

# Proteolytic processing of human growth hormone by multiple tissue kallikreins and regulation by the serine protease inhibitor *Kazal-Type5 (SPINK5)* protein

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## Abstract

**Background:** Human growth hormone (hGH) is naturally present in numerous isoforms, some of which arise from proteolytic processing in both the pituitary and periphery. The nature of the enzymes that proteolytically cleave hGH and the regulation of this process are not fully understood. Our objective is to examine if members of a newly discovered human tissue kallikrein family (KLKs) are expressed in the pituitary and if these enzymes can cleave hGH *in-vitro*.

**Methods:** Expression of 12 of the *KLKs* (*KLKs* 4–15) and *serine protease inhibitor Kazal-type 5 (SPINK5)* genes and their proteins in the pituitary was examined by RT-PCR and immunohistochemistry. Recombinant hGH was digested by various recombinant *KLKs* and fragments were characterized by N-terminal sequencing. *SPINK5* recombinant fragments were used for inhibition of *KLK* activities.

**Results:** We here describe for the first time expression of numerous *KLKs* (*KLKs* 5–8, 10–14) and *SPINK5* in the pituitary. *KLK6* and *SPINK5* appeared to be localized to hGH-producing cells. *KLKs* 4–6, 8, 13 and 14 were able to cleave hGH, yielding various isoforms, *in vitro*. Inhibitor *SPINK5* fragments were able to suppress activity of *KLKs* 4, 5 and 14 *in vitro*. Based on these data, we propose a model for the proteolytic processing of hGH in the pituitary and the regulation of this system by *SPINK5* inhibitory domains. We speculate that loss of *SPINK5* inhibitory domains, as in the case of Netherton syndrome, may lead to proteolytic over-processing of hGH and to growth retardation.

**Conclusion:** We conclude that many *KLKs* and *SPINK5* are expressed in the pituitary. This serine protease-inhibitor system is likely to participate in the regulated proteolytic processing of hGH in the pituitary, leading to generation of hGH fragments. Our data suggest that *KLKs* 5, 6 and 14 might be involved in this process.

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**Keywords:** Human growth hormone; Protease inhibitors; *SPINK5*

**Abbreviations:** hGH, human growth hormone; *KLK*, human tissue kallikrein gene; *KLK*, human tissue kallikrein protein; *SPINK5*, serine protease inhibitor Kazal-type 5; Ab, antibody; ELISA, enzyme-linked immunosorbent assay; RT-PCR, reverse transcription-polymerase chain reaction; SD, standard deviation; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PBS, phosphate-buffered saline.

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## 1. Introduction

Human growth hormone (hGH) is known to exist as a heterogeneous population of molecules in the human body [1]. In the periphery, 70–75% of hGH exists as the classic, 22 kDa, 191-amino acid single-chain form (single-hGH). Single-hGH can be post-translationally processed by proteolytic cleavage

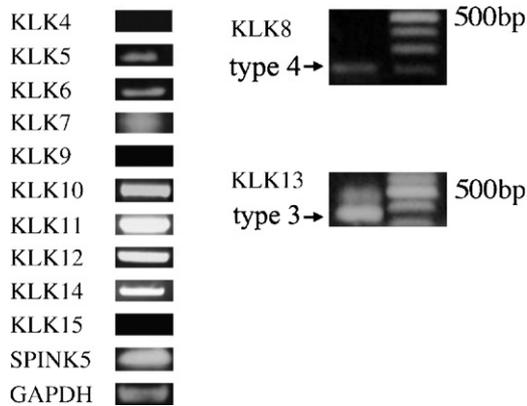


Fig. 1. Expression of (mRNA) *KLKs* and *SPINK5* mRNA in normal human pituitary. Although *KLK* genes are known to encode for various splice variants [46], in normal human pituitary, only classic forms were amplified for *KLKs* 5–7, 10–12, 14 and *SPINK5*. (mRNAs) *KLK* 10–12 and *SPINK5* mRNA showed the highest expression. Although the PCR product for (mRNA) *KLK7* was smeared, a band corresponding to the classic form was confirmed by sequencing. (mRNAs) *KLK* 4, 9 and 15 were not detected. Splice variants were detected for (mRNAs) *KLK8* and 13; (mRNA) *KLK8* type 4 (GenBank Accession no. AF251125) and (mRNA) *KLK13* type 3 (GenBank Accession no. AB108823).

not only in the pituitary but also in plasma [2,3]. Putative cleavage sites for numerous proteases, such as thrombin, plasmin, collagenase, subtilisin and chymotrypsin-like serine proteases are located around residues 134–150 [1,4,5]. These modifications transform single-hGH to a 2-chain form, linked by a disulfide bond [6], significantly slowing its rate of metabolism in the circulation [7]. Besides the 22 kDa form, the translated product of a splice variant and fragments of 17 (or 16), 12 and 5 kDa have also been detected both in the pituitary and the periphery [8]. Upon reduction of the disulfide bond in the 2-chain form, the generated 16 kDa N-terminal hGH fragment inhibits endothelial cell proliferation and angiogenesis in the early stage chick chorioallantoic membrane assay [9]. The proportion of circulating non-22 kDa hGH isoforms, especially 2-chain form, increases in some children with growth retardation, without a decrease in total hGH levels [10]. These reports suggest that hGH not only requires proteolytic cleavage to exert its full biological activity [2], but also that hGH isoforms are potential endogenous regulators of physiological and pathological processes such as angiogenesis [11]. However, hGH isoforms are not distinguishable from single-hGH or from each other by routine assays of hGH levels in plasma or other biological fluids [8].

Human tissue kallikreins are secreted serine proteases, encoded by a group of 15 genes that are tandemly located on chromosome 19q.13.4 [12–14]. With the new nomenclature [15], the genes are designated *KLK1* through *KLK15* and the encoded proteins *KLK1* through *KLK15*. By using RT-PCR, immunohistochemistry and ELISA assays, previous studies have shown that these enzymes are expressed in diverse tissues, mainly steroid hormone-regulated tissues, including the pituitary [12,16–21]. One member of this family, *KLK3*, better known as prostate-specific antigen (PSA), is the primary biomarker for diagnosis and monitoring of prostate cancer [22]. Many other kallikreins have already been shown to be useful biomarkers for pituitary tumors and ovarian, breast,

prostate, testicular and other cancers [12–14,23–26]. Despite the wealth of literature on kallikrein over-expression in many different types of cancers, the function and regulation of these enzymes in normal and cancerous tissues are largely unknown. A recent review describes possible links between kallikreins and receptor, hormone or cytokine processing, angiogenesis, metastasis, cell growth, etc. [13].

*SPINK5* (also known as LEKTI for its protein product) is a gene encoding a 125 kDa secretory serine protease inhibitor pre-proprotein, containing 15 potential inhibitory domains [27,28]. *SPINK5* protein is thought to be cleaved by furin to yield at least 14 independently working serine protease inhibitory domains [29,30]. Since *SPINK5* protein and many tissue kallikreins co-localize in the skin (in lamellar bodies of the uppermost epidermis and the pilosebaceous units of normal human skin tissue) [20,31–34], it has been hypothesized that these proteins may be part of a proteolytic enzyme-inhibitor system that controls skin desquamation and shedding [29,34–38]. Indeed, recent evidence suggests that Netherton syndrome patients, who lack *SPINK5* inhibitory domains due to gene mutations, suffer from severe erythroderma, hair shaft defects and other atopic features [28,39,40]. These features are attributed to elevated stratum corneum trypsin-like activity which leads to over-desquamation and severe skin permeability barrier dysfunction in Netherton syndrome patients [29] and *SPINK5*-deficient mice, mimicking Netherton syndrome [41].

One consistent feature of Netherton syndrome patients is growth retardation [39,40]. Based on this and the other findings mentioned above, we hypothesized that the tissue kallikrein-

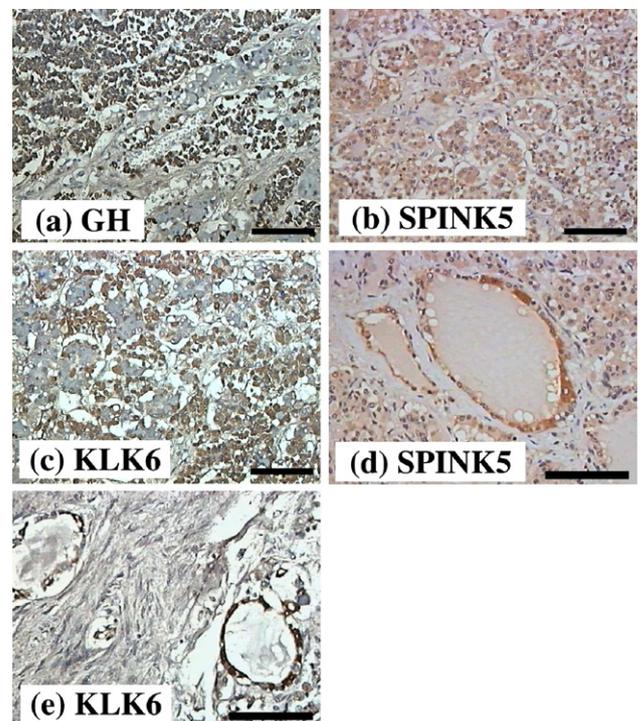


Fig. 2. Immunohistochemistry for hGH, *KLK6* and *SPINK5* proteins in normal human anterior pituitary. Scale bar indicates 100  $\mu$ m. hGH producing cells seemed to also produce *KLK6* and *SPINK5* proteins (a,b,c). The endothelium of small veins in the pituitary intensely expressed *KLK6* and *SPINK5* protein, but not hGH (d,e).

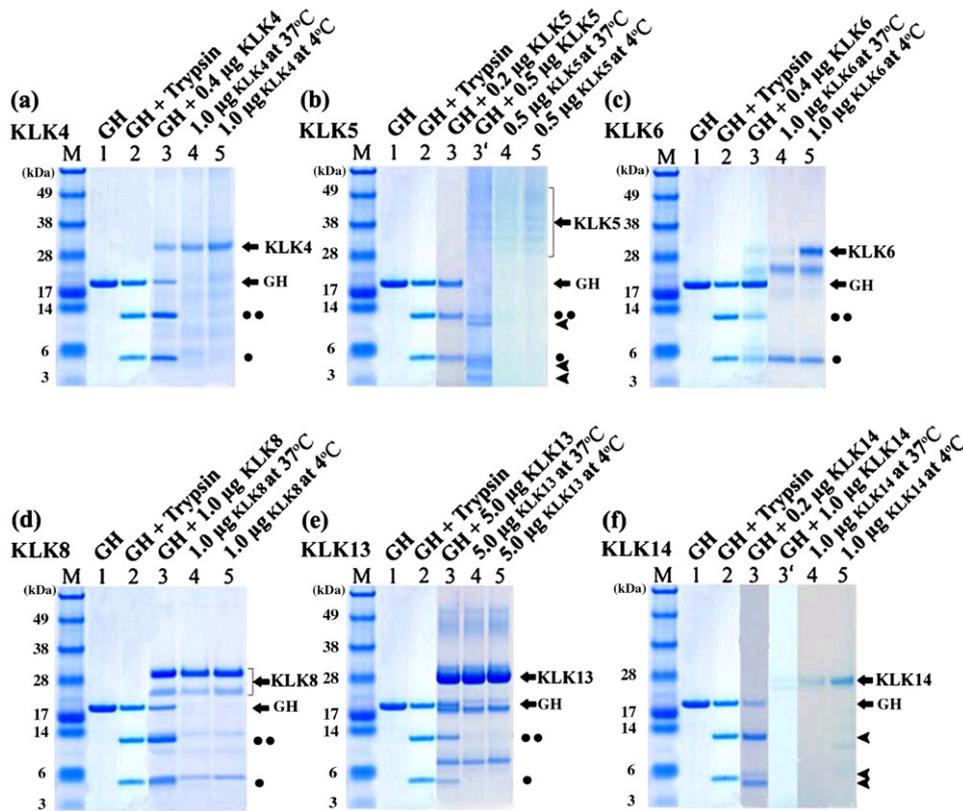


Fig. 3. hGH proteolytic processing by KLKs. Recombinant hGH (1.0  $\mu\text{g}$ ) kept at 37  $^{\circ}\text{C}$  for 24 h without enzymes (lane 1), digested hGH with 0.1 ng of trypsin at 37  $^{\circ}\text{C}$  for 24 h (lane 2), and digested hGH with each KLK at 37  $^{\circ}\text{C}$  for 24 h (lane 3 and 3'), and each KLK (0.5–5.0  $\mu\text{g}$ ) incubated at 37  $^{\circ}\text{C}$  (lane 4) and at 4  $^{\circ}\text{C}$  (lane 5) for 24 h were separated on gradient 4–12% Bis–Tris gels and stained with Coomassie blue. M, molecular mass markers. (●●), N-terminal Phe<sup>1</sup>–Arg<sup>134</sup> 12 kDa hGH fragment; (●), Thr<sup>135</sup>–Phe<sup>191</sup> 5 kDa hGH fragment (both confirmed by sequencing). Arrowheads, other hGH fragments. Recombinant KLK5 appears as several bands around 35–50 kDa (panel b, lanes 3–5) due to glycosylation [57]. KLK4 (0.4  $\mu\text{g}$ ), KLK5 (0.2  $\mu\text{g}$ ), KLK6 (0.4  $\mu\text{g}$ ), KLK8 (1.0  $\mu\text{g}$ ) and KLK13 (5.0  $\mu\text{g}$ ) generated the same Phe<sup>1</sup>–Arg<sup>134</sup> (●●) and Thr<sup>135</sup>–Phe<sup>191</sup> (●) (panels a–e, lane 3) fragments. KLK5 (0.5  $\mu\text{g}$ ) also cleaved after Arg<sup>8</sup> and Arg<sup>94</sup>, generating 2 new Leu<sup>9</sup>–Arg<sup>134</sup> (~11 kDa) and Ser<sup>95</sup>–Arg<sup>134</sup> (~3 kDa) fragments (panel b, lane 3'). A C-terminal 4.5 kDa fragment was also estimated to be Thr<sup>135</sup>–Arg<sup>178</sup> (or Arg<sup>181</sup>) from its size. The rest of the predicted fragments were not visible (panel b lane 3'). Chymotrypsin-like activity of KLK14 cleaved at Tyr<sup>143</sup>/Ser<sup>144</sup>, and trypsin-like activity of KLK14 cleaved at Arg<sup>64</sup>/Glu<sup>65</sup> (panel f, lane 3). No visible bands remained when 1.0  $\mu\text{g}$  of KLK14 was added (panel f, lane 3'). The released peptides are Phe<sup>1</sup>–Tyr<sup>143</sup> (or Arg<sup>134</sup>) (~12 kDa), a mix of Phe<sup>1</sup>–Arg<sup>64</sup> and Glu<sup>65</sup>–Tyr<sup>143</sup> (or Arg<sup>134</sup>) (~6 kDa), and Ser<sup>144</sup>–Phe<sup>191</sup> (~4.5 kDa). Autolysis of KLKs 4, 5, 8 and 13 was minimal, while KLK6 and KLK14 were autolyzed by incubation at 37  $^{\circ}\text{C}$  for 24 h (panels a–c, e, lane 4). Non-proteolyzed hGH and the kallikreins are shown by arrows in all panels.

*SPINK5* protein proteolytic cascade pathway, which operates in the skin, may also function similarly in the pituitary, controlling hGH and other hormone processing in this tissue. The objectives of this study were (a) to examine kallikrein and *SPINK5* gene and protein expression in the human pituitary, (b) to investigate if serine proteases of the kallikrein family can proteolytically digest hGH and characterize the cleavage sites, (c) to examine if this proteolysis can be inhibited by *SPINK5* protein fragments and (d) to develop a model for hGH processing by multiple kallikrein enzymes.

## 2. Materials and methods

### 2.1. Reverse transcription-polymerase chain reaction

Normal human pituitary cDNA was purchased from Clontech (Palo Alto, CA). One nanogram of cDNA was used as template for PCR amplification. The primers and PCR conditions for (mRNAs) *KLK4* (amplification from exon 2 to 5), *KLK5* (exon 2 to 5), *KLK6* (exon 2 to 5), *KLK7* (exon 2 to 5), *KLK8* [non-coding region (upstream of exon 1) to exon 5], *KLK9* [exon 1 to non-coding region (downstream of exon 5)], *KLK11* (exon 3 to 4), *KLK14* (exon 3 to 5),

*SPINK5* and glutaraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNAs were described elsewhere [28]. Our experiments have been performed under the following conditions: 1) we followed the experimental procedures described in detail in Ref. [42]. 2) All PCR products presented in Fig. 1 have been fully confirmed by DNA sequencing. 3) PCR products for which we could not confirm the DNA sequence were not included in our data. In addition, the following primers were used for amplification of *KLK10*; Forward (F) 5'-GGAAACAAGCCACTGTGGGC-3' (on exon 2), Reverse (R) 5'-GAG-GATGCCTTGGAGGGTCTC-3' (on exon 5), annealing temperature at 60  $^{\circ}\text{C}$ , product size 468 bp, *KLK12*; F 5'-TTGACCACAGGTGGTCTCTCA-3' (on exon 2), R 5'-GTGTAGACTCCAGGATGCCA-3' (on exon 5), 61  $^{\circ}\text{C}$ , 542 bp, *KLK15*; F 5'-CTACGGACCACGTCTCGGGTC-3' (on exon 3), R 5'-GACACCAGGCTTGGTGGTGTG-3' (on exon 5), 65  $^{\circ}\text{C}$ , 459 bp. Forty amplification cycles were used for all (mRNA) *KLKs* and *SPINK5* mRNA and 26 cycles for *GAPDH* mRNA.

### 2.2. Immunohistochemistry

Anti-KLK6 rabbit polyclonal antibody (Ab) was developed in-house, using full-length recombinant protein as immunogen. No cross-reactivity of this Ab against all other kallikreins (KLKs 3–15) [20], and (KLKs 1–2) (data not shown) was observed by Western blotting. Since the specificity of Abs towards other KLKs has not as yet been determined, immunohistochemistry was restricted to

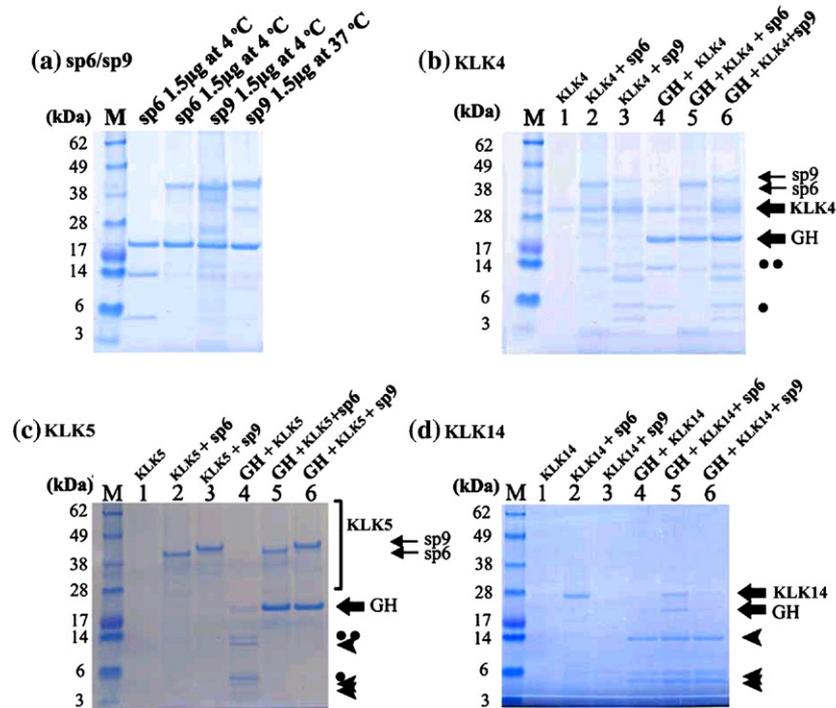


Fig. 4. Inhibition of KLK activity by *SPINK5* protein fragments. a; sp6/sp9: sp6 and sp9 (1.5 µg) incubated at either 37 °C or 4 °C for 24 h. No significant autolysis was observed. b, c and d; KLK alone or GH/KLK mixtures were incubated at 37 °C for 24 h in the presence and absence of sp6 or sp9. According to the results of Fig. 3, 0.4 µg of KLK4 and KLK14, and 0.2 µg of KLK5 were applied, along with 1.0 µg of recombinant hGH. (●●) and (●), 12 kDa Phe<sup>1</sup>–Arg<sup>134</sup> and 5 kDa Thr<sup>135</sup>–Phe<sup>191</sup> fragments, respectively. Arrowheads indicate other hGH fragments produced by each KLK. Panel b; KLK4 digested both sp6 and sp9 into at least 5 fragments (lanes 2–3). When hGH was incubated with KLK4 in the presence of sp6/sp9, fragments were derived only from sp6 and sp9, but not from hGH (lanes 5–6). Panel c; KLK5 proteolytic activity against hGH was completely suppressed by both sp6 and sp9 (lanes 5–6), although sp6 and sp9 were not cleaved by KLK5. Panel d; both sp6 and sp9 were entirely digested by 0.4 µg of KLK14, while sp6 alone prevented autolysis of KLK14. In addition, sp6 alone partially inhibited hGH digestion by KLK14 (lanes 2 and 5).

KLK6 only. Anti-*SPINK5* protein mouse monoclonal antibody (clone 1C11G6) was developed as described elsewhere [43]. Formalin-fixed paraffin-embedded human pituitaries were sectioned. Anti-hGH Ab was purchased (Pituitary hormones and antisera center, Harbor-UCLA Medical Center, Torrance, CA). Staining procedures included deparaffinization in xylene and rehydration through graded ethanols. Endogenous peroxidase activity was blocked with 0.3% H<sub>2</sub>O<sub>2</sub> in methanol for 20 min, and the sections were pretreated with 10 mmol/l citrate buffer (pH 6.0) at 95 °C for 10 min. The primary Abs were diluted in 0.1% bovine serum albumin/phosphate-buffered saline. The normal serum blocking reagent, secondary biotinylated Ab and peroxidase-labeled streptavidin complex were purchased from ID Labs (Ontario, Canada). Samples were stained with 3,3'-diaminobenzidine substrate (DAB substrate kit for peroxidase, Vector Laboratories, Inc., Burlingame, CA) for 5 min. The sections were counterstained with hematoxylin for 1 min. Non-immune rabbit or mouse serum was substituted as the primary antibody for negative control staining.

### 2.3. hGH proteolytic processing by KLKs and inhibition by *SPINK5* protein fragments

KLKs [20], *SPINK5* protein fragment 6–9 (sp6) [44], and *SPINK5* protein fragment 9–12 (sp9) [38] were recombinantly expressed in insect cells and purified. Recombinant hGH (HUMATROPE®) was purchased from Eli Lilly Canada Inc. Each protein was diluted in 0.01% phosphosaline buffer (PBS) solution. Equal volumes of samples in 0.01% PBS containing hGH, KLKs and/or sp6/9 were incubated for 24 h at 37 °C or 4 °C, then samples were separated on 4–12% Bis–Tris gels (Invitrogen) by sodium dodecyl sulphate–polyacrylamide gel electrophoresis. After electrophoresis, proteins were either stained with Simply Blue™ SafeStain (Invitrogen) or electrotransferred to PVDF transfer membranes (Hybond™-P, Amersham Pharmacia Biotech, Buckinghamshire, England) at 30 V for 90 min for N-terminal amino acid sequencing.

N-terminal amino acid sequencing was performed by the University of Victoria Proteomics Center (British Columbia, Canada).

## 3. Results

Reverse transcription-polymerase chain reaction (RT-PCR) was performed to examine the expression of 12 (mRNA) *KLKs* and *SPINK5* mRNAs in normal human pituitary (Fig. 1). mRNAs for *KLKs* 1–3 were previously identified in the pituitary [16]. We detected classic forms of (mRNA) *KLKs* 5–7, 10–12, and 14, as well as *SPINK5* mRNA. (mRNA) *KLK8* splice variant type 4 [45] and (mRNA) *KLK13* type 3 [42] were also detected. These splice variant forms are predicted to encode for truncated proteins devoid of enzymatic activity [46]. These data may not necessarily represent expression in disease states since the ratio of splice forms may change in certain diseases [46–48].

Growth hormone-producing cells were identified by immunohistochemistry. These cells seem to be randomly distributed and have a small round nucleus and compact cytoplasm (Fig. 2a). In contrast, non-hGH producing cells exhibit an irregular (flattened) nuclear shape and a wider cytoplasm. Non-hGH producing cells more frequently displayed a “lobular-like” formation in which the nucleus was found in the outer periphery (Fig. 2a). Staining with specific antibodies against KLK6 and *SPINK5* proteins revealed cells similar to those stained with GH antibodies. Thus, hGH hormone-producing cells seem to

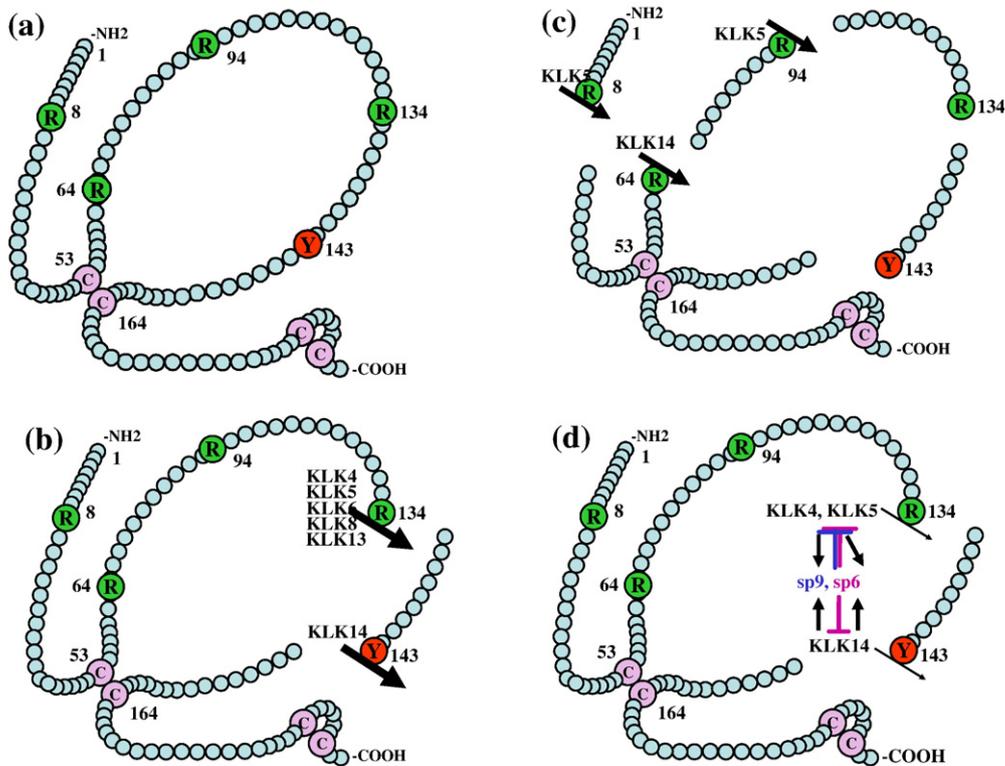


Fig. 5. A model for hGH proteolytic processing by KLKs and regulation by *SPINK5* protein inhibitory fragments. a; The hGH single-chain form. Cys<sup>53</sup>–Cys<sup>164</sup> form a disulphide bond. b; The Arg<sup>134</sup>/Thr<sup>135</sup> is the most preferred cleavage site by KLKs 4–6, 8 and 13. KLK14, with chymotrypsin-like activity cleaves at Tyr<sup>143</sup>/Ser<sup>144</sup>. With such proteolysis, hGH single-chain form is modified to a hGH 2-chain form. c; The identified minor cleavage sites by KLK5 and KLK14. d; When sp6 or sp9 fragments are mixed with KLKs and hGH, they can either act as competitive substrates to hGH, directly suppress KLK activities or the digested fragments of sp6 or sp9 could themselves suppress KLK activity. Only sp6 (but not sp9) was capable to suppress KLK14 activity; thus, sp6 may contain a domain that can specifically inhibit KLK14 (⊥ indicates inhibition). Black arrows indicate digestion of sp6 or sp9 by the indicated kallikreins. For more details, see Figs. 3 and 4.

produce KLK6 and *SPINK5* proteins as well (Fig. 2b, c). In addition, the intense KLK and *SPINK5* protein staining in the endothelial cells of pituitary small veins (Fig. 2d, e) suggests that the released pituitary hormones could undergo proteolytic processing during passage through pituitary veins, prior to their appearance in the peripheral circulation.

To examine whether KLKs can cleave hGH *in-vitro*, the recombinant 22 kDa hGH single-chain form was incubated individually with each KLK or trypsin (Fig. 3). Approximately 12 and 5 kDa fragments were produced by trypsin (Fig. 3 lane 2). Judging from the size of the 2 fragments and by amino acid sequencing, they were identified as N-terminal Phe<sup>1</sup>–Arg<sup>134</sup> (●●) and C-terminal Thr<sup>135</sup>–Phe<sup>191</sup> (●) fragments (Fig. 3). Arg<sup>134</sup>/Thr<sup>135</sup> is known to be a site also cleaved by thrombin and plasmin [5].

All KLKs examined here possess trypsin-like activity [12]. Thus, excluding KLK14, each KLK produced 12 kDa Phe<sup>1</sup>–Arg<sup>134</sup> (●●) and 5 kDa Thr<sup>135</sup>–Phe<sup>191</sup> (●) fragments (Fig. 3a–e lane 3). In addition to identical cleavage of hGH by trypsin and KLK5 (0.2 μg) (Fig. 3b lane 3), 0.5 μg of KLK5 cleaved also at Arg<sup>8</sup>/Leu<sup>9</sup> and Arg<sup>94</sup>/Ser<sup>95</sup> (Fig. 3b lane 3'; data confirmed by N-terminal sequencing). These cleavages additionally yielded 6 and 3 kD hGH fragments. Although KLK14 has been predicted to be a trypsin-like serine protease [12], in this assay, KLK14 (0.2 μg) acted as both chymotrypsin-like (Tyr<sup>143</sup>/Ser<sup>144</sup>) and trypsin-like enzyme (Arg<sup>64</sup>/Glu<sup>65</sup>) (Fig. 3f lane 3). The trypsin

and chymotrypsin-like activity of KLK14 was recently confirmed by using a substrate phage display library [49]. No visible bands remained when 1.0 μg of KLK14 was added (Fig. 3f lane 3'). The results of Fig. 3 show that many KLK proteins are capable of proteolytically modifying hGH to the 2-chain form and that an excess of KLKs can further digest hGH to smaller fragments.

The inhibition of KLK activity by *SPINK5* protein fragment derivatives was examined by using the recombinant *SPINK5* protein fragments, sp6 (*SPINK5* protein domains 6–9) and sp9 (*SPINK5* protein domains 9–12) [27,38,44], which are frequently absent in Netherton syndrome patients (Fig. 4) [29]. KLK4 efficiently digested sp6/sp9, even when hGH was also present, but hGH cleavage was significantly suppressed (Fig. 4b). KLK5 did not digest sp6 and sp9 nor hGH in the presence of sp6 and sp9 (Fig. 4c). Despite the fact that sp6 and sp9 were substrates for KLK4 but not for KLK5, they had a specific inhibitory effect on hGH digestion by both KLK4 and KLK5. Both sp6 and sp9 were entirely digested by KLK14, while sp6 alone prevented autolysis of KLK14 (Fig. 4d). In addition, sp6 alone partially inhibited hGH proteolysis by KLK14.

#### 4. Discussion

Previously, it was reported that rat KLK2 proteolytically cleaved rat prolactin [50], suggesting that rat KLK2 is a putative

prolactin-processing protease [51]. Rat KLK1 catalyzes the release of kinins, which are capable of stimulating prolactin and GH secretion [52]. In human, KLK1 was co-localized with prolactin in prolactin-secreting adenomas of the human anterior pituitary [23]. KLK1 was associated with prolactin-secreting cells within human GH-secreting adenomas [24]. To complement these preceding studies, our study demonstrates for the

first time that many human tissue kallikrein enzymes are expressed in the normal human pituitary gland, and that they are able to generate various proteolytically modified hGH isoforms, such as the 22 kDa 2-chain form and 15, 14, 6, 5 and 3 kDa fragments. Based on these data, we developed a model describing the proteolytic processing of hGH by KLKs (Fig. 5). The preferred cleavage site for most of the KLKs was Arg<sup>134</sup>/Thr<sup>135</sup>,

### (a) Normal

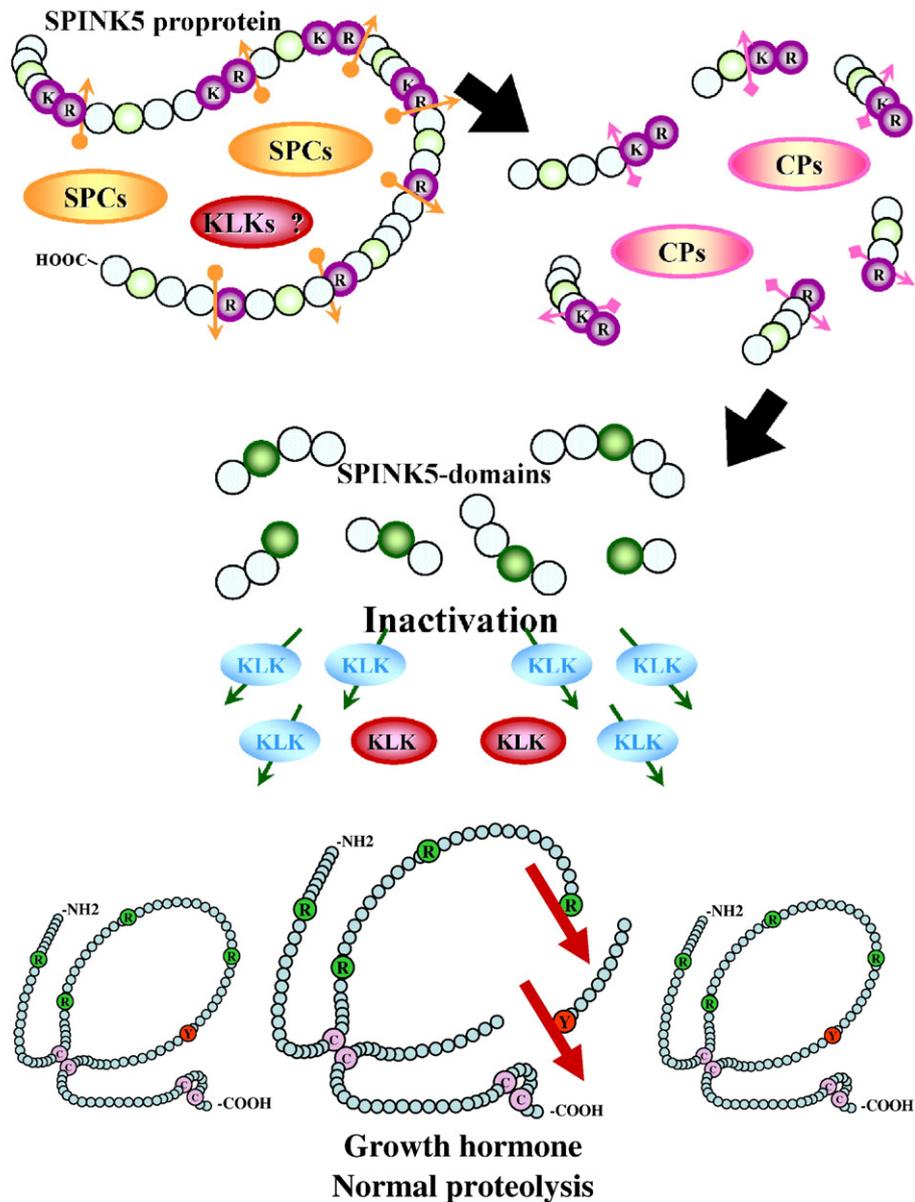


Fig. 6. A model for regulation of growth hormone proteolysis in normal individuals and Netherton syndrome patients (modified from [29]). (a) Upper panel: In normal pituitary *SPINK5* proprotein is proteolytically processed to at least 14 individual bioactive domains. Cleavage occurs at Lys/Arg (K/R)–X<sub>n</sub>–Lys/Arg (K/R)↓ (*n*=0, 2, 4, and 6) motifs, which are repeatedly found in-between an inhibitory domain and other domains of *SPINK5* proprotein, by subtilisin-like proprotein convertases (SPCs), such as furin [29,30,58,59]. Since it has not been conclusively determined whether KLKs are involved in *SPINK5* proprotein processing KLKs are indicated with a question mark. Cleaved domains may be further processed by carboxypeptidases (CPs) [29] to bioactive *SPINK5* domains. Middle panel: *SPINK5* domains are capable of inhibiting KLK serine protease activities, (blue circles with arrows), leaving some in active form (red circles). Thus, *SPINK5* domains are believed to be negative regulators of KLKs [29,41]. Bottom panel: KLKs may contribute to hGH proteolytic processing, yielding a 2-chain form in pituitary and/or periphery. (b) In Netherton syndrome patients, all mutations in *SPINK5* reported so far [29,54,55], lead to premature translation termination in and truncated protein. Consequently, Netherton syndrome patients possess fewer *SPINK5* inhibitory domains. This leads to higher KLK enzymatic activity and higher proteolytic processing of GH which may become biologically inactive. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

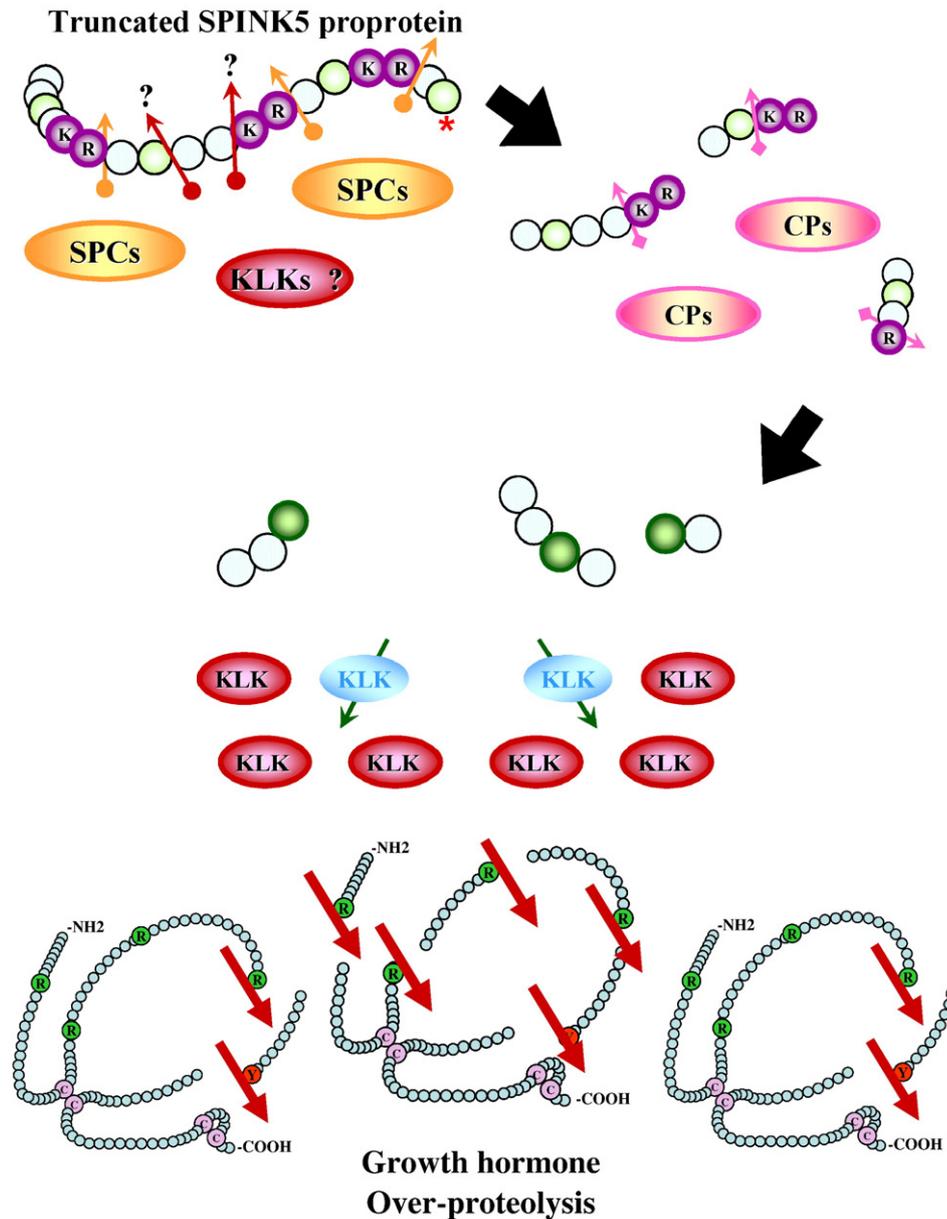
**(b) Netherton syndrome**

Fig. 6 (continued).

or Tyr<sup>143</sup>/Ser<sup>144</sup> for KLK14. Both cleavages convert hGH to the 2-chain form (Fig. 5b). Several other Arg residues are also targeted, probably as a pathway that ultimately leads to hGH degradation (Fig. 5c). Furthermore, *SPINK5* protein fragments can suppress KLK protease activity, leading to decreased hGH proteolytic processing (Fig. 5d).

Our preliminary immunohistochemical data suggest that human kallikreins are produced by cells that also produce hGH, as well as by endothelial cells of pituitary small veins. It is thus possible that hGH, and possibly other pituitary hormones, are proteolytically modified by KLKs before entering the systemic circulation. This proteolysis may be under the control of inhibitors such as *SPINK5* protein, which was also localized in hGH-producing cells and endothelial cells (Fig. 2). These

preliminary data need confirmation with double-staining techniques. We further demonstrated that excess KLKs can digest hGH into smaller, and likely inactive fragments (Fig. 3), and that *SPINK5* protein fragments can inhibit this process (Fig. 4).

Groups of human kallikrein enzymes, their inhibitors, and probably other proteases, may participate in cascade enzymatic pathways, e.g., in the skin and cancer tissues [13,39,53] (Fig. 6a). *SPINK5* pro-protein may be proteolytically processed by furin (and possibly some kallikreins) to generate at least 14 inhibitory domains [29,30]. *SPINK5* inhibitory domains may then control multiple kallikrein activities [37,38] (Figs. 4 and 6a). It is likely that similar pathways are operating in the pituitary and/or the periphery, leading to hormone processing and degradation (Fig. 6a).

In Netherton syndrome, all *SPINK5* gene mutations identified so far lead to premature stop codons [29,54,55] (Fig. 6b). These mutations result in the production of truncated *SPINK5* proteins, lacking inhibitory domains downstream of the mutations. The reduction of *SPINK5*-derived inhibitory domains would explain the elevated corneocyte trypsin-like activity in Netherton syndrome and the clinical picture, which is associated with increased desquamation [29,41]. We hypothesize that in the same patients, proteolytic over-processing of hGH in the pituitary may be responsible for the growth retardation [40], which is a consistent finding in these patients (Fig. 6b). Further examination of this hypothesis may lead to design of rational therapies of Netherton syndrome, including development of selective inhibitors for certain human kallikreins. Strategies for designing such inhibitors have recently been published [56].

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