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Short Communication

**Kallikrein-related peptidase 14 is the second
KLK protease targeted by the serpin vaspin**

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Abstract

Kallikrein-related peptidases KLK5, KLK7 and KLK14 are important proteases in skin desquamation and aberrant KLK activity is associated with inflammatory skin diseases such as Netherton syndrome but also with various serious forms of cancer. Previously, we have identified KLK7 as the first protease target of vaspin (Serpine A12). Here, we report KLK14 as a second KLK protease to be inhibited by vaspin. In conclusion, vaspin represents a multi-specific serpin targeting the kallikrein proteases KLK7 and KLK14, with distinct exosites regulating recognition of these target proteases and opposing effects of heparin binding on the inhibition reaction.

Keywords: adipokine, exosite, kallikrein, obesity, diabetes, metabolic syndrome, inflammation, serine protease, serpin A12, site - directed mutagenesis, structural biology

Visceral adipose tissue-derived serpin (vaspin) is a member of the serine protease inhibitor family (Serpine A12) and exhibits various beneficial effects on obesity-related diseases (Heiker, 2014). Inhibitory serpins form covalent complexes with serine proteases under incorporation of the reactive center loop (RCL) into their central β -sheet A (Stratikos and Gettins, 1999). Vaspin does not inhibit common serine proteases such as trypsin, elastase, urokinase, factor Xa, collagenase and dipeptidyl peptidase (Hida *et al.*, 2005). Kallikrein 7 (KLK7), a protease with chymotrypsin-like specificity (Egelrud and Lundstrom, 1991), was identified as the first vaspin target protease (Heiker *et al.*, 2013). Vaspin did not inhibit the closely related kallikrein proteases KLK4 and KLK5 (Heiker *et al.*, 2013), which are both proteases with trypsin-like specificity (Debela *et al.*, 2006). KLK7 is the most abundantly expressed KLK in the stratum corneum (Komatsu *et al.*, 2006) and, with contributions of KLK5 and KLK14, the major enzyme involved in corneocyte shedding (de Veer *et al.*, 2017). Aberrant activities of these KLKs are related to inflammatory skin diseases such as Netherton syndrome (Kasperek *et al.*, 2017), psoriasis (Ekholm and Egelrud, 1999) and acne rosacea (Yamasaki *et al.*, 2007). KLK7 and vaspin have been shown to be co-expressed in human skin (Schultz *et al.*, 2013) as well as in pancreatic islets (Heiker *et al.*, 2013). KLK14 is a member of the kallikrein family that displays both trypsin- and chymotrypsin-like activity (Felber *et al.*, 2005) and was tested here as a potential target protease of vaspin.

Figure 1 demonstrates the inhibitory activity of different vaspin variants against the known protease target KLK7 and the newly identified target KLK14. As previously shown, vaspin wildtype (wt) forms stable serpin-protease complexes with KLK7 (Figure 1A) with an apparent molecular weight of ~ 70 kDa. In comparison, complex formation of vaspin with KLK14 was also observed (Figure 1B), though complex formation is much slower. Additional bands at a molecular weight of ~ 44 kDa and ~ 42 kDa represent fragments derived by proteolytic cleavage of the N-terminus and of the reactive center loop (RCL) of vaspin, respectively. For KLK7, N-terminal processing after Y³⁰ of vaspin results in release of a short decapeptide vaspin(L²⁰-Y³⁰) (Ulbricht *et al.*, 2017). Also with KLK14 we observed specific N-terminal cleavage of vaspin, but after R²⁸ (Supplementary Figure 1, Supplementary Table 1), which is in agreement with the strong trypsin-like arginine preference of KLK14 at the P1 position (Felber *et al.*, 2005). It is unknown, whether these peptides inherit any biological function, as e.g. described for a peptide derived from the N-terminal sequence of PEDF (Serpine F1) (Koskimaki *et al.*, 2012). The N-terminally truncated vaspin retains full inhibitory activity (Ulbricht *et al.*, 2017). Incubation of vaspin with KLK14 yielded an

additional protein band with an apparent molecular weight of ~35 kDa (Figure 1B, 180 min). Trypsin in-gel digestion of the protein band and subsequent mass spectrometry (MS) identified multiple vaspin-derived peptides covering the middle of the molecule (residues 130 – 280; data not shown). By using a substrate variant of vaspin bearing the A369P mutation, we could demonstrate that this vaspin fragment results from KLK14 cleavage of native or cleaved vaspin but not from specific degradation of the serpin-protease complex (Figure 1C). As previously shown, the A369P mutation retards RCL integration into the central β -sheet A and enables the protease to escape the serpin-mechanism by facilitating RCL cleavage (Pippel *et al.*, 2016).

Chymotrypsin-like KLK7 cleavage in the vaspin RCL has been shown to occur after M³⁷⁸ (Heiker *et al.*, 2013), which is in line with reported subsite preferences of KLK7 (Debela *et al.*, 2006). MS analysis of KLK14-cleaved vaspin revealed the same scissile bond within the RCL sequence as targeted by KLK7 (Supplementary Figure 1, Supplementary Table 1), which is in agreement with previously reported cleavage of the antitrypsin RCL for KLK14 (Felber *et al.*, 2006).

Thus, both proteases attack the same peptide bond between P1 residue M³⁷⁸ and P1' residue E³⁷⁹. For KLK7, we have demonstrated the repressing effect of E³⁷⁹ on vaspin activity, as the P1' site mutation of glutamate to serine (E379S) resulted in substantial acceleration of KLK7 inhibition (Ulbricht *et al.*, 2015). A scissile bond of P1 Met – P1' Ser resembles the cleavage site in antitrypsin (Serpine A1), a very good inhibitor of KLK7 and KLK14 (Felber *et al.*, 2006; Luo and Jiang, 2006). Mutation of the P1 site to a more preferred Tyr (M378Y) only resulted in minor improvement of KLK7 inhibition (Pippel *et al.*, 2016), demonstrating the P1' site as the decisive regulatory element of inhibition rate within the RCL.

KLK7 is unable to cleave the synthetic RCL-derived peptide comprising vaspin residues T³⁶⁵-K³⁸⁸ and thus exosite interactions are essential for the efficient inhibition of KLK7 by vaspin (Ulbricht *et al.*, 2015). To investigate whether exosites are equally important for vaspin activity towards KLK14, the same vaspin RCL-derived peptide was incubated with KLK14. As also observed for KLK7, this peptide was not cleaved by KLK14 (Supplementary Figure 2) indicating that the P1' residue E³⁷⁹ is also a negative regulatory element for KLK14. This was supported by complex formation analysis using the E379S vaspin mutant (Figure 1D). Exchange of P1' Glu did clearly accelerate the inhibition reaction, though not in the same extent as it does affect the KLK7 inhibition reaction (Figure 1D). Together, these data suggest

the P1' glutamate as the primary determinant of specificity for vaspin. Furthermore, these results indicate the requirement of exosite interactions for efficient inhibition of KLK14 by vaspin.

Vaspin residue R³⁰², located in the β -sheet C and close to the RCL cleavage site, was identified as the crucial exosite for KLK7 recognition and inhibition (Ulbricht *et al.*, 2015). Complex formation is much slower and the inhibition rate is significantly decreased for vaspin mutants lacking R³⁰² (Figure 1A and Table 1).

For the inhibition of KLK14, striking differences for the vaspin wt and variants were observed. First and as mentioned before, inhibition of KLK14 by vaspin wt is a significantly slower than inhibition of KLK7, with inhibition rates of $1.0 \text{ mM}^{-1} \text{ s}^{-1}$ compared to $12.3 \text{ mM}^{-1} \text{ s}^{-1}$ (Table 1). Furthermore, while mutation of the exosite residue R³⁰² (R302A) dramatically decreased the inhibition rate for KLK7 (down to $0.3 \text{ mM}^{-1} \text{ s}^{-1}$, Table 1), kinetic analysis revealed a small increase in KLK14 inhibition rate for R302A compared to the wt ($1.5 \text{ mM}^{-1} \text{ s}^{-1}$). These data imply that R³⁰² is not an essential exosite for KLK14 recognition. Instead, the arginine side chain in the wt seems to slightly hinder serpin protease interaction, affecting both the inhibitory and substrate pathway (Figure 1B).

Previously, an artificial disulfide mutant of vaspin (D305C/V383C) showed a 5-fold increase in inhibitory activity towards KLK7 (Ulbricht *et al.*, 2015). We generated the vaspin mutant D305A to investigate this aspartate residue as a potential second exosite regulating or fine-tuning protease specificity. Indeed, the D305A mutant exhibited a significantly accelerated inhibition rate for KLK7 ($89.7 \text{ mM}^{-1} \text{ s}^{-1}$, Table 1), suggesting a repulsive effect of the negatively charged side chain on KLK7. The stoichiometry of inhibition (SI) for KLK7 and vaspin D305A was also decreased, indicating a more efficient inhibition reaction compared to the wt (1.8 vs 3.6, Table 1). In contrast, the mutation of D³⁰⁵ to alanine did not affect KLK14 inhibition and kinetic parameters were similar to the vaspin wt (Figure 1B, Table 1). Densitometric estimation of the SI from SDS-gels also gave comparable values for vaspin wt and variants (Table 1). Notably, serpin-protease complexes of both mutants, R302A and D305A, were stable for at least 24 h, but KLK14 complexes with vaspin wt were only stable up to 7 h (data not shown) and were not detected after prolonged incubation (Figure 1E). Serpin-enzyme complexes are cleared from the circulation rather rapidly (Pizzo *et al.*, 1988; Mast *et al.*, 1991), and the stability time of vaspin-KLK14 complexes should therefore allow potential *in vivo* relevance. Together, these results demonstrate that vaspin is a multi-specific

serpin which utilizes individual exosites for the inhibition of its target proteases despite its unfavored RCL residue on the prime site of the scissile bond. Yet, specific exosite(s) important for KLK14 recognition and inhibition remain unknown.

Alike other common serpins such as antithrombin (Serpine C1), heparin-cofactor II (Serpine D1) or protein C inhibitor (Serpine A5), vaspin is a heparin-binding serpin and heparin accelerates KLK7 inhibition by vaspin via the template-bridging mechanism (Ulbricht *et al.*, 2017). To evaluate the effect of heparin on KLK14 inhibition, vaspin and KLK14 were incubated in the presence of increasing concentrations of heparin. In contrast to the inhibition of KLK7, the complex formation of KLK14 and vaspin wt was inhibited by heparin for all concentrations investigated (Figure 2A), yet RCL cleavage still occurred. KLK14-cleaved vaspin in the presence of heparin migrated slightly lower than without heparin which may indicate additional and specific processing in the presence of heparin.

Altered migration of vaspin molecules (full length or cleaved) in the presence of heparin was not observed before (Ulbricht *et al.*, 2017). We used mass spectrometry to analyze the appearance of additional vaspin peptides after cleavage by KLK14 in the presence of increasing heparin concentrations. While acquisition of peptide and protein spectra was not feasible for higher concentrations of heparin ($>1 \times \text{mol/mol}$ to vaspin), we did detect additional peptide fragments after incubation of vaspin with KLK14 in the presence of equimolar heparin (data not shown). These peptides were in the range of 5 kDa which is in line with the band shift observed in the SDS-PAGE gels. Yet, we could not detect the corresponding full protein peaks, and due to the size we could not sequence the peptides by MS/MS. We conclude that in the presence of heparin there is additional vaspin cleavage by KLK14, but the exact cleavage site remains unclear.

Thus, for vaspin-KLK14 complex formation, heparin binding may block or interfere with crucial, yet unknown exosite interactions. Similar findings have been reported for the reaction of kallistatin (Serpine A4) and tissue kallikrein (KLK1), with heparin preventing protease inhibition by the serpin (Chen *et al.*, 2000). For kallistatin, a positively charged loop functions as both the heparin binding site (Chen *et al.*, 2001) and as exosite contact for KLK1 (Chen *et al.*, 2000). Yet, while the location of these basic residues in kallistatin is corresponding to R³⁰¹ and R³⁰² of vaspin, our data demonstrated that this position, while essential for KLK7 inhibition, is not contributing to heparin binding (Ulbricht *et al.*, 2017), nor is it important for KLK14 inhibition by vaspin. Furthermore, as KLK7 is also inhibited by kallistatin (Luo and

Jiang, 2006), it would be interesting to see how heparin affects the inhibition of KLK7 by kallistatin. The presence of heparin may have similarly opposing effects as observed for vaspin inhibition of KLK7 and KLK14, respectively.

Finally, glycosylation of vaspin has been reported at three different sites (N221, N233 and N267), two of them in close proximity of the RCL (Oertwig *et al.*, 2017). As already demonstrated for KLK7, glycosylation of vaspin also slowed down inhibition of KLK14 compared to unglycosylated vaspin (Figure 2B) but did not prevent complex formation.

Recent evidences indicate anti-inflammatory actions as a major function of vaspin. These protective functions of vaspin in diverse tissues are exerted via both, regulation of protease activity and interaction with cell surface molecules. Overexpression of vaspin in adipose tissue protects mice from obesity-induced adipose tissue inflammation and insulin resistance and vice versa, with the knock-out of the vaspin gene metabolic functions deteriorate under high fat diet (Nakatsuka *et al.*, 2012). Interestingly, inactive vaspin mutant A369P failed to improve glucose tolerance in high fat diet-induced insulin resistant mice (Heiker *et al.*, 2013), suggesting the regulation of protease activity to contribute to these effects. Following the identification of KLK7 as a protease target of vaspin, we have recently shown, that knock out of the *Klk7* gene in adipose tissue reduced obesity-induced local and systemic inflammation and improved insulin sensitivity in mice (Zieger *et al.*, 2017).

Thus, the identification of target proteases, such as KLK7 and also KLK14 will contribute to deeper mechanistic understanding of the protective effects of vaspin in obesity and metabolic diseases. Furthermore, they also may lead to new findings on KLK functions in tissues that have not been in the focus of KLK research yet. For instance, KLK14 is expressed in significant amounts in pancreas and adipose tissue according to the human protein atlas (Uhlén *et al.*, 2015) and it is tempting to speculate on potential functional relevance of KLK14 in obesity and/or diabetes similar to KLK7. Finally, efforts to identify the physiological substrates of KLK7 and KLK14 are key to the detailed understanding of molecular pathways regulated by these proteases. This may lead to new pharmacologic strategies to treat obesity associated diseases.

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Conflict of interest statement

The authors declare that they have no conflicts of interest regarding the contents of this article.

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Tables and figures

Table 1 Kinetic parameters for KLK inhibition by vaspin wt and variants.

Vaspin variant / Protease	KLK7		KLK14	
	k_a	SI	k_a	SI
wt	12.3 ± 0.3^a	3.6 ± 0.3^a	1.0 ± 0.1	3.6 ± 0.3^c
R302A	0.3 ± 0.1^a	2.7 ± 0.6^a	1.5 ± 0.2	3.1^c
D305A	89.7 ± 14.7	1.8 ± 0.4	1.2^b	3.0^c

^aData from Ulbricht *et al.* (2015); k_a ($\text{mM}^{-1} \text{s}^{-1}$); SI (I / E (mol / mol)); I Inhibitor; E Enzyme; data presented as means of at least three experiments $\pm$ S.D.; ^bdetermined once; ^cdensitometric estimation. A continuous method was applied for vaspin variant D305A using 10 nM KLK7, 25 μM fluorogenic peptide (Mca-Arg-Pro-Lys-Pro-Val-Glu~Nva-Trp-Arg-Lys(Dnp)-NH₂ trifluoroacetate salt) and various vaspin concentrations (vaspin: KLK7 ratio 2.5-25:1). SI determination was performed using 19.2 nM KLK7, 10 μM NFF3 and various vaspin concentrations (vaspin: KLK7 ratio 0.25-3:1). For KLK14, a discontinuous method was applied using 4 nM KLK14, 100 μM fluorogenic peptide (Boc-Val-Pro-Arg-AMC) and 100-fold excess of vaspin. Measurements were made after 20 min of incubation time. Residual KLK7 activity was measured on a FlexStation3 Multi-Mode Microplate Reader (Molecular Devices). Second-order rate constants for both proteases were determined as described previously (Ulbricht *et al.*, 2015). SI were estimated using the band intensity of complexed and cleaved vaspin divided by the band intensity of complexed vaspin as described previously (Ulbricht *et al.*, 2015).

Figure 1 Complex formation analyses of vaspin wt and variants with KLK7 and KLK14.

SDS-analysis of inhibition complex formation reveals vaspin as a multi-specific serpin which utilizes individual exosites for the inhibition of its target proteases. Vaspin wt or mutants (R302A (RA) and D305A (DA), respectively) were incubated with (A) KLK7 or (B) KLK14 for indicated times. (C) SDS-PAGE analysis of the vaspin-KLK14 reaction using a vaspin substrate variant. Vaspin substrate variant R211A/K359A/A369P (P10 Pro) was incubated with KLK14 for three and 24 h. (D) Analysis of complex formation of vaspin RCL-mutant E379S (ES, P1' residue) with KLK14 in comparison to the reaction with the wt vaspin and with the inhibition of KLK7 (note the much shorter reaction time for KLK7). (E) Vaspin variants were incubated with KLK14 for 24 h. Human vaspin wt and variants were expressed in and purified from *E.coli* as described previously (Ulbricht *et al.*, 2015). KLK7 and KLK14 (both from R&D) were activated with thermolysin (R&D) according to manufacturer's protocol. 0.5 μ M protease was incubated with vaspin wt or variant (protease: serpin molar ratio of 2:1) in TBS (20 mM Tris, 150 mM NaCl, pH 7.5) for indicated times. Reactions were stopped by adding reducing SDS sample buffer and immediately heated at 95°C for 5 min. SDS-PAGE was performed as previously described (Ulbricht *et al.*, 2015) and gels were

silver stained for protein detection. Indicated bands are: 1 – vaspin-protease complex; 2 – full-length vaspin; 3 – N-terminally cleaved vaspin; 4 – RCL-cleaved vaspin; 5 – vaspin fragment; 6 – protease; 7– thermolysin; con – vaspin only.

Figure 2 Complex formation analyses of vaspin wt with KLK14 and heparin.

SDS-analysis of vaspin: KLK14 complex formation with (A) heparin and (B) in HEK-cells. Human vaspin wt expression, purification and activation was performed as described in Figure 1. (A) 0.5 μ M protease was incubated with vaspin wt (protease: serpin molar ratio 2:1) in TBS (20 mM Tris, 150 mM NaCl, pH 7.5) for 180 minutes and indicated vaspin: heparin ratios (unfractionated heparin from Sigma Aldrich). In contrast to KLK7, complex formation of KLK14 and vaspin wt was inhibited by heparin for all concentrations investigated. (B) 366 nM vaspin wt expressed in HEK-cells (BioLegend) was incubated with KLK14 (serpin: protease ratio of 1:1) for indicated times. Glycosylation of vaspin did not prevent complex formation. Samples were analyzed as described in Figure 1. Indicated bands are: 1 – vaspin-KLK14 complex; 2 – full-length vaspin; 3 – N-terminally cleaved vaspin; 4 – RCL-cleaved vaspin; 5 – vaspin fragment; 6 – KLK14; con – vaspin only.

Supplementary table and figures

Supplementary Table 1 Recombinant vaspin-derived peptides identified by MS analysis (as in Supplementary Figure 1).

m/z expected	m/z observed	Mono	AA	Sequence
2270.022	2269.993	-0.029	1-19	GHHHHHHHHHHSSGHIEGR
3337.556	3337.417	-0.139	1-28	GHHHHHHHHHHSSGHIEGRHMKPSFSPR
4037.407	4037.512	0.105	379-414	ETPLVVKIDKPYLLLIYSEKIPSVLFLGKIVNPIGK

Sequence analysis after MALDI-TOF-MS was performed using Sequence Editor (Bruker Daltonics software).

Supplementary Figure 1 MALDI-TOF MS analysis of vaspin peptides generated by KLK14 cleavage.

MALDI-TOF MS results showing the relative abundance (a.u.) of N-terminal and C-terminal vaspin wt peptides obtained from vaspin protein cleavage by KLK14. Vaspin protein was generated and KLK14 was activated as described in Figure 1. 5.8 μ M vaspin wt was incubated with KLK14 at a molar ratio of 5:1 for 4h. For MS analysis, samples were desalted using C18 ZipTipTM Pipette Tips C18 (10 μ l, Merck, Darmstadt, Germany) according to the manufacturer’s protocol. MS was performed using the matrix SDHB (super 2,5-dihydroxybenzoic acid) on an Ultraflex III mass spectrometer (Bruker Daltonics, Billerica,

USA) in the reflector modus. Measurements were analyzed by FlexAnalysis 3.0 (Bruker Daltonics software).

Supplementary Figure 2 HPLC analysis of RCL-derived peptide (365-388) after KLK14 incubation.

Synthetic RCL-derived vaspin peptide (AA 365-388) was incubated at a molar ratio of 700:1 with KLK7 for 4 h and 24 h at room temperature. The RCL-derived peptide was synthesized as previously described [1]. KLK14 was activated as described in Figure 1. After incubation, samples were analyzed by RP-HPLC (ProZap Expedite MSC18 Alltech column 10 mm x 2,1 mm, 1,5 μ m) using a water/acetonitrile (ACN) gradient with 0.1% TFA from 10-50% in 5 min.

Supplementary Reference

Ulbricht, D., *et al.*, A unique serpin P1' glutamate and a conserved beta-sheet C arginine are key residues for activity, protease recognition and stability of serpinA12 (vaspin). *Biochem J*, 2015. 470(3): p. 357-67.