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PHOSPHORYLATION IN VIVO OF RAT HEPATIC GLUCOCORTICOID RECEPTOR[1]

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Summary. Rat liver glucocorticoid receptors were labeled in vivo with [^{32}P]orthophosphate. In the last two fractionation procedures leading to purified, molybdate-stabilized, unactivated receptor complex, bound [^{32}P] coeluted with peaks of bound [^{3}H]triamcinolone acetonide. SDS-gel electrophoresis revealed [^{32}P]labeled 90K and 24K bands. The lower molecular weight band is heavily phosphorylated and it could be either a component of the unactivated receptor or a degradation product.

Several reports have suggested that the steroid binding activity as well as activation of the glucocorticoid receptor may be regulated by phosphorylation/dephosphorylation (1-5). Recent reports have demonstrated in vivo phosphorylation of both the progesterone (6) and glucocorticoid (7) receptors. In both instances, Mr = 90,000 proteins, possessing the steroid binding activity of the receptor, have been found to be phosphorylated on serine residues. Recently, we have purified the unactivated rat liver glucocorticoid receptor to near homogeneity (8).

These studies have indicated that a heterogeneity in the molecular composition of the unactivated glucocorticoid receptor may exist. Mr = 90,000; 41,000; 40,000, and 24,000 components have been found to be related to the unactivated complex (8). Inclusion of triamcinolone acetonide in the starting cytosol which

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[3]Address all correspondence to this Author. This paper is dedicated to the memory of Dr. Thomas I. Diamondstone, former student, colleague and friend.

The Abbreviations used are: [^{3}H]TA: [6,7 - ^{3}H(N)]Triamcinolone acetonide, trivial name for 9α-fluoro-11β,21-dihydroxy-16α,17-[1-methyl-ethylidene bis-(oxy)] pregna-1,4-diene-3,20-dione. SDS: Sodium dodecyl sulfate

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preempted binding of the receptor to the affinity gel, caused the removal not only of the 90,000 dalton protein but also of the Mr = 41,000; 40,000, and 24,000 components of the final preparation. This indicated the relationship of all of these forms to the purified glucocorticoid receptor. This heterogeneity may also be due to a proteolytic breakdown of the Mr = 90,000 protein. We reasoned that using an *in vivo* [^{32}P]-labeled receptor, the distribution of the phosphorous isotope among the above four components might decide whether the possible spontaneous proteolytic cleavage of the Mr = 90,000 protein occurs. The results are suggestive that a proteolytic mechanism may not be responsible for the heterogeneity in molecular composition of the unactivated glucocorticoid receptor complex if resulting molecular weights from SDS gels are taken literally.

Experimental Procedures

Chemicals - [6,7-^{3}H(N)]Triamcinolone acetonide ([^{3}H]TA, 37Ci/mmol) and EN^{3}HANCE were purchased from New England Nuclear; carrier free [^{32}P]orthophosphate from ICN. All other reagents were obtained from Fisher Scientific.

In vivo [^{32}P]-labeling - Five male Wistar rats weighing 150-200g and adrenalectomized 2-3 days prior to injection with 10 mCi [^{32}P]orthophosphate were used. They were sacrificed 18 hr after injection and livers were then perfused *in situ* via the portal vein. The perfusion of livers and preparation of cytosol were performed as reported previously (8).

Receptor purification - Glucocorticoid receptor was purified by the method of Grandics *et al.*, (8). Briefly, crude liver cytosol was incubated with a deoxycorticosterone-derivatized agarose (9) at 0°C for 2 hr. The resin was then extensively washed and gel-bound receptors were eluted with 2 μM [^{3}H]-triamcinolone acetonide at 0°C overnight. The affinity gel eluate was chromatographed on a Bio-Gel A-1.5 m agarose column. Peak fractions of receptor were combined and applied to a DEAE-cellulose column. Bound receptors were eluted with a linear potassium phosphate gradient (50-500 mM) in the presence of 10 mM sodium molybdate. The unactivated complexes were eluted at a potassium phosphate concentration of 0.23-0.26 M.

Gel electrophoresis - SDS-polyacrylamide gel electrophoresis of the receptor fractions was carried out on 10% acrylamide slab gels as reported previously (8). Gels were fixed and stained overnight in 45% methanol, 10% acetic acid and 0.24% (w/v) Coomassie Blue R-250 in distilled water. Destaining was performed in the above fixative mixture without Coomassie Blue. The gel slab was then impregnated with EN^{3}HANCE according to the manufacturer's suggestions before drying. The dried gel was autoradiographed at - 70°C using regular enhancing screens.

Results and Discussion

Rats were injected with [^{32}P]orthophosphate (10 mCi into each animal) in order to label the glucocorticoid receptor *in vivo*. Hepatic glucocorticoid-receptor complexes were then purified by the standard three-step procedure uti-

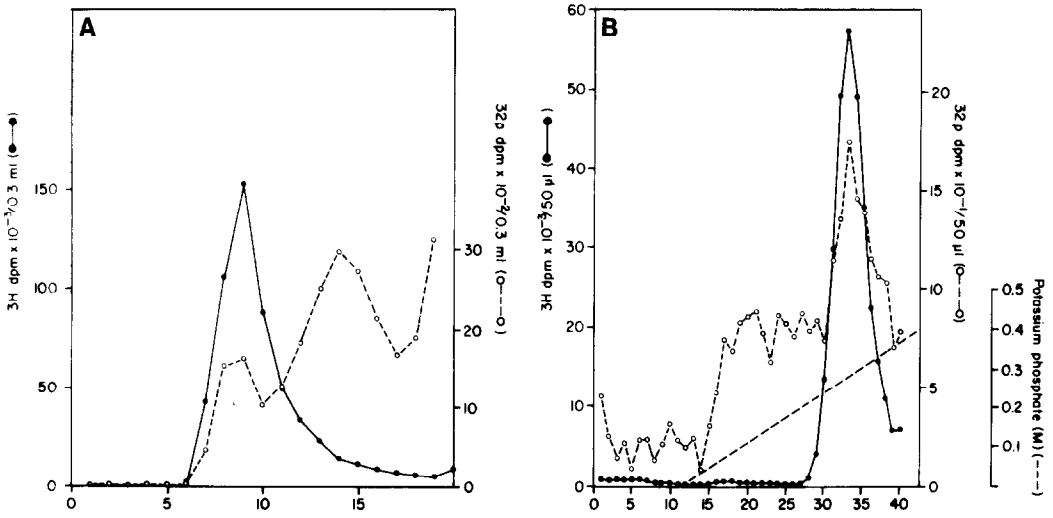

Figure 1. Purification of affinity gel eluted, in vivo [32P]-labeled [3H]TA - receptor complexes. (A) Affinity eluate was chromatographed on a Bio-Gel A-1.5m column, equilibrated with a 50 mM potassium phosphate-10 mM sodium molybdate-10 mM thioglycerol buffer, pH 7.0. Eight ml fractions were collected and 0.3 ml aliquots were taken for counting radioactivity. (B) DEAE-cellulose chromatography of Bio-Gel purified, [3H]TA-receptor complexes. Combined receptor-peak fractions from Bio-Gel A-1.5 m chromatography were applied to a 3 ml DEAE-cellulose column and then eluted with a linear 50-500 mM potassium phosphate gradient containing 10 mM sodium molybdate and 10 mM thioglycerol, pH 7.0 (8). One ml fractions were collected and 50 µl aliquots were taken for measurement of radioactivity.

lizing affinity chromatography, agarose gel filtration, and DEAE-cellulose chromatography (8). [32P]-Containing material coeluted with peaks of [3H]triamcinolone acetonide during the final DEAE-purification step (Fig. 1B) and previous Bio-Gel purification step (Fig. 1A). Occasionally, other phosphorylated components with Stokes radii higher than that of the unactivated complexes were also observed (data not shown). Aliquots of receptor-peak fractions obtained from DEAE-cellulose chromatography were precipitated with trichloroacetic acid and subjected to SDS-polyacrylamide gel electrophoresis (8) and staining with Coomassie Blue. The protein-stained gel displayed a single Mr = 90,000 band in the receptor-peak fractions of the DEAE-cellulose chromatogram (Fig. 2A) and the intensity of this protein band closely followed the distribution of bound [3H]triamcinolone acetonide (see Fig. 1B). The gel was then treated with EN3HANCE, dried and autoradiographed at-70°C for 1 month. The autoradiogram displayed a distribution pattern of the Mr = 90,000 protein identical to that of the Coomassie stain (Fig. 2B). Thus, our data, obtained on a near homogeneously

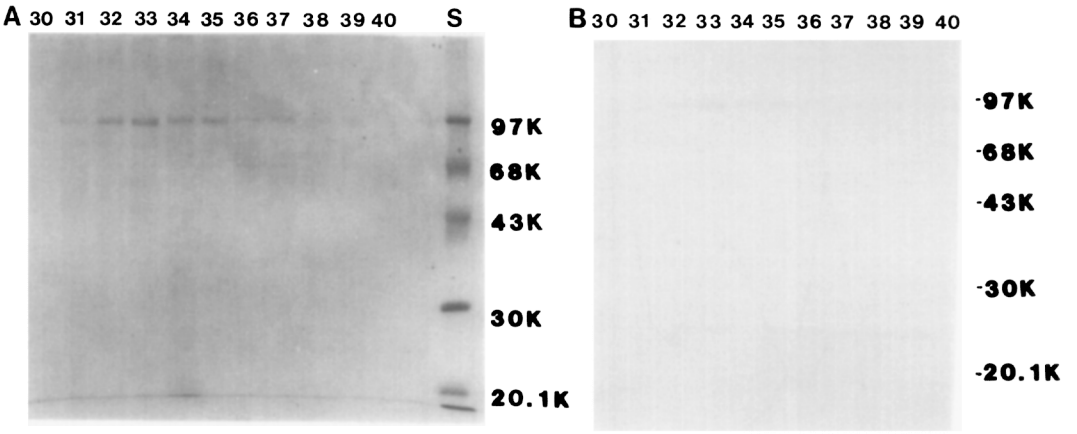

Figure 2. SDS-polyacrylamide gel electrophoresis of receptor fractions from DEAE-cellulose chromatography. (A) Receptor-peak fractions were precipitated with trichloroacetic acid and electrophoresed on a 10% acrylamide slab gel (8). Proteins were stained with Coomassie Blue. The molecular weight standards are shown in kilodaltons. The fraction numbers of the samples are displayed at the top of the gel; S stands for standard proteins. (B) Autoradiograph of the same gel.

purified receptor, are in accord with those reported by Housley and Pratt in L cells (7).

Interestingly, another component, which remained undetected by the Coomassie stain, also incorporated phosphorous isotope. This phosphorylated component was eluted from DEAE-cellulose at a salt concentration higher than that of the bound [^{3}H]-radioactivity peak itself and exhibited an apparent molecular weight of 24,000. This band is identical to the Mr = 24,000 component, easily detectable by the double staining method, which has also been shown to be related to the glucocorticoid receptor (8). The Mr = 24,000 entity, as based on protein stain (8), is much more heavily phosphorylated than the 90,000 dalton protein indicating that this component may contain tightly bound nucleic acid or may be a small polynucleotide itself. When the final receptor preparations were stained by the double staining procedure (8), this component always appeared as a characteristic dark grey band, whereas all of the standard proteins and other components of the receptor preparation exhibited an uniform, golden-brownish color. This may also be indicative of a special chemical composition peculiar to this Mr = 24,000 component.

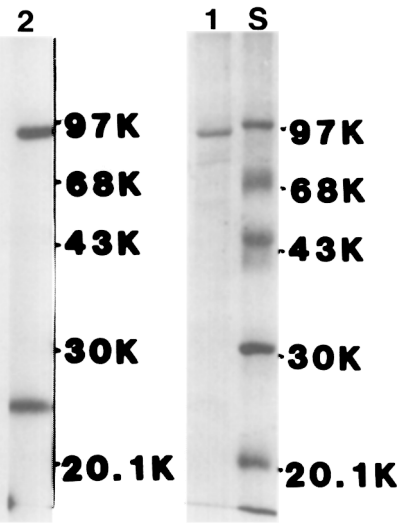

Figure 3. SDS-polyacrylamide gel electrophoresis of [^{32}P]-labeled receptors of the pooled DEAE-fractions. Receptor-containing fractions from DEAE-cellulose chromatography were combined, processed for gel electrophoresis and run on a 10% acrylamide gel. Staining and autoradiography were performed as indicated in Experimental Procedures, and Results and Discussion. S, standard proteins; lane 1, Coomassie Blue stained final receptor; lane 2, autoradiograph of lane 1.

We have found that no phosphorylated components other than the Mr = 90,000, and 24,000 were present in the final preparation (Fig.3, lane 2). It is of particular interest that, according to this observation, the previously reported Mr = 41,000 and 40,000 proteins (8), also related to the unactivated complex, are not phosphorylated *in vivo*. As we have demonstrated, the Mr = 90,000; 41,000, and 40,000 proteins coelute from the DEAE-cellulose column in the final purification step (8). This suggests that if the 41,000 and 40,000 dalton components were degradation products that of the Mr = 90,000, they might be formed during elution from the DEAE-cellulose column, or in the tubes of collected fractions. Since these two proteins account for 90% of the total molecular weight of the 90,000 dalton component, the observation of a small, approximately 10,000 dalton phosphorylated fragment would support the theory of spontaneous proteolytic breakdown of steroid-receptor complexes. This small fragment should carry all the phosphorylated residues of the Mr = 90,000 protein and hence it could be so acidic that it may not be eluted from the DEAE-cellulose column by the 50-500 mM potassium phosphate gradient. Therefore, after eluting steroid-receptor

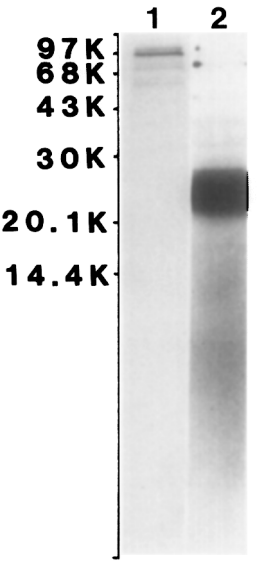

Figure 4. SDS-polyacrylamide gel electrophoresis of a 3M KCl eluate of the DEAE-cellulose column. After eluting steroid-receptor complexes by the 50-500 mM phosphate gradient, 10 ml of 3M KCl was applied to the DEAE-cellulose column.

complexes by the salt gradient, 10 ml of 3M KCl was passed through the DEAE-cellulose column in an attempt to recover this postulated small receptor fragment. The components of the eluate were then precipitated by trichloroacetic acid and analyzed on a linear 15% SDS-polyacrylamide gel (8). The autoradiogram displayed only a heavy ~ 24,000 dalton band (Fig. 4, lane 2) which remained undetected by the Coomassie stain (Fig. 4, lane 1). This aligns with previous observations that the Mr = 24,000 component cannot be eluted completely from DEAE-cellulose by the routinely used 50-500 mM phosphate gradient (8) and that its band also remained undetected by the Coomassie stain (see Fig 3). However, the hypothetical, small, Mr ~ 10,000 phosphorylated fragment has not been detected. Similarly, if proteolytic cleavage of steroid receptor complexes had occurred in the fraction tubes, a heavy phosphorylated band representing the Mr ~ 10,000 receptor fragment should have appeared at the dyefront upon SDS-polyacrylamide gel electrophoresis (see Fig.3, lane 1). This, however, has not been observed.

Taken collectively, our studies point to the limitations of the Coomassie Blue staining method when it is used to analyze the molecular composition of an

unactivated glucocorticoid receptor preparation. The data obtained suggest that the previously observed heterogeneity in the composition of the unactivated complex (8) is not due to a spontaneous proteolytic breakdown of the glucocorticoid receptor. Alternatively, the 24K phosphorylated product could be showing an abnormal position on the gel. Further work needs to be done to establish the precise subunit composition of the unactivated glucocorticoid-receptor complex.

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