Pepsin-Mediated Processing of the Cytoplasmic Histone H2A to Strong Antimicrobial Peptide Buforin I[1]

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The intestinal epithelium forms a first line of innate host defense by secretion of proteins with antimicrobial activity against microbial infection. Despite the extensive studies on the antimicrobial host defense in many gastrointestinal tracts, little is known about the antimicrobial defense system of the stomach. The potent antimicrobial peptide buforin I, consisting of 39 aa, was isolated recently from the stomach tissue of an Asian toad, *Bufo bufo gargarizans*. In this study we examined the mechanism of buforin I production in toad stomach tissue. Buforin I is produced by the action of pepsin isozymes, named pepsin Ca and Cb, cleaving the Tyr[39]-Ala[40] bond of histone H2A. Immunohistochemical analysis revealed that buforin I is present extracellularly on the mucosal surface, and unacetylated histone H2A, a precursor of buforin I, is localized in the cytoplasm of gastric gland cells. Furthermore, Western blot analysis showed that buforin I is also present in the gastric fluids, and immunoelectron microscopy detected localization of the unacetylated histone H2A in the cytoplasmic granules of gastric gland cells. The distinct subcellular distribution of the unacetylated histone H2A and the detection of the unacetylated buforin I both on the mucosal surface and in the lumen suggest that buforin I is produced from the cytoplasmic unacetylated histone H2A secreted into the gastric lumen and subsequently processed by pepsins. Our results indicate that buforin I along with pepsins in the vertebrate stomach may contribute to the innate host defense of the stomach against invading microorganisms. *The Journal of Immunology*, **2000, 165: 3268–3274.**

Vertebrates have natural defense mechanisms that protect against invading microorganisms that can easily penetrate the epithelia of the respiratory, gastrointestinal, and genitourinary tract (1). Most of the exposure to bacteria occurs in the gastrointestinal tract. Because most mucosal surfaces do not normally contain abundant phagocytic cells, surface epithelial cells are of critical importance in mediating the innate protection of the host from microbial infection. This requires a pre-existing or rapidly responsive antimicrobial defense against microbial invasion. The role of gastric acid and peristaltic activity has long been recognized in the maintenance of relative sterility of the gastrointestinal tract (2, 3). However, recent studies have shown that enteric bacteria are able to survive the acidic conditions encountered in the stomach (4, 5), suggesting that the present concept of the role of gastric acid in the bacterial clearance is not sufficient and needs to be modified. Antimicrobial peptides are important components of the natural defenses of most living organisms against microbial infection (6). Antimicrobial peptides play an important role in mucosal defense and are often found in high concentrations on damaged mucosal surfaces, including the tongue, trachea, and intestine (7). The expression of individual members of gene families that encode antimicrobial peptides is tissue specific. Antimicrobial peptides can function intracellularly, as in circulating leukocytes or in the external environment after release by secretory cells and other granulated epithelia (8). Certain antimicrobial peptides have diverse functions beyond microbicidal activity, such as promotion of wound healing (9) and chemotaxis of dendritic and memory T cells (10).

Although peptide antimicrobials have been purified and characterized extensively, their biological functions and the nature of their post-translational processing are not yet well understood. The most studied antimicrobial peptides are cecropins (11), bactenecins (12), and defensins (13, 14). The delineation of biosynthetic and maturation processes of antimicrobial peptides may help to define more accurately their in vivo functions. Recently, a potent antimicrobial peptide consisting of 39 aa, termed buforin I, was isolated from the stomach of an Asian toad (15). Sequencing of the cDNA encoding buforin I revealed its precursor to be histone H2A (16). However, the protease that is responsible for the generation of buforin I from histone H2A has not been identified. Furthermore, the involvement of histone H2A as a precursor of potent antimicrobial peptide raises many interesting questions about the cellular processing mechanism of histone H2A in the innate host defense of vertebrate stomach. In this study we describe the mechanism for the generation of buforin I from histone H2A. We also show that buforin I constitutes an innate host defense system that is operative at the mucosal layer and lumen of the vertebrate gastrointestinal tract.

Materials and Methods

Materials

Calf thymus histone H2A, pepstatin A, leupeptin, and E-64 were purchased from Roche (Mannheim, Germany). PMSF, pepstatin A-Agarose, and porcine pepsin A (crystallized and prepared chromatographically) were obtained from Sigma (St. Louis, MO). To remove any contaminating pepsin

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C, pepsin A was subjected to chromatography on a Mono Q HR 5/5 (Pharmacia LKB, Uppsala, Sweden) column that had been equilibrated with pyridine-acetate buffer, pH 6.0. All other reagents used in this study were of analytical grade.

Assay for proteolytic activity

Crude extracts of toad stomachs were prepared as described by Kim et al. (16). Proteolytic cleavage of histone H2A was examined by incubating calf thymus histone H2A (10 μg) with crude stomach extracts (5 μg) in reaction buffers ranging in pH from 1.0 to 11.0 (total reaction volume, 50 μl). After incubation for 1 h at room temperature, the reactions were stopped by boiling for 5 min and were analyzed by tricine SDS-PAGE. For protease inhibition assays, crude stomach extracts (5 μg) were preincubated for 1 h at room temperature with each of the following protease inhibitors: PMSF (10 mM), leupeptin (50 μM), pepstatin A (50 μM), EDTA (5 mM), and E-64 (50 μM). Histone H2A (10 μg) was then added, and the protease reactions were allowed to proceed for an additional hour (final reaction volume, 50 μl in reaction buffer).

Purification of specific proteases

Crude stomach extracts that contained ~50 mg of proteins (5 mg/ml) were acidified to pH 3.0 and loaded into a column of pepstatin A-Agarose, which had been equilibrated at 4°C with 50 mM sodium acetate (pH 3.0) containing 0.2 M NaCl. The column was washed with the same buffer at a flow rate of 0.2 ml/min until the absorbance at 280 nm fell to the baseline. Proteins of interest were then eluted at the same flow rate with 50 mM Tris-HCl (pH 8.5) containing 1 M NaCl. Fractions (1 ml) were collected and analyzed for proteolytic activity at pH 3.0 by tricine SDS-PAGE. Active fractions were concentrated and desalted using a Centricon microconcentrator (Amicon, Beverly, MA) with a 10-kDa molecular mass cut-off membrane. A Mono Q (HR 5/5) column (0.5 × 5.0 cm) from the Pharmacia fast protein liquid chromatography (FPLC)[3] system was equilibrated with 0.05 M pyridine-acetate buffer (pH 6.0), loaded with the pooled active fractions from the pepstatin A-Agarose step, and eluted with a gradient of 0–1.0 M ammonium acetate in a total volume of 50 ml of the starting buffer. Two separate components with buforin I-producing activity were isolated, and each component was further purified by a second pass over the Mono Q column. The purified proteases were concentrated to ~1 mg/ml using the Centricon microconcentrator. Protein concentration was determined using a bicinchoninic acid protein assay (Pierce, Rockford, IL) with BSA as a standard.

Purification of the peptide generated from histone H2A by specific proteases

Calf thymus histone H2A (100 μg) was incubated for 1 h at room temperature with each of the purified toad stomach proteases (3 μg/reaction) and porcine pepsin A in 100 μl of 50 mM sodium acetate (pH 3.0) containing 50 mM NaCl. Samples from the protease reactions were injected into a Waters HPLC system equipped with a C_{18} reversed-phase column (3.9 × 300 mm, Delta Pak, Millipore, Milford, MA). A linear gradient of 0–100% buffer B (buffer A, 0.1% trifluoroacetic acid (TFA) in H_2O; buffer B, 0.1% TFA in acetonitrile) was established over a period of 2 h at a flow rate of 1 ml/min. Fractions corresponding to each peak were lyophilized, resuspended in water, and analyzed by tricine SDS-PAGE.

Antimicrobial activity assay

The antimicrobial activity of HPLC fractions of protease-digested histone H2A was examined by the radial diffusion assay on a *Bacillus subtilis* lawn as described by Lehrer et al. (17).

Structural analyses

The m.w. of the processed peptide presumably corresponding to buforin I was determined by matrix-associated laser desorption ionization mass spectroscopy (Kratos Kompact MALDI, Manchester, U.K.). Lyophilized peptide (~1 nmol) was dissolved in 50% acetonitrile containing 7% (w/v) sinapinic acid and mixed with a platinum probe. After removing the solvent in warm air, the peptide that had been adsorbed to the platinum probe was applied to a vacuum chamber and analyzed. Peptides were hydrolyzed by incubation in evacuated tubes with 6 M HCl containing 0.1% phenol at 110°C for 24 h. The total composition of the peptide was determined using a Pico-tag analysis system on a Beckman 121 MB amino acid analyzer (Fullerton, CA). The N-terminal sequence analyses of the processed peptide and purified proteases were performed by automatic Edman degradation in a gas phase sequencer (Applied Biosystems, Foster City, CA).

Ab preparation

The mouse mAbs to histone H2A (BWA3) and histone H2B (LG2-2) were obtained from Monestier et al. (18). We purchased a rabbit polyclonal Ab to acetylated (Ac-Lys[5]) histone H2A (Serotec, Oxford, U.K.) and a mouse mAb to histone H1 (clone AE-4, Santa Cruz Biotechnology, Santa Cruz, CA). The Ab to buforin I was elicited in rabbits by injection of a synthetic peptide after conjugation via an added C-terminal cysteine residue to keyhole limpet hemocyanin (Pierce). The peptide used to generate the buforin I Ab was prepared on the basis of the following amino acid sequence: RAGLQFPVGRVHRLLRKG (aa 20–37 of histone H2A). This sequence includes the C-terminal 18 residues of buforin I. New Zealand White rabbits were immunized at 2-wk intervals with four s.c. injections of 200 μg of the peptide conjugate mixed with Freund's adjuvant (Sigma). The Ab directed against toad pepsin C was produced in rabbits by injection of 20 μg of purified toad pepsin C mixed with Freund's adjuvant. Ab specificity was determined by ELISA using the peptide as a competitor (Pierce).

Immunohistochemistry

Fresh toad stomach tissue was placed in 4% paraformaldehyde dissolved in PBS, incubated overnight at 4°C, and embedded in paraffin. Serial cross-sections (4 μm) of the embedded toad stomach were deparaffinized, treated with 0.3% hydrogen peroxide in methanol for 30 min, and washed extensively, first with water and then with PBS. Immunostaining was performed using a biotin-avidin-peroxidase method (Vectastain ABC Elite kit, Vector, Burlingame, CA) according to the manufacturer's protocol. Briefly, the sections were incubated with 1% normal blocking serum for 20 min at room temperature and then incubated for 1 h with the respective Abs: anti-unacetylated histone H2A (20 μg/ml), anti-buforin I (1/2000 dilution), anti-pepsin C (1/1000 dilution), anti-acetylated histone H2A (1/3000 dilution), anti-histone H1 (10 μg/ml), and anti-histone H2B (10 μg/ml). Sections were developed with chromogen diaminobenzidine for 10 min and then counterstained with Mayer's hematoxylin, dehydrated, and mounted. Reagents for the hematoxylin and eosin stain and the periodic acid-Schiff (PAS) stain were obtained from Poly Scientific (Bay Shore, NY). Control incubations were performed using equivalent dilutions of rabbit preimmune serum as a primary Ab.

Immunoelectron microscopy

Fresh toad stomach tissue was fixed in 4% paraformaldehyde and 0.5% glutaraldehyde in PBS, incubated overnight at 4°C, and washed in PBS. Sections (50 μm) were incubated with anti-histone H2A mAb (50 μg/ml) or anti-buforin I polyclonal Ab (1/1000 dilution). Labeled Abs were detected with the Vectastain Elite ABC kit described above. After development of peroxidase activity, sections were postfixed, dehydrated in ascending grades of alcohol, embedded in Epon resin, and allowed to polymerize at 60°C for 2 days. Thin sections were cut and stained with 1% toluidine blue. The regions of interest were selected, and ultrathin sections, silver colored, were examined by transmission electron microscope.

Preparation of peptide samples from gastric luminal lavage

Anesthetized toads were surgically prepared for the collection of gastric luminal lavage samples. The lumen of each resected stomach was perfused with a 0.9% saline (NaCl) solution in the presence of pepstatin A (50 μM). After cells and particulates were removed by centrifugation, the perfusate was acidified with glacial acetic acid to a final concentration of 5%. The lavage was then subjected to reversed-phase concentration with a Sep-Pak C_{18} cartridge (Millipore) that had been activated with 80% (v/v) acetonitrile containing 0.1% (v/v) TFA and flushed with 0.1% (v/v) TFA to remove excess acetonitrile. After being loaded with the lavage fluid, the cartridge was washed with 20 ml of 0.1% (v/v) TFA, and the peptides trapped in the cartridge were eluted with 5 ml of 80% (v/v) acetonitrile containing 0.1% (v/v) TFA. The eluate was then lyophilized and subsequently resuspended in 10 mM Tris-HCl (pH 7.5) containing 10 mM NaCl.

Western blot analysis

For immunoblot analysis of buforin I and histone H2A, the samples containing an equal amount of protein (10 μg) were separated by discontinuous 16.5% tricine SDS-PAGE and blotted onto nitrocellulose membranes (0.2 μm pore size; Bio-Rad, Hercules, CA) by semidry electrophoretic transfer (Bio-Rad). Nonspecific protein binding sites were blocked with 25 mM Tris-HCl (pH 7.2), 150 mM sodium chloride, and 0.05% Tween-20 containing 3% BSA. Membranes were incubated with either a mAb to histone H2A (2 μg/ml) or a polyclonal Ab to buforin I (1/4000 dilution) for

[3] Abbreviations used in this paper: FPLC, fast protein liquid chromatography; TFA, trifluoroacetic acid; PAS, periodic acid-Schiff.

1 h at room temperature in 25 mM Tris-HCl (pH 7.2), 150 mM sodium chloride, and 0.05% Tween-20 containing 0.5% BSA. The second Ab was an anti-mouse IgG or anti-rabbit IgG conjugated with HRP (Amersham, Aylesbury, U.K.). Immunoreactivity was visualized by enhanced chemiluminescence (ECL kit, Amersham).

Results

Purification of specific proteases responsible for buforin I production

As a preliminary experiment we studied the specific proteolytic activity in crude toad stomach extracts that converts histone H2A to buforin I. The crude extracts showed a buforin I-producing activity that functioned best under acidic conditions (pH range, 1.0–5.0) and displayed optimal activity at a pH of about 3.0 (data not shown). Enzyme inhibition studies showed that the production of buforin I from histone H2A by the crude toad stomach extracts was completely abolished by pepstatin A, a strong aspartic acid protease inhibitor (data not shown). However, serine protease inhibitors such as PMSF and leupeptin, metalloprotease inhibitor EDTA, and cysteine protease inhibitor E-64 had no effect on the buforin I-producing activity. The pH optimum of the reaction and the fact that pepstatin A inhibited buforin I production suggested the involvement of acid protease(s). Therefore, a pepstatin A-Agarose column was used as a first step in the purification of the buforin I-producing protease(s). The fraction from the pepstatin A-Agarose column that contained proteolytic activity was separated further into two discrete protein fractions by chromatography on an FPLC Mono Q column (Fig. 1*A*). Each of the fractions contained a single protein band of identical size (m.w. of ~30,000), as determined by SDS-PAGE (Fig. 1*B*). Both protein bands displayed buforin I-producing proteolytic activity.

The two purified proteins showing the buforin I-producing activity were subjected to automated Edman degradation for amino acid sequence analysis, which allowed identification of the N-terminal 20 aa residues (Fig. 1*C*). We searched the National Center for Biotechnology Information data bank using the BLAST network service and found that the amino acid sequences from the amino terminus of the buforin I-producing enzymes closely matched the N-terminal sequence of pepsin C, especially that of bullfrog pepsin C (19). Therefore, we named the two purified buforin I-producing proteases pepsin Ca and Cb. The N-terminal amino acid sequences of pepsin Ca and Cb were identical with 18 of 20 and 19 of 20 amino-terminal residues of the bullfrog pepsin C, respectively. The proteolytic activities of pepsin Ca and Cb were tracked throughout the purification by determination of the molecular size and antimicrobial activity of the processed buforin I peptide (Fig. 2, *A* and *B*). The amino acid composition and sequence of the processed peptide were identical with those of buforin I. In repeated mass analysis, the active peptide was found to have a mean mass value of 4279.9 Da, which was in good agreement with the theoretical molecular mass of buforin I. On the basis of the above results, we concluded that the pepsin isozymes, termed pepsin Ca and Cb, cleaved the Tyr^{39}-Ala^{40} bond of histone H2A to produce buforin I.

Proteolytic processing of histone H2A by porcine pepsin A

Pepsin A, a principal class of pepsins in vertebrate stomachs, was tested for its ability to process histone H2A to yield buforin I. Purified porcine pepsin A (purchased from Sigma) was further purified to remove any contaminants by chromatography over a Mono Q column. Digestion of calf thymus histone H2A with the purified porcine pepsin A resulted in one distinct peptide band on

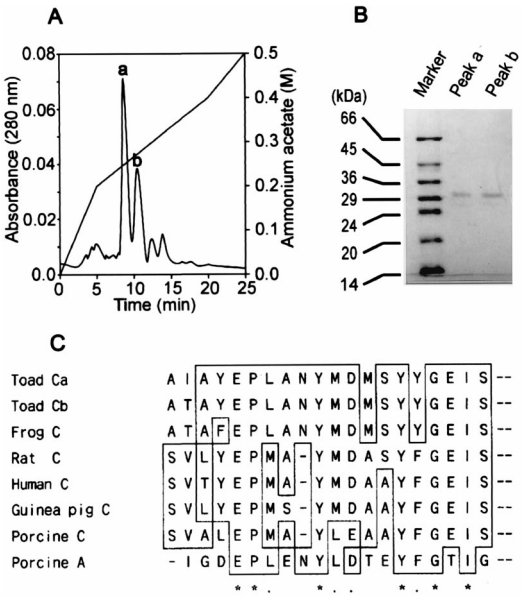

FIGURE 1. Purification of specific proteases. *A*, Chromatography on an FPLC Mono Q column of active fractions obtained from the pepstatin A-Agarose affinity purification step. The column was equilibrated with 0.05 M pyridine-acetate buffer, pH 6.0, and eluted with a gradient of 0–1.0 M ammonium acetate in the same buffer. *B*, SDS-PAGE analysis of purified specific proteases. FPLC fractions with buforin I-producing activity were subjected to 12.5% SDS-PAGE. *C*, Alignment of amino acid sequences of purified specific proteases. The multiple sequence alignment was obtained using the CLUSTAL W program with default parameters. The amino acid sequences of specific toad proteases (designated pepsin Ca and Cb) are aligned with those of bullfrog pepsin C, rat pepsin C, human pepsin C, guinea pig pepsin C, porcine pepsin C, and porcine pepsin A. Deletions of amino acids are indicated with hyphens. Asterisks and dots indicate perfect matches of amino acids and conservative substitutions, respectively. Residues identical in two or more sequences are boxed.

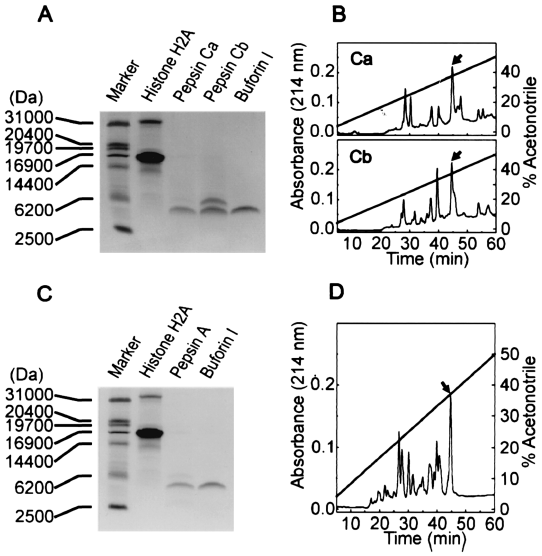

FIGURE 2. In vitro production of buforin I. *A*, Tricine SDS-PAGE analysis of processed histone H2A by toad pepsins Ca and Cb. Histone H2A and buforin I were loaded as controls. *B*, Reversed-phase HPLC of the processed histone H2A on a Delta Pak C_{18} column. The column was eluted using a linear gradient of acetonitrile (0–100%) containing 0.1% TFA at a flow rate of 1 ml/min. The peak indicated by an arrow was found by structural analyses to represent buforin I. *C*, Tricine SDS-PAGE analysis of processed histone H2A by porcine pepsin A. *D*, Reversed-phase HPLC of the processed histone H2A on a Delta Pak C_{18} column.

a tricine SDS-polyacrylamide gel (Fig. 2*C*) whose m.w. was similar to that of buforin I. The proteolytic reaction mixture was also fractionated by reversed-phase HPLC to isolate the processed peptide (Fig. 2*D*). The major peptide peak showing strong antimicrobial activity was then subjected to structural analyses to characterize the processed peptide. We obtained results identical with those of experiments with the purified toad pepsin Ca and Cb. Taken together, these findings indicate that porcine pepsin A can also produce buforin I from histone H2A by cleaving preferentially the Tyr^{39}-Ala^{40} bond.

Buforin I production in the toad gastric tissue

Immunohistochemical experiments were performed to determine the histological location of histone H2A and buforin I in the cross-section of the toad stomach (Fig. 3). To distinguish the histone H2A present in cytoplasm from that in nucleus, two specific Abs, anti-acetylated (Ac-Lys^5) histone H2A and anti-unacetylated histone H2A, were used. As shown in Fig. 3*D*, the mAb to unacetylated histone H2A stained the cytoplasm of gastric gland cells with a granular pattern, indicating that the unacetylated histone H2A protein, the precursor of buforin I, was present in the cytoplasm of the gland cells of toad gastric mucosa. The mAb used was specific for the unacetylated histone H2A in the cytoplasm and did not recognize the acetylated histone H2A in the nucleus (Fig. 3, *D* and *G*) or buforin I (Fig. 3*E*). The stomach section subjected to PAS staining indicates that cells immunopositive for histone H2A were not the PAS-positive cells that are responsible for the generation of mucus present in the gastric pit and the neck region of the gastric gland (Fig. 3*B*). Incubation of the stomach sections with a polyclonal Ab to buforin I localized the immunoreactivity to the mucosal surface of the stomach (Fig. 3*E*). The polyclonal Ab was specific for buforin I and did not recognize nuclear or cytoplasmic histone H2A (Fig. 3*E*). Control sections of the toad stomach incubated with preimmune serum showed no immunoreactivity (Fig. 3*C*). A polyclonal Ab specific to toad pepsin C showed the same strong, punctate staining pattern as that of the unacetylated histone H2A (Fig. 3*F*), which suggests the possible secretion of histone H2A into the lumen, similar to the secretion of pepsinogen C by gastric gland cells. This suggests that histone H2A may be secreted into the lumen in a similar way as pepsinogen C. To study the presence of other histone proteins in the cytoplasm of the toad stomach, the parallel sections of the toad stomach were stained using mAbs to histone H1 (clone AE-4) and histone H2B (clone LG2-2). Of special interest is the finding that histone H1 and H2B were localized in the cytoplasm of toad gastric gland cells in addition to the nucleus (Fig. 3, *H* and *I*), but the granularities of staining were much finer than that of histone H2A.

Our observations made by immunohistochemistry were further confirmed using immunoelectron microscopy on the toad gastric mucosa. Examination under an electron microscope showed histone H2A over endoplasmic reticulum of some gastric gland cells (Fig. 4*A*) and also located in the granule (Fig. 4*B*). In contrast, buforin I was associated with epithelial surface (Fig. 4*C*).

Buforin I secretion in the vertebrate gastric lumen

Western blot analysis was then performed to examine the presence of buforin I in the gastric lumen. Analysis of gastric luminal lavage fluids identified an immunoreactive peptide corresponding to the

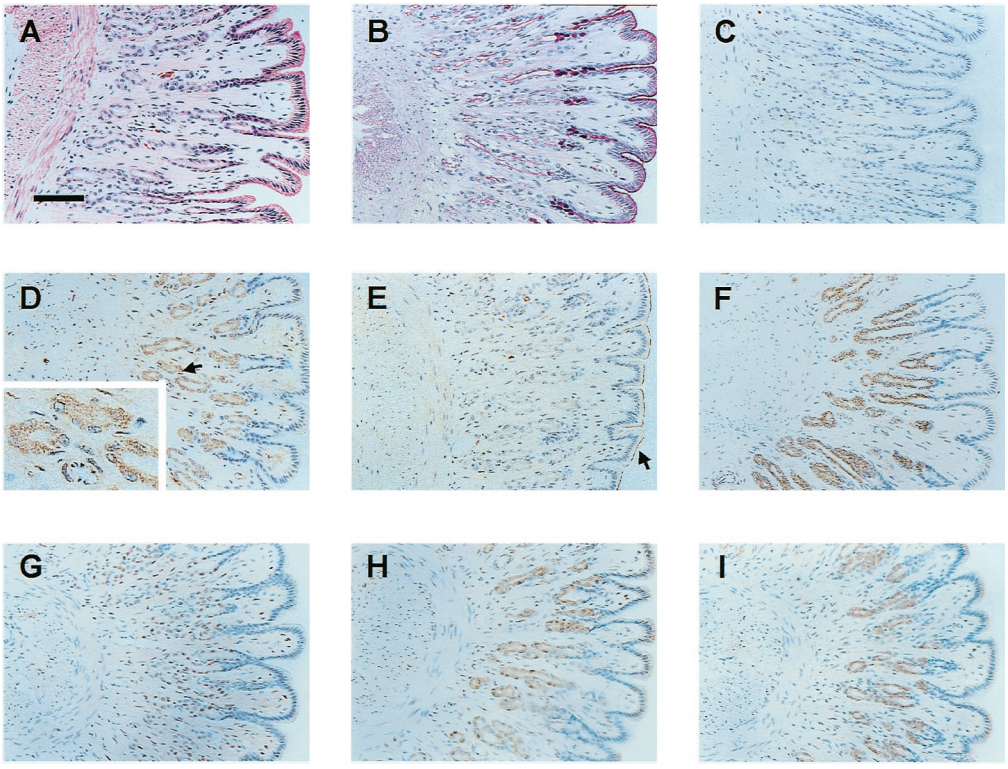

FIGURE 3. Immunohistochemistry of the cross-sectioned toad stomach. *A*, Hematoxylin-eosin staining was included to show gastric mucosal morphology. *B*, PAS reaction stained carbohydrate with a pink color in a mucous gland. *C*, There was no immunoreactivity detected with preimmune serum. *D*, Strong immunostaining for histone H2A, indicated by arrows, was seen in the cytoplasm of gland cells of toad gastric mucosa. The inset clearly indicates a granular staining pattern for histone H2A in the cytoplasm of gastric gland cells. *E*, Intense dark staining for buforin I, indicated by arrows, was seen on the mucous surface of the stomach. *F*, Pepsinogen C showed the same immunolocalization as that of histone H2A. *G*, Strong immunostaining for acetylated (Ac-Lys^5) histone H2A was seen in the nucleus of gastric gland cells. *H* and *I*, Histone H1 (*H*) and histone H2B (*I*) immunoreactivities were seen in the cytoplasm as well as in nucleus of gastric gland cells. Bar = 100 μm.

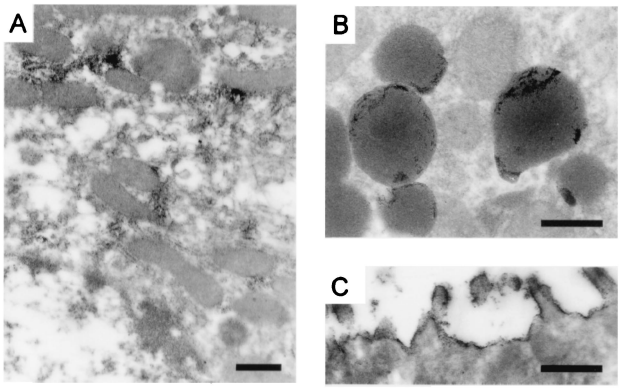

FIGURE 4. Immunoelectron microscopy of a section of toad gastric mucosa. *A*, Extensive immunoreactivity to histone H2A was seen over endoplasmic reticulum of gastric gland cells. *B*, Immunoreactive histone H2A was present in the granule of gastric gland cell. *C*, Immunoreactive buforin I was present on the surface epithelium. Bar = 0.1 μm.

purified buforin I, indicating that the peptide is released from histone H2A by pepsin C cleavage and resists degradation in the gastric lumen (Fig. 5). Additional experiments were performed to determine whether buforin I could also be found in the stomach of other vertebrates. Gastric tissues from porcine, bovine, and human species were subjected to lavage, and the fluid was collected for partial purification by reversed-phase concentration. Fig. 6 shows the presence of an immunoreactive peptide in porcine, bovine, and human stomachs that comigrated with the purified buforin I. In bovine and human gastric lavage fluids, one band corresponding to buforin I was detected, whereas two forms of the peptide were detected in porcine gastric fluid, indicating the presence of buforin I and a further processed form of the antimicrobial peptide.

Discussion

In this study we have demonstrated that the antimicrobial peptide buforin I is generated from the unacetylated histone H2A in the cytoplasm of gastric gland cells by the action of pepsin C isozymes in the toad stomach. Pepsin C isozymes, Ca and Cb, carry out the production of buforin I from histone H2A by cleaving specifically the Tyr^{39}-Ala^{40} bond of histone H2A, as confirmed by structural and immunological analyses of the processed peptide. The vertebrate stomach contains two immunologically and biochemically distinct groups of pepsin precursors, namely pepsinogens A and C. Pepsinogen A is localized mainly in the fundus, whereas pepsinogen C is distributed throughout the stomach and proximal duodenum (20–22). Those pepsinogens are secreted into the gastric lumen and activated autocatalytically to active pepsins under the acidic condition (23, 24). The relative contents of pepsins A and C in the vertebrate stomach differ depending on the species of the animal. In frogs and rodents pepsin C is the major protease found in gastric fluid, while in other vertebrates pepsin A is more abundant (19–22). Therefore, we also examined the buforin I-producing ability of pepsin A, which is present as a major protease in the gastric fluids of most vertebrates. Pepsin A also cleaved preferentially the Tyr^{39}-Ala^{40} bond of histone H2A to produce buforin I, even though the buforin I-producing activity was less than that of the toad pepsin C isozymes.

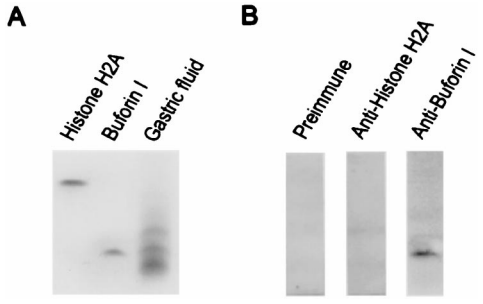

FIGURE 5. Western blot analysis of gastric lavage fluids from the perfused toad stomach. *A*, Tricine SDS-PAGE analysis of gastric lavage fluids. The standards are 1 μg of histone H2A and 0.4 μg of purified buforin I. *B*, Western blot analysis demonstrated the presence of immunoreactive buforin I in the gastric luminal lavage fluid. The Western blots were probed with preimmune serum, anti-histone H2A mAb, or anti-buforin I polyclonal Ab. Each lane contains 10 μg of extracts.

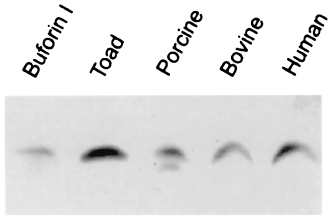

FIGURE 6. Western blot analysis of gastric lavage fluids from porcine, bovine, and human stomachs. Gastric lavage fluids were collected and concentrated using a Sep-Pak C_{18} cartridge as described in *Materials and Methods*. The standard is 50 ng of purified buforin I. Western blot analysis demonstrated the presence of immunoreactive buforin I in porcine, bovine, and human gastric lavage fluids. Each lane contains 10 μg of extracts.

To elucidate the mechanism of buforin I generation in the toad stomach, we used immunohistochemical methods and an Ab raised against buforin I in rabbits immunized with a synthetic peptide containing residues 20–37 of buforin I. This synthetic peptide was suitable to elicit an Ab that was specific for buforin I. The buforin I-specific Ab was immunologically unreactive with respect to both nuclear and cytoplasmic histone H2As. A mapping study of the linear histone H2A regions revealed that the peptide sequence of the synthetic congener buforin I is not localized on the surface of histone H2A protein, and therefore histone H2A is not accessible to the buforin I Ab (25). A mAb (BWA3) to unacetylated histone H2A was used for the detection of histone H2A in the cytoplasm. The specificity of BWA3 was previously reported (18). Immunohistochemical and immunoelectron microscopic analyses revealed that buforin I is present extracellularly on the mucosal surface, whereas unacetylated histone H2A is distributed in both the cytoplasmic granules and the cytoplasm of gastric gland cells (Fig. 4, *A* and *B*), and Western blot analysis confirmed the presence of buforin I in luminal lavage from the stomach. The anti-acetylated histone H2A, whose epitope lies on SGRG*Ac*KQGGK (aa 1–9 of histone H2A and buforin I; AcK indicates the acetylated lysine), stained the nucleus only, not the cytoplasm. This further indicates that histone H2A in the cytoplasm is the unacetylated one. If buforin I is derived from the acetylated histone H2A in the nucleus, buforin I on the mucosal surface also should be immunostained by the anti-acetylated histone H2A, and acetylated lysine residue should be detected in the amino acid analysis of buforin I obtained from the lumen. However, buforin I on the mucosal surface was not immunostained by the anti-acetylated histone H2A, and the acetylated lysine residue was not detected by the amino acid analysis of buforin I. The distinct subcellular distribution of the unacetylated histone H2A and the detection of the unacetylated buforin I both on the mucosal surface and in the lumen suggest that buforin I is produced from the cytoplasmic unacetylated histone H2A secreted into the gastric lumen and subsequently processed by pepsins. This idea was further supported by the observation that toad

pepsinogen C showed the same immunolocalization in the cytoplasm as that of the unacetylated histone H2A (Fig. 3*F*), which indirectly indicates that the unacetylated histone H2A is secreted into the lumen in a similar way as pepsinogen C.

It seems that a large amount of unacetylated histone H2A is available in the cytoplasm of toad gastric gland cells in part because of the rapid regeneration of the gastric mucosa and active transcription of gastric gland cells causing high expression of histone proteins. Of the histone H2A synthesized, a limited amount moves into the nucleus and is acetylated (26), and the excess unacetylated histone H2A accumulates in the cytoplasm, eventually secreted into the lumen. While the functional role of acetylation is poorly understood, it is thought to play a role in the formation of either newly synthesized or transcriptionally competent chromatin (27–29). We also observed the immunoreactivity to unacetylated histone H2A in the endoplasmic reticulum of some gastric gland cells, which would be wholly consistent with de novo histone synthesis in the cytoplasm.

Recently, the presence of histones in both the cytoplasm and the nucleus has been reported (3, 30–34), and secretion of histone proteins into extracellular milieu was also observed (35–38). Now it is clear that histone proteins are present in both the cytoplasm and the nucleus of rapidly regenerating or transcriptionally active cells such as stomach epithelial cells.

Until recently, the functional role of histones has been viewed mainly in connection with DNA stabilization and gene expression. However, there is growing evidence that histones may be involved in a multitude of biological functions (39–43), including host defense (33, 39). The generation of antimicrobial peptide buforin I may be one of its secondary biological functions.

It seems that the expression of buforin I is not up-regulated during infections, and cytokines do not play a role in the production of buforin I. Instead, buforin I production is closely related to the secretion of hydrochloric acid and pepsinogen. The toad, in common with other anurans, swallows its prey intact, and stores its food for some period of time before digestion begins, so the secretion of antimicrobial peptides into the stomach lumen may serve to inhibit bacterial growth. Once the food is ingested into the stomach, it signals cells in the stomach wall to secrete the hydrochloric acid and pepsinogen, thus contributing to the production of buforin I from histone H2A.

Of note is the finding that the immunoreactivity of buforin I is extracellular on the mucosal surface. This indicates that buforin I coats the mucosal surface, where it may contribute to the establishment of a local antimicrobial milieu. It is suggested that antimicrobial peptides present on the mucosal surfaces of vertebrates may serve as a defense of the gastrointestinal tract against microbial invasion or to aid in wound repair (44). For pathogens that invade the host, surface epithelium is the first site of contact with the host. Microbial pathogens that breach the epithelial barrier can cause an acute inflammatory response that is characterized by increased expression of cytokines with chemoattractant and proinflammatory functions. Thus, the presence of buforin I on the mucosal surfaces may contribute to maintain mucosal barrier function in conjunction with mucosal immune cells. Further studies are necessary to examine the relationship between buforin I and mucosal immune cells.

It is well established that most amphibian functional peptides have either identical or closely related counterparts in the mammalian brain and gastrointestinal tract (45). Whether buforin I is ubiquitous in the stomachs of vertebrates is not yet clear. However, we confirmed its presence in the lavage fluids of porcine, bovine, and human stomachs by Western immunoblot analysis (Fig. 6). Our results in this study suggest that antimicrobial peptides, such as buforin, act in conjunction with gastric hydrochloric acid to enable the stomach to contribute to the innate defense of more distal intestinal organs.

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