Structural Organization of the Human Glucocorticoid Receptor Determined by One- and Two-Dimensional Gel Electrophoresis of Proteolytic Receptor Fragments†

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Received July 15, 1986; Revised Manuscript Received October 1, 1986

ABSTRACT: The structural organization of the steroid-binding protein of the IM-9 cell glucocorticoid receptor was investigated by using one- and two-dimensional gel electrophoresis of proteolytic receptor fragments. One-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of receptor fragments isolated after trypsin digestion of immunopurified [^{3}H]dexamethasone 21-mesylate ([^{3}H]DM-) labeled receptor revealed the presence of a stable 26.5-kilodalton (kDa) steroid-containing, non-DNA-binding fragment, derived from a larger, less stable, 29-kDa fragment. The 26.5-kDa tryptic fragment appeared to be completely contained within a 41-kDa, steroid-containing, DNA-binding species isolated after chymotrypsin digestion of the intact protein. Two-dimensional electrophoretic analysis of the [^{3}H]DM-labeled tryptic fragments resolved two (p$I \simeq$ 5.7 and 7.0) 26.5-kDa and two (p$I \simeq$ 5.7 and 6.8) 29-kDa components. This was the same number of isoforms seen in the intact protein, indicating that the charge heterogeneity of the steroid-binding protein is the result of modification within the steroid-containing, non-DNA-binding, 26.5-kDa tryptic fragment. Two-dimensional analysis of the 41-kDa [^{3}H]DM-labeled chymotryptic species revealed a pattern of isoforms more complex than that seen either in the intact protein or in the steroid-containing tryptic fragments. These results suggest that the 41-kDa [^{3}H]DM-labeled species resolved by one-dimensional SDS–PAGE after chymotrypsin digestion may be composed of several distinct proteolytic fragments.

The steroid-binding protein of the glucocorticoid receptor (GR)[1] must perform at least two distinct functions. It must be able to recognize and bind glucocorticoidal steroids with high affinity and specificity. It must also, when occupied and activated, be able to interact with chromatin, or chromatin-associated proteins, to influence the expression of hormonally regulated genes. Examination of the structure of the glucocorticoid binding protein of the rat and mouse GR suggests that each of these functions can be attributed to specific domains of the protein which are bounded by protease-sensitive regions (Wrange & Gustafsson, 1978; Carlstedt-Duke et al., 1982; Vedeckis, 1983).

The steroid-binding domain of the steroid-binding protein of the rat liver GR is located within the smallest steroid-containing species ("mero-receptor") which can be identified after digestion with trypsin. When analyzed by gel filtration chromatography, this species has an apparent Stokes radius of 1.9 nm (Carlstedt-Duke et al., 1977; Wrange & Gustafsson, 1978). However, SDS–PAGE analysis of affinity-labeled receptors has resolved the 1.9-nm component into two steroid-containing fragments of 25–27 and 27–31 kDa (Reichman et al., 1984; Wrange et al., 1984). The DNA-binding domain of the steroid-binding protein of the rat liver GR is located within an internal 16-kDa, non-steroid-containing, tryptic fragment of the protein (Eisen et al., 1985). This fragment is derived from a larger, 46-kDa, tryptic fragment which contains both the DNA-binding and steroid-binding domains of the protein (Eisen et al., 1985). Similarly, both the steroid-binding and DNA-binding domains of the protein are found in a 3.6-nm, 38–40-kDa chymotryptic fragment of the receptor (Wrange & Gustafsson, 1978; Reichman et al., 1984; Wrange et al., 1984).

Carlstedt-Duke et al. (1982) have also identified a third receptor domain suggested to be responsible for the normal regulation of receptor function. This portion of the steroid-binding protein appears to be absent from the receptors of steroid-resistant S49 *nt*^{i} (Nordeen et al., 1981; Gehring, 1983; Westphal et al., 1984; Northrop et al., 1985, 1986) and PR1798 cells (Stevens et al., 1981; Okret et al., 1983) and thus is apparently necessary for normal receptor function. This domain is released by limited chymotryptic digestion as a putative 55-kDa non-steroid-binding, non-DNA-binding, fragment which cannot be isolated intact but appears to be rapidly digested through a more stable 2.6-nm, 31–32-kDa, component which is further cleaved to very small fragments not resolved by SDS–PAGE (Wrange et al., 1984). This region of the steroid-binding protein is also the most antigenic of the three domains; the majority of antibodies raised against the rat liver receptor have been directed against this domain (Eisen, 1980, 1982; Okret et al., 1981, 1984; Stevens et al., 1981; Westphal et al., 1982; Gustafsson et al., 1983).

The recent cloning and sequencing of human, rat, and mouse GR cDNAs suggest that the steroid-binding, DNA-binding, and immunogenic domains of the steroid-binding protein are located in the carboxyl, central, and amino portions, respec-

† This work was supported by USPHS Grant CA32226 awarded by the National Cancer Institute to J.M.H.

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[1] Abbreviations: DM, dexamethasone 21-mesylate; EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NEPHGE, nonequilibrium pH gradient electrophoresis; NP-40, Nonidet-P40; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; GR, glucocorticoid receptor; DSCT–agarose CL-4B, (diaminoethylsuccinylthioethylamino-2-thiopyridyl)–agarose CL-4B; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; kDa, kilodalton(s).

tively, of the protein (Hollenberg et al., 1985; Northrop et al., 1986; Meisfeld et al., 1986). However, it is clear that post-translational modification of the steroid-binding protein can play an important role in receptor function. Sando et al. (1979) and Wheeler et al. (1981) have suggested that the unoccupied form of the receptor must be phosphorylated in order to bind ligand. In addition, it is clear that modification of sulfhydryl groups can influence the capacity of the receptor to bind steroid and DNA (Rees & Bell, 1975; Bodwell et al., 1984a,b; Grippo et al., 1985). More recently, we have shown that there are at least two immunoreactive isoforms of the steroid-binding protein of the human GR (Smith & Harmon, 1985), only the more basic of which binds to DNA after receptor activation (Smith et al., 1986).

In the present study, we have used limited proteolytic digestion of the steroid-binding protein of the human GR to define the functional domains of this protein. In addition, we have used denaturing high-resolution two-dimensional gel electrophoresis to map the charge heterogeneity responsible for differential DNA binding to a 26.5-kDa steroid-containing, non-DNA-binding tryptic fragment of the steroid-binding protein.

Experimental Procedures

Cells and Cell Culture. IM-9 human lymphoid cells were grown in RPMI 1640 in the presence of 5% fetal bovine serum as stationary suspensions in a humidified atmosphere of 95% air and 5% CO_2 as previously described (Harmon et al., 1984). Cells were maintained at a density of from 10^5 to 2×10^6 cells/mL.

Materials. [^{3}H]Dexamethasone 21-mesylate ([^{3}H]DM, 40–48 Ci/mmol) was purchased from New England Nuclear, Boston, MA. "AMPLIFY" was obtained from Amersham Corp., Arlington Heights, IL. TLCK–chymotrypsin was obtained from Sigma Chemical Co., St. Louis, MO, and TPCK–trypsin was obtained from Cooper Biomedical, Freehold, NY. Soybean trypsin inhibitor was purchased from Boehringer Mannheim, Indianapolis, IN. Protein A–Sepharose CL-4B and ampholytes (Pharmalyte pH 3–10 and pH 5–8) were obtained from Pharmacia Fine Chemicals, Piscataway, NJ. Other reagents for electrophoresis were purchased from Bio-Rad Laboratories, Richmond, CA. DNA–cellulose was prepared as previously described (Eisen et al., 1986). HEPES was purchased from Calbiochem, San Diego, CA. Hydrofluor was obtained from National Diagnostics, South Sommerville, NJ, and X-ray film (Kodak XAR-5) was obtained from Eastman Kodak, Rochester, NY. All other chemicals were at least of reagent grade and were purchased from either Sigma Chemical Co. or J. T. Baker Chemical Co., Philipsburg, NJ. Anti-human glucocorticoid receptor antiserum 884 was obtained after immunization of female New Zealand white rabbits with highly purified preparations of IM-9 glucocorticoid receptor. The preparation and characterization of this antisera have been previously reported (Harmon et al., 1984).

Affinity Labeling. IM-9 cytosol was prepared in buffer A (10 mM HEPES, 1 mM EDTA, 0.1 M NaCl, and 10% glycerol, pH 7.6) as previously described (Harmon et al., 1984). Affinity labeling was accomplished by incubation of cytosol in the presence of 200 nM [^{3}H]DM (Simons et al., 1980; Eisen et al., 1981; Simons & Thompson, 1981) for 3 h at 0–4 °C. Unreacted [^{3}H]DM was inactivated by the addition of dithiothreitol (1 mM) for 30 min at 0–4 °C.

Immunopurification of Labeled Receptor. For immunopurification of [^{3}H]DM-labeled receptors, 250 μL of labeled cytosol was incubated with 20 μL of antiserum 884 for 18 h at 0–4 °C. Antibody–receptor complexes were adsorbed to Sepharose CL-4B immobilized protein A as previously described (Smith & Harmon, 1985).

Protease Digestion. [^{3}H]DM-labeled receptor was proteolyzed either prior to or after immunopurification. For trypsin digestion, samples were treated with TPCK–trypsin (280 μg/mL, or 10-fold dilutions of that concentration) for 10 min at 10 °C. For chymotrypsin digestion, samples were treated with TLCK–chymotrypsin (6 μg/mL or 10-fold dilutions of that concentration) for either 10 or 50 min at 10 °C. Reactions were halted by the addition of soybean trypsin inhibitor (1.4 mg/mL).

DNA–Cellulose Chromatography. DNA–cellulose chromatography was performed in buffer A as previously described (Eisen et al., 1986). Bound [^{3}H]DM-labeled material was eluted with buffer A containing 0.5 M NaCl.

SDS–PAGE. Samples to be analyzed by SDS–PAGE were diluted with an equal volume of 2× Laemmli buffer (Laemmli, 1970) and heated at 95 °C for 5 min. Alternatively, immunopurified samples were eluted directly from protein A–Sepharose beads into 1X Laemmli sample buffer as described previously (Smith & Harmon, 1985). SDS–PAGE was performed as described by Laemmli (1970) in 12.5% polyacrylamide gels with a 3% polyacrylamide stacking gel. Following electrophoresis, gels were fixed in 10% acetic acid and impregnated with AMPLIFY. Gels were dried, and radioactivity was visualized by fluorography at −70 °C with Kodak XAR film. Molecular weights were determined from standard curves constructed from the mobilities of the ^{14}C-methylated proteins: phosphorylase *b* ($M_r \simeq 97\,000$), bovine serum albumin ($M_r \simeq 69\,000$), ovalbumin ($M_r \simeq 46\,000$), carbonic anhydrase ($M_r \simeq 30\,000$), and lysozyme ($M_r \simeq 14\,500$).

Two-Dimensional Electrophoresis. Affinity-labeled, immunopurified, receptor was eluted from protein A–Sepharose CL-4B as previously described (Smith & Harmon, 1985). Alternatively, chymotryptic or tryptic receptor fragments were diluted with an equal volume of sample solution (urea, 9.5 M; ampholytes, 1.6% pH 5–8 and 0.4% pH 3.10; β-mercaptoethanol, 5% v/v; and NP-40, 2% v/v). Samples were then subjected to two-dimensional electrophoresis using NEPHGE to achieve first-dimensional separations essentially as previously described (Smith & Harmon, 1985). The only differences were that an additional 30 min of electrophoresis at 800 V was used for first-dimension separations and a 2-cm stacking gel was used for second-dimension separations.

Results

Identification of Steroid-Containing Proteolytic Fragments. As we have previously shown (Smith & Harmon, 1985), there are two (p*I* ≃ 5.7 and 6.0–6.5) immunoreactive isoforms of the 92-kDa steroid-binding protein of the IM-9 glucocorticoid receptor (Figure 1). More recently, we have demonstrated that after activation of [^{3}H]DM-labeled GR only the more basic of these two isoforms is capable of DNA binding (Smith et al., 1986). Thus, it appears that covalent charge modification of the steroid-binding protein can alter the ability of the steroid-binding protein of the human GR to bind to DNA. In order to identify in which region of the steroid-binding protein this structural modification occurs, it was first necessary to define the structural organization of the IM-9 GR steroid-binding protein.

Accordingly, [^{3}H]DM-labeled IM-9 GR was incubated with anti-human GR antiserum and immune complexes adsorbed onto protein A–Sepharose beads. The adsorbed complexes were then digested with various concentrations of either trypsin

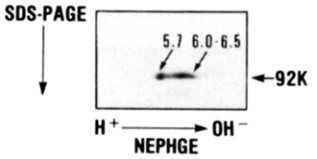

FIGURE 1: Two-dimensional electrophoresis of the immunopurified, [^{3}H]DM-labeled receptor. [^{3}H]DM-labeled receptor was immunopurified and subjected to two-dimensional gel electrophoresis as described under Experimental Procedures. Labeled protein was visualized by fluorography.

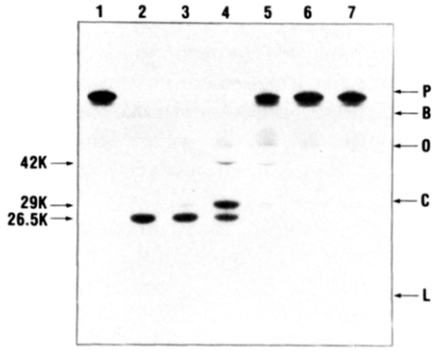

FIGURE 2: Trypsin digestion of immunopurified, [^{3}H]DM-labeled receptor. [^{3}H]DM-labeled, immunopurified, receptor was subjected to one-dimensional SDS–PAGE either before (lane 1) or after digestion with 280 μg/mL (lane 2), 28 μg/mL, (lane 3), 2.8 μg/mL (lane 4), 280 ng/mL (lane 5), 28 ng/mL (lane 6), or 2.8 ng/mL (lane 7) TPCK–trypsin, as described under Experimental Procedures. Arrows to the left of the figure indicate the positions of stable tryptic fragments. Arrows to the right indicate the positions of the molecular markers phosphorylase *b* (P), bovine serum albumin (B), ovalbumin (O), carbonic anhydrase (C), and lysozyme (L).

or chymotrypsin and the steroid-containing digestion products analyzed by SDS–PAGE and fluorography. The results of digestion with trypsin are presented in Figure 2. In lane 1 is shown the intact 92-kDa steroid-binding protein. Lanes 2–7 are the results of digestion with various concentrations of trypsin. At the highest concentration of trypsin employed (280 μg/mL), a single steroid-containing fragment of 26.5 kDa was resolved (lane 2). At lower concentrations of trypsin, both the 26.5- and a larger 29-kDa fragment were observed (lanes 3–5). In addition, under these more mild digestion conditions, a distinct 42-kDa steroid-containing fragment could be clearly visualized (lanes 4 and 5) as well as a series of less well-resolved, somewhat larger, fragments (lanes 4–7). The progressive nature of these digestions with increasing amounts of trypsin suggests that the 26.5-kDa fragment is generated by sequential digestion of the 92-kDa precursor through a series of intermediate fragments. The size of these fragments was comparable to those reported for tryptic fragments of rat receptor (Reichman et al., 1984; Wrange et al., 1984).

The results of digestion of the [^{3}H]DM-labeled 92-kDa steroid-binding protein with chymotrypsin are presented in Figure 3. At the highest concentration of chymotrypsin employed (6 μg/mL), a stable 41-kDa component was resolved (Figure 3, lane 2). At lower concentrations, multiple poorly resolved higher molecular weight forms were visible (lanes 3–6). As was the case for the steroid-containing tryptic fragments of the IM-9 steroid-binding protein, the size of the major 41-kDa steroid-containing chymotrypic fragment, as well as the sizes of the intermediate, higher molecular weight forms, was extremely similar to those reported after chymotryptic digestion of the rat liver and HTC cell GR (Reichman et al., 1984; Wrange et al., 1984).

To determine the physical relationship between the steroid-containing 26.5-kDa tryptic fragment and the 41-kDa

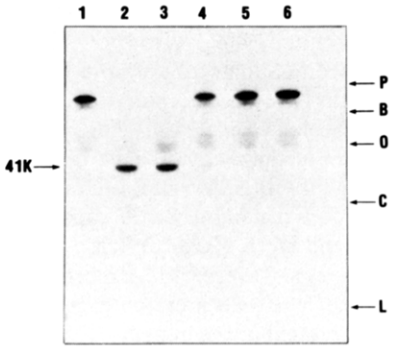

FIGURE 3: Chymotrypsin digestion of immunopurified, [^{3}H]DM-labeled receptor. [^{3}H]DM-labeled, immunopurified receptor was subjected to one-dimensional SDS–PAGE either before (lane 1) or after digestion with 6 μg/mL (lane 2), 600 ng/mL (lane 3), 60 ng/mL (lane 4), 6 ng/mL (lane 5), or 600 pg/mL TLCK–chymotrypsin for 50 min as described under Experimental Procedures. Arrows to the right indicate the positions of the molecular weight markers phosphorylase *b* (P), bovine serum albumin (B), ovalbumin (O), carbonic anhydrase (C), and lysozyme (L).

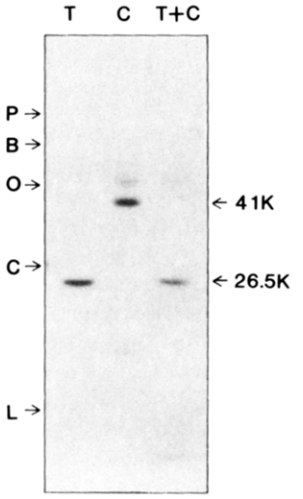

FIGURE 4: Sequential digestion of immunopurified, [^{3}H]DM-labeled receptor. Immunopurified, [^{3}H]DM-labeled receptor was subjected to SDS–PAGE after digestion with 280 μg/mL TPCK–trypsin for 10 min (T), 6 μg/mL TLCK–chymotrypsin for 50 min (C), or 6 μg/mL TLCK–chymotrypsin for 50 min followed by 280 μg/mL TPCK–trypsin for an additional 10 min (T+C) as described under Experimental Procedures. Arrows to the right of the figure indicate the positions of the 41-kDa chymotryptic fragment and the 26.5-kDa tryptic fragment. Arrows to the left indicate the positions of the molecular weight markers phosphorylase *b* (P), bovine serum albumin (B), ovalbumin (O), carbonic anhydrase (C), and lysozyme (L).

chymotryptic fragment, the intact 92-kDa steroid-binding protein was digested sequentially with chymotrypsin and then trypsin. The results of these experiments (Figure 4) showed that the steroid-containing sequential digestion product was precisely the same size (26.5 kDa) as the tryptic digestion product. Thus, the 26.5-kDa steroid-containing tryptic fragment appears to be completely contained within the larger 41-kDa chymotryptic fragment.

DNA-Binding Characteristics of [^{3}H]DM-Labeled Proteolytic Fragments. The ability of the [^{3}H]DM-labeled proteolytic fragments to bind to DNA was assessed by DNA–cellulose chromatography. Immunopurified samples were digested with either trypsin or chymotrypsin and then applied to 3.0-mL columns of DNA–cellulose. Bound material was eluted with buffer containing 0.5 M NaCl and examined by SDS–PAGE and fluorography. The results of these experiments (Figure 5) show that after trypsin digestion there was no retention of either the 26.5- or the 29-kDa steroid-con-

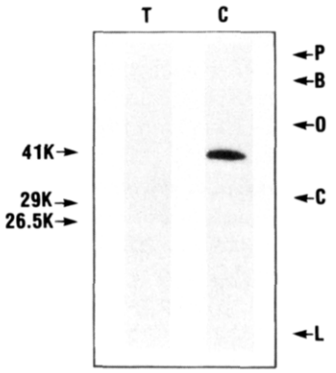

FIGURE 5: DNA–cellulose binding of [^{3}H]DM-labeled proteolytic fragments. [^{3}H]DM-labeled receptor was digested with 280 μg/mL TPCK–trypsin for 10 min (T) or 6 μg/mL TLCK–chymotrypsin for 10 min (C) and subjected to DNA–cellulose chromatography as described under Experimental Procedures. Samples were then subjected to SDS–PAGE as in Figure 4.

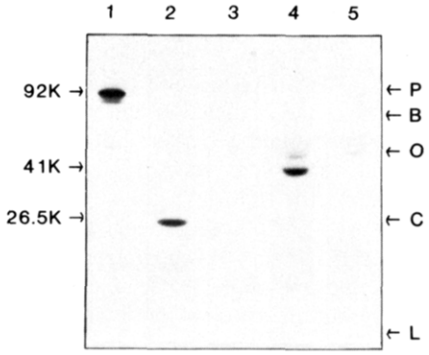

FIGURE 6: Immunoreactivity of [^{3}H]DM-labeled proteolytic fragments. [^{3}H]DM-labeled receptor was subjected to proteolysis with TPCK–trypsin (280 μg/mL) (lanes 2 and 3) or TLCK–chymotrypsin (6 μg/mL) (lanes 4 and 5) either before (lanes 3 and 5) or after (lanes 2 and 4) immunopurification. Samples were then analyzed by SDS–PAGE. A control, undigested, sample (lane 1) was subjected to SDS–PAGE after immunopurification. Arrows to the left of the figure indicate the positions of intact receptor and the major chymotryptic (41 kDa) and tryptic (26.5 kDa) steroid-containing fragments. Arrows to the right of the figure indicate the positions of the molecular weight markers phosphorylase *b* (P), bovine serum albumin (B), ovalbumin (O), carbonic anhydrase (C), and lysozyme (L).

taining fragments, indicating that neither fragment contains the DNA-binding region of the IM-9 GR steroid-binding protein. In contrast, a considerable portion (≃50%) of the 41-kDa [^{3}H]DM-labeled chymotryptic fragment was retained on DNA–cellulose (Figure 5), indicating that the DNA-binding domain of the steroid-binding protein is located within this fragment. These results are analogous to those obtained for the rat and mouse GR steroid-binding proteins (Carlstedt-Duke et al., 1982; Vedeckis, 1983, 1984; Reichman et al., 1984; Wrange et al., 1984) and suggest that the structural organization of the human GR steroid-binding protein is extremely similar to that of the rat.

Immunoreactivity of [^{3}H]DM-Labeled Proteolytic Fragments. In the experiments described above, the [^{3}H]DM-labeled steroid-binding protein was subjected to proteolytic digestion after immunoadsorption. Therefore, it was not obvious with which region(s) of the steroid-binding protein the anti-human GR antiserum was reacting. To resolve this question, the 92-kDa [^{3}H]DM-labeled GR was treated with trypsin or chymotrypsin either prior to or after immunoadsorption with anti-human GR antiserum. The results showed that only when the receptor was digested after immunoadsorption could the 26.5-kDa tryptic and 41-kDa chymotryptic [^{3}H]DM-labeled fragments be identified (Figure 6, lanes 2 and 4). If instead [^{3}H]DM-labeled receptor was digested prior to immunoadsorption, no labeled material smaller than 41 kDa was recovered (Figure 6, lanes 3 and 5). Thus, as we have previously suggested (Harmon et al., 1984), our anti-human GR antiserum appears to be directed against determinants in the non-steroid-binding, non-DNA-binding portion of the steroid-binding protein. This specificity is consistent with the observation that most anti-rat GR antibodies have been shown to recognize the analogous region of the rat GR (Okret et al., 1981; Stevens et al., 1982; Eisen, 1982; Westphal et al., 1982; Gustafsson et al., 1983; Wrange et al., 1984). Additional support for this conclusion is the observation that several faint bands larger than 41 kDa can be seen after immunoadsorption of chymotrypsin-cleaved GR (Figure 6, lane 5). These most probably represent intermediate cleavage products of the steroid-binding protein which contain a portion of the immunogenic domain of the receptor.

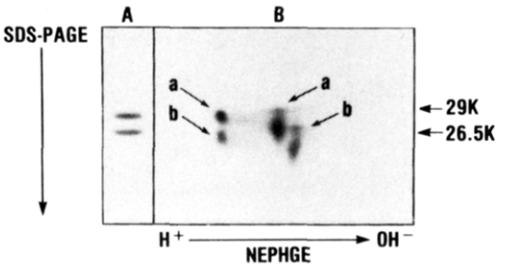

FIGURE 7: Two-dimensional electrophoresis of [^{3}H]DM-labeled tryptic fragments. Immunopurified, [^{3}H]DM-labeled receptor was digested with 2.8 μg/mL TPCK–trypsin. Samples were then subjected to one-dimensional SDS–PAGE (panel A) or two-dimensional electrophoresis (panel B) as described under Experimental Procedures. Arrows to the right of the figure indicate the positions of the 26.5- and 29-kDa tryptic fragments. Diagonal arrows indicate the positions of the two isoforms of the 29-kDa fragment (a) and the 26.5-kDa fragment (b).

Charge Heterogeneity of [^{3}H]DM-Labeled Proteolytic Fragments. Having characterized the functional domains of the IM-9 GR steroid-binding protein by one-dimensional SDS–PAGE, we sought to determine the location of the charge heterogeneity we have previously identified in the 92-kDa steroid-binding protein (Smith & Harmon, 1985). Accordingly, immunoadsorbed, [^{3}H]DM-labeled receptors were digested with trypsin or chymotrypsin and then subjected to high-resolution two-dimensional gel electrophoresis. When labeled IM-9 GR was digested with trypsin, the results presented in Figure 7 were obtained. Under the conditions employed in the experiment presented, both the 26.5- and 29-kDa tryptic fragments were present (Figure 7A). Two-dimensional electrophoresis of these fragments revealed the presence of two isoelectric species of each fragment (Figure 7B). The apparent p*I*'s of the 29-kDa tryptic fragment were ≃5.7 and 6.8, while the apparent p*I*'s of the smaller 26.5-kDa fragment were ≃5.7 and 7.0. From these results, it is clear that each of the [^{3}H]DM-labeled tryptic fragments displays comparable charge heterogeneity to that present in the intact 92-kDa steroid-binding protein (Smith & Harmon, 1985; Smith et al., 1986). It therefore appears that the charge heterogeneity seen in the intact protein is the consequence of covalent charge modification within the steroid-binding domain of the protein.

In many experiments, the p*I* 6.8 species of the 29-kDa fragment and the p*I* 7.0 species of the 26.5-kDa fragment displayed some vertical streaking and appeared to run somewhat faster in the second dimension than their more acidic counterparts (Figure 7B). Since no such size heterogeneity was seen on one-dimensional gels (Figure 7A), this result may

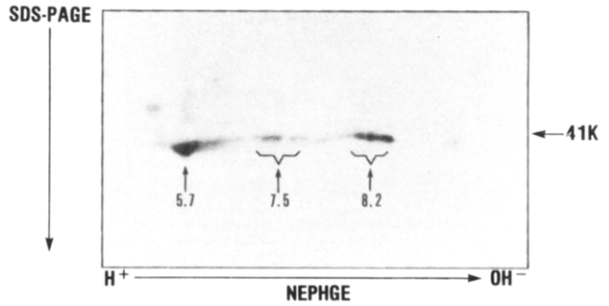

FIGURE 8: Two-dimensional electrophoresis of [^{3}H]DM-labeled chymotryptic fragments. Immunopurified, [^{3}H]DM-labeled receptor was digested with TLCK–chymotrypsin (6.0 μg/mL for 10 min) and subjected to two-dimensional electrophoresis as described under Experimental Procedures. The arrow to the right of the figure indicates the position of the 41-kDa chymotryptic fragments. Vertical arrows indicate the positions of the three major regions of isoelectric species.

simply reflect incomplete denaturation of, and/or SDS binding to, the basic isoforms during equilibration of first-dimension gels prior to electrophoresis in the second dimension. Alternatively, it may reflect actual differences in the conformations, or precise sites of trypsin cleavage, between the two isoforms due to the nature of the covalent modification between the two isoforms.

The results presented in Figure 7 demonstrate that the charge heterogeneity seen in the immunopurified 92-kDa steroid-binding protein is the result of covalent charge modification within the 26.5-kDa steroid-binding domain. It would, therefore, be expected that two-dimensional analysis of the 41-kDa steroid-containing chymotryptic fragment of the receptor would also yield a pattern composed of two isoforms. However, when immunopurified [^{3}H]DM-labeled GR was subjected to two-dimensional separation after chymotrypsin digestion, a complex pattern of acidic and basic isoforms was resolved (Figure 8). [^{3}H]DM-labeled material of $\simeq$41 kDa was identified in three distinct regions: a poorly resolved species of apparent pI $\simeq$ 5.7, a set of species at apparent pI $\simeq$ 7.5, and a pair of species at apparent pI $\simeq$ 8.2. It is difficult to see how a single cut in the 92-kDa steroid-binding protein could produce a pattern of isoelectric species significantly more complex than that present in the intact protein itself. Indeed, the appearance of each region of chymotryptic isoforms is more similar to the charge heterogeneity seen in the intact protein (Figure 1) than is the total chymotryptic pattern. In particular, both the pI $\simeq$ 7.5 and 8.2 regions appear to be composed of pairs of isoforms.

There are several potential explanations for the unexpected complexity of the two-dimensional chymotryptic pattern. It could represent the presence of contaminating proteolytic activities. However, several different lots of chymotrypsin gave essentially the same result. More probable, in our view, is the presence of at least three different chymotryptic digestion products of extremely similar size, but distinctly different charge. Indeed, on the basis of one-dimensional gradient gel electrophoresis, Reichman et al. (1984) suggested that the 3.6-nm, steroid-containing, DNA-binding chymotryptic fragment of the rat liver GR is actually composed of a number of closely spaced fragments. Thus, it appears that although chymotrypsin digestion of the intact steroid-binding protein yields a product with both steroid- and DNA-binding activity, this product is not a single peptide but a group of peptides.

Discussion

Many proteins contain functional regions separated into structurally distinct domains. This has been shown for the steroid-binding protein of the rat liver glucocorticoid receptor which can be separated by limited proteolysis into a steroid-binding domain, a DNA-binding domain, and a third domain, postulated to be involved in the regulation of normal receptor function (Wrange & Gustafsson 1978; Carlstedt-Duke et al., 1982; Reichman et al., 1984). More recently, on the basis of comparison of the nucleotide sequences of the human estrogen and glucocorticoid receptors, Krust et al. (1986) have postulated that the DNA-binding domain of the human GR is located between amino acids 421 and 480. In addition, they identified a region of 159 N-terminal amino acids homologous to the human estrogen receptor which may represent conservation of the third domain postulated by Carlstedt-Duke et al. (1982).

In the present study, we have examined the structural organization of the glucocorticoid receptor of the human lymphoid cell line IM-9, the cell line from which human GR cDNA clones were originally obtained (Weinberger et al., 1985). Not surprisingly, it was found that the general organization of the IM-9 steroid-binding protein was extremely similar to that of the rat protein (Reichman et al., 1984; Wrange et al., 1984). When examined by one-dimensional SDS–PAGE of affinity-labeled receptor, both the steroid-binding and DNA-binding domains of the IM-9 steroid-binding protein were contained in fragments similar in size to those of the rat. In addition, the data in Figure 4 demonstrate that the stable 26.5-kDa steroid-containing fragment derived after trypsin digestion is completely contained within the 41-kDa, steroid-containing, DNA-binding species obtained after chymotrypsin digestion of the intact protein.

Examination of affinity-labeled receptor fragments by two-dimensional electrophoresis was more revealing. The 26.5-kDa steroid-containing tryptic fragment was resolved into two isoelectric species. This was the same extent of charge heterogeneity previously demonstrated in the intact, immunopurified, 92-kDa steroid-binding protein of the IM-9 GR (Figure 1; Smith & Harmon, 1985) and indicates that the charge heterogeneity of the intact protein is the result of modification within the steroid-binding domain. Posttranslational modification of the steroid binding protein does not appear necessary for steroid-binding activity since Hollenberg et al. (1985) have recently shown that the primary in vitro translation product of the steroid-binding protein mRNA is capable of steroid binding. However, we have recently shown that only the basic isoform of the steroid-binding protein is able to bind to DNA–cellulose after receptor activation (Smith et al., 1986). Thus, it appears that covalent modification within the steroid-binding domain of the steroid-binding protein is able to influence the overall ability of the intact protein to bind to DNA. Bodwell et al. (1984b) have shown that the ability of activated steroid receptor complexes to bind to DSCT–agarose CL-4B was inhibited by the presence of soluble DNA. The interaction of the activated GR with this matrix appeared to be through a sulfhydryl located in the non-DNA-binding, steroid-containing, tryptic fragment of the receptor. On the basis of these results, they suggested that the tryptic fragment (mero-receptor) might contain a portion of the DNA-binding domain of the protein. Alternatively, both their results and the results presented here are consistent with the idea that modifications in one domain of the steroid-binding protein of the GR can regulate the functional capacity of other regions of the protein.

Two-dimensional analysis of the steroid-containing chymotryptic receptor fragments was also informative. The large degree of heterogeneity seen in these fragments compared to

that observed in either the intact protein (Figure 1; Smith & Harmon, 1985; Smith et al., 1986) or the smaller tryptic fragments (Figure 7) suggests that the 41-kDa band resolved by one-dimensional SDS–PAGE is actually composed of several different fragments. Inspection of the amino acid sequence of the steroid-binding protein of the human GR (Hollenberg et al., 1985) suggests several possible explanations for this apparent multiplicity of chymotryptic fragments. The carboxy-terminal portion of this 777 amino acid protein contains aromatic residues at positions 764 and 774. Between residues 764 and 774 are two lysine residues (positions 770 and 771). Two additional basic residues (His-775 and Lys-777) are located between Phe-774 and the carboxy terminus. Thus, it would be possible to generate multiple chymotryptic fragments of distinctly different charge without an obvious alteration in molecular weight. In addition, given the large number of basic residues between residues 442 and 498, it is possible that the multiplicity of fragments results from alternate cutting in this region of the protein. This latter possibility seems less likely in view of the fact that if, as has been suggested (Hollenberg et al., 1985; Krust et al., 1986), the steroid-binding domain of the protein is located near the carboxy terminus, then chymotryptic cleavage in the region of 442–498 would yield steroid-containing fragments significantly smaller than 41 kDa. Alternatively, it is possible that chymotrypsin is cleaving at other than aromatic residues, in which case it is not possible to predict potential cleavage sites. In any case, it does not appear that there is differential DNA binding of the multiple chymotryptic fragments; two-dimensional analysis of fragments obtained after DNA–cellulose chromatography yielded isoelectric patterns similar to those obtained in Figure 8 (data not shown).

The origin of the charge heterogeneity which we have mapped to the 26.5-kDa steroid-containing tryptic fragment remains undetermined. We have previously suggested that this heterogeneity is not the result of differential phosphorylation (Smith et al., 1986). This suggestion was based on the fact that the difference in apparent p*I*'s between the two isoforms of the intact 92-kDa steroid-binding protein is approximately equivalent to twice that contributed by a single phosphate group. In addition, the apparent p*I* of neither isoform was altered after alkaline phosphatase treatment (Smith et al., 1986). Nor is it likely that this heterogeneity reflects receptor polymorphism since only the α form of the protein is present in IM-9 cells (Hollenberg et al., 1985). Other possibilities, including ADP-ribosylation and glycosylation, are currently under investigation. Indeed, the inferred amino acid sequence of the steroid-binding protein contains several potential sites for N-glycosylation, including one at Asn-707. Regardless of the nature of the modification in the steroid-containing tryptic fragment responsible for the presence of two immunoreactive steroid-binding protein isoforms, it is clear that this modification can affect the ability of the intact protein to bind to DNA (Smith et al., 1986). Thus, post-translational modification of the steroid-binding protein within the steroid-binding domain may play an important role in the regulation of the functional activity of the glucocorticoid receptor.

ACKNOWLEDGMENTS

We gratefully acknowledge the technical assistance of Marian Elsasser in the execution of these experiments and the editorial assistance of Patsy Amoss and Jeane McWilliams in the preparation of the manuscript. The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the view of the Department of Defense or the USUHS.

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Pactamycin Binding Site on Archaebacterial and Eukaryotic Ribosomes†

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Received July 29, 1986; Revised Manuscript Received September 22, 1986

ABSTRACT: The presence of a photoreactive acetophenone group in the protein synthesis inhibitor pactamycin and the possibility of obtaining active iodinated derivatives that retain full biological activity allow the antibiotic binding site on *Saccharomyces cerevisiae* and archaebacterium *Sulfolobus solfataricus* ribosomes to be photoaffinity labeled. Four major labeled proteins have been identified in the yeast ribosome, i.e., YS10, YS18, YS21/24, and YS30, while proteins AL1a, AS10/L8, AS18/20, and AS21/22 appeared as radioactive spots in *S. solfataricus*. There seems to be a correlation between some of the proteins labeled in yeast and those previously reported in *Escherichia coli* [Tejedor, F., Amils, R., & Ballesta, J. P. G. (1985) *Biochemistry 24*, 3667–3672], indicating that the pactamycin binding sites of both species, which are in the small subunit close to the initiation factors and mRNA binding sites, must have similar characteristics.

Although the basic steps of protein synthesis have been maintained throughout evolution, significant structural differences prevent a direct extrapolation of the data from prokaryotic to eukaryotic ribosomes.

The situation is further complicated by the description of the archaebacterial kingdom, comprised of unicellular organisms, thus far classified as bacteria, with peculiar properties (Woese, 1982). From the point of view of protein synthesis, archaebacteria have characteristics, such as ribosome structure (Lake et al., 1984) and sensitivity to antibiotics (Elhardt & Böck, 1982; Cammarano et al., 1985), differentiating them from eukaryotic and prokaryotic cells.

The mechanism of protein synthesis and the structure of the different components involved (ribosomes, tRNAs, soluble factors, etc.) are starting to be reasonably well understood in eubacteria. In spite of recent advances, data are relatively scarce for eukaryotic cells (Bielka, 1982) and almost nonexistent for archaebacteria. It would therefore be of great interest to establish correlations among the three systems that would facilitate the use of the wealth of data available on eubacteria to understand the process in the other two systems.

Antibiotics have been used as powerful tools in the investigation of the protein synthesis mechanism and the structure of its components, especially the ribosomes. The characterization of the antibiotic's target in the protein synthesis machinery allows the correlation of its components with the functions inhibited by the drug. Ribosomal components involved in peptide bond formation and interaction with elongation factors have been located by identifying the binding sites of antibiotics that inhibit these activities, such as chloramphenicol (Pongs & Messer, 1976), macrolides (Tejedor & Ballesta, 1985), puromycin (Nicholson et al., 1982), thiostrepton (Thompson et al., 1979), and tetracycline (Goldman et al., 1983).

Antibiotics are known to be specific inhibitors of either eukaryotic or eubacterial protein synthesis (Vâzquez, 1979), although some of them are able to affect both systems. The use of the last class of drugs is especially suited for comparative studies since the identification of their respective targets allows a correlation between their components in the different cell types to be established.

Pactamycin, a representative of these wide-spectrum antibiotics, was initially reported as an antitumor drug that also inhibited the growth of bacterial cells (Goldberg, 1974). It has been suggested that pactamycin inhibits the initiation step of protein synthesis by interacting with the small ribosomal subunit (Goldberg, 1974). The identification of the components of the pactamycin binding site in ribosomes from different species may thus allow the correlation of the ribosomal components involved in the initiation of protein synthesis in different systems.

The pactamycin binding site can be easily studied by affinity-labeling methods, since its molecule contains an acetophenone group that is photoreactive as well as a phenolic group that is susceptible to radioactive labeling by iodination (Figure

† This work has been supported by an institutional grant from the Fondo de Investigaciones Sanitarias (FIS) and by personal grants from the Comisión Asesora de Investigación Científica y Técnica and from the Fundación Eugenio Rodriguez Pascual.

0006-2960/87/0426-0652$01.50/0