New functions for the components of glucocorticoid receptor apparatus?

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Received 4 April 1984

By ammonium sulphate precipitation, phosphocellulose and DEAE–Sephacel chromatography and Sephadex G-75 gel filtration, a factor was separated from rat liver cytosol which was shown to suppress the inhibitory effect of the steroid deoxycorticosterone (DOC) on the specific [^{3}H]glucocorticoid binding to cytoplasmic receptors. By SDS-polyacrylamide gel electrophoresis (SDS–PAGE) of factor-containing fractions its M_r was suggested to be about 40000. The possible role of this factor in the regulation of glucocorticoid receptor apparatus function is discussed.

Heart *Myocardium* *Liver* *Hormone* *Glucocorticoid* *Glucocorticoid receptor*

1. INTRODUCTION

Cytoplasmic glucocorticoid receptors from various tissues and organs are quite similar in a number of physical and chemical characteristics. Nevertheless, tissue specificity was indicated in their interaction with some steroids [1]. For example, there is a significant difference in the ability of steroid DOC to suppress specific binding of [^{3}H]glucocorticoids to liver and heart cytosols [2]. Liver cytosol was reported to contain a protein fraction which decreases sensitivity of the heart glucocorticoid receptor system to DOC [3]. We here present data on the separation of a receptor-modifying factor (RMF) from glucocorticoid receptors in liver cytosol and its partial purification by chromatography and gel filtration.

2. MATERIALS AND METHODS

Intact and adrenalectomized Wistar rats were used and maintained as described previously [4]. [^{3}H]Triamcinolone acetonide ([^{3}H]TA) (spec. act. 29 Ci/mmol) was purchased from Amersham (England), DEAE–Sephacel and Sephadex G-75 from Pharmacia (Sweden), and electrophoresis reagents were from Bio-Rad (USA). All other chemicals used were of A-grade purity.

To estimate [^{3}H]glucocorticoid receptor binding liver and heart cytosols were prepared from adrenalectomized rats as in [4] using buffer solution A [20 mM Tris, 1 mM EDTA, 2 mM dithiothreitol (DTT), 10% (w/v) glycerol, 30 mM KCl (pH 7.4)] at 4°C. Precipitation by $(NH_4)_2SO_4$ of liver glucocorticoid receptors was performed in buffer B (buffer A containing additionally 5 mM DTT and 10 mM Na_2MoO_4). [^{3}H]TA specific binding to cytosol preparations was estimated as in [4]. To separate and purify RMF, liver cytosol from intact rats was prepared using buffer C [20 mM Tris, 1 mM EDTA, 30 mM KCl (pH 7.4)] at 4°C.

To test liver cytosol fractions for the presence of RMF activity 0.15 ml of the liver fraction was added to 0.15 ml of adrenalectomized rat heart cytosol (protein concentration 8–10 mg/ml) and specific [^{3}H]TA binding was estimated in the presence of 20-fold excess (10^{-6} M) unlabeled DOC. That quantity of RMF which increased specific [^{3}H]TA binding by heart cytosol in the presence of DOC to the value of 1 pmol was arbitrarily taken as one unit of RMF activity. From the

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00145793/84/$3.00

fractions obtained after different steps of purification proteins were precipitated by 10% trichloroacetic acid. Fifty μg of proteins precipitated were analyzed on 10% SDS–PAGE as in [5]. Protein was determined according to [6] as modified in [7]. Radiometry was carried out on an LKB-1215 Rackbeta scintillation spectrometer with a ^{3}H counting efficiency of 40%.

3. RESULTS AND DISCUSSION

Fig.1,2 show suppression of specific [^{3}H]TA binding to glucocorticoid receptors of heart and liver cytosols by 20-fold excess unlabeled DOC. The suppressive effect of DOC was weak in the non-fractionated liver cytosol (fig.1a) but increased greatly after fractionation of liver glucocorticoid receptors by precipitation at 0–30% $(NH_4)_2SO_4$ saturation as in [8] (fig.1b). Recombination of the glucocorticoid receptor fraction (0–30% precipitable fraction) with the 50–70% $(NH_4)_2SO_4$-precipitable fraction from the same liver cytosol again made the glucocorticoid receptor system less sensitive to DOC (fig.1c). Fig.2a shows that 20-fold excess unlabeled DOC sharply suppressed specific [^{3}H]TA binding to non-fractionated heart cytosol. The sensitivity of the heart glucocorticoid receptor system to DOC significantly decreased after the addition of the 50–70% $(NH_4)_2SO_4$-precipitable fraction from liver cytosol (fig.2b). After preliminary treatment of this fraction with trypsin its ability to influence the DOC effect was lost (fig.2c). The data obtained show that the 50–70% $(NH_4)_2SO_4$-precipitable fraction from liver cytosol contains a protein factor which is able to affect the interaction of steroids with glucocorticoid receptors. We call it RMF.

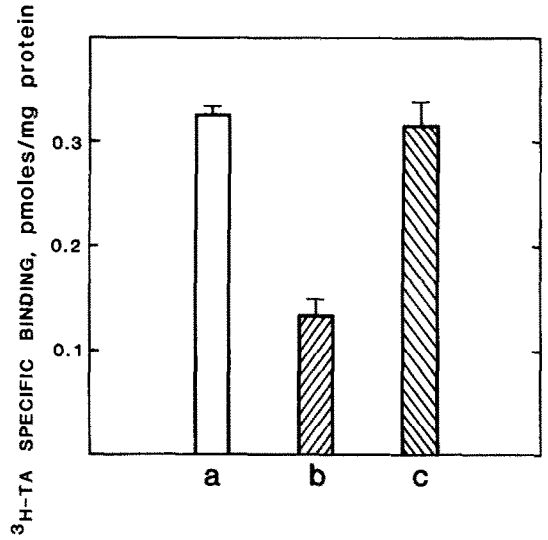

Fig.1. Specific [^{3}H]TA binding to glucocorticoid receptors in non-fractionated and fractionated liver cytosol in the presence of 20-fold excess unlabeled DOC. (a) Binding of [^{3}H]TA (5×10^{-8} M) to non-fractionated liver cytosol in the presence of DOC (10^{-6} M). (b) [^{3}H]TA specific binding to the 0–30% $(NH_4)_2SO_4$-precipitable fraction in the presence of DOC. (c) The same as in b but after the addition of the 50–70% $(NH_4)_2SO_4$-precipitable fraction. Values are means ± SE of 4 independent measurements.

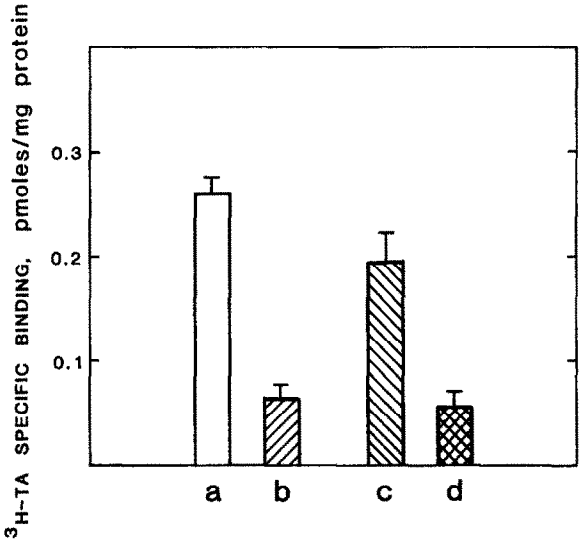

Fig.2. Suppression by DOC of [^{3}H]TA specific binding to heart cytosol glucocorticoid receptors in the absence or presence of the 50–70% $(NH_4)_2SO_4$-precipitable fraction from liver cytosol. (a) [^{3}H]TA (5×10^{-8} M) binding to heart cytosol in the absence of DOC. (b) The same as in a but in the presence of DOC (10^{-6} M). (c) The same as in b but in the presence of the 50–70% $(NH_4)_2SO_4$-precipitable fraction from liver. (d) The same as in c but the 50–70% $(NH_4)_2SO_4$-precipitable fraction was treated with 1 $\mu g/A_{280}$ trypsin for 15 min at 10°C, and soybean trypsin inhibitor (10-fold excess) before addition to heart cytosol. Values are means ± SE of 6 independent measurements.

Table 1 presents data on a stepwise fractionation of cytosol [obtained from livers (197 g wet wt) of

Table 1

Steps of liver cytosol RMF purification

Step	Volume (ml)	Total proteins (mg)	Total activity (relative units)[a]	Purification factor[b]	Yield (%)
Cytosol	300	7140	406.98	–	100
50–70% $(NH_4)_2SO_4$	77	2009	372.44	3.25	91.5
Breakthrough from PC-column	85	1854	351.13	3.32	86.3
DEAE–Sephacel chromatography (0.06 M)	25	8.3	242.56	512.70	59.6

[a] See section 2

[b] Ratio of RMF activity/mg fraction protein to RMF activity/mg non-fractionated cytosol protein

intact rats] performed for the characterization and partial purification of RMF. At the step of RMF precipitation at 50–70% saturation by $(NH_4)_2SO_4$ the loss of its activity was not more than 15% (table 1) and a 3.25-fold enrichment of RMF activity relative to protein content was achieved. The 50–70% $(NH_4)_2SO_4$-precipitable fraction was dissolved in buffer C and dialyzed twice against the same buffer (1:50, v/v). It was then applied to a phosphocellulose P-II column (2.0 cm^2 × 15 cm) and eluted with buffer C. This permitted separation of RMF activity from positively charged proteins including activated glucocorticoid-receptor

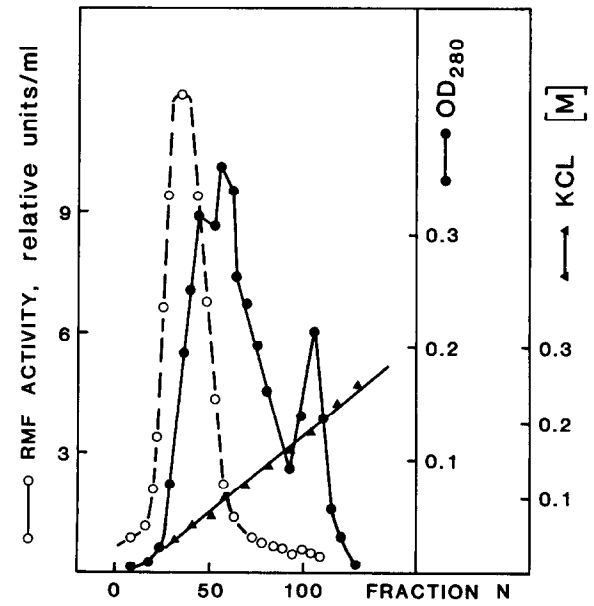

Fig.3. DEAE–Sephacel chromatography of phosphocellulose breakthrough fraction (liver cytosol).

complexes which were absorbed on the column. The breakthrough fraction contained up to 86% of initial RMF activity (table 1). It was collected, applied to a DEAE–Sephacel column (5.0 cm^2 × 10 cm) equilibrated with buffer C and the absorbed proteins were eluted by a linear KCl gradient (60 ml 30 mM KCl–60 ml 300 mM KCl). As can be seen from fig.3, maximal RMF activity was found at 0.06 M KCl in the eluate. RMF-containing fractions were collected, lyophilized and used for further investigations. At this step of purification the loss of RMF activity was about 40% (table 1).

Lyophilized preparations of RMF were dissolved in buffer C to a final protein concentration of 3–5 mg/ml. Samples (1 ml) were applied to a Sephadex G-75 column (10 cm^2 × 60 cm) and eluted with buffer C. The Stokes radius (R_s) of RMF was estimated by linear regression of R_s from the K_{av} of protein standards [9] listed in the legend to fig.4. The fraction corresponding to an R_s of 31.25 Å was analyzed on the SDS–PAGE in parallel with the samples obtained after earlier steps of purification. In preliminary experiments we found that on gel filtration of the 50–70% $(NH_4)_2SO_4$-precipitable fraction on Sephadex G-75 (at about 20 mg/ml protein) the maximal RMF activity was eluted in samples corresponding to R_s 31.25 ± 3.75 Å or roughly to M_r 40000 (fig.4). SDS–PAGE patterns (fig.5) show that one band corresponding to M_r 40000 is absent in the DEAE–Sephacel breakthrough samples where RMF activity was also not detected. This band is also present in samples of the third (DEAE–Sephacel) and fourth (Sephadex G-75) purification steps. The last two samples also reveal a band cor-

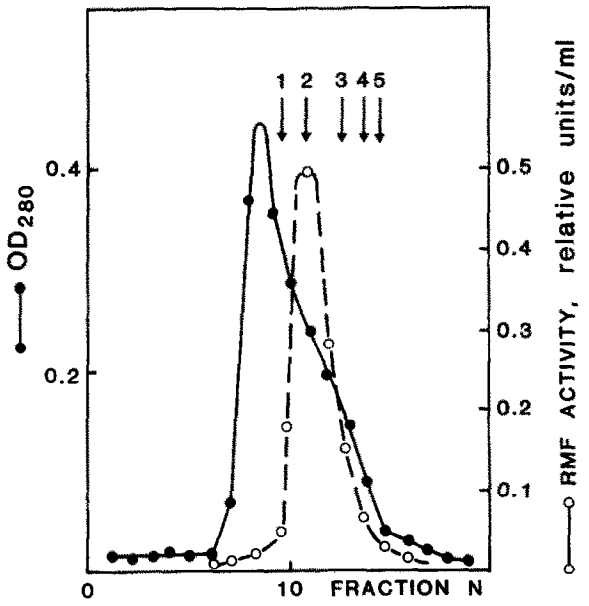

Fig.4. Gel filtration on Sephadex G-75 of the 50–70% $(NH_4)_2SO_4$-precipitable fraction (liver cytosol). Arrows denote elution positions of protein standards: (1) BSA, (2) ovalbumin, (3) chymotrypsinogen, (4) myoglobin, (5) cytochrome *c*. Volume of elution probes is 1.86 ml. Representative results of one out of 3 separate experiments.

responding to M_r 14000. We cannot exclude the possibility that the latter appears as a result of splitting of native RMF during the preparation of samples for electrophoresis. Other data indirectly support the notion that the band with M_r ~40000 corresponds to RMF. Electrophoresis of the third-purification-step samples from kidney, lung, skeletal muscle and heart cytosols did not reveal this band (fig.6). Also, we were not able to detect any RMF activity in cytosol fractions from these organs (not shown).

These data show that the differences in glucocorticoid receptor sensitivity to DOC in various organs can be due to the presence, absence or different concentrations of protein factor (M_r 40000) dissimilar to steroidophilic components of receptor apparatus. We cannot rule out that the factor described is a non-steroidophilic regulatory subunit of glucocorticoid receptors. However, this suggestion requires further experimental support. It is also necessary to elucidate whether there are any relations between RMF and high-molecular-

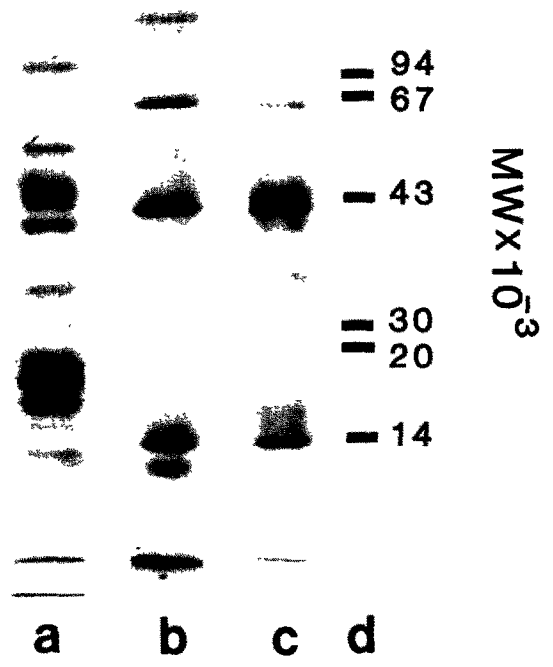

Fig.5. SDS–PAGE patterns of samples obtained after various steps of liver RMF purification. (a) DEAE–Sephacel breakthrough fraction, (b) DEAE–Sephacel chromatography step fraction (eluted at 0.06 M KCl), (c) 31 Å Sephadex G-75 fraction (4th step of purification), (d) protein standards.

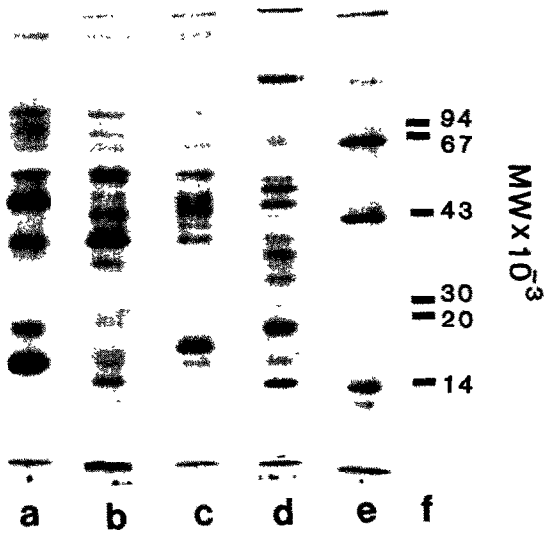

Fig.6. SDS–PAGE patterns of samples obtained after 3-step fractionations (last step: DEAE–Sephacel) of various cytosols: (a) Lung, (b) skeletal muscle, (c) heart, (d) kidney, (e) liver, (f) protein standards.

mass modulators of glucocorticoid receptor complex activation which were described recently [10,11].

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