

Proteolytic Activation Cascade of the Netherton Syndrome–Defective Protein, LEKTI, in the Epidermis: Implications for Skin Homeostasis

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Lympho-epithelial Kazal-type-related inhibitor (LEKTI) is the defective protein of the ichthyosiform condition Netherton syndrome (NS). Strongly expressed in the most differentiated epidermal layers, LEKTI is a serine protease inhibitor synthesized as three different high-molecular-weight precursors, which are rapidly processed into shorter fragments and secreted extracellularly. LEKTI polypeptides interact with several proteases to regulate skin barrier homeostasis as well as inflammatory and/or immunoallergic responses. Here, by combining antibody mapping, N-terminal sequencing, and site-specific mutagenesis, we defined the amino-acid sequence of most of the LEKTI polypeptides physiologically generated in human epidermis. We also identified three processing intermediates not described so far. Hence, a proteolytic cascade model for LEKTI activation is proposed. We then pinpointed the most effective fragments against the desquamation-related kallikreins (KLKs) and we proved that LEKTI is involved in stratum corneum shedding as some of its polypeptides inhibit the KLK-mediated proteolysis of desmoglein-1. Finally, we quantified the individual LEKTI fragments in the uppermost epidermis, showing that the ratios between LEKTI polypeptides and active KLK5 are compatible with a fine-tuned inhibition. These findings are relevant both to the understanding of skin homeostasis regulation and to the design of novel therapeutic strategies for NS.

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INTRODUCTION

Serine proteases and their specific inhibitors have recently been shown to have a pivotal role in maintaining a perfect balance between epidermal cell proliferation and desquamation. Alterations of this equilibrium lead to defective epidermal differentiation and barrier function, as attested by inherited diseases such as Netherton syndrome (NS, OMIM 266500) and autosomal recessive ichthyosis with hypotrichosis (OMIM 610765), and by several animal models (Ovaere *et al.*, 2009). In particular, NS is caused by lack of

expression of the lympho-epithelial Kazal-type-related inhibitor (LEKTI) and is characterized by congenital erythrodermic ichthyosis, hair defects, and atopic manifestations (Comel, 1949; Netherton, 1958; Chavanas *et al.*, 2000). Severe complications, such as recurrent bacterial infections, hypernatremic dehydration, and failure to thrive, frequently compromise NS prognosis in infants.

LEKTI is a serine protease inhibitor whose primary structure recalls that of the Kazal-type family. However, unlike its related molecules, LEKTI shows as many as 15 inhibitory domains (D1–D15) (Magert *et al.*, 1999). LEKTI D2 and D15 contain a typical Kazal motif with the canonical six cysteine residues, whereas the remaining 13 domains present a Kazal-related structure that misses one of the three conserved disulfide bridges. However, all LEKTI domains are still able to generate the typical Kazal-binding loop hairpin structure (Lauber *et al.*, 2003). In human, alternative pre-mRNA processing generates three LEKTI isoforms differing only in their C-terminal portion: LEKTI full-length with 15 domains (LEKTI_{FL}, 145-kDa), a long isoform (LEKTI_L, 148 kDa) carrying a 30 amino-acid residue insertion between D13 and D14, and a short isoform (LEKTI_{SH}, 125 kDa) composed of the first 13 domains (Tartaglia-Polcini *et al.*, 2006). All LEKTI isoforms contain a signal peptide and the inhibitory domains are separated by linker regions that can be cleaved by subtilisin-like proprotein convertases, such as

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Abbreviations: CM, conditioned medium; DSG1, desmoglein-1; K_i, inhibition constant; KLK, kallikrein; LEKTI, lympho-epithelial Kazal-type-related inhibitor; NHK, normal human keratinocytes; NS, Netherton syndrome; NSK, Netherton syndrome patient-derived keratinocytes; pAb, polyclonal antibody; rLEKTI, recombinant LEKTI; SC, stratum corneum

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furin (Bitoun *et al.*, 2003; Jayakumar *et al.*, 2005; Deraison *et al.*, 2007).

On the basis of *in vitro* and *in vivo* evidences, the following model for LEKTI functioning in the epidermis has been proposed: LEKTI is at first synthesized as precursor proteins by differentiating keratinocytes of the upper spinous/granular layers, and then cleaved by furin to generate several single or multi-domain polypeptides in a post-endoplasmic reticulum compartment (Bitoun *et al.*, 2003; Tartaglia-Polcini *et al.*, 2006). Thereafter, these fragments are transported by specific intracellular lamellar granule cargoes to the extracellular space between granular and horny layers (Ishida-Yamamoto *et al.*, 2005); there they prevent premature corneodesmosome degradation by forming inhibitory complexes with serine proteases involved in epidermal desquamation, such as kallikreins (KLKs) (Borgono *et al.*, 2007; Deraison *et al.*, 2007). Finally, progressive stratum corneum (SC) acidification mediates the release of active KLKs from LEKTI fragments (Deraison *et al.*, 2007), allowing degradation of corneodesmosomal components and the physiological exfoliation of the SC outermost layers. Accordingly, LEKTI deficiency results, both in human and mouse, in increased serine protease activity at the granular and transitional cell layers, premature corneodesmosome cleavage, and loss of skin barrier function. Nevertheless, the analysis of LEKTI-deficient skin showed several additional alterations such as abnormal profilaggrin processing, SC lipid disorganization, and overexpression of proinflammatory and proallergic molecules (Yang *et al.*, 2004; Descargues *et al.*, 2005, 2006; Hewett *et al.*, 2005). These data allowed the recognition of the involvement of LEKTI in other biological pathways (Bonnart *et al.*, 2010; Briot *et al.*, 2010). In light of the LEKTI multi-functional role and its peculiar primary structure, it can be hypothesized that both transcriptional and post-transcriptional modifications contribute to the generation of an ample number of fragments with related but distinct structure and role. Consistently, several *in vitro* studies have shown that each single or multi-domain LEKTI fragment displays a specific inhibitory profile toward epidermal proteases (Borgono *et al.*, 2007; Deraison *et al.*, 2007). However, most of the polypeptides tested represented "putative" bioactive fragments. In fact, although the activation of LEKTI precursors by furin has been demonstrated by different groups, thus so far, only few physiological fragments have been identified.

In this study, by combining antibody (Ab) mapping, N-terminal sequencing, and site-specific mutagenesis, we defined the amino-acid sequence of most of the LEKTI polypeptides physiologically generated in human keratinocytes (HKs). We also showed that some of these fragments are powerful inhibitors of KLK5 and 14, and are able to prevent KLK-mediated proteolysis of desmoglein-1 (DSG1). Finally, we demonstrated that, in the epidermal granular/horny layers, the relative amounts of LEKTI fragments and active KLK5 are compatible with a fine-tuned inhibition. All together, our findings increase the molecular understanding of LEKTI function and provide the basis for previously unreported therapeutic interventions for NS and, likely, other

skin disorders presenting deregulated KLKs activity and skin barrier defects.

RESULTS

Several LEKTI fragments are generated in epidermis, cultured keratinocytes, and transfected HEK293 cells

To define LEKTI physiological proteolysis, Ab mapping was at first performed using a newly generated anti-D7D12 polyclonal Ab in combination with previously described ones (Figure 1a). According to previous data (Deraison *et al.*, 2007), LEKTI precursors could be detected only in the intracellular fraction of differentiated normal HKs (NHKs) (not shown), while several LEKTI fragments (65, 68, 42, 37, 30, and 23 kDa) were visualized both in human epidermis and conditioned medium (CM) of differentiated NHK (Figure 1b). The same signals were also detected in the HEK293 epithelial cell line ectopically expressing C-terminal-tagged LEKTI proteins, indicating that the proproteins follow the same processing in both cell types (Figure 1b). Absence of signals in cells which do not express LEKTI, as proliferating NHK, NS keratinocytes and HEK 293 transfected with the empty vector, confirmed Ab specificity (Figure 1b and c). The molecular weight of the detected bands also excluded cross-reactivity with the recently discovered single-domain serine protease inhibitors SPINK6 (~6-kDa) and LEKTI-2 (7.7 and 15 kDa as monomer and dimer, respectively) (Brattsand *et al.*, 2009; Meyer-Hoffert *et al.*, 2009, 2010). In our experimental conditions, LEKTI single domains (~7 kDa) could be detected exclusively by the mAb 1C11G6 in the HEK293 over-expressing the recombinant protein (Figure 1c). Interestingly, three additional previously undescribed fragments of 102, 105, or 79 kDa were detected in CM of cells ectopically expressing LEKTI_{FL}, LEKTI_L, and LEKTI_{sh}, respectively, (Figure 1c). As they were also recognized by antibodies directed to the C-terminal histidine-tag (Figure 1c), it was inferred that each of them represents a processing intermediate that includes the respective isoform-specific C-terminal domain. Likely, in human epidermis and NHK, these fragments are rapidly cleaved to generate the more stable C-terminal fragments of 65, 68, and 42 kDa (Figure 1b and c) (Bitoun *et al.*, 2003). As to the 37, 30, and 23-kDa fragments, they were visualized using the anti-D7D12 and 1C11G6, but not the C-terminal-directed antibodies (Figure 1b and c), indicating that they do not comprise this portion of the protein.

LEKTI fragments are generated by cleavage at furin consensus sequences

In silico analysis of LEKTI primary structure (Swiss-Prot Q9NQ38) (Duckert *et al.*, 2004) revealed three potential furin consensus sequences with a significant score (Arg355, Arg425, and Arg625), as well as several others with lower values, all located in the linker regions between the inhibitory domains (Figure 2a). To identify the physiological cleavage sites, two different strategies were used: N-terminal sequencing of purified polypeptides and mutagenesis of the predicted positions. In particular, as immunoblotting analysis indicated that the isoform-specific C-terminal fragments of

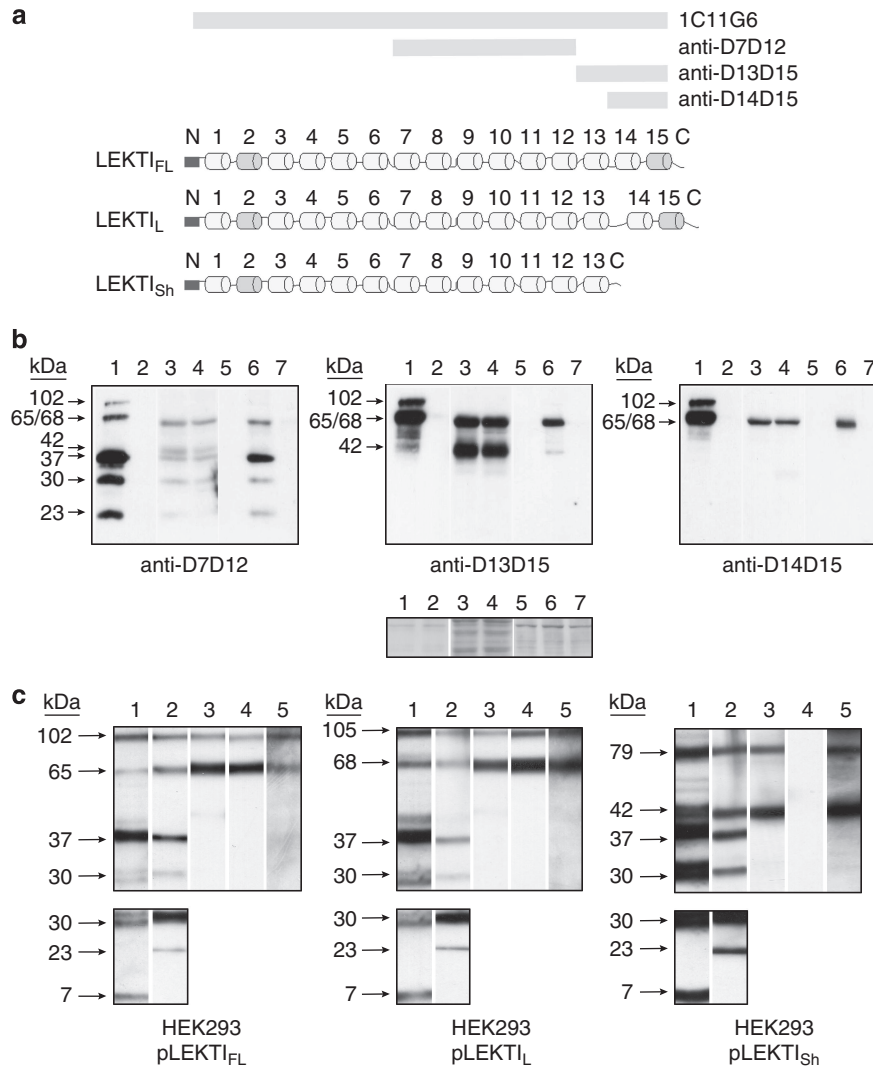


Figure 1. LEKTI proteolytic fragments in the epidermis, human keratinocytes, and transfected HEK293. (a) Schematic representation of lympho-epithelial Kazal-type-related inhibitor (LEKTI) isoforms with location of the antigenic polypeptides used to generate the indicated antibodies. (b) Immunoblotting detection of LEKTI in: conditioned medium (CM) of HEK293 transfected with pLEKTI_{FL} (1) or empty vector (2), total extract from human epidermis of two healthy donors (3, 4), CM of proliferating natural human keratinocytes (NHKs) (5), CM of differentiated NHK (6), and Netherton syndrome (NS) keratinocytes (7). Note that the experimental conditions used did not allow separation of the 65- and 68-kDa fragments, and that the band corresponding to the 65-kDa fragment displays a higher molecular weight in HEK293 expressing LEKTI_{FL} because of the C-terminal tag. Ponceau S staining was used to ensure even protein loading among similar samples (lower panel). Different exposures of the same blot (lanes 1–2, 3–4, 5–7) were juxtaposed. (c) Immunoblotting detection of LEKTI in CM of HEK293 transfected with pLEKTI_{FL}, pLEKTI_L, or pLEKTI_{Sh} using the antibodies 1C11G6 (1), anti-D7D12 (2), anti-D13D15 (3), anti-D14D15 (4), or anti-His₆ (5). Lower panels: 1C11G6 and anti-D7D12 immunoblot longer exposures revealing the 23- and 7-kDa fragments. Sizes of immunoreactive bands are indicated.

65, 68, and 42 kDa are generated by the usage of the same cleavage site (Figure 1c), the 42-kDa polypeptide was chosen as prototype. It was purified from CM of HEK293 expressing pLEKTI_{Sh}, and subjected to N-terminal sequencing, which revealed a perfect match with residues 626–634 of LEKTI (Figure 2b). This result demonstrated that the 42-kDa C-terminal fragment originates from the usage of the Arg625 furin cleavage site located in the linker region between D9 and D10. To confirm our assumption that the 65 and 68-kDa C-terminal fragments are also generated by cleavage at Arg625 of the LEKTI longer isoforms, this residue was mutagenized in the LEKTI_{FL} sequence and the mutated protein (R625A) was ectopically expressed in the

LEKTI-deficient cells NS keratinocytes and HEK293. Immunoblotting of CM from both transfected cell types showed that processing of the mutated protein does not generate the 65-kDa polypeptide and results in accumulation of the intermediate C-terminal fragment of 102-kDa (Figure 2c). Noteworthy, the presence of this fragment in CM of pLEKTI_{FL}-transfected NS keratinocytes (Figure 2c) demonstrates that also in HKs, LEKTI cleavage proceeds through the generation of intermediate products. These findings allowed to conclude that in HKs, all three LEKTI isoforms are cleaved at Arg625, thus, generating the C-terminal fragments of 65, 68, and 42 kDa, corresponding to D10-D15_{FL}, D10D15_L, and D10D13_{Sh}, respectively. Furthermore, in R625A LEKTI_{FL}-expressing cells

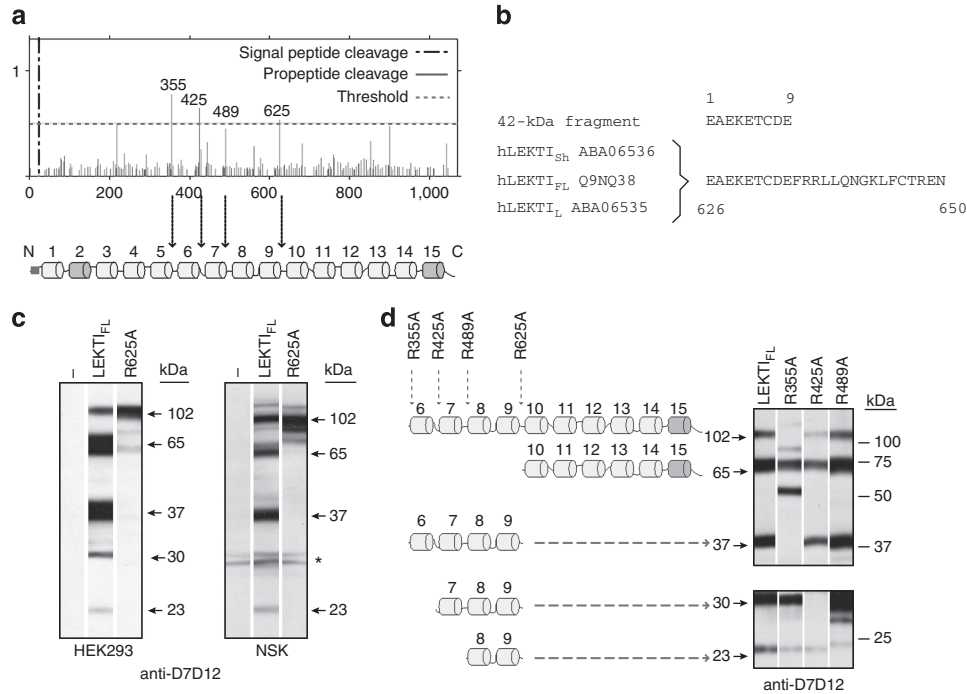


Figure 2. Mapping of LEKTI proteolytic fragments. (a) Graphic illustration of predicted proprotein convertase cleavage sites in lympho-epithelial Kazal-type-related inhibitor (LEKTI) protein. (b) Partial N-terminal amino-acid sequence of the 42-kDa fragment isolated from conditioned medium (CM) of pLEKTI_{SH} transfected HEK293 in alignment with the three human LEKTI isoform sequences. (c) Immunoblotting detection of LEKTI fragments in CM from HEK293 or Netherton syndrome (NS) keratinocytes transfected with LEKTI_{FL}, LEKTI R625A mutant, or the empty vector. The asterisk indicates nonspecific signals. (d) Immunoblotting detection of LEKTI fragments in CM from HEK293 transfected with either wild type or mutant LEKTI. Samples were separated on 12% (upper panel) or 15% (lower panel) SDS-PAGE. On the left, schematic representation of LEKTI processing as deduced from N-terminal sequencing and mutagenesis results. Sizes of immunoreactive bands are indicated. Different exposures of the same blot were juxtaposed.

also the generation of the newly characterized 37, 30, and 23-kDa fragments was compromised, indicating that the usage of the Arg625 cleavage site produces either the N- or the C-terminal end of these polypeptides (Figure 2c).

To verify whether the other predicted furin cleavage sites are actually used for LEKTI processing, the Arg355, Arg425, and Arg489 residues were mutagenized in the LEKTI_{FL} sequence. Although predicted with a low score, the Arg489 was included in the study as a LEKTI fragment starting from residue Glu490 had been previously described (Ahmed *et al.*, 2001). Expression of mutant LEKTI_{FL} proteins in HEK293 showed that all the potential furin cleavage sites analyzed are indeed used for LEKTI processing (Figure 2d). Specifically, substitution of Arg355 with A (R355A) resulted in loss of both the 102 and 37-kDa fragments. Mutagenesis of Arg425 (R425A) affected only the 30-kDa polypeptide. Finally, the substitution R489A resulted in loss of the 23-kDa fragment (Figure 2d). In some instances, additional bands were observed, suggesting that impairment of the physiological cleavage sites prompts usage of alternative sequences (Figure 2d). Analogous results were obtained expressing the mutants in NS keratinocytes (not shown). These findings, in combination with those obtained using the R625A mutant, allowed to conclude that the 102, 37, 30, and 23-kDa proteins correspond to D6D15_{FL}, D6D9, D7D9, and D8D9, respectively.

LEKTI fragments inhibit epidermal proteases

The capability of the newly identified LEKTI polypeptides to function *in vitro* as serine protease inhibitors was assessed against KLK5, KLK7, and KLK14, as they were previously suggested to represent LEKTI targets being involved in skin desquamation, cleavage of profilaggrin, and, more in general, epidermal barrier maintenance, all processes altered in NS (Brattsand and Egelrud, 1999). Information on the recombinant LEKTI fragments used throughout the present study is reported in the Supplementary Table S1 and S2 online. The inhibitory profiles and inhibition constants (K_i) of the LEKTI fragments that displayed a strong dose-dependent inhibitory activity are shown in Figure 3. KLK5 and KLK14, which are the major contributors to the trypsin-like activity in SC, were indeed inhibited to various extents by the different fragments. In particular, the strongest inhibitory activity toward KLK14 was observed with D7D9 ($K_i = 0.16$ nM) and toward KLK5 with D8D9 ($K_i = 5.38$ nM) (Figure 3a). In general, fragments generated from the LEKTI region comprised between D6 and D9 resulted to be more effective compared with the C-terminal polypeptides, that, under the specific *in vitro* assay conditions, inhibited at most 77% of KLK14 ($V_i/V_0 > 0.33$ at [D10D15_{FL}] = 12 nM) or 55% of the KLK5 activity ($V_i/V_0 > 0.45$ at [D10D15_L] = 25 nM) (not shown). In addition, the single domains D6 and D7 were weaker inhibitors as compared with the corresponding multi-domain fragments

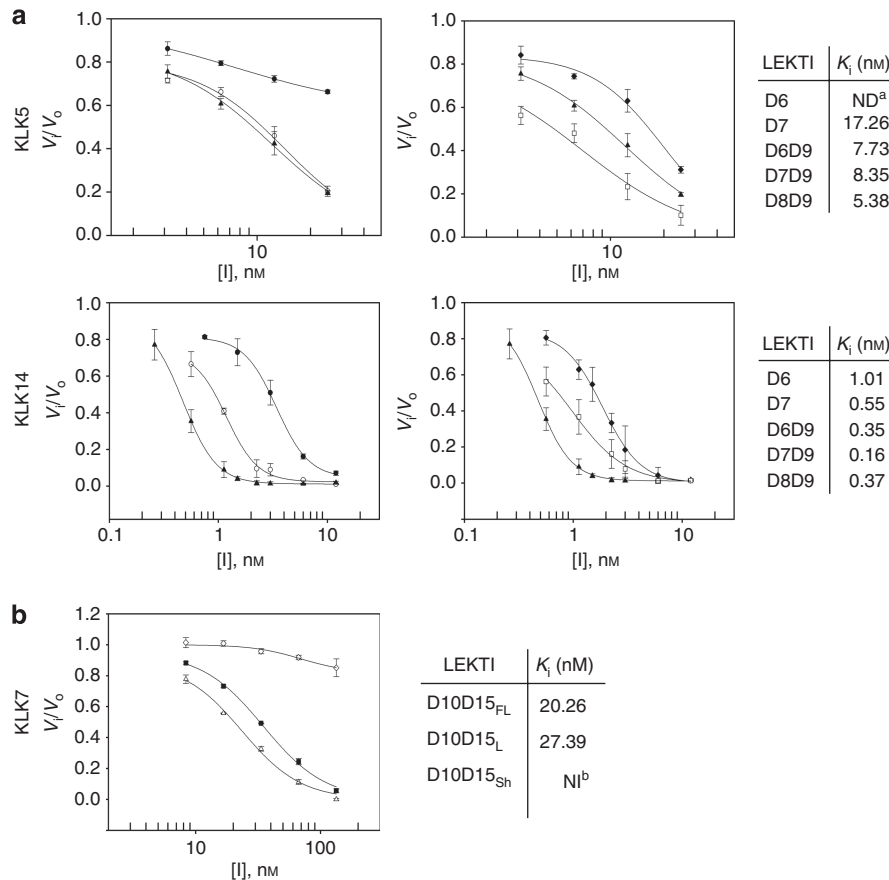


Figure 3. Dose-dependent inhibition of KLK activity by recombinant LEKTI fragments. (a) Dose-response inhibition curves of GST-D6 (●), GST-D7 (◆), GST-D6D9 (○), GST-D7D9 (▲), and GST-D8D9 (□) toward the trypsin-like serine proteases kallikrein (KLK)5 and KLK14. (b) Dose-response inhibition curves of D10D15_{FL}-His (△), D10D15_L-His (■), and D10D15_{Sh}-His (◇) toward the chymotrypsin-like KLK7. (a, b) KLKs were preincubated with increasing concentrations of inhibitors before addition of the specific substrates described in Supplementary Table S3 online. Residual KLK activity is expressed relative to control with no inhibitors (V_i/V_0). Each data point represents means \pm SD of three independent assays. K_i values are indicated on the right. ^aThe single domain fragment D6 inhibited at most 35% of KLK5 activity ($V_i/V_0 > 0.65$ at [D6] = 25 nM) and the relative K_i value could not be determined (ND). ^bNI, no inhibition.

(i.e., D6D9 and D7D9), and D6 was less effective than D7 (Figure 3a). For what concerns the chymotrypsin-like activity of KLK7, although to a lesser extent, it was also inhibited by LEKTI bioactive fragments. In particular the C-terminal polypeptides D10D15_{FL} and D10D15_L presented the strongest inhibitory activity (Figure 3b), with K_i values of 20.26 and 27.39 nM, respectively. Fragments derived from the LEKTI region D6–D9 instead showed a low inhibitory activity, regardless of their concentration (not shown). The isoform-specific fragment D10D13_{Sh} did not inhibit any of the tested proteases (not shown).

As recombinant LEKTI degradation by KLKs had previously been reported (Borgono *et al.*, 2007), the stability of the naturally occurring LEKTI fragments in presence of epidermal KLKs was evaluated. CM of differentiated NHK was incubated with the different peptidases and proteolysis of each LEKTI fragment assessed over time by immunoblotting. All LEKTI polypeptides were resistant to KLK5-mediated hydrolysis and slowly degraded by KLK14 (Figure 4a and b). Conversely, they were rapidly digested by the chymotrypsin-like protease KLK7 (Figure 4c). Interestingly, the 42-kDa LEKTI_{Sh} C-terminal fragment (D10D13_{Sh}) that did

not show any inhibitory activity against KLK7, was not digested by this enzyme. Of note, in some instances digestion of fragments resulted in the generation of discrete polypeptides (Figure 4b and c).

LEKTI fragments regulate skin barrier homeostasis by inhibiting DSG1 degradation

The loss of SC adhesion observed in the skin of both NS patients and mouse models is mainly because of the proteolytic degradation of DSG1 concurrent with a significant increase of trypsin-like activity in the epidermis (Descargues *et al.*, 2005, 2006). As DSG1 had been reported to represent a substrate for both KLK5 and KLK14 (Caubet *et al.*, 2004; Borgono *et al.*, 2007), the ability of the LEKTI fragments most effective toward these enzymes to inhibit DSG1 proteolysis was verified *in vitro*. In our experimental conditions, KLK5-mediated proteolysis of DSG1 was quite slow and reached its completion after 24 hours incubation at 37 °C, whereas almost all the protein was degraded after 1-hour incubation with KLK14 (not shown). However, when the enzymes were preincubated with different LEKTI fragments, various extents of inhibition were observed (Figure 5).

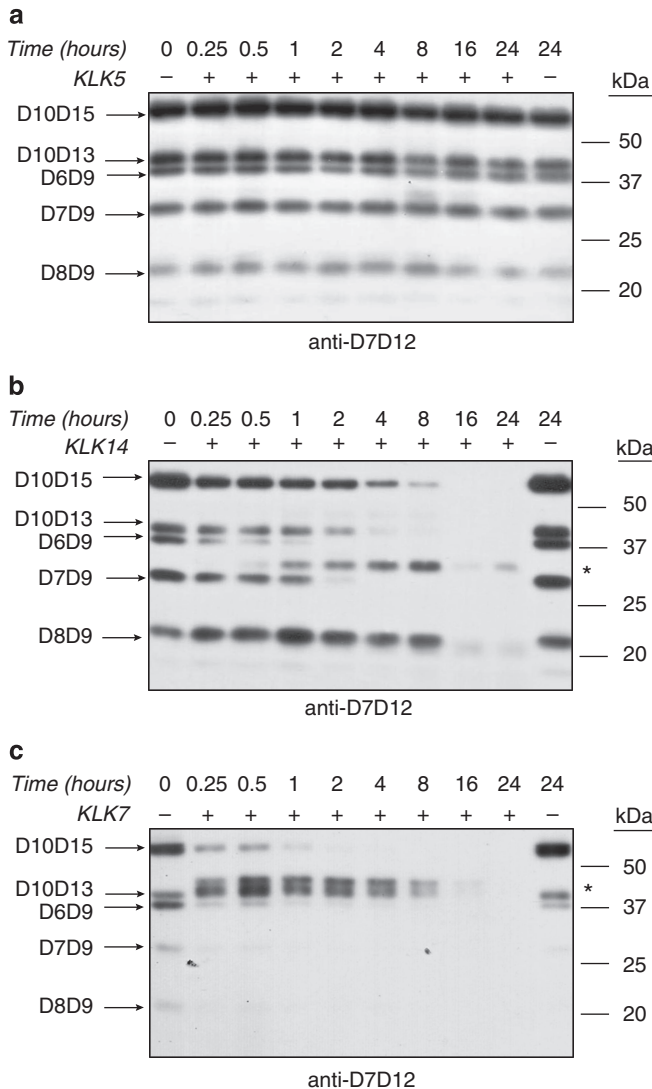


Figure 4. KLK-mediated hydrolysis of LEKTI fragments. (a-c) Immunoblotting detection of lympho-epithelial Kazal-type-related inhibitor (LEKTI) fragments in natural human keratinocyte (NHK) conditioned medium (CM) incubated for up to 24 hours in presence of kallikrein (KLK)5 (a), KLK14 (b), or KLK7 (c), and analyzed at various time points. Asterisks indicate sub-products derived from KLK-mediated cleavage.

In particular, the multi-domain fragments D6D9, D7D9, and D8D9 resulted to be, in a quite similar manner, very effective inhibitors of KLK14-mediated DSG1 proteolysis by preserving over 80% of DSG1 molecules from cleavage. As to the KLK5-mediated DSG1 cleavage, a significant inhibitory effect (~80%) was achieved by the addition of D6D9, whereas a medium to low inhibitory effectiveness, ranging from 60% (D7) to 20% (D8D9), was observed with the other fragments (Figure 5).

Concentration of LEKTI fragments in human epidermis is congruent with KLK5 inhibition

To further investigate the regulative role of LEKTI toward the epidermal KLKs, the relative amounts of each LEKTI bioactive polypeptide and of KLK5 in the epidermis were determined.

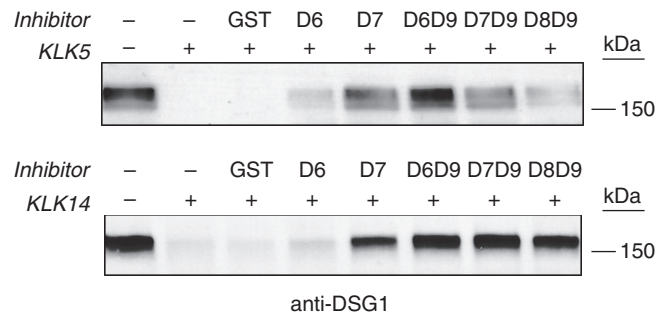


Figure 5. Inhibition of KLK-mediated DSG1 degradation by LEKTI fragments. Immunoblotting detection of desmoglein-1 (DSG1) in epidermal extracts incubated with kallikrein (KLK)5 (upper panel) or KLK14 (lower panel). Proteases were preincubated with the indicated recombinant polypeptides.

As several evidences demonstrated colocalization of LEKTI with epidermal KLKs at the granular/horny layers (Ishida-Yamamoto *et al.*, 2005), human skin biopsy samples were mechanically and chemically treated to obtain epidermal specimens enriched for these highly differentiated layers (Figure 6a). Individual LEKTI fragments and KLK5 were then quantitatively measured by western blot analysis of protein extracts. Defined amounts of the corresponding recombinant proteins were used to generate reference standard dilution curves (Figure 6b). The molar concentrations of LEKTI fragments D6D9, D7D9, and D8D9 turned out to be comparable to that of the KLK5 active form (Figure 6b), in keeping with a finely regulated inhibition. LEKTI C-terminal fragments, instead, were present in 4- to 7-fold molar excess. These data indicate that, along with their different inhibitory capability, these LEKTI polypeptides show a diverse stability *in vivo*.

DISCUSSION

The defective expression of the multi-domain serine protease inhibitor LEKTI and the consequent unrestrained protease activity result in the life-threatening syndromic skin disease NS. No curative therapies are currently available for this complex disorder. Topical delivery of LEKTI bioactive fragments represent the most promising therapeutic approach for NS. With this thought in mind, we have characterized the LEKTI polypeptides generated in human epidermis. In particular, by mapping four physiologically utilized cleavage sites (Arg355, Arg425, Arg489, and Arg625) we portrayed eight of the LEKTI bioactive polypeptides (D6, D7, D6D9, D7D9, D8D9, D10D15_{FL}, D10D15_L, and D10D13_{SH}). We also identified in cells exogenously expressing LEKTI, three processing intermediates (D6D15_{FL}, D6D15_L, and D6D13_{SH}) not described so far. Of note, some of the polypeptides identified in this study partly differ from previously inferred ones. In particular, our site-specific mutagenesis experiments demonstrate that the 23-kDa fragment detected in the epidermis originates from the cleavages at the Arg489 and Arg625 furin recognition sites and, thus, corresponds to LEKTI D8D9. This data partially conforms with the earlier description of a LEKTI fragment with the N-terminal sequence initiating with residue Glu490 (Ahmed *et al.*, 2001). However, the authors estimated the molecular weight of this polypeptide as

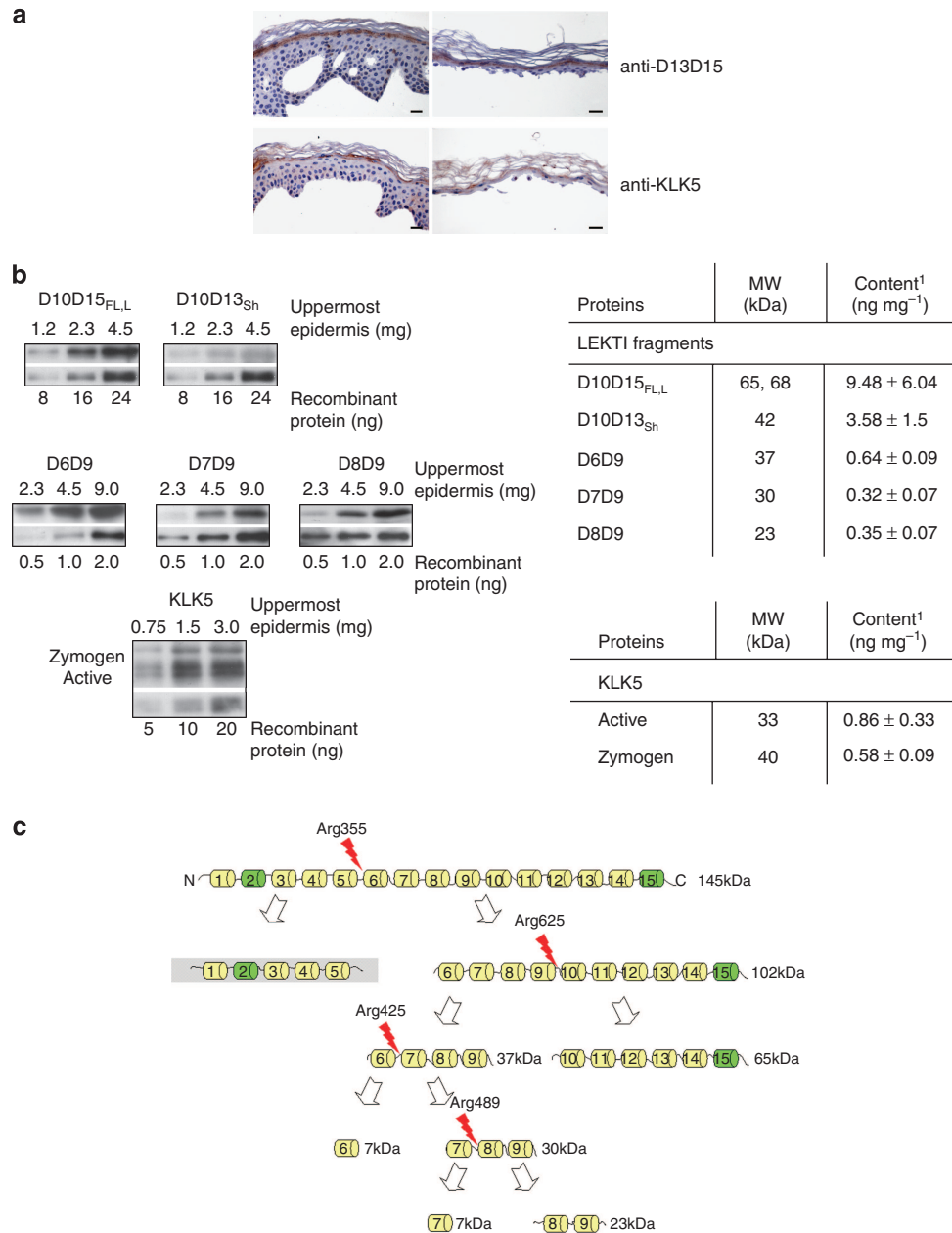


Figure 6. LEKTI fragments in human epidermis. (a) Immunohistochemical analysis for lympho-epithelial Kazal-type-related inhibitor (LETKI) and kallikrein (KLK5) in human epidermis (left panels) and epidermal specimens enriched for granular/horny layers (right panels). Bar = 40 μ m. (b) Quantitative analysis of LEKTI fragments and KLK5 in epidermis enriched for granular/horny layers by immunoblotting. Increasing amounts of protein extracts (expressed as microgram of uppermost epidermis dry weight) are compared with defined amounts of the corresponding recombinant proteins (expressed in ng). Note that the LEKTI weight values are relative to the sole polypeptides and not to the entire fusion proteins. The tables on the right show the protein content in the epidermis, measured as ng per mg of uppermost epidermis dry weight. ¹The values indicate the mean of three independent experiments \pm standard deviation. (c) Model of LEKTI proteolytic activation cascade as deduced from bioactive peptide mapping. Generation of the D1D5 fragment is inferred on the basis of previously published data.

30 kDa, likely because of the protein separation methodology used. Afterward this claim rebounded on the deductive analysis carried out by Deraison *et al.* (2007), who incorrectly defined the 30-kDa fragment as D8D11. In addition, here several evidences concur in demonstrating that the isoform-specific C-terminal fragments are generated by cleavage at residue Arg625 within the linker region between D9 and D10. Conversely, Deraison *et al.* (2007) proposed cleavage

between D8 and D9. Incomplete deglycosylation might account for this misinterpretation.

The identification of LEKTI sequential cleavage products allowed us to draw an activation cascade model, as depicted in Figure 6c for LEKTI_{FL}. The first cut takes place between D5 and D6, generating the C-terminal intermediate fragment of 102-kDa (D6D15_{FL}). Subsequently, cleavage between D9 and D10 generates both the 37-kDa fragment D6D9 and the

isoform-specific 65-kDa polypeptide D10D15_{FL}. Although the latter fragment is not further proteolyzed, D6D9 is cleaved at Arg425, within the linker region between D6 and D7, generating the 30-kDa fragment D7D9 and the previously characterized single-domain D6 (Magert *et al.*, 1999). Finally, D7D9 cleavage at Arg489 generates the 23-kDa fragment D8D9 and the single-domain D7. As to the N-terminal region, although the antibodies used did not visualize any processing product, we assumed that the previously described 37-kDa fragment starting from Lys23 (Jayakumar *et al.*, 2005) represents the D1D5 polypeptide generated by this cleavage. Further cleavages of this LEKTI portion could not be investigated.

Next, we studied the inhibitory specificities/activities of the identified LEKTI fragments. Specifically, we analyzed the epidermal serine proteases KLK5, KLK7, and KLK14, as they represented the best LEKTI target candidates. Indeed, they colocalize with the inhibitor (Ishida-Yamamoto *et al.*, 2005; Komatsu *et al.*, 2005b) and are involved in processes deregulated in LEKTI-deficient epidermis, in particular skin desquamation and profilaggrin processing (Brattsand and Egelrud, 1999; Bonnart *et al.*, 2010; Sales *et al.*, 2010). All KLKs tested were inhibited by LEKTI polypeptides with K_i in the nanomolar range. In agreement with the major increase of trypsin versus chymotrypsin-like activity observed in SC of NS patient (Komatsu *et al.*, 2008), the strongest inhibition was achieved toward KLK5 and KLK14, in particular by fragments derived from the LEKTI region D6–D9. The same polypeptides were also very effective inhibitors of KLK-mediated DSG1 degradation, a key step in corneocyte shedding. These results are in line with a NS genotype/phenotype correlation study by Komatsu *et al.* (2008) that highlights the important role of LEKTI D6–D12 region in determining NS severity. On the basis of the assumption that NS patients synthesize truncated forms of LEKTI, reliant on the location of mutations, the authors suggested that expression of this portion of the protein provides for inhibition of trypsin-like activity and consequent reduction of the NS clinical manifestations correlated to SC over-desquamation. For what concerns the activity toward KLK7, the best inhibition was achieved by LEKTI fragments D10D15_{FL} and D10D15_L. Chymotrypsin-like inhibitory capability has not been previously described for the LEKTI C-terminal region (Egelrud *et al.*, 2005; Schechter *et al.*, 2005; Deraison *et al.*, 2007), possibly due to structural differences or post-translational modifications of the LEKTI fragments used. Indeed, we expressed the C-terminal fragments in mammalian cells to ensure their correct glycosylation/functionality. However, we also observed that LEKTI fragments are rapidly degraded by KLK7, suggesting that this protease might not be their main target *in vivo*. Altogether, our results formally prove that the long-stated concept that each LEKTI fragment displays a specific inhibitory profile holds true for the LEKTI polypeptides physiologically generated in the epidermis. However, some differences between our inhibition assay results and findings by other groups (Borgono *et al.*, 2007; Deraison *et al.*, 2007) were noticed. Likely, the nature of the recombinant proteins tested as well as the experimental conditions used account for these discrepancies.

In summary, by showing that the herein identified LEKTI fragments exhibit differential specificity/activity, we demonstrate that each domain contributes to modulate the interaction with target proteases. We also prove that the multi-domain polypeptides are not merely processing intermediates on the way to single-domain inhibitors, but represent the truly active forms. Finally and most importantly, we demonstrate that the LEKTI polypeptides derived from the central portion of the proprotein are capable to block the KLK-mediated DSG1 degradation. Moreover, at the site of their potential interaction, these LEKTI fragments and KLK5 are present in a nearly 1:1 molar ratio, which is essential for a fine-tuned inhibition. Of note, the amount of KLK5 measured in our samples is comparable to that previously calculated by Komatsu *et al.* (2005a) in human SC using a different methodology. This correspondence allows to widen the considerations about the LEKTI fragments/KLK5 ratio to other KLKs described in that study.

These findings are relevant to the understanding of skin homeostasis regulation. In addition, they gain upon the design of a previously unreported and effective therapeutic intervention for NS, based on the replacement of the missing LEKTI polypeptides. The currently utilized therapies for this life-threatening and disabling disease are only aimed at reducing the clinical symptoms (Saif and Al-Khenaizan, 2007; Renner *et al.*, 2009). Nevertheless, in spite of the encouraging results (Di *et al.*, 2010; Roedl *et al.*, 2011), the *ex-vivo* gene transfer cannot be envisaged as an imminent therapeutic option. Local delivery through the horny layer, by means of topical application or non-invasive mini-gene transfer (Foldvari *et al.*, 2006) of LEKTI bioactive fragments could attenuate unopposed KLKs activity and counteract the skin barrier impairment of NS patients. Although the efficacy and safety *in vivo* of the “putative” LEKTI fragments studied by others cannot be predicted, it is fair to expect for a beneficial effect by the LEKTI physiological fragments. Fine-tuning of serine proteases activity could represent an important pharmacological tool also for more common skin diseases presenting increased SC serine proteases activity and altered epidermal barrier function, such as atopic dermatitis. The analysis of the inhibitory properties of the herein identified LEKTI fragments toward other epidermal serine proteases, shown to be involved in the pathways altered in NS, represents an important area for future studies.

MATERIALS AND METHODS

Purification of the 42-kDa LEKTI fragment

LEKTI_{Sh} 42-kDa fragment was purified from the CM of HEK293 transfected with pLEKTI_{Sh} using Ni-NTA Agarose (Qiagen, Hilden, Germany). The fragment was processed for N-terminal sequencing by Primm-srl (Milano, Italy).

Protein extract preparation

The study was conducted according to the Declaration of Helsinki Principles. Approval from the IDI-IRCCS Ethics Committee and informed consent from NS patient/healthy donors were obtained for all described studies that used human materials.

Cultured cells were lysed in RIPA buffer containing Complete (Roche Applied Science, Mannheim, Germany). Lysates were clarified by centrifugation at 13,000 *g*, 4 °C for 15 minutes. The CM was concentrated by acetone precipitation. Skin biopsy samples of three healthy volunteers undergoing abdominal plastic surgery were processed for epidermal protein extracts generation. Following dermal-epidermal cleavage by heating 5 minutes at 56 °C in PBS, the epidermis was homogenized on ice with Ultra-Turrax in RIPA buffer with Complete. Epidermal extracts used for DSG1 degradation assays were prepared as described (Caubet *et al.*, 2004).

LEKTI fragment proteolysis assay

Secreted proteins, concentrated from CM of differentiated NHK by acetone precipitation, were incubated with 50 ng of proteases up to 24 hours at 37 °C. Samples, boiled for 2 minutes in Laemmli buffer, were separated on 12% SDS-PAGE and immunoblotted.

DSG1 degradation inhibitory assay

Fifty nanogram of KLK5 or KLK14 was pre-incubated for 10 minutes at RT with 4-fold molar excess of recombinant LEKTI fragments or equivalent buffer volume. The mixture enzyme inhibitor, or equivalent volume of proteolysis buffer (10 mM sodium phosphate buffer, pH 7.2, 0.15 M NaCl), was added to 70 µg of epidermal extracts and incubated at 37 °C for 24 hours for KLK5 assays or for 90 minutes for KLK14 assays. To evaluate the inhibitory activity of recombinant LEKTI fragments, the amount of intact DSG1 detected by immunoblotting was assessed by densitometric analysis. The inhibition efficacy was given by the ratio between the DSG1 molecules still detected after incubation with both protease and inhibitors relative to protease-free controls.

Preparation of epidermal specimens enriched for granular/horny layers

Skin biopsy samples obtained from healthy volunteers were mechanically and chemically treated following a previously published protocol (Toulza *et al.*, 2007), with minor modifications. Procedure details are described in the Supplementary Material online.

Additional information regarding the antibodies, cell culturing and DNA transfection, enzyme, substrates and kinetic inhibition assays is available in the Supplementary Material online.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/jid>

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