Heteromeric nature of glucocorticoid receptors

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The wild-type and a mutant receptor of S49.1 lymphoma cells have been shown by photoaffinity labelling to contain steroid-binding polypeptides of M_r 94000 and 40000, respectively. We investigated the molybdate-stabilized forms of these receptors and obtained M_r 325000 and 285000, respectively, by gel filtration and sedimentation analysis. Mild chymotrypsin treatment of the large wild-type receptor resulted in a form of about M_r 290000 which contained a steroid-binding polypeptide of M_r 40000. The data suggest that the high-M_r forms of glucocorticoid receptors are heteromeric in nature and contain one steroid-binding polypeptide per complex

Glucocorticoid receptor *Steroid-binding polypeptide* *Subunit structure*

1. INTRODUCTION

Glucocorticoid receptors are known to occur in different molecular forms. Receptors of responsive tissues were found by affinity labelling and analysis under denaturing conditions to contain a steroid-binding polypeptide of M_r 90000–95000 [1–6]. Under non-denaturing conditions, however, receptor forms as large as M_r 320000–340000 were detected [7–12] and it was postulated [10–13] that these are tetramers of either 4 steroid-binding polypeptides or, alternatively, contain 2 such steroid-binding polypeptides associated with 2 somehow related subunits. Here we addressed the problem of receptor subunit structure by comparing the high-M_r forms of wild-type receptors of S49.1 mouse lymphoma cells and a receptor mutant. The mutant receptor was of the nt^i ('increased nuclear transfer') type [14,15] and has been found by photoaffinity labelling and SDS gel electrophoresis to contain a steroid-binding polypeptide of only M_r 40000 [5].

2. MATERIALS AND METHODS

2.1. *Cell lines and cell culture*

The S49.1 mouse lymphoma sublines S49.1G.3 (wild-type) and S49.1TB.4.143R (nt^i type) were grown and harvested as in [5].

2.2. *Receptor complexes*

Cell extracts were prepared in 20 mM potassium phosphate (pH 7.4) containing 20 mM sodium molybdate, 2 mM mercaptoethanol, 1 mM EDTA, and 10% glycerol, and incubated with 40 nM [^{3}H]triamcinolone acetonide (New England Nuclear; 33 Ci/mmol) as in [5]. All procedures were carried out in the cold.

2.3. *Gel filtration*

A column of Sephacryl S-300 (Pharmacia) of 200–210 ml bed volume and 100–105 cm length was equilibrated with the above buffer. Receptor preparations containing about 500000 dpm specifically bound ^{3}H-labelled steroid in 1–2 ml were applied without removal of excess free steroid and the column was developed at a flow rate of 8 ml/h. Fractions were assayed for radioactivity. For calibration the following markers were used: Blue Dextran 2000 (void volume, V_o), thyroglobulin (R_S 86.1 Å), β-galactosidase (R_S 68.5 Å), catalase (R_S 52.3 Å), hemoglobin (R_S 32.1 Å), myoglobin (R_S 19.0 Å), and [^{3}H]valine (included volume, V_i). The distribution coefficient

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$K_D = (V_e - V_o)/V_i$ [16], where V_e is the elution volume of receptor complexes or marker proteins.

2.4. *Sedimentation analysis*

Following removal of excess free steroid by charcoal treatment, receptor preparations containing about 100000 dpm specifically bound ^{3}H-labelled hormone in 0.2 ml were applied onto 3.8 ml 16–41% linear glycerol gradients in the above buffer and spun in a Spinco SW 60 Ti rotor for 16 h at 47000 rpm. Fractions of about 100 μl were collected from the bottom of the tubes. Internal markers were β-galactosidase ($s_{20,w}$ 15.9 S), catalase ($s_{20,w}$ 11.3 S), aldolase from rabbit skeletal muscle ($s_{20,w}$ 7.9 S), and hemoglobin ($s_{20,w}$ 4.4 S).

2.5. *Chymotrypsin treatment*

Wild-type receptor complexes were submitted to a 10 min treatment with 10 μg/ml α-chymotrypsin (Serva, Heidelberg) in the cold followed by the addition of 100 μg/ml chymostatin (Sigma, St. Louis).

2.6. *Photoaffinity labelling and SDS gel electrophoresis*

The procedures were those previously described [5]. Gel slices of 2 mm were counted for tritium. Polypeptide markers were glycogen phosphorylase *b* (M_r 97400), bovine serum albumin (M_r 66100), carbonic anhydrase (M_r 29000), and soybean trypsin inhibitor (M_r 21500).

3. RESULTS AND DISCUSSION

Wild-type and mutant receptor complexes with tritiated triamcinolone acetonide were stabilized in their non-activated forms by the addition of sodium molybdate [17] and their hydrodynamic

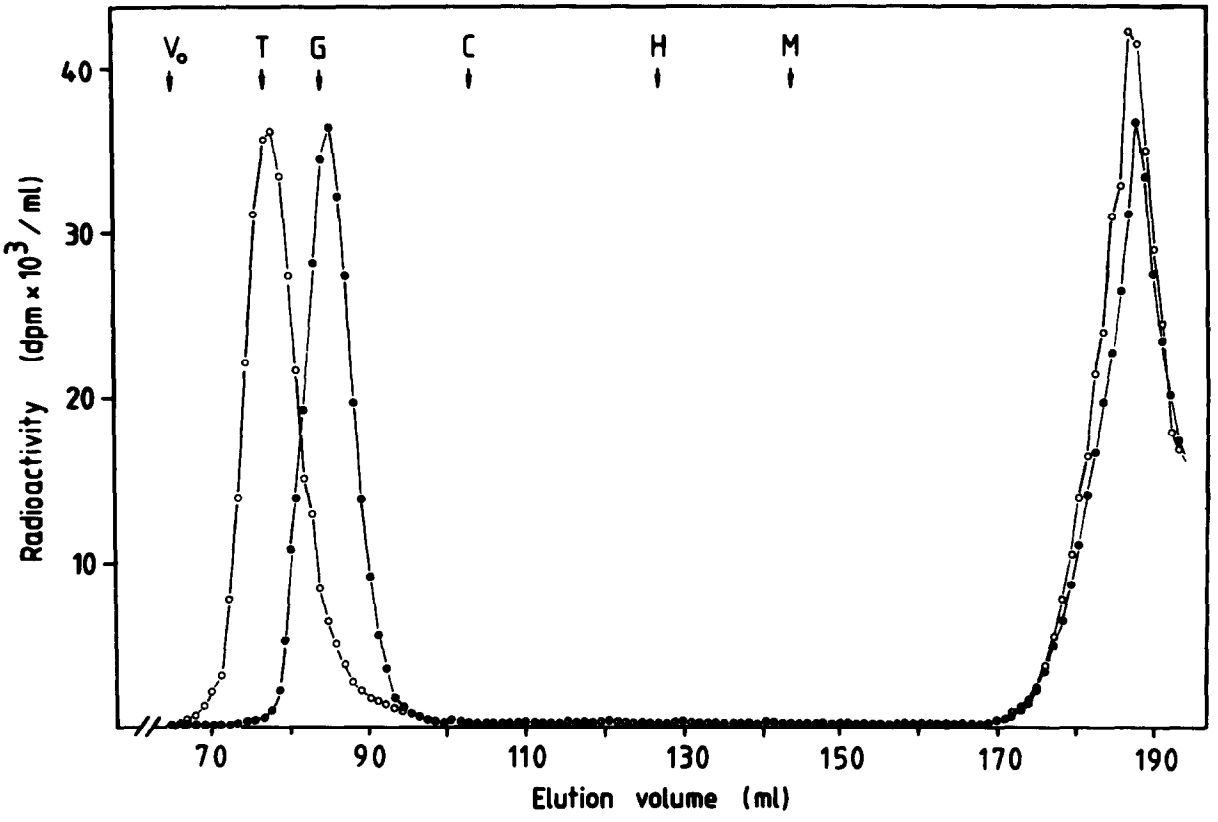

Fig.1. Gel filtration of glucocorticoid receptors. Wild-type (○) and mutant (●) receptor complexes were chromatographed on Sephacryl S-300. Protein markers were thyroglobulin (T), β-galactosidase (G), catalase (C), hemoglobin (H), and myoglobin (M). Stokes radii (R_S) of receptor complexes were determined from the linear correlation of K_D with log R_S [16].

properties were determined. Gel permeation chromatography of receptor complexes resulted in symmetrical and sharp peaks suggesting that unspecific aggregation is negligible. As shown in fig.1, wild-type receptors had a Stokes radius of 81 Å while the mutant receptors eluted at a markedly later position from the column corresponding to a Stokes radius of 71 Å. By contrast, there was no difference in the sedimentation behaviour of the two types of receptor complexes (fig.2) both sedimenting at a velocity of 9.5 S. The data are compiled in table 1. From the Stokes radii and sedimentation coefficients we computed M_r values of 325000 and 285000 for the wild-type and nt^i mutant receptors, respectively.

Thus we find a difference in molecular masses of roughly 50 kDa between wild-type and mutant receptors in both the high molecular mass receptor forms described here and the steroid-binding polypeptides [5]. We therefore suggest that the high-M_r receptors of both wild-type and mutant contain one steroid-binding subunit per complex. Accordingly, both the 94- and 40-kDa steroid-binding polypeptides are expected to be associated within the large complexes with non-hormone binding subunits totalling about 230–250 kDa in size.

The wild-type steroid binding polypeptide of M_r 94000 has previously been shown to harbour 3 distinct domains: one for steroid binding, another for interaction with nuclei, chromatin, or DNA, and a third domain that was found to function as a modulator for nuclear interaction [5,18] and to contain the main antigenic sites of the polypeptide [19–21]. The modulation domain is missing from nt^i mutant receptors and is easily cleaved off by proteolysis from the wild-type polypeptide of M_r 94000. We therefore submitted the large wild-type receptor complex of M_r 325000 to mild proteolysis.

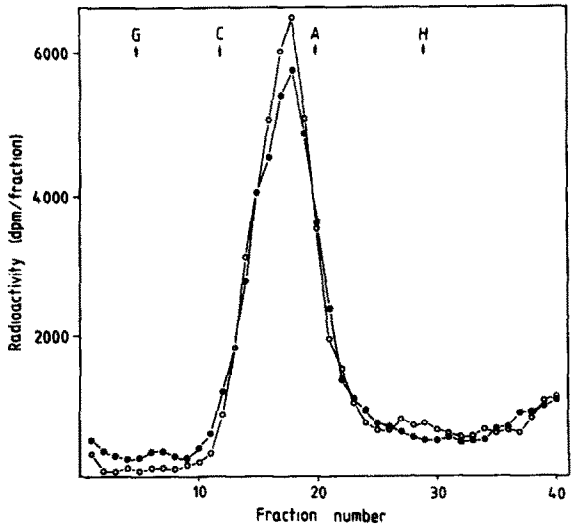

Fig.2. Sedimentation analysis of glucocorticoid receptors. Wild-type (○) and mutant (●) receptor complexes were sedimented in glycerol gradients. Protein markers were β-galactosidase (G), catalase (C), aldolase (A), and hemoglobin (H). Sedimentation coefficients of receptor complexes were determined relative to those of the markers.

Table 1

Molecular parameters of receptors

Receptor type (treatment)	R_S (Å)	$s_{20,w}$ (S)	M_r[a]	Axial ratio[a]	M_r of affinity labelled polypeptide
Wild-type (native)	81 ± 1	9.5 ± 0.3	325000	12	94000[b]
Wild-type (after chymotrypsin)	69 ± 2	10.0 ± 0.2	291000	9	39600 ± 3000
nt^i mutant (native)	71 ± 1	9.5 ± 0.5	285000	9	40000[b]

[a] M_r values and axial ratios were calculated from Stokes radii and sedimentation coefficients [16] assuming $\bar{V}$ = 0.732 ml/g [7]

[b] Data from [5]

Stokes radii and sedimentation coefficients were determined in 3–6 independent experiments; means ± SD are given

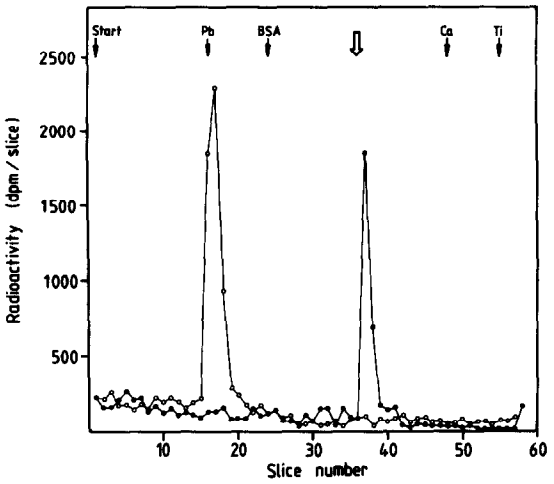

Fig.3. Photoaffinity labelling and SDS gel electrophoresis of wild-type receptors. Wild-type receptor complexes with (●) or without (○) chymotrypsin treatment as described in section 2 were subjected to photolabelling and SDS gel electrophoresis [5]. The position to which the photoaffinity labelled nt[1] receptor complex migrates is indicated by an open arrow. Polypeptide markers were phosphorylase *b* (Pb), bovine serum albumin (BSA), carbonic anhydrase (Ca), and trypsin inhibitor (Ti).

Following chymotrypsin treatment we recovered a receptor form with a Stokes radius of 69 Å and an M_r of 291000 (table 1). Under the conditions of this experiment the steroid binding polypeptide was indeed degraded to about 40 kDa as revealed by photoaffinity labelling and subsequent analysis in SDS polyacrylamide gels (fig.3, table 1). This suggests that the large size complexes of wild-type and mutant receptors indeed contain the same non-steroid binding subunits. While the cleaved-off fragment of the 94-kDa polypeptide is released from the complex the remainder stays associated suggesting that the large wild-type receptor complex is held together by the 40-kDa moiety of the steroid-binding subunit. Within the large complex the wild-type steroid-binding subunit appears to be rather exposed so that it is accessible to enzymatic cleavage. By contrast, the additional subunits of the large receptor complex are less susceptible to proteolysis.

The experiments described here suggest a structure for the high-M_r form of glucocorticoid receptors in which one steroid-binding polypeptide is associated with other subunits. The biochemical identities and the biological functions of these associated components remain unknown at present. They may be mere packaging material or may be involved in receptor activation to a form that interacts with the cellular genome and hence specifically affects gene expression. This question is of particular interest in view of a recent report [22] which describes the immunochemical detection of a non-hormone binding polypeptide common to the high-M_r forms of 4 different steroid hormone receptors including glucocorticoid receptors.

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