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Steroid Receptors and DNA Interaction

SUBUNIT STRUCTURE OF THE GLUCOCORTICOID RECEPTOR AND ACTIVATION TO THE DNA-BINDING STATE

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Summary—Glucocorticoid receptors of S49.1 mouse lymphoma cells were analyzed under a variety of conditions. The complexes with an agonist or a steroidal antagonist can be formed in cytosolic extracts, they are of high molecular weight, $M_r \sim 330,000$ and have a Stokes radius of 82 Å. Cross-linking by several agents stabilized this structure against subunit dissociation which produces the activated receptor form of 60 Å and DNA-binding ability. Careful analysis of intermediate cross-linked forms lead to the conclusion that the large receptor structure is a hetero-tetramer consisting of one hormone-bearing polypeptide of $M_r \sim 94,000$, two 90 kDa subunits and a protein component of $M_r \sim 50,000$. The 90 kDa subunits are the heat shock protein hsp90. The high molecular weight receptor form also exists in intact cells as revealed again by cross-linking. The cytosolic complex with the antagonist can become activated to the DNA-binding form upon warming but simultaneously looses the ligand. Ligand rebinding does not occur subsequent to receptor dissociation. Upon incubation of intact cells at 37°C with agonist or antagonist the respective receptor–ligand complexes are formed. The agonist complex is immediately activated, however, the antagonist complex remains stable in the undissociated state. This explains the biological effect of the antagonist.

INTRODUCTION

The molecular actions of glucocorticoids and other steroid hormones depend on specific receptors which are contained within target cells and which function as intracellular mediators. The pivotal role of the glucocorticoid receptor became particularly obvious by the *in vitro* isolation of a large series of resistant cell variants from glucocorticoid responsive lymphoid cells in culture [1, 2]. All of these variants harbour mutations in the receptor polypeptide and some of the receptor mutants became very useful tools for the elucidation of receptor actions. The glucocorticoid receptor has been cloned from several mammalian species [3–5] and was found to contain about 780 amino acids in a polypeptide chain of $M_r \sim 94,000$. This receptor polypeptide comprises several functional domains in a linear arrangement [1, 2, 6–8], one of which is involved in binding the hormonal ligand, and another in interacting with specific response elements on the DNA.

Within target cells or in extracts of such cells the glucocorticoid receptor may exist in different molecular forms. One of these is of high molecular weight, $M_r \sim 330,000$ and consists of several subunits. It has been shown to contain only one hormone-binding subunit [9, 10], the receptor polypeptide mentioned above. It is important to note that this receptor form does bind the hormone but is unable to interact with DNA or chromatin. Activation to the DNA-binding state occurs *in vivo* under physiological conditions subsequent to steroid binding [11]. In cell extracts activation can be brought about by various treatments, most notably by exposure to high ionic strength or to increased temperature. During recent years it became clear that activation of the receptor–hormone complex to DNA-binding ability occurs concomitantly with dissociation of the heteromeric receptor structure of $M_r \sim 330,000$ [12–14]. The steroid-bearing receptor polypeptide is thus released. This dissociated receptor may then translocate to the nucleus and specific nuclear localization signals

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Trivial names: triamcinolone acetonide, 9α-fluoro-11β,16α, 17α,21-tetrahydroxypregna-1,4-diene-3,20-dione cyclic 16,17-ketal with acetone; RU 38486, 17β-hydroxy-11β-(4-dimethylaminophenyl)-17α-(1-propynyl)-estra-4,9-diene-3-one.

have been detected within the glucocorticoid receptor polypeptide which require the presence of hormone in order to be activated [15]. Within the nucleus, the activated receptor may have to dimerize in order to specifically interact with the appropriate contact points on the DNA.

Here we summarize our recent studies on the subunit structure of the unactivated, high molecular weight glucocorticoid receptor. In particular, we show that this receptor form pre-exists in intact cells. It is a heterotetramer consisting of three different types of subunits. We also present experiments which show that the steroidal ligand profoundly influences the ability of the receptor structure to become activated.

COVALENT CROSS-LINKING STABILIZES THE HIGH MOLECULAR WEIGHT RECEPTOR STRUCTURE

If receptor dissociation and activation to DNA-binding are indeed tightly coupled events it should be possible to inhibit activation by preventing dissociation through chemical cross-linking of receptor subunits. This has been achieved by use of several reagents [16]. Bi-functional *N*-hydroxysuccinimidyl-esters and bisimidates, both of which react with ϵ-amino-groups of lysine residues, turned out to be particularly useful [17]. In addition, sulfhydryl groups in protein subunits can be linked to each other by mild oxidation with atmospheric oxygen in conjunction with the complex of cupric ions with *o*-phenanthroline [18].

Figure 1 shows an experiment in which the glucocorticoid receptor of wild-type S49.1 mouse lymphoma cells was complexed with radiolabeled triamcinolone acetonide and subsequently reacted with dithiobis(succinimidyl propionate). Gel filtration on Sephacryl S-300 was used at high ionic strength in order to analyze the products under conditions which favour dissociation. Two separate peaks of 82 and 60 Å

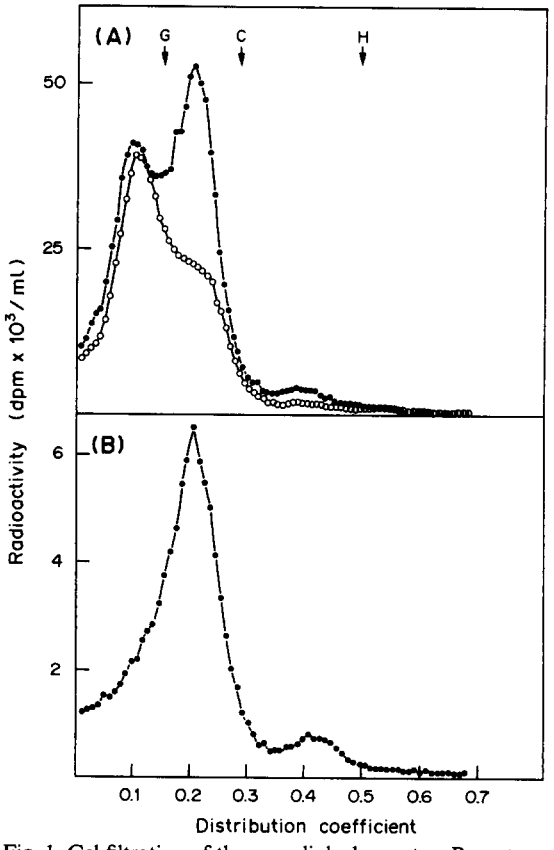

Fig. 1. Gel filtration of the cross-linked receptor. Receptors contained in a cytosolic extract of S49.1 cells were complexed with [^{3}H]triamcinolone acetonide for 2 h at 0°C, excess unbound hormone was removed with charcoal and the complex was treated with dithiobis (succinimidyl propionate) for 30 min at 0°C. Cross-linking was stopped by the addition of excess lysing. The material was chromatographed on Sephacryl S-300 in 300 mM KCl either directly (A, ●) or after filtration through DNA-cellulose (○). In B, the high molecular weight complex was isolated by DEAE-cellulose chromatography, incubated over night with mercaptoethanol and submitted to gel filtration. Marker proteins were E. coli β-galactosidase (G; $R_s = 68.5$ Å), bovine liver catalase (C; $R_s = 52.3$ Å), and human hemoglobin (H; $R_s = 32.1$ Å). (Data from Ref. [16])

Stokes radii, respectively, were obtained. The 60 Å form corresponds to the activated receptor as demonstrated by the fact that prior filtration through DNA-cellulose removed most of this

Table 1. Cross-linking and cleavage of the high molecular weight receptor complex

	Treatment	Stokes radius (Å)	Molecular weight (M_r)	DNA-binding
Control	—	80.6[a]	328,000[a]	–
	—	59.6	116,000	+
Cross-linking in cell extracts				
	Dithiobis (succinimidyl propionate)	82.3	316,000	–
	Dithiobis (succinimidyl propionate), cleavage	60.2	~120,000	+
	Cu^{++}/*o*-phenanthroline	80.2	335,000	–
	Cu^{++}/*o*-phenanthroline, cleavage	61.6	~125,000	+
	Diethylpyrocarbonate	80.9	~330,000	–
Cross-linking in intact cells				
	Dithiobis (succinimidyl propionate)	81.2	~320,000	
	Dithiobis (succinimidyl propionate), cleavage	60.0	~120,000	

Analysis was in the presence of 300 mM KCl except in [a].

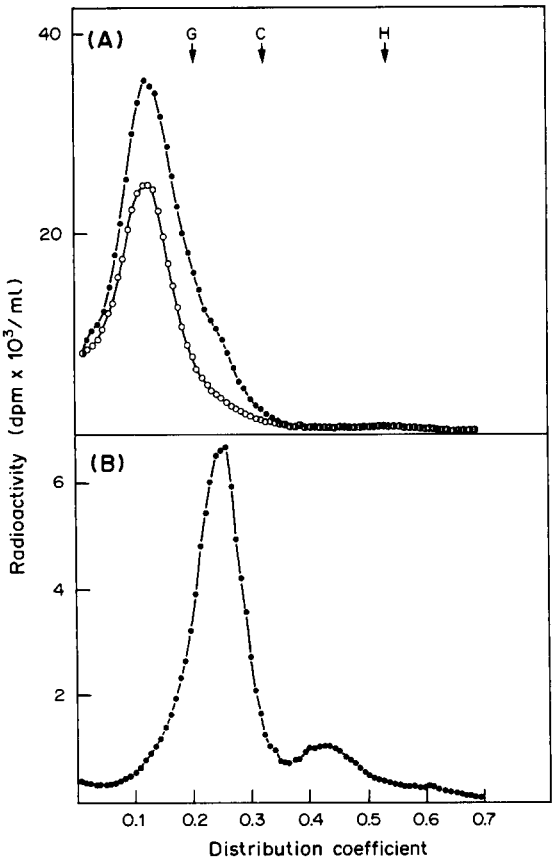

Fig. 2. Gel filtration of the oxidized receptor. Cytosolic S49.1 receptor complexes with [^{3}H]triamcinolone acetonide were treated with 0.25 mM $CuSO_4$ and 1.3 mM *o*-phenanthroline for 30 min at 20°C. The material was chromatographed as in Fig. 1. A: gel filtration either directly (●) or after filtration through DNA-cellulose (○); and B: gel filtration after cleavage with mercaptoethanol. (Data from Ref. [16])

material [Fig. 1(A), ○]. By contrast, the 82 Å material is the undissociated high molecular weight structure of $M_r \sim 330{,}000$ (Table 1). In the experiment of Fig. 1 this material is unable to dissociate by salt treatment due to cross-linking and is consequently unable to adsorb to DNA-cellulose. Cross-linking with dithiobis(succinimidyl propionate) has the advantage that the molecule bridging the receptor subunits can subsequently be cleaved by reduction with mercaptoethanol. As shown in Fig. 1(B) this treatment of the previously stabilized 82 Å receptor form again allowed dissociation by high salt to yield the receptor of 60 Å Stokes radius. This clearly shows that covalent cross-linking of the subunits within the large receptor structure prevents activation to DNA-binding by solely blocking dissociation. Subsequent cleavage of the cross-links again permits receptor dissociation and activation.

Similar experiments were also carried out with the high molecular weight complex of a receptor mutant of S49.1 cells. In this particular mutant, called nt^i, the receptor polypeptide chain is truncated in the amino terminal half of the molecule but still contains the hormone-binding and DNA-binding domains perfectly intact [1, 19]. The high molecular weight form of the nt^i receptor has a Stokes radius of ~72 Å; it dissociates to the activated DNA-binding form of ~38 Å [13]. When we exposed the unactivated nt^i receptor to the bifunctional agent dithiobis-(succinimidyl propionate) we similarly obtained cross-linking and hence stabilization of the high molecular weight structure [16]. Again reductive cleavage of the cross-links produced the lower molecular weight form of 38 Å Stokes radius and DNA-binding ability.

Cross-linking of receptor subunits was also achieved by introducing disulfide bridges. This is shown in Fig. 2. In this case we easily obtained complete cross-linking of the wild-type receptor complex in its high molecular weight state as revealed by the fact that only the 82 Å peak was obtained [Fig. 2(A)]. Subsequent treatment with mercaptoethanol again resulted in cleavage and thus allowed subunit dissociation at high ionic strength to produce the 60 Å receptor form [Fig. 2(B)]. These cross-linking and cleavage experiments are summarized in Table 1. Similar data were also obtained for the nt^i receptor mutant [16].

PROGRESSIVE CROSS-LINKING OF THE HIGH MOLECULAR WEIGHT RECEPTOR

In an alternative approach we used dimethyl suberimidate as the bifunctional cross-linking reagent and SDS polyacrylamide gel electrophoresis as the analytical technique. For detection of the cross-linking products we used immunoblotting with a monoclonal antibody which specifically detects the glucocorticoid receptor polypeptide [20, 21]. Figure 3 shows that treatment with the cross-linker for various lengths of time resulted in progressive cross-linking. Starting out with a major band of $M_r \sim 100{,}000$, the receptor polypeptide itself, higher molecular weight bands were obtained which finally shifted towards a major species of ~330,000 Da (Fig. 3, lane 5). This corresponds to the fully cross-linked receptor described above. Two intermediate immunoreactive bands of $M_r \sim 200{,}000$ and 245,000 were clearly detected. As this method, however, produced relatively diffuse signals we decided to try a different approach using radiochemical rather than immunochemical detection of the cross-linking products [22].

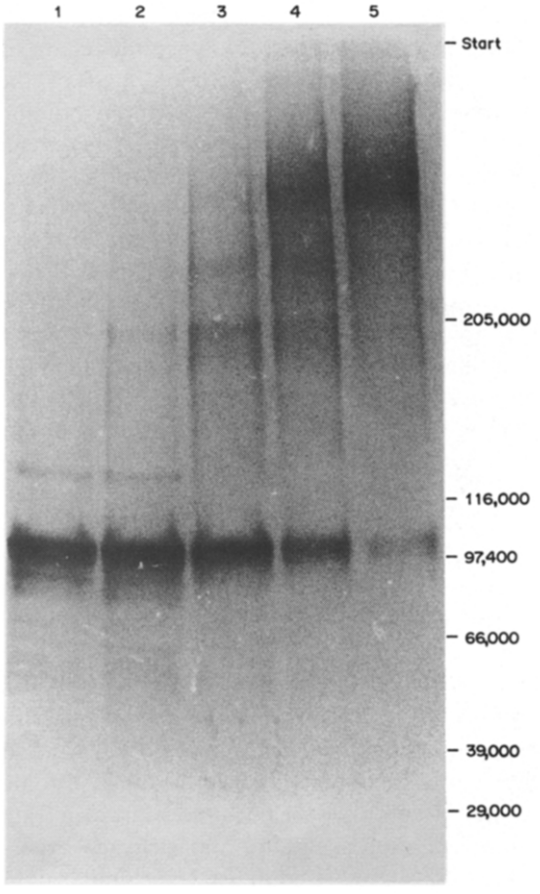

Fig. 3. Immunoblot of the progressively cross-linked receptor. Cytosolic S49.1 receptor complexes with triamcinolone acetonide were treated for 0 (lane 1), 5 (lane 2), 15 (lane 3) 30 (lane 4) and 45 (lane 5) min with 20 mM dimethyl suberimidate in the cold. Samples were subjected to immunoaffinity purification [16] and run on a 4 to 8% gradient polyacrylamide gel. Proteins were transferred to an Immobilon PVDF membrane and analyzed with receptor-specific monoclonal antibody mab49 [16]. Molecular weight markers were rabbit myosine heavy chain (205,000), E. coli β-galactosidase (116,000), rabbit muscle phosphorylase b (97,400), bovine serum albumin (66,000), rabbit muscle aldolase (39,000) and bovine carbonic anhydrase (29,000).

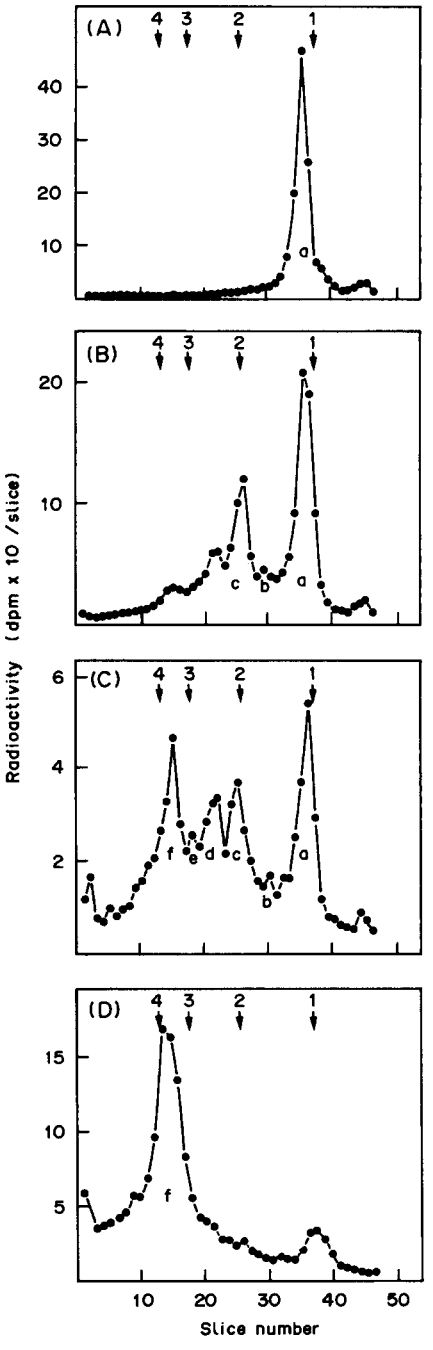

Fig. 4. SDS gel electrophoresis of the progressively cross-linked receptor. Cytosolic S49.1 receptor complexes with [^{3}H]dexamethasone mesylate were treated for 0 (A), 20 (B), 40 (C) and 60 (D) min with 20 mM dimethyl suberimidate in the cold, subjected to immunoaffinity purification and analyzed on 3.3% polyacrylamide gels. Gels were sliced and analyzed for radioactivity. Multimers of the rabbit muscle phosphorylase a subunit (97,400) are indicated by arrows. (Data from Ref. [22])

For this purpose the receptor polypeptide was tagged by covalent affinity labeling with radioactive dexamethasone mesylate. Figure 4 again shows progressive cross-linking with dimethyl suberimidate. A total of six radiolabeled peaks were consistently observed. In addition to the fully cross-linked material of ~350 kDa (peak **f**) we detected four intermediate forms of ~150 kDa (peak **b**), ~195 kDa (peak **c**), ~240 kDa (peak **d**), and ~300 kDa (peak **e**). Peaks **c** and **d** clearly correspond to the intermediate bands detected in the immunoblot of Fig. 3 while peaks **b** and **e** are clearly minor species and therefore not seen in the immunoblot.

It is important to emphasize that a total of four intermediate cross-linked forms were detected. This demonstrates directly that the large receptor structure is not trimeric in composition. We rather have to conclude that it is a hetero-tetramer which contains one steroid-binding polypeptide of M_r ~ 100,000 associated

with two very similar subunits of M_r 90,000 and one additional component of ~50,000 Da. The cross-linked receptor forms of intermediate sizes correspond to all combinations of the receptor polypeptide chain R with the other subunits, i.e. R + 50 kDa (peak **b**), R + 90 kDa (peak **c**), R + 90 kDa + 50 kDa (peak **d**), and R + 2 × 90 kDa (peak **e**).

Similar results were also obtained upon progressive cross-linking of the high molecular weight nt^i receptor complex with dimethyl suberimidate. Again a total of six radiolabeled peaks were seen on SDS gels [22]. In these experiments the bands were shifted by about 50 kDa towards lower molecular weights corresponding to the lower molecular weight of the nt^i receptor polypeptide itself.

THE HIGH MOLECULAR WEIGHT RECEPTOR FORM EXISTS IN INTACT CELLS

The above described experiments for characterizing the high molecular weight receptor structure were carried out with receptor–steroid complexes contained in cell extracts. This raised the possibility that the receptor polypeptide might associate artifactually with other cellular components as a consequence of cell homogenization. We, therefore, carried out cross-linking experiments with intact cells in order to find out whether the high molecular weight structure preexists in these cells. To this end wild-type S49.1 lymphoma cells were pretreated with radiolabeled triamcinolone acetonide in the cold in order to avoid receptor activation. The cells were then exposed to the cross-linking reagent and after extensive washing they were ruptured. The cell extract was analyzed by gel filtration. Figure 5 shows an experiment with dithiobis-(succinimidyl propionate). Clearly, the major portion of the receptor complex was stabilized in the 82 Å form and was thus unable to dissociate in the presence of high salt concentrations. Subsequent reductive cleavage again allowed receptor dissociation. These experiments are also summarized in Table 1. Corresponding *in vitro* cross-linking was similarly observed when we used mutant S49.1 cells of the nt^i type [16].

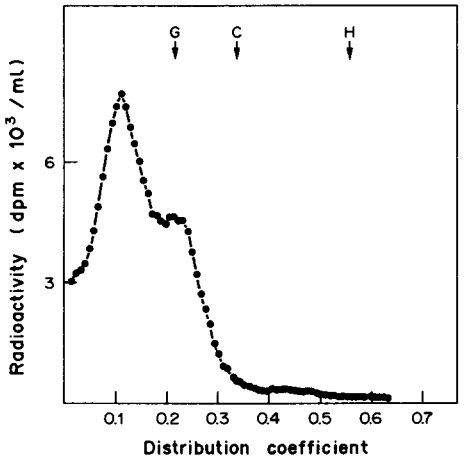

Fig. 5. Gel filtration of the receptor cross-linked in intact cells. Intact S49.1 cells were incubated with [^{3}H]triamcinolone acetonide for 1 h in the cold, subsequently treated with diethiobis(succinimidyl propionate) for another 1 h and then washed with saline. Cells were ruptured and the extract submitted to gel filtration as in Fig. 1. (Data from Ref [16])

In other experiments we used intact cells and exposed them to the cross-linking reagent dimethyl suberimidate. Again intermediate forms similar to those depicted in Fig. 4 were obtained [22].

Taken together these data prove that the heteromeric M_r ~ 330,000 receptor–steroid complex certainly preexists in intact cells subsequent to hormone addition. This, however, still leaves open possible questions about the effects of the hormonal ligand on the formation of this complex structure and on its subunit dissociation. Indeed, the 82 Å receptor does exist in the absence of steroid. This was shown by passing a steroid-free extract of S49.1 cells through Sephacryl S-300 under low salt conditions and fractionating the effluent according to the positions of the marker proteins. The 82 and 60 Å regions were then pooled, run on an SDS gel and the immunoblot was stained with the anti-receptor antibody mentioned above. As shown in Fig. 6 only the 82 Å region gave a positive signal. In another experiment we exposed intact cells in the absence of steroid to dithiobis(succinimidyl propionate), chromatographed the cell extract on Sephacryl S-300 in high salt and again detected the immunochemical signal only in the 82 Å region of the effluent. This clearly shows that the high molecular weight heteromeric receptor structure preexists in intact cells, even in the absence of hormone.

COMPONENTS OF THE HIGH MOLECULAR WEIGHT RECEPTOR

There is ample evidence in the literature for the heat shock protein hsp90 being one of the components of the high molecular weight glucocorticoid receptor [14, 16, 23–27]. We produced an antiserum directed against hsp90 from mouse lymphoma cells and used it for the detection of

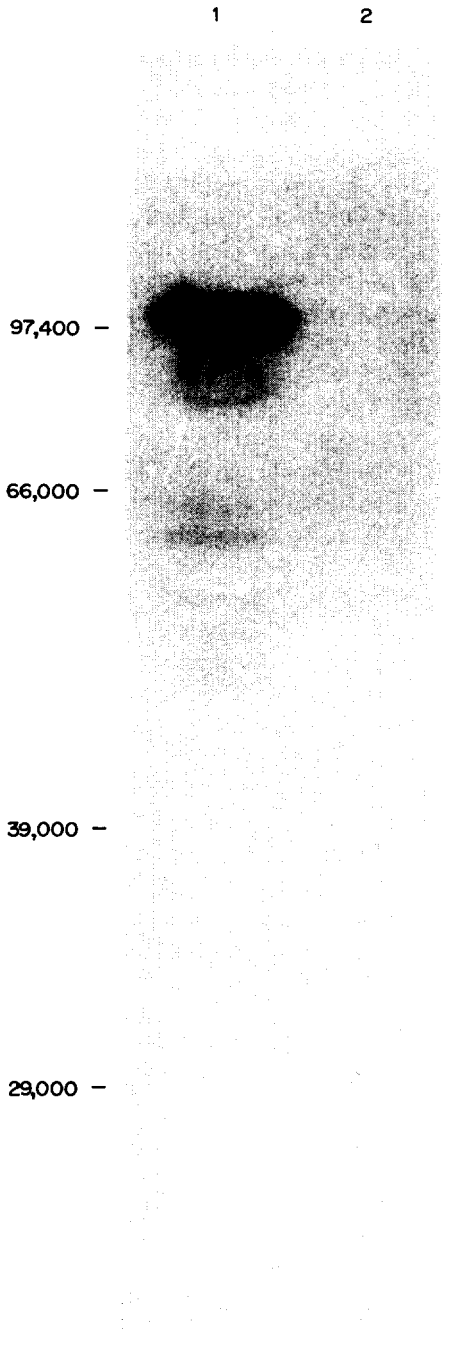

Fig. 6. Gel filtration and immunoblot of the unliganded receptor. Cytosolic extract of S49.1 cells was submitted to gel filtration as in Fig. 1 in the presence of 20 mM sodium molybdate. The fractions corresponding to the 82 Å (lane 1) and 60 Å (lane 2) regions were pooled, concentrated by immunoaffinity chromatography, subjected to SDS gel electrophoresis in a 10% gel, and analyzed by immunoblotting with the receptor specific monoclonal antibody [16]. Markers were as in Fig. 3.

hsp90 in cross-linked receptor complexes [16]. Since hsp90 is a very abundant protein, we had to highly purify the receptor. This was achieved by immunoaffinity chromatography with the receptor-specific monoclonal antibody. In the experiment of Fig. 7 the receptor structure was either cross-linked by dithiobis(succinimidyl propionate) in cell extracts or in intact S49.1 cells. In both instances a strong signal at 90,000 Da was obtained in the immunoblot. Omitting the cross-linker resulted in practically no hsp90 signal even though the receptor signal was as strong as in the other samples (Fig. 7, lane 5 vs 1). This is due to the fact that the antibody column was thoroughly washed with high salt buffers in order to remove the large excess of non-receptor associated hsp90 as well as hsp90 not covalently linked to the receptor polypeptide. Figure 7 (lane 8 vs 4) also shows that the high molecular weight receptor stabilized by disulfide bridges likewise contains hsp90 as a structural component.

These data, taken together with the above described results obtained by incomplete cross-linking with dimethyl suberimidate, unequivocally show that two molecules of hsp90 are contained in the large receptor structure of $M_r \sim 330{,}000$. This conclusion has also been arrived at by others using a completely different approach [26]. The 330 kDa receptor structure may, however, be contained in cytosol in a much larger complex in association with further hsp90 molecules and other cellular components [28].

As to the identity of the additional receptor subunit of $M_r \sim 50{,}000$ the situation is far less clear at present. We need to mention at this point that the molecular weight of this component as determined by a subtractive method (see above) is certainly only a rough estimate. Data in the literature point to two completely different structural components: RNA of the approximate size of tRNA [29–31] or a polypeptide of $M_r \sim 59{,}000$ [32–34].

In a series of experiments we first set out to test the possibility of RNA being involved. As in a previous publication [29] we used u.v. irradiation in order to introduce cross-links between protein and nucleic acid. As a precaution against ubiquitous RNases we included in these experiments diethyl pyrocarbonate. We indeed observed cross-linking and stabilization of the 82 Å wild-type receptor form [16]. Careful control experiments, however, revealed that cross-linking was not caused by u.v. light but rather by exposure to diethyl pyrocarbonate (Table 1). This is apparently due to the formation of isopeptide linkages between subunits in the receptor structure. In another approach we labeled the high molecular weight receptor with radioactive

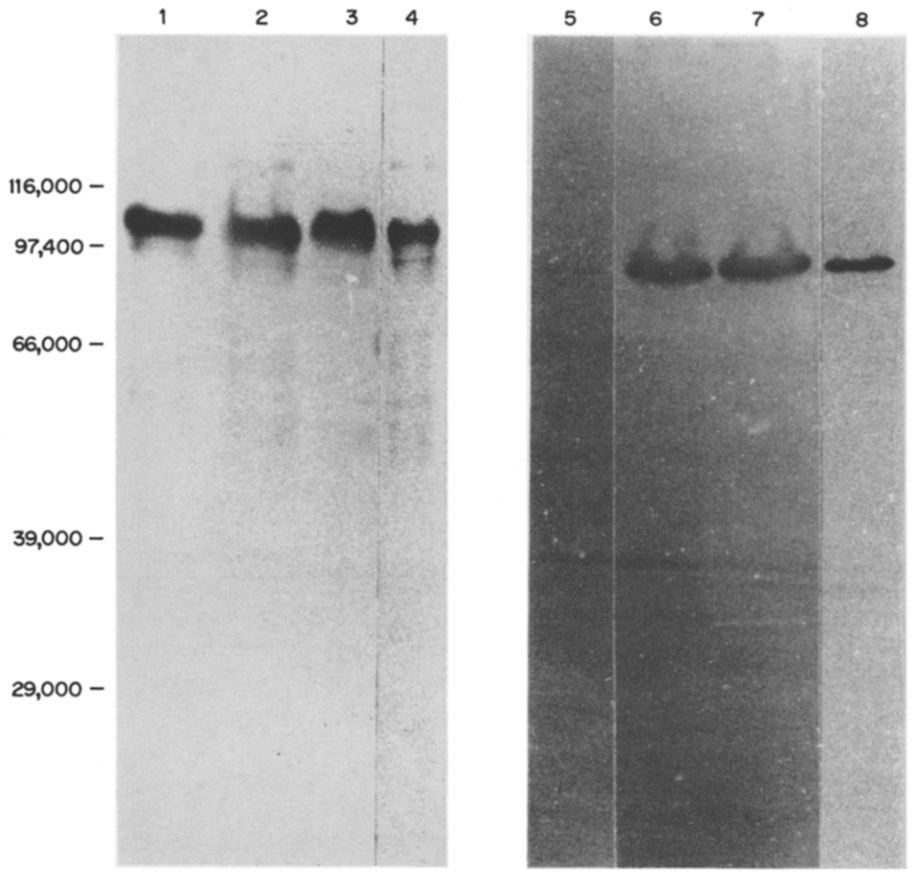

Fig. 7. Immunoblot of the completely cross-linked receptor. Cytosolic S49.1 receptor complexes with triamcinolone acetonide were either kept as controls (lanes 1 and 5), treated with dithiobis(succinimidyl propionate) (lanes 2 and 6) or oxidized with cupric *o*-phenanthroline (lanes 4 and 8). Intact cells were similarly treated with dithiobis(succinimidyl propionate) (lanes 3 and 7) and subsequently extracted. Duplicate samples were purified by immunoaffinity chromatography, run on a 10% SDS gel, and blotted onto Immobilon. Analysis was either with receptor-specific monoclonal antibody (lanes 1 to 4) or with an antiserum against mouse hsp90 (lanes 5 to 8). Markers were as in Fig. 3. (Data modified from Ref [16])

dexamethasone mesylate and used formaldehyde as the cross-linking agent. Analysis was in isopycnic CsCl gradients. The receptor material consistently banded at a density of 1.33 g/ml [35], a value very similar to that of pure proteins. The very same density was also obtained after prior treatment of the receptor complex with ribonuclease A or after activation to the DNA-binding state. We, therefore, have to conclude that the high molecular weight form of the glucocorticoid receptor of S49.1 mouse lymphoma cells does not contain any significant amount of RNA.

In fact, the above described cross-linking experiments all employed reagents which are known to react with amino acid side groups of proteins. In particular, *N*-hydroxysuccinimidyl-esters and imidates are very unlikely to react with functional groups in nucleic acids. We thus argue that the forth subunit of the high molecular weight receptor form must be of protein nature. In order to prove its identity we now have to purify the cross-linked receptor structure to homogeneity and analyze this material on SDS polyacrylamide gels subsequent to cleavage of the cross-links. This approach is in progress. It is, however, not easy to distinguish by staining of SDS gels between a very persistent impurity and the specific receptor component. For example, tubulins turned out to be very sticky contaminants in our hands. In any case, it will be interesting to see whether the above mentioned protein of 59 kDa is detectable after extensive purification.

EFFECT OF THE STEROIDAL LIGAND ON RECEPTOR ACTIVATION AND STABILITY

In order to approach the problem of how the steroid itself influences the structure and functions of the receptor we decided to study the effects of an antiglucocorticoid relative to a

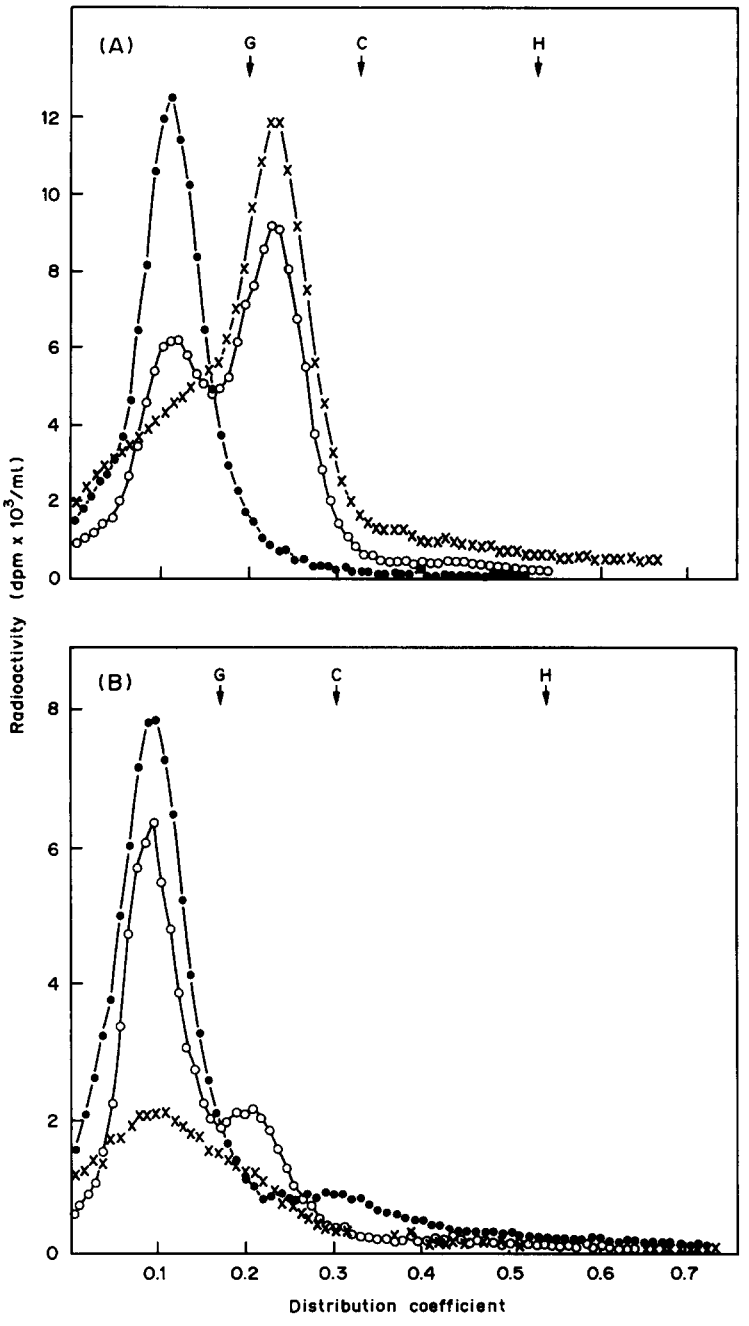

Fig. 8. Gel filtration of temperature activated receptor complexes. Cytosolic S49.1 receptor complexes with [^{3}H]triamcinolone acetonide (A) or [^{3}H]RU 38486 (B) were chromatographed on Sephacryl S-300 in the presence of 20 mM molybdate either directly (●) or after a 28°C treatment for 15 min (A, o), 30 min (A, x), 30 min (B, o), or 60 min (B, x). Markers were as in Fig. 1. (Data from Ref. [36])

glucocorticoid [36]. We used the antihormone RU 38486 [37, 38] and compared its effects to those of the potent glucocorticoid triamcinolone acetonide.

We observed that the receptor extracted from S49.1 cells and complexed with either steroid was easily activated by treatment with high salt to the dissociated form of 60 Å Stokes radius and DNA-binding ability. However, when we attempted receptor activation by warming we obtained a puzzling result. Figure 8 shows that the triamcinolone acetonide complex is progressively shifted from the 82 Å form to the 60 Å receptor species. By contrast, we observed very little of a corresponding shift with the RU 38486 complex but the yield of the labeled 82 Å species decreased drastically with the time at increased temperature. Similarly, we obtained only very

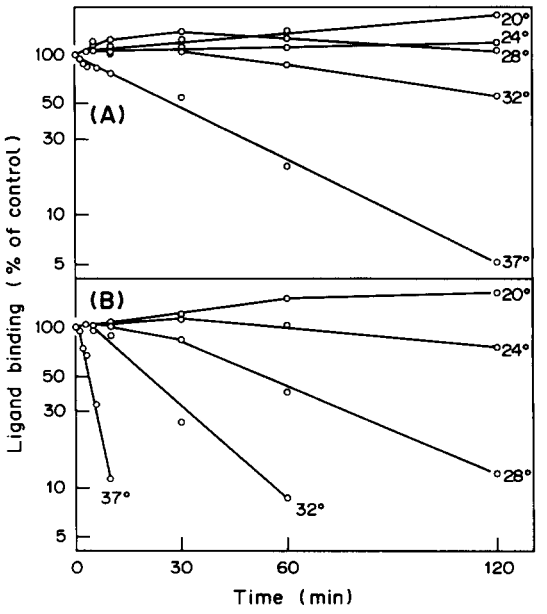

Fig. 9. Stability of receptor complexes at different temperatures. Cytosolic S49.1 receptor complexes with [^{3}H]triamcinolone acetonide (A) or [^{3}H]RU 38486 (B) were exposed to different temperatures for various lengths of time as indicated and subsequently cooled. Binding was assessed by the charcoal assay.

little binding of radiolabeled RU 38486 complex to DNA after 60 min at 28°C [36]. This suggested to us that the antagonist complex might be unstable at temperatures of 28°C and above. Figure 9 shows kinetic experiments at different temperatures which in fact prove this point. While the triamcinolone acetonide–receptor complex was stable at temperatures of up to 30°C it showed disintegration at 37°C. However, the RU 38486–receptor complex was quite unstable above 25°C and was almost completely lost within 10 min at 37°C. Thus the antagonist–receptor complex shows pronounced instability under the conditions of thermal activation but there is by far less instability if the ligand is an agonist. We observed even greater thermal instability, for example at 28°C, if the receptor–RU 38486 complex had previously been activated by salt treatment [36].

In order to find out whether the above described temperature instability is due to ligand dissociation only or whether the receptor polypeptide is simultaneously degraded we used again immunoblotting with the monoclonal antibody described above. In the experiment of Fig. 10 equal amounts of receptor were complexed either with triamcinolone acetonide or RU 38486 and subjected to either salt or temperature activation. All four samples were then applied to DNA-cellulose and the eluates with 300 mM KCl were used for immunoblotting. The same polypeptide molecular weights were obtained in each instance. Therefore, proteolysis does not explain receptor instability in our system at increased temperature. More importantly, we detected significant amounts of immunoreactive material under conditions which cause almost complete destruction of both agonist and antagonist complexes (cf. Figs 9 and 10, lanes 2 and 4). This shows that the ligand-free but activated receptor binds efficiently to DNA. Interestingly, similar quantities of receptor bound to DNA independent of the activation procedure and of the type of ligand used.

We also used the immunoblotting technique to check for receptor material in the 60 Å region of our Sephacryl S-300 gel filtration of the RU 38486 complex after 60 min at 28°C (cf. Fig. 8). We indeed detected significant amounts of the intact receptor polypeptide. This suggests that the receptor complex at elevated temperature dissociates into subunits and simultaneously or subsequently looses the bound RU 38486 ligand.

LACK OF STEROID BINDING AFTER RECEPTOR DISSOCIATION

We asked whether rebinding of triamcinolone acetonide or RU 38486 could be achieved after thermal dissociation of the ligand from the receptor. We treated agonist and antagonist complexes at 37°C for 1 h or 10 min, respectively, cooled the samples without prior removal of excess labeled steroid, and then simply assayed for the amount of bound ligand during a period of up to 24 h. We did not observe any significant rebinding over the background level which we observed immediately after warming [36].

In another experiment we preincubated the receptor with a saturating concentration of unlabeled cortisol (1 μM), removed excess hormone by adsorption to charcoal, and divided the sample. One half of the material was activated at 28°C for 30 min and subsequently incubated for up to 20 h at 0°C with 30 nM radiolabeled triamcinolone acetonide, the other half was maintained at 0°C and similarly treated with the labeled agonist. We found no binding of labeled steroid in the case of the temperature treated sample but significant exchange of cortisol by triamcinolone acetonide in the unactivated material [36]. This suggests that after receptor subunit dissociation the hormonal ligand is not freely exchangeable. Once the hormonal ligand has dissociated from the activated receptor,

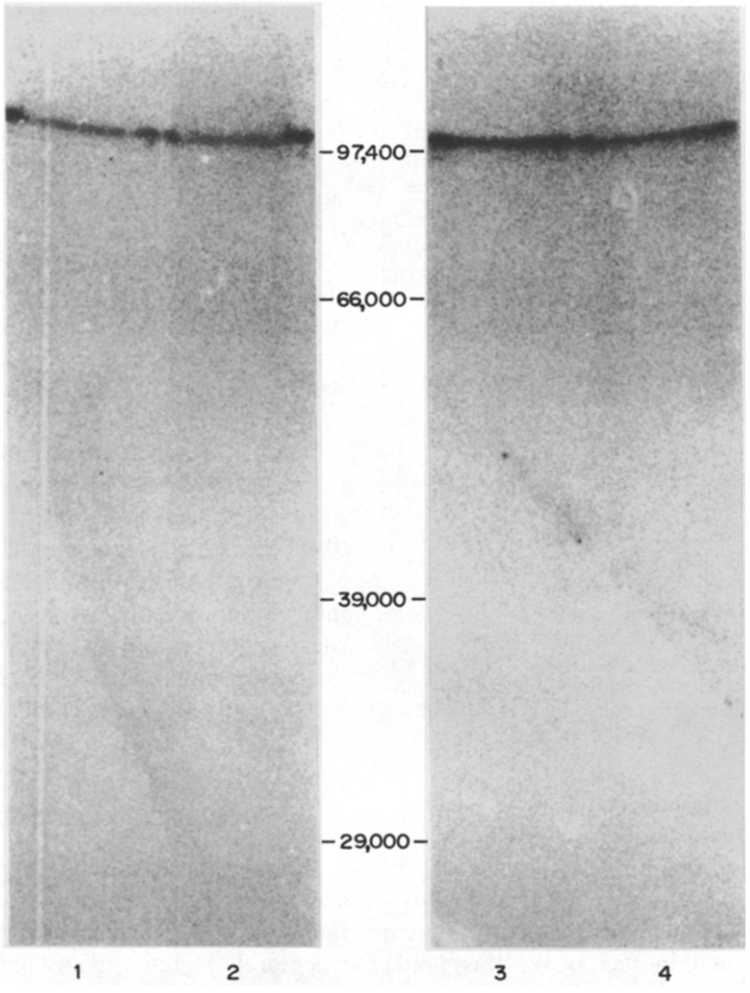

Fig. 10. Immunoblot of activated receptor complexes. Equal amount of S49.1 cytosol receptor complexes with triamcinolone acetonide (lanes 1 and 2) or RU 38486 (lanes 3 and 4) were either treated with 400 mM KCl for 1 h at 0°C (lanes 1 and 3) or exposed to 37°C (lane 2, 1 h; lane 4, 10 min). Samples were chromatographed on DNA-cellulose and step eluted with 300 mM KCl. They were purified by immunoaffinity chromatography, run on a SDS gel, blotted onto Immobilon and analyzed as before with the receptor-specific antibody. Markers were as in Fig. 3. (Data from Ref. [36])

another molecule of steroid cannot be bound again. Our observation in the S49.1 system agrees well with recent data in a different cell system [39] in which the hormone-binding activity was found to decay in parallel with the dissociation of the unliganded heteromeric receptor complex by various treatments.

RECEPTOR LOCALIZATION IN INTACT CELLS AFTER STEROID TREATMENT

The above described instability of the receptor–RU 38486 complex made us wonder whether it would provide a reasonable explanation for RU 38486 functioning as antihormone. This appears very unlikely since the experiment of Fig. 10 clearly demonstrates that the receptor complex in the cell extract is activated upon warming and binds to DNA even though the RU 38486 ligand is lost. Close inspection of Fig. 8, however, reveals that the receptor–antagonist complex certainly changes much more slowly at elevated temperature than the agonist complex, i.e. it is less readily activated. We thus wondered how the receptor–RU 38486 complex behaves in intact cells and first checked the distribution of the receptor in intact S49.1 cells after exposure to agonist and antagonist for 1 h at 37°C. Following incubation, the cells were lysed in the cold, the crude nuclear and cytosolic fractions were recovered, and the nuclei were extracted. The immunoblot of Fig. 11 shows that following treatment with triamcinolone acetonide a significant portion of immunoreactive receptor is

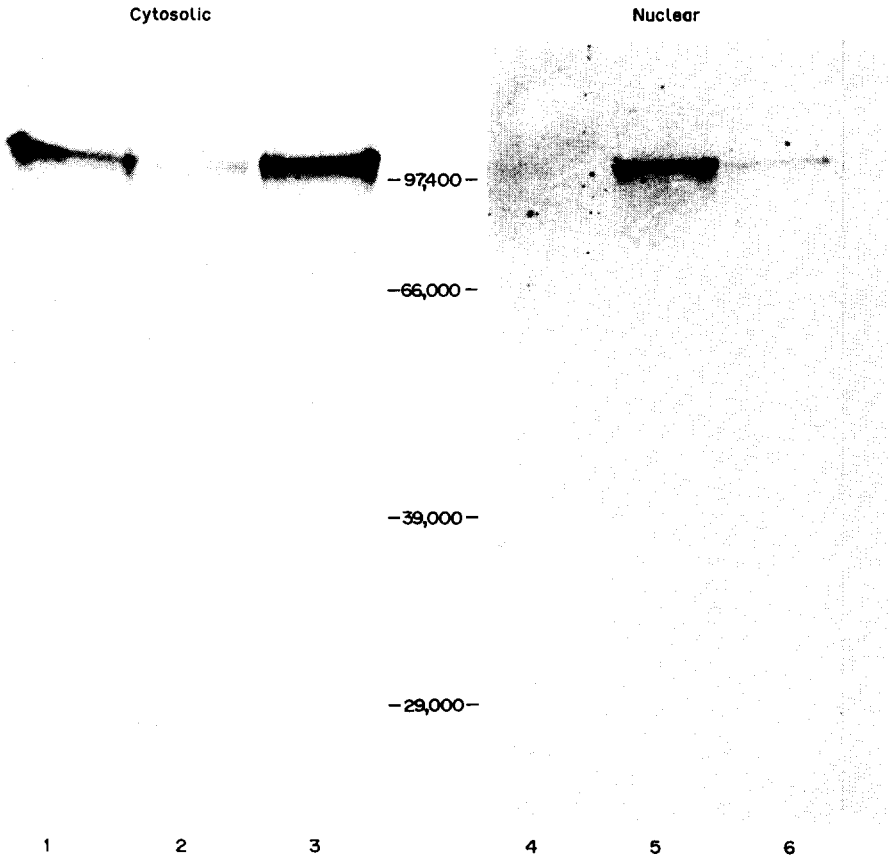

Fig. 11. Immunoblot of the receptor in cellular compartments. Intact S49.1 cells were incubated for 1 h at 37°C either without added steroid (lanes 1 and 4) or with triamcinolone acetonide (lanes 2 and 5) or RU 38486 (lanes 3 and 6). Cells were ruptured by freezing and thawing and crude nuclear and cytosolic fractions were obtained by centrifugation at 5000 *g*. The nuclei were extracted with 300 mM KCl at 20°C. Samples were purified by immunoaffinity chromatography, run on a SDS gel, blotted onto Immobilon and analyzed as before with the receptor-specific antibody. Markers were as in Fig. 3. (Data from Ref. [36])

detected in the nuclear extract while in untreated cells the receptor is present only in the soluble fraction. Most importantly, after incubating cells with RU 38486 there was very little receptor associated with the nuclei but immunoreactive material was found in the soluble cellular fraction to about the same level as in control cells.

We next asked in which molecular state the receptor exists after incubating the cells at 37°C with the antagonist. The cells were extracted in the cold, the cytosolic extract was submitted to gel filtration and the fractions corresponding to the 82 and 60 Å regions were pooled. Figure 12 shows immunoblots of these regions. Clearly, the receptor is present only in the high molecular weight form of 82 Å. In a similar experiment we treated cells under the same conditions with the radiolabeled antihormone and analyzed the cell extract by gel filtration. We observed a prominent peak of receptor–ligand complex at 82 Å with only a small shoulder at 60 Å [36]. These data unequivocally prove that the large receptor–RU 38486 complex is perfectly stable in intact cells at 37°C and is not activated.

The discrepancy between the receptor behaviour in intact cells and in cell extracts needs to be explained. Most significantly, the cytosol within an intact cell gets highly diluted upon preparing an extract. With this, endogenous inhibitors of activation would also become diluted no matter whether they are of phosphoglyceride [40] or metal ion nature [41] or perhaps a complex of both. We suppose that this is the reason why in the cell the large complex with RU 38486 remains stable but easily disintegrates into subunits under experimental conditions used with cell extracts. Clearly, the antagonist RU 38486 and hormonal agonists must bind somewhat differently to the hormone binding site of the receptor and thus affect the dissociation behaviour of the heteromeric structure in a different way.

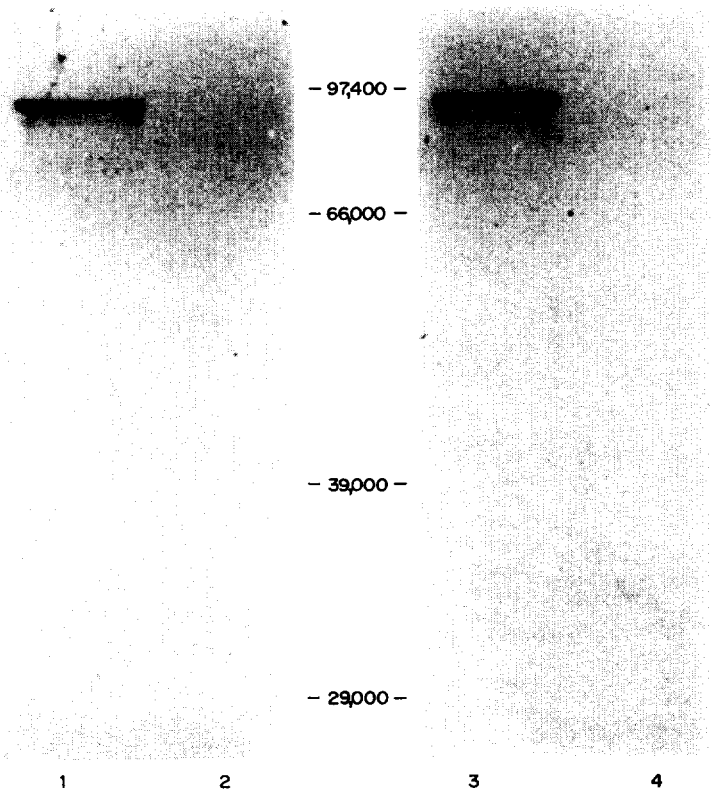

Fig. 12. Gel filtration and immunoblot of cellular receptor forms. Intact S49.1 cells were incubated for 1 h at 37°C either without added steroid (lanes 1 and 2) or with RU 38486 (lanes 3 and 4). Cells were extracted in the cold in the presence of 20 mM sodium molybdate and extracts were submitted to gel filtration on Sephacry S-300 in the presence of 20 mM sodium molybdate. The 82 Å regions (lanes 1 and 3) and 60 Å regions (lanes 2 and 4) were pooled according to the positions of the marker proteins. Samples were again purified by immunoaffinity chromatography, run on a SDS gel, blotted onto Immobilon and analyzed as before with the receptor-specific antibody. Markers were as in Fig. 3. (Data from Ref. [36])

The above data provide a plausible explanation for the fact that RU 38486 behaves as a biological antagonist in S49.1 lymphoma cells as well as in other cell systems. The antihormone simply occupies the ligand-binding site of the high molecular weight receptor but blocks dissociation of the multimeric structure while it is contained in the cell. Lack of ligand and receptor subunit dissociation within the living cell is of considerable biological importance since the ligand-free 60 Å receptor form would have to be expected to convert back in the cell to the original high molecular weight structure [42] which in turn would rebind steroid. With agonist and antagonist simultaneously present, as in a physiologically relevant competition situation, after a few cycles of binding, dissociation, and reassociation this would convert all of the cell's receptor into the activated agonist complex. This is certainly not the case.

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