

Identification of a Novel Glycoprotein (AGp110) Involved in Interactions of Rat Liver Parenchymal Cells with Fibronectin

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Abstract. We have identified an integral membrane glycoprotein in rat liver that mediates adhesion of cultured hepatocytes on fibronectin substrata. The protein was isolated by affinity chromatography of detergent extracts on wheat germ lectin-Agarose followed by chromatography of the WGA binding fraction on fibronectin-Sepharose. The glycoprotein (AGp110), eluted at high salt concentrations from the fibronectin column, has a molecular mass of 110 kD and a pI of 4.2. Binding of immobilized AGp110 to soluble rat plasma fibronectin required Ca^{2+} ions but was not inhibited by RGD peptides. Fab' fragments of immunoglobulins raised in rabbits against AGp110 reversed the spreading of primary hepatocytes attached onto fibronectin-coated substrata, but had no effect on cells spread on type IV collagen or laminin substrata. The effect of the antiserum on cell spreading was reversible. AGp110 was detected by immunofluorescence around the periphery of the ventral surface of substratum attached hepatocytes, and scattered on the dorsal

surface. Immunohistochemical evidence and Western blotting of fractionated liver plasma membranes indicated a bile canalicular (apical) localization of AGp110 in the liver parenchyma. Expression of AGp110 is tissue specific: it was found mainly in liver, kidney, pancreas, and small intestine but was not detected in stomach, skeletal muscle, heart, and large intestine. AGp110 could be labeled by lactoperoxidase-catalyzed surface iodination of intact liver cells and, after phase partitioning of liver plasma membranes with the detergent Triton X-114, it was preferentially distributed in the hydrophobic phase. Treatment with glycosidases indicated extensive sialic acid substitution in at least 10 O-linked carbohydrate chains and 1-2 N-linked glycans. Immunological comparisons suggest that AGp110, the integrin fibronectin receptor and dipeptidyl peptidase IV, an enzyme involved in fibronectin-mediated adhesion of hepatocytes on collagen, are distinct proteins.

ADHESIVE interactions between the hepatic parenchyma and the surrounding biomatrix are important in maintaining the morphological and functional integrity of the liver. The extracellular matrix can modulate the expression of liver-specific proteins (5, 25, 46, 55) and, in pathological conditions such as cirrhosis and fibrosis, impaired liver function may be related to significant alterations in the distribution and synthesis of extracellular matrix components (20, 47, 48).

The most abundant noncollagenous matrix constituent in adult rat liver is fibronectin, a major component of biomatrices of connective tissue or epithelial origin (1, 25, 49). In liver parenchyma, fibronectin is mostly present in the perisinusoidal space (39), but can also be detected on the

lateral and bile canalicular cell surface domains (18, 25). Basement membrane proteins such as type IV collagen and laminin are sparsely distributed on the sinusoidal surface only (25, 39), but a typical basement membrane structure, as seen in other epithelia, has not been observed at the basal surface of liver parenchymal cells.

Hepatocytes in vitro attach and spread on extracellular matrix proteins including fibronectin (7, 30, 31) and a putative fibronectin receptor of rat hepatocytes has recently been isolated (32). This receptor belongs to the integrin family of proteins (29, 49) and binds to the Arg-Gly-Asp (RGD) sequence in the cell-binding domain of fibronectin, in common with some other integrins (49, 51). In this study we describe another protein from rat liver which mediates adhesion of hepatocytes on fibronectin matrices. This protein, AGp110, for apical glycoprotein of molecular mass 110 kD, is not related to the integrin-receptor family but its properties suggest strongly that it makes an important contribution to the adhesive interactions of liver parenchymal cells with fibronectin matrices.

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Materials and Methods

Cells

Hepatocytes were isolated from young adult female Sprague-Dawley rats by liver perfusion with collagenase (6). Viable cells were further purified by Percoll isopycnic centrifugation (37), and cultured in arginine-free DME (56) supplemented with 0.5 mM ornithine, 0.1 μ M insulin, 0.025 μ M dexamethasone, 60 μ g/ml penicillin, 100 μ g/ml streptomycin, and 50 μ g/ml gentamycin. Cell culture substrata were treated with either 25 μ g/ml fibronectin, 40 μ g/ml type IV collagen, or 40 μ g/ml laminin in PBS for at least 1 h and washed with PBS; cells were then seeded and grown in serum-free medium.

Proteins and Enzymes

Fibronectin was purified from rat plasma on a gelatin-Sepharose column (17). Type IV collagen and laminin were isolated from the Engelbreth-Holm-Swarm (EHS) sarcoma implanted in lathyritic mice as described (36). Collagenase (type IV; Sigma Chemical Co., St. Louis, MO), ovalbumin, bovine albumin, lactoperoxidase, and glucose oxidase were purchased from Sigma Chemical Co. Wheat germ agglutinin was obtained from Pharmacia Fine Chemicals (Piscataway, NJ). GRGDS peptide was obtained from Calbiochem-Behring Corp. (La Jolla, CA).

Glycosidase F (N-glycanase), endoglycosidase F (Endo F), and endo- α -N-acetylgalactosaminidase (O-glycanase) were purchased from Boehringer (Mannheim, FRG) and O-glycanase was also obtained from Genzyme Corp. (Boston, MA); results using O-glycanase from these two sources were indistinguishable. *Vibrio cholerae* neuraminidase was from Calbiochem-Behring Corp.

Radioactive Labeling

For metabolic labeling, cell cultures in petri dishes were incubated in low-methionine medium for 1 h and then grown for 1–16 h in low-methionine medium containing 100 μ Ci/ml of [35 S]methionine. In some experiments cell cultures were treated with 1 μ M tunicamycin for 3 h followed by addition of [35 S]methionine and labeling over a further 16 h.

Surface labeling was carried out according to the lactoperoxidase-catalyzed iodination procedure using carrier-free 125 I (0.5 mCi/ml) as described (12, 24).

Electrophoretic Techniques

SDS-PAGE and two-dimensional electrophoresis were carried out as described (38, 44). Western blotting of proteins on nitrocellulose and detection using antibody probes was performed according to established procedures (10, 15) as described (54). For preparative electrophoresis, proteins were run on 7–12% SDS polyacrylamide gels under reducing conditions, stained for 30 min with 0.1% Coomassie blue and destained with 30% methanol in 10% acetic acid. The bands of interest were cut out and destained further at 37°C with 50% methanol in 0.1 M Tris, pH 8.8. The destained bands were washed exhaustively with distilled H₂O and before elution, were incubated for 30 min in a minimal volume of SDS-PAGE sample buffer. The protein was eluted from the gel in a 10-ml disposable pipette that was cut to appropriate size and fitted with a dialysis membrane bag at the tip.

Affinity Chromatography

Rat plasma fibronectin and WGA were coupled to CNBr-activated Sepharose (5 mg/ml gel) as recommended (Pharmacia Fine Chemicals). Affinity chromatography on both WGA and fibronectin columns was performed at 4°C. Hepatocytes or purified canalicular membranes were extracted with 25 mM octyl glucoside in Hepes-saline (10 mM Hepes, pH 7.1, 150 mM NaCl) containing 1 mM PMSF, 1 mM NEM, 2 mM CaCl₂, and 2 mM MgCl₂. The extract was centrifuged for 30 min at 25,000 rpm in a rotor (model 42.1; Beckman Instruments, Fullerton, CA) and the clear supernatant was applied to a WGA-Sepharose column (5 \times 2 cm) equilibrated with the same extraction buffer. Unbound material was removed by extensive washing with the same buffer and bound proteins were eluted with 1 M *N*-acetylglucosamine in buffer. Fractions (2 ml) were collected and peak fractions were dialysed versus Hepes-saline pH 7.1, 1 mM PMSF, 1 mM *N*-ethylmaleimide, 1 mM octyl glucoside. This fraction was made up to 25 mM octyl glucoside, 2 mM CaCl₂, 2 mM MgCl₂ and applied to a fibro-

nectin-Sepharose column (5 \times 2 cm) equilibrated in the same buffer as above. Elution of bound material was effected by 1 M NaCl in 10 mM Hepes, pH 7.1 containing 25 mM octyl glucoside or by 20 mM EDTA in the same buffer. 2-ml fractions were collected and peak fractions were pooled for further analysis.

Ion Exchange Chromatography

AGp110, isolated from bile canalicular membranes by WGA affinity chromatography followed by fibronectin affinity chromatography, was purified further by ion exchange chromatography on a Mono Q column using the Pharmacia FPLC system. AGp110 eluted from a fibronectin column with 1 M NaCl was dialysed versus distilled H₂O containing 1 mM octyl glucoside and then versus 20 mM piperazine. HCl, pH 5, containing 25 mM octyl glucoside. The glycoprotein was then loaded onto the column and eluted with a linear NaCl concentration gradient (0–1 M NaCl) in 20 mM piperazine, HCl pH 5, 25 mM octyl glucoside. AGp110 eluted at \sim 0.3 M NaCl.

Antibodies and Isolation of IgG Fragments

Antisera against rat plasma fibronectin and AGp110 were raised in rabbits by intradermal injection at several sites in Freund's adjuvant followed by weekly booster injections 5 wk after the primary injection. To obtain sufficient amounts of AGp110 to raise antibodies, we extracted crude liver plasma membranes with 0.3% deoxycholate and applied the extract to a WGA Sepharose column equilibrated in the same buffer. The binding fraction that eluted with 1 M *N*-acetyl-D-glucosamine was dialysed versus distilled H₂O and lyophilized. The lyophilized sample was dissolved in electrophoresis sample buffer and run on preparatory SDS gels. The bands with *M_r* 110 kD were excised and electroeluted. This electroeluted antigen was used for injecting rabbits. Subsequent booster injections were with AGp110 isolated from bile canalicular membranes by WGA-agarose and fibronectin-Sepharose affinity chromatography. Rabbit antisera against the β -subunit of fibronectin receptor integrin and dipeptidyl peptidase IV (DPPIV)¹ were kind gifts from Dr. S. Johansson (Biomedical Center, Uppsala, Sweden) and Dr. D. Doyle (State University of New York at Buffalo, NY), respectively. Anti-rabbit IgG/rhodamine was purchased from Cappel Laboratories (Cochranville, PA), anti-rabbit IgG/horseradish peroxidase and anti-rabbit IgG/alkaline phosphatase from Sigma Chemical Co.

Immunoglobulin G was isolated from AGp110 immune sera on a protein A-Agarose (Pharmacia Fine Chemicals) column equilibrated in PBS. Bound IgG was eluted with 0.6% glacial acetic acid in 0.15 M NaCl (19). The pH of the eluate was neutralized with solid Tris. F(ab')₂ fragments were obtained as described (19): briefly, IgG solutions were dialysed versus 0.2 M acetate buffer, pH 4.5, and digested with 2% pepsin (weight enzyme/weight IgG) overnight at 37°C. The digested IgG was dialysed versus PBS, passed through the protein A-Sepharose column and the F(ab')₂ fragments were recovered in the flowthrough fraction. Fab' fragments were obtained after reduction and alkylation of F(ab')₂ dimers (13): reduction was effected with 0.11 vol of 0.1 M DTT in 0.2 M Tris HCl, pH 8.0 containing 10 mM EDTA for 1 h at room temperature; 0.11 vol of 0.22 M iodoacetamide in Tris/EDTA were then added and alkylation was allowed to proceed for 30 min at 4°C. Fab' fragments were used in adhesion experiments after extensive dialysis against 10 mM Hepes, pH 7.2.

ELISA

The binding of AGp110 to fibronectin was investigated by an ELISA. In these experiments, we used AGp110 isolated from purified bile canalicular membranes by WGA-Agarose affinity chromatography, Fn-Sepharose affinity chromatography, and Mono Q FPLC ion exchange. Fibronectin was isolated from rat plasma. The purity of both proteins was confirmed by PAGE followed by silver staining.

Purified AGp110 was suspended in "sensitizing" buffer (4.5 ml of 0.2 M Na₂CO₃ and 8 ml of 0.2 M Na₂HCO₃, made up to 50 ml with distilled water) containing 0.05% Tween 20 and dispensed in microtiter plate wells: \sim 0.1 μ g/100 μ l AGp110 was used per well. The plates were then incubated overnight at 4°C. The wells were washed with PBS and unoccupied binding sites on polystyrene were blocked by incubation with 10% BSA in PBS pH 7.3 for 1 h at 37°C. A stock solution of fibronectin (630 μ g/ml) was used at dilutions ranging from 1:5 to 1:5,000 and dispensed in 100- μ l amounts in individual wells. The incubation buffer used was PBS pH 7.3 containing 1% BSA, 4 mM CaCl₂, and 0.05% Tween 20. This buffer (incubation

1. *Abbreviations used in this paper:* DPPIV, dipeptidyl peptidase IV; NEM, *N*-ethylmaleimide.

buffer) was used for all subsequent reactions and washes. The plates were incubated with fibronectin for 1 h at 37°C and then extensively washed. anti-fibronectin serum was added at a dilution of 1:200 and left for 1 h at 37°C. After extensive washing, the plates were incubated for 1 h at 37°C with anti-rabbit IgG conjugated to alkaline phosphatase (1:400). The wells were again exhaustively washed and alkaline phosphatase substrate (*p*-nitrophenyl phosphate from Sigma in sensitizing buffer containing 0.1% of 0.5 M MgCl₂) was added (100 μl/well). Wells were developed with this chromogen for 2 h at 37°C. Absorbance at 400 nm was read in a Titertek Multiskan (Flow Laboratories, Maclean, VA).

Controls for these experiments consisted of wells with no adsorbed AGp110 but otherwise processed as described above. These gave very low absorbance readings. The amount of AGp110 adsorbed onto polystyrene was found to be ~90% of added protein: solutions of AGp110 were allowed to adsorb onto polystyrene wells as described and then transferred to new wells on the same plate and incubated further under the same conditions. The plates were developed using anti-AGp110 and anti-rabbit IgG-alkaline phosphatase followed by chromogenic substrate as described above. The ratio of absorbances (primary over secondary incubation) was ~10:1 with no significant variation among triplicate experiments. A third incubation did not give detectable absorbance. The amount of fibronectin linked to AGp110 was determined by referring to a standard curve: we found that, as in the case of AGp110, ~80–90% of fibronectin in solution adsorbed onto the polystyrene wells after overnight incubation. Fibronectin at different dilutions was incubated in microtiter plate wells and the wells were processed with anti-Fn serum, alkaline phosphatase-linked secondary antibody and chromogen. Estimated amounts of bound fibronectin were plotted against OD readings. A fibronectin standard curve was plotted with every experiment and incubation with chromogenic substrate was for identical times as for plates with preadsorbed AGp110. In experiments designed to study the affinity of AGp110 for the RGD sequence on fibronectin, wells with adsorbed AGp110 were incubated with solutions of GRGDS for 30 min at 37°C and the peptide was also included in the incubation buffer during the reaction with fibronectin. In experiments describing the effect of calcium, the ion was omitted from all incubation buffers.

Adhesion Experiments

Nunc four-well multidishes (Nunc, Roskilde, Denmark) were coated with fibronectin (30 μg/ml), type IV collagen (40 μg/ml), or laminin (40 μg/ml) for 1 h at 37°C. Free-binding sites on the surface of these substrata were blocked with albumin (5 mg/ml, heat treated to 56°C) for 1 h at 37°C. Hepatocytes, freshly isolated by collagenase liver perfusion, were treated with 20 μM cycloheximide in oxygenated serum-free medium for 1 h at 37°C and seeded in the presence of cycloheximide and 0.1% heat-treated albumin onto the prepared substrata. Cell attachment and spreading was allowed to proceed for 3 h at 37°C. To test the effects of the AGp110 antiserum on cell spreading, solutions of Fab' fragments (100–1,000 μg/ml) in 10 mM Hepes, pH 7.2, containing 1 mg/ml albumin were mixed with equal volumes of twice-concentrated DME medium and added to the cultures. The effect of these antibodies was monitored with an inverted Zeiss microscope after further incubation at 37°C.

Immunoprecipitation

Hepatocyte primary cultures that were either metabolically labeled or surface-iodinated were extracted with detergent and immunoprecipitated according to an established protocol (55). The procedure was modified to include a preclearing step as follows: hepatocytes were extracted by boiling in 0.2 M Tris buffer, pH 8.0, containing 0.2% SDS, 1 mM PMSF, and 1 mM NEM. Preimmune rabbit serum at a final dilution of 1:20 was then added to 2 vol of cell extract, 1 vol of 2% NP-40, 2% sodium deoxycholate, and 1 vol of 1% BSA. The mixture was incubated with agitation for 1 h at 37°C and then a suspension of protein A-Sepharose 6MB was added at a final dilution of 1:5. The cell extract was incubated for 1 h at 37°C, the beads were spun off and the supernatant was reextracted with fresh protein A-Sepharose. These pre-clearing steps did not remove any AGp110 from the detergent extracts. The supernatants, after the two preclearing steps, were used for immunoprecipitation with the antisera of interest (1:20 dilution), followed by incubation with protein A-Sepharose at 1:5 dilution. The beads were washed four times in 0.1 M Tris, pH 8, containing 0.1% SDS, 0.5% NP-40, and 0.5% DOC and suspended in sample buffer for electrophoresis.

Radioiodinated hepatocytes used for immunoprecipitations under non-dissociating conditions were extracted overnight in cold Hepes-saline (10 mM Hepes, 150 mM NaCl) pH 7.3 containing 10 mM CHAPS, 2 mM PMSF/NEM, and 1 mM CaCl₂/MgCl₂. The soluble extract was separated

from the residue by centrifugation, precleared twice with nonimmune rabbit serum and protein A-Sepharose and once with protein A-Sepharose alone and used for immunoprecipitations. Incubations and dilutions of antisera were as before but the nondissociating buffer, Hepes-saline pH 7.3 containing 10 mM CHAPS, was used throughout in incubations and washes.

Immunoprecipitated proteins in SDS gels, labeled with either [³⁵S]methionine or ¹²⁵I were detected by fluorography or indirect autoradiography respectively. For fluorography, gels were impregnated with 1M sodium salicylate before drying. For indirect autoradiography, high-speed X intensifying screens (X-ray Accessories Ltd., Watford, UK) were used on dried gels. Kodak XAR-5 x-ray film was used throughout.

Immunofluorescence

Frozen sections of rat liver were dried, fixed for 3 min in a chloroform:acetone (1:1) mixture, drained and stored at -20°C. Before immunofluorescent staining sections were washed in PBS for at least 1 h. Both primary and secondary, fluorophore-conjugated, antibodies were diluted 1:50 in PBS containing 1% albumin. Incubations were for 1 h at 37°C.

Live hepatocytes were stained for AGp110 by incubating for 100 min with anti-AGp110 IgG (1:50), rinsing and then incubating for 10 min with rhodamine-conjugated anti-rabbit IgG (1:50).

Glycosidase Digestion of Glycoproteins

Glycosidase treatments were performed on preparations obtained by immunoprecipitation. Protein A-Sepharose beads carrying immunoprecipitate (~1,000 cpm ¹²⁵I) were incubated with various glycosidases overnight at 37°C. For neuraminidase treatment, the beads were suspended in 100 μl of 25 mM sodium acetate buffer, pH 6.1, containing 20 mU of *Vibrio cholerae* neuraminidase. The beads were then washed with 0.5 M Tris-HCl, pH 6.8. For endoglycosidase F treatment, the protein A-Sepharose beads were incubated overnight at 37°C in 100 μl of 0.25 M sodium acetate, pH 6.5, containing 10 mM β-mercaptoethanol and 0.2 units of Endo F. For O-glycanase treatment, protein A-Sepharose beads were incubated first with neuraminidase as above. The beads were then washed with 10 mM sodium acetate, 20 mM sodium cacodylate, pH 6.1, containing 1% octyl glucoside and finally incubated overnight in 100 μl of the same buffer containing 5 mU of O-glycanase. For N-glycanase treatment, protein A-Sepharose beads with bound AGp110 were washed with PBS, pH 7.2, containing 10 mM EDTA, 10 mM β-mercaptoethanol, and 1% octyl glucoside and incubated overnight in 100 μl of the same buffer containing 2 U of N-glycanase. All the reactions with glycosidases were stopped by boiling in PAGE sample buffer.

Controls for the glycanase treatments mentioned above included digestion of fetuin (5 μg) with the various enzymes and incubation of AGp110 with the relevant buffers in the absence of enzymes. The result confirmed enzymatic cleavage of glycans from fetuin and no degradation of AGp110 during incubations in the absence of glycanases.

Plasma Membrane Isolation and Fractionation

Crude membranes were isolated from liver homogenate in 1 mM NaHCO₃, pH 8. The homogenate was centrifuged at 5,000 rpm and the pellet was recovered. Specific domains of parenchymal plasma membranes, i.e., sinusoidal, lateral, and canalicular were isolated from liver homogenates on sucrose gradients (59). Purified membrane fractions were partitioned in Triton X-114 as described (3, 8). The detergent Triton X-114 achieves an aqueous and hydrophobic phase partition in solution and integral membrane proteins having abundant nonpolar residues distribute in the hydrophobic (detergent) phase. About 200 μg of membrane proteins were used in this fractionation. Aliquots of the aqueous and detergent phases were electrophoresed, transferred onto nitrocellulose and overlaid with anti-AGp110 serum. In other experiments, plasma membranes were treated with 2 M NaCl to remove peripheral proteins. Membrane pellets were homogenized in 50 mM Tris, pH 7.5 containing 2 M NaCl and agitated for 1 h at 4°C. The suspension was centrifuged in a rotor (42.1; Beckman Instruments) for 2 h at 40,000 rpm. This salt extraction was repeated once more, the final membrane pellet was extracted with 1% deoxycholate and the residue was centrifuged down and discarded. The content of AGp110 in the detergent and pooled high salt extracts was compared by running SDS gels, blotting, and overlaying with anti-AGp110 antiserum.

Tissue Extractions

Tissues were obtained by dissection of an adult female rat. The tissues were

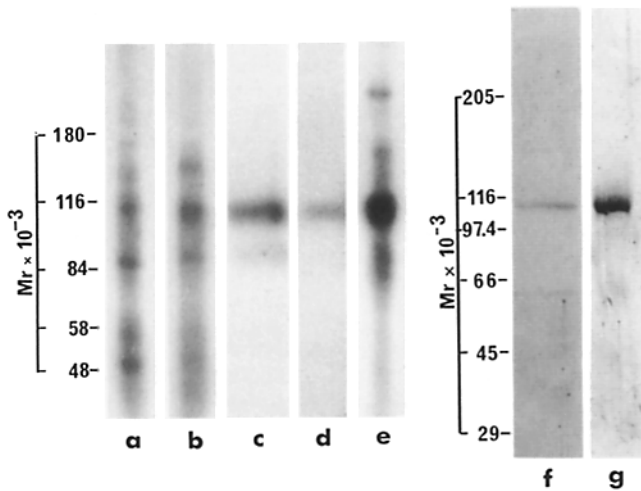


Figure 1. Isolation of AGp110. Autoradiograms of 7.5% gels (a-e) and silver-stained 5-12% gradient gels (f and g). (a) Octyl glucoside extract of radioiodinated hepatocytes. (b) WGA-bound cell surface glycoproteins of iodinated hepatocytes. (c) Fraction of WGA-bound cell surface iodinated glycoproteins eluted from a fibronectin-Sepharose affinity column with 1 M NaCl. (d) Cell surface iodinated glycoproteins eluted from a fibronectin-Sepharose column by 20 mM EDTA. (e) WGA-bound iodinated cell surface glycoproteins that did not bind to fibronectin Sepharose. (f) Fibronectin-binding fraction of WGA-bound glycoproteins from canalicular plasma membranes extracted with octyl glucoside. This fraction was eluted from a fibronectin-Sepharose column with 1 M NaCl. (g) AGp110 purified from canalicular membranes by WGA and Fn affinity chromatography, followed by Mono Q FPLC.

thoroughly rinsed with PBS and then extracted in Tris-saline pH 7.4 containing 200 mM octyl glucoside, 2 mM EDTA, 2 mM PMSF, and 2 mM NEM. The tissues were chopped in small pieces and left in the detergent-containing buffer for 4 h on ice before spinning out the remnants at 10,000 *g* for 20 min at 2°C. The amount of protein in the supernatants was estimated by the Pierce BCA assay (Pierce Chemical Co., Rockford, IL). For electrophoresis, equal amounts (100 μ g) of protein were applied to 7.5% gels, transferred to nitrocellulose and probed with specific antibodies.

Results

Identification of AGp110

Rat hepatocytes seeded onto substrata derivatized with fibronectin were surface-labeled with 125 I and extracted with octyl glucoside (see Materials and Methods). The detergent extract (Fig. 1 a) was chromatographed on a wheat germ agglutinin-Agarose column and glycoproteins bound to the lectin were eluted with 1 M *N*-acetyl-D-glucosamine. This fraction (Fig. 1 b) was applied to a fibronectin-Sepharose affinity column and proteins eluted with 1 M NaCl and 20 mM EDTA were analyzed by SDS electrophoresis (Figs. 1, c and d, respectively). The results indicated that a glycoprotein of molecular mass 110 kD was retained by the fibronectin column. Binding of AGp110 to fibronectin seemed to be specific under the experimental conditions described above since the protein was not retained in affinity columns of Sepharose derivatized with type IV collagen, laminin, albumin, or protein A or in columns of underivatized Sepharose. Elution of the fibronectin-Sepharose column by EDTA, i.e., by divalent ion chelation, indicated that binding of the glyco-

protein to fibronectin required calcium and/or magnesium ions. Neither the high salt nor EDTA eluates contained proteins migrating in SDS gels with molecular weights similar to those observed (32) for the α and β subunits of the liver integrin fibronectin receptor, 145 and 130 kD, respectively. We conclude that integrin does not bind to whole rat fibronectin under the experimental conditions used in our affinity chromatography and is presumably eluted with the flow-through fraction (Fig. 1 e). This fraction also appears to contain a major band of \sim 110 kD proteins. Rechromatography of the flowthrough fraction on the fibronectin column yielded additional amounts of the 110-kD component in the high salt and EDTA eluates and essentially complete depletion was reached only after three rounds of chromatography (results not shown). In all cases the flowthrough fraction contained other proteins of molecular mass \sim 110-kD, whose identity is discussed later.

Larger amounts of an AGp110 fraction were prepared from liver membranes and used to raise antibodies in rabbits. The antiserum reacted strongly with AGp110 purified from a fibronectin-Sepharose affinity column (Fig. 2 a). In Western blots of two-dimensional gels of WGA-bound glycoproteins of liver membranes, reaction with anti-AGp110 revealed a single major spot at an indicated isoelectric point value of 4.2 (Fig. 2 b).

In addition to chromatography, evidence for affinity of AGp110 for fibronectin was provided by ELISA experiments in which AGp110 was immobilized on microtiter wells and its association with rat plasma fibronectin was assessed using fibronectin-specific antibody probes. For these experiments AGp110 was purified to homogeneity from bile canalicular membrane preparations using WGA-Agarose and fibronectin-Sepharose affinity chromatography (Fig. 1 f) followed by Mono Q FPLC (Fig. 1 g). Fibronectin bound to immobilized AGp110 in a saturable manner (Fig. 3 a). Binding was not RGD-directed since association was not significantly reduced by addition of the pentapeptide GRGDS (Fig. 3 b). However,

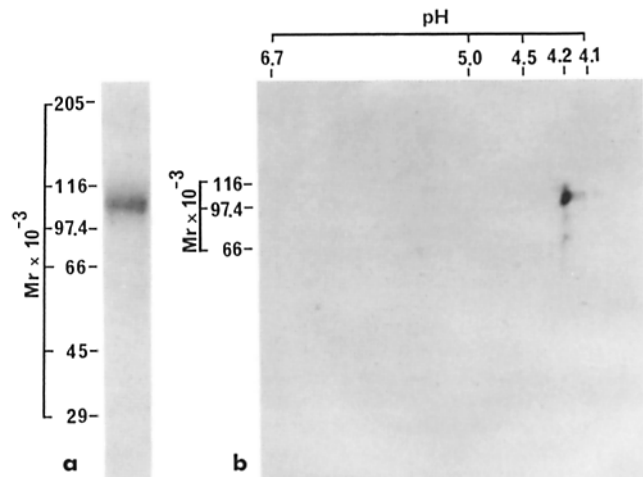


Figure 2. Characterization of anti-AGp110 serum. (a) Western blot of purified AGp110 stained with anti-AGp serum followed by horseradish peroxidase-goat anti-rabbit IgG. (b) Western blot of two-dimensional gel of liver membrane proteins probed with anti-AGp110 serum. The spot detected at the molecular mass position of 110 kD has a pI of 4.2.

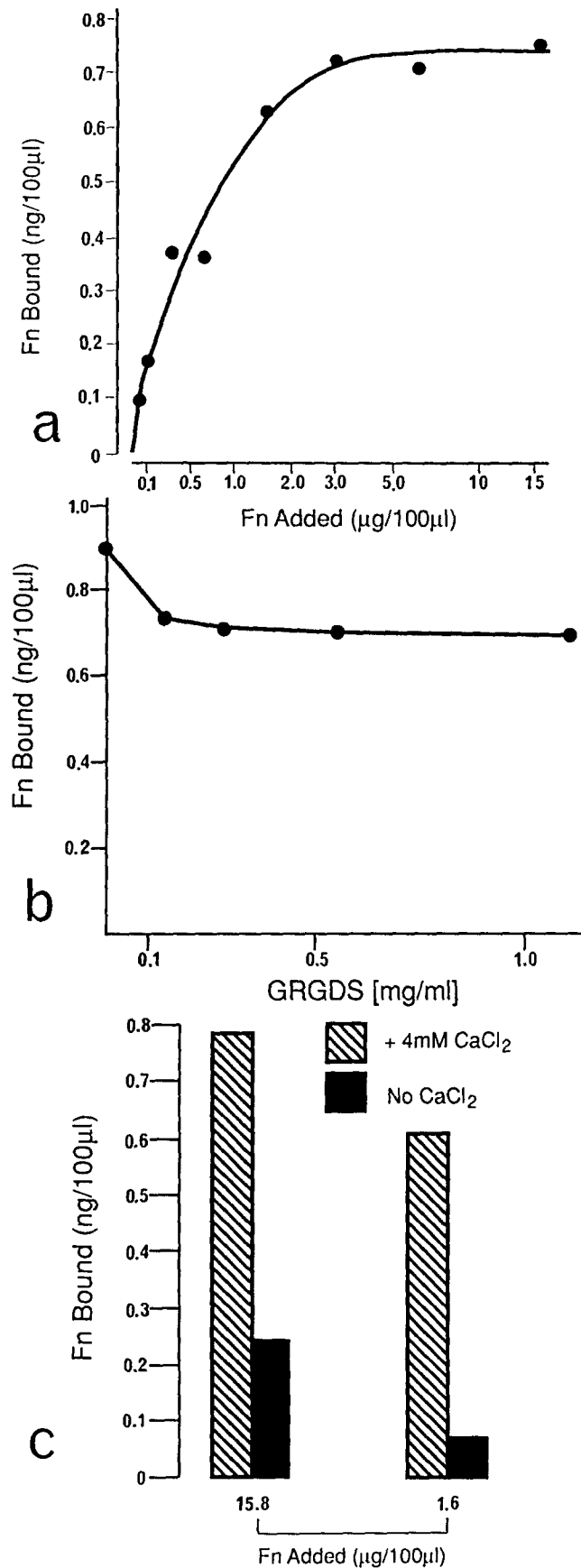


Figure 3. Characteristics of AGp110 binding to rat plasma fibronectin. Points on graphs are mean values from triplicate experiments. Deviations from mean values are $\sim 10\%$. Constant amounts of

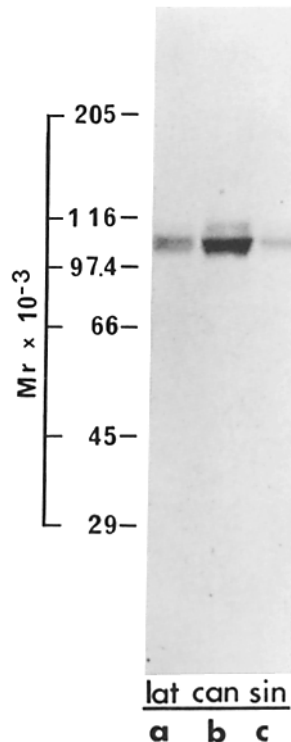


Figure 4. Relative quantities of AGp110 in fractionated liver plasma membranes. 100 μg of membrane proteins from each membrane fraction were separated by SDS-PAGE, blotted onto nitrocellulose and treated with anti-AGp110 serum. (a) Lateral membranes; (b) canalicular membranes; (c) sinusoidal membranes.

omission of calcium ions from incubation buffers resulted in decreased binding (Fig. 3 c).

Localization in Rat Liver Membranes

Western blotting of purified plasma membranes from the sinusoidal, lateral and canalicular cell surface domains of the liver parenchyma using anti-AGp110 serum indicated that the protein is located predominantly in the bile canalicular plasma membrane domain (Fig. 4). Secretion of AGp110 into the bile canaliculus does not seem to occur since the protein could not be detected in bile fluid by Western blotting (result not shown). Immunofluorescent staining of frozen liver sections (Fig. 5 a) affirmed antigen residency exclusively on the bile canalicular surface and provided no evidence for sinusoidal or lateral membrane localization, thus suggesting that the low level of expression of AGp110 found in Western blots of sinusoidal and lateral membranes (Fig.

AGp110 ($\sim 0.1 \mu\text{g}$) were immobilized on microtiter plate wells and incubated with fibronectin solutions of various concentrations. The wells were then incubated with rabbit anti-rat plasma fibronectin followed by anti-rabbit IgG conjugated with alkaline phosphatase. The OD₄₀₀ values obtained after reaction with chromogen were compared with those of a standard curve for fibronectin, and the amount of fibronectin attached to immobilized AGp110 was quantitated. (a) Semi-log plot of added versus bound fibronectin. Saturable binding was observed. (b) Plot of fibronectin bound to AGp110 versus amount of soluble GRGDS peptide in incubation buffer. The result indicates minimal inhibition of binding. (c) Effect of calcium on fibronectin binding to AGp110. A significant reduction in the amount of fibronectin bound to AGp110 was observed in buffers lacking calcium.

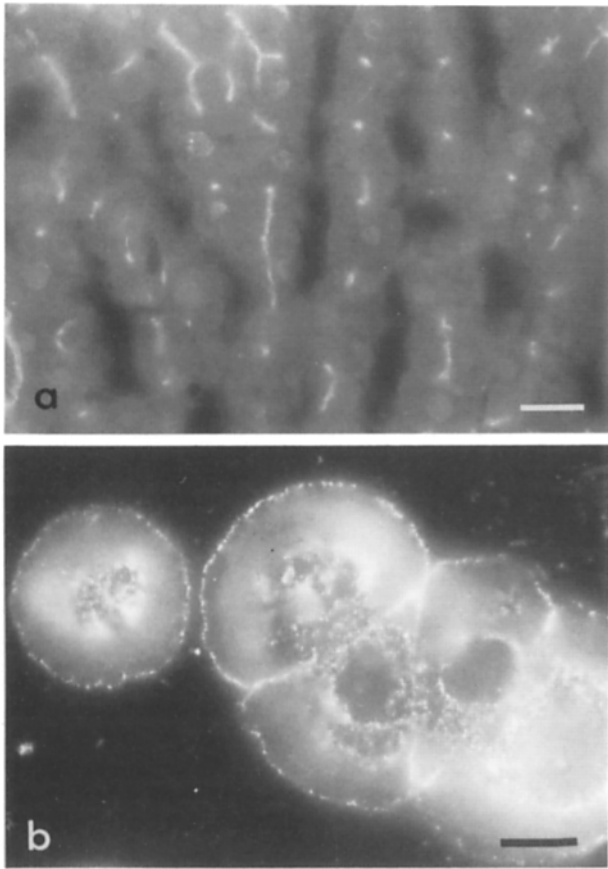


Figure 5. Localization of AGp110 in frozen liver sections and in cultured hepatocytes by immunofluorescence. Immunofluorescent image of AGp110 localization using anti-AGp110 serum on frozen liver sections. The antigen is seen along the bile canaliculi. Bar, 20 μm . (b) Localization of AGp110 on live hepatocytes by immunofluorescence. Hepatocytes were cultured on fibronectin in serum-free medium at 37°C for 3 h before exposure to anti-AGp110 antibodies. The photograph depicts a double exposure at different focal planes. Focusing was on the substrate plane and close to the top cell surface. AGp110 is visualized along the periphery of the ventral surface close to the substratum and scattered over the dorsal cell surface. Bar, 10 μm .

4) is due to cross-contamination with membranes from the canalicular domain.

In vitro AGp110 was found on the substrate contact area along the periphery of the hepatocyte ventral surface, but was also scattered over the entire dorsal surface (Fig. 5 b). The protein was neither incorporated into the fibronectin/fibrin matrix that hepatocytes synthesize in vitro (54) nor absorbed onto the fibronectin-coated culture substratum as shown by immunofluorescence experiments (results not shown).

AGp110 is exposed on the cell surface, since it was labeled by lactoperoxidase-catalyzed iodination of intact hepatocytes. To investigate the interaction of AGp110 with the membrane lipid bilayer, purified bile canalicular membranes were extracted with Triton X-114 or with buffers of high ionic strength, and the presence of the protein in each extract was probed with the antiserum in Western blots. AGp110 was found mainly in the detergent (hydrophobic) phase after Tri-

ton X-114 extraction and phase separation of canalicular membranes (Fig. 6, a and b) and could not be dissociated from the plasma membrane after treatment with 2 M NaCl (Fig. 6, c and d). On the basis of these experiments we conclude that AGp110 is an integral membrane protein of hepatocytes with an extracellular domain.

Role of AGp110 in the Adhesion of Rat Hepatocytes on Fibronectin

Since AGp110 was shown to be exposed on the hepatocyte cell surface and to interact with fibronectin, we investigated its possible role in hepatocyte adhesion to fibronectin by studying the effect of the AGp110 specific antiserum on hepatocytes cultured on fibronectin substrata.

Hepatocytes were allowed to spread on fibronectin substrata in serum-free medium in the presence of the protein synthesis inhibitor cycloheximide. The use of cycloheximide was deemed necessary in view of the fact that hepatocytes in vitro synthesize copious amounts of fibronectin and lesser amounts of other adhesive matrix proteins (such as type IV collagen and laminin) whose deposition on the substratum would have invalidated comparison of results from cells on different substrata (54–56). 3 h after inoculation monovalent Fab' fragments of anti-AGp110 IgG were added in the culture medium and caused rapid rounding of the cells (Fig. 7 b) compared with control cultures (Fig. 7 a). Control experiments using Fab' fragments of nonimmune rabbit IgG were totally negative. Ruffling activity at the peripheral lamella of spread hepatocytes could be noticed under the microscope just 10 min after adding the AGp110 specific antibodies. The adverse effect of anti-AGp110 Fab' fragments on hepatocyte spreading was independent of cell density and intercellular contacts: experiments in which hepatocytes were inoculated on fibronectin-coated substrata at different cell densities demonstrated that single cells or colonies of hepatocytes were equally prone to reversal of spreading after antibody treatment (results not shown). Fab' concentrations ranging from 500 to 50 $\mu\text{g}/\text{ml}$ were tested and found to be effective in rounding up hepatocytes spread on fibronectin substrata. The cells remained viable throughout the antibody

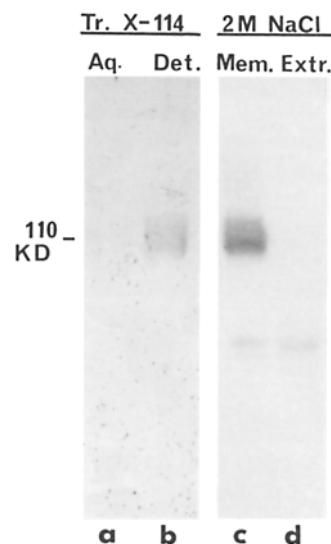


Figure 6. Membrane association of AGp110. (a) Western blot of liver membrane proteins distributed in the aqueous phase after Triton X-114 fractionation. Blot treated with anti-AGp110. (b) Western blot of liver membrane proteins distributed in the detergent phase after Triton X-114 fractionation. Treatment with AGp110 antiserum reveals AGp110 in this phase. (c) Western blot of liver membrane residue after 2 M NaCl extraction. (d) Western blot of high-salt extract of liver membranes.

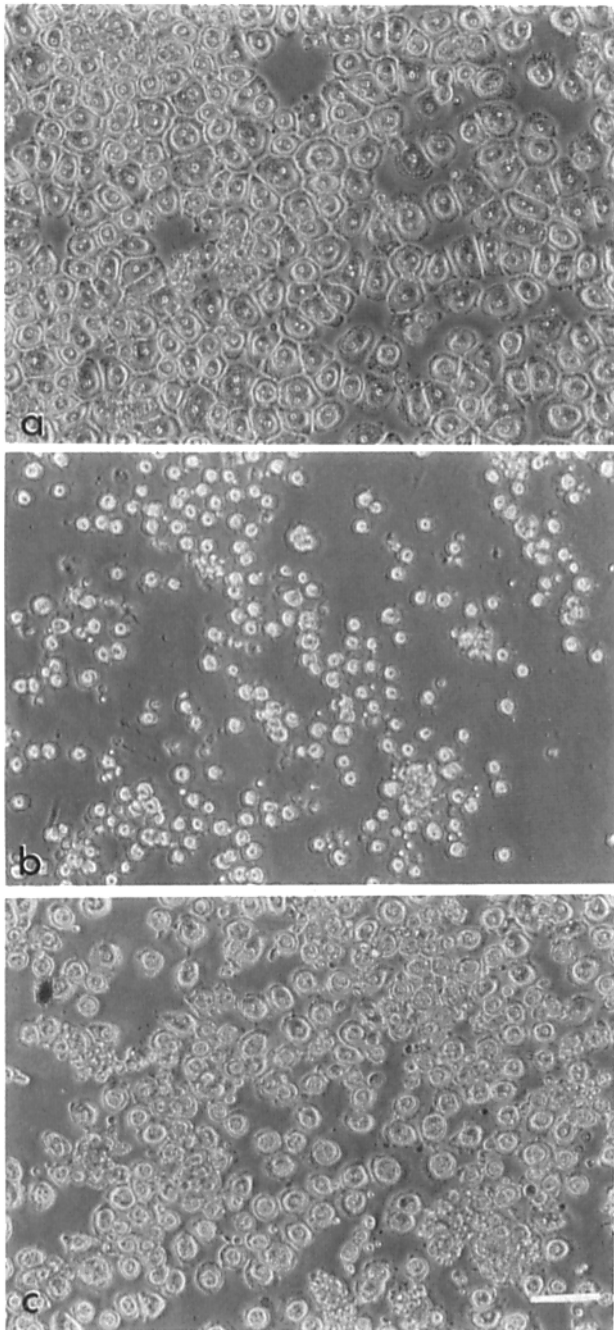


Figure 7. Effect of AGp110 antibody on hepatocytes spreading on fibronectin substrata. Bar, 50 μm . (a) Hepatocytes seeded onto a fibronectin-coated substratum in the presence of cycloheximide and allowed to spread for 3 h. (b) Hepatocytes were allowed to spread for 3 h on a fibronectin substratum as in a and then incubated for 1 h in medium containing 500 $\mu\text{g/ml}$ monovalent Fab' fragments of AGp110 antibodies. (c) Hepatocyte cultures were treated with Fab' fragments as in b, the culture medium was replaced with medium without Fab' and the cells were incubated for a further hour.

treatment as shown by the fact that cellular spreading on fibronectin substrata was reestablished after removal of the Fab fragments from the culture medium (Fig. 7 c). The Fab' fragments did not induce detachment of the cells, even after prolonged exposure and initial attachment of hepatocytes

onto fibronectin could not be inhibited with Fab' concentrations up to 500 $\mu\text{g/ml}$ (results not shown).

We also examined the effect of the antiserum on cells spread on type IV collagen or laminin substrata. As shown in Fig. 8, the anti-AGp110 Fab' fragments caused no noticeable reversal of cell spreading on either type IV collagen (Fig. 8 b) or laminin (Fig. 8 d) when compared with control cultures (Fig. 8, a and c, respectively).

Glycosylation of AGp110

Since AGp110 binds to WGA, it is clearly a glycoprotein. Further information on the extent and type of glycosylation was sought by making use of specific glycosidases. Hepatocytes spread on fibronectin were radioiodinated, extracted in 0.2% SDS (see Materials and Methods) and AGp110 was specifically immunoprecipitated. The immunoprecipitates were then digested with various glycosidases. Endo F, which cleaves high-mannose, hybrid, and some complex oligosaccharides attached through an N-linkage to asparagine, produced a small but reproducible molecular mass shift of $\sim 2\text{--}3$ kD (Fig. 9 b; compare with control, a). On the other hand, removal of sialic acid with neuraminidase reduced the molecular mass to 94 kD (Fig. 9 c), a reduction of 16 kD. Assuming complete removal of N-linked chains by Endo F, these results indicate an abundance of terminal sialic acid residues on carbohydrate chains O-linked to serine or threonine. However, since the protein may contain N-linked saccharides that are not cleaved by Endo F, we repeated the experiment using N-glycanase, which removes a broader range of complex asparagine-linked carbohydrate structures than endo F and O-glycanase, which releases O-glycans from serine or threonine. Since substitution with sialic acid at the core Gal($\beta 1\rightarrow 3$)GalNAc of O-glycans prevents release of this disaccharide, we first treated the immunoprecipitated AGp110 with neuraminidase: the asialoprotein migrated to 94 kD (Fig. 9 e) as noted in the previous experiment (Fig. 9 c). Subsequent hydrolysis with O-glycanase resulted in a molecular weight shift of ~ 5 kD (Fig. 9 f), confirming the presence of O-glycans. Digestion with N-glycanase increased the electrophoretic mobility of the protein (Fig. 9 g) by a magnitude similar to that obtained after Endo F hydrolysis (Fig. 9 b). These results indicate the presence of $\sim 1\text{--}2$ N-glycans and an abundance (at least 10 chains) of O-glycans. The number of carbohydrate chains cannot be determined accurately since removal of polysaccharides may alter the association of SDS with the protein, thus disproportionately affecting its mobility in SDS gels. Further proof on the extent of N-glycosylation was provided by examining the inhibitory effect of tunicamycin. Tunicamycin inhibits the first step of N-glycan assembly and hence blocks N-glycosylation completely. Metabolic labeling of hepatocytes with [^{35}S]methionine in the presence of tunicamycin and subsequent immunoprecipitation of detergent-extracted proteins with anti-AGp110 yielded a band in SDS-gels at a molecular weight position of 107 kD (Fig. 9 k), consistent with the results obtained after Endo F (Fig. 9 b), and N-glycanase (Fig. 9 g) hydrolysis. Experiments in which cultures were pulsed for varying times (1–4 h) with isotope indicated the presence of a precursor of ~ 91 kD (Fig. 9, j–l) that is processed to yield the mature protein, as indicated after equilibrium labeling (Fig. 9. i).

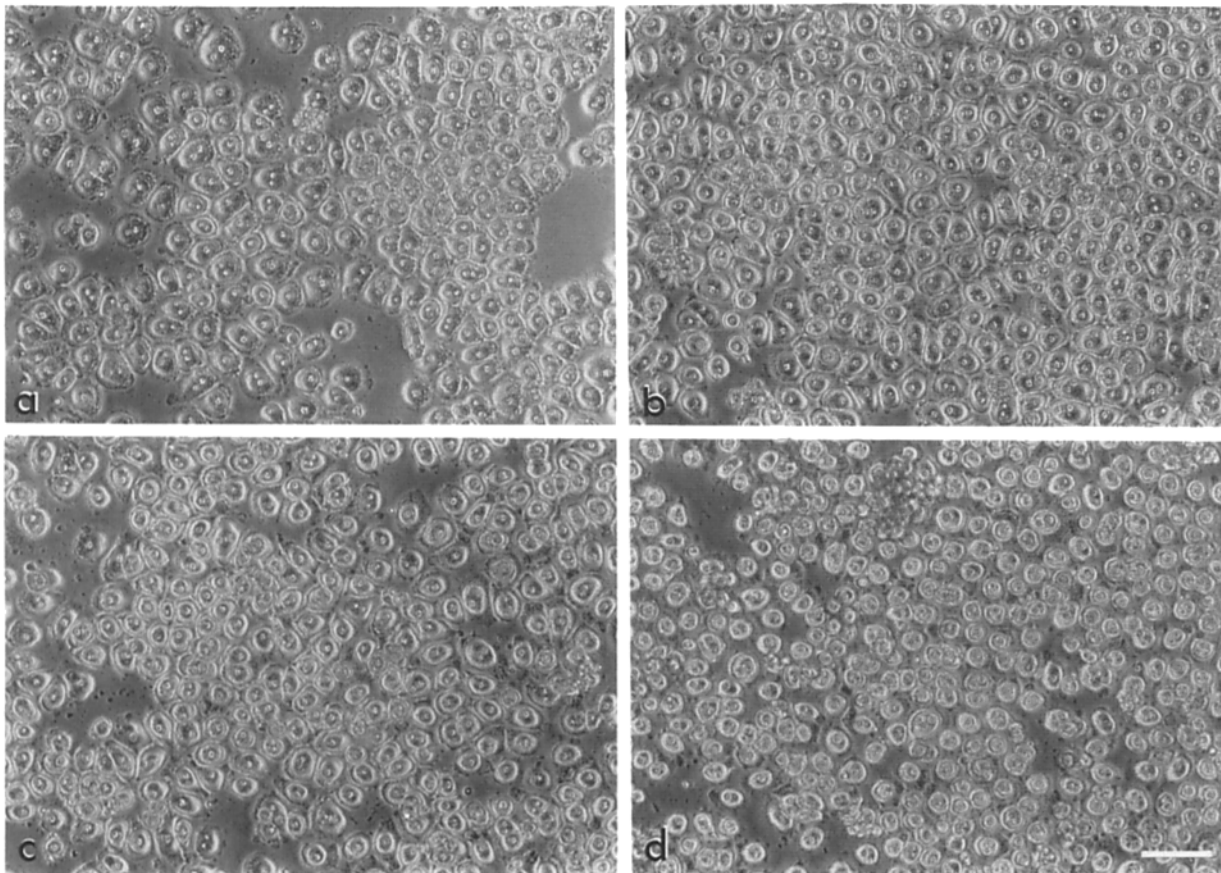


Figure 8. Effect of AGp110 antibody on hepatocyte spreading on type IV collagen- or laminin-coated substrata. Bar, 50 μm . (a) Hepatocytes inoculated on type IV collagen and incubated for 3 h. (b) Cells incubated for 3 h on type IV collagen and then treated with medium containing 500 $\mu\text{g/ml}$ Fab' fragments of anti-AGp110 antibodies for 1 h. (c) Hepatocytes spread on laminin after 3 h incubation. (d) Hepatocytes spread on laminin and then treated with anti-AGp110 Fab'.

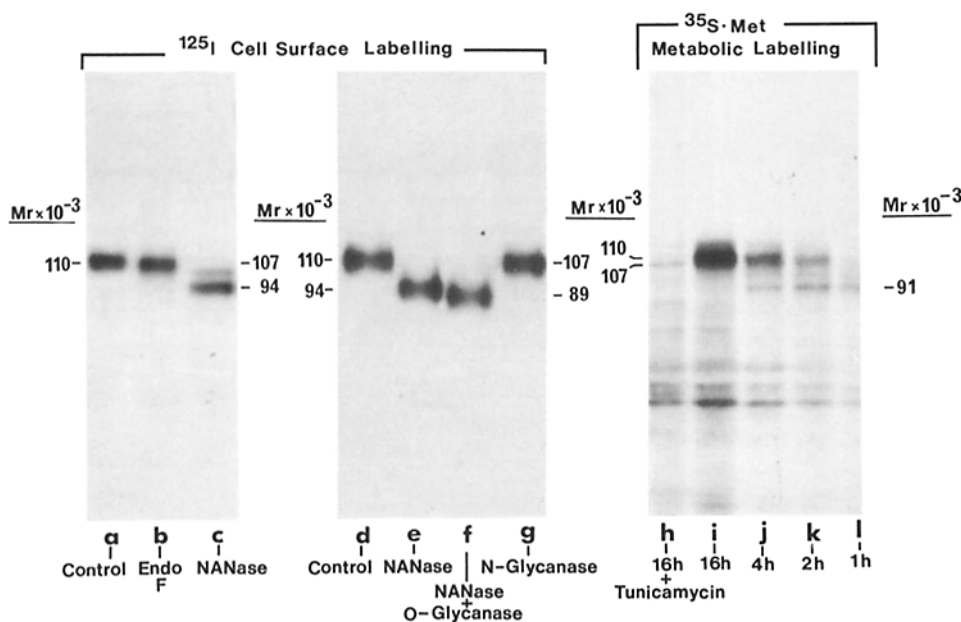


Figure 9. Glycosylation and processing of AGp110. Autoradiograms of 7.5% SDS gels of immunoprecipitates of detergent-extracted surface-radioiodinated hepatocytes (a-g) and fluorograms of SDS gels of immunoprecipitates of detergent-extracted hepatocytes labeled metabolically with [^{35}S]-methionine (h-m). (a) Immunoprecipitation of detergent extracts of surface iodinated hepatocytes by specific AGp110 antibodies. (b) Endo F treatment of iodinated immunoprecipitated AGp110. (c) Neuraminidase treatment of iodinated AGp110. (d) AGp110 immunoprecipitated from surface-iodinated hepatocytes (same as a). (e) Neuraminidase-treated AGp110 (same as c). (f) Neuraminidase followed by O-glycanase treatment of AGp110. (g) N-glycanase treatment of AGp110. (h) AGp110 immunoprecipitated from extracts of cells that were metabolically labeled with [^{35}S]-methionine in the presence of tunicamycin. (i-l) Immunoprecipitation of AGp110 from hepatocytes labeled with [^{35}S]-methionine for 16 (i), 4 (j), 2 (k) and 1 h (l). A 91-kD precursor detected in short pulses is indicated.

AGp110 immunoprecipitated from extracts of cells that were metabolically labeled with [^{35}S]-methionine in the presence of tunicamycin. (i-l) Immunoprecipitation of AGp110 from hepatocytes labeled with [^{35}S]-methionine for 16 (i), 4 (j), 2 (k) and 1 h (l). A 91-kD precursor detected in short pulses is indicated.

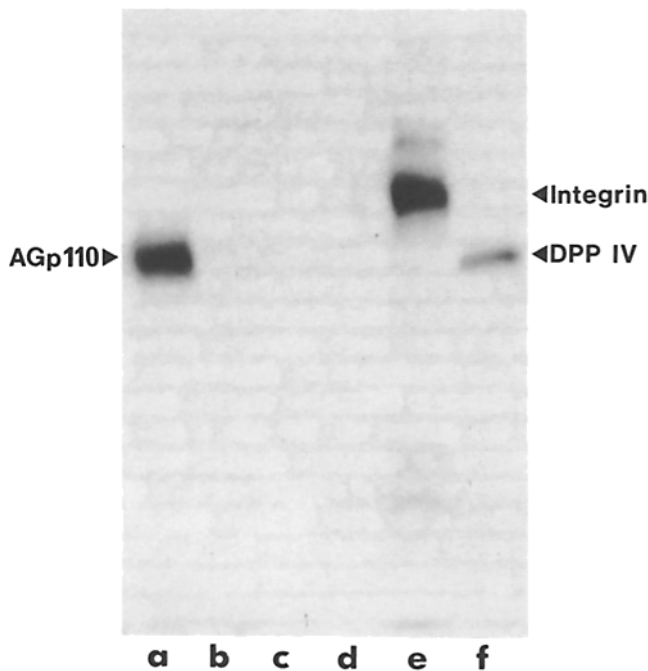


Figure 10. Comparison of AGp110 with other liver proteins. Autoradiograms of reduced 7.5% SDS gels showing immunoprecipitations of surface-radioiodinated hepatocytes extracted with CHAPS-containing buffer under nondissociating conditions. (a-d) Serial immunoprecipitations with AGp110 specific antiserum resulting in immunodepletion of AGp110 (b-d). (e) Immunoprecipitation of integrin from AGp110-immunodepleted cell extract. (f) Immunoprecipitation of DPPIV from AGp110-depleted cell extract.

Comparison of AGp110 with Other Parenchymal Cell Surface Glycoproteins

We compared AGp110 with the liver fibronectin receptor integrin (32) and DPPIV, an enzyme that is involved in fibronectin-mediated attachment of hepatocytes on collagen (21). Hepatocytes spread on fibronectin substrata were surface labeled by lactoperoxidase-catalyzed iodination and extracted under nondissociating conditions (see Materials and Methods). Aliquots of this extract were successively immunoprecipitated with anti-AGp110 serum (Fig. 10, a-d). The extracts thus immunodepleted of AGp110 were immunoprecipitated with antisera against integrin (Fig. 10 e) or DPPIV (Fig. 10 f). The results show that these proteins were present in the extract after AGp110 was removed, thereby indicating that AGp110 is a distinct glycoprotein.

In immunoprecipitations of AGp110 and integrin under nonreducing and nondissociating conditions (Fig. 11), we did not detect any change in the electrophoretic mobility of AGp110 in the absence of reducing agents (Fig. 11 a). The fact that a single band was observed in AGp110 immunoprecipitations under nondissociating conditions either in the presence (Fig. 10 a) or absence (Fig. 11 a) of reducing agents, suggests that the protein is a monomer. In contrast, the dimeric structure of the fibronectin receptor integrin was clearly revealed in nonreducing gels: the α and β subunits (Fig. 11 b, arrows) ran at indicated molecular masses of 155 and 118 kD respectively, in agreement with previous observations (32). The bands of higher molecular weight (Fig. 11 b, arrowheads) that were precipitated with the anti- β integrin

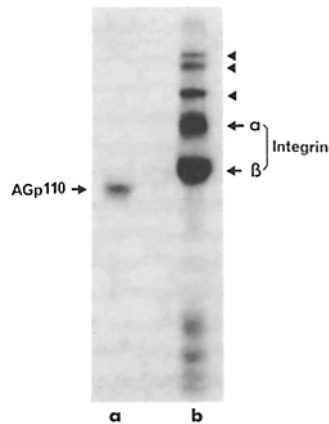


Figure 11. Immunoprecipitation of AGp110 (a) and integrin (b) from surface radioiodinated cells extracted under nondissociating conditions. The gels (7.5%) were run in the absence of reducing agents. The arrows in b indicate the α and β subunits of fibronectin receptor integrin. Arrowheads indicate other proteins immunoprecipitated with the anti- β serum that could possibly be different integrin α subunits.

antiserum could be different α subunits associated with the same β_1 subunit of the fibronectin receptor.

Tissue Distribution of AGp110

The distribution of AGp110 in different tissues was determined by Western blotting of detergent extracts of tissues obtained from an adult rat. Equal amounts of protein from various tissues extracted with octyl glucoside (see Materials and Methods) were separated by SDS-PAGE, blotted onto nitrocellulose, and probed with anti-AGp110 (Fig. 12). The protein was found mainly in liver, pancreas, kidney, and small intestine. In the small intestine the protein migrated at a lower molecular mass (~ 90 kD), possibly due to proteolytic cleavage from digestive enzymes found in that organ. Lesser amounts of AGp110 were observed in lung, thymus, and spleen, but it was absent in stomach, large intestine, heart, and thigh muscle (Fig. 12). The results demonstrate that expression of AGp110 is tissue specific.

Discussion

We have identified and partially characterized a hepatic bile canalicular protein, AGp110, that interacts with fibronectin.

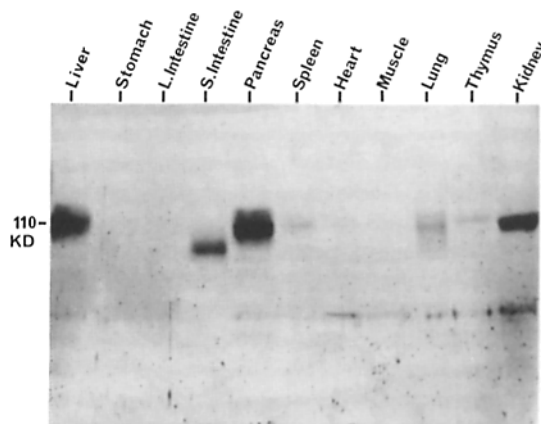


Figure 12. Relative amounts of AGp110 in various rat tissues. Equal amounts of protein (100 μ g) from tissues extracted with octyl glucoside were subjected to SDS-PAGE. The separated proteins were transferred to nitrocellulose and probed with anti-AGp110 and peroxidase-conjugated anti-rabbit IgG.

AGp110 is an integral membrane glycoprotein with an extracellular domain that is extensively glycosylated with sialylated O-glycans and one or two N-glycan chains. The functional significance of the binding to fibronectin was demonstrated in adhesion assays in which spreading of hepatocytes was arrested and reversed by antibodies to AGp110. In that respect therefore, the protein functions as a fibronectin receptor. The fibronectin receptor integrin of rat liver (32) and AGp110 seem to be distinct proteins: they migrate to different molecular weight positions in SDS gels, immunodepletion of AGp110 does not remove integrin from cell extracts and anti-AGp110 does not recognize the parenchymal integrin. Furthermore, the fact that our experiments indicate that the protein is a monomer without extensive intrachain disulfide bonding argues against it being a heterodimer or a β -subunit from the integrin groups discovered so far (14, 29, 34).

Integrins are the major cell surface receptors for extracellular matrix components including fibronectin, but proteoglycans (50), gangliosides (11, 35), and other glycoproteins (2, 57) have also been shown to exhibit receptor-like activities in cell-fibronectin interactions. As regards the integrin family of macromolecules, fibronectin receptor integrins from liver parenchyma and endothelium have different α subunits but share a common β subunit (32, 33). T lymphocytes have two integrin receptors (58), which are dimers of the $\alpha_4\beta_1$ and $\alpha_5\beta_1$ type. In agreement with this, our experiments suggest that the β subunit (β_1 type) of hepatocyte integrin (32) may associate with different α subunits to form multiple heterodimers. This plurality of cellular receptors corroborates findings on multiple ligand sites on the fibronectin molecule: the main cell adhesion fibronectin fragment contains the RGD cell recognition signal, while the heparin-binding domains activate adhesion by linkage to cell surface heparan sulfate (1, 29, 49, 51). The alternatively-spliced type III connecting segment (IIICS) that lies between the COOH-terminal heparin and fibrin domains has two adhesion-promoting sequences (26, 28): one of these (CS5) contains the peptide REDV (in human fibronectin—in the rat it mutates to RGDV [52]), which supports melanoma but not fibroblast cell attachment (26, 28), while the other (denoted CS1) induces neurite extension (27) and contains a peptide sequence recognized by the $\alpha_4\beta_1$ integrin of peripheral blood and cultured T lymphocytes (58). The latter integrin has no affinity for RGD. In addition to these adhesion domains, evidence exists for yet another sequence operating in synergy with RGD (16). In this context, the discovery of a dual fibronectin receptor system in liver parenchyma, comprising AGp110 as well as integrin(s), although novel, follows the concept of fibronectin receptor plurality as discussed above.

Our adhesion experiments show that initial attachment of hepatocytes on fibronectin are not blocked with anti-AGp110, whereas spreading of cells in 3-h-old cultures can be reversed. This indicates a minor (if any) role of AGp110 in initial contact to fibronectin, but a significant contribution in initiating or maintaining cell spreading. Since inhibition of integrin function with specific antisera also reverses spreading (30), active spreading may require co-operative action from both receptors, whereas integrin alone may suffice to effect initial attachment.

Among nonintegrin hepatic glycoproteins with molecular weight similar to AGp110, DPPIV is involved in fibronectin-mediated adhesion of hepatocytes on collagen (21). Apparently, DPPIV is found on the bile canalicular surface (22),

although other data suggests a less polarized distribution (9). Our immunodepletion experiments (Fig. 10) and other serological comparisons using our anti-AGp110 serum and monoclonal antibodies against DPPIV indicate that DPPIV and AGp110 are distinct proteins. Cell-CAM 105, an intercellular adhesion molecule (41, 43) shows a low level of antigenic cross-reactivity with AGp110, but behaves differently under nonreducing conditions in SDS-PAGE and is N-glycosylated to a significantly higher degree than AGp110 (43), so identity is unlikely. A transformation-sensitive 110-kD glycoprotein has also been localized in bile canalicular plasma membranes, but has a slightly more acidic isoelectric point and displays a different tissue distribution (4). AGp110 may, however, be related to a monocyte fibronectin receptor described previously (23): this protein (M_r 105 kD) is also a monomer with no indication of intrachain disulfide bonds.

The physiological role of a double-receptor system in the liver parenchyma is not clear. Multiple receptors for fibronectin could confer tissue specificity in adhesive interactions or modulate phenomena such as cell migration and selective adhesion in either normal programmed events, such as development, or in pathological conditions, such as the neoplastic state in which the spectrum of integrins is known to be altered (45). Fibronectin in rat liver shows an uneven, but essentially nonpolarized distribution with respect to the hepatocellular surface: the protein can be found on the canalicular and lateral surfaces (25) but is most prominent in the perisinusoidal space (25, 39). Recently, different fibronectin isoforms have been found on each hepatocellular surface domain (18), thus necessitating a sorting mechanism perhaps provided by different receptors. Such a putative role for AGp110 would not exclude a functional, perhaps transient, involvement in adhesive interactions in vivo under conditions where the strict polarization of the hepatocellular surface is perturbed such as during cell division in expansion of the liver parenchyma. Antigens found on the bile canalicular surface in adult liver have been shown to exhibit a different distribution in the embryonic liver (40), in hepatocarcinogenesis (53) and during liver regeneration after partial hepatectomy (42).

In conclusion, we have demonstrated the existence of a novel glycoprotein in liver parenchyma that is active in mediating spreading of cultured hepatocytes on fibronectin substrata. AGp110 and integrin fibronectin receptor are distinct proteins suggesting that a dual receptor system mediates cell-fibronectin interactions in the liver parenchyma.

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