Identification of cytosolic peroxisome proliferator binding protein as a member of the heat shock protein HSP70 family

(peroxisomes/drug-binding protein)

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ABSTRACT   Clofibrate and many of its structural analogues induce proliferation of peroxisomes in the hepatic parenchymal cells of rodents and certain nonrodent species including primates. This induction is tissue specific, occurring mainly in the liver parenchymal cells and to a lesser extent in the kidney cortical epithelium. The induction of peroxisomes is associated with a predictable pleiotropic response, characterized by hepatomegaly, and increased activities and mRNA levels of certain peroxisomal enzymes. Using affinity chromatography, we had previously isolated a protein that binds to clofibric acid. We now show that this protein is homologous with the heat shock protein HSP70 family by analysis of amino acid sequences of isolated peptides from trypsin-treated clofibric acid binding protein and by cross-reactivity with a monoclonal antibody raised against the conserved region of the 70-kDa heat shock proteins. The clofibric acid-Sepharose column could bind HSP70 proteins isolated from various species, which could then be eluted with either clofibric acid or ATP. Conversely, when a rat liver cytosol containing multiple members of the HSP70 family was passed through an ATP-agarose column, and eluted with clofibric acid, only P72 (HSC70) was eluated. These results suggest that clofibric acid, a peroxisome proliferator, preferentially interacts with P72 at or near the ATP binding site.

Clofibrate, including many of its structural analogues and certain other compounds that are not structurally related to clofibrate, induces proliferation of peroxisomes in hepatic parenchymal cells of rats and mice (1–3). This induction is associated with a predictable pleiotropic response that is characterized by hepatomegaly, increase in the number of peroxisomes in liver cells, and marked increase in the mRNA levels and the activities of certain peroxisomal enzymes (3–5). Chronic exposure to peroxisome proliferators results in the development of hepatocellular carcinomas in rats and mice, despite the fact that these compounds are nonmutagenic (3, 6). Available evidence indicates that xenobiotic-induced peroxisome proliferation occurs to a maximum extent mainly in the parenchymal cells of the liver and to a limited degree in the epithelial cells of the kidney cortex, though other tissues appear essentially unresponsive (7, 8). Given the structural diversity of peroxisome proliferators, it would appear that these agents exert their cell-specific pleiotropic effects by a common pathway (8). The demonstration of a reversible, specific, albeit weak binding of nafenopin, a peroxisome proliferator, to a cytosolic protein in liver (9) led to the proposal that peroxisome proliferators evoke their action possibly by a receptor-mediated mechanism (3, 8). However, the identity and nature of this postulated receptor

remain unknown and some have questioned the existence of such a receptor (10). In an effort to address this issue, we have undertaken studies to identify and characterize peroxisome proliferator binding protein(s) (PPbPs) in rat liver with the anticipation that such proteins might shed some light on the mechanism of tissue-specific induction of peroxisome proliferation. A PPbP from rat liver cytosol was isolated by affinity chromatography using three different affinity ligands—clofibric acid, ciprofibrate, and nafenopin—each bound to Sepharose (11). The present study demonstrates that the PPbP is identical with the constitutively expressed member (P72, HSC72) of the heat shock protein HSP70 family.

MATERIALS AND METHODS

Purification of Clofibric Acid Binding Protein. Clofibric acid was immobilized on Sepharose by coupling its -COOH group to the amino group of AH-Sepharose using the carbodiimide reaction (12). An anti-albumin-Sepharose column was made by linking anti-albumin IgG to CnBr-activated Sepharose (12).

F344 male rats, weighing 120–150 g, were starved overnight. Under ether anesthesia, the liver was perfused via the portal vein with ice-cold saline and homogenized in 4 vol of 10 mM Hepes, pH 7.5/1 mM EDTA/1 mM dithiothreitol/10% glycerol (HEDG buffer) containing 0.3 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine hydrochloride, 0.4 M KCl, and the 100,000 × g supernatant prepared as described earlier (9, 11). The clofibric acid binding protein from liver cytosol was prepared as described (11), with minor modifications. The protein eluted from the clofibric acid-Sepharose column was diluted with HEDG buffer so as to reduce the KCl concentration to 0.2 M. The eluate was then passed through an anti-albumin-Sepharose column, to remove traces of albumin present in the eluate, as albumin is known to bind nonspecifically to clofibric acid and certain other compounds. The protein that was unbound to the anti-albumin-Sepharose column was dialyzed against deionized water and then concentrated by lyophilization.

Partial Amino Acid Sequence of Clofibric Acid Binding Protein. The lyophilized protein was analyzed on NaDod-SO₄/PAGE according to Laemmli (13), stained briefly with Coomassie blue R, and destained with 50% methanol/7% acetic acid. The 70-kDa band was cut out from the gel, and the protein was extracted according to Hunkapiller et al. (14). This protein was lyophilized, dissolved in 50 µl of water, and precipitated with 500 µl of ethanol. The precipitated protein was then dissolved in 100 µl of NH₄HCO₃ and digested with 1.5 µg of trypsin. The resulting peptides were separated on reverse-phase HPLC; three of the peptides were subjected to

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Abbreviations: PPbP, peroxisome proliferator binding protein; HSP, heat shock protein.

automated Edman chemistry on an Applied Biosystems model 477 pulsed liquid-phase sequencer equipped with an on-line model 120A phenylthiohydantoin analyzer (Applied Biosystems).

Purification of HSPs. The HSP70-related HSPs from rabbit reticulocytes, chicken, human, and *Escherichia coli* were purified by DEAE and ATP-Sepharose affinity chromatography (15). Purified HSPs were then passed over clofibric acid-Sepharose, and the column was washed sequentially with HEDG buffer containing 0.4 M and 1 M KCl. The column was then eluted with either 3 mM clofibric acid or 3 mM ATP. The eluted proteins were dialyzed, lyophilized, and subjected to NaDodSO$_4$/PAGE. A 100,000 × g supernatant prepared from a 10% rat liver homogenate in 10 mM Tris·HCl (pH 7.4) containing 20 mM NaCl, 3 mM MgCl$_2$, 0.1 mM EDTA, and 15 mM dithiothreitol was passed through an ATP-Sepharose column (15). The column was washed with homogenization buffer containing 0.5 M NaCl and then eluted with buffer containing 3 mM clofibric acid.

Other Procedures. Immunoblotting was done according to Towbin (16), using an anti-HSP70 antibody, 7.10, which recognizes the conserved epitope of the HSP70 protein family (17, 18). The antibody bound to the filter was detected by either anti-IgG tagged with peroxidase or ^{125}I-labeled protein A as the secondary antibody. Protein concentration was determined by the method of Bradford (19).

RESULTS

Purification of PPbP. A protein from rat liver cytosol that bound to the clofibric acid-Sepharose affinity column was purified to apparent homogeneity (Fig. 1). This purified protein was digested with trypsin and peptide fragments were separated as described in *Materials and Methods*. Analysis of three peptides (peptides A, B, and C) revealed the amino acid sequence, shown in Fig. 2. Peptides A, B, and C are identical to segments of the amino acid sequence derived from the nucleotide sequence of a cloned rat gene encoding a constitutively expressed member of the HSP70 protein family referred to in the literature as HSP72, P72, or HSC70. We will use P72 herein hereafter. Amino acid residues 169–171 and 221–236 of the published sequence are identical to peptides A and B, respectively (20, 21). Peptide C also is identical from amino acid residues 138 to amino acid 152. Based on this
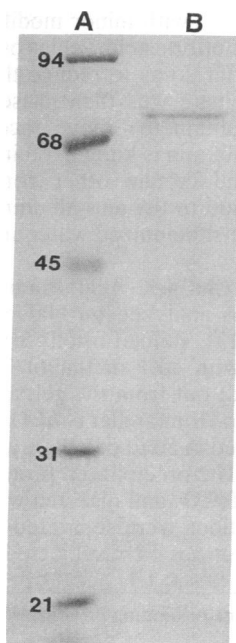
Peptide A: 5'- D A G T H A G L N V L R - 3'
HSP 72:   5'- D A G T H A G L N V L R - 3'

Peptide B: 5'- S T A G D T H L G G E D F D N R - 3'
HSP 72:   5 - S T A G D T H L G G E D F D N R - 3'

Peptide C: 5'- T V T N A V V T V P A Y F N D - 3'
HSP 72:   5'- T V T N A V V T V P A Y F N D - 3'

FIG. 2.   Amino acid sequence of three peptides of the clofibric acid binding protein. The clofibric acid binding protein was digested with trypsin (see text); three of the peptides were subjected to automated Edman chemistry. The matching peptides of HSP72 are shown for comparison. Peptide A is from amino acid residues 160–171; peptide B is from residues 221–236; peptide C is from residues 138–152.

identity, we conclude that the PPbP corresponds to a member of the HSP70 gene family and is most closely related to P72.

Binding of HSP70 Protein Family to Clofibric Acid. The identity of clofibric acid binding protein with that of P72 was further established as follows. First, we ascertained that anti-HSP antibodies cross-react with purified clofibric acid binding protein. On Western blot analysis, the purified clofibric acid binding protein (Fig. 3) reacted with monoclonal antibody raised against the conserved epitope of the HSP70 protein family (17, 18). This antibody also recognizes the glucose-regulated protein GRP78, present in mouse liver (Fig. 3, lane B). The purified clofibric acid binding protein is recognized by the monoclonal antibody and corresponds to a position similar to P72.

We next determined whether other members of the HSP70 protein family bind to clofibric acid affinity matrix, since studies on affinity chromatography using ATP as the ligand have shown that all members of the HSP70 family, including P72, the inducible HSP70, and GRP78, can bind and be coeluted. Fig. 4 shows the chromatographic profile of purified rabbit reticulocyte HSP on passage through the clofibric acid-Sepharose column. Most of the rabbit reticulocyte HSP that was applied bound to the column and was eluted with clofibric acid (Fig. 4*A*). These results also indicated that the

FIG. 1. NaDodSO$_4$/PAGE of purified PPbP used for amino acid sequence analysis. The protein (5 μg) eluted from the clofibric acid-Sepharose column followed by preparative gel electrophoresis (lane B) was electrophoresed in a 10% polyacrylamide gel in the presence of NaDodSO$_4$ under reducing conditions and stained with Coomassie blue R. Lane A shows molecular weight markers: phosphorylase B (M_r 94,000), bovine serum albumin (M_r 68,000), ovalbumin (M_r 45,000), carbonic anhydrase (M_r 31,000), and soybean trypsin inhibitor (M_r 21,500).
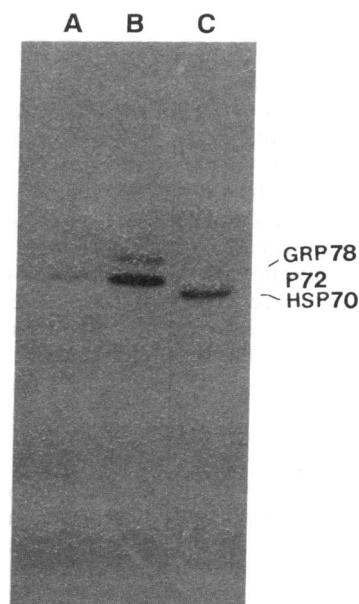

FIG. 3.   Immunoblot analysis using anti-HSP70 antibody. Purified clofibric acid binding protein (lane A) and HSP70 protein family proteins purified from mouse (lane B) and human 293 cell line (lane C) were electrophoresed and transferred to nitrocellulose and allowed to react with anti-HSP70 antibody. The antigen–antibody complexes were visualized by ^{125}I-labeled protein A. The filter was then subjected to autoradiography. The positions of HSP70, P72, and GRP78 are indicated.

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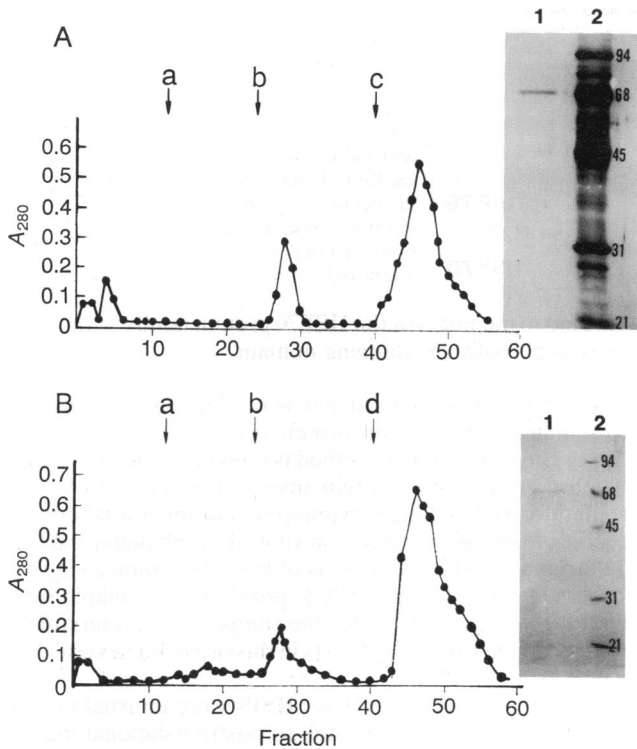*Proc. Natl. Acad. Sci. USA 87 (1990)* 5295

FIG. 4. (*A*) Elution profile of purified rabbit reticulocyte HSP70 on clofibric acid-Sepharose affinity column. Rabbit reticulocyte HSP was dialyzed against HEDG buffer/0.4 M KCl and passed through the clofibric acid-Sepharose column. The arrows indicate the positions where changes in washing conditions occurred: (a) HEDG buffer/0.4 M KCl at room temperature; (b) HEDG buffer/1 M KCl at room temperature; (c) HEDG buffer/0.4 M KCl containing 3 mM clofibric acid. In *B*, the column was eluted with 3 mM ATP (arrow d). (*Insets*) NaDodSO$_4$/PAGE of the protein eluted with clofibric acid (*A*, lane 1) and ATP (*B*, lane 1); lanes 2 show molecular weight standards (shown as $M_r \times 10^{-3}$).

interaction between P72 and clofibrate was not specific to liver P72. The purified rabbit HSP, which was bound to clofibric acid-Sepharose, could also be eluted with 3 mM ATP (Fig. 4*B*), suggesting that clofibric acid and ATP compete with one another for binding to HSP. The protein that eluted from the clofibric acid-Sepharose column with either clofibric acid or ATP, when subjected to NaDodSO$_4$/PAGE,

showed the presence of a single protein band corresponding to an apparent molecular mass of 72 kDa (Fig. 4 *A* and *B*).

To determine whether clofibrate would interact with other members of the HSP70 family, we examined the binding of the HSP70 protein family to the clofibric acid-Sepharose column. HSP70 proteins corresponding to HSP70 and P72, purified from chicken reticulocytes and a human 293 cell line, bound to the clofibric acid-Sepharose column, whereas the HSP70 homologue from *E. coli* dnaK did not bind (data not shown).

The proteins comprising the HSP70 family have an ATP binding site and can be easily purified by affinity chromatography on ATP-agarose (15). Fig. 5*A* shows the chromatographic profile obtained on passage of a 100,000 × *g* rat liver supernatant through an ATP-agarose column (linked through C-8). On immunoblotting with the monoclonal antibody raised against the conserved region of the HSP family, the protein that eluted from the ATP-agarose column with 3 mM clofibric acid showed the presence of P72 (Fig. 5*B*), demonstrating that clofibric acid is capable of eluting P72 bound to ATP-agarose, indicating once again that clofibric acid and ATP may possibly be competing for the same site on the protein molecule. However, clofibric acid, unlike ATP, did not eluate GRP78 from the ATP agarose column.

Tissue Distribution of the HSP70 Protein Family. The distribution of the HSP70 protein family in 10 different organs of normal rat and a rat treated for 12 weeks with ciprofibrate (0.025%, wt/wt), a peroxisome proliferator, was ascertained by immunoblotting (Fig. 6) to determine whether the tissue specificity of peroxisome proliferator response could be attributed to differences in P72 levels in different tissues. P72 was an abundant protein in all tissues and was found in liver, lung, spleen, intestine, heart, testis, and brain of normal rats. In brain only P72 was detected, whereas in most other tissues GRP78 was also present at variable levels. Testis contained GRP78, P72, and very high levels of HSP70 (Fig. 6). Chronic administrations of ciprofibrate did not cause any detectable change in the amount of these proteins in any given tissue when compared with controls.

DISCUSSION

Several structurally dissimilar peroxisome proliferators induce a highly predictable pleiotropic response—the hallmark of which is the remarkable increase in the number of peroxisomes in liver parenchymal cells (1–6). These agents also induce proliferation of peroxisomes in hepatocytes trans-
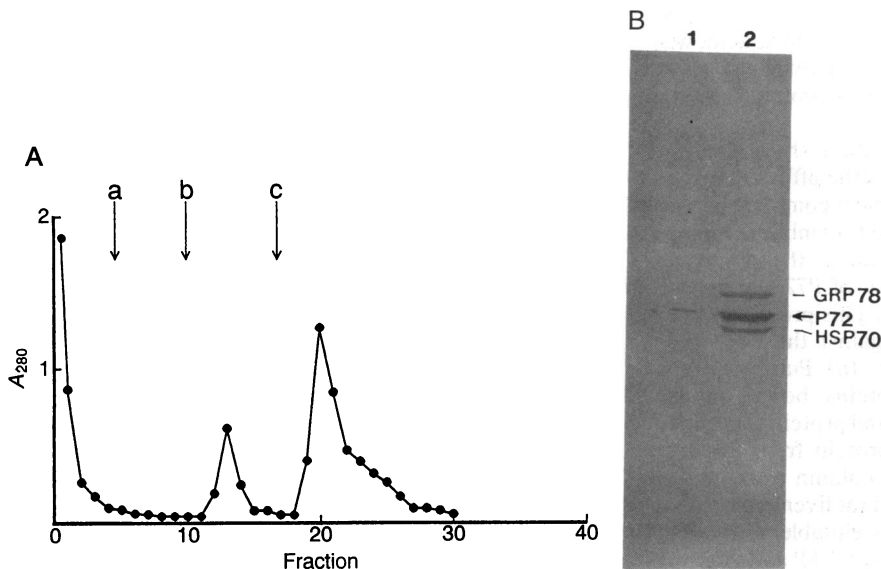

FIG. 5. (*A*) Elution profile of rat liver 100,000 × *g* supernatant on an ATP-agarose column. A rat liver 100,000 × *g* supernatant was applied on an ATP-agarose column. The column was then washed with 10 mM Tris·HCl (pH 7.4) containing 20 mM NaCl, 3 mM MgCl$_2$, 0.1 mM EDTA, and 15 mM dithiothreitol (a); this was followed by a wash with the same buffer containing 0.5 M NaCl (b). The column was then eluted with 3 mM clofibric acid (c), and the eluted protein was immunoblotted with anti-HSP70 (*B*, lane 1). Lane 2 shows human 293 cell line HSP70 proteins. The arrow indicates the position of P72.
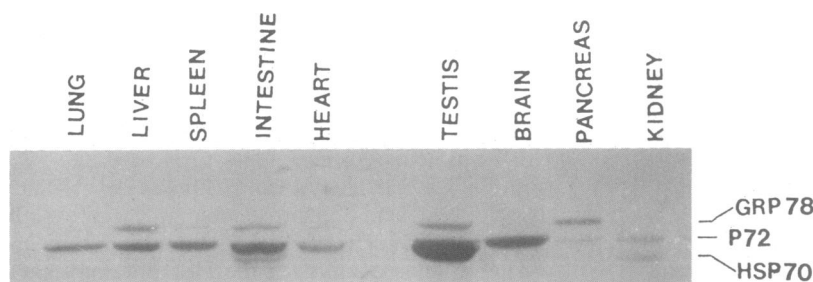
FIG. 6. Immunoblot of different tissues from normal rat. Seventy-five micrograms of total protein from lung, liver, spleen, intestine, heart, testis, brain, pancreas, and kidney was subjected to NaDodSO₄/PAGE, transferred onto nitrocellulose, and immunoblotted with antibody 7.10. The positions of GRP78, P72, and HSP70 are noted.

planted at extrahepatic locations, such as the anterior chamber of the eye and the interscapular fat pads (8, 22). In addition, these agents also increase the number of peroxisomes in pancreatic hepatocytes that differentiate in the pancreas under certain experimental conditions, whereas the pancreatic cells that are adjacent and in direct contact with the hepatocytes do not respond to the inductive effects of peroxisome proliferators (23).

Based on hepatocyte specificity of peroxisome proliferator-induced alterations, it was proposed that these chemicals exert their biological effects by a common pathway—namely, a receptor-mediated mechanism (3, 24). Although specific binding of nafenopin, a potent peroxisome proliferator to liver cytosol, has been observed (9), little progress has been made toward purification and molecular characterization of this moiety. This is, in part, due to the remarkable structural diversity and potency of identified peroxisome proliferators (2, 3), the low specific activity of the currently available radiolabeled ligand, nafenopin (9), and the difficulties in using such a ligand in the receptor assays in classical density gradient ultracentrifugation.

Since, in recent years, affinity chromatography has been proven to be effective in the purification of receptors and drug binding proteins (25), we began studies on the identification of PPbPs in an effort to elucidate the mechanism of their action. Using the affinity chromatographic approach, we have previously described the purification of a PPbP from rat liver cytosol (11). This involved the coupling of the -COOH group of the ligand clofibric acid with the -NH₂ group on AH-Sepharose 4B beads by the carbodiimide reaction (12). A M_r 70,000 protein present in the rat liver cytosol that adsorbed to this affinity matrix was eluted with clofibric acid (11). A similar M_r 70,000 protein was isolated when two other peroxisome proliferators—nafenopin and ciprofibrate, the structural analogues of clofibrate—were used as the affinity ligands (11). The protein bound to the affinity matrix displays specificity with regard to elution in that Wy-14643, a peroxisome proliferator structurally unrelated to clofibric acid, was ineffective in eluting this protein (K.A., unpublished). Likewise, phenobarbital also failed to elute this protein from the affinity matrix.

The results of the present investigation show that the peptide sequences of three fragments of the affinity-purified clofibric acid binding protein exhibit almost complete homology to P72, the constitutively expressed member of the rat HSP70 protein family (20, 21). Furthermore, the identity of the clofibric acid binding protein as that of P72 was further established by the following criteria. (*i*) This protein reacted with a monoclonal antibody raised against the conserved epitope of the HSP70 protein family. (*ii*) Purified rabbit reticulocyte and human HSP70 proteins bound to the clofibric acid affinity matrix and the bound protein was eluted with clofibric acid. (*iii*) The HSP70 protein from rat liver cytosol that bound to the ATP affinity column was elutable with clofibric acid. (*iv*) The protein from rat liver cytosol that bound to the clofibric acid column was elutable with either ATP or clofibric acid. Thus it appears that ATP and clofibric

acid bind to members of the HSP70 protein family at the same site or a partially overlapping domain.

The biological significance of the binding *in vitro* of P72 to the clofibric acid affinity matrix is not known. The clofibric acid-binding liver cytosolic protein (i.e., P72) isolated by this affinity chromatographic method is unlikely to be the specific receptor or the only protein involved in the peroxisome proliferator-induced gene expression and the attendant cell-specific pleiotropic response, in view of the abundance of this P72 protein in almost all organs of the rat examined (Fig. 6). The tissue distribution of [¹⁴C]ciprofibrate, by whole body autoradiography, showed that the compound is preferentially localized to the liver and persists in this organ for several days (26).

To examine whether the liver HSPs were unusual in their binding properties, perhaps due to posttranslational modifications including methylation (27) and fatty acid modifications (28) that are known to occur on HSP proteins, we examined whether HSP70 proteins from other organisms, including rabbit, chicken, and human, bound to clofibric acid-Sepharose. Our results clearly indicate that P72 and HSP70 from these sources can bind to the clofibric acid affinity matrix. If the specific binding of this drug to P72 is responsible for the retention in the liver, then one should expect the presence of this drug in a variety of tissues in view of the rather generalized tissue distribution of HSP70 family proteins assuming that the *in vitro* binding of this protein to clofibric acid truly reflects the *in vivo* situation. It is conceivable that this rather liver-specific distribution of a peroxisome proliferator may be due to the intracytoplasmic binding of these ligands to P72, provided the absence in nonhepatic tissues reflects differences in the uptake or internalization of the compound attributable to a specific plasma membrane moiety. Alternatively, it is likely that the postulated peroxisome proliferator receptor is found probably in minute quantities in responsive tissues and that the P72 associates with this receptor *in vivo*. The inability to detect and isolate the putative receptor with the clofibric acid affinity column may be due to the blocking of the -COOH of this ligand as it is linked to the -NH₂ of AH-Sepharose. The -COOH could be responsible for binding to the postulated receptor. Under the present experimental conditions, the clofibric acid affinity matrix is, therefore, binding to the P72 but not the receptor. Attempts to generate affinity columns leaving the -COOH of the ligand free are required to clarify this matter.

It is pertinent to recall that the glucocorticoid receptor (29), steroid hormone receptor (30), and the 2,3,7,8-tetrachlorodibenzo-*p*-dioxin receptor (31) are associated with another HSP, HSP90. It is postulated that receptor–HSP90 complex dissociates upon the binding of the ligand to this receptor *in vivo*, thus allowing for the translocation of the receptor–ligand complex to the nucleus where the binding to the specific regulatory regions of the gene occurs (29, 30). If the peroxisome proliferator binds to the specific receptor in target cells, then it is likely that the P72 may complex with this receptor and thus play a critical role in the interaction of the receptor with the DNA. Additional studies are also

required to ascertain whether any of the hepatocyte plasma membrane proteins selectively recognize the peroxisome proliferator. Recent evidence indicates that peroxisome proliferators alter and modify the expression of certain hepatocyte plasma membrane proteins (32).

In eukaryotes, the major HSP, HSP70, belongs to a class of related proteins that includes proteins present in the cytoplasm of normal cells and are constitutively expressed—HSP72, the glucose-regulated protein GRP78, which is located in the endoplasmic reticulum, and the heat-inducible protein HSP70 (17). In rat liver, members of the HSP70 protein family represent up to ≈1% of cytosolic proteins. The precise function of this protein is not well understood, but it has been shown to catalyze the ATP-dependent disruption of clathrin coats of clathrin-coated vesicles *in vitro* (33) and in the assembly of a variety of protein complexes *in vivo* (34). Recently it has been shown to play a major role in the translocation of proteins into the endoplasmic reticulum and mitochondria (35, 36).

Another possibility based on the involvement of P72 and HSP70 in the transport of proteins to the endoplasmic reticulum and the mitochondria (35, 36) is that binding of clofibric acid to P72 or HSP70 at the ATP binding site or an overlapping site may prevent the binding of ATP and in so doing disrupt the normal function of the protein, leading to the proliferation of the peroxisomes. It should be noted that the family of HSPs is known to be translocated from the cytoplasm to the nucleus during the cell cycle and into the nucleolus on heat shock (37). The 72-kDa species associated with the nuclei in control and heat shock cells can be rapidly released by ATP, with the concomitant hydrolysis of ATP. The inhibition of binding of ATP, or the binding of clofibric acid itself, might trigger events that lead to the transcriptional activation of a number of genes, resulting in the increased synthesis of the related proteins.

Finally, when a 100,000 × *g* supernatant of human 293 cells (in which the inducible HSP70 is the predominant species of the HSP protein family) was passed through the clofibric acid-Sepharose column, P72 and HSP70 were isolated. This is in contrast to the rat liver cytosol, where P72 was the major protein isolated. It should be noted that the ATP binding sites of HSP70 and P72 are localized to a highly conserved region of the protein (38); thus both forms can bind to clofibric acid affinity matrix. Eukaryotic cells contain GRP78 (immunoglobulin binding protein), P75 (mitochondrial), P72 (major constitutive), and HSP70 (major inducible). Of these, the two proteins that are most closely related are P72 and HSP70 (85% identity). Interestingly, the clofibric acid affinity column binds preferentially to these two proteins and not P75 and GRP78, whereas all four members of the HSP70 family bind to the ATP affinity columns. In human cells, HSP70 is often the predominant protein, whereas in rat, P72 predominates, and flooding the system with any one of the proteins may lead to the isolation of the predominant form.

This study demonstrates that pharmacological agents such as the peroxisome proliferators can specifically bind to and perhaps affect the normal function of a subset of the HSP70 protein family. It remains to be shown whether these interactions are directly linked to the events that lead to peroxisome proliferator-induced liver pathophysiology.

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