ISOLATION OF SPINK 6 IN HUMAN SKIN: A SELECTIVE INHIBITOR OF KALLIKREIN-RELATED PEPTIDASES

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Running Title: Spink6, a selective KLK Inhibitor

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Kallikrein-related peptidases (KLKs) play a central role in skin desquamation. They are tightly controlled by specific inhibitors including the lympho-epithelial Kazal-type inhibitor (LEKTI) encoded by serine protease inhibitor Kazal type 5 (Spink5) and LEKTI-2 encoded by Spink9. Herein, we identified Spink6 as a selective inhibitor of KLKs in the skin. Unlike LEKTI but similar to LEKTI-2, Spink6 possesses only one typical Kazal domain. Its mRNA was detected to be expressed at low levels in several tissues and was induced during keratinocyte differentiation. Natural Spink6 was purified from human plantar stratum corneum extracts. Immunohistochemical analyses revealed Spink6 expression in the stratum granulosum of human skin at various anatomical localisations and in the skin appendages, including sebaceous glands and sweat glands. Spink6 expression was decreased in lesions of atopic dermatitis. Using KLK5, KLK7, KLK8, and KLK14, thrombin, trypsin, plasmin, matriptase, prostatin, mast cell chymase, cathepsin G, neutrophil elastase and chymotrypsin, inhibition with recombinant SPINK6 was detected only for KLK5, KLK7 and KLK14 with an apparent K(i) of 1.33 nM, 1070 nM and 0.5 nM, respectively. Spink6 inhibited desquamation of human plantar callus in an ex vivo model. Our findings suggest that Spink6 plays a role in modulating the activity of KLKs in human skin. A selective inhibition of KLKs by Spink6 might have therapeutic potential when KLK activity is elevated.

The skin protects us from water loss and mechanical damage. The surface-exposed epidermis, a self-renewing stratified squamous epithelium composed of several layers of keratinocytes, is most important for the barrier defense against these challenges. Recent discoveries have highlighted the balance of proteases and protease-inhibitors as key players in both, desquamation processes and epidermal barrier functions (1).

Human tissue kallikreins, or kallikrein-related peptidases (KLK), are the largest family of trypsin- or chymotrypsin-like secreted serine proteases, which are encoded by 15 genes on chromosome region 19q13.4 (2). At least eight KLKs are expressed in normal skin, among which KLK5, KLK7, KLK8 and KLK14 have been reported to be most important (3-6). KLKs are capable of cleaving corneodesmosomes (7-10) and are thought to be key regulators of the desquamation process. The activity of the KLKs is regulated by the pH and specific protease inhibitors. The importance of epithelial protease inhibitors has been revealed impressively in Netherton Syndrome (NS; OMIM 256500), an autosomal recessive disorder caused by mutations in the gene serine protease inhibitor Kazal-type 5 (Spink5) (11). NS presents as an ichthyosiform dermatosis with variable erythroderma, hair-shaft defects (bamboo hair), atopic features, and growth retardation (12). Lymphoepithelial Kazal-type-related inhibitor (LEKTI) (13), the product of Spink5, includes in its primary structure 15 different serine protease inhibitory domains (13). Domain 15 and 2 each comprises a typical Kazal-type structure.
whereas the other domains miss a disulfide bridge. Recently, LEKTI-2, encoded by Spink9, was reported as a KLK5 selective inhibitor expressed at palmoplantar sites (14;15). LEKTI-2 consists of a single typical Kazal-type domain, which exhibits the highest homology to LEKTI/Spink5 domain 15. This suggests that a complex balance exists between the KLK cascades and Spink family members in human skin, maintaining normal epithelial barrier functions. Taking the multiple skin-expressed KLKs members into account, we hypothesized that more Spink members are present in human skin. Herein, we identified Spink6 as a selective inhibitor of KLKs in human skin.

**EXPERIMENTAL PROCEDURES**

**Materials-** All experiments were performed according to the Declaration of Helsinki protocols and under protocols approved by the ethics committee of the Medical Faculty of the Christian Albrechts University Kiel (AZ A 104/06) and all patients and healthy control persons gave written informed consent. Normal skin specimens were taken from routine clinical work at the Department of Dermatology, UKSH Kiel, and represent tumor-free margins of benign melanocytic tumors surgically removed from patients. Restriction endonucleases were from New England Biolabs (Frankfurt, Germany). KLKs were purchased from R&D Systems Inc. (Minneapolis, MN). All other proteases, primers, substrates and chemicals were purchased from Sigma–Aldrich (Taufkirchen, Germany), if not otherwise indicated.

**Bioinformatics** Homology- search was done using the tBLASTn algorithm as provided by the Ensemble BlastView server (http://www.ensembl.org). Determination of gene structure was done using the BLAT algorithm (16) as provided by the Ensemble UCSC Genome Browser (http://genome.ucsc.edu/cgi-bin/hgBlat). Subsequent sequence manipulations utilized the online BLAST 2 Sequences (17). Protein domains were discovered on the SMART server (18). Multiple sequence alignments were performed using the ClustalW2 program and edited with GeneDoc (http://www.psc.edu/biomed/genedoc).

**Rapid amplification of cDNA ends (RACE-** Total RNA was obtained from cultured human foreskin-derived keratinocytes by using TRIZol reagent (Invitrogen, Hamburg, Germany). After treatment with RNase-free DNase I (Roche Diagnostics, Mannheim, Germany) to exclude contamination with genomic DNA, 2 µg of DNA-free total RNA was used for the first-strand cDNA synthesis for RACE using SMART RACE cDNA Amplification Kit (BD Bioscience Clontech, Heidelberg, Germany) according to the manufacturer’s protocol. 5'- RACE was performed with a gene-specific antisense primer (5'-AGG CAC ATT TAT TGC CAT ATG TCT GGC CAT C-3') while 3'- RACE was done with a gene-specific sense primer (5'-GTG AGT TCC AGG ACC AGG TCT ACT G-3') essentially according to the manufacturer’s protocol. PCR reaction cycles were performed under the following conditions: 1 min at 95°C, 5 cycles of 20 s at 95°C and 3 min at 72°C, 5 cycles of 20 s at 95°C and 3 min at 70°C, 25 cycles of 20 s at 95°C and 3 min at 68°C, and a final extension of 10 min at 72°C. Subsequently, the PCR product was diluted 50-fold into Milli-Q water and used as a template for a nested PCR with a nested primer (For 5'-nest: 5'-GCC ACA GTG TGG GTT AGA TTC CGG AGC G-3'; for 3'-nest: 5'-CCA CAC TGT GGC TCT GAT GCC CAG A-3') under the following conditions: 1 min at 95°C, 30 cycles of 20 s at 95°C and 3 min at 70°C, and a final extension of 10 min at 70°C. The amplified fragment was gel purified and subcloned into the pGEM-T vector (Promega, Mannheim, Germany) followed by fully sequencing in both directions.

**RT-PCR-** Total RNA was isolated from cultured human foreskin-derived keratinocytes, HaCaT cell line and skin by using TRIZol reagent (Invitrogen, Hamburg, Germany). Other total RNAs from different tissues were obtained from BD Bioscience Clontech (Heidelberg, Germany). A total of 2 µg of total RNA from human tissues or cultured was reverse transcribed with an oligo(dT)18 primer and Superscript II RNaseH reverse transcriptase (Invitrogen). One pair of gene-specific PCR primers (forward primer: 5’- ACC TCA GCT GGA CAA AGC AG -3’; reverse primer: 5’- TGG CAA GTC ACC AAG AAA CA -3’) were
designed to amplify a 322-bp product and to span all three exon-intron boundaries. PCR was carried out with the Advantage 2 PCR polymerase (BD Bioscience Clontech, Heidelberg, Germany) and amplicons were analyzed by 2.0% agarose gel electrophoresis. As an internal control of cDNA templates, the housekeeping gene GAPDH (glyceraldehyde phosphodehydrogenase) was assessed with each cDNA in a separate PCR reaction as described (14). For quantitative real-time RT-PCR, assay was carried out with the same primer pair as above and the SYBR® Premix Ex Taq™ Kit (Takara Bio, Heidelberg, Germany) in a fluorescence thermocycler following the instructions of the manufacturer (LightCycler, Roche Molecular Biochemicals, Hamburg, Germany). Cycling conditions were as follows: with 45 cycles at 95°C for 5 s, 69°C for 20 s ('touchdown' of -1°C/cycle to 63°C), 72°C for 20 s. To calculate the relative transcript amplification, the housekeeping gene GAPDH was performed with each cDNA in a separate PCR reaction. The data from triplicate samples were analyzed with software (GraphPad Prism 4). For absolute DNA quantification, reactions were carried out with different concentrations of linearized plasmid DNA containing the insert in parallel to the samples that should be quantified. The data from three independent observations were analyzed with the software and expressed as mean ± SD of mRNA copies relative to 10 ng of total RNA.

Electrospray ionization mass spectrometry (ESI-MS)-analyse- Protein and peptide mass determinations were performed by electrospray mass spectrometry (ESI-MS)-analyses using a Quadrupol-Time-of-Flight-Hybrid-Mass-Spectrometer (Q-TOF™ II, Waters Micromass, Milford, Massachusetts) equipped with an orthogonal electrospray source (Z-spray) operated in positive ionisation mode. For MS analysis aliquots (2–10 µl) of HPLC fractions containing the sample were diluted with 100µl carrier solvent (50:50 acetonitrile: water containing 0.2% formic acid or 60:40 methanol: 10 mM ammonium formate, pH 6.5) and infused into the electrospray source at a rate of 10 - 20 µL/min. Sodium iodide was used for mass calibration for a calibration range of m/z 100–2,000. The capillary potential was set to 3.5 or 4 kV and cone voltages between 25 and 75 V were chosen; cone temperature was set to 80°C; desolvation temperature was 150 °C; the ESI gas was nitrogen. The charge-to-mass ratio of ions was scanned in the range of 280 to 2000. Acquisition and data analysis were all performed using the MassLynx 4 software package supplied by Waters Micromass, Milford, Massachusetts. Mass Spectra were averaged typically over 2-10 scans (2-20s/scan). The multiply charged raw data of intact proteins were background-subtracted and deconvoluted using Maximum Entropy 1 (“MaxEnt1”) to obtain singly charged ion mass spectra to determine average molecular masses of intact proteins. The raw combined spectral data from small peptides or obtained after MS-MS fragmentation of selected precursor ions were background-subtracted and subjected to Maximum Entropy 3 (“MaxEnt3”) deconvolution to determine monoisotopic molecular masses. Sample identity was determined by database search analysis of peptide fragment mass patterns and/or after de novo sequencing of tryptic peptide fragments. All mass fingerprint and MS/MS data were searched against the human protein database using the Mascot program (Matrix Science, Boston, Massachusetts). Peptide sequences were directly determined from MS/MS data using the software program PepSeq from the MassLynx4 software package (Waters Micromass, Milford, Massachusetts). PepSeq-derived peptide sequences were analysed with the NCBI-BLAST protein database search program.

Recombinant protein production- The recombinant expression of Spink6 cDNA (rSpink6; residues 25 to 80) in Escherichia coli (E. coli) was performed by molecular subcloning of Spink6 cDNA into the prokaryotic expression vectors pET-32a (Novagen, North Ryde, Australia) and pET-SUMO (Invitrogen), as previously described (19). The SUMO-Histagged fusion protein was digested with SUMO protease 1 according to the manufacturer’s suggestions (Lifesensors Inc., Pennsylvania, USA). To subclone into the expression vector pET-32a (Novagen, North Ryde, Australia), PCR was performed with Pfu DNA polymerase.
Promega, Mannhein, Germany. The inserts were gel purified and inserted into the pET-SUMO vector. Specific primer pairs used in this study are p32-Spink6-forward: ACT GAG ATC TGG GTA CCG ACG ACG ACA AGC AGG TTG ACT GTG GTG AGT TC; p32-Spink6-reverse ATT TGC GGC CGC TCA GCA TTT TCC AGG ATG CTT; pSUMO-Spink6-forward GGA GGA CAG GTT GAC TGT G; pSUMO-Spink6-reverse TCA GCA TTT TCC AGG ATG CTT. All positive clones were identified and verified by sequencing. The plasmids were introduced into the E. coli host strain BL21(DE3)pLysS (Novagen). Subsequently, these were grown at 37°C in tryptic soy broth (TSB) medium containing appropriate antibiotics. Expression of the recombinant protein was induced with 1 mM isopropyl thio-β-D-galactoside (IPTG) for 3 h at 37°C. Bacteria were harvested by centrifugation at 5,000 x g for 5 min at 4°C, lysed by sonication and then centrifugated at 18,000 x g for 30 min at 4°C (Beckman Coulter, Krefeld, Germany). Recombinant proteins were trapped with Ni²⁺-prepared columns (Macherey-Nagel, Düren, Germany) and Ni²⁺-affinity column-bound proteins were subjected to preparative reversed phase high-performance liquid chromatography (RP-HPLC) with a column (SP250/10 Nucleosil 300-7 C8; Macherey-Nagel) that was previously equilibrated with 0.1% (v/v) TFA in HPLC-grade water containing 10% acetonitrile. The polyhistidine-tagged fusion proteins were eluted with a gradient of increasing concentrations of acetonitrile containing 0.1% (v/v) TFA (flow rate, 0.5 ml/min). Fractions of each peak were collected. Purity of recombinant fusion-proteins was determined by SDS-PAGE. Masses of fusion proteins and recombinant Spink6 peptides were analyzed by ESI-QTOF-mass spectrometry. Concentrations of proteins present in HPLC fractions were estimated by ultraviolet absorbance integration at 215 nm using ubiquitin for calibration.

Production of antibody- Polyclonal antiserum was generated in a goat against human recombinant Spink6 (rSpink6; residues 25 to 80) similar as described (14). Briefly, a total of 1.0 mg of fusion protein (pET-32a-Spink6) was conjugated to maleimide-activated keyhole limpet hemocyanin (1:1, w/w) and subsequently mixed with 500 µg of pET-32a-Spink6 for use as immunogens. Antisera were affinity-purified by absorption first against protein G. Unspecific antibodies were eliminated by collecting the flowthrough after given the antibody to a pET-tag coupled column. Finally, the antibody was affinity-purified against rSpink6 that was covalently bound to the HiTrap NHS-activated HP 1 ml column, respectively (Amersham Biosciences, Freiburg, Germany). Specificity was tested by Western blot analyses using rSpink6, naturally isolated LEKTI-2 (data not shown) and Spink6 as well as SC extracts.

Isolation of Spink6 from SC extracts- Pooled heel stratum corneum (SC) was extracted similarly as described (14). After diafiltration (Amicon filters, cut off: 3 kDa) against 10 mM Tris/citrate buffer, pH 8.0, extracts were applied to an anti-Spink6 affinity column. Affinity-purified polyclonal human Spink6 antibodies (anti-Spink6) were covalently bound to HiTrap NHS-activated HP 1 ml columns (Amersham Biosciences). Unspecific material which was weakly bound to the affinity-column was first eluted with 2 M NaCl. Strong bound material was eluted by pH shift using 0.1 M Glycine-HCl (pH 3). Strong affinity-column-bound material was further purified by reversed-phase HPLC (Jupiter C18 column, Phenomenex). Each HPLC-fraction was analyzed by ESI-MS. Lyophilized fractions were subjected to tributylphosphine/vinylpyridine gas-phase reduction and alkylation (20). After dissolving sample in 20 µl NH₄HCO₃ with 2 µl acetonitrile
peptides were digested with 4.5 ng/µl trypsin at 37°C for 2 h and then further analysed by MS/MS.

**Western blot analyses-** For Western-blot analysis, healthy persons’ SC extract, rSpink6 and HPLC-fractions containing natural SPINK6, were loaded onto a 12% SDS-tricine polyacrylamide gel. Proteins were transferred to a Protran-nitrocellulose membrane (Schleicher & Schuell BioScience, Dassel, Germany), blocked for 1 h in blocking buffer (5% (w/v) bovine serum albumine (BSA) in PBS containing 0.05% Tween (PBST) and then incubated for 18 h at 4°C in 3% (w/v) BSA milk in PBST containing affinity-purified anti-Spink6 antibody (1 µg/ml). The membrane was washed with PBST six times for 5 min each and then incubated for 45 min in PBST containing 1:10,000 dilution of goat anti-mouse IgG HRP conjugate (Dianova, Hamburg, Germany). After further washing the membrane was incubated for 5 min with chemiluminescent peroxidase substrate (Roche) and visualized using a Diana III cooled CCD-camera imaging system (Raytest, Straubenhardt, Germany).

**Immunohistochemistry-** Immunohistochemical staining of paraffin-embedded tissue was performed as described previously (21), using affinity-purified polyclonal goat anti-Spink6 antibody (final concentration of 10 ng/µl), followed by a biotinylated secondary rabbit anti-goat IgG (1:100, Dako Cytomation) antibody and subsequently incubated with Vector Universal DAB Kit (Vector, Burlingame, CA, USA). Counterstaining was done with hematoxylin. Specificity test of the anti-Spink6 was performed by using rSpink6 peptide to block the primary antibody. Negative controls were established by using preimmune goat sera to stain sections.

**Protease inhibition assays-** All protease assays were performed measuring chromogenic substrate release by proteases in the buffer recommended by the manufacturer, as previously described (14). Specific concentrations of proteases, substrates and inhibitors were indicated in Table 1. Recombinant human kallikreins were purchased from R&D systems and activated according to the manufacturer’s protocol. All enzymes were incubated with increasing concentrations of recombinant Spink6 for 5 minutes in 0.1 M Tris, 150 mM NaCl, 5 mM EDTA, 0.05% Tween-20, pH 8.0. After incubation, solution of substrate in the same buffer was added, resulting in a final concentration of 2 nM KLK, 1 mM Tosyl-Gly-Pro-Arg-pNa (KLK5, 8, 14) or S-2586 (KLK7) and Spink6 in a total volume of 200 µl. The reaction progress was monitored by measuring absorbance OD405nm using Tecan Sunrise microplate reader in kinetic mode. Initial reaction velocities for each tested inhibitor concentration were determined by linear regression and were subsequently plotted against inhibitor concentration. Data were further analysed according to tight-binding inhibition model by Morrison. For determination of K_m, enzymes were mixed with varying substrate concentrations. Mixtures contained 2 nM KLK and respective substrate in a concentration range of 0.2 to 6 mM. Initial velocities were determined by linear regression and plotted against respective substrate concentrations. Collected data were analysed by nonlinear regression to the Michaelis-Menten equation, using GraphPad Prism 5 built-in model.

**Cell Shedding from Human Plantar Stratum Corneum-** The procedure of Lundström and Egelrud (22) was applied. About 0.5-mm-thick flakes of stratum corneum were cut parallel to the skin surface from the heels of healthy volunteers. After the tissue had been soaked in 0.01 M sodium phosphate, 0.14 M NaCl, 0.1% NaN3, 1% Triton X-100, pH 7.2, for 5 h at room temperature, loosely attached cells were scraped off with a scalpel. Stratum corneum cylinders with a defined surface area were prepared with a 3-mm biopsy punch. Cylinders were incubated in 1.5-ml Eppendorf tubes with 1 ml of 0.1 M Tris-HCl, 5 mM EDTA, 0.1% NaNs, pH 8.0, for 18 h at 37°C. After vortex agitation (30 s), the tissue pieces were removed from the incubation solution. The released cells were collected by centrifugation (10,000 × g, 10 min), washed in phosphate-buffered saline, and extracted with 1 M NaOH). Solubilized protein was quantified using Nanodrop with bovine serum albumin treated with 1 M NaOH as standard.
RESULTS

*Spink6 is expressed in human skin.* To search for genes encoding putative functional domains homologous to atypical kazal-like domains of LEKTI, the amino acid sequence of LEKTI domain 6 (D-6) was used as a query to blast against the human genome (NCBI builder 34, 2003). A gene locus was identified and localized between the two known genes *Spink5* (GenBank NM_001127698) and *Spink7* (GenBank NM_032566) (Fig. 1A). To identify the gene, RACE and RT-PCR were performed. Two full-length cDNA sequences were obtained from cultured foreskin-derived keratinocytes and were registered under the GenBank accession number GQ504704 and GQ504705, respectively.

Although this gene had been predicted and designated as serine protease inhibitor kazal-type 6 (gene symbol *Spink6*; GenBank NM_205841), to the best of our knowledge the identification of this gene has not been reported. Comparison between the cDNA and genomic sequences revealed that the *Spink6* gene contains 4 conserved exons, while it has two different transcription starting sites (TSSs) at bp position 1 and 64, but only one termination site (Fig. 1B). All of the splice donor and acceptor sites conform to the GT/AG rule. These two transcripts, Spink6-v1 (947 bp) and Spink6-v2 (883 bp) contain a common open-reading frame (ORF) of 243 bp and a polyadenylation signal (AAUAAA) situated 214 nucleotides 5' of the polyadenylation tail (Fig. 1C). The deduced protein chain consists of 80 amino acids with a leader sequence containing a putative signal peptide (residues 1–23) and a typical kazal domain with 6 cysteine residues (residues 28–80). Comparison of Spink6 with LEKTI/Spink5 domains and Spink9 revealed that Spink6 showed high homology to the atypical kazal domains of LEKTI/Spink5 especially in the putative reactive centre residues, P2 (Thr), P1 (Arg) and P1' (Glu) (Fig. 2), whereas LEKTI-2/Spink9 exhibits the highest degree of sequence homology to Spink5-d2.

Given the fact that *Spink6* cDNA was cloned from cultured human keratinocytes, we tried to identify Spink6 protein in the skin using an affinity column of polyclonal Spink6 antibody to capture natural Spink6 from human callus extracts. After affinity chromatography the eluting fraction was further purified by C18-reversed phase-HPLC resulting in two peaks (Fig. 3A) with masses of 6045.03 Da and 6062.57 Da, which fitted well with a Spink6 containing three disulfide bonds starting with Gln24 and its derivative starting with pyroglutamine24 (Fig. 1C underlined AA sequence). The identity of these peptides as Spink6 was confirmed by MS/MS analyses (data not shown) and Western blot analyses (Fig. 3B). Immunoblotting analyses exhibited immunoreactivity of the purified pyroglutamine24 (panel 1), purified Gln24 Spink6 and the recombinant Spink6.

To further investigate whether *Spink6* is expressed in other tissues and organs, RT-PCR analyses were performed. Low levels of *Spink6* mRNA were detected in all tissues and cells examined using RT-PCR (data not shown). Realtime-PCR analyses revealed that *Spink6* expression was much higher in cultured primary keratinocytes and HaCat keratinocytes compared to tissue samples (Fig. 4A). Spink6 expression was induced during keratinocyte differentiation (Fig. 4B).

To localize the Spink6 protein expression in human skin, immunohistochemical analyses of paraffin-embedded sections were performed. Spink6 immunoreactivity was detected in the upper epidermal layer of the skin (Fig. 5). In skin, Spink6 was detected at different body sites, including face, arms, trunk, legs, while it exhibited the most prominent expression level at palmoplantar sites (Fig. 5, D). Furthermore, Spink6 immunoreactivity was also detected in sebaceous glands and in some sweat glands (Fig. 5, E, F). The specificity of the antibody was demonstrated by blocking the antibody with recombinant antigen (Fig. 5 B). Spink6 expression was markedly decreased in atopic dermatitis lesions and slightly in psoriasis lesions (Fig. 6).

*Spink6 is a selective inhibitor of KLKs.* As Spink6 possesses a typical Kazal-type domain, inhibition against different serine proteases was tested. Spink6 exhibited inhibition against KLK5, KLK7 and KLK14, while it had no obvious activity against all other serine proteases.


tested, including trypsin, cathepsin G, mast cell chymase, leukocyte elastase, plasmin, matriptase, prostatin, chymotrypsin and thrombin (Table 1). Next, inhibition against the relevant members of skin KLKs, KLK5, KLK7, KLK8 and KLK14, was analysed in more detail. The strongest inhibitory activity was observed against KLK5 and KLK14 with $K_i$ values of 1.33 and 0.5 nM, respectively (Fig. 7, Table 2), whereas KLK7 was just slightly inhibited and that KLK8 was not inhibited by Spink6. Recombinant Spink6 inhibited spontaneous desquamation of human plantar skin using an ex vivo model in a concentration dependent manner (Table 3).

**DISCUSSION**

In the present study we have identified and purified the protease inhibitor Spink6 from human stratum corneum extracts. We have cloned its full length cDNA from cultured human keratinocytes and show its expression in human epidermis. The most relevant result of the study is that Spink6 is a selective inhibitor of several KLKs, which are known to be important in epidermal homeostasis. Our finding suggests that Spink6 plays a role in modulating activities of KLKs in human skin. As we could detect Spink6 mRNA expression in various tissues, we suggest that the biological function of Spink6 might not be limited to the epidermis.

Expression of Spink6 mRNA was detectable at low levels in all tissue samples investigated. Spink6 mRNA expression was much higher in cultured keratinocytes compared to skin extracts. Spink6 protein was identified in human callus. Whether Spink6 is expressed in other tissues remains to be investigated in more detail. Immunohistochemical analyses of terminal ileum as well as colon tissue samples revealed no Spink6 expression (data not shown) though mRNA levels were comparable to those of Spink6 in the skin. The expression pattern of Spink6 in the skin is similar to that of LEKTI/Spink5. Ultrastructural analyses revealed that LEKTI/Spink5 and KLK7 are transported separately in the lamellar granule system and are co-localized in the extracellular spaces (23), which was demonstrated to be expressed in lamellar bodies and secreted into the intercellular space, in the uppermost stratum granulosum (23-25). However, the expression of Spink6 differs from that of LEKTI/Spink5 in skin appendages. The latter is present in the inner root sheath of the hair follicle, where we could not detect Spink6 (data not shown). Instead, we could find Spink6 immunoreactivity in sebaceous glands and in some sweat glands. Its expression in sweat glands was not always present in different skin samples and showed no homogenous level in specific sweat glands (Fig.5), suggesting a potential regulatory mechanism of Spink6 expression in sweat glands, though further experiments are required.

The other skin-expressed Spink member, Spink9, was only described at palmoplantar sites, where we could also detect Spink6 expression. Slightly varying expression patterns of LEKTI/Spink5, Spink6 and Spink9 in human skin suggest localized functional differences. Their function is thought to act as protease inhibitors. Spink6 shows structural similarities to the atypical domains of LEKTI/Spink5 (Fig. 2). Especially the P1 site exhibits high similarity to these domains suggesting a similar inhibition profile. Like LEKTI/Spink5, Spink6 inhibits KLK5, KLK7 and KLK14, but it does not inhibit other serine proteases tested so far. LEKTI/Spink5 has been reported to have a much broader activity profile. The LEKTI/Spink5 domains inhibit trypsin, plasmin, subtilisin A, cathepsin G, human neutrophil elastase and KLK5, 7 and 14 (26). The determined $K_i$ of LEKTI domains to inhibit KLK5 was in the range of 3 nM (domain 8-11) to 120 nM (domain 9-15) (9;27;28). Spink9 exhibited only inhibition activity against KLK5 with a determined $K_i$ of 65 -230 nM (14;15). Interestingly, KLK8 is not inhibited by any of these Spink members. The inhibition of KLK5 and KLK14 by Spink6 in the nanomolar scale might have pathophysiological relevance. KLK5 and KLK14, but not KLK8, have been reported to activate the protease activated receptor (PAR)-2 (29), a signaling receptor in epidermal inflammation (30) and regulator of epidermal barrier function (31). Recently, KLK5 was identified to induce AD-like lesions through activation of the PAR-2 by inducing thymic stromal lymphopoietin expression in NS

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In the present study we have identified and purified the protease inhibitor Spink6 from human stratum corneum extracts. We have cloned its full length cDNA from cultured human keratinocytes and show its expression in human epidermis. The most relevant result of the study is that Spink6 is a selective inhibitor of several KLKs, which are known to be important in epidermal homeostasis. Our finding suggests that Spink6 plays a role in modulating activities of KLKs in human skin. As we could detect Spink6 mRNA expression in various tissues, we suggest that the biological function of Spink6 might not be limited to the epidermis.

Expression of Spink6 mRNA was detectable at low levels in all tissue samples investigated. Spink6 mRNA expression was much higher in cultured keratinocytes compared to skin extracts. Spink6 protein was identified in human callus. Whether Spink6 is expressed in other tissues remains to be investigated in more detail. Immunohistochemical analyses of terminal ileum as well as colon tissue samples revealed no Spink6 expression (data not shown) though mRNA levels were comparable to those of Spink6 in the skin. The expression pattern of Spink6 in the skin is similar to that of LEKTI/Spink5. Ultrastructural analyses revealed that LEKTI/Spink5 and KLK7 are transported separately in the lamellar granule system and are co-localized in the extracellular spaces (23), which was demonstrated to be expressed in lamellar bodies and secreted into the intercellular space, in the uppermost stratum granulosum (23-25). However, the expression of Spink6 differs from that of LEKTI/Spink5 in skin appendages. The latter is present in the inner root sheath of the hair follicle, where we could not detect Spink6 (data not shown). Instead, we could find Spink6 immunoreactivity in sebaceous glands and in some sweat glands. Its expression in sweat glands was not always present in different skin samples and showed no homogenous level in specific sweat glands (Fig.5), suggesting a potential regulatory mechanism of Spink6 expression in sweat glands, though further experiments are required. The other skin-expressed Spink member, Spink9, was only described at palmoplantar sites, where we could also detect Spink6 expression. Slightly varying expression patterns of LEKTI/Spink5, Spink6 and Spink9 in human skin suggest localized functional differences. Their function is thought to act as protease inhibitors. Spink6 shows structural similarities to the atypical domains of LEKTI/Spink5 (Fig. 2). Especially the P1 site exhibits high similarity to these domains suggesting a similar inhibition profile. Like LEKTI/Spink5, Spink6 inhibits KLK5, KLK7 and KLK14, but it does not inhibit other serine proteases tested so far. LEKTI/Spink5 has been reported to have a much broader activity profile. The LEKTI/Spink5 domains inhibit trypsin, plasmin, subtilisin A, cathepsin G, human neutrophil elastase and KLK5, 7 and 14 (26). The determined $K_i$ of LEKTI domains to inhibit KLK5 was in the range of 3 nM (domain 8-11) to 120 nM (domain 9-15) (9;27;28). Spink9 exhibited only inhibition activity against KLK5 with a determined $K_i$ of 65 -230 nM (14;15). Interestingly, KLK8 is not inhibited by any of these Spink members. The inhibition of KLK5 and KLK14 by Spink6 in the nanomolar scale might have pathophysiological relevance. KLK5 and KLK14, but not KLK8, have been reported to activate the protease activated receptor (PAR)-2 (29), a signaling receptor in epidermal inflammation (30) and regulator of epidermal barrier function (31). Recently, KLK5 was identified to induce AD-like lesions through activation of the PAR-2 by inducing thymic stromal lymphopoietin expression in NS
Thymic stromal lymphopoietin is an epithelial cell-derived cytokine and a potential key player in the induction of allergic inflammation. Thymic stromal lymphopoietin-activated human dendritic cells produce Th2-attracting chemokines, but no IL-12, and induce naïve CD4+ and CD8+ T cell differentiation into effector cells with a typical pro-allergic phenotype (33). Increased trypsin-like proteolytic activity was reported in atopic dermatitis lesions (34), which might be the result of decreased Spink6 expression as demonstrated herein. As Spink6 is a very potent and selective inhibitor of KLKs, it could have therapeutic potential to modulate increased KLKs activity. We propose that the members of the Spink family LEKTI/Spink5, Spink6 and Spink9 are natural inhibitors of KLKs in human skin showing a specific expression and inhibition profile. As Spink6 mRNA is detectable in other tissues, the biological function of Spink6 might not be limited to the skin. Indeed, KLK5 and KLK14 expression has also been reported in other tissues. KLK5 was reported to be expressed at high levels (100-1000 ng/g tissue) in breast, testis, salivary glands and thyroids, whereas KLK14 showed high concentrations in bladder, breast and vagina tissue samples (35). Further studies are needed to clarify whether Spink6 plays a functional role in other tissues than the skin. In conclusion, Spink6 is present in human skin and inhibits a selective repertoire of skin-expressed KLKs.

REFERENCES

FOOTNOTES

We thank Christel Martensen-Kerl and Jutta Quitzau for excellent technical assistance. This work was supported by Deutsche Forschungsgemeinschaft, SCHR305/4-1.

Abbreviations used are: AD, atopic dermatitis; DTT, dithiothreitol; ESI-MS, electrospray ionization mass spectrometry; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; KLKs, kallikrein-related peptidases; LEKTI, lympho-epithelial Kazal-type inhibitor; PBS, phosphate buffered saline; QTOF, quadrupol-time-of-flight; RACE, rapid amplification of CDNA ends; RP-HPLC, reversed-phase high-performance liquid chromatography; RT-PCR, reverse transcription polymerase chain reaction; SC, stratum corneum; Spink, serine protease inhibitor kazal-type; TFA, trifluoroacetic acid;
The nucleotide sequences reported in this paper have been submitted to the Gen-Bank™ Bank with accession numbers GQ504704 and GQ504705.

FIGURE LEGENDS

Fig. 1. Nucleotide and amino acid sequences of human Spink6. (A) Schematic physical map of human SPINK genes locus (5q33.1). Genes are ordered from centromere (left hand side) to telomere (right hand side). (B) Schematic diagram of the Spink6 gene, based on its cDNA isolated from foreskin-derived keratinocytes. It consists of four exons and three introns. The positions of the exons (boxes) and introns (curve lines) of Spink6 are deduced by comparing its full-length cDNA sequence with the corresponding genomic DNA. (C) The two full-length cDNA sequences of Spink6 and the deduced protein sequence.

Fig. 2. Amino acid sequence alignment of Spink6 in comparison to LEKTI/Spink5 domains and Spink6. The alignment of the Kazal domains of Spink6, LEKTI-2/Spink9 and LEKTI/Spink5 domains was generated by ClustalW and displayed using GeneDoc. Identical residues are boxed in black while gray boxes mark partially conserved residues. The darker the shading, the more the amino acids are conserved among the family members. The different LEKTI/LEKTI-2 domains are ordered by their homology to Spink6. The P1 and P1’ sites are indicated.

Fig. 3. Purification of Spink6 from human stratum corneum extracts. (A) Natural Spink6 was isolated from human plantar stratum corneum extracts by using first an anti-Spink6 affinity-column (not shown) followed by a C18-reversed-phase HPLC. ESI-MS analyses of UV-absorbing peak fractions revealed masses of 6045.03 Da and 6062.57 Da, which fitted well with a truncated Spink6 containing three disulfide bonds starting with Gln24 (calc. 6062.8 Da) and its pyroglutamine24 form (calc. 6045.8 Da). For both peptides sequences were confirmed by MS/MS. The dotted line marks the eluting acetonitrile concentration. (B) Immunoblotting analyses exhibited immunoreactivity of the purified pyroglutamine24 (panel 1), purified Gln24 Spink6 and the recombinant Spink6.

Fig. 4. Spink6 mRNA is ubiquitously expressed in human tissues and cultured keratinocytes. qPCR analyses revealed expression of Spink6 mRNA in all cDNA samples investigated (A). Spink6 mRNA expression was induced in cultured primary keratinocytes during differentiation (B).

Fig. 5. Spink6 is expressed in skin and its appendages. Immunohistochemical analyses using an affinity purified, polyclonal goat anti-Spink6 antibody revealed localization of Spink6 within the stratum granulosum of epidermis and stratum corneum (A, C, D). Exemplarily anatomical localizations of the samples were: (A) face, (C) plantar site, (D) upper arm. In (B) the control is shown when recombinant Spink6 (0.5 µg/ml) was used to block the antibody (same paraffin sample as (A)). Sebaceous glands showed positive staining (E) and some sweat glands exhibited immunoreactive Spink6 (F).

Fig. 6. Spink6 expression in atopic dermatitis and psoriasis. Immunohistochemical analyses of atopic dermatitis (B) and psoriasis (C) lesions compared to healthy skin (same patient as (B)). Exemplary results of three independent investigations are shown.

Fig. 7. Inhibition of human kallikreins by recombinant Spink6. Inhibition of KLKs (KLK5, 7, 8 and 14) was performed using increasing amounts of recombinant Spink6. The reaction progress was monitored by measuring absorbance OD405nm. Initial reaction velocities for each tested inhibitor concentration were determined by linear regression and were subsequently plotted against inhibitor concentration. Data were further analysed according to the tight-binding inhibition model by Morrison. Presented graphs include result of at least 3 replicates for each enzyme ± SEM.
### Table 1  Protease Inhibition by recombinant Spink6

<table>
<thead>
<tr>
<th>Proteinase</th>
<th>SPINK6 (nM)</th>
<th>Inhibition (%)</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine Trypsin (2 nM)</td>
<td>400</td>
<td>0</td>
<td>N-(p-Tosyl)-Arg-Gly-Val 5-NA</td>
</tr>
<tr>
<td>Cathepsin G (1 nM)</td>
<td>666</td>
<td>0</td>
<td>N-Succinyl-Ala-Ala-Pro-Phe pNA</td>
</tr>
<tr>
<td>Chymase (2 nM)</td>
<td>666</td>
<td>0</td>
<td>N-Succinyl-Ala-Ala-Pro-Phe pNA</td>
</tr>
<tr>
<td>Human Chymotrypsin (2 nM)</td>
<td>400</td>
<td>0</td>
<td>3-MeO-Arg-Pro-Tyr pNA</td>
</tr>
<tr>
<td>KLK14 (2 nM)</td>
<td>400</td>
<td>99.9</td>
<td>N(p-Tosyl)-Arg-Gly-Val 5-NA</td>
</tr>
<tr>
<td>KLK5 (5.3 nM)</td>
<td>400</td>
<td>99.9</td>
<td>N(p-Tosyl)-Arg-Gly-Val 5-NA</td>
</tr>
<tr>
<td>KLK7 (15.8 nM)</td>
<td>400</td>
<td>88.3</td>
<td>MeO-Suc-Arg-Pro-Tyr-pNA</td>
</tr>
<tr>
<td>Human leukocyte elastase (2 nM)</td>
<td>400</td>
<td>0</td>
<td>N-MeO-Ala-Ala-Pro-Val pNA</td>
</tr>
<tr>
<td>Human Plasmin (2 nM)</td>
<td>400</td>
<td>0</td>
<td>N-(p-Tosyl)-Gly-Pro-Lys 4-NA</td>
</tr>
<tr>
<td>Human Thrombin (1 nM)</td>
<td>400</td>
<td>0</td>
<td>N-(p-Tosyl)-Gly-Pro-Arg p-NA</td>
</tr>
<tr>
<td>Matriptase (0.5 nM)</td>
<td>400</td>
<td>0</td>
<td>H-D-Ile-Pro-Arg p-NA</td>
</tr>
<tr>
<td>Prostasin/Prss8 (3.5 nM)</td>
<td>1750</td>
<td>3.3</td>
<td>Tos-Gly-Pro-Arg-7-amino-4-methylcoumarin</td>
</tr>
</tbody>
</table>

### Table 2  KLK Inhibition by Spink6

Kinetic parameters of KLKs interaction with recombinant Spink6. Data include results of at least 3 replicates for each enzyme ± SEM. NI indicates no inhibition.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>$K_m$ [mM]</th>
<th>$K_i$ [nM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>KLK5</td>
<td>T-Gly-Pro-Arg-pNa</td>
<td>0.6 ± 0.06</td>
<td>1.33 ± 0.04</td>
</tr>
<tr>
<td>KLK7</td>
<td>MeO-Suc-Arg-Pro-Tyr-pNA</td>
<td>2.0 ± 0.4</td>
<td>1070 ± 80</td>
</tr>
<tr>
<td>KLK8</td>
<td>T-Gly-Pro-Arg-pNa</td>
<td>5.8 ± 0.8</td>
<td>NI</td>
</tr>
<tr>
<td>KLK14</td>
<td>T-Gly-Pro-Arg-pNa</td>
<td>1.675 ± 0.27</td>
<td>0.5 ± 0.03</td>
</tr>
</tbody>
</table>

### Table 3  Effects of Spink6 on cell shedding from human plantar callus

Single 3-mm callus cylinders, thickness about 0.5 mm, were incubated for 18 h at 37°C in 0.1 M Tris-HCl, 5 mM EDTA, pH 8.0 with protease inhibitors as indicated.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration (µM)</th>
<th>Inhibition ± SE</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td></td>
<td>0 ± 12.9</td>
<td>5</td>
</tr>
<tr>
<td>Spink6</td>
<td>10</td>
<td>98.7 ± 0.5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>90.7 ± 2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>78.1 ± 8.8</td>
<td>2</td>
</tr>
<tr>
<td>aprotinin</td>
<td>10</td>
<td>96.4 ± 2.2</td>
<td>3</td>
</tr>
<tr>
<td>α-Antitrypsin</td>
<td>10</td>
<td>95 ± 3.7</td>
<td>3</td>
</tr>
</tbody>
</table>
Figure 1

A. Human chromosome 5q32

B. exon 1 (315/379 bp) exon 2 (23 bp) exon 3 (116 bp) exon 4 (429 bp)

C. M K L S G M F L L S L A

Exons:
- Exon 1: 1-601
- Exon 2: 602-615
- Exon 3: 616-675
- Exon 4: 676-725

Intron lengths:
- Intron 1: 2881 bp
- Intron 2: 7851 bp
- Intron 3: 879 bp

Spink5 Spink6 Spink7 Spink9 FBXO38

Human chromosome 5q32
Figure 3

(A) Chromatogram showing the retention times of two peaks at 6062.57 Da and 6045.03 Da. The graph plots the absorbance at 215 nm against retention time in minutes.

(B) Western blot analysis showing bands at approximately 26 kDa, 17 kDa, and 10 kDa.
Figure 7

KLK5

KLK7

KLK8

KLK14

Spink6 [M]