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CYCLIC SOMATOSTATIN ANALOGS BIND SPECIFICALLY TO pI 6.1 CARBOXYLESTERASE OF RAT LIVER CELLS

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Abstract—The hydrophobic cyclohexapeptide cyclo(Phe-Thr-Lys-Trp-Phe-DPro) (008), an analog of somatostatin with retro sequence, was previously shown to competitively inhibit the uptake of cholate and taurocholate into isolated rat liver cells. Conversely, the competitive uptake inhibition of 008 into isolated rat hepatocytes by bile acids confirmed the observation of common binding and transport sites by bile acids and cyclosomatostatin. Furthermore the transport characteristics of 008 uptake revealed a significant and rapid binding to cell membranes. In this context it was of special interest to investigate the specificity of the binding component since specific binding of the substrate to membrane proteins could be responsible for the low K_m of 008-transport. Therefore, the cyclohexapeptide 008 could be used as the ligand in affinity chromatography in order to isolate such binding proteins. The gel matrix used did not interact non-specifically with octylglucoside-solubilized proteins from isolated rat liver plasma membranes. In affinity chromatography of octylglucoside-solubilized plasma membranes, two dominant proteins with apparent molecular masses of 60 and 58 kDa bound specifically to the 008 ligand. When used as ligands in affinity chromatography, these membrane-associated 60 and 58 kDa proteins bound exclusively to aromatic cyclopeptides, e.g. cyclosomatostatin 008, but not to linear peptides or taurocholate derivatives. The amino acid sequences of tryptic digests of the 008-affinity-purified 58 kDa protein were identical to the sequence of a microsomal pI 6.1 carboxylesterase. Immunofluorescence of intact hepatocytes showed that this xenobiotic metabolizing enzyme is also located in sinusoidal rat liver plasma membranes and could therefore account for the extensive and specific binding of the cyclosomatostatin to sinusoidal plasma membranes of rat liver.

Key words: cyclosomatostatin; binding proteins; bile acid transport system; hepatocytes; rat liver

The development of drugs with peptide structure, e.g. analogs of somatostatin, is facilitated by manipulation of the peptide conformation and structure. For example, cyclization leads to the formation of compounds that are stable to proteolytic cleavage (cyclosomatostatins) [1]. Nevertheless, these compounds often have a short half-life *in vivo* due to rapid biliary elimination. Pharmacokinetic studies in the rat have shown that somatostatin and especially its cyclic analogs are rapidly taken up by the liver and are subsequently excreted as intact peptides in the bile [2].

The cyclosomatostatin analog 008§, competitively inhibits the uptake of both taurocholate and cholate into isolated rat hepatocytes, with K_i values of 2 and 3 μM, respectively [3]. On the other hand the uptake of 008 is competitively inhibited by taurocholate and cholate, with K_i values of 13 and 7 μM, respectively [3]. The existence of mutual competitive inhibition of bile acid and 008 transport is the main evidence for a common transport system of these chemically different compounds and for their recognition by a common transport protein. As a consequence, however, it makes the clinical usefulness of such drugs questionable, since they show high first-pass effects and cause cholestasis. Besides efficient and specific inhibition of bile acid transporters, the cyclosomatostatin 008 displayed a rapid and significant binding to the hepatocyte plasma membranes, as was previously shown [3].

It was of special interest to clarify whether this large binding component is due to the hydrophobic properties of 008, leading to unspecific adsorption to the plasma membranes in addition to its specific binding to the bile acid transporters, or whether the binding is exclusively specific, since a specific binding could probably account for the low K_m of 1.5 μM [3]. To proceed on the assumption that transport of a substrate through the sinusoidal plasma membrane of the hepatocyte first requires the binding of the compound to the carrier protein or to proteins which are related to the transporter, the aim of the present study was to identify and isolate these binding proteins for cyclosomatostatins in isolated rat liver basolateral plasma membranes. To this end, the cyclohexapeptide 008 was used as the ligand in affinity chromatography.

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§ Abbreviations: ABATC, 4′-amino-7-benzamido-taurocholate c, cyclol; CHAPS, 3-[(cholamidopropyl)-dimethylammonia]-1-propanesulfonate; 008, cyclo(Phe-Thr-Lys-Trp-Phe-DPro); blPm, basolateral plasma membranes; PMSF, phenylmethylsulfonyl fluoride.

MATERIALS AND METHODS

Materials

008 was a generous gift from Prof. Dr H. Kessler (Munich, Germany). The following compounds were also kindly provided as gifts: ABATC from Prof. Dr H. Fasold and Dr S. Müllner (Frankfurt/M., Germany); the linear renin inhibitor EMD 55068 from Merck (Darmstadt, Germany); and cyclodecapeptide c(Phe-Ala-Lys-Pro-Phe-Phe-Ala-Lys-Pro-Phe) from Dr G. Zannotti (Rome, Italy). Affi-Gel 10 was obtained from Bio-Rad (Munich, Germany). Octylglucoside was acquired from Merck (Darmstadt, Germany). Marker proteins for SDS–PAGE were from Pharmacia (Heidelberg, Germany). Trypsin of sequencing grade was from Boehringer (Mannheim, Germany). The pI 6.0 carboxylesterase antibody was a generous gift from Prof. Dr R. Mentlein (Kiel, Germany). The second antibody used for immunofluorescence (fluorescein-isothiocyanate-conjugated) was purchased from Sigma (Deisenhofen, Germany). The reagents for cell culture were from GIBCO-BRL (Deisenhofen, Germany). All other chemicals were of analytical grade purity or better.

Methods

Isolation of rat hepatocytes. Rat liver parenchymal cells were isolated from male Wistar rats by the perfusion technique of Berry and Friend [4]. Only preparations with less than 15% non-viable cells, as determined by Trypan Blue exclusion, were used for immunofluorescence studies.

Immunofluorescence. Immunofluorescence was performed as described by Honscha *et al.* [5]. Accordingly, freshly isolated hepatocytes were grown on 12 mm cover slips for 2 hr and then fixed with 3% freshly prepared paraformaldehyde in 100 mM PBS, pH 7.4, for 1 hr at 4°. The cells were washed three times for 10 min with PBS, pH 7.4 and subsequently incubated with rabbit antisera to rat liver microsomal carboxylesterase p*I* 6.0 [6] at a dilution of 1:50 overnight at 4°. After three additional 10 min washings with PBS, pH 7.4, the cells were incubated with fluorescein-isothiocyanate conjugated goat anti-rabbit IgG at a dilution of 1:200 for 2 hr at room temperature in the dark. After mounting the cover slips onto slides with a glycerol-based medium containing *p*-phenylene-diamine to reduce photobleaching [7], the hepatocytes were visualized with a Zeiss Universal microscope, equipped with appropriate fluorescence filters. Hepatocytes incubated with preimmune serum served as control.

Preparation of blPm from rat liver. Sinusoidal plasma membranes were prepared from livers of male Wistar rats according to the method of Blitzer and Donovan [8], 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 PMSF, and was centrifuged at 2500 *g* for 15 min using a GSA-rotor in a Sorvall RC5C centrifuge. A continuous Percoll gradient was performed by centrifugation in an SS 34-rotor (Sorvall) at 24,000 *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 −196° in liquid nitrogen.

Analysis of enzyme activities. Purity of plasma membrane fractions was characterized by determination of marker enzymes. Na^+/K^+-ATPase was determined according to the method of Scharschmidt *et al.* [9]. Glucose-6-phosphatase was determined according to Harper [10] and 5′-nucleotidase according to Michell and Hawthorne [11]. Release of an organic phosphate by the latter two enzymes was measured according to the method of Chen *et al.* [12], modified by Ames and Dubin [13]. The quality of the membrane preparation was tested by measuring marker enzyme enrichments. The blPm marker Na^+/K^+-ATPase was enriched 18–22-fold, the canalicular marker 5′-nucleotidase 3.2–3.5-fold and the marker for microsomes, glucose-6-phosphatase, was enriched 1.5–1.7-fold, indicating a microsomal contamination of blPm. Esterase activity of the 008-affinity-purified proteins was determined spectrophotometrically by the release of 4-nitrophenol from 4-nitrophenylacetate at 405 nm according to the method of Mentlein *et al.* [14]. The concentration of the substrate was 200 μM. The inhibitors cyclohexapeptide 008, cyclodecapeptide c(Phe-Ala-Lys-Pro-Phe-Phe-Ala-Lys-Pro-Phe) and linear renin inhibitor 55068 [15] were used at a concentration of 100 μM in 50 mM Tris–HCl buffer, pH 7.4, 2% methanol. The reaction was started by addition of 10 μg of the 008-affinity-purified protein.

Separation of hydrophilic membrane associated proteins from integral hydrophobic proteins. Plasma membrane vesicles were dialysed overnight at 4° against 10 mM EDTA at pH 8.0. Thereafter, membrane-associated hydrophilic proteins were separated from the more hydrophobic proteins by centrifugation at 100,000 *g* for 30 min at 4°.

Solubilization of the plasma membranes. The blPm pretreated with EDTA pH 8.0 were solubilized for 1 hr at 4° with 2% octylglucoside at a protein to detergent ratio of 1:10. After a centrifugation step for 1 hr at 100,000 *g* the supernatant was used for affinity chromatography.

Preparation of 008 *affinity columns.* After washing the gel bed (Affi-Gel 10) several times with ethanol, 17 μmol 008 per mL of gel, dissolved in ethanol, were added and coupled by gentle agitation at room temperature overnight. The coupling reaction of the cyclosomatostatin 008 to the Affi-Gel 10 matrix is shown in Fig. 1. Subsequently, 100 μL 1 M ethanolamine (pH 8.0) per mL gel were added and stirred for 1 hr at room temperature to block free binding sites. The gel was then washed with an excess of PBS (pH 7.4), containing 1 mM PMSF, and stored at 4°. The ethanolamine-blocked Affi-Gel 10 matrix without ligand was used as control. After packing the 0.5 × 10 cm columns with the gel matrices the gel was degassed prior to use.

Affinity chromatography. All steps in affinity chromatography were performed at 4°. The solubilized plasma membrane proteins were applied onto the column and allowed to penetrate into the gelbed. After an incubation period of 1 hr the column was

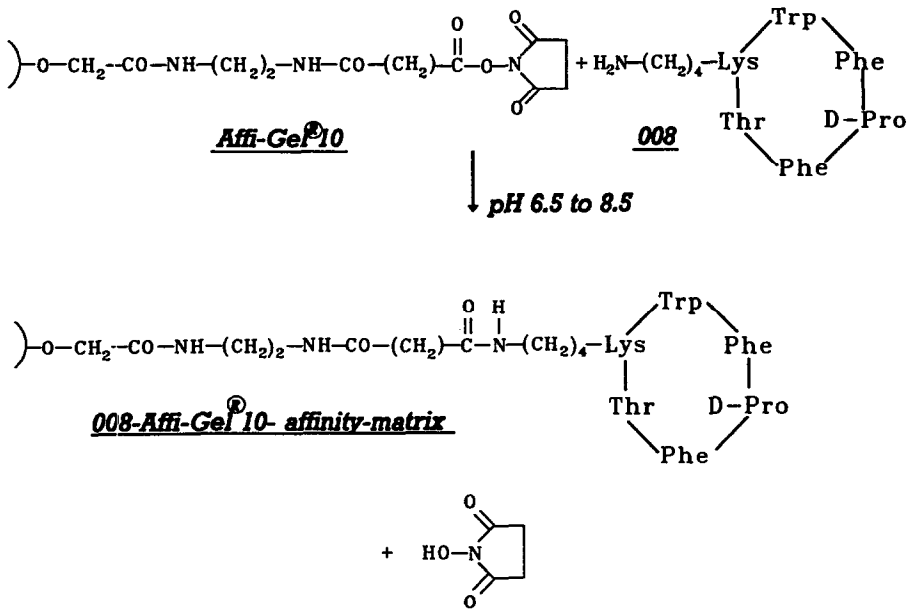

Fig. 1. Coupling reaction of cyclosomatostatin 008 with Affi-Gel 10. The 008 affinity resin was synthesized under anhydrous conditions as described in the Materials and Methods.

washed with PBS to elute unbound proteins followed by a linear 0–1 M KCl gradient to elute proteins bound via electrostatic interactions. To increase further the specificity of the column, an additional washing step with a linear 0–500 μM piperonyl-butoxide gradient in 30% ethanol was applied to elute cytochrome P450 dependent enzymes. These enzymes are known to represent contaminating microsomal proteins in the blPm fraction. Since their photolabelling by bile acid derivatives has been demonstrated previously [16], they could possibly also display affinity to 008. Elution of proteins bound specifically to the cyclosomatostatin ligand via hydrophobic interactions was carried out with a linear 0–1 M KSCN gradient. Finally a linear 0–10 mM taurocholate gradient was applied to elute proteins which could not be desorbed by weakening the hydrophobic interactions but which display a high affinity to bile acids. All elution steps were performed in the presence of a detergent concentration above the critical micellar concentration (cmc). Elution was done at a flow rate of 0.17 mL/min. The protein-containing fractions, as determined by the method of Lowry *et al.* [17], were pooled and concentrated with Centricon™ microconcentrators (exclusion limit 30 kDa) prior to analysis by SDS–PAGE according to the method of Laemmli [18]. If no protein was detectable by the Lowry method, the whole gradient was concentrated prior to SDS–PAGE. In Fig. 2 a typical protein elution profile of octylglucoside-solubilized blPm on the 008-column is shown.

In-gel digestion of proteins with trypsin for internal sequence analysis after SDS–PAGE. 008-affinity-purified proteins of 20 chromatographic runs were pooled and concentrated prior to separation on a preparative 10% SDS–polyacrylamide gel. The gel was stained in 0.2% Coomassie Brilliant Blue G-250/20% methanol/0.5% acetic acid for 20 min and destained in 30% methanol until the background became colorless. The stained protein bands were excised and washed for 2 days in aqua bidest. The water was changed frequently. The bands were cut into 1 mm pieces and lyophilised for 4 hr in a WKF-L2 freeze-dryer. For in-gel digestion trypsin was dissolved in 1 mM HCl (1 μg/μL). Trypsin (10% of protein weight) was made up to 100 μL with 0.2 M *N*-methylmorpholine-acetate/1 M urea, pH 7.8, and added to the gel pieces. Subsequently the gel pieces were soaked in the same buffer until no more fluid was taken up and shaken gently overnight. The supernatant was taken off, the gel pieces washed with 500 μL of buffer shaken for 5 min and

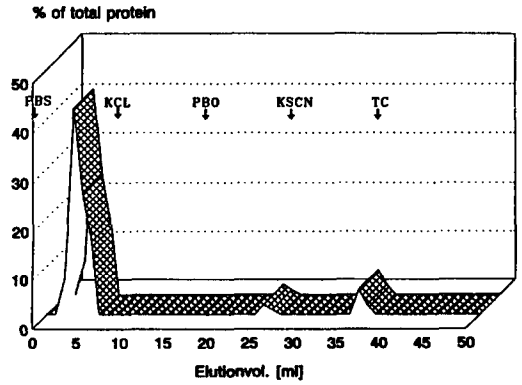

Fig. 2. Protein distribution of octylglucoside-solubilized blPm in 008 affinity chromatography. After washing the column with PBS, pH 7.4, the linear gradients were applied where indicated (↓). The gradients were: KCl, 0–1 M; piperonylbutoxide (PBO), 0–500 μM; KSCN, 0–1 M; taurocholate (TC), 0–10 mM.

centrifuged for 3 hr in an Eppendorf microfuge at 12,000 rpm. The supernatant was again taken off, the gel pieces washed with 500 μL trifluoroacetic acid, shaken for 5 min and centrifuged for 1 hr. The last washing step was repeated with 20% acetonitrile.

Separation of tryptic peptides and amino acid sequencing. The combined supernatants from the in-gel digestion procedure were injected on an Aquapore®, C8 reversed phase HPLC column (250 mm × 1 mm). The tryptic peptides were eluted from the column using a linear gradient formed from buffer A (5% acetonitrile in water, 0.1% trifluoroacetic acid) and buffer B (90% acetonitrile in water, 0.1% trifluoroacetic acid). The gradient was performed from 0–55% buffer B in 76 min, at a flow rate of 50 μL/min. The peptides were collected and submitted to automatic sequence analysis using a pulsed liquid protein sequencer (477A, Applied Biosystems).

RESULTS

Affinity chromatography of EDTA-extracted octylglucoside solubilized blPm proteins on 008 *affinity columns*

As the first step in purification of integral plasma membrane proteins, the blPm were dialysed against 10 mM EDTA, pH 8.0 to remove proteins loosely bound to membrane bilayer via Ca^{2+} ions. The resulting more hydrophobic integral proteins were solubilized with the detergent octylglucoside. Octylglucoside-solubilized proteins did not bind to the gel matrix Affi Gel 10 without ligand. Solubilized plasma membrane proteins of 58 and 60 kDa, however, bound to the 008 affinity matrix and were specifically eluted by KSCN. Proteins of 56, 43 and 37 kDa were found in the fraction of taurocholate, a competitive inhibitor of 008 uptake into isolated rat hepatocytes (Fig. 3A). The massively silver-stained 67 kDa protein of the piperonylbutoxide (Fig. 3A, lane d) and KSCN fraction (Fig. 3A, lane e) is likely to be albumin since this protein was identified by chemically reactive and photolabile bile acid analogs [19, 20]. A 43 kDa protein (taurocholate fraction: Fig. 3A, lane f) was identified as actin [21]. The role of albumin in bile acid uptake is controversial [22, 23], the manner by which this hydrophilic protein adsorbs to the hydrophobic parts of the membrane during EDTA extraction remains unclear. The 43 kDa protein actin does not seem to be directly involved in bile acid transport [24, 25].

It must be noted that the number of the cyclosomatostatin binding proteins could not be calculated from silver-stained PAGE, since the intensity of the sensitive silver stain is more dependent on the kind of protein than on its quantity. Therefore, the proteins specifically eluted from the 008 column were separated by SDS–PAGE, stained with Coomassie Brilliant Blue G-250 and quantified by elution of the dye from the stained protein bands [26]. It became evident that only the two binding proteins eluted by KSCN with apparent molecular masses of 58 and 60 kDa were present in an amount allowing reliable N-terminal sequencing, whereas the other silver-stained proteins with affinity to 008 were almost undetectable by Coomassie Brilliant Blue G-250 staining (Fig. 3B).

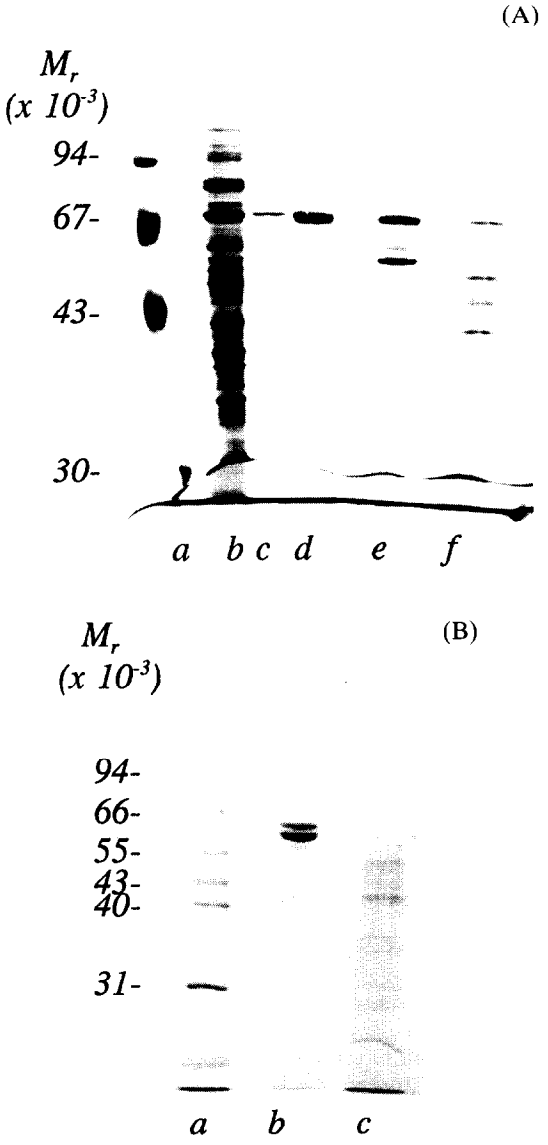

Fig. 3. 008 affinity chromatography of EDTA-extracted octylglucoside-solubilized blPm proteins. (A) Silver-stained 10% SDS gel. a, marker proteins; b, flow through fraction; c, proteins eluted with a KCl gradient; d, proteins eluted by piperonylbutoxide; e, proteins bound to the affinity column and eluted by KSCN; f, proteins eluted from the 008-affinity-ligand by the competitive inhibitor of 008 uptake, taurocholate. (B) Coomassie-stained 10% SDS gel. a, marker proteins; b, proteins eluted by KSCN; c, proteins desorbed by taurocholate.

Specificity of the interaction between the 58 *and* 60 *kDa proteins and cyclosomatostatin* 008

Affinity chromatography with substrates of a bile acid transport system as ligands. The supposition that the interaction of the 60 and 58 kDa proteins with the 008 affinity matrix is rather specific than attributable to non-specific hydrophobic interactions

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  1 MLRYPLVWLF LAACTAWGYP SSPPVVNTVK GKVLGKYVNL EGFAQPYAUF

 51 LGIPFAKPPL GSLRFAPPQP AEPWNFVKNT TSYPPMCSQD AVGGQVLSEL

101 FTNRKENIPL QFSEDCLYLN VYTPADLTKN SRLPVMVWIH GGGLVVGGAS

151 TYDGQVLSAH ENVVVVTIQY RLGIWGFFST GDEHSRGNWG HLDQVAALHW

201 VQDNIANFGG NPGSVTIFGE SAGGFSVSAL VLSPLAKNLF HRAISESGVV

251 LISALITTDS KPIAKLIATL SGCKTTTSAV MVHCLRQKTE DELLETSLKL

301 NLFKLDLLGN PKESYPFLPT VIDGVVLPKT PEEILAEKSF NTVPYIVGIN

351 KQEFGWIIPT LMGYPLSEGK LDQKTAKSLL WKSYPTLKIS EKMIPVVAEK

401 YFGGTDDPAK RKDLFQDLVA DVIFGVPSVM VSRSHRDAGA PTFMYEFEYR

451 PSFVSAMRPK TVIGDHGDEL FSVFGSPFLK DGASEEETNL SKMVMKYWAN

501 FARNGSPNGG GLPHWPEYDQ KEGYLKIGAS TQAAQRLKDK EVASWSELRA

551 KEAAEEPSHW KHVEL
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Fig. 4. Amino acid sequence of the microsomal pI 6.1 carboxylesterase. The determined sequences of five peptides from the tryptic digest of the 008-affinity-purified 58 kDa protein are in bold print and underlined. Comparison of the obtained sequences with known amino acid sequences of proteins was done by Swiss Prot Data bank.

is supported by the fact that these proteins neither bind to ABATC nor the linear renin inhibitor EMD 55068, nor the immunosuppressant cyclosporine A (data not shown), all of which are hydrophobic in nature. ABATC is a precursor of azidobenzamidotaurocholate, which was shown to competitively inhibit the uptake of taurocholate into isolated rat hepatocytes as well as the uptake of cholate [27]. EMD 55068 and cyclosporine A inhibit taurocholate uptake into isolated rat liver cells competitively, but they inhibit cholate uptake noncompetitively [28, 29].

Identification of the 58 kDa binding protein as a carboxylesterase

In order to identify the two dominant 008 binding proteins, pooled KSCN fractions from the 008 affinity chromatography of octylglucoside-solubilized blPm were separated on a preparative 10% SDS gel, the Coomassie G-250 stained 58 kDa bands were excised and the proteins digested with trypsin as described in the Materials and Methods. Five of the resulting peptides were sequenced. The amino acid sequences thus obtained (Fig. 4) were identical to that of a microsomal p*I* 6.1 carboxylesterase (E.C. 3.1.1.1). Evidence that the 60 kDa protein is most likely similar to the 58 kDa carboxylesterase comes from their very similar behavior in various purification steps. This includes the coincident elution in Concanavalin A-affinity-, hydroxylapatite- or anion-exchange-chromatography (data not shown). In addition, both structural microheterogeneity concerning different glycosylations [30] as well as generation of a second polypeptide from the native enzyme during the purification procedure have been described [31]. Therefore, sequencing of the 60 kDa 008 binding protein was not performed, since this protein also appears to be a carboxylesterase.

Specificity of the 008–carboxylesterase interaction

The specificity of the binding of the 60 and 58 kDa proteins to 008 was demonstrated by the fact that these two proteins bound to cyclosomatostatin but not to ABATC, EMD 55068 or cyclosporine A. It could be demonstrated further by inhibition of the affinity-purified esterase activity by the aromatic cyclohexapeptide 008 (63.4 ± 7.0% esterase activity compared to control without inhibitor). A cyclodecapeptide c(Phe-Ala-Lys-Pro-Phe-Phe-Ala-Lys-Pro-Phe) that gave identical results to 008 in affinity chromatography also inhibited the esterase activity of the affinity-purified enzyme (74.3 ± 10.2% of control). In contrast, the linear peptide EMD 55068 did not inhibit esterase activity (101.2 ± 4.8% of control).

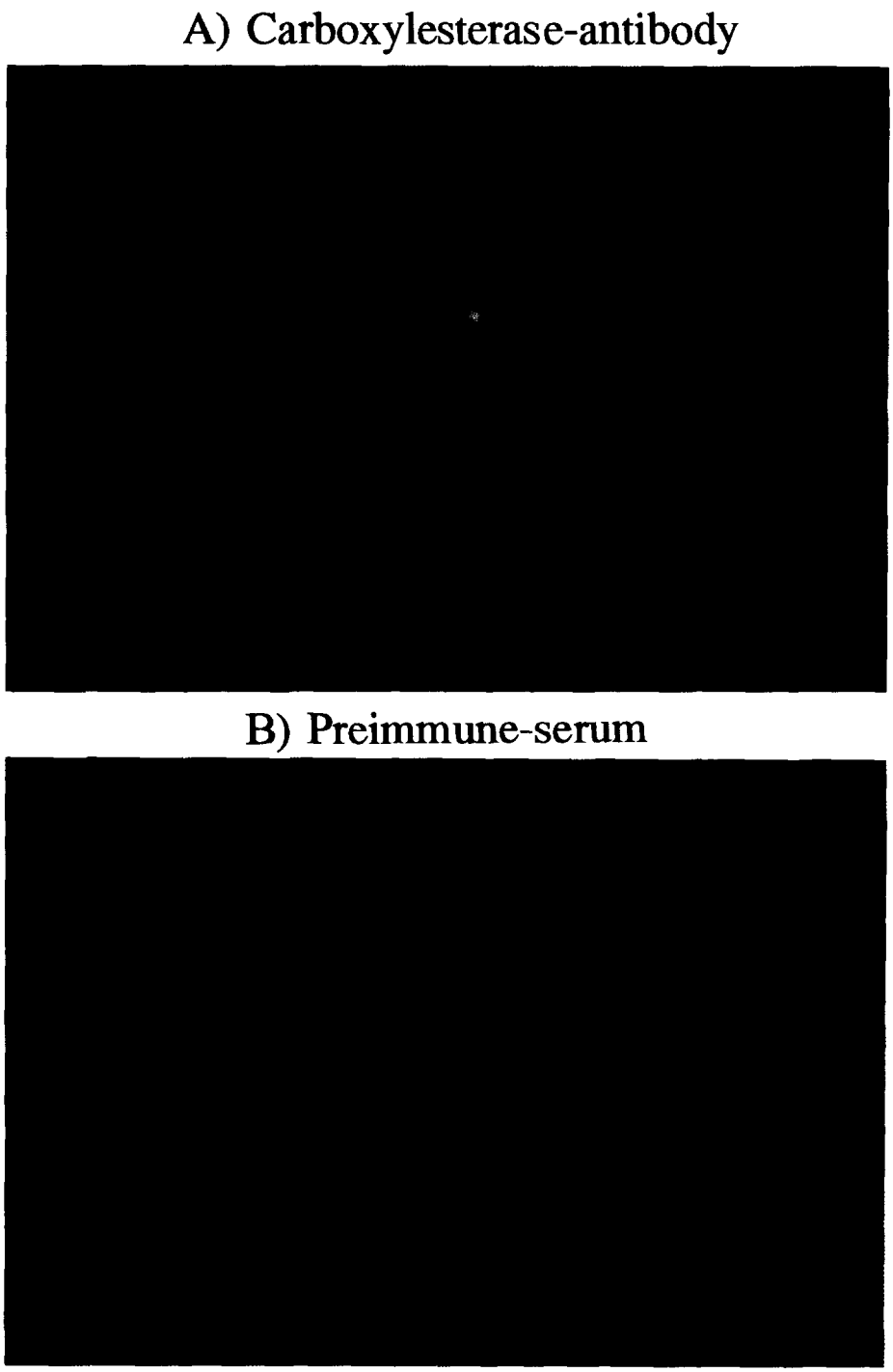

Fig. 5. Immunofluorescence of isolated rat hepatocytes. Freshly isolated liver cells were fixed and incubated with anti-pI 6.0 carboxylesterase antibody (A) at a dilution of 1:50 as described in Materials and Methods. Preimmune serum was used as control (B). The second antibody, fluorescein-isothiocyanate conjugated goat anti-rabbit IgG, was used at a dilution of 1:200. The fluorescent hepatocytes were visualized with a Zeiss Universal microscope, equipped with a fluorescence filter.

Localization of the carboxylesterase

The rat liver pI 6.1 carboxylesterase has been described to be located in the endoplasmic reticulum, bound to the inner side of the membrane [32]. In order to test whether the affinity-purified carboxylesterase could be responsible for a large and specific plasma membrane binding component of rat liver cells with regard to 008-binding, or whether the enzyme originates from microsomal contamination of isolated blPm, immunofluorescence of isolated rat hepatocytes was measured by means of a specific antibody directed against pI 6.0 carboxylesterase. The antibody was found to recognize the antigen on the hepatocyte surface shown by the clusters of fluorescent spots in the case of carboxylesterase–antibody treated cells (Fig. 5A). In contrast, cells treated with preimmune serum only showed minimal fluorescence (Fig. 5B). That the differences in the pIs of the affinity-purified carboxylesterase and the carboxylesterase against which the antibody was directed are due to methodological limits is more than suggestive since: the antibody directed against carboxylesterase pI 6.0 is specific [6] for this isoenzyme; and the molecular masses of the affinity-purified carboxylesterase and the carboxylesterase used for immunization is identical in SDS–PAGE.

DISCUSSION

From kinetic studies it became evident that the cyclohexapeptide 008, a somatostatin analog with retro sequence, is cleared from the circulation by transport systems that are located on the sinusoidal surface of the hepatocyte membrane. These transport systems physiologically transport bile acids. Moreover the hydrophobic 008 displayed a rapid binding to the hepatocyte plasma membranes. The aim of the present study, therefore, was to identify and characterize this binding component. Based on its high affinity with bile acid transporters, the cyclosomatostatin 008 should be a suitable tool for the identification and isolation of binding and/or transport proteins in isolated basolateral plasma membranes by affinity chromatography. In this regard, octylglucoside seemed to be an appropriate detergent for the solubilization of blPm, since proteins solubilized by the detergent did show specific binding to the Affi-Gel 10 matrix. A pre-purification of basolateral plasma membranes was attained by EDTA extraction at pH 8.0, removing loosely-attached proteins. A 60 and 58 kDa protein bound specifically to the 008 affinity matrix. The 58 kDa protein could be identified as a microsomal pI 6.1 carboxylesterase (E.C.3.1.1.1.). The enzyme is involved in detoxication of xenobiotics and in the activation of prodrugs of the ester as well as of the amide type, but probably also in the metabolism of natural substrates [14]. The 60 kDa protein behaved in the same manner as the 58 kDa carboxylesterase in all purification steps. The occurrence of microheterogeneity leading to isoenzymes of slightly different molecular masses further supports the assumption that the 60 kDa protein is also carboxylesterase. The specific interaction of 008 with the enzyme was demonstrated by the inhibition of the affinity-purified carboxylesterase by aromatic cyclopeptides (008 and cyclodecapeptide) but not by the linear renin inhibitor EMD 55068. However, from the results obtained, it remains unclear whether 008 binds to the active site of the enzyme or whether it alters the activity of the enzyme by binding to another site of the protein. The latter case is also described for glutathione-*S*-transferase (unpublished results), a cytosolic bile acid and aromatic cyclopeptide binding enzyme that has different binding sites with respect to glutathione and bile acids. It should be noted that bile acids are not a substrate of the enzyme [33]. Although the carboxylesterases hydrolyze aromatic amides, the rate of cleavage is usually low [34]. We were not able to detect any 008 metabolites in rat bile after perfusion of the liver with 008 nor in the incubation medium after incubation of 008 with microsomes or blPm, as shown by thin layer chromatography- and HPLC-techniques.

Immunofluorescence showed that the pI 6.1 carboxylesterase, in addition to the microsomal localization, was also present in the plasma membrane of the hepatocyte. Such a distribution in the plasma membrane as well as in the endoplasmic reticulum of the rat hepatocyte was also described for the xenobiotic metabolizing enzyme epoxide hydrolase [35] and for the protein disulfide isomerase [5].

The 58 kDa carboxylesterase as well as the 60 kDa 008 binding protein did not have any affinity with substrates which have been described as competitive inhibitors of the Na^+/taurocholate co-transporter, e.g. EMD 55068 or cyclosporine A, when they were used as ligands in affinity chromatography. On one hand this implies the specificity of the binding to 008, but on the other, the 008 transport is probably not related to Na^+/taurocholate transport, although competitive transport inhibition has been shown [3]. The carboxylesterase is probably involved as binding protein in cholate transport. Evidence for this hypothesis comes from studies using CHAPS as detergent for solubilization of plasma membrane proteins. CHAPS, a bile acid derivative, has been shown to inhibit cholate uptake into isolated rat liver cells with an IC_{50} 2.4-fold lower than that for taurocholate transport (data not shown; IC_{50} cholate $= 562 \pm 47\ \mu M, r = 0.97$; IC_{50} taurocholate $= 1339 \pm 187\ \mu M, r = 0.96$). In 008 affinity chromatography with CHAPS solubilized proteins the 60 and 58 kDa 008 binding proteins were found in the flow-through fraction. It seems that CHAPS prevents the binding of the proteins to 008. Therefore, we suggest that CHAPS itself has affinity with these proteins. The specificity of this interaction between CHAPS and the 008 binding proteins was evident by the re-acquirement of binding capacity when 008 re-chromatography of the flow-through fraction was carried out with octylglucoside.

In conclusion, our studies suggest that the carboxylesterase purified by affinity chromatography represents a specific binding protein for the cyclosomatostatin 008 in the sinusoidal plasma membrane of rat hepatocytes. According to the affinities of the enzyme with substrates of basolateral localized bile acid transporters, it could be related

to the cholate transport system but not to the taurocholate transporter. In this regard, the membrane-associated carboxylesterase could possibly function as part of a binding protein dependent transport system, as has been described for solute transporters in bacteria [36, 37].

In future, reconstitution studies will be necessary to elucidate these functional properties of the 008-affinity-purified carboxylesterase.

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