Activation of Proteinase-Activated Receptor-2 by Human Kallikrein-Related Peptidases

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Proteinase-activated receptor-2 (PAR_2) is a seven transmembrane spanning, G-protein-coupled receptor, present on the membrane of many cell types including keratinocytes. In skin, PAR_2 is suggested to play a regulatory role during inflammation, epidermal barrier function, and pruritus. PAR_2 is activated by trypsin-like proteases by a unique mechanism where cleavage of the receptor leads to the release of a small peptide, which activates the receptor as a tethered ligand. The endogenous activators of PAR_2 on keratinocytes have not been identified as of yet. Potential candidates are kallikrein-related peptidases (KLKs) expressed by epidermal cells. Therefore, the ability of four human skin-derived KLKs was examined with regard to their capacity to activate PAR_2 *in vitro*. PAR_2 cleavage was followed by immunofluorescence analysis and functional activation by measurements of changes in intracellular calcium levels. We found that KLK5 and KLK14, but neither KLK7 nor KLK8, induced PAR_2 signalling. We conclude that certain, but not all, epidermal KLKs are capable of activating PAR_2. We could also show the coexpression of KLK14 and PAR_2 receptor in inflammatory skin disorders. These *in vitro* results suggest that KLKs may take part in PAR_2 activation in the epidermis and thereby in PAR_2-mediated inflammatory responses, including epidermal barrier repair and pruritus. The role of KLKs in PAR_2 activation *in vivo* remains to be elucidated.

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INTRODUCTION

The skin forms a protective barrier not only by means of constituting an efficient physico-chemical barrier but also via its highly active and specialized participation in immune and inflammatory reactions. The major cellular constituent of the epidermis, the keratinocyte, has been described as a ''cytokine factory'' and plays an important role in our immune defence (reviewed by Steinhoff *et al.*, 2001). A new concept in skin immunology is that proteolytic enzymes may act as key modulators of biological functions in a cytokine-like manner. Endogenous or exogenous proteinases may influence cells through the activation of proteinase-activated receptors (PARs) (reviewed by Steinhoff *et al.*, 2005).

Proteinase-activated receptor-2 (PAR_2) is a member of the PAR family, which encompasses four PARs, designated PAR1–4 (Vu *et al.*, 1991; Nystedt *et al.*, 1995; Ishihara *et al.*, 1997; Xu *et al.*, 1998). These are G-protein-coupled receptors with seven transmembrane domains, which are activated by a unique mechanism dependent on proteolytic cleavage. Upon cleavage within the N-terminal extracellular part of the receptor, a peptide is released that binds to and irreversibly activates the receptor while still tethered to it. Activation leads to interaction with heterotrimeric G-proteins in the plasma membrane and downstream signalling events. Proteinases capable of activating PARs belong to the serine protease family and include thrombin, trypsin, and cathepsin G. PAR_2 is not activated by thrombin but has been shown to be activated by the following proteases *in vitro*: trypsin, mast cell tryptase, factor Xa, acrosin, gingipain, and neuronal serine proteases (reviewed by Dery *et al.*, 1998; Macfarlane *et al.*, 2001; Ossovskaya and Bunnett, 2004; Steinhoff *et al.*, 2005). PAR_2 is expressed by keratinocytes (Santulli *et al.*, 1995; D'Andrea *et al.*, 1998; Steinhoff *et al.*, 1999) where it may function as a regulator of growth and differentiation (Derian *et al.*, 1997) and is highly implicated in inflammatory reactions (Steinhoff *et al.*, 2005). Recently, PAR_2 was identified as a novel signalling mechanism of the epidermal permeability barrier (Hachem *et al.*, 2006). The endogenous activator of PAR_2 in the epidermis, however, has not been identified yet. It has been suggested that mast cell tryptase may activate PAR_2 during inflammatory conditions upon mast cell infiltration and degranulation (Steinhoff *et al.*, 1999). Other possibilities include endogenous trypsin-like proteases present in the epidermis.

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Abbreviations: KLK, *human kallikrein-related peptidase gene; KLK, human kallikrein-related peptidase protein; KNRK, Kirsten Murine Sarcoma Virus transformed rat kidney epithelial; PAR_2, proteinase-activated receptor-2; PBS, phosphate-buffered saline; rEK, recombinant enterokinase; RT, room temperature*

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We have previously purified, cloned, and characterized three epidermal serine proteases; human kallikrein-related peptidase (KLK) 5 (hK5, stratum corneum tryptic enzyme, SCTE), KLK7 (hK7, stratum corneum chymotryptic enzyme, SCCE), and KLK14 (hK14) (Hansson *et al.*, 1994; Brattsand and Egelrud, 1999; Brattsand *et al.*, 2005; Stefansson *et al.*, 2006). KLK8 is another human KLK, which is abundantly present in the stratum corneum (Komatsu *et al.*, 2005a). These four enzymes all belong to the human kallikrein gene family of 15 related serine proteases (Clements *et al.*, 2001; Yousef and Diamandis, 2001) and have different substrate specificities. KLK5 and KLK8 have trypsin-like activity (Brattsand *et al.*, 2005; Rajapakse *et al.*, 2005), whereas KLK7 has chymotrypsin-like activity (Skytt *et al.*, 1995). KLK14 has mainly trypsin-like but also a significant chymotrypsin-like activity (Brattsand *et al.*, 2005; Felber *et al.*, 2005). These enzymes are here denoted as human kallikrein-related peptidases according to the new nomenclature proposed by Lundwall *et al.* (2006).

Given the presence of both PAR_2 and KLKs in the epidermis, it is plausible that one or several KLKs may function as activators of PAR_2 and thus play a role in inflammatory skin diseases. In this work, we investigated whether KLKs 5, 7, 8, or 14 may be capable of activating PAR_2 *in vitro*. We selected KLKs 5, 7, and 14 because these enzymes are known to be present in the skin in catalytically active form (Egelrud, 1993; Brattsand and Egelrud, 1999; Stefansson *et al.*, 2006). KLK8 may, besides KLK11, be the most abundant trypsin-like KLK in normal human skin (Komatsu *et al.*, 2005a). We show that KLK5 and KLK14, but neither KLK7 nor KLK8, could activate PAR_2. We also show the colocalization of KLK14 and PAR_2 in the epidermis of inflammatory skin disorders. Thus, certain but not all KLKs expressed by keratinocytes are potential endogenous activators of PAR_2 within the epidermis, and may play a role in epidermal barrier physiology/pathophysiology and itching.

RESULTS

Production and activation of recombinant KLKs

Production of active KLK5 and KLK14 has been published elsewhere (Brattsand *et al.*, 2005). Purified recombinant pro^{EK}-KLK7 could, as expected, be activated by recombinant enterokinase (rEK). Recombinant pro-KLK8, having the native propeptide sequence, was also easily activated by rEK. Activation was verified by a mobility shift on SDS-PAGE (Figure 1) and by measuring activity toward chromogenic peptide substrates. Active KLK8 fusion protein had an apparent molecular mass of 38.6 kDa according to SDS-PAGE analysis (Figure 1). Active-site titration of enzyme preparations showed that around 30% ($KLK7^{EK}$) and 60% ($KLK5^{EK}$) of the respective enzyme was active when compared with expected activity according to protein concentration determined with the DC Protein Assay. Close to 100% of the KLK14 preparation was in active form. In Figures 2 and 3, all KLK8 was assumed to be in active form. However, as KLK8 does not react with α_1-antitrypsin, it was not possible to perform active-site titration of the enzyme in the same way as for KLKs 5, 7, and 14, but taking into account the proportion of active enzyme in the other preparations, and the fact that electrophoretic analyses showed no or negligible degradation of KLK8 (Figure 1), an assumption was made that at least 30% of the KLK8 preparation should be functional. The active KLK8 preparation also showed high activity toward chromogenic peptide substrates (see below).

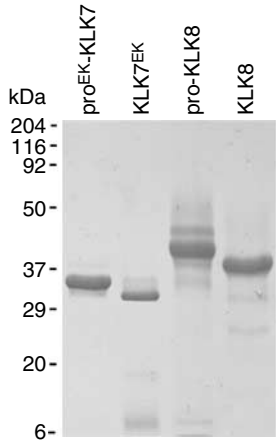

Figure 1. Preparations of recombinant human tissue KLK protein precursors and active enzymes. Coomassie brilliant blue stained SDS-PAGE.

Effects of KLKs 5, 7, 8, and 14 on Ca^{2+} mobilization

Trypsin, $KLK5^{EK}$, and KLK14 stimulated Ca^{2+} mobilization in Kirsten Murine Sarcoma Virus transformed rat kidney epithelial (KNRK)-PAR_2 cells (Figure 2a, b, and e), interpreted as PAR_2 signalling. No reaction was obtained when $KLK7^{EK}$ or KLK8, in concentrations from 0.1 nM up to 10 μM, was used (Figure 2c and d). However, subsequent addition of 100 nM trypsin to the same cells gave an expected reaction, confirming the integrity of cells (Figure 2c and d). Addition of rEK in concentrations similar or higher to that present in preparations of $KLK5^{EK}$, $KLK7^{EK}$, and KLK8 did not lead to any detectable Ca^{2+} mobilization (data not shown). Figure 3a shows dose response curves. Both trypsin and KLK14 were far more potent activators of PAR_2 than $KLK5^{EK}$. The lowest detectable signal was obtained at 50 nM concentration for KLK14. $KLK5^{EK}$ gave a detectable signal at 0.1 μM. Even at 1 μM, the signal obtained with KLK5 was lower than that for KLK14 at 50 nM. Neither KLK7 nor KLK8 could activate PAR_2. The ability of KLK5 and KLK14 to induce Ca^{2+} mobilization in cells via the PAR_2 signal system was confirmed using human dermal microvascular endothelial cells naturally expressing functional PAR_2 receptors (Figure 3b; Shpacovitch *et al.*, 2002).

Immunofluorescence analyses

By using antibodies to the Flag and the HA11 epitopes of the PAR_2 receptor, we were able to discriminate between intact and cleaved PAR_2. The antibody to the extracellular Flag would detect only uncleaved receptor, because proteolytic cleavage removes the proximal Flag epitope. Results from immunofluorescence analyses (Figure 4) were in accordance

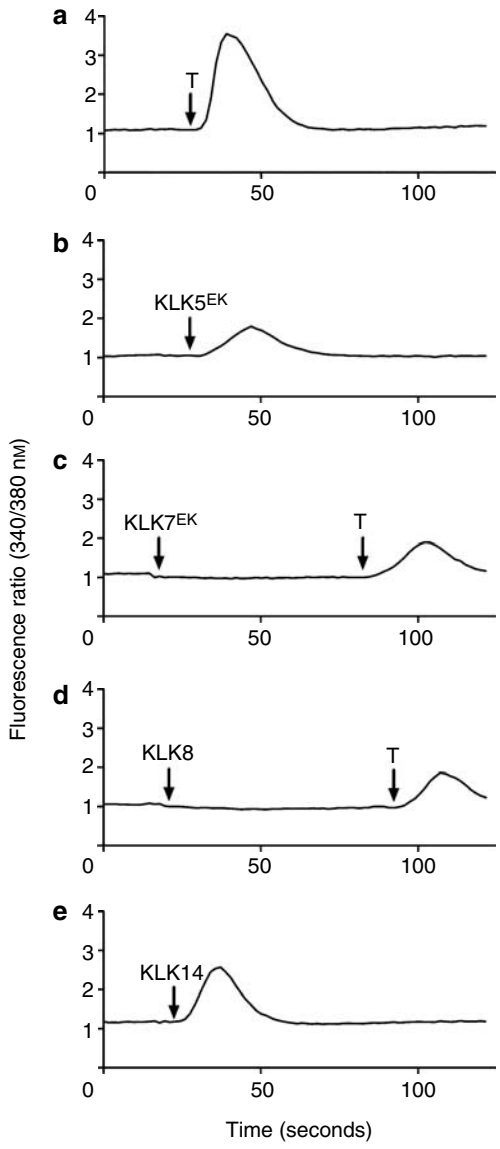

Figure 2. Ca^{2+} responses to trypsin and KLK in KNRK-PAR2 cells. KNRK-PAR$_2$ cells were treated with (**a**) 1 μM trypsin (T), (**b**) 10 μM KLK5^{EK}, (**c**) 10 μM KLK7^{EK}, (**d**) 10 μM KLK8, and (**e**) 1 μM KLK14. Trypsin or KLK was added at indicated time points (arrows). If no response could be detected, the integrity of the cells was verified by addition of 100 nM trypsin (T).

with the results obtained in Ca^{2+} mobilization assays. After treatment with 1 μM recombinant KLK5^{EK} or KLK14, no staining of the Flag epitope could be detected around the cell membrane, indicating cleavage and loss of the extracellular part of the PAR$_2$ receptor. Treatment with 1 μM KLK7^{EK} or KLK8 did not abolish staining of the Flag epitope, that is, with these enzymes results similar to the negative control with cells treated with vehicle only were obtained. rEK treatment did not have any effect on immunofluorescence patterns (results not shown).

Kinetic properties of KLK8

Despite its trypsin-like properties, KLK8 did not give any detectable PAR$_2$ activation. Therefore, we wanted to test this

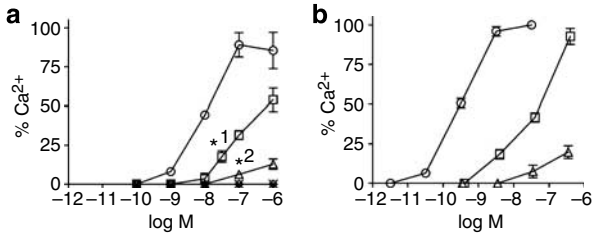

Figure 3. Dose response curves for Ca^{2+} mobilization in response to trypsin and KLK. (**a**) KNRK-PAR$_2$ cells were treated with active trypsin (circles), KLK5^{EK} (triangles), KLK7^{EK} (crosses), KLK8 (diamonds) or KLK14 (squares). Means ± SEM, $n \geq 3$ for trypsin, KLK5^{EK}, and KLK14. For KLK7^{EK} and KLK8, experiments were carried out in duplicate at 1 μM, at lower concentrations single experiments were performed. *1: statistically significantly different from baseline ($n = 3$, $P = 0.04$); *2: statistically significantly different from baseline ($n = 4$, $P = 0.003$). (**b**) Human dermal microvascular endothelial cells (hDMEC) cells were treated with active trypsin (circles), KLK5^{EK} (triangles), or KLK14 (squares) with concentrations as indicated in the figure. 100% Ca^{2+} corresponds to fluorescence ratio 340/380 nm = 2.

enzyme against a series of chromogenic peptide substrates and compare its kinetic properties to that of KLK14. The results with the chromogenic peptide substrates S-2251, S-2288, and S-2302 (see Materials and Methods for structures) are shown in Table 1. KLK8 had a catalytic rate (k_{cat}) and catalytic efficiency (k_{cat}/K_m) 3–5 times lower than that of KLK14 for S-2288 and S-2302. Whereas S-2251 was readily cleaved by KLK8, it was not cleaved at detectable rates by KLK14.

Immunolocalization of KLK14 and PAR$_2$ in inflammatory skin disorders

Staining of inflamed skin from patients with atopic dermatitis and rosacea shows immunoreactivity of both KLK14 and PAR$_2$ widely distributed in the upper squamous and granular layers (Figure 5).

DISCUSSION

PAR$_2$ has been implicated in immunological and inflammatory responses of many tissues. This receptor may contribute to the pathophysiology of inflammatory skin diseases such as atopic dermatitis and psoriasis (Steinhoff *et al.*, 1999, 2005). It has also been depicted as a novel pathway for pruritus in human skin (Steinhoff *et al.*, 2003) and for events linked to lamellar body secretion in response to epidermal barrier injuries (Hachem *et al.*, 2006). The nature of the proteinases which activate PAR$_2$ in the skin under physiological and pathophysiological conditions is, however, still uncertain. Therefore, the aim of this study was to identify possible endogenous activators of PAR$_2$ in the epidermis. Candidates were four serine proteinases within the family of KLKs. Several KLKs have been shown to be produced by epidermal cells (Sondell *et al.*, 1994; Brattsand and Egelrud, 1999; Komatsu *et al.*, 2003, 2005a, b; Stefansson *et al.*, 2006). In this work, we focused on KLKs 5, 7, 8, and 14. So far, epidermal KLKs 5 and 7 have been predominantly implicated in the process of desquamation (Egelrud and Lundström, 1991; Lundström and Egelrud, 1991; Egelrud, 1993; Ekholm

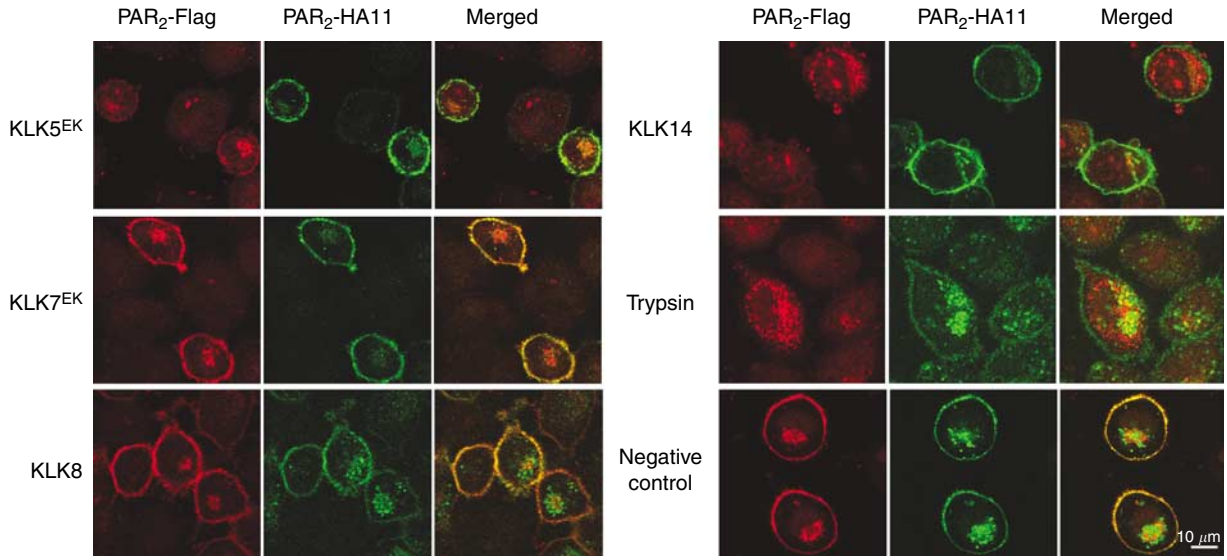

Figure 4. Confocal photomicrographs of KNRK-PAR2 cells stimulated with trypsin or KLK. KNRK-PAR$_2$ cells were stimulated with KLK5^{EK}, KLK7^{EK}, KLK8, KLK14, or trypsin, all at 1 μM. Negative control shows cells incubated in DMEM/0.1% BSA only. Cells incubated with rEK looked like cells in negative control (data not shown). Cells were stained with anti-Flag antibody toward extracellular Flag epitope and anti-HA antibody toward intracellular HA11 epitope. Merged: superimposition of images in the same row. Yellow color in superimposed images indicates no degradation of extracellular parts of PAR$_2$.

Table 1. Kinetic parameters of recombinant human KLK8 and KLK14

Enzyme	Substrate	Enzyme concentration (nM)	K_m (mM)	V_{max}[1] (nM s^{-1})	k_{cat}[1] (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})
KLK8[2]	S-2251	50	0.37	99	2.0	5.3×10^3
	S-2288	50	0.16	160	3.2	2.0×10^4
	S-2302	50	0.13	100	2.1	1.7×10^4
KLK14[2]	S-2251	50	—	—	—	—
	S-2288	10	0.18	120	12	7.0×10^4
	S-2302	10	0.081	61	6.1	7.5×10^4

k_{cat}, catalytic rate; k_{cat}/K_m, catalytic efficiency; KLK, human kallikrein-related peptidase.
[1]Values for K_m and V_{max} represents means from four different experiments.
[2]For KLK8, all enzyme protein was assumed to be catalytically active; the concentration of KLK14 was calculated from active-site titration.
''—'', no enzyme activity could be detected.

et al., 2000; Caubet *et al.*, 2004). However, similar as PAR$_2$, both KLKs 5 and 7 show the highest expression in the stratum granulosum, suggesting a possible interaction between these proteinases and PAR$_2$ (Sondell *et al.*, 1994; Steinhoff *et al.*, 1999; Ekholm *et al* 2000). In this context, also KLK14 may be of interest. In normal skin, the highest expression of this enzyme is seen in sweat ducts (Stefansson *et al.*, 2006). It exhibits trypsin-like specificity and a high catalytic efficiency (Brattsand *et al.*, 2005). Thus, KLK14 may also be associated with PAR$_2$ activation via a paracrine mechanism. KLK8, another trypsin-like KLK (Rajapakse *et al.*, 2005), is abundantly present in the stratum corneum (Komatsu *et al.*, 2005a) and may therefore also be a possible activator of PAR$_2$ in the epidermis.

In the dermis, a likely activator of PAR$_2$ is mast cell tryptase. Steinhoff *et al.* (1999) showed that tryptase stimulated a rapid increase of intracellular calcium concentration in cultured human keratinocytes. Mast cell tryptase may constitute as much as 20% of the total protein content of mast cells. Under inflammatory conditions such as atopic dermatitis, the number of mast cells in the dermis is dramatically increased, and mast cells can be found close to the dermal–epidermal border. Also in psoriasis, there is an increased number of dermal mast cells, sometimes apparently invading the epidermis (Steinhoff *et al.*, 1999). Considering the fact that keratinocytes in high suprabasal epidermal layers show the highest expression of PAR$_2$ under normal conditions (Steinhoff *et al.*, 1999), it seems less likely, however, that tryptase would be the only PAR$_2$-activating enzyme in the skin. Besides mast cell tryptase, another possible activator of PAR$_2$ in the epidermis is trypsin IV (Cottrell *et al.*, 2004). Trypsin IV generated by keratinocytes in human skin can

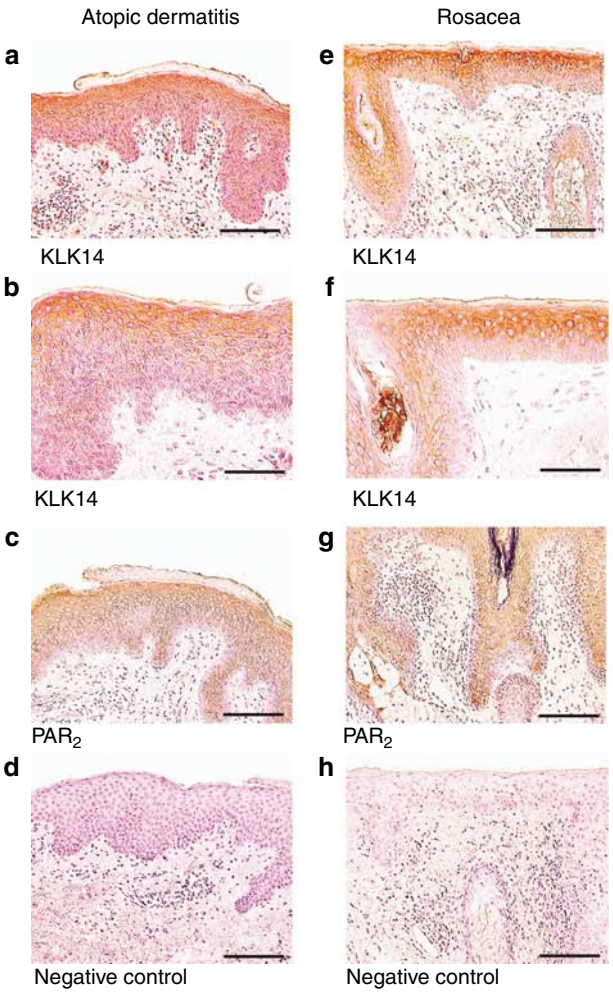

Figure 5. Immunoreactivity of KLK14 and PAR2 in inflamed skin tissues. Immunoreactivity of KLK14 as well as PAR$_2$ was found widely distributed in the upper squamous and granular layer in patients with inflammatory skin disorders. (**a**–**d**) Atopic dermatitis, (**e**–**h**) rosacea. Staining was performed with antisera specific for (**a**, **b**, **e**, **f**) KLK14 or (**c**, **g**) PAR$_2$. (**d**, **h**) Negative controls. Inset size bars = 50 μM (**b**, **f**) or 100 μM.

activate PAR$_2$ *in vitro* (M Steinhoff, unpublished observation). Cottrell *et al.* (2004) showed that trypsin IV is able to activate PAR$_2$ in KNRK-PAR$_2$ cells as well as in the epithelial cell lines PC-3 (prostate) and SW480 (colon). These results were, however, questioned by Grishina *et al.* (2005), who claimed that trypsin IV cannot activate PAR$_2$ in epithelial cells. The opposing results may be due to different glycosylation or transactivation mechanisms (Hollenberg and Compton, 2002).

In normal skin, PAR$_2$ is highly expressed by keratinocytes. The highest levels can be found in the stratum granulosum. PAR$_2$ is also localized in keratinocytes of hair follicles and sebaceous glands, as well as endothelial cells, myoepithelial cells of sweat glands, and dermal dendritic cells (Steinhoff *et al.*, 1999). Under inflammatory conditions, staining for PAR$_2$ can also be observed in primary afferent nerve fibers (Steinhoff *et al.*, 2000, 2003). In atopic dermatitis, differing from normal conditions and psoriasis, PAR$_2$ is expressed also by keratinocytes in lower epidermal layers (Steinhoff *et al.*, 1999, 2003). In this work, we show that KLK14 and the PAR$_2$ receptor are coexpressed in the upper squamous and granular layers of inflammatory skin (Figure 5).

We found that KLK5 and KLK14, but not KLK7 nor KLK8, were able to activate PAR$_2$. The positive results of KLK5 and KLK14 were confirmed in a recent study of Oikonomopoulou *et al.* (2006), where they also found that KLK6 could activate PAR$_2$. Owing to its chymotrypsin-like primary substrate specificity (Skytt *et al.*, 1995), the inability of KLK7 to activate PAR$_2$ was expected. Trypsin-like KLKs show different substrate specificities, which may reflect different functions among these enzymes in the skin. Considering the trypsin-like primary substrate specificity of KLK8 (Rajapakse *et al.*, 2005), its abundance in the stratum corneum (Komatsu *et al.*, 2005a) and the capacity of KLKs 5 and 14 to activate PAR$_2$, the inability of KLK8 to activate PAR$_2$ was unexpected. In order to confirm that our KLK8 preparation was active, we compared the catalytic properties of KLKs 8 and 14. We found that KLK8 had a catalytic efficiency 3–6 times lower than KLK14 against the chromogenic substrates S-2288 and S-2302. On the other hand, KLK8 was able to cleave S-2251, a substrate not cleaved by KLK14 (Table 1). Hence, KLK8 is active but differs in its substrate specificity from KLK14. This difference may be reflected in the inability of KLK8 to activate PAR$_2$. A functional difference among KLKs in the skin is supported also by the fact that KLK5, which has a catalytic efficiency around 100-times lower than KLK14 toward S-2288 (Brattsand *et al.*, 2005), could activate PAR$_2$. In this context, it should be noted that an inactivation of PAR$_2$ by KLK7 and KLK8 could be ruled out by control experiments (cf. Figure 2c and d).

PAR$_2$ activation in the skin may be involved in the elicitation of itch. PAR$_2$ as well as tryptase has been shown to be highly expressed in lesional skin in atopic dermatitis (Steinhoff *et al.*, 2003 and Figure 5). Neuronal PAR$_2$ can be activated by tryptase (Steinhoff *et al.*, 2000), and PAR$_2$ agonists induce pruritus in atopic dermatitis. It has therefore been suggested that PAR$_2$ activation may play a major role in pruritus of atopic dermatitis (Steinhoff *et al.*, 2003). The presence of KLKs in sweat, and the fact that sweating is the most common itch-triggering factor in atopic dermatitis, may be of relevance in this context. Komatsu *et al.* (2006) showed that among other KLKs, KLKs 5, 7, 8, and 14 are present in sweat. We have earlier found that KLK14 is preferentially detected in sweat ducts and glands by immunohistochemistry of normal skin (Stefansson *et al.*, 2006). Although the concentration of KLK14 may be low in sweat (Komatsu *et al.*, 2006), its high catalytic efficiency (Brattsand *et al.*, 2005) and its ability to activate PAR$_2$ could make this protease (together with other proteases) an important sweat-mediated itch inducer. It may be speculated that skin barrier defects in atopic dermatitis (Werner and Lindberg, 1985; Linde, 1992) may provide access to pruritogenic nerve fibers for sweat-carried KLKs such as KLK14. Proteinases, as itch mediators, have been proposed for a long time (reviewed by Ständer and Steinhoff, 2002; Steinhoff *et al.*, 2006).

In conclusion, we clearly demonstrated that at least two (but not all) KLKs present in human epidermis can act as PAR$_2$

activators *in vitro*. The situation *in vivo* remains to be elucidated. Our results give further evidence that epidermal proteases may play important roles not only under normal conditions but also under pathophysiological conditions including skin inflammation, epidermal barrier function, and pruritus.

MATERIALS AND METHODS

All described studies were approved by the medical ethical committee of Umeå University and performed according to the Declaration of Helsinki Principles. All participants gave their written informed consent.

Cell lines

KNRK cells stably expressing human PAR_2 with N-terminal Flag epitope and C-terminal HA11 epitope (Bohm *et al.*, 1996; Dery *et al.*, 1999; DeFea *et al.*, 2000) were propagated in DMEM high glucose (Invitrogen, Groningen, The Netherlands), 10% bovine calf serum, 200 μg/ml geneticin (G-418) (Invitrogen, 10131). For passage of cells, cell dissociation buffer enzyme-free Hank's-based (Invitrogen, 13150-016) was used. Human dermal microvascular endothelial cells, derived from dermis at the proliferating state, expressing functional PAR_2 receptors were grown in Endothelial Cell Basal Medium (PromoCell, Heidelberg, Germany), as described (Shpacovitch *et al.*, 2002).

Production and activation of recombinant proteins

$KLK5^{EK}$ was produced as pro^{EK}-KLK5 in High Five insect cells and activated as described by Brattsand *et al.* (2005). KLK14 was produced in active form in *Pichia pastoris* KM71H as described by Brattsand *et al.* (2005).

Active $KLK7^{EK}$ was produced by enterokinase treatment of pro-KLK7 with the activation site replaced by an enterokinase cleavage site (Huang *et al.*, 1997), pro^{EK}-KLK7. Pro^{EK}-KLK7 was produced by site-directed mutagenesis, using the Quick-Change Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) with pS500 plasmid as template (Hansson *et al.*, 1994), and the primer pair $KLK7^{EK}$s (5′-CTGCAGGAGAAGAAGCCGATGATGACAAGATTATTGATGGCGCC-3′) and $KLK7^{EK}$as (5′-GGCGCCATCAATAATCTTGTCATCATCGGCTTCTTCTCCTGCAG-3′). As this rendered an incomplete activation site (DDDK), one additional D was inserted through amplification with primers KLK7FD4K (5′-GAAGAAGACGATGATGACAAGATTA-3′) and KLK7R2 (5′-TCTAGATTAGCGATGCTTTTTCATGGTGTCAT-3′). The reverse primer included a stop codon and an *Xba1* site. The resulting fragment was cloned into a TOPO TA vector (Invitrogen) and then transferred into the pPICZαA vector through cleavage with restriction enzymes *Eco*R1 and *Xba1* and subsequent ligation. *P. pastoris* strain X-33 was transformed with the pPICZαA-$KLK7^{EK}$ vector using the Easy Comp Transformation kit (Invitrogen). Recombinant protein was produced essentially as described in the EasySelect Pichia Expression Kit manual, purified, and activated as described for KLK5 and KLK14 by Brattsand *et al.* (2005). Pro^{EK}-KLK7 activation was performed by incubation with 0.006 U of rEK per μg recombinant proenzyme for 5 hours at room temperature (RT) under conditions as described (Brattsand *et al.*, 2005).

(cDNA)*KLK8* was cloned from total RNA prepared from human epidermis (Brattsand and Egelrud, 1999), using the primer pair KLK8F1 (5′-CAGGAGGACAAGGTGCTGGGG-3′) and KLK8R1 (5′-CCCTTGCTGATGATCTTCTTG-3′). (cDNA)*KLK8* fragment was ligated into a TOPO TA vector and thereafter transferred into the pPICZαA vector. The construct was used to transform *P. pastoris* strain KM71H using the Easy Comp Transformation kit. Recombinant protein was produced according to manual. As the reverse primer does not contain a stop codon, this results in a protein containing C-terminal His- and V5 tags. Purification of pro-KLK8 was made in several steps. First, conditioned cell media were concentrated by precipitation with 80% saturated ammonium sulfate. The pellet was dissolved in 1/10 of the original volume in 20 mM Tris-HCl pH 8.0. The mixture was filtered through a 0.45 μM Millipore filter and then subjected to reversed phase chromatography. The first chromatography step was followed by ion exchange chromatography and a second run of reversed phase chromatography (Brattsand and Egelrud, 1999). Recombinant pro-KLK8 was activated by incubation with rEK, 0.02 U per μg protein as described for pro^{EK}-KLK5 by Brattsand *et al.* (2005).

Accuracy of desired nucleotide sequences was verified by sequencing using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and analyzed using an ABI377 automated DNA sequencer (Applied Biosystems). The sequence of (cDNA)*KLK8* was compared and matched to the published sequence of *KLK8* transcript variant 1 (accession no. NM_007196). The sequence of (cDNA)*KLK7EK* was compared to the published sequence of *KLK7* transcript variant 1 (GenBank NM_005046). Protein concentration was determined with the DC Protein Assay (Bio-Rad Laboratories, Hercules, CA). All active protein preparations used, with the exception of KLK8, were active-site titrated with α_1-antitrypsin (Sigma, St Louis, MO, A-9024), essentially according to Salvesen and Nagase (1994). Known amounts of bovine pancreatic trypsin (Sigma, T-8253) were used for standardization.

Measurement of intracellular calcium concentration

PAR_2 signalling was assessed by measuring cytosolic $[Ca^{2+}]$ with fura 2-AM (Molecular Probes, Eugene, OR, F-1201) (Bohm *et al.*, 1996; Dery *et al.*, 1999; DeFea *et al.*, 2000). Cells growing on glass coverslips were loaded with 2.5 μM fura 2-AM in Hank's buffer/0.1% BSA (PAA Laboratories GmbH, Cölbe, Germany, Fraction V K41-001-100) at 37°C for minimum 25 minutes. Extracellular fura 2-AM was removed by rinsing in Hank's buffer/0.1% BSA. Cells were transferred to a cuvette with 37°C Hank's buffer/0.1% BSA and placed in a spectrofluorometer (Fluoro-Max2, Yobin Yvon GmbH, Munich, Germany). Cells were challenged with recombinant KLK or trypsin (Sigma, T-4665) at 0.1 nM to 10 μM concentrations. Fluorescence was measured at 340 and 380 nm excitation wavelengths. The ratio of the fluorescence signal at the two wavelengths, which is proportional to the intracellular calcium ion concentration, was calculated.

Immunofluorescence and confocal microscopy

Cells were grown on glass coverslips in DMEM until near confluence. The coverslips were transferred into an incubation chamber and washed once with DMEM/0.1% BSA preheated to 37°C. Cells were incubated with 1 μM recombinant KLK or trypsin (Sigma, T-4665), diluted in DMEM/0.1% BSA for 10 minutes at 37°C. Negative controls were treated with rEK in amounts corresponding to rEK content in activated enzyme preparations or appropriate buffer only. Cells were fixed with ice-cold methanol for 20 minutes on ice.

Cells were washed and subsequently treated with 0.3% Triton-X 100 in phosphate-buffered saline (PBS)/0.1% BSA for 15 minutes. The cells were washed before blocking in blocking solution (PBS/2% goat serum (DakoCytomation, Glostrup, Denmark, X0907)) for 60 minutes at RT. Cells were incubated with mouse anti-Flag M1 mAb (Sigma, F 3040) diluted 1:200 and rabbit anti-HA antibody (Sigma, H 6908) diluted 1:200 over night at 4°C. After washing in blocking solution, cells were incubated with secondary antibodies: FITC-conjugated AffiniPure goat anti-rabbit antibody (Jackson Immuno Research, Cambridgeshire, UK, 111-095-003) and Cy3 AffiniPure anti-mouse antibody (Jackson Immuno Research, 715-166-151) diluted 1:200 in blocking solution, for 4 hours in the dark at RT. The cells were again washed and mounted on superfrost slides using Vectashield mounting medium (Vector Laboratories, Burlingame, CA, H-1000). Specimens were observed using a Bio-Rad MRC 1.000 confocal microscope. Images were collected at 0.68 μM intervals using a Zeiss *100 Plan Apo 1.4 NA objective and a zoom from 1.5–2 X.

Enzyme kinetics

Buffer (37.5 mM Tris-HCl pH 8.0/0.1 M NaCl/0.0075% Tween-20), enzymes (final concentrations as specified in Table 1), and chromogenic peptide substrates (Haemochrom Diagnostica AB, Mölndal, Sweden) at final concentrations ranging from 50 to 900 μM (S-2251 (H-D-Val-Leu-Lys-pNA × 2HCl), S-2302 (H-D-Pro-Phe-Arg-pNA × 2HCl)) or 1,000 μM (S-2288 (H-D-Ile-Pro-Arg-pNA × 2HCl)) were mixed in a 96-well plate. Total volume in each well was 77.5 μl. Mixtures were incubated in duplicate at RT for 10 minutes in an ELISA reader (SpectraMAX 340, Molecular Devices, Berkshire, UK). The apparatus was calibrated using known amounts of pNA (Haemochrom Diagnostica AB). K_m and V_{max} values were calculated using a Lineweaver Burk plot. rEK in amounts corresponding to that used for activation of KLK8 gave no detectable activity toward any of the substrates used.

Immunohistochemistry

Tissues were fixed in 10% (w/v) buffered formalin and embedded in paraffin. Slides (5–6 μm) were cut and incubated for 30 minutes at 60°C. Afterwards, sections were dewaxed, rehydrated, and heated in a steamer (MultiGourmet plus FS20, Braun, Kronberg, Germany) for 25 minutes in 0.1 M ChemMate Target Retrieval Solution (DakoCytomation) in a plastic cuvette. Sections were allowed to cool in this buffer for 20 minutes. Endogenous peroxidase activity was quenched with 100 mM NaN_3/0.1% (w/v) H_2O_2 in PBS for 20 minutes at RT. After washing with PBS, sections were blocked with 2% (w/v) BSA in PBS for 25 minutes at RT. Sections were incubated with first specific polyclonal antibody diluted in 1% (w/v) BSA overnight at 4°C in a humid chamber. For KLK14, Em-14 (1.6 μg/ml; Stefansson *et al.*, 2006) and for PAR_2, H-99 at final concentration 0.4 μg/ml (Santa Cruz Biotechnology, Santa Cruz, CA) was used. Negative controls were prepared by omission of the primary antibodies. Specificity of Em-14 antibody to KLK14 has been shown through adsorption experiments (Stefansson *et al.*, 2006). After rinsing with PBS, slides were incubated with secondary antibodies for 1 hour at RT in a humid chamber (EnVision + System Labeled Polymer-HRP, anti-rabbit; DakoCytomation). The immunoreactivity was detected with the Liquid DAB + Substrate Chromogen System (DakoCytomation). Sections were counterstained with Hematoxylin QS (Vector Laboratories) and mounted with Aqua-mount (BDH, Poole, UK). Stainings were examined using a DM LB microscope (Leica, Solms, Germany), equipped with a HV-C20M CCD camera (Hitachi, Rodgau, Germany) and Diskus 4.20 software (Carl H. Hilgers, Königswinter, Germany).

CONFLICT OF INTEREST

Torbjörn Egelrud has a patent on KLK7.

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