Keratinocytes Store the Antimicrobial Peptide Cathelicidin in Lamellar Bodies

Marissa H. Braff,* Anna Di Nardo,† and Richard L. Gallo*

*University of California, San Diego, La Jolla, California, USA and Department of Dermatology, VAMC, La Jolla, California, USA; †San Gallicano Dermatological Institute (IRCCS), Rome, Italy

Innate immune defense against microbial pathogens occurs by physical barriers, by recruitment of cells such as neutrophils, NK cells, and macrophages, and by secretion of molecules with antimicrobial activity. Such molecules are produced by various epithelia including skin. The importance of antimicrobial peptides has been shown in cathelicidin-deficient mice, which have increased susceptibility to skin infection by Streptococcus. Although keratinocytes increase cathelicidin expression upon injury, their role relative to neutrophil cathelicidin and their sites of peptide storage and activation have not been elucidated. Herein, it is reported that cathelicidin predominantly resides in granules of the superficial epidermis and partially localizes in lamellar bodies as determined by immunogold electron microscopy and immunoblot of lamellar bodies isolated from mice. In cultured keratinocytes, cathelicidin displays a granular distribution and partially localizes within the Golgi apparatus. Cathelicidin processing can be observed by western blot analysis in keratinocyte extracts but not in conditioned media. Further, fluorescent bacteria colocalize with cathelicidin in granules both intracellularly and at the cell surface. These observations illustrate the immune defense potential of keratinocytes acting directly through storage and processing of antimicrobial peptides.

Key words: keratinocytes/cathelicidin/innate immunity/lamellar bodies/antimicrobial peptides


The skin is positioned at the interface between an organism’s internal milieu and an external environment characterized by constant assault with potential microbial pathogens. Once considered merely as a physical protective barrier, it is now apparent that a major role of the skin is to defend the body by rapidly mounting an innate immune response to injury and microbial insult. Both resident skin cells and recruited inflammatory cells, including keratinocytes and neutrophils, respectively, synthesize antimicrobial peptides and various other host defense molecules that together demonstrate broad-spectrum activity against bacteria, fungi, and viruses.

Two major families of small cationic antimicrobial peptides have been best studied in mammalian skin: β-defensins and cathelicidins. β-defensins form β-sheet structures and contain multiple cysteine residues that form intermolecular disulfide bridges (Lehrer and Ganz, 1999). Cathelicidins share a conserved cathelin pro-domain and a variable antimicrobial peptide domain that is released from the parent molecule by proteolytic processing (Zanetti et al., 2000). Both infiltrating immune cells and resident epithelial cells express these antimicrobial peptides. For example, human β-defensin-1 (hBD-1) is constitutively made by keratinocytes, whereas hBD-2, hBD-3, and the human cathelicidin hCAP18/LL-37 are typically upregulated in keratinocytes during inflammation and accumulate in the skin in part through release by neutrophils.

The essential role of antimicrobial peptides in defense of the skin against bacterial infection has been demonstrated in mice deficient in the murine cathelicidin gene (Nizet et al., 2001) and in pig wounds infected from the pig cathelicidins to their active peptide form (Cole et al., 2001). Cathelicidins are derived from several cell types present in the skin including keratinocytes (Frohm et al., 1997; Sorensen et al., 2003), neutrophils (Turner et al., 1998), mast cells (Di Nardo et al., 2003), and eccrine glands (Murakami et al., 2002). Because of the various cellular sources of cathelicidin and the multiple antimicrobial peptide families present in skin, the relative contribution of keratinocyte cathelicidin to epidermal barrier defense against infection is unclear.

Understanding the role of antimicrobial peptides in the skin requires careful assessment of their production by the epidermis. This stratified epithelial structure is comprised of multiple keratinocyte layers in various states of differentiation and with distinct potential functions. The granular layer and stratum corneum consist of terminally differentiated cells involved in superficial barrier formation (Norlen et al., 2003) and contain secretory granules called lamellar bodies. These lipid-rich disks are bounded by a single membrane, range in size from 0.1 to 0.6 μm and release their contents into intercellular spaces where stored hydrolytic enzymes are activated and process polar lipids into the hydrophobic products that constitute the stratum corneum (Madison et al., 1998; Rassner et al., 1999). Lamellar bodies are secreted within 30 min and for at least 6 h following disruption.
of the permeability barrier (Menon et al., 1992; Nickoloff, 1999; Rassner et al., 1999). This timing is consistent with antimicrobial peptide production following injury, and suggests that an additional function of keratinocyte granules may be to participate in innate immune defense. Consistent with this hypothesis, lamellar bodies have recently been shown to provide storage sites for β-defensins (Oren et al., 2003).

This study sought to characterize the intracellular sites of keratinocyte cathelicidin expression and to evaluate the antimicrobial function of keratinocytes in epidermal immune defense. The results indicate that cathelicidin is present in keratinocyte lamellar bodies, undergoes proteolytic processing by keratinocytes, and associates with internalized and cell surface bacteria. Keratinocyte synthesis of cathelicidin may therefore play an important role in skin immunity by forming an active part of the antimicrobial defense barrier that is independent of circulating immune cells.

Results

The intracellular localization of cathelicidin in human skin was investigated by immunofluorescence microscopy of cultured human skin constructs. These constructs consist of normal human keratinocytes (NHK) grown and differentiated on a fibroblast-populated collagen gel matrix and resemble in vivo skin structure, including stratum corneum formation. Under these culture conditions, hCAP18/LL-37 reactivity was apparent in both basal and suprabasal keratinocyte layers, although expression increased in differentiated cell layers (Fig 1a). Expression in these layers appeared to be localized mainly within cells, but scattered expression in intercellular spaces within the stratum corneum could also be appreciated. The keratinocyte differentiation marker involucrin showed reactivity only in the suprabasal layers of the epidermis and in the stratum corneum (Fig 1b). When these images were merged, hCAP18/LL-37 was found to partially colocalize with involucrin in suprabasal keratinocytes by confocal microscopy (Fig 1c).

To further investigate hCAP18/LL-37 subcellular localization in keratinocytes, immunogold electron microscopy was performed using the Apligraf human skin equivalent. This commercial construct is similarly comprised of differentiated keratinocytes grown on a fibroblast-populated matrix. Immunogold electron microscopy revealed specific labeling of hCAP18/LL-37 in granules, some of which displayed a characteristic lamellar structure (Fig 2). Identical skin sections incubated with rabbit pre-immune serum were negative (data not shown). Because of the electron microscopy processing techniques required to preserve cathelicidin antigenicity, the lamellar structure could not be observed in all cathelicidin-containing granules. Biochemical lamellar body isolation techniques were therefore undertaken to confirm the localization of cathelicidin in these keratinocyte granules.

Lamellar bodies were enriched from adult wild-type mouse skin by selective sequential filtration (Grayson et al., 1983, 1985). Granule preparations were then concentrated and subjected to immunoblot analysis for detection of the murine ortholog of hCAP18/LL-37 (mCRAMP) using a polyclonal rabbit anti-mCRAMP antibody. Filtrates were simultaneously assayed for total and acid phosphatase activity to confirm lamellar body enrichment in each fraction. The mCRAMP antibody was reactive with lamellar body-containing fractions, particularly the concentrated >30 kDa filtrates, from mouse epidermis (Fig 3). Mouse bone marrow, which expresses high levels of mCRAMP, along with synthetic mCRAMP peptide, served as positive controls for immunoblotting (data not shown). Since lamellar bodies are...
enriched in acid hydrolases, detection of increased total phosphatase activity in the concentrated >30 kDa filtrates confirmed that lamellar bodies were most abundant in these mCRAMP-reactive filtrates (Fig 3). Additional experiments to specifically evaluate acid phosphatase activity confirmed that the total phosphatase detected in mCRAMP-reactive fractions shown in Fig 3 represented acid hydrolase activity (data not shown). These assays further support localization of cathelicidin in keratinocyte lamellar bodies.

The subcellular distribution of cathelicidin in keratinocytes grown on tissue culture plates was also investigated. Immunofluorescence microscopy was performed on monolayer cultures of undifferentiated NHK or immortalized HaCaT keratinocytes. Using antibodies against either hCAP18/LL-37 or its conserved cathelin domain, cathelicidin was observed in a punctate perinuclear distribution pattern in cultured keratinocytes (Fig 4a). No differences in localization were detected between hCAP18/LL-37 and the cathelin domain or between NHK and HaCaT cells (data not shown). Pre-immune chicken IgY controls were negative. The perinuclear distribution pattern of cathelicidin resembled that of the Golgi apparatus, so colocalization studies were performed on NHK using a combination of antibodies recognizing either hCAP18/LL-37 or the cytoplasmic Golgi 58K Protein, a microtubule-binding protein that serves as a marker for the Golgi apparatus. Immunofluorescence microscopy suggested partial localization of keratinocyte cathelicidin within the Golgi apparatus (Fig 4b).

The expression and processing of cathelicidin by cultured keratinocytes was further studied by western blot analysis of keratinocyte extracts and their conditioned media. Analysis of dilute keratinocyte extracts by direct
application to SDS-PAGE gels was limited by the sensitivity of the Western blot and by the sample volume. Therefore, extracts were immunoprecipitated using a polyclonal rabbit anti-hCAP18/LL-37 antibody. Analysis of these immunoprecipitated samples, as well as pure synthetic peptide or recombinant protein, revealed expected bands corresponding to the IgG used for the immunoprecipitation in addition to bands at \( \sim 18 \) and \( \sim 5 \) kDa corresponding to the precursor form of cathelicidin (hCAP18) and the mature peptide (LL-37), respectively (Fig 5). Cathelicidin was not detected in immunoprecipitations of conditioned media. Based on the LL-37 peptide control precipitated from an identical volume of extraction buffer, the concentration of LL-37 in keratinocyte extracts was estimated to be approximately 32 nM.

Upon confirmation of cathelicidin localization and processing in keratinocytes, cathelicidin expression was assessed upon incubation of keratinocytes with bacteria. By confocal microscopy, keratinocytes were found to internalize heat-killed, fluorescent Escherichia coli BioParticles, which colocalized with cathelicidin both intracellularly and near the cell surface (Fig 6). The cell surface colocalization may represent lamellar body secretion into intercellular spaces near sites of bacterial contact with keratinocytes.

**Discussion**

Under inflammatory conditions, cathelicidin is deposited at sites of injury by both neutrophils and keratinocytes. In neutrophils, which contain up to 1.34 \( \mu \)M hCAP18/LL-37 during inflammation, cathelicidin is stored in secondary (specific) granules. Neutrophil primary (azurophil) granules contain various other antimicrobial peptides and processing enzymes, including defensins, elastase, and proteinase 3 (Cowland et al, 1995; Sorensen et al, 1997; Turner et al, 1998). Specific granules generally secrete their contents extracellularly, whereas azurophil granules deliver their contents to intracellular phagolysosomes. Separation of cathelicidins from their corresponding processing enzymes may function to maintain the peptides as an inactive pool until proteolysis occurs during inflammation. Since keratinocytes are not considered to be a major source of cathelicidin in normal skin, their relative contribution to cutaneous host defense in comparison with traditional immune effector cells such as neutrophils is unclear (Sorensen et al, 1997). Therefore, this study set out to identify sites of subcellular localization and activation of cathelicidin in keratinocytes.

In previous studies, involucrin reactivity has primarily been shown in granular layer keratinocytes and in the inner stratum corneum; involucrin has also been detected in cytoplasmic granules of both the suprabasal epidermis and cultured cells (Warhol et al, 1985; Murthy et al, 1993). Further, lamellar granule-associated protein (LGP) is expressed in coordination with the cornified cell envelope precursor proteins involucrin and loricrin during epidermal development (Akiyama et al, 1999). hCAP18/LL-37 colocalization with involucrin suggests that cathelicidin is stored in granules, possibly lamellar bodies, and that cathelicidin expression increases during keratinocyte differentiation. Of note, keratinocytes cultured as monolayers and stimulated to differentiate solely by increasing calcium concentration do not increase cathelicidin expression relative to cells grown in low calcium conditions (Sorensen et al, 2003). Keratinocytes must be cultured as three-dimensional skin constructs in order to achieve terminal differentiation and to
form lipid profiles that resemble in vivo skin (Uchida et al., 2001).

Some of the granules in which cathelicidin was detected by immunogold electron microscopy displayed stacks of lamellae, whereas other granules were the appropriate size of lamellar bodies but lacked the characteristic internal structure. The loss of lipid structures during cryopreservation and antigen retrieval is not uncommon during processing for electron microscopy, so further experiments were performed to confirm the presence of cathelicidin in lamellar bodies isolated from skin. Since lamellar bodies are enriched in acid hydrolases, particularly glucosidase, acid phosphatase, phospholipase A, and sphingomyelinase, these enzymes serve as useful markers for identification of these granules (Freinkel and Traczyk, 1983). Therefore, phosphatase assays measuring hydrolysis of p-nitrophenyl phosphate (pNPP), a phosphatase substrate, were performed to complement the detection of cathelicidin in isolated granules by immunoblot (Okawara et al., 1972; Freinkel and Traczyk, 1981). Although these techniques are useful for detecting cathelicidin expression and acid phosphatase activity associated with lamellar bodies, they do not distinguish whether cathelicidin is bound to lamellar body membranes or fully contained within these granules.

These studies suggest that cathelicidin partially localizes to keratinocyte granules, including lamellar bodies. Some staining is also seen in the intercellular spaces, which is consistent with prior studies showing that cathelicidin is deposited in the extracellular spaces of the stratum corneum in a pattern similar to that shown for hBD-2 (Dorschner et al., 2001; Liu et al., 2002; Oren et al., 2003). Lamellar bodies may therefore act as storage sites for cathelicidin and offer a potential mechanism for extracellular secretion of this antimicrobial peptide upon injury or even during normal keratinocyte differentiation and stratum corneum formation. The recent detection of β-defensin storage in lamellar bodies suggests that these granules comprise an important source of skin antimicrobial peptides (Oren et al., 2003). The Golgi origin of lamellar bodies further supports the finding that cathelicidin localizes in both the Golgi apparatus of cultured keratinocytes and in the lamellar bodies of differentiated keratinocytes that comprise the skin (Madison et al., 1998).

The detection of proteolytically processed cathelicidin in keratinocyte extracts suggests that keratinocytes have the enzymatic machinery necessary for intracellular activation of this antimicrobial peptide for potential function in skin defense. Prior analysis of stratum corneum from normal and virally infected skin has in fact shown that cathelicidin present in this compartment is processed to the mature ~5 kDa form that is presumably LL-37 or a recently described cathelicidin of similar size such as KS-30 or RK-31 (Conner et al., 2002; Murakami et al., 2004). The absence of detectable levels of processed LL-37 in keratinocyte conditioned medium may be because of the predominance of intracellular activation and antimicrobial activity, the presence of protease inhibitors or LL-37 binding proteins in the intercellular space, or the degradation of LL-37 upon secretion from keratinocytes. Further, intracellular LL-37 secretion and processing may only occur in response to an inflammatory stimulus such as skin injury or bacterial infection. The confocal microscopy data showing that keratinocytes internalize bacteria, which then partially colocalize with granular cathelicidin, suggests a direct role for intracellular cathelicidin processing in keratinocytes antimicrobial activity. Experiments aimed to determine the relative contribution of cathelicidin to keratinocyte antimicrobial activity are currently underway.

In conclusion, keratinocytes synthesize, store and activate cathelicidin antimicrobial peptides. Keratinocytes also internalize bacteria, which colocalize with endogenous cathelicidin. These observations illustrate the potential contribution of keratinocytes to cutaneous defense and confirm the role of the epidermis as an independent direct immune barrier. The clinical consequences of defective antimicrobial peptide production by the epidermis are evident in atopic dermatitis patients, who lack the ability to normally increase antimicrobial peptide release during inflammation and have a greatly increased risk of infection (Ong et al., 2002). Therefore, understanding the regulation of keratinocyte-derived antimicrobial peptides may have an important role in the treatment of skin disease.

Materials and Methods

Immunofluorescence microscopy of skin constructs Skin constructs were generated by growing and differentiating primary keratinocytes on a collagen-embedded fibroblast matrix for 10 d as previously described (Watson et al., 1994; Ponec et al., 2002). Constructs were paraffin-embedded, fixed in paraformaldehyde, and sectioned for immunohistochemistry. Following hydration and antigen retrieval steps, sections were blocked with 5% donkey serum/goat serum and then incubated simultaneously with polyclonal chicken anti-hCAP18/LL-37 and monoclonal mouse anti-involucrin primary antibodies. Donkey anti-chicken tetramethylrhodamine isothiocyanate (TRITC) and goat anti-mouse IgG conjugated to AlexaFluor488 (Molecular Probes, Eugene, Oregon) were used as secondary antibodies, respectively. Sections were mounted in ProLong Anti-Fade reagent (Molecular Probes). Images were obtained using a Zeiss LSM510 laser scanning confocal microscope (Carl Zeiss Light Microscopy, Göttingen, Germany) coupled with an Axiovert 100 (Carl Zeiss) inverted stage microscope.

Immunogold electron microscopy Immunogold electron microscopy was performed using Apilgraf human skin constructs (Organogenesis, Canton, Massachusetts). Briefly, samples were fixed overnight at 4°C in Immunofix containing 0.5% glutaraldehyde and then transferred to 0.1 M cacodylate buffer (pH 7.4) containing 0.1 M glycine for 15 min at room temperature. Samples were dehydrated to 50% ethanol, incubated for 30 min in 70% ethanol containing 0.2% uranyl acetate, and further dehydrated to 95% ethanol. Samples were then placed in a 1:1 mixture of LR White resin (Ted Pella Inc., Redding, California) in 95% ethanol, rotated overnight at room temperature, and infiltrated with 100% LR White resin for another 7 h. Strips of Apilgraf were placed in gelatin capsules that were quickly filled with resin to which LR White Accelerator had been added (one drop accelerator per 10 mL resin). Blocks hardened within 15 min and sections were collected on 1% parlodion-coated nickel grids. Immunogold labeling was performed as previously described (Deftos et al., 1993; Springer and Haywood-Reid, 1993), using a 1:5 dilution of polyclonal rabbit anti-hCAP18/LL-37 antibody and goat anti-rabbit IgG conjugated to colloidal gold. Rabbit pre-immune serum served as a negative control. Immunogold-labeled grids were examined using a Zeiss EM10B electron microscope at an accelerating voltage of 60 kV (VADSHS Core Imaging Facility, San Diego, California).
Lamellar body isolation by selective sequential filtration Lamellar bodies were isolated by selective sequential filtration as previously described (Grayson et al, 1983, 1985). Briefly, skin was removed from adult mice and the epidermis was separated from the dermis by overnight treatment with dispase (Roche, Indianapolis, Indiana). The isolated intact epidermis was then homogenized in a ground glass Dounce homogenizer, sonicated, and centrifuged at 700 × g for 10 min to pellet cell debris. The lamellar body-containing supernatant was then passed through a series of membrane filters, including 10, 5, 3, 1.2, 0.8, 0.6, 0.4, and 0.2 μm sizes (Millipore, Bedford, Massachusetts). Filters were collected following passage through the 0.6, 0.4, and 0.2 μm filters. BCA assays were performed to confirm equal protein loading among filters. These filters were then applied to 30 kDa molecular weight cut-off Microcon centrifugal filter devices (Millipore) in order to trap lamellar bodies whereas allowing passage of soluble cathelicidin peptides that were not associated with lamellar bodies. Fractions from the 0.6, 0.4, 0.2 μm, and 30 kDa filters were assayed by immunodot blot analysis to detect cathelicidin protein expression and by acid phosphatase assay to confirm lamellar body isolation. Animal use was done with approval of the VA San Diego Animal Use Subcommittee.

Immunodot blot analysis Expression of cathelicidin was evaluated in sequential size filtrates by immunodot blot analysis. mCRAMP synthetic peptide and mouse bone marrow served as positive controls. Briefly, filtrates were diluted in 1 mM TBS and blotted onto a nitrocellulose membrane. The membrane was blocked with 5% non-fat milk for 1 h at room temperature and incubated with polyclonal rabbit anti-mCRAMP antibody diluted in blocking solution for an additional hour at room temperature. The membrane was then incubated with goat anti-rabbit horseradish peroxidase antibody in blocking solution for 1 h at room temperature. Three 5-min washes were performed after each antibody step using 0.1% TBS-Tween. Blots were developed using standard chemiluminescence reagents (Perkin-Elmer Life Sciences, Boston, Massachusetts). Densitometry measurements were obtained using Chemilager 4400 (Alpha Innotech, San Leandro, California).

Acid phosphatase assay General phosphatase activity was assessed in sequential size filtrates by measuring pNPP hydrolysis according to the manufacturer's instructions (Upstate Biotechnology, Lake Placid, New York). Briefly, each reaction mixture contained 40 mM NiCl₂, 5 mg/mL BSA, lamellar body preparation (enriched in lamellar bodies) and pNPP substrate. Reaction mixtures were incubated at 37 °C for 20 min prior to substrate addition and then washed, fixed in cold methanol and immunostained as described above using either polyclonal chicken anti-hCAP18/LL-37 or monoclonal mouse anti-Golgi 58K Protein (Sigma, St Louis, Missouri). The Golgi 58 K Protein is a 58 kDa microtubule-binding protein located on the peripheral Golgi membrane. Donkey anti-chicken TRITC and goat anti-mouse IgG conjugated to AlexaFluor488 were used as secondary antibodies, respectively. Images were obtained using an Olympus BX41 fluorescent microscope (Scientific Instrument Company, Temecula, California).

Immunoprecipitation and western blot analysis To evaluate the expression of cathelicidin in keratinocytes, HaCaT keratinocytes were cultured to confluence in a 10-cm dish. Conditioned media was removed and stored on ice whereas cells were scraped from the plastic and extracted in 1% Triton-X 100/phosphate-buffered saline (PBS). Both keratinocyte extracts and conditioned media were sonicated on ice and spun down at 14,000 × g for 10 min. To immunoprecipitate, 1 mL keratinocyte extract and conditioned media were incubated for 1 h on ice with 1 μg rabbit anti-LL-37 polyclonal antibody, followed by overnight incubation at 4°C with Protein A-Agarose beads (Roche, Indianapolis, Indiana). Both full-length hCAP18/LL-37 keratinocyte extract and conditioned media were incubated for 1 h on ice with 1 μg rabbit anti-LL-37 polyclonal antibody, followed by overnight incubation at 4°C with Protein A-Agarose beads (Roche, Indianapolis, Indiana). Both full-length hCAP18/LL-37 and LL-37 peptide were used as positive controls. After several washes, samples were resuspended in SDS loading buffer and boiled for 10 min prior to loading on a 16% Tris- Tricine gel. The gel was run at 100 V for 1 h at room temperature and then transferred onto a nitrocellulose membrane at 100 V on ice for another hour. The membrane was blocked and then probed with 1:5000 rabbit anti-LL-37 and 1:5000 goat anti-rabbit horseradish peroxidase for 1 h each prior to developing with standard chemiluminescence reagents.

Bacterial internalization by confocal microscopy NHK were grown to 50% confluence and incubated overnight with E. coli TRITC BioParticles bacteria (Molecular Probes). Cells were washed, fixed in cold methanol and immunostained as described above using either polyclonal chicken anti-hCAP18/LL-37 or polyclonal chicken anti-cathelin domain, followed by goat anti-chicken fluorescein isothiocyanate. Images were obtained using a Zeiss LSM510 laser scanning confocal microscope coupled with an Axiovert 100 inverted stage microscope.

We wish to acknowledge Patricia Reid at the VASDHS Core Imaging Facility for her electron microscopy expertise. We also wish to thank Dr. Peter Elias at UCSF for providing valuable experimental insight. Funding for this research was provided by a VA Merit Award, NIH AI052453 and AR45676 (R. L. G.), NIH Training Grant DK07202 (M. H. B.).

DOI: 10.1111/j.0022-2052.2004.23443.x

Manuscript received January 22, 2004; revised June 2, 2004; accepted for publication June 8, 2004

Address correspondence to: Richard L. Gallo, 3350 La Jolla Village Drive, Mail Code 151, San Diego, California 92161, USA. Email: rgallo@vapop.ucsd.edu

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