Nagai, K., Kitagawa, T., & Morimoto, H. (1980) *J. Mol. Biol. 136*, 271-289.
Naqui, A., Kumar, C., Ching, Y., Powers, L., & Chance, B. (1984) *Biochemistry 23*, 6222-6227.
Ogura, T., Hon-nami, K., Oshima, T., Yoshikawa, S., & Kitagawa, T. (1983) *J. Am. Chem. Soc. 105*, 7781-7783.
Ogura, T., Sone, N., Tagawa, N., & Kitagawa, T. (1984) *Biochemistry 23*, 2826-2831.
Ondrias, M. R., & Babcock, G. T. (1980) *Biochem. Biophys. Res. Commun. 93*, 29-35.
Rousseau, D. L. (1981) *J. Raman Spectros. 10*, 94-99.
Rousseau, D. L., Tan, S. L., Ondrias, M. R., Ogawa, S., & Noble, R. W. (1984) *Biochemistry 23*, 2857-2865.
Salmeen, I., Rimai, L., & Babcock, G. T. (1978) *Biochemistry 17*, 800-806.
Seybert, D. W., Moffat, K., Gibson, Q. H., & Chang, C. K. (1977) *J. Biol. Chem. 252*, 4225-4231.
Van Buuren, K. J. H., Nicolas, P., & Van Gelder, B. F. (1972) *Biochim. Biophys. Acta 256*, 258-276.
Van Steelandt-Frentrup, J., Salmeen, I., & Babcock, G. T. (1981) *J. Am. Chem. Soc. 103*, 5982-5984.
Wikström, M., Krab, K., & Saraste, M. (1981) *Cytochrome Oxidase: A Synthesis*, Academic Press, New York.
Woodruff, W. H., Dallinger, R. F., Antalis, T. M., & Palmer, G. (1981) *Biochemistry 20*, 1332-1338.

Multiple Forms of the Glucocorticoid Receptor Steroid Binding Protein Identified by Affinity Labeling and High-Resolution Two-Dimensional Electrophoresis†

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ABSTRACT: Potential charge heterogeneity within the glucocorticoid binding protein (GBP) of the glucocorticoid receptor was examined by a combination of affinity labeling, immunopurification, and high-resolution two-dimensional (2D) gel electrophoresis. One-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of [^{3}H]dexamethasone 21-mesylate ([^{3}H]DM) labeled cytosol identified a major, competable, component of M_r ≃92 000 (92K). This component was recognized by anti-human glucocorticoid receptor antibodies but not by nonimmune serum, indicating that the 92K component was the reduced denatured GBP. Examination of [^{3}H]DM-labeled GBP by conventional 2D electrophoresis utilizing equilibrium isoelectric focusing in the first dimension failed to resolve the 92K GBP into discrete isoelectric components. This behavior was not representative of other, nonspecifically [^{3}H]DM-labeled proteins or proteins in general. Nonequilibrium pH gradient electrophoresis (NEPHGE) was therefore employed to achieve separation in the first dimension. Immunopurified, [^{3}H]DM-labeled GBP subjected to NEPHGE reached isoelectric equilibrium after 6 h of electrophoresis at 400 V. A single, broad peak of radioactivity was identified at pH ≃6.3. Second-dimension analysis of the NEPHGE-separated GBP by SDS-PAGE resolved this peak into two discrete, 92K, isoforms of apparent pI = 5.7 and 6.0-6.5. The GBP charge heterogeneity was confirmed by NEPHGE 2D analysis of [^{3}H]DM-labeled GBP prepared directly from crude cytosol. Two isoforms indistinguishable from those observed in immunopurified samples were identified. An additional, more acidic, isoform (apparent pI ≃ 5.2) was also identified. Thus, there are at least two, and perhaps three, isoforms of the GBP. These data therefore suggest that there is significant charge heterogeneity in the GBP of the glucocorticoid receptor.

Glucocorticoid steroid hormone receptors can exist in at least four biochemically relevant forms. These include the active and inactive steroid binding forms of the unoccupied receptor (Sando et al., 1977) and the unactivated and activated forms of the glucocorticoid receptor (GR)[1] complex (Munck et al., 1972; Baxter et al., 1972). Activation of the GR complex has been defined in intact cells as the temperature-dependent conversion of cytoplasmic GR complexes to a form capable of nuclear translocation (Baxter et al., 1972). In vitro, activation of GR complexes results in their increased affinity for nuclei and DNA (Baxter et al., 1972; Milgrom et al., 1973; Kalimi et al., 1975; LeFevre et al., 1979) and their altered chromatographic behavior on DEAE-Sephadex, DEAE-cellulose, and phosphocellulose (Parchman & Litwack, 1977; Sakaue & Thompson, 1977). This altered chromatographic behavior has been used to demonstrate that in intact cells activation precedes nuclear translocation of GR complexes (Munck & Foley, 1979; Markovič & Litwack, 1980). In addition, we have shown that mutants defective in activation of GR complexes are completely resistant to the biological activity of glucocorticoids (Schmidt et al., 1980; Harmon et

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[1] Abbreviations: β-ME, β-mercaptoethanol; DM, dexamethasone 21-mesylate; EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NEPHGE, nonequilibrium pH gradient electrophoresis; NP-40, Nonidet P-40; GBP, glucocorticoid binding protein; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; GR, glucocorticoid receptor. The trival names for the steroids used are triamcinolone acetonide for 9-fluoro-11β,21-dihydroxy-16α,17-[(1-methylethylidene)bis(oxy)]pregna-1,4-diene-3,20-dione and dexamethasone for 9-fluoro-11β,17,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione.

al., 1984a). Thus, activation is an obligatory step in the pathway of glucocorticoid hormone action.

Several lines of indirect evidence suggest that covalent charge modification of the GR complex may be involved in activation. Activation of GR complexes is inhibited by some phosphatase inhibitors, most notably sodium molybdate (Sando et al., 1979). It has also been shown that addition of exogenous phosphatase activity promotes GR complex activation (Barnett et al., 1980). Thus, activation may involve GR complex dephosphorylation. In addition, the increased affinity of activated GR complexes for DNA, as well as other polyanions, and the decreased affinity of activated GR complexes for DEAE-cellulose and DEAE-Sephadex suggest that activation is accompanied by the exposure of positively charged residues or the removal of negatively charged residues from the surface of the receptor (Baxter et al., 1972; Milgrom et al., 1973; Parchman & Litwack, 1979; Sakaue & Thompson, 1977). Results obtained from nondenaturing isoelectric focusing of unactivated and activated GR complexes are also consistent with charge modification during activation. Unactivated GR complexes have been reported to have p*I*s in the range of 5.0–6.3, while activated GR complexes have been reported to be more basic (Wrange, 1979; Hansson et al., 1981; Leiferman et al., 1983; Ben-Or & Chrombach, 1983; Cidlowski & Richon, 1984).

In addition to potential charge modification during GR complex activation, it has recently been suggested that there may be charge heterogeneity within a given form of the receptor. Using nondenaturing isoelectric focusing followed by SDS–PAGE to analyze affinity-labeled mouse L cell glucocorticoid receptors, Housely & Pratt (1983) found a heterogeneous charge species of approximately 92000 daltons. Using virtually identical methods, Cidlowski & Richon (1984) suggested that the unactivated GR complex obtained from HeLa cells may actually be composed of five distinct isoelectric forms.

Despite the large amount of indirect evidence that covalent charge modification may regulate glucocorticoid receptor function, definitive proof has not yet been obtained. All of the data are equally consistent with a multimeric model of the glucocorticoid receptor in which the various apparent charge differences are the result of subunit dissociation. Indeed, several laboratories have recently suggested that glucocorticoid receptor activation involves the dissociation of a multimeric complex (Vedeckis, 1983; Holbrook et al., 1983; Sherman et al., 1983). If this is the case, then the multiple isoelectric forms seen under nondenaturing conditions could simply reflect different combinations of subunit association. To investigate the question of receptor charge heterogeneity and potential regulation through covalent charge modification, we have employed a denaturing two-dimensional gel electrophoretic procedure for the analysis of affinity-labeled glucocorticoid receptors. This procedure is based on the nonequilibrium pH gradient electrophoretic (NEPHGE) method of O'Farrell et al. (1977) and allows direct examination of the denatured steroid binding protein of the glucocorticoid receptor.

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., 1984b). Cells were maintained at a density of 10^5 to 2×10^6 cells/mL.

Materials. Ampholytes (Pharmalyte pH 5–8 and pH 3–10) were purchased from Pharmacia Fine Chemicals, Piscataway, NJ. [^{3}H]Dexamethasone 21-mesylate ([^{3}H]DM, 40–48 Ci/mmol) and EN^{3}HANCE were obtained from New England Nuclear, Boston, MA. Anti-human glucocorticoid receptor antibodies were obtained after immunization of female New Zealand white rabbits with highly purified preparations of IM-9 glucocorticoid receptor. The preparation and characterization of these antisera have been previously reported (Harmon et al., 1984b).

Sample Preparation. IM-9 cytosol in buffer A (10 mM HEPES, 1 mM EDTA, 0.1 M NaCl, 10% glycerol, pH 7.6) was prepared as previously described (Harmon et al., 1984b). Affinity labeling was accomplished by incubation of cytosol with 200 nM [^{3}H]DM (Simons & Thompson, 1981; Simons et al., 1981; Eisen et al., 1981) in the presence or absence of 50 μM unlabeled triamcinolone acetonide (TA) 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. Samples were then prepared for either one-dimensional SDS–PAGE, isoelectric focusing, NEPHGE, or immunoadsorption.

Samples for one-dimensional SDS–PAGE were diluted with an equal volume of 2× Laemmli sample buffer and heated at 95 °C for 5 min (Laemmli, 1970). Samples to be subjected to isoelectric focusing or NEPHGE were diluted with an equal volume of 2D sample solution 1 [9.5 M urea, 2.0% ampholytes (1.6% pH 5–8, 0.4% pH 3–10), 5% β-mercaptoethanol, 2.0% NP-40].

For immunopurification of [^{3}H]DM-labeled receptors, 250 μL of labeled cytosol was incubated with 20 μL of immune or nonimmune anti-human glucocorticoid receptor antiserum for 18 h at 0–4 °C. Antibody–receptor complexes were adsorbed to Sepharose CL-4B immobilized protein A for 30 min followed by centrifugation at 12000*g* for 2 min. The resulting pellets were washed 4 times with 1 mL of buffer A containing 0.5 M NaCl and 2 times with 1 mL of buffer A. [^{3}H]DM–receptor complexes were eluted with 1× Laemmli sample buffer prior to SDS–PAGE. Alternatively, [^{3}H]DM-labeled receptors were eluted by incubation in 50 μL of 2D sample solution 2 [0.5% SDS, 9.5 M urea, 5% β-ME, 2% ampholytes (pH 3–10)] for 10 min at room temperature followed by addition of an equal volume of 2D sample solution 1. All samples were either submitted directly to electrophoresis (isoelectric focusing or NEPHGE) or quick frozen and stored at −70 °C.

Isoelectric Focusing. Isoelectric focusing was performed, with minor modification, by the method of O'Farrell (1975). Polyacrylamide gels (3% T, 5% C) containing 9.2 M urea, 2.0% NP-40, and a mixture of 1.6% pH 5–7 and 0.4% pH 3–10 ampholytes were cast in glass tubes (125 mm × 3 mm) to a height of 11.5 cm. Gels were preelectrophoresed (15 min at 200 V, 30 min at 300 V, and 30 min at 400 V), and sample focusing was carried out from cathode to anode for 16 h at 300 V and an additional h at 800 V.

Nonequilibrium pH Gradient Electrophoresis. NEPHGE was performed according to the method of O'Farrell et al. (1977). Modification of gel composition was as stated above for isoelectric focusing. Electrophoresis of samples was performed on non-preelectrophoresed gels from anode to cathode at 400 V for 2–12 h.

Measurement of pH Gradient. The pH gradient formed by ampholyte migration during IEF or NEPHGE was measured immediately after electrophoresis. Gels were sliced into 6-mm sections and incubated in distilled H_2O for 30 min. The pH of the water was then determined.

Determination of Radioactivity. Immediately after electrophoresis, NEPHGE gels were sliced into 6-mm sections and incubated for 18 h with 0.5 mL of Protosol at 37 °C. Acetic

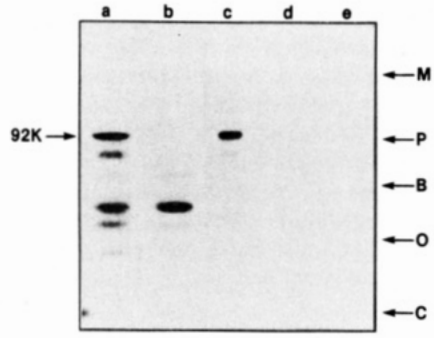

FIGURE 1: Identification of GBP by one-dimensional SDS–PAGE. Crude cytosol labeled with 200 nm [^{3}H]DM in the absence (lanes a, c, and e) or presence (lanes b and d) of 50 μM unlabeled TA was subjected to SDS–PAGE either directly (lanes a and b) or after immunoadsorbtion with immune (lanes c and d) or nonimmune sera (lane e). The arrows on the right of the figure indicate the mobility of the molecular weight markers myosin (M), phosphorylase *b* (P), bovine serum albumin (B), ovalbumin (O), and carbonic anhydrase (C).

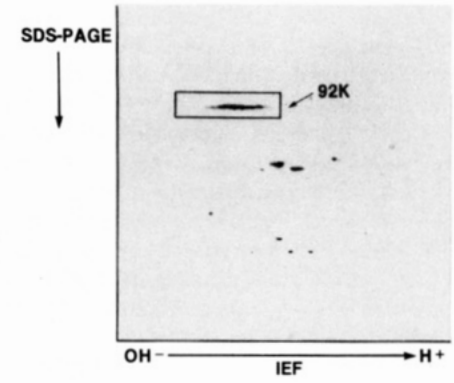

FIGURE 2: Two-dimensional electrophoresis of IM-9 cytosol using equilibrium isoelectric focusing in the first dimension. [^{3}H]DM-labeled cytosol was denatured and subjected to two-dimensional electrophoresis with preelectrophoresed isoelectric focusing gels as described under Experimental Procedures. Separated proteins were visualized by fluorography, in order to observe [^{3}H]DM-labeled species. The arrow indicates the position of the 92K GBP.

acid (17 μL) was added to neutralize the solution, and scintillation fluid was then added. Radioactivity was determined in a Beckman LS 7800.

SDS–PAGE. SDS–PAGE was performed as described by Laemmli (1970). For one-dimensional electrophoresis, samples were analyzed in 8% polyacrylamide gels with 3% polyacrylamide stacking gels. For second-dimension analysis, isoelectric focusing or NEPHGE gels were placed in 5 mL of Laemmli sample buffer (Laemmli, 1970) and either quick frozen for storage at −70 °C or immediately equilibrated by gentle rocking for 30 min at room temperature. After equilibration, gels were rinsed with water and sealed with 1% agarose in Laemmli sample buffer (without β-ME) onto 8% polyacrylamide gels. A small piece of agarose containing [^{14}C]methylated protein markers was included at one end to facilitate determination of molecular weight. A 1-cm 3% polyacrylamide stacking gel was routinely employed.

Following electrophoresis, gels were fixed in 10% acetic acid and then impregnated with EN^{3}HANCE. Gels were then dried, and radioactivity was visualized by fluorography at −70 °C with Kodak XAR-5 film. Molecular weights were determined from standard curves constructed from the mobilities of the [^{14}C]methylated proteins myosin ($M_r \simeq 200\,000$), phosphorylase *b* ($M_r \simeq 97\,000$), bovine serum albumin ($M_r \simeq 69\,000$), ovalbumin ($M_r \simeq 46\,000$), and carbonic anhydrase ($M_r \simeq 30\,000$).

RESULTS

Identification of the GBP by One-Dimensional SDS–PAGE. Identification of IM-9 glucocorticoid receptors under denaturing conditions was achieved through use of the covalent affinity ligand [^{3}H]DM (Simons & Thompson, 1981; Eisen et al., 1981). Incubation of IM-9 cytosol with 200 nM [^{3}H]DM for 3 h at 0–4 °C results in the covalent labeling of multiple cytosolic proteins. However, the major labeled component had an apparent molecular weight of 92 000, and the labeling of this component was completely abolished, if an excess of unlabeled TA was included during the labeling reaction (Figure 1). On the basis of the specificity of labeling and the fact that the molecular weight of this component corresponds to that of highly purified rat liver glucocorticoid receptor (Wrange et al., 1984; Grandics et al., 1984), the 92K protein is almost certainly the reduced denatured glucocorticoid binding protein (GBP) of the IM-9 glucocorticoid receptor.

Additional evidence that the 92K-dalton component is the reduced denatured GBP was obtained by immunoadsorption of the [^{3}H]DM-labeled cytosol with anti-human glucocorticoid receptor antibodies. When labeled cytosol was adsorbed with immune serum and immune complexes subjected to SDS–PAGE, a major component of 92K daltons and a minor component of 78K daltons were resolved (Figure 1). In contrast, incubation of labeled cytosol with nonimmune serum or adsorption with immune serum of cytosol labeled in the presence of an excess of unlabeled TA failed to identify any [^{3}H]DM-labeled material (Figure 1). Thus, identification of the 92K component is immune serum dependent. The origin of the 78K protein is uncertain. However, on the basis of the known susceptibility of the glucocorticoid receptor to proteolysis (Carlstedt-Duke et al., 1979; Cidlowski, 1980; Sherman et al., 1976, 1981; Stevens & Stevens, 1981; Vedeckis, 1983), it is probable that the 78K protein is a proteolytic fragment of the 92K GBP.

Analysis by Two-Dimensional Electrophoresis. Initial experiments to resolve the charge characteristics of the GBP were performed by the widely used method of O'Farrell (1975) for two-dimensional separation of proteins. When [^{3}H]DM-labeled cytosol was subjected to this procedure, the results presented in Figure 2 were obtained. Although the 92K-dalton GBP could be identified, it could not be resolved into a discrete spot or series of spots (Figure 2). Instead, marked streaking was observed in the isoelectric focusing dimension. In addition, in many experiments, a substantial portion of the competable [^{3}H]DM binding remained at the origin. This was in contrast to IM-9 cytosolic proteins in general, which could be readily seen as discrete spots after Coomassie staining of two-dimensional gels (data not shown). Moreover, the inability to resolve the 92K-dalton GBP was not a general characteristic of nonspecifically [^{3}H]DM-labeled proteins. As seen in Figure 2, many nonreceptor [^{3}H]DM-labeled proteins appeared as discrete spots after two-dimensional separation. These proteins are presumably labeled by the nonspecific interaction of [^{3}H]DM with exposed reduced sulfhydryls (Simons et al., 1980). As can be seen from the data in Figure 1, they are not immunoreactive, nor do they display saturable binding for glucocorticoids. Thus, it appears that the behavior of the 92K-dalton GBP in this electrophoretic system is a characteristic of the particular protein.

Numerous modifications were attempted to improve the resolution of the GBP. Use of immunoadsorbed receptor, alteration of the conditions of sample preparation, and variation of gel concentration all failed to provide increased resolution of the GBP. We therefore concluded that this system

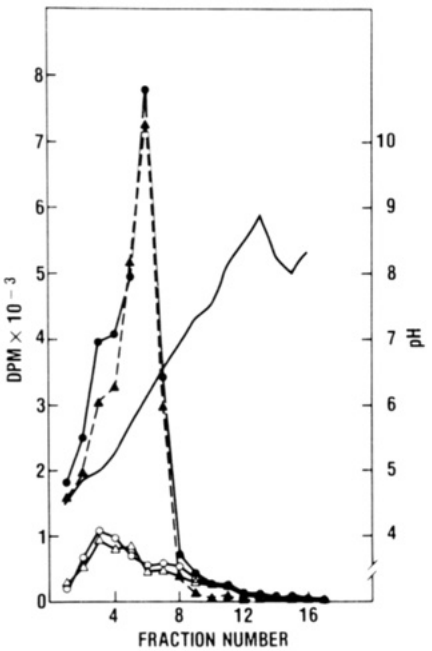

FIGURE 3: NEPHGE of immunopurified, denatured [^{3}H]DM–GBP. IM-9 cytosol labeled with 200 nM [^{3}H]DM in the presence (△) or absence (●, ○) of competing nonradioactive steroid (50 μM TA) was adsorbed with immune (●) or nonimmune (○, △) serum and subjected to NEPHGE. Electrophoresis was carried out for 6 h at 400 V. Gels were sliced and analyzed for radioactivity as well as pH (solid line). The dashed line (– –) indicates competable radioactivity, calculated by subtraction of the results obtained for samples labeled in the presence of competing, unlabeled, steroid from the results obtained for samples labeled in the absence of competing steroid.

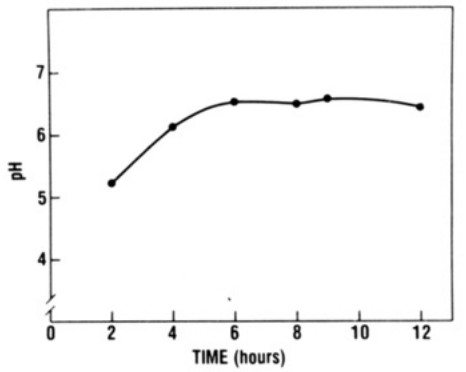

FIGURE 4: Time course of [^{3}H]DM–GBP migration in NEPHGE gels. Immunoadosrbed [^{3}H]DM–GBP was subjected to electrophoresis in NEPHGE gels for various lengths of time at 400 V. After electrophoresis, gels were sliced, and radioactivity and pH were determined as described under Experimental Procedures. Data were analyzed as in Figure 3 to determine the apparent pH of the steroid-competable peak. A stable pH gradient was achieved by 4 h and was invariant thereafter.

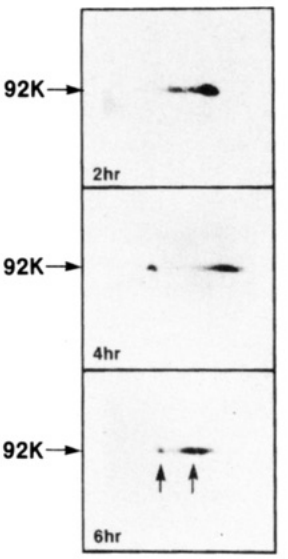

FIGURE 5: Two-dimensional electrophoresis of immunopurified GBP using NEPHGE in the first dimension. [^{3}H]DM-labeled, immunopurified, denatured GBP was subjected to electrophoresis in NEPHGE gels for 2, 4, and 6 h at 400 V, followed by second-dimension separation by SDS–PAGE. Vertical arrows indicate the positions of the 92K charged species.

was inappropriate for the analysis of the GBP.

Two-Dimensional Analysis Using NEPHGE. In order to obtain adequate first dimension separations and thus better resolve the affinity labeled GBP, we adopted the NEPHGE procedure of O'Farrell et al. (1977). To maximize the amount of GBP loaded onto each gel, initial experiments were performed with immunoadsorbed [^{3}H]DM-labeled receptor. When immunoadsorbed [^{3}H]DM-labeled receptor was electrophoresed for 6 h at 400 V and the gels were sliced and counted for determination of radioactivity, the results presented in Figure 3 were obtained. A single peak of [^{3}H]DM radioactivity was identified at pH $\simeq$6.3. Labeling of this peak was virtually abolished if an excess of unlabeled TA was included during the affinity labeling reaction, indicating that the radioactivity was associated with the GBP (Figure 3). Similarly, when [^{3}H]DM-labeled cytosol was adsorbed with nonimmune serum prior to electrophoresis, no significant radioactivity was detected in the NEPHGE gels (Figure 3). Thus, it appears that all of the radioactivity seen in the pH 6.3 peak is associated with the GBP. Of equal importance is the fact that no significant radioactivity was detected at the origin of the gel, indicating that all of the GBP had entered.

The optimal time of elecrophoresis for the [^{3}H]DM-labeled GBP was determined by evaluating the electrophoreric profiles obtained after various times of electrophoresis. The results of these experiments demonstrated that isoelectric equilibrium of the GBP was achieved after 6 h of electrophoresis at 400 V (2400 V h). Under these conditions, the GBP exhibited an apparent p*I* of $\simeq$6.5 (Figure 4). Shorter times resulted in incomplete migration of the GBP (Figure 4). Increased periods of electrophoresis did not alter the apparent p*I* of the GBP (Figure 4), nor did they improve the sharpness of the profile (data not shown). Thus, although the short electrophoresis times used in NEPHGE are often insufficient to allow migration of some proteins (particularly basic proteins) to their apparent isoelectric points (O'Farrell et al., 1977), it appears that 6 h was sufficient to allow the [^{3}H]DM-labeled GBP to reach its apparent p*I*.

To determine whether the width of the electrophoretic profiles seen at apparent equilibrium was the result of poor resolution or charge heterogeneity within the GBP, NEPHGE gels of immunoadsorbed [^{3}H]DM-labeled cytosol were subjected to second-dimensional analysis by SDS–PAGE. The results of these experiments demonstrate that there is significant charge heterogeneity within the GBP (Figure 5). Analysis of second-dimension gels revealed that during NEPHGE the 92K-dalton GBP was progressively resolved into at least two discrete isoelectric species. At equilibrium (6 h), the [^{3}H]DM-labeled GBP was resolved into a major, more basic component ($6.0 \leq pI \leq 6.5$) and a minor, more acidic component ($pI \simeq 5.7$). Both components are clearly derived from the GBP since inclusion of an excess of unlabeled TA during the affinity labeling reaction or adsorption of affinity-labeled complexes with nonimmune serum resulted in the

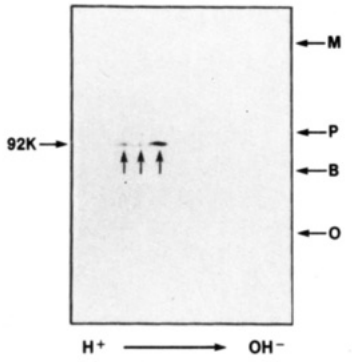

FIGURE 6: Two-dimensional electrophoresis of crude cytosol using NEPHGE in the first dimension. [^{3}H]DM-labeled cytosol was analyzed by two-dimensional electrophoresis as described under Experimental Procedures. NEPHGE was carried out for 6 h at 400 V. Vertical arrows indicate the positions of the three 92K charged species that are resolved. Arrows to the right of the figure indicate the positions of the molecular weight markers myosin (M), phosphorylase *b* (P), bovine serum albumin (B), and ovalbumin (O).

elimination of both components (data not shown). In some experiments, the major component appeared to be composed of more than one spot (data not shown), suggesting additional charge heterogeneity. However, it is clear from the data presented in Figure 5 that the GBP of IM-9 glucocorticoid receptors is composed of more than one isoelectric specie.

Two-Dimensional Analysis of Affinity-Labeled Cytosol. Affinity-labeled GBP was also examined directly from crude cytosol without immunopurification. Under these conditions, the 92K GBP was resolved into three isoelectric forms (Figure 6). The two most basic forms (p*I* $\simeq$ 5.7 and 6.0–6.5) were indistinguishable from the two GBP isoforms seen when immunopurified samples were examined. However, the third, most acidic form (p*I* $\simeq$ 5.2), was unique to cytosol preparations. The labeling of each of these forms was abolished if an excess of unlabeled TA was present during incubation with [^{3}H]DM, indicating that all three forms are derived from the GBP of the IM-9 glucocorticoid receptor (data not shown). Thus, two-dimensional analysis of crude cytosol appears to resolve an additional isoelectric species (p*I* $\simeq$ 5.2) not seen when immunopurified complexes were analyzed. Comparison of the data in Figure 2 with those in Figure 6, both obtained from crude affinity-labeled cytosol, suggests that fewer nonspecifically labeled proteins are resolved if NEPHGE is used for first-dimensional separation. Indeed, in the experiment shown in Figure 6, there was little evidence that any [^{3}H]-DM-labeled proteins other than the GBP had entered the gel. It therefore appears that not only does the use of NEPHGE to achieve first-dimensioin separation improve the resolution of the GBP but it also results to increased specificity.

Discussion

Posttranslational modification is an important mechanism for the biological regulation of protein function. In particular, recent evidence suggests that such modification may be involved in controlling the activity of glucocorticoid receptors. Both Housley & Pratt (1983) and Cidlowski & Richon (1984) have reported that when [^{3}H]DM-labeled glucocorticoid receptors are subjected to two-dimensional electrophoresis, the GBP appears as a heterogeneous charge species of approximately 90 000 daltons rather than as a discrete spot. However, in both of these reports the isoelectric focusing procedure of Wrange (1979) was utilized to achieve isoelectric separation. This procedure was designed to focus native (nondenatured) glucocorticoid receptors (Wrange, 1979) and thus cannot differentiate between charge heterogeneity that results from direct covalent modification of the GBP and apparent charge heterogeneity resulting from interaction of the GBP with other receptor associated components.

In order to directly examine potential charge heterogeneity within the GBP, we analyzed affinity-labeled GBP by using two-dimensional separations in which the GBP was denatured in both dimensions. The conventional two-dimensional electrophoresis system of O'Farrell (1975) achieved good resolution of nonreceptor proteins in stained as well as fluorographed gels. However, consistently poor focusing of the [^{3}H]DM–GBP was obtained, precluding estimation of a p*I* for the GBP or the determination of a specific number of receptor charge species. We therefore chose to analyze the GBP by the alternate two-dimensional system of O'Farrell et al. (1977) in which NEPHGE is used to achieve separation in the first dimension. In this system, there is no preelectrophoresis of focusing gels prior to sample application. In addition, the direction of migration is from anode to cathode rather than the opposite direction in standard isoelectric focusing.

When immunopurified, denatured [^{3}H]DM–GBP was analyzed by NEPHGE, the GBP electrophoresed to a pH in the range of 5.7–6.5 in 6 h. The apparent p*I* of the GBP did not change after additional electrophoresis, indicating that the [^{3}H]DM–GBP had attained isoelectric equilibrium. Two-dimensional analysis revealed that the peak seen in one-dimensional separations was actually composed of at least two isoelectric species (p*I* $\simeq$ 5.7 and 6.0–6.5). The presence of both species was competable and immune serum dependent, establishing that each was derived from the glucocorticoid receptor. Interestingly, two-dimensional analysis of crude affinity-labeled cytosol revealed the presence of three isoforms (p*I* $\simeq$ 5.2, 5.7, and 6.0–6.5). Each was competable with unlabeled steroid, establishing their receptor origin and suggesting the presence of an additional charge form of the steroid binding protein. It is unclear why the third, most acidic form of the GBP was not detected in immunopurified samples. It is possible that the antiserum used does not recognize this form of the receptor. Alternatively, the antiserum may contain phosphatase or other enzymic activity that may act on the GBP during immunoadsorption, resulting in the loss of the p*I* = 5.2 isoform.

Regardless of the precise reason for the difference in the number of charged species detected when crude or immunopurified samples are examined, it is clear that use of NEPHGE to achieve first-dimension separations is vastly superior to the equilibrium method of O'Farrell. It also appears that the denatured GBP of the IM-9 glucocorticoid receptor contains significant charge heterogeneity. The source of this heterogeneity is at this point undetermined. Recent evidence suggests that the glucocorticoid receptor is a phosphoprotein and in fact may even possess intrinsic protein kinase activity (Housely & Pratt, 1983; Kurl & Jacob, 1984; Schmidt & Litwack, 1985). Using nondenaturing isoelectric focusing to achieve first-dimension separations, Housley & Pratt (1983) found that when receptors were purified from cells grown in the presence of [^{32}P]orthophosphate two receptor-specific charge species could be identified. Although it is difficult to compare their results to those reported here, it is tempting to speculate that the charge heterogeneity that we have observed reflects different phosphorylation states of the receptor.

It is also possible that the apparent heterogeneity of the GBP is due to the presence of a small amount of sample contamination with activated receptor. However, given the fact that three isoforms were identified from crude cytosol it seems unlikely that this could account for all of the heterogeneity

observed. In addition, inclusion of sodium molybdate during the labeling reaction and subsequent sample preparation did not alter the number or distribution of the species observed (data not shown). Thus, it appears that the charge heterogeneity in the GBP is intrinsic to the unactivated form of the occupied glucocorticoid receptor.

An alternative explanation for the apparent charge heterogeneity in the GBP is the presence of multiple GBP alleles. If this is the case, it is not reflected in the presence of more than one affinity class of glucocorticoid binding site. Analysis of equilibrium binding of [^{3}H]dexamethasone to intact cells indicated the presence of a single affinity class of receptors (A. C. Smith and J. M. Harmon, unpublished results). However, Bourgeois & Newby (1977) have shown that the complete loss of steroid binding activity in murine W7 cells requires two mutational events. In addition, the mouse glucocorticoid receptor has been mapped to chromosome 18 and is thus autosomal (Franke & Gehring, 1980). Thus, resolution of the origin of the charge heterogeneity in the IM-9 GBP will require not only careful biochemical characterization of the GBP but the analysis of glucocorticoid receptor mutants defective in various aspects of steroid binding and activation. These experiments are currently in progress.

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REFERENCES

Baxter, J. D., Rousseau, G. G., Benson, M. C., Garcea, R. L., Ito, J., & Tomkins, G. M. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1892–1896.

Ben-Or, S., & Chrombach, A. (1983) *Arch. Biochem. Biophys. 221*, 343–353.

Bourgeois, S., & Newby, R. F. (1977) *Cell (Cambridge, Mass.) 11*, 423–430.

Carlstedt-Duke, J., Wrange, Ö., Dahlberg, E., Gustafsson, J.-Å., & Högberg, B. (1979) *J. Biol. Chem. 254*, 1537–1539.

Cidlowski, J. A. (1980) *Biochemistry 19*, 6162–6170.

Cidlowski, J. A., & Richon, V. (1984) *Endocrinology (Baltimore) 115*, 1588–1597.

Eisen, H. J., Schleenbaker, R. E., & Simons, S. S., Jr. (1981) *J. Biol. Chem. 256*, 12920–12925.

Francke, U., & Gehring, U. (1980) *Cell (Cambridge, Mass.) 22*, 657–664.

Grandics, D., Miller, A., Schmidt, T. J., Mittman, D., & Litwack, G. (1984) *J. Biol. Chem. 259*, 3173–3180.

Hansson, L. A., Gustafsson, S. A., Carlstedt-Duke, J., Gahrton, G., Högberg, B., & Gustafsson, J.-Å (1981) *J. Steroid Biochem. 14*, 757–764.

Harmon, J. M., Schmidt, T. J., & Thompson, E. B. (1984a) *J. Steroid Biochem. 21*, 227–236.

Harmon, J. M., Eisen, H. J., Brower, S. T., Simons, S. S., Jr., Langley, C. L., & Thompson, E. B. (1984b) *Cancer Res. 44*, 4540–4547.

Holbrook, N. J., Bodwell, J. E., Jeffries, M., & Munck, A. (1983) *J. Biol. Chem. 258*, 6477–6485.

Housley, P. R., & Pratt, W. B. (1983) *J. Biol. Chem. 258*, 4630–4635.

Kalimi, M., Coleman, P., & Feigelson, P. (1975) *J. Biol. Chem. 250*, 1080–1086.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

LeFevre, B., Bailly, A., Sallas, N., & Milgrom, E. (1979) *Biochim. Biophys. Acta 585*, 266–272.

Leiferman, K. M., Schroeter, A., Kirschner, M. K., & Spelsberg, T. C. (1983) *J. Invest. Dermatol. 81*, 355–360.

Markovič, R. D., & Litwack, G. (1980) *Arch. Biochem. Biophys. 202*, 374–379.

Milgrom, E., Atger, M., & Baulieu, E.-E. (1973) *Biochemistry 12*, 5198–5205.

Munck, A., & Foley, R. (1979) *Nature (London) 278*, 752–754.

Munck, A., Wira, C., Young, D. A., Mosher, K. M., Hallahan, C., & Bell, P. (1972) *J. Steroid Biochem. 3*, 567–578.

O'Farrell, P. H. (1975) *J. Biol. Chem. 250*, 4007–4021.

O'Farrell, P. Z., Goodman, H. M., & O'Farrell, P. H. (1977) *Cell (Cambridge, Mass.) 12*, 1133–1142.

Parchman, L. G., & Litwack, G. (1977) *Arch. Biochem. Biophys. 183*, 374–382.

Sakaue, Y., & Thompson, E. B. (1977) *Biochem. Biophys. Res. Commun. 77*, 533–541.

Sando, J. J., Nielson, C. J., & Pratt, W. B. (1977) *J. Biol. Chem. 252*, 7579–7582.

Sando, J. J., LaForest, A. C., & Pratt, W. B. (1979) *J. Biol. Chem. 254*, 4772–4778.

Schmidt, T. J., & Litwack, G. (1985) in *Molecular Mechanisms of Steroid Hormone Action* (Moudgil, V. K., Ed.) de Gruyter, New York (in press).

Schmidt, T. J., Harmon, J. M., & Thompson, E. B. (1980) *Nature (London) 286*, 507–510.

Sherman, M. R., Pickering, L. A., Rollwagen, F. M., & Miller, L. K. (1976) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37*, 167–173.

Sherman, M. R., Tuazon, F. B., & Sömjen, G. J. (1981) in *Physiopathology of Endocrine Diseases and Mechanisms of Hormone Action* (Soto, R. J., DeNicola, A., & Blaquier, J., Eds.) pp 321–337, Liss, New York.

Sherman, M. R., Moran, M. C., Tuazon, F. B., & Stevens, Y.-W. (1983) *J. Biol. Chem. 258*, 10366–10377.

Simons, S. S., Jr., & Thompson, E. B. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 3541–3545.

Simons, S. S., Jr., Pons, M., & Johnson, P. F. (1980) *J. Org. Chem. 45*, 3084–3088.

Stevens, J., & Stevens, Y.-W. (1981) *Cancer Res. 41*, 125–133.

Vedeckis, W. V. (1983) *Biochemistry 22*, 1983–1989.

Wrange, Ö. (1979) *Biochim. Biophys. Acta 582*, 346–357.

Wrange, Ö., Okret, S., Radojčič, M., Carlstedt-Duke, J., & Gustafsson, J.-Å., (1984) *J. Biol. Chem. 259*, 4534–4541.