PARTIAL PURIFICATION OF THE ACTIVATED GLUCOCORTICOID RECEPTOR OF RAT LIVER

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1. Introduction

Studies on the mechanism of action of steroid hormones are presently concerned with the role of hormone receptors in enzyme induction and, more specifically, in the transcription of particular genes. The use of crude receptor preparations leads to artefactual results, due to the presence of nucleases and inhibitors which interfer with receptor binding to the nuclear acceptor sites [1–4]. Therefore, a prerequisite for this type of investigations is the availability of techniques for the purification of the receptor proteins. In this paper we describe a simple procedure for the partial purification of the activated glucocorticoid receptor of rat liver, based on the differential affinity for phosphocellulose of the activated and the non-activated receptor–steroid complex. The partially purified receptor–steroid complex is free of deoxyribonucleases and binds efficiently to chromatin and DNA. Procedures similar to the one reported here have been briefly proposed by other workers [3,5].

2. Materials and methods

Triamcinolone acetomide-(6,7-H), [^{3}H]TA, specific activity 33.7 Ci/mmol, was obtained from New England nuclear Inc., and crystallized bovine serum albumin (BSA) from the Behringwerke, Marburg. Phosphocellulose (P-11) was obtained from Whatman Inc. Male Wistar II rats weighing 120–140 g were used throughout. The animals were adrenalectomized 5 to 10 days before the beginning of the experiments.

For each preparation of cytosol 30 to 32 g of either freshly obtained or frozen liver were used. The livers had been perfused in situ through the portal vein with 20 ml of cold TSS-buffer (0.25 M sucrose, 25 mM KCl, 5 mM $MgCl_2$, 1 mM mercaptoethanol in 50 mM Tris-HCl, pH 7.55) and the preparation of the cytosol was as previously described [6]. Unless otherwise stated all the subsequent procedures were carried out at 0–2°C. [^{3}H]TA, was added to the cytosol at a final concentration of 50 nM (specific activity 6.7 Ci/mmol) and binding was allowed to proceed at 0°C for 30 min. The cytosol was applied to a phosphocellulose column (12 × 2.7 cm) equilibrated with TSS-buffer, and the column was eluted with the same buffer. The break-through of the column was applied to a second phosphocellulose column (7 × 2 cm) equilibrated with the same buffer. The material eluting in the break-through of the second phosphocellulose column was incubated at 20°C for 30 min [7], and applied at 0°C and 20 ml/h to a third phosphocellulose column (1 × 2 cm) equilibrated with TSS-buffer containing 0.1% BSA. The column was then washed with 5 to 6 volumes of TGA-buffer (10 mM Tris-HCl, pH 7.55, 10% glycerol, 1 mM $EDTANa_2$, 1 mM mercaptoethanol and 0.1% BSA), containing 0.1 M NaCl, and the activated receptor–steroid complex (R-TA) was eluted with TGA-buffer containing 0.5 M NaCl. In order to separate the receptor from BSA, saturated ammonium sulphate (neutralized to pH 7 with NH_4OH), was added to a final saturation of 30%. After standing for 1 h at 0°C, the sample was centrifuged at 15 000 *g* for 10 min, the supernatant discarded and the pellet resuspended in small volume of TGA-buffer without BSA and containing 25 mM

NaCl. The precipitation with ammonium sulphate was repeated, and the final pellet resuspended in a very small volumn (100 μl) of the same buffer and stored at 0°C. The concentration of (R-TA) was determined by the charcoal technique [6]. For preparations containing less than 1 mg protein/ml, BSA was added to the samples (final concentration of 1 mg/ml) before mixing with a 10-fold diluted dextran-charcoal solution. Radioactivity was measured by adding 100 μl aliquots of the supernatant to 5 ml of Bray's solution [8].

Procedures for the preparation of rat liver chromatin and DNA-cellulose, as well as the conditions for the binding assay of the partially purified (R-TA) to this preparations have been reported in detail [4,9,10]. Determination of DNA was carried out by the procedure of Burton [11] and proteins were estimated with the technique of Lowry et al. [12] scaled down to a sensitivity of 4–5 μg.

For the detection of deoxyribonuclease activity a very sensitive probe was used, consisting of highly labelled globin cDNA (4×10^5 cpm/μg). This DNA was prepared with AMV reverse transcriptase and purified rabbit globin mRNA in the absence of actinomycin D, using [^{3}H]tymidine as label. Aliquots of cDNA were incubated with different amounts of partially purified (R-TA) in TGA-buffer containing 25 mM NaCl and 2 mM $MgCl_2$, in a final volume of 200 μl. After mixing in the ice bath, an aliquot was taken for the determination of TCA-insoluble radioactivity, and the samples were incubated in a water bath at 25°C for 60 min. At the end of the incubation, another aliquot was taken for determination of TCA-insoluble radioactivity, using the filter paper disk procedure [13].

3. Results

Column chromatography on phosphocellulose of hepatic cytosol incubated with [^{3}H]TA and precipitated with ammonium sulphate yields a pattern which is depicted in fig.1. Around half of the protein-bound radioactivity does not bind to the resin, and appears

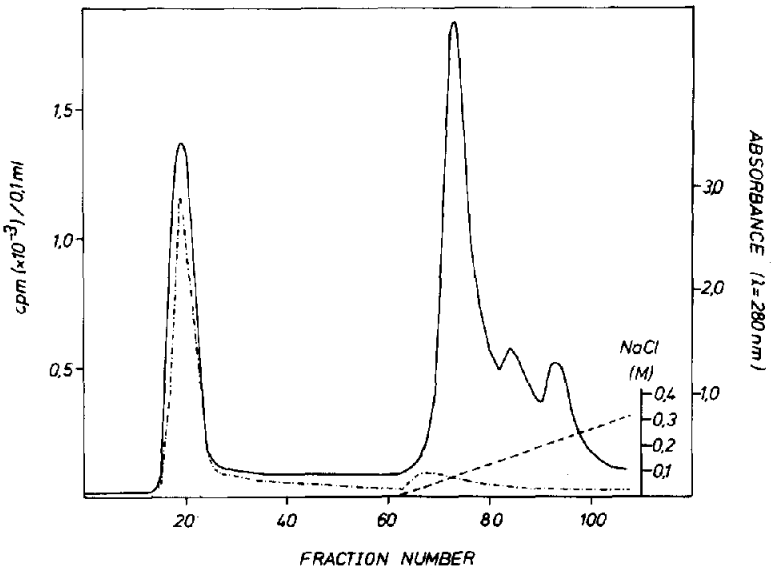

Fig.1. Column chromatography on phosphocellulose of liver cytosol labeled with [^{3}H]TA. Liver cytosol (46 ml) was incubated with [^{3}H]TA (50 nM, 20°C for 30 min) and the (R-TA) was precipitated by 30% saturated ammonium sulphate. After passing through a column of Sephadex G-25 to eliminate the ammonium sulphate, the protein peak was applied to a phosphocellulose column (2.5 × 10 cm), equilibrated with 0.1 M Na phosphate buffer pH 7.2, containing 10% glycerol, 1 mM $EDTANa_2$ and 2 mM mercaptoethanol. The column was washed with 100 ml of the starting buffer and eluted with a 400 ml linear gradient of NaCl (0-0.6 M) in the same buffer. The radioactivity (—·—·—·—), the absorbancy at 280 nm (–·–·–·–) and the Cl^- content. (- - - - -) were determined in the eluate.

Table 1
Partial purification of (R-TA)

Fraction	Protein (mg)	Specific activity (cpm (× 10^{-3})/mg)	Purification (fold)	Yield (%)
Cytosol	2283	3.41	1	100
1st Break-through	1830	3.45	1.01	81
2nd Break-through	1450	4.30	1.26	80
Phosphocellulose Eluate[a]	4.5	602.10	176.0	35
Ammonium Sulphate	0.17	11 278.0	3301.0	25

[a]This fraction includes the added BSA (1 mg/ml). The NaCl concentration was brought to 25 mM by passing through a Biogel P-10 column.

in the break-through of the column. Elution with a linear gradient of NaCl, results in a heterogeneous radioactivity profile, with a major peak eluting at 0.25 M NaCl, and two minor peaks eluting at 0.34 M and 0.38 M respectively. The reason for this heterogeneity is unknown yet. For the present studies, elution of the bound (R-TA) was carried out in a single step with 0.5 M NaCl.

A comparison of the break-through and the retained material, in respect to their ability to bind to DNA-cellulose, shows that the two fractions correspond to non-activated and thermaly activated (R-TA) respectively, as only the latter exhibits affinity for DNA [2,14–16]. An evaluation of numerous experiments has shown that around 50% of the total (R-TA) formed in cytosol at 20°C and low ionic strength is activated and binds to phosphocellulose under the conditions used in these experiments.

Our procedure for the purification of the activated receptor takes advantage of this differencial affinity for phosphocellulose of the activated and the non-activated (R-TA). The receptor-steroid complex is first formed at low ionic strength and 0°C, in order to prevent activation of the (R-TA), and the cytosol is passed through two consecutive phosphocellulose columns, to which all proteins having affinity for the resine are bound. The break-through of the second phosphocellulose column is activated by raising the temperature to 20°C, and applied to a third small

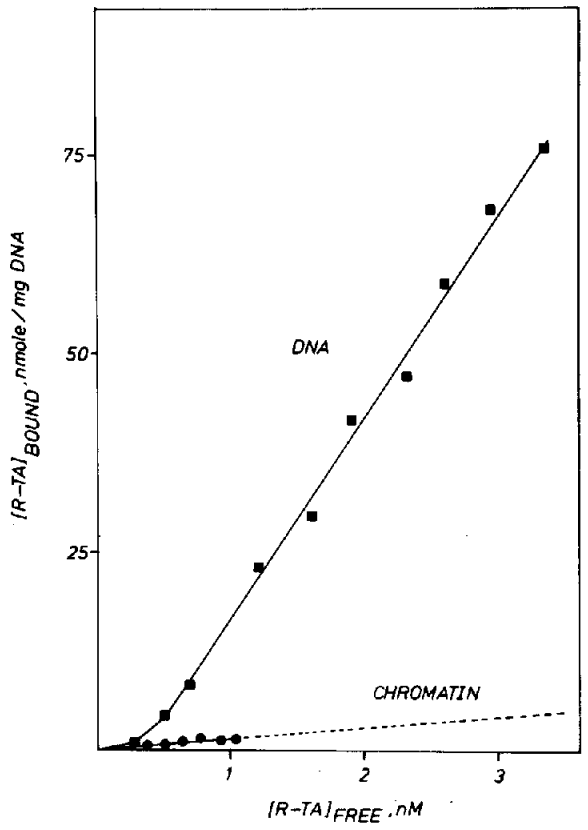

Fig.2. Binding of partially purified (R-TA) to rat liver chromatin and DNA-cellulose. Different amounts of partially purified (R-TA) were incubated with either rat liver chromatin (210 μg DNA), native rat liver DNA-cellulose (30 μg DNA) or an equivalent amount of cellulose. Incubations were performed at 20°C for 60 min in 500 μl assays containing 0.1 M NaCl and 0.1% BSA [4]. The amount of (R-TA) bound to chromatin (●——●) and DNA (■——■) was determined by centrifugation and washing of the pellets, followed by resuspension in Bray's solution [8] as described [4].

phosphocellulose column. The activated (R-TA) is bound and can be eluted by raising the NaCl concentration of the buffer to 0.5 M. Finally, the receptor is separated from BSA by two consecutive precipitations with 30% saturated ammonium sulphate. The results of such a purification procedure are summarized in table 1. As can be seen, the degree of purification of the (R-TA) is around 3000-fold, and twice this value if we consider that only half of the total receptor–steroid complex in cytosol is activated. The final yield is around 25% of total (R-TA), that means, 50% of the activated (R-TA). Analysis of the partially purified (R-TA) on polyacrylamide gels containing 0.1% SDS [17] showed an heterogeneous pattern with some bands corresponding to residual BSA (data not shown). The mol. wt. of the partially purified (R-TA) is 33 000 as determined by Sephadex gel filtration and sucrose gradient centrifugation. A detailed analysis of the physio-chemical properties of this (R-TA) will be published elsewhere (F. Climent and M. Beato, in preparation).

The partially purified (R-TA) was able to bind to rat liver chromatin and DNA in vitro (fig.2). The amount of (R-TA) bound per mg DNA was a linear function of the concentration of free (R-TA), and DNA showed a 10-fold higher binding capacity than chromatin [4]. A comparison with the results obtained using crude receptor preparations shows that the partially purified (R-TA) binds much more efficiently and is deprived of cytosol factors, which interfere with the nuclear binding of (R-TA) [4].

Of special importance was to prove that the partially purified receptor preparation does not contain deoxyribonuclease activity. Table 2 shows that a second chromatography on phosphocellulose before activation is essential in order to obtain a final preparation which does not degrade globin cDNA to TCA-soluble products. Analysis of the incubated cDNA on sucrose gradients gave no indication of endonuclease activity (data not shown).

4. Discussion

The purification procedure described in this paper represents a rapid and relatively simple technique for the preparation of large quantitatives of activated (R-TA). The final preparation, which can be obtained in 12–14 h, is free of deoxyribonucleases and binds efficiently to liver chromatin and DNA-cellulose [4]. Some aspects of the procedure deserve a comment.

The use of TA as the radioactive steroid offers certain advantages, as the complex of receptor and TA is much more stable than the complex of receptor with natural glucocorticoids or dexamethasone. The $t_{1/2}$ of the receptor–dexamethasone–complex is around 10 h at 0°C [18], whereas the receptor–TA complex exhibits at $t_{1/2}$ of several days at this temperature [4]. This slower rate of dissociation leads to a higher stability of the receptor resulting in better yields and making unnecessary a reincubation of the receptor with steroid after each purification step.

In our initial experiments we used DNA-cellulose instead of phosphocellulose throughout the purification procedure. Although DNA appears to be a more

Table 2
Deoxyribonuclease activity of partially purified (R-TA)

Sample	(R-TA) added	cDNA added	TCA-soluble cpm[b]
Cytosol	5 nM	50 000 cpm	21 000
(R-TA)[a]	5 nM	50 000 cpm	2850
(R-TA) final step	5 nM	50 000 cpm	910
BSA (1 mg/ml)	–	50 000 cpm	850
Buffer	–	50 000 cpm	760
Pancreatic DNase (50 ug/ml)	–	50 000 cpm	36 000

[a]This preparation was obtained as indicated in Materials and methods, but omitting the second phosphocellulose column.

[b]Incubation was at 25°C for 60 min, as indicated in Materials and methods.

physiological acceptor for the receptor, it has the disadvantage of being attacked by deoxyribonucleases present in crude cytosol; thus leading to partial release of DNA from the cellulose and poor retention of the activated (R-TA) in the last DNA-cellulose column.

Addition of BSA is necessary to prevent losses of the partially purified (R-TA) during column chromatography or during the charcoal assay. Added BSA can be partially removed by precipitation with 30% saturated ammonium sulphate, but still some BSA is present after the final purification step. This means, that our estimate purification represents a minimal figure. Assuming that 1 molecule of TA binds per molecule of receptor (mol. wt. 33 000), the final preparation is about 5% pure, and therefore still far for homogeneity. Nevertheless, the partial purification reported here may prove to be sufficient for the studies of the interaction of the (R-TA) with the cellular genome, as it is free of deoxyribonuclease and bind very efficiently to DNA and chromatin [4].

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