

LOCALIZATION OF THE ~12 kDa M_r DISCREPANCY IN GEL MIGRATION OF THE MOUSE GLUCOCORTICOID RECEPTOR TO THE MAJOR PHOSPHORYLATED CYANOGEN BROMIDE FRAGMENT IN THE TRANSACTIVATING DOMAIN

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Summary—The intact wild-type mouse glucocorticoid receptor has a theoretical molecular weight of ~86 kDa based on amino acid sequence, but on SDS–polyacrylamide gel electrophoresis it migrates as a protein of ~98 kDa. It is not known where the unusual primary structure or covalent modification responsible for this anomalous migration is located within the amino acid chain. In the course of examining the pattern of fragmentation of ³²P-labeled glucocorticoid receptors from Chinese hamster ovary (CHO) cells containing amplified mouse receptor cDNA, we have found a localized region in the amino-terminal half of the receptor that accounts for this anomalous behavior. Cyanogen bromide treatment of the intact receptor produces a 23.4 kDa (theoretical) fragment consisting of residues 108–324 and containing all of the identified phosphorylated serines within the receptor. We find that the only large resolvable ³²P-labeled receptor fragment produced after complete cyanogen bromide cleavage of intact receptors migrates with an apparent molecular weight of ~35 kDa. Because the apparent difference between the theoretical and the experimentally observed molecular weights of this cyanogen bromide fragment is essentially the same as the difference between the theoretical and experimental molecular weights of the intact mouse glucocorticoid receptor, we propose that some feature lying within this fragment accounts for slower migration. Although the existence of an additional phosphorylation site lying within the 15 kDa tryptic receptor fragment containing the DNA-binding domain has been contested, we also demonstrate that this fragment of the mouse glucocorticoid receptor is phosphorylated *in vivo* upon incubation of CHO cells in growth medium containing [³²P]orthophosphate.

INTRODUCTION

When cDNAs for glucocorticoid receptors (GR) were cloned, it became apparent that significant discrepancies existed between their molecular weights predicted from sequence and their apparent M_r on SDS–polyacrylamide gel electrophoresis (SDS–PAGE). For example, the human and rat GR cDNAs encode proteins of 777 and 795 amino acids, respectively, yet the M_r for both of these ~86–87,000 Da proteins is ~94 kDa on SDS–PAGE [1, 2]. The 783 amino acid mouse GR [3] migrates with an M_r of ~98 kDa on SDS–PAGE [4], and the basis for this ~12 kDa discrepancy is unknown.

Such deviations from theoretical molecular weight have been explained for other proteins

by the attachment of carbohydrate moieties, or by the acylation or phosphorylation of amino acids, or by the presence of an highly unusual primary amino acid sequence within the polypeptide chain. Because the only known covalent modification of the mouse glucocorticoid receptor [4–12], and indeed of all steroid receptors (for review, see Ref. [13]), is phosphorylation, one could presume that this might account for the M_r discrepancy. However, we have previously demonstrated that digestion of the mouse GR with calf intestine alkaline phosphatase removes all but a trace amount of the receptor phosphate without altering the apparent receptor M_r on SDS–PAGE (cf. Fig. 9 in Ref. [10]). Thus, the M_r discrepancy cannot be due to receptor phosphorylation. However, during the course of such a study of receptor phosphorylation, we have found that the

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~12 kDa M_r discrepancy is determined by a region in the transactivation domain of the mouse GR.

In this work we have labeled mouse GR overexpressed in Chinese hamster ovary (CHO) cells by incubating cells with [^{32}P]orthophosphate and have examined the pattern of fragmentation produced after exhaustive cyanogen bromide cleavage. The predicted molecular weight for the largest receptor fragment produced by cyanogen bromide cleavage (containing much of the transactivating domain) is 23.4 kDa, and it contains all of the phosphorylation sites thus far identified in the mouse receptor, representing 81% of the total ^{32}P recovered by Bodwell *et al.* [12]. Cyanogen bromide cleavage of ^{32}P -labeled mouse GR reveals a single resolvable ^{32}P -labeled receptor fragment migrating at ~35 kDa. As the apparent difference between the theoretical and experimentally observed molecular weights of the cyanogen bromide fragment is approximately the same as the difference between the theoretical and observed molecular weights of the intact mouse GR, we propose that this region of the receptor determines the anomalous migration of the intact receptor on SDS-PAGE.

EXPERIMENTAL

Chemicals

[^{32}P]Orthophosphate (carrier free) was from Amersham Corp, and cyanogen bromide was from Pierce. Methotrexate, proline, TPCK-treated trypsin and TLCK-treated chymotrypsin, TPCK, soybean trypsin inhibitor, protein A-Sepharose 4B, molecular weight markers, affinity-purified horseradish peroxidase conjugate of goat anti-mouse IgG, and phosphate-free and phosphate-containing Dulbecco's modified Eagle medium (high glucose) were from Sigma Chemical Co. BuGR2 anti-receptor monoclonal antibody was from Affinity Bioreagents; nitrocellulose membranes were from Bio-Rad; Immobilon-P membranes were from Millipore; calf serum was from HyClone; and dialysed calf serum was from Gibco.

Cell culture, ^{32}P -labeling conditions and cytosol preparation

The WCL2 line of CHO cells overexpressing the mouse GR was established by Hirst *et al.* [14] and the receptor expressed in this cell line has been described previously [15]. The cells

were grown in monolayer culture using Dulbecco's modified Eagle medium plus 3 μM methotrexate, 30 $\mu\text{g}/\text{ml}$ proline and 10% iron-supplemented calf serum at 37°C. For ^{32}P -labeling experiments, cells in log phase of growth were washed with phosphate-free medium containing dialysed calf serum and then incubated in this medium containing 1 mCi [^{32}P]orthophosphate per flask for 15–18 h. All subsequent steps were done at 0–4°C. Cells were scraped into Earle's balanced saline, centrifuged, and washed again before suspension and dounce homogenization in 1.5 vol of Hepes buffer (10 mM Hepes, 1 mM EDTA, 10 mM NaF, pH 7.35 at 4°C). The homogenate was centrifuged at 100,000 g for 1 h, and the clear supernatant (minus the floating lipid layer) was removed and is referred to as "cytosol".

Proteolysis and chemical cleavage

Receptors were digested with proteases before adsorption to protein-A Sepharose with BuGR2. TPCK-treated trypsin or TLCK-treated chymotrypsin solutions were freshly prepared in Hepes buffer. Proteins in cytosol were digested with 100 $\mu\text{g}/\text{ml}$ trypsin or 1 $\mu\text{g}/\text{ml}$ chymotrypsin for 1 h at 0°C. To terminate the proteolysis, 1 mg/ml soybean trypsin inhibitor in Hepes buffer (trypsin digestions) or 100 $\mu\text{g}/\text{ml}$ TPCK in methanol (chymotrypsin digestions) were added to the mixtures.

For cleavage with cyanogen bromide, receptors were first immunoadsorbed to protein A-Sepharose, washed (6 \times 1 ml) with TEGNT buffer (10 mM TES, 4 mM EDTA, 550 mM NaCl, 10% w/v glycerol, 0.4% Triton X-100, pH 7.6 at 4°C) to remove nonspecifically adsorbed proteins. The immunoadsorbed proteins were then resolved by SDS-PAGE, electrophoretically transferred to nitrocellulose membranes, and the nitrocellulose strip corresponding to the intact ^{32}P -labeled receptor was localized by autoradiography of the dried nitrocellulose membrane. The strip was then excized with a razor blade and put into a microfuge tube with 100 μg of bovine serum albumin (BSA) and 800 μl of 100 mg/ml solution of cyanogen bromide in 70% formic acid under nitrogen gas and mixed overnight at room temperature. The resultant supernatant was removed, evaporated to dryness, and the cleaved proteins in the dried supernatant were then resolved using 12% SDS-PAGE. The ^{32}P -labeled receptor fragments were visualized by autoradiography.

Incubation with antibodies and adsorption to protein A-Sepharose

Aliquots of untreated or protease-digested cytosol were mixed with equal volumes of TEG buffer (10 mM TES, 50 mM NaCl, 4 mM EDTA, 10% w/v glycerol, pH 7.6 at 4°C). BuGR2 antibody was added at 2% of the final volume. The mixtures were incubated 2–4 h on ice and then added to a 50 μ l bed volume of protein A-Sepharose and mixed at 4°C for 1 h. The Sepharose beads were pelleted and washed 5–8 times by resuspension in TEGNT buffer, and the pellet was washed three additional times with TEG buffer. The immunoadsorbed proteins were extracted by boiling the pellet in SDS sample buffer plus 10% β -mercaptoethanol.

Gel electrophoresis and Western blotting

SDS-PAGE was performed in 10–12% acrylamide gels according to Laemmli [16]. In Fig. 3, a gradient polyacrylamide gel of 5–20% polyacrylamide was used to resolve the protein mixture. Gels were cooled to 4°C during electrophoresis. All samples were taken up in 2 \times SDS sample buffer containing 10% β -mercaptoethanol and boiled. Molecular weight standards were myosin, $M_r = 205,000$, β -galactosidase, $M_r = 116,000$; phosphorylase *b*, $M_r = 97,000$; BSA, $M_r = 66,000$; ovalbumin,

$M_r = 45,000$; glyceraldehyde-3-phosphate dehydrogenase, $M_r = 36,000$; carbonic anhydrase, $M_r = 29,000$; trypsinogen, $M_r = 24,000$; trypsin inhibitor, $M_r = 20,000$, and α -lactalbumin, $M_r = 14,200$. After electrophoresis, proteins were transferred to Immobilon-P membranes and Western blotted by probing with 1% BuGR2 antibody followed by a second incubation with goat anti-mouse IgG coupled to horseradish peroxidase. Autoradiography to visualize 32 P was performed directly from the Western blot.

RESULTS AND DISCUSSION

As reported by Danielson *et al.* [3], the intact mouse GR is a 783 amino acid chain with a calculated molecular mass of ~ 86 kDa. Figure 1 shows a diagram of the mouse GR indicating the functional domains and the expected pattern of cleavage produced by chymotrypsin and trypsin [17]. Cyanogen bromide cleavage of the receptor produces a number of receptor fragments, all smaller than 10 kDa except for one, which is 23.4 kDa in size and contains residues 108–324. Hoek and Groner [11] showed that this fragment is heavily phosphorylated and it contains all of the GR

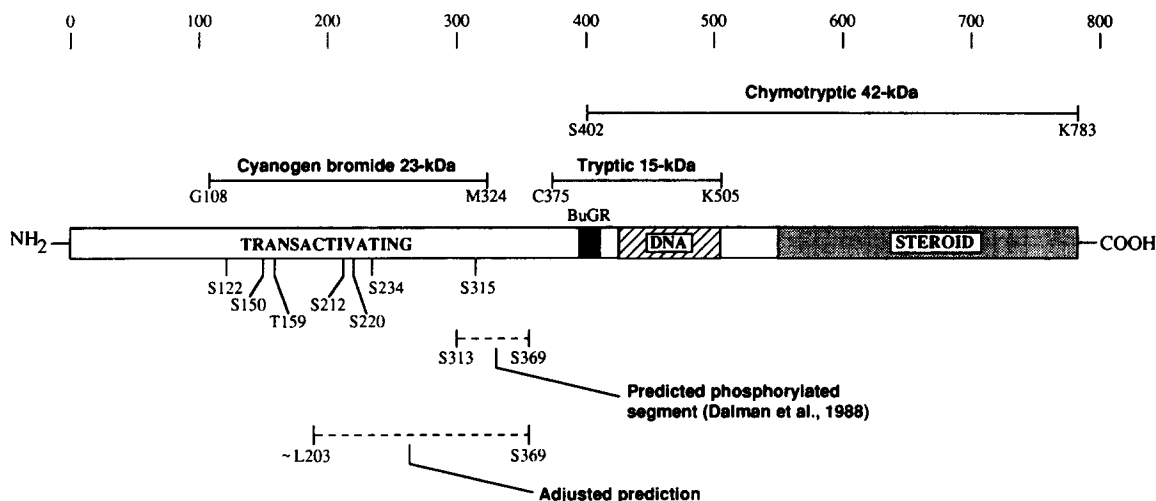


Fig. 1. Localization of phosphorylation sites, cyanogen bromide fragments and proteolytic fragments within the mouse GR. The phosphorylation sites identified by Bodwell *et al.* [12] in the mouse GR are indicated in a linear representation of the mouse receptor with placement of steroid- and DNA-binding domains as reported by Danielson *et al.* [3]. The location of the chymotryptic 42 kDa and tryptic 15 kDa fragments are as reported from fragment sequencing by Carlstedt-Duke *et al.* [17]. The solid region defines the BuGR epitope according to Rusconi and Yamamoto [18]. The 23 kDa receptor cyanogen bromide fragment was identified using the PEPLOT program from the Wisconsin Genetics Computer Group software package on the mouse GR (GENBANK accession number = X04435). The dashed lines below the receptor show the major phosphorylated segment predicted by Dalman *et al.* [10] as well as our adjusted prediction taking into account the ~ 12 kDa M_r discrepancy lying within the cyanogen bromide fragment.

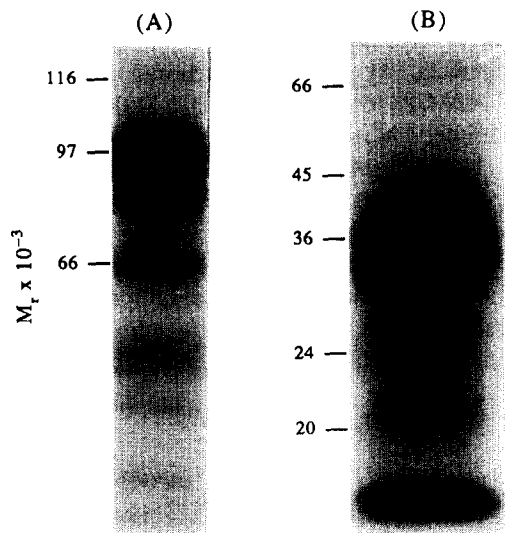


Fig. 2. The major ^{32}P -labeled cyanogen bromide fragment of the mouse GR is ~ 12 kDa larger than the calculated molecular weight. WCL2 cells were labeled for 15 h with [^{32}P]orthophosphate, and GR were immunopurified from cell lysates with BuGR2 antibody. The immunopurified ^{32}P -labeled receptor was resolved by SDS-PAGE, electrophoretically transferred to nitrocellulose, and the region of the nitrocellulose membrane corresponding to the intact ~ 98 kDa receptor was excized with a razor blade. The nitrocellulose strip was then treated overnight with cyanogen bromide at room temperature. The resultant supernatant was lyophilized, resolved on 10% SDS-PAGE, and the receptor fragments were visualized by autoradiography. (A) Autoradiographic profile of immunopurified GR from ^{32}P -labeled WCL2 cells. (B) Autoradiographic profile of CNBR-cleaved receptor showing the 23.4 kDa (calculated) receptor fragment migrating at ~ 35 kDa.

phosphorylation sites subsequently identified by Bodwell *et al.* [12].

In the experiment shown in Fig. 2, WCL2 cells were labeled for roughly a cell generation time with [^{32}P]orthophosphate, lysed, and the intact GR was immunoabsorbed with BuGR2. The immune pellet was washed extensively with a buffer containing both detergent and salt to remove extraneous proteins. The immunopurified ^{32}P -labeled intact receptor is shown in Fig. 2(A) and the cyanogen bromide-cleaved receptor fragments obtained from the gel-purified intact receptor are shown in Fig. 2(B). There is a major ^{32}P -labeled band migrating at ~ 35 kDa, with the remainder of the labeled fragments running at the gel dye front. Of the 25 cyanogen bromide fragments, all of the rest (9.4 kDa or less) are at the dye front. The lack of resolvable fragments between the ~ 35 kDa band and the dye front is evidence for cleavage to completion in the experiment of Fig. 2, and longer cyanogen bromide treatment did not yield smaller fragments. To achieve an ~ 35 kDa fragment extending into the DNA-

binding domain region, the 23.4 kDa fragment would have to be extended through cyanogen bromide cleavage sites at residues 324, 339, 344 and 389 without showing ^{32}P -labeled bands indicating partial cleavage at any of these sites. Thus, we conclude that this ~ 35 kDa fragment contains residues 108–324. The ~ 12 kDa difference between the theoretical molecular weight (23.4 kDa) and experimentally observed M_r (~ 35 kDa) of this cyanogen bromide fragment accounts for most of the difference between the theoretical (~ 86 kDa) and experimental (~ 98 kDa) values for the intact mouse GR. Thus, we propose that this fragment of the receptor contains the residues that cause anomalous migration of the intact receptor on SDS-PAGE.

Because the mouse GR can be nearly completely dephosphorylated without change in M_r on SDS-PAGE [10], the anomalous M_r cannot be explained on the basis of receptor phosphorylation. Numerous attempts by our lab and others have failed to demonstrate a reduction in M_r by treatment of the GR with glycohydrolases, and it is the impression in the field that the GR does not contain carbohydrate moieties. Thus, at the moment, the basis for the M_r differential is unknown, but its localization to residues 108–324 should be taken into account when calculations are made from receptor fragments identified on SDS-PAGE.

An example of the error that can arise was published in a previous study from our lab by Dalman *et al.* [10] in which the location of the ~ 12 kDa differential was not known and the major sites of phosphorylation were predicted from proteolytic fragments to lie between residues 313 and 369 of the mouse GR. This predicted phosphorylated segment would have been extended about 110 amino acids toward the amino-terminus to yield the adjusted prediction shown in Fig. 1 had we known that the ~ 12 kDa differential lay within the cyanogen bromide fragment. The adjusted prediction includes four of the seven phosphorylated sites identified by Bodwell *et al.* [12] in the mouse GR (Fig. 1). These four sites (Ser 212, 220, 234, 315) account for $\sim 75\%$ of the ^{32}P recovered in the seven phosphorylation sites (see Table I in Bodwell *et al.* [12]) and after this adjustment, the predictions made by Dalman *et al.* [10] from proteolytic fragments without sequence data become much more accurate.

Five phosphorylated peptides (phosphopeptides 5, 6, 11, 16 and 23) containing 19% of the

total ^{32}P recovered by Bodwell *et al.* [12] were not sequenced. Our cyanogen bromide experiments also reveal small ^{32}P -labeled peptides on SDS-PAGE [Fig. 2(B)] suggesting the presence of other phosphorylated sites within the receptor. Both the Pratt and the Groner laboratories have previously reported that the tryptic 15 kDa fragment containing the DNA binding domain (Fig. 1) is phosphorylated in GR isolated from mouse L cells [10] and rat hepatoma cells [11]. These reports have been challenged by van der Weijden Benjamin *et al.* [19] who concluded from a study of GR phosphorylation in cultured AtT-20 cells that the 15 kDa phosphorylated tryptic fragment is similar in size to the DNA-binding tryptic fragment containing the BuGR epitope (C375-K505) but is not the DNA-binding, BuGR-reactive fragment itself. The data of Fig. 3 show that the 15 kDa tryptic fragment containing the DNA-binding domain of the mouse GR is phosphorylated.

To determine if the ~ 15 kDa DNA-binding

domain fragment of the GR is phosphorylated in WCL2 cells, we cultured the cells in [^{32}P]orthophosphate containing growth medium for 15 h, lysed the cells under hypotonic conditions, and then cleaved receptors in cytosol before immunoadsorption to protein A-Sepharose with BuGR2. The immunoreactive intact receptor is shown in lane 3 of Fig. 3(A), while lane 3 [Fig. 3(B)] shows [^{32}P]phosphate associated with the intact receptor. Lane 1, in A shows the 42 kDa chymotryptic immunoreactive fragment containing the DNA- and steroid-binding domains, and as reported for L cell receptors [10], lane 1, in B shows that the 42 Da chymotryptic fragment contains radiolabeled phosphate. Lane 2, in A shows the immunoreactive 15 kDa tryptic receptor fragment containing the DNA-binding domain, while lane 2, in B, shows the immunoadsorbable [^{32}P]phosphate signal in the trypsin-cleaved cytosol. It is clear that the 15 kDa immunoreactive fragment produced from the intact receptor

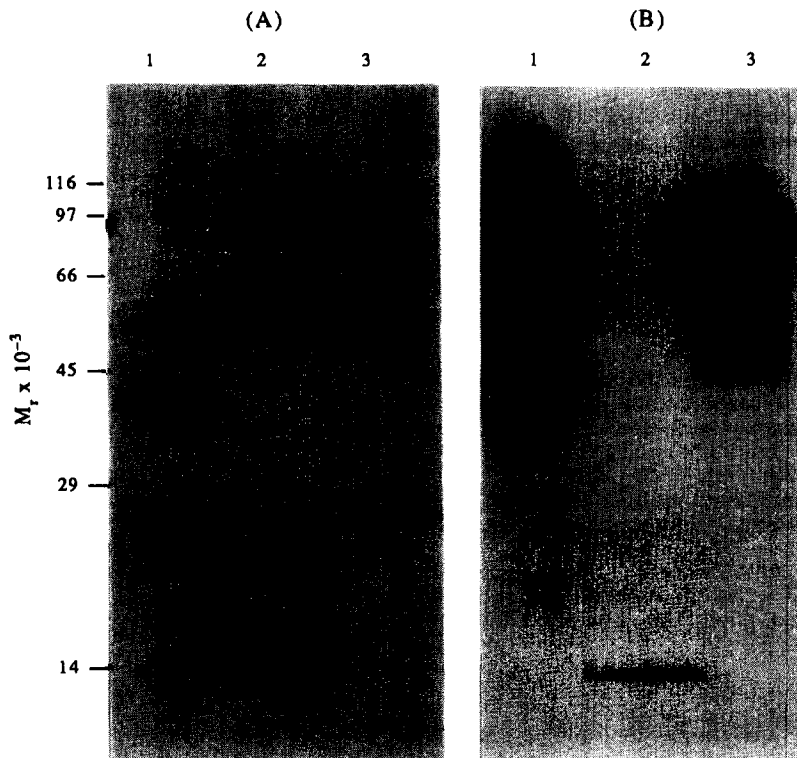


Fig. 3. The 15 kDa tryptic fragment containing DNA-binding domain of the mouse GR is phosphorylated after incubation of WCL2 cells in [^{32}P]orthophosphate-containing growth medium. WCL2 cells were labeled for 15 h with phosphate-free medium (DMEM) containing [^{32}P]orthophosphate. Cell lysates were harvested and treated with buffer (lane 3), chymotrypsin (lane 1) or trypsin (lane 2). Receptors were then immunopurified from treated lysates with BuGR2 adsorbed to protein A-Sepharose, resolved on a gradient SDS-polyacrylamide gel and electrophoretically transferred to Immobilon-P membranes. (A) Shows an immunoblot with BuGR2 to visualize the intact receptor and receptor fragments, and (B) is an autoradiogram of the same immunoblot. The two bands present in all three lanes of the Western blot migrating at ~ 55 and ~ 25 kDa are the heavy and light chains, respectively, of the BuGR antibody.

comigrates exactly with the 15 kDa ³²P-labeled receptor fragment. As this 15 kDa tryptic ³²P-labeled fragment was immunoadsorbed, it must contain the BuGR epitope and it must be the C375-K505 peptide. Indeed, we have demonstrated previously that this BuGR-adsorbed 15 kDa fragment is fully competent in binding DNA [20]. Thus, we must disagree with van der Weijden Benjamin *et al.* [19] who concluded that the mouse GR DNA-binding domain is not phosphorylated *in vivo*. However, it is clear that the 15 kDa tryptic fragment is not a major site for phosphorylation, as we estimate that this fragment contains only ~5% of the total receptor phosphate.

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