53790-13-9; porphobilinogen deaminase, 9074-91-3; uroporphyrinogen III cosynthase, 37340-55-9.

References

Battersby, A. R., & McDonald, E. (1976) *Philos. Trans. R. Soc. London, Ser. B 273*, 161.

Battersby, A. R., Fookes, C. J. R., Meegan, M. J., McDonald, E., & Wurziger, H. K. W. (1981a) *J. Chem. Soc., Perkin Trans. 1*, 2786.

Battersby, A. R., Fookes, C. J. R., Matcham, G. W. J., & Pandey, P. S. (1981b) *Angew. Chem., Int. Ed. Engl. 21*, 293.

Battersby, A. R., Fookes, C. J. R., Gustafson-Potter, K. E., McDonald, E., & Matcham, G. W. J. (1982a) *J. Chem. Soc., Perkin Trans. 1*, 2413.

Battersby, A. R., Fookes, C. J. R., Gustafson-Potter, K. E., McDonald, E., & Matcham, G. W. J. (1982b) *J. Chem. Soc., Perkin Trans. 1*, 2427.

Bogorad, L. (1958a) *J. Biol. Chem. 233*, 501.

Bogorad, L. (1958b) *J. Biol. Chem. 233*, 510.

Díaz, L., Frydman, R. B., Valasinas, A., & Frydman, B. (1979) *J. Am. Chem. Soc. 101*, 2710.

Díaz, L., Valasinas, A., & Frydman, B. (1981) *J. Org. Chem. 46*, 864.

Franck, B., Bock, W., Bringmann, G., Fels, G., Grubenbecker, F., Marsman, M., Pietschmann, R., Schapers, K., Spiegel, V., Steinkamp, R., Ufer, G., & Wegner, Ch. (1980) *Int. J. Biochem. 12*, 671.

Frydman, R. B., & Frydman, B. (1970) *Arch. Biochem. Biophys. 136*, 193.

Frydman, R. B., & Frydman, B. (1973) *Biochim. Biophys. Acta 293*, 506.

Frydman, R. B., & Feinstein, G. (1974) *Biochim. Biophys. Acta 350*, 358.

Frydman, R. B., & Frydman, B. (1975) *FEBS Lett. 52*, 317.

Frydman, B., Reil, S., Despuy, M. E., & Rapoport, H. (1969) *J. Am. Chem. Soc. 91*, 2738.

Frydman, R. B., Valasinas, A., & Frydman, B. (1973) *Biochemistry 12*, 80.

Frydman, B., Frydman, R. B., Valasinas, A., Levy, E. S., & Feinstein, G. (1976) *Philos. Trans. R. Soc. London, Ser. B 273*, 137.

Frydman, R. B., Levy, E. S., Valasinas, A., & Frydman, B. (1978a) *Biochemistry 17*, 110.

Frydman, R. B., Levy, E. S., Valasinas, A., & Frydman, B. (1978b) *Biochemistry 17*, 115.

Frydman, R. B., Frydman, B., & Valasinas, A. (1979) in *The Porphyrins* (Dolphin, D., Ed.) Vol. VI, pp 1–123, Academic Press, New York.

Gellert, M. (1981) *Annu. Rev. Biochem. 50*, 879–910.

Higuchi, M., & Bogorad, L. (1975) *Ann. N.Y. Acad. Sci. 244*, 401.

Lascelles, J. (1956) *Biochem. J. 62*, 78.

Radmer, R., & Bogorad, L. (1972) *Biochemistry 11*, 904.

Scott, A. I., Ho, K. S., Kajiwara, M., & Takahashi, T. (1976) *J. Am. Chem. Soc. 98*, 1589.

Shemin, D. (1975) *Ann. N.Y. Acad. Sci. 244*, 348.

Wayne, A. W., Straight, R. C., Wales, E. F., & Englert, E., Jr. (1979) *HCC CC, J. High Resolut. Chromatogr. Chromatogr. Commun. 2*, 621.

Photoaffinity Labeling and Partial Proteolysis of Wild-Type and Variant Glucocorticoid Receptors†

Ulrich Gehring* and Agnes Hotz

ABSTRACT: Glucocorticoid receptors of wild-type lymphoid cells and of two classes of glucocorticoid-resistant variants of "nuclear transfer deficient" (nt^-) and "increased nuclear transfer" (nt^i) phenotypes, respectively, were investigated. Photoaffinity labeling of receptor complexes with a radiolabeled glucocorticoid of high affinity was used to analyze these receptor types by electrophoresis in sodium dodecyl sulfate containing gels. Wild-type and nt^--variant receptors yielded radiolabeled polypeptide bands of M_r 94 000 ± 5000 while nt^i-variant receptors had a molecular weight of 40 000 ± 2000. Partial proteolysis of wild-type and nt^- receptors with α-chymotrypsin resulted in steroid-labeled receptor fragments of M_r 37 000–38 000 while nt^i-variant receptors remained unchanged. In the case of wild-type receptors, the chymotryptic fragment had increased affinity for DNA indistinguishable from that of native nt^i-variant receptors. Depending on the nt^- cell clone, the chymotryptic receptor fragments containing the steroid binding site had either the same low affinity for DNA as the undigested receptors or a slightly increased affinity. Partial proteolysis with trypsin of wild-type, nt^-, and nt^i receptors resulted in steroid-labeled fragments of M_r 29 000 as major products and some fragments of M_r 27 000. These tryptic receptor fragments were devoid of DNA binding ability regardless of the original receptor types. With a lysine-specific protease, similar fragments were obtained from wild-type, nt^-, and nt^i receptors. In contrast, a protease specific for arginine residues did not produce receptor fragments detectable by our techniques. A model of the wild-type receptor is discussed.

Specific receptors play a pivotal role in the mechanism by which steroid hormones elicit physiological responses in target cells [for reviews, see Agarwal (1978), Higgins & Gehring (1978), Baxter & Rousseau (1979), and Katzenellenbogen (1980)]. Thus many cell culture lines of murine thymic lymphoid cells that contain functionally active glucocorticoid receptors respond to this class of steroids by growth inhibition and cell lysis. On the other hand, unresponsive cell variants are either deficient in receptors or contain defective receptors [for a review, see Gehring (1980a)].

Two types of receptor defects have thus far been identified in which hormone binding is roughly normal, but interaction of the receptor–glucocorticoid complexes with cell nuclei,

† From the Institut für Biologische Chemie, Universität Heidelberg, D-6900 Heidelberg, West Germany. *Received March 21, 1983.* This work was supported by the Deutsche Forschungsgemeinschaft and Fonds der Chemischen Industrie.

0006-2960/83/0422-4013$01.50/0

chromatin, or DNA is affected. Nuclear binding is abnormally low with receptors of the "nuclear transfer deficient" (nt^-) type, while increased binding to nuclei or chromatin and abnormally high affinity for DNA is seen with receptors of the "increased nuclear transfer" (nt^i) type. The preliminary characterization of these receptor defects, as well as many biochemical studies with normal receptors, has led to the view that the wild-type receptor contains two distinct but functionally linked active domains: one for steroid binding and the other for nuclear interaction (Gehring, 1980a). More recently, however, at least one other domain within the receptor structure has been postulated from biochemical and immunological experiments with wild-type and variant receptors (Dellweg et al., 1982; Carlstedt-Duke et al., 1982). This particular area of the receptor molecule is involved in modulating DNA- and nuclear-binding of receptor–hormone complexes such that a biological response is triggered, and it appears to carry the main antigenic determinants.

Affinity labeling of glucocorticoid receptors with radiolabeled steroid ligands has recently become a powerful tool for studying the molecular structure of the receptors (Govindan & Manz, 1980; Nordeen et al., 1981; Simons & Thompson, 1981; Westphal et al., 1981; Dellweg et al., 1982) since crude or partially purified receptor preparations can be analyzed by gel electrophoresis under denaturing conditions. In the present study we used this technique in combination with chromatography on DNA–cellulose. Limited proteolysis of various receptor types with several endoproteinases allows us now to more accurately define the functional domains within glucocorticoid receptors of lymphoid cells.

Experimental Procedures

Buffers. Buffer A contained 20 mM potassium phosphate (pH 7.4 or 8.5, at 20 °C), 20 mM KCl, 2 mM mercaptoethanol, 1 mM EDTA,[1] and 10% glycerol. Buffer B contained 10 mM *N*-[tris(hydroxymethyl)methyl]glycine (pH 7.8 at 20 °C), 30 mM KCl, 2 mM mercaptoethanol, 1 mM EDTA, and 10% glycerol. $NaDodSO_4$ buffer contained 70 mM Tris-HCl, pH 6.8, 100 mM mercaptoethanol, 0.005% bromophenol blue, 3% $NaDodSO_4$, and 20% glycerol. All procedures were carried out at 0–6 °C unless otherwise noted.

Cell Cultures. The S49.1 mouse lymphoma sublines S49.1G.3 (wild type), S49.1G.3.83R (nt^- type), S49.1TB.4.41.22R (nt^- type), and S49.1TB.4.55R (nt^i type) were those previously used (Gehring, 1979). The subline S49.1TB.4.143R (nt^i type) was kindly provided by Dr. O. Pongs. The human lymphoblastic leukemia cell line CEM-C7 (Norman & Thompson, 1977) was kindly provided by Dr. C. Bird. All cell lines were recloned prior to use. Cells were grown in suspension culture as previously described (Gehring, 1980b) except that line CEM-C7 was grown in medium supplemented with 10% fetal calf serum instead of horse serum. Cells were harvested and washed as described (Gehring, 1980b), quickly frozen in liquid nitrogen, and stored at −90 °C.

Tumors. The mouse lymphomas CS P1798 and CR P1798 (Stevens et al., 1978) were kindly provided by Drs. J. Stevens and S. Okret. They were grown as subcutaneous tumors in female Balb/c mice. About 2 weeks after inoculation, the tumors were excised from the animals and squeezed through a tissue press. The cells were washed twice with ice-cold saline (buffered to pH 7.4), collected by centrifugation at 1000*g*, quickly frozen in liquid nitrogen, and stored at −90 °C.

Cytosol Preparations. Cytosols were prepared at 0 °C from frozen cell pellets in buffer A, pH 7.4, as previously described (Gehring, 1980b). The protease inhibitors aprotinin and leupeptin (Sigma, St. Louis, MO) were added to final concentrations of 0.6 and 1 μg/mL, respectively, except in those experiments in which receptors were subsequently submitted to proteolysis with trypsin, Lys-C endoproteinase, or Arg-C endoproteinase. Cytosol preparations were incubated at 0 °C for 2 h with 48 nM [^{3}H]triamcinolone acetonide (New England Nuclear, Boston, MA; 31 Ci/mmol). Sodium molybdate, 10 mM, was added to cytosols prior to the steroid in those experiments in which receptor complexes were to be preserved in the nonactivated state (Leach et al., 1979). Binding of the hormone was assessed by the charcoal assay, and radioactivity was determined as previously (Spindler-Barth & Gehring, 1982).

Use of Proteases. Receptor complexes were routinely treated with proteases either prior to DNA–cellulose chromatography or after photoaffinity labeling. Bovine α-chymotrypsin (Serva, Heidelberg) was routinely used at a concn. of 10 μg/mL for 5 min in the cold. Bovine trypsin (Sigma, St. Louis, MO; treated with diphenylcarbamyl chloride) was routinely used at 20 μg/mL for 30 min in the cold. Endoproteinase Lys-C from *Lysobacter enzymogenes* was kindly provided by Dr. P. Wunderwald; endoproteinase Arg-C was from Boehringer (Mannheim). Both enzymes were used at concentrations of 60–80 μg/mL for 30 min in the cold.

DNA–Cellulose Chromatography. Cytosol preparations containing 100 000–300 000 cpm of specifically bound [^{3}H]-triamcinolone acetonide were activated either by a 30-min incubation at 20 °C or by incubating with 300 mM KCl for 1 h at 0 °C followed by 10-fold dilution into buffer B without KCl. Samples were chromatographed as described (Gehring, 1980b) except that DNA–cellulose was equilibrated with buffer B containing 100 μg/mL bovine serum albumin. After being extensively rinsed, the columns were eluted with a linear 30–300 mM KCl gradient in buffer B.

Photoaffinity Labeling. Cytosol receptor preparations containing up to 10^6 cpm bound [^{3}H]triamcinolone acetonide were preserved in the nonactivated state by addition of molybdate, adjusted to pH 8.5, and applied onto 3-mL columns of DEAE-cellulose (DE-52, Whatman) equilibrated with buffer A, pH 8.5. After an extensive rinsing with buffer, the columns were washed with 5–10 mL of 110 mM KCl in buffer A and the receptor complexes eluted with 200 mM KCl. Eluates were irradiated for 50–60 min as previously described (Dellweg et al., 1982) with a XBO 450-W high-pressure xenon lamp (LX 501, Zeiss). Protein was precipitated with 10% TCA, collected on glass filters (Whatman GF/F), washed with acetone, and redissolved in $NaDodSO_4$ buffer by boiling for 5 min.

Gel Electrophoresis. Electrophoresis in $NaDodSO_4$-containing polyacrylamide gels [10% acrylamide and 0.26% bis(acrylamide) or 7% acrylamide and 0.19% bis(acrylamide)] was carried out as described previously (Laemmli, 1970). At least 8000 dpm of protein-bound ^{3}H were applied per slot. For protein fixation, gels were treated with TCA/acetic acid/methanol/water (1:1:3:5), soaked in EN^{3}HANCE (New England Nuclear, Boston, MA), dried and submitted to fluorography with Kodak XR5 film at −90 °C for 2–4 weeks. In several instances, some radiolabeled material did not enter the gel. This varied among preparations from the same cell

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate; DEAE, diethylaminoethyl; TCA, trichloroacetic acid; triamcinolone acetonide, 9α-fluoro-11β,16α,17α,21-tetrahydroxypregna-1,4-diene-3,20-dione cyclic 16,17-ketal with acetone.

Table I: DNA Binding Properties and Molecular Weights of Receptor Types[a]

receptor type	molecular weight		DNA–cellulose (mM KCl required for elution)	
	native	after chymotrypsin	native	after chymotrypsin
S49.1 wild type	94 700 ± 5000 (9)	38 000	175 ± 5 (2)	236 ± 9 (2)
S49.1 nt$^-$ (clone 22R)	94 000 ± 4000 (6)	37 400	75 ± 13 (3)	129 ± 9 (3)
S49.1 nt$^-$ (clone 82R)	94 000 ± 3000 (3)	37 400	86 ± 15 (3)	87 ± 8 (3)
S49.1 nt^i (clone 55R)	40 300 ± 2000 (10)	39 000	225 ± 9 (2)	229 ± 9 (2)
S49.1 nt^i (clone 143R)	40 800 ± 2000 (5)	41 000	210 ± 10 (2)	209 ± 10 (2)
CS P1798	93 000 ± 3000 (2)		193 ± 10 (3)	
CR P1798	38 500 ± 2000 (2)		212 ± 9 (2)	
CEM-C7	95 000		187 ± 8 (3)	

[a] Mean values and range are reported (number of separate experiments in parentheses).

clones; the results, however, were not affected.

Marker Proteins. For $NaDodSO_4$ gel electrophoresis, the following polypeptide markers were used: β-galactosidase from *Escherichia coli* (M_r 116 300; Fowler & Zabin, 1978), rabbit muscle glycogen phosphorylase *b* (M_r 97 400; Titani et al., 1977), bovine serum albumin (M_r 66 100; Brown, 1975), rabbit muscle aldolase (M_r 39 000; Lai, 1975), and bovine erythrocyte carbonic anhydrase (M_r 29 000; Sciaky et al., 1976). Serum albumin was obtained from Behringwerke (Marburg), the other marker proteins were from Boehringer (Mannheim). A total of 1 mg of each marker protein was dissolved in 0.25 mL of 8 M guanidine hydrochloride in 50 mM Tris-HCl, pH 7.8, iodo[1-^{14}C]acetamide (New England Nuclear, Boston, MA; 24 mCi/mmol) was added to a final concentration of 0.8 mM, and the mixture was incubated for 24 h at room temperature. Proteins were precipitated with TCA, collected, and washed several times with 5% TCA followed by acetone. The pellets were dissolved in $NaDodSO_4$ buffer, and 400 dpm of protein-bound ^{14}C of each marker protein were applied per slot. ^{14}C-Labeled β-galactosidase was used as a marker only in 7% polyacrylamide gels.

Results

DNA–Cellulose Chromatography. Chromatography on DNA–cellulose as a matrix has become a convenient method for the distinction of wild-type, nt$^-$, and nt^i receptor types (Yamamoto et al., 1976). As shown in Table I, wild-type receptor complexes of S49.1 mouse lymphoma cells eluted with 170–190 mM KCl while nt$^-$ and nt^i receptor complexes required lower (70–90 mM) and higher (210–230 mM) salt concentrations, respectively. Wild-type receptors of other glucocorticoid-sensitive lymphoid cells behaved similarly, as shown in Table I for human lymphoblastic leukemia cells of line CEM-C7 and for the mouse lymphoma CS P1798. In the case of the lymphoma P1798, a cortisol-resistant tumor variant (CR P1798) had been obtained by long-term steroid treatment of the carrier animals (Stevens et al., 1978). Receptor complexes from these CR P1798 tumors had previously been found to bind more tightly to DNA than those of the CS P1798 strain (Stevens & Stevens, 1981). By use of DNA–cellulose chromatography, we now show that CR P1798 receptor complexes require 212 mM KCl for elution (Table I) and thus behave identical with the nt^i variants of S49.1 origin.

Photoaffinity Labeling. Photoactivation at $\lambda \geq 320$ nm of receptor complexes with the high-affinity glucocorticoid triamcinolone acetonide has recently been used to covalently tag receptor polypeptides (Dellweg et al., 1982). We applied this method to receptor preparations partially purified by DEAE-cellulose chromatography and analyzed the cross-linked polypeptides by $NaDodSO_4$ gel electrophoresis (Figure 1). Wild-type receptors of mouse lymphomas S49.1 and CS P1798 and of human leukemia cells CEM-C7 revealed a major band

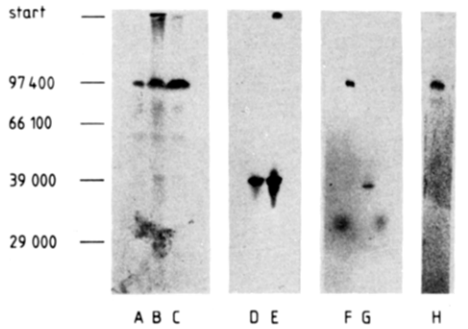

FIGURE 1: $NaDodSO_4$ gel electrophoresis of photoaffinity-labeled receptors. Cytosol receptor complexes with [^{3}H]triamcinolone acetonide were irradiated as described under Experimental Procedures. (A) S49.1 wild type; (B) S49.1 nt$^-$ (clone 22R); (C) S49.1 nt$^-$ (clone 83R); (D) S49.1 nt^i (clone 55R); (E) S49.1 nt^i (clone 143R); (F) CS P1798; (G) CR P1798; (H) CEM-C7.

of M_r 94 000 ● 5000 (Table I). Variant receptors of the nt$^-$ type were investigated with use of two independent isolates within the S49.1 cell system. In both instances (Figure 1, lanes B and C), the molecular weight was identical with that of wild-type receptors. The same result was also obtained when electrophoresis was carried out in 7% polyacrylamide gels (data not shown). In contrast, nt^i-variant receptors revealed radiolabeled polypeptide bands of M_r 40 000 ● 2000 as shown in Figure 1 for two independent S49.1 subclones (lanes D and E) and the CR P1798 lymphoma (lane G). Within the limits of our method (Table I), these nt^i-variant receptors had the same molecular weights. In some nt^i receptor preparations, a minor labeled band of about M_r 37 000 was observed in addition to the main band of 40 000 (for example, Figure 1, lane E).

In order to address the question whether or not receptor–steroid complexes retain their functional integrity upon photoactivation, we carried out the following experiment. Wild-type S49.1 receptor was complexed with ^{3}H-labeled steroid, passed over DEAE-cellulose, and irradiated as in our standard procedure. Following dilution to 30 mM KCl and a 30-min incubation at 20 °C, the material was submitted to DNA–cellulose chromatography; the eluate was precipitated with TCA and analyzed by gel electrophoresis. A labeled polypeptide band of M_r 95 000 was obtained (data not shown), demonstrating that the receptor–steroid complex is still able to interact with DNA following covalent cross-linking.

Partial Proteolysis. Mild proteolysis with α-chymotrypsin of wild-type receptors of rat liver (Wrange & Gustafsson, 1978) and mouse lymphoma cells (Andreasen & Gehring, 1981; Stevens & Stevens, 1981) has previously been shown to create receptor forms with abnormal DNA binding properties. By use of DNA–cellulose chromatography, it became clear that the wild-type receptor treated with α-chymotrypsin binds to DNA with an affinity indistinguishable from that of

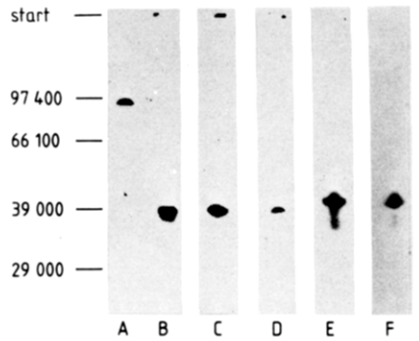

FIGURE 2: NaDodSO$_4$ gel electrophoresis of chymotrypsin-treated receptors. Cytosol receptor complexes with [^{3}H]triamcinolone acetonide were subjected to photoaffinity labeling followed by a 5-min treatment with 10 μg/mL α-chymotrypsin. (A) Control of S49.1 wild type without chymotrypsin treatment; (B) S49.1 wild type; (C) S49.1 nt$^-$ (clone 22R); (D) S49.1 nt$^-$ (clone 83R); (E) S49.1 nt^i (clone 55R); (F) S49.1 nt^i (clone 143R).

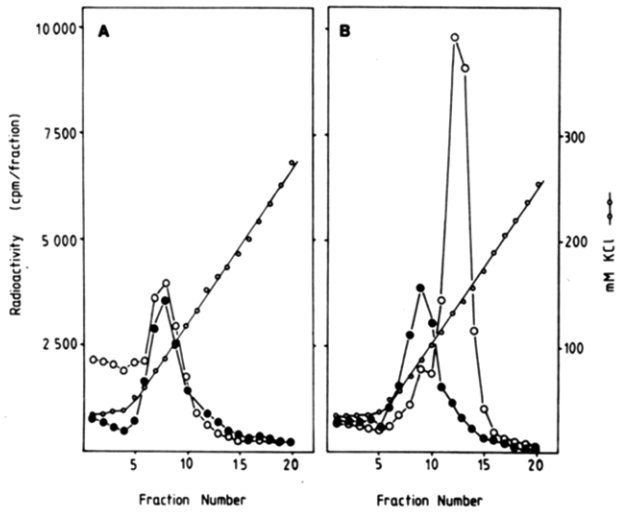

FIGURE 3: DNA–cellulose chromatography of chymotrypsin-treated nt$^-$ receptors. Cytosol preparations of S49.1 nt$^-$ clone 83R (A) and clone 22R (B) were incubated with [^{3}H]triamcinolone acetonide, activated at 20 °C, and chromatographed on DNA–cellulose either in the native state (●) or following a 5-min treatment with 10 μg/mL α-chymotrypsin (○). Fractions of about 700 μL were collected.

the native nt^i receptor (Dellweg et al., 1982; Table I). NaDodSO$_4$ gel electrophoresis revealed a major steroid-labeled polypeptide of about M_r 38 000 (Figure 2, lane B); in some experiments, a faint band of about M_r 29 000 was also observed. The same result was obtained whether chymotrypsin treatment was after irradiation as in our standard procedure or prior to photoactivation (data not shown).

Similar chymotryptic receptor fragments of about M_r 38 000 were also recovered from two types of S49.1 nt$^-$-variant receptors (Figure 2, lanes C and D). These receptor fragments had either the same low affinity for DNA as the native receptor (clone 83R) or slightly increased affinity (clone 22R) as judged from DNA–cellulose elution profiles (Figure 3, Table I). Variant receptors of the nt^i type proved to be resistant to chymotrypsin; the molecular size of the labeled polypeptides did not change (Figure 2, lanes E and F), and DNA–cellulose chromatography did not reveal any differences (Table I).

Mild proteolysis of S49.1 wild-type receptors with trypsin resulted in the complete loss of DNA binding ability of the steroid-labeled receptor (Figure 4), suggesting separation of the active domains for steroid binding and for DNA interaction. The same result was also obtained with nt$^-$- and nt^i-variant receptors (data not shown). Under these conditions

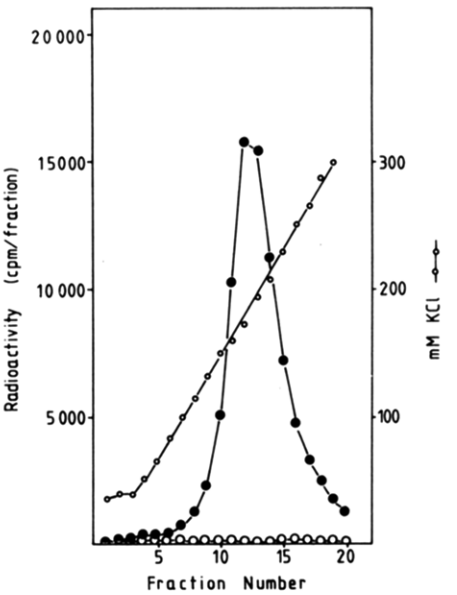

FIGURE 4: DNA–cellulose chromatography of native and trypsin-treated receptors. A cytosol preparation of S49.1 wild type was incubated with [^{3}H]triamcinolone acetonide, activated at 20 °C, split in halves, and chromatographed on DNA–cellulose either in the native state (●) or following a 30-min treatment with 20 μg/mL trypsin (○). Fractions of about 700 μL were collected.

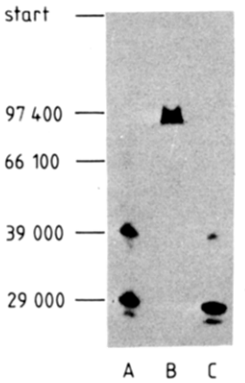

FIGURE 5: NaDodSO$_4$ gel electrophoresis of trypsin-treated receptors. S49.1 wild-type cytosol receptor complexes with [^{3}H]triamcinolone acetonide were subjected to photoaffinity labeling followed by a 30-min treatment with trypsin. (A) Treatment with 10 μg/mL trypsin; (B) control without trypsin; (C) treatment with 20 μg/mL trypsin.

(20 μg of trypsin/mL), steroid-labeled receptor fragments of M_r 29 000 ± 2000 and 27 000 ± 2000 were produced from the wild-type receptor (Figure 5C). The same fragments were obtained when proteolysis was prior to rather than after irradiation (data not shown). When trypsin was used at a lower concentration (10 μg/mL), the receptor band of M_r 27 000 was very faint; instead, a labeled fragment of M_r 38 000 ± 2000 (Figure 5A) appeared, which is very similar in size to the chymotryptic fragment described above.

Endoproteinase Lys-C is of exquisite specificity since it cleaves polypeptides only at lysine residues (Steffens et al., 1982). When we used this enzyme with S49.1 wild-type, nt$^-$, and nt^i receptors, we observed patterns of steroid-labeled receptor fragments that were similar to those produced by trypsin (Figure 6) except that an additional minor band of M_r 26 000 appeared. In contrast, the arginine-specific endoproteinase Arg-C (Schenkein et al., 1969) did not produce any labeled receptor fragments (Figure 6, lanes B, E, and H). Receptors digested with endoproteinase Lys-C did not bind to DNA–cellulose (data not shown) as would be expected from the similarity with the fragments obtained by tryptic cleavage.

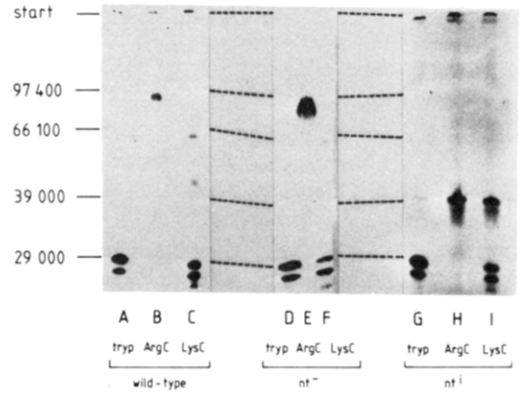

FIGURE 6: $NaDodSO_4$ gel electrophoresis of receptors treated with proteases. Cytosol receptor complexes with [^{3}H]triamcinolone acetonide were subjected to photoaffinity labeling followed by a 30-min treatment with 20 μg/mL trypsin (lanes A, D, and G), 80 μg/mL endoproteinase Arg-C (lanes B, E, and H), or 60 μg/mL endoproteinase Lys-C (lanes C, F, and I). (Lanes A–C) S49.1 wild type; (lanes D–F) S49.1 nt⁻ (clone 22R); (lanes G–I) S49.1 nt^i (clone 55R).

Discussion

DNA has previously been used as a model for nuclear interaction of receptor–glucocorticoid complexes (Baxter et al., 1972; Gehring & Tomkins, 1974; Yamamoto et al., 1974, 1976; Simons et al., 1976); in this respect, DNA is particularly useful when adsorbed onto cellulose (Alberts & Herrick, 1971). Using this system of affinity chromatography, it is possible to distinguish between wild-type receptors and the nt⁻ and nt^i receptor variants by the salt concentrations required for elution (see Table I). The differences in elution profiles reflect decreased and increased affinities for DNA of nt⁻- and nt^i-variant receptors, respectively, as compared to the wild-type (Yamamoto et al., 1976; Andreasen & Gehring, 1981). Trypsin treatment of glucocorticoid receptors of rat liver and of lymphoid cells has previously been shown to produce receptor forms devoid of DNA binding (Wrange & Gustafsson, 1978; Andreasen & Gehring, 1981; Stevens & Stevens, 1981). By use of DNA–cellulose chromatography, we now show that mild tryptic digestion of S49.1 wild-type and of both types of variant receptors completely abolishes their ability to bind to DNA, thus suggesting that trypsin either separates the domains for steroid binding and for DNA interaction or destroys that part of the receptor molecule that is involved in DNA binding. By contrast, proteolysis with α-chymotrypsin of the wild type caused a dramatic increase in the affinity for DNA; this partially digested receptor form behaves identical with the nt^i variant upon DNA–cellulose chromatography. While chymotrypsin treatment did not further increase the DNA binding ability of native nt^i receptors, we found some increase in the affinity for DNA with one of our nt⁻ receptors but not with the other. It is not clear at present what molecular differences there are between these two nt⁻-variant receptors since they have the same polypeptide molecular weights and were degraded by chymotrypsin to fragments of the same size. Interestingly, they also showed some differences in the dissociation rate of their complexes with steroid (Spindler-Barth & Gehring, 1982).

Affinity labeling of glucocorticoid receptors has in recent years become a valuable analytical method [as reviewed by Simons & Thompson (1982)]. We have previously used photoactivation of receptor complexes with triamcinolone acetonide to covalently label receptors and determine their molecular weights (Dellweg et al., 1982). In the present investigation, a more accurate standardization of the method was achieved by use of radiolabeled marker polypeptides. We now find a molecular weight of 94 000 ● 5000 for wild-type receptors of mouse and human lymphoid cells, as well as for nt⁻-variant receptors of two independent cell isolates. On the other hand, nt^i-variant receptors of three independent isolates showed molecular weights of 40 000 ± 2000. Partial proteolysis with trypsin of wild-type, nt⁻, and nt^i receptors yielded steroid-labeled fragments of M_r 29 000 and 27 000. Digestion with a lysine-specific protease of bacterial origin gave rise to the same fragments while treatment with an arginine-specific enzyme from mouse submaxillary glands did not cleave the native receptors. Chymotryptic fragments of wild-type and nt⁻-variant receptors were found to be slightly smaller (by 2000–3000 daltons) than native nt^i receptors. These results suggest that glucocorticoid receptors of molecular weights in the range of 40 000 have abnormally high DNA binding affinity, independent of whether they are of mutational origin (nt^i receptors) or proteolytic products of the wild-type. The eliminated portion of about 54 000 daltons must be involved in modulating the binding to DNA in a biologically meaningful way. It is noteworthy that Carlstedt-Duke et al. (1982) were able to detect by immunochemical methods a fragment of roughly that size in chymotryptic digests of rat liver glucocorticoid receptors. Also, antibodies raised against wild-type receptors of rat liver did not react with the nt^i receptor variant of CR P1798 lymphoma cells (Stevens et al., 1981).

Glucocorticoid receptors of various tissues including lymphoid cells have also been analyzed by gel-permeation chromatography, and molecular weights ranging from 240 000 to 320 000 have been reported (Cidlowski, 1980; Niu et al., 1981; Weatherill & Bell, 1982). Considering the fact that the steroid-labeled polypeptide is of M_r 94 000, we regard these higher molecular weight receptor forms as polymeric in nature. It remains open, however, what other polypeptides are involved and how the 94 000 receptor polypeptide is organized in such complexes. At high ionic strength (400–500 mM salt), these large complexes appear to dissociate since receptor forms of 90 000–95 000 have been detected by gel-permeation chromatography and sedimentation analysis (Yamamoto et al., 1976; Weatherill & Bell, 1982). Also, molecular weights of about 90 000 were reported for the highly purified glucocorticoid receptor of rat liver and for one of the receptor forms of rat thymus (Govindan, 1979; Wrange et al., 1979; Tsawdaroglou et al., 1981).

The characteristics of wild-type and variant glucocorticoid receptors reported and discussed in the present paper lead to the following molecular model for the wild-type receptor: In addition to the sites for steroid binding and for nuclear interaction, the 94 000-dalton polypeptide chain contains a third domain that modulates nuclear interaction and DNA binding. The nt^i mutation results in a receptor molecule from which the modulating domain is missing. Similarly, proteolysis with chymotrypsin eliminates the modulating domain but leaves the steroid binding site and the nuclear interaction domain covalently linked. Tryptic digestion, in contrast, separates these domains such that a receptor fragment is recovered that only contains the steroid binding site. Tryptic cleavage occurs at lysine residues within the polypeptide chain since a lysine-specific enzyme produced similar fragments. This view is in accordance with the recent finding that pyridoxal phosphate, which supposedly reacts with ε-amino groups of lysine residues, partially protects receptors against trypsin such that the steroid binding site and the nuclear interaction domain remain linked (Ninh et al., 1982). The sequential order of the three active domains along the 94 000-dalton polypeptide chain of the wild-type receptor remains unknown except that the modu-

lating domain must be located distal to the cluster of steroid binding and nuclear interaction sites. As to the cellular origin of the nt^{i}-variant receptors, there are several possibilities that have been discussed recently (Nordeen et al., 1981; Dellweg et al., 1982). Variant receptors of the nt^{-} type most probably harbor defects in the nuclear interaction domain itself since removal of the modulating domain does not significantly alter DNA binding.

Acknowledgments

We thank Bernd Segnitz for his excellent technical assistance.

References

Agarwal, M. K. (1978) *FEBS Lett. 85*, 1–8.
Alberts, B., & Herrick, G. (1971) *Methods Enzymol. 21*, 198–217.
Andreasen, P. A., & Gehring, U. (1981) *Eur. J. Biochem. 120*, 443–449.
Baxter, J. D., & Rousseau, G. G. (1979) *Monogr. Endocrinol. 12*, 1.
Baxter, J. D., Rousseau, G. G., Benson, M. C., Garcea, R. L., Ito, J., & Tomkins, G. M. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1892–1896.
Brown, J. R. (1975) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 34*, 591.
Carlstedt-Duke, J., Okret, S., Wrange, Ö., & Gustafsson, J.-Å. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 4260–4264.
Cidlowski, J. A. (1980) *Biochemistry 19*, 6162–6170.
Dellweg, H.-G., Hotz, A., Mugele, K., & Gehring, U. (1982) *EMBO J. 1*, 285–289.
Fowler, A. V., & Zabin, I. (1978) *J. Biol. Chem. 253*, 5521–5525.
Gehring, U. (1979) *Adv. Enzyme Regul. 17*, 343–361.
Gehring, U. (1980a) *Biochem. Actions Horm. 7*, 205–232.
Gehring, U. (1980b) *Mol. Cell. Endocrinol. 20*, 261–274.
Gehring, U., & Tomkins, G. M. (1974) *Cell (Cambridge, Mass.) 3*, 301–306.
Govindan, M. V. (1979) *J. Steroid Biochem. 11*, 323–332.
Govindan, M. V., & Manz, B. (1980) *Allergologie 3*, 204–213.
Higgins, S. J., & Gehring, U. (1978) *Adv. Cancer Res. 28*, 313–397.
Katzenellenbogen, B. S. (1980) *Annu. Rev. Physiol. 42*, 17–35.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Lai, C. Y. (1975) *Arch. Biochem. Biophys. 166*, 358–368.
Leach, K. L., Dahmer, M. K., Hammond, N. D., Sando, J. J., & Pratt, W. B. (1979) *J. Biol. Chem. 254*, 11884–11890.
Ninh, N. V., Arányi, P., & Horváth, I. (1982) *J. Steroid Biochem. 17*, 599–601.
Niu, E.-M., Neal, R. M., Pierce, V. K., & Sherman, M. R. (1981) *J. Steroid Biochem. 15*, 1–10.
Nordeen, S. K., Lan, N. C., Showers, M. O., & Baxter, J. D. (1981) *J. Biol. Chem. 256*, 10503–10508.
Norman, M. R., & Thompson, E. B. (1977) *Cancer Res. 37*, 3785–3791.
Schenkein, I., Boesman, M., Tokarsky, E., Fishman, L., & Levy, M. (1969) *Biochem. Biophys. Res. Commun. 36*, 156–165.
Sciaky, M., Limozin, N., Filippi-Foveau, D., Gulian, J.-M., & Laurent-Tabusse, G. (1976) *Biochimie 58*, 1071–1082.
Simons, S. S., & Thompson, E. B. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 3541–3545.
Simons, S. S., & Thompson, E. B. (1982) *Biochem. Actions Horm. 9*, 221–254.
Simons, S. S., Martinez, H. M., Garcea, R. L., Baxter, J. D., & Tomkins, G. M. (1976) *J. Biol. Chem. 251*, 334–343.
Spindler-Barth, M., & Gehring, U. (1982) *FEBS Lett. 138*, 91–94.
Steffens, G. J., Günzler, W. A., Ötting, F., Frankus, E., & Flohé, L. (1982) *Hoppe-Seyler's Z. Physiol. Chem. 363*, 1043–1058.
Stevens, J., & Stevens, Y.-W (1981) *Cancer Res. 41*, 125–133.
Stevens, J., Stevens, Y.-W., Rhodes, J., & Steiner, G. (1978) *J. Natl. Cancer Inst. (U.S.) 61*, 1477–1485.
Stevens, J., Eisen, H. J., Stevens, Y.-W., Haubenstock, H., Rosenthal, R. L., & Artishevsky, A. (1981) *Cancer Res. 41*, 134–137.
Titani, K., Koide, A., Hermann, J., Ericsson, L. H., Kumar, S., Wade, R. D., Walsh, K. A., Neurath, H., & Fischer, E. H. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4762–4766.
Tsawdaroglou, N. G., Govindan, M. V., Schmid, W., & Sekeris, C. E. (1981) *Eur. J. Biochem. 114*, 305–313.
Weatherill, P. J., & Bell, P. A. (1982) *Biochem. J. 206*, 633–640.
Westphal, H. M., Fleischmann, G., & Beato, M. (1981) *Eur. J. Biochem. 119*, 101–106.
Wrange, Ö., & Gustafsson, J.-Å. (1978) *J. Biol. Chem. 253*, 856–865.
Wrange, Ö., Carlstedt-Duke, J., & Gustafsson, J.-Å. (1979) *J. Biol. Chem. 254*, 9284–9290.
Yamamoto, K. R., Stampfer, M. R., & Tomkins, G. M. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 3901–3905.
Yamamoto, K. R., Gehring, U., Stampfer, M. R., & Sibley, C. H. (1976) *Recent Prog. Horm. Res. 32*, 3–32.