Cathelicidin antimicrobial peptides are expressed in salivary glands and saliva

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ABSTRACT

The expression of antimicrobial peptides at epithelial surfaces such as skin, lung, and intestine is thought to provide protection against infection. Cathelicidin antimicrobial peptides are essential for the protection of skin against invasive bacterial infection. To determine if cathelicidins are also present in the oral cavity, we examined the expression of both mRNA and protein in mice and human saliva. The murine cathelicidin (CRAMP) was detected in the adult by reverse-transcription/polymerase-chain-reaction (RT-PCR), and in embryonic, newborn, and adult tissues by in situ hybridization and immunohistochemistry. CRAMP mRNA and protein were localized to the salivary glands, specifically in acinar cells of the submandibular gland and palatine minor glands, as well as in lingual epithelium and palatal mucosa. In man, the human cathelicidin LL-37 was detected in human saliva by Western blotting. These results indicate that cathelicidins are present in the salivary system, in some oral epithelia, and in saliva, contributing to broad-spectrum defense of the oral cavity.

KEY WORDS: oral mucosa, CRAMP, LL-37, innate immunity.

INTRODUCTION

Many peptides have been discovered based on their ability to kill or inhibit the proliferation of pathogenic micro-organisms (Zasloff, 2002). Antimicrobial peptides are widely known in plants, insects, and animals, where they provide a first line of defense against invading pathogens. In humans, antimicrobial peptides of three families have received considerable attention: the cathelicidins (Frohm et al., 1997; Zaiou and Gallo, 2002), the defensins (α- and β-) (Zhao et al., 1996), and the histatins (Oppenheim et al., 1988). When studied in vitro, these peptides have distinct and overlapping antimicrobial activity (Bals, 2000; Yang et al., 2001).

Cathelicidins are a widely expressed family of mammalian antimicrobial peptides that contain a highly conserved signal sequence and cathelin domain but show substantial heterogeneity in the C-terminal domain encoding the mature active peptide (Zanetti et al., 1995). Cathelicidins are found in many mammals, including primates, ungulates, rodents, and rabbits (Zaiou and Gallo, 2002). The murine cathelicidin CRAMP (Cathelin-related Antimicrobial Peptide) (Gallo et al., 1997) closely resembles human LL-37, first isolated from human granulocytes (Gudmundsson et al., 1996). Both CRAMP and LL-37 antimicrobial domains show considerable structural homology, are synthesized as preproteins (Zanetti et al., 1995), and are processed from an inactive precursor form to an active mature peptide to kill bacteria. In skin, these antimicrobial peptides show identical patterns of expression and are greatly increased after wounding occurs (Dorschner et al., 2001).

Cathelicidins have broad-spectrum activity against Gram-positive and Gram-negative bacteria as well as fungi and envelope viruses (Bals et al., 1998; Johansson et al., 1998; Turner et al., 1998; Dorschner et al., 2001). Histatin peptides are microbicidal, possibly involved in maintaining the acquired enamel pellicle, and enhance the glycolytic activity of certain oral micro-organisms. Histatin and calprotectin have antimicrobial activity against Candida (Oppenheim et al., 1988; Eversole et al., 1993). β-defensins have antimicrobial activity against Gram-negative bacteria such as E. coli, some Gram-positive bacteria, and Candida albicans (Dale and Krisanaprakornkit, 2001; Lehrer and Ganz, 2002). β-defensins have also been specifically investigated with respect to their activity against oral bacteria; Capnocytophaga spp. are sensitive to oxygen-independent killing by human defensins, whereas the A. actinomycetemcomitans and E. corrodens strains were resistant (Miyasaki et al., 1990). Therefore, the co-expression of cathelicidins and β-defensins in saliva with peptides such as histatin and calprotectin provides a powerful combined natural antibiotic barrier.

In the oral cavity, antimicrobial peptides of the β-defensin and histatin families have been described (vanderSpek et al., 1990; Weinberg et al., 1998). β-defensins and histatins have antimicrobial activity distinct from that of cathelicidins, but are also active against...
In situ Hybridization

For in situ probes, a PCR product of CRAMP mature form (CRmf, 159 bp) was amplified and sequenced for confirmation of identity, then inserted into the PCR IL-TOPO vector (TOPO TA Cloning version M, Invitrogen, Carlsbad, CA, USA). Plasmid DNA was purified by means of a DNA Extraction Maxi Kit (Qiagen, Valencia, CA, USA) and linearized with restriction enzymes (BglII and XbaI) for anti-sense and sense RNA probe. Digoxigenin-labeled riboprobes were prepared on linearized plasmid by means of the digoxigenin (DIG) RNA labeling kit (SP6/T7, Roche Molecular Biochemicals, Mannheim, Germany). To confirm the specificity of the labeled probes, we blotted 2 μL of the plasmid cDNA for CRmf on nitrocellulose filters (OSMONICS, Westborough, MA, USA).

Fresh-frozen sections were fixed with 4% PFA for 10 min at room temperature, and pre-treated with 1 μg/mL proteinase K in PBS for 15 min at 37°C. Sections were treated with a 1 M triethanolamine solution (pH 8.0) containing 0.25% acetic anhydride for 15 min at 37°C, washed with PBS at least 3 times, then treated with 100% ethanol for 5 min, and dried. Pre-hybridization was performed with 50% formamide in 2x sodium saline citrate (SSC: 3 M sodium chloride, 0.03 M sodium citrate) for 30 min at 45°C. Sections were then hybridized for 16 hrs at 45°C with sense or antisense DIG-labeled cRNA probes (1 μg/mL) in hybridization solution (1 mg/mL yeast tRNA, 20 mM Tris-HCl buffer [pH 8.0], 2.5 mM EDTA, 1x Denhardt's solution, 0.3 M NaCl, 50% de-ionized formamide, 50% dextran sulfate). Stringent washing was done for 60 min at 45°C with 50% formamide in 2x SSC, and for 10 min at 37°C with 2x SSC; samples were then treated with RNase-A (Roche Molecular Biochemicals, Mannheim, Germany) for 30 min at 37°C. After being washed with the 2x SSC for 30 min at 37°C, sections were reacted with anti-DIG alkaline phosphate Fab fragment antibody (1:500, in PBS, Roche Molecular Biochemicals, Mannheim, Germany) for 5 hrs at RT. Alkaline phosphate was visualized by incubation with 5-bromo-4-chloro-3-indolyl phosphate (X-phosphate) and nitroblue tetrazolium (NBT) with addition of levamisole solution (DAKO, Carpinteria, CA, USA) overnight at room temperature (RT). Methyl green stain was used for nuclear counterstain. RNA preservation in tissues was confirmed by similar hybridizations by means of a keratin 14 antisense probe (insert 175 bp).

Immunohistochemistry

For CRAMP protein, rabbit anti-CRAMP polyclonal antibody was used as previously described (Dorschner et al., 2001; Pestonjamasp et al., 2001). Tissue sections were immersed in PBS after 4% PFA fixation for 20 min, and endogenous peroxidase activity blocked with a 30-minute incubation in 0.3% H2O2 in methanol. Sections were blocked with 2% goat serum in PBS for 30 min, and incubated with primary antibody, rabbit anti-CRAMP polyclonal antibody (0.8 μg/mL) in PBS, 3% bovine serum albumin for 60 min. Signal was detected with a Vectorstain ABC Elite Rabbit kit (Vector Laboratories, Burlingame, CA, USA) and processed by means of an ABC kit (Vector Laboratories, Burlingame, CA, USA). As a negative control, the polyclonal antibody was replaced by normal rabbit pre-immune IgG diluted with PBS containing 3% BSA at the same protein concentration as the primary antibody. Finally, sections were incubated in 0.02% diaminobenzidine with 0.05% H2O2 in PBS for 5 min and counterstained with Mayer's hematoxylin for 30 sec. The specificity of the primary antibody reaction has been confirmed in separate experiments by absorption of the anti-CRAMP with excess amounts of synthetic peptide.
Western-blot Analysis

Rabbit anti LL-37 polyclonal antibody for immunostaining was kindly provided by Dr. Birgitta Agerberth (Microbiology and Tumorbiology Center, Stockholm, Sweden). This antibody was derived against the entire LL-37 peptide (Agerberth et al., 1995; Frohm et al., 1997).

Chicken polyclonal antibodies were derived against LL-37 mature form and cathelin domain (CATH) peptides by Aves Labs. (Tigard, OR, USA). Peptide amino acid sequences used for immunization were CZQRIKD-FLRNLVPRTES and CZNLVR-LLDLPRPTMD for LL-37 and CATH, thus identifying domains in the C-terminal antimicrobial peptide and the precursor protein, respectively. IgY fractions were affinity-purified with the respective immunizing peptides. The stock concentrations of the antibodies were 1.25 mg/mL and 1.62 mg/mL for LL-37 and cathelin.

Samples were separated by 4-20% gradient SDS/polyacrylamide gel electrophoresis (Express gels, ISC BioExpress, Kaysville, VT, USA), and transferred to nitrocellulose membrane (OSMONTICS, Westborough, MA, USA). Membrane was blocked (0.1% TTBS: 5% non-fat milk in 0.1% Tween 20/Tris buffered saline (BS: 150 mM NaCl, 10 mM Tris Base, pH 7.4)) for 60 min at RT, and then rabbit anti-LL37 polyclonal antibody (1:3000 in the blocking solution) was incubated with the membrane for 60 min at RT. After the membrane was washed 3x with 0.1% TTBS, horseradish-peroxidase-labeled anti-rabbit goat polyclonal antibody (1:5000 in the blocking solution) was incubated with the membrane for 60 min at RT. After the membrane was washed with 0.1% TTBS, it was immersed in ECL solution (Western Lightning Chemiluminescence Reagents Plus, New Lifescience Products, Boston, MA, USA) for 60 sec, then exposed to x-ray film (Kodak). Synthetic LL-37 peptide derived by Synpep Corporation (Dublin, OR, USA) was used for positive control of Western blot.

For confirmation of the identity of the band detected by Western blot, the filter was stripped of antibody (62.5 mM Tris-HCl [pH 6.8], 2% SDS, 100 mM β-mercaptoethanol) at 50°C for 30 min, then reacted with a newly derived chicken anti-cathelin antibody (1:10000) and horseradish-peroxidase-labeled goat anti-chicken IgY (1:10000; Aves Labs.). The filter was again stripped of antibody, then reacted with a newly derived chicken anti-LL37 antibody (1:2000) and horseradish-peroxidase-labeled goat anti-chicken IgY according to the same method described above.

RESULTS

CRAMP mRNA is Expressed in the Salivary Gland and Oral Mucosa

To determine if CRAMP mRNA could be detected in the oral cavity of the adult mouse, we first performed RT-PCR on microdissected tissues. CRAMP mRNA was detected in the salivary gland (submandibular gland), tongue, gingival, and palatal mucosal tissues, respectively (Fig. 1). No CRAMP was detected in buccal mucosa.

For further exploration and specific confirmation of the expression of cathelicidin in the oral cavity, in situ hybridization was performed. Non-radioactive in situ hybridization with digoxigenin-labeled cRNA anti-sense probes for CRmf
CRAMP protein is expressed in the submandibular (salivary) gland, and oral mucosa. CRAMP protein in submandibular glands of adult mouse (A), E-17 embryonic mouse (B), newborn Day-4 mouse (C), and palatine minor salivary gland of the newborn (G). Newborn gingiva (H) and tongue (I, lower layer) also show CRAMP expression. In newborn palatal mucosa, CRAMP is located in the basal cell layer and a few suprabasal cells (I, upper layer). For negative controls, pre-immune rabbit IgG (D-F, J-L) was used. Original magnifications: ×400.

CRAMP Protein is Expressed in the Salivary Glands and Oral Mucosal Tissues

For further exploration and confirmation of cathelicidin expression at the protein level, oral tissue was examined for CRAMP by immunohistochemistry. Specific anti-CRAMP antibody showed abundant localization in the submandibular glands of the adult (Fig. 3A), D-4 newborn (Fig. 3C), and the E-17 embryo (Fig. 3B). D-4 mouse palatine minor salivary gland also showed expression (Fig. 3G). CRAMP was mainly located in the cytoplasm, especially around the nucleus in serous-type acini and mucinous-type acini. Some ductal cells of the intercalated and striated duct epithelium also showed protein expression. CRAMP was also expressed in the whole epidermis of gingiva (Fig. 3H), palatal mucosa, and the dorsum of the tongue (Fig. 3I) in newborns.

Human Cathelicidin LL-37 is Expressed in Saliva

The detection of cathelicidin protein and mRNA in the murine oral cavity suggested that this antimicrobial peptide may also be present in human saliva. To study this, we evaluated normal human saliva by Western blot with three antibodies independently derived against distinct domains of the full-length cathelicidin gene product (anti-CATH and anti-LL-37).

LL-37 protein expression was detectable in crude extracts of human saliva (Fig. 4). Three bands were detected with rabbit anti-LL37, two with chicken anti-cathelin domain, and two with chicken anti-LL37 used for further confirmation of the identity of the proteins seen by Western blot. Bands at 18 kDa correspond to the full-length cathelicidin, and additional 14-kDa bands were detectable with all three antibodies. Bands at 5 kDa correspond with the mature LL-37 peptide. Chicken anti-LL-37 had lower sensitivity than rabbit anti-LL-37, as seen by comparison of reactivity to the LL-37 synthetic peptide.

DISCUSSION

Oral epithelium is constantly exposed to a variety of microbial pathogens. Antimicrobial peptides are essential elements of epithelial defense participating in a variety of immune mechanisms, both direct and indirect (Zasloff, 2002). The expression of defensins, in particular hBD-1 and hBD-2, has previously been described in major salivary glands, tongue, gingiva, and buccal mucosa (Dale and Krisanaprakornkit, 2001). Cathelicidins are expressed in various epithelia under both constitutive and inflamed conditions such as wound-healing (Frohm et al., 1997; Dorschner et al., 2001). This family of antimicrobial peptides can inhibit the growth of oral pathogens in vitro (Guthmiller et al., 2001) but has previously been described as a relatively minor part of the antimicrobial milieu of the mouth (Dale and Krisanaprakornkit, 2001). In this investigation, we sought to use the mouse model to explore whether cathelicidins are expressed in significant amounts in the oral cavity, thus providing an additional innate antimicrobial defense system. Our results show that the murine cathelicidin CRAMP is expressed primarily in the salivary
glands. Furthermore, analysis of human saliva shows that cathelicidin is also present in the human mouth, as demonstrated by the presence of LL-37 in both its mature and immature forms.

To explore the expression of cathelicidin, we micro-dissected individual tissues from the mouth and performed semi-quantitative PCR. These results suggested that cathelicidin mRNA was present in the oral cavities of mice from the late embryonic period to adult. The high sensitivity and potential for false-positive results with PCR necessitated further analysis by in situ hybridization and immunohistochemistry. Both techniques confirmed expression of cathelicidin in secretory epithelia and mucosal structures. CRAMP mRNA was detected only in acinar cells, and CRAMP protein in both acinar and ductal cells. In contrast, hBD-1 had been previously shown in the minor salivary gland and ductal cells, with no expression found in acinar cells (Saahsabudhe et al., 2000). These results show that secretion of cathelicidin and defensin differs in the salivary glands, with a possibility of distinct regulation as well as mutual cooperation against oral pathogens.

To confirm that these observations in mouse salivary glands are relevant to human oral defense, we investigated human saliva. Using a combination of distinct antibodies against different domains of human cathelicidin LL-37, we showed that this peptide is abundantly present and apparently processed into several forms. Using antibodies derived against the mature peptide and the 18-kDa immature form, we could see a 14-kDa band and the mature 37-amino-acid peptide. However, analysis of human saliva with a chicken anti-LL-37 antibody directed against the cathelin domain detected both the mature peptide and the 18-kDa immature form, we could likely reflect epitope masking in the mature form, since both antibodies could detect the mature form of LL-37, and a third antibody directed against the cathelin domain detected both the 18- and 14-kDa forms. The function of these various processed forms of cathelicidin remains unknown.

In the oral cavity, multiple peptides and proteins with antimicrobial action have been described. For example, lysozyme (Tobgi et al., 1988), calprotectin (Eversole et al., 1993), β-defensins (Mathews et al., 1999; Dale and Krisanaparakornkit, 2001; Dunsche et al., 2001), and histatin (Oppenheim et al., 1988; vanderSpek et al., 1990) have been identified. Cathelicidin expression has been previously described in tongue and buccal mucosa (Frohm Nilsson et al., 1999). However, although cathelicidins are essential in the defense of skin against bacterial invasion (Nizet et al., 2001), there have been no reports of cathelicidin expression in salivary glands. The significance of the current observations of cathelicidin expression is therefore the recognition of a natural antibiotic previously shown to be essential to skin defense as an additional antimicrobial peptide participating in oral protection.

Like all reports of in vitro antimicrobial activity, the interpretation of antimicrobial results depends on the assay system used. For example, oral bacteria such as A. actinomycetemcomitans, C. sputigena, C. gingivalis, and C. ochracea have been shown to be sensitive to LL-37 in 10 mM sodium phosphate with 1% TSB solution, but not in high salt (Tanaka et al., 2000). LL-37 has been reported as ineffective in tryptic soy broth (TSB)-based medium for oral bacteria such as A. actinomycetemcomitans, F. nucleatum, P. gingivalis, S. sanguis, C. albicans, C. kurusei, and C. tropicalis, with no correlation with salt concentration (Guthmiller et al., 2001). Such conflicting results suggest that caution be applied to extrapolation of data from the in vitro system to conclusions regarding in vivo immune defense of the mouth. Nevertheless, the synergistic action of the previously described oral antimicrobial peptides supports their role in host defense (Nagaoka et al., 2000; Dale and Krisanaparakornkit, 2001).

Further study of the combined activities and regulation of oral antimicrobial peptides will greatly advance our understanding of this system of epithelial defense. Direct analysis of the individual contribution of each peptide to antimicrobial action is needed. As information continues to accumulate, it has become clear, however, that unique systems of gene regulation and protein processing contribute to the functions of these molecules in different tissues. In mammals, cathelicidins are likely to play an important role in host defense at multiple epithelial surfaces and may provide important diagnostic and therapeutic advances toward disease resistance. These observations have led to an associated human periodontal disease. A deficiency in salivary LL-37 correlates with severe periodontal disease in morbus Kostmann (Putsep et al., 2002).

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REFERENCES


