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A 50 kDa, actin-binding protein in plasma membranes of rat hepatocytes and of rat liver tumors

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Plasma membranes from normal rat livers and rat liver tumors were compared by SDS-gel electrophoresis, and analyzed for actin-binding proteins by an ^{125}I-labelled actin gel-overlay assay and by actin-affinity blotting After treatment of rats with α-hexachlorocyclohexane and after induction of liver tumors by combined treatment with *N*-nitrosomorpholine and phenobarbital, liver plasma membranes prepared from these animals were found to be highly enriched in an actin-binding, 50 kDa polypeptide. This polypeptide seemed to be an integral protein of the plasma membrane as judged by Triton X-114-phase separation. Microsomes did not contain an actin-binding polypeptide in the 50 kDa region. Therefore, the 50 kDa protein is a candidate for interaction of actin with the liver cell plasma membrane. A possible relationship of this protein with the multi-specific, cholate transporting system of the rat liver plasma membrane is discussed

Introduction

In liver and other non-muscular cells [1,2], actin in filamentous form is mainly located in the cytocortex, close to the plasma membrane [3] In particular, a contractile system exists around the bile canaliculi as demonstrated by time-lapse cinematography [4] The congruent location of actin, myosin, and tropomyosin beneath the plasma membrane of bile canaliculi has been shown in our laboratory by immunofluorescence microscopy, and by staining with rhodaminyl phalloidin [5] The actin-specific poisons, phalloidin and cytochalasin B and D interrupt contractile movements of this system in cultured hepatocytes [6,7], and they lead to dramatic changes of the surface of isolated liver cells [8,9], presumably because they disturb the ordered interaction between microfilaments and the plasma membrane [10,11] Nothing is known about the insertion of F-actin in the liver plasma membrane This led us to look for actin-binding proteins associated with the plasma membrane of hepatocytes In the present study, we examined the binding of ^{125}I-labelled actin to partially renatured protein bands of SDS-polyacrylamide gels with the gel-overlay technique [12] and by means of an actin-affinity blot In addition, we report preliminary results of applying both methods to the comparison of different plasma membrane preparations from control livers, from livers of rats treated with α-hexachlorocyclohexane, from rat liver tumors induced by *N*-nitrosomorpholine as initiator and phenobarbital as promotor, and from a rat hepatoma cell line (AS-30 D) which originally was induced by treatment with 3'-methyl-4-dimethylaminoazobenzene [13]

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Materials

Bolton-Hunter reagent, mono-^{125}I-substituted, was obtained from Amersham Buchler, Braunschweig, F R G , chemicals for gel-electrophoresis, PMSF, Triton X-114, ATP from Serva, Heidelberg, F R G , enzyme substrates from Boehringer Mannheim, Mannheim, F R G , enzymes and other proteins from Sigma, Taufkirchen, F R G , nitrocellulose from Schleicher & Schuell, Dassel, F R G All other reagents used were of the highest purity grade available commercially

Methods

Membrane preparations Plasma membranes were prepared from perfused rat livers, solid rat liver tumors, or AS-30 D cells, usually according to Touster et al [14], with the modified sucrose gradient [15] the supernatant obtained after centrifugation ($1000 \times g$, 10 min, $33000 \times g$, 7 5 min) of liver or hepatocytes homogenate was spinned down at $75000 \times g$ and the 'microsomal pellet' (Touster nomenclature) resuspended in 49% sucrose (final concentration) to be separated through a stepwise gradient of 8 5, 26, 34, 49% sucrose overnight This method has been shown to enrich the sinusoidal plasma membranes as vesicles [14] In some cases, membranes were prepared according to Hubbard et al [16], or Inoue et al [17] The Hubbard method purifies plasma membranes from the 'nuclear pellet' by a different sucrose gradient leading to membranes as sheets and no enrichment of one domain of the hepatocyte plasma membrane [16], whereas the method of Inoue et al [17] uses a mixed Ficoll/sucrose gradient as an alternative way for the enrichment of the sinusoidal domain 5′-Nucleotidase and glucose-6-phosphatase were determined as marker enzymes for plasma membranes and microsomes, respectively [18] With AS-30 D cells, which have only low 5′-nucleotidase activity, $(Na^+ + K^+)$-ATPase was determined instead [19] In all cases, 10 μM PMSF was added at the moment of tissue homogenization, and the plasma membrane fractions taken from the sucrose gradient were washed once before further analysis

Microsomes were prepared according to De Duve et al [20], and the final pellet resuspended and split into several fractions by the same sucrose gradient as in Ref 15

Pretreatment of rats For induction of the cytochrome *P*-450 system and liver growth, Wistar rats were given doses of 150 mg/kg body weight α-hexachlorocyclohexane in olive oil on the 1st, 3rd and 5th day Membranes were prepared 36 h after the last dose For tumor induction, rats were given a single dose of *N*-nitrosomorpholine (275 mg/kg), and then over a period of six month 50 mg/kg phenobarbital daily Hepatectomy was performed according to Higgins and Anderson [21], 12 h before sacrificing the animals

Radioiodination of actin Highly purified rabbit skeletal muscle actin was prepared according to Pardee and Spudich [22] The final purification step was performed by gel filtration over Ultrogel AcA 44 Only the trailing fractions were used in order to avoid contamination by 'capping proteins' G-actin was dialyzed 6 h against borate buffer (see below) Bolton and Hunter reagent [23] was coupled to 30–50 μg protein, contained in not more than 50 μl of borate buffer, according to the instructions of the producer The reaction was stopped with 9 volumes 2 M glycine in borate buffer after 20 min, kept on ice for 30 min, and then taken up in one volume 0 2% bovine serum albumin in borate buffer This solution was dialyzed against G-buffer (see below), and then stored at 4°C

Gel-overlay and affinity-blot SDS-polyacrylamide minigels (0 5 mm thick) were run according to the method of Laemmli [24], in a miniaturized system as described by Matsudaira and Burgess [25] Both gels and running buffer contained 1 mM EDTA

The gel-overlay procedure was essentially that of Snabes et al [12] with the modifications of Schleicher et al [26] After electrophoresis, the gels were fixed in 25% 2-propanol/10% acetic acid, then washed with 10% ethanol, blocked with overlay buffer (see below) containing 5% bovine serum albumin, incubated with ^{125}I-actin overnight, then washed with overlay buffer and Tris-buffered saline, dried and exposed to Kodak XAR-5 X-ray film

The actin-affinity electroblot was performed as described by Towbin et al [27], using Tween 20 as blocking agent [28], with iodinated actin replacing

the antibodies Depending on the gel area, nitrocellulose sheets were incubated overnight with 50 000–200 000 cpm ^{125}I-actin

In this first approach, we employed two renaturing methods from SDS gels, one by washing out denaturing agents from the gel without removing the separated polypeptide bands from their location, the other by stripping off SDS from polypeptides transferred to the nitrocellulose membrane Only when both methods gave coinciding results, we regarded this as preliminary indication of an actin-binding protein

Solubilization of membrane proteins Hydrophilic and hydrophobic membrane proteins were phase separated according to the Triton X-114-method of Bordier [29] Plasma membranes were solubilized with 1% Triton X-114 in Tris-buffered saline at 0°C and centrifuged 10 min at 100 000 × *g* The supernatant was incubated at 37°C, then centrifuged at 6000 × *g* at room temperature, resulting in the separation of an upper hydrophilic and a lower hydrophobic phase Both phases were restored to about the original sample volume, the upper phase with 1% Triton X-114, the lower phase with Tris-buffered saline, redissolved in the cold, incubated again at 37°C, and centrifuged at 6000 × *g* These washed hydrophilic and hydrophobic phases were used for further analysis

Buffers G-buffer 2 mM Tris-HCl (pH 8 0)/0 2 mM ATP/0 5 mM β-mercaptoethanol/0 2 mM $CaCl_2$/0 005% NaN_3 Overlay buffer 50 mM Tris-HCl (pH 7 6)/0 2% (w/v) bovine serum albumin/0 25% (w/v) gelatin/0 2 M NaCl/0 05% NaN_3 As blocking solution, the same buffer contained 5% bovine serum albumin Borate buffer 0 1 M sodium borate (pH 8 4)/0 2 mM CaCl/ 0 02% NaN_3

Results

Rat liver plasma membranes prepared by different methods as described in Methods, and from rats with different pretreatment, could be compared all on one gel (Fig 1) Three such gels, identical in composition because they were cast in one block, were run in parallel for (a) protein staining, (b) gel-overlay with ^{125}I-actin, and (c) affinity-blot with ^{125}I-actin

There is a major difference in Coomassie staining between membranes prepared by different methods (Fig 1A, lanes b–e) In particular, two additional bands at 32 kDa (which is strongly actin-binding) and about 160 kDa (which is weakly actin-binding, Fig 1C, lanes b–e) were prominently observed with 'Hubbard membranes' Coomassie staining of all other samples of plasma membranes appears rather similar But there is an enrichment of the 50 kDa-band in membranes from the *N*-nitrosomorpholine/ phenobarbital-induced (solid tumor, Fig 1A, lane i) as well as in

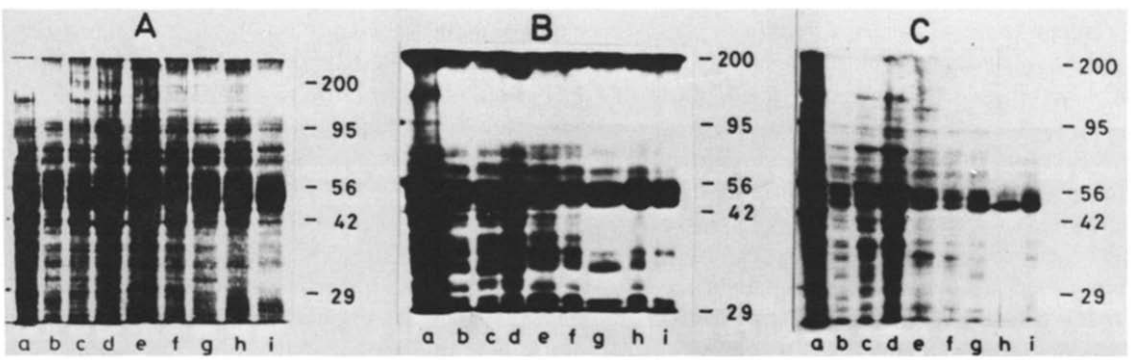

Fig 1 Comparison of different plasma membrane preparations Three 10%-gels were loaded with identical samples, each lane with the same amount of protein (20 μg) (A) Coomassie stain (B) Gel-overlay with ^{125}I-labelled actin (C) Actin-affinity blot (a) Isolated hepatocytes (b) Membrane vesicles according to Inoue et al [17] (c) Membrane sheets according to Hubbard et al [16] (d) Same as in (c), double amount of protein (e) Membrane vesicles according to Touster et al [14] (f) Touster membranes from 2/3-hepatectomized rats, 12 h after operation (g) Touster-membranes from α-hexachlorocyclohexane-treated rat (h) Same treatment as in g but another animal (i) Touster membranes from solid rat liver tumor (induction by *N*-nitrosomorpholine, promotion by phenobarbitol) Further details see Methods Sample b was kindly provided by Dr M Taefler

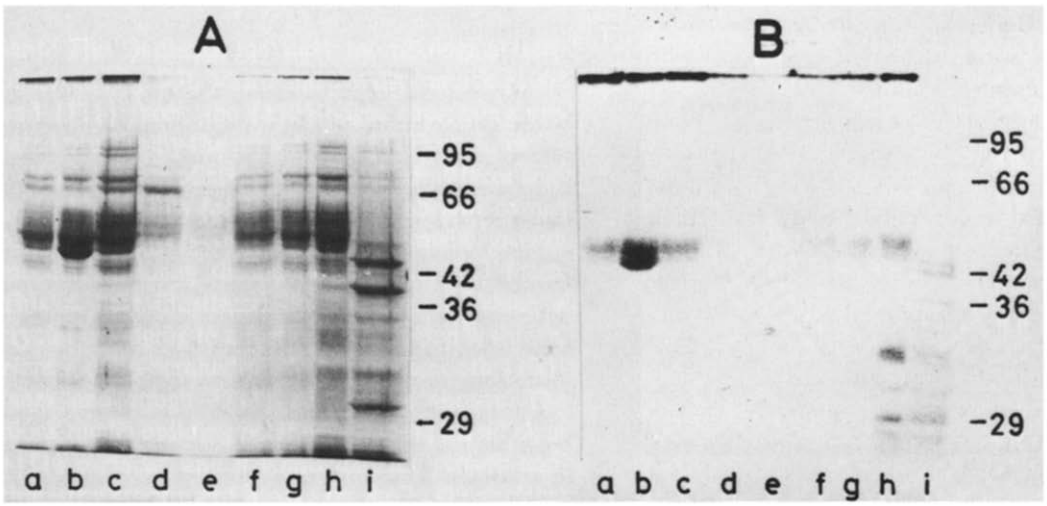

Fig 2 Comparison of plasma membranes with microsomal membranes (A) Coomassie stain (B) ^{125}I-Actin-affinity blot (a) Touster membranes from normal rat liver (b) Touster membranes from *N*-nitrosomorpholine-induced rat liver tumor (c–i) Fractions 1–7 from sucrose gradient centrifugation of the microsomal pellet prepared from normal rat liver Activity of glucose-6-phosphatase relative to 5′-nucleotidase was (lane in brackets) 0 6 (c) 36 7 (d) 9 8 (e), 1 6 (f), 1 7 (g), 3 1 (h) 0 0 (i) Electrophoresis was performed as described in Fig 1 20 μg protein per lane

one sample from α-hexachlorocyclohexane-treated animals (no visible tumor, lane h) This slight difference in general protein staining comes out very strong both with the gel-overlay and the affinity-blot assay of actin-binding (Fig 1B, C)

A control with rat liver microsomes was necessary because our plasma membrane preparations were not totally free from microsomal enzyme activity Phenobarbital and possibly also α-hexachlorocyclohexane induce cytochrome *P*-450 species in the range of 52–53 kDa [30,31] We had to exclude the possibility that the 50 kDa band could be ascribed to a contamination with these or similar microsomal proteins After sucrose gradient centrifugation of the microsomal pellet, the fraction with the highest relative activity of glucose-6-phosphatase contained only a faint 50 kDa band with little or no actin binding (Fig 2A, B, lane d) Reversely, in those fractions which contain a higher proportion of 5′-nucleotidase, attributable to plasma membranes, a 50 kDa band with actin-binding property is clearly to be seen (details in legend to Fig 2) That the enrichment of the 50 kDa-band is not necessarily a property of transformed liver cells can be inferred from Fig 3, which compares plasma membranes from normal rat livers with those from AS-30 D ascites

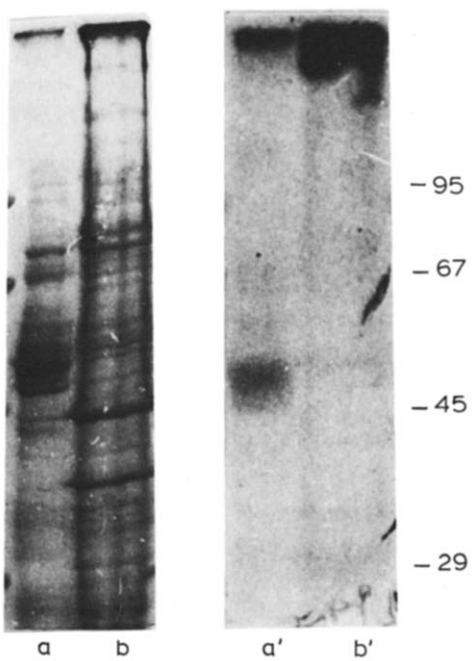

Fig 3 Plasma membranes from rat liver and AS-30 D ascites hepatoma cells (a, a′) Normla rat liver plasma membranes (b, b′) AS-30 D plasma membranes Both samples were prepared according to Touster et al [14] with the only difference that AS-30 D cells were homogenized with the aid of a French press prior to differential centrifugation (a b) Coomassie stain (a′, b′) Gel-overlay with ^{125}I-actin Electrophoresis was performed as in Fig 1, with 20 μg protein per lane

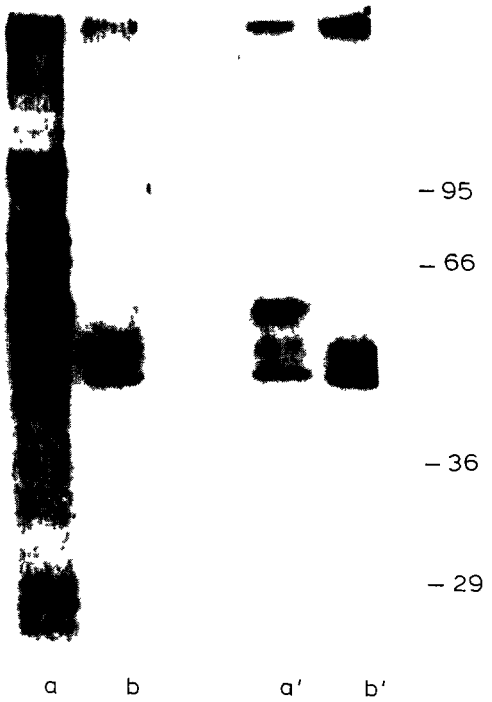

Fig 4 Gel-overlay assay with Triton X-114 fractionated rat liver tumor plasma membranes Phase separation was performed according to Bordier [29] 10% gels were loaded with 20 μg protein per lane (a, a') Detergent phase (b, b') Hydrophilic phase (a, b) Coomassie-stained gel (a', b') Gel-overlay with ^{125}I-labelled actin

hepatoma cells, a more dedifferentiated cell line, originally induced by 3'-methyl-4-dimethylaminoazobenzene [13] These plasma membranes neither show any enrichment of the 50 kDa band, nor any actin-binding to be detected in the corresponding actin-overlaid gel (Fig 3, lanes a, a')

Preliminary investigations of the location of the actin-binding 50 kDa protein (peripheral/intrinsic) by phase separation with Triton X-114 were indicative of an integral, detergent-soluble membrane component (Fig 4) Analyzing plasma membranes from the *N*-nitrosomorpholine/phenobarbital-induced tumor, we found an actin-binding band at 50 kDa in the hydrophobic as well as in the hydrophilic phase (Fig 4, lanes a', b') The same splitting was observed with Coomassie staining (lanes a, b) At the same time, however, the hydrophilic subfraction seems to be slightly larger (lanes a, a')

Discussion

As we have demonstrated, the mode of membrane preparation significantly influences the content of actin-binding proteins in the plasma membranes analyzed Therefore, we generally used only one preparation method (Touster et al [14]) for comparisons between normal and pretreated rats In plasma membranes of livers from rats treated with the tumor promoting α-hexachlorocyclohexane and from rat liver tumors deduced from an initiation/promotion treatment with *N*-nitrosomorpholine plus phenobarbital, the relative proportion of the 50 kDa band was much higher than in controls This cannot be due generally to cell proliferation, because there is little difference between membranes from normal rats and from those that have been 2/3-hepatectomized in order to induce liver regeneration (Fig 1 A–C, lane f) It is unlikely, that the 50 kDa actin-binding polypeptide band enriched in α-hexachlorocyclohexane-treated rats represents a microsomal protein (Fig 2) As demonstrated by phase separation, the 50 kDa band, including it's actin-binding activity, seems to be an integral membrane protein Thus, though the function of the 50 kDa actin-binding protein in liver plasma membranes is not at all understood, we suspect it may be a candidate for a site of direct actin-binding to the plasma membrane

There are other actin-binding polypeptide bands on the gels shown, most of which seem to disappear from the plasma membranes of rat livers chemically induced with α-hexachlorocyclohexane and also from those of the *N*-nitrosomorpholine/phenobarbital-induced liver tumor This may be due to the relative enrichment of the 50 kDa band On the other hand, the Coomassie staining pattern does not show such drastic differences in protein content It should be noted, that high-molecular-weight proteins are misrepresented, because the density of the gels used for the gel-overlay method imposes restrictions on the renaturing process of longer polypeptide chains Also, high-molecular-weight polypeptides are eluted less easily by electrophoresis during the blotting procedure, as can be demonstrated with lower-percentage gels (data not shown)

Further experiments will have to be done on

the nature of actin-binding in these tests in the presence of ATP and/or in the absence of Ca^{2+} and other agents influencing the interaction of actin with other proteins For biochemical and functional characterization of an identified actin-binding protein it will be necessary to purify it from membrane preparations

Another possibility for the function of the 50 kDa actin-binding protein is raised by the comparison of normal rat liver plasma membranes and plasma membranes from As-30 D cells These cells, though deduced from liver tumor, apparently lost several properties of hepatocytes In earlier studies, a total deficiency of cholate (and phallotoxin) transport in As-30 D cells was found [32] A 50 kDa (48 kDa, according to other workers [33]) polypeptide is suspected to be a component of the Na^+-dependent multi-specific bile salt transport system of hepatocytes [34] This system is absent in AS-30 D hepatoma cells, and is reduced in regenerating livers [35] An affinity label for this transport system labelled mainly proteins in the 50–54 kDa region of plasma membranes from isolated hepatocytes but not those from AS-30 D cells [34] At present, speculations concerning a participation of actin in bile acid transport are premature It cannot be excluded, that the 50 kDa region contains additional, non actin-binding polypeptide bands, that could account for cholate affinity labelling

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