Introduction of a Gonadotropin Receptor Expression Plasmid into Immortalized Granulosa Cells Leads to Reconstitution of Hormone-dependent Steroidogenesis

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Abstract. We have recently succeeded in immortalizing rat granulosa cells by co-transfection with SV-40 DNA and the Ha-*ras* oncogene. These cells lost their response to gonadotropins, but expressed the cytochrome $P450_{scc}$ mitochondrial system enzymes and produced progesterone and 20α-hydroxy-4-pregnan-3-one (20α-OH-P) upon cAMP stimulation (Suh, B. S., and A. Amsterdam. 1990. *Endocrinology.* 127:2489–2500; Hanukoglu, I., B. S. Suh, S. Himmelhoch, and A. Amsterdam. 1990. *J. Cell Biol.* 111:1973–1981). In an attempt to restore the steroidogenic response to gonadotropins in immortalized cells, lutropin/choriogonadotropin (LH/CG-R) receptor expression plasmid was prepared by introducing the complete coding region of LH receptor cDNA (McFarland, K. C., R. Sprengel, H. S. Phillips, M. Köhler, N. Rosemblit, K. Nikolics, D. L. Segaloff, and P. H. Seeburg. 1989. *Science (Wash. DC).* 245:494–499) into a SV-40 early promoter based eucaryotic expression vector. Granulosa cells from preovulatory follicles were transfected with this LH receptor expression plasmid, together with SV-40 DNA and the Ha-*ras* oncogene. Cell lines obtained after this triple transfection accumulated cAMP in a dose-dependent manner in response to hCG. Moreover, they produced progesterone and 20α-OH-P upon hCG stimulation with an ED_{50} of 125 pM and 75 pM, respectively, which is within the physiological range. Concomitantly with hCG induced differentiation, inhibition of cell proliferation was evident following stimulation with hormone concentrations as low as 40 pM. The number of hCG receptor sites per cell after numerous passages and several freezing and thawing cycles was 1.9×10^4, they showed a K_d of 180 pM. Stimulation with hCG induced pronounced morphological and biochemical changes in these cells including formation of mitochondrial located adrenodoxin, a marker enzyme for enhanced steroidogenesis. These findings make possible the expression in immortalized granulosa cells, of selectively mutated receptor molecules which preserve their steroidogenic potential, thereby opening the way to analysis of structure–function relationships of the receptor molecule.

STEROID hormones are produced in steroidogenic cells containing the enzymes that are capable of metabolizing cholesterol and converting it to hormones which possess important physiological functions such as maintenance of salt balance in the body, control of fertility and the immune response, and even induction of behavioral changes (for review see Naftolin et al., 1975; Clark et al., 1985; Rose, 1985). Progesterone, which is crucial for control of the estrous cycle and pregnancy, is synthesized and secreted by ovarian granulosa cells. The secretion of this steroid hormone is stimulated by pituitary glycoprotein hormones, gonadotropins, which interact with specific membrane receptor molecules (Amsterdam and Lindner, 1984). Valuable information about the molecular properties of the receptors to gonadotropins was obtained as a result of the development of an affinity purification method for the lutropin/choriogonadotropin (LH/CG)[1] receptors, indicating that it is a single polypeptide chain in the range of 75–95 kD (Wimalasena et al., 1985; Saxena et al., 1986; Keinänen et al., 1987; Kusuda and Dufau, 1988; Sojar and Bahl, 1989; Zhang and Menon, 1989; Alpaugh et al., 1990). The genes coding for the receptor molecules to LH/CG and to follicle stimulating hormone (FSH) were recently cloned (McFarland et al., 1989; Loosfelt et al., 1989; Sprengel et al., 1990). The receptor for LH/CG is a 93-kD glycoprotein molecule and

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1. *Abbreviations used in this paper*: ECL, enhanced chemiluminescence; FSH, follicle stimulating hormone; IBMX, 3-isobutyl-1-methylxanthine; LH/CG, lutropin/choriogonadotropin; LH/CG-R, LH/CG-receptor; RIA, radioimmunoassay; 20α-OH-P, 20α-hydroxy-4-pregnan-3-one.

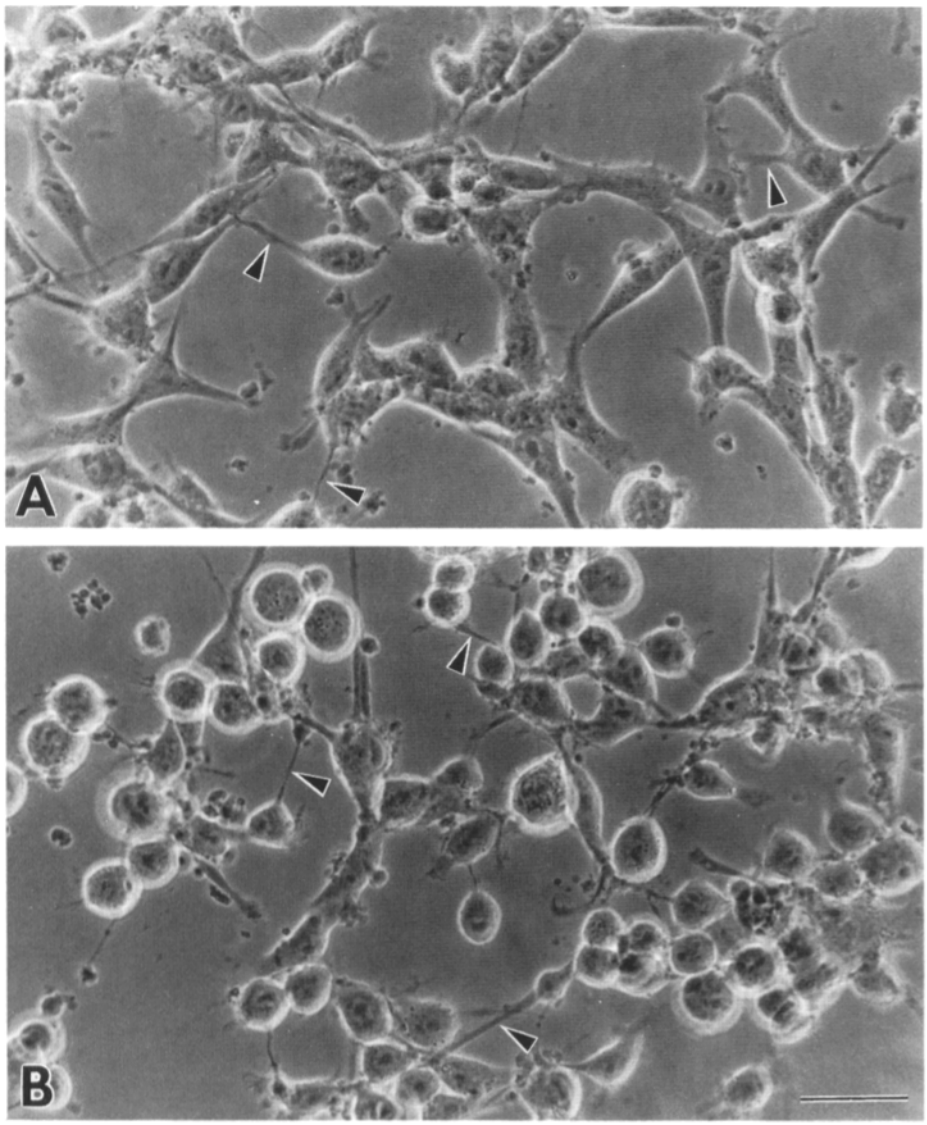

Figure 1. Morphological changes induced by hCG in a hormone-sensitive cell line. GLHR-15 cells grown in the absence (*A*) and in the presence (*B*) of 1.2 nM hCG for 1 h. Unstimulated cells show an elongated shape. In contrast, hCG-treated cells show rounding of the cell body. Cells seem occasionally to be interconnected by elongated cell processes (*arrowheads*). Bar, 20 μm.

the receptor to FSH is a 75-kD glycoprotein: they are both believed to be members of the G protein–coupled receptor family (McFarland et al., 1989; Loosfelt et al., 1989; Sprengel et al., 1990; Braun et al., 1991).

It has been clearly demonstrated that the interaction between gonadotropic receptor molecules and gonadotropic hormones lead to activation of adenylate cyclase and the stimulation of steroidogenesis (for review see Hsueh et al., 1984; Amsterdam and Rotmensch, 1987). Nevertheless, the coupling between these two metabolic events is not well understood and recent studies in cultured primary granulosa cells suggest alternative signal transduction mechanisms associated with gonadotropin stimulation such as inositol phosphate formation, calcium mobilization (Davis et al., 1987; Flores et al., 1990, 1992; Gudermann et al., 1992), and modulation of potassium and chloride channels (Mattioli et al., 1991; Morley et al., 1991). Since primary granulosa cells cease to divide upon prolonged stimulation with gonadotropins (Hsueh et al., 1984; Amsterdam and Rotmensch, 1987), the necessity to immortalize granulosa cells which could preserve their gonadotropin-stimulated steroidogenesis became evident.

We have recently established transformed granulosa cell lines after co-transfection of primary cells with SV-40 DNA and the Ha-*ras* oncogene (Amsterdam et al., 1988; Suh and Amsterdam, 1990; Hanukoglu et al., 1990; Baum et al., 1990). These lines retain their cAMP-mediated steroidogenic response but unfortunately lose their gonadotropic response. In this work, we succeeded in restoring the steroidogenic response to gonadotropins by additional transfection of the primary granulosa cells with LH/CG receptor (LH/CG-R) expression plasmid (McFarland et al., 1989). These new lines, which constitutively express the LH/CG-R, can be stimulated by hCG to produce progesterone and its metabolite 20α-hydroxy-4-pregnan-3-one (20α-OH-P) to a level comparable to that produced by gonadotropin-stimulated primary cells. Moreover, in the transfected, as in the normal cell, this stimulation is attributed to upregulation of the expression of the cytochrome $P450_{scc}$ mitochondrial system enzymes, which is the first and rate-limiting step in steroid hormone biosynthesis.

This work represents the first case where the complete steroidogenic response was reconstituted in a steroid hormone producing cell. Furthermore, in this system the induc-

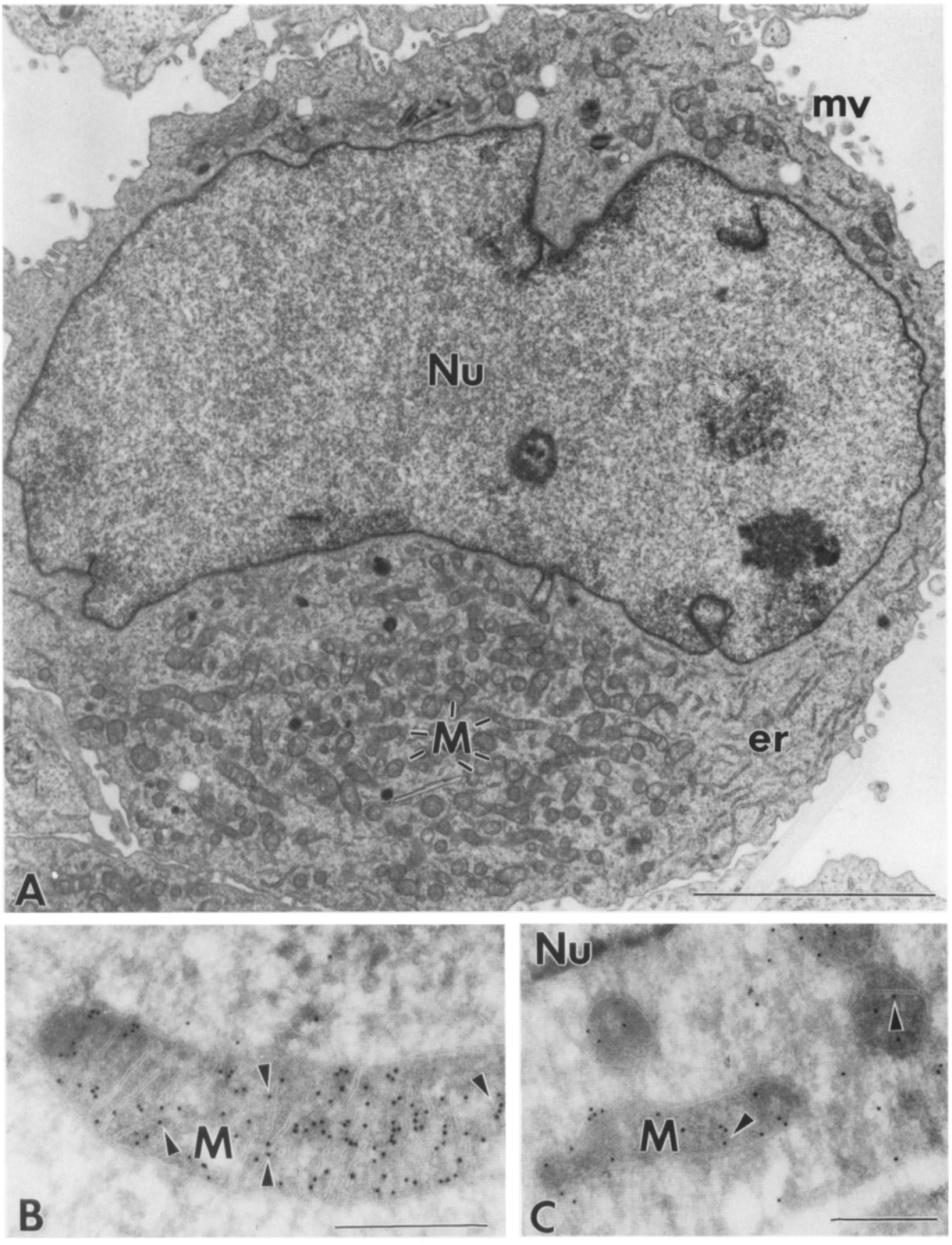

Figure 2. Morphology of GLHR-15 cells (*A*) and mitochondrial localization of adrenodoxin (*B* and *C*). (*A*) Ultrastructure of a GLHR-15 cell after 48-h incubation with 6 nM hCG. The round cell is covered with microvilli (*mv*). Endoplasmic reticulum (*er*) is well-developed, and the cytoplasm is loaded with mitochondria (*M*); *Nu*, nucleus. (*B* and *C*) Ultrathin cryo-sections were stained with rabbit anti-adrenodoxin and goat anti-rabbit antibodies coupled to 15 nm gold particles, as described in Materials and Methods. (*B*) Cells were incubated with 6 nM hCG for 48 h. (*C*) Cells were cultured in hormone-free medium. Gold particles mark the location of adrenodoxin (*arrowheads*). The vast majority of the gold particles are located on mitochondrial christae. Bar, (*A*) 5 μm; (*B* and *C*) 0.5 μm.

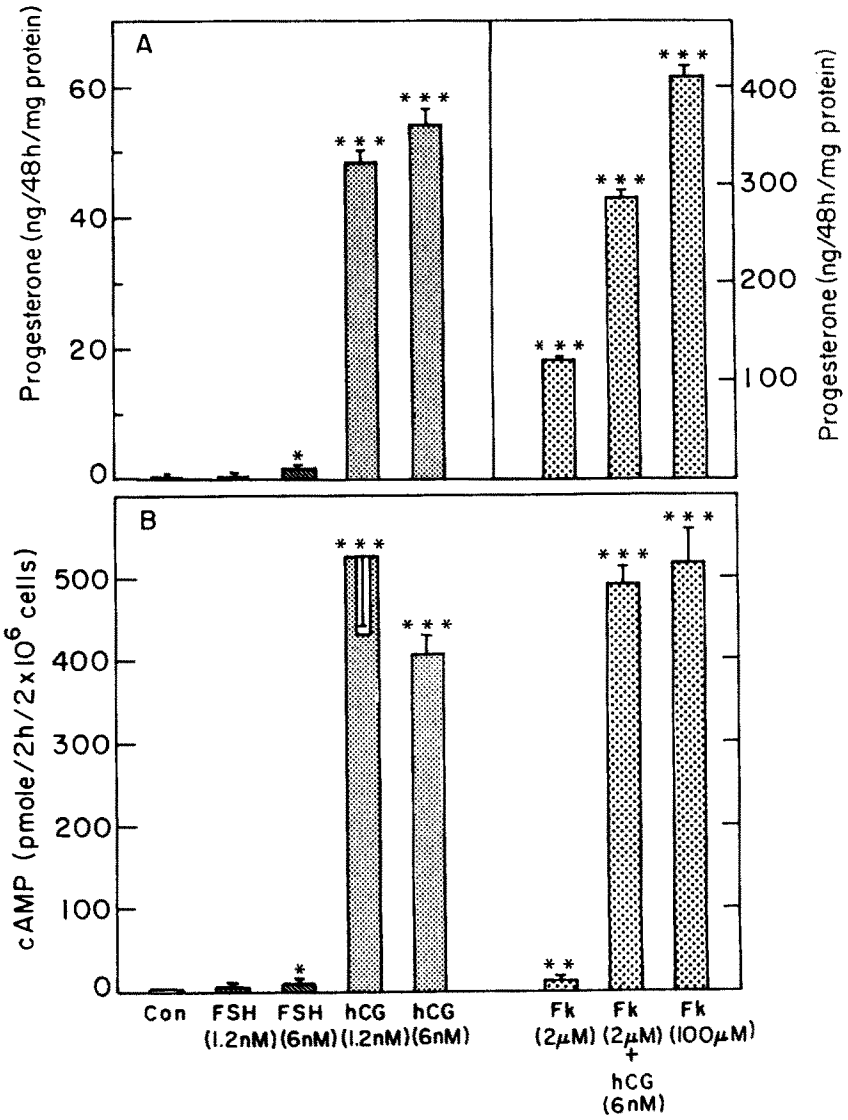

Figure 3. Progesterone (*A*) and cAMP (*B*) secretion from GLHR-15 cells. cAMP and progesterone concentrations in media were measured in the same cultures (after 2 and 48 h, respectively, in the presence and absence of the various stimuli). Progesterone and cAMP concentrations in nonstimulated cultures were 0.4 ng/10^6 cells and <10 pmoles/10^6 cells, respectively (10^6 cells are equivalent to 0.26 mg protein). Data are means ± SEM (n = 3). *Con*, control; *Fk*, Forskolin; t test of experimental vs. control values: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

tion of steroidogenesis is very high (>100-fold), due to minimal steroidogenic activity in nonstimulated cells. It is proposed that the newly established steroidogenic cell lines can serve as a useful model for studying the cellular and the molecular mechanisms involved in gonadotropin-stimulated steroidogenesis and the effect of oncogenes on this process.

Materials and Methods

Reagents

Highly purified hCG CR-123 was kindly provided by Dr. E. Canfield (Columbia University, NY). Highly purified ovine FSH-13 and ovine LH-23 were kindly provided by the National Institutes of Health (Bethesda, MD).

Plasmids

The SV-40 genome was from plasmid pSVBam, the Ha-*ras* from plasmid pEJ 6.6 (Amsterdam et al., 1988; Suh and Amsterdam, 1990), and the rat LH/CG-R from plasmid pSVLHR. In pSVLHR most of the cytomegalovirus (CMV) promoter sequence of pCLHR (McFarland et al., 1989) was replaced by SV-40 early promoter sequences. The plasmid encodes a chimeric LH/CG-R transcription unit with a 5' end of the CMV 1E1 cDNA (X17403) (~50 nucleotides) followed by 164 nucleotides synthetic intron sequences, the LH/CG-R cDNA (M26199) and the 3' end of the SV-40 early polyadenylation signal (M24914).

Preparation of Rat Granulosa Cells and Lines of Transfected Cells

Granulosa cells were isolated from immature female rats treated with pregnant mare serum gonadotropin (Amsterdam et al., 1979). Primary cultures were transfected simultaneously with 2 μg of pSVBam, 5 μg pEJ 6.6 (Amsterdam et al., 1988; Suh and Amsterdam, 1990), and 5 μg of pSVLHR, using the calcium phosphate precipitation procedure (Amsterdam et al., 1988). Densely growing foci of transformed cells were visualized, selected, and expanded into cell lines. The detailed procedures have been described previously (Amsterdam et al., 1988; Suh and Amsterdam, 1990). Initial screening for lines expressing a functional LH/CG-R was performed by stimulation of the cells with 6 nM hCG for 2 h and screening for cell rounding which occurs as a consequence of elevation of intracellular cAMP (see Fig. 1). The hCG responsive cell lines (6 of 50 lines, named GLHR lines) were further characterized by ^{125}I-hCG binding and cAMP and steroidogenic responses to hCG.

Immunoprecipitation of SV-40 T Antigen and the ras Oncogene

Cells grown in 100-mm dishes were labeled for 4 h with [^{35}S]methionine (40 μCi per dish; 1 Ci = 37 GBq) in 4 ml of methionine-free medium. Cell

extracts containing equal amounts of acid-insoluble radioactive material were prepared from cells labeled with [^{35}S]methionine for 12 h and immunoprecipitated with either mAb PAb419 directed against SV-40 large T antigen (Maltzman et al., 1981), together with mAb Pab 431 directed against p53 (Suh and Amsterdam, 1990), or mAb Y13-259 specific for ras p21 (Oncogene Science, Manhasset, NY). Proteins immunoprecipitated with the ras antibody were resolved on 15% polyacrylamide gels, while the T-antigen immunoprecipitates were resolved on 10% polyacrylamide gels. The gels were fluorographed with 2,5-diphenyloxazole in DMSO solution (PPO/DMSO, 20% wt/vol) and exposed to Agfa Curix X-ray film for 7 d at −70°C (Maltzman et al., 1981; Amsterdam et al., 1988; Suh and Amsterdam, 1990).

Binding Assay Using Radiolabeled Ligand (^{125}I-hCG)

LH/CG-R receptors expressed in GLHR-15 cells were measured by a binding assay using radiolabeled ligand. 20 μg of highly purified hCG (CR-123) was used for iodination, using the Iodogen method (Fraker and Speck, 1978). 0.5 mCi of Na^{125}I were added to 20 μg of hCG in an Iodogen-coated tube and reacted for 3 min on ice. Radiolabeled hCG (^{125}I-hCG) was eluted from a sephadex G-50 column by 1% BSA-PBS and distributed for binding assay into plastic test tubes (10 × 75 mm) at 2.6 × 10^7 cpm/ml. Specific activity of the radiolabeled ligand (I^{125}-hCG) was 4 × 10^4 cpm/ng protein. The binding assay was carried out by both saturation binding and displacement binding. In the saturation binding assay, different concentrations of ^{125}I-hCG were used for binding to 1.2 × 10^6 cells. For displacement binding, a fixed amount of ^{125}I-hCG (2 × 10^5 cpm) was used for binding to 1.2 × 10^6 cells in the presence of different concentrations of unlabeled hCG. Cells were removed from tissue culture plates by brief incubation (2 min) with calcium- and magnesium-free PBS containing 2 mM EDTA, pH 7.4. Medium was removed after a 5-min centrifugation at 600 *g* and cells were resuspended in PBS containing calcium, magnesium, and 0.1% BSA (PBS-BSA). Samples of cells (1.2 × 10^6 in 50 μl) were lysed at 4°C by adding 250 μl of 10 mM phosphate buffer, pH 7.4, containing 0.1% BSA and 0.5 mM PMSF to inhibit any traces of proteolytic activity (lysis buffer) (Amsterdam and Suh, 1991). Overnight incubation at 4°C of cell lysates with the hormone was carried out in a final volume of 0.30 ml in Eppendorf tubes pre-coated with 1% BSA solution. After incubation, bound and free ligand was separated by 5-min centrifugation in an Eppendorf centrifuge (Brinkman Instruments Inc., Westbury, NY) (12,000 *g*) at 4°C. The pellets containing the cell membranes were washed three times at 4°C by resuspension and recentrifugation with the lysis buffer. Total and non-specific counts in the cell pellets were measured in a γ-counter (1277 Gammamaster; LKB Instruments, Inc., Gaithersburg, MD) with efficiency of 52%. Specific counts were determined by subtraction of non-specific counts from total counts. Scatchard analysis was carried out as described earlier (Furman et al., 1986).

Steroid Hormone, cAMP, and Protein Assays and [^{3}H]Thymidine Incorporation into DNA

Progesterone and 20α-OH-P, the two major progestins accumulating in the culture medium of granulosa cells, were determined by radioimmunoassay (RIA) (Erlanger et al., 1959; Kohen et al., 1975; Amsterdam et al., 1979; Suh and Amsterdam, 1990). cAMP from medium and cells were measured by a protein binding method (Gilman, 1970; Amsterdam et al., 1979, 1988). Cells were cultured in serum-free media as described below. For measurement of intracellular cAMP, cells were washed twice with PBS, lysed with 1 ml of 50 mM sodium acetate (pH 4.5) containing 0.1 mM 3-isobutyl-1-methylxanthine (IBMX), harvested into Eppendorf tubes (Brinkman Instruments Inc.) using a rubber policeman, and heated for 10 min at 90°C. The cell debris was precipitated by centrifugation for 5 min (12,000 *g*), and cAMP in the supernatant was measured. Significant amounts of cAMP were found inside the transformed cells and in the medium (secreted by the cells) when measured after a 2-h stimulation with either hCG or forskolin (see also Fig. 5 *A* and Fig. 8). Therefore measuring of extracellular levels of cAMP at this point also faithfully reflects the intracellular levels (see Fig. 3). Antisera and binding protein were generous gifts of Drs. F. Kohen and E. Hanski (Department of Hormone Research, Weizmann Institute).

Protein was determined by the Bradford method (Bradford, 1976). Incorporation of [^{3}H]thymidine into TCA insoluble fractions of cells was measured in a β counter as described earlier (Suh and Amsterdam, 1990).

Western Blot

Cells were plated in 100-mm Nunc petri dishes (800,000 cells/dish) in DME/F12 containing 5% FCS. After 24 h the medium was replaced by serum-free DME/F12 containing the desired stimulants. 48 h after stimulation, the medium was collected for progesterone assay and the cells were washed twice with PBS before being collected with 250 μl of phosphate buffer (6.5 mM Na_2HPO_4, 2.5 mM KCl, 1.5 mM KH_2PO_4) in Eppendorf tubes (Brinkman Instruments, Inc.), and stored at −20°C. A portion of each sample was taken for protein determination. The remaining cells were lysed by freezing in liquid nitrogen and thawing. The Eppendorf tubes were centrifuged for 10 min at 1,000 rpm to remove intact nuclei and cell debris. The pellet was discarded and 30 μg protein of each suspension was subjected to electrophoresis overnight at 10 mA using a 5% polyacrylamide upper gel and a 15% polyacrylamide lower gel. A Western blot was prepared by electrotransfer (2 h at 400 mA at 4°C). The nitrocellulose membrane was incubated in blocking solution (10% milk containing 0.1% fat, 0.05% Tween-20 in PBS) overnight at 4°C. It was first reacted with antibody to adrenodoxin (1:500 dilution for 5 h at room temperature) (see also Hanukoglu and Hanukoglu, 1986; Hanukoglu, 1990), and then with protein A coupled to HRP (Amersham Corp., Arlington Heights, IL; 1:4,000 dilution for 2 h at room temperature). The detection was carried out by the ECL (enhanced chemiluminescence) method, using Amersham Corp. products. A computing densitometer (no. 300A; Molecular Dynamics, Sunnyvale, CA) was used to quantitate the intensity of the bands on the film. Since adrenodoxin appeared on Western blot as a double band (see Fig. 9), both bands were quantitated together.

Cell Culture

Cells were maintained on Nunc petri dishes (100 mm) containing 10 ml DME/F12 medium (1:1) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% FCS. For biochemical assays, cells (0.5 × 10^6, 35% confluent) were plated on Nunc petri dishes (35 mm) in the same medium, and on the following day the medium was replaced by serum-free medium containing insulin (2 μg/ml), transferrin (5 μg/ml), and hydrocortisone (40 ng/ml). Experiments described in Fig. 8 *A* were performed in the presence of 5% FCS.

Phase Contrast, EM, and Immunocytochemistry

Cultures were fixed with 2.5% glutaraldehyde in PBS and photographed at 400× magnification, using a Photomicroscope III (Carl Zeiss, Oberkochen, Germany), equipped with a water immersion lens. For examination of cell

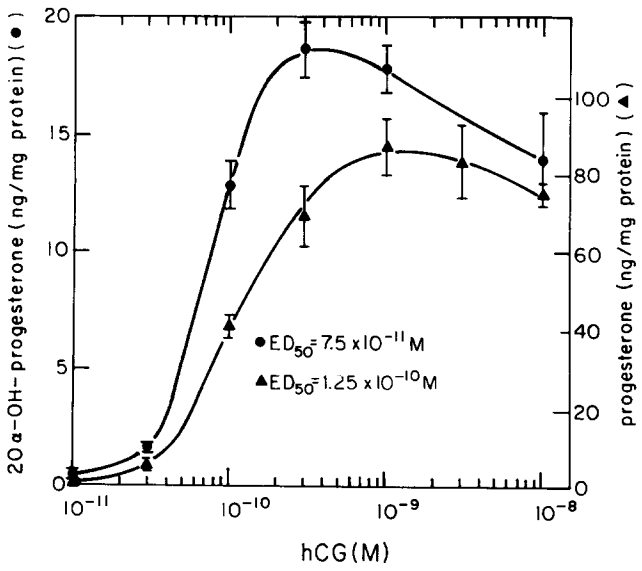

Figure 4. Concentration dependence for the formation of progesterone (●) and 20α-OH-P (▲) after incubation of GLHR-15 cells with hCG for 48 h. Data are means ± SEM (n = 3).

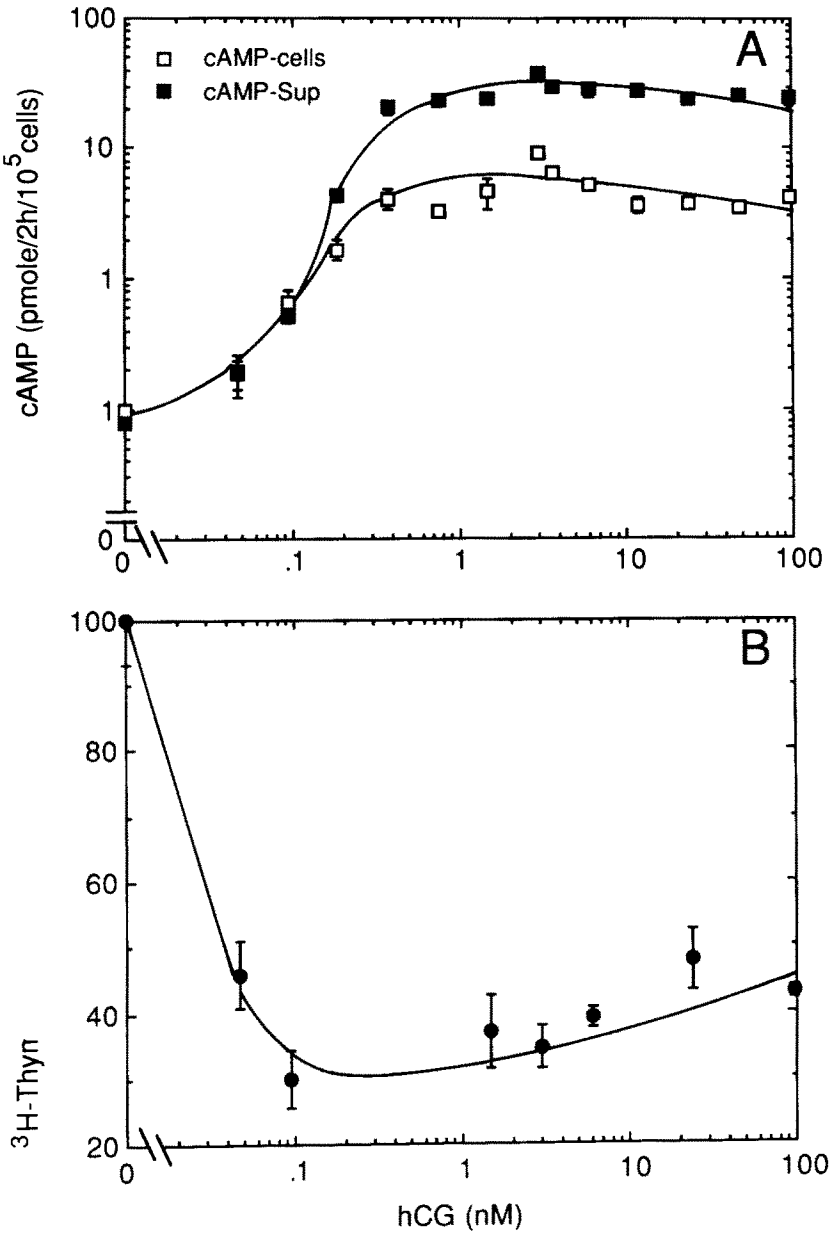

Figure 5. Concentration effect dependence of the formation of cAMP (*A*) and [^{3}H]thymidine incorporation into DNA after hCG stimulation of GLHR-15 cells. Cells were incubated with hCG for 43 h and subsequently with 1 μCi/ml [^{3}H]-thymidine for 5 h. *cAMP-cells*, intracellular levels of cAMP; *cAMP-Sup*, extracellular levels of cAMP. Data are means ± SEM (n = 3).

ultrastructure, pellets of glutaraldehyde-fixed cells were post-fixed with 1% osmium tetroxide, stained en bloc with 2% aqueous uranyl acetate, and embedded in polybed 812 embedding medium (Polyscience Inc., Warrington, PA). Ultrathin sections were stained with uranyl acetate and lead acetate.

For electron microscopic immunocytochemistry, the cultured cells were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 2 h. The cells were then harvested using a rubber policeman and pelleted by centrifugation (12,000 *g*) for 30 min in an Eppendorf centrifuge. The pellet was cut into small squares (1–2 mm) and infiltrated with 2.3 M sucrose in PBS, sectioned using an ultracut E microtome (Reichert Jung, Vienna, Austria) with a FC4D cryo-attachment and labeled as described (Tokuyasu, 1980; Griffiths et al., 1984). Sections were first incubated with rabbit antisera to adrenodoxin generously provided by Dr. I. Hanukoglu. The second antibody used was goat anti-rabbit IgG coupled to 15-nm gold particles (Jansen Pharmaceutica, Beerse, Belgium). Electron micrographs were taken either on a EM 410 or EM 300 at 80 kV (Philips Electronic Instruments, Mahwah, NJ).

Statistical Analysis

Analysis of concentrations of progesterone, 20α-OH-P, and cAMP was performed using the t test for comparison of means. Differences between treatment groups were considered statistically significant at $P < 0.05$.

Results

General Morphology of GLHR Lines

To preserve the steroidogenic response to the gonadotropic hormone (hCG) in the transformed granulosa cells, primary cells from preovulatory follicles were transfected simultaneously with SV-40 DNA, Ha-*ras* oncogene, and a plasmid coding for LH/CG-R transcriptionally controlled by SV-40 early promoter sequences. Preliminary selection of the appropriate lines (GLHR lines) was based on the presence of the typical elongated *ras* morphology and morphological changes (e.g., cell rounding) induced by hCG stimulation of intracellular cAMP accumulation (Fig. 1). Similar morphological changes were also observed when cells were stimulated with 1.2 nM oLH, but not with 1.2 nM oFSH. Cells treated with hCG for 48 h showed pronounced accumulation of mitochondria in the cytoplasm, which contained a more extensive network of inner membranes (christae) compared

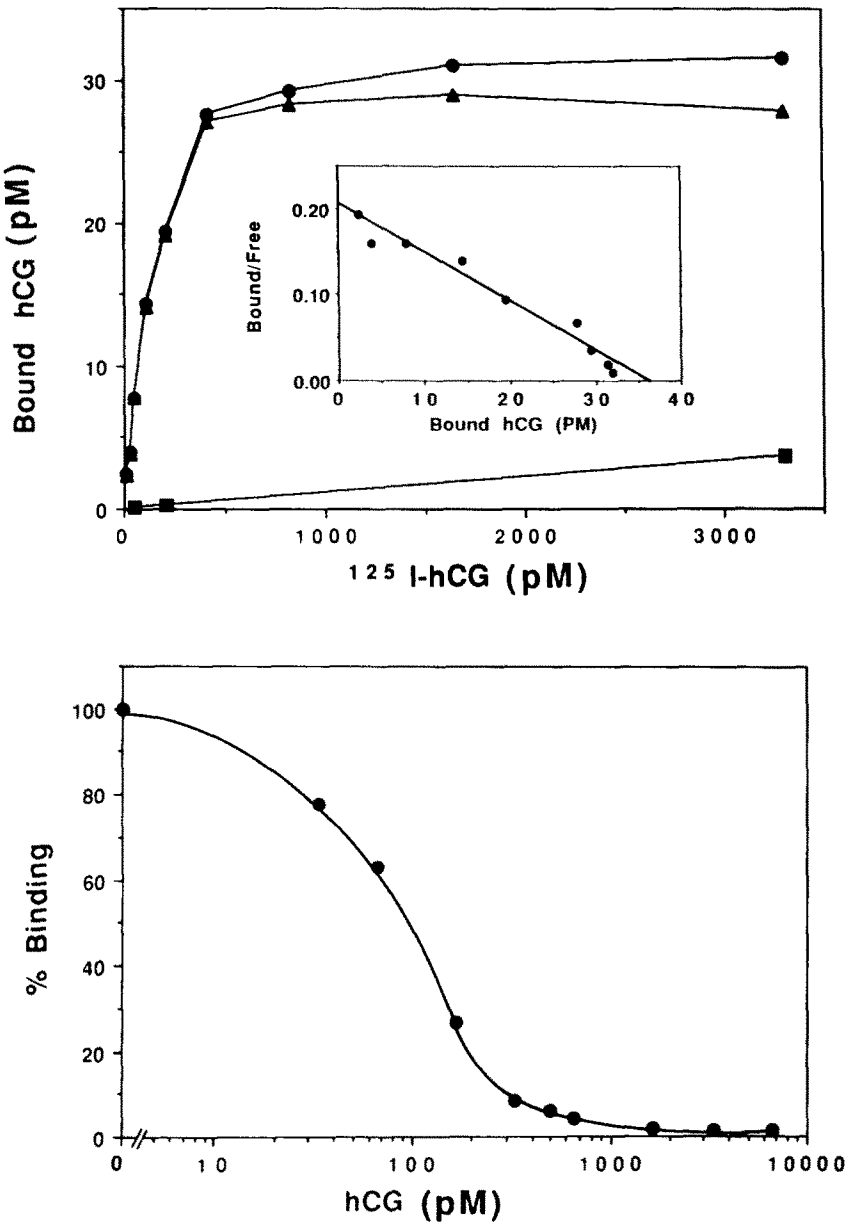

Figure 6. Binding of ^{125}I-hCG to GLHR-15 cells. (*A*) Saturation curve and Scatchard analysis (*inset*). Details are specified in Materials and Methods. Non-specific binding (■—■) was determined in the presence of 1,000 times excess of unlabeled hCG. (●—●) Total binding; (▲—▲) specific binding (after subtracting the values of nonspecific binding from total binding). (*B*) Displacement of ^{125}I-hCG binding by unlabeled hormone (see Materials and Methods for details). Data for *A* and *B* are means of triplicate determinations. The standard error of the means did not exceed 7%.

to mitochondria of untreated cells (Fig. 2). The accumulation of mitochondria with a more extensive network of inner membranes is also a characteristic of normal rat and human granulosa cells luteinized either in vivo or in vitro reflecting the accumulation of the mitochondrial cytochrome p450scc enzyme system including adrenodoxin and adrenodoxin reductase (for recent reviews see Amsterdam et al., 1991, 1992).

Characterization of Gonadotropic Response

The steroidogenic response to hCG was characterized in the cell lines. 48 h of stimulation with 1.2 or 6.0 nM hCG yielded pronounced progesterone production (50–60 ng/48 h/mg cell protein) in the representative GLHR-15 line (Fig. 3). Similar progesterone production levels were induced by 1.2 nM oLH. In unstimulated cells, only trace amounts of progesterone secretion were detected (1–2 ng/48 h/mg protein). oFSH at 1.2 nM concentration was without effect but at 6 nM a slight stimulation of progesterone was detected, probably due to LH contamination of the FSH preparation (Fig. 3). After a 2-h incubation with hCG or 100 μM forskolin, (a potent stimulant of adenylate cyclase) (Laurenza et al., 1989), cAMP levels in the medium were elevated to values similar to those after hCG stimulation. However, progesterone production measured 48 h after onset of the stimulation was substantially greater in forskolin-treated cells (400 ng/48 h/mg cell protein) than in hCG-treated cells (60 ng/48 h/mg protein). Stimulation of cells with hCG in the presence of 2 μM forskolin yielded a much higher production of progesterone (280 ng/48 h/mg protein), that was only slightly lower than the stimulation by 100 μM forskolin. Similar high levels of cAMP were observed in cultures stimulated for 2 h with 1.2 mM hCG alone or with hCG + 2 μM forskolin (Fig. 3 *B*). Interestingly, stimulation of the cells with 2 μM forskolin resulted in a very modest cAMP accumulation compared to hCG stimulation (20 vs. 480 pmoles/2 h/2 $\times$ 10^6 cells), while progesterone production, after 48 h, was significantly higher (110 ng/mg protein).

hCG stimulated progesterone and 20α-OH-P production

in a concentration-dependent manner with an ED_{50} of 125 pM and 75 pM, respectively (Fig. 4). hCG stimulation of cAMP production showed a similar concentration-dependent response (i.e., maximum stimulation at 1 nM and ED_{50} of 90 pM) (Fig. 5 *A*). A tendency toward reduction in steroid hormone and cAMP production was evident at hCG concentrations greater than 1.0 nM (Figs. 4 and 5 *A*).

When cell proliferation in the presence of increasing concentrations of hCG was tested, it was found that there was an inverse relationship between [^{3}H]thymidine incorporation into DNA and the cAMP/steroidogenic response, with maximum inhibition of DNA synthesis (~70% of non-stimulated cells) at concentrations as low as 100 pM of hCG (Figs. 4 and 5).

Expression of LH/CG Receptors

Expression of LH/CG-R in GLHR-15 transformed cells was evident by typical saturation and displacement curves, using ^{125}I-hCG as a tracer (Fig. 6). The affinity of hCG for the specific receptors on the transformed cells (GLHR-15) and the number of binding sites were calculated by Scatchard analysis of binding studies using ^{125}I-hCG. The Kd value calculated from the saturation curve was 180 ± 30 pM (mean ± SEM, n = 5) and the number of binding sites per cell was 19,000 ± 1,500 (mean ± SEM, n = 4). The K_d value representing the affinity of hCG to the specific receptors on GLHR-15 was in agreement with the ED_{50} of the cAMP response (150 pM) and the ED_{50} of the steroidogenic response calculated for progesterone (125 pM) and for 20α-OH-P (75 pM). Five other GLHR cell lines isolated after the triple transfection procedure (SV-40-DNA + Ha-*ras* oncogene + LH/CG-R expression plasmid) showed a similar number of hCG receptors (15,000–25,000 receptors/cell) with similar affinities.

GLHR-15 cells express the transfected synthetic LH/CG receptor gene as indicated by the Scatchard analysis (Fig. 6) and the typical displacement curve (Fig. 6). This analysis does not exclude an expression of the endogenous LH/CG-R. However, Ha-*ras* - SV-40 immortalized granulosa cells lose their hCG binding capacity as well as their cAMP and steroidogenic response to the gonadotropic hormone (Suh and Amsterdam, 1990), which suggests that immortalization is accompanied by a shut-down of endogenous LH/CG-R gene expression.

Expression of T Antigen and p21

Since isolated cell lines expressing the LH/CG receptor and responding to hormonal stimulation by producing cAMP and progesterone were also co-transfected with SV-40 DNA and the Ha-*ras* oncogene, we tested whether these cells express similar amounts of the oncogene products as do cells transfected with SV-40 and Ha-*ras* alone. Indeed, we demonstrated (Fig. 7), by immunoprecipitation of the large T antigen, together with the associated p53 (Oren et al., 1981), and the p21 from double transfected cells (PO-GRS1) and triple transfected cells (GLH-R15) that the amount of the oncogene products remains essentially the same; therefore we can conclude that the expression of these proteins does not interfere with the cellular response to hCG mediated by the LH/CG-R expressed by the pSVLHR introduced into these cells.

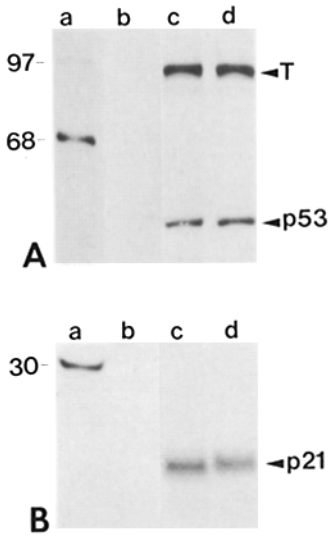

Figure 7. Expression of SV-40 large T antigen (*A*) and Ha-*ras* oncogene (*B*) in cells transfected with SV-40 DNA and Ha-*ras* oncogene alone (POGRS-1 line, lane *c*) or together with LH/CG-R expression plasmid (GLHR-15, lane *d*). Metabolically labeled proteins were extracted from cells and immunoprecipitated with monoclonal antibody Pab 419 directed against SV-40 large T-antigen + mAb Pab 421 directed against p53 (*A*). Labeled proteins were also immunoprecipitated with monoclonal antibody Y13-259 to the ras oncogene product p21 (*B*). Lane *a*, molecular markers; lane *b*, treatment with control hybridoma culture medium.

Kinetics of cAMP and Progesterone Production in Gonadotropin-stimulated Cells

Formation of progesterone after hCG stimulation was first evident at 6 h and the rate of steroid production was progressively elevated during the 96 h of stimulation investigated (Fig. 8 *A*). In contrast, the intracellular cAMP was sharply elevated within the first 15 min after hCG stimulation, gradually fell to 20% of the peak levels within the next 3–6 h (Fig. 8 *B*), and returned to basal levels 12 h after the onset of stimulation. High concentrations of cAMP persist in the medium for at least 12 h after the onset of gonadotropin stimulation. The kinetics of intracellular cAMP accumulation after forskolin stimulation showed a pattern essentially similar to that of hCG-stimulated cells, but with a twofold higher level of the cyclic nucleotide during the first hour of stimulation (Fig. 8 *B*). In contrast, progesterone secretion showed different kinetics reaching a plateau of maximum secretion at 30 ng/10^6 cells within 48 h after forskolin stimulation. hCG-treated cells secreted only 15% of this amount at 48 h after stimulation and at 96 h the progesterone production in the medium of hCG-stimulated cells was more than 50% that of forskolin-stimulated cells (Fig. 8 *A*).

Expression of P450$_{scc}$ System Enzymes

We examined whether induction of progesterone-production by hCG is due to stimulation of the expression of the steroidogenic enzymes, using adrenodoxin, a member of the mitochondrial cytochrome P450$_{scc}$, as a marker for the steroidogenic enzymes. After 48 h of stimulation by 1.2 nM hCG, or by 2 μM forskolin, the amount of adrenodoxin revealed by Western blot analysis and densitometer tracing (Fig. 9) was five times higher than in nonstimulated cells. Incubation of cells with the hormone plus 2 μM of forskolin doubled adrenodoxin formation. Similar induction of the enzyme was observed using 50 μM of forskolin.

A complementary method for examining the induction of expression of the steroidogenic enzymes as well as their in-

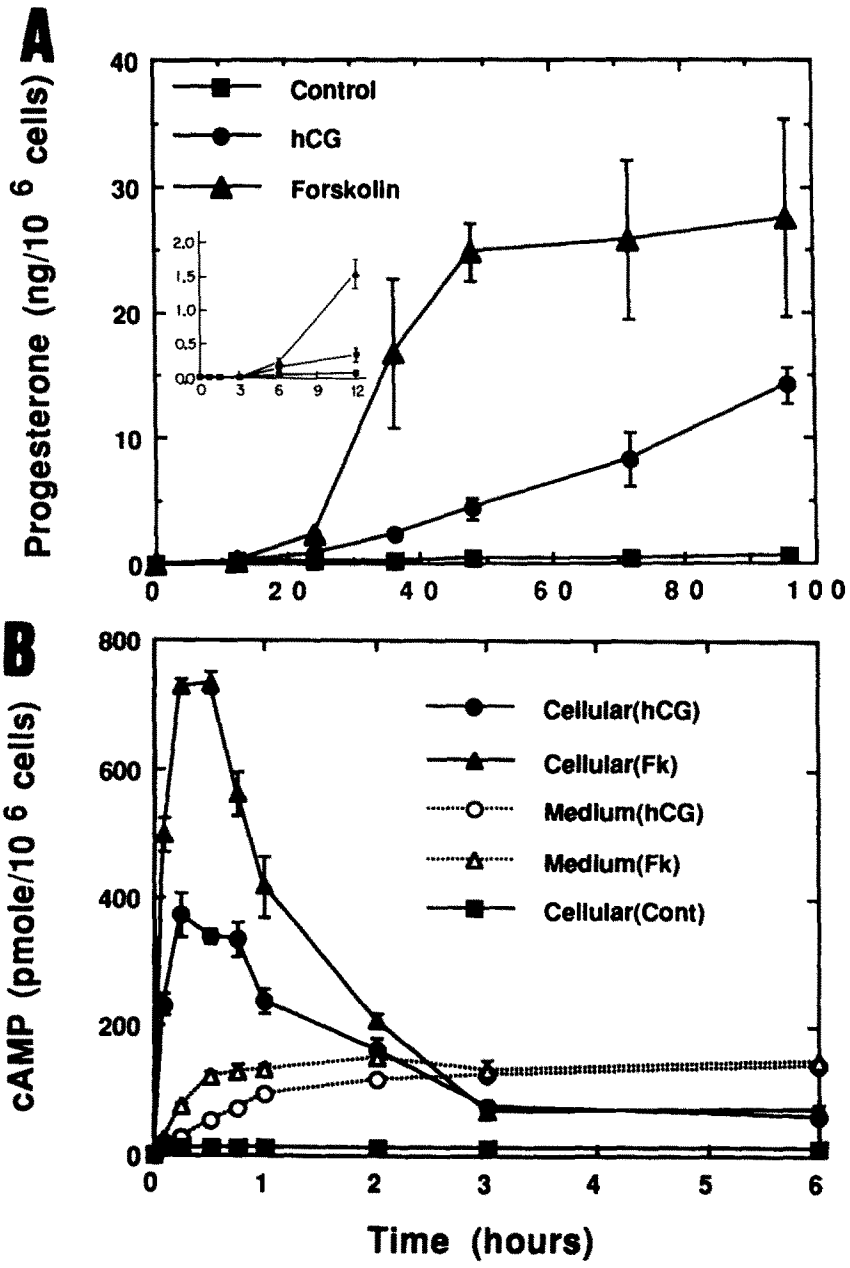

Figure 8. Formation of cAMP and progesterone in GLHR-15 cells in the presence of 6.0 nM hCG and 100 μM forskolin. Data are means of triplicate determinations ± SEM.

tracellular localization was high resolution immunocytochemistry using specific antibodies to adrenodoxin and the immunogold labeling technique. After a 48 h stimulation by hCG, transformed cells showed numerous mitochondria containing adrenodoxin (Fig. 2). In non-stimulated cells, however, this enzyme is detected in only very low amounts. The density of labeling of this enzyme in mitochondria of hCG-stimulated cells was five times higher than in mitochondria of non-stimulated cells and 30–40% of that in forskolin-stimulated cells (not shown). Although there was some fluctuation in the density of labeling of the enzyme among individual cells, all cells stimulated with either hCG or forskolin show significantly higher labeling within mitochondria, compared to mitochondria of nonstimulated cells, suggesting the monoclonal origin of the cell line.

Discussion

In this work, the establishment of transformed steroidogenic cell lines expressing the LH receptor was achieved by co-transfection of primary granulosa cells with SV-40 DNA, Ha-*ras* oncogene, and LH/CG-R expression plasmid.

In earlier work, the cloned LH/CG-R was inserted into an expression plasmid and was transfected into 293 cells (McFarland et al., 1989) and COS-7 cells (Loosfelt et al., 1989). This transfection resulted in expression of the receptor molecule which was able to bind ^{125}I-hCG and to evoke a cAMP response upon gonadotropin stimulation (McFarland et al., 1989). However, these experiments were performed on non-steroidogenic cells. In the present study we succeeded in transfecting steroidogenic granulosa cells with

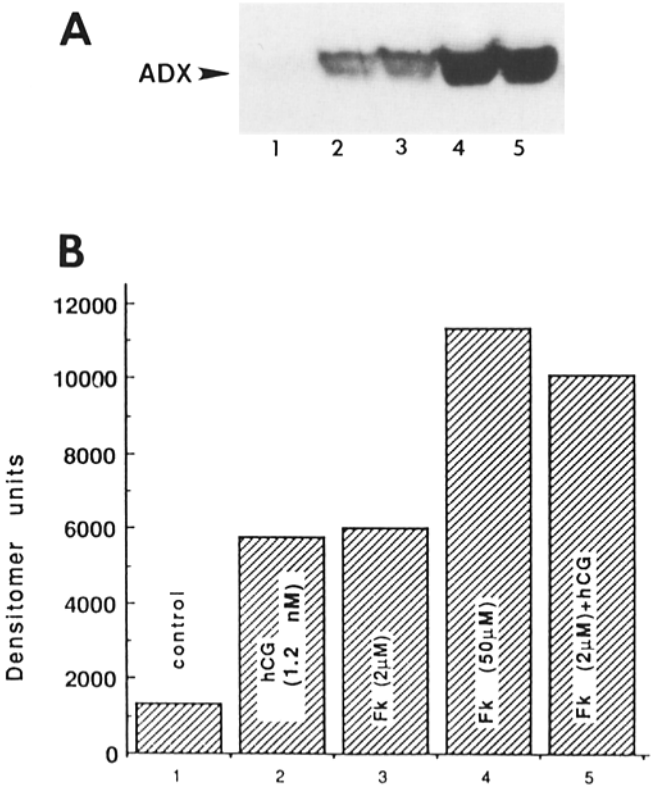

Figure 9. Western blot (*A*) and its densitometric analysis (*B*) of adrenodoxin, in non-stimulated and 48 h stimulated GLHR-15 cells. The numbers under the individual lanes in the blot correspond to those under the columns of the densitometric analysis.

the LH/CG-R expression plasmid and in demonstrating that the complete steroidogenic response can be preserved in immortalized cells.

Triple transfection with SV-40 DNA + Ha-*ras* oncogene + LH/CG-R expression plasmid proved to be essential for the maintenance of the steroidogenic response to LH and hCG in the newly established granulosa cell lines. Transfection with SV-40 DNA is essential for immortalization of the primary granulosa cells (Amsterdam et al., 1988), by a mechanism which involves a burst of SV-40 gene expression including formation of the SV-40 large T antigen responsible for cell transformation (Khandjian and Gauchat, 1988; Hraba-Renevey et al., 1989; Borowiec et al., 1990). Transfection with Ha-*ras* oncogene is essential for preserving cAMP-stimulated steroidogenesis in the cells by a mechanism which is not yet understood (Amsterdam et al., 1988; Suh and Amsterdam, 1990; Hanukoglu et al., 1990; Baum et al., 1990). However, the cotransfection with these DNA sequences is not sufficient to restore the gonadotropic response in granulosa cells since they lose their intrinsic LH receptors, as can be demonstrated by the lack of both specific binding by ^{125}I-hCG and cAMP-mediated steroidogenic response to LH and hCG (Suh and Amsterdam, 1990). Transfection with the LH/CG-R expression plasmid seems to be obligatory to restore a functional LH receptor in these cells. This system is particularly attractive since it permits the testing of the activity of a transfected wild type or mutant receptor in a natural cell environment without interference by the endogenous receptor.

The expressed receptor molecule in the transformed lines exhibits an affinity to the hormone similar to that of the native receptor molecule in normal granulosa cells (for review see Amsterdam et al., 1981). In addition, the ED_{50} for the cAMP and the steroidogenic response to hCG in the new lines is comparable to that of primary cells. Moreover, desensitization to the hormone (although modest) is observed upon stimulation of the GLHR-line with a high dose of hCG (see Fig. 4). This phenomenon is well characterized in normal granulosa cells (Amsterdam et al., 1979; Hsueh et al., 1984; Amsterdam and Rotmensch, 1987). The genetically engineered DNA coding for the LH/CG-R molecule is lacking the authentic promoter region of the native receptor and therefore was coupled to the SV-40 early promoter region, which probably results in constitutive expression of the receptor molecule. Therefore, the observed refractoriness to high doses of hCG is probably due to clustering or internalization of the receptor molecule (Conn et al., 1978; Rao and Mitra, 1979; Amsterdam et al., 1980, 1981) rather than to down regulation of its expression, upon chronic hCG stimulation.

The steroidogenic response of the cells to hCG was relatively slow (6–12 h after the onset of the stimulation) and was only 15% of the maximal stimulation achieved by 100 μM forskolin after 48 h (see Fig. 8). Nevertheless, stimulation by 2 μM forskolin and 1.2 nM hCG resulted in a synergistic effect on steroidogenesis which was almost as high as after stimulation by a saturating dose of forskolin. This suggests that elevation of intracellular cAMP may serve as an important mediator in the steroidogenic response. However, there was no direct quantitative correlation between cAMP levels and the steroidogenic response evoked by the saturating doses of hCG or forskolin. The level of cAMP during the first hour of stimulation was increased twofold in forskolin-treated cells compared to hCG-treated cells and after 2 h the levels of the cyclic nucleotide were similar in both treatments, although the steroidogenic response was eight times higher in the forskolin-stimulated cells, than in the hCG stimulated cells. In addition, combined stimulation by hCG and forskolin did not elevate further cAMP, yet there was a sixfold elevation of the steroidogenic response compared to hCG stimulation alone (see Fig. 3). The sharp rise of intracellular cAMP during the first 15 min of stimulation by hCG, and its subsequent fall to basal levels in the next 3-6 h, before any stimulation of progesterone synthesis could be observed, further suggest that hCG may not exert its effect on the steroidogenic response exclusively via activation of adenylate cyclase (Davis et al., 1987; Flores et al., 1990; Gudermann et al., 1992). The progressive elevation of progesterone production in the presence of the hormone on the 3rd and 4th day of culture, when intracellular cAMP levels remained extremely low, support such an hypothesis. Stimulation of steroidogenesis by 2 μM forskolin was as high as by a saturating dose of hCG while cAMP levels rose to only 5% of those of hCG stimulated cells. This would suggest that the effect of forskolin, as well, is not exerted exclusively by the activation of adenylate cyclase and alternative mechanisms such as modulation of membrane ion channels (Laurenza et al., 1989) should be considered.

The lag period in the steroidogenic response following hCG stimulation suggests de novo synthesis of the cytochrome $P450_{scc}$ system enzymes in the cell line. Indeed, both by

immuno-EM and Western blot techniques using antibodies to adrenodoxin, a key enzyme of this system, a dramatic increase in the level of this enzyme was observed in mitochondria of hCG-stimulated cells compared to nonstimulated cells (Figs. 2 and 9). Interestingly, higher levels of adrenodoxin were detected when cells were costimulated with 1.2 nM hCG and 2 μM forskolin, in agreement with the higher levels of progesterone produced by the costimulated cells compared to cells stimulated with hCG alone (Figs. 3 and 9).

The stimulation of the newly established cell lines with hCG leads to inhibition of cell proliferation in a dose-dependent manner (Fig. 5). This inhibitory effect was evident even at a concentration as low as 40 pM hCG which was sufficient to induce a significant increase in intracellular levels of cAMP in these cells (Fig. 5). This phenomenon was observed in spite of the fact that these cells were transformed by SV-40 DNA and the Ha-*ras* oncogene and in line with the well-known phenomenon that an inverse relationship exists between growth and differentiation of normal granulosa cells (for review see Hsueh et al., 1984; Amsterdam and Rotmensch, 1987). Further studies may shed light on the mechanism by which cell proliferation can be controlled by pituitary glycoprotein hormones both in normal and in oncogene transformed cells expressing the receptor molecules for the appropriate hormone.

The unique established cell lines described here can serve as a convenient model for studying the cellular and molecular mechanisms associated with gonadotropin stimulated steroidogenesis. Moreover, site-directed mutagenesis of the receptor molecule and expression of the mutated molecules in immortalized steroidogenic cells may facilitate structure-function analysis of the receptor molecule.

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