Reduced Pepsin A Processing of Sonic Hedgehog in Parietal Cells Precedes Gastric Atrophy and Transformation*

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Sonic hedgehog (Shh) is not only essential to the development of the gastrointestinal tract, but is also necessary to maintain the characteristic acid-secreting phenotype of the adult stomach. Gastrin is the only hormone capable of stimulating gastric acid and is thus required to maintain functional parietal cells. We have shown previously that gastrin-null mice display gastric atrophy and metaplasia prior to progression to distal, intestinal-type gastric cancer. Because reduced levels of Shh peptide correlate with gastric atrophy, we examined whether gastrin regulates Shh expression in parietal cells. We show here that gastrin stimulates Shh gene expression and acid-dependent processing of the 45-kDa Shh precursor to the 19-kDa secreted peptide in primary parietal cell cultures. This cleavage was blocked by the proton pump inhibitor omeprazole and mediated by the acid-activated protease pepsin A. Pepsin A was also the protease responsible for processing Shh in tissue extracts from human stomach. By contrast, extracts prepared from neoplastic gastric mucosa had reduced levels of pepsin A and did not process Shh. Therefore processing of Shh in the normal stomach is hormonally regulated, acid-dependent, and mediated by theaspatic protease pepsin A. Moreover parietal cell atrophy, a known pre-neoplastic change in the stomach, follows loss of Shh processing.

Intestinal type gastric cancer develops in a chronically inflamed stomach (1). Prior to the development of dysplasia, the gastric mucosa is typically atrophic in which the acid-producing parietal cells are drastically reduced or absent (2). In human subjects, the presence of an atrophic stomach can be confirmed by documenting a loss of gastric acid or serologically by measuring the pepsinogen (Pg) A to C ratio (3–6). It has been suggested that the presence of gastric atrophy is a reliable indicator of pre-neoplastic changes in the stomach (2). Loss of mature parietal cells from the body of the stomach (atrophy) by either genetic or pharmacologic mechanisms results in severe abnormalities in the differentiation and development of gastric cell lineages characterized by the appearance of intestinal or gastric metaplastic cells (7–10). Thus, understanding the mucosal signals that promote loss of parietal cells will reveal important targets for therapeutic intervention.

Gastrin is the only hormone capable of regulating acid secretion by increasing parietal cell mass and stimulating expression of the acid pump, H⁺,K⁺-ATPase (11). Although parietal cells express H⁺,K⁺-ATPase protein, gastrin-deficient mice have severely impaired acid secretion, demonstrating a requirement for gastrin hormone in the functional maturation and maintenance of the parietal cell (12, 13). Thus, gastrin is required to sustain the differentiated, acid-producing phenotype of the stomach. Recently, we reported that these gastrin-null mice develop bacterial overgrowth by 4 months of age (14) and intestinal-type gastric cancer by 12 months (15). In addition, gastric atrophy characterized by the drastic reduction in the number of parietal cells preceded the development of the tumors (15).

Like gastrin, the morphogen sonic hedgehog (Shh) also regulates epithelial cell differentiation in the adult stomach (16, 17). Normally, Shh is expressed in the mature acid-secreting glands of the mammalian stomach, primarily within parietal cells (16–20). In addition, we have shown that Shh stimulates H⁺,K⁺-ATPase gene expression (18). Interestingly, the gastric mucosa of newborn Shh-deficient mice also exhibit abnormalities in glandular differentiation (21, 22), raising the possibility that Shh levels contribute to the dysfunction or loss of the parietal cell. Thus to determine if Shh levels are modulated by gastrin and contribute to parietal cell function, we examined the expression of Shh in primary cultures of parietal cells.

We show here that gastrin stimulates the processing of Shh peptide by increasing parietal cell acid production. The increase in acid facilitates the intramolecular conversion of PgA to the active protease pepsin A. Pepsin A subsequently cleaves Shh to the active 19-kDa form (ShhN). Thus, Shh processing in the parietal cell depends upon processing by pepsin A. In addition, we show that the processing of Shh by pepsin A also occurs in the normal human stomach and is severely restricted in the atrophic neoplastic stomach. Therefore, a consequence of hypochlorhydria is the absence of activated pepsin available to process Shh to its biologically active form in the parietal cell.

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§ The abbreviations used are: Shh, sonic hedgehog; WT, wild type mice; G⁻/⁻, gastrin-deficient mice; G⁻/⁻ GAST, gastrin-infused gastrin-deficient mice; G cells, gastrin-expressing cells; Ptc1, patched receptor; PgA, pepsinogen A; HA, hemagglutinin; FITC, fluorescein isothiocyanate; OM, omeprazole; WCE, whole cell extract; ANOVA, analysis of variance; ELISA, enzyme-linked immunosorbent assay.
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EXPERIMENTAL PROCEDURES

Animals—Gastrin-deficient (G−/−) and strain-matched (C57BL/6×129/Sv) wild-type control mice (WT) were bred by homozygous mating. WT (n = 8) and G−/− (n = 8) mice were maintained in individual, sterile microisolator cages in non-barrier mouse rooms (conventional housing) for 4 months. All mice were fasted overnight with access to water ad libitum before analysis. The study was performed with the approval of the University of Michigan Animal Care and Use Committee that maintains an American Association for the Accreditation of Laboratory Animal Care (AAALAC) facility.

Gastrin Infusions—Both amidated (G-17) and non-amidated (G-17gly) forms of gastrin are secretagogues of acid secretion (23, 24) and prevent parietal cell atrophy in the gastrin-deficient mice (25). Thus, the infusion of G-17 and G-17gly (GAST infusion) into 8-week-old G−/− mice was performed using a microosmotic pump (Alzet, model 1002) inserted into the peritoneum, delivering 5 μg/kg/h of a mixture of rat gastrin (G-17) (Bachem) plus gastrin-17gly (G-17gly) (from Dr. Chris Dickinson, University of Michigan). Mice were continuously infused for 14 days prior to sacrifice and collection of plasma for radioimmunoassay. Protein was extracted from gastric mucosal scrapings by homogenization of the tissue in lysis buffer (300 mmol/liter NaCl, 30 mmol/liter Tris, 2 mmol/liter MgCl2, 2 mmol/liter CaCl2, 1% Triton X-100, pH 7.4, supplemented with protease inhibitor tablets (without pepstatin, Roche Applied Science)) then analyzed for Shh and Ptch expression by immunoblot. Total RNA was isolated from gastric mucosal scrapings using TRIzol Reagent according to the manufacturer’s protocol (Invitrogen) and analyzed for Shh and Ptch mRNA abundance by RT-PCR.

Gastrin Radioimmunoassay—After euthanization, ~1 ml of blood was collected by cardiac puncture, aliquoted into lithium-heparin tubes (Sarstedt, Germany) and centrifuged at 15,000 rpm for 15 min at 4 °C. The plasma was collected immediately and stored at −20 °C until assayed for gastrin by radioimmunoassay as previously described (14). Rabbit antisera number 1296 was from the Center for Ulcer Research and Education (CURE) at the University of California, Los Angeles.

Gastric Acidity—After an overnight fast, the stomach was opened along the greater curvature and washed with 2 ml of normal saline (pH 7.0). Gastric debris was removed by centrifugation at 3,000 rpm for 5 min, and the supernatant collected. The acid concentration of the supernatant was determined by titration using 0.005 N NaOH. The gastric acidity was expressed as μEq (14).

Quantitative RT-PCR—Total RNA was isolated from gastric mucosal scrapings using TRIzol Reagent according to the manufacturer’s protocol (Invitrogen). Using the iScript cDNA synthesis kit (BioRad), cDNA was synthesized from 1 μg of total RNA. RT-PCR was then performed based on previously published primer sequences and conditions (26).

Primary Parietal Cell Preparation and Culture—Primary cells were isolated from the gastric mucosa of four wild type C57BL/6 and gastrin-deficient (G−/−) mouse stomachs according to a previously modified method using dispase (14). Gastric cells (2 × 10^6 cells/well) were cultured in RPMI medium containing 10% fetal calf serum and 1% penicillin-streptomycin on 35-mm round culture dishes coated with 150 μl of growth factor reduced Matrigel (BD Biosciences) in a total media volume of 5 ml. The cells were treated with either phosphate-buffered saline or G-17 and G-17gly (GAST, 10 nM) for 6 h. The media was then collected to size fractionate the secreted forms of Shh by gel filtration chromatography. Whole cell extracts were prepared from the entire 5-ml plate of cells using M-Per extraction lysis buffer (Pierce) then analyzed by immunoblot.

Canine parietal cells were isolated based on a modified elution method by Soll et al. (27). The isolated parietal cells (2 × 10^6 cells/well) were cultured in Ham’s F-12/Dulbecco’s modified Eagle’s medium (1:1) containing 0.1 mg/ml gentamicin, 50 units/ml penicillin G, 0.01 mg/ml ciprofloxacin, and 2% Me2SO (Sigma) on 35-mm round culture dishes coated with 150 μl of growth factor reduced Matrigel in 5 ml of media (18). After an overnight culture and removal of non-adherent cells, the remaining adherent cells were 95 to 98% homogenous for parietal cells (28). Omeprazole was dissolved in Me2SO/PEG (4.5/0.5 v/v). Parietal cells were treated with either vehicle (Veh, Me2SO/PEG), the G-17 and G-17gly mix (GAST, 10 nM), omeprazole alone (OM, 100 nM), histamine alone (HIST, 10−4 M), isobutylmethylxanthine (IBMX) (10−5 M), HIST plus IBMX, HIST plus OM, HIST plus IBMX. The medium was collected to size fractionate secreted Shh by gel filtration chromatography as described below, and the corresponding adherent cells were lysed in radiolabel precipitation assay buffer (Sigma) to analyze Shh in the whole cell extracts by immunoblot.

Gel Filtration Chromatography—Shh in the culture media of primary cells was detected using previously published methods (18, 29). Briefly, the entire 5 ml of media from each plate was concentrated to 0.5 ml using a Centricron YM-10 spin column (Millipore, Bedford, MA) then loaded onto a Superose 12 gel filtration column (Amersham Biosciences) that was equilibrated with 0.01% Nonidet P-40 in phosphate-buffered saline. The molecular mass standards used to calibrate the column were albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa). Twenty 0.5-ml fractions were collected after the void volume. The three 0.5-ml fractions corresponding to the 19-kDa molecular weight marker were combined, concentrated to 50 μl using the Centricron YM-10 columns, then 20 μl was mixed with sample buffer and resolved by SDS-PAGE for immunoblot analysis.

Shh Expression Vectors—Wild-type mouse Shh expressed in pBlueScript II SK− (a gift from Dr. Philip Beachy, Johns Hopkins University) was subcloned into pcDNA (Invitrogen) for transfection into AGS cells, a human gastric adenocarcinoma cell line. After the FuGENE transfection, cells were allowed to settle for 16 h. Subsequent to replenishing the media, the cells were allowed to incubate for 48 h prior to collect the media for analysis. Two copies of the hemagglutinin (HA) epitope (TAC-CTTACGACGTTCCTGATTAGCT TACCTTAACACGT- GTTCCTGATTAGCT) were inserted after residue 32 by PCR. Mutation of residue 200 from F to H and residue 271 from H to A was performed using the QuikChange kit (Invitrogen).

Primary parietal cell preparation and culture—Primary cells were isolated from the gastric mucosa of four wild type C57BL/6 and gastrin-deficient (G−/−) mouse stomachs according to a previously modified method using dispase (14).
and the respective primers, respectively: GCCAAATCCGGCG-GCTGTTCACCAGCCACCCGTG, WT; GCCAAAT-CCGGCGGCTGTACCCGGATCCGCCACCCGTG, F200H Sense; TGCTACCCGGCGGCACTTCCTTCTGCGGC, WT; TGCTACCCGGCGGCCTTCTTCTTGC, H271A Sense. All mutations and modifications of the Shh expression vector were confirmed by sequencing. HA-tagged ShhN was detected on immunoblots using anti-HA-conjugated horseradish peroxidase (HA-Probe (Y-11) horseradish peroxidase cath). SC-805HRP, Santa Cruz Biotechnology).

Aminopyrine Uptake—Aminopyrine (Amersham) was used to detect acid production by the canine parietal cells. Cultured parietal cells were washed once with Earle’s balanced salt solution and incubated with 0.1 μCi of [14C]aminopyrine. The cells were then treated with either (Veh, MeSO4/PEG), G-17 and G-17gly (GAST, 10 nM), omeprazole alone (OM, 100 nM) or OM plus GAST for 30 min. Parietal cells were then lysed with 500 μl of 1% Triton X-100, and the radioactivity in the lysate was quantified in a liquid scintillation counter.

Mouse Shh ELISA—Shh was measured using 100 μl of cell culture media collected from WT or G-/- mouse cell cultures treated with either vehicle (Veh) or gastrin (GAST). A mouse-specific Shh ELISA kit (R&D Systems) was used according to the manufacturer’s protocol.

Luciferase Assay—AGS cells were transfected with a hedgehog responsive reporter construct in which 8 Gli binding sites were subcloned upstream of a luciferase reporter construct in a reticulocyte lysate system (Promega). A mutation was introduced into the mouse Shh cDNA using the QuikChange kit at residue 200 (F200H) as described above. In vitro translated protein was then incubated with whole cell extract (10 μg) collected from canine parietal cells treated with vehicle, gastrin, omeprazole, or omeprazole plus gastrin for 4 h at pH 2–3 at 37 °C. Whole cell extracts were immunodepleted of Shh, PgA, or PgC by incubating 50 μg of protein with a 1:200 dilution of the goat polyclonal anti-Shh (Santa Cruz Biotechnology, sc-1194), 1:200 rabbit anti-PgA or PgC (Acris, Germany), 1:100 anti-HA-conjugated horseradish peroxidase (Santa Cruz Biotechnology) or 1:500 GAPDH (Molecular Probes) antibody. The membranes were washed three times for 5 min and incubated for an additional 1 h with a 1:2000 dilution of the horse-radish peroxidase-conjugated secondary anti-goat, rabbit, or mouse antibodies. Proteins were visualized using enhanced chemiluminescence (Lumilight substrate, Roche Applied Science, Mannheim, Germany).

Protease Activity—The protease fluorescent detection kit (Sigma, PF0100) was used to measure the pepsin A concentration in extracts collected from canine parietal cells treated with vehicle, gastrin, omeprazole, or omeprazole plus gastrin according to the manufacturer’s protocol. Briefly, the pepsin A standard curve was generated using known concentrations of the protease between 0.5 and 20 ng and FITC-tagged casein as the substrate. The pepsin A concentration was then measured in 10 μl of parietal cells extracts. The fluorescence intensity was detected at an excitation wavelength of 485 nm and an emission wavelength of 525 nm using a Wallac, Victor3 1420 Multilabel Counter (PerkinElmer Life Sciences).

Statistical Analysis—The significance of the results was tested using the unpaired t test for the in vivo mouse studies or one-way ANOVA for cell culture experiments using a commercially available software (GraphPad Prism, GraphPad Software, San Diego, CA). A p value <0.05 was considered significant.

RESULTS

Gastrin Regulates Shh Expression in Vivo—Gastric atrophy is a pre-neoplastic lesion that is intimately linked to the presence of the parietal cell and its ability to produce acid. We have shown previously that Shh stimulates H⁺,K⁺-ATPase gene expression and in this way is able to regulate the content of this parietal cell-specific enzyme (18). In addition, Shh transcrip-
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**TABLE 1**

| Plasma gastrin and gastric acid levels in WT, G^-/-, and G^-/-Gast mice |
|--------------------------|-------------------|
| **Group**    | Plasma gastrin | Gastric acidity |
|              | pmol/liter     | μEq            |
| WT           | 31 ± 6         | 6 ± 1          |
| G^-/-        | ND^a           | 1 ± 0.5^b      |
| G^-/-Gast    | 562 ± 57^b     | 8 ± 0.6^c      |

^a ND, not detected.
^b p < 0.05 versus WT.
^c p < 0.05 versus G^-/-.

When the mice are hypochlorhydric, but have not yet developed parietal cell atrophy, both the 45-kDa nascent Shh protein and the 19-kDa processed form were detected in tissue homogenates from WT mice (Fig. 1, A and B). However, there was a significant decrease in the level of Shh peptides in the (G^-/-) mice suggesting that gastrin-regulated Shh expression. To test this hypothesis, we infused gastrin into G^-/- mice for 2 weeks (G^-/-Gast) and restored gastric acid secretion (Table 1). Gastrin infusion also induced re-expression of Shh (Fig. 1, A and B) that was accompanied by parallel changes in the Shh receptor Patched (Ptc). A known transcriptional target of hedgehog signaling (Fig. 1, A and C). The dramatic loss of Shh peptide suggested that there might be a decrease in Shh mRNA in the gastrin-deficient mice. Indeed, qRT-PCR analysis of RNA extracted from the WT, G^-/-, and G^-/-Gast demonstrated essentially the same changes observed at the protein level (Fig. 1, D and E). These results showed that gastrin induces Shh expression and correlates with restoration of acid secretion in vivo.

To study the mechanism by which Shh levels are regulated, we developed a primary gastric culture system and determined whether the induction of Shh by gastrin observed in vivo could be recapitulated in the cultures. Primary gastric cells dissociated from the stomachs of WT or G^-/- mice, were treated with gastrin for 6 h prior to generating WCE for immunoblot analysis. We found that the 45-kDa peptide was induced in both the WT and G^-/- mouse primary cultures. However, the 19-kDa processed form of Shh (ShhN) was not readily detected in the extracts despite induction of the Ptc protein, the transcriptional target and receptor for Shh (Fig. 2A). Because prior studies have indicated that Shh is autocatalytically processed and the biologically active form secreted from the cell, we collected the culture media and performed an ELISA, which detected Shh reactive material (Fig. 2B). To confirm that the immunoreactive material detected by ELISA was indeed the processed form of Shh, we concentrated the entire 5 ml of media from each plate of cells and resolved the protein on a 4–20% SDS gradient gel. The results confirmed that the 19-kDa ShhN peptide was indeed present in the media and clearly showed that secretion of Shh into the media was in response to the gastrin treatment. Moreover, these results demonstrated that induction of Shh expression could be studied acutely (6 h) in a primary culture system. A somewhat surpris-
Effect of both gastrin and OM on the secretion of the 19-kDa primary parietal cell cultures (Fig. 3). Because OM inhibited both basal and gastrin-induced secretion of the 19-kDa peptide by about 75%, we concluded that production of acid was required for effective processing of Shh by the parietal cell. Because histamine also stimulates gastric acid secretion, we treated parietal cell cultures with histamine and the phosphodiesterase inhibitor IBMX to sustain cAMP levels. We found that histamine and IBMX also increased the presence of the processed Shh form in the media and that OM blocks this process (Fig. 3D).

To confirm that the media containing the secreted 19-kDa fragment was biologically active, we treated a transiently transfected cell line (AGS) expressing a Shh reporter (Fig. 3E) with media from the canine cultures or recombinant human Shh (rShh). The three 0.5-ml fractions corresponding to the 19-kDa molecular weight marker were combined, concentrated to 50 μl and then 20 μl were resolved by SDS-PAGE for immunoblot analysis. Shh protein expression was quantified using the Odyssey Infrared Imaging System software. The mean ± S.E. for three cell preps, one-way ANOVA. *, p < 0.05 compared with Veh-treated cells

FIGURE 2. Shh protein expression in WT and G-/- mouse gastric cells. A, immunoblot analysis of whole cell extracts collected from WT and gastrin-deficient (G-/-) mouse gastric cells treated with vehicle (Veh), or 10 nM gastrin (Gast) for 6 h. ImmunobLOTS representative of three separate cell preps are shown. B, gel-purified media from WT/Veh, WT/Gast, G-/-/Veh, and G-/-/Gast treated gastric cells was analyzed by immunoblot. Media from treated gastric cells was collected and mouse Shh (mShh) concentrations measured by ELISA. The mean ± S.E. for mShh (pg/ml) is shown. *, p < 0.05 compared with WT/Veh-treated cells for n = 3 cell preps, one-way ANOVA.

FIGURE 3. Shh protein expression in canine parietal cells. A, acid production from canine parietal cells treated with gastrin. Aminopyrine (AP)-uptake in response to vehicle (Veh), gastrin (Gast), omeprazole (OM), or OM plus Gast treatment was performed for 30 min. The data are expressed as the mean-fold induction over Veh-treated cells + S.E., n = 3 experiments. *, p < 0.05 compared with Veh. B, immunoblot analysis of whole cell extracts collected from canine parietal cells treated with vehicle (Veh), 10 nM Gast, 100 nM omeprazole (OM), or OM plus Gast for 6 h. The immunoblot for Shh was re-blotted for Ptch. C, gel-purified media from canine parietal cell cultures was analyzed by immunoblot. The three 0.5-ml fractions corresponding to the 19-kDa molecular weight marker were combined, concentrated to 50 μl and then 20 μl were resolved by SDS-PAGE for immunoblot analysis. Shh protein expression was quantified using the Odyssey Infrared Imaging System software. The mean ± S.E. for mShh (pg/ml) is shown. *, p < 0.05 compared with Veh-treated cells by one-way ANOVA. D, media from parietal cells treated with Veh, 100 μM histamine (HIST), 10 μM isobutylmethylxanthine (IBMX), HIST plus IBMX, 100 nM OM, HIST plus OM, OM, HIST plus OM plus IBMX for 6 h was analyzed by immunoblot. E, AGS cells transiently transfected with the 8X3 Gli-BS-Luc (Shh reporter) plasmid were treated with either recombinant human Shh (rShh) or 19 kDa (fractions 14, 15, and 16) Shh immunoreactive protein from column extracts of 5 ml of media collected from canine parietal cells (cShh). The mean ± S.E. for RLU (fold induction) is shown. *, p < 0.05 compared with untreated cells, #, p < 0.05 compared with cShh-treated cell, n = 3 cell preps, one-way ANOVA.
recombinant peptide. This result is consistent with studies using native Shh peptide, which is more active than the recombinant peptide because of post-translational modification with cholesterol and palmitate (29, 34). Thus, we concluded from these results that processing of Shh from the nascent 45-kDa form to the secreted 19-kDa peptide in parietal cells is regulated and requires acid.

**Pepsin A CLEAVES 45-KDA SHH PRECURSOR TO THE 19-KDA SHhN—** Shh processing was shown previously in cell lines to be autocatalytic (33, 35, 36). Nevertheless, our results in primary cell culture suggested that Shh processing occurred primarily through a regulated mechanism. Therefore, to study Shh processing directly, a cell-free assay was developed using *in vitro* translated Shh. WCE were prepared from canine parietal cells pretreated with gastrin or OM then incubated with the 45-kDa *in vitro* translated Shh protein. Parietal cells pretreated with gastrin prior to the preparation of WCE, showed processing of the 45-kDa to the 19-kDa form (Fig. 4A, lane 7). However when the WCE were pretreated with OM, Shh processing was blocked (Fig. 4A, lane 8). To eliminate the possibility that endogenous Shh present in the WCE cleaved the *in vitro* translated 45-kDa protein, the endogenous protein was removed by immunodepletion (ID) (Fig. 4B, lanes 1–3). Processing was observed with WCE immunodepleted of endogenous Shh (Fig. 4B, lane 7). These results suggested that gastrin might induce acid-dependent peptidase activity because the cleavage of Shh was blocked by OM. To test directly if a protease inhibitor blocked Shh cleavage, WCE collected from parietal cells pretreated with gastrin were incubated with the acid peptidase inhibitor pepstatin. Pepstatin treatment abolished Shh processing induced by gastrin (Fig. 4B, lane 8), raising the possibility that proteolytic processing of Shh protein depended on a protease and an acidic pH.

Because pepsinogen A (PgA) and PgC are the most abundant acid-activated proteases in the stomach and there is a preferred cleavage site for the aspartic proteinase pepsin A at residue 200 (Phe<sup>200</sup>-Pro), we compared the levels of pepsin A and C in parietal cell extracts. We found that there was a significant increase in the expression of pepsin A in gastrin-treated parietal cells (Fig. 5A). Moreover, conversion of PgA to pepsin A was inhibited by OM explaining the requirement for acid to maximize the activity of the enzyme. While there is pepsin C in the corpus mucosa, we did not detect pepsin C in WCE from purified parietal cells (Fig. 5B). This result confirmed that PgC is not present in parietal cells, but is likely the major pepsinogen isoform secreted from chief and mucous cells as previously reported (37). Next, we determined the concentration of pepsin A in the extracts of canine parietal cells treated with vehicle, gastrin, OM, or OM plus gastrin. Using a standard curve generated with pure enzyme (Fig. 5C), we observed that there was abundant pepsin A activity only in gastrin-treated parietal cell extracts compared with vehicle- or OM-treated (Fig. 4D). We did not observe processing of *in vitro*-translated Shh protein with small amounts (<10 μg) of extracts from vehicle- or OM-treated parietal cells because of the significantly lower levels of pepsin A activity under basal conditions (Veh) and in the absence of acid.

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**FIGURE 4. Immunoblot analysis of *in vitro* translated mouse Shh protein.**

* A. Processing of the 45 kDa *in vitro* translated Shh protein to the 19-kDa form after incubation with WCE (10 μg) collected from canine parietal cells treated with vehicle (Veh), 10 nM Gast, 100 nM omeprazole (OM), or OM plus Gast for either 0 h (lanes 1–4) or 4 h (lanes 5–8). B. WCE collected from canine parietal cells treated with gastrin (Gast, lane 1) were immunodepleted of endogenous Shh (ID<sub>SHH</sub>). Immunocomplexes were separated using protein A/G. Bound (B/ID<sub>SHH</sub>, lane 2) and unbound (UB/ID<sub>SHH</sub>, lane 3) fractions were analyzed by immunoblot. Processing of the 45 kDa *in vitro*-translated Shh protein to the 19-kDa peptide after incubation without WCE (lane 4) with WCE collected from canine parietal cells treated with vehicle (Veh, lane 5) or 10 nM OM (lane 6). WCE immunodepleted (ID) with Shh antibody (GAST/ID<sub>SHH</sub>, lane 7) or WCE treated with pepstatin (PS, lane 8).

**FIGURE 5. Expression and concentration of pepsin A in canine parietal cell extracts.**

A. Immunoblot analysis of pepsin A (35 kDa) protein expression in canine parietal cells treated with Veh, Gast, OM, or OM plus Gast. The same blot was re-probed for GAPDH. B. Immunoblot analysis of pepsin C (36 kDa) protein expression in canine parietal cells treated as in A and homogenate prepared from canine corpus mucosa. C. Pepsin A standard curve (ng) generated using a protease fluorescence kit and FITC-tagged casein substrate. D. Pepsin A concentrations (ng) assayed in 0, 5, 10, 25, 50, and 100 μg of WCE collected from Veh-, Gast-, OM-, and OM + Gast-treated parietal cells.
Processing of in vitro translated Shh by pepsin A. A, processing of the 45 kDa in vitro translated Shh protein to the 19-kDa protein after a 4 h of incubation with WCE (100 μg): collected from canine parietal cells treated with Veh (lane 1), Gast (lane 2), WCE immunodepleted (ID) with PgA (lane 3), or PgC (lane 4) antibody (IDαPg or IDβPg). B, in vitro-translated Shh 45-kDa protein incubated with buffer at pH 2. Cleavage of wild type (C) or mutated (D) (mShh) 45 kDa in vitro translated Shh protein to the 19-kDa peptide after treatment by pepsin A at pH 2.0 for 0, 20, 40 min (lanes 1–3). E, conditioned media (100 μg) from AGS cells transfected with vector (Vect), wild type Shh (WT), F200H mutant or H271A mutant was resolved on a 12% SDS-PAGE gel and blotted with anti-HA antibody. ShhN, processed N-terminal Shh. F, incubation of the 45 kDa in vitro-translated Shh protein with chymotrypsin (Chym, lane 1), pepsin A (PsA, lane 2) cathepsin D (CathD, lane 3), trypsin (Tryp, lane 4), or plasmin (Plas, lane 5) for 1 h at 37 °C. ( — , blank lane 6). mShh, recombinant human Shh protein (rhShh, 0.25 μg, lane 7).

(OM-treated) (Fig. 5D). However when 100 μg of WCE from all treatments were compared, a basal level of processing was observed even in the absence of gastrin or with OM alone at 60 min.

To demonstrate that the Shh processing activity within the parietal cell extracts was indeed pepsin A, we used PgA antibodies to immunodeplete parietal cell extracts of pepsin A and tested whether the depleted extract retained Shh processing activity. Immunodepletion with the PgC antibody was used as a negative control. We found that extracts depleted of PgA but not PgC lost the ability to process Shh after gastrin treatment clearly demonstrating that it is pepsin A that provides the acid-dependent processing activity in the parietal cells (Fig. 6A).

To demonstrate that pepsin A cleaves at the residues necessary to generate the 19-kDa N-terminal fragment, commercially available pepsin A was incubated with the 45-kDa in vitro translated substrate. In the absence of pepsin, processing was not observed at pH 2 without exogenous protease (Fig. 6B). Pepsin A cleaved the 45-kDa precursor to the 19-kDa protein within 20 min (Fig. 6C). Based on the size of the processed protein, cleavage by pepsin A was expected to occur near resi-
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A. Human Stomach
   Normal Tumor
   Pepsin A
   GAPDH

B. Human Stomach
   Normal Tumor
   Shh 45kDa
   Shh 19kDa
   GAPDH

C. Human Stomach
   Normal Tumor
   Shh 45kDa
   Shh 19kDa

D. Human Stomach
   N/PS TIPS
   Shh 45kDa
   Shh 19kDa

E. Human Stomach
   NID/PID
   Shh 45kDa
   Shh 19kDa

F. Human Stomach
   NID/PID
   Shh 45kDa
   Shh 19kDa

FIGURE 7. Processing of in vitro-translated Shh by pepsin A activity within human gastric tissue extracts. A, immunoblot analysis of pepsin A (35 kDa) protein expression in tissue extracts collected from normal (lane 1) and tumor (lane 2) tissue. The same blot was re-probed for GAPDH. B, protein expression of 45-kDa precursor Shh and 19-kDa processed protein in normal (lane 1) and tumor (lane 2) human tissue extracts. C, expression toward gastric neoplasia. However, it is not understood whether reduced PgA levels simply correlate with parietal cell atrophy or might be causative. We show here that acid secretion and subsequently the conversion of PgA to pepsin A is required for Shh processing. It has been shown previously that Shh is processed autocatalytically from a 45-kDa precursor in which the C-terminal domain is removed leaving the biologically active N-terminal domain available to bind the hedgehog (Hh) receptor Ptc. The N-terminal domain is a 19-kDa fragment that is cholesterol-modified at its C terminus and palmitoylated at its N terminus (41). We show here that a biologically active ShhN is generated in the stomach by the protease pepsin A. When acid secretion was blocked preventing activation of the proenzyme pepsinogen A, ShhN was not generated. Moreover, we show that the pepsin A protease has functional significance, since pepsin A activity is drastically reduced or absent in gastric mucosa prepared from patients with gastric cancer. In gastric cancer samples from human subjects, we found that the 45-kDa Shh precursor is clearly not processed to the 19-kDa ShhN because of insufficient levels of pepsin A.

The absence of Shh processing in human gastric cancer extracts was surprising, because many gastric cell lines have elevated hedgehog signaling activity (42, 43) and would therefore be expected to process Shh appropriately. Prior studies demonstrated using xenografts of some human gastric cell lines that Hh signaling is required for gastric cancer cell growth. It was assumed that the Shh ligand mediating the activation was ShhN, but the form of Shh produced by the xenografts was not evaluated directly. Our results would suggest that the major form of the Shh ligand present in the hypochlorhydric stomach is the 45-kDa peptide. However, it remains to be determined whether the full-length peptide has any biologic activity that differs from the processed 19-kDa ShhN.

Although autacatalytic processing of Shh might occur in the native parietal cell, the abundance of the aspartic proteinases in the acid-producing stomach suggests that pepsin A cleavage of Shh is the predominant mechanism for processing this ligand. Moreover, we have shown previously that Shh stimulates H+,K+-ATPase gene expression (18). Therefore, we suggest that generating the 19-kDa Shh ligand by parietal cells is essential to its ability to produce acid. Several studies have demonstrated that targeted deletion of parietal cell H+,K+-ATPase subunits or its inability to make acid will induce atrophy and metaplasia (9, 44–46). Thus, regulation of H+,K+-ATPase content (47). Furthermore, Shh stimulation of H+,K+-ATPase gene expression (18) suggested to us that gastrin is linked to Shh expression through its ability to stimulate acid production. Collectively, these studies indicate that Shh is essential to the proximal stomach where parietal
cells reside and secrete acid, since their absence sets the stage for neoplastic transformation (48). We also show in this study that gastrin stimulates Shh gene expression, although its acute effect is on Shh processing through activation of PgA to pepsin A. Interestingly, processing of Shh in the parietal cell due to activation of acid-dependent proteases generates considerable amounts of the 19-kDa ShhN form that exhibit at least 25-fold greater hedgehog (Hh) signaling activity. Because the ShhN form is found only in the media, one would predict that it is capable of diffusion in an aqueous medium such as the gastric lumen and may form aggregates (34).

In the stomach, Pgs are abundant and available to the parietal cell (49). We have shown that Shh is exported from the parietal cell efficiently, suggesting that Shh is available for processing by pepsin A. The current study presents a unique mechanism by which Shh is processed in gastric parietal cells. Analysis of membrane fractions from canine parietal cells showed that the 45-kDa Shh precursor and pepsin A were found within the same subcellular fraction as the H⁺,K⁺-ATPase.³ Because the H⁺,K⁺-ATPase enzyme becomes activated upon insertion into the elaborate, apical canalicular membrane, this initial result suggests that Shh and pepsin A are located on that parietal cell surface. A prior report by Maity et al. (50) alluded to Shh processing being located within Golgi after cholesterol esterification then shuttling to the plasma membrane where it either remains or is secreted from the cell. We suggest that Shh may be processed at the canalicular membrane surface where the activated H⁺,K⁺-ATPase has been inserted and pumps acid so that it is available to convert PgA to pepsin A. Nevertheless, it is important to note that the endoplasmic reticulum is also present in the H⁺,K⁺-ATPase-containing microsomal fraction. Therefore, additional characterization is required to determine the precise location of Shh processing by the parietal cell. Whether Shh secreted from the parietal cells is cholesterol modified is not known, and requires further investigation considering that the activity of the Shh ligand we identified in the culture media is orders of magnitude greater than the activity of the recombinant form.

Having established that Shh processing can be regulated in adult stomach, this result raises the question as to whether protease-dependent processing occurs in other tissues. A limited screen of extracts from other gastrointestinal and non-gastrointestinal tissues suggests that there is peptstatin-inhibitable activity in other tissues.³ Indeed, pepstatin-sensitive proteases are a discrete family of aspartic proteinases defined by their activity at an acidic pH. This family of proteases includes the cathepsins, chymosin and renins in addition to the Pgs. A wide variety of normal and neoplastic tissues specifically express these proteases but whether Hh proteins are processed in these tissues by a similar mechanism remains to be determined.

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³ Y. Zavros and J. L. Merchant, unpublished data.

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