Proc. Natl. Acad. Sci. USA
Vol. 75, No. 6, pp. 2785–2789, June 1978
Cell Biology

Immunofluorescent localization of a serine protease in rat small intestine

(group-specific protease/intracellular protease/atypical mast cell)

RICHARD G. WOODBURY*, GREGORY M. GRUZENSKI*, AND DAVID LAGUNOFF†

Departments of *Biochemistry and †Pathology, University of Washington, Seattle, Washington 98195

Communicated by Hans Neurath, March 6, 1978

ABSTRACT **An intracellular serine protease, which is believed to initiate the degradation of several intracellular pyridoxal phosphate-dependent enzymes, was localized by immunofluorescence in atypical mast cells of the lamina propria and in intraepithelial cells of the rat small intestine. Some mucus-secreting goblet cells also contained the protease antigen. Atypical mast cells containing the enzyme were present in large numbers beneath the epithelium of bronchioles. All atypical mast cells also contained low levels of the chymotrypsin-like protease of normal mast cells. Both enzymes were consistently present in normal connective tissue mast cells. Amino acid content, molecular weight, and lack of immunologic cross-reactivity indicate that the two enzymes are similar but not identical. The cell-specific localization of the intestinal serine protease makes it unlikely that the enzyme has any general role in the degradation of pyridoxal phosphate-dependent enzymes. The function of the enzyme in mast cells, atypical mast cells, and intestinal goblet cells is not known.**

Katunuma and coworkers (1) isolated a serine protease from rat small intestine while investigating the *in vivo* turnover of ornithine aminotransferase (EC 2.6.1.13). They observed that among rats maintained on different diets, levels of the protease were inversely proportional to ornithine aminotransferase activity. Katunuma *et al.* (2) have further shown that the protease selectively inactivates the apo forms of several intracellular pyridoxal phosphate-dependent enzymes, but does not attack the holo forms of these enzymes or the apo forms of several enzymes requiring other cofactors. On the basis of these observations, Katunuma has proposed that the enzyme is a group-specific protease (GSP) responsible for initiating the intracellular degradation of certain pyridoxal phosphate-dependent enzymes. Proteases with similar activities have been obtained from rat liver and skeletal muscle (2). Each of these group-specific enzymes exhibits maximal activity between pH 8 and pH 9 and possesses chymotrypsin-like specificity toward ester substrates. The primary structure of small intestine GSP indicates homology with other known mammalian serine proteases (3).

In an attempt to clarify the possible role of the protease in intracellular protein degradation, we have determined the localization of GSP by the method of immunofluorescence.

MATERIALS AND METHODS

Normal, adult, female, outbred white rats (Tyler Laboratories, Bellevue, WA) were used to obtain tissues for isolating and localizing GSP.

Purification of Antigens. GSP was isolated from thoroughly washed small intestine by an affinity adsorption procedure (to be described in detail elsewhere) using a chymotrypsin inhibitor from potato, P.I.-1 (4), covalently attached to an insoluble support. Protease isolated by this method was identical in chemical, physical, enzymatic, and immunologic properties to that purified by the more laborious procedure of Katunuma *et al.* (2). The chymotrypsin-like mast cell protease (MCP) (5) was isolated from mast cells collected from rat peritoneal cavities. Granules prepared from these cells were extracted with 3 M KCl. The extract was chromatographed sequentially over Sephadex G-100 and CM-cellulose, and eluted from the latter in a salt gradient. The molecular weights of GSP and MCP were determined by gel electrophoresis in the presence of sodium dodecyl sulfate and dithiothreitol (6).

Relative Amino Acid Content. Duplicate samples of GSP and MCP were hydrolyzed in 6 M HCl at 110° for 24 hr. Amino acid analyses were performed on a Durrum model D-500 amino acid analyzer. Tryptophan and half-cystine were not determined.

Antisera. Antisera to GSP or MCP were prepared by injecting into rabbits (New Zealand Whites), at 4-wk intervals, 50–100 μg of antigen following an initial intramuscular inoculation of antigen with complete Freund's adjuvant. The specificities of the antisera were determined by immunodiffusion tests. Goat and sheep anti-rabbit immunoglobulin (Ig) coupled with fluorescein isothiocyanante (FITC) were obtained from Miles Laboratories. Normal rabbit sera were substituted for anti-GSP and anti-MCP sera in control tests.

Tissue Fixation. Small intestine removed from a freshly killed rat was cut open lengthwise and 1-cm samples were pinned flat (with villi upward) to small cork boards. The tissue was immediately placed in ice-cold fixative. Fixatives used in this study included ethanol (96%, vol/vol), Carnoy's fluid (7), Newcomer's solution (7), buffered (0.1 M sodium phosphate, pH 7.6) 4% (wt/vol) paraformaldehyde solution, and Enerbäck's solution (8). In addition, tissues were obtained from rats perfused (5 min) with buffered (0.2 M sodium cacodylate, pH 7.2) 4% paraformaldehyde, and placed in Enerbäck's solution or 0.2 M sodium cacodylate buffer, pH 7.2.

Tissues were dehydrated and embedded in paraffin, usually after overnight (16–20 hr) fixation at 4°. Sections of tissue (2–4 μm) were cut on a JB4 microtome (DuPont, Sorvall), deparaffined, and rehydrated immediately before use.

Immunofluorescent and Histochemical Staining. GSP and MCP were localized by indirect immunofluorescence. Sections of tissue were incubated in a 1:20 dilution of phosphate buffer (4.3 mM $Na_2HPO_4 \cdot 7H_2O$/1.5 mM KH_2PO_4/2.7 mM KCl/138 mM NaCl), pH 7.4, of specific antisera for GSP or for MCP for 5–60 min at room temperature. The sections were rinsed with several changes of phosphate buffer (pH 7.2) for 30 min and then incubated in a 1:20 dilution of FITC-conjugated goat or sheep anti-rabbit Ig. Finally, the sections were rinsed with several changes of phosphate buffer (pH 7.2) and mounted in

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: GSP, group-specific protease; MCP, mast cell protease; FITC, fluorescein isothiocyanate.

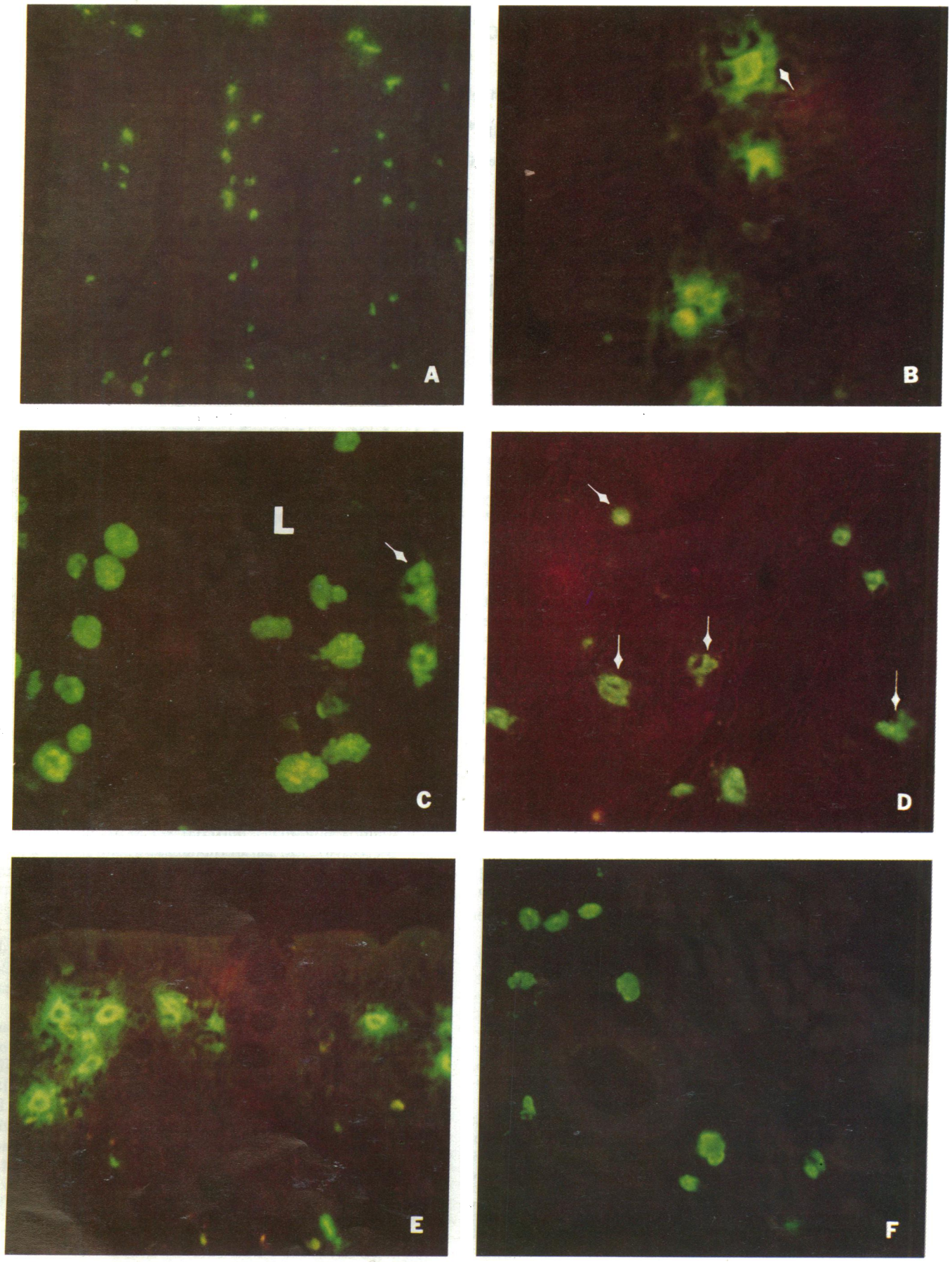

FIG. 1. (*Legend appears at bottom of the next page.*)

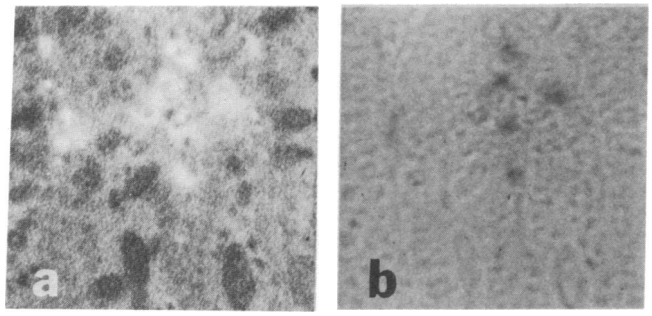

FIG. 2. (×250.) (*a*) Section (4 μm thick) of small intestine fixed in Carnoy's fluid and treated with anti-GSP serum showing several fluorescent GSP-containing cells clustered in the lamina propria. The section previously was incubated in alcian blue solution (pH 1.0) for 2 min, resulting in some decrease in the antigenicity of GSP. (*b*) Light microscopy of the same area as in *a* showing that the GSP-containing cells are alcian blue-positive "atypical" mast cells. Positive staining was not as intense as normal because the incubation time was reduced from 30 min to 2 min.

a 1:1 solution of buffer and glycerol. Sections were examined for fluorescence with a Ploem illumination system.

Standard histochemical procedures (7) were performed in order to identify various cell types: methyl green/pyronin for plasma cells, periodic acid/Schiff reaction for macrophages, acidic toluidine blue for mast cells, and Lendrum's stain (9) for eosinophils.

The alcian blue and safranin stain (8) was also applied to tissues to characterize mast cells. Both Lendrum's stain and alcian blue (2- to 3-min stain) could be used on the sections without interfering with subsequent immunofluorescent staining. With the other histochemical reagents, the immunohistochemical stain was applied to alternate serial sections (2–4 μm).

Photomicroscopy. Fluorescent sections were photographed with Kodak Ektachrome 64 or Kodacolor II film (2- to 6-sec exposures) using a K510 filter on the fluorescence microscope. Sections stained with histochemical reagents were examined and photographed (Ektachrome 64) on an Olympus (Vanox) microscope.

RESULTS

The immunofluorescent localization of GSP in small intestine fixed in Carnoy's fluid indicated that the protease was located in cells scattered throughout the lamina propria (Fig. 1*A*). Many stained subepithelial cells were observed in the crypt region. Fluorescence of cells within the epithelium was rarely seen. In contrast, small intestine obtained from rats perfused with paraformaldehyde showed immunofluorescent localization of GSP both in the lamina propria (Fig. 1*B*) and within the epithelium, where it appeared to be associated with mucus secretory droplets of goblet cells (Fig. 1*C*) and in smaller cells lacking mucus (Fig. 1*D*). The positive small epithelial cells were present

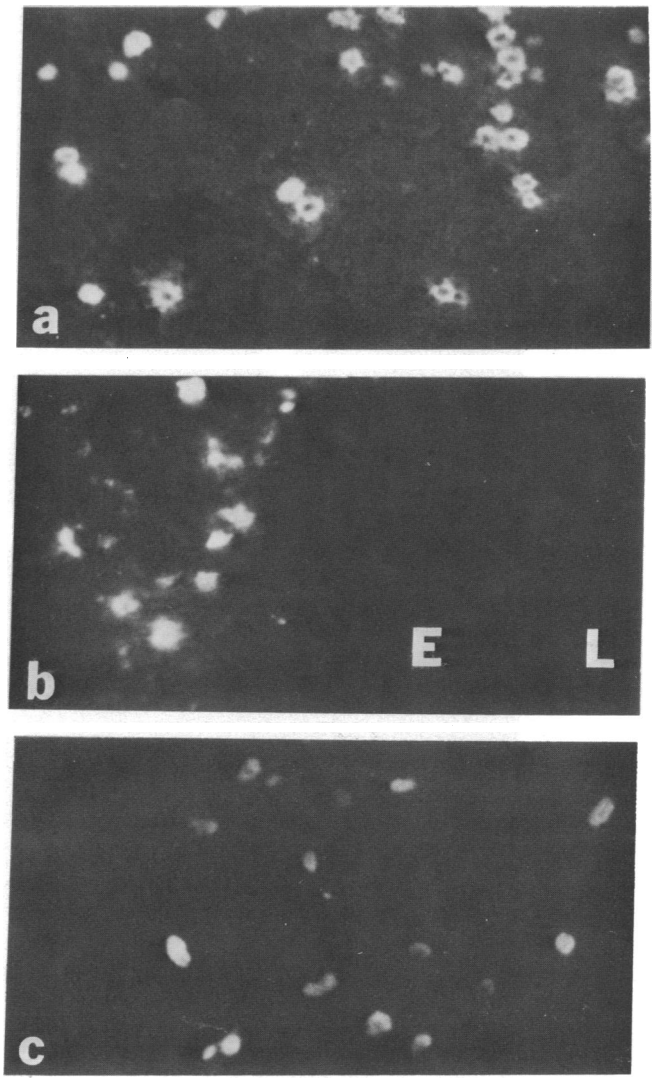

FIG. 3. Immunofluorescent localization of GSP in sections of tissue obtained from a rat perfused with paraformaldehyde (4%). (×250.) (*a*) Connective tissue of popliteal lymph node. Few fluorescent cells were observed in the germinal regions of the node. (*b*) Subepithelial GSP-containing cells surrounding a bronchus. E, epithelium; L, bronchus lumen. (*c*) GSP-containing cells scattered throughout connective tissue of skin.

only in the crypt region. In intestine fixed in Carnoy's fluid most of the fluorescence appeared confined to cells, whereas considerable extracellular fluorescence consistently was observed in sections of perfusion-fixed small intestine. Incubation of rehydrated sections in phosphate buffer (pH 7.4) at 37° for 6 hr prior to the immunofluorescent staining did not wash out the extracellular GSP from the tissue. Numerous GSP-containing

FIG. 1 (*on preceding page*). Fluorescence micrographs. (*A*, ×100; *B–F*, ×250.) (*A*) Immunofluorescent localization (indirect method using FITC-coupled Ig) of GSP in a section of rat small intestine fixed in Carnoy's fluid. The tissue was dehydrated, embedded in paraffin, and cut into 2-μm thick sections. GSP-containing cells are distributed throughout the connective tissue (lamina propria) beneath the epithelium. (*B*) GSP-containing cells within the lamina propria of an intestinal villus (enlarged). Small intestine was obtained from a rat fixed by perfusion with 4% paraformaldehyde buffered (pH 7.2) in 0.2 M sodium cacodylate. Yellow fluorescence indicates high concentration of antigen and outlines the approximate cell boundaries. Green fluorescence (arrow) indicates lower concentration of antigen, which appears to be extracellular. (*C*) Numerous epithelial goblet cells exhibiting positive immunofluorescent staining of GSP. Dark area indicated by L is the gut lumen between two villi. Several subepithelial cells (arrow) can be seen. Small intestine was obtained from a rat fixed by perfusion with paraformaldehyde. (*D*) Several small GSP-containing cells (arrows) within the crypt epithelium of small intestine from a rat fixed by perfusion with paraformaldehyde. These cells are not goblet cells, but are similar to those in the lamina propria containing GSP. (*E*) Section (2 μm thick) of colon obtained from a rat fixed by perfusion with paraformaldehyde. Gut lumen is at the top of the picture. Numerous GSP-containing cells are observed beneath the epithelium. Yellow areas indicate intense fluorescence and outline the approximate cell boundaries. Considerable extracellular antigen surrounding the cells is indicated by green fluorescence. (*F*) Section (2 μm thick) of tongue from a perfusion-fixed rat. Numerous cells containing the GSP antigen are observed in connective tissue near a blood vessel.

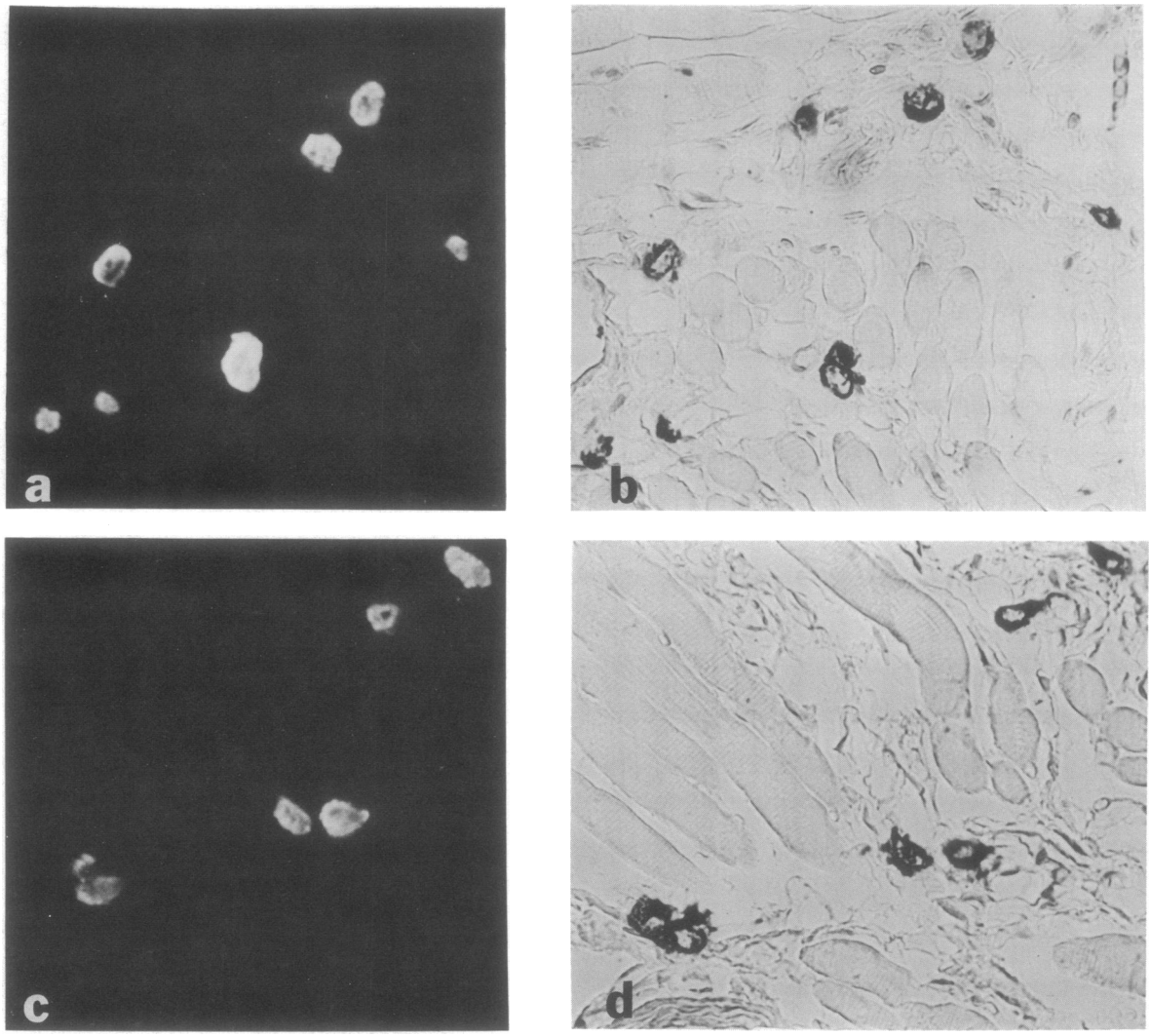

FIG. 4. Immunofluorescent localization of GSP and MCP in connective tissue mast cells of tongue. (*a*) Immunofluorescent staining for GSP. (×250.) (*b*) Light microscopy of the same area as shown in *a* after removing the coverslip and staining the section with toluidine blue to visualize mast cells. (×300.) (*c*) Immunofluorescent staining for MCP. (×250.) (*d*) Light microscopy of the same area as shown in *c* after staining with toluidine blue. (×300.)

cells also were observed in the connective tissue of the colon (Fig. 1*E*) and tongue (Fig. 1*F*). No specific fluorescence was observed when normal rabbit serum was substituted for anti-GSP serum. The yellow autofluorescence of macrophages was easily distinguished from the green fluorescence of fluorescein-labeled antibody.

Systematic histochemical examination of small intestine resulted in the identification of the GSP-containing cells as alcian blue-reactive atypical mast cells (Fig. 2). The intraepithelial fluorescent cells were also alcian blue-positive, indicating that these cells are intraepithelial mast cells. No other cell type in intestine was observed to contain specific fluorescent material.

When sections of perfusion-fixed small intestine were incubated with anti-MCP serum, numerous cells in the lamina propria exhibited weak fluorescence. Intraepithelial cells were stained as well. The fluorescent cells corresponded to alcian blue-staining cells, and all alcian blue-positive cells appeared to contain some fluorescence. No specific fluorescence was observed in the goblet cells when treated with anti-MCP serum. In both perfusion-fixed and Carnoy's-fixed tissue the specific fluorescence was entirely intracellular.

Immunofluorescent localization of GSP was examined in tongue, esophagus, stomach, colon, submaxillary gland, lymph nodes (popliteal and mesenteric), skin, thymus, bladder, skeletal muscle, fat (mesenteric), and lung. In all cases, GSP-containing cells were present in the connective tissue (Fig. 3) and were identified as normal mast cells by staining with toluidine blue. Neither GSP-containing cells nor mast cells were observed in spleen.

Sections of tongue were first incubated with either anti-GSP or anti-MCP serum, then incubated with FITC-anti-rabbit Ig, and photographed to record fluorescence. The coverslips were removed and the sections were stained with toluidine blue for mast cells. The same areas of the tongue that had been photographed for fluorescence were photographed again to record the mast cell distribution. The results of this experiment (Fig. 4) indicate that normal connective tissue mast cells contain both GSP and MCP.

Lung differed from other organs in having two distinct populations of mast cells identified by staining properties with alcian blue and safranin. Safranin-staining (red) cells and alcian blue-staining cells were observed scattered throughout the lung connective tissue. Beneath the epithelium of the bronchioles,

Table 1. Relative amino acid contents of group-specific protease and mast cell protease

Amino acid	mol amino acid/mol protein GSP	MCP
Aspartate	14.0	18.0
Threonine	12.0	16.4
Serine	12.6	12.4
Glutamate	17.6	20.7
Proline	14.7	17.0
Glycine	18.4	21.9
Alanine	15.8	16.3
Valine	19.2	21.5
Methionine	4.6	5.7
Isoleucine	13.2	12.6
Leucine	11.7	13.0
Tyrosine	8.2	10.7
Phenylalanine	6.0	8.0
Histidine	8.8	8.8
Lysine	12.8	23.3
Arginine	12.0	13.0

Duplicate samples were hydrolyzed for 24 hr in 6 M HCl at 100°. Half-cystine and tryptophan were not determined. The amino acid concentrations were referred to molecular weights of 25,000 and 29,000 for GSP and MCP, respectively.

however, the mast cell population consisted almost exclusively of the atypical variety. Both types of mast cells contained both GSP and MCP antigens. The antigens associated with the subepithelial cells did not have a pronounced granular distribution and some GSP was apparent outside of the cells. In contrast, the distribution of fluorescence observed in safranin-staining mast cells indicated that both GSP and MCP were associated with granules.

The molecular weights of GSP and MCP as estimated by gel electrophoresis in sodium dodecyl sulfate are 25,200 and 29,000, respectively. Their relative amino acid contents, following hydrolysis in 6 M HCl for 24 hr, are presented in Table 1. In immunodiffusion tests, no crossreaction occurred between MCP and GSP and their respective antibodies (Fig. 5).

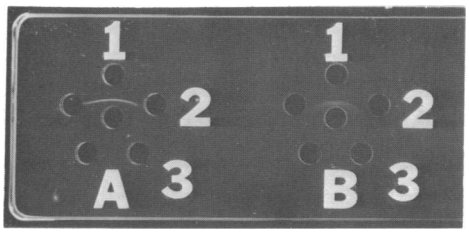

FIG. 5. Immunodiffusion test of anti-GSP and anti-MCP sera specificity. (*A*) Center well, 5 μl of anti-GSP serum; *1*, 5 μl of GSP solution (0.1 mg/ml); *2* and *3*, 5 μl of MCP solution (0.1 mg/ml). (*B*) Center well, 5 μl of anti-MCP serum; *1*, 5 μl of MCP solution (0.1 mg/ml); *2* and *3*, 5 μl of GSP solution (0.1 mg/ml).

DISCUSSION

The present results indicate that the major source of the serine protease (GSP) of rat small intestine is the atypical mast cells occurring in the epithelium and the lamina propria.

The diffuse localization of the enzyme around atypical mast cells in sections of perfusion-fixed small intestine and lung could be due either to failure to adequately fix the enzyme in its exclusively intracellular site or to its normal secretion into the surrounding connective tissue. Failure to extract the extracellular GSP from tissues by preincubation of sections in buffer indicated that the extracellular protease was fixed adequately. The possibility remains, however, that some GSP leaked from the mast cells during the early stages of fixation. The significance of positive immunofluorescent localization of GSP in numerous goblet cells is unknown; these cells synthesize GSP, or sequester the protease obtained from mast cells, or contain a distinct protein that shares with GSP one or more antigenic sites. The observations that intraepithelial atypical mast cells often appear closely associated with goblet cells in the crypts and that less than half of the goblet cells show specific fluorescence favor the hypothesis that some goblet cells acquire GSP from atypical mast cells.

The typical connective tissue mast cells contain both GSP and MCP, and in these cells both enzymes appear to be associated with the secretory granules. It was not clearly evident that either protease was similarly distributed in atypical mast cells, primarily because the granules are small and difficult to resolve.

The two enzymes possess notable similarities in relative amino acid content and esterase specificity (2, 10). MCP is several times more active on a molar basis in hydrolyzing benzoyltyrosine ethyl ester, and MCP can inactivate holoornithine aminotransferase, whereas GSP cannot (R. G. Woodbury and D. Lagunoff, unpublished results). The two proteins show no immunologic crossreactivity whatever. Determination of the amino acid sequence of MCP will establish whether or not GSP can be derived from MCP by limited proteolysis. It is proposed that, in the absence of a more systematic basis for distinguishing the two proteases, the chymotrypsin-like mast cell protease be called MCP I and the group-specific protease of small intestine, MCP II.

The localization of GSP in mast cells generally and in atypical mast cells of the gut and lung in particular would seem to make untenable any theory involving a general action of the enzyme in the control of pyridoxal phosphate-dependent enzymes as proposed by Katunuma and colleagues (1, 2). What functions the two mast cell proteases may play in the inflammatory response or the overall economy of connective tissue is not known.

The authors wish to thank Pamela Pritzl for preparing several of the antisera and Dr. Hans Neurath for his interest and encouragement during this study. This work was supported in part by grants from the National Institutes of Health (GM-15731 and HL-03174). R.G.W. is a recipient of a postdoctoral award from the National Institutes of Health (AM-05144).

1. Katunuma, N., Kominami, E. & Kominami, S. (1971) *Biochem. Biophys. Res. Comm.* **45**, 70–75.
2. Katunuma, N., Kominami, E., Kobayashi, K., Banno, Y., Suzuki, K., Chichibu, K., Gamaguchi, Y. & Katsunuma, T. (1975) *Eur. J. Biochem.* **52**, 37–50.
3. Woodbury, R., Katunuma, N., Ericsson, L., Neurath, H. & Titani, K. (1976) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **35**, 1622.
4. Melville, J. C. & Ryan, C. A. (1972) *J. Biol. Chem.* **247**, 3445–3453.
5. Lagunoff, D. & Pritzl, P. (1976) *Arch. Biochem. Biophys.* **173**, 554–563.
6. Weber, K. & Osborne, M. (1969) *J. Biol. Chem.* **244**, 4406–4412.
7. Lillie, R. D. & Fullmer, H. M. (1976) *Histopathologic Technic and Practical Histochemistry* (McGraw-Hill, New York), 4th Ed.
8. Enerbäck, L. (1966) *Acta Pathol. Microbiol. Scand.* **66**, 289–302.
9. Lendrum, A. C. (1944) *J. Pathol. Bacteriol.* **56**, 441.
10. Benditt, E. P. & Arase, M. (1959) *J. Exp. Med.* **110**, 451–460.