Phosphorylated Sites within the Functional Domains of the ~100-kDa Steroid-Binding Subunit of Glucocorticoid Receptors†

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ABSTRACT: The steroid-binding subunit of the glucocorticoid receptor is known to be a ~100-kDa phosphoprotein composed of an immunogenic, DNA-binding, and steroid-binding domain. When isolated from WEHI-7 cells, this protein contains between two and three phosphoryl groups per steroid-binding site (Mendel et al., 1987). To identify the domains that contain these phosphorylated sites, we have analyzed the phosphate content of selected proteolytic fragments of the ~100-kDa steroid-binding protein from nonactivated and activated receptors. The ~100-kDa steroid-binding protein from WEHI-7 cells grown in the presence of [^{32}P]orthophosphate was covalently labeled with [^{3}H]dexamethasone 21-mesylate, purified with the BuGR2 monoclonal antibody, digested with chymotrypsin or trypsin, and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).[1] Chymotrypsin digestion of this protein yields a ~45-kDa fragment containing both the steroid-binding and DNA-binding domains, which contained both ^{32}P and ^{3}H. Trypsin digestion of the protein yields a ~29-kDa fragment encompassing the steroid-binding domain but not the DNA-binding domain of the ~100-kDa protein, which also contained both ^{32}P and ^{3}H. The ^{32}P/^{3}H ratio of each fragment provides a measure of phosphate content per steroid-binding site and indicated that each fragment has approximately 30% of the phosphate content of the intact protein. This is sufficient to account for one of the three receptor phosphoryl groups. Since the entire tryptic fragment is contained within the chymotryptic fragment, we conclude that this phosphoryl group is in the steroid-binding domain of the ~100-kDa protein and, therefore, that the DNA-binding domain is not phosphorylated. To determine more directly the phosphate content of the DNA-binding domain of the ~100-kDa protein, we isolated a ~16-kDa tryptic fragment from cytosol of WEHI-7 cells grown in the presence of [^{32}P]orthophosphate and [^{35}S]cysteine. This fragment contains the BuGR2 epitope and has DNA-binding, but not steroid-binding, activity. Comparison of the ^{32}P/^{35}S ratio of this fragment to that of the intact protein indicated that it contains less than 0.2 mol of phosphoserine/mol of protein. This is insufficient to account for a single phosphorylated residue within the DNA-binding domain, a result that is consistent with our previous conclusion. These data, in conjunction with our phosphoamino acid determination, indicate that there is one phosphoserine residue within the steroid-binding domain of the ~100-kDa subunit of the glucocorticoid receptor in WEHI-7 cells, none in the DNA-binding domain, and therefore, probably one or two in the immunogenic domain.

The hypothesis that glucocorticoid receptor action involves a phosphorylation–dephosphorylation cycle was based on the observation that receptor-binding capacity was reduced in rat thymocytes depleted of ATP by exposure to anaerobic conditions and could be rapidly restored, even in the absence of protein synthesis, by returing ATP levels to normal. According to this model, the dephosphorylated receptor was assumed to be unable to bind hormone (Munck & Brinck-Johnsen, 1968; Munck et al., 1972; Bell & Munck, 1973). Subsequent studies investigating the effects of phosphatases and phosphatase inhibitors on receptor action provided indirect evidence for a role of phosphorylation in the maintenance of steroid-binding capacity [reviewed by Housley et al. (1984)].

It has been demonstrated that the ~100-kDa steroid-binding subunit of glucocorticoid receptors isolated from mouse fibroblasts (Housley et al., 1985), WEHI-7 mouse thymoma cells (Mendel et al., 1986a), and rat liver (Grandics et al., 1984; Singh & Moudgil, 1985) is a phosphoprotein. However, there is still no direct evidence that a change in phosphorylation state is involved in receptor action. Activation of the WEHI-7 receptor causes no change in net phosphorylation of either the ~100-kDa subunit obtained from cytosol or from salt-extracted nuclei (Mendel et al., 1987; Tienrungroj et al., 1987), nor in the receptor-associated ~90-kDa heat shock protein (Orti et al., 1989). Given the fact that there are only two or

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[1] Abbreviations: DM, dexamethasone 21-mesylate (9α-fluoro-11β,17,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione 21-methanesulfonate); TA, triamcinolone acetonide (9α-fluoro-11β,16α,17α,21-tetrahydroxypregna-1,4-diene-3,20-dione 16,17-acetonide); HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DMEM, Dulbecco's modified Eagle's medium; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; TLCK, N^{α}-tosyl-L-lysine chloromethyl ketone; BIS, *N*,*N*-methylenebis(acrylamide); BAC, bis(acrylylcystamine); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TEMED, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine.

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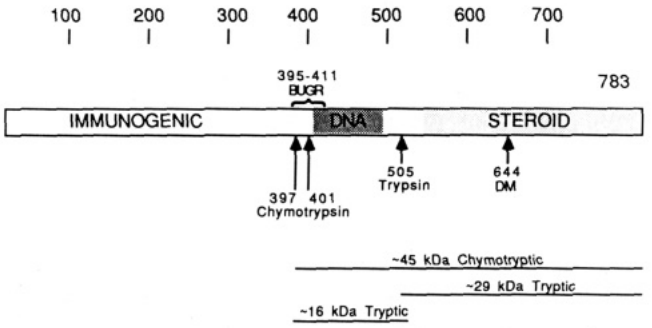

FIGURE 1: Location of selected proteolytic fragments of the ~100-kDa steroid-binding protein within the functional domains of the murine glucocorticoid receptor. Positions of the functional domains of the ~100-kDa protein are essentially as described by Giguere et al. (1986). The location of the BuGR epitope has been determined by Rusconi and Yamamoto (1987). The chymotryptic (Phe_{397}, Tyr_{401}) and tryptic (Lys_{505}) sites that give rise to the ~45- and ~29-kDa fragments, respectively, were determined by Carlstedt-Duke et al. (1987). The rat receptor was used to determine the sites listed above. Corresponding locations within the murine receptor were derived from the published murine receptor sequence (Danielson et al., 1986). The DM binding site was determined by Smith et al. (1988). The proposed placement of the ~16-kDa fragment is based on the data presented in this paper.

three phosphoryl groups on the steroid-binding protein, a change of even a single net phosphate would have been detected (Mendel et al., 1987).

Early proteolysis studies indicated that the receptor is composed of three major functional domains: the immunogenic, the DNA-binding, and the steroid-binding domains (Wrange & Gustafsson, 1978; Carlstedt-Duke et al., 1982). Subsequent cloning of the receptor cDNA (Hollenberg et al., 1985; Miesfeld et al., 1986; Danielson et al., 1986) confirmed these observations and more precisely defined the location and nature of each domain (see Figure 1). An important conclusion derived from a number of more recent studies is that under certain circumstances the different domains can function as autonomous units (Green & Chambon, 1987; Picard & Yamamoto, 1987; Miesfeld et al., 1987; Godowski et al., 1988). Identification of a phosphorylated site within one of these functional domains may prove to be an initial step in demonstrating a role of phosphorylation in receptor action.

To localize the phosphorylated sites, we have purified dual-labeled receptors using the BuGR2 monoclonal antibody, subjected them to partial proteolysis, and then analyzed selected fragments for phosphate content. Chymotrypsin digestion yields a ~42-kDa fragment encompassing the DNA-binding and steroid-binding domains. Within this fragment is a ~29-kDa tryptic fragment that contains solely the steroid-binding domain (Wrange & Gustafsson, 1978, Carlstedt-Duke et al., 1987). Using higher concentrations of trypsin, we obtain a ~16-kDa fragment that contains the BuGR epitopes. This fragment must contain all, or at least part, of the DNA-binding domain since it reportedly has DNA-binding ability and recognizes glucocorticoid responsive elements (Eisen et al., 1985; Sanchez et al., 1987; Dalman et al., 1988; Harrison, 1988) (see Figure 1).

A study of these proteolytic fragments generated from the intact ~100-kDa protein labeled either with [^{32}P]orthophosphate and [^{3}H]dexamethasone 21-mesylate or with [^{32}P]orthophosphate and [^{35}S]cysteine, coupled with our knowledge of the receptor sequence (Hollenberg et al., 1985; Danielson et al., 1986) and the steroid-binding site (Simons et al., 1987; Smith et al., 1988), has provided us with a measure of phosphate content for both the DNA- and steroid-binding domains. On the basis of our results we conclude that there is a single phosphoserine in the ~29-kDa steroid-binding tryptic fragment and none in the ~16-kDa DNA-binding tryptic fragment of the receptor. The other one or two phosphorylated residues must be located within the amino-terminal region of the receptor.

Materials and Methods

BuGR2 monoclonal antibody in hybridoma culture medium and BuGR1 antibody in ascites fluid directed against the glucocorticoid receptor (Gametchu & Harrison, 1984) were generously provided by Dr. Robert Harrison (University of Arkansas Medical Center). DM, [6,7-^{3}H]DM (38–50 Ci/mmol), Protosol, and Econofluor were purchased from New England Nuclear (Boston, MA). [^{32}P]Orthophosphate (carrier free) was obtained from ICN (Irvine, CA). ^{125}I sheep anti-mouse immunoglobulin F(ab')$_2$ fragment (16 μCi/μg) and L-[^{35}S]cysteine (600 Ci/mmol) were from Amersham (Arlington Heights, IL). Triamcinolone acetonide was purchased from Steraloids (Wilton, NH), tissue culture media were from KC Biologicals (Lenexa, KS), defined supplemented bovine calf serum was from Hyclone Sterile Systems (Logan, UT), dialyzed fetal bovine serum was from Hazelton (Lenexa, KS), and dialyzed bovine serum was from Gibco (Grand Island, NY). SDS–PAGE reagents and cellulose (Cellex 410) were products of Bio-Rad (Richmond, CA). HEPES, TES, Tris, protein A, Sepharose CL-4B, soybean trypsin inhibitor, TLCK, Ponceau S, iodoacetamide, SDS–PAGE standards, and reagents used for the phosphophosphoamino acid determinations were from Sigma (St. Louis, MO). TPCK-treated trypsin and chymotrypsin were obtained from Worthington Biochemicals (Freehold, NJ). Constant boiling 6 N HCl was obtained from Pierce (Rockford, IL). Calf thymus DNA was purchased from P-L Biochemicals (Milwaukee, WI), and X-Omat film was from Kodak (Rochester, NY).

Buffers. The following buffers and solutions were used: freeze–thaw (FT) buffer, 25 mM TES, pH 8.2 at 3 °C, 2 mM EGTA, 2 mM EDTA, 50 mM NaF, 20 mM sodium molybdate, and 10% (v/v) glycerol; TEG buffer, 10 mM Tris, pH 7.6 at 3 °C, 1 mM EDTA, 10% (v/v) glycerol, 20 mM sodium molybdate, and 50 mM NaCl; "mock" SDS–PAGE sample buffer, 62.5 mM Tris, pH 6.8 at 25 °C, 10% glycerol, and 20 mM sodium molybdate; SDS–PAGE sample buffer, 0.125 M Tris-base, pH 6.8, with 4% SDS, 20% glycerol, 20 mM molybdate, and 1.25 M β-mercaptoethanol; fixing solution, 50% methanol–10% acetic acid; staining solution, 0.12% Coomassie Blue in 50% methanol–10% acetic acid; destaining solution, 5% methanol–10% acetic acid; transfer buffer, 25 mM Tris, pH 8.0, containing 192 mM glycine; wash buffer, phosphate-buffered saline, pH 7.4, containing 1 mM EDTA and 0.1% Triton X-100.

Cell Culture and Whole Cell Labeling Techniques. WEHI-7 cells were maintained in RPMI 1640 culture medium containing 10% defined supplemented calf serum at 37 °C in a humidified incubator equilibrated with 5% CO_2 in air. Cells were harvested by centrifugation for 3 min at 400g. Cells to be labeled with [^{32}P]orthophosphate were resuspended in phosphate-free DMEM containing 10% dialyzed calf serum (Gibco), incubated at 37 °C for 1 h to remove endogenous phosphate, and then collected by another centrifugation. After resuspension in phosphate-free DMEM containing 10% dialyzed fetal bovine serum (Hazelton) and [^{32}P]orthophosphate (25 μCi/mL) at a final density of 10^6 cells/mL, the cells were incubated in the absence of steroid for 18 h. We have found these conditions sufficient to obtain steady-state labeling of the WEHI-7 cells (Mendel et al., 1987). Cells to be labeled with [^{35}S]cysteine were harvested by a 3-min centrifugation

at 400*g*, resuspended at a final density of 10^6 cells/mL in RPMI 1640 containing 10% defined supplemented bovine calf serum and [^{35}S]cysteine (20 μCi/mL), and incubated in the absence of steroid for 24 h. In experiments where cells were labeled with both [^{32}P]orthophosphate and [^{35}S]cysteine, the labeling was done separately, and the cells were combined at the end of the labeling period prior to the preparation of cytosol.

Preparation of Cytosol from WEHI-7 Cells. Cytosol was prepared essentially as described previously (Mendel et al., 1986a). Briefly, cells were harvested, resuspended in 3 volumes of FT buffer, immersed in liquid nitrogen for 10 min, and then thawed in a circulating water bath at 3 °C to lyse the cells. All subsequent procedures were carried out at 3 °C. The broken cell suspension was centrifuged at 11000*g* for 4 min to remove nuclei and large cellular debris and the supernatant centrifuged again at 100000*g* for 60 min to obtain the cytosol. This cytosol was then used in the procedures described below.

Purification of Activated Glucocorticoid Receptors and Formation of the ~42-kDa Chymotryptic and ~29-kDa Tryptic Receptor Fragments Labeled with ^{32}P and ^{3}H. Cytosol obtained from cells labeled only with [^{32}P]orthophosphate was incubated with a final concentration of 2 μM [^{3}H]dexamethasone 21-mesylate (diluted with DM to produce a final specific activity of 9.98 Ci/mmol) for 2 h at 3 °C to form nonactivated glucocorticoid receptor complexes. The complexes were heat activated by warming for 15–20 min at 25 °C. The activated cytosol was then cooled to 3 °C, and DTT was added to a final concentration of 2.5 mM. All subsequent procedures, unless otherwise noted, were carried out at 3 °C. Three equal aliquots of the activated cytosol were loaded onto 2-mL DNA–cellulose columns that had been preequilibrated with 10 volumes of TEG buffer containing 2 mg/mL BSA and 5 mM DTT. The columns were washed with 10 volumes of this same buffer. Activated complexes were eluted from the DNA column with 5 volumes of TEG buffer containing 2 mg/mL BSA and 400 mM NaCl and adsorbed directly onto 0.2-mL protein A–Sepharose–BuGR2 columns prepared as described below. Each column was washed successively with 3 mL of TEG buffer containing 400 mM NaCl, 3 mL of TEG buffer containing 400 mM NaCl and 0.2% Triton X-100, and 3 mL of FT buffer. The contents of each column was then transferred to a 2.0-mL Eppendorf tube, and 130 μL of FT buffer (control), 0.75 μg of chymotrypsin, or 0.5 μg of TPCK-treated trypsin was added. Digestions were carried out at 3 °C for 1 h with constant mixing. At the end of the digestion, 130 μL of SDS–PAGE sample buffer was added, and the tubes were heated in a boiling water bath for 5 min. The samples were then centrifuged at 400*g* for 10 min through a 1-cm^3 syringe fitted with a filter disk and the soluble fractions recovered and subjected to SDS–PAGE.

The protein A–Sepharose–BuGR2 columns used above were prepared by washing 0.2 mL of protein A–Sepharose in a 1-cm^3 tuberculin syringe with 3 mL of TEG buffer and 1 mL of TEG buffer containing 2 mg/mL BSA. After addition of 200 μL of the BuGR2 antibody, the column was washed again with 3 mL of TEG buffer to remove any unbound antibody.

Formation of the ~16-kDa Immunoreactive Fragment from the ~100-kDa Receptor and the ~45-kDa Receptor Fragment. Cytosol prepared as described above was incubated with 2 μM DM for 2 h at 3 °C and divided into six equal aliquots. The first aliquot was a control, with buffer added in place of enzyme. The second aliquot was digested with 200 μg/mL trypsin for 30 min at 3 °C, and the reaction was stopped by the addition of an equivalent volume of soybean trypsin inhibitor (1 mg/mL). The other four aliquots were digested with 20 μg/mL chymotrypsin for 60 min at 3 °C, and the reaction was stopped with the addition of TPCK (1 mg/mL). BuGR2 antibody (200 μL) was added to all six samples, which were incubated for 16–18 h at 3 °C. The immunoreactive species were adsorbed onto Protein A–Sepharose columns that had been washed with 3 mL of TEG buffer, 1 mL of TEG buffer containing 2 mg/mL BSA, and again with 3 mL of TEG buffer. In each experiment a sample of buffer containing antibody but no cytosol was also adsorbed onto a column. The columns with adsorbed immunoreactive species were washed successively with 5 volumes of TEG buffer, TEG buffer containing 0.2% Triton X-100, 5 volumes of TEG buffer containing 400 mM NaCl and 0.2% Triton X-100, 5 volumes of TEG buffer containing 400 mM NaCl, and 5 volumes of mock SDS–PAGE sample buffer.

The column contents containing three of the four chymotrypsin digests were then treated with 130 μL of 25, 50, or 100 μg/mL trypsin for 1 h at 3 °C. The column contents of the sample containing antibody but no cytosol was also treated with 130 μL of 50 μg/mL trypsin. SDS–PAGE sample buffer (130 μL) was added to each sample and each tube heated in boiling water for 5 min. The soluble fractions were recovered by centrifugation at 400*g* for 10 min. Following electrophoresis the proteins were transferred to nitrocellulose and analyzed as described below.

Formation and Purification of the ~16-kDa Tryptic Receptor Fragment from Cytosol Labeled with ^{32}P and ^{35}S. Cytosol prepared from cells labeled with [^{32}P]orthophosphate and [^{35}S]cysteine was usually not exposed to steroid. The cytosol was divided into two aliquots to which either buffer (control) or TPCK-treated trypsin was added to a final concentration of 200 μg/mL. After 30 min at 3 °C, 200 μL of a 1 mg/mL solution of soybean trypsin inhibitor was added to each sample. BuGR2 monoclonal antibody (200 μL) was then added and the solution incubated for 16–18 h at 3 °C. The immunoreactive species were then purified on 0.2-mL protein A–Sepharose columns prepared and subsequently washed as described in the previous section. After washing, the column contents were transferred to a 2.0-mL Eppendorf tube containing 175 μL of SDS–PAGE sample buffer and heated for 5 min in boiling water, and following centrifugation the soluble fraction was analyzed by SDS–PAGE.

In one experiment, the [^{32}P]orthophosphate- and [^{35}S]-cysteine-labeled cytosol was prepared with FT buffer without molybdate. After a 2-h incubation with 100 nM TA, the cytosol was warmed at 25 °C for 15–20 min and digested with 200 μg/mL TPCK-treated trypsin, and soybean trypsin inhibitor was added to inactivate the enzyme. The ~16-kDa fragment was bound to a DNA–cellulose column, eluted onto a protein A–Sepharose–BuGR2 column, and immunopurified as described in the section detailing the purification of the ~45-kDa chymotryptic and ~29-kDa tryptic fragments.

Polyacrylamide Gel Electrophoresis. Vertical slab gels (14 cm × 16 cm × 1.5 mm) of 7% or 10% polyacrylamide were prepared according to a modification of the procedure of Laemmli (1970) in which SDS is present not within the gel matrix but at 0.03% in the upper electrode buffer (Bodwell & Meyer, 1981). Gels were electrophoresed at 10–15 mA in a Bio-Rad Protean cell cooled to approximately 10 °C with running water. They were then fixed for approximately 1 h, stained for 1 h, and destained. Sample lanes were cut into 2-mm slices and the slices incubated in a solution of 6% Protosol in Econofluor at 45 °C for 14–18 h. The radioactivity in each slice was determined with a Packard 3390 liquid

Table I: Analysis of the Phosphate Content of the ~45-kDa Chymotryptic and ~29-kDa Tryptic Fragments Obtained from Purified Activated ^{32}P- and ^{3}H-Labeled ~100-kDa Steroid-Binding Protein

	expt 1	expt 2	expt 3	$X \pm SE$
intact				
^{32}P cpm	411	807	667	
^{3}H cpm	2579	5632	4649	
$^{32}P/^{3}H$	0.159	0.143	0.144	
chymotryptic				
^{32}P cpm	139	140	79	
^{3}H cpm	3182	3144	2588	
$^{32}P/^{3}H$	0.044	0.044	0.031	
% phosphate[a] in fragment	27.7	30.7	21.5	26.6 ± 2.7
no. of phosphates[b] in fragment	0.83	0.92	0.65	0.80 ± 0.08
tryptic				
^{32}P cpm	205	156	81	
^{3}H cpm	3721	3438	2363	
$^{32}P/^{3}H$	0.055	0.045	0.034	
% phosphate[a] in fragment	34.6	31.4	23.6	29.9 ± 3.3
no. of phosphates[b] in fragment	1.04	0.94	0.71	0.90 ± 0.10

[a] The percent phosphate in the fragment is calculated relative to the phosphate present in the intact ~100-kDa steroid-binding protein. That is, % phosphate in fragment = [($^{32}P/^{3}H$ fragment)/($^{32}P/^{3}H$ intact receptor)] × 100. [b] The number of phosphates in the fragment was calculated on the assumption that there are three phosphates in the intact ~100-kDa protein from the formula 3(% phosphate)/100.

Table II: Analysis of the Phosphate Content of the Immunoreactive ~16-kDa Tryptic Fragment Obtained from ^{32}P- and ^{35}S-Labeled ~100-kDa Steroid-Binding Protein[a]

	expt 1	expt 2	expt 3	expt 4	$X \pm SE$
intact					
^{32}P cpm	3310	2165	2897	535	
^{35}S cpm	45	230	219	423	
$^{32}P/^{35}S$	73.5	9.41	13.2	1.27	
fragment					
^{32}P cpm	276	126	225	17	
^{35}S cpm	43	132	176	167	
$^{32}P/^{35}S$	0.16	1.05	0.78	0.04	
% phosphate[a] in fragment	4.6	5.3	5.1	3.4	4.6 ± 0.4
no. of phosphates[b] in fragment	0.14	0.16	0.15	0.10	0.14 ± 0.01

[a] To obtain a relative measure of the phosphate content in the ~16-kDa fragment relative to the intact ~100-kDa steroid-binding protein, this equation was used: % ^{32}P in fragment relative to the intact protein = {[$^{32}P/^{35}S$ (fragment)]/[$^{32}P/^{35}S$ (intact)]} × (10/19) × 100, where 10 and 19 are the number of cysteine residues in the ~16-kDa fragment and the intact ~100-kDa protein, respectively. [b] The number of phosphates in the fragment was calculated on the assumption that there are three phosphates in the intact ~100-kDa protein from the formula 3(% phosphate in fragment)/100.

scintillation counter. The dual-labeled samples were counted at the settings described previously by Mendel et al. (1987). The amount of each isotope present in a specific protein peak was then determined. After background subtraction for each protein peak, the ratio of ^{32}P cpm to ^{3}H cpm was calculated (Table I legend). This value provides a relative measure of phosphate content normalized to the number of DM binding sites. To obtain a relative measure of phosphate content for the ~16-kDa receptor fragment (which does not contain the DM-binding site), the ^{32}P cpm was normalized to the ^{35}S cpm as described in the legend to Table II.

Molecular weights were estimated according to the method of Weber and Osborn (1968) using the mixture of proteins in the Sigma molecular weight marker kits.

Western Blot Analysis and Immunooverlay. The procedures described in this section have been previously reported in detail (Mendel & Orti, 1988). Briefly, proteins were first separated by SDS–PAGE and then transferred to nitrocellulose paper over an 18-h period at 100 mA in a Bio-Rad Trans Blot apparatus in transfer buffer. Following transfer, the molecular weight standards were visualized with Ponceau S. The nitrocellulose was incubated with a blocking solution (Johnson et al., 1984) to reduce nonspecific protein binding. The nitrocellulose was then incubated with BuGR1 from ascites fluid diluted 1:500 in the above blocking solution, washed several times in wash buffer, and incubated with (1–1.5) × 10^5 cpm/mL ^{125}I sheep anti-mouse immunoglobulin (Fab'_2 fragment) in blocking solution for 3 h at room temperature. After washing away free ^{125}I-labeled antibody, immunoreactive proteins were identified by autoradiography for approximately 18 h at −80 °C by using X-Omat film in the presence of an intensifying screen.

Phosphoamino Acid Analysis. The BuGR2 antibody was used to purify nonactivated receptors from cytosol of WEHI-7 cells that had been grown in the presence of [^{32}P]orthophosphate and then labeled with [^{3}H]dexamethasone 21-mesylate. The purified receptors were then electrophoresed on 7% polyacrylamide gels containing a mixture of BIS and BAC cross-linkers (1:1 ratio) at a final concentration of 1%. This gel system allows complete separation of the ~100- and ~90-kDa components of the nonactivated complex so that each protein can be eluted separately with high recovery (Smith et al., 1988).

To determine which amino acid residues in the receptor are phosphorylated, the method of Copper et al. (1983) was used. The purified ~100- and ~90-kDa subunits obtained from the gel slices were lyophilized, and 50 μg of bovine serum albumin was added as carrier. The protein was precipitated with a final concentration of 10% TCA, resuspended in constant boiling 6 N HCl in a Pierce Reactivial, and hydrolyzed at 110 °C for 2 h in a heating block. After lyophilization the hydrolysate was resuspended in a solution of pyridine–acetic acid–distilled water (1:10:189 by volume) containing phosphoserine, phosphothreonine, and phosphotyrosine markers. The amino acids were then separated by one-dimensional electrophoresis at pH 3.5 on 100-μm cellulose TLC plates for 36 min at 1000 V. A control sample obtained from gel slices that contained no ~100- or ~90-kDa protein was also analyzed to establish a background pattern. The TLC plates were stained with 0.2% ninhydrin in acetone to determine the migration of the unlabeled standards. The phosphoamino acids were detected by autoradiography after exposure of the TLC plates to Kodak X-Omat film for 48 h at −80 °C in the presence of an intensifying screen.

Results and Discussion

Phosphoamino Acid Analysis of the Nonactivated Receptor Subunits. Although it has been demonstrated that the glucocorticoid receptor from mouse fibroblast L-cells (Housley & Pratt, 1983) and mouse AtT-20 pituitary tumor cells (Kovacic-Millivojevic & Vedeckis, 1986) is phosphorylated on serine residues, there have been reports that tyrosine residues in the human breast epithelial and rat liver receptor are also phosphorylated (Rao & Fox, 1987; Auricchio et al., 1987). These earlier phosphoamino acid determinations were carried out on preparations in which the ~100-kDa steroid-binding and ~90-kDa non-steroid-binding proteins were not separated. We therefore performed phosphoamino acid analyses on nonactivated complexes purified from WEHI-7 cells grown in the presence of [^{32}P]orthophosphate. The ~100- and ~90-kDa proteins were completely separated by SDS–poly-

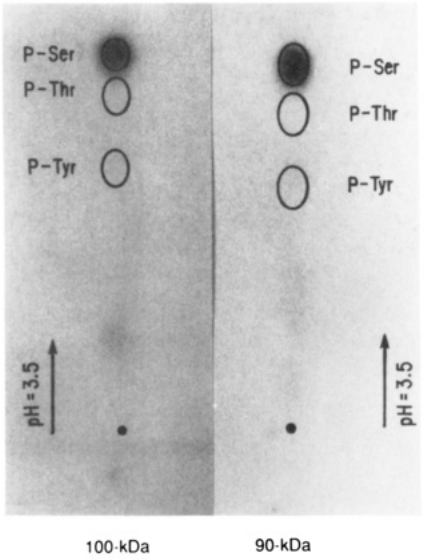

FIGURE 2: Phosphoamino acid analysis of the ~100-kDa and ~90-kDa subunits of nonactivated glucocorticoid receptors. Cytosol from WEHI-7 cells grown in the presence of [^{32}P]orthophosphate was labeled with 2 μM [^{3}H]dexamethasone 21-mesylate at 3 °C for 2 h. The nonactivated complexes were purified by using the BuGR2 monoclonal antibody and subjected to SDS–PAGE on a wide-pore gel, and the 100-kDa steroid-binding and 90-kDa non-steroid-binding subunits were eluted separately from the gel as described under Materials and Methods. Peak samples were pooled, TCA-precipitated, and subjected to acid hydrolysis at 110 °C for 2 h. Following lyophilization, the samples were resuspended in a solution of pyridine–acetic acid–distilled water (1:10:189) at pH = 3.5 and electrophoresed on cellulose TLC plates at 1000 V for 36 min. The phosphoamino acids were detected by autoradiography after 48 h. The circles indicate the migration positions of the phosphoamino acid standards visualized by ninhydrin staining (P-Ser, phosphoserine; P-Thr, phosphothreonine; P-Tyr, phosphotyrosine).

acrylamide electrophoresis and eluted independently from the gel as previously described (Smith et al., 1988). The phosphoamino acid analyses were carried out under conditions that should minimize the loss of phosphotyrosine residues. For example, our cell lysis buffer contains molybdate, a potent inhibitor of tyrosine phosphatases (Tonks et al., 1988), and sodium fluoride and EDTA, which inhibit non-tyrosine phosphatases (Antoniw & Cohen, 1976; Garrison, 1983). In addition, acid hydrolysis of the proteins was performed under conditions that would be expected to result in a high recovery of phosphotyrosine (Cooper et al., 1983).

After analysis the only phosphoamino acid detected in either the ~100-kDa steroid-binding protein or the receptor-associated ~90-kDa protein was phosphoserine (Figure 2). The absence of phosphotyrosine in both these proteins is also indicated by independent experiments in which we attempted to immunoprecipitate either nonactivated or activated receptors with an anti-phosphotyrosine antibody. Although the antibody we used recognizes insulin receptors that contain phosphotyrosine (Gould et al., 1989), it did not react with either form of the glucocorticoid receptor.

Our results with WEHI-7 cells agree with earlier studies in which the two subunits were not resolved (Housley & Pratt, 1983; Kovacic-Millivojevic & Vedeckis 1986) and with a more recent determination on the purified ~100-kDa steroid-binding subunit from mouse L-cells (Dalman et al., 1988). In addition, our analysis indicates that the receptor-associated ~90-kDa heat shock protein is phosphorylated only on serine residues. The non-receptor-associated HSP-90 has previously been shown to be phosphorylated on serine residues in other systems (Oppermann et al., 1981; Schlesinger et al., 1982).

Analysis of Phosphorylated Sites in the Fragments Containing the Steroid-Binding Domain. We previously determined that the ~100-kDa steroid-binding subunit of the glucocorticoid receptor contains two to three phosphoryl groups per steroid-binding site (Mendel et al., 1987). Since there is a single steroid-binding site per ~100-kDa protein as determined by the analysis of receptors covalently labeled with different synthetic steroids (Simons et al., 1987; Smith et al., 1988; Carlstedt-Duke et al., 1988), there are two to three phosphoserines in the steroid-binding protein. Our interest was in determining the location of these phosphoserines within the functional domains of the ~100-kDa protein.

The DNA- and steroid-binding domains of the ~100-kDa subunit can be analyzed by performing partial proteolysis on purified activated receptors. Chymotryptic cleavage of the steroid-binding subunit of the receptor yields a ~45-kDa fragment that includes the DNA- and steroid-binding domains (Wrange & Gustaffson, 1978, Wrange et al., 1984). According to the sequence analysis performed by Carlstedt-Duke et al. (1987) this chymotryptic fragment, which is heterogeneous at its amino-terminal end, begins at Ser_{410} and Ser_{414} of the rat liver glucocorticoid receptor (due to cleavage at Phe_{409} and Tyr_{413}) and spans to the carboxy terminus of the protein. The corresponding fragments in the mouse receptor begin at Ser_{398} and Ser_{402} [derived from Danielson et al. (1986)]. This fragment therefore contains all of the DNA-binding domain as defined by mutational analysis (Giguere et al., 1986) and by functional analysis of receptor fragments translated in vitro from cloned receptor sequences (Rusconi & Yamamoto, 1987). Trypsin cleavage yields a smaller ~29-kDa fragment containing the steroid-binding domain (Wrange & Gustaffson, 1978, Wrange et al., 1984), which was localized to the carboxy-terminal portion of the protein by mutagenesis experiments (Giguere et al., 1986) and by direct identification of amino acid residues to which bound glucocorticoids can be linked covalently (Simons et al., 1987; Smith et al., 1988; Carlstedt-Duke et al., 1988). This tryptic fragment begins at Gly_{518} in the rat liver receptor (Carlstedt et al., 1987), which corresponds to Gly_{506} in the mouse receptor [derived from Danielson et al. (1986)]. If this fragment extends completely to the carboxy terminus of the protein (i.e., amino acids 506–783), then it would be expected to have a molecular weight of approximately 31 000–32 000, which is close to the size of the phosphorylated fragment (~29 kDa) we obtain on SDS–PAGE (see Figure 1).

To obtain a relative measure of phosphate content of these receptor fragments, cytosolic receptors from cells grown in the absence of steroid in [^{32}P]orthophosphate were then labeled with [^{3}H]DM, activated, and bound to DNA–cellulose. Activation, either in whole cells or under cell-free conditions, has been shown not to affect net phosphorylation of the ~100-kDa protein (Mendel et al., 1987; Tienrungroj et al., 1987; Orti et al., 1989) and was included in the procedure to remove the ~90-kDa phosphoprotein component of the nonactivated complex. The ~100-kDa steroid-binding protein eluted from the DNA–cellulose column was purified with the BuGR2 monoclonal antibody and subjected to partial proteolysis with chymotrypsin and trypsin. As illustrated in Figure 3, the intact ~100-kDa protein (panel A), the ~45-kDa chymotryptic fragment (panel B), and the ~29-kDa tryptic fragment (panel C) all contain both [^{32}P]phosphate and [^{3}H]DM. The ^{32}P/^{3}H ratio for the two proteolytic fragments can be compared to that of the intact ~100-kDa protein to determine the extent of phosphorylation, relative to steroid-binding capacity, within these fragments. In the three different experiments we have

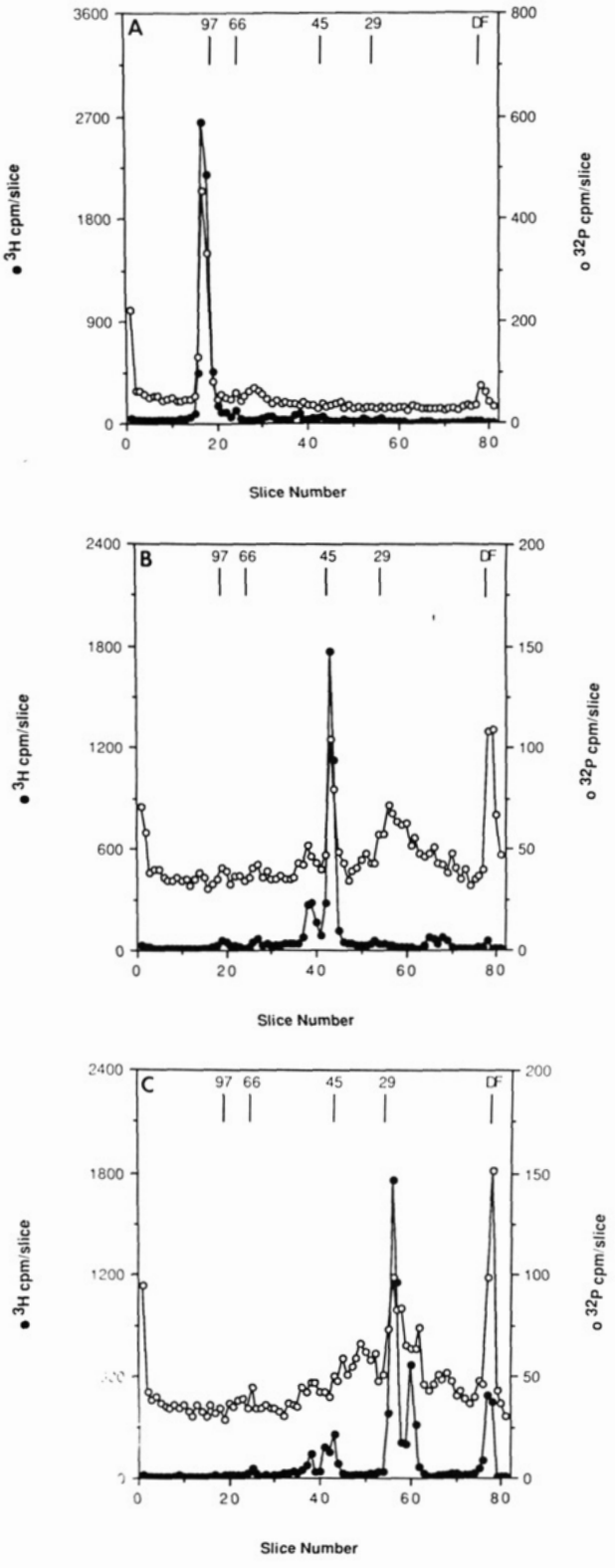

FIGURE 3: Digestion of the purified ^{32}P- and ^{3}H-labeled ~100-kDa protein from activated receptors by chymotrypsin and trypsin. Cytosol from WEHI-7 cells grown in the presence of [^{32}P]orthophosphate was incubated with 2 μM [^{3}H]dexamethasone 21-mesylate at 3 °C for 2 h. The cytosol was heat-activated at 25 °C for 15–20 min and bound to a DNA–cellulose column. After extensive washing, the receptor complexes were eluted with 400 mM NaCl onto a BuGR2–protein A–Sepharose column. After washing, the column contents were incubated with buffer (A), 0.75 μg/mL chymotrypsin (B), or 0.5 μg/mL TPCK-treated trypsin (C) at 3 °C for 1 h. The samples were then boiled in SDS–PAGE sample buffer and electrophoresed on 10% polyacrylamide gels. The sample lanes were then cut into 2-mm slices, and the amount of ^{32}P and ^{3}H in each slice was determined. The closed circles represent the ^{3}H cpm and the open circles the ^{32}P cpm.

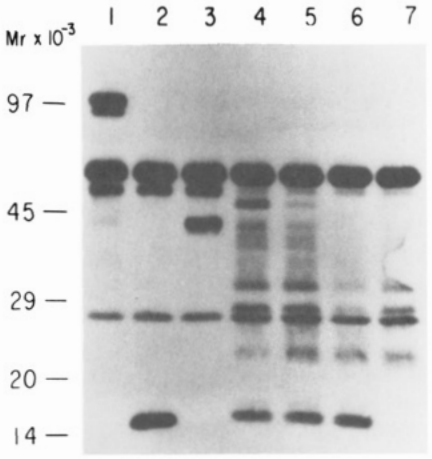

FIGURE 4: Western blot of intact and chymotrypsin- and trypsin-digested nonactivated glucocorticoid receptors. Cytosol from WEHI-7 cells was treated with buffer as a control (lane 1), 200 μg/mL trypsin for 30 min at 3 °C (lane 2), or 20 μg/mL chymotrypsin for 60 min at 3 °C (lanes 3–6). The intact ~100-kDa protein, the ~16-kDa receptor fragments, and the ~45-kDa receptor fragments were purified with the BuGR2 monoclonal antibody. The ~45-kDa fragments were then further digested with 25 (lane 4), 50 (lane 5), or 100 μg/mL (lane 6) trypsin for 60 min at 3 °C. In lane 7, a buffer sample containing antibody only was treated the same as the sample in lane 5. Following electrophoresis on 10% SDS–PAGE gel, the proteins were transferred to nitrocellulose. The immunoreactive species were detected by incubation with the BuGR2 antibody, followed by a ^{125}I-labeled sheep anti-mouse (Fab'_2 fragment) second antibody. The immunoblot was then exposed to X-ray film for 18 h at −80 °C prior to development.

done (Table I), the chymotryptic fragments have 21–31% of the phosphate content of the intact protein purified from the same cytosol. In each experiment the corresponding tryptic fragments, although not as clean as the chymotryptic fragments, contain an equivalent amount of phosphate. Because there are two to three phosphoserines in the intact ~100-kDa steroid-binding protein, it follows that, to the nearest integer, there is one phosphoserine in either the chymotryptic or tryptic fragments. Since the tryptic fragment, which encompasses the steroid-binding domain, is contained in the chymotryptic fragment (Figure 1), we conclude that this phosphoserine is within the steroid-binding, not the DNA-binding, domain. The absence of phosphoserine in the DNA-binding domain is established directly in the next experiments.

Formation and Purification of a ~16-kDa Tryptic Fragment from the Intact ~100-kDa Protein and the ~45-kDa Chymotryptic Fragment. Several lines of evidence indicate that the ~16-kDa fragment contains some, if not all, of the DNA-binding domain. First, as we also demonstrate, this fragment has been shown to bind to DNA–cellulose (Eisen et al., 1985; Sanchez et al., 1987; Dalman et al., 1988). Harrison et al. (1988) have further demonstrated that this fragment binds with high affinity to glucocorticoid response elements. Finally, this fragment contains the BuGR epitope, which is within amino acids 395–411 of the mouse receptor [derived from Rusconi and Yamamoto (1987)]. These amino acids are near the border of the immunogenic and DNA-binding domains as determined by Carlstedt-Duke et al. (1987).

The exact tryptic cleavage sites that give rise to this ~16-kDa fragment are, to our knowledge, still unknown. To define more precisely the location of this fragment within the steroid-binding protein, we digested the ~100-kDa protein under various conditions and, after electrophoresis and transfer to nitrocellulose, probed with the BuGR antibody (Figure 4). In lane 1 the intact protein is present slightly above the ~97-kDa

phosphorylase *b* standard. Antibody purification after digestion of cytosol with trypsin (lane 2) or chymotrypsin (lane 3) yields ~16- and ~45-kDa immunoreactive fragments, respectively. The purified ~45-kDa chymotryptic fragment was then further digested with increasing concentrations of trypsin (lanes 4–6) to yield an immunoreactive fragment very similar to the ~16-kDa fragment generated with trypsin alone. The last lane indicates the fragments that are formed by digestion of the BuGR antibody itself.

These results indicate that a ~16-kDa tryptic fragment is contained within the amino-terminal portion of the ~45-kDa chymotryptic fragment (Figure 1). Since the ~16-kDa fragment is also BuGR reactive, it must have the same amino terminus as the larger ~45-kDa fragment, which contains the BuGR epitope at its amino terminus (Carlstedt-Duke et al., 1987). The other 29 000 Da not visible by Western blotting must comprise the steroid-binding carboxy terminus of the chymotryptic fragment. Although this experiment may not unequivocally localize the ~16-kDa fragment within the receptor sequence, we think that the cleavage site that gives rise to the ~16-kDa fragment upon trypsin digestion of the intact ~100-kDa protein in cytosol is most likely Arg_{394} [as suggested by Dalman et al. (1988)] or Arg_{374}. These are the only trypsin-sensitive sites in the receptor that are close to the residue(s) that give rise to the chymotryptic fragment. At the very least, the two ~16-kDa fragments (generated from the intact receptor and the chymotryptic fragment) must overlap to a significant extent since they both contain the BuGR epitope and the entire DNA-binding domain. The carboxy-terminal border of the ~16-kDa fragment has not been determined, but on the basis of apparent molecular weights it seems likely that this fragment does not overlap with the amino-terminal region of the steroid-binding domain.

Analysis of the Phosphate Content of the ~16-kDa Receptor Fragment Containing the DNA-Binding Domain. To obtain an independent measure of the phosphorylation state of the DNA-binding domain, we analyzed the ~16-kDa receptor fragment generated from cytosol, which contains this domain. On the basis of the reported sequence of the mouse receptor (Danielson et al., 1987) and localization of the DNA-binding domain (Giguere et al., 1986; Rusconi & Yamamoto, 1987), it is evident that 10 of the 19 cysteine residues in the ~100-kDa protein are in the DNA-binding domain (Severne et al., 1988). Nine of these ten cysteines are conserved and are presumably involved in the formation of zinc fingers (Weinberger et al., 1985; Severne et al., 1988; Freedman et al., 1988). Knowing the cysteine content of DNA-binding domain, which is contained within the ~16-kDa fragment, allows us to quantitate the amount of phosphoserine in this fragment relative to the phosphoserine in the intact protein.

The intact ~100-kDa protein and the ~16-kDa fragment were purified from cytosol labeled with [^{32}P]orthophosphate and [^{35}S]cysteine in the absence of steroid and then analyzed by SDS-PAGE. The ~16-kDa tryptic fragment clearly contains [^{35}S]cysteine (Figure 5B), but unlike the intact ~100-kDa protein, which contains both ^{35}S and ^{32}P (Figure 5A), it contains very little ^{32}P. The ^{32}P/^{35}S ratio of the ~16-kDa fragment and the intact ~100-kDa protein was determined from the peak protein slices in the gel. The ratio for the fragment was then compared to that of the intact protein and a correction made for the number of cysteines in the fragment as explained in the legend of Table II. In Table II, experiments 1–3 were done with nonactivated preparations. In experiment 4, the cytosol was treated with steroid and

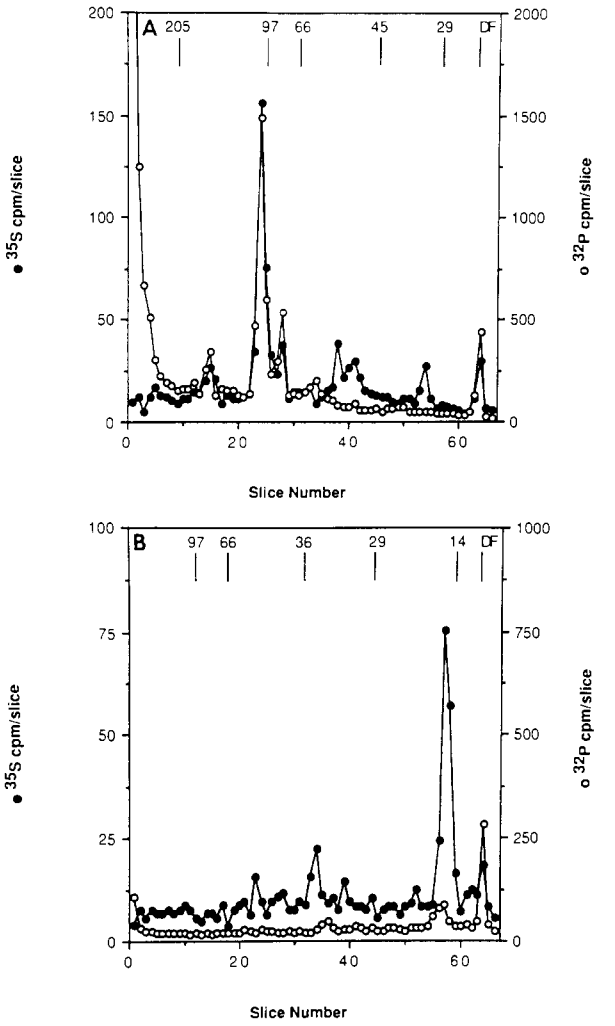

FIGURE 5: Trypsin digestion of the ^{32}P- and ^{35}S-labeled ~100-kDa protein from cytosolic nonactivated glucocorticoid receptors. Cytosol from WEHI-7 cells grown in medium containing [^{32}P]orthophosphate and [^{35}S]cysteine was incubated with buffer (A) or 200 μg/mL TPCK-treated trypsin (B) at 3 °C for 30 min. An amount of soybean trypsin inhibitor equivalent to five times the concentration of trypsin (w/w) was added to both samples. After an overnight incubation with the BuGR2 antibody, the receptor–antibody complexes were purified on protein A–Sepharose. After extensive washing, the samples were boiled in SDS sample buffer and electrophoresed on 7% (A) or 10% (B) polyacrylamide gels. The sample lanes were cut into 2-mm slices, and the amount of ^{32}P and ^{35}S in each slice was determined. The closed circles represent the ^{35}S cpm and the open circles the ^{32}P cpm.

heat-activated, and both the intact receptor and ~16-kDa fragment bound to and eluted from DNA–cellulose prior to immunopurification. Although the absolute ratios of ^{32}P/^{35}S for the fragment and the intact ~100-kDa protein vary with each experiment, comparisons are made within a single experiment, and therefore the relative ratio of the fragment to the receptor reflects the phosphoserine content. The variability of the ratios is due to different amounts of incorporation of each isotope during the metabolic labeling. Our results indicate that although there is some ^{32}P in the ~16-kDa region of the gel, there is only enough to account for about 0.15 phosphoserine, assuming there are three phosphoserines in the intact steroid-binding protein. This observation that the DNA-binding domain does not contain a phosphorylated site is consistent with the results described above.

In all four experiments we consistently observed that the ^{32}P peak does not coincide completely with the ^{35}S (protein) peak (Figure 5B) but seems to be associated with only the first

half of the peak. Thus, there may be two different species present, which suggests that the ^{32}P present is not within the ~16-kDa receptor fragment but is due to a contaminant.

General Discussion

Our conclusion that the ~100-kDa steroid-binding subunit of the glucocorticoid receptor from WEHI-7 mouse thymoma cells has one phosphoserine within the steroid-binding domain differs from that of Dalman et al. (1988), who have reported that they find no phosphorylated residues in the steroid-binding domain contained within a ~27-kDa tryptic fragment from murine fibroblast L-cells. This discrepancy could be due to differences between the cells, as it has not been shown that glucocorticoid receptors in all cells are phosphorylated at the same locations, or even to the same extent. Alternatively, as Dalman et al. (1988) point out, the smaller apparent molecular weight of the tryptic fragment they generate suggests that some amino acids are missing from the carboxy terminus of the L-cell glucocorticoid receptor. In the murine glucocorticoid receptor sequence there are lysine residues at positions 749 and 769 that could be susceptible to tryptic cleavage, and these are followed by potential serine phosphorylation sites. From this we would expect either Ser_{750}, Ser_{752}, or Ser_{771} to be the phosphorylated residue in the mouse glucocorticoid receptor, since these are the only serines present after residue 749. Such a result would be consistent with the recent demonstration that the chick progesterone receptor (787 amino acids in length) is phosphorylated on Ser_{767} (L. Denner, N. Weigel, W. Schrader, and B. O'Malley, personal communication).

Our conclusion, derived from two different experimental approaches, that the DNA-binding domain is not phosphorylated also differs from that of Dalman et al. (1988), who report that the ~16-kDa DNA-binding fragment from L-cells is phosphorylated. In our studies, though we find some ^{32}P in the ~16-kDa fragment, there is much less (<0.2) than can account for a single phosphoserine residue. Furthermore, as noted above, the consistent lack of coincidence of the ^{32}P peak with the ^{35}S peak obtained by slicing the gels suggests that the ^{32}P is due to a contaminant. Since Dalman et al. (1988) quantitated the amount of the ~16-kDa tryptic fragment by a counter-antibody technique on an autoradiograph following transfer to nitrocellulose, it is possible that they might not have observed a discrepancy between the migration of the immunoreactive and the phosphorylated fragment because of limited resolution. Alternatively, the tryptic cleavage sites that yield the ~16-kDa fragment may be different from those in our system, thus producing a slightly different fragment containing more phosphorylated sites.

The assignment of the phosphorylated sites to the domains of the receptor presented in this paper was, by necessity, based on the assumption that the receptor is phosphorylated at discrete sites. To date, this has not been shown directly by sequence analysis of phosphopeptides. The immunogenic domain, located at the amino terminus of the protein, has not been considered in this study because at present we have no means of selectively purifying this region. However, it is reasonable to suggest that the remaining one or two phosphoserines are in this region, before amino acid 397, which is a proposed start of the ~45-kDa chymotryptic fragment in the mouse receptor. We have observed that digestion of purified receptors with chymotrypsin yields a heterogeneous peak of ^{32}P-labeled fragments that are not labeled with ^{3}H (slices 52–62 in Figure 2B), which probably represents at least a portion of the immunogenic domain.

Although it has been known for quite some time that the glucocorticoid, as well as progesterone and estrogen, receptors are phosphorylated, the functional significance of receptor phosphorylation has not been determined. Recently, a non-hormone-binding form of the glucocorticoid receptor has been identified in the nuclei of ATP-depleted WEHI-7 cells (Mendel et al., 1986b) that appears to have lost one phosphoryl group (Mendel et al., 1989). Identification of the labile phosphoryl group within this receptor form using the methods described in this paper may provide the first direct correlation between receptor phosphorylation and steroid-binding capacity.

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