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Binding Proteins for Cyclic and Linear Oligopeptides in Plasma Membranes and the Cytosol of Rat Hepatocytes

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ABSTRACT. Using a cyclolinopeptide A analogue, the hydrophobic cyclic peptide c(-Ala-Lys-Pro-Phe-Phe-Ala-Lys-Pro-Phe-Phe-), termed CDP (cyclodecapeptide), as ligand in affinity chromatography, hepatocellular peptide binding proteins were isolated from the integral part of plasma membranes and the cytosol. The sequence of the isolated protein with MW of 50 kDa from the integral part of the plasma membrane fraction was identical to cytochrome P450 II C13 and cytochrome P450 II C22, whereas the sequence of the 54 kDa protein was identical to 3-hydroxyandrogen-UDP-glucuronosyltransferase. These proteins have also been described as binding proteins for bile acids. As shown in earlier studies, bile acids and CDP also compete for uptake into hepatocytes. In the cytosol, a further known bile acid binding protein, the glutathione-S-transferase (G-S-T) subunit Yb1, was isolated and sequenced as binding protein for CDP and also for a further cyclopeptide, the somatostatin analogue OO8, and a linear peptide with renin-inhibiting activity, EMD 55068. As shown in uptake studies using isolated basolateral plasma membrane vesicles, G-S-T was able to increase the uptake of EMD 51921, a linear peptide with renin-inhibiting potency, into the vesicles when the latter were preloaded with G-S-T. The binding of the substrate to the outside of the preloaded vesicles was not different than binding to unloaded vesicles. The maximal transport rate of the carrier-mediated/facilitated diffusion and the rate of permeation, however, were doubled in the presence of G-S-T, pointing to the involvement of intracellular binding proteins such as G-S-T in the unloading of the carrier protein and in the reduction of the free substrate concentration. BIOCHEM PHARMACOL **54**;4:481–490, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. cyclolinopeptide A analogues; binding proteins; rat hepatocytes; bile acid transport system; affinity chromatography

Two classes of hydrophobic cyclic peptides, the somatostatin analogues and the cyclolinopeptide A analogues, have been developed as cytoprotective substances [1, 2] to prevent hepatocellular lesions caused by phalloidin. This organotropic toxin is known to be taken up via a bile acid transport system. Therefore, the prevention of toxicity is due to uptake inhibition by these peptides [3, 4] and not to a cytoprotective process. All peptides preventing phalloidin intoxication of hepatocytes inhibit the uptake of bile acids into hepatocytes as well [3, 4]. On the basis of the mutually competitive transport inhibition of the bile acid cholate and cyclolinopeptide A analogues, it was suggested that both substrates may also have affinity to common binding proteins in basolateral plasma membranes and the cytosol of liver cells. To allow the isolation of hepatocellular binding proteins for these peptides via affinity chromatography, small amino acid sequences of cyclolinopeptide A, a natural cyclic nonapeptide isolated from linseed [5] c(-Pro-Pro-Phe-Phe-Leu-Ile-Ile-Leu-Val-), were synthesized [6]. All active cyclic peptides possess two aromatic residues adjacent to a proline [1, 2]. The conformation of this subsequence is stabilized by the cyclisation of the peptides [7]. The strongest competitive inhibitor, CDP#, with IC_{50} values of approximately 5 μM for cholate and 17 μM for taurocholate uptake inhibition, was produced in sufficient amounts to be used in affinity chromatography. These binding proteins should be able to recognize the steroidal structure of bile acids and the aromatic amino acid residues of the cyclopeptides [8], with hydrophobic interactions occurring between the binding proteins and the reference substrate. To isolate these binding proteins, the high-affinity hydrophobic peptide cyclodecapeptide (CDP) was

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Abbreviations: blPm(v), basolateral plasma membrane (vesicles); CDP, cyclodecapeptide; EMD, Emanuel Merck Darmstadt; G-S-T, glutathione-S-transferase; NEPHGE, nonequilibrium pH gel electrophoresis; PDI, proteindisulfide isomerase; PMSF, phenylmethylsulfonylfluoride.

coupled to a gel matrix (Affi-Gel® 10). This matrix does not bind plasma membrane or cytosolic proteins in the absence of the ligand. It was used to identify and isolate cytosolic and integral basolateral membrane proteins of rat liver cells that bind cyclopeptides.

MATERIALS AND METHODS

Materials

The cyclolinopeptide A analogue c(-Ala-Lys-Pro-Phe-Phe-Ala-Lys-Pro-Phe-Phe-) was synthesized by Dr. G. Zanotti and Anna Maria Maione (Rome, Italy). Antibodies against UDP-glucuronosyltransferase were a gift from Dr. Axel Hoffmann (Frankfurt/M., Germany). AffiGel® 10 was from Bio-Rad (Munich, Germany). Marker proteins for SDS polyacrylamide gel electrophoresis and liver UDP-glucuronosyltransferase were from Sigma (Deisenhofen, Germany). Reagents for electrophoresis were from Roth (Karlsruhe, Germany). Cellulose nitrate (Protran) was from Schleicher and Schuell (Dassel, Germany). Triton X-100 and X-114 were purchased from Serva (Heidelberg, Germany). Lys C of sequencing grade was from Boehringer (Mannheim, Germany). All other chemicals were of analytical grade.

Methods

PREPARATION OF BASOLATERAL PLASMA MEMBRANES (BLPM) FROM RAT LIVER. For the isolation of binding proteins for CDP, basolateral plasma membranes from rat liver were prepared according to the method of Blitzer and Donovan [9], 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 with 10 mM Tris HCl (pH 7.6) containing 250 mM sucrose and 1 mM phenylmethylsulfonylfluoride (PMSF) and was centrifuged at 2500 $\times g$ for 15 min. The continuous Percoll gradient was centrifuged at 24,000 $\times g$ for 35 min. The final pellet containing the plasma membranes was resuspended in NaCl/P_i (NaCl 137 mM, KCl 2.7 mM, $MgCl_2$ 0.5 mM, KH_2PO_4 1.5 mM, Na_2PO_4 4 mM, with 1 mM PMSF, pH 7.4) to a protein concentration of 4 mg/mL, determined by the method of Lowry, [10] and stored at −196°C in liquid nitrogen. The cytosol was obtained in the supernatant after ultracentrifugation of disintegrated rat liver cells at 100,000 $\times g$ for 1 hr at 4°C.

Analysis of Enzyme Activities

The purity of the prepared basolateral plasma membrane fraction was defined by the assay of marker enzymes. Na^+/K^+-ATPase (E.C. 3.6.1.37) was determined according to Scharschmidt [11], glucose-6-phosphatase (E.C. 3.1.3.9.) according to Harper [12], and 5′-nucleotidase (E.C. 3.1.3.5.) according to Michell and Hawthorne [13]. The release of inorganic phosphate by the two latter enzymes was measured according to the method of Chen [14], modified by Ames [15].

Solubilisation of Plasma Membrane Proteins and Phase Separation of Hydrophobic from Hydrophilic Proteins

The two-phase nonionic detergent Triton X-114 was used to separate hydrophobic from hydrophilic proteins according to the method of Bordier [16], slightly modified by Honscha *et al.* [17]

Preparation of the CDP-Affinity Columns and Affinity Chromatography

CDP was dissolved in methanol with an equimolar concentration of triethylamine to cleave the trifluoric acetate, which protected the free lysine residue of the cyclodecapeptide during storage. CDP was coupled to the gel bed (Affi-Gel® 10) as described by Wenzel *et al.* [18]. All steps in affinity chromatography were carried out at 4°C as previously described [18].

Two-Dimensional Gel Electrophoresis

Preparative nonequilibrium pH gel electrophoresis (NEPHGE) was performed according to the method of Honscha *et al.* [17]. For the second dimension of the 2D gel electrophoresis, the method according to O'Farell [19] was used.

Production of Polyclonal Antibodies Against the 54 kDa Membrane Protein

Polyclonal antibodies were produced in rabbits according to Harlow and Lane [20], with minor modifications. The denatured 54 kDa antigen was purified from SDS-PAGE. Protein (15 μg) was necessary for immunization. The 54 kDa bands were cut out and fragmented prior to homogenization with 1 mL NaCl/P_i (pH 7.4) in a Wheaton glass homogenizer with a total capacity of 3 mL. Immunization was carried out with an emulsion of the protein solution in an equal volume of Freund's adjuvant. Male New Zealand rabbits were primed with the antigen and complete Freund's adjuvant and subsequently boostered with antigen and incomplete Freund's adjuvant. In total, a maximal volume of 2 mL of the emulsion, deposited at five sites, was applied subcutanously. The rabbits were boostered at intervals of 3 weeks. Ten days after each boostering, blood samples were taken and the quality of the antiserum assessed.

WESTERN BLOTTING. The proteins from SDS-gel electrophoresis (one- or two-dimensional) were transferred onto nitrocellulose membranes using the semidry electroblotting method. Unspecific binding sites were blocked with 25 mM sodium phosphate, 0.9% sodium chloride, pH 7.2 (NaCl/P_i) containing 1% gelatin for 2 h. The blot was incubated

overnight with a polyclonal antiserum against rat UDP-glucuronosyltransferase and washed in NaCl/P_i before incubation with a peroxidase-conjugated antirabbit antibody for 2 h. Thereafter, the blot was washed once again and developed in 25 mL NaCl/P_i, pH 7.2 containing 15 mg 4-chloro-1-naphthol dissolved in 5 mL methanol and 200 μL 30% aqueous solution of hydrogen peroxide.

In-Gel Digestion of Proteins with Lys C for Internal Sequence Analysis After SDS-PAGE

The affinity purified proteins were collected and concentrated; the concentration of binding proteins in the whole elution fractions was determined using the quantitation method according to Ball [21]. Proceeding from these results, ca. 15 μg of protein were separated in a preparative 10% SDS-PAGE. The gel was stained with 0.2% Coomassie Brilliant Blue G-250 (mass/vol.)/20% methanol/0.5% acetic acid for 20 min and destained in 30% methanol until the background was clear. The stained protein bands were excised and gently washed in twice-distilled water with several changes for 2 days at 4°C. For in-gel digestion with the endoproteinase Lys C (E.C. 3.4.21.50.), the method according to Rosenfeld [22] was modified as follows. The protein-containing gel fragments were washed twice for 20 min at 30°C with 50% acetonitrile in 0.2 M *N*-methylmorpholine-acetate, pH 7.8. Subsequently, they were cut into pieces of 1 mm^3 and dried for 15 min at room temperature. Endoproteinase Lys C (10% of protein weight) was dissolved in 0.2 M *N*-methylmorpholine-acetate, pH 7.8 containing 0.02% Tween 20 and added to the gel pieces in a total volume of 20–50 μL. Afterwards, buffer without Lys C was added until the gel pieces were saturated. They were shaken at 30°C for 4 h, followed by addition of 6 μL 25% trifluoroacetic acid. After digestion the peptides were extracted by soaking the gel pieces twice in 60% acetonitrile/0.1% trifluoroacetic acid at 30°C for 20 min. The supernatants were transferred to a fresh tube and concentrated to a maximal volume of 50 μL in a SpeedVac. Remaining gel debris was removed by centrifugation.

SEPARATION OF LYS C DIGESTED PEPTIDES AND AMINO ACID SEQUENCING. The supernatant from the in-gel digestion procedure was injected onto an Aquapore® C8 reversed-phase HPLC column (250 × 1 mm). The peptide fragments were eluted from the column using an acetonitrile gradient of 5 to 56% in 0.1% triflouroacetic acid in twice-distilled water at a flow rate of 50 μL/min. The peptides were collected and submitted to automatic sequence analysis using a pulsed liquid protein sequencer (477 A, Applied Biosystems).

Enzyme Inhibition

The enzyme activity determinations for the 3-OH-androgen-UDP-glucuronosyl-transferase with 4-aminobiphenyl according to Green and Tephly [23] and for the glutathione-*S*-transferase (G-S-T, E.C. 2.5.1.18.) with 1-chloro-2,4-dinitrobenzene according to Lai and Tu [24] were generously performed by a collaborator of Dr. A. Bock (Tübingen, Germany). Inhibition experiments were carried out in the presence of CDP to concentrations as high as 50 μM.

Preparation of Basolateral Plasma Membrane Vesicles from Rat Liver (blPmv)

Because the plasma membranes isolated as described by Blitzer [9] turned out not to be suitable for measuring the uptake of substrates, basolateral plasma membrane vesicles for uptake studies were isolated according to the method of Meier *et al.* [25]. The blPm pellet of the last centrifugation step (100,000 × *g* for 45 min at 4°C) was resuspended in 250 mM sucrose, 0.2 mM $CaCl_2$, 5 mM $MgSO_4$, and 10 mM Hepes/Tris pH, 7.5 and then vesiculated through a 25 G needle by 10–15 strokes. The blPmv with protein concentrations of 3.0–4.0 mg/mL were stored in liquid nitrogen until use.

Uptake Studies of Emanuel Merck Darmstadt (EMD) 51921 in blPmv under Various Conditions

Different conditions inside the vesicles were achieved by vesiculation in buffer solutions containing either additional 18 μM bovine serum albumin, 18 μM G-S-T, or cytosol. To remove the remaining external buffer solution, the vesicles were centrifuged at 25,000 × *g* for 30 min at 4°C and resuspended in standard vesicle buffer. The transport studies were performed according to the rapid filtration technique of Meier *et al.* [26]. Aliquots of 25 μL vesicle solution were preincubated at 37°C for 2 min followed by the addition of 100 μL incubation medium (100 mM NaCl, 100 mM sucrose, 0.2 $CaCl_2$, 5 mM $MgSO_4$, and 10 mM Hepes/Tris, pH 7.5) containing 3 μM [^{3}H] EMD 51921. At defined time intervals, the incubations were stopped with 1 mL 100 mM KCl, 100 mM sucrose, 0.2 mM $CaCl_2$, 5 mM $MgSO_4$, and 10 mM Hepes/Tris pH, 7.5. The supernatant was withdrawn by suction through a Millipore membrane filter, and the vesicles were rinsed with another 5 mL of the stop solution. The membrane filters were transferred into mini vials, 3.0 mL of scintillator solution were added, and radioactivity was measured after 4 hr at 40°C in a liquid scintillation counter (Wallac 1409/11). To determine the unspecific binding of [^{3}H] EMD 51921 to the filter, the 25 μL blPmv were replaced by 25 μL twice-distilled water, and the values were substracted from uptake data.

RESULTS

Membrane Preparation

To test the purity of blPm, the enrichment of marker enzymes was measured. In the preparation according to Blitzer and Donovan [9], the blPm marker Na^+/K^+-ATPase was increased 22–24 (28-)-fold, the canalicular marker 5′-nucleotidase 8–10 (13-)-fold, and the microsomal

marker glucose-6-phosphatase 0.6–1.2 (0.2-)-fold, indicating a minor contamination with microsomal proteins. The enrichment values for the membranes prepared according to Meier *et al.* [25] are indicated in round brackets.

CDP Affinity Chromatography of Triton X-114 Phase-Separated Integral Membrane Proteins

After application of a fraction of hydrophobic integral proteins of the isolated basolateral plasma membranes, two proteins specifically bound to the cyclolinopeptide ligand in affinity chromatography. As shown in Fig. 1, proteins with apparent molecular masses of 50 and 54 kDa (Fig. 1f and g) were not washed from the affinity gel matrix in the first two elution steps (Fig. 1d and e). Apart from the 50 and 54 kDa proteins, there was only a smear of proteins detectable in SDS-PAGE in the chaotropic KSCN and the taurocholate fraction, indicating a strong hydrophobic interaction between the ligand and the desorbed proteins. As shown in Fig. 1, the proteins desorbed by the last two elution steps seemed to be identical in SDS-PAGE. The amount of protein in the taurocholate fraction (Fig. 1g) was not sufficient for sequencing; therefore, the last elution step was omitted and the proteins of the KSCN fraction (Fig. 1f) were sequenced.

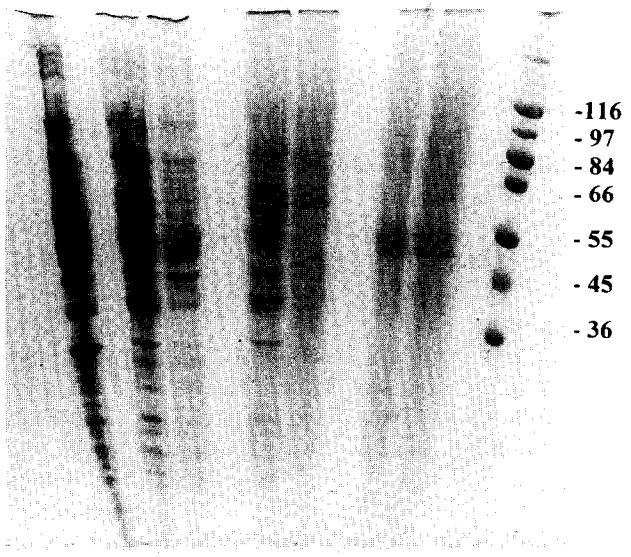

FIG. 1. CDP affinity chromatography of hydrophobic integral rat liver blPm proteins. Shown is a Coomassie-stained 10% SDS-gel. BIPm proteins were solubilized with Triton X-114 and phase-separated as described. The hydrophobic integral blPm proteins were separated by CDP affinity. Ten micrograms of protein per slot were applied onto SDS-PAGE: (a) blPm proteins; (b) associated blPm proteins; (c) integral blPm proteins; (d) integral blPm proteins from the affinity matrix washed with NaCl/P_i; integral blPm proteins bound to the affinity matrix and eluted with a KCL (e) a KSCN (f) and a taurocholate gradient (g); (h) marker proteins.

Sequencing of the 50 and 54 kDa Proteins Isolated via Affinity Chromatography

The 50 and 54 kDa CDP-binding proteins of the integral part of the membranes were isolated from the KSCN fractions. The sequenced peptide fragment of the 50 kDa protein possessed 67% identity in a six amino acid overlap to two members of the cytochrome P 450 II C subfamily. Similarities to the sequenced amino acids F-I-N-Y-F-I were present in the region of the amino acids 264 to 269 of rat cytochrome P 450 II C 13 (F-I-D-Y-F-L) and in the region of the amino acids 262 to 267 of rat cytochrome P 450 II C 22 (F-I-D-Y-F-L). The 54 kDa protein seems to be related to the UDP-glucuronosyltransferase. A 12 amino acid sequence of the integral 54 kDa protein was identical to the amino acids 344 to 355 of rat liver 3-OH-androgen-UDP-glucuronosyltransferase.

As further proof for specific affinity of the UDP-glucuronosyltransferase to the coupled CDP ligand, immunoblotting with a specific antibody against this protein was performed. Distinct protein fractions were separated by SDS-PAGE and electrotransferred onto cellulose nitrate membranes. In the amino acid sequence analysis, the partially sequenced 54 kDa protein of the KSCN elution (Fig. 2e) was found to be related to UDP-glucuronosyltransferase. Figure 2 shows that the UDP-glucuronosyltransferase-related 54 kDa protein was strongly bound to the CDP affinity matrix (Fig. 2e). No UDP-glucuronosyltransferase could be detected in the NaCl/P_i- fraction (Fig. 2d). Only the subsequent KSCN elution detached this protein (Fig. 2e). As a control, bovine UDP-glucuronosyltransferase from Sigma confirmed the quality of the antiserum (Fig. 2f).

By using a polyclonal antiserum against the isolated 54 kDa cyclolinopeptide binding protein raised in our laboratory, we once again observed specific binding of rat UDP-glucuronosyltransferase to the affinity matrix. The specificity was demonstrated by Western blots of two-dimensional PAGE with the NEPHGE system (see Fig. 3). The whole blPm proteins were separated and then electrotransferred onto cellulose nitrate membranes. Figure 3 shows the detection of the UDP-glucuronosyltransferase with an apparent molecular mass of 54 kDa by this antiserum.

Inhibition of the Activity of the 3-OH-Androgen-UDP-Glucuronosyltransferase with the Cyclolinopeptide A Analogue CDP

To test whether the cyclolinopeptide A analogue CDP blocks the enzymatic activity of the UDP-glucuronosyltransferase in microsomes, the enzyme assay for the 3-OH-androgen-UDP-glucuronosyltransferase with 4-aminobiphenyl as the specific substrate was performed according to Green and Tephly [23]. No inhibition of the reaction occurred up to a concentration of 50 μM of added CDP.

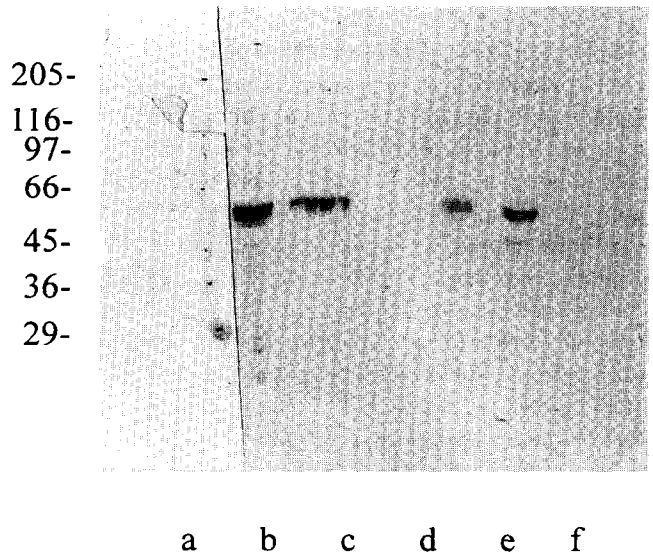

FIG. 2. Immunoblot of a 10% SDS-gel after electrotransfer onto cellulose nitrate. The UDP-glucuronosyltransferase was specifically detected in the separated protein fractions by the polyclonal antiserum from Dr. A. Hoffmann. The antibody dilution used was 1:2000. (a) Marker proteins; (b) rat liver blPm proteins; (c) integral blPm proteins after Triton X-114 phase separation; (d) NaCl/P_i-fraction of the integral blPm proteins that were applied onto the CDP affinity matrix. UDP-glucuronosyltransferase was not detected in the unbound protein fraction; (e) KSCN eluate of the integral blPm proteins specifically bound to the CDP affinity matrix; (f) bovine UDP-glucuronosyltransferase (1 μg) from Sigma.

Identification of the Cytosolic 28 kDa Protein Isolated via Affinity Chromatography

In affinity chromatography with the coupled CDP ligand, we isolated, as shown in SDS-PAGE, one major cytosolic protein with strong binding capacity to the affinity matrix (see Fig. 4). In addition to the cyclolinopeptide, we used another cyclic hydrophobic peptide, the somatostatin analogue with retrosequence called 008. Furthermore, we coupled the hydrophobic linear peptide EMD 55068 with renin-inhibiting property as ligand to the Affigel® 10 matrix. As shown in Fig. 5, it seems that each of the hydrophobic substrates binds strongly to the same protein in the molecular weight range of 28 kDa. This 28 kDa cytosolic protein was identified by sequencing internal fragments as the glutathione-S-transferase subunit Yb1.

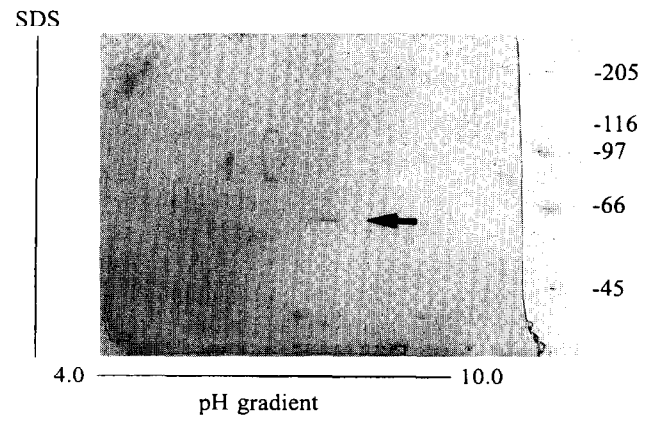

FIG. 3. The blPm proteins (1 mg) were separated by preparative nonequilibrium-pH-gel-electrophoresis (NEPHGE) and then electrotransferred onto cellulose nitrate. The polyclonal antisera specifically recognized one protein spot with an apparent molecular mass of 54 kDa, the UDP-glucuronosyltransferase, indicated by an arrow.

Checking the Uniformity of the 28 kDa Protein

To determine whether this 28 kDa protein band in SDS-PAGE is really a single protein, we analyzed the KSCN fraction in a two-dimensional PAGE. As illustrated in Fig. 6, the 28 kDa band was split off in a few spots differing only slightly in their pI.

Inhibition of the Activity of the Glutathione-S-Transferases of Rat Liver Cells by the Cyclolinopeptide A Analogue CDP

To determine whether the G-S-T activity in rat liver cell cytosol is inhibited by the presence of the cyclolinopeptide A analogue, we added 50 μM CDP and measured the conversion of the specific substrate 1-chloro-2,4-dinitrobenzene according to Lai and Tu [24]. We noted a significant increase in the enzymatic activity in the presence of CDP rather than an inhibition. In liver cell homogenate, there was a small increase in G-S-T activity from 6.6 to 7.0 nmol substrate · mg protein^{-1} · min^{-1}, but the enzyme activity measured in the cytosol increased from 7.4 to 9.5 nmol substrate · mg protein^{-1} · min^{-1}.

Uptake of the Radioactive Labelled Linear Hydrophobic Peptide EMD 51921 by Basolateral Rat Liver Plasma Membrane Vesicles Under Various Conditions

Having identified a subunit of the G-S-T as an intracellular binding protein for hydrophobic peptides, we tried to prove the significance of G-S-T for the uptake or intracellular accumulation of the peptides into hepatocytes. The uptake of cyclopeptides such as 008 and linear peptides such as the renin-inhibiting peptide EMD 51921 into isolated hepatocytes is carrier mediated. The driving forces for peptide uptake into hepatocytes are not known. It seems that the peptides tested are taken up via facilitated diffusion. In this case, intracellular binding proteins may be involved in unloading of the carrier protein and lowering the free substrate concentration. To answer this question precisely, it was necessary to conduct the experiments under defined and reproducible conditions; hence, the uptake studies were performed using blPm vesicles.

In vesicles preloaded with G-S-T or cytosolic proteins during their preparation and vesiculation, the uptake of EMD 51921 was significantly increased (Fig. 7). The equilibrium values at 300 sec were doubled by the presence of cytosolic proteins or G-S-T inside the vesicles. Binding

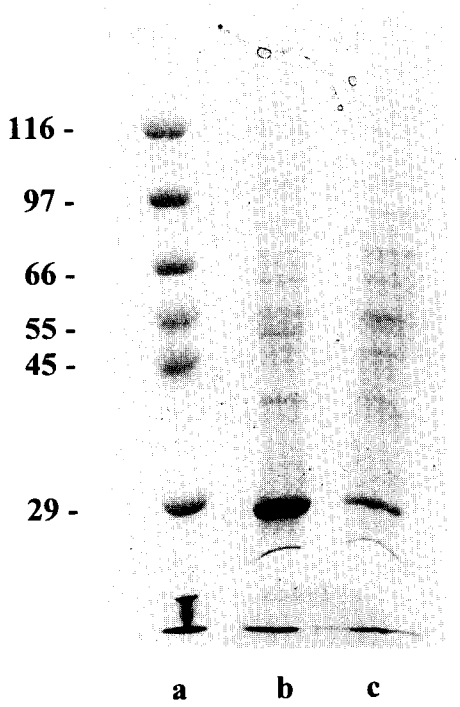

FIG. 4. Two milligrams of cytosolic liver cell proteins were applied to the CDP affinity matrix. After washing with NaCl/P_i and elution with KCl up to 1 M, the KSCN eluate was concentrated and separated in a 10% SDS-gel. (a) Marker proteins; (b, c) KSCN eluate. The Coomassie-stained gel shows that in the KSCN there was one major cytosolic protein with an apparent molecular weight of 28 kDa, which was bound to the CDP and thereafter eluted with the chaotropic salt. Lane c demonstrates that the 28 kDa protein was isolated in only minor amounts on reusing the CDP affinity matrix. This could be due to the loss of ligand after several extensive washing procedures. In addition, strongly bound proteins that were not completely detached limit the accessibility of the masked ligand.

of EMD 51921 to the outside of the vesicles was not affected by preloading the vesicles. This became evident in studies increasing the osmolarity of the incubation medium, which leads to shrinking of the vesicles. Measuring initial uptake rates at 20 sec and plotting these rates against 1/osmolarity showed that the binding to the membrane was not different under those conditions (preloaded vesicles 36%; unloaded vesicles 37%; data not shown). The K_m value for the affinity of the peptide to the as yet unidentified carrier protein determined according to Hanes [27] increased from 14.3 μM in standard vesicles to 25.3 μM in preloaded vesicles. The maximal transport rate of the carrier-mediated facilitated diffusion (V_{max} of 115.4 pmol · mg^{-1} · min^{-1} in standard vesicles and 348.5 pmol · mg^{-1} · min^{-1}/in preloaded vesicles; see Fig. 8) and the rate of permeation, however, were doubled in preloaded vesicles ($2.318 \cdot 10^{-7}$ cm/sec and $4.794 \cdot 10^{-7}$ cm/sec). The stimulation of uptake was inhibited by adding the bile acid taurocholate and, more intensively, cholate to the outside of the vesicles (data not shown).

DISCUSSION

In kinetic studies it has been shown that cyclolinopeptide A-related peptides as well as other cyclic peptides, such as somatostatin analogues and linear renin-inhibiting peptides, are mutual competitive inhibitors of bile acid uptake into isolated rat liver cells [4]. This indicates common transport systems for these substrates [28, 29]. No information, however, was available on the nature of the binding proteins of cyclolinopeptide analogues in hepatocytes. To identify such binding components, the cyclolinopeptide A analogue CDP was used in affinity chromatography. Two integral high-affinity binding proteins with apparent molecular masses of 50 and 54 kDa could be isolated from the plasma membrane fraction. The amino acid sequence of the 50 kDa binding protein is identical to members of the cytochrome P450 superfamily. Cytochromes P 450 II C are members of one subfamily involved in the metabolism of steroids. Approximately 17% of the total male rat liver microsomal cytochromes are CYP 2 C 13, an active steroid 6β-hydroxylase for testosterone, androstenedione, bile acids (e.g. lithocholic and chenodeoxycholic acid), and xenobiotics [30–32]. The amino acid sequence of the 54 kDa protein is identical to 3-hydroxyandrogen-UDP-glucuronosyltransferase, which also takes part in steroid metabolism [33], whereby it glucuronidates the free 3-OH position of several bile acids, e.g. lithocholic, deoxycholic, chenodeoxycholic and ursodeoxycholic acid, detoxifying them and facilitating their elimination by increased urinary excretion, especially during cholestasis [34, 35].

Although it seems obvious that these isolated enzymes function in the metabolizing of xenobiotics, the peptides used in affinity chromatography are not metabolized by cytochrome P450 enzymes or glucuronyltransferases [36]. Furthermore, there is no inhibition of the activity of cytochrome P450 isoenzymes or glucuronyltransferases in microsomal preparations in the presence of up to 50 μM CDP. Nevertheless, binding between the ligands and the isolated proteins is clearly demonstrated. There is a structural relationship between these peptides and bile acids, because the common uptake mechanism in rat hepatocytes is dependent upon recognition of a similar region of these compounds: the steroidal structure of the bile acids on the one hand and aromatic amino acid residues (in a special conformation) of the cyclic peptides on the other [1, 2, 6, 7]. Substrates transported via a common transport mechanism into rat hepatocytes share a common feature with the endogenous substrate cholate. As was demonstrated for the cyclic somatostatin analogues [37], cyclolinopeptide A [38], bumetanide and cholate [39], the cyclolinopeptide CDP also shows two distinct surfaces: one hydrophilic, comprising mainly the peptide backbone, and the other lipophilic, formed by protruding aromatic side chains [40]. Our results are supported by this common feature, because the microsomal enzymes, isolated as cyclolinopeptide binding proteins, are described as taking part in steroid metabolism or in binding bile acids. Aromatic residues of the cyclic

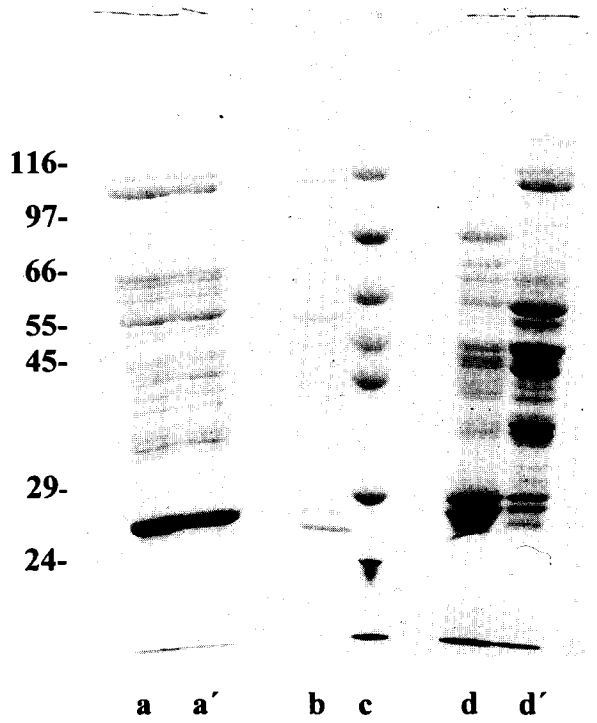

FIG. 5. Different hydrophobic peptide ligands were used in affinity chromatography. All are known to be taken up into rat hepatocytes as competitive inhibitors of bile acids. One cyclic peptide was 008, belonging to the group of somatostatin analogues with retrosequence. In addition, we used the linear hydrophobic peptide EMD 55068, which is an inhibitor of renin. Cytosolic proteins of rat liver cells were applied to various affinity columns. The KSCN eluates were concentrated and separated on a 10% SDS-gel. In all affinity chromatograms, there was one main protein detectable, which bound strongly to the peptides and was detached by KSCN elution. Lanes a, a'—KSCN eluates from the 008 affinity matrix; lane b—KSCN eluates from the CDP affinity matrix; and lanes d, d'—KSCN eluates from the EMD 55068 affinity matrix. Lanes a', b and d' show the proteins in the KSCN fraction after the reuse of the same affinity matrix for chromatographic separation. Lane c—marker proteins.

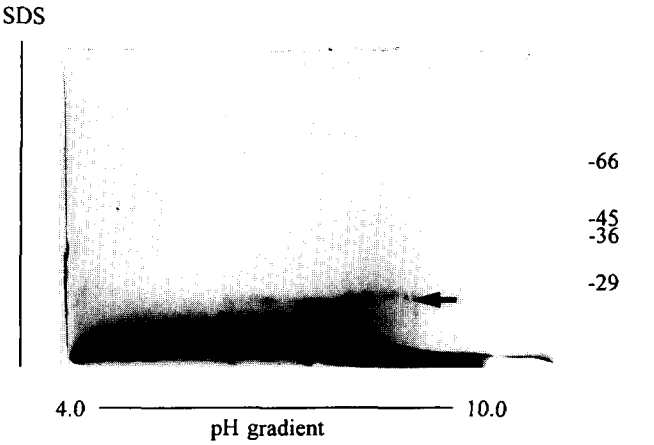

FIG. 6. Separation of KSCN eluates from CDP affinity chromatography of cytosolic rat hepatocytes by two-dimensional gel electrophoresis. The arrow shows that there are indeed several protein spots of the 28 kDa protein, which differ only slightly in pI.

peptide, such as phenylalanine and neighbouring proline residues, are probably responsible for mimicking a steroidal structure. Fasold and Hoffmann [41] have coisolated a cytochrome, P450 h, which is closely related to cytochrome P450 g and UDP-glucuronosyltransferase, as binding proteins for photoreactive bile acid analogues in affinity chromatography. In a subsequent affinity chromatography study, Wenzel and Ziegler [42] used the photoreactive bile acid derivative 4'amino-7-benzamidotaurocholate from Hoffmann and alternatively, another cyclopeptide 008, as ligands. The cyclopeptide 008 belongs to the somatostatin analogues with retrosequence and possesses high affinity for the transporter responsible for sinusoidal bile acid uptake. Two integral proteins with apparent molecular weights of 48 and 52 kDa and high ligand affinity were isolated from basolateral plasma membranes of rat hepatocytes. It was demonstrated that the 52 kDa 4'amino-7-benzamidotaurocholate binding protein was localized exclusively in blPm, not in microsomes. Unfortunately, protein sequencing was unsuccessful. Considering, however, the conformity of the purification methods and the relationship of the ligands used in affinity chromatography, it is likely that the 52 kDa protein above, purified with the 008 affinity matrix, corresponds to the UDP-glucuronosyltransferase isolated in this work, and that the 48 kDa protein is a member of the cytochrome family. The affinity chromatographic method, however, is unsuitable for predicting the functional connections between the ligands and their corresponding binding proteins.

There was no binding of antibodies to the isolated and partially sequenced integral binding proteins in immunofluorescence studies using intact isolated hepatocytes (data not shown). Hence, we assume these proteins are mainly located in microsomes. On the other hand, the similarities to the described enzymes concern only partial sequences, so it is conceivable that these proteins may be bifunctional, depending on different processing and localization subsequent to the sorting procedures, as was described for the epoxide hydrolase (E.C. 3.3.2.3.) by Levy et al. [43, 44]. Distinct forms of the enzyme are postulated as being responsible for some part of the sodium-dependent sinusoidal taurocholate uptake and for the sodium-independent bile acid transport in smooth endoplasmic reticulum. Studies on the microsomal uptake of cyclopeptides, however, have yet to be performed. Loeper *et al.* [45] were able to detect cytochrome P 450 species (of undefined function) on the outer surface of plasma membranes of rat hepatocytes. Petzinger *et al.* demonstrated the localization of protein disulfide isomerase (PDI) (E.C. 5.3.4.1.) [46] and catalase (E.C.1.11.1.6.) [47] in basolateral plasma membranes of rat hepatocytes. In addition, the 3,5,3'-tri-iodo-L-thyronine receptor in hepatocyte membranes is 93% homologous to PDI [48]. Waddell *et al.* [49] sequenced a glucose-transporting 52 kDa membrane protein of the endoplasmic reticulum. This transporter, GLUT-7, shows 68% similarity to

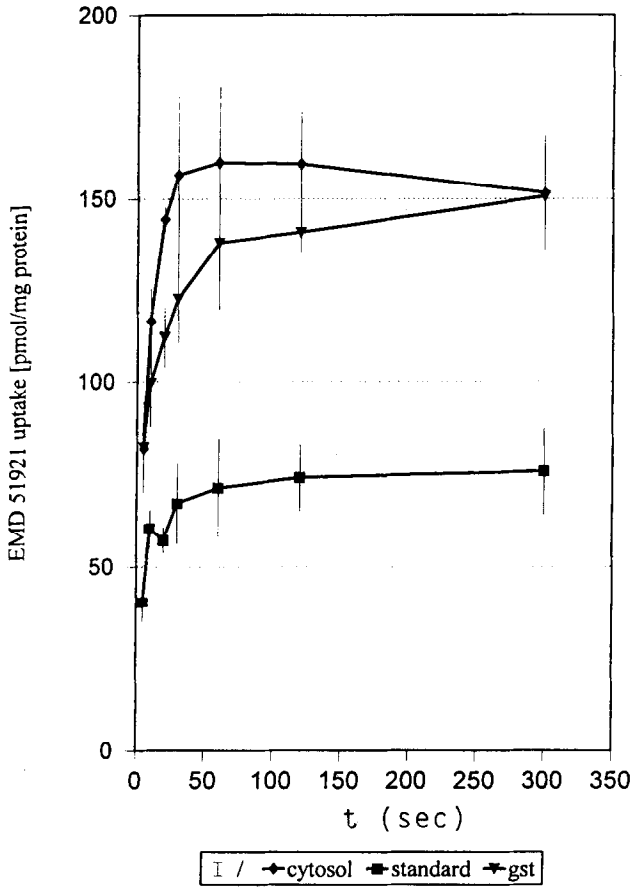

FIG. 7. EMD 51921 uptake into blPmv. The time-dependent uptake of EMD 51921 into vesicles containing either cytosol (◆), glutathione-S-transferase (▽) or standard vesicle buffer (■). EMD 51921 uptake after 60 sec was increased to 169% in cytosolic vesicles and 154% in G-S-T vesicles compared to standard vesicles.

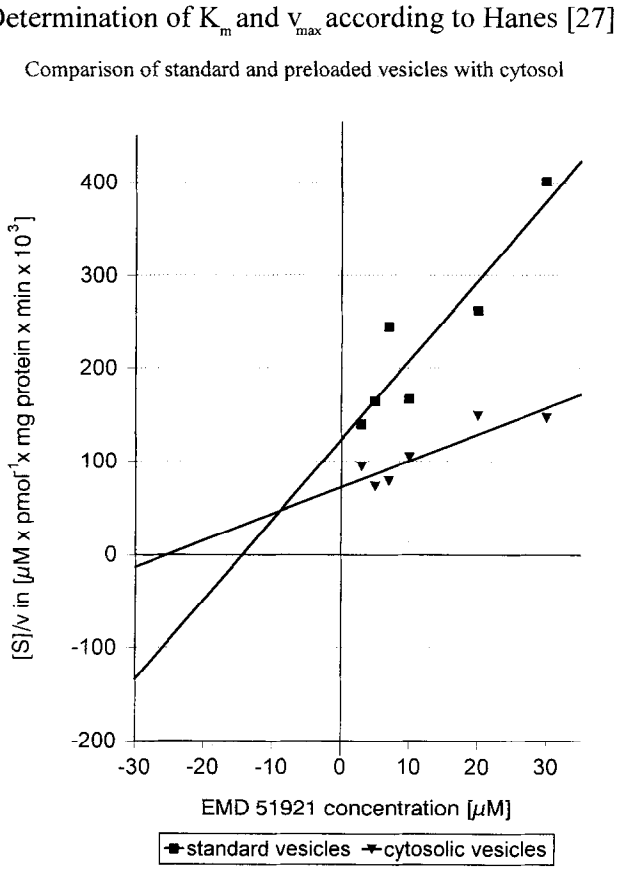

FIG. 8. Calculation of K_m and V_{max} of the EMD 51921 uptake into standard vesicles and vesicles containing cytosol according to Hanes [27]: K_m was 14.3 µM and V_{max} was 115.4 pmol · mg^{-1} · min^{-1} in standard vesicles. In cytosolic vesicles, we determined a K_m of 25.3 µM and V_{max} of 348.5 pmol · mg^{-1} · min^{-1}.

the GLUT-2 of the plasma membrane and has sequence homologies to UDP-glucuronosyltransferase. In contrast to the GLUT-2, the GLUT-7 has 6 additional amino acids at the C-terminus to anchor the protein in the membrane of the endoplasmic reticulum. This indicates a possible relationship between microsomal enzymes and their modifications leading to completely different functions for these proteins, including even roles in transport.

In our study, we have identified at least one subunit of the glutathione-S-transferase as an intracellular binding protein for small hydrophobic peptides, such as the cyclolinopeptide A analogue CDP, the somatostatin analogue 008, and even the linear hydrophobic peptide EMD 55068. The superfamily of the glutathione-S-transferases comprises homo- or heterodimeric proteins with different subunits [50, 51]. In addition to their enzymatic function they have distinct high-affinity binding sites for organic anions, bile acids or carcinogens [50, 52–55]. These are important in the neutralization of carcinogens, either enzymatically or nonenzymatically, or for the prevention of a possible cholestatic effect of hydrophobic bile acids, e.g. chenodeoxycholic acid or lithocholic acid. These bile acids are taken up into hepatocytes mainly via passive diffusion with intracellular binding, thereby causing their massive intracellular accumulation and the reduction of toxic free bile acids. Surprisingly, the enzymatic activity of the glutathione-S-transferases was increased in the presence of CDP. We, thus, presume that the binding of the cyclolinopeptide A analogue CDP to the G-S-T at a distinct binding site causes an alteration of its allosteric conformation, leading to a better accessibility of the substrate to the enzyme's active center. The influence of G-S-T-bound glutathione on additional binding of bile acids or organic anions was described by Takikawa *et al.* [55]. In parallel, we assume that physiologically glutathione at millimolar concentrations is permanently present with these enzymes, causing a reduction of their affinity for other ligands such as the peptides tested here. This is especially true for the partially sequenced subunit Yb1. In our artificial isolation system, we did not focus on this problem, but *in vivo* the affinity of the subunit Yb1 to hydrophobic peptides might very well be lower. We demonstrated by two-dimensional NEPHGE that other subunits of the glutathione-S-transferase family

also participate in intracellular peptide binding. The influence of physiological glutathione saturation may, consequently, be of minor importance. Similar to the case of accumulation of hydrophobic bile acids, we propose that these hydrophobic peptides, which permeate the hepatocytes' basolateral plasma membrane with high permeability constants (cyclic peptides: 10^{-4} cm/sec and linear peptides $8 \cdot 10^{-6}$ cm/sec) are enriched in liver cells by binding to the cytosolic glutathione-S-transferases. This may be one reason for the enormous 250-fold intrahepatocellular concentration of hydrophobic in contrast to hydrophilic peptides [56, 57]. To support this hypothesis, we performed vesicle studies under various conditions. We measured the uptake of radioactively labelled EMD 51921, another linear hydrophobic peptide with renin-inhibiting activity, into standard vesicles with a defined buffer solution and compared these results with its uptake into vesicles loaded with cytosolic proteins, with G-S-T and, as another control, with bovine serum albumin. These experiments clearly demonstrated the uptake stimulation of EMD by G-S-T and the cytosolic proteins. This effect was inhibited by the addition of taurocholate or cholate, indicating that there may not only be competitive uptake but competition for intracellular binding sites as well.

We have tried, thus far unsuccessfully, to characterize the driving forces of EMD uptake. As there was no dependence on ATP, on membrane potential, or on a H^+- or OH^--gradient (data not shown), we thus propose that there is an Na^+-independent, saturable, carrier-mediated facilitated diffusion. Cytosolic proteins, predominantly the G-S-T, serve to enrich cyclic and linear hydrophobic peptides, either by binding and accordingly minimizing free peptide concentrations, or by stimulating the transporter by an as yet undisclosed mechanism.

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References

1. Kessler H, Klein M, Mueller A, Wagner K, Bats JW, Ziegler K and Frimmer M, Conformational prerequisites for the *in vitro* inhibition of cholate uptake in hepatocytes by cyclic analogues of antamanide and somatostatin. *Angew Chem Int* **25:** 997–999, 1986.
2. Zanotti G, Rossi F, Di Blasio B, Pedone C, Benedetti E, Ziegler K and Tancredi T, Structure activity relationship in cytoprotective peptides. In: *Peptides* (Eds. Rivier JE and Marshall GK), pp. 118–119, 1990.
3. Ziegler K and Frimmer M, Molecular aspects of cytoprotection by modified somatostatins. *Klin Wochenschr* **64:** 87–89, 1986.
4. Ziegler K, Frimmer M, Kessler H, Damm I, Eiermann V, Koll S and Zarbock J, Modified somatostatins of a multispecific transport system for bile acids and phallotoxins in isolated hepatocytes. *Biochim Biophys Acta* **845:** 86–93, 1985.
5. Kaufmann HP and Tobschirbel A, Über ein Oligopeptid aus Leinsamen. *Chem Ber* **92:** 2805–2809, 1959.
6. Rossi F, Saviano M, Di Blasio B, Zanotti G, Maione AM, Tancredi T and Pedone C, Bioactive peptides: Solid state, solution and molecular dynamics studies of a cyclolinopeptide A-related cystinyl cyclopentapeptide. *Biopolymers* **34:** 273–284, 1994.
7. Kessler H, Klein M, Müller A, Wagner K, Bats JW, Ziegler K and Frimmer M, Konformative Voraussetzungen für die biologische Aktivität von cytoprotektiv wirksamen Cyclopeptiden. *Angew Chem* **13:** 34–39, 1987.
8. Ziegler K, Pedone C and Kessler H, Common structural features of cyclopeptides that inhibit bile acid uptake. *Biol Chem Hoppe-Seyler* **371:** 754–755, 1990.
9. Blitzer BL and Donovan CB, A new method for the rapid isolation of basolateral plasma membrane vesicles from rat liver. *J Biol Chem* **259:** 9295–9301, 1984.
10. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193:** 265–275, 1951.
11. Scharschmidt BF, Keefe EB, Blankenship NM and Ockner RK, Validation of a recording spectrophotometric method for measurement of membrane-associated Mg^{2+} and Na^+/K^+-ATPase activity. *J Lab Clin Med* **93:** 790–799, 1979.
12. Harper AE, *Methoden der enzymatischen Analyse*, pp. 788–792. Verlag Chemie, Weinheim, 1962.
13. Michell RH and Hawthorne JN, The site of diphosphoinositide synthesis in rat liver. *Biochem Biophys Res Commun* **21:** 33–38, 1965.
14. Chen PS, Toribara TY and Warner H, Microdetermination of phosphorus. *Anal Chem* **28:** 1756–1758, 1956.
15. Ames BN and Dubin DT, The role of polyamines in the neutralization of bacteriophage desoxyribonucleic acid. *J Biol Chem* **235:** 769–775, 1960.
16. Bordier C, Phase separation of integral membrane proteins in Triton X-114 solution. *J Biol Chem* **256:** 1604–1607, 1981.
17. Honscha W, Ottallah M, Schenk A, Schuh K and Petzinger E, Separation and purification by two-dimensional gel electrophoresis of a 52–54 kDa bumetanide binding protein from rat liver plasma membrane. *Eur J Pharmacol* **226:** 215–223, 1992.
18. Wenzel U, Jouvenal K, Tripier D and Ziegler K, Cyclic somatostatin analogs bind specifically to 6.1 carboxylesterase of rat liver cells. *Biochem Pharmacol* **49:** 479–487, 1995.
19. O'Farrell PH, High resolution two-dimensional electrophoresis of proteins. *J Biol Chem* **250:** 4007–4021, 1975.
20. Harlow E and Lane E, *Antibodies, a Laboratory Manual*, pp. 61–119. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988.
21. Ball EH, Quantitation of proteins by elution of Coomassie Brilliant Blue R from stained bands after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Anal Biochem* **155:** 23–27, 1986.
22. Rosenfeld J, Capdevielle J, Guillemot JC and Ferrara P, In-gel digestion of proteins for internal sequence analysis after one- or two-dimensional gel electrophoresis. *Anal Biochem* **203:** 173–179, 1992.
23. Green MD and Tephly TR, N-glucuronidation of carcinogenic aromatic amines catalyzed by rat hepatic microsomal preparations and purified rat liver uridine 5'-diphosphate-glucuronosyltransferases. *Cancer Res* **47:** 2028–2031, 1987.
24. Lai H-CJ and Tu C-PD, Rat glutathione S-transferases supergene family. *J Biol Chem* **261:** 13793–13799, 1986.
25. Meier PJ, Sztul ES, Reuben A and Boyer JL, Structural and functional polarity of canalicular and basolateral plasma

membrane vesicles isolated in high yield from rat liver. *J Cell Biol* **98:** 991–1000, 1984a.
26. Meier PJ, Meier-Abt AS, Barrett C and Boyer JL, Mechanism of taurocholate transport in canalicular and basolateral plasma membrane vesicles. *J Biol Chem* **259:** 10614–10622, 1984b.
27. Hanes CS, Studies of plant amylases. The effect of starch concentration upon the velocity of hydrolysis by amylase of germinated barley. *Biochem J* **26:** 1406–1421, 1932.
28. Petzinger E, Ziegler K and Frimmer M, Occurrence of a multispecific transporter for the hepatocellular accumulation of bile acids and various cyclopeptides. In: *Bile Acids and the Liver* (Eds. Paumgartner G, Stiehl A and Gerok W), pp. 111–124. MTP Press, Lancaster, UK, 1987.
29. Ziegler K, Lins W and Frimmer M, Hepatocellular transport of cyclosomatostatins: Evidence for a carrier system related to the multispecific bile acid transporter. *Biochim Biophys Acta* **1061:** 287–297, 1991.
30. McClellan-Green P, Waxman DJ, Caveness M and Goldstein JA, Phenotypic differences in expression of cytochrome P450g but not its mRNA in outbred male Sprague–Dawley rats. *Arch Biochem Biophys* **253:** 13–25, 1987.
31. Zimniak P, Holsztynska EJ, Radominska A, Iscan M, Lester R and Waxman DJ, Distinct forms of cytochrome P-450 are responsible for 6 beta-hydroxylation of bile acids and neutral steroids. *Biochem J* **275:** 105–111, 1991.
32. Zimniak P, Radominska A, Zimniak M and Lester R, Formation of three types of glucuronides of 6-hydroxy bile acids by rat liver microsomes. *J Lipid Res* **29:** 183–190, 1988.
33. Mackenzie PI, Rat liver UDP-glucuronosyltransferase. cDNA sequence and expression of a form glucuronidating 3-hydroxyandrogens. *J Biol Chem* **261:** 14112–14117, 1986.
34. Stiehl A, Becker M, Czygan P, Fröhlich W, Kommerell B, Rotthauwe HW and Senn M, Bile acids and their sulfated and glucuronidated derivatives in bile, plasma and urine of children with intrahepatic cholestasis: effects of phenobarbital treatment. *Eur J Clin Invest* **10:** 307–316, 1980.
35. Kirkpatrick RB, Falany CN and Tephly TR, Glucuronidation of bile acids by rat liver 3-OH androgen UDP-glucuronosyltransferase. *J Biol Chem* **259:** 6176–6180, 1984.
36. Hruby VJ, Design of peptide hormone and neurotransmitter analogues. *Trends Pharmacol Sci* **6:** 259–262, 1985.
37. Kessler H, Gehrke M, Haupt A, Klein M, Müller A and Wagner K, Common structural features for cytoprotection activities of somatostatin, antamanide and related peptides. *Klin Wochenschr* **64:** 74–78, 1986.
38. Tancredi T, Benedetti E, Grimaldi M, Pedone C, Rossi F, Saviano M, Temussi PA and Zanotti G, Ion binding of cyclolinopeptide A: An NMR and CD conformational study. *Biopolymers* **31:** 761–767, 1991.
39. Petzinger E, Föllmann W, Blumrich M, Schermuly R, Schulz S, Hahnen J and Feit PW, Interaction of bumetanide derivatives with hepatocellular bile acid uptake. *Am Physiol Soc* **28:** G942–G954, 1993.
40. Zanotti G, Maione A, Rossi F, Saviano M, Pedone C, Saviano G and Tancredi T, Solution structure of cyclopeptides inhibiting the cholate uptake in hepatocytes. In: *Peptides 1994*, Proceedings of the 23 European Peptide Symposium (Ed. H.L.S. Maia), pp. 559–560, 1994.
41. Hoffmann A, Müllner S, Ziegler K and Fasold H, Purification and partial sequence of proteins involved in the cholic acid transport into rat liver hepatocytes. *J Protein Chem* **12:** 763–767, 1993.
42. Wenzel U and Ziegler K, Binding proteins for cyclosomatostatins and bile acids in basolateral plasma membranes of rat liver. *Biochim Biophys Acta* **1193:** 17–23, 1994.
43. Von Dippe P, Amoui M, Alves C and Levy D, Sodium dependent bile acid transport by hepatocytes is mediated by a protein similar to microsomal epoxide hydrolase. *Am J Physiol* **264:** G528–534, 1993.
44. Alves C, von Dippe P, Amoui M and Levy D, Bile acid transport into hepatocyte smooth endoplasmic reticulum vesicles is mediated by microsomal epoxide hydrolase, a membrane protein exhibiting two distinct topological orientations. *J Biol Chem* **268:** 20148–20155, 1993.
45. Loeper J, Descartoire V, Maurice M, Beaune P, Feldmann G, Larrey D and Pessayre D, Presence of a functional cytochrome P-450 on isolated rat hepatocyte plasma membrane. *Hepatology* **11:** 850–855, 1990.
46. Honscha W, Ottallah M, Kistner A, Platte HD and Petzinger E, A membrane-bound form of protein disulfide isomerase (PDI) and the hepatic uptake of organic anions. *Biochim Biophys Acta* **1153:** 175–183, 1993.
47. Ottallah-Kolac M, Tripier D, Brühl B, Platte H, Jouvenal BK, Schuh K, Kemmer H and Petzinger E, The 60 kDa bumetanide-binding protein from rat liver membranes is a catalase. *Eur J Biochem* **228:** 506–514, 1995.
48. Horiuchi R, Yamauchi K, Hayashi H, Koya S, Takeuchi Y, Kato K, Kobyashi M and Takikawa H, Purification and characterization of a 55 kDa protein with 3,5,3′-triiodo-L-thyronine binding activity and protein disulfide isomerase activity from beef liver membrane. *Eur J Biochem* **183:** 529–538, 1989.
49. Wadell ID, Zomerschoe AG, Voice MW and Burchell A, Cloning and expression of a hepatic microsomal glucose transport protein. Comparison with liver plasma-membrane glucose-transport protein GLUT 2. *Biochem J* **286:** 173–177, 1992.
50. Kaplowitz N, Physiological significance of glutathione-S-transferases. *Am J Physiol* **239:** 439–444, 1980.
51. Pickett CB and Lu AYH, Glutathione-*S*-transferases: Gene structure, regulation and biological function. *Annu Rev Biochem* **58:** 743–764, 1986.
52. Stolz A, Sugiyama Y, Kuhlenkamp J, Osadchey B, Yamada T, Belnkamp W, Balistreri W, Kaplowitz N, Cytosolic bile acid binding protein in rat liver. *Hepatology* **6:** 433–439, 1986.
53. Stolz A, Takikawa H, Ookthens M and Kaplowitz N, The role of cytoplasmic proteins in hepatic bile acid transport. *Annu Rev Physiol* **51:** 161–176, 1989.
54. Takikawa H, Sugiyama Y and Kaplowitz N, Binding of bile acids by glutathione-S-transferases from rat liver. *J Lipid Res* **27:** 955–966, 1986.
55. Takikawa H and Kaplowitz N, Comparison of the binding sites of GSH S-transferases of the Ya- and Yb-subunit classes: Effect of glutathione on the binding of bile acids. *J Lipid Res* **29:** 279–286, 1988.
56. Ziegler K and Seeberger A, Hepatocellular uptake of peptides-I: Carrier-mediated uptake of hydrophilic linear peptides with renin-inhibitory activity into isolated rat liver cells. *Biochem Pharmacol* **45:** 909–916, 1993.
57. Seeberger A and Ziegler K, Hepatocellular uptake of peptides—II: Interactions between hydrophilic linear renin-inhibiting peptides and transport systems for endogenous substrates in liver cells. *Biochem Pharmacol* **45:** 917–925, 1993.