Multiple human tissue kallikreins are regulated by lympho-epithelial Kazal-type inhibitor and digest desmoglein 1

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Background

- Stratum corneum (SC) desquamation requires proteolysis of desmosomes by trypsin-like and chymotrypsin-like serine proteases (SP)[1]
- Serine protease inhibitors (SPI) regulate SC desquamation as evidenced by the SP/SPI imbalance observed in Netherton syndrome (NS)[2,3,4]
  - NS is caused by nonsense mutations in *serine protease inhibitor Kazal-type 5* [*SPINK5*; encodes lympho-epithelial Kazal-type inhibitor (LEKTI)]
  - The decrease in LEKTI expression/activity leads to unopposed SP activity resulting in over-desquamation
- Among SC proteases implicated in desquamation are human tissue kallikreins (hKs), 15 secreted SP with trypsin-like and/or chymotrypsin-like specificity[5]
- Most kallikreins (i.e. kallikreins 1, 4-8, 10, 13, 14) are expressed in the stratum granulosum (SG) at the mRNA and/or protein level[6,7] within lamellar granules (e.g. hK5, hK7, hK8)[8,9] and secreted to the intercellular spaces of the SC
- To date, hK5 and hK7 have been shown to digest desmosomal proteins[10]:
  - corneodesmosin (CDSN)
  - desmocollin 1 (DSC1)
  - desmoglein 1 (DSG1)
- hK5 and hK7 are inhibited by SPI that co-localize to the SG and SC[11,12,13]:
  - Lympho-epithelial Kazal-type inhibitor (LEKTI)
  - Secretory leukocyte protease inhibitor (SLPI)
  - Elafin / protease inhibitor 3
  - α_2-macroglobulin like-1 (A2ML1)

Aim

To examine the potential contribution of multiple hKs (hK1, hK5, hK6, hK13 and hK14) present in the SC to desquamation by assessing their: 1) regulation by SC serine protease inhibitors and 2) digestion of desmoglein 1

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Materials & Methods

1. *Materials.* 7-Amino-4-methylcoumarin (AMC) peptide substrates Boc-Val-Pro-Arg-AMC, H-Pro-Phe-Arg-AMC, Boc-Gln-Ala-Arg-AMC, were purchased from Bachem Bioscience and MeOSuc-Ala-Ala-Pro-Val-AMC was obtained from Calbiochem. Recombinant mature hK1 and hK5 were expressed and purified from a baculovirus/insect cell line system as previously described[23,14]. Recombinant pro-hK5, pro-hK13 and mature hK14 were produced in the *P.pastoris* expression system as described in detail elsewhere [15-17]. Recombinant neutrophil elastase and elafin were purchased from Sigma-Aldrich. Recombinant LEKTI fragments containing intact domains 1-6 (rLEKTI1-6), 6-8 and partial domain 9 (rLEKTI6-9'), 9-12 (rLEKTI9-12) and 12-15 (rLEKTI12-15) were produced in a baculovirus/insect cell line system as previously reported[14,18]. Recombinant SLPI and a recombinant DSG1/Fc chimera were obtained from R&D Systems Inc. Anti-LEKTI monoclonal antibody 1C11G6 was produced as previously described[20].
2. *Kinetic inhibition assays.* Individual hKs (12nM of hK1, hK2, hK5, hK13 and hK14 or 30nM of hK6) were pre-incubated with varying concentrations of LEKTI fragments (0-60nM), SLPI (0-1.2 μM) and elafin (0-1.2 μM) in 10μl of optimal buffer for different time points. The mixtures were subsequently added to 90 μl of optimal buffer containing several fixed AMC peptide concentrations (4-3000μM) within microtitre plate wells. Initial rates of hK-mediated peptide hydrolysis was monitored by measuring free AMC fluorescence on the Wallac 1420 Victor^2 fluorometer with excitation and emission filters of 380nm and 480nm, respectively. Activities were expressed relative to control incubations from which inhibitors were excluded. Michaelis-Menten parameters (Km, Vmax), the equilibrium inhibition constant (Ki) and inhibitory mechanisms were determined by linear and non-linear regression analysis using the Enzyme Kinetics Module 1.1.
3. *In vitro digestion experiments.* Individual hKs were incubated with SLPI, rLEKTI fragments and DSG1/Fc at 37°C. Reactions were monitored over time by reducing SDS-PAGE followed by Silver-staining (for SLPI and DSG1/Fc) or transferred to nitrocellulose membranes and probed with anti-LEKTI mAb 1C11G6 (for rLEKTI fragments).
4. *N-terminal sequencing.* N-terminal sequence analysis was performed to identify hK cleavage sites within SLPI and DSG1/Fc. hKs (250ng) were incubated with LEKTI, SLPI or DSG1 (2.5 μg), separated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane and stained with Coomassie blue R-250. SLPI, rLEKTI and DSG1 fragments were excised and subjected to automated N-terminal Edman degradation consisting of 5 cycles of Edman chemistry on a Porton/Beckman Gas-phase Microsequencer, followed by phenylthiohydantoin (PTH) analysis on an HPLC column.

Results

I. Secretory leukocyte protease inhibitor (SLPI) and Elafin do not inhibit hKs

Table 1. Inhibitory profile of SLPI

Protease (nM)	Molar ratio (Protease:SLPI)	SLPI (nM)	Residual Activity (%)	Inhibition (%)
Elastase (18)	1:5	90	0.2	99.8
hK1 (12)	1:100	1200	100.6	-0.6
hK5 (12)	1:100	1200	103.0	-3.0
hK6 (12)	1:100	1200	94.0	6.0
hK13 (12)	1:100	1200	120.0	-20.0
hK14 (12)	1:100	1200	91.9	8.1

Table 2. Inhibitory profile of elafin

Protease (nM)	Molar ratio (Protease:Elafin)	Elafin (nM)	Residual Activity (%)	Inhibition (%)
Elastase (12)	1:2	24	0.2	99.8
hK1 (12)	1:100	1200	103.2	-3.2
hK5 (12)	1:100	1200	102.4	-2.4
hK6 (12)	1:100	1200	100.3	-0.3
hK13 (12)	1:100	1200	101.4	-1.4
hK14 (12)	1:100	1200	99.6	0.4

II. Digestion of SLPI by hKs

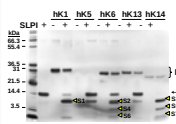

Figure 1. SDS-PAGE analysis of SLPI (500ng) after incubation with hK1, hK5, hK6, hK13 and hK14 (50ng) for 24 hours at 37°C. SLPI and individual hKs were alone incubated alone (*"SLPI -" lanes*). Reactions were terminated and separated by SDS-PAGE followed by Silver staining. *Yellow arrowheads* (◂) indicate SLPI fragments. The position of molecular weight standards (kDa) is indicated on the *left*.

Table 3. hK cleavage sites within SLPI

hK	hK-generated SLPI fragment	N-terminal sequence	hK cleavage site	Location of cleavage site within SLPI[1]
hK1, hK6, hK14	S1/2/3/4/5	Y^{21}KKPE	R$^{20}\downarrow$Y^{21}	1st WAP[2] domain
hK6, hK14	S6/7	M^{73}LNPPN	L$^{72}\downarrow$M^{73}	2nd WAP domain

1. Numbering starts from mature SLPI sequence (Genbank accession no. NP_003055)
2. WAP: whey acidic protein domain, four-disulfide core domain

III. Digestion of rLEKTI by hKs

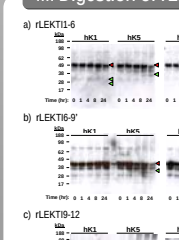

Figure 2. Immunodetection of a) rLEKTI1-6, b) rLEKTI6-9' and c) rLEKTI9-12 after incubation with individual hKs. rLEKTI fragments (10ng) were incubated with hKs (1ng) in hK reaction buffer for various time points (0, 1, 4, 8, 24 hours) at 37°C. Reactions were terminated and separated by SDS-PAGE followed by Western blotting using α-LEKTI mAb 1C11G6. rLEKTI fragments were incubated alone for 24 hours in each hK reaction buffer ("-hK" lanes). *Red arrowheads* (◂) indicate intact rLEKTI fragments; *green arrowheads* (◂) denote degraded rLEKTI fragments. The position of molecular weight standards (kDa) is indicated on the *left*.

IV. rLEKTI inhibition of hKs

Table 4. Inhibitory profile of rLEKTI1-6

hK	Mechanism	rLEKTI1-6 (nM)	Vmax (nmol L^{-1} min^{-1})	Km (mM)	Ki (nM)	R^2
hK1	No inhibition	-	-	-	-	-
hK5	Mixed	0	2884 ± 35	0.46 ± 1.91E-02	2.35 ± 0.22	0.99
		12	1140 ± 18	0.93 ± 4.00E-02		
		24	1028 ± 44	1.88 ± 0.16		
		60	350 ± 15	3.72 ± 0.26		
hK6	Non-competitive	0	912 ± 9	0.23 ± 1.21E-02	21.59 ± 1.24	0.97
		12	653 ± 11	0.29 ± 1.94E-02		
		24	580 ± 9	0.33 ± 2.03E-02		
		60	266 ± 6	0.43 ± 2.97E-02		
hK13	Mixed	0	777 ± 12	0.48 ± 2.54E-02	24.13 ± 3.82	0.98
		12	712 ± 10	0.57 ± 2.50E-02		
		24	534 ± 7	0.64 ± 2.46E-02		
		60	506 ± 8	0.82 ± 3.50E-02		

Table 5. Inhibitory profile of rLEKTI6-9'

hK	Mechanism	rLEKTI6-9' (nM)	Vmax (nmol L^{-1} min^{-1})	Km (mM)	Ki (nM)	R^2
hK1	No inhibition	-	-	-	-	-
hK5	Mixed	0	2829 ± 34	0.44 ± 2.06E-02	4.68 ± 0.66	0.98
		12	1640 ± 20	0.67 ± 2.68E-02		
		24	1079 ± 24	1.41 ± 7.21E-02		
		60	451 ± 21	2.96 ± 2.37E-02		
hK6	Non-competitive	0	1186 ±15	0.44 ± 2.13E-02	47.58 ± 1.40	0.99
		12	958 ±14	0.36 ± 2.06E-02		
		24	709 ± 13	0.32 ± 2.47E-02		
		60	501 ± 4	0.32 ±1.07E-02		
hK13	Non-competitive	0	1069 ± 24	0.51 ± 3.83E-02	222.12 ± 9.51	0.98
		12	905 ± 20	0.42 ± 3.44E-02		
		24	788 ± 8	0.40 ± 1.45E-02		
		60	757 ± 12	0.51 ± 2.68E-02		
hK14	Non-competitive	0	4186 ± 123	0.25 ± 2.07E-02	6.26 ± 0.37	0.98
		12	1578 ± 24	0.18 ± 1.13E-02		
		24	739 ± 17	0.32 ± 2.74E-02		
		60	171 ± 3	0.33 ± 1.78E-02		

Table 6. Inhibitory profile of rLEKTI9-12

hK	Mechanism	rLEKTI9-12 (nM)	Vmax (nmol L^{-1} min^{-1})	Km (mM)	Ki (nM)	R^2
hK1	No inhibition	-	-	-	-	-
hK5	Mixed	0	2795 ± 33	0.44 ± 1.87E-02	2.75 ± 0.24	0.99
		12	1144 ± 26	0.75 ± 4.85E-02		
		24	823 ± 24	1.66 ± 1.10E-02		
		60	707 ± 62	5.09 ± 6.44E-02		
hK6	Non-competitive	0	937 ± 16	0.14 ± 7.68E-03	195.32 ± 11.66	0.99
		12	893 ± 17	0.28 ± 1.96E-02		
		24	698 ± 11	0.24 ± 1.76E-02		
		60	593 ± 8	0.25 ± 1.77E-02		
hK13	Non-competitive	0	1064 ± 19	0.50 ± 2.98E-02	408.63 ± 16.47	0.99
		60	868 ± 11	0.40 ± 2.06E-02		
		120	832 ± 17	0.52 ± 3.41E-02		
hK14	Mixed	0	3480 ± 34	0.18 ± 6.07E-03	10.26 ± 1.25	0.98
		12	2608 ± 61	0.23 ± 1.57E-02		
		24	1901 ± 20	0.38 ± 1.08E-02		
		60	1326 ± 29	0.68 ± 4.05E-02		

Table 7. Inhibitory profile of rLEKTI12-15

hK	Mechanism	rLEKTI12-15 (nM)	Vmax (nmol L^{-1} min^{-1})	Km (mM)	Ki (nM)	R^2
hK1	No inhibition	-	-	-	-	-
hK5	Mixed	0	6279 ±113	0.27 ± 0.015	21.80 ± 2.40	0.99
		12	5991 ± 77	0.32 ± 0.012		
		24	5598 ± 92	0.47 ± 0.024		
		60	4796 ± 66	0.65 ± 0.026		
hK6	No inhibition	-	-	-	-	-
hK13	No inhibition	-	-	-	-	-
hK14	No inhibition	-	-	-	-	-

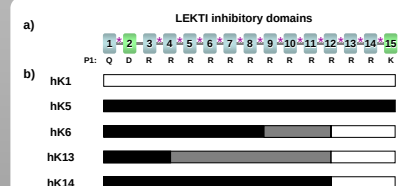

Figure 3. Summary of rLEKTI inhibition of hKs. a) Organization of LEKTI inhibitory domains (Genbank accession no. NP_006837). *Blue boxes* denote Kazal-type inhibitory domains; *green boxes* represent non-Kazal-type domains. The * symbol indicates the location of putative pro-protein convertase motifs (R-Xn-KR↓). The identity of the amino acid at the P1 position is indicated below each domain. b) *Boxes* indicate strength of LEKTI inhibition of individual hKs based on Ki values. *White* boxes indicate no inhibition; *gray* boxes indicate moderate inhibition (Ki > 200nM); *black* boxes represent strong inhibition (Ki < 50nM).

For further information

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V. Digestion of DSG1 by hKs

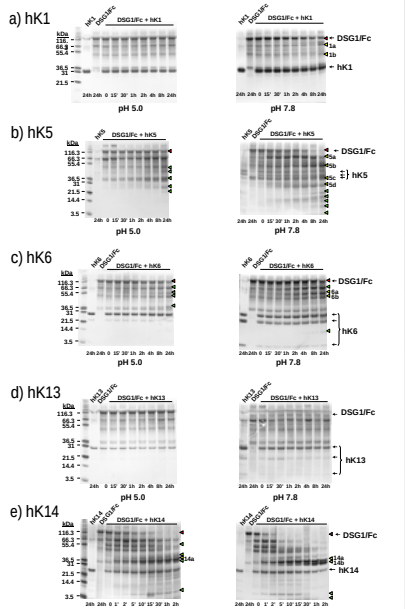

Figure 4. SDS-PAGE analysis of DSG1/Fc after incubation with a) hK1, b) hK5, c) hK6, d) hK13 and e) hK14 at pH 5.0 and 7.8. DSG1/Fc (500ng) was incubated with hKs (50ng) in hK reaction buffer for various time points at 37°C. Reactions were terminated and separated by SDS-PAGE followed by Silver-staining. DSG1/Fc and individual hKs were incubated alone for 24 hours in each hK reaction buffer (lanes 1 and 2). *Red arrowheads* (◂) indicate intact DSG1/Fc; *green arrowheads* (◂) denote DSG1/Fc fragments; *yellow arrowheads* (◂) indicate DSG1/Fc fragments sent for N-terminal sequencing. The position of molecular weight standards (kDa) is indicated on the *left*. Note: **DSG1/Fc** is a chimeric protein formed of the 4 extracellular cadherin followed by a peptide linker and 2 immunoglobulin constant region domains.

Table 8. hK cleavage sites within DSG1

hK	hK-generated DSG1/Fc fragment	N-terminal sequence	hK cleavage site[1]	Location of cleavage site within DSG1
hK1	1a	I^{198}IRQEP	K$^{197}\downarrow$I^{198}	2nd CAD[2] domain
	1b	N^{446}KVTKE	K$^{445}\downarrow$N^{446}	4th CAD domain
hK5	5a/d	D^{243}GGADG	R$^{242}\downarrow$D^{243}	2nd CAD domain
	5b	Y^{423}VMGNN	R$^{422}\downarrow$Y^{423}	4th CAD domain
	5c	V^{147}LDIND	R$^{146}\downarrow$V^{147}	1st CAD domain
hK6	6a	A^{369}SKI	K$^{368}\downarrow$A^{369}	3rd CAD domain
	6b	Y^{423}VMGNN	R$^{422}\downarrow$D^{423}	4th CAD domain
hK14	14a/b	S^{529}SEPGN	Y$^{528}\downarrow$S^{529}	Between 4th CAD domain & TM[3] region

1. Numbering starts from desmoglein 1 preproprotein sequence (Genbank accession no. NP_001933)
2. CAD: cadherin domain, DSG1 contains 4 extracellular CAD domains
3. TM: transmembrane region

Summary

1. Multiple hKs represent specific targets of LEKTI, but not of SLPI or elafin
   - rLEKTI1-6, 6-9' and 9-12 strongly inhibit hK5 and hK14 and to a slightly lesser extent hK6 and hK13, but do not affect hK1 activity
   - rLEKTI12-15 only inhibits hK5 and not other hKs studied
2. rLEKTI6-9' is degraded by hK6 and rLEKTI9-12 by hK6 and hK14, which may lead to inactivation
3. hK6 and hK14 may inactivate SLPI via cleavage at L^{72}
4. Desmoglein 1 is a potential *in vivo* substrate of hK5, hK6 and hK14, but not of hK1 or hK13

Conclusion

In addition to hK5 and hK7, other hKs including **hK6**, **hK13** and **hK14** are implicated in SC desquamation due to their specific inhibition by LEKTI and/or digestion of desmoglein 1