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Glycosylation of human vaspin (SERPINA12) and its impact on serpin activity, heparin binding and thermal stability

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Abstract

Vaspin is a glycoprotein with three predicted glycosylation sites at asparagine residues located in proximity to the reactive center loop and close to domains that play important roles in conformational changes underlying serpin function. In this study, we have investigated the glycosylation of human vaspin and its effects on biochemical properties relevant to vaspin function. We show that vaspin is modified at all three sites and biochemical data demonstrate that glycosylation does not hinder inhibition of the target protease kallikrein 7. Although binding affinity to heparin is slightly decreased, the protease inhibition reaction is still significantly accelerated in the presence of heparin. Glycosylation did not affect thermal stability.

Keywords. adipokine, glycosylation, kallikrein 7, serine protease, serpin, vaspin

Abbreviations ER - endoplasmatic reticulum, GAG – glycosaminoglycan, KLK7 – kallikrein 7, RCL – reactive center loop, TAMRA – tetramethylrhodamine

Introduction

Obesity significantly increases the risk of type-2 diabetes, hypertension, cardiovascular diseases, cancer, inflammation and non-alcoholic fatty liver disease [1]. Adipokines are adipose tissue-secreted bioactive molecules that play a major role in obesity and the mentioned related co-morbidities [2-4]. Human visceral adipose tissue-derived serpin (vaspin; serpinA12 according to the serpin nomenclature [5]) is a promising anti-diabetic, anti-atherogenic and appetite suppressing protein [6]. It was first identified in visceral adipose tissue of the Otsuka Long-Evans Tokushima Fatty rat model of type-2 diabetes [7]. Vaspin mRNA expression in human adipose tissue and vaspin serum concentrations are associated with obesity and insulin resistance [8-10]. Vaspin treatment of obese and insulin-resistant mice significantly increases glucose tolerance and decreases expression of insulin resistance genes and pro-inflammatory genes in white adipose tissues [11]. Vaspin belongs to the large family of serine protease inhibitors (serpins) and we have identified kallikrein 7 (KLK7) as the first protease inhibited by vaspin [12]. Intraperitoneal and central (intracerebroventricular) vaspin administration reduces food intake and blood glucose in mice [13] and in rats, where central vaspin decreased neuronal neuropeptide Y expression and increased proopiomelanocortin expression [14]. The impact of central vaspin on blood glucose levels is based on the inhibition of hepatic glucose production and enhanced insulin signaling which is conveyed from the brain to the liver via the dorsal vagal complex [15]. Vaspin is also a promising anti-atherogenic factor and studies have reported anti-apoptotic and anti-inflammatory effects on endothelial [16-20], vascular smooth muscle [21-23] and skin cells [24, 25]. While the improvement of glucose tolerance and the reduction of food intake were found to be dependent on protease inhibition [12, 26], some anti-inflammatory effects in the liver and the vasculature have been linked to interaction with a membrane associated ER chaperone protein [19, 27].

The serpin family shares a highly conserved tertiary structure with 8-9 α-helices (A – H/I), three β-sheets (A - C) and the exposed reactive center loop (RCL), which serves as a substrate bait for target proteases on top of the molecule (Figure 1A). The protease inhibition mechanism of serpins demands extensive conformational flexibility [28]. After protease attack, the RCL is inserted into the core β-sheet A and the covalently bound protease is translocated to the bottom of the serpin molecule. On the protease side, this conformational change results in distortion of its active site and covalent inhibition [29]. For the serpin, the complementation of the central β-sheet results in its most stable but inactive conformation. Thus, functional activity requires serpins to initially fold into a metastable (but active) conformation. As a consequence, autonomous serpin inactivation can occur via complementation of the central β-sheet with the

own uncleaved RCL in the absence of a target protease (latency transition) or by swapping domains with a second serpin molecule (polymerization).

Vaspin features a unique but rather unfavorable glutamate Glu379 at the P1' position within its RCL, resulting in moderate inhibition rates for KLK7 [30]. This glutamate represses inhibitory activity of vaspin towards KLK7 if not for a crucial exosite in Arg302, which enables inhibition of KLK7 by vaspin despite the detrimental cleavage site (Figure 1A). We have also reported the exceptional thermostability of vaspin [31] and have identified the vaspin binding site for heparin that provides high affinity binding of this activator and enables heparin induced rate-acceleration of KLK7 inhibition [32]. The heparin binding site is located at the central β-sheet A and differs from other known binding sites in serpins (residues Arg211 and Lys359 in Figure 1A, B).

Many serpins are post-translationally modified by glycosylation [33-35]. These glycan modifications are of great importance as they can regulate secretion or plasma stability, serpin activity or inactivation and the interaction with activators such as glucosaminoglycans. Human vaspin has three potential sites for N-linked glycosylation with the consensus motif Asn-X-Ser/Thr. Two of the potential glycosylation sites reside on the top of the serpin molecule (Figure 1B), in regions playing important roles in the conformational changes underlying serpin function (activity) and dysfunction (polymerization).

In many of the studies on the physiologic functions of vaspin mentioned above, recombinant human vaspin has been used that was derived either from *E.coli* or from HEK (human embryonic kidney) cells. Notably, while murine vaspin lacks the glycosylation sites Asn221 and Asn233, the Asn267 site is conserved. Therefore, we were interested in the glycosylation of human vaspin and potential effects on vaspin function. Thus, the aims of this study were to experimentally identify utilized glycosylation sites and investigate the consequences of glycosylation on inhibition of the known protease target KLK7, on heparin binding and acceleration of protease inhibition as well as thermal stability.

Materials and Methods

Material - Recombinant expression and purification of human KLK7 and human vaspin from *E.coli* were performed as previously described [30]. HEK-derived human vaspin was from BioLegend (San Diego, CA, USA). Unfractionated heparin was from Sigma-Aldrich (St. Louis, MO, USA). Cell culture material was from PAA Laboratories (Pasching, Austria). Polyethylenimine (PEI), 25 kD linear form, was from Polysciences (Warrington, PA, USA). PNGase F and Endo H were from New England Biolabs (Ipswich, MA, USA). Potential N-

glycosylation sites were predicted using NetNGlyc server 1.0 [36]. Figures were prepared using PyMOL (http://www.pymol.org).

Cell culture and plasmid transfection - HEK293T cells were maintained in DMEM/Ham's F-12 (1:1) containing 10 % heat-inactivated FCS at 37 °C in a humidified atmosphere with 5 % CO_2. Human vaspin in the pcDNA3 expression vector (Thermo Fisher Scientific, Waltham, MA, USA) was a kind gift of Dr. Jana Breitfeld (IFB Adiposity Diseases, University of Leipzig, Leipzig, Germany). Vaspin mutants (N221A, N233A, N267A, double and triple mutants) were generated by QuikChange site-directed mutagenesis (Agilent Technologies, Santa Clara, CA, USA). For transfection, 5 µg of plasmid was transfected into near-confluent HEK293T cells in 60-mm plates using 15 µg PEI in 500 µl of serum-free DMEM. After 24 h, cells were washed twice with PBS and incubated with serum-free medium for 12 h. Cell supernatants were concentrated using AMICON-Ultra filters with a 10 kDa molecular weight cut-off (Merck Millipore, Billerica, MA, USA). For deglycosylation, 50 µg total protein were incubated with PNGase F or Endo H according to the manufacturers protocols. For analysis, 25 or 50 µg total protein were separated by SDS-PAGE and detected via Western Blot using anti-vaspin antibody (Adipogen International, San Diego, CA, USA), anti-GAPDH antibody (Santa Cruz Biotechnology, Dallas, TX, USA) or anti-tetramethylrhodamine antibody (Thermo Fisher Scientific) as previously described [12].

Complex formation analysis and determination of inhibition parameters - Recombinant KLK7 and vaspin (mutants) were incubated at a ratio of 3:1 (protease:serpin; KLK7 concentration was 3.5 µM) in TBS buffer (50 mM Tris, 150 mM NaCl, pH 8.5) with or without heparin at a molar ratio of GAG to vaspin of 12.5:1. Proteins were separated by SDS-PAGE using BOLT 4-12 % Bis-Tris Plus precast gels and NuPAGE MES/SDS running buffer (Thermo Fisher Scientific). Bands were visualized with Coomassie Blue or silver stain. Pseudo-first-order rate constants (k_{obs}) were determined as previously described [30].

Heparin affinity chromatography - Heparin affinity chromatography was performed using 1 ml HiTrap Heparin HP columns on an ÄKTA protein purification system (both from GE Healthcare, Little Chalfont, United Kingdom). 25 µg of HEK-derived vaspin (from Biolegend) was loaded onto the column. Elution was performed by a sodium chloride gradient from 150 mM to 2 M (flow rate: 0.75 ml/min) and monitored at 220 nm.

Thermal stability - For temperature-induced polymerization, glycosylated vaspin (from Biolegend) was heated for up to 60 min at 70 °C in TBS buffer. During heating, samples were taken at indicated time points and non-reducing SDS-sample buffer was added. Polymerization was analyzed by SDS-PAGE and Coomassie or silver stain [30]. Thermal unfolding was measured by nano differential scanning fluorimetry (nanoDSF) using the Prometheus NT.48 (NanoTemper Technologies, Munich, Germany). Unfolding was monitored by intrinsic tryptophan and tyrosine fluorescence changes at 330 and 350 nm upon heating from 20-95 °C (rate 1 °C/min) using standard capillaries (NanoTemper). *E.coli* vaspin (35 µM) was measured in TBS buffer, HEK vaspin (1.7 µM) in TBS containing 10 mM $MgCl_2$ and 0.05 % (w/v) Tween-20. The principles of nanoDSF are described elsewhere [37].

MALDI-TOF Mass Spectrometry - Samples of recombinant protein were concentrated and desalted using ZipTip C18-filter tips (Merck Millipore) and analyzed by MALDI-TOF MS on a Bruker Ultraflex III MALDI TOF/TOF mass spectrometer.

In vitro stability assay of vaspin in blood plasma - Recombinant *E.coli*-derived vaspin was labelled via cysteine residues using 20-fold molar excess of tetramethylrhodamine (TAMRA)-5- (and -6) C2 maleimide (AnaSpec, Fremont, CA, USA) in Tris-buffered saline (pH 7.0) for 16 h at 4 °C. Residual free labelling reagent was removed by extensive dialysis. TAMRA-labelled vaspin was stored at 4 °C and protected from exposure to light. Citrate stabilized human blood plasma was purchased from the Blutbank (Universitätsklinikum Leipzig, Leipzig, Germany). 190 µL of pre-warmed human blood plasma was added to 10 µL of 1 mg/mL tetramethylrhodamine (TAMRA)-labeled vaspin (derived from *E.coli*) in PBS and incubated for indicated time points at 37 °C. The reaction was stopped by precipitation of plasma proteins with the addition of 800 µL of 10 % trichloroacetic acid in acetone. As a negative control, 10 µL PBS were used instead of the protein solution. Precipitates were centrifuged at 13,000 g for 1 min, washed thrice with PBS, boiled for 5 min with 50 µl of SDS-sample buffer and subsequently used for Western Blot analysis.

Results and Discussion

Sequence analysis revealed three potential glycosylation sites in human vaspin. Commercial HEK-derived vaspin migrated at an apparent molecular weight of ~60 kDa on an SDS-PAGE gel under denaturing and reducing conditions compared to *E.coli*-derived vaspin at the predicted 47.5 kDa (Figure 2A). Mass spectrometry showed a mass increase of ~7.5 kDa for

the HEK-derived vaspin indicating glycosylation at ~2-3 Asn residues (Figure 2B). Two potential glycosylation sites in vaspin are of the Asn-X-Thr type (Asn221 and Asn267) and Asn233 is of the Asn-X-Ser type. Mutation of these three potential sites for N-linked glycosylation to alanine and expression in HEK cells resulted in mass shifts for every single mutant (Figure 2C). The triple mutant glycan-null variant (N221A/N233A/N267A) and PNGase F-digested HEK-derived vaspin migrated at the molecular weight of the unglycosylated *E.coli*-derived protein (Figure 2C). Single mutants were detected at levels in the cell supernatants that are comparable to wild type (wt) vaspin. Secretion of the glycan-null variant was very low, but analysis of intracellular protein expression revealed comparable protein synthesis (Figure 2D). Misfolding, intracellular degradation or protein aggregation after prolonged intracellular residence in consequence of improper glycosylation has been previously shown to contribute to poor secretion of mutant serpin proteins, such as antitrypsin and neuroserpin [38, 39]. For neuroserpin, mutagenesis studies have investigated the roles of glycosylation on protein secretion by analyzing protein stability with respect to intracellular aggregation/polymerization and also degradation [39]. Glycosylation of two efficiently utilized sites prevents polymerization and thus increases protein stability, while glycosylation of a usually unmodified site leads to increased intracellular protein degradation. While neuroserpin and vaspin do not share conserved glycosylation sites, proper glycosylation of vaspin may enable unrestricted secretion via similar contributions to protein stability. We did not analyze intracellular protein degradation or aggregation in detail and thus both mechanisms may contribute to poor secretion of unglycosylated vaspin. With respect to protein folding, the expression of soluble active vaspin protein in *E.coli* and the high thermal stability of unglycosylated vaspin indicate that glycosylation does not significantly affect vaspin folding. Constitutive secretory proteins progress through the secretory pathway going from the rough ER to the cisternae of the Golgi apparatus via ER-to-Golgi transport vesicles and subsequently via transport vesicles from the trans Golgi network to the plasma membrane. There they are released from the cell via exocytosis. Lack of vaspin glycosylation may also prevent ER to Golgi transport or sorting into transport vesicles for exocytosis later on. Altogether, these results indicate that N-linked glycosylation is required for delivery of vaspin to the cell surface but the mechanisms underlying these effects remains unknown.

To more closely determine the type of N-linked glycosylation, vaspin (as unpurified protein from HEK cell supernatants) was also digested with the endoglycosidase Endo H. As shown in Figure 2E, single mutants N221A and N233A display residual intermediate Endo H resistant bands indicating in part complex glycosylation. Also, N267A seems to be resistant to Endo H

treatment. Together this indicates a complex glycosylation at Asn221 and Asn233 sites. This is also in agreement with the minor shift of the Endo H digested wt protein. Asn267 in turn, seems to be modified by a high-mannose type glycan, as all mutants still comprising the Asn267 glycosylation site, display a clear shift after Endo H treatment, while mutants bearing the Asn221 or Asn233 glycosylation site (single or double mutants) display residual intermediate Endo H resistant bands (Figure 2E, F). Analysis of double mutants showed that only N221A/N233A, comprising just the Asn267 glycosylation site, displayed a shift after Endo H treatment (Figure 2F) and thus confirms the conclusions drawn from the single mutants. We also analyzed commercial murine vaspin (produced in CHO cells) which only exhibits the conserved Asn267 glycosylation site (in addition to a second site at Asn92 that is not present in human vaspin). While PNGase F treatment resulted in full removal of glycan modifications, ~50 % of murine vaspin was clearly fully resistant to Endo H treatment and the other ~50 % exhibited a shift indicating removal of one of the two glycan modifications (Supplementary Figure 1A).

Notably, for the N221A mutant we always detected a single strong band. Also, an additional minor band is present for single mutants exhibiting the Asn221 glycosylation site (N233A and N267A). Also the double mutant N233A/N267A (Figure 2F) shows a minor fraction of a hypoglycosylated vaspin form. These data support Asn221 to represent an inefficiently used sequon. The amino acid following the Asn-X-Ser/Thr motif has been shown to influence core glycosylation efficiency and these effects are much more pronounced for Asn-X-Ser sequons [40]. In human vaspin, Asn221-X-Thr is followed by a Lys224. For Asn-X-Thr glycosylation sites, only a subsequent Pro residue had a strong inhibitory effect (core glycosylation efficiency <10 %), while all other amino acids yielded core glycosylation efficiencies >80 % [40] which is in line with the minor hypoglycosylated bands observed for vaspin mutants comprising Asn221.

For the classical serpin members antitrypsin (SERPINA1) and antithrombin (SERPINC1) it has been shown that these are modified by bi- and tri-antennary complex N-glycans [41-44]. Whether all three vaspin sites are utilized with similar efficiency *in vivo* and if there are tissue or cell-specific differences remains to be investigated in future studies.

Very often, N-linked glycosylations regulate biological functions of proteins by influencing protein stability and flexibility. These effects depend on the number and positions of the glycosylation sites. This is very important for serpins as they are critically dependent on their metastable active conformation and conformational flexibility to execute protease inhibition via the serpin mechanism. The glycosylation site at Asn267 is located in an α-turn linking β-strands

s2B and s3B. An asparagine is a common residue at this position in the serpin family. SERPINA1 (antitrypsin) is also glycosylated at this position [42, 45-47] and various human serpins exhibit a glycosylation there, such as serpins A3 (antichymotrypsin) [46-48], A5 (protein C inhibitor) [46], A10 (protein Z-dependent protease inhibitor) [46], F2 (alpha-2-antiplasmin) [46] and G1 (plasma protease C1 inhibitor) [46, 47]. In contrast, the other two glycan modifications of vaspin are more remarkable as they are located on top of the serpin molecule in regions that play important roles in conformational changes underlying serpin function (and dysfunction) (Figure 1A). Asn221 is located in a very short helical region above the central β-sheet A and Asn233 is located in the gate region within the β-turn linking β-strands s3C and s4C. Carbohydrate moieties in these positions will likely affect RCL mobility across the top of β-sheet C (Figure 1B). We have recently shown that efficient KLK7 inhibition by vaspin is dependent on an exosite interaction located at Arg302 of vaspin [30]. This exosite is located close to the C-terminal part of the RCL on the back side of the vaspin molecule and away from the glycosylation sites at the front of β-sheet C (Figure 1A). In line with these observations, we found that the inhibition reaction with the protease target KLK7 was only slightly affected for unglycosylated and glycosylated vaspin. Complex stability was not altered and complexes were stable for >14 h (Figure 3A), as previously shown for unglycosylated vaspin [12]. SDS-PAGE analysis demonstrated that the occurrence of serpin-protease complex as well as cleaved serpin after incubation of vaspin with KLK7 was comparable for *E.coli*- and HEK-derived vaspin (Figure 3B). For heparin cofactor II and antithrombin it is known that glycosylation influences heparin binding strength [49, 50]. We recently identified basic residues on top of the central β-sheet A to contribute to heparin binding and heparin-mediated acceleration of protease inhibition [32]. With respect to the heparin binding site, steric hindrance and charge effects of the N-glycan chain especially at Asn221 may affect the interaction of vaspin with the glycosaminoglycan chain (Figure 1B). Indeed, HEK-derived vaspin eluted at ~450 mM NaCl from heparin-sepharose in affinity chromatography experiments (Figure 3C) and thus exhibited a shift of ~100 mM in relative heparin affinity compared to unglycosylated vaspin [30]. Also, heparin-induced acceleration of the KLK7 inhibition reaction was slightly affected. Heparin clearly increased complex band formation after 5 min and 10 min (Figure 3B), and the reaction rate (k_{obs} = 3.3 ± 0.2 $mM^{-1} \cdot s^{-1}$) was increased ~2.5-fold by heparin (wt: $k_{obs,UFH}$ = 8.2 ± 0.8 $mM^{-1} \cdot s^{-1}$; Figure 3D). Complex formation with human KLK7 and heparin-mediated acceleration were slightly increased for murine vaspin (with only the conserved Asn267 glycosylation site) compared to glycosylated human vaspin and similar to unglycosylated human vaspin (Supplementary Figure 1B). Taken

together, inhibition of the protease KLK7 is not substantially affected by glycosylation of vaspin and although glycosylation does reduce relative heparin affinity, it clearly does not prevent heparin activation of vaspin.

As mentioned above, Asn221 and Asn233 are located in or close to the gate region. The gate region of serpins is relevant in self-inactivation via the latency transition, where the intact and uncleaved RCL is inserted into β-sheet A. Recently, the simulation of the latency transition of plasminogen activator inhibitor-1 (PAI-1, serpinE1) at atomic resolution has provided insights into the structural intermediates along the way [51]. Thereby, β-strand s1C separates from β-sheet C and this enables the RCL to leap over the gate region and to insert the N-terminal part of the RCL into sheet A. In contrast to PAI-1, antitrypsin is reluctant to undergo the latency transition and is also glycosylated close to the gate region [42]. We have found unglycosylated vaspin to be very reluctant to undergo the latent transition as well [27] and glycosylation at both Asn233 and Asn221 may further affect RCL movement around the gate and into the latent conformation.

Glycosylation of the serpin antitrypsin leads to increased stability with respect to chemical and thermal denaturation [52]. As mentioned above, glycosylation of two sites in neuroserpin prevents polymerization and increases protein stability [39]. Unglycosylated vaspin already exhibits a remarkable thermal stability [30] but we did not observe a further increase in T_m for glycosylated vaspin (Figure 4A). Though, when analyzing heat-induced polymerization by non-heated and non-reducing SDS-PAGE, glycosylated vaspin was found slightly more prone to polymerization. Incubation at 60 °C resulted in minimal polymer formation for unglycosylated and even less for glycosylated vaspin (Figure 4B). However, at 70 °C, glycosylated vaspin showed very prominent oligomer bands and the appearance of polymer bands was reduced when vaspin was deglycosylated (Figure 4B). Prolonged heating resulted in increased protein aggregation and precipitation. Glycosylated murine vaspin showed a polymerization pattern similar to unglycosylated human vaspin (Supplementary Figure 1C).

In other studies, to investigate the beneficial effects on glucose tolerance and reduction of food intake *in vivo*, where anti-protease activity has been shown to be relevant, both glycosylated and unglycosylated vaspin has been administered. HEK-derived vaspin has been used in studies exploring central effects of vaspin on food intake and glucose disposal [13, 15], while *E.coli*-derived protein was used to investigate vaspin effects on glucose tolerance after peripheral administration [11, 12]. With respect to the various anti-inflammatory effects observed for vaspin, where the underlying mechanisms still remain elusive, recombinant vaspin in these studies has been more or less in equal shares derived from *E.coli* or HEK cells. Importantly,

altogether consistent and matching *in vitro* findings have been reported using both *E.coli*- [19, 20, 22, 23] as well as HEK-derived recombinant vaspin [16-18, 21, 25]. Antitrypsin represents an example, as glycosylation of this serpin seems of greater relevance for its anti-inflammatory functions than for anti-protease properties [53] but this remains to be investigated for vaspin in future studies.

Finally, glycosylation often increases *in vivo* plasma stability of serpins, e.g. antitrypsin [54] and antithrombin [55]. As plasma stability of vaspin has not been investigated until now, we analyzed the *in vitro* stability of vaspin in human plasma using TAMRA-labeled unglycosylated *E.coli*-derived human vaspin. Western Blot analysis revealed that fully intact vaspin was still present after 6 days and no fragments of vaspin were detected using both anti-TAMRA and anti-vaspin antibodies (Figure 5A, B). Detailed analysis of protein half-life of both the unglycosylated and fully glycosylated vaspin proteins (preferably *in vivo*) is needed to get a clear picture on the contribution of glycosylation on vaspin plasma stability and whether these are similar to other serpins such as antithrombin. Although we did not investigate plasma stability of glycosylated vaspin here, these first data indicate a high plasma stability for human vaspin and the glycan modifications may be especially relevant to further increase stability vaspin also *in vivo*.

Conclusion

Together, our data demonstrate that glycosylation at all three sites does not attenuate anti-protease or more specifically anti-KLK7 activity. Although affinity for heparin is slightly reduced, heparin binding still accelerated the protease-inhibition reaction. And while previous results using glycosylated and unglycosylated vaspin are mostly consistent, interactions with yet unknown target proteases or other interacting molecules may be specifically dependent, controlled or regulated by glycosylation of human vaspin.

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Conflict of interest

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

Author contributions

KO, DU and JTH conceived the study, designed and conducted experiments and analyzed data. SH, JP and NS expressed and purified the recombinant KLK7. KBS performed stability tests. All authors discussed results, edited and commented on the manuscript. JTH supervised the project and wrote the paper.

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Figures

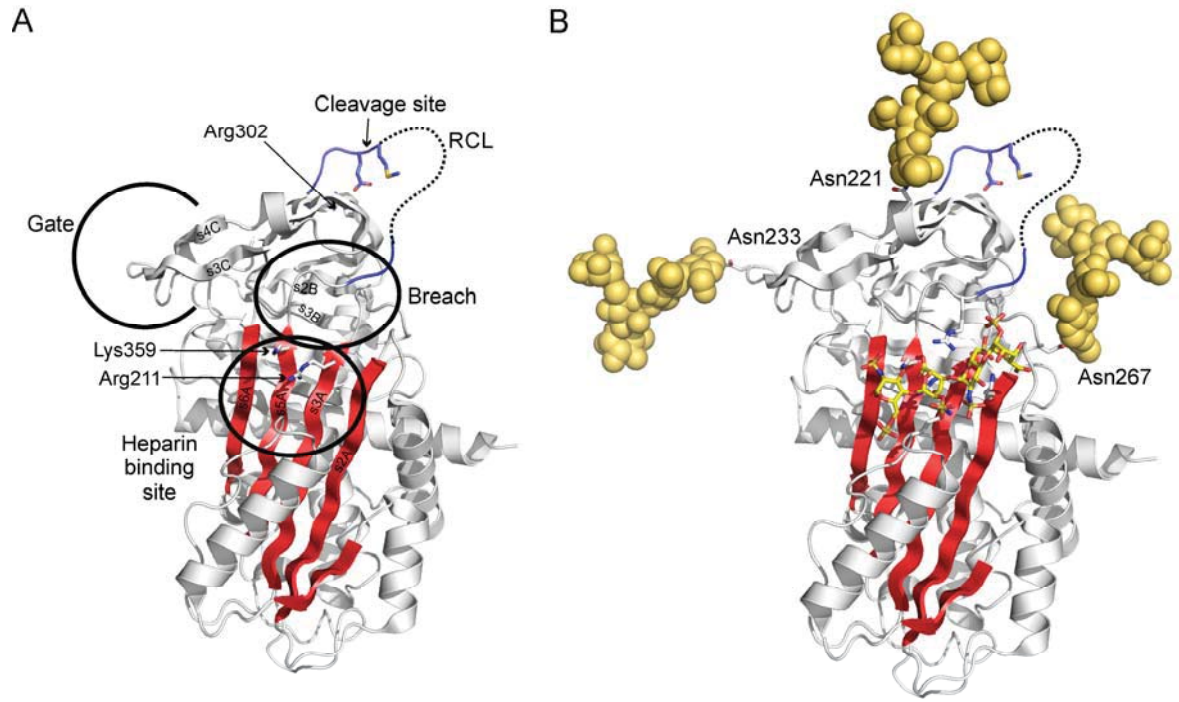

Figure 1. Glycosylation sites in human vaspin. A) Structure of human vaspin with important regions and residues labeled. Arg302 represents the major exosite residue enabling KLK7 inhibition despite the unfavorable cleavage site between Met378 – Glu379 [30]. The heparin binding site comprises Arg211 and Lys359 as essential residues for specific heparin binding and heparin-accelerated KLK7 inhibition [32]. B) Structure of vaspin with modeled basic oligosaccharide modifications and a bound heparin tetra-saccharide. N-glycosylation sites were predicted by NetNGlyc 1.0 server and the structure was produced with GlyProt [56] based on pdb:4IF8. The binding of a heparin tetra-saccharide dp4 was modeled using the ClusPro servers heparin docking method [57] based on pdb:4IF8. The figures were prepared with PyMOL (www.pymol.org).

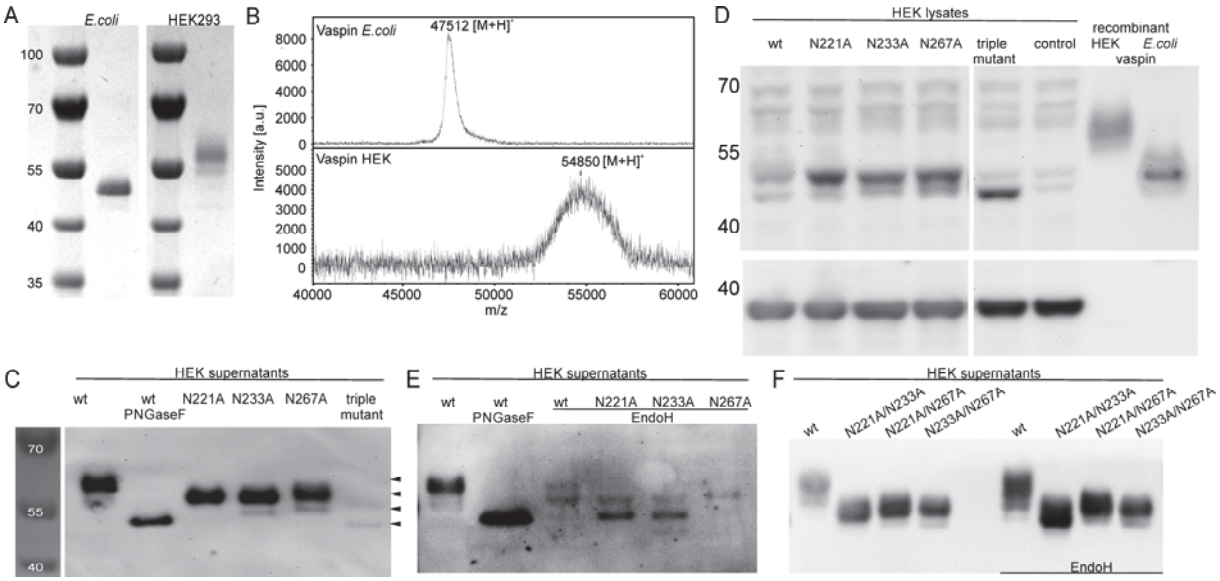

Figure 2. Identification of utilized glycosylation sites in HEK-derived vaspin. A) Coomassie stained SDS-PAGE gels of *E.coli* or commercial HEK-derived vaspin. B) MALDI-TOF MS spectrum of recombinant vaspin from *E.coli* or commercial HEK-derived vaspin in linear mode ($[M_{avg} + H]^+_{theoretical}$= 47512 for *E.coli* derived vaspin). Glycosylation increases the molecular weight by ~7.5 kDa. C) Western Blot analysis of HEK-supernatants secreting vaspin and mutants using an anti-vaspin antibody shows glycosylation at all three asparagine residues. Lanes: 1 - wt vaspin; 2 - wt vaspin after PNGase F treatment; 3-5 - alanine mutants N221A, N233A and N267A; 6 - triple mutant N221A/N233A/N267A; 7 – double mutant N233A/N267A. D) Analysis of protein expression in HEK lysates corresponding to supernatants analyzed in C) and recombinant vaspin proteins from HEK or *E.coli* as controls. GAPDH served as loading control. All vaspin mutants are expressed at similar levels in HEK cells. E) Incubation of HEK-derived vaspin and mutants with PNGase F removes all glycosylations and the deglycosylated vaspin migrates at the predicted MW of 47.5 kDa. Incubation with Endo H of wt and vaspin mutants indicates that both Asn221 and Asn233 are modified by complex glycans, whereas a hybrid or high mannose type glycan seems to be attached to Asn267. F) Western Blot analysis of HEK-derived vaspin and double mutants using an anti-vaspin antibody before and after Endo H treatment confirmed that Asn267 likely is modified by hybrid or high mannose type glycan.

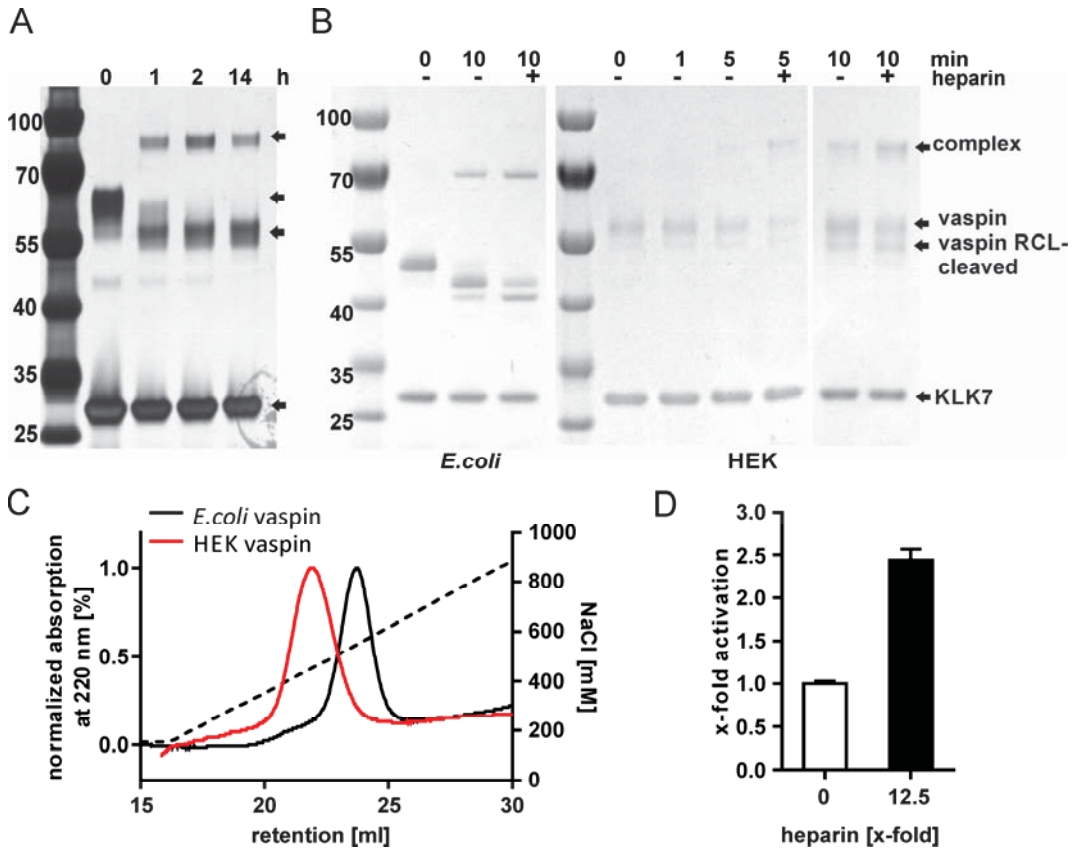

Figure 3. Biochemical properties of HEK-derived vaspin: KLK7 inhibition, heparin activation and heparin binding. Coomassie stained SDS-PAGE gels of *E.coli* or commercial HEK-derived vaspin incubated with KLK7 for various times with or without heparin as indicated. A) Glycosylation of vaspin does not affect complex stability as vaspin-KLK7 complexes are stable for >14 h. B) Inhibition of KLK7 by glycosylated vaspin is comparable to *E.coli* derived vaspin and can be accelerated by heparin. C) Heparin binding of glycosylated vaspin was assessed by heparin-affinity chromatography using a NaCl gradient (black dotted line). Elution profiles of HEK- and *E.coli*-derived vaspin were monitored at 220 nm. Glycosylated vaspin elutes with ~450 mM NaCl and thus ~100 mM earlier than unglycosylated vaspin. D) Inhibition of KLK7 by glycosylated vaspin with 12.5-fold excess of unfractionated heparin was measured under pseudo-first-order conditions in a discontinuous assay. Shown is the increase as x-fold over control (without heparin).

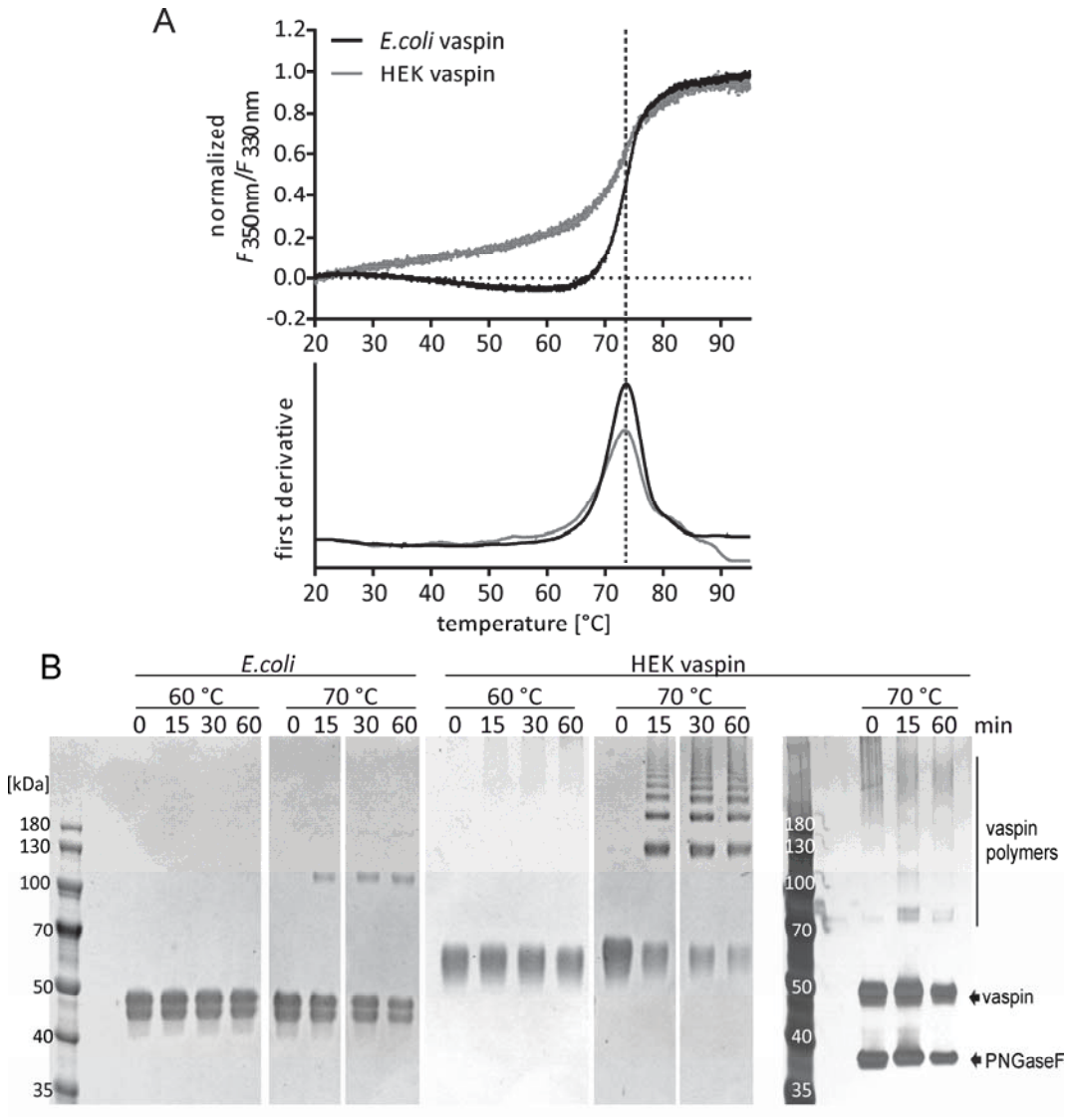

Figure 4. Thermal stability of glycosylated vaspin. A) Thermal denaturation of glycosylated and unglycosylated vaspin was observed by nanoDSF. The ratio of 350/330 nm (top) or its first derivative (bottom) are plotted against temperature to visualize the fold transitions. B) Heat-induced polymerization of unglycosylated (left), glycosylated (middle) and deglycosylated (right) vaspin after incubation at 60 and 70 °C for indicated times is analyzed by non-heated and non-reducing SDS-PAGE.

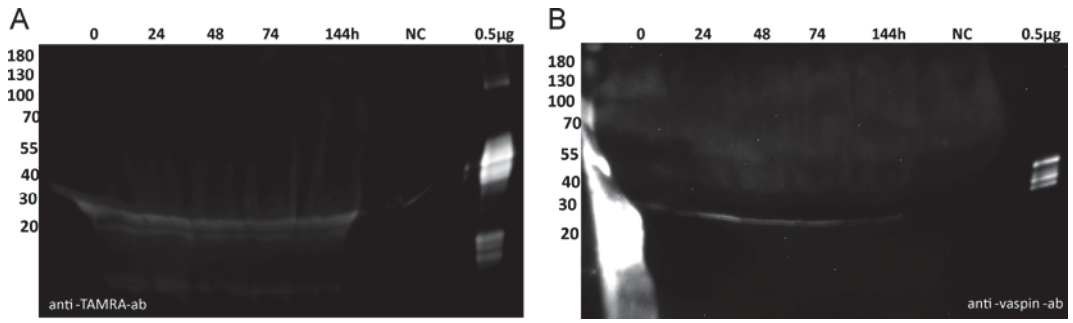

Figure 5. *In vitro* plasma stability of unglycosylated human vaspin. A) and B) TAMRA-labeled human vaspin was incubated in human plasma at 37°C for indicated times. After protein precipitation, vaspin was detected by anti-TAMRA (A) and, after stripping of the blot, by anti-vaspin antibodies (B). Intact vaspin bands are detected upon incubation for as long as 6 days. Note, that the high amount of serum albumin and immunoglobulins in the plasma samples considerably affected protein migration resulting in the vaspin band apparently migrating below the control protein (NC: negative control).