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NEW SITE-DIRECTED POLYCLONAL ANTIBODY MAPS N-TERMINUS OF OCCLUDED REGION OF THE NON-TRANSFORMED GLUCOCORTICOID RECEPTOR OLIGOMER TO WITHIN BUGR EPITOPE

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Summary—Using a 17-mer synthetic peptide for immunization, a polyclonal antibody (WS933) directed against amino acid residues 395–411 of the mouse glucocorticoid receptor (GCR) has been raised and used to probe the significance of this region in forming the receptor oligomer and to localize the truncation site of the mutant GCR of the P1798 lymphosarcoma. This region of the receptor, which encompasses the BUGR epitope, is amino-terminal of and immediately adjacent to the DNA-binding domain. The polyclonal antibody WS933 reacted with both native and denatured forms of the wild-type mouse GCR as judged by its ability to shift the transformed receptor peak on Sephacryl S300 columns, to immunoadsorb the receptor to protein A Sepharose, and by immunoblot analysis where it identified the 98 kDa receptor protein in the cortisol-sensitive line of the P1798 mouse lymphosarcoma. WS933 also reacted with rat and rabbit GCR, but not human GCR. These characteristics were shared by the BUGR-2 monoclonal antibody. Unexpectedly, there were two highly significant differences between WS933 and BUGR-2. The first was the ability of WS933 to bind to the mutant 45 kDa GCR of the cortisol-resistant P1798 lymphosarcoma as judged by its capability of shifting the receptor peak on Sephacryl S300 columns. BUGR-2, in contrast, was unable to shift this mutant receptor peak. Secondly, WS933 was unable to react with the non-DNA-binding form of the wild-type (or mutant) GCR, whereas BUGR-2 could react with the non-DNA-binding form of the wild-type GCR. The first observation suggests that the truncation site of the mutant receptor may lie within a portion of the BUGR domain. Additionally, the second observation implies that at least part of the region lying within amino acid residues 395–411 of the mouse GCR is occluded in the receptor oligomer and that this site only becomes available upon transformation of the GCR to the DNA-binding form. This data provides the first mapping of the amino-terminus of the occluded region of the non-transformed receptor, and suggests that WS933 will be a useful probe for characterizing mutant as well as wild type glucocorticoid receptors.

INTRODUCTION

The glucocorticoid receptor (GCR) is a member of a large receptor superfamily which has been shown to regulate gene transcription via a direct interaction with specific hormone responsive elements on DNA. The GCR is organized into three well-characterized structural domains [1–3] (see Fig. 1) and has a molecular weight of approx. 98 kDa. The domains can be operationally defined based on the chymotrypsin/trypsin limited proteolysis cleavage sites [4]: (i) amino-terminal "modulatory" or "immunological" domain, mouse GCR residues 1–397 or 1–401; (ii) DNA-binding domain, mouse GCR residues 398–505; and (iii) steroid-binding domain, mouse GCR residues 506–783. The amino-terminal domain has been shown to carry the major immunological determinants of the receptor, and the majority of antibodies prepared against the purified GCR interact with epitopes in this region. There is also a transcriptional activation site in this domain [5]. The central DNA-binding region (amino acid residues 428–488) is rich in cysteine, lysine and arginine residues which are thought to be arranged as two zinc fingers which bind to the major groove of the DNA double helix [6–8]. Immediately upstream of the DNA-binding site is the BUGR epitope, which has been mapped to the area residing between amino acid residues

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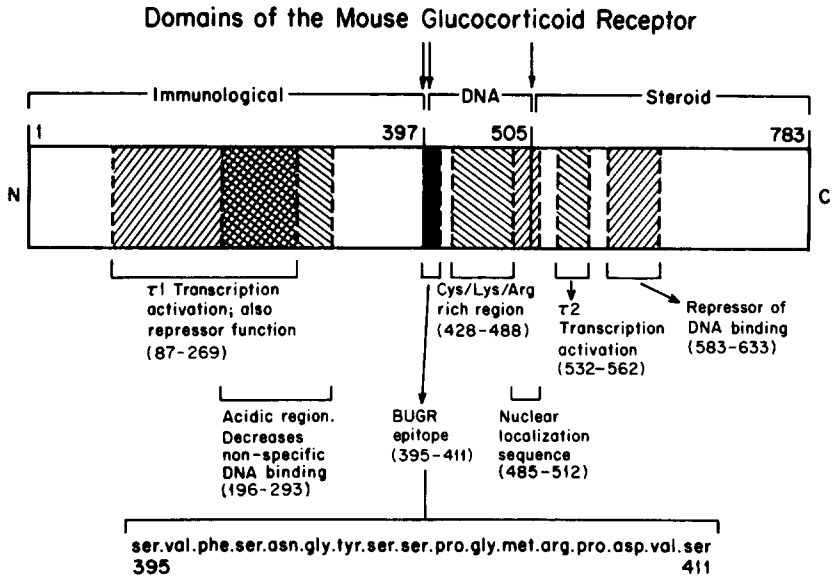

Fig. 1. Domain structure of the mouse GCR. This figure was compiled based on the data of Oro *et al.* [40] for the human GCR (tau 1 and repressor region in amino-terminal domain), Danielsen *et al.* [2] for the mouse GCR (acidic region, repressor region in carboxy-terminal domain), Rusconi and Yamamoto [3] for the rat GCR (BUGR epitope), Carlsted-Duke *et al.* [4] for the rat GCR (chymotrypsin and trypsin cleavage sites), Picard and Yamamoto [41] for the rat GCR (nuclear localization sequence), Hollenberg and Evans [5] for the human GCR (tau 2). Sequences are expressed in terms of the equivalent mouse amino acid residues. The arrows at the top represent the chymotrypsin (397, 401) and trypsin (505) cleavage sites, respectively. The peptide used for immunization (putative BUGR epitope) is shown at the bottom of the figure. The rat GCR has an identical BUGR sequence (amino acid residues 407–423), while the human GCR (amino acid equivalents 387–403) differs at two sites: mouse GCR ser 395 is replaced by human GCR thr 387 and mouse GCR gly 405 is replaced by human GCR ser 397.

395 and 411 of the mouse GCR [3]. The carboxy-terminal domain of the GCR contains the glucocorticoid-binding site, as well as a "repressor" region thought to inhibit GCR function in the absence of hormone [2] and a transcription activation region [5]. In the absence of steroid, the GCR exists as an oligomer (reviewed by Pratt *et al.* [9]), composed of one subunit of the GCR [10, 11] and 2 subunits of the 90 kDa heat shock protein (hsp90) [11–13], as well as other proteins including hsp56 [14–16] and hsp70 [14, 17]. Transformation to the DNA-binding form involves a dissociation of the subunits as well as a conformational change in the receptor subunit.

We have recently reported that the glucocorticoid-resistant line of the P1798 mouse lymphosarcoma contains a mutant 45 kDa GCR which appears to be truncated in the amino-terminal region [18]. This receptor is of considerable interest since it is functional in terms of its ability to induce specific proteins (glucocortin, glutamine synthetase and 5′-nucleotidase), but is unable to effect glucocorticoid-induced lysis [18]. Both the DNA- and steroid-binding domains appear to be intact in this mutant receptor [18, 19]; however it does not react with an antibody having determinants to the amino-terminal region [20] and is not detected by the BUGR-2 monoclonal antibody on Western blots [18]. The latter antibody, developed against the purified GCR by Gametchu and Harrison [21], was one of the first to recognize determinants of the receptor in regions other than the "immunological" domain. Interestingly, both BUGR-1 and BUGR-2 antibodies have been shown to react with the 40–45 kDa receptor fragment resulting from limited chymotrypsin digestion of the wild-type GCR [22–24] or from chymotrypsin-like tissue proteases [25, 26]. However, similar to the GCR from the cortisol-resistant (CR) line of the P1798 lymphosarcoma, there was no reactivity with the 45 kDa GCR of the nt^i mutant of the S49 lymphoma [24, 27]. Since the chymotrypsin cleavage sites lie within the BUGR epitope, both the CR-GCR of the P1798 lymphosarcoma and the nt^i-GCR of the S49 lymphoma may be truncated at an amino acid residue on the carboxy-terminal side of the BUGR recognition site. Alternatively, there may be an alteration in amino acid sequence at the truncated end of the mutant GCRs, as has been suggested by Dieken

et al. [28] for one of the nt[i]-mutant receptors of the S49 lymphoma.

The current report describes a new polyclonal antibody, WS933, which was developed against a 17-mer peptide synthesized to duplicate the amino acid sequence which encompasses the BUGR epitope region, amino acid residues 395–411, of the mouse GCR. Unexpectedly, this new antibody has been shown to react with both mutant and wild-type forms of the GCR in cortisol-resistant and -sensitive (CS) lines of the P1798 lymphosarcoma, suggesting that it will be a useful tool to probe structural differences between these two receptors. Moreover, this new antibody, unlike BUGR-2, was not reactive with the non-transformed, oligomeric form of the GCR, suggesting that the amino-terminus of the occluded site on the receptor oligomer extends to a site within the BUGR epitope.

MATERIALS AND METHODS

Reagents

[1,2,4-^{3}H(N)]Triamcinolone acetonide (TA) (29 Ci/mmol), [2,4,6,7,16,17-^{3}H]estradiol (150 Ci/mmol), 5α-dihydro[1,2,4,5,6,7-^{3}H]testosterone (DHT) (107 Ci/mmol), and [1,2,6,7-^{3}H]-progesterone (85 Ci/mmol) were purchased from Amersham Corp. (Arlington Heights, IL) and [6,7-^{3}H(N)]dexamethasone mesylate (49.9 Ci/mmol) from New England Nuclear/Dupont (Boston, MA). The BUGR-2 monoclonal antibody was generously provided by Dr Robert Harrison. Sephacryl S-300, CNBr-activated Sepharose 4B and protein A Sepharose were purchased from Pharmacia (Gaithersburg, MD), the reagents for electrophoresis from BioRad (Richmond, CA), nitrocellulose from Schleicher and Schuell (Keene, NH), and peroxidase-conjugated affinity purified rabbit anti-mouse and goat anti-rabbit IgG from Cappel/Cooper Biomedical (Malvern, PA) and ABTS (2,2′-azinodi-[3-ethylbenzthiazolinsulfonate]) from Boehringer Mannheim (Indianapolis, IN). All other reagents used were of the highest quality available.

Tumors, animals and cell lines

The glucocorticoid-sensitive and -resistant lines of the P1798 lymphosarcoma [29] are carried routinely in CDF_1 male mice bred in our laboratory from BALB/cAnNCr1BR female and DBA/2NCr1BR male mice (Charles River, Wilmington, MA). Tumors were transplanted as described previously [30]. The sensitivity or resistance of both tumor lines to glucocorticoids was checked at every passage, and for all studies reported in this paper, the tumor was verified to be either sensitive or resistant, as appropriate, to *in vivo* glucocorticoid treatment. Mice were kept in an air-conditioned room with a 12-h light–dark schedule. They were housed 5 per cage and given food (Teklad mouse chow) and water *ad libitum*. The well-differentiated R3327 Dunning rat adenocarcinoma was obtained initially from the Papanicoleau Cancer Research Foundation in Miami, FL. It was passaged at approx. 4 month intervals in male Copenhagen-Fischer F1 rats bred at the Springville facility of Roswell Park from female Fischer F344/NHsd and male Copenhagen (COP/Hsd) rats (Harlan Sprague–Dawley, Indianapolis, IN). Uterus for assay of estrogen and progesterone receptors was obtained either from female Copenhagen-Fischer F1 rats obtained from the breeding program of the Springville Laboratories or from CDF_1 female mice. Rats were housed 3–4 per cage and given food (Teklad rat chow) and water *ad libitum*.

All cell lines were carried in RPMI 1640 tissue culture medium containing 10% fetal calf serum (FCS), 50 μg/ml gentamycin and 25 mM HEPES. Cells were fed twice weekly.

Buffers

Buffers used in these experiments were as follows. (1) TEDGM: 10 mM Tris–HCl, pH 7.4 at 4°C, 1.5 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol, 20 mM sodium molybdate. (2) TEDG: TEDGM buffer with molybdate omitted. (3) TEG: TEDG buffer with dithiothreitol omitted. (4) Sephacryl running buffer (SRB): 20 mM Tris–HCl, pH 7.8 at 4°C, containing 20 mM sodium molybdate, 1 mM EDTA, 2 mM β-mercaptoethanol, 10% (v/v) glycerol, 0.02% (w/v) sodium azide and NaCl (100 mM for non-transformed and 400 mM for transformed receptors). (5) SDS sample buffer: 60 mM Tris–HCl, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol. (6) Transfer buffer: 12.5 mM Tris, pH 8.3 at 24°C, 96 mM glycine, 15% (v/v) methanol and 0.1% (w/v) SDS. (7) Buffer A: 10 mM Tris–HCl, pH 7.4, 0.9% (w/v) NaCl, 1% (w/v) bovine serum albumin (BSA). (8) TDM: 10 mM Tris–HCl, pH 7.8 at 4°C, 0.5 mM DTT, 10 mM sodium molybdate. (9) Borate buffer: 5 mM boric acid, 1.25 mM Na tetraborate, 3.75 mM NaCl, pH 8.4.

GCR Assays

Cytosol isolation and radiolabeling of GCR. P1798 tumors were homogenized in 3 vol of TEDGM buffer containing the following protease inhibitors unless noted otherwise: aprotinin, 60 μg/ml; soybean trypsin inhibitor, 100 μg/ml; PMSF, 0.3 mM; leupeptin, 0.1 mM; benzamidine, 25 mM; pepstatin A, 0.25 μg/ml; EGTA, 5 mM; and bacitracin, 100 μg/ml. Molybdate was omitted when samples were to be transformed. When samples were to be used for gel filtration or for Western blotting, homogenates were centrifuged at 12,000*g* for 10 min, 20 nM [^{3}H]TA ($\pm$ a 100-fold excess of unlabeled TA) was added, and the sample further centrifuged at 105,000*g* for 90 min at 4°C. Samples for fluorography were instead labeled with 50 nM [^{3}H]dexamethasone mesylate and DTT was omitted during homogenization. Following centrifugation, the radiolabeled cytosol was further incubated for 60 min at 4°C either in the presence of buffer (non-transformed samples) or NaCl to a final concentration of 0.4 M (transformed samples), treated with dextran-coated charcoal [18], and the supernate used for gel filtration. To determine if incubation with antibody changed the gel filtration pattern, transformed or non-transformed cytosols were diluted with TEDG (transformed) or TEDGM (non-transformed) buffer to reduce the salt concentration in the transformed sample to 0.1 M, and an equal volume of antibody in TEDG or TEDGM buffer added. Controls were incubated with either TEDG(M) buffer or with preimmune serum at a final dilution of 1:4, (v/v). Samples [at a final antibody dilution of 1:4, (v/v) and salt concentration of 50 mM] were then incubated for 1 h at 4°C prior to application to the gel filtration column.

Sephacryl S-300 gel filtration. Gel filtration was performed at 4°C using 1.6 x 82 cm columns of Sephacryl S-300 or Sephacryl S-300 HR equilibrated with SRB buffer. 2–5 mg of cytosol protein were applied to the column and the column eluted with SRB buffer at a rate of 10–12 ml/h. The columns were calibrated with 3 or more of the following standards: bovine thyroglobulin, Rs (Stokes radius) 8.6 nm; horse spleen apoferritin, Rs 6.1 nm; sweet potato β-amylase, Rs 4.8 nm; yeast alcohol dehydrogenase, Rs 4.6 nm; BSA, Rs, 3.6 nm; bovine erythrocyte carbonic anhydrase, Rs 2.3 nm.

Immunoadsorption, SDS–polyacrylamide gel electrophoresis, autoradiography and immunoblotting. In some experiments, the GCR was immunoadsorbed to protein A Sepharose using WS933 or BUGR-2 antibodies as follows. Protein A Sepharose (1 mg/ml) was added in a ratio of 1 ml protein A to 4 ml salt transformed cytosol (data shown in Fig. 6) or partially purified GCR (data shown in Figs 7 and 8), the mixture was incubated for 30 min at 4°C, and then centrifuged at 1075*g* for 10 min to remove non-specifically bound proteins. The supernate was then mixed with WS933 or BUGR-2 at final dilutions of 1:6 (v/v; data in Figs 6 and 7) or with BUGR-2 at a final dilution of 1:100 (v/v, data in Fig. 8). The samples were then incubated for 2 h at 4°C (Figs 6 and 7) or 16 h at 4°C (Fig. 8), followed by addition of protein A Sepharose at a dilution of 1:4 (v/v, data in Figs 6 and 8) or 1:6 (v/v, data in Fig. 7). After a further incubation for 30–60 min at 4°C, the sample was centrifuged and the pellet washed 3 times with TEG buffer and once with TEG buffer containing 0.3 M KCl. The pellet was then extracted by boiling in an equal volume of SDS sample buffer containing 10% (v/v) β-mercaptoethanol for 2 min, centrifuged, and the supernate applied to SDS-8% (w/v) full-length [Figs 6, 7 and 8(B)] or SDS-12% (w/v) mini [Fig. 8(A)] polyacrylamide gels essentially according to Laemmli [31]. For fluorography (Fig. 6), the gel was soaked in Fluor-Hance for 30 min, dried and subjected to fluorography using intensifying screens and Dupont Cronex 4 X-ray film at -70°C for 3 weeks. The following molecular weight standards were used for calibration of the SDS–PAGE gels: lysozyme, 14.4 kDa; soybean trypsin inhibitor, 21.5 kDa; carbonic anhydrase, 31 kDa; ovalbumin, 45 kDa; BSA 66.2 kDa; phosphorylase B, 97.4 kDa. Alternatively, samples to be used for immunoblotting were prepared and subjected to electrophoresis as described above, then the gels were transferred electrophoretically to nitrocellulose using an EC TransBlot or BioRad Mini TransBlot apparatus and transfer buffer. After transfer, the paper was washed in Buffer A containing 0.05% (v/v) Tween-20 and 1% (w/v) skim milk powder for 1 h at 24°C. The filter was then incubated with the WS933 antibody [1:4 (v/v) final dilution, Fig. 7 or 1:100 (v/v) final dilution, Fig. 8(A)] or with BUGR-2 [1:100 (v/v) final dilution, Fig. 8(B)] dissolved in buffer A/Tween for 2 h at 24°C and 16 h at 4°C, washed 5 times in Buffer A/Tween, then incubated for 2 h at 24°C with peroxidase-linked rabbit anti-mouse second antibody. The filter was then washed 5 times as above, and colour

developed using 3'3'diaminobenzidine [2 mg in 100 ml of 10 mM Tris–HCl, pH 7.5, 0.15 M NaCl, 1 mM EDTA and 0.1% (v/v) Triton X-100 buffer, followed by 10 μl of 30% (v/v) H_2O_2 per 50 ml DAB solution] for 10 min. Where noted, gels were scanned with a Molecular Dynamics Model 300A scanning densitometer, and the relative density of the bands quantitated by "Fast Scan" (ImageQuant Software), v 3.0.

Synthesis and purification of peptide

A 17-mer peptide encompassing the BUGR epitope of the mouse GCR, amino acid residues 395–411 (see Fig. 1), was synthesized by the Department of Biological Chemistry at the State University of New York at Buffalo, using a Biosearch SAM2 peptide synthesizer. The crude peptide was purified with a preparative HPLC System (Waters Chromatography Division of Millipore, Milford, MA). For purification, 104 mg peptide was dissolved in 20 ml 0.1% (v/v) TFA, filtered and injected onto a 5 × 30 cm radial compression cartridge (RCC) (Waters) packed with Vydac C18, 15–20 μ (The Separations Group, Hesperia, CA). The sample was eluted with 0.1% (v/v) TFA in acetonitrile–water (17:83, v/v) at a flow rate of 45 ml/min (μ = 0.055 cm/s). The major peak was collected at 49 min (identified by absorbance at 214 nm), lyophilized, then checked for purity on a 0.8 × 10 cm RCC packed with Vydac C18 (15–20 μ). A single peak was observed. The amino acid composition of the purified peptide was determined with a PICOTAG System (Waters) and found to be consistent with the primary structure of the 17-mer. The molecular weight of the purified peptide was determined in a Finnegan MAT90 (Finnigen MAT, San Jose, CA) and found to be 1787.1 (expected 1786.7).

Antiserum production and purification

The purified 17-mer was conjugated to KLH using glutaraldehyde [32], and for immunization, suspended in Freund's complete adjuvant at a concentration of 1 mg/ml. 2 ml was injected intradermally at multiple sites into each of 2 New Zealand white male rabbits (Becken Farm, Sanborn, NY). The rabbits were boosted 3 times at 4 week intervals using the same amount of peptide, but suspended in Freund's incomplete adjuvant. Rabbits were bled prior to immunization (pre-immune serum) and at 4 week intervals thereafter. The resulting WS933 antibody was purified by immunoaffinity chromatography on a peptide affinity column as described by Weigel *et al.* [33]. To prepare the column, the purified 17-mer (50 mg) was conjugated to 5 g CNBr-activated Sepharose 4B according to the instructions from the manufacturer (Pharmacia, Piscataway, NJ). Active fractions from the column were pooled and lyophilized. The purified antibody was resuspended at a concentration of 20 mg protein/ml buffer for use in the experiments described herein.

ELISA assay

To 96-well plates, 10 or 50 ng 17-mer peptide, dissolved in 100 μl borate buffer containing 0.05% (v/v) Tween-20 was added as a standard curve and incubated overnight at 4°C. The plate was washed 5 times with borate buffer/Tween and blocked for 2 h with 2% (w/v) BSA in borate buffer/Tween. After washing 5 times with borate buffer/Tween, 100 μl antiserum (dilutions of 1:2–1:200,000, v/v), preimmune serum (control), BSA (control) or buffer (control) was added to each well and the plate was incubated for 4 h at room temperature, washed again 5 times with borate buffer/Tween, then peroxidase-labeled second antibody (for BUGR-goat anti-mouse and for WS933 antibody-goat anti-rabbit) was added, followed by a 2 h incubation at room temperature. The plate was then washed with borate buffer/Tween, the color developed with ABTS/H_2O_2 [100 μl per well of a solution of 12.5 mg ABTS/50 ml 0.1 M Na citrate, pH 4.8 to which 16.7 μl of 30 (v/v) H_2O_2 was added], and the plate read on a Bio-Tek model EL311 microplate reader at 405 nm after a 30 min incubation.

DNA-cellulose-binding assay

Transformed and non-transformed GCRs were incubated with TEDG, TEDGM or antibody for 1–2 h at 4°C as described above. To test the ability of the GCR to bind to DNA-cellulose, 200 μl cytosol (final salt concentration was 0.05 M) containing approx. 500 μg protein was added to 200 μl DNA–cellulose (40 μg DNA) prepared in TDM buffer. The mixture was incubated at 4°C for 1 h, centrifuged, and the pellet washed 3 times, then resuspended in 0.5 mM TDM buffer, transferred to a scintillation vial, and counted in 10 ml aqueous scintillation fluid.

Progesterone, estrogen and androgen receptor assays

Cytosol prepared from mouse or rat uteri in TEDG buffer, was labeled with 50 nM

[^{3}H]progesterone (in the presence of a 1000-fold excess of unlabeled cortisol and in the presence or absence of a 100-fold excess of unlabeled progesterone) or 50 nM [^{3}H]estradiol (in the presence or absence of a 100-fold excess of unlabeled 17β-estradiol). Cytosol prepared in TEDG buffer from the Dunning rat R3327 prostate adenocarcinoma or the DMBA-induced rat mammary tumor was labeled with 5–50 nM [^{3}H]DHT in the presence or absence of a 200-fold excess of unlabeled DHT, or in the presence of a 200-fold molar excess of unlabeled dexamethasone. The labeled samples were transformed with NaCl as described above, treated with dextran-coated charcoal and aliquots incubated with antibody (1:4 final dilution, v/v) prior to application to Sephacryl S-300 columns as described above.

Statistics

Data are presented as means ± SEM. Statistical comparisons were carried out using Student's t-test.

RESULTS

The 17-mer peptide used for immunization is shown in the lower portion of Fig. 1. The structure encompasses the putative BUGR epitope, as defined by Rusconi and Yamamoto [3], and is identical in mouse and rat GCRs, but differs in the human GCR at mouse 395 (human 387) where thr is substituted for ser, and at mouse 405 (human 397) where ser is substituted for gly. HPLC analysis of the synthesized 17-mer showed one major and 2 minor peaks. Purification yielded a single HPLC peak, confirmed by amino acid analysis and mass spectroscopy to be the desired peptide (data not shown). This purified material was used for immunization.

Since this peptide was designed to encompass the putative BUGR epitope, initial studies were undertaken to compare the resulting new polyclonal antibody WS933, with the BUGR-2 monoclonal antibody. ELISA assays demonstrated that the WS933 antiserum had a higher titer against the 17-mer immunizing peptide than did BUGR-2 [Fig. 2(A) and (B)]. With WS933, a 1:500 dilution of antibody was needed to reduce the absorbance to 50% of the non-diluted values. In contrast, when BUGR-2 was diluted 1:7.5, the absorbance was < 50% of the original value, even though 50 ng of 17-mer was used in this ELISA instead of 10 ng used

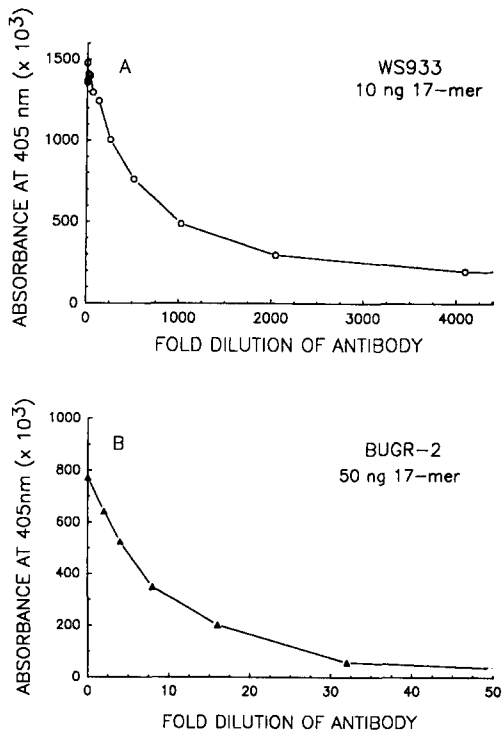

Fig. 2. The effect of antibody dilution on ELISA of WS933 (A) and BUGR-2 (B) antibodies against a fixed concentration of the 17-mer synthetic peptide. WS933 was tested against 10 ng 17-mer (A) and BUGR-2 against 50 ng 17-mer (B); little reactivity was observed when BUGR-2 was tested against 10 ng 17-mer.

with the WS933 antibody. This could reflect the fact that BUGR-2 was a hybridoma supernate in contrast to WS933 which was affinity purified using the 17-mer, or that the affinity or specificity of BUGR-2 for the 17-mer was decreased in comparison to WS933.

Antibody reactivity with DNA-binding and non-DNA-binding forms of the GCR as assessed by Sephacryl S300 chromatography

Wild-type GCR. It has been demonstrated by Wilson *et al.* [34] that an antibody raised against a portion (mouse GCR equivalent amino acid residues 457–471) of the second zinc finger of the DNA-binding domain does not interact with the non-transformed GCR, implying that this region of the GCR is occluded in the receptor oligomer. Since the BUGR epitope is adjacent to the DNA-binding domain (see Fig. 1), it was of interest to determine whether the limits of this occlusion might extend to the BUGR region of the GCR (amino acid residues 395–411). To examine this, cytosol and antibody were incubated as described in Materials and Methods, and the Stokes radius of the complex compared with that of the free GCR by chromatography

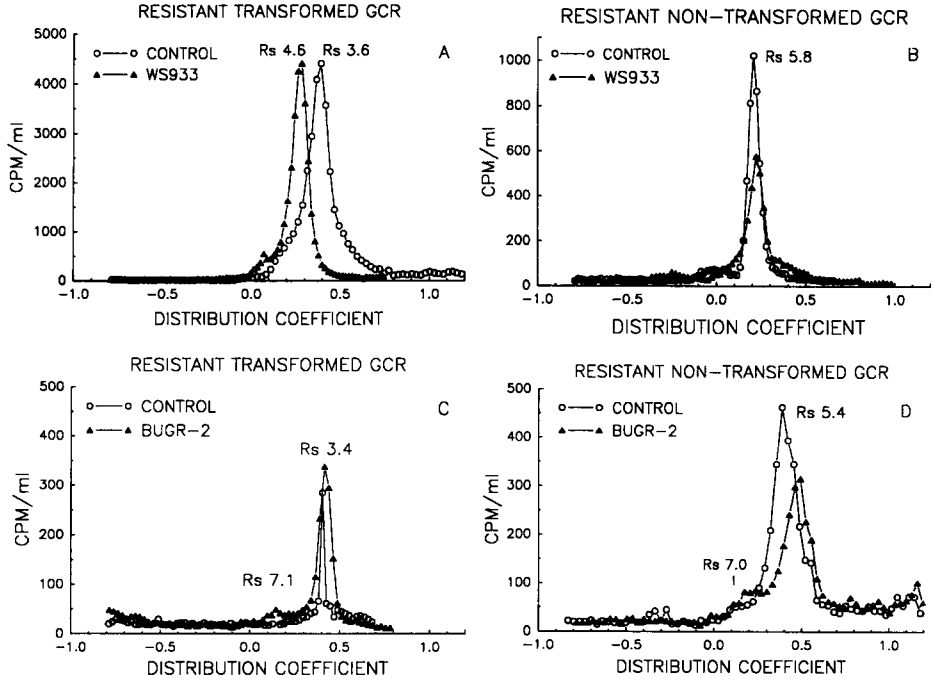

Fig. 3. The effect of the WS933 and BUGR-2 antibodies on the Sephacryl S300 gel filtration pattern of [^{3}H]TA labeled GCRs from CS P1798 lymphosarcoma. Tumors were homogenized in the presence of protease inhibitors, transformed or non-transformed as described in Materials and Methods, and labeled cytosol incubated with WS933 or BUGR-2 at a final dilution of 1:4, or, as control, preimmune serum at a final dilution of 1:4 (A) or TEDGM buffer (B, C, D).

on Sephacryl S300 gel filtration columns. Fig. 3(A) demonstrates that WS933 reacts with the transformed, DNA-binding form of the native GCR in CS P1798 tumor cytosol, as indicated by an increased Stokes radius in the presence of antibody. WS933 did not react, however, with the non-transformed, non-DNA-binding form of the CS-GCR [Fig. 3(B)], suggesting that its epitope is blocked in this receptor form. In contrast, BUGR-2 reacted with both transformed and non-transformed GCRs from the CS tumor line [Fig. 3(C) and (D)].

Mutant GCR. Since the mutant GCR of the CR line of the P1798 lymphosarcoma has not yet been sequenced, the truncation site of this DNA- and steroid-binding mutant receptor is not known. However, if WS933 were to bind to this receptor, this would suggest that at least a portion of the domain lying between amino acid residues 395–411 were present in this mutant. As can be seen in Fig. 4(A), WS933 does indeed bind to the transformed GCR from the CR tumor line, as evidenced by the increased Stokes radius in the presence of antibody. This figure also demonstrates the smaller size of the GCR from this tumor line when compared to that from the corresponding CS tumor [Fig. 3(A)]. Consistent with the observation with the CS-GCR, WS933 did not bind to the non-transformed, non-DNA-binding form of the CR-GCR [Fig. 4(B)]. In contrast to WS933, BUGR-2 was unable to shift the majority of the CR-GCR peak [Fig. 4(C)], although the small peak at Rs 7.1 in Fig. 4(C), which was a generally consistent finding, suggests that BUGR may interact with the GCR with low affinity, resulting in only a small percentage remaining bound during the overnight gel filtration. A similar minimal reactivity of BUGR-2 with the non-transformed GCR from the CR tumor was also observed [Fig. 4(D)]. A summary of the effect of both antibodies on the shifting of the Sephacryl peaks is presented in Table 1.

Binding of WS933 to the native GCR appeared to be site-specific, since preincubation of the antibody with the 17-mer immunizing peptide completely inhibited its ability to interact with the GCR (Fig. 5).

Immunoadsorption of [^{3}H]dexamethasone mesylate-labeled CS and CR GCRs with WS933 and BUGR-2

The ability of both WS933 and BUGR-2 to immunoadsorb the GCR to protein A Sepharose was next examined using SDS–PAGE fol-

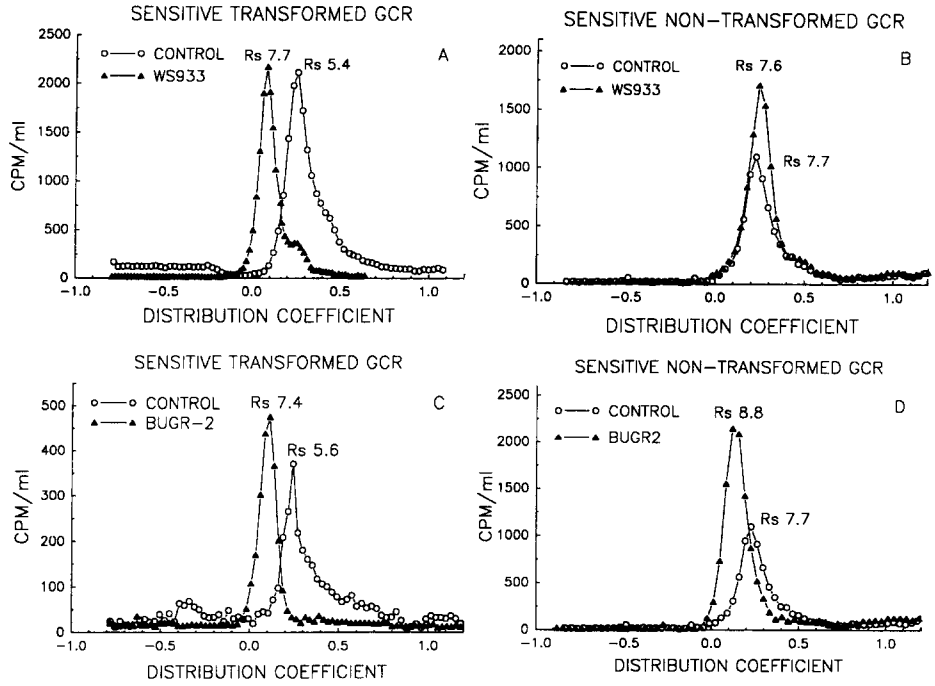

Fig. 4. The effect of the WS933 and BUGR-2 antibodies on the Sephacryl S300 gel filtration pattern of [^{3}H]TA labeled GCRs from CR P1798 lymphosarcoma. Tumors were homogenized in the presence of protease inhibitors, transformed or non-transformed as described in Materials and Methods and labeled cytosol incubated with WS933 or BUGR-2 at a final dilution of 1:4, or, as control, preimmune serum at a final dilution of 1:4 (A) or TEDGM buffer (B, C, D).

lowed by fluorography. As can be seen in Fig. 6, both the CS and CR-GCRs are immunoadsorbed by WS933 and BUGR-2. This figure also demonstrates that the steroid-binding GCR is a ~98 kDa species in the CS tumor and a ~45 kDa species in the CR tumor. That the latter is not a proteolytic artifact is suggested by the observation that the 45 kDa band was observed in both the presence or absence of proteolytic inhibitors, although interestingly, the 45 kDa band was stronger in the presence of the inhibitors. A steroid-binding 98 kDa band has not been observed in the CR tumor under any conditions [18]. Other data has shown that the 98 and 45 kDa bands are specifically labeled with [^{3}H]dexamethasone mesylate and that the bands seen at other molecular weights are nonspecifically labeled [18]. It is important to point out that in this figure, 50% more of the CR receptor was applied to the SDS–PAGE gel after immunoprecipitation with WS933, and twice as much after immunoprecipitation with BUGR-2, when compared to the CS receptor.

Immunoblotting of CS and CR GCRs

Using Western blot analysis, we have previously demonstrated [18] that BUGR-2 reacts with the 98 kDa GCR from the CS tumor. However, only minimal reactivity was observed with the 45 kDa steroid-binding GCR from the CR tumor. Additionally, BUGR-2 also identified a non-steroid-binding GCR of ~98 kDa

Table 1. Effect of WS933 and BUGR-2 antibodies on Stokes radius of GCRs from CS and CR P1798 lymphosarcoma

	Stokes radius (nm)		
	GCR	GCR + WS933	GCR + BUGR-2
CS-non-transformed	7.4 ± 0.1[a] (6)	7.5 ± 0.1 (4)	8.1 ± 0.3* (4)
CS-transformed	5.6 ± 0.0 (26)	7.2 ± 0.1* (9)	6.8 ± 0.6* (2)
CR-non-transformed	5.8 ± 0.1 (8)	5.8 ± 0.2 (5)	5.3 ± 0.2[b] (2)
CR-transformed	3.5 ± 0.0 (18)	4.8 ± 0.1* (5)	3.6 ± 0.1[b] (3)

[a]Data is presented as mean ± SEM. Number is in parentheses. [b]R_s of major species.
*$P < 0.05$ compared to corresponding control.

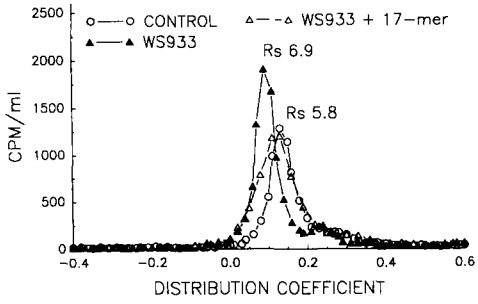

Fig. 5. The effect of the 17-mer synthetic peptide on the ability of WS933 to shift the [^{3}H]TA labeled GCR peak. The receptor was obtained from the CS P1798 lymphosarcoma. 1 ml of WS933 (20 mg protein/ml) was incubated with 200 μg purified 17-mer for 2 h at 4°C. This mixture was then incubated with labeled cytosol for 1 h at 4°C and the gel filtration pattern compared with that of antibody–cytosol and cytosol alone.

in the CR tumor [18]. When denatured GCRs from both tumors were transferred to nitrocellulose from SDS–PAGE gels and probed with the WS933 antibody, a 98 kDa receptor species was identified in both CS and CR tumors (Fig. 7). The antibody also recognized proteins with

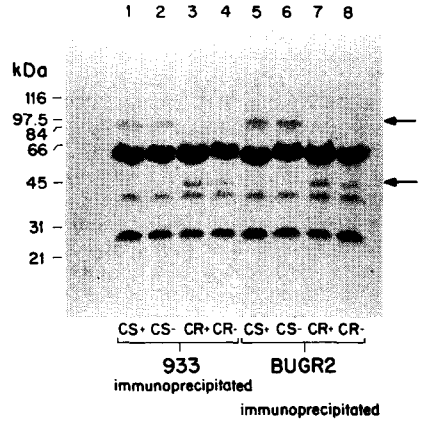

Fig. 6. Fluorogram of [^{3}H]dexamethasone mesylate labeled GCRs from CS and CR P1798 lymphosarcoma. CS and CR tumor samples were each homogenized in the presence of TEDG buffer in the absence (−) or presence (+) of the protease inhibitors described in Materials and Methods. After centrifugation and labeling with [^{3}H]dexamethasone mesylate as described in Materials and Methods, the samples were transformed by addition of NaCl to a final concentration of 0.3 M and incubated at 15°C for 30 min. Each sample was then diluted with TEG buffer (plus or minus protease inhibitors, as appropriate) to reduce the NaCl concentration to 0.2 M, immunoadsorbed to protein A Sepharose with either WS933 (labeled 933 in figure) or BUGR-2 as described in Materials and Methods, then applied to SDS–PAGE and fluorograms of the resulting gels prepared. Sample application to each lane was as follows (in μl): 1, CS+, 60; 2, CS−, 60; 3, CR+, 90; 4, CR−, 90; 5, CS+, 50; 6, CS−, 50; 7, CR+, 100; 8, and CR−, 100. Arrows indicate the positions of the 98 and 45 kDa steroid-binding receptor species. All other protein bands have been shown previously to be non-specifically bound by [^{3}H]dexamethasone mesylate [18].

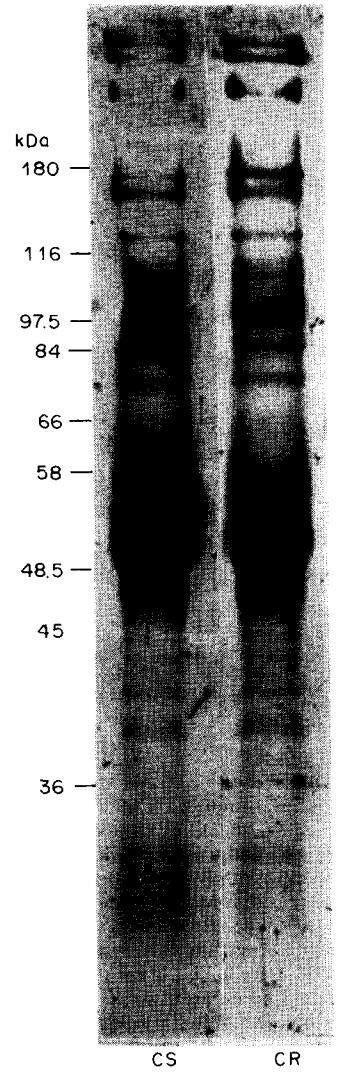

Fig. 7. Immunoblot of GCRs from CS and CR P1798 lymphosarcoma. CS and CR GCRs were partially purified for use in this experiment by ammonium sulfate treatment [50% (w/v), for 30 min at 4°C] of dexamethasone-labeled cytosol (prepared in TEDG buffer containing protease inhibitors), followed by application of the resuspended (in TEDG buffer containing protease inhibitors) pellet to a DNA–cellulose column. The column was washed with TDM buffer and the GCR eluted with TEG buffer containing 0.5 M KCl. After desalting on a Biorad 10 DG desalting column, CS and CR GCRs were immunoadsorbed to protein A Sepharose using WS933, extracted and applied directly to SDS–PAGE gels. After electrophoresis, the gel was electroblotted to nitrocellulose and the filters probed with WS933. Lane 1, CS-GCR. Lane 2, CR-GCR. The heavy band at 50–55 kDa is the heavy chain of the antibody used for immunoprecipitation (as demonstrated in control experiments; data not shown). The numbers at the left indicate the positions and molecular weights of the standards run concurrently.

molecular weights of 84, 70–72 and 47 kDa, possibly degradation products of the GCR, as well as some higher molecular weight species, most notably, one at ~180 kDa. Only faint

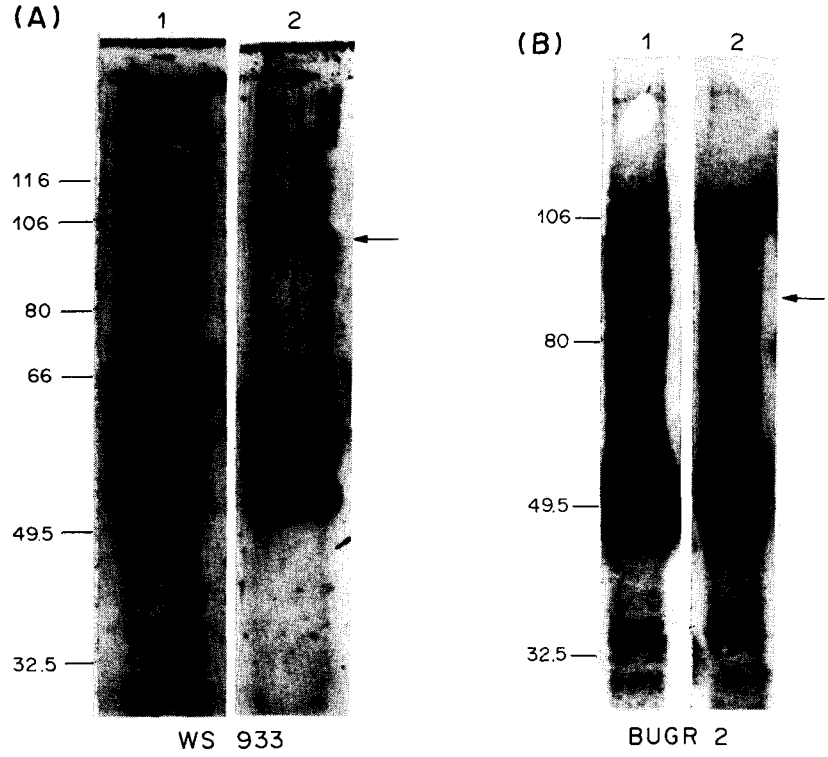

Fig. 8. Effect of the 17-mer immunizing peptide on the reactivity of WS933 and BUGR-2 on immunoblots. Cytosol was prepared from CS tumors, incubated with 50 nM unlabeled TA, precipitated with 35% (w/v) ammonium sulfate by incubation for 30 min at 4°C, centrifuged and the pellet resuspended in TEDG. Following dialysis against TEDG buffer, the sample was immunoadsorbed to protein A Sepharose by incubation with BUGR-2 at a final dilution of 1:100 (v/v) for 16 h at 4°C, then with protein A Sepharose as described in Materials and Methods. The samples were then resuspended in SDS–sample buffer, and applied in equal volumes to SDS–PAGE minigels (A) or SDS–PAGE full-length gels (B). After electrophoresis, the gels were electroblotted to nitrocellulose and the filters probed with WS933 (A) or BUGR-2 (B). Lane 1 in each case was probed with antibody alone, while lane 2 was probed with antibody that had been preincubated with the 17-mer peptide. For immunoblotting, each antibody (at a final dilution of 1:100, v/v) was preincubated either in the absence or presence of 100 μg 17-mer peptide (total volume 10 ml) for 2 h at room temperature, and then used to probe the nitrocellulose filter. Densitometric scanning of the 98 kDa bands was carried out as described in Materials and Methods. In A, the integrated volume absorbance was 2490 for WS933 alone, and 1089 for WS933 + 17-mer. In B, the integrated volume absorbance was 1165 for BUGR-2 alone and 94 for BUGR-2 + 17-mer. Molecular weight standards were run in parallel lanes, and the positions of the standards are marked on the ordinate. The arrows indicate the position of the 98 kDa GCR band.

reactivity with the steroid-binding 45 kDa GCR from the CR tumor was observed, however. Moreover, a similar faint band was noted in the CS tumor. In an attempt to determine the specificity of WS933 on immunoblots, CS tumor cytosol was partially purified with ammonium sulfate and BUGR-2 immunoprecipitation, electrophoresed, transferred to nitrocellulose and then probed with WS933 antibody that had been preincubated either in the absence or the presence of 100 μg of the 17-mer peptide used for immunization. As can be seen in Fig. 8(A) there was partial competition of the 17-mer for WS933 binding to the 98 kDa GCR species (the lane 2 band is reduced by 56%). We were unable to titrate this further because of an insufficient supply of the peptide. For comparative purposes, a parallel sample was probed with BUGR-2 which had been preincubated in the absence or presence of 100 μg of the 17-mer peptide. Fig. 8(B) shows that this concentration of the 17-mer competed very effectively for BUGR-2 binding to the 98 kDa GCR (the lane 2 band is reduced by 92%). The greater efficacy of the 17-mer in competing for BUGR-2 in this experiment may result from the fact that there are fewer GCR binding sites in the BUGR-2 preparation (which is a tissue culture supernate) than in the more concentrated affinity-purified WS933 antibody preparation; as a consequence, the 17-mer may more readily saturate the BUGR-2 GCR sites.

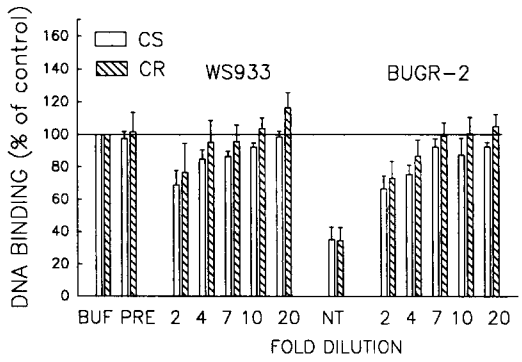

Fig. 9. Effect of WS933 or BUGR-2 on the ability of CS and CR-GCRs to bind to DNA–cellulose. Radiolabeled cytosol was incubated with buffer (BUF), pre-immune serum (PRE, at a 1:4 final dilution) or antibody at the various dilutions indicated on the figure, and then tested for binding to DNA–cellulose as described in Materials and Methods. NT represents the non-transformed control samples. Each point represents the mean ± SEM of 4–7 samples.

Effect of WS933 and BUGR-2 on DNA- and steroid-binding ability of GCR

Since the peptide sequence to which WS933 is directed is near the DNA-binding region of the GCR, it was possible that binding of antibody to the GCR would interfere with the subsequent ability of the GCR to bind to DNA. This was not the case, however, as can be seen in Fig. 9 which demonstrates that neither WS933 nor BUGR-2 interfered with the ability of the GCR from either tumor line to bind to DNA–cellulose. The slight inhibitory effect seen at the 1:2 dilution was not statistically significant, and is probably non-specific. Further studies demonstrated that preincubation of either CS- or CR-GCR with WS933 or BUGR-2 did not interfere with binding of [^{3}H]TA to the GCR at any of the dilutions tested above 1:2 (data not shown).

Reactivity of WS933 and BUGR-2 with GCRs from other species and with other steroid receptors

Both WS933 and BUGR-2 reacted with the GCR from rat and rabbit liver based on the ability of the antibodies to shift the GCR peak on Sephacryl S300 columns. In this series of experiments, the Stokes radius of the transformed rat liver GCR in the absence of antibody was 5.2 ± 0.1 nm and that of the rabbit liver GCR 5.1 ± 0.3 nm. In the presence of WS933, the rat and rabbit liver peaks were shifted to 6.1 ± 0.2 and 7.2 ± 0.6 nm, respectively. BUGR-2 shifted the peaks to 7.0 ± 0.5 and 7.5 ± 0.5 nm, respectively ($N = 2$–3 in each experiment). Neither WS933 nor BUGR-2 reacted with the GCR from human IM-9 lymphoma, human CRCC-CEM leukemia, or human MCF-7 breast cancer cells (data not shown). In addition, neither WS933 nor BUGR-2 reacted with the rat or mouse uterine estrogen or progesterone receptors as judged by their inability to shift the receptor peak on Sephacryl columns (data not shown). Using the same assay, apparent reactivity of both antibodies was seen with the androgen receptor from the Dunning R3327 rat prostate adenocarcinoma. This was judged to be non-specific, however, since unlabeled dexamethasone (as well as unlabeled DHT) could partially compete for the 4.9 nm (Stokes radius) [^{3}H]DHT peak on the Sephacryl column as well as in the charcoal-dextran assay (data not shown). Furthermore, in follow-up studies, WS933 did not bind to the androgen receptor in the DMBA-induced rat mammary tumor as judged by its inability to shift the peak on Sephacryl S300 columns. Additionally, dexamethasone was unable to compete for [^{3}H]DHT binding to the androgen receptor in this tumor (data not shown).

DISCUSSION

In this paper we describe a new site-directed polyclonal antibody which should be useful in characterizing mutant GCRs lacking the amino-terminal immunological domain. This antibody was developed by immunizing rabbits with a 17-mer synthetic peptide representing amino acid residues 395–411 of the mouse GCR, which has been reported by others to encompass the BUGR epitope [3]. Our initial goal in these studies was to produce an antibody with similar reactivity to the BUGR monoclonal antibody, since preliminary studies using immunoadsorption to protein A Sepharose had suggested that BUGR could identify both CS and CR-GCRs. Indeed, the resultant antibody would appear to be specifically directed to the amino acid residue 395–411 region of the GCR since the 17-mer immunizing peptide blocked the interaction of WS933 with the GCR. Unexpectedly, however, we developed an antibody with properties quite different from the monoclonal after which it was modeled. The two most significant differences are: (i) that WS933 reacts with the GCR from the CR line of the P1798 mouse lymphosarcoma as judged by its ability to shift the receptor peak on Sephacryl S300 columns, whereas BUGR-2 demonstrates only limited reactivity with this receptor by the same assay, and (ii) that WS933

reacts only with the DNA-binding form of the (CS and CR) receptor, whereas BUGR-2 reacts with both DNA- and non-DNA-binding forms of the (CS) receptor. As discussed in more detail below, these observations allow us to make two novel proposals regarding GCR structure: first, that the truncation site of the mutant GCR of the CR line of the P1798 lymphosarcoma lies within amino acid sequence 395–411 and secondly, that the portion of the GCR that is occluded in the non-DNA-binding receptor oligomer extends in the amino-terminal direction to a site within the peptide sequence between 395–411.

An explanation for the differences between WS933 and BUGR-2 is not readily apparent, although several possibilities can be considered. The first of these, which we consider the most likely, is that since deletion mutant analysis was used to infer that the BUGR epitope resided between mouse equivalent amino acid residues 395 and 411 [3], the exact epitope may be only a small portion of this region. This is discussed in more detail below. Secondly, in the studies in which the epitope characterization was undertaken [3], BUGR-1 rather than BUGR-2 was used. It is thus possible that the epitopes for these two monoclonal antibodies differ, although it should be noted that both fail to detect the GCR from the nt^i mutant of S49 lymphoma [24, 27] while still recognizing the chymotrypsin fragment of the wild-type GCR [22–24], suggesting that if a difference does exist, it is a subtle one. As noted above, limited chymotrypsin digestion is thought to cleave the GCR at mouse equivalent amino acid residues 397 and 401, both within the BUGR region. Thirdly, a trivial explanation of the difference between the two antibodies could be that the titer was very different; this might explain the very minimal reactivity of BUGR with the CR-GCR using Sephacryl analysis. This hypothesis is unlikely, however, since a low BUGR titer would not explain the difference in reactivity of the two antibodies with the non-transformed form of the CS receptor (BUGR reacts and WS933 does not).

Two questions are raised by the results of these experiments. The first is the ability of BUGR-2 to immunoadsorb the CR-GCR to protein A Sepharose, but not to recognize this receptor to any significant extent upon Sephacryl analysis. A possible reason for this apparent discrepancy is suggested by the observation of a consistent although minor fraction of a CR-GCR: BUGR-2 complex during Sephacryl gel filtration. It is possible that BUGR-2 may interact with the CR-GCR but with low affinity, perhaps because only a part of the epitope is present in this truncated receptor. As a result, during the overnight (up to 16 h) chromatographic step, the majority of the complex may dissociate, leading to the apparent conclusion that BUGR-2 does not bind to CR-GCR, when in fact it does, as the immunoadsorption data would suggest. Dissociation of the CR-GCR–BUGR-2 complex may not occur so readily during the immunoprecipitation step since the incubation time is much shorter, dilution does not occur and subsequent addition of protein A Sepharose may stabilize the complex.

A second interesting question arises from the observation that whereas WS933 can recognize the CR-GCR when the interaction is assessed by a shift in the Stokes radius of the GCR–antibody complex, or during immunoadsorption of the GCR to protein A Sepharose, this 45 kDa GCR species is not recognized on immunoblots. Based on the fact that the positive reactivities are with the native receptor, and the negative with the denatured receptor, it could be hypothesized that the antibody is conformation-specific, recognizing the CR-GCR only in its native conformation. It should be noted, however, that WS933 can recognize the ~98 kDa GCR in both its native and denatured forms; this would therefore suggest that there may be major conformational differences in the amino acid residue 395–411 regions of the CS and CR-GCRs. This in fact is likely, since the truncation site may be adjacent to or within this region and it would not be unexpected that the structures of the 2 proteins would be different. We also considered the possibility that since immunoadsorption of the 45 kDa GCR was detected under conditions in which [^{3}H]dexamethasone mesylate was used as a ligand, whereas the immunoblot analysis was done on GCRs labeled with TA or dexamethasone, that the covalent binding of dexamethasone mesylate might alter the conformation of this GCR species in such a way that it would be recognized by the antibody, whereas a non-covalently bound ligand (such as TA or dexamethasone) would not be. Preliminary immunoblot studies in our laboratory, however, could still not detect a 45 kDa receptor species when the immunoprecipitation step prior to SDS–PAGE and immunoblot analysis was carried out on dexa-

methasone mesylate labeled receptors. We are thus left with the hypothesis that WS933 reactivity with the 45 kDa receptor may be conformation specific. Indeed, there may even be a degree of conformation specificity in the interaction of WS933 with the wild-type receptor, based on the greater efficacy of the 17-mer to compete for WS933 binding to the native GCR (Fig. 5) than for the denatured GCR [Fig. 8(A)].

The data in this paper suggests that the WS933 antibody will be an important tool for characterizing the mutant CR receptor of the P1798 lymphosarcoma, in addition, possibly, to the nt^i mutant of S49 lymphoma. Moreover, the different reactivity between WS933 and BUGR-2 yields some important clues as to the actual epitope recognized by each antibody. Specifically, based on our data, as well as the report by Dieken *et al.* [28] that the S49 nt^i GCR may diverge from the wild-type receptor upstream of mouse equivalent amino acid residue 405, we hypothesize that the major portion of the BUGR-2 epitope lies between mouse amino acid residues 395–404, whereas the WS933 antibody recognizes a region or regions within amino acid residues 405–411 (as a polyclonal, WS933 may have multiple, possibly overlapping epitopes within the region for which it was designed). Additional support for this hypothesis is provided by the fact that amino acid residues 405–411 are very hydrophilic, suggesting that the latter half of the 17-mer used for immunization may have been more antigenic than the amino acid residues in the first half. Amino acid residue 405 is probably included within the WS933 epitope since WS933 does not recognize the human GCR, which differs at this site. Finally, based on the fact that BUGR-2 reacts with the non-transformed, oligomeric form of the native GCR (Fig. 3, also Refs [23, 35]), whereas WS933 does not react with the non-transformed CS receptor oligomer, we propose that residues 405–411 are occluded in the non-DNA-binding form of the receptor but that residues 395–404 are exposed and remain available to the antibody. It has been suggested that in the non-transformed receptor oligomer, hsp90 may associate with a specific domain in the steroid-binding domain of the receptor [36–38], thus blocking the DNA-binding site directly, by steric hindrance, or indirectly, by a conformational change in the receptor subunit. Support for this hypothesis comes from recent studies in which antibodies raised against specific sequences in the DNA-binding [34, 39] or hinge [33, 35], regions of the steroid receptor were unable to interact with the non-transformed form of the receptor. Results reported herein with WS933 extend the known limit of this occlusion by a component of the receptor oligomer and/or conformational change, to at least amino acid residue 405 of the mouse receptor which, as shown in Fig. 1, is on the amino terminal side of the DNA-binding site. This data provides the first mapping of the amino terminus of the occluded region of the non-transformed receptor.

The results from this study confirm our earlier observation [18] that the GCR from the CS P1798 lymphosarcoma is a 98 kDa steroid-binding protein which is recognized by the BUGR-2 monoclonal antibody, as well as by the new antibody, WS933, that we have described herein. In contrast, the CR tumor contains 2 mutant GCR species, a non-steroid-binding 98 kDa molecule which is recognized by both antibodies, and a steroid-binding 45 kDa molecule which, on Sephacryl analysis, is bound only by WS933.

In conclusion, using the new antibody WS933 directed against amino acid residues 395–411 of the mouse GCR, we report two novel observations on the GCR. First, the portion of the receptor that is occluded in the non-DNA-binding configuration extends in the amino-terminal direction at least as far as amino acid residue 405. Previous studies had mapped the occluded region only as far as the second zinc finger [34]. Secondly, a comparison of the data obtained with WS933 and BUGR-2 suggests that the truncation site of the mutant GCR in the P1798 lymphosarcoma lies within the 395–411 sequence. Although cloning of the CR-GCR will be required to precisely determine structural differences between the wild-type and mutant receptors, the current studies have provided us with important insights into the differences between these two proteins. We conclude that the polyclonal antibody WS933 will be a useful probe to further investigate structural and functional differences between mutant and wild-type receptors.

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