Review

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# Sweetened kallikrein-related peptidases (KLKs): glycan trees as potential regulators of activation and activity

**Abstract:** Most kallikrein-related peptidases (KLKs) are N-glycosylated with N-acetylglucosamine$_2$-mannose$_9$ units at Asn-Xaa-Ser/Thr sequons during protein synthesis and translocation into the endoplasmic reticulum. These N-glycans are modified in the Golgi machinery, where additional O-glycosylation at Ser and Thr takes place, before exocytotic release of the KLKs into the extracellular space. Sequons are present in all 15 members of the KLKs and comparative studies for KLKs from natural and recombinant sources elucidated some aspects of glycosylation. Although glycosylation of mammalian KLKs 1, 3, 4, 6, and 8 has been analyzed in great detail, e.g., by crystal structures, the respective function remains largely unclear. In some cases, altered enzymatic activity was observed for KLKs upon glycosylation. Remarkably, for KLK3/PSA, changes in the glycosylation pattern were observed in samples of benign prostatic hyperplasia and prostate cancer with respect to healthy individuals. Potential functions of KLK glycosylation in structural stabilization, protection against degradation, and activity modulation of substrate specificity can be deduced from a comparison with other glycosylated proteins and their regulation. According to the new concept of protein sectors, glycosylation distant from the active site might significantly influence the activity of proteases. Novel pharmacological approaches can exploit engineered glycans in the therapeutical context.

**Keywords:** β-turn; N-glycosylation; O-glycosylation; post-translational modification; protein sector; surface loops.

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## Introduction

As tissue kallikrein (KLK1) and all other kallikrein-related peptidases (KLKs) are secreted from cells into various tissues and body fluids, and harbor numerous glycosylation sites, they are glycosylation targets of the cellular export machinery. Since protein glycosylation has a significant influence on folding, stability, molecular recognition, and specific activity, it is worth to investigate the current knowledge and to illuminate the largely unknown functions of KLK glycosylation. Also, *in vitro* studies might benefit from employing KLKs that carry defined, homogeneous glycan trees similar to their natural counterparts, in particular with respect to the structure-function relationship. It is noteworthy that recombinant expression in bacteria generates no glycosylation at all, whereas eukaryotic cells such as *Sf9* or *Leishmania* produce short homogeneous glycan cores of five to six sugars (Figure 1). In contrast, yeast expression, e.g., in *Pichia* or *Saccharomyces*, yields rather inhomogeneous, large and more branched glycans than those obtained from mammalian cell cultures, such as Chinese hamster ovary (CHO) and HEK293 cells (Figure 1) (Hamilton and Gerngross, 2007). Up to now, no systematic analyses of the biological and biochemical properties have been performed for most glycosylated KLKs. However, for individual KLKs, notably KLK3/PSA, extensive studies investigated its highly variable glycan trees with a focus on the patho-physiological role in prostate cancer.

All 15 members of the KLK family carry at least one typical N-glycosylation motif or so-called sequon of the type Asn-Xaa-Ser/Thr, where Xaa can be any amino acid (but very rarely Pro), while in about 1% of cases a Cys is found at position 3 instead of Ser or Thr (Bause, 1983; Satomi et al., 2004). Slightly more than 50% of the

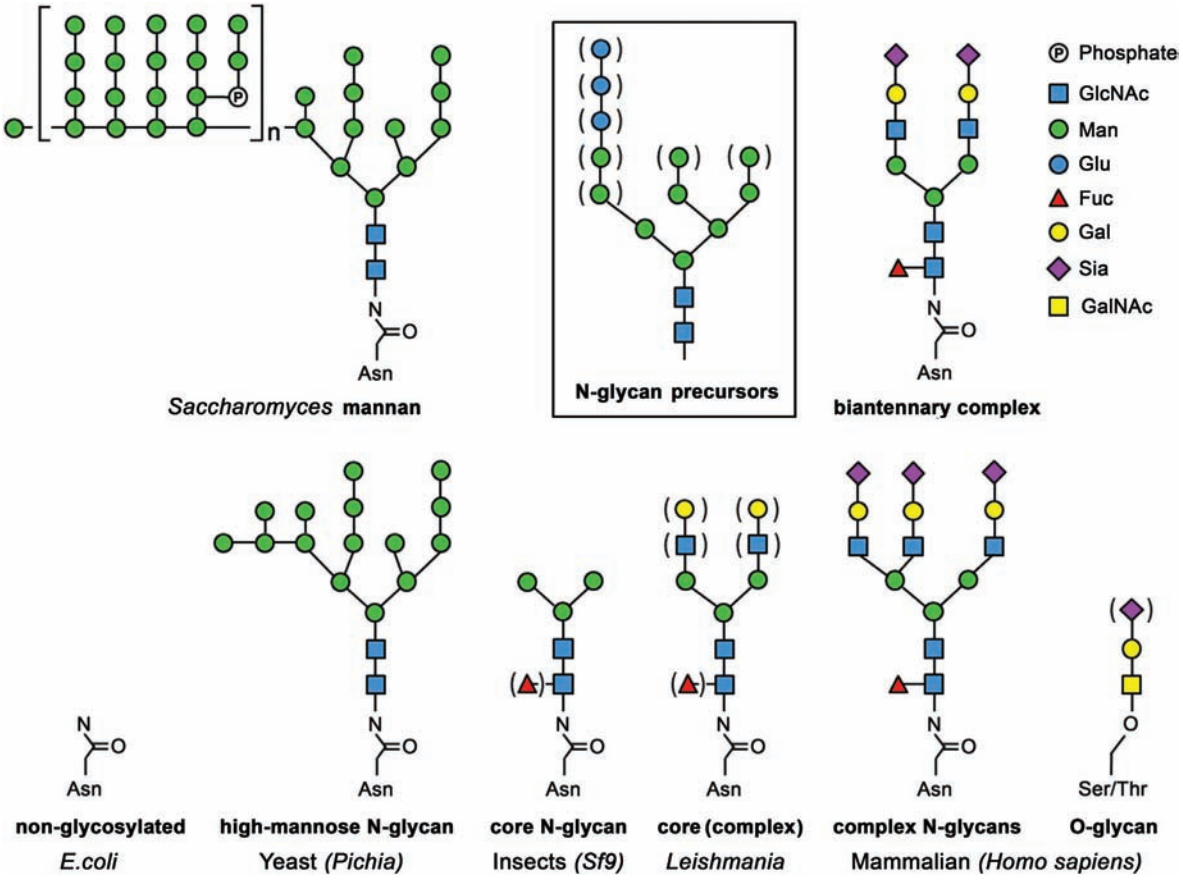



**Figure 1** Glycosylation patterns of natural and recombinant expression.
N-glycosylation of Asn differs considerably between domains, kingdoms and classes (Varki et al., 2009). However, the common eukaryotic precursor molecules are $GlcNAc_2Man_9Glu_3$ and $GlcNAc_2Man_5$. Prokaryotes, such as *Escherichia coli* do not glycosylate sequons or potential O-glycosylation sites, while yeast, such as *Pichia* and, in particular, *Saccharomyces* attach large, multi-antennary $GlcNAc_2Man_{>15}$ sugar trees. In contrast, mutated strains of *Pichia* may produce relatively small and more homogeneous N-glycans (Krainer et al., 2013). Protozoa, such as *Leishmania tarentolae*, and insects, e.g., *Spodoptera frugiperda 9* cells, generate mostly short $GlcNAc_2(Fuc)Man_3$ core glycans. Sometimes *Leishmania* glycans are nearly as extended as in mammalian complex biantennary N-glycans (Breitling et al., 2002). Human HEK293T cells attach regular multi-antennary glycans, e.g., $GlcNAc_2(Fuc)\ Man_3(GlcNAcGalSia)_2$, whereas HEK293S cells produce rather short $GlcNAc_2(Fuc)Man_5$ cores. The most common type of O-glycosylation at Ser or Thr side-chains is the mucin type, with O-GalNAcGal cores that are often extended with Sia or other sugars. Also, single O-glycosylation Fuc, GlcNac and Man are found in mammals.

standard sequons carry glycan trees. Moreover, recent glyco-proteomic studies indicate that unusual motifs, such as Asn-Xaa-Val, or simply Asn-Gly are occasionally glycosylated as well (Zielinska et al., 2010; Lam et al., 2013). Notably, protein folding often determines which sequons are N-glycosylation targets, similar to the less frequent O-glycosylation sites (Pless and Lennarz, 1977; Thanka Christlet and Veluraja, 2001). No distinct sequon can be specified for O-glycosylation at Ser or Thr. Still, surrounding Pro residues, such as Pro(-1)-Ser/Thr-Xaa-Xaa-Pro(+3), are favorable as observed in the O-glycosylation sequence Pro-$^{125}$Thr-Gln-Glu-Pro of KLK3 (Hansen et al., 1996; Thanka Christlet and Veluraja, 2001).

Although the cellular post-translational modification processes of KLKs have not been studied in detail, we can assume that they follow the standard secretory pathway of eukaryotes. The signal peptide (pre-peptide) of a KLK polypeptide emerging from a ribosome is bound by the signal recognition particle (SRP), which results in delayed translation and association with a membrane-bound SRP receptor (Akopian et al., 2013). Subsequently, this complex assembles with the Sec complex of the rough endoplasmic reticulum (ER) and the elongating polypeptide inserts into the Sec61 transmembrane channel (Deshaies et al., 1991; Nyathi et al., 2013). The so-called translocon complex consists of the Sec61 core and regulators, such as TRAM, TRAP, RAMP4, the oligosaccharyltransferase complex (OST), and the signal peptidase complex (SPC) (Wang and Dobberstein, 1999). Signal peptidase cleaves hydrophobic signal peptides that are inserted into the membrane with

their N-terminus at the luminal side of the ER (Kalies et al., 1998; Paetzel et al., 2002; Liang et al., 2003). The substrate specificity of signal peptidase is largely restricted to Ala, Gly, Ser, Thr, and small hydrophobic residues in P1 and P3 position, as seen in all 15 human pre-pro-KLKs (Dalbey et al., 1997; Karla et al., 2005). Signal peptide removal and the first step of N-glycosylation by oligosaccharyltransferase often proceed in a cotranslational manner, before the translocon complex releases the protein into the ER lumen (Haguenauer-Tsapis, 1992; Ruiz-Canada et al., 2009).

The N-linked glycosylation of proteins in the ER is an important prerequisite for protein targeting (Hubbard and Ivatt 1981; Aebi, 2013). Nearly all eukaryotic OSTs use the membrane-bound dolichol pyrophosphate $GlcNAc_2Man_9Glc_3$ as substrate (Kelleher et al., 2003). Overall, eukaryotic OSTs prefer N-X-T sequons by a factor of about 1.5 over N-X-S, with sites in turns or loops and β-sheets significantly more frequent than in α-helices (Zielinska et al., 2012). Concomitantly, disulfide formation by protein disulfide isomerase (PDI) is another crucial step for protein folding in the ER, in particular for pre-pro-KLKs that contain five to six disulfide bonds (Kim et al., 1998; Ojore B. V. Oka and Bulleid 2013). For more complicated multidomain proteins folding can be completed by chaperones of the ER, followed by selection of properly folded proteins (Anelli and Sitia, 2008; Gidalevitz et al., 2013). Glucosidases and a glucosyltransferase cleave the glucose molecules of the glycan a-branch, which is combined with folding quality checks in the CNX/CRT cycle (Parodi, 2000). Further trimming by ER mannosidases leaves N-linked $GlcNAc_2Man_9$ trees with three branches on folded proteins that can enter the ER Golgi intermediate compartment (ERGIC) for sorting, further processing and quality control (Appenzeller-Herzog and Hauri, 2006; Anelli and Sitia, 2008).

The secretory proteins enter the cis-Golgi region for post-translational modifications, such as trimming of N-glycans by α-mannosidases (Hubbard and Ivatt, 1981). Also, the maturation process of N-glycans in the Golgi apparatus involves elongation reactions that are performed by N-acetylglucosaminyl-transferase, galactosyl-, fucosyl- and sialyltransferases, resulting in biantennary, triantennary or tetraantennary oligosaccharides (Breton et al., 1998; Grabenhorst et al., 1998; Roth 2002). These types of glycosylation have been reported for KLKs, while the unbranched N-glycosaminglycan (GAG, mucopolysaccharide) trees have not yet been observed for them.

Seven types of O-glycosylation are known: the most common mucin type of secretory proteins requires a Ser or Thr-Oγ atom linked to N-acetylgalactosamine (GalNAc), which can be either connected to another galactose or N-acteylglucosamine in an α/β1-3 or α/β1-6 bond and extended by sialic acid or various branches (Figure 1) (Wopereis et al., 2006). The already mentioned GAG trees can be O-linked via a tetrasaccharide with an O-xylose-Ser link, while O-GlcNAc, -Glu, -Fuc, and -Man links might not be relevant for KLKs (Steen et al., 1998; Lommel and Strahl, 2009). Phospho-Ser glycans, C-links and glypation (GPI-anchoring in membranes), do not seem to play a role for KLKs. Glycosylated KLKs are packed into vesicles budding from the trans-Golgi network for trafficking to the cell membrane (Ishida-Yamamoto et al., 2004). Finally, the release of KLKs from the secretory vesicles by exocytosis into the extracellular environment might be triggered by specific signals, which have to be elucidated in the future (Yahiro and Nagato 2002).

## Current status of glyco-KLK site analysis

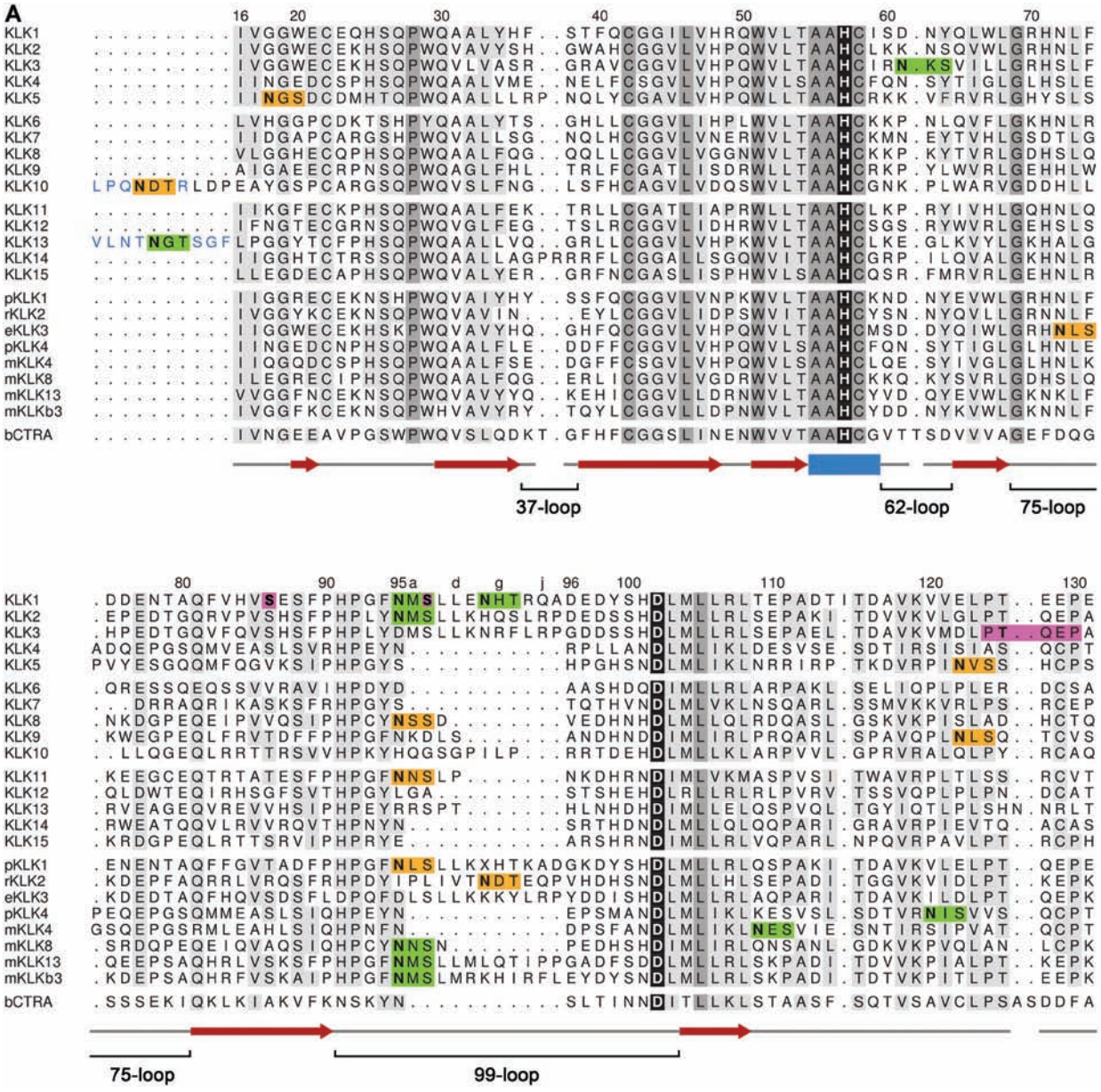
### Tissue kallikrein (KLK1)

Human kallikrein-1 (KLK1), the true tissue kallikrein, is responsible for the regulation of blood pressure by cleaving kininogens, thereby displaying a dual tryptic and chymotryptic specificity (Frey and Kraut, 1928; Kraut et al., 1930; Bourgeois et al., 1997; Chao et al., 2006). KLK1 exhibits three sequons of the N-X-S/T type at Asn95, Asn95F and Asn148 (Figure 2). Using protein-chemical methods, six carbohydrate side chains were identified in human urinary KLK1, whereby the three N-glycosidic links to Asn were confirmed together with three O-glycosidic links at Ser86, Ser95C, and Ser150 (Kellermann et al., 1988). Remarkably, the six glycan trees cluster around the active site cleft, mainly in the extended kallikrein/99-loop and the 148-loop, which may have a significant effect on substrate specificity and turnover. In contrast to KLK1 expressed in *Escherichia coli*, natural urinary KLK1 was confirmed to be glycosylated, as well as the variant expressed in the yeast *Pichia pastoris* (Angermann et al., 1989; Chan et al., 1998). This expression system with heterogeneous glycosylation caused problems in crystallization of human KLK1, even after enzymatic deglycosylation, requiring the mutation of the sequons for a successful X-ray structure determination (Katz et al., 1998). In the crystal structure of human apo-KLK1 only a single GlcNAc at Asn95 was visible in the electron density, despite the presence of up to three $GlcNAc_2Man_3$(Fuc) trees (Figure 3) (Laxmikanthan et al., 2005).

Recombinant human KLK1 expression in CHO cells yielded heterogeneous samples, possibly due to partial glycosylation at Asn148 and variation of mono-, di-, tri-, and tetrasialylated glycan trees, resulting in a maximum molecular weight of 41 kDa (Watson et al., 1994; Hsieng S. Lu et al., 1996b). This glycosylation has only a minor effect on the enzymatic activity towards small substrates, such as D-Val-Leu-Arg-pNA, when compared to the non-glycosylated KLK1 from *E. coli* expression (Lu et al., 1996a). Interestingly, rat KLK1 contains only a single N-glycosylation site with an N-X-S/T sequon at position Asn95F (Figure 2). Full-length active rat tissue kallikrein was expressed previously in *E. coli* and, as a glycosylated form, in the yeast *Saccharomyces cerevisiae*, yielding equally active KLK1 (Wang et al., 1991).

## The prostatic KLKs 2 and 3

Seminal plasma contains the highest concentration of KLK2, which is thought to be the activator of the semen liquefying KLK3/PSA (Lovgren et al., 1997; Takayama et al., 1997). KLK2 possesses tryptic specificity, cleaves the propeptide APLILSR from KLK3 and may support KLK3 in




**(Figure 2** Continued)

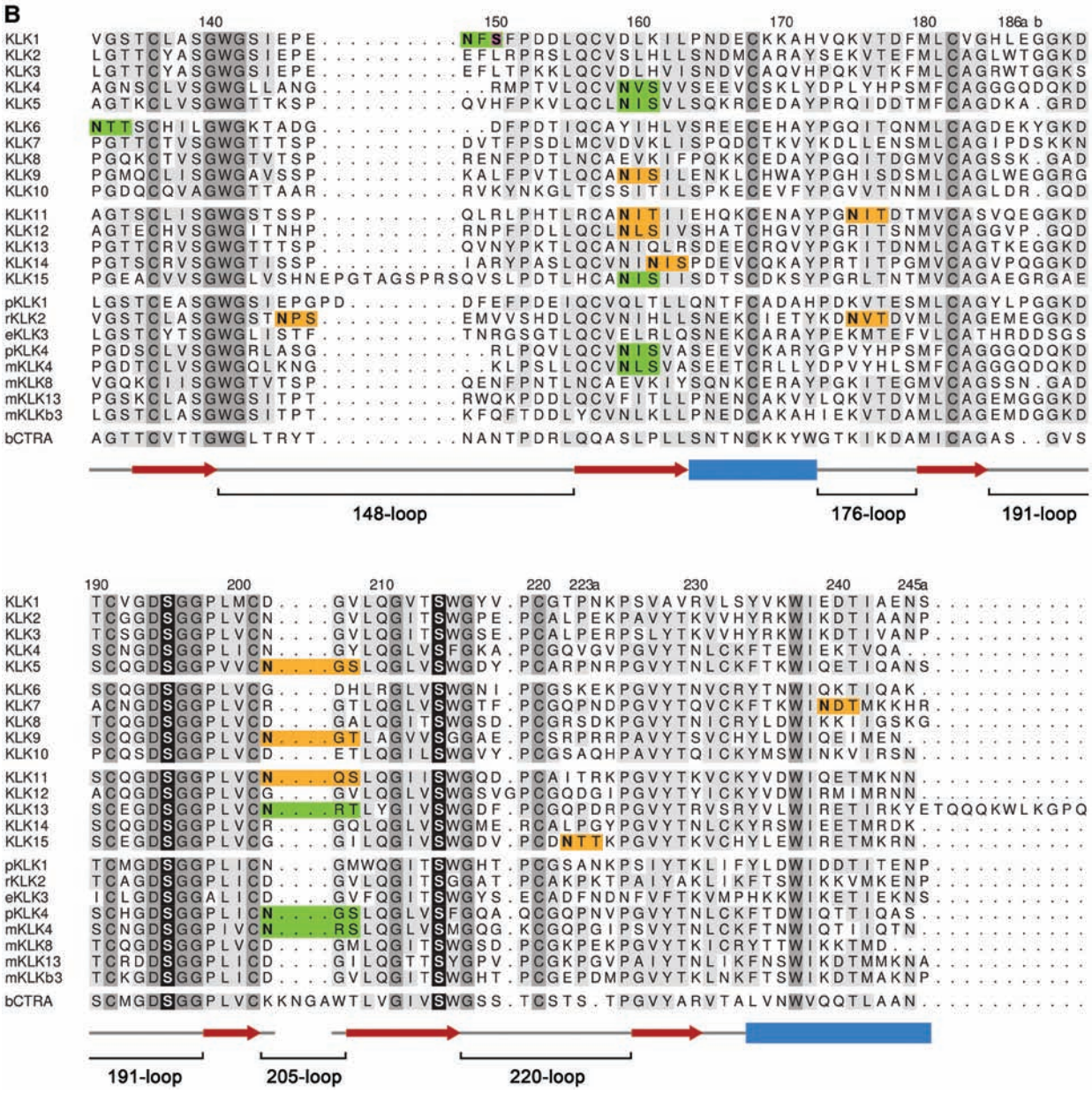



**Figure 2** Alignment of human and other relevant KLKs.
Structure-based alignment of the mature protease domains of the 15 human KLKs, porcine (pKLK1, pKLK4), horse (eKLK3), rat (rKLK2/rKLKc2), murine (mKLKb3, mKLK4, mKLK13/mKLKb13) KLKs and bovine chymotrypsin (bCTRA) as numbering reference. The degree of conservation is depicted in increasingly darker shades of grey, with the catalytic triad residues in white with black background, including Ser214 that stabilizes Asp102. β-strands are depicted as red arrows and α-helices as blue rectangles. Confirmed and putative sequons are displayed with green and orange backgrounds, respectively. O-glycosylation sites are highlighted in magenta, e.g., Ser86 in KLK1, while the two other O-glycosylation sites coincide with sequons (Ser95C and Ser150). KLK10 and KLK13 possess sequons in their propeptides, which are shown as blue letters. The alignment was prepared with STRAP and visualized with TeXshade (Beitz, 2000; Gille and Frömmel 2001).

degrading semenogelins (Lovgren et al., 1999). According to its sequence, KLK2 contains a single sequon at Asn95, which is most likely glycosylated in natural KLK2, displaying a molecular weight of 31 kDa (Figure 1) (Frenette et al., 1997). Also, KLK2 expressed in hamster cells exhibited an additional mass of 2038 Da; PNGase F treatment, which removes N-glycans completely and turns Asn into Asp, as well as lectin interaction indicated the presence of mannose, sialic acid and fucose (Mikolajczyk et al., 1997). This form of KLK2 was highly active against tryptic substrates, e.g., H-D-Pro-Phe-Arg-pNA, and bound suitable serpin inhibitors, such as PI-6 (Mikolajczyk et al., 1998;

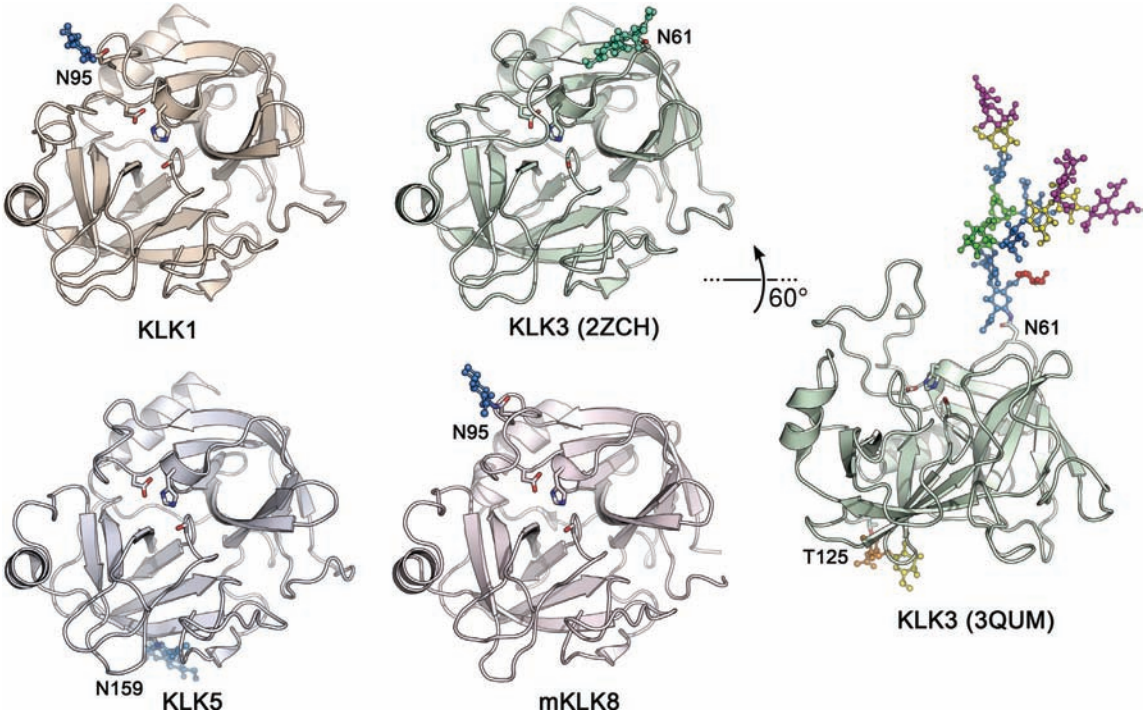



**Figure 3** Structures of glycosylated KLKs.
Glycans in KLK1 (PDB ID 1SPJ), KLK3 (PDB IDs 2ZCH and 3QUM), KLK5 (PDB ID 2PSX) and mKLK8 (PDB ID 1NPM) are indicated. The extended triantennary polysaccharide in the KLK3 structure (right) illustrates the remarkable size of a typical complex N-linked glycan compared to the size of the protease. Glycosylated residues and members of the catalytic triad (Asp102, His57 and Ser195) are drawn as sticks. Carbohydrates are shown in ball-and-stick representation: blue, N-acetyl-β-D-glucosamine (GlcNAc); red, α-L-fucose (Fuc); green, α-D-mannose (Man); yellow, β-D-galactose (Gal); magenta, O-sialic acid (Sia); orange, N-acetyl-2-deoxy-2-amino-galactose (GalNAc); cyan, 2-(acetylamino)-2-deoxy-α-D-glucopyranose (N-acetyl-α-D-glucosamine, α-GlcNAc).

Saedi et al., 2001). Recently, we used *Leishmania tarentolae* (LEXSY) and *E. coli* cells to produce human KLK2 variants that exhibited a significant mass difference, which was confirmed by mass spectrometry to be predominantly a $GlcNAc_2Hexose_3$ core at Asn95 in case of the LEXSY-KLK2 (Guo, unpublished).

KLK3/PSA is widely used as biomarker for prostate cancer screening, diagnosis and/or monitoring. Due to its clinical use, the glycosylation pattern of KLK3/PSA has been more thoroughly investigated than for any other KLK. The major physiological function of KLK3 is chymotryptic cleavage of semenogelins, which results in semen liquefaction, a crucial step in fertilization (Denmeade et al., 1997; Malm et al., 2000). N-glycosylation of the KLK3/PSA sequon N-K-S was confirmed at Asn61 by mass spectrometry, displaying additional 2351 Da, which is in agreement with a biantennary N-linked oligosaccharide, terminal sialic acid groups, and a core fucose (Bélanger et al., 1995). An enzymatic glycan analysis identified a mixture of mono- and biantennary N-glycosylation including mono- and disialylated KLK3, which becomes more heterogeneous by truncation of the glycan tree and internal proteolytic clipping (Okada et al., 2001; Mattsson et al., 2008). Analyses of human urinary and seminal KLK3 samples corroborated varying N-glycosylation and O-glycosylation, which had a significant effect on the enzymatic activity against even small substrates, such as suc-Leu-Leu-Val-Tyr-AMC (Shibata et al., 1997). Whereas one KLK3 crystal structure (2ZCH, Figure 3) exhibited only two GlcNAc molecules linked to Asn61, another structure (3QUM) showed a triantennary N-glycan with terminal sialic acids and a mucin-type O-glycan (GlcNAc-Gal) at Thr125 (Menez et al., 2008; Stura et al., 2011). Similar to seminal KLK3, non-glycosylated KLK3 from *E. coli* expression reacts with anti-chymotrypsin. Non-glycosylated and glycosylated KLK3 forms display chymotryptic specificity profiles, however, with relatively large differences, in particular in the P1 position: Tyr > Leu > Phe (sem-KLK3), Tyr > Phe > Pro (insect-derived ins-KLK3), Met > Ala > Tyr (eco-KLK3) (Takayama et al., 1997; Coombs et al., 1998; Mekdes Debela et al., 2006b). Specific activity against MeO-suc-Arg-Pro-Tyr-pNA was 100% for sem-KLK3, 70%

for non-glycosylated eco-KLK3, and nearly 100% for yeast-expressed KLK3 variants, which carried either no glycan or a $GlcNAc_2Man_{10}$ tree (Hsieh and Cooperman 2000; Habeck et al., 2001). However, a larger effect of different glycosylation types on substrate turnover cannot be excluded, as observed for mammalian cell (BHK-21) expressed KLK3 that was <50% active against Lys-Gly-Ile-Ser-Ser-Glu-Tyr-AMC (Lovgren et al., 1997). As the large multi-antennary glycan at Asn61 resides in the prime side region of KLK3 close to the active site, it might interfere with substrate recognition and turnover, especially when larger protein substrates are involved.

Aberrant glycosylation in prostate cancer and potential diagnostic applications have been reviewed recently by Gilgunn and coworkers and the group of Vermassen (Vermassen et al., 2012; Gilgunn et al., 2013). Mass spectrometrical analyses identified at least 40 different glycan structures on KLK3 both from healthy and prostate cancer samples (White et al., 2009; Behnken et al., 2014). Earlier studies observed that a large portion of KLK3 from plasma of prostate cancer patients was non-glycosylated, whereas malignant tumor cells generate KLK3 with more antennae than the common biantennary N-glycan of normal KLK3 (Barak et al., 1989; Prakash and Robbins, 2000). Also, the glycosylation of prostate cancer KLK3 was reduced in benign prostate hyperplasia (BPH) and exhibited a different composition (Basu et al., 2003; Ohyama et al., 2004) KLK3 from prostate tumor cell lines shows an altered sugar composition, with a significant lack of sialic acid in the N-glycan tree (Peracaula et al., 2003). Seemingly, differences exist in fucosylation and sialylation of KLK3 from prostate cancer patients, although they do not appear to be sufficient for reliable prognosis of the disease (Tabares et al., 2006, 2007). Nevertheless, these differences have the potential to discriminate between benign and malignant prostate disease, e.g., by monitoring the complex of KLK3 with anti-chymotrypsin, by using fucosylation analysis, mass spectrometry or lectin microarrays and immunosorbent assays (Tajiri et al., 2008; Dwek et al., 2010; Sarrats et al., 2010; Yan Li et al., 2011).

## The prostatic and dental KLK4

KLK4 plays an important and clearly defined role in higher mammalian tooth development. As protease with a strong tryptic preference, KLK4 degrades matrix proteins during enamel formation and could be an activator of KLK2 and 3 in seminal fluid and of several other KLKs (Lu et al., 2008; Yoon et al., 2009). A nonsense mutation at the Trp141 codon to a stop codon in the fourth exon and a frameshift mutation at the Gly69 codon of the human *KLK4* gene result in truncated KLK4 variants lacking the catalytic Ser195 and subsequently in improper enamel formation, termed *amelogenesis imperfecta* (Hart et al., 2004; Wang et al., 2013). In addition, KLK4 is expressed in the normal prostate at a low level and its expression is increased in prostate cancer (Seiz et al., 2010). Human KLK4 has one potential N-glycosylation site at Asn159, which is conserved in five other KLKs (Figure 2). There is some evidence for glycosylation of KLK4: anti-KLK4 antibodies detected 32–45 kDa proteins in seminal plasma, which is considerably larger than the 27 kDa of pre-pro-KLK4 or 24 kDa of the active KLK4; pre-pro-KLK4 from *in vitro* reticulocyte lysate transcription system loses about 2 kDa mass upon treatment with PNGase F; yeast-expressed KLK4 loses about 5 kDa upon PNGase F treatment (Dong et al., 2005; Obiezu et al., 2006). Non-glycosylated KLK4 expressed in *E. coli* is more active against X-Arg-pNA substrates and in pro-KLK3 activation than the corresponding KLK2 (Takayama et al., 2001b). It is highly active as monomer, but nearly inactive as oligomer and crystallized in various oligomeric arrays that contained tetrameric ring-like subunits (Debela et al., 2006a).

Similar to human KLK4, the mouse and pig homologues (enamel matrix serine proteinase 1) are required for proper tooth formation (Simmer et al., 2009; Hu et al., 2011). Murine mKLK4 possesses three sequons at Asn109, Asn159, and Asn202; it shares the latter two with porcine pKLK4 that has a third sequon at Asn120 (Figure 2). Recombinant pKLK4 from *E. coli*, insect cells and HEK293 cells, as well as natural samples from pig teeth displayed increasing molecular weights of 28 (eco), 31 (insect), 34 (HEK293), and 34 to 37 kDa (natural), indicating increasing degrees of glycosylation (Ryu et al., 2002). However, only natural pKLK4 was significantly active against small fluorogenic compounds and the physiological substrate amelogelin. Deglycosylation with PNGase F reduced the apparent molecular from 34 to 37 kDa to about 28 kDa and rendered the enzyme virtually inactive. This observation cannot be easily explained, since these three potential N-glycosylation sites are located at the molecular surface opposite to the active site, which was suggested previously and is illustrated in the structure-based alignment (Figure 2) (Scully et al., 1998). Possibly, the N-glycans protect pKLK4 from auto-degradation (Lu et al., 2008). Recently, a systematic analysis of porcine and murine glyco-KLK4 isolated from teeth found bi- and triantennary N-glycan trees on both enzymes (Yamakoshi et al., 2011). Yamakoshi and coworkers identified typical mammalian glycans, consisting of the $GlcNAc_2Man_3$ core, sometimes with a core fucose attached, and with further extensions

of GlcNAc-Gal-Sia. Seemingly, pKLK4 and mKLK4 share two of the trees, while the third one differs with up to three terminal sialic acids.

## The epidermal KLKs 5, 7 and 14

Initially designated as stratum corneum trypsin-like enzyme, the physiological role of KLK5 is relatively well defined in an enzymatic concert with chymotryptic KLK7 and tryptic 14, which is responsible for skin desquamation (Brattsand et al., 2005) Exceeding activity of KLK5 is connected to skin diseases, such as Netherton syndrome and atopic dermatitis. Unbalanced expression also occurs in various cancer types (Goettig and Magdolen, 2012). Active human KLK5, with four sequons at Asn18, 122, 159, and 202 (Figure 2), was isolated from skin and exhibited a relatively broad range of apparent molecular weights above 30 kDa, indicating inhomogeneous glycosylation, which was reduced to 28 kDa upon deglycosylation with PNGase F (Brattsand and Egelrud, 1999). Yeast-expressed KLK5 appeared to be glycosylated with molecular masses from 30 to 44 kDa, which turned over tryptic substrates like VPR-AMC; PNGase F treatment reduced the mass to 26 or 28 kDa, respectively (Yousef et al., 2003; Michael et al., 2005). The two GlcNAc residues that are well defined in the electron density in the insect cell-expressed KLK5 crystal structure are located in a solvent exposed region of a β-sheet distant from the active site, similar to the three other potential sites (2PSX, Figure 3) (Mekdes Debela et al., 2007a).

Studies on the stratum corneum chymotryptic enzyme, KLK7, isolated from human skin and from recombinant expression in murine C127 cells, indicated N-glycosylation, as at least two distinct forms of the active protease were observed (Hansson et al., 1994; Ekholm et al., 2000). Although the KLK7 sequence contains a sequon at Asn239 in the terminal α-helix, according to the crystal structure of the insect cell-expressed enzyme, no glycosylation was observed even at 1.0 resolution (2QXI) (Mekdes Debela et al., 2007b). This phenomenon can be explained by the fact that sequons close to the N-terminus and in α-helices are rarely glycosylated. Furthermore, according to the NetOGlyc 4.0 Server (http://www.cbs.dtu.dk/services/NetOGlyc/) and structural considerations a single potential O-glycosylation site at Thr144 is present in KLK7, which may account for the partial glycosylation. KLK14 possesses an N-I-S sequon at Asn161 that was apparently not glycosylated upon expression in yeast, insect cells, primate COS-7 cells, and human HEK293 cells (Figure 2) (Brattsand et al., 2005; Borgoño et al., 2007; Rajapakse and Takahashi 2007).

## The brain-located KLKs 6 and 8

Both KLK6, neurosin, and KLK8, neuropsin, are highly expressed in human brain and to some extent in skin (Shaw and Diamandis, 2007). Nevertheless, more studies have been carried out with the homologous KLKs from rats or mice. KLK6 proteases are tryptic enzymes and, besides involvement in inflammation and skin diseases, their physiological role could be the de- and re-myelination of glia cells, in particular during neural growth after injuries (Scarisbrick et al., 2006; Mekdes Debela et al., 2006b; Bayani and Diamandis, 2012). Human KLK6 exhibits a single and otherwise not conserved N-glycosylation site Asn132 (Figure 2). KLK6 has been found to be upregulated in ovarian cancer (Seiz et al., 2012). Interestingly, glycosylation patterns of KLK6 from cerebrospinal fluid and ovarian cancer ascites differ significantly, in particular, by an increased heterogeneity and higher degree of terminal sialylation in the cancer samples, as shown by mass spectrometry, raising hopes for a new diagnostic tool in oncology (Kuzmanov et al., 2009, 2012). Insect cell-expressed human KLK6 was highly active against tryptic fluorogenic substrates, similar to the *E. coli* expressed variant (Angelo et al., 2006; Mekdes Debela et al., 2006b). A mass spectrometric analysis confirmed a heterogeneous glycosylation of active ins-KLK6, which may carry a typical N-glycan tree of up to six sugar moieties (Bernett et al., 2002). Bernett and coworkers solved the crystal structure of this KLK6 variant and noticed additional electron density at Asn132, but it was insufficient for accurate modeling even at 1.6 Å resolution (1LO6).

Besides occurring in skin, expression of mammalian KLK8 appears to be mostly restricted to the hippocampus, where it contributes to long term potentiation (LTP) and memory acquisition by rebuilding of synaptic connections (Tamura et al., 2006; Shaw and Diamandis, 2007; Eissa et al., 2011). In line with this proposed function, genetic variations of KLK8 in the 3′ regulatory region of the gene have been observed in patients with manic-depressive disorder and cognitive impairment (Izumi et al., 2008). Human and mouse KLK8 share about 80% identical residues in the active protease domain harboring the conserved sequon at Asn95 (Figure 2). Recombinant mKLK8 from insect cells, which is most likely glycosylated, displayed a different specific activity compared with natural mKLK8 (Shimizu et al., 1998; Takahashi et al., 1999). Recombinant human KLK8 expressed in insect cells had an additional mass of about 8 kDa with respect to the non-glycosylated polypeptide of 27.3 kDa (Mitsui et al., 1999). N-glycosylation at Asn95 was confirmed by the crystal structure of mKLK8, which exhibited two GlcNAc residues (1NPM, Figure 3) (Kishi et al., 1999). A mutational analysis of the mKLK8 99-loop that included

deletion of Asn95 and Asn95Ala and Asn95Ser mutations confirmed that the N-glycan had an influence on the S2 specificity for fluorogenic substrates, e.g., the $K_M$ for P2-Pro was not affected, but reduced for P2-Phe, indicating a size exclusion effect of the linked sugar tree (Oka et al., 2002).

### KLKs 13 and 15

Human KLK13 and KLK15 are expressed in various tissues at medium levels. The physiological function of these KLKs is not yet clearly defined (Shaw and Diamandis, 2007). As the majority of the family members, KLK13 prefers tryptic substrates, e.g., VPR-AMC, and was characterized as recombinant protein from yeast expression carrying an extended N-terminus (Kapadia et al., 2003; Sotiropoulou et al., 2003). The groups of Kapadia and Sotiropoulou observed a broad molecular weight range from 38 to 50 kDa for this construct, which was reduced to <28 kDa by PNGase F treatment. Both sequons at Asn10 and Asn202 appear to be N-glycosylated, while the standard propeptide QESSK$^{8}$VLNTNGTSG$^{15}$F can be cleaved either after Lys8 or Phe15, resulting in a long and short KLK13 variant, which are both active (Viktor Magdolen, unpublished). Possibly, N-glycosylation at Asn10 prevents the standard activation via generation of an N-terminal Leu16.

Seemingly, recombinant human KLK15 from *E. coli* expression does not require N-glycans for its tryptic activity during cleavage of the KLK3/PSA propeptide (Thomas K. Takayama et al., 2001a; Yoon et al., 2009). Expression of KLK15 in HEK293 cells yielded a protein with a mass 8 kDa higher than the polypeptide alone, which could be removed by PNGase F (Shaw et al., 2007). Interestingly, this study found equally high molecular weight KLK15 in human body fluids, such as milk, saliva and semen, indicating physiological glycosylation probably at Asn159 and perhaps Asn222 (Figure 2).

### Potential N-glycosylation sites in KLKs 9, 10, 11, and 12

Both function and glycosylation of the following KLKs have been investigated less extensively than for the other KLKs. KLK9 is strongly expressed in heart and seems to play a role in neural injuries (Shaw and Diamandis, 2007; Radulovic et al., 2013). Sequons of the presumably chymotryptic KLK9 are found at Asn122, Asn159, and Asn202, as in KLK5, which are most likely glycosylated upon expression in CHO cells (Memari et al., 2006; Goettig et al., 2010). Both KLK10 and KLK11 exhibit an unusual mixed chymotryptic tryptic enzymatic specificity (Mekdes Debela et al., 2006b). KLK10 is found in various tissues at lower expression levels and may act as tumor suppressor, while KLK11 is strongly expressed in prostate and found in semen (Shaw and Diamandis, 2007; Luo and Diamandis, 2012). Rather unusually, KLK10 has a single sequon at Asn9 in the propeptide, similar to KLK13 with Asn10, whereas three of the four sequons of KLK11 at Asn95, 159, 175 and 202 are conserved in several members of the KLK family (Figure 2). Eventually, KLK12, which is distinctly expressed in bones, lungs and other tissues, exhibits the conserved sequon Asn159. A study with murine NS0 myeloma cell-expressed KLK11 and KLK12 confirmed the tryptic specificity of the latter enzyme and showed molecular masses above 40 and 35 kDa, respectively, which hints to glycosylation of both KLKs (Memari et al., 2007).

### Other mammalian kallikrein-related peptidases

In contrast to human tissue kallikrein KLK1 with three N-glycosylation sites, only one standard sequon at Asn95 is present in the porcine homologue pKLK1 (Figure 2). Until now, no evidence for glycosylation in this position is available; the extended flexible 99/kallikrein-loop is not well defined in the three known crystal structures of pKLK1 (2PKA, 2KAI, and 1HIA). Similarly, no glycosylation of the sequon at Asn72 was observed in the crystal structure of horse kallikrein-1 related peptidase, eKLK3 (1GVZ, Figure 2). Despite similar numbering, the following mouse and rat KLKs are not homologues of the human KLKs. For example, rat KLK1c2 is a paralogue of KLK1 and corresponds to the angiotensin-converting enzyme tonin. It has one potential N-glycosylation site at Asn95F, which is located in an undefined region of the crystal structure, and a second at Asn175, which apparently carried no sugar moiety (1TON) (Fujinaga and James, 1987). In addition, rKLKc2 possesses a very rarely glycosylated N-P-S sequon at Asn145. Three KLK-related peptidases from mouse, mKLK13 (orthologue of human KLK13), the pro-renin converting enzyme (1AO5), and two mouse KLK1-paralogues, the active mKLKb3 (γ) and inactive mKLKb4 (α) subunits from the nerve growth factor complex (1SGF), share N-glycosylation at the conserved sequon at Asn95, as corroborated by the electron density maps, which show one (α) to two (γ) sugar moieties (Bax et al., 1997; Timm, 1997).

## Functional analogies and differences to other proteases

Seemingly, proteases with a wide range of substrate specificity and high turnover do not require glycosylation,

as exemplified by the digestive enzymes from stomach and pancreas. N- and O-glycosylation of pepsin, chymotrypsin, trypsin, and pancreatic elastases is either missing or scarce, although in elastase 3B Asn95 is glycosylated and supposed to protect the 99-loop from proteolysis (Wendorf et al., 1989). However, the intestinal activator of trypsin, enterokinase, is heavily glycosylated, which is mainly required for integral membrane localization (Zheng et al., 1999). Although four sequons are present in enterokinase, none of them belong to conserved and confirmed ones seen in KLKs, suggesting that activator KLKs may require less or different glycosylation than KLKs that are activation targets, while several KLKs may play a dual role as zymogen and mature protease in activation cascades (Yoon et al., 2009). The introduction of N-glycosylation sites in porcine pepsin enhanced the thermal stability with respect to the glycan-free wild type (Yoshimasu et al., 2004). These novel N-glycans shifted the pH that allowed autoactivation of pepsin to less acidic values, which was explained by increased rigidity of the structure. Moreover, the glyco-mutation Thr77Asn, located in the flap over the active site cleft, caused a strong increase of $K_M$ and decrease of $k_{cat}$, which is most likely due to rigidifying and enlarging the flap on the nearby S1 and S2 subsites. Thus, the conserved glycosylation sites in the similarly arranged 99-loop of KLKs 1, 2, 8 and 11 may stabilize this flexible region and regulate substrate access and turnover.

The heavy glycosylation of some blood coagulation factors, such as FVIII and von Willebrand factor, stands in contrast to the previous examples. Both factors circulate in blood as tight complex and each one is linked to more than 20 glycans, which is thought to maintain their structural integrity and prevent premature reactions before the coagulation cascade starts (Lenting et al., 2010). Patients with single additional N-glycosylation sites in mutated FVIII suffer from severe hemophilia A, which is caused by an FVII activity decrease to <1%, due to minimal activation by thrombin (Aly et al., 1992). While the catalytic domains of coagulation factors IX and X exhibit no sequons for N-glycosylation, they both have two N- and two O-glycans attached to their activation peptides. In FX, these glycans appear to be crucial for proper activation by the activation complexes of the instrinsic and extrinsic pathway and prevent cofactor independent zymogen activation (Sinha and Wolf, 1993; Yang et al., 2009). Seemingly, there is no comparable glycosylation of the rather short KLK propeptides, although KLK10 and KLK13 possess each one sequon at Asn9 and Asn10, respectively. Interestingly, for both KLKs the native N-terminal residue of the active protease appears to be ambiguous. Nevertheless, active recombinant KLK10 and 13 were obtained with longer N-termini than the standard residue 16, in case of KLK13 with an N-glycan carrying Asn10 (Figure 2) (Sotiropoulou et al., 2003; Mekdes Debela et al., 2006b). Also, the unusual sequon at Asn18 in KLK5 might have a significant influence on activation, if it is really glycosylated.

In a ternary complex between the serpin antithrombin, thrombin and a heparin mimetic that binds the inhibitor and the anion binding exosite II of thrombin, the natural N-glycan at Asn60G appears to orient the glycosaminoglycan in a way that prevents improper binding to exosite I (1TB6) (Li et al., 2004). As KLK3 possesses similar positively charged regions and a triantennary N-glycan at Asn61 as thrombin, a comparable role of the sugar moiety as guiding module for heparin binding can be assumed. Intriguingly, the anticoagulant heparin, which resembles glycosaminoglycans in the female genital tract, mediates and modulates the binding of KLK3 to its seminal plasma substrates fibronectin and semenogelin I and stimulates the activity (Andrade et al., 2010; Kumar et al., 2012). Additional effects of different glycosylation were observed for natural antithrombin, where the β-form lacks an N-glycan at Asn135, increasing the affinity of heparin binding (Mccoy et al., 2003). The multidomain LEKTI-1 inhibitor of skin-derived KLKs 5, 7, and 14 contains N- and O-glycosylation sites in three inhibitory domains, which are thought to regulate fragment processing (Deraison et al., 2007). Glycan trees on protease inhibitors seem to regulate the association with the protease, as demonstrated by the accelerated reaction of the deglycosylated serpin PCI with thrombin (Sun et al., 2008). Even the extent and composition of glycan trees can have a marked influence on the function, as shown for the PCI inhibition of thrombin and plasma kallikrein, which was strongly decreased by the complete removal of N-acetylneuraminic acid and galactose and the trimannosyl core from biantennary N-glycans (Izutani et al., 2001).

## Functional analogies to other glycoproteins

Frequently, glycan trees serve as distinct and specific modules in molecular recognition, either in intramolecular interaction, as seen in various antibody Fc fragments, or in intermolecular interactions, as observed in the neonatal Fc receptor complex with heterodimeric Fc (hdFc) (Nagae and Yamaguchi, 2012). In the case of multiple glycosylation sites within one molecule, e.g., KLK1 with three N-glycans and three O-glycans surrounding

the active site cleft, their interplay may strongly affect substrate access, possibly like a filter, while additional conformational changes of the 99- and 148-loop may considerably influence the substrate turnover. Also, single glycan trees at certain positions of monomer subunits can serve as guides and stabilizers upon oligomerization, as in the hexameric insect storage protein arylphorin (Nagae and Yamaguchi, 2012). N-glycosylation at Asn159 in KLK4 could serve as such stabilizer for the tetrameric and octameric building blocks observed in the crystal structures, as it fits well in the gap between subunits (2BDI) (Mekdes Debela et al., 2006a). More generally, mobile or disordered glycan trees enable or support protein interaction or ligand docking, as observed for membrane receptors and particularly large binding partners (Nagae and Yamaguchi, 2012). Thus, glyco-KLK interaction with substrates and regulators, which might themselves represent carriers of glycans, may depend to a great extent on the carbohydrates involved.

## Conclusions and outlook

The more conserved KLK sequons in the 99-loop and at the molecular surface opposite to the active site, represent two common structural motifs associated with N-glycosylation (Figure 2). Asn95 in KLK1 and KLK8 is located in a typical β-turn with a Pro as second and a Gly as third residue, while Asn159 belongs to a surface exposed β-strand. Intriguingly, sequons are only present in KLK 99-loops that have an insertion with respect to the short chymotrypsin loop (Figure 2). Most likely, N-glycosylation serves as protector from clipping and regulator of substrate access and turnover at the S2 to S4 subsite region. The function of N-glycosylation at Asn159, Asn175, Asn202 or near the N-terminus is less clear. N-glycosylated Asn159 might support folding in some cases, as bacterial expression of KLK5 failed, however, it succeeded for KLK4 (Schechter et al., 2005; Mekdes Debela et al., 2006b). According to the protein sector concept, which explained allosteric effects by spatial long-range interaction in rat trypsin, Asn159 would possibly mark the border between two sectors and could serve as stabilizing element (Halabi et al., 2009).

Interestingly, the KLK O-glycosylation site at Thr125 of PSA is highly conserved with the sequence P-T-X-E-P in the classical and closely related mammalian KLKs (Figure 2). Apparently, the O-glycan rigidifies the surrounding surface loop region (Stura et al., 2011). Surprisingly, the corresponding site in KLK1 was not glycosylated, while not predictable O-glycosylation was observed in all Ser/Thr residues of the N-glycosylated sequons (Kellermann et al., 1988). Unfortunately, the O-glycoproteome has been investigated far less extensively than the N-glycoproteome, although O-glycosylation is widespread in secreted proteins and an important modifier of proteolytic processing (Schjoldager and Clausen, 2012).

The requirement for intact, natural glycosylation in KLKs is emphasized by the fact that an array of seven active mature KLKs mostly degraded *E. coli* expressed zymogen KLKs 4-8, 10, 13, and 15, while it activated only KLK11 (Beaufort et al., 2010). However, the influence of natural substrate glycosylation and their potential interaction with the KLKs has been investigated only in a few cases: Corneodesmosomal matrix proteins from skin are equally degraded upon deglycosylation by KLK5, although the kinetic of the degradation and the interplay with KLKs 7 and 14 remains to be elucidated (Caubet et al., 2004).

Better differentiating glyco-biomarkers for cancer may revolutionize the field of medical KLK research in the future (Kuzmanov et al., 2013). Also, recent advances in isotope labeling by mammalian expression may facilitate future glycosylation analyses by NMR measurements (Sastry et al., 2012). Novel therapeutic approaches involving proteases employ glycan modifications and may be applicable to KLKs and their inhibitors, in particular to those present in skin. For example, an engineered factor IX with an N-glycan that was extended by PEG units showed an improved pharmacokinetic behavior (Negrier et al., 2011). Artificial glycosylation of proteases and their inhibitors appears to be a useful approach for the extension of their biostability and lifetime.

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