Kex2 family endoprotease furin is expressed specifically in pit-region parietal cells of the rat gastric mucosa

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Kamimura, Hitoshi, Yoshitaka Konda, Hiromi Yokota, Sei-ichi Takenoshita, Yukio Nagamachi, Hiroyuki Kuwano, and Toshiyuki Takeuchi. Kex2 family endoprotease furin is expressed specifically in pit-region parietal cells of the rat gastric mucosa. Am. J. Physiol. 277 (Gastrointest. Liver Physiol. 40): G183–G190, 1999.—The proprotein-processing endoprotease furin is localized in the gastric epithelial cells of the pit region in the rat gastric gland. The gastric pit is composed of several cell types, including gastric surface mucous (GSM) cells and parietal cells. Furin converts many growth- or differentiation-related proproteins to their active forms. We examined identification of furin-positive cells by immunostaining of rat gastric mucosa and regulators of the furin expression by measuring the furin promoter activity by luciferase assay. Furin-positive cells were stained for H +K + ATPase, indicating that they are parietal cells. Furin-positive parietal cells were not stained for transforming growth factor-α (TGF-α) but were surrounded by TGF-α-positive GSM cells. In contrast, parietal cells below the proliferative zone were positive for TGF-α but not for furin. Furin-positive parietal cells expressed a high level of epidermal growth factor receptor (EGFR). TGF-α stimulated the furin promoter activity highly in a mouse GSM cell line GSM06. Thus we suggest that the parietal cells of the pit region have furin-mediated functions that can be stimulated by EGFR signaling.

nonproliferative functions in the gastric gland.

Recently, we found that the proprotein-processing endoprotease furin is distributed around the upper one-fourth of the gastric gland of the adult rat (21). This furin-positive cell zone is a little higher than the proliferative zone. Furin cleaves the carboxy-terminal end of -Arg4-Lys/Arg2-Arg1, which is found in many growth-related precursor proteins (14, 28). These precursor proteins include adhesion molecules such as a procadherin family and integrin subunits α6 and αvβ6, matrix metalloproteinases (MMP) such as stromelysin-3 and membrane-type MMP, several growth factor precursors including platelet-derived growth factor, human HB-EGF (27), transforming growth factor-β, and parathyroid hormone-related protein, and, in some growth factor proreceptors such as insulin receptor, insulin-like growth factor-1 receptor and hepatocyte growth factor receptor (oncoprotein MET). Because many of these precursor proteins with furin-degradable sites are expressed in the gastric mucosa, furin may be involved in the growth and differentiation of mucosal cells.

We previously investigated the expression of furin and TGF-α using a GSM cell line, GSM06 (21). This cell line is derived from the GSM cells of a transgenic mouse that was transformed with the temperature-sensitive SV40 T antigen (35). At high temperature (39°C), at which the SV40 T antigen is inactivated, the GSM06 cells exhibit growth arrest and exhibit differentiated features, such as the production of periodic acid-Schiff-positive materials, secretory granules, and
expression of TGF-α. In contrast, when the cells are exposed to 33°C, they start growing by acquiring a characteristic of T antigen-transformed cells and express a high level of furin and a diminished low level of TGF-α.

In the rat gastric pit, furin-positive cells are localized a little higher than the proliferative zone (21). Because furin-positive GSM06 cells become TGF-α-positive when placed in a differentiation-inducing condition (39°C and/or confluent state), we presumed that furin-positive cells may become TGF-α-positive cells during the pit cell lineage differentiation in the normal gastric mucosa. In this paper, we investigated the topological relation of furin-positive and TGF-α-positive cells in the rat gastric glands, the cellular characteristics of the furin-positive cells, and the effect of TGF-α as well as other growth factors on the expression of furin.

MATERIALS AND METHODS

Morphological studies. Male Wistar rats (150–200 g) were purchased from Imai Experimental Animal Farm (Saitama, Japan). The rats were maintained under controlled light (7:00 AM to 7:00 PM) with food and water provided ad libitum. When their stomachs were used for morphological studies, they were fasted for 24 h and then killed. Stomachs were removed and subjected to the above-described saccharose replacement and were embedded in buffer, pH 7.4, at 4°C for 24 h. Small pieces of minced tissue were minced and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer to 7:00 AM and 7:00 PM) with food and water provided ad libitum.

Physiological saline (100 µl) was injected subcutaneously into rats. After 30 min, the stomachs were removed and subjected to the above-described fixation and immunostaining process.

Plasmid construction. A human furin 5’-upstream sequence was cloned from the human genomic library using oligonucleotide probes based on the previously reported sequence (2). The furin gene possesses the three alternative 5’-noncoding exons. Of the three, only exon 1 has a TATA box and is activated by transcription factor CAAT enhancer binding protein-β (C/EBP-β). The other two, exons 1A and 1B, have characteristics of promoters for housekeeping genes. Thus we used the TATA box containing furin promoter in this study. Four 5’-upstream DNA fragments, −363/−55 (Pst I-Pst I), −1092/−55 (Bgl II-Pst I), −612/−55 (Xba I-Pst I), and −56/−55 (BamH I-Pst I), were placed before a firefly luciferase gene supplied with the Dual-Luciferase reporter assay system (Promega, Madison, WI).

Cell culture and luciferase assay. As a culture model for the surface mucosal cells, we used a GSM06 cell line (35). The cells were cultured on a collagen type I-coated plastic plate (Iwaki, Tokyo, Japan) in DMEM-Ham’s F-12 medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (GIBCO, Grand Island, NY) and 1% ITS (100% ITS: 2 mg/l insulin, 2 mg/l transferrin, 0.122 mg/l ethanolamine, and 9.14 µg/l sodium selenite) in a humidified 5% CO2 atmosphere at 33°C unless otherwise indicated. Luciferase activity was assayed by transient gene expression in GSM06 cells. GSM06 cells were seeded initially at 5 × 103 cells/10-cm plate and cultured for 2 days. Transfection was carried out with each of the five luciferase gene constructs, including a promoterless gene, together with the sea pansy luciferase gene placed under several ubiquitous promoters as an internal standard, using a DOSPER liposomal transfection reagent (Boehringer Mannheim, Mannheim, Germany). Twenty-four hours after the transfection, the medium was changed to a new one and the culture was continued for another 6 h. The cells were then harvested to prepare cell lysates using the cell lysis buffer supplied with the Dual-Luciferase reporter assay system (Promega). Stimulants were obtained from the following suppliers: phorbol 12-myristate 13-acetate (PMA) from Sigma Chemical, TGF-α and EGF from Collaborative Biomedical Products (Bedford, MA), and platelet-derived growth factor-BB from Genzyme (Cambridge, MA). Mitogen-activated protein (MAP) kinase kinase (MEK) inhibitor PD-098059 was obtained from Research Biochemicals International (Natick, MA). Protein kinase C (PKC) inhibitor H-7 was obtained from Sekagaku (Tokyo, Japan). Each of these stimulants was added at the change of medium, 24 h after transfection; the cells were then incubated for another 6 h before harvest. The reaction was initiated by the addition of 100 µl of the luciferin solution to 50 µl of cell lysate (−100 µg protein), and light emission was measured for 10 s using a luminometer. Luciferase activity was measured as arbitrary light units per 10 s per 100 µg protein. Protein concentrations were determined by the Bradford method. Luciferase activity is expressed as multiplicity (-fold) against the value obtained by the promotorless luciferase gene or as a percentage against the control value obtained without stimulants.

RESULTS

Distribution of furin-positive cells in the rat gastric mucosa. We examined the distribution of PCNA-positive cells, furin-positive cells, and TGF-α-positive cells in the rat gastric mucosa (Fig. 1, A–F). Black staining of PCNA was localized to nuclei in the gastric mucosal cells, which have a relatively small cell size (Fig. 1, D and E). Most PCNA-positive cells were distributed around one-third down the gastric gland relative to the luminal surface, although there were several positive cells in the glandular region (Fig. 1, A and B). Overlapping with a higher level of the PCNA-positive cell layer, the furin-positive cells were distributed upward in a scatter pattern around one-fourth down the gastric gland relative to the luminal surface.
Furin-positive cells looked larger than PCNA-positive cells (Fig. 1, D and F). In Fig. 1E, large TGF-α-negative cells appeared to be furin-positive when compared with furin-positive cells in Fig. 1, D and F. The furin-positive cells decreased in number toward the luminal surface, but staining became heavier and occupied the entire cytoplasm (Fig. 1, D and F). The furin-positive cells were distributed further upward in the pit region, but the luminal top-pit region was negative for TGF-α (Fig. 1, B and C). TGF-α-staining, apparently in the parietal cells of the glandular region, was distinctly weaker than that of GSM cells in the pit (Fig. 1, B and C). Furin-positive but TGF-α-negative cells were surrounded by TGF-α-positive GSM cells (Fig. 1, E and F). Thus furin-positive cells were scattered between the PCNA-positive and TGF-α-positive cell layers in the pit region.

Immunohistochemical characterization of furin-positive cells. Because furin-positive cells looked larger in size and because parietal cells were distributed in the pit region as well as the glandular region, we suspected that the furin-positive cells in the gastric pit might be parietal cells. To examine this possibility, we performed double immunostaining of furin and H⁺-K⁺-ATPase because H⁺-K⁺-ATPase is specifically localized to the tubulovesicles of the resting parietal cells. The H⁺-K⁺-ATPase-positive parietal cells distributed from the pit region to the glandular region of the gastric gland (Fig. 2A). As expected, furin-positive cells were also positive for H⁺-K⁺-ATPase, although H⁺-K⁺-ATPase-positive cells were not always positive for furin in the pit region (Fig. 2). The population ratio of furin-positive parietal cells to the total parietal cells located up from the middle of the growth zone was 10.220.33.6 by 10.220.33.6 on June 24, 2017 http://ajpgi.physiology.org/ Downloaded from
The staining of H⁺-K⁺-ATPase in the pit-region parietal cells indicated a complex tubulovesicle-canaliculus system composed of many small curly formations (Fig. 2D). Furin staining distributed along with H⁺-K⁺-ATPase and furin were overlapped together and displayed a horseshoe-shape formation (Fig. 3). Thus, by light microscopic resolution, furin appears to be colocalized with H⁺-K⁺-ATPase in the pit-region parietal cells.

Because tubulovesicles fuse with intracellular canaliculi when the parietal cells have been stimulated with acid secretagogues, we stimulated acid secretion with histamine and carbachol. After stimulation, both H⁺-K⁺-ATPase and furin were overlapped together and displayed a horseshoe-shape formation (Fig. 3). Thus, by light microscopic resolution, furin appears to be colocalized with H⁺-K⁺-ATPase in the pit-region parietal cells.

Furin-positive parietal cells stain positively for H⁺-K⁺-ATPase in a curly formation. We compared the appearance of parietal cells over and below the proliferative zone using H⁺-K⁺-ATPase and phalloidin stainings because a tubulovesicle-canaliculus system is associated with actin filament (F-actin) cytoskeletons in parietal cells and FITC-phalloidin specifically stains actin filaments (19). Phalloidin staining as well as H⁺-K⁺-ATPase staining were spread to the entire cytoplasm in the pit-region parietal cells (Fig. 4, A and B). In contrast, in the parietal cells of the glandular region, H⁺-K⁺-ATPase distributed with many punctate stainings around the nucleus (Fig. 4E). Actin filament cytoskeletons surrounded the H⁺-K⁺-ATPase-positive circular formation with a partial overlap inside (Fig. 4F). Thus parietal cells can be classified into two subtypes: the furin-positive but TGF-α-negative cell population that is typically found in the pit region and the TGF-α-positive but furin-negative cell population in the glandular region, with the former looking larger than the latter.

EGF receptor localization in the pit region. Because furin-positive parietal cells were surrounded by TGF-α-positive GSM cells in the pit region (Fig. 1, C and F) and because TGF-α is situated extracellularly on its membrane-bound precursor (24), we examined the distribution of its receptor EGF in the gastric pit. EGF was stained over the whole gastric mucosa, especially in the top-pit and glandular regions (Fig. 5B), as reported previously (30, 34). In the pit, parietal cells were stained strongly for EGF (Fig. 5, D and E). The H⁺-K⁺-ATPase-containing tubulovesicle-canaliculus system looked surrounded by EGF-positive stainings (Fig. 5F). GSM cells were apparently stained for EGF in a punctate manner but much weaker than the staining of parietal cells (Fig. 5, D and E). In contrast, the parietal cells of the glandular region were stained for EGF less strongly than the pit-region parietal cells (Fig. 5, G and H). Instead, we found strong EGF-positive cells distributed next to parietal cells (Fig. 5C and arrows in Fig. 5, G–I). By their distribution pattern, these EGF-positive cells appeared to be chief cells. In general, EGF-positive parietal cells are surrounded by TGF-α-positive GSM cells in the gastric pit, whereas TGF-α-positive parietal cells were neighbored by strongly EGF-positive cells in the glandular region.

Effect of TGF-α and other stimulants on the expression of furin. GSM cells turn over with a lifetime of ~3 days, whereas parietal cells possess a lifetime of more than 50 days (17, 19). Thus, during the movement of TGF-α-positive GSM cells upward among EGF-positive and furin-positive parietal cells in the gastric pit, the EGF-positive parietal cells may receive juxta-crine signals from TGF-α-positive GSM cells by cell-to-cell interaction. To examine this possibility, we measured furin expression by TGF-α and other stimulants with a furin promoter plus luciferase reporter gene. Although the parietal cell is the best choice as a target to investigate furin promoter activity, it is hard to isolate and introduce DNA into parietal cells. To circumvent this difficulty, we used a GSM06 cell line that expresses EGF (data not shown).

Initially, we characterized a promoter activity of furin gene by using five lengths of 5′-upstream sequence and found that the luciferase activity was highest for the −612/+55-length promoter (Fig. 6A). The fact that the two longer furin promoters presented lower luciferase activity suggests the presence of inhibitory elements between −1092 and −612. Furthermore, the rapid decrease of luciferase activity by the −56/−55 promoter may suggest the presence of enhancing elements between −612 and −55. We used the −612/+55-length promoter for the following study. Among five stimulants, including TGF-α, EGF, insulin-like growth factor I, hepatocyte growth factor, and PMA, TGF-α and PMA were highest and EGF was significantly higher in inducing luciferase activity (Fig. 6B). Further-
more, luciferase activity was increased dose dependently by both TGF-α and PMA up to 10 nM, then plateaued to a 100 nM point (Fig. 6C). It is suggested that furin promoter activity is regulated by the EGFR-mediated signaling and PKC-mediated signaling, probably through a Ras/MAP kinase signaling pathway (9, 23). We examined the signaling through the Ras/MAP kinase pathway by using the MEK inhibitor PD-098059 (8) and confirmed that the increase of furin promoter activity by TGF-α was suppressed by this inhibitor in a dose-dependent manner (Fig. 6D). The furin promoter activity without TGF-α also decreased gradually, suggesting that the Ras/MAP kinase pathway is activated to some extent in GSM06 cells, possibly by endogenously produced TGF-α (21). We further examined the functional role of PKC on furin expression by a PKC inhibitor H-7 (11) and PKC desensitization by PMA. As shown in Fig. 6E, in the presence of H-7, luciferase activity decreased 25.0% even in nonstimulated GSM06 cells, suggesting the production of endogenous stimulants including TGF-α from GSM06 cells. The activity by TGF-α and PMA stimulation decreased 48.3% and 68.4%, respectively, in the presence of H-7 (Fig. 6E). After pretreatment of the cells with 1 µM PMA for 24 h, TGF-α increased the luciferase activity only 12.6% relative to the value without PMA (data not shown). Thus TGF-α signal appears to be mediated partly by PKC. Recently, Polk (32) showed that PMA pretreatment of mouse small intestine epithelium-derived culture cells blocked EGF-induced cell migration and demonstrated that phospholipase C is involved in EGF signaling by producing diacylglycerol for PKC activation in a Ca2+ dependent manner.

DISCUSSION

The present study demonstrates that furin-positive cells localized in the pit region were identified as parietal cells by the presence of H+-K+-ATPase. The furin-positive parietal cells were not stained for TGF-α, in contrast to the parietal cells in the glandular region. Instead, the furin-positive parietal cells were surrounded by TGF-α-positive GSM cells, suggesting signaling from TGF-α-positive GSM cells to EGFR-positive parietal cells in the pit region. We further demonstrated that furin expression was upregulated by TGF-α using a GSM cell-derived cell line, GSM06.

Most parietal cells are derived from the granule-free precursor cells localized in the isthmus, from where GSM cells are also derived (17). By morphological
appearance, parietal cells are apparently similar in any region, except those in the lower base. Karam et al. (20) reported functional heterogeneity of parietal cells along the pit-gland axis by showing very little morphological change in response to acid secretagogues, in contrast to the typical morphological transformation of parietal cells in other regions. In this study, we demonstrate that parietal cells displayed different features over and

Fig. 6. Furin promoter activity assay by TGF-α and other stimulants. A: activity of furin promoter; 5'-flanking region of the human furin gene was deleted by restriction endonucleases and fused to the firefly luciferase cDNA. DNA constructs were transfected to the gastric surface mucosal cell line, GSM06, and furin promoter activity was measured 12 h later. Data are presented as multiplicity (fold) of increase against the promoterless luciferase DNA construct. Values represent means ± SD. B: furin promoter activity by TGF-α and other stimulants; 5'-flanking region of the human furin promoter (−612/+55) was utilized for this experiment. Stimulants used were TGF-α, EGF, insulin-like growth factor I (IGF-I), hepatocyte growth factor (HGF), and phorbol 12-myristate 13-acetate (PMA) at concentrations of 10 nM each. Data are presented as fold against the promoter activity without stimulants. Values represent means ± SD. *P < 0.05, **P < 0.01 against the control value. C: dose-response curves of luciferase activity by TGF-α and PMA. Luciferase activity without stimulation was taken as 100. Values represent means ± SD. **P < 0.01 against the control value. D: suppression of luciferase activity by mitogen-activated protein kinase kinase inhibitor PD-098059. Luciferase activity was assayed in the presence or absence of 10 nM TGF-α together with an increasing dose of PD-098059 (0, 10, 30, and 100 µM). Luciferase activity without PD-098059 in the absence of TGF-α was taken as 100. *P < 0.05, **P < 0.01 against the control value. E: suppression of luciferase activity by protein kinase C inhibitor H-7. Luciferase activity was assayed in the presence or absence of 10 µM H-7. Luciferase activity without H-7 in the absence of stimulants was taken as 100. Stimulants used were TGF-α (10 ng/ml) and PMA (100 nM). Open bars, in the absence of H-7. Solid bars, in the presence of H-7. Values represent means ± SD.
below the proliferative zone in the isthmus. The distinct features of parietal cells in the pit region were their larger size, the complex form of the tubulovesicle-canaliculus system, expression of furin, and absence of TGF-α, compared with those in the neck to base regions.

Parietal cells that possess EGFR stay in the pit for 50–60 days, whereas TGF-α-positive GSM cells migrate upward from the proliferative zone to the top-pit region by 3 days (17, 19). Thus, when GSM cells pass by parietal cells, both cell types may exchange important signals for cell differentiation by cell-to-cell interaction. Using canine oxyntic mucosal cell culture, Chen et al. (4) demonstrated that GSM cells that are in contact with parietal cells display DNA synthesis and suggested that TGF-α from parietal cells stimulates the growth of GSM cells in vivo. However, in the rat gastric pit, parietal cells do not express TGF-α but express EGFR extensively, whereas GSM cells express TGF-α strongly and EGFR weakly. Thus we think that the TGF-α signal moves from GSM cells to parietal cells in the pit. In the glandular region, parietal cells express TGF-α to a moderate extent, so TGF-α-positive parietal cells can stimulate EGFR-positive GSM cells in the mixed culture of gastric mucosal cells (4).

The absence of PCNA-positive staining suggests that parietal cells and GSM cells were not proliferating and thus that TGF-α-to-EGFR signaling in the pit may not be growth promoting but rather cell differentiating. Recently, Miyoshi et al. (25) demonstrated that the membrane-bound form of HB-EGF suppresses cell growth and promotes survival of rat hepatoma-derived AH66tc cells; in contrast, a soluble form of HB-EGF cleaved from the membrane-bound form increases cell growth of AH66tc cells. Takemura et al. (36) also showed a distinct feature of soluble and membrane-bound forms of HB-EGF using a renal epithelial cell line, NRK-52E. HB-EGF as well as TGF-α precursors are thought to be processed to their active forms by MMP-like endoproteases (12). Thus the effect of membrane-bound TGF-α on GSM cell membranes may maintain the survival of EGFR-expressing parietal cells, resulting in a long lifespan for parietal cells.

TGF-α is known to suppress acid secretion acutely from parietal cells (33, 38), but its chronic effect increases acid secretion in isolated parietal cells (6). We suspected that parietal cells in the pit display similar acid secretion function to those in the glandular region. The parietal cells displayed horseshoe-shaped morphological transformation when stimulated with acid secretagogues. Thus we think that acid secretion is possible in the pit-region parietal cells under strong TGF-α signaling. Interestingly, Kaise et al. (16) demonstrated that EGF increases the promoter activity of H+K+-ATPase α-subunit gene expression in isolated canine parietal cells (16). We also measured a furin promoter activity using luciferase as a reporter gene and found that TGF-α and the phorbol ester PMA stimulate furin gene expression in GSM06 cells. Although caution is required when extrapolating this result to in vivo gastric mucosa, we presume that TGF-α signaling from GSM cell membranes stimulates furin gene expression as well as H+K+-ATPase α-subunit gene expression in parietal cells (16).

TGF-α is released from its membrane-bound precursor by metalloproteinases (1), which are stimulated by PMA through a PKC-mediated pathway (10, 31). It is important to identify the natural stimulants leading to metalloproteinase activation through this PKC-mediated pathway in the gastric mucosa. Although furin does not activate TGF-α processing and also TGF-α-activating metalloproteinase processing, furin expressed by EGFR signaling may activate other growth-related proteins to their active forms that facilitate cell growth or differentiation in an autocrine and/or paracrine fashion. Several candidate proproteins can be nominated, including procadherin, integrin subunits α3 and α6, membrane-type MMP MT1-MMP, and parathyroid hormone-related protein, as described in the introduction (14, 28). The pit-region parietal cells may produce furin-activated proteins and/or secrete a truncated form of furin itself that activates proproteins in extracellular space (37). After activation by furin, these proteins may exert their effect on GSM cells. At present, these substrate proteins remain to be identified.

Parietal cells play a pivotal role in maintaining gastric mucosal cell structure because parietal cell-deleted mouse mucosa exhibit atrophic gastritis-like appearance (3, 22). In this study, we found that parietal cells are distinct in the expression of furin and TGF-α over and below the proliferative zone. We suggest that pit-region parietal cells play an essential role in gastric pit structure formation by producing furin-mediated growth and differentiation factors in contact with TGF-α-positive GSM cells.

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REFERENCES


