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Peroxisome proliferator-binding protein: Identification and partial characterization of nafenopin-, clofibric acid-, and ciprofibrate-binding proteins from rat liver

(receptor/peroxisome proliferation/affinity chromatography)

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ABSTRACT **Peroxisome proliferators (PP) induce a highly predictable pleiotropic response in rat and mouse liver that is characterized by hepatomegaly, increase in peroxisome number in hepatocytes, and induction of certain peroxisomal enzymes. The PP-binding protein (PPbP) was purified from rat liver cytosol by a two-step procedure involving affinity chromatography and ion-exchange chromatography. Three PP, nafenopin and its structural analogs clofibric acid and ciprofibrate, were used as affinity ligands and eluting agents. This procedure yields a major protein with an apparent M_r of 70,000 on NaDodSO$_4$/PAGE in the presence of reducing agent and M_r 140,000 (M_r 140,000–160,000) on gel filtration and polyacrylamide gradient gel electrophoresis under nondenaturing conditions, indicating that the active protein is a dimer. This protein has an acidic pI of 4.2 under nondenaturing conditions, which rises to 5.6 under denaturing conditions. The isolation of the same M_r 70,000 protein with three different, but structurally related, agents as affinity ligands and the immunological identity of the isolated proteins constitute strong evidence that this protein is the PPbP capable of recognizing PP that are structurally related to clofibrate. The PPbP probably plays an important role in the regulation of PP-induced pleiotropic response.**

The hypolipidemic compound clofibrate and several of its structural analogs, such as ciprofibrate and nafenopin, induce remarkable proliferation of the cytoplasmic organelle peroxisome in the hepatic parenchymal cells of rodents and certain nonrodent species including primates (1–4). The induction of peroxisome proliferation by structurally unrelated peroxisome proliferators (PP) is associated with a typical pleiotropic response characterized by hepatomegaly and significant increases in the activities of certain peroxisomal enzymes (3–6). Recent evidence indicates that PP coordinately regulate the induction of the enzymes of the peroxisomal β-oxidation system at the transcriptional level but do not alter the transcriptional rate of catalase, the marker enzyme of this organelle (7). In addition, all PP tested so far in long-term studies have been found to induce hepatocellular carcinomas in rats and mice despite the inability of these compounds to interact with and damage DNA directly (4, 8). Thus, hepatocarcinogenicity is also considered as a delayed component of the PP-induced pleiotropic response (9). Elucidation of the mechanism by which structurally diverse PP induce the highly predictable biological effects is, therefore, of prime consideration in understanding the role of peroxisome proliferation in hepatocarcinogenesis.

A cell-specific PP recognition site is suggested by the tissue and cell specificity of the PP-induced pleiotropic response (9). Further, a cytosolic protein displaying reversible, stereospecific binding to nafenopin has been identified in rat liver (10). Based on these findings, we postulated that PP evoke the characteristic pleiotropic response by a receptor-mediated mechanism (4, 9, 10). The purification, characterization, and comparison of PP-binding protein (PPbP) from varying sources is essential for understanding the tissue-specific induction as well as species differences in the magnitude of the observed pleiotropic response. In this paper we present evidence for a PPbP(s) in the hepatic cytosol of rats. A rapid two-step purification procedure described here yields a PPbP preparation that appears to be essentially homogeneous. We also show that three structurally related PP bind to the same protein moiety, as demonstrated by the immunological identity, by using a specific polyclonal antiserum directed against the purified nafenopin-binding protein.

MATERIALS AND METHODS

Preparation of Liver Cytosol and Assay for Nafenopin Binding. F344 male rats, weighing 120–150 g (body weight), were starved overnight for 18 hr, and under light ether anesthesia their livers were perfused *in situ* by way of hepatic portal vein with ice-cold 0.15 M NaCl. The livers were homogenized in 4 vol of 10 mM Hepes, pH 7.5/1 mM EDTA/1 mM dithiothreitol/10% (vol/vol) glycerol (HEDG buffer) containing 0.3 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 0.4 M KCl, and the 105,000 × *g* cytosol was prepared as described (10).

Cytosol (≈10 mg of protein per ml) was incubated with varying concentrations (50–500 pmol) of [^{3}H]nafenopin (13 Ci/mmol; purity > 99%; 1 Ci = 37 GBq) in the presence or absence of 100 nmol of unlabeled nafenopin at 4°C for 1 hr, and the specific binding was determined as described (10). Protein concentration was determined by the procedure of Bradford (11).

Purification of PPbP by Affinity Chromatography. The ligands, nafenopin, ciprofibrate, and clofibric acid, were individually immobilized on AH-Sepharose 4B by carbodiimide reaction, coupling the -COOH group of the ligands and -NH_2 groups on Sepharose beads (12, 13). The column or batch elution procedure was used to isolate the [^{3}H]-nafenopin-binding protein. Approximately 100 ml of cytosol was passed through a 5-ml nafenopin-AH-Sepharose 4B affinity column. The column was washed at 4°C with 5 vol of

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Abbreviations: PP, peroxisome proliferator(s); PPbP, PP-binding protein.

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HEDG buffer containing 0.4 M KCl and then washed with HEDG containing 1.0 M KCl. The affinity column was brought to room temperature and the two washing steps were repeated. The bound protein was eluted with HEDG buffer/0.4 M KCl containing 1 mM nafenopin and an aliquot of [^{3}H]nafenopin. Fractions (3 ml each) were collected and 0.5-ml aliquots were incubated at 4°C for 1 hr. The unbound nafenopin was removed by treatment with dextran-coated charcoal, as described (10). For batch procedure, about 300 ml of rat liver cytosol and 50 ml of affinity resin were gently mixed overnight at 4°C. The affinity resin with bound protein was collected on a sintered glass funnel and washed with 5 vol of HEDG buffer containing 0.4 M KCl at 4°C; this was followed by washing with 5 vol of HEDG buffer containing 1 M KCl and elution at room temperature with HEDG buffer containing 0.4 M KCl and 1 mM free ligand. The procedure was repeated for ciprofibrate- and clofibric acid-Sepharose affinity gels. The eluate was dialyzed against cold water at neutral pH and lyophilized by freeze-drying.

DEAE-Cellulose Chromatography. A 5-ml DEAE-cellulose gel was equilibrated in HEDG buffer and packed in the (0.8 × 15 cm) column. Affinity-purified proteins with nafenopin- or clofibric acid-Sepharose gels were incubated with [^{3}H]-nafenopin (100 pmol/ml) at 4°C for 1 hr and passed through the ion-exchange column. The column was washed with equilibration buffer and then eluted with a 0–0.5 M KCl (30 ml) gradient in HEDG buffer. Fractions (1.5 ml each) were collected at 4°C and aliquots of these fractions were analyzed for radioactivity. The protein in radioactive peak was dialyzed against water, lyophilized, and subjected to NaDodSO$_4$/PAGE. Alternatively, the affinity-purified protein was further purified on HPLC by using a Mono Q (Pharmacia) column.

NaDodSO$_4$/PAGE and Isoelectric Focusing. The electrophoresis was performed on polyacrylamide slab gels by the method of Laemmli (14). Isoelectric focusing of purified protein samples was performed at pH 3.5–10 under denaturing conditions in the presence of 9 M urea/1% Nonidet P-40 or under nondenaturing conditions (15).

Molecular Weight Estimation of PPbP. Cytosol labeled with [^{3}H]nafenopin was subjected to electrophoresis under nondenaturing conditions as described by Gillner *et al.* (16). Polyacrylamide gradient (2.5–20% acrylamide) slab gels (12 cm in length) were prepared. An ≈50-μl sample containing 200 μg of cytosolic protein was analyzed along with molecular weight marker proteins. The electrophoresis was carried out overnight for 18 hr at 4°C and 167 V. The running buffer consisted of 90 mM Tris·HCl (pH 8.35, 25°C), 80 mM boric acid, and 2.5 mM EDTA. After electrophoresis, gels were sliced (5-mm sections) and soaked in scintillation cocktail for 24 hr in the dark, and the radioactivity was measured.

Sephacryl S-300 Chromatography. A Sephacryl S-300 column (1.0 cm × 50 cm) was equilibrated with HEDG buffer/0.4 M KCl at 4°C. The affinity-purified protein was labeled with [^{3}H]nafenopin as described above and loaded under gravity flow (5 ml/hr). Fractions (0.6 ml) were collected and the radioactivity was determined. Molecular weight markers (blue dextran, alcohol dehydrogenase, bovine serum albumin, and cytochrome *c*) were used for calibrating the column.

Immunological Methods. Antibodies to purified nafenopin-binding protein were raised in a male New Zealand White rabbit. Nafenopin-binding protein preparation (containing >80% M_r 70,000 protein) was injected subcutaneously with complete adjuvant (RiBi Immunochem Research, Hamilton, MT) once weekly for 4 wk. Immunodiffusion analysis was performed as described by Ouchterlony and Nilsson (17). Immunoblotting was done by using a 1:1000 dilution of antinafenopin-binding protein antiserum and a 1:5000 dilutionof anti-rabbit IgG goat serum as described by Towbin *et al.* (18).

RESULTS

Affinity Purification of Nafenopin-Binding Protein. Approximately 0.1–0.2 μmol of nafenopin was bound to 1 ml of AH-Sepharose 4B beads when immobilized by the carbodiimide reaction. The column or batch affinity chromatography demonstrated rapid binding and high capacity for retaining >70% PPbP from rat liver cytosol. The elution profile of the [^{3}H]nafenopin-binding protein from the affinity column is presented in Fig. 1. For further studies, a batch procedure was preferred over the column procedure since it facilitated rapid and extensive washing. Washing of affinity gel with high salt buffer (1 M KCl) at room temperature enabled the removal of most of the nonspecifically bound protein. Elution of the bound protein was accomplished by incubating the affinity gel at room temperature for 1 hr with 1 mM free ligand in HEDG buffer containing 0.4 M KCl. The eluted fractions from this batch procedure also demonstrated ligand binding, when [^{3}H]nafenopin was included in the elution mixture. This single-step affinity procedure resulted in a 10,000- to 15,000-fold increase in the level of nafenopin binding (data not presented). Quantitative analysis of the exact recovery of binding activity was not completely reliable due to the presence of high concentration of free ligand in elution buffer. The eluate was dialyzed extensively and lyophilized. Lyophilization enabled quantitation of protein and subsequent electrophoresis but resulted in a substantial loss of ligand-binding activity.

NaDodSO$_4$/PAGE analysis of a typical preparation of affinity-purified nafenopin-binding protein is shown in Fig. 2. The eluate contains a major Coomassie blue-staining band with an apparent M_r of 70,000. A densitometric evaluation of the gel indicated that this band represents 70–80% of the eluted proteins in various batches. Minor bands with M_rs 79,000, 55,000, and 31,000 were also present. When liver cytosol, presaturated with the ligand, was applied to the affinity gel, the M_r 70,000 protein did not bind to the column and was present in the breakthrough fractions. The selective removal of the M_r 70,000 band by this procedure suggests the specificity of PPbP.

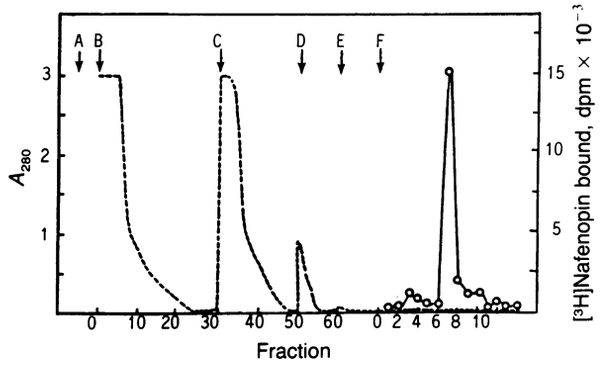

FIG. 1. Elution profile of nafenopin-binding protein on nafenopin affinity column. Approximately 150 ml of a 105,000 × *g* cytosol (A) was passed through the nafenopin-AH-Sepharose 4B affinity column. The arrows indicate the positions where changes in washing conditions occurred as indicated. B, HEDG buffer/0.4 M KCl at 4°C; C, HEDG buffer/1.0 KCl at 4°C; D, HEDG buffer/1.0 M KCl at room temperature; E, HEDG buffer/0.4 M KCl at room temperature. The column was eluted with HEDG buffer/0.4 M KCl containing 1 mM nafenopin (F). The dotted line shows the protein profile at different stages. These fractions did not reveal substantial nafenopin-binding activity. For assay of the binding activity, an aliquot of [^{3}H]nafenopin (3.8 × 10^6 dpm/μmol of nafenopin) was added to the elution buffer. Fractions (3 ml) were collected and incubated at 4°C for 1 hr; the unbound nafenopin was then removed by treatment with dextran-coated charcoal. The solid line (○—○) shows the elution profile of [^{3}H]nafenopin-bound protein.

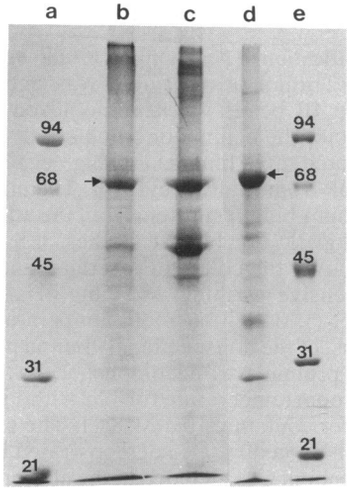

FIG. 2. NaDodSO$_4$/PAGE of PPbP. Proteins eluted from the nafenopin (lane b), ciprofibrate (lane c), and clofibric acid (lane d) affinity columns were electrophoresed in 10% polyacrylamide gels in the presence of NaDodSO$_4$ under reducing conditions. Lanes a and e contain molecular weight markers (shown as $M_r \times 10^{-3}$): phosphorylase B (M_r 94,000), bovine serum albumin (M_r 68,000), ovalbumin (M_r 45,000), carbonic anhydrase (M_r 31,000), soybean trypsin inhibitor (M_r 21,500), and lysozyme (M_r 14,000). The arrow indicates the M_r 70,000 protein present in all three preparations.

Comparison of Affinity-Purified Clofibric Acid-, Ciprofibrate-, and Nafenopin-Binding Proteins. Clofibric acid and ciprofibrate, the structural analogs of nafenopin, have been shown to competitively inhibit the specific binding of [^{3}H]-nafenopin to the protein moiety in rat liver cytosol (10). When used as affinity ligands, clofibric acid and ciprofibrate bound to the same M_r 70,000 protein as did nafenopin (Fig. 2). Cross-elution of proteins bound to clofibric acid affinity gel, with either ciprofibrate or nafenopin, also yielded the same major M_r 70,000 protein, as judged by NaDodSO$_4$/PAGE (data not presented). We are thus able to verify the identity of the M_r 70,000 band as the PPbP.

DEAE-Cellulose Chromatography of Affinity-Purified PPbP. Protein eluted from nafenopin affinity gel with excess [^{3}H]nafenopin was subjected to DEAE-cellulose chromatography for further purification of PPbP. The affinity-purified protein was loaded onto a DE-52 column, washed extensively with HEDG buffer until no detectable protein or radioactivity was recovered, and then eluted by a 0–0.5 M KCl gradient in HEDG buffer. A radioactive peak coinciding with protein peak was detected at 0.17–0.2 M KCl (Fig. 3*A*). This peak contained an enriched M_r 70,000 protein band when analyzed by NaDodSO$_4$/PAGE (Fig. 3*B*). Alternatively, the affinity-purified protein was further enriched on Mono Q column; the protein eluted at 0.23 M NaCl displayed a M_r 70,000 band on NaDodSO$_4$/PAGE (Fig. 4).

Molecular Weight Determination of PPbP. When [^{3}H]-nafenopin-labeled rat liver cytosol was electrophoresed under nondenaturing conditions using a polyacrylamide gradient gel (2.5 to 20% acrylamide), the native form of the PP-binding species migrated as a distinct radioactive band (data not presented). This band corresponds to a protein of $M_r \approx 140{,}000$. When similarly analyzed, the cytosol incubated with [^{3}H]nafenopin in the presence of excess unlabeled nafenopin demonstrated a discernible inhibition of binding. Likewise, the affinity-purified protein also revealed a single protein band at M_r 140,000 under nondenaturing electrophoretic conditions (data not presented).

The gel-filtration profile on a Sephacryl S-300 column of affinity-purified [^{3}H]nafenopin protein showed a radioactive peak corresponding to an M_r of $\approx 158{,}000$. Further analysis of the protein in this peak by NaDodSO$_4$/PAGE demonstrated a distinct M_r 70,000 Coomassie blue-stained band (data not presented).

The pI of the nafenopin-binding protein under nondenaturing conditions was estimated at 4.2 and under denaturing conditions the pI was estimated at 5.6.

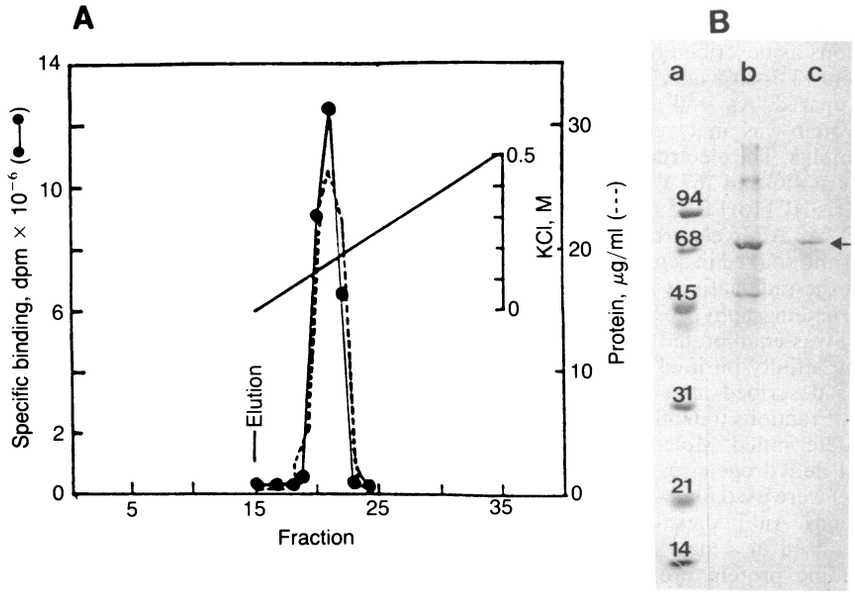

FIG. 3. Elution profile on DEAE-cellulose (DE-52) chromatography. (*A*) The affinity-bound protein was eluted from the nafenopin affinity column in HEDG buffer containing 1 mM nafenopin and ≈100 pmol of [^{3}H]nafenopin per ml. The eluate was kept on ice for 1 hr and passed through a 5-ml DE-52 column preequilibrated in HEDG buffer. The column was washed with HEDG buffer and eluted with 0–0.5 M KCl in HEDG buffer (30-ml elution gradient), and 1.5-ml fractions were collected and analyzed for protein (---) and radioactivity (●—●). (*B*) Peak fractions from the DE-52 eluate were dialyzed, concentrated, and electrophoresed in 10% polyacrylamide gels in the presence of NaDodSO$_4$. Lane a, molecular weight markers (shown as $M_r \times 10^{-3}$); lane b, nafenopin affinity gel-purified proteins; lane c, proteins eluted from the DE-52 column.

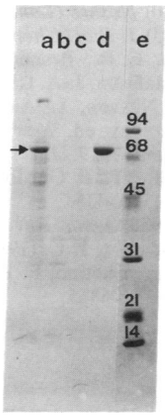

FIG. 4. $NaDodSO_4$/PAGE profile of proteins eluted from Mono-Q HPLC column. The protein eluted from the clofibric acid affinity column (lane a) was loaded on a Mono Q column equilibrated with Tris·HCl (pH 7.4) and eluted with a NaCl gradient; lane b, unbound fraction; lane c, proteins eluted at 0.1 M NaCl; lane d, proteins eluted at 0.23 M NaCl; lane e, molecular weight standards (shown as $M_r \times 10^{-3}$). The electrophoresis was carried out under reducing conditions.

Immunological Properties. The polyclonal antibodies raised against nafenopin-binding protein gave a single precipitin line with affinity-purified binding protein when analyzed by the Ouchterlony double-diffusion method (not illustrated). Immunoblotting of rat liver cytosol revealed that the antibodies reacted with a M_r 70,000 protein. Immunoblotting using antinafenopin-binding protein antibodies revealed cross-reactivity with the affinity-purified clofibric acid- and ciprofibrate-binding proteins (Fig. 5). The PPbP did not react with the antibody against rat albumin.

DISCUSSION

The present paper describes a two-step procedure for the isolation of the nafenopin-binding protein from rat liver cytosol. The isolation of the same protein using two other PP as affinity ligands constitutes strong evidence that this protein is a PPbP that may be capable of recognizing PP that are the structural analogs of the hypolipidemic drug clofibrate. These drugs are readily coupled to $-NH_2$ groups on Sepharose beads to generate an affinity matrix. The adsorption of a specific protein(s) from rat liver cytosol to the immobilized PP and the selective elution of this protein from affinity columns by structurally similar analogs facilitated the identification and further purification of the PPbP. The acidic pI of PPbP allows its retention on the DEAE-cellulose or Mono Q column, thus enabling further purification of this protein to virtual homogeneity.

FIG. 5. Immunoblot (immunoperoxidase stained) of affinity-purified proteins. Samples of affinity-purified proteins from rat liver cytosol were separated on $NaDodSO_4$/PAGE, transferred electrophoretically onto a nitrocellulose paper, and immunoblotted against antiserum raised against PPbP. The immunoblots show an immunoperoxidase-stained band of M_r 70,000 (arrow) in clofibric acid-binding protein (lane a), ciprofibrate-binding protein (lane b), and nafenopin-binding protein (lane c).

The two-step purification protocol yielded a preparation that was markedly enriched for a protein of M_r 70,000, as evaluated by $NaDodSO_4$/PAGE. The native M_r of the PPbP was estimated to be in the range of 140,000–160,000 by gel filtration and by electrophoresis under nondenaturing conditions. Thus, the PPbP appears to be a dimer with a subunit M_r of 70,000. The pI (4.2–5.6) of nafenopin-binding protein suggests that it has a strong negative charge and this feature may be important for ligand binding. Prebinding of nafenopin to the PPbP in rat liver cytosol blocked the adsorption of this protein to the nafenopin-Sepharose affinity column, suggesting that PPbP binds specifically to the immobilized nafenopin. The specificity of this binding was further established by cross-eluting the same protein with closely related ligands. The similarity of the binding proteins isolated from nafenopin, clofibric acid, and ciprofibrate affinity columns was also confirmed by immunoblotting using the polyclonal antibody raised against nafenopin-binding protein. Further studies are, however, required to establish that the molecular parameters of binding proteins isolated with three different affinity ligands are identical.

It is now increasingly recognized that the nature and extent of biological responsiveness of an organism to certain classes of chemicals and biological molecules depend upon the presence of specific molecules or receptors in responsive cells that are capable of recognizing such ligands (19–21). On the basis of the available data, we suggested that the predictable pleiotropic response induced by PP in liver of rodents may be mediated by a specific cytosolic-binding protein or receptor (9, 10). Progress in delineating the physicochemical properties of the postulated PP receptor has been slow, due, in part, to the remarkable variability in the potency and structure of the known PP (2, 4) and to the relatively low-affinity binding of the ligand to the receptor (10). This may be due, in part, to the low specific activity of the currently available radiolabeled ligand nafenopin. As reviewed elsewhere (9), the diverse nature of the chemical structures of the agents known to induce essentially the same pleiotropic response makes it difficult to postulate that all PP act by interacting with a single type of binding protein with a single recognition site, unless molecular modeling of PP were to demonstrate similarities in shape. The possibility that more than one binding site may be present on the PPbP or that more than one type of PPbP may exist in the liver, however, cannot be excluded.

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