Purification and Characterization of Lutropin Receptor from Membranes of Pig Follicular Fluid†

T. A. Yarney, M. R. Sairam,* G. N. Bhargavi, and S. K. Mohapatra
Reproduction Research Laboratory, Clinical Research Institute of Montreal, Montreal, Quebec, Canada H2W 1R7
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ABSTRACT: Membranes derived from free floating granulosa cells in porcine ovarian follicular fluid were used as a starting material for structural characterization of both LH/hCG and FSH receptors. The receptors were highly hormone-specific and showed single classes of high-affinity binding sites (K_d = 19–74 pM). Their molecular weights as determined by affinity cross-linking with their respective ^{125}I-ligands were similarly 70 000. The membrane-localized receptors could be solubilized with reduced Triton X-100 in the presence of 20% glycerol with good retention of hormone binding activity. The Triton extracts of membranes also showed hormone specificity and equilibrium binding constants similar to the membrane receptors (K_d = 32–48 pM). Affinity chromatography on divinylsulfonyl-Sepharose–oLH columns was utilized to purify the solubilized LH/hCG receptor to a specific activity of 2000 pmol/mg of protein. The purified receptor exhibited a high specificity for hCG and hLH but not for hFSH nor bTSH. The purified receptor was iodinated and visualized to be composed of a major protein of $M_r \approx 70\,000$ and other minor proteins of molecular weights ranging from 14 000 to 40 000. Except for the M_r 14 000 protein, all other protein species bound to the concanavalin A–Sepharose column. The data suggest that the ovarian LH/hCG and FSH receptors are structurally similar and consist of a single polypeptide chain, as recently documented for the LH/hCG receptor (Loosefelt et al., 1989; McFarland et al., 1989).

Gonadotropins regulate ovarian functions following specific interaction(s) with receptors on the cell membrane (Roche & Ryan, 1985). In order to understand the molecular events that underlie gonadotropin–receptor interactions, the structures of both components would be essential. While the structures of the hormones are well established (Sairam, 1983), there are controversies as to the exact chemical makeup of the receptors. Thus, for a number of years, several investigators have focused attention on the purification and characterization of receptors from different species, in spite of their low abundance in gonadal tissues. Although there is evidence in support of a multimeric structure for the luteinizing hormone (LH)[1] receptor (Bruch et al., 1986; Dattatreyamurty et al., 1983; Shin et al., 1986a), the majority of investigators agree that the LH receptor consists of a single polypeptide chain (Ascoli & Segaloff, 1989). Consistent with this, the entire amino acid sequence of a single polypeptide chain constituting the LH/hCG receptor in pig testis and the rat ovary has recently been deduced by cDNA cloning (Loosefelt et al., 1989; McFarland et al., 1989). By contrast, the molecular structure of FSH receptor from any source is yet to be reported. Available data suggest that the FSH receptor from either the calf testis (Reichert & Dattatreyamurty, 1989) or the pig granulosa cells (Shin et al., 1986b) might be composed of disulfide-linked subunits.

A consideration of the above reports revealed that nearly all of the studies have utilized the corpus luteum as the source of LH/hCG receptor. There are no reports in the literature on attempts to purify the LH/hCG receptor from granulosa cells which contain FSH receptors as well (Channing & Ledwitz-Rigby, 1975). Since these cells are freely floating or loosely attached in the mature graafian follicle, it occurred to us that they may be an appropriately clean source of starting material from which to purify LH/hCG and/or FSH receptor(s), which would allow more reliable comparisons to be made between structures of these receptors. In a preliminary study, we have recently reported on the relative abundance of specific LH/hCG binding sites (Yarney et al., 1988) as well as FSH binding sites (Sebok et al., 1987; Yarney et al., 1988) in particulate fractions of pig follicular fluid collected from commercial suppliers. In the present report, we describe the purification and characterization of a single LH/hCG receptor protein from this source and compare it to the FSH receptor found in the same source.

Materials and Methods

Hormones and Chemicals. Purified hCG (CR125, iodination grade) was supplied by the NIADDK. Ovine LH (Sairam, 1976), hLH (Sairam et al., 1978), oFSH (Sairam, 1979), and hFSH (Sairam & Li, 1979) were isolated in this laboratory. Bovine TSH was a gift from Dr. J. G. Pierce (California). Bovine γ-globulin, BSA, Triton X-100, lactoperoxidase, PEG (M_r 8000), and PMSF were purchased from Sigma Chemical Co. (St. Louis, MO). Reduced Triton X-100 (Triton X-100R) was from Aldrich Chemicals. Bicinchoninic acid (BCA) protein assay reagents and DSS were purchased from Pierce Chemical Co. (Rockford, IL). Heparin–Sepharose CL-6B and concanavalin A–Sepharose 4B were from Pharmacia Fine Chemicals (Uppsala, Sweden). Reagents for SDS–PAGE and molecular weight standards were from

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* Correspondence should be addressed to this author at the Reproduction Research Laboratory, Clinical Research Institute of Montreal, 110 Pine Ave. West, Montreal, Quebec, Canada H2W 1R7.

[1] Abbreviations: AMP, adenosine monophosphate; BSA, bovine serum albumin; Con A, concanavalin A; DG-hCG, deglycosylated hCG; DMSO, dimethyl sulfoxide; DSS, disuccinimidyl suberate; DTT, dithiothreitol; EDTA, disodium ethylenediaminetetraacetate; FSH, follicle-stimulating hormone; hCG, human chorionic gonadotropin; LH, luteinizing hormone; PEG, poly(ethylene glycol); PMSF, phenylmethanesulfonyl fluoride; PRL, prolactin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone.

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Bio-Rad (Richmond, CA). The colloidal gold protein assay reagent (Auro Dye Forte) was purchased from Janssen Biotech N.V. (Olen, Belgium). All other chemicals used were of reagent grade or higher purity.

Preparation of Membrane Fraction. Membranes were prepared from particulate fractions of pFF (consisting mainly of free floating granulosa cells and other cellular debris). The pFF, supplied by the Contraceptive Development Branch of the NICHD, had been obtained as frozen aliquots (500 mL) from J. R. Scientific (Woodland, CA) and kept frozen at −20 to −70 °C. At the time of processing in the laboratory, the pFF was thawed and centrifuged at 4 °C at 30000*g* for 1 h. The pellet was homogenized in 5 volumes of 25 mM sodium phosphate (PO_4, pH 7.4) containing 300 mM sucrose and 100 μM PMSF and centrifuged at 4 °C at 30000*g* for 1 h. The final pellet was washed twice and finally dispersed in 2 volumes of the above buffer containing 100 μM PMSF for storage at −70 °C.

Solubilization of Receptor from the pFF Membrane Fraction. Membranes prepared from pFF pellets were thawed and rehomogenized in 2 volumes of 25 mM PO_4 buffer containing 100 μM PMSF and then centrifuged at 4 °C at 30000*g* for 30 min. The pellet was suspended in 2 volumes of 25 mM PO_4 containing 20% glycerol, 150 mM KCl, 100 μM PMSF (TSR buffer), and 1% Triton X-100R. The mixture was stirred on ice for 1 h and then centrifuged at 4 °C at 30000*g* for 1 h. The supernatant was recovered and filtered serially through 3- and 0.65-μm and through 0.45-μm Millipore filters (Millipore Corp., Bedford, MA). The solubilized receptor was stored in aliquots at −70 °C until needed. These conditions optimized for the solubilization of LH/hCG receptor activity also allowed detection of significant FSH receptor activity in the extracts (see Results).

Preparation of DVS-Sepharose–oLH Affinity Columns. Purified oLH was immobilized on divinyl sulfone (DVS)-activated Sepharose 4B or 6B according to published procedures (Sairam, 1981). The column was then washed extensively at 4 °C with ≈6 L of 25 mM Tris-HCl. Column washes were carefully checked for the absence of oLH activity in a porcine ovarian membrane radioreceptor assay before use. Each batch of DVS-Sepharose–oLH was used for up to five cycles of affinity chromatography, although binding capacity declined gradually with successive cycles. In control experiments, we have used similar columns with myoglobin immobilized on DVS-Sepharose.

Purification of Solubilized Receptor by Affinity Chromatography. The solubilized receptor (10 mL) was diluted 10-fold in TSR buffer and added to the DVS-Sepharose–oLH gel (20 mL) preequilibrated with TSR buffer containing 0.1% Triton X-100R. The mixture was agitated in a plastic bottle for 3 h at 4 °C after which it was poured into a column and the unadsorbed fraction collected. The column was washed with 250–500 mL of TSR buffer containing 500 mM NaCl and 0.05% Triton X-100R, until the washings were free of detectable ^{125}I-hCG binding activity. Elution of receptor was effected with 25 mM acetic acid containing 20% glycerol, 1 M NaCl, 0.05% Triton X-100R, and 100 μM PMSF. Fractions of 3 mL were collected and immediately neutralized with drops of NH_4OH or NaOH. Aliquots (50 or 100 μL) of fractions were assayed for ^{125}I-hCG binding, keeping final Triton X-100R concentrations to 0.01%. Appropriate controls were always run in each binding experiment. Fractions were frozen at −70 °C until further processing.

For repurification of the first affinity-purified receptor, several batches of active fractions (frozen) or entire eluates from columns (nonfrozen) were concentrated at least 4-fold by ultrafiltration through an Amicon (Lexington, MA) ultrafiltration unit with a PM-10 membrane having an exclusion limit of M_r 10000. The concentrate was diluted with an equal volume of TSR buffer containing 0.05% Triton X-100R mixed with preequilibrated gel and agitated end-over-end for 3 h at 4 °C. The second affinity column was packed, washed, and eluted as indicated for the first column. Aliquots (100 μL) of fractions were assayed for ^{125}I-hCG binding, and the remainder was stored at −70 °C.

As necessary, aliquots of affinity-purified receptors were thawed and concentrated with the Amicon Centricon-10 (M_r 10000 cutoff) unit, while pooled batches were concentrated with the Amicon Centriprep-10 unit or ultrafiltration cells with the PM-10 membrane.

Radioiodination of Hormone and Receptors. The hCG (CR125), oFSH, and solubilized and purified receptors were labeled with ^{125}I by the lactoperoxidase method as previously described (Sairam, 1979). Free Na^{125}I was separated from iodinated protein by gel filtration on Sephadex G-50 or on a Bio-Rad Econo-column PD 10 (Bio-Gel P6 gel) desalting columns. For hormone–receptor cross-linking studies, the elution buffer for ^{125}I-hCG purification contained 0.05% Triton X-100R instead of 0.1% BSA. Radiolabeled receptors were eluted in the TSR buffer containing 0.2% NaN_3 and 0.05% Triton X-100R. The specific activity ranged from 50 to 80 μCi/μg for ^{125}I-hCG and from 60 to 110 μCi/μg for ^{125}I-oFSH.

Binding Assays. Assays were performed in duplicate in 12 × 75 mm polystyrene tubes containing 25 mM Tris-HCl, pH 7.2, 10 mM $MgCl_2$, and 0.1% BSA. For single-point assays, 1 ng of ^{125}I-hCG was added to appropriate aliquots of membrane, solubilized, or purified receptor samples in a 500-μL final reaction volume. Concentrations of ^{125}I-hCG were varied from 0.12 to 15 ng for saturation analysis, and in competition studies, increasing concentrations of hormones were added to 1 ng of ^{125}I-hCG. Nonspecific binding determined with 1 μg of hCG was no more than 5–8% of added ^{125}I-hCG for membrane, solubilized, and purified receptor preparations.

Following overnight incubation at 25 °C, membrane receptor assays were terminated by the addition of 2 mL of chilled assay buffer and centrifugation at 2900*g* for 15 min. Soluble ^{125}I-hCG–receptor complexes were sedimented by adding 500 μL of bovine γ-globulin (2 mg/mL in assay buffer) and 1 mL of 25% PEG (w/v in H_2O) to tubes followed by vortexing, 20-min incubation at 25 °C, and centrifugation at 5500*g* for 30 min at 4 °C. Pellets were reprecipitated with 12.5% final PEG concentration after solubilization with 0.1% Triton X-100 (in 10 mM PO_4 buffer). Precipitates were counted in a Rackgamma II counter with 55% counting efficiency. The same procedures described above were employed for assaying ^{125}I-oFSH binding activity in membranes and Triton extracts. Nonspecific binding was determined with 1 μg oFSH and was no more than 8% of added ^{125}I-oFSH.

Protein Assays. Aliquots of membranes were solubilized with equal volume of 500 mM NaOH, diluted with water, and assayed directly in the BCA protein assay (Smith et al., 1985). Protein contents of purified receptors were measured by the colloidal gold method of Stoscheck (1987). BSA was used as standard in both assays.

Cross-Linking of hCG– and oFSH–Receptor Complexes. Membranes (≈600 μg of protein) were incubated with ^{125}I-hCG or ^{125}I-oFSH in the absence or presence of 1 μg of homologous hormone overnight at 25 °C in 25 mM PO_4 buffer (assay volume = 500 μL). Purified receptor was incubated

with ^{125}I-hCG in the absence or presence of 10–100 ng of hCG or 100 ng of oLH for 12 h at 25 °C in 25 mM PO_4 buffer (volume = 270 μL). Final Triton X-100R concentration in soluble receptor incubates was maintained at 0.01% (no BSA). Following hormone–receptor complex formation, membranes were washed twice and dispersed again in fresh buffer. With purified receptor, there was no separation of bound and free hormone. To resuspended membrane or pure receptor preparation, DSS (dissolved in DMSO) was added to a final concentration of 500 μM for the LH/hCG receptor and to 1 mM for the FSH receptor, and the mixtures were incubated for 15 min at 25 °C. The reaction was quenched by addition of Tris-HCl (pH 7.4) to a final concentration of 140 mM, followed by an additional 10-min incubation at 25 °C. Cross-linked membranes were washed and then treated with 4% SDS and prepared for SDS–PAGE. Aliquots of the purified receptor–^{125}I-hCG complex following cross-linking were directly used for SDS–PAGE.

For comparison, cross-linking studies were also performed with mouse Leydig tumor cells (MA-10 clone donated by Dr. M. Ascoli, New York).

Con A–Sepharose Chromatography of Receptors. About 3 mL of Con A–Sepharose was packed in a column and washed with 25 mM Tris-HCl buffer containing 1 mM $CaCl_2$, 1 mM $MnCl_2$, 500 mM NaCl, and 0.05% Triton X-100R (Con A buffer), followed by washing with Con A buffer plus 1 M methyl α-D-glucoside (MEG) and finally with Con A buffer alone. The ^{125}I-purified receptor was diluted to 200 000 cpm/500 μL of Con A buffer and loaded onto column. The column was stopped for 0.5–1 h and then washed with Con A buffer and eluted with Con A buffer plus 1 M MEG. Stock, unadsorbed, and eluted fractions were dialyzed (M_r 6000–8000 cutoff) against water; samples were lyophilized and analyzed by SDS–PAGE.

Heparin–Sepharose Chromatography of Receptors. About 3 mL of swollen heparin–Sepharose was poured into a column and washed extensively. Solubilized receptor (2 mL) was diluted 10-fold in TSR buffer containing 100 mM NaCl and mixed with the equilibrated gel for 16 h. The unadsorbed fraction was collected, and the column was washed extensively with the TSR buffer containing 100 mM NaCl and 0.05% Triton X-100R. Bound receptor was eluted with TSR buffer containing 1.5 M NaCl and 0.05% Triton X-100R. Stock, unadsorbed, and eluted fractions were assayed for ^{125}I-hCG binding activity with inclusion of appropriate controls. Labeled purified receptor (200 000 cpm) was diluted in TSR buffer containing 100 mM NaCl and 0.05% Triton X-100R and incubated with gel overnight and processed as above.

SDS–PAGE. The discontinuous gel system of Laemmli (1970) was used with 4–5% stacking gels and a gradient of 5–15% separating gels. Samples with DTT were boiled for 20 min prior to being loaded on gels. Electrophoresis was performed at 60 mA for 6 h with a Protean II apparatus (Bio-Rad) which was cooled with circulating water. Following fixation, the gels were stained in Coomassie Brilliant Blue R-250, destained, dried, and subjected to autoradiography at −70 °C using a Dupont Cronex Lightning plus intensifying screen and Eastman Kodak X-Omat XRP or XAR film.

Data Analysis. The data from saturation studies were analyzed with the LIGAND program modified for the IBM microcomputer (McPherson, 1985). In these calculations, M_r 37 000 was used for hCG, and M_r 28 300 was used for oFSH (Sairam, 1983).

RESULTS

LH/hCG and FSH Receptor in Pig Ovarian Membranes.

Table I: LH/hCG and FSH Receptor Binding Activities in Membranes and Triton-Soluble Extracts of Membranes of Porcine Follicular Fluid[a]

receptor	fraction	dissociation constant ($\times 10^{-12}$ M)	binding capacity (fmol/mg of protein)
LH/hCG	membrane	18.7 ± 1.9 (6)	119.3 ± 10.3 (6)
	Triton X-100R soluble	31.6 ± 2.6 (4)	300.6 ± 36.5 (4)
FSH	membrane	73.6 ± 8.1 (6)	292.1 ± 74.7 (6)
	Triton X-100R soluble	48.2 ± 5.9 (4)	133.5 ± 50.4 (4)

[a] Values represent mean ± SEM of number of determinations indicated in parentheses. All measurements were done using ^{125}I-hCG or ^{125}I-oFSH.

As the follicular fluid was aspirated from all visible large follicles in the ovaries of pigs, it can be assumed that they were also from antral follicles. At this stage, the granulosa cells which are found in the fluid contain both LH/hCG and FSH receptor (Channing & Ledwitz-Rigby, 1975). The hormone binding capacity for membranes collected over the last 4–5 years for different batches was rather consistent, showing 119.3 ± 10.3 and 292.1 ± 74.7 fmol of ^{125}I-hCG and ^{125}I-FSH binding activity, respectively, per milligram of membrane protein (Table I). The dissociation constants were 18.7 ± 1.9 and 73.6 ± 8.1 pM for hCG and FSH, respectively. Both these binding activities were highly hormone-specific (data not shown). These membrane fractions could be stored in the form of lyophilized powders at 4 °C for at least 2 years without any significant loss of binding activity. Although hormone-specific binding activity (receptor) could be extracted from these dry powders after storage, we have chosen to employ frozen membranes as quickly as possible for solubilization and subsequent purifications.

Solubilization of LH/hCG and FSH Receptors. The reduced Triton X-100 extract contained up to 65% of the original hCG binding activity in membranes. The specific activity of the soluble receptor preparation was assessed to be 300.6 ± 36.5 fmol/mg of protein, and the dissociation constant was 31.6 ± 2.6 pM (Table I). The hormone specificities of membrane and solubilized receptors were similar. With hCG as reference, DG-hCG and oLH were 3.4 and 0.8 times active in the membrane assay and 7.1 and 1.8 times active in the soluble receptor assay. Ovine FSH showed little reactivity (≤0.03 times) in both assays.

About 70–80% of the ^{125}I-oFSH binding activity was extractable from the membranes. However, only about 20% of the initial activity in the membranes was assayable in the final soluble extract after several days, and the activity appeared to be maintained at this level following storage at −70 °C. It is possible that slightly different conditions may have to be utilized for the preservation of FSH receptor activity upon solubilization (Reichert & Dattatreyamurty, 1989) or that this receptor is relatively more unstable than the LH receptor from the same source. Similar to the membranes, the Triton extract showed high binding affinity (Table I) and was also highly hormone-specific (data not included).

Compared to the membrane, the solubilized hCG receptor showed some degree of instability during storage even in the presence of glycerol and storage at −70 °C. The solubilized receptor could maintain about 100% of initial hCG binding activity for up to 1 year although in some batches some decline in activity was observed from 80 to 50% of initial in 5 months. Soluble receptor preparations were generally used for purification of LH/hCG receptor within 6 weeks of extraction.

Cross-Linking of ^{125}I-hCG to Membrane Receptor. For structural characterization of the LH/hCG receptor as present in the membranes, the bifunctional cross-linker DSS was used

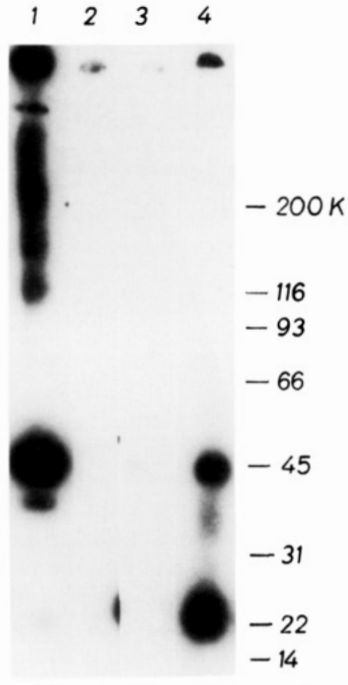

FIGURE 1: Cross-linking of ^{125}I-hCG to porcine membrane-bound LH/hCG receptor. Membrane receptors were incubated with ^{125}I-hCG in the absence (lane 1) or presence of 1 μg of hCG (lane 2) or 1 μg of oLH (lane 3). The mixtures were then cross-linked with DSS. Lane 4 represents cross-linked ^{125}I-hCG. Samples were analyzed by SDS–PAGE, and gels were dried and subjected to autoradiography. The positions of the molecular weight standards are indicated on the right of the autoradiogram.

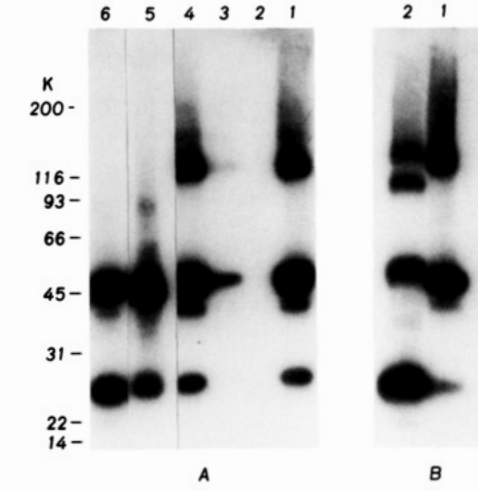

FIGURE 2: Cross-linking of ^{125}I-hCG to mouse Leydig tumor cell LH/hCG receptor. (A) Cells in medium were incubated with ^{125}I-hCG in the absence (lane 1) or presence of 10 μg/mL hCG (lane 2), 20 μg/mL oLH (lane 3), and 20 μg/mL oFSH (lane 4). Excess ligand was aspirated, and the cells were washed twice with PBS (pH 7.4). Bound ligand was cross-linked with DSS. Receptors were solubilized with SDS and analyzed with SDS–PAGE under nonreducing conditions. The gel was dried and subjected to autoradiography. Lane 5 represents cross-linked ^{125}I-hCG, and lane 6 represents non-cross-linked ^{125}I-hCG sample. (B) Sample analyzed in lane 1 was reanalyzed under nonreducing (lane 1) and reducing (lane 2) conditions. The positions of the molecular weight standards are indicated on the left of the autoradiogram.

to chemically couple ^{12}I-hCG to the receptor. With ^{125}I-hCG, the reaction yielded some cross-linking between hCG subunits, a proportion of which was unable to penetrate the gel (lane 4, Figure 1). The fraction that penetrated the gel gave bands at M_r 45 000 and 23 000. These represent hCG-α,β and hCG-α, respectively; a faint band representing hCG-β was sometimes seen at M_r 33 000–40 000, confirming that labeling occurs primarily in the hCG α-subunit (Morgan et al., 1974). Cross-linking of ^{125}I-hCG to the membrane receptor yielded some aggregates of cross-linked components which were unable to penetrate the gel. Those penetrating the gel separated into components of molecular weight ranging from 40 000 to 206 000. The M_r 40 000–52 000 bands correspond to hCG; the M_r 22 000 is hCG-α. The larger molecular weight bands in the range greater than 120 000 might represent varying ratios of ^{125}I-hCG–receptor complexes. The band at about M_r 120 000 likely represents a 1:1 ratio of ^{125}I-hCG and receptor. That these bands correspond to the ^{125}I-hCG–receptor complexes is supported by the ability to abolish their appearance altogether with excess unlabeled hCG or oLH (lanes 2 and 3). Thus, accounting for the size of the radiolabel, the molecular weight of the receptor is estimated to be 70 000–90 000.

For comparison, Figure 2 demonstrates that when similar conditions for cross-linking were applied with the mouse tumor Leydig cell (MA-10) receptor, four prominent bands were obtained at M_r 120 000, 45 000, 40 000, and 24 000 positions (lane 1); lighter bands were also present at the M_r >120 000–200 000 region. Again, the bands at M_r 24 000, 40 000, and 45 000 are hCG-α, hCG-β, and hCG-α,β. The larger molecular weight bands are likely ^{125}I-hCG–receptor complexes with the prominent band at $M_r \approx 120\,000$ of a 1:1 ratio of hormone and receptor. The faint complexes in the M_r 120 000–200 000 region may represent extra cross-linking of ^{125}I-hCG subunits with ^{125}I-hCG–receptor complexes, but this occurred only to a minor degree with this receptor. Again, only LH/hCG (lanes 2 and 3) but not FSH (lane 4) could compete for binding to these sites. Under reduced conditions, the M_r 120 000 band separated into two bands at $M_r \approx 100\,000$ and $M_r \approx 125\,000$. The hCG β-subunit bound to receptor would not be readily visible on the gel under reduced conditions as this subunit contains little or no radioactivity. Thus, under reduced conditions, the M_r 100 000 and 120 000 can only correspond to receptor–hCG-α and receptor–hCG-α,β cross-linked complexes. Thus, the molecular weight of MA-10 receptor was also judged to be similar to that seen above in porcine granulosa cell membranes, providing supporting evidence that the binding components examined in the pig follicular fluid pellet were of cellular origin.

Cross-Linking of ^{125}I-oFSH to Membrane Receptor. As specific oFSH binding was detected in every batch of the particulate fraction (Sebok et al., 1987; Yarney et al., 1988) studied thus far, it was of interest to ascertain the approximate size(s) of the molecular species involved in this interaction. This was examined by employing the same cross-linking methods as utilized for ^{125}I-hCG in Figure 1, with the exception that 1 mM DSS was used. These data are shown in Figure 3. There were some notable similarities as well as differences in comparison to the cross-linking experiments with ^{125}I-hCG. First, the autoradiograms were less complex as fewer high molecular weight components were detected. In contrast to the ^{125}I-hCG, the oFSH (M_r 33 000) was labeled to a detectable extent in the hormone-specific β-subunit also (lane 4). The major protein band representing the membrane component plus the radioactive hormone appeared to be of M_r 103 000, in addition to another minor but detectable species of M_r 165 000 (lane 1). The third band of M_r 33 000 was concluded to be the same as ^{125}I-oFSH which was not cross-linked. When excess (1 μg) unlabeled oFSH was present in the incubation (lane 2), radioactive components noted above were completely absent. However, 1 μg of oLH did not compete for these binding sites and hence cross-linking (lane

Table II: Purification of the Porcine LH/hCG Receptor from Membranes of Porcine Follicular Fluid[a]

fraction	protein	activity (pmol)	specific activity (pmol/mg)	purification (x-fold)	recovery (%)
crude membrane	176–659 mg	47.6–52.0	0.079–0.271	1	100
Triton X-100R extract	66–207 mg	21.3–25.7	0.125–0.324	1.2–1.6	45–47
first-cycle affinity column eluate	1.64–1.82 μg	2.59–4.4	1580–2420	9000–20000	5–9

[a] Range of values obtained for representative batches is shown.

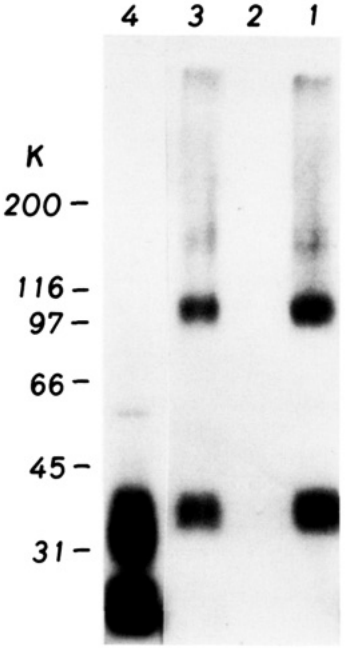

FIGURE 3: Cross-linking of ^{125}I-oFSH to porcine membrane-bound FSH receptor. Membrane receptors were incubated with ^{125}I-oFSH in the absence (lane 1) or presence of 1 μg of oFSH (lane 2) or 1 μg of oLH (lane 3). The mixtures were then cross-linked with DSS. Lane 4 represents ^{125}I-oFSH. Samples were analyzed by SDS–PAGE, and gels were dried and subjected to autoradiography.

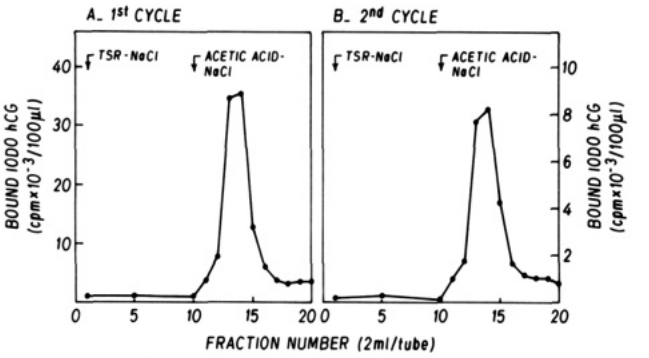

FIGURE 4: Purification of porcine LH/hCG receptor by sequential affinity chromatography on DVS-Sepharose–oLH (10-mL packed gel). The column was extensively washed and verified as described under Materials and Methods before interaction with Triton-solubilized receptor for 3 h at 4 °C. The unadsorbed fraction was saved and the column extensively washed with the starting buffer containing 20% glycerol. In this figure, only the last few milliliters of the wash fraction is depicted after the first arrow indicating TSR–NaCl. Bound receptor was eluted by 25 mM acetic acid, 1 M NaCl, 20% glycerol, and 100 μM PMSF, pH 4.0. Following neutralization and assay, appropriate fractions were pooled and submitted to a second cycle of affinity chromatography on a duplicate column of DVS-Sepharose–oLH (panel B).

3), establishing that these components were FSH specific. Considering that the apparent molecular weight of ^{125}I-oFSH (lane 4) is 33 000, the species detected at M_r 103 000 (lane 1) could represent a 1:1 complex of hormone and receptor, the latter contributing a mass of 70 000 daltons. The minor component observed at about M_r 165 000 could be due to an aggregate of the receptor. Thus, it would appear that the ^{125}I-FSH binding component seen in pFF membranes is similar in size to that of LH/hCG receptor (Figure 1).

Purification of LH/hCG Receptor. Data on representative purifications and recoveries are shown in Figure 4 and Table II. During preliminary trials, the soluble receptor extract was incubated with DVS-Sepharose–oLH or with hCG–Affigel 10 gel overnight at 4 °C. This protocol resulted in tight binding of solubilized receptor to the hormone on the column such that less than 10% of adsorbed receptor was eventually elutable with the acetic acid buffer described under Materials and Methods. Alkaline buffer (100 mM NH_4OH, 20% glycerol, 1 M NaCl, 0.05% Triton X-100R, and 100 μM PMSF, pH 10) was also effective, but we have preferred to use the acidic elution conditions as they were more consistent. High-salt buffer (3 M NaCl in TSR, pH 7.0) was however ineffective in eluting receptor. Generally, the yield of receptors recovered from the DVS-Sepharose–oLH columns was more than from hCG–Affigel 10 columns. Incubating receptor with the DVS-Sepharose–oLH gel for 3 h at 4 °C or for 2 h at 25 °C enabled 20–45% of adsorbed material (10–20% of activity loaded) to be eluted from the column as estimated by actual determination of activity not accounting for any possible losses due to partial inactivation. The receptor was eluted in the first few fractions where the pH changed from 7.0 to 4.0. Further purification of the receptor could be obtained by incubating concentrated eluates from the first column with a new gel. During this step, 44–73% of the first cycle eluate bound to the second column. Of the loaded activity (20–60% of adsorbed), 15–32% during the second cycle chromatography was recovered in the eluates. We assessed that handling of the first cycle eluates prior to incubating with the second affinity column or freezing led to a loss of 50–65% of activity. The recovery of the first cycle receptors was up to about 10% of initial activity in crude membranes. The specific activity of the first cycle purified receptor averaged 2000 pmol/mg of protein.

Characterization of Purified Receptor. SDS–PAGE Analysis of Iodinated Receptor. The degree of purity of the first- and second-cycle affinity-purified receptors was analyzed by SDS–PAGE following iodination. We chose to perform these experiments with ^{125}I-labeled materials to enhance the detection of components (related or unrelated if any) in the fraction. Affinity-purified receptor fractions (see Figure 4A,B) concentrated by Amicon centricon units could be adequately labeled by lactoperoxidase (see Materials and Methods) and fractionation on Bio-Gel P6 DG columns. Under nonreducing and nondenaturing conditions (Figure 5) (lanes 2 and 3, panel B, and lane 2, panel A), the second-cycle receptor revealed esssentially a single component with a molecular weight around 66 000. However, when gels were deliberately overloaded to permit detection, some other components could be revealed (lane 1 in panels A and B). In the reduced state, the major component migrated with an approximate molecular weight of 93 000. The appearance of fainter bands varied slightly from batch to batch (two extreme examples are depicted in Figure 5A and Figure 5B, to emphasize this), with components varying mostly from M_r 45 000 to M_r 14 000. By densitometric

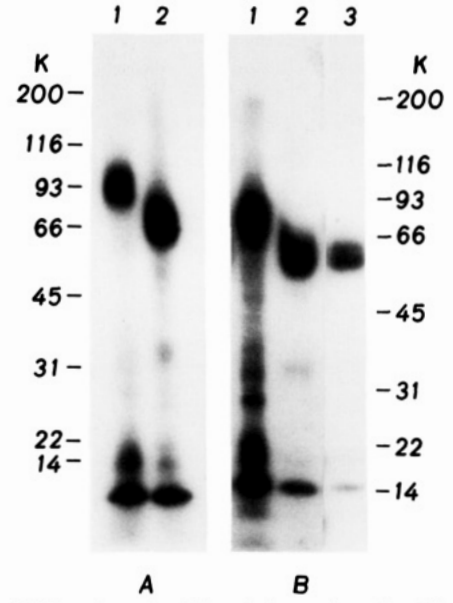

FIGURE 5: SDS–polyacrylamide gel electrophoresis of the iodinated purified receptor. Samples were concentrated and iodinated as described under Materials and Methods and analyzed by SDS–PAGE under reducing (lane 1) and nonreducing (lanes 2 and 3) conditions. Gels were dried and subjected to autoradiography. Two separate runs of the second-cycle receptor are depicted in panels A and B. Lane 3 (in panel B) depicts analysis with less radioactivity to enable visualization of doublet of bands. The positions of the molecular weight standards are shown on the sides of each panel. In lane 1 (panel B) excess radioactivity was deliberately overloaded to visualize other components.

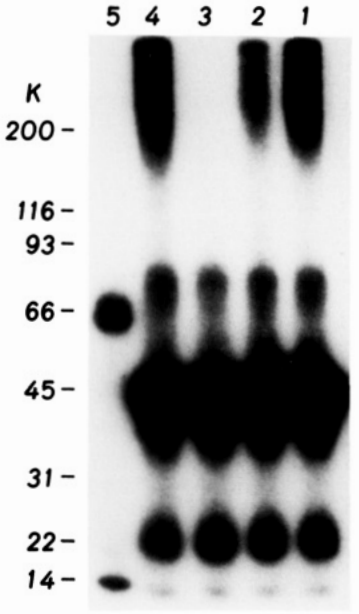

FIGURE 6: Cross-linking of ^{125}I-hCG to purified LH/hCG receptor. LH/hCG receptors eluted from the first affinity column were concentrated by ultrafiltration through a PM-10 membrane. Aliquots of concentrated receptor were incubated with ^{125}I-hCG in the absence (lane 1) or presence of 10 ng of hCG (lane 2), 100 ng of hCG (lane 3), and 100 ng of hFSH (lane 4). Cross-linking was carried out with DSS. Aliquots of samples were then analyzed by SDS–PAGE under nonreducing conditions. The gel was dried and subjected to autoradiography. Iodinated one-cycle purified receptor is depicted in lane 5 for comparison. Note that in this batch of receptor other components except for M_r 14000 are absent. The positions of the molecular weight standards are shown on the left of the autoradiogram.

analysis of various batches of iodinated receptor, we could estimate that in the reduced condition the M_r 93000 component accounted for at least 80% of the total protein present in the fraction. This, however, assumes that all the protein contained therein had tyrosine residues accessible for radiolabeling.

When the first-cycle receptor was analyzed under the same conditions (data not shown), the results were similar, with the M_r 66000 (unreduced) and 93000 (reduced) being the major detectable components. In some instances, the M_r 22000 and 14000 bands were also evident. Prolonged storage of the iodinated second-cycle purified receptor at −70 °C (6 months) showed a definite increase in the appearance of the M_r 50000 band, as well as the M_r 22000 band. The M_r 14000 component appeared to have been reduced and presumably transformed to one of lower molecular weight now migrating closer to the dye front. These components, in addition to the original M_r 66000 and 93000 bands, were seen in the unreduced and reduced conditions. Thus, slow degradation of the M_r 66000–93000 protein could give rise to other components under different conditions.

Upon chromatography of the solubilized extract on columns of DVS-Sepharose–myoglobin, all the LH/hCG binding activity was completely excluded, with no detectable activity being present in the acid eluates of this column. Additionally, when a known quantity of solubilized extract was pretreated with unlabeled LH/hCG prior to affinity chromatography, again, no ^{125}I-hCG bindable activity was eluted in the elution buffer. Such experiments provided convincing data of the specific adsorption of the LH/hCG receptor in the extract on DVS-Sepharose–LH columns.

In order to further establish the origin of the smaller components and to ascertain if these have hormone binding ability, the crude Triton-solubilized receptor was itself labeled. Following this, it was subjected to affinity chromatographic purification as described above (Figure 4). Radioactivity bound to DVS-Sepharose–LH column was analyzed on SDS–PAGE. The electrophoretic patterns of the principal components (i.e., M_r 66000 and 93000) were identical (not shown) with that seen in Figure 5 for the purified receptor under nonreduced and reduced conditions. There was also another component of M_r 45000, but this was of much lower intensity. That this could be eluted from the affinity column provided clear indication of the hormone binding ability of the M_r 45000 component. Some of the other components, noticeable in Figure 5 following iodination of purified receptor, were conspicuously absent in this experiment.

Cross-Linking of ^{125}I-hCG to Affinity-Purified Receptor. Figure 6 depicts the cross-linking of ^{125}I-hCG to the affinity-purified receptor emerging from columns shown in Figure 4. In lane 5 is the SDS–PAGE of receptor following its iodination. Again, the predominant M_r 66000 component is evident with the minor M_r 14000 band being detectable again in this preparation. When affinity-purified receptors were cross-linked by using DSS to bound ^{125}I-hCG, the gels were run without separation of the free ^{125}I-hCG. Thus, the region of migration of the hormone, M_r 52000–45000, is relatively darker than the M_r 24000 band due to the α-subunit arising during the electrophoresis. The M_r 80000 band is a product of cross-linked hCG molecules which is also present in lane 3. The hormone–receptor complexes generated in this experiment migrated as very high aggregates showing molecular weight values of 200000 and above. Accounting for the size of the ligand (^{125}I-hCG-α or ^{125}I-hCG-α,β), the size of the H–R complex must be at least 160000 daltons. These high molecular weight complexes were completely displaceable by hCG (varying concentrations in lanes 2 and 3) but not by 100 ng of purified hFSH (lane 4). The high molecular weight cross-linked aggregates did not dissociate (data not shown)

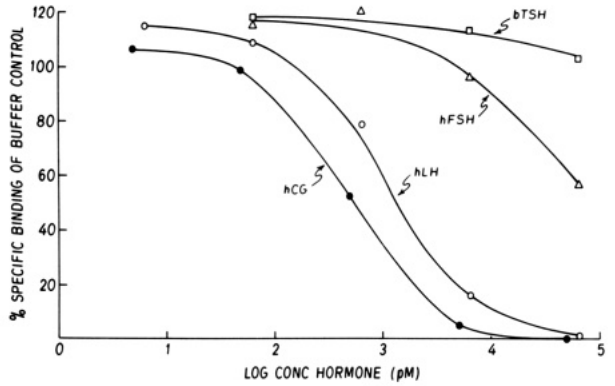

FIGURE 7: Competitive displacement of ^{125}I-hCG binding to purified receptor by unlabeled hormones. First-cycle purified receptor was incubated with a constant amount (1 ng) of ^{125}I-hCG and various amounts of unlabeled hormones. Each point represents the average of duplicate determinations. The molecular weight of hCG was considered as 37 000 and those for hLH, hFSH, and bTSH as 28 300.

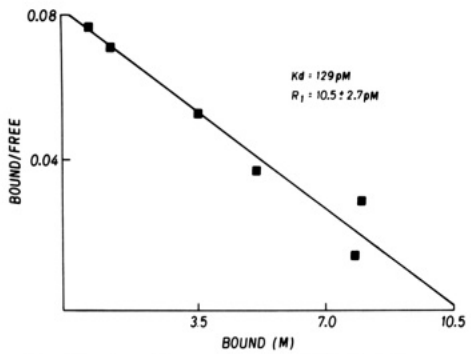

FIGURE 8: Scatchard analysis of purified LH/hCG receptor. Concentrated first-cycle purified receptor was assayed with increasing amounts of ^{125}I-hCG, in the absence or presence of 1 μg of hCG. Data were analyzed and plotted by the LIGAND program modified for the IBM microcomputer (McPherson, 1985).

upon reduction, indicating tight cross-linking in the soluble form.

Hormone Specificity and Binding Affinity of Purified Receptor. The material obtained from the first-cycle affinity column showed high specificity for hCG and hLH; hFSH and bTSH were poor competitors for ^{125}I-hCG binding (Figure 7) according to the degree of their LH contamination. The dissociation constant of the first-cycle-purified receptor was determined to be 1.29×10^{-10} M (Figure 8).

Binding of Purified Receptor to Con A- and Heparin-Sepharose. The iodinated first- and second-cycle affinity-purified receptors were evaluated for their ability to bind Con A- and heparin-Sepharose. Of the radioactivity loaded onto the column, 50–75% was bound to Con A under the conditions used and was subsequently displaced with 1 M methyl α-D-glucoside. The unbound fraction consisted of only the M_r 14 000 protein described above (see autoradiographic patterns in the inset of Figure 9). The components bound to the Con A column were proteins migrating at M_r 66 000 and 22 000 and 40 000 (for the second-cycle receptor). The iodinated purified receptors did not bind to heparin. However, the crude solubilized receptor bound to heparin-Sepharose when incubated overnight at 4 °C (39%). Bound receptor was recovered (50–100% of adsorbed) with 1.5 M NaCl in TSR buffer. As there was no significant decrease in protein concentration, this step was judged to be unsuitable for routine purification.

Discussion

One of the primary reasons for undertaking this investigation was to be able to utilize a single source as a potential starting

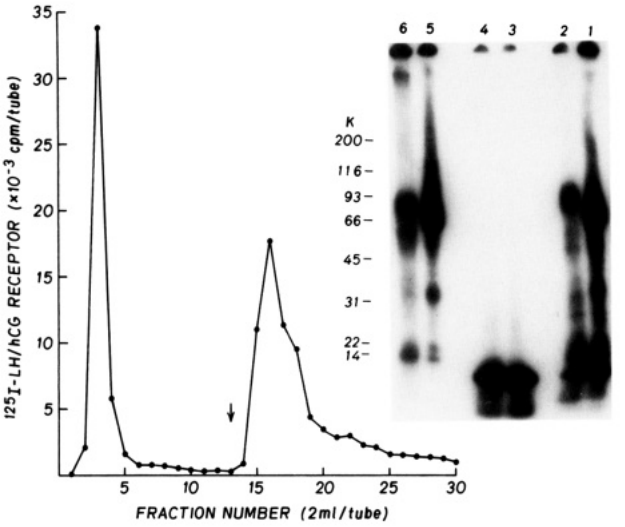

FIGURE 9: Chromatography of iodinated purified LH/hCG receptor on Con A-Sepharose. Iodinated purified LH/hCG receptor was diluted in Con A buffer described under Materials and Methods and loaded onto the column. The column was stopped for about 30 min and washed with Con A buffer (fractions 1–13). Iodoreceptor bound to the gel was eluted (start of elution indicated by arrow) with 1 M MeG in Con A buffer. Fractions were counted for radioactivity. Peak flow-through and MeG-eluted fractions were collected separately. Aliquot of stock loaded onto column and pooled fractions were desalted by dialysis and lyophilized and then analyzed by SDS-PAGE. Gels were dried and subjected to autoradiography. The inset shows the SDS-PAGE pattern of the stock sample (lanes 1 and 2) and flow-through (lanes 3 and 4) and MeG-eluted (lanes 5 and 6) fractions analyzed under nonreducing (lanes 1, 3, and 5) and reducing (lanes 2, 4, and 6) conditions. The positions of the molecular weight standards are shown on the left side of the autoradiogram.

material for eventual isolation of both LH and FSH receptors. As demonstrated in this study, material of pig ovarian origin, collected by commercial suppliers (Table I), contained an appreciable quantity of receptors, although for the LH/hCG receptors the concentration is surpassed only by the corpus luteum (Roche & Ryan, 1985; Ascoli & Segaloff, 1989). On the other hand, the calf testes used to study FSH receptors has a very low content of LH/hCG receptors (Reichert & Dattatreyamurty, 1989; our unpublished results), while the corpus luteum lacks FSH receptor altogether.

Before undertaking detailed purification studies, we deduced the molecular nature of the two interacting components (receptors) by making affinity cross-links with appropriate labeled hormones (^{125}I-hCG and ^{125}I-FSH). Interestingly, in both cases, the hormone binding component, as studied in the membranes (Figures 1 and 3), had a molecular weight of about 70 000. Considering that both the hormone (ligands) and receptors are glycoproteins and the atypical behavior of such proteins on SDS-PAGE, the molecular weight approximations reported here are very close to the LH/hCG receptor reported in the recent literature (Roche & Ryan, 1985; Ascoli & Segaloff, 1989). Others studying FSH receptors have suggested a $M_r \approx 70\,000$ for the porcine granulosa cell receptor (Shin et al., 1986b) and M_r 240 000 for the calf testis receptor (Reichert & Dattatreyamurty, 1989).

Because the LH/hCG receptor was relatively more stable than the FSH receptor in the soluble form, we focused on its purification. As described in this report, these data represent the first detailed investigation on the purification of LH/hCG receptor from granulosa cells as opposed to all others utilizing either the corpus luteum tissue (Dattatreyamurty et al., 1983; Wimalasena et al., 1985; Bruch et al., 1986; Kusuda & Dufau, 1986; Keinanen et al., 1987a; Roche & Ryan, 1989; Sojar &

Bahl, 1989) or the testicular Leydig cells (Aubry et al., 1982; Minegishi et al., 1987).

Detailed structural investigations on the granulosa cell LH/hCG receptor and the corpus luteum receptor might reveal if there are any differences between the two associated with the process of follicular development and corpus luteum formation (differentiation). Isolation and purification of both LH and FSH receptors from granulosa cells would enable direct and reliable comparisons to be made between the two. The granulosa cells from ovaries of domestic animals offer a suitable source for possible simultaneous characterization of FSH and LH residing on the same cell. The specificity of the LH/hCG and FSH binding sites found in the membranes of commercially obtained follicular fluid was identical with those of cells harvested from individual follicles in the laboratory (data not shown), allowing us to conclude that the high-speed pellet obtained during centrifugation of pig follicular fluid indeed represents a pool of granulosa cell membranes. These hormonal specificities were also maintained upon solubilization.

In previous studies, the LH/hCG receptor of gonadal tissue of a variety of species [see Ascoli and Segaloff (1989)] was solubilized almost exclusively with Triton X-100 or other nonionic detergent. In the present study, reduced Triton X-100 which is similar to Triton X-100 (but contains lesser amounts of peroxides and shows little UV absorption) was used to efficiently solubilize (65%) the porcine LH/hCG receptor with good retention of binding activity. Less than 100% recovery was perhaps due to losses of receptor protein during the intermediary processing steps following solubilization. The specific activity of the soluble receptor extract was about 2-fold higher than that of the membrane fraction, but the affinity was somewhat lower than that of the membrane fraction. In contrast to LH/hCG receptors, the recovery of FSH receptors in the Triton X-100R extracts (Table I) was less than optimal, and specific binding capacity was reduced. Although the reason for such a discrepancy is not clear at present, it is perhaps indicative of the marked susceptibility of the FSH receptor to inactivation even in presence of the added stabilizing agent, namely, glycerol. As emphasized for the calf testicular receptor (Reichert & Dattatreyamurty, 1989), a careful selection of protein to detergent concentration might improve solubilization and stability. In addition, we must also point out that the PEG precipitation step might allow recovery of only a fraction of FSH receptor in solution.

As we have successfully applied the DVS-Sepharose–oLH affinity columns for purification of antibodies (Sairam, 1981), this method has been extended to purify the LH/hCG receptor with acceptable recoveries. However, it was not without its problems. The oLH–receptor complex appeared difficult to dissociate (elute) following protracted incubation at 4 °C. Only short-term incubations facilitated subsequent elution of receptor, thus clearly confirming the contention that the hormone–receptor complex progresses from a loose- to a tight-fitting state with time (Katikineni et al., 1980). Therefore, we had to compromise by using shorter incubation times even though it reduced the overall yield in each chromatographic purification.

We successfully utilized the two-cycle affinity chromatographic approach on these columns to purify the porcine follicular LH/hCG receptor to a specific activity of 2000 pmol/mg of protein, a value somewhat lower than that previously reported for the porcine corpus luteum receptor (Wimalasena et al., 1985). The difference between our data from granulosa cells and those of Wimalasena et al. (1985) based on the corpus luteum can be ascribed to certain notable differences in methodology. This study employed a chromatographic protocol similar to that used by Minegishi et al. (1987) for purification of the rat Leydig cell LH/hCG receptor, wherein 100 μM PMSF was incorporated in buffers to improve recoveries. In the study of Wimalasena et al. (1985), soybean trypsin inhibitor, EDTA, and PMSF were added to buffers. In view of the experience of other investigators with the use of a cocktail of enzyme inhibitors, we performed the entire purification in the presence of compounds such as *N*-ethylmaleimide, EDTA, PMSF, TLCK, and 1,10-phenanthroline hydrate. The protein (receptor) components found in the eluates with or without the use of these inhibitors (data not included) were more or less identical, suggesting that the observed heterogeneity (Figure 5) is directly related to the starting material or a consequence of iodination.

It should be noted that the detection of smaller components (fragments) in our study (Figure 5) is not unique to pig granulosa cells. Others working with the hCG receptor of rat (Kusuda & Dufau, 1986; Keinanen et al., 1987a; Rosemblit et al., 1988; Roche & Ryan, 1989) and pig corpus luteum (Wimalasena et al., 1985, 1986) have reported similar findings although to varying degrees.

Structural characterization of the porcine follicular LH/hCG receptor by cross-linking with hormone suggested a M_r $\approx$70 000 for the receptor. This value compares favorably with that of the major protein band in the iodinated purified receptor and that of the MA-10 cell receptor assessed by cross-linking studies. These data confirm and extend previous observations comparing porcine granulosa cells and Leydig tumor cells (Ascoli & Segaloff, 1986). Our estimation of molecular weight is also in close agreement with that previously reported for the pig (Wimalasena et al., 1985, 1986) and the rat (Kusuda & Dufau, 1986, 1988). That estimates for the pig agrees well with our estimates for the MA-10 cell receptor and values previously reported for the rat concurs with the suggestion that the receptor structure is conserved among species (Keinanen & Rajaniemi, 1988). However, it should be noted that a value of $\approx$90 000 has been reported by others for a number of species (Keinanen et al., 1987b, 1988; Rosemblit et al., 1988; Roche & Ryan, 1989). It is possible that this difference in value is due to methodology. Indeed, upon reduction, the purified pig granulosa cell LH/hCG receptor showed a molecular weight of 93 000 (Figure 5), reflecting differences in migration patterns typical of glycoproteins on SDS–PAGE.

In some experiments, the M_r $\approx$70 000 protein band was visualized as a doublet especially when lower counts of radioactivity were loaded onto SDS–PAGE gels (Figure 5). The presence of a doublet of bands was not reported in earlier investigations on the pig corpus luteum LH/hCG receptor (Wimalasena et al., 1985, 1986). The human corpus luteum LH/hCG receptor is, however, present as a doublet (Keinanen & Rajaniemi, 1988); the lighter molecular weight species of the pair was said to represent a proteolytic fragment or a covalently modified form of the heavier molecular weight species. This and/or minor differences in glycosylation could explain the presence of closely migrating M_r 70 000 components in our purified preparation. If any such alterations did occur to a significant extent, they did not seem to matter for affinity purification.

The data available at present suggest that all the molecular species of protein observed, except perhaps the M_r 14 000 component, were glycosylated since they bound to lectin–Sepharose (Figure 9). Further work is obviously required to conclusively establish whether the M_r 14 000 protein is not

glycosylated since the lack of binding to Con A only indicates the absence of branched mannose sugar residues (Goldstein & Hayes, 1978). At present, we are uncertain as to why the iodinated receptor does not bind to heparin whereas the crude soluble receptor binds. The glycoprotein nature of the LH/hCG receptor has been alluded to previously by other investigators (Wimalasena et al., 1985; Kusuda & Dufau, 1988), and our data suggest that the purified pig granulosa cell LH/hCG receptor is also a glycoprotein.

Analysis of iodinated purified receptor on the SDS–PAGE gel sometimes yielded $M_r \approx 120\,000$ bands. Cross-linking of labeled hCG to purified receptor also suggested the presence of this molecular weight form of receptor ($M_r \approx 160\,000$). The M_r 120 000–160 000 is roughly 2-fold greater than the size of the major protein band in our purified receptor material and may perhaps be a dimeric form of the major protein of $M_r \approx 70\,000$. The presence of dimeric forms of the LH/hCG receptor in both iodinated (Kusuda & Dufau, 1988) and noniodinated (Wimalasena et al., 1985; Kusuda & Dufau, 1988; Roche & Ryan, 1989) purified receptors has been reported. Interestingly, the dimeric form of the receptor has been suggested as the physiologically functional form of the receptor (Kusuda & Dufau, 1988).

In conclusion, in this study, we have shown that both the LH/hCG and FSH receptors residing in the membranes of granulosa cells have similar molecular weights. The purified LH receptor is composed of a major glycosylated species of $M_r \approx 70\,000$, although other minor glycosylated proteins were evident. These data in conjunction with emerging structural information as recently reported (Loosefelt et al., 1989; McFarland et al., 1989) should contribute to our understanding of gonadotropin receptors. Of particular interest in the context of our findings are the recent observations of Loosefelt et al. (1989) indicating three mRNA transcripts for the LH receptor, arising probably by alternate splicing. If such events are developmentally regulated, they may offer an explanation for the multiplicity of receptor components.

Acknowledgments

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