Distribution of Urokinase-type Plasminogen Activator Immunoreactivity in the Mouse

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ABSTRACT   Immunocytochemistry, using rabbit antibodies to a urokinase-type 48-Kdalton M_r mouse plasminogen activator, showed that enzyme immunoreactivity is widely distributed in the normal mouse. Strong staining was obtained in widely disseminated connective tissue cells with a fibroblast-like morphology. Such cells occurred in high numbers in the lamina propria mucosae of the gastrointestinal tract, and in moderate numbers in the connective tissue septa of the pancreas. A few such cells were detected around the larynx, trachea, and bronchi. Immunoreactivity also occurred in epithelial cells of the proximal and distal kidney tubules, the ductus deferens, and in pulmonary pneumocytes. In addition, presumably extracellular staining was seen irregularly along the basement membrane and fibrillar structures in the lamina propria of the small and large intestines. Moreover, decidual cells of the mouse placenta stained strongly, and a moderate staining was observed in epithelial cells of involuting mammary glands, but not in those of noninvoluting glands. No immunoreactivity was observed in endothelial cells.

Control experiments included absorption of the antibodies against highly-purified mouse plasminogen activator and the corresponding proenzyme, and the finding of a good correspondence between the number of immunoreactive cells and measurable enzymatic activity determined in adjacent tissue sections. Separation by SDS PAGE followed by immunoblotting revealed only one immunochemically stainable protein band with M_r ~48 Kdaltons in extracts from tissues showing immunoreactivity.

The trypsin-like serine protease plasmin degrades most proteins. It is formed from the zymogen plasminogen, present in many extracellular fluids. Plasminogen is activated by another group of serine proteases (for reviews, see references 1–3). At least two types of plasminogen-activating enzymes are produced by animal cells, differing in M_r (~70 and 50 Kdaltons, respectively), immunological reactivity, and interaction with other proteins (4–19). At least some plasminogen activators are themselves produced as inactive proenzymes (20–23). The process leading to generation of plasmin is thus a cascade reaction characterized by amplification of the proteolytic activity and regulated by enhancing and inhibitory factors (7, 9, 24–26).

The chemistry of plasminogen, plasmin, and plasminogen activation is partly clarified (9, 27, 28). The biological function of plasmin and the regulation of the enzyme generation is less well understood. Clearly, plasmin plays a role in a variety of normal and pathological processes; its thrombolytic activity

is the most studied (2). Recent findings indicate that the M_r ~70 kdalton type of plasminogen activator (often designated tissue-type plasminogen activator) is a key enzyme in this process (29–31). Much attention has also been directed toward a possible role of plasmin in tissue degradation under normal and pathological conditions, e.g., involution of the mammary gland, inflammation, arthritis, and invasive growth of trophoblasts and cancer cells (3, 5, 32–41, 59–62). In most of these cases, the plasminogen activators involved seem to be of the M_r ~50-kdalton type (often designated the urokinase-type). A role for plasmin in various other biological processes, e.g., cell migration (42, 43) and conversion of prohormones to hormones (44) has also been proposed.

Little or no information is available about the identities of cells in the intact organism producing the two types of plasminogen activating enzymes. Both types are produced by a variety of cultured cell types, derived from normal as well as neoplastic tissue (6, 10, 15, 22, 26, 32–35, 43–45). It is

doubtful, however, whether the cultured cells in this respect are always representative of the cells in the intact organism. Enzyme histochemical studies have been performed using an agarose overlay technique with plasminogen-rich fibrin (7, 8, 46–48). This method often has shortcomings, however, related to a low degree of resolution, a lack of distinction between the two types of activators, the occurrence of inactive proenzymes of the plasminogen activators, and the interference of inhibitors of plasminogen activators and plasmin (9, 20–26).

Immunocytochemical detection of plasminogen activators has hitherto been hampered by the fact that they are present in cells in very low concentrations. We have previously reported the derivation of rabbit antibodies against a murine M_r ~48-kdalton urokinase-type plasminogen activator (13). These antibodies do not cross-react with the M_r ~70-kdalton enzyme (13) and have now been employed for immunocytochemical studies in the normal mouse.

MATERIALS AND METHODS

Materials: The following materials were obtained from the indicated sources: Cyanogen-bromide-activated Sepharose (Pharmacia, Inc., Uppsala, Sweden); swine IgG anti-rabbit immunoglobulins and peroxidase-antiperoxidase complex (Dakopatts, Copenhagen, Denmark); Millipore nitrocellulose paper GSWP 000 10 (Millipore, Molsheim, France); urokinase (Leo Pharmaceutical, Ballerup, Denmark). All other materials were those described previously (10, 13, 18, 19, 20, 22, 49, 53), or of the best commercially available grade.

Animals: Female and male BALB/c mice, 6–10 wk old, were anesthetized with diethylether and perfusion-fixed with cold (4°C) 0.01 M sodium phosphate buffer, pH 7.4, containing 0.14 M NaCl (PBS), followed by a cold (4°C) 4% (wt/vol) paraformaldehyde solution in 0.1 M sodium phosphate buffer, pH 7.4. In typical experiments, 20 ml of PBS and an equal amount of the fixative were used. In addition, tissue specimens were obtained from the placenta of pregnant BALB/c mice (19–20 d of pregnancy) and from mammary glands of lactating mice deprived of their litters for 4 d after 5 d lactation, or from mice lactating for 9 d. Tissue used for enzyme assay and electroblotting was removed from animals perfused with 20 ml of PBS, after diethyl ether anesthesia.

Tissue Treatments: Specimens from animals perfused with paraformaldehyde were cut into 1–2-mm cubes and postfixed overnight (typically, 16 h) at 4°C. This was followed by a 24-h rinsing in 0.1 M sodium phosphate buffer, pH 7.3, containing 20% sucrose. The tissue-cubes were then frozen in melting Freon-22, sectioned at 4–8 μm on a cryostat at −20°C, and the sections mounted on chromealum-gelatin-coated slides.

Specimens from the stomach (oxyntic and antropyloric mucosa) of animals perfused with 20 ml of PBS were flat-mounted on cardboard, frozen in melting Freon-22, and sectioned horizontally in a cryostat at −20°C. Alternating sections were cut at 40- and 5-μm thickness. The 5-μm sections were stained with haematoxylin and eosin for identification of morphology and depth within the mucosa, and the 40-μm sections were individually collected in small testtubes and kept frozen at −20°C for extraction and enzyme assay.

Extracts from frozen tissue samples of freshly PBS-perfused tissue were prepared as described (33) with 0.1 M Tris/HCl, pH 8.1, containing 0.5% (wt/vol) Triton X-100, 10 μl per mg wet tissue weight. The samples were homogenized and centrifuged (4,000 g) at 4°C for 10 min.

Antibodies: Antibodies against highly-purified 48-Kdalton M_r murine plasminogen activator (MPA48)[1] were produced by immunization of rabbits, as described (13). The IgG fraction was purified by sodium sulphate precipitation and DEAE-Sephadex ion exchange chromatography (63). The IgG was further purified in one of two ways: (a) absorption against a glutaraldehyde polymer of murine proteins (50). The glutaraldehyde polymer was prepared from a mixture of conditioned medium from mouse sarcoma-virus transformed plasminogen activator-producing 3T3 cells (3T3/MSV-LO) depleted of plasminogen activator by three passages through a 4-aminobenzamidine aminododecyl cellulose column (49); conditioned medium from murine embryonal fibroblasts (10); and normal mouse serum. All three parts were further depleted of MPA48 by being passed through a column of anti-MPA48 IgG coupled to

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[1] *Abbreviations used in this paper:* MPA48, 48 kdalton M_r murine plasminogen activator; TBS, Tris-buffered saline.

Sepharose 4B. The glutaraldehyde polymer was prepared from 30 ml protein solution in 0.2 M sodium acetate buffer, pH 5.0, containing BSA (25 mg/ml) by slowly adding 6 ml of 2.5% glutaraldehyde (wt/vol). The IgG preparations were applied to and eluted from the polymer in PBS at 4°C (50). (b) Affinity chromatography on a column containing purified MPA48 coupled to CNBr-activated Sepharose 4B. The column (bed volume 2 ml) contained 2 mg of purified enzyme (fraction IV, see reference 49), and was equilibrated with 0.1 M Tris HCl, pH 8.1, 0.1% (wt/vol) Triton X-100. The IgG was applied in this buffer (20 ml), and the column washed with 20 ml 0.1 M Tris HCl, pH 8.1, 0.7 M NaCl, 0.1% wt/vol Triton X-100. Elution (14 ml/h) was carried out with 0.05 M glycine HCl, pH 2.5, 0.4 M NaCl, 0.01% Triton, and the emerging fractions were immediately mixed with sufficient Tris HCl-buffer to re-establish a pH of 8.1.

Preimmune and nonimmune IgG was purified and absorbed in the same way. The purified IgG preparations were screened for their ability to stain spots of MPA48 and its proenzyme form (pro-MPA48) (20) with an immunocytochemical model system (51), in which spots of the enzyme preparations were applied to Whatman No. 1 paper (Whatman Laboratory Products, Inc., Clifton, NJ).

Immunocytochemistry: Cryostat sections or paper models were soaked in 0.05 M Tris HCl, pH 7.4, with 0.15 M NaCl and 1% (wt/vol) Triton X-100 (TBS-Triton) for 15 min, exposed to 1% (wt/vol) human serum albumin or 10% normal goat serum in TBS-Triton for 30 min at room temperature, briefly rinsed in TBS-Triton, and exposed to varying concentrations of antibodies purified as described above. For both affinity-purified antibodies and antibodies cross-absorbed against irrelevant mouse proteins, optimal IgG concentrations for staining were found to be 5–10 μg/ml (dilutions performed in TBS with 0.25% BSA) using 20 h of incubation at 4°C followed by 2 h of re-equilibration at room temperature. The site of antigen-antibody reaction was revealed with the peroxidase-antiperoxidase method of Sternberger, as described (52, 53). Peroxidase activity was demonstrated by the diaminobenzidine-H_2O_2 method (52) and sections were occasionally counterstained lightly with haematoxylin.

In some experiments, endogenous peroxidase activity of mouse tissues was quenched by exposure to methanol/H_2O_2 prior to immunocytochemical staining (53). This treatment somewhat compromised, but did not totally inhibit, immunocytochemical staining, and was therefore not used routinely. Whenever employed, adjacent sections not exposed to the methanol/H_2O_2 reagent were analyzed in parallel.

Controls were those recommended by Sternberger (52) and included (a) omission of either the first, second, or third layer of antiserum; (b) substitution of the primary antibodies by preimmune or nonimmune IgG (at concentrations of 5–50 μg/ml), or by control hyperimmune sera (antigastrin serum 4652, antisomatostatin serum R 213/3); (c) absorption of the primary antibodies against varying concentrations (4.2–63.0 kU/ml), of highly-purified pro-MPA48 or MPA48 (20, 49). The pro-MPA48 used for antibody absorption showed one Coomassie Blue stainable band corresponding to M_r ~48 Kdaltons after polyacrylamide gel electrophoresis under reducing as well as nonreducing conditions (20) (detection limit for possible contaminating proteins was ~5%). The MPA48 for absorption studies was generated from the pro-MPA by incubation with catalytic amounts of plasmin (20). Further controls for establishing that the staining was due to pro-MPA48/MPA48 present in the tissue included: (d) comparisons among the distribution of immunocytochemically stained structures and levels of extractable enzyme activity in various organs; (e) comparisons between the topography of immunocytochemically stained cells and enzyme activity in the mouse stomach using horizontal sections; and (f) separation of extracted tissue proteins on gradient SDS PAGE electroblotting to nitrocellulose paper and immunocytochemical staining of the blots (see below).

Plasminogen Activator Assay: The plasminogen activator content in tissue samples was determined by a modification of the ^{125}I-labeled fibrin plate assay, in which catalytic amounts of plasmin were added to allow for the simultaneous determination of pro-MPA48 and MPA48 (20). Each assay well contained 0.5 ml of 0.1 M Tris HCl, pH 8.1, 0.1% Triton X-100, 0.25% gelatin, 1 μg human glu-plasminogen, and 7 ng human plasmin. In parallel assays, 0.25 μg affinity-purified anti-MPA48 IgG (13) was added. This completely inhibited the enzymatic activity of purified pro-MPA48 and MPA48, although it did not inhibit M_r ~70-Kdalton murine plasminogen activator (13). Pro-MPA48/MPA48 in the samples was calculated as that part of the activity that was inhibited by the anti-MPA48 IgG. In all assays, a highly purified pro-MPA48 standard preparation (20, 0.5–2 U/ml, see reference 10 for definition of 1 U MPA48) was used for calibration. The same preparation was used as an internal standard to check for the possible inhibition of the assay by inhibitors of plasmin and/or plasminogen activators (20). In some tissue extracts, a strong inhibition of the internal standard was observed under these conditions. For the assays described in Table I, the assay conditions were therefore modified so that the incubation period was 20 h and the concentration of plasmin was 660

pg/ml. This modification allowed a higher dilution of the samples before the assay (5,000–200,000-fold) and resulted in the inhibition being nondetectable or moderate in all cases (see legend for Table I). In the 20-h assays, a pro-MPA48 standard at the concentrations 21–210 mU/ml was used for calibration.

Electrophoresis and Immunoblotting: SDS PAGE was performed in a stacking system using slab gels with a gradient of 6–16% (wt/vol) polyacrylamide (10) and a potential difference of 60 V, for 16–18 h. Following SDS PAGE of tissue extracts, the proteins were transferred electrophoretically (10 V, 250 mA) from the polyacrylamide gel onto nitrocellulose paper (54) for 16 h, in a buffer containing 0.125 M Tris, pH 8.6, 0.19 M glycine, 20% (vol/vol) methanol and 0.1% (wt/vol) SDS, or alternatively a 50 mM phosphate buffer, pH 7.4. The nitrocellulose paper was air-dried, fixed in 4% (wt/vol) paraformaldehyde solution in 0.1 M sodium phosphate buffer, pH 7.4, and washed in TBS-Triton for 2 × 10 min. Longitudinal lanes were cut out and some lanes were then stained with amido black and destained in 10% acetic acid. Other lanes were incubated for 30 min with TBS-Triton containing human serum albumin (10 mg/ml). The paper was then washed 2 × 15 min in TBS-Triton and incubated overnight (4°C) with anti-MPA48 IgG, control IgG, or BSA, all diluted in TBS-Triton containing 0.25% (wt/vol) BSA. After temperature re-equilibration for 1 h, lanes were washed in TBS-Triton 3 × 20 min and incubated for 30 min with swine anti-rabbit immunoglobulins (diluted 1:30 in PBS with normal swine IgG (4 mg/ml)). The lanes were further washed 3 × 10 min in TBS-Triton and incubated with peroxidase-antiperoxidase complex (diluted 1:70 in TBS) for 30 min. Before the final peroxidase reaction (52), lanes were extensively washed in the following order: TBS-Triton (5 min), 8 M urea in TBS-Triton (1 h at 37°C, while shaken), TBS-Triton (2 × 5 min), and 0.05 M Tris-HCl, pH 7.6 (2 × 5 min). All incubations and washing procedures were performed at room temperature unless otherwise stated.

In each gel, the following mixture of marker proteins was electrophoresed and transferred by electroblotting: rabbit phosphorylase (M_r 94 Kdaltons), BSA (M_r 67 Kdaltons), ovalbumin (M_r 43 Kdaltons), carbonic anhydrase (M_r 30 Kdaltons), soybean trypsin inhibitor (M_r 20.1 Kdaltons), and α-lactalbumin (M_r 14.4 Kdaltons).

RESULTS

Cytochemical Paper Models

When applied to paper models containing spots of pro-MPA48 or MPA48, both affinity-purified antibodies and antibodies absorbed against mouse proteins produced strong staining at IgG concentrations varying between 1–10 μg/ml. The antibodies were equally effective in staining both pro-MPA48 and MPA48, but did not stain BSA, plasminogen, and plasmin. Unfixed models as well as models fixed for 1 h in 4% paraformaldehyde were stained equally well. Absorption of the antibodies (at 5 μg IgG per ml with 21 KU per ml of pro-MPA48 or MPA48) abolished staining of the paper models. Control IgG and control hyperimmune sera did not stain the pro-MPA48 or MPA48 spots.

Tissue Distribution

In tissue sections, both affinity-purified antibodies and antibodies absorbed against a mixture of mouse proteins depleted of MPA48 produced exactly the same pattern of staining. In all localizations to be reported below, both types of antibodies were used.

Gastrointestinal Tract

MPA48-like immunoreactivity occurred in a widespread system of connective tissue cells distributed in the lamina propria mucosae along the entire gastrointestinal tract. Other layers of the gut wall were devoid of immunoreactive cells. In the oxyntic gland area of the stomach, immunoreactive cells occurred in the uppermost portion of the lamina propria mucosae, surrounding the neck of the glands (Fig. 1). Deeper parts of the lamina propria were devoid of immunoreactive cells. The cells displayed a fibroblast-like morphology and were often spindle-shaped or triangular, occasionally with

TABLE I

Enzyme Activity of MPA48 in Murine Tissues

Tissue	Total plasminogen activating activity	Inhibition by anti-MPA48 IgG	MPA48 activity
	U/mg*	% total activity	U/mg*
Stomach[†]	310[§]	38[§]	120[§]
Stomach[‖]	190[§]	56[§]	110[§]
Vas deferens	64 ± 1	96 ± 3	59 ± 3
Kidney	42 ± 1	80 ± 1	34 ± 1
Lung	73 ± 3	25 ± 6	18 ± 4
Placenta	7 ± 1	92 ± 5	6 ± 1
Bladder	8.1 ± 0.2	38 ± 5	3.1 ± 0.5
Pancreas	8.2 ± 0.3	17 ± 5	1.4 ± 0.4
Duodenum	11.4 ± 0.4	13 ± 3	1.3 ± 0.3
Liver	2.2 ± 0.1	44 ± 2	0.9 ± 0.1

Tissues were removed, weighed, and extracted with 0.1 M Tris HCl containing 0.5% Triton X-100. Extracts from five mice were pooled and assayed by the ^{125}I-labeled fibrin plate method for 20 h in the presence of plasminogen and catalytic amounts of plasmin. This method determines the combined content of MPA48 and its inactive proenzyme (pro-MPA48). Extracts with purified pro-MPA added as an internal standard were assayed in parallel. No inhibition of the internal standard was observed except with the extract of duodenum. Numbers are corrected for this inhibition, which was 28%. Anti-MPA48 IgG (affinity purified) was added in a concentration (0.5 μg/ml) that completely inhibited the activity of purified MPA48 and pro-MPA48. The MPA48 activity was calculated as that part of the activity that was inhibited by the antibodies. All determinations were performed in quadruplicate. Means ± standard errors on the means are indicated.
* Milligrams of wet weight of tissue.
† Superficial layer of antral mucosa.
§ Plasminogen activator activity in the uppermost 300 μm of the mucosa, determined by adding results of assays of tissue sections performed as described in the legend to Fig. 1.
‖ Superficial layer of oxyntic mucosa.

blunt processes extending towards the surface or neck epithelial cells. Similar cells were detected in the antropyloric mucosa of the stomach. In this region, however, the cells extended deeper between the pyloric glands, sometimes reaching the muscularis mucosae. The antropyloric cells were more elongated than the oxyntic cells and frequently gave off long processes that came into close contact with the epithelial cells. In the stomach, most or all immunoreactivity was localized to the cytoplasm of the fibroblast-like cells, with little evidence of extracellular localization. No immunoreactivity was detected in the nuclei of the cells.

In the small intestines, much immunoreactivity was distributed in the connective tissue core of the villi, and smaller amounts occurred in the lamina propria surrounding the crypts (Fig. 2). Most immunoreactivity was associated with slender fibrillar structures and with the basement membrane. Without the aid of electron microscopy it was not possible to decide whether the long fibrillar structures represented fibroblastoid cells. A fair amount of the immunoreactivity detected in the small intestines, particularly that associated with the basement membrane, may represent extracellular stores of pro-MPA48 or MPA48.

In the colon, immunoreactive fibroblast-like cells morphologically similar to those observed in the stomach were again encountered (Fig. 3). Typically, the colonic cells were located superficially in the lamina propria mucosae beneath the surface epithelium, but were occasionally seen to extend down between the colonic crypts. The basement membrane under the colonic epithelium also displayed strong immunoreactivity. The staining was often patchy, but sometimes followed the contour of the entire basement membrane down to the
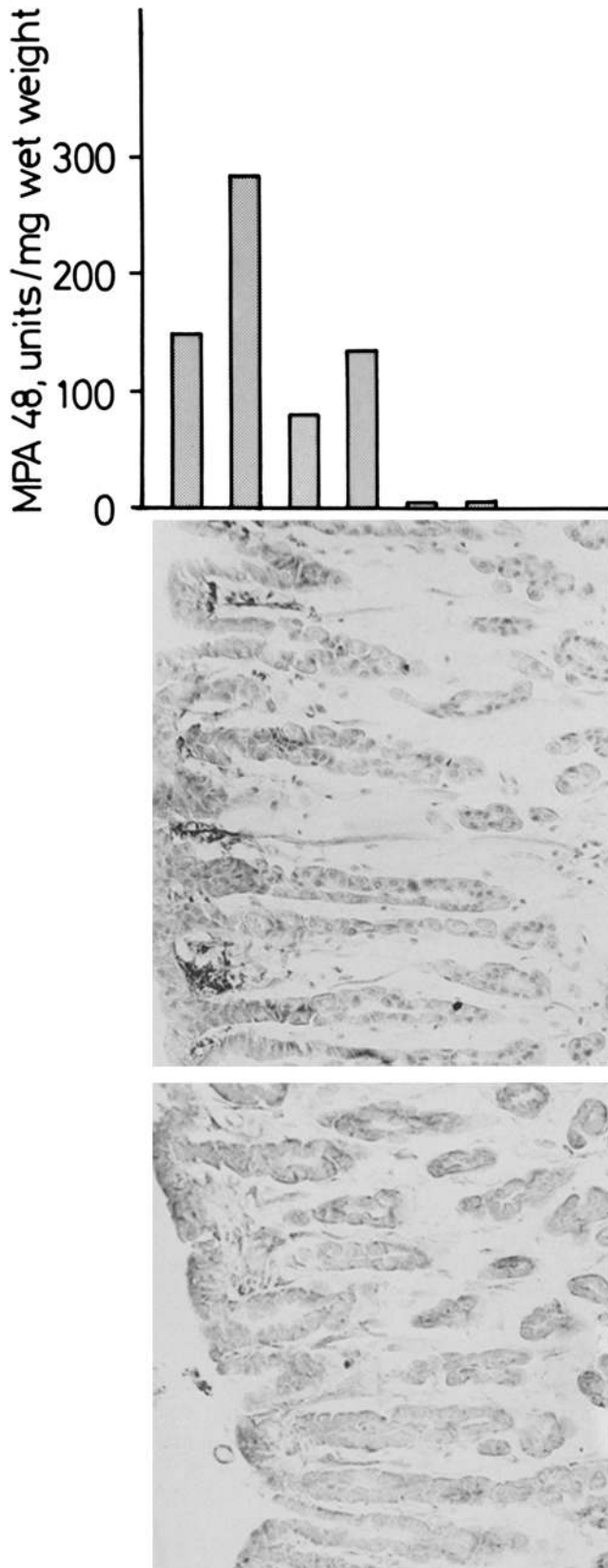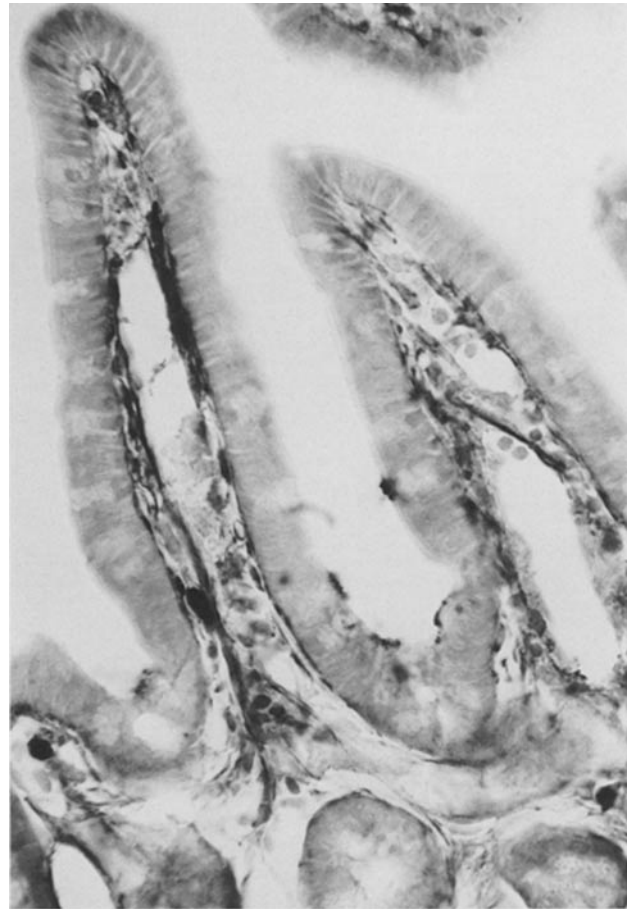
FIGURE 2 Mouse duodenum stained with antiserum to MPA48. Reaction product occurs in fibrillar structures in the villi cores and along the basement membrane. In this region, it is difficult to decide whether the stained material is intracellular or not.

bottom of the crypts. Again, it is likely that this staining represents extracellular material. It is impossible to decide by light microscopy whether it was the basement membrane proper or a structure closely associated with it that was stained. The colonic fibroblast-like cells were of a more rounded shape than those detected in the stomach and contained abundant immunoreactivity in their cytoplasm with unstained nuclei. They were typically positioned close to the overlying epithelium.

Few and scattered immunoreactive cells were also observed in the connective tissue septa separating the pancreatic lobules. Again, the cells were fibroblast-like and displayed a triangular shape, occasionally with long processes. No other pancreatic structure (ducts, acini, islets, blood vessels) was stained.

Thus, in the gastrointestinal tract, immunoreactive MPA48 is confined to cells that, from their morphology and position in the connective tissue stroma are reminiscent of fibroblasts. It is important to point out, however, that most gastrointes-

FIGURE 1 Distribution of MPA48 enzyme activity (a) and immunoreactivity (b) in mouse oxyntic mucosa. As described in the text, horizontal 40-μm cryostat sections were cut and assayed for enzyme activity (top). The distribution of this activity agrees closely with the distribution of MPA48 immunoreactivity as shown by immunocytochemistry (middle). Thus, as shown by the diagram the upper 180 μm of the mucosa contains abundant enzyme activity as well as

immunocytochemically stained cells, whereas deeper portions contain only trace amounts of enzyme activity and no positive cells. In the bottom panel an adjacent section has been stained with MPA48-preabsorbed anti-MPA48. Note absence of staining of the superficially located fibroblastoid cells. Enzyme activity was determined by the ^{125}I-labeled fibrin plate assay with incubation for 1 h at 37°C.
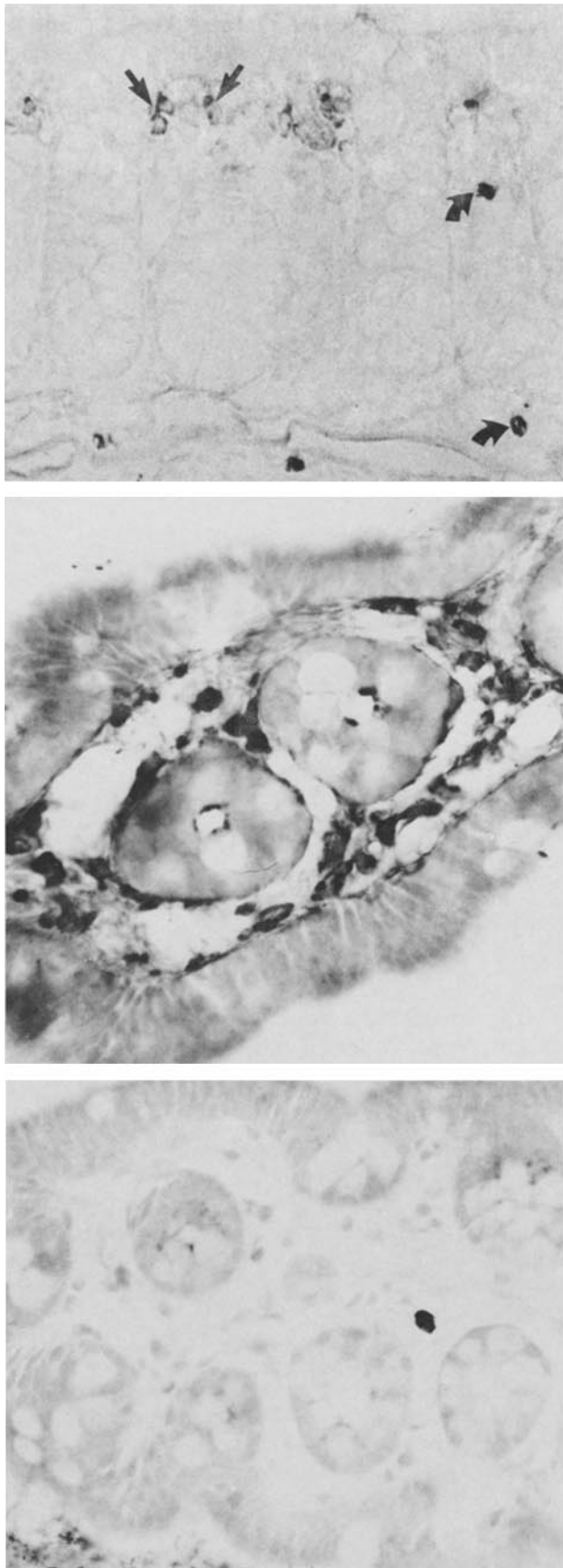
tinal fibroblasts are nonreactive. In the small and large intestines, but not in the stomach or pancreas, part of the MPA48-like immunoreactivity may be localized to the extracellular compartment, particularly in association with the basement membrane.

Other Organs

Structures containing MPA48-like immunoreactivity could not be detected in the brain, pituitary gland, or liver. In the lung, virtually all pneumocytes were weakly to moderately stained, producing a typical reticulated staining pattern (Fig. 4). In addition, the connective tissue surrounding the cartilage occurring in the larger bronchi and, particularly, in the trachea and larynx, contained scattered, strongly immunoreactive, fibroblast-like cells. The tracheal and bronchial epithelium, glands, cartilage, and most of the connective tissue stroma were unstained. The staining of the pneumocytes was dependent upon effective perfusion-fixation of the lung through the pulmonary arteries.

Very intense and widespread staining was observed in the kidneys. Portions of both proximal and distal tubules and medullary rays were stained. In the tubules (Fig. 5), the staining was distinctly granular. Immunopositive granules were often seen apically in the tubule cells, and occasionally also surrounded the nuclei. Few or no immunoreactive granules were seen in the basal portion of the cells. In some tubules, immunoreactivity was also seen on the luminal surfaces of the epithelial cells. In the urinary bladder, immunoreactive cells occurred in the epithelium, situated below the surface epithelial cells and displaying a granular staining pattern (Fig. 6). The epithelial cells of the vas deferens also displayed strong immunoreactivity. Here the immunostaining was confined to apical granules of the stereociliated cells in a fashion suggesting active secretion into the lumen. In the lumen, weak to moderate immunoreactivity was detected, often associated with spermatozoa (Fig. 7).

In the placenta in late pregnancy (19–21 d), numerous strongly immunoreactive cells were detected in the decidua (Fig. 8). In the mammary glands of mice that had been lactating for 9 d, no immunoreactivity could be detected. If, however, the mice had been deprived of their litters after 5 d of lactation and then left for 4 d before sacrifice, weak to moderate immunoreactivity could be detected in intracytoplasmic vacuoles of the involuting gland cells (Fig. 9). In all organs investigated, no staining was found in the endothelial cells of any vessel.

Control Experiments

Staining controls, including the use of up to 50 μg/ml nonimmune or preimmune IgG and unrelated hyperimmune sera, were negative, as were experiments in which the various antibody layers were deleted. Absorption of the anti-MPA48 antibodies against highly-purified pro-MPA48 or MPA48

colonic mucosa. Unspecific peroxidase activity occurs in granulocytes scattered all over the mucosa (curved arrows). In tangential sections (middle), the fibroblastoid cells are seen to surround the apical portions of the colonic crypts. Occasionally, as in this picture, staining associated with the basement membrane is also observed. Preabsorption of the antiserum with MPA48 (bottom) prevents staining of the fibroblastoid cells and basement membrane, whereas the unspecific peroxidase activity of the granulocytes persists.

FIGURE 3   Mouse colonic mucosa stained with anti-MPA48 (top and middle) or with MPA48-preabsorbed anti-MPA48 (bottom). The immunoreactive fibroblastoid cells (marked with straight arrows, top) occur in great number in the most superficial part of the
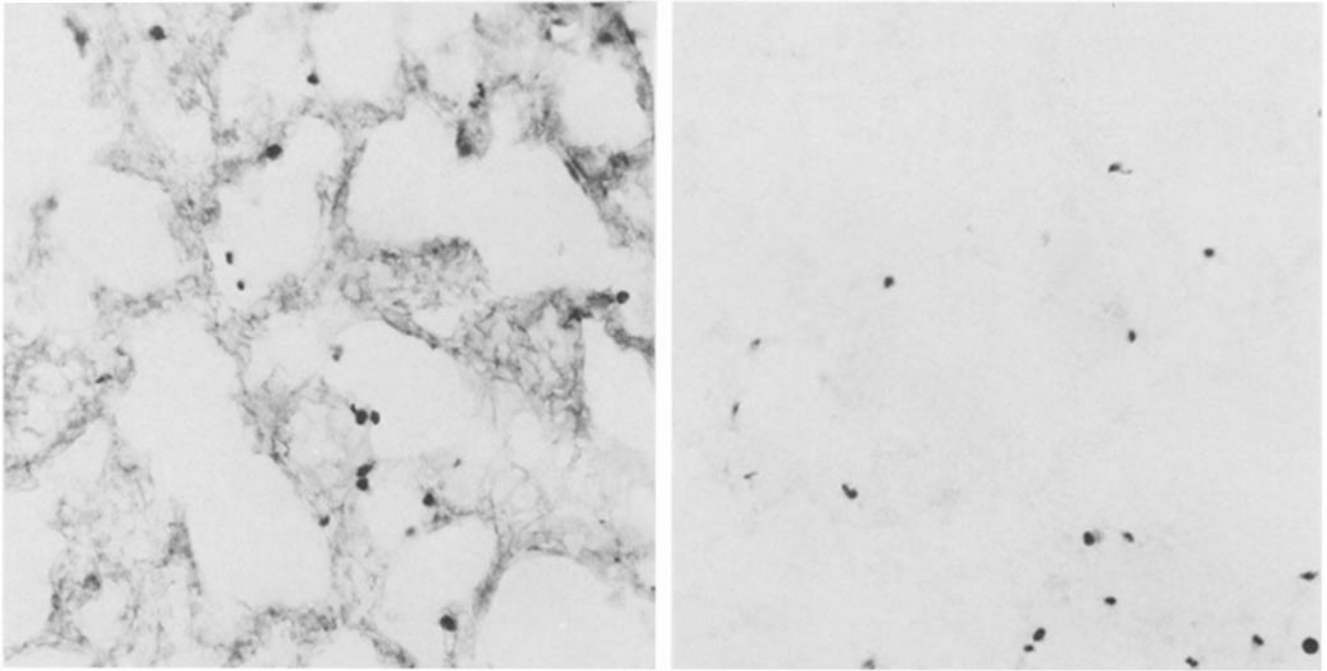
FIGURE 4 Mouse lung stained with anti-MPA48 (*left*) or with MPA48-preabsorbed anti-MPA48 (*right*). Specific staining occurs in the pneumocytes and is completely abolished in the preabsorbed control. Scattered erythrocytes display pseudoperoxidase activity.
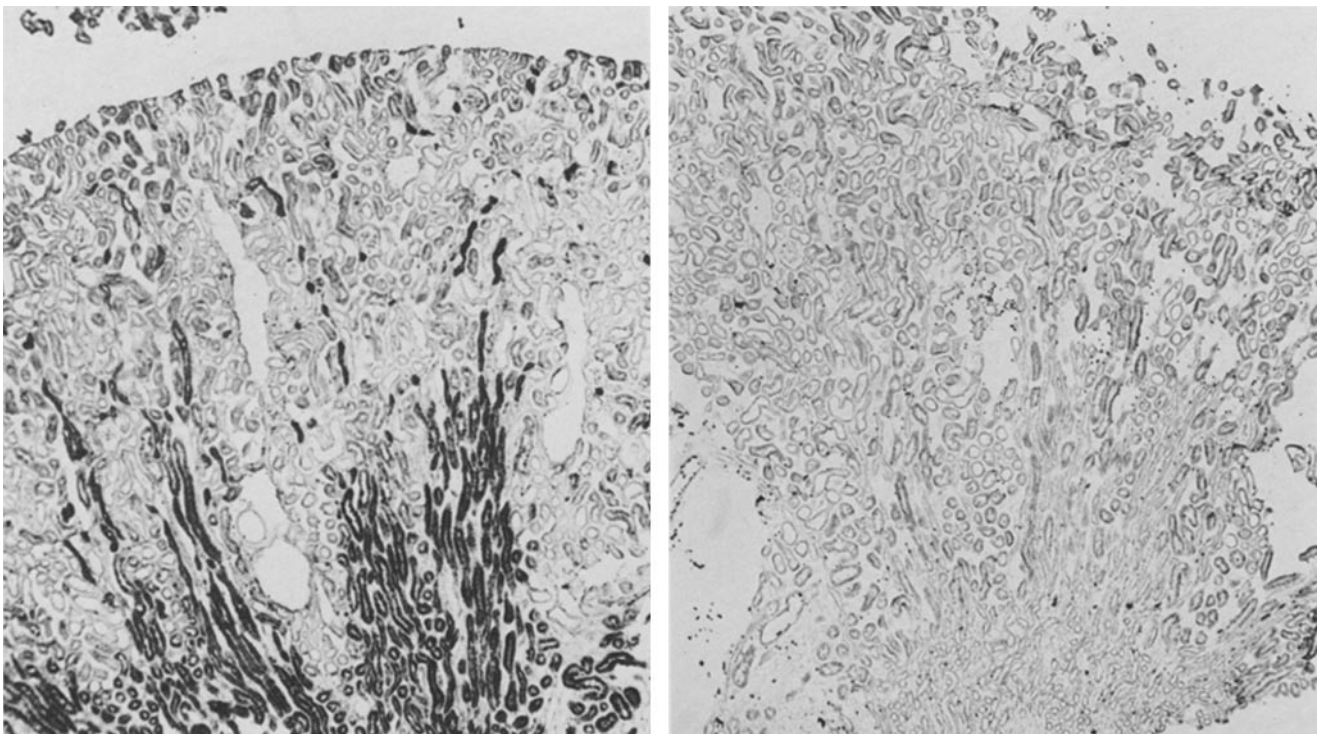

FIGURE 5 Mouse kidney stained with anti-MPA48 (*left*) or with MPA48-preabsorbed anti-MPA48 (*right*). Note the occurrence of specific staining in part of the tubules.

completely abolished staining of all structures reported above. In all specimens, erythrocytes and granulocytes were the only cell types displaying significant endogenous peroxidase activity. As noted above, blocking of endogenous peroxidase activity was associated with some decrease in specific immuno-

reactivity. However, after such blocking treatment, both erythrocytes and granulocytes were completely unstained.

The distribution of immunoreactive cells was compared with enzyme activity in tissue extracts (Table I). The MPA48 activity was calculated as that part of the total plasminogen

activating activity that was inhibited by antibodies against MPA48. For extracts where the MPA48 activity constituted a large part of the total activity, this led to rather precise determinations, whereas the determinations for extracts in which MPA48 constituted a small part of the activity showed

FIGURE 6 Mouse urinary bladder stained with anti-MPA48. Note the presence of MPA48 immunoreactivity in cytoplasmic granules of some, but not all, epithelial cells (arrows).

great statistical uncertainty. In the tissues for which fairly precise enzyme measurements were obtained, there was a good correspondence between enzyme activity and immunoreactivity. Thus, in descending order both in terms of number of cells stained and in enzyme activity, came the superficial mucosa of stomach, vas deferens, kidney, lung, and placenta.

Within the gastric mucosa, the topographic distribution of immunoreactive cells and of enzyme activity also corresponded. Thus, in the oxyntic mucosa, where the immunoreactive fibroblast-like cells were oriented very superficially, enzyme activity was also located superficially (see Fig. 1). In the antropyloric mucosa, in which the immunoreactive cells extended deeper between the glands, the enzyme activity also extended deeper (data not shown).

Extracts of superficial sections of oxyntic mucosa were separated by SDS polyacrylamide gradient gel electrophoresis, electroblotted, and the nitrocellulose replicas stained immunocytochemically. Only one immunoreactive band, corresponding in electrophoretic mobility to pure MPA48, was observed (under nonreducing conditions, pro-MPA48 and MPA48 display similar mobilities in this system [20; Fig. 10]). In deeper sections of oxyntic mucosa (not containing immunoreactive cells), no immunoreactive band was detected on the nitrocellulose replicas. Similar analysis of extracts of lung, vas deferens, placenta, and kidney also disclosed one single band with the same electrophoretic mobility as pro-MPA48 and MPA48 (data not shown).
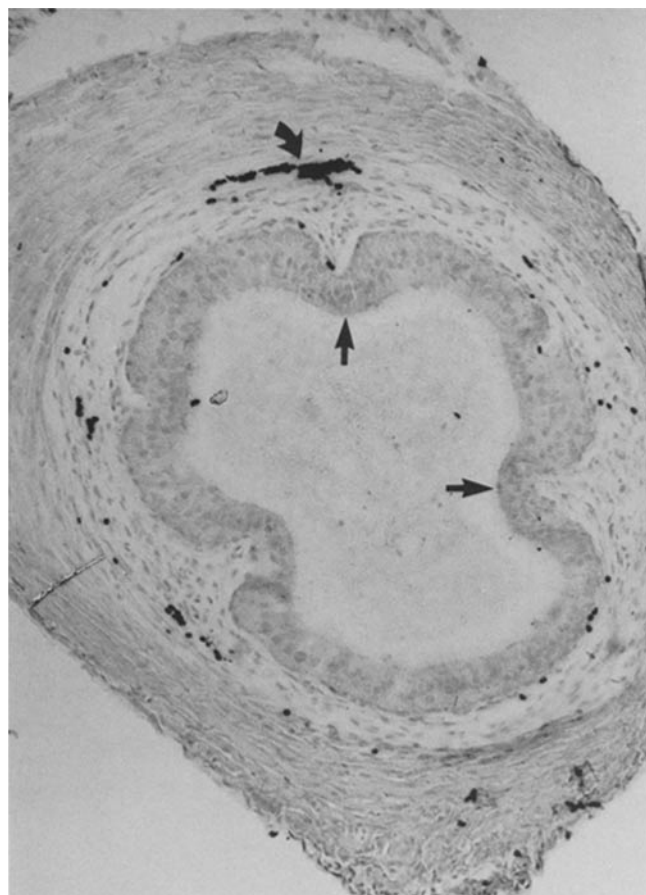

FIGURE 7 Mouse vas deferens stained with anti-MPA48 (left) or with MPA48-preabsorbed anti-MPA48 (right). Note the presence of immunoreactive MPA48 in apical granules of the stereociliated epithelial cells (straight arrows) and weaker immunoreactivity in luminal material surrounding spermatozoa. Nonspecific pseudoperoxidase activity is displayed by erythrocytes (curved arrows).
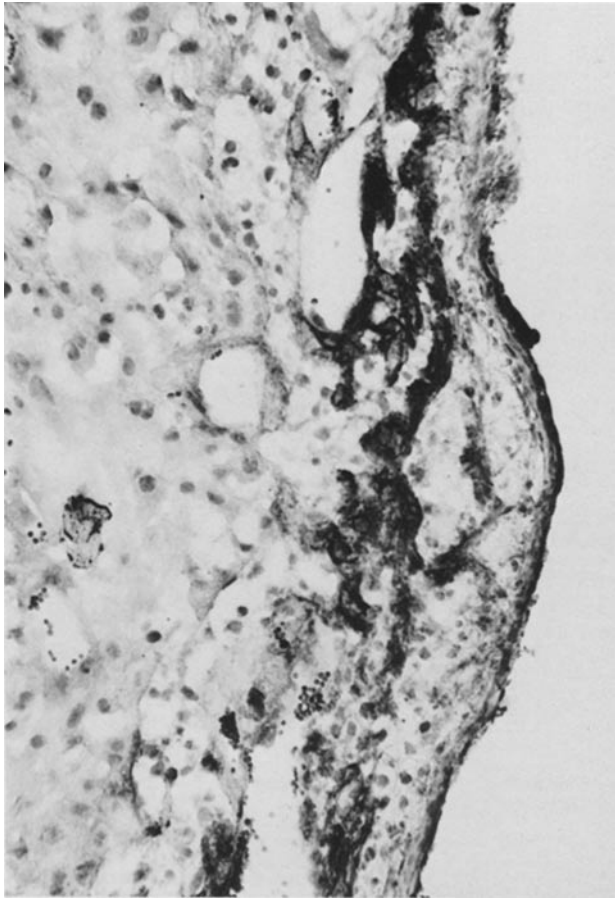
FIGURE 8   Mouse placenta (day 19 of pregnancy) stained with anti-MPA48. Scattered cells situated at the border of the decidua and the uterine wall display strong immunoreactivity.
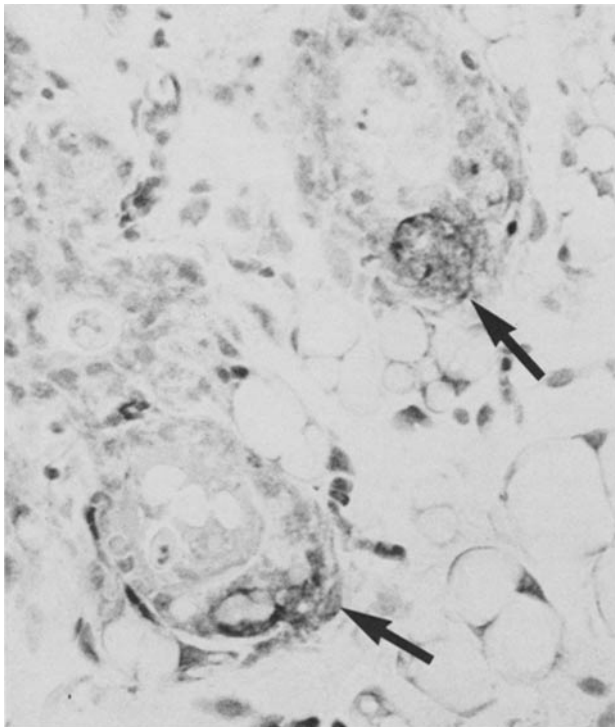

FIGURE 9   Mouse involuting breast. Specific MPA48 immunoreactivity occurs in intracellular vacuoles of degenerating gland cells (arrows).
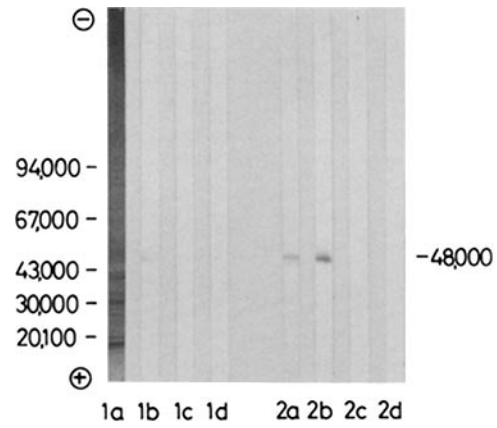

FIGURE 10   Immunoblotting analysis of MPA48 from Triton X-100 extracts of fundus (1) and of purified pro-MPA48 (2). Whole fundus was extracted with 0.1 M Tris-HCl, pH 8.1, containing 0.5% Triton X-100, and 350 μg of proteins were applied to each of 4 lanes (1, a–d). Highly purified pro-MPA48 were applied to each of four other lanes (21 kunits to lane 2a and 105 U to lane 2, b–d). Following SDS polyacrylamide gel electrophoresis (10), the proteins were electrophoretically transferred onto nitrocellulose paper. The paper was cut longitudinally and the lanes were either stained with amido black (a) or by the PAP method using anti-MPA48 IgG (5 μg/ml) (b), anti-MPA48 IgG (5 μg/ml) preabsorbed with pro-MPA48 (21 kunits/ml) (c), or nonimmune IgG (5 μg/ml) (d).

DISCUSSION

We have utilized polyclonal rabbit antibodies to highly purified MPA48 for immunocytochemistry. Our staining and absorption controls clearly demonstrate that nonimmunological binding of the purified IgG preparations to tissue components can be excluded. The results of these control experiments direct our considerations towards the possible presence of contaminating antibodies or cross-reactive antigens. The enzyme preparations used for absorption controls were electrophoretically pure (detection limit for contaminating proteins was ~5%). It is therefore unlikely, although not completely excluded, that the staining was due to the presence of contaminating antibodies. The possible staining of cross-reacting antigens in the various cell types and tissue compartments could not be evaluated by the absorption experiments, but a number of other findings indicate that the staining was most likely due to the presence of pro-MPA48/MPA48. Comparisons between the distributions of enzyme activity and immunocytochemically-stained cells in the different organs correlate well. Both pro-MPA48 and MPA48 are detected by the enzymatic assay used (20), and our paper cytochemical models demonstrate that the antibodies employed bind equally well to pro-MPA48 and MPA48. Furthermore, pretreatment of the paper models with the fixative used (4% paraformaldehyde) showed that this treatment did not compromise the antigenicity. We also find that the results of our studies on the topography of extractable enzyme activity in the oxyntic mucosa (see Fig. 1) provide convincing evidence that the staining, at least in this area, in reality corresponds to authentic pro-MPA48/MPA48.

By extraction with Triton X-100 followed by SDS PAGE and immunoblotting, we have demonstrated, in a number of tissues, the selective staining of a protein band with an electrophoretic mobility in SDS PAGE identical to that of pro-MPA48/MPA48. To diminish the possibility of a hypothetical cross-reactive antigen not being transferred in the electroblot-

ting cell, this process was run at two different pH and with or without SDS, yielding identical results. However, a hypothetical cross-reacting antigen could still be unseen, if it remained tissue-bound after Triton-solubilization, or if it had a molecular weight too large to pass into the polyacrylamide gel. The consistency of our staining results and our biochemical findings, however, argues against this possibility. We thus find that, although not completely conclusive, it is very likely that the staining demonstrated in this study was due to authentic pro-MPA48/MPA48.

However, pro-MPA48 or MPA48 may be present in other cells or organs than those found in this study, in amounts below our detection limit. Furthermore, our results only determine the presence of a certain amount of pro-MPA48 or MPA48 in cells or extracellular space at the time of fixation, and they do not necessarily reflect the site or amount of production of the enzyme.

We have previously reported the immunocytochemical localization of the human 52-kdalton M_r plasminogen activator in the cytoplasm of cultured glioblastoma cells grown in culture (55). In the present study, immunoreactivity was also mainly located in the cytoplasm. This contrasts to an earlier report of plasminogen activator immunoreactivity primarily associated with the cell membrane in an electron microscopical study (56). This report, however, lacks details about the purity of the enzyme preparation used to elicit the antibodies, and apart from negative results with nonimmune serum, cites no control experiments verifying the specificity of the staining. Moreover, the staining conditions used presumably did not permit intracytoplasmic penetration of the antibodies. It should be noted that the distribution of the MPA48 immunoreactivity in the cytoplasm is different in different cell types; immunoreactivity is distinctly granular in kidney tubular cells and epithelial cells of the vas deferens, reticulated in pneumocytes, and more diffusely distributed in the MPA48-immunoreactive fibroblast-like cell type and the decidual cells.

Localization of plasminogen activators was previously done by histochemical studies, seldom distinguishing between the two types of plasminogen activators. The original studies demonstrated plasminogen-dependent fibrinolysis primarily in the venous endothelium of a number of human organs (47, 48). Furthermore, lysis was seen over renal tubules and lung tissue.

The 50-Kdalton type plasminogen activator is found in large quantities in urine (49). The demonstration of a great amount of MPA48 immunoreactivity in the kidney tubule cells suggests that the urine plasminogen activator is excreted or synthesized by these cells. Our results from mammary glands agree with previous findings and lend support to the hypothesis that the urokinase-type plasminogen activator plays a role in postlactational involution (32). The finding of MPA48 immunoreactivity in what appears to be secretory granules in the ductus deferens correlates with a report on plasminogen activators in semen (57).

It is noteworthy that no MPA48 immunoreactivity was found in endothelial cells of any vessels. The plasminogen activator found histochemically in these cells (47, 48), at least in human tissue, is of the 70-kdalton M_r type (17, 31). These findings support the hypothesis that the type of plasminogen activator playing a natural role in thrombolysis is of the 70-kdalton M_r type (tissue-type) and not of the 50-kdalton M_r (urokinase-type), although the latter type of plasminogen activator has been widely used as a thrombolytic agent (58).

The possible role of plasminogen activator in the alimentary canal still remains to be investigated. It is tempting to link the localization beneath the epithelium in these organs with the continuous renewal of this epithelium.

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