Influence of Limited Proteolysis on the Physicochemical and DNA-binding Properties of Glucocorticoid Receptors from Corticoid-sensitive and -resistant Mouse Lymphoma P1798

John Stevens and Yee-Wan Stevens
Research Institute, Joint Diseases North General Hospital, Mount Sinai School of Medicine, New York, New York 10035

ABSTRACT

We have compared the DNA-cellulose-binding properties of cytosolic ([3H]triamcinolone acetonide ([3H]TA)-receptor complexes from the corticoid-sensitive (CS) and corticoid-resistant (CR) strains of mouse lymphoma P1798. About 50% of heat-activated 61-Å (Stokes radius) CS complexes and close to 60% of heat-activated 28-Å (Stokes radius) CR complexes bound to DNA-cellulose. [3H]TA complexes from the CR lymphocytes were more refractory to extraction from DNA-cellulose with MgCl2, spermidine, and KCl than were complexes from the CS cells. This behavior closely mimics the type of interaction previously observed after nuclear binding of [3H]TA in intact P1798 cells at 37°C.

Limited digestion (30 min at 10°C) of the 61-Å CS P1798 [3H]TA complex with chymotrypsin converted it to a 28-Å form in which interaction with DNA-cellulose (i.e., extractability with MgCl2, spermidine, and KCl) was indistinguishable from the interaction with DNA-cellulose of undigested 28-Å CR P1798 [3H]TA-receptor complexes. Chymotrypsin treatment (30 min; 10°C) of [3H]TA complexes from CR lymphocytes did not alter their Stokes radius or their extractability from DNA-cellulose. Proteolysis (30 min; 10°C) of CS and CR P1798 [3H]TA complexes with trypsin gave rise to a discrete 19-Å (Stokes radius) fragment that did not bind to DNA-cellulose. In all instances, the Stokes radii were determined on heat-activated (1 hr; 20°C) [3H]TA-receptor complexes.

Our results suggest that differences in glucocorticoid-receptor complex interaction with the nucleus between CS and CR P1798 lymphocytes may be determined by receptor properties. Furthermore, our findings are consistent with the idea that DNA and/or other anionic species may be an important part of nuclear acceptor sites in both strains of tumor. Our data also support the hypothesis that the portion of the CS P1798 complex that is digested by chymotrypsin, i.e., the 61 → 28 Å region, may play an important role in modulating glucocorticoid-receptor complex interaction with the nucleus in CS tumor lymphocytes.

INTRODUCTION

Many steroid effects on target tissues appear to require binding of the hormone to specific intracellular receptors and interaction of the resulting hormone-receptor (HR) complexes with the nucleus (5, 16, 18, 23, 41). However, nuclear binding of HR complexes is not always followed by the expected phenotypic changes. This has been particularly well documented in the case of glucocorticoid action on malignant lymphocytes. For example, Sibley and Tomkins (35) isolated variant clones of mouse lymphoma S49 that displayed appreciable levels of nuclear glucocorticoid binding but were unaffected by concentrations of dexamethasone that caused extensive lysis of CS lymphocytes. Nuclear glucocorticoid binding has also been described in CR mouse lymphoma P1798 (21, 42), in CR mouse thymoma W7 (3), and in a variety of CR human lymphoproliferative disorders (8, 11).

We recently found that low concentrations of KCl, MgCl2, and spermidine extracted [3H]TA-receptor complexes more efficiently from CS than from CR P1798 nuclei (42). In subsequent work, we showed that CR P1798 lymphocytes contained a 28-Å (Rg) 3.3-S glucocorticoid receptor that was considerably smaller and more symmetrical than the 61-Å (Rg) 3.7-S receptor present in CS P1798 cells (40). Wränge and Gustafsson (48) reported that limited proteolysis of the 61-Å (Rg) rat liver glucocorticoid receptor gave rise to a 36-Å (Rg) fragment that bound more tightly to DNA-cellulose than did native dexamethasone-receptor complexes. Based on these observations, we speculated that the CS P1798 [3H]TA-binding component might consist of a portion that resembled the CR P1798 receptor plus an additional region that enabled it to bind "normally" to the nucleus (40).

In this paper, we show that interaction of CS and CR P1798 [3H]TA-receptor complexes with DNA-cellulose closely mimicked their previously described mode of interaction with the nucleus and that chymotrypsin digestion of the 61-Å CS P1798 complex gave rise to a fragment the physicochemical and DNA-binding properties of which were indistinguishable from those of the 28-Å CR P1798 complex. These results are consistent with the idea that DNA and/or other anionic species may be an important part of nuclear acceptor sites for CS and CR P1798 glucocorticoid-receptor complexes, and they support the hypothesis that the portion of the CS P1798 receptor that is digested by chymotrypsin (the 61 → 28 Å region) may play an important role in modulating HR complex interaction with the nucleus in CS lymphocytes.

MATERIALS AND METHODS

Chemicals and Radioisotopes. Highly polymerized double-stranded calf thymus DNA, Coomassie brilliant blue G250, chymotrypsin (crystallized and lyophilized 3 times; 50 units/mg protein), DFP-chymotrypsin (crystallized and lyophilized 3 L-tosylamide-2-phenylethyl chloromethyl ketone; SBTI, soy bean trypsin inhibitor; LBTI, lima bean trypsin inhibitor; DTT, dithiothreitol; TES, tris(hydroxymethyl)aminomethylphenyl)phosphoglycerate; TLCK, p-tosyl-L-lysine chloromethyl ketone; TPCK; and/or other anionic species may be an important part of nuclear acceptor sites in both strains of tumor. Our data also support the hypothesis that the portion of the CS P1798 complex that is digested by chymotrypsin, i.e., the 61 → 28 Å region, may play an important role in modulating glucocorticoid-receptor complex interaction with the nucleus in CS tumor lymphocytes.

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3 The abbreviations used are: HR, hormone-receptor; CS, corticoid-sensitive; CR, corticoid-resistant; TA, triamcinolone acetonide; Rg, Stokes radius; DFP, dizopropylfluorophosphate; TLCK, p-tosyl-L-lysine chloromethyl ketone; TPCK,

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together with trypsin, this amount of SBTI completely inhibited the addition of a 5-fold excess, by weight, of SBTI. When added to the tubes to an ice water bath followed by the immediate digestion with trypsin was stopped by transferring the samples was incubated for 30 min was added after which the samples were incubated for 30 min to 10°, free steroid was adsorbed with activated charcoal (24). Close to 4°C, free steroid was adsorbed with activated charcoal (24). Close to maximal binding was achieved under these conditions. Specific binding was calculated as the difference, after charcoal treatment, between dpm/mg protein in cytosol incubated with [3H]TA alone and dpm/mg protein in cytosol incubated with radioactive plus nonradioactive steroid. Nonspecific binding was 2 to 5% of the total binding level in CS cytosol and 5 to 10% in CR cytosol. In a typical experiment, specific binding was 0.75 pmol of [3H]TA per mg protein in CS cytosol and 0.3 pmol of [3H]TA per mg protein in CR cytosol. Protein was measured with Coomassie blue according to Sedmak and Grossberg (30). DTT does not interfere with this assay. The method of Lowry et al. (20) gave comparable results after protein concentration of undiluted cytosol was 2 to 5% of the total binding level in CS cytosol and 5 to 7 mg/ml. The sources of all other chemicals and radioisotopes were the same as in our earlier publications (40, 43). The specific activity of the [3H]TA used for these studies was either 31.3 or 37.1 Ci/mmol.

Buffers. The pH of the buffers was 7.5 at 4°C. On the day of experiment, DTT was added to all buffers at a final concentration of 0.5 mM (for column elution and glycerol gradient centrifugation), 1 mM (for washing DNA-cellulose), or 10 mM (for preparing cytosol or extracting bound [3H]TA complexes from DNA-cellulose).

Source of Tumor and Preparation of HR Complexes. The CS and CR strains of mouse lymphoma P1798 were maintained in female BALB/c mice (Mammalian Genetics and Animal Production Section, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md.) as described previously (42). Cell suspensions were prepared from the solid tumors as in an earlier publication (42). The cells were homogenized in a 4°C cold room with 10 strokes of a motor-driven glass-Teflon Potter-Elvehjem homogenizer in 5 volumes of 8 mM Na2HPO4:4 mM EDTA containing 10 mM DTT and either 50 mM [3H]TA alone or 50 mM [3H]TA plus 5 μM nonradioactive TA. The homogenizer was constantly immersed in an ice water bath, and 10-sec intervals were allowed between strokes to ensure rigorous temperature control. Cytosol was obtained by spinning the homogenate for 15 min at 27,000 x g and recentrifuging the 27,000 x g supernatant for 30 min at 100,000 x g. After incubation, 1 ml of 8 mM Na2HPO4:4 mM EDTA containing 1 mM DTT was added to each tube. The tubes were vortexed and spun 5 min at 3000 rpm. The pellet was washed twice in the same fashion and then extracted overnight at room temperature with 0.7 ml of ethanol. The samples were centrifuged for 10 min at 3000 rpm, and an aliquot of the supernatant was counted. In these and in all other DNA-cellulose binding experiments, the amount of binding to plain cellulose (6 to 10% of input bound counts) has been subtracted from all values. Binding of free [3H]TA to DNA-cellulose was negligible.

Extractability of HR Complexes from DNA-Cellulose. All tubes contained 10 μg of DNA. After 90 min of incubation at 15°C, the samples were washed at 4°C as described above. The washed pellets were resuspended in 1 ml of 10 mM TES containing 10 mM EDTA and varying concentrations of MgCl2, in 1 ml of 10 mM TES:4 mM EDTA plus 10 mM DTT and varying concentrations of spermidine, or in 1 ml of 10 mM TES:4 mM EDTA containing 10 mM DTT and varying concentrations of KCl. All these buffers were supplemented with 2 mg of bovine serum albumin per ml and 10% (v/v) glycerol. The samples were extracted for 15 min at 4°C with gentle manual shaking and centrifuged for 10 min at 3000 rpm, and an aliquot of the supernatant was counted. The pellet was extracted overnight at room temperature with 0.7 ml of 95% ethanol. The amount of radioactivity extracted with each cation is expressed as a percentage of the total amount of radioactivity initially bound to DNA-cellulose (i.e., radioactivity in the MgCl2, spermidine, or KCl supernatants plus radioactivity in the ethanol extract of the corresponding DNA-cellulose pellet).

Gel Filtration and Ultracentrifugal Analysis. Agarose gel filtration on calibrated columns of Bio-Gel A-0.5m (100 to 200 mesh) and A-1.5m (100 to 200 mesh) and glycerol density gradient centrifugation were performed exactly as described in our earlier work (40), except that some of the gradients contained 4 to 17.5% (w/v) glycerol instead of 8 to 35%. In previous work, we used a Rf of 30.5 Å for the ovalbumin marker as indicated in the Pharmacia calibration kit. However,


28.6 Å appears to be a more accurate estimate (34), and we have used this value in the present studies. Therefore, we now report a slightly smaller Rf for the CR [3H]TA-binding component (28 Å instead of 30 Å). The Rf of carbonic anhydrase was taken as 22.8 Å (34). Apparent molecular weights and frictional ratios were calculated from the Rf and sedimentation coefficient as described by Siegel and Monty (36), using the previous assumptions (38, 44).

**Measurement of Radioactivity.** Radioactivity was counted as described earlier (40).

**RESULTS**

**Interaction of Native [3H]TA-Receptor Complexes with DNA-Cellulose.** Both CS and CR P1798 [3H]TA-receptor complexes bound efficiently to DNA-cellulose (Chart 1). Close to maximum uptake was achieved by incubating labeled cytosol with 10 to 20 μg of DNA. The time course and temperature dependence of this process are illustrated in Chart 2. As in other systems (12, 49), very little binding took place at 3° over the time period studied. Optimal binding was achieved after 90 min at 15° for both strains of tumor. Under these conditions, about 50% of input CS complexes and close to 60% of input CR complexes bound to DNA-cellulose. The slightly higher binding of CR complexes was a consistent observation. The decrease in binding after prolonged incubation at 25° was always more pronounced for CS than for CR HR complexes. This may be due to the tighter interaction of CR [3H]TA complexes with DNA-cellulose described below. Alternatively, CS complexes may be less stable than CR complexes at 25°.

Chart 3 shows that 40 to 50% of CS P1 798 complexes could be extracted from DNA-cellulose with 2 to 4.5 mM MgCl2 and 50 to 60% with 1 mM spermidine. In contrast, 10% or less of radioactivity was released with these concentrations of divalent or trivalent cation when CR cytosol was used as the source of HR complex. Raising the concentration of spermidine to 5 or 10 mM eliminated the difference between the 2 strains of tumor, and 70% or more of [3H]TA complexes were extracted regardless of which cytosol was used. A similar, although less pronounced, difference was observed when DNA-cellulose was extracted with 0.1 M KCl. Thus, there is a remarkable similarity between MgCl2, spermidine, and KCl extractability of the complexes from DNA-cellulose and the previously described ability of these agents to release bound steroid from prelabeled homologous nuclei (42).

The greater release of [3H]TA from DNA-cellulose with 4.5 mM MgCl2 or 1 mM spermidine, in the case of CS cytosol, was not due to a difference in the stability of CS versus CR complexes, since we found that 80 to 90% of radioactivity in the extracts was bound to macromolecules (excluded from Sephadex G-25) when the extraction buffer was supplemented with 2 mg bovine serum albumin per ml and 10% glycerol. The presence of albumin and glycerol did not affect the extractability of either CS or CR complexes. Only 30 to 40% of the radioactivity was excluded from Sephadex G-25 when nonsupplemented buffer was used. We also established by analytical agarose gel filtration that the Rf of HR complexes extracted from DNA-cellulose with 10 mM spermidine was 61 Å for CS cytosol and 28 Å for CR cytosol. These values are in excellent agreement with our previous data on the size of cytosolic and nuclear-associated P1798 glucocorticoid receptors (40). We also explored the possibility that CS cytosol might contain a factor that promoted release of [3H]TA complexes from DNA-cellulose or that CR cytosol might lack this factor or contain an inhibitory component. For this purpose, labeled CS cytosol was mixed with unlabeled CR cytosol and vice versa. The mixtures were incubated for 90 min at 15° with DNA-cellulose, which was then washed and extracted with 4.5 mM MgCl2 or 1 mM spermidine. For each mixture, the amount of radioactivity extracted was that expected for the [3H]TA-labeled species alone, thereby ruling out the influence of nonreceptor components present in one type of cytosol but not in the other (Table 1).

![Chart 1](chart1.png)

**Chart 1.** Relationship between DNA concentration and binding of HR complexes. After incubation of [3H]TA-labeled cytosol with the indicated amounts of DNA-cellulose for 1 hr at 4° or 20°, the samples were washed with buffer, and radioactivity was extracted from the pellets with ethanol. Results are expressed as the percentage of input bound counts that became attached to DNA-cellulose. Points, mean of 3 experiments; bars, S.E. Average input was 13,164 dpm for CS cytosol and 6,813 dpm for CR cytosol. The lower input with CR cytosol is a reflection of the lower level of glucocorticoid binding in the CR lymphocytes (42).

![Chart 2](chart2.png)

**Chart 2.** Temperature dependence and time course of HR complex binding to DNA-cellulose. All tubes contained 10 μg of DNA. Points, average of 2 experiments, except for the CR cytosol at 10° (1 experiment); bars, range. Other details as in Chart 1. Average input was 10,203 dpm for CS cytosol and 6,466 dpm for CR cytosol.
A 5-fold higher concentration of chymotrypsin (0.60 unit/mg of protein) caused no further decrease in $R_s$. In contrast to its effect on CS P1798 $[\text{H}]\text{TA}$-receptor complexes, chymotrypsin had no effect on the $R_s$ of CR complexes. However, as shown in Chart 5, CR P1798 complexes sedimented at 3.0S after incubation with 0.12 unit of chymotrypsin per mg of cytosol protein, compared to 3.3S before enzyme treatment. As illustrated in the same chart, the chymotrypsin fragment of CS $[\text{H}]\text{TA}$ complex also had a sedimentation coefficient of 3.0S, compared to 3.7S of the undigested species. These physicochemical parameters are summarized in Table 2 together with the corresponding statistical data. Inasmuch as identical results were obtained whether digestion was performed with plain chymotrypsin, with TLCK-chymotrypsin, or with a mixture of chymotrypsin plus SBTI, it is unlikely that these changes were due to a trypsin contaminant. Inactivated (DFP-treated) chymotrypsin had no effect on either CS or CR $[\text{H}]\text{TA}$-receptor complexes.

Chart 6 shows that, when the 3.0S peak of CS chymotrypsin-treated cytosol was subjected to gel filtration, the peak of bound $[\text{H}]\text{TA}$ was broader and shifted to the right compared to the peak of bound $[\text{H}]\text{TA}$ in a portion of the same cytosol that had not been analyzed by gradient centrifugation. This was most probably due to further cleavage of the $[\text{H}]\text{TA}$ complexes because of continued exposure to chymotrypsin during the 21 hr ultracentrifugation, particularly since the same broadening and shift occurred when cytosol was digested with TLCK-chymotrypsin or chymotrypsin plus SBTI. Identical results were obtained with chymotrypsin-treated cytosol from CR lymphocytes (not shown).

Effect of Chymotrypsin. Incubation of $[\text{H}]\text{TA}$-labeled CS cytosol for 30 min at 10$^\circ$C with increasing concentrations of chymotrypsin resulted in progressive conversion of the 61-Å $[\text{H}]\text{TA}$ complex to a discrete 28-Å fragment (Chart 4). Quantitative conversion was achieved with 0.12 unit of chymotrypsin in the absence of added cation has been subtracted from all values (5 to 10% of the initially bound radioactivity). Average binding of input HR complexes to DNA-cellulose was 54% for CS cytosol and 63% for CR cytosol.

Table 1

Extractability of \textasciitilde"mixed\textasciitilde\textasciitilde" cytosols from DNA-cellulose.

<table>
<thead>
<tr>
<th>Cytosol</th>
<th>$[\text{H}]\text{TA}$ bound to DNA-cellulose (% of input)</th>
<th>$\text{MgCl}_2$ (4.5 mM)</th>
<th>Spermidine (1 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Labeled CS + unlabeled CS Experiment 1</td>
<td>50</td>
<td>50</td>
<td>56</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>54</td>
<td>48</td>
<td>51</td>
</tr>
<tr>
<td>Labeled CR + unlabeled CR Experiment 1</td>
<td>60</td>
<td>8</td>
<td>13</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>62</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td>Labeled CS + unlabeled CR Experiment 1</td>
<td>53</td>
<td>51</td>
<td>52</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>54</td>
<td>48</td>
<td>51</td>
</tr>
<tr>
<td>Labeled CR + unlabeled CR Experiment 1</td>
<td>61</td>
<td>7</td>
<td>14</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>68</td>
<td>8</td>
<td>12</td>
</tr>
</tbody>
</table>

Effect of Trypsin. Incubation of $[\text{H}]\text{TA}$-labeled CS cytosol for 30 min at 10$^\circ$C with increasing concentrations of trypsin converted the 61-Å ($R_s$) complex to a 19-Å ($R_s$) species via formation of what appears to be a 27- to 28-Å ($R_s$) intermediate. Complete 61 → 19 Å conversion was achieved with 2.72 units of trypsin per mg of cytosol protein (Chart 7). There was no $28 \text{ Å}$ transition took place without appreciable dissociation of $[\text{H}]\text{TA}$ from its binding site(s). A 5-fold higher concentration of chymotrypsin (0.60 unit/mg of protein) caused no further decrease in $R_s$. In contrast to its effect on CS P1798 $[\text{H}]\text{TA}$-receptor complexes, chymotrypsin had no effect on the $R_s$ of CR complexes. However, as shown in Chart 5, CR P1798 complexes sedimented at 3.0S after incubation with 0.12 unit of chymotrypsin per mg of cytosol protein, compared to 3.3S before enzyme treatment. As illustrated in the same chart, the chymotrypsin fragment of CS $[\text{H}]\text{TA}$ complex also had a sedimentation coefficient of 3.0S, compared to 3.7S of the undigested species. These physicochemical parameters are summarized in Table 2 together with the corresponding statistical data. Inasmuch as identical results were obtained whether digestion was performed with plain chymotrypsin, with TLCK-chymotrypsin, or with a mixture of chymotrypsin plus SBTI, it is unlikely that these changes were due to a trypsin contaminant. Inactivated (DFP-treated) chymotrypsin had no effect on either CS or CR $[\text{H}]\text{TA}$-receptor complexes.

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Effect of Chymotrypsin. Incubation of $[\text{H}]\text{TA}$-labeled CS cytosol for 30 min at 10$^\circ$C with increasing concentrations of chymotrypsin resulted in progressive conversion of the 61-Å $[\text{H}]\text{TA}$ complex to a discrete 28-Å fragment (Chart 4). Quantitative conversion was achieved with 0.12 unit of chymotrypsin per mg of cytosol protein. No species of intermediate size was detected with lower concentrations of the enzyme. The portion of the 61-Å ($R_s$) CS P1798 $[\text{H}]\text{TA}$-receptor complex that is digested by chymotrypsin, thereby reducing the $R_s$ of the complex to 28 Å, is termed the 61 → 28 Å region of the receptor. It may include all or part of what Sherman et al. (32) have defined as the distal segment of the receptor. The 61 →
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Chart 5. Ultracentrifugai analysis of chymotrypsin-treated CS (a) and CR (b) P1798 [3H]TA-receptor complexes. [3H]TA-labeled cytosol was incubated with 0.12 unit of chymotrypsin per mg of cytosol protein. Control samples were incubated with an equivalent amount, by weight, of DFP-treated (inactive) chymotrypsin. Glycerol concentration was 8 to 35%. Arrow, position of the internal [14C]ovalbumin marker.

Table 2

<table>
<thead>
<tr>
<th>Cytosol</th>
<th>Protease</th>
<th>Rs (Å)</th>
<th>Sedimentation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS</td>
<td>None</td>
<td>60.7 ± 1.3 (6)</td>
<td>3.72 ± 0.05 (6)</td>
</tr>
<tr>
<td>CR</td>
<td></td>
<td>27.5 ± 0.4 (6)</td>
<td>3.30 ± 0.03 (6)</td>
</tr>
<tr>
<td>CS</td>
<td>Chymotrypsin</td>
<td>27.5 ± 0.4 (3)</td>
<td>3.0 ± 0.04 (3)</td>
</tr>
<tr>
<td>CR</td>
<td></td>
<td>27.7 ± 0.4 (3)</td>
<td>3.05 ± 0.03 (3)</td>
</tr>
<tr>
<td>CS</td>
<td>Trypsin</td>
<td>19.3 ± 0.4 (5)</td>
<td>2.76 ± 0.04 (3)</td>
</tr>
<tr>
<td>CR</td>
<td></td>
<td>18.8 ± 0.5 (4)</td>
<td>2.75 ± 0.04 (3)</td>
</tr>
</tbody>
</table>

Mean ± S.D.

Numbers in parentheses, number of experiments.

p < 0.001 versus CS Rs.

p < 0.001 versus untreated CS sedimentation coefficient.

p < 0.001 versus untreated CS and CR sedimentation coefficient.

p < 0.001 versus chymotrypsin-treated CS and CR Rs.

p < 0.001 versus chymotrypsin-treated CS and CR sedimentation coefficient.

appreciable loss of bound steroid, and 5-fold higher concentrations of trypsin (13.60 units/mg of protein) caused no additional reduction in Rs. The same chart shows that incubation of CR cytosol with 2.72 units of trypsin per mg of protein quantitatively converted the 28-Å [3H]TA complex to the 19-Å (Rs) form. There was no further decrease in size when a 5-fold higher concentration of the protease was used. Chart 8 shows that the trypsin fragment of both CS and CR P1798 [3H]TA-receptor complexes had a sedimentation coefficient of 2.8S, significantly smaller than the value for undigested or chymotrypsin-treated complexes (Table 2). All the above results were obtained with TPCK-trypsin, thereby making it unlikely that these effects were due to chymotrypsin contamination of the enzyme. Furthermore, a mixture of 5-fold excess by weight of SBTI with trypsin abolished the cleaving ability of the enzyme. When the 2.8S peak from enzyme-treated CS or CR cytosol was analyzed by agarose gel filtration, over 80% of the radioactivity eluted as a sharp symmetrical peak with a Rs of 19 to 20 Å, in a manner indistinguishable from separate portions of trypsinized cytosol that were chromatographed without prior gradient centrifugation (not shown).

Because of the ability of chymotrypsin to convert the 61-Å CS [3H]TA complex to a form similar in size to the smaller CR [3H]TA complex, it was important to determine whether CR lymphocytes contained cleaving activity that could artifactualy give rise to the 28-Å complex during homogenization of the cells and subsequent labeling of the cytosol. We therefore performed the following experiment. Equal numbers of CS and CR lymphocytes were mixed together and homogenized with [3H]TA as described in “Materials and Methods.” Separate [3H]TA-labeled CS and CR cytosols were prepared in parallel from the same cell suspensions that were used for making the “mixed” cytosol. The CS, CR, and mixed cytosols were analyzed by agarose gel filtration, and the amounts of the 61-Å and 28-Å components were determined. If CR cytosol had contained activity that was able to cleave the 61-Å complex, the amount of the 28-Å component in the mixed cytosol should have increased at the expense of the 61-Å component. However, this did not occur, and the amount of radioactivity recovered in each peak was identical to that observed in the corresponding cytosol prepared and run in parallel by itself.

Interaction of Chymotrypsin- and Trypsin-treated [3H]TA-Receptor Complexes with DNA-Cellulose. Table 3 shows that the chymotrypsin fragment of heat-activated CS [3H]TA com-
plex bound to DNA-cellulose to approximately the same extent as intact [3H]TA-receptor complexes from CS and CR P1798 lymphocytes and to the same extent as chymotrypsin-treated complexes from CR cells. However, the interaction of the CS chymotrypsin fragment with DNA-cellulose was quite different from that of intact [3H]TA complexes from CS lymphocytes. While 4.5 mM MgCl2 and 1 mM spermidine extracted 40 to 50% of undigested CS [3H]TA complexes, these concentrations of divalent and trivalent cations were practically ineffective in releasing the CS chymotrypsin fragment from DNA-cellulose. In this respect, the chymotrypsin fragment of the CS receptor behaved exactly like the intact and chymotrypsin-treated [3H]TA complexes from CR lymphocytes. Finally, the data in Table 3 show that the 19-A fragment produced by treating [3H]TA-receptor complexes from CS or CR P1798 lymphocytes with trypsin did not bind to DNA-cellulose.

**DISCUSSION**

Practically every nuclear structure has been proposed as a possible "acceptor" site for steroid HR complexes, including the nuclear membrane (15), ribonucleoprotein particles (18), DNA (14), chromosomal proteins (39), or combinations of these (29). The nuclear matrix has also been invoked (2). The possible role of DNA in glucocorticoid-receptor complex interaction with the nucleus was also suggested by the ability of DNase digestion to release the complexes from thymus (6) and leukemic myeloblast nuclei (17), as well as by the reduced capacity of nuclei that were previously digested with DNase to bind activated glucocorticoid-receptor complexes (14). Substitution of thymidine for bromodeoxyuridine in intact hepatoma tissue culture cells increased the affinity of glucocorticoid-receptor complexes for their nuclear binding sites as judged by the higher concentration of salt required to extract the complexes from the nuclei (26).

We have found a striking similarity between the interaction of P1798 [3H]TA-receptor complexes with DNA-cellulose and the interaction of these complexes with the nucleus after whole-cell binding of [3H]TA at 37°. Thus, low concentrations of KCl and spermidine were much more effective in extracting CS P1798 than in extracting CR P1798 [3H]TA complexes from DNA-cellulose, in exactly the same way that these cations were shown earlier to cause greater release of [3H]TA complexes from nuclei of CS P1798 lymphocytes than from nuclei of CR P1798 lymphocytes (42). Similarly, the extractability of CS but not of CR [3H]TA complexes from DNA-cellulose with mM concentrations of MgCl2 paralleled the previously reported extractability of bound [3H]TA from sensitive but not from resistant P1798 nuclei with this divalent cation (42). These
results are consistent with the idea that binding to DNA may be a major factor in determining the mode of HR complex interaction with the nucleus in intact P1798 tumor lymphocytes. However, other polyanions may also play a role (7, 19). Our data also suggest that the differences in nuclear-glucocorticoid interactions between CS and CR P1798 cells most probably stem from the altered physicochemical properties of the CR HR complex (40).

It is of interest to compare the properties of protease-treated CS P1798, CR P1798, and rat liver glucocorticoid-receptor complexes. Chymotrypsin reduced the $R_s$ of CS P1798 [3H]TA complexes from 61 to 28 Å, a value identical to that of undigested CR P1798 [3H]TA complexes. The $R_s$ of CR complexes was not modified by chymotrypsin. The chymotrypsin fragment of CS P1798 glucocorticoid receptor appears to be smaller than the chymotrypsin fragment of rat liver dexamethasone-receptor complexes described by Wrange and Gustafsson (48), which has a $R_s$ of 36 Å and an apparent molecular weight of 46,000. However, we found that chymotrypsin digestion converted the 59- to 61-Å rat liver [3H]TA-receptor complex to a 28- to 29-Å form (range of 2 experiments) that eluted from Bio-Gel A-1.5m coincidentally with the internal [14C]ovalbumin marker. We were unable to calculate the apparent molecular weight of chymotrypsin-treated P1798 [3H]TA-receptor complexes because of the discrepancy in $R_s$ before and after glycerol gradient centrifugation. A molecular weight range of 36,000 to 40,000 would seem reasonable if one assumes a $R_s$ of 28 Å and a sedimentation coefficient of 3.0 to 3.3 S.

The fragment produced by chymotrypsin digestion of [3H]TA complexes from the CS P1798 tumor and rat liver shared with undigested and chymotrypsin-treated CR P1798 receptor complexes the ability to bind more tightly to DNA-cellulose than did intact 61-Å [3H]TA complexes. In this respect, the 28-Å fragment of the CS P1798 [3H]TA-receptor complex and the intact and chymotrypsin-treated CR P1798 [3H]TA complexes clearly resemble the M.W. 50,000 [3H]dexamethasone-receptor complexes of nuclear transfer-increased CR S49 lymphocytes, which, as shown by Yamamoto et al. (49), also bound more tightly to DNA-cellulose than did M.W. 90,000 complexes from CS S49 lymphocytes. These authors suggested that this might account for the greater degree of nuclear localization of bound steroid in the CR S49 cells. However, they did not examine receptor extractability from the nucleus itself. Our own data indicate that tighter binding of glucocorticoid-receptor complexes to DNA-cellulose does not necessarily correlate with increased nuclear transfer in the intact cell, since we previously found no difference in the intracellular distribution (80% nuclear; 20% cytoplasmic) of HR complexes between CS and CR P1798 lymphocytes (40).

The 19-Å 2.8S trypsin fragment obtained from CS and CR P1798 [3H]TA complexes appears to be similar in size to the 19-Å 2.5S trypsin fragment of rat liver glucocorticoid receptor described by Wrangle and Gustafsson (48). We obtained a higher sedimentation coefficient for the P1798 trypptic fragments because we assigned a sedimentation coefficient of 1.87S to cytochrome c (22) rather than the value of 1.73S used by Carlsstedt-Duke et al. (4). Thus, the steroid-binding trypsin fragment of P1798 [3H]TA-receptor complex is a globular polypeptide with a molecular weight of approximately 23,000, a frictional ratio (due to shape) of 1.0, and an axial ratio of 1.0 (28) and conforms to the definition of meroreceptor of Sherman et al. (33). Trypsin-treated [3H]TA-receptor complexes from the P1798 tumor (this paper) or rat liver (48) did not bind appreciably to DNA-cellulose. Rat kidney glucocorticoid meroreceptor also lacked the determinants of nuclear binding (33). Trypsinized estrogen-receptor complexes did not bind to nuclear constituents either (27). In contrast, the smallest form of androgen receptor identified thus far (23 Å; 3.0S) was reported to have retained both DNA- and steroid-binding properties (46).

Cleavage of either the A or B subunit of chick oviduct progesterone receptor by an endogenous calcium-activated protease gave rise to a 27-Å fragment (M.W. 43,000; Form IV) that contained the hormone-binding region of the receptor (45). Although there are similarities between the physicochemical properties of Form IV and those of the chymotrypsin fragment of CS P1798 glucocorticoid receptor and of undigested or chymotrypsin-treated CR P1798 [3H]TA-receptor complexes, the functional properties of Form IV and of chymotrypsin-treated lymphoma P1798 glucocorticoid receptors are quite different. Thus, Form IV of the progesterone receptor did not bind to DNA-cellulose, whereas undigested and chymotrypsin-treated CR P1798 [3H]TA-receptor complexes, as well as the chymotrypsin fragment of CS P1798 glucocorticoid receptor, bound to DNA-cellulose quite avidly.

The cytosol mixing experiment described in this paper argues strongly against the presence in CR cytosol of an activity that can cleave the 61-Å CS [3H]TA-receptor complex to a 28-Å fragment. This conclusion is reinforced by the fact that even prolonged incubation of a mixture of CS and CR cytosol at 15°C did not modify the interaction of CS [3H]TA complexes with DNA-cellulose. This would not have been the case had the CS complexes been converted to the smaller more tightly binding form. Unless the CR receptor is derived from a larger precursor
with a susceptibility to proteolysis that is entirely different from that of 61-Â CS receptor, it seems reasonable to suggest that the 28-Â [H]TA complex that we have isolated may represent the native form of CR P1798 glucocorticoid receptor. Our previous results also indicated that the 28-Â CR P1798 [H]TA-binding component was unlikely to be an artifactual cleavage product of a 61-Â CS-like glucocorticoid receptor (40). Thus, we were unable to detect a larger CR form even when CR nuclei were extracted with low-ionic-strength buffer in the presence of high concentrations of leupeptin, conditions under which proteolytic activity would be at a minimum. In addition, isolation of cytosolic [H]TA complexes from prelabeled CR P1798 lymphocytes with buffers containing 20 mg leupeptin yielded only the small form of receptor. Furthermore, coextraction of a mixture of CS and CR nuclei did not convert the 61-Â CS complex to a 28-Â species. On the other hand, the remarkable ability of limited proteolysis with chymotrypsin to confer ‘resistant’-like properties on the CS P1798 [H]TA-receptor complex raises the possibility that the CR binding component may be synthesized as a larger precursor which then undergoes rapid intracellular processing to the 28-Â form. Alternatively, the CR P1798 glucocorticoid receptor may be synthesized directly as a 28-Â polypeptide because of a defect at the level of the genome that permits transcription only of “small” receptor mRNA. Abnormal processing or defective synthesis directly as a 28-Â polypeptide because of a defect.

REFERENCES


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John Stevens and Yee-Wan Stevens


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