Identification of Cysteine 656 as the Amino Acid of Hepatoma Tissue Culture Cell Glucocorticoid Receptors That Is Covalently Labeled by Dexamethasone 21-Mesy late

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Recent results using proteases suggest that dexamethasone 21-mesy late (Dex-Mes) labeling of the rat hepatoma tissue culture (HTC) cell glucocorticoid receptor occurs at one or a few closely grouped cysteine residues (Simons, S. S., Jr. (1987) J. Biol. Chem. 262, 9669–9675). In this study, a more direct approach was used both to establish that only one cysteine is labeled by [3H]Dex-Mes and to identify the amino acid sequence containing this labeled cysteine. Various analytical procedures did not provide the purification of the extremely hydrophobic Staphylococcus aureus V8 protease digestion fragment that is required for unique amino acid sequencing data. Therefore, Edman degradation was performed on the limit protease digest mixtures which appeared to contain only one 3H-labeled peptide. These degradation experiments revealed the number of amino acid residues between the NH2 terminus of each peptide and the [3H]Dex-Mes-labeled cysteine. A comparison of these amino acid spacings with the published amino acid sequence of the HTC cell glucocorticoid receptor (Miesfeld, R., Rusconi, S., Godowski, P. J., Maler, B. A., Okret, S., Wikstom, A-C., Gustafsson, J-A., and Yamamoto, K. R. (1986) Cell 46, 389–399) indicated that the one cysteine labeled by [3H]Dex-Mes is Cys-656. Further analysis of the receptor sequence for the presence of the observed grouping of proteolytic cleavage sites, but without any preconditions as to which amino acid was labeled, gave Asp-122 and Cys-656 as the only two possibilities. Potential labeling of Asp-122 could be eliminated on the basis of immunological and genetic evidence. We, therefore, conclude that the single Dex-Mes-labeled site of the HTC cell glucocorticoid receptor has been identified as Cys-656. Since several lines of evidence indicate that [3H]Dex-Mes labeling of the receptor occurs in the steroid binding site, Cys-656 is the first amino acid which can be directly associated with a particular property of the glucocorticoid receptor.

Three functional domains have been identified in the receptor protein for glucocorticoid steroid hormones: a steroid binding domain, a DNA binding domain, and an antigenic domain (1, 2). Detailed information regarding the steroid binding domain is needed to answer questions such as 1) why intact steroid-free receptors are devoid of activity, 2) how the steroid binding cavity determines which ligands display high affinity binding to the receptor, and 3) how the seemingly subtle variations in steroid ligand structure are translated into one of the three major activity classes of glucocorticoids, antiglucocorticoid, or inactive steroid.

Of the various methods available to define the composition of the steroid binding site of glucocorticoid receptors, the most comprehensive is x-ray crystallography. This is the only method capable of providing details, at a resolution of ~2 Å, of the interactions of the steroid with each amino acid in the binding cavity. Genetic engineering can give variant receptor proteins with altered steroid binding properties; however, this method is unable to distinguish between direct and indirect effects of changes in receptor sequence on the binding of steroid. Electrophilic affinity labeling can give high yields of covalent receptor-steroid complexes (3–5) which usually contain only 1–3 labeled amino acids (6, 7). Thus, this approach is well suited to defining some of the amino acids that comprise the steroid binding cavity of receptors.

In the preceding paper (7), we demonstrated that the electrophilic affinity label dexamethasone1 21-mesy late (Dex-Mes) (8, 9) selectively labels cysteines in the HTC cell glucocorticoid receptor. In the present paper, we have determined that only one cysteine of the receptor is labeled by [3H]Dex-Mes and have conclusively identified this cysteine which is in the steroid binding cavity of the rat HTC cell glucocorticoid receptor.

MATERIALS AND METHODS

The various reagents, the TAPS buffer, the preparation of [3H] Dex-Mes-labeled receptors (10), and the details of the 15% SDS-polyacrylamide gels are described in the preceding paper (7). Antidepressant antibodies were obtained from Dr. George Chrousos (National Institutes of Health). Purification of the activated [3H] Dex-Mes-labeled complexes to essentially radiochemical homogeneity by DNA-cellulose chromatography has been previously reported (4).

Limit protease digest of the radiochemically pure activated complexes was conducted as detailed in the preceding paper (7). Briefly, [3H]Dex-Mes-labeled complexes in pH 8.2 TAPS buffer containing 500 mM NaCl were diluted with an equal volume of pH 8.8 TAPS buffer, de natured at ~22 °C for 30 min with 0.2% SDS, 0.5 mM dithiothreitol, and digested with 150 μg/ml trypsin, chymotrypsin, or Staphylococcus aureus V8 protease for 16–18 h at ~22 °C.

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Chromatography of the limit protease digests was performed on Sep-pak C18 cartridges (Waters) that were pre-wet with methanol and then water containing 0.1% trifluoroacetic acid (Fisher Chemical Co.). The [3H]Dex-Mes-labeled limit digests (100–200 µl) were diluted to 0.5 ml with water, boiled for 5 min, and applied to the cartridges, which were washed with 10 ml of water, 0.1% trifluoroacetic acid. The sample was eluted with 90% MeOH, 10% water, 0.1% trifluoroacetic acid, and 0.5-ml fractions were collected. The peak fractions were determined by scintillation counting. If further chromatography on C8 or C18 HPLC columns (Altek) was desired, the Sep-pak-eluted material was diluted with 10 volumes of water, 0.1% trifluoroacetic acid, applied to columns equilibrated in 10% MeOH, 90% water, 0.1% trifluoroacetic acid, and eluted by a linear gradient of 90% MeOH, 10% water, 0.1% trifluoroacetic acid.

The computer search of the glucocorticoid receptor amino acid sequence for possible protease cleavage sites was performed at the National Biomedical Research Foundation (Washington, D. C.).

RESULTS

Purification of V8 Limit Digest Fragment(s) for Sequencing—The 1790-dalton [3H]labeled material obtained by S. aureus V8 protease limit digestion of the intact, radiochemically pure, affinity-labeled 98-kDa receptor (Dex-Mes complex (7)) purified on DNA-cellulose columns was determined by scintillation counting. If further chromatography was chosen for purification for two reasons. First, it appears to be the most homogeneous of the [3H]-labeled limit digest fragments obtained from digestion of the intact 98-kDa complex with trypsin, chymotrypsin, or V8 protease (7). Second, the most homogeneous of the "H-labeled limit digest fragments obtained from digestion of the intact 98-kDa complex with trypsin, chymotrypsin, or V8 protease (7). The 1.8-kDa V8 limit digest material is known to contain all of the [3H]Dex-Mes-labeled cysteine(s) present in the intact 98-kDa labeled complex (7).

The 1.8-kDa [3H]-labeled V8 limit digest fragment(s) could be separated on 15% SDS-polyacrylamide gels (7) from most of the other protein and peptides in the crude digestion mixture (Fig. 1). However, the further purification to homogeneity that was desirable for the amino acid sequencing of this labeled material proved to be problematical. Lysophilization of the digested samples caused most of the [3H]Dex-Mes-labeled peptide to stick tightly to the walls of the tube and to resist resolubilization by most solvents (data not shown). The labeled material was retained equally well by nonspecific and anti-dexamethasone antibodies and stuck to Amicon "Centricon 10" ultrafiltration membranes (data not shown). All of the radioactivity in crude V8 protease limit digest samples appeared in the void volume of Sephadex columns, regardless of whether or not the limit digest solution was first heated at 100 °C for 5 min before being chromatographed (a) on LH-20 columns with pH 8.2 TAPS buffer containing 10 mM β-mercaptoethanol, (b) on LH-20 columns with TAPS-β-mercaptoethanol buffer containing 4 M guanidinium hydrochloride, or (c) on either LH-20 or G-25 columns with TAPS buffer containing 1% SDS and 0.1 M dithiothreitol (data not shown). All of the [3H]Dex-Mes-labeled digest was irreversibly bound to C18 HPLC columns. Reasonable recoveries (~60%) were obtained from a C8 HPLC column if the crude limit digest was heated (100 °C for 5 min) before being applied to the column and then eluted with a water/acetonitrile or water/methanol gradient (data not shown), but considerable amounts of contaminating peptides co-eluted with the [3H]-labeled fragment(s) at a solvent composition of 40% acetonitrile (Fig. 1). Good recoveries (60–70%) of the labeled material were obtained from Sep-pak C18 cartridges by eluting with methanol, 0.1% trifluoroacetic acid; the amount of purifica-

![FIG. 1. Fractionation of V8 protease limit digest of activated [3H]Dex-Mes-labeled HTC cell glucocorticoid receptors.](image)

The covalently bound steroid appears to reduce the size of the various limit digest fragments, we have not accounted for the 680-dalton difference due to the covalently bound steroid but refer, instead, to the apparent size of the peptide (see Table II in the preceding paper (7)).

1 The covalently bound steroid appears to reduce the size of all [3H]Dex-Mes-labeled peptides by ~680 daltons (7). When discussing the size of the various limit digest fragments, we have not accounted for the 680-dalton difference due to the covalently bound steroid but refer, instead, to the apparent size of the peptide (see Table II in the preceding paper (7)).

3 After correcting for the effect of the covalently bound steroid, the V8 protease fragment was calculated to contain 18 amino acids (7). It was eventually determined (see below) that this fragment contains 21 amino acids.
bound [3H]steroid is attached to cysteine (7) in the 1.8-kDa V8 limit digest peptide(s). An examination of the amino acid sequence of the HTC cell glucocorticoid receptor (11) revealed that only five of the 20 cysteines are seven positions downstream (i.e. closer to the COOH terminus) from an acidic amino acid residue that could be cleaved by V8 protease.

Trypsin digestion of the [3H]Dex-Mes-labeled 98-kDa receptor yields a 1580-dalton fragment(s) that appears to be slowly cleaved to still smaller peptides. These smaller peptides do not remain in the 15% SDS-polyacrylamide gels during staining and destaining (7). Analysis of the first 20 sequencing cycles of the 1.6-kDa trypsin limit digest material showed that essentially all of the radioactivity was found in cycle 5 (Fig. 2B). These data also indicate that the proposed second trypsin cleavage site must be on the carboxyl terminal side of the labeled cysteine. There are three cysteines in the HTC cell glucocorticoid receptor which are five amino acids downstream from a basic amino acid residue that would be cleaved by trypsin. Of these three cysteines, only Cys-656 is also compatible with the V8 protease limit digest data of Fig. 2A (see also Fig. 3). It should be noted that there is a second trypsin site within 5-10 amino acids downstream of Cys-656 that would account for the further cleavage to a fragment that is too small to remain on 15% gels.

Sequential Edman degradation was also performed on the 1470-dalton chymotrypsin limit digest material (7) in order to confirm the labeling of Cys-656. A major peak of radioactivity was seen in cycle 17 (Fig. 2C). The yield of radioactivity here was lower than with the trypsin and V8 protease digestion fragments, as expected from the cumulative effect of less than quantitative yields for each step of the Edman degradation. There are four cysteines that are 17 amino acids downstream from a conventional chymotrypsin cutting site (i.e. the aromatic amino acids phenylalanine, tyrosine, or tryptophan and leucine (15)). In all cases, the cleavage site for chymotrypsin would be a leucine. Of these four cysteines, only Cys-
656 is also compatible with the trypsin data. Three of these four cysteines were also identified from the V8 limit digestion data as candidates for covalent labeling: Cys-265, Cys-492, and Cys-666. Collectively, the sequential Edman degradation data for the trypsin, chymotrypsin, and V8 protease limit digests uniquely implicate Cys-656 as the single cysteine of the HTC cell glucocorticoid receptor that is affinity labeled by Dex-Mes (Fig. 3).

Identification of [³H]Dex-Mes-labeled Amino Acid by Computer Pattern Analysis of the Edman Degradation Data—The above conclusions that Cys-656 of the HTC cell glucocorticoid receptor is affinity-labeled relied on previous data indicating that Dex-Mes was specifically labeling a cysteine (3, 4, 7, 8, 16). For confirmation of the location of the labeled amino acid in the receptor molecule, we used an approach that did not require knowing the chemical identity of the labeled residue. The above Edman degradation data of the limit digest fragments revealed the existence of a chymotrypsin site 17 amino acids upstream, a V8 protease site 7 amino acids upstream, and a trypsin site 5 amino acids upstream from the 

\[ ^3 \text{H} \] labeled amino acid. Thus, the receptor sequence in the vicinity of the labeled amino acid(s) should be: (Phe, Tyr, Trp, or Leu) - - - - - - - (Asp or Glu) - (Lys or Arg) - - - - - - - ([³H]Dex-Mes-labeled amino acid). A computer search of the entire HTC cell glucocorticoid receptor sequence (11) for this pattern yielded two possible sites: positions 105-122 with Asp-122 being the labeled amino acid and positions 639-656 with Cys-656 being the labeled amino acid. Asp-122 is a chemically viable candidate for labeling since Dex-Mes does react with carboxylic anions, although at rates that are >500 times slower than the reaction with thiol anions (16). However, a combination of genetic and immunological evidence eliminates Asp-122 as the affinity-labeled amino acid. The antigenic regions in the rat liver glucocorticoid receptor for the monoclonal antibodies 250 (17) and BUGR1 (18) have been localized to the amino-terminal end of the receptor, which includes Asp-122 (11). Since Asp-122 can be removed from dexamethasone-bound, or Dex-Mes-labeled, receptors by mild-chymotrypsin digestion to give a 42-kDa species that still binds to DNA and contains the initially bound, or labeled, steroid (1, 10, 17, 19), Asp-122 cannot be the amino acid that is labeled by Dex-Mes. Thus, we can identify Cys-656 as the affinity-labeled amino acid in the HTC cell glucocorticoid receptor without any prior conclusions regarding the particular amino acid(s) that is labeled.

DISCUSSION

Using sequential Edman degradation of small radioactive limit protease digest fragments of the intact [³H]Dex-Mes-labeled 98-kDa receptor, we have demonstrated that Dex-Mes selectively labels only one amino acid in the HTC cell glucocorticoid receptor. Since V8 protease, trypsin, and chymotrypsin appear to retain their usual cleavage specificity under our digestion conditions (7), we were able to identify the chemical nature of several amino acids and specify their position relative to the [³H]labeled amino acid. A search of the HTC cell glucocorticoid receptor sequence (11) for these appropriately spaced amino acids identified Cys-656 as the one affinity-labeled amino acid. This identification was most readily established if one started with the extensively supported premise (3, 4, 7, 8, 16) that Dex-Mes covalently labels only cysteines. However, the current data allowed us to unambiguously conclude that it is Cys-656 that is affinity labeled even if no assumption was made about the chemical identity of the labeled amino acid. These results also extend our previous conclusions that the reactions of Dex-Mes are highly specific for thiols (4, 7, 16) and that each limit protease digest of [³H]Dex-Mes-labeled receptors observed on SDS-polyacrylamide gels contains only one labeled peptide (7).

This technique of using sequential Edman degradation of radioactive limit protease digestion fragments to identify affinity-labeled amino acids does require knowledge of the amino acid sequence of the protein. However, as seen for steroid receptors (11, 13, 14, 20, 21), protein sequences are increasingly likely to be deduced from the cloning of genes sooner than from the sequencing of isolated proteins. Our approach to identifying affinity-labeled amino acids and their position in intact proteins should thus be of general utility and may be the only viable method with peptides that are difficult to purify or are available only in small quantities.

Several lines of evidence have established that Dex-Mes covalently labels glucocorticoid receptors in the binding cavity of the steroid binding domain. Dex-Mes is an irreversible antiglucocorticoid in HTC cells (8) and can covalently label glucocorticoid receptors in both whole cell (4, 5) and broken cell preparations (4, 8-10, 22). This whole cell antagonist activity, whole cell labeling of receptors, and cell-free labeling of receptors are all inhibited by excess glucocorticoid (3-5, 8-10, 22). The whole cell and cell-free activities of Dex-Mes and cortisol 21-mesyate exhibit the same relative potencies as do the parent steroids dexamethasone and cortisol, as expected from structure-activity correlations. Furthermore, covalently labeled receptor Dex-Mes can be activated to an apparently normal DNA binding complex (4, 5, 22, 23). Since activation of the receptor to a high affinity DNA binding species requires the prior binding of steroid to receptors (24, 25), it can be concluded that Dex-Mes labeling occurs in the steroid binding cavity. Thus, the affinity labeling of Cys-656 by Dex-Mes means that at least this amino acid is part of the steroid binding site of HTC cell glucocorticoid receptors. These results also directly support the conclusions of Bodwell et al. (26) that at least one -SH group necessary for steroid binding to the receptor is different from the -SH group(s) required for receptor-steroid complex binding to DNA.

Due to a lack of information regarding the tertiary structure of the receptor, it is not yet possible to say whether all of the amino acids in the steroid binding cavity are in one contiguous region of the receptor. The 95.8% homology between human (15) glucocorticoid receptors, and 96.5% homology between rat (11) and mouse (14), in the 120 amino acids on either side of Cys-656 of the rat receptor (e.g. Fig. 3) does not help to define the critical elements of the steroid binding site. This homology does, however, permit us to predict that Cys-638 and Cys-644 are the sites of Dex-Mes labeling in the human and mouse receptors, respectively. These sites are also consistent with the data from deletion mapping which indicate that ~200 amino acids at the carboxyl-terminal end of the receptor constitute the steroid binding domain (11, 13, 14, 27). The size of this domain suggests that the correct tertiary structure is critical for steroid binding activity. While information is not yet available concerning this tertiary structure, it is probable that the bulk of the steroid binding site is somewhat distant from Cys-656 since the reactive C-21 position of Dex-Mes is at one extremity of the steroid.

Regions of amino acid homology have been noted between the human (and rat) glucocorticoid receptor, the human and chicken estrogen receptor (28), and the chicken (20, 29) and rabbit (30) progesterone receptor. A glycine instead of a cysteine occurs at that position of the estrogen receptor which corresponds to Cys-656 of the rat glucocorticoid receptor (28).

\[ ^4 \text{K. R. Yamamoto, personal communication.} \]
Dex-Mes bound to, but did not affinity label, the chicken progesterone receptor.² Consistent with this is the fact that progesterone receptor contains a serine in place of the Cys-656 of rat glucocorticoid receptors (30).² Similarly Dex-Mes displayed little if any binding or labeling of rat mineralocorticoid receptors;³ and human mineralocorticoid receptors have an alanine instead of the Cys-656 of rat glucocorticoid receptors.

The receptor sequence in the vicinity of the labeled Cys-656 (Fig. 3) can account for all of the limit protease digestion patterns with [³H]Dex-Mes-labeled 98-kD receptors (7). The ~50% recovery of radioactivity in the trypsin limit digest fragment of Met-652 to Arg-673 would result from slow cleavage at the internal trypsin site (Lys-662) to give an 11-residue fragment that would not remain on the gel during staining and destaining (7). Predictions of which chymotrypsin sites will actually be cleaved by chymotrypsin are known to be difficult (7, 15). The data of Fig. 2C indicate that chymotrypsin cuts inefficiently at Leu-638, Phe-641, and Met-652 (see Ref. 15 for infrequent cleavage after methionine) and not at all after Leu-645; the lack of cleavage at Leu-654 is expected due to the following proline (15). There are also numerous chymotrypsin sites outside of the limit digest fragment (Cys-640 to Tyr-658) that could give rise to the multiple small chymotrypsin fragments that are seen (7). Further cleavage of the V8 protease limit digest peptide (Gln-650 to Glu-670) of the V8 protease limit digest fragment at Leu-665 by added chymotrypsin would yield the still smaller fragments that were occasionally observed during double protease digestion (7). The fact that all of these peptides are larger than was observed (7) could easily derive from the observed extreme hydrophobicity of the V8 limit digest fragment and probably also of the chymotrypsin and trypsin limit digest fragments.

The present data allow the precise identification of the first components of any of the three functional domains of the glucocorticoid receptor, i.e., antigenic domain, DNA binding domain, and steroid binding domain. Homology with the DNA binding protein erb A, or T₃ receptor (31, 32), has led to proposing the cysteine-rich region corresponding to positions 440-500 of the rat receptor (11) as being intimately involved in DNA binding (27, 33). The facts that 41% of the overlapping sequence of 486-517 is composed of basic amino acids, which could form ionic bonds with the DNA phosphate backbone, and that structures in this sequence can be formed that are similar to the proposed DNA-binding "fingers" of TFIIIA (34) further strengthen this tentative assignment. An antigenic site that is missing in a biologically inactive variant

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