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The broad distribution of GP2 in mucous glands and secretory products

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ABSTRACT

GP2, a GPI-anchored glycoprotein that is a useful marker for M cells of Peyer's patches, is functionally related to the uptake of pathogenic bacteria in the gut lumen. Our immunostaining throughout the whole body of mice detected a broader localization than previously found of GP2 in various mucous glands and secretory cells. In the oral cavity, the palatine gland and lingual gland intensely expressed GP2 with immunolabeling along the basolateral membrane of acini and in luminal secretions of ducts. Secretory portions of the duodenal gland as well as the pancreas were immunoreactive for GP2 in the digestive tract. Luminal contents in the small intestine contained aggregations of GP2-immunoreactive substances which mixed with bacteria. The bulbourethral gland of Cowper displayed the GP2 immunoreactivity among the male reproductive organs. The vaginal epithelium contained many GP2-immunoreactive goblet-like cells, the occurrence of which dramatically changed according to the estrous cycle. These findings show that GP2 is a popular secretory product released from mucous glands and secretory cells and may support defense mechanisms against pathogenic bacteria in the tubular organs open to the external milieu.

GP2, a heavily glycosylated protein with a molecular weight of 75~92,000, was originally identified as a major granular component of pancreatic acinar cells (17). GP2 accounts for up to 40% of all membrane proteins of zymogen granules in pancreatic acinar cells. It is produced via the Golgi apparatus, bound to the limiting membrane of zymogen granules, targeted to the apical plasma membrane, and released into secretions (3, 4). Most researchers hypothesized the predominant functions of GP2 to be the formation of secretory granules or packaging and sorting of digestive enzymes. However, GP2 *null* mice displayed no detectable anomalies in either protein secretion or pancreatic acinar cell morphology (22). On the other hand, GP2 appeared as a

candidate of transcytotic receptors specific for M cells of Peyer's patches (6, 20), which are known to actively ingest macromolecules and microbes residing in the gut lumen. GP2 in M cells has been shown to function in the detection and uptake of a group of bacteria in the intestinal lumen (7, 23). GP2 was also identified as an antigen of pancreatic autoantibodies in Crohn's disease, and its increased expression in colonic enterocytes was related to the pathogenesis of this disease (16).

Although GP2 is one of the main secretory products of the pancreas, other secretory cells, such as conjunctival goblet cells, have an ability to produce GP2 (12). Epithelial cells dispersed in the respiratory epithelium of the paranasal sinuses were weakly positive for periodic acid-Schiff (PAS) reaction but intensely immunolabeled with the GP2 antibody (11). Furthermore, epithelial cells distributed in the lacrimal canaliculi, lacrimal sac, and nasolacrimal duct contained GP2 along the basolateral membrane and showed a sign of the release of GP2 into the lumen (12). These findings encouraged us to examine

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the distribution of GP2 in various organs, especially exocrine glands. The present study reports a broad distribution of GP2 in various exocrine glands of mucous types, and suggests a possible function related to bacteria.

MATERIALS AND METHODS

Tissue samplings. Ten-week-old adult ddY mice were supplied by Japan SLC (Shizuoka, Japan). The estrous cycle of female mice was determined by vaginal smears. Fresh samples were obtained from male and female mice, which were deeply anesthetized by an intraperitoneal injection of pentobarbital and sacrificed by bloodletting from the heart. The samples were directly embedded in a freezing medium (OCT compound; Sakura FineTechnical Co. Ltd., Tokyo, Japan) and quickly frozen in liquid nitrogen for *in situ* hybridization analyses. For immunohistochemistry, deeply anesthetized mice ($n = 5$ for each sex) were perfused via the aorta with a physiological saline, followed with 4% formaldehyde in 0.1 M phosphate buffer, pH 7.4. Various tissues were removed and immersed in the same fixative for an additional 6 h at 4°C. All experiments using animals were performed under protocols following the Guidelines for Animal Experimentation, Hokkaido University Graduate School of Medicine.

***In situ* hybridization.** Two non-overlapping antisense oligonucleotide DNA probes (45 mer in length) were designed for the mRNA of mouse glycoprotein 2 (Gp2): 421–465 and 1331–1375 of Gp2 mRNA (accession number: NM_025989). The probes were labeled with ^{33}P -dATP using terminal deoxynucleotidyl transferase (Invitrogen, Carlsbad, CA). Fresh frozen sections, 14- μm -thick, were fixed with 4% formaldehyde in 0.1 M phosphate buffer for 15 min and then acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine-HCl (pH 8.0) for 10 min. Hybridization was performed at 42°C for 10 h with a hybridization buffer containing ^{33}P -labeled oligonucleotide probes (10,000 cpm/mL in concentration). The sections were rinsed twice at 55°C for 40 min in $0.1 \times \text{SSC}$ ($1 \times \text{SSC}$: 150 mM sodium chloride, 15 mM sodium citrate) containing 0.1% *N*-lauroylsarcosine sodium, dehydrated through a graded series of ethanol, and air-dried. Sections were dipped in an autoradiographic emulsion (Kodak NTB-2; Carestream Health, Inc., Rochester, NY) at 4°C for 8–10 weeks. The hybridized sections used for autoradiography were counterstained with hematoxylin after development.

The *in situ* hybridization technique using the two non-overlapping antisense probes for the mRNA exhibited identical labeling in all the tissues examined. The specificity of the hybridization was also confirmed by the disappearance of signals upon the addition of an excess of an unlabeled antisense probe.

Immunohistochemistry. Fixed tissues were immersed in 30% sucrose and quickly frozen in liquid nitrogen. Cryostat sections, 14 μm in thickness, were prepared and mounted on poly-L-lysine-coated glass slides. Samples for luminal contents of the intestine were obtained by washing the lumen of the jejunum with a physiological saline and dropped on a glass slide. For immunofluorescence staining, the sections were incubated with the rat anti-GP2 antibody (D278, 1 $\mu\text{g}/\text{mL}$; MBL, Nagoya, Japan) overnight, followed by incubation with Cy3-labeled anti-rat IgG (Jackson ImmunoResearch, West Grove, PA). Some of the immunostained sections were counterstained with SyTO 13 (SYTOX; Invitrogen) for observation of the nuclei. The stained sections were mounted with glycerin-PBS and observed under a confocal laser scanning microscope (FV1000; Olympus, Tokyo, Japan).

Silver-intensified immunogold method for electron microscopy. Frozen sections from the formaldehyde-fixed tissues were mounted on poly-L-lysine-coated glass slides, incubated with the rat anti-GP2 antibody (1 $\mu\text{g}/\text{mL}$) overnight, and subsequently reacted with goat anti-rat IgG covalently linked with 1-nm gold particles (1 : 200 in dilution; Nanoprobes, Yaphank, NY). Following silver enhancement using a kit (HQ silver; Nanoprobes), the sections were osmicated, dehydrated, and directly embedded in Epon (Nissin EM, Tokyo, Japan). Ultrathin sections were prepared and stained with both uranyl acetate and lead citrate for observation under an electron microscope (H-7100; Hitachi, Tokyo, Japan). The specificity of the immunoreactions was confirmed by the disappearance of the immunolabeling when the antibody was pre-incubated with the antigen.

RESULTS

Distribution of GP2-expressing cells

The oral cavity is associated with many excretory glands of various sizes. The parotid gland, submandibular gland, and sublingual gland are classified as major salivary glands. In this study, their secretory portions were free from the immunoreactivities of GP2, except for the sublingual gland—where only the luminal surface of secretory ducts was immuno-

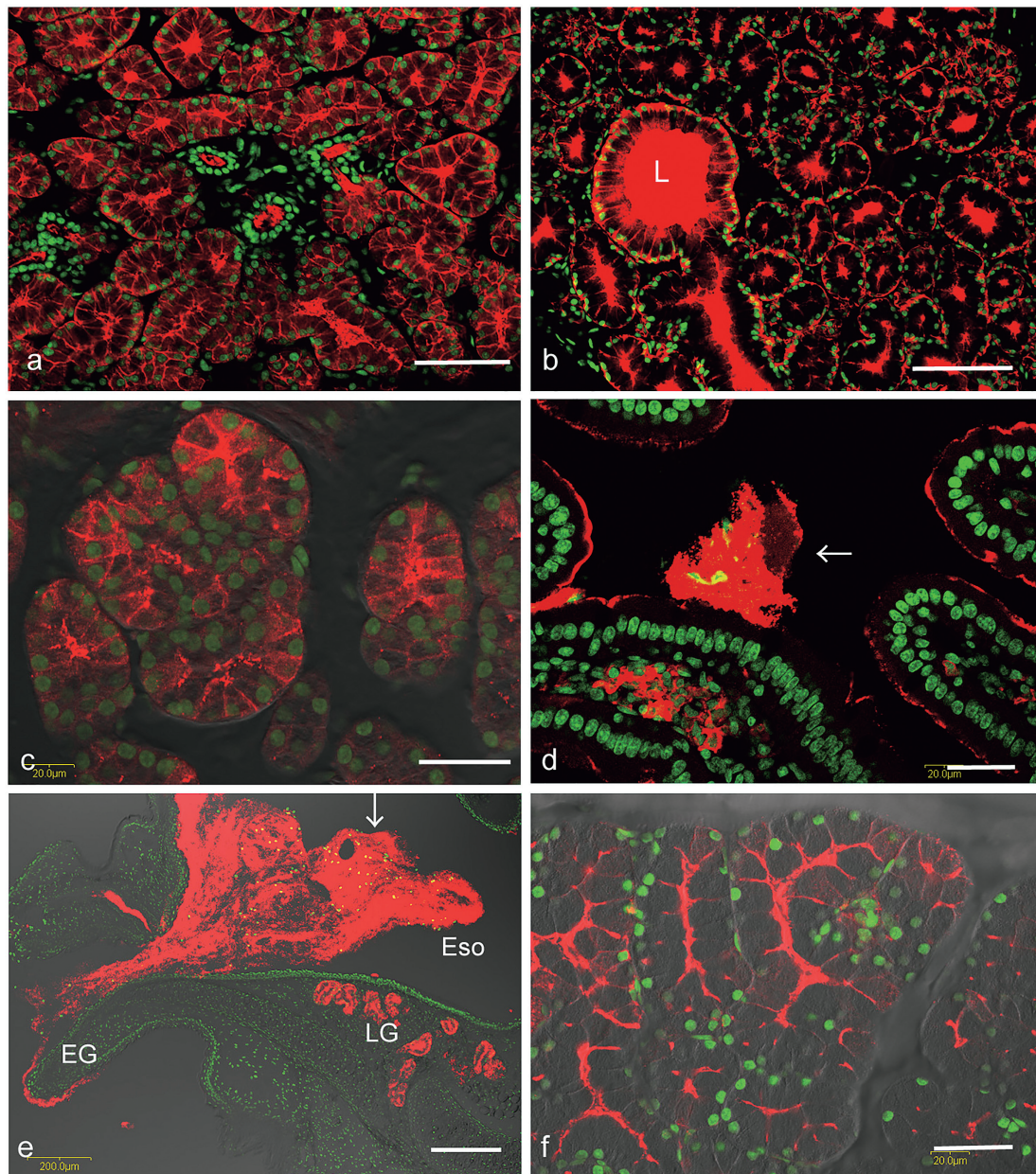


Fig. 1 Immunohistochemistry of GP2. The basolateral membrane of mucous secretory cells are immunoreactive for GP2 in the palatine gland (a), lingual gland (b), and duodenal gland (c). In the lingual gland, luminal contents of the excretory duct are heavily immunolabeled (b). L: lumen. The lumen of the jejunum contains immunoreactive substances (arrow in d). In addition, the brush border and a small group of cells in the lamina propria are immunoreactive. In the larynx, luminal contents (arrow) as well as the laryngeal gland (LG) are immunoreactive (e). EG: epiglottis, Eso: esophagus. The bulbourethral gland displays an immunoreactivity in the lateral cell membrane and luminal contents (f). Bars: 40 μ m (c, d, f), 100 μ m (a, b), 200 μ m (e)

labeled (data not shown). In contrast, small salivary glands of the mucous type including the palatine glands and lingual glands displayed intense immunoreactivities for GP2 (Fig. 1a, b). Mucous glands at the proximal part of the tongue exhibited an intense immunoreactivity for GP2, while lingual glands

of the serous type closely located to circumvallate papillae were not immunolabeled for GP2. The duodenal gland as well as the pancreas was immunoreactive in the gastrointestinal tract (Fig. 1c). Surface mucous cells of the stomach, pyloric gland cells, and intestinal goblet cells were negative in immuno-

reaction. Luminal contents in the esophagus and the small intestine (Fig. 1d, e) contained GP2-immunoreactive substances, possibly derived mainly from the oral cavity and pancreatic duct, respectively. In the airway, the nasal gland, laryngeal gland (Fig. 1e), and tracheal gland contained glandular portions of the mucous type showing an intense immunoreactivity for GP2. Luminal contents from the pharynx to larynx presented a GP2 immunoreactivity (Fig. 1e). In all the mucous gland cells mentioned above (Fig. 1a–c), the lateral membrane and contents of the lumina were heavily immunolabeled, while positive staining of the basal cell membrane was inconsistent.

The Cowper's gland, a large mucous gland situated at the proximal region of the penis, showed an intense immunoreactivity for GP2 (Fig. 1f). Here again, the lateral membrane of glandular cells and luminal contents were immunoreactive for GP2.

Mucosa of the vagina exhibited various features according to the sexual cycle. At the diestrus, the surface epithelium is covered by PAS-positive goblet-like cells, which were immunoreactive for GP2 (Fig. 2a). Essentially, the plasma membrane of the vaginal mucous cells including the apical membrane was selectively labeled with the GP2 antibody. However, thick stratified squamous epithelium lacking goblet-like cells at estrus was completely immunonegative for GP2. The vagina also displayed GP2-immunoreactivity in the PAS-positive luminal content and surface mucous layer above the epithelium (Fig. 2b, c).

An *in situ* hybridization analysis detected mRNA expression in several tissues and confirmed the existence of GP2 at the mRNA level (Fig. 3). A representative tissue is the lingual gland, where serous types and mucous types are mixed on sections of the proximal tongue. Signals for GP2 mRNA were restricted to the mucous type of lingual gland (Fig. 3a). A significant expression of GP2 mRNA was also confirmed in the Cowper's gland (Fig. 3b).

Subcellular localization of GP2

The subcellular localization of GP2 was examined in the lingual gland (Fig. 4a), palatine gland, laryngeal gland (Fig. 4b), and Cowper's gland. Electron microscopically, the basolateral membrane of these secretory cells was immunolabeled with gold particles for GP2. When the immunoreactivities were light in intensity, the labeling was restricted to the basal half of the lateral membrane, especially the ruffled border provided with irregular microvilli. The luminal side of the cell membrane was also immunolabeled in secretory cells of the laryngeal gland

(Fig. 4b) and Cowper's gland. The cytoplasm and any intracellular structures were largely negative in reaction.

Relationship of GP2 with bacteria in the intestine

Secretory products released from secretory glands were frequently immunolabeled; the lumina of the esophagus (Fig. 1e), larynx, and vagina (Fig. 2b) contained heavily labeled secretory contents. The intestine very frequently contained immunoreactive substances in the lumen (Fig. 1d). In addition, the brush border and the surface mucous layer on the intestinal villi of the duodenum and jejunum were immunoreactive for GP2, but no surface immunolabeling was visible in the terminal ileum or large intestine. When smear preparations of the intestinal luminal contents were double-stained with the GP2 antibody and a DNA marker (SYTOX green), the samples contained GP2-immunoreactive substances intermingled with bacteria (Fig. 5a). Electron microscopically, the immunoreactive substances attached to bacteria which resided freely in the lumen or were partially embedded in the epithelium (Fig. 5b).

DISCUSSION

The production of GP2 is not unique to the pancreas or some specialized cells such as intestinal M cells, since the present study revealed an extensive expression of GP2 in many mucous glands in the digestive, respiratory, and genital tracts. In the oral cavity, small salivary glands of the mucous type are the main sources of GP2 production. In contrast, glandular portions of three major salivary glands which possess both mucous and serous characteristics did not contain GP2. Interestingly, the sublingual gland, predominantly mucous in nature, exhibited an immunoreactivity for GP2 on the luminal side of the ducts. Goblet cells are mucous cells dispersed in various types of the mucosal epithelium. They are negative in the immunoreaction for GP2 in the digestive tract and respiratory tract, but conjunctival goblet cells were found to express abundant GP2 (12). The vaginal mucosa is unique as the mucosal epithelium changes during the estrous cycle under the control of estrogen and progesterone. Progesterone induces differentiation to a mucus-secreting epithelium of the vagina. The goblet-like cells appear in the surface layer of the vaginal epithelium during diestrus but detach from the epithelium by the subsequent proestrus period (5). These goblet-like cells were found in the present study to be positive in PAS reaction and intensely immunoreactive for GP2. The bulbo-

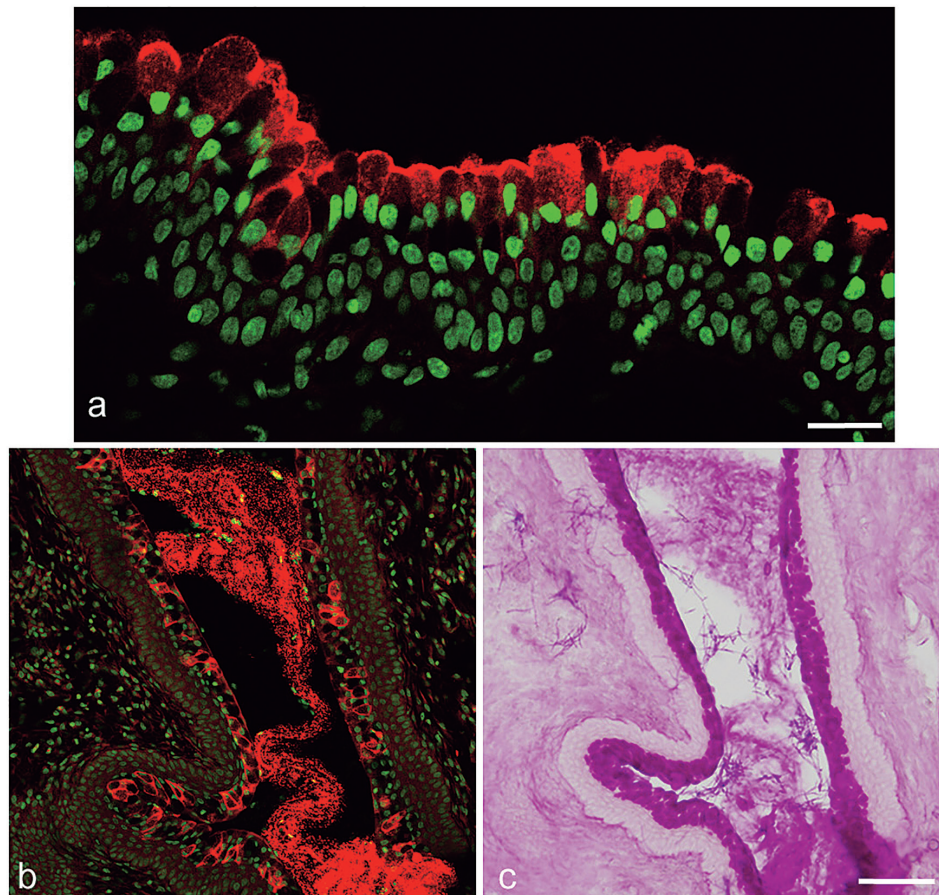


Fig. 2 GP2 immunoreactivity in the vagina. The surface goblet-like cells show positive immunoreactivity along apical and lateral cell membranes (a). Observation of the same section shows that the luminal contents with GP2 immunoreactivity (b) correspond to PAS-positive substances (c). Bars: 20 μm (a), 100 μm (b, c)

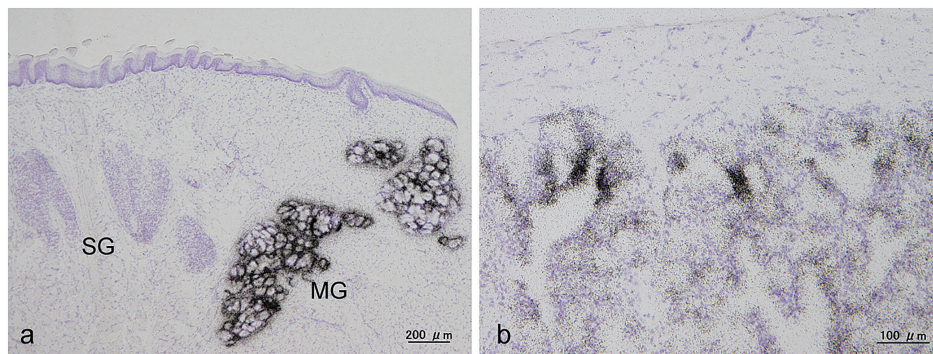


Fig. 3 An *in situ* hybridization analysis for GP2 mRNA in the tongue and bulbourethral gland. Intense signals are seen in the mucous gland (MG) of the tongue but not the serous gland (SG) (a). In the bulbourethral gland, the glandular portion expresses GP2 mRNA (b).

urethral gland of Cowper is a typical mucous gland which produces GP2. It is worth noting that the tubular organs rich in GP2 are all exposed to the external milieu.

The subcellular localization of GP2 in the mucous glands examined in the present study corresponds to the membrane-associated localization of GP2 in the pancreatic acinar cells (8) and M cells (7). Besides

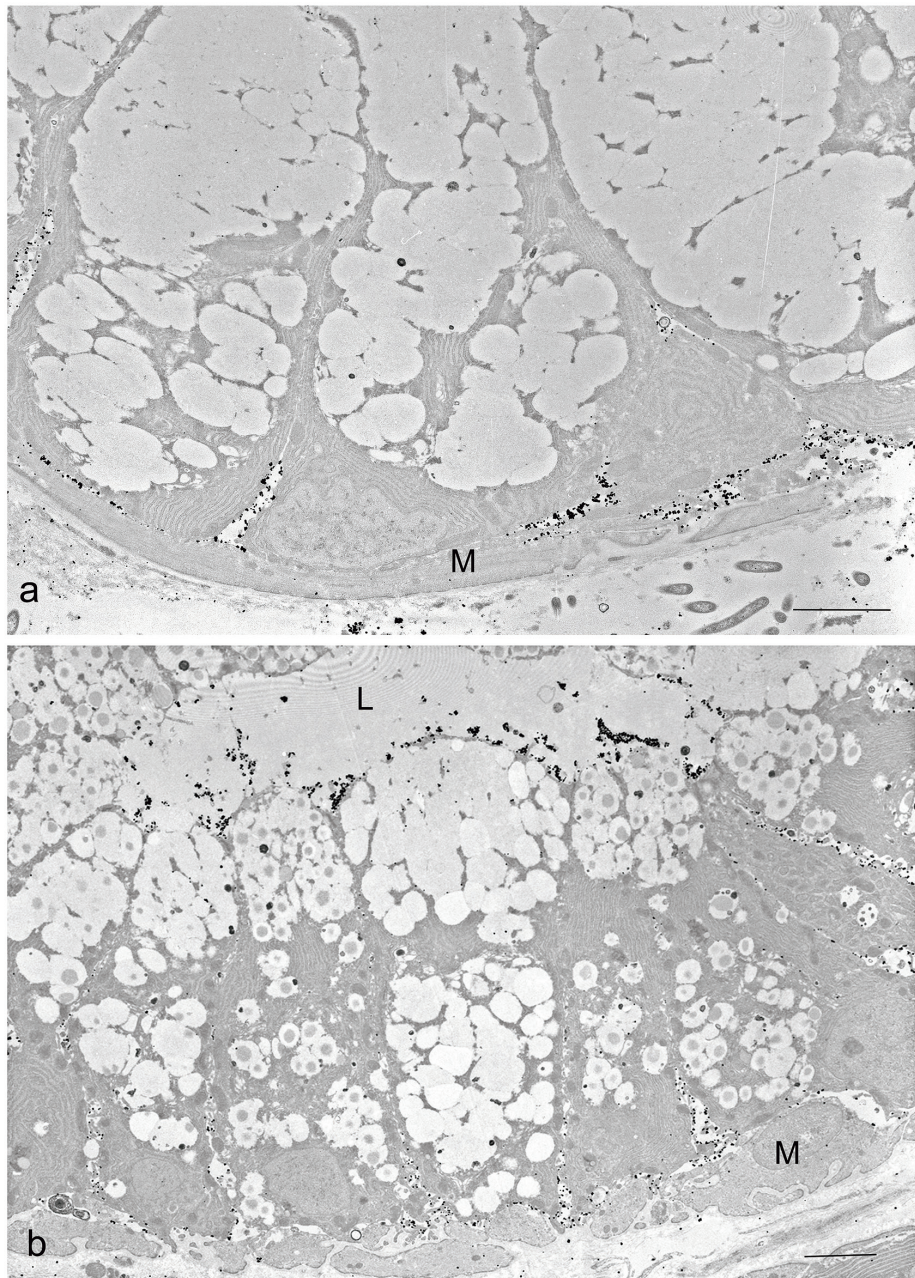


Fig. 4 Silver-intensified immunogold method for electron microscopy. Immunogolds are distributed along the lateral and basal membrane of the laryngeal gland (a). In the lingual gland, heavy labeling is seen on the apical membrane, with moderate labeling along the basolateral membrane. L: lumen, M: myoepithelial cells. Bars: 2 μ m

the membrane protein form, GP2 in the pancreatic acinar cells occurs as a soluble form and is copiously released into the pancreatic juice (8, 15). One characteristic feature in the present study is the rich existence of immunoreactive GP2 in the luminal contents, clearly shown in the oral cavity, esophagus, pharynx, intestine, and vagina. The luminal contents holding GP2 in the oral cavity and esophagus may

be derived from the palatine and lingual glands. A part of the luminal GP2 in the pharynx must be secreted from the pharyngeal gland. In addition, the paranasal sinuses possess numerous GP2-secreting cells in the epithelium (18) and may secrete GP2 into paranasal sinuses, further flowing into the nasal/pharyngeal cavities. Goblets cells in the conjunctiva excrete GP2 into the conjunctival space. Our previ-

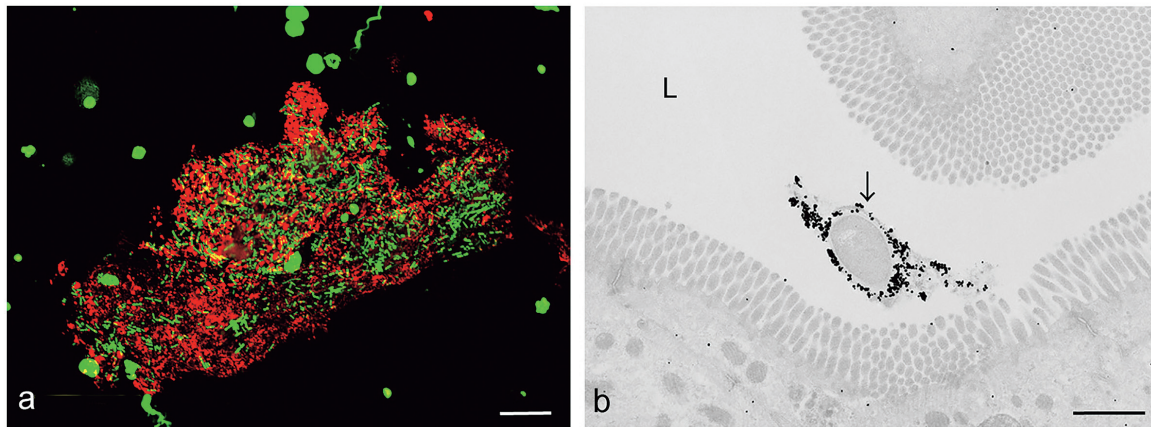


Fig. 5 Relationship of GP2 and bacteria in the lumen of the intestine. Samples were obtained by washing the lumen of the jejunum with a physiological saline and dropped on a glass slide. The glass slides were stained with anti-GP2 (red) and SYTOX green for DNA marker. In the aggregates of various sizes, GP2 and bacteria are mixed (a). Immunoelectron microscopy by using immunogold method shows an aggregation of gold particles for GP2 around bacteria (arrow) in the lumen (L) of the intestine. Bars: 20 μ m (a), 1 μ m (b)

ous study (12) has shown that GP2-immunoreactive cells of peculiar shapes release their products into the lacrimal canaliculi, lacrimal sac, and nasolacrimal duct. In this tear duct system, the volume of secreted GP2 is sufficient to irrigate the narrow space of the tear duct up to the nasal cavity. GP2 in the pancreas abundantly pours into the duodenum, resulting in the delivery of a great amount of GP2 into the lumen of the upper part of the small intestine. This is sufficient to coat the surface of intestinal villi, namely the brush border, as we observed positive labeling for GP2 there in the present study. Vaginal goblet-like cells supply GP2 in the vaginal lumen only during diestrus, in contrast to estrus when the vaginal mucosa is covered by thick stratified epithelium. The lumen of the male urethra contained GP2 derived from the Cowper's gland. Thus, the airway, tear duct, alimentary tract, and genital tract all have an ability to hold abundant amounts of secreted GP2 in the lumen. What is the biological function of the secreted GP2?

GP2 in M cells was localized predominantly on the apical plasma membrane and possessed a binding ability against particular enterobacteria to promote the internalization of the bacteria (7). The closest homologue of GP2 is Tamm-Horsfall protein (THP, also termed uromodulin), which is structurally related to GP2 with about a 50% homology. THP has been identified as a predominant glycoprotein present in human urine and is localized on the luminal plasma membrane and vesicular elements in the apical cytoplasm of renal urinary tubules (1, 4, 9, 19). The two proteins have a common tendency to self-aggregate

and form a fibrillar network, a characteristic important for trapping bacteria (8, 10). THP specifically binds to uropathogenic *E. coli* with Type 1 fimbriae and promotes bacterial clearance in the bladder (13). Two independent laboratories have examined THP null mice and showed increased sensitivities to urinary tract infections (2, 13). *E. coli* with Type I fimbriae may represent a major pathogen in both the gastrointestinal and urogenital tracts. The present observation in the intestine morphologically showed an intimate relation with bacteria there. The similarities between GP2 and THP in biochemical structure, subcellular localization, and binding to Type 1 fimbriae indicate a role for GP2 in the defense system. Thus, there is a possibility that the released GP2 makes bacteria innocuous or 'opsonizes' them for effective uptake such as by M cell-like cells or professional scavenger cells.

Some studies have focused on the involvement of GP2 in inflammatory bowel diseases. An analysis of pancreatic autoantibodies from Crohn's disease detected GP2 as an autoantigen, being a candidate for novel serologic parameters (16). The immunofluorescence method for GP2 stained the brush border of colonic enterocytes in Crohn's disease (16). The localization in the brush border may be supported by the proteomic characterization of lipid raft proteins from the brush border membrane of the rat jejunum (14). However, since there is no direct evidence showing mRNA expression of enterocytes at cellular levels, we cannot deny that GP2 detected in the brush border of enterocytes is derived from GP2 released from the pancreas. A flow cytometry analy-

sis of human samples using a GP2 antibody detected the existence of GP2 in various types of cells including T/B cells, monocytes, and isolated intestinal epithelial cells, with only modest expression in epithelial cell lines (21). When we stained the small intestine of mice with the GP2 monoclonal antibody used in this study, the lamina propria of intestinal villi was found to contain many GP2-immunoreactive cells of irregular shapes, which may be macrophages or dendritic cells (Fig. 1d). Identification of GP2 in immune cells requires careful staining with respect to the expression levels and specificity of the immunoreactions.

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