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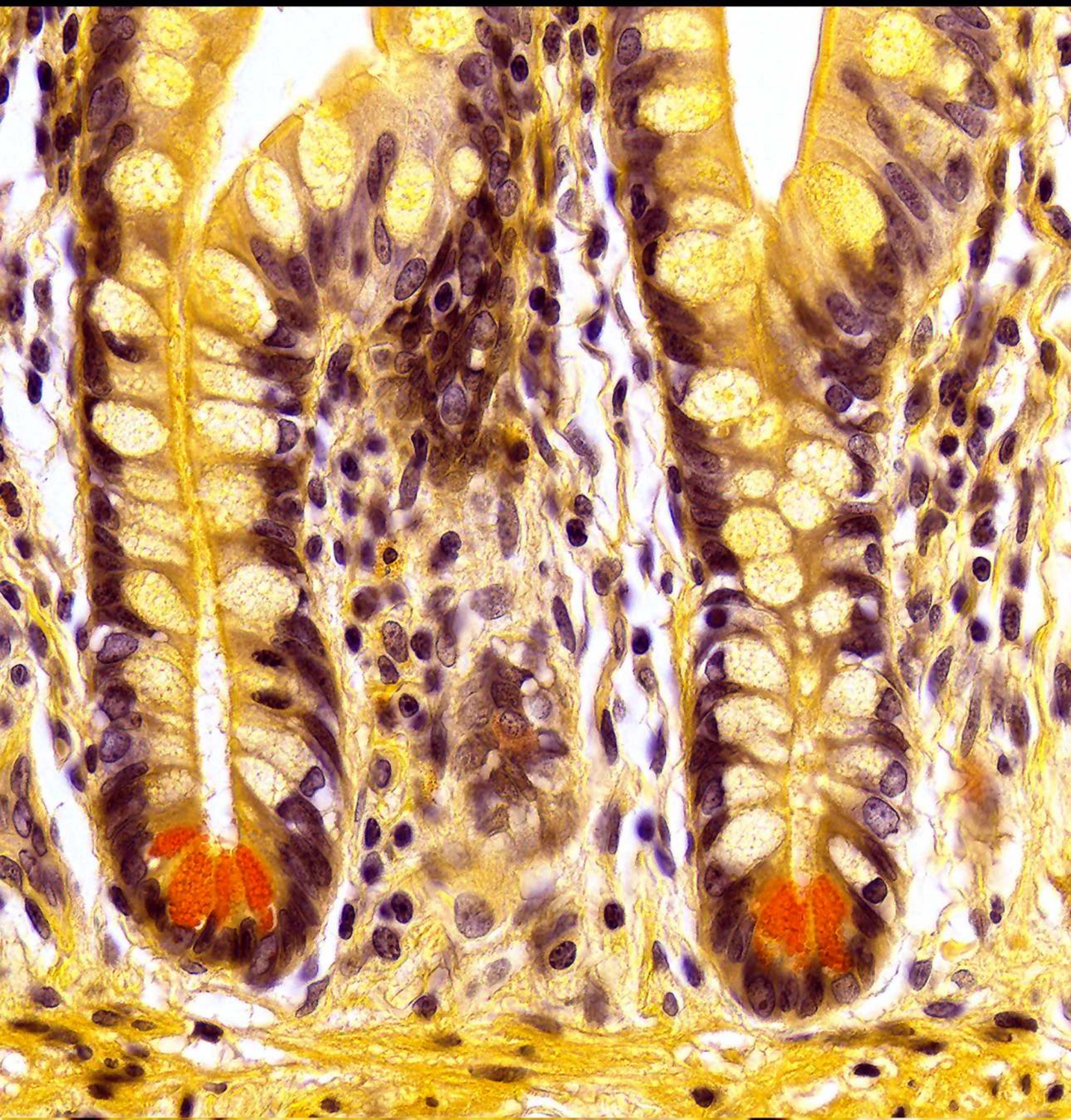

Paneth cell trypsin is the processing enzyme for human defensin-5

Dipankar Ghosh[1,2], Edith Porter[3,4], Bo Shen[1,5], Sarah K. Lee[1,2], Dennis Wilk[1,2], Judith Drazba[2], Satya P. Yadav[2], John W. Crabb[2,6], Tomas Ganz[3] and Charles L. Bevins[1,2,5,7]

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The antimicrobial peptide human α-defensin 5 (HD5) is expressed in Paneth cells, secretory epithelial cells in the small intestine. Unlike other characterized defensins, HD5 is stored in secretory vesicles as a propeptide. The storage quantities of HD5 are ~90–450 μg per cm² of mucosal surface area, which is sufficient to generate microbicidal concentrations in the intestinal lumen. HD5 peptides isolated from the intestinal lumen are proteolytically processed forms—HD5(56–94) and HD5(63–94)—that are cleaved at the Arg⁵⁵-Ala⁵⁶ and Arg⁶²-Thr⁶³ sites, respectively. We show here that a specific pattern of trypsin isozymes is expressed in Paneth cells, that trypsin colocalizes with HD5 and that this protease can efficiently cleave HD5 propeptide to forms identical to those isolated *in vivo*. By acting as a prodefensin convertase in human Paneth cells, trypsin is involved in the regulation of innate immunity in the small intestine.

Because mucosal surfaces are continually confronted with microbes, effective host defense at these sites is critical to host survival. Several recent lines of investigation highlight that epithelial cells actively contribute to the recognition of and coordinated defensive responses against microbes. In particular, the discovery that many epithelial cells synthesize and secrete antimicrobial polypeptides has broadened the scope of their contributions to mucosal host defense[1–7]. However, the molecular details of this dynamic process remain incompletely understood.

From a host defense standpoint, the mammalian small intestine presents formidable challenges. First, there is an expansive epithelial surface required for adequate nutrient absorption. This epithelium with its multitude of villi and narrow invaginations (crypts) is a potential portal of microbial invasion. Second, the epithelium turns over every 2–5 days and the stem cells that replenish this surface epithelium with fresh cells require continuous antimicrobial protection[8,9]. Third, the lumenal environment is nutrient-rich and seems to provide an ideal medium for microbial proliferation. Finally, there is a continuous exposure to microbes, from the adjacent colon with its heavy bacterial colonization and from ingested food and water that frequently has bacterial contamination. Despite all these factors, microbial density in the healthy small intestine is low[10]; in addition, the defense mechanisms are able to maintain the crucial barrier and absorptive functions of this mucosa.

Paneth cells are likely key contributors to effective host defense in the intestine[11,12]. These epithelial cells are located in clusters at the base of the small intestinal crypts of Lieberkühn, which are narrow invaginations distributed throughout this surface epithelium. Paneth cells are most numerous in the ileum and have an abundance of large apically located eosinophilic secretory granules. These secretory granules are rich with antibiotic polypeptides, including lysozyme[13], secretory phospholipase-A_2[14,15] and defensins[16,17]. Stimulation of Paneth cells with cholinergic agonists[18,19] and bacterial stimuli[20,21] causes the release of these granules into the crypt lumen.

Defensins are a group of gene-encoded, cysteine-rich cationic peptides that effect a broad spectrum of antibiotic activity, primarily by disrupting microbial cell membranes[22,23]. Defensins are expressed by phagocytic leukocytes and by various epithelial cells, including Paneth cells; they express α-defensins[2,24–27], a subfamily of defensin peptides defined by their cysteine spacing and disulfide connectivity. Six human α-defensins have been identified. In neutrophils, α-defensin 1, α-defensin 2, α-defensin 3 and α-defensin 4 (which are also known as, and are referred to hereafter as, human neutrophil peptide 1 (HNP1), HNP2, HNP3 and HNP4, respectively) are stored in the azurophilic granules as fully processed ~3-kD mature peptides[28,29]. These defensins exert their antibiotic function primarily in an intracellular compartment, the phagolysosome. In Paneth cells, human α-defensin 5 (HD5) and HD6 are stored in secretory granules that are destined for extracellular activities in the intestinal lumen[17]. In contrast to the detailed understanding we have of neutrophil α-defensin precursor processing, vesicular storage and antimicrobial activity[22,23,28,29], less is known about their Paneth cell counterparts.

Unlike neutrophils, Paneth cells do not store defensins as processed mature peptides, rather they store them as propeptides[30,31]. This was an unexpected finding, given that all previously isolated α-defensins were fully processed active peptides and the neutrophil α-defensin propeptides are inactive *in vitro*[32]. Because HD5 is a major defensin in human Paneth cells, we investigated this phenomenon further. This issue gained significance when studies in mice found that disrupted α-defensin processing in murine Paneth cells led to marked vulnerability

Departments of [1]Immunology, [5]Gastroenterology and [7]Colorectal Surgery and [2]The Lerner Research Institute and [6]The Cole Eye Institute, The Cleveland Clinic Foundation, 9500 Euclid Ave., Cleveland, OH 44195, USA. Department of Medicine[3], UCLA School of Medicine, 10833 Le Conte Ave., Los Angeles, CA 90095, USA. [4]Department of Biological Sciences, California State University, 5151 State University Dr., Los Angeles, CA 90032, USA. Correspondence should be addressed to C. L. B. (bevinsc@ccf.org).

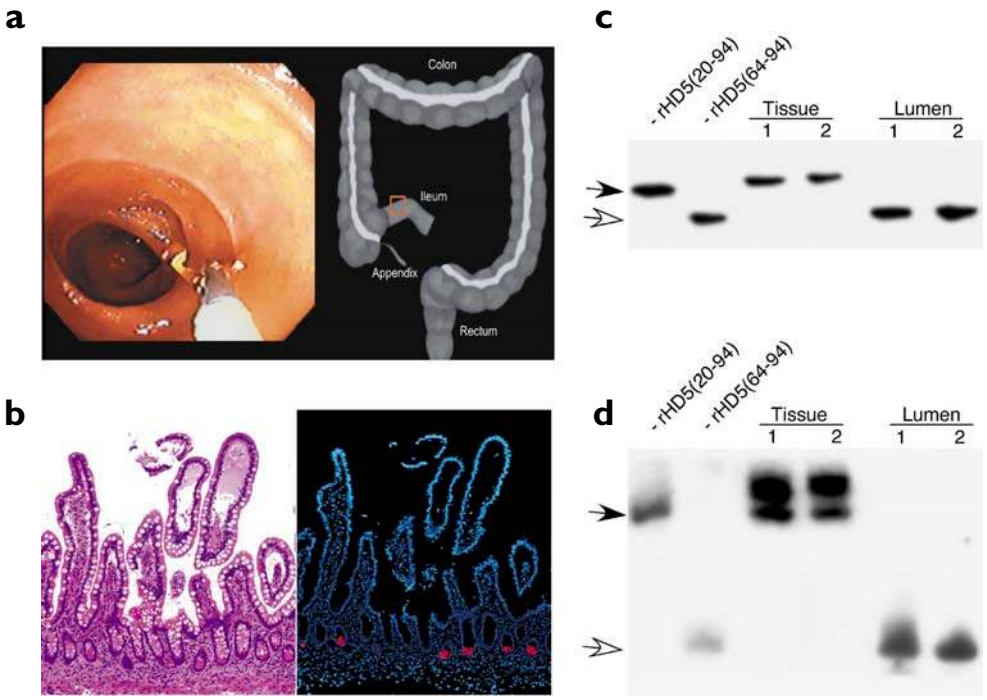

Figure 1. Analysis of tissue and lumenal forms of HD5. (**a**) Samples of ileal tissue and the adjacent lumen were obtained by endoscopies done for non-inflammatory clinical conditions. The box indicates the anatomic site of endoscopic sampling. (**b**) (Left) Terminal ileal tissues were stained with hematoxylin and eosin. (Right) Tissue sections were also stained with polyclonal rabbit antiserum to HD5[17], with Alexa Fluor 568 (red) immunofluorescent detection and DAPI (blue) as a nucleic counterstain. Control slides incubated with preimmune serum showed no staining (data not shown). (**c**) Samples of tissue and lumen, along with HD5 peptides, were resolved by 12.5–20% Tris-tricine SDS-PAGE under nonreducing conditions, electroblotted onto PVDF membranes and probed with an polyclonal antiserum to HD5. (**d**) Portions of the same samples and standards used in **b** were resolved by 12.5% AU-PAGE, transferred to a PVDF membrane and analyzed for HD5 as in **c**. Tissue, an extract from 0.04 mg of ileal tissue; lumen, equivalent of 1 ml of diluted (unnormalized) lumenal aspirate; rHD5(20–94), 20 ng of rproHD5; rHD5(64–94), 20 ng of rHD5(64–94). Twenty-three specimens of tissue and 16 specimens of lumen were analyzed and representative data are shown.

to enteric infection[33]. The matrix metalloproteinase (MMP) matrilysin mediated the proteolytic processing of murine enteric α-defensins (called cryptdins in mice). Although activity of matrilysin is crucial for the innate immune functions of cryptdins[33], there is no evidence for the expression of homologous MMP-7 in the human small intestinal mucosa. We show here that one function of Paneth cell trypsin is to act as a prodefensin-processing enzyme and implicate this well studied serine protease in the regulation of innate immunity in the human small intestine.

Results

Analysis of tissue and lumenal HD5

Nondiseased specimens of human distal small intestinal tissue derived from either surgical resection or endoscopically obtained biopsies (**Fig. 1a**) were examined by routine histology and immunohistochemistry to confirm the presence of normal surface mucosa with Paneth cells (**Fig. 1b**). Specific staining of Paneth cells with this antibody was detected at the base of the small intestinal crypts, confirming published data[17] (**Fig. 1b**). Protein was extracted from the samples in the presence of protease inhibitors and subjected to immunoblot analysis with HD5 antiserum or further purified (see below). Tissue-derived HD5 migrated in a similar manner to recombinant HD5 propeptide amino acids 20–94 (referred to hereafter as rproHD5) on Tris-tricine SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (**Fig. 1c**) and acid urea–PAGE (AU-PAGE) (**Fig. 1d**). Thus, Paneth cell HD5 appears as a propeptide when extracted from intestinal tissue, confirming published data[30,31]. Tissue concentrations of HD5, estimated based on quantitative AU-PAGE immunoblotting and rproHD5 standards, were ~0.5–2.5 mg/g of terminal ileum mucosa (**Fig. 1d** and data not shown). Lumenal aspirates obtained by endoscopy were subjected to the same analysis as tissue samples. Lumenal HD5 migrated in a similar manner to the recombinant HD5 amino acids 64–94 (referred to hereafter as rHD5(64–94)) on Tris-tricine SDS-PAGE (**Fig. 1c**) and AU-PAGE (**Fig. 1d**), indicating that during or after secretion HD5 propeptide was cleaved.

Isolation and characterization of HD5

HD5 peptides were purified by cation-exchange chromatography followed by reverse-phase high-performance liquid chromatography (RP-HPLC). Fractions were monitored for HD5 immunoreactivity and isolated peptides were subjected to mass spectral and NH_2-terminal amino acid sequence analyses. Tissue specimens consistently yielded proforms of HD5 (**Table 1** and **Fig. 2**). The most abundant form consisted of HD5 amino acids 20–94 (referred to hereafter as HD5(20–94)), which confirmed published findings[31]; less abundant forms observed were HD5(23–94) and HD5(29–94). Mass spectral analysis indicated that the HD5 propeptides isolated from tissue exist, in part, as unmodified proteins. Our analysis showed that a substantial portion of tissue HD5 was glycosylated (unpublished data); this post-translational modification likely explains the heterogeneity of

Table 1. Biochemical characterization of HD5 forms purified from ileal tissue

Origin	Form	Mass[a]	NH_2-terminal sequence
Tissue	**20–94**	**8107/8102**	**ESLQE...**
	23–04	7770/7773	QERAD...
	29–94	7044/7044	ATTQK...
Lumen	**63–94**	**3582/3582**	**ATCYCR...**
	56–94	4271/4270	TSGSQ

Amino acid sequences are in standard single letter code. Expected molecular masses were deduced from the cDNA sequence[24]; it was assumed that all cysteines participate in intramolecular disulfide bonds. The predominant HD5 forms from these sources are shown in bold. [a]MALDI-TOF experimental values/expected values.

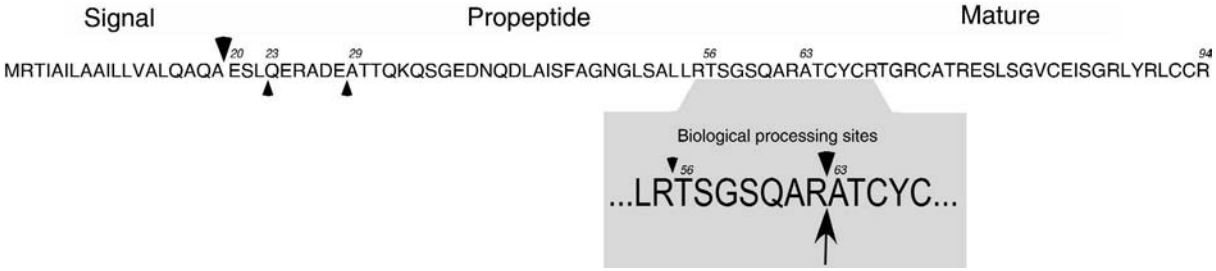

Figure 2. Primary structure of preproHD5 deduced from the cDNA sequence[24]. Arrowheads designate the HD5 NH_2-termini that were isolated from biological sources. Large arrowhead designates the predominant form, HD5(20–94), and smaller arrowheads designate the minor forms, HD5(23–94) and HD5(29–94), that were isolated from intestinal tissue. Shaded box highlights the biological processing sites of preproHD5; the large arrowhead designates the predominant form, HD5(63–94), and small arrowhead designates the less abundant form, HD5(56–94), that were isolated from intestinal lumen. Arrow designates the *in vitro* processing site of proHD5, where trypsin generates a stable HD5(63–94) product.

AU-PAGE analysis (**Fig. 1d**). Lumenal samples yielded processed HD5 peptides: HD5(63–94) was the predominant form (**Table 1** and **Fig. 2**). The processed forms of HD5 were not detected in any tissue specimen, and the tissue-derived propeptides were not detected in samples of intestinal lumen. The forms of HD5 that were recovered from the intestinal lumen have been identified previously in ileal neobladder urine[30], suggesting that the protease responsible for processing in that context was likely derived from the ileal mucosa rather than from the urinary tract. We did not detect the HD5(36–94) form that was characterized in the ileal neobladder urine[30]. The HD5(36–94) form, but not the HD5(63–94) or HD5(56–94) forms, was detected during the analysis of secretions from isolated terminal ileal crypts *in vitro*[31]. These differences may reflect the differing environments of the *in vivo* and *in vitro* systems.

Antimicrobial activity of HD5

Standard antimicrobial colony-forming unit (CFU) assays were done against Gram-negative *Salmonella typhimurium* with various HD5 peptides, proHNP1 and mature HNP1 at equal molar concentrations (3.3 μM). Lumenal HD5(63–94), effected a 99.9% reduction of the bacterial inoculum during 2-h incubation and HD5(56–94) a 99.0% reduction (**Fig. 3a**). When tested against Gram-positive *Listeria monocytogenes*, these HD5 forms effected a 90.0% and 99.9% reductions of bacteria, respectively (**Fig. 3b**). However, unprocessed proHD5 showed antimicrobial activity against both bacterial strains, reaching a 99.0% reduction of *L. monocytogenes* (**Fig. 3**). In comparison, proHNP1 was inactive against these bacteria and mature HNP1 was active only against *L. monocytogenes*, which supported published data[32]. Thus, all observed HD5 forms exerted antimicrobial activity, but their potency was affected by peptide processing.

In vitro processing of HD5

NH_2-terminal analysis of the two lumenal forms of HD5 revealed a cleavage site COOH-terminal to an arginine residue (**Table 1** and **Fig. 2**), which suggested the serine protease trypsin as a candidate processing enzyme. This possibility was tested *in vitro* by incubating rproHD5 with bovine trypsin (at a 2:1 molar ratio of peptide to enzyme); the products were analyzed by AU-PAGE and mass spectral analysis. Trypsin cleaved rproHD5 and rHD5(56–94) quantitatively to a homogeneous product, which was identified by mass spectrometry as HD5(63–94) (**Fig. 4a**). Next, rproHD5 was cleaved within minutes to HD5(63–94) by human trypsin at a molar ratio of peptide to enzyme of 300:1 (**Fig. 4b**). This cleavage was blocked by the serine protease inhibitor (serpin) α_1-antiprotease (α_1-AP, also known as α_1-antitrypsin) (**Fig. 4b**). Trypsin was also partially purified from extracts of ileal tissue and incubated with tissue-derived proHD5; it cleaved proHD5 to the lumenal peptide form HD5(63–94) (**Fig. 4c**). In other experiments, lysates of ileal mucosa incubated at pH 7.4 without protease inhibitors completely converted the propeptide to the arginine-rich, but trypsin-resistant, HD5(63–94) form (**Fig. 4d**). Inclusion of a serine protease inhibitor (APMSF) with the lysate blocked the processing, whereas cleavage proceeded unabated in the presence of cysteine, aspartyl or MMP inhibitors (**Fig. 4d**). Mature HD5 is resistant to cleavage upon prolonged incubation with trypsin[34].

Trypsin expression in human Paneth cells

Our earlier finding that HD5 processing occurred in ileal neobladders suggested a local production of the processing enzyme(s) in the ileal mucosa[30]. We therefore examined trypsin expression in the intestinal mucosa and determined its location in relation to HD5 production sites. Using northern blot analysis, we detected abundant trypsin mRNA

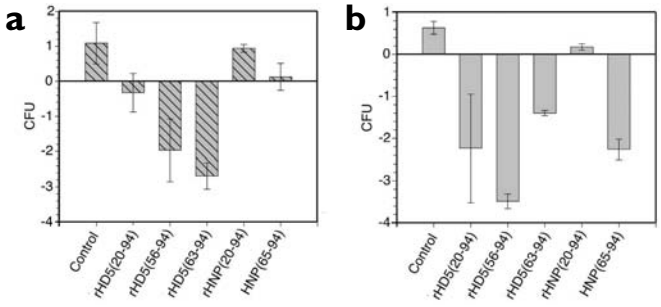

Figure 3. Antibacterial activity of HD5 peptides. Total CFU (log (CFU_{t2}/CFU_{t0})) of test bacteria were determined after 2 h incubation in presence or absence of 3.3 μM of the indicated forms of HD5 peptides in 10 mM sodium phosphate buffer (pH 7.4) supplemented with 1% (v/v) trypticase soy broth. The inoculum was 2×10^6 CFU/ml of (**a**) wild-type *S. typhimurium* strain 14028s and (**b**) *L. monocytogenes*. For comparison, rproHNP1(20–94) and HNP1(65–94) were included at the same molarity.

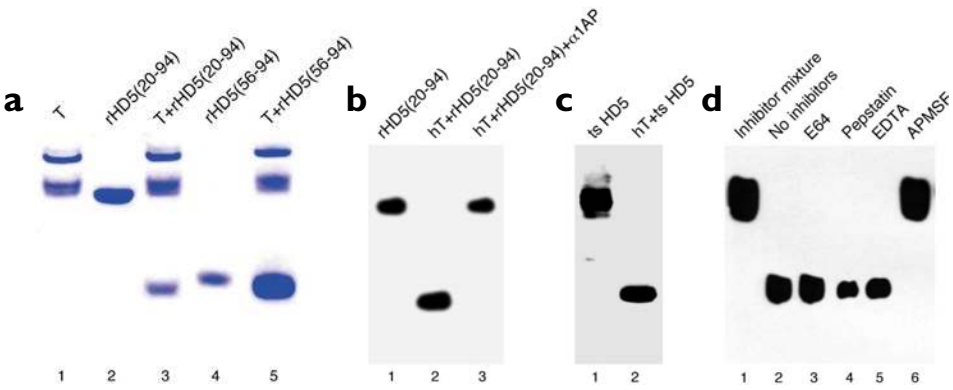

Figure 4. *In vitro* cleavage of proHD5 by trypsin. (**a**) Coomassie blue–stained AU-PAGE of rHD5(20–94) and rHD5(56–94) after incubation for 2 h at 37 °C with purified bovine pancreatic trypsin (lanes 3 and 5, respectively) or without trypsin (lanes 2 and 4, respectively). Trypsin was also incubated in the absence of HD5 (lane 1). (**b**) AU-PAGE immunoblotting of rproHD5 (lane 1), which was incubated for 5 min at 37 °C with human trypsin in the absence (lane 2) or presence of α_1-AP (lane 3). (**c**) AU-PAGE immunoblotting of human intestinal tissue HD5 (lane 1) incubated under same conditions as in **b** with trypsin purified from human intestinal tissue (lane 2).The HD5 products generated by trypsin proteolysis (**a–c**) were isolated and characterized by mass spectral analysis. (**d**) AU-immunoblotting of human ileal tissue lysates incubated at 37 °C for 30 min with various classes of protease inhibitors. Lysates were treated with either protease inhibitor mixture (lane 1), 2 mM E64 (lane 3), 2 mM pepstatin (lane 4), 20 mM EDTA (lane 5), 50 μM APMSF (lane 6) or with no inhibitors (lane 2).

expression in the ileal mucosa (**Fig. 5a**). By northern blot analysis, abundant mRNA were detected in the ileum encoding two inhibitors of trypsin, the serpin α1-AP and the Kazal-type inhibitor, pancreatic secretory trypsin inhibitor (PSTI, **Fig. 5a**).

Using immunofluorescence histochemistry and confocal microscopy on sections of human terminal ileum, we found that trypsin was colocalized with HD5 in Paneth cells at the base of the crypts of Lieberkühn (**Fig. 5b**). Trypsin immunoreactivity was observed in Paneth cells (**Fig. 5b**, middle panel and **Fig. 5c**, right panel), consistent with published studies that used a polyclonal antibody[35]. The staining pattern indicated colocalization with HD5 in secretory granules (**Fig. 5b**, right panel). Whereas HD5 immunoreactivity was uniformly distributed, the trypsin signal was more apparent in basally located granules, suggesting a complexity to trypsin epitopes in the secretory granules. In parallel tissue sections, α_1-AP was similarly detected in Paneth cell secretory granules (**Fig. 5c**, left panel).

Three isoforms of human trypsin have been characterized in human pancreatic tissue[36,37]. The forms of trypsin that were expressed in the mucosa of terminal ileum were analyzed with a highly specific hybridization analysis of reverse-transcribed mRNA (**Fig. 6**). Consistent with published data[36,37], the pancreatic sample contained mRNA from all three trypsin isoform—cationic, anionic and mesotrypsin—at a ratio of ~24:22:2, respectively. In contrast, none of the four samples of terminal ileum contained detectable cationic trypsin

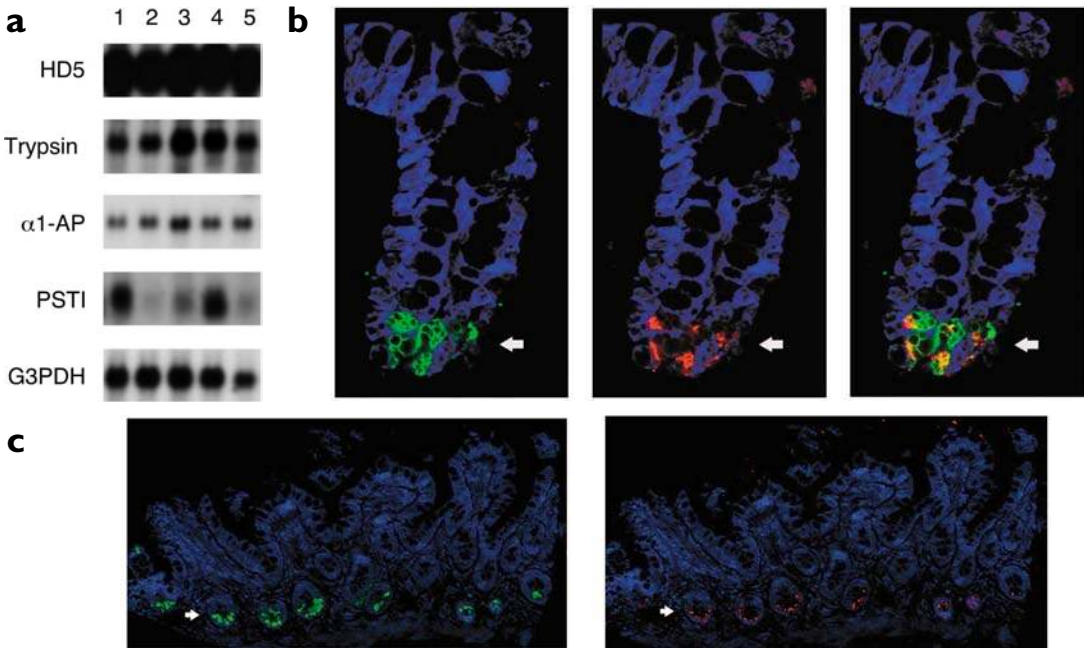

Figure 5. Expression of trypsin in Paneth cells. (**a**) Northern blot hybridization of trypsin expression in human small intestine. Total RNA (10 μg) from adult human small intestinal ileum was analyzed with probes for HD5, trypsin, α_1-AP, PSTI and glyceraldehyde-3-phosphate dehydrogenase. The autoradiographic exposure was 3 days. (**b**) Immunohistochemical colocalization of HD5 and trypsin in human small intestine. Terminal ileum tissue sections were incubated with polyclonal rabbit antiserum to HD5[17] and a pooled monoclonal anti–trypsin IgG. Alexa Fluor 488–conjugated anti–rabbit IgG (green) or biotinylated anti–mouse IgG and Alexa Fluor 568–conjugated streptavidin (red) were used for immunodetection, respectively; DAPI was used as a counterstain (blue). Arrows denotes the clusters of Paneth cells at the base of an intestinal crypt. (**c**) Immunohistochemical localization of α_1-AP (left panel) and trypsin (right panel) in human small intestine. Terminal ileum tissue sections were incubated with a rabbit polyconal α_1-AP antiserum and a pooled monoclonal anti–trypsin IgG. Alexa Fluor 488–conjugated (green) anti–rabbit IgG or biotinylated anti–mouse IgG and Alexa Fluor 568–conjugated streptavidin (red) were used for immunodetection, respectively; DAPI was used as a counterstain (blue). Arrows denote the base of intestinal crypts.

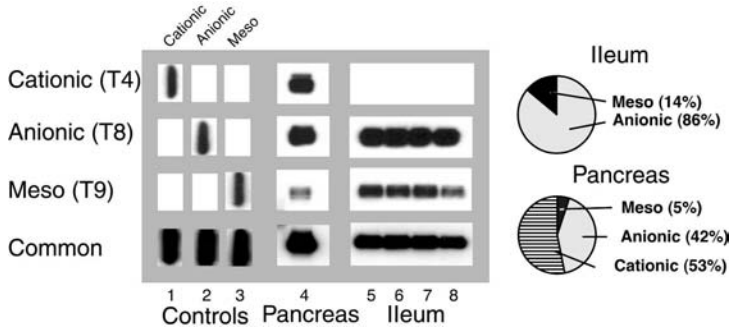

Figure 6. Analysis of trypsin isoform expression in human pancreas and ileum. RT-PCR analysis of pancreatic and terminal ileal mRNA was done with PCR primers hTryp-2s and hTryp-3a, whose sequences correspond to regions of sequence identity in cationic, anionic and mesotrypsin mRNA. A single PCR product of expected size (0.6 kb) was identified in all five samples and was blotted to a nylon membrane. Included on the membrane were samples of cloned plasmid DNA encoding each of the three trypsin isoforms as hybridization controls. The membranes were sequentially hybridized with ^{32}P-labeled oligonucleotide probes for each trypsin isoform under high stringency conditions, and signal intensity was quantified with phosphorimager analysis. An oligonucleotide (hTryp-1a) whose sequence was identical to the corresponding sequences in all three trypsin isoforms (common probe) was used to normalize for equivalent loading of each sample. The pie diagrams indicate the relative proportion of each detected trypsin isoform in the pancreatic and ileal (average of four) samples. **T4**, cationic; **T8**, anionic; **T9**, mesotrypsin[51].

mRNA. Rather, only anionic and mesotrypsin mRNA were detected in ileal samples, at a ratio of ~6:1, respectively (**Fig. 6**). Hence, Paneth cells express a distinct pattern of trypsin mRNA isoforms.

Discussion

Epithelial cells are a source of antimicrobial peptides that contribute to the defense of mucosal tissues in humans and other mammals[4,5,7]. In the small intestine, Paneth cells secrete their apically oriented, antimicrobial-laden granules into the crypt lumen[18,20,21,25]. Defensins are a prominent antimicrobial in these secretions[21]. We showed here that HD5 is stored in Paneth cells of the human ileal mucosa at quantities of ~90–450 μg per cm^2 of ileal surface area. Assuming complete secretion, and given an estimated ileal diameter of ~3.75 cm, steady-state storage quantities of HD5 could generate concentrations of 50–250 μg/ml in the intestinal lumen. Thus, significant quantities of α-defensin peptides are available for delivery into the lumen. Through analysis of isolated crypt preparations *ex vivo*, others have found that mouse Paneth cells in a single crypt secrete α-defensin peptides to concentrations of ~25 mg/ml in the minute volume (~3–10 pl) of the crypt lumen[21]. Given that defensins have antimicrobial activity at concentrations of μg/ml, Paneth cell secretions provide a formidable antimicrobial capacity. We[12,27] and others[11,25,31] envisage that enteric α-defensins contribute to host defense of the small intestine through selective antibiotic activity. This influences the composition and limits the numbers of transient and resident lumenal microbes in the crypt and lumen, thereby providing protection that preserves the structural integrity and critical physiological functions of this vital epithelium.

Unlike all other characterized α-defensins in mammals, we have shown that HD5 is stored exclusively as a propeptide in healthy ileal mucosa, extending published findings[30,31], and that it is proteolytically processed after secretion. Together, our biochemical, inhibition profile and localization data provide evidence that Paneth cell–derived trypsin is the protease responsible for the processing of HD5 *in vivo*. The storage of HD5 exclusively as a propeptide, and the colocalization with its processing enzyme, indicates that the enzymatic activity of Paneth cell trypsin is tightly regulated. We envisage several complementary mechanisms achieve this control. First, we observed that Paneth cell trypsin, like the pancreatic counterparts, is stored as an inactive zymogen (trypsinogen) and recovered the processed active form from ileal lumen (unpublished observations). This indicates that zymogen activation occurs either during or after secretion. Second, Paneth cells make abundant quantities of serine protease inhibitors[38,39]. We showed that the serpin α_1-AP is found in human Paneth cells secretory granules, and the Kazal-type trypsin inhibitor, PSTI, is similarly expressed in Paneth cells[39]. This suggests that there is an intracellular balance between trypsin and its inhibitors and that the enzymatic activity is liberated after extracellular release and dilution of its inhibitors, a regulatory theme also inherent to pancreatic trypsin. We speculate that activation of Paneth cell trypsin may be mediated by a number of processes, including the action of host-derived enteroprotease-like enzymes, the activity of microbially derived proteases, or autoactivation characteristic of trypsin-like serine proteases that may be related to changes in ionic composition or pH changes accompanying lumenal release.

Paneth cell prodefensin processing seems to be vital to intestinal immunity. Mice deficient in the α-defensin processing enzyme do not produce mature α-defensins (cryptdins) and are highly susceptible to oral challenges with *S. typhimurium*[33]. However, the processing pathways of enteric α-defensins do not appear to be fully conserved between species. The protease responsible for cleavage of murine prodefensins in Paneth cells is the MMP matrilysin[25,33,40]. Analysis of 23 human (inflamed and uninflamed) ileal samples by RNA blot analysis, under experimental conditions that readily permitted detection of the enzyme in mouse intestinal tissues, did not detect homologous human MMP-7 (data not shown). Also, immunoblots of these human samples did not detect this enzyme (data not shown). Although MMP-7 can cleave rproHD5 *in vitro* upon overnight incubation and generates a processed peptide that was chemically characterized as HD5(54–94) (data not shown), this 41–amino acid peptide was never detected in any of our tissue or lumenal samples. Thus, mice and humans use different enzymes to cleave their enteric α-defensin peptides.

The processing of proHD5 also differs from that of the human α-defensins proHNP1, proHNP2 and proHNP3 in neutrophil precursors: the predominant mature forms of HNP1, HNP2 and HNP3 are not directly generated by trypsin or trypsin-like enzymes and their processing takes place intracellularly during granulogenesis[28–29]. Although new to the defensin family, extracellular proteolytic processing of host defense peptides from NH_2-terminally extended precursors is emerging as a common theme in innate immunity. Cathelicidins are a second family of abundant antimicrobial peptides in mammals[41,42]. In these peptides, the active antimicrobial peptide resides at the COOH-terminus of 70–amino acid cathepsin L inhibitor (cathelin)-like domain. Neutrophil serine proteases cleave the larger precursor polypeptide in the extracellular milieu to release and activate the antimicrobial peptide[43–45]. In lower vertebrates and insects, similar examples of extracellular proteolytic activation of antimicrobial polypeptides have been described[1,46–48], which suggests a conserved strategy in host defense.

We determined here the primary structure of HD5 from the intestinal lumen and found that it is a potent antibacterial agent against both Gram-positive and Gram-negative test bacteria. Although we found that its antimicrobial activity is enhanced by proteolytic processing, unmodified proHD5 also has antimicrobial activity, especially when tested against Gram-positive *L. monocytogenes*. The differential

antibacterial activity between the HD5 forms that exist *in vivo* suggests that proteolytic processing could be a mechanism that diversifies the spectrum of antibiotic activity from a single antimicrobial gene product. Consistent with this idea, the NH_2-terminal sequence has marked effects on the antimicrobial activity of some mouse cryptdins[49]. Also, many defensins have biological activities in addition to their antibiotic activity[22], and the NH_2-terminal sequence can markedly affect that activity. For example, HNP1 and HNP3, which have an identical primary sequence except for a single residue at the NH_2-terminus, have markedly different potency in their ability to serve as chemoattractants for lymphocytes[50]. It will be interesting to determine whether the proteolytically processed fragments of HD5 could modulate such additional activities as well.

Trypsin is among the best characterized serine proteases. Three distinct isoforms of trypsin have been identified in human tissues and are commonly referred to as cationic, anionic and mesotrypsin[37]. The genes encoding cationic and anionic trypsin are on chromosome 7 and both are embedded within the T cell receptor gene cluster[51]. In contrast, the gene for mesotrypsin is found on chromosome 9[51]. Pancreatic trypsin, which we confirmed consists of a mixture of all three isoforms[37], is involved in the activation of digestive proteinases and breakdown of ingested dietary proteins. Our studies suggest that Paneth cells express anionic and meso isoforms of trypsin, but not the cationic isoform. The presumed charge characteristics of Paneth cell trypsin may facilitate its interactions with its cationic substrate, proHD5.

Other reports of extrapancreatic expression of trypsin highlight other nondigestive functions of this enzyme[52–54]. For example, trypsin executes signaling functions through interaction with protease-activated receptor 2 (PAR-2)[55–56], which is expressed on the lumenal surface of enterocytes lining the small intestinal crypts and villi[57] as well as in other cell types[58,59]. The extracellular NH_2-terminal domain of PARs contain a proteolytic cleavage site for their cognate activating protease and, upon cleavage, a latent tethered ligand is unmasked, enabling it to activate the signal-transducing domains of the receptor[55–56]. Signaling through PAR-2 has been linked to the activation of both stress-activated protein kinases and inhibitory κB kinases, with a likely role in inflammatory responses[60,61].

We envision that the release of granules from Paneth cells is followed by the activation of trypsinogen to trypsin, the cleavage of prodefensin to defensin and the release of self-protective protease inhibitors that terminate further proteolysis. The key to the sequential coordination of these events may lie in the physical structure and chemical composition of Paneth cell granules as well as the changes in pH and ionic composition take place during degranulation. By acting as a prodefensin convertase, and perhaps through signaling effects, trypsin released from Paneth cells after microbial or inflammatory stimuli may be central to the activation of innate immune responses in the human small intestine.

Methods

Recombinant HD5 peptides. HD5 was biosynthesized in Hi5 insect cells infected with baculovirus carrying cDNA for preproHD5, as described[17]. To obtain rproHD5, cell culture supernatants were collected 56 h after infection. During prolonged incubation, further processing of HD5 occurred and culture supernatants that contained the predominantly processed HD5, rHD5(56–94), were collected 72 h after infection. Cell culture supernatants were supplemented with 2 mM phenylmethylsulfonyl fluoride (PMSF) and 5 mM EDTA, centrifuged at 500*g* for 5 min. The resulting supernatants admixed with the cation-exchange matrix CM-Macroprep (BioRad, Richmond, CA) equilibrated in 25 mM ammonium acetate (pH 6.4) at a supernatant:matrix ratio of 10:1. After overnight incubation at 4 °C with constant agitation, the matrix with the bound cationic peptides was separated by centrifugation at 500*g* for 10 min and washed three times for 5 min at room temperature in 25 mM ammonium acetate (pH 6.4). Cationic peptides were then batch-eluted once with two matrix volumes of 10% acetic acid and twice with five matrix volumes of 5% acetic acid for 30 min at 4 °C, both with constant agitation. Acetic acid eluates were pooled and subjected to C18 RP-HPLC with an acetonitrile gradient and 0.3% trifluoroacetic acid (TFA) as pairing agent, as described[30]. rHD5(63–94) was prepared by trypsin cleavage of rproHD5, as described below.

Tissue specimens. Biopsy and lumenal ileum specimens were from healthy individuals who underwent colonoscopy for screening of colon polyps. Patients with inflammatory or neoplastic processes of the colon were excluded. Colonoscopy was done by a gastroenterologist using a CF-100T video colonoscope (Olympus America, Melville, NY). The Cleveland Clinic Foundation Institutional Review Board approved these experiments. Endoscopic biopsy samples, taken from patients who underwent clinically indicated colonoscopy and who gave informed consent, followed protocol 3672. The terminal ileum was intubated if there were no pathological findings for the entire colon. Biopsies of ileum mucosa were obtained with BARD-coated disposable biopsy forceps (BARD Endoscopic Technologies, Billerica, MA). Sterile water (20 ml) was flushed into the intestinal lumen of the ileum through the biopsy channel of the colonoscope. The water was then aspirated into a collection bottle. One ileal biopsy specimen was fixed in 4% (w/v) paraformaldehyde for routine histological and immunofluorescent examination. The other biopsy specimen and the ileal wash were immediately frozen with liquid nitrogen and then stored at –80 °C until further analysis.

Isolation of HD5 from intestinal tissue. Redundant, surgically resected human small intestinal tissues (IRB Protocol EX0078) were washed with Hank's buffer. The mucosa was removed by resection[62], snap frozen in liquid nitrogen and stored at –80 °C. Approximately 180 mg of mucosa was isolated from 1 cm^2 of ileal tissue by this technique. A portion of all specimens was also processed for routine histological evaluation. Ileal tissue samples with normal histology, which were resected from patients with colon cancer or intestinal obstruction, were used. Tissue specimens were homogenized with a Brinkmann Polytron homogenizer in ice-cold 20% aqueous acetic acid (1:20 w/v) that contained 1:100 (v/v) Protease Inhibitor Cocktail III, which was composed of 100 mM 4-(2-aminoethyl)benzenesulfonylfluoride HCl, 80 μM aprotinin, 1.5 mM bestatin, 1.5 mM E64, 1 mM pepstatin A and 2 mM leupeptin hemisulfate (Calbiochem, La Jolla, CA). The extracts were then sonicated (Heat Systems-Ultrasonics, Plainview, NY) for 1 min on ice and was left stirring overnight at 4 °C. Biopsy specimens (3–7 mg wet weight) were placed in ice-cold 20% acetic acid (1:10 w/v) that contained the same protease inhibitor mixture, sonicated (3×5-s bursts) in a Branson Sonifier 450 with a double-tipped microtip (Branson Ultrasonics, Danbury, CT) and then extracted overnight. The next day, all extracts were centrifuged at 19,800*g* for 30 min at 4 °C, passed through Mirah Cloth (Calbiochem) and then ultracentrifuged at 110,000*g* for 30 min at 4 °C. The clarified extract was analyzed for total protein by the Bradford method (BioRad), snap frozen and stored at –80 °C. For isolation of tissue HD5, the extract was thawed on ice, a fresh aliquot of protease inhibitors was added and the pH was adjusted to 6.0 with ammonium hydroxide. The precipitates that formed were removed by centrifugation (19,800*g* for 30 min at 4 °C), and the supernatant was then dialyzed against 5 mM ammonium acetate (pH 6.0) overnight in 1-kD dialysis bags (Spectra/Por, Spectrum, Rancho Dominguez, CA). The sample was reduced to 1/4 volume with a Speed Vac (Savant, Molbrook, NY). The resulting sample was loaded on a 4.6×200 mm Poly Cat A weak cation exchange column (Poly LC, Columbia, MD) that was equilibrated in 20 mM ammonium acetate buffer (pH 6). Cationic proteins and peptides were eluted on a 0–40% linear gradient of acetic acid (1 ml/min) over 160 min. HD5 elution was analyzed by dot-blot immunoreactivity with the anti-HD5 polyclonal sera[30]. The fractions of interest were purified with a 2.1×250 mm C18 RP-HPLC column (Vydac, Hesperia, CA) with a linear 5–80% acetonitrile gradient in 0.1% TFA.

Mass spectrometry and Edman degradation. Immunopositive peaks were analyzed by MALDI-TOF mass spectrometry. Mass analysis was done with a Voyager DE Pro Biospectrometry Workstation (Applied Biosystems, Foster City, CA) equipped with a nitrogen laser (337 nm). It was operated in the delayed extraction and linear mode with a matrix of 7 mg/ml of 3,5-dimethoxy-4-hydroxycinnamic acid (Acros Organics, Lane Fairlawn, NJ) in acetonitrile/water/0.3% trifluoroacetic acid, 3:6:1 (v/v/v). Internal standards were used for calibration; they included insulin ($(M+H)^+$ 5734.59) and thioredoxin ($(M+H)^+$ 11674.47). Samples of interest were blotted on Immobilon PS-Q PVDF (Millipore, Bedford MA) and subjected to NH_2-terminal sequencing with a Procise Model 492 Microsequencer (Applied Biosystems) in the LRI Biotechnology Core Facility.

Isolation of the secreted form of HD5 from small intestinal lumen. Ileal lumenal aspirates from healthy small intestines were thawed after storage at –80 °C, acidified with acetic acid to a final concentration of 20% (v/v), thoroughly mixed by vortex and kept on ice for 30 min. A fresh aliquot of the protease inhibitor mixture was added, the pH was adjusted to 6.0 with ammonium hydroxide and the precipitates were removed by centrifugation (19,800*g*). The supernatant was diluted 1:15 (v/v) with 5 mM ammonium acetate (pH 6.0) and the resulting supernatant was admixed with 1 ml of CM-Macroprep resin equilibrated in the same ammonium acetate buffer. After overnight incubation on a roller bottle platform at 4 °C, the resin was precipitated by centrifugation (800*g*) and then washed with 5 mM ammonium acetate (pH 6.0). Cationic peptides were batch-eluted with the addition of two matrix volumes of 20% acetic acid. The resulting eluate was analyzed by immunoblotting and purified by RP-HPLC. The fractions of interest were analyzed by AU-PAGE immunoblots, MALDI-TOF mass spectroscopy and NH_2-terminal sequence determination.

***In vitro* processing of rproHD5 by trypsin.** rproHD5 and rHD5(56–94) were incubated with bovine pancreatic trypsin (Pierce, Rockford, IL) at a 2:1 molar ratio (substrate:enzyme) in 10 mM sodium phosphate buffer with 100 mM NaCl (pH 8.0) at 37 °C for 2 h. The reaction was stopped by the addition of 1 volume 5% acetic acid. Samples were dialyzed against 2% acetic acid, lyophilized, resuspended in 5% acetic acid and a portion was analyzed by AU-PAGE. The HD5 reaction product was purified with RP-HPLC and subsequently used for antimicrobial assays and as a positive control immunoblot analysis. In other experiments, rproHD5 was treated with human pancreatic trypsin (Calbiochem) at a 300:1 substrate:enzyme molar ratio in the absence or presence of equimolar (inhibitor:enzyme) amounts of α1-AP (Athens Research, Athens, GA) at 37 °C for 5 min and subsequently analyzed. For isolation of trypsin from human ileal tissue, ileal mucosa was extracted in 50 mM Tris-HCl (pH 8.0), 100 mM NaCl and 50 mM benzamidine with a modified protocol[54]. The extract was clarified by centrifugation (19,800*g*) and concentrated with an Amicon 10-kD ultrafiltration unit with 10 mM Tris-HCl (pH 8.0) and 4 mM benzamidine (buffer B). The retentate was admixed with a Sepharose Q (Amersham Pharmacia, Piscataway, NJ) matrix equilibrated in buffer B and stirred for 2 h at 4 °C. The matrix was washed once with buffer B and anionic proteins were eluted with buffer B supplemented with 500 mM NaCl. The eluate was again subjected to ultrafiltration in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl and 1 mM $CaCl_2$ with an Amicon 10-kD ultrafiltration unit at 4 °C. Total protein in the retentate was estimated by the Bradford method, and trypsin immunoreactivity was analyzed by immunoblot analysis with a polyclonal rabbit antibody to trypsin (Athens Research). Purified HD5 propeptides from human ileal tissue were added to the retentate in a 1:1 HD5:total retentate proteins ratio and the mixture was incubated at 37 °C for 10 min. The reaction was stopped by the addition of p-amidinophenylmethylsulfonylfluoride-HCl (APMSF) at a final concentration of 50 μM. For protease inhibition experiments, human ileal tissue was extracted in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl and 1 mM $CaCl_2$, and aliquots were immediately treated with protease inhibitor cocktail III (1:100 v/v), 50 μM APMSF, 2 mM pepstatin, 20 mM EDTA or 2 mM E64 or with no inhibitors. The aliquots were then incubated at 37 °C for 30 min and the reaction products assessed by AU-PAGE immunoblot analysis.

Quantitative AU-PAGE immunoblot analysis. AU-PAGE immunoblotting was done with the protocol described elsewhere[30] with minor modifications. Multiple defined amounts of clarified tissue extract(s) or CM batch eluants from human lumenal aspirates were analyzed on AU-PAGE gels composed of 12.5% acrylamide with 2% (w/v) bis-acrylamide as cross-linker, 8 M urea and 5% (v/v) acetic acid. rHD5(64–94) and rproHD5 were used as positive controls (20 ng/lane respectively). After electrophoresis, proteins were transferred to an Immobilon PS-Q membrane in 5% acetic acid with a semidry apparatus (Fisher Scientific, Pittsburgh PA) at 1.5 mA/cm^2 for 30 min. The membrane was fixed with 0.01% glutaraldehyde (in PBS) for 20 min, blocked in 5% nonfat milk and probed with an HD5 rabbit polyclonal antibody (1:10, 000)[17]. The blots were processed with West-Pico Chemiluminescence reagent (Pierce). Proteins were quantified by the Bradford method.

SDS tricine immunoblot analysis. Electrophoresis was done with 12.5–20% SDS tricine gels, with 2% (w/v) bis-acrylamide as a cross-linker, under nonreducing conditions[63]. After electrophoresis, the gels were transferred on an Immobilon PS-Q membrane with a semidry apparatus (Fisher) at 0.8 mA/cm^2 for 20 min; the membranes were then analyzed as above.

Antimicrobial assays. The CFU assay was similar to that described[37]. Briefly, various HD5 and HNP1 forms were adjusted to 33 μM in 0.01% acetic acid. To verify the correct concentrations, aliquots were subjected to Coomassie blue–stained AU-PAGE and band intensity was compared to serial dilutions of known peptide standards. *L. monocytogenes* and wild-type *S. typhimurium* strain 14028s were adjusted visually to McFarland standard 0.5 (~10^8 *L. monocytogenes* per ml and 5×10^7 *S. typhimurium* per ml) and further diluted to 2×10^6 CFU/ml in 10 mM sodium phosphate buffer (pH 7.4) supplemented with 0.03% (w/v) Tryptic Soy Broth powder (Difco, Detroit, MI). The bacterial suspension (90 μl) was admixed with 10 μl of defensin stock solution (33 μM) or buffer only, as a control. At t_0 (control only) and t_2 (after 2 h incubation at 37 °C) samples were placed on ice, diluted 100-fold in assay buffer, plated on TSA plates with a spiral plater and incubated for 18 h at 37 °C. Colonies were counted and the CFU/ml calculated.

Immunohistochemistry of human small intestinal tissue. Ileal tissue samples were fixed overnight in 4% (w/v) paraformaldehyde, dehydrated in a graded alcohol series and paraffin-embedded. Sections (10 μm) were deparaffinated, rehydrated and treated with 20 μM APMSF in PBS for 30 min. The sections were then treated with the Glyca Antigen Retrieval System (Biogenex, San Ramon, CA) in a microwave pressure cooker for 30 min according to the manufacturer's protocol. The slides were transferred to PBS that contained 20 μM APMSF and 1:100 (v/v) Protease Inhibitor Cocktail Set III for 5 min at room temperature. The tissue sections were blocked with 0.15% horse serum in PBS for 20 min. Anti-HD5 rabbit polyclonal IgG (1:20k), anti–α1-AP (1:5k) (Sigma, St. Louis, MO) and pooled monoclonal anti–trypsin IgG (1:6k) (Chemicon International, CA and QED Bioscience, CA) were used as primary antibodies and were incubated overnight at 4 °C. The tissue sections were then washed in PBS and treated either with Alexa Fluor 568–conjugated anti-rabbit or Alexa Fluor 488–conjugated anti-rabbit (Molecular Probes, Eugene, OR). Trypsin staining was done by treating the sections with biotin–anti-mouse (Vector Laboratories, Burlingame, CA) followed by Alexa Fluor 568–streptavidin (Molecular Probes). Parallel histological sections were stained with hematoxylin and eosin. Sections for confocal microscopy were mounted with Vectashield with DAPI (Vector Laboratories) and the edges of the coverslips were sealed with clear nail polish. Confocal images were obtained with a Leica TCS-SP spectral laser scanning confocal microscope (Leica Microsystems GmbH, Heidelberg, Germany) with HCX Plan Apo ×10, 1.32 NA and HCX Plan Apo ×63 1.32 NA objectives. The specimens were excited with separate Argon lasers at 364 nm (ultraviolet) for DAPI, 488 nm for Alexa Fluor 488 and with a Krypton laser at 568 nm for Alexa Fluor 568. The emitted fluorescence from each of the three probes was detected with three separate photomultiplier detectors. Images were collected sequentially at each level of the specimen to prevent cross-talk between the fluorophores.

Northern blot analysis of human small intestinal samples. Total RNA was isolated from healthy human small intestinal epithelium and prepared for northern blot analysis as described[24]. The oligonucleotide hybridization probes (Biosource international, Camarillo, CA) were as follows. Trypsin (hTryp-1a) 5′-GTTGTAGACCTTGGTGTAGACTCCAGGC TTGTTCTTC-3′; HD5 (HSIA-309a) 5′-TGCTTTGGTTTCTATCTAGGAAGCTCAGCGA CAGCAGAGTCTGTAGAG-3′; α-1 antiprotease (ha1AT-1a) 5′-CATTTTCCAGGTGCTG TAGTTTCCCCTCATCAGGTAG-3′; pancreatic secretory trypsin inhibitor (hPSTI-2a) 5′-AACACGCATTCATTGGGATAAGTATTTCCATCAGTCC-3′. A glyceraldehyde-3-phosphate dehydrogenase probe (G3PDH-1a) 5′-AGCCCCRGCCTTCTCCATGGTRGTGAA GACVCCR-3′ was used to assess RNA levels and integrity. All probes were end-labeled to a specific activity of ~10^7 DPM/pmol with 3000 Ci/mmol γ-[^{32}P]ATP (DuPont, Wilmington, DE) and T4 polynucleotide kinase (Roche, Mannheim, Germany)[64]. Labeled probes were hybridized overnight to immobilized RNA in 35% (v/v) formamide, 5× SSC, 5% Denhardt's solution and 1% (w/v) SDS at 42 °C and then washed at high stringency in 2× SSC and 0.1% SDS at 55 °C for 30 min[64]. The washed filters were exposed to film with an intensifying screen at –80 °C for 3 days. After each analysis, the filter was stripped of oligonucleotide label by incubation in 0.1× SSC and 0.1% SDS at 70 °C for 30 min and exposed to film to ensure the probe had been removed before hybridization with another probe.

Analysis of trypsin mRNA in ileal mucosa. Total RNA from adult human small intestinal ileum was isolated, and RNA from human pancreas was obtained from a commercial source (Clontech, Palo Alto, CA). Single-strand cDNA synthesis with these RNA species as templates used a modified oligo-dT primer (TTCTAGAATTCAGCGGCCGC$(T)_{30}$VN, Marathon cDNA synthesis primer, Clontech), according to the supplier's recommended modification of published methods[65]. The resulting cDNA product was used as a template in a PCR (34 cycles, 94 °C for 25 s, 58 °C for 30 s and 72 °C for 1 min; 0.2 μM primer concentration; 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$ and 0.2 μM dNTP) with oligonucleotide primers hTryp-2s (5′-GTGAGACTGGGAGAGCACAACA-3′) and hTryp-3A (5′-CACTTTATTGGTATAGAGACTG-3′), whose sequences are identical to corresponding sequences of cationic, anionic and mesotrypsin. The DNA products were recovered by chromatography (Qiagen, Valencia, CA). A single PCR product of expected size (0.6 kb) was identified in all reactions and was blotted onto a nylon membrane. Plasmid DNA containing cDNA of cationic, anionic and mesotrypsin as hybridization controls was also blotted onto the membrane. Equal loading of each control plasmid was checked by ethidium bromide staining. The membrane was sequentially hybridized with ^{32}P-labeled oligonucleotide probes under high stringency conditions, and signal intensity was quantified with phosphorimager analysis. The filter hybridization and wash conditions with hTryp-1a—whose sequence was identical to the corresponding sequences in all three trypsin isoforms (common probe)—were hybridization, 35% (v/v) formamide, 5× SSC, 1× Denhardt's solution, 1% SDS and 100 μg/ml of yeast RNA at 42 °C; wash, 2× SSC/0.1% SDS at room temperature for 1 h and in 2× SSC/0.1% SDS at 55 °C for 30 min. This probe normalized the total content of trypsin sequence in each sample. For specific detection of cationic trypsin, the probe hTryp1-4a (5′-TCAGAGTCTTCCTGTCGTATTG-3′) was used under identical conditions except that the formamide concentration was 33% and the final wash was at 53 °C. For anionic trypsin, the probe hTryp2-14a (5′-CCAGAGTCCGGCT GTTGTATTTG-3′) was used under identical conditions except that the formamide concentration was 37.5% and the final wash was at 58 °C. For mesotrypsin, the probe hTryp4-13a (5′-TACACTCAGCCTGGGTCAGCACC-3′) was used under identical conditions except that the formamide concentration was increased to 45% and the final wash was at 62 °C. The washed filters were exposed to film for a time that was sufficient to yield comparable signals in the positive control. Signal intensities were quantified by Phosphorimager analysis (Molecular Dynamics, Sunnyvale, CA). The relative signal intensity for each specific trypsin probe was calculated by normalizing to control plasmid and the control probe hTryp-1a. The filter was stripped of residual probe after each hybridization experiment by washing in 0.5 M NaOH and 1.5 M NaCl at room temperature for 30 min. Efficient stripping of the probe was documented before subsequent probe hybridization.

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Competing interests statement

The authors declare that they have no competing financial interests.

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Paneth cell trypsin is the processing enzyme for human defensin-5

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News & Views

Trypsin, for the defense

Michael Zasloff

Nature Immunology. 3 : 508-510

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The α-defensins from Paneth cells in intestinal crypts need processing to be fully functional. Unlike for mice, the cleaving enzyme for human HD5 turns out to be trypsin.

The human small intestine would seem to pose something of a challenge for our immune system. It is lined by a single epithelial layer with an enormous surface area that consists of villi, microvilli and crypts. Microbes enter with food and during digestion abrasions must occur; both these wounds and the delicate surface must be protected. The lining cells are replaced every 2–3 days from stem cells that lie within the crypts, which are perfect hiding places for microbes. The lumen is filled with warm, concentrated and digested nutrients. Yet the normal surfaces of the small bowel show little sign of inflammation. Furthermore, the density of microbes in the human small bowel is remarkably sparse, considering the environment. In this issue of *Nature Immunology*, Ghosh *et al.* report that trypsin—a well studied digestive enzyme—contributes to the innate defense of crypts against bacterial outgrowth and therefore helps maintain the epithelial integrity of the intestine and preserve stem cell viability[1].

Antimicrobial peptides help to defend the epithelial barrier against microbial assault and play a role in the control of microbial densities in the gut lumen[2]. They are expressed both by enterocytes and by the specialized granule-laden cells, called Paneth cells, that lie at the base of the crypts[3]. The enterocytes express peptides both constitutively and upon induction by bacteria or cytokines[4]. The peptides

are secreted onto the lumenal surface or are retained in a cell-associated manner. The Paneth cells secrete the contents of their granules into the base of the cryptal well, filling the crypt with high concentrations of antimicrobial peptides, which are subsequently flushed into the lumen. The secretion within the crypt provides antimicrobial protection for stem cells of the gut epithelial layer, which lie within the crypt. As expected for components of host defense, Paneth cells degranulate upon exposure to heat-killed or live bacteria or to microbial products such as lipoteichoic acid and lipopolysaccharide[5] (**Fig. 1**). Transgenic mice that overexpress human α-defensin-5 (HD5), a peptide that is more active against *Salmonella typhimurium* than the murine defensins, in the Paneth cells are dramatically less sensitive to oral *S. typhimurium* challenge[6] (N. H. Salzman, D. Ghosh, K. M. Huttner & C. L. Bevins, personal communication). For an organism to cause infectious disease by entering the body through the gastrointestinal tract, it must escape the challenge of passage through the defenses mounted by the Paneth cells.

The Paneth cells store and secrete several antimicrobial substances, including secretory phospholipase 2, lysozyme and defensins, the latter being the most abundant of the proteins produced by this cell[3,6]. The Paneth cell defensins—like those expressed by phagocytic cells—are of the α family, based on the spatial distribution of the three intramolecular disulfide bond linkages that characterize the mammalian defensin family. In the early 1980s α-defensins were discovered in phagocytic leukocytes and four (HNP1–HNP4) are now known to be present in the primary granules of neutrophils and are responsible for the neutrophil's nonoxidative killing activity[7]. Some years later, α-defensins were found to be abundantly expressed in the Paneth cells of the mouse intestine (where they are called cryptdins) and the human small bowel (as HD5 and HD6)[3]. Within the white blood cell, the defensins are stored as processed mature species in granules, which deliver their contents upon fusion with a phagolysosome. The human Paneth cell defensins, on the other hand, appear to be stored as precursor species, requiring processing after secretion. The discoveries that, in mice, the metalloproteinase (MMP) matrilysin is required for Paneth cell defensin processing and that mice deficient in this enzyme lack functional cryptdins and are highly susceptible to orally administered *Salmonella* highlighted the importance of this processing step in the production of functional Paneth cell defensins[8]. It also confirmed the importance of these peptides in defense against gut associated pathogens[8]. Humans, however, do not make matrilysin in their small intestines, so another proteolytic enzyme must be responsible for maturation of the human Paneth cell defensin from its stored precursor.

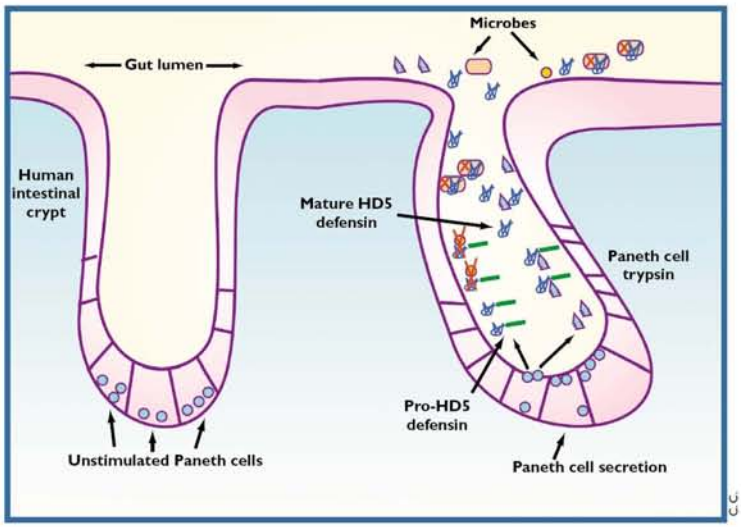

Figure 1. Proteolytic maturation of the human Paneth cell antimicrobial peptide HD5. Two intestinal crypts are shown: one in an unstimulated state, the other provoked by the presence of bacteria. HD5 is stored as a precursor in the granules of the Paneth cell (proHD5), along with trypsinogen. Exocytosis—induced by stimuli such as bacteria or cholinergic agonists—is followed by cleavage of the secreted proHD5 to mature HD5 within the crypt lumen by the action of trypsin. Although both precursor and mature defensin peptides are antibiotic, they differ in their spectrum of activity.

To identify the enzyme involved in processing HD5, Ghosh *et al.* extracted human ileal biopsy samples, purified the stored form of HD5 and determined its sequence[1]. The most abundant peptide was a signal peptide–free propeptide (corresponding to amino acids (aa) 20–94 of the prepropeptide); it consists of a 43-aa NH_2-terminal leader attached to the mature peptide[1] (**Fig. 1**). The lumenal species they found were exclusively mature, mostly HD5(63–94) with some HD5(56–94). Both the propeptide and the mature species exhibited antimicrobial activity *in vitro*, differing somewhat in potency against *Listeria monocytogenes* and *S. typhimurium*. Analysis of the NH_2-terminal residues of the processed, mature, lumenal forms suggested that a trypsin-like enzyme might be responsible. Indeed, cleavage of recombinant proHD5 with trypsin yielded mature HD5. Ghosh *et al.* then showed trypsin activity and mRNA could be found in intestinal extracts. Using immunohistochemistry, they found that trypsin was colocalized with HD5 in Paneth cells within secretory granules. Pancreatic secretory trypsin inhibitor and the serpin α1-AP were also present in the Paneth cells[1]. Surprisingly, unlike in the pancreas, only anionic and mesotrypsin were expressed in the terminal ileum, suggesting that Paneth cells express a distinct trypsin isoform pattern[1]

The storage of HD5 as a propeptide and its colocalization with its processing enzyme requires tight control over trypsin's activity. Ghosh *et al.* note that Paneth cell trypsin is stored as the inactive zymogen, suggesting that trypsin activation occurs either during or after secretion[1]. The storage of several protease inhibitors within the same cells suggests that the activity is carefully controlled. Just how trypsinogen is activated is unknown, but the possible roles for novel enteroproteases or even microbial-derived proteases, as suggested by Ghosh *et al.*[1], are intriguing. It is surprising, then, that the enzymes used in Paneth cell defensin processing are not conserved between mice and humans. However, efforts on the part of Ghosh *et al.* to detect intestinal MMP-7—the human analog of mouse matrilysin—or an equivalent activity failed. In addition, MMP-7 digestion of proHD5 does not yield mature HD5[1].

The role played by trypsin in the processing of the Paneth cell defensin has a striking parallel to another recently discovered scenario that takes place in the gastric mucosa of most vertebrates, including humans, pigs and cows. The cells of the gastric glands accumulate histone 2A in cytoplasmic granules. Upon secretion, histone 2A is processed by cosecreted pepsin A to the potent antimicrobial peptide buforin I, which comprises the 39-aa NH_2-terminus of histone 2A. Buforin I remains adherent to the mucous layer coating the stomach wall, providing a powerful antimicrobial barrier[9]. In

addition, the expression of trypsin by the Paneth cell highlights new functions for this well studied digestive enzyme. Of particular relevance is the observation that the lumenal surfaces of enterocytes of the human intestinal crypts and villi express protease-activated receptor 2 (PAR-2), a G protein–coupled seven membrane–spanning protein. Upon trypsin cleavage, PAR-2 releases a latent, tethered ligand, generating inositol-1,4,5-triphosphate, arachidonic acid release and the secretion of prostaglandins E_2 and $F_{1\alpha}$, which can affect numerous gut functions[10]. Thus, not only does trypsin act as a prodefensin convertase in the setting of the intestinal crypt, but also it likely initiates specific patterns of gene expression in the neighboring enterocytes. How these two trypsin-dependent events interrelate within the context of the small intestine remains to be determined.

The issues raised by the findings of Ghosh *et al.* are many. Further research is needed to determine why mice and humans use a different enzyme for Paneth cell defensin processing. The possibility exists that some diseases in humans could be associated with defects in defensin processing due to impaired trypsin expression or activation. In addition, trypsin variants could be associated with bowel dysfunction. Other questions that remain unanswered include how the system evolves with development, how it behaves in states of inflammatory bowel disease or intestinal infection, what role the cosecreted trypsin inhibitors play in this pathway and what are the possible consequences of disturbances in their expression.

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Georgetown University, Medical Center, Med-Dent Building 103 NW, 3900 Reservoir Road NW, Washington, DC 20007, USA. (maz5@georgetown.edu)