Anti-Human FSH Receptor Monoclonal Antibodies: Immunochemical and Immunocytochemical Characterization of the Receptor†

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ABSTRACT: The extracellular domain of the human FSH receptor was expressed in *Escherichia coli* as a fusion protein with ubiquitin. It was tagged with a poly-His tract which was used for its purification. Immunization of mice allowed the preparation of high affinity antireceptor monoclonal antibodies. The latter fell into two categories: some of them were inhibited hormone binding and adenylate cyclase activation whereas others were devoid of these properties. None of the antibodies had agonistic activity (i.e., stimulated adenylate cyclase). Immunoaffinity chromatography allowed us to purify the native receptor in a single step either from a permanently transfected L cell line (75% recovery) or from human ovaries (33% recovery). Immunoblotting of the receptor in human ovaries showed the presence of a major band of 87 kDa and of a minor band of 81 kDa. Endoglycosidase digestion and pulse−chase experiments showed the former to be the mature receptor and the latter the precursor containing mannose-rich carbohydrates. Thus, as in the case for the LH receptor, there was an accumulation (albeit to a lower degree) of the precursor in target cells. We did not detect variant forms of the protein corresponding to the alternative mRNA transcripts previously described. Additive binding to the receptor of several antibodies, but not of the same antibody, allowed us to establish a sandwich-type ELISA for the receptor (sensitivity ∼1 fmol) and to obtain evidence against the existence of previously described oligomeric forms of the protein. All monoclonal antibodies were able to label the receptor immunocytochemically in transfected cells, and two of them were also able to detect it at the markedly lower physiological concentrations, i.e., in human Sertoli and granulosa cells.

FSH is a glycoprotein secreted by the pituitary which acts through a specific receptor on Sertoli cells in the male and on granulosa cells in the female [reviewed in Orth and Christensen (1977) and Hsueh et al. (1989)]. It thus plays a major role in spermatogenesis and in ovarian follicular development. Its receptor has been the subject of numerous studies [for reviews, see in Dias et al. (1982), Shin and Ji (1985), and Abou-issa et al. (1987)] but remained elusive in many aspects up to the cloning of its cDNA in 1990 (Sprengel et al., 1990). The cloning of rat (Sprengel et al., 1990) and human (Minesgish et al., 1991) FSH receptors was permitted by their homologies with the LH receptor, the first member of this family of proteins, which also includes the TSH receptor to be cloned (McFarland et al., 1989; Loosfelt et al., 1989; Parmentier et al., 1989; Misrahi et al., 1990). The sequence of the FSH receptor as deduced from the cDNA showed it to span the membrane seven times, as has been found for other G protein coupled receptors. The extracellular N-terminal segment is very large and has been shown to be the site of hormone binding (Braun et al., 1991; Dattatreyamurty et al., 1992). It is composed of leucine-rich repeats, similar to those found in cell-adhesion or protein−protein interaction domains of a family of proteins (Braun et al., 1991). The cloning of the cDNA has not completely solved the problem of the structure of the protein. Several reports have described immunoblotting using antibodies raised against receptor peptides: Dattatreyamurty et al. (1992) have studied the human granulosa cell receptor in nondenaturing conditions and observed a 240 kDa species which could bind ^{125}I-FSH and a 160 kDa species which did not bind the hormone. Christophe et al. (1993) have expressed the rat receptor in insect cells and observed 130 and 77 kDa immunoreactive species. Quintana et al. (1993) expressed the rat FSH receptor in 293 human kidney cells and observed several 58−83 kDa immunoreactive proteins in nonreducing conditions and a set of 69−81 kDa reactive species in reducing conditions. Ligand blotting has yielded a molecular mass of 240 kDa for the porcine granulosa cell receptor in nonreducing conditions and of 60 kDa in reducing conditions (Dattatreyamurty et al., 1993). Identical results were obtained for purified receptor from calf testes (Dattatreyamurty et al., 1990).

We considered that the preparation of well-defined monoclonal antibodies might allow us to resolve these discrepancies. Furthermore, such antibodies could be used for immunopurification of the receptor, for the establishment of ELISA tests allowing an easy quantification of the protein, and for immunocytochemical determinations of the cells expressing the receptor. We describe here the preparation of such antibodies and their use for the immunochemical and immunocytochemical characterization of the receptor. We have chosen to study the human receptor due to its

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potential importance both in pathology and as a possible target of contraception (Simoni et al., 1993).

MATERIALS AND METHODS

Materials. I^{125}-FSH was from Du Pont New England Nuclear (Specific Radioactivity, 90 μCi/μg; Biological Activity, 50%); peptide N-glycosidase F (PNGase F; EC 3.2.2.18) and endo-β-*N*-acetylglucosaminidase H (endoglycosidase H or Endo H 3.2.1.96) were obtained from Boehringer Mannheim (Mannheim Germany). G418 (geneticin), Dulbecco's-modified Eagle's medium (DMEM) and fetal calf serum were from Gibco-BRL (Paisley, Great Britain).

Construction of an Expression Vector Encoding the Full-Length hFSH Receptor. The cDNA encoding the human FSH receptor was isolated from a human testis cDNA library and sequenced (H. Loosfelt, and M. Atger, unpublished results). The sequence diverged slightly from that published initially by Minesgish et al. (1991) at codons 112 (AAC = Asn instead of ACC = Thr) and 197−198 (GAGCTG = Glu-Leu instead of GCAGTG = Ala-Val). These amino acids were thus identical to those found in the rat FSH receptor (Sprengel et al., 1990). Silent mutations were also observed at codons 146 (AAA instead of AAG = Lys) and 471 (ATA instead of ATC = Ile). An insert encompassing the cDNA of the full-length hFSHR (including 4 bp in the 5′ noncoding region, 3 bp in the 3′ noncoding region and *Eco*RI sites at the two extremities) was initially subcloned in the Bluescript vector (Stratagene). This insert was subsequently introduced into the *Eco*RI site of the pSG5 vector (Stratagene). Similar vectors were constructed so as to contain fragments of the human LH receptor and were used for control experiments (Atger et al., 1995; H. Loosfelt, unpublished results).

L Cells Permanently Expressing the hFSH Receptor. Mouse L cells were cotransfected, using the calcium phosphate precipitate method, with the plasmid encoding the hFSHR (pSG5-hFSHR) and with the plasmid pSV-Neo, a vector which confers resistance to the antibiotic G 418 (Southern & Berg, 1982). Neomycin-resistant cells were selected in DMEM supplemented with 10% fetal calf serum and G 418 (geneticin, Sigma) (1 mg/mL). The resistant clones were then screened for the hFSH receptor by an immunocytochemical test using the antireceptor antibody 323. The transfected cells were maintained in medium containing G 418 (200 μg/mL) for further studies.

Expression of hFSHR in Escherichia coli. cDNA fragments encoding amino acids 23−358, 23−171, or 172−358 of the human FSHR were introduced into the polylinker of the vector pUR 292 (Rüther & Müller-Hill, 1983) or pNMhUb (Monia et al., 1989). A short sequence coding for a 6-histidine tag was introduced at the C-terminal tail of the fusion protein Ub-hFSHR(23−358) allowing its purification by immobilized metal ion affinity chromatography on Ni-NTA Resin (Quiagen). All expression vectors were sequenced. Fusion proteins of hFSHR were produced in *E. coli* as described (Loosfelt et al., 1992). Cells were resuspended in phosphate buffered saline (PBS) containing 1% Triton X-100 and 1 mg/mL lyzozyme. After two freeze−thawing cycles, the cell lysates were treated with 50 μg/mL DNase for 20 min at 20 °C. After centrifugation at 10000*g* for 30 min, the pellets were washed with PBS containing 1% Triton X-100 and solubilized in buffer A (6 M guanidinium hydrochloride, 100 mM NaH_2PO_4, 10 mM Tris, pH 8) containing 20 mM β-mercaptoethanol. After centrifugation at 10000*g* for 30 min at 4 °C, the supernatant was applied to the Ni-NTA resin. The Ni-NTA resin was washed with 15 volumes of buffer A, 15 volumes of buffer B (8 M urea, 60 mM Tris, 100 mM H_2PO_4, 50 mM sodium citrate, HCl, pH 8), and 15 volumes of buffer B at pH 6.3. The fusion protein was eluted in buffer B at pH 4.5. The eluate was neutralized with 1 M NaOH and concentrated on Centriprep-10 ultrafiltration units (Amicon). For immunization, the samples were dialyzed with 4 M urea and PBS, pH 7.4. The purified fusion proteins were analyzed by polyacrylamide−SDS gel electrophoresis and Coomassie blue staining. The concentration of protein was estimated by comparison with known amounts of β-galactosidase.

Immunization and Preparation of Hybridomas. Balb/c mice were immunized with four subcutaneous injections of purified Ub-hFSHR(23−358) fusion protein (100 μg/injection) at 15 day intervals. One week later, 50 μg of antigen was injected intravenously. Mice were killed 4 days later, and hybridomas were produced by fusion with Sp_2O-Ag cells as previously described (Buttin et al., 1978). To detect hybridomas secreting antibodies against hFSHR, clones were screened by ELISA using the antigen Ub-hFSHR(23−358) and the βgal-hFSHR(23−171) and βgal-hFSHR(172−358) fusion proteins. Ub-hLHR(21−229) and βgal-hLHR(21−229) fusion proteins were used as negative controls. Of the 371 hybridomas, 19 secreted antibodies against the Ub-hFSHR(23−358) and βgal-hFSHR(172−358) fusion proteins. These were repeatedly cloned. The five antibodies 18 (IgG1), 156 (IgG1), 190 (IgG2b), 225 (IgG2b), and 323 (IgG2a) were produced in ascites and purified as described (Vu Hai et al., 1990).

Effect of Anti-hFSHR Monoclonal Antibodies on Adenylate Cyclase Activity. L cells permanently expressing hFSHR were plated on six-well plates (2×10^6 cells/well) in DMEM. After 2 days of growth, the cells were washed twice with DMEM medium containing 25 mM Hepes, pH 7.4, and gelatin (500 μg/mL) at 37 °C. Each dish was then incubated with the same medium containing 3-isobutyl-1-methylxanthine (IBMX) (0.5 mM) for 30 min at 37 °C. The cells were incubated for 30 min at 37 °C in the same medium with increasing concentrations of monoclonal antibodies or hFSH (Serono). The incubation was stopped by aspiration of the medium and addition of 200 μL/well of 1 M perchloric acid. The cell debris was then collected by centrifugation at 2000*g* for 15 min at 4 °C. The resulting supernatants were neutralized with 0.72 M KOH and 0.6 M $KHCO_3$. cAMP was measured by radioimmunoassay using the Amersham kit.

Effect of Antibodies on FSH-Mediated Stimulation of Adenylate Cyclase. The cells expressing the receptor were incubated for 1 h at 37 °C with various concentrations of monoclonal antibodies in DMEM and 0.5 mM IBMX. FSH (100 nM) was added for 15 min at 37 °C. cAMP was measured as described above. The results were expressed as percent of FSH stimulation measured in the absence of antibodies.

Effect of Antibodies on FSH Binding to the Receptor. Membranes were prepared from L cells expressing permanently hFSHR and were resuspended in 10 mM Tris, 5 mM $MgCl_2$, and 0.25% BSA at a final concentration of protein

of 1 mg/mL. The membrane suspension (100 μL containing 50 fmol of hFSHR) was incubated with ^{125}I-FSH (10 000 cpm, 35 fmol) and increasing concentrations of monoclonal antibodies for 18 h at 4 °C. The amount of nonspecifically bound radioactivity was measured in reactions containing a 1000-fold excess of unlabeled hormone. The incubation was stopped by the addition of 1 mL of ice-cold buffer and centrifugation of the tube at 15000*g* for 10 min. The supernatant was removed by suction, and the pellet was washed twice with 1 mL of ice-cold buffer. The tubes containing membrane pellets were counted in a γ-counter. Results were expressed as percent of specific binding of ^{125}I-FSH to its receptor in the absence of antibodies.

Immunoprecipitation of ^{125}I-FSH−Receptor Complexes with Monoclonal Antibodies. Membranes of L cells expressing hFSHR (50 μg of total proteins) were prepared and incubated with ^{125}I-FSH (20 nM) as described above. After three washes, the membranes were solubilized in 10 mM Tris, 10 mM $MgCl_2$, and 1% Triton buffer, pH 7.5. After centrifugation at 100000*g* for 1 h, the solubilized ^{125}I-FSH−receptor complexes (5000 cpm in a total volume of 200 μL) were incubated with increasing concentrations of purified monoclonal antibodies for 4 h at 4 °C. Anti-mouse immunoglobulin antibodies (200 μg per μg of monoclonal antibodies) were then added and incubated for 18 h at 4 °C. The tubes were centrifuged at 15000*g* for 10 min at 4 °C, and the pellets were washed once with 1 mL of incubation buffer. The immunoprecipitated complexes were counted for radioactivity and compared to background values obtained with a nonrelated monoclonal antibody IDA 10 (Legrain et al., 1983). The total concentration of ^{125}I-FSH−receptor complexes in the membrane extract was measured by precipitation with 15% poly(ethylene glycol) 6000.

Metabolic Labeling and Immunoprecipitation of the Human FSH Receptor. After 2 days of growth, subconfluent L cells expressing the human FSH receptor were washed twice with DMEM free of methionine and cysteine. Cells were pulse-labeled for 1 h with the same medium containing 100 μCi/mL of [^{35}S]methionine and [^{35}S]cysteine (Du Pont New England Nuclear). Chases with unlabeled amino acids were performed for various periods of time using complete DMEM supplemented with 10% fetal calf serum. At the end of the incubation, the cells were washed twice with PBS and scraped in the presence of protease inhibitors, and the cell pellets after centrifugation were frozen at −20 °C. All subsequent steps were performed at 4 °C in the presence of protease inhibitors: 1 mM benzamidine (Sigma), 100 μg/mL bacitracin (Sigma), 40 μg/mL aprotinin (Calbiochem), 5 mM leupeptin (Sigma), 1 μg/mL pepstatin (Sigma), and 1 mM phenylmethanesulfonyl fluoride (Sigma). Cell pellets were resuspended in 20 mM Tris, 50 mM NaCl, and 20% glycerol, pH 7.5, buffer and centrifuged at 30000*g* for 30 min. The membrane pellet was then resuspended in solubilization buffer: 20 mM Tris, 0.4 M NaCl, 10 mM EDTA, 5 mM *N*-ethylmaleimide (Sigma), and 1.2% Triton X-100 (Sigma). After centrifugation at 100000*g* for 30 min, the supernatant was incubated with 50 μL of protein A-Sepharose 4B (1 mg/mL) and 10 μg of antibody 323 for 16 h at 4 °C. The protein A-Sepharose 4B was then washed extensively with solubilization buffer. The FSH receptor was eluted in 50 mM sodium citrate, pH 2.5, and 0.05% Triton X-100 and immediately neutralized with 1 M Tris, pH 10. After denaturation and reduction, the FSH receptor was electrophoresed on 8% SDS−PAGE as described (Misrahi et al., 1994). The polyacrylamide gel was incubated in Amplify (Amersham), dried under vacuum at 60 °C, and exposed at −80 °C with βmax Hyperfilm (Amersham) overnight.

Immunopurification of the hFSH Receptor. Monoclonal antireceptor antibody 323 directed against the extracellular domain of hFSHR was coupled to Affi-Gel 10 (Bio-Rad) at a concentration of 10 mg of antibody per mL of gel as recommended by the manufacturer. All buffers contained protease inhibitors as described above.

Immunopurification from Human Ovaries. Human ovaries (30 g) were obtained at surgery and frozen in liquid nitrogen. After thawing, the ovaries were homogenized in a Waring-Blendor (2 × 10 s) and in a glass-Teflon homogenizer (10 strokes) in two volumes of 20 mM Tris, 50 mM NaCl, and 20% glycerol, pH 7.5 (buffer C), and then centrifuged at 800*g* for 15 min. The supernatant was centrifuged at 30000*g* for 30 min. The membrane pellet was washed twice in buffer C, centrifuged at 30000*g* for 30 min [for some experiments the pellet was incubated with ^{125}I-FSH (20 nM, NEN-Dupont)], and extracted with a glass-Teflon homogenizer in 20 mM Tris, 0.4 M NaCl, 10 mM EDTA, 5 mM *N*-ethylmaleimide (Sigma), and 1.2% Triton X-100 (Sigma) (buffer D) at a final concentration of protein of 8 mg/mL. The extract was centrifuged at 100000*g* for 30 min. The supernatant was applied at a flow rate of 2 mL/h on a 300 μL column of Affi-Gel 10 coupled to antibody 323. After the gel was washed with 50 volumes of buffer D, the hFSHR was eluted in 50 mM sodium citrate and 0.05% Triton X-100, pH 2.5, neutralized with 1 M Tris, pH 10, and conserved at −80 °C. hFSHR concentration was measured by ELISA.

Immunopurification from L Cells Permanently Expressing the FSH Receptor. L cells (200 mg) were scraped in PBS containing protease inhibitors. After centrifugation at 800*g* for 10 min at 4 °C, the pellet of cells was stocked at −80 °C. The cell pellet was homogenized in buffer C and centrifuged at 30000*g* for 30 min at 4 °C. The membrane pellet was then resuspended in the solubilization buffer (see above). After centrifugation at 100000*g* for 30 min at 4 °C, the supernatant was applied on a 50 μL column of Affi-Gel 10 coupled to the antibody 323. All the subsequent steps were the same as for the immunopurification of the receptor from human ovaries.

Immunoenzymatic Assay of FSH receptor. Antibodies 18 or 323 (1 μg/well) were coated onto 96-well plates (Maxisorb) in 50 mM potassium phosphate and 8 M urea, pH 7.4, buffer for 16 h at 4 °C. The plates were incubated with PBS containing 0.1% Tween (PBS Tween buffer) and 1% BSA (blocking buffer) for 1 h at 20 °C and then washed with PBS Tween buffer. The extract (50 μL) containing the receptor was added at various dilutions with the blocking buffer. After incubation for 2 h at 20 °C and three washes with PBS, the plates were incubated with biotinylated antibody 323 (1 μg/mL in the blocking buffer) at 20 °C for 1 h. After washing, the plates were incubated with streptavidin biotinylated horseradish peroxidase linked antibody (Amersham) at 1/1000 dilution for 1 h at 20 °C. The immunocomplexes were colored with ABTS (Amersham). Optical density was read at 492 nm. The concentration of the immunopurified receptor was measured by reference to known concentrations of hFSHR fusion proteins purified from *E. coli*.

Treatment of Purified FSH Receptor with Peptide N-Glycosidase F (PNGase F) and Endoglycosidase H (Endo H). Purified receptor (4 pmol/50 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 sodium citrate, pH 5.5, buffer in the presence of 1 mM phenylmethanesulfonyl fluoride at 37 °C for 16 h. The enzymes were omitted for control incubations. The reaction products were detected by immunoblotting.

Immunoblotting of FSH Receptor. The immunopurified hFSHR receptor (4 pmol/well) was electrophoresed on an 8% SDS−polyacrylamide gel under reducing and denaturing conditions as described (Misrahi et al., 1994). Proteins were electrotransferred to nitrocellulose at 80 V for 5 h in a 25 mM Tris, 195 mM glycine, pH 8.2, 10% methanol buffer. The nitrocellulose membrane was blocked for 16 h at 4 °C in PBS containing 5% nonfat dry milk and 0.1% Tween (Sigma). Antireceptor monoclonal antibody 323 directed against the extracellular domain of hFSHR was added for 1 h at 20 °C in the same buffer at a final concentration of 5 μg/mL. A sheep anti-mouse horseradish peroxidase linked antibody (Amersham) was used at 1/10 000 dilution as a secondary antibody. Bound immunoglobulins were revealed using the ECL system (Amersham).

Immunocytochemistry. Testes fragments were obtained from patients undergoing castration for prostatic cancer or testicular biopsy during vasectomy. Nine surgical biopsies of ovarian tissue were also examined; they were removed from premenopausal women undergoing hysterectomy. Six-micron-thick frozen sections were cut with an OTM 56 bright cryomicrotome, air dried for 30 min at room temperature, and then fixed in cold (−20 °C) acetone and left to dry for 20 min. The sections were individually wrapped in cellulose and tin foil and stored at −20 °C till further use. Before immunohistochemistry, the slides were left unwrapped to thaw for 10 min at room temperature and were then rehydrated in two successive baths of PBS, pH 7.2. After 20 min pretreatment with preimmune sheep serum diluted 20-fold in PBS containing 5% bovine serum albumin, the slides were incubated with the primary antibody 323 (3 μg/mL), overnight, in a humid chamber at 4 °C. The bound immunoglobulins were subsequently revealed with biotinylated anti-mouse secondary antibodies and then with streptavidin−biotin−peroxydase complexes according to the manufacturer's instructions (Amersham Int., Bucks, England). Aminoethilcarbazole (Sigma Chemical Co., St. Louis, MO) was used as a chromogen. The sections were slightly counterstained with Meyer's hematoxylin (Merck Diagnostica). Replacement of the specific primary monoclonal antibody with preimmune mouse immunoglobulin of the same subclass (IgG2a) at the same concentration (Sigma Chemical) on serial sections resulted in the absence of staining.

RESULTS

Production of Antihuman FSH Receptor Monoclonal Antibodies. Mice were immunized with the fusion protein Ub-hFSHR (23−358) (Figure 1). This protein contains the extracellular domain of the FSH receptor (excluding the signal peptide and the following six amino acids) fused to ubiquitin and containing at its C-terminal end a polyhistidine tract to allow its purification. The resulting hybridomas were screened with ELISA tests using the antigen but also βgal-hFSHR (23−171) and βgal-hFSHR (172−358) fusion proteins. The βgal fusion proteins were devoid of the ubiquitin moiety and thus only shared fragments of the receptor with the Ub-hFSHR protein. Fusion proteins with the human LH receptor Ub-hLHR (21−229) and βgal-hLHR (21−229) (see Figure 1) were also used as negative controls. Of the 371 hybridomas which were tested, 56 reacted with the antigen and 19 were specific for the FSH moiety of it. It was later confirmed that they reacted with the native immunopurified receptor.

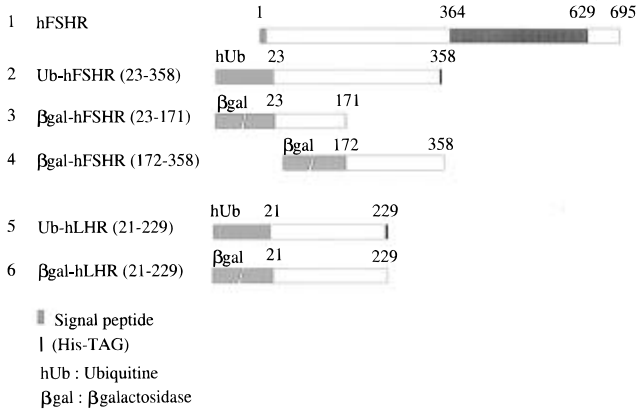

FIGURE 1: Receptor fragments expressed in *E. coli*. (1) The entire hFSHR is composed of the signal peptide (amino acids 1−17), the extracellular domain (amino acids 18−364), the transmembrane domain (amino acids 365−629), and the intracellular domain (amino acids 630−695). (2) The protein Ub-hFSHR (23−358) was used for immunization of mice. It is tagged at its C-terminus with a poly-His tract which permits its purification (see Materials and Methods). (3, 4) The proteins βgal-hFSHR (23−171) and βgal-hFSHR (172−358) were used for the selection of antibodies directed against the extracellular domain of the hFSHR. (5, 6) The proteins Ub-hLHR (21−229) and βgal-hLHR (21−229) were used as negative controls.

All these hybridomas recognized the 172−358 fragment of the receptor and none the 23−171 fragment. It thus seems that the N-terminal part of the receptor is poorly antigenic. Five hybridomas were selected for further study, based mainly on the intensity of the reaction given in the ELISA tests.

Double Determinant Immunoassay of the Human FSH Receptor. Although all five monoclonals recognized the 172−358 fragment of the receptor, most of them bound to different epitopes and could therefore interact simultaneously with the receptor [only binding of antibodies 156 and 323 was mutually exclusive (not shown)].

A sandwich ELISA was established where plates were coated with antibody 18 and biotinylated antibody 323 was used to detect the bound antigen. The assay allowed us to detect 1 fmol of receptor (Figure 2).

It was also remarked that no signal was obtained if plates were coated with an antibody, then reacted with membrane extracts containing the receptor, and then probed with the same biotinylated antibody (Figure 2). The results of this experiment argue strongly against the presence of polymeric forms of the receptor in membrane extracts.

Immunoprecipitation and Immunopurification of the Receptor. The antigen used to prepare the monoclonal antibodies was the extracellular domain of the receptor expressed in *E. coli*. The epitopes recognized by the antibodies could

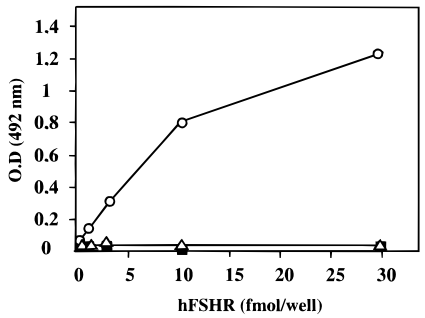

FIGURE 2: Double determinant immunoassay of the FSH receptor. The monoclonal antibodies 18 (○) or 323 (△) were coated onto 96-well plates. The membrane extracts (50 μL) containing the FSH receptor were incubated in the wells. After three washes, the biotinylated antibody 323 was used to detect the bound receptor. The immunocomplexes were revealed with a streptavidin biotinylated horseradish peroxidase/ABTS system. OD was measured at 492 nm. The values were compared with those obtained with a nonrelated antibody IDA 10 (■) (see Materials and Methods).

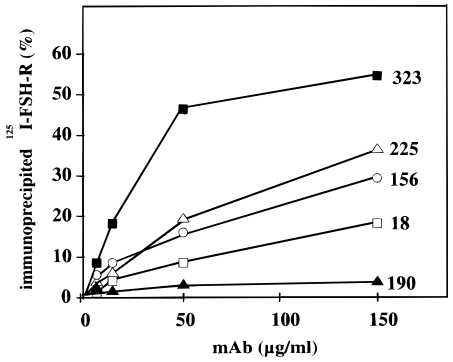

FIGURE 3: Immunoprecipitation of ^{125}I-FSH−receptor complexes with monoclonal antibodies. Triton X-100 membrane extracts of L cells (see Materials and Methods) containing ^{125}I-FSH−receptor complexes (5000 cpm in a total volume of 200 μL) were incubated with increasing concentrations of monoclonal antibodies (mAb 18, 156, 190, 225, 323). Anti-mouse immunoglobulin antibodies were added (200 μg per μg of mouse antibodies). After centrifugation, the immunoprecipitated complexes were counted for radioactivity. Results were expressed as percent of radioactivity which was precipitated at a range of concentrations of each antibody.

thus either be buried inside the native protein or be expressed at its surface. Only antibodies recognizing the latter would immunoprecipitate hormone−receptor complexes and would be useful for immunopurification of native receptor.

We thus prepared membranes from an L cell line expressing the hFSHR (see Materials and Methods), incubated them with ^{125}I-FSH, and solubilized the hormone receptor complexes. The complexes were then incubated with the different antibodies, and the resulting antigen−antibody complexes were precipitated with anti-mouse immunoglobulin antiserum. As shown in Figure 3, antibody 323 was the antibody most capable of precipitating hormone−receptor complexes in these immunoprecipitation experiments. It was thus selected for the immunopurification of the receptor.

Initial experiments involved human FSH receptor permanently expressed in a L cell line (see Materials and Methods). Triton X-100 extracts from membranes of these cells contained 0.62 pmol of receptor/mg of protein. A single-step purification through an immunomatrix containing antibody 323 allowed the purification of the receptor with a recovery of 75% (Table 1A).

The concentration of FSH receptor in human ovaries was markedly lower (0.054 fmol/mg of protein). It was thus necessary to establish that immunopurification could be carried out in these conditions. Indeed, as shown in Table 1B, chromatography through the immunomatrix resulted in purification of the receptor with a 33% yield.

The specific activities of receptor purified from the transfected L cells and from human ovaries were 11.7 and 7.4 nmol/mg of protein, respectively. The theoretical specific activity of a completely pure monomeric receptor of a molecular mass of 75 kDa would be ∼13.3 nmol/mg of protein.

Effect of the Monoclonal Antibodies on the Binding of the Hormone and on the Activation of Adenylate Cyclase. Membranes were prepared from L cells expressing the FSH receptor. They were incubated with ^{125}I-FSH and increasing concentrations of antibodies for 18 h at 4 °C. As shown in Figure 4 antibody 323 inhibited the specific binding of ^{125}I-FSH by 75%, and antibody 156 was slightly less efficient. Antibodies 18, 190, and 225 had a very low inhibitory efficiency.

A possible agonistic effect of the antibodies was then examined by incubating them with transfected L cells. As shown in Figure 5, they had a very limited effect on cAMP accumulation, especially when compared with the activity of FSH.

An antagonistic effect of the antibodies was then examined by incubating first the membranes with the antibodies and then adding FSH. Figure 6 shows that antibodies 323 and 156 inhibited adenylate cyclase activation by FSH. This activity was in agreement with their ability to inhibit FSH binding to the receptor (see Figure 4).

FSH Receptor Species Present in Transfected L Cells and in Human Ovaries. Various transcripts have been described for FSH receptor mRNA in numerous species including humans (Gromoll et al., 1992, 1993; Lapolt et al., 1992; Kahn et al., 1993). For the LH receptor, a mannose-rich precursor glycoprotein is present in high concentrations in target organs (Vu Hai et al., 1990; Vu Hai et al., 1992; Hipkin et al., 1992). The TSH receptor undergoes a cleavage reaction in the human thyroid (Loosfelt et al., 1992; Misrahi et al., 1994). It was important to analyze the FSH receptor protein species present in target organs and to compare them with the species present in transfected L cells. As shown in Figure 7, a very similar immunoblot pattern was observed using either extracts of ovary or the transfected L cells. The major immunoreactive protein species had a molecular mass of ∼87 kDa. A minor species of ∼81 kDa was also observed. To further identify these molecules, we submitted them to endoglycosidase digestion (Figure 8). When endoglycosidase F, which digests all carbohydrate moieties, was used, both receptor species were transformed into a 76 kDa protein moiety. When endoglycosidase H, which only digests mannose-rich carbohydrates, was used, the 87 kDa receptor was unaltered whereas the 81 kDa receptor was transformed into the 76 kDa protein. These results thus suggested that the 87 kDa species is the mature receptor containing complex carbohydrates, whereas the 81 kDa species is the precursor containing immature mannose-rich carbohydrates.

To further confirm this conclusion, a pulse−chase experiment was performed using the permanently transfected L cells. After 1 h of labeling, immunoprecipitation with

Table 1: Immunopurification of FSH Receptor from L Cells (A) and from Human Ovaries (B)[a]

	volume (mL)	protein (μg/mL)	receptor concentration (pmol/mL)	I^{125}-FSH (pmol/mL)	specific activity (pmol/mg of protein)
A					
membranes (30000*g* pellet)	4	970	ND	0.7	
Triton-X100 extract (supernatant of 100000*g* pellet)	4	240	0.7	0.5	2.9
flow-through	4	240	0.21	0.18	0.9
column wash	30	6.7	0	0	0
eluate	0.2	0.9	10.5	5.1	11 700
B					
membranes (30000*g* pellet)	200	8000	ND	4	
Triton-X100 extract (supernatant of 100000*g* pellet)	200	1500	5	3	0.003
flow-through	200	1500	1.5	1.2	0.001
column wash	250	6	0	0	0
eluate	1.5	0.03	222	63	7400

[a] The membranes were extacted with Triton X-100. The extract was applied to an immunomatrix. The flow-through and the wash fractions were collected. Finally the receptor was eluted from the immunoaffinity column. The protein concentration was measured by a BCA kit (Pierce). The receptor concentration was measured by a double determinant immunoassay using the antibody 18 and the biotinylated antibody 323 (see Materials and Methods). ^{125}I-FSH (2×10^6 cpm/mL, specific activity 90 μCi/μg) was incubated with the membranes, and the recovery was calculated at the different steps of the purification procedure.

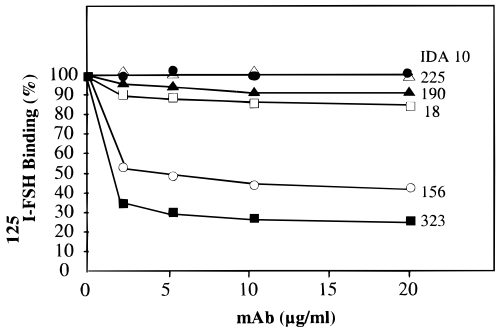

FIGURE 4: Inhibition of ^{125}I-FSH binding to FSHR by monoclonal antibodies. Membranes were prepared from L cells permanently expressing hFSHR. Membrane suspensions (50 fmol of hFSHR in 100 μL) were incubated with ^{125}I-FSH (10 000 cpm, 35 fmol) and increasing concentrations of monoclonal antibodies. The incubations were stopped by addition of 1 mL of ice-cold buffer. After centrifugation, the tubes containing the membrane pellets were counted for radioactivity. IDA 10 is a nonspecific antibody (see Materials and Methods). Results are presented as the percent reduction in ^{125}I-FSH binding induced at different concentrations of each antibody.

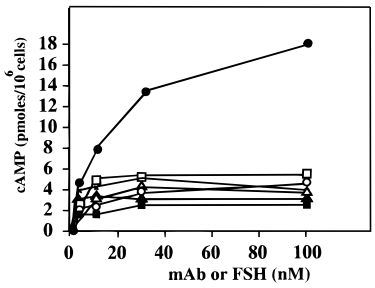

FIGURE 5: Stimulation of cAMP production by anti-hFSHR monoclonal antibodies. L cells permanently expressing hFSHR were plated and incubated with increasing concentrations of antibodies: 18 (□), 156 (○), 190 (▲), 225 (△), and 323 (■). For each concentration of antibodies, adenylate cyclase activity was measured in the presence of the same concentration of FSH (●). Control adenylate cyclase activity was measured by incubating the cells with hCG (these L cells are devoid of hCG/LH receptors) (×). The incubations were stopped by aspiration of the medium and addition of perchloric acid to the cells. cAMP was measured by radioimmunoassay in the cell extracts (see Materials and Methods).

antibody 323 yielded only the 81 kDa species. A chase of 2, 4, and 6 h allowed us to observe a progressive reduction of the intensity of labeling of the 81 kDa glycoprotein and

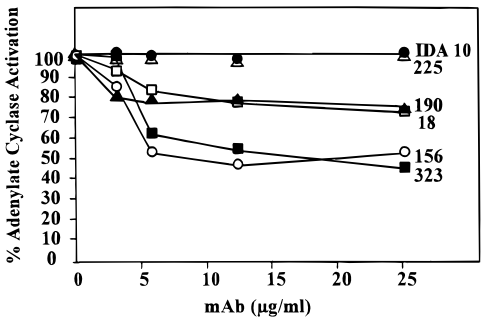

FIGURE 6: Inhibition of activation of adenylate cyclase by anti-FSHR antibodies. L cells permanently expressing the hFSHR were plated and incubated with various concentrations of monoclonal antibodies in DMEM and 0.5 mM IBMX. FSH (100 nM) was added. cAMP was measured by radioimmunoassay (see Materials and Methods). The results were expressed as percent of FSH stimulation measured in the absence of antibody.

the appearance of the 87 kDa species (Figure 9). These results were compatible with the 81 kDa species being a precursor of the 87 kDa receptor.

Immunocytochemical Detection of the Receptor. Initial experiments were performed on COS-7 cells transfected with an expression vector encoding the receptor. All the antibodies used reacted with these cells whereas they did not react with cells transfected with the control vector lacking the receptor sequence (not shown). However, such transfected cells express unphysiologically high levels of protein. It was thus necessary to verify if these antibodies were also able to detect the receptor in physiological conditions.

Antibodies 156 and 323 gave the strongest immunolabeling in the testes and ovaries (Figure 10). In the human ovary, granulosa cells of large antral follicles were labeled. This labeling was localized on the membrane (Figure 10A). Endothelial cells of the vessels of the thecal capillary plexus surrounding the larger follicles were also stained. This staining was not observed when anti-FSH receptor antibodies were replaced by a nonrelated antibody of the same class. It was not decreased by 0.15% H_2O_2 treatment which extinguishes endogenous peroxidases. The presence of LH receptors in vascular endothelial cells of target organs has been described (Ghinea et al., 1994).

All Sertoli cells of the human seminiferous epithelium were strongly immunostained. The labeling was accentuated

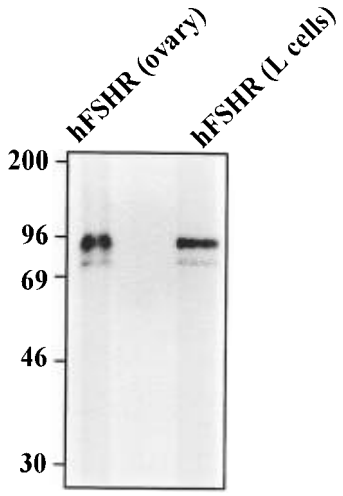

FIGURE 7: Immunoblot of FSHR in human ovaries and in L cells permanently expressing hFSHR. The immunopurified receptor preparations (see Materials and Methods) were resolved by SDS−PAGE in reducing conditions and detected by immunoblot with the monoclonal antibody 323. The size of the molecular mass markers is indicated on the left in kDa.

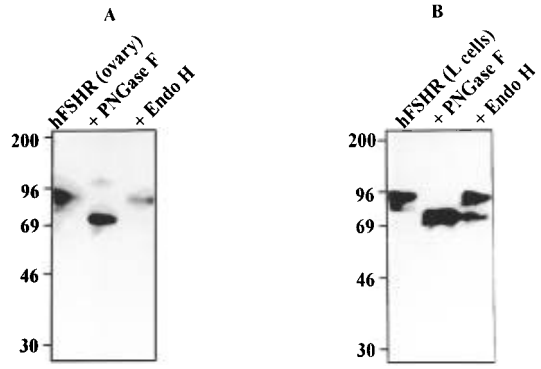

FIGURE 8: Endoglycosidase digestion of hFSHR in human ovaries and in L cells permanently expressing hFSHR. The receptor was immunopurified from human ovaries (A) or from L cells (B). Samples were either deglycosylated with N-glycosidase F (PNGase F) or with endoglycosidase H (Endo H) (see Materials and Methods). Control samples (hFSHR) were incubated in the absence of enzymes. After SDS−polyacrylamide gel electrophoresis, the reaction products were detected by immunoblotting with monoclonal antibody 323. Sizes of molecular mass markers are shown on the left in kDa.

at the basal pole of the cells and around the spermatogonia (Figure 10B,C). The endothelial cells of the small vessels in the interstitial spaces were also stained (not shown).

DISCUSSION

We have purified the extracellular domain of the human FSH receptor after expression in *E. coli*. Immunization of mice allowed us to prepare a panel of monoclonal antibodies. These antibodies were used to design a sandwich ELISA for the receptor, to immunopurify the receptor in a single step from both a permanently transformed L cell line and from human ovaries, and to study the receptor by immunoblotting and by immunocytochemistry. Some of the antibodies inhibited the binding of hormone to the receptor and partially inhibited the activation of adenylate cyclase. However, these antibodies (especially antibody 323) were very effective for the immunoprecipitation of hormone−

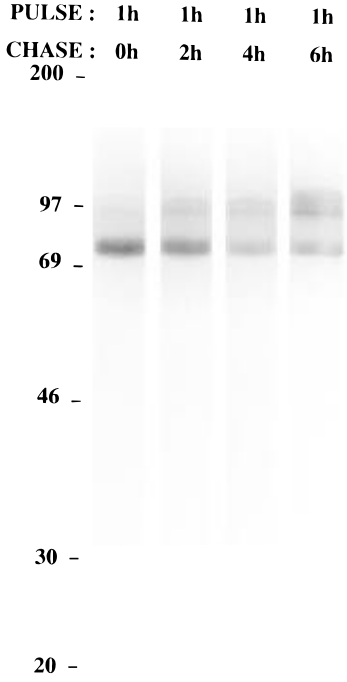

FIGURE 9: Pulse−chase of the hFSHR permanently expressed in an L cell line. L cells were pulse labeled with [^{35}S]methionine and [^{35}S]cysteine for 1 h and chased with a medium containing unlabeled amino acids. Total membrane extracts were prepared. The FSH receptor was immunoprecipited with monoclonal antibody 323 (see Materials and Methods). Purified proteins were resolved by SDS−PAGE in reducing conditions. When the nonrelated antibody IDA 10 was used there was no precipitation of radioactive proteins (not shown).

receptor complexes, showing that fixation of antibody and hormone was not mutually exclusive. Thus, either the binding sites for hormone and antibody only partially overlap or binding of either of them favors a receptor conformation yielding a lower affinity for the other. None of the antibodies had appreciable agonistic activity, i.e., was able to activate adenylate cyclase through binding to the FSH receptor.

Immunoblot experiments were particularly important since they allowed us to define the receptor species present in target cells. The main receptor species found in the ovary was a ~87 kDa glycoprotein containing complex mature carbohydrates. There was also a minor species of ~81 kDa which was shown to be the mannose-rich precursor glycoprotein. The accumulation of such a precursor has also been shown to occur for the LH receptor in pig (Vu Hai et al., 1990, 1992) and rat (Hipkin et al., 1992) testes. It thus seems to be a characteristic of gonadotropin receptors but does not occur in physiological conditions for other G-protein coupled receptors, including the TSH receptor (Malbon et al., 1991; Loosfelt et al., 1992; Misrahi et al., 1994). In transfected cells, the accumulation of such a precursor is more frequently observed probably because the biosynthetic capacity of such cells is overwhelmed by the extremely high level of synthesis of the protein. Expression of rat receptor in 293 cells yielded a 89 kDa mature receptor and a 82 kDa precursor protein (Davis et al., 1995). The reason for the presence of such precursors in relatively high concentrations in the gonads is not understood. It may be a source of receptor which can be rapidly mobilized in specific physiological situations. It has been shown that the precursor for LH receptor does not bind the hormone (Vu Hai et al., 1990; Hipkin et al., 1992)

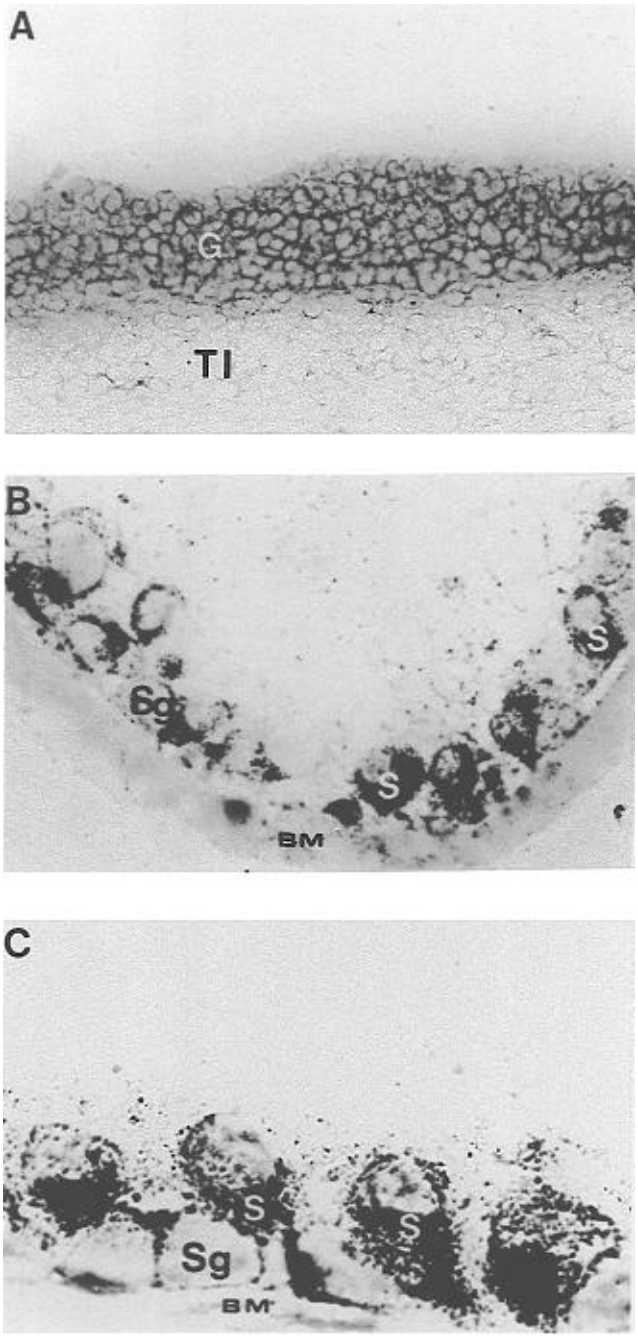

FIGURE 10: (A) Large antral follicle: immunolabeling with anti-FSH receptor antibody 323. All granulosa cells are stained, the labeling is uniformely distributed on the cellular membranes (×125). G, granulosa; TI, theca interna. (B and C) Seminiferous tubule: all Sertoli cells are immunostained with anti-FSH receptor antibody 323. The staining is stronger on the basal pole of the cells and around the spermatogonia. S, Sertoli cells; Sg, spermatogonia; BM, basement membrane (B, ×250; C, ×1200).

and is localized inside the cells in the Golgi−endoplasmic reticulum membranes (Hipkin et al., 1992).

RNA analysis has shown the presence of several FSHR transcripts arising from alternative splicing in target organs (Gromoll et al., 1992, 1993; Lapolt et al., 1992; Kahn et al., 1993). Immunoblot experiments were thus especially important in order to establish if variant proteins were expressed. No such variant could be detected. Thus it seems that either these variant proteins are secreted from the cells or their concentration is very low and could not be detected by immunoblot. In the case of the pig LH receptor, the variants, although secreted for the major part, were detectable by immunoblot in the same conditions as these employed here for FSH receptors (Vu Hai et al., 1992).

Several reports have described glycosylated FSH receptors of molecular masses lower than the 76.5 kDa calculated from the amino acid composition (Dattareyamurty et al., 1993; Quintana et al., 1993). It was thought possible that the FSH receptor underwent cleavage, similarly to that described for the TSH receptor (Loosfelt et al., 1992; Misrahi et al., 1994). However, the immunoblot experiments performed here did not reveal any evidence of cleaved receptor. Oligomeric structures (especially tetramers) have been described for solubilized FSH receptor (Dattatreyamurty et al., 1990, 1993). The results of our ELISA experiments strongly argue against this possibility. When the receptor was bound by an antibody adsorbed to the plastic plates, the same antibody which had been biotinylated could not attach to the complex. The contrary would have been expected from oligomeric structures.

The immunoblot experiments also showed a difference in cell-specific glycosylation with LH and TSH receptors. The mature LH and TSH receptors contain more carbohydrates when transfected into L cells than when expressed in their target cells (Vu Hai et al., 1990, 1992; Loosfelt et al., 1992; Misrahi et al., 1994). Such differences in glycosylation have been previously described for several proteins (Hugues et al., 1990). In the case of the FSH receptor, no such difference was observed.

The development of a sensitive ELISA for the extracellular domain of the receptor should facilitate the search for secreted variants of the receptor in blood or in follicular fluid (Dattatreyamurty et al., 1994; Anasti et al., 1995). Furthermore easy immunopurification of the native receptor from the permanent L cell line should allow us to examine the possibility of the existence of an autoimmune disease involving the FSH receptor (Dias et al., 1982; Chiauzzi et al., 1982). The existence of stimulatory or inhibitory anti-TSH receptor antibodies in various thyroid diseases (Weetman et al., 1994) evokes the possibility of similar situations for gonadotropin receptors.

The recombinant protein used here to immunize mice has also be shown to provoke the formation of antireceptor antibodies in monkeys (G. F. Weinbauer, E. Nieschlag, H. Loosfelt, B. Vannier, and E. Milgrom, unpublished observations). It thus opens an interesting possibility towards immunization with the FSH receptor in humans, especially as a means of contraception in men (Simoni et al., 1993).

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