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PHOSPHORYLATION OF PURIFIED GLUCOCORTICOID RECEPTOR FROM RAT LIVER BY AN ENDOGENOUS PROTEIN KINASE

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SUMMARY: Glucocorticoid receptor was purified from rat liver cytosol using a dexamethasone affinity column. The receptor thus purified displayed a single protein band when subjected to SDS-polyacrylamide gel electrophoresis. It had a molecular weight of 90,000 which was consistent with the reported value for other glucocorticoid receptor preparations. Incubation of the purified preparation with [γ^{32}P] ATP and Mg^{2+} resulted in transfer of [^{32}P] to the receptor protein indicating the presence of an endogeneous protein kinase activity capable of phosphorylating the receptor molecule. Phosphorylation of the glucocorticoid receptor by the endogenous protein kinase might serve as a direct mechanism for the activation of the receptor.

Studies in several laboratories have suggested that phosphorylation of the hormone receptors is involved in the action of steroid hormones. Early studies showed that specific glucocorticoid binding in rat thymocytes is reduced by glucose and oxygen deprivation but is reversed when aerobic conditions are restored. These alterations in binding of glucocorticoids paralleled the levels of cellular ATP (1). Moreover, 2,4-dinitrophenol which reduces ATP levels has been shown to reduce glucocorticoid binding in the mouse fibroblasts (2) and chick embryo retina cells (3). On the other hand, treatment of cytosol with alkaline phosphatase leads to a decrease in the binding ability of either glucocorticoids (4) or estrogens (5).

The purified progesterone receptor can be phosphorylated in vitro by a cAMP-dependent protein kinase (6,7) and these studies have been corroborated by purification of ^{32}P-labelled progesterone receptor from the oviducts of chickens injected with [^{32}P] orthophosphate (8). Incubation of intact mouse fibroblasts with [^{32}P] orthophosphate is known to phosphorylate the glucocorticoid receptor (9). However, it has not been established whether the

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glucocorticoid receptor itself contains a protein kinase activity as has been observed for the progesterone (6,7) and the insulin receptors (10,11). Our laboratory has partially purified protein kinase NI (12,13) from rat liver and characterized an "N_1-type" protein kinase (Seidel, Stetler, Jacob, unpublished data) and protein kinase NII (14) from a rat hepatoma, the two tissues that are known to contain the glucocorticoid receptor. Although protein kinases NI and NII are largely localized in the nucleus, they do resemble cytoplasmic casein kinases I and II in several respects (15). We have now initiated studies to determine (a) whether purified glucocorticoid receptor can function as a protein kinase and (b) if so, whether it is related to the protein kinases NI, NII or other well-characterized protein kinases. The present report provides evidence that the purified glucocorticoid receptor contains protein kinase activity which is capable of phosphorylating the receptor.

METHODS

Purification of rat liver glucocorticoid receptor (GR): GR was purified from adrenalectomized rat livers as described earlier (16,17). Essentially, livers from animals adrenalectomized for 3-4 days were perfused in situ with isotonic saline and homogenized in 2 volumes of Buffer A [(20 mM Tris HCl) (pH 7.4) 50 mM KCl, 2 mM dithiothreitol 20% (v/v) glycerol and 0.3 mM phenylmethylsulfonyl fluoride)]. The homogenate was centrifuged for 1h at 105,000 x g and the supernatant (cytosol) was applied on a dexamethasone affinity column. After extensive washing of the column with Buffer A, the gel matrix was incubated with 10^{-6}M ^{3}H-dexamethasone (specific activity 4Ci/mmol). The labeled GR was eluted from the column with Buffer A + 0.5 M NaSCN and the eluate desalted on a Sephadex G-50 column. The flow through from the latter column was incubated with 10^{-7} M ^{3}H-dexamethasone (specific activity 44 Ci/mmol) for 30 min at room temperature and then applied to a DEAE-Cellulose column equilibrated with Buffer A (pH 8.5) without salt. After extensive washing, the receptor was eluted with a linear gradient of 0-500 mM KCl in the same buffer. The radioactive peak that eluted between 60 mM and 120 mM KCl was pooled and glycerol was added to final concentration of 50% (v/v) before storage at -70°C in aliquots.

Phosphorylation of the GR: Aliquots of the GR were incubated in the presence of $MgCl_2$ (5mM), dithiothreitol (2mM) and [γ^{32}P] ATP (30μM) at 25°C for 30 minutes. The reaction was terminated by cooling the tubes rapidly and the contents were applied to a DEAE-cellulose column (bed volume = 1.5 ml). After extensive washing of the column, the GR was eluted with 0.2M KCl. Aliquots of the wash fractions as well as salt-eluted fractions were analyzed for ^{3}H and ^{32}P. The bulk of the radioactivity that eluted with salt was precipitated with TCA and subjected to electrophoresis as described below.

Polyacrylamide gel electrophoresis under denaturing conditions: GR was precipitated with trichloroacetic acid [10%(w/v) final concentration] for 16-30h at 4°C. The acid precipitate was collected by centrifugation in a microfuge for 15 minutes and washed once with 0.5ml acetone. After the residual acetone had been dried off, the precipitate was dissolved in 10μl of buffer containing 65mM Tris-HCl (pH 7.0), 2% (w/v) sodium dodecyl sulfate, 10%

(v/v) glycerol and 5% (v/v) β-mercaptoethanol. The sample and appropriate molecular weight markers were heated in a boiling water bath for 2 minutes and applied to a 4-30% linear gradient slab gel and subjected to electrophoresis as described by Laemmli (18). Electrophoresis was carried out at 75V for an initial 15 minutes and then at 120V until the tracking dye reached the bottom of the gel. The gels were fixed and then stained with Coomassie Brilliant Blue. The destained gels were subjected to autoradiography at -70°C using XAR-5 X-ray (Kodak) film and an intensifying screen.

RESULTS AND DISCUSSION

Purification of the glucocorticoid receptor was achieved using a previously described protocol which included chromatographic fractionation with a dexamethasone affinity column (16,17). In the final purification step the receptor was eluted between 60-120mM salt from a DEAE-cellulose column (Fig. 1). The receptor, thus purified, displayed a single protein band when subjected to electrophoresis under denaturing conditions (Fig. 2). When the receptor preparation was incubated with [γ^{32}P] ATP in the presence of Mg^{2+} and subsequently rechromatographed on DEAE-cellulose, the ^{32}P-label co-migrated with ^{3}H (Fig. 3), which indicates that the GR was phosphorylated under these conditions. When the purified receptor was analyzed by SDS-polyacrylamide

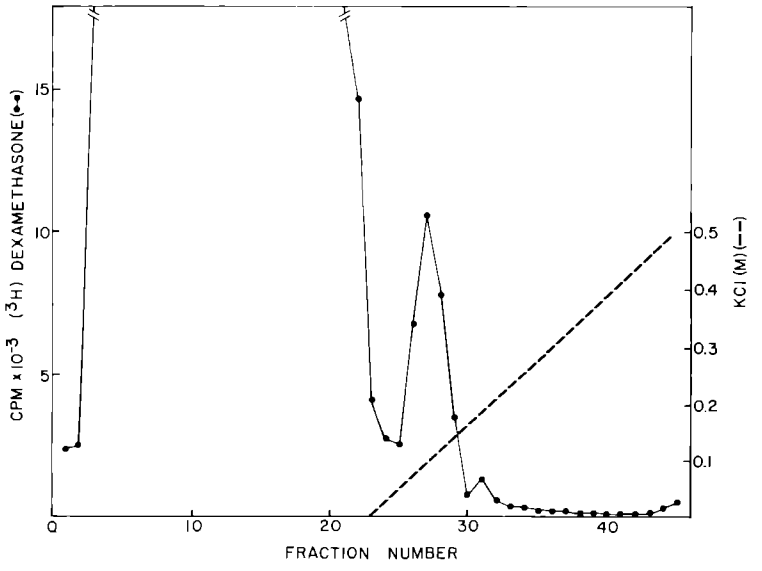

Figure 1: DEAE-Cellulose elution profile of purified glucocorticoid receptor from rat liver. Receptor initially purified by affinity chromatography was desalted on Sephadex G-50 column and applied to 10 ml of DEAE-Cellulose column. After extensive washing of the column, the receptor was eluted with a linear gradient of 0-500 mM KCl in the equilibration buffer. Radioactivity was measured in 100 µl of 3 ml fractions.

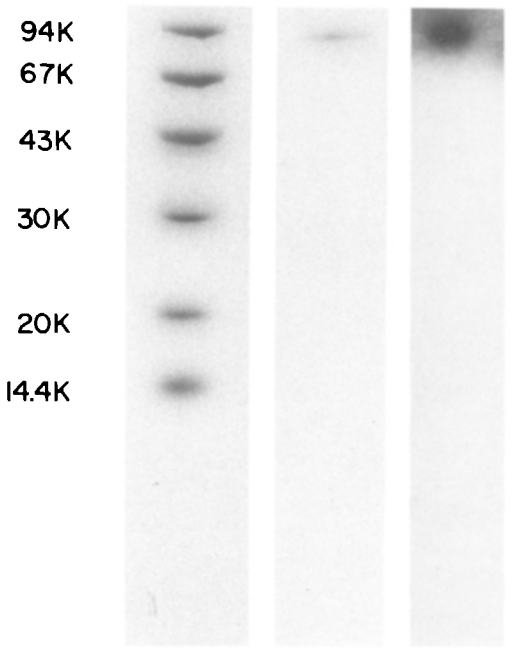

Figure 2: Phosphorylation of the glucocorticoid receptor. Aliquots of pooled receptor fractions from DEAE-Cellulose column eluted with salt were phosphorylated as described in the Methods Section precipitated with 10% (w/v) trichloroacetic acid, applied to a 4-30% linear gradient slab gel and stained with Coomassie blue (lane 1). The corresponding autoradiograph after 3 days exposure is shown in lane 2.

gel electrophoresis, a single protein with a molecular weight of about 90,000 band was observed following staining with Coomassie Blue (Fig. 2). No other protein was visible in the gel which suggests the high purity of the receptor preparation. Autoradiography of the gel revealed that the radioactive band was superimposable with the stained protein (Fig. 2).

Since the receptor and the kinase were bound to the dexamethasone affinity column and no visible protein besides the receptor was detected on the SDS gel, it seems likely that the protein kinase is an integral component of the glucocorticoid receptor. Moreover, the receptor exhibited a molecular weight of 90,000 which is consistent with the reported molecular weight for other glucocorticoid receptor preparations (16,19). In addition, preliminary studies have shown that the receptor and kinase activity co-migrate on a sucrose density gradient.

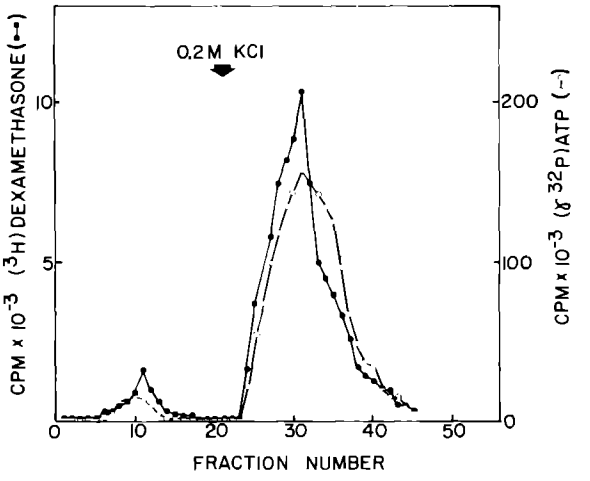

Figure 3: Profile of phosphorylated glucocorticoid receptor on DEAE cellulose column. The glucocorticoid receptor was phosphorylated as described in legend to Figure 2 and layered on 1.5 ml of DEAE-Cellulose column. After extensive washing of the column, the receptor was eluted with equilibration buffer containing 0.2 M KCl. Radioactivity was measured in 25 µl aliquots of each fraction.

To our knowledge, this is the first report on the presence of an endogenous protein kinase in the purified glucocorticoid receptor. Studies are in progress to characterize further the kinase associated with the glucocorticoid receptor. Since phosphorylation of receptor appears to be required for its binding to the hormone (20), the presence of an endogenous kinase in the receptor molecule capable of phosphorylating the receptor offers an attractive mechanism for achieving phosphorylation in response to rapid physiological stimuli and consequently, facilitating the biological action of the steroid hormone.

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