Biol. Chem., Vol. 391, pp. 299–310, April 2010 • Copyright © by Walter de Gruyter • Berlin • New York. DOI 10.1515/BC.2010.038

Review

Kallikrein-related peptidases: proteolysis and signaling in cancer, the new frontier*

Katerina Oikonomopoulou[1,2,a,], Eleftherios P. Diamandis[2,3,b] and Morley D. Hollenberg[1,b]**

[1] Department of Physiology and Pharmacology, University of Calgary, Calgary, Alberta T2N 4N1, Canada
[2] Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, Toronto, Ontario M5T 3L9, Canada
[3] Department of Clinical Biochemistry, University Health Network, Toronto, Ontario M5G 2C4, Canada

**Corresponding author
e-mail: oikoa@mail.med.upenn.edu

Abstract

The exact mechanism(s) by which kallikrein-related peptidases (KLKs) function, their levels of activity and their potential endogenous targets *in vivo* have only recently begun to be revealed. Our group and others have shown that KLKs can have hormonal properties by signaling via proteinase-activated receptors (PARs), a family of G-protein-coupled receptors. Signals by PAR_1, PAR_2, and PAR_4 can regulate calcium release or mitogen-activated protein kinase activation and lead to platelet aggregation, vascular relaxation, cell proliferation, cytokine release, and inflammation. We have further documented the presence of active KLK6 and 10 (by activity-based ELISA or proteomics) and the presence of proteinase inhibitors, such as α_1-antitrypsin, in cancer-derived fluids. We suggest that tumors and inflamed tissues can release active KLKs, which are under tight regulation by proteinase inhibitors. These enzymes can potentially control cell/tissue behavior by regulating PAR activation in specific settings and disease stages.

Keywords: hormone; inflammation; kallikrein-related peptidases; proteases; proteinase-activated receptors; proteinases; signaling; trypsin.

Introduction

The term hormone, by definition, refers to a messenger substance secreted by one tissue into the blood circulation to reach another distant cell, tissue or organ and generate a chemical signal able to regulate tissue function. These characteristics of secretin led Bayliss and Starling (1902) to use the term hormone, possibly inspired by the Greek verb for excite or arouse, *ormao* (Henderson, 2005). Thereafter, several ligands such as amine-derived hormones (e.g., catecholamines), peptides/proteins (e.g., vasopressin and insulin), and lipid/phospholipid-derived mediators (e.g., steroids and prostaglandins) have been categorized as hormones.

According to this classical definition, even proteolytic enzymes can qualify as hormones. For example, thrombin, a well-recognized serine proteinase, is synthesized as a proenzyme in the liver and circulates through the bloodstream to signal to its target tissues (e.g., endothelium and platelets) (Barnhart, 1965). In addition, from a functional perspective, proteolytic enzymes can have other hormone-like characteristics. Apart from their roles in protein catabolism, which are well appreciated, several studies have shown that proteinases may mimic the action of traditional hormones. For example, work by Rieser and Rieser (1964) and Rieser (1967) showed that pepsin and chymotrypsin can mimic insulin action to promote glycogen formation in a rat diaphragm preparation. Soon thereafter it was shown that trypsin, like insulin, can stimulate glucose oxidation and inhibit lipolysis in isolated fat cells (Kono and Barham, 1971). This action of trypsin has been rationalized in terms of the tryptic cleavage of a regulatory domain of the insulin receptor (Shoelson et al., 1988). Furthermore, studies on thrombin and trypsin have revealed that these proteinases, like insulin and epidermal growth factor, can stimulate mitogenesis in cultured cell systems (Burger, 1970; Sefton and Rubin, 1970; Chen and Buchanan, 1975). Thus, the ability of proteinases to fulfill the criteria expected of a hormone and to trigger hormone-like signals in target tissues has been known for almost half a century, but it is only recently that several pieces of the puzzle relating to the mechanisms responsible for the hormonal actions of proteinases have been put together.

Kallikrein-related peptidases as hormones

Kallikrein-related peptidases (KLKs) are synthesized in several tissues and are secreted into the circulation and other major biological fluids, such as cerebrospinal fluid, vaginal fluid, nipple aspirate fluid, and tumor ascites fluid (Borgoño et al., 2004; Borgoño and Diamandis, 2004). Subsequently, KLKs can travel to surrounding and distant cells to exert their biological actions by means of proteolysis. For example, many KLKs, such as KLKs 4, 6, 8 and 10, are secreted by ovarian tumors and/or surrounding stromal cells and are

*This review presents information presented by K.O. as the E.K. Frey-E. Werle Young Investigator Award Address at the 3rd International Symposium on Kallikreins and Kallikrein-related Peptidases, Munich, Germany, August 30–September 2, 2009.

[a] Present address: University of Pennsylvania, School of Medicine, Department of Pathology and Laboratory Medicine, 401 Stellar-Chance labs, 422 Curie Boulevard, Philadelphia, PA 19104-6100, USA.

[b] These authors contributed equally to this work.

thus overexpressed in ascites fluid and/or serum of ovarian cancer patients compared to non-malignant pathologies or normal controls (Borgoño et al., 2004; Borgoño and Diamandis, 2004; Obiezu and Diamandis, 2005). Likewise, KLK2, PSA/KLK3, and KLK11 are similarly highly expressed in prostate cancer patients, whereas KLK5 and 14 are overexpressed in breast carcinomas.

It has been shown *in vitro* that some of these KLKs can proteolytically activate hormones and growth factors, other proteinases or extracellular matrix components (Borgoño et al., 2004; Borgoño and Diamandis, 2004), possibly affecting tissue function (Figure 1). The KLKs can also induce cell proliferation, as demonstrated in the case of keratinocytes (Klucky et al., 2007), prostate-derived tumor cells (Klokk et al., 2007; Mize et al., 2008), and non-small-cell lung carcinoma cells (Heuzé-Vourc'h et al., 2009). Furthermore, it has long been known that kallikrein 1 (KLK1; formerly known as tissue kallikrein) has mitogenic effects by generating active kinin peptides from their kininogen precursor (Figure 1). The kinins can subsequently trigger the activation of the bradykinin B2 G-protein-coupled receptor (Roberts and Gullick, 1989; Bhoola et al., 1992) (Figure 1). These KLKs should not be confused with plasma kallikrein, which can also liberate active kinin peptides from kininogen, but does not belong to the kallikrein-related peptidase family (Schmaier and McCrae, 2007).

Although it is very likely that KLK1 is the only KLK that can signal via the generation of active kinin peptides *in vivo*, our recent studies have shown that KLKs can also regulate cell signaling by cleaving and triggering the activation of proteinase-activated receptors (PARs; Oikonomopoulou et al., 2006a,b, 2007; Figure 1). PAR 1–4 are members of the G-protein-coupled receptor superfamily (Macfarlane et al., 2001; Hollenberg and Compton, 2002). They have been implicated in a number of physiological and pathological signaling pathways in a variety of tissues (Coughlin, 2005; Steinhoff et al., 2005; Ramachandran and Hollenberg, 2008). Thus, we can now consider these receptors as additional targets by which KLKs, like hormones, can transmit their chemical messages in an endocrine, paracrine or autocrine manner.

Pharmacology of PAR signaling by KLKs

We used a pharmacological approach to show that KLKs, represented by KLKs 5, 6, and 14, can mediate activation of PARs 1, 2, and 4 (Oikonomopoulou et al., 2006a,b). The main finding of our study was that signaling via human, rat, and mouse PARs can be regulated by human KLKs (Table 1). More specifically, KLKs 5, 6 and 14 caused PAR-dependent calcium signaling responses in cells expressing PAR_2, as well as endothelium-derived vascular relaxation via PAR_2. Interestingly, we found that among these three KLKs, it was only KLK14 that could also target the disarming/inhibition of PAR_1 and activation of PAR_4, and it could also cause platelet aggregation by means of PAR_4 activation.

Our studies principally focused on PAR_2, a major player in the settings of inflammation and nociception (Vergnolle et al., 2001a,b; Vergnolle, 2004), and on KLK14, a prognostic and diagnostic marker of ovarian and breast carcinoma (Borgoño et al., 2003, 2007b). KLK6 exhibited a comparable but unique potency for activation of PAR_2 compared with KLK14, whereas KLK5 was less efficient as an activator of this receptor (Oikonomopoulou et al., 2006a,b). Furthermore, the actions of these three KLKs were distinct from the effect of plasma kallikrein (which is a related proteinase but not a member of the KLK family), as well as tissue kallikrein (KLK1). It has been reported that these KLKs do not activate PAR_2 (Molino et al., 1997a,b), even though they can both act as agonists to trigger PAR_4 signaling (Houle et al., 2005). It has been suggested that this effect may involve activation of the bradykinin B2 receptor as a result of the KLK1-mediated release of active kinin peptides.

Later work by Stefansson et al. (2008) demonstrated that KLK14 is a strong and KLK5 a moderate activator of PAR_2 (Table 1). However, these authors could not conclude that KLK7 or KLK8 can induce a similar signal via this receptor.

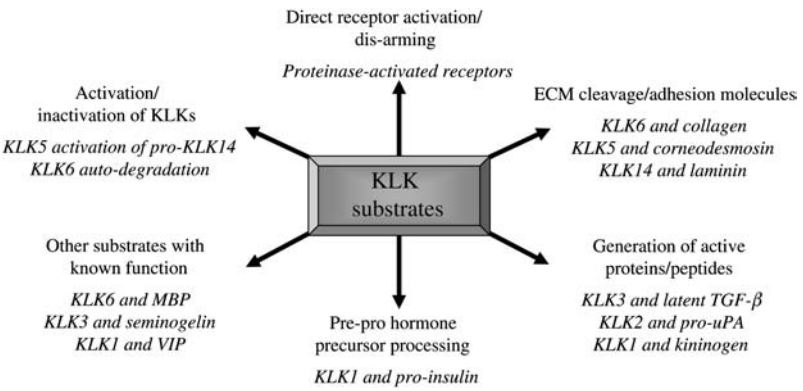

Figure 1 Potential substrates via which KLKs can regulate tissue function.
Selected examples are given for each suggested substrate group. MBP stands for myelin basic protein, VIP for vasoactive intestinal peptide, TGF-β for tissue growth factor type β, and pro-uPA for the zymogen of urokinase plasminogen activator. For details about the potential KLK substrates, see Borgoño et al. (2004), Borgoño and Diamandis (2004) and Sotiropoulou et al. (2009).

Table 1 Kallikrein-related peptidases as regulators of proteinase-activated receptor signalling.

KLK	PAR[a]	Response	Tissue/cell line[b]	Comment	Reference
KLK1	PAR_2	Ca^{2+}; phosphoinositide hydrolysis	HUVEC; COS-1-PAR_2 cells	Negative assay	Molino et al., 1997b
KLK1	PAR_1	Ca^{2+}	NSC34 neurons; Neu7 astrocytes	Negative assay	Vandell et al., 2008
KLK1	PAR_4	Edema	Mouse paw	Indirect activation via kinin-dependent triggering of B2 receptor	Houle et al., 2005
KLK2	PAR_2	ERK	DU145 prostate cancer cells; KOLF-PAR_2 cells	Proliferation	Mize et al., 2008
KLK4	PAR_1	Ca^{2+}	LMF-PAR_1 cells	Possible PAR_1 disarming at high KLK4 concentrations	Ramsay et al., 2008a
KLK4	PAR_2	Ca^{2+} and ERK	LMF-PAR_2 cells	PAR_2 ERK>PAR_2 Ca^{2+}>PAR_1 Ca^{2+}	Ramsay et al., 2008a
KLK4	PAR_2	Ca^{2+}	PC3 prostate cancer cells	PAR_2 and KLK4 co-localization in primary and bone metastatic cancer	Ramsay et al., 2008a
KLK4	PARs 1 and 2	ERK	DU145 prostate cancer cells; KOLF-PAR_1 and KOLF-PAR_2 cells	Proliferation	Mize et al., 2008
KLK4	PAR_1	ERK	WPMY-1 prostatic stromal cells; CHO-TO-PAR_1 cells	Stromal-epithelial interaction: KLK4 (prostatic epithelium) triggers PAR_1 (stroma) to produce IL-6→ secretion of PSA and KLK4 from prostate cancer cells	Wang et al., 2010
KLK5	PAR_2	Ca^{2+}	HEK/KNRK-PAR_2 cells	Different KLK potencies dependent on the expressing system and PAR species	Oikonomopoulou et al., 2006a,b; Stefansson et al., 2008
KLK5	PAR_2	Relaxation	Rat aorta	Endothelium-dependent	Oikonomopoulou et al., 2006a,b
KLK5	PAR_4	Aggregation	Platelets	Negative assay	Oikonomopoulou et al., 2006a,b
KLK5	PAR_2	NF-κβ	Keratinocytes	Upregulation of TLSP confirmed in Netherton syndrome patients	Briot et al., 2009
KLK6	PAR_2	Ca^{2+}	HEK/KNRK-PAR_2 cells	Possible disarming of PAR_2 at high KLK6 concentrations	Oikonomopoulou et al., 2006a,b
KLK6	PAR_2	Relaxation	Rat aorta	Endothelium-dependent	Oikonomopoulou et al., 2006a,b
KLK6	PAR_4	Aggregation	Platelets	Negative assay	Oikonomopoulou et al., 2006a,b
KLK6	PAR_1	Ca^{2+}/ERK	NSC34 neurons	Major part of signaling is mediated by B2 receptor; possible ERK/AKT activation favoring neuronal cell death	Vandell et al., 2008
KLK6	PAR_1 and PAR_2	Ca^{2+}	Neu7 astrocytes	Possible ERK/AKT activation favoring astrocyte survival	Vandell et al., 2008
KLK7	PAR_2	Ca^{2+}	KNRK-PAR_2 cells	Negative assay	Stefansson et al., 2008
KLK8	PAR_2	Ca^{2+}	KNRK-PAR_2 cells	Negative assay	Stefansson et al., 2008
KLK14	PAR_1 and PAR_2	Ca^{2+}	HEK/KNRK-PAR_2 cells	Disarming of PAR_1 at lower KLK14 concentrations	Oikonomopoulou et al., 2006a,b

Table 1 (Continued)

KLK	PAR[a]	Response	Tissue/cell line[b]	Comment	Reference
KLK14	PAR_2	Ca^{2+}	KNRK-PAR_2 cells	KLK14 more potent than KLK5; co-localization of KLK14 and PAR_2 in inflammatory skin disorders (atopic dermatitis and rosacea)	Stefánsson et al., 2008
KLK14	PAR_2	Relaxation	Rat/mouse aorta	Endothelium-dependent; absent in $PAR_2^{-/-}$	Oikonomopoulou et al., 2006a,b
KLK14	PAR_4	Aggregation/Ca^{2+}	Platelets/HEK-PAR_4 cells	KLK14 is the only KLK thus far shown to activate PAR_4 and trigger platelet aggregation	Oikonomopoulou et al., 2006a,b

[a] For the majority of the studies summarized in this Table, investigation of whether the activation or inhibition of a PAR is the result of direct or indirect KLK-mediated proteolytic activity is not always a straightforward procedure. Therefore, the intermediate involvement of other receptors or other KLK-related substrates within the KLK-PAR signaling pathways cannot be excluded.
[b] CHO-TO, Chinese hamster ovary Tet-On; HEK, human embryonic kidney epithelial cells; HUVECs, human umbilical vein endothelial cells; KNRK, kirsten murine sarcoma virus-transformed rat kidney epithelial cells; KOLF, PAR_1 knockout mouse lung fibroblast cells (lacking PAR_1 and PAR_2); LMF, lung murine fibroblasts. HEK cells naturally express PARs 1 and 2, but no other PAR unless otherwise indicated. CHO-TO, COS-1, KOLF, and LMF cells were transfected to overexpress the PAR indicated. KNRK cells were either transfected to express rat PAR_2 (Oikonomopoulou et al., 2006a,b) or human PAR_2 (Stefansson et al., 2008).

In addition, it has been shown that KLK4 can induce signals via PAR_2 in PAR-transfected cells [Ca^{2+} and extracellular signal-regulated kinase (ERK) signaling] and prostate cancer cultured cells (Ca^{2+} signaling), and could also immobilize a lower Ca^{2+} response via PAR_1, but not PAR_4 (Ramsay et al., 2008a; Table 1). This study also showed that KLK4 can act as a more potent activator of PAR_2-mediated ERK1/2 signaling at a lower concentration of the enzyme compared with the Ca^{2+} response triggered by higher levels of the proteinase. Similar work on KLK1 and 6 has also shown that KLK6 can trigger Ca^{2+} release from both cultured neurons (PAR_1-mediated) and astrocytes (PAR_1 or PAR_2-mediated), whereas both KLKs exhibited a variable pattern of regulation of ERK, Jun N-terminal kinase (JNK) and AKT (Vandell et al., 2008; Table 1). Notably, the neuronal KLK1-triggered ERK response and a major part of KLK6 neuronal signaling were attributed to activation of the bradykin B2 receptor.

The data derived from these pharmacological studies, and summarized in Table 1, suggest that the KLKs not only target different members of the G-protein coupled proteinase-activated receptor family or other G-protein coupled receptors, but also exhibit variable pharmacological potencies for cleaving at the same tethered ligand site of catalysis and can trigger distinct G-protein-coupled mechanisms (e.g., G_q-coupled increases in intracellular calcium versus $G_i/G_{12,13}$-coupled activation of mitogen-activated protein kinase). It is therefore important to note the variable signaling potential of the different members of the same enzyme family. We suggest that all human KLKs, as well as KLKs from other species, can have both common and distinct actions via the different PARs in cells and tissues expressing these receptors.

KLKs and PARs in cancer and inflammation

During the past few years, interest in the field of KLK signaling via PARs has increased and it has been confirmed that there is an association between KLK levels and processes associated with both inflammation and cancer. This finding is not surprising, since inflammation and carcinogenesis share important features and should therefore be considered as biochemically and biophysically related pathologies. This idea was first introduced in 1863 by Rudolf Virchow, who pointed out the increased presence of inflammatory cells (leukocytes) in neoplastic tissue (reviewed by Macarthur et al., 2004). The hypothesis linking inflammation to the development of cancer has been regularly revisited, and there are currently several experimental, clinical and epidemiological studies that support a relationship between chronic inflammation and cancer and a possible signaling pathway crosstalk between these two pathological conditions (Coussens and Werb, 2002; Macarthur et al., 2004; Rogers and Fox, 2004; Allavena et al., 2008a,b).

It has also been noted that the extracellular tumor microenvironment can be characterized by the presence of mediators found in inflamed tissues (Macarthur et al., 2004; Allavena et al., 2008a,b). These common mediators include growth factors, proteinases and cytokines, which can pro-

mote cell survival, regulate adaptive immunity, increase angiogenesis, and initiate tissue destruction either to facilitate tumor invasion and metastasis (Macarthur et al., 2004; Allavena et al., 2008b) or to promote the resolution of inflammation. A number of cell-derived proteinases are expressed by either the tumor tissue or stromal cells (including invading leukocytes and macrophages) that share the tumor microenvironment. Apart from the well-appreciated roles of matrix metalloproteinases (MMPs) in either promoting or inhibiting cancer spread (Seiki, 2003; López-Otín and Matrisian, 2007), serine proteinases can also be of considerable importance in the tumor microenvironment. Many of these serine proteinases and metalloproteinases have been implicated in the pathology of ovarian cancer (Stack et al., 1998; Camerer, 2007), including urokinase plasminogen activator (uPA) (Choong and Nadesapillai, 2003), a serine proteinase that converts plasminogen to plasmin.

The pharmacological work mentioned above indicates that KLK4–6 and 14, which are highly expressed tumor-associated proteinases, can be potential stimulators of PARs in the settings of cancer and inflammation (Table 1). Much of the data supporting a role for KLKs in regulating cell function have come from studying the effects of KLKs on PAR-expressing cell lines *in vitro* (either naturally occurring PARs, as in human HEK cells, or as recombinantly expressed receptors in background cells, such as rat KNRK cells). Thus, the next step in testing the hypothesis that PARs might play a role in cancer and inflammation was to design experiments performed with cultured cells and *in vivo* systems related to the settings of a specific disease.

Confirmation of KLK signaling *in vivo*

Data obtained from pharmacological studies using PAR-derived receptor-activating peptides and agonists, known to activate cells and tissues *in vitro*, are of considerable value in predicting what can *potentially* happen in a physiological setting. However, it would be misleading to predict the *in vivo* role of PAR-activating enzymes, such as KLKs, based solely on data obtained *in vitro*. This reservation arises for three main reasons: (a) all of the *in vitro* protocols include an unavoidable bias, since the experiments target only one or two receptors, which are often abundant in the cell expression systems; (b) many of the studies of PAR-mediated calcium signaling have used cells in suspension, so that the impact of the extracellular matrix network on PAR responses has not been taken into account; and (c) cell lines, platelets, and tissues are assayed after extensive washing, which in principle might underestimate a potential role for serine proteinase inhibitors in regulating proteinase-mediated PAR signaling. Therefore, the next important step was to investigate if KLKs can have a functional role *in vivo*.

We used a murine paw edema inflammation model to study the response of KLKs *in vivo* (Vergnolle et al., 1999). Based on our data showing that KLKs mimic the action of trypsin to activate PAR_2 in rodent and human cell systems expressing this receptor, we anticipated that KLKs would also mimic the inflammatory action of trypsin mediated via PAR_2 (Vergnolle et al., 1999, 2001b; Cenac et al., 2002, Nguyen et al., 2003) in this murine paw edema model. KLK14 was able to trigger a murine inflammatory response in this model *in vivo*, an effect that is very likely due to PAR activation (Oikonomopoulou et al., 2006b, 2007). These preliminary data suggest that a more in-depth evaluation of the role played by KLKs and the participation of proteinase inhibitors in this interesting model of inflammatory response triggered either by PARs or via a non-specific inflammatory stimulus, such as carrageenan, will be fruitful in the future.

Prostate cancer

Among the different tumors, prostate cancer is one of the best-studied types of carcinoma in which KLKs are highly expressed (Borgoño et al., 2004; Borgoño and Diamandis, 2004). Of the 15 members of the KLK family, PSA/KLK3 (prostate-specific antigen) has served as the most valuable biomarker for monitoring prostate cancer patients and has been extensively used for the diagnosis and prognosis of the disease (Loeb and Catalona, 2007). Apart from PSA, the closely-related enzyme, KLK2, as well as other members of the family, are promising biomarkers for the detection of prostate cancer (Becker et al., 2001; Borgoño et al., 2004; Borgoño and Diamandis, 2004; Obiezu and Diamandis, 2005). Despite the suggested clinical applicability of monitoring levels of several members of the KLK family by immunoassay for the detection or prognosis of prostate cancer, their biological roles and their levels of enzyme activity are essentially unknown. The same caveat applies for the PARs, which are also highly expressed in primary prostate cancer and prostate tumor-derived cell lines (Ramsay et al., 2008b).

Recent data have pointed for the first time to one of the KLKs as a major potential regulator of prostate tumor signaling (Ramsay et al., 2008a). More specifically, it was shown that KLK4 can initiate Ca^{2+} responses in prostate tumor-derived PC3 cells expressing PARs 1 and 2. It is possible that these signals were generated by preferential PAR_2 activation. KLK4 has also been co-localized with PAR_2 in primary prostate cancer and metastatic prostate cancer bone lesions, pointing to a role for this enzyme in the progression of prostate cancer (Ramsay et al., 2008a). Similar observations have subsequently been reported by another group documenting the proliferative role of KLK2 and 4 in the aggressive and androgen-independent DU145 prostate cancer cells (Mize et al., 2008). This study concluded that KLK4 can trigger ERK-activating signals via both PAR 1 and 2. Interestingly, KLK2 was able to initiate phosphorylation of ERK1/2 only via PAR_2.

A more important observation followed: the KLK4 interaction with PAR_1 and the subsequent initiation of ERK1/2 signaling can play a major role in tumor-stroma interactions in androgen-independent prostate cancer (Wang et al., 2010). Thus far, most of the studies that have evaluated KLKs as potential regulators of PAR signaling have considered tumor cells as the primary targets of KLK action. However, it is possible that stromal cells, rather than the tumor, play the major regulatory role in the tumor microenvironment. In this regard, the study by Wang et al. (2010) provided molecular

and immunohistochemical evidence that KLK4 produced by prostate cancer epithelial cells can trigger, in a paracrine manner, a reaction from the surrounding stroma by means of PAR_1 activation. This action resulted in cytokine release. One of the cytokine molecules released, interleukin (IL)-6, was able to stimulate ERK-dependent signaling by the epithelial cancer cells. This signal in turn caused secretion of KLKs 4 and 3 and resulted in cell proliferation.

Multiple sclerosis

Among the KLKs expressed in the central nervous system (CNS), KLK6 has been implicated in multiple sclerosis (Scarisbrick et al., 2002; Blaber et al., 2004), Alzheimer's disease (Little et al., 1997; Diamandis et al., 2000; Ogawa et al., 2000; Mitsui et al., 2002; Zarghooni et al., 2002), and Parkinson's disease (Ogawa et al., 2000; Iwata et al., 2003). Furthermore, KLK8 (also known as brain serine peptidase or neuropsin) may play a role in synaptic plasticity (Shimizu et al., 1998; Yousef et al., 2003), long-term potentiation (Tamura et al., 2006), and neurodegeneration (Terayama et al., 2007). Several KLKs are also highly expressed by immune cells, such as T-cells and macrophages, in inflammatory conditions like multiple sclerosis, and are under steroid hormone regulation (Scarisbrick et al., 2002, 2006). Recent data suggest that KLKs 1 and 6 can serve as serological markers of progressive multiple sclerosis and contribute directly to the development of neurodegeneration (Scarisbrick et al., 2008). Given that the KLKs can regulate PAR_2 activity and that PAR_2 has been implicated in a murine experimental autoimmune encephalitis model of multiple sclerosis (Noorbakhsh et al., 2006), we can predict that a PAR_2-KLK interaction may play an important role not only in multiple sclerosis, but also in other types of neurodegenerative diseases. It has also been shown that many of the PARs are highly expressed both in the central and peripheral nervous system by neurons and their associated cells, such as astrocytes (Steinhoff et al., 2005; Luo et al., 2007). PARs 1 and 2, specifically expressed in neuronal cells, are primarily responsible for triggering inflammation (Steinhoff et al., 2000; de Garavilla et al., 2001). Therefore, interactions of KLKs with many members of the PAR family in neurological disease are likely.

Inspired by these observations, a recent study has investigated the hypothesis that KLKs 1 and 6 are physiological regulators of PAR signaling in neurons and astrocytes (Vandell et al., 2008). The authors found that KLK6 could evoke PAR_1-dependent signaling responses in neurons and astrocytes, whereas activation of PAR_2 occurred only in the case of astrocytes, which express low levels of PAR_1. Furthermore, it was found that KLK1 acts primarily via the bradykinin B2 receptor. It is intriguing that the KLK6 responses were the result of the combined activation of both PAR_1 and B2 receptors. These findings point to a potential major regulatory role for the KLKs and the bradykinin receptor in the CNS, complementary to the role of the PARs, possibly involving a synergistic interaction between the two receptors. The authors postulated that signaling via these mechanisms could promote astrocyte survival while triggering neuronal death (Vandell et al., 2008).

Skin pathology

Three KLKs, KLK5, 7 and 14, have been isolated in their active forms from the outermost layers of the stratum corneum (Hansson et al., 1994; Brattsand and Egelrud, 1999; Stefansson et al., 2006). Other KLKs are also expressed in the epidermis (Komatsu et al., 2005, 2006). Despite the fact that accumulating data indicate the involvement of many KLKs in skin (patho)physiology, knowledge of their specific contributions to trypsin- or chymotrypsin-like activity in the skin is still limited. A KLK cascade involving KLKs 5 and 7 has been identified and may effect skin desquamation via the degradation of intercellular (corneo)desmosomal adhesion molecules (Caubet et al., 2004; Brattsand et al., 2005). Moreover, it has been suggested that KLK5, 6, and 14 are also involved in this process owing to their immunolocalization and ability to degrade desmoglein-1, one of the adhesive proteins in the corneodesmosome (Borgoño et al., 2007a). Finally, of the skin-abundant KLKs, KLK5 regulates signaling responses via PAR_2 (Oikonomopoulou et al., 2006a,b; Stefansson et al., 2008). The activity of several of the aforementioned KLKs can be regulated by the proteinase inhibitor lymphoepithelial Kazal-type-related inhibitor (LEKTI; Borgoño et al., 2007a; Deraison et al., 2007). Genetic mutations of LEKTI diminish its inhibitory activity and therefore increase constitutive proteinase activity, resulting in stratum corneum overdesquamation, which can lead to pathological conditions of the skin, such as Netherton syndrome, in which several KLKs are also overexpressed (Chavanas et al., 2000; Komatsu et al., 2008).

Recent data have shed additional light on the potential role of one of these KLKs in the pathobiology of Netherton syndrome (Briot et al., 2009). The authors proposed a signaling pathway in which KLK5 has the leading role and activates skin PAR_2 to increase expression of proinflammatory cytokines and chemokines. More specifically, KLK5 caused a nuclear factor-κB (NF-κβ)-mediated induction of thymic stromal lymphopoietin (TSLP), tumor necrosis factor α, and IL-8, as well as an increase in intercellular adhesion molecule 1 in human primary keratinocytes. TSLP secretion was further confirmed in keratinocytes from patients with Netherton syndrome. These data indicated that KLK5 hyperactivity in the epidermis of patients can trigger skin inflammation via PAR_2, independently of environmental stimuli and the adaptive immune system (Briot et al., 2009).

The data summarized in this manuscript have now added KLKs 1, 2, 4–6 and 14 to the list of potential regulators of PARs. The studies discussed indicate that active KLKs can single out these receptors as targets in the settings of cancer and inflammation *in vivo*. However, the exact roles assumed by KLKs within a specific (patho)physiological setting will depend on the absolute amount of enzyme activity. This issue raises a very important question: what are the levels of *enzymatically active* KLKs released by tumor or stromal cells into biological fluids related to inflammation and cancer? These secretions may be a significant reservoir of active pro-

teinases capable of regulating signaling in tumor and inflammatory cells.

Activity of KLKs in cancer-derived fluids

Activity-based probe ELISA

To investigate the levels of KLK activity in cancer-derived fluids with high sensitivity and specificity, we used a biotinylated activity-based probe (ABP) with selectivity for trypsin-like serine proteinases (Bio-PK-DPP 4; Pan et al., 2006; Figure 2A). Since KLKs are all secreted proteins, we reasoned that this type of ABP would be an efficient reagent to target KLK activity in biological fluids. This approach, targeting secreted enzymes, circumvents problems encountered with the use of biotin-tagged reagents for cell-based analyses, because in cells or tissues the high levels of endogenous background biotin-containing proteins can be problematic (Sadaghiani et al., 2007; Fonović et al., 2007). The ABP we used was designed in accordance with the strategy for tagging serine proteinases with a biotinylated diphenylphosphonate probe (Hawthorne et al., 2004). The phosphonate reactive group (which alkylates the serine residue within the active site of any serine proteinase) is preceded by a proline-lysine sequence in the P2/P1 enzyme target site to restrict the probe specificity to trypsin-like serine proteinases (Figure 2A). This enzyme target is separated from the biotin tag with an N-terminal-attached spacer (Bio-PK-DPP 4; Pan et al., 2006; Paulick and Bogyo, 2008). The interaction between the probe and the enzyme is characterized as a suicide inhibitor mechanism, such that the inhibitor is covalently and irreversibly bound to the proteinase. This reaction mimics the interaction of serine proteinases with many of their naturally occurring inhibitors belonging to the family of serpins (Law et al., 2006).

In a previous study, we used immunological isolation to capture KLK6 reactive to the ABP from cerebrospinal fluids, cultured cancer cell supernatants, and ovarian cancer-associated ascites fluids (Oikonomopoulou et al., 2008). By detecting the biotin moiety of the probe, we showed that only a low proportion (approx. 0.1–5% of the total enzyme) of the immunoreactive KLK6 in such samples represents active enzyme not complexed to inhibitors. However, even though the absolute concentrations of active enzyme in our clinical and cell-derived samples were low, the total amount of enzyme, if generated in a restricted environment, would in principle be sufficient to cause cell and tissue signals. Furthermore, the *total* levels of immunoreactive KLK6 (possibly in zymogen form) in all the samples we surveyed were relatively high. Thus, a change in the microenvironment (e.g., an increase in pH) or an increase in the levels of a KLK-activating proteinase could potentially trigger the generation of active KLK6 from the zymogen reservoir.

Therefore, the active enzyme produced by tissues at a localized site could be present in concentrations able to cleave and regulate PARs, as well as cleave extracellular matrix molecules, all of which have been established as targets of KLK6 *in vitro*. This proteolytic activity could contribute to disease pathogenesis (Borgoño and Diamandis, 2004).

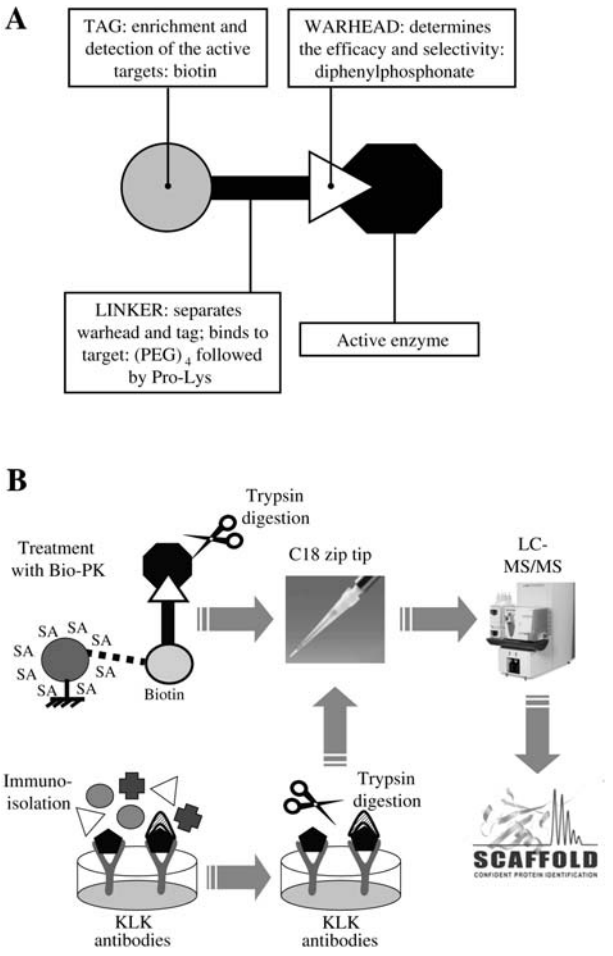

Figure 2 Activity-based probe for serine proteinases with trypsin-like activity.
(A) Structure of the Bio-PK-DPP 4 probe. The probe consists of three groups: a biotin molecule followed by a linker moiety poly(ethylene glycol)$_4$ or (PEG)$_4$ joined to a P2/P1 enzyme target site, Pro-Lys-peptide, followed by a warhead, diphenylphosphonate, which alkylates the active serine of the proteinase. The biotin tag facilitates enrichment and detection of active trypsin-like serine proteinases covalently bound to the probe. For details, see Pan et al. (2006). (B) Functional proteomics analysis of ovarian cancer ascites fluid. Samples were enriched either (1) for active proteinases using the activity-based probe followed by immobilization to Streptavidin beads (SA), or (2) for the total content of kallikrein-related peptidases, captured by KLK-specific monoclonal antibodies. Captured proteins (KLKs: black pentagons; KLK complexes: grey arrowheads) were fragmented with trypsin and subjected to mass spectrometric identification. For details, see Oikonomopoulou et al. (2010).

ABP proteomics

Having examined the activity of KLK6 in cancer-derived fluids, question to address was whether several members of the KLK family and other enzyme classes can be present and active in the same microenvironment. These proteinases can

form potential cascades that may lead to activation of the effector enzyme and progression of the carcinogenic or inflammatory effect. To test this hypothesis, we coupled the ABP used for the immunofluorometric detection assay with tandem mass spectrometry to investigate the presence of active proteinases in ovarian cancer ascites, one of the best-studied cancer-related fluids (Figure 2B).

This protocol took advantage of the chemical properties of the probe to facilitate sample enrichment and specific isolation of active proteinases, with preference for cleavage after Lys, from the complex proteome of a biological fluid such as ovarian cancer ascites (Oikonomopoulou et al., 2010). We also included parallel enrichment of the same fluids using KLK-specific antibodies to replace the probes (Figure 2B). We anticipated that the isolated proteins would be either active KLKs or partners of these peptidases in complex with the enzymes.

Despite the low overall serine proteinase activity of ovarian cancer ascites, our preliminary work using the ABP proteomics approach singled out KLK10 among the active serine proteinases of ovarian cancer ascites fluid (Oikonomopoulou et al., 2010). By contrast, other KLKs abundantly expressed in ovarian cancer ascites as indicated by immunoassay results (such as KLKs 5, 6, and 8) remained undetected by the ABP assay and were therefore presumed to be enzymatically inactive (zymogens, fragmented proteinases or proteinases complexed to inhibitors). In keeping with this conclusion, the four members of the family detected in ovarian cancer ascites fluid by immunoassay (KLKs 5, 6, 8, and 10) were also detected by mass spectral analysis, along with proteinase inhibitors. This finding suggests that enzymatically active KLKs can be generated in the setting of cancer, but the enzymes are rapidly sequestered by inhibitors in the environment of tumors.

Conclusions and future directions

Our work to date has focused on the KLK family of serine proteinases, which are highly expressed in various types of cancer and inflammation. As summarized in Figure 1, there are many potential target substrates for these enzymes that can lead to their biological effects *in vivo* in the settings of a tumor or an inflamed tissue. In this regard, an extensive body of literature suggests a role for the measurement of immunoreactive KLK levels in the diagnosis, prognosis or monitoring of several types of cancer, including ovarian, prostate, and breast cancers (Borgoño et al., 2004; Borgoño and Diamandis, 2004). For example, KLK3 is an established biomarker for monitoring of prostate cancer patients. Another member of the family, KLK6, is a putative biomarker of ovarian cancer prognosis and response to treatment. However, apart from KLK3, no other KLK, measured either alone or in combination with other members of the KLK family, has thus far served as an optimal tumor marker with clinical utility for cancer diagnosis and prognosis. Furthermore, despite recent reports documenting KLK expression in inflammatory diseases (Scarisbrick et al., 2008), strong evidence of the role of KLKs in these settings is only starting to emerge.

We suggest that identification of the functional roles of KLKs will facilitate their use as potential disease markers in the settings of inflammation and cancer. It will therefore be important to investigate the signaling pathways that KLKs activate downstream of PARs, or other as yet unknown receptors, to trigger inflammatory and tumorigenic responses *in vivo*. PAR-null animals are a valuable tool that can be used to investigate the role of these receptors in KLK-mediated inflammation and carcinogenesis.

Furthermore, it is essential to determine the proportion of KLKs detected by immunoassay that are catalytically active *in vivo* in inflamed tissues, tumor cells and related fluids, or in samples derived from patients experiencing chronic inflammation and pain. As knowledge on the catalytic specificity of serine proteinases and their endogenous substrates is rapidly increasing, selection of an optimal probe for activity-based assays will significantly increase both the sensitivity and specificity of proteinase detection. Despite the restriction of such activity-based tools to specific groups of proteinases, they have the potential to be used in clinical practice. In this regard, we foresee a major clinical importance for our recently developed high-throughput ABP proteomics concept, which may facilitate rapid identification or quantification of all active proteinases (e.g., serine, cysteine, metalloproteinases) in clinical samples.

The levels of proteinase inhibitors in pathological settings may also be used as clinical markers of disease. Supporting this proposal is the finding that the percentage of free KLK3 (not complexed to inhibitors but mainly enzymatically inactive) is lower in serum samples from patients with prostate cancer, which, with the use of the appropriate set of probes, may be associated with a more aggressive form of prostate cancer for reasons that are yet unknown (Carter et al., 1997; Arcangeli et al., 1998; Catalona et al., 1998).

The activity-based proteomics approach can also serve to identify simultaneously active KLKs or other serine proteinases in several inflammatory or tumor settings. Targeting the analysis of multiple active proteinases (and their inhibitors) may provide insights into the network of proteinases critical for the inflammatory response or for tumor survival and progression. This information may point the way to appropriately customized therapeutic modalities.

Finally, it is of major importance to delineate the specific components that act both upstream and downstream of KLKs and PARs. Identification of naturally occurring specific activators or inhibitors of KLK activity *in vivo* may lead to the development of regimens for the treatment of diseases such as cancer, multiple sclerosis and Netherton syndrome. Furthermore, as discussed, the different KLKs may have differential effects on cells and tissues via the activation of multiple signaling pathways. These effects may also depend on the presence of several co-factors. Identification of these distinct signal pathways will also contribute to the selection of therapeutic targets for the treatment of patients suffering from inflammatory diseases or cancer in which KLKs may be involved.

In summary, the future of KLK research will involve delineation of the components of the signaling pathways

upstream or downstream of the active proteinases and their receptors, as well as their levels of function and activity. These findings may lead to the development of clinical assays for disease detection, prognosis or monitoring, as well as to the production of therapeutic compounds for pathological conditions, such as cancer and inflammation.

Acknowledgments

We are most grateful to the E.K. Frey-E. Werle Foundation and the IKS 2009 organizing committee for a Young Investigator Award to K.O. The award led to the presentation of much of the data summarized in this article, as presented at the 3rd International Symposium on Kallikreins and Kallikrein-Related Peptidases (IKS 2009), Munich, Germany (August 30–September 2, 2009). Some of the data in the article are also found in the Doctoral Thesis of K.O. (Kallikrein-related peptidase signaling via proteinase-activated receptors, University of Toronto, 2008) based on work carried out under the joint mentorship of Dr. E.P. Diamandis and Dr. M.D. Hollenberg. Contributions to this work in a number of ways by the following are gratefully acknowledged: Dr. N. Vergnolle, Dr. A. Baruch, Dr. K.K. Hansen, Dr. M. Saiffedine, A. Soosaipillai, K. Chapman, B. Renaux, T. Earle, L. Grass, Dr. R. Ramachadran, and Dr. J. Yu. Part of the studies described in this manuscript were made possible by term grants from the Canadian Institutes of Health Research (CIHR; E.P.D. and M.D.H.) and by support for K.O. from an Alberta Heritage Foundation for Medical Research (AHFMR) Postdoctoral Fellowship, an Ontario Graduate Scholarship (OGS) and the Peterborough K.M. Hunter Graduate Studentship.

References

Allavena, P., Garlanda, C., Borrello, M.G., Sica, A., and Mantovani, A. (2008a). Pathways connecting inflammation and cancer. Curr. Opin. Genet. Dev. *18*, 3–10.

Allavena, P., Sica, A., Solinas, G., Porta, C., and Mantovani, A. (2008b). The inflammatory micro-environment in tumor progression: the role of tumor-associated macrophages. Crit. Rev. Oncol. Hematol. *66*, 1–9.

Arcangeli, C.G., Humphrey, P.A., Smith, D.S., Harmon, T.J., Shepherd, D.L., Keetch, D.W., and Catalona, W.J. (1998). Percentage of free serum prostate-specific antigen as a predictor of pathological features of prostate cancer in a screening population. Urology *51*, 558–564.

Barnhart, M.I. (1965). Prothrombin synthesis: an example of hepatic function. J. Histochem. Cytochem. *13*, 740–751.

Bayliss, W.M. and Starling, E.H. (1902). The mechanism of pancreatic secretion. J. Physiol. *28*, 325–353.

Becker, C., Noldus, J., Diamandis, E., and Lilja, H. (2001). The role of molecular forms of prostate-specific antigen (PSA or hK3) and of human glandular kallikrein 2 (hK2) in the diagnosis and monitoring of prostate cancer and in extra-prostatic disease. Crit. Rev. Clin. Lab. Sci. *38*, 357–399.

Bhoola, K.D., Figueroa, C.D., and Worthy, K. (1992). Bioregulation of kinins: kallikreins, kininogens, and kininases. Pharmacol. Rev. *44*, 1–80.

Blaber, S.I., Ciric, B., Christophi, G.P., Bernett, M.J., Blaber, M., Rodriguez, M., and Scarisbrick, I.A. (2004). Targeting kallikrein 6 proteolysis attenuates CNS inflammatory disease. FASEB J. *18*, 920–922.

Borgoño, C.A. and Diamandis, E.P. (2004). The emerging roles of human tissue kallikreins in cancer. Nat. Rev. Cancer *4*, 876–890.

Borgoño, C.A., Grass, L., Soosaipillai, A., Yousef, G.M., Petraki, C.D., Howarth, D.H., Fracchioli, S., Katsaros, D., and Diamandis, E.P. (2003). Human kallikrein 14: a new potential biomarker for ovarian and breast cancer. Cancer Res. *63*, 9032–9041.

Borgoño, C.A., Michael, I.P., and Diamandis, E.P. (2004). Human tissue kallikreins: physiologic roles and applications in cancer. Mol. Cancer Res. *2*, 257–280.

Borgoño, C.A., Michael, I.P., Komatsu, N., Jayakumar, A., Kapadia, R., Clayman, G.L., Sotiropoulou, G., and Diamandis, E.P. (2007a). A potential role for multiple tissue kallikrein serine proteases in epidermal desquamation. J. Biol. Chem. *282*, 3640–3652.

Borgoño, C.A., Michael, I.P., Shaw, J.L., Luo, L.Y., Ghosh, M.C., Soosaipillai, A., Grass, L., Katsaros, D., and Diamandis, E.P. (2007b). Expression and functional characterization of the cancer-related serine protease, human tissue kallikrein 14. J. Biol. Chem. *282*, 2405–2422.

Brattsand, M. and Egelrud, T. (1999). Purification, molecular cloning, and expression of a human stratum corneum trypsin-like serine protease with possible function in desquamation. J. Biol. Chem. *274*, 30033–30040.

Brattsand, M., Stefansson, K., Lundh, C., Haasum, Y., and Egelrud, T. (2005). A proteolytic cascade of kallikreins in the stratum corneum. J. Invest. Dermatol. *124*, 198–203.

Briot, A., Deraison, C., Lacroix, M., Bonnart, C., Robin, A., Besson, C., Dubus, P., and Hovnanian, A. (2009). Kallikrein 5 induces atopic dermatitis-like lesions through PAR2-mediated thymic stromal lymphopoietin expression in Netherton syndrome. J. Exp. Med. *206*, 1135–1147.

Burger, M.M. (1970). Proteolytic enzymes initiating cell division and escape from contact inhibition of growth. Nature *227*, 170–171.

Camerer, E. (2007). Protease signaling in tumor progression. Thromb. Res. *120*, S75–S81.

Carter, H.B., Partin, A.W., Luderer, A.A., Metter, E.J., Landis, P., Chan, D.W., Fozard, J.L., and Pearson, J.D. (1997). Percentage of free prostate-specific antigen in sera predicts aggressiveness of prostate cancer a decade before diagnosis. Urology *49*, 379–384.

Catalona, W.J., Partin, A.W., Slawin, K.M., Brawer, M.K., Flanigan, R.C., Patel, A., Richie, J.P., deKernion, J.B., Walsh, P.C., Scardino, P.T., et al. (1998). Use of the percentage of free prostate-specific antigen to enhance differentiation of prostate cancer from benign prostatic disease: a prospective multicenter clinical trial. J. Am. Med. Assoc. *279*, 1542–1547.

Caubet, C., Jonca, N., Brattsand, M., Guerrin, M., Bernard, D., Schmidt, R., Egelrud, T., Simon, M., and Serre, G. (2004). Degradation of corneodesmosome proteins by two serine proteases of the kallikrein family, SCTE/KLK5/hK5 and SCCE/KLK7/hK7. J. Invest. Dermatol. *122*, 1235–1244.

Cenac, N., Coelho, A.M., Nguyen, C., Compton, S., Andrade-Gordon, P., MacNaughton, W.K., Wallace, J.L., Hollenberg, M.D., Bunnett, N.W., Garcia-Villar, R., et al. (2002). Induction of intestinal inflammation in mouse by activation of proteinase-activated receptor-2. Am. J. Pathol. *161*, 1903–1915.

Chavanas, S., Bodemer, C., Rochat, A., Hamel-Teillac, D., Ali, M., Irvine, A.D., Bonafé, J.L., Wilkinson, J., Taïeb, A., Barrandon, Y., et al. (2000). Mutations in *SPINK5*, encoding a serine protease inhibitor, cause Netherton syndrome. Nat. Genet. *25*, 141–142.

Chen, L.B. and Buchanan, J.M. (1975). Mitogenic activity of blood components. I. Thrombin and prothrombin. Proc. Natl. Acad. Sci. USA *72*, 131–135.

Choong, P.F. and Nadesapillai, A.P. (2003). Urokinase plasminogen activator system: a multifunctional role in tumor progression and metastasis. Clin. Orthop. Relat. Res. *415*, S46–S58.

Coughlin, S.R. (2005). Protease-activated receptors in hemostasis, thrombosis and vascular biology. J. Thromb. Haemost. *3*, 1800–1814.

Coussens, L.M. and Werb, Z. (2002). Inflammation and cancer. Nature *420*, 860–867.

de Garavilla, L., Vergnolle, N., Young, S.H., Ennes, H., Steinhoff, M., Ossovskaya, V.S., D'Andrea, M.R., Mayer, E.A., Wallace, J.L., Hollenberg, M.D., et al. (2001). Agonists of proteinase-activated receptor 1 induce plasma extravasation by a neurogenic mechanism. Br. J. Pharmacol. *133*, 975–987.

Deraison, C., Bonnart, C., Lopez, F., Besson, C., Robinson, R., Jayakumar, A., Wagberg, F., Brattsand, M., Hachem, J.P., Leonardsson, G., et al. (2007). LEKTI fragments specifically inhibit KLK5, KLK7, and KLK14 and control desquamation through a pH-dependent interaction. Mol. Biol. Cell *18*, 3607–3619.

Diamandis, E.P., Yousef, G.M., Petraki, C., and Soosaipillai, A.R. (2000). Human kallikrein 6 as a biomarker of Alzheimer's disease. Clin. Biochem. *33*, 663–667.

Fonović, M., Verhelst, S.H., Sorum, M.T., and Bogyo, M. (2007). Proteomics evaluation of chemically cleavable activity-based probes. Mol. Cell. Proteomics *6*, 1761–1770.

Hansson, L., Strömqvist, M., Bäckman, A., Wallbrandt, P., Carlstein, A., and Egelrud, T. (1994). Cloning, expression, and characterization of stratum corneum chymotryptic enzyme. A skin-specific human serine proteinase. J. Biol. Chem. *269*, 19420–19426.

Hawthorne, S., Hamilton, R., Walker, B.J., and Walker, B. (2004). Utilization of biotinylated diphenyl phosphonates for disclosure of serine proteases. Anal. Biochem. *326*, 273–275.

Henderson, J. (2005). Ernest Starling and 'hormones': an historical commentary. J. Endocrinol. *184*, 5–10.

Heuzé-Vourc'h, N., Planque, C., Guyetant, S., Coco, C., Brillet, B., Blechet, C., Parent, C., Laurent, B., Reverdiau, P., Jourdan, M.L., et al. (2009). High kallikrein-related peptidase 6 in non-small cell lung cancer cells: an indicator of tumor proliferation and poor prognosis. J. Cell. Mol. Med., in press. DOI: 10.1111/j.1582-4934.2009.00763.x.

Hollenberg, M.D. and Compton, S.J. (2002). International Union of Pharmacology. XXVIII. Proteinase-activated receptors. Pharmacol. Rev. *54*, 203–217.

Houle, S., Papez, M.D., Ferazzini, M., Hollenberg, M.D., and Vergnolle, N. (2005) Neutrophils and the kallikrein-kinin system in proteinase-activated receptor 4-mediated inflammation in rodents. Br. J. Pharmacol. *146*, 670–678.

Iwata, A., Maruyama, M., Akagi, T., Hashikawa, T., Kanazawa, I., Tsuji, S., and Nukina, N. (2003). α-Synuclein degradation by serine protease neurosin: implication for pathogenesis of synucleinopathies. Hum. Mol. Genet. *12*, 2625–2635.

Klokk, T.I., Kilander, A., Xi, Z., Waehre, H., Risberg, B., Danielsen, H.E., and Saatcioglu, F. (2007). Kallikrein 4 is a proliferative factor that is overexpressed in prostate cancer. Cancer Res. *67*, 5221–5230.

Klucky, B., Mueller, R., Vogt, I., Teurich, S., Hartenstein, B., Breuhahn, K., Flechtenmacher, C., Angel, P., and Hess, J. (2007). Kallikrein 6 induces E-cadherin shedding and promotes cell proliferation, migration, and invasion. Cancer Res. *67*, 8198–8206.

Komatsu, N., Saijoh, K., Sidiropoulos, M., Tsai, B., Levesque, M.A., Elliott, M.B., Takehara, K., and Diamandis, E.P. (2005). Quantification of human tissue kallikreins in the stratum corneum: dependence on age and gender. J. Invest. Dermatol. *125*, 1182–1189.

Komatsu, N., Tsai, B., Sidiropoulos, M., Saijoh, K., Levesque, M.A., Takehara, K., and Diamandis, E.P. (2006). Quantification of eight tissue kallikreins in the stratum corneum and sweat. J. Invest. Dermatol. *126*, 925–929.

Komatsu, N., Saijoh, K., Jayakumar, A., Clayman, G.L., Tohyama, M., Suga, Y., Mizuno, Y., Tsukamoto, K., Taniuchi, K., Takehara, K., et al. (2008). Correlation between SPINK5 gene mutations and clinical manifestations in Netherton syndrome patients. J. Invest. Dermatol. *128*, 1148–1159.

Kono, T. and Barham, F.W. (1971). Insulin-like effects of trypsin on fat cells. Localization of the metabolic steps and the cellular site affected by the enzyme. J. Biol. Chem. *246*, 6204–6209.

Law, R.H., Zhang, Q., McGowan, S., Buckle, A.M., Silverman, G.A., Wong, W., Rosado, C.J., Langendorf, C.G., Pike, R.N., Bird, P.I., et al. (2006). An overview of the serpin superfamily. Genome Biol. *7*, 216.

Little, S.P., Dixon, E.P., Norris, F., Buckley, W., Becker, G.W., Johnson, M., Dobbins, J.R., Wyrick, T., Miller, J.R., MacKellar, W., et al. (1997). Zyme, a novel and potentially amyloidogenic enzyme cDNA isolated from Alzheimer's disease brain. J. Biol. Chem. *272*, 25135–25142.

Loeb, S. and Catalona, W.J. (2007). Prostate-specific antigen in clinical practice. Cancer Lett. *249*, 30–39.

López-Otín, C. and Matrisian, L.M. (2007). Emerging roles of proteases in tumour suppression. Nat. Rev. Cancer *7*, 800–808.

Luo, W., Wang, Y., and Reiser, G. (2007). Protease-activated receptors in the brain: receptor expression, activation, and functions in neurodegeneration and neuroprotection. Brain Res. Rev. *56*, 331–345.

Macarthur, M., Hold, G.L., and El-Omar, E.M. (2004). Inflammation and cancer II. Role of chronic inflammation and cytokine gene polymorphisms in the pathogenesis of gastrointestinal malignancy. Am. J. Physiol. Gastrointest. Liver Physiol. *286*, G515–G520.

Macfarlane, S.R., Seatter, M.J., Kanke, T., Hunter, G.D., and Plevin, R. (2001). Proteinase-activated receptors. Pharmacol. Rev. *53*, 245–282.

Mitsui, S., Okui, A., Uemura, H., Mizuno, T., Yamada, T., Yamamura, Y., and Yamaguchi, N. (2002). Decreased cerebrospinal fluid levels of neurosin (KLK6), an aging-related protease, as a possible new risk factor for Alzheimer's disease. Ann. N.Y. Acad. Sci. *977*, 216–223.

Mize, G.J., Wang, W., and Takayama, T.K. (2008). Prostate-specific kallikreins-2 and -4 enhance the proliferation of DU-145 prostate cancer cells through protease-activated receptors-1 and -2. Mol. Cancer Res. *6*, 1043–1051.

Molino, M., Barnathan, E.S., Numerof, R., Clark, J., Dreyer, M., Cumashi, A., Hoxie, J.A., Schechter, N., Woolkalis, M., and Brass, L.F. (1997a). Interactions of mast cell tryptase with thrombin receptors and PAR-2. J. Biol. Chem. *272*, 4043–4049.

Molino, M., Woolkalis, M.J., Reavey-Cantwell, J., Pratico, D., Andrade-Gordon, P., Barnathan, E.S., and Brass, L.F. (1997b). Endothelial cell thrombin receptors and PAR-2. Two protease-activated receptors located in a single cellular environment. J. Biol. Chem. *272*, 11133–11141.

Nguyen, C., Coelho, A.M., Grady, E., Compton, S.J., Wallace, J.L., Hollenberg, M.D., Cenac, N., Garcia-Villar, R., Bueno, L., Steinhoff, M., et al. (2003). Colitis induced by proteinase-activated receptor-2 agonists is mediated by a neurogenic mechanism. Can. J. Physiol. Pharmacol. *81*, 920–927.

Noorbakhsh, F., Tsutsui, S., Vergnolle, N., Boven, L.A., Shariat, N., Vodjgani, M., Warren, K.G., Andrade-Gordon, P., Hollenberg,

M.D., and Power, C. (2006). Proteinase-activated receptor 2 modulates neuroinflammation in experimental autoimmune encephalomyelitis and multiple sclerosis. J. Exp. Med. *203*, 425–435.

Obiezu, C.V. and Diamandis, E.P. (2005). Human tissue kallikrein gene family: applications in cancer. Cancer Lett. *224*, 1–22.

Ogawa, K., Yamada, T., Tsujioka, Y., Taguchi, J., Takahashi, M., Tsuboi, Y., Fujino, Y., Nakajima, M., Yamamoto, T., Akatsu, H., et al. (2000). Localization of a novel type trypsin-like serine protease, neurosin, in brain tissues of Alzheimer's disease and Parkinson's disease. Psychiatry Clin. Neurosci. *54*, 419–426.

Oikonomopoulou, K., Hansen, K.K., Saifeddine, M., Tea, I., Blaber, M., Blaber, S.I., Scarisbrick, I., Andrade-Gordon, P., Cottrell, G.S., Bunnett, N.W., et al. (2006a) Proteinase-activated receptors, targets for kallikrein signaling. J. Biol. Chem. *281*, 32095–32112.

Oikonomopoulou, K., Hansen, K.K., Saifeddine, M., Vergnolle, N., Tea, I., Blaber, M., Blaber, S.I., Scarisbrick, I., Diamandis, E.P., and Hollenberg, M.D. (2006b). Kallikrein-mediated cell signalling: targeting proteinase-activated receptors (PARs). Biol. Chem. *387*, 817–824.

Oikonomopoulou, K., Hansen, K.K., Chapman, K., Vergnolle, N., Diamandis, E.P., and Hollenberg, M.D. (2007). Kallikrein-mediated activation of PARs in inflammation and nociception. Inflamm. Res. *56*, S499–S502.

Oikonomopoulou, K., Hansen, K.K., Baruch, A., Hollenberg, M.D., and Diamandis, E.P. (2008). Immunofluorometric activity-based probe analysis of active KLK6 in biological fluids. Biol. Chem. *389*, 747–756.

Oikonomopoulou, K., Batruch, I., Smith, C.R., Soosaipillai, A., Diamandis, E.P., and Hollenberg, M.D. (2010). Functional proteomics of kallikrein-related peptidases in ovarian cancer ascites fluid. Biol. Chem. *391*, 381–390.

Pan, Z., Jeffery, D.A., Chehade, K., Beltman, J., Clark, J.M., Grothaus, P., Bogyo, M., and Baruch, A. (2006). Development of activity-based probes for trypsin-family serine proteases. Bioorg. Med. Chem. Lett. *16*, 2882–2885.

Paulick, M.G. and Bogyo, M. (2008). Application of activity-based probes to the study of enzymes involved in cancer progression. Curr. Opin. Genet. Dev. *18*, 97–106.

Ramachandran, R. and Hollenberg, M.D. (2008). Proteinases and signalling: pathophysiological and therapeutic implications via PARs and more. Br. J. Pharmacol. *153*, S263–S282.

Ramsay, A.J., Dong, Y., Hunt, M.L., Linn, M., Samaratunga, H., Clements, J.A., and Hooper, J.D. (2008a). Kallikrein-related peptidase 4 (KLK4) initiates intracellular signaling via protease-activated receptors (PARs). KLK4 and PAR-2 are co-expressed during prostate cancer progression. J. Biol. Chem. *283*, 12293–12304.

Ramsay, A.J., Reid, J.C., Adams, M.N., Samaratunga, H., Dong, Y., Clements, J.A., and Hooper, J.D. (2008b). Prostatic trypsin-like kallikrein-related peptidases (KLKs) and other prostate-expressed tryptic proteinases as regulators of signalling via proteinase-activated receptors (PARs). Biol. Chem. *389*, 653–668.

Rieser, P. (1967). The insulin-like action of pepsin and pepsinogen. Acta Endocrinol. (Copenh.) *54*, 375–379.

Rieser, P. and Rieser, C.H. (1964). Anabolic responses of diaphragm muscle to insulin and to other pancreatic proteins. Proc. Soc. Exp. Biol. Med. *116*, 669–671.

Roberts, R.A. and Gullick, W.J. (1989). Bradykinin receptor number and sensitivity to ligand stimulation of mitogenesis is increased by expression of a mutant *ras* oncogene. J. Cell Sci. *94*, 527–535.

Rogers, A.B. and Fox, J.G. (2004). Inflammation and Cancer. I. Rodent models of infectious gastrointestinal and liver cancer. Am. J. Physiol. Gastrointest. Liver Physiol. *286*, G361–G366.

Sadaghiani, A.M., Verhelst, S.H., and Bogyo, M. (2007). Tagging and detection strategies for activity-based proteomics. Curr. Opin. Chem. Biol. *11*, 20–28.

Scarisbrick, I.A., Blaber, S.I., Lucchinetti, C.F., Genain, C.P., Blaber, M., and Rodriguez, M. (2002). Activity of a newly identified serine protease in CNS demyelination. Brain *125*, 1283–1296.

Scarisbrick, I.A., Blaber, S.I., Tingling, J.T., Rodriguez, M., Blaber, M., and Christophi, G.P. (2006). Potential scope of action of tissue kallikreins in CNS immune-mediated disease. J. Neuroimmunol. *178*, 167–176.

Scarisbrick, I.A., Linbo, R., Vandell, A.G., Keegan, M., Blaber, S.I., Blaber, M., Sneve, D., Lucchinetti, C.F., Rodriguez, M., and Diamandis, E.P. (2008). Kallikreins are associated with secondary progressive multiple sclerosis and promote neurodegeneration. Biol. Chem. *389*, 739–745.

Schmaier, A.H. and McCrae, K.R. (2007). The plasma kallikrein-kinin system: its evolution from contact activation. J. Thromb. Haemost. *5*, 2323–2329.

Sefton, B.M. and Rubin, H. (1970). Release from density dependent growth inhibition by proteolytic enzymes. Nature *227*, 843–845.

Seiki, M. (2003). Membrane-type 1 matrix metalloproteinase: a key enzyme for tumor invasion. Cancer Lett. *194*, 1–11.

Shimizu, C., Yoshida, S., Shibata, M., Kato, K., Momota, Y., Matsumoto, K., Shiosaka, T., Midorikawa, R., Kamachi, T., Kawabe, A., et al. (1998). Characterization of recombinant and brain neuropsin, a plasticity-related serine protease. J. Biol. Chem. *273*, 11189–11196.

Shoelson, S.E., White, M.F., and Kahn, C.R. (1988). Tryptic activation of the insulin receptor. Proteolytic truncation of the α-subunit releases the β-subunit from inhibitory control. J. Biol. Chem. *263*, 4852–4860.

Sotiropoulou, G., Pampalakis, G., and Diamandis, E.P. (2009). Functional role of human kallikrein-related peptidases. J. Biol. Chem. *284*, 32989–32994.

Stack, M.S., Ellerbroek, S.M., and Fishman, D.A. (1998). The role of proteolytic enzymes in the pathology of epithelial ovarian carcinoma. Int. J. Oncol. *12*, 569–576.

Stefansson, K., Brattsand, M., Ny, A., Glas, B., and Egelrud, T. (2006). Kallikrein-related peptidase 14 may be a major contributor to trypsin-like proteolytic activity in human stratum corneum. Biol. Chem. *387*, 761–768.

Stefansson, K., Brattsand, M., Roosterman, D., Kempkes, C., Bocheva, G., Steinhoff, M., and Egelrud, T. (2008). Activation of proteinase-activated receptor-2 by human kallikrein-related peptidases. J. Invest. Dermatol. *128*, 18–25.

Steinhoff, M., Buddenkotte, J., Shpacovitch, V., Rattenholl, A., Moormann, C., Vergnolle, N., Luger, T.A., and Hollenberg, M.D. (2005). Proteinase-activated receptors: transducers of proteinase-mediated signaling in inflammation and immune response. Endocr. Rev. *26*, 1–43.

Steinhoff, M., Vergnolle, N., Young, S.H., Tognetto, M., Amadesi, S., Ennes, H.S., Trevisani, M., Hollenberg, M.D., Wallace, J.L., Caughey, G.H., et al. (2000). Agonists of proteinase-activated receptor 2 induce inflammation by a neurogenic mechanism. Nat. Med. *6*, 151–158.

Tamura, H., Ishikawa, Y., Hino, N., Maeda, M., Yoshida, S., Kaku, S., and Shiosaka, S. (2006). Neuropsin is essential for early processes of memory acquisition and Schaffer collateral long-term potentiation in adult mouse hippocampus *in vivo*. J. Physiol. *570*, 541–551.

Terayama, R., Bando, Y., Murakami, K., Kato, K., Kishibe, M., and Yoshida, S. (2007). Neuropsin promotes oligodendrocyte death,

demyelination and axonal degeneration after spinal cord injury. Neuroscience *148*, 175–187.

Vandell, A.G., Larson, N., Laxmikanthan, G., Panos, M., Blaber, S.I., Blaber, M., and Scarisbrick, I.A. (2008). Protease-activated receptor dependent and independent signaling by kallikreins 1 and 6 in CNS neuron and astroglial cell lines. J. Neurochem. *107*, 855–870.

Vergnolle, N. (2004). Modulation of visceral pain and inflammation by protease-activated receptors. Br. J. Pharmacol. *141*, 1264–1274.

Vergnolle, N., Hollenberg, M.D., Sharkey, K.A., and Wallace, J.L. (1999). Characterization of the inflammatory response to proteinase-activated receptor-2 (PAR2)-activating peptides in the rat paw. Br. J. Pharmacol. *127*, 1083–1090.

Vergnolle, N., Bunnett, N.W., Sharkey, K.A., Brussee, V., Compton, S.J., Grady, E.F., Cirino, G., Gerard, N., Basbaum, A.I., Andrade-Gordon, P., et al. (2001a). Proteinase-activated receptor-2 and hyperalgesia: a novel pain pathway. Nat. Med. *7*, 821–826.

Vergnolle, N., Wallace, J.L., Bunnett, N.W., and Hollenberg, M.D. (2001b). Protease-activated receptors in inflammation, neuronal signaling and pain. Trends Pharmacol. Sci. *22*, 146–152.

Wang, W., Mize, G.J., Zhang, X., and Takayama, T.K. (2010). Kallikrein-related peptidase-4 initiates tumor-stroma interactions in prostate cancer through protease-activated receptor-1. Int. J. Cancer *126*, 599–610.

Yousef, G.M., Kishi, T., and Diamandis, E.P. (2003). Role of kallikrein enzymes in the central nervous system. Clin. Chim. Acta *329*, 1–8.

Zarghooni, M., Soosaipillai, A., Grass, L., Scorilas, A., Mirazimi, N., and Diamandis, E.P. (2002). Decreased concentration of human kallikrein 6 in brain extracts of Alzheimer's disease patients. Clin. Biochem. *35*, 225–231.

Received November 2, 2009; accepted January 11, 2010