ANTI-LIVER-KIDNEY MICROSOME ANTIBODY RECOGNIZES A 50,000 MOLECULAR WEIGHT PROTEIN OF THE ENDOPLASMIC RETICULUM

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Children with autoimmune chronic active hepatitis can be separated into two groups depending on the presence in the serum of either smooth muscle antibody (SMA) or liver-kidney microsome antibody (LKMA) (1). The latter can be detected by immunofluorescence as a cytoplasmic staining of hepatocytes and of kidney tubular cells in sections from the respective rat organs (2). Indeed, it has been shown by immunoelectron microscopy that LKMA binds to constitutents of the endoplasmic reticulum of rat hepatocytes (3). Using recent developments in cell fractionation and immunological techniques, we have determined that the antigen recognized by LKMA is an integral membrane protein of 50,000 mol wt located primarily in the smooth endoplasmic reticulum.

Materials and Methods

Sera. LKMA-positive sera were obtained from five children with chronic active hepatitis as proven by liver biopsy (immunofluorescence LKMA titer, 1:500 to 1:100,000; serum gamma globulin levels, 13.5–43 g/l). As a control we studied the sera from 20 children with various chronic inflammatory liver diseases whose sera were negative for LKMA by immunofluorescence: five children with active hepatitis, verified by liver biopsy, whose sera contained high (>1:100) titers of SMA (serum gamma globulin levels, 19.5–44 g/l); five children with chronic active hepatitis related to hepatitis B virus infection, proven by liver biopsy; five children with Wilson's disease whose liver biopsy exhibited signs of aggressivity and liver cirrhosis; and five patients with a similar histologic pattern due to alpha-1 antitrypsin deficiency. Sera were collected before any treatment, except from one child of the LKMA group whose serum was collected during a relapse, 4 mo after interruption of immunosuppressive therapy.

Preparation of Rat Microsomes and Microsomal Subfractions. Female Wistar rats (150 g), fasted for 18 h, were sacrificed and the livers homogenized. The microsomal fraction obtained by differential centrifugation (4) was further divided into 12 subfractions according to their densities on a preformed sucrose gradient (5). These subfractions have been characterized previously (6); 100 μg of each of two fractions were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), in a 6–11% gradient of polyacrylamide, and stained with Coomassie Blue (Fig. 1, *a* and *b*).

Rough microsomes (RM) and smooth microsomes (SM) were also prepared from livers of fasted female Sprague-Dawley rats (150 g) according to Kruppa and Sabatini (7).

Treatment of Microsomal Fractions. SM washed with 0.25 M sucrose were resuspended

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(2.5 mg/ml) in (*a*) phosphate-buffered saline (PBS), (*b*) 50 mM Hepes, pH 7.5, containing 1% Emulgen 913 (KAO Atlas, Tokyo), (*c*) PBS, containing 0.25 M sucrose and 0.05% DOC (deoxycholic acid, sodium salt [Calbiochem-Behring Corp., La Jolla, CA]) (8), or (*d*) 100 mM Na_2CO_3, pH 11 (9). After 20 min incubation at 4°C, supernatant and pellet fractions were obtained (5 min at 4°C, rotor 30 lbs/in^2) (Airfuge; Beckman Instruments, Inc., Fullerton, CA). The pellets were resuspended to the original volume of the microsomal suspension and 8-μl aliquots of supernatant and pellet fractions were analyzed by SDS-PAGE (10% polyacrylamide) followed by Western blot analysis (10).

SM (1.3 mg/ml in 50 mM Tris-HCl buffer, pH 7.4) were proteolyzed in the absence or presence of Triton X-100 (0.2% final concentration) by adding proteinase K (Boehringer Mannheim Biochemicals, Indianapolis, IN) at a final concentration of 75 μg/ml (final volume, 40 μl). After incubation for 15, 30, 45, 60, 90, and 180 min at 22°C, proteinase K was inhibited by the addition of phenylmethylsulfonyl fluoride (PMSF), and 15 μl of each sample was analyzed by SDS-PAGE followed by Western blot analysis (10).

Western Blot Analysis of Microsomes and Microsomal Subfractions. This was done essentially as described by Towbin et al. (10). Patient sera were used as a first antibody at dilutions of 1:100 to 1:1000. A peroxidase-conjugated F(ab')$_2$ fragment of goat anti–human IgG heavy and light chain (E-Y Laboratories, San Mateo, CA) was used as a second antibody at a final dilution of 1:1000. In the proteolysis experiment, an additional control was performed by incubating samples that had been digested first for 60 and 180 min, with rabbit anti–rat albumin antibodies and subsequently with peroxidase-conjugated goat anti–rabbit IgG (Cappel Laboratories, Cochranville, PA). The immunoblots were developed for 30 s with diaminobenzidine in 50 mM Tris-HCl, pH 7.4 (0.5 mg/ml) and H_2O_2 (0.01% final concentration).

Immunoprecipitation of the 50,000 Mol Wt Protein from [^{35}S]Methionine-labeled Rat Hepatocytes. Hepatocytes from 150-g Sprague-Dawley rats were isolated and cultured for 48 h as previously described (11). Two cultures (10^6 cells in 60-mm dishes) were preincubated with methionine-free medium. Tunicamycin (2 μg/ml) was added to one of the dishes. After 1 h of preincubation both cultures received 125 μCi of [^{35}S]methionine (sp act, 800 Ci/mmol; New England Nuclear, Boston, MA). After 5 h, the culture media were saved and the cells were rinsed with Moscona's PBS. The cells were scraped and extracted with 100 μl of lysis buffer (10 mM NaCl, 10 mM Tris-HCl, pH 7.4, and 1.5 mM $MgCl_2$) containing 0.5% DOC and 0.5% Nonidet P-40 (Sigma Chemical Co., St. Louis, MO). After a 5 min incubation on ice the nuclei were removed by sedimentation (5 min, 4°C, in Eppendorf centrifuge). The 50,000 mol wt protein was then immunoprecipitated from media or cell extracts (10^6 cpm) by adding 5 μl of LKMA-positive or control serum (for details see reference 12). The immunoprecipitates were analyzed by SDS-PAGE followed by fluorography (1 wk exposure).

Results

It has been previously demonstrated (3) that the LKM antigen is localized in microsomal fractions prepared from the liver and kidney of rat. In attempts to identify the antigen, patient sera were used for Western blot analysis of rat liver microsomes. As shown in Fig. 1, the only stained band corresponds in relative mobility to a molecular weight of 50,000. The same result was obtained using the sera from any of the five LKMA-positive patients. This band was not stained using control sera tested at similar dilutions.

Using highly purified microsomal subfractions of decreasing isopycnic density (Fig. 1, *a–g*), which is largely related to the amounts of ribosomes attached to the microsomal vesicles, we determined the distribution of a 50,000 mol wt protein between the rough (RER) and smooth endoplasmic reticulum (SER). The fractions of lower density (Fig. 1, *f* and *g*) contain a much higher concentration of the antigen compared with the heavier microsomal subfractions (*c* and *d*), which are mainly derived from the RER (6). This finding cannot be attributed to the presence of ribosomal proteins in RER-derived fractions, since they

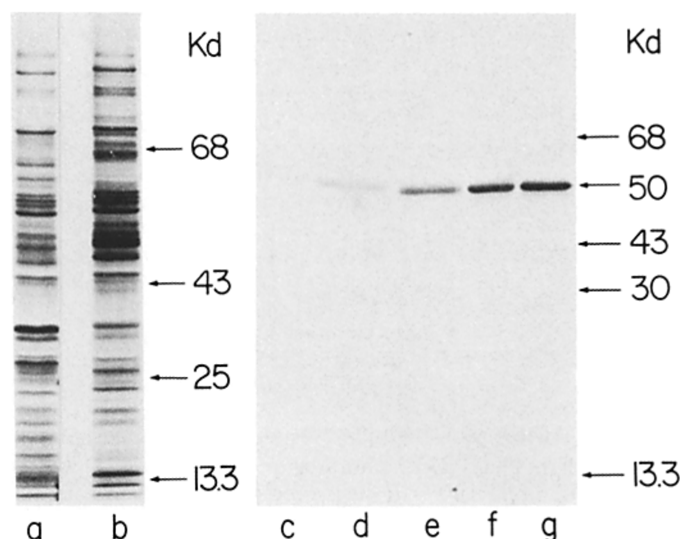

FIGURE 1. Rat liver microsomal fractions analyzed by SDS-PAGE were either stained by Coomassie Blue (*a* and *b*) or subjected to Western blot analysis (*c–g*) using serum from a patient with autoimmune chronic active hepatitis. In *c–g*, microsomal subfractions with decreasing ribosome contents were loaded (*a* and *c*, 236 μg RNA/mg protein; *b* and *g*, 37 μg RNA/mg protein). For details concerning the characterization of fractions, see Ref. 6. Molecular weight markers: bovine serum albumin (68,000), ovalbumin (43,000), carbonic anhydrase (30,000), chymotrypsinogen A (25,000), and cytochrome *c* (13,300).

represent ≤25% of the total protein content. The conclusion that the 50,000 mol wt protein is enriched in SER membranes is further supported by the fact that lighter fractions (1.06 g/cm^3), which mainly contain elements of the Golgi apparatus, plasma membranes, and smooth surface membranes from other organelles, contain very little, if any, of this protein (data not shown).

To test whether LKM antigen is an integral membrane protein, a peripheral protein attached to the outside or inside of the microsomal vesicle, or a luminal content protein, SM were extracted with detergents or with alkaline buffer (pH 11). After treatment with Emulgen 913, a neutral detergent that solubilizes SM completely, the 50,000 mol wt protein was found in the supernatant after centrifugation. On the other hand, DOC (0.05%), which permeabilizes microsomal vesicles but does not dissolve the membranes (8), did not release the 50,000 mol wt protein (Fig. 2) and, after alkaline treatment of microsomes (9), the 50,000 mol wt protein remained sedimentable with the membrane fraction (Fig. 2). It appears therefore, that this antigen is an integral membrane protein of the endoplasmic reticulum. No change in electrophoretic mobility was observed when reducing agents were omitted from the sample, demonstrating that this protein does not form homopolymers or heteropolymers linked by disulfide bridges (results not shown).

To determine the exposure of the 50,000 mol wt protein in microsomal membranes, intact SM were proteolyzed with proteinase K. The 50,000 mol wt protein was digestable at rather high protease concentrations (Fig. 3). Solubilization of membranes with Triton X-100 enhanced digestion of this protein. No smaller size digestion products were detected. On the other hand, albumin was

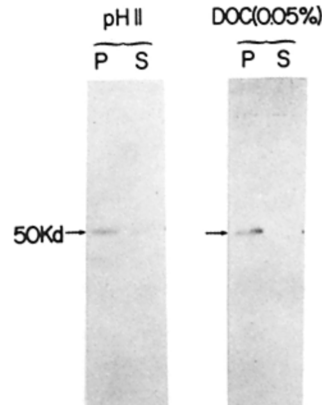

FIGURE 2. Rat liver SM were treated with low concentration of DOC or alkaline buffer (see Materials and Methods). After both treatments, the 50,000 mol wt protein was recovered in the pellet (*P*); only small amounts of the protein were found in the supernatant (*S*).

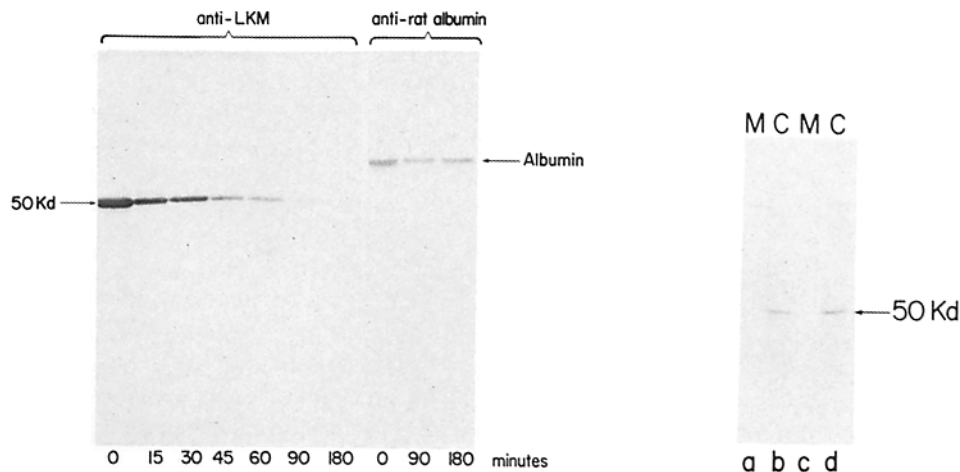

FIGURE 3. Rat liver microsomes were digested with proteinase K. Samples digested for up to 180 min were analyzed by SDS-PAGE followed by Western blot using LKMA or anti-rat serum albumin.

FIGURE 4. Rat hepatocytes were labeled in vitro for 5 h with [^{35}S]methionine in the absence (*a* and *b*) or presence (*c* and *d*) of tunicamycin. *M*, media; *C*, cell extracts.

only digested when detergent was added to microsomal samples.

As expected for an integral membrane protein of the endoplasmic reticulum, the 50,000 mol wt protein was immunoprecipitated only from cell extracts (Fig. 4). When cultures of primary hepatocytes were pretreated with tunicamycin, a drug that inhibits cotranslational glycosylation of asparagine residues, no changes in electrophoretic mobility were observed (Fig. 4*d*), indicating that it is not a glycoprotein. As expected, following the same tunicamycin treatment, alpha-1 antitrypsin, a serum glycoprotein, showed a marked increase in electrophoretic mobility (results not shown).

Discussion

The results show that one or more factors present in sera of children with LKMA specifically recognize a microsomal protein of 50,000 mol wt that is in

higher concentration in rat liver SER than in RER. This result agrees with the finding of a more intense immunofluorescence staining using LKMA on hepatocytes with an SER hypertrophy induced by hepatitis B virus (13).

Our results demonstrate that the 50,000 mol wt protein is an integral membrane protein at least partially exposed on the cytoplasmic face of the endoplasmic reticulum. A harsh digestion condition using a rather unspecific protease was required to digest the protein recognized by LKMA. A previous report (3) showed that the LKM antigen is relatively resistant to trypsin treatment; only 50% of the antigenicity was lost when microsomal membranes were treated with 200 μg/ml of enzyme for 2 h at 37°C (3). This relative resistance to protease treatment may account in part for the antigenicity of this protein after lysis of hepatocytes. The absence of identifiable digestion products in Western blots may be due to the fact that LKMA is directed against a small segment of the protein exposed at the cytoplasmic side of microsomal vesicles or that the small fragments resulting from digestion cannot be resolved in our electrophoretic system.

It remains unclear why patients with autoimmune chronic active hepatitis develop either LKMA- or SMA-positive sera, although in both cases extensive lysis of hepatocytes occurs. The 50,000 mol wt protein is not present in plasma membranes nor secreted by the cells, indicating that normally it is not exposed to the immune system. On the other hand, the exposure of the 50,000 mol wt protein to immune competent cells upon cell lysis cannot be a sufficient condition for the development of LKMA in a particular group of patients, since none of the sera from the children of similar ages with chronic inflammatory liver diseases of different etiology and similar severity recognize this protein.

LKMA are also found in patients with hepatitis caused by tielinic acid, but the immunofluorescence patterns in liver and kidney sections from rats and mice are different than those shown by LKMA from patients with autoimmune chronic active hepatitis (14). It appears, therefore, that the LKMA recognizes different antigens in these two types of hepatitis. This could also be the case in chronic infection with hepatitis B surface antigen (HBsAg) associated delta agent, where a low titer of LKMA can be demonstrated by immunofluorescence, since this LKMA reacts preferentially with liver and kidney of human compared with the corresponding rat and mouse organs. This difference is not observed in LKMA from autoimmune chronic active hepatitis (15).

Our results suggest that the 50,000 mol wt protein was highly conserved during evolution, allowing its detection in mouse and rat cells using human serum. Studies currently in progress will better characterize the 50,000 mol wt protein and help define its physiological role.

Summary

Children with autoimmune chronic active hepatitis may have high titers of antibodies detected by immunofluorescence staining of hepatocytes and tubular cells in rat liver and kidney sections, respectively. These antibodies are directed against antigens contained in microsomal fractions prepared from these two organs. We have found that sera from these patients recognized a 50,000 mol wt protein present in higher concentration in smooth microsome subfractions compared with rough microsome subfractions. This protein is an integral membrane protein and is not glycosylated. It is exposed on the cytoplasmic face of

the endoplasmic reticulum and is rather resistant to proteolysis with proteinase K. Since patients with liver disease of different etiology and similar severity of cell lysis do not give rise to liver-kidney microsome antibody (LKMA), lysis of hepatocytes is apparently not a sufficient condition for their development.

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