

The 420K LEKTI variant alters LEKTI proteolytic activation and results in protease deregulation: implications for atopic dermatitis

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Lymphoepithelial Kazal-type related inhibitor (LEKTI) is a multidomain serine protease inhibitor which plays a central role in skin permeability barrier and allergy. Loss-of-function mutations in the LEKTI encoding gene *SPINK5* cause Netherton syndrome, a rare and severe genetic skin disease with a profound skin barrier defect and atopic manifestations. Several studies also reported genetic association between the multifactorial disease atopic dermatitis (AD) and a frequent and non-conservative LEKTI variant, E420K, in different populations. Here, we provide evidence that the 420K variant impacts on LEKTI function by increasing the likelihood of furin-dependent LEKTI precursor cleavage within the linker region D6–D7. This results in the reversal of the cleavage priorities for LEKTI proteolytic activation and prevents the formation of the LEKTI fragment D6D9 known to display the strongest inhibitory activity against kallikrein (KLK) 5-mediated desmoglein-1 (DSG1) degradation. Using *in situ* and gel zymographies, we show that the modification of the subtle balance in LEKTI inhibitory fragments leads to enhanced KLK5, KLK7 and elastase-2 (ELA-2) activities in 420KK epidermis. By immunohistochemistry and western blot analyses, we found that increased epidermal protease activity correlates with reduced DSG1 protein expression and accelerated profilaggrin proteolysis. All changes determined by the presence of residue 420K within the LEKTI sequence likely contribute to defective skin barrier permeability. Remarkably, LEKTI 420KK epidermis displays an increased expression of the proallergic cytokine thymic stromal lymphopoietin (TSLP). This is the first functional evidence supporting association studies which identified the 420K LEKTI variant as a predisposing factor to AD, in combination with other genetic and environmental factors.

INTRODUCTION

The epidermal barrier protects the organism from chemical and physical assaults, antagonizes pathogen invasion and prevents unregulated water and solute loss. This barrier results from a differentiation process that crosses all the epidermis starting from the basal layer, in which keratinocytes proliferate, and culminating in the stratum corneum (SC) formation,

with terminally differentiated flattened cells called corneocytes. In the SC, a meshwork of these anucleated and keratin-filled cells is embedded in a highly organized extracellular lipid compartment that prevents internal water loss and penetration of water-soluble materials. Moreover, an insoluble shell of structural proteins extensively cross-linked to each other, the cornified envelope (CE), confers strength to the corneocytes and acts as a scaffold for the attachment of lipids.

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Finally, the SC structural integrity, which blocks penetration of irritants and allergens, is maintained by specialized cell–cell junctions, the corneodesmosomes. Yet, the epidermal barrier is not an inert structure: throughout all life it undergoes a continuous tightly regulated remodelling attained by balancing the shedding of corneocytes at the skin surface (desquamation) and the cell repopulation from the inner layers (1).

Serine proteases are well-recognized leader elements in skin barrier homeostasis, participating in SC desquamation, lipid barrier construction and CE formation. In particular, a serine protease cascade, which principally involves kallikreins (KLKs) 5, 7 and 14, is implicated in corneocyte shedding by cleaving corneodesmosomal structural components (2–4). At the same time, a complex network of serine proteases, such as furin, profilaggrin endopeptidase-1 (PEP1), matriptase, prostatic, elastase-2 (ELA-2) and other proteolytic enzymes, including the recently discovered skin-specific retroviral-like aspartic protease (SASPase) (5), act in concert with the epidermal KLKs to mediate the multistep cleavage of the profilaggrin (FLG) precursor, one of the major contributors to the epidermal barrier function (6,7). Specifically, synthesized as a ~400 kDa molecule, profilaggrin is at first cleaved into the FLG monomers that aggregate the corneocyte keratin intermediate filaments to form macrofibril bundles. Then, as the terminally differentiating cells move towards the outer layer of the skin, proteolytic enzymes break down the keratin–FLG complex, thus releasing the FLG monomers that are incorporated into the CE. Finally, in the uppermost SC layers, FLG monomers are further degraded up to free amino acids that contribute to the production of the natural moisturizing factor of the skin (6).

The importance of a precise control of these proteolytic activities is supported by the presence of a broad range of protease inhibitors in the epidermis (4). Among them, the serine protease inhibitor lymphoepithelial Kazal-type related inhibitor (LEKTI) is of special interest because of its pathophysiological relevance. Indeed, loss-of-function mutations in the LEKTI encoding gene, *SPINK5*, result in the ichthyosiform condition Netherton syndrome (NS, OMIM 266500) (8). This autosomal recessive disorder is characterized by congenital scaly erythroderma, a specific hair shaft abnormality and severe atopic manifestations including eczema-like rashes, intense itching, high levels of immunoglobulin E in the serum, frequent asthma and food allergies (9,10).

Studies in NS patients and murine models have shown that unopposed serine protease activity secondary to LEKTI deficiency results in a severe skin barrier defect mainly because of excessive degradation of the corneodesmosomal component desmoglein-1 (DSG1) leading to abnormal SC detachment. In addition, lack of LEKTI causes premature profilaggrin processing and SC lipid disorganization attributable to unrestrained activity of proteases during CE formation (11–15).

The ability of LEKTI to exert multiple inhibitory actions relies on its peculiar structure, in that it is composed of as many as 15 potential inhibitory domains (D1–D15) (16). Initially synthesized as three precursor isoforms (LEKTI_{FL}, LEKTI_L and LEKTI_{Sh}) (17), LEKTI is subjected to a furin-driven proteolytic activation cascade which results in a significant number of single or multidomain polypeptides, at time overlapping (18). As to the major LEKTI inhibition targets,

several evidence point out to the most abundant KLKs within the SC, KLK5, KLK7 and KLK14 (3,18,19). In fact, aside from cleaving the corneodesmosomal components, these KLKs act as catalysts of the proteolysis of enzymes involved in the synthesis of SC specific lipids, thus regulating the CE lipid deposition (20). They also play an indirect role in profilaggrin processing, at least through the proteolytic activation of ELA-2 (7). Finally, KLK5 and KLK14 have been shown to activate the signaling of the inflammatory protease-activated receptor-2 (PAR-2) (21–23), thus explaining, at least in part, the inflammatory and atopic manifestations of NS. We recently demonstrated that LEKTI physiological proteolytic fragments derived from the central portion of the proprotein are capable of blocking the KLK-mediated DSG1 degradation *in vitro*, with D6D9 showing the strongest inhibitory capacity (18). In contrast, the role of LEKTI in the regulation of profilaggrin processing appears to be indirect. Indeed, LEKTI does not inhibit ELA-2 directly, but is likely to modulate profilaggrin processing through KLK5-mediated ELA-2 activation (7,12,13).

Besides the well proven direct cause–effect relationship between LEKTI deficiency and NS, several genetic association studies suggested a contribution of LEKTI to the pathogenesis of atopic dermatitis (AD), a frequent and chronic-relapsing inflammatory skin disease caused by complex interactions between genetic and environmental factors (24). Specifically, an early candidate gene approach found an association between the non-synonymous and frequent c.1258G>A (p. E420K) polymorphism in the *SPINK5* gene and AD in two British cohorts (25). This observation fitted in with the emerging view that permeability barrier abnormalities could represent primary events in AD pathogenesis (26). Although the LEKTI 420K/AD association could be replicated in several studies in different populations (27–31), other studies found a weak (32) or no association in different populations (33–36), thus questioning the functional significance of the 420K variant.

In contrast, FLG stop mutations have since been identified as the strongest genetic risk factors for AD in widely replicated studies and in all populations tested (37–40). These results have placed the breakdown of the epidermal barrier at the centre of the development of eczema. They have pointed out to actors of the FLG cascade and other important players of epidermal integrity, as potential candidate genes for predisposition to AD. In order to address the potential contribution of LEKTI to AD predisposition, we examined the consequences of the LEKTI 420K variant in the LEKTI protein activation process and the inhibitory activity of the resulting bioactive fragments.

RESULTS

Keratinocytes carrying the 420K LEKTI variant display a distinct proteolytic pattern of LEKTI bioactive fragments

LEKTI E420K polymorphism is situated nearby one of the furin cleavage sites used for LEKTI processing, R425, located in the linker region D6–D7. Thus, the potential effect of the missense variant on the precursor protein activation was investigated. At first, *in silico* furin-specific cleavage

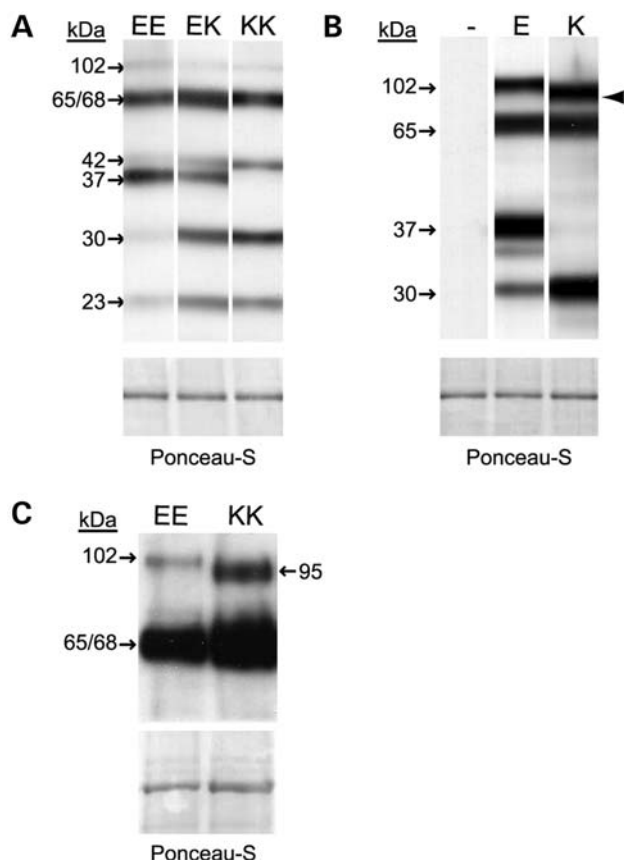


Figure 1. Distinct LEKTI proteolytic patterns from cells expressing different LEKTI 420 residue genotypes. (A) Immunoblotting detection of LEKTI in the CM of differentiated HK from individuals with 420EE (EE), 420EK (EK) or 420KK (KK) genotypes. The image is representative of at least five different keratinocyte strains for each genotype. (B) Immunoblotting detection of LEKTI in the CM of HEK293 transfected with an empty vector (–), pLEKTI_{420E} (E) or pLEKTI_{420K} (K). Under our experimental conditions, the 23 kDa D8D9 LEKTI fragment could only be detected as a very faint signal, regardless of the LEKTI genotype and it is not shown. (C) Immunoblotting detection of LEKTI high molecular weight processing intermediates in the CM of differentiated HK from individuals with either 420EE (EE) or 420KK (KK) genotypes. Proteins were separated on a 12% (A–B) or 8% (C) SDS-PAGE, and were detected with the anti-LEKTI-D7D12 polyclonal antibody. Ponceau S staining was used to ensure even protein loading among samples (lower panels). Sizes of immunoreactive bands are indicated.

site prediction was carried out using LEKTI sequences (Swiss-Prot Q9NQ38) with either glutamic acid (E) or lysine (K) at position 420 (41) (<http://www.cbs.dtu.dk/services/ProP/>). This computational analysis indicated that the E420K substitution slightly increases the likelihood of cleavage within the LEKTI D6–D7 linker region. Next, to determine the functional impact of this predicted alteration, a panel of human keratinocytes (HK) from healthy donors was genotyped at LEKTI codon-420. Their expression profile for LEKTI proteolytic fragments was then analyzed by western blotting with the anti-LEKTI-D7D12 polyclonal antibody. In the conditioned media (CM) from *in vitro* differentiated LEKTI 420E homozygous cells (420EE), polypeptides of 65/68, 42, 37, 30 and 23 kDa were visualized (Fig. 1A). As previously reported, these fragments represent the LEKTI processing bioactive products D10D15_{FL/L}, D10D13_{Sh}, D6D9, D7D9 and

D8D9, respectively (18). In the same samples, a 102 kDa band, which corresponds to the D6D15_{FL} intermediate product, was also seen as a faint signal (Fig. 1A). When compared with the LEKTI 420EE cells, the 420KK samples lacked the 37 kDa fragment and showed increased amounts of the 30 and 23 kDa bands (Fig. 1A). Intermediate quantities of the 37, 30 and 23 kDa polypeptides were visualized in the CM of 420EK heterozygous cells (Fig. 1A). No differences were noticed with regard to the other fragments (Fig. 1A). Similar results were observed using protein extracts from the epidermis of healthy volunteers with different codon-420 genotypes (not shown).

These data indicate that the E420K substitution, by increasing the likelihood of cleavage in the LEKTI D6–D7 linker region, results in the absence of the 37 kDa fragment D6D9 with consequent accumulation of its byproducts D7D9 (30 kDa) and D8D9 (23 kDa). Because the three LEKTI isoforms differ from each other only in their C-terminal portions (17), the LEKTI processing alterations secondary to the E420K substitution pertain to all of them.

The ectopically expressed E420K substitution alters LEKTI proteolytic activation

To verify whether the diverse LEKTI fragment profiles observed in HK and epidermal samples with different genotypes at codon-420 are actually due to the E420K substitution, the 420E residue of recombinant LEKTI_{FL} was mutagenized. The proteolytic processing of both LEKTI_{FL-420E} and LEKTI_{FL-420K} was then analyzed by ectopically expressing the recombinant proteins in the LEKTI deficient epithelial cells HEK293, followed by western blot detection. According to previous data (18), the 102 kDa LEKTI_{FL} processing intermediate D6D15_{FL} and the 65, 37 and 30 kDa proteolytic end-products were secreted by the cells expressing the LEKTI_{FL-420E} form (Fig. 1B). Conversely, processing of the ectopically expressed LEKTI_{FL-420K} did not generate the 37 kDa fragment while a greater amount of the 30 kDa band could be visualized (Fig. 1B). Finally, the high molecular weight processing intermediate, resulting from the proteolysis of the LEKTI_{FL-420K} form, exhibited a slightly faster migration rate, compared with the 102 kDa D6D15_{FL} fragment generated by LEKTI_{FL-420E} (Fig. 1B, arrowhead).

To confirm this additional variation, which had not been taken into account in HK, the CM of differentiated HK with either 420EE or 420KK homozygous genotypes were analyzed by high-resolution gel electrophoresis. In order to detect LEKTI processing intermediate fragments, high amounts of proteins were used. Similarly to what was observed in the ectopically expressing cells, the higher molecular weight LEKTI polypeptide generated by the LEKTI 420KK HK exhibited a faster migration rate, when compared with the fragment of the LEKTI 420EE cells (Fig. 1C). The molecular weight of this novel LEKTI polypeptide was estimated to be 95 kDa and is likely to correspond to a C-terminal intermediate polypeptide D7D15_{FL}.

All together, the herein reported results demonstrate that, in agreement with the *in silico* prediction, the E420K substitution increases the furin cleavage rate at the LEKTI linker region D6–D7 and elicits a reversal of the proprotein cleavages

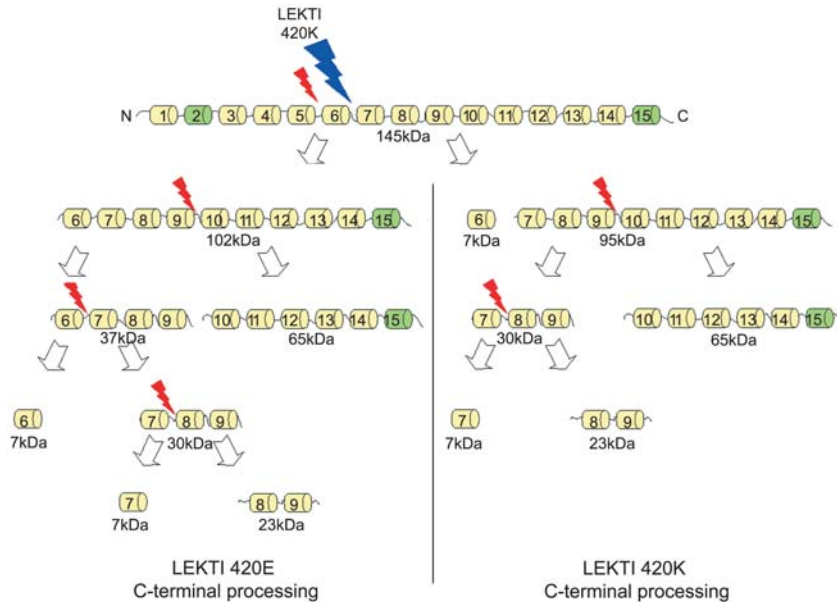


Figure 2. Model of the proteolytic activation cascade of LEKTI 420E versus 420K. Schematic representation of LEKTI sequential proteolytic cleavage in the presence of a glutamic acid (left panel) or a lysine (right panel) at residue 420. In addition to the physiological cleavage within the LEKTI region D5–D6, LEKTI_{420K} is precociously cleaved within the linker region D6–D7, preventing the formation of the D6D9 fragment. Processing of the LEKTI region D1D5 is not shown. LEKTI fragment sizes are indicated below each fragment. Adapted from Fortugno *et al.*, (18), doi:10.1038/jid.2011.174.

priority. Specifically, in the presence of the 420K residue, LEKTI_{FL} activation begins with the production of a C-terminal D7D15_{FL} intermediate fragment of 95 kDa, which replaces the canonical 102 kDa D6D15_{FL} and prevents the subsequent formation of the 37 kDa D6D9 polypeptide (Fig. 2). Afterwards, the sequential recognition by furin of the R625 and R489 physiological cleavage sites generates the 30 kDa polypeptide D7D9, the 65 kDa isoform-specific C-terminal D10D15_{FL}, the 23 kDa D8D9 fragment and the single-domain D7 (Fig. 2). In spite of the massive amounts of HK CM used, the assessment of the E420K substitution outcome on LEKTI_L and LEKTI_{Sh} isoform intermediate precursors was hampered by their physiological low expression level (17).

The 420K LEKTI variant leads to enhanced epidermal protease activity

To determine whether the altered proteolytic processing caused by the E420K substitution impacts on LEKTI inhibitory function, skin sections and epidermal protein extracts from individuals with different genotypes at codon-420 were tested for protease activity. By *in situ* zymography with a casein-conjugated substrate, skin samples from 420KK individuals showed a moderate increase of protease activity which extended throughout the most differentiated epidermal layers (Fig. 3A). In addition, casein gel zymography of epidermal protein extracts showed the presence of several proteolytic activities, some of which were noticeably increased in the 420KK samples, when compared with the 420EE ones (Fig. 3B). The molecular weights of three major bands (31, 28 and 20 kDa) are suggestive of KLK5, ELA-2 and KLK7, respectively. This hypothesis was strengthened by *in situ*

zymographies carried out with specific substrates for trypsin-like, chymotrypsin-like and elastolytic activities which showed a moderate increase in each of the activities tested (Supplementary Material, Fig. S1). These results are consistent with defective protease inhibition secondary to the absence of the 37 kDa D6D9 polypeptide, with an appreciable effect on KLK5, KLK7 and ELA-2 activities.

The 420K LEKTI variant correlates with lower protein levels of the corneodesmosomal component DSG1

We recently demonstrated that the 37 kDa D6D9 bioactive peptide was the most effective LEKTI fragment inhibiting KLK5-mediated proteolysis of DSG1 *in vitro* (18). To investigate a possible effect of the LEKTI 420K variant expression on corneodesmosome stability, DSG1 level in the epidermis of individuals with different codon-420 genotypes was evaluated by immunofluorescence. A slight to medium reduction of signal intensity was observed in the epidermis of 420KK individuals, compared with the 420EE ones (Fig. 4A). A similar result was also obtained from western blot experiments on epidermal protein extracts (Fig. 4B). Densitometric analysis of the detected bands revealed an ~50% reduction of the DSG1 amount in the epidermis of 420KK individuals compared with the 420EE samples (Fig. 4C). To ascertain the expression level of DSG1 transcripts, total RNA isolated from epidermal specimens derived from the same individuals was subjected to real-time RT-PCR analysis. As shown in Fig. 4B, equal amounts of DSG1 mRNA could be detected in all samples, regardless of the LEKTI genotype. All together, these data indicate that the presence of the 420K residue results in a reduction of the corneodesmosomal component

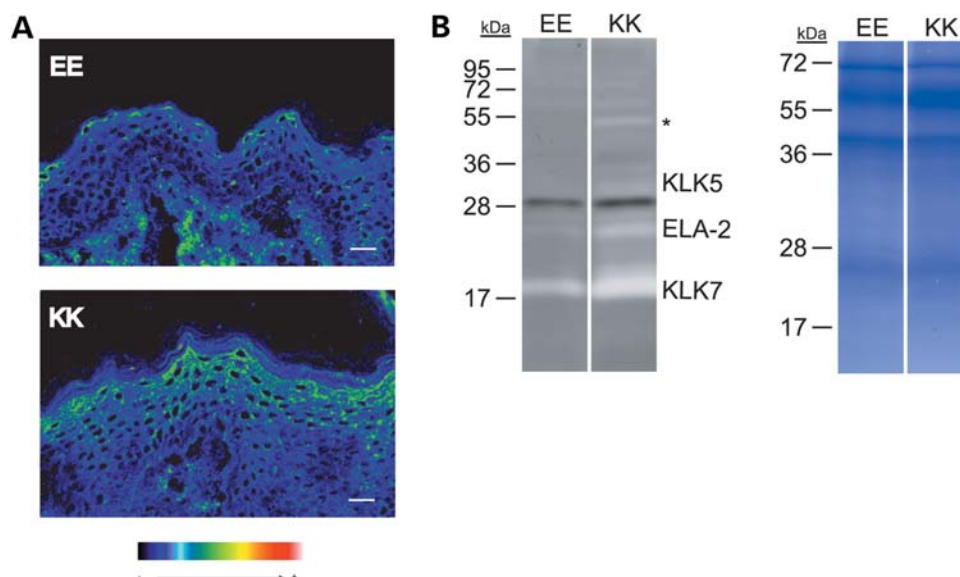


Figure 3. Serine protease activity in the epidermis from individuals with different LEKTI residue 420 genotypes. (A) Caseinolytic activity of 420EE and 420KK epidermis by *in situ* zymography analysis. In 420EE epidermis, a weak caseinolytic activity, mainly localized in the granular layer, is detected by the degradation of the 4,4-difluoro-5,7 dimethyl-4 bora-3a,4a-diaza-s-indacene-3 propionic acid green fluorescent labeled casein substrate. In contrast, in 420KK epidermis, the intensity of the caseinolytic activity is increased in the granular layer and extends to the uppermost layers of the spinous compartment. Scale bar = 80 μ m. (B) Proteolytic activity in 420EE and 420KK epidermis analyzed by casein gel zymography. The 420KK epidermal samples showed increasing of several caseinolytic bands. The 31 kDa band is referable to KLK5 activity, the 28 kDa band to ELA-2 activity and the 20 kDa band to KLK7. The asterisk indicates an additional unidentified $\sim$ 55 kDa proteinase whose activity is noticeably increased in 420KK epidermis. Coomassie staining of a corresponding SDS-PAGE showed even protein loading among samples (right panel).

DSG1 likely due to KLK over-activity secondary to LEKTI D6D9 deficiency.

The 420K LEKTI variant results in increased profilaggrin processing

The features so far described in 420KK healthy individuals are reminiscent, even though in a much milder form, of NS epidermis (11–14,20,42). Since lack of LEKTI expression also results in altered profilaggrin processing (12–14), we addressed the hypothesis that the LEKTI 420K variant could also affect profilaggrin cleavage. Epidermal and differentiated HK extracts from healthy individuals with either the LEKTI 420EE or 420KK genotype were therefore analyzed for FLG expression. In conformity with our working hypothesis, immunoblot analysis of high-salt protein extracts from 420KK epidermis (Fig. 5A) and HK (data not shown) showed reduced amounts of both profilaggrin and FLG monomers, when compared with the 420EE samples. In all samples analyzed, FLG protein decrease did not correlate with a significant variation of the profilaggrin mRNA steady-state level, as judged by real-time RT-PCR analysis (Fig. 5A). The presence of recurrent mutations in the *FLG* gene was also excluded. The decrease in protein expression was validated by a reduction of the intensity of the immunofluorescence FLG signal in the epidermis of 420KK individuals compared with 420EE samples (Fig. 5B). Quantitative analysis of both western blotting and immunofluorescence results revealed that, despite some variability among analyzed individuals, 420KK epidermis display an $\sim$ 50% reduction of FLG expression, compared with 420EE specimens (Fig. 5C). As ELA-2 has recently been shown to

participate in profilaggrin maturation (7), the here described results may be due, at least in part, to increased ELA-2 activity associated with the expression of the LEKTI 420K variant. However, within the context of a LEKTI fragment imbalance, a contribution of other deregulated protease activities to the overall FLG content decrease cannot be ruled out.

In parallel to the analysis of FLG expression in epidermis with LEKTI 420EE or 420KK genotypes, *in vitro* assays were carried out to determine the potential impact of 420-codon polymorphism on profilaggrin processing regulation. Specifically, a flag-tagged recombinant N-terminal portion of the FLG precursor (N-ProFLG) was co-transfected with either LEKTI_{FL-420E} or LEKTI_{FL-420K} in cultured proliferating keratinocytes. In the experimental condition used for transfection, endogenous LEKTI protein is expected not to be expressed. Nevertheless, to prevent any unascertainable endogenous protein expression, Netherton syndrome keratinocytes (NSK) were used as receiving cells. Following transient protein expression, cell lysates were analyzed by immunoblot with a monoclonal antibody directed to the N-terminal tag of the recombinant N-ProFLG construct. As shown in Fig. 5D, a signal corresponding to the entire N-ProFLG polypeptide could be clearly detected in the NSK co-transfected with LEKTI_{FL-420E}, whilst only a faint signal was detectable in the control samples where N-ProFLG was co-transfected with the empty vector. Interestingly, intermediate quantities were observed in the cells expressing the LEKTI_{FL-420K} form (Fig. 5D). This result shows that, while in LEKTI deficient keratinocytes the recombinant N-ProFLG protein is rapidly cleaved by endogenous proteases, ectopic expression of LEKTI is able to prevent this proteolysis.

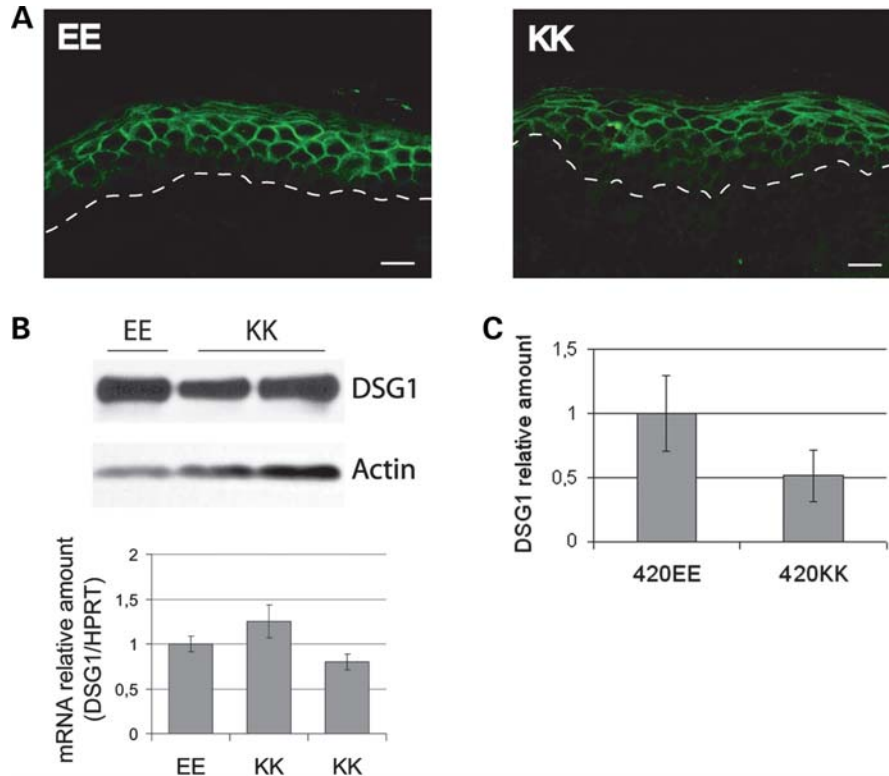


Figure 4. Decreased DSG1 protein levels in the presence of the LEKTI_{420K} genotype. (A) Immunofluorescence staining of DSG1 in human epidermis with different LEKTI codon-420 genotypes. Scale bar = 20 μ m. (B) Immunoblotting detection of DSG1 protein in epidermal extracts from individuals with either 420 EE or KK genotypes. Protein loading was normalized by actin protein levels. DSG1 transcriptional levels were assessed by real-time RT-PCR (lower panel). (C) Densitometric analysis of DSG1 protein levels in individuals with either 420EE or 420KK genotypes. The results shown are the average of three independent experiments carried out with three different individuals for each genotype at time.

Furthermore, it shows that the LEKTI_{FL-420K} protein exerts a reduced inhibitory action, compared with the LEKTI_{FL-420E}, thus pointing out to the biological implications of the E420K LEKTI polymorphism. Of note, this is the first direct evidence for a LEKTI inhibitory action on the initial cleavage of the FLG precursor.

The 420K LEKTI variant associates with increased expression of the pro-Th2 cytokine TSLP

As we have learnt from NS, unrestrained KLKs activity and epidermal barrier defects secondary to LEKTI deficiency induce the priming of a cutaneous allergic inflammation (22,23). Indeed, the imbalance between proteases and protease inhibitors leads to aberrant proteolytic activation of the PAR-2 receptor (22). This, in turn, primes a T-helper type 2 (Th2)-mediated immune response through the transcriptional activation of the cytokine thymic stromal lymphopoietin (TSLP) (23). In this study, we investigated whether the presence of the LEKTI 420K variant impacts also on the expression of this pro-Th2 mediator. Real-time RT-PCR analysis on RNA samples from differentiated keratinocytes with different LEKTI 420 genotypes was carried out. Interestingly, the expression level of TSLP transcripts in 420KK keratinocytes was higher when compared with the LEKTI 420E homozygous cells in which only a very small amount of constitutively expressed TSLP mRNA could be detected (Fig. 6A).

Consistently, although TSLP immunolabeling of LEKTI 420EE epidermis results in a barely detectable signal, TSLP protein could be clearly visualized in the epidermis of individuals with the 420KK genotypes (Fig. 6B).

DISCUSSION

In the last 10 years, LEKTI has been the subject of a number of studies aiming at deciphering the NS disease mechanism. This allowed to ascribe to this molecule a multifaceted regulatory function. By interacting with the epidermal serine proteases KLKs, LEKTI takes part in many physiological processes such as desquamation, lipid permeability barrier formation and CE deposition (13,14,20). In parallel, LEKTI exerts its inhibitory function both in pro-inflammatory and innate immune defence pathways (22,23,43). To play this variety of functions, LEKTI undergoes a complex post-transcriptional and post-translational regulation. Specifically, alternative processing of the LEKTI encoding pre-mRNA generates three isoforms (17). Thereafter, these three multidomain precursors follow a sequential furin-driven cleavage activation process that generates a balanced mixture of single- and multi-domain polypeptides with related but distinct structure and inhibition properties (18,19,44).

In this study, we hypothesized that sequence variations abolishing or creating furin cleavage sites within the LEKTI sequence may impact its proteolytic processing and possibly

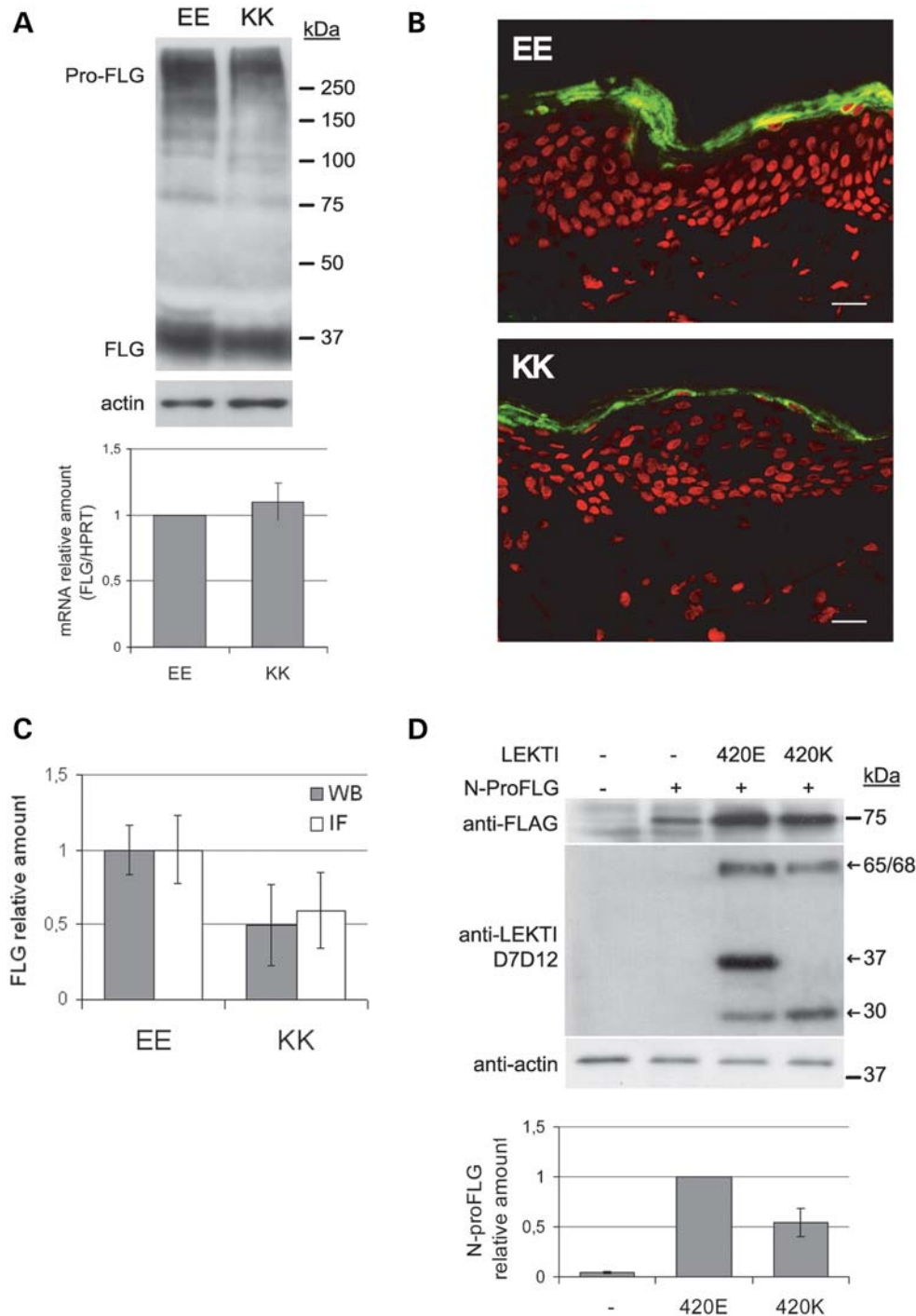


Figure 5. Increased FLG processing in the presence of LEKTI_{420K} genotype. (A) FLG expression analysis in epidermis from individuals with different LEKTI codon-420 genotypes. Immunoblot of high-salt epidermal protein extracts from individuals with either 420 EE or KK genotypes probed with a monoclonal antibody that detects both profilaggrin (Pro-FLG) and processed FLG. Actin immunoblot was performed to ensure equal protein loading. FLG mRNA expression was assessed by real-time RT-PCR (lower panel). (B) Immunofluorescence staining of FLG in human epidermis with different LEKTI codon-420 genotypes. Scale bar 20 μ m. (C) Densitometric analysis of total profilaggrin and FLG protein levels (calculated as total pixel density of each western blot lane, starting from the 37 kDa FLG band to the ~400 kDa high molecular weight profilaggrin molecules and including the processing intermediate forms) in individuals with either 420EE or 420KK genotypes. The results shown are the average of three independent experiments every time carried out with three different individuals for each genotype. (D) *In vitro* N-ProFLG processing assay. Immunoblotting detection of N-ProFLG recombinant protein in NSK transfected with pN-ProFLG in combination with either an empty vector (-), pLEKTI_{420E} (420E) or pLEKTI_{420K} (420K). N-ProFLG was detected using anti-FLAG monoclonal antibody. NSK transfected with the empty vector alone were used as a negative control. Recombinant LEKTI expression was verified using the anti-LEKTI-D7D12 polyclonal antibody. Even protein loading was verified using the anti-actin polyclonal antibody. Results of the densitometric analysis of total N-ProFLG protein levels of three independent experiments are shown (lower panel).

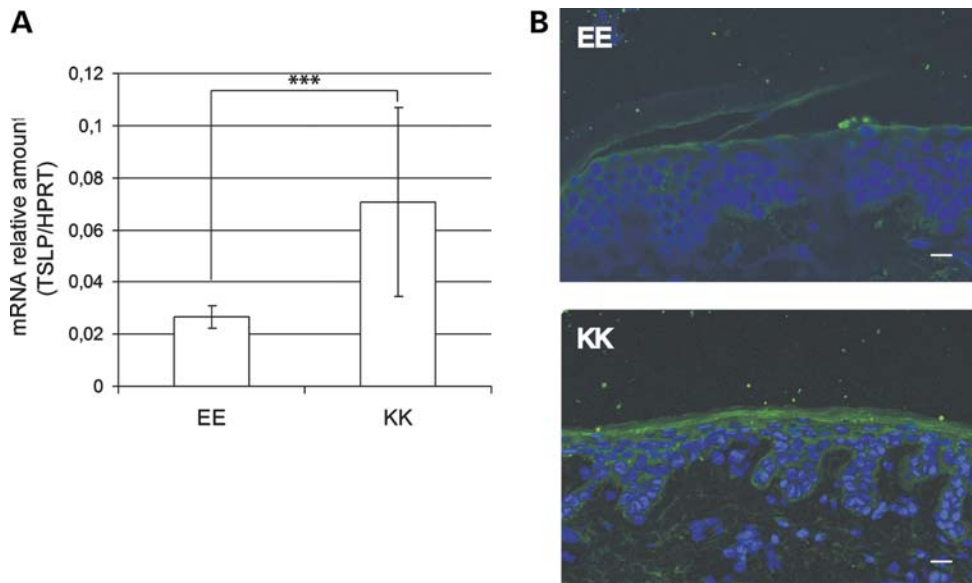


Figure 6. Increased pro-Th2 cytokine TSLP expression in keratinocytes with LEKTI_{420K} genotype. (A) Quantitative RT-PCR analysis on cultured primary keratinocytes with either 420KK or 420EE LEKTI genotype. The result is the mean $\pm$ SD of two independent experiments with three 420KK and three different 420EE keratinocytes. *** $P > 0.0001$. Note, under the experimental conditions used, the TSLP mRNA expression level in NSK showed a 10-fold increase, when compared with the 420EE cells. (B) Immunofluorescence staining of TSLP in human epidermis with different LEKTI codon-420 genotypes. TSLP expression is not detectable in the epidermis from individuals with LEKTI 420EE genotype, whereas it is expressed in the epidermis of individuals with 420KK LEKTI genotype. Scale bar, 20 μ m.

one or more LEKTI functions. In particular, since the LEKTI variant 420K identified by several studies as an AD risk factor (25,27,28,30,31) locates in linker 6 near the physiological furin-dependent R425 cleavage site, we theorized that this polymorphic variant could alter LEKTI proprotein activation. In fact, even though LEKTI residue 420 is not within the sequence motif strictly required for furin recognition, R-X-[K/R]-R↓ (P4–P1), it is part of a more recently described consensus region that widens up to 20 residues (P14–P6') (45). In principle, the remarkable degree of cross-species conservation of the amino acid sequence of this LEKTI region (Fig. 7) also corroborates our hypothesis in that it suggests that any amino acid change within this sequence might have a detrimental effect on the proprotein activation, both in terms of compartmental specificity and on the sequential ordering of the furin cleavage events. Accordingly, *in silico* furin cleavage site prediction indicated that the E420K substitution increases the likelihood of furin cleavage in the linker region between D6 and D7. Consistent with this prediction, immunoblot analysis of differentiated HK from individuals with different genotypes at residue 420 showed that the 420K variant associates with an atypical LEKTI expression profile, compared with the previously described one (18). The formal demonstration that the E420K substitution alters LEKTI proteolytic processing was provided by analysis of LEKTI deficient HEK293 cells ectopically expressing recombinant LEKTI proteins which reproduced the same fragment profiles as the ones observed in HK. Specifically, as the E420K substitution hastens the furin cleavage within the D6–D7 linker region, this LEKTI processing step occurs prior to the physiologically scheduled LEKTI D9–D10 cleavage at residue 625 and generates a novel D7D15 (95 kDa)

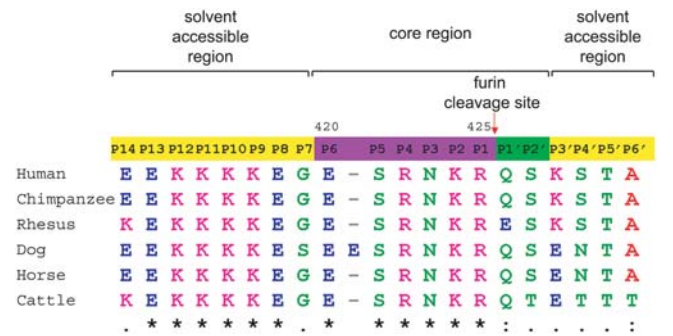


Figure 7. Motif length and physical properties of LEKTI D6–D7 furin cleavage site. Alignment of amino acid sequences from the mammalian LEKTI linker region D6–D7 (www.ebi.ac.uk/Tools/msa/clustalw2) (63) comprising 20 residues of the furin cleavage site motif (P14–P6'). Amino acids are colour coded according to their physicochemical properties: basic (magenta), acidic (blue), uncharged polar (green) and nonpolar (red). An asterisk (*) indicates position that have a single, fully conserved residue; a colon (:) indicates conservation between groups of strongly similar properties; a period (.) indicates conservation between the groups of weakly similar properties. The physiological R425 furin cleavage site, as previously determined by site-specific mutagenesis, is indicated with a red arrow. The physical properties of the furin consensus sequence are schematically summarized: the core region, which contributes to the binding strength of the substrate, is represented by purple and green boxes (positions P6–P2); yellow boxes represent the two polar regions (positions P7–P14 and P3'–P6') outside the furin binding pocket that mainly contribute to the solvent accessibility of the substrate.

intermediate fragment with no D6D15 (102 kDa) fragment (Fig. 2). As a consequence, processing of the LEKTI 420K variant results in both shortage of the 37 kDa D6D9 bioactive fragment and increase of the 30 kDa D7D9 and 23 kDa D8D9 fragments (Fig. 2).

Next, we investigated whether the altered LEKTI cleavage secondary to the E420K substitution results in deregulated activity of one or more serine proteases. By *in situ* and casein gel zymographies, we provided evidence that LEKTI 420K expression in the epidermis associates with enhanced proteolytic activities referable to KLK5, KLK7 and ELA-2, the most deregulated proteases in NS (13,14). This result is in agreement with previous reports describing higher KLKs activity in the SC of AD patients, compared with healthy controls (46–48). Casein gel zymographies also revealed the presence of few other overactive proteases, the identity of which was not investigated. Interestingly, one of these proteases has an apparent molecular weight (~55 kDa) which could correspond to urokinase (tissue-type plasminogen activator, tPA) activity. This hypothesis, which is consistent with previous studies describing higher urokinase activity in barrier-damaged skin (49,50) and increased plasmin activity in patients with NS (51), suggests that the LEKTI 420K variant might impact on tPA activation which could contribute to skin inflammation. Within the context of a LEKTI inhibitory domain imbalance, it is also reasonable to hypothesize overactivation of additional LEKTI direct/indirect targets.

Interestingly, the overall picture associated with a LEKTI 420 KK genotype recalls, in a milder form, those of NS patients and *Spink5*-deficient mouse epidermis (7,11–14,20,42,51) and is in line with a recent report describing a NS patient with a single heterozygous *SPINK5* null mutation combined with the homozygous 420K variation (52). Likely, our findings are referable to the lack of the 37 kDa LEKTI D6D9 fragment in LEKTI 420KK epidermis. This is consistent with disruption of a fine-tuned regulation between this LEKTI fragment and the active form of KLK5, which we recently showed to interact in a 1:1 molar ratio (18). Here, we also demonstrate that homozygosity for LEKTI 420K associates with reduced amounts of the corneodesmosomal component DSG1 in the epidermis. These data conform to the previous demonstration that the LEKTI D6D9 fragment displays the strongest inhibitory activity on KLK5-dependent degradation of DSG1, *in vitro* (18). Similarly to NS epidermis, in which LEKTI deficiency determines several alterations additional to over-desquamation, skin samples of individuals carrying the LEKTI 420K variant also display increased activity of ELA-2, a recently discovered epidermal serine protease that plays a relevant role in epidermal barrier integrity (7).

LEKTI 420K also associates with reduced FLG expression in the epidermis. Increased ELA-2 activity found in LEKTI 420K homozygous individuals could play a major role in these alterations. Indeed, transgenic ELA-2 mouse model studies have identified ELA-2 as one of the major players in profilaggrin maturation (7). Of note, even though not directly inhibited by LEKTI, ELA-2 is thought to contribute to NS clinical manifestations in that activation of its zymogen is determined by KLK5 cleavage (7).

By *in vitro* transfection experiments, we also demonstrated that the recombinant LEKTI 420K variant displays a weaker inhibitory activity on profilaggrin proteolysis compared with LEKTI 420E. To our knowledge, this is the first direct experimental evidence for LEKTI involvement in N-profilaggrin cleavage. Since undifferentiated transfected keratinocytes should not express any active KLKs, this result must be referred

to other proteases additional to those directly or indirectly regulated by KLK5. The multifunctional inhibitory role of LEKTI in the epidermis makes reasonable to hypothesize that the expression of LEKTI 420K variant elicits increased profilaggrin/FLG cleavages via additionally deregulated proteolytic steps.

Nevertheless, the extent to which the subtle serine protease deregulation associated with LEKTI 420K variant impacts on the skin permeability barrier is unknown. Interestingly, recent studies on human subjects and murine models have shown that FLG haploinsufficiency confers a risk of AD through SC structural and functional alterations, most of which are mediated by the pH increase secondary to reduced concentration of acidic FLG breakdown products (53,54). Indeed, elevated skin-surface pH accelerates lamellar bodies maturation and secretion and enhances the activity of several proteases that not only increases corneocyte shedding and degrades lipid processing enzymes, but also activates pro-inflammatory pathways (20,22,55).

In view of the current AD model that places epidermal barrier defects in the centre of converging pathogenic events ('outside-inside-outside' model) in which the regulation of protease activity plays a major role (26,56), the results that we report point to a link between the LEKTI 420K variant and AD pathogenesis, via subtle epidermal barrier dysfunction. They shed light on controversial genetic association study results. Indeed, the early report by Walley described a significant association of maternally derived alleles carrying 420K with AD and atopy in two independent panels of British families ($P = 0.002$ and $P = 0.005$) (25). Since then, other genetic association studies confirmed a potential contribution of this LEKTI variant to the AD pathogenesis in German and Japanese cohorts, even though the association was often weak and sometimes restricted to sub-phenotypes (27–30). In contrast, three other studies in European populations did not reproduce this genetic association (33–35), possibly because of the study design (case-control studies that cannot detect a maternal effect versus family-based studies), population and ethnic differences, the lack of power to detect association, differences in phenotypic characteristics, including age (children versus adults), severity and asthma, and the synergic interaction of 420K with additional genetic risk variants and environmental factors.

To determine the real effect of the 420K variant, the analysis of large AD cohorts was required. This type of analysis was performed by Weidinger who studied a significant number of AD patients from German and Irish/English family-based and case-control cohorts (32). While no association with 420K was found in the case-control analysis, an association with a maternal effect was identified in the family-based study. Analysis in the pooled AD population (2774 cases and 10 607 control subjects) showed a weak association ($OR = 1.13$ and $P = 0.045$), and the authors concluded that LEKTI 420K confers a risk of AD, but it is not a major genetic contributor. Although the authors found no evidence for an epistatic effect between 420K and FLG mutations, they suggested that LEKTI is a potential player within the FLG cascade, thus anticipating the results of our functional studies.

Here, we demonstrate that the modification of the subtle balance in LEKTI inhibitory fragments determined by the presence of the 420K residue impacts on protease regulation,

leads to enhanced DSG1 degradation and increases profilaggrin proteolytic processing, which are likely to contribute to defective skin barrier permeability. It is also possible that LEKTI altered proteolysis affects its inhibition capacities towards external proteases derived from allergens and *Staphylococcus aureus*, as well as lipid processing and PAR-2-mediated inflammation, all features which are relevant to AD pathogenesis. Unrestrained KLK5 activity plays a major role in allergic manifestations in NS through an intrinsic KLK5-PAR2-TSLP pro-allergic pathway (22,23). Consistent with KLK5 overactivity associated with the expression of LEKTI 420K variant, a weak but still detectable increase in TSLP expression was observed in 420KK epidermis. While TSLP overexpression is a major characteristic of lesional atopic skin (57), the extent to which the 420K associated LEKTI abnormal processing favors the development of AD remains to be determined. In fact, considering the high frequency of the E420K variation and the weak genetic association with AD reported, it is possible that other determinants compensate for the effects of LEKTI abnormal proteolytic processing. The variable reduction of the FLG and DSG1 content in 420KK epidermis is consistent with this possibility. Moreover, as underscored by the fact that AD develops in only ~42% of all *FLG* null mutation heterozygous carriers (58), the combined effects of several genetic and/or environmental factors are required for the development of AD.

It is also conceivable that 420K could favor the development or modify the course of other frequent skin diseases. In particular, it could influence those disorders in which the skin barrier and proteases are likely to play important roles. Protease deregulation could impact on cathelicidin processing, epidermal structural integrity, Toll-like receptors and/or gap junctions and thereby influence skin conditions such as acne rosacea (59,60), xerosis (dry skin), skin aging, contact dermatitis and possibly susceptibility to skin infections (40). These possibilities, together with the development of innovative therapeutic approaches for AD aimed at restoring LEKTI inhibitory function, would certainly represent new and promising future fields of investigation.

MATERIALS AND METHODS

The study was conducted according to the Declaration of Helsinki Principles, it was approved by the local ethics committee and a written informed consent was obtained from all NS patients and healthy volunteers involved.

Cell cultures

Isolation and culturing of normal and NS human primary keratinocytes (HK and NSK, respectively) have been carried out, as previously described (44). Cell differentiation was induced by culturing confluent cells for 5 days in keratinocyte growth medium (KGM, Invitrogen, Palo Alto, CA, USA) containing 1.2 mM Ca^{2+} . To analyze profilaggrin processing, HK were cultured on a feeder layer of lethally irradiated 3T3-J2 mouse fibroblasts in Rheinwald and Green keratinocyte growth medium, as described (44), and were harvested for 3 days after cells reached confluence. At least five HK strains for each

codon-420 genotype and two different NSK strains have been used for each experiment. Details of the *SPINK5* gene mutations in NS patients are described elsewhere (61). HEK293 cells were grown in Dulbecco's modified Eagle's Medium with 10% fetal calf serum, 100 U/ml penicillin/streptomycin and 2 mM glutamine (Invitrogen). To study TSLP expression, normal HKs were seeded on 6-well plates and grown in half EpiLife 0.06 mM CaCl_2 (Invitrogen) and half Green medium (62) until confluency. Cells were then washed and grown in Green medium without fetal calf serum for 3 days.

SPINK5 and *FLG* genotyping

For *SPINK5* and *FLG* gene analyses, all the HK strains and skin biopsies used in this study were subjected to total genomic DNA isolation. Genotype determination at the *SPINK5* g.1258G>A (p. E420K) single nucleotide polymorphic site was carried out by direct sequencing of the corresponding genomic region PCR amplification products, as elsewhere described (61). Each keratinocyte strain was genotyped for the eight most prevalent *FLG* null mutations in Europe (424del17, 621del4, R501X, 2282del4, R2447X, 2974delGA, S3247X and 3702delG). R501X, R2447X and S3247X were analyzed by Taqman allelic discrimination (ABI 7500, Applied Biosystems, Foster, CA, USA). Typing for 424del17, 621del4, 2282del4, 2974delGA and 3702delG was performed by sizing of the fluorescently labeled PCR product on ABI 3130 sequencer (Applied Biosystems).

Protein extract preparation

Cultured cells were lysed in radio immunoprecipitation assay (RIPA) buffer containing Complete[®] (Roche Applied Science, Mannheim, Germany). Lysates were clarified by centrifugation at 13 000g, at 4°C for 15 min. The CM was concentrated by acetone precipitation. Skin biopsies of healthy volunteers undergoing plastic surgery were processed for epidermal protein extract preparation. Following dermal-epidermal cleavage by heating for 5 min at 56°C in phosphate buffered saline (PBS), the epidermis was homogenized on ice with Ultra-Turrax in RIPA buffer with Complete[®]. Lysates were clarified by centrifugation at 13 000g, 4°C for 15 min. To analyze profilaggrin processing, both epidermal and HK total soluble proteins were extracted in Tris/urea buffer (8 M urea, 50 mM Tris-HCl, pH 7.6, Complete[®], Roche Applied Science). HK were cultured in parallel with those used for RNA isolation for quantitative PCR analysis. Skin biopsies from at least three different donors for each codon-420 genotype have been used.

Western blotting and densitometric analysis

For LEKTI fragments analysis, CM (400 μl) of differentiated HK or LEKTI transfected HEK293 cells were separated on a 12% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a PVDF membrane (Amersham Biosciences, Inc., Piscataway, NJ, USA). An 8% SDS-PAGE was used for better resolution of higher molecular weight LEKTI fragments. Membranes were incubated with anti-LEKTI-D7D12 polyclonal antibody (18). Ponceau S staining (Sigma

Aldrich, St Louis, MO, USA) was used to ensure equal protein loading among samples.

For DSG1 and FLG analysis, 20 µg of Tris/urea epidermal protein extracts were separated on an 8% or 12% SDS-PAGE, respectively, transferred to a PVDF membrane and blotted with specific monoclonal antibodies.

For N-ProFLG analysis, 50 µg of transfected NSK total protein extracts were separated on a 12% SDS-PAGE transferred to a PVDF membrane and blotted with anti-FLAG monoclonal antibody or anti-actin polyclonal antibody. Transfection efficiency was evaluated by western blotting analysis of equal amounts of the corresponding CM using the anti-LEKTI-D7D12 polyclonal antibody.

Densitometric analysis was carried out to evaluate relative protein amounts using a GS-710 imaging densitometer and the Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA). For FLG analysis, pixel density for each sample was calculated on the entire lane (from 37 to 400 kDa), while for DSG1, N-ProFLG and actin on the specific bands. Protein amounts were normalized to actin and plotted.

RNA isolation and analysis

For FLG and DSG1 mRNA expression level evaluation, skin biopsies were incubated for 1 h in 0.5% dispase at 37°C and the epidermis was mechanically separated. Total RNA was isolated from separated epidermis using TriZol[®] according to the manufacturer's guidelines (Invitrogen). RNA was reverse transcribed into complementary DNA (cDNA) using Super Script III and oligo dT (Invitrogen). Quantitative real-time PCR was realized on an ABI Prism 7500 sequence detection system (Applied Biosystems) using an ABI TaqMan[®] gene expression master mix (Applied Biosystems). Primers and FAM-conjugated probes for *FLG* (Hs00863478_g1), *DSG1* (Hs.PT.51.20624927) and *HPRT1* (Hs.PT.51.2145446) were purchased from both Applied Biosystems and Integrated DNA Technologies (IDT, Coralville, IA, USA). To allow for comparisons between samples, target gene expression was normalized to the corresponding *HPRT1* levels. For TSLP expression, total RNA was isolated using the RNeasy mini kit (QIAGEN). cDNA was synthesized using MMLV-RT with oligo-dT (Invitrogen). Quantitative real-time PCR was realized using the qPCR Mesagreen mix (Eurogentec). Results were normalized with the *HPRT* gene and analyzed using sequence detection system version 1.2 (Applied Biosystems). Sequences of primers used are h-*HPRT*-F GCCCTGGC GTCGTGATTAGT, h-*HPRT*-R AGCAAGACGTTTCAGTCC TGTC, h-TSLP-F CGCGTCGCTCGCCAAAGAAA, h-TSLP-R TGAAGCGACGCCACAATCCTTG.

Construct generation

The construct for expression in eukaryotic systems of the LEKTI_{FL} isoform carrying the most common variant 420E (pLEKTI_{FL-420E}) was previously described (18). The pLEKTI_{FL-420K} variant was generated by mutagenic PCR using pLEKTI_{FL-420E} as template and the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) following manufacturer's instructions. To generate the flag-tagged pN-ProFLG expression vector, profilaggrin N-terminal sequence, corresponding to

residues 2-467 (NP_002007.1), was amplified from differentiated normal HK cDNA using primers N-ProFLG_FOR (cccgaattctctactctcctggaaacatctttgccc) and N-ProFLG_REV (cccctcgagagaccctgaacgtccagaccgttcccc), and cloned into the pFLAG-CMV-6 vector (Sigma Aldrich).

DNA transfection

Confluent HEK293 cells were transiently transfected with 8 µl of Lipofectamine 2000 reagent (Invitrogen) and 10 µg of DNA/9.6 cm² well in serum-free medium. Thirty-six hours after transfection, CM of transfected cells was recovered and secreted proteins were concentrated by acetone precipitation. For *in vitro* N-ProFLG processing assays, the day before transfection NSK were seeded in a 6-well plate in KGM containing 0.15 mM Ca²⁺ at a cell density of 2 × 10⁵ per well. Double transfections were performed using 8 µl of Lipofectin reagent (Invitrogen) and 1 µg of total plasmid DNA for each well. These experiments were carried out using two different NSK strains.

Antibodies

The affinity-purified rabbit polyclonal antibody directed to the LEKTI region D7D12 was previously described (18). Mouse monoclonal antibodies anti-DSG1 (P23), anti-FLG (AKH1) and anti-FLAG (M2) were purchased from PROGEN Biotechnik GmbH (Heidelberg, Germany), Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Sigma, respectively. Rabbit polyclonal anti-actin antibody was purchased from Santa Cruz Biotechnology. Rabbit polyclonal antibody anti-TSLP was purchased from Abcam.

Casein gel zymography

Epidermis from skin biopsies of healthy volunteers undergoing plastic surgery, previously genotyped for *SPINK5* g.1258G > A, were crushed in 1 M acetic acid solution with an Ultra-Turrax. After overnight extraction at 4°C, soluble proteins were lyophilized and resuspended in water. Proteins were then assayed (Bradford, Bio-Rad Laboratories, Hercules, CA, USA), and 12 µg of soluble fractions were mixed in a non-denaturing loading buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol and 0.1% bromophenol blue) and loaded onto casein copolymerized with acrylamide gels (15% acrylamide, 0.05% α-casein, Sigma-Aldrich) for electrophoresis. Gels were washed with 2.5% Triton X-100 for 1 h to remove SDS and incubated for 72 h at 37°C in a reaction buffer containing 50 mM Tris, pH 8. Gels were stained with 1% Coomassie brilliant blue for 30 min. Areas of caseinolytic activity appeared as clear zones against a dark blue background. Twelve micrograms of soluble fractions were also loaded onto a 12% denaturing SDS-PAGE and stained with 1% Coomassie brilliant blue for 30 min for loading control.

In situ zymography

Frozen sections (5 µm thickness) were rinsed with a washing solution (2% Tween 20 in PBS) and incubated at 37°C

overnight with 100 μ l of 4,4-difluoro-5,7 dimethyl-4 bora-3a,4a-diaza-s-indacene-3 propionic acid green fluorescent labeled casein (10 μ g/ml) to assess total protease activity, or FITC-conjugated elastin (50 μ g/ml) using the EnzChek-elastase assay kit (Invitrogen) in 50 mM Tris-HCl, pH 8, in order to visualize elastolytic activity. Cryostat sections were incubated under the same conditions with Boc-Val-Pro-Arg-AMC at 100 μ M or Suc-Leu-Leu-Val-Tyr-AMC (Sigma-Aldrich, St Louis, MO) at 100 μ M in 50 mM Tris-HCl, pH8, CaCl₂ 10 mM for the detection of trypsin- and chymotrypsin-like activity, respectively. All sections were rinsed with PBS solution and visualized either with the confocal microscope Leica TCS SP5 AOBS or with the Zeiss Axio Observer. Frozen sections from LEKTI 420EE or 420KK individuals were photographed at equal time points and exposure time. Images were captured and analyzed with the ImageJ software. The intensity of the fluorescence signals was coded as color gradient, ranging from 0 (dark) to 255 (white).

Immunofluorescence staining

Frozen sections (5 μ m thickness) were air-dried for 15 min and fixed in ice-cold methanol:acetone 1:1 for 10 min before the staining procedure. After 1 h pre-incubation with 1% bovine serum albumin, the sections were incubated with anti-FLG (2 μ g/ml), anti-DSG1 (non-diluted) monoclonal antibodies or anti-TSLP (1 μ g/ml) for 1 h at room temperature. Specimens were subsequently incubated with Dylight 488 anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) or Alexa-Fluor 488 goat anti-rabbit IgG (Life technologies, France) for 1 h at room temperature and mounted in Vectashield mounting medium with 4',6 diamino-2-phenylindole (Vector Laboratories, Burlingame, CA, USA). Slides were evaluated using a Zeiss Axioskop 2 (Carl Zeiss, Jena, Germany) or a confocal microscope Leica TCS SP5 AOBS.

FLG image analysis was performed using ImageJ software (<http://rsb.info.nih.gov/ij>). Five to ten fields for each FLG stained section were imaged in one session under identical settings. Average signal intensity was measured on defined regions of interest.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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