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Tetrameric structure of the nonactivated glucocorticoid receptor in cell extracts and intact cells

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Mouse lymphoma cells contain a nonactivated glucocorticoid receptor of $M_r \sim 330000$ which is heteromeric in nature and is unable to bind to DNA. Following affinity labeling of the steroid-binding subunit and subsequent cross-linking with dimethyl suberimidate at various times either in cell extracts or in intact cells, a series of labeled bands was detected in SDS gels. From the molecular masses of completely and partially cross-linked complexes we conclude that the large nonactivated receptor is a tetramer composed of two 90 kDa subunits, one 50 kDa polypeptide and one steroid-binding subunit.

Chemical crosslinking; Dexamethasone mesylate; Dimethyl suberimidate; Glucocorticoid receptor; Subunit structure

1. INTRODUCTION

The physiological effects of steroid hormones are mediated by intracellular receptors which may exist in different molecular forms in extracts of target cells (reviews [1,2]). During recent years it became clear that high molecular mass glucocorticoid receptors are unable to interact with DNA or chromatin but become activated to a DNA-binding state upon warming or exposure to high ionic strength: this process involves subunit dissociation [3–6]. The large receptor form of $M_r \sim 330000$ has recently been shown by chemical cross-linking to exist not only in cell extracts but also in intact cells [6]. It contains only one hormone-binding polypeptide of $M_r \sim 100000$ per complex [7,8]. We now know that the heat shock protein hsp90 is a constituent of the heteromeric glucocorticoid receptor structure [4,6,9,10]: it may be present as a dimer [5,11,12]. The involvement of other molecular components is less clear. A polypeptide of M_r 59000 was detected in association with several steroid hormone receptors [13]. RNA has also been envisaged as part of large glucocorticoid receptors (review [14]). In the present study we investigated high molecular mass glucocorticoid receptors in terms of numbers of macromolecular subunits and used cross-linking with a bifunctional reagent that allows subsequent analysis in SDS gels. The detection of several intermediate forms provides compelling evidence for the high molecular mass receptor being a hetero-tetramer.

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Abbreviations: DMS, dimethyl suberimidate; nt^i, receptor of 'increased nuclear transfer'; PAGE, polyacrylamide gel electrophoresis

2. MATERIALS AND METHODS

2.1. *Cell culture and cell extracts*

The S49.1 mouse lymphoma sublines S49.1G.3 (wild-type) and S49.1TB.4.143R (nt^i mutant) were those previously used [15]. Cells were grown and harvested as described [5] and cell pellets were stored frozen. Extracts were prepared from frozen cells [6] and were incubated with 100 nM [^{3}H]dexamethasone mesylate (NEN Research Products; 1.8 TBq/mmol) for 2.5 h at 0°C. Extracts of wild-type cells were then used for cross-linking while those of nt^i cells were first submitted to gel filtration on Sephacryl S-300 in a buffer containing 20 mM sodium molybdate and 150 mM KCl [5]. Peak fractions corresponding to 70 Å Stokes radius were combined and submitted to cross-

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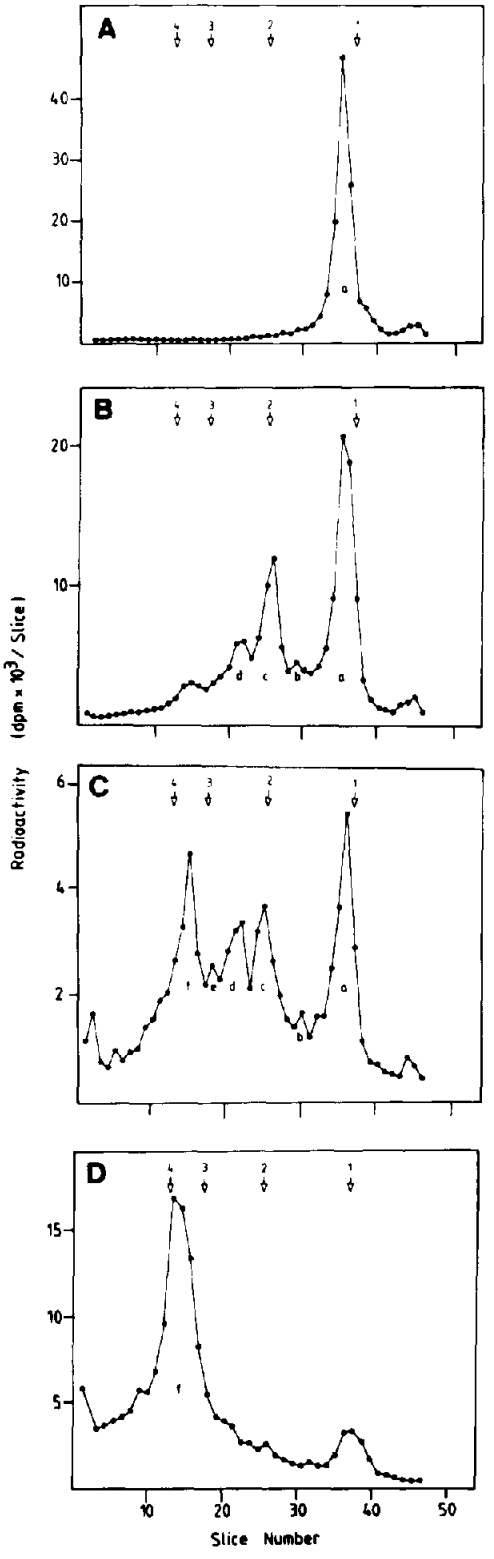


linking. Intact cells were incubated with labeled hormone as previously described [6] except that dexamethasone mesylate was used, extracts were prepared subsequently to cross-linking and extensive washing of cells.

2.2. *Chemical cross-linking*

Cross-linking with DMS (Pierce Chemical Co.) was carried out according to a protocol described by Aránvi et al. [16]. Reactions were at 6–8°C for the times given. Intact cells were treated in the same way except that cell suspensions were adjusted to pH 8.0 by KOH. Wild-type receptors were subsequently purified on Sepharose to which the receptor specific antibody mab 49 had been coupled [6]. The eluate with sodium thiocyanate was precipitated with trichloroacetic acid and prepared for SDS-PAGE [6]. Cross-linked nt^i-receptor fractions were passed over Sephadex G-25 in order to remove excess reagent as well as molybdate prior to precipitation with trichloroacetic acid.

2.3. *SDS-PAGE*

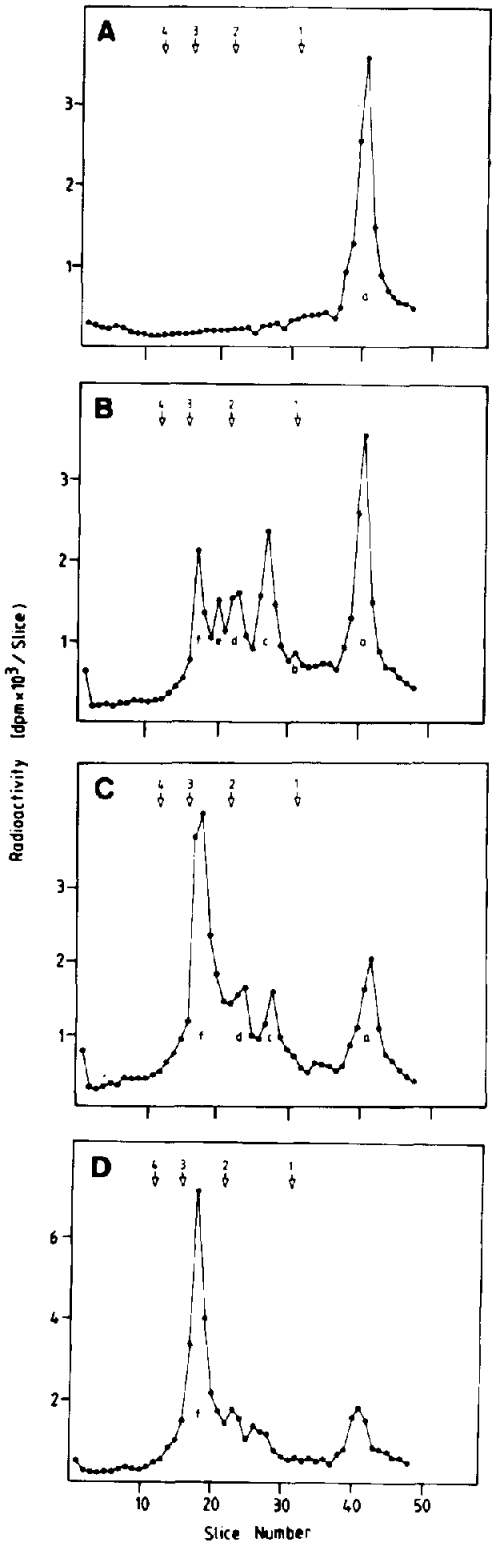
We used polyacrylamide slab gels (1.5 mm thickness) in a continuous buffer system [17]. Gels were prepared from 3.3% acrylamide and 0.11% methylenebisacrylamide in buffer (50 mM Tris phosphate, pH 7.2, 0.1% SDS) and run at a constant voltage of 100 V. Samples of 40 μl were applied to slots of 1.5 cm. As markers we used rabbit muscle phosphorylase *a* (subunit M_r 97400) which had been incompletely cross-linked with DMS. Gels were stained with Coomassie and dried onto filter paper. Routinely, slices of 2 mm were taken, dissolved in 30% H_2O_2, and radioactivity measured by liquid scintillation counting; in some experiments the gels were cut in slices of about 600 μm.

3. RESULTS

3.1. *Cross-linking in extracts of wild-type cells*

The hormone-binding polypeptide of large receptor complexes was labeled covalently with the steroid [^{3}H]dexamethasone mesylate which is known to affinity label glucocorticoid receptors with high yield [18]. Since, however, other cellular material is non-specifically labeled with this reagent [19], a receptor specific purification procedure was necessary. For the wild-type receptor we used immunoaffinity chromatography with the monoclonal antibody mab 49 which recognizes a domain of the steroid-binding polypeptide that

Fig.1. SDS-PAGE of wild-type receptors cross-linked in cell extracts. Cross-linking was for 0 (A), 20 (B), 40 (C) and 60 (D) min. Gels were run for 3.5 h. Multimers of the phosphorylase *a* subunit are indicated by arrows (1–4).



does not participate in cross-linking [6] and is missing from the nt^{i} mutant receptor [20]. Fig.1 shows a series of SDS gels in which wild-type receptors were cross-linked with DMS for various lengths of time. Starting out with a single labeled peak of M_r ~100000 (fig.1A) increasingly complex patterns were obtained which finally shifted towards a major species of M_r ~350000 (fig.1D). This corresponds to the fully cross-linked receptor previously obtained with other methods [6]. A total of 6 peaks was observed (table 1). Even though forms b and e were less prominent than the other intermediate species c and d they were consistently seen: they became particularly obvious when gels were cut into 600 μm slices (not shown).

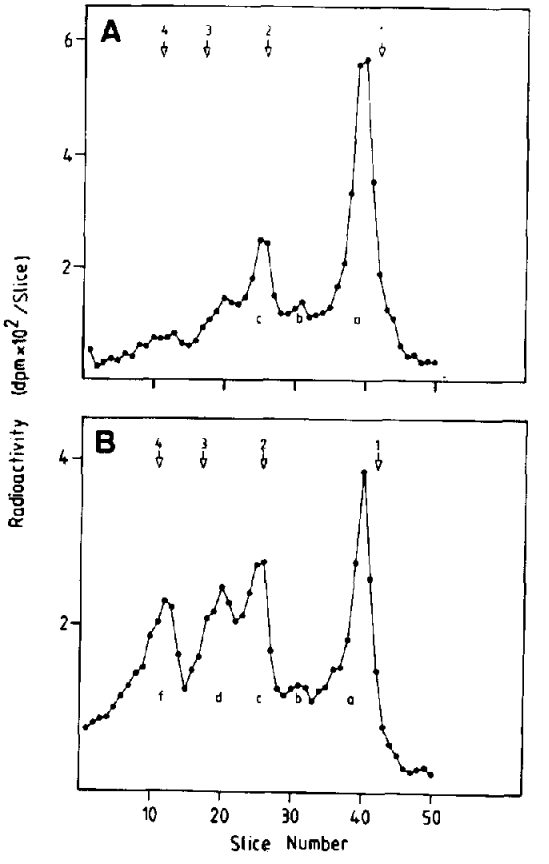


Fig.3. SDS-PAGE of wild-type receptors cross-linked in intact cells. Cross-linking was for 30 (A) and 90 (B) min; other details as in fig.1.

Fig.2. SDS-PAGE of nt^{i} receptors cross-linked in cell extracts. Symbols as in fig.1. Gels were run for 2.5 h.

Table 1

Cross-linking of receptors

Receptor type	Molecular masses of labeled receptor species					
	a	b	c	d	e	f
Wild-type	104000 ± 5000 (11)	149000 ± 5000 (4)	194000 ± 10000 (9)	241000 ± 9000 (6)	301000 ± 12000 (5)	349000 ± 8000 (6)
nt^i mutant	51000 ± 4000 (8)	95000 ± 4000 (5)	136000 ± 7000 (8)	178000 ± 7000 (7)	214000 ± 6000 (2)	264000 ± 10000 (7)

Molecular masses were determined in SDS gels. Mean values and ranges are reported (number of experiments in parentheses)

3.2. *Cross-linking in extracts of nt^i mutant cells*

In order to confirm the above data we carried out similar experiments with the nt^i receptor which has a glucocorticoid-binding polypeptide of about half the wild-type size [15] and a high molecular mass form of about 290 kDa [5–7]. This receptor form was separated from nonspecifically labeled material by gel filtration in the presence of molybdate [5]. As shown in fig.2 we again observed a series of 6 labeled receptor species. The M_r data are summarized in table 1.

3.3. *Receptor cross-linking in intact cells*

In other experiments we incubated wild-type cells in the cold with the affinity label and subsequently with DMS. Cells were broken and receptors were immunoaffinity purified and subjected to SDS-PAGE. Fig.3 shows that cross-linking is less efficient than in cell extracts, however, the same labeled receptor species were observed.

4. DISCUSSION

In previous experiments we achieved chemical cross-linking between receptor subunits by using bifunctional *N*-hydroxysuccinimide esters [6]. These compounds, however, give rise to covalent links which are unstable under the conditions commonly used for SDS-PAGE. We therefore now turned to bis-imidates like DMS which produce more stable amidine cross-links. With both wild-type and nt^i receptors we obtained in SDS gels sets of 6 labeled receptor species with varying yields depending largely on the extent of cross-linking. The molecular mass data, compiled in table 1, strongly suggest a tetrameric structure for the high molecular mass forms of both receptor types. Table 2 presents our interpretation of the data. We conclude that the large receptors contain two subunits of M_r 90000 and one polypeptide of M_r ~50000 in association with one steroid-binding subunit of either M_r ~100000 (wild-type) or 50000 (nt^i).

As to the identity of the 90 kDa subunits it is clear that hsp90 is at least one of these components. In fact, Mendel and Orti [12] recently provided evidence for a roughly 1:2 ratio of steroid-binding subunit to hsp90. In contrast, the identity of the subunit of M_r ~50000 is still unknown. Since the cross-linker DMS has a high

Table 2

Interpretation of M_r data

Wild-type	nt^i mutant type
a M_r ~105000: R	M_r ~50000: R
b M_r ~150000: R + p50	M_r ~95000: R + p50
c M_r ~195000: R + p90	M_r ~135000: R + p90
d M_r ~240000: R + p90 + p50	M_r ~180000: R + p90 + p50
e M_r ~300000: R + p90 + p90	M_r ~215000: R + p90 + p90
f M_r ~350000: R + p90 + p90 + p50	M_r ~265000: R + p90 + p90 + p50

R refers to the steroid-binding polypeptides of M_r ~100000 (wild-type) and 50000 (nt^i); p90 and p50 refer to associated polypeptides of M_r 90000 and 50000, respectively. The letters a to f relate to the labeled peaks in figs 1–3

degree of selectivity for amino groups in proteins [21] we assume that all four receptor subunits are of polypeptide nature.

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