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Identification of Cysteine-644 as the Covalent Site of Attachment of Dexamethasone 21-Mesylate to Murine Glucocorticoid Receptors in WEHI-7 Cells†

Lynda I. Smith,*,‡,§ Jack E. Bodwell,‡ Dirk B. Mendel,‡,∥ Thomas Ciardelli,⊥,# William G. North,‡ and Allan Munck‡

Departments of Physiology and Pharmacology, Dartmouth Medical School, Hanover, New Hampshire 03756

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ABSTRACT: Dexamethasone 21-mesylate is a highly specific synthetic glucocorticoid derivative that binds covalently to glucocorticoid receptors via sulfhydryl groups. We have identified the amino acid that reacts with the dexamethasone 21-mesylate by using enzymatic digestion and microsequencing for radiolabel. Nonactivated glucocorticoid receptors obtained from labeling intact WEHI-7 mouse thymoma cells with [^{3}H]dexamethasone 21-mesylate were immunopurified and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The purified ~100-kDa steroid-binding subunit was eluted from gel slices and subjected to enzymatic digestion. Trypsin digestion followed by reversed-phase high-performance liquid chromatography (reversed-phase HPLC) produced a single [^{3}H]dexamethasone 21-mesylate labeled peptide. Automated Edman degradation of this peptide revealed that the [^{3}H]dexamethasone 21-mesylate was located at position 5 from the amino terminus. Dual-isotope labeling studies with [^{3}H]dexamethasone 21-mesylate and [^{35}S]methionine demonstrated that this peptide contained methionine. *Staphylococcus aureus* V8 protease digestion of [^{3}H]dexamethasone 21-mesylate labeled steroid-binding subunits generated a different radiolabeled peptide containing label at position 7 from the amino terminus. On the basis of the published amino acid sequence of the murine glucocorticoid receptor, our data clearly identify cysteine-644 as the single residue in the steroid-binding domain that covalently binds dexamethasone 21-mesylate. We have confirmed this finding by demonstrating that a synthetic peptide representing the amino acid sequence 640–650 of the murine glucocorticoid receptor behaves in an identical manner on reversed-phase HPLC as the trypsin-generated peptide from intact cells.

Most effects of glucocorticoids appear to be mediated through specific cellular proteins known as glucocorticoid receptors. These receptors bind steroid and under physiological conditions undergo a process termed activation. The activated glucocorticoid receptor complex binds with affinity to certain consensus sequences on the DNA, altering transcription of genes thought to code for proteins that give rise to the observed physiological effects of glucocorticoids (Yamamoto, 1985).

Dexamethasone 21-mesylate (DM),[1] the α-keto mesylate derivative of dexamethasone, is a synthetic steroid that binds

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* Address correspondence to this author.
‡ Department of Physiology.
§ Sponsored by a Predoctoral Endocrine Training Grant from the National Institutes of Health.
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⊥ Department of Pharmacology.
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[1] Abbreviations: DM, dexamethasone 21-mesylate; dexamethasone, 9α-fluoro-11β,17,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione; reversed-phase HPLC, reversed-phase high-performance liquid chromatography; TEMED, *N,N,N′,N′*-tetramethylethylenediamine; HEPES, *N*-(2-hydroxyethyl)piperazine-*N′*-2-ethanesulfonic acid; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; BIS, bis(acrylamide); BAC, bis(acrylylcystamine); TFA, trifluoroacetic acid; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; PTC, phenyl isothiocyanate; Tris, tris(hydroxymethyl)aminomethane; TCA, trichloroacetic acid; Boc, *tert*-butoxycarbonyl; Tos, tosyl; HTC, rat hepatoma cell line; GR, glucocorticoid receptor.

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to receptors in intact cells as well as in cell-free systems and possesses both agonist and antagonist activity (Simons & Thompson, 1981). Under appropriate conditions, DM binds covalently to the receptor with 90% efficiency and remains attached to the receptor under denaturing conditions (Simons et al., 1983). For this reason, DM has proved invaluable in the characterization of glucocorticoid receptors from a number of different systems (Eisen et al., 1981; Housley et al., 1985; Mendel et al., 1986).

The established sensitivity of glucocorticoid receptors to sulfhydryl-modifying reagents (Baxter & Tomkins, 1971; Koblinsky et al., 1972; Rees & Bell, 1975), coupled with the reported reactivity of the α-keto mesylate group to thiol anions, led to the proposal that covalent binding of DM to the receptor involved the formation of a thioether bond with a cysteine residue. Indirect support for this idea comes from the recent demonstration that DM interacts with bovine serum albumin exclusively through binding to a cysteine residue and that binding of DM to this protein and HTC glucocorticoid receptors is blocked by the thiol-specific reagent methyl methanethiolsulfonate (Simons, 1987). This cysteine residue is presumably located within the glucocorticoid binding site of the receptor, as glucocorticoids such as dexamethasone will compete with DM in binding assays (Simons & Thompson, 1981).

In the present study we labeled the glucocorticoid receptor in intact WEHI-7 mouse thymoma cells with [^{3}H]DM and purified the nonactivated complexes with the BuGR2 monoclonal antibody (Gametchu & Harrison, 1984). Following SDS–PAGE, the [^{3}H]DM binding protein was selectively eluted from the gel and enzymatically digested, and the peptide fragments were analyzed by reversed-phase HPLC. Radioisotopically pure peptides were then analyzed by Edman degradation. Using this approach, in conjunction with the published amino acid sequence of the murine glucocorticoid receptor (Danielson et al., 1986), we have identified a single cysteine residue, located within the steroid binding domain of the protein, which covalently binds DM. Our results with the murine receptor agree well with the recently published results of Simons et al., (1987), who have studied the DM binding site in the activated form of the rat glucocorticoid receptor.

Materials and Methods

DM, [6,7-^{3}H]DM (38–50 Ci/mmol), Protosol, and Econofluor were purchased from New England Nuclear (Boston, MA). L-[^{35}S]Methionine (>1000 Ci/mmol) was obtained from Amersham (Arlington Heights, IL). Tissue culture media and dialyzed calf serum were purchased from KC Biologicals (Lexena, KS); supplemented calf serum was purchased from Hyclone Sterile Systems (Logan, UT). Acrylamide, bis(acrylamide), bis(acrylylcystamine), ammonium persulfate, and TEMED were products of Bio-Rad (Richmond, CA). HEPES, TES, Tris, DTT, iodoacetamide, protein A–Sepharose CL-4B, and SDS–PAGE standards were from Sigma (St. Louis, MO). TPCK-treated trypsin and *Staphylococcus aureus* V8 protease were obtained from Cooper Biomedical (Malvern, PA). Hydrofluor was purchased from National Diagnostics (Manville, NJ). C-8 columns and packing materials were obtained from Alltech (Deerfield, IL); the C-4 column was obtained from Vydac (Hesperia, CA) and the Dynamax column from Rainin (Emeryville, CA). HPLC solvents were purchased from J. T. Baker (Muskego, MI). Boc-His(Tos)OH and Boc-Lys(Cl_2)OH were from Biosearch (San Rafael, CA). All other amino acid derivatives used in the peptide synthesis were obtained from Peptides International (Louisville, KY). BuGR2 monoclonal antibody to the glucocorticoid receptor (Gametchu & Harrison, 1984) was provided by Dr. Robert Harrison (University of Arkansas Medical Center).

Tissue Culture and Whole Cell Labeling Techniques. WEHI-7 mouse thymoma cells were maintained in RPMI-1640 tissue culture medium containing 10% supplemented bovine calf serum at 37 °C in a humidified incubator equilibrated with 5% CO_2/95% air. Prior to labeling, cells were harvested by centrifugation for 3 min at 400*g* and washed 2× in Krebs-Ringer buffer, pH 7.4, containing 15 mM sodium bicarbonate, 5.5 mM glucose, and 10 mM HEPES. The procedures for labeling WEHI-7 cells with [^{35}S]methionine have been described previously (Mendel et al., 1987). Briefly, cells were resuspended at a final density of 10^6 cells/mL in methionine-free RPMI-1640 containing 20 μM methionine (20% of the methionine normally present in RPMI-1640 tissue culture medium) and 10% dialyzed calf serum. Upon addition of [^{35}S]methionine to a final concentration of 30 μCi/mL, the cells were incubated at 37 °C for 12–14 h.

Formation and Purification of Affinity-Labeled Glucocorticoid Receptor Complexes. Cells to be labeled with [^{3}H]DM were adjusted to 0.3–0.4 mL of packed cells/mL of cell suspension. Subsequent purification steps, unless otherwise noted, were carried out at 3 °C. Routinely, about one-fourth of the cells were incubated for 2 h with [^{3}H]DM at a final concentration of 2 μM. The remaining cells were incubated with 2 μM unlabeled DM. At the end of the labeling period, the cells from both incubations were mixed in a 15-mL conical tube and centrifuged at 400*g* for 5 min. After resuspension in 3 volumes of freeze–thaw (FT) buffer [25 mM TES, pH 8.2 at 3 °C, 1 mM EDTA, 50 mM NaCl, 20 mM sodium molybdate, and 10% (v/v) glycerol], the cells were frozen in liquid nitrogen for 10 min. Upon thawing an additional 2 volumes of FT buffer was added, and the broken cell suspension was centrifuged at 11000*g* for 4 min. The supernatant was removed and centrifuged at 100000*g* for 90 min. Ascites fluid containing the BuGR2 monoclonal antibody was added to the cytosol, which remained at 3 °C overnight.

Receptor–antibody complexes were isolated by passing the cytosol over a 0.25-mL protein A–Sepharose column. After extensive washing to remove nonspecifically bound proteins (Mendel et al., 1986), the complexes were eluted by boiling the column contents in 2× SDS–PAGE sample buffer (0.6 M Tris, pH 8.0, 20 mM sodium molybdate, 4% SDS, 20% (v/v) glycerol, and 25 mM DTT). The soluble fraction was obtained by centrifugation of the mixture for 10 min at 500*g*.

Gel Electrophoresis and Subsequent Elution of the Purified 100-kDa Subunit. Vertical slab gels (14 cm × 16 cm × 0.75 mm) of 7% polyacrylamide (1% cross-linker) were formed by cross-linking acrylamide with a 50:50 mixture of bis(acrylamide) (BIS) and bis(acrylylcystamine) (BAC). Due to the sensitivity of the BAC cross-linker to sulfhydryl reagents, prior to electrophoresis samples were S-carboxymethylated with 100 mM iodoacetamide for 1 h at 25 °C to block reduced sulfhydryl groups. The samples were then adjusted to pH 6.5–7.0 at room temperature with HCl and electrophoresis was performed at 25 mA in a Bio-Rad Protean cell maintained at approximately 10 °C with running water. Following electrophoresis, gels were fixed and stained with 0.12% Coomassie blue in a solution of 50% methanol/10% acetic acid and destained in a solution of 10% methanol/10% acetic acid. Samples lanes were cut into 2-mm slices and the slices incubated in 6% Protosol in Econofluor for 20 h at 37 °C; the radioactivity in each slice was determined in a Packard 3390 liquid scintillation counter. Alternatively, to elute the protein

from the gel for enzymatic digestion, sample lanes were cut into 2-mm slices immediately after electrophoresis, and each slice was incubated in 1.0 mL of 10 mM DTT in deionized water for 20–24 h at 37 °C. After removal of the swollen gel slices, an aliquot of each solution was assayed for radioactivity. Peak fractions of radioactivity were combined and lyophilized.

Proteolytic Digestion of the ~100-kDa Steroid-Binding Subunit. Freeze-dried samples were resuspended in 200 μL of distilled water, and the protein was precipitated free of buffer salts by the addition of trichloroacetic acid to a final concentration of 10%. After several hours on ice, the protein was pelleted by centrifugation at 11000*g* for 10 min and washed once with cold acetone. Samples to be digested with TPCK-treated trypsin were resuspended in 50 μL of 0.4% Triton X-100 and brought to a final volume of 200 μL with 50 mM HEPES, pH 7.8. Digestion was carried out at 37 °C by the sequential addition of 10 μg of trypsin over a 24–26-h period (2 μg of trypsin was added at 0, 2, and 4 h, and then another 4 μg was added at 16–18 h of incubation and the digestion continued for the remaining time). For digestion with *S. aureus* V8 protease, samples were resuspended in 100 μL of 0.4% SDS and brought to a final volume of 200 μL with 200 mM ammonium bicarbonate, pH 7.8. Excess SDS was sequestered by addition of Triton X-100 to a final concentration of 2.0%. Samples were then digested by the sequential addition of 20 μg of enzyme over a 30-h period at 37 °C (4 μg added at 0, 2, and 4 h and then 8 μg added at 16–18 h). The digests were lyophilized and stored at −80 °C until further analysis.

Purification of [^{3}H]DM-Labeled Peptides by Reversed-Phase HPLC. The trypsin-generated peptides were dissolved in 0.03% trifluoroacetic acid (TFA) in 30% acetonitrile in preparation for chromatography on a Waters 840 HPLC work station equipped with two Model 510 pumps, a U6K injector, and a Beckman Model 165 multiwavelength detector. Separation of peptides was carried out on two different C-8 columns (5 μm, 2.1 mm × 250 mm). The Macrosphere C-8 column was obtained from Alltech Associates. The Adsorbosphere C-8 column was packed according to the procedure of Verzele (1984) with material also obtained from Alltech. The products of trypsin digestion of [^{3}H]DM-labeled receptors were eluted from the Macrosphere C-8 column with 0.03% TFA in distilled water as the ion-pairing reagent (solvent A) and 75% acetonitrile containing 0.03% TFA as the organic modifier (solvent B) (linear gradient from 10% to 90% solvent B over 64 min at a flow rate of 0.2 mL/min). Dual-labeled peptides, formed by growing cells in [^{35}S]methionine-containing medium prior to labeling with [^{3}H]DM, were separated on an Adsorbosphere C-8 column by performing a linear gradient ranging from 30% to 95% solvent B over 45 min at a flow rate of 0.2 mL/min. The peptides from the *S. aureus* V8 protease digests were subjected to gradient elution on the Adsorbosphere C-8 column with a 45-min linear gradient that ranged from 40% to 90% solvent B. In each case 100-μL fractions were collected at 30-s intervals and assayed for radioactivity. Alternatively, an aliquot of each fraction was counted and the peak fraction(s) of radioactivity lyophilized and used for radiosequence analysis.

Microsequencing of Radiolabeled Peptides by Edman Degradation. ^{3}H-Labeled, S-carboxymethylated peptides obtained from the reversed-phase HPLC fractionation were reconstituted in 400 μL of 50% aqueous acetic acid for automated sequencing on a Beckman 89 protein/peptide sequencer utilizing a 0.5 M Quadrol buffer system (North & Mitchell, 1981). Prior to addition of the peptide, the spinning cup was prepared by adding 3 mg of polybrene and 200 nmol of L-Leu-GlyOH and performing three complete cycles of sequencing at the end of each analysis (Hunkapillar & Hood, 1978). Peptide sequencing was carried out for 20 cycles for the initial analysis of the tryptic peptide and for 10 cycles for subsequent analyses. Sequencing of each peptide was performed in duplicate. Phenyl isothiocyanate (PTC) amino acids obtained from the sequencing were resuspended in 300 μL of methanol and assayed for radioactivity. The repetitive recovery calculations were based on the corrected counts per minute (cpm) present within the single peak fraction after subtraction of the cpm remaining in the spinning cup from the total cpm applied.

Synthesis and Purification of [^{3}H]DM-Labeled Peptide. A peptide representing the sequence 636–650 of the mouse glucocorticoid receptor was synthesized by automated Merrifield solid-phase synthesis (Merrifield, 1963) on a Biosearch 9500 peptide synthesizer. N-Terminal protection was afforded by *tert*-butoxycarbonyl groups (Boc), and side-chain functionalities were appropriately blocked [i.e., Arg (tosyl), Asp (cxyclohexyl ester), Cys (*p*-methoxybenzyl), Cyc (acetamidomethyl), Glu (benzyl ester), Lys (2-chlorocarbonylbenzoxyl), Thr (*o*-benzyl), and Tyr (2,6-dichlorobenzyl)]. All couplings were mediated by diisopropylcarbodiimide. This peptide corresponded to a tryptic fragment of the receptor with four additional amino acid residues on the amino terminus. These four additional amino acids were added to facilitate the solubility and enhance the immunogenicity of the peptide.

The peptide was synthesized with the sulfhydryl group on the cysteine closest to the carboxyl terminus (corresponding to cysteine-649 of the glucocorticoid receptor) blocked with an acetamidomethyl group while the cysteine positioned closer to the amino terminus (corresponding to cysteine-644 of the glucocorticoid receptor) was blocked with *p*-methoxybenzyl. Cleavage and deblocking of the peptide from the resin were done with liquid hydrofluoric acid and cresol (1 h, 0 °C). This treatment released the (*p*-methoxybenzyl)hydrylamine blocking group, but not the acetamidomethyl blocking group, enabling us to selectively label cysteine-644 in a subsequent procedure.

The peptide was purified on a C-8 Dynamax column (12 μm, 2.1 cm × 10 cm) with 0.1% aqueous TFA (solvent A) as the ion-pairing reagent and 70% aqueous acetonitrile in the presence of 0.1% TFA (solvent B) as the organic modifier. The crude peptide (55 mg) was dissolved in 16.5 mL of a 60:40 mixture of solvents A and B. Four separate injections of 1.5 mL each were made into the HPLC with the flow rate held at 1.0 mL/min. As soon as the pressure normalized the flow rate was increased to 5.0 mL/min over a 7-min period. A 40-min linear gradient ranging from 58% to 70% solvent B was performed, and the major peak eluting at 32 min was collected. This fraction was concentrated 2× under a stream of nitrogen and was then lyophilized.

In order to couple DM to the peptide, 20 mg of the purified material was dissolved in 1.0 mL of 50% aqueous acetonitrile. An equimolar amount of DM containing a trace amount (5.2×10^{-13} mol) of [^{3}H]DM was dissolved in 100% acetonitrile, and the two solutions were mixed. Upon addition of triethylamine, the pH approached 7.0–8.0 and the solution turned cloudy as a precipitate formed. This precipitate disappeared upon the addition of 1.0 mL of 50% aqueous acetonitrile. The pH at this point was 9.0 and was maintained as such for the duration of the coupling reaction. After 3 h the reaction mixture was concentrated with nitrogen to a volume of 1.0 mL and diluted 10× with 0.1% aqueous TFA. An aliquot of 2.0 mL was injected into a C-4 Vydac column (5 μm, 4.6 mm ×

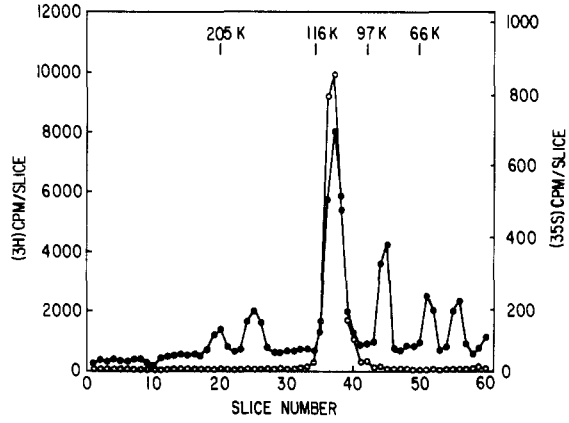

FIGURE 1: SDS gel electrophoresis of immunopurified [^{3}H]DM- and [^{35}S]methionine-labeled glucocorticoid receptors. Glucocorticoid receptors from WEHI-7 cells grown for 12–14 h in the presence of [^{35}S]methionine and subsequently labeled with 2 μM DM were purified with the BuGR2 monoclonal antibody. Following immunopurification the sample was separated on a 7.0% gel (1.0% cross-linker). The sample lane was cut into 2-mm slices, and the samples were incubated in a solution of 6.0% Protosol in Econofluor for 20 h prior to scintillation counting. The open circles (○) represent ^{3}H cpm and the closed circles (●) ^{35}S cpm.

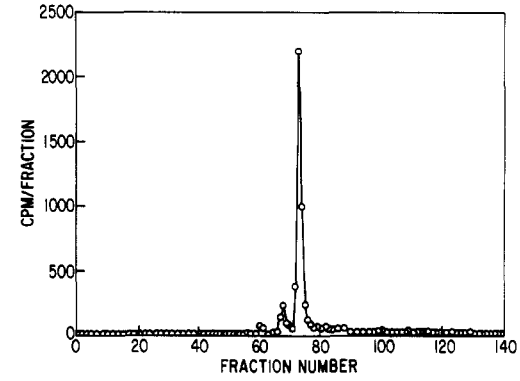

FIGURE 2: Reversed-phase HPLC analysis of purified [^{3}H]DM-labeled glucocorticoid receptors following trypsin digestion. Immunopurified glucocorticoid receptors labeled with 2 μM [^{3}H]DM were digested with 10 μg of trypsin for 24–26 h as described. The sample was injected onto a Macrosphere C-8 column and eluted with a 64-min linear gradient ranging from 7.5% to 67.5% aqueous acetonitrile in the presence of 0.03% TFA. Fractions were collected at 30-s intervals and assayed for radiolabel.

250 mm). A linear gradient from 40% to 95% solvent B (60% aqueous acetonitrile/0.1% TFA) was applied over 30 min at a flow rate of 1.0 mL/min. The peak eluting 20 min into the gradient was collected, concentrated to half its volume under nitrogen, and then lyophilized.

The acetamidomethyl blocking group on the cysteine equivalent to position 649 in the receptor sequence was removed according to the method of Gray et al. (1984). The deblocked and DM-conjugated peptide (500 μg in 2.4 mL of 60% aqueous acetonitrile) was reduced overnight by the addition of 0.24 mL of 250 mM dithiothreitol in 10 mM HEPES, pH 7.8. S-Alkylation of the peptide was performed by adding iodoacetamide from a concentrated stock solution to 100 mM. After 90 min at room temperature, the reaction was quenched by the addition of 25 mM dithiothreitol. The iodoacetamide-treated and DM-conjugated peptides were digested with trypsin and analyzed by reversed-phase HPLC on the Adsorbosphere C-8 column by the method described earlier. Elution of the synthetic peptide(s) from the column was monitored by absorbance at 210 nm as well as by assaying fractions for radioactivity.

RESULTS

Elution of the ~100-kDa Steroid-Binding Protein from Polyacrylamide Gels. Glucocorticoid receptors affinity-labeled with [^{3}H]DM can be substantially purified by using the BuGR2 monoclonal antibody. Analysis of the receptor by SDS–PAGE electrophoresis according to the system of Laemmli (1970) indicates that the nonactivated receptor is composed of at least two subunits, an ~100-kDa steroid-binding protein and two ~90-kDa non-steroid-binding proteins (Mendel et al., 1986; Mendel & Ortí, 1988). Our initial attempts to elute the steroid-binding subunit from gels prepared according to the procedure of Laemmli (1970) were unsuccessful. We have subsequently solved this problem by utilizing a combination of two acrylamide cross-linkers, BIS and BAC, to develop a stable poly(acrylamide) matrix, which is useful for several reasons. This matrix, which has a low total percent cross-linker (approximately 1.0% of the total acrylamide), is quite porous and therefore provides complete separation of the ~100-kDa steroid-binding protein and the ~90-kDa non-steroid-binding components of the nonactivated receptor (Figure 1). Furthermore, the steroid-binding protein is separated from other non-steroid-binding, methionine-labeled proteins that may be components of the ~330-kDa nonactivated complex or minor contaminants not removed during the purification procedure. In addition, reducing agents can be used to cleave the BAC cross-linker, which disrupts the structure of the polyacrylamide gel matrix allowing the receptor subunits to be efficiently eluted. Treatment with 10 mM DTT at 37 °C for 18–20 h disrupts the gel matrix sufficiently to elute 80–95% of the covalently labeled ~100-kDa subunits. Routinely, 20–30 pmol of glucocorticoid receptor was electrophoresed, as estimated from the moles of bound [^{3}H]DM assuming one steroid binding site per subunit.

Localization of the Dexamethasone 21-Mesylate Binding Cysteine by Edman Degradation of HPLC-Purified Tryptic Peptides. The [^{3}H]DM-labeled ~100-kDa subunits selectively eluted from SDS–PAGE gels were precipitated with TCA. After resuspension in the appropriate buffer, the protein was digested by the sequential addition of trypsin, which cleaves after the basic amino acids lysine and arginine. Initial time course studies in which the steroid-binding subunit was digested for 8, 24, 30, and 48 h indicated that a limit digest of the receptor subunit (500 ng) could be attained if 10 μg of trypsin was added in 2-μg aliquots over a 24–26-h period. This conclusion was based on the generation of a single [^{3}H]DM-labeled peak on reversed-phase HPLC that contained a significant proportion of the total cpm (Figure 2). The overall recovery from the column was calculated by summing the tritium cpm in all the fractions after background subtraction and then dividing by the number of cpm that were injected. The overall recovery for the experiment shown in Figure 2 was 82%, with 47% of the recovered counts present within the major peak eluting in fractions 72–75 (36 min). Digestion of the receptor for a longer period (up to 48 h) results in a shift in the peptide elution profiles (data not shown). This may be the result of the generation of trypsin autolysis products, which have strikingly different specificities from intact trypsin (Kiel, 1982).

In two separate experiments, automated Edman degradation of the [^{3}H]DM-labeled tryptic peptides eluted from reversed-phase HPLC resulted in the release of a single peak of radioactivity associated with the PTC amino acid generated by five cycles of degradation (see Table I). In the reported amino acid sequence of the murine GR (Danielson et al., 1986), 3 of the 20 cysteines lie 5 residues in from a trypsin

Table I: Microsequence Analysis of Reversed-Phase HPLC Purified [^{3}H]Dexamethasone 21-Mesylate Labeled Peptides[a]

cycle	tryptic peptide	*S. aureus* peptide	cycle	tryptic peptide	*S. aureus* peptide
1	43.4	44.7	6	221.1	70.2
2	38.2	28.9	7	95.4	549.0
3	25.2	16.3	8	58.9	483.8
4	60.5	48.9	9	39.1	78.8
5	788.6	30.8	10	26.7	50.8

[a] Values are cpm corrected for quench, assuming a 34% efficiency for tritium. A representative analysis from duplicate experiments is shown. In each instance the repetitive recovery per cycle averaged 70%.

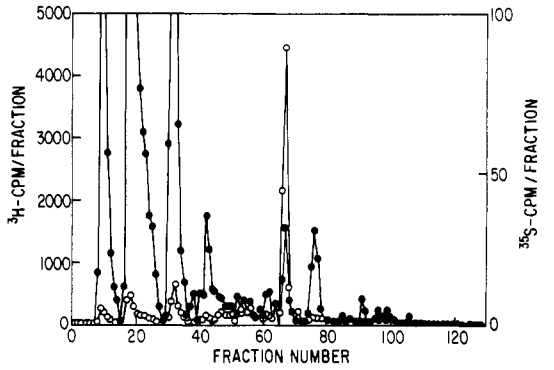

FIGURE 3: Reversed-phase HPLC analysis of purified [^{3}H]DM- and [^{35}S]methionine-labeled glucocorticoid receptors following trypsin digestion. Dual-labeled glucocorticoid receptors were immunopurified and digested with 10 μg of trypsin for 24 h. Peptides were separated on an Adsorbosphere C-8 column with a 45-min linear gradient ranging from 22.5% to 72% aqueous acetonitrile in the presence of 0.03% TFA. Fractions were collected at 30-s intervals and radioactive content was determined. The open circles (○) represented ^{3}H cpm and the closed circles (●) ^{35}S cpm.

cleavage site. On the basis of these data, the cysteine that reacts with the DM could lie within the tryptic peptides encompassing amino acids 327–349, 379–384, or 640–650.

A dual-isotope experiment was performed to discriminate between the above three possibilities. Of the three tryptic peptides, only the one encompassing amino acids 640–650 contains a methionine residue. Since we have previously demonstrated that the ~100-kDa protein can be metabolically labeled with [^{35}S]methionine (Mendel et al., 1986), we repeated the experiment described above using WEHI-7 cells that were grown in medium containing [^{35}S]methionine. As can be seen in Figure 3, the ^{35}S label comigrates on reversed-phase HPLC with the [^{3}H]DM-labeled peptide generated by trypsin digestion. The substantial amount of ^{35}S that elutes early in the gradient is due to other methionine-containing peptides, which would be expected since the receptor contains a total of 24 methionine residues (Danielson et al., 1986). The data shown in Figure 3 depict a representative experiment from a total of three separate experiments. Although there was not enough ^{35}S incorporated into the [^{3}H]DM-labeled peptide to determine the location of the labeled residue(s) by Edman degradation, the presence of the ^{35}S label does suggest that cysteine-644, present within tryptic peptide 640–650, is the residue that covalently binds DM.

Localization of the Dexamethasone 21-Mesylate Binding Site by Edman Degradation of HPLC-Purified Staphylococcus V8 Protease Peptides. In order to confirm that the cysteine-644 covalently binds DM, we digested the ~100-kDa receptor subunit with *S. aureus* V8 protease. In the presence of ammonium ions, this enzyme cleaves preferentially on the carboxyl side of glutamic acid residues (Houmard & Drapeau, 1972). Digestion of the ~100-kDa receptor with this enzyme

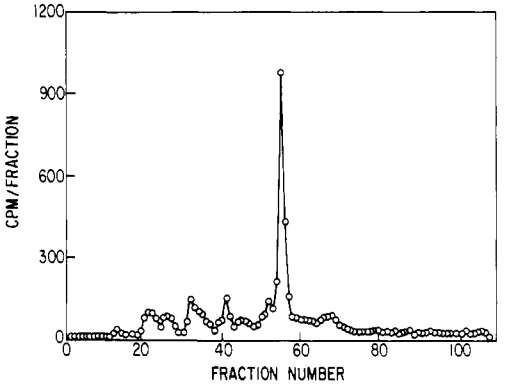

FIGURE 4: Reversed-phase HPLC analysis of immunopurified [^{3}H]DM-labeled glucocorticoid receptors following *S. aureus* V8 protease digestion. Glucocorticoid receptors from WEHI-7 cells were labeled with 2 μM [^{3}H]DM, immunopurified, and digested with 20 μg of *S. aureus* V8 protease for 30 h. The resulting peptides were separated on an Adsorbosphere C-8 column with a 45-min linear gradient ranging from 30% to 75% aqueous acetonitrile in the presence of 0.03% TFA. Fractions were collected at 30-s intervals and assayed for radioactivity.

would be expected to result in a different [^{3}H]DM-labeled peptide from which we could identify the location of the [^{3}H]DM-labeled cysteine residue. Early experiments indicated that the [^{3}H]DM-labeled peptide generated by this enzyme was very hydrophobic, so in subsequent experiments a slightly different gradient than that used for the tryptic peptides was performed. As illustrated in Figure 4, we were able to obtain a single major [^{3}H]DM peak on reversed-phase HPLC after 28–30 h of digestion with *S. aureus* V8 protease. The total recovery of tritium cpm from the column in this experiment was 90%, with 23% of the recovered cpm present within the single peak eluting in fractions 54–56. The observation that only 23% of the recovered cpm is present in the major peak indicates that digestion with *S. aureus* V8 protease is less complete than with trypsin.

Edman degradation of this peptide in two separate experiments gave a single peak of radioactivity on the PTC amino acid generated at cycle 7 (see Table I). According to the published receptor sequence (Danielson et al., 1986), *S. aureus* V8 protease digestion would generate three peptides in which a cysteine residue is separated by six residues from a glutamic acid residue. Of these three peptides, which correspond to amino acids 304–370, 458–496, and 638–658, only the one encompassing amino acids 638–658 overlaps and is consistent with the results of tryptic digestion. Therefore, on the basis of two separate enzymatic digestions and subsequent radiosequencing, plus the demonstrated methionine content, it appears that cysteine-644 is uniquely labeled by DM.

Analysis of the Synthetic Peptide Representing the GR Sequence 640–650 by Reversed-Phase HPLC. The synthetic peptide corresponding to amino acid residues 636–650 of the mouse GR was labeled with [^{3}H]DM and digested with trypsin according to the procedure described above. This treatment removes the amino-terminal tetrapeptide structure, producing the [^{3}H]DM-labeled tryptic fragment 640–650. The resulting peptide and the in vivo labeled tryptic peptide both eluted at the same position of the gradient on the C-8 column when analyzed under the same conditions (Figure 5). In a separate experiment the acetamidomethyl protecting group on cysteine-649 was removed and the peptide alkylated with iodoacetamide before trypsin digestion. Analysis by reversed-phase HPLC revealed several peaks, which were presumably oxidation products formed during the deprotection procedure. However, one of the peaks eluted at the same position as the

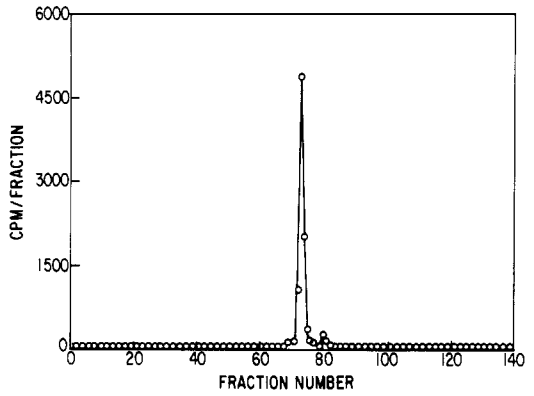

FIGURE 5: Reversed-phase HPLC analysis of [^{3}H]DM-labeled synthetic peptide representing the glucocorticoid receptor sequence 640–650. The synthetic peptide representing amino acids 640–650 of the murine glucocorticoid receptor was prepared as described under Materials and Methods. The sample was analyzed on the Macrosphere C-8 column employing a linear gradient identical with the one described in Figure 2. Fractions were collected at 30-s intervals and assayed for radioactivity.

tryptic peptide generated from intact cells (data not shown). Therefore, we have demonstrated that a synthetic peptide corresponding to tryptic fragment 640–650 of the mouse glucocorticoid receptor behaves in a manner identical with that of the single DM-binding peptide obtained from labeling the receptor in intact WEHI-7 cells.

DISCUSSION

In the present study we have determined that the synthetic steroid DM covalently binds to cysteine-644 of the mouse glucocorticoid receptor, which is near the carboxy terminus of the protein. Our results are in excellent agreement with those of Simons et al. (1987), who localized the DM binding site to an analogous position within the rat glucocorticoid receptor sequence. These investigators labeled glucocorticoid receptors in cytosol prepared from rat HTC cells with [^{3}H]-DM, purified activated complexes by DNA–cellulose chromatography, digested them with trypsin, chymotrypsin, or *S. aureus* V8 protease, separated the [^{3}H]DM-labeled peptides on SDS–PAGE gels, and microsequenced them for radiolabel. A computer search of the published rat receptor sequence then identified a single DM-binding cysteine residue.

Our approach has been somewhat different. Nonactivated [^{3}H]DM-labeled glucocorticoid receptors were generated in vivo in WEHI-7 mouse thymoma cells. The receptors were purified by using the BuGR2 monoclonal antibody followed by SDS–PAGE. After digestion with trypsin or *S. aureus* V8 protease, the resulting [^{3}H]DM-labeled peptides were purified by reversed-phase HPLC. The data obtained from microsequence analysis enabled us to identify a single site of DM attachment. By dual-isotope labeling of glucocorticoid receptors with [^{3}H]DM and [^{35}S]methionine we obtained evidence for the presence of methionine within the tryptic peptide encompassing the DM binding site, further supporting our initial identification. Finally, we synthesized a peptide representing amino acids 640–650 of the murine glucocorticoid receptor and demonstrated that it behaved on reversed-phase HPLC in a manner identical with the [^{3}H]DM-labeled tryptic peptide we isolated from intact cells.

Early proteolysis studies suggested that the glucocorticoid receptor is composed of three discrete functional domains: an immunogenic domain, a DNA-binding domain, and a steroid-binding domain (Wrange & Gustaffson, 1978; Carlstedt-Duke et al., 1982; Okret et al., 1983). The location of these domains has become more clearly defined by analysis of the amino acid sequence deduced by cloning of the human glucocorticoid receptor cDNA (Hollenberg et al., 1985; Weinberger et al., 1985). In particular, Hollenberg et al. obtained a clone that lacked C-terminal residues and was incapable of binding steroid, which led to the proposal that residues important in conferring steroid-binding capacity are localized near the carboxy terminus of the receptor (Weinberger et al., 1985). Further evidence to support this proposal has been obtained by demonstrating that insertional mutations occurring within the last 200 amino acids of the human GR completely abolish steroid binding (Giguere et al., 1986). Our results in the present study further support this proposal, as cysteine-644 is located near the carboxy terminus of the murine glucocorticoid receptor. Finally, Danielson et al. (1986) have demonstrated by cDNA cloning experiments that a hormone-binding-deficient receptor from S49 cells contains a single amino acid substitution at position 546. It is worth noting that the cysteine residue we have implicated in the binding of DM is approximately 100 amino acids away from this position. This disparity may be explained by the fact that the covalent binding we studied occurs through the mesylate group at carbon-21, which is distant from the A ring of the steroid, which may be more intimately associated with the steroid binding cavity. Our result, therefore, confirms the finding that residues important to steroid binding are primarily localized near the carboxy terminus and provides some insight into the three-dimensional nature of the steroid binding cavity, which may be composed of residues that are not closely associated in a linear fashion.

To date there is only indirect evidence, based on densitometric analysis of Coomassie blue stained SDS–PAGE gels of purified glucocorticoid receptors, to suggest that there is a single steroid-binding site per ~100-kDa steroid-binding protein (Wrange et al., 1979). This evidence, though well supported, is based on the assumption that the ~100-kDa protein binds Coomassie blue to the same extent as bovine serum albumin. However, the validity of this assumption is questionable, since it has been shown that different proteins can vary as much as 10-fold in terms of their capacity to bind Coomassie blue in dye binding assays (Read & Northcote, 1981). Our demonstration in this study that the ~100-kDa protein covalently binds a single DM molecule is a more direct confirmation of the existence of a single steroid-binding site. It must be noted, though, that there could still be glucocorticoid-binding sites that lacked an appropriately located sulfhydryl group for covalent linkage of DM; such sites would not have been detected in this study.

In a previous study (Mendel et al., 1987), we reported that the ~100-kDa steroid-binding protein contains two to three phosphates per steroid-binding site. Our current identification of a single steroid-binding site per ~100-kDa protein enables us to assume that there are the same number of phosphates per steroid-binding protein. Although the current study was undertaken to determine the covalent site of attachment of DM to the receptor, the methods developed for the preparation, analysis, and identification of peptide fragments of the purified steroid-binding protein can also be applied to the study of other covalent modifications of the receptor. In particular, we are in the process of using this methodology to determine the sites of phosphorylation within the receptor.

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Registry No. DM, 2265-22-7; Cys, 52-90-4.

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Structural Studies on the Interaction between Ferredoxin and Ferredoxin–NADP$^+$ Reductase†

Giuliana Zanetti,*,‡ Daniele Morelli,‡ Severino Ronchi,§ Armando Negri,§ Alessandro Aliverti,‡ and Bruno Curti‡

Dipartimento di Fisiologia e Biochimica Generali and Istituto di Fisiologia Veterinaria e Biochimica, Università di Milano, 20133 Milano, Italy

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ABSTRACT: Structural studies carried out on a cross-linked complex between spinach ferredoxin–NADP$^+$ reductase (EC 1.18.1.2) and its protein substrate ferredoxin allowed the identification of peptide regions involved in the interaction between the two proteins. Carboxyl groups of ferredoxin were shown to interact with amino groups of the reductase. Two types of cross-links could be located within a few residues. The major one was found between the peptide segment 72–91 of the reductase, where Lys-85 and/or Lys-88 were identified as the amino donors in the carbodiimide-promoted linkage, and the ferredoxin region 76–97, which contains Asp-84, Glu-88, Glu-92, Glu-93, and Glu-94. Carboxypeptidase Y digestions would suggest an involvement of a glutamic residue of the acidic cluster 92–94. The minor cross-link was found between the α-NH_2 of the N-terminal residue of the reductase and the peptide region 5–37 of ferredoxin, which contains Glu-15, Asp-20, Asp-21, Asp-26, Glu-29, Glu-30, Glu-31, and Asp-34. Glu-15, Glu-31, and Asp-34 could be ruled out. Lack of chymotrypsin digestion at Tyr-23 of the cross-linked peptide would indicate Asp-20, -21, or -26 as the most likely COOH donor in the linkage. These results point out the importance of the N-terminal moiety of the reductase, where the flavin binding domain is located, for the interaction with ferredoxin.

In photosynthesis, NADP$^+$ photoreduction requires the interaction between the two terminal components of the chloroplast electron-transport chain, namely, ferredoxin and ferredoxin–NADP$^+$ reductase. The cross-linked complex between the two purified proteins, obtained through the aid of a carbodiimide (Zanetti et al., 1984), is considered to be a valid model of the in vivo noncovalent electron-transfer complex on the following bases. (a) The cross-linked complex has been

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‡ Dipartimento di Fisiologia e Biochimica Generali.

§ Istituto di Fisiologia Veterinaria e Biochimica.

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