicular receptor. In this case, the existence of a large pool of precursor molecules suggest possible posttranslational regulations. Interestingly, the 68-kDa immature receptor of COS-7 cells interacted with hCG and was retained on D_1E_8 anti-hCG antibody immunomatrix, whereas the 68-kDa testicular species was not. This suggests that although these receptor forms have similar molecular weights and have their carbohydrate moieties digested by endoglycosidase H, they must, however, have some discrete differences in their glycosylation patterns and that these differences may be responsible for changes in hormone binding properties. In rat LH receptor, conversion of Asn-173 to Gln resulted in the total loss of hormone binding. Substitutions in putative glycosylation sites 77, 152, 269, 277, and 291 were without effect (Zhang et al.,

Table II: Additivity of Binding of Monoclonal Antibodies to the LH/hCG Receptor[a]

antibodies coated onto plates	biotinylated antibodies					
	LHR 38	LHR 285	LHR 346	LHR 436	LHR 442	LHR 775
LHR 38	1	5	4	54	3	29
LHR 285	11	2	7	35	7	20
LHR 346	4	0	1	53	4	35
LHR 436	84	61	79	0	76	64
LHR 442	4	0	1	100	1	42
LHR 775	26	6	21	49	21	0

[a] Antibodies were coated onto ELISA plates and incubated with purified testicular receptor and then with biotinylated antireceptor antibodies. Binding of the latter (see Materials and Methods) was determined. Results were expressed as the percent of the strongest ELISA reaction obtained with every biotinylated antibody (the maximal optical densities were 0.76, 0.82, 0.70, 1.9, 0.75, and 0.55 for LHR 38, LHR 285, LHR 346, LHR 436, LHR 442 and LHR 775, respectively).

1991). Russo et al. (1991) have shown for the TSH receptor that mutation of the Asn-77 putative glycosylation site abolished high-affinity hormone binding and mutation of Asn-113 decreased the affinity whereas mutations of Asn-99, -177, -198, and -302 (also putative glycosylation sites) had no effect. Thus, discrete differences in glycosylation patterns at specific sites may modify hormone binding properties of this type of receptor. However, it is still unclear if these mutated receptors had normal membrane distribution distribution and were not prone to premature proteolysis.

Transfection experiments confirmed that the shorter variants of LH receptor mRNA, devoid of the transmembrane domain, encoded the 45–48-kDa protein species. These experiments allowed confirmation (Xie et al., 1990; Tsai-Morris et al., 1990; Braun et al., 1991; Ji & Ji, 1991) that the N-terminal region of the receptor was responsible for hormone binding. Studies of variants of LH receptor in rat have shown them to bind hormone. However, discrepant results have been obtained on their secretion or not from the cells (Tsai-Morris et al., 1990; Xie et al., 1990). In the case of the porcine receptor, we have observed clear-cut secretion of the variants from transfected cells. Further experiments will be necessary to search for the presence of these soluble LH receptors in the blood or in other biological fluids as has been observed in the case of the growth hormone (Leung & al., 1987; Baumbach et al., 1989), the prolactin (Postel-Vinay et al., 1991), and the transferrin (Shih et al., 1990) receptors. The 45–48-kDa receptor species contained high-mannose oligosaccharides (see digestion with endoglycosidase H). Such an unusual pattern of glycosylation has also been observed for the truncated secreted form of the epidermal growth factor (EGF) receptor (Carpenter, 1987).

The function of these soluble variants of the LH receptor remains unknown, although they seem to have some enhancing effect on the activity of the full-length receptor. This effect is not linked to a decrease in the concentration of hormone necessary to elicit a half-maximal stimulation but consists of an apparent increase of the maximal hormone-induced activity of adenylate cyclase. In other models and especially in the case of interleukin receptors, soluble variant receptors yielded either stimulatory (Hibi et al., 1990) or inhibitory (Mosley et al., 1989) effects. In the case of the platelet-derived growth factor (PDGF) receptor, inactive complexes have been observed between the full-length receptor and its soluble variant (Ueno et al., 1991). The formation of such complexes in the case of the LH receptor could not be tested directly due to the lack of antibodies interacting with only one of the species, allowing study of the possible coprecipitation of the other species. However, soluble hormone–receptor complexes were shown to be monomeric. Thus, either the bonds between receptor monomers are unstable or such interactions only occur in the cell membranes and cannot be observed in solubilized receptors. It has been suggested that LH may also act by coupling to phospholipase A (Minegishi et al., 1989) and phospholipase C (Cooke, 1990). The role of variant forms of the receptor with respect to the stimulation of these enzymes remains to be established.

Specific regulations of alternatively processed LH receptor transcripts different from those of the full-length receptor have been described (Segaloff et al., 1990b; Hu et al., 1990; LaPolt et al., 1991). Cooke and co-workers (West & Cooke, 1991) have observed the release of a soluble hCG binding protein by hormone-dependent truncation of the receptor in mice and rats. The existence of variant forms of LH receptor messenger RNAs in human has been described (Frazier et al., 1990; Minegish et al., 1990), but it is not known if they are translated into actual proteins.

ACKNOWLEDGMENT

We thank Jean-Michel Bidart for the anti-hCG antibody D_1E_8. M. Guerrois typed the manuscript.

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