Biochimie 92 (2010) 1546–1567

Contents lists available at ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi

Review

Natural and synthetic inhibitors of kallikrein-related peptidases (KLKs)

Peter Goettig [a,*], Viktor Magdolen [b], Hans Brandstetter [a]

[a] Division of Structural Biology, Department of Molecular Biology, University of Salzburg, Billrothstrasse 11, 5020 Salzburg, Austria
[b] Klinische Forschergruppe der Frauenklinik, Klinikum rechts der Isar der TU München, Ismaninger Strasse 22, 81675 München, Germany

ARTICLE INFO

Article history:
Received 24 February 2010
Accepted 29 June 2010
Available online 6 July 2010

Keywords:
Tissue kallikrein
Specificity pockets
Inhibitory compound
Zinc
Rule of five

ABSTRACT

Including the true tissue kallikrein KLK1, kallikrein-related peptidases (KLKs) represent a family of fifteen mammalian serine proteases. While the physiological roles of several KLKs have been at least partially elucidated, their activation and regulation remain largely unclear. This obscurity may be related to the fact that a given KLK fulfills many different tasks in diverse fetal and adult tissues, and consequently, the timescale of some of their physiological actions varies significantly. To date, a variety of endogenous inhibitors that target distinct KLKs have been identified. Among them are the attenuating Zn^{2+} ions, active site-directed proteinaceous inhibitors, such as serpins and the Kazal-type inhibitors, or the huge, unspecific compartment forming α_2-macroglobulin. Failure of these inhibitory systems can lead to certain pathophysiological conditions. One of the most prominent examples is the Netherton syndrome, which is caused by dysfunctional domains of the Kazal-type inhibitor LEKTI-1 which fail to appropriately regulate KLKs in the skin. Small synthetic inhibitory compounds and natural polypeptidic exogenous inhibitors have been widely employed to characterize the activity and substrate specificity of KLKs and to further investigate their structures and biophysical properties. Overall, this knowledge leads not only to a better understanding of the physiological tasks of KLKs, but is also a strong fundament for the synthesis of small compound drugs and engineered biomolecules for pharmaceutical approaches. In several types of cancer, KLKs have been found to be overexpressed, which makes them clinically relevant biomarkers for prognosis and monitoring. Thus, down regulation of excessive KLK activity in cancer and in skin diseases by small inhibitor compounds may represent attractive therapeutical approaches.

1. Introduction

Regulation of protease activity in the living organism is a highly complex task that involves all levels of cellular organization. Control and timing of protease activity starts with gene expression, transcription and translation, and continues with protein targeting and zymogen activation. Once activated, the protease is often kept in check by endogenous inhibitors, while the last steps of protease regulation may be limited proteolysis and final degradation. The emerging research in the field of tissue kallikrein-related peptidases (KLKs) provides many diverse examples for nearly all aspects of protease regulation by inhibitors.

Tissue kallikrein (Kallikrein 1, KLK1) and the kallikrein-related peptidases are (chymo)trypsin-like serine proteases, belonging to family S1A of clan PA(S) according to the MEROPS classification [1]. Prior to the introduction of a new nomenclature in 2006, the KLKs were often referred to as hKs or rKs for human and rat proteins, respectively [2]. Fully sequenced genomes of placental mammals, such as primates or rodents, and even of marsupials (e.g. the opossum), exhibit at least eleven *KLK* genes, but usually lack the counterparts of human *KLK2* and *KLK3* [3,4]. However, the numbers of corresponding proteases vary from ten KLKs in cows, eleven in dogs, and 26 ones in mice. The latter possess a series of functional KLK1 paralogs [2,5,6]. Kallikrein 1 and the kallikrein-related peptidase genes are organized in a single cluster on chromosome locus 19q13.4 [7,8]. The 15 human KLK members are only distantly related to plasma kallikrein, which shares 38% identical residues with KLK1 in the catalytic domain, while KLK1 and trypsin share 46% identity [9,10].

One or more *KLK* genes are expressed in nearly all tissues and fluids of the human body. They fulfill a diverse range of tasks throughout one's lifetime from embryonic development to processes in adulthood [8,11–13]. KLKs are intracellulary synthesized as precursors with a signal peptide (15–34 amino acids) that is cleaved off upon secretion into the endoplasmatic reticulum. The proform or zymogen of the KLK protease is extracellularly activated by the removal of the propeptide (3–37 amino acids), resulting in active proteases of 223–238 residues (Fig. 1), and in some cases reaching molecular weights of up to 50 kDa due to heavy glycosylation [14]. The activation process of KLKs may involve autoactivation [15–17],

* Corresponding author. Tel.: +43 662 8044 7283; fax: +43 662 8044 7209.
E-mail address: peter.goettig@sbg.ac.at (P. Goettig).

0300-9084/$ – see front matter © 2010 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.biochi.2010.06.022

KLK activation cascades [18–20], serine proteases from the thrombostasis axis, such as plasmin, plasma kallikrein, and factor Xa [21], or the proteolytic activity of other proteases, such as urokinase-type plasminogen activator (uPA), matrix metalloproteinases (MMPs), and dipeptidyl peptidase I [22–24]. However, the KLK activity is not restricted to regulation by steroid-dependent expression [25–27] or by fine-tuned zymogen activation. In the case of KLKs 6, 12, and 14, from example, regulation is likely, at least in part, achieved by autolysis [17,28–31]. Furthermore, in some cases an interplay of KLKs and their corresponding natural inhibitors has been established, even with pathophysiological significance [32]. However, many potential inhibitors of KLKs have not yet been unambiguously assigned to a given KLK. Another unusual feature of certain KLKs is the timescale of their activity, which can reach months, if not years, as seen with KLK4 in tooth development, which was also observed in a comparison of *Klk4/lacZ* knockin mice and the wild type [33,34].

Overall, the human KLKs can be subdivided into in several tissue-specific groups with distinct physiological substrates and functions. In the 1920s and 1930s, the first kallikrein (KLK1) was discovered and characterized as a proteolytic enzyme, mainly occurring in urine, kidney, and the pancreas, the latter being the inspiration for the protein's name which is derived from the Greek word for pancreas (καλλικρεας, Kallikreas) [35–37]. A major function of KLK1 is the reduction of blood pressure by releasing the peptide hormone Lys-bradykinin (kallidin) from low molecular weight kininogen, which effects muscle relaxation and inflammatory processes [38]. Knockout of the *Klk1* gene in mice causes both cardiovascular abnormalities and a defect of efficient renal tubular calcium absorption [39,40]. Intriguingly, administration of this protease can reduce cardiac and renal injuries, restenosis, and ischemic stroke and promotes angiogenesis and skin wound healing [41]. Similar to the other "classical" KLKs, KLK 2 and 3, which were discovered in the late 1980s, KLK1 features an extended "99-loop" (also called kallikrein loop) of 11 inserted amino residues with respect to chymotrypsin. Among the "new" KLKs, KLK 4–15 that were gradually characterized from the mid-1990s onwards, KLKs 8–13 possess 99-loop insertions from two to eight residues (see alignment in Fig. 1).

In the prostate, KLKs 2, 3, 4, 11, and to some extent KLKs 14 and 15, are produced for secretion into seminal plasma [8,11]. There, they most likely activate each other in a cascade-like manner resulting in the degradation of semenogelins and fibronectin, mainly by KLK3 and KLK14, for semen liquefaction [42,43]. Since KLK3 (PSA, "prostate specific antigen") blood plasma levels correlate with prostate cancer progression, an immunoassay for PSA has become a widespread medical application, despite its moderate reliability as prognostic biomarker for malignant processes. Thus, there still remains the need for additional specific KLK tumor markers [44–46]. One of these promising markers is KLK4, which is distinctly expressed in the early stages of prostate cancer [47]. Interestingly, KLK4 has the capacity to activate the proform of the urokinase-type plasminogen activator (pro-uPA) and to modulate the activity of its receptor uPAR, both of which play a significant role in prostate and ovarian cancer [48,49]. In addition, KLKs 2, 4, 5, 6, and 14 seem to be potential players in signal transduction via activation of G-coupled protease receptors, such as PARs 1, 2, and 4, resulting, e.g. in inflammation or in tumor cell proliferation and migration [50–56].

In addition to its role in the prostate, which is not yet fully understood, KLK4 is imperative for tooth development, particularly in formation of enamel, which also depends on MMP-20 [57]. Under normal circumstances, both proteases degrade the extracellular matrix required for the growth of dentin crystallites. Single mutations, however, result in either the malfunctioning of MMP-20 or KLK4, causing the hereditary disease *amelogenesis imperfecta*, which is characterized by very fragile teeth [58,59]. More specifically, KLK4 seems to be crucial for the formation of large coherent enamel crystallites, as seen in *Klk4* knockout mice [34].

A larger subset of kallikrein-related peptidases, namely KLKs 5, 7, 11, and 14, is highly expressed in human skin, mainly in the outermost layer, the *stratum corneum*, while KLKs 6, 8, 10, and 13 are found at medium expression levels [11,13,60]. KLKs 5, 7, and 14 are capable of degrading proteins of the corneodesmosomes, leading to desquamation, the shedding of cornified skin cells [61–63]. In contrast, KLK8 is involved in cellular differentiation and healing of the skin [64], similar to KLK6, which induced rapid wound healing by promoting keratinocyte proliferation and migration in a mouse model based on the shedding of E-cadherin by Klk6 [65]. Also, KLKs 4, 5, and 8 specifically activate the metalloproteinases meprin α and/or β, which are located in separate layers of the epidermis [66]. Tight activity regulation of these KLKs by several types of inhibitors is necessary, otherwise diverse skin diseases will develop [67].

Intriguingly, two KLKs, KLK6 and KLK8 (also termed neurosin and neuropsin, respectively), are expressed at higher levels in human brain [8,68]. KLK6 accumulates at brain lesions of humans and investigations in mice suggest that excessive KLK6 activity causes inflammation of the central nervous system and promotes multiple sclerosis through demyelinating activity [69,70]. The physiological role of KLK6 seems to be both de- and remyelination of glia cells, contributing to neurite and axon growth after injuries [71]. In contrast, KLK8, which mostly occurs in the hippocampus, is involved in long term potentiation (LTP) and memory acquisition by restructuring synapses, as shown by mouse models [72–75]. Furthermore, in human brains with Alzheimer's disease a more than 10-fold expression of KLK8 was observed [76]. On the other hand, *Klk8* knockout mice were shown to be susceptible to epileptic seizure [77]. Furthermore, single nucleotide polymorphisms in the human *KLK8* gene are associated with manic-depressive disorder and cognitive impairment [78].

Although the KLK9 protease is present in many tissues and dominates among all KLKs in fetal and adult heart [11], no physiological function has been defined so far, however, it may serve as an ovarian and breast cancer marker [79]. Similarly, KLKs 10, 12, 13 and 15 are associated with distinct cancers without established (patho)physiological roles [17,80,81]. Nevertheless, there are some indications of a tumor suppressor role for KLK10 in breast cancer [82], KLK12 may be involved in angiogenesis regulation [83], KLK13 in ovary tissue remodelling and interleukin processing [84,85], and KLK15 in KLK3/PSA activation [86]. Intriguingly, expression of KLKs in the female reproductive system appears to be complementary to the expression pattern of KLKs in prostate, suggesting an activation cascade that probably involves all KLKs during impregnation [87].

As we will see later for the KLKs, natural inhibitors of proteases often bind directly to the active site, exploiting some degree of complementarity at the interaction surface [88–90]. Expectedly, the more the components of the inhibitor bind to distinct specificity pockets of the protease, the more specificity and affinity can be gained for inhibition [91], which in some cases may be enhanced by additional binding of the inhibitor to so-called exosites [92–94]. Also, knowledge of the substrate specificity of the KLKs will be a guideline for the identification of endogenous (and perhaps exogenous) inhibitors, and for the design of synthetic substrates, as well as of highly specific inhibitors, which may eventually yield powerful pharmaceutical compounds. Numerous studies have investigated the specificity for all KLKs, using either individual chromogenic and fluorogenic substrates, such as in the case of KLKs 8, 12, 15 [17,95–97] or systematic positional scanning approaches for KLKs 3, 4, 5, 6, 7, 10, 11, 13, 14 [98–101], phage display for KLKs 1, 2, 3, 4, 6, 14 [102–107], or peptide libraries for KLKs 1, 2, and 3 [108–110]. In addition, KLK cleavage sites in natural substrates have also been analyzed to a large extent. Since these studies show many differences, sometimes stark