Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 790 (2003) 349–353
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Purification of a full-length recombinant glucocorticoid receptor

Kazuki Okamoto, Naoya Suematsu, Fumihide Isohashi*

Department of Biochemistry, St. Marianna University School of Medicine, 2-16-1 Sugao, Miyamae-ku, Kawasaki, Kanagawa 216-8511, Japan

Abstract

We described a novel purification method for a recombinant glucocorticoid receptor (GR) in detail. The purification procedure consists of sequential chromatographies using common ion-exchange columns (Mono Q and Mono S). This procedure is based upon a new finding that the activated GR binds both to a Mono Q column and to a Mono S column at the same pH. The entire chromatographies took about 3 h and GR represented 97% of the purified protein sample. This purification protocol will be applicable to the purification of native GR, point-mutated recombinant GR and other nuclear receptors.

Keywords: Glucocorticoid receptors; Proteins

1. Introduction

Glucocorticoid receptor (GR), one of ligand-dependent transcription factors, exists ubiquitously in all mammalian tissues in very low amounts (about 30 pmol/g wet mass of rat liver [1]). In addition to this, GR is a very fragile protein, will form insoluble aggregation if its tertiary structure is no longer maintained. Inability to obtain sufficient quantities of purified GR causes difficulties in studying in vitro the interactions of GR with the other proteins that contribute to transcriptional activation of chromatin. Attempts to express a full-length, functional GR have not been successful in bacterial hosts [2], but successful in insect cells using the baculovirus system [2–4]. The recombinant GR expressed in the insect cells is indistinguishable from the bona fide GR with respect to immunogenic reactivity, cytoplasmic localization, ligand-binding activity, sedimentation profiles in sucrose density gradient, electrophoretic mobility, and DNA binding [2–4]. The recombinant GR is expressed as a functional protein judged by its ability to translocate from the cytoplasm to the nucleus upon hormone-binding, and to act as a transcriptional enhancer in vivo [3,5]. Furthermore, the recombinant GR induces transcription from a mouse mammary tumor virus long terminal repeat-containing DNA template in an in vitro system [6,7]. Thus, the baculovirus system appeared to be a proper system for overexpression of the functional full-length GR. However, only partial purification of the full-length recombinant GR from baculovirus system has been reported [2,6,7].

We have attempted to purify the insect cell-expressed recombinant GR using the protocols for the native GR from rabbit liver [8], and WCL2 cells [9]. These protocols are based upon the observation 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

*Corresponding author. Tel.: +81-44-977-8111; fax: +81-44-976-7553.
E-mail address: k2oka@marianna-u.ac.jp (F. Isohashi).

1570-0232/03/$ – see front matter
doi:10.1016/S1570-0232(03)00166-1

than that with which the monomeric activated GR (GR dissociating from hsp90) is eluted [8,9]. Unfortunately, our attempt using these protocols failed [4]. The unactivated recombinant GR was eluted from the column with a salt concentration only slightly higher than that with which the activated recombinant GR was eluted [4], and sufficient degree of purification was not obtained. The insect equivalent of the hsp90 and/or unknown proteins in the insect cytosol may affect the elution profile of the unactivated recombinant GR from the Mono Q column. Thus, we had to develop a new purification procedure for the recombinant GR [4]. In this review article, we show the novel and simple two-step method for the purification of the recombinant GR in detail. This method is based upon a new finding that the activated GR binds both to a Mono Q column and to a Mono S column at the same pH (pH 8.4). This method accommodates GR of 97% purity within 3 h. The purified GR is functional, although not proof, according to the abilities to bind hormone ligand and to interact specifically with the DNA containing GRE motif [4].

2. Expression of the recombinant GR in insect cells

The full-length rat GR cDNA was obtained as described previously [4,10]. The full-length cDNA was inserted into the *Bam*HI–*Sma*I site of the transfer vector pVL1393 (BD PharMingen, San Diego, CA, USA). The recombinant transfer vector (pVL1393/GR) and the linearized *Autographa californica* nuclear polyhedrosis virus (AcNPV) DNA (BaculoGold) were cotransfected into *Spodoptera frugiperda* (Sf9) insect cells, then the recombinant baculovirus, AcNPV/GR, was produced by in vivo homogenous recombination as described previously [4]. Sf9 cells in monolayer culture, maintained in the serum-free ESF921 insect medium from Nieffenegger (Woodland, CA, USA), were infected with the recombinant virus, AcNPV/GR, at a multiplicity of infection of about 4. Western blot analysis showed that the recombinant GR protein was seen with the maximum accumulation at 72 h post-infection [4]. Thus, we used the cytosol from the insect cells at 72 h post-infection for the following experiments.

At 72 h post-infection, the cells were harvested and washed twice with the ice-cold cell lysis buffer consist of 20 m*M* Tris–HCl, pH 7.8 at 25 °C, 1 m*M* Na_2EDTA, 1 m*M* dithiothreitol and protease inhibitor cocktail (Roche, 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 the 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 then it was heat-activated for 30 min at 20 °C followed by immediate cooling in an ice-bath for 5 min. After the heat-activation, we removed denatured proteins by microcentrifugation at 16 000 *g* (maximum speed) for 10 min at 4 °C.

3. Step 1, Mono Q column chromatography

A Mono Q (HR 5/5) column chromatography was performed using a SMART system. 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 A pump and buffer B (buffer A plus 500 m*M* NaCl) for B pump. It should be emphasized here that CHAPS, the zwitterionic derivative of deoxycholate, is essential. CHAPS stabilizes GR and prevents the nonspecific adsorption of GR to tubes and mono-beads [9]. The gradient program (Table 1) controlled the flow-rate and the concentration of salt. The cytosol containing heat-activated GR was loaded onto the Mono Q column equilibrated with buffer A containing 75 m*M* NaCl at 4 °C. The column was washed with 5 ml of the same buffer at a flow-rate of 250 μl/min and eluted with a linear gradient of 75–500 m*M* NaCl in buffer A at a flow-rate of 100 μl/min at 4 °C. Fractions of 250 μl were collected and aliquots (5 μl) of each fraction were used for enzyme-linked immunosorbent assay (ELISA) detection of GR protein using anti-GR antibody [11]. We found three peaks of immunoreactive material (Fig. 1). The first

Table 1
Program for MonoQ column chromatography

Volume (ml)	Method	
0.00	Inject	
0.00	Dual wavelength	280, 260
0.00	Autozero	
0.00	Conc B	15.0
0.00	Flow	250.0
3.90	Load	
4.00	Conc B	15.0
5.00	Flow	100.0
6.50	Needle position	Down
6.50	Goto tube	1
6.50	Fraction size	250
13.00	Conc B	50.0
13.00	Conc B	100.0
14.00	Flow	250
15.00	Fraction stop	
20.00	Conc B	100.0

peak (peak A) was eluted with about 170 m*M* NaCl (fraction 9), the second (peak B) with about 180–190 m*M* NaCl (fraction 11–14), and the third (peak C) with about 250 m*M* NaCl (fraction 21–23). The peak B was the major peak and eluted with the same NaCl concentration as that with which the activated native GR was eluted [8,9]. From the assay of radioactivity, peak A and B represent protein with ligand binding ability, however, protein in peak C has little ligand binding ability [4]. Western blot analysis of the material in peak A and C showed that both peaks contain an intense immunoreactive band at $M_r \approx 94\,000$ and that peak A contains an additional minor band at about $M_r \approx 135\,000$ [4]. Thus, we abandoned peaks A and C and used the peak B for the further purification.

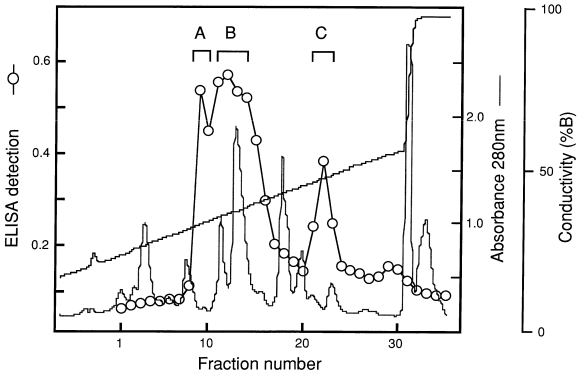

Fig. 1. Elution of the activated recombinant GR from a Mono Q column. Sf9 cytosol was incubated with TA on ice for 2 h, and then heat-activated for 30 min at 20 °C. The heat-activated cytosol was loaded onto a Mono Q column. The activated recombinant GR was eluted with a linear gradient of 75–500 m*M* NaCl. The recombinant GR was detected with ELISA.

4. Step 2, Mono S column chromatography

A Mono S (PC 1.6/5) column chromatography was also performed using the SMART system. The buffers used were the same as those in the Mono Q column chromatography. The program used is detailed in Table 2. We mixed the pooled fractions from the Mono Q column (peak B) with the same volume of the ice-cold buffer A to decrease the NaCl concentration of the sample and immediately applied it to the Mono S column. The column was washed with 3 ml of the buffer A at a flow-rate of 100 μl/min and eluted with a linear gradient of 0–500 m*M* NaCl in the buffer A at a flow-rate of 50 μl/min at 4 °C. Fractions of 100 μl were collected and aliquots (5 μl) of each fraction were used for ELISA detection of the GR protein. As shown in Fig. 2, the activated recombinant GR bound to the Mono S column and was eluted with about 175 m*M* NaCl (fraction 6), whereas most other proteins did not bind to the column and were recovered in the flow-

Table 2
Program for MonoS column chromatography

Volume (ml)	Method	
0.00	Inject	
0.00	Dual wavelength	280, 260
0.00	Autozero	
0.00	Conc B	0.0
0.00	Flow	100.0
3.00	Load	
3.00	Conc B	0.0
3.00	Flow	50.0
4.00	Needle position	Down
4.00	Goto tube	1
4.00	Fraction size	100
5.00	Fraction stop	
5.50	Flow	100.0
5.50	Conc B	50.0
5.50	Conc B	100.0
6.50	Conc B	100.0

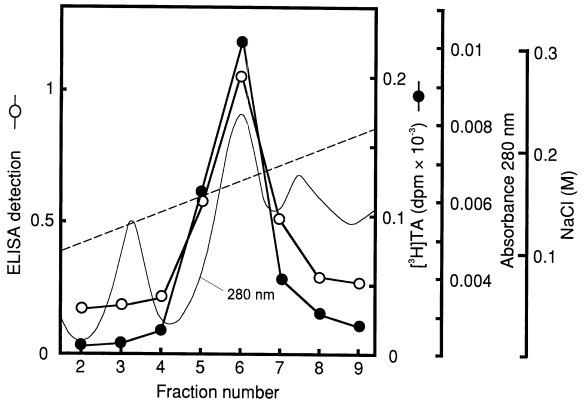

Fig. 2. Elution of the activated recombinant GR from a Mono S column. The pooled fractions (peak B from the Mono Q column) were loaded onto a Mono S column. The activated recombinant GR was eluted with a linear gradient of 0–500 m*M* NaCl. The recombinant GR was detected with ELISA (open circles) or with radioactivity (closed circles). From Ref. [4], with permission.

through fraction (compare the lane MQ with the lane FT in Fig. 3A). The purification parameters are summarized in Table 3. The proteins eluted from the column were analyzed by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE). When the polyacrylamide gel was silver stained (Fig. 3A), a major band ($M_r \approx 94\,000$) was clearly seen in the fraction 6. Western blot analysis of the fraction 6 showed a major band of immunoreactive protein corresponding to a molecular mass of about 94 000 (Fig. 3B). The lane of the fraction 6 in the silver stained gel was analyzed by densitometric scanning software (Kodak digital science 1D). The software found two bands with molecular masses of about 94 000 and about 64 000 (Fig. 3C). The calculated intensities of these bands were 20 252 and 607, respectively. Thus, the recombinant GR protein represented 97% of the proteins eluted in the fraction 6. We estimate that we will be able to purify about 0.3 μg of the activated GR from a monolayer culture in a 150-mm plate.

5. Discussion

In this study, we have demonstrated the expression of a full-length GR in Sf9 cells and its simple two-step purification procedure. The purification

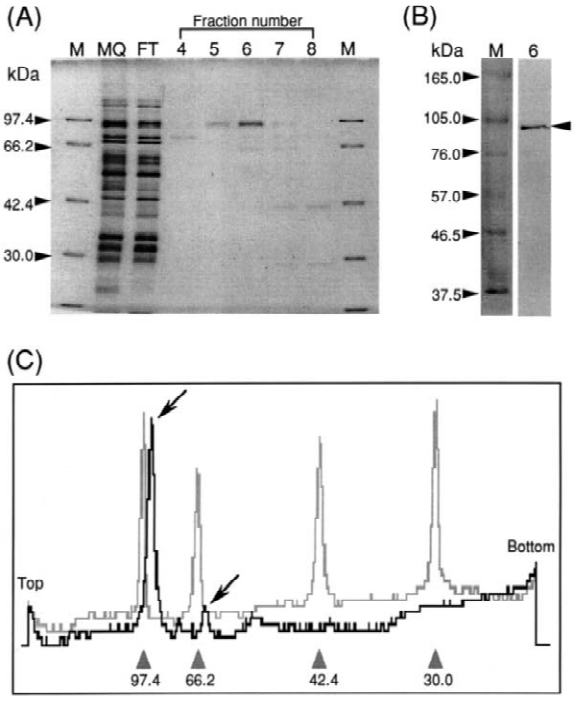

Fig. 3. SDS–PAGE and western blot analysis of the fractions from the Mono S column. (A) A constant volume (10 μl) of the fraction was loaded onto each lane of a 10–20% acrylamide gradient gel. The gel was silver stained. Lane MQ, pooled fractions (peak B) from the Mono Q column. Lane FT, pooled flow-through fractions from the Mono S column. The molecular mass standards (lane M) are phosphorylase b (97 400), bovine serum albumin (66 200), aldorase (42 400), and carbonic anhydrolase (30 000). kDa=kilodalton. (B) The fraction 6 from the Mono S column was analyzed by the Western blot as described above. Lane M, biotinylated molecular mass markers. (C) The lane of the fraction 6 in the silver stained gel was analyzed by the densitometric scanning (black line). The lane M was also analyzed (gray line) and the data were overlapped. The arrows indicate the bands recognized by the image analysis software. From Ref. [4], with permission.

Table 3
Purification of the 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. [4], with permission.

procedure consists of sequential chromatographies of the common ion-exchange columns. The entire chromatographies take about 3 h and the purified GR represented 97% of the eluted proteins. The binding of the purified recombinant GR to GRE motif provides strong evidence, although not proof, that the purified recombinant GR is functional [4].

The purification protocol reviewed in this article is based upon the new finding that the activated GR binds both to an anion-exchange column and to a cation-exchange column at the same pH (pH 8.4). It is well known that the charge of a protein depends on the pH of its environment (operating pH). When the operating pH is greater than the isoelectric point (p*I*) of the protein, the protein will have a net negative charge, and should bind to an anion-exchange column and not to a cation-exchange column. Vice versa, when the protein binds to the cation-exchange column, the operating pH should be lower than the pI of the protein. Therefore, at our operating pH (about 8.4 at 4 °C), most proteins which bind to the Mono Q column do not bind to the Mono S column and are recovered in the flow-through fraction from the column, whereas the recombinant GR binds to the Mono S column. This would be because of the bipolar nature of the GR protein. The net p*I* of the recombinant GR is calculated to be 6.21, however, the p*I* of the C-terminal half (containing the DNA binding domain and steroid binding domain) of the GR (amino acid residues 426–795) is 8.50 and the p*I* of the N-terminal half (amino acid residues 1–425) is 4.64. Therefore, we speculated that the alkaline p*I* of the C-terminal half of the GR may be responsible for the GR binding to the Mono S column under nearly physiological pH. The C-terminal half of the GR has a good homology to the other steroid receptors. Indeed, the p*I* values 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 calculated to be 8.35, 8.57 and 8.93, respectively. Thus, our purification procedures would be applied to these steroid receptors, in addition to the native GR and the point-mutated recombinant GR.

References

[1] F. Isohashi, M. Terada, Y. Nakanishi, Y. Sakamoto, Cancer Res. 36 (1976) 4382.
[2] E.S. Alnemri, A.B. Maksymowych, N.M. Robertson, G. Litwack, J. Biol. Chem. 266 (1991) 3925.
[3] G. Srinivasan, E.B. Thompson, Mol. Endocrinol. 4 (1990) 209.
[4] M. Hyodo, K. Okamoto, K. Shibata, N. Suematsu, F. Isohashi, J. Chromatogr. B 765 (2001) 89.
[5] G. Srinivasan, J.F. Post, E.B. Thompson, J. Steroid Biochem. Molec. Biol. 60 (1997) 1.
[6] S.Y. Tsai, G. Srinivasan, G.F. Allan, E.B. Thompson, B.W. O'Malley, M.J. Tsai, J. Biol. Chem. 265 (1990) 17055.
[7] G.F. Allan, N.H. Ing, S.Y. Tsai, G. Srinivasan, N.L. Weigel, E.B. Thompson, M.J. Tsai, B.W. O'Malley, J. Biol. Chem. 266 (1991) 5905.
[8] M. Denis, P. Blanchardie, J.L. Orsonneau, P. Lustenberger, J. Chromatogr. 508 (1990) 97.
[9] B.S. Warren, P. Kusk, R.G. Wolford, G.L. Hager, J. Biol. Chem. 271 (1996) 11434.
[10] K. Okamoto, K. Shibata, F. Isohashi, BioFactors 11 (2000) 39.
[11] K. Okamoto, G. Liu, W.G. Yu, T. Ochiai, F. Isohashi, J. Biochem. 119 (1996) 920.