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Binding proteins for cyclosomatostatins and bile acids in basolateral plasma membranes of rat liver

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Abstract

The bile acids cholate and taurocholate on the one hand and the cyclopeptide c(Phe-Thr-Lys-Trp-Phe-D-Pro) (008), an analog of somatostatin with retro sequence, on the other hand, display mutually competitive transport inhibition into isolated rat hepatocytes. This indicates a common transport system for bile acids and cyclosomatostatins in sinusoidal rat liver plasma membranes. In order to identify and isolate common binding and/or transport proteins for bile acids and the cyclopeptides by affinity chromatography, the bile acid derivative 4′-amino-7-benzamidotaurocholate (ABATC) and the cyclosomatostatin-analog 008 were attached to a gel matrix. Two methods were used to prepare integral membrane proteins: (1) alkaline EDTA extraction and (2) Triton X-114 phase separation. Octyl glycoside solubilized, alkaline EDTA-extracted integral basolateral membrane proteins with apparent molecular masses of 52 and 48 kDa bound specifically to the ABATC affinity matrix. Two-phase Triton X-114 separated integral membrane proteins of the same molecular masses bound specifically to the cyclosomatostatin ligand. The 48 kDa ABATC and 008 binding protein was shown to be present in the basolateral plasma membrane fraction and in the microsomal fraction. The isolated 52 kDa ABATC binding protein was localized only in basolateral plasma membranes and could not be found in isolated microsomes.

Key words: Bile acid transport system; Binding protein; Cyclosomatostatin; Two-phase separation; EDTA extraction; (Rat liver)

1. Introduction

Several peptide drugs of pharmacological interest, such as the cyclic derivatives of somatostatin, are thought to share a common transport system with bile acids in the sinusoidal membrane of hepatocytes. The affinity of such drugs to bile acid transport proteins leads to an unfavourable high first-pass effect and is responsible for the development of cholestasis. Besides a mutual competitive inhibition of cholate uptake and the uptake of the cyclosomatostatin c(Phe-Thr-Lys-Trp-Phe-D-Pro), termed 008, into isolated rat hepatocytes, the cyclohexapeptide 008 competitively inhibits the uptake of taurocholate and vice versa [1]. As was the case for the bile acids taurocholate and cholate [2], the cyclosomatostatin 008 therefore has affinity to the so called 'multispecific bile acid transporter' [3], which physiologically transports cholate, and to the sodium-taurocholate cotransporter. Therefore, these two bile acid transport systems in the sinusoidal plasma membrane of the hepatocyte display obviously overlapping substrate specificities. This assumption is further supported by the identification of binding and/or transporting proteins with identical molecular masses using chemically reactive or photoreactive bile acid or cyclopeptide analogs [4–10].

The aim of the present study was the identification and isolation of binding and/or transport proteins derived from the sinusoidal surface of the rat hepatocyte that bind to both substrates of a bile acid transport system, the bile acid derivative 4′-amino-7-benzamidotaurocholate (ABATC) and the cyclosomatostatin 008 (for structures, see Fig. 1). Therefore, affinity matrices which specifically interact with binding

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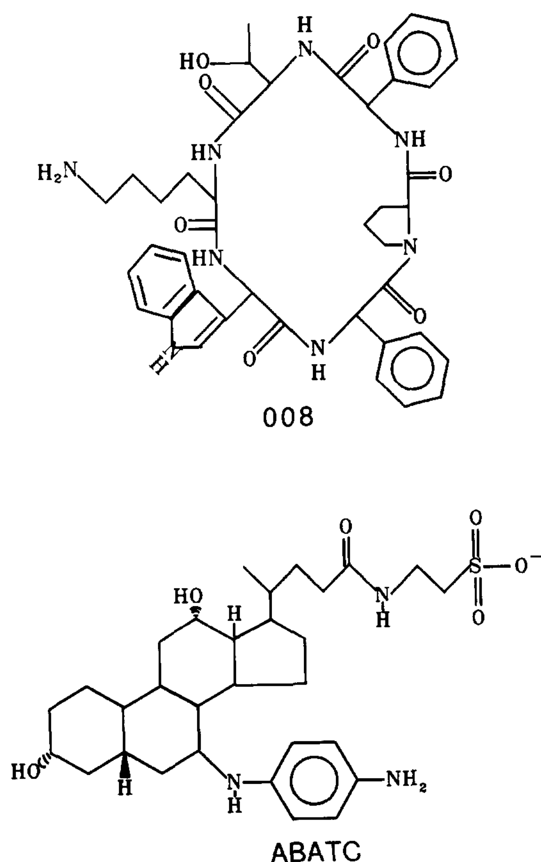

Fig. 1. Structures of cyclosomatostatin 008 and 4′-amino-7-benzamidotaurocholate (ABATC).

proteins for the taurocholate or somatostatin analog respectively were prepared.

2. Material and methods

2.1. Materials

[14]C-labeled azidobenzamido-008 was synthesized as described previously [11]. The non-radioactive cyclic somatostatin analog 008 was a generous gift from Prof. Dr. H. Kessler, Munich, Germany and the 4′-amino-7-benzamido-analog of taurocholate a generous gift from Prof. Dr. H. Fasold and Dr. S. Müllner, Frankfurt/Main, Germany. The structures of the chemically different compounds are shown in Fig. 1. Marker proteins for SDS-gel electrophoresis were purchased from Promega/Serva, Heidelberg, Germany. The reagents for gel electrophoresis were from Roth, Karlsruhe, Germany. Triton X-114 and CHAPS were from Serva, Heidelberg, Germany, octyl glycoside from Merck, Darmstadt, Germany. All other chemicals were of analytical grade purity or better.

2.2. Methods

Preparation of sinusoidal plasma membranes (blPm) from rat liver

Sinusoidal plasma membranes were prepared from livers of male Wistar rats according to the method of Blitzer and Donovan [12] with the following modifications: after homogenization of the livers with a loose Dounce homogenizer and a motor driven teflon pestle the homogenate was diluted 10-fold (w/v) with 10 mM Tris-HCl (pH 7.6), containing 250 mM sucrose and 1 mM phenylmethylsulfonyl fluoride (PMSF) and was centrifuged at $2500 \times g$ for 15 min in a GSA-rotor (Sorvall). The continous Percoll gradient was performed by centrifugation in a SS 34-rotor (Sorvall) at $24000 \times g$ for 35 min. The final pellet containing the blPm was resuspended in PBS (pH 7.4, containing 1 mM PMSF) with a protein concentration of 4 mg/ml and stored at −70°C in liquid nitrogen.

Preparation of rat liver microsomes

Microsomes of rat liver cells were isolated by centrifugation of the fluffy layer supernatant from the preparation of blPm (see above) at $100000 \times g$ for 1 h at 4°C. The centrifuged supernatant was free of Na^+/K^+-ATPase activity [12]. The pellet was resuspended in PBS and designated as microsomal fraction. For the use in affinity chromatography the isolated microsomes were pretreated as described for blPm (see below).

Analysis of enzyme activities

The plasma membrane fractions were characterized by determination of the marker enzymes. Na^+/K^+-ATPase was determined according to the method of Scharschmidt [13]. Glucose-6-phosphatase was determined according to Harper [14] and 5′-nucleotidase according to Michell and Hawthorne [15].

The release of anorganic phosphate by the two latter enzymes was measured according to the method of Chen [16], modified by Ames [17].

Photoaffinity labeling of isolated blPm with ^{14}C-azidobenzamido-008

Isolated blPm in PBS were labeled with cyclo(-Phe-(p-NH[1-^{14}C]Ac)-Thr-Lys-(CO(p-N_3)C_6H_4)-Trp-Phe-D-Pro), named azidobenzamido-008, as described [18]. Protein concentration was 0.6 mg/ml, concentration of the label 2 μM. Isolated blPm were preincubated for 3 min in the dark at 37°C before photolysis. Radioactive labeled proteins were identified after electrophoresis by fluorography. Bound radioactivity was quantitated after slicing SDS rod gels.

Separation of hydrophilic membrane associated proteins from integral hydrophobic proteins

Plasma membranes were dialysed overnight at 4°C against 10 mM EDTA at pH 9.1. Thereafter membrane associated hydrophilic proteins were separated from the integral hydrophobic proteins by centrifugation at $100000 \times g$ for 30 min at 4°C. Alternatively, hydrophilic and hydrophobic proteins were separated by Triton X-114 phase separation as described below.

Solubilization of the plasma membranes

Triton X-114. Solubilisation of blPm and the separation of hydrophilic and hydrophobic proteins by means of the two-phase detergent Triton X-114 was performed according to the method of Bordier [19] with slight modifications according to Honscha et al. [20]. Therefore, isolated blPm were pelleted by centrifugation at $100000 \times g$ for 30 min at 4°C. Subsequently, the pellet was resuspended in 10 mM Tris, 150 mM NaCl, 1% Triton X-114, 0.1 mM PMSF, pH 7.4 at a protein concentration of 5 mg/ml and solubilized by gentle agitation at 4°C overnight. Insoluble membrane components were pelleted by a centrifugation step at $100000 \times g$ for 1 h. 200 μl aliquots of the supernatant were carefully overlayed onto 300 μl 10 mM Tris, 150 mM NaCl, 6% sucrose, 0.06% Triton X-100, pH 7.4 in each case, and after an incubation period of 3 min at room temperature the Eppendorf centrifugation vials were centrifuged at $600 \times g$ for 3 min. During this centrifugation step, the separation of the lipophilic detergent phase at the bottom and the aqueous phase on top of the vials occurred. The two phases were carefully separated by means of a pipette prior to use in affinity chromatography.

Octyl glycoside and CHAPS. Isolated plasma membranes were dialysed against 10 mM EDTA, pH 9.1 at 4°C overnight to dissolve associated hydrophilic membrane proteins. After centrifugation at $100000 \times g$ for 30 min, the pellet was resuspended in PBS (pH 7.4) containing 1 mM PMSF and 2% of the respective detergent at a protein to detergent ratio of 1:10. The sample was stirred for 1 h at 4°C, centrifuged at $100000 \times g$ for 1 h and the supernatant was used for affinity chromatography.

Preparation of 008 and ABATC affinity columns

After washing the gel bed (Affi-Gel 10) several times with ethanol for 008-column preparation and with methanol for ABATC-column preparation, 17 μmol 008 or ABATC respectively per ml of gel, dissolved in ethanol or methanol, were added and coupled by gentle agitation at room temperature overnight. Subsequently, 100 μl 1 M ethanolamine (pH 8.0) per ml gel were added and stirred for 1 h at room temperature to block free binding sites. The gel was then washed extensively with PBS (pH 7.4), containing 1 mM PMSF and stored at 4°C. The ethanolamine blocked Affi-Gel 10 matrix without ligand was used as control. After packing the 0.5 × 10 cm colums with the gel matrices the gel was degassed prior to use.

Affinity chromatography

All steps in affinity chromatography were done at 4°C. The solubilized plasma membrane proteins were applied to the column and allowed to penetrate. After an incubation period of 1 h the column was washed with PBS to elute unbound proteins followed by a 0–1 M KCl gradient to elute proteins bound via electrostatic interactions. To further increase the specificity of the column, an additional washing step with a 0–500 μM piperonylbutoxide gradient in 30% ethanol was performed to elute cytochrome *P*-450 dependent enzymes, which are known to represent contaminating microsomal proteins that were photolabeled with bile acid derivatives [21]. However, several cytochrome *P*-450 isoenzymes are probably also present, active and inducible, on the plasma membrane surface of hepatocytes [22]. Elution of proteins specifically bound to the cyclosomatostatin ligand was carried out with a 0–1 M KSCN gradient to elute proteins bound via hydrophobic interactions and a 0–10 mM taurocholate gradient to elute proteins belonging to a taurocholate and/or 'multispecific bile acid transport system'. The elution steps in affinity chromatography were carried out with 0.4% octyl glycoside in the case of octyl glycoside solubilized proteins and with 0.05% Triton X-100 in the case Triton X-114 solubilized proteins. Elution was done with a flow rate of 0.17 ml/min. Peak fractions were pooled and concentrated with Centricon™ microconcentrators (exclusion limit 30 kDa) prior to analysis by SDS-PAGE according to the method of Laemmli [23].

3. Results

3.1. Membrane preparation

The quality of the membrane preparation was assessed by measuring the enrichments of marker enzymes. The marker for blPm, Na^+/K^+-ATPase was enriched 18–22-fold; 5′-nucleotidase, a marker enzyme for canalicular membranes 3.2–3.5-fold and the microsomal marker enzyme glucose-6-phosphatase was enriched 1.5–1.7-fold.

3.2. Photoaffinity labeling of isolated blPm with ^{14}C-azidobenzamido-008

Photoaffinity labeling of freshly isolated blPm with ^{14}C-azidobenzamido-008 resulted in the preferential labeling of proteins in a molecular mass range of 48–60 kDa. The highest amount of radioactivity was bound to 52 and 48 kDa proteins (Fig. 2). The labeling of these

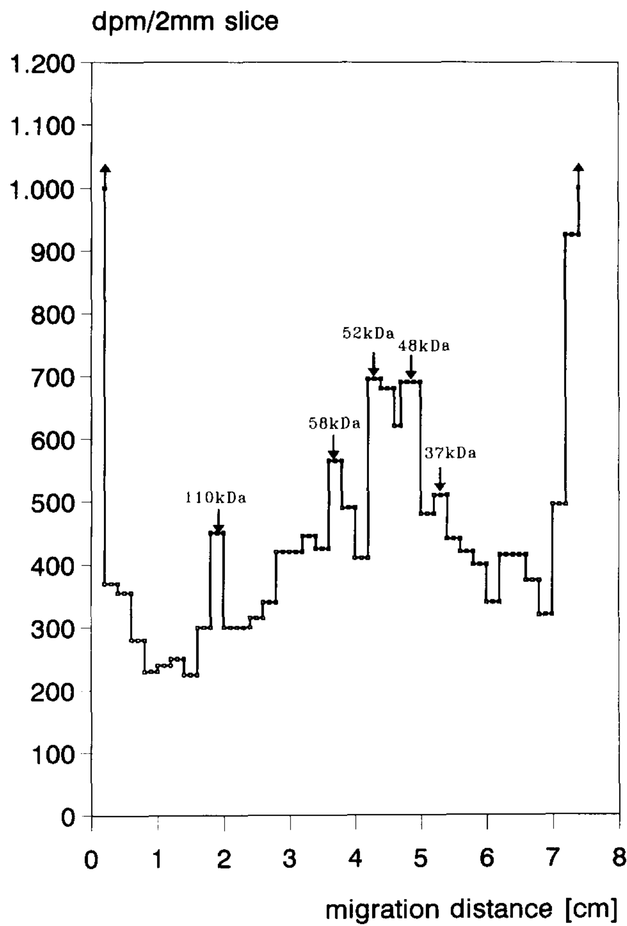

Fig. 2. Photoaffinity labeling of isolated blPm with ^{14}C-azidobenzamido-008. Labeled blPm were separated from unbound radioactivity by centrifugation at 100000 × *g* for 15 min at 4°C in 30 mM Tris, containing proteinase inhibitors. The pellet was resuspended in the same buffer and the proteins subjected to SDS rod gel electrophoresis (7.5%). The protein associated radioactivity was quantitated by slicing the rod gels and counting the radioactivity in a Packard scintillation counter after incubation in Lipoluma/Lumasolve/water for 4 h at 40°C.

proteins by the photolabile 008-analog and also by the photoreactive bile acid analog ^{14}C-azidobenzamido-taurocholate was demonstrated in a previous report [10].

3.3. Choice of an appropriate detergent for affinity chromatography

In order to identify and isolate binding and/or transport proteins for cyclosomatostatins and bile acids, Triton X-114 was chosen as an appropriate detergent for affinity chromatography. The two-phase detergent separates integral and associated membrane proteins, delivering the hydrophobic integral proteins in a solubilized state. This seems to be of great advantage since transporting proteins should span the bilayer of the plasma membrane hence showing hydrophobic characteristics. Therefore, the use of Triton X-114 results in a pre-purification of transport proteins. Because there was no binding of Triton X-114 solubilized integral plasma membrane proteins to the ABATC affinity matrix (data not shown), two further detergents, octyl glycoside and CHAPS, which were known to be the most effective in solubilizing functional receptors [24], were used. In this case isolated blPm were depleted of associated proteins by EDTA extraction at pH 9.1. CHAPS solubilized integral blPm did not bind to the ABATC affinity matrix (data not shown), possibly indicating an interaction of the bile acid analog 3-((cholamidopropyl)dimethylammonio)propanesulfonate (CHAPS) with the bile acid binding proteins. All detergents tested showed no non-specific binding to the Affi-Gel 10 matrix without coupled ligand. Because of the obtained results, octyl glycoside was used as detergent for the solubilization of integral membrane proteins with respect to ABATC affinity chromatography and Triton X-114 was used to obtain integral blPm proteins for 008 affinity chromatography.

3.4. 008 Affinity chromatography of Triton X-114 phase-separated integral blPm proteins

Integral proteins with apparent molecular masses of 52 and 48 kDa that were obtained by Triton X-114 two-phase separation of isolated blPm bound specifi-

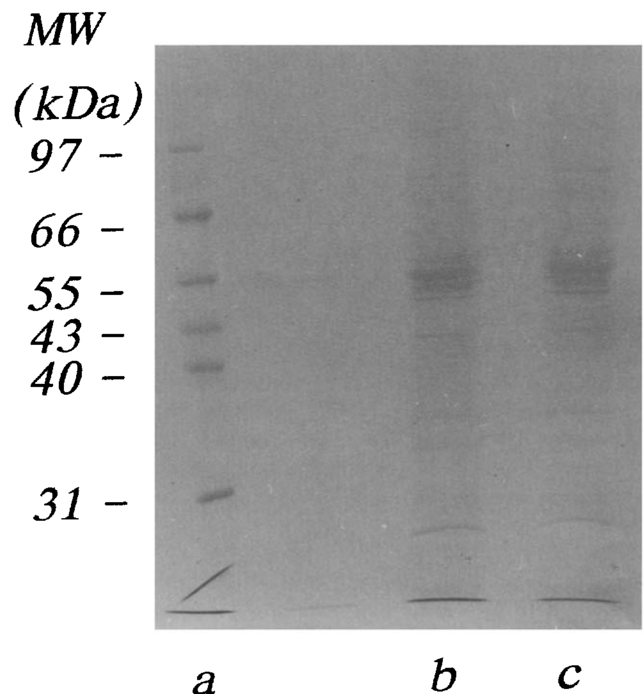

Fig. 3. 008 Affinity chromatography of hydrophobic integral blPm and microsomal proteins. Shown is a Coomassie G-250 stained 10% SDS gel. BlPm and microsomal proteins were solubilized with Triton X-114. Hydrophobic proteins were separated from hydrophilic ones by phase separation as described under Materials and methods. a, marker proteins; b, integral blPm proteins bound to the affinity matrix and eluted with a KSCN gradient; c, integral microsomal proteins bound to the affinity matrix and desorbed by KSCN.

cally to the cyclosomatostatin affinity matrix. They could be desorbed by KSCN (Fig. 3), a chaotropic salt that reduces hydrophobic interactions. Subsequent elution with 10 mM taurocholate, a competitive inhibitor of 008 uptake, resulted in no further elution of 008 binding proteins. The 008-binding proteins of 52 and 48 kDa displayed the same apparent molecular masses as those labeled with photolabile 008 (Fig. 2) and bile acid analogs [10]. It is also shown that 008 binding proteins of identical molecular masses were present in the microsomal fraction (Fig. 3), including two faintly stained 56 and 45 kDa proteins.

3.5. *ABATC affinity chromatography of EDTA (pH 9.1) extracted octyl glycoside solubilized blPm proteins*

The resistance of proteins to the release from the membrane by exposure to very alkaline pH is a commonly used criterion for an integral membrane protein [25–27], indicating that it interacts strongly and hydrophobically with the lipid bilayer.

Therefore, the blPm were depleted from associated membrane proteins by EDTA extraction at pH 9.1 prior to octyl glycoside solubilization. Two integral membrane proteins with apparent molecular masses of 52 and 48 kDa bound to the ABATC affinity matrix and could be specifically eluted by taurocholate (Fig. 4). It is also shown that these two proteins were clearly absent in the fraction of associated membrane proteins. In contrast to the 52 kDa 008-binding protein, which was also present in the microsomal fraction, no 52 kDa ABATC-binding protein of microsomal localization could be detected. The ABATC binding 48 kDa protein, however, was present in the blPm and microsomal fraction (Fig. 5). A further more faintly stained ABATC binding protein with an apparent molecular mass of 56 kDa was also present in both membrane fractions.

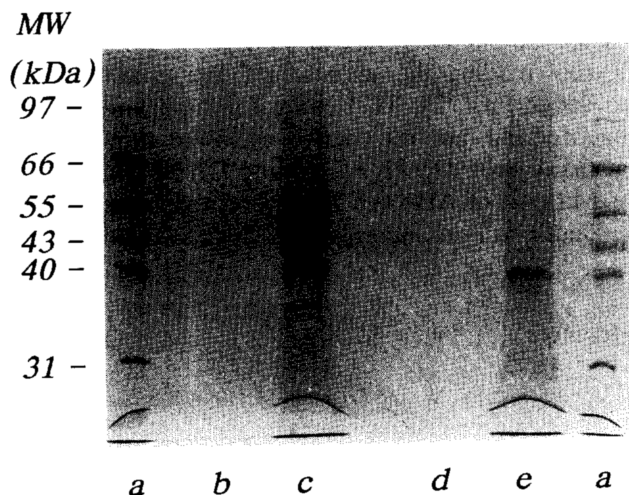

Fig. 4. ABATC affinity chromatography of integral octyl glycoside solubilized and associated blPm proteins. Shown is a Coomassie G-250 stained SDS gel (10%). Hydrophobic proteins were separated from hydrophilic ones by EDTA extraction of blPm at pH 9.1 as described under Materials and methods. The 100000 × *g* pellet, containing the integral membrane proteins, was resuspended with PBS and solubilized with 2% octyl glycoside at a protein to detergent ratio of 1:10. The supernatant of the 100000 × *g* centrifugation, containing the associated membrane proteins, was concentrated in Centricon™ 30 microconcentrators prior to pouring it on the affinity column. a, marker proteins; b, KSCN eluate of integral blPm proteins; c, integral blPm proteins bound to the affinity matrix and eluted with a taurocholate gradient; d, KSCN eluate of associated blPm proteins; e, associated blPm proteins bound to the affinity matrix and desorbed by taurocholate.

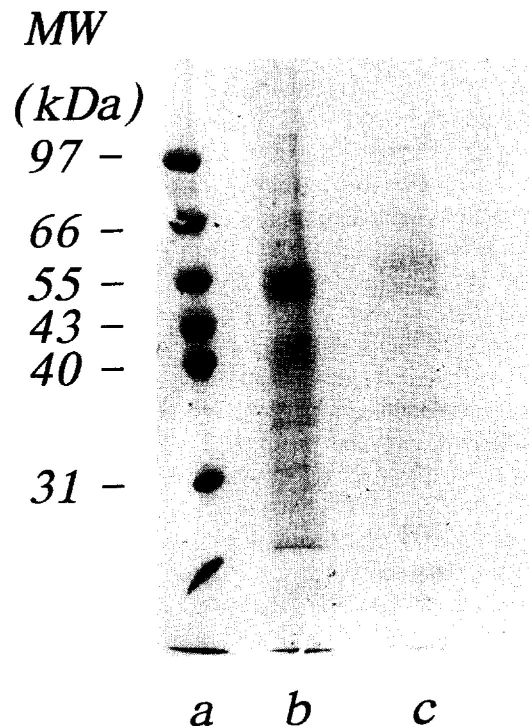

Fig. 5. ABATC affinity chromatography of hydrophobic integral blPm and microsomal proteins. Shown is a Coomassie G-250 stained 10% SDS gel. Hydrophobic proteins were separated from hydrophilic ones by EDTA extraction of isolated blPm or microsomes at pH 9.1 as described under Materials and methods. The 100000 × *g* pellet, containing the integral membrane proteins, was resuspended with PBS and solubilized with 2% octyl glycoside at a protein to detergent ratio of 1:10 prior to pouring it on the affinity column. a, marker proteins; b, integral blPm proteins bound to the affinity matrix and eluted by a taurocholate gradient; c, integral microsomal proteins bound to the affinity matrix and eluted with taurocholate.

4. Discussion

From kinetic and photoaffinity labeling studies it became evident that the cyclosomatostatin 008, a somatostatin analog with retro sequence, shares a common transport system with bile acids in the sinusoidal plasma membrane of the hepatocyte. Since proteins with apparently identical molecular masses were la-

beled by photoreactive taurocholate and 008 derivatives, it should be possible to isolate such binding and/or transport proteins using 008 and the taurocholate derivative ABATC as ligands in affinity chromatography. On the basis that transport of a substrate across the membrane requires first its binding to specific receptors or transport molecules, it becomes more likely that binding and/or transport proteins which are involved directly in the translocation process are located in the integral part of the plasma membrane. Therefore, the isolated sinusoidal plasma membranes were first depleted from associated membrane proteins by two-phase separation with Triton X-114. Because the integral membrane proteins obtained by the phase separation technique did not bind to the ABATC ligand, an alkaline EDTA extraction was used to remove associated membrane proteins in this case, followed by octyl glycoside solubilization. Electrophoresis of the concentrated eluted KCl and piperonylbutoxide fractions from both affinity columns gave negative results. The elution patterns of specifically eluted proteins from the 008 affinity matrix as well as the ABATC affinity matrix revealed two dominant binding proteins with apparent molecular masses of 52 and 48 kDa. These molecular masses are in agreement with those of plasma membrane proteins photolabeled with azidobenzamido-008 or -taurocholate. The integral binding proteins are clearly absent in the hydrophilic membrane fraction, indicating an efficient separation. The 48 kDa 008- as well as the 48 kDa ABATC-binding protein were detected in the blPm and the microsomal fraction. The presence of a 48 kDa binding protein in the blPm could be the consequence of a minor contamination of the isolated plasma membranes with microsomal structures. This assumption is supported by the enhanced specific activity of glucose-6-phosphatase in the plasma membrane fraction when compared to the liver homogenate. This contamination can be observed frequently for the preparation method used and can only be prevented by refined techniques [28]. As an alternative explanation for the presence of a microsomal 48 kDa binding protein in the plasma membrane could serve the demonstration, that the 49 kDa microsomal epoxide hydrolase (EC 3.3.2.3) is functioning as a Na^+-dependent taurocholate transporter in the basolateral hepatocyte plasma membrane [29] as shown by reconstitution studies by means of monoclonal antibodies against the 49 kDa protein [30–32]. In contrast to the 48 kDa binding protein the localization of the 52 kDa ABATC-binding protein is confined to the basolateral plasma membrane fraction. The fact that the 52 kDa 008-binding protein was also detected in the microsomal fraction makes it probable that in this case two proteins of different subcellular origin bound to the cyclosomatostatin ligand. Interestingly, a localization confined to the plasma membrane of hepatocytes was also described for a high-affinity cholate binding protein functioning presumably as cholate transporter [33]. In this case binding of cholate was inhibited by bile acids and other organic anions competitively, indicating the 'multispecific' binding properties of this protein. The fact that the 52 kDa ABATC-binding protein is limited to the plasma membrane is of special interest since all proteins identified by affinity techniques with bile acid derivatives and thereafter sequenced have a microsomal localization [21,34]. The supposition that a 52 kDa protein is involved in Na^+-independent transport of cholate and other organic anions is further supported by photolabeling of such a protein with a taurocholate derivative in little skate (*Raja erinacea*) hepatocytes and isolated hepatocyte plasma membranes because only a Na^+-independent transport system for bile acids and organic anions exists in the primitive vertebrate [35]. In accordance with this result no 48 kDa protein could be labeled by the photoreactive taurocholate derivative.

In order to identify the isolated 52 and 48 kDa ABATC and 008 binding proteins amino acid sequencing and functional reconstitution will be necessary in the future.

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