PURIFICATION OF HUMAN CHORIONIC GONADOTROPIN–RECEPTOR COMPLEX BY IMMUNOAFFINITY CHROMATOGRAPHY

Kalervo METSIKKÖ and Hannu RAJANIEMI
Department of Anatomy, University of Oulu, 90220 Oulu 22, Finland

Received 30 July 1979

1. Introduction

Affinity chromatography methods have been applied to purification of several peptide hormone receptors, including insulin [1], prolactin [2] and LH(hCG) receptors [3,4]. However, in these methods a good displacement of the receptor from the ligand hormone cannot be achieved because of the almost irreversible nature of the binding between these two entities. Especially, the direct affinity chromatography methods used in LH receptor purification require extreme conditions for dissociation of the receptor and thus LH receptor with all of its original properties is not obtained [3].

We have found that anti-hCG gamma-globulins, covalently linked to CNBr-activated Sepharose 4B, bind solubilized ^{125}I-labeled hCG–receptor complex without significant dissociation of the complex. The bound hormone–receptor complex can specifically be displaced from the immunoaffinity matrix with an excess of hCG. Here we describe a purification procedure for hCG–receptor complex by the immunoaffinity chromatography developed.

2. Materials and methods

hCG–receptor complex was purified from immature pseudo pregnant rat ovaries. The pseudo-pregnancy was induced in immature day 25–27 Wistar rats by sequential PMSG (Diosynth, Holland) and hCG (Diosynth) treatment as in [5].

LH(hCG) receptors were prelabeled with radio-iodinated hCG as follows. Two ovaries (200 mg) were homogenized in 2 ml 0.01 M phosphate buffer (pH 7.0), 0.14 M NaCl (PBS) containing 1 mg/ml Trasylol (Bayer, FRG) and *N*-ethylmaleimide (Sigma, St Louis, MO) with 30 strokes in a glass homogenizer. The homogenate was centrifuged at 20 000 × *g* for 30 min and the pellet obtained was suspended in 2 ml PBS containing the enzyme inhibitors and incubated with 2 × 10^6 cpm of ^{125}I-labeled hCG for 30 min at 37°C. hCG was labeled with carrier free Na^{125}I (Radiochemical Center, Amersham, England) to spec. act. 20 μCi/μg using chloramine T method as in [6]. The binding reaction was stopped by adding 3 ml ice-cold PBS and the membrane particles were sedimented by centrifuging at 20 000 × *g* for 30 min. The pellet was suspended in 5 ml ice-cold PBS and centrifuged as above. The final pellet was suspended in 2 ml 1% Triton X-100 (BDH Chemicals, England) in PBS containing the enzyme inhibitors with 100 strokes in a glass homogenizer and incubated subsequently at 4°C for 30 min, mixing by a magnetic stirrer. The mixture was diluted with cold PBS to 0.5% towards Triton X-100 and centrigued at 100 000 × *g* for 2 h to obtain a soluble ^{125}I-labeled hCG–receptor complex.

The hormone–receptor complex was subsequently attached to anti-hCG Sepharose 4B immunoaffinity matrix which was prepared as follows. Gamma-globulins of rabbit anti-hCG serum were precipitated with saturated ammonium sulphate essentially as in [7]. Gamma-globulins were covalently coupled to CNBr-activated Sepharose 4B (Pharmacia, Uppsala) according to the instructions of the manufacturer. The binding capacity of anti-hCG Sepharose was 3 μg hCG/ml and K_a av. was 0.5 × 10^7 mol/l. hCG–receptor

complex was attached to the gel by incubating the 100 000 X g supernatant with 20 μl immunoaffinity matrix in a syringe for 4 h at 22°C by mixing end-over-end. After the incubation the column was washed with 10 ml cold 0.5% Triton–PBS (0.4 ml/min) and the ^{125}I-labeled hCG–receptor complex was displaced specifically with 200 μl 20 μM hCG in 0.5% Triton–PBS (4 h, 22°C). The displaced radioactivity was analyzed by gel filtration on a Sepharose 6B column. Free hCG was separated from the hormone–receptor complex by gel filtration on Sephadex G-150 (Pharmacia, Uppsala). Proteins were determined by the Lowry method [8] using bovine serum albumin (Sigma, St Louis) as standard. Precipitation of Triton X-100 in the analyzed aliquots was inhibited with sodium dodecyl sulfate [9].

3. Results

Over 90% of the ^{125}I-labeled hCG–receptor complex was obtained into soluble form with a single Triton X-100 extraction of the prelabeled membrane particles of the ovaries. The anti-hCG Sepharose bound 90% of the soluble radioactivity during subsequent incubation of the extract with the immunoaffinity gel. When the washed gel was incubated with an excess of hCG, 30% of the bound radioactivity was released and another 30% could be released with renewed incubation. Accordingly, a 40% recovery of ^{125}I-labeled hCG–receptor complex was achieved with two hCG displacements. The displacement of the ^{125}I-labeled hCG–receptor complex appeared to be specific since neither 0.5 M NaCl nor 0.1% albumin in 0.5% Triton-PBS could cause detachment of the hormone–receptor complex. The gel filtration of the displaced material on Sepharose 6B showed one radioactive peak eluting in the same volume with the ^{125}I-labeled hCG–receptor complex present in the original Triton extract (fig.1). Molecularly intact hCG was released from the complex when lowered to pH 3.

A possibility remains that the immunoaffinity gel leaks antibody and therefore the displaced material could represent ^{125}I-labeled hCG–antibody complex. However, the controls performed exclude this possibility. When ^{125}I-labeled hCG alone was attached to anti-hCG Sepharose and subsequently displaced with an excess of hCG only free labeled hCG was

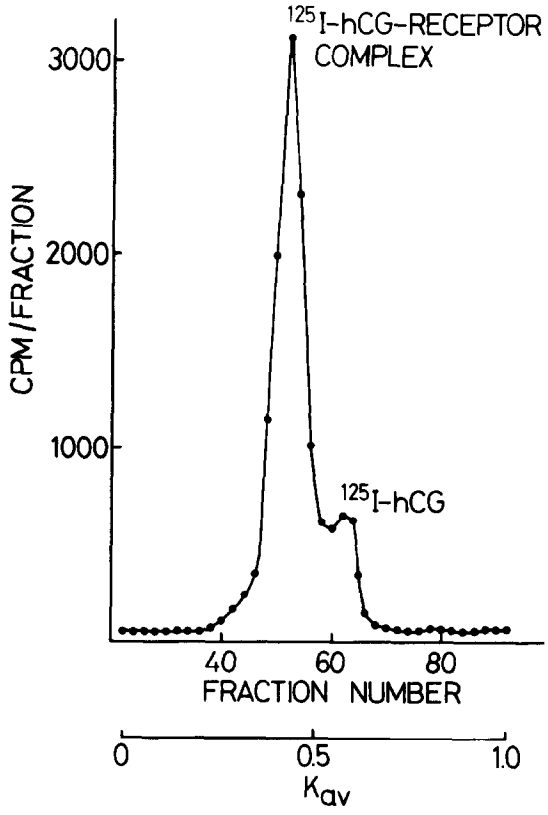


Fig.1. Gel filtration on a Sepharose 6B column (2.6 X 70 cm) of the ^{125}I-labeled hCG–receptor complex displaced from the immunoaffinity matrix. The column was eluted at 4°C with 0.5% Triton–PBS 20 ml/h and 4.5 ml fractions were collected.

obtained in the gel filtration of the displaced material. In addition, when ^{131}I-labeled hCG was attached to the gel together with ^{125}I-labeled hCG–receptor complex no exchange of ^{131}I radioactivity to the higher molecular form occurred (fig.2). On the other hand, no aggregation of the purified ^{125}I-labeled complex took place when the displaced material was incubated with anti-rabbit gammaglobulin serum (fig.3).

Elution of the immunoaffinity gel containing the ^{125}I-labeled hCG–receptor complex with 0.1 M acetic acid (pH 4) for 1 min released only a negligible amount of radioactivity. The subsequent treatment of the gel with an excess of hCG displaced only free ^{125}I-labeled hCG indicating that the pH treatment had dissociated the receptor from the hormone. However, no specific hCG-binding could be detected in the

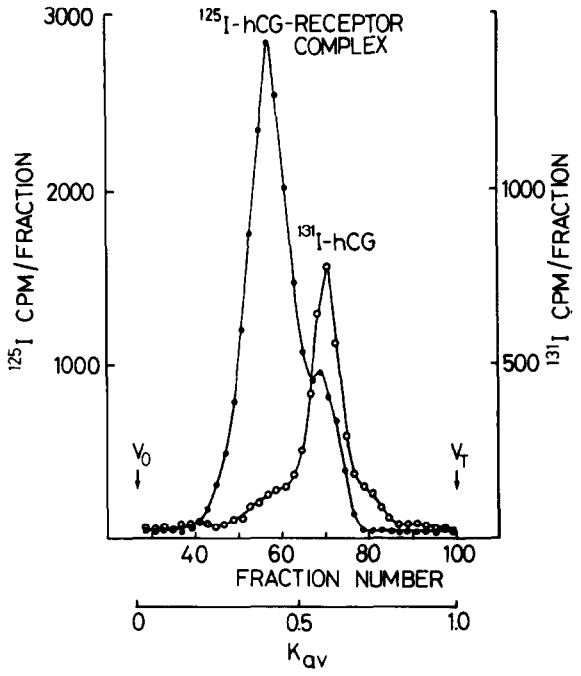
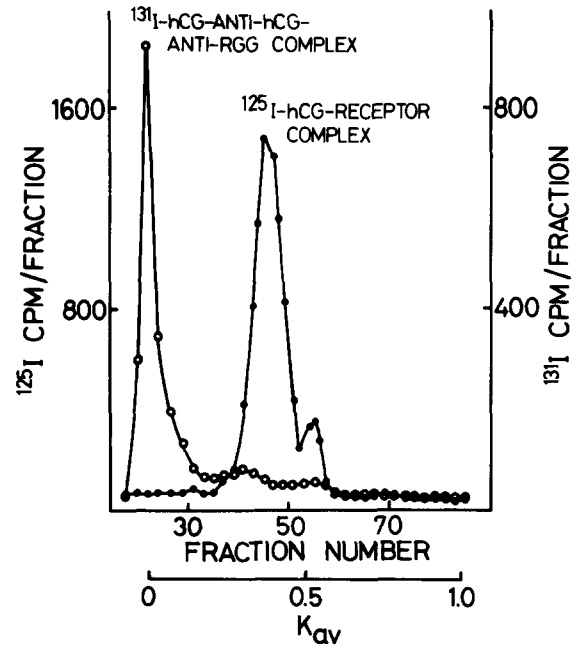


Fig.2. Triton-solubilized ^{125}I-labeled hCG–receptor complex and ^{131}I-labeled hCG were simultaneously attached to anti-hCG Sepharose and subsequently displaced with an excess of unlabeled hCG. The displaced material was fractionated on a Sepharose 6B column (2.6 X 70 cm). ^{131}I radioactivity was solely eluted at the volume of free labeled hCG (K_{av} = 0.58).



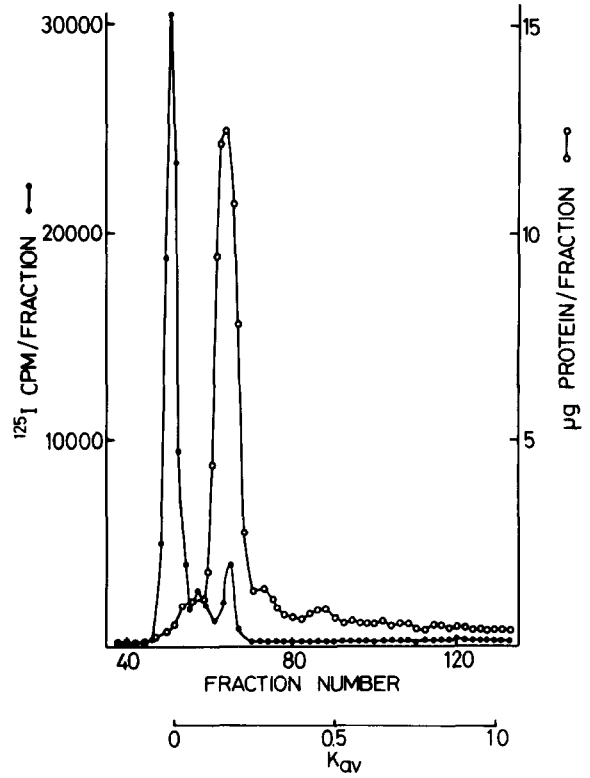


Fig.4. Removal of hCG by a Sephadex G-150 column (1.5 X 60 cm) from the ^{125}I-labeled hCG–receptor complex purified with anti-hCG Sepharose. The column was equilibrated and eluted with 0.1% Triton-PBS (3 ml/h) and 0.6 ml fractions were collected.

neutralized pH 4 eluate suggesting that the receptor was denatured.

hCG from the immunoaffinity gel eluate was removed by gel filtration on Sephadex G-150 (fig.4). A purification coefficient of $\geqslant$1500 was achieved (table 1).

Fig.3. ^{131}I-labeled hCG was incubated with soluble anti-hCG gamma-globulin in 0.5% Triton-PBS for 4 h at 20°C and subsequently 16 h with goat anti-rabbit gamma-globulin serum. The ^{125}I-labeled hCG–receptor complex purified with the anti-hCG Sepharose was also incubated with goat anti-rabbit gamma-globulin serum for 16 h. The two incubates were mixed and fractionated on a Sepharose 6B column. No aggregation of the ^{125}I-labeled hCG complex occurred during the incubation with anti-rabbit gamma-globulin serum.

Table 1
Calculation of the recovery and purification coefficient of the ^{125}I-labeled hCG–receptor complex

Purification stage	Protein (μg)	cpm X10^{-6}	cpm/μg protein	Recovery (%)	Purification coefficient
Homogenate of 6 ovaries	23 430	12.97[a]	554	100	1
Triton X-100 extract	3530	11.69	3312	90.1	6.0
Immunoaffinity gel	–	10.43	–	80.4	–
Eluate of two hCG displacements	–	4.82	–	37.2	–
Top fractions of Sephadex G-150	<2	1.75	875 000	13.5	>1579

[a] In the ovarian pellet after washing off free ^{125}I-labeled hCG

4. Discussion

This study demonstrates that anti-hCG gamma-globulins conjugated to CNBr-activated Sepharose can be used to purify hCG–receptor complex with rather high recovery and in sufficient quantity to facilitate further studies. The method is based on biological as well as immunological specificity regarding the attachment and detachment from the immunoaffinity matrix. Several attempts have been made to purify LH(hCG) receptor by a direct affinity chromatography using hCG as a ligand hormone coupled to an agarose matrix [3,4]. However, we have found that the receptor is virtually irreversibly bound to hCG-affinity matrix and thus, it is very difficult to dissociate the receptor in active form. In addition, the agarose matrices with a long spacer arm leak conjugated ligand hormone. The elution at low pH also causes a release of hCG binding substance from agarose whose hCG complex precipitates with poly(ethyleneglycol). It has been shown [10] that several non-receptor materials also bind insulin specificially and to saturation.

Conventional systems generally used for protein purification are not applicable to receptor purification because of the low receptor concentration in the starting material. Direct affinity methods have not proved applicable and it appears that the immuno-affinity method remains the only possible. Antiserum raised towards the whole hCG–receptor complex probably contains antibodies which bind also to non-occupied receptor. This could enable the purification of the LH receptor with antibodies raised against the hormone–receptor complex.

Acknowledgements

We wish to thank Miss Helena Jakkula for excellent technical asistance. This work was supported by grants from The Academy of Finland and The Cultural Foundation of Finland.

References

[1] Cuatrecasas, P. (1972) Proc. Natl. Acad. Sci. USA 69, 1277–1281.
[2] Shiu, R. P. C. and Friesen, H. G. (1974) J. Biol. Chem. 249, 7902–7911.
[3] Dufau, M. L., Ryan, D. W., Baukal, A. J. and Catt, K. J. (1975) J. Biol. Chem. 250, 4822–4824.
[4] Saxena, B. J. (1976) in: Methods in Receptor Research I (Blecher, M. ed) pp. 251–299, Marcel Dekker, Basel, New York.
[5] Parlow, A. F. (1961) in: Human Pituitary Gonadotropins (Albert, A. ed) pp. 300–310, C. C. Thomas, Springfield, IL.
[6] Leidenberger, F. A. and Reichert, L. E., jr (1972) Endocrinology 91, 135–143.
[7] Kendall, F. E. (1937) J. Clin. Invest. 16, 921–931.
[8] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[9] Dulley, J. R. and Grieve, P. A. (1975) Anal. Biochem. 64, 136–141.
[10] Cuatrecasas, P. and Hollenberg, M. D. (1975) Biochem. Biophys. Res. Commun. 62, 31–41.