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Identification of Y_b-glutathione-*S*-transferase as a major rat liver protein labeled with dexamethasone 21-methanesulfonate

(glucocorticoid/affinity label/glucocorticoid-binding protein)

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ABSTRACT **Dexamethasone 21-methanesulfonate, an affinity label for glucocorticoid-binding proteins, was incubated with rat liver cytosol preparations. The predominant covalently labeled component was identified as Y_b-glutathione-*S*-transferase on the basis of chromatographic properties, electrophoretic mobility, and specific retention by an anti-Y_b-immunoadsorbant. Affinity labeling of this protein was blocked by excess dexamethasone. Preferential reactivity of dexamethasone 21-methanesulfonate with the Y_b subclass of glutathione-*S*-transferase (glutathione transferase, EC 2.5.1.18) was also evident with mixtures containing the multiple forms of the enzyme. Y_b-glutathione-*S*-transferase, the nonsaturable glucocorticoid-binding component of rat liver cytosol should, therefore, be reclassified; because of its high concentration and selective interaction with steroids, this enzyme may be an intracellular glucocorticoid-binding protein and, thereby, influence transport, metabolism, and action of the steroids.**

Dexamethasone-21-methanesulfonate (dexamethasone 21-mesylate) is an effective affinity label for glucocorticoid-binding proteins (1–3). The methanesulfonate substituent at C-21 of the steroid derivative is a reactive electrophile that reacts preferentially with thiols, more slowly with amino and carboxylate moieties, but not with hydroxyl and imidazole groups (4). Rat and human glucocorticoid receptors, covalently labeled with dexamethasone 21-mesylate, have been identified by immunological methods (2, 5), and have been characterized. Dexamethasone 21-mesylate forms covalent linkages with glucocorticoid receptors in both cell extracts and whole cells (6). This steroid derivative also acts as a long-term irreversible anti-glucocorticoid in HTC cells (1–3). The covalently bound steroid–receptor complexes are activated to DNA binding forms (6–8) and then bind to the same long-terminal-repeat regions of mouse mammary tumor virus DNA (9) as the noncovalent complexes.

Glutathione *S*-transferases (glutathione transferase, EC 2.5.1.18) are a family of enzymes that catalyze reactions of glutathione (GSH) with compounds that contain electrophilic centers. Through this type of reaction, the enzyme can detoxify toxic compounds (10–15). Because these enzymes have the capacity to bind a large number of lipophilic substances including endogenous metabolites (e.g., bilirubin, heme, and bile acids), carcinogens, and various other xenobiotics (16–24) an intracellular transport function has also been ascribed to the glutathione-*S*-transferases. Multiple forms of the rat enzyme arise from dimeric combinations of subunit types designated as Y_a, Y_b, and Y_c (18, 24–35). Reports that the Y_b form of the protein selectively binds certain steroids (36) prompted this study to explore possible reactions of the protein with steroid affinity probes and to determine whether the protein retains steroid-binding capacity in the presence of other cytosolic proteins, notably glucocorticoid receptors. Evidence is presented that the Y_b subgroup of rat liver glutathione-*S*-transferase is the major cytosolic protein covalently labeled with dexamethasone 21-mesylate.

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MATERIALS AND METHODS

[6,7-^{3}H(N)]Dexamethasone 21-mesylate in methanol with a specific activity of 48.9 Ci/mmol (1 Ci = 37 GBq) and dexamethasone 21-mesylate powder (unlabeled) were obtained from New England Nuclear. Sephadex G-100, DEAE-Sephacel, protein-A Sepharose CL-6B, and CNBr-activated Sepharose-4B were obtained from Pharmacia. GSH, *S*-methyl-GSH and dexamethasone were obtained from Sigma and 1-chloro-2,4-dinitrobenzene from Aldrich. Acrylamide and other reagents used for gel electrophoresis were obtained from Bio-Rad.

Protein Preparations. Glutathione-*S*-transferases were prepared as described (35, 36). In brief, cytosol prepared from livers of male Sprague–Dawley rats (175–200 g) (105,000 × *g* supernatants) in 10 mM Tris·HCl/1.0 mM EDTA, pH 8.0 (Tris/EDTA buffer) was fractionated by chromatography on Sephadex G-100. The fractions containing glutathione-*S*-transferase activity were pooled, applied to a lysyl-GSH affinity column (35), and eluted with 10 mM *S*-methyl glutathione. The multiple forms of the enzyme were resolved by chromatography using DEAE-Sephacel (35, 36). Homogeneous preparations of Y_b forms with and without bound GSH were separated as described (37).

The IgG fraction of rabbit anti-rat Y_b-glutathione-*S*-transferase antisera (36) was purified by a protein A-Sepharose CL-6B column, and it was coupled to CNBr-activated Sepharose 4B to prepare the immunoadsorbant.

Enzymatic activities were measured using 1.0 mM 1-chloro-2,4-dinitrobenzene and 1.0 mM GSH as substrates. Absorbance at 343 nm was measured at 25°C in 0.1 M sodium phosphate, pH 6.5 (10).

Dexamethasone 21-Mesylate Labeling. Rat liver cytosol preparations (3 ml), adjusted to a protein concentration of 38 mg/ml with Tris/EDTA buffer, pH 8.0, were incubated with 10 μl of [^{3}H]dexamethasone 21-mesylate at a final concentration of 4 nM. Experiments were repeated with at least three different cytosol preparations. Reactions were allowed to proceed for 12 hr at 4°C. Aliquots of this mixture were applied to Sephadex G-100 columns or anti-Y_b immunoadsorbants. Aliquots were also analyzed directly by $NaDodSO_4$/polyacrylamide gel electrophoresis. Some cytosol preparations were diluted 1:100 in the Tris/EDTA buffer and labeled with dexamethasone 21-mesylate in the presence or absence of 1 μM dexamethasone.

Abbreviations: GSH, glutathione; dexamethasone 21-mesylate, 9-fluoro-11β,17,21-trihydroxy-16α-methylpregna-1,4-diene 3,20-dione 21-methanesulfonate.

To label glutathione-S-transferase preparations, 0.05 ml aliquots of 2.7–5.8 × 1 μM protein in Tris/EDTA buffer were incubated with [^{3}H]dexamethasone 21-mesylate (final concentrations 5–10 nM) for 12 hr at 4°C. Covalently labeled proteins were analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis.

Gel Electrophoresis. NaDodSO$_4$/polyacrylamide gels were prepared (35, 36). Gels 13 cm in length containing 10.5% acrylamide were stained with Coomassie brilliant blue R250 and destained in 10% methanol/7.5% acetic acid (vol/vol). To identify bands that were covalently labeled, gel lanes were cut into 1.0-mm-thick slices, and slices incubated with 0.6 ml of 30% hydrogen peroxide at 40°C for 12 hr. Hydrofluor solution was then added, and radioactivity was measured using an LKB Rackbeta liquid scintillation counter.

RESULTS

Rat liver cytosol preparations were incubated with dexamethasone 21-mesylate and covalently labeled proteins analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis. The predominant labeled band had a molecular weight of 24,000, but other polypeptides with apparent molecular weights of about 50,000, 67,000, and >150,000 were also labeled (Fig. 1). Minor labeled components included bands in the region of glucocorticoid receptors of M_r ≈90,000.

The distribution of [^{3}H]dexamethasone 21-mesylate among liver cytosolic proteins was examined after fractionation by gel chromatography. Small amounts of the steroid derivative were associated with the excluded, high molecular weight fractions and with lower molecular weight fractions in this system, but the major radioactive component copurified with fractions that contained glutathione-S-transferase activity (Fig. 2). This labeled fraction contained many proteins, including residual serum albumin, but analysis of NaDodSO$_4$/polyacrylamide gels (Fig. 2 *Inset*) and calculation of specific activities indicated that members of the family of glutathione-S-transferases were the major constituents of these fractions. Indeed, the principal covalently labeled component had an electrophoretic mobility coincident with that of the Y_b form of glutathione-S-transferase (Fig. 2 *Inset*).

For some affinity-labeling experiments a higher ratio of dexamethasone 21-mesylate to protein was used. Fractions containing glutathione-S-transferase activity were also labeled under these conditions (Fig. 3). The affinity reaction of this component was effectively blocked with excess dexamethasone and the labeling pattern on gels was shifted to lower molecular weight components (Fig. 3).

A substantial portion of the cytosolic fraction covalently labeled with dexamethasone 21-mesylate adhered to an immunoadsorbant resin prepared from rabbit anti-rat Y_b-glutathione-S-transferase IgG fractions (Fig. 4). The family of rat glutathione-S-transferases with subunits Y_a, Y_b, and Y_c was isolated by means of the lysyl-GSH affinity resin (35). This mixture of proteins was incubated with [^{3}H]dexamethasone 21-mesylate, and the covalently labeled products were analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis. The radioactive ligand preferentially reacted with forms of the enzyme containing Y_b subunits (Fig. 5*A*). The Y_b-type glutathione-S-transferases were purified and the enzyme with bound GSH was separated from the enzyme alone by chromatography on DEAE-Sephacel (37). The enzyme with bound GSH effectively did not react with dexamethasone 21-mesylate (Fig. 5*B*). The labeled component in this experiment was enzymatically inactive and did not adhere to the lysyl-GSH affinity resin.

DISCUSSION

The Y_b form of rat liver glutathione-S-transferase is a major protein of rat liver cytosol that reacts with [^{3}H]dexamethasone 21-mesylate. This compound has a highly reactive methanesulfonate moiety that can react with numerous cellular nucleophiles but selectively affinity labels glucocorticoid-binding proteins such as the glucocorticoid receptor. The primary species labeled in rat liver cytosol has been identified as the Y_b-glutathione-S-transferase on the basis of its electrophoretic mobility (Figs. 1 and 2) and its retention by a specific anti-Y_b-immunoadsorbant (Fig. 4). In studies on the reaction of HTC cell glucocorticoid receptors with [^{3}H]-dexamethasone 21-mesylate, Simons and co-workers (3, 6–8) observed significant labeling of a M_r 25,500 polypeptide which is about the same size as Y_b subunits. Those workers considered this protein among the nonspecific binding proteins because a dexamethasone excess of about 60-fold failed to prevent its covalent labeling. That concentration of dexamethasone may, however, be far less than the amount required to saturate this abundant protein (12–15), and thus it would not be expected to interfere with [^{3}H]dexamethasone 21-mesylate labeling. In dilute cytosolic solutions, saturating concentrations of dexamethasone blocked the affinity labeling of Y_b-glutathione-S-transferase (Fig. 3). These results suggest competitive interaction of glucocorticoids with this enzyme and indicate that affinity labeling of this protein probably reflects specific binding even though high concentrations of dexamethasone may also inhibit enzymatic activity.

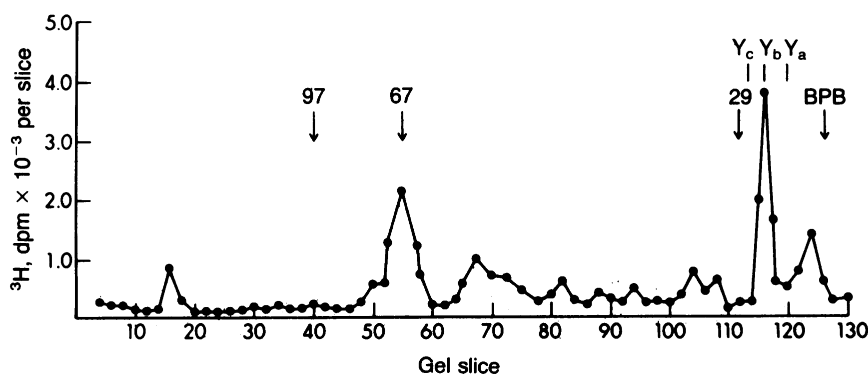

FIG. 1. NaDodSO$_4$/polyacrylamide gel electrophoresis of cytosol labeled with dexamethasone 21-mesylate. In this representative experiment, rat liver cytosol (protein concentration of 38 mg/ml) in Tris/EDTA buffer was incubated with 1.0 nM [^{3}H]dexamethasone 21-mesylate for 12 hr at 4°C, and 100 μl of the sample was applied to a NaDodSO$_4$/polyacrylamide slab gel. The molecular weight markers (×10^{-3}) were phosphorylase b (97.4), bovine serum albumin (67.0), and carbonic anhydrase (29.0), and the tracking dye was bromphenol blue (BPB). Electrophoretic mobilities of purified preparations of Y_a, Y_b, and Y_c forms of rat glutathione-S-transferase are indicated.

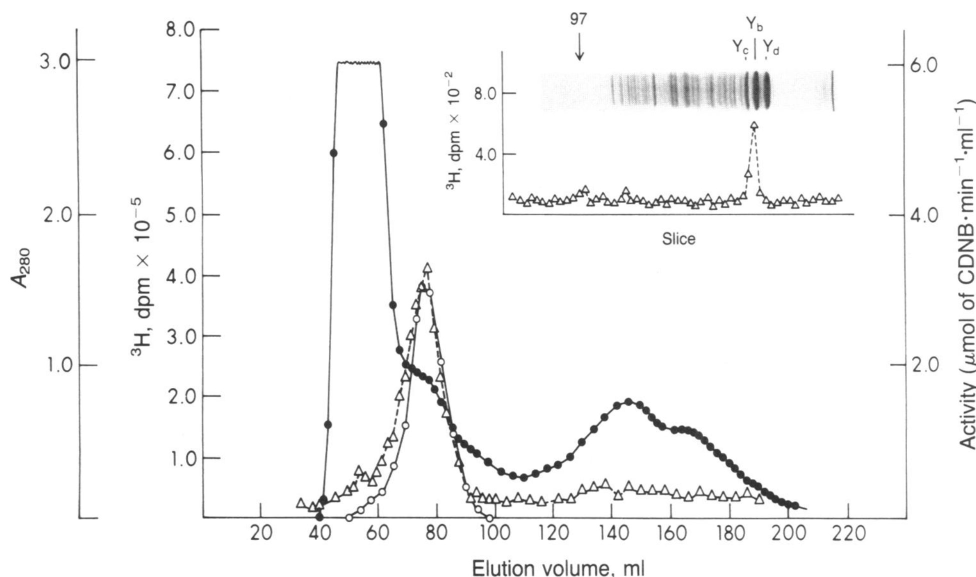

FIG. 2. Gel chromatography of cytosol affinity labeled with dexamethasone 21-mesylate. A 3.0-ml sample of rat liver cytosol adjusted to a protein concentration of 38 mg/ml in Tris/EDTA buffer was incubated with 10 μl of [^{3}H]dexamethasone 21-mesylate at 4 nM at 4°C for 12 hr. The sample was then applied to a 1.6 × 90 cm column of Sephadex G-100 and eluted with the same buffer. Fractions 70–90 ml were pooled and 100 μl was applied to a NaDodSO$_4$/polyacrylamide slab gel. (*Inset*) Photograph of the gel, which was subsequently cut into 1.0-mm slices. The radioactivity in the slices (△) was aligned with the picture of the gel. The 97,000 marker is the mobility of phosphorylase b, and electrophoretic positions of Y_a, Y_b, and Y_c glutathione-*S*-transferases are indicated. ●, A_{280}; ○, enzymatic activity; △, radioactivity.

In a mixture that contained a full complement of the multiple forms of rat liver glutathione-*S*-transferases, [^{3}H]dexamethasone 21-mesylate reacted with the Y_b polypeptide exclusively (Fig. 5*A*). The Y_a–Y_c glutathione-*S*-transferases have broad binding specificities and have been shown to be glucocorticoid binding proteins in rat liver (38). Steroid binding to Y_a and Y_c forms, however, is of lower affinity than Y_b (36). It is also possible that the cysteine residues of Y_a and Y_c are not in a proper orientation in the binding domains of these proteins to react with dexamethasone 21-mesylate.

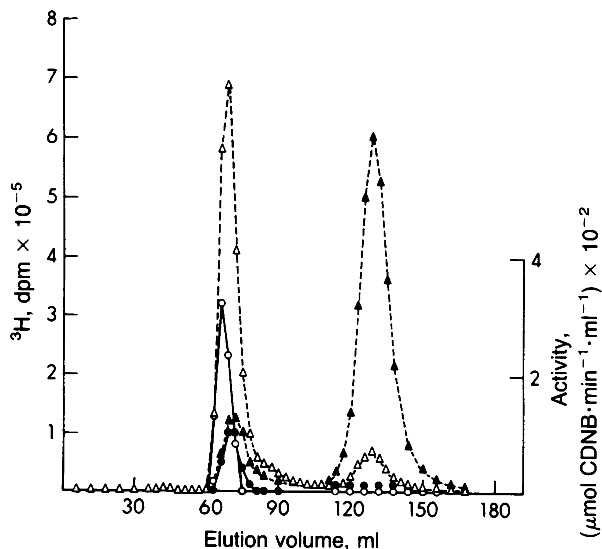

FIG. 3. Competitive effects of dexamethasone on affinity labeling patterns of cytosolic proteins. Diluted samples of rat liver cytosol preparations (3.0 ml containing protein at 0.4 mg/ml in Tris/EDTA buffer) were incubated with 10 nM [^{3}H]dexamethasone 21-mesylate and applied to a 1.6 × 90 cm column of Sephadex G-100. ----, radioactivity; ——, enzymatic activities. △ and ○, Cytosol preparations incubated with [^{3}H]dexamethasone 21-mesylate in the absence of dexamethasone; ▲ and ●, cytosol fractions preincubated with 10 μM dexamethasone.

GSH bound to the Y_b form blocked covalent labeling of the protein (Fig. 5*B*). It is possible that the bound GSH competes with protein thiols for reaction with the α-keto mesylate moiety. In the gel chromatographic fraction that contained [^{3}H]dexamethasone 21-mesylate (Figs. 2 and 3), some of the label was covalently linked to an unidentified, low molecular weight component.

The glutathione-*S*-transferases are abundant proteins with widespread tissue distribution. The purified preparations of the Y_b subclass in particular have high affinity binding capacity for steroid hormones (36). Under the conditions described in this report the extent of the reaction of dexa-

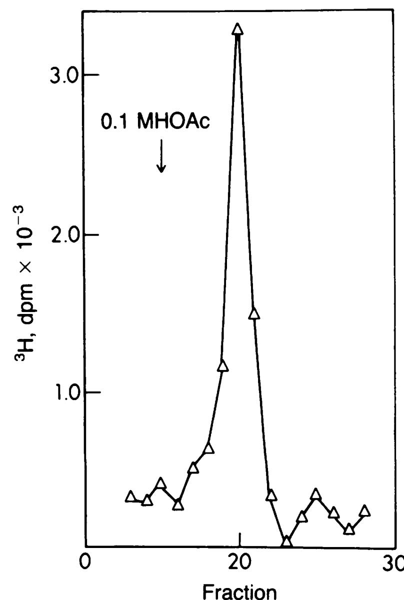

FIG. 4. Immunoadsorbant analysis of dexamethasone 21-mesylate labeling patterns of cytosolic proteins. A 1.0-ml sample of rat liver cytosol labeled with [^{3}H]dexamethasone 21-mesylate at 1.6 × 10^5 dpm per ml. Aliquots of 1.0 ml were applied to an 0.8 × 10 cm column of rabbit anti-rat Y_b glutathione IgG immunoadsorbant. Protein retained by this column was eluted with 0.1 M acetic acid.

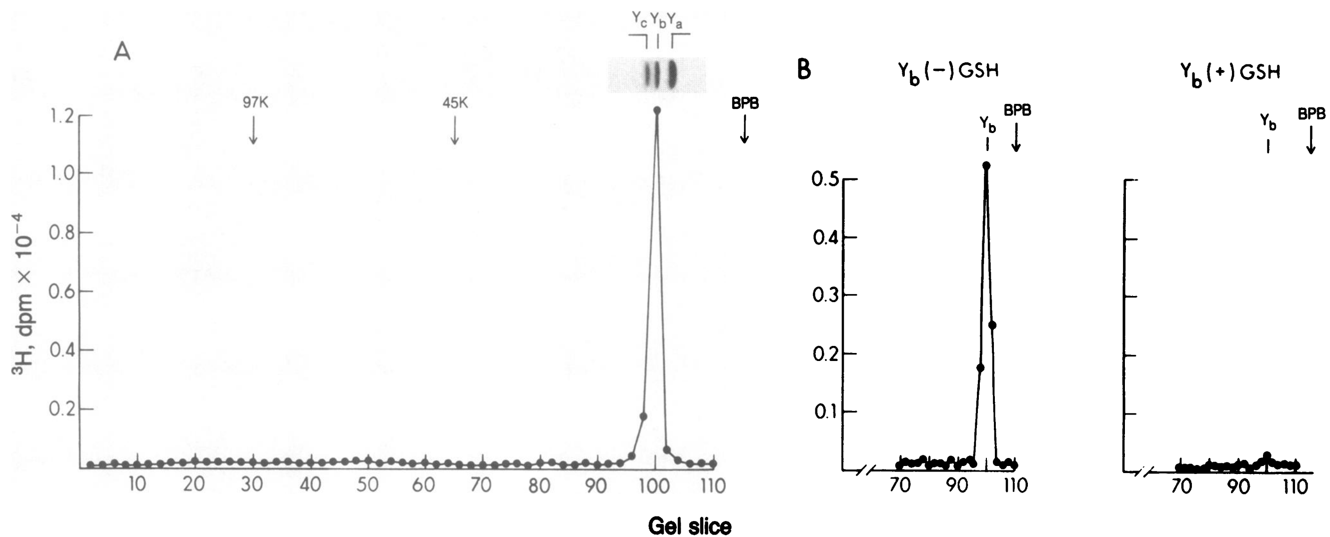

FIG. 5. NaDodSO$_4$/polyacrylamide gel electrophoresis of dexamethasone 21-mesylate labeling of rat glutathione-*S*-transferases. (*A*) A mixture with approximately equal concentrations (2.6 mg/ml) of the Y_a, Y_b, and Y_c forms of glutathione-*S*-transferase, as indicated in the stained gel, was incubated with 5.0 nM [^{3}H]dexamethasone 21-mesylate. After 12 hr at 4°C, 100 μl of the solution was applied to a NaDodSO$_4$/polyacrylamide gel and radioactivity of slices was analyzed. (*B*) A purified preparation of Y_b type glutathione-*S*-transferase after removal of bound GSH [Y_b(−)GSH] (37) (protein concentration = 1.2 mg/ml) and a fraction isolated with bound GSH [Y_b(+)GSH] (protein concentration at 2.5 mg/ml) are shown. Each sample was incubated with 5.0 nM [^{3}H]dexamethasone 21-mesylate and NaDodSO$_4$/polyacrylamide gel electrophoretic analyses were performed as in *A*. Labeling and molecular weight markers are as in Fig. 1.

methasone 21-mesylate with putative steroid receptors of rat liver cytosol is quantitatively insignificant relative to its reaction with Y_b-glutathione-*S*-transferase. This protein should not be defined as a nonspecific glucocorticoid-binding protein because subsaturating amounts of dexamethasone failed to displace trace amounts of labeled steroid. Irreversible covalent labeling may not accurately reflect cellular processes where relative dissociation rates of steroids from steroid binding proteins and receptors are important. It is also not clear whether Y_b-glutathione-*S*-transferases can govern the accessibility of glucocorticoids for interaction with receptors or whether this protein may even have a more direct role in steroid hormone action. Preferential binding to the Y_b forms may in part reflect the large quantities of the protein present in cytosol, but it is for this reason that the protein has the potential to function prominently in intracellular steroid hormone transport, metabolism, and action.

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