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Identification and Localization of Steroid-binding and Nonsteroid-binding Forms of the Glucocorticoid Receptor in the Mouse P1798 Lymphosarcoma

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Glucocorticoid receptors (GCRs) were characterized in sublines of the mouse P1798 lymphosarcoma that are sensitive (S) or resistant (R) to glucocorticoid-mediated apoptosis. Previous work had identified two steroid-binding GCRs in S and R cells: a 97 kDa wild-type GCR in S cells (WT-GCR), and a 45 kDa truncated GCR in R cells (TR-GCR). A third GCR, a 97 kDa nonsteroid-binding GCR (NSB-GCR), was also identified in R cells. Using subcellular fractionation and Western blotting, we now show that in contrast to the WT-GCR which is localized in both the cytoplasm and nucleus of S cells, the NSB-GCR is localized predominantly in R cell nuclei. Moreover, gel filtration chromatography revealed that treatment with 400 mM NaCl and heat did not significantly alter the Stokes radius of the NSB-GCR suggesting that this receptor is not present in a heterooligomeric complex with other proteins. The TR-GCR was localized predominantly in the soluble cytoplasmic fraction but also in the crude membrane fractions of R cell nuclei, suggesting that this receptor is tightly associated with nuclear structures. It was not detected in the soluble nuclear fraction. Unexpectedly, a 45 kDa nonsteroid-binding immunoreactive protein was detected in crude membrane fractions of S cells. These studies describe a complex GCR system in the P1798 lymphosarcoma that necessitates a further consideration of glucocorticoid signaling in S and R cells.

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INTRODUCTION

Glucocorticoids (GCs) are used successfully as part of multidrug regimens in the treatment of various leukemias and lymphomas [1]. A functional glucocorticoid receptor (GCR) pathway is necessary, but not sufficient [2–8] for GCs to induce apoptosis of leukemic cells. Upon binding GC, cytosolic GCRs dissociate from a heterooligomeric complex and translocate into the nucleus where they interact with specific sequences of DNA in steroid-responsive genes to alter transcription of these genes. Alteration in gene expression subsequent to GC treatment results in a series of changes that culminate in apoptotic cell death. However, the precise mechanism by which GCs induce apoptosis is not known. Consequently, in GC-resistant models, it can be difficult to fully understand the mechanism(s) underlying the resistance phenotype.

A consideration of the potential functioning of variant GCRs is especially important when examining GC-resistance of lymphoid-derived cells. N-terminally-deleted variant GCRs in steroid-resistant lymphoma lines have been shown to maintain several GCR functions including steroid binding activity [9, 10], DNA binding activity [10, 11], and the ability to activate transcription of reporter plasmids [12]. Similarly, since there are many examples of unliganded WT-GCRs binding to glucocorticoid responsive elements (GREs) [13–17], this suggests that nonsteroid-binding variant GCRs may have the potential to interact with DNA. Finally, GC-resistant cells of the P1798 lymphosarcoma which express variant GCRs retain some characteristic GC-inducible functions, although the lysis function has been lost [18]. To fully describe the resistance mechanism in GC-resistant

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models, there needs to be a more complete characterization of mutant receptors as well as delineation of other cellular factors which might contribute to the resistance phenotype.

The mouse P1798 lymphosarcoma provides a suitable model system for such an investigation. There are two sublines of the P1798 lymphosarcoma; one subline is sensitive (S) while the other is resistant (R) to the lytic effects of GC. The S subline expresses the 97 kDa wild-type steroid-binding GCR (WT-GCR). The R subline lacks the WT-GCR, but contains two variant GCRs: a 45 kDa truncated steroid-binding receptor (TR-GCR) [9, 11, 18, 19], and a 97 kDa nonsteroid-binding receptor (NSB-GCR) [18, 20]. The present study was undertaken to more completely characterize the types of steroid-binding and nonsteroid-binding GCRs in the P1798 lymphosarcoma and their subcellular localization.

MATERIALS AND METHODS

Reagents

Bovine calf serum was purchased from Hyclone (Logan, UT). Newborn calf serum and gentamicin were purchased from Gibco (Grand Island, NY). Aprotinin, benzamidine HCl, pepstatin, phenylmethylsulfonyl fluoride (PMSF), sodium molybdate, Tween 20, 3′,3′-diaminobenzidine, dexamethasone (dex), triamcinolone acetonide (TA), gel filtration molecular weight standards, 2-mercaptoethanol and dithiothreitol (DTT) were purchased from Sigma Chemical Co. (St Louis, MO). Leupeptin was purchased from Boehringer Mannheim (Indianapolis, IN). Soybean trypsin inhibitor was purchased from Calbiochem (La Jolla, CA). Sephacryl S-300 column matrix was purchased from Pharmacia (Piscataway, NY). Acrylamide, *N,N′*-methylene-bis acrylamide (BIS), electrophoresis grade urea and molecular weight markers for electrophoresis were purchased from BioRad (Richmond, CA). Immobilon-P membrane was purchased from Millipore (Bedford, MA). The BUGR2 monoclonal antibody was a generous gift from Dr R. W. Harrison, University of Rochester (Rochester, NY). The 710 monoclonal anti-peptide antibody was a generous gift from Dr H. M. Westphal, Institut für Molekularbiologie und Tumorforschung (Marburg, Germany). PA1-511 and synthetic peptide PEP-001 (DQKPIFNVIPPIPVGSENWNRC) were purchased from Affinity BioReagents (Neshanic Station, NJ). Monoclonal antibody cell culture supernatant against a brain tumor antigen that was used as a control antibody was a generous gift from Drs G. Klaich and P. Kanter, Roswell Park Cancer Institute (Buffalo, NY). Biotinylated goat anti-mouse and goat anti-rabbit IgG and IgM secondary antibodies, and streptavidin peroxidase were purchased from Chemicon (Temecula, CA). [1,2,4(N)^{3}H]Triamcinolone acetonide ([^{3}H]TA) (17.0 Ci/mmol) and ECL Western blotting detection reagents were purchased from Amersham Corp. (Arlington Heights, IL). [6,7-^{3}H(N)]Dexamethasone mesylate ([^{3}H]DM) (35.0 Ci/mmol) was purchased from New England Nuclear/Dupont (Boston, MA). All other reagents used were of the highest quality available.

Isolation and culture of cells

Cells were isolated from the P1798 lymphosarcoma as previously described [21]. Isolated tumor cells were grown as suspension cultures in RPMI 1640 medium supplemented with 10% serum (8% bovine calf serum, 2% newborn calf serum), 25 mM HEPES-OH pH 7.55 and 0.1 mg/ml gentamicin. Serum was stripped with dextran-coated charcoal to remove endogenous steroids [22]. Freshly isolated tumor cells were frozen after 2–3 weeks in culture in medium containing 20% serum and 10% dimethylsulfoxide and stored in liquid nitrogen until used. The cells reestablished in culture were incubated at 37°C in a humidified atmosphere (5% CO_2) and cultured at a density between 1.5 and 3.0×10^6 cells/ml (log phase growth) for 4 weeks whereupon the cultures were discarded and fresh cultures were established from stocks stored in liquid nitrogen. Both S and R cell lines showed similar growth characteristics (19 and 23 h doubling time, respectively) and cell viability was greater than 95% throughout the 4 week culture period.

Formation of steroid–receptor complexes

Steroid–receptor complexes were formed either by incubating intact cells or cell extracts with a tritiated or nonradioactive GC. For labeling GCRs in intact cells with [^{3}H]TA, cells were suspended at 2×10^7 cells/ml in medium, and [^{3}H]TA, ±a 500-fold molar excess of nonradioactive TA, was added to a final concentration of 20 nM. Cells were then incubated at 37°C for 2–3 h. For labeling GCRs in intact cells with [^{3}H]DM, cells were suspended at 2×10^7 cells/ml in PBS (4°C), and [^{3}H]DM, ±a 500-fold molar excess of nonradioactive dex, was added to a final concentration of 100 nM. Cells were then incubated at 4°C for 2 h. For labeling GCRs in intact cells with nonradioactive TA or dex for localization studies, cells were suspended to 2×10^6 cells/ml in medium and TA or dex was added to a final concentration of 200 nM. Cells were then incubated at 37°C for 2–3 h. For labeling GCRs in cell extracts, [^{3}H]DM or [^{3}H]TA was added to the supernatant of the 10,000 *g* centrifugation step (see below) to a final concentration of 50–100 nM or 20 nM, respectively.

Soluble extracts from whole cells

To prepare the extracts, steroid treated or untreated S and R cells (1×10^8–1×10^9) were centrifuged at 500 *g* for 5 min, and cell pellets were washed 2 times with PBS. Cell pellets were then resuspended in TEEDG buffer (10 mM Tris–Cl, 1.5 mM EDTA, 1.5 mM EGTA, 0.5 mM DTT, 10% glycerol, pH 7.8

at 4°C) with protease inhibitors (0.01 μM aprotinin, 25 mM benzamidine HCl, 100 μM leupeptin, 0.36 μM pepstatin, 0.3 mM PMSF, 100 μg/ml soybean trypsin inhibitor), and with or without 20 mM sodium molybdate, subjected to one freeze–thaw cycle, and centrifuged at 10,000 *g* for 10 min at 4°C. This procedure lyses the nuclei. The supernatant was recentrifuged at 100,000 *g* for 1.5 h at 4°C followed by a 1 h incubation at 4°C. The 100,000 *g* supernatant was then cleared of free steroid by DCC treatment (0.1 vol of 10% w/v acid treated charcoal, 1% w/v dextran in TEEG) for 10 min at 4°C. The supernatant from the 100,000 *g* centrifugation step (whether it is from the whole cell lysates, the nuclear fraction, or the cytoplasmic fraction) is referred to as *soluble* extract throughout the text.

Nuclear extract preparation

S and R cells (1×10^8 cells) were incubated in culture medium with either 200 nM TA or ethanol vehicle for 2 h at 37°C. Cells were pelleted and washed twice with PBS as described above. Cell pellets were resuspended with 0.5 ml of nuclear preparation buffer (10 mM Tris–Cl, 250 mM sucrose, 2 mM EGTA, 5 mM $MgCl_2$, pH 7.8 at 4°C) with protease inhibitors (as described above), and subjected to one freeze–thaw cycle. Light microscopy and trypan blue dye exclusion were used to verify that the plasma membrane was disrupted and the nuclei remained intact. Intact nuclei were separated from the cytosolic fraction by centrifugation at 300 *g* for 10 min at 4°C and the crude nuclear pellet was washed two times with nuclear preparation buffer. Pelleted nuclei were lysed with 1.0 ml of NP40 lysis buffer (50 mM Tris–Cl, 400 mM NaCl and 1% NP40, pH 8.0 at 4°C), the resulting DNA mass was sheared using a Thomas (Philadelphia, PA) teflon pestle/glass homogenizer and the entire extract was centrifuged at 10,000 *g* for 5 min at 4°C. The resulting supernatant was recentrifuged at 100,000 *g* for 1 h at 4°C. The supernatant from this step was then prepared for SDS–PAGE. The 10,000 and 100,000 *g* pellets were washed twice with NP40 lysis buffer and extracted with 1% SDS in 50 mM Tris–Cl (pH 6.8 at 23°C) for 10 min at 100°C. Extracted proteins were then prepared for SDS–PAGE and Western blotting.

Cytosolic extract preparation

The cytosolic fraction prepared above was centrifuged at 300 *g* (10 min) two more times to ensure that no nuclei were present. The cytosol was then centrifuged at 10,000 *g* for 10 min at 4°C and the supernatant was recentrifuged at 100,000 *g* for 1 h at 4°C. The resulting supernatant was prepared for SDS–PAGE. Proteins were extracted from the 10,000 *g* and 100,000 *g* pellets as described above.

Activation of GCRs

For the purpose of this manuscript, the term "activated GCRs" refers to GCRs that were dissociated from a heterooligomeric complex with other proteins. Activated GCRs were prepared using two different procedures: (1) GCRs were artificially activated by adding NaCl to a final concentration of 400 mM to the 100,000 *g* supernatant, followed by incubation at 15°C for 30 min; and (2) GCRs were activated in a more physiologically relevant manner by treating intact cells in culture with [^{3}H]TA for 2–3 h at 37°C, followed by preparation of cytoplasmic extracts from these cells. Nonactivated receptors were prepared by lysing cells in a TEEG buffer containing 20 mM sodium molybdate and protease inhibitors, labeling GCRs with steroid after cell lysis, and by omitting the 400 mM NaCl treatment at 15°C.

Partial purification of GCRs by ammonium sulfate precipitation

In some experiments, receptors were partially purified by the slow addition of saturated ammonium sulfate to the 100,000 *g* supernatant to a final concentration of 35% (v/v), followed by centrifugation at 10,000 *g* for 20 min at 4°C. The resulting pellets were then prepared for Western blotting by resuspension in 1 × Laemmli sample buffer (see below).

Gel filtration chromatography

1–3 mg protein from soluble whole cell extract in 1.0 ml of TEEDG buffer with protease inhibitors (prepared for either activated or nonactivated GCR) were loaded directly onto 1.5 × 100 cm Sephacryl S300 gel filtration columns equilibrated with TEEG buffer, pH 7.8 at 4°C containing 150 mM NaCl, 2 mM 2-mercaptoethanol and 20 mM sodium molybdate. The standard proteins used to calibrate the columns were as follows: bovine thyroglobulin, Stokes radius (R_s) 8.6 nm; horse spleen apoferritin, R_s 6.1 nm; yeast alcohol dehydrogenase, R_s 4.6; bovine serum albumin, R_s 3.6 nm; and bovine erythrocyte carbonic anhydrase, R_s 2.3 nm. Proteins were chromatographed at 4°C at a flow rate of 10 ml/h. Column fractions (3 ml) from one column were assessed for [^{3}H]TA by scintillation counting, while selected fractions from a duplicate column were made 35% with saturated ammonium sulfate to precipitate the GCR. Protein from each precipitated fraction was resuspended in 1 × Laemmli sample buffer and prepared for SDS–PAGE and Western blotting as described below.

SDS–PAGE and Western blotting

Samples were resuspended in an equal volume of 2 × Laemmli sample buffer, boiled for 5 min, and electrophoresed on 7.5, 10 or 12% polyacrylamide minigels (25 μg protein per lane) at 100 V for 2 h by the method of Laemmli [23]. Protein was electrophoretically transferred to Immobilon-P membranes at 100 V for 2 h. Membranes were blocked with 5% non-fat dried milk in Tris-buffered saline (TBS)-Tween (10 mM Tris–Cl, 150 mM NaCl, 0.05% Tween 20 pH 7.5 at 23°C) for

2 h at room temperature. Western blotting was performed using three different anti-GCR antibodies: (1) BUGR2 monoclonal antibody which recognizes an epitope corresponding to amino acid sequence 395–411 in the mouse GCR [24]; (2) polyclonal anti-peptide PA1-511 raised against a synthetic peptide corresponding to amino acids 346–367 of the human GCR [25]; and (3) monoclonal anti-peptide 710 antibody (710 Ab) raised against a synthetic peptide corresponding to amino acids 710–716 of the murine GCR [26]. Primary antibodies were diluted in TBS-Tween and incubated with membranes at 4°C (BUGR2 for 12 h at a 1:50 dilution) or 23°C (PA1-511 for 2 h at a concentration of 0.5 μg/ml and 710 Ab for 2 h at 1:20 dilution of cell culture supernatant), followed by addition of either biotinylated goat anti-mouse or biotinylated goat anti-rabbit IgG and IgM (1 h, 23°C at a 1:1000 dilution), then streptavidin peroxidase (30 min, 23°C at a 1:2000 dilution). Immunoreactive bands were detected by two methods: (1) by incubating membranes with 0.2 mg/ml diaminobenzidine for 5 min at 23°C; or (2) by incubating membranes with chemiluminescent substrate, and subsequently exposing membranes to X-ray film (Kodak) for 1–60 s. Peptide competition experiments were performed by preincubating PA1-511 (0.5 μg/ml) with a final concentration of 1.0 μg/ml of the corresponding peptide for 1 h at room temperature prior to incubating the membrane with the peptide-competed antibody. Quantitation of immunoreactive bands following Western blotting with chemiluminescent substrate, was performed with a Molecular Dynamics Computing Densitometer (Model 300A).

Western blots that had been previously probed, were stripped in 62.5 mM Tris–Cl (pH 6.8 at 23°C) containing 0.1 M 2-mercaptoethanol and 2% SDS at 70°C for 45 min with gentle shaking. Stripped blots were briefly rinsed in TBS-Tween prior to blocking and Western blotting as described above.

SDS–PAGE and [^{3}H]fluorography

After SDS–PAGE, gels were washed two times with DMSO (30 min each wash), and then incubated with 20% (w/v) PPO in DMSO for 3 h at room temperature, followed by incubation with deionized H_2O for 1 h [27]. Gels were then dried, loaded into cassettes with X-ray film, and stored at −70°C for a period of 3–14 days prior to developing films.

RESULTS

Quantitation of a 97 kDa nonsteroid-binding immunoreactive receptor (NSB-GCR)

A 97 kDa NSB-GCR in R tumors of the P1798 lymphosarcoma was first identified by Ip *et al.* [18] (see Fig. 1). To quantitate the level of NSB-GCR in R cells relative to the level of the WT-GCR in S cells, we performed Western blotting of soluble extracts of S and R cells using the polyclonal antibody PA1-511 (Fig. 1). Densitometric analysis of Western blots revealed that the NSB-GCR in R cells was present at 28 ± 1% (mean ± SEM, $n = 3$) the level of WT-GCR in S cells (Fig. 2, lanes 1 and 2, bottom arrow). The PA1-511 Ab also detected two higher molecular weight proteins at 115 and 140 kDa (Fig. 2, lanes 1 and 2, upper arrows), previously identified by Cidlowski *et al.* [25] in HeLa S_3 cells.

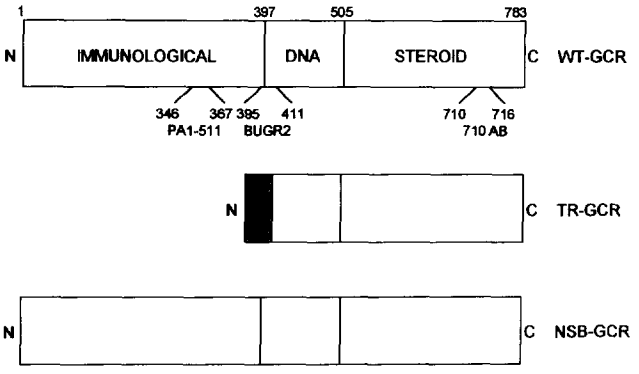

Fig. 1. Glucocorticoid receptors in the mouse P1798 lymphosarcoma. Amino acid epitopes for antibodies used in this study are shown. The N-terminal sequence of the TR-GCR is not known but is thought to include 41 amino acids (hatched region) derived from exon 1 of the GCR mRNA [12, 20].

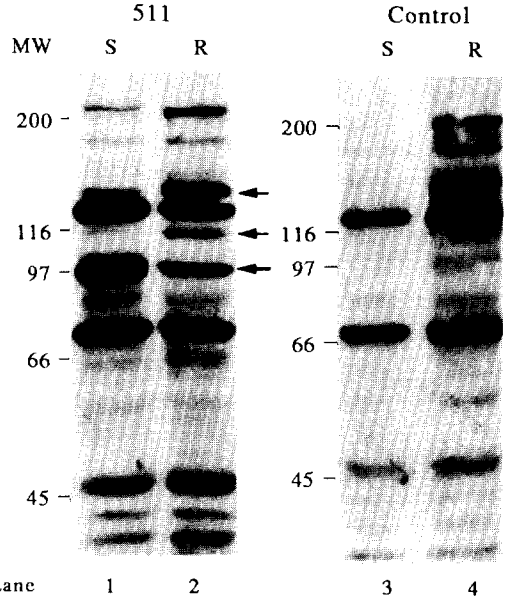

Fig. 2. Western blot quantitation of 97 kDa immunoreactive receptor in S and R cells. Equal amounts (25 μg protein) of soluble whole cell extracts from S cells (lanes 1 and 3) and R cells (lanes 2 and 4) were electrophoresed on SDS–PAGE minigels and prepared for Western blotting with PA1-511 (lanes 1 and 2) or peptide-competed PA1-511 (lanes 3 and 4). The bottom arrow indicates the position of the WT-GCR and the NSB-GCR. The upper arrows indicate the positions of the 115 and 140 kDa immunoreactive proteins.

R cell extracts do not inhibit steroid binding or induce proteolysis of the WT-GCR

It is possible that there is some factor in R cell extracts that could prevent binding of GCs to the NSB-GCR. It is also possible that there is a proteolytic activity in R cell extracts that results in proteolysis of the 97 kDa GCR to produce the 45 kDa TR-GCR. To address these questions, mixing experiments were performed with soluble extracts of S and R cells. As shown in Fig. 3, the 97 kDa WT-GCR in S cells and the 45 kDa TR-GCR in R cells were labeled with [^{3}H]DM (lanes 1 and 3, respectively) in unmixed extracts. Both bands were absent when soluble extracts were incubated with [^{3}H]DM plus a 500-fold molar excess of nonradioactive dex (Fig. 3, lanes 2 and 4). When S and R extracts were mixed prior to labeling with [^{3}H]DM, both the 97 and 45 kDa-labeled GCRs were detected at comparable levels (Fig. 3, lane 5). Since lane 5 was loaded with 25 μg of S extract mixed with 25 μg of R extract, the 97 and 45 kDa bands in lane 5 are present at approximately one half the intensity of the corresponding bands in lanes 1 and 3 which were loaded with 50 μg of S and R extract, respectively. These findings demonstrated that R cell extracts did not prevent binding of [^{3}H]DM to the WT-GCR suggesting that lack of GC-binding by the NSB-GCR is a property of the NSB-GCR protein itself. Secondly, R cell extracts did not induce proteolysis of the [^{3}H]DM-labeled WT-GCR.

Interestingly, a minor [^{3}H]DM-labeled-band at 46 kDa was detected in R cell extracts (Fig. 3, lane 3, arrow). It is possible that this protein represents a posttranslationally-modified isoform of the TR-GCR.

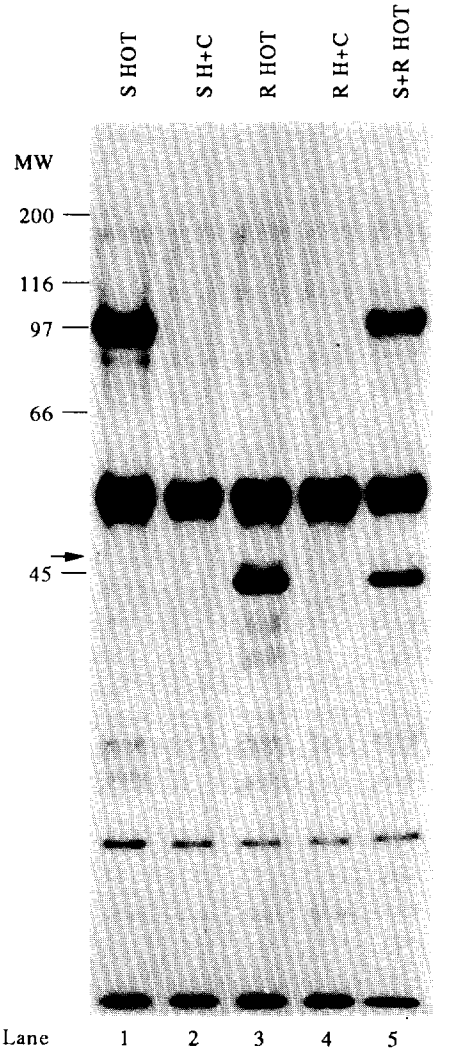

Fig. 3. SDS–PAGE fluorography of [^{3}H]DM-labeled receptors from S and R cells. S and R whole cell extracts were incubated with a final concentration of 100 nM [^{3}H]DM for 2–3 h in the absence (HOT; lanes 1, 3 and 5) or presence (H + C; lanes 2 and 4) of 500-fold molar excess of nonradioactive dex. Protein was precipitated with ammonium sulfate and 50 μg of partially purified receptors were resuspended in 1× Laemmli sample buffer and electrophoresed on SDS–PAGE gels prior to fluorography. In lane 5, equal amounts of S and R soluble whole cell extracts were mixed prior to incubation with [^{3}H]DM. The combined extract was prepared as above for SDS–PAGE and 50 μg total protein was loaded in lane 5. Arrow indicates a minor [^{3}H]DM-labeled isoform of the TR-GCR. Migration of molecular weight markers is indicated on the left hand side.

Unlike the WT-GCR and TR-GCR, the Stokes radius of the NSB-GCR is similar under nonactivating and activating conditions

The WT-GCR and the TR-GCR are present in a heterooligomeric complex with other proteins (nonactivated receptor) that can be dissociated by the addition of 400 mM NaCl yielding the monomeric GCR (activated receptor) [18]. When assessed by Sephacryl S300 chromatography, activation was found to reduce the R_s of the WT-GCR from 8.1 to 5.4 and the TR-GCR from 7.0 to 2.3 (see Table 1). To at least partially answer the question of whether the NSB-GCR is part of a nonactivated heterooligomeric complex, we chromatographed extracts of R cells, which had been prepared under nonactivating or activating conditions, and subsequently analyzed column fractions by SDS–PAGE and Western blotting with an antibody (BUGR2) that recognizes the NSB-GCR (Fig. 1). To establish the validity of the procedure, we first compared the R_s values of the WT-GCR determined either by analyzing column fractions by Western blotting with BUGR2, or by counting column fractions for [^{3}H]TA. The peak immunoreactivity for the nonactivated WT-GCR occurred at R_s 7.6 [Fig. 4 (A, fraction 25)] and the peak immunoreactivity for the activated WT-GCR occurred at R_s 5.3 [Fig. 4 (B, fraction 29)]. This was in close agreement with values of R_s 8.1 and 5.7 determined for the nonactivated and activated [^{3}H]TA-labeled WT-GCR, respectively, chromatographed on separate columns (data not shown).

Having established the validity of analyzing column fractions by Western blotting, we determined the R_S

Table 1. R_S values for GCRs in S and R cells of the mouse P1798 lymphosarcoma

Cell line	Receptor type	[^{3}H]TA binding	Average R_S nonactivated	Average R_S activated
S	WT-GCR	+	8.1 ± 0.1 (5)	5.4 ± 0.1 (8)
R	TR-GCR	+	7.0 ± 0.3 (2)	2.3 ± 0.1 (7)
R	NSB-GCR	−	6.9 ± 0.2 (4)	6.6 ± 0.1 (6)

Reported R_S values are the average of 2–8 determinations ± SEM in nm (number of determinations in parentheses). Values for the NSB-GCR were determined using Western blotting (immunoreactivity of the 97 kDa band) following Sephacryl S300 chromatography, values for the TR-GCR were determined by scintillation counting of Sephacryl S300 fractions, and values for the WT-GCR were determined using both Western blotting and scintillation counting. GCRs were activated by adding NaCl to a final concentration of 400 mM to the 100,000 *g* supernatant, followed by incubation at 15°C for 30 min.

values for the NSB-GCR under nonactivating and activating conditions. In contrast to the WT-GCR, there was no significant difference in the R_S of the NSB-GCR prepared under nonactivating [Fig. 5 (A, fraction 26)] or activating conditions [Fig. 5 (B, fraction 26)]. Mean values determined for the nonactivated and activated NSB-GCR were R_S 6.9 and R_S 6.6, respectively (Table 1). These data suggest that the NSB-GCR is not present in a heterooligomeric complex comparable to that of the WT-GCR and TR-GCR. However, it is also possible that the NSB-GCR is so tightly associated with other proteins, that the complex cannot be dissociated under conditions which dissociate the heterooligomeric complexes of nonactivated WT-GCR and TR-GCR.

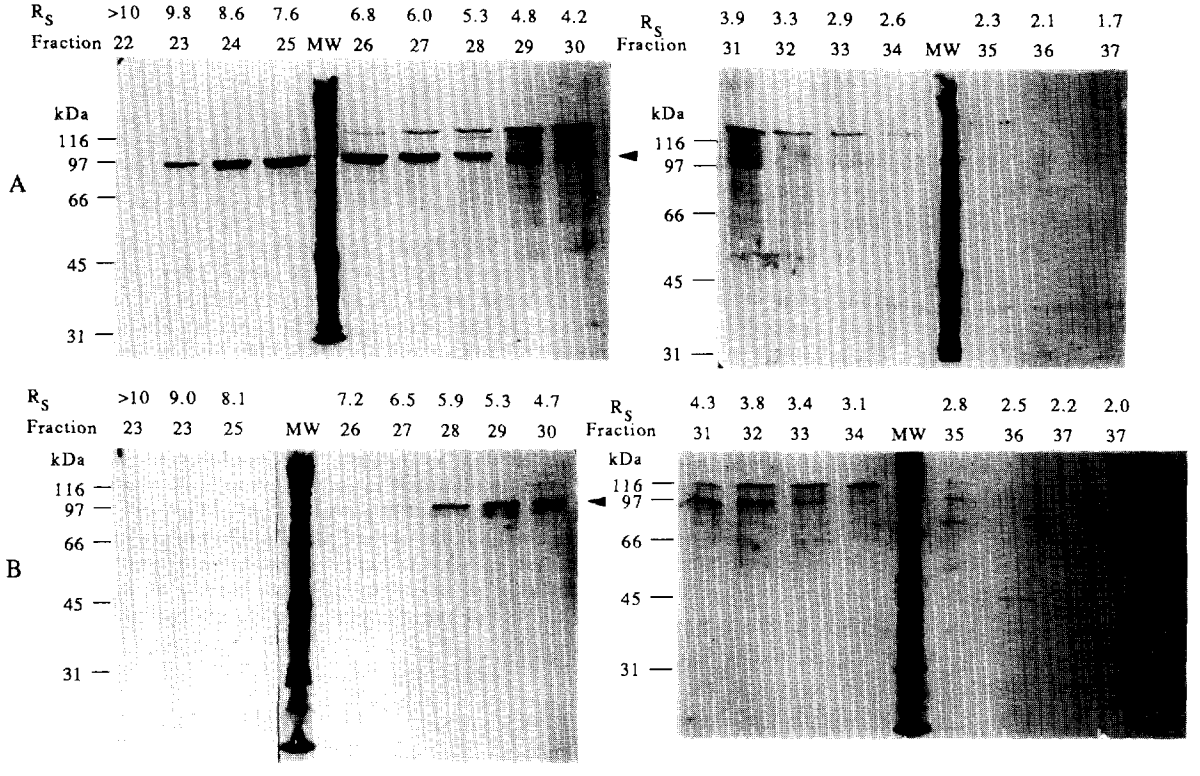

Fig. 4. Sephacryl S300 gel filtration chromatography followed by Western blotting of nonactivated and activated WT-GCR from S cells. GCRs from S cells were prepared under nonactivated conditions (A), or activated conditions (B) and then were directly loaded onto Sephacryl S300 columns. GCR was activated by treating the 100,000 *g* supernatant with 400 mM NaCl and incubating the extract at 15°C for 30 min as described in Materials and Methods. WT-GCR in each fraction was precipitated with ammonium sulfate and then prepared for Western blotting with BUGR2. The immunoreactive band at 97 kDa is the WT-GCR (arrowhead); the band above the 97 kDa band is nonspecific (data not shown). Stokes radius (R_S) in nm is shown above each column fraction. Biotinylated molecular weight markers are shown under the heading MW, and the molecular weight of each standard is shown on the left.

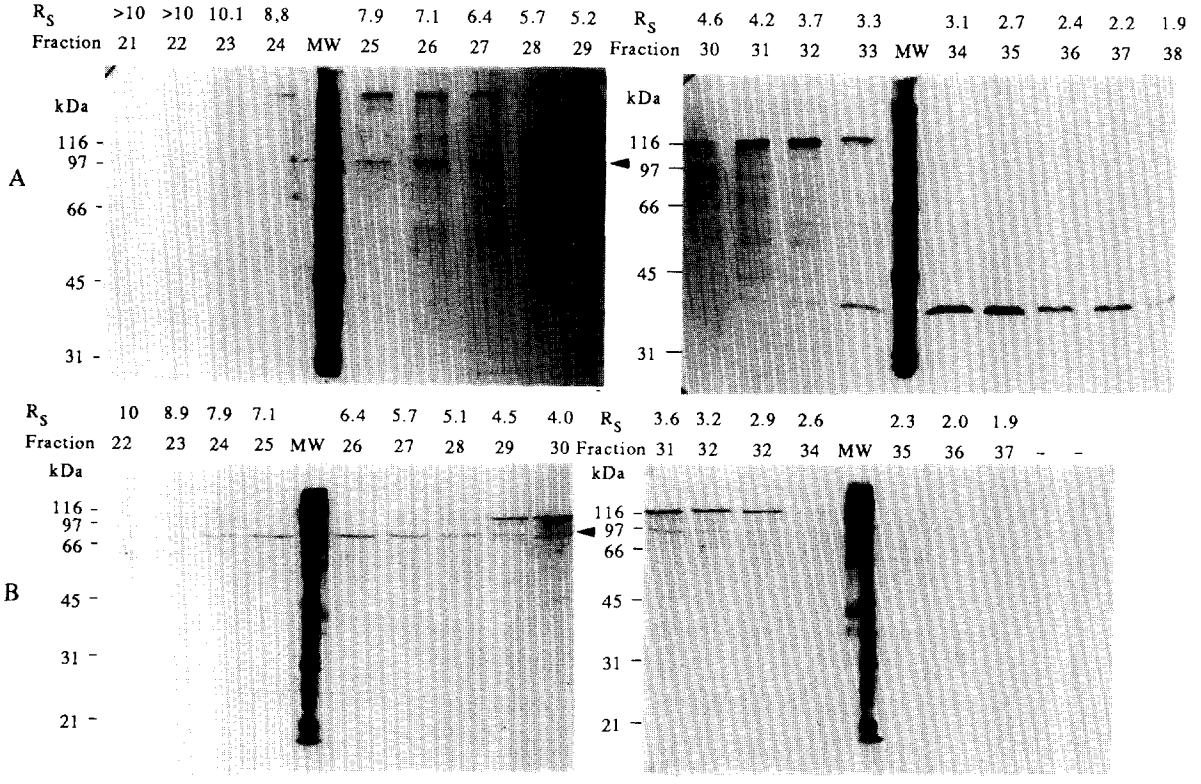

Fig. 5. Sephacryl S300 gel filtration chromatography followed by Western blotting of activated and nonactivated NSB-GCR from R cells. Identical experiment as described in Fig. 4 except R cells were used. GCRs from R cells were prepared under nonactivated (A) or activated (B) conditions and then were directly loaded onto Sephacryl S300 columns. Western blotting was performed with BUGR2. The immunoreactive band at 97 kDa is the NSB-GCR (arrowhead); other bands above and below the 97 kDa band are nonspecific (data not shown). Stokes radius (R_S) in nm is shown above each column fraction. Biotinylated molecular weight markers are shown under the heading MW, and the molecular weight of each standard is shown on the left.

The unliganded WT-GCR is detected in both the cytoplasmic and nuclear fraction of S cells

Controversy exists over the subcellular localization of unliganded GCR. Most studies have reported that the majority of the unliganded GCR resides in the cytoplasm [25, 28–33]. However, other studies have found that the GCR is nuclear in the absence of ligand [34–36]. It was thus of interest to determine the subcellular localization of the WT-GCR in S cells and to compare the pattern to that of the NSB-GCR and TR-GCR in R cells. Figure 6 shows the subcellular localization of WT-GCR in S cells which had been incubated in the absence or presence of TA for 2 h at 37°C. WT-GCR was recovered predominantly in the cytoplasmic fraction of untreated S cells [Fig. 6 (A, lane 3, arrow)]. However, a significant amount of immunoreactive receptor was also present in the nuclear fraction of untreated S cells [Fig. 6 (A, lane 5)]. As expected, treatment of S cells with TA resulted in a decrease in the amount of cytoplasmic WT-GCR [Fig. 6 (A, lane 2 vs lane 3)] and a concomitant increase in the amount of nuclear WT-GCR [Fig. 6 (A, lane 4 vs lane 5)].

The NSB-GCR is localized predominantly to the nuclear fraction of R cells

In contrast to the WT-GCR, there was no significant difference between the distribution of the NSB-GCR in TA-treated and -untreated R cells [Fig. 6 (C, compare lane 2 vs lane 3 and lane 4 vs lane 5, arrow)]. The majority of the NSB-GCR (75%) was recovered in the nuclear fraction of R cells [Fig. 6 (C, lanes 4 and 5, arrow)].

Note that it is not possible to report an absolute receptor distribution in intact cells from these data because of protein leakage from the nucleus during the procedure, differences in the way each extract was prepared and the exclusion of protein recovered in the crude membrane fractions. However, the data do show steroid-mediated GCR redistribution and show clear differences between the subcellular localization of GCR in S and R cells. These data are summarized in Table 2.

The TR-GCR is not detected in the soluble nuclear fraction of R cells

To localize the TR-GCR on Western blots, we used an antibody (710 Ab) directed against the C-terminal

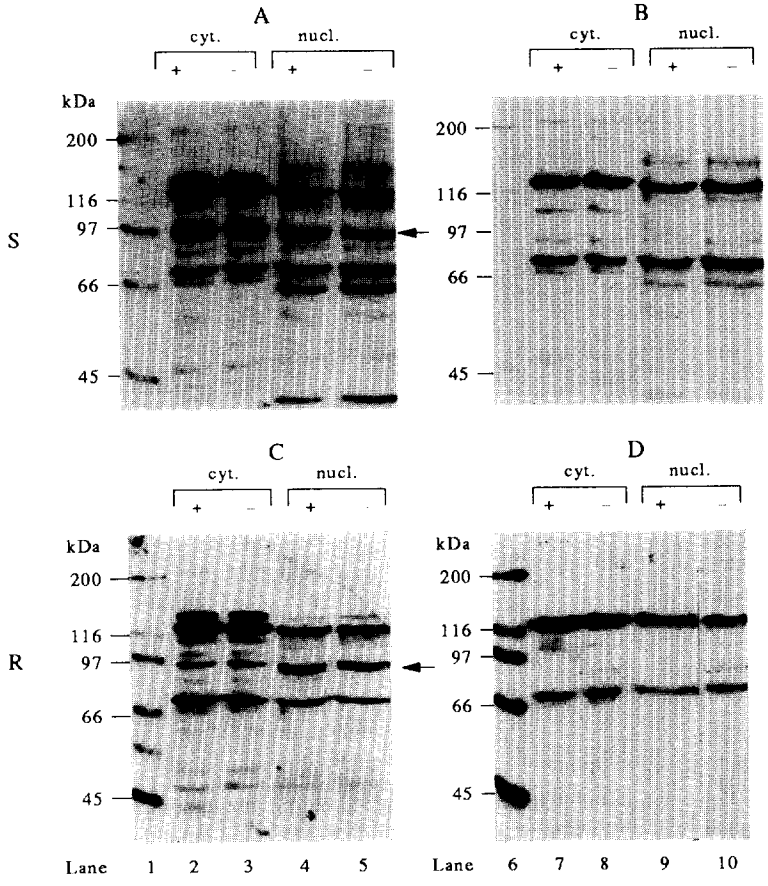

Fig. 6. Subcellular localization of the WT-GCR and NSB-GCR. S cells (A and B) and R cells (C and D) were incubated with 200 nM TA (+) or vehicle (−) for 2 h at 37°C prior to separation of the cytoplasmic (cyt.) and nuclear (nucl.) fractions for Western blotting with PA1-511 (A and C) or peptide-competed PA1-511 (B and D). Equal amounts (30 μg protein) of soluble cytoplasmic and nuclear protein were loaded per lane. Arrows indicate the migration of the WT-GCR (A) and the NSB-GCR (C). Biotinylated molecular weight markers are shown in lanes 1 and 6.

domain of the GCR (Fig. 1). This antibody also reacts well with the WT-GCR but reacts less strongly with the NSB-GCR. Steroid treatment of R cells in culture resulted in a reduction in the amount of cytoplasmic TR-GCR (Fig. 7, lane 1 vs lane 2). This indicates that the TR-GCR is responsive to steroid-induced redistribution of GCR in the cell. However, in contrast to the WT-GCR, the TR-GCR was not detected in the soluble nuclear fraction of either steroid-treated or -untreated R cells (Fig. 7, lanes 3 and 4).

The TR-GCR is detected in the crude membrane fractions of R cells

Since immunoreactive TR-GCR was not detected in the soluble nuclear fraction of R cells (Fig. 7, lanes 3 and 4), we wanted to determine whether it could be detected in the crude membrane fractions of R cells. As seen in Fig. 8 (bottom left, lanes 2–9, arrow), a 45 kDa immunoreactive band was detected in the crude membrane fractions of the cytoplasmic as well as the nuclear fractions of R cells regardless of whether cells were

Table 2. Levels of immunoreactive WT-GCR and NSB-GCR in the soluble cytoplasmic and nuclear fractions of S and R cells

	(−) TA		(+) TA	
	Cytoplasmic	Nuclear	Cytoplasmic	Nuclear
WT-GCR in S cells	84 ± 4	16 ± 4	59 ± 1	42 ± 1
NSB-GCR in R cells	26 ± 1	75 ± 1	26 ± 2	75 ± 2

The contribution of the cytoplasmic and nuclear immunoreactivity to the total immunoreactivity (cytoplasmic + nuclear = 100%) is reported for S and R cells incubated with (+) or without (−) 200 nM TA for 2 h in culture as described in Materials and Methods. Reported values are the mean of densitometric scans of the 97 kDa immunoreactive bands on Western blots ± SEM in % ($n = 2$). TA, triamcinolone acetonide.

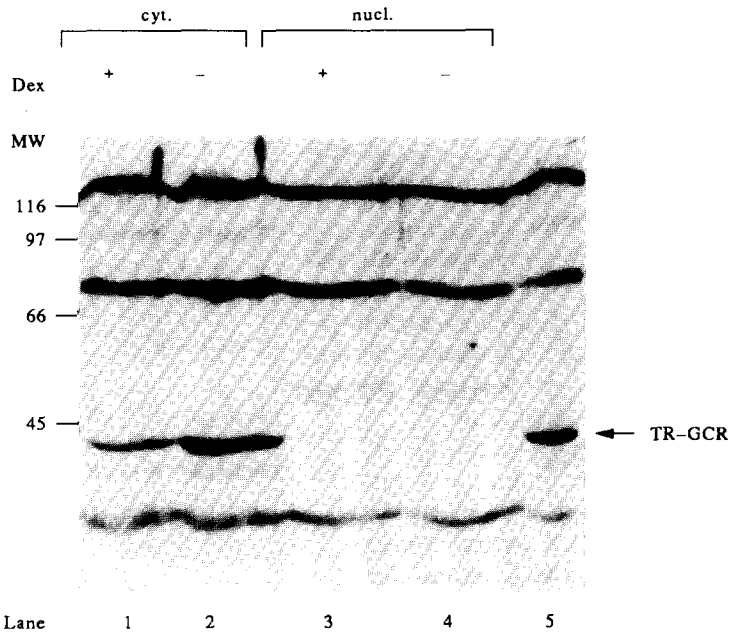

Fig. 7. Subcellular localization of the TR-GCR. Similar experiment as described in Fig. 6 except Western blotting was performed with the 710 Ab. R cells (R) were incubated with 200 nM dex (+) or vehicle (−) prior to separation of the cytoplasmic (cyt.) and nuclear (nucl.) fractions. Soluble cytoplasmic extract (100 μg protein) was loaded in lanes 1 and 2 and nuclear extract (20 μg) was loaded in lanes 3 and 4. Lane 5 was loaded with 30 μg soluble whole cell extract protein from R cells to show the migration of the TR-GCR (arrow).

incubated with (+) or without (−) dex in culture. The immunoreactive band in the crude membrane fractions (Fig. 8, bottom left, lanes 2–9) had a molecular weight identical to that of the TR-GCR in soluble extracts of R cells (Fig. 8, bottom left, lane 10, arrow). This suggests that the TR-GCR is tightly associated with

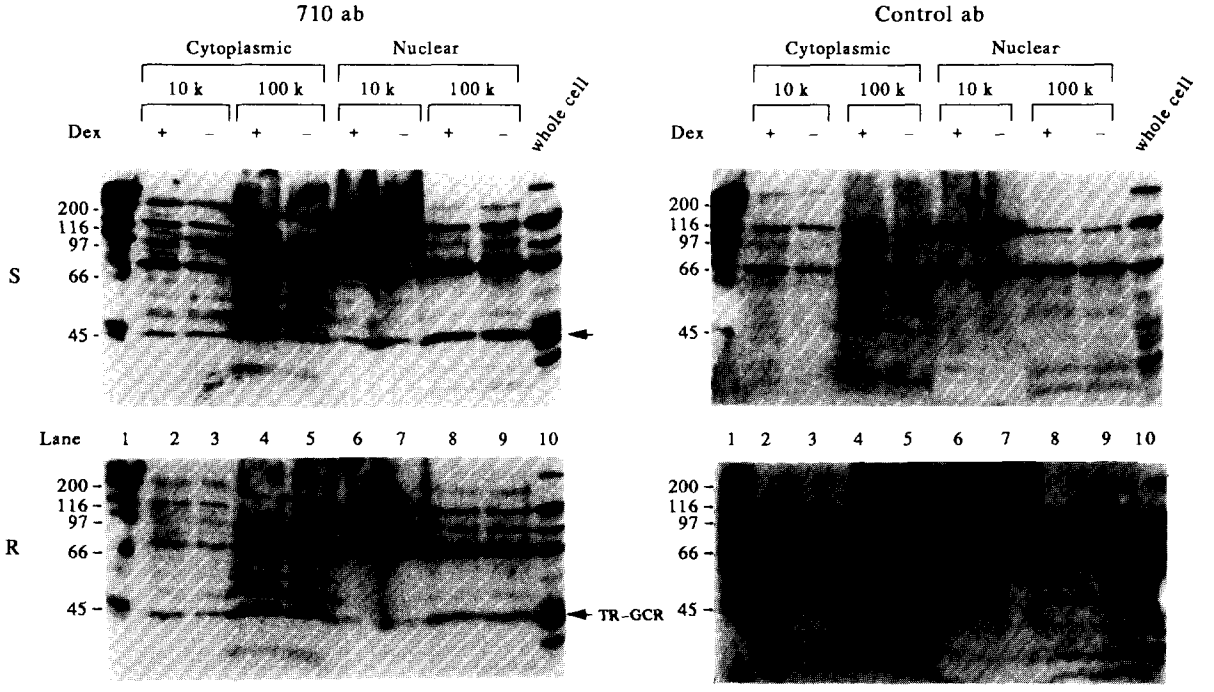

Fig. 8. Identification of 45 kDa immunoreactive receptor in the crude membrane fractions of S and R cells. S cells (top) and R cells (bottom) in culture medium were incubated with 200 nM dex (+) or vehicle (−) for 2 h at 37°C. Proteins were extracted from the 10,000 *g* (10 k) and the 100,000 *g* (100 k) pellets of the cytoplasmic (C) and nuclear (N) fractions and equal volumes of extracted proteins were separated on 10% SDS–PAGE gels prior to Western blotting with the 710 Ab (left) or with a control monoclonal antibody tissue culture supernatant (right). Soluble, whole cell extract from R cells was loaded in lane 10 in each gel to show migration of the TR-GCR for comparison (arrow). Biotinylated molecular weight markers are shown in lane 1.

the crude membrane fractions of the cytoplasm and nuclei.

Quite unexpectedly, a 45 kDa immunoreactive band was also detected in the crude membrane fractions of S cells (Fig. 8, top left, lanes 2–9). Soluble extract from R cells was loaded in lane 10 (Fig. 8, top left) to show migration of the TR-GCR (arrow) for comparison. The 45 kDa band in the crude membrane fractions of S cells (Fig. 8, top left, lanes 2–9) had an identical molecular weight to that of the TR-GCR in soluble extracts of R cells (Fig. 8, top left, lane 10). Clearly, a 45 kDa [^{3}H]DM-labeled band was not detected in soluble extracts from S cells (Fig. 3, lane 1).

Since we detected a 45 kDa immunoreactive GCR band in the crude membrane fractions of S cells, it was of interest to determine if there was a nonsteroid-binding form of the 45 kDa TR-GCR in the *soluble* fraction of S cells. If a nonsteroid-binding form of the TR-GCR was present in soluble S cell extracts, it could potentially modulate functioning of the WT-GCR. Using the 710 Ab, the TR-GCR was detected on Western blots of R cells, in both crude (Fig. 9, lane 3) and ammonium sulfate partially purified (Fig. 9, lane 1) soluble extracts. In S cells, the 710 Ab detected the WT-GCR at 97 kDa (Fig. 9, lanes 2 and 4); however, a 45 kDa band was *not* detected in *soluble* extracts (Fig. 9, lanes 2 and 4).

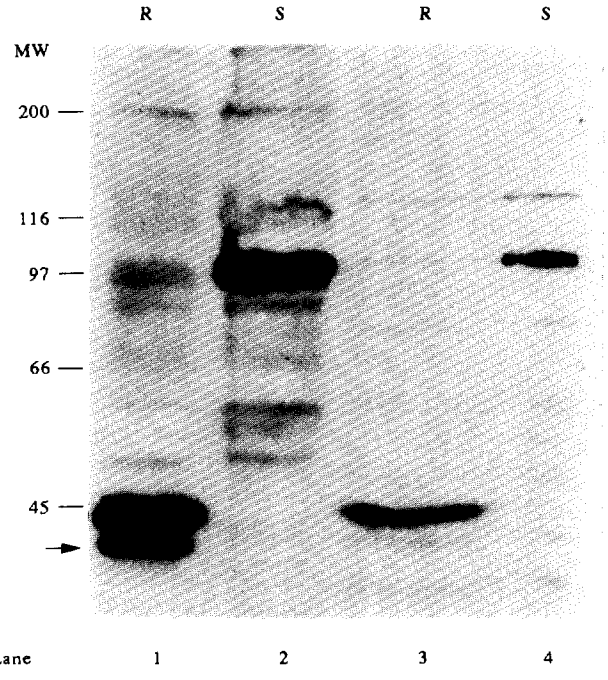

Fig. 9. Western blot of receptors in S and R cells using the 710 Ab. Equal amounts (30 μg protein) of crude extracts (lanes 3 and 4) and ammonium sulfate partially purified extracts (lanes 1 and 2) from S cells (lanes 2 and 4) and R cells (lanes 1 and 3) were electrophoresed on SDS–PAGE minigels and prepared for Western blotting with the 710 Ab. Arrow indicates the position of the 41 kDa immunoreactive protein.

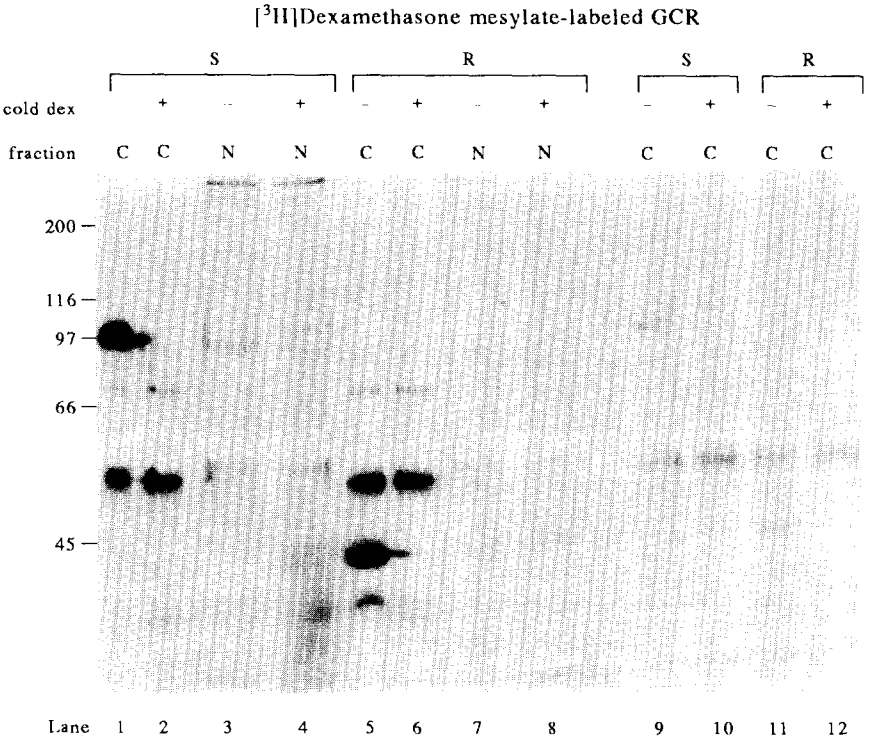

Fig. 10. Identification of [^{3}H]DM-labeled receptors in the crude membrane fractions of S and R cells. S and R cells in PBS were incubated with 100 nM [^{3}H]DM for 2 h at 4°C with (+) or without (−) a 500-fold molar excess of nonradioactive dex as described in Materials and Methods. Soluble extracts from the cytoplasmic (C) and nuclear (N) fractions were loaded in lanes 1–8 (100 μg protein per lane). Proteins extracted from the 10,000 *g* pellet of the cytoplasmic (C) fraction were loaded in lanes 9–12 (100 μl per lane). The gel was prepared for fluorography of [^{3}H]DM.

A 41 kDa immunoreactive protein was detected in ammonium sulfate-purified extracts of R cells (Fig. 9, lane 1, arrow). This protein was not an artifact of the SDS–PAGE gel since it was also detected on 2D Western blots [36a]. It is possible that this band is a degradation product of the TR-GCR, a post-translationally modified isoform, or possibly the product of a truncated GCR message (see Discussion).

[^{3}H]DM-labeled WT-GCR and TR-GCR are detected in crude membrane fractions

Recent reports have demonstrated the presence of GCRs and GCR-like proteins in the plasma membrane of cells [37–43]. Furthermore, Gametchu [40] found that the presence of membrane GCR in S49 lymphoma correlated with the effectiveness of steroid-induced apoptosis. It was of interest to determine whether there were any steroid-binding proteins in the membrane fractions of R cells which could potentially contribute to GC signaling. When whole cells were incubated with [^{3}H]DM and protein was extracted from the 10,000 *g* and the 100,000 *g* pellets from the cytoplasmic and nuclear fractions, protein specifically labeled with [^{3}H]DM was detected only in the 10,000 *g* cytoplasmic pellets. A 97 kDa band was detected in the S cell pellet (Fig. 10, lane 9) and a 45 kDa band was detected in the R cell pellet (Fig. 10, lane 11). Both bands were absent when cells were incubated with [^{3}H]DM plus a 500-fold molar excess of nonradioactive dex (Fig. 10, lanes 10 and 12). These bands were at the same molecular weight as bands in the soluble cytoplasmic fraction of cells corresponding to the [^{3}H]DM-labeled WT-GCR (Fig. 10, lane 1, 97 kDa) and the TR-GCR (Fig. 10, lane 5, 45 kDa). These data suggest that only the 45 kDa TR-GCR in R cells and the 97 kDa WT-GCR in S cells are the steroid-binding receptors through which the steroid signal is transduced. Specific labeling was not detected in the soluble nuclear fraction of S and R cells (Fig. 10, lanes 3 and 7) presumably because the labeling procedure (2 h at 4°C with the antiglucocorticoid [^{3}H]DM) favored maintenance of the nonactivated cytoplasmic GCR complex.

DISCUSSION

We have characterized the steroid-binding and the nonsteroid-binding GCRs present in S and R cells of the mouse P1798 lymphosarcoma. The NSB-GCR in R cells was present at 28% of the level of WT-GCR in S cells. Although this shows that the NSB-GCR in R cells is present at significantly lower levels than the WT-GCR in S cells, we cannot rule out the possibility that PA1-511 reacts less avidly with the NSB-GCR than with the WT-GCR.

There were clear differences in the subcellular distribution of all receptor forms in S and R cells. Interestingly, the 97 kDa NSB-GCR in R cells was localized predominantly in the nuclear fraction of R cells. Although we did not find immunoreactive TR-GCR in the *soluble* nuclear fraction of steroid-treated cells, we did detect immunoreactive TR-GCR in the crude membrane fraction of the nucleus. Surprisingly, we also found a 45 kDa immunoreactive protein in the crude membrane fraction of S cell nuclei.

Subcellular localization of GCRs in the mouse P1798 lymphosarcoma

A significant amount of 97 kDa immunoreactive GCR was found in the soluble nuclear fraction of dex-untreated S cells suggesting that a proportion of the unliganded GCR resides in the nucleus. A question that remains is whether any of this nuclear receptor in untreated S cells is competent for steroid binding. Interestingly, we did not detect [^{3}H]DM-labeled proteins in the soluble nuclear fraction of S cells (Fig. 10, lane 3) suggesting that the 97 kDa immunoreactive GCR in the soluble nuclear fraction is a NSB-GCR form. However, it is possible that [^{3}H]DM-labeled receptor is present below the level of sensitivity of the fluorography procedure. Another possibility is that other factors in the serum-containing culture medium induce a cytoplasmic to nuclear shift of unliganded GCR.

In addition to its nuclear localization shown here, in other work we have found that the NSB-GCR in R cells binds to the single GRE of the tyrosine aminotransferase gene (Rowan and Ip, submitted). Nuclear localization and GRE binding ability of the NSB-GCR thus necessitates a consideration that this protein may affect transcription. Although other nonsteroid-binding or reduced steroid-binding GCRs have been identified in clones of the mouse S49 lymphoma [44–46], human CEM C7 leukemia [33, 47, 48] and mouse WEHI-7 lymphoma [49], the subcellular localization and functional capacity of these receptors has not been fully assessed. One clone (ICR-27) of CEM C7 cells contains a nonsteroid-binding GCR that is localized in the cytoplasmic fraction of cells and is not translocated to the nucleus after steroid treatment in culture [33]. Given this lack of nuclear localization, it is unlikely that this form of the GCR could affect nuclear transcription.

In contrast to the predominantly nuclear localization of the NSB-GCR, TR-GCR immunoreactivity was not detected in the soluble nuclear fraction of R cells. It is possible that our inability to detect the TR-GCR in this fraction resulted from the fact that the amount of protein per unit volume of nuclear extract permitted loading of only 20 μg of protein per lane. On the same Western blots, we had to load 100 μg of cytoplasmic extract per lane to detect an immunoreactive signal. Hence, it is possible that if more nuclear extract could have been loaded an immunoreactive signal in the soluble nuclear fraction would have been detected. It is also possible that the TR-GCR is especially prone to

proteolysis in soluble nuclear extracts. We did, however, detect a 45 kDa immunoreactive band in the crude membrane fractions of R cell nuclei suggesting that the TR-GCR binds tightly to nuclear structures. This is in agreement with previous work showing that the 45 kDa nt^i receptor of S49 lymphoma [10] and the TR-GCR in the P1798 lymphosarcoma [11, 21] demonstrated an increased binding to nuclei and a higher affinity for DNA-cellulose when compared to WT-GCR. We also have evidence that the TR-GCR has a high isoelectric point (pI 8.1) under native conditions [36a]. This could also contribute to tight binding of the TR-GCR to nuclear structures. Tight binding of the TR-GCR to nuclear structures could markedly affect the availability of this receptor to interact with GRE-containing sequences.

Gametchu's laboratory [40, 42] has identified a 97 kDa [^{3}H]DM-labeled protein as well as several higher molecular weight [^{3}H]DM-labeled proteins in the membrane fractions of S49 lymphoma cells. These membrane proteins were postulated to be a component of GC signaling in these cells. Although we did not detect any [^{3}H]DM-labeled proteins in a pellet containing the plasma membrane (cytoplasmic 100,000 *g* pellet, data not shown), we clearly detected immunoreactive GCR in all the crude membrane fractions of cells. Further experimentation using GCs that bind noncovalently to the GCR will be necessary before we can rule out localization of steroid-binding GCR in the membrane fractions of P1798 lymphosarcoma cells.

The WT-GCR and the TR-GCR, but not the NSB-GCR, are responsive to temperature and heat-induced activation

There was little difference in the R_S of the NSB-GCR under activating and nonactivating conditions. However, a R_S of 6.6 nm for the NSB-GCR prepared under activating conditions, is higher than the R_S of 5.4 nm for the WT-GCR prepared in a similar manner. This suggests that either the NSB-GCR has a different conformation than the WT-GCR, or that some factor is bound very tightly to NSB-GCR resulting in the higher Stokes radius.

It is possible that the majority of the NSB-GCR resides in the nuclear fraction of R cells uncomplexed with hsp90. Recent work has shown that under non-physiological conditions of ATP depletion, the GCR in WEHI-7 cells loses steroid binding capacity and is recovered exclusively in the nuclear fraction of the cells as a dephosphorylated "null" receptor [50, 51]. Loss of steroid binding capacity of the "null" receptor is proposed to be the result of failure of these receptors to be reconstituted with hsp90 [52] since reconstitution of GCR with hsp90 is necessary for high affinity steroid binding [53, 54]. Our data suggest that the NSB-GCR is not in a heterooligomeric complex that could be dissociated with heat and NaCl. Further experimentation using antibodies directed against hsp90 will be necessary to examine whether this protein forms a complex with the NSB-GCR.

Generation of variant receptors in the P1798 lymphosarcoma

The primary sequences of the TR-GCR and the NSB-GCR in the P1798 lymphosarcoma are not known. Northern blot analysis of GCR mRNAs from the P1798 tumor revealed that while both S and R tumors contained large mRNA species, the R tumor also contained several smaller mRNA species [20]. Smaller mRNA species were also detected in the steroid-resistant nt^i variant of S49 lymphoma when compared to the parental, steroid-sensitive cell line [12, 55]. Recent work from our laboratory suggests that alternative splicing of GCR mRNA (exon 2 deletion) produces a smaller mRNA species that may code for the 45 kDa TR-GCR [20 and W. K. Shea and M. M. Ip, unpublished observations].

We also detected a 41 kDa immunoreactive protein in R cell extracts. This protein could be generated by an alternatively spliced transcript or by proteolysis of the TR-GCR during preparation of receptors. Interestingly, although the 41 kDa protein was detected on Western blots, a corresponding [^{3}H]DM-labeled protein was not detected on fluorographs. The same observation was made on 2D Western blots and 2D fluorographs [36a]. Given the intensity of the immunoreactive band for this protein relative to the intensity of the immunoreactive band for the TR-GCR, it would be expected that if the 41 kDa protein was labeled with [^{3}H]DM, a labeled band would be detected on fluorographs.

There are several possibilities for the generation of the NSB-GCR that have precedence in the literature, including point mutation in the steroid-binding domain [44, 47, 48] and alternative splicing resulting in C-terminal truncation [56]. It is also possible that posttranslational modifications of the WT-GCR generate the NSB-GCR. Future work in this laboratory is directed at cloning and sequencing mRNA transcripts in R cells that code for the NSB-GCR and the TR-GCR.

Steroid resistance in the P1798 lymphosarcoma

If the 45 kDa protein detected in the crude membrane fraction of S cells is truly the TR-GCR, then it would presumably be produced from a truncated mRNA species present at low levels in S cells. In other work, we have detected a 97 kDa nonsteroid-binding GCR in S cells that is not labeled with [^{3}H]DM [36a]. This suggests that P1798 cells may contain both WT and (presumably) alternatively spliced messages present in the cell and the level and/or stability of one message over the other would dictate whether the combination of WT-GCR + NSB-GCR or TR-GCR + NSB-GCR was expressed in a given cell, which in turn would dictate the responsiveness to GC.

This scenario is supported by the finding that switching between sensitivity and resistance in the P1798 lymphosarcoma occurs *in vivo* (W. K. Shea, D. Robinson and M. M. Ip, unpublished observations). While this is an attractive hypothesis, one must also consider that there are other differences in proteins between S and R cells that could potentially contribute to sensitivity or resistance to GC [18, 57].

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