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Purification of gonadotropin releasing hormone receptors using the avidin–biotin technique

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ABSTRACT

The avidin–biotin technique has been applied to the purification of gonadotropin releasing hormone (GnRH) receptors from other solubilized membrane proteins. The following steps were involved in this approach: (a) solubilization of rat pituitary GnRH receptor with the zwitterionic detergent CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate, (b) equilibration of the solubilized GnRH receptor with [biotinyl-D-Lys^6]GnRH immobilized on avidin-agarose; and (c) elution of the receptors with high salt and GnRH analogues. Following two cycles of affinity chromatography the GnRH receptor was purified to homogeneity. The overall recovery of the purified receptor was 4–10% of the initial activity in the CHAPS extract and the calculated purification was approximately 10 000 to 15 000 fold. The development of a two step affinity chromatography for the purification of GnRH receptors can be used for detailed studies on the structure and function of the receptor. These studies will advance our understanding of the molecular basis of GnRH action.

INTRODUCTION

The hypothalamic decapeptide gonadotropin releasing hormone (GnRH) stimulates gonadotropin release from the anterior pituitary and thus has a pivotal role in the regulation of reproduction[1,2]. The GnRH receptor is the initial site of action of the hormone in the mammalian pituitary and represents a family of hormone receptors that has not been investigated at the molecular level. Therefore, elucidation of the GnRH-receptor structure has theoretical and practical implications both in reproductive biology and cancer[1,2]. For most peptide hormones, such studies are greatly facilitated by developing techniques for solubilization and purification of the hormone binding protein in an active form.

The high affinity constant (10^{15} *M*) between the glycoprotein avidin and the vitamin biotin provides an important exprimental tool for a wide variety of biological applications. The avidin–biotin complex has been used as a meduator in localization,

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isolation and immunological studies[3–8]. Recently, biotinylated peptide hormones have been used for the localization and isolation of receptors on cell surfaces. Here, we report on the synthesis and the application of biotinylated GnRH to highlight the potential use of this method for GnRH receptor purification.

EXPERIMENTAL

Synthesis of [biotinyl-D-Lys^6]GnRH

[Biotinyl-D-Lys^6]GnRH was synthesized as previously described[9,10]. Briefly, [D-Lys^6]GnRH (0.6 mg; 0.45 mmol) (Peninsula, Belmont, CA, U.S.A.) was reacted with 2 molar equivalents of *d*-biotin-*p*-nitrophenyl ester (Sigma, St. Louis, MO, U.S.A.) in methanol–dimethylformamide (10:1, v/v) in the presence of 1.2 equivalents of triethylamine. After standing at 24°C for 3 h, the product was precipitated by the addition of ether and washed three times with ethyl acetate in order to remove unreacted *d*-biotin-*p*-nitrophenyl ester. The product was then purified by preparative high-voltage paper electrophoresis (Whatman No. 3 paper, 60 min at 60 V/cm) in pyridine–acetate buffer (pH 3.5); elctrophoretic mobility, 0.62.

Cell culture and assay of biological activity

Rat pituitary cell cultures were prepared as previously described[11]. After 48 h in culture, the medium was discarded, the cells were washed twice and incubated with the GnRH analogues (4 h at 37°C). The biological activity was assessed by the quantitation of luteinizing hormone (LH) released from the cells[11].

Iodination and pituitary membrane preparations

[D-Ser(t-Bu)^6, des-Gly^{10}, ethylamide]GnRH (Buserelin) was iodinated by the lactoperoxidase method[12]. Specific activity of the labeled peptide was approximately 1.0 mCi/μg, as measured by self-displacement in the pituitary radioligand receptor assay. Pituitary membranes were prepared from 25- to 28-day old Wistar derived female rats[13]. Briefly, the glands were homogenized gently with a Dounce homogenizer at 4°C in 10 m*M* Tris · HCl, pH 7.4, and centrifuged for 10 min at 1000 *g*. The supernatant was then centrifuged for 20 min at 20 000 *g*. The pellet was resuspended in 10 m*M* Tris · HCl buffer, centrifuged at 20 000 *g* for 20 min and the pellet stored at −20°C.

Solubilization of GnRH receptors

Solubilization of GnRH receptors was performed as described previously[13,14]. Briefly, pituitary membrane preparations were washed with 10 m*M* Tris · HCl, pH 7.4 by centrifugation (20 min at 20 000 *g*). The pellet was resuspended in 10 m*M* Tris buffer containing 5 m*M* 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS) (Sigma), shaken for 60 min at 4°C and centrifuged (60 min at 100 000 *g*). This procedure was repeated, the supernatants were combined and used to measure binding. Usually, the solubilized receptor was kept in 1 m*M* CHAPS–10 m*M* Tris containing 10% glycerol and 1 m*M* phenylmethylsulfonyl fluoride (Sigma).

Binding assays to solubilized receptors

Solubilized receptors (25 to 50 μg protein) were incubated with labeled Buserelin

(50 000 cpm) in 0.5 ml of 10 m*M* Tris–0.1% bovine serum albumin containing 1 m*M* CHAPS for 2.5 h at 4°C. The reaction was stopped by the addition of 0.3 ml ice-cold dextran-coated charcoal [0.5 g Dextran T-70 (Pharmacia, Uppsala, Sweden) and 5.0 g activated charcoal (Norit A, Fisher, Raleigh, NC, U.S.A.) dissolved in 1000 ml of phosphate-buffered saline]. The tubes were left on ice for 10 min and then centrifuged for 20 min at 2000 *g* at 4°C. The supernatants were collected and counted in a γ-counter. Specific binding represents the bound radioactivity in the presence of 10^{-7} *M* unlabeled Buserelin subtracted from the total bound radioactivity.

Affinity chromatography on GnRH resin

Avidin–agarose (Sigma), 1.5 ml containing 2.5 g of avidin) was incubated with 10^{-6} *M* [biotinyl-D-Lys^6]GnRH in 10 m*M* Tris · HCl buffer for 5 h at 24°C. The resin was washed extensively with 10 m*M* Tris · HCl buffer (10 times, 20 ml each wash) and subsequently equilibrated with 1 m*M* CHAPS–10 m*M* Tris · HCl–10% glycerol. The resin was incubated with the solubilized receptors (1 to 2 mg) in 5 to 10 ml of 1 m*M* CHAPS–10 m*M* Tris · HCl–10% glycerol for 12 h at 4°C and poured into a column (5 cm × 0.4 cm I.D.). The eluate was collected by centrifugation and the column (kept at 4°C) was washed with 13 ml of the above buffer. The solubilized receptor was eluted by using two procedures: (i) with 0.5 *M* NaCl in 1 m*M* CHAPS–10 m*M* Tris–10% glycerol (pH 7.4) and the samples were dialyzed against 1 m*M* CHAPS–10 m*M* Tris–10% glycerol to remove excess of NaCl; or (ii) with $5 \cdot 10^{-9}$ *M* Buserelin and the samples were diluted (1:5) and dialyzed before assays. GnRH binding activity was detected by charcoal assay.

RESULTS

[Biotinyl-D-Lys^6]GnRH (Fig. 1) was prepared by chemical modification of the ε-amino group in position 6 of [D-Lys^6]GnRH with *d*-biotin-*p*-nitrophenyl ester. [D-Lys^6]GnRH was selected as the starting material for derivatization since: (i) substitution of D-amino acids in position 6 of GnRH results in more potent and metabolically stable derivatives, and (ii) the ε-amino group of lysine serves as a spacer for substitution reactions and thus the GnRH conformation is less likely to be disturbed. Table I lists the biological potency and binding affinity of GnRH, [D-Lys^6]GnRN, and the biotinylated GnRH analogue. [biotinyl-D-Lys^6]GnRH ex-

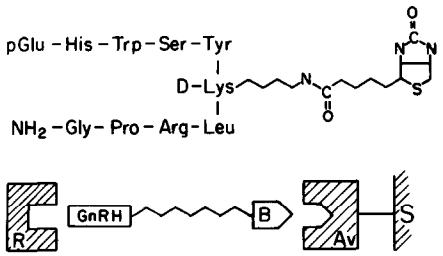

Fig. 1. Schematic representation illustrating the bifunctional ligand [biotinyl-D-Lys^6]GnRH (upper) as the mediator for absorption of GnRH receptor to avidin–Sepharose. Av = avidin; B = biotin; S = Sepharose and R = receptor.

TABLE I

BIOLOGICAL POTENCY AND BINDING AFFINITY OF GnRH, [D-Lys^6]GnRH OR [BIOTINYL-D-Lys^6]

IC_{50}: The concentration of unlabeled ligand at which the specific binding of labeled ligand is displaced by 50%. ED_{50}: The concentration required to stimulate 50% of the maximal LH release from pituitary cells.

Analogue	*IC_{50} (nM)*	*ED_{50} (nM)*
GnRH	10.0	0.5
[D-Lys^6]GnRH	3.0	0.5
[Biotinyl-D-Lys^6]GnRH	0.7	0.075

hibits a 4-fold and 14-fold higher binding affinity than that of [D-Lys^6]GnRH and GnRH, respectively and was 7-fold more potent in stimulating LH release from pituitary cells.

Purification of GnRH receptors by affinity chromatography was performed by immobilization of [biotinyl-D-Lys^6]GnRH on avidin–agarose (Fig. 1). The resin was equilibrated with the solubilized GnRH receptor, washed and subsequently eluted with 0.5 *M* NaCl in 1 m*M* CHAPS–10 m*M* Tris–10% glycerol (Fig. 2A). The samples were then dialyzed to remove NaCl and binding assays were conducted. Using this protocol, about 40–60% of the receptor was recovered and a purification fold of 7 to 12 was achieved. Recovery (*R*) of receptors was calculated according to the following equation R = (fmol[^{125}I]Buserelin bound/mg of protein) × (total mg of protein) and

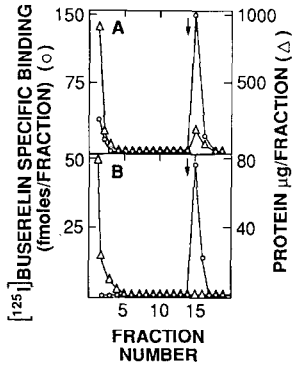

Fig. 2. Typical elution of rat GnRH receptor from affinity column. (A) [Biotinyl-D-Lys^6]GnRH immobilized on avidin–agarose resin (1.5 ml) was incubated with the solubilized receptor (1.8 mg/6.5 ml) in 1 m*M* CHAPS–10 m*M* Tris–10% glycerol for 12 h at 4°C and poured into a column (5 cm × 0.4 cm I.D.). The supernatant was collected by centrifugation and the column was washed with 13 ml of the same buffer. The resin was then incubated (60 min at 4°C) with 2 ml of 0.5 *M* NaCl in the chromatography buffer (arrow). The supernatant was collected by centrifugation and eluated fractions (1 ml) were dialyzed. All fractions were sampled for protein and GnRH binding activity. (B) The fractions with the highest receptor activity from NaCl eluted affinity column were pooled (0.3 mg/1.3 ml) and rechromatographed in a second affinity purification step. The experimental details are as in A, except that elution of the receptor was carried out by incubating the resin (3 h at 4°C) with $5 \cdot 10^{-9}$ *M* Buserelin (arrow). The fractions (0.3 ml) were diluted (1:5), dialyzed and sampled for protein and binding activity.

CHAPS extracts were taken as 100%. Purification-fold was calculated as follows: (fmol[^{125}I]Buserelin bound/mg of protein in fraction) divided by (fmol[^{125}I]Buserelin bound/mg of protein in soluble extract). For the second purification step, the fractions exhibiting the highest binding activity from NaCl eluted column were pooled and rechromatographed. The column (after washing) was then incubated with $5 \cdot 10^{-9}$ *M* Buserelin for 3 h at 4°C in 1 m*M* CHAPS–10 m*M* Tris–10% glycerol. The eluted fractions were diluted (1:5), dialyzed and binding assays conducted (Fig. 2B). The overall recovery of the purified receptor was 4–10% of the initial activity in the CHAPS extract and the calculated purification was approximately 10 000 to 15 000 fold. Iodination of this affinity purified receptor and subsequent autoradiographic analysis by sodium dodecyl sulphate–polyacrylamide gel electrophoresis revealed no other bands except that of the GnRH receptor (data not shown). This suggests that following two cycles of affinity chromatography the GnRH receptor was purified to homogeneity.

DISCUSSION

The avidin–biotin complex system has been used widely to study biological interactions that involve the specific binding between a protein and a ligand. Recently, immobilized forms of avidin have been used for the isolation of receptors. This can be accomplished either by binding of the biotinylated hormone to avidin columns, followed by subsequent interaction with the solubilized receptor or, by prior incubation of the biotinylated hormone with the solubilized receptor and then immobilization on avidin columns (shown schematically in Fig. 1). The receptor can either be eluted directly from the column or the biotinylated hormone–receptor complex can be eluted with biotin and subsequently the receptor can be dissociated from the hormone. The advantages of the method are: (i) the hormone can be attached to the support via a single defined site that is not involved in its biological function; (ii) the anchoring of the hormone to the support is unequivocal and proceeds in high yield; (iii) the chemical manipulations are performed with the free hormone and thus its effect on binding and biological activity can be readily assessed; and (iv) the technique is highly reproducible and can be readily scaled up for production of larger quantities of receptors.

In the present study, this novel procedure has been applied to the purification of GnRH receptors from other solubilized membrane proteins. The following steps were involved in this approach: (a) solubilization of rat pituitary GnRH receptor with CHAPS without alteration of binding affinity; (b) immobilization of [biotinyl-D-Lys^6]GnRH on avidin–agarose; (c) equilibration of the solubilized GnRH receptor with the affinity resin; and (d) elution of the receptors with high salt and GnRH analogues. Following two cycles of affinity chromatography the GnRH receptor was purified to homogeneity.

The development of a simple two step affinity chromatography for the purification of GnRH receptors permits preparation of a large quantity of the pure receptor. This can be used for detailed studies on the structure and function of the receptor, for the development of monospecific antibodies, as well as for partial amino acid sequencing. These studies will advance our understanding of the molecular basis for the action of GnRH, which has a pivotal role in the regulation of reproduction.

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