Immunohistochemical relationships of huntingtin-associated protein 1 with enteroendocrine cells in the pyloric mucosa of the rat stomach

（ラット胃の幽門部粘膜における腸管内分泌細胞と

ハンチンチン関連タンパク質 HAP1 の免疫組織化学的検討）

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Contents

1. ABSTRACT

Huntingtin-associated protein 1 (HAP1) is a neuronal cytoplasmic protein that is predominantly expressed in the brain and spinal cord. In addition to the central nervous system, HAP1 is also expressed in the peripheral organs including endocrine system. Different types of enteroendocrine cells (EEC) are present in the digestive organs. To date, the characterization of HAP1-immunoreactive (ir) cells remains unreported there. In the present study, the expression of HAP1 in pyloric stomach in adult male rats and its relationships with different chemical markers for EEC [gastrin, marker of gastrin (G) cells; somatostatin, marker of delta (D) cells; 5-HT, marker of enterochromaffin (EC) cells; histamine, marker of enterochromaffin-like (ECL) cells] were examined employing single- or double-labelled immunohistochemistry and with light-, fluorescence- or electron-microscopy. HAP1-ir cells were abundantly expressed in the glandular mucosa but were very few or none in the surface epithelium. Double-labelled immunofluorescence staining for HAP1 and markers for EECs showed that almost all the G-cells expressed HAP1. In contrast, HAP1 was completely lacking in D-cells, EC-cells or ECL-cells. Our current study is the first to clarify that HAP1 is selectively expressed in G-cells in rat pyloric stomach, which probably reflects HAP1's involvement in regulation of the secretion of gastrin.

Keywords: huntingtin-associated protein 1, gastrin, somatostatin D cells; enterochromaffin cells; enterochromaffin-like cells; immunohistochemistry

Abbreviations

DAB, diaminobenzidine

EC, enterochromaffin

EEC, enteroendocrine cells

ECL, enterochromaffin-like

G-cells, gastrin cells

HAP1, Huntingtin-associated protein 1

htt, huntingtin

NDS, normal donkey serum

polyQ, polyglutamine

SBMA, spinal and bulbar muscular atrophy

STB, stigmoid body

2. Introduction

Huntingtin-associated protein 1 (HAP1) is a cytoplasmic protein and considered as a determinant marker for stigmoid body (STB; Li et al., 1996; Gutekunst et al., 1998; Fujinaga et al., 2004, 2007, 2009; Islam et al., 2012, 2017, 2020a; Wroblewski et al., 2018; Chen et al., 2020). STB was originally identified in our previous studies as a spherical-to oval-shaped, non-membranous physiological cytoplasmic inclusion (0.5 to 3 mm in diameter) of granular to fuzzy texture with low-moderate electron density (Shinoda et al., 1992, 1993).

HAP1 was originally recognized as a polyglutamine (polyQ) length-dependent interactor of huntingtin (htt), the gene product responsible for Huntington's disease (Li et al., 1995). STB/HAP1 can protect against apoptosis and cell death induced by htt with an expanded polyQ sequence (Li et al., 2003; Metzger et al., 2008). In addition, STB/HAP1 can also bind to a polyQ-expanded androgen receptor derived from spinal and bulbar muscular atrophy (SBMA), and over expression of HAP1 suppresses polyQ androgen receptor-induced apoptosis (Takeshita et al., 2006). STB/HAP1 can also interact with the causal agents of some other polyQ diseases, such as with Abelson helper integration site 1 in Joubert syndrome (Sheng et al., 2006), TATA binding protein in spinocerebellar ataxia type 17 (Prigge and Schmidt, 2007) and ataxin 3 in Machado-Joseph disease (Takeshita et al., 2011). In addition, a number of biochemical, in situ hybridization, histochemical and immunohistochemical studies on STB/HAP1-expression and distribution have clarified that HAP1 is abundantly expressed in the limbic-hypothalamic regions of brain and in the dorsal horn of spinal cord in normal rodents (Fujinaga et al., 2004; Takeshita et al., 2006, 2011; Islam et al., 2012, 2017; Wroblewski et al., 2018; Chen et al., 2020). Interestingly, these regions of the brain and spinal cord are usually spared from neurodegeneration, whereas the regions lacking STB/HAP1 or with little expression such as neocortex, striatum, thalamus, cerebellum and spinal motoneurons are major targets in different neurodegenerative diseases

(Fujinaga et al., 2004; Islam et al., 2017). Taken together, STB/HAP1 is thought to augment the threshold of vulnerability to neurodegenerative apoptosis, confer increased neuronal stability, and subsequently protect against cell death and apoptosis in several neurodegenerative diseases. This has been referred to as the "STB/HAP1 protection hypothesis" (Fujinaga et al., 2001; Metzger et al., 2008; Takeshita et al., 2006; Islam et al., 2017; Wroblewski et al., 2018).

In terms of physiological functions, several studies have reported that hypothalamic HAP1 can act as a mediator of feeding behaviors and modulate hypothalamic function for stress response (Chan et al., 2002; Dragatsis et al., 2004; Sheng et al., 2006; Lin et al., 2010; Niu et al., 2011; Chen et al., 2020). However, using in situ hybridization and immunohistochemistry, it has also been reported that HAP1 is expressed not only in the central nervous system but also in the peripheral organs including pituitary gland, thyroid gland, and pancreatic islets (Dragatsis et al. 2000; Liao et al., 2005). In the rat pituitary, HAP1 is selectively expressed only in thiotrophs, whereas in pancreatic islets, HAP1 is expressed in the B cells (Liao et al., 2010). In thyroid gland, HAP1 is believed to express in parafollicular cells (Liao et al., 2005). In addition to these peripheral organs, STB/HAP1 is also expressed in the mucosa of gastrointestinal tract (Liao et al., 2005; Li et al., 2019). The expression of HAP1 is higher in the mucosa of stomach than that of other regions of the gastrointestinal tract (Li et al., 2019). In the gastric mucosa, HAP1-immunoreactive (ir) cells are densely distributed in the glands, and they are numerous in the pyloric region, few to moderate in the fundic region, and very few in the cardiac region (Liao et al., 2005; Li et al., 2019). Abundant expression of HAP1 in the mucosa of pyloric stomach imply that STB/HAP1 may be involved in processing or modification of certain gastric functions. In this context, it becomes important to clarify the characterization of HAP1-ir cells in the mucosa of stomach.

3. Purpose of the present study

Gastrointestinal hormones secreted from enteroendocrine cells (EEC) play a vital role in modulating metabolic and digestive functions (Furness et al., 2013; Worthington et al., 2018; Hunne et al., 2019). The major types of chemically defined distinct endocrine cells in the gastric mucosa are gastrin (G) cells, delta (D) cells, enterochromaffin (EC) cells and enterochromaffin-like (ECL) cells (Solcia et al., 2000). Gastrin is a well-known marker of G-cells), somatostatin is used as marker of D-cells, 5-HT (serotonin) is treated as marker of EC-cells and histamine is used as marker of ECL-cells (Solcia et al., 2000; Hunne et al., 2019). To date, however, elucidation of immunohistochemical relationships of HAP1-ir cells with the above-mentioned markers has not been conducted.

In the present study, using immunohistochemistry and electron microscopy, first we set out to confirm the expression and subcellular distribution of HAP1 in the mucosa of pyloric stomach and then we aim to carry out double-label immunofluorescence immunohistochemistry of HAP1 with above-mentioned four markers to reveal the immunohistochemical relationships of HAP1 with EECs.

4. Materials and methods

4.1. Primary antibodies

The details of the primary antibodies used in the current study are listed in Table 1, most of which are commercially available. The characterization of these primary antibodies was clarified in our previous studies or earlier by others (Table 1). In addition, the characterization of the rabbit polyclonal anti-HAP1 primary antibody was also determined in the present study (Fig. 1).

4.2. Animals and ethics

A total of 12 adult male Wistar rats (8-10-week-old) were purchased from Japan SLC Inc., (Shizuoka, Japan) for the current study. The animals were kept in groups (3-4 rats) at 22-24^{0}C temperature with a 12 to 12-h light dark cycle (lights on 08:00-20:00) and provided sufficient water and food. Experimental protocols used in this study were approved by the Yamaguchi University School of Medicine Committee on the Ethics of Animal Experimentation and carried out according to the guidelines for Animal Research of the Government of Japan (Law No. 105, Notification No. 6). All efforts were employed to reduce the number of rats used and their suffering.

4.3. Tissue preparation for immunohistochemistry

Rats were transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4) under anesthesia with pentobarbital sodium (60-80 mg/kg, intraperitoneal injection). Pyloric region of the stomach was collected and immediately post-fixed for overnight in the same fixative used for perfusion and then transferred to 0.1 M PB containing 30% sucrose solution for one weeks. Finally, the samples were frozen in powdered dry ice and then sectioned at a thickness of 30 □m on a cryostat.

4.4. Immunohistochemistry

4.4.1. Single immunoperoxidase staining

Single immunoperoxidase immunohistochemistry was performed as described in our previous studies (Jahan et al., 2015; Islam et al., 2020b). In brief, free floating sections of pyloric stomach were blocked with 10% normal goat serum (NGS; Millipore, Temecula, CA, USA) containing 0.3% Triton X-100 at room temperature for 2h, pretreated with 1.5% hydrogen peroxide and 50% methanol at 4 °C for 30 min and then incubated with rabbit polyclonal primary antibody to HAP1 (1: 500) at 20 °C for 3 days. For negative control, pre-immune normal rabbit serum was used instead of primary antibody. Then, after three washing the sections were incubated at 20 °C for 2 h with biotinylated goat anti-rabbit secondary antibody (Millipore; 1:1,000 dilution) followed by incubation at 20 °C for 2h with peroxidase-conjugated streptavidin (1:1,000 dilution; Dako, Glostrup, Denmark). After washing three times with 0.05 M Tris-HCl buffer (pH 7.6), the sections were processed for nickel-enhanced diaminobenzidine (DAB) reaction at 4 °C for 10-20 min with a mixture of 0.02% 3, 3/ DAB; (Dojinbo Laboratories, Kumamoto, Japan) and 0.6% nickel ammonium sulfate (Sigma-Aldrich, Tokyo, Japan) in 0.05 M Tris-HCl buffer containing 0.0008% H_2O_2. Finally, the sections were mounted on glass slides, air-dried for 30 min, dehydrated using graded series of alcohol and Xylene and lastly embedded with Entellan New (Millipore).

4.4.2. Double-label immunofluorescence histochemical staining

Double-label immunofluorescence immunohistochemistry was carried out as described in our previous reports (Islam et al., 2017, 2020a; Wroblewski et al., 2018). In brief, free floating sections of pyloric stomach were blocked with 10% NDS containing 0.3% Triton X-100 at 4 °C for 2 h and incubated with goat polyclonal anti-HAP1 (1:5,000) antibody in combination

with a rabbit anti-histamine (1:500), mouse anti-somatostatin (1:25), rabbit anti-5HT (1:200) antibody at 20 °C for 3 days (Table 1 for detail about primary antibodies). After washing, the sections were incubated with a mixture of Alexa Fluor 594-conjugated donkey anti-goat IgG (Invitrogen, Eugene, OR, USA; 1:1,000) and Alexa Fluor 488-conjugated donkey anti-rabbit IgG or anti-mouse IgG (Invitrogen, Rockford, IL, USA; 1:1,000) secondary antibodies at 20 °C for 3 h. Similarly, double-staining of HAP1 and gastrin was performed using rabbit anti-HAP1 antibody (1:500) and goat-anti gastrin antibody (1:2000). In this case, mixture of Alexa Fluor 594-conjugated donkey anti-rabbit (Invitrogen, Eugene, OR, USA; 1:1,000) and Alexa Fluor 488-conjugated donkey anti-goat IgG (Invitrogen, Rockford, IL, USA; 1:1,000) secondary antibodies were used. The sections were then mounted on glass slides, air-dried for 30 min and finally embedded with Fluoromount/Plus (K048, Diagonostic Biosystems, Pleasanton, CA, USA).

4.5. **Electron microscopy**

Tissue preparation for immunoelectron microscopy was performed, as previously described (Islam et al., 2012; 2017). Adult male Wistar rats were anesthetized, as described above, intracardially perfused with 0.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M PB. The trimmed slabs of stomach blocks containing the pylorus of stomach were cut into 30-μm-thick sections on a vibrating blade microtome (Leica VT 1000S; Leica Microsystems, Nussloch, Germany) in 0.1 M PB. The free-floating sections were stained by the nickel-enhanced DAB reaction method, as described above, using the rabbit polyclonal anti-HAP1 antibody. HAP1-stained sections were postfixed for 1 h with 1% osmium tetroxide in 0.1 M PB, block-stained for 1 h with 2% uranyl acetate in distilled water, dehydrated with a graded series of ethanol rinses, infiltrated in propylene oxide, placed in a mixture of propylene oxide and Epoxy resin (Quetol 812; Nissin EM, Tokyo, Japan; 1:1), and then embedded on Aclar

film (Nisshin EM, Tokyo, Japan) in Epon. Ultrathin sections were obtained from the trimmed slab containing the mucosa of pylorus with HAP1-ir structures using ultramicrotome (Ultracut OmU4; Reichert-Jung, Vienna, Austria) and mounted on copper grids. The sections were observed under electron microscope (H-7500; Hitachi Ltd., Tokyo, Japan) operated at 80 kV without uranyl acetate or lead staining.

4.6. Tissue analyses and Cell counting

Co-expression ratios for HAP1/EEC markers (Table 2) were calculated from the actual number of HAP1-ir cells and EEC markers-ir cells, and from those double-stained for HAP1 and EEC markers following the counting procedure described in previous studies (Nagano and Shinoda, 1994; Palus et al., 2018). From one rat, at least 300 positive cells were counted for every combination of HAP1 and an EEC marker. Values were shown as mean ± SEM (n = 4). HAP1-ir cells with a clearly visible-nucleus were counted in our current study.

4.7. Photomicrographs

A color digital Lumenera USB 2.0 camera (Lumenera Corporation, Ottawa, Canada) equipped with an Eclipse E80i photomicroscope (Nikon) was used to capture the immunoperoxidase staining images. A laser-scanning confocal microscope (LSM510 Meta; Carl Zeiss, Oberkochen, Germany) was used to obtain a single optical section (2048 × 2048 pixels) for immunofluorescence staining images. Finally, Adobe Photoshop Elements 2018 (Adobe Systems, Inc., San Jose, CA, USA) were used to modify the images only for contrast and brightness.

Table1. Antibodies used in the present study

Antibody		Immunogen	Host /clonality	Code/Source	Source	Dilution	References
Primary antibody	Anti- HAP1 (R19)	Rat HAP1 C-terminus	Goat polyclonal	Cat# sc-8770, RRID: AB_647322	Santa Cruz Biotechnology, Santa Cruz, CA, USA	1:5,000	Islam et al., 2017
	Anti- HAP1 (R12)	Rat HAP1 full length	Rabbit polyclonal	Cat# HAP1R12, RRID: AB_2571562	Shinoda lab, Yamaguchi University, Japan	1:500	Fujinaga et al., 2009
	Anti-gastrin (C-20)	Human gastrin	Goat polyclonal	Cat# sc-7783, RRID: AB_2108261	Santa Cruz Biotechnology, Santa Cruz, CA, USA	1:2,000	Bonnavion et al., 2015
	Anti-somatostatin	Human somatostatin	Mouse monoclonal	Cat# V1169, RRID: AB_2313730	Biomeda, Foster City, CA, USA	1:25	Chang et al., 2007
	Anti-5HT	5HT conjugated to Bovine serum albumin	Rabbit polyclonal	Cat# 066D, RRID: AB_2313879	Biomeda, Foster City, CA, USA	1:200	Bartel et al., 2006
	Anti-histamine	Hstamine coupled to succinylated KLH with carbodiimide linker.	Rabbit polyclonal	Cat# 22939, RRID: AB_572245	Immunostar, Hudson, WI, USA	1:500	Webber et al., 2017
Secondary antibody	biotinylated anti-goat	IgG, isolated from donkey serum	Polyclonal	Cat# AP180B, RRID: AB_11214009	Millipore, Temecula, CA, USA	1:1,000	Islam et al., 2012
	Alexa Fluor 594 donkey anti-goat	IgG (H + L) goat	Polyclonal	Cat# A11058, RRID: AB_2534105	Invitrogen, Eugene, OR, USA	1:1,000	Wroblewski et al., 2018
	Alexa Fluor 594 donkey anti-rabbit	IgG (H + L) rabbit	Polyclonal	Cat# A21207, RRID: AB_141637	Invitrogen, Eugene, OR, USA	1:1,000	Fujinaga et al., 2011
	Alexa Fluor 488 donkey anti-rabbit	IgG (H + L) rabbit	Polyclonal	Cat# A32790, RRID: AB_2534105	Invitrogen, Eugene, OR, USA	1:1,000	Jahan et al., 2015
	Alexa Fluor 488 donkey anti-goat	IgG (H + L) goat	Polyclonal	Cat# A11055, RRID: AB_2534102	Invitrogen, Eugene, OR, USA	1:1,000	Takeshita et al., 2011
	Alexa Fluor 488 donkey anti-mouse	IgG (H + L) mouse	Polyclonal	Cat# A21202, RRID: AB_141607	Invitrogen, Eugene, OR, USA	1:1,000	Islam et al., 2017

AR, Androgen receptor; HRP, horseradish peroxidase, IgG, Immunoglobulin G, RRID, Research Resource Identifier; KLH, keyhole limpet hemocyanin

5. Results

5.1. Distribution and subcellular localization of HAP1-immunoreactivity in the gastric mucosa

In immunohistochemistry, abundant expression of HAP1-ir cells were observed in the pyloric part of gastric mucosa (Fig. 1A-B). HAP1-ir cells were mainly distributed in the glandular mucosa but were very few or none in the surface epithelium.

In the current study, majority of the HAP1-ir cells had dot-like structures in their cytoplasm (type A), whereas few cells showed only diffuse HAP1-immunoreactivity in the cytoplasm with no clear dot-like structures (type B). Using pre-immune rabbit serum instead of primary antibody, both types of HAP1-immunoreactivity were completely eliminated (Fig. 1C). In the present study, using immuno-electron microscopy, the HAP1-ir dot like structures were proven to be STBs (Fig. D-F). In the present study, the diameter of the STB was about 0.7 mm.

5.2. Immunohistochemical relationships of STB/HAP1 with different EECs

To elucidate the morphological relationships between HAP1 and EECs (Gastrin cells, somatostatin D cells, 5HT-ir (serotonin) EC cells and histamine-ir ECL cells), first the expression and distribution of the markers of EEC were examined immunohistochemically in the mucosa of pyloric part of stomach (Fig. 2A-D). All the EECs were prominently expressed in the glandular mucosa of pyloric stomach. Most of the histamine-ir cells (2A), somatostatin-ir cells (2B), 5HT-ir cells (2C), and gastrin-ir cells were located mainly at the base of the epithelial layer. HAP1 showed similar distribution pattern as that of EECs in the pyloric stomach (Fig. 1-2).

Next, we performed double-label immunofluorescence staining for HAP1 with gastrin (marker of G cells), somatostatin (marker of D cells), 5-HT (marker of EC cells) and

histamine (marker of ECL cells). Our current double-immunofluorescence staining results revealed that HAP1 was never detected in somatostatin- (Fig. 3A-D), 5-HT- (Fig. 3E-H), or histamine-expressing cells (Fig. 3I-L) (Table. 2). Intriguingly, in contrast, the cell counting of our double-staining data showed that almost all the HAP1-ir cells co-expressed with gastrin-expressing cells (Fig. 4A-L; Table. 2). Cell counting revealed that the co-expression ratio of HAP1 in gastrin-ir cells was 98.6 ± 1.2% and that of gastrin in HAP1-ir cells was 93.2 ± 2.6% (Table 2). Our current results confirmed that HAP1 is expressed exclusively in the G cells but not in the D cells, EC cells or ECL cells in the pyloric part of gastric mucosa.

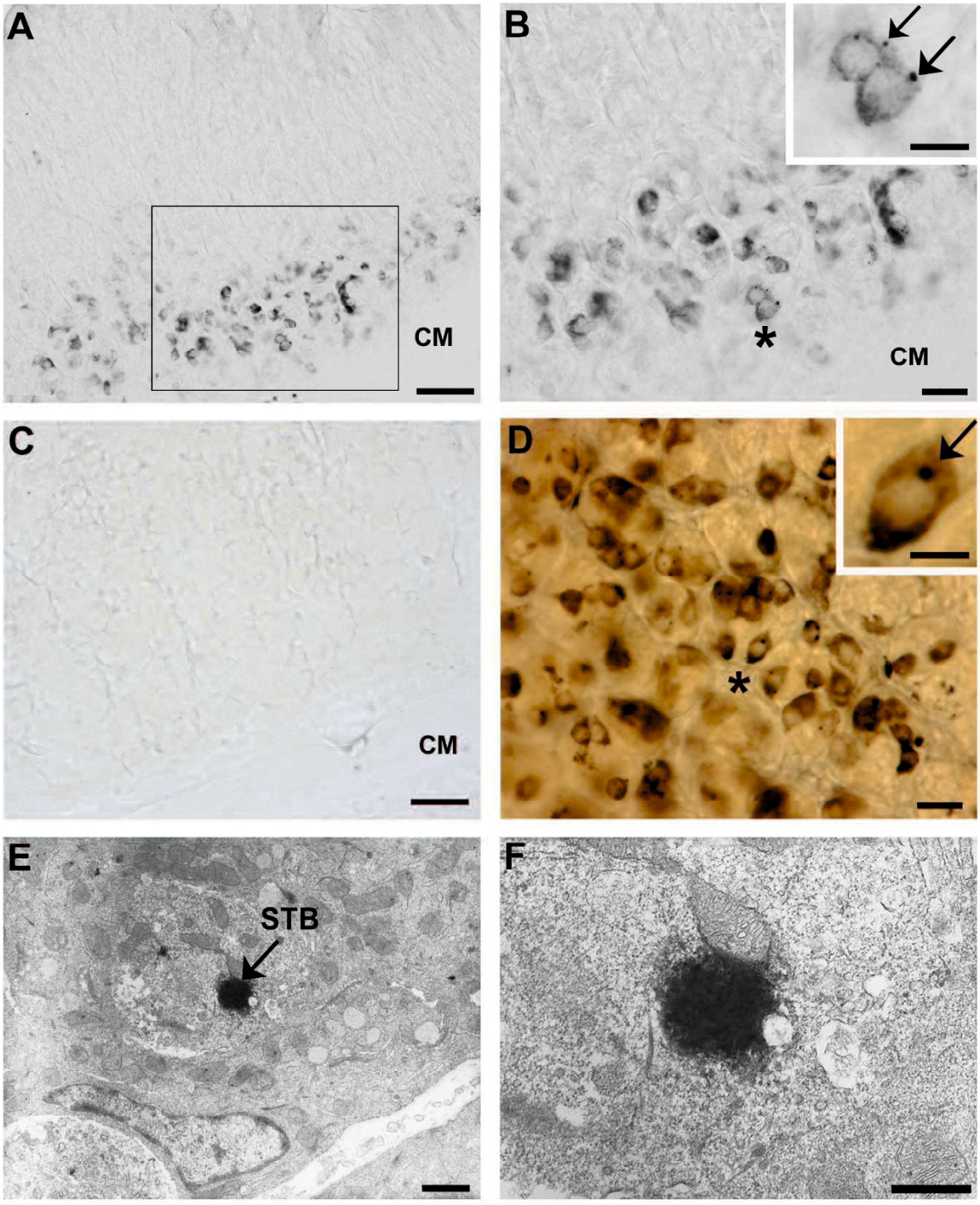

Fig. 1. Immunohistochemical distribution and immuno-electron microscopy of huntingtin-associated protein 1 (HAP1) in pylorus of the stomach.

(A) Immunohistochemistry showing the presence of HAP1-immunoreactive (ir) cells in the pylorus. (B) Enlargement of Box in A. (C) Using of pre-immune rabbit serum instead of rabbit polyclonal anti-HAP1 antibody eliminated the HAP1-immunoreactivity. (D) Flat-

embedded section in epoxy resin. (E-F) Electron microscopy of HAP1-ir cells in pyloric stomach. Insets are the enragement of * in B and D showing sub-cellular expression of HAP1. Arrows indicate stigmoid body (STB). CM, circular muscle. Scale bar = 50 μm in A, C; 20 μm in B, D; 10 μm in insets of B, D; 1μm in E, and 500 nm in F.

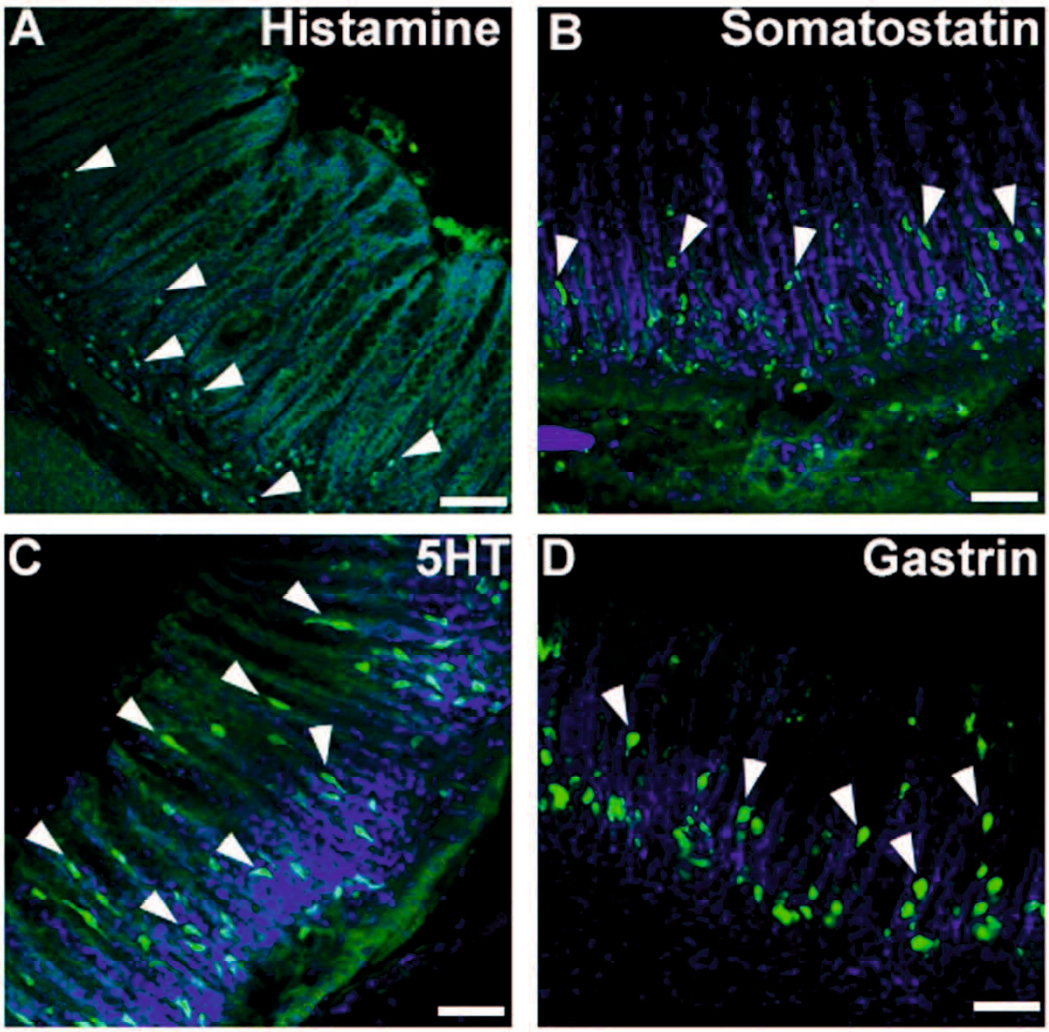

Fig. 2. Comparative distribution of the enteroendocrine cell markers in the pylorus of the stomach.

Arrowheads showing the immunoreactions for (A) histamine, (B) somatostatin, (C) 5 H T and (D) gastrin. Scale bar = 50 μm in A–D.

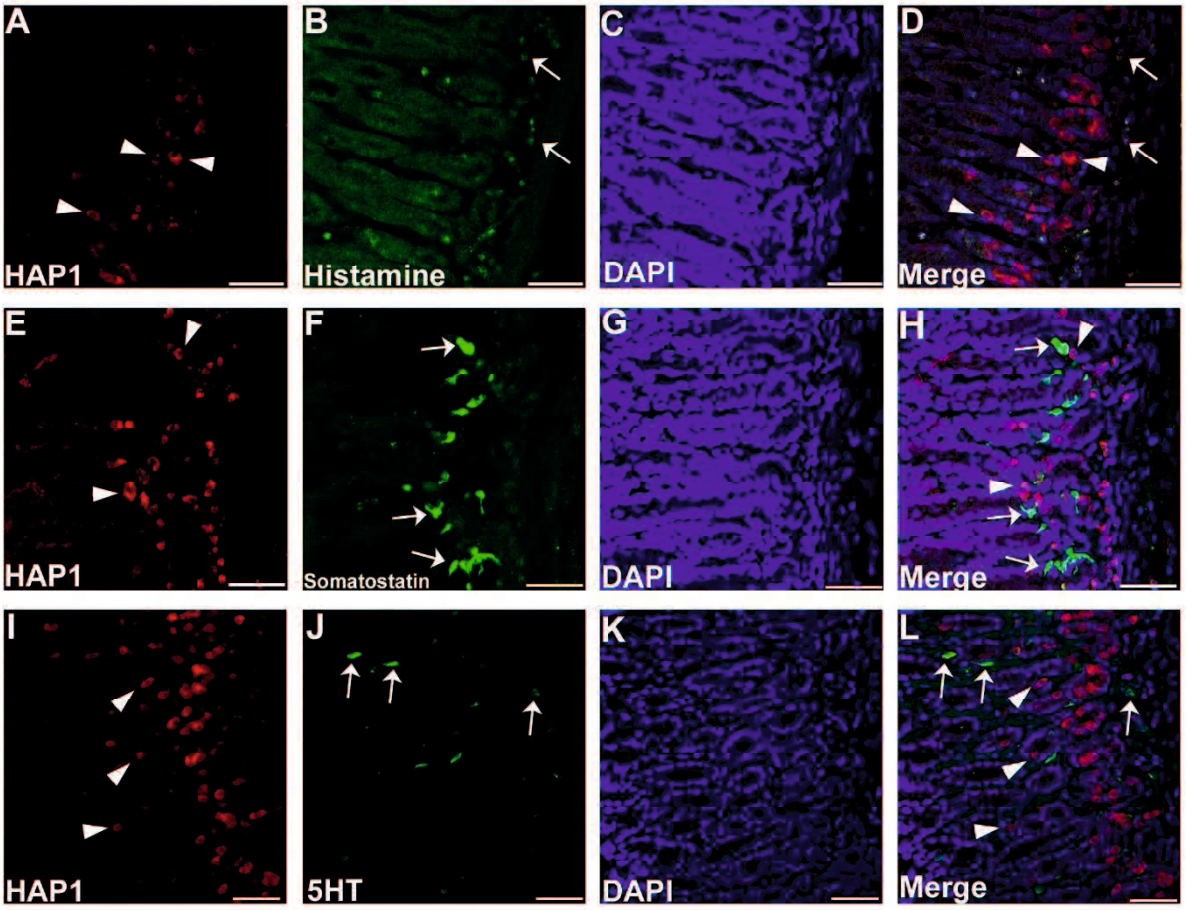

Fig. 3. Double-label immunofluorescence immunohistochemistry for HAP1 with Histamine, somatostatin and 5HT.

Photomicrograph showing double-label immunofluorescence staining of HAP1 and histamine (A–D), somatostatin (E–H) and 5HT (I–L) in the pylorus of the stomach. Arrowsheads indicate cells for single-positive for HAP1. Arrows indicate cells single-positive for histamine, somatostatin or 5HT. Scale bar = 50 μm in A–L.

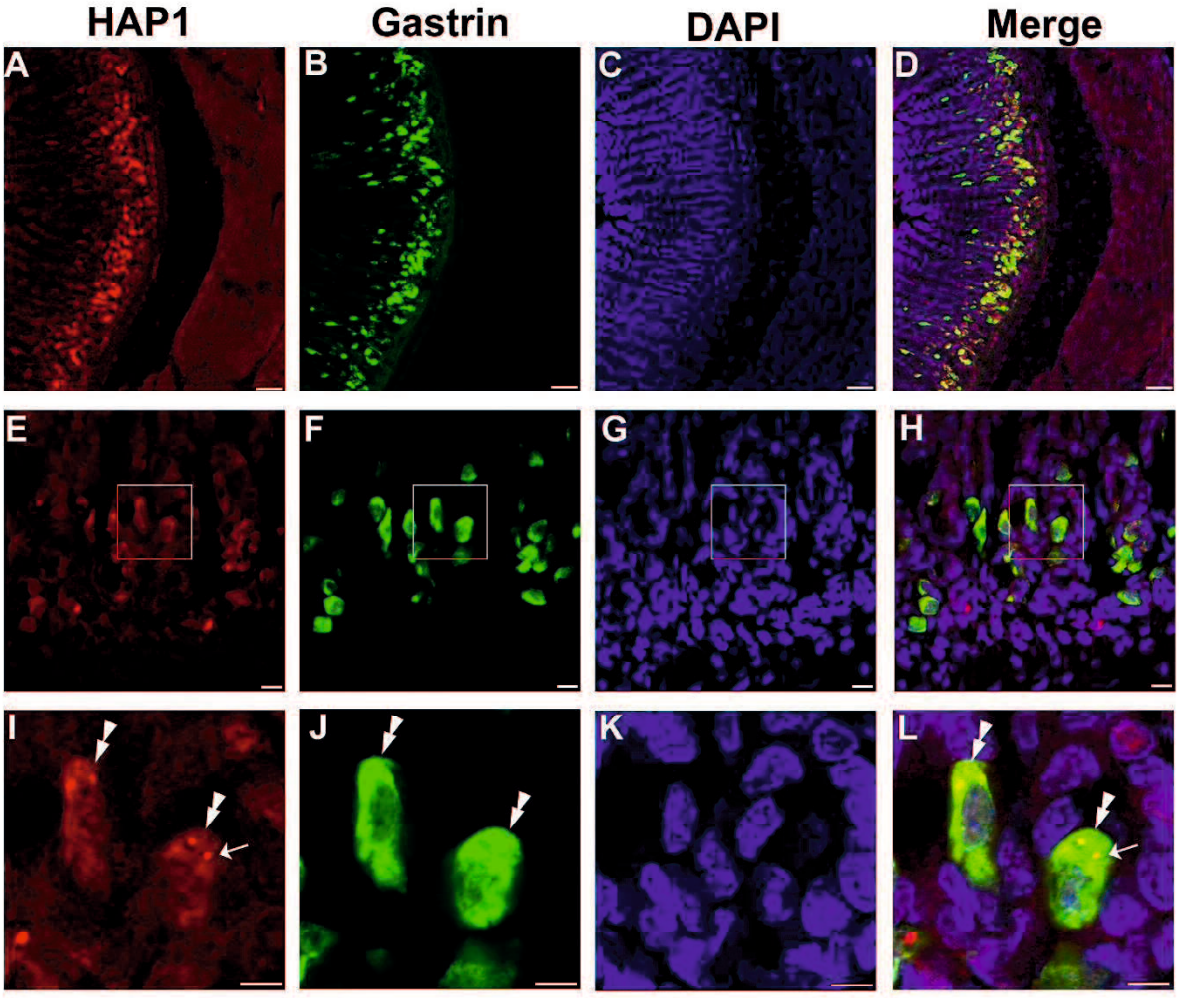

Fig. 4. Double-label immunofluorescence immunohistochemistry for HAP1 with gastrin. Photomicrograph showing double-label immunofluorescence staining of HAP1 and gastrin (low magnification A–D, high magnification E–H) in the pylorus of the stomach. I–L are the enlargements of boxes in E-H respectively. Double arrowheads indicate cells positive for both HAP1 and gastrin. Double arrowheads indicate cells positive both for HAP1 and gastrin. Arrows indicate STB. Scale bar = 50 μm in A–D, 10 μm in E–L.

Table 2. Co-expression ratios of HAP1/EEC markers and EEC markers/HAP1 in the pyloric part of gastric mucosa

Relationship of HAP1 with different EEC markers (ratio %)	Somatostatin	5HT	Histamine	Gastrin
HAP1/Marker	0	0	0	98.6 ± 1.2
Marker/HAP1	0	0	0	93.2 ± 2.6

Values represent the mean ± SEM (n = 4). From one rat, at least 300 positive cells were counted for every combination of HAP1 and an EEC marker. EEC, enteroendocrine cells; HAP1, Huntingtin-associated protein 1

6. Discussion

In the current study employing single- or double-label immunohistochemistry and electron microscopy, we determined the expression and subcellular distribution of HAP1 in the pyloric part of the rat stomach. The present study is the first to characterize HAP1-immunoreactivity in relation to the EEC subpopulations. Although the expression of HAP1 has been reported in the central nervous system previously in a number of studies (Li et al., 1996; Gutekunst et al., 1998; Dragatsis et al., 2004; Sheng et al., 2006, 2008; Lin et al., 2010; Niu et al., 2011; Fujinaga et al., 2004, 2007, 2009; Islam et al., 2012, 2017, 2020a; Wroblewski et al., 2018; Chen et al., 2020), its expression has been cursorily reported in the stomach (Liao et al., 2005; Li et al., 2019). Our current results clearly showed that in stomach most of the HAP1-ir cells had STBs in their cytoplasm (type A), while few other HAP1-ir cells had only diffuse staining in their cytoplasm with undetectable STBs (type B), as previously observed in the brain and spinal cord (Li et al., 1998a; Fujinaga et al., 2007; Islam et al., 2012, 2017). There are two HAP1 isoforms, HAP1A and HAP1B, which have different C-terminal amino acid sequences (Li et al., 1998a; Fujinaga et al., 2007). The emergence of STBs depends on the intracellular expression ratio of HAP1A and HAP1B. Cells with comparatively more HAP1A induce the STB in their cytoplasm while those with comparatively more HAP1B display diffuse HAP1-immunoreaction in their cytoplasm (Fujinaga et al., 2007; Islam et al., 2017, 2020a; Wroblewski et al., 2018). In the current study, it is thus somewhat difficult to discern the staining of HAP1A from that of HAP1B in immunohistochemistry.

In our present study, we observed that HAP1-ir cells were mainly expressed in the glandular mucosa of the rat pyloric stomach. Our current results are consistent with a previous study (Liao et al., 2005). Different types of EECs are present in the glandular mucosa of pyloric stomach. The major types of chemically defined distinct endocrine cells in

the gastric mucosa are G-cells, D-cells, EC-cells and ECL-cells (Solcia et al., 2000; Hunne et al., 2019). In the present study, we observed these EECs are located mainly at the base of the epithelial layer. These results are also consistent with a previous study (Hunne et al., 2019). To characterize the HAP1-ir cells, we performed double-label immunofluorescence staining for HAP1 with gastrin (marker of G-cells), somatostatin (marker of D-cells), 5-HT (marker of EC-cells) and histamine (marker of ECL-cells). The most striking finding in the current study, however, is that almost all of the G cells express HAP1-immunoreaction, while the D cells, EC cells or EEC cells are completely devoid of HAP1-immunoreaction. G-cells are neuroendocrine cells responsible for the synthesis and secretion of gastrin. G-cells are mainly expressed in the pyloric antrum but can also be found in the duodenum and the pancreas (Schubert, 2016; Xiaoli et al., 2017). Our current results indicate that HAP1 is selectively expressed in G-cells, which probably reflects HAP1's involvement in regulation of the secretion of gastrin. It has been suggested that HAP1 can participate in intracellular trafficking in cells, including transport of molecules, vesicles or organelles, and endocytosis of membrane receptors (Gauthier et al. 2004; Kittler et al. 2004; McGuire et al. 2006; Rong et al. 2007). The expression of HAP1 in G cells strongly suggests that HAP1 may play a vital role in the trafficking of membrane receptors, channel proteins or secretory granules in G-cells.

In the present study, another intriguing data is that the somatostatin D-cells, EC-cells and histamine ECL-cells are devoid of HAP1-immunoreaction. According to the STB/HAP1 protection hypothesis, lack of HAP1-immunoreaction in these cells might make them more vulnerable to certain stresses than HAP1-ir G-cells. It has been hypothesized that HAP1 expression can raise the threshold of vulnerability to degeneration and confer increased stability to cells expressing HAP1, protecting against apoptosis or cell death. Brain or spinal cord regions that are rich in HAP1, including limbic-hypothalamic regions of brain and the

dorsal horn of the spinal cord, are usually spared from neurodegeneration. On the other hand, regions with little or no STB/HAP1 expression such as the neocortex, striatum, thalamus, cerebellum, and spinal motoneurons are major targets in the aforementioned neurodegenerative diseases (Fujinaga et al., 2004; Takeshita et al., 2006, 2011; Islam et al., 2012, 2017). Moreover, it has recently been reported that overexpression of HAP1 reduced cell growth in breast cancer cell lines (Zhu et al., 2013). In addition, HAP1 is dramatically decreased in pancreatic cancer tissues (Li et al., 2019). Taken together, our current results may suggest that HAP1 plausibly protect G-cells from cancerous growth in certain stress conditions. Future studies should include detailed physiological and morphological experiments to elucidate the effects of HAP1 on the vulnerability of EECs.

7. Conclusion

In conclusion, the current study is the first to characterize the HAP1-ir cells in the pyloric stomach in adult rats. Our current results suggest that HAP1 is selectively expressed in G-cells, which probably reflects HAP1's involvement in regulation of the secretion of gastrin.

8. Acknowledgements

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Conflict of interest statement

The authors have no conflicts of interest to declare.

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