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Novel and simple two-step purification of a full-length rat glucocorticoid-receptor expressed in a baculovirus system

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Abstract

We purified the activated recombinant glucicorticoid receptor (GR) overexpressed in insect cells by sequential chromatographies using Mono Q and Mono S columns. This procedure was 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). The entire chromatographies took about 3 h and GR represented 97% of the purified protein sample. The purified GR was able to bind specifically to a DNA fragment containing the glucocorticoid response element. This purification protocol will be applicable to the purification of native GR, point-mutated recombinant GR and other nuclear receptors.

Keywords: Glucocorticoid receptors

1. Introduction

Glucocorticoid receptor (GR), a member of the family of ligand-dependent transcription factors, exerts its functions by binding to *cis*-acting glucocorticoid-response element (GRE) sequences. After binding to the GRE sequences, GR recruits coactivators, such as SRC-1/NcoA1, CBP/p300, and GRIP-1/TIF-2/NcoA2 which contribute to the transcriptional activation of organized chromatin template (for a review, see Ref. [1]). These interactions between GR and the coactivators are important regulatory steps for ligand-dependent gene transcription [1]. To investigate the GR-coactivator interactions in vitro, we need to obtain highly purified GR protein in abundance.

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 [2,3]. The recombinant GR expressed in insect cells associates with the insect equivalent of mammalian heat shock protein 90 (hsp 90) and hsp 70 [3–5], suggesting that the recombinant GR associates with its protein chaperons. The recombinant GR is indistinguishable from the bona fide GR with respect to immunogenic reactivity, cytoplasmic localization, ligand-binding activity, sedimentation profiles in sucrose density gradient, chromatographic

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and electrophoretic mobility, and DNA binding [2,3]. 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 [2,5,6].

On the other hand, complete purification procedures of the native GR from rat liver [8], rabbit liver [9], and WCL2 cells [10] have been demonstrated. We have attempted to purify the insect cell-expressed recombinant GR using the protocols for the native GR from rabbit liver [9], and WCL2 cells [10]. These protocols are based upon the observation that unactivated GR (GR associating with hsp90) binds to a Mono Q anion-exchange column and is eluted with a higher salt concentration than that with which the activated GR (GR dissociating from hsp90) is eluted [9,10]. Unfortunately, our attempt using these protocols failed. 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. Thus, the protocols for the purification of the native GR could not be applied to the purification of the insect cell-expressed recombinant GR. In this paper, we report a novel and simple two-step purification procedure for the recombinant GR. The purified GR represented 97% in the purified protein sample, and was able to interact specifically with the DNA containing GRE motif.

2. Experimental

2.1. Materials

The transfer vector pVL1393, the linearized *Autographa californica* nuclear polyhedrosis virus (AcNPV) DNA (BaculoGold), a positive control vector pVL1392-XylE and *Spodoptera frugiperda* (Sf9) insect cells were from BD PharMingen (San Diego, CA, USA). The serum-free ESF921 insect medium was from Nieffenegger (Woodland, CA, USA). [^{3}H]Triamcinolone acetonide (TA, 35.40 Ci/mmol) was from DuPont NEN (Boston, MA, USA). Complete protease inhibitor cocktail set was from Roche Diagnostics (Mannheim, Germany). Mono Q (HR 5/5), Mono S (PC 1.6/5) columns and the AutoSeq G-50 spin column were from Amersham Pharmacia Biotech (Uppsala, Sweden). Restriction enzymes, pUC118 DNA and bovine serum albumin (BSA) (molecular biology grade) were from Takara (Kyoto, Japan). The protein detector enzyme-linked immunosorbent assay (ELISA) kit (HRP anti-rabbit, ABTS system) was from Kirkegaard & Perry Labs. (Gaithersburg, MD, USA). 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) and all other chemicals (reagent grade) were from Sigma.

2.2. Construction of pVL1393-GR

Total RNA from the livers of Donryu albino male rats was isolated after homogenization in guanidine thiocyanate. Messenger RNA was purified using oligo(dT)-cellulose column and subjected to cDNA synthesis with an oligo-dT primer. The cDNAs coding the three distinct domains of rat GR were separately amplified by polymerase chain reaction (PCR) using primer sets corresponding to nucleotides 42-65/999-1022, 972-995/1827-1850, 1782-1805/2457-2480 of rat GR cDNA [11]. The amplified cDNA fragments were subcloned into pUC118 and sequenced. The cDNA fragments were then fused to construct the full-length GR. The full-length cDNA was inserted into the *Bam*H I–*Sma* I site of the transfer vector pVL1393.

2.3. Generation of recombinant baculovirus

The recombinant transfer vector (pVL1393-GR) and the linearized virus DNA (BaculoGold) were cotransfected into Sf9 cells. Sf9 cells were cultured at 27°C in ESF921 serum-free medium and maintained as monolayers. The recombinant baculovirus, AcNPV-GR, was produced by in vivo homogenous recombination and purified by the end-point-dilution assay according to the manufacturer's specifications.

2.4. Expression of GR

Sf9 cells in monolayer culture were infected with the recombinant virus at a multiplicity of infection (MOI) of about 4. At 72 h post-infection, the cells were harvested and washed twice with ice-cold buffer A (20 m*M* Tris–HCl, pH 7.8 at 25°C, 1 m*M* Na_2EDTA, 1 m*M* dithiothreitol, protease inhibitor cocktail). The cells were lysed by freeze–thawing in buffer A at a density of $2.5 \cdot 10^7$ cells/ml. The cell lysate was centrifuged at 105 000 *g* for 30 min at 4°C and the clear supernatant (cytosol) was used for further experiments.

2.5. Western blot analysis

The cytosol was heated for 5 min at 100°C with Laemmli sample buffer and run on a 10–20% acrylamide gradient gel. The proteins were electro-blotted from the gel to a nitrocellulose membrane in the transfer buffer (25 m*M* Tris, 192 m*M* glycine, 10% methanol). The membrane was blocked with 5% goat serum in buffer B (50 m*M* Tris–HCl, pH 7.8 at 25°C, 200 m*M* NaCl) for 30 min at room temperature, and then washed three times with buffer B. The membrane was incubated for 16 h at 4°C with anti-(rat GR) rabbit antiserum [12] diluted 1:1000 in buffer B containing 2% goat serum. The reaction products were developed by the avidin–biotin–peroxidase complex method as described previously [13]. Molecular mass protein markers used are biotinylated protein marker 7711BTS (New England BioLabs, MA, USA).

2.6. Steroid binding and competition analysis

The amount of expressed GR in the cytosol was determined by incubating the cytosol with 120 n*M* [^{3}H]TA in the presence or absence of a 500-fold excess of non-radioactive TA on ice for 2 h. The free steroid was removed by gel filtration using the AutoSeq G-50 spin column equilibrated with buffer C (10 m*M* Tris–HCl, pH 7.8 at 25°C, 7.5 m*M* CHAPS, 1 m*M* dithiothreitol, 10% glycerol) containing 150 m*M* NaCl and 2 mg/ml BSA at 4°C. Radioactivity was determined by liquid scintillation counting. For the competition analysis, the cytosol was incubated with 30 n*M* [^{3}H]TA in the presence or absence of a 1-, 10-, 100-, or 1000-fold excess non-radioactive TA, progesterone, 17β-estradiol, testosterone on ice for 2 h.

2.7. Purification of the recombinant GR

The clear cytosol (950 μl) was incubated with 120 n*M* non-radioactive or radioactive TA in an ice-bath for 2 h, and then it was heat-activated for 30 min at 20°C followed by immediate cooling in an ice-bath. The cytosol containing heat-activated GR was loaded onto a Mono Q column (HR 5/5) equilibrated with buffer C 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 C 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 ELISA of GR protein. The fractions containing GR were combined and mixed with the same volume of ice-cold buffer C. The mixture was immediately loaded onto a Mono S column (PC 1.6/5) equilibrated with buffer C at 4°C. The column was washed with 2 ml of buffer C at a flow-rate of 100 μl/min and eluted with a linear gradient of 0–500 m*M* NaCl in buffer C 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 of the GR protein. Fractions were promptly frozen in liquid nitrogen and stored at −80°C.

2.8. ELISA

The recombinant GR was detected by the protein detector ELISA kit (HRP anti-rabbit, ABTS system). All procedures were performed according to the manufacturer's specifications. The anti-(rat GR) rabbit antiserum [12] was diluted 1:1000 in BSA dilution/blocking solution in the kit. After color development, the absorbancies were quantitated at 405 nm on an ELISA plate reader (Bio-Rad).

2.9. Glycerol density gradient analysis

The purified recombinant GR was layered onto a 3.8-ml gradient of 12–25% glycerol prepared in buffer D (20 m*M* Tris–HCl, pH 7.8 at 25°C, 25 m*M* NaCl, 1 m*M* dithiothreitol). The gradients were

centrifuged in a vertical rotor (SRP83VT, Hitachi) at 549 000 *g* for 30 min at 4°C. After centrifugation, fractions (160 μl) were collected from the bottom as described previously [12,14].

2.10. Preparation of DNA containing GRE motif

A 251-base pair (bp) DNA containing the GRE sequence was amplified by PCR from pMSG-CAT (Amersham Pharmacia Biotech, GeneBank accession number U13861) using a primer set corresponding to nucleotides 7869–7892 and 8096–8119. The amplified 251-bp DNA was purified by Mono Q column (PC 1.6/5) chromatography, followed by ethanol precipitation as described previously [15]. To construct a negative control DNA, we digested the 251-bp DNA with restriction enzyme *Tru*9I (Promega, Madison, WI, USA). The resulting 71-bp and 151-bp fragments were purified by Mono Q column chromatography and ligated together. The resulting 222-bp DNA lacks the following sequence: TAAGTAAGTTTTTGGTTACAAACTGTTCT (underlined nucleotides correspond to the GRE motif). The 222-bp DNA was amplified by PCR using the same primer set and purified from the PCR product by Mono Q column chromatography.

2.11. Protein determination

Protein concentrations were determined by the method of Bradford [16] using BSA as a standard.

3. Results and discussion

3.1. Expression and characterization of recombinant GR

The expression of recombinant GR was tested by Western blot analysis of the cytosols from Sf9 cells at 24–96 h post-infection. A polyclonal antibody raised against the synthetic peptide representing amino acid 366–387 of rat GR [13] was used. As shown in Fig. 1, an intense immunoreactive band at about 94 000 was seen with the maximum signal at 72 h post-infection. Thus, we used the cytosol from the insect cells at 72 h post-infection for the following experiments. To test whether the recombinant GR can bind glucocorticoids, we incubate the cytosol from the Sf9 cells at 72 h post-infection with [^{3}H]TA in the presence or absence of the non-radioactive TA. Specific TA-binding was observed (26 600 dpm/10 μl of the cytosol). Specific TA-binding was scarcely observed in cytosols from uninfected Sf9 cells. Based on the specific activity of [^{3}H]TA (35.40 Ci/mmol), it appears that the Sf9 cells express about 7.4 pmol receptor/mg protein, or about $8 \cdot 10^5$ receptor molecules/cell. These concentrations are in good agreement with the previously reported concentrations of the expressed recombinant GR [2,3]. Next, we examined whether the recombinant GR can specifically bind a glucocorticoid agonist. Thus, we incubated the cytosol with [^{3}H]TA in the presence or absence of non-radioactive steroids. Fig. 2 shows that 17β-estradiol and testosterone are poor com-

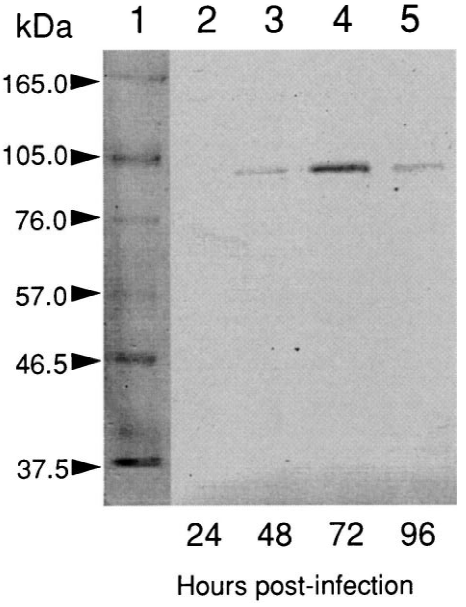

Fig. 1. Western blot analysis of the recombinant GR from baculovirus-infected Sf9 cells. A constant volume (5 μl) of the cytosol from the baculovirus-infected Sf9 cells was loaded onto each lane of a 10–20% acrylamide gradient gel. The proteins were then blotted onto a nitrocellulose membrane and detected with the anti-GR antibody as described in Experimental. Lane 1, biotinylated molecular mass markers. Lanes 2–5, the cytosols from the Sf9 cells at 24–96 h post-infection, respectively.

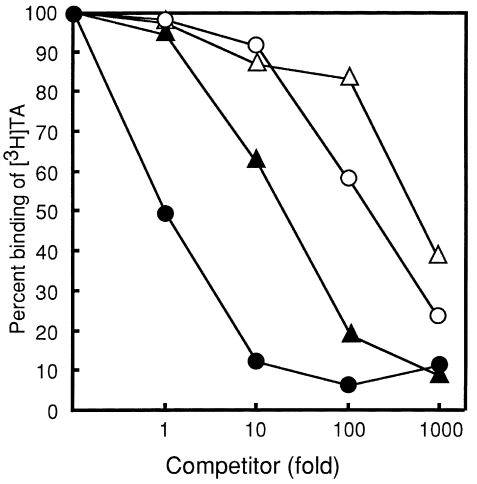

Fig. 2. Competition binding analysis of the recombinant GR. Sf9 cytosols (4.6 mg protein/ml) were labeled with 30 n*M* [^{3}H]TA in the absence or presence of a 1-, 10-, 100-, or 1000-fold excess nonradioactive TA (closed circles), progesterone (closed triangles), testosterone (open circles), and 17β-estradiol (open triangles) on ice for 2 h. Bound radioactivity in the absence of competitors was taken as 100%.

petitors and progesterone is a moderate competitor, similar to that observed with the native rat GR [3].

3.2. Activation (transformation) of recombinant GR

The [^{3}H]TA-labeled unactivated recombinant GR formed a peak sedimenting at about 8 S (data not shown). After heat activation, the [^{3}H]TA-labeled recombinant GR formed a peak sedimenting at about 4 S identical to that of the partially purified activated GR from rat liver [15]. These data suggest that like the native rat liver GR the recombinant GR is synthesized in the insect cells as a large oligomeric complex (8 S) and in vitro heat activation releases the 4 S monomeric GR from the complex.

3.3. Elution profiles of recombinant GR from the anion-exchange column

The TA-labeled unactivated (molybdate stabilized) GR was loaded onto a Mono Q column and eluted with a NaCl gradient. The GR in the eluate was detected by ELISA. Previous studies on the purification of the native GR [9,10] showed that the liganded unactivated GR can be eluted with a NaCl concentration of about 320 m*M*. As shown in Fig. 3A, the major peak of the liganded unactivated recombinant GR was eluted with about 220 m*M* NaCl, which is about 100 m*M* lower salt concentration than that with which the unactivated native GR was eluted. Several lines of evidence [9,10,17] show that the hsp90 contained in the oligomeric unactivated GR complex is responsible for the behavior of the unactivated GR on the anion exchangers. The oligomeric complex of the unactivated recombinant GR will contain the insect equivalent of the mammalian hsp90 [2,3]. Therefore, the insect equivalent of the hsp90 and/or unknown proteins in the insect cytosol

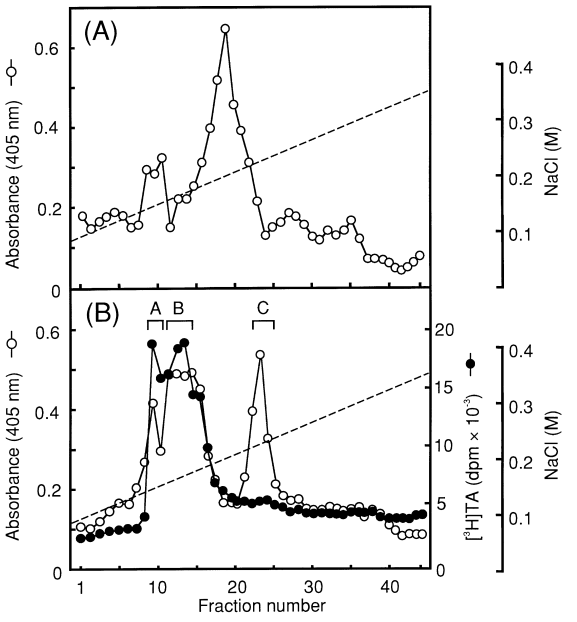

Fig. 3. Elution profile of the unactivated (A) and the activated (B) recombinant GR from a Mono Q column. (A) Sf9 cytosol was incubated with nonradioactive TA in the presence of 20 m*M* Na_2MoO_4 on ice for 2 h, and then loaded onto a Mono Q column. The unactivated recombinant GR was eluted with a linear gradient of 75–500 m*M* NaCl. The recombinant GR was detected with ELISA as described in Experimental. (B) 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. 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 (open circles) or with radioactivity (closed circles).

will affect the elution profile of the unactivated recombinant GR from the Mono Q column.

The radioactive TA-labeled heat-activated recombinant GR was loaded onto a Mono Q column and eluted with a NaCl gradient (Fig. 3B). The recombinant GR in the eluate was detected by ELISA and radioactivity. We found three peaks of immunoreactive material. The first peak (peak A) was eluted with about 170 m*M* NaCl, the second (peak B) with about 180 m*M* NaCl, and the third (peak C) with about 250 m*M* NaCl. Peak B was the major peak and eluted with the same NaCl concentration as that with which the activated native GR was eluted [9,10]. From the assay of radioactivity, peaks A and B represent protein with ligand binding ability, however, 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 about 94 000 and that peak A contains an additional minor band at about 135 000 (data not shown). Thus, we abandoned peaks A and C.

It should be noted that the difference between the salt concentrations with which the unactivated and the activated native GR are eluted is about 140 m*M* in previous studies by others [9,10], whereas it is only 40 m*M* in our case of the recombinant GR. Thus, it was difficult to purify the recombinant GR according to the procedure described for the native GR [9,10].

3.4. Purification of recombinant GR

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. The recombinant GR (p*I* 6.54) bound to the Mono Q anion-exchange column at our operating pH (about 8.4 at 4°C). We calculated the p*I* of the C-terminal half of the GR (amino acid residues 401–795). That is 8.62, while the p*I* of the N-terminal half (amino acid residues 1–400) is 4.67. Therefore, we speculated that the C-terminal half of the GR may bind to a cation-exchange column at our operating pH. We mixed the pooled fractions from the Mono Q column (peak B) with the same volume

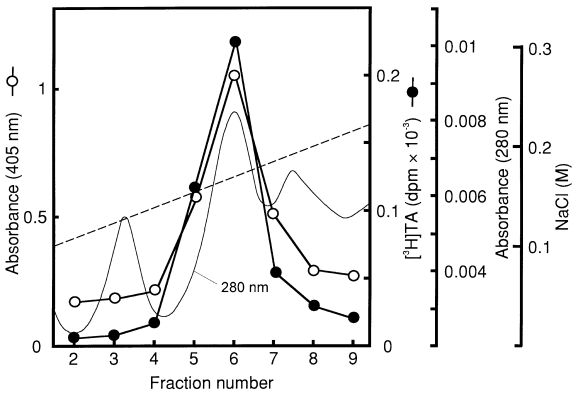

Fig. 4. Elution profile of the activated recombinant GR from a Mono S column. The pooled fractions (peak B from the Mono Q column) were loaded onto a Mono S column. The activated recombinant GR was eluted with a linear gradient of 0–500 m*M* NaCl. The recombinant GR was detected with ELISA (open circles)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

of the buffer to decrease the NaCl concentration of the sample and immediately applied it to the Mono S column. As shown in Fig. 4, the activated recombinant GR expectedly 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-through fraction (compare the lane MQ with the lane FT in Fig. 5A). The purification parameters are summarized in Table 1. The proteins eluted from the column were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). When the polyacrylamide gel was silver stained (Fig. 5A), a major band (about 94 000) was clearly seen in fraction 6. Western blot analysis of fraction 6 showed a major band of immunoreactive protein corresponding to a molecular mass of about 94 000 (Fig. 5B). The lane of 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. 5C). The calculated intensities of these bands were 20 252 and 607, respectively. The recombinant GR protein represented 97% of the eluted proteins. Thus, the activated recombinant GR was purified in two steps. 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.

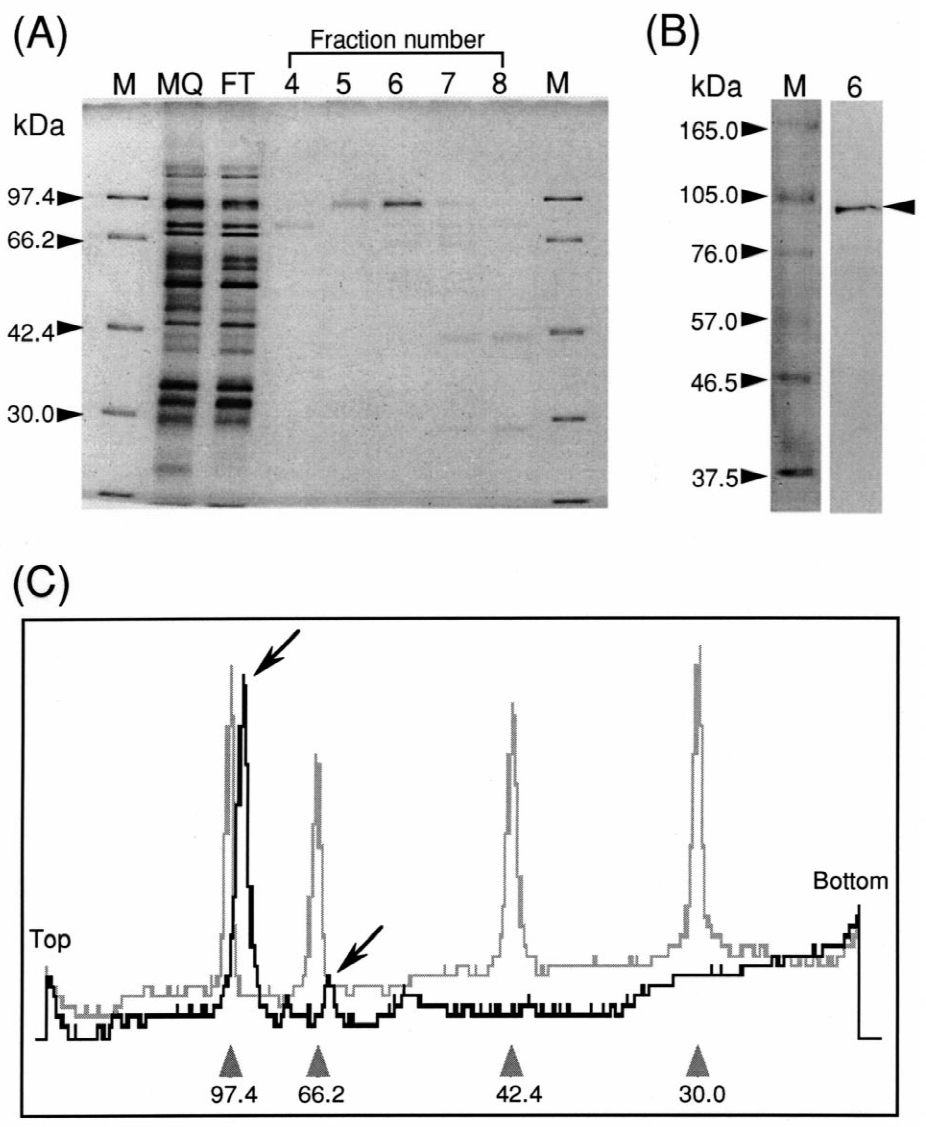

Fig. 5. SDS–PAGE and Western blot analysis of the fractions from the Mono S column. (A) A constant volume (10 μl) of the fraction was loaded onto each lane of a 10–20% acrylamide gradient gel. The gel was silver stained. Lane MQ, pooled fractions (peak B) from the Mono Q column. Lane FT, pooled flow-through fractions from the Mono S column. The molecular mass standards (lane M) are phosphorylase b (97 400), BSA (66 200), aldorase (42 400), and carbonic anhydrolase (30 000). (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). Lane M was also analyzed (gray line) and the data were overlapped. The arrows indicate the bands recognized by the image analysis software.

3.5. Characterization of the purified recombinant GR

The sedimentation coefficient of the purified recombinant GR was determined by the glycerol density gradient analysis. As shown in Fig. 6, the sedimentation coefficient of the purified recombinant GR is about 4 S, which is identical to that of the activated form of the partially purified (about 3000-fold) native GR [12,13,15]. Next, we examined

Table 1
Purification of the activated recombinant GR expressed in Sf9 cells

Step	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

whether the purified recombinant GR can specifically bind to the GRE motif. The binding was analyzed by sedimentation through a 12–25% glycerol gradient. As shown in Fig. 7, the purified recombinant GR bound to the DNA containing the GRE sequence and formed a peak sedimenting at about 7 S, which is similar to the results of the previous studies on the partially purified native GR [12,13,15]. Moreover, the purified recombinant GR bound more strongly to the DNA containing the GRE sequence than to the DNA containing no GRE sequences. This observation shows that the purified recombinant GR can bind specifically to the GRE motif.

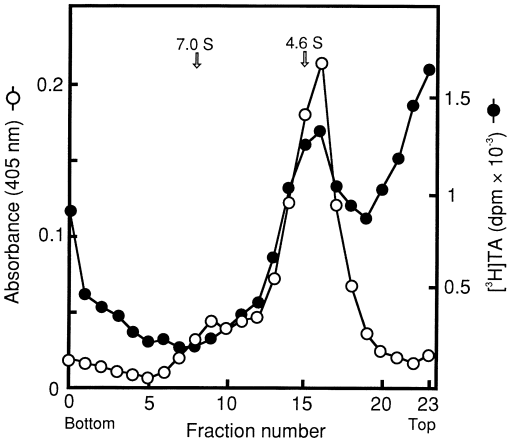

Fig. 6. Glycerol-density-gradient analysis of the purified recombinant GR. A portion (12.5 μl) of the fraction 6 from the Mono S was layered onto a 12–25% glycerol gradient (open circles). The gradient was centrifuged in a vertical rotor at 549 000 *g* for 30 min at 4°C. After centrifugation, fractions were collected from the bottom. The recombinant GR was detected with ELISA. As for the comparison, the partially purified native GR from rat liver (labeled with [^{3}H]TA) was layered onto a 12–25% glycerol gradient (closed circles). The tritium at the top of the gradient represents dissociated hormone ligand. The ELISA activity and radioactivity remaining in the tube were counted as the ''zero'' fraction. Sedimentation coefficients were estimated using external standards, human gamma-globulin (7.0 S) and BSA (4.6 S).

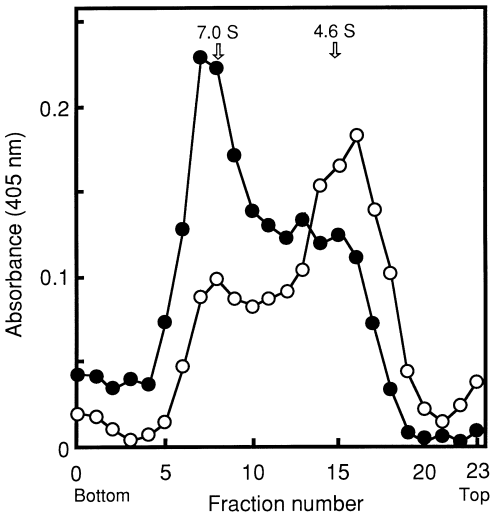

Fig. 7. Glycerol-density-gradient analysis of the purified recombinant GR binding to GRE. The purified recombinant GR was incubated with the 251-bp DNA containing the GRE sequence (closed circles) or with the 222-bp DNA containing no GRE (open circles) for 15 min at 4°C. The mixture was layered onto a 12–25% glycerol gradient. After centrifugation, fractions were collected from the bottom. The recombinant GR was detected with ELISA.

3.6. General 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 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.

As mentioned above, 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 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 point-mutated recombinant GR.

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