Limited Proteolysis of Covalently Labeled Glucocorticoid Receptors as a Probe of Receptor Structure†

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ABSTRACT: [^{3}H]Dexamethasone 21-mesylate affinity-labeled glucocorticoid receptors were subjected to controlled proteolysis by trypsin, chymotrypsin, and *Staphylococcus aureus* V8 protease and then analyzed on denaturing constant percentage or gradient polyacrylamide gels. The molecular weights (M_r ≃98 000) and cleavage patterns for rat liver and HTC cell receptors indicated extensive homology between the glucocorticoid receptors from normal rat liver and a transformed rat liver cell line. The major DNA-binding species generated by chymotrypsin treatment was found to be a 42K fragment that was accompanied by several unresolved, slightly lower molecular weight fragments. The meroreceptors obtained after trypsinization were comprised of two species of M_r 30 000 and 28 000. Each of the three proteases, despite their differing specificities, generated fragments with molecular weights close to 42 500, 30 500, and 27 000. Nevertheless, each of the three proteases gave rise to a distinctive "ladder" of labeled fragments. No differences could be detected in the digestion patterns of unactivated and activated HTC cell complexes for all three proteases. Also, native and denatured receptor–steroid complexes yielded surprisingly similar digestion patterns with each enzyme. Digestion of denatured complexes readily generated large amounts of a fragment of $M_r \simeq 15\,000$ that was much smaller than the protease-resistant meroreceptors formed from native complexes. The presence of these ~15K fragments suggested that the [^{3}H]dexamethasone 21-mesylate labeling of the steroid-binding cavity is restricted to a relatively small segment of the receptor.

Steroid receptors are absolutely required for steroid hormone action and appear to provide excellent model systems for studying the regulation of gene expression in eukaryotes. Nevertheless, details about receptor molecules such as a description of the functional domains and the changes that accompany activation of the receptor–steroid complex to a DNA binding molecule remain largely unknown. In theory, proteases are useful probes of receptor structure and function, and some such studies have been reported. The role of endogenous proteases has been considered in the appearance of certain phenotypes of variant receptors (Stevens & Stevens, 1981; Nordeen et al., 1981; Yamamato et al., 1975). Exogenous proteases have been used to define three different domains of the glucocorticoid receptor that are involved in (1) steroid binding, (2) DNA binding, and (3) the expression of biological activity (Wrange & Gustafsson, 1978). However, the utility of proteolytic analysis has been limited by the two major obstacles of needing purified receptor for further studies and of being unable to identify specific portions of the receptor under conditions that permit higher resolution of the fragments. With the recent development by one of us (Simons, 1981, 1982; Simons & Thompson, 1981) and others (Dure et al., 1980; Nordeen et al., 1981; Westphal et al., 1981; Holmes et al., 1981; Katzenellenbogen et al., 1983) of affinity labels for steroid receptors, it is now possible to overcome both of these problems. The high selectivity of affinity labels enables one to obtain minimally purified receptor preparations in which only the receptor is covalently labeled. The formation of a covalent steroid–receptor bond permits one to follow the steroid binding portion of the receptor even under strongly denaturing conditions.

The types of information that can be obtained from proteolysis of affinity-labeled receptors have just begun to emerge. Birnbaumer et al. (1983) have concluded that the A and B subunits of the chick oviduct progesterone receptor are derived from separate genes. Nordeen et al. (1981) and Gehring & Hotz (1983) have determined that the glucocorticoid receptor of nt$^+$, but not nt$^-$, variant S49 mouse lymphoma cells is smaller than the wild-type receptor and similar in properties to a major chymotrypsin fragment of the wild-type receptor. In this study, we have used proteolysis of affinity-labeled receptors to examine the structural homology between glucocorticoid receptors in rat liver and rat hepatoma tissue culture (HTC) cells. In addition, aided by the near quantitative yield of labeled receptors with the electrophilic affinity label dexamethasone 21-mesylate (Simons & Thompson, 1981; Eisen et al., 1981; Simons et al., 1983), we have identified the denatured, covalently labeled fragments that correspond to the trypsin-generated meroreceptors (Sherman et al., 1976; Wrange & Gustafsson, 1978; Sherman et al., 1983; Vedeckis, 1983a) and to the DNA-binding species observed after chymotrypsin digestion (Wrange & Gustafsson, 1978; Gehring & Hotz, 1983). We have also used exogenous proteases to examine the primary and higher order structures of glucocorticoid receptors in different functional (i.e., activated vs. unactivated) and physical (i.e., native vs. denatured) states.

Materials and Methods

Chemicals. [^{3}H]Triamcinolone acetonide (33 Ci/mmol), [^{3}H]dexamethasone (46 Ci/mmol), [^{3}H]dexamethasone 21-mesylate (46 Ci/mmol), and Aquasol were from New England Nuclear. Hydrofluor was supplied by National Diagnostics. Tricine,[1] triamcinolone acetonide, dexamethasone, and sodium

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[1] Abbreviations: Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]glycine; TAPS, 3-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-amino]-1-propanesulfonic acid; NaDodSO$_4$–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TPCK, tosylphenylalanine chloromethyl ketone; TLCK, tosyllysine chloromethyl ketone; Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol.

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molybdate were from Sigma. TAPS was obtained from Sigma or Cal-Biochem. Reagents for $NaDodSO_4$–PAGE including molecular weight standards were from Bio-Rad. Sephacryl S-200, Sephadex G-25, and hydroxylapatite were from Pharmacia. The counting efficiency of all ^3H-labeled samples was 40–55%. Trypsin (TPCK treated) was obtained from Worthington or Sigma, chymotrypsin (TLCK treated) was from Sigma, and *Staphylococcus aureus* V8 protease was from Miles. The molecular weight standards (myosin, M_r 200 000; β-galactosidase, M_r 116 250; phosphorylase *b*, M_r 97 400; albumin, M_r 66 300; ovalbumin, M_r 45 000; carbonic anhydrase, M_r 30 600; soybean trypsin inhibitor, M_r 21 500; lysozyme, M_r 14 400) were from Bio-Rad. Fluorescent UltEmit autoradiography marker was from New England Nuclear.

Buffers and Solutions. $TAPS_{50}$ buffer was composed of 25 mM TAPS, 50 mM NaCl, 20 mM Na_2MoO_4, 1 mM ethylene diaminetetraacetic acid (EDTA), and 10% glycerol, pH 8.2 at 0 °C. $TAPS_0$ buffers used with HTC cell preparations differed from $TAPS_{50}$ buffer in containing no NaCl or Na_2MoO_4 and in having higher pH values at 0 °C, i.e., pH 8.8 and 9.5. The pH 8.8 $TAPS_0$ buffer is identical with the pH 8.5 buffer reported in an earlier publication (Simons et al., 1983). The higher pH here was due to the use of an Altex electrode (531167 combination electrode with internal silver/silver chloride reference electrode) and the use of temperature-corrected standard buffers at 0 °C (Beckman) instead of room temperature standard buffers with external temperature compensation. Twofold concentrated $NaDodSO_4$ sample buffer contained 0.6 M Tris (pH 8.85), 2% $NaDodSO_4$, 0.2 M dithiothreitol, 20% glycerol, and bromphenol blue.

Cells and Preparation of Cytosols. Preparation of rat liver cytosol and $(NH_4)_2SO_4$ precipitation of rat liver glucocorticoid receptors have been reported previously (Eisen et al., 1981). Growth of HTC cells in spinner and monolayer cultures has been described (Thompson, 1979). The preparation of HTC cell cytosol has been modified from our previous procedure (Simons et al., 1979) as follows. After the cells were washed in phosphate-buffered saline (without calcium), the cell pellet was quick frozen in dry ice for 45 min and then stored at −20 °C. After the pellet was thawed, an equal volume of pH 9.5 $TAPS_0$ buffer was added, and the solution was gently refluxed 20 times in a pipet. At this point >95% of the cells were ruptured, as determined by staining with Azur A. Subsequent centrifugation steps were as previously described (Simons et al., 1979), i.e., 15 min at 17000*g* followed by 90 min at 200000*g*. The 200000*g* supernatant, or cytosol, was used immediately or quick frozen in liquid nitrogen. Cytosol frozen in this manner retained its original receptor binding capacity for at least $6^1/_2$ months (data not shown) when the frozen cytosol was rapidly thawed and used immediately.

Covalent Labeling of Cytosols. The labeling of $(NH_4)_2SO_4$-precipitated rat liver receptors has been previously described (Eisen et al., 1981). The published procedures for HTC cell receptors were modified only by using $TAPS_0$ buffers instead of the Tricine-based buffer A. Thus, the receptors in the thawed, HTC cell cytosol were treated with [^3H]dexamethasone 21-mesylate ± 80-fold excess unlabeled dexamethasone in 0.67 volume of pH 9.5 $TAPS_0$ buffer. At this point the pH of the cytosol solution was usually 8.8 or slightly less. All further dilutions were with pH 8.8 $TAPS_0$ buffer unless otherwise indicated.

Procedures for Partial Purification of Labeled Receptors. Sephacryl S-200 chromatography was carried out at 4 °C in $TAPS_{50}$ buffer plus 1 mM dithiothreitol on columns with dimensions of 80 cm length and 2 cm diameter. One column was used for initial size fractionation of crude cytosol and a second for trypsin-treated material.

DNA–cellulose chromatography was conducted as previously described (Simons et al., 1983).

DEAE-cellulose (Whatman DE52) chromatography was performed on 2.5 (diameter) × 3 (length) cm columns using cytosol solutions that first had been chromatographed on Sephadex G-25 PD-10 columns (Pharmacia) equilibrated with 5 mM potassium phosphate buffer containing 20 mM Na_2MoO_4 (pH 7.8 at 0 °C). This material (2.1–2.8 mL) was then loaded onto the DEAE-cellulose columns equilibrated with the same buffer. The column was developed with two sample volumes of the above phosphate buffer followed by 10 mL each of 50 mM potassium phosphate/10 mM Na_2MoO_4, 75 mM potassium phosphate/10 mM Na_2MoO_4, and 300 mM potassium phosphate/20 mM Na_2MoO_4 all at pH 7.8–7.9 at 0 °C. Noncovalent [^3H]dexamethasone and covalent [^3H]-dexamethasone 21-mesylate labeled complexes exhibited identical step-elution and gradient-elution profiles (data not shown). Under these conditions, [^3H]dexamethasone-bound receptors contained only 6% activated complexes (data not shown). It should be pointed out, however, that increasing concentrations of Na_2MoO_4 in the potassium phosphate buffers were found to decrease the potassium phosphate concentrations required to elute activated and unactivated complexes (data not shown).

$(NH_4)_2SO_4$ precipitation of affinity-labeled HTC cell receptors was performed as previously described (Eisen et al., 1981) except that all of the $(NH_4)_2SO_4$ was added at once to samples in $TAPS_0$ buffer (final pH 8.8) containing 20 mM Na_2MoO_4 in 1.5-mL screw-capped conical centrifuge tubes (Sarstedt no. 72.692). After incubation at 0 °C for 30 min, the precipitate was centrifuged (0 °C/1 min in a microfuge), resuspended in pH 8.2 $TAPS_{50}$ buffer containing 20 mM Na_2MoO_4, quick frozen at −80 °C, and stored at −20 °C. In view of the fact that all of the activated complexes and only some of the unactivated complexes are precipitated by 40% $(NH_4)_2SO_4$ (Dahmer et al., 1981), it could be calculated from the yield of receptor–steroid complex precipitation (~40%) and from the amount of activated complexes initially present (~6%; see above) that the unactivated complexes constituted ≥80% of the total complexes isolated by $(NH_4)_2SO_4$ precipitation of unactivated, labeled cytosols. By the criterion of binding to DNA–cellulose columns in pH 8.2 $TAPS_0$ buffer containing 20 mM Na_2MoO_4, $(NH_4)_2SO_4$ precipitated, unactivated HTC cell complexes contained <2% activated complexes (data not shown).

Conditions for Limited Proteolysis. Unless otherwise stated, incubations with protease were conducted at 10 °C for 20 min and stopped either by (i) addition of soybean trypsin inhibitor (Sigma) and cooling to 4 °C, (ii) freezing in dry ice followed by resuspension in 2× $NaDodSO_4$ sample buffer, or (iii) addition of 2× $NaDodSO_4$ sample buffer followed by freezing at −80 °C. For the analalysis of DNA binding properties of chymotryptic fragments, DNA–cellulose chromatography was performed either immediately upon termination of digestion or after thawing digested samples previously frozen in dry ice.

Polyacrylamide Gel Electrophoresis. Preparation of samples has been described previously (Simons et al., 1983). Procedures for electrophoresis are as described previously (Simons et al., 1983) with the following modifications. Constant percentage acrylamide gels [10.5–11% with 0.26–0.28% *N,N'*-methylenebis(acrylamide)] were run in a water-cooled (15 °C) Protean slab gel apparatus (Bio-Rad) at 30 mA/gel. Gradient gels [7–15% acrylamide with

0.18–0.39% *N,N'*-methylenebis(acrylamide)] were run at 70 V for 16 h or at 80 V until the dye front entered the running gel and then at 100 V. Gels were fixed and stained in 50% methanol/7.5% acetic acid containing 0.01% Coomassie Blue (R-250), destained in 10% methanol/7.5% acetic acid, washed briefly in distilled water, incubated for 1–2 h in Autofluor or Enhance (New England Nuclear) with constant shaking at room temperature, dried by using either a Bio-Rad gel drier or vacuum suction between sheets of PD-150 noncoated cellophane (a gift from Du Pont), and autoradiographed for 5–8 days with Kodak X-OMAT XAR-5 film.

Results

Partial Purification of [^{3}H]Dexamethasone 21-Mesylate Labeled HTC and Rat Liver Receptors. Dexamethasone 21-mesylate forms specific, covalent steroid–protein complexes only with the glucocorticoid receptor in rat liver and HTC cell cytosols. With HTC cell cytosols, however, fragments of the covalently labeled receptor are produced along with numerous nonspecifically labeled species (Simons, 1981; Simons & Thompson, 1981; Eisen et al., 1981; Simons et al., 1983) both of which could complicate our analysis of receptor cleavage patterns by exogenous proteases. Freeze–thaw rupture of HTC cells yielded receptor preparations that could be labeled with [^{3}H]dexamethasone 21-mesylate to give full-sized receptor–steroid complexes ($M_r \simeq 98\,000$)[2,3] while achieving a marked reduction of nonspecifically labeled species when compared with cytosols prepared by mechanical homogenization (Figure 1). Nordeen et al. (1981) have reported that similar procedures yielded only an $M_r \simeq 37\,000$ species that was probably a proteolytic fragment of the full-sized complex. This could be due to their use of a subsequent homogenization step which we omit.

In spite of the above decrease in nonspecific labeling of HTC cell cytosol, it was desirable to purify further the affinity-labeled HTC cell cytosol receptors before studying the effects of proteases. DNA–cellulose chromatography (Eisen and Glinsmann, 1978; Simons et al., 1983) of the labeled, freeze–thaw prepared receptor afforded activated, covalently labeled HTC cell glucocorticoid receptors that were virtually free of receptor fragments and nonspecifically labeled proteins [Figure 2A, lane 1, vs. Figure 7B of Simons et al. (1983)]. As would be expected, this decrease in fragments of the labeled receptors in cytosols prepared by freeze–thaw was accompanied by a 2-fold increase in the yield of intact 98K, labeled receptors (Figure 1; data not shown).

Reaction of [^{3}H]dexamethasone 21-mesylate with 40% $(NH_4)_2SO_4$-precipitated rat liver cytosol followed by Sephacryl S-200 and DNA–cellulose chromatography (Simons et al., 1983) afforded activated, covalently labeled rat liver receptors that consisted predominantly of the intact 98K receptor. It

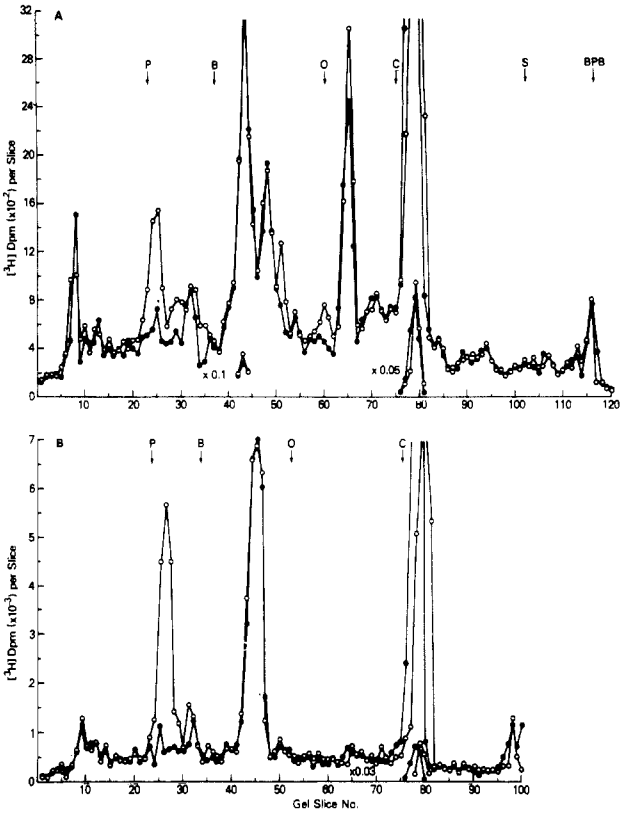

FIGURE 1: [^{3}H]Dexamethasone 21-mesylate labeling of HTC cell cytosol receptors prepared by different methods. (A) Cytosol was prepared by mechanical homogenization of frozen cells in buffer A [20 mM Tricine, 2 mM $CaCl_2$, 1 mM $MgCl_2$ (pH 8.0 at 0 °C)] as previously described (Simons et al., 1979). (B) Cytosol was prepared by freeze–thaw in pH 9.5 $TAPS_0$ buffer as described under Materials and Methods. For both cytosol preparations, covalent labeling of the receptors [i.e., 2×10^{-8} M [^{3}H]dexamethasone 21-mesylate without (○) and with (●) 1.6×10^{-6} [^{1}H]dexamethasone at 0 °C, pH ~8.8, for 2–3 h) and analysis of the solutions by $NaDodSO_4$–polyacrylamide gel electrophoresis were conducted under similar conditions [see Simons et al. (1983) and Materials and Methods]. P, phosphorylase *b*; B, bovine serum albumin; O, ovalbumin; C, carbonic anhydrase; S, soybean trypsin inhibitor; BPB, bromophenol blue.

should be noted that the fluorographs shown (see Figures 3 and 4) have been overexposed to reveal minor labeled species. Labeling of many of these minor bands is competed by excess unlabeled dexamethasone. Quantitation of radioactivity on the gels (by densitometry for fluorographs or by slicing and counting) indicated that the 98K glucocorticoid receptor band represented greater than 90% and 80% of the specifically bound radioactivity in gels of rat liver and HTC cell cytosolic receptor preparations, respectively.

Limited Proteolysis of Activated [^{3}H]Dexamethasone 21-Mesylate Labeled Receptors: Comparison of Digestion Patterns Obtained Using Trypsin and Chymotrypsin. Covalent, activated receptor–steroid complex preparations were treated with various concentrations of trypsin or chymotrypsin as described under Materials and Methods and analyzed by $NaDodSO_4$–PAGE. Each enzyme had been treated with a specific inhibitor of the other protease.

Figure 2A shows [^{3}H]dexamethasone 21-mesylate labeled HTC cell receptors that were treated with trypsin (0.063–100 μg/mL). In addition to the full-sized 98K moiety, numerous discrete fragments could be identified (Table I). With increasing concentrations of trypsin, a "ladder" of fragments derived from the labeled 98K receptor was obtained, which is consistent with a precursor–product relationship. Unfortunately we cannot yet deduce the positions of each fragment

[2] By fluorographic analysis of the gels, the molecular weight of HTC cell receptors was 98 600 ± 1500 (SD) as determined for 28 separate preparations. This observed molecular weight is slightly higher than that previously reported by us for HTC cell receptors (M_r 89 900 ± 3000, n = 39) (Simons et al., 1983). Relatively minor changes in the gel electrophoresis protocol, including water cooling (15 °C) of gels (see Materials and Methods), appear to be responsible for this difference. In a larger study which included sliced and counted gels, the molecular weight of HTC cell receptors was 97 300 ± 3000 (n = 71), and denatured activated and unactivated complexes still exhibited identical molecular weights [97 200 ± 3100 (n = 42) and 97 400 ± 2900 (n = 29), respectively].

[3] The molecular weights of the same fragments in a given sample often had slightly different values, depending on whether constant percentage or gradient polyacrylamide gels were run (data not shown). Unless specified, all molecular weights were determined from constant percentage gels.

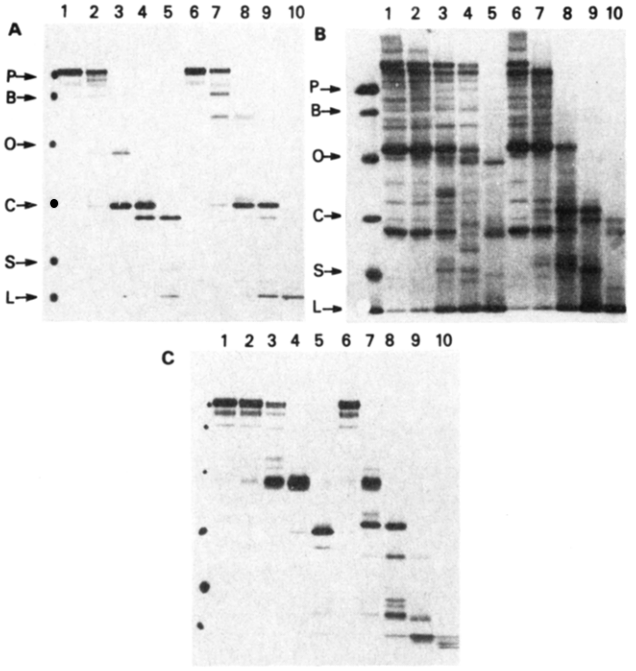

FIGURE 2: Digestion of activated, [^{3}H]dexamethasone 21-mesylate labeled, HTC cell receptors by trypsin and chymotrypsin. (A) Fluorograph of trypsin digestion products. Activated, covalently labeled receptors [prepared as in Figure 1B followed by activation at 20 °C for 30 min with 50 mM β-mercaptoethanol, chromatography on a DNA–cellulose column, and elution with pH 8.2 $TAPS_0$ buffer with 0.5 M NaCl (Simons et al., 1983)] were treated in the native state (lanes 1–5) or the denatured state (30 min/22 °C in $NaDodSO_4$/DTT) (lanes 6–10) with trypsin for 20 min at 10 °C, at which point the samples were quick frozen in dry ice and thawed only long enough to permit mixing of an equal volume of 2× $NaDodSO_4$ sample buffer. These samples were then treated at 100 °C for 5 min before being analyzed on $NaDodSO_4$–polyacrylamide gels. The gels were subsequently stained with Coomassie Blue and fluorographed as described under Materials and Methods. The concentrations of trypsin used to digest the native, activated complexes in lanes 1–5 were 0, 0.063, 0.5, 4, and 100 μg/mL, respectively; the trypsin concentrations used to digest the denatured, activated complexes in lanes 6–10 were 0, 0.5, 4, 20, and 100 μg/mL, respectively. (B) Coomassie Blue staining of trypsin digestion products. The Coomassie Blue stained, enhanced, dried gel that was fluorographed to give (A) is shown here. (C) Fluorograph of chymotyrpsin digestion products. The procedure and concentrations of protease were exactly the same as in (A) except that the protease used here was chymotrypsin. The digestion of native complexes is shown in lanes 1–5; the digestion of denatured complexes is shown in lanes 6–10. In both sets, the concentrations of protease increase in going from left to right as in (A). Dots at edge of fluorographs were made by overlaying a fluorescent marker (UltEmit) on the bands of the molecular weight markers in the dried gels. P, phosphorylase *b*; B, bovine serum albumin; O, ovalbumin; C, carbonic anhydrase; S, soybean trypsin inhibitor; L, lysozyme.

in the intact receptor molecule. The recovery of radiolabel in the tryptic products appeared to be quantitative at trypsin concentrations less than 20 μg/mL. With large amounts of trypsin ($\sim$100 μg/mL), only a portion of the radiolabel was recovered. Labeled peptides with $M_r \sim$ <10000 appear to be soluble under the conditions used to fix and destain the gel.

Chymotrypsin treatment of covalently labeled HTC cell receptors resulted in a different pattern of fragments, some of which were similar in molecular weight to the tryptic fragments (Figure 2C vs. Figure 2A and Table I). For both proteases, it is of interest that the 98K receptor–steroid complex was more susceptible to proteolysis than were the major, Coomassie Blue stained proteins (Figure 2A vs. Figure 2B; data not shown).

When labeled rat liver receptors were treated with trypsin or chymotrypsin and analyzed by $NaDodSO_4$–PAGE, the

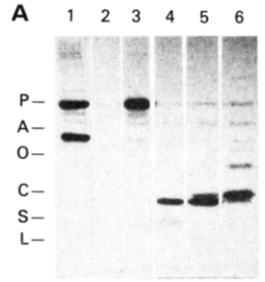

FIGURE 3: Fluorograph of partially purified covalent, [^{3}H]dexamethasone 21-mesylate labeled, rat liver receptor–steroid complex before and after trypsin treatment. Ammonium sulfate precipitated rat liver cytosol was labeled with [^{3}H]dexamethasone 21-mesylate as described under Materials and Methods and subjected to chromatography on Sephacryl S-200. The peak of radioactivity in the void fractions (lane 1) was chromatographed on DNA–cellulose (bed volume $\sim$ 3 mL) as described in text. The eluate (lane 3) was digested with trypsin for 20 min at 10 °C at the doses indicated: lane 4, 100 μg/mL; lane 5, 10 μg/mL; lane 6, 1 μg/mL. Digestion was stopped by addition of 2× $NaDodSO_4$ sample buffer, and samples were treated at 100 °C for 3 min and applied to 7–15% gradient $NaDodSO_4$–polyacrylamide gels. Standards (lane 2) are indicated by letters on left of figure.

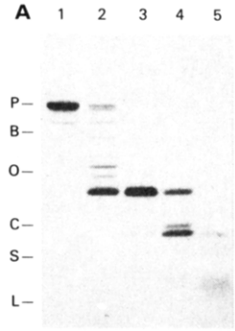

FIGURE 4: Fluorograph of activated, [^{3}H]dexamethasone 21-mesylate labeled, rat liver glucocorticoid receptors digested by chymotrypsin. Activated, covalently labeled receptors were prepared, digested with various amounts of chymotrypsin, and analyzed by electrophoresis as described in Figure 3. The DNA–cellulose purified receptor preparation (lane 1) was digested with chymotrypsin at the indicated doses: lane 2, 0.1 μg/mL; lane 3, 1 μg/mL; lane 4, 10 μg/mL; lane 5, 100 μg/mL.

molecular weights of the major fragments obtained were essentially the same as those obtained by proteolysis of HTC cell receptors (Figures 3 and 4). Gradient gels were used for analysis of rat liver preparations in order to provide better resolution in a range of M_r 98 000–45 000. Analysis of the trypsin digests of HTC cell receptors on gradient gels gave digestion patterns that were very similar to those of rat liver receptors,[3] although quantitative differences were observed among the intensities of the labeled fragments (data not shown). HTC receptor preparations appeared more resistant to trypsin digestion than the rat liver preparations. The "stepladder" of fragments with M_r >45 000 was more prominent in HTC preparations. The chymotrypsin digestion patterns of unactivated rat liver and HTC cell receptors were also qualitatively the same when analyzed on the same constant percentage gel; the molecular weights of the fragments were identical (data not shown). The differences in digestion patterns appear to reflect different *rates* of digestion, which would be expected due to the presence of unequal amounts of other proteins (see Figure 2B), and possibly protease inhibitors, in the two receptor preparations.

Limited Proteolysis of [^{3}*H*]*Dexamethasone 21-Mesylate Receptor Complexes by Staphylococcus aureus V8 Protease.* *Staphylococcus aureus* V8 protease required higher concen-

Table I: Molecular Weight of Major Proteolytic Fragments of Covalently Labeled HTC Cell Glucocorticoid Receptors[a]

	unactivated		activated	
protease	native	denatured	native	denatured
trypsin	85 000	83 000	84 800	
	79 400	77 300	78 500	78 300
	65 500	64 900	67 200	66 900
		54 600		55 300
	42 500		41 900	
	30 400	30 400	30 500	30 400
	28 400	28 200	28 300	28 200
	15 000	14 800	15 000	15 100
chymotrypsin	65 400		64 500	
	49 100		49 400	
	46 200	45 600	46 000	46 000
	42 000	41 900	42 000	42 100
		34 300		34 300
	32 100	31 800	32 000	31 900
	30 700		30 500	
	27 700		27 700	
		26 000		26 300
		19 000		19 200
		17 800		
	17 200	16 100	16 300	16 200
		15 700		15 700
			<14 400	<14 400
V8 protease	77 900	77 000	79 800	77 900
	62 500	62 300	62 700	62 300
	58 800	58 700	59 000	58 900
	56 100	55 800	56 300	56 200
	51 100		50 500	
	43 400		42 900	
	31 200	30 700	30 800	31 100
	26 800	26 200	26 000	26 500
		14 900		15 700
		14 400		14 400

[a] Each group of molecular weight values was determined from the fluorographs of two gels (three for V8 protease digestion of native, activated complexes) such as shown in Figures 2, 5, and 6. The individual values in each group represent the average of the molecular weight values calculated from semilog plots of the molecular weights of the protein standards vs. R_f. Due to the resolution of the constant percentage gels, these values are least accurate at molecular weight of values >66 000. At lower molecular weight values, the reproducibility was usually ± ≤400. The molecular weight values of the fragments are listed in the table so that the same fragment of different receptor preparations (as determined by intensity and relative positions in the ladder) is aligned. Those bands that are underlined represent the most intense bands that are seen at various stages of proteolysis.

trations of enzyme and more forcing digestion conditions than either trypsin or chymotrypsin to give the same percentage digestion of the 98K HTC cell receptor (Figure 5). Table I lists the molecular weights of the observed proteolytic fragments. Over the range of the enzyme concentrations used, V8 protease tended to yield a higher proportion of large molecular weight fragments than did the other two enzymes. As with trypsin and chymotrypsin, the labeled 98K complex was preferentially digested before most of the more abundant proteins, as visualized with Coomassie Blue (data not shown).

Proteolysis of HTC Cell Glucocorticoid Receptors in Different Functional and Physical States. All of the above experiments were conducted with native, activated complexes. An examination of the proteolysis of activated and unactivated HTC cell complexes, both before and after denaturation, was highly desirable since potential chemical or conformational changes accompanying activation or changes in three-dimensional structure occurring during denaturation could affect proteolysis of the complexes.

Na_2MoO_4 blocks activation of glucocorticoid receptor–steroid complexes under a variety of conditions including DEAE-cellulose chromatography and $(NH_4)_2SO_4$ precipitation

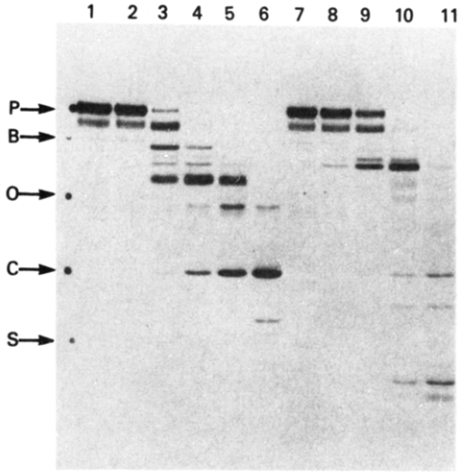

FIGURE 5: Fluorograph of V8 protease digestion products of activated, [^{3}H]dexamethasone 21-mesylate labeled, HTC cell receptors. The procedure was exactly the same as in Figure 2 except that the protease used here was V8 protease and digestion was conducted at 22 °C for 90 min. Native complexes that were kept at 0 °C are shown in lane 1; lanes 2–6 show the digestion of native complexes; lanes 7–11 show the digestion of denatured (30 min/22 °C in $NaDodSO_4$/DTT) complexes. The concentrations of enzyme used in lanes 2–6 and lanes 7–11, was 0, 1.6, 8, 40, and 200 μg/mL, respectively. Dots at edge of flurograph were made by overlaying fluorescent marker (UltEmit) on the bands of the molecular weight markers on the dried gels. P, phosphorylase *b*; B, bovine serum albumin; O, ovalbumin; C, carbonic anhydrase; S, soybean trypsin inhibitor.

(Leach et al., 1979; Barnett et al., 1980; Moudgil & John, 1980; Noma et al., 1980; Dahmer et al., 1981; Vedeckis, 1981; Holbrook et al., 1983). We therefore could partially purify the unactivated [^{3}H]dexamethasone 21-mesylate labeled complexes by DEAE-cellulose chromatography (Sakaue & Thompson, 1977) followed by precipitation with 40% $(NH_4)_2SO_4$ in the presence of 20 mM Na_2MoO_4 (Dahmer et al., 1981; Eisen et al., 1981). Somewhat surprisingly, this preparation was not significantly purer than that obtained simply by $(NH_4)_2SO_4$ precipitation of crude labeled complexes in 20 mM Na_2MoO_4 before DEAE-cellulose chromatography [note: complexes that were precipitated with $(NH_4)_2SO_4$ were still unactivated as shown by their inability to bind to DNA–cellulose (data not shown)]. Both preparations were devoid of the nonspecifically labeled species at $M_r \simeq 29\,000$ (Figure 1B vs. Figure 6A). Both preparations also gave virtually identical results in the protease studies described below. These preparations contained labeled, nonreceptor proteins, but when analyzed in parallel with identically prepared nonspecifically labeled cytosols, one could easily identify the labeled receptor at $M_r \simeq 98\,000$ and the generated proteolytic fragments (see Figure 6).

Denaturation of receptor–steroid complexes could facilitate the cleavage of labile functional groups. Since one of the possible chemical changes accompanying activation is dephosphorylation (Leach et al., 1979; Barnett et al., 1980; Housely & Pratt, 1983), relatively mild denaturing conditions were sought in order to avoid inadvertent dephosphorylation by chemical hydrolysis. Complete denaturation normally is assured by boiling the sample in $NaDodSO_4$. Denaturation by heating at 22 °C for 30 min, or 100 °C for 3 min, with 0.2% $NaDodSO_4$ and 0.5 mM DTT produced the same digestion pattern for each protease; thus, most of the complexes in these studies were denatured by treatment with 0.2% $NaDodSO_4$ and 0.5 mM DTT at 22 °C for 30 min.

Within the limits of $NaDodSO_4$–PAGE resolution, the digestion fragments obtained with each of the three enzymes were found to be identical for native unactivated and activated

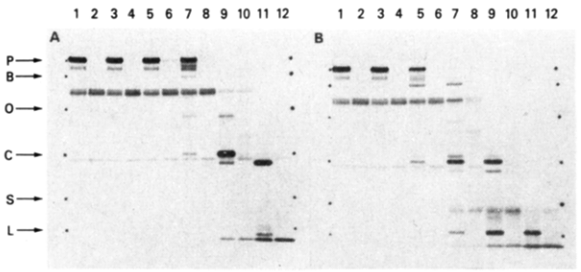

FIGURE 6: Digestion of unactivated, [^{3}H]dexamethasone 21-mesylate labeled HTC cell receptors by trypsin. Paired samples of unactivated receptors that had been covalently labeled by [^{3}H]dexamethasone 21-mesylate without (odd numbered lanes) or with (even numbered lanes) an excess of [^{1}H]dexamethasone were kept at 0 °C (lanes 1 and 2) or were digested at 10 °C for 20 min (lanes 3–12) in the native (A) and denatured (30 min/22 °C in $NaDodSO_4$/DTT) (B) state. Enzyme concentrations (μg/mL) used are given in parentheses after the lane numbers: (for A) lanes 1–4 (0); 5, 6 (0.063); 7, 8 (0.5); 9, 10 (4); 11, 12 (100); (for B) 1–4 (0); 5, 6 (0.8); 7, 8 (4); 9, 10 (20); 11, 12 (100). Gels were processed as described in Figure 2A. (A) Fluorograph of digest of native complexes; (B) fluorograph of digest of denatured complexes. Dots at edge of fluorograph were made by overlaying a fluorescent marker (UltEmit) on the band of the molecular weight markers on the dried gels. P, phosphorylase *b*; B, bovine serum albumin; O, ovalbumin; C, carbonic anhydrase; S, soybean trypsin inhibitor; L, lysozyme.

complexes (Figure 2A vs. Figure 6A; data not shown). This similarity occurs even though the salt and protein concentrations, and types of proteins present, are quite different in the partially purified, unactivated and activated preparations. Na_2MoO_4, a well-known inhibitor of thermal activation (Leach et al., 1979; Barnett et al., 1980; Noma et al., 1980) even with $(NH_4)_2SO_4$-precipitated receptors (Dahmer et al., 1981; Moudgil & John, 1980; H. J. Eisen et al., unpublished results), was present at 18 mM during the protease digestion of unactivated complexes in order to prevent activation during the incubation. Na_2MoO_4 is also an effective inhibitor of receptor cleavage by endogenous proteases (Hazato & Murayama, 1981; Miller et al., 1981; Vedeckis, 1983a; Sherman et al., 1983) but did not prevent cleavage by the exogenous proteases used here. Likewise, the digestion patterns of native vs. denatured complexes (activated or unactivated) were qualitatively similar (Figures 2A,C, 5, and 6A,B and Table I). Although there were many quantitative differences in the intensities of the various bands, to a surprising extent, most of the bands seen for the native complex were also detected for the denatured complex, especially at $M_r \geq 28\,000$. A distinct ladder of fragments was obtained when the denatured complexes were treated with each enzyme, indicating that even in the denatured protein all cleavage sites in the 98K complex are not equally susceptible to proteases and proteolysis still occurs in an ordered fashion.

Two major differences in the digestion patterns of native vs. denatured complexes were observed with all three proteases. First, the relatively protease resistant ~30.5K "cores" of the native receptors were, with the denatured complexes, readily digested to smaller species (Figures 2A,C, 5, and 6A,B). Second, while the native covalently labeled receptors were preferentially digested by the proteases before the major Coomassie Blue stained proteins, the denatured major proteins and labeled receptors were digested at about equal rates (Figure 2A vs. Figure 2B; data not shown). These differences are further evidence that the conditions we have employed were effective in denaturing protein and the receptor–steroid complexes.

Endogenous Proteases in HTC Cell Cytosol. DNA–cellulose-purified, [^{3}H]dexamethasone 21-mesylate labeled complexes, comparable to those used for enzymic digestions, were incubated at 0 °C for 48 h, and crude labeled complexes were incubated either for 8 h at 23 °C in the presence of 20 mM Na_2MoO_4 (added to block activation) or for 5 h at 25 °C in the absence of Na_2MoO_4. In all cases, we observed no further digestion to yield specifically labeled fragments (data not shown). Because of this apparent lack of endogenous protease activity, we cannot say whether the few fragments that are observed in samples free of exogenous proteases were formed during cytosol preparation or represent whole cell degradation products of the steroid-free receptors.

Characterization of Major Tryptic and Chymotryptic Fragments. Previous studies with [^{3}H]triamcinolone acetonide receptors under nondenaturing conditions demonstrated that trypsin treatment produces a 19-Å meroreceptor fragment that does not bind to DNA and, therefore, does not contain the "DNA-binding" domain of the receptor (Wrange & Gustafsson, 1978). Similarly, chymotrypsin treatment produces a 28-Å fragment that contains both the steroid and the DNA-binding domains (Wrange & Gustafsson, 1978; Gehring & Hotz, 1983). Because many studies of these forms have been performed with rat liver receptors, we felt it was important to identify the tryptic meroreceptor and chymotryptic DNA-binding fragment among the various [^{3}H]dexamethasone 21-mesylate labeled fragments observed in this study. For identification of the tryptic meroreceptor we developed a procedure that utilizes two Sephacryl S-200 chromatographic steps (without a DNA–cellulose step) in order to separate directly the smaller tryptic fragments from other labeled species. The untreated and trypsin-treated [^{3}H]-triamcinolone–receptor complexes could be resolved readily by Sephacryl S-200 chromatography (Figure 7A). Because [^{3}H]dexamethasone 21-mesylate treated, $(NH_4)_2SO_4$-precipitated rat liver cytosol fractions contain other labeled moieties, its elution profile was more complex (Figure 7B); however, the void fractions contained the 98K receptor (Figure 8A). When these void fractions were treated with trypsin and rechromatographed, the 31K and 27K fragments (on gradient gels; equals 30K and 28K on constant percentage gels) eluted at the position of the [^{3}H]triamcinolone acetonide labeled meroreceptor (Figures 7C and 8). These moieties did not bind to DNA–cellulose or DEAE-cellulose. They did bind to hydroxylapatite. Thus, they had the physicochemical characteristics of meroreceptors (Sherman et al., 1976; Wrange & Gustafsson, 1978; Sherman et al., 1983; Vedeckis, 1983a).

Chymotrypsin treatment of rat liver receptors produced a group of fragments of $M_r \simeq 38\,000$–$40\,000$ on gradient gels (Figure 4; M_r 39 000–42 000 on constant percentage gels; data not shown). A similar result was observed with HTC cell receptors (see Figure 2C). As shown in Figure 8B, the rat liver 38–40K fragments (gradient gel), produced by chymotrypsin treatment of the 98K complexes in S-200 void fractions (Figure 8A), bound to DNA–cellulose just like the previously identified 28-Å fragment (Wrange & Gustafsson, 1978).

Discussion

We have used controlled proteolysis of covalent, dexamethasone 21-mesylate labeled receptors to examine the glucocorticoid receptors in rat liver and HTC cells with regard to (1) the degree of homology between receptors from normal and transformed rat liver cells, (2) the degree of homology between unactivated and activated receptors, (3) the presence and significance of receptor fragments in whole cells, (4) the denatured molecular weight of the limited trypsin and chymotrypsin fragments that have been described in the literature, and (5) the number of positions on the receptor that are labeled

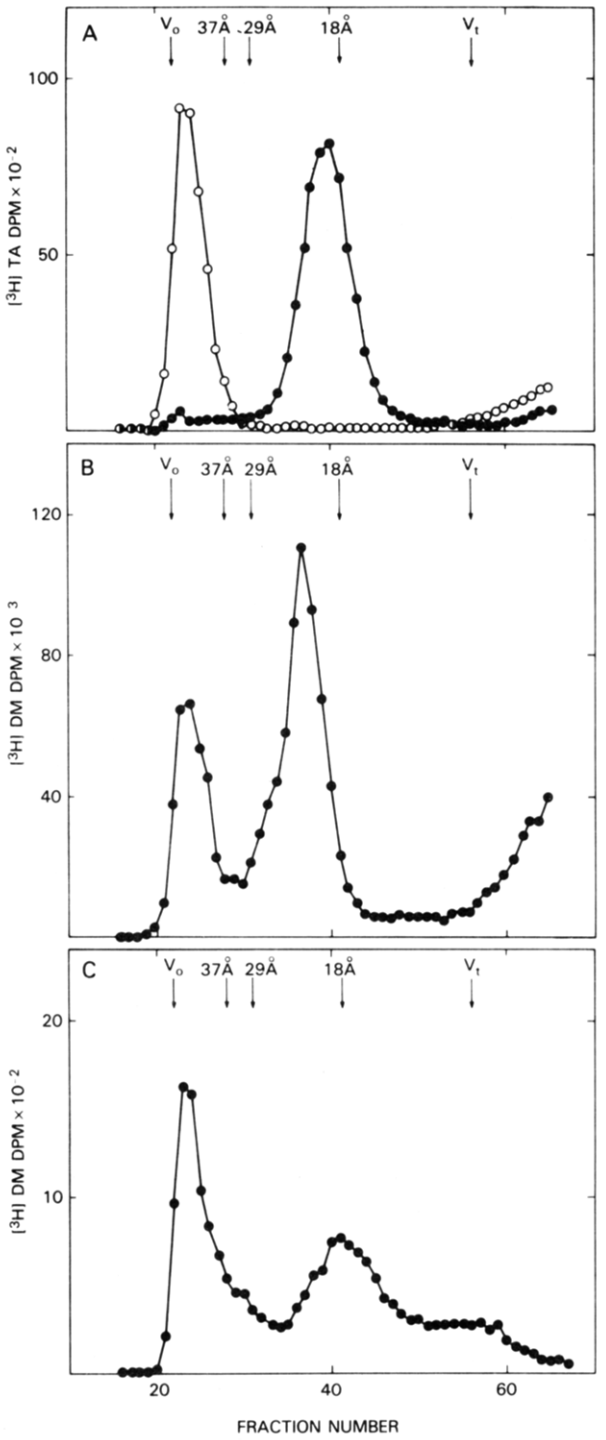

FIGURE 7: Gel filtration of native and trypsin treated rat liver glucocorticoid receptor-steroid complexes. (A) Rat liver cytosol (5 mL) was labeled for 2 h at 0 °C with 50 nM [^{3}H]triamcinolone acetonide and then chromatographed on a Sephacryl S-200 column (dimensions 80 × 2 cm). Radioactivity is expressed as dpm per 100-μL aliquot of each 2.5-mL fraction of the eluate. The void fractions (5 mL) were pooled, treated with trypsin (20 μg/mL for 20 min at 10 °C), and chromatographed on a second S-200 column with the same column dimensions as above. Radioactivity is expressed as above. (○) Native [^{3}H]triamcinolone acetonide labeled receptors; (●) trypsin-treated receptor–steroid complexes. (B) Ammonium sulfate precipitated rat liver cytosol (10 mL) was labeled with 100 nM [^{3}H]dexamethasone 21-mesylate for 2 h at 0 °C and then chromatographed on a Sephacryl S-200 column as above (50-μL aliquots from each 2.5-L fraction were counted). (C) The void fractions from the column eluate in (B) were pooled, and a 6-mL portion was treated with trypsin (20 μg/mL for 20 min at 10 °C). The sample was chromatographed on a Sephacryl S-200 column as above (100-μL aliquots of each 2.5-mL fraction were counted). Column standards and the Stokes radii were the following: bovine serum albumin, 37 Å; ovalbumin, 29 Å; cytochrome *c*, 18 Å.

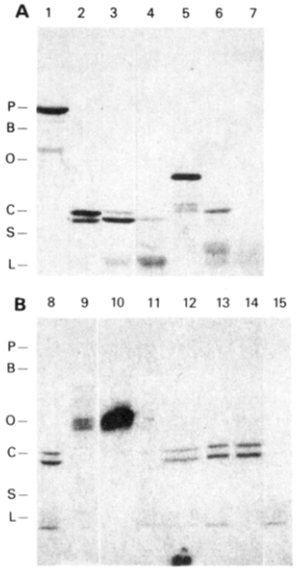

FIGURE 8: Characterization of [^{3}H]dexamethasone 21-mesylate labeled, proteolytic fragments of rat liver glucocorticoid receptor. (A) Fluorograph of partially purified affinity-labeled receptors before and after digestion with trypsin and chymotrypsin. $(NH_4)_2SO_4$-precipitated [^{3}H]dexamethasone 21-mesylate labeled rat liver receptors were chromatographed on a Sephacryl S-200 column; the pooled void fractions (20–24 of Figure 7B) were treated with trypsin or chymotrypsin as indicated below for 20 min at 10 °C. Lane 1, pooled void fractions; lane 2, 50 μg/mL trypsin; lane 3, 100 μg/mL trypsin; lane 4, 500 μg/mL trypsin; lane 5, 5 μg/mL chymotrypsin; lane 6, 100 μg/mL chymotrypsin; lane 7, 500 μg/mL chymotrypsin. (B) Analysis of tryptic and chymotrypsin fragments by DNA–cellulose and DEAE-cellulose chromatography. Aliquots of the pooled void fractions of Figure 7B were treated with trypsin (20 μg/mL) (lane 8). Digestion was terminated by addition of 200 μg/mL soybean trypsin inhibitor, and this material was immediately chromatographed on a second Sephacryl S-200 column (see Figure 7C). Fractions 44–48 (of Figure 7C) were pooled and either electrophoresed (lane 13) or applied to a DNA–cellulose column (3 mL bed volume) or to a DEAE–cellulose column (5 mL bed volume). The pooled flow-through and wash fractions from these columns are shown in lane 12 (DNA–cellulose) and lane 14 (DEAE-cellulose). The pooled flow-through and wash fractions contained all of the applied radioactivity. The DNA–cellulose column was eluted with 0.5 NaCl (lane 15). The pooled void fractions from the Sephacryl S-200 chromatography [lane 1 of (A)] were digested for 20 min at 0 °C with chymotrypsin at 5 μg/mL (lane 9). This material was immediately applied to a 5 mL bed volume DNA–cellulose column. The wash (lane 11) and 0.5 M NaCl elute (lane 10) of this column were obtained as described under Materials and Methods.

by dexamethasone 21-mesylate. The dexamethasone 21-mesylate labeled fragments were examined under denaturing conditions by using $NaDodSO_4$–polyacrylamide gel electrophoresis. These techniques generate a large number of receptor fragments, but only those with covalently attached [^{3}H]-dexamethasone 21-mesylate will be detected.

The tryptic and chymotryptic digestion patterns of partially purified, activated [^{3}H]dexamethasone 21-mesylate labeled, rat liver and HTC cell receptors are very similar (Figures 2–4). The few differences that are observed could easily be due to varied experimental conditions. This close similarity of proteolytic digestion patterns, coupled with the same molecular weights of the intact, denatured, labeled receptors from rat liver and HTC cells (this report; Simons et al., 1983), supports the conclusion that the glucocorticoid receptors from normal hepatocytes and a transformed hepatocyte (i.e., HTC cells) are identical. However, it should be appreciated that these analyses cannot detect differences between the receptors that involve small insertions or deletions (<1000–2000 daltons) or amino acid changes at positions far removed from the sites

of proteolysis. We also cannot detect altered proteolysis of those receptor fragments lacking the covalently bound ^{3}H-steroid.

Activation of glucocorticoid receptor–steroid complexes is a process that is defined operationally by the increased affinity of complexes for nuclear binding sites. This process has been found to be accompanied by very few physicochemical changes (Simons, 1979; Schmidt & Litwack, 1982). In this study, we have used receptor–steroid complex binding to DNA to define activated complexes. While evidence is accumulating that there are discrete subgroups of activated glucocorticoid complexes (Simons, 1977; Munck & Foley, 1980; Simons et al., 1983; Svec & Williams, 1983; Hirose et al., 1983), the ability of receptor–steroid complexes to bind to DNA is the common definition of activation (Schmidt & Litwack, 1982) and does serve to distinguish between two distinct populations of glucocorticoid complexes. An examination of the kinetics of activation (Atger & Milgrom, 1976) and the observed increase in affinity of activated complexes for polyanions (Schmidt & Litwack, 1982) have led many people to consider that activation involves a conformational change in the complex. Recent data suggest that activation may simply involve dissociation of an oligomeric unactivated complex (Vedeckis, 1981; Raaka & Samuels, 1983; Sherman et al., 1983 Vedeckis, 1983b). Either mechanism of activation would involve reorganizations at the level of the exposed surface area of the receptor molecule. Nevertheless, the fragments produced by digestion of native unactivated and activated HTC cell receptor–steroid complexes were virtually identical (Figure 2A vs. Figure 6A and Table I). Likewise, the activated and unactivated complexes that were denatured under relatively mild conditions gave indistingusihable digestion fragments (Figure 2A vs. Figure 6B and Table I). The differences in intensities of individual fragments are most likely due to different protein (and perhaps protease inhibitor) concentrations in the two preparations, which affect the *rates* of digestion. Thus, as determined by the denatured molecular weights of affinity-labeled HTC cell receptors before and after varying amounts of proteolysis by trypsin, chymotrypsin, or V8 protease, the unactivated and activated complexes are identical. It is possible, though, that such putative conformational changes occur in structurally "open" or "hinge" regions of the receptor far from the covalently bound steroid and are rapidly cleaved by proteases in both unactivated and activated complexes. Alternatively, other proteases might detect such conformational changes (Mornet et al., 1984).

Despite the very different specificities of the three proteases used in this study, they each produced several similarly sized fragments of the native complexes, i.e., $M_r \simeq 42\,500$, $\simeq 30\,500$, and $\simeq 27\,000$ (see Table I). If most of each similarly sized fragment contains the same segment of the receptor, this would indicate the presence in the receptor of proteolytic "hot spots" containing high concentrations of aromatic, basic, and acidic amino acids. In view of the increased proteolytic sensitivity of receptors, compared to the abundant cytosolic proteins, these hot spots could be involved in in vivo receptor–steroid complex degradation. A single amino acid change in the appropriate hot spot may facilitate the generation of the smaller (i.e., 42.5K) receptor found in several lines of glucocorticoid-resistant cells (Yamamoto et al., 1974; Stevens & Stevens, 1981; Nordeen et al., 1981; Gehring & Hotz, 1983; Okret et al., 1983).

The major chymotrypsin fragment of activated rat liver glucocorticoid receptors is a group of fragments of $M_r \simeq 38\,000$–$40\,000$ on gradient gels. Very similar values ($M_r \simeq 37\,000$–$38\,000$) have recently been reported for chymotrypsin-treated S49 mouse lymphoma receptor (Gehring & Hotz, 1983). The trypsin-generated meroreceptor (Wrange & Gustaffson, 1978; Miller et al., 1981) was found on gradient gels to consist of two species of $M_r \simeq 31\,000$ and $\simeq 27\,000$.

In model systems, the reaction of dexamethasone 21-mesylate is highly specific for thiol groups (Simons et al., 1980; Eisen et al., 1981). Nevertheless, in the local environment of the steroid-binding site, other groups could react or more than one thiol group could be involved. The fact that, for each protease, most of the initial radioactivity at $M_r \simeq 98\,000$ in the denatured complexes could be digested down to a much smaller fragment ($M_r \simeq 15\,000$; see Figures 2, 5, and 6 and Table I) suggests that the covalent labeling by dexamethasone 21-mesylate is limited to a relatively small region in the primary structure of the receptor.

In the current study, we have used an affinity-labeling steroid to identify the steroid-binding portion of the receptor during progressive proteolysis. The use of monoclonal antibodies should greatly expand the utility of this technique since other portions of the proteolyzed receptors could then be examined. A potential problem with this technique stems from the dynamic nature of the digestion reaction. Each lane in the above gels represents one instant in a constantly changing solution. Furthermore, the effects of unknown contaminating proteins and different buffers are unpredictable. Therefore, to base conclusions on the intensity, or even the presence or absence, of a few bands can be misleading. For example, the intensity of the 32K species obtained from chymotrypsin digestion of native HTC cell complexes varied from being barely detectable (Figure 2C) to being a major band (data not shown), presumably because this species is a short-lived intermediate. Given these precautions the ability to follow defined segments of glucocorticoid (and other) receptor molecules during controlled proteolysis, especially when crude receptor preparations are used, should greatly facilitate future structure/function studies of receptors.

Acknowledgments

We thank Linda A. Hamilton for expert secretarial assistance.

Registry No. Trypsin, 9002-07-7; chymotrypsin, 9004-07-3; *Staphylococcus aureus* V8 protease, 66676-43-5; dexamethasone 21-mesylate, 2265-22-7; triamcinolone acetonide, 76-25-5.

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