A Domain-specific Marker for the Hepatocyte Plasma Membrane: Localization of Leucine Aminopeptidase to the Bile Canalicular Domain

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ABSTRACT Indirect immunofluorescence was used to establish a domain-specific marker for hepatocyte plasma membranes. In frozen sections of fixed rat liver (0.5–4 μm), antibodies directed against rat intestinal leucine aminopeptidase (LAP) recognized an antigen that was restricted to the bile canalicular plasma membrane. Fluorescence was not observed on the sinusoidal or lateral membranes, and intracellular staining was not detected. The liver antigen was identified as LAP, based on its chemical similarity to intestinal LAP. First, immunoprecipitation experiments using trypsin-solubilized intestinal LAP (G-200 fraction, 91% pure) established a correlation between the loss of LAP enzyme activity from the soluble fraction and the appearance in the specific immunoprecipitates of polypeptides migrating on SDS PAGE between 110,000 and 130,000 daltons. The antigen precipitated from a detergent extract of liver plasma membranes had the same electrophoretic mobility. Second, the chymotryptic map of the major band in the liver immunoprecipitate was similar to that of purified intestinal LAP.

The hepatocyte is a polarized epithelial cell whose plasma membrane is differentiated into three morphologically and functionally distinct domains. The sinusoidal surface, characterized by irregular microvilli and coated pits, is specialized for the exchange of metabolites with the blood. The lateral surface, marked by junctional elements, functions in cell-cell adhesion and communication. The bile canalicular domain, formed by the outpocketing of two adjacent cell membranes, is separated from the lateral surface by tight junctions and is involved in the secretion of bile constituents. These functional differences among the domains are most likely reflected in compositional differences. However, the successful purification and subsequent characterization of the three membrane regions have yet to be achieved.

The apical and basolateral membranes of both kidney and intestinal epithelial cells have been separated into relatively enriched fractions through the use of domain-specific "markers," that is, enzymes localized predominantly to a particular membrane surface. For example, alkaline phosphatase (AP),[1] leucine aminopeptidase (LAP), and sucrase-isomaltase (SI) have been localized to the apical brush borders of these cells (1–5) and Na^+, K^+-ATPase to the basolateral surface (6, 7). The distributions of these activities have been used to follow the isolation of the two membrane domains. Several problems arise when such an approach is applied to the hepatocyte plasma membrane: (*a*) unlike the brush borders of kidney and intestinal cells, the hepatocyte apical membrane (bile canalicular domain) does not fragment as an intact entity but yields vesicles similar to those of sinusoidal membrane upon homogenization (8, 9); (*b*) several of these enzymes are absent from the liver (e.g., SI); and (*c*) some of these enzymes use rather nonspecific substrates towards which other nonplasmalemmal enzymes are active. For example, several aminopeptidases, having different forms (soluble vs. membrane-bound) and different subcellular locations (plasma membrane, lysosomes, cytosol), have been shown to act on naphthylamide and nitroanilide substrates (10–13).

[1] Abbreviations used: LAP, leucine aminopeptidase EC 3.4.11.2; DAB, diaminobenzidine; HRP, horseradish peroxidase; PMSF, phenylmethyl sulfonyl fluoride; SBTI, soybean trypsin inhibitor; TAME, tosylargnine methyl ester; TMRITC, tetramethylrhodamine isothiocyanate; PBS, phosphate-buffered saline; TEA, triethanolamine; AP, alkaline phosphatase; S-I, sucrase-isomaltase; TMP, trimetaphosphatase; NC, nitrocellulose; WB, wash buffer; MDCK, Madin-Darby canine kidney.

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With the goal of establishing a specific marker for one domain of the hepatocyte plasma membrane, we used an immunocytochemical approach to localize membrane-LAP in the liver. As stated above, LAP is an integral glycoprotein that is localized predominantly to the apical domain of kidney and intestinal epithelial cells. LAP activity is also present in isolated rat liver plasma membrane (14), but its distribution among the surface domains (canalicular, lateral, sinusoidal) is not known. We found, by indirect immunofluorescent staining of frozen sections of rat liver, that antibodies directed against intestinal LAP recognize an antigen that is localized to the canalicular domain. In addition, immunoprecipitation, electrophoretic blotting, and peptide mapping studies reveal that the liver antigen recognized by anti-intestinal LAP antibodies is similar to the intestinal enzyme. Preliminary portions of this work have been presented elsewhere (15).

MATERIALS AND METHODS

Reagents: Reagents were obtained from the following sources: 3-amino-1,2,4-triazole, diaminobenzidine (DAB), horseradish peroxidase (HRP, Type VI), leucine p-nitroanilide-HCl, phenylmethylsulfonyl fluoride (PMSF), soybean trypsin inhibitor (SBTI), trypsin, and protein A from the Sigma Chemical Co. (St. Louis, MO); Emulphogene BC-720 from GAF Co. (NY); tosylarginine methyl ester (TAME) from Calbiochem-Behring Corp. (San Diego, CA); Trasylol from Mobay Chemical Corp. (NY); tetramethylrhodamine isothiocyanate (TMRITC), 479 mol wt, from Bethesda Biological Laboratories, (Bethesda, MD); α-chymotrypsin from Worthington Biochemical Corp. (Freehold, NJ); carrier-free $Na^{125}I$ from Amersham Corp. (Arlington Heights, IL); whole goat serum and rhodaminated goat anti-guinea pig IgG from Cappel Laboratories (Cochranville, PA); DEAE (DE-52 pre-swollen microgranular) from Whatman Ltd. (Maidenstone Kent, England); Sepharose 4B, Sephadex G-25 and G-200, and protein A from Pharmacia Fine Chemicals (Piscataway, NJ); Ultrogel Ac34 from LKB Instrument Inc. (Rockville, MD); nitrocellulose paper from Schleicher and Schuell (Keene, NH); and chloramine-T from the Eastman Kodak Co. (Rochester, NY). All other chemicals were of reagent grade.

LAP Purification: The purification of rat intestinal LAP was initially based on the protocols of Maroux et al. (16, 17) for the isolation of LAP from porcine intestine. Small intestines were removed from decapitated rats (male, Sprague-Dawley, from Charles River Breeding Laboratories, Wilmington, MA) and flushed with cold 0.15 M NaCl containing 1 mM TAME, 0.5 mM PMSF, 1 μg/ml antipain, 1 μg/ml pepstatin, and 25 U/ml Trasylol. Each intestine was slit along its length and the mucosal surface scraped with a glass slide. The scrapings (~3 g/350 g rat) were collected in 50-ml conical tubes and frozen at −20°C for 1 wk to 4 mo before further processing.

Approximately 100–130 g of tissue were processed for each purification. All procedures were carried out at 4°C and aliquots were saved at each step for LAP activity and protein measurements. The frozen mucosa was thawed by adding 2 vol of Buffer A (10 mM Na phosphate, pH 6.0, 0.02% NaN_3), with 1 mM TAME, 0.5 mM PMSF, 1 μg/ml antipain, 1 μg/ml pepstatin, 0.5 mg/ml SBTI, and 25 U/ml Trasylol, to 1 g (wet weight) of tissue and stirring for 1 h. This slurry (H) was centrifuged at 22,000 *g* for 1 h (JA-14 rotor, Beckman J-21) and the resulting pellet (P1) resuspended in Buffer A containing 2% Emulphogene and protease inhibitors. After stirring for 2 h, the slurry was centrifuged for 4 h (23,000 *g*). The supernate (S2) was collected and DEAE (equilibrated in Buffer A, 1% Emulphogene) was added in a 30-fold excess of S2 protein (by weight). The mixture was gently stirred for 20 min and then filtered on a Buchner funnel (Whatman #2). One-third of the initial amount of DEAE was added to the filtrate and the resulting slurry incubated and filtered as described above. Equilibration buffer was added to the combined filtercakes and the degassed slurry packed into a 2.5 × 60 cm column at a flow rate of 140 ml/h. After the absorbance at 280 nm reached 0, the bound material was eluted with a linear salt gradient of 0–0.3 M NaCl in equilibration buffer at a flow rate of 43 ml/h; 8-ml fractions were collected. The volume of the salt gradient was four times the packed DEAE column volume (mean volume 450 ml, range 350–500 ml). LAP activity eluted from the column in a peak between 0.02 and 0.06 M NaCl comprised of 12 fractions. The eluted material was divided into two (early and late) pools which were concentrated (Amicon, PM-10 membrane) separately and were further divided into several aliquots so as not to overload subsequent columns. The early pool had approximately two to four times the specific activity of the late pool.

The DEAE fraction (5 ml, 65 mg protein) was chromatographed by reverse flow on an Ultrogel Ac34 column (LKB Instruments: 2.5 × 100 cm, Buffer A, 0.5% Emulphogene). LAP eluted at a relative elution volume of 1.2 (Ve = 300 ml with Blue Dextran used as a void volume marker). Trypsin[2] was added to the concentrated Ultrogel pool in a protein:protease ratio of 50:1 (wt/wt) and, after a 4-h incubation at 4°C, the sample was chromatographed on a G-200 column (1.5 × 100 cm, in 10 mM Na phosphate, pH 7.0, 0.02% NaN_3). LAP activity eluted in the void volume, and the peak was divided into early and late pools to insure that at least half of the preparation would be free of lower molecular weight contaminants that were sometimes observed.

The purification of LAP was monitored by measurements of enzyme activity and SDS PAGE analysis. The results are presented in Table I and Fig. 1. The DEAE step yielded the greatest enrichment in both enzyme activity (70-fold, Table I) and Coomassie Blue staining material at ~120 kdaltons (Fig. 1, lane *D*). The success of this chromatographic step depended on three factors: (*a*) the inclusion of protease inhibitors in all steps prior to the ion exchange column; (*b*) the binding of >85% of the enzyme activity to the resin which was achieved by adding a 40-fold excess of DEAE (by weight) to S2 protein; and (*c*) the dividing of the DEAE peak into early and late pools. The early pool had three to four times the total activity of the late peak while the protein was equally divided between the two pools.

The final fraction (G-200), whose specific activity was 1,421 μmol/h/mg of protein, was 302-fold enriched in LAP activity over the starting material (Table I) and consisted predominantly of a polypeptide migrating at ~120 kdalton (Fig. 1, lane *G*). This band represented 82–90% of the total Coomassie-Blue-staining material in the G-200 fraction (four preparations). An additional band directly below the major component was sometimes observed (Fig. 1, lane *G*); however, chymotryptic maps of this polypeptide and the 120-kdalton band showed correspondence among many of the peptides (data not shown). We analyzed all G-200 pools by SDS PAGE prior to use as an antigen. Only those pools demonstrating >85% of the total Coomassie Blue material at the 120-kdalton band were used to produce anti-LAP antibodies.

Antibody Preparations: Antibodies to intestinal LAP were raised in chickens and guinea pigs.

CHICKENS: We injected female white Leghorn chickens (4–6 mo) with 130 μg of antigen (G-200 fraction) emulsified in an equal volume of Freund's adjuvant (total volume = 1 ml). The immunization schedule of Gerace et al. (18) was followed and precipitating antibodies were obtained 10 d after the third injection. The total amount of antigen injected was 390 μg. The whole immune serum was stored at −20° or −70°C.

GUINEA PIGS: We injected 25 μg of intestinal antigen in complete adjuvant into the footpads of male guinea pigs (500 g). We administered intraperitoneal and intramuscular boost injections according to the schedule cited (18) except that precipitating antibodies were obtained after the second inoculation (total amount of injected protein = 50 μg). Serum was stored at −20° or −70°C.

Precipitating antibodies were detected in all chickens and guinea pigs immunized with intestinal LAP (Fig. 2 *a* and *b*). No precipitation line was formed with preimmune serum. Although we used the chicken for most of the localization studies described below, we have verified the results obtained with the guinea pig antibodies. These are noted.

AFFINITY PURIFICATION OF ANTI-LAP ANTIBODIES: LAP-specific antibodies were purified from whole serum using a LAP-Sepharose column. 2 mg of the G-200 fraction was linked to 2 ml of packed CNBr-activated Sepharose 4B (19) in the presence of 0.1 M Na phosphate, pH 8.0, with a coupling efficiency of 80–90% (monitored by enzyme activity). A 2-ml column was prepared and equilibrated with phosphate-buffered saline (PBS) containing 2% Triton X-100. An aliquot of serum (1 ml) was clarified by centrifugation (100,000 *g*, 15 min) and Triton X-100 was added to the resulting supernatant (final concentration 2%). This material was applied to the affinity column at 4°C at a rate of 1.5 ml/h. After extensive washing at 4°C, the column was equilibrated to room temperature and bound IgG was eluted with 1.5 column volumes of 3.0 M KSCN in 0.1 M triethanolamine (TEA-HCl), pH 8.5. The eluted fraction was immediately desalted on a G-25 column equilibrated with PBS or dialyzed overnight against PBS. The pooled fractions or dialyzed samples were concentrated to 0.5–1.0 mg protein/ml and stored on ice at 4°C. Protein concentrations were determined by measuring OD_{280} nm with $E^{1\%}_{1cm} = 14$ (20).

ADSORPTION AGAINST A MUCOSAL PELLET: Preliminary fluorescent localization studies revealed that, in addition to the brush-border staining, the content of goblet cells reacted with immune whole serum. Therefore, a mucosal fraction was prepared according to a modification of the brush border isolation of Louvard et al. (1). That is, the pellet obtained after centrifugation of the homogenate at 1,800 *g* for 10 min was resuspended in 10 mM Tris-HCl, 1 mM $CaCl_2$, 10 mM $MgCl_2$, 0.25 M sucrose, 1 μg/ml antipain, 1 μg/ml pepstatin, 1

[2] Trypsin was shown by Maroux et al. (16) to hydrolyze the detergent form of LAP to a smaller fragment that no longer aggregated in aqueous buffer and, thus, presumably was devoid of a hydrophobic peptide segment.

TABLE I

*Summary of LAP Purification**

Fraction	Total activity	Yield	Total protein	Specific activity	Purification
	μmol/h	*%*	*mg*	*μmol/h/mg*	*-fold*
Homogenate	46,140	100	9,760	4.1	1
Low salt pellet	38,220	83	4,260	9	1.9
Detergent extract	32,093	70	2,025	16	3.4
DEAE peak 0.04-.06 M NaCl‡	15,790	34	50	330	70
Ultrogel§	6,444	14	12	537	114
Sephadex G-200‖	3,980	9	2.8	1,421	302

* The values presented are from one LAP purification. The amounts of LAP activity and protein were measured in fractions enriched in LAP activity, and the specific activity, percent recovery, and purification of LAP were calculated. The yield of LAP in each fraction represents the percent of homogenate activity recovered in that fraction. The purification was calculated in each fraction and normalized to homogenate specific activity.

‡ The values given are for the early pool of the DEAE peak that contained 3.5 times more LAP activity than the second portion of the peak.

§ These values are the sum of the activities in the material obtained from the 1 Ultrogel column on which the early DEAE material was chromatographed.

‖ These values are the sum of the activities obtained from the 2 G-200 columns on which the above mentioned Ultrogel material was chromatographed. We chose the highest specific activity that we obtained for the G-200 fraction, 1,640 μmol/h/mg protein, as the value for our pure enzyme.

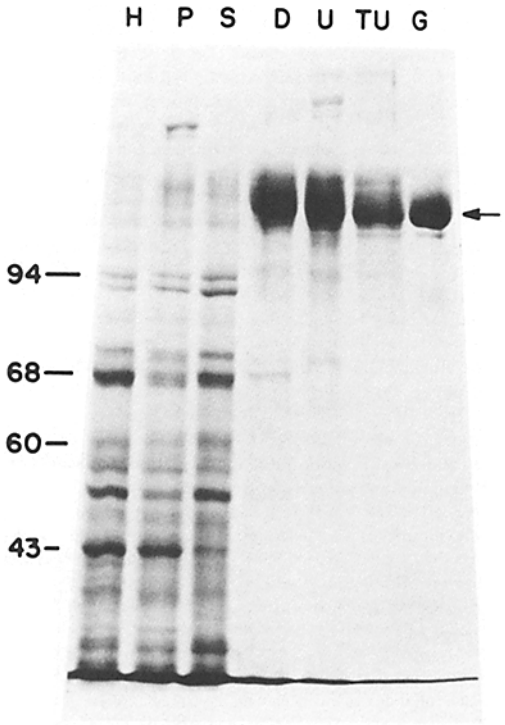

FIGURE 1 SDS PAGE of fractions from an intestinal LAP purification. Samples were prepared, electrophoresed in 8% polyacrylamide gels, stained with Coomassie Blue R, and destained as described in Materials and Methods. (*H*) Homogenate, 100 μg. (*P*) Pellet, P1, 100 μg. (*S*) Detergent extract, 100 μg. (*D*) DEAE, 70 μg. (*U*) Ultrogel, 50 μg. (*TU*) Trypsinized Ultrogel, 50 μg. (*G*) G-200, 25 μg. The arrow points to the 120-mol wt region. Molecular weights, 10^{-3}.

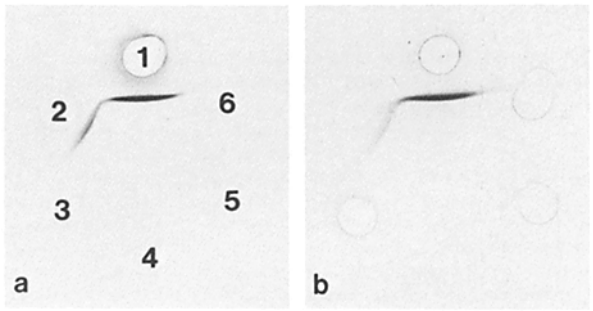

FIGURE 2 Double immunodiffusion of chicken and guinea pig antibodies to intestinal LAP. (*a*) 2.56 μg of G-200 protein was added to the center well and 360 and 180 μg of immune whole chicken serum were added to wells *1* and *2*. (*b*) 3.6 μg of G-200 proteins added to the center well and 250 and 125 μg of mucin-adsorbed whole guinea pig serum were added to wells *1* and *2*. Equivalent amounts of nonimmune chicken serum (*a*, 360 and 180 μg) or guinea pig serum (*b*, 250 and 125 μg) were added to wells *3* and *4*, respectively.

mM benzamidine, 1 mM TAME, 0.5 mM PMSF, and 25 U/ml Trasylol and subjected to three rounds of resuspension and centrifugation (1,800 *g* × 10 min). Removal of brush-border material was monitored by disappearance of LAP activity from the pellet. The final mucosal pellet contained no detectable enzyme activity and was found by phase-contrast microscopy to be composed of clumped nuclei and fibrous material. An aliquot of this material (0.15 ml) was incubated with whole anti-LAP serum (1 ml) for 12–15 h at 4°C, after which the mucosal material was removed by sedimentation (100,000 *g*, 15 min). The adsorbed-serum exhibited only brush-border immunofluorescence (see below). Commercial preparations of submaxillary mucin (Sigma Chemical Co., bovine Type I) were not effective in removing goblet cell staining.

RHODAMINE-CONJUGATED GUINEA PIG ANTI-CHICKEN: Antibodies to chicken IgG were raised in guinea pigs. An IgG fraction was prepared from immune serum by sodium sulfate precipitation followed by DEAE-cellulose chromatography (20, 21). The material eluting at the void volume was collected, and electrophoresis on a 5% polyacrylamide gel (nonreducing conditions) demonstrated that this material was devoid of contaminants. Double immunodiffusion (18, 22) demonstrated its reactivity with chicken IgG.

Guinea pig anti-chicken IgG (6–11 mg) was conjugated with TMRITC (120–240 μg) and then fractionated on DEAE as described by Brandtzaeg (23). Material eluting at 0.05 and 0.1 M NaCl was collected, concentrated, aliquoted, and frozen at −20°C. The final conjugate had a fluorochrome-to-protein ratio (OD_{515}/OD_{280}) of 0.95–1.3 and its reactivity with chicken IgG was demonstrated by double immunodiffusion. The rhodaminated second antibody was pre-adsorbed against either fixed-quenched intestinal mucosa or liver homogenate before use in localization studies. For preparation of the adsorbents, intestinal or liver homogenates (0.5 ml) were fixed with 4% formaldehyde, freshly prepared from paraformaldehyde in 0.1 M Na phosphate, pH 7.4, for 1 h on ice and then quenched for 15 min on ice with three changes of a solution containing 0.5 mg $NaBH_4$/ml 0.1 M Tris-HCl, pH 7.5. The quenched homogenates were then centrifuged for 5 min in a microfuge (Eppendorf 5412), and the resulting pellet was resuspended in 0.5 ml of 20 mM Na phosphate, pH 7.4. 500 μl of rhodaminated guinea-pig anti-chicken was incubated with 200 μl of quenched homogenate for 16–18 h at 4°C with constant agitation. The particulate material was removed by centrifugation (100,000 *g*, 30 min) and the resulting supernatant divided into aliquots and frozen at −20°C.

Rhodaminated goat anti-guinea pig IgG was purchased from Cappel Laboratories and pre-adsorbed against intestinal or liver homogenates prior to use.

Immunofluorescent Localization of LAP

INTESTINES: Small intestines were removed from decapitated rats and were flushed with the saline-inhibitor mix as described above. A 20-cm segment was filled with 4% formaldehyde in 0.1 M Na phosphate, pH 7.4, placed in a beaker containing the same fixative, and incubated for 30 min on ice. All subsequent steps were carried out at 0–4°C. The tissue was then quenched for 10 min with a solution containing 0.5 mg $NaBH_4$/ml 0.1 M Tris-HCl pH 7.4, infused with 1

M sucrose in 0.1 M Tris-HCl, pH 7.4, filled with O.T.C. embedding compound (Lab-Tek Div., Miles Laboratories, Inc., Naperville, IL), and frozen in isopentane cooled in liquid nitrogen. The tissue blocks were stored at −70°C.

Sections were cut at the 2–4-μm setting of the cryostat (American Optical, −20 to −22°C) and mounted on gelatin-coated slides. Sections were quenched for 15 min at 22°C with three changes of $NaBH_4$/Tris-HCl. The sections were rinsed in Tris and then 20 mM Na phosphate, 0.5 M NaCl, pH 7.4. The first antibody (chicken anti-LAP or nonimmune preparations) was appropriately diluted with 10 mM Na phosphate, 0.25 M NaCl pH 7.4, nonimmune guinea pig serum was added to a final concentration of 10% and the mixture (200–300 μl) applied to the slides. After incubating the sections for 1 h in a humidified box at 37°C, we washed the slides in 20 mM Na phosphate, 0.5 M NaCl for 15 min at room temperature. The slides were then incubated with rhodaminated second antibody (guinea pig anti-chicken) under the conditions described above. The rinsed slides were mounted with PBS:glycerol (3:1) and sections observed by epifluorescence in a Leitz photomicroscope. Photographs were taken with Kodak Tri-X film at an ASA setting of 800 and exposures of 15–60 s for the intestine and 30–120 s for the liver.

LIVERS: Rat livers were fixed by perfusion (24) for 5 min with 4% formaldehyde in 0.1 M Na phosphate, pH 7.4. After removal of the liver, ~1–2-mm slices were cut and fixed for an additional hour on ice. The slices were then rinsed with several changes of 0.1 M Tris-HCl, pH 7.5, prior to quenching and freezing as described above. The immunofluorescent staining for LAP in liver tissue was carried out as described for the intestine except that the second antibody was preadsorbed against fixed-quenched liver homogenate. Experiments with the guinea pig anti-LAP preparations followed the same protocol except that antibodies were diluted with PBS and 10% nonimmune goat serum.

We used HRP to mark the blood sinusoids. We injected HRP (25 mg/100 g body weight in 10 mM Na phosphate, pH 7.4) into the saphenous vein of an anesthetized rat, and after 5 min the liver was fixed by perfusion. The preparation of the tissue for freezing and the subsequent handling of the cryostat sections were carried out as described above for liver tissue incubated with chicken antibodies. To visualize the peroxidase, we incubated cryostat sections with a DAB-amino-triazole mixture (25) for 10 min at 22°C between applications of the first and second antibodies.

Cryostat sections were also reacted for alkaline phosphatase (AP) according to the metal-salt procedure of Gomori (26) as described by Lojda et al. (27). Trimetaphosphatase (TMP) staining of cryostat sections was carried out using the protocol of Oliver (28), except that: (*a*) sucrose was omitted; (*b*) tissue sections were incubated with 2% $CaCl_2$ for 10 min at 22°C prior to their treatment with ammonium sulfide; and (*c*) sections were treated with 1% OsO_4 in 0.1 M cacodylate buffer, pH 7.4 (45–60 min 22°C), after ammonium sulfide (29). Controls for AP and TMP reactions consisted of omission of substrate from the incubation mix and were blank.

FROZEN THIN SECTIONS: Liver tissue was fixed as described above and either processed immediately or stored up to 1 wk in 2% formaldehyde, 1 M sucrose in 0.1 M Na phosphate, pH 7.4. Prior to freezing in liquid nitrogen, the tissue blocks were infused for 20–30 min at 22°C with 1.2 M sucrose in 0.1 M Na phosphate, pH 7.4. ~200–500-nm sections (estimated by the color of the section) were cut on a MT-2B Ultramicrotome (Sorval Instruments, Spinco Div., Palo Alto, CA) fitted with a cryokit attachment. The sections were reacted for immunofluorescence according to the methods of Tokuyasu (30), except that: (*a*) the cut sections on polylysine-coated slides (0.1 mg/ml) were fixed for 5 min with 2% formaldehyde in 0.1 M Na phosphate, pH 7.4; (*b*) the 2% gelatin-PBS wash during the quenching steps was omitted; and (*c*) both antibodies were diluted with PBS to which nonimmune goat serum was added to a final concentration of 10%. All incubations were carried out for 30 min at 22°C in a humidified box.

Analytical Procedures

ASSAYS: LAP. The activity was measured by following the release of nitroaniline from leucine *p*-nitroanilide. We added 0.05 ml of sample to 0.45 ml of buffer-substrate mix containing 50 mM Na phosphate pH 8.0 and 4 mM leucine *p*-nitroanilide. The reaction was carried out for 30 and 60 min in a shaking waterbath at 37°C. The reaction was stopped with 0.05 ml of 40% TCA, and 0.45 ml of an alkaline reagent (31), pH 10.7, was then added and absorbance measured at 410 nm. A molar extinction coefficient of 8,800 M^{-1} for nitroaniline was used to calculate activity (16). Protein determinations were carried out by the method of Lowry et al. (32) with bovine serum albumin as the standard, or of Bradford (33) using a standard of bovine gamma globulin and Bio-Rad dye reagent.

SDS PAGE: One-dimensional gel electrophoresis was performed by the method of Maizel (34). Phosphorylase A (94 kdalton), albumin (68 kdalton), catalase (60 kdalton), muscle actin (43 kdalton), chymotrypsinogen (26 kdalton), and cytochrome *c* (11 kdalton) were run as standards. Gels were scanned on a Gilford 260 spectrophotometer. The percent contribution of a given polypeptide to the total Coomassie-Blue-staining material was determined from the gel scans by weighing the peak of interest and the total 560-nm profile of the electrophoresed sample and taking a weight ratio.

IODINATION: Protein A was iodinated by the Chloramine-T procedure of Greenwood et al. (35) to a specific activity of 3.6–6.8 × 10^7 counts/min/μg.

PEPTIDE MAPS: Chymotryptic maps were prepared according to the procedure of Elder et al. (36).

PROTEIN A-SEPHAROSE: Protein A was coupled to CNBr-activated Sepharose 4B by the protocol of March et al. (19). Phosphate buffer at pH 8.0 (0.1 M) was used and the coupling efficiency, determined by the addition of ^{125}I-protein A (1 μCi), ranged from 93% to 97%.

Immunoprecipitations

SOLUBILIZATION OF INTESTINAL MUCOSA AND LIVER PM: An Emulphogene extract was prepared from intestinal mucosa (10 g) as described in the LAP purification protocol. Liver PM (10 mg), prepared according to Hubbard et al. (37), was extracted with 50 mM carbonate buffer, pH 10.5 (37). The resulting pellet was resuspended in 10 mM Na phosphate, pH 6.0, 2% Emulphogene, 0.02% NaN_3, 1 mM benzamidine, 1 μg/ml antipain, 1 μg/ml pepstatin, 1 mM PMSF, 10 U/ml Trasylol. After stirring for 2 h at 4°C, the suspension was centrifuged at 100,000 *g* for 30 min. The supernatant was collected and the pellet resuspended in the above buffer for enzyme and protein determination.

IMMUNOPRECIPITATION: Immunoprecipitations were carried out as described by Mellman and Unkeless (38) with the following modifications: (*a*) the detergent extracts were adjusted to 0.15 M NaCl and pH 7.4; (*b*) the total volume of the reaction mix ranged from 400 to 450 μl; (*c*) antibody incubations (25–200 μg of LAP-specific or nonimmune guinea pig IgG) were carried out for 2–3 h at 4°C with constant agitation; (*d*) the mixed detergent solution contained 0.05% Triton X-100, 0.1% SDS, 0.3 M NaCl, and 10 mM Tris-HCl, pH 8.6; (*e*) the beads were recovered by centrifugation for 5 min in a microfuge; and (*f*) the antigen was eluted from the Sepharose beads as described by Gerace and Blobel (39). We assessed the efficiency of immunoprecipitation by monitoring the loss of enzyme activity from the supernate following incubation of the antigen-antibody complex with protein A-Sepharose. We monitored loss of enzyme activity from the soluble fraction to assess antigen-antibody-protein A-Sepharose binding, because the adherence of the Sepharose beads to the walls of the tubes and pipettes made accurate measurements of bead-associated activity difficult. The amount of liver PM extract or intestinal mucosa used in each experiment contained the equivalent of 20 μg of LAP protein. This amount was calculated from the specific activities of the LAP in a particular fraction and of purified LAP (1,640 μmol/h/mg protein).

BLOT TRANSFER: Polypeptides that had been separated were transferred to nitrocellulose for 4 h (400 mA) according to the protocol of Towbin et al. (40). Following transfer, the nitrocellulose (NC) was either dried between two pieces of filter paper and stored for later use or incubated for 1 h at 22°C in a filtered solution (Millipore, GS 0.22 μm) of wash buffer (WB: 0.15 M NaCl, 0.2% Triton X-100, 10 mM Tris-HCl pH 7.4, 0.05% NaN_3) containing 4% BSA. The NC was incubated with affinity-purified antibodies (2.5–75 μg in 10 ml) in the same buffer for 11–15 h at 4°C with constant agitation. The strips were washed for 20 min with four changes of WB, 15 min in 2 M urea, 1% Triton X-100, 0.1 M glycine, and then 5 min with 1 change of WB. ^{125}I-protein A (0.5–1 × 10^5 counts/min/ml, 10 ml) in filtered 4% BSA-WB was added and the strips were incubated for an additional 4 h at 4°C. The labeled strips were washed as described above, dried, and exposed to Kodak X-Omat AR film for 4–48 h at −70°C using an intensifying screen (DuPont Instruments, Wilmington, DE, Lightning Plus). Transfers were stained after autoradiography in 0.1% Amido-black (Bio-Rad Laboratories, Richmond, CA) in 50% methanol, 10% acetic acid, and destained in 90% methanol followed by 25% methanol, 10% acetic acid.

RESULTS

Intestinal LAP was chosen as the antigen for our studies because: (*a*) a significant amount of LAP protein could be obtained from a small amount of intestinal tissue, since the enzyme represents ~8% of the brush-border protein (41) (in liver, we estimate that LAP constitutes 0.2–0.5% of the plasma membrane protein); (*b*) a purification scheme for intestinal LAP had been published (16, 17); and (*c*) antibodies directed against intestinal LAP cross-react with the kidney enzyme (3), suggesting that similarities exist among LAPs in different tissues.

Localization of LAP in Intestinal Tissue

The localization of LAP was first determined on cryostat sections of fixed-quenched small intestine because the antigen

was obtained from this source, and the exclusive localization of LAP to the brush-border membrane had been previously established (4). When tissue sections were reacted with immune whole chicken serum, we observed an intense staining of the brush borders (Fig. 3*a*). Fluorescent labeling of the other surfaces of the enterocytes or of intracellular structures was not detected.

Localization of the Liver Antigen Recognized by Anti-LAP Antibodies

IMMUNOFLUORESCENT STAINING OF CRYOSTAT SECTIONS: The immunofluorescent localization of the liver antigen is presented in Fig. 4*b* and the corresponding bright field image in Fig. 4*a*. The fluorescent pattern was one of anastomosing networks of branched channels as well as circular profiles restricted to regions between adjacent cells. To more clearly identify the surface domains, we marked the blood sinusoids by injecting high doses (25 mg/100 g body wt) of HRP into the circulation of a rat 5 min before killing. The cells lining the blood space pinocytose this molecule which can be detected cytochemically. Erythrocytes present in the sinusoids also react and appear black. Cryostat sections incubated with anti-LAP antibodies and reacted for peroxidase activity (Fig. 4*a* and *b*) revealed that the immunofluorescent pattern *did not* correspond to that of the internalized peroxidase. Less than 3% of all fluorescent images seen appeared associated with peroxidase-positive regions.

The fluorescent image of branched channels and circular profiles was the overwhelming one observed in livers from 15 rats (four of which were injected with HRP). We observed little to no intracellular staining (Fig. 4*b*), and nonimmune controls were blank (Fig. 4*e*). Removal of non-LAP antibodies by adsorption against intestinal mucin did not alter the fluorescent pattern. The liver staining pattern suggested that the antigen was highly concentrated in one domain of the hepatocyte which appeared morphologically to be the bile canaliculus. The sinusoidal and lateral surfaces appeared to be devoid of the antigen.

ALKALINE PHOSPHATASE AND TRIMETAPHOSPHATASE: The cytochemical localization of alkaline phosphatase (AP) to the bile canalicular domain of the hepatocyte plasma membrane has been documented (42). To confirm that the liver antigen recognized by the anti-LAP antibodies was localized to the canalicular membrane, we compared the fluorescent image described above to the image obtained after reacting cryostat sections for AP. Fig. 4*b* and *c* demonstrates the general similarity of the two patterns. In tissue sections reacted for AP, the reaction product was restricted to circular areas between hepatocytes and channels coursing over the sections.

Reaction of cryostat sections for TMP provided evidence that the antigen was *not* associated with the lysosomes found near the bile canaliculus (Fig. 4*f*). In contrast to the AP and immunofluorescent patterns observed, the TMP reaction product was granular and showed a more cytoplasmic distribution. While many cells demonstrated a concentration of precipitate near the surface, the dense staining region appeared to be inside the cells.

FROZEN THIN SECTIONS: The results of the localization studies described above suggested that the antigen recognized by the anti-intestinal antibodies was specifically localized to the canalicular membrane domain. However, to demonstrate this localization more conclusively, we carried out indirect immunofluorescent localization studies on 0.5-μm frozen sections. For these studies and the immunoprecipitation experiments, the chicken antibody proved difficult to work with, requiring high salt concentrations (0.5 M) and high concentrations of affinity-purified antibodies to obtain positive staining. Therefore, we subsequently used guinea pig anti-LAP preparations.

First, the cellular organization seen in 0.5-μm sections was more defined than that seen in thicker sections. The three domains of the hepatocyte surface were clearly identifiable (Fig. 5*a*). The sinusoidal front faced large blood spaces containing lining cells and an occasional erythrocyte. The lateral surface appeared as an organelle-free line between adjacent cells. The bile front domain was characterized by a clear circular or elliptical space between adjacent cells. The immunofluorescent staining pattern was also more distinct in 0.5-μm sections using affinity-purified guinea pig anti-LAP antibodies. We found the immunostaining to be restricted to the canalicular surface of the hepatocytes (Fig. 5*a* and *b*). We did not detect fluorescence in regions identified as sinusoidal by size, the presence of erythrocytes or sinusoidal lining cells. Furthermore, we detected no intracellular staining.

When whole serum or adsorbed serum was used for localization studies, a similar staining pattern was obtained (data not shown). The material not bound to the affinity column was also collected. No specific fluorescence was noted in 0.5-μm sections incubated with this unbound fraction, even when concentrations 10–20 times those of whole serum were used (data not shown). No brush-border staining was observed when this same adsorbed antibody preparation was incubated with cryostat sections of intestine (data not shown). The loss of intestinal and hepatocyte staining from immune serum after passage over the affinity column suggested that antibodies directed against an intestinal antigen recognized a similar

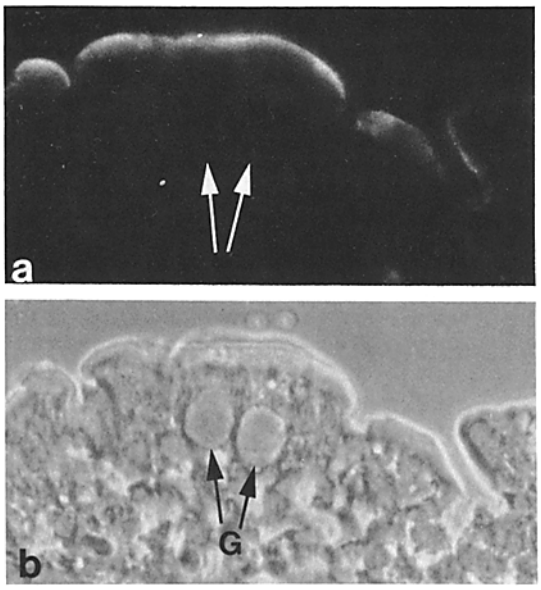

FIGURE 3 Immunofluorescent localization of LAP in cryostat sections of fixed intestine. Cryostat sections were first incubated with adsorbed chicken serum serum and then with rhodaminated guinea pig anti-chicken. The intense fluorescence staining (*a*) corresponds to the brush border, seen as a refractile zone in the corresponding phase micrograph (*b*). Incubations of the antibodies with a mucosal extract from intestine removed the goblet cell staining (arrows) without diminishing the staining of the brush border (see Materials and Methods). × 850.

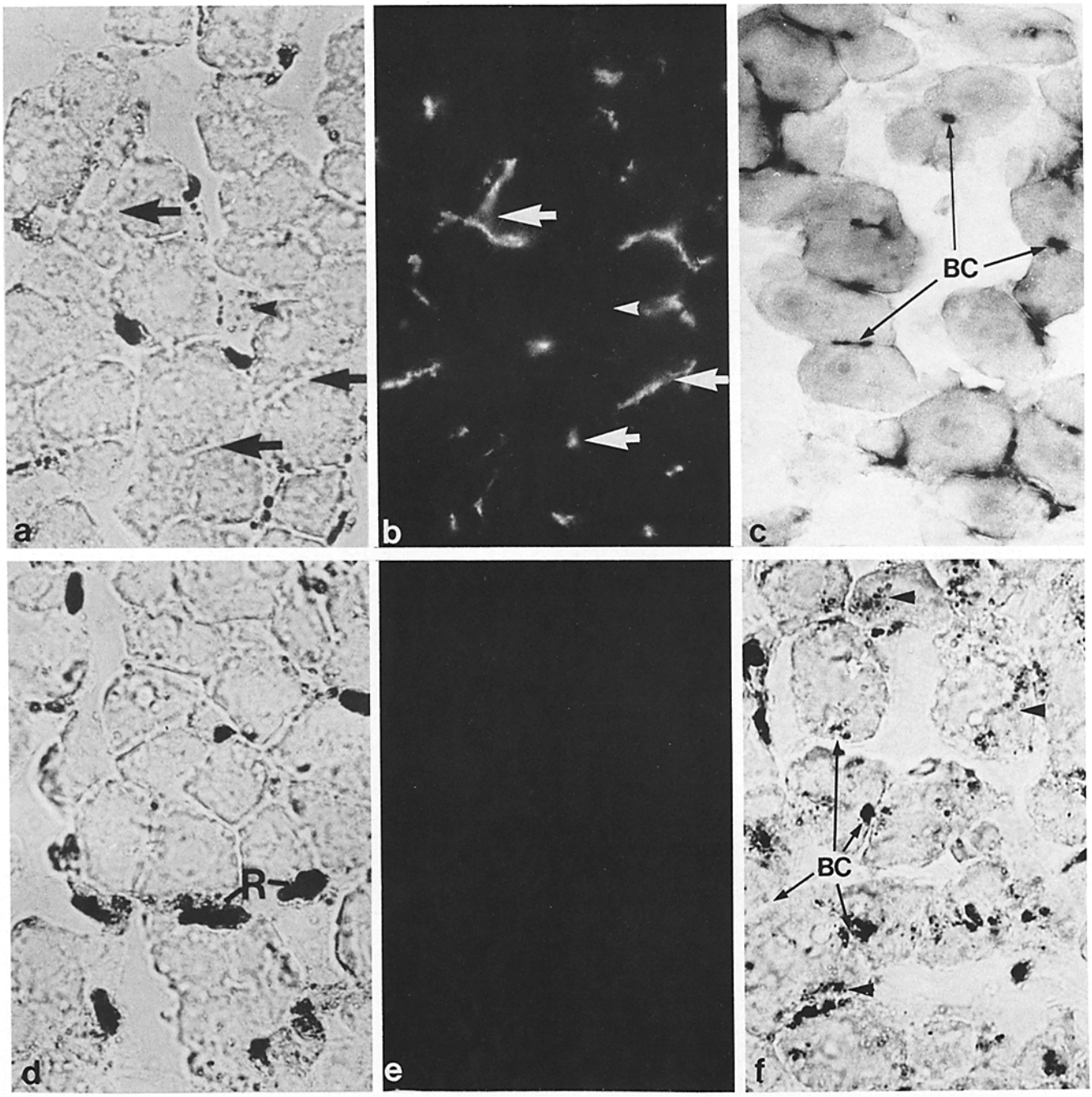

FIGURE 4 Immunofluorescent localization of the liver antigen in cryostat sections of fixed liver. Cryostat liver sections from HRP-injected rats were incubated with either immune (*a* and *b*) or nonimmune (*d* and *e*) whole chicken serum. After incubation with this first antibody, slides were reacted for peroxidase activity and then incubated with the rhodaminated second antibody. Arrows indicate corresponding regions on the fluorescence and light micrographs. Arrowheads mark HRP reaction product, and RBC (R) are identified. × 820. (*c*) Cryostat section of liver tissue reacted for alkaline phosphatase. The stained areas between adjacent cells have been marked as bile canaliculi (*BC*). × 870. (*f*) Cryostat section from the same liver used in *c* incubated for lysosomal trimetaphosphatase activity. The staining appeared to be concentrated in regions morphologically identified as bile canaliculi (*BC*) and also was seen within the cell (arrowheads). × 890.

antigen on the bile canalicular membrane. However, these studies did not identify the liver antigen as LAP.

Immunoprecipitation

Having established the presence of a domain specific antigen(s) in the liver, we next carried out immunoprecipitation experiments to identify the reactive component(s) in the liver. For all of the immunoprecipitation studies, the amount of starting material (mucosal detergent extract, liver PM detergent extract, etc.) represented 20 μg of LAP protein. This value was derived from the specific LAP activity of the fraction to be immunoprecipitated (which varied from 11 to 1,581 μmol/h/mg) and the specific activity of the purified intestinal mucosal enzyme (1,640 μmol/h/mg protein). First, fractions from intestinal mucosa that were highly-enriched in LAP (SA = 1581 μmol/h/mg protein) were incubated with affinity-purified anti-LAP antibodies and protein A-Sepharose, followed by LAP measurements and SDS PAGE of the various fractions. Loss

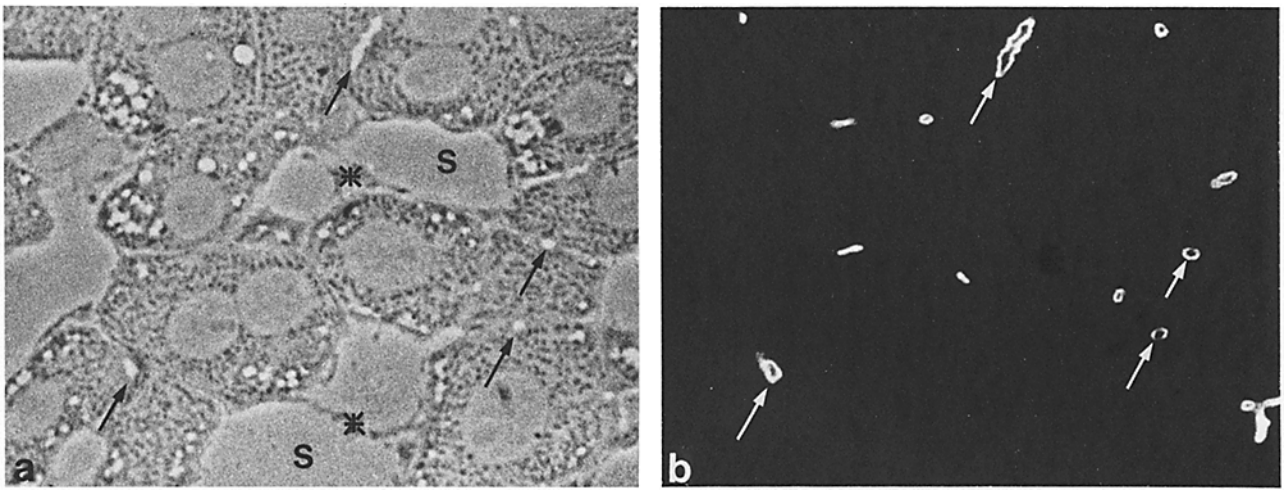

FIGURE 5 Immunofluorescent localization of the liver antigen in 0.5-μm sections. 0.5-μm sections were incubated with affinity-purified antibodies. Sinusoidal lining cells (*) and sinusoids (*S*) are easily identified in the light micrograph. Arrows indicate corresponding regions between light (*a*) and fluorescence (*b*) micrographs. × 1,000.

of enzyme activity from the soluble (unbound) fraction was correlated with loss from this same fraction of polypeptide bands migrating at 110–130 kdaltons on SDS PAGE and the appearance of this material in the specific immunoprecipitates (Fig. 6, lanes *P1* and *P2* and Table II, lines *1* and *2*). That is, the addition of 25 μg of affinity-purified guinea pig anti-LAP removed ~10-μg equivalents (52%) of LAP protein and yielded ~46% of the initial 120-kdalton material in the final immunoprecipitate (Fig. 6, *P1*). Increasing the antibody input to 200 μg resulted in removal of nearly all (91%) the activity from the soluble fraction and appearance of 92% of the 120-kdalton band in the immunoprecipitate (Fig. 6, lane *P2*). A similar comparison could not be made with any other band on the SDS gel, suggesting that the 120-kdalton material (or some multimer of it) was responsible for all the enzyme activity. In samples incubated with nonimmune guinea pig IgG, 10% of the initial activity was removed from the soluble fraction (Table II, line *3*), but no material migrating at the 120-kdalton region in the immunoprecipitates was detected (Fig. 6, lane *P3*). At present, the identities of material migrating at 150 and 94 kdaltons in both immune and nonimmune samples (Fig. 6, lanes *P2* and *P3*) are not known.

A comparison of the immunoprecipitates from detergent extracts of intestinal mucosa and liver PM is presented in Fig. 7. We chose liver plasma membrane as the starting material for the following reasons: (*a*) detergent extraction of liver PM yielded a fraction that had nearly the same specific activity as the intestinal extracts (11–16 μmol/h/mg protein), so that equivalent amounts of total protein could be used; and (*b*) indirect immunofluorescence demonstrated that the liver antigen was localized to the canalicular plasma membrane and showed no intracellular distribution.

The major Coomassie-Blue-staining material in the immunoprecipitate from the Emulphogene extract of intestinal mucosa migrated as a broad band between 110 and 130 kdaltons; three bands could often be resolved in this region (Fig. 7, intestine-IP). Additional bands (*2–4*) of higher molecular weight were often seen in this sample but the number and intensity of these multiple bands varied among preparations. The factors responsible for their generation are not known. Bands migrating between 110 and 130 kdaltons were not detected in intestinal samples precipitated by nonimmune IgG (intestine-NP). The presence of additional bands at ~94 and 60 kdaltons were sometimes noted (Fig. 7), but their identity is unknown.

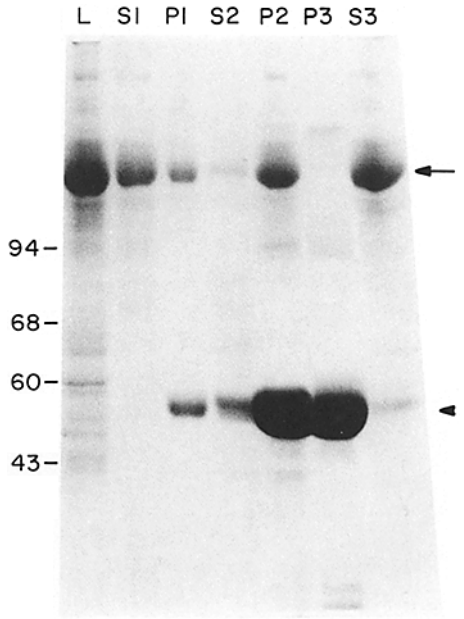

FIGURE 6 Immunoprecipitation of LAP from intestinal samples. Samples estimated to contain 20 μg of LAP protein (G-200 fractions) were incubated first with either 25 μg or 200 μg of affinity-purified guinea pig anti-LAP antibodies or 200 μg of nonimmune IgG and then protein A-Sepharose (50 μl, at 1 mg protein A/ml Sepharose). After elution from the beads, the immunoprecipitated samples and the unbound material were electrophoresed on a 8% polyacrylamide gel. (*L*) Load (33 μg G-200 fraction). (*S1*) Supernate from 25 μg Ab incubation (total). (*P1*) Immunoprecipitate 25 μg Ab (total). (*S2*) Supernate from 200 μg Ab incubation (total). (*P2*) Immunoprecipitate 200 μg Ab (total). (*P3*) Immunoprecipitate from 200 μg NI IgG, (total). (*S3*) Supernate from 200 μg NI incubation (total). The arrow denotes the region of the gel where protein appears in the immunoprecipitate; the arrowhead indicates the heavy chain of IgG. The residual IgG heavy chain band in the unbound fraction suggests that the antibody binding capacity (at 200 μg antibody input) of protein A-Sepharose had been exceeded. Molecular weights, $\times 10^{-3}$.

Only one polypeptide with a molecular weight of ~130-kdaltons was present in the liver PM (Fig. 7, Liver-IP). No

TABLE II

Correlation between Precipitation of Enzyme Activity and Coomassie-Blue-Staining Material from Intestinal G-200 Fraction*

Input‡	Antibody§	% Precipitation of activity‖	% 120 kdalton peak weight¶/% load peak weight
20 μg LAP	I 25 μg	52	46
20 μg LAP	I 200 μg	91	92
20 μg LAP	NI 200 μg	10	0

* These data were obtained from the immunoprecipitation experiment and densitometric scan of the gel seen in Fig. 6.

‡ 33 μg of G-200 fraction was actually used, in which 60% (or 20 μg) of the fraction protein was LAP.

§ The immune (*I*) guinea pig antibodies used in these studies were affinity purified on LAP-Sepharose. The nonimmune (*NI*) guinea pig antibodies used were on IgG fraction prepared by Na_2SO_4 precipitation and chromatography on DEAE and G-200.

‖ The percent precipitation was calculated by (1 − supe/total) × 100.

¶ These values were derived from the scanned gels and calculated as follows: weight of 120-kdalton peak in immunoprecipitate/weight of 120-kdalton peak in initial load × 100 on a scale of 0.6 OD_{560}. Since we were not able to accurately measure the amount of activity precipitated, we do not know whether the 10% of the activity removed from the supernatant in the N1 sample represents bead-associated activity or an inactivation of 10% of the enzyme.

such material was detected in nonimmune samples (Liver-NP). The comparable intensity of Coomassie Blue staining in liver and intestinal immunoprecipitates, as well as the similar levels of enzyme activity that were precipitated (intestine: 54.1 ± 13.5%, 48–63%, 16 experiments; liver: 61.5 ± 8.9%, 52–87%, 13 experiments), demonstrated that the anti-intestinal LAP antibodies efficiently recognized a liver protein migrating in SDS PAGE at approximately the same molecular weight as the purified LAP.

Identification of Additional Bands in the Immunoprecipitates

As described above, although the predominant material recognized by the anti-LAP antibodies migrated in SDS PAGE at 110–130 kdaltons, several bands of higher molecular weight were often observed in the immunoprecipitates of intestinal extracts. We were concerned that the presence of these additional bands, as well as the appearance of three bands in the 110–130-kdalton region, could be due to the presence of non-LAP antibodies. We therefore asked whether the other molecular weight species were related to the 120-kdalton component by selectively adsorbing our antibody preparations against only the 120-kdalton band and testing the remaining preparation against all the bands in the immunoprecipitate. The general protocol used was as follows: (*a*) an Emulphogene extract of intestinal mucosa was immunoprecipitated, solubilized, and applied across the entire width of an SDS gel and electrophoresed; (*b*) 0.5-cm wide gel strips were cut from each end and stained with Coomassie Blue to determine the banding pattern; (*c*) the sample in the remaining gel was electrophoretically transferred to NC; (*d*) strips were incubated with affinity-purified antibody and ^{125}I-protein A to determine the staining pattern; (*e*) the 120-kdalton region was identified and then the corresponding regions from unreacted NC strips were incubated with limiting amounts (2.5 μg)[3] of affinity-purified antibodies followed by ^{125}I-protein A to determine how much IgG had been bound to the NC; (*f*) the "120-kdalton-adsorbed" antibody preparation was incubated with an unreacted nitrocellulose strip and the banding pattern determined. The results of this adsorption experiment are seen in Fig. 8. First, the banding pattern of the intestinal immunoprecipitate seen on the SDS polyacrylamide gels (Fig. 8, lane *CB*) was reproduced on Amido black-stained NC (Fig. 8, lane *AB*) and in autoradiograms of NC strips incubated with affinity-purified guinea pig anti-LAP antibodies (Fig. 8, lane *B*). Second, the banding pattern of an NC strip, from which a segment had been removed for incubation with anti-LAP antibodies, demonstrated that only the 120-kdalton region had been used as the adsorbent (Fig. 8, lane *B'*). Finally, removal of antibodies directed against the 120-kdalton region eliminated staining of all bands in our specific immunoprecipitate (Fig. 8, lane *A2*). Fig. 8, lane *A1*, shows that a partially adsorbed antibody preparation (75% as determined by the amount of ^{125}I-protein A which bound to the nitrocellulose adsorbent) resulted in diminished staining of all the bands (Fig. 8, lane *A1*). A longer autoradiographic exposure of the same ^{125}I-labeled strip revealed that all of the bands seen in the initial sample (Fig. 8,

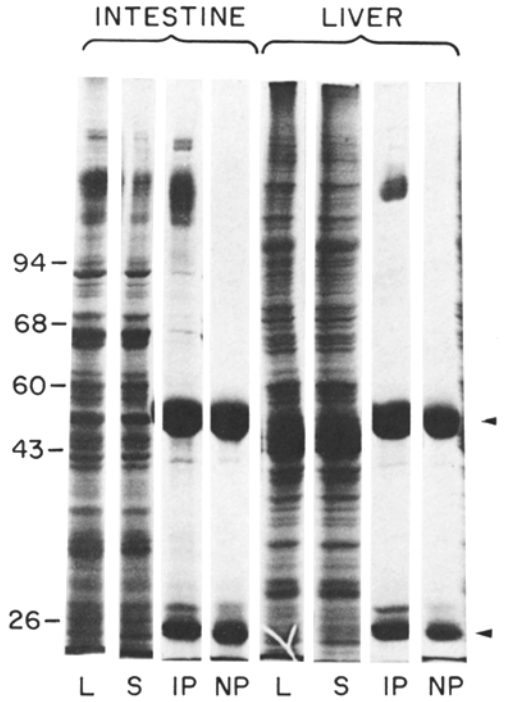

FIGURE 7 Comparison of the immunoprecipitates from detergent extracts of intestinal mucosa and liver PM. Emulphogene extracts of the intestinal mucosa and liver PM, prepared as described in Materials and Methods, were incubated with either 200 μg of LAP-specific guinea pig antibodies or nonimmune IgG followed by protein A-Sepharose. The first four lanes are intestinal samples and the second four are from liver. (*L*) Detergent extracts (load) 200 μg. (*S*) Unbound material from samples incubated with LAP-specific antibodies, 200 μg (~7% of total sample). (*IP*) Specific immunoprecipitates (total). (*NP*) Material precipitated by nonimmune guinea pig IgG (total). The arrowheads indicate heavy and light chains. Molecular weights, × 10^{-3}.

[3] The amount of antibody chosen (2.5 μg) was based on the calculation of 3 μg of LAP protein per 0.5-cm wide strip. This number was derived from the amount of enzyme activity immunoprecipitated, the specific activity of purified LAP, the width of the gel, and the assumption that all of the enzyme activity was associated with the 120-kdalton component.

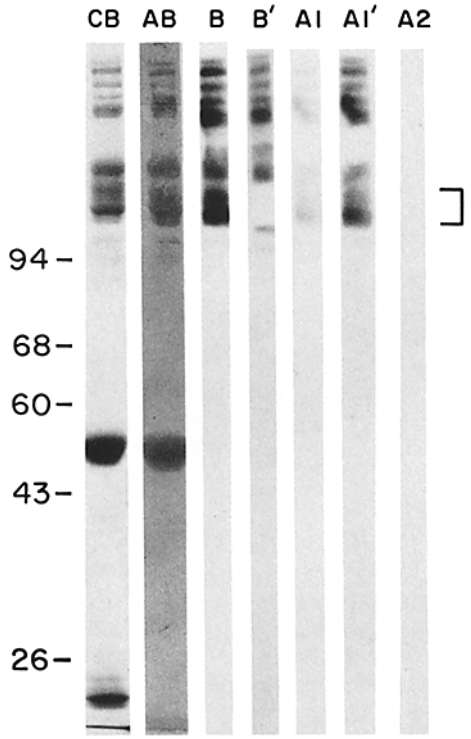

FIGURE 8 Electrophoretic transfer of the intestinal immunoprecipitate to nitrocellulose and adsorption of affinity-purified antibodies against the 120-mol wt region. The detergent extract of intestinal mucosa was immunoprecipitated as described, electrophoresed and transferred to NC. 0.5-cm strips were then incubated with affinity-purified guinea pig anti-LAP antibodies (2.5 μg) or antibody samples that had been adsorbed against the 120-mol wt region of the transferred immunoprecipitate. The strips were reacted with ^{125}I-protein A (8–8.5 × 10^5 cpm, total) and then autoradiographed. (*CB*) Coomassie-Blue-stained immunoprecipitate. (*AB*) Amido black-stained nitrocellulose. (*B*) Autoradiogram of blot reacted with 2.5 μg of antibody and ^{125}I-protein A (exposed for 15 h). (*B′*) Autoradiograph of strip from which the 120-mol wt region had been removed and then reacted with 2.5 μg of antibody and ^{125}I-protein A (15 h). (*A1*) Partially adsorbed sample (see Results) (15 h). (*A1′*) *A1* exposed for 35 h. (*A2*) Adsorbed anti-LAP preparation from which antibodies directed against the 120-mol wt component had been removed (35 h). Molecular weights, × 10^{-3}.

lane *B*) could be accounted for (Fig. 8, lane *A1′*). These findings indicate that all bands present in the specific immunoprecipitate are immunologically related to the 120-kdalton region.

Peptide Maps

We next prepared chymotryptic maps of purified intestinal LAP (G-200) and the various bands from specific intestinal and liver immunoprecipitates after iodination, so as to compare their polypeptide patterns. Fig. 9, *L* demonstrates that the overall polypeptide pattern of the liver antigen was very similar but not identical to that of purified LAP (Fig. 9, *G*) and to the intestinal immunoprecipitates (Fig. 9, *1* and *5*). Several spots appear to be unique (bars) and several intestinal spots appear to be absent. However, on the basis of the similarity between the intestinal and liver antigens, we propose that the liver antigen is LAP.

The overall pattern of ^{125}I-chymotryptic peptides in the immunoprecipitated samples (Fig. 9, *1* and *5*) was similar to that of our purest intestinal antigen (Fig. 9, *G*). These results suggested that the additional bands share polypeptide sequences with the intestinal LAP.

DISCUSSION

The use of immunofluorescence has allowed us to define a domain-specific marker for the hepatocyte plasma membrane. Within the limits of this technique, we have determined that the liver antigen recognized by the anti-intestinal LAP antibodies is concentrated in the bile canalicular plasma membrane. In addition, on the basis of the similarities in electrophoretic mobility and polypeptide composition of the intestinal and liver antigens, we propose that the liver antigen is LAP.

LAP Is a Domain-specific Marker for the Apical Membrane of Polarized Epithelial Cells

The integral membrane glycoprotein LAP has been localized to the apical domain of several different epithelial cells (3, 4, 43). Therefore, our finding that the liver membrane LAP is confined to the bile canalicular membrane of the hepatocyte, which is the apical domain of this epithelial cell, is not unexpected. Nonetheless, the function of this membrane-bound aminopeptidase is epithelia such as kidney and liver is presently not known.

From our own results and those of others, it appears that the LAPs in different tissues are immunologically related. For instance, antibodies directed against intestinal LAP recognize the enzyme purified from the brush border of kidney epithelial cells (3) and hepatocyte plasma membranes (our results). In addition, the peptides of liver and intestinal antigens show homology. The similar locations and molecular characteristics of LAPs from different epithelia, together with the convenience of LAP enzymatic or immunological detection, make LAP a useful marker for apical plasma membrane.

Markers for the bile canalicular membrane other than LAP have been suggested and used in subcellular fractionation studies (5′-nucleotidase: see references 8, 44, 45; alkaline phosphatase: see references 8, 46, 47; alkaline phosphodiesterase: see references 8, 46). However, enzyme activities were followed in these experiments, not antigens, which raises uncertainties regarding the specificity of the substrate for only one enzyme. Furthermore, the initial evidence for their localization to the biliary domain was based on histochemistry, a technique which is rather insensitive and often not specific for a single protein. There has been a particular problem with regard to the histochemical localization of LAP in tissue sections of liver due to the presence of cytosolic, lysosomal, and plasma membrane-associated activities that hydrolyze amino acid naphthylamide substrates. Whether these activities even represent LAP has not been established. Patterson et al. (48) demonstrated that LAP was not the only enzyme responsible for the hydrolysis of the chromogenic substrates (aminoacyl naphthylamides) by tissue sections. Sylvén and Bois (49) compared the histochemical and biochemical localization of LAP in liver and found that different cellular structures were stained depending upon the ion composition and pH of the incubation mixture. In addition, their results suggested that any or all of the following enzymes could be responsible for the hydrolysis of the chromogenic substrate: (*a*) a metal-dependent group of naphthylamidases; (*b*) a leucinamide-splitting, Mn^{++} activated enzyme, regarded as LAP; (*c*) α-chymotrypsin; (*d*) prolinase; (*e*) carboxypeptidase B; and (*f*) cathepsin B. Therefore, our results indicate that an immunological approach is more specific.

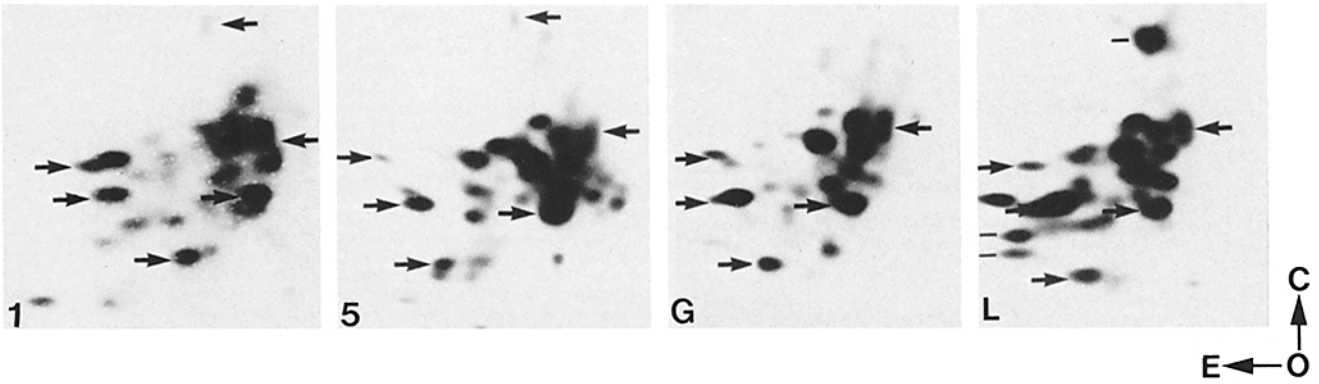

FIGURE 9 Autoradiograms of the peptide maps of the bands obtained from intestine and liver immunoprecipitates. The major bands seen in the immunoprecipitates of intestinal mucosa and liver PM and in purified intestinal LAP were iodinated and digested with α-chymotrypsin. The gel from which the intestinal immunoprecipitate was obtained is seen in Fig. 8, *CB*. The bands were numbered *1–7* starting at the top (highest molecular weight). The maps of bands *1* (minor) and *5* (major) are presented. The major band at 120 mol wt in the liver immunoprecipitate was excised (see Fig. 7, Liver-IP). For the G-200 sample, see Fig. 1, *G*. (*1*) Intestine, IP #1. (*5*) Intestine, IP #5. (*G*) Intestine, G-200. (*L*) Liver, IP. Arrows indicate corresponding spots; bars indicate spots that appear to be unique. The directions of electrophoresis (*E*) and chromatography (*C*) and the origin (*O*) are indicated in lower right corner. The maps of bands *3*, *6*, and *7* are similar to those presented.

An Intracellular Pool of Hepatocyte LAP Is Not Detected by Immunofluorescence

Incubation of 0.5-μm sections of liver with anti-LAP antibodies resulted in an intense fluorescence of the canalicular membrane, but staining of intracellular membranes was not detected. In contrast, Louvard (43), also using immunofluorescence, found intracellular pools of LAP in MDCK cells. Widnell et al. (50), using antibodies to 5′-nucleotidase, have reported an intracellular distribution of this plasma membrane enzyme in cultured rat fibroblasts and suggested that the intracellular pool of enzyme was in equilibrium with the cell surface. Since we were studying liver in situ, and since the others were investigating the recycling of membrane proteins in cultured systems, the discrepancies in our results could be due to: (*a*) differences in pathways taken by molecules in liver, fibroblastic and kidney cells; or (*b*) differences in the distribution of plasma membrane proteins in tissue culture cells vs. those in situ. Recently, Feracci et al. (51) noted staining of an intracellular structure, tentatively identified as Golgi complex, in 0.2-μm frozen sections of intestines incubated with anti-aminopeptidase-N antibodies. If intracellular LAP is present in liver, it must represent a very small pool because staining over the cytoplasm was equivalent to that over the sinusoids (i.e., background). However, LAP follows the same biosynthetic pathway taken by other plasma membrane glycoproteins (41, 52); therefore, we would expect to find small amounts of this protein in the various membrane compartments it passes through en route to the canalicular membrane. Immunofluorescence appears not to be a sensitive enough technique to detect this intracellular distribution of LAP. However, because a small amount of this antigen must be present on other membranes, use of LAP as a liver bile canalicular domain marker should be accompanied by the appropriate controls to evaluate the nature of any LAP-containing fraction (e.g., determinations of LAP sidedness, carbohydrate composition, etc.).

Staining of the Lateral Surface Is Not Detected by Immunofluorescence

Several reports on the biosynthesis of S-I (5) and the recycling of LAP (43) have suggested that these integral membrane glycoproteins first appear in the basolateral domain before their concentration in the apical domain. Using a kinetic approach and subcellular fractionation, Quaroni et al. (53, 54) and Hauri et al. (5) demonstrated that ~60% of both [^{3}H] fucose-labeled glycoproteins and the sucrase-isomaltase precursor were incorporated into the basolateral membrane before reaching the apical surface. However, in neither of these studies was the basolateral membrane preparation free of apical or Golgi-membrane contamination, making their findings inconclusive. Feracci et al. (51) addressed the question of whether LAP was present on the lateral surface of enterocytes through a combined histochemical and immunocytochemical approach. With the use of a chromogenic substrate, strong staining of the brush border was noted with additional reaction product found in the apical region of the lateral membrane. However, by immunofluorescence, these investigators (51) observed only brush border staining. It is possible that the lateral staining observed by histochemistry was the result of diffusion of reaction product from the brush border, or that the intensity of the brush border fluorescence obscured the detection of the antigen on the lateral membrane. We did not detect LAP on the lateral surface of hepatocytes by immunofluorescence. However, the intense canalicular staining and our inability to resolve the lateral surface immediately beyond the tight junctions may account for the lack of staining in this region. Immunocytochemical localization at the ultrastructural level in combination with kinetic studies is needed to determine whether LAP is located on the lateral membrane, and whether this population of antigens represents molecules in transit to the bile canalicular membrane.

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