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Review

New purification method for glucocorticoid receptors

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Abstract

In this report, we describe a new purification method for activated recombinant glucocorticoid receptor (GR) utilizing a cation-exchanger (Mono S) at pH 8.4. This method is based upon a new finding that activated GR binds to both Mono Q and Mono S columns at the same pH. This method enables us to purify recombinant GR within 3 h. The purified GR represents more than 97% of the eluted proteins. Purified recombinant GR is able to bind specifically to a DNA fragment containing the glucocorticoid response element. Recombinant GR has no tag sequence that can be utilized for purification. Thus, this separation method is also applicable to purification of native GR.

Keywords: Reviews; Purification; Glucocorticoid receptor

Contents

1. Introduction

Glucocorticoid receptor (GR) protein is hard to refine. Besides its low content (~30 pmol/g wet weight of rat liver) in mammalian tissues [1], it is also very fragile. It has a tertiary structure that is constructed and maintained by a protein chaperon system in vivo [2]. The tertiary structure of GR is easily denatured in vitro by rising temperature, rapid pH shift, dilution of the sample and lengthy procedures. Once denatured in vitro, GR loses its steroid binding ability and forms insoluble aggregates; we could not recover functional GR from the precipitants. GR protein is also adsorbed non-specifically to the surfaces of the glass tubes, the plastic tubes and the matrix resins of the columns. Thus, great care and rapid procedures are required for purification of GR. The first purification method, which achieved at least 80% pure GR preparation, was demonstrated from rat liver [3,4]. Thereafter, several more convenient procedures were demonstrated from rabbit liver [5], and WCL2 cells [6,7]. These protocols are based upon the observation

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that oligomeric unactivated GR complex (GR associating with hsp90) binds to a Mono Q anion-exchange column and is eluted with a higher salt concentration (∼325 m*M*) than the monomeric activated GR (GR dissociating from hsp90) (∼180 m*M*). Thus, the cytosol is loaded onto the Mono Q column under high salt concentration (260 m*M* NaCl) in the presence of molybdate which stabilizes the unactivated state of GR. Then, by washing the column in the absence of salt and molybdate, GR is activated (on-column transformation) and eluted from the column at ∼180 m*M* NaCl [5–7]. This protocol achieved ∼28% purity in one column.

In order to overcome the disadvantage of the low content of GR in mammalian cells, we employed a baculovirus–insect cell expression system. The baculovirus system is a proper system for overexpression of functional full-length GR. Several lines of evidence show that recombinant GR expressed in the insect cells is indistinguishable from native GR and is functional [8–13]. However, only partial purification of full-length recombinant GR from the baculovirus system has been reported [9–11]. We have attempted to purify recombinant GR expressed in insect cells using the protocols for native GR [14]. Unfortunately, our first attempt failed. Unactivated recombinant GR was eluted from the column with a salt concentration (∼220 m*M*) only slightly higher than that for recombinant GR (∼180 m*M*) [14], and sufficient degree of purification was not obtained. The reason why unactivated recombinant GR was eluted with 100-m*M* lower salt concentration than unactivated native GR, is still unknown. However, the insect equivalent of hsp90 may affect the elution profile of unactivated recombinant GR from the Mono Q column. Nevertheless, we had to develop a new purification procedure for recombinant GR [14].

In this report, we describe a novel and simple two-step purification procedure for recombinant GR. This method is based upon a new finding that activated GR binds to both Mono Q and Mono S columns at the same pH (pH 8.4). The reason why the GR (calculated p*I* = 6.21) binds to a Mono S column under pH 8.4 still remains to be elucidated. However, it is speculated that GR protein has a bipolar nature. The net p*I* of recombinant GR is 6.21, but the p*I* of the C-terminal half (amino acid residues 426–795, containing the DNA binding domain and steroid binding domain) of the GR is 8.50 and the p*I* of the N-terminal half (amino acid residues 1–425) is 4.64. Therefore, the alkaline p*I* of the C-terminal half of the GR may be responsible for GR binding to the Mono S column under pH 8.4. The C-terminal half of the GR has good homology to the other steroid receptors. Indeed, the p*I*s of the C-terminal regions (400 amino acid residues from the C-terminal) of the androgen receptor, the mineralocorticoid receptor and the progesterone receptor are 8.35, 8.57 and 8.93, respectively. Thus, our purification procedures are applicable to purification of these steroid receptors, in addition to purification of native GR and point-mutated recombinant GR.

2. Chromatographic methods

2.1. Preparation of the insect cell cytosol

Full-length rat GR cDNA was obtained [15] and inserted into the *Autographa californica* nuclear polyhedrosis virus (AcNPV) as described previously [14]. GR cDNA has no tag sequence utilized for purification. The expressed GR has the same molecular weight as native GR with respect to SDS–PAGE analysis [14]. *Spodoptera frugiperda* (Sf9) insect cells in monolayer culture were infected with the recombinant virus, AcNPV/GR. The cells were maintained in serum-free ESF921 insect medium from Nieffenegger (Woodland, CA, USA). At 72 h post-infection, the expression of GR was maximum. Sf9 cells express ∼7.4 pmol GR/mg protein, or $\sim 8 \times 10^5$ GR molecules/cell [14]. This concentration is in good agreement with previously reported concentrations of the expressed recombinant GR [8,10]. Although the expression is not enough to show the intense band of GR when the cytosol is analyzed on SDS–PAGE, the GR concentration in the insect cell cytosol increased ∼25-fold in comparison with that in liver cytosol. The Sf9 cells were harvested and washed twice with ice-cold cell lysis buffer (20 m*M* Tris–HCl, pH 7.8 at 25 °C, 1 m*M* Na_2EDTA, 1 m*M* dithiothreitol) containing protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). The cells were lysed by a single freeze–thawing cycle in the buffer at a density of 2.5×10^7 cells/ml. The cell lysate was centrifuged at 105 000 *g* for 30 min at 4 °C and a clear supernatant (cytosol) was obtained. The clear cytosol (950 μl) was incubated with 120 n*M* non-radioactive or radioactive TA (triamcinolone acetonide) in an ice-bath for 2 h with periodic shaking, and was then heat-activated for 30 min at 20 °C followed by immediate cooling in an ice-bath for 5 min. After heat activation, we removed denatured proteins by microcentrifugation at 16 000 *g* (maximum speed) for 10 min at 4 °C.

2.2. Preparation of columns and buffers

The Mono Q column (1 ml, HR 5/5) and the Mono S (0.1 ml, PC 1.6/5) column chromatographies were performed at 4 °C using a SMART system (Amersham Biosciences, Uppsala, Sweden). The buffers used were buffer A (10 m*M* Tris–HCl, pH 7.8 at 25 °C, 7.5 m*M* CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate), 1 m*M* dithiothreitol and 10% glycerol) for pump A and buffer B (buffer A plus 500 m*M* NaCl) for pump B. The observed pH value at 4 °C of the buffers (pH 7.8 at 25 °C) was 8.4. CHAPS, the zwitterionic derivative of deoxycholate, is essential. It is reported that CHAPS stabilizes GR and prevents non-specific adsorption of GR to tubes and mono-beads [6,7]. CHAPS and DTT were added daily. After the addition, the buffers were passed through a 0.22-μm filter. The Mono Q column and the Mono S column were pre-equilibrated with 75 m*M* NaCl buffer A (15% B) and buffer A (0% B) at 4 °C, respectively.

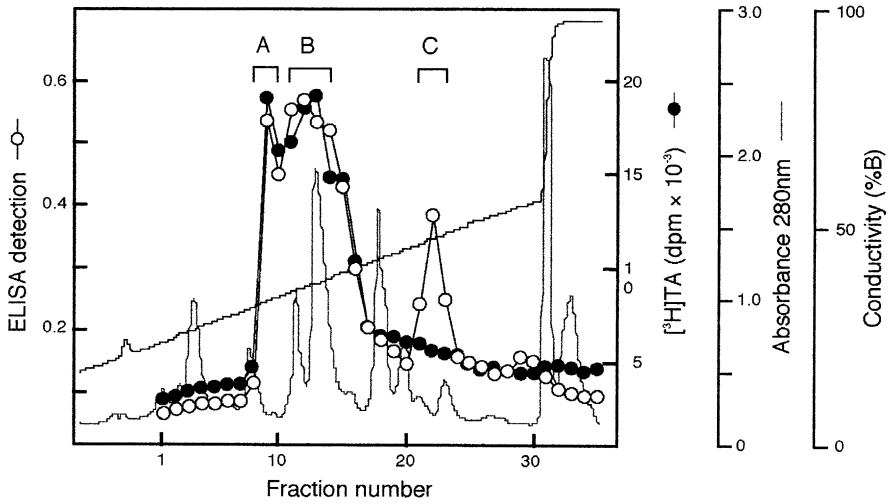

Fig. 1. Elution of recombinant activated GR from a Mono Q column. Sf9 cytosol was incubated with non-radioactive or radioactive TA on ice for 2 h, and then heat-activated for 30 min at 20 °C. Heat-activated cytosol was loaded onto a Mono Q column. Activated recombinant GR was eluted with a linear gradient of NaCl. Recombinant GR was detected with ELISA (open circles) or with radioactivity (closed circles). The data from the experiments using non-radioactive TA and radioactive TA overlapped.

2.3. Elution profiles of recombinant GR from the Mono Q column

The cytosol containing heat-activated GR was loaded onto the Mono Q column at a flow rate of 250 μl/min. The column was washed with 4 ml of the 75 m*M* NaCl buffer A at the same flow rate and eluted with a linear gradient of 75–250 m*M* NaCl in 9 ml of buffer A at a flow rate of 100 μl/min. Collection of the fraction (250 μl) was started at 125 m*M* NaCl (25% B). Aliquots (5 μl) of each fraction were used for ELISA detection of GR protein using anti-GR antibody [16] or 10 μl for radioactivity (when radioactive TA was used). Fig. 1 shows the typical and reproducible result of the elution profile from the Mono Q column. We found three peaks of immunoreactive material. The first peak (peak A) was eluted with ∼170 m*M* NaCl, the second (peak B) with ∼180 m*M* NaCl, and the third (peak C) with ∼250 m*M* NaCl. Peak B was the major peak and eluted with the same NaCl concentration as that for activated native GR [5–7]. From the assay of radioactivity, peaks A and B represent protein with ligand binding ability, while the protein in peak C has little ligand binding ability. Western blot analysis of the material in peaks A and C showed that both peaks contain an intense immunoreactive band at M_r∼94 000 and that peak A contains an additional minor band at ∼135 000 [14]. Thus, we abandoned peaks A and C.

2.4. Elution profiles of recombinant GR from the Mono S column

The pooled fractions (peak B) from the Mono Q column were mixed with the same volume of ice-cold buffer A to decrease the NaCl concentration of the sample and immediately applied to the Mono S column. The column was washed with 3 ml of buffer A at a flow rate of 100 μl/min and eluted with a linear gradient of 0–250 m*M* NaCl in 2.5 ml of buffer A at a flow rate of 50 μl/min. Collection of the fraction (100 μl) was started at 100 m*M* NaCl (20% B). As shown in Fig. 2, activated recombinant GR bound to the Mono S column at pH 8.4 and was eluted with ∼175 m*M* NaCl (fraction 6), whereas most other proteins did not bind to the column and were recovered in the flow-through fraction (compare lane MQ with lane FT in Fig. 3A). The purification parameters are summarized in Table 1. The proteins eluted from the column were analyzed by SDS–PAGE. When the polyacrylamide gel was silver stained (Fig. 3A), a major band (M_r∼94 000) was clearly seen in fraction 6. Western blot analysis of fraction 6 showed a major band of immunoreactive protein corresponding to M_r∼94 000 [14]. The arrow heads in Fig. 3A show the other proteins recognized except for GR on the SDS–PAGE. Protein of M_r∼74 000 is seen only in fraction 4. In fractions 6 and 7 M_r∼64 000 protein is seen, M_r∼41 000 and M_r∼28 000 proteins are in fraction 7 and 8, and M_r∼76 000 protein is in fraction 8. The lane of fraction 6 in the silver stained gel was analyzed by densitometric scanning software (Kodak digital science 1D). The calculated intensities of these bands (M_r∼94 000 and M_r∼64 000) were 20 252 and 607, respectively (Fig. 3B). Thus, recombinant GR protein represented 97% of the proteins eluted in fraction 6.

Table 1
Purification of activated recombinant GR expressed in Sf9 cells

Stage	Protein (μg)	GR (pmol)	Yield (%)	Purification (-fold)
Cytosol	4370	32.4	100	1
Mono Q	175	8.1	25	6.2
Mono S	0.49	4.4	13.5	1211

From Ref. [14], with permission.

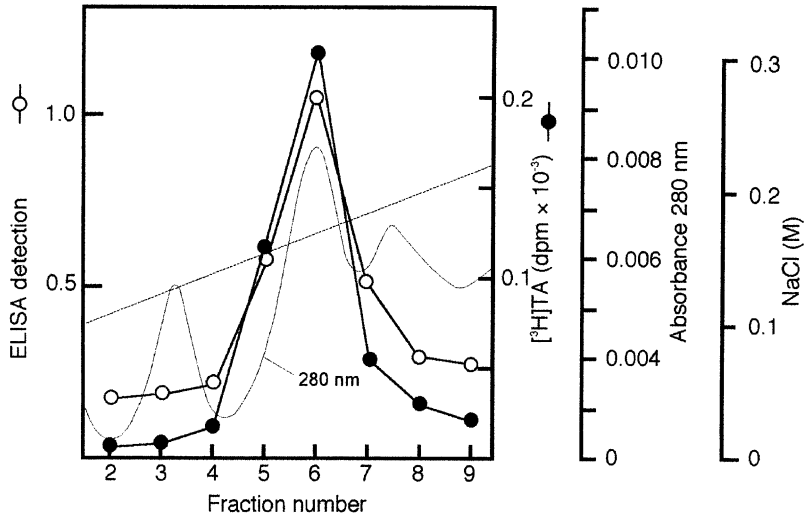

Fig. 2. Elution of activated recombinant GR from a Mono S column. The pooled fractions (peak B) from the Mono Q column were loaded onto a Mono S column. Activated recombinant GR was eluted with a linear gradient of NaCl. NaCl concentration was calculated from the conductivity. Recombinant GR was detected with ELISA (open circles) or with radioactivity (closed circles). From Ref. [14], with permission.

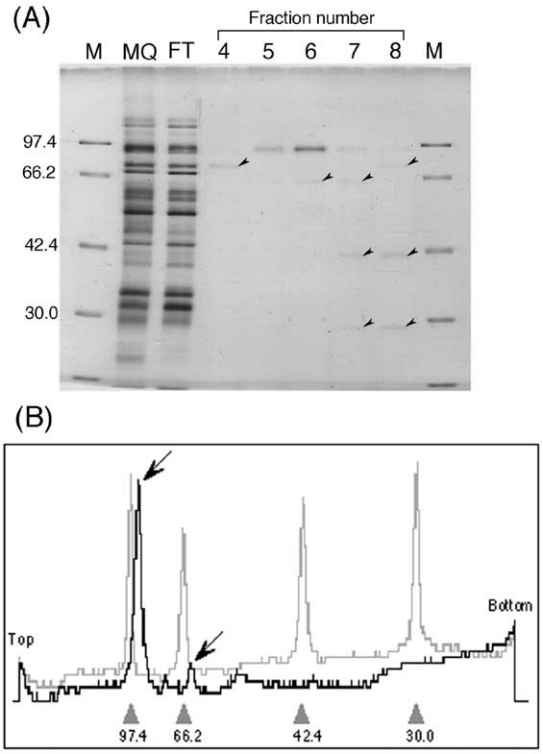

Fig. 3. SDS–PAGE analysis of the fractions from the Mono S column. (A) A constant volume (10 μl) of the fraction was loaded onto each lane of a 10–20% acrylamide gradient gel. The gel was silver stained. Lane MQ, pooled fractions (peak B) from the Mono Q column. Lane FT, pooled flow-through fractions from the Mono S column. The relative molecular mass standards (lane M) are phosphorylase b (97 400), BSA (66 200), aldorase (42 400), and carbonic anhydrolase (30 000). The arrow heads are the recognized proteins except for GR on the SDS–PAGE. (B) The lane of fraction 6 in the silver stained gel was analyzed by densitometric scanning (black line). Lane M was also analyzed (gray line) and the data overlapped. The arrows indicate the bands recognized by the image analysis software. From Ref. [14], with permission.

2.5. Characterization of purified recombinant GR

The sedimentation coefficient of purified recombinant GR was determined by glycerol density gradient analysis. As shown in Fig. 4A, the sedimentation coefficient of purified recombinant GR is ∼4 S, which is identical to that of the activated form of the partially purified (∼3000-fold) native GR [16,17]. Next, we examined whether purified recombinant GR can specifically bind to the glucocorticoid response element (GRE). The binding was analyzed by sedimentation through a 12–25% glycerol gradient. As shown in Fig. 4B, purified recombinant GR bound more strongly to the DNA containing the GRE sequence than to the DNA containing no GRE sequences and formed a peak sedimenting at ∼7 S, which is similar to the results of previous studies on the partially purified native GR [16–18]. The binding of purified recombinant GR to the GRE motif provides strong evidence, although not proof, that purified recombinant GR is functional.

3. Evaluation of the methods

In this report, we have demonstrated a simple two-step procedure for purifying full-length recombinant GR from Sf9 insect cells. The purification procedure consists of sequential chromatographies of the common ion-exchange columns. The entire chromatographies take ∼3 h and purified GR represents more than 97% of the eluted proteins (Fig. 3B). Purified recombinant GR binds to the hormone ligand (Fig. 2) and binds to the GRE motif (Fig. 4B), and thus will be functional.

Mono Q and Mono S column chromatographies automatically controlled by a SMART system show reproducible results. Especially, fraction 6 of the Mono S column is always

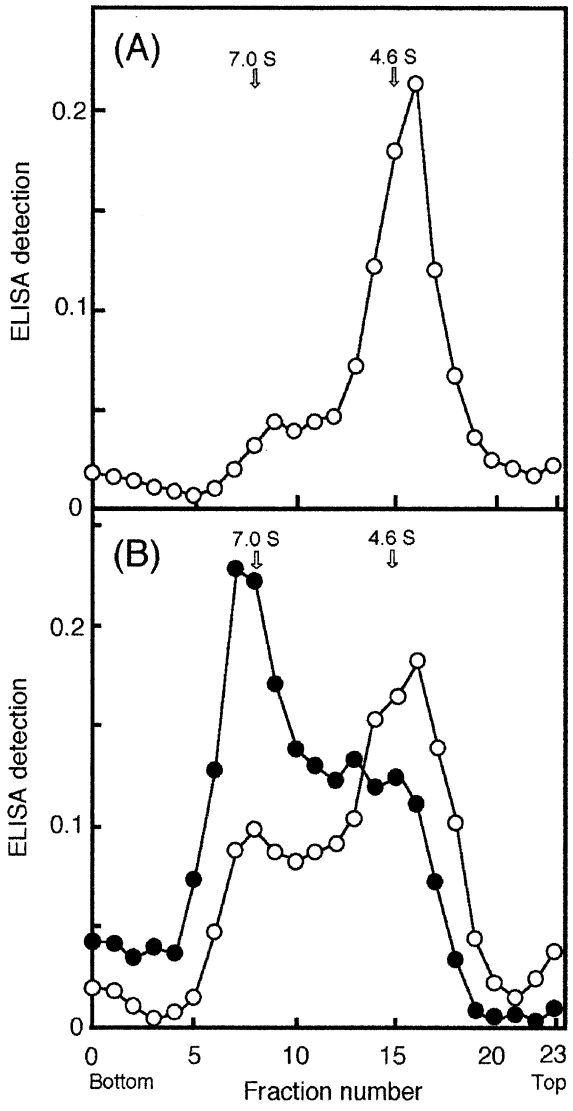

Fig. 4. Glycerol-density-gradient analysis of purified recombinant GR and its binding to GRE. (A) A portion (12.5 μl) of fraction 6 from the Mono S was layered onto a 12–25% glycerol gradient. The gradient was centrifuged in a vertical rotor at 549 000 *g* for 30 min at 4 °C. After centrifugation, fractions were collected from the bottom. Recombinant GR was detected with ELISA. ELISA activity remaining in the tube was counted as the "zero" fraction. Sedimentation coefficients were estimated using external standards, human gamma-globulin (7.0 S) and BSA (4.6 S). (B) Purified recombinant GR was incubated with the 251-bp DNA containing the GRE sequence (closed circles) or with the 222-bp DNA not containing the GRE sequence (open circles) for 15 min at 4 °C. The mixture was layered onto a 12–25% glycerol gradient. From Ref. [14], with permission.

the peak fraction of GR. Among the other proteins eluted from the Mono S column (Fig. 3A, arrow heads), only the elution of M_r~64 000 protein is overlapped with the elution of GR. We have no idea what the M_r 64 000 protein is, so far. However, the elution peak of the M_r 64 000 protein does not seem to synchronize exactly with that of GR. Therefore, it is presumed that the M_r 64 000 protein is not the protein combined with GR. Use of a longer Mono S column, if available, may improve the purity of GR.

Purified GR frozen in liquid nitrogen and stored at −70 °C is stable for at least 2 years. Without addition of BSA or insulin which reduces loss by non-specific adsorption to surfaces [3,4,6,7], purified GR is stable for at least one freezing and thawing cycle. However, if the subsequent assay is not disturbed by BSA, purified GR is mixed with protease- and nuclease-free BSA (2 mg/ml at final concentration) and divided into aliquots to prevent repeated freeze–thaws.

As mentioned above, recombinant GR has no tag sequence for purification. The purification procedure does not involve particular affinity columns. The basic principle of this purification technique originates from the property of the GR protein. This purification procedure uses two common ion-exchange columns and always provides a reliable and reproducible result. Thus, we believe this procedure is applicable to purification of native GR and point-mutated recombinant GR.

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