Hepatocyte Attachment to Laminin Is Mediated through Multiple Receptors

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Abstract. The interaction of hepatocytes with the basement membrane glycoprotein laminin was studied using synthetic peptides derived from laminin sequences. Rat hepatocytes bind to laminin and three different sites within the A and B1 chains of laminin were identified. Active laminin peptides include the PA22-2 peptide (close to the carboxyl end of the long arm in the A chain), the RGD-containing peptide, PA21 (in the short arm of the A chain) and the pentapeptide YIGSR (in the short arm of the B1 chain). PA22-2 was the most potent peptide, whereas the other two peptides had somewhat lower activity. Furthermore, hepatocyte attachment to laminin was inhibited by the three peptides, with PA22-2 being the most active. Various proteins from isolated membranes of cell-surface iodinated hepatocytes bound to a laminin affinity column including three immunologically related binding proteins : M_r = 67,000, 45,000, and 32,000. Several proteins—M_r = 80,000, 55,000, and 38,000–36,000—with a lower affinity for laminin were also identified. Affinity chromatography on peptide columns revealed that the PA22-2 peptide specifically bound the M_r = 80,000, 67,000, 45,000, and 32,000 proteins, the PA21 peptide bound the M_r = 45,000 and 38,000–36,000 proteins and the YIGSR peptide column bound the 38,000–36,000 protein. Antisera to a bacterial fusion protein of the 32-kD laminin-binding protein (LBP-32) reacted strongly with the three laminin-binding proteins, M_r = 67,000, 45,000, and 32,000, showing that they are immunologically related. Immunoperoxidase microscopy studies confirmed that these proteins are present within the plasma membrane of the hepatocyte. The antisera inhibited the adhesion of hepatocytes to laminin by 30%, supporting the finding that these receptors and others mediate the attachment of hepatocytes to several regions of laminin.

LAMININ is the major glycoprotein in all basement membranes, the thin extracellular matrices that underlie epithelial cells and endothelial cells and surround nerve, muscle, and fat cells (Martin and Timpl, 1987). It promotes the adhesion, migration, growth, and differentiation of a variety of cells (Kleinman et al., 1985; Timpl et al., 1983*a*). Considerable progress has been made in determining the structure of laminin. It is composed of three chains, A (M_r = 400,000); B1 (M_r = 210,000); and B2 (M_r = 200,000); which are led in a cross-like structure by disulfide bonds (Barlow et al., 1984; Palm et al., 1985; Paulsson et al., 1985; Sasaki et al., 1988). These chains have been cloned and sequenced, and biologically active sites responsible for cell-binding have been identified using both proteolytic fragments and synthetic peptides (Fig. 1) (Aumailley et al., 1987; Goodman et al., 1987; Rao et al., 1982; Terranova et al., 1983; Timpl et al., 1983*b*). A pentapeptide, YIGSR-NH_2, from the cysteine-rich domain III in the B1 chain has been shown to promote cell attachment and migration and to prevent melanoma colonization of lungs (Graf et al., 1987; Iwamoto et al., 1987). Another peptide, F9, from the globule in domain III of the B1 chain has also been reported being active for cell attachment and for heparin binding (Charonis et al., 1988). In addition, a peptide containing an RGD sequence from the short arm of the A chain is also active for cell attachment (Grant et al, 1989).[1] The peptide can promote neurite outgrowth of NG108-15, murine neural-glial hybrid cells, when conjugated to keyhole limpet hemoglobin (KLH).[2] More recently, a 19-mer peptide from the carboxy-terminal end of the α-helical region of the long arm of the A chain was shown to promote cell attachment and migration (Tashiro et al., 1989). This peptide itself, without being conjugated to KLH, can promote neurite outgrowth on PC12, rat pheochromocytoma cells (Sephel et al., 1989; Tashiro et al., 1989).

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1. Tashiro, Sephel, Greatorex, Sasaki, Martin, Kleinman, and Yamada, manuscript in preparation.

2. *Abbreviations used in this paper*: CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate; KLH, keyhole limpet hemoglobin; TEA, triethanolamine.

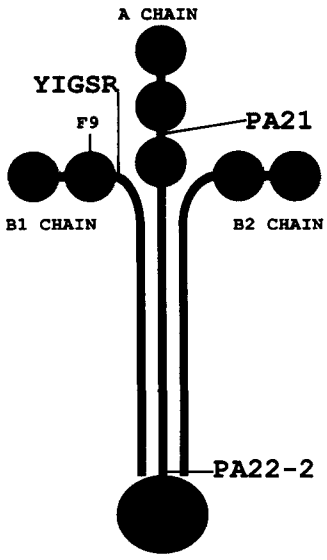


Figure 1. A schematic drawing of laminin. The location of the peptides used in this study are indicated. The peptides and their position are: peptide PA21, residues 1,115–1,129 in the A chain sequence (Sasaki et al., 1988); PA22-2, residues 2,091–2,108 in the A chain sequence (Sasaki et al., 1988); YIGSR, residues 928–933 in the B1 chain sequence (Graf et al., 1987); F9 site is from Charonis et al. (1988).

Because laminin has multiple cell adhesion sites, it is expected that several different cellular receptors will bind to laminin. Indeed various laminin-binding proteins have been isolated. Initially, a 67-kD protein has been identified as a laminin receptor in tumor cells and muscle cells (Rao et al., 1983; Lesot et al., 1983; Malinoff and Wicha, 1983; von der Mark and Kuhl, 1985). Wewer et al., (1986) have reported a partial cDNA for the human 67-kD protein. Subsequently, several groups (Yow et al., 1988)[3] have isolated human and mouse full-length cDNA whose deduced amino acids sequence matches the cDNA clone described by Wewer et al. (1986). However, these full-length cDNA clones have a coding capacity for a 32-, but not 67-kD protein. Indeed, the 32-kD protein has identified as a cell surface laminin-binding protein in various cells (Mercurio and Shaw, 1988).[3] Antibodies to a bacterial fusion protein from a full-length clone for the 32-kD protein (LBP-32) recognize 32-, 45-, and 67-kD proteins. Although the relationship between these proteins is unclear at the present, studies using cell-free translation and sequence specific antibodies suggest that these proteins could share a common epitope, but be encoded by distinct genes (Segui-Real, B., unpublished results).[3] Other laminin-binding proteins have also been described including several members of the integrin receptor family (Gehlsen et al., 1988; Hemler et al., 1988; Ignatius and Reichardt, 1988; Tomaselli et al., 1988).

In the adult liver, laminin is present mainly in the basement membrane surrounding vascular endothelial and bile duct cells. Its presence in the space of Disse is controversial, for while there is no basement membrane in this area, some immunoreactivity for laminin has been observed (Abrahamson et al., 1985; Bissell et al., 1987; Clément et al., 1988; Hahn et al., 1980; Martinez-Hernandez, 1984). Laminin alters the morphology and the function of hepatocytes in culture (Sudhakaran et al., 1986; Sawada et al., 1987; Bissell et al., 1986, 1987). Specifically, hepatocytes remain in a globular shape and only slowly spread on laminin in comparison with either collagen or fibronectin substrates. The decrease in albumin synthesis is less pronounced when hepatocytes are cultured on laminin instead of on collagen substrates. Hepatocytes on fibronectin synthesizes DNA at twice the rate observed with cells on laminin. Thus, hepatocytes interact and respond to laminin with a more differentiated phenotype. We report here that normal adult hepatocytes attach to multiple sites on laminin through several surface-binding proteins.

3. Segui-Real, Savagner, Reich, Ogle, Martin, and Yamada, manuscript in preparation.

Materials and Methods

Materials

Laminin was extracted from the Engelbreth-Holm-Swarm tumor (Timpl et al., 1979). Horseradish peroxidase-conjugated goat anti-rabbit antibodies were obtained from Bio-Rad Laboratories (Oxnard, CA). Immobilon were purchased from Millipore Corp. (Bedford, MA). $Na^{125}I$ was from Amersham Corp. (Arlington Heights, IL). Protein A-Sepharose 4B was obtained from Pharmacia Fine Chemicals (Piscataway, NJ). *n*-Octyl b-D-glucopyranoside (octylglucoside), triethanolamine (TEA), and lactoperoxidase were from Sigma Chemical Co. (St. Louis, MO). 3-[(3-Chloroamidopropyl)-dimethylammino]-1-propane sulfonate (CHAPS) was from Calbiochem-Behring Corp. (La Jolla, CA). Bacterial collagenase from *Clostridium histolyticum* was purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN).

Synthetic Laminin Peptides

Peptides were synthesized with an automated synthesizer (model 430A; Applied Biosystems, Inc., Foster City, CA). All peptides were prepared as amides (Graf et al., 1987) and the composition of all peptides was verified by amino acid analyses. Peptides possessing biological activity were further purified using preparative HPLC. Cyclic YIGSR was prepared according to the method described by Robey and Fields (1989). The specific sequence of the peptides used was: PA21 from the A chain residues 1,115–1,129, CQAGTFALRGDNPQG-amide; PA22-2 from the A chain residues 2,091–2,108, CSRARKQAASIKVAVSADR-amide; YIGSR, residues 929–933, from the B1 chain (Fig. 1), YIGSK-amide, served as a control peptide with a similar charge. Cysteine (C) of PA 21 and PA 22-2 was included to facilitate coupling, but is not part of the actual sequence.

Cell Isolation and Culture

Hepatocytes were isolated from 2-mo-old Sprague-Dawley rats using the two-step collagenase perfusion method (Guguen-Guillouzo and Guillouzo, 1986), and were then transferred to L15 Leibovitz medium. The cells were either studied after a 20-min incubation in L15 medium or immediately plated in M-199 medium supplemented with 10 mg/ml porcine insulin and 0.02% bovine albumin.

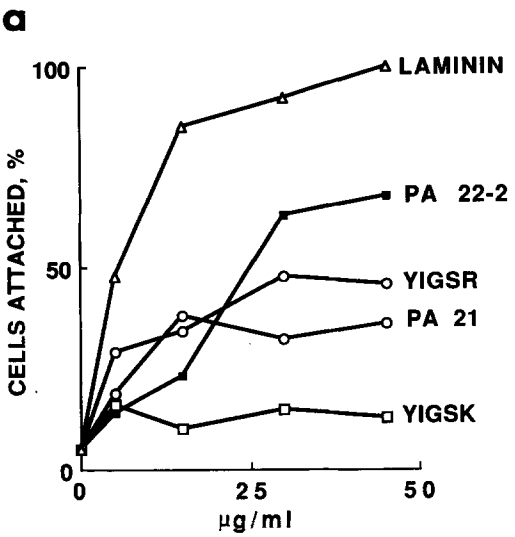
Antibody Production

LBP-32 cDNA has been previously cloned from a mouse F9 teratocarcinoma cell cDNA library;[3] using an oligonucleotide probe prepared from the published sequence for human 67-kD laminin receptor (Wewer et al., 1983). The 1.1-kb-long cDNA codes for a 32-kD protein (LBP 32). Two antibodies against LBP-32 were used in this study. Antisera were made against a bacterial fusion protein coded for by the β-galactosidase gene plus the 0.9-kb cDNA sequence encoding the nearly entire molecule, (FP 0.9) and were found to recognize several proteins including the LBP-32, the 67-kD laminin receptor and a 45-kD laminin binding protein (Mercurio and Shaw, 1988; Segui-Real et al., 1989). Antisera were raised against a 17-mer synthetic peptide from the NH_2-terminal region of LBP-32 (residues 25–41) and were found to recognize the 32- and the 45-kD laminin-binding protein but not the 67-kD protein in M2 mouse melanoma cells (Segui-Real et al., 1989).

Cell Adhesion Assay

Various amounts of laminin or peptides were coated on 0.32-cm^2 well tissue-culture plates containing 100 μl of serum-free Eagle's MEM at 37°C in a 5% CO_2 atmosphere. After 2 h, 3% bovine albumin in Eagle's MEM was added to a final concentration of 1.5% for a further 30 min. Medium

was removed, then hepatocytes were added in serum-free M-199 medium containing 0.02% bovine albumin and 10 μg/ml porcine insulin. After 1 h, plates were gently washed twice with 0.1 M phosphate buffer, pH 7.5. Attached cells were counted using an Olympus CK2 microscope. Adhesion assays were carried out in duplicate in three independent experiments, and the results within each assay did not differ by >15%. For the inhibition of attachment to laminin by the peptides, each well was coated with either 4 or 2 μg of laminin for 2 h. Before addition of the hepatocytes, varying concentrations of peptides solubilized in serum-free Eagle's MEM containing 0.02% BSA were added to each well. Adhesion assay was carried out in duplicate as described above. FP0.9 antisera were tested for their ability to inhibit cell attachment to laminin. Laminin (20 μg/ml) was added to tissue culture plates as described above. Various amounts of affinity-purified antisera were mixed with the cells for 15 min before seeding. The adhesion assay was performed as described above. Control cells were first incubated with preimmune serum, then assayed for their adhesion to laminin. Each assay was carried out in duplicate.



Radiolabeling of Hepatocytes

Only preparations of hepatocytes that were >95% viable as judged by their exclusion of trypan blue were radioactively labeled. Suspensions of freshly isolated hepatocytes (10^7 cells) were extensively washed with PBS at 4°C, then labeled for 30 min with 2 mCi of $Na^{125}I$ by lactoperoxidase-catalyzed iodination at 4°C in a final volume of 1 ml. Dead cells were removed by low-speed centrifugation. Hepatocytes were then washed three times with cold PBS and immediately processed for membrane extraction.

Preparation of Membranes

Freshly isolated hepatocytes were homogenized in cold 5 mM Tris-HCl, pH 7.4, containing 0.25 M sucrose, and then centrifuged at 1,000 *g* to separate the nuclear fraction. Plasma membranes were obtained using the method of Aronson and Touster (1978). Briefly, the supernatant fraction was clarified at 33,000 *g* for 7.5 min. Membrane fractions were pelleted at 78,000 *g* for 100 min, and then homogenized in a 57% sucrose solution. The homogenate was then placed at the bottom of a discontinuous sucrose gradient (57%/34%/8.5%), which was centrifuged for 18 h at 75,500 *g*. Membrane fractions were diluted with 4 vol of cold 5 mM Tris-HCl, pH 8, then centrifuged for 1 h at 78,000 *g*.

Affinity Chromatography

^{125}I-labeled hepatocytes were homogenized in 0.01 M TEA, pH 7.5, containing 8.5% sucrose and 2 mM PMSF at 4°C, and centrifuged at 25,000 *g* to prepare crude membranes. The cell membrane pellet was then extracted with 1% CHAPS and 1% octylglucoside in 0.01 M TEA, pH 7.5, containing 2 mM PMSF for 2 h at 4°C. After centrifugation at 25,000 *g*, the supernatant fraction was incubated overnight at 4°C with laminin- or peptide- Sepharose beads equilibrated with 0.1% CHAPS in 0.01 M Tris-HCl, pH 7.5, containing 1 mM PMSF. The beads were packed in a column and washed extensively with the same buffer containing 0.15 M NaCl. Proteins were eluted

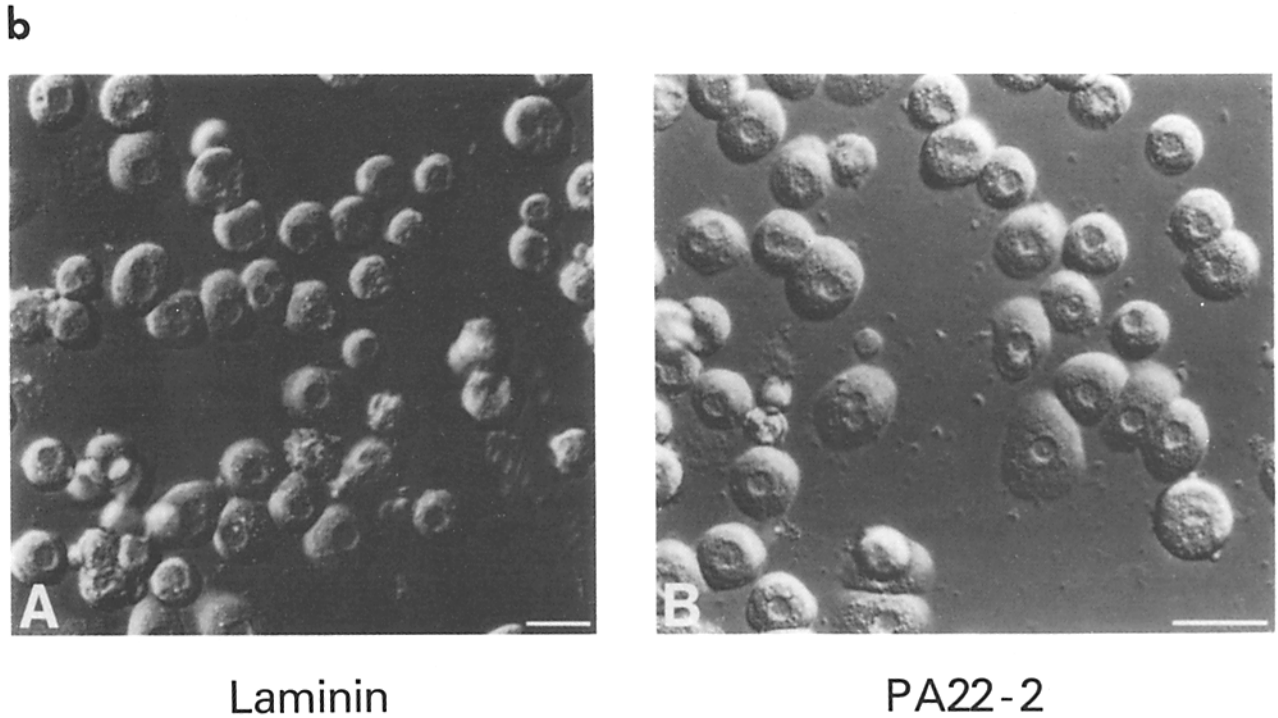


Figure 2. Cell attachment of hepatocytes to laminin and to laminin-derived peptides. (*a*) The attachment assay was carried out in duplicate as described in Materials and Methods. Activity with 50 μg/ml laminin is taken as 100%. No significant cell adhesion was observed when hepatocytes were seeded on YIGSK, a control peptide. (*b*) Hepatocytes were incubated on either laminin or peptide PA22-2 for 60 min and photographed using Hoffman modulation contrast on a Nikon inverted light microscope. Hepatocytes remained rounded on laminin and on peptide PA22-2. Bar, 20 mm.

first with an increasing linear NaCl gradient (0.25-1 M) in the presence of detergent and then with 8 M urea containing 0.15 M NaCl and 0.1 M acetic acid. Fraction activity was counted in a gamma counter, then extensively dialyzed against distilled water and dried using a Speed-Vac apparatus (Savant Instruments, Hicksville, NY). Proteins were reduced and resolved on SDS polyacrylamide gels. The gels were then dried and exposed to x-ray film. In another experiment, ^{125}I-labeled hepatocytes were homogenized in a lysis buffer, containing 25 mM octylglucoside, 0.15 M NaCl, 2 mM PMSF, 1 mM $MnCl_2$, in 0.01 M Tris-HCl, pH 7.5, and were then incubated with affinity beads. After a 2-h incubation at 4°C, the beads were packed into columns and extensively washed with the same buffer. Binding proteins were eluted in a similar buffer but lacking $MnCl_2$ and containing 20 mM EDTA.

Western Blotting

Membrane proteins eluted from affinity columns were separated by electrophoresis on a polyacrylamide gel and transferred onto Immobilon membranes. The filters were incubated for 2 h at room temperature with 50 mM Tris-HCl, pH 7.5, containing 0.15 M NaCl, 0.2% NP-40, 3% bovine albumin, and 3% nonfat dry milk. Rabbit antisera were then added in fresh buffer, containing bovine albumin and powdered milk. After 2 h, the filter was washed four times (15 min each) with the same buffer. Horseradish peroxidase-conjugated goat anti-rabbit antibodies were incubated in the presence of BSA and milk for a further 2 h. The filter was washed extensively before incubation in 4-chloro-1-naphthol/H_2O_2.

Immunocytochemistry

Freshly isolated hepatocytes were plated in M-199 medium supplemented with 10% FCS, 10 μg/ml porcine insulin, and 0.02% BSA, in 35-mm tissue-culture dishes. After adhesion, cells were washed with cold PBS at 4°C for 2 min. Hepatocytes were incubated for 1 h at 4°C in the presence of either affinity-purified FP 0.9 antisera (70 μg/ml), or preimmune sera in PBS (dilution 1:10). Cells were washed in cold PBS and incubated with peroxidase-conjugated goat anti-rabbit IgG (Institut Pasteur, Paris, France) in PBS (dilution 1:100) for 1 h at 4°C. After washing in PBS, cells were fixed in a 4% paraformaldehyde/PBS solution for 30 min at 4°C. Staining was performed with 3,3′-diaminobenzidine/H_2O_2 for 20 min.

Results

Attachment of Hepatocytes to Laminin- or Peptide-coated Substrata

We have examined the attachment of freshly isolated hepatocytes to laminin substrates. Few if any cells bound to the uncoated plastic in 1 h. Laminin promoted the attachment of the hepatocytes in a dose-dependent manner, reaching a maximum at the concentration of 40 μg/ml (Fig. 2 *a*) with 65% of the initial cells seeded adhering (an average of 2×10^4 cells per 0.32 cm² well culture plate). The hepatocytes remained round and did not spread on a laminin substrate even after 6 h (Fig. 2 *b*).

Several synthetic peptides from sequences in the laminin chains have been identified as active for the attachment of a variety of cells (Graf et al., 1987; Grant et al., 1989; Tashiro et al., 1989).[2] We tested their activity as substrates for hepatocytes. These include the peptides PA21 (Grant et al., 1989)[2] and PA22-2 from the A chain (Tashiro et al., 1989), and the cyclic YIGSR located in the cysteine-rich domain of the B1 chain of laminin (Graf et al., 1987; Fig. 1). Cells attached to plastic coated with the peptides (YIGSR, PA21, and PA22-2) in a dose-dependent manner (Fig. 2 *a*). Peptide PA22-2 was the most active with the hepatocytes, with 70% of the activity of laminin while cyclic YIGSR and PA21 showed ~50 and 40% of the activity of laminin. Hepatocytes attaching to these peptides remained rounded during the first 6 h (Fig. 2 *b*). No significant cell adhesion was observed when hepatocytes were seeded on YIGSK, a control peptide.

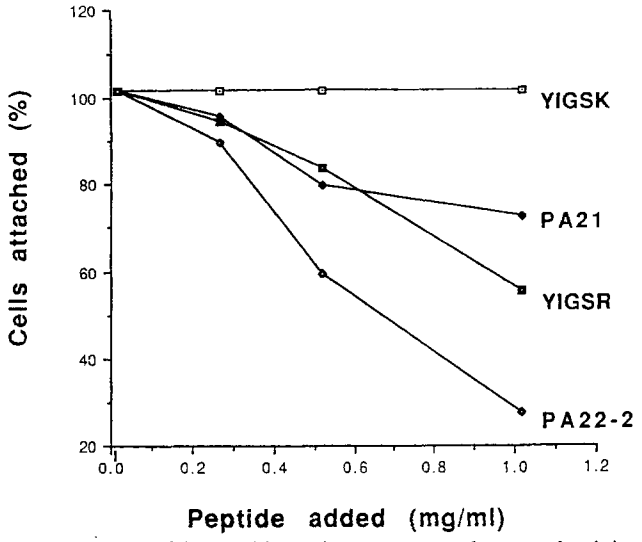


Figure 3. Effect of the peptides on hepatocyte attachment to laminin. 0.32-cm² well tissue-culture plates were coated with 2 μg of laminin per well. Before addition of hepatocytes, varying concentrations of the peptide solubilized in the medium were added to the wells.

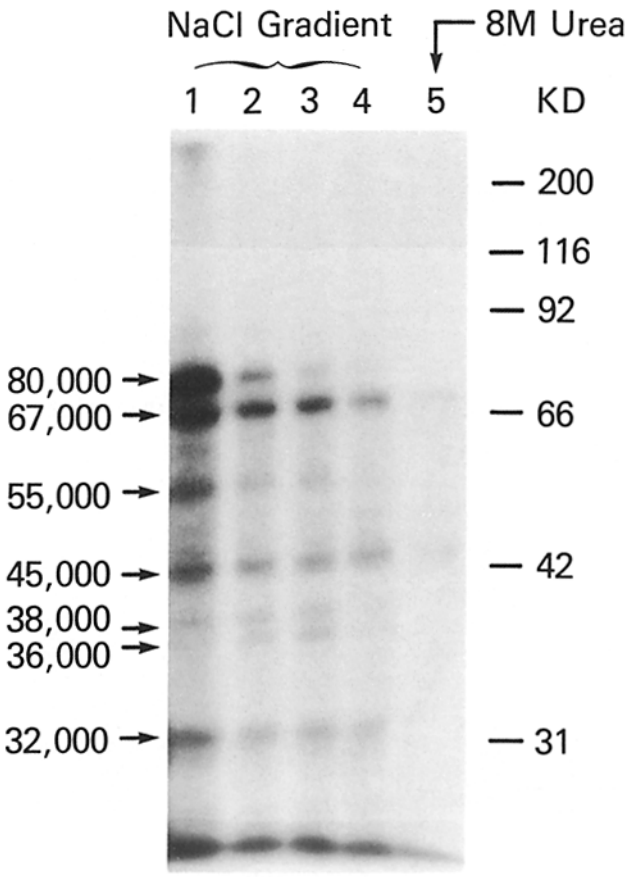


Figure 4. Laminin affinity chromatography of iodinated hepatocyte surface proteins. Iodinated membrane proteins were eluted from a laminin affinity column using a NaCl gradient and electrophoresed on a 10% polyacrylamide gel. Lanes *1–4* represent elution with 0.25-1 M NaCl with an increment of 0.25 M NaCl in each lane. Lane *5* represents elution with 8 M urea.

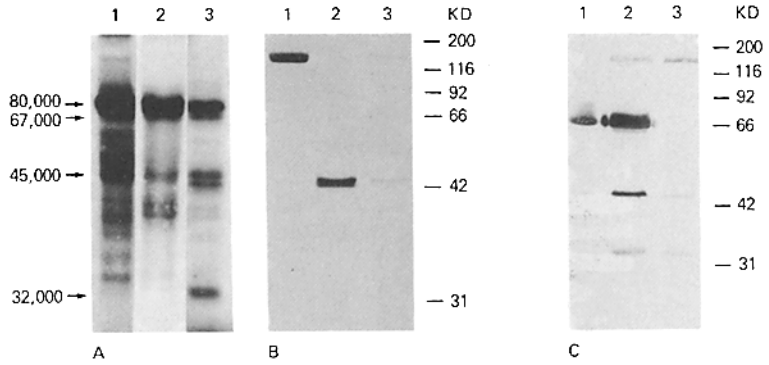


Figure 5. PA22-2 affinity chromatography of iodinated hepatocyte surface proteins. Iodinated membrane proteins eluted from PA22-2 affinity column were electrophoresed on a 10% polyacrylamide gel and transferred onto Immobilon membranes. The filters were exposed to x-ray film (*A*) and subsequently incubated with either: (*B*), antisera against a 17-mer synthetic peptide from the N-terminal region of LBP-32 (residues 25–41); or (*C*), antisera against a bacterial fusion protein coded for by β-galactosidase gene plus the entire 0.9-kb LBP-32 cDNA clone (FP 0.9) (*A*) and (*B*) are from the same filter. Lane *1*, starting material; lane *2*, bound proteins eluted with 1 M NaCl; lane *3*, bound proteins eluted with 8 M urea.

We also assessed the ability of the peptides to block laminin-mediated adhesion (Fig. 3). Wells were coated with laminin, then peptides and hepatocytes were added to the dishes. Peptide PA22-2 was the most active and inhibited the adhesion of hepatocytes on laminin by 75%. YIGSR and PA21 showed less but significant activity, with 50 and 70% inhibition, respectively. No inhibition was found when YIGSK peptide was added to the medium.

Affinity Chromatography of Detergent Extracts of Hepatocyte Membranes

Freshly isolated hepatocytes were surface-labeled with ^{125}I by the lactoperoxidase method and the ^{125}I-labeled proteins were solubilized with detergent and passed over a laminin-affinity column to identify specific membrane proteins involved in the attachment to laminin. Four major labeled proteins, M_r = 80,000, 67,000, 45,000, and 32,000, were eluted by the NaCl gradient (Fig. 4). Other proteins, M_r = 55,000, 38,000, and 36,000, were observed to bind but these were less abundant than the major binding proteins. 8 M urea eluted mainly the 67- and 45-kD proteins.

Identification of Binding Proteins for the Peptide PA22-2

Although the 19-mer peptide PA22-2 from the long arm of the A chain is known to be active in the attachment of a variety of cells (Tashiro et al., 1989), it is not known which cell surface receptor is involved. To identify the surface proteins that bind PA22-2, ^{125}I-labeled purified cell membranes were solubilized in detergent and passed over a PA22-2-affinity column. Eluted proteins were resolved by PAGE and transferred onto Immobilon filters (Millipore Corp., Bedford, MA) that were exposed to x-ray films (Fig. 5 *a*). The fraction eluted with 1 M NaCl contained four major iodinated proteins (M_r = 80,000; 67,000; 45,000; 36,000). Subsequently, 8 M urea eluted more of these proteins plus a M_r = 32,000 protein. The filters were subsequently incubated with antisera against a synthetic peptide from the NH_2-terminal part of LBP-32. Only a 150-kD protein was stained in the starting material, whereas the 1-M NaCl and 8-M urea fractions contained mainly the 45-kD protein (Fig. 5 *b*). Overloading of the gel was necessary to reveal the 32-kD protein (data not shown).

When the proteins in the 1-M NaCl eluate from the PA22-2-affinity column were incubated with antisera against the entire LBP-32-fusion protein, four bands (M_r = 150,000, 67,000, 45,000, and 32,000) were stained in the fraction eluted with 1 M NaCl, with the M_r = 67,000 being the most abundant (Fig. 5 *c*, lane *2*). The 8-M urea fraction lacked the 67-kD protein but contained the others. Although the 150-kD protein was weakly stained, this protein was not

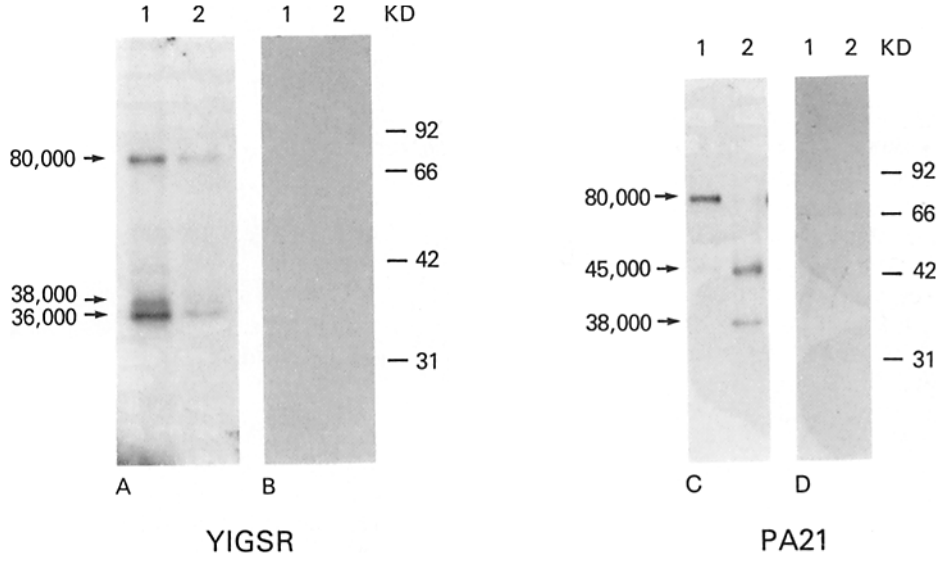


Figure 6. YIGSR and PA21 affinity chromatography of iodinated hepatocyte surface proteins. Iodinated membrane proteins eluted from YIGSR (Fig. 5, *A* and *B*) or PA21 (Fig. 5, *C* and *D*) affinity columns were electrophoresed on a 10% polyacrylamide gel and transferred onto Immobilon membranes. The filters were exposed to x-ray films (Fig. 5, *A* and *C*) and subsequently incubated with antisera against a 17-mer synthetic peptide from the NH_2-terminal region of LBP-32 (residues 25–41) (Fig. 5, *B* and *C*). *A* and *B* and *C* and *D* are, respectively, from the same filters. Lane *1*, bound proteins eluted with 1 M NaCl; lane *2*, bound proteins eluted with 8 M urea.

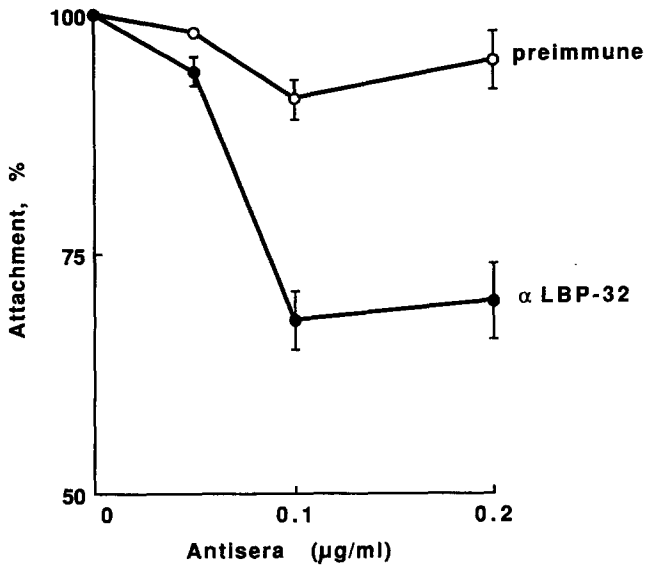


Figure 7. Inhibition of hepatocyte attachment by antisera against a bacterial fusion protein coded for by β-galactosidase gene plus the entire 0.9-kb LBP-32 cDNA clone (FP 0.9). Tissue-culture plates were coated with laminin (20 μg/ml). Various amounts of affinity-purified antisera were mixed with the cells for 15 min before seeding. Control cells were first incubated with pre-immune serum, then assayed for their adhesion on laminin. Each assay was carried out in duplicate in three independent experiments.

detectable by autoradiography (Fig. 5 *a*). The 67-kD protein appeared weakly radiolabeled on a 6% polyacrylamide gel, which allowed a better separation from the strongly radiolabeled 80-kD protein (data not shown). The 32- and 45-kD protein were both immunostained and radiolabeled. The iodinated 80-kD protein was not stained by the antisera. These observations suggest that the 150- and 67-kD proteins are localized mainly intracellularly and not extracellularly. We conclude that peptide PA22-2 binds to multiple proteins, including the 80- and the related 67-, 45-, and 32-kD proteins that share a common epitope.

Identification of Binding Proteins for Peptides YIGSR and PA21

Two other cell-binding peptides, YIGSR from the B1 chain and PA21 from the A chain, were also examined for their interaction with cell-surface receptors. Three major ^{125}I-labeled membrane proteins (M_r = 80,000, 36,000, and 38,000) were eluted from a YIGSR-column with either 1 M NaCl or 8 M urea (Fig. 6 *a*). These proteins were not stained with the antisera to either FP0.9 (data not shown) or the 17-mer synthetic peptide from the N-terminal region of LBP-32 (Fig. 6 *b*), indicating that they were distinct from the LBP-32 family. 1 M NaCl eluted mainly an 80-kD protein, and 8 M urea eluted two proteins (M_r = 45,000 and 38,000) from a PA21-column (Fig. 6 *c*). Filters were negative when incubated with either antibodies to the LBP-32 (Fig. 6 *d*). However, because the 45-kD protein was detected only after autoradiography for 48 h, it is possible that it was present in too small amounts to be detected by Western blotting.

Because PA-21 contains an RGD sequence, it is expected that integrin may interact with this peptide (Ruoslahti and Pierschbacher, 1986). Radiolabeled membrane proteins were prepared in the presence of $MnCl_2$, then passed over columns made with either laminin, PA22-2, YIGSR, or PA21. EDTA eluted a major M_r = 80,000 protein from these columns (data not shown). Overloading of the gels and long-time exposure (up to 1 wk) allowed the detection of minor proteins with integrin-like migration behavior in SDS gels (M_r = 200,000, 180,000, 95,000, and 90,000) (data not shown). This suggests that integrin(s), if any, are not primarily involved in the attachment of hepatocytes to laminin.

Inhibition of Hepatocyte Attachment by the LBP-32 Antisera

To confirm that the protein family of LBP-32 is involved in attachment of hepatocytes to laminin, the cells were incubated with the affinity-purified antisera to the LBP-32-fusion protein, then assayed for their attachment on laminin substrate (Fig. 7). Attachment of hepatocytes was inhibited in a dose-dependent manner by this protein. At a concentration of 0.15 mg/ml, the antisera inhibited the adhesion of hepatocytes by 30% when compared with cells first incubated with preimmune serum. Because the adhesion of hepatocytes was inhibited by the antisera by only 30%, it is likely that these cells use additional receptors not detected by this antisera to adhere to laminin.

Immunocytochemistry

Indirect immunoperoxidase microscopy studies were performed to examine cell surface localization of proteins that react with affinity-purified antisera to the LBP-32-fusion protein. Incubation of unfixed hepatocytes with FP 0.9 affinity-purified antisera allowed the localization of antigens on the surface of the cells (Fig. 8 *A*). Faint and punctuated staining was found around all the hepatocytes. Cells incubated with preimmune sera were negative (Fig. 8 *B*).

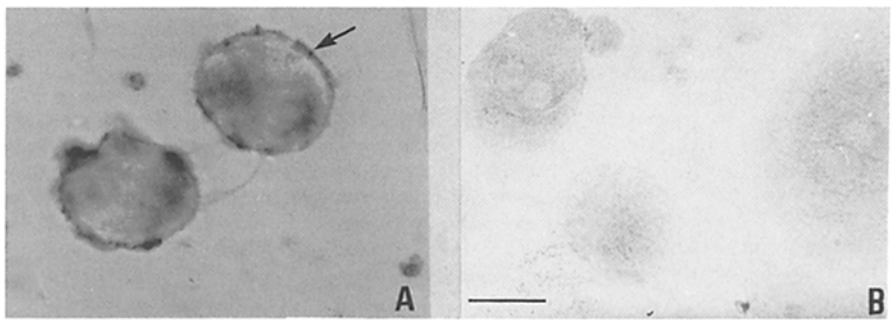


Figure 8. Immunoperoxidase localization of laminin-binding proteins on hepatocytes. Cells were plated on tissue culture dishes for 6 h. Unfixed hepatocytes were incubated in the presence of affinity-purified FP 0.9 antisera (*A*) or preimmune sera (*B*), and then a peroxidase-conjugated goat anti-rabbit IgG. Staining is present on the plasma membrane of the cells (*arrows*). Bar, 10 μm.

Discussion

Hepatocytes adhere in vitro to the major constituents of extracellular matrix, including collagens, fibronectin, and basement membrane proteoglycan, through specific cell surface binding proteins (Rubin et al., 1986; Johansson et al., 1981, 1987; Clément et al., 1989; Bissell et al., 1986). Previously, Timpl et al. (1983*b*) used protein fragments of laminin to localize the interaction of hepatocytes to the disulfide-bonded center of the cross-shaped molecule and its short arm. Those fragments would include two of the cell attachment sites studied here. Using synthetic peptides, we found that different sites on the A and B1 chains of laminin are responsible for the attachment of hepatocytes. These include the pentapeptide YIGSR in the cysteine-rich domain of the B1 chain of laminin (Graf et al., 1987) and the PA21 containing RGD sequence in the A chain which is active in promoting the adhesion of a variety of cells (Grant et al., 1989).[2] In addition, we found that hepatocytes bind even more strongly to a peptide from a sequence in the A chain (PA22-2) close to the carboxyl end of the helical domain of the long arm. Cell attachment activity was previously found in proteolytic fragments containing the PA22-2 sequence (Aumailley et al., 1987). The region of the molecule also promotes neurite outgrowth of neuronal cells as does the synthetic peptide PA22-2 (Goodman et al., 1987; Edgar et al., 1984; Sephel et al., 1989). Thus, hepatocytes adhere to laminin using different attachment sites, suggesting that several specific binding-proteins are involved in this process.

Using affinity chromatography, we found that hepatocytes bind laminin strongly through two groups of cell-surface proteins. The first group of receptors (M_r = 67,000, 45,000, and 32,000) has been described in other cells including mouse melanoma cells[3] and mouse macrophages (Mercurio and Shaw, 1988). These proteins are immunologically related but their exact relationship is not known. We found that in the case of hepatocytes, this receptor family binds mainly the PA22-2 peptide. Antisera prepared against a fusion protein expressed by bacteria containing β-galactosidase plus the entire LBP-32 c-DNA sequence recognize these proteins in plasma membrane extracts. However, antisera against the NH_2-terminal end of LBP-32 recognized mainly the 45- and the 32-kD proteins, plus a 150-kD intracellular protein. Both the 32- and 45-kD proteins were heavily iodinated, suggesting that they are both exposed to the extracellular milieu.

The adhesion of hepatocytes to laminin is inhibited by only 30% by anti-LBP-32 affinity-purified antibodies suggesting the possibility that other cell surface proteins are likely to be involved in this process. Indeed, we found a second group of proteins (i.e., 80, 55, 38, and 36 kD) that bind laminin. The 80-kD protein appeared to be a major binding protein for the adhesion of hepatocytes to laminin, to all the laminin peptide listed and to collagen IV (Clément et al., manuscript in preparation). The 38- and 36-kD proteins bind laminin through the YIGSR site, whereas the 38- and 45-kD proteins recognize the PA21 sequence.

Several other laminin receptors have been described including the integrin family (Gehlsen et al., 1988; Hemler et al., 1988; Ignatius and Reichard, 1988; Tomaselli et al., 1988). The PA21 peptide in the A chain of laminin contains a RGD sequence and it is possible that hepatocytes bind to laminin through an integrin as recently shown in glioblastoma cells (Gehlsen et al., 1988; Sonnenberg et al., 1988). We have indeed detected proteins that bind laminin in a divalent cation-dependent manner in the iodinated cell surface proteins eluted from the peptide- or laminin-affinity columns. Because these proteins were not easily detectable, it is likely that integrin(s) are minor binding proteins for the attachment of hepatocytes to laminin. Clearly integrins are involved in fibronectin-mediated attachment of hepatocytes (Johansson et al., 1987).

From these data, we conclude that hepatocytes bind to laminin through several sites mainly on the A chain, using multiple receptors. That adult normal liver contains mainly the B1 and B2 chains of laminin (Kleinman et al., 1987; Clément et al., 1988) may explain the low amount of laminin normally deposited in the space of Disse, at the contact of hepatocytes. Whether accumulation of laminin in the space of Disse during embryogenesis (Rescan et al., 1989) or fibrosis (Albrechtsen et al., 1988; Bianchi et al., 1986; Clément et al., 1988) is correlated with changes in the expression of the different chains of laminin and laminin receptor(s) remains to be elucidated.

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