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LEKTI proteolytic processing in human primary keratinocytes, tissue distribution and defective expression in Netherton syndrome

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***SPINK5*, encoding the putative multi-domain serine protease inhibitor LEKTI, was recently identified as the defective gene in the severe autosomal recessive ichthyosiform skin condition, Netherton syndrome (NS). Using monoclonal and polyclonal antibodies, we show that LEKTI is a marker of epithelial differentiation, strongly expressed in the granular and uppermost spinous layers of the epidermis, and in differentiated layers of stratified epithelia. LEKTI expression was also demonstrated in normal differentiated human primary keratinocytes (HK) through detection of a 145 kDa full-length protein and a shorter isoform of 125 kDa. Both proteins are N-glycosylated and rapidly processed in a post-endoplasmic reticulum compartment into at least three C-terminal fragments of 42, 65 and 68 kDa, also identified in conditioned media. Processing of the 145 and 125 kDa precursors was prevented in HK by treatment with a furin inhibitor. In addition, *in vitro* cleavage of the recombinant 145 kDa precursor by furin generated C-terminal fragments of 65 and 68 kDa, further supporting the involvement of furin in LEKTI processing. In contrast, LEKTI precursors and proteolytic fragments were not detected in differentiated HK from NS patients. Defective expression of LEKTI in skin sections was a constant feature in NS patients, whilst an extended reactivity pattern was observed in samples from other keratinizing disorders, demonstrating that loss of LEKTI expression in the epidermis is a diagnostic feature of NS. The identification of novel processed forms of LEKTI provides the basis for future functional and structural studies of fragments with physiological relevance.**

INTRODUCTION

Netherton syndrome (NS; MIM 256500) is a severe autosomal recessive skin disorder characterized by ichthyosiform erythroderma, a specific bamboo hair defect (trichorrhexis invaginata) and atopy. NS infants typically present at birth or soon after with generalized exfoliative erythroderma, which persists throughout life in the most severe cases or gradually evolves into a milder condition known as ichthyosis linearis circumflexa (1–3). Trichorrhexis invaginata is detectable by light microscopy on a variable proportion of scalp and eyebrow hairs, and corresponds to the atrophy and invagination of their distal parts (2,4). Patients display a broad range of allergic manifestations including atopic dermatitis, and markedly elevated serum IgE levels (5,6). Most patients also experience recurrent, if not persistent, bacterial infections (7). Histological and

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ultrastructural studies reveal an incomplete keratinization of the epidermis and severely impaired cornification. The granular layer is greatly reduced and may be lacking completely. The highly variable clinical presentation, together with the commonly delayed appearance of the pathognomonic bamboo hair after infancy, makes NS difficult to diagnose in early life. In the absence of specific treatment, NS prognosis is poor. The neonatal period is often associated with a profound failure to thrive, which leads to a fatal course in up to 20% of the cases (8).

We recently identified *SPINK5* (serine protease inhibitor Kazal-type 5) as the defective gene in NS (9,10). A total of 34 *SPINK5* mutations have been reported in patients, all of which create premature termination codons of translation (10–13). Northern blot analyses revealed a dramatic reduction of the 3.7 kb *SPINK5* transcript levels in cultured human primary keratinocytes (HK) from the majority of patients examined (10,12), suggesting defective protein expression. *SPINK5* encodes LEKTI (lympho-epithelial Kazal-type related inhibitor), a predicted serine protease inhibitor. The protein consists of 1064 amino acids organized into 15 potential inhibitory Kazal-type domains (D1–D15), preceded by a signal peptide. Only D2 and D15 perfectly match the typical Kazal motif [C-$(X)_n$-C-$(X)_7$-C-$(X)_{10}$-C-$(X)_{2/3}$-C-$(X)_m$-C], whilst other domains exhibit a Kazal-type-derived four-cysteine residue pattern. The isolation of individual domains D1, D5 and D6 from human hemofiltrate (14,15) revealed that LEKTI full-length protein most likely represents an inactive precursor, the proteolytic processing of which would be required for the release of bioactive forms. A 30 kDa polypeptide with an N-terminal sequence matching D8 of LEKTI was also purified from HK conditioned medium (16).

Subtilisin-like proprotein convertases (SPC) are a family of endoproteases responsible for the processing of numerous inactive prohormones and other proproteins into their biologically active forms (17). SPCs display a specific tissue distribution and cellular localization (17), and cleave their substrates at the general motif (K/R)-X_n-(K/R)↓($n = 0$, 2, 4 or 6; X-any amino acid) (18). A number of these consensus sequences have been identified within the amino-acid sequence of LEKTI, suggesting that SPCs may be involved in the proteolytic processing of LEKTI (13).

Analysis of the human tissue distribution of *SPINK5* transcripts predicts high expression levels of LEKTI in thymus, vaginal epithelium, oral mucosa, tonsils and Bartholin's and parathyroid glands (14). More recently, *in situ* hybridization has revealed the localization of *SPINK5* transcripts to the uppermost spinous layers and the granular layer of normal human epidermis (13). The authors also reported a marked increase in trypsin-like hydrolytic activity in the cornified layer of NS patients. This finding, together with the demonstration of a trypsin-inhibiting activity for D5 and D6 domains (14,15), supports a key role for LEKTI in the regulation of proteolytic events involved in skin barrier formation and maintenance.

To further characterize LEKTI human tissue distribution and investigate protein expression in HK from normal controls and NS patients, we developed monoclonal and polyclonal anti-N, and polyclonal anti-C terminal, antibodies. We report the first detection of the full-length protein, together with the identification of a shorter isoform in normal HK. We show that both precursors are N-glycosylated and rapidly cleaved in a post-endoplasmic reticulum compartment to generate at least three C-terminal fragments, and provide evidence that furin is involved in LEKTI processing. We also describe the protein expression in normal stratified epithelia, and demonstrate that loss of LEKTI expression in the epidermis is a diagnostic feature of NS.

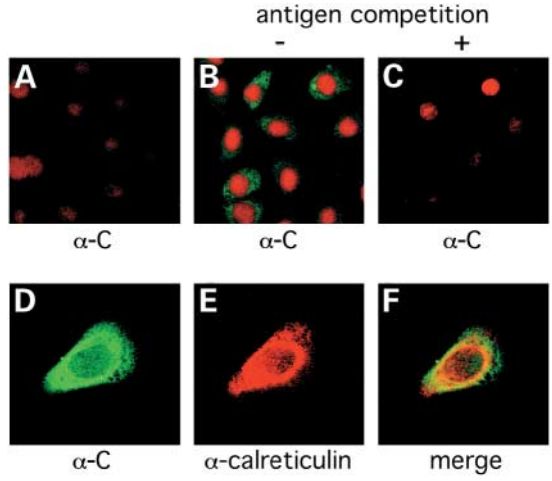

Figure 1. Immunolocalization of LEKTI in HK. HK were cultured in proliferating (low-calcium medium; **A**) or differentiating (high-calcium medium; **B**–**F**) conditions for 24 h, and processed for immunofluorescence microscopy analysis as described in Materials and Methods. (A–C) Cells were immunostained with LEKTI polyclonal α-C antibodies (green) pre-incubated in the presence (+) or absence (−) of the recombinant antigen GST-D13-D15 prior to immunodetection. Cells were counterstained with propidium iodide to reveal nuclei (red). (D–F) Cells were double stained with α-C (D) and anti-calreticulin (E) antibodies. The degree of overlap between the two proteins is represented by the yellow signal in the merged image (F). The results shown are representative of three to five independent experiments.

RESULTS

Characterization of LEKTI expression in normal HK

Given the predicted expression of LEKTI in the uppermost differentiated layers of the epidermis, we assessed the immunospecificity of the newly generated antibodies on proliferating and differentiated cultured HK. Terminal differentiation was induced by switching the ionic calcium content of the culture medium from low (<0.1 mM) to high (1.2 mM) concentrations, as previously described (19,20).

Immunolocalization of LEKTI. HK were cultured for 24 h in low or high calcium conditions and analysed by immunofluorescence microscopy using antibodies generated against the C-terminus of LEKTI (α-C). A strong labelling was observed in differentiated cells (Fig. 1B; green) whilst only very low protein levels were present in cells grown in proliferating conditions (Fig. 1A). No labelling was observed using pre-immune serum (data not shown). The specificity of labelling was subsequently confirmed by antigen competition experiment. Incubation of antibodies with recombinant antigen, prior to immunodetection, led to a dramatic reduction in signal

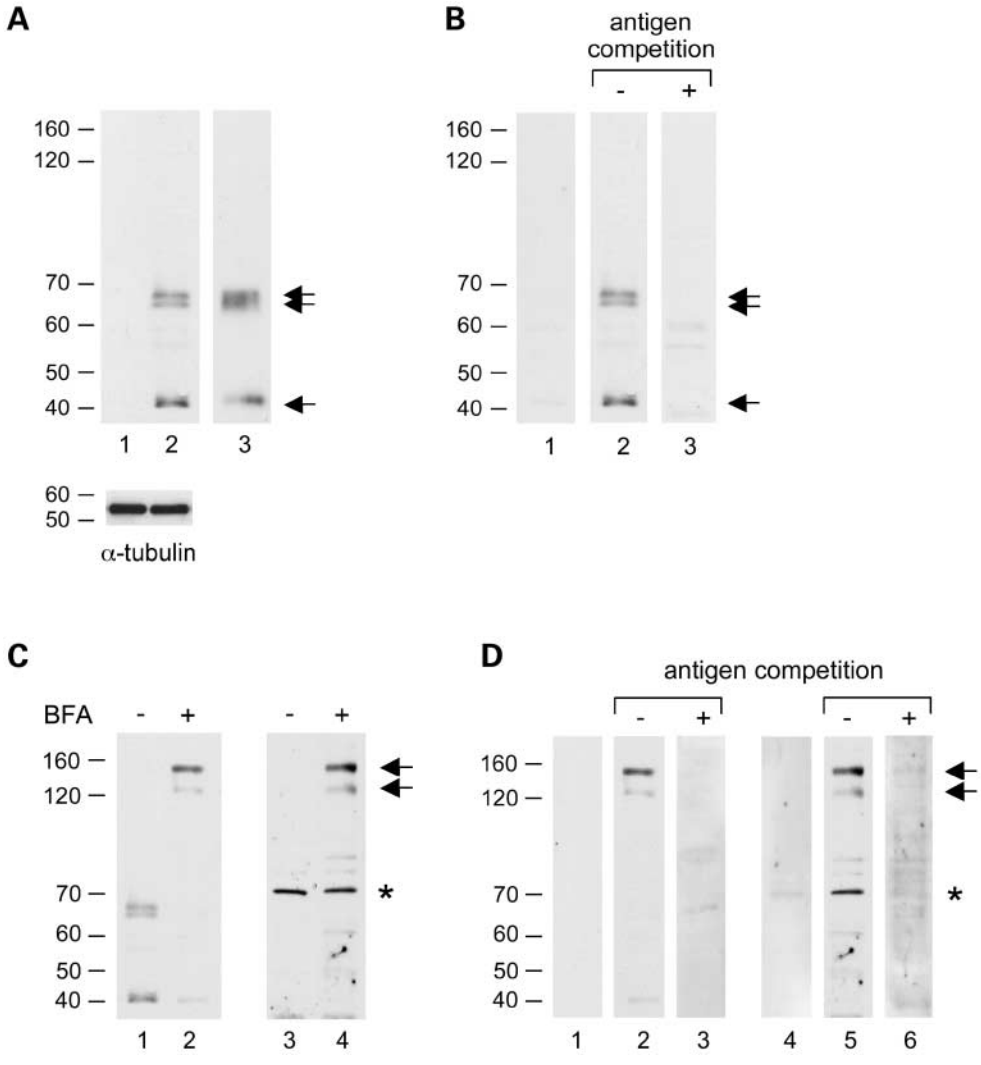

Figure 2. Molecular detection of LEKTI in HK. HK were cultured in proliferating (low-calcium medium) or differentiating (high-calcium medium) conditions for 24 h. Twenty microgram protein samples were separated by SDS–PAGE (7.5%) and analysed by western blot. (**A**) Total cell extracts (lanes 1 and 2) and conditioned medium (lane 3) from proliferating (lane 1) and differentiated (lanes 2 and 3) cells were analysed using LEKTI polyclonal α-C antibodies. Anti-tubulin detection shows equal loading of cell extracts. (**B**) Total cell extracts from differentiated cells were analysed using α-C antibodies (lanes 2 and 3) pre-incubated in the presence (+) or absence (−) of the recombinant antigen, or using the pre-immune serum (lane 1). (**C**) Total cell extracts from differentiated cells cultured for an additional 6 h in the presence (+) or absence (−) of BFA at 10 μg/ml were analysed using LEKTI polyclonal α-C (lanes 1 and 2) and α-N (lanes 3 and 4) antibodies. (**D**) Total cell extracts from differentiated cells treated with BFA were analysed using LEKTI polyclonal α-C (lanes 2 and 3) and α-N (lanes 5 and 6) antibodies pre-incubated in the presence (+) or absence (−) of the respective recombinant antigens (GST-D13–D15 and GST-D1–D6), or using the corresponding pre-immune sera (lanes 1 and 4, respectively). The asterisk indicates an unrelated protein cross-reacting with α-N antibodies, which is also present in samples from NS patients (data not shown). Arrows indicate the positions of the 42, 65, 68, 125 and 145 kDa protein bands. Molecular weight markers are indicated on the left in kDa. The results shown are representative of three to five independent experiments.

intensity (Fig. 1C). Since the labelling showed an extensive reticular pattern characteristic of the endoplasmic reticulum (ER), we performed double staining using α-C antibodies (Fig. 1D) and an antibody specific for the ER marker protein, calreticulin (Fig. 1E). Superimposition showed significant overlap (Fig. 1F), indicating that the majority of LEKTI is localized in the ER. Similar results were obtained using antibodies generated against the N-terminus of LEKTI (α-N; data not shown).

Molecular detection of LEKTI. HK were cultured as above, and analysed by western blot using α-C antibodies (Fig. 2A). Three bands of ∼42, 65 and 68 kDa were detected in total cell extract (lane 2) and conditioned medium (lane 3) from differentiated cells, but not in undifferentiated cells (lane 1). These signals were not detected using pre-immune serum (Fig. 2B; lane 1) or α-N antibodies (data not shown). As above, we confirmed the signal specificity by antigen competition experiment (Fig. 2B; lanes 2 and 3). We also examined a lower protein molecular weight range, but no additional N- or C-terminal-specific fragments were identified by the corresponding antibodies (data not shown).

The molecular weight of LEKTI predicted by the amino acid sequence is 118 kDa. Although both α-N and α-C antibodies detected LEKTI in the ER of differentiated HK, only 42, 65 and 68 kDa proteins were identified in the corresponding cell

extracts by α-C antibodies. These data suggest that LEKTI is rapidly processed in a post-ER compartment and that the 42, 65 and 68 kDa proteins represent C-terminal proteolytic forms. In an attempt to detect the full-length protein, we treated differentiated HK with brefeldin A (BFA), a drug that blocks ER to Golgi protein transport (21). Western blot analysis of these cell extracts (Fig. 2C) revealed two high molecular weight bands of ~125 and 145 kDa using α-C (lane 2) and α-N (lane 4) polyclonal antibodies, that were not detected in the absence of BFA (lanes 1 and 3, respectively). Note that only very low levels of the three C-terminal fragments were detected under such conditions (lane 2). In contrast, pre-immune sera failed to recognize the 125 or 145 kDa proteins (Fig. 2D; lane 1, α-C and lane 4, α-N). The specificity of these bands was further confirmed by antigen competition experiments (Fig. 2D; lanes 2 and 3, α-C and lanes 5 and 6, α-N), indicating that both the 125 and 145 kDa proteins were derived from LEKTI.

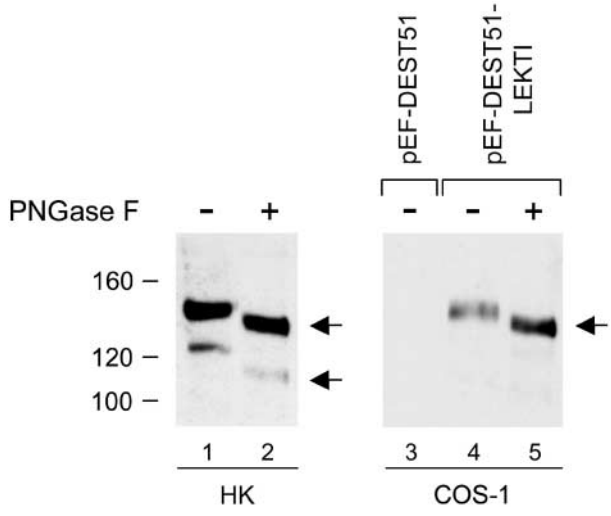

Figure 3. LEKTI glycosylation pattern. HK were differentiated for 24 h in high-calcium medium and cultured for an additional 6 h in the presence of BFA at 10 μg/ml (lanes 1 and 2). COS-1 cells were transiently transfected for 48 h with the pEF-DEST51-LEKTI construct containing LEKTI full-length cDNA (lanes 4 and 5), or with the empty pEF-DEST51 vector as control (lane 3). Twenty micrograms of total cell extracts from HK and COS-1 cells were incubated for 1 h at 37°C in the presence (+) or absence (−) of PNGase F. Samples were resolved by SDS–PAGE (5%) and analysed by western blot using LEKTI polyclonal α-C antibodies. Arrows indicate the positions of the 115 and 135 kDa de-glycosylated protein forms. Molecular weight markers are indicated on the left in kDa. The results shown are representative of three to five independent experiments.

LEKTI is expressed as two N-glycosylated precursor isoforms in HK

We previously detected the expression of a single 3.7 kb *SPINK5* transcript in cultured HK (10,12), whose size is consistent with the full-length protein (14). Since BFA treatment revealed two specific signals, we tested whether the 145 kDa band could correspond to the glycosylated form of the 125 kDa protein, which approximately matches LEKTI predicted size. Total cell extracts from differentiated HK treated with BFA were incubated in the presence or absence of the peptide N-glycosidase F (PGNaseF) and analysed by western blot using α-C antibodies. PGNase F treatment resulted in a molecular weight shift of ~10 kDa for both proteins (Fig. 3; compare lanes 1 and 2). These results indicate that the 125 and 145 kDa bands correspond to N-glycosylated forms of two separate precursors of LEKTI, with apparent molecular weights of ~115 and 135 kDa, respectively.

To confirm the identity of the 145 kDa band as the N-glycosylated form of LEKTI full-length precursor, COS-1 cells were transfected with full-length LEKTI cDNA. Western blot analysis using α-N (data not shown) or α-C (Fig. 3) antibodies showed expression of a 145 kDa band in total protein extract from LEKTI cDNA transfected cells (lane 4), but not from mock transfected cells (lane 3). A 135 kDa protein was detected after PGNaseF treatment (lane 5), consistent with the result obtained in HK (lane 2).

Furin is involved in the processing of LEKTI in HK

The absence of cleavage of the recombinant full-length protein in COS-1 cells prompted us to test furin, a subtilisin-like proprotein convertase expressed in the epidermis (22), as a potential candidate for the endoproteolytic processing of LEKTI.

Protein extracts from COS-1 cells overexpressing LEKTI were incubated with human recombinant furin for increasing times, and analysed by western blot using α-C antibodies (Fig. 4A). Interestingly, the appearance of 65 and 68 kDa fragments were first detected 7 min after furin addition (lane 3), and were not observed in total cell extracts (lane 1), or after a short 3 min incubation (lane 2), a time when the 145 kDa full-length protein was evident. Importantly, an increase in intensity of the 65 and 68 kDa proteolytic fragments was observed with increasing incubation times, and was mirrored by a loss in signal of the full-length protein (compare lanes 2–6). Processing reached a maximum at 60 min, when very little precursor protein was detected (lane 6). In contrast, mock-treated cells showed no processing of LEKTI after 60 min (lane 7). The 65 and 68 kDa proteolytic products generated from incubation of over-expressed LEKTI with furin in these experiments migrated at the same size as two of the C-terminal proteolytic forms identified in HK (Fig. 2).

To confirm the involvement of furin in the physiological processing of LEKTI, differentiated HK were cultured for 6 h with increasing concentrations of the furin inhibitor, Dec-RVKR-CMK, or in the presence of brefeldin A (BFA) as a control (Fig. 4B). Consistent with our previous results (Fig. 2), incubation with BFA led to the detection of the 125 and 145 kDa proteins, with the concomitant loss of 42, 65 and 68 kDa proteolytic fragments (lane 4), whilst the precursors were not observed in untreated cells (lane 1). Significantly, incubation with the furin inhibitor (lanes 2 and 3) had a similar effect to that of BFA: there was a strong inhibition of processing of the native 125 and 145 kDa precursors into the 42, 65 and 68 kDa proteolytic forms. These results further implicate furin in the processing of LEKTI precursors into the three C-terminal proteolytic forms identified in HK extracts.

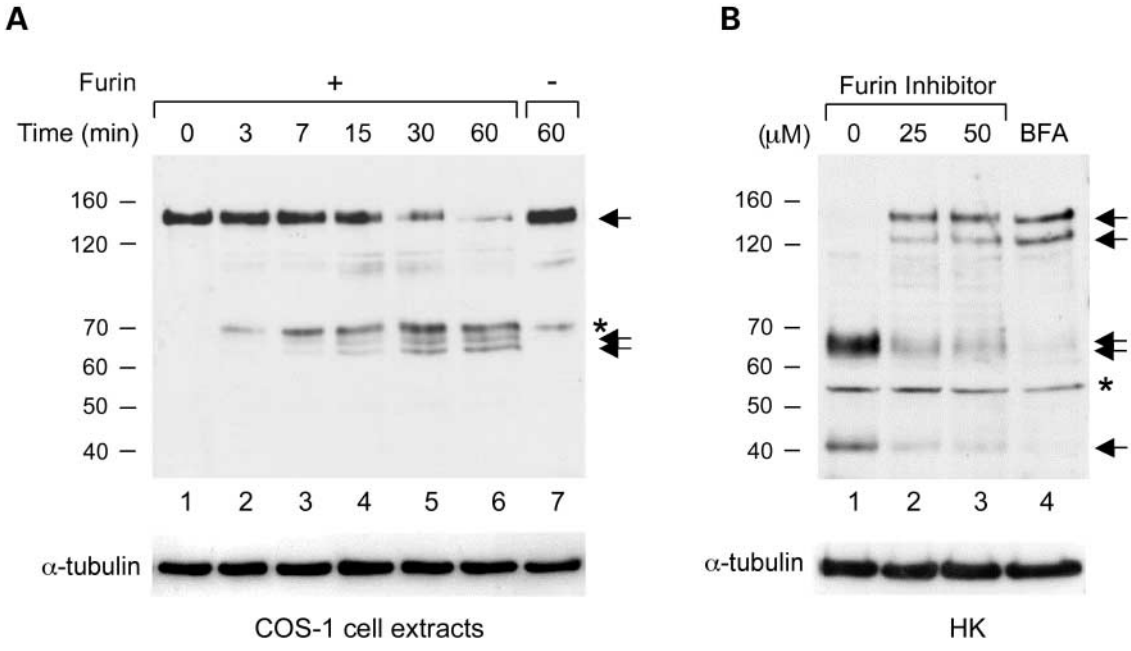

Figure 4. Involvement of furin in LEKTI intracellular processing. (**A**) COS-1 cells were transiently transfected for 48 h with the pEF-DEST51-LEKTI construct containing LEKTI full-length cDNA. Twenty micrograms of total cell extracts were treated for the indicated times in the presence (+) or absence (−) of human recombinant furin (2 units), at 30°C. Samples were resolved by SDS–PAGE (7.5%) and analysed by western blot using LEKTI polyclonal α-C antibodies. The asterisk indicates a 70 kDa protein band, detected in all samples incubated at 30°C (lanes 2–7) including furin untreated extract (lane 7), which most likely represents a degradation product of the 145 kDa protein. (**B**) HK were differentiated for 24 h in high-calcium medium, and cultured for an additional 6 h in the absence (lane 1) or presence of the indicated concentrations of furin inhibitor I (lanes 2 and 3) or BFA (10 μg/ml; lane 4). Twenty micrograms of total cell extracts were separated and analysed as above. The asterisk indicates an unrelated protein cross-reacting with α-C antibodies. Anti-tubulin detection shows equal loading. Arrows indicate the positions of the 42, 65, 68, 125 and 145 kDa protein bands. Molecular weight markers are indicated on the left in kDa. The results shown are representative of three to five independent experiments.

LEKTI expression is impaired in HK from NS patients

We investigated LEKTI expression in HK from three NS patients, termed NS1, NS2 and NS3, whose *SPINK5* mutations introduce premature termination codons of translation [(12); patients 10, 11 and 12, respectively].

We first compared the relative expression levels of *SPINK5* transcript in normal (N) and NS patient (NS1-3) differentiated HK by northern blot analysis. A dramatic reduction of the 3.7 kb signal was observed for all three patients compared to control (Fig. 5A), suggesting nonsense-mediated mRNA decay and predicting severe impairment of LEKTI expression.

LEKTI protein levels were subsequently examined by western blot analysis of cell extracts from normal (N) and NS (NS1-3) differentiated HK treated in the presence (Fig. 5B) or absence (Fig. 5C) of BFA. Importantly, the full-length protein was only detected in normal controls incubated with BFA, and not in NS patient cell extracts (Fig. 5B). Similarly, the 42, 65 and 68 kDa proteolytic fragments were not observed in NS samples, but were present in the control (Fig. 5C). These data confirm that the 145 and 125 kDa full length proteins, and the 68, 65 and 42 kDa fragments, are encoded by *SPINK5*, and provide the first conclusive evidence for defective expression of LEKTI in NS.

LEKTI expression in normal tissues

Paraffin wax-embedded sections of a variety of normal human tissues were tested for reactivity with LEKTI antibodies by immunohistochemistry. α-C polyclonal, and α-N monoclonal and polyclonal antibodies showed similar labelling patterns throughout the tissues investigated (Table 1). A strong immunoreactivity was detected in interfollicular epidermis of the skin and was localized to the cytoplasm of keratinocytes of the granular and uppermost spinous layers; the horny layer was negative. In the granular layer, the labelling often appeared more intense at the cell periphery and in the upper cytoplasm, forming a cuff above the nucleus and resulting in a polarized appearance (Fig. 6A). Antigen competition completely abolished the labelling, confirming its specificity (Fig. 6B). Staining was also observed in cells lining acrosyringeal ducts of hair follicles (Fig. 7A–C), and in the duct of sebaceous glands (Fig. 7D). In hair follicles, the labelling of the hair bulb was restricted to matrical cells that differentiate into the inner root sheath and hair shaft (Fig. 7A-4 and C). Above the hair bulb, the inner root sheath layers and hair cuticle were stained, whilst the outer root sheath was negative (Fig. 7B-3 and C). In the hair isthmus, reactivity was limited to inner cells of the outer root sheath surrounding the hair shaft (Fig. 7A-2). The follicular infundibulum displayed a staining pattern similar to that observed in interfollicular epidermis (Fig. 7A-1). In the thymus, LEKTI was abundantly expressed in Hassall's corpuscles, which represent the terminal differentiation stages of the thymic medullary epithelium (Fig. 7E). Strong expression was also detected in the gingival mucosa, mainly localized to the upper half of the stratum spinosum (Fig. 7F). An extended labelling was observed throughout suprabasal layers of the vaginal mucosa (Fig. 7G)

Table 1. Reactivity of LEKTI antibodies on normal human tissues

Positive		Negative			
Stratified epithelia		Simple epithelia		Others	
Skin	(10/10)	Lung	(0/10)	Heart	(0/4)
Thymus	(11/11)	Liver	(0/6)	Central nervous system	(0/2)
Gingiva	(4/4)	Kidney	(0/12)	Cerebellum	(0/1)
Tonsil	(2/2)	Stomach	(0/7)	Peripheral nerves	(0/3)
Oesophagus	(4/4)	Duodenum	(0/5)	Ovary	(0/2)
Vagina	(4/4)	Colon	(0/11)	Testis	(0/4)
Uterine ectocervix	(4/4)	Appendix	(0/2)	Uterus	(0/5)
		Rectum	(0/1)	Prostate	(0/4)
				Seminal vesicle	(0/2)
				Breast	(0/5)
				Adrenal gland	(0/4)
				Pancreas	(0/3)
				Thyroid	(0/8)
				Salivary gland	(0/4)
				Bone marrow	(0/4)
				Lymph node	(0/5)
				Spleen	(0/9)

Sections of various tissues were analysed by immunohistochemistry, as described in Materials and Methods. For each tissue, the number of positive samples out of the total tested is indicated in parentheses.

and uterine ectocervix (Fig. 7H). The tonsillar epithelium (Fig. 7I) and the oesophagus (data not shown) also showed positive staining. In contrast, no labelling was found in the epithelia of the gastrointestinal tract, liver, lung and kidney (data not shown). No specific staining was detected in the remainder of the organs tested (Table 1). In all tissues examined, no labelling was observed using the corresponding pre-immune sera (data not shown). Further confirmation of antibody specificity was provided by antigen competition (data not shown).

LEKTI expression in congenital erythrodermas, keratinizing and inflammatory skin diseases

Skin sections from patients affected with congenital erythrodermas, inherited disorders of keratinization and inflammatory skin diseases were tested for reactivity with LEKTI antibodies (Table 2). Immunostaining of skin sections from 21 patients with proven NS showed no detectable protein expression in 20 patients, including NS1, NS2 and NS3 (Fig. 8A), and very weak and discontinuous staining in the residual granular layer of the remaining (data not shown). In contrast, LEKTI was detected in the epidermis of sections from all other congenital erythrodermas, keratinizing and inflammatory skin diseases tested. All 18 samples of neonatal and infantile erythrodermas due to immune deficiency, atopic dermatitis or psoriasis, clearly showed expression of LEKTI. However the staining pattern was irregular, with areas of reduced staining alternating with areas of normal or increased reactivity in the upper spinous layers (Fig. 8B, C and E). In all genetic keratinizing disorders in children or adults tested, LEKTI expression pattern was abnormally extended to a variable number of cell layers of the stratum spinosum. This was particularly marked in specimens from bullous (Fig. 8D) and non-bullous (data not shown) congenital ichthyosiform erythrodermas, lamellar (Fig. 8F) and X-linked (data not shown) ichthyoses, and Darier's (Fig. 8H) and Hailey-Hailey (data not shown) diseases. In ichthyosis vulgaris, LEKTI was expressed in the reduced granular layer and in the uppermost cell layer of the stratum spinosum (Fig. 8G).

Among inflammatory skin disorders, psoriasis was chosen both in view of the well-characterized alterations in the expression pattern of several epidermal differentiation markers and as a possible cause of neonatal erythroderma, which can be misdiagnosed as NS. LEKTI expression was detected in all 12 samples of chronic plaque psoriasis examined (Fig. 8I). However, an irregular expression pattern was observed, with areas of markedly reduced staining alternating with areas of normal labelling intensity and extended reactivity pattern. Decreased labelling was mainly detected in thinned suprapapillary epidermis showing a highly diminished granular layer, marked parakeratosis with neutrophil microabscesses and leukocyte infiltration. In interpapillary epidermis of elongated ridges, LEKTI immunodetection often appeared of normal intensity but extended to several spinous layers. Lastly, we analysed samples from five adult patients affected with atopic dermatitis, all of which also proved positive for LEKTI expression with decreased staining in spongiotic areas (data not shown).

DISCUSSION

In this study, we report the first cellular and tissue detection of lympho-epithelial Kazal-type related inhibitor (LEKTI) using anti-C and anti-N terminal specific antibodies. We demonstrate that LEKTI is a marker of epithelial differentiation whose expression, like most differentiation-specific proteins (23,24), is regulated in cultured keratinocytes by external calcium concentrations. We have shown that LEKTI is expressed in differentiated HK as two N-glycosylated precursor proteins of

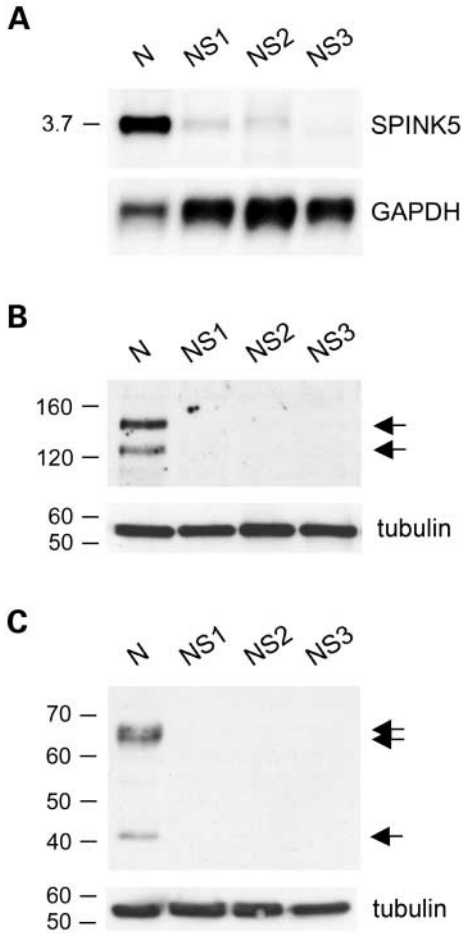

Figure 5. Comparative analysis of *SPINK5* and LEKTI expression levels in HK from normal (N) and NS patients (1–3). HK were differentiated for 24 h in high-calcium medium. (**A**) Twenty micrograms of total RNA were analysed by northern blot, as described in Materials and Methods. Hybridization with a ^{32}P-labelled *SPINK5*-cDNA specific probe shows a 3.7 kb signal. GAPDH detection allows the comparison between samples loading. (**B** and **C**) Differentiated HK were cultured for an additional 6 h in the presence (B) or absence (C) of BFA (10 µg/ml). Twenty micrograms of total cell extracts were separated by SDS–PAGE (7.5%) and analysed by western blot using LEKTI polyclonal α-C antibodies. Arrows indicate the positions of the 42, 65, 68, 125 and 145 kDa protein bands. RNA size (A) and molecular weight (B and C) markers are indicated on the left in kb and kDa, respectively. The results shown are representative of three to five independent experiments.

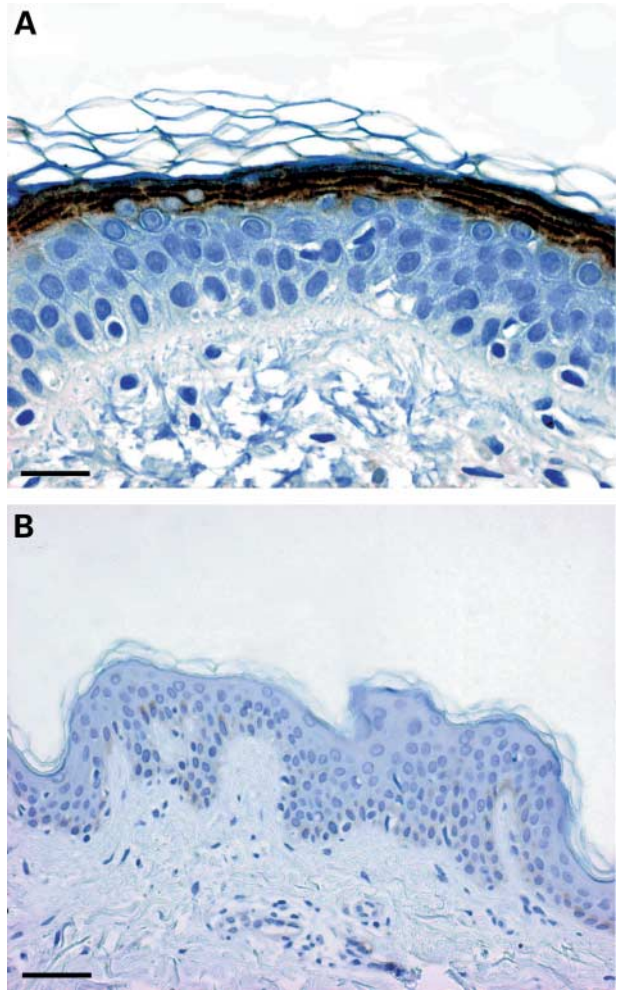

Figure 6. LEKTI expression in normal human skin. Immunohistochemical staining of paraffin embedded sections with LEKTI polyclonal α-N antibodies was performed as described in Materials and Methods. LEKTI is strongly expressed in the cytoplasm of keratinocytes of the epidermal granular and uppermost spinous layers; the polarized appearance of the labelling in the granular layer, resulting from a more intense reactivity in the upper cytoplasm, is evident (**A**). Preincubation of α-N antibodies with the recombinant antigen GST-D1-D6 prior to immunodetection results in a complete absence of reactivity, confirming the specificity of labelling (**B**). Bars: 25 µm (A); 50 µm (B).

145 and 125 kDa. However, under the experimental conditions used, these proteins were only detected when ER to Golgi transport was inhibited with brefeldin A, indicating rapid intracellular processing in a post-ER compartment of the secretory pathway. In addition, we identified three C-terminal proteolytic fragments of 42, 65 and 68 kDa in both cell extracts and conditioned medium. Time course analysis over 72 h of cell differentiation showed no further processing of these proteolytic forms (data not shown). These data suggest that the 42, 65 and 68 kDa polypeptides represent stable, and thereby potentially biologically active, forms of LEKTI. They also indicate that the inhibitory action of LEKTI-derived peptides/polypeptides (14,15) could target secreted and/or cell-surface-exposed serine proteases. In contrast to HK, no processing of the overexpressed 145 kDa full-length protein was observed in COS-1 cells, further indicating that the proteolytic forms identified in HK do not result from auto-catalytic cleavage events. This result also suggested that the 125 kDa protein was not generated through auto-cleavage of the 145 kDa isoform in the ER of HK.

We have also shown that *in vitro* cleavage of the overexpressed 145 kDa precursor by furin generates proteins of 65 and 68 kDa, whose sizes match those of two C-terminal proteolytic forms identified in HK. This finding suggests that the 65 and 68 kDa proteins, but not those of 125 kDa and 42 kDa, originate from the cleavage of the 145 kDa precursor in HK. Interestingly, preliminary data indicate that the 125 kDa isoform results from alternative processing of the *SPINK5* pre-mRNA in keratinocytes (A. Tartaglia-Polcini *et al.*, manuscript in preparation), suggesting that the 42 kDa fragment could originate from the cleavage of this isoform. We have also

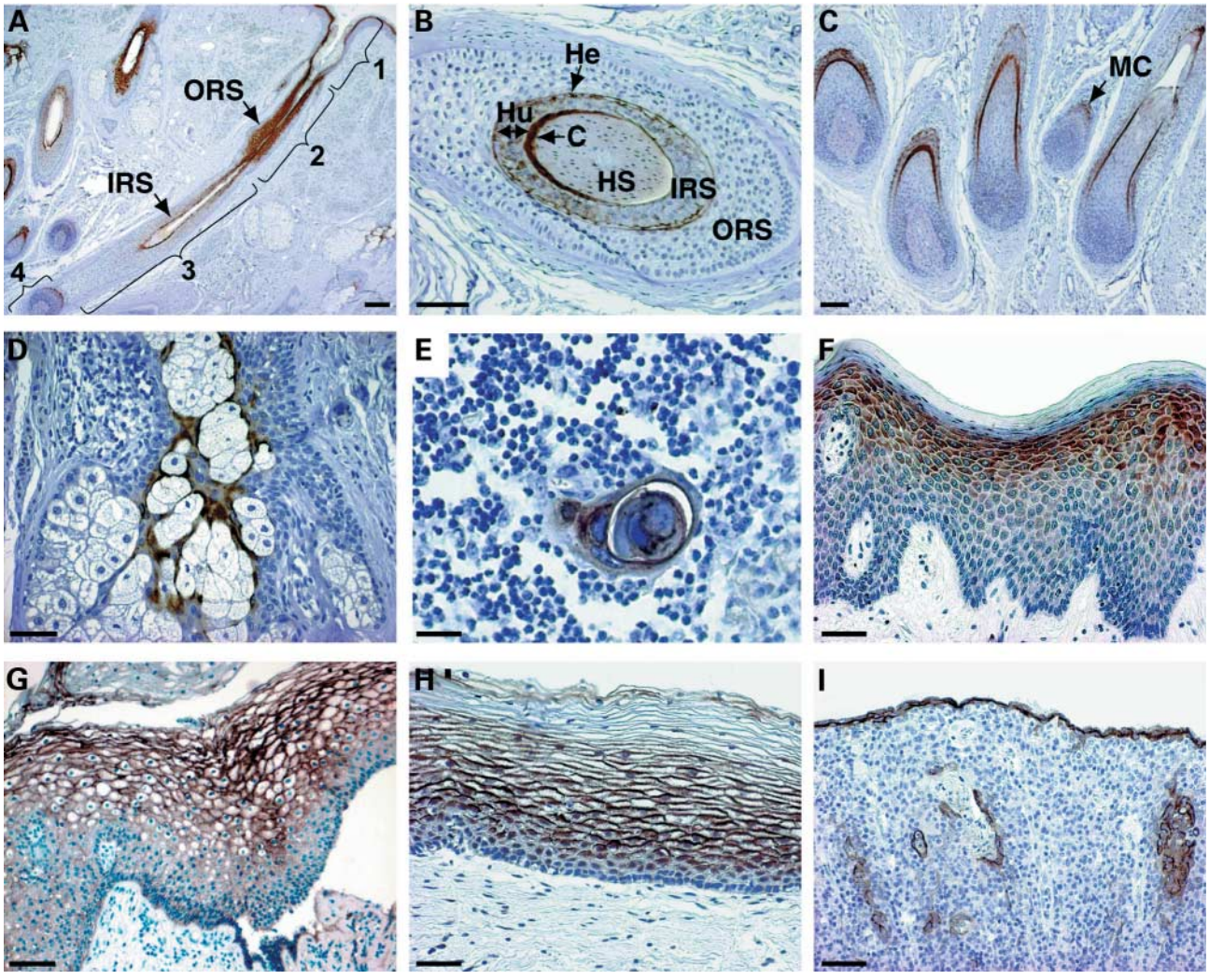

Figure 7. Human tissue distribution of LEKTI. Immunohistochemical staining of paraffin sections from normal human tissues (Table 1) with LEKTI polyclonal α-N antibodies was performed as described in Materials and Methods. In the hair follicle (**A**–**C**), a strong labelling is evident in the matrical cells of the anagen bulb (A-4 and C), in all internal root sheath layers (cuticle, Huxley's and Henle's layers), in the hair shaft cuticle in the lower portion of the follicle (A-3, B and C), in the innermost layers of the outer root sheath at the level of the isthmus (A-2) and in the infundibulum (A-1). Intense LEKTI expression is also observed in the duct of sebaceous glands (**D**), in thymic Hassall's corpuscles (**E**), suprabasal epithelial layers of the gingival (**F**), vaginal (**G**) and uterine ectocervix (**H**) mucosa, as well as in the tonsillar epithelium (**I**). ORS, outer root sheath; IRS, inner root sheath; HS, hair shaft; C, cuticle; He, Henle's layer; Hu, Huxley's layer; MC, matrical cells. Bars: 25 μm (E); 50 μm (D, F–I); 100 μm (B, C); 165 μm (A).

shown that treatment of HK with the furin inhibitor, Dec-RVKR-CMK, prevents the processing of both endogenous precursors, further implicating furin in the intracellular processing of LEKTI. This is likely to occur in the *trans*-Golgi network where the active form of furin resides (25). Co-localization of furin and LEKTI in the granular layer of the epidermis (22) further supports this hypothesis. However, expression of other subtilisin-like proprotein convertases (SPC), including PACE4, PC5/PC6, PC7/8 and PC8, have been demonstrated in human epidermis (22,26), raising the possibility that these endo-proteases could also be involved in the proteolytic processing of LEKTI.

The number of consensus sequences for SPC cleavage identified within LEKTI could generate at least 14 polypeptides (13). However, identification of the 42, 65 and 68 kDa C-terminally processed forms in this study, together with a 30 kDa polypeptide (16), reveals that only a selected proportion of these sites are used during the *in vivo* processing of LEKTI. Interestingly, processing of the N-terminal half of LEKTI seems to generate single domains (D1, D5, D6) (14,15), whilst the C-terminal half produces larger multi-domain fragments. These fragments may result from selective post-translational proteolysis (27) with a different processing in various tissues depending on the enzymes involved. The reasons we could not detect D1, D5 and D6 in HK using α-N antibodies raised against D1–D6 remain unclear; however, this could be due to the lack of epitope recognition within these domains and/or the rapid degradation of single protein domains under the experimental conditions used.

LEKTI tissue distribution pattern clearly demonstrates a specific expression in the most differentiated viable layers of stratified epithelial tissues (Table 1). These included

Table 2. Congenital erythrodermas and other keratinizing disorders examined in this study for LEKTI expression by immunohistochemical analysis of skin sections

Disease	Cases examined
Netherton syndrome	21
Other neonatal/infantile erythrodermas	18
SCID	5
Omenn syndrome	3
Atopic dermatitis	5
Psoriasis	5
Keratinizing disorders in children or adults	51
Ichthyosis vulgaris	3
Lamellar ichthyosis[a]	9
Non-bullous congenital ichthyosiform erythroderma[a]	5
Bullous congenital ichthyosiform erythroderma	2
X-linked ichthyosis	2
Other rare ichthyoses[b]	3
Darier's disease	5
Hailey-Hailey disease	5
Atopic dermatitis	5
Psoriasis	12

[a]Two cases due to TGM1 mutations.
[b]Ichthyosis of Siemens (1), ichthyosis hystrix (1) and Sjögren–Larsson syndrome (1).

keratinizing (skin and its appendages, gingiva and thymus), as well as non-keratinizing (tonsil, oesophagus, vagina and uterine ectocervix) epithelia. In the epidermis, LEKTI expression was mainly restricted to the granular layer, where critical biochemical and morphological changes of terminal differentiation lead to cornification (28). In hair follicle, the strong expression of LEKTI in matrical cells of the bulb, the hair shaft cuticle and inner root sheath, suggest a possible role in hair growth and differentiation. In non-keratinizing epithelia, LEKTI expression was more diffuse throughout the upper half of the spinous layers, supporting a role for the protein in earlier stages of epithelial differentiation.

We have shown that the absence of LEKTI expression in HK and epidermis is a common feature of NS. Although very low levels of LEKTI could be detected in skin sections of one patient, these results demonstrate that loss of LEKTI expression is the major molecular mechanism underlying NS. In contrast, normal or slightly reduced levels of LEKTI were detected in all other inherited and acquired skin diseases examined, including a number of clinically resembling skin disorders (Table 2). This finding reveals that defective expression of LEKTI in the epidermis is a diagnostic feature of NS, thus providing the basis for the powerful use of LEKTI antibodies for rapid, early and reliable diagnosis of NS. In a variety of other inherited keratinizing disorders tested, LEKTI expression appears to be upregulated and is extended to several spinous layers. The various epidermal responses mounted to compensate for altered epidermal permeability barrier in these diseases, from cytokine production by keratinocytes to the modulation of epidermal calcium gradient (23,29,30), could be involved in driving increased expression of LEKTI. Interestingly, LEKTI expression pattern in these diseases does not match that of any known epithelial differentiation protein, such as loricrin, involucrin or filaggrin (31,32). This indicates that specific mechanisms are involved in the transcriptional/post-transcriptional regulation of LEKTI expression. In psoriasis and neonatal/infantile erythrodermas (due to immune deficiency or atopy), an irregular LEKTI staining pattern is detected. In particular, decreased expression corresponds to the presence of an intense lympho-mononuclear inflammatory infiltrate invading the epidermis, suggesting that some inflammatory cytokines could downregulate LEKTI expression.

Although the biological function(s) of LEKTI is still unknown, its specific expression in highly differentiated regions of lympho-epithelial tissues, together with the clinical features of NS patients, predicts key roles in a number of physiological processes. It is likely that LEKTI plays a role in terminal epidermal differentiation and/or corneocyte desquamation (13), as suggested by its restricted expression in the granular layer of the epidermis, and impaired keratinization and cornification in NS. Among possible targets are the stratum corneum trypsin- and chymotrypsin-like enzymes (SCTE and SCCE, respectively) (33), whose defective inhibition by LEKTI would result in over desquamation of corneocytes (13). Other putative targets are trypsin-like serine proteases, including the membrane-type serine protease 1 (MT-SP1) (34), which could mediate inhibition of keratinocyte differentiation through activation of PAR-2 (protease-activated receptor-2) at the keratinocyte surface (35). The extent of atopic manifestations in NS, which are not seen in other congenital ichthyoses, also predicts a role for LEKTI as a downregulator of inflammatory and/or immune allergic responses (36). Two recent independent studies reporting association between *SPINK5* missense variants and atopic dermatitis (37,38) further support this assumption. Among the serine proteases secreted during the inflammation process, the trypsin-like mast cell tryptase is a major mediator of numerous allergic and inflammatory conditions (39). Induction of inflammatory cytokines cell secretion (40), mast cell activation (41), and eosinophil and neutrophil recruitment (42) by the mast cell tryptase, via PAR-2 activation (43), indicates that this serine protease may mediate an amplification mechanism of the inflammatory response. Lack of downregulation by LEKTI could thus trigger a cycle of chronic allergen-induced inflammation in NS, and potentially, in common atopic diseases. Finally, many allergens themselves are serine proteases (44), including the major house dust-mite and pollen (45). It is thus possible that lack of inhibition of some of these activities by LEKTI may also contribute to the allergen hyper-responsiveness associated with NS pathology. Despite absence of significant immune function abnormalities in NS patients (6), the strong and localized expression of LEKTI in thymic Hassall's corpuscles suggests that the protein could be involved in the regulation of T cell maturation. Although the functional significance of Hassall's corpuscles remains to be determined, recent findings suggest that these are involved both in maturation of developing thymocytes (46), and activation and tolerization of mature T cells (47). For its specific expression in Hassall's corpuscles (48), the trypsin-like kallikrein 6 (KLK6) serine protease represents a potential target of LEKTI inhibitory activity. LEKTI expression in differentiated and keratinizing areas of the hair follicle (Fig. 7A–C) and sebaceous glands (Fig. 7D) suggests a possible role in growth and differentiation of pilosebaceous units. This assumption is further supported by the specific hair shaft abnormality, trichorrhexis invaginata (TI), and slow growing hair feature

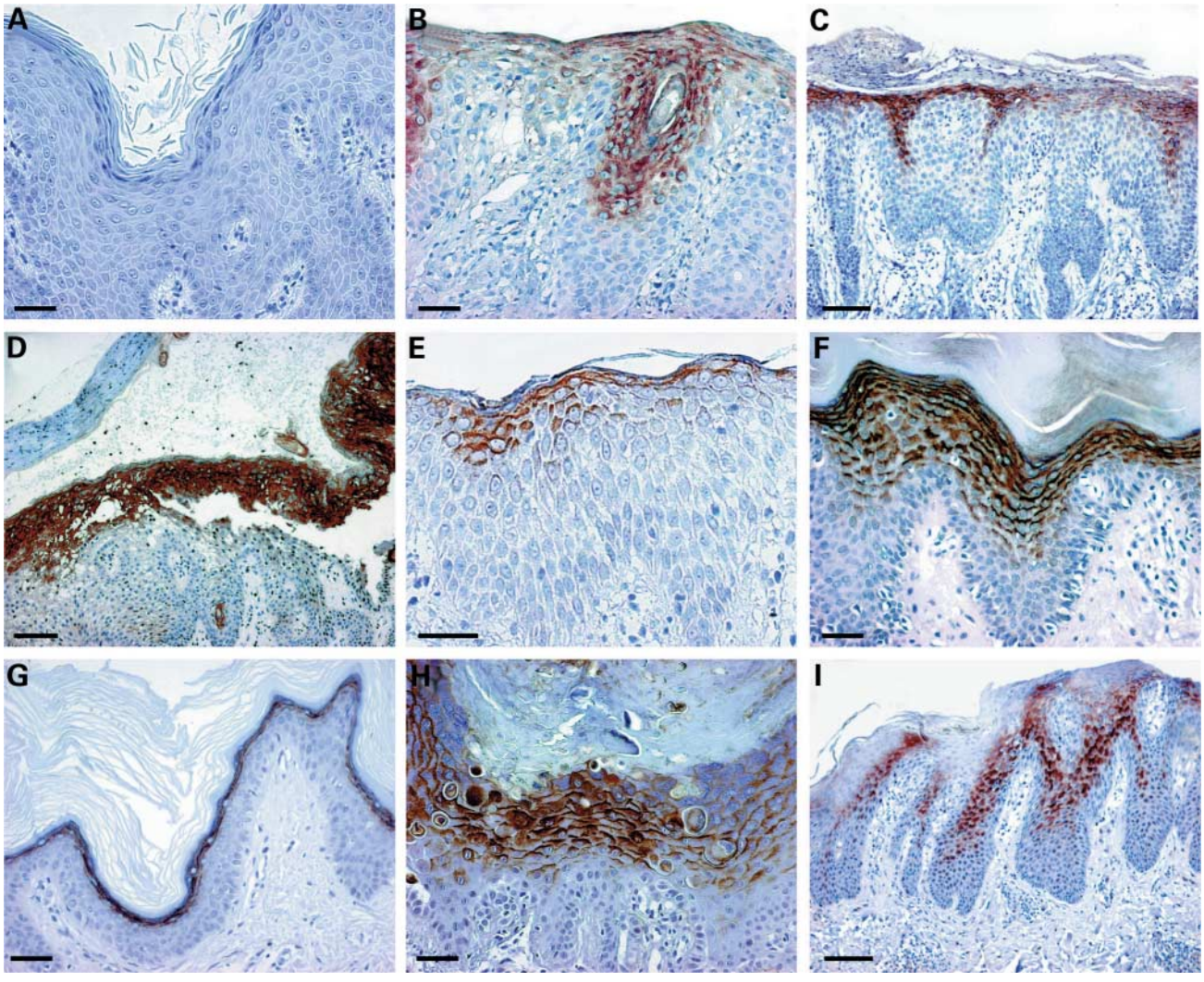

Figure 8. Expression of LEKTI in Netherton syndrome compared to inherited and acquired skin diseases (Table 2). Immunohistochemical staining of skin sections from patients affected with Netherton syndrome (**A**) and other skin diseases (**B**–**I**) were performed with LEKTI polyclonal α-N antibodies, as described in Materials and Methods: severe combined immunodeficiency syndrome (SCID) (B); Omenn syndrome (C); bullous congenital ichthyosiform erythroderma (D); erythroderma due to atopic dermatitis (E); lamellar ichthyosis (F); ichthyosis vulgaris (G); Darier's disease (H); and psoriasis (I). Note the intense staining of an increased number of spinous cell layers in bullous congenital ichthyosiform erythroderma (D), lamellar ichthyosis (F), and Darier's disease (H). In SCID (B), Omenn syndrome (C), atopic dermatitis (E) and psoriasis (I), areas of reduced staining are evident and often match the thinned parakeratotic suprapapillary epidermis where exocytosis of the inflammatory infiltrate is more prominent, while areas of staining extension to suprabasal cell layers are observed. In ichthyosis vulgarilis (G), LEKTI detection was similar to that obtained in normal skin. Bars: 50 μm (A, B, E–H); 100 μm (C, D, I).

observed in NS patients. Interestingly, LEKTI co-localizes with SCCE and SCTE in hair (inner root sheath and innermost layers of the outer root sheath in the uppermost follicle) and sebaceous (duct) follicles (33,49). This observation raises the possibility that, like in epidermis, LEKTI may regulate the activity of these serine proteases in pilosebaceous follicles. Lack of LEKTI expression in NS hair may lead to impaired keratinization of structures of the hair shaft, which could account for softening and collapse of its distal part into its proximal portion when driven upward by the growing force, resulting in the formation of TI bamboo nodes (50). Reports of morphological abnormalities in the keratogenous zone of the hair cortex, and unkeratinized hair cuticle cells in TI (50), also correlate with the predicted lack of LEKTI expression in this location. The predisposition of NS patients to cutaneous infections of bacterial and viral origins could be attributed to the severe skin permeability barrier dysfunction, facilitating invasion by microorganisms. However, their persistent/recurrent character, unique to NS among ichthyoses (6), suggests additional loss of an important host-defense mechanism in the skin. Anti-microbial and anti-viral activities have been described for a number of serine protease inhibitors (51), including the antileukoprotease (ALP) (52), and β defensins secreted by keratinocytes (53). It is thus possible that LEKTI-derived bioactive peptides/polypeptides share similar activities as part of the innate immune response in the skin. Given its tissue distribution, a role for LEKTI in the general anti-microbial protection of mucous epithelia is also anticipated. Surprisingly, particular organs relevant to NS pathology such as lung, kidney and digestive tract were negative for LEKTI

expression in normal individuals (Table 1). This suggests that life-threatening complications associated with NS, such as bronchopneumonia, malnutrition and metabolic disorders (6), are not primarily due to defective LEKTI function(s) in these organs, and may reflect a secondary effect resulting from failure to produce LEKTI in other tissues.

LEKTI full-length recombinant protein has recently been shown to inhibit the enzymatic activities of plasmin, trypsin, subtilisin A, cathepsin G and elastase (54). This result supports the involvement of LEKTI in multiple biological pathways relevant to tissue homeostasis, inflammation and anti-microbial defense. Interestingly, the native D6 peptide shows a selective and more potent trypsin inhibition than the full-length recombinant protein, suggesting that the inhibitory potency of processed LEKTI domains against specific proteinases could be significantly higher than that of the precursor(s). These findings, together with our present data, further suggest that LEKTI proteolytic polypeptides represent bioactive forms, with different target specificities. The identification of the novel processed forms of LEKTI described in this study provides the basis for future functional and structural analysis of fragments with physiological relevance.

MATERIALS AND METHODS

Materials

All reagents, chemicals and antibodies were purchased from Sigma (Poole, UK) unless otherwise stated. Cell culture media were obtained from Invitrogen (Paisley, UK).

Tissue samples

A wide variety of normal human tissues, listed in Table 1, were retrieved from the archives of the Department of Pathology of Purpan Hospital in Toulouse (France). Skin specimens from 21 patients affected with Netherton syndrome (NS) and other genetic and acquired disorders of keratinization, listed in Table 2, were obtained from the archives of the Service of Histopathology of the IDI-IRCCS in Rome (Italy), the Department of Pathology in Toulouse, and the Department of Dermatology of Necker Hospital in Paris (France). Tissue samples had been obtained for diagnostic or research purposes. The Central Oxfordshire Research Ethic Committee approved the study and all patients gave informed consent.

Cloning of LEKTI full-length cDNA into pEF-DEST51 expression vector

LEKTI full-length cDNA (GenBank AJ228139) was generated by long-range PCR (Expand Long Template PCR System; Roche Molecular Biochemicals, E. Sussex, UK) using total cDNA from differentiated HK (obtained as described below) as a template. The PCR product was subcloned into pCRII-TOPO vector (TOPO TA Cloning Kit; Invitrogen) and transferred into the mammalian expression vector pEF-DEST51 using the Gateway technology, according to the manufacturer's instructions (Invitrogen). pEF-DEST51-LEKTI construct was fully sequenced using the Big Dye Terminator Sequencing Kit and an ABI377 automated sequencer (Applied Biosystem, Cheshire, UK).

Cell culture and transfections

Normal and NS human primary keratinocytes (HK) were isolated from skin biopsies as previously described (55). HK were expanded on a feeder layer of lethally irradiated 3T3-J2 mouse fibroblasts in keratinocyte-growth medium, following the method described by Rheinwald and Green (56,57). For all experiments, HK were grown in the absence of 3T3 feeder layers in low calcium (<0.1 mM) keratinocyte serum-free medium (K-SFM) supplemented with epidermal growth factor (0.4 ng/ml) and bovine pituitary extract (25 μg/ml). Cells were seeded in low calcium K-SFM at a density corresponding to 80% confluence in 60 × 15 mm tissue culture dishes or eight-well chamber slides (Nalge Nunc International, Naperville, IL, USA) for northern/western blot and immunofluorescence microscopy analysis, respectively. Cell differentiation was induced for 24 h in high calcium (1.2 mM) K-SFM. Further treatments with brefeldin A (BFA) and furin inhibitor I (Dec-RVKR-CMK; Calbiochem, Nottingham, UK) were performed for 6 h at 10 μg/ml and 25–50 μM respectively, in high calcium K-SFM.

COS-1 cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, 100 U/l penicillin/streptomycin and 10 ng/ml L-glutamine. Cells were plated in 60 × 15 mm tissue culture dishes at 70% confluence and transiently transfected with pEF-DEST51 or pEF-DEST51-LEKTI vectors (2.5 μg DNA), using FuGENE™ 6 Transfection Reagent according to the manufacturer's recommendations (Roche Molecular Biochemicals). Transfected cells were maintained in culture for 48 h before proceeding to western blot analysis.

RNA extraction, reverse transcriptase–PCR and northern blotting

Total RNA was isolated from normal and NS differentiated HK using Trizol (Invitrogen) according to the manufacturer's instructions. cDNA was generated from normal HK total RNA by reverse-transcription using the AMV reverse transcriptase (Roche Diagnostic Spa, Monza, Italy) and random hexamers. It was subsequently used as a template for the PCR amplification of a 273 bp cDNA product comprised within the 3′ untranslated region of LEKTI cDNA sequence. PCR conditions were as described below with an annealing temperature of 59°C, and primers used were as follows: 5′-CAGGAAGATTGTTGAAAG CCA-3′ (sense, nucleotides 3239–3259) and 5′-ATTGAACA GGCAGTTGGACAG-3′ (antisense, nucleotides 3491–3511). The PCR product was radiolabelled and used as a probe for the northern blot analysis of 20 μg of total RNA extracts, following standard methods (58). To assess uniformity of RNA loading and transfer, the membrane was further hybridized with a radiolabelled probe corresponding to the ubiquitously expressed gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH; GenBank NM002046). Quantification of the hybridization signals was performed by densitometric scanning using a GS-750 densitometer (Bio-Rad, Hercules, CA, USA).

Polyclonal LEKTI antibodies production

Rabbit polyclonal antibodies were raised against the recombinant N-terminal (D1–D6) and C-terminal (D13–D15) parts of LEKTI, and were termed α-N and α-C antibodies, respectively.

Construction of pGEX4T1-D1–D6 and pGEX3X-D13–D15 bacterial expression vectors. Fragments of LEKTI cDNA encoding the protein domains D1–D6 (nucleotides 52–1278) and D13–D15 (nucleotides 2483–3241) were generated by PCR using the following primers: D1 sense, 5′-CCGCTCGA GCAAGATGCTGCCAGTAAGAATGAA-3′; D6 antisense, 5′-CCGCTCGAGTTGTCTTTTGTTTCTTGATTCGCC-3′; D13 sense, 5′-GCGGGATCCTGGAAAGGGAAGCAGCTG-3′, and D15 antisense, 5′-CGGAATTCTGTCATTCGTCAGAC GGG-3′. PCR cycling conditions were: 94°C for 10 min, 35 cycles comprising 94°C for 30 s, 66°C (D1–D6) or 55°C (D13–D15) for 45 s, and 72°C for 1 min, followed by a final extension at 72°C for 10 min. PCR products were fully sequenced as decribed above, and subcloned into the Glutathione-S-Transferase (GST) gene fusion vectors pGEX-4T-1 and pGEX-3X (Amersham Pharmacia Biotech, Amersham, UK) using *Xho*I and *Eco*RI/*Bam*HI restriction sites for D1–D6 and D13–D15, respectively. pGEX-D1–D6 and pGEX-D13–D15 constructs were transformed into BL21 and TOPO10 *E. coli* strains (Stratagene, Amsterdam, Netherlands), respectively.

Expression and purification of recombinant GST-fusion proteins. Procedures were essentially performed as previously described (59). Briefly, cell cultures were grown at 37°C in Luria Broth base (Invitrogen) medium containing 100 μg/ml ampicillin to an OD_{600nm} of 0.8. Protein expression was then induced by addition of IPTG to 0.1 mM, and cultures were grown overnight at room temperature. Cells were harvested, and lysed by sonication. Sonicates were clarified from insoluble material by centrifugation at 16 000*g*, 4°C for 30 min. GST-D1–D6 (74.6 kDa) and GST-D13–D15 (55.6 kDa) were affinity purified using glutathione sepharose 4B beads, according to the manufacturer's recommendations (Amersham Pharmacia Biotech).

Immunizations. Immunizations of rabbits with the recombinant antigens GST-D1–D6 and GST-D13–D15 were performed by Eurogentec Bel S.A (Herstal, Belgium) and Primm (Milan, Italy), respectively.

Serum purification. α-C and α-N crude antisera were purified by affinity chromatography for the respective antigens, using NHS-activated Sepharose® 4 Fast Flow (Amersham Pharmacia Biotech) and the AminoLink Plus Immobilization Trial purification procedure (Pierce, Rockford, IL, USA), respectively, following manufacturers' instructions.

Monoclonal LEKTI antibody production

BALB/c mice were immunized four times with 50 μg of recombinant GST-D1–D6 at 2 week intervals, once subcutaneously and three times intraperitoneally, in association with complete (first injection), incomplete (second and third injections) and without (fourth injection) Freund's adjuvant (Calbiochem, La Jolla, CA, USA). Three days after the fourth immunization, fusion of the spleen cells with the non-Ig-producing myeloma cell line X63 Ag8 653 was performed using standard techniques (60). When hybridoma growth could be detected, supernatants were tested for antibody-binding activity using an enzyme linked immunosorbent assay (ELISA). Of the 295 clones generated, one was found to secrete anti-D1–D6 antibody, as determined by ELISA and immunohistochemistry (see below). The selected hybridoma was cloned by limiting dilution. Isotope characterization showed that the anti-D1–D6 monoclonal antibody belongs to an IgG1 subclass.

Immunofluorescence microscopy

Cells were fixed in ice-cold methanol for 30 min and processed for immunofluorescence analysis, as previously described (61). The primary antibodies used were LEKTI polyclonal α-N (11 μg/ml) and α-C (4 μg/ml) antibodies, the corresponding rabbit pre-immune sera used at the same dilutions, and the monoclonal anti-calreticulin antibody (Calbiochem). Secondary antibodies were goat anti-rabbit conjugated to FITC and sheep anti-mouse conjugated to TRITC. For competition experiments, α-N and α-C antibodies were pre-incubated for 1 h at room temperature with 2.5-fold excess (weight) of the respective recombinant antigens GST-D1–D6 and GST-D13–D15, prior to immunodetection. Cells were mounted in Vectorshield in the presence or absence of propidium iodide (Vector Laboratories, Peterborough, UK), and examined under a Nikon Optiphot with a 60× oil objective. Images were captured using an MRC 1024 confocal laser microscope and collected using Lasersharp software (Bio-Rad).

Western blotting

Cells were lysed in a suitable volume of ice-cold lysis buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 1 mM PMSF and 1 μg/ml each of antipaine, chymostatin, leupeptin and pepstatin). Lysates were incubated for 30 min on ice and clarified from the insoluble materiel by centrifugation at 16 000*g*, 4°C for 3 min. Conditioned media were concentrated by overnight precipitation at −20°C in the presence, per ml of medium, of 3.6 ml of ethanol and 100 μl of the solution mix 20 mM N-ethylmalaeimide, 10 mM EDTA, 1 mM PMSF, 100 mM 2-mercaptoethanol–acetic acid in 0.1 M Tris–HCl buffer pH 7.4. Proteins were recovered by centrifugation at 13 000*g*, 4°C for 30 min and resuspended in a suitable volume of ice-cold lysis buffer. Protein samples were quantified using the BCA protein assay kit (Pierce, Rockford, IL, USA), and 20 μg were fractionated by SDS–polyacrylamide gel electrophoresis (PAGE). Proteins were transferred onto Immobilon-P membranes (Millipore, Herts, UK) using the Hoefer Semiphor semi-dry transfer unit (Amersham Pharmacia Biotech). Blots were immunostained following standard protocols (60). First antibodies were LEKTI polyclonal α-N (1.9 μg/ml), α-C (1.2 μg/ml), and monoclonal α-N (1 μg/ml) antibodies, and the corresponding rabbit pre-immune sera used at the same

dilutions. Secondary antibodies were donkey anti-rabbit or sheep anti-mouse antibodies conjugated to horseradish peroxidase (Amersham Pharmacia Biotech). Proteins were visualized using the ECL detection system according to the manufacturer's instructions (Amersham Pharmacia Biotech). Competition experiments were performed as described above using 2.5-fold excess (weight) of the recombinant antigens. To verify equal sample loading, membranes were stripped following Amersham's ECL detection system instructions, and reprobed using the anti-tubulin monoclonal antibody. PNGase F (New England Biolabs, Hitchin, UK) and human recombinant furin sample treatments were performed for 1 h at 37 and 30°C, respectively, according to the manufacturer's instructions.

Immunohistochemistry

LEKTI α-N polyclonal and monoclonal antibodies worked on paraffin sections only, whilst α-C polyclonal antibodies also worked on frozen sections. These antibodies were used on a large panel of normal human tissue samples (Table 1), as well as on skin biopsies from patients affected with NS or other skin disorders (Table 2). Tissue samples were fixed in 10% neutral buffered formalin. Four-micrometer sections were prepared from paraffin-wax-embedded tissues and their reactivity with LEKTI antibodies was investigated by immunohistochemistry. Prior to immunodetection, specimens were deparaffinized, rehydrated and processed as described elsewhere (62). Antigen retrieval of dewaxed sections was performed by heat-treatment for 40 min using a water bath at 95°C in 10 mM citrate buffer pH 6.0 supplemented with 1% Tween 20, or using the Target Retrieval Solution (Dako, Trappes, France). Sections were immunostained for 30 min at room temperature with polyclonal α-N (11 µg/ml) or α-C (4 µg/ml) antibodies diluted in PBS containing 0.3% BSA, or with the undiluted α-N monoclonal antibody (5 µg/ml). Tissue sections were incubated with the streptavidin-biotin-peroxidase complex (ABC method) using the StrepABComplex/HRP Duet (mouse/rabbit) kit (Dako). Extensive washing with PBS containing 0.3% BSA was performed between each step. Labelling was revealed using diaminobenzidine tetrahydrochloride and hydrogen peroxide, and nuclei were counterstained with hematoxylin. Negative controls were included for each sample by omitting the primary antibody. The labelling specificity was verified using the corresponding pre-immune sera at the same concentrations and by competition experiments, as described above, using 10-fold excess (weight) of the recombinant antigens.

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REFERENCES

1. Comèl, M. (1949) Ichthyosis linearis circumflexa. *Dermatologica*, **98**, 133–136.
2. Netherton, E.W. (1958) A unique case of trichorrhexis nodosa—'bamboo hairs'. *Arch. Dermatol.*, **78**, 483–487.
3. Traupe, H. (1989) The Comèl–Netherton syndrome. In Traupe, H (ed.), *The Ichthyoses: a Guide to Clinical Diagnosis, Genetic Counselling, and Therapy.* Springer, Berlin, pp. 168–178.
4. Stevanovic, D.V. (1969) Multiple defects of the hair shaft in Netherton's disease. Association with ichthyosis linearis circumflexa. *Br. J. Dermatol.*, **81**, 851–857.
5. Greene, S.L. and Müller, S.A. (1985) Netherton's syndrome: report of a case and review of the literature. *J. Am. Acad. Dermatol.*, **13**, 329–337.
6. Judge, M.R., Morgan, G. and Harper, J.I. (1994) A clinical and immunological study of Netherton's syndrome. *Br. J. Dermatol.*, **131**, 615–621.
7. Smith, D.L., Smith, J.G., Wong, S.W. and deShazo, R.D. (1995) Netherton's syndrome: a syndrome of elevated IgE and characteristic skin and hair findings. *J. Allergy Clin. Immunol.*, **95**, 116–123.
8. Jones, S.K., Thomason, L.M., Surbrugg, S.K. and Weston, W.L. (1986) Neonatal hypernatraemia in two siblings with Netherton's syndrome. *Br. J. Dermatol.*, **114**, 741–743.
9. Chavanas, S., Garner, C., Bodemer, C., Ali, M., Teillac, D.H., Wilkinson, J., Bonafe, J.L., Paradisi, M., Kelsell, D.P., Ansai, S. *et al.* (2000) Localization of the Netherton syndrome gene to chromosome 5q32, by linkage analysis and homozygosity mapping. *Am. J. Hum. Genet.*, **66**, 914–921.
10. Chavanas, S., Bodemer, C., Rochat, A., Hamel-Teillac, D., Ali, M., Irvine, A.D., Bonafe, J.L., Wilkinson, J., Taieb, A., Barrandon, Y. *et al.* (2000) Mutations in SPINK5, encoding a serine protease inhibitor, cause Netherton syndrome. *Nat. Genet.*, **25**, 141–142.
11. Sprecher, E., Chavanas, S., DiGiovanna, J.J., Amin, S., Nielsen, K., Prendiville, J.S., Silverman, R., Esterly, N.B., Spraker, M.K., Guelig, E. *et al.* (2001) The spectrum of pathogenic mutations in SPINK5 in 19 families with Netherton syndrome: implications for mutation detection and first case of prenatal diagnosis. *J. Invest. Dermatol.*, **117**, 179–187.
12. Bitoun, E., Chavanas, S., Irvine, A.D., Lonie, L., Bodemer, C., Paradisi, M., Hamel-Teillac, D., Ansai, S., Mitsuhashi, Y., Taieb, A. *et al.* (2002) Netherton syndrome: disease expression and spectrum of SPINK5 mutations in 21 families. *J. Invest. Dermatol.*, **118**, 352–361.
13. Komatsu, N., Takata, M., Otsuki, N., Ohka, R., Amano, O., Takehara, K. and Saijoh, K. (2002) Elevated stratum corneum hydrolytic activity in Netherton syndrome suggests an inhibitory regulation of desquamation by SPINK5-derived peptides. *J. Invest. Dermatol.*, **118**, 436–443.
14. Magert, H.J., Standker, L., Kreutzmann, P., Zucht, H.D., Reinecke, M., Sommerhoff, C.P., Fritz, H. and Forssmann, W.G. (1999) LEKTI, a novel 15-domain type of human serine proteinase inhibitor. *J. Biol. Chem.*, **274**, 21499–21502.
15. Magert, H.J., Kreutzmann, P., Standker, L., Walden, M., Drogemuller, K. and Forssmann, W.G. (2002) LEKTI: a multidomain serine protease inhibitor with pathophysiological relevance. *Int. J. Biochem. Cell Biol.*, **34**, 573–576.
16. Ahmed, A., Kandola, P., Ziada, G. and Parenteau, N. (2001) Purification and partial amino acid sequence of proteins from human epidermal keratinocyte conditioned medium. *J. Prot. Chem.*, **20**, 273–278.
17. Bergeron, F., Leduc, R. and Day, R. (2000) Subtilase-like pro-protein convertases: from molecular specificity to therapeutic applications. *J. Mol. Endocrinol.*, **24**, 1–22.
18. Seidah, N.G. and Chretien, M. (1999) Proprotein and prohormone convertases: a family of subtilases generating diverse bioactive polypeptides. *Brain Res.*, **848**, 45–62.
19. Hennings, H., Michael, D., Cheng, C., Steinert, P., Holbrook, K. and Yuspa, S.H. (1980) Calcium regulation of growth and differentiation of mouse epidermal cells in culture. *Cell*, **19**, 245–254.
20. Wille, J.J., Pittelkow, M.R., Shipley, G.D. and Scott, R.E. (1984) Integrated control of growth and differentiation of normal human prokeratinocytes cultured in serum-free medium: clonal analyses, growth kinetics, and cell cycle studies. *J. Cell Physiol.*, **121**, 31–44.
21. Lippincott-Schwartz, J., Yuan, L.C., Bonifacino, J.S. and Klausner, R.D. (1989) Rapid redistribution of Golgi proteins into the ER in cells treated with brefeldin A: evidence for membrane cycling from Golgi to ER. *Cell*, **56**, 801–813.

22. Pearton, D.J., Nirunsuksiri, W., Rehemtulla, A., Lewis, S.P., Presland, R.B. and Dale, B.A. (2001) Proprotein convertase expression and localization in epidermis: evidence for multiple roles and substrates. *Exp. Dermatol.*, **10**, 193–203.
23. Elias, P.M. and Feingold, K.R. (2001) Coordinate regulation of epidermal differentiation and barrier homeostasis. *Skin Pharmac. Appl. Skin Physiol.*, **14**, 28–34.
24. Elias, P.M., Ahn, S.K., Denda, M., Brown, B.E., Crumrine, D., Kimutai, L.K., Komuves, L., Lee, S.H. and Feingold, K.R. (2002) Modulations in epidermal calcium regulate the expression of differentiation-specific markers. *J. Invest. Dermatol.*, **119**, 1128–1136.
25. Molloy, S.S., Thomas, L., VanSlyke, J.K., Stenberg, P.E. and Thomas, G. (1994) Intracellular trafficking and activation of the furin proprotein convertase: localization to the TGN and recycling from the cell surface. *EMBO J.*, **13**, 18–33.
26. Bruzzaniti, A., Goodge, K., Jay, P., Taviaux, S.A., Lam, M.H., Berta, P., Martin, T.J., Moseley, J.M. and Gillespie, M.T. (1996) PC8 [corrected], a new member of the convertase family. *Biochem. J.*, **314**, 727–731.
27. Hopper, N.M., Karran, E.H. and Turner, A.J. (1997) Membrane protein secretases. *Biochem. J.*, **321**, 265–279.
28. Eckert, R.L. (1989) Structure, function, and differentiation of the keratinocyte. *Physiol. Rev.*, **69**, 1316–1346.
29. Lavrijsen, A.P., Oestmann, E., Hermans, J., Bodde, H.E., Vermeer, B.J. and Ponec, M. (1993) Barrier function parameters in various keratinization disorders: transepidermal water loss and vascular response to hexyl nicotinate. *Br. J. Dermatol.*, **129**, 547–553.
30. Schmuth, M., Yosipovitch, G., Williams, M.L., Weber, F., Hintner, H., Ortiz-Urda, S., Rappersberger, K., Crumrine, D., Feingold, K.R. and Elias, P.M. (2001) Pathogenesis of the permeability barrier abnormality in epidermolytic hyperkeratosis. *J. Invest. Dermatol.*, **117**, 837–847.
31. Hohl, D. (1993) Expression patterns of loricrin in dermatological disorders. *Am. J. Dermatopathol.*, **15**, 20–27.
32. De Heller-Milev, M., Huber, M., Panizzon, R. and Hohl, D. (2000) Expression of small proline rich proteins in neoplastic and inflammatory skin diseases. *Br. J. Dermatol.*, **143**, 733–744.
33. Ekholm, I.E., Brattsand, M. and Egelrud, T. (2000) Stratum corneum tryptic enzyme in normal epidermis: a missing link in the desquamation process? *J. Invest. Dermatol.*, **114**, 56–63.
34. Takeuchi, T., Harris, J.L., Huang, W., Yan, K.W., Coughlin, S.R. and Craik, C.S. (2000) Cellular localization of membrane-type serine protease 1 and identification of protease-activated receptor-2 and single-chain urokinase-type plasminogen activator as substrates. *J. Biol. Chem.*, **275**, 26333–26342.
35. Derian, C.K., Eckardt, A.J. and Andrade-Gordon, P. (1997) Differential regulation of human keratinocyte growth and differentiation by a novel family of protease-activated receptors. *Cell Growth Differ.*, **8**, 743–749.
36. Cookson, W.O. and Moffatt, M.F. (2000) Genetics of asthma and allergic disease. *Hum. Mol. Genet.*, **9**, 2359–2364.
37. Walley, A.J., Chavanas, S., Moffatt, M.F., Esnouf, R.M., Ubhi, B., Lawrence, R., Wong, K., Abecasis, G.R., Jones, E.Y., Harper, J.I. *et al.* (2001) Gene polymorphism in Netherton and common atopic disease. *Nat. Genet.*, **29**, 175–178.
38. Kato, A., Fukai, K., Oiso, N., Hosomi, N., Murakami, T. and Ishii, M. (2003) Association of SPINK5 gene polymorphisms with atopic dermatitis in the Japanese population. *Br. J. Dermatol.*, **148**, 665–669.
39. Rice, K.D., Tanaka, R.D., Katz, B.A., Numerof, R.P. and Moore, W.R. (1998) Inhibitors of tryptase for the treatment of mast cell-mediated diseases. *Curr. Pharm. Res.*, **4**, 381–396.
40. Hou, L., Kapas, S., Cruchley, A.T., Macey, M.G., Harriott, P., Chinni, C., Stone, S.R. and Howells, G.L. (1998) Immunolocalization of protease-activated receptor-2 in skin: receptor activation stimulates interleukin-8 secretion by keratinocytes *in vitro*. *Immunology*, **94**, 356–362.
41. He, S., Gaca, M.D. and Walls, A.F. (1998) A role for tryptase in the activation of human mast cells: modulation of histamine release by tryptase and inhibitors of tryptase. *J. Pharmacol. Exp. Ther.*, **286**, 289–297.
42. He, S., Peng, Q. and Walls, A.F. (1997) Potent induction of a neutrophil and eosinophil-rich infiltrate *in vivo* by human mast cell tryptase: selective enhancement of eosinophil recruitment by histamine. *J. Immunol.*, **159**, 6216–6225.
43. Schechter, N.M., Brass, L.F., Lavker, R.M. and Jensen, P.J. (1998) Reaction of mast cell proteases tryptase and chymase with protease activated receptors (PARs) on keratinocytes and fibroblasts. *J. Cell Physiol.*, **176**, 365–373.
44. Thomas, W.R. (1993) Mite allergens group I–VII. A catalogue of enzymes. *Clin. Exp. Allergy*, **23**, 350–353.
45. Bagarozzi, D.A., Jr and Travis, J. (1998) Ragweed pollen proteolytic enzymes: possible roles in allergies and asthma. *Phytochemistry*, **47**, 593–598.
46. Bodey, B., Bodey, B., Jr, Siegel, S.E. and Kaiser, H.E. (2000) Novel insights into the function of the thymic Hassall's bodies. *In Vivo*, **14**, 407–418.
47. Douek, D.C. and Altmann, D.M. (2000) T-cell apoptosis and differential human leucocyte antigen class II expression in human thymus. *Immunology*, **99**, 249–256.
48. Petraki, C.D., Karavana, V.N., Skoufogiannis, P.T., Little, S.P., Howarth, D.J., Yousef, G.M. and Diamandis, E.P. (2001) The spectrum of human kallikrein 6 (zyme/protease M/neurosin) expression in human tissues as assessed by immunohistochemistry. *J. Histochem. Cytochem.*, **49**, 1431–1441.
49. Ekholm, E., Sondell, B., Stranden, P., Brattsand, M. and Egelrud, T. (1998) Expression of stratum corneum chymotryptic enzyme in human sebaceous follicles. *Acta. Derm. Venereol.*, **78**, 343–347.
50. Ito, M., Ito, K. and Hashimoto, K. (1984) Pathogenesis in trichorrhexis invaginata (bamboo hair). *J. Invest. Dermatol.*, **83**, 1–6.
51. Sallenave, J.M. (2002) Antimicrobial activity of antiproteinases. *Biochem. Soc. Trans.*, **30**, 111–115.
52. Wiedow, O., Harder, J., Bartels, J., Streit, V. and Christophers, E. (1998) Antileukoprotease in human skin: an antibiotic peptide constitutively produced by keratinocytes. *Biochem. Biophys. Res. Commun.*, **248**, 904–909.
53. Liu, L., Wang, L., Jia, H.P., Zhao, C., Heng, H.H., Schutte, B.C., McCray, P.B., Jr and Ganz, T. (1998) Structure and mapping of the human beta-defensin HBD-2 gene and its expression at sites of inflammation. *Gene*, **222**, 237–244.
54. Mitsudo, K., Jayakumar, A., Henderson, Y., Frederick, M.J., Kang, Y., Wang, M., El-Naggar, A.K. and Clayman, G.L. (2003) Inhibition of serine proteases plasmin, trypsin, subtilisin A, cathepsin G, and elastase by LEKTI: a kinetic analysis. *Biochemistry*, **42**, 3874–3881.
55. Watt, F.M., Boukamp, P., Hornung, J. and Fusenig, N.E. (1987) Effect of growth environment on spatial expression of involucrin by human epidermal keratinocytes. *Arch. Dermatol. Res.*, **279**, 335–340.
56. Rheinwald, J.G. and Green, H. (1975) Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. *Cell*, **6**, 331–343.
57. Simon, M. and Green, H. (1985) Enzymatic cross-linking of involucrin and other proteins by keratinocytes particulates *in vitro*. *Cell*, **40**, 677–683.
58. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
59. Frangioni, J.V. and Neel, B.G. (1993) Solubilization and purification of enzymatically active glutathione S-transferase (pGEX) fusion proteins. *Anal. Biochem.*, **210**, 179–187.
60. Harlow, E. and Lane, D. (1988) *Antibodies: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
61. Goodyer, I.D., Jones, E.E., Monaco, A.P. and Francis, M.J. (1999) Characterization of the Menkes protein copper-binding domains and their role in copper-induced protein relocalization. *Hum. Mol. Genet.*, **8**, 1473–1478.
62. Failla, C.M., Odorisio, T., Cianfarani, F., Schietroma, C., Puddu, P. and Zambruno, G. (2000) Placenta growth factor is induced in human keratinocytes during wound healing. *J. Invest. Dermatol.*, **115**, 388–395.