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Functional, Structural, and Distribution Analysis of the Chorionic Gonadotropin Receptor Using Murine Monoclonal Antibodies

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LH and human chorionic gonadotropin (hCG) control steroid production and gametogenesis. They also function as growth factors through interaction with a specific receptor that is a member of the seven-transmembrane receptor family coupled via G proteins to signal pathways involving cAMP and phospholipase C/inositol 3 phosphate.

For this study, monoclonal antibodies (mAbs) were raised against the human LH receptor (LHR)/hCG receptor (hCGR), using Chinese hamster ovary LHR-transfected cells as the immunogen. Two reagents were then selected on the basis of their ability to recognize the full-length transmembrane receptor expressed both by Chinese hamster ovary LHR-transfected cells and by a limited number of tumor cell lines.

One of these mAbs reacts with the LHR/hCGR in tissue sections of both frozen and paraffin-embedded specimens. This unique feature allowed us to map the cytological distribution of LHR/hCGR in human breast tissues at different stages of development in physiological and benign pathological conditions. The same mAb proved to be agonistic: receptor ligation elicits signals that modulate the growth of selected breast tumor cell lines. This observation suggests that the mAb recognizes an epitope that is included in the domain of the receptor involved in the interaction with the natural ligand. (*J Clin Endocrinol Metab* 88: 5537–5546, 2003)

THE LH/HUMAN chorionic gonadotropin (hCG) receptor (LH/hCGR) is a member of the glycoprotein hormone receptor family, which includes FSH and TSH receptors (1). The LHR is made up of two distinct domains of roughly the same size, the N-terminal ligand-binding domain and the C-terminal domain, and contains seven-transmembrane helices and their connecting loops (2, 3). The LHR is coupled to the cAMP (4, 5) and to the phospholipase C/inositol 3 phosphate signal transduction pathways (6).

LH and hCG (the ligands of LHR) are members of a single dimeric glycoprotein hormone family and share a common α-subunit noncovalently bound to a hormone-specific β-subunit that differs from hormone to hormone (1). The structural similarity of the β-subunits mirrors the functional similarity between LH and hCG pituitary hormones that bind the same receptors and exert similar functions (7).

Until recently, LH and hCG were presumed only to regulate ovarian and testicular steroidogenesis (1). Current evidence reveals that the endocrine effects of LH and hCG are flanked by paracrine effects influencing the behavior of a number of tissues that express the specific receptors (*e.g.* the female and male reproductive tracts, the fetoplacental unit, brain, hippocampal cells, skin, breast, urinary bladder, and sperm) (8–13).

Abbreviations: CHO, Chinese hamster ovary; CHO-LHR, CHO cells transfected with the cDNA of human LHR; GaMIgG, goat antimouse IgG; hCG, human chorionic gonadotropin; hCGR, hCG receptor; IMDM, Iscove's MEM; LHR, LH receptor; mAb, monoclonal antibody; rLH, human recombinant LH.

Strategy for the prevention of breast cancer have been devised on the basis of observation that pregnancy at a young age offers protection against the development of breast cancer (14, 15). Russo *et al.* (16–18) have observed that the induction of mammary tumors in normal rats by dimethylbenzanthracene can be delayed or inhibited by treatment with hCG. The *in vivo* results have been confirmed *in vitro*, where hCG exerts direct antiproliferative effects on human breast epithelial cells in culture (19). Contrasting results were reported over 20 yr ago, with hCG featuring growth-promoting activities on breast cancer cells in a different mouse model (20).

Dual effects have also been reported in other models. It is thought that hCG may also offer protection against prostate cancer (21), whereas it may enhance the development of epithelial ovarian carcinoma (22) and the growth of bladder (23) and lung cancer cell lines (24).

Like other hormone receptors, such as estrogen and progesterone receptors, LH/hCGR is characterized by heterogeneous expression by tumors of the same histotype. The stimulating or inhibitory effect of LH/hCG on the growth of tumors of different origins implies that the corresponding tissues express the appropriate receptors.

A means of rapidly identifying *in vitro* expression of the LH/hCGR would represent a clear asset to research and would also have tremendous diagnostic potential.

In the current study, mAbs specific for the LH/hCGR were raised and two specific reagents adopted. One of these mAbs reacts with the LH/hCGR even on formalin-fixed and par-

affin-embedded tissues and displays agonistic activities *in vitro*.

Materials and Methods

Production and selection of murine mAbs specific for LH/hCGR

Female Balb/c mice (Charles River, Calco, Italy) were injected ip with 10^7 CHO cells transfected with the cDNA of human LHR (CHO-LHR) (kindly provided by T. Arulanandam, Serono Research Biotechnology Institute, Rockland, ME) and the injection was repeated four times every 15 d. Four days after the last injection, splenocytes were fused with the murine HGPRT$^-$ myeloma P3.X63.Ag8.653 (25). The selected primary cultures were cloned at least three times and expanded *in vitro* in conventional culture conditions and *in vivo* in Pristane-primed Balb/c mice.

Purification of endotoxin-free mAb

Ascitic fluids collected from the mice were purified by HPLC (Beckman Gold 125/166 and 126/166NM, Beckman, Milan, Italy). Briefly, samples were passed through a 100 × 25 mm Affi-Prep protein A MAPS column (Bio-Rad, Milano, Italy). Bound IgG were then eluted with citric acid (pH 4) and dialyzed against sodium phosphate buffer (pH 6.7). The eluted Ig were bound to the hydroxylapatite on a high performance hydroxylapatite preparative column and eluted with the appropriate gradient. The final purification step involved detoxi-gel chromatography on Polimyxin B-Sepharose (Pharmacia, Uppsala, Sweden). The presence of endotoxins in the final product was determined using the *Limulus Amebocyte Lysate* Pyrogent test (BioWhittaker, Inc., Walkersville, MD) (26).

Immunoprecipitation of the target antigen

Iodo-Gen (Pierce Chemical Co., Pero, Italy) was dissolved in chloroform at 0.25 mg/ml and the solvent evaporated. The CHO-LHR and CHO-mock cells (5×10^7) were added to 0.5 ml of PBS containing 1.85 MBq of carrier-free Na ^{125}I (Amersham, Milano, Italy) for 15 min at 20 C. Samples were then transferred to saturated tyrosine-PBS solution and washed to remove unbound [^{125}I]iodide. Immunoprecipitation was performed as described in (27). Briefly, ^{125}I-surface-labeled cells were lysed and after preclearing postnuclear cell lysate with goat antimouse IgG (GaMIgG) bound to Sepharose, the target molecule was immunoprecipitated for 12 h at 4 C with 5 μg of purified mAb followed by the addition of GaMIgG bound to Sepharose for 2 h at 4 C. The immunocomplexes were then subjected to SDS-PAGE and the gels exposed for autoradiography.

Western blot analysis

It was performed using CHO-LHR and CHO-mock cells membranes isolated after cell lysis. Twenty micrograms of protein were electrophoresed on 10% SDS-PAGE under reducing conditions and electrotransferred onto polyvinylidene difluoride membranes. Membranes were incubated with 5% BLOTTO (Amersham) in Tris-HCl (pH 7.4), 0.15% Tween 20, for 2 h at room temperature. The selected mAbs (5 μg/ml) were applied onto the membrane overnight at 4 C. After rinsing with Tris-HCl, the membranes were incubated with peroxidase-conjugated GaMIgG antibody for 1 h at room temperature. Specific signals were detected using the enhanced chemiluminescence method. The molecular size of the proteins of interest was determined by running molecular size marker proteins in an adjacent line.

Characterization of the reactivity of the selected mAbs

The selected mAbs were analyzed on a panel of viable normal cells peripheral blood mononuclear cells and endothelial cells and tumor cell lines including MCF-7, MDA/MB-231, SKBR-3, and T47-D (human breast cancer), MCF-10 (nonmalignant breast), TSA and AG12 (murine breast cancer), NIH-OVCAR 3 (ovary cancer), HEC-1-B (endometrial cancer), Hep G2 (hepatocellular cancer), CF-PAC1 (pancreatic cancer), LoVo (colon cancer), Kato III (gastric cancer), PC-3 (prostatic cancer), U-251 and Lan-1 (neuroblastoma) and G4 (glioma). Briefly, cells (3×10^5/sample) resuspended in PBS were incubated for 30 min at 4 C with 100 μl of the supernatants from the hybridoma cultures. After washing, cells were incubated for 30 min at 4 C with fluorescein isothiocyanate-labeled GaMIg (LiStarFISH, Milano, Italy). Fluorescence was analyzed by means of a FACSort (Becton Dickinson, Milano, Italy). Background mAb binding was estimated by means of isotype-matched negative control mAbs.

Immunohistochemical analysis of the distribution of the LH/hCGR

Ovary and testis (positive controls) as well as breast biopsies were obtained from the file of the local Department of Pathology. Tissues were fixed in 10% formaldehyde buffer (pH 7.4) for 24 h, paraffin embedded, and processed for immunohistochemical analysis. Endogenous peroxidase and endogenous biotin were blocked by means of incubation with 6% H_2O_2 and with specific avidin- and biotin-blocking reagents (Biogenex, San Ramon, CA), respectively. Sections were incubated with normal goat serum (1 : 20) for 2 h at 37 C and successively with the primary 20C3 mAb (0.5 μg/ml) for 2 h at 37 C. After washing, sections were incubated for 13 min at 20 C with biotinilated GaMIgG (StrAviGen Multilink Kit, BioGenex), then washed and incubated with peroxidase-conjugated streptavidin for 13 min at 20 C. The reaction was developed in a solution containing 3,3′-diaminobenzidine (LiquidDAB Substrate Pack, BioGenex). Nuclei were counterstained with Mayer hemallum.

CHO-LHR and CHO-mock cells were centrifuged, and the pellets were processed either for cryostatic or paraffin sections which were used to test the mAb reactivity and specificity. Furthermore, the specificity of the selected mAb was assessed after adsorption with CHO-LHR cells and successively used on tissue sections.

Competition between mAbs and ligands to target hCGR

Purified mAbs and human recombinant LH (rLH) (Serono International, Geneva, Switzerland) were iodinated using the chloramine-T technique. The binding properties were characterized using a radioligand receptor assay with CHO-LHR cells as the target and CHO-mock cells as the negative control. Competition experiments were performed by coincubating cells (2×10^6/ml) with the relevant ^{125}I-labeled ligand in the presence (or in the absence) of the selected mAb for 2 h at 4 C. After extensive washings, the bound radioactivity was evaluated in a γ-counter.

Analysis of signals mediated by LHR ligation

Intracellular cAMP assay. CHO-LHR cells were plated in 24-well plates (4×10^5/well) and incubated for 24 h in Iscove's MEM (IMDM) (Sigma, Milano, Italy) supplemented with 5% FCS. Before treatment, cells were washed twice with serum-free IMDM and incubated in IMDM containing isobutylmethylxantine (0.1 mg/ml) for 15 min at 37 C. After adding increasing amounts of recombinant hCG (Serono International, Geneva, Switzerland) or purified mAbs, the incubation was increased to 90 min at 37 C. Then the medium was removed, the cells rinsed once with IMDM without isobutylmethylxantine and lysed in 0.1 M HCl. Intracellular cAMP concentrations were determined after acetylation of the samples using a direct cAMP enzyme immunoassay kit (sensitivity = 0.037 pmol/ml) (Sigma), according to the manufacturer's protocol.

Cell proliferation assay. Cell proliferation was assessed by a ^{3}[H]thymidine (Amersham) incorporation test. 1.2×10^4 cells/well were cultured in 96-well plates in the presence or in the absence of increasing amounts (range: 0.1 ng/ml–10 μg/ml) of soluble recombinant hCG or affinity purified pyrogen-free mAbs. Cultures were pulsed at predetermined times with 1 μCi/well ^{3}[H]thymidine, incubated for another 12 h, harvested onto filter plates, and the counts analyzed in a β-counter (Perkin-Elmer, Milano, Italy).

Results

Production and characterization of hCGR-specific mAb

The hybridomas obtained were selected on the basis of their ability to react on CHO-LHR cells. The positive clones

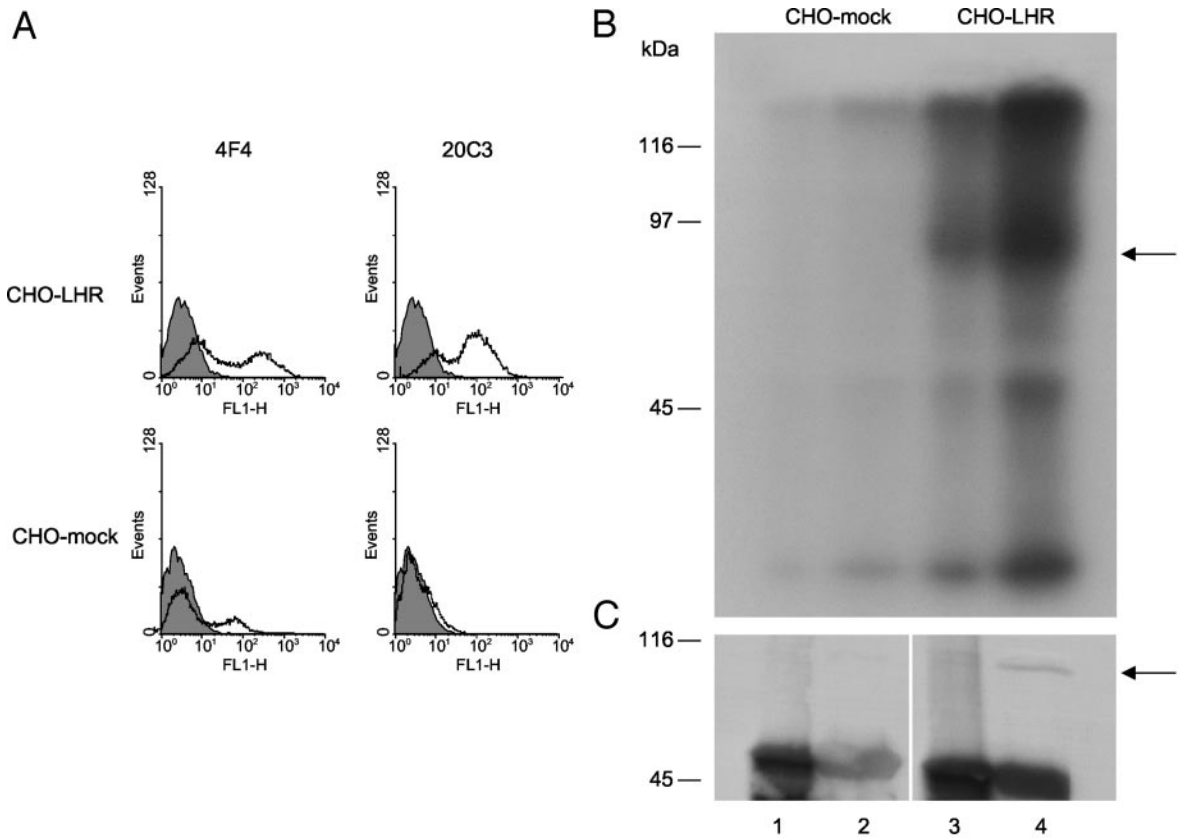

FIG. 1. A, Analysis of the reactivity of 20C3 and 4F4 mAbs on CHO-LHR and CHO-mock cells by means of FACS analysis. X-axis, Fluorescence intensity/cell; y-axis, number of cells registered/channel. Number of cells tested = 5000. B, Immunoprecipitation of LHR from ^{125}I-surface-labeled CHO-LHR and CHO-mock cells by means of 20C3 and 4F4 mAbs. SDS-PAGE profile of proteins precipitated with 4F4 mAb (lanes 1 and 3) and 20C3 mAb (lanes 2 and 4). Molecular mass of protein standards are indicated in kilodaltons. C, Western blot analysis. CHO-LHR and CHO-mock lysates were immunoprecipitated with 20C3 mAb, whereas proteins separated by SDS-PAGE were blotted onto nitrocellulose. The membranes were probed with 4F4 mAb (lanes 1 and 3) or 20C3 (lanes 2 and 4).

selected were unable to bind the CHO-mock cells. Two mAbs that showed the proper reactivity, namely 20C3 (IgG_{2a}) and 4F4 (IgG_1) (Fig. 1A), underwent three cycles of cloning by limiting dilution. The hybridomas were expanded *in vitro* and the culture supernatant was used for all the staining in suspension cells. The hybridomas were also grown *in vivo* as tumor ascites, used for purification. The purified and depyrogenized mAbs were used for staining tissue sections and for signal transduction experiments.

Immunoprecipitation experiments were carried out on ^{125}I-surface-labeled CHO-LHR and CHO-mock cells using both 20C3 and 4F4 mAb and analyzed with SDS-PAGE, revealing a predominant band with an apparent molecular mass of approximately 90 kDa and a minor band of approximately 50 kDa. The bands were not detected on CHO-mock cells (Fig. 1B). Moreover, immunoprecipitation obtained with either an irrelevant mAb or in the absence of the specific antibody failed to reveal the bands (data not shown), indicating that 20C3 and 4F4 specifically bind the LHR.

Immunoblotting analysis of CHO-LHR cells highlighted the presence of a major protein band of approximately 90 kDa identified by 20C3 and at a very low level by 4F4 mAb (Fig. 1C, lanes 3 and 4), whereas no reactivity was apparent on CHO-mock cells (Fig. 1C, lanes 1 and 2).

The binding of the selected mAbs was then analyzed on a panel of viable normal cells and tumor cell lines of different origins by means of the indirect immunofluorescence technique and FACS analysis. The results are summarized in Table 1. 20C3 and 4F4 mAbs displayed weak to moderate reactivity with all of the selected breast cancer cell lines so far analyzed and with the NIH-OVCAR-3 ovary carcinoma. The selected mAbs did not react with peripheral blood mononuclear cells (resting or activated), human keratinocytes, or tumor lines of the gastrointestinal tract. The characteristics of reactivity and fluorescence intensity on selected cell types were notably different for the selected mAbs. The range of 4F4 reactivity was much wider and included normal umbilical endothelial cells and selected brain tumors, *i.e.* LAN-1 (neuroblastoma) and G4 (glioma) cell lines.

Immunohistochemical analysis of LH/hCGR distribution in human breast

After noting that the 20C3 and 4F4 mAbs are reactive with breast cancer cell lines, the next step was to evaluate the presence of the LHR in normal and pathological human breast tissues. The analytical conditions were optimized in CHO-LHR cells processed as cryostatic samples and as paraffin-embedded tissues. On fixed CHO-LHR cells 4F4 showed a weak reactivity, whereas 20C3 showed a strong

TABLE 1. Reactivity of the 20C3 and 4F4 mAbs on selected tumor cell lines

Cells	20C3 mAb	4F4 mAb
CHO-LHR	+++	+++
CHO-mock	−	−
PBMC	−	−
HEC (endothelial cells)	−	+
NCTC (human keratinocytes)	−	−
MCF-7 (human breast cancer)	+	++
MDA/MB-231 (human breast cancer)	−	++
SKBR-3 (human breast cancer)	+	++
T47-D (human breast cancer)	−	+
MCF-10 (nonmalignant breast)	−	−
TSA (murine breast cancer)	−	−
AG-12 (murine breast cancer)	−	−
NIH-OVCAR 3 (ovary cancer)	−	−
HEC-1-B (endometrial cells)	−	+
Hep G2 (hepatocellular cancer)	−	−
CF-PAC1 (pancreatic cancer)	−	−
LoVo (colon cancer)	−	−
Kato III (gastric cancer)	−	−
PC-3 (prostatic cancer)	−	−
U-251 (neuroblastoma)	−	−
LAN-1 (neuroblastoma)	−	+
G4 (glioma)	−	+

−, Less than 10% positive cells; +, 10–30% positive cells; ++, 31–60% positive cells; +++, more than 61% positive cells.

and reproducible reactivity at both the membrane and cytoplasmic levels (Fig. 2, A and C). No reactivity was observed in the CHO-mock cells (Fig. 2, B and D). 20C3 mAb reactivity on CHO-LHR cells was completely abolished by adsorption of the mAb with the same cells (data not shown).

The reactivity of the 20C3 mAb on human ovary was restricted to theca interna cells (Fig. 2, E and F), whereas on testis it was restricted to the Leydig's cells (Fig. 2, G and H).

The analysis of hCGR expression was performed in normal human mammary gland (tested in discrete differentiative steps) and benign breast lesions. The ductal structures diverging from the nipple were not stained in fetal breast tissues (two cases at 28 and 26 wk gestation, respectively) (Fig. 3A). In more developed mammary gland, the expression of LH/hCGR was mainly restricted to basal cells of lobular structures. In adolescent mammary tissues obtained from a 13-yr-old girl affected by juvenile fibroadenoma, hCGR was expressed on basal cells, on scattered epithelial luminal cells and on stromal cells of the fibroepithelial lesion (Fig. 3B).

Normal tissues and benign breast lesions obtained from seven women of premenopausal age (ranging from 29–47 yr) showed diffuse membrane immunostaining of basal cells and of occasional luminal cells in the majority of lobular structures (Fig. 3, C and D). The intensity of mAb staining varied among the lobules and the reactivity was completely abolished when sections were tested with adsorbed mAb (data not shown).

In normal lactating gland the reactivity was restricted to occasional basal cells (Fig. 3E). In postmenopausal women, basal cell expression of hCGR was barely visible around lobules (Fig. 3F).

Tissue samples of five fibroadenomas, two benign papillomas, and one sclerosing adenosis obtained from pre- and postmenopausal women displayed intense membrane immunostaining of basal cells, and of occasional luminal epithelial cells (Fig. 3, G and H).

Epitope analysis

The epitope analysis of the determinants recognized by 20C3 and 4F4 mAbs was performed by assaying the ability of 4F4 mAb to compete with the binding of ^{125}I-labeled 20C3 mAb to CHO-LHR target cells, and, *vice versa*, the ability of 20C3 mAb to compete with ^{125}I-labeled 4F4 mAb. The results demonstrated that 4F4 mAb was partially able to interfere with 20C3 binding in a dose-dependent manner when used at high concentrations. However, the competition never exceeded 35–40% of the total binding (Fig. 4A). On the contrary, 20C3 inhibited approximately 20% of the binding of ^{125}I-labeled 4F4 mAb only when used at concentrations ranging from 10–20 μg/ml (data not shown).

Both mAbs were able to interfere with the binding of the natural ligands to LH/hCGR. Indeed, incubation of CHO-LHR target cells with purified 20C3 or 4F4 mAbs was followed by partial inhibition of ^{125}I-LH binding even when the mAbs were used at high concentrations (Fig. 4B). Expression of the 20C3 and 4F4 epitopes was increased by the addition of recombinant LH, as witnessed by the binding of the ^{125}I-mAbs in the presence of LH. The effect was weak (ranging from 10–25%) but dose-dependent and reproducible in several experiments (Fig. 4C).

Analysis of signals mediated by hCGR

Intracellular cAMP assay. Treatment of CHO-LHR cells with hCG resulted in a significant dose-dependent production of cAMP compared with CHO-mock cells. Ligation of LH/hCGR by the 20C3 mAb was followed by the induction of intracellular cAMP, although at lower levels than after hormone binding (Fig. 5). The addition of the 4F4 mAb in the same experimental conditions was unable to induce a detectable cAMP production.

Effects of hCG and of anti-hCGR mAbs on cell growth. The effects induced by the addition of hCG (10 ng/ml) were analyzed on the growth of MCF-7 and SKBR-3 breast cancer cell lines cultured in 5% FCS. These conditions did not influence the growth rate of the cells, which showed no apparent response to hCG treatment. The next step was to reduce the supply of the FCS. The MCF-7 and SKBR-3 cells were cultured in 0.5% FCS, thereafter becoming susceptible to the addition of hCG. Indeed, the growth rate was lower than that of the control cells cultured in similar conditions but without hCG. The next step was the evaluation of the effects induced by ligation of hCGR in the same cells by means of the mAbs. Experiments were done using 10 μg/ml of purified 20C3, 4F4 or isotype-matched control mAbs; both hCG and mAbs were added every 48 h. Growth inhibitory effects were seen on SKBR-3 and MCF-7 cells after the addition of the 20C3 mAb, whereas 4F4 mAb and other isotype-matched mAbs did not affect the cell growth. A kinetic analysis of the mAb-mediated effects demonstrated that they become apparent after 24 h in culture, peaking at d 3 (Fig. 6, A and B).

The effects induced by hCGR ligation by the 20C3 mAb on breast cancer cell proliferation were confirmed by monitoring [^{3}H]thymidine incorporation of MCF-7 and SKBR-3 lines

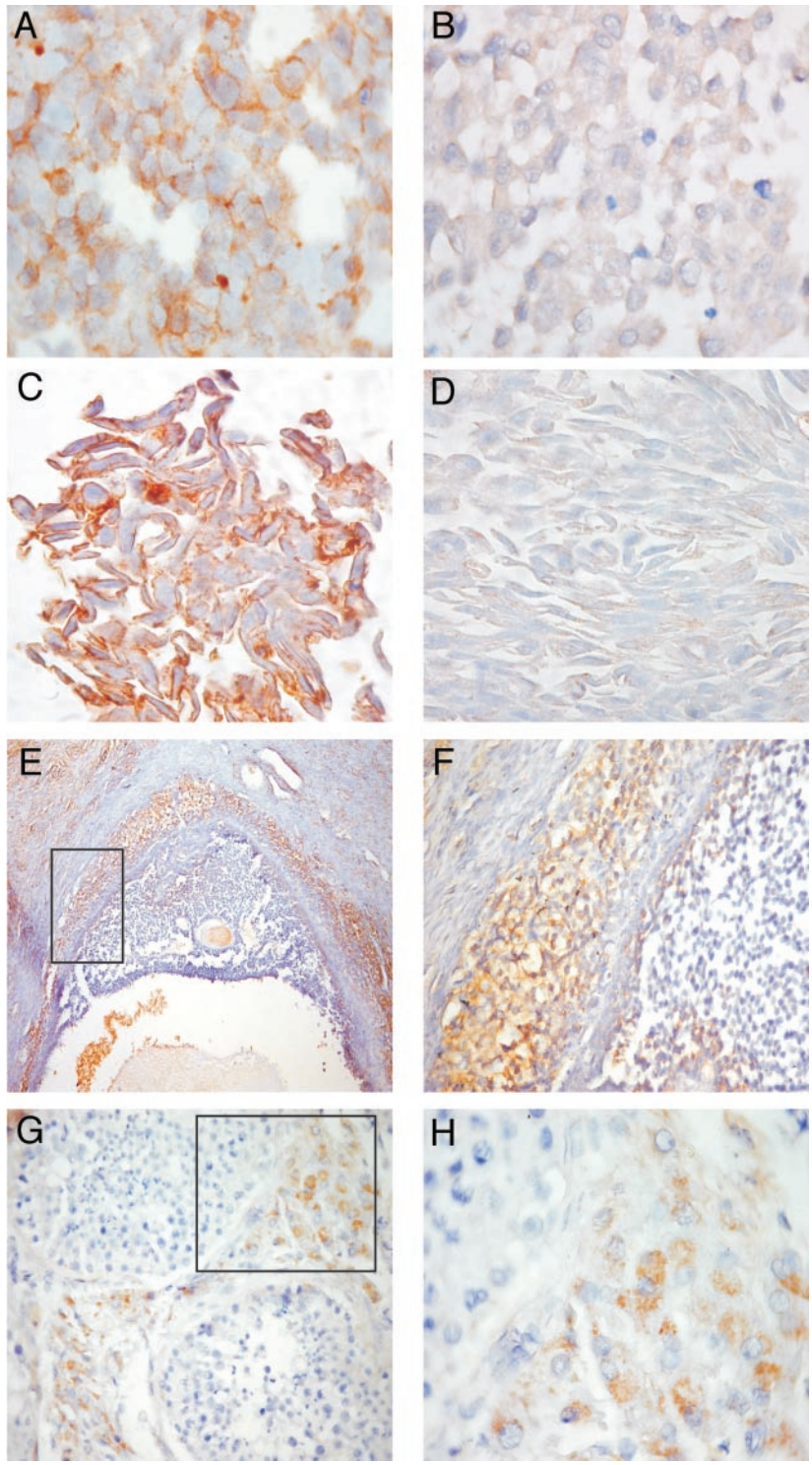

FIG. 2. Immunostaining with 20C3 mAb shows LHR expression (*brown staining*) on the cell membrane of CHO-LHR cells both on cryostatic sections (A) and on sections obtained from formalin-fixed paraffin-embedded cells (C). Frozen (B) and fixed (D) CHO-mock cells do not express LHR (original magnification, panels A–D, ×100). 20C3 mAb clearly reacts with LH/hCGR in normal formalin-fixed paraffin-embedded ovary (E and F) and testis (G and H) tissue sections. In ovary, the receptors are mainly expressed by *techa interna* cells (E, *inset*) (original magnification of panel, ×10). The immunostaining is localized on the cell membrane (F) (original magnification, ×40). In the testis, LH/hCGR is only expressed by Leydig cells (G, *inset*) (original magnification, ×20) and is localized both on the cell membrane and within the cytoplasm (H) (original magnification, ×100).

cultured in the presence of increasing amounts of 20C3 mAb. The experiments were performed in parallel with hCG, with the 4F4 mAb included as the negative control. The results obtained indicate that hCGR ligation by the 20C3 mAb is followed by a reduction of [^{3}H]thymidine incorporation by d 4 of culture for the MCF-7 and SKBR-3 lines. The inhibition of [^{3}H]thymidine uptake was dose-dependent and was effective even with 1 ng/ml of 20C3 mAb. No effects on cell proliferation were induced by ligation of the receptor by 4F4 mAb or by the control mAbs (Fig. 6C).

Discussion

The gonadotropin LH/hCG hormones play a fundamental physiological role in the control of steroid production and gametogenesis. The gonadal actions of LH/hCG result in

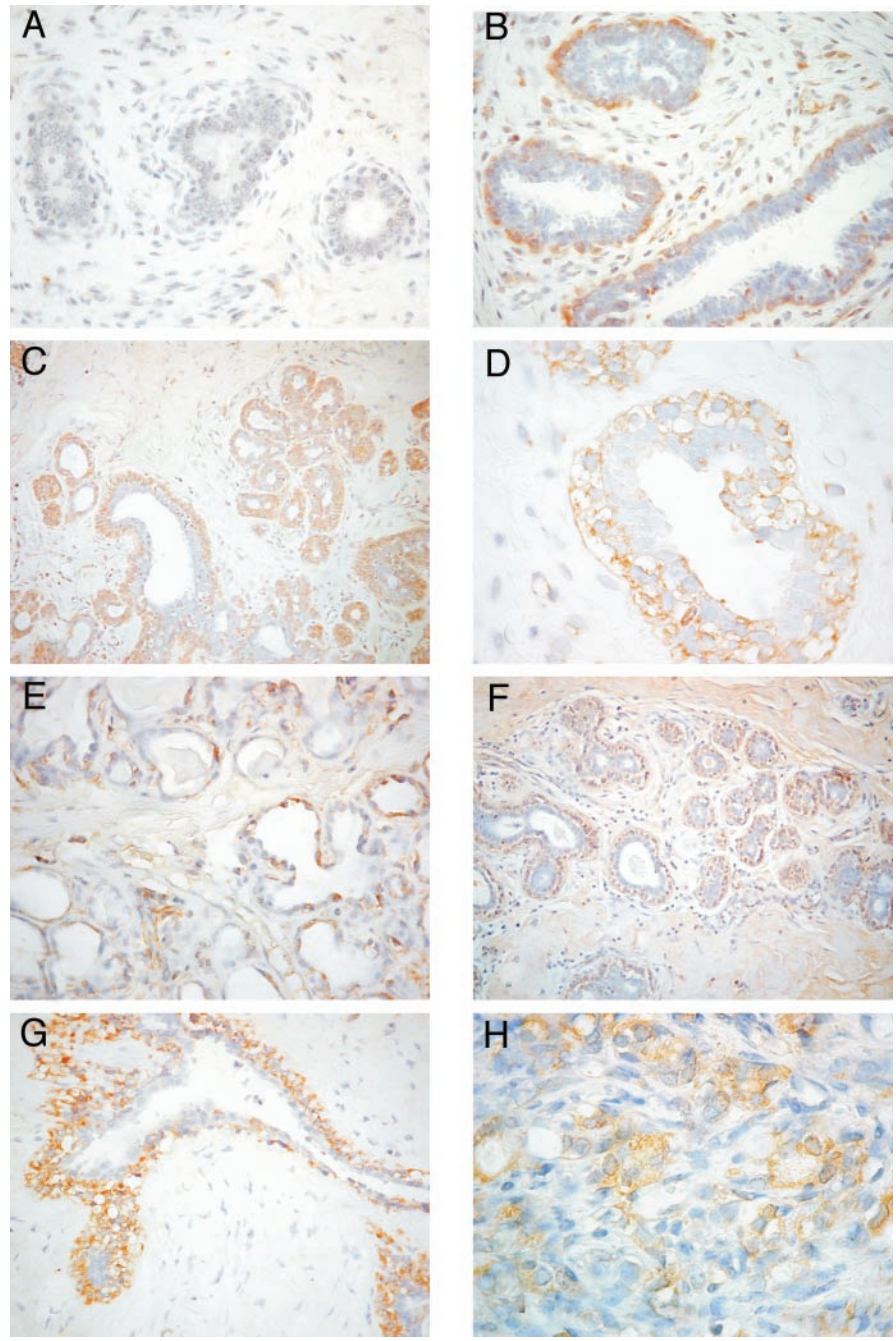

FIG. 3. Expression of LH/hCGR evaluated using 20C3 mAb in normal human mammary gland at various differentiative steps (A and C–F) and in benign breast lesions (B, G, H). Fetal tissue does not express LH/hCGR (A) (original magnification, ×40). Samples obtained from adolescent mammary gland affected by fibroadenoma shows LH/hCGR expression mostly in basal cells and in rare epithelial luminal and stromal cells (B) (original magnification, ×40). Premenopausal normal breast tissue expresses LH/hCGR in cells of lobular structures (C) (original magnification, ×10). The expression is localized on the membrane of basal cells and of occasional epithelial luminal cells (D) (original magnification, ×100). A sample of lactating mammary gland shows LH/hCGR expression restricted to rare basal cells of lobules (E) (original magnification, ×40). In premenopausal breast tissue, occasional basal cells of lobular structures express LH/hCGR (F) (original magnification, ×20). In benign breast pathology samples obtained from fibroadenoma (G) (original magnification, × 20) and sclerosing adenosis (H) (original magnification, ×100) receptor expression is restricted to the membrane of basal cells.

increased synthesis of steroid hormones, which in turn interact with different targets (28). The nongonadal actions of these hormones are not fully defined and vary according to the target organs and their physiological conditions (29). The biological effects mediated by gonadotropin hormones are modulated through their interaction with receptors expressed by a variety of tissues (30, 31). The density of the receptor as expressed by different tissues represents the principal mechanism of hCG modulation (32).

The focus of the present work was initially on the hCGR, which has been studied until now by exploiting hCG itself. The only mAbs reported to be specific for the receptor were raised against a chimeric protein (33) and are difficult to procure. The difficulties inherent in the direct use of hCG, together with the lack of a simple means of studying the distribution of the receptor or the signals channeled in normal and pathological samples prompted us to raise a panel of conventional mAbs specific for the human LHR/hCGR.

The strategy adopted allowed us to produce reagents recognizing the actual molecule expressed by various tissues, and was proven successful given that 1) one of the mAbs selected reacts with the hCGR in tissue sections, and, more importantly, 2) this occurs even when the tissues are fixed with conventional techniques. This makes it now possible to evaluate the samples previously stored in the Pathology Department files. The second major result of this work is that

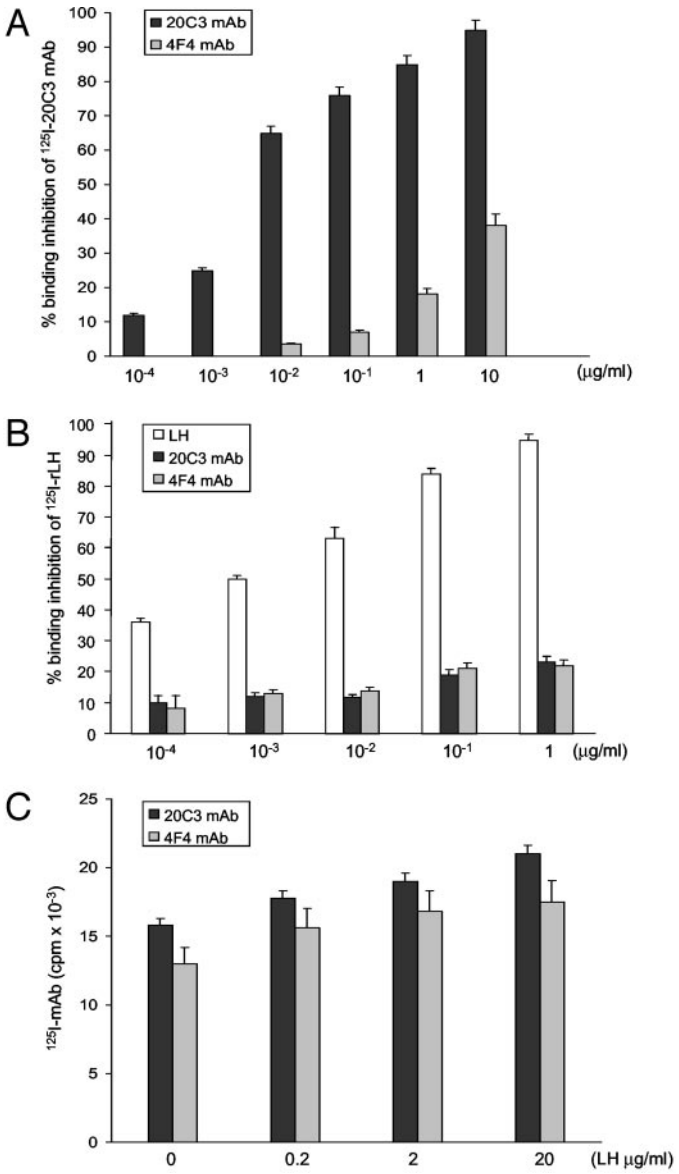

FIG. 4. A, Epitope mapping of 4F4 and 20C3 mAbs. CHO-LHR cells were incubated (2 h at 4 C) with 10^5 cpm of ^{125}I-20C3 mAb (specific activity, ~25 μCi/μg) in the presence of increasing concentrations of purified 20C3 or 4F4 mAb. All samples were performed in triplicate and results represent the percentage of ^{125}I-20C3 binding inhibition and are expressed as means ± SD of three experiments performed. B, Effects of 4F4 and 20C3 mAb on rLH binding to LHR. CHO-LHR cells were incubated with 5×10^4 cpm of ^{125}I-rLH (specific activity, ~18 μCi/μg) in the presence of increasing concentration of rLH or purified 4F4 and 20C3 mAbs. All samples were performed in triplicate and results represent the percentage of binding inhibition of ^{125}I-rLH and are expressed as means ± SD of three experiments performed. C, Effects of rLH on ^{125}I-20C3 and ^{125}I-4F4 mAb binding to CHO-LHR cells. CHO-LHR cells were incubated with 2×10^5 cpm of ^{125}I-20C3 (specific activity, ~25 μCi/μg) or ^{125}I-4F4 (specific activity, ~20 μCi/μg) in the presence of increasing concentrations of rLH. All samples were performed in triplicate and results represent the mAb binding and are expressed as means ± SD of three experiments performed.

the epitope of one of the mAbs produced (namely 20C3) is involved in (or includes) the domain of the receptor specific for the interaction with the ligand. Indeed, the binding between 20C3 mAb and the receptor is followed by the delivery

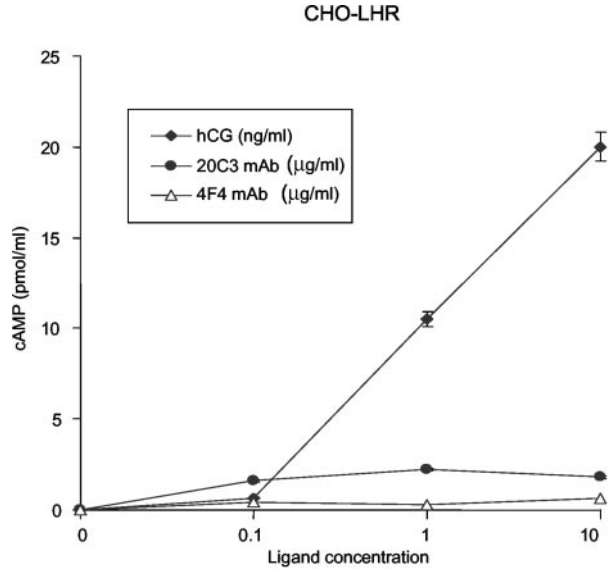

FIG. 5. LHR binding and cAMP induction. CHO-LHR cells were cultured in IMDM medium in the presence of isobutylmethylxantine (0.1 mg/ml) and incubated with increasing concentrations of hCG, 20C3 or 4F4 mAb as described in *Materials and Methods* and intracellular cAMP was evaluated after cell lysis using a direct cAMP enzyme immunoassay kit.

of signals, which are identical in some aspect to those channeled through the physiological interactions between receptor and ligand.

The target antigen immunoprecipitated from ^{125}I-labeled CHO-LHR cells demonstrates that 20C3 and 4F4 mAbs recognize a dominant surface protein with an apparent molecular mass of approximately 90 kDa, close to that predicted from the amino acid sequence of the mature transmembrane receptor (34). The protein of lower molecular mass (~50 kDa) may reflect a partial degradation of the larger protein.

LH/hCGRs have been found in man, monkey, mouse, rat, pig, cow, sheep, turkey, and fish (35–37), confirming that their expression is not species specific. The expression of LH/hCGR has been investigated by means of a wide range of technical approaches, including gene transcription, radio binding, signaling pathways, and biological responses (38–40). The availability of specific mAbs allowed the immunohistochemical localization of the LH/hCGR in human tissues even after fixation, which is the routine approach used in pathology. The tissue fixation procedure appears to be a crucial step in regulating successive immunostaining: indeed, the hCGR proves to be labile and easily degraded. Based on these findings, 20C3 mAb was selected as the analytical probe for the study of the tissue distribution of hCGR. The reactivity of 20C3 mAb was initially evaluated on ovary and testis to compare the results obtained in this work with the localization of the receptor determined with different techniques by other groups (41, 42). The results indicate that the hCGR was detected on theca interna cells of the antral follicles and on the Leydig cells of testis. The present results fit perfectly with those reported so far following different technical approaches (43), indirectly confirming the specificity of 20C3 mAb for the LH/hCGR.

The reactivity of 20C3 mAb on formalin-fixed tissues al-

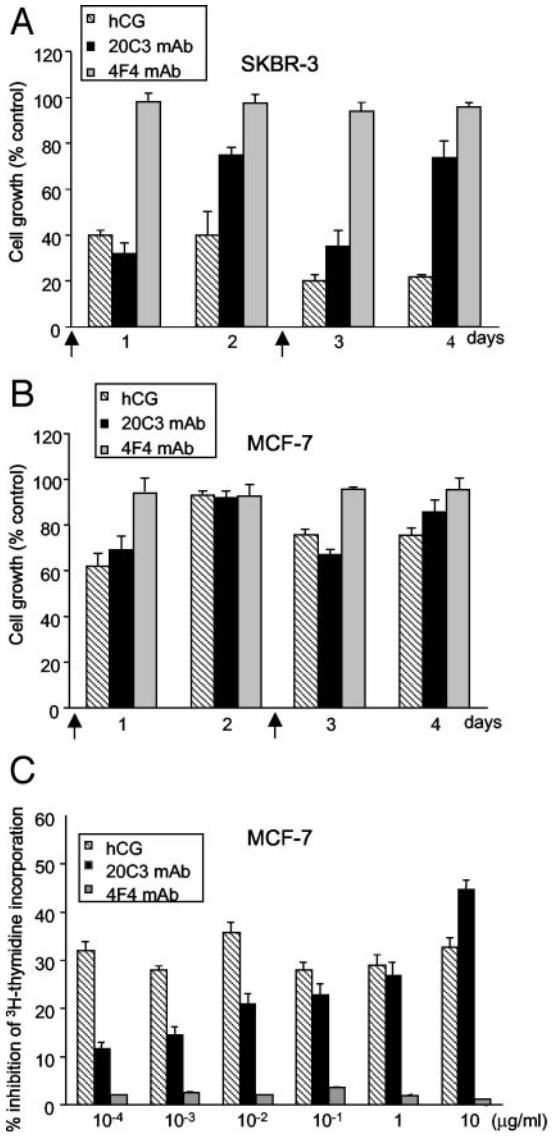

FIG. 6. Effect of 20C3 mAb on the growth of SKBR-3 (A) and MCF-7 (B) breast tumor cells. The cells (10^4/well) were seeded in 24-well plates and cultured for increasing lengths of time in the presence or in the absence of hCG (10 ng/ml), 20C3 (10 μg/ml), or 4F4 (10 μg/ml). HCG and mAbs were added at time zero and after 48 h (*arrow*). Cells were detached with a trypsin-EDTA solution and counted using a hemocytometer. Comparisons between treated cells and control cells were made at each time point. The control bars, which are considered 100%, are not presented. Results are expressed as means ± SD of three experiments performed. C, Effects of hCG and 20C3 mAb on MCF-7 breast tumor cell proliferation. Cells were cultured in the presence or in the absence of increasing concentrations of hCG, 20C3 or 4F4 mAb for 4 d. HCG and 20C3 mAb were added at time zero and after 48 h. Cultures were pulsed with 1 μCi/well ^{3}[H]thymidine and incubated for an additional 12 h. Counts were evaluated in a β-counter. The ^{3}[H]thymidine incorporation obtained in the control is considered 100%. Results represent the percentage of inhibition of ^{3}[H]thymidine uptake and are expressed as means ± SD of three experiments performed.

lowed us to map the cytological distribution of hCGR in human breast tissue at different stages of development. hCGR could not be detected in the mammary gland of female fetus. This is in line with the observation that women with hCG resistance have spontaneous breast development, whereas hCG stimulation is necessary for normal ovarian steroidogenesis and ovulation (44). In physiological and pathological conditions of the adult life, hCGR expression is detectable in breast tissue, as previously reported (33). The difference from previous reports is that 20C3 mAb detects the target molecule on the membrane of the basal cell layer, which is constituted of non differentiated cells and myoepithelial cells. Only occasional luminal epithelial cells are stained. The expression of the hCGR by basal cells is lower in postmenopausal samples. This result is in line with the notion that the hCGR is generally not detectable in the postmenopausal ovary, and down-modulated in other target organs during the same phase of life (45). Analysis of the distribution of hCGR on breast cancers using the 20C3 mAb is currently underway.

The results obtained thus far using the two mAbs suggest the existence of two discrete epitopes on the receptor. Binding competition experiments showed that 4F4 mAb can partially interfere with the binding of 20C3 mAb on the target cells. This suggests that the reagents likely recognize two different epitopes of the receptor and that steric hindrance is likely responsible for the partial displacement observed. The hypothesis of steric hindrance might also explain the interference observed in mAb and LH binding. An alternative explanation is that 20C3 mAb ligation induces conformational changes that affect the interaction of LH with its binding site. However, a difference in affinity of the mAbs compared with that of the natural ligand may be responsible for the effects observed.

Several breast cancer cell lines reportedly show a diminished growth when cultured in the presence of hCG (46). In the present work, the growth inhibitory effects mediated by the addition of hCG in culture are reproduced by the addition of the 20C3 mAb to the SKBR-3 and MCF-7 cell lines. It is reasonable to think that a mAb/receptor engagement is related to the hCGR-mediated signaling. This is consistent with the premise that hCG exerts a protective role against breast cancer in humans. It has been proposed that hCG induces the proliferative terminal end buds of mammary glands to differentiate into secretory type lobules *in vivo*, rendering the cells less susceptible to neoplastic transformation (47, 48). This hypothesis is supported by epidemiological studies indicating that early parity, late menarche and early menopause have protective effects against breast cancer (15). However, contradictory results were obtained by another group in a different mouse mammary adenocarcinoma model (20). The reasons for these experimental discrepancies are still unknown.

hCG binding to the LH/hCGR is coupled to the adenylyl cyclase and PLC signal pathways, thus leading to the production of cAMP and IP3 as intracellular signal molecules (34, 49). Engagement of the hCGR by the 20C3 mAb is followed by increased intracellular concentrations of cAMP, although significantly lower than those induced by ligand binding. This observation likely reflects the lower efficiency of mAb/receptor binding compared with the physiological hormone/receptor interactions. This finding is not surprising in light of existing evidence that hCG and its receptor undergo multistep interactions with alteration of the recep-

tor geometry, physical shape, and flexibility, leading eventually to total activation of the receptor (50, 51). It is likely that mAb ligation of the receptor is unable *per se* to induce the structural modifications necessary for eliciting efficient production of cAMP. Alternatively, the affinity of the mAb might be too low to elicit efficacious intracellular signals. The resolution of the crystal structure of hCG has provided clues about the early steps of hormone/receptor interactions and has made it possible to localize the binding domains of the receptor (52). Nonetheless, detailed information about gonadotropin pharmacology is limited, hampered by the difficulties in crystallizing the gonadotropin receptors, which are seven-transmembrane amphipathic proteins. However, large-scale production of the receptor ectodomain-hormone soluble complexes was recently proposed as a realistic strategy for obtaining soluble and secreted molecules stable enough to allow structural analysis and crystallization (53). This approach will offer new insights into the physiological mechanisms regulating the cross-talk of hormone and receptor.

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References

1. **Pierce JG, Parsons TF** 1981 Glycoprotein hormones: structure and function. Annu Rev Biochem 50:465–495
2. **Dufau ML** 1998 The luteinizing hormone receptor. Annu Rev Physiol 60: 461–496
3. **Xie YB, Wang H, Segaloff DL** 1990 Extracellular domain of lutropin/choriogonadotropin receptor expressed in transfected cells binds choriogonadotropin with high affinity. J Biol Chem 265:21411–21414
4. **Ji IH, Ji TH** 1991 Human choriogonadotropin binds to a lutropin receptor with essentially no N-terminal extension and stimulates cAMP synthesis. J Biol Chem 266:13076–13079
5. **Remy JJ, Couture L, Pantel J, Haertle T, Rabesona H, Bozon V, Pajot-Augy E, Robert P, Troalen F, Salesse R, Bidart JM** 1996 Mapping of HCG-receptor complexes. Mol Cell Endocrinol 125:79–91
6. **Davis JS, West LA, Farese RV** 1984 Effects of luteinizing hormone on phosphoinositide metabolism in rat granulosa cells. J Biol Chem 259:15028–15034
7. **McFarland KC, Sprengel R, Phillips HS, Kohler M, Rosemblit N, Nikolics K, Segaloff DL, Seeburg PH** 1989 Lutropin-choriogonadotropin receptor: an unusual member of the G protein-coupled receptor family. Science 245: 494–499
8. **Al-Hader AA, Lei ZM, Rao CV** 1997 Novel expression of functional luteinizing hormone/chorionic gonadotropin receptors in cultured glial cells from neonatal brains. Biol Reprod 56:501–507
9. **Bird J, Li X, Lei ZM, Sanfilippo J, Yussman MA, Rao CV** 1998 Luteinizing hormone and human chorionic gonadotropin decrease type 2 5 α-reductase and androgen receptor protein levels in women's skin. J Clin Endocrinol Metab 83:1776–1782
10. **Dirnhofer S, Berger C, Hermann M, Steiner G, Madersbacher S, Berger P** 1998 Coexpression of gonadotropic hormones and their corresponding FSH- and LH/CG-receptors in the human prostate. Prostate 35:212–220
11. **Pabon JE, Li X, Lei ZM, Sanfilippo JS, Yussman MA, Rao CV** 1996 Novel presence of luteinizing hormone/chorionic gonadotropin receptors in human adrenal glands. J Clin Endocrinol Metab 81:2397–2400
12. **Tao YX, Heit M, Lei ZM, Rao CV** 1998 The urinary bladder of a woman is a novel site of luteinizing hormone-human chorionic gonadotropin receptor gene expression. Am J Obstet Gynecol 179:1026–1031
13. **Eblen A, Bao S, Lei ZM, Nakajima ST, Rao CV** 2001 The presence of functional luteinizing hormone/chorionic gonadotropin receptors in human sperm. J Clin Endocrinol Metab 86:2643–2648
14. **Kelsey JL, Gammon MD, John EM** 1993 Reproductive factors and breast cancer. Epidemiol Rev 15:36–47
15. **Guzman RC, Yang J, Rajkumar L, Thordarson G, Chen X, Nandi S** 1999 Hormonal prevention of breast cancer: mimicking the protective effect of pregnancy. Proc Natl Acad Sci USA 96:2520–2525
16. **Russo IH, Koszalka M, Russo J** 1990 Human chorionic gonadotropin and rat mammary cancer prevention. J Natl Cancer Inst 82:1286–1289
17. **Russo IH, Koszalka M, Russo J 1991** Comparative study of the influence of pregnancy and hormonal treatment on mammary carcinogenesis. Br J Cancer 64:481–484
18. **Russo J, Russo IH** 1995 The etiopathogenesis of breast cancer prevention. Cancer Lett 90:81–89
19. **Alvarado MV, Alvarado NE, Russo J, Russo IH** 1994 Human chorionic gonadotropin inhibits proliferation and induces expression of inhibin in human breast epithelial cells in vitro. In Vitro Cell Dev Biol Anim 30A:4–8
20. **Kellen JA, Kolin A, Mirakian A, Acevedo HF** 1982 Effects of antibodies to choriogonadotropin in malignant growth. II. Solid transplantable rat tumors. Cancer Immunol Immunother 13:2–4
21. **Tao YX, Bao S, Ackermann DM, Lei ZM, Rao CV** 1997 Expression of luteinizing hormone/human chorionic gonadotropin receptor gene in benign prostatic hyperplasia and in prostate carcinoma in humans. Biol Reprod 56:67–72
22. **Kuroda H, Mandai M, Konishi I, Tsuruta Y, Kusakari T, Kariya M, Fujii S** 2001 Human ovarian surface epithelial (OSE) cells express LH/hCG receptors, and hCG inhibits apoptosis of OSE cells via up-regulation of insulin-like growth factor-1. Int J Cancer 91:309–315
23. **Gillott DJ, Iles RK, Chard T** 1996 The effects of β-human chorionic gonadotrophin on the in vitro growth of bladder cancer cell lines. Br J Cancer 73: 323–326
24. **Kumar S, Talwar GP, Biswas DK** 1992 Necrosis and inhibition of growth of human lung tumor by anti-α-human chorionic gonadotropin antibody. J Natl Cancer Inst 84:42–47
25. **Malavasi F, Bellone G, Matera L, Ferrero E, Funaro A, Demaria S, Caligaris Cappio F, Camussi G, Dellabona P** 1985 Murine monoclonal antibodies as probes for the phenotypical, functional and molecular analysis of a discrete peripheral blood lymphocyte population exerting natural killer activity in vitro. Hum Immunol 14:87–102
26. **Petsch D, Anspatch FB** 2000 Endotoxin removal from protein solution. J Biochem 76:97–119
27. **Horenstein AL, Stockinger H, Imhof BA, Malavasi F** 1998 CD38 binding to human myeloid cells is mediated by mouse and human CD31. Biochem J 330:1129–1135
28. **Rao CV** 1999 A paradigm shift on the targets of luteinizing hormone/human chorionic gonadotropin actions in the body. J Bellevue Obstet Gynecol Soc 15:26–32
29. **Hai MV, De Roux N, Ghinea N, Beau I, Loosfelt H, Vannier B, Meduri G, Misrahi M, Milgrom E** 1999 Gonadotropin receptors. Ann Endocrinol 60: 86–92
30. **Zhu X, Gilbert S, Birnbaumer M, Birnbaumer L** 1994 Dual signaling potential is common among Gs-coupled receptors and depend on receptor density. Mol Pharmacol 46:460–469
31. **Lei ZM, Rao CV** 2001 Neural action of luteinizing hormone and human chorionic gonadotropin. Semin Reprod Med 19:103–109
32. **Ji TH, Ryu KS, Gilchrist R, Ji I** 1997 Interaction, signal generation, signal divergence, and signal transduction of LH/CG and the receptor. Recent Prog Horm Res 52:431–453
33. **Meduri G, Charnaux N, Loosfelt H, Jolivet A, Spyratos F, Brailly S, Milgrom E** 1997 Luteinizing hormone/human chorionic gonadotropin receptors in breast cancer. Cancer Res 57:857–864
34. **Ascoli M, Fanelli F, Segaloff DL** 2002 The lutotropin/choriogonadotropin receptor, a 2002 perspective. Endocr Rev 23:141–174
35. **Jia XC, Oikawa M, Bo M, Tanaka T, Ny T, Boime I, Hsueh AJ** 1991 Expression of human luteinizing hormone (LH) receptor: interaction with LH and chorionic gonadotropin from human but not equine, rat, and ovine species. Mol Endocrinol 5:759–768
36. **You S, Kim H, El Halawani ME, Foster DN** 2000 Three different turkey luteinizing hormone receptor (tLH-R) isoforms II: characterization of differentially regulated tLH-R messenger ribonucleic acid isoforms in the ovary. Biol Reprod 62:117–124
37. **Kumar RS, Trant JM** 2001 Piscine glycoprotein hormone (gonadotropin and thyrotropin) receptors: a review of recent developments. Comp Biochem Physiol Biochem Mol Biol 129:347–355
38. **Puett D, Bhowmick N, Fernandez LM, Huang J, Wu C, Narayan P** 1996 hCG-receptor binding and transmembrane signaling. Mol Cell Endocrinol 20:55–64
39. **Paukku T, Ahtiainen P, Haavisto AM, Huhtainiemi IT** 1998 Persistence of biological activity of biotinylated human chorionic gonadotropin and its use

for visualization of rat luteinizing hormone receptors in tissue sections. J Histochem Cytochem 46:993–998
40. **Rao CV** 2001 An overview of the past, present, and future of nongonadal LH/hCG actions in reproductive biology and medicine. Semin Reprod Med 19:7–17
41. **Takao Y, Honda T, Ueda M, Hattori N, Yamada S, Maeda M, Fujiwara H, Mori T, Wimalasena J** 1997 Immunohistochemical localization of the LH/hCG receptor in human ovary: hCG enhances surface expression of LH/hCG receptor on luteinizing granulosa cells in vitro. Mol Hum Reprod 3:569–578
42. **Parrott JA, Doraiswamy V, Kim G, Mosher R, Skinner MK** 2001 Expression and action of both follicle stimulating and luteinizing hormone receptor in normal ovarian surface epithelium and ovarian cancer. Mol Cell Endocrinol 172:213–222
43. **Lejeune, H, Habert R, Saez JM** 1998 Origin, proliferation and differentiation of Leydig cells. J Mol Endocrinol 20:1–25
44. **Arnhold IJ, Latronico AC, Batista MC, Izzo CR, Mendonca BB** 1999 Clinical features of women with resistance to luteinizing hormone. Clin Endocrinol (Oxf) 51:701–707
45. **Coutinet B, Meduri G, Lecce MG, Young J, Brailly S, Loosfelt H, Milgrom E, Schaison G** 2001 The postmenopausal ovary is not a major androgen-producing gland. J Clin Endocrinol Metab 86:5060–5066
46. **Lojun S, Bao S, Lei ZM, Rao CV** 1997 Presence of functional luteinizing hormone/chorionic gonadotropin (hCG) receptors in human breast cell lines: implications supporting the premise that hCG protects women against breast cancer. Biol Reprod 57:1202–1210
47. **Russo IH, Russo J** 2000 Hormonal approach to breast cancer prevention. J Cell Biochem Suppl 34:1–6
48. **Popnikolov N, Yang J, Liu A, Guzman R, Nandi S** 2001 Reconstituted normal human breast in nude mice: effect of host pregnancy environment and human chorionic gonadotropin on proliferation. J Endocrinol 168:487–496
49. **Gilchrist RL, Ryu KS, Ji I, Ji TH** 1996 The luteinizing hormone/chorionic gonadotropin receptor has distinct transmembrane conductors for cAMP and inositol phosphate signals. J Biol Chem 271:19283–19287
50. **Bernard MP, Myers R, Moyle R** 1998 Lutropins appears to contact two independent sites in the extracellular domain of their receptor. Biochem J 335: 611–617
51. **Zeng H, Phang T, Sang Song Y, Ji S, Tae JH** 2001 The role of the hinge region of the luteinizing hormone receptor in hormone interaction and signal generation. J Biol Chem 276:3451–3458
52. **Lapthorn AJ, Harris DC, Littlejohn A, Lustbader JW, Canfield RE, Machin KJ, Morgan FJ, Isaacs NW** 1994 Crystal structure of human chorionic gonadotropin. Nature 369:455–461
53. **Remy JJ, Nespoulous C, Grosclaude J, Grebert D, Couture L, Pajot E, Salesse R** 2001 Purification and structural analysis of a soluble human chorionogonadotropin hormone-receptor complex. J Biol Chem 276:1681–1687