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Immunochemical analysis of the glucocorticoid receptor: Identification of a third domain separate from the steroid-binding and DNA-binding domains

(limited proteolysis/antiglucocortieoid receptor antibody/enzyme-linked immunosorbent assay)

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ABSTRACT **The glucocorticoid–receptor complex can be subdivided into three separate domains by limited proteolysis with trypsin or α-chymotrypsin. The following characteristics can be separated: steroid-binding activity (domain A), DNA-binding activity (domain B), and immunoactivity (domain C). We have previously reported the separation of the steroid-binding domain from the DNA-binding domain by limited proteolysis of the receptor with trypsin. In this paper, we report the detection by immunochemical analysis of a third domain of the glucocorticoid receptor, which does not bind hormone. Immunoactivity was detected by using specific antiglucocorticoid receptor antibodies raised in rabbits against purified rat liver glucocorticoid receptor and the assay used was an enzyme-linked immunosorbent assay. After digestion with α-chymotrypsin, the immunoactive region of the receptor (domain C) was separated from the other two domains (A and B). The immunoactive fragment was found to have a Stokes radius of 2.6 nm. Further digestion with α-chymotrypsin resulted in separation of the immunoactive fragment to give a fragment having a Stokes radius of 1.4 nm. The immunoactive domain could be separated from the half of the glucocorticoid receptor containing the steroid-binding and the DNA-binding domains (Stokes radius, 3.3 nm), by limited proteolysis of the receptor by α-chymotrypsin followed by gel filtration or chromatography on DNA-cellulose.**

Glucocorticoid–receptor complexes (GR) have been physicochemically characterized in a variety of tissues from various species and two predominant forms have been described. The larger of the two forms has a Stokes radius of ≈6.0 nm and a M_r of ≈90,000 (1–23). The smaller of the two forms is reported to have a Stokes radius of ≈3 nm and a M_r of ≈40,000 (3–12, 16–18, 20–24). In some of the earlier studies, the smaller form of the GR was found predominantly in the cell nucleus whereas the larger form was found in the cytosol (3–5, 7, 25). However, this was not the case in the rabbit lung (1, 2). It was later shown that the larger form of the GR could be recovered from liver cell nuclei under suitable conditions when proteolysis was reduced (7, 14). Thus, the appearance of the smaller form of the GR did not have any function with regard to the activation and nuclear uptake of the GR *in vitro*.

It has been shown that the larger (6-nm) form of the GR can be converted into the smaller (3-nm) form by limited proteolysis (4–9, 20, 21, 23). Extended proteolysis resulted in the formation of a third GR complex with a Stokes radius of ≈2 nm. The 6-nm GR form was susceptible to limited proteolysis by a variety of enzymes, such as trypsin, chymotrypsin, and papain (4, 5, 7, 8, 20, 21), as well as by endogenous proteases (4, 7, 9, 23), by extracts of purified lysosomes (4, 6, 7), and by incubation with purified nuclei (4, 7).

Conversion of the 6-nm GR (domains A, B, and C; cf. Fig. 5) into the 3-nm form (domains A and B) results in a complex that binds more tightly to DNA (5, 7, 20). The conversion of the 3-nm fragment into the 2-nm fragment (domain A)—e.g., by trypsin-digestion—results in loss of the DNA-binding activity (domain B). The only fragment that can then be detected, using radiolabeled hormone, is the ligand-binding fragment (domain A).

The conversion of the 6-nm complex (domains A, B, and C) into the 3-nm form (domains A and B) results in loss of about half of the receptor molecule (domain C) that was previously not possible to trace. The function(s) of this fragment is as yet unknown. However, results obtained by studies of corticosteroid-sensitive and -resistant clones of S49 or P1798 mouse lymphoma cells (16–22) indicate that the loss of a part of the glucocorticoid receptor similar or identical to domain C described in this paper results in corticosteroid resistance. This paper describes an immunochemical analysis of the structure of the GR carried out by using specific antibodies raised against purified rat liver GR (26) and directed toward this interesting domain of the receptor protein.

MATERIALS AND METHODS

Preparation of the GR. Rat liver cytosol was prepared using EPG buffer [20 mM sodium phosphate, pH 7.4/2 mM 2-mercaptoethanol/1 mM Na_2EDTA/10% (wt/vol) glycerol] as described (4). The cytosol was incubated for 60 min at 0°C with 9α-fluoro-11β,21-dihydroxy-16α,17α-(1-methylethylidinebis-[oxy])[6,7-^{3}H]pregna-1,4-diene-3,20-dione (triamcinolone acetonide; New England Nuclear; specific activity, 1.1–1.85 TBq/mmol). Prior to use, the labeled triamcinolone acetonide was diluted with unlabeled steroid to a specific activity of 333 GBq/mmol.

Proteolysis. Limited proteolysis of the GR was carried out as described (4, 5, 7) using α-chymotrypsin [Worthington; 81 units/mg of protein; 0.8 μg/(A_{280}–A_{310}) unit (5)] and incubating for 30 min at 10°C. The reaction was terminated by addition of lima bean trypsin inhibitor at 1:20 (mol/mol) (Worthington).

Gel Filtration. Gel filtration was carried out on Sephadex G-150 (Pharmacia Fine Chemicals, Uppsala, Sweden) or on agarose A-0.5m (Bio-Rad). Samples of up to 6 ml were applied on the Sephadex G-150 columns (60 × 2.6 cm) and eluted at 2 to 3 cm/hr. Samples of 2 ml were applied on the agarose A-0.5m

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Abbreviations: triamcinolone acetonide, 9α-fluoro-11β,21-dihydroxy-16α,17α-(1-methylethylidenebis[oxy])pregna-1,4-diene-3,20-dione; GR, glucocorticoid-receptor complexes; ELISA, enzyme-linked immunosorbent assay.

(90 × 1.6 cm) and eluted at 6 cm/hr. Prior to chromatography, the concentration of NaCl in the sample was adjusted to 0.15 M by addition of 5 M NaCl. Chromatography was carried out in EPG buffer/0.15 M NaCl/0.02% NaN_3. Some of the separations on Sephadex G-150 were also carried out in the presence of 20 mM Na_2MoO_4. In these cases, molybdate was added to the sample 30 min after the addition of tritiated steroid and 1 hr before application to the column. The columns were calibrated by using the following proteins: horse spleen ferritin, [^{14}C]methylated bovine serum albumin, ovalbumin, and whale skeletal muscle myoglobin (Sigma). The Stokes radii for these proteins were 6.15, 3.59, 2.86, and 2.01 nm, respectively (27).

DNA-Cellulose Chromatography. DNA-cellulose was prepared as described by Alberts and Herrick (28). Cytosol was diluted with EPG buffer to 5 ($A_{280} - A_{310}$) units/ml prior to incubation with triamcinolone acetonide. After incubation as described above, the cytosol was further incubated for 30 min at 25°C to activate the GR (5, 29, 30). The cytosol was then treated with dextran-coated charcoal and, when appropriate, incubated with α-chymotrypsin. Samples of 30 ml of cytosol were applied on 10 ml DNA-cellulose columns. After washing with three column volumes of EPG buffer, the columns were eluted with either a linear 0–0.5 M NaCl gradient in EPG buffer or a discontinuous gradient consisting of three column volumes of 0.09, 0.3, and 0.5 M NaCl in EPG buffer.

Antiglucocorticoid Receptor Antibodies. Antibodies against purified rat liver GR were raised in rabbits. After elution of the GR from the second DNA-cellulose column (15), the preparation was purified to apparent homogeneity by $NaDodSO_4$/polyacrylamide gel electrophoresis. The GR band was localized by staining parallel tracks, and the corresponding bands from unstained tracks were cut out and homogenized in Freund's complete or incomplete adjuvant (Difco). The rabbits were injected subcutaneously at 10–15 sites with a total of 10 μg of GR in Freund's complete adjuvant. After 4 and 6 wk, the rabbits received booster injections of 10 μg of GR in Freund's incomplete adjuvant. The specificity of the resulting antiserum was as described (26) and the titers were of the same order of magnitude.

Enzyme-Linked Immunosorbent Assay (ELISA). After chromatography, aliquots were taken from each fraction and assayed for radioactivity or for immunoactivity using an indirect competitive ELISA based on specific antibodies against the GR raised in rabbits as described above (26). Samples of 0.2 ml were incubated at 4°C overnight with 0.05 ml of antiserum purified on protein A-Sepharose (26) and diluted 1:60. After incubation, the amount of antibody not bound to antigen was measured on micro-ELISA plates coated with 40 ng of purified GR (15) in each well. Thus, color development in the well was inversely proportional to the amount of GR in the test sample.

The assay of GR using specific antibodies against it by ELISA has proved to be a useful and sensitive method for its detection (26). The minimum detection level is ≈0.2 nM GR with a maximum sample volume of 0.2 ml. The assay is specific. The assay is slightly affected by high concentrations of salt or by extreme pH. No interference of the ELISA was seen when α-chymotrypsin or lima bean trypsin inhibitor was added to the cytosol immediately prior to chromatography on agarose A-0.5m. The immunoactivity in these samples was identical to that of parallel samples of cytosol without the addition of either α-chymotrypsin or lima bean trypsin inhibitor (cf. Fig. 1).

RESULTS

Chromatography of untreated labeled cytosol on agarose A-0.5m (Fig. 1) gave a complex that eluted close to the void volume

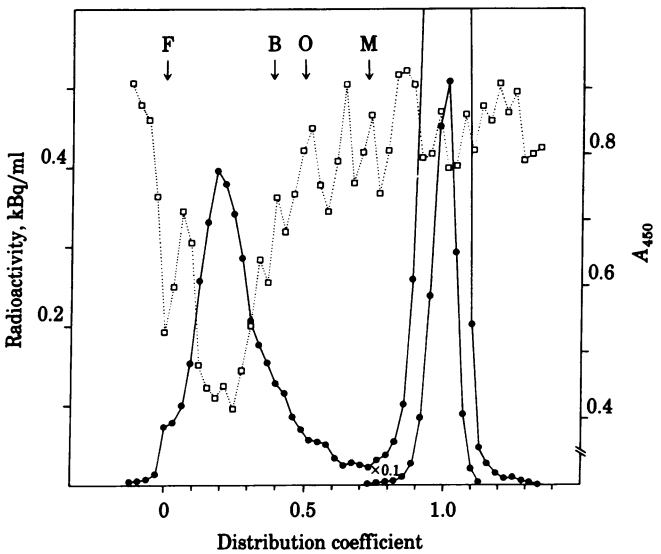

FIG. 1. Gel filtration of [^{3}H]triamcinolone acetonide-labeled cytosol on agarose A-0.5m. Cytosol was incubated with triamcinolone acetonide, the concentration of NaCl was adjusted to 0.15 M, and the sample was applied to the column and eluted with EPG buffer/0.15 M NaCl/0.02% NaN_3. After chromatography, the fractions were analyzed for radioactivity (●) and for immunoactivity (A_{450}; □). Markers: F, ferritin; B, bovine serum albumin; O, ovalbumin; M, myoglobin.

(domains A, B, and C; cf. Fig. 5). The size of the complex was above the linear part of the standard curve. Cochromatography of identical samples on Sephadex G-150 resulted in a peak corresponding to the 6.1-nm complex previously described (4–7). Analysis of the chromatogram after either agarose A-0.5m (Fig. 1) or Sephadex G-150 chromatography of the untreated labeled cytosol showed that the immunoactivity, as assayed by ELISA, cochromatographed with the radioactivity. A small varying amount of immunoactivity was also found in the void volume.

Treatment of the labeled cytosol with α-chymotrypsin prior

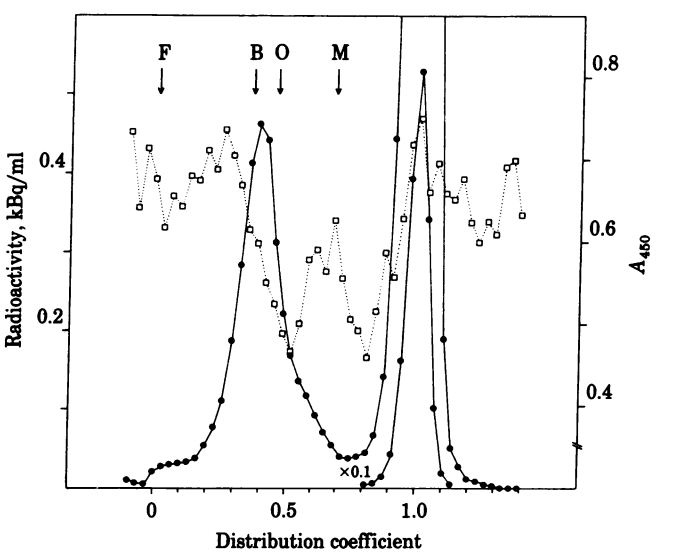

FIG. 2. Gel filtration of [^{3}H]triamcinolone acetonide-labeled α-chymotrypsin-treated cytosol. Cytosol was labeled with triamcinolone acetonide, labeled cytosol was incubated with α-chymotrypsin, and the reaction was terminated by addition of lima bean trypsin inhibitor. Chromatography on agarose A-0.5m was carried out as described in Fig. 1 and *Materials and Methods*. Fractions were analyzed for radioactivity (●) and for immunoactivity (A_{450}; □).

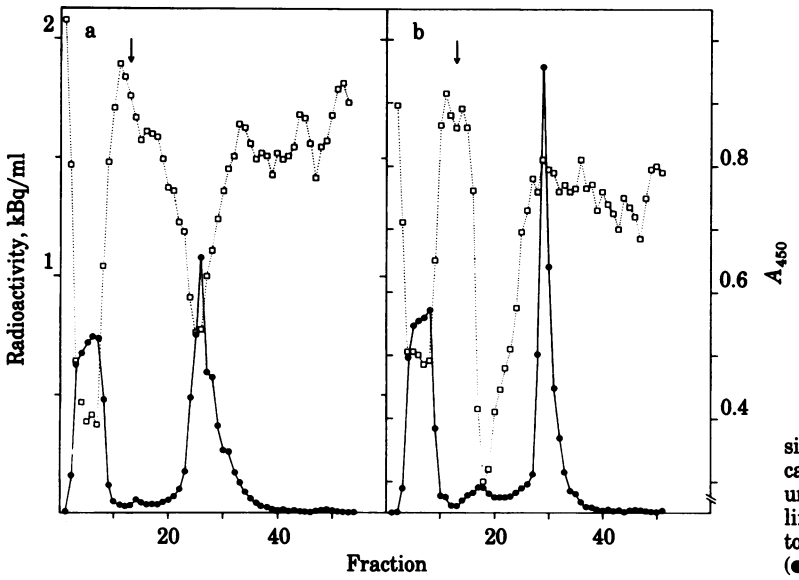

FIG. 3. DNA-cellulose chromatography of α-chymotrypsin-treated (*b*) or untreated (*a*) labeled cytosol. After application of dextran-coated charcoal-treated samples, the columns were washed with EPG buffer and then eluted with a linear 0–0.5 M NaCl gradient. ↓, Start of the gradient at the top of the column. Fractions were analyzed for radioactivity (●) and for immunoactivity (A_{450}; □).

to chromatography on agarose A-0.5m resulted in a radioactive GR fragment (domains A and B) that eluted at a volume corresponding to a Stokes radius of 3.29 ± 0.11 nm (mean ± SD; $n = 7$) (Fig. 2). The immunoactivity (domain C) did not cochromatograph with the radioactivity but eluted later as two distinct peaks with Stokes radii of 2.61 ± 0.02 nm and 1.44 ± 0.08 nm ($n = 3$). Prolonged proteolysis with α-chymotrypsin reduced the amount of the 2.6-nm peak and increased the amount of the 1.4-nm peak.

The addition of molybdate to untreated labeled cytosol followed by chromatography on Sephadex G-150 in the presence of molybdate resulted in elution of both the radioactive peak and the immunoactivity in the void volume (data not shown). If the cytosol was incubated with α-chymotrypsin prior to chromatography on Sephadex G-150 in the presence of molybdate, the radioactive complex (domains A and B) eluted close to the void volume whereas the immunoactivity (domain C) eluted at the same volume as in the absence of molybdate, corresponding to a Stokes radius of 2.6 nm. The immunoactivity was thus totally separated from the radioactivity in these chromatograms (data not shown). If the GR was first heat activated, addition of molybdate had no effect on the elution of radioactivity or immunoactivity, both when the cytosol had been treated with α-chymotrypsin and when it had not.

The radioactive GR fragment (domains A and B) could also be separated from the immunoactive fragment (domain C), after limited proteolysis with α-chymotrypsin, by chromatography on DNA-cellulose (Fig. 3). Chromatography of untreated labeled cytosol on DNA-cellulose followed by elution with a linear salt gradient resulted in the coelution of radioactivity and immunoactivity (domains A, B, and C) at 0.17 M NaCl (Fig. 3*a*). If, however, the cytosol was first incubated with α-chymotrypsin, the immunoactivity eluted at ≈0.06 M NaCl whereas the radioactive peak eluted at 0.25 M NaCl (Fig. 3*b*). Elution of the DNA-cellulose columns with a discontinuous salt gradient resulted in more specific elution of both the radioactivity and immunoactivity.

Cytosol proteins, after limited proteolysis, were separated on DNA-cellulose by elution with a discontinuous salt gradient and then analyzed by gel filtration on agarose A-0.5m (Fig. 4). Chromatography of the first peak from DNA-cellulose (0.09 M NaCl) gave a peak of immunoactivity eluting at a volume corresponding to a Stokes radius of 2.79 ± 0.15 nm ($n = 3$) and a small peak of radioactivity eluting at a volume corresponding

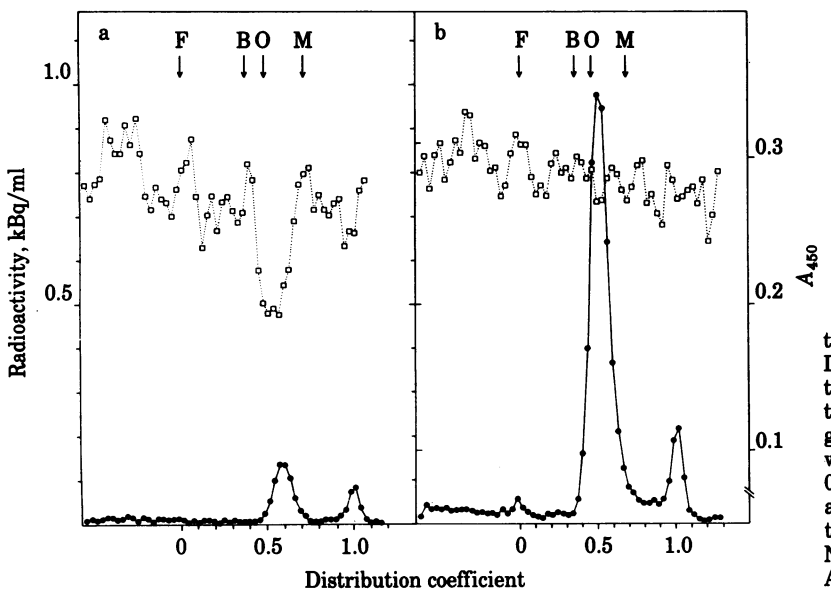

FIG. 4. Gel filtration on agarose A-0.5m of chymotrypsin-treated labeled cytosol previously separated on DNA-cellulose. After incubation with triamcinolone acetonide and treatment with chymotrypsin, cytosol was treated with dextran-coated charcoal and chromatographed on DNA-cellulose. The DNA-cellulose column was eluted with a discontinuous gradient consisting of 0.09 M, 0.3 M, and 0.5 M NaCl. After analysis for radioactivity (●) and immunoactivity (A_{450}; □), the peak fractions corresponding to the 0.09 M NaCl (*a*) and the 0.3 M NaCl (*b*) eluates were analyzed by gel filtration on agarose A-0.5m. Marker proteins are as in Fig. 1.

to 2.52 ± 0.06 nm. Chromatography of the second peak from DNA-cellulose (0.3 M NaCl) gave a peak of radioactivity eluting at a volume corresponding to a Stokes radius of 2.72 nm (n = 2). No immunoactivity at all was found in this chromatogram.

Labeled cytosol that was not heat activated was compared with heat-activated labeled cytosol by chromatography on DNA-cellulose after treatment with α-chymotrypsin. No difference was seen in the amount of immunoactivity eluting at 0.09 M NaCl in the two samples. However, no radioactivity eluted at either 0.09 M NaCl or 0.3 M NaCl if the cytosol was not heat activated prior to treatment with α-chymotrypsin (data not shown).

DISCUSSION

In this study, some differences were found when comparing the Stokes radii of the different forms of the GR obtained by analysis on two different matrices for gel filtration. Gel filtration on Sephadex G-150 gave complexes of the sizes previously reported, 6.1 and 3.6 nm (4–7). However, chromatography of identical samples of chymotrypsin-treated cytosol on agarose A-0.5m gave a complex that eluted at a volume corresponding to a Stokes radius of 3.3 nm. This difference can partially explain the large variation in sizes previously reported for the GR (1–24). However, regardless of the variations found for the different forms of the GR, it is clear that limited proteolysis of the native 6-nm form (domains A, B, and C) results in the formation of specific fragments. In other words, the receptor is not split into many small fragments by proteolytic digestion at the protease concentrations used here but is preferentially cleaved at a small number of sites highly sensitive for proteolysis. This is patent with regard to proteolysis by α-chymotrypsin (Figs. 2 and 3). In this case, two distinct fragments can be seen following cleavage of the 6-nm form of the GR. The larger form, in this study found to have a Stokes radius of 3.3 nm, retains the capacity to bind both the steroid and DNA (domains A and B) (5, 7). Another distinct fragment (domain C), having a Stokes radius of 2.6 nm (Fig. 2), can be demonstrated by using an ELISA. This fragment contains the immunological determinant(s) for the GR. We have succeeded in raising antibodies against purified rat liver GR in several rabbits. Of the seven positive antisera tested, all are directed against the same fragment of the GR (domain C) and do not react with the 3.3-nm form (unpublished results). The 2.6-nm fragment has very low affinity for DNA (Fig. 3*b*) and can be digested to give a smaller immunoactive fragment that has a Stokes radius of 1.4 nm. We cannot, at this stage, exclude that other small fragments are formed following treatment with α-chymotrypsin that do not react with our antibodies. The affinity of the 2.6-nm fragment for DNA appears to be nonspecific, as it is eluted at a low concentration of NaCl. Furthermore, heat activation is not a requirement for DNA binding of this fragment. DNA-cellulose chromatography of cytosol that has not been heat activated but has been treated with α-chymotrypsin enables the 2.6-nm fragment to be totally separated from the 3.3-nm fragment.

Molybdate has previously been shown to interact with the nonactivated receptor but not with the activated GR (23). The result of this interaction is the formation of GR complexes that have larger Stokes radii. The addition of molybdate to the 3- to 3.6-nm form results in the formation of a GR complex that has a Stokes radius of 7.1 nm (23). The addition of molybdate to the native 6-nm form results in a complex that has a Stokes radius of 8 nm. In this study, addition of molybdate to labeled cytosol resulted in elution of radiolabeled GR in the Sephadex G-150 void volume (Stokes radius, >7 nm), even when the labeled cytosol had been incubated with α-chymotrypsin (domains A, B, and C or A and B). However, after limited proteolysis of the labeled cytosol, addition of molybdate had no effect on the 2.6-nm fragment containing the immunoactivity. After heat activation of the chymotrypsin-treated labeled cytosol, addition of molybdate had no effect on either the radioactive 3- to 3.6-nm fragment (domains A and B) or on the immunoactive 2.6-nm complex.

After chromatography of the chymotrypsin-treated labeled cytosol on DNA-cellulose, the radiolabeled fragment was found to have a smaller (2.5–2.7 nm) size when rechromatographed on agarose A-0.5m (Fig. 4*b*). The reason for this difference is unclear. However, it was previously found that GR complexes extracted from purified nuclei or DNA-cellulose were a little (≈0.5 nm) smaller than the corresponding forms of the GR found in the cytosol (4, 5, 7). It is possible that the larger size of the GR in the cytosol is the result of its nonspecific interaction with other proteins. The addition of rat liver cytosol to GR purified on DNA-cellulose results in an increase (≈0.5 nm) in Stokes radius to the size normally found in the cytosol (unpublished results). No change in size was seen for the immunoactive 2.6-nm fragment (domain C) after chromatography on DNA-cellulose.

The peak of radioactivity eluting from DNA-cellulose at 0.09 M NaCl and having a Stokes radius of 2.5 nm on agarose A-0.5m (Fig. 4*a*) appears to represent the radioactive fragment recovered in the 0.3 M NaCl peak from DNA-cellulose (domains A and B). Neither of these two radioactive peaks is found if the cytosol is not first heat activated, whereas the immunoactivity found in the 0.09 M NaCl peak is recovered irrespective of prior heat activation. Thus, the small amount of radioactivity eluting in the first peak on DNA-cellulose is not associated with the immunoactive fragment but probably represents loosely bound radioactive GR (domains A and B). Alternatively, it may represent a minor fraction (8–9%) of a receptor fragment slightly smaller than domains A and B and having similar functions in terms of steroid binding but less affinity for DNA.

A functional difference between the 6-nm and the 3-nm forms of the GR has been described in the mouse lymphomas S49 and P1798 (16–20). In the normal wild-type corticosteroid-sensitive strain, the GR has a Stokes radius of ≈6 nm and a M_r of 87,000–90,000. However, in the corticosteroid-resistant P1798 and S49 lines that demonstrate increased nuclear uptake of the GR (nt^i; corticosteroid resistant), the GR has a Stokes radius of ≈3 nm and a M_r of 39,000–50,000. This difference does not appear to be the result of increased proteolysis of the GR in the resistant cell lines, although, in sensitive cell lines, it can be cleaved to a form similar to that from the resistant cell lines by limited proteolysis with α-chymotrypsin (18, 31). It has previously been reported that GR from corticosteroid-sensitive P1798 cells or from human chronic leukemia cells interacts with specific antibodies against the GR whereas that from corticosteroid-resistant cell lines does not bind to antibodies raised against purified GR (22). In contrast to the studies reported here, in which limited proteolysis of the GR results in the formation of a separate complex that is immunoactive (domain C), no such immunoactivity can be found in the cytosol from corticosteroid-resistant cells (32). Limited proteolysis of the GR from corticosteroid-sensitive cells, however, gives rise to an immunoactive complex (domain C), just as in this study.

Chymotrypsin-treated GR or GR from resistant cell lines binds more tightly to DNA or to purified cell nuclei than native GR from sensitive cell lines does (19, 20). This is analogous to the DNA binding of the different forms of GR in rat liver (5). However, this DNA-binding represents primarily nonspecific binding of the GR to any form single or double stranded, of DNA (5, 30).

A model for the functional domains of the glucocorticoid re-

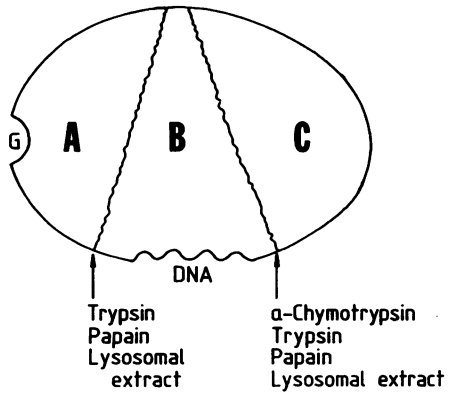

FIG. 5. Model for the functional domains of the GR. Domain A contains the steroid binding site, domain B contains the DNA binding site, and domain C contains the immunological determinant(s).

ceptor is shown in Fig. 5. The receptor protein can be cleaved into three specific fragments by limited proteolysis. After treatment with α-chymotrypsin, two fragments are obtained. The larger fragment, the 3-nm form of the GR (domains A and B), contains both the steroid- and the DNA-binding sites. The smaller fragment, the 2.6-nm fragment (domain C), contains the immunological determinant(s). Domain C can be further cleaved by prolonged proteolysis with α-chymotrypsin. Further proteolysis of domains A and B with trypsin results in the formation of a 1.9-nm form of the GR (4–7) that only contains the steroid binding site (domain A). The function of domain C is still unclear. However, in light of the results obtained previously with S49 and the P1798 cells (see above), it appears that this domain is required to retain the biological activity of the GR.

The purified 6-nm form of rat liver GR has been shown to specifically interact with the mouse mammary tumor virus gene (33), a gene that is specifically induced by glucocorticoids (34, 35). The roles of the various domains of the GR complex described above in the specific interaction with DNA should provide further information concerning the biological functions of the domains.

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