

# A Plasma Membrane Protein Is Involved in Cell Contact-mediated Regulation of Tissue-specific Genes in Adult Hepatocytes

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**Abstract.** We have identified the liver-regulating protein (LRP), a cell surface protein involved in the maintenance of hepatocyte differentiation when cocultured with rat liver epithelial cells (RLEC). LRP was defined by immunoreactivity to a monoclonal antibody (mAb L8) prepared from RLEC. mAb L8 specifically detected two polypeptides of 85 and 73 kD in immunoprecipitation of both hepatocyte- and RLEC-iodinated plasma membranes. The involvement of these polypeptides, which are integral membrane proteins, in cell interaction-mediated regulation of hepatocytes was assessed by evaluating the perturbing effects of the antibody on cocultures with RLEC. Several parameters characteristic of differentiated hepatocytes were studied, such as liver-specific and house-keeping

gene expression, cytoskeletal organization and deposition of extracellular matrix (ECM). An early cytoskeletal disturbance was evidenced and a marked alteration of hepatocyte functional capacity was observed in the presence of the antibody, together with a loss of ECM deposition. By contrast, cell-cell aggregation or cell adhesion to various extracellular matrix components were not affected. These findings suggest that LRP is distinct from an extracellular matrix receptor. The fact that early addition of mAb L8 during cell contact establishment was necessary to be effective may indicate that LRP is a novel plasma membrane protein that plays an early pivotal role in the coordinated metabolic changes which lead to the differentiated phenotype of mature hepatocytes.

CELL-cell interactions are of fundamental importance in the development and organization of multicellular organisms and in their physiology and pathology. The general assumption is that these interactions are mediated by cell adhesion molecules, substrate adhesion molecules, and cell junctional molecules. All are morpho-regulatory molecules during the early stages of development and permissively regulate primary processes such as cell movement and division. They give rise to the signals which lead to differential gene expression and thus to embryonic induction. Various histogenetic examples support this proposal (Gallin et al., 1986; Edelman, 1987; Jessel, 1988). However, the molecular mechanisms involved in these coordinated regulations are still poorly understood.

Cell-cell communications also play a very important role in maintaining the differential gene expression of the mature cells of adult tissues. Pertinent examples are provided by the central and peripheral nervous systems (Rathjen et al., 1987; Seilheimer et al., 1989). An interesting example is also provided by the adult liver. Disrupting liver tissue to isolate hepatocytes leads to a decline in the transcription of most liver-specific genes (Clayton et al., 1985). Furthermore, establishment in culture of homotypic interactions between hepatocytes fails to preserve their adult phenotype (Jefferson et al., 1984), while proteoglycans induce gap junction ex-

pression and restore transcription of tissue-specific mRNAs in primary cultures (Fujita et al., 1987). This suggested a potential role for nonparenchymal cells on the differential transcription of liver-specific genes in adult hepatocytes.

Different nonparenchymal epithelial cell lines have been established from both neonatal and adult rat livers that show phenotypic similarities to oval cells (Grisham, 1980; Fausto et al., 1987). Moreover, they can express some of the functions characteristic of hepatocytes (Germain et al., 1988). The exact origin of these cells in the liver is still incompletely solved. However, according to Fausto (1990) they can be defined as a population of stem-like cells, most likely originating from bile ductules, that have retained their broad developmental capacities. Several cell lines (rat liver epithelial cell [RLEC]<sup>1</sup>) with similar characteristics were obtained in our laboratory (Morel-Chany et al., 1978). We have shown that the establishment of cell-cell contacts between adult hepatocytes and these nonparenchymal epithelial cells, allows hepatocytes to restore cell polarity and to maintain their functional activities for several weeks (Guguen-Guillouzo et al., 1983). An increased expression of liver-specific

1. *Abbreviations used in this paper:* CAM, cell adhesion molecule; ECM, Extracellular matrix; EHS, Engelbreth-Holm-Swarm mouse sarcoma tumor; LRP, liver regulating protein; RLEC, rat liver epithelial cell.

genes was observed, resulting, at least in part, from activation of transcription (Fraslin et al., 1985). In addition, secretion and deposition of various matrix components gave rise to a complex extracellular matrix (ECM) network which surrounded and covered hepatocyte colonies. Interestingly, both liver-specific gene activation and matrix deposition occurred in a coordinated manner (Baffet et al., 1982). Furthermore, it has been established that cell-cell contacts with RLEC, but not via gap-junction communications, are required while soluble factors did not seem to be involved to any major extent (Mesnil et al., 1987). Therefore, this hepatocyte coculture system represents a useful model to study the causal relationship between the different biological events leading to stable differentiation in adult liver.

In the present study, we have explored the possibility that a plasma membrane protein expressed on RLEC might play a critical role in mediating cell-cell recognition and leading to increased tissue-specific gene transcription. We found that a mAb directed against an RLEC cell surface protein inhibited liver-specific gene activation when added early to hepatocyte-RLEC cocultures, and concomitantly altered matrix deposition and cytoskeleton organization in hepatocytes. This report describes in some detail this biological phenomenon and analyzes its specificity. It presents evidence for the involvement of a novel plasma membrane liver-regulating protein (LRP).

## Materials and Methods

### Reagents

Laminin-entactin complex, laminin, heparan-sulfate proteoglycan, and collagen IV were extracted from the Engelbreth-Holm-Swarm mouse sarcoma tumor (EHS) (Kleinman et al., 1982) according to Timpl et al. (1979) with slight modifications (Hassel et al., 1985). Fibronectin was from Boehringer Mannheim (Meylan, France).

Hybridization probes for mRNA analysis were as follows: pRSA8 for albumin gene (Sargent et al., 1979), A4C9 for aldolase B gene (Simon et al., 1983) and procollagen  $\alpha_1(I)$  (Clavel et al., 1989).

Anticytokeratin 8 and 18 antibodies (anti-CK 55 and anti-CK 49) of known specificity (Leroux-Nicollet et al., 1983) were a gift of Dr. N. Marceau (Hotel Dieu, Quebec).

### Cell Isolation and Culture

Adult normal hepatocytes were obtained by perfusing rat livers (Sprague-Dawley; 150–200 g) with 0.025% collagenase solution (Boehringer Mannheim) buffered with 0.1 M Hepes (pH 7.4) according to the method of Seglen (1973) with slight modifications (Guguen et al., 1975).

Adult hepatocytes were maintained either in pure culture or in coculture. For pure culture, hepatocytes were plated in a mixture of 75% MEM and 25% medium 199, supplemented with 10% FCS and containing per ml: penicillin (100 IU), streptomycin sulfate (100  $\mu$ g), bovine insulin (5  $\mu$ g), and BSA (1 mg). The medium, added with  $7 \times 10^{-5}$  M hydrocortisone hemisuccinate, was renewed 4 h later and every day thereafter. In some assays, pure cultures of hepatocytes were maintained in a medium containing 25 mM nicotinamide (Paine et al., 1979).

Cocultures were prepared according to the conditions previously described (Guguen-Guillouzo et al., 1983). Briefly, 4 h after hepatocyte seeding, the medium was discarded and nontransformed RLEC or other cell types, suspended in a fresh medium, were added. 24 h later, the medium was supplemented with  $7 \times 10^{-5}$  M hydrocortisone hemisuccinate and renewed every day thereafter.

Mouse 3T3 fibroblasts (Rheinwald et al., 1975), bovine corneal endothelial cells (Gospodarowicz et al., 1979), human skin fibroblasts, and rat liver myofibroblasts were maintained in the serum-supplemented medium described above, without insulin, corticosteroids, and albumin. Two RLEC cell lines (SDIII, SDVI) were established from the liver of 10-d-old Sprague-Dawley rats in our laboratory, according to the procedure of Williams et al. (1971) and used between passages 10 and 30. In addition,

a woodchuck liver epithelial cell line (WLEC) was obtained from an imported eastern American woodchuck. RLEC, WLEC, and bovine lens epithelial cells (Arruti and Courtois, 1978) were grown in the Williams' E medium supplemented with 10% FCS.

For RNA analysis, hepatocytes from cocultures were selectively separated from RLEC by incubation for 10 min with a calcium-free collagenase solution (0.05%, pH 7.6) buffered with 0.1 M Hepes (Fraslin et al., 1985). Hepatocytes that were more sensitive to low concentration of  $Ca^{++}$  became rounded and detached in clumps, whereas RLEC remained well spread.

### Production and Selection of mAbs

Balb/c mice were immunized with a cell suspension of  $10^7$  live RLEC, and spleen cells were fused with the SP<sub>2</sub>/O-Ag 14 myeloma cells (Bourel et al., 1984). Culture supernatants were screened for the production of an Ig by using an indirect immunofluorescent assay on both RLEC and hepatocytes. Subsequently, the positive clones were selected according to both their ability to recognize a plasma membrane protein and their inability to react with nonhepatic cells, e.g., human skin fibroblasts and bovine corneal endothelial cells. Cell suspensions were fixed in a 4% paraformaldehyde solution buffered with 0.1 M sodium cacodylate (pH 7.5) for 15 min at 4°C.

The ability of mAbs to modify the functional capacities of hepatocytes in coculture was checked by adding 100, 200, and 500  $\mu$ l of supernatants from the previously selected hybridoma clones to the cocultures upon RLEC seeding, and then every day at each medium renewal. Viability of hepatocytes was tested and albumin secretion was measured after 5 or 6 d.

In some assays, euglobulin-precipitated IgM L8 prepared by dialyzing 2 ml of ascitic fluid against distilled water at pH 5 was used as described elsewhere (Goding, 1986; Garcia-Gonzalez et al., 1988).

Albumin secretion rate was quantified in the culture medium by laser immunonephelometry (Lescoat et al., 1985).

### Indirect Immunolocalization

Hepatocyte pure cultures and cocultures were fixed in a 4% paraformaldehyde solution buffered with sodium cacodylate 0.1 M (pH 7.4) for 30 min at 4°C. mAbs were localized using either the indirect immunoperoxidase or immunofluorescence techniques (Guillouzo et al., 1982). Incubations were carried out with or without 0.2% saponin. Second antibody was a peroxidase-labeled anti-mouse IgM (Nordic Immunological Laboratories, Tilburg, The Netherlands) or an FITC anti-mouse Ig (Diagnostics Pasteur, Marnes-la-Coquette, France).

For EM, cells were postfixed with 2.5% glutaraldehyde and subsequently incubated for 30 min in 1% osmium solution in 0.1 M cacodylate buffer. The cells were then dehydrated in graded ethanol and embedded in Epon.

### ECM Deposition

The reticulin staining by silver impregnation of the extracellular matrix, was carried out according to the method of Gordon and Sweets (1936) in cocultures fixed with a mixture of 4% paraformaldehyde and 2.5% glutaraldehyde in cacodylate buffer (pH 7.4) for 15 min at 4°C.

### Time-lapse Cinephotomicroscopy

A 1-d-old coculture, grown on a glass coverslip coated with fibronectin was mounted in a Rose chamber. The chamber was put onto the microscope stage 90 min after epithelial cell seeding and under a controlled constant temperature of 37°C. Cell behavior was monitored with a Zeiss ICM 405 microscope equipped with Normarsky differential interference contrast, a Zeiss objective 100  $\times$  NA 1.25 plan and a 16-mm Arriflex camera. The time-lapse device gave an impulse every 2 s, and a photomicrograph was taken with Agfa copex pan rapid film.

### Cell-Cell and Cell-Substratum Adhesion Assays

The effect of mAb L8 on hepatocyte attachment to RLEC was examined. Dishes of 3.5 cm in diameter covered by a confluent RLEC monolayer were preincubated with 2  $\mu$ l of either SP<sub>2</sub>/O-Ag or L8 ascitic fluid per ml of serum-free medium for 1 h. Freshly isolated hepatocytes ( $10^6$  cells) were then seeded and maintained at 37°C in the same medium containing SP<sub>2</sub>/O-Ag or mAb L8. Media of vials in duplicate were harvested at 2, 3, 4, 7, and 22 h, and the number of unattached hepatocytes was estimated by measuring the lactate dehydrogenase activity after cell lysis with PBS containing 0.2% Triton X-100 (Rubin et al., 1986).

The effect of mAb L8 on hepatocyte attachment to various substrata in-

cluding EHS gel, fibronectin, collagen IV, laminin, laminin-ectactin complex, and heparan-sulfate proteoglycan, was also examined. 2  $\mu$ g of protein were coated on 0.32-cm<sup>2</sup> well tissue culture plates containing 100  $\mu$ l of serum-free medium. After 2 h, 3% BSA was added to a final concentration of 1.5% for a further 30 min. Medium was removed and hepatocytes previously incubated for 30 min in a serum-free medium containing increasing amounts of mAb L8 or SP<sub>2</sub>/O-Ag ascitic fluid and 0.02% BSA were seeded. After 1 h, the plates were gently washed twice with PBS. The number of attached and unattached hepatocytes was measured as described above.

### Extraction of RNA and Northern Blot Hybridization

Total RNA was prepared from hepatocytes, using 5 M guanidium thiocyanate/CsCl technique (Chirgwin et al., 1979).

Total RNA (20  $\mu$ g) was resolved by electrophoresis and transferred onto a nitrocellulose filter (Thomas, 1980). The filter was prehybridized according to Andrews et al. (1982) and hybridized with  $3 \times 10^6$  cpm/ml of <sup>32</sup>P nick-translated cDNA probe for 18 h at 65°C.

### In Vitro Translation of mRNA

Total RNA (5  $\mu$ g) was added to 25  $\mu$ l of rabbit nuclease-treated reticulocyte lysate (Pelham and Jackson, 1976) containing 50  $\mu$ Ci of [<sup>35</sup>S]-methionine (Amersham, Les Ulis, France). Translation was carried out 2 h at 30°C. TCA-precipitable material ( $4 \times 10^5$  cpm) was run on SDS-PAGE.

### Labeling of Plasma Membrane Proteins

Radioiodination of cell surface proteins was performed according to a procedure described elsewhere (Clément et al., 1989) with some modifications. Monolayer cultures (150-cm<sup>2</sup> Petri dishes containing  $18 \times 10^6$  hepatocytes and  $50 \times 10^6$  epithelial or endothelial cells) were washed three times with cold PBS and labeled at room temperature with 3 ml of PBS containing 2 mCi carrier-free Na<sup>125</sup>I (Amersham) by lactoperoxidase-catalyzed iodination.

### Plasma Membrane Protein Extraction

Cells were washed three times with PBS, scraped off the Petri dish, pelleted in the microfuge at 6,500 rpm for 10 s and solubilized in either 1% Triton X-100, 0.01% SDS, 2 mM EDTA, 130 mM NaCl, 10 mM Tris-HCl (pH 7.4), or in 1% Triton X-114, 130 mM NaCl, 10 mM Tris-HCl (pH 7.4), by passing five times through a G26 needle with a syringe and maintained 30 min at 4°C. Two protease inhibitors, aprotinin (100 IU/ml) and phenylmethylsulfoxide (2 mM), were included in all buffers. Cell lysates were then centrifuged for 10 min at 13,000 rpm.

When extracted in Triton X-114, the soluble material was submitted to phase separation according to Bordier (1981), and then the two phases were adjusted by adding either Triton X-114 or the buffer in order to obtain the same salt and surfactant contents in the different samples for immunoprecipitation.

### Immunoprecipitation

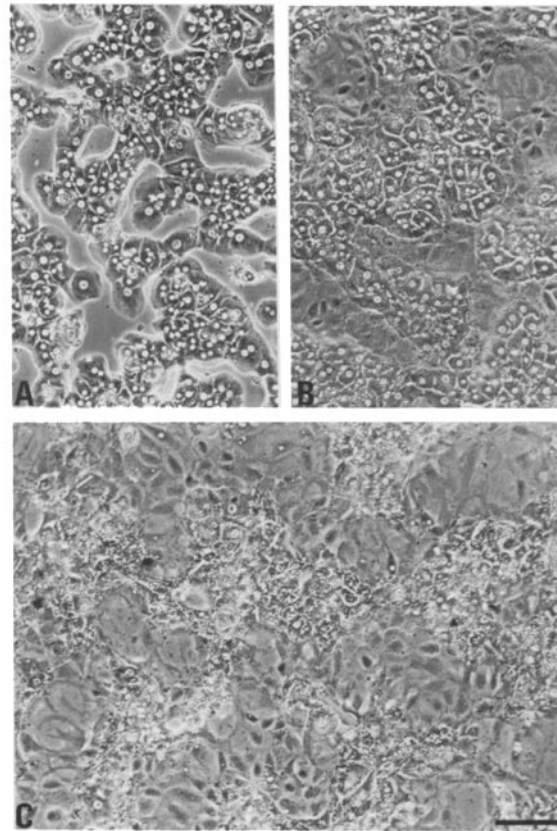
Lysates were incubated 90 min with 3  $\mu$ l of ascitic fluid. To eliminate aspecific boundaries, ascitic fluid was treated by protein A-Sepharose before use. Subsequently, the samples were incubated with goat anti-mouse IgM (Nordic Immunological Laboratories) followed by protein A-Sepharose (Pharmacia, St Quentin-Les-Yvelines, France). The affinity beads were washed with lysis buffer and bound material was eluted with 100  $\mu$ l of sample buffer according to Laemmli (1970) for molecular weight determination or with 100  $\mu$ l of lysis buffer according to O'Farrell (1975) for pI determination. SDS-PAGE electrophoresis was performed in 4–15% or 7.5–15% polyacrylamide linear gradient slabs in a Laemmli buffer system. IEF was performed with Ampholines pH 3–10 (Pharmacia) in O'Farrell buffer system.

Gels were stained by Coomassie blue, dried, and autoradiographed using Kodak X-Omat AR films.

## Results

### Selection of a mAb (mAb L8) Able to Alter the Hepatocyte-differentiated Phenotype in Coculture

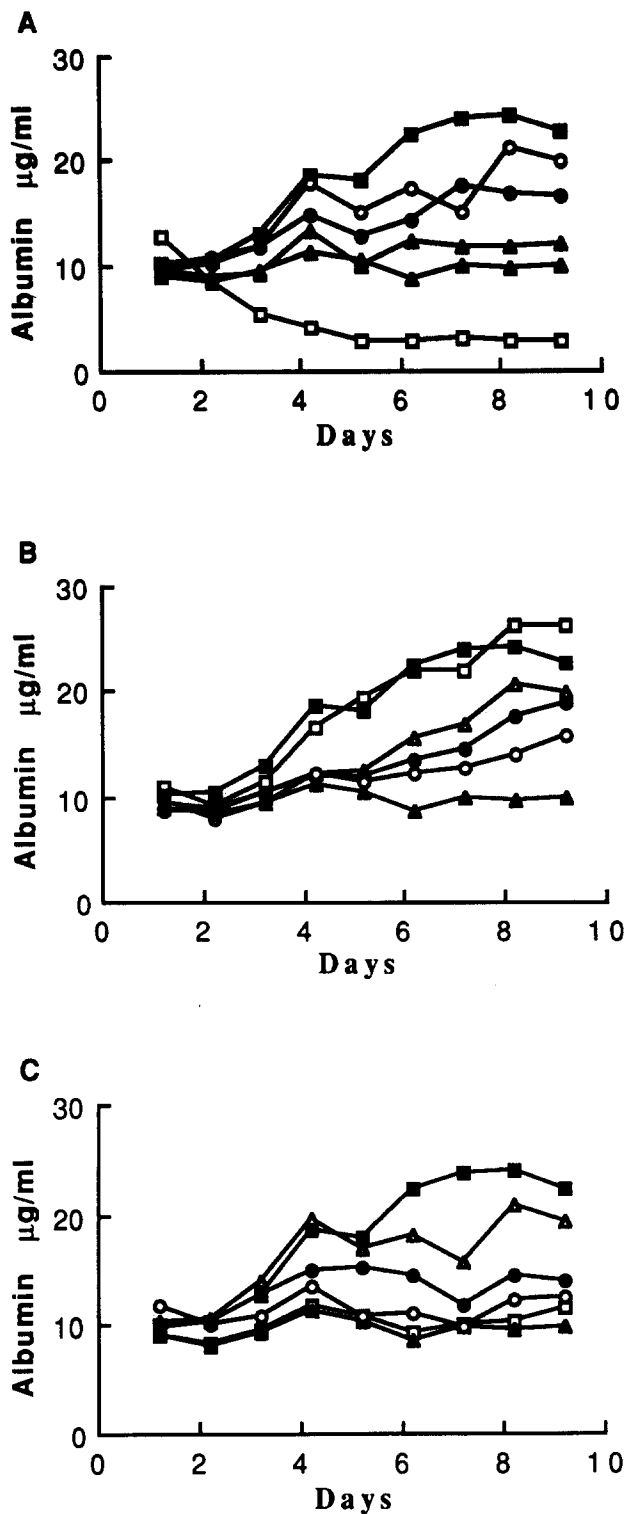
To identify a cell surface protein involved in the interactions



**Figure 1.** Specific effect of mAb L8 on the survival of rat hepatocytes cultured with RLEC. Phase-contrast micrographs of (A) 2-d-old hepatocyte pure culture treated with L8 hybridoma supernatant; (B) 5-d-old hepatocyte coculture; (C) 5-d-old hepatocyte coculture treated with L8 hybridoma supernatant. mAb L8, 500  $\mu$ l per ml of medium, was added upon starting the culture and then every day at each medium renewal. Note that hepatocytes died in coculture with mAb L8, whereas RLEC remained alive and well spread. Bar, 50  $\mu$ m.

of hepatocytes with RLEC in coculture, mAbs were generated after five different fusions, using live RLEC as immunogen. Hybridoma culture supernatants were first screened for positive immunofluorescence with freshly isolated hepatocytes and RLEC. From 400 positive hybridoma cultures, 95% were positive with RLEC and hepatocytes and 5% were positive only with RLEC. Antibodies from both groups were selected according to their ability to bind antigens located on plasma membranes. mAbs that reacted with human skin fibroblasts and bovine corneal endothelial cells were discarded. Finally, 24 hybridomas actively secreting liver plasma membrane antibodies were selectively cloned and grown for further analysis.

The ability of mAbs to inhibit the coculture effect was tested by adding every day for one week increasing concentrations of hybridoma culture supernatants into the media of hepatocyte pure cultures and cocultures. Only mAb L8 was able to markedly reduce the survival of hepatocytes in cocultures. By day 3, the typical cuboidal shape of hepatocytes maintained in cocultures was not observed in the presence of this antibody. Most hepatocytes died after 5 or 6 d as in pure cultures, whereas RLECs were unaffected (Fig. 1). No effect on hepatocyte viability was detected with time in pure cultures. These results indicated that mAb L8 did not induce



**Figure 2.** Effect of mAb L8 on serum albumin secretion by hepatocytes in coculture under the following conditions. (A) The antibody was added at different concentrations. Serum albumin was measured in the medium collected daily from pure cultures (□) and cocultures (■) maintained without mAb L8, as controls, and from cocultures added with 2.5 (○), 5 (●), 10 (△), and 20 µg/ml (▲) of partially purified mAb L8. Antibody was added upon RLEC seeding and every day at each medium renewal. Serum albumin was measured by immunonephelometry. (B) The antibody was removed early or late from the coculture. Serum albumin was measured in the medium from cocultures maintained without mAb

toxic effects, but might alter cell-cell interactions between hepatocytes and RLEC.

The capacity of the cells to secrete albumin was investigated in cultures treated daily with mAb L8. While highly maintained in untreated cocultures, albumin secretion dropped in those to which 10 or 20 µg of partially purified antibody per ml of medium had been added (Fig. 2 A). The remaining production observed in mAb L8-treated coculture could correspond to a release of the protein from cells that did not detach as fast as in pure cultures or resulted from another hepatocyte regulating process.

mAb L8 was characterized as an IgM by the Ouchterlony technique. The effects of mAb L8 on hepatocyte cocultures were verified to be unrelated to the globulin isotype by treating the cocultures with the supernatant of another hybridoma selected for its ability to secrete mAb of the same isotype (data not shown).

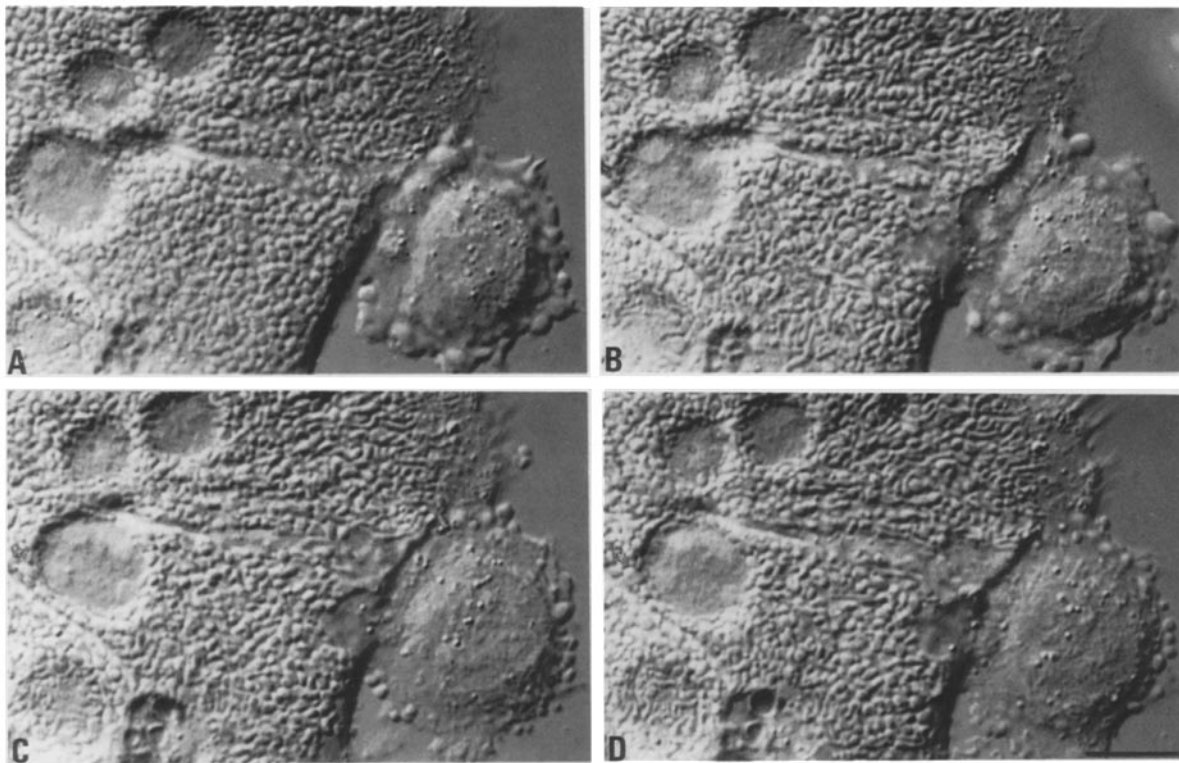
#### *The mAb L8-related Effect Takes Place during Cell Contacts Establishment*

To determine the optimal time of mAb L8 addition that affects hepatocyte cocultures, two experiments were performed. Firstly, mAb L8 was added at the onset of cell seeding and the treatment was stopped 1, 2, 3, or 4 d thereafter (Fig. 2 B). Secondly, the coculture was initiated without antibody and the treatment was begun only after 1, 2, or 3 d (Fig. 2 C). The mAb L8 effect was evaluated by measuring the capacity of hepatocytes to survive and to secrete albumin in the medium. We found that suppression of mAb L8 treatment by day 1, as well as late addition of antibody at day 3, had no significant influence on cell viability and albumin production. An inhibitory effect was only evidenced when the antibody was present between days 1 and 2 after RLEC seeding, corresponding to the establishment of cell-cell contacts. The first day lag-phase represented the time needed by RLECs to attach, grow, and reach confluency with hepatocyte colonies.

The effect of mAb L8 appeared fully reversible when the coculture was treated for 1 d (Fig. 2 B). However, the albumin secretion levels did not completely reach those of untreated cocultures after 2 d or more of mAb L8 treatment, even when the cells were allowed to recover for up to 12 d. This correlated with previous data showing the existence of a critical state between days 3 and 4, beyond which initiation of coculture was no longer possible (Fraslin et al., 1985).

In addition, microcinematography was performed to explore the hepatocyte behavior during establishment of cell contacts with RLEC. A 1-h film of a liver epithelial cell making contact with hepatocytes revealed that a flat and clear

L8 as control (■) and from cocultures added with 20 µg/ml of partially purified mAb L8 at RLEC seeding and removed at days 1 (□), 2 (△), 3 (●), or 4 (○), or maintained during all the culture time (▲). (C) The antibody was added early or late to the coculture. Serum albumin was measured in the medium from cocultures without mAb L8 as control (■), and from cocultures to which 20 µg/ml of partially purified mAb L8 were added either upon hepatocytes seeding (▲) or RLEC seeding (□), or at day 1 (○), day 2 (●), or day 3 (△), and every day thereafter.



**Figure 3.** Normarsky micrographs of hepatocytes when establishing contacts with RLEC. One epithelial cell just establishes contact with hepatocytes (A). A flat and clear zone at the contact site started to form within 20 min (B) and was wider and more evident 50 min (C) and 60 min later (D). Note that cytoplasmic organelles gradually rearranged more radially toward the contacting site. Bar, 10  $\mu\text{m}$ .

zone was formed at the contact site within 20 min while intracytoplasmic organelles were reoriented in a radial manner toward this site (Figs. 3, A–D). This indicated that establishment of cell–cell contacts with RLEC induced very early changes in hepatocyte organization.

#### ***The Molecule Localized by mAb L8 Is Expressed by Both Hepatocytes and RLEC***

Immunopositive reaction with mAb L8 was located all along the plasma membrane of both freshly isolated hepatocytes and hepatocyte pure cultures for only the 4 first days and then disappeared. By contrast, mAb L8 immunoreacted with hepatocyte plasma membranes in cocultures for 2 wk (Fig. 4 B). The reaction in these cells gradually decreased with time thereafter. Immunopositive reaction was also observed on RLEC, at a lower intensity. It was slightly increased in cells close to hepatocytes. Electron microscopic examination revealed electron-dense deposits distributed all along the cell surface of both hepatocytes and RLEC (Fig. 4 C).

The antigen recognized by mAb L8 was present in the normal liver. Antigenic sites were generally distributed in the hepatic lobule, but restricted to the sinusoids (Fig. 4 A).

#### ***Cells Immunoreacting with mAb L8 Are Capable of Interacting with Hepatocytes in Coculture***

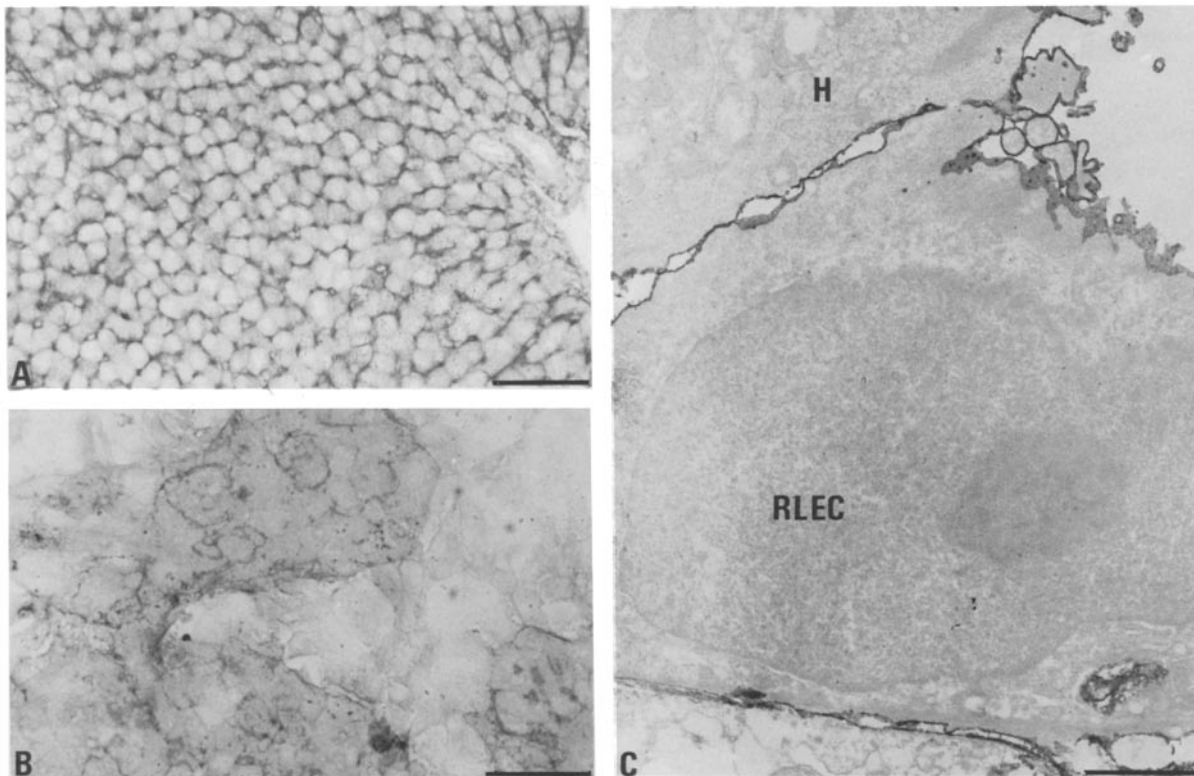
Two different RLEC cell lines (SDIII and SDVI) and one woodchuck liver epithelial cell line (WLEC) interacted with rat hepatocytes in favoring survival and albumin secretion. Furthermore, addition of mAb L8 strongly reduced these

effects. In parallel, these cells were immunoreactive to mAb L8 (Table I). This feature was not characteristic of all cells with an epithelial origin or all cells from liver tissue since bovine lens epithelial cells and rat liver myofibroblasts failed to interact with hepatocytes in coculture and that mouse 3T3 fibroblasts succeeded in it (Kuri-Harcuch and Mendoza-Figueroa, 1989). Moreover, immunoreactivity of mAb L8 on cells was limited to those from rat and woodchuck origin. Immunopositive reaction was not detected on mouse 3T3 cells and cocultures with 3T3 cells were not affected by mAb L8 in consequence of the species specificity expected of this mAb.

In addition, assays were performed which combined hepatocytes from different species with RLEC. Hepatocytes from mouse, woodchuck, dog, baboon, and man were all able to interact with RLEC and to maintain a higher functional stability in coculture.

#### ***mAb L8 Specifically Suppresses the Liver-specific Gene Expression in Coculture***

Total hepatocyte RNA was extracted from pure hepatocyte cultures and from untreated and mAb L8–treated cocultures at different times and was analyzed by Northern blot. Hybridization was performed with cDNA probes corresponding to rat albumin, aldolase B, and procollagen  $\alpha_1$  (I). As expected from previous data (Fraslin et al., 1985), the steady state of albumin mRNA level gradually decreased with time in pure cultures, while that of procollagen  $\alpha_1$  (I) mRNA strongly increased after 4 d of culture (Fig. 5 B). The steady-



**Figure 4.** Immunolocalization of the mAb L8 reacting protein. (A) Intralobular distribution of the protein in adult rat liver using the immunoperoxidase staining. Formaldehyde-fixed frozen sections were incubated for 1 h with mAb L8 in the presence of 0.2% saponin. The protein appeared to be mainly expressed in the sinusoids. (B) Localization of the protein by indirect immunoperoxidase staining in a 3-d-old coculture. It is expressed by hepatocytes and by RLEC at a lower intensity; it appears located exclusively on the plasma membrane and distributed all along the cell surface. (C) Electron micrograph of 3-d-old coculture. The molecule is detected by indirect immunoperoxidase staining. The protein is observed on the plasma membranes of both hepatocytes (H) and epithelial cells (RLEC). No intracellular staining is visible. Bars: (A) 100  $\mu\text{m}$ ; (B) 50  $\mu\text{m}$ ; (C) 1  $\mu\text{m}$ .

state levels of albumin and aldolase B mRNAs were strongly increased from day 2 to day 5 in untreated cocultures, whereas these levels dramatically decreased in the presence of mAb L8 by day 5. In contrast, increased amounts of procollagen  $\alpha_1$  (I) were observed in the presence of mAb L8 as in pure cultures (Fig. 5 A).

**Table I.** Ability of Various Cell Types to Interact with Rat Hepatocytes in Coculture and Immunoreactivity with mAb L8

Cells	Species origin	Cell interaction	mAb L8 reactivity
<b>Epithelial cells</b>			
Liver RLEC SDIII	Rat	+	+
Liver RLEC SDVI	Rat	+	+
Liver WLEC	Woodchuck	+	+
Lens epithelial cells	Bovine	-	ND
<b>Other cell types</b>			
Liver myofibroblasts	Rat	-	ND
Skin fibroblasts	Human	-	ND
3T3 fibroblasts	Mouse	+	ND
Corneal endothelial cells	Bovine	-	ND

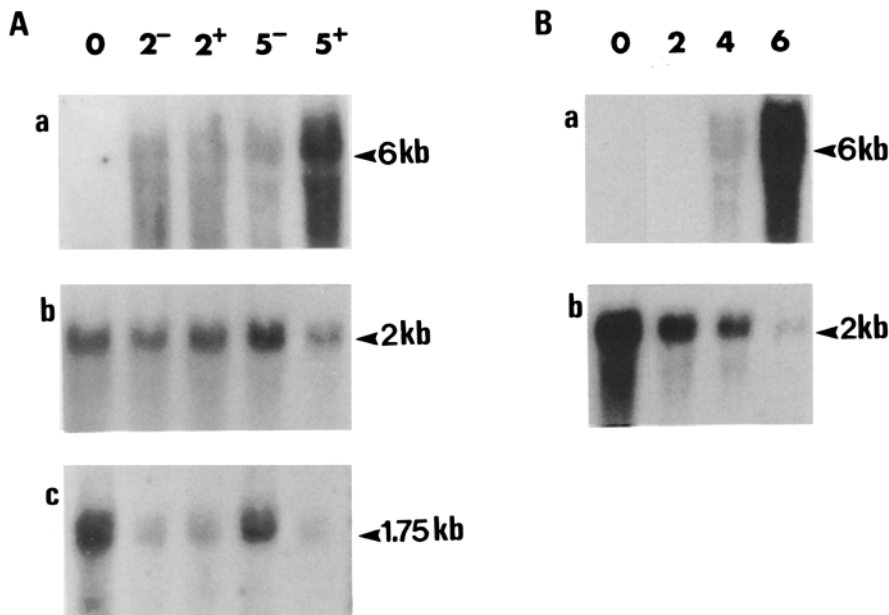
Cocultures of different cell types with rat hepatocytes were performed. Those cells that were able to improve both cell survival and albumin secretion were considered to establish cell interactions with hepatocytes. Immunoreactivity with mAb L8 was evidenced by indirect immunoperoxidase localization. ND indicated that no reactivity could be detected.

To demonstrate that mAb L8 specifically altered the cell-cell contact-mediated regulation of liver-specific genes, pure cultures of hepatocytes were prolonged for up to 8 d in the presence of 25 mM nicotinamide (Paine et al., 1979) and continuously exposed to mAb L8. Neither the morphology nor albumin secretion was modified in these cells when mAb L8 was present. In contrast, cocultures with or without nicotinamide lost the ability to secrete high levels of albumin in the presence of mAb L8 (data not shown).

To further verify that mAb L8 had no direct effect on hepatocyte pure cultures, we analyzed the in vitro translation products of hepatocytes untreated or daily treated with mAb L8 and maintained in pure culture or in coculture. The pattern of proteins synthesized by pure cultures was strikingly similar during the culture period regardless of the presence of mAb L8. In contrast, treatment of cocultures with mAb L8 for 3 d specifically induced changes in the synthesis level of various proteins (data not shown).

#### **mAb L8 Alters Hepatocyte Cytoskeleton Organization and Perturbs Deposition of ECM Components**

Cell shape and cytoskeleton are closely associated with the hepatocyte-differentiated phenotype (Ben-Ze'Ev et al., 1988). We have examined whether cell-cell contacts with RLEC induced early or late cytoskeletal changes. Cytokeratins CK8 and CK18, the two components that form the intermediate-



**Figure 5.** Effect of mAb L8 on the liver-specific mRNA of cocultured hepatocytes. (A) Freshly isolated hepatocytes (0) and hepatocytes maintained in coculture for 2 or 5 d with (+) or without (-) 20 µg/ml of partially purified mAb L8, were analyzed for procollagen  $\alpha_1$  (I) (a), albumin (b), and aldolase B (c) mRNA levels. Equal amounts of total RNA (20 µg) from the different sources were used and hybridized with an excess of specific cDNA probes. For the assay, hepatocytes were selectively detached and collected from RLEC using a calcium-deprived collagenase solution (0.05%). (B) Freshly isolated hepatocytes (0) and hepatocytes maintained in pure culture for 2, 4, and 6 d, were analyzed for procollagen  $\alpha_1$  (I) (a) and albumin (b) mRNA levels.

sized filaments of hepatocytes, are expressed in coordinated manner (Baffet et al., 1991). They were analyzed by immunolocalization in both pure cultures and cocultures, in the presence or absence of mAb L8. In pure cultures, cytokeratin filaments formed a complex network uniformly distributed in the cytoplasm of hepatocytes with both CK8 and CK18 antibodies (Fig. 6 A). In cocultures, they were principally located at the cell periphery, just beneath the plasma membrane as observed *in vivo* (Fig. 6 B). Daily addition of mAb L8 from the moment the coculture was started, strongly perturbed this typical organization: filaments became randomly distributed into the cytoplasm as in pure cultures (Fig. 6 C).

The presence of ECM has been found to be associated with the maintenance of hepatocyte functions in coculture (Baffet et al., 1982). We have investigated the role of mAb L8 on the ECM deposition by using the reticulin staining. In untreated cocultured cells, ECM was primarily located between the two cell populations. This material gradually covered the hepatocyte cords within 1 wk. Early and daily addition of 10 µg/ml and 20 µg/ml of mAb L8 to cocultures strongly inhibited deposition of ECM (Figs. 7, A and B).

#### **mAb L8 Does Not Alter Cell-Cell and Cell-Substratum Adhesion**

Since mAb L8-related effects started during cell contact establishment, we sought firstly whether the antibody might alter attachment to RLEC. Hepatocytes were set to attach onto confluent RLEC monolayer, in the presence or absence of mAb L8. No significant difference in hepatocyte attachment to RLEC was observed in the presence of mAb L8. Differences between treated and untreated cells were found only after 3 d consisting of a delay in hepatocyte spreading in mAb L8 cocultures while untreated cells were well spread. Finally, the deposition of ECM was strongly reduced (Fig. 7, C and D) and the viability of hepatocytes was limited to a few days as described above.

Secondly, we determined whether mAb L8 was able to alter hepatocyte adhesion to various ECM components. Seeding hepatocytes in a medium containing increasing con-

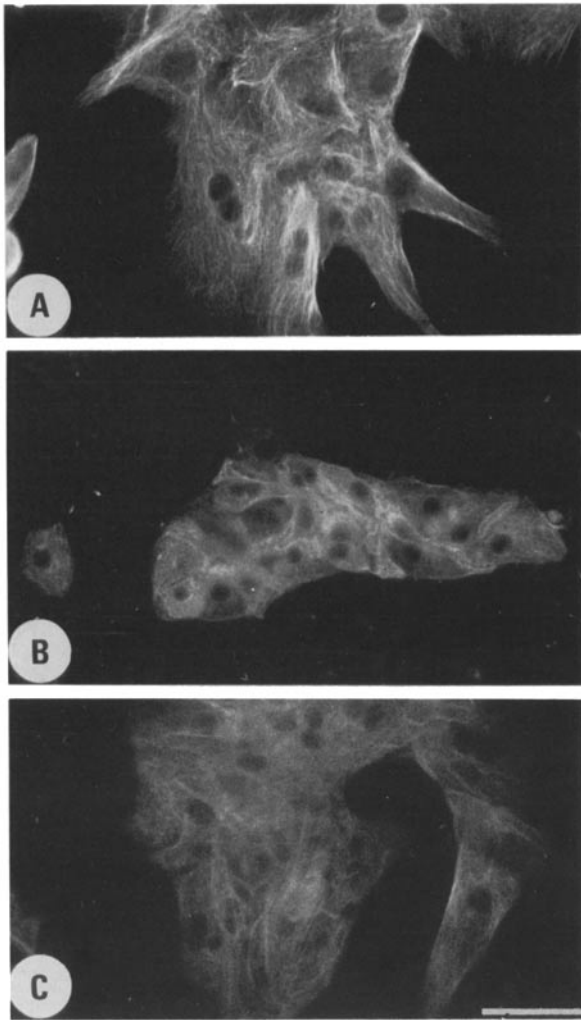
centrations of mAb L8 or SP<sub>2</sub>/O-Ag ascitic fluid did not prevent or delay their attachment to plastic, fibronectin, laminin, laminin/entactin complex, collagen IV, heparan sulfate proteoglycan, or EHS gel substrata (Table II). Preincubation of cells with mAb L8 did not affect these results.

Furthermore, we have verified that mAb L8-treated hepatocytes seeded on EHS gel-precoated dishes, did not show any change in their survival and functional capacity (data not shown). Taking together, these data suggest that the epitope recognized by mAb L8 is unlikely involved in the reactivity to ECM.

#### **Characterization of the Polypeptides Recognized by mAb L8**

Immunoprecipitation was performed on detergent cell lysates after cell surface iodination of hepatocytes and RLEC. SDS-PAGE of mAb L8 immunoprecipitates was qualitatively analyzed. It revealed two peptide chains having apparent molecular masses of 85 and 73 kD from both hepatocytes and RLEC (Fig. 8 A). These two bands were neither detected after immunoprecipitation with the ascitic fluid of SP<sub>2</sub>/O-Ag myeloma cells used for the fusion nor when iodinated detergent lysates were prepared from bovine corneal endothelial cells. The two polypeptides were constantly present under both reducing and nonreducing conditions, suggesting that the two chains were not disulfide-linked (Fig. 8 B). The heavier chain (85 kD) appeared to migrate slightly faster in nonreducing conditions, with an apparent molecular mass of 80 kD. The pI of 4.9–5.1 and 5–5.2 for the 85 kD and 73 kD polypeptides, respectively, were slightly different (Fig. 8 C). The possibility that one chain derived from the other by limited proteolysis is unlikely since the two bands were regularly and strikingly detected in both hepatocytes and RLEC.

Hepatocytes were submitted to Triton X-114 extraction and the lysate was partitioned at 30°C as described by Bordier (1981). The two polypeptides were preferentially recovered from the hydrophobic phase, suggesting that both were integral plasma membrane proteins (Fig. 8 D). They could

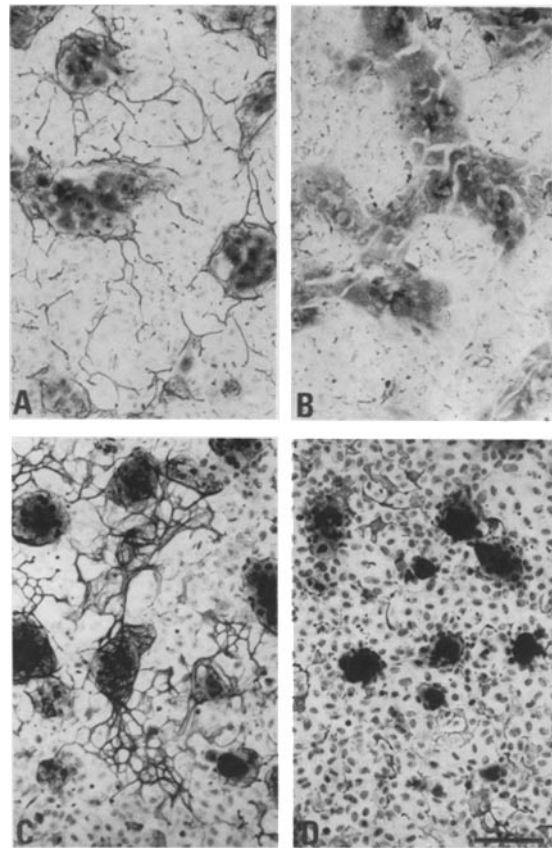


**Figure 6.** Indirect immunofluorescence localization of cytokeratin 18 in 3-d-old hepatocyte pure culture (A); 3-d-old coculture treated daily with 2  $\mu$ l/ml of SP<sub>2</sub>/O-Ag myeloma cell ascitic fluid as control (B) and 3-d-old coculture treated daily with 2  $\mu$ l/ml of L8 hybridoma ascitic fluid (C). In the presence of mAb L8, the intermediate filaments become randomly distributed into the cytoplasm, as in pure culture. Bar, 50  $\mu$ m.

not be extracted from the plasma membrane after treatment with PBS or EDTA.

### Discussion

In this study, we show that a plasma membrane protein of 85/73 kD apparent molecular mass, plays a pivotal role in the functional activity of mature hepatocytes when cocultured with bile ductule epithelial cells. For this reason, this molecule was named LRP. The existence of novel factors that involve heterotypic interactions and participate in hepatocyte differentiation was suggested by previous observations (Guguen-Guillouzo and Guillouzo, 1983; Reid et al., 1986). In particular, difficulty in maintaining functionally stable hepatocytes in vitro as a consequence of their separation from other hepatic cells and from their pericellular matrix has been observed (Clayton et al., 1985; Bissell et al., 1987; Ben-Ze'Ev et al., 1988). In addition, coculturing hepato-



**Figure 7.** mAb L8-inhibited deposition of extracellular matrix components in cocultures established either by seeding RLEC 4 h after hepatocyte plating in order to get a cell monolayer (A and B) or by seeding hepatocytes on a layer of RLEC (C and D). Reticulin staining of 3-d-old cocultures treated daily with 2  $\mu$ l/ml of SP<sub>2</sub>/O-Ag myeloma cell ascitic fluid as controls (A and C) and L8 hybridoma ascitic fluid (B and D). Note that when mAb L8 is present, only few extracellular matrix fibers are observed in both coculture conditions and hepatocytes remain aggregated on the RLEC layer. Bar, 100  $\mu$ m.

cytes with RLEC dramatically enhanced long-term functional capacities of the adult parenchymal cells (Guguen-Guillouzo, 1986).

The role of this protein has been determined by mAb immunoreaction experiments and by analysis of the hepatocyte-differentiated phenotype in normal and in mAb L8-treated cocultures. The parameters chosen as indicative of well-preserved differentiation stage in coculture included long-term cell survival, active serum albumin production, presence of high levels of liver-specific mRNAs, characteristic organization of cytoskeletal components, and deposition of ECM fibers. Although hepatocytes in pure cultures were not affected, mAb L8 induced modifications on behavior of cocultured cells that failed to stabilize their functions and exhibited the same pattern as in pure cultures: cell survival was reduced, serum albumin secretion quickly diminished in parallel with a decreased level in the corresponding mRNA and mRNAs from other liver-specific genes. As in pure cultures (Clément et al., 1988), the mAb L8-treated cocultured hepatocytes also actively synthesized procollagen  $\alpha_1$  (I), which did not polymerize. Since long-term functional stabil-



**Table II. Hepatocyte Attachment Assays (% of Attached Cells) on Different Extracellular Matrix Components in the Presence or Absence of mAb L8**

Extracellular matrix components		Antibody ( $\mu\text{g/ml}$ )				
		0	10	20	50	100
Fibronectin	Assay	60	65	66	61	74
	Control	62	63	68	65	70
Laminin	Assay	61	63	67	74	75
	Control	63	60	68	70	72
Laminin/Entactin complex	Assay	66	68	64	74	81
	Control	67	66	69	73	79
Collagen IV	Assay	60	62	62	68	73
	Control	62	62	65	69	70
Heparan-sulfate proteoglycan	Assay	50	52	51	53	55
	Control	52	50	53	58	57
EHS gel	Assay	80	86	87	84	ND
	Control	78	83	85	86	89

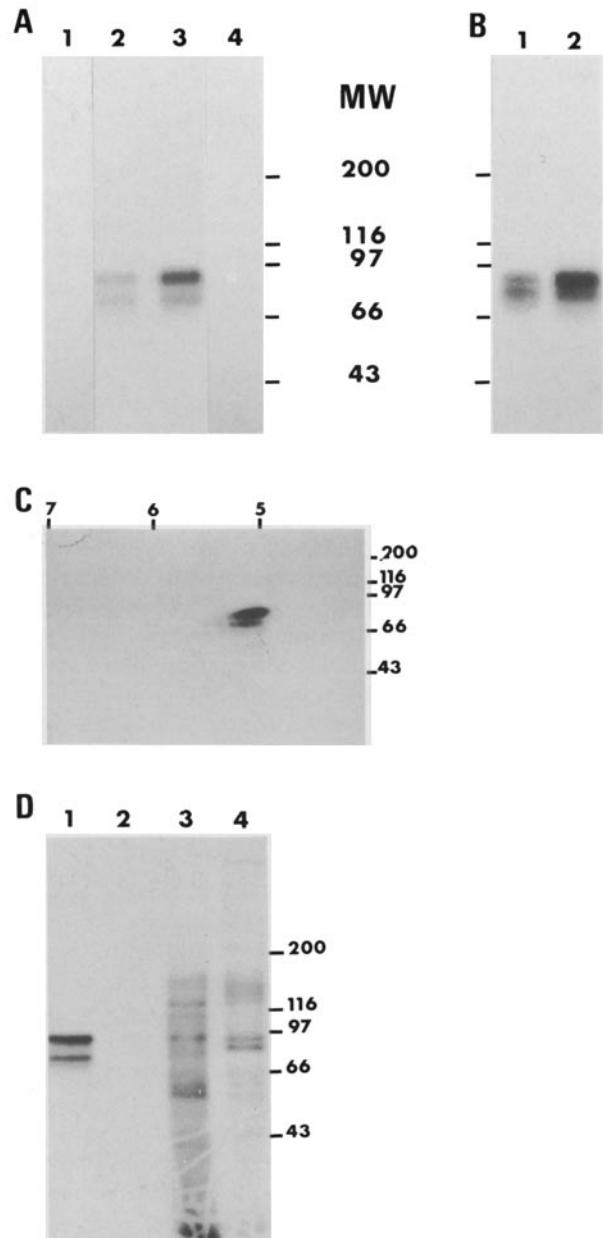
Hepatocyte attachment assays were performed on 0.32-cm<sup>2</sup> well tissue plates coated with 2  $\mu\text{g}$  of different extracellular matrix components in the absence or presence of increasing amounts of L8 hybridoma ascitic fluid (10 mg antibody/ml). Assays were carried out in duplicate and the percentage of attached hepatocytes was measured as described in Material and Methods. Controls corresponded to cultures added with equal amounts of SP<sub>2</sub>/O-Ag ascitic fluid to those of mAb L8. No significant variation of cell adhesion was observed with the different extracellular matrix components in the assays compared to the controls. SD did not exceed 5%.

ity in coculture was found to result from both increased gene transcription and stabilization of liver-specific mRNAs, it may be postulated that mAb L8 could inhibit the coculture effect by acting at these two levels of regulation. In addition, these mAb L8-related changes correlated with the nearly complete loss of ECM deposition. They also correlated with the inability of hepatocytes to develop a cytoskeletal architecture characteristic of the differentiated phenotype, as described by Katsuma et al. (1988). These results provide a dramatic example of the coordinate regulation of tissue-specific gene expression, cytoskeletal protein organization and ECM deposition, which is dictated by cell-cell interac-

**Figure 8.** Analysis of mAb L8 immunoprecipitation. Cells were labeled with Na<sup>[125I]</sup> by the lactoperoxidase-catalyzed reaction. Lysates of the different sources were precipitated with L8 hybridoma ascitic fluid. (A) Immunoprecipitates from bovine corneal endothelial cells (lane 1), hepatocytes (lane 2), and RLEC (lane 3) lysates were analyzed on SDS-PAGE (4–15% polyacrylamide linear gradient slabs) in reducing conditions. Two chains of 85 and 73 kD were specifically precipitated from hepatocytes and RLEC, but not from bovine corneal endothelial cells. Immunoprecipitate of RLEC lysate with the SP<sub>2</sub>/O-Ag myeloma cell ascitic fluid, as control (lane 4). Samples of RLEC and endothelial cells were derived from  $50 \times 10^6$  cells and samples of hepatocytes were derived from  $18 \times 10^6$  cells. (B) Immunoprecipitates obtained with hepatocytes (lane 1) and RLEC (lane 2) lysates when analyzed on SDS-PAGE in nonreducing conditions. The 85-kD peptide migrated faster with an apparent molecular mass of 80 kD. Molecular mass is given in kilodaltons. (C) Isoelectrofocusing of LRP followed by SDS-PAGE (7.5–15% polyacrylamide linear gradient slab) in the second dimension. LRP was immunoprecipitated from RLEC lysate. The pH values of control gels are shown at the top. (D) Membrane associa-

tions. The data also reveal that LRP, the plasma membrane protein immunoreactive to mAb L8, is involved in one of the critical steps of this coordinate process.

The question arises whether LRP acts on the liver-specific gene transcription by mediation of an ECM component. It is clearly established that systems containing a complex ECM or EHS matrix (Caron, 1990), support a higher level of transcription than do those without ECM (Bissell et al., 1990). The matrix deposited in coculture between hepatocyte colonies and RLEC contains basement membrane com-



tion of LRP. Hepatocytes were solubilized in the Triton X-114 detergent and submitted to phase fractionation. Membrane proteins distributed in the detergent phase (lane 1) and the aqueous phase (lane 2) after immunoprecipitation with mAb L8 were analyzed by SDS-PAGE (4–15% polyacrylamide linear gradient slab) in reducing conditions. The detergent phase (lane 3) and the aqueous phase (lane 4) before mAb L8 treatment were loaded as controls.

ponents including laminin, collagen IV, and heparan sulfate proteoglycan (Clément et al., 1988). Moreover, its deposition correlates with enhanced gene expression and we have shown that it is inhibited by addition of mAb L8. However, the data presented also indicate that mAb L8 does not modify hepatocyte attachment to different matrix components and fails to alter their functional capacities on EHS gel. These findings strongly support the idea that the LRP epitope recognized by mAb L8 differs from an ECM receptor. It is nevertheless reasonable to postulate that LRP participates to the control of ECM deposition in coculture and, consequently, indirectly to the liver-specific gene transcription. LRP influences the cytoskeleton organization. Indeed, addition of mAb L8 perturbs cytoke-  
 ratin distribution. This is in good agreement with the general assumption that cell shape and cytoskeleton are closely associated with the hepatocyte-differentiated phenotype (Ben-Ze'Ev et al., 1988).

It is noteworthy that the inhibitory effect of mAb L8 was only evidenced in coculture when the antibody was present between days 1 and 2 or so after RLEC seeding. It corresponds specifically to the establishment of heterologous cell contacts. This argues for a critical role of the mAb L8 immunoreactive protein in the early key regulating processes during cell contact establishment, which engage hepatocytes to maintain or to restore their differentiated phenotype and which include transcription of liver-specific genes, morphological changes and ECM deposition. Exploration by microcinematographic analysis of the hepatocyte behavior when making contact with RLEC has revealed early changes which support this hypothesis.

The biological properties of LRP also suggest that it is distinct from the two adhesion molecules described in the liver, L-CAM (Gallin et al., 1983) and cell CAM 105 (Odin et al., 1986). Thus, in contrast to mAb L8, polyclonal anti-cell CAM 105 greatly affects adhesion and aggregation of hepatocytes and fails to alter their functional capacities in coculture (1988, unpublished observations). The protein appears mainly expressed in the sinusoids of adult liver. This intralobular distribution is also distinct from that of the two cell adhesion molecules. Moreover, it indicates that LRP-positive cells could be present in the sinusoids. This is in agreement with data of Goulet et al. (1988) showing coculture with sinusoidal endothelial cells.

Immunoaffinity isolation revealed two peptide bands of 85 and 73 kD, respectively, that are not associated by disulfide bonds. These two bands were both preferentially distributed in the hydrophobic phase after Triton X114 partition, suggesting that they are both integral proteins that may connect the extracellular domain to the intracellular one.

These two polypeptides, reacting with the same monoclonal antibody could be explained in several ways: (a) the presence of one truncated form of the same molecule as described for N-CAM (Cunningham et al., 1987); (b) protein made of two types of polypeptide chains with analogous structure and associated through nondisulfide bond; or (c) two independent molecules sharing the same epitope.

Taken together, our results lead to the conclusion that LRP must be expressed by both cell types to induce the coculture. The mechanism by which the hepatocyte-LRP molecules and RLEC-LRP molecules interact is still unknown. It could be either homophilic or heterophilic as found with the Ig superfamily. More direct assays along with chemical and mo-

lecular analysis are in progress. They should provide new insights into the characterization of LRP and its role in hepatocyte differentiation.

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