Protein Kinase Activity Can Be Separated from the Purified Activated Rat Liver Glucocorticoid Receptor*

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Glucocorticoid receptor (GR) has been purified and characterized in its nonactivated (non DNA binding) and activated (DNA binding) form, from both anabolic and catabolic target tissues (1–4). The complete understanding of GR structure has not yet been achieved although it appears that independently of tissue source GR always contains a 90- to 98-kDa steroid-binding protein (1–4). Aside from the 94-kDa steroid-binding protein, the nonactivated form of GR contains a 90-kDa protein which seems to be a member of the heat shock protein family (4, 5), while the activated form of GR contains a 72-kDa protein of yet not well-characterized function (2, 6, 7). The 94-kDa steroid-binding protein is phosphorylated in vivo in a number of tissues (3, 4, 8–10).

Indirect evidence suggests that phosphorylation of GR might play a potential role in the regulation of GR functions such as hormone binding and activation, as reviewed by Schmidt et al. (1) and Houseley et al. (11), but direct evidence has not yet been obtained. Besides, the protein kinase(s) involved in the phosphorylation of GR have not been identified. Several authors have reported that the purified GR preparations obtained from rat liver by steroid affinity chromatography display a protein kinase activity (12–14) and it was proposed that the 94-kDa GR protein itself has intrinsic protein kinase activity (1). However, the GR protein purified from mouse fibroblasts by immunoabsorption to the monoclonal antibody against GR, does not show the intrinsic kinase activity (15).

In order to clarify whether the kinase activity present in the liver GR preparation is merely associated with, or is intrinsic to the 94-kDa GR protein itself, we have used a different purification method based on DNA affinity chromatography (2, 7) to obtain a highly purified activated form of GR.

The data presented in this report show that the kinase activity can be separated from the 94-kDa steroid-binding protein by differential elution from a DEAE-Sepharose column.

EXPERIMENTAL PROCEDURES

Materials—Glassware was siliconized with silicone solution from Serva. Dithiothreitol was obtained from Sigma. Single peak pig insulin was from Galenika, Beograd. Mixed histones type-Ia was from Sigma, histone 1 from Boehringer Mannheim, and histone 2b from Worthington. Phosphocellulose P11 and cellulose powder CF11 were obtained from Whatman. DEAE-Sepharose CL-6B was from Pharmacia. Calf thymus DNA was obtained from Sigma. [1,2,4-3H]triamcinolone acetonide (TA) (28 Ci/mmol) was purchased from Amersham Corp. (England). It was routinely diluted to a specific radioactivity of 9 Ci/mmole from Sigma. [γ-32P]ATP (10 Ci/mmol or 3000 Ci/mmol) was from Amersham Corp. (England). ATP was obtained from P-L Biochemicals.

Glucocorticoid Receptor Purification—GR was purified from livers of 2-month-old male Wistar rats 5 days after adrenalectomy. The livers were perfused in situ with ice-cold EPB buffer (20 mM sodium phosphate buffer, pH 7.0, 1 mM EDTA, 10% (v/v) glycerol, 2 mM dithiothreitol) containing 50 mM NaCl. After homogenization in the same buffer and ultracentrifugation, the cytosol fraction was incubated with 100 nM [1,2,4-3H]triamcinolone acetonide at 0 °C for 45 min. The phosphorylation was carried out using three chromatographic steps. The first step was chromatography on a phosphocellulose column after which the GR was activated by 2-fold dilution in EPB buffer and incubation at 25 °C for 30 min. The preparation was further chromatographed on DNA-cellulose and DEAE-Sepharose columns equilibrated in ETG buffer (20 mM Tris-HCl, pH 7.8, 1 mM EDTA, 10% glycerol, 10–20 mM dithiothreitol), according to the method described by Wrange et al. (2, 7). Purified GR was stored at −70 °C in the presence of 250 µg/ml insulin as a carrier. 10% glycerol and 10 mM triamcinolone acetonide.

GR Quantitation—The amount of GR was calculated from the quantitation of 3H radioactivity ([3H]triamcinolone acetonide, 9 Ci/mmol) assuming one steroid-binding site per GR molecule and M1 = 94,000 D (2).

Assay for Protein Kinase Activity—Aliquots (0.045 ml) from DEAE-Sepharose fractions were immediately assayed for protein kinase activity. The reaction mixtures (0.060 ml) contained 10 mM MgCl2, 0.2 mg/ml mixed histones, and 0.05 mM [γ-32P]ATP (2000 cpm/pmol). The assays were carried out at 25 °C for 30 min, and the reactions were terminated by the addition of trichloroacetic acid in a final concentration of 20%. The peak fraction of protein kinase activity obtained from the DEAE-Sepharose column was used in all subsequent phosphorylation experiments. The kinase was further

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1 The abbreviations and trivial names used are: GR, glucocorticoid receptor; TA, triamcinolone acetonide; 8-fluoro-11β,21-dihydroxy-16α,17α-(1-methyl-3-oxo-1-adrenenedene)pregna-1,4-diene-3,20-dione; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

2 In the text 90- to 98-kDa steroid-binding protein will be referred to as a 94-kDa according to Wrange et al. (2).
characterized for its substrate specificity using either endogenous substrate (GR itself at the concentrations indicated in the figure legends) or various exogenous substrates, i.e. mixed histones, histones H1, H2b, H4, protamine sulfate, or dephosphorylated casein. All the substrates were at a final concentration of 0.2 mg/ml. Concentrations of divalent ions and of ATP are indicated in the figure legends. SDSPolyacrylamide Gel Electrophoresis—Samples for SDSPAGE were precipitated using ice-cold 20% trichloroacetic acid (final concentration) overnight, and the precipitates were washed twice with ice-cold acetone. The pellets were dissolved in gel electrophoresis sample buffer, incubated for 20 min at 65 °C, and electrophoresed on 10% or 14% SDS-polyacrylamide gel at 4 °C (16). Gels were stained by the silver nitrate method (17). A molecular weight calibration kit (Bio-Rad) consisting of phosphorylase b (M, 97,400), bovine serum albumin (M, 67,000), ovalbumin (M, 45,000), carbonic anhydrase (M, 30,000), soybean trypsin inhibitor (M, 20,100), and lysozyme (M, 14,400) was used. Gels were dried and autoradiographed at -70 °C using Kodak X-Omat AR-5 films and intensifying screens. The length of exposure is indicated in the figure legends.

RESULTS

The activated form of GR was purified to near homogeneity from rat liver cytosol using a three-step purification procedure (2, 7). After chromatography on phosphocellulose the [3H]TA GR preparation was activated and further chromatographed on DNA-cellulose from which the GR was eluted with MgCl2-containing buffer. Peak fractions of GR-bound radioactivity were pooled and chromatographed on a DEAE-Sepharose column. Aliquots of each DEAE-Sepharose fraction were assayed for [3H]TA radioactivity bound to GR (Fig. 1, panel b) for protein content using SDS-PAGE (Fig. 1, panel a) and for protein kinase activity tested under standard reaction conditions as described under "Experimental Procedures" (Fig. 1, panel c). SDS-PAGE analysis shows that the 94-kDa GR protein peak which corresponds to the maximum of [3H]TA GR-bound radioactivity can be separated from the peak of protein kinase activity by means of its differential binding to the DEAE-Sepharose column. The 94-kDa GR protein was eluted from DEAE-Sepharose at 0.12 M NaCl, while the copurifying kinase eluted at 0.14 M NaCl. The GR-copurifying kinase was assayed further assayed for its substrate specificity and ion dependence. Various exogenous substrates were tested including some typical basic substrates such as mixed histones, purified histone 1, histone 2b, histone 4, and protamine, as well as casein as an acidic substrate. Casein appeared to be a poor substrate for GR-copurifying kinase (data not shown), while basic substrates were phosphorylated under standard reaction conditions (Fig. 2, lanes 2 and 3).

The ion dependence of the kinase was further tested using mixed histones as a substrate. As shown in Fig. 2 (lanes 7 and 8), the GR-copurifying kinase activity was strictly dependent on the presence of MgCl2, while in the presence of 0.1–1 mM CaCl2 no phosphorylation of mixed histones was observed. Calcium ions, when present together with magnesium, did not inhibit kinase activity (data not shown). The presence of sodium chloride in the range of 8–80 mM did not affect the phosphorylation. When the amino acid residues phosphorylated by this GR-copurifying kinase were analyzed, phosphothreonine and less phosphoserine were detected.

It was also of interest to test whether the GR-copurifying kinase phosphorylates 94-kDa GR protein. Therefore, the DEAE-Sepharose fraction with the maximal kinase activity was incubated with the GR as a substrate. As shown in Fig. 3 (lane 2), when the protein kinase fraction was incubated with the precipitated GR as an exogenous substrate, the phosphorylation of 94-kDa GR protein was observed. The protein kinase fraction alone showed a low level of the GR phosphorylation as a consequence of incomplete separation of the GR and the kinase (Fig. 3, lane 1). No phosphorylation was observed with the precipitated GR alone (Fig. 3, lane 3).
the studies, histones were phosphorylated by the kinase and with the GR prepared with different methods and from different cell types has similar characteristics (12-15). In all of these tests for kinase activity. The results presented in this study indicate that the kinase activity of GR preparation was tested with mixed histones as substrate in the absence of divalent cations (lane 6) and in the presence of 10 mM MgCl₂ (lane 7) or 1 mM CaCl₂ (lane 8). Reactions were terminated by precipitation with trichloroacetic acid, analyzed on 14% gels, and autoradiographed at -70°C for 20 h. An autoradiogram of the gel is shown.

**DISCUSSION**

In the present study, we have found a protein kinase activity associated with the purified activated rat liver GR. However, the kinase can be partially separated from the 94-kDa steroid-binding protein by chromatography on a DEAE-Sepharose column from which it elutes at a higher salt concentration than the GR itself.

The kinase has a broad specificity toward basic substrates such as various histone fractions and protamine. The phosphorylation occurs in the presence of Mg²⁺ ions and is not supported by Ca²⁺ ions. The amino acid residues phosphorylated by the GR-copurifying kinase are threonine and serine. The native structure of the GR does not seem to be necessary for the phosphorylation by the kinase because the denatured GR (precipitated by trichloroacetic acid) can also be phosphorylated.

Other authors have reported the presence of a protein kinase activity in the GR preparations obtained from anabolic and catabolic target tissues by different purification procedures (12-15). Only in the case of mouse fibroblast GR was it reported that the kinase activity is not intrinsic to the steroid-binding 94-kDa protein or to the 90-kDa receptor-associated protein (15). In that study the loss of GR phosphorylation was observed when monoclonal antibody was used, instead of polyclonal antibodies, for formation of immunocomplexes which were tested for kinase activity. The results presented in this study indicate that the kinase activity can be separated from the 94-kDa GR protein also in the case of an anabolic target tissue such as liver. It seems that the kinase copurifying with the GR prepared with different methods and from different cell types has similar characteristics (12-15). In all of the studies, histones were phosphorylated by the kinase and required the presence of Mg²⁺ ions.

We report here that the GR-copurifying kinase in addition to histones also phosphorylates the 94-kDa GR protein. This is in accordance with other reports concerning phosphorylation of GR by the GR-associated kinase in the presence of Mg²⁺ ions (12, 15), except in one case when Ca²⁺-dependent receptor phosphorylation was reported (14).

Our data suggest that the additional step(s) in GR purification might lead to the complete separation of the copurifying kinase from the 94-kDa GR protein.

Although the GR kinase seems to be distinct from the 94-kDa GR protein, it might be tightly associated with it, as it is present in GR preparations obtained by different purification methods (12-15). Those findings suggest that the association might be of the physiological relevance for the GR function. The kinase might be involved in phosphorylation of the 94-kDa GR protein that is observed in vivo in a number of tissues (3, 4, 9, 10). The GR-copurifying kinase could also participate directly or indirectly in the increased phosphorylation of rat liver ribosomal protein S6 observed after glucocorticoid administration in vivo (20, 21).

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**Addendum**—While this paper was under review, a report appeared by Hapgood et al. (22) confirming that the protein kinase activity is not intrinsic to the 94-kDa GR protein purified from rat liver.
REFERENCES


