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ORIGINAL ARTICLE

Degradation by Stratum Corneum Proteases Prevents Endogenous RNase Inhibitor from Blocking Antimicrobial Activities of RNase 5 and RNase 7

Arby Abtin[1], Leopold Eckhart[1], Michael Mildner[1], Minoo Ghannadan[1], Jürgen Harder[2], Jens-Michael Schröder[2] and Erwin Tschachler[1,3]

The antimicrobial defense of the skin is partially mediated by RNase 7, an abundant ribonuclease of the stratum corneum (SC). Here, we investigated the expression and regulation of members of the RNase A family and of the endogenous RNase inhibitor (RI) protein in epidermal keratinocytes (KCs). Reverse transcription-PCR screening revealed that KCs expressed not only RNase 7 but also RNase 5, which was shown earlier to kill the yeast *Candida albicans*, as well as RNase 1, RNase 4, and RI. The mRNA and protein levels of RNase 5, RNase 7, and RI increased during KC differentiation. When RNase 5 and RNase 7 were incubated with RI *in vitro*, not only their ribonucleolytic activities but also their antimicrobial activities were strongly suppressed. Immunochemical analyses revealed that SC contains RNase 5, whereas RI was not detectable. Unlike recombinant RNase 5, recombinant RI was degraded when exposed to SC extract. The addition of aprotinin prevented the degradation of RI, indicating that serine proteases of the SC cleave RI. Taken together, this study adds RNase 5 to the list of antimicrobial factors present in the SC and suggests that proteases contribute indirectly to the defense function of the SC by releasing the RI-mediated inhibition of RNase 5 and RNase 7.

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INTRODUCTION

The fact that the surface of human skin contains strong ribonuclease (RNase) activity is well known (Tabachnick and Freed, 1961; Steigleder and Raab, 1962). However, the physiological role of the skin ribonuclease activity and its control has not yet been characterized in depth. Recently, RNase 7 was isolated from human stratum corneum (SC), and was shown to exert antimicrobial activity (Harder and Schröder, 2002).

RNase 7 is a member of the human RNase A superfamily, which is encoded by a cluster of 13 genes located on chromosome 14q11.2. Only eight genes (*RNASE1–RNASE8*) of this family encode for proteins that are catalytically active to varying degrees against standard RNA substrates (Dyer and Rosenberg, 2006). In addition to their ribonuclease activity, some members of this family such as RNase 2 (eosinophil-derived neurotoxin), RNase 3 (eosinophil cationic protein), RNase 5, the above-mentioned RNase 7 and RNase 8 have antimicrobial properties as well (Lehrer *et al.*, 1989; Domachowske *et al.*, 1998; Harder and Schröder, 2002; Hooper *et al.*, 2003; Rudolph *et al.*, 2006), indicating a role in the innate immune defense. RNase 7 is active especially against *Enterococcus faecium*, *Pseudomonas aeruginosa*, and *Pichia pastoris*, and to a lesser degree against *Propionibacterium acnes* and *Escherichia coli* (Harder and Schröder, 2002; Huang *et al.*, 2007). It has been suggested that the antimicrobial activity of RNase 7 is due to pore formation and disruption of the bacterial membrane and is independent of the ribonuclease activity (Huang *et al.*, 2007). RNase 5, also known as angiogenin, has been implicated in blood vessel formation (Fett *et al.*, 1985), but a recent study revealed an antimicrobial activity of RNase 5, especially against *Candida albicans* (Hooper *et al.*, 2003). RNase 5 is secreted by the reconstructed human epidermis *in vitro* (Rendl *et al.*, 2001); however, its contribution to the skin defense is unknown.

Ribonuclease inhibitor (RI) is a horseshoe-shaped leucine-rich repeat protein (Kobe and Deisenhofer, 1993), which constitutes about 0.1% of the total protein in the cytosol of mammalian cells (Leland and Raines, 2001). Through its concave cavity, RI binds at a 1:1 stoichiometry to several members of the RNase A superfamily (Shapiro *et al.*, 1986; Shapiro and Vallee, 1987; Papageorgiou *et al.*, 1997; Maeda

[1]Department of Dermatology, Medical University of Vienna, Vienna, Austria; [2]Department of Dermatology, University Hospital Schleswig-Holstein, University of Kiel, Kiel, Germany and [3]Centre de Recherches et d'Investigations Epidermiques et Sensorielles, Neuilly-sur-Seine, France

Correspondence: Dr Leopold Eckhart, Department of Dermatology, Medical University of Vienna, Waehringer Guertel 18-20, Vienna 1090, Austria. E-mail: leopold.eckhart@meduniwien.ac.at

Abbreviations: Ab, antibody; CFUs, colony-forming units; DEPC, diethylpyrocarbonate; KCs, keratinocytes; PBS, phosphate-buffered saline; qRT-PCR, quantitative real-time PCR; RI, ribonuclease inhibitor; RNase, ribonuclease; SC, stratum corneum; TSB, tryptic soy broth

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et al., 2002; Iyer *et al.*, 2005; Johnson *et al.*, 2007). RI has been shown to inhibit the enzymatic activity of RNases, as well as the antiretroviral activity of RNase 2 (Domachowske and Rosenberg, 1997) and the angiogenic activity of RNase 5 (Shapiro and Vallee, 1987). By contrast, the killing activity of RNase 3 toward microfilaria and trypanosomes is not blocked by RI (Molina *et al.*, 1988; Hamann *et al.*, 1990).

Here, we have investigated the regulation of RNase–RI interactions in the outer layers of the epidermis. We show that *in vitro*, the ribonuclease and antimicrobial activities of RNase 5 and RNase 7, which are both expressed by KCs and present in the SC, are sensitive to RI. Despite the fact that, similar to RNase 5 and RNase 7, RI is also upregulated during terminal KC differentiation, it is virtually absent from normal SC. Co-incubation of RI and SC extracts revealed the existence of SC proteolytic activity that is able to degrade RI. We propose that by degrading RI, proteases of the SC contribute to the antimicrobial defense of the skin.

RESULTS

Epidermal KCs express RNases 1, 4, 5, and 7, as well as RI

To provide the basis for a better understanding of the potential roles of RNase–RI interactions in the skin, we determined the mRNA expression profile of RNases 1–8 and of RI in cultured epidermal KCs. RNases 1, 4, 5, and 7, as well as RI were detected by reverse transcription-PCR, whereas other RNases were not. RNases 4, 5, and 7 were expressed in both proliferating (data not shown) and differentiated KCs (Figure 1a). Differentiated KCs also expressed RNase 1 splice variants, 2 (lower band, GenBank acc. no. NM198234) and 4 (upper band, GenBank acc. no. NM002933), which differ in the 5′-untranslated region but code for the same protein. For RNase 4, splice variant 2 (GenBank acc. no. NM002937) and for RNase 5, splice variant 1 (GenBank acc. no. NM001145) could be amplified (Figure 1a, upper panel). The identity of the PCR products was determined by direct sequencing, and the integrity of the assays for RNases 2, 3, 6, and 8, which were not detected at the cDNA level, was confirmed by PCR amplification using genomic DNA as a template (Figure 1a, lower panel). The expression of RNases 9–13 was not investigated, as they do not have all the elements to support ribonuclease activity (Dyer and Rosenberg, 2006).

Focusing on RNases with antimicrobial activities (Harder and Schröder, 2002; Hooper *et al.*, 2003), we investigated the regulation of RNase 5 and RNase 7 during KC differentiation. By quantitative real-time PCR (qRT-PCR), we found that the expressions of RNase 5 and RNase 7 mRNA were upregulated four- and nine-fold, respectively, in differentiated cultures compared with proliferating KCs (Figure 1b and c). The concentrations of secreted RNase 5 and RNase 7 proteins were determined by ELISA. Culture medium conditioned by differentiated KCs for 24 hours contained approximately four- and six-fold higher levels of RNase 5 and RNase 7, respectively, than medium conditioned by proliferating cells (Figure 1d and e). As the cell biomass, estimated by protein quantification, was elevated by only 1.7-fold (data not shown), these data show that the production of RNase 5 and RNase 7 was upregulated during differentiation of KCs. The absolute concentration of secreted RNase 5 was smaller than that of RNase 7 (Figure 1d and e). Similarly, the amount of soluble RNase 5 that we could extract from the SC of human heels, that is, 57 ± 38 pg per mg SC, was much smaller than the amount of RNase 7 that has been extracted from the SC of different donors, that is, up to 6,000 pg per mg SC (Harder and Schröder, 2002).

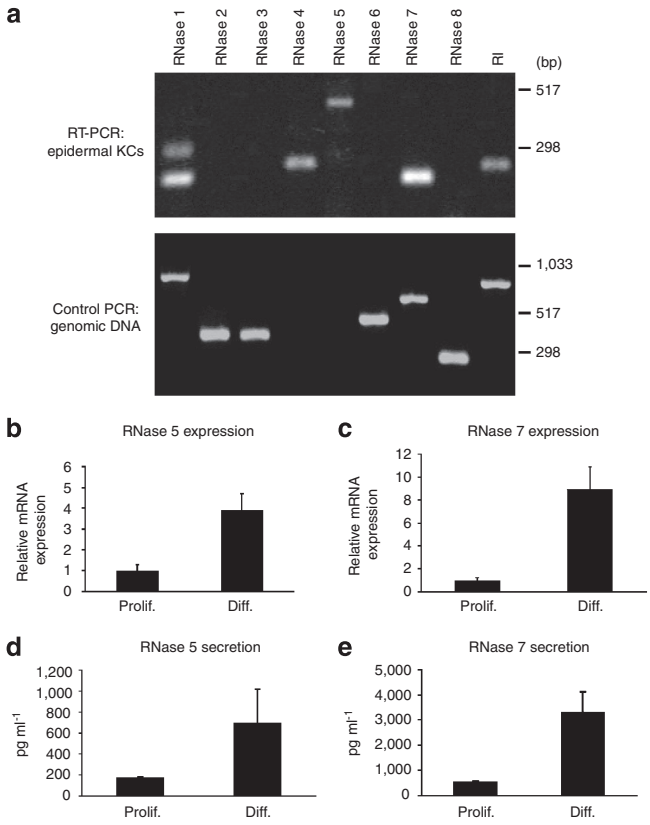

Figure 1. KCs express RNases 1, 4, 5, and 7, as well as RI. Total RNA was isolated from proliferating (prolif.) and differentiated (diff.) human KC cultures and reverse-transcribed to cDNA. (**a**) cDNAs of diff. KCs were amplified by PCRs specific for RNases 1–8 and for RI. Amplicons were separated on a 1.5% agarose gel containing ethidium bromide. To confirm the efficiency of each PCR, reactions with the same primer combinations were performed on genomic DNA. In these control reactions, exonic and intronic sequences of all genes except RNases 4 and 5, in which the primer annealing sites are separated by a long intron, were successfully amplified (lower panel). (**b**) RNase 5 and (**c**) RNase 7 expressions in KCs were determined by qRT-PCR. Relative gene expression levels were normalized to the expression of the housekeeping gene ALAS. The mean values of the expression levels relative to the expression level in proliferating cells are displayed. Secretion of (**d**) RNase 5 and (**e**) RNase 7 of proliferating and differentiated KCs was determined by ELISAs. Data represent the mean ± SD. bp, base pair.

RI is expressed in the epidermis but is absent in the SC

We next investigated the expression of RI in human skin ($n = 5$) by immunohistochemistry. RI was expressed in all viable layers of the epidermis, with the strongest expression being detectable in the uppermost layers of the epidermis. By contrast, RI immunoreactivity was not detectable in the SC (Figure 2a).

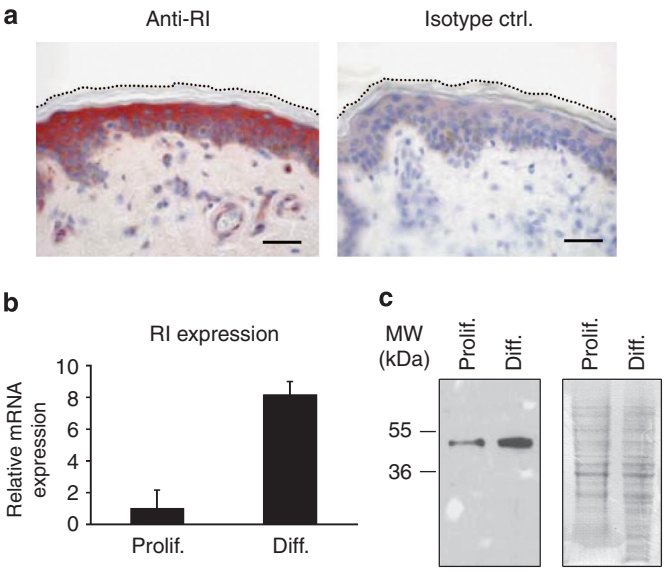

Figure 2. RI is expressed in the epidermis and in cultured KCs. (**a**) RI was detected by immunohistochemistry in normal human skin. An isotype antibody was used as negative control (ctrl.). The outer border of the SC is marked by a dotted line. Bars = 40 μm. (**b**) qRT-PCR of RI in proliferating (prolif.) and differentiated (diff.) KCs. Relative gene expression levels were normalized to the expression of the housekeeping gene *ALAS*. Data represent the mean ± SD. (**c**) Western blot analysis of RI in prolif. and diff. KCs (left panel). Ponceau staining of the membrane served as loading control of protein lysates (right panel). MW, molecular weight; RI, ribonuclease inhibitor.

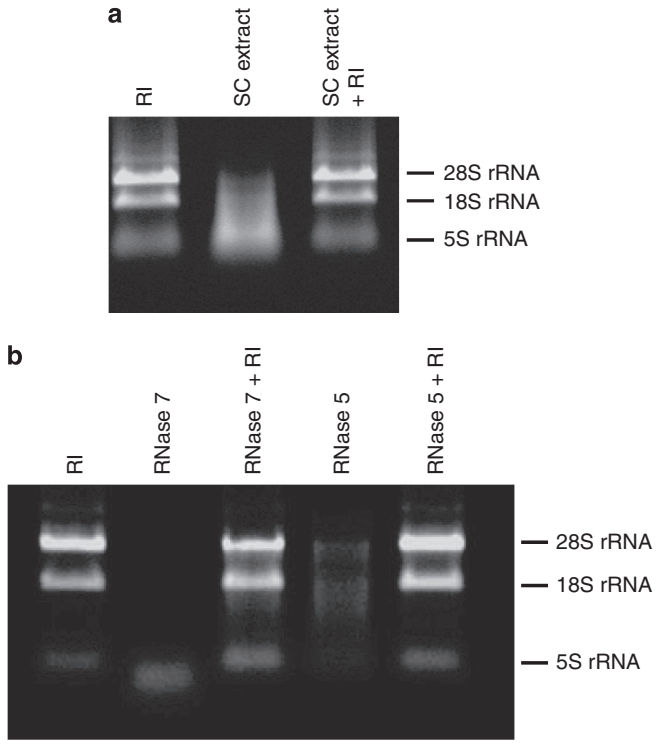

Figure 3. Ribonuclease activities of SC extract, RNase 5, and RNase 7 are blocked by RI. (**a**) SC extract (30 ng), (**b**) RNase 5 (25 ng), and RNase 7 (5 ng) were pre-incubated with or without RI (30 U for SC extract and 40 U for RNase 5 and RNase 7). Subsequently, the mixtures were incubated with total RNA (1.5 μg) prepared from KCs as described in the Materials and Methods section. Thereafter, the samples were electrophoresed through an RNase-free agarose gel containing ethidium bromide. Positions of the 28S, 18S, and 5S rRNA bands are indicated. U, units.

The expression of RI during KC differentiation *in vitro* was investigated by qRT-PCR and western blot analysis. RI was upregulated at both the mRNA (Figure 2b) and protein levels (Figure 2c) in differentiated versus proliferating KCs.

RI blocks ribonuclease activity of SC as well as the antimicrobial activity of RNase 5 and RNase 7

In accordance with an earlier report (Probst *et al.*, 2006), we readily detected ribonuclease activity in SC extracts. The addition of recombinant human RI virtually abrogated this activity, suggesting that the RNases of the SC can be bound and neutralized by RI (Figure 3a). Moreover, RI blocked the ribonuclease activity of recombinant RNase 5 and purified RNase 7 from SC *in vitro*, confirming the functionality of our proteins (Figure 3b).

We next tested whether RI is also able to interfere with the antimicrobial activities of RNase 5 and RNase 7 against *C. albicans* and *E. faecium*, respectively (Harder and Schröder, 2002; Hooper *et al.*, 2003). In the absence of RI, antimicrobial activity of RNase 5 and RNase 7 strongly reduced the colony-forming units (CFUs) of *C. albicans* and *E. faecium*, respectively. Pre-incubation with RI strongly suppressed the antimicrobial activity of both RNases, whereas the addition of RI alone had no effect on either microbe (Figure 4a and b). These results show that RI not only blocks ribonuclease activity but also suppresses the antimicrobial activity of RNase 5 and RNase 7, which to our knowledge has not been reported earlier.

The antimicrobial effect of RNase 5 is blocked by chemical inhibitors of its ribonuclease activity

As RI interacts with a large portion of the surface of target RNases (Papageorgiou *et al.*, 1997) and not specifically with the catalytic center, the blockade of antimicrobial activities of RNase 5 and RNase 7 may be mediated by interference with the RNase catalytic activity or by sterical blockade of a bactericidal protein domain. The antimicrobial activity of RNase 7 is mediated by a cluster of lysine residues and does not depend on catalytic activity (Huang *et al.*, 2007). Amino-acid sequence alignment showed that the residues critical for the antibacterial activity of RNase 7 are not conserved in RNase 5 (Figure S1). Therefore, we hypothesized that RNase 5 utilizes a different mechanism, possibly involving its ribonucleolytic activity to kill *Candida*. RNase 5 was pre-incubated with two chemical inhibitors of its ribonuclease activity, namely, diethylpyrocarbonate (DEPC) (Shapiro *et al.*, 1987) and benzopurpurin B (Jenkins and Shapiro, 2003). As expected, both substances inhibited the degradation of RNA by RNase 5 in a dose-dependent manner (Figure 5a and b). In close correlation with this blockade, both DEPC and benzopurpurin B also inhibited the candida-cidal activity of RNase 5 (Figure 5c and d), indicating that the antimicrobial effect of RNase 5 depends on its ribonucleolytic activity.

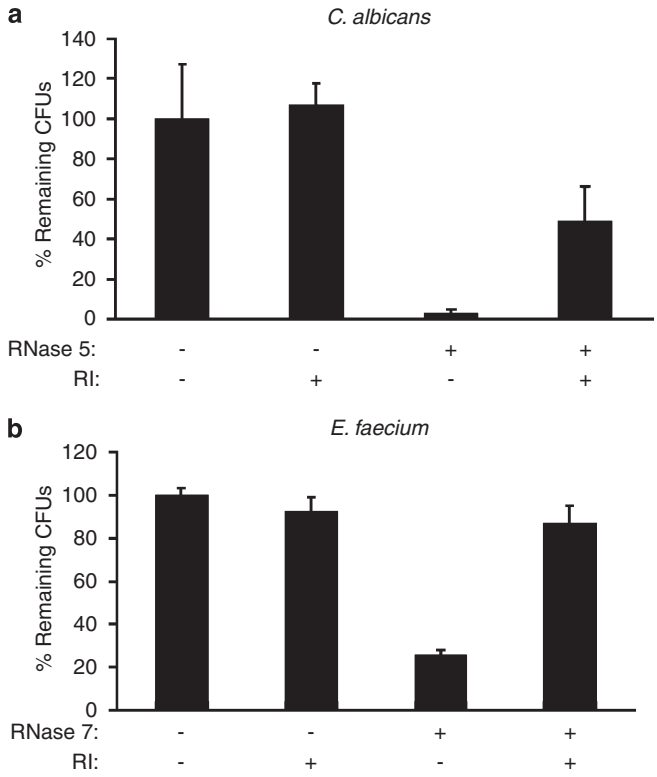

Figure 4. RI inhibits antimicrobial activity of RNase 5 and RNase 7. (**a**) RNase 5 (0.2 μM) or (**b**) RNase 7 (0.33 μM) was incubated with and without RI (40 and 20 U for RNase 5 and RNase 7, respectively) and then tested for their ability to inhibit the growth of *C. albicans* or *E. faecium*, respectively. In addition, *C. albicans* or *E. faecium* was treated with buffer and RI only. The results are displayed as the percentage of the remaining CFUs in relation to untreated controls (100%). Data represent the mean of triplicates ± SD. CFU, colony-forming units.

To further characterize the antimicrobial potential of RNase 5, we also tested *Pseudomonas aeruginosa*, *E. coli*, methicillin-resistant *Staphylococcus aureus*, *Enterococcus faecium*, and group A *Streptococcus* for their sensitivity toward RNase 5 treatment. However, among the microorganisms investigated, RNase 5 was only effective in killing *C. albicans* (Figure S2). To evaluate whether enhancement of the expression of RNase 5 might contribute to the control of its antimicrobial activity, KCs were stimulated with heat-inactivated *C. albicans* or with the proinflammatory cytokines, IL-1α and IL-6. Neither treatment altered the expression of RNase 5 in KCs (Figure S3). Similarly, the expression of the RNase 5 inhibitor, RI, was not changed by proinflammatory stimuli (Figure S3).

RI is degraded by SC extract

As immunohistochemistry suggested that RI, although present in the viable epidermal layers, was absent from SC (Figure 2a), we extracted proteins from the total epidermis and from SC alone and performed western blot analysis. RI was detected in lysates of the epidermis but, confirming the immunohistochemistry data, was absent in SC extracts (Figure 6a). The integrity of SC extract was confirmed by western blot analysis of caspase-14, which was readily detectable in its mature form (Figure 6a) (Fischer *et al.*, 2004).

The fact that only the viable layers of the epidermis but not the SC contained RI, indicated the existence of a mechanism to eliminate RI during conversion of KCs into corneocytes. To investigate this hypothesis, recombinant human RI was co-incubated with SC extracts and then analyzed by western blot. Under these conditions, RI protein was degraded, with an intermediate cleavage product of RI being detectable (Figure 6b). Incubation of RI alone with the extraction buffer

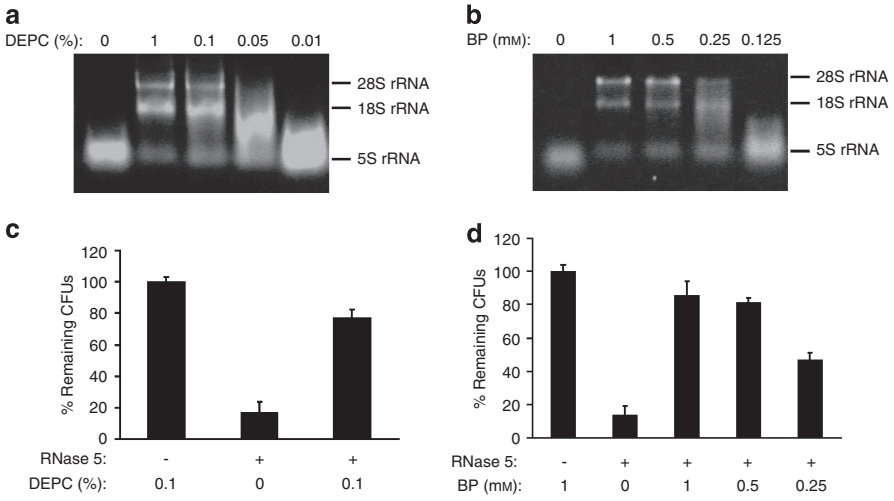

Figure 5. Antimicrobial effect of RNase 5 depends on its ribonuclease activity. (**a**) Ribonuclease activity of RNase 5 (0.6 μM) was tested in the presence of diethylpyrocarbonate (DEPC) or (**b**) benzopurpurin B (BP) as described in the Materials and Methods section. Positions of the 28S, 18S, and 5S rRNA bands are indicated. (**c**) RNase 5 (0.2 μM) was pre-incubated with DEPC or (**d**) BP and thereafter tested for its ability to inhibit the growth of *C. albicans*. The results are displayed as the percentage of the remaining CFUs in relation to only DEPC- or BP-treated controls (100%). Data represent the mean of triplicates ± SD.

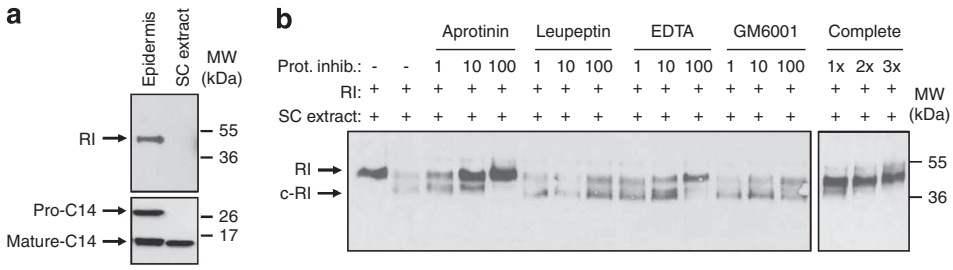

Figure 6. RI is absent in the SC and is degraded by SC extract. (**a**) Epidermis and SC were extracted as described in the Materials and Methods section and subjected to western blot analysis for RI and caspase-14 (C14). Positions of a molecular weight (MW) marker are indicated on the right. Note that the SC contains only the mature form of caspase-14 (mature-C14) (Fischer *et al.*, 2004) whereas both pro-caspase-14 (pro-C14) and mature-C14 are present in total epidermis. (**b**) SC extract were pre-incubated with increasing concentrations (1–100 μM) of the protease inhibitors aprotinin, leupeptin, EDTA, GM6001, or complete protease inhibitor cocktail, EDTA free (complete; 1 × to 3 ×) and subsequently incubated with RI, as described in the Materials and Methods section. RI was detected by western blot analysis. prot. inhib., protease inhibitor; c-RI, cleaved RI protein.

showed the stability of the protein under assay conditions. To investigate the types of enzymes that are responsible for RI degradation, SC extracts were pretreated with various protease inhibitors (that is, aprotinin for serine proteases, leupeptin for thiol proteases, EDTA and GM6001 for metalloproteases, and a protease inhibitor cocktail (EDTA free) for serine and cysteine proteases) and then analyzed for their ability to degrade RI. The inhibitor cocktail, as well as aprotinin and EDTA, inhibited proteases from degrading RI (Figure 6b). It can be noted that EDTA was not effective at a concentration that is considered sufficient to block metalloproteases, but only at a concentration at which EDTA also interferes with the activity of some serine proteases (Beynon and Bond, 2001). The metalloprotease-specific inhibitor, GM6001, showed virtually no inhibition. This inhibition profile suggested that serine proteases are mainly accountable for RI degradation by SC extracts. In contrast to RI, recombinant RNase 5 was stable when co-incubated with SC extracts (Figure S4).

DISCUSSION

In this study, we have explored the expression of all functional members of the RNase A superfamily, as well as that of RI in epidermal KCs, and propose that differential breakdown of RI during SC formation contributes to the antimicrobial activity of the skin surface. Our expression screening showed that four members of the RNase A family are expressed by KCs. Two of these RNases, namely, RNase 5 and RNase 7, act as antimicrobial proteins, whereas no such activity has been reported for RNase 1 and RNase 4. This study shows the presence of RNase 5 in human SC and thereby establishes another antimicrobial factor of the skin defense against microbes. Similar to RNase 7, RNase 5 was upregulated during differentiation. However, proinflammatory or microbial inducers of expression could thus far be identified for RNase 7 only (Harder and Schröder, 2002). The rate of production of RNase 7 was approximately five times higher than that of RNase 5 in cultured KCs, and much higher amounts of RNase 7 than RNase 5 could be extracted from human SC, indicating that RNase 7 contributes more to the ribonucleolytic and antimicrobial activities of the skin surface. Nevertheless, it is conceivable that the local concentration of RNase 5, within distinct SC microcompartments, may be sufficient for effective antimicrobial activity.

Importantly, the antimicrobial activity spectra of RNase 5 and RNase 7 appear to differ significantly. In particular, *C. albicans* is killed by low concentrations of RNase 5 (Hooper *et al.*, 2003; this study) but much less effectively by RNase 7 (Harder and Schröder, 2002), whereas several bacteria are killed by RNase 7 (Harder and Schröder, 2002) and not by RNase 5 (this study). The relative contribution of RNase 5 to the innate immune defense of the skin, which is mediated by a plethora of peptides and other substances (Schröder and Harder, 2006), remains to be determined in future studies.

Our study provides a characterization of endogenous RI in the epidermis and, importantly, extends its range of functions to the inhibition of RNase antimicrobial activities. Both the candida–cidal activity of RNase 5 and the enterococcus–cidal activity of RNase 7 were effectively blocked by recombinant RI. Earlier reports have shown that RI suppresses the ribonuclease activity, which is low compared with other members of the RNase A family, and the angiogenic activity of RNase 5 (Shapiro *et al.*, 1986; Shapiro and Vallee, 1987; Papageorgiou *et al.*, 1997; Maeda *et al.*, 2002; Iyer *et al.*, 2005; Johnson *et al.*, 2007). Two small chemical substances, DEPC and benzopurpurin B, which inhibit the catalytic activity of RNase 5, also suppressed its antimicrobial activity. The binding site of benzopurpurin B on RNase 5 has been determined (Jenkins and Shapiro, 2003), whereas the various RNase 5 subsites, likely to be targeted by DEPC through the modification of lysine and histidine residues, have not been investigated for their individual contribution to the inhibitory effect of DEPC. Therefore, it appears likely that RI suppresses the antimicrobial function of RNase 5 by inhibiting its catalytic center. However, further studies using point-mutated variants of RNase 5 are required to substantiate this hypothesis.

Although the antimicrobial activity of RNase 7 does not depend on ribonucleolytic activity (Huang *et al.*, 2007), our data show that it is suppressed by RI. There are two possible explanations for this finding: (1) It is possible that RI, which has a horseshoe-like shape and binds RNases within its concave cavity, masks the amino-acid residues responsible

for antimicrobial action. (2) The interaction of RI with RNase 1 has recently been shown to induce conformational changes of RNase 1 (Johnson *et al.*, 2007). As the interaction of RI and RNase 7 is likely to occur in a similar manner, a conformational change might also abrogate the antimicrobial activity of RNase 7.

The finding that RI is expressed strongly in the uppermost layers of the epidermis but is absent in SC, and the fact that SC contains ribonuclease activity and antimicrobial activity of RNase 7 (Harder and Schröder, 2002), indicated that a physiological mechanism exists to prevent the inhibition of skin surface ribonuclease functions. In support of this hypothesis, the results of our co-incubation experiments using recombinant RI and SC extract showed degradation of RI. Our finding that aprotinin blocked the degradation of RI indicates that this breakdown is mediated by serine proteases, such as SCTE (SC tryptic enzyme, kallikrein 5) or SCCE (SC chymotryptic protease, kallikrein 7). EDTA, which scavenges divalent cations, also blocked the reaction; however, only when it was used at a concentration higher than that required to block metalloproteases but sufficient to also inhibit some serine proteases (Beynon and Bond, 2001). Further studies are necessary to determine the molecular identity of the RI-degrading protease(s).

The proteolysis of RI by SC proteases establishes a role of proteases in the antimicrobial defense function of the skin. Conceptually, this control mechanism resembles the control of the pro-apoptotic DNase, caspase-activated DNase (CAD)/DNA fragmentation factor B (DFFB) by the inhibitor of CAD (ICAD)/DNA fragmentation factor A (DFFA). ICAD inhibits CAD unless it is cleaved by the cysteine protease caspase-3 (Sakahira *et al.*, 1998). It remains to be investigated whether proteolysis of RI enhances the breakdown of cellular RNAs during differentiation-associated programmed cell death of KCs. Irrespective of this potential role in cellular remodeling, breakdown of RI appears to facilitate the activities of RNase after corneocyte formation. A more direct dependence on proteases of protein activation in the SC has been shown for cathelicidin, which is converted into the antimicrobial peptide, LL-37, by the action of the serine proteases, kallikrein 5 (SCTE) and kallikrein 7 (SCCE) (Yamasaki *et al.*, 2006). The processing of cathelicidin by these proteases does not require a distinct stimulus and leads to the constitutive presence of LL-37 on the skin surface (Yamasaki *et al.*, 2006). Similarly, the proteolytic degradation of RI is likely to occur in a constitutive manner. Taken together, the disturbance of SC proteolytic activities, either caused by genetic defects or by environmental influences, may compromise antimicrobial skin defense.

In summary, this study identifies RNase 5 as a component of the innate immune system of the skin and reveals an additional level of the regulation of antimicrobial RNases in human SC, that is, the removal of their inhibitor by proteases.

MATERIALS AND METHODS

Cell culture

Human primary KCs prepared from neonatal foreskin were obtained from Clonetics (San Diego, CA) and cultured in serum-free keratinocyte growth medium (Clonetics) as described earlier (Rendl *et al.*, 2002). Third-passage KCs were cultured in 12-well tissue culture plates (Corning Incorporated, Corning, NY) and used at a confluence of 60–70% (designated as proliferating) or maintained for 4 days after reaching confluence (designated as differentiated) by replacing the medium every 24 hours. The differentiation state of KCs was confirmed by determination of the differentiation marker, filaggrin. Cultures were grown in triplicates. For *in vitro* stimulation, recombinant IL-1α and IL-6 (R&D Systems, Minneapolis, MN) were used.

RNA isolation, reverse transcription, and PCR

RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. cDNAs were synthesized as described earlier (Abtin *et al.*, 2008). Briefly, RNA was reverse-transcribed with murine leukemia virus reverse transcriptase using the Gene Amp RNA PCR kit (Applied Biosystems, Foster City, CA) and oligo dT primers (Roche Diagnostics, Basel, Switzerland). cDNA sequences of the genes under investigation were obtained from the GenBank. Primers were designed using the PRIMER3 software from the Whitehead Institute for Biomedical Research (Cambridge, MA). The following forward (F) and reverse (R) intron-spanning primers were synthesized by VBC Genomics (VBC Genomics, Vienna, Austria):

RNase 1: F, 5′-GATTGCAGAAACTGGCCTTC-3′ and R, 5′-CTG GGGGAACTGTCTGAGTC-3′; RNase 2: F, 5′-CTGAACCCCAGAAC AACCAG-3′and R, 5′-GAGCCCAGGTAAACTGTGGA-3′; RNase 3: F, 5′-AACAACCAGCTGGATCAGTTC-3′ and R, 5′-CCACTGAGCCCTCGTA AACT-3′; RNase 4: F, 5′-AGAAGCGGGTGAGAAACAAA-3′ and R, 5′-AGTAGCGATCACTGCCACCT-3′; RNase 5: F, 5′-AGAAGCGGG TGAGAAACAAA-3′ and R, 5′-TGTGGCTCGGTACTGGCATG-3′; RNase 6: F, 5′-ATACACACAGGGCTCGAAGG-3′ and R, 5′-GGAC ACACTGGTCCCCATAG-3′; RNase 7: F, 5′-GAGTCACAGCACGAA GACCA-3′ and R, 5′-GGCTGCATGTGCTGAATTT-3′; RNase 8: F, 5′-CAGTGGTTTAAAACTCAGCATG-3′ and R, 5′-ACTATGTAAG GTGTGTTCAGGT-3′; RI: F, 5′-CATCAGCTCTGCACTTCGAG-3′ and R, 5′-CAAGAGGTTGTCGCTGAGGT-3′; β2-microglobulin (B2M): F, 5′-GATGAGTATGCCTGCCGTGTG-3′ and R, 5′-CAATCC AAATGCGGCATCT-3′; ALAS: F, 5′-CCACTGGAAGAGCTGTGT GA-3′ and R, 5′-ACCCTCCAACACAACCAAAG-3′; and human β-defensin-2 (hBD-2): F, 5′-ATCAGCCATGAGGGTCTTGT-3′ and R, 5′-GAGACCACAGGTGCCATTTT-3′.

PCRs were performed in a volume of 50 µl containing 0.2 µl Red Hot DNA polymerase (ABgene, Hamburg, Germany), 5 µl 10 × reaction buffer IV (ABgene), 3 µl $MgCl_2$ (25 mM), 4 µl dNTPs (2.5 mM), 2.5 µl of each primer (10 µM), and 31.8 µl H_2O. The PCR included 5 minutes at 94 °C for initial denaturing, followed by 38 cycles of 1 minute at 94 °C, 45 seconds at 60 °C, 45 seconds at 72 °C, and a final extension step at 72 °C for 5 minutes. A negative control (omitting cDNA) and a genomic DNA control PCR were performed for each primer pair. Amplicons were subjected to electrophoresis in a 1.5% agarose gel containing ethidium bromide.

qRT-PCR was performed by the LightCycler technology using the Fast Start SYBR Green I kit for amplification and detection (Roche Diagnostics). In all assays, cDNA was amplified using a standardized program (10 minutes denaturing step and 55 cycles of 5 seconds at 95 °C; 15 seconds at 65 °C and 15 seconds at 72 °C; melting point analysis in 0.1 °C steps; final cooling step). Each LightCycler

capillary was loaded with 1.5 µl DNA master mix, 1.8 µl $MgCl_2$ (25 mM), 10.2 µl H_2O, and 0.5 µl of each primer (10 µM). Relative quantification of target gene expression and amplification efficiencies were performed using a mathematical model as described earlier (Pfaffl, 2001). The expression of the target gene was normalized to the expression of the housekeeping genes 5-aminolevulinic acid synthase (ALAS) and/or B2M. Similar target mRNA quantifications were obtained with either housekeeping gene as standard. All qRT-PCRs were performed in triplicate. The specificity of PCR reactions was confirmed by sequencing of the PCR products. To identify RNase 1 variants, PCR products were cloned into a pCR2.1-TOPO plasmid (Invitrogen) according to the manufacturer's instructions, and clones with different insert lengths were sequenced.

Quantification of RNase 5 and RNase 7

Culture supernatants of KCs or SC extracts were depleted by centrifugation of insoluble cell fragments and stored at −20 °C until analysis. The concentration of RNase 5 was determined by ELISA (R&D Systems) according to the manufacturer's instructions.

The concentration of RNase 7 was determined with an in-house RNase 7 ELISA, which has been developed using affinity-purified goat RNase 7 antibodies (Abs), as will be described elsewhere (Könten B, Gläser R, Schröder JM, Harder J, unpublished data).

Immunohistochemical analysis

Normal human skin was kindly provided by the Department of Plastic Surgery (Medical University of Vienna, Austria). Immunohistochemical analysis was performed on paraffin-embedded, formalin-fixed skin sections (4 µm) using the indirect immunoperoxidase staining technique. Briefly, endogenous peroxidase activity of sections were blocked by 0.3% H_2O_2/phosphate-buffered saline (PBS) (pH 7.5) for 15 minutes, washed with PBS, and then blocked for 30 minutes with 10% goat serum (PAA, Linz, Austria) diluted in 2% BSA/PBS. A polyclonal rabbit IgG anti-porcine RI Ab (kindly provided by J. Hofsteenge, Friedrich Miescher-Institut, Basel, Switzerland) was used. This Ab crossreacts with human RI (Blazquez *et al.*, 1996). Before immunolabeling with anti-RI or a rabbit IgG isotype control (Santa Cruz Biotechnology, Santa Cruz, CA), the sections were pretreated in ChemMate Target Retrieval Solution (Dako Cytomation, Glostrup, Denmark) in a microwave oven for 2 × 5 minutes at 500 W. The anti-RI Ab and/or rabbit IgG isotype control (both at 2 µg ml^{-1}) were diluted in 2% BSA/PBS and applied for 1 h on sections at room temperature. After washing in PBS, the slides were incubated with biotinylated goat anti-rabbit IgG (1:100, Vector Laboratories, Burlingame, CA) for 30 minutes, washed in PBS, and then exposed to StreptABComplex/HRP (Dako Cytomation) for 30 minutes. The AEC (aminoethylcarbazole) substrate system (Dako Cytomation) was used as the chromogen. The sections were counterstained with hematoxylin.

Ribonuclease activity assay

Recombinant RNase 5 (R&D Systems), purified RNase 7 (Harder and Schröder, 2002), or SC extract were pre-incubated with RI in 30 mM Tris (pH 7.5) containing 30 mM NaCl in a total volume of 15 µl for 10 minutes at 37 °C. Later, 1.5 µg of total RNA was added to the reactions and incubated for 5 minutes at 37 °C. Then, RNA loading buffer (Fermentas, Glen Burnie, MD) was added, and the mixture was heated for 5 minutes at 65 °C, cooled on ice, and subsequently electrophoresed through a 1% agarose gel containing ethidium bromide.

Ribonuclease activity of RNase 5 was blocked by pre-incubation with DEPC or benzopurpurin B (both from Sigma-Aldrich, Vienna, Austria) in 10 mM sodium phosphate buffer (pH 7.4) in a total volume of 15 µl for 1 h at 37 °C.

Antimicrobial activity assay

C. albicans ATCC 90028 and a vancomycin-resistant *E. faecium* clinical isolate were cultured overnight in tryptic soy broth (TSB; Fluka, Buchs, Switzerland) with agitation at 37 °C, subcultured the next day in fresh TSB, and then grown to optical densities of 1.0 at 600 nm for *E. faecium* or at 450 nm for *C. albicans*. Cells were washed twice with PBS and diluted to 10^4 CFU per ml in 10 mM sodium phosphate buffer (pH 7.4) containing 1% (v/v) TSB.

Recombinant human RNase 5 (>95% purity) was purchased from R&D Systems and natural RNase 7 was purified from heel SC as described earlier (Harder and Schröder, 2002). Recombinant human RI was purchased from Ambion (Warrington, UK). The antimicrobial activity was tested using a microdilution assay system. Tubes were pre-incubated with 2% BSA/PBS for 1 hour at 37 °C and were then rinsed with 10 mM sodium phosphate buffer (pH 7.4) containing 1% (v/v) TSB. As RI is provided with a storage buffer containing 20 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)-KOH (pH 7.6), 50 mM KCl, 5 mM dithiothreitol, and 50% (v/v) glycerol, the same buffer without RI was used in control reactions. For inhibitory activity experiments, RNase 5 or RNase 7 was pre-incubated with buffer alone or with RI in 10 µl volumes of 10 mM sodium phosphate buffer (pH 7.4) for 1 hour at 37 °C. An aliquot of 100 µl of the above-mentioned *C. albicans* or *E. faecium* suspensions were then added and incubated for 3 hours at 37 °C with agitation. Later, 20 µl aliquots were plated on Luria-Bertani agar or Sabouraud-Dextrose agar for *E. faecium* and *C. albicans*, respectively. The plates were incubated overnight at 37 °C. On the following day, the number of CFUs was determined. The growth inhibitory activities of the substances were calculated as follows: (CFUs after incubation with substance)/(CFUs after incubation without substance) × 100, which represents the percentage of remaining CFUs after treatment.

Antimicrobial activity and calculation of growth inhibition of recombinant RNase 5 toward *P. aeruginosa* ATCC 27853, methicillin-resistant *S. aureus* ATCC 33592, *E. coli* ATCC 35218, and a clinical isolate of group A *Streptococcus* were performed as described above, except that group A *Streptococcus* was grown in TSB supplemented with 10% heat-inactivated fetal calf serum. To determine CFUs, group A *Streptococcus* was plated on Columbia agar, and *P. aeruginosa*, *S. aureus*, and *E. coli* were plated on Luria-Bertani agar.

To test whether RNase 5 antimicrobial activity is blocked by DEPC or benzopurpurin B, RNase 5 was pre-incubated in 10 µl volumes of sodium phosphate buffer (10 mM, pH 7.4) for 1 hour at 37 °C with increasing concentrations of DEPC or benzopurpurin B. An aliquot of 100 µl of the above-mentioned *C. albicans* suspension was then added and incubated for 3 hours at 37 °C with agitation. The growth inhibitory effect was calculated as described above.

SC extraction and incubation with RI

SC obtained from the heels of healthy volunteers was extracted with 100 mM MES (2-(*N*-morpholino)ethanesulfonic acid) buffer pH 7.5 (Sigma-Aldrich). A ratio of 25 mg SC to 200 µl buffer was used. SC was homogenized in 1.5 ml tubes (Eppendorf, Hamburg, Germany) with a micropestle (Eppendorf) on ice (1–2 minutes) and then incubated for 1 h rotating at 4 °C. Thereafter, the tubes were centrifuged with 15,000 *g* for 20 minutes at 4 °C. Subsequently, the supernatant was aliquoted and stored at −20 °C until analysis. Sterility was verified by plating extracts on blood agar. Protein concentrations of SC extracts were measured by the bicinchoninic acid method (Pierce, Rockford, IL).

SC extract of 10 µg was co-incubated with 20 U recombinant human RI or 50 ng recombinant RNase 5 (R&D Systems) at 37 °C with agitation. The final reaction volume was 30 µl. Aliquots of SC were pre-incubated for 90 minutes at 37 °C with the following protease inhibitors: complete protease inhibitor cocktail (EDTA free), leupeptin, aprotinin (all from Roche Diagnostic), EDTA pH 7.5 (Merck, Darmstadt, Germany), and GM6001 (Calbiochem Inc., La Jolla, CA).

Western blot analysis

For analysis of RI expression, KC monolayer cultures were lysed in 1% Nonidet P-40 (Igepal CA-630; Sigma-Aldrich), and insoluble cell debris was removed by centrifugation. The epidermis of skin biopsies was separated by dispase II (30 minutes at 37 °C; Roche Diagnostic) from the dermis. Later, epidermis and/or heel scales were quick-frozen with liquid nitrogen, ground in a mortar and suspended in 100 mM MES (pH 7.5), and left rotating for 1 hour at 4 °C. Thereafter, samples were centrifuged (15,000 *g* for 20 minutes at 4 °C) and the supernatant was aliquoted. The protein concentration was measured by the bicinchoninic acid method (Pierce). Western blot analysis was performed as described earlier (Mildner *et al.*, 2006). Equal loading of protein lysates was confirmed by Ponceau staining of the membrane. Rabbit anti-RI Ab was used to detect RI protein (dilution 1:3,000). A peroxidase-conjugated goat anti-rabbit IgG Ab (dilution 1:10,000, Pierce) was used as a secondary Ab. Mouse anti-caspase-14 (dilution 1:1,000; MBL, Naka-ku Nagoya, Japan) was used to detect the proform and the active form of caspase-14 with a secondary peroxidase-conjugated sheep anti-mouse IgG Ab (dilution 1:10,000, Amersham, Buckinghamshire, UK). To detect RNase 5, a polyclonal goat Ab (R&D Systems) and a secondary peroxidase-conjugated rabbit anti-goat (dilution 1:10,000, Dako Cytomation) Ab were used. The membranes were developed using the Chemiglow reagent (Alpha Innotech, San Leandro, CA) according to the manufacturer's instructions.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Figure S1. Amino-acid sequence alignment of RNase 5 and RNase 7.

Figure S2. Antimicrobial activity of RNase 5 against potential skin pathogens.

Figure S3. RNase 5 and/or RI are not induced by IL-1α, IL-6, or *C. albicans*.

Figure S4. RNase 5 is not degraded by SC extract.

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