Rat ovarian lutropin receptor is a transmembrane protein

Evidence for an M_r -20000 cytoplasmic domain

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Membrane topography of the rat ovarian lutropin receptor was studied by two different approaches. Ovarian membrane preparation, labelled with ^{125}I -labelled human choriogonadotropin *in vivo*, was subjected to extensive chymotryptic digestion. The soluble and membrane-bound radioactive complexes were cross-linked with glutaraldehyde, and analysed by SDS/polyacrylamide-gel electrophoresis and autoradiography. Chymotrypsin solubilized 70–75% of the radioactivity as M_r -96000, M_r -74000 and M_r -61000 complexes, and decreased the size of the membrane-bound ^{125}I -labelled human choriogonadotropin–receptor complex from M_r 130000 to M_r 110000. The M_r -110000 complex was not observed when 0.1% Triton X-100 was present in the proteolytic digestion. Enrichment of inside-out-oriented plasma-membrane vesicles by concanavalin A affinity chromatography increased by 70% the fraction of radioactivity that remained in the membrane fraction after chymotrypsin treatment. Chymotrypsin also diminished the size of the membrane-bound unoccupied receptor from M_r 90000 to M_r 70000, as detected by ligand (^{125}I -labelled human choriogonadotropin) blotting. These results suggest that the lutropin receptor is a transmembrane protein with a cytoplasmic domain of M_r 20000 that is sensitive to proteolytic digestion in the inside-out-oriented plasma-membrane vesicles.

INTRODUCTION

The gonadal lutropin receptor consists of a single integral plasma-membrane polypeptide of M_r 90000–100000 (Dufau *et al.*, 1975; Metsikkö & Rajaniemi, 1982; Metsikkö, 1984; Rapoport *et al.*, 1984). Radiolabelled hCG can be covalently cross-linked to its membrane receptor to give an M_r -130000 complex (Rebois *et al.*, 1981; Metsikkö & Rajaniemi, 1982). Contradictory views of the structure of the lutropin receptor have also been reported (Ji *et al.*, 1981, 1985; Dattatreya Murthy *et al.*, 1983; Hwang & Menon, 1984; Wimalasena *et al.*, 1985).

The binding of lutropin or choriogonadotropin to its receptor leads to guanine-nucleotide-mediated activation of adenylate cyclase on the inner surface of the plasma membrane (Dufau *et al.*, 1980; Gilman, 1984). In order to understand the mechanism of transmembrane signalling by the receptor, information about the receptor's membrane topography would be necessary. Until now, no structural studies concerning the membrane topography of any adenylate cyclase activating receptor have been reported.

In this paper we provide evidence that the rat ovarian lutropin receptor is a transmembrane protein with an M_r -20000 cytoplasmically oriented domain.

MATERIALS AND METHODS

Pregnant-mare serum gonadotropin (2000 i.u./mg) and hCG (12100 i.u./mg) were obtained from Diosynth, Oss, The Netherlands. Na^{125}I (carrier-free) was obtained from Amersham International, Amersham, Bucks., U.K.

hCG was radioiodinated by the chloramine-T method (Hunter & Greenwood, 1962) to a specific radioactivity of 60–80 Ci/g, as described by Markkanen *et al.* (1980). Bovine serum albumin, haemoglobin, PMSF and α -chymotrypsin were purchased from Sigma. Concanavalin A covalently coupled to CNBr-activated Sepharose 4B was purchased from Pharmacia. The nitrocellulose paper was from Bio-Rad Laboratories, Richmond, CA, U.S.A. M_r standards (myosin heavy chain, M_r 205000; β -galactosidase, M_r 116000; phosphorylase *b*, M_r 94000; albumin, M_r 66000; ovalbumin, M_r 45000; carbonic anhydrase, M_r 29000) for electrophoresis were purchased from Sigma.

Female 27-day-old Wistar rats were rendered pseudo-pregnant by successive treatment with 30 i.u. of pregnant-mare serum gonadotropin and 25 i.u. of hCG (Parlow, 1961). They were killed 7 days after the hCG injection.

Radiolabelling of the ovarian lutropin receptor *in vivo*

Radioiodinated hCG (10×10^6 c.p.m.; 200 μl) was injected into the tail vein of a pseudopregnant rat, and the animal was killed 1 h later. The 1 h uptake of ^{125}I -hCG was about 1×10^6 c.p.m./ovary. One ovary (about 100 mg of tissue) was homogenized in 1 ml of PBS containing 5 mM-*N*-ethylmaleimide, and centrifuged for 5 min at 120 *g*. The supernatant was further centrifuged for 30 min at 27000 *g*. The pellet was washed once by suspension in PBS containing 5 mM-*N*-ethylmaleimide, and centrifuging at 27000 *g* for 30 min. The crude membrane pellet was resuspended in the same buffer and used for the subsequent experiments. The membrane preparation contained 1–2 mg of protein/ml. The protein was assayed as described by Lowry *et al.* (1951).

Abbreviations used: Con A, concanavalin A; hCG, human choriogonadotropin; PBS, phosphate-buffered saline (10 mM-sodium phosphate, pH 7.4, containing 140 mM-NaCl; PMSF, phenylmethanesulphonyl fluoride.

Proteolytic digestion of the membrane preparation

Samples (0.45 ml) of crude membrane preparation, labelled with ^{125}I -hCG *in vivo*, were treated with α -chymotrypsin (1 mg/ml) for 5–120 min at 37 °C in the presence or absence of 0.1% (v/v) Triton X-100. The reactions were terminated by chilling on ice and adding 20 μl of 0.2 M-PMSF (in ethanol). The chymotrypsin-released material was separated by centrifuging at 100000 *g* for 30 min. The membrane pellet and the supernatant were counted for radioactivity in an automatic gamma counter (LKB Wallac Ultrogamma). The soluble degradation products were cross-linked with glutaraldehyde and analysed electrophoretically as described below. The pellets were washed once with 2 ml of PBS containing 5 mM-*N*-ethylmaleimide and 2 mM-PMSF, and then solubilized in 1 ml of PBS containing 5 mM-*N*-ethylmaleimide, 0.2 mM-PMSF and 1% Triton X-100, by stirring on ice for 30 min. The soluble fraction was obtained by centrifuging at 100000 *g* for 1 h. The solubilized radioactive complexes were analysed by SDS/polyacrylamide-gel electrophoresis after glutaraldehyde cross-linking as described below.

Enrichment of inside-out-oriented plasma-membrane vesicles

The inside-out-oriented plasma-membrane vesicles were enriched by Con A-Sepharose affinity chromatography (Walsh *et al.*, 1976). The crude membrane preparation (in PBS containing 5 mM-*N*-ethylmaleimide) was passed through a column (3.3 cm $\times$ 1 cm) of Con A-Sepharose 4B. The unretarded radioactivity was eluted in the void volume, and was treated with chymotrypsin (1 mg/ml) for 60 min at 37 °C. The control preparation (not passed through Con A-Sepharose) was diluted to the same concentration of radioactivity and treated identically. The membrane-bound radioactivity was solubilized after the chymotrypsin treatment and analysed as described below.

Cross-linking and electrophoresis

The chymotrypsin-released and detergent-solubilized radioactive complexes were cross-linked with glutaraldehyde as previously described (Metsikkö & Rajaniemi, 1982), with minor modifications. Briefly, glutaraldehyde (0.1 M) was added to a final concentration of 2.5 mM, and the samples were incubated for 1 h at room temperature. Thereafter 1 M-Tris/HCl, pH 7.5, was added to a final concentration of 25 mM. The radioactive products were analysed by SDS/polyacrylamide-gel electrophoresis after dialysis against SDS sample buffer (Laemmli, 1970). The electrophoresis was performed under non-reducing conditions, with 5%-acrylamide stacking and 7.5%-acrylamide separating gels. The samples were not reduced. The gels were stained with Coomassie Brilliant Blue R-250, dried, and exposed for 1–7 days on Kodak XAR-5 film at -70 °C.

Proteolytic digestion of the unoccupied lutropin receptor, and detection of the membrane-bound products by ligand blotting

Crude ovarian membrane preparation from a pseudo-pregnant rat (not injected with radiolabelled hormone) was treated with chymotrypsin (1 mg/ml) for 60 min at 37 °C, as described above. The chymotrypsin-treated and

control membrane pellets were solubilized in 0.5 ml of PBS containing 5 mM-*N*-ethylmaleimide, 0.2 mM-PMSF, 20% (v/v) glycerol and 1% Triton X-100 for 30 min on ice. The suspensions were centrifuged for 1 h at 100000 *g*, and 50 μl samples of the supernatants were mixed with an equal amount of SDS sample buffer (Laemmli, 1970), and electrophoresed as described above. The lutropin receptor and its proteolytic products were detected by a ligand-blotting procedure. The proteins were electrophoretically transferred from the gel to nitrocellulose paper in 25 mM-Tris/192 mM-glycine at 100 mA overnight in a Bio-Rad Trans-Blot apparatus placed in an ice bath. The nitrocellulose paper was cut into strips, and the strips containing the M_r standards were stained with Amido Black, whereas the strips containing the samples were rinsed in PBS, and incubated for 4 h at room temperature in PBS containing 0.1% bovine serum albumin, 0.25% gelatin, 1% haemoglobin and 10% glycerol. Thereafter the nitrocellulose strips were incubated for 16 h in PBS containing 0.1% albumin and 200000 c.p.m. of ^{125}I -hCG/ml, in the presence or absence of unlabelled hCG (50 i.u./ml). The strips were washed for 30 min in PBS containing 0.1% Triton X-100, rinsed in PBS, and autoradiographed on Kodak XAR-5 film at -70 °C for 5 days. By the procedure described above, we have demonstrated the presence of the M_r -90000 lutropin receptor in pseudopregnant-rat ovaries and mouse Leydig cells, and its absence from non-target tissues and rat ovaries after hormone-induced down-regulation of the receptor (K. P. Keinänen, S. Kellokumpu & H. J. Rajaniemi, unpublished work).

RESULTS AND DISCUSSION

Proteolytic digestion of the membrane-bound ^{125}I -hCG-receptor complex

Rat ovarian lutropin receptor was labelled *in vivo* with ^{125}I -hCG, administered intravenously 1 h before the animal was killed. The uptake of the radioactivity was

Fig. 1. Time course of the release of membrane-bound radioactivity by chymotrypsin

Rat ovarian membrane vesicles, labelled with ^{125}I -hCG *in vivo*, were incubated with (●) or without (○) chymotrypsin (1 mg/ml) for the indicated times at 37 °C. Thereafter the membrane-bound radioactivity was determined as described in the Materials and methods section.

Fig. 2. SDS/polyacrylamide-gel electrophoresis of the cross-linked proteolytic products of the ^{125}I -hCG-receptor complex

Rat ovarian membrane vesicles, labelled with ^{125}I -hCG *in vivo*, were incubated with chymotrypsin (1 mg/ml) for 60 min at 37 °C in the presence or absence of 0.1% Triton X-100. The chymotrypsin-released and membrane-bound degradation products were subjected to SDS/polyacrylamide-gel electrophoresis after glutaraldehyde cross-linking as described in the Materials and methods section. Samples: membrane-bound hormone-receptor complex from the control incubation (chymotrypsin omitted) in the absence (a) and presence (b) of Triton X-100; membrane-bound complex produced by chymotrypsin in the absence (c) and presence (d) of Triton X-100; soluble complex from control incubation (e); soluble complexes produced by chymotrypsin in the absence (f) and presence (g) of Triton X-100.

about 10% of the total amount given. This radioactivity represents receptor-bound hormone on the luteal cell plasma membrane, since studies *in vivo* (Markkanen & Rajaniemi, 1980) and *in vitro* (Rajan & Menon, 1985) have shown that internalization of ^{125}I -hCG by the luteal cells is negligible, at least during the first few hours after hormone administration.

Crude membrane fraction (27000 g, 30 min), prepared from ovaries labelled with ^{125}I -hCG *in vivo*, was treated with chymotrypsin (1 mg/ml) for up to 120 min at 37 °C. Some 70–75% of the radioactivity was released into the solution during the first 60 min of incubation (Fig. 1). Further digestion for 60 min did not detach any more radioactivity from the membrane fraction, indicating that 25–30% of the membrane-bound radioactivity was protected from chymotrypsin, most likely by a membrane barrier. To analyse the proteolytic products of the hormone-receptor complex, the membrane vesicles were subjected to chymotrypsin digestion (1 mg/ml) for

60 min at 37 °C in the presence or absence of 0.1% Triton X-100. The chymotrypsin-released radioactivity (corresponding to 70–75% of the total radioactivity) was cross-linked with glutaraldehyde and analysed by SDS/polyacrylamide-gel electrophoresis. Autoradiography of the dried gels revealed complexes with M_r values of 96000, 74000 and 61000 (Figs. 2f and 2g). In addition, cross-linked hCG (M_r 48000), its α -subunit (in the dye front), and an M_r -40000 species, representing degradation of the hormone, were present. In the control incubation (chymotrypsin omitted) only 5–8% of the radioactivity was released from the membrane vesicles, and it consisted of an M_r -74000 complex (Fig. 2e). This complex is produced by the endogenous proteolysis of the hormone-receptor complex (Kellokumpu & Rajaniemi, 1985). The radioactivity remaining in the membrane vesicles after extensive chymotryptic digestion (corresponding to 25–30% of the total radioactivity) was solubilized, cross-linked with glutaraldehyde and analysed electrophoretically. Autoradiography revealed an M_r -110000 band (Fig. 2c), and the intact ^{125}I -hCG-receptor complex solubilized from control vesicles appeared as an M_r -130000 band (Fig. 2a), in accordance with previous results (Metsikkö & Rajaniemi, 1982). When 0.1% Triton X-100 was present in the proteolytic digestion, the M_r -110000 band was absent (Fig. 2d). This is probably due to alteration in the membrane permeability in the presence of detergent. Triton had no effect on the molecular size of the intact hormone-receptor complex (Fig. 2b). A trace of the M_r -74000 complex, generated by endogenous proteolysis, was also present in variable amounts in all samples. No signs of degradation of hCG were observed (Fig. 2c), indicating that the decrease in the size of the ^{125}I -hCG-receptor complex is not caused by proteolysis of the hormone. The intact hormone-receptor complex produced often, but not constantly, more aggregation in the interface between the concentrating and separating gels than did the chymotrypsin-treated complex (Figs. 2a and 2b). The significance of this finding is at present unclear.

Con A affinity chromatography of the membrane preparation

The partial resistance of the membrane-bound ^{125}I -hCG-receptor complex to chymotrypsin suggested that the membrane preparation includes a fraction of inverted vesicles with the extracellular surface oriented to the interior. Thus the decrease in the M_r of the hormone-receptor complex by 20000 could be due to cleavage of the receptor's cytoplasmic extension. If this were true, the enrichment of inside-out-oriented vesicles would increase the fraction of chymotrypsin-resistant membrane-bound radioactivity (i.e. M_r -110000 complex). To test this, the ^{125}I -hCG-labelled crude membrane vesicles were passed through a Con A-Sepharose 4B column (Walsh *et al.*, 1976). About 50–60% of the total radioactivity failed to bind to the Con A matrix. When the Con A-Sepharose-unretarded vesicles were subjected to chymotrypsin (1 mg/ml, 60 min, 37 °C), 46% of the total radioactivity remained associated with the vesicles (Table 1). This radioactivity consisted of the M_r -110000 complex and a trace of the M_r -74000 complex, as revealed by SDS/polyacrylamide-gel electrophoresis (results not shown). In the control vesicles (not passed through Con A-Sepharose), only 28% of the radioactivity resisted digestion by chymotrypsin in identical conditions

Table 1. Proteolysis of the Con A-Sepharose-unretarded vesicles

Rat ovarian membrane preparation, labelled with ^{125}I -hCG *in vivo*, was passed through a Con A-Sepharose column, and incubated with or without chymotrypsin (1 mg/ml) for 60 min at 37 °C. Thereafter the membrane-bound radioactivity was determined as described in the Materials and methods section. Results are means $\pm$ S.D. from three similar experiments.

Vesicle preparation	Membrane-bound radioactivity (% of total)	
	Control	Chymotrypsin
Crude membrane vesicles	94.5 $\pm$ 1.6	28.1 $\pm$ 0.1
Con A-Sepharose-unretarded vesicles	91.1 $\pm$ 1.5	48.6 $\pm$ 3.5

(Table 1). Thus, passage of the vesicles through Con A-Sepharose led to 1.7-fold enrichment of the chymotrypsin-resistant M_r -110 000 complex. The rather low enrichment observed is in accordance with the findings by Walsh *et al.* (1976), that most of the vesicles that fail to bind to Con A-Sepharose are permeable to trypsin. Attempts to elute significant amounts of bound radioactivity with 0.5 M α -methyl mannoside were not successful, probably because of the large amount of specifically bound glycoside groups in the vesicles (Walsh *et al.*, 1976).

Proteolytic digestion of the membrane-bound unoccupied receptor

The proteolytic products of the unoccupied receptor were analysed by ligand blotting. Rat ovarian membrane preparation was treated with chymotrypsin (1 mg/ml, 1 h, 37 °C), and the membrane-bound products were subjected to SDS/polyacrylamide-gel electrophoresis and transferred to nitrocellulose paper. After the free protein-binding sites had been blocked with a protein mixture, the blots were incubated with radioiodinated hCG. Autoradiography revealed an intensely labelled M_r -90 000 band produced by the untreated membrane preparation and corresponding to the intact receptor (Fig. 3a). Chymotrypsin treatment abolished the M_r -90 000 band, but produced a faint M_r -70 000 band (Fig. 3b). The binding of ^{125}I -hCG to both bands was displaced by an excess of unlabelled hCG (Figs. 3c and 3d). The supernatants from the proteolytic incubation did not produce any bands in the ligand blotting (results not shown), suggesting that the soluble proteolytic products do not retain or regain their binding activity during the procedure. The observation that the M_r of the membrane-bound receptor is diminished by 20 000 in the presence of chymotrypsin is in accordance with the above findings concerning the proteolysis of the ^{125}I -hCG-receptor complex.

Conclusions

The most plausible explanation of the above findings is that the M_r -110 000 hormone-receptor complex that resists solubilization by chymotrypsin is generated by

Fig. 3. Detection of the proteolytic products of the unoccupied lutropin receptor by ligand blotting

Rat ovarian membrane preparation was incubated with chymotrypsin (1 mg/ml) for 1 h at 37 °C. Thereafter the membrane fraction was recovered, and the membrane proteins were solubilized with detergent and subjected to SDS/polyacrylamide-gel electrophoresis. The size-separated proteins were transferred to nitrocellulose, and the hCG-binding proteins were detected by incubation with radioiodinated hormone in the absence or presence of an excess of unlabelled hormone as described in the Materials and methods section. The dried nitrocellulose sheets were subjected to autoradiography. Samples: intact receptor incubated in the absence (a) or presence (c) of unlabelled hCG; chymotrypsin-treated receptor incubated in the absence (b) and presence (d) of unlabelled hCG.

cleavage of the cytoplasmic extension of the receptor from the inside-out-oriented vesicles. Several facts support this notion. First, consistently one-quarter of the radiolabelled receptor resisted solubilization by excess chymotrypsin. The chymotrypsin-resistant hormone-receptor complex was not intact, but consisted of an M_r -110 000 complex. Second, this chymotrypsin-resistant fraction of radioactivity was increased by passing the vesicles through Con A-Sepharose before the proteolytic treatment, suggesting that the M_r -110 000 complex resides in inside-out-oriented vesicles. Third, when the proteolysis was carried out in the presence of 0.1% Triton X-100, the M_r -110 000 complex was not present in the membrane fraction. This indicates that perturbing the integrity of a membrane barrier facilitates further degradation of the complex. Finally, chymotrypsin diminished the size of the unoccupied membrane-bound lutropin receptor also by M_r 20 000, excluding the

possibility that dissociation or proteolysis of the ^{125}I -hCG gives rise to the M_r -110000 complex.

In conclusion, the above results strongly suggest that rat ovarian lutropin receptor is a transmembrane protein. The extracellular domain of the receptor contains the ligand-binding site, but the function of the M_r -20000 cytoplasmic domain remains to be elucidated.

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